AdipoR1 and AdipoR2 Maintain Membrane Fluidity in Most Human Cell Types and Independently of Adiponectin

Authors: Mario Ruiz¹, Marcus Ståhlman², Jan Borén² and Marc Pilon¹*  
#These authors contributed equally.

Affiliations: ¹Department of Chemistry and Molecular Biology, University of Gothenburg, Gothenburg, Sweden, ²Department of Molecular and Clinical Medicine/Wallenberg Laboratory, Institute of Medicine, University of Gothenburg, Gothenburg, Sweden.

*Corresponding author

Contact information for corresponding author

Marc Pilon  
Dept Chemistry and Molecular Biology  
University of Gothenburg, Box 462  
S-405 30 Gothenburg  
Sweden

Tel: +46 31 7863279  
Email: marc.pilon@cmb.gu.se

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Abbreviations: FRAP, fluorescence recovery after photobleaching; HUVEC, human umbilical vein endothelial cells; LCPUFA, long-chain PUFA; OA, oleic acid; PA, palmitic acid, PC; phosphatidylcholine, PE, phosphatidylethanolamine; SFA, saturated fatty acid; TAG, triacylglyceride,
ABSTRACT

The fatty acid composition of phospholipids must be tightly regulated to maintain optimal cell membrane properties and compensate for a highly variable supply of dietary fatty acids. Earlier studies have shown that AdipoR2 and its homolog PAQR-2 are important regulators of phospholipid fatty acid composition in HEK293 cells and C. elegans, respectively. Here we show that both AdipoR1 and AdipoR2 are essential to sustain desaturase expression and high levels of unsaturated fatty acids in membrane phospholipids of many human cell types, including primary human umbilical vein endothelial cells (HUVEC), and to prevent membrane rigidification in cells challenged with exogenous palmitate, a saturated fatty acid. Three independent methods confirm the role of the AdipoRs as regulators of membrane composition and fluidity: fluorescence recovery after photobleaching (FRAP), measurements of Laurdan dye generalized polarization, and mass spectrometry to determine the fatty acid composition of phospholipids. Furthermore, we show that AdipoRs can prevent lipotoxicity in the complete absence of adiponectin, their putative ligand. We propose that the primary cellular function of AdipoR1 and AdipoR2 is to maintain membrane fluidity in most human cell types and that adiponectin is not required for this function.

Keywords: fatty acid/desaturases, membranes/fluidity, phospholipids/metabolism, lipotoxicity, receptors/plasma membrane, adiponectin receptor, palmitate.
INTRODUCTION

The AdipoR1 and AdipoR2 proteins were initially identified as putative adiponectin receptors using fluorescent labelled recombinant adiponectin as bait when screening a cDNA library expressed in Ba/F3 murine cells (1). These proteins are expressed in most/all tissues, localize to the plasma membrane and contain seven transmembrane domains oriented such that their N-terminus is cytosolic and their C-terminus is extracellular (1). Since their discovery, several high-profile reports using AdipoR1/2 single or double knockout mice have shown that the AdipoRs regulate metabolism, and in particular may improve insulin response and generally protect against metabolic syndrome complications, especially during high fat diet challenges (2-4). However, other reports have not reproduced these findings. In particular, one study found that AdipoR1 and AdipoR2 may have opposite effects on metabolism (5), another showed that the double mutants are embryonic lethal (6), and other studies noted specific defects only in the retina of AdipoR1 mutant mice (7, 8). Finally, a careful study of recombinant adiponectin, which is often used as a mean of activating the AdipoRs in published experiments, suggested that any detected biological effect may be caused by the presence of contaminants such as LPS (9), which adds to the confusion regarding the AdipoR literature.

More recently, the crystal structure of the AdipoRs has been solved and suggests that they may be hydrolases: a cavity opening towards the cytoplasm is a likely site for substrate entry and product exit (10). By homology with a yeast homolog, Izh2 (11, 12), the AdipoRs were proposed to act as ceramidases (13, 14). However, the ceramidase activity is at best a slow reaction (15) and it is possible that the AdipoRs may have other substrate specificities. Nevertheless, that the AdipoRs are hydrolases of some sort seems very likely since that is a conserved activity of the large CREST family of related proteins that include the AdipoRs as well as phospholipases such as the yeast Per1p, which has phospholipase A2 activity (16).

Several years ago, we began studying the *C. elegans* homologs of the AdipoRs with the hope that leveraging forward genetics in this model organism would lead to new, unbiased insights into their functions. Our findings so far may be briefly summarized as follows: 1) *C. elegans* has two AdipoR homologs, namely PAQR-1 and PAQR-2 (17); 2) Worms lacking PAQR-2 show a morphology defect in the thin membranous tail tip, and are intolerant to cold (15°C) or to saturated fatty acid (SFA)-rich diets (17-19); 3) The primary function of PAQR-2 is to maintain membrane fluidity in response to membrane-rigidifying challenges such as cold or SFA-rich diets (18-20); 4) PAQR-2 function depends on a dedicated protein partner, namely the single-pass transmembrane protein IGLR-2 with which it colocalizes (18); 5) PAQR-2 can maintain systemic membrane homeostasis cell nonautonomously via lipid exchange among *C. elegans* tissues (21); and 6) PAQR-2 deficient worms can be chemically rescued by small amounts of detergents or genetically...
rescued by mutations that promote fatty acid desaturation or that increase the levels of phospholipids containing long-chain polyunsaturated fatty acids (LCPUFAs) (20, 22). It is also important to note here that there no *C. elegans* homolog of adiponectin has yet been identified and that after several screens for *paqr* 2 mutant suppressors or genocopi erers, we have found no evidence that PAQR-2 depends on a ligand for its function.

Recently, we found that several of our observations in *C. elegans* hold true also in mammalian cells. In particular, AdipoR2 is essential to maintain membrane fluidity in HEK293 cells challenged with the SFA palmitic acid (PA; 16:0) and is also able to maintain membrane homeostasis cell nonautonomously (19, 21). Furthermore, and just as in *C. elegans*, HEK293 cells where AdipoR2 has been knocked down can be rescued by providing LCPUFAs exogenously or by inhibiting expression of the plasma-membrane localized TLCD1 or TLCD2 proteins (22). The TLCDs function as limiters of LCPUFA incorporation into phospholipids, and their inhibition therefore leads to enhanced incorporation of LCPUFAs which promotes fluidity (22).

The present study focuses on exploring further the roles of AdipoR1 and AdipoR2 in regulating membrane homeostasis in human cells. Specifically, we ask whether both AdipoR1 and AdipoR2 act as fluidity regulators, and whether many cell types, including primary human cells, require the AdipoRs to regulate membrane fluidity. Finally, we investigate whether adiponectin is required for the ability of the AdipoRs to regulate membrane homeostasis.
MATERIALS AND METHODS

Cell Culture
HEK293, HepG2 and 1321N1 cells were grown in DMEM containing glucose 1 g/l, pyruvate and GlutaMAX and supplemented with 10% fetal bovine serum, 1% non-essential amino acids, HEPES 10 mM and 1% penicillin and streptomycin (all from Life Technologies) at 37°C in a water humidified 5% CO2 incubator. Cells were sub-cultured twice a week at 90% confluence. HEK293, HepG2 and 1321N1 cell lines were authenticated by Eurofins. HUVEC (passages 1 to 5) were obtained from Gibco and cultivated as described in (23). Briefly, cells were grown in M200 medium (Gibco) containing the Low Serum Growth Supplement (Gibco) and 1% penicillin and streptomycin. Cells were sub-cultured twice a week at 90% confluence. TrypLE Express reagent (Gibco) was used to detach HUVEC, HEK293 and 1321N1 cells and Accutase (GE Healthcare) to detach HepG2 cells. All cell types were cultivated on treated plastic flask and multi-dish plates (Nunc). For microscopy experiments, cells were seeded in glass bottom dishes (Ibidi) pre-coated with 0.1% porcine gelatin (Sigma).

Fatty acid treatment
PA, OA and EPA were dissolved/diluted in sterile DMSO (Sigma) then mixed with fatty acid-free BSA (Sigma) in serum-free medium for 15 min at room temperature. The molecular ratio of BSA to PA was 1 to 5.3 in experiments using 400 µM PA, 1 to 2.65 when 200 µM PA was used, and 1 to 0.66 when 50 µM PA. Cells were then cultivated in serum-free media containing the fatty acids for 24 h prior to analysis.

siRNA treatment
The following pre-designed siRNAs were purchased from Dharmacon: AdipoR1 J-007800-10, AdipoR2 J-007801-10, Non-target D-001810-10 and SCD J-005061-07. HEK293, HepG2 and 1321N1 cells transfection was performed in complete media using 25 nM siRNA and Viromer Blue according to the manufacturer’s instructions 1X (Lipocalyx). HUVEC were transfected using 10 nM siRNA and Lipofectamine™ RNAiMAX Transfection Reagent following the HUVEC optimized protocol from the manufacturer (Invitrogen). Knockdown gene expression was verified 24 h or 48 h after transfection.

Glucose treatment
Cells were grown in complete media at 5, 25 or 100 mM glucose (Sigma) for 48 h and then switched to serum-free medium supplemented with BSA (0.5%), but maintaining the glucose concentration constant at 5, 25 or 100 mM for another 24 h and then analysed.

Quantitative PCR
Total cellular RNA was isolated using RNeasy Kit according to the manufacturer’s instructions (Qiagen) and quantified using a NanoDrop spectrophotometer (ND-1000; Thermo Scientific). cDNA was obtained using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) with random hexamers. qPCR was performed with a CFX Connect thermal cycler (Bio Rad) using Hot FIREpol EvaGreen qPCR SuperMix (Solis Biodyne) and standard primers. Samples were measured as triplicates. The relative expression of each gene was calculated according to the ΔΔCT method (24) Expression of the housekeeping gene PPIA was used to normalize for variations in RNA input. Primers used were: AdipoR1-For (CCATCTGCTTGGTTTTCGTGC) and -Rev (AGACGGTGGAAGAGCCAG), AdipoR2-For (TCATCTGTGTGCTGGGCCATT) and -Rev (CTATCTGCCCATATGGTTGGCG), PPIA-For (GTCTCCTTTGAGCTGTGGTCAG) and -Rev (GGACAAGATGCCAGAACCC), SCD-For (TTCGGTGCCACTTCTTGGCG) and -Rev (TGGTGAGTAGTTGGGAAGGCC), FADS-1-For (TGGCTAGTGATCGACGGTAA) and -Rev (GGCCCTTTGTGTGATGGAAG), FADS-2-For (GGGCCGTAGCTACTACATCG) and -Rev (ACAAACCAGGGCGCTCCTCCAG). qPCR for Adiponectin was executed on a QuantStudio7 Flex Real-Time PCR System thermal cycler using Power SYBR Green PCR Master Mix (Applied Biosystems). Two sets of primers for Adiponectin were used (i) Adiponectin-For (AGGGCATCCGGGCCATAAT) and -Rev (CTCCGGTTTCACCGATGTCT) and (ii) Adiponectin-For (TGGTGAGAAGGGTGAGAAAGG) and -Rev (CTCCAATCCCACACTGAATGC). The second set of Adiponectin primers was kindly shared by Matthew Harms. cDNA from human subcutaneous abdomen adipose tissue and human breast adipose tissue was a kind gift of Xiao-Rong Peng and Henrik Palmgren (AstraZeneca, Gothenburg); samples of adipose tissues were collected from patients undergoing elective surgery at Sahlgrenska University Hospital in Gothenburg, Sweden. All study subjects received written and oral information before giving written informed consent for the use of the tissue. The studies were approved by The Regional Ethical Review Board in Gothenburg, Sweden.

Protein Samples and Western Blot

Cellular proteins were extracted using a lysis buffer (recipe: 1% Nonidet P-40, 0.1% SDS, 10% Glycerol, 1% Na-deoxycholate, 1 mM DTT, 1 mM EDTA, 100 mM HEPES, 100 mM KCl) containing Halt Protease Inhibitor Cocktail (1x, Pierce) on ice for 10 min. Upon lysis completion, cell lysates were centrifuged at 13,000 rpm for 10 min at 4 °C. The soluble fraction was kept for further analysis and the protein sample concentration was quantified using the BCA protein assay kit (Pierce) according manufacturer’s instructions. 20 µg of protein were mixed with Laemmli Sample Loading Buffer (Bio Rad), heated to 37 °C for 10 minutes and loaded in in 4-15% gradient pre-casted SDS-gels (Bio-Rad). After electrophoresis, the proteins were transferred to nitrocellulose membranes using Trans Blot Turbo Transfer Packs and a Trans
Blot Turbo apparatus - predefined Mixed-MW program- (Bio Rad). Blots were blocked with 5% non-fat dry milk in PBS for 1 hour at room temperature. Blot were incubated with anti-human AdipoR1 rabbit IgG (IBL #18993) at 1 µg/ml in 5% non-fat dry milk in PBS-T (0.05% Tween-20) overnight at 4 °C (following Sluch et al. 2018 recommendations (8)). Then, blots were washed with PBS-T and incubated with a swine anti-rabbit immunoglobulins/HRP (Dako #P0399, 1:3000 dilution) and washed again with PBS-T. Blots were developed with ECL (ImmobilonTM Western, Millipore), and the signal visualized with a digital camera (VersaDoc, BioRad). Then, blots were stripped and reproved with anti GAPDH (14C10) rabbit IgG (1:2500 dilution, Cell Signaling #2118). PageRuller Plus prestained protein ladder was used to assess molecular weight (Thermo Scientific). Western blots were quantified by densitometry using Image Lab v.6 software.

Lipidomics
Samples were prepared as previously described in (19, 22). Briefly, cells were cultivated in serum-free media with or without fatty acids for 24 h prior to harvesting using TrypLE Express (Gibco). For lipid extraction, the pellet was sonicated for 10 minutes in methanol and then extracted according to published methods (25). Internal standards were added during the extraction. Lipid extracts were evaporated and reconstituted in chloroform:methanol [1:2] with 5 mM ammonium acetate. This solution was infused directly (shotgun approach) into a QTRAP 5500 mass spectrophotometer (Sciex) equipped with a Nanomate Triversa (Advion Bioscience) as described previously (26). Phospholipids were measured using multiple precursor ion scanning, which gathers information about individual phospholipids by scoring many fatty acid fragments in the Q3 of the mass spectrometer (27, 28), and triacylglycerols (TAGs) were measured using neutral loss scanning (29). The data was evaluated using the LipidView software (Sciex). The complete lipidomics dataset is provided in the supplementary Table S1.

FRAP
Live HEK293 cells were stained with BODIPY 500/510 C1, C12 (4,4-difluoro-5-methyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoic acid; BODIPY-C12) (Invitrogen). FRAP images were acquired with an LSM880 confocal microscope equipped with a live cell chamber (set at 37° and 5% CO₂) and ZEN software (Zeiss) with a 40× water immersion objective as previously described (19, 21, 22).

Laurdan dye measurement of membrane fluidity
Live cells were stained with Laurdan dye (6-dodecanoyl-2-dimethylaminonaphthalene) (Thermo Fisher) at 15 µM (HEK293 and HepG2 cells) or 10 µM (HUVEC) for 45 min. Images were acquired with an LSM880 confocal microscope equipped with a live cell chamber (set at 37° and 5% CO₂) and ZEN software (Zeiss).
with a 40× water immersion objective as described before (21). Cells were excited with a 405 nm laser and the emission recorded between 410 and 461 nm (ordered phase) and between 470 and 530 nm (disordered phase). Pictures were acquired with 16 bits image depth and 1024 × 1024 resolution, using a pixel dwell of ∼1.02 µsec. Images were analyzed using ImageJ version 1.47 software (30), following published guidelines (31).

**Trypan Blue staining**

After 24 h of treatment, cell supernatant was collected, cells detached and mixed again with their respective supernatant. Then, the cell suspension was mixed 1:1 with a 0.4 % trypan blue solution (Gibco) and loaded in a hemacytometer and examined immediately under the microscope. The percentage of positive and negative cells in at least 4 quadrants was registered.

**Adiponectin (aka AdipoQ) treatment**

Recombinant full-length adiponectin (produced in HEK293 and forming high molecular weight and hexameric species) was purchased from Enzo (ALX-522-063) and used at 5 (as in (14)) or 10 µg/ml. Cells were then cultivated in serum-free media containing adiponectin and 400 µM PA for 24 h prior to analysis.

**Statistics**

t-tests were used to identify significant differences between treatments/genotypes. Error bars show the standard deviation (SD) in histograms and standard error of the means (SEM) in FRAP curves. Asterisks (*) and hash signs (#) are used in the figures to indicate various degrees of significance, where *: p<0.05; **: p<0.01; and ***: p<0.001.
RESULTS

Both AdipoR1 and AdipoR2 regulate membrane fluidity in HEK293 cells

We used siRNA to efficiently knock down the expression of AdipoR1 and AdipoR2 singly or simultaneously in HEK293 cells (derived from human embryonic kidney cells); qPCR confirmed knockdown of the transcripts (Fig. 1A) and a Western blot confirmed protein downregulation for AdipoR1 (Supplemental Fig. S1A); we could not obtain a useful antibody for AdipoR2. Knockdown of AdipoR1 and/or AdipoR2 had no effect on membrane fluidity of the cells under basal culture condition as determined using Fluorescence Recovery After Photobleaching (FRAP; Supplemental Figure S1B-E). Note that the FRAP method measures the lateral diffusion rate of the BODIPY-C12 fluorophore used in these experiments; throughout this article we use the term “membrane fluidity” as a proxy for what is likely a complex phenomenon reflecting several distinct membrane biophysical properties such as fluidity, phase behaviour, thickness or compressibility (32-34). siRNA knockdown of AdipoR1 also had little effect on the membrane fluidity of HEK293 cells challenged with 200 µM palmitic acid (PA), but inhibiting AdipoR2 caused a clear loss of membrane fluidity under these conditions (Fig. 1B-C), while inhibiting both AdipoR1 and AdipoR2 had the most dramatic effect (Fig. 1D-E). These results demonstrate that AdipoR2 is more important to protect against the rigidifying effects of PA but that AdipoR1 also contributes to maintenance of membrane fluidity. In other words, AdipoR1 and AdipoR2 have partially redundant functions in membrane homeostasis. Most studies of the effects of PA on cells require relatively high concentrations to elicit a detectable effect, with 400 µM being a typical concentration. The pronounced sensitivity of the double AdipoR1/2 siRNA treated cells to 200 µM PA suggest that these proteins are a primary mechanism protecting cells against PA lipotoxicity. Indeed, HEK293 cells treated with double AdipoR1/2 siRNA show a dramatic loss of membrane fluidity even when challenged with as little as 50 µM PA (Fig. 1F-G), which is exceptionally low compared to other published studies. Note that AdipoR2 knockdown causes membrane rigidification when cells are challenged with 200 µM palmitate but not when treated with vehicle alone, and that this palmitate-induced rigidification in AdipoR2 knockdown cells can be prevented by including equimolar amounts of the monounsaturated fatty acid (MUFA) oleic acid (OA), suggesting that a function of the AdipoRs during palmitate challenge is to restore SFA/UFA balance (Supplemental Figure S1F-J). The loss of membrane fluidity in AdipoR1/2 siRNA-treated cells challenged with 200 µM PA can also be entirely suppressed by the inclusion of as little as 5 µM of the LCPUFA eicosapentaenoic acid (EPA; 20:5) in the culture media (Fig. 1H-I); EPA is a potent fluidizing fatty acid and its ability to restore membrane fluidity in the AdipoR1/2 siRNA-treated cells is consistent with these proteins playing a role in regulating the FA composition of phospholipids.

The Laurdan dye method confirms the roles of AdipoR1/2 in membrane homeostasis
Our measurements of membrane fluidity have so far relied heavily on the FRAP method. To guard against any misleading interpretations, it is important to verify critical results with independent methods. Therefore, we also made use of the Laurdan dye method to monitor membrane fluidity. This method relies on the fact that the membrane-bound Laurdan dye emits fluorescent light at different wavelengths when water is present within the phospholipid bilayer, which happens more readily in fluid membranes. This method has the additional advantages that multiple cells are imaged simultaneously, that subcellular regions with increased rigidity can be identified and that the images can be scored quantitatively using an automated ImageJ script (31). Analysis of membrane fluidity using the Laurdan dye method corroborates the findings using the FRAP method with the exception that it can now detect a role for AdipoR1. Specifically, we found that siRNA knockdown of AdipoR1 or AdipoR2 singly or together leads only to a minor membrane rigidification under basal conditions (Supplemental Figure S1K-G), but that both AdipoR1 and AdipoR2 are required to maintain membrane fluidity when HEK293 cells are challenged with 200 µM PA (Fig. 2A-C). Furthermore, inhibiting both simultaneously leads to a much more severe rigidifying effect of PA (Fig. 2A-C), which indicates that AdipoR1 and AdipoR2 have overlapping functions. Also, we noted that the plasma membrane appears to be most affected by rigidification when AdipoR1/2 are inhibited. This is particularly interesting because AdipoR1 and AdipoR2 are localized to the plasma membrane and may have an especially important function in maintaining fluidity in that membrane.

AdipoR1 and AdipoR2 promote membrane fluidity via several desaturases

We have previously shown that the C. elegans paqr-2 mutant has an excessively high SFA/UFA ratio among phospholipids and is unable to stimulate FA desaturation upon membrane-rigidifying challenges (cold or SFA-rich diets). This role in membrane homeostasis is also conserved for AdipoR1 and AdipoR2 in human cells. siRNA against AdipoR1 or AdipoR2 causes HEK293 cells to have an excess SFAs in their phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs) both under basal conditions and even more so when challenged with 200 µM PA, and this effect is increased when AdipoR1 and AdipoR2 are simultaneously inhibited (Fig. 3A-B and Supplemental Figure S2). The change in FA composition of the phospholipids correlates with reduced desaturase expression: AdipoR1 siRNA-treated cells have reduced expression of SCD and FADS2 while AdipoR2 siRNA-treated cells have reduced expression of SCD, FADS1 and FADS2 (Fig. 3C). Not surprisingly then, knockdown of AdipoR1 and /or AdipoR2 increases the lipotoxicity of PA: while 200 µM palmitate is well-tolerated by HEK293 cells treated with non-target siRNA, knockdown of the AdipoRs greatly increases the number of dead cells in the presence of PA (but not in basal media; Fig. 3D). Altogether these results indicate that the AdipoRs redundantly maintain membrane fluidity in PA-challenged HEK293 cells by regulating the expression of desaturases, and that this activity is essential to prevent lipotoxicity by SFAs. Accordingly, siRNA against AdipoR1 or AdipoR2
also causes HEK293 cells to have an excess SFAs in triacylglycerides (TAGs) (Fig. S2C-D), which again likely reflects decreased desaturase activity.

The *C. elegans paqr-2* mutant, which lack a functional PAQR-2 protein that is homologous to the AdipoRs, is unable to grow in the presence of glucose, even at concentrations as low as 4 mM. This is because the glucose is converted to SFAs by the dietary *E. coli*, which leads to membrane rigidification in the worms. In other words, glucose in itself has no effect on the *paqr-2* mutant. Similarly, glucose has no effect on the membrane fluidity of AdipoR1 and/or AdipoR2 siRNA-treated HEK293 cells even when applied at concentrations of 100 mM, and also had no effect on the SFA and MUFA content in the PCs, PEs or TAGs of cells treated with AdipoR2 siRNA (Supplemental Figure S3). We conclude that, as with the *C. elegans* PAQR-2, the AdipoRs are required specifically to respond to the rigidifying challenge posed by exogenous SFAs, and not for glucose tolerance.

**AdipoR1 and AdipoR2 maintain membrane fluidity in several cell types**

The AdipoRs are widely expressed and it is therefore possible that they are important regulators of membrane fluidity in many, and perhaps even most cell types. To explore this possibility, we investigated the effect of AdipoR1 and/or AdipoR2 siRNA on hepatocyte-derived HepG2 cells and astrocyte-like 1321N1 cells. siRNA knockdown in HepG2 cells reduced AdipoR1 mRNA levels by nearly 90% and AdipoR2 levels by more than 50% (Supplemental Figure S4A); siRNA knockdown of AdipoR1 caused a ~60% reduction in protein levels (Supplemental Figure S4B-C). Knockdown of AdipoR1 alone had little effect on the membrane fluidity (measured with the Laurdan dye method) of HepG2 cells in basal media or media supplemented with 200 µM PA (Fig. 4A-C and Supplemental Figure S4D-F). In contrast, AdipoR2 knockdown caused a reduced membrane fluidity both in basal and PA-supplemented media, and this effect was greatly increased when AdipoR1 and AdipoR2 were simultaneously knocked down (Fig. 4A-C and Supplemental Figure S4D-F). As in HEK293 cells, knockdown of AdipoR1 and/or AdipoR2 resulted loss of cell viability upon PA challenge (Supplemental Figure S4G) and an increase in SFAs at the expense of MUFAs in PCs, PEs and TAGs, especially when the cells are challenged with PA (Supplemental Figure S5H-M). Very similar results were obtained with the 1321N1 cell line (Supplemental Figure S4N-U). In summary, these results suggest that AdipoR1 and AdipoR2 are redundantly essential to maintain membrane fluidity in all three cell lines tested, each representing a different type of cells, namely embryonic kidney cells, hepatocytes and astrocytes. Maintenance of membrane fluidity is likely the fundamental function of the AdipoRs, and is particularly required upon membrane-rigidifying challenges such as when SFAs are provided exogenously.
The AdipoRs maintain membrane fluidity in human primary cells independently of adiponectin

Established cell lines harbour a multitude of mutations and are abnormal in many ways, including in lipid metabolism. It was therefore important to verify our key findings in human primary cells. We began by optimizing knockdown of AdipoR1 and/or AdipoR2 in HUVEC (human umbilical vein endothelial cells) cells, being able to inhibit >90% of their expression of (Fig. 5A). We then also optimized the Laurdan dye method for HUVEC cells and found that it clearly detected membrane rigidification when these cells were challenged with 400 µM PA (Supplemental Figure S5A-C), which is the high concentration most often used to rigidify cell membranes in control cells. Knockdown of AdipoR1 alone had little effect on the HUVEC cells challenged with 200 µM PA (Fig. 5B-D). In contrast, AdipoR2 knockdown caused a reduced membrane fluidity in 200 µM PA-supplemented media, and this effect was greatly increased when AdipoR1 and AdipoR2 were simultaneously knocked down (Fig. 5B-D). Knockdown of AdipoR1 and/or AdipoR2 had no effect on the membrane fluidity of HUVEC cells grown in basal media (Supplemental Figure S5D-F). We conclude that AdipoR1 and AdipoR2 are essential to maintain membrane fluidity in primary human endothelial cells, especially in the presence of exogenous SFAs.

As with the HEK293 cells, inhibition of either AdipoR1 and AdipoR2 led to reduced expression of the FA desaturases SCD, FADS1 and FADS2 (only SCD was not downregulated by AdipoR1 siRNA; Fig. 5E). Desaturase activity is essential for the maintenance of membrane fluidity upon a PA challenge. In particular, SCD inhibition by siRNA (Supplemental Figure S5G) led to decreased membrane fluidity of HUVEC cells incubated with 200 µM PA, but not in basal media (Supplemental Figure S5H-M).

Finally, we note that adiponectin, a proposed ligand for the AdipoRs, was never included in any of our experiments. Indeed, most experiments involved long period of incubations in serum-free media (basal) or serum-free media supplemented with PA and there is likely no expression of adiponectin from the tested cells themselves since this is an adipocyte-specific protein. As definitive test we performed qPCR analysis using mRNA from the three studied cell lines (HEK293, HepG2 and 1321N1), HUVEC cells and human adipose tissue. Using this assay, we could detect strong adiponectin expression in the adipocyte sample and no expression in the cell lines or HUVEC cells; the house-keeping control gene PPIA was detected in all samples (Table 1 and Supplemental Figure S6A). Supplementing HUVEC cells with 5 (as in (14)) or 10 µg/ml recombinant adiponectin (produced from a mammalian cell expression system) did not improve their ability to prevent membrane rigidification by 400 µM PA (the lowest concentration that reduced fluidity in control HEK293 cells), suggesting that adiponectin does not potentiate the activity of the AdipoRs, at least in this context (Supplemental Figure S6B-C). We conclude from this that adiponectin is not required for the ability of the AdipoRs to act as regulators of membrane fluidity in human cells.
DISCUSSION
The three most important conclusions from the present work are: 1) Both AdipoR1 and AdipoR2 maintain membrane fluidity by promoting desaturase activity, hence MUFA levels in phospholipids; 2) AdipoR1 and AdipoR2 help maintain membrane fluidity in multiple cell types, including primary human endothelial cells; and 3) Adiponectin is not required for the function of the AdipoRs as fluidity regulators. These conclusions lead to a simple model of AdipoR function (Fig. 6) and will now be discussed in the broader context of existing literature.

That both AdipoR1 and AdipoR2 have similar functions is not surprising given their high degree of high degree of amino acid identity (~66%; (1)). Many tissues express both proteins and it is likely that many cell types also express both, as was the case for all four cell types studied here (HEK293, HepG2, 1321N1 and primary HUVEC cells). Co-expression of two proteins with similar functions provides redundancy, hence robustness, in the maintenance of membrane homeostasis. We note however that in all cell types it was inhibition of AdipoR2 that had the strongest effect on membrane fluidity, suggesting that AdipoR2 plays a larger role than AdipoR1. This is interesting because the AdipoR2 knock out mice are reported to have a more severe phenotype, including an enlarged brain, male sterility, underweight and increased risk of diabetes (5, 35) than the AdipoR1 mice, which primarily show a retina defect that is also found in humans with mutations in AdipoR1 (7, 8, 36, 37), as well as a tendency to gain weight (5). That the two genes have overlapping functions is clear from the fact that double mutant mice lacking both AdipoR1 and AdipoR2 have more severe metabolic defects than either single mutants (2), and may even be embryonic lethal according to another study (6). The situation in mice therefore echoes that in C. elegans where the two AdipoR homologs, paqr-1 and paqr-2, are also partially redundant (the double mutant is more severe than the single mutant) though paqr-2 is clearly more important since it shows strong phenotypes as a single mutant (cold and SFA intolerance, and a morphology defect in the thin membranous tail tip) that are accompanied by loss of membrane fluidity (17).

The knowledge that AdipoR1 and AdipoR2 are important for membrane homeostasis, primarily promoting fatty acid desaturation to restore fluidity, may help better understand several of the mouse mutant phenotypes. For example, the enlarged brain of the AdipoR2 knockout mice may be secondary to an inability to sustain membrane fluidity in the mutant (5). The brain is exceedingly dependent on membrane fluidity for the process of neurotransmitter vesicle trafficking and fusion at synapses (38). Similarly, spermatogenesis is critically dependent on an abundance of fluid membrane, and desaturase mutants are therefore sterile (39). It will be interesting to revisit these and other AdipoR2 knockout mouse phenotypes...
and test the specific hypothesis that they are the result of failures in membrane fluidity homeostasis. Further, several articles have implicated the AdipoRs as being important to prevent metabolic syndrome complications, especially on high fat diets. Given the extensive literature documenting high SFA content and membrane rigidity in diabetics (40, 41), it will be very interesting to see if the alleged protective effects of the AdipoRs may be explained precisely because they help delay or even prevent such membrane rigidification. In particular, pancreatic beta cell function depends on delicate membrane trafficking and fusion events essential for insulin secretion (42). Such processes are very sensitive to membrane rigidification and maintenance of membrane fluidity in beta cells may be an important function of the AdipoRs in the context of the metabolic syndrome.

The AdipoRs were initially identified as adiponectin receptors in a cDNA expression screen to identify clones that would bind fluorescently labelled recombinant adiponectin (1). Later, several studies have suggested that the activity of the AdipoRs, for example in preventing metabolic defects upon high fat diets, were dependent on adiponectin, usually provided in its recombinant, E. coli-expressed form (2). The functionality of recombinant adiponectin has however been questioned (9), and the hypothesis that adiponectin could act as a hormone may be questioned on the grounds that it is simply too abundant a serum protein to serve such a function (adiponectin is present at ug/ml levels compared to other hormones such as insulin or leptin that are present at ng/ml levels), though it is possible that much less abundant multimeric forms with dynamically regulated levels are the true nature of adiponectin as a hormone (43, 44). In any case, the three different cell lines and human primary cells do not express adiponectin and yet we could demonstrate clear functions for both AdipoR1 and AdipoR2 in preventing membrane rigidification by PA; addition of recombinant adiponectin also did not improve the ability of HEK293 cells to maintain membrane fluidity when challenged with palmitate, suggesting that it is not limiting for AdipoR function in these assays. This again echoes the situation in C. elegans, where no adiponectin homolog has so far been identified and where several forward genetic screens for suppressors or genocopiers of paqr-2 have failed to identify a putative ligand (17, 20, 22, 45).

One limitation of our experiments is that we do not know the extent of protein activity reduction achieved by the siRNA treatments. Good antibodies to these proteins have been difficult to produce. For AdipoR1, more than 15 different antibodies were recently rigorously tested and a single one was found to specifically recognize AdipoR1 in a useful way (8). Using this antibody in Western blot shows a >90% and ~50% reduction in AdipoR1 levels in siRNA-treated HEK293 and HepG2 cells, respectively (Supplemental Figures S1A and S4B). We also tried two different AdipoR2 antibodies without success: both produced a very high level of background bands. This likely explains why AdipoR2 antibodies have so rarely been used
in publications to detect the endogenous protein. However we are confident in the siRNA successfully inhibiting AdipoR2 in our experiments for several reasons: 1) Two different siRNA oligo pairs are producing the same membrane rigidification effect (19); 2) The qPCR clearly shows efficient downregulation of the AdipoR2 mRNA in cell lines and primary cells (Fig. 3C and Fig. 5E); 3) The effects of siRNA against AdipoR2 are additive with that of AdipoR1 knockdown; and 4) The membrane rigidification caused by siRNA against AdipoR2 is accompanied by excess SFAs in phospholipids in all cell types tested, just as it is in *C. elegans* (19, 20).

Finally, it is interesting to note that the methods employed to evaluate membrane properties (FRAP and Laurdan dye fluorescence) and composition (lipidomics) did not distinguish among the various organelar membranes of the cells examined. Rather, all three methods scored the entire cells. Of the three methods, it is the Laurdan dye fluorescence that provides the best spatial resolution. Examination of the GP index pseudocolor images (e.g. Fig. 2A for HEK 293, Fig. 4A for HepG2 and Fig. 5B for HUVEC cells) indicates membrane rigidification across the entire cells but clearly most pronounced in the plasma membranes. This is interesting because the AdipoRs are the only sense-and-response membrane sensors localized to the plasma membrane and may therefore be especially important for plasma membrane homeostasis. The AdipoRs may therefore complement the activities of other guardians of cellular membranes, such as PCYT1A and Tafazzin (sense and repairs packing defects in the inner nuclear membrane and mitochondria, respectively; (46, 47)), IRE1 (senses bilayer stress in the ER; (48)) or the SREBPs (sense cholesterol and phosphatidylethanolamine levels; (49, 50)). It will be interesting in the future to perform cell fractionation experiments in AdipoR deficient cells to better understand the organelle-specific roles of these proteins.

In conclusion, the present findings indicate that the AdipoRs are important regulators of membrane fluidity in most cell types, including human primary endothelial cells, and that they can carry out this function without any ligand being present.
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Table 1. qPCR results for the detection of adiponectin (primer set I) and PPIA expression in all cell types studied in the present work. The CT values are indicated for each sample/gene. Results from different cDNA preparations are shown for several samples (numbered #1, #2 or #3). NTC refers to the non-template control.

| Sample         | Adiponectin | PPIA |
|----------------|-------------|------|
| Subcutaneous fat | 17.01       | 20.47|
| Breast fat      | 16.76       | 19.91|
| HUVEC #1        | non-detected | 15.13|
| HUVEC #2        | non-detected | 16.75|
| HUVEC #3        | non-detected | 17.82|
| HEK293 #1       | non-detected | 15.80|
| HEK293 #2       | non-detected | 15.96|
| HepG2           | non-detected | 16.98|
| 1321N1 #1       | non-detected | 15.30|
| 1321N1 #2       | non-detected | 15.19|
| NTC             | non-detected | 34.63|
Fig. 1. FRAP analysis showing that AdipoR1 and AdipoR2 redundantly maintain membrane fluidity in HEK293 cells. (A) qPCR results showing the efficiency of the knockdown using nontarget (NT), AdipoR1 and/or AdipoR2 siRNA. The expression levels are normalized to the NT value. (B-D) FRAP results in HEK293 cells challenged with 200 µM PA and treated with NT, AdipoR1, AdipoR2 or a combination of AdipoR1 and AdipoR2 (AdipoR1/2) siRNA. (n=6-14) (E) Average T\textsubscript{1/2} values (the time by which half of the maximum fluorescence recovery is reached) from multiple experiments as in B-D. (F) FRAP results in HEK293 cells challenged with 50 µM PA and treated with NT, AdipoR2 or combined AdipoR1/2 siRNA. (n=10-13) (G) Average T\textsubscript{1/2} values from F. (H-I) FRAP results in HEK293 cells challenged with 200 µM PA and treated with either vehicle (DMSO) or 5 µM EPA (n=10). Error bars show the SD in histograms and SEM in FRAP panels, and significance is indicated as follows: *, ** and *** indicate significant differences from the control treatment with p<0.05, 0.01 and 0.001 respectively; # symbols similarly indicate significant differences from the AdipoR1/2 combined siRNA treatment.
Fig. 2. The Laurdan dye method confirms that AdipoR1 and AdipoR2 are required to maintain membrane fluidity in HEK293 cells. (A) Pseudocolor images showing the Laurdan dye generalized polarization (GP) index at each pixel position in HEK293 cells challenged with 200 µM PA and treated with NT, AdipoR1 and/or AdipoR2 siRNA. Note the pronounced rigidification of the plasma membrane in the AdipoR1/2 siRNA treated cells. (B) Average GP index from several images as in A (n=10-15). (C) Distribution of the GP index values in representative images for each treatment. Error bars show the SD and significance is indicated as follows: *** indicate significant differences from the control treatment with $p<0.001$ and ### symbols similarly indicate significant differences from the AdipoR1/2 combined siRNA treatment.
Fig. 3. The AdipoRs are required to maintain MUFA levels in PCs, sustain desaturase gene expression and prevent lipotoxicity by PA. (A-B) SFA and MUFA abundance (mol%) in the PCs of HEK293 cells cultivated in the presence of either vehicle (DMSO) or 200 µM PA and treated with NT, AdipoR1 and/or AdipoR2 siRNA (n=3). (C) qPCR results showing the expression of three desaturases in HEK293 cells following knockdown using NT, AdipoR1 and/or AdipoR2 siRNA. The expression levels are normalized to the NT value. (D) Percentage of dead HEK293 cells (Trypan Blue-positive) following cultivation in the presence of either vehicle (DMSO) or 200 µM PA and treated with NT, AdipoR1 and/or AdipoR2 siRNA. Error bars show the SD and significance is indicated as follows: *, ** and *** indicate significant differences from the control treatment with \( p<0.05 \), 0.01 and 0.001 respectively; # symbols similarly indicate significant differences from the AdipoR1/2 combined siRNA treatment.
Fig. 4. The Laurdan dye method shows that AdipoR1 and AdipoR2 are required to maintain membrane fluidity in HepG2 cells. (A) Pseudocolor images showing the Laurdan dye GP index at each pixel position in HepG2 cells challenged with 200 µM PA and treated with NT, AdipoR1 and/or AdipoR2 siRNA. Note the pronounced rigidification of the plasma membrane in the AdipoR1/2 siRNA treated cells. (B) Average GP index from several images as in A (n=15). (C) Distribution of the GP index values in representative images for each treatment. Error bars show the SD and significance is indicated as follows: * and *** indicate significant differences from the control treatment with $p<0.05$ and 0.001 respectively; # symbols similarly indicate significant differences from the AdipoR1/2 combined siRNA treatment.
Fig. 5. The AdipoRs maintain membrane fluidity and desaturase expression in primary human cells (HUVEC). (A) qPCR results showing the efficiency of the knockdown in HUVEC cells using NT, AdipoR1 and/or AdipoR2 siRNA. The expression levels are normalized to the NT value. (B) Pseudocolor images showing the Laurdan dye GP index at each pixel position in HUVEC cells challenged with 200 µM PA and treated with NT, AdipoR1 and/or AdipoR2 siRNA. Note the pronounced rigidification of the plasma membrane in the AdipoR1/2 siRNA treated cells. (C) Average GP index from several images as in A (n=10-15). (D) Distribution of the GP index values in representative images for each treatment. (E) qPCR results showing the expression of three desaturases in HEK293 cells following knockdown using NT, AdipoR1 and/or AdipoR2 siRNA. The expression levels are normalized to the NT value. Error bars show the SD and significance is indicated as follows: *, ** and *** indicate significant differences from the control treatment with p<0.05, 0.01 and 0.001 respectively; # symbols similarly indicate significant differences from the AdipoR1/2 combined siRNA treatment.
Fig. 6. Model for the role of the AdipoRs in membrane homeostasis. In healthy cells challenged with exogenous SFAs, the AdipoRs sense membrane rigidification and signals to promote desaturase enzyme expression, resulting in increased incorporation of fluidizing UFAs into phospholipids. AdipoR-deficient cells fail to promote desaturase activity to compensate for the exogenously provided SFAs that become incorporated into phospholipids, leading to membrane rigidification hence lipotoxicity.