Decreasing cytosolic translation is beneficial to yeast and human Tafazzin-deficient cells

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ABSTRACT Cardiolipin (CL) optimizes diverse mitochondrial processes, including oxidative phosphorylation (OXPHOS). To function properly, CL needs to be unsaturated, which requires the acyltransferase Tafazzin (TAZ). Loss-of-function mutations in the TAZ gene are responsible for the Barth syndrome (BTHS), a rare X-linked cardiomyopathy, presumably because of a diminished OXPHOS capacity. Herein we show that a partial inhibition of cytosolic protein synthesis, either chemically with the use of cycloheximide or by specific genetic mutations, fully restores biogenesis and the activity of the oxidative phosphorylation system in a yeast BTHS model (tazΔ). Interestingly, the defects in CL were not suppressed, indicating that they are not primarily responsible for the OXPHOS deficiency in tazΔ yeast. Low concentrations of cycloheximide in the picomolar range were beneficial to TAZ-deficient HeLa cells, as evidenced by the recovery of a good proliferative capacity. These findings reveal that a diminished capacity of CL remodeling deficient cells to preserve protein homeostasis is likely an important factor contributing to the pathogenesis of BTHS. This in turn, identifies cytosolic translation as a potential therapeutic target for the treatment of this disease.

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Abbreviations:
BTHS – Barth syndrome,
CHX – cycloheximide,
CL – cardiolipin,
OXPHOS – oxidative phosphorylation,
ROS – reactive oxygen species,
WT – wild type.

INTRODUCTION
The Barth Syndrome (BTHS) is a rare X-linked recessive mitochondrial disorder that is characterized by cardiac and skeletal myopathies, growth retardation, hypercholesterolemia, increased urine levels of 3-methylglutaconic acid and high susceptibility to bacterial infections, due to cyclic neutropenia [1-3]. This disease is caused by mutations in Tafazzin, a mitochondrial protein involved in the remodeling of cardiolipin (CL). This phospholipid is mainly found in mitochondria, [4-10] where it optimizes numerous processes including oxidative phosphorylation (OXPHOS) [11-13], fusion [14], fission [15, 16], protein import [17, 18], iron-sulfur cluster biogenesis [19], mitophagy [20-23], apoptosis [7, 23-28] and the transport of metabolites across the mitochondrial inner membrane [6, 17, 29-36]. Tafazzin is an acyltransferase required for the maintenance of unsaturated carbon-carbon bonds in CL fatty acyl chains [1, 37-41]. Loss-of-function mutations in Tafazzin lead to reduced levels of unsaturated CL and the accumulation of CL species with an incomplete set of fatty acyl chains (such
as monolysocardiolipin, MLCL) [42-44]. This in turn results in multiple mitochondrial alterations that ultimately compromise the OXPHOS capacity [24, 45-48].

Simple model organisms such as Saccharomyces cerevisiae or baker’s yeast are an important resource for the study of mitochondrial diseases. Mitochondria from this single-celled fungus and humans show many similarities [49-53]. Being easily amenable to genetic manipulation of mitochondrial function [54, 55], and owing to the ability of yeast to survive the loss of oxidative phosphorylation; yeast models of human mitochondrial diseases can be easily created and kept alive when provided with fermentable substrates [56, 57]. The common respiratory growth defect of these models enables large-scale screening of genetic and pharmacological suppressors [57-59]. Yeast has in this way already pointed to several potential druggable therapeutic intervention points, such as the oxidicarboxylic acid carrier [60] and mitochondrial protein import [61], among others.

Herein we report that reducing cytosolic protein synthesis preserves OXPHOS in CL remodeling deficient yeast and improves the growth rate and viability of human HeLa cells lacking Tafazzin. This study sheds new light on the pathogenesis of BTHS and identifies cytosolic protein synthesis as a potential intervention point for the treatment of the disease.

RESULTS

Decreasing cytosolic protein synthesis improves respiratory growth of taz1Δ yeast

We [60] and others [62] showed that yeast cells lacking the gene encoding Taz1p (taz1Δ) grow poorly on respiratory carbon sources at 36°C, compared to the wild-type (WT) TAZ1 strain. Using a drug screening procedure we previously described [58], we found that cytosolic protein synthesis inhibitors such as, cycloheximide (hereafter abbreviated as CHX), anisomycin and emetine suppressed this phenotype in a dose-dependent manner. In the tests shown in Fig. 1A, taz1Δ cells freshly grown by fermentation in glucose were spread on plates containing ethanol (respiratory carbon source) and then exposed to paper disks saturated with the drugs dissolved in DMSO. After five days of incubation at 36°C, halos of enhanced growth appeared around the filters, whereas DMSO alone had no effect. In this assay the compounds diffused into the medium, explaining why growth was improved only at some distance around the filters, below which it was totally absent due to a too high concentration of the protein synthesis inhibitors. CHX was active at 20-30 fold lower concentrations compared to anisomycin and emetine (Fig. 1A). The optimal rescuing concentration range of CHX was determined by growth tests in liquid media containing 2% ethanol and 0.5% galactose, at 36°C. After consumption of the galactose, which is a fermentable substrate, growth of taz1Δ yeast was much less efficient compared to the WT, owing to its failure to properly express mitochondrial function (Fig. 1B). The best growth improvement of taz1Δ yeast was observed in the presence of 10 nM CHX. At this concentration, growth of the wild type was unaffected (Fig. 1B). Pulse labeling of proteins with $^{35}$-methionine and $^{35}$-cysteine revealed that the rate of cytosolic protein synthesis was decreased by about 50% in taz1Δ yeast grown in the presence of 10 nM CHX, in comparison to the WT (Fig. 1C). Interestingly, cytosolic translation was already decreased in the mutant grown in the absence of the drug by about 35%, possibly as a means to attenuate a protein stress induced by a lack in CL remodeling (see below).

If CHX is a well-known inhibitor of cytosolic translation, one cannot exclude that it has other effects in cells that could be responsible for the improved respiratory growth of taz1Δ yeast. We therefore tested the effects on taz1Δ yeast of null mutations in the genes REI1 and RPL6B that are known to partially inhibit cytosolic protein synthesis by 20% and 30% respectively [63, 64]. The double mutants taz1Δ rei1Δ and taz1Δ rpl6bΔ grew efficiently on respiratory carbon sources (Fig. 1D, E), and showed a 50% drop in the rate of protein synthesis (Fig. 1C). These data confirmed that the beneficial effect of CHX in taz1Δ yeast resulted from a decreased rate of protein synthesis.

Decreasing cytosolic protein synthesis improves mtDNA maintenance in taz1Δyeast

We previously showed that taz1Δ yeast grown by fermentation at 28°C, i.e. in conditions where the presence of functional mtDNA is not indispensable, has an increased propensity to produce $\rho^0$ cells issued from large deletions in the mitochondrial genome (60% vs 5% in the WT) [60]. The double mutants taz1Δ rei1Δ and taz1Δ rpl6bΔ produced five to ten times less $\rho^0$ cells than taz1Δ yeast in glucose cultures (Fig. 1F). Thus, partially decreasing cytosolic translation preserves a proper maintenance of mtDNA in CL deficient yeast cells.

Reducing cytosolic translation does not restore CL remodel- ing in taz1Δ yeast

As reported [39, 60, 62], mitochondria from taz1Δ yeast, compared to those from the WT, have 50% less CL, a 2-fold higher content in phosphatidylinositol (PI), whereas phosphatidylethanolamine (PE) and phosphatidylcholine (PC) accumulated normally (Fig. 2A). Additionally, the remaining CL species are less unsaturated as suggested by the decreased levels in oleic acid chains (C18:1) and increased stearic (C18:0) and palmitic (C16:0) groups compared to CL molecules extracted from the WT (Fig. 2B). Strains taz1Δ rei1Δ and taz1Δ rpl6bΔ showed very similar phospholipid profiles (Fig. 2A, B), indicating that mitochondrial function recovery in taz1Δ yeast upon partial inhibition of cytosolic translation did not result from an enhanced production of mature CL species.

Partially decreasing cytosolic protein synthesis fully re- stores OXPHOS in taz1Δ yeast

As we have shown [60], the reduced ability of taz1Δ yeast to grow at 36°C in 2% ethanol + 0.5% galactose (shown in Fig. 1B) correlated with a decreased rate of oxygen con-
FIGURE 1: Partially decreasing cytosolic translation in Tafazzin-deficient (taz1Δ) yeast improves respiration-dependent growth and mtDNA maintenance. (A) taz1Δ yeast cells were spread as dense layers onto rich ethanol solid media and then exposed to sterile filters spotted with cycloheximide, anisomycin or emetine (dissolved in DMSO). The plates were scanned after 5 days of incubation at 36°C. The filter at the top left was spotted with DMSO alone to provide a negative control. (B) Determination in liquid cultures of CHX concentrations that optimally rescue taz1Δ yeast. Complete synthetic media (CSM) containing 0.5% galactose + 2% ethanol supplemented or not with CHX at the indicated concentrations were inoculated with WT and taz1Δ cells pre-grown in CSM containing 2% glucose at 28°C. The cultures were performed at 36°C and cells densities (OD<sub>600nm</sub>) taken over a period of 36 hours. (C) Rate of cytosolic protein synthesis. Total proteins and mitochondrial proteins were labeled with a mixture of [35S]-methionine and [35S]-cysteine for 20 min in whole cells from wild type, taz1Δ rei1Δ, taz1Δ rpl6bΔ and taz1Δ yeast grown for 24 hours in rich 0.5% galactose + 2% ethanol at 36°C, and taz1Δ cells grown in the same conditions in presence of 10 nM cycloheximide (CHX). After the labeling reactions, total protein extracts were prepared and separated by SDS-PAGE on a 12% polyacrylamide gel (75 µg per lane). The gels were dried and analyzed with a PhosphorImager. Quantification was performed using Image J. Data are expressed in % relative to the WT (n=3). The shown data are cytosolic protein synthesis rates (total minus mitochondrial protein synthesis rates). Statistical analysis was done with Tukey's test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001). (D) Genetic ablation of REI1 (rei1Δ) or RPL6B (rpl6bΔ) improves respiratory growth of taz1Δ yeast. WT, taz1Δ, taz1Δ rei1Δ and taz1Δ rpl6bΔ cells freshly grown at 28°C in rich glucose were serially diluted and spotted onto rich ethanol and glucose plates. The plates were scanned after 4 days of incubation at the indicated temperature. (E) Growth of WT, taz1Δ, taz1Δ rei1Δ and taz1Δ rpl6bΔ strains in liquid complete synthetic media containing 0.5% galactose + 2% ethanol at 36°C. The cultures were inoculated with cells grown in CSM containing 2% glucose at 28°C. The cultures were performed at 36°C and cell densities (OD<sub>600nm</sub>) taken over a period of 60 hours. (F) Genetic ablation of REI1 (rei1Δ) or RPL6B (rpl6bΔ) in taz1Δ yeast preserves mtDNA maintenance. Proportions of ρ<sup>-</sup>/ρ<sup>0</sup> cells produced in glucose cultures at 28°C in strains WT, taz1Δ, taz1Δ rei1Δ, and taz1Δ rpl6bΔ were determined using the procedure described in [60] (n=3). Data are expressed in % relative to the WT and were statistically analyzed using Tukey's test (*P<0.05; **P<0.01; ***P<0.001; ****P<0.0001).
lipid remodeling. TP synthesis was restored to almost normal levels (ΔΨ).

These observations were corroborated by monitoring changes in mitochondrial membrane potential (ΔΨ) with Rhodamine 123. As we showed [60], adding ADP to respiring taz1Δ mitochondria did not decrease ΔΨ due to their poor capacity to synthesize ATP. Consistent with their good ability to produce ATP, those from strains taz1Δ rei1Δ and taz1Δ rpl6bΔ efficiently responded to ADP as WT mitochondria (Fig. 4). Furthermore, after a subsequent addition of KCN, to block the respiratory chain, ΔΨ collapsed in one single rapid phase in taz1Δ mitochondria, whereas a residual potential was preserved in those from taz1Δ rei1Δ and taz1Δ rpl6bΔ and WT yeasts. This residual potential is due to the pumping of protons by ATP synthase, coupled by the hydrolysis of the ATP that accumulated in the mitochondrial matrix during phosphorylation of the added ADP, as evidenced by the loss of this potential by inhibiting ATP synthase with oligomycin (Fig. 4).

Taken together, these observations demonstrate that partially decreasing cytosolic translation preserves the biogenesis and activity of the OXPHOS system in CL remodeling deficient yeast.

**Partially decreasing cytosolic protein synthesis suppresses the enhanced production of ROS in taz1Δ yeast**

Defects in the mitochondrial respiratory chain often result in a higher production/accumulation of reactive oxygen species (ROS) owing to an enhanced diversion of electrons from their normal pathway to oxygen, which was observed in CL remodeling deficient cells [65]. Thus, it was expected that taz1Δ yeast should produce less ROS after deleting REI1 or RPL6B or during growth in the presence of 10 nM CHX, which was indeed observed (Fig. 5).

**Decreasing cytosolic protein synthesis is also beneficial to tafazzin-deficient human cells**

We aimed to understand whether partially decreasing cytosolic translation could also benefit human cells lacking Tafazzin. To this end, we used our previously described HeLa cells, in which the TAZ gene was knocked down by RNA interference (shTaz1) and two control cell lines, shWT1 and shTaz1R in which the expression of TAZ1 was not inhibited. As reported, ShTaz1 poorly accumulates Tafazzin, is defective in CL maturation, has a reduced capacity to associate respiratory chain complexes into ‘respirosomes’, produces abnormally enlarged cells and has a higher content in mitochondria compared to shWT1 and shTaz1R, as do cells from BTHS patients [24, 48, 66].

Herein we report that ShTaz1 cells proliferate four times slower and die more rapidly, in comparison to ShWT1 and ShTazR1 cells (Fig. 6 A, B). We took advantage of these differences to test the capacity of CHX at countering the detrimental effects of a lack in CL maturation in human cells. A large beneficial effect was observed at a 50 pM concentration of CHX. In the experiment shown in Fig. 6C, the drug was added 24-25 hours after inoculating 200 µl of media with 5000 cells. CHX induced a 48 h lag phase, after which the cells grew for a long-lasting period of 170 hours, before dying and detaching from their support. In the absence of CHX, death was observed much more rapidly, after 72 hours of continuous growth. At the concentration of 50 pM, CHX had no effect on the proliferation of ShWT1 (Figure 6D). These observations indicate that a partial decrease in cytosolic translation is, as in taz1Δ yeast, beneficial to human cells defective in CL remodeling.
DISCUSSION

While a general inhibition of cytosolic protein synthesis would obviously be detrimental to the cell, our study reveals that a partial (50%) decrease in this activity preserves mtDNA maintenance and the biogenesis and activity of the oxidative phosphorylation (OXPHOS) system in a yeast model of the Barth syndrome, a mitochondrial disease associated to defects in the remodeling of cardiolipin (CL). The decreased mtDNA stability in tazΔ yeast occurred in fermenting (glucose) cultures where the presence of this DNA is not dispensable. With a respiratory carbon source (glycerol), tazΔ yeast cells lacking functional mtDNA were much less abundant owing to their incapacity to proliferate in these conditions. Thus, the respiratory deficiency of tazΔ yeast does not result from a lack in mtDNA.

Importantly, CL remodeling was still deficient in tazΔ cells rescued by a partial inhibition of cytosolic translation. This finding has two important corollaries: (i) the CL species remaining in the mutant (50% vs the WT) are sufficient for a proper biogenesis and functioning of the OXPHOS system, and (ii) the OXPHOS deficit is secondary to some other cellular dysfunction(s) that can be suppressed by decreasing cytosolic translation. At low concentration (50 pM), CHX also improved proliferation of HeLa cells deficient in tafazzin whereas that of WT HeLa cells was not modified, which clearly demonstrates that growth improvement resulted from a compensation of the lack in tafazzin. Thus yeast and human CL remodeling deficient cells face similar difficulties that can be attenuated by targeting cytosolic translation. These finding reveal that a diminished capacity of CL remodeling deficient cells to preserve protein homeostasis is likely an important factor contributing to the pathogenesis of the Barth syndrome. This in turn, identifies cytosolic translation as a potential therapeutic target for the treatment of this disease.

Previous work revealed that decreasing cytosolic translation can also rescue yeast models of adPEO (autosomal dominant progressive external ophthalmoplegia) caused by mutations in ANT, a protein that exchanges adenine nucleotides across the mitochondrial inner membrane [68]. In addition to a defective exchange of adenine nucleotides, these mutations compromise the impermeability to protons of the inner membrane. Consequently, a sufficient
electrical potential cannot be maintained across this membrane and this impedes many proteins to reach the organelle. This has deleterious consequences, not only for mitochondria, but also for the cytosol that is then confronted with the over-accumulation and the misfolding of mitochondrial precursor proteins [63]. This protein stress, which was termed mitochondrial precursor over-accumulation stress (mPOS), was shown to induce a cellular response, named unfolded protein response activated mitochondrial protein (Mito), 10 μl ethanol (EtOH), 2 mM potassium cyanide (KCN), 4 μM CCCP (carbonyl cyanide-m-chlorophenyl hydrazone) and 4 μg/ml oligomycin (oligo). The shown tracings are representative of four experimental trials. The data for WT and taz1Δ strains were reported previously [60].

because these assays used minute amounts of preproteins it might be difficult to detect in this way a partially diminished protein import capacity.

The recovery of mitochondrial function in taz1Δ yeast by a partial decrease of cytosolic translation is in line with a recent study showing that CL has an important role in promoting the induction of a mitochondrial-to-cytosolic stress response (MCSR) that enables the cell to improve protein homeostasis in both compartments [71]. Consistently also, it was shown that rapamycin, a specific inhibitor of the mTOR signaling pathway that regulates several extra-mitochondrial cellular pathways among which protein synthesis, robustly enhances survival and attenuates the disease’s progression in a mouse model and patient cells of the Leigh Syndrome, one of the most devastating mitochondrial disorders [72, 73].

Clearly, beyond a certain level of mitochondrial damage, the protein stress responsive pathways may not be sufficient. This may explain that in addition to its spontaneous 35% drop in protein synthesis, taz1Δ yeast requires a further (15%) decrease in this activity to be effectively rescued. Whether a general protein synthesis inhibition or a reduced production of specific proteins is beneficial to CL remodeling deficient cells is an interesting and important issue. In this respect, it is interesting to note that Gerst et al. found that ribosomal protein paralogs specifically modulate translation of mitochondrial precursor proteins [74]. Such observations hold promise for the development of more targeted therapeutic approaches with less undesirable side-effects to preserve protein homeostasis in cells poisoned by the over-accumulation in the cytosol of mitochondrial protein precursors.

FIGURE 4: Mitochondrial membrane potential. Variations in mitochondrial ΔΨ were monitored by fluorescence quenching of Rhodamine 123, using intact, osmotically-protected, mitochondria isolated from WT, taz1Δ, taz1Δ rei1Δ and taz1Δ rpl6bΔ cells grown in CSM containing 0.5% galactose + 2% ethanol at 36°C until a density of 2-3 OD600. The additions were 75 μM ADP, 0.5 μg/ml Rhodamine 123, 75 μg/ml mitochondrial proteins (Mito), 10 μl ethanol (EtOH), 2 mM potassium cyanide (KCN), 4 μM CCCP (carbonyl cyanide-m-chlorophenyl hydrazone) and 4 μg/ml oligomycin (oligo). The shown tracings are representative of four experimental trials. The data for WT and taz1Δ strains were reported previously [60].
Yeast-based drug assay

0.125 OD of exponentially growing cell were homogeneously spread with sterile glass beads on a square Petri dish (12 cm x 12 cm) containing solid YPA ethanol medium. Sterile filters were deposited on the plate and spotted with cycloheximide (purchased from Sigma), anisomycin (purchased from Sigma), and emetine (purchased from Sigma) dissolved in DMSO. Growth improvement was assessed after several days of incubation at 36°C.

Bienergetics experiments

The mitochondria were prepared by the enzymatic method as described [76]. Protein concentrations were determined by the Lowry method [77] in the presence of 5% SDS. Oxygen consumption rates were measured on 75 μg of fresh mitochondria using a Clarke electrode in the respiration buffer (0.65 M mannitol, 0.36 mM ethylene glycol-bis(Z-aminoethylether)-N,N,N’,N’-tetraacetic, 5 mM tri-s-phosphosphate, 10 mM tris-maleate, pH 6.8) as described [78] (see legend of Fig. 3 for the concentrations of reagents used). Variations in mitochondrial transmembrane potential (ΔΨ) were evaluated in the same respiration buffer, by monitoring the quenching of rhodamine 123 fluorescence (0.5 mg/ml) using a λexc of 485 nm and a λem of 525 nm under constant stirring, using a FLX Spectrofluorimeter (SAFAS, Monaco), as described [79]. ATP synthesis rates were measured using 75 μg of fresh mitochondria in a 2-ml thermostatically controlled chamber at 28°C in respiration buffer, in the presence of 4 mM NADH and 1mM ADP as described [80]. Aliquots were withdrawn from the oxygraph cuvette every 15 seconds and supplemented with 2.5% (w/v) perchloric acid, 8.5 mM EDTA to stop the reaction and then neutralized to pH 6.8 by adding 2N KOH, 0.3 M MOPS. ATP was quantified using a luciferin/luciferase assay (ATPlite kit from Perkin Elmer) on a LKB bioluminometer. The participation of the F1,F0-ATP synthase in ATP production was assessed using the same protocol, in the presence of oligomycin (3 μg/ml).

BN/CN-PAGE & SDS-PAGE

Blue native BN-PAGE and clear native CN-PAGE experiments were carried out as described [81], using mitochondrial extracts solubilized with digitoxin (2 gr per gr protein) run in 3–12% continuous gradient acrylamide gels. The in-gel complex IV activity was revealed using a solution of Tris 5mM pH 7.4, diaminobenzidine 0.5 mg/ml, cytochrome c 0.05 mM. The proteins were also analyzed by Western blotting on poly(vinylidene difluoride) membranes as described [82]. Polyclonal antibodies raised against yeast ATP synthase were used at a dilution of 1:50000 for subunit α (gift from J. Veilours, IBGC, Bordeaux, France); 1:10000 for cytochrome c (gift from S. Manon, IBGC, Bordeaux, France); 1:5000 for succinate dehydrogenase Sdh2 subunit (gift from C. Dallabona, University of Parma, Italy). Monoclonal antibodies against porin and Cox2 (from Molecular Probes) were used at a dilution of 1:2500 dilution, and analyzed by electrochemiluminescence. Quantification of the protein signals was performed with the ImageJ software.
In vivo labeling of mitochondrial translation products

The indicated strains were grown to early exponential phase (OD/ml of 2) in 20 ml of rich ethanol/galactose media at 36°C. The cells were harvested by centrifugation and washed twice with a minimum medium containing 2% ethanol and 0.5% galactose, supplemented with histidine, tryptophan, leucine, uracil and adenine (50 mg/liter each). To evaluate total protein synthesis, cells were resuspended in 1 ml of the same medium with the addition of 55 µCi of [35S] methionine plus [35S] cysteine (Amersham Biosciences) and incubated for 20 min at 36°C. To evaluate mitochondrial protein synthesis the same procedure was followed but before adding [35S] methionine plus [35S] cysteine, cells were first treated with 7.5 mg/ml cycloheximide during 5 minutes. After the labeling reactions, total protein extracts were prepared and quantified using the Lowry method. The proteins were separated by SDS-PAGE on a 12% polyacrylamide gel, transferred onto a nitrocellulose membrane and analyzed with a PhosphorImager. Quantification of radioactive proteins was performed using the software ImageJ.

Lipid analyses

Mitochondrial lipids were analyzed as described [60]. In summary, the lipids were extracted with 2 ml of chloro-
form/methanol (2:1, v/v). After centrifugation, the organic phase was isolated and the remaining lipids were further extracted twice by adding 2 ml of chloroform to the aqueous phase. The organic phases were pooled and evaporated to dryness. The lipids were then resuspended in chloroform/methanol (2:1, v/v). Respective volumes equivalent to 50 μg of acyl chains were spotted on silica plates, four times for each strain. Polar lipids were separated by one-dimensional TLC using chloroform/methanol/1-propanol/methyl acetate/0.25% KCl (10:4:10:3.6, by vol.) as a solvent [84]. The lipids were located by immersing the plates in a solution of 0.001% (w/v) primuline in PBS, followed by visualization under UV light. The zones of the gel corresponding to PE, Cl, PI and PC were then scraped and added to 1 ml of methanol/2.5% H2SO4 containing 5 μg of heptadecanoic acid methyl ester as a standard. The lipid mixtures were incubated at 80°C for 1 h, and 1.5 ml of water and 400 μl of hexane were then added. After centrifugation, the hexane phase containing FAMES (fatty acid methyl esters) was isolated. Separation of FAMES was performed as described [39].

**ROS analysis**

Cells at 0.4 OD units were taken from liquid cultures, pelleted in a microcentrifuge, resuspended in 1 ml of phosphate-buffered saline (PBS) containing 50 μM dihydroethidium (DHE; Molecular Probes) and incubated at room temperature for 5 min. Flow cytometry was carried out on a Becton-Dickinson Accuri C6 model flow cytometer. The DHE fluorescence indicated was the direct output of the FL2A (red fluorescence) channel without compensation. A total of 100,000 cells were analyzed for each curve.

**HeLa cells culture and transfection**

The cervical carcinoma HeLa cell lines were cultured in DME supplemented with 10% FCS and L-glutamine. Transfection of HeLa cells was performed using Lipofectamine 2000 (Invitrogen). Bcl-xL, shTaz, and shCont stable HeLa cell lines were generated by transfection with pcDNA3/Bcl-xL, pSUPER/shTaz, or pSUPER/shCont, respectively, and selected in G418 [67]. The revertant shTaz1R cell line was generated by cotransfecting shTaz1 HeLa cells with pLpC vector (carrying a puromycin resistance gene), and pcDNA3/Taz mut and stable clones were selected in the presence of puromycin [67].

**xCELLigence real time cellular proliferation measurements**

Experiments were carried out using the xCELLigence RTCA DP instrument (ACEA Biosciences, Ozyme, France) placed in a humidified incubator at 37°C and 5% CO2. Cell proliferation and cytotoxicity experiments were performed using 16-well plates (E-plate, Ozyme, Montigny le Bretonneux, France). The microelectrodes attached at the bottom of the wells allowed for impedance-based detection of the attachment, spreading and proliferation of the cells. Initially, 180 μl of cell-free growth medium (10% FBS) was added to the wells. After leaving the devices at room temperature for 30 min, the background impedance for each well was measured. Cells were harvested from exponential phase cultures by a standardized detachment procedure using 0.05% Trypsin-EDTA (Invitrogen). Flow cytometry was used to count the cells and test their viability (FSC versus propidium iodide staining). 5000 or 7500 cells in 20 μl were added in each well. After leaving the plates at room temperature for 30 min to allow early cell attachment, in accordance with the manufacturer’s guidelines, they were locked in the RTCA DP device in the incubator and the impedance value of each well was automatically monitored by the xCELLigence system and expressed as a Cell Index value (CI). Water was added to the space surrounding the wells of the E-plate to avoid interference from evaporation. For proliferation assays, the cells were incubated during 120h for toxicity in growth medium (10% FBS) and CI was monitored every 15 min during the whole duration of the experiment. Four replicates of each conditions were used in each test. After an initial assessment of the concentration of cycloheximide which would be non-toxic to the ShWT1 cells, we used a test of proliferation in presence and absence of low doses of cycloheximide (1 pM to 100 pM) either prior to cell seeding or after an initial adhesion phase and at an early proliferative step (at 24-26 hours). All experiments were conducted over 240 hours. All plots were normalized to the Cell Index.
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