C. elegans PAQR-2
A Regulator of Membrane Homeostasis

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To my family

ABSTRACT

The progestin and adipoQ receptor (PAQR) protein family is characterized by a 7-transmembrane domain, and a topology reversed that of G-protein coupled receptors, i.e. the N-terminus resides in the cytoplasm. Despite the presence of this class of receptors in humans, as well as in the established model organisms, the intracellular signaling pathway has not been adequately elucidated. The most extensively researched PAQR proteins are the mammalian adiponectin receptors, ADIPOR1/2, which mediate the insulin-sensitizing actions of adiponectin on glucose uptake, fatty acid oxidation and gluconeogenesis. AMPK and PPARα are downstream targets of the ADIPORs, and ceramide signaling has also been implicated in mice and yeast. The aim of our studies of the PAQR protein family in *C. elegans* is to further elucidate their downstream signaling pathway using a model organism well suited for the generation of unbiased knowledge through forward genetics screens.

We have focused our research on the *C. elegans* loss of function mutant of *paqr-2*. This protein is closely related to the mammalian ADIPORs and the mutant displays several interesting phenotypes. A forward genetics screen led us to identify IGLR-2 as a protein that physically interacts with PAQR-2 on cell membranes. The *paqr-2* and *iglr-2* mutants display identical phenotypes: sensitivity to cold and exogenous glucose as well as a withered tail tip morphology defect. All three phenotypes can be suppressed by mutations that directly or indirectly increase expression of Δ9 desaturases, enzymes that convert saturated fatty acids (SFA) into unsaturated fatty acids; conversely, *paqr-2* and *iglr-2* mutants have increased levels of SFA and decreased expression of the Δ9 desaturase reporter *pfat-7::GFP*. Poikilotherm organisms, such as *C. elegans,* adapt to a decreased environmental temperature in part by adjusting the fluidity of their cellular membranes. We hypothesized that PAQR-2 and IGLR-2 may act as regulators of membrane fluidity, and measured this property using fluorescence recovery after photobleaching, FRAP. The results reveal that *paqr-2* and *iglr-2*, unlike wild type, do have reduced membrane fluidity upon challenge with low temperature or glucose supplementation, and that this defect can be suppressed by mutations know to promote Δ9 desaturase activity or rescued by detergents provided at membrane-fluidizing concentrations.

We conclude that the adiponectin receptor homolog PAQR-2, and its partner IGLR-2, are involved in the *C. elegans* homeoviscous adaptation response and regulate membrane fluidity through activation of Δ9 desaturases.

Keywords: PAQR, LRRIG, glucose, membrane fluidity, desaturase, homeoviscous adaptation

PUBLICATIONS

This thesis is based on the following publications, referred to by their roman numerals in the text:

I. The Adiponectin Receptor Homologs in *C. elegans* Promote Energy Utilization and Homeostasis
Svensson E., Olsen L., Mörck C., Brackmann C., Enejder A., Faergeman N.J., Pilon M.,
PLoS One 2011 6(6): e21343

II. PAQR-2 Regulates Fatty Acid Desaturation during Cold Adaptation in *C. elegans*
Svensk E., Ståhlman M., Andersson C-H., Johansson M., Borén J., Pilon M.,
PLoS Genetics 2013 9(9): e1003801

III. *Caenorhabditis elegans* PAQR-2 and IGLR-2 Protect Against Glucose Toxicity by Modulating Membrane Lipid Composition
Svensk E., Devkota R., Ståhlman M., Ranji P., Rauthan M., Magnusson F., Hammarsten S., Johansson M., Borén J., Pilon M.,
PLoS Genetics 2016 12(4): e1005982

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INTRODUCTION

The introduction to this thesis is divided into four main sections: the PAQR protein family, adiponectin, *Caenorhabditis elegans* fatty acid metabolism and finally homeoviscous adaptation. This largely reflects the sequence of the research where, from the beginning, our theoretical framework was mainly that of the existing literature on the most well-known members of the PAQR protein family, i.e. the adiponectin receptors. As the study progressed, our theoretical framework shifted increasingly towards a foundation based on our own results, with fatty acids, phospholipids and the regulation of membrane fluidity upon challenge, or homeoviscous adaptation response, as the main focus. However, the closest homologs of the protein of study, *C. elegans* PAQR-2, are still the mammalian adiponectin receptors and the present knowledge on their function must be described in detail.

THE PAQR PROTEIN FAMILY

The **p**rogestin and **a**dipo**Q** **r**eceptor (PAQR) protein family is relatively novel, the name being coined in 2005 by Tang *et al.* and research intensifying only following the cloning of the adiponectin receptors, ADIPOR1 and ADIPOR2 (i.e. the mammalian PAQR1 and 2 respectively) in 2003 (Yamauchi et al., 2003a). However, these proteins are far from novel in the evolutionary sense since the family is represented in humans (11 members) as well as in all the established model organisms: *Escherichia coli* (1), *Saccharomyces cerevisiae* (4), *Arabidopsis thaliana* (6), *C. elegans* (5), *Drosophila melanogaster* (5) and *Mus musculus* (11 members) (Yamauchi et al., 2003a; Tang et al., 2005).

The most well-known and researched PAQR proteins are the mammalian adiponectin receptors, which will be further discussed below. The focus of this thesis lies on the PAQR protein family in *C. elegans*, and more specifically on the function of one member of this family, namely PAQR-2, which will be introduced in later sections.

PAQR PROTEIN STRUCTURE

All PAQR proteins have a conserved overall structure consisting of a 7-transmembrane (TM) domain with less conserved intracellular N-, and extracellular C-termini (Figure 1A). There is no amino acid sequence homology between PAQR proteins and the well-known G-protein coupled

receptor (GPCR) family of 7-TM domain proteins, and the N-, C-terminus topology is reversed between the two families (Yamauchi et al., 2003a; Tang et al., 2005). Several smaller motifs within the PAQR protein sequence have been described such as a proposed GxxxG type dimerization motif within TM5 (Kosel et al., 2010) and a possible di-leucine motif ($D(X)_3LL$) and cluster of hydrophobic amino acids ($F(X)_3F(X)_3F$) in the cytoplasmic tail close to TM1 that are both thought to be involved in protein transport and localization (Figure 1B) (Ding et al., 2009; Juhl et al., 2012; Keshvari and Whitehead, 2015).

The crystal structure of the human adiponectin receptors (PAQR1 and 2), minus their N-termini, was recently determined revealing, seen from the outside of the cell, a clockwise circular formation of the helixes in the 7-TM domain and several features that again sets the PAQRs apart from GPCRs, such as the absence of proline-induced kinks in the TM domains. A zinc ion, with a proposed structure-stabilizing effect, was found to be residing in the intracellular layer of the membrane, coordinated by histidine residues in TM2 and 7 and an aspartic acid residue in TM3 (via a water molecule). The circular formation of the TM domain helixes surrounds a large cavity stretching from the cytoplasm to the middle of the membrane outer lipid layer (Tanabe et al., 2015). A yeast two-hybrid assay has previously demonstrated that adiponectin can bind to the extracellular C-terminus of ADIPOR1 (Mao et al., 2006), while the present crystal structure rather points towards binding of adiponectin to a larger area of the extracellular surface, including the extracellular loops (Tanabe et al., 2015).

PAQR PROTEINS IN OTHER MODEL ORGANISMS

S. cerevisiae

The genome of *S. cerevisiae* encodes four members of the PAQR family, IZH1-4, and these proteins may be involved in zinc homeostasis (Lyons et al., 2004). However, the expression of *Izh1*, *2* and *4* is also regulated by fatty acids (FA) (Karpichev et al., 2002; Lyons et al., 2004). The most extensively studied yeast PAQR protein is IZH2 and expression of the *Izh2* mRNA is induced by growth in presence of glucose or saturated fatty acids (SFA, C14-18:0) while being reduced by growth in presence of oleic acid, an unsaturated fatty acid (UFA, C18:1) (Karpichev and Small, 1998; Karpichev et al., 2002). The *Izh2* deletion strain has increased resistance to nystatin (Karpichev et al., 2002), an antifungal molecule that can bind to sterols in the cell membrane (Marini et al., 1961; Kinsky, 1962), which suggests an altered membrane composition in the mutant (Karpichev et al., 2002).

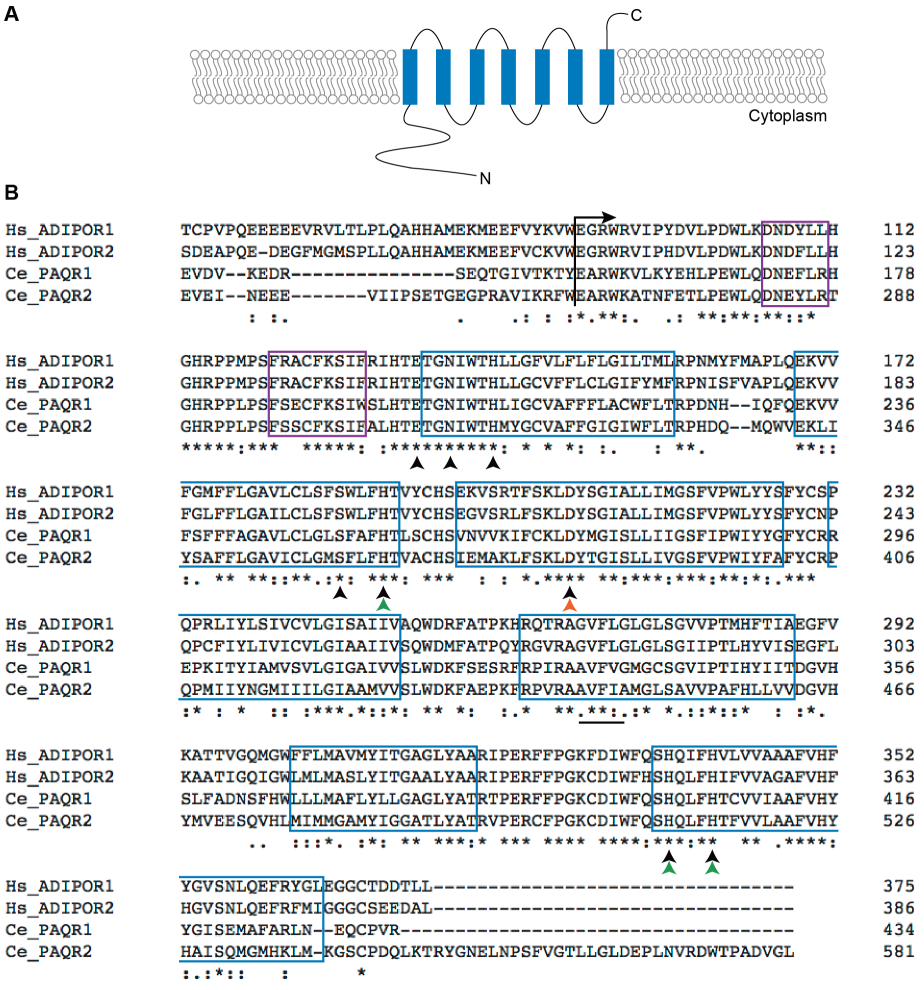

Figure 1. Overall structure of PAQR proteins and an alignment of the human ADIPORs to their *C. elegans* homologs. A, Schematic drawing of a PAQR protein and, **B**, protein sequence alignment for human ADIPOR1 and 2 (PAQR1 and 2) and *C. elegans* PAQR-1 and -2 with known sequence motifs indicated. Black arrow indicates start of the truncated ADIPOR1/2 versions used for crystallography and blue boxes indicates the helixes of the 7-TM domain as per the crystal structure (Tanabe et al., 2015). Black arrowheads show the putative ceramidase motif discussed in the section on *S. cerevisiae* (Villa et al., 2009) and green/orange arrowheads indicate the Zn^{2+}-coordinating histidine (ADIPOR1/2) and aspartic acid (ADIPOR2) residues in ADIPOR1/2 (Tanabe et al., 2015). Purple boxes in the N-terminal domain indicate motifs important for receptor transport and localization (Ding et al., 2009; Juhl et al., 2012; Keshvari and Whitehead, 2015) and the black line under TM5 shows the putative dimerization motif (Kosel et al., 2010).

Overexpression of IZH2 inhibits induction of *FET3* (part of an iron uptake complex) during iron limitation in *S. cerevisiae*. This effect is dependent on protein kinase A and can be mimicked by overexpression of human ADIPOR1, in the same conditions, and ADIPOR2 if also treating with adiponectin (Kupchak et al., 2007). IZH2 can bind the antifungal tobacco protein osmotin *in vitro*, and osmotin can induce phosphorylation of AMP-activated protein kinase (AMPK) in C2C12 myocytes in an ADIPOR1/2 dependent manner, thus mimicking adiponectin (Narasimhan et al., 2005).

Villa *et al.* used the above mentioned repression of *FET3* as a reporter for IZH2 activity to further research the signaling mechanism of this PAQR protein and in 2009 published the first paper connecting the PAQR family to ceramidase activity. They discovered homology of certain short amino acid sequences within PAQR proteins to a class of alkaline ceramidases and furthermore showed that overexpression of a known alkaline ceramidase or treatment with an exogenous product from the ceramidase reaction, a primary sphingoid base, can inhibit *FET3* induction upon iron deprivation, hence mimicking the activity of IZH2. Overexpression of IZH2 mediates a small but significant accumulation of the primary sphingoid base. This effect can be seen also in a strain lacking the two known yeast ceramidases and can be blocked by myriocin (Villa et al., 2009), an inhibitor of serine palmitoyltransferase that stops production of sphingoid base (Miyake et al., 1995). Myriocin can also restore the iron limitation induced *FET3* expression in the IZH2 overexpression strain. The authors tried, but could not detect *in vitro* ceramidase activity from membrane preparations of IZH2 overexpressing cells (Villa et al., 2009). Nevertheless, a known inhibitor of alkaline ceramidases, D-*erythro*-MAPP (Bielawska et al., 1996), could inhibit activity of IZH2 as well as ADIPOR1 in the previously mentioned *FET3* expression assay (Kupchak et al., 2009; Villa et al., 2009).

To summarize, studies on the *S. cerevisiae* PAQR protein IZH2 show that it may signal through an intrinsic or associated ceramidase activity (Villa et al., 2009) and that it can bind the antifungal ADIPOR1/2 agonist osmotin (Narasimhan et al., 2005). Importantly for the studies presented in this thesis, *Izh2* expression is induced by SFA or glucose while being repressed by UFA, and the *Izh2* loss off function mutant strain may have an aberrant membrane composition (Karpichev and Small, 1998; Karpichev et al., 2002).

D. melanogaster

The *D. melanogaster* ADIPOR homolog, dADIPOR (66% similarity), is expressed in several regions of the fly brain including the insulin producing cells (IPC). RNAi treatment of *dAdipoR* (partial effect) specifically in these cells results in several metabolic phenotypes, such as increased levels of

glucose in the hemolymph, increased levels of triglycerides, shorter lifespan, increased sensitivity to high fat (HF) feeding and resistance to starvation (Kwak et al., 2013). Upon starvation, *Drosophila* insulin like peptides (DILPs) accumulate in the IPCs and are then secreted upon re-feeding, however, in the previously mentioned IPC-specific *dAdipoR* RNAi system this response is not normal and the assayed DILP, DILP2, is retained in the IPCs thus indicating a role for dADIPOR in DILP secretion (Kwak et al., 2013).

A *dAdipoR* null allele, $AdipoR^{27}$, was recently published and determined to be lethal (Laws et al., 2015). The authors thus perform their study of dADIPOR function in germline stem cells (GSC) using genetically mosaic flies recognizing the homozygous $AdipoR^{27}$ cells by loss of GFP. In this system loss of dADIPOR has no effect on the insulin dependent GSC proliferation while the diet dependent GSC maintenance is affected, indicating a role for dADIPOR in this process (Laws et al., 2015). On a rich diet the GSCs proliferate faster and are less frequently lost, as a response to nutrient availability, a signal potentially conferred via the target of rapamycin (TOR) pathway (Ables et al., 2012).

Thus, although little is known about the *D. melanogaster* ADIPOR homolog, both insulin and non-insulin related functions have been described (Kwak et al., 2013; Laws et al., 2015) and the lethality of the homozygous *dAdipoR* knockout fly (Laws et al., 2015) indicates an important function of this protein.

THE MAMMALIAN ADIPONECTIN RECEPTORS

As mentioned earlier there are 11 members of the PAQR protein family in mice and humans (Yamauchi et al., 2003a; Tang et al., 2005). In 2003 Yamauchi *et al.* cloned the receptors of the adipokine adiponectin (ADIPOR1 and 2) and these turned out to belong to the PAQR protein family (PAQR1 and 2). Since then, two single labs have contributed most of the fundamental research towards elucidating the downstream signaling pathways of these receptors in mammals: the Kadowaki lab in Tokyo and the Scherer lab in Texas.

ADIPOR1/2 expression pattern and mouse knockout models

The mouse *AdipoR1* mRNA is expressed ubiquitously, with the highest abundance in skeletal muscle, while *AdipoR2* is most abundantly expressed in liver, with low levels in other tissues. In human the *AdipoR2* mRNA is highly expressed in liver as well as skeletal muscle while *AdipoR1* mRNA is

again more broadly expressed with high levels in skeletal muscle, heart, placenta, kidney and liver (Yamauchi et al., 2003a).

The expression level of *AdipoR1* and *2* in mouse liver and skeletal muscle (as quantified by real-time PCR) increases upon fasting and is again reduced with re-feeding. Plasma insulin levels behave in the opposite manner, being reduced with fasting and strongly induced upon re-feeding, and analogously *AdipoR1* and *2* expression in skeletal muscle is increased when streptozotocin is used to cause insulin deficiency, but again reduced with addition of insulin (Tsuchida et al., 2004). The expression level of *AdipoR1* and *2* is also decreased in insulin-resistant *ob/ob* mice, which have notoriously high levels of insulin in their plasma, and in the obesity model *db/db* mice, which also have high fasting insulin levels (Tsuchida et al., 2004; Yamauchi et al., 2007).

AdipoR1 knockout mice (*AdipoR1*$^{-/-}$) generated in the Kadowaki lab (removing exon 2, 3 and 4 of *AdipoR1*) are viable and fertile with normal body weight and food intake (Yamauchi et al., 2007). However when challenged in an oral glucose tolerance test, the *AdipoR1*$^{-/-}$ mice show increased plasma glucose and insulin levels as compared to wild type (WT), indicative of insulin resistance. The Kadowaki group *AdipoR2* knockout mice (removing exon 3 of *AdipoR2*) express an aberrantly spliced *AdipoR2* mRNA, which the authors state is not translated. These *AdipoR2*$^{-/-}$ mice are viable and fertile with a normal body weight and display increased plasma insulin, but not glucose, in the oral glucose tolerance test. The double knockout of the above-mentioned mutant alleles, i.e. *AdipoR1*$^{-/-}$ *AdipoR2*$^{-/-}$ mice, show signs of insulin resistance (plasma glucose and insulin levels increased) and when calculating the insulin resistance index of WT and mutant strains the effect is enhanced in the double, as compared to the single knockouts (Yamauchi et al., 2007).

In contrast, studies from three different groups using *AdipoR1* and *2* knockout mice constructed by Deltagen (San Carlos, CA, removing part of exon 2 of *AdipoR1* and exon 5 of *AdipoR2*) show in part opposing phenotypes. These *AdipoR1*$^{-/-}$ mice have increased adiposity (due to decreased energy expenditure) (Bjursell et al., 2007; Parker-Duffen et al., 2014) and the males are glucose intolerant but with a normal insulin response (Bjursell et al., 2007). The females have increased fasting glucose levels, again with normal levels of insulin (Bjursell et al., 2007) and develop insulin resistance when fed a HF diet (Parker-Duffen et al., 2014). In contrast to the *AdipoR1*$^{-/-}$ mice, the Deltagen *AdipoR2*$^{-/-}$ mice have lower body weight (not as a consequence of decreased food intake), less body fat mass as compared to WT (Bjursell et al., 2007; Parker-Duffen et al., 2014) and are also resistant

to weight gain from feeding with a HF diet (Bjursell et al., 2007; Liu et al., 2007; Parker-Duffen et al., 2014). On HF diet, *AdipoR2*$^{-/-}$ mice have an improved glucose tolerance with lower levels of glucose and insulin in the plasma, as compared to WT, following an oral glucose tolerance test, as well as increased energy expenditure and locomotor activity (Bjursell et al., 2007; Liu et al., 2007). However, when fed a HF diet over a longer period of time (16-20 weeks) the level of fasting plasma glucose starts to increase in the *AdipoR2*$^{-/-}$ mice, while the increase in fasting plasma insulin is still quite small, indicating an inability of the *AdipoR2*$^{-/-}$ mice to compensate for this moderate glucose intolerance (Liu et al., 2007). Furthermore, this *AdipoR2*$^{-/-}$ allele slows down progression of atherosclerosis in the atherosclerosis model *ApoE*$^{-/-}$ background, and double knockout mice, i.e. *AdipoR1*$^{-/-}$ *AdipoR2*$^{-/-}$, generated from the Deltagen strains die before embryonic day 16.5 (Lindgren et al., 2013).

All in all, there are several discrepancies between the different mouse models: 1) the double *AdipoR1/2* knockout mice generated by the Kadowaki group is alive (Yamauchi et al., 2007), while the double knockout of the Deltagen mice is embryonic lethal (Lindgren et al., 2013); 2) the single knockouts from the Kadowaki lab show a similar trend of insulin resistance, which is worse in the double knockout (Yamauchi et al., 2007), while in the Deltagen mice the *AdipoR1*$^{-/-}$ is insulin resistant on a HF diet (Parker-Duffen et al., 2014), while the *AdipoR2*$^{-/-}$ show opposite phenotypes, with improved glucose tolerance on a HF diet, increased energy expenditure (Bjursell et al., 2007; Liu et al., 2007) and decreased body weight and adiposity (Bjursell et al., 2007; Parker-Duffen et al., 2014). Thus, the discrepancies are mainly with the *AdipoR2*$^{-/-}$ knockouts, where the Deltagen *AdipoR2*$^{-/-}$ show opposite phenotypes, compared to the other knockout strains, and phenotypes that actually resemble those of a mouse model overexpressing globular adiponectin i.e. improved glucose tolerance and increased energy expenditure when on a HF diet, as well as inhibition of atherosclerosis progression in the *ApoE*$^{-/-}$ background (Yamauchi et al., 2003b; Bjursell et al., 2007; Liu et al., 2007; Lindgren et al., 2013).

The discrepancies between the different mouse models makes it hard to draw specific conclusions from this body of work, although one should be safe to conclude that the ADIPORs are involved in metabolism, whether having similar or opposing effects. A new, and separately generated, set of knockout mice could potentially resolve the present discrepancies.

ADIPOR1/2 signaling

The two most well-known targets of ADIPOR1/2 signaling are AMPK, a driver of catabolic pathways to produce ATP expressed ubiquitously in

eukaryotic cells (Hardie et al., 2016), and PPARα (**p**eroxisome **p**roliferator-**a**ctivated **r**eceptor **α**), a transcription factor involved in expression of β-oxidation genes in response to nutritional status (Pawlak et al., 2015).

Treatment of C2C12 myocytes, mouse soleus muscle or rat extensor digitorum longus (EDL) muscle strips with adiponectin for 5-30 minutes results in an increase in phosphorylation of AMPK and stimulates FA oxidation and glucose uptake (Tomas et al., 2002; Yamauchi et al., 2002). Further enhancement of these effects in C2C12 myocytes can be seen when simultaneously expressing ADIPOR1 (Yamauchi et al., 2003a) and the stimulation of FA oxidation and glucose uptake, can be partially inhibited by use of a dominant negative version of AMPK (Yamauchi et al., 2002). The effect on phosphorylation of AMPK with adiponectin treatment can also be seen in mouse liver (Yamauchi et al., 2002) and this effect can be enhanced in hepatocytes by simultaneous expression of ADIPOR1 (Yamauchi et al., 2003a). Treatment with adiponectin reduces glucose output and expression of gluconeogenic genes in liver/primary hepatocytes (Combs et al., 2001; Yamauchi et al., 2002; Miller et al., 2011) and while Yamauchi *et al.* 2002 attributes this effect to AMPK in liver, Miller *et al.* 2011 states that, in primary hepatocytes, other pathways separate from LKB1-AMPK are also involved.

Using a GAL4/UAS reporter system expressing a chimera of GAL4 and the PPARα ligand binding domain (Murakami et al., 1998), an increase in PPARα ligand activity upon adiponectin treatment was initially shown to be present in C2C12 myocytes but not in primary hepatocytes (Yamauchi et al., 2003b). The effect is reduced by expression of *AdipoR1* siRNA, while *AdipoR2* siRNA has partial effects. The combination of both siRNAs is very effective at abolishing the induction of PPARα ligand activity as well as the effect of adiponectin treatment on FA oxidation and glucose uptake in C2C12 myocytes (Yamauchi et al., 2003a).

Expression of ADIPOR1, but not ADIPOR2, in the liver of *db/db* mice, increases the level of AMPK phosphorylation seen after treatment with adiponectin while expression of ADIPOR2, but not ADIPOR1 in the same tissue, can instead increase expression of PPARα and its target genes. However, expression of either ADIPOR1/2 in the liver of *db/db* mice confers an increase in FA oxidation and a decrease in tissue triglyceride content, potentially then via different targets (Yamauchi et al., 2007).

Thus, ADIPOR1 is thought to activate AMPK in skeletal muscle, to increase glucose uptake and FA oxidation (Tomas et al., 2002; Yamauchi et al., 2002; Yamauchi et al., 2003a), and in liver to decreased triglyceride content and

increase FA oxidation (Yamauchi et al., 2007). A decreased triglyceride content and increased FA oxidation is also seen from ADIPOR2 via PPARα in liver (Yamauchi et al., 2007), while the potential activation of PPARα in C2C12 myocytes (Yamauchi et al., 2003a; Yamauchi et al., 2003b) has yet to be connected to physiological effects.

Administration of adiponectin to WT mice increases the level of *PGC-1α* (**p**eroxisome proliferator-activated receptor **γ** **c**o-activator **1α**) mRNA in skeletal muscle (Iwabu et al., 2010), a sign of increased mitochondrial biogenesis (Wu et al., 1999). Muscle specific *AdipoR1* knockout mice have decreased levels of mitochondrial DNA in their skeletal muscles accompanied by a decrease in markers of mitochondrial biogenesis, such as *PGC-1α*, and unlike WT the levels cannot be increased by administration of adiponectin. The muscle-specific *AdipoR1* knockout mice have a reduction of type I fibers in the soleus muscle, as compared to WT, and lower exercise endurance when challenged on a treadmill (Iwabu et al., 2010).

In C2C12 myocytes treatment with adiponectin causes an increase in mitochondrial DNA content and this effect can be blunted by expression of siRNA towards *AdipoR1*, *CaMKKβ* (**Ca**$^{2+}$/cal**m**odulin-dependent protein **k**inase **k**inase **β**, a kinase upstream of AMPK (Hardie et al., 2016)) or both isoforms of *AMPKα*, but not by expressing siRNA targeting *AdipoR2*. Adiponectin treatment also causes influx of Ca^{2+} into C2C12 myocytes in an ADIPOR1-dependent manner, and the upregulation of *PGC-1α* after adiponectin treatment can be blocked by removal of Ca^{2+} (by EDTA) or by treatment with a CAMKK inhibitor (Iwabu et al., 2010). The Ca^{2+} signal in itself, upon stimulation of C2C12 myoblasts with adiponectin, occurs also in the presence of EGTA, thus indicating use of intracellular Ca^{2+} stores, and the resulting phosphorylation of AMPK in this setting is reduced upon treatment with inhibitors of CAMKK or phospholipase C (PLC) (Zhou et al., 2009). Furthermore the increase in cytoplasmic Ca^{2+} upon adiponectin stimulation is completely blocked by a PLC inhibitor, indicating a mechanism where adiponectin activates PLC to promote influx of Ca^{2+} from the endoplasmatic reticulum (ER) (Zhou et al., 2009) via the known PLC pathway of inositol triphosphate (IP_3) production and subsequent activation of the IP_3 receptors (Mikoshiba and Hattori, 2000).

In summary, ADIPOR1 seems to mediate the positive effect of adiponectin on skeletal muscle mitochondrial content (Iwabu et al., 2010) in a PLC-Ca^{2+}-CAMKK-AMPK dependent fashion (Zhou et al., 2009).

Administration of adiponectin to *ob/ob* mice, or mice on a HF diet, can decrease ceramide content in liver, which is otherwise elevated in these

mouse models. Similarly, transgenic expression of adiponectin is protective against liver ceramide accumulation, caused by a HF diet, and an adiponectin knockout mouse (*AdipoQ*$^{-/-}$) accumulates increased levels of liver ceramides, as compared to WT, upon feeding with a HF diet, a relation that correlates with the insulin sensitivity of the different strains (Holland et al., 2011). *Lkb1*$^{-/-}$ mice were used to assay whether the effect on ceramides is exerted via AMPK, since LKB1 (**l**iver **k**inase **B1**) is a kinase well-known to activate AMPK (Hardie et al., 2016). In such mice, adiponectin can still lower plasma glucose levels and liver ceramide content, and these effects are consequently independent of LKB1-activated AMPK (Holland et al., 2011).

As previously mentioned, a yeast protein in the PAQR family has an associated, or potentially intrinsic, ceramidase activity (Villa et al., 2009). This knowledge was used to further assay the ADIPORs. Expression of ADIPOR1/2 in HEK293T cells (derived from human kidney) increases ceramidase activity and this effect can be further stimulated by addition of adiponectin, or blunted by expressing versions of ADIPOR1/2 with mutations in the putative ceramidase domain. Furthermore, ceramidase activity cannot be stimulated by adiponectin in mouse embryonic fibroblasts (MEF) obtained from double *AdipoR* knockout mice, though it can in WT derived MEFs (Holland et al., 2011).

A ceramidase converts ceramide to sphingosine, which can then in turn be phosphorylated into sphingosine 1-phosphate (S1P, by sphingosine kinase) (Holland and Summers, 2008). Ceramide and S1P have different effects on cells: ceramide impairs insulin signaling (Holland and Summers, 2008) while S1P is an inhibitor of apoptosis and a positive regulator of growth (Takabe et al., 2008). As a second messenger S1P functions intracellularly, as well as via secretion and signaling through the S1P receptors (GPCRs) (Takabe et al., 2008). The potential ceramidase activity of the ADIPORs could be intrinsic or associated (Villa et al., 2009; Holland et al., 2011); the present knowledge can not really distinguish between these two possibilities, and while LKB1 is not needed to confer physiological effects i.e. to lower plasma blood glucose levels and liver ceramide content (Holland et al., 2011), CAMKK-AMPK signaling can not be excluded.

In 2011, when the Nature paper on adiponectin receptors and ceramides was published from the Scherer lab both they themselves and the Kadowaki lab made some effort to combine the knowledge on AMPK, PPARα, ceramides etc. into one potential signaling pathway downstream of the ADIPORs, the outline of which is depicted in Figure 2.

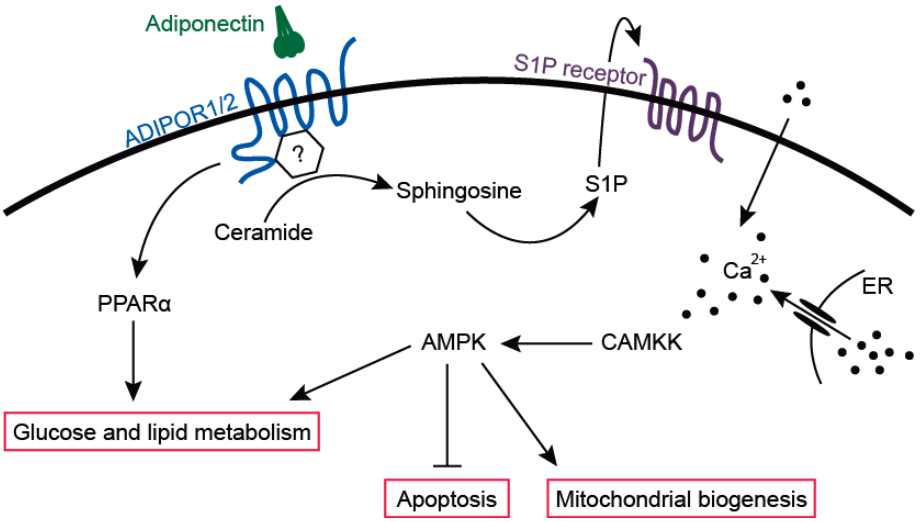

Figure 2. A proposed signaling pathway downstream of the mammalian adiponectin receptors. Adiponectin binding to ADIPOR2 triggers activation of PPARα, and stimulates glucose uptake and FA oxidation, while adiponectin binding to either ADIPOR1 or 2 also activates a ceramidase activity converting ceramide to sphingosine. Sphingosine can in turn be converted to S1P (via sphingosine kinase) which signals, via the S1P receptors, to promote Ca^{2+} influx into the cytoplasm. An increased level of intracellular Ca^{2+} activates CAMKK, which in turn phosphorylates and activates AMPK to promote glucose uptake, FA oxidation and mitochondrial biogenesis while preventing apoptosis. S1P, sphingosine 1-phosphate; CAMKK, Ca^{2+}/calmodulin-dependent protein kinase kinase. Adapted from (Holland et al., 2011; Kadowaki and Yamauchi, 2011). Note that this pathway excludes many findings, such as the proposed ADIPOR1 interaction partners, discussed below, and that the illustration also does not indicate tissue specificity.

ADIPOR1/2 interaction partners

Several candidate ADIPOR1/2 interaction partners have been identified, mainly using yeast two-hybrid screens. However, none seem to be recognized by the main groups working on the signaling pathway: they are rarely mentioned in publications by the Kadowaki or Scherer groups.

APPL1 (**a**daptor protein containing **p**leckstrin homology domain, **p**hosphotyrosine-binding domains and **l**eucine zipper motif) was found in yeast two-hybrid screens towards a human fetal brain cDNA library using the cytoplasmic domain of mouse ADIPOR1 or 2 as bait, catching the phosphotyrosine-binding domain of APPL1. ADIPOR1 can be co-immunoprecipitated with APPL1 from C2C12 myocytes and the interaction is enhanced by treatment with adiponectin. Treatment of C2C12 myocytes

with siRNA against *APPL1* also blunts phosphorylation of AMPK upon stimulation with adiponectin (Mao et al., 2006). This effect can be explained by a function of APPL1 for LKB1 localization since APPL1 and LKB1 can interact physically in co-immunoprecipitation, *in vitro* binding and yeast two-hybrid experiments, and since LKB1 fails to translocate from nucleus to cytoplasm upon stimulation with adiponectin in C2C12 myoblasts treated with siRNA towards *APPL1* (Zhou et al., 2009).

The nuclear localization of LKB1 is dependent on phosphorylation of Ser307 by protein kinase Cζ (PKCζ) (Xie et al., 2009). The phosphorylation status of LKB1 is in turn dependent on adiponectin, with decreased levels of phosphorylation and increased levels of cytosolic localization upon treatment. Furthermore, stimulation of C2C12 myotubes with adiponectin reduces the activity of PKCζ (in an APPL1-dependent manner) while increasing the activity of protein phosphatase 2A (PP2A), and both the kinase and the phosphatase can be co-immunoprecipitated with APPL1 (Deepa et al., 2011). Specific inhibition of PP2A using low concentrations of cantharidin (Honkanen, 1993) results in increased levels of phosphorylation of PKCζ and consequently also of LKB1, and these results led the authors to suggest a mechanism where the adiponectin-induced phosphorylation of AMPK is dependent on LKB1 being translocated to the cytosol due to a PP2A-dependent decrease in activity of PKCζ, mediated by the ADIPOR1/2 interacting protein APPL1 (Deepa et al., 2011).

An isoform of APPL1, APPL2, can also bind to ADIPOR1 and 2 as evidenced by pull-down of endogenous receptor with GST-tagged APPL2 (Wang et al., 2009). The interaction of APPL2 and ADIPOR1 can be further confirmed by co-immunoprecipitation in C2C12 myotubes, but contrary to the interaction with APPL1, this interaction is reduced upon stimulation with adiponectin (Mao et al., 2006; Wang et al., 2009). Overexpression of APPL2 in C2C12 myoblasts reduces the induction of AMPK phosphorylation seen upon stimulation with adiponectin and knockdown of *APPL2* using shRNA increases levels of phosphorylated AMPK in stimulated and unstimulated conditions. Visualisation of the cellular localization of APPL1 and 2, using Myc-tagged versions of the proteins, show that in the unstimulated condition APPL1 is localized in the cytoplasm while APPL2 is at the plasma membrane. Upon stimulation with adiponectin the two proteins change location and APPL1 associates with the plasma membrane while APPL2 is localized in the cytoplasm. Knockdown of *APPL2* using shRNA in C2C12 myotubes results in an increased ADIPOR1-APPL1 interaction and also in increased glucose uptake and FA oxidation in adiponectin stimulated and unstimulated conditions (Wang et al., 2009). The proposed ADIPOR1/2-APPL1-LKB1-AMPK signalling axis could thus be dependent on

replacement of APPL2 with APPL1 at the ADIPOR1/2 intracellular N-terminus.

RACK1 (**r**eceptor for **ac**tivated protein **k**inase C**1**) was found interacting with the full-length ADIPOR1 in yet another yeast two-hybrid screen towards a human liver cDNA library. Tagged versions of the two proteins do co-immunoprecipitate with each other and knockdown of *RACK1* in HepG2 cells using siRNA inhibits adiponectin-stimulated uptake of a fluorescent derivative of glucose, 2-NBDG (Xu et al., 2009). The authors speculate, based on the role of RACK1 in interaction with other receptors and signalling molecules (Chang et al., 1998; Geijsen et al., 1999; Lopez-Bergami et al., 2005) that it may act as a scaffold protein recruiting cytosolic downstream targets to ADIPOR1 (Xu et al., 2009).

CK2β, the regulatory subunit of the protein serine/threonine kinase CK2, has been found interacting with ADIPOR1 in a yeast two-hybrid screen towards a human testis cDNA library. The interaction can be narrowed down to amino acid 113-132 in the ADIPOR1 N-terminal domain, very close to TM1, where amino acid 113-119 constitutes a putative Src homology 3 (SH3) domain. CK2β can be co-immunoprecipitated with EYFP-tagged ADIPOR1 from MCF7 cells, if also crosslinking proteins prior to the assay, indicating that the interaction is short lived or dependent on the cellular environment (Heiker et al., 2009). Using a similar setup with overexpression of tagged ADIPOR1, crosslinking and co-immunoprecipitation, interaction between the receptor and the catalytic subunit of protein serine/threonine kinase CK2 (CK2α) can also be detected, and confirmed using the independent method of **bi**molecular **f**luorescence **c**omplementation (BiFC) visualizing the interaction at the membrane of HEK293 cells (Juhl et al., 2011). Functionality of the interaction can be scored with an inhibitory phosphorylation of acetyl-CoA carboxylase (ACC) as readout, where the CK2β inhibitor DMAT (Pagano et al., 2004) decreases the levels of phosphorylation otherwise seen upon stimulation with adiponectin (Heiker et al., 2009). Several proteins of the insulin signaling pathway, including the insulin receptor and AKT are on the list of the numerous known CK2 substrates (Meggio and Pinna, 2003), and Heiker *et al*. thus speculate on a role for CK2 in the crosstalk between the signaling pathways.

ERp46, a thioredoxin-like protein which may function as a chaperone, has been found interacting with ADIPOR1 after transient transfection of a C-terminally FLAG-tagged version of the later in CHO cells, immunoprecipitation using the tag and identification of the extra band from SDS-PAGE/silver stain using mass spectrometry. The interaction is specific to ADIPOR1: the same assay with transient transfection of ADIPOR2 can

not co-immunoprecipitate ERp46 and the site of interaction can be narrowed down to amino acid 1-70 of the ADIPOR1 N-terminal domain, a region with low sequence similarity even between ADIPOR1 and 2. ADIPOR1 and ERp46 expressed as C- terminally tagged proteins can both be detected in the plasma membrane of non-permeabilized cells, even though ERp46 is mainly localized to the ER. Knockdown of *ERp46* using shRNA in HeLa cells results in increased levels of ADIPOR1 and 2 in the plasma membrane and increased levels of phosphorylation of AMPK upon stimulation with adiponectin. Being an ER protein with potential chaperone function one could speculate that ERp46 would be in involved in folding and transport of ADIPOR1 to the plasma membrane. However, the increased levels of ADIPOR1 in the plasma membrane seen after knockdown of *ERp46* could instead indicate that ERp46 is involved in internalization of ADIPOR1 (Charlton et al., 2010).

Multimerization of ADIPOR1/2

When cloning the adiponectin receptors, the authors also showed that HA-tagged ADIPOR1 can be co-immunoprecipitated with both FLAG-tagged ADIPOR1 and ADIPOR2, indicating that the proteins can form homo-, and heteromultimers (Yamauchi et al., 2003a). This property of the receptors has been further studied for ADIPOR1 where endogenous homodimers can be detected in several human cell lines as well as in femoral muscle tissue (Kosel et al., 2010). Dimerization of ADIPOR1 can be assayed and visualized in the cell membrane of HEK293 cells using BiFC, and the signal quantified using flow cytometry. The dimerization is dependent on the previously mentioned GxxxG motif in TM5, since substitution of the two neutral glycines to the polar amino acid glutamic acid will substantially decrease the amount of dimer detected by western blot or flow cytometry. Stimulation of the HEK293 cells with adiponectin decreases the level of endogenous ADIPOR1 dimer detected while the amount of monomer, as quantified by western blot, is not changed, thus probably not reflecting degradation, and this effect is as potent when using only amino acids 60-89 of the adiponectin collagenous domain as when using the full-length adiponectin; the globular domain has no effect (Kosel et al., 2010). These results suggest a signaling mechanism where stimulation with adiponectin dissociates the ADIPOR1 dimers, hence that the receptors may signal as monomers.

More recently FRET (**f**luorescence **r**esonance **e**nergy **t**ransfer) has been used to assay homo-, as well as heterodimerization of ADIPOR1/2 in HEK293AD cells and all three types of complexes (ADIPOR1/2 homodimers and ADIPOR1/ADIPOR2 heterodimers) can be detected on the cell membrane and potentially also in the ER (Almabouada et al., 2013).

Anterograde transport of ADIPOR1/2

The ADIPOR1 N-terminus harbors a sequence of hydrophobic amino acids, $^{121}F(X)_3F(X)_3F^{129}$ close to TM1 (Juhl et al., 2012) and this type of motif has previously been shown to regulate ER export of GPCRs (Bermak et al., 2001; Dong et al., 2007). Substitution of all three phenylalanine (F) residues in the motif to alanine prominently decreases the amount of ADIPOR1 expressed on the cell membrane, without changes in actual protein levels, and the mutant version instead co-localizes with the ER marker pDsRed2-ER (Juhl et al., 2012). A truncated version of ADIPOR1 lacking the N-terminal amino acids 1-117 has the previously mentioned motif intact but still localizes predominantly to the ER indicating that yet other sequences must be important. An acidic dileucine motif, $^{106}D(X)_3LL^{111}$, can also be found in the ADIPOR1 N-terminus (Juhl et al., 2012) and such motifs have previously been shown to regulate exit from ER (Schulein et al., 1998). Mutation of D106 and L110 to alanine decreases levels of ADIPOR1 on the cell membrane to the same extent as substitution of all three phenylalanines of the $^{121}F(X)_3F(X)_3F^{129}$ domain, and also in this case, co-localization can instead be seen with the ER marker. Mutation of both domains do not further enhance the effect, indicating that both domains are important for, and together regulate, anterograde transport of ADIPOR1 (Juhl et al., 2012).

The $^{121}F(X)_3F(X)_3F^{129}$ domain is also implicated in protein folding since lowering of the temperature, a treatment known to facilitate correct folding and subsequent ER exit of misfolded GPCRs (Dong and Wu, 2006), can overcome the ER retention of mutant versions with either of the three phenylalanine residues substituted to alanine (Juhl et al., 2012). Other functions of these types of short amino acid sequences in the process of receptor transport can be binding of chaperones or transport proteins or receptor dimerization, which is known to be important for ER exit of some GPCRs (Pagano et al., 2001; Dong et al., 2007).

In HeLa and HEK cells, ADIPOR1 is mainly present at the cell membrane while ADIPOR2 is, to a large extent, retained in the ER (Charlton et al., 2010; Keshvari et al., 2013). Experiments swapping the most N-terminal non-conserved parts of ADIPOR1 (amino acid 1-70) and ADIPOR2 (amino acid 1-81) with each other result in detection of only ADIPOR2, carrying the ADIPOR1 N-terminus, on the cell membrane. Removal of the non-conserved N-terminal sequences instead confers detection of ADIPOR1 as well as ADIPOR2 on the cell membrane, thus indicating that the non-conserved N-terminal sequence conveys the distinct properties (Keshvari et al., 2013). Co-transfection of tagged ADIPOR1 and ADIPOR2 confers increased levels of ADIPOR2 on the cell membrane, implicating that heterodimerization may

play a role in ER exit of the receptors (Keshvari et al., 2013) a mechanism used by the GPCR gamma-aminobutyric acid type B1 (Pagano et al., 2001).

An increase in the amount of HA-tagged ADIPOR1 and 2 on the cell membrane can also be seen after serum starvation in HEK239 cells (Keshvari and Whitehead, 2015), and the receptors are again internalized after treatment with adiponectin (Almabouada et al., 2013; Keshvari and Whitehead, 2015) or serum from WT but not from adiponectin knockout mice (Keshvari and Whitehead, 2015).

In summary, several short motifs have been found in the ADIPOR1/2 N-terminal domains (Juhl et al., 2012). The part closest to TM1 has higher homology between the two proteins and motifs in that region are proposed to regulate exit from ER (Juhl et al., 2012), while the less homologous and more N-terminal sequences, in cell culture, confer differential expression on the cell membrane (Keshvari et al., 2013).

Endocytosis of ADIPOR1

Co-localization experiments in C2C12 myotubes show that Myc-tagged ADIPOR1 and Alexa 555-labeled adiponectin co-localizes with transferrin but not caveolin positive endosomes (Ding et al., 2009), indicating a role for the clathrin-mediated pathway of endocytosis (Le Roy and Wrana, 2005). Blocking of clathrin-mediated endocytosis in HEK293T cells by expression of a mutant version of EPS15, known to specifically inhibit this process (Benmerah et al., 1999), depletion of K^+ or growth in hypertonic medium (Larkin et al., 1986; Heuser and Anderson, 1989) can greatly reduce internalization of ADIPOR1 and the same effect can be seen on adiponectin upon K^+ depletion of C2C12 myotubes (Ding et al., 2009). Furthermore, in HeLa cells and C2C12 myotubes, endocytosed ADIPOR1 co-localizes with RAB5, a GTPase involved in clathrin-mediated endocytosis (McLauchlan et al., 1998), and a dominant negative version of RAB5 (Li and Stahl, 1993) can block endocytosis of ADIPOR1 (Ding et al., 2009). Pretreatment of C2C12 myotubes to deplete K^+, and thus prevent endocytosis of ADIPOR1, enhances the effect of stimulation with adiponectin on phosphorylation of AMPK (Ding et al., 2009).

As previously mentioned, ADIPOR1 is internalized upon adiponectin stimulation in HEK293 cells (Almabouada et al., 2013; Keshvari and Whitehead, 2015) and one could thus speculate on a role for the clathrin-mediated endocytosis pathway in down-regulation of the adiponectin-ADIPOR1 signaling response, a mechanism well described for receptor tyrosine kinases (Goh and Sorkin, 2013).

ADIPONECTIN

The discovery of adiponectin

The discovery of adiponectin was independently published by four different groups in 1995-1996, seven years before the cloning of the adiponectin receptors. The Lodish lab (Cambrigde, Massachusetts) published their report on an abundant serum protein with similarity to complement factor C1q that is secreted only by adipocytes and named the protein ACRP30 (**a**dipocyte **c**omplement-**r**elated **p**rotein of **30**kDA) (Scherer et al., 1995). The Spiegelman lab (Boston, Massachusetts) studied differentiation of adipocytes and found what they called the *AdipoQ* mRNA to be highly regulated during this process (Hu et al., 1996). The Matsubara lab (Osaka, Japan) isolated the most abundant mRNA from human adipose tissue and called it *apM1* (**a**di**p**ose **m**ost abundant gene transcript **1**) (Maeda et al., 1996) and finally the Tomita lab (Tokyo, Japan) isolated proteins from human plasma on the basis of binding to gelatin, and found what they called GBP28 (**g**elatin-**b**inding **p**rotein of **28**kDA) (Nakano et al., 1996). Today the name "adiponectin", coined by the Matsuzawa lab (Osaka, Japan) in 1999 (Arita et al., 1999), is almost exclusively used.

Adiponectin consists of an N-terminal signal sequence followed by a non-homologous region, 22 collagen repeats and a globular C-terminal domain (Figure 3A) with homology to complement factor C1q, the globular domain of collagen type VIII and X (Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996), HIB27 expressed during the summer months in hibernation capable animals (Scherer et al., 1995), multimerin (Nakano et al., 1996) and the brain specific protein cerebellin (Hu et al., 1996). Proteins with similar overall structure and size include lung surfactant protein and the mannose-binding lectin (Scherer et al., 1995; Hu et al., 1996). More recently adiponectin was grouped together with other homologous proteins into the CTRP family of **C**1q/**t**umor necrosis factor-α-**r**elated **p**roteins (Wong et al., 2004; Wong et al., 2008).

Adiponectin circulates in the blood as homotrimers (Scherer et al., 1995; Nakano et al., 1996) and higher order structures such as hexamers (Nakano et al., 1996), nonamers, dodecamers (Scherer et al., 1995) and octadecamers (Nakano et al., 1996). Two of the subunits in a trimer are bound together by disulfide bonds (Scherer et al., 1995) and disulfide bonds also hold the hexamers together, amino acid residue C39 being especially important in both cases (Pajvani et al., 2003; Tsao et al., 2003). Both the trimeric form of adiponectin and the high molecular weight forms (12-18 subunits) have been determined to be biologically active (Pajvani et al., 2003; Pajvani et al., 2004). Some reports speculate on the trimeric form being an active form of

adiponectin, or potentially an intermediate formed during an activation process (Pajvani et al., 2003), while others attribute the adiponectin effects to the high molecular weight forms (Pajvani et al., 2004).

The globular domain of adiponectin (amino acid 111-247) has been crystalized and the structure solved, revealing a trimeric structure of β sheets held together by hydrophobic interactions at the base, the overall structure being very similar to proteins of the tumor necrosis factor (TNF) family (Shapiro and Scherer, 1998). Furthermore freeze etch electron microscopy show that the adiponectin trimer is associated in a "ball-and-stick" shape while the hexamer conformation resembles the letter Y (Figure 3B) (Tsao et al., 2003).

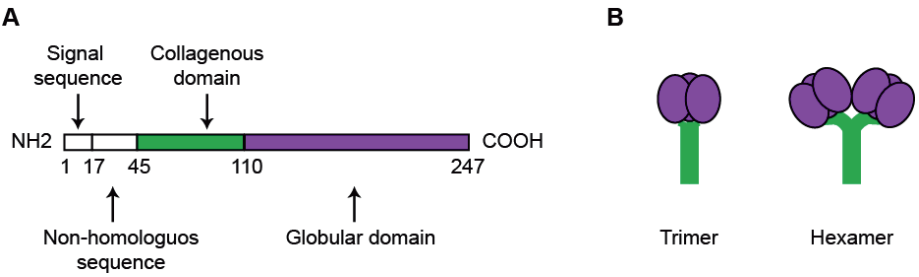

Figure 3. Structure of the adiponectin protein. A, The 247 amino acid protein adiponectin consists of an N-terminal signal sequence followed by a non-homologous sequence, a collagenous domain of 22 collagen repeats and a C-terminal globular domain (Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996). **B**, Adiponectin circulates in plasma as homotrimers, hexamers and higher order complexes where the collagenous domains group together to form a bouquet-like appearance (Scherer et al., 1995; Nakano et al., 1996; Shapiro and Scherer, 1998; Pajvani et al., 2003; Tsao et al., 2003).

The initial reports on adiponectin expression include findings of adiponectin being expressed only by adipocytes (Scherer et al., 1995; Hu et al., 1996; Maeda et al., 1996), with expression levels being highly induced during differentiation (Scherer et al., 1995; Hu et al., 1996). Adiponectin is secreted into the blood (Scherer et al., 1995; Hu et al., 1996; Nakano et al., 1996), and the secretion is increased upon stimulation with insulin (Scherer et al., 1995). Circulating levels can, in mice, account for 0.05% of total plasma protein (Scherer et al., 1995) and is higher in females then males in both mice and humans (Arita et al., 1999; Pajvani et al., 2003).

Examination of adiponectin mRNA levels in fat pads from *ob/ob* mice versus lean *ob/+* mice, human adipose tissue from obese and normal weight individuals (Hu et al., 1996) and *db/db* mice fed a HF diet (Yamauchi et al., 2001) shows that transcript levels are reduced in the obese condition (Hu et al., 1996). The mRNA levels correlate with decreased levels of plasma adiponectin in obese individuals (Arita et al., 1999), a circumstance also observed in type-2 diabetes (Hotta et al., 2000) and coronary heart disease (Ouchi et al., 1999). Dietary restriction (Berg et al., 2001) or treatment with rosiglitazone (a thiazolidinedione antidiabetic agent) increases plasma adiponectin levels in mice (Berg et al., 2001; Yamauchi et al., 2001).

T-cadherin has been identified as a receptor for high molecular weight forms of adiponectin (Hug et al., 2004). This GPI-anchored membrane protein (Ranscht and Dours-Zimmermann, 1991) is expressed at high levels in the cardiovascular system (Ivanov et al., 2001) and has been implicated in adiponectin mediated cardioprotection and revascularization (Denzel et al., 2010; Parker-Duffen et al., 2013; Parker-Duffen and Walsh, 2014). The role of T-cadherin in adiponectin signaling will not be further discussed in this thesis since the main focus lies on the PAQR protein family, to which the ADIPORs belong, and not on adiponectin *per se*.

An overview of the physiological effects of adiponectin is presented in Figure 4, and more thoroughly described in the following sections.

Physiological response to recombinant full-length adiponectin

Treatment of C2C12 myocytes with recombinant full-length adiponectin increases glucose uptake and FA oxidation *in vitro* (Yamauchi et al., 2002). In *in vivo* experiments using WT, *ob/ob*, non-obese diabetic or streptozotocin-treated mice, intraperitoneal injection of recombinant full-length adiponectin mediates a decrease in plasma glucose levels 4 h post injection, with a prolonged duration of the effect in the diabetes models as compared to WT, and without changes in insulin levels (Berg et al., 2001). The later can be explained by experiments in primary rat hepatocytes showing that adiponectin potentiates the repressive effect of insulin on hepatic glucose production (Berg et al., 2001), and pancreatic euglycemic clamp studies showing that adiponectin treatment do suppress hepatic glucose production with a concomitant decrease in mRNA levels of gluconeogenic enzymes (Combs et al., 2001).

Continuous infusion of recombinant adiponectin can significantly improve hyperglycemia and hyperinsulinemia in *db/db* mice or WT mice on a HF diet (Yamauchi et al., 2001). Food intake is higher in adiponectin-treated WT mice on a HF diet but the treatment nevertheless tends to decrease the gain of

weight and the size of white adipose tissue depots associated with HF diet (Yamauchi et al., 2001).

On the subject of atherosclerosis, preincubation of human aortic endothelial cells (HAEC) with adiponectin (full-length produced in *E. coli*) protects against TNFα induced expression of adhesion proteins, VCAM-1, E-selectin and ICAM-1, and adhesion of THP-1 cells (human monocytic cell line) to the HAECs, thus modulating the proinflammatory response (Ouchi et al., 1999).

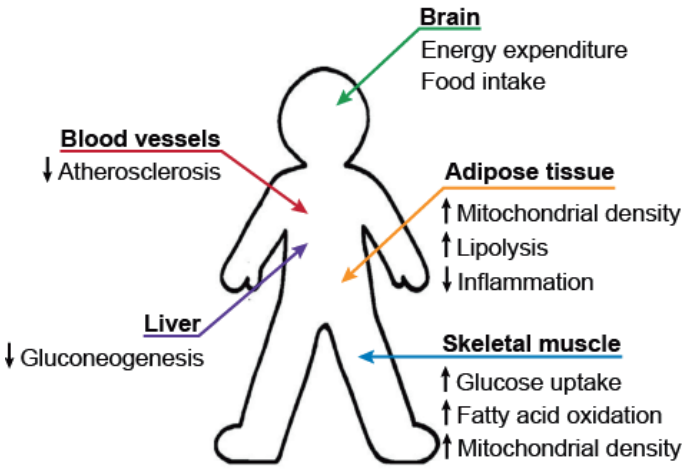

Figure 4. Physiological response to adiponectin. Several physiological responses to adiponectin are known, including: increased glucose uptake, fatty acid oxidation (Fruebis et al., 2001; Yamauchi et al., 2002) and mitochondrial density in skeletal muscle (Iwabu et al., 2010), decreased gluconeogenesis in liver (Berg et al., 2001; Combs et al., 2001), decreased atherosclerotic lesions in blood vessels (Yamauchi et al., 2003b), increased mitochondrial density and lipolysis in subcutaneous adipose tissue (Kim et al., 2007; Asterholm and Scherer, 2010) and a less well understood role in regulation of energy expenditure and food intake via the hypothalamus (Qi et al., 2004; Kubota et al., 2007; Coope et al., 2008).

Physiological response to recombinant globular adiponectin

A smaller protein encompassing the C-terminal globular domain of adiponectin can be detected at low levels in human plasma (Fruebis et al., 2001; Yamauchi et al., 2001; Maeda et al., 2002) and may potentially be generated through cleavage of full-length adiponectin by leukocyte elastase (Waki et al., 2005). Treatment of C2C12 myocytes, or isolated soleus and EDL muscle, with globular adiponectin results in increased levels of oxidation of the assayed substrate oleate (C18:1) (Fruebis et al., 2001) and

increased mRNA levels of several genes involved in handling of FA (transport, combustion, energy dissipation) (Yamauchi et al., 2001). *In vivo*, injection of globular adiponectin into WT mice at the time of a force-fed HF/sucrose meal (with consequent injections at 45 and 105 minutes) blunts the increase in free FA, glucose, and to a smaller extent also triglycerides, normally seen in the plasma, without mediating significant changes in insulin levels as compared to control-injected animals (Fruebis et al., 2001), and can also alleviate hyperglycemia and hyperinsulinemia in a lipoatrophic PPAR$\gamma^{+/-}$ mouse model (Yamauchi et al., 2001). Moreover, treatment of HF fed obese mice with globular adiponectin over a period of 10-16 days reduces body weight despite continued HF feeding (Fruebis et al., 2001).

In some of the studies on globular adiponectin, the full-length form had less potent, or no effect on the parameters assayed (Fruebis et al., 2001; Yamauchi et al., 2001) however, note that in these studies, the full-length and globular adiponectin used were produced in *E. coli.* Other reports have seen no effect on plasma glucose levels from bacterially-produced globular adiponectin and conclude that mammalian expression systems, such as overexpression in HEK293T cells and purification of protein from culture media, is needed to obtain a functional full-length protein (Berg et al., 2001). It is known that endotoxins, often found as contaminants in batches of recombinantly produced proteins, can reduce blood glucose levels in mice (Harizi et al., 2007) and that contaminating glycerol can cause phosphorylation of ACC. Using the later as a readout for AMPK activity could thus potentially be problematic (Tullin et al., 2012). The whole publication by Tullin *et al.* 2012 is dedicated to the production and study of recombinant adiponectin, and while this group, based on Novo Nordisk A/S, produces a full-length human adiponectin in CHO cells that can mediate an anti-inflammatory phenotype, it can not replicate the phosphorylation of AMPK *in vitro* and lowering of plasma glucose *in vivo* (Tullin et al., 2012) as originally published by the Kadowaki (Yamauchi et al., 2002) and Scherer (Berg et al., 2001) groups respectively. The study by Tullin *et al.* documents the difficulties and pitfalls related to producing active recombinant adiponectin, and casts a shadow on published studies that rely heavily on such reagents.

Adiponectin has been detected in cerebrospinal fluid (Qi et al., 2004; Kubota et al., 2007), and the adiponectin receptors are expressed in the brain (Yamauchi et al., 2003a; Qi et al., 2004). Injection of adiponectin (mammalian, globular or full-length) into the lateral cerebral ventricles (i.c.v) of WT mice mediates a transient weight loss, during relatively normal levels of food intake, accompanied by increased oxygen consumption, thus indicating an increase in energy expenditure (Qi et al., 2004). Adiponectin

injected i.c.v. does not result in increased levels in plasma meaning that the effect is via the brain, not directly on peripheral tissue (Qi et al., 2004), while an increase in adiponectin in cerebrospinal fluid can be detected after systemic injection (Qi et al., 2004; Kubota et al., 2007). Performing the i.c.v. treatment on agouti mice, which are known to be resistant to leptin, partly through deficient melanocortin-3 and -4 signaling (Marsh et al., 1999; Butler et al., 2000; Zhang et al., 2005), does not confer any effect (Masaki et al., 2003; Qi et al., 2004) indicating an important role for signaling via this pathway, known to regulate feeding behavior, thermogenesis and glucose metabolism (Marsh et al., 1999; Butler et al., 2000). Yet other studies pinpoints ADIPOR1, and not ADIPOR2, in mediating the effects of adiponectin in the brain (Kubota et al., 2007; Coope et al., 2008), but also report on opposite effects on food intake with adiponectin causing a decrease (Coope et al., 2008) or an increase of food intake together with a decrease in energy expenditure (Kubota et al., 2007).

Adiponectin mouse models

The first studies of adiponectin knockout mice were published in 2002 (Kubota et al., 2002; Maeda et al., 2002). These mice develop with normal body weight, plasma insulin levels (Kubota et al., 2002; Maeda et al., 2002) and food intake (Maeda et al., 2002). The study by Kubota *et al.* reports on increased levels of plasma glucose in the oral glucose tolerance test, as well as reduced ability to remove glucose from plasma in the insulin tolerance test in adiponectin knockout mice at six weeks of age. Maeda *et al.* reports a normal performance in glucose and insulin tolerance tests but delayed removal of free FA from plasma (upon Intrafat injection) in the knockout mice fed normal chow. However, when fed a HF/high sucrose diet the Maeda *et al.* adiponectin knockout mice develop hyperglycemia and hyperinsulinemia with increased levels of free FA in plasma, but with retention of normal body weight and adiposity, and these effects can be ameliorated by adenoviral production of adiponectin.

To study the possible role of adiponectin in atherosclerosis, inflammation can be induced in the femoral artery by a cuff: such a treatment results in increased intimal thickness in adiponectin knockout mice as compared to WT, indicating a protective role for adiponectin *in vivo* (Kubota et al., 2002).

Chronically elevated levels of adiponectin have been studied through expression of low levels of ΔGly-adiponectin in mouse. This mutant form lacks 13 of the 22 collagen repeats and is expressed from the aP2 adipose specific promoter. This results in more efficient secretion of endogenous adiponectin, after blocking of the adiponectin degradation cycle by trimers formed from a mix of endogenous and ΔGly-adiponectin, and a 3-fold

increase in endogenous adiponectin in plasma (Combs et al., 2004). The ΔGly-adiponectin transgenic female WT or *ob/ob* mice have increased body weight and adiposity but normal food intake, improved clearance of triglycerides after feeding and increased insulin sensitivity with decreased gluconeogenesis in liver (Combs et al., 2004; Kim et al., 2007; Asterholm and Scherer, 2010). The increased adiposity consists of subcutaneous fat with small adipocyte size, increased sensitivity to lipolytic stimuli (Kim et al., 2007; Asterholm and Scherer, 2010), increased expression of mitochondrial markers (Combs et al., 2004; Kim et al., 2007; Asterholm and Scherer, 2010) and amount of mitochondria (Asterholm and Scherer, 2010) as well as decreased macrophage infiltration and expression of inflammatory markers (Kim et al., 2007).

A transgenic mouse model overexpressing globular adiponectin exhibits normal plasma glucose and insulin levels as well as body weight comparable to WT. Feeding the transgenic mice with a HF diet does not cause changes in their body weight, as compared to WT, although the transgenic mice display several beneficial responses, including reduced levels of glucose and insulin in plasma upon glucose and insulin tolerance tests as well as decreased triglyceride content in muscle and liver. Crossing the transgene into an *ob/ob* background results in increased food intake with no change in obesity levels, thus indicating elevated energy expenditure, and an increase in FA oxidation can be found in skeletal muscle but not in liver. The transgene also increases insulin sensitivity and glucose tolerance in *ob/ob* mice on a HF diet. Crossing the transgene instead into *ApoE*$^{-/-}$ background reduces formation of atherosclerotic lesions (Yamauchi et al., 2003b).

In summary, the studies of the different adiponectin mouse models shows that while the adiponectin knockout mice are insulin resistant on a HF diet (Maeda et al., 2002) and develop increased intimal thickness upon atherosclerotic inflammation (Kubota et al., 2002), the mouse models with increased secretion of endogenous adiponectin or overexpression of globular adiponectin, both in *ob/ob* genetic background, display increased insulin sensitivity (Yamauchi et al., 2003b; Combs et al., 2004; Kim et al., 2007; Asterholm and Scherer, 2010) with increased mitochondrial content and decreased inflammation in adipose tissue (Kim et al., 2007; Asterholm and Scherer, 2010), or reduced progression of atherosclerosis in the *ApoE*$^{-/-}$ genetic background (Yamauchi et al., 2003b) respectively.

Adiponectin mimetics

The first ADIPOR agonist, the antifungal tobacco protein osmotin, was found through its interaction with the yeast PAQR protein IZH2 and shown to also interact with ADIPOR1/2 and thereby increase phosphorylation of AMKP in

C2C12 myocytes (Narasimhan et al., 2005). More recently, several screens for identification of adiponectin mimetics, i.e. ADIPOR1/2 agonists, have been performed. Otvos *et al.*, with the aim of finding receptor agonists for cancer treatment, identified a fragment of adiponectin, amino acids 149-166, as the ADIPOR1/2 activating site and generated a 10 amino acid peptide mimetic, ADP 355, which can increase phosphorylation of AMPK in an ADIPOR1/2-dependent manner, and exert cytostatic effects, in MCF-7 breast cancer cells (Otvos et al., 2011). The Kadowaki lab, who earlier cloned the adiponectin receptors, published their agonist, called AdipoRon, in 2013. AdipoRon was identified through screening of libraries of small molecules for increased ADIPOR-dependent phosphorylation of AMPK in C2C12 myocytes, and the compound can mediate many of the adiponectin effects *in vivo* such as increased insulin sensitivity, increased mitochondrial content in skeletal muscle, increased PPARα activity in liver and reduced inflammation in white adipose tissue (Okada-Iwabu et al., 2013). Furthermore, Singh *et al.* identified their previously characterized osteoanabolic compound 6-*C*-β-D-glucopyranosyl-(2S,3S)-(+)-3',4',5,7-tetrahydroxyflavonol (GTDF) (Khan et al., 2013) as an ADIPOR1 agonist after noting substantial overlap in physiological response between GTDF and adiponectin treatment (Singh et al., 2014).

Different screening strategies have also been developed, including a luciferase-based protocol for detection of ADIPOR-ligand interaction in *S. cerevisiae* (Aouida et al., 2013) and a fluorescence polarization-based method (Lea and Simeonov, 2011) assaying competition in binding to the adiponectin receptors using a FITC labeled version of the adiponectin peptide mimetic, ADP 355, mentioned above. The later strategy was used to screen a library of natural products and identified three compounds from the plant *Arctium lappa* that induce phosphorylation of AMPK and display cytostatic effects in the MDA-MB-231 human breast adenocarcinoma cell line (Sun et al., 2013).

Adiponectin/AdipoR polymorphisms

The adiponectin locus on 3q27 has been identified as a susceptibility locus for type 2-diabetes following genome wide scans in French whites (Vionnet et al., 2000), Indo-Mauritians (Francke et al., 2001) and Japanese (Mori et al., 2002) as well as for metabolic syndrome in US Caucasians (Kissebah et al., 2000).

Several single nucleotide polymorphisms (SNPs) in the adiponectin gene, promoter and 3'UTR have been identified, and while one should be aware that there are discrepancies in the literature, some of the SNPs have been associated with plasma adiponectin levels or insulin resistance (Menzaghi et

al., 2007). The Kadowaki lab initially reported the SNPs +45 and +276, or most probably a combination of the two, to be associated with type 2-diabetes, and SNP +276 to be associated with insulin resistance in a Japanese population (Hara et al., 2002). More recently an association between plasma adiponectin levels and SNPs in the adiponectin promoter (Hivert et al., 2008; Henneman et al., 2010) and 3' UTR has been found (Hivert et al., 2008).

Waki *et al.* in 2003 published experimental assessments of the following non-synonymous mutations previously found in the adiponectin gene G84R, G90S, R92X, Y111H, R112C, I164T, R221S and H241P (Takahashi et al., 2000; Hara et al., 2002; Kondo et al., 2002; Vasseur et al., 2002) out of which the G84R, G90S, R112C and I164T mutations has been associated with low plasma adiponectin levels (Takahashi et al., 2000; Kondo et al., 2002; Vasseur et al., 2002) and the G84R, G90S, Y111H and I164T mutations with type 2-diabetes (Kondo et al., 2002; Vasseur et al., 2002; Hivert et al., 2008). The R112C and I164T mutations have been shown to confer plasma adiponectin levels of ~2 µg/ml compared to ~7 µg/ml in subjects with no mutations (Kondo et al., 2002) and the minor allele C of the Y111H mutation has an increased hazard ratio of 1.94 (p=0.01) for type 2-diabetes (Hivert et al., 2008). The G84R and G90S mutations perturb formation of high molecular weight adiponectin complexes, and the R112C and I164T lack all forms of multimers when expressed in NIH-3T3 fibroblast and analyzed by non-reducing and non-heat-denaturing SDS-PAGE. The lack of complex formation may hinder secretion since the R112C and I164T versions cannot be detected in the growth media, while monomers are present in cell lysates (Waki et al., 2003). A similar but more recent study identified another set of three non-synonymous mutations, R55H, R112H and R131H, out of which the mutations at position 55 and 131 were only found in type 2-diabetic patients and not in controls. After expression in HEK239T cells the R112H and R131H mutations prevent secretion into the culture media also of WT adiponectin, while the R55H mutation confers lower levels of high molecular weight complexes (Jungtrakoon et al., 2011).

A study investigating SNPs in adiponectin, as well as the adiponectin receptors, found an association between several SNPs in adiponectin and serum adiponectin levels as well as three SNPs associated with type 2-diabetes, while no association was found for the *AdipoR* SNPs investigated (Peters et al., 2013). As with the adiponectin polymorphisms, there are many discrepancies in the literature on the importance of SNPs in the *AdipoR* genes, and low reproducibility between studies (Crimmins and Martin, 2007). Nevertheless, several reports on associations between *AdipoR* promoter/gene SNPs and type 2-diabetes (Mather et al., 2012; Jin et al., 2014) or the level of insulin sensitivity (Stefan et al., 2005; Kim et al., 2009) are available.

Recently a homozygous *AdipoR1* frameshift, and most probably loss of function mutation was found in a patient with syndromic retinitis pigmentosa (Xu et al., 2016), a group of disorders with abnormalities in the retinal photoreceptors or retinal pigment epithelium that concludes with progressive visual loss (Ferrari et al., 2011) and, in a separate study, an intronic *AdipoR1* SNP was associated with age-related macular degeneration (Kaarniranta et al., 2012). ADIPOR1 is expressed in photoreceptor cells (Lin et al., 2013; Rice et al., 2015; Xu et al., 2016) and retinal pigment epithelium of mice (Lin et al., 2013; Xu et al., 2016) as well as in human retinal pigment epithelium (Lin et al., 2013). *AdipoR1*, but not adiponectin, knockout mice display a flecked retinal syndrome and progressive degeneration of the retina with only a thin layer of photoreceptors remaining at 33 weeks of age (Rice et al., 2015). An essential omega-3 (ω-3) FA, docosahexaenoic acid (DHA, 22:6n-3), is highly enriched in the phospholipids of the outer segment cell membrane in retinal photoreceptor cells (Bazan et al., 2011). Systemic delivery of labeled DHA to WT and *AdipoR1*$^{-/-}$ mice and later examination of DHA content in retina shows that ADIPOR1 is important for DHA uptake, and in *AdipoR1*$^{-/-}$ retinas the levels of DHA and its elongation products, the very long chain polyunsaturated fatty acids (PUFA), are greatly reduced (Rice et al., 2015).

C. ELEGANS LIPID METABOLISM

The following paragraphs will encompass a very short summary of the current knowledge on the *C. elegans* lipid metabolism relevant for this thesis. Thorough and recent reviews on the topic can be found in (Watts, 2009; Zheng and Greenway, 2012; Srinivasan, 2015; Witting and Schmitt-Kopplin, 2016).

The main tissue for fat storage in *C. elegans* is the intestine, where triglycerides (Figure 5A) are stored in lipid droplets, but hypodermal lipid stores also exists (Kimura et al., 1997), and the β-oxidation enzymes for utilization of fat stores for energy production are expressed in both tissues (Srinivasan et al., 2008). The FA synthesis pathway (Figure 5B) from acetyl-CoA is present in *C. elegans* and the nematode can synthesize ω-3 as well as ω-6 FA (Watts and Browse, 2000; Napier and Michaelson, 2001; Watts and Browse, 2002). The most well-known and researched *C. elegans* desaturase enzymes are the Δ9 desaturases FAT-5, FAT-6 and FAT-7, enzymes with the function of introducing the first double bond into a SFA of 16 (FAT-5) or 18 (FAT-6, -7) carbons (Watts and Browse, 2000; Brock et al., 2006, 2007). Experiments using ^{13}C-labeled bacteria have shown that ~7% of the *C. elegans* total lipid C16:0 is derived from FA synthesis, and the remaining >90% comes from the bacterial diet. The synthesized fraction is larger for

mono-, and polyunsaturated fatty acids with up to 19% originating from synthesis (Perez and Van Gilst, 2008). Thus, the standard laboratory *E. coli* OP50, or other bacterial diets, have a large impact on the *C. elegans* lipidome (Perez and Van Gilst, 2008; Brooks et al., 2009). Exceptions to this being the monomethyl branched-chain fatty acids (mmBCFA), which are exclusively produced within the nematode (Kniazeva et al., 2004; Perez and Van Gilst, 2008) and the FA C18:0 (stearic acid), C18:1n-9 (oleic acid) and C18:2n-6 (linoleic acid) which are not present in the standard laboratory diet and thus needs to be synthesized by the worms through modification of C16:0 (Watts and Browse, 2002; Perez and Van Gilst, 2008).

The fat storage in *C. elegans* is governed by several pathways, including the homolog of sterol regulatory element-binding protein SREBP, SBP-1 (McKay et al., 2003; Yang et al., 2006; Nomura et al., 2010), the insulin-like signaling pathway, as well as the TGF-β (**t**ransforming **g**rowth **f**actor-**β**) signaling pathway (Kimura et al., 1997). β-oxidation of FA is regulated by a worm homolog of PPARα/HNF4 (**h**epatocyte **n**uclear **f**actor **4**), NHR-49 (Van Gilst et al., 2005a), and serotonin signaling (Srinivasan et al., 2008). The degree of conservation in fat metabolism between mammals and *C. elegans* is evident at several levels, including: 1) the *C. elegans* SBP-1 is regulated by phosphatidylcholine (PC) levels, just as the mammalian SREBP1 (Walker et al., 2011); 2) NHR-49 is sequence-wise a homolog of HNF4 but acts as a functional homolog of the mammalian PPARα (Bertrand et al., 2004; Van Gilst et al., 2005a; Van Gilst et al., 2005b; Atherton et al., 2008); 3) the insulin-like signaling pathway is extremely conserved between *C. elegans* and mammals, from receptor to downstream targets such as DAF-16/FOXO (Kimura et al., 1997; Jones and Ashrafi, 2009); and 4) over 70% of human lipid metabolism genes have orthologs in *C. elegans* (Zhang et al., 2013).

Synthesis of phospholipids (Figure 5C) has not been extensively studied in *C. elegans*. However, enzymes of the Kennedy pathway for PC synthesis from choline, and of the phosphobase methylation pathway for PC synthesis from serine and ethanolamine (Vance and Vance, 2004) are present in the nematode (Figure 5D) (Friesen et al., 2001; Gee and Kent, 2003; Lochnit and Geyer, 2003; Palavalli et al., 2006; Brendza et al., 2007; Schwudke et al., 2007; Li et al., 2011; Walker et al., 2011), as well as the enzyme required for the synthesis of phosphatidylserine from PC by head group exchange (Nilsson et al., 2011). Recently, the rate of FA turnover in *C. elegans* was determined to be almost twice as high in the membrane phoshpolipids (4.5%) as in the triglycerides (2.7%). At least 60% of the membrane phospholipids are replaced every 24 h and the replacement rate is reduced upon Δ9 desaturase RNAi, with FAT-6 being the main contributor, indicating that

these enzymes, but not other desaturases, i.e. FAT-1, -2, -3 and -4, are important regulators of this process. The fastest dynamics is seen in the phosphatidylethanolamine (PE) lipid fraction, for FA as well as head group remodeling, and one can therefore conclude that it can be important to assay each lipid fraction separately when attempting to monitor phospholipid metabolism or composition (Dancy et al., 2015).

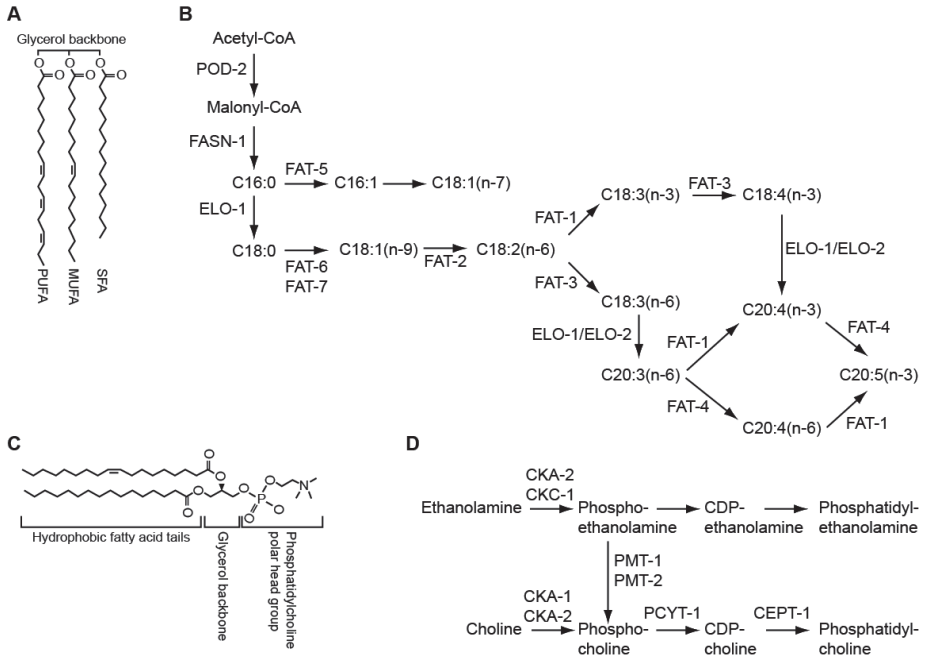

Figure 5. The structure of triglycerides and phospholipids, and synthesis pathways in *C. elegans*. **A**, Schematic drawing of a triglyceride. **B**, The FA synthesis pathway in *C. elegans* with FA products and enzymes indicated, adapted from (Watts, 2009; Witting and Schmitt-Kopplin, 2016). **C**, Schematic drawing of a phospholipid and **D**, synthesis pathway for the main classes of phospholipids in *C. elegans*, adapted from (Walker et al., 2011; Witting and Schmitt-Kopplin, 2016). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid. Fatty acids are denoted by number of carbons (C18), number of double bonds (C18:2) and the position of the first double bond as counted from the methyl end (C18:2n-6).

WT *C. elegans* do, in the laboratory setting, grow at temperatures ranging from 12 to 25°C (Sulston and Hodgkin, 1988), with 20°C being the normal laboratory condition. The homeoviscous adaptation response has previously been studied in this organism through examination of membrane lipid

composition of WT *C. elegans* grown at 15, 20 and 25°C (Tanaka et al., 1996) or 10 and 25°C (Murray et al., 2007). These studies show that the fraction of SFA is markedly reduced at 10 or 15°C compared to 20 or 25°C, mainly in the PC fraction while the PUFA 18:2n-6 (linoleic acid) and 20:5n-3 (eicosapentaenoic acid) are increased in both PC and PE fractions (Tanaka et al., 1996; Murray et al., 2007), which together makes up more than 80% of *C. elegans* phospholipids (Satouchi et al., 1993). In accordance with this, double mutants of the *C. elegans* Δ9 desaturases (Watts and Browse, 2000) display reduced survival at 10°C (Brock et al., 2007; Murray et al., 2007), and the most severely affected double mutant in terms of FA composition, *fat-6;fat-7* also at 15°C (Brock et al., 2007) while the triple mutant *fat-5;fat-6;fat-7* is lethal at all temperatures (Brock et al., 2006). Recently, the PC/PE ratio was determined for WT and the *fat-6;fat-7* double mutant revealing a ratio of 1.51 in WT and 1.13 in the mutant, potentially a compensatory response of the *fat-6;fat-7* mutant to maintain membrane fluidity despite severely reduced levels of UFA (Shi et al., 2013).

Response to glucose supplementation

Generally *C. elegans* tolerates very high amounts of glucose supplemented in the culture plates with no effect seen on brood size for concentrations up to 250 mM (Mondoux et al., 2011). However, glucose supplementation has been shown to induce apoptosis (Choi, 2011) and is known to reduce lifespan at concentrations ranging from 2.8 mM (0.05%) to 111 mM (2%) (Schulz et al., 2007; Lee et al., 2009). The lifespan reduction caused by 111 mM glucose is dependent on the transcription factors DAF-16 (FOXO), HSF-1 (heat shock transcription factor) and a downstream target, the aquaporin glycerol channel AQP-1 (Lee et al., 2009). Conversely, knockdown of the glycolytic enzyme glucose 6-phosphate isomerase, *gpi-1*, the only known functional *C. elegans* glucose transporter, *fgt-1*, or treatment with the glucose analog 2-deoxy-D-glucose, which can not be metabolized and thus blocks glucose metabolism (Sols and Crane, 1954), confers an extension of lifespan (Hansen et al., 2005; Schulz et al., 2007; Feng et al., 2013). The longevity and high temperature constitutive dauer phenotype of *daf-2* (insulin/IGF-1 receptor) are suppressed by glucose supplementation, indicating that glucose activates the *C. elegans* insulin-like signaling pathway in a way that bypasses the insulin/IGF-1 receptor (Lee et al., 2009). A shortening of the lifespan of WT worms can also bee seen if supplementing the worm plates with glycerol, and glycerol, as well as glucose levels are increased in glucose fed worms, but not vice versa, indicating that the effect of glucose on lifespan could be via conversion to glycerol (Lee et al., 2009).

The reduction in lifespan seen upon glucose supplementation can be further enhanced by RNAi against *sbp-1* or the mediator subunit *mdt-15* (Lee et al.,

2015), where MDT-15 is known to work together with SBP-1 as well as NHR-49 (Taubert et al., 2006; Yang et al., 2006). Conversely overexpression of SBP-1, a gain of function allele of *mdt-15* or a loss of function mutation in S-adenosyl methionine synthetase, *sams-1* (Hansen et al., 2005), known to activate SBP-1 (Walker et al., 2011), can suppress the defect (Lee et al., 2015). Expression of the *C. elegans* Δ9 desaturases is induced by glucose feeding (Nomura et al., 2010; Lee et al., 2015), and this treatment also increases levels of fat storage (Schulz et al., 2007; Garcia et al., 2015; Lee et al., 2015). Lee *et al.* assayed FA composition in total lipid extracts and found that glucose supplementation mediates accumulation of SFA, which is further enhanced by knockdown of *sbp-1* or *mdt-15*, and that the glucose-mediated reduction in lifespan is greatly enhanced by co-supplementation with SFA. In their study Lee *et al.* do not connect the increased SFA content upon glucose supplementation to the possibility of reduced membrane fluidity. Rather, they conclude that dihydroxyacetonephosphate (DHAP), a metabolite produced during glycolysis and that mediates a reduction in lifespan similar to that of glucose, is a potential cause; this is consistent with their observation that RNAi against the enzymes required for DHAP production, i.e. the aldolases *aldo-1* and *-2*, is protective with respect to the reduction in lifespan caused by glucose supplementation (Lee et al., 2015).

The study of glucose by Lee *et al.* 2009, as well as the study of 2-deoxy-D-glucose by Schulz *et al.* 2007, both confirmed that the glucose effect on lifespan is directly on the worm, and not via the *E. coli* food source, by using metabolically arrested bacteria or by rendering the *E. coli* deficient for glucose uptake (Schulz et al., 2007; Lee et al., 2009). However, the effect of glucose on anoxia survival, where a concentration of 28 mM reduces the survival after 24 h of anoxia from almost 100% down to around 10%, has been shown to depend on the bacterial diet: the standard OP50 and HT115 diets both confer the glucose effect, while the effect is quite strongly suppressed when a OP50 strained deficient in glucose uptake (Lee et al., 2009) is used as food source (Garcia et al., 2015). The effect of 28 mM glucose on anoxia survival is suppressed by a *daf-2* mutation, and can be re-suppressed by mutation of *daf-16* or by RNAi of several enzymes of the FA synthesis pathway, including the Δ9 desaturases and the ceramide synthase *hyl-2*. Conversely, the double mutant *fat-6;fat-7* shows an increased sensitivity to 1 day of anoxia, and the ceramide synthase mutant *hyl-2* to 2 days of anoxia on normal growth media (Garcia et al., 2015).

HOMEOVISCOUS ADAPTATION

The term homeoviscous adaptation was coined in 1974, and at that time it referred to the process where *E. coli* increase the proportion of SFA in their

phospholipids upon increasing cultivation temperature in order to maintain constant membrane fluidity (Sinensky, 1974). However, a homeoviscous adaptation response is needed in all poikilotherm organisms to allow growth in an environment where the temperature is not constant. Lowering of environmental temperature without regulation of membrane fluidity causes transition of membranes from the fluid phase to a more rigid gel phase, which in turn increases the permeability for cations and water and affects the localization and function of integral and membrane-associated proteins (Hazel, 1995).

To achieve membrane fluidity homeostasis and counter rigidity, poikilotherm organisms remodel their membrane lipid composition, increasing the proportion of UFA as well as PE head groups when temperature decreases (Marr and Ingraham, 1962; Pruitt, 1988). Some organisms also incorporate branched-chain FA into their membrane lipids to maintain fluidity (Suutari and Laakso, 1992). The homeoviscous adaptation response has been studied extensively in microorganisms but also in fish (Hazel, 1984), where a response to high hydrostatic pressure also comes into play and where membrane fluidity is increased in response to increased pressure (Avrova, 1984). Recent studies in the ectotherm *D. melanogaster* show that this organism also changes the proportion of UFA and the PE to PC ratio to maintain membrane homeostasis upon temperature changes (Overgaard et al., 2008; Cooper et al., 2014). It is interesting to note that neither *D. melanogaster* (Sang and King, 1961) nor *C. elegans* (Hieb and Rothstein, 1968; Merris et al., 2003) has the capacity to synthesize cholesterol, a lipid that greatly influences the fluidity of membranes in mammals (Yeagle, 1985).

Mammals, being homotherms do not need to adjust membrane fluidity upon changing environmental temperature. The impact of cholesterol on cell membranes is bi-functional: higher levels of cholesterol render the membrane less flexible while simultaneously preventing tight packaging within the membrane, which promotes fluidity (Yeagle, 1985). The composition of lipids and cholesterol is known to vary widely among the organelles of mammalian cells, and this property must therefore be tightly regulated (van Meer et al., 2008; Holthuis and Menon, 2014). SREBP, residing in the ER has an important function in regulation of FA and cholesterol synthesis (Brown and Goldstein, 1997), and the ER unfolded protein response via IRE-1, is involved in sensing of the SFA/UFA ratio and can trigger synthesis of unsaturated lipids (Schuck et al., 2009; Deguil et al., 2011; Surma et al., 2013). Mammalian SREBP2 is regulated by ER sterol levels (Brown and Goldstein, 1997; Radhakrishnan et al., 2008) via the SREBP cleavage-activating protein, SCAP, while another SREBP isoform, SREBP1 instead

respond to changes in PC levels (Walker et al., 2011), and both forms can be activated by glucose (Cheng et al., 2015). The rate limiting enzyme in PC synthesis, cytidine triphosphate:phosphocholine cytidylyltransferase (CCT or PCYT-1 in *C. elegans*) is regulated by association to membranes (Clement and Kent, 1999). CCT binds to membranes with high proportion of conically shaped lipids, PE or diacylglycerol, and this increases substrate affinity, and thus activity, resulting in increased PC synthesis (Attard et al., 2000; Lee et al., 2014), and CCT can therefore by thought of as a mammalian sensor of membrane composition.

Despite the fact that homeoviscous adaptation is an important and well-known phenomenon, the mechanisms by which membrane fluidity *per se* is sensed and regulated are not well-known. Such a sensor and regulator of membrane fluidity, DesK, has been found in *Bacillus subtilis* and will be discussed in the next section. From our own work we conclude that in *C. elegans* the adiponectin receptor homolog PAQR-2 may be a regulator of membrane fluidity, while Cooper *et al.* 2014 speculate that SREBP, known to regulate PE synthesis (Dobrosotskaya et al., 2002), may play a similar role in *D. melanogaster* (Cooper et al., 2014).

The DesK regulator of membrane fluidity

The *B. subtilis desK* (histidine kinase) and *desR* (DNA binding response regulator) genes were initially described in 2001 and regulate the transcription of the desaturase gene, *des*. Upon high cultivation temperature (37°C) DesK acts as a phosphatase to dephosphorylate DesR, while at a lower cultivation temperature (25°C) DesK instead phosphorylates and activates DesR to induce expression of *des* (Aguilar et al., 2001). More specifically the DesK C-terminal domain has autokinase activity, phosphorylates itself on H188 and the phosphate group is further transferred to D54 of DesR (Albanesi et al., 2004).

The actual sensing of temperature or membrane fluidity has been attributed to the transmembrane domain of DesK, since expression of the cytosolic fragment activates *des* transcription at 37°C as well as 15°C (Hunger et al., 2004). DesK has a transmembrane domain consisting of 5 TM helixes with an extracellular N-, and intracellular C-terminus. Deletion of the most N-terminal TM helix results in very high levels of expression of *des* at 37°C as well as 25°C, indicating that the temperature-sensing capacity is lost, while an engineered variant of DesK with one TM domain, the N-terminal part of which is identical to TM1 and the C-terminal part identical to TM5 and connected to the intracellular C-terminus, results in activities very similar to that of the WT DesK. The most N-terminal part of TM1 is unusual in amino acid composition: while transmembrane helixes usually consist of

hydrophobic amino acid residues that match the hydrophobic core of the cell membrane (Killian and von Heijne, 2000), this helix contains three hydrophilic amino acids (glutamine, lysine, asparagine) close to the extracellular surface (Cybulski et al., 2010). A hypothesis has been proposed where this inbuilt instability center would be exposed to the extracellular environment and hydrated at high temperature, which causes increased fluidity and a thinning of the membrane, but dehydrated and buried in the membrane at low temperature, where the membrane is more rigid and thicker (Pan et al., 2008; Cybulski et al., 2010; Szekely et al., 2011). This proposed fluidity-dependent conformational change likely regulates the phosphatase/kinase activity (Cybulski et al., 2010). In support of this hypothesis, mutation of the hydrophilic amino acids to hydrophobic leucine or alanine residues abolishes sensing of temperature or, rather, of lipid bilayer thickness (Cybulski et al., 2010). Assays of DesK activity in proteoliposomes, with well defined lipid composition, and *in vivo* experiments, modulating membrane lipid composition to contain short chain FA or long chain FA, further supports the hypothesis of DesK reacting to membrane thickness (Martin and de Mendoza, 2013; Porrini et al., 2014).

RESULTS AND DISCUSSION

The starting point of our research on PAQR proteins was influenced by an awareness of several discrepancies in the literature on adiponectin and its receptors, including 1) the viability of the Kadowaki lab *AdipoR* double knockout mice contrasted with the lethality of the Deltagen double knockouts; 2) the opposing phenotypes seen from the two published *AdipoR2*$^{-/-}$ strains; and 3) the difficulties in production of recombinant adiponectin, and controversies regarding their biological activity. Our experimental study of PAQR proteins in *C. elegans* was based on the belief that the use of a simpler model system, and a research strategy relying primarily on forward genetics, would have the opportunity to yield new, unbiased knowledge of the signaling pathway downstream of this class of receptors, and which may be conserved with mammals.

One last note regarding adiponectin should be made before discussing the results of the thesis work. Sequence-wise, there is no putative adiponectin homolog in *C. elegans*. However, the possibility of ligand-dependent activation of the *C. elegans* ADIPOR homologs should not be excluded since conservation of a ligand at the level of an important epitope for receptor activation, in combination with remodeling of the remaining protein domains, on an evolutionary time scale, may have produced a functional PAQR ligand in *C. elegans* that is not recognizable as an adiponectin homolog.

A preview of the results and discussion section: In Paper I, we identify PAQR proteins in *C. elegans* and characterize the phenotypes of the corresponding loss of function mutants; Paper II is focused on the mutant *paqr-2* and a forward genetics screen for suppressors of its phenotypes; and Paper III describes another forward genetics screen for novel mutants that genocopy *paqr-2*. The papers convey a progressive study of the *C. elegans* PAQR proteins, with focus on *paqr-2*, and consequently the discussions with respect to each paper are somewhat continuous with one another.

PAPER I - THE ADIPONECTIN RECEPTOR HOMOLOGS IN *C. ELEGANS* PROMOTE ENERGY UTILIZATION AND HOMEOSTASIS

In this initial study we identify five *C. elegans* proteins that show homology to the mammalian PAQR proteins. Two out of the five are more closely related to the mammalian PAQR1 and 2, the adiponectin receptors (Tang et al., 2005), one is related to PAQR3, while the two remaining group together with the mammalian PAQR7/8 and PAQR10/11 respectively (Figure 6A).

We obtained *C. elegans* loss of function mutants of the three first-mentioned genes and generated all combinations of double and triple mutants.

On gross morphological examination only one phenotype can be recognized within the set of mutants, namely a withered tail tip defect in *paqr-2* mutant animals (Figure 6B). The phenotype is more related to maintenance than to growth or development since the defect becomes pronounced only in adult worms, with a penetrance of 100%.

The mutants (single, double and triple) were quantitatively scored with respect to several health traits: lifespan, growth rate, self-brood size and locomotion. This study allowed us to group the mutants into three groups. The single mutants *paqr-1* and *paqr-3* and the double mutant *paqr-1 paqr-3*, in most assays, behave like WT (N2), *paqr-2* and the double mutant *paqr-2;paqr-3* display reduced performance in all assays while the double mutant *paqr-1;paqr-2* and the triple mutant *paqr-1 paqr-3;paqr-2* have aggravated phenotypes. This data points to *paqr-2* being the most important *C. elegans paqr* gene, showing some functional redundancy to *paqr-1*.

The most striking phenotype found in the mutant set is the inability of *paqr-2* to develop from L1 larvae to fertile adult at 15°C, where the phenotype is again worse in *paqr-1;paqr-2* with a low frequency of growing worms already at 20°C (Figure 6C). Such a defect could potentially be related to aberrant regulation of membrane fluidity since *C. elegans*, being a poikilotherm, must adjust its membrane composition in response to changes in environmental temperature (Tanaka et al., 1996; Murray et al., 2007). Growth at a lower temperature favors an increase in UFA and PE head groups within the membrane lipid pool of poikilotherm organisms in order to maintain stable membrane properties (Hazel, 1995), a well-known phenomenon termed homeoviscous adaptation (Sinensky, 1974). Previous studies on *C. elegans* have shown that the nematode reacts to a lowering of the cultivation temperature (from 20-25°C to 10-15°C) by reducing the proportion of SFA predominantly in the PC fraction while increasing the amount of 18:2n-6 (linoleic acid) and 20:5n-3 (eicosapentaenoic acid) in both PC and PE fractions (Tanaka et al., 1996; Murray et al., 2007). Importantly for this thesis, the regulatory mechanisms responsible for homeoviscous adaptation are not well understood in any animal.

Transcriptional GFP reporters reveal consistent expression of *paqr-1* in several tissues including the intestine, vulva muscle and gonad sheath cells, *paqr-2* is expressed in many neurons as well as the gonad sheath cells while *paqr-3* (using a longer stretch of upstream sequence) shows expression in several cells including the hypodermis and gonad sheath. A translational

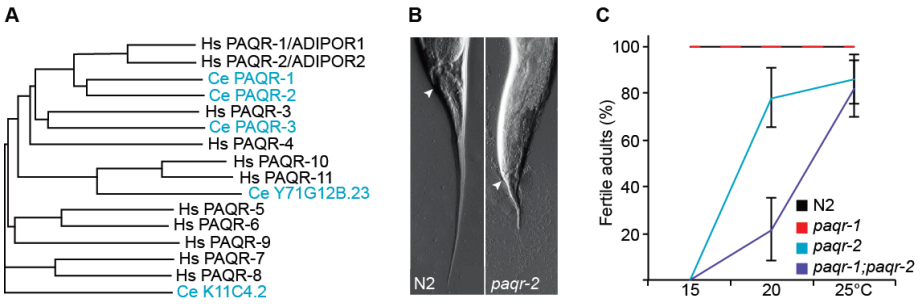

Figure 6. Relationship between human and *C. elegans* PAQR proteins and the most prominent phenotypes of *paqr-2*. **A**, Dendogram depicting the relationship between the 11 human and 5 *C. elegans* PAQR proteins based on sequence homology. **B**, The withered tail tip phenotype of *paqr-2*, with a penetrance of 100% in adults. **C**, The fraction of L1s that develop into fertile adults at different temperatures. The arrowheads in B point to the anus and the error bars in C show the 95% confidence interval.

PAQR-2 reporter tagged with GFP at the C-terminus can rescue growth of the mutant at 15°C, while not producing any fluorescence, thus indirectly confirming the topology of the protein with the C-terminus being outside of the cell. When the GFP is instead placed between amino acids R195 and R196 in the cytoplasmic N-terminal domain, the construct again rescues growth of the *paqr-2* mutant at 15°C while also giving rise to fluorescence in a smaller subset of neurons in the head and tail, as well as in the gonad sheath cells. Taken together, and based on multi copy array GFP reporter constructs, PAQR-2 may function in neurons and gonad sheath cells, the later being a common site of expression for all three *paqr* genes assayed. The gonad sheath is part of the somatic gonad and consists of five pairs of cells per gonadal arm that envelops the germline (Kimble and Hirsh, 1979) and its function is, among other things, to engulf apoptotic germ cells (Gumienny et al., 1999) and enable transport of yolk proteins to oocytes (Grant and Hirsh, 1999; Hall et al., 1999).

To assay the mutant set in relation to lipid metabolism we used gas chromatography of total lipid extracts and **c**oherent **a**nti-stokes **r**aman **s**cattering (CARS) microscopy to assess the FA composition and amount of lipids stored. CARS microscopy has previously been used on *C. elegans* and allows visualization of lipid depots without any kind of labeling, through probing of the characteristic vibrational properties of the carbon-hydrogen bond (Cheng and Xie, 2004; Burkacky et al., 2006; Hellerer et al., 2007). Our results show that the *paqr-1;paqr-2* double mutant has increased lipid stores, at a level comparable to that of a mutant of the *C. elegans* insulin receptor,

daf-2, which is known to store excess fat (Kimura et al., 1997). Regarding total FA composition the mutants previously grouped together with WT show only small changes, while *paqr-2* and the double and triple mutants bearing this mutation show disturbed profiles. The increase in the SFA C14:0 and C18:0 in *paqr-2* total lipids is interesting with respect to the cold sensitivity phenotype and the reported decrease in membrane lipid SFA in WT *C. elegans* upon growth at 10 or 15°C (Tanaka et al., 1996; Murray et al., 2007) as well as with respect to the phospholipid profiling performed in Paper II and III.

To further define the metabolic defects in *paqr-2*, and potentially connect its function to already known pathways or processes, we examined the localization of the translational *pDAF-16::gfp* reporter (Henderson and Johnson, 2001) in the *paqr-2* background, and also monitored the ability of the *paqr-2* mutant to grow at 15° or 20°C when combined with loss of function mutations of known metabolic regulators/enzymes. DAF-16 is the *C. elegans* homolog of FOXO, which in the fed state is repressed by insulin-like signaling via DAF-2, but upon starvation or in a *daf-2* mutant background is de-repressed and translocates to the nucleus where it upregulates expression of genes involved in increased fat storage, among other processes (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001). We examined DAF-16::GFP localization in *paqr-2* background and found the same diffuse cytoplasmic pattern as in WT, indicating that *paqr-2* induces excess fat storage in the *paqr-1;paqr-2* double mutant separately from the insulin-like signaling pathway. For the genetic interaction study, a set of mutants was chosen to represent metabolic regulators and enzymes, including the *C. elegans* homolog of SREBP1, *sbp-1* (McKay et al., 2003; Yang et al., 2006; Nomura et al., 2010), two transcription factors of the nuclear hormone receptor family, *nhr-49* and *-80*, where the former is a homolog of HNF4/PPARα (Bertrand et al., 2004; Van Gilst et al., 2005a; Van Gilst et al., 2005b; Atherton et al., 2008), a *C. elegans* homolog of the catalytic α subunit of AMPK, *aak-2* (Apfeld et al., 2004), the three *C. elegans* Δ9 desaturases, *fat-5*, *-6*, *-7* (Watts and Browse, 2000), the β-oxidation acyl-CoA synthetase, *acs-2* (Van Gilst et al., 2005a), as well as three ceramide synthases, *hyl-1*, *hyl-2*, *lagr-1* (Tedesco et al., 2008) and the putative acid ceramidase F27E5.1.

Synthetic lethality at 20°C was found in the double mutants *paqr-2;nhr-49* and *paqr-2 sbp-1*, while double homozygous *paqr-2;fat-6* usually grow to adults but are sterile. The analysis was further extended to include *paqr-1*, which is also synthetic lethal with *sbp-1* but not with *nhr-49*. These interactions indicate that PAQR-2 has redundant functions with SBP-1 and NHR-49, the outcome of which could potentially include the enzymatic

activity of FAT-6. On the other hand, the loss of function mutations of *aak-2* or *nhr-80* can partially suppress the inability of *paqr-2* to develop at 15°C. Finally, changing the diet from the standard laboratory *E. coli* OP50 strain to HT115 also confers a partial suppression of the defect, however, compared to the suppressors found in Paper II these effects of *aak-2*, *nhr-80* or dietary changes are very modest and nowhere near complete suppression.

C. elegans SBP-1 is known to promote fat storage (McKay et al., 2003; Yang et al., 2006; Nomura et al., 2010) and increased mRNA expression of the Δ9 desaturases *fat-5* (Nomura et al., 2010), *fat-6* and *fat-7* (Yang et al., 2006; Nomura et al., 2010), while NHR-49 positively regulates not only the expression of Δ9 desaturases but also that of enzymes involved in β-oxidation (Van Gilst et al., 2005a). The regulatory function common to both SBP-1 and NHR-49 is thus to increase expression of the *C. elegans* Δ9 desaturases, and the genetic interaction study as well as the increase in SFA seen in total lipid extracts from *paqr-2* mutant worms suggests that regulation of this class of enzymes is an important function also of PAQR-2.

C. elegans AAK-2 inhibits adipose triglyceride lipase, ATGL-1, to keep lipid stores intact during the dauer stage (Narbonne and Roy, 2009). One could thus hypothesize that the partial suppressive effect of the *aak-2* mutation is via activation of ATGL-1 and subsequently more FA released from lipid stores as substrates for Δ9 desaturases or β-oxidation. NHR-80, like NHR-49, positively regulates the Δ9 desaturases, with the *nhr-80* mutant having very low levels of *fat-7* mRNA, a moderate reduction of *fat-5* and only a small reduction in the mRNA levels of *fat-6* (Brock et al., 2006). One could thus speculate that a compensatory increase in the expression of *fat-6*, via other transcription factors, could be occurring in the *paqr-2 nhr-80* double mutant and, if true, this would fit the genetic interaction study where FAT-6 seems to be especially important for the viability of *paqr-2* mutants, since only *paqr-2;fat-6* is synthetic sterile while mutation of *fat-5* or *fat-7* do not result in such a phenotype. Mutation of the β-oxidation enzyme *acs-2*, the ceramide synthases and the putative acid ceramidase did not grossly affect the phenotype of *paqr-2* and therefore their functions are probably not central to PAQR-2 activity.

PAPER II - PAQR-2 REGULATES FATTY ACID DESATURATION DURING COLD ADAPTATION IN *C. ELEGANS*

In Paper II we performed a forward genetics screen to identify suppressors of the cold adaptation defect in the *paqr-2* mutant. The screening procedure is depicted in Figure 7. *paqr-2* worms were treated with the mutagen EMS (**e**thyl **m**ethane **s**ulfonate) then allowed to grow at 20°C. The F2 generation

was incubated at 15°C from the L1 stage and worms that could now grow and reproduce at this temperature were isolated. We screened about 15 000 haploid genomes and found nine new mutations in six different genes.

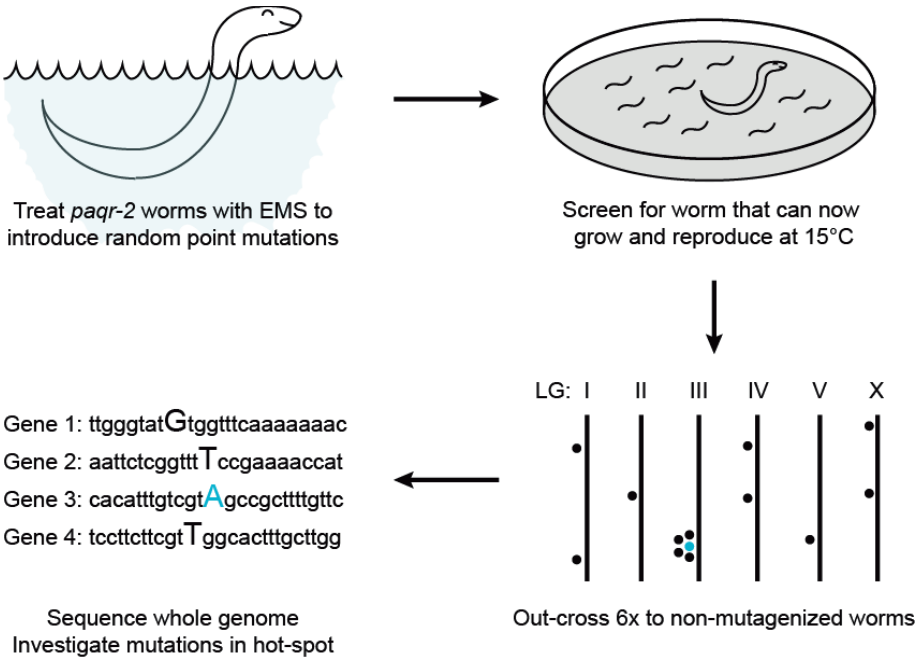

Figure 7. Forward genetics screen for *paqr-2* suppressors. EMS-mutagenized *paqr-2* worms were grown at 20°C and the F2 generation was subjected to 15°C as L1s. Worms that could now grow and reproduce at the lower temperature were identified and outcrossed 6 times to non-mutagenized worms before preparation of genomic DNA and subsequent whole genome sequencing.

The ease by which forward genetics screens can be performed is one of the many merits with *C. elegans* as a model system. Any recessive mutation introduced into the germline of the mutagenized hermaphrodite worms will be present in heterozygous form in the F1 generation and in homozygous form, displaying the accompanying phenotype, in the F2 generation (Brenner, 1974). Traditionally the identity of the new mutations was found by mapping experiments narrowing down the location of the mutation with respect to the recombination frequency to known genetic markers (Brenner, 1974), and later with PCR-based SNP mapping using the polymorphic Hawaiian *C. elegans* isolate (Williams et al., 1992; Wicks et al., 2001; Davis et al., 2005). More recently techniques for mutant identification through

whole genome sequencing have been developed and rely on comparisons to a reference WT genome and, if applicable, between genomes of simultaneously obtained mutants. The strategy relies on outcrossing of the mutant genome 4-6 times before whole genome sequencing, a process that will remove many of the mutations introduced by the EMS treatment but still retain mutations linked to the causative one, resulting in a cluster of unique mutations, including the interesting one, in each mutant strain (Sarin et al., 2008; Zuryn et al., 2010).

The suppressor mutations obtained can be assigned to three different groups: 1) gain of function mutations in *nhr-49* or the mediator subunit *mdt-15*; 2) loss of function mutations in the synthesis pathway for PC; and 3) loss of function mutations in β-oxidation enzymes. By a targeted search, based on the identification of the suppressor mutations isolated in the screen, we found that an overexpression transgene of GFP-tagged SBP-1 (Walker et al., 2011) and a loss of function mutant of the *C. elegans* S-adenosyl methionine synthetase, *sams-1* (Hansen et al., 2005), also act as *paqr-2* suppressors. Interestingly the suppressors of the cold adaptation defect also, to a very large extent, suppress the tail tip morphology defect of *paqr-2*, indicating that the suppression is not merely related to cold but to *paqr-2* in general.

In Paper II we also performed more extensive lipidomics profiling on *paqr-2*, now separating the PC, PE and triglyceride fractions. The analysis shows that the level of SFA is increased in *paqr-2*, as compared to WT, in both PC and PE fractions and that this is corrected, or overcompensated for, by a suppressor mutation in *nhr-49* or in the choline/ethanolamine phosphotransferase, *cept-1* (Walker et al., 2011). In relation to this we also examined expression levels of an integrated *fat-7* promoter reporter, *pfat-7::GFP* (Walker et al., 2011), as an indicator of expression levels of the *C. elegans* Δ9 desaturases, and found that the level of fluorescence from this reporter is reduced in *paqr-2* background, and that this can also be corrected/overcompensated for by the above mentioned suppressor mutations in *nhr-49* or *cept-1*. Furthermore, suppression of the cold adaptation defect by these same suppressors, as well as by a suppressor mutation in the β-oxidation hydroxyacyl-CoA dehydrogenase, *hacd-1* (Van Gilst et al., 2005b), is completely abolished if reducing the expression of *fat-6* or *fat-7*, but not *fat-5*, using RNAi feeding. Thus, despite potential differences in mechanism, there is a common output of all three groups of suppressors which heavily relies on the C18:0 Δ9 desaturases FAT-6 and FAT-7.

If activity of the Δ9 desaturases to produce MUFA is important for the suppression of the *paqr-2* cold adaptation defect, this should then also, by extension, be an important function of PAQR-2. NHR-49 and SBP-1,

together with MDT-15, are already known to directly regulate expression of the Δ9 desaturases (Van Gilst et al., 2005a; Taubert et al., 2006; Yang et al., 2006; Nomura et al., 2010). PC levels are known to regulate the activity of SBP-1 (Walker et al., 2011), and suppression by loss of function mutations in the PC synthesis pathway could thus act on the Δ9 desaturases via activation of SBP-1. As for the suppression by loss of function mutations in β-oxidation enzymes we hypothesize that a decrease in the flow of FA through this pathway may increase the amount of FA substrates available for desaturation, a hypothesis similar to the one presented for the partial suppression by *aak-2* in Paper I.

Using a different allele of *paqr-2* and also a different *fat-7::GFP* construct, the Horvitz lab recently published a forward genetics screen for mutations that increase expression of the *fat-7::GFP* reporter in the *paqr-2* mutant background where they identified several loss of function alleles of *acdh-11*, potentially an acyl-CoA dehydrogenase in β-oxidation (Ma et al., 2015). We have later (in Paper III) confirmed that a loss of function mutation in *acdh-11* functions as a suppressor of the withered tail tip and cold sensitivity phenotypes of *paqr-2*, analogously to the loss of function mutations in β-oxidation enzymes found in our own suppressor screen.

With respect to the above mentioned data and hypothesis, we assayed whether oleic acid, C18:1, the product of the FAT-6 and FAT-7 Δ9 desaturase activity, could rescue the cold adaptation and tail tip phenotype of *paqr-2*, as it is known to rescue phenotypes of *sbp-1* and *mdt-15* (Yang et al., 2006). Oleic acid supplemented to the growth media can only marginally rescue the *paqr-2* phenotypes, while a potent rescue is obtained from supplementation with very low amounts (0.005-0.05%) of detergents, NP-40 or Triton X-100. Note that the detergents do not rescue the fertility of *paqr-2* worms grown at 15°C, but that this can be achieved by providing both oleic acid and NP-40 in the culture plates.

Taking the supplementation experiments at face value, and assuming that the oleic acid properly enters the worm even without the solubilizing NP-40, suggests that the activity of the Δ9 desaturases alone is not enough to rescue the *paqr-2* phenotypes. Detergents, at non-solubilizing concentrations, are known to fluidize membranes (Ahyayauch et al., 2010; Henriksen et al., 2010) hence the defects in the *paqr-2* mutants may not solely be with expression of Δ9 desaturases but rather with regulation of membrane fluidity, where the Δ9 desaturase activity is but one cellular resource contributing to the maintenance of homeostasis under changing environmental conditions. In Paper III we directly measure membrane fluidity in WT and *paqr-2* mutant

worms, and the discussion on this topic will thus be continued in the following section on Paper III.

Despite having isolated and identified several *paqr-2* suppressor mutations, and the finding that they all act in different aspects of lipid metabolism (Figure 4 in Paper II), we still do not know the exact role of PAQR-2 in this process. In other words, we do not know by what mechanism or signal PAQR-2 regulates lipid metabolism, or what the specific targets of the PAQR-2 pathway are. The fact that loss of function mutations in β-oxidation enzymes suppress *paqr-2* strengthens the hypothesis from Paper I that among the functions of NHR-49 it is specifically the activation of Δ9 desaturases, and not β-oxidation enzymes, that is important. Furthermore a loss of function mutation in *cept-1*, assumed to activate SBP-1 and thereby suppress *paqr-2*, can also suppress the synthetic lethality of *paqr-2;nhr-49* found in Paper I, while a gain of function mutation in *nhr-49* can not suppress the synthetic lethality of *paqr-2 sbp-1*. One could thus speculate, based on the genetic interactions, that a function of PAQR-2 is to activate NHR-49, when needed, to increase the amount of UFA available for membrane phospholipid turnover, while SBP-1 being regulated by PC levels has somewhat the same function but in a separate pathway, making a double *paqr-2 sbp-1* mutant worse off than a double *paqr-2;nhr-49* mutant. Yet another hypothesis could be that PAQR-2 regulates PC levels to activate SBP-1. In connection to the later, the PAQR family has been grouped together with other 7-TM proteins into a large superfamily of putative membrane bound hydrolases, dubbed CREST (alkaline **c**eramidase, PAQR **r**eceptor, P**E**R1, **S**ID-1 and **T**MEM8), where several members have enzymatic ability to remodel lipids (Pei et al., 2011). The homology between PAQR proteins and alkaline ceramidases, and the putative intrinsic or associated ceramidase activity of the yeast IZH2 and the mammalian ADIPORs (Villa et al., 2009; Holland et al., 2011) has been mentioned previously in this thesis. In addition, the yeast CREST family member PER1 has an intrinsic or associated phospholipase A_2 activity for remodeling of the FA in glycosylphosphatidylinositol (GPI) anchored proteins, necessary for their maturation (Fujita et al., 2006). Thus, in theory there are potential mechanisms for an intrinsic or associated activity of PAQR-2 in remodeling of membrane lipids, something that could either generate lipid messengers to activate transcription factors, such as the *C. elegans* NHRs, or directly remodel the FA composition of the membrane phospholipids.

PAPER III - *CAENORHABDITIS ELEGANS* PAQR-2 AND IGLR-2 PROTECT AGAINST GLUCOSE TOXICITY BY MODULATING MEMBRANE LIPID COMPOSITION

In Paper III we performed yet another forward genetics screen, now with the aim of identifying new mutants that genocopy (Zori and Williams, 1991) *paqr-2*, meaning that they confer the same phenotypes as the *paqr-2* mutation does. Such genocopy mutations could potentially affect proteins working closely together with PAQR-2 in the receptor signaling pathway, or mutations in the PAQR-2 ligand. The screen was performed largely as in Paper II, but now WT worms were mutagenized and the F2s screened for presence of a tail tip phenotype similar to *paqr-2*. All worms with a tail tip defect were further screened for the inability to grow from L1s to adults at 15°C, and mutants that displayed both phenotypes were candidate *paqr-2* genocopies. A parallel screen was also performed looking first for the inability to grow at 15°C and then for presence of a *paqr-2*-like tail tip phenotype. We screened ~11 000 haploid genomes using the first mentioned strategy and ~70 000 using the second strategy and together the two screens yielded five new mutations that genocopy the original *paqr-2* loss of function.

Out of the five new mutations, two turned out to be new alleles of *paqr-2* itself, while the remaining three are novel alleles of the gene *iglr-2*. The *iglr-2* alleles are most probably loss of function mutations since two of them result in premature stop codons, one as early as in amino acid 83 of the 773 amino acid *iglr-2* gene product. IGLR-2 is, like PAQR-2, a transmembrane protein, and thus a likely candidate for close interaction with PAQR-2. IGLR-2 belongs to the LRRIG family of proteins (**l**eucine **r**ich **r**epeat and **Ig** containing proteins) (Homma et al., 2009) and is predicted to have an intracellular C-terminus, one transmembrane domain, one Ig (immunoglobulin) domain and five leucine rich repeats (LRRs) in the extracellular N-terminal part. The proteins of the LRRIG family have diverse functions (Homma et al., 2009). Domain-wise, *C. elegans* IGLR-2 is most similar to the AMIGO or LINGO proteins, which also have one transmembrane domain, one Ig domain and 6 (AMIGO) or 12 (LINGO) LRRs but no additional known domains (Homma et al., 2009). AMIGO is a subunit of the neuronal potassium channel Kv2.1 complex, and known to increase the activity of the channel (Peltola et al., 2011; Peltola et al., 2016) while LINGO1, also in the central nervous system, is part of the NgR1/p75 signaling complex involved in axon regeneration (Mi et al., 2004) and known to bind to, and negatively regulate, receptor tyrosine kinases, a function also of other LRRIG proteins such as LRIG1 (Gur et al., 2004; Laederich et al., 2004; Mandai et al., 2009; Meabon et al., 2016).

The *iglr-2* mutants display phenotypes very similar to *paqr-2* mutants in all assays tested, including an increase in SFA in PC and PE phospholipids, decreased expression of the *pfat-7::GFP* reporter and also the cold sensitivity and withered tail tip defects, which were the initial screening criteria. A translational GFP reporter shows that *iglr-2* is predominantly expressed in the gonad sheath cells, a tissue where *paqr-2* has also been detected. Using BiFC, a method where the YFP variant VENUS (Nagai et al., 2002) is split into two non-fluorescent fragments and tagged to the proteins of interest to reconstitute the full fluorescent VENUS protein only if the two carrier proteins come into very close proximity of each other (Hu et al., 2002; Shyu et al., 2006), we show that PAQR-2 and IGLR-2 localize to the cell membrane (expression driven by an intestine specific heat shock promoter (Hiatt et al., 2008; Shyu et al., 2008)) and are capable of forming a complex.

With respect to the function of IGLR-2 what we know so far is that localization of PAQR-2 to the gonad sheath cell membrane is somewhat impaired in the *iglr-2* mutant background, but since the *paqr-2* characteristic phenotypes are as strong in *iglr-2* one could predict that the function of IGLR-2 is not limited only to PAQR-2 localization.

During the process of characterization of the *iglr-2* alleles we discovered that *iglr-2* and *paqr-2* mutants are sensitive to glucose. The mutants cannot grow from L1s to fertile adults if the OP50-seeded worm plates are supplemented with 20 mM glucose. The mutants are also somewhat sensitive to other sugars at 20 mM, while L-glucose, the biologically inert enantiomer of the natural D-glucose variant, does not have any effect, indicating a biological and not a physical/chemical effect of glucose on the worms. The double mutant *paqr-2 iglr-2* is phenotypically very similar to either single mutant in all assays tested, including glucose sensitivity, showing that *paqr-2* and *iglr-2* are indeed working in the same pathway.

Glucose supplementation in *C. elegans* has been studied most extensively in context of the reduced lifespan caused by glucose concentrations ranging from 2.8 to 111 mM (Schulz et al., 2007; Lee et al., 2009). Glucose supplementation is also known to increase levels of triglycerides (Schulz et al., 2007; Garcia et al., 2015; Lee et al., 2015) and to influence the lipid composition, resulting in increased amounts of SFA in total lipids of WT worms (Lee et al., 2015) even though glucose has also been shown to induce increased expression of the *C. elegans* Δ9 desaturases (Nomura et al., 2010; Lee et al., 2015). In Paper III we assayed lipid composition in WT and *paqr-2*, *iglr-2* and *paqr-2 iglr-2* mutant strains upon glucose treatment more specifically in the PC and PE membrane fractions and found that, in accordance with previous reports, the level of SFA is increased in WT worms

upon glucose treatment and that this phenotype is further enhance in the single and double mutants, consistent with the decreased expression of the Δ9 desaturase *pfat-7::GFP* reporter in these strains.

We tested if the *paqr-2* suppressors from Paper II could also suppress the glucose intolerance and, using the same groups as in Paper II, found that the gain of function mutations in *nhr-49* and *mdt-15* as well as the SBP-1 overexpression transgene can suppress *paqr-2* with ≥ 80% of the worms now developing into fertile adults. The loss of function mutations in the PC synthesis pathway can partially suppress the glucose intolerance with ≤ 35% developing into fertile adults, while the loss of function mutations in the β-oxidation pathway do not suppress the glucose intolerance of *paqr-2*. Similar results were obtained when assaying a subset of the suppressors in *iglr-2* and *paqr-2 iglr-2* background, while the cold adaptation and tail tip defects of these mutant strains are more efficiently suppressed. Inclusion of 0.1% detergent (NP-40) in the plates can also partially rescue the glucose sensitivity of the single and double mutants.

The above findings indicate that the three major phenotypes of *paqr-2* and *iglr-2*, i.e. the glucose intolerance, cold adaptation defect and withered tail tip, arise from the same underlying problem, which we have previously hypothesized to be a lack of membrane fluidity regulation in the absence of these proteins. In Paper III we directly measure membrane fluidity of *paqr-2* and *iglr-2* mutants under normal growth condition as well as after cold or glucose treatment using **f**luorescence **r**ecovery **a**fter **p**hotobleaching (FRAP). In this method, a high power laser is used to photobleach a fluorescent molecule and the subsequent movement of fluorescent molecules into the bleached area is recorded and quantified (Reits and Neefjes, 2001). For our purposes we use a prenylated, and thus membrane-bound, version of GFP expressed in the intestine (Morck et al., 2009), photobleach a region in the cell membrane of an intestinal cell, and follow the movement of GFP into that area. Our results show that under normal growth conditions (NGM plates at 20°C) neither *paqr-2* nor *iglr-2* have decreased membrane fluidity. However, when challenged at 15°C or on glucose-supplemented plates, the membrane fluidity of the mutants is decreased while that of WT worms is unaffected. The membrane fluidity of the mutants can be rescued by a suppressor mutation in *nhr-49* or by supplementation with detergents.

Our data thus shows that PAQR-2 and IGLR-2 work together in the same pathway and that they can form a complex on cell membranes. Lack of PAQR-2 or IGLR-2 confers increased levels of SFA in the PC and PE membrane lipid fractions, and decreased levels of expression of the *C. elegans* Δ9 desaturase *pfat-7::GFP* reporter. Despite the increased levels of

SFA, *paqr-2* and *iglr-2* have normal membrane fluidity at standard growth conditions, but lack of these proteins leads to decreased membrane fluidity upon challenge with a lower temperature or glucose supplementation. The decreased membrane fluidity of the mutants upon challenge can be suppressed by mutations known to increase expression of Δ9 desaturases, or rescued by detergents at membrane-fluidizing concentrations. See Figure 8 for a proposed model of the function of PAQR-2 and IGLR-2 in the regulation of membrane fluidity.

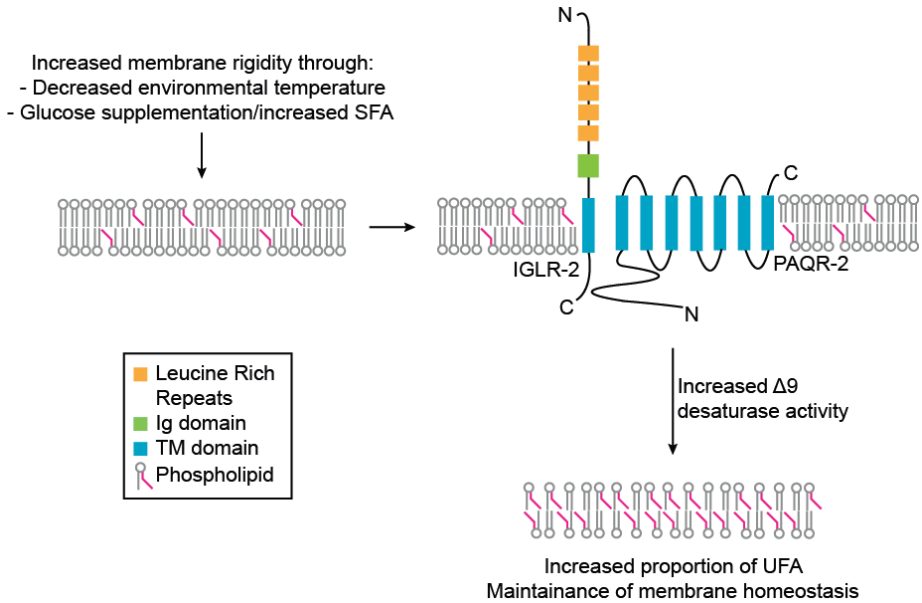

Figure 8. Proposed model for the regulation of membrane fluidity by PAQR-2 and IGLR-2. PAQR-2 and IGLR-2 are not needed to maintain membrane fluidity under standard laboratory growth condition. However, when the worms are challenged by treatments that require the homeoviscous adaptation response, worms lacking PAQR-2 or IGLR-2 suffer from decreased membrane fluidity. We propose a model where the PAQR-2/IGLR-2 complex is activated upon membrane rigidity, obtained either physically through decreased temperature or biologically through glucose supplementation/increased saturated fatty acids (SFA), and that their activation promotes Δ9 desaturase activity to increase the proportion of unsaturated fatty acids (UFA) in the cell membrane and thus maintain membrane fluidity.

PAQR-2 and IGLR-2 may thus constitute a membrane fluidity sensor, which is activated under conditions where membrane fluidity must be adjusted to maintain homeostasis. As previously mentioned, such a membrane fluidity

sensor and regulator, the DesKR system, has previously been described in *B. subtilis* where the 5-TM domain histidine kinase DesK regulates the activity of DesR to promote expression of the desaturase gene *des* (Aguilar et al., 2001). The proposed mechanism for DesK activation involves the physical properties of the cell membrane where a less fluid, more ordered and thus thicker membrane at low temperature (Pan et al., 2008; Szekely et al., 2011) hides a hydrophilic stretch of amino acid residues within TM1 of DesK in the hydrophobic core of the cell membrane and mediates conformational changes within the protein that activates the intrinsic kinase activity (Cybulski et al., 2010; Martin and de Mendoza, 2013; Porrini et al., 2014; Saita et al., 2015). One could thus speculate that PAQR-2, IGLR-2 or the complex has a similar, though potentially mechanistically different, function in sensing and regulating membrane fluidity.

The genocopy screen performed in Paper III yielded new alleles of only two genes, *paqr-2* and *iglr-2*, and thus did not identify a PAQR-2 ligand. At present we do not even know if there is a PAQR-2 ligand, and there is indeed no evident adiponectin homolog in worms. In this context, it is interesting to note that even the mammalian adiponectin receptors are sometimes reported to display activity from mere overexpression (Kupchak et al., 2007; Holland et al., 2011), and to keep in mind that the above-mentioned bacterial sensor of membrane fluidity, DesK, is activated in response to membrane properties, and not by binding a ligand. That PAQR-2 may have no *bona fide* ligand is therefore not mere far-fetched speculations.

That cold temperature constitutes a condition where the process of homeoviscous adaptation comes into play is well-known. This is however not the case for increased glucose availability, which is less well recognized as a condition where such mechanisms are important. We (Paper III) and others have recently reported that the amount of SFA in total lipids and membrane lipid fractions are increased in WT *C. elegans* upon glucose supplementation, and that this effect is enhanced by RNAi of *sbp-1* and *mdt-15* (Lee et al., 2015), or loss of function mutations of *paqr-2* and *iglr-2*. Since glucose supplementation also confers increased triglyceride stores (Schulz et al., 2007; Garcia et al., 2015; Lee et al., 2015), lipogenesis is most probably playing an important role in the handling of glucose within the worm. The lipogenesis pathway uses acetyl-CoA to produce SFA, which can then be elongated, desaturated and incorporated into triglycerides or phospholipids (Strable and Ntambi, 2010; Holthuis and Menon, 2014). It could thus be expected that in mutants with reduced level of Δ9 desaturase activity the increased proportion of SFA in the newly synthesized FA pool would be increased, and via FA replacement in phospholipids, the rate of which was measured by Dancy *et al.* 2015, the composition of the membranes (as well

as the triglycerides) would soon be affected, since at least 60% of the membrane phospholipids are replaced within 24 h (Dancy et al., 2015). Another possibility is that the glucose rich diet mediates changes in the *E. coli* OP50, which in turn affects the composition of the FA pool in the worms.

Membrane fluidity in WT worms is not changed upon glucose treatment, despite a measurable increase in the levels of SFA, indicating that WT worms have the capacity to compensate for the changes in lipid composition induced by the glucose-rich diet. However, the *paqr-2* and *iglr-2* mutants display a greatly enhanced accumulation of SFA accompanied by a decrease in membrane fluidity, indicating that the regulatory mechanisms are ineffective in the mutants We do not know how the enhanced levels of SFA in *sbp-1* and *mdt-15* upon glucose supplementation (Lee et al., 2015) influence membrane fluidity, but since these worms are able to grow, albeit with slow growth for *sbp-1* and embryonic lethality in progeny produced on glucose plates for *mdt-15*, the hypothesis would be that their membrane fluidity is within the physiologically acceptable range. One could speculate that, in presence of PAQR-2 and IGLR-2 as in the *sbp-1* and *mdt-15* mutants, the available UFA could be targeted specifically to the membrane and less so to the triglyceride stores. In this context it is interesting to note that, unlike in *paqr-2* or *iglr-2* mutants, the level of C18:0 is actually decreased in *sbp-1* and *mdt-15* RNAi-treated worms upon glucose supplementation, while still being increased as compared to WT (Lee et al., 2015). This indicates that the activation of FAT-6 and FAT-7 upon glucose supplementation, at least in part, is dependent on other pathways (besides SBP-1 and MDT-15) or that the reduced lipogenesis conferred by theses genetic backgrounds is protective.

The glucose toxicity seen in the *C. elegans paqr-2* and *iglr-2* mutants is likely due to increased levels of SFA and decreased membrane fluidity. Can these finding illuminate some aspects of human physiology or disease states? Glucose toxicity is a hallmark of diabetes, but the underlying mechanism is not well understood and no unifying theory has been presented (Giaccari et al., 2009). Glucose levels in plasma are normally kept within a very narrow range, and even a small increase confer higher incidence of microvascular (retinopathy, neuropathy, nephropathy) and macrovascular (myocardial infarction, stroke etc.) complications (Kawahito et al., 2009; Laakso and Cederberg, 2012). Glucose toxicity from chronic hyperglycemia leads to reduced insulin secretion from the pancreatic β-cells and increased insulin resistance of liver, skeletal muscle and adipose tissue (Giaccari et al., 2009; Kawahito et al., 2009). Some of the proposed causative mechanisms are oxidative stress (Nishikawa et al., 2000), lipotoxicity (McGarry and Dobbins,

1999), advanced glycation end products (AGE) (Nowotny et al., 2015) and increased glucose flux through the hexosamine pathway (Marshall et al., 1991).

Chronic hyperglycemia is also associated with increased cell membrane rigidity in erythrocytes (Kamada et al., 1992; Kroger et al., 2011; Mahendran et al., 2014; Kroger et al., 2015). Decreased levels of UFA in erythrocyte cell membranes has been associated with an increased risk for the development of diabetes (Kroger et al., 2015) and reduced membrane fluidity upon chronic hyperglycemia has been proposed to decrease glucose uptake and, as with the other glucose toxicity mechanisms listed above, to be a causative factor in the diabetes pathophysiology (Weijers, 2012). Thus, there is a connection from lipid composition and membrane fluidity to diabetes, and our findings regarding PAQR-2 are especially interesting given that its mammalian homologs, the adiponectin receptors, have demonstrated anti-diabetic functions. Is fluidity regulation the missing link that can explain glucose toxicity and the anti-diabetic properties of the ADIPORs? Only further research will tell if the mammalian adiponectin receptors also regulate membrane fluidity, and whether our findings regarding membrane fluidity and PAQR proteins in *C. elegans* are relevant in explaining the pathologies seen in diabetic patients.

In summary, the adiponectin receptor homolog PAQR-2 and its partner IGLR-2 are involved in the *C. elegans* homeoviscous adaptation response, and regulate membrane fluidity by promoting FA desaturation.

FUTURE PERSPECTIVES

We have studied the PAQR protein family in *C. elegans* with a focus on PAQR-2 and found that this protein, together with its partner IGLR-2, have a role in regulation of membrane fluidity in the homeoviscous adaptation response. This is evidenced by decreased membrane fluidity of the *paqr-2* and *iglr-2* mutant worms, not under normal growth conditions, but when challenged by cold temperature or exogenous glucose. Furthermore, *paqr-2* and *iglr-2* have increased levels of SFA in the PC and PE membrane lipid fractions, and decreased expression of the Δ9 desaturase reporter *pfat-7::GFP* already under normal growth conditions, and the mutant phenotypes can be suppressed by mutations known to directly or indirectly increase Δ9 desaturase activity, or rescued by detergents at membrane-fluidizing concentrations.

Naturally, the most interesting continuation of the work presented in this thesis would be to find out if there is any bearing of the membrane fluidity hypothesis on PAQR proteins in general and on the mammalian ADIPORs in particular. That the mRNA level of the yeast PAQR protein IZH2 is induced upon treatment with SFA or glucose, and reduced in presence of the MUFA oleic acid (Karpichev and Small, 1998; Karpichev et al., 2002) supports our findings. Furthermore, Karpichev *et al.* 2002 speculates about aberrant membrane composition in the *Izh2* deletion strain due to increased resistance to the antifungal compound nystatin, know to interact with sterols in the cell membrane (Marini et al., 1961; Kinsky, 1962). The intrinsic or associated ceramidase activity suggested for the yeast IZH2 (Villa et al., 2009) as well as for the mammalian ADIPORs (Holland et al., 2011) is interesting with respect to membrane composition and the possibility of generation of a lipid messenger downstream of the PAQRs. Also, in the case of *C. elegans* where PAQR-2 and IGLR-2 seems to be expressed in only a few cells, while regulating membrane fluidity of others, the ceramide second messenger, S1P, is appealing since it makes it easy to envision a model for cell non-autonomous signaling. However, we have so far not found any genetic interaction between *paqr-2* and the known *C. elegans* ceramidases, and also we did not isolate any ceramidase mutant is our forward genetic screens. Initial studies of the connection between membrane fluidity and the mammalian ADIPORs could include examination of membrane lipid composition of different cell types from diabetic, and hyperglycemic, mouse models as well as from the *AdipoR* knockout mice. Studies in cell culture would also allow direct examination of membrane fluidity, by FRAP, after knockdown of the *AdipoRs*.

However, despite the compelling possibility of extending our findings in *C. elegans* to mammals, there are still so many questions to be answered in the worms. The effect of glucose on *C. elegans* lifespan has been shown to be independent of the *E. coli* diet (Schulz et al., 2007; Lee et al., 2009), however, we do not know how the glucose confers the toxicity seen in *paqr-2* or *iglr-2* mutants. The outcome is increased SFA and decreased membrane fluidity, but what is the mechanism? Is it increased lipogenesis leading to too low levels of UFA, and if so is the bacterial diet involved in this process? Or is it accumulation of metabolites in the glycolysis pathway that is toxic as suggested by Lee *et al.* 2015, and could one also then wonder if these are produced solely by the worm or also by the bacteria? Sydney Brenner, whose work (Brenner, 1974) has made *C. elegans* the well-known and widely used model organism it is today, was originally introduced to the *Caenorhabditis* nematodes by the French biologist Ellsworth C. Dougherty who was studying the nematode in relation to nutrition and metabolism (Dougherty and Calhoun, 1948; Dougherty et al., 1959) and, recently, the possibility of using *C. elegans* as a model for research into micronutrients and the composition of diets has been revisited (Macneil and Walhout, 2013; Yilmaz and Walhout, 2014). It will certainly be interesting to further characterize the glucose toxicity of the *paqr-2* and *iglr-2* mutants to find out where the increased SFA originate from, to what extent the bacterial diet is involved, and whether the diet could be changed, or supplemented with other molecules than the detergents, to alleviate the glucose toxicity.

Throughout the studies included in this thesis we have used the *pfat-7::GFP* reporter as a general readout for Δ9 desaturase activity. However, our genetic interaction studies from Paper I indicate that FAT-6 is the more important Δ9 desaturase with respect to PAQR-2 function. Also, from the study of phospholipid replacement rate in *C. elegans* by Dancy *et al.* 2015, among the desaturases FAT-6 may potentially be the more important regulator of this process. Thus, construction and integration of a *pfat-6::GFP* reporter may be of importance for continued studies.

Some, but not all of the suppressors of the *paqr-2* cold adaptation defect isolated in Paper II can also suppress the glucose toxicity of this mutant. However, even the most potent suppressors do not confer a WT growth rate and 100% fertility on 20 mM glucose-supplemented plates. Therefore, additional *paqr-2* and/or *iglr-2* suppressor screens to identify new mutations that suppress the glucose toxicity phenotype to WT performance may identify new proteins that connect to PAQR-2 function.

Eventually though, there is a limit to what can be found through genetic screens. If the mechanism through which PAQR-2 functions turns out to be

the generation of a lipid messenger, such a molecule will never be found in a genetic screen. Chakravarthy *et al.* 2009 identified an endogenous PPARα phospholipid ligand in mouse liver by extraction of lipids bound to PPARα isolated from the liver of WT mice and liver specific fatty acid synthase (*Fas*) knockout mice (where FAS is suspected to be involved in generation of the PPARα ligand), mass spectrometric analysis of the extracted lipids and comparison between genotypes (Chakravarthy et al., 2009). Such methods would be applicable also in *C. elegans* to identify potential ligands generated by PAQR-2 itself, or by downstream enzymes.

There is also a wealth of experiments to be performed regarding the function of IGLR-2, whether it has a function besides localization of PAQR-2 to cell membranes and what part of PAQR-2 and IGLR-2 actually interact. The later could be assayed through deletion of protein domains of the PAQR-2 and IGLR-2 BiFC constructs used in Paper III. It would of course also be interesting to find out if there is a mammalian protein equivalent to IGLR-2 for the ADIPORs, and since we do not know the interaction site between PAQR-2 and IGLR-2 it is hard to guess whether such a protein should have been picked up by the already published yeast two-hybrid screens.

The common expression site of the *C. elegans* PAQR-2 and IGLR-2 proteins, the gonad sheath cells, is also intriguing since many of the other actors in *C. elegans* lipid metabolism are predominantly expressed in the intestine (McKay et al., 2003; Brock et al., 2006; Taubert et al., 2006; Srinivasan, 2015). A crude mosaic analysis of *iglr-2* (Paper III) using a previously described method based on expression of SUR-5GFP(NLS) (Yochem et al., 1998) and the rescuing pIGLR-2 transgene has so far revealed that expression in the intestine, while most often seen, is not necessary to rescue the glucose toxicity phenotype of *iglr-2*, while expression in the hypoderm is sufficient to rescue the phenotype. We have for a long time been limited in this aspect of the research on *paqr-2* by toxicity effects from tissue specific translational reporters. The mosaic analysis, in combination with tissue specific RNAi, may resolve the question of where PAQR-2 and IGLR-2 are expressed and exert their effects. It should also be noted here, and in connection to the potential importance of FAT-6 over the other Δ9 desaturases for PAQR-2 function, that while all three *C. elegans* Δ9 desaturases are expressed in the intestine, FAT-6 is also expressed in the hypodermis (Brock et al., 2006).

Finally, we have shown that the *C. elegans* adiponectin receptor homolog PAQR-2 and its partner IGLR-2 are involved in the homeoviscous adaptation response required when the nematode is cultivated at a low temperature or in the presence of excess environmental glucose. While the direct downstream

signaling pathway remains unresolved, an outcome of PAQR-2 signaling seems to be increased Δ9 desaturase activity, and such a signal could potentially be transferred via SBP-1, NHR-49 or MDT-15. With the predominant site of expression of PAQR-2 and IGLR-2 being the gonad sheath cells, and expression of IGLR-2 in the hypodermis, where also FAT-6 is expressed, being sufficient to rescue the glucose sensitivity of *iglr-2*, pathways for cell autonomous as well as whole body, cell non-autonomous, regulation of membrane fluidity could be envisioned.

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