Supporting Information

Detailed Protocol for the Yeast-Based Receptor Assay

The details for the following assay, as well as the procedures for cloning human PAQRs, the identity of expression plasmids and the yeast strains used, have all been reported previously (*1-6*). All yeast and human PAQR genes were cloned from either genomic DNA or cDNAs (OpenBiosystems) into pRS316-*GAL1*, pYES260 or pGREG536 via homologous recombination as previously described. All of these expression plasmids use the *GAL1* promoter to drive expression in yeast. All genes were sequenced to ensure that no errors occurred during cloning. To induce expression from the *GAL1* promoter, galactose was added as the sole carbon source in the medium. Changing the amount of galactose can vary expression levels. Maximal expression was induced by adding 2% galactose. At this level of expression, many PAQRs have enough basal signaling capability to induce constitutive signaling. For reduced expression levels, the amount of galactose was limited to 0.05%. To ensure that the medium contains 2% total sugar, medium with 0.05% galactose was augmented with 1.95% raffinose. At this lower level of expression, none of the PAQRs posses enough basal signaling to affect the downstream signaling pathway in the absence of agonist ligand.

In yeast, PAQR receptors activate a pathway that results in the negative regulation of several genes. One of these genes, *FET3*, encodes a ferroxidase involved in iron uptake. When yeast are grown in medium that is made iron-limiting using EDTA as a chelator (Low Iron Medium, LIM), the *FET3* gene is induced. We discovered that, when PAQR receptors are overexpressed or activated, the *FET3* gene was repressed even under iron-limited conditions where it is supposed to be induced. We have obtained a *FET3-lacZ* promoter-reporter fusion construct that can be used as an indirect reporter for receptor activity. When the signaling pathway is on, β-galactosidase activity produced from the *FET3-lacZ* reporter is low. When the signaling pathway is off, β-galactosidase activity produced from the *FET3-lacZ* reporter is high. We demonstrated that β-galactosidase activity responds to agonist levels in a dose-dependent manner.

Expression vector and *FET3-lacZ* were co-transformed into wild type yeast (BY4742, Euroscarf). Overnights of these cells were inoculated into iron-limited LIM an allowed to grow to mid-log phase (~20 hours) at which time cells were harvested, washed and permeabilized. Then β-galactosidase activity in the supernatant was measured. Globular adiponectin (Biovendor Laboratory Medicine, Inc. #RD172029100), TNFα (Chemicon International #GF023) and progesterone were added to the cultures at various concentrations upon inoculation of the cells. Progesterone was added from an ethanol stock. In experiments where progesterone was added, untreated control cells were actually treated with an equivalent volume of ethanol to control for vehicle effects. All experiments were performed at least three times to ensure consistency and the figures show a representative experiment. For individual experiments, three separate colonies were picked and cultured identically to generate error bars, which indicate ± one standard deviation. β-galactosidase activity was generally plotted as a percentage of β-galactosidase activity measured in untreated cells carrying *FET3-lacZ* and an empty expression vector. ID_{50} and ED_{50} values were calculated using the web-based software, BioDataFit 1.02 (www.changbioscience.com) using a sigmoidal fit.

References:

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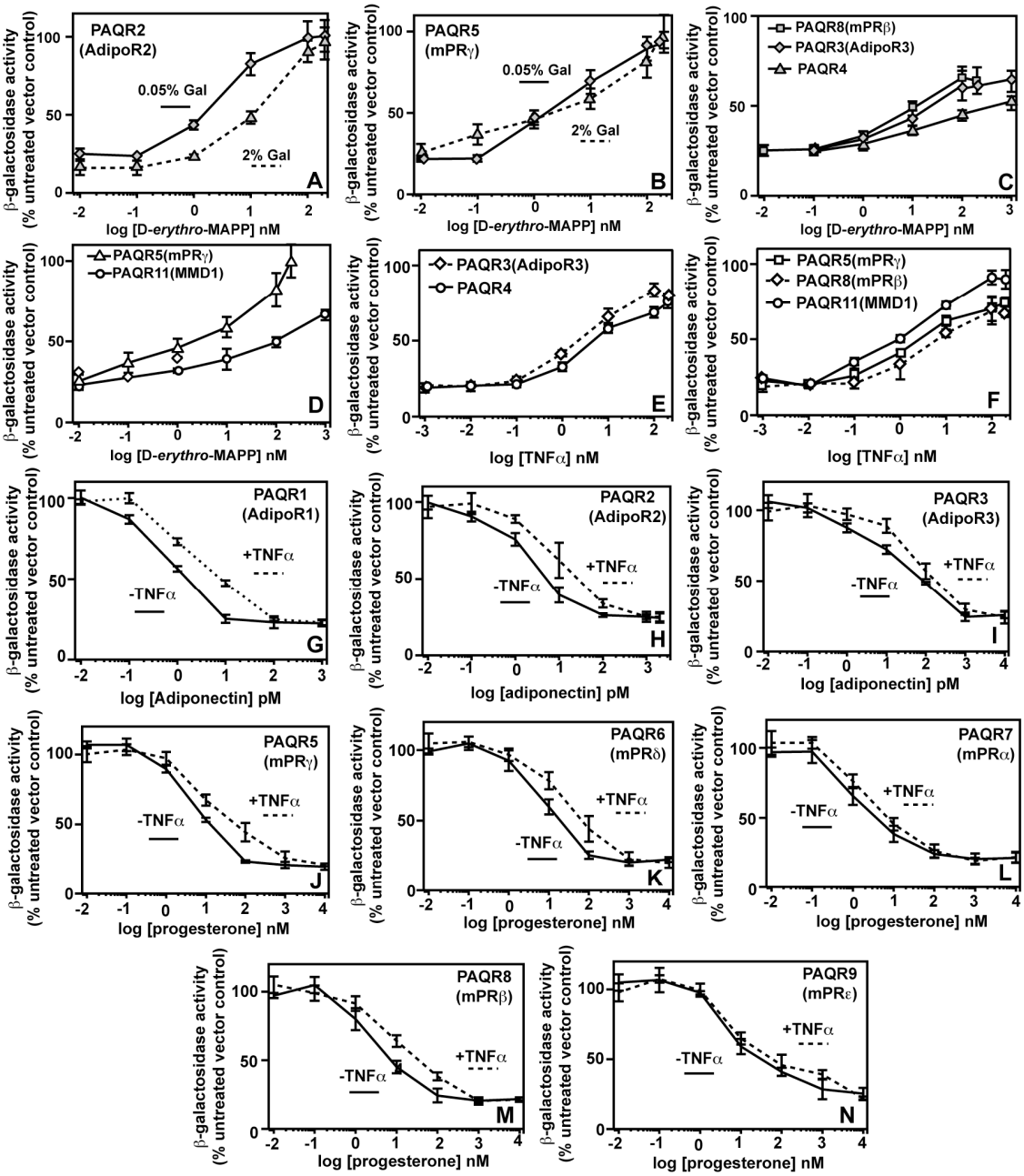
Supporting Figure



Supporting Figure: (A)-(D) MAPP inhibits basal and agonist-dependent signaling of human PAQRs. **(A)** MAPP inhibition of adiponectin-dependent signaling of PAQR2/AdipoR2 at different expression levels. Medium contains either 2% galactose for maximal AdipoR2 expression or 0.05% galactose for low-level AdipoR2 expression. AdipoR2 does not possess basal signaling at any expression level so 100 pM adiponectin is added in each case to induce signal transduction. **(B)** MAPP inhibition of basal signaling of PAQR5/mPRγ at different expression levels. Medium contains either 2% galactose for maximal mPRγ expression or 0.05% galactose for low-level mPRγ expression. At maximal expression, mPRγ does not need agonist at low expression levels, 100 nM progesterone is added to induce signal transduction. **(C)-(D)** MAPP inhibition of basal signaling via PAQR3/AdipoR3, PAQR4, PAQR5/mPRγ, PAQR8/mPRβ and PAQR11/MMD1. Medium contains 2% galactose to maximally induce PAQR expression. No agonist is added. **(E)-(F)** TNFα inhibits basal signaling of human PAQRs. Medium contains 2% galactose for maximal PAQR expression. No agonist added. **(E)** PAQR3/AdipoR3 and PAQR4 **(F)** PAQR5/mPRγ, PAQR8/mPRβ and PAQR11. **(G)-(N)** TNFα antagonizes agonist-dependent signaling of human PAQRs. Medium contains 0.05% galactose for low-level PAQR expression. At this expression level all PAQRs require agonist to activate the downstream signaling pathway. Graphs show dose-dependent agonist induction of each PAQR in the absence of TNFα or in the presence of 100 nM TNFα. Agonist is adiponectin for PAQR1/AdipoR1 **(G)**, PAQR2/AdipoR2 **(H)** and PAQR3/AdipoR3 **(I)**. Agonist is progesterone for PAQR5/mPRγ **(J)**, PAQR6/mPRδ **(K)**, PAQR7/mPRα **(L)**, PAQR8/mPRβ **(M)** and PAQR9/mPRε **(N)**.