PAQR-2 may be a regulator of membrane fluidity during cold adaptation

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PAQR-2 is a *C. elegans* homolog of the mammalian adiponectin receptors. We have recently shown that PAQR-2 is essential for the ability of *C. elegans* to grow at its lower temperature range, i.e., 15 °C, and that the likely role of PAQR-2 during cold adaptation is to regulate membrane fluidity by promoting fatty acid desaturation. Here we present a summary of this work, with an emphasis on placing our *C. elegans* findings in the context of mammalian biology.

Why Study Adiponectin Receptor Homologs in *C. elegans*?

Adiponectin

Adiponectin, discovered in the mid-'90s, is a hormone expressed specifically by adipocytes.[1-3] It is composed of 244 amino acids, with a collagenous domain that mediates multimerization, and a globular domain that interacts with the receptors. In mice, administration of adiponectin enhances insulin sensitivity, fatty acid oxidation[4-7] and energy expenditure,[8,9] protects from atherosclerosis,[10] and causes decreased body weight.[5,11] In human, serum concentration of adiponectin correlates negatively with BMI and insulin resistance,[12] and low serum adiponectin level is a well-established risk factor for type 2 diabetes[13,14] and myocardial infarction.[15] Importantly, polymorphisms in adiponectin or its receptors are associated with an increased risk of type 2 diabetes and other metabolic syndrome complications.[16-20] What emerges from the literature is that adiponectin is a key regulator of metabolism, and that pharmacomodulation of the adiponectin pathway has great potential in the context of the metabolic syndrome. How adiponectin signals to cause its important effects remains however far from clear.

Adiponectin receptors

Human adiponectin likely acts via two homologous receptors: AdipoR1 and AdipoR2.[21] These are members of the poorly understood PAQR (progestin and adipoQ receptors) protein family characterized by seven transmembrane domains with a topology inverse that of G protein-coupled receptors (GPCRs): in PAQR proteins the N terminus is intracellular.[22] Both receptors are expressed in the hypothalamus and many peripheral tissues.[9,21] Knockout mice have confirmed the insulin-sensitizing roles of these receptors: they exhibit increased adiposity, insulin resistance, excess gluconeogenesis, and reduced fatty acid oxidation.[23,24] Adiponectin receptors clearly regulate the balance between energy utilization and storage, but how?

Events immediately downstream of AdipoR1/2 are ill-defined, and several discrepancies exist in the literature. Recent work inspired by studying a distant yeast homolog indicates that the AdipoR1/2 receptors may have an associated ceramidase activity, but this remains to be experimentally confirmed in other organisms.[25-27] Some groups also reported that adiponectin induces AMPK in liver,[28] that globular and recombinant adiponectin are biologically active,[10,23] that double knockout mice lacking AdipoR1 and AdipoR2 are viable,[23] and that adiponectin regulates apetite.[9] Other groups have contrary findings.[24,25,29-32] It is not known whether the receptors act as dimers or monomers,
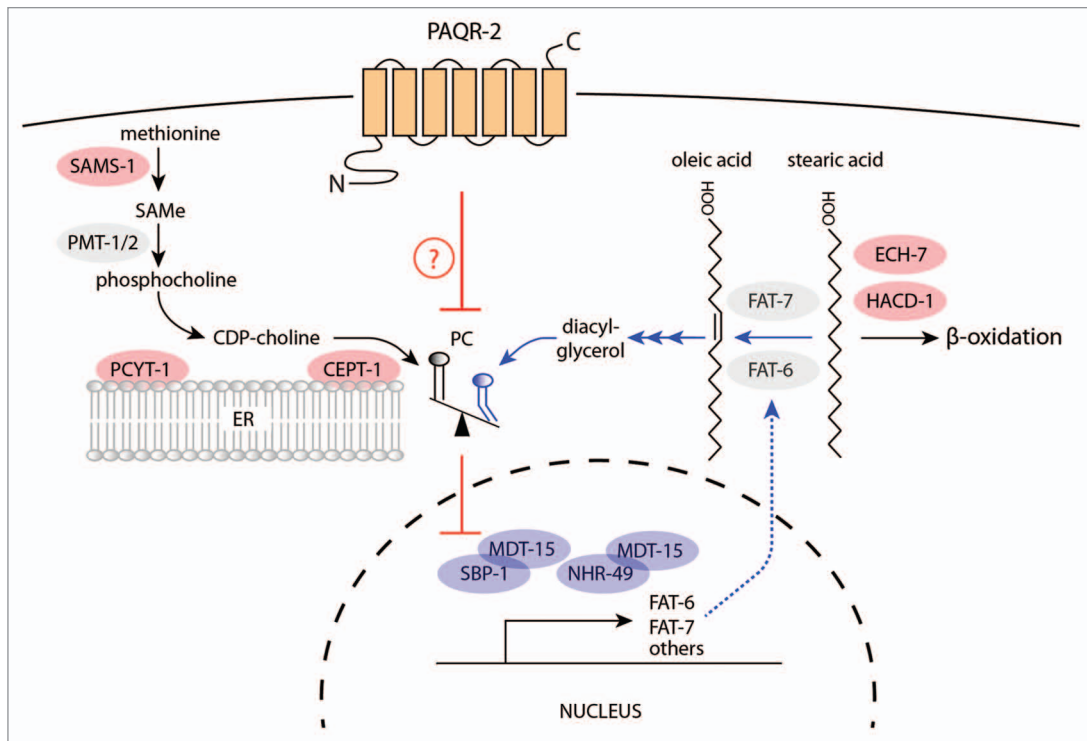
Figure 1. Simplified pathway for *paqr-2* suppressors. *lof* mutations affecting the proteins depicted in red, or *gof* mutations in proteins depicted in blue, can suppress the cold adaptation and withered tail tip phenotypes of the *paqr-2(tm3410)*-null mutant. Two main pathways are at work: on the left is the pathway for PC biosynthesis, and on the right are reactions important for FA metabolism. Lowering PC biosynthesis is expected to promote SBP-1 activation, which in turn activates Δ9 desaturases such as FAT-6 and FAT-7, ultimately resulting in more unsaturated fatty acids and increasing their relative abundance within membrane phospholipids. This increases membrane fluidity, which is adaptive at low temperatures. The role of PAQR-2 itself is unknown but one speculation is that it may regulate PC levels. *lof* mutations in *aak-2* and *nhr-80* can also partially suppress *paqr-2* phenotypes (not shown).

and some investigators even suggest that ligand binding causes monomerization of inactive receptor dimers.[33] This confusing situation about an important metabolic hormone calls for an unbiased approach to elucidate the adiponectin signaling pathway, which is one of our goals.

Adiponectin Receptor Homologs in *C. elegans*

The road to isolating *paqr-2* suppressors in *C. elegans*

There is no obvious homolog of adiponectin in *C. elegans*. However, in 2011 we published a description of the two closest *C. elegans* adiponectin receptor homologs, which we named PAQR-1 and PAQR-2.[34] These are expressed in metabolically important tissues, and *paqr-2* is the most important of the two genes: the *paqr-2* mutant exhibits a cold adaptation defect (inability to grow at 15 °C), a distinctive tail tip morphology defect, and an excess fat storage phenotype when combined

with a *paqr-1* mutation. Furthermore, *paqr-2* is sterile or synthetic lethal with mutations in regulators of fatty acid metabolism, including the Δ9 desaturase *fat-6*, the SREBP homolog *sbp-1*, and the HNF4/PPARα homolog NHR-49.[34]

The greatest merit of using *C. elegans* is that it can be used as a tool to discover new molecular components that regulate or mediate the effects of proteins, such as AdipoR1 and AdipoR2. A powerful and unbiased approach is to use random mutagenesis screens to identify modifier mutations. In work recently published, we screened 15 000 mutagenized haploid genomes and identified nine novel mutations (alleles *et6* to *et14*) that can suppress the growth arrest phenotype of *paqr-2* mutants at 15 °C.[35] Importantly, all nine suppressors alleviated the *paqr-2* tail phenotype, at least to some degree. This indicates that they compensate specifically for loss of *paqr-2* function, rather than generally improving cold tolerance. The suppressor mutations therefore likely

affect genes downstream of PAQR-2, or genes acting in parallel pathways. This mutant collection is potentially an important resource to elucidate the adiponectin pathway, of which many components will certainly be conserved between mammals and worms.

Identity of the *paqr-2* suppressors

Whole genome sequencing and experimental confirmation allowed us to identify all nine suppressor alleles. The novel alleles affect genes belonging to two metabolic pathways: some mutations decrease phosphatidylcholine biosynthesis (loss of function [*lof*] mutations in *pcyt-1* and *cept-1*) while other mutations likely result in increased fatty acid elongation and desaturation (gain-of-function [*gof*] mutations in *mdt-15* and *nhr-49* or loss of function mutations in *ech-7* or *hacd-1*). Subsequent testing of a *lof* allele of *sams-1* and of an overexpression *sbp-1* transgene also revealed these to be *paqr-2* suppressors. The following summarizes some of the literature that led us to construct the

model shown in **Figure 1**, and discusses some implications of our findings.

paqr-2 suppressors and PC biosynthesis

SAMS-1 is the homolog of the mammalian methionine adenosyltransferase, while SAMe is the methyl donor for hundreds of methylation reactions, including RNA, DNA, proteins, and lipids,[36] and methylation represents one of the earliest steps in de novo PC biosynthesis. PCs are produced by two alternative pathways: they can be synthesized either from choline (Kennedy pathway) or by methylation of phosphatidylethanolamine (PE) by PEMT in mammals, or of phosphoethanolamine by the PMT-1 and PMT-2 orthologs in *C. elegans* and plants.[37-39] The Kennedy pathway includes the reactions performed by the *C. elegans* proteins PCYT-1 and CEPT-1.[40] In mammals, these enzymes are named phosphocholine cytidylyltransferase (CCT) and cholinephosphotransferase (CPT), respectively, with CCT carrying out the rate-limiting step.[41] The PC biosynthesis enzymes just mentioned have the following intracellular localizations: SAMS-1 is cytosolic;[42,43] PMT-1 and PMT-2 work together and are also likely cytosolic;[38,44] PCYT-1 is present in the nucleus where it may be connected to lipid signaling or simply sequestered in its inactive form, and associates with ER membranes where it may be regulated by bilayer elastic stress;[45-48] CEPT-1 is associated with the ER and nuclear membranes.[45]

paqr-2 suppressors and FA metabolism

SBP-1 activation has two primary outcomes: the production of elongated and unsaturated fatty acids, and the accumulation of stored fat.[49,50] The positive regulation of *elo-2*, *fat-5*, *fat-6*, and *fat-7* by SBP-1 is supported by quantitative PCR and in vivo GFP reporters,[49] and a role for SBP-1 as promoter of fat storage is evidenced by visualization of fat depots in *sbp-1* mutants.[49,50] The mediator subunit MDT-15 is required for SBP-1's ability to control lipid homeostasis in *C. elegans*,[51] and also interacts with NHR-49 to mediate its effects on fatty acid metabolism and response to dietary stress that, like SBP-1, include activation of the Δ9 desaturases.[52,53] ECH-7 and HACD-1 are the worm homologs of the mammalian enoyl CoA hydratase and L-3-hydroxyacyl CoA dehydrogenase, mitrochondrial enzymes responsible for two of the four recurring steps of β-oxidation of fatty acids.[54,55] *lof* mutations in these genes would be expected to decrease the rate of FA oxidation, hence increasing the time during which they are available to be modified by Δ9 desaturases. AAK-2 (the *C. elegans* AMP-activated kinase α2 catalytic subunit) acts to inhibit ATGL, hence slowing down energy utilization during periods of starvation;[56,57] *lof* mutations in this gene also partially suppress *paqr-2* phenotypes,[34] and could act by releasing stored FAs for further exposure to Δ9 desaturases.

Connecting PC biosynthesis and FA metabolism

The profound regulatory effects of PC levels on SREBP activity have recently been discovered and are conserved between *C. elegans* and mammalian cells,[58] although the precise mechanism is not yet clear. Our own work on PAQR-2 and its role in cold adaptation suggest that decreasing PC synthesis may be essential for cold adaptation by allowing adaptive remodeling of the structural lipids via elongases and desaturases regulated by SBP-1 and NHR-49.

The identity of the *paqr-2* suppressors suggest that this adiponectin receptor homolog is a regulator of fatty acid metabolism in *C. elegans*. Furthermore, our results suggest a specific role for PAQR-2 in the regulation of membrane fluidity during cold adaptation in *C. elegans*: in the suppressor mutants there is a dramatic increase in the expression of a *fat-7* transcriptional reporter that is accompanied by an increase in the abundance of unsaturated FAs.[35] A greater proportion of unsaturated FAs is expected to increase membrane fluidity, which is an important adaptation to cold in poikilotherms. The role of *paqr-2* as a regulator of membrane fluidity via influencing unsaturated FA levels could also explain the tail phenotype: the tail tip, being such a fine and delicate structure, may be particularly sensitive to defects in membrane fluidity. Remarkably, growth of the *paqr-2* mutant was rescued by cultivating the worms in the presence of mild detergents used at concentrations expected to increase membrane fluidity.[35] This observation provides elegant support for the hypothesis that one essential function of *paqr-2* is to increase membrane fluidity during cold adaptation. Modulation of membrane fluidity is of great importance for many cellular processes, including regulating the function and clustering of membrane proteins, such as receptors and ion channels.[59,60]

PAQR-2, Adiponectin, and Cold Adaption

Human subjects wearing a 10 °C liquid-conditioned suit for 2 h have shown a near-doubling of circulating adiponectin levels.[61] Although based on a relatively small study, cold exposure is the only known condition that can cause an acute increase in adiponectin levels. Furthermore, the increase was highest among individuals with the lowest basal levels of adiponectin, i.e., subjects with the highest BMI. Cold-induction of serum adiponectin levels has also been observed in rats kept at 4 °C for 24 h, with adiponectin mRNA levels also becoming elevated in brown adipose tissue (BAT).[62] The induction of adiponectin by exposure to cold and the specific and significant increase of mRNA in BAT suggests that adiponectin might play a role in maintaining body temperature and basal metabolic rate in response to changing environmental conditions. Interestingly, as noted by Scherer and co-workers,[3,63,64] adiponectin shows sequence homology with hibernation-associated plasma proteins (HP-27, HP-25, and HP20) and p88 HRP in the blood of Asian chipmunks (*T. asiaticus*) and hibernating woodchucks, respectively, which suggests that adiponectin may be part of a protein family that functions during cold adaptation, possibly by acting via the AdipoRs. Very few studies have been done that monitor the levels of adiponectin or its several CTRP homologs during hibernation. Based on scant evidence however, it does seem that the levels of adiponectin are lowest during hibernation, just as is the case for the hibernation-associated proteins;[65,66] one effect of this may be to facilitate a drop in body temperature and cause a slow utilization of the fat stores during the many months of hibernation. Interestingly,

some authors have suggested that the common ancestor to all mammals may have been a hibernator.[67,68]

In any case, adiponectin-type proteins may have been regulating fat storage vs. utilization in response to body temperature changes since the dawn of mammals, and probably even before that. It is therefore very interesting that an adiponectin receptor homolog is essential for cold adaptation in *C. elegans*, a poikilotherm. In *C. elegans*, adaptation to cold requires changes to the lipid composition so as to maintain membrane fluidity, as well as other changes that are currently not well defined.[69-71] The *paqr-2* suppressor mutants that we have identified suggest that homeostasis of lipid fluidity during cold adaptation is a primary function of this adiponectin receptor homolog. Given that the last common ancestor between worms and mammals was also a poikilotherm, the ancient function of the adiponectin receptors may perhaps, quite speculatively, be inferred from that of *paqr-2* in *C. elegans*: to maintain membrane fluidity by promoting fatty acid desaturation, with these effects being essential for growth at lower temperature. In homeotherms such as mammals, the AdipoR1 and AdipoR2 have evidently retained their roles as metabolic regulators, and may even have retained a role in cold adaptation if the acute rise in adiponectin serum levels in response to cold exposure have a functional relevance.

Outstanding Question: What Does PAQR-2 Actually Do?

In spite of having identified many *paqr-2* suppressors, we still have no idea of the actual biochemical function of the PAQR-2 protein, nor of its immediate downstream target. A PAQR-type protein exists in yeast, and was found to be associated with a ceramidase activity,[27] a finding that prompted some investigators to examine this possible function for the mammalian AdipoR1/2 proteins.[25] However, our own studies in *C. elegans* give no hint of a connection to ceramides, although it is certainly interesting to point out that ceramides are well known to have profound effects on membrane fluidity. A bioinformatics analysis of PAQR-type proteins led to the proposal that they are part of a much larger protein family, termed CREST (alkaline ceramidase, PAQR receptor, Per1, SID-1, and TMEM8).[72] CREST proteins all have seven transmembrane domains inserted in an inverse orientation compared with GPCRs, i.e., having their N terminus facing the intracellular space, and all seem to act as hydrolases, including proteins with ceramidase and phospholipase A2 activities. One speculates, therefore, that PAQR-2 may also be a hydrolase. Specifically, and with an eye to the pathway that emerges from the study of *paqr-2* suppressors, one interesting possibility is that PAQR-2 may be a phospholipase that uses PCs as its substrates. In this scenario, the end effect of PAQR-2 would be similar to those mutations that inhibit PC synthesis, i.e., reducing PC levels. This hypothesis is particularly seducing in view of the fact that PCYT-1, which is responsible for the rate limiting step in PC synthesis, is regulated by membrane properties, such as curvature and/or fluidity that reflect a need for increased PCs.[48] Thus, PAQR-2 and PCYT-1 could have opposite and complementary roles to fine-tune membrane properties. One way to test this is to examine the potential phospholipase activity of PAQR-2 using in vitro assays. Another is to identify new mutants that phenocopy the *paqr-2* mutant, hoping that they will be more directly informative about the point of contact between *paqr-2* and its downstream pathway. We are pursuing both avenues.

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