HIGHLIGHTS

- A mechanism for selectivity of ETC disruption in cancer is proposed
- In contrast to cancer cells, most somatic cells are non-proliferating
- They are protected from ETC-induced cell death when glucose is not limiting
- Protection is conferred by antioxidant defense that eliminates ETC-derived ROS
- In limiting glucose conditions (as in a tumor) protection is no longer effective

Antioxidant defense in quiescent cells determines selectivity of electron transport chain inhibition-induced cell death

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Running title: Selectivity of electron transport chain inhibition

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ABSTRACT

Mitochondrial electron transport chain (ETC) targeting shows a great promise in cancer therapy. It is particularly effective in tumors with high ETC activity where ETC-derived reactive oxygen species (ROS) are efficiently induced. Why modern ETC-targeted compounds are tolerated on the organismal level remains unclear. As most somatic cells are in non-proliferative state, the features associated with the ETC in quiescence could account for some of the specificity observed. Here we report that quiescent cells, despite increased utilization of the ETC and enhanced supercomplex assembly, are less susceptible to cell death induced by ETC disruption when glucose is not limiting. Mechanistically, this is mediated by the increased detoxification of ETC-derived ROS by mitochondrial antioxidant defense, principally by the superoxide dismutase 2 – thioredoxin axes. In contrast, under conditions of glucose limitation, cell death is induced preferentially in quiescent cells and is correlated with intracellular ATP depletion but not with ROS. This is related to the inability of quiescent cells to compensate for the lost mitochondrial ATP production by the upregulation of glucose uptake. Hence, elevated ROS, not the loss of mitochondrially-generated ATP, are responsible for cell death induction by ETC disruption in ample nutrients condition, *e.g.* in well perfused healthy tissues, where antioxidant defense imparts specificity. However, in conditions of limited glucose, *e.g.* in poorly perfused tumors, ETC disruption causes rapid depletion of cellular ATP, optimizing impact towards tumor-associated dormant cells. In summary, we propose that antioxidant defense in quiescent cells is aided by local glucose limitations to ensure selectivity of ETC inhibition-induced cell death.

Key words: Electron transport chain, supercomplexes, antioxidant defense, SOD2, thioredoxin

INTRODUCTION

Mitochondria play a key role in multiple cellular processes, including energy production, metabolism and cell death. Functional mitochondria are essential in tumorigenesis [1-3], and targeting of the mitochondrial electron transport chain (ETC) emerges as an effective strategy to suppress and eliminate cancer cells [4-8]. ETC consists of four macromolecular protein complexes (referred to as CI, CII, CIII and CIV) that associate further into supercomplexes (SCs) [9, 10], and work together to maintain mitochondrial inner membrane potential, used by the mitochondrial ATP synthase (a.k.a complex V, CV) to produce ATP. Because of high electron traffic and presence of redox centers, the ETC is a significant source of reactive oxygen species (ROS) [11, 12]. To counter ETC-derived ROS, mitochondria are equipped with several antioxidant mechanisms, such as manganese superoxide dismutase (SOD2), as well as the glutathione and thioredoxin systems [13-15]. Inhibition of the ETC interferes with mitochondrial ATP synthesis, but also substantially increases ETC-derived ROS production, leading to cell death [4]. The susceptibility to cell death upon ETC inhibition will therefore be governed by the degree of cell's reliance on mitochondrial ATP production, and by the balance between the propensity to form and detoxify ETC-derived ROS.

Recent findings indicate that ETC targeting is particularly effective in cancer cells and subtypes with increased dependence on mitochondrial respiration and higher SC assembly [5, 16-18]. At the same time, pharmacologically relevant ETC inhibitors, such as metformin or the recently developed mitochondrial-targeted agents such as MitoTam and MitoVES, are well tolerated in animals, suggesting selectivity for cancer cells [5, 19]. However, despite its obvious translational relevance, the molecular reasons for this selectivity are unclear.

While cancer cells are hard-wired for proliferation, the majority of normal somatic cells are in a non-proliferative, post-mitotic state. Features associated with quiescence and non-proliferative state could therefore partially account for the specificity we observed previously: quiescent cells were resistant to agents that induce ROS from the ETC [20, 21], but molecular reasons remained uncharacterized. Hence, specific configuration of the ETC in quiescent cells, different handling of ROS, or changes in the dependence on mitochondrial ATP production could all significantly contribute to the therapeutic

window associated with the ETC inhibition in cancer. Understanding the paradigms that govern sensitivity to cell death in quiescent cells under various circumstances is therefore of high importance for the development of efficient and non-toxic ETC-directed cancer therapy.

In the current study we attempted to resolve the selectivity issue by characterizing the ETC and the ability to form and detoxify ROS upon ETC inhibition in proliferating and quiescent, non-transformed cells. Using pharmacological and genetic manipulations, we show that the capacity to detoxify ROS is increased in quiescent cells, significantly contributing to the resistance of these cells to ETC inhibition, despite their increased reliance on the ETC. Furthermore, under conditions of nutrient limitations expected to occur in poorly vascularized areas of tumors, ETC inhibition brings an additional benefit by interfering with mitochondrial ATP production, further improving specificity.

MATERIAL AND METHODS

Reagents

All reagents were purchased from Sigma Aldrich (St. Louis, MO, USA), unless stated otherwise.

Cell culture

The cells used in the study were obtained from ATCC and were authenticated. The Ea.hy926 cells were cultured in high glucose (4.5 g/l) (Lonza, Basel, Switzerland, BE12-604F) or low glucose (1 g/l) (Lonza, BE12-707F) DMEM media supplemented with 10% fetal bovine serum (ThermoFisher, Waltham, MA, USA, 10270106), HAT supplement 2% (ThermoFisher, 21060017) and 1% antibiotics (penicilin G 100U/ml and streptomycin 100 µg/ml) and, for low glucose media only, L-glutamine 0,03% in a 5% CO₂, 37°C incubator. MCF10A cells were cultured in DMEM:F12 (Lonza, BE12-719f) media supplemented with 5% horse serum (Sigma-Aldrich, H1138), human insulin 0.1% (I9278), 20 ng/mL epidermal growth factor (ThermoFisher, PHG0311), hydrocortisone 0.5 mg/ml (H0888), cholera toxin 100 ng/mL (C8052). For the experiments the media was changed for low glucose or high glucose DMEM supplemented as DMEM:F12 above. All cell lines were regularly screened for mycoplasma contamination using the MycoAlert kit (Lonza, LT07-318). For experiments requiring pharmacological treatments, equal numbers of cells ($5-7 \times 10^5$) were exposed to the studied compounds. For quiescent cultures, the cells were seeded into 24-well plates at 80% density, and cultured for 4 days post-confluence. For proliferating cultures, cells were seeded into 6-well plates at 2×10^5 cells per well two days before the experiment. 1 ml of medium per well were used for both proliferating and quiescent cultures.

Lentiviral production and RNA interference

pLKO1 plasmids containing shRNA sequences against the targeted transcripts were obtained from Sigma-Aldrich. Lentiviral particles were produced in Hek293-FT cells using second generation psPAX and pMD.2G plasmids and calcium phosphate or Lipofectamine 3000 (ThermoFisher, L3000008) transfections. Virus-containing medium was collected after 48 h, centrifuged at $3,000 \times g$ for 15 min, and in some cases the viruses were concentrated by overnight incubation with PEG-it

(SBI-System Biosciences, Palo Alto, CA, USA, LV825A-1). The supernatants were aliquoted and stored at -80°C. To induce silencing, the lentivirus-containing supernatants were added to cells grown in normal culture medium overnight. The transduced cells were selected by puromycin, and the level of silencing was assessed by western blotting (WB). The specific shRNA used were as follows: SOD2 - TRCN0000320739, GPX1TRCN0000046231, TXNRD2 - TRCN0000433391, GSR - TRCN0000046425

Respiration assessment

Cellular respiration was assessed by Seahorse XF24 analyzer (Seahorse Biosciences, Santa Clara, CA, USA). For quiescent cultures the cells were seeded at 80% density in the Seahorse XF 24-well microplates and cultured 4 days post-confluence. Proliferating cells were seeded at 20,000 per well two days before the experiment. Medium was switched before the experiment for the base medium (D5030) of pH 7.4, containing 0.2% BSA and 10 mM glucose. Oxygen consumption rate (OCR) was measured in the basal state and after sequential addition of 10 μ M oligomycin, 1 and 2 μ M carbonyl cyanide p-(trifluoromethoxyl)-phenyl-hydrozone (FCCP) and combined 10 μ M rotenone plus 25 μ M antimycin. Data were normalized to DNA content measured by the Quant-iT™ PicoGreen® dsDNA reagent (ThermoFisher, P7581). To determine the efficacy of ETC inhibition by the tested compounds, the respiration of proliferating endothelial Ea.hy926 cells under both low glucose (1 g/l) and high glucose (4.5 g/l) conditions was monitored by the Oroboros Oxygraph 2-K instrument (Oroboros Instruments, Innsbruck, Austria) as described [22], using 2×10^6 of non-permeabilized cells per chamber. Basal routine respiration was established after adding succinate (10 mM) and ADP (3 mM), after that pharmacological agents were added as shown below.

SDS-PAGE and Western blot analysis

SDS-PAGE and WB analysis were performed using standard methods as detailed elsewhere [1]. Antibodies used were: anti-peroxiredoxin III (SantaCruz, Dallas, TX, USA, sc-23973), anti-thioredoxin 2 (Abcam, Cambridge, United Kingdom, ab185544), anti-SOD1 (SantaCruz, sc-11407), anti-catalase (Abcam, ab1877), anti-GPX-1 (Abcam, ab108427), anti-SOD2 (Acris, Herford,

Germany, AP03024-PU-N), anti-GSR (Proteintech, Manchester, United Kingdom, 18257-1-AP), anti-TXNRD2 (Abcam, ab180493). The anti- β -Actin HRP conjugate (CellSignaling, Danvers, MA, USA, #5125) was used for visualization. Quantification was done in the ImageJ software.

Blue native electrophoresis (BNE) and high-resolution clear native electrophoresis (hrCNE)

Mitochondria were isolated using a Balch-style homogenizer as detailed elsewhere [4, 23]. The published protocols were modified by using hand-driven 1 ml disposable syringes (Becton Dickinson, Franklin Lakes, NJ, USA, 309628). BNE followed by immunoblotting and hrCNE followed by in-gel activity assays was performed using digitonine-solubilised mitochondria as described [24]. Primary antibodies used were as follows. CI - anti-NDUFB8 (Abcam, ab110242), CII - anti-SDHA (Abcam, ab14715), CIII - anti-UQCRCF1 (Abcam, ab14746), CIV - anti-MTCO2 (Abcam, ab110258), CV - anti-ATP5A (Abcam, ab110273). Samples used for native gels were also run on SDS-PAGE/WB to verify equal mitochondrial loading using anti-mtHSP70 antibody (ThermoFisher, MA3-028).

Mitochondrial NADH measurements

Mitochondrial NADH was measured by two photon microscopy using Zeiss LSM880 NLO inverted microscope, equipped with Chameleon Ultra II and Compact OPO MP lasers (Coherent, Santa Clara, CA, USA), internal 32 channel spectral GaAsP detector and two non-descanned BiG-2 GaAsP detectors. We used 63x/1.4 Plan-Apochromat oil immersion objective, 740 nm laser line at 8 mW power (6 mW in scanning mode during image acquisition which includes blanking), and emission range 390-480 nm. The NADH emission spectrum was first confirmed on internal spectral detector, for the final image acquisition we used non-discanned BiG detector. The cells were grown in glass-bottom microscopy plates (In Vitro Scientific, Mountain View, CA, USA, D35-14-1.5-n) in normal culture medium (see the Cell Culture part), pre-treated with 1 nM TMRM for 30 min and examined either untreated, exposed to 5 μ M CCCP, or to 1.25 μ M rotenone. Mitochondrial NADH was quantified via the Fiji software by segmenting cells using thresholding and averaging signal over mitochondrial ROIs. 3-4 independent experiments were performed, with more than 150 cells per condition imaged in each experiment.

Detection of cell death and ROS

For the detection of cell death, cells were seeded before the experiment as described above. If required, the cells were pre-incubated with acetylcysteine 1 mM (NAC, A0150000) or auranofin 0.75 μ M (Enzo Life Sciences, Farmingdale, NY, USA, BML-EL206-0100) for 1 or 2 h, respectively. Incubation time with the tested compounds was 22 h, after which cell death was assessed by flow cytometry using Annexin V/propidium iodide (PI) staining as described [4], using BD FACSCalibur or BD LSRFortessa flow cytometers (Becton Dickinson). Cells positive for Annexin V, PI, or Annexin V/PI were considered dead. The identity of the tested agents was as follows: H₂O₂ (516813), phenethyl isothiocyanate (PEITC, 253731), rotenone (R8875), piericidin A (Enzo, ALX-380-235-M002), myxothiazol (T5580), oligomycin A (75351), and CCCP (C2759). MitoVES and MitoTam were synthesized in-house [5, 6]. For ROS measurements, cell cultured as described were exposed to the tested compounds for one hour. For the last 20 min, the cells were co-incubated with 5 μ M 2',7'-dichlorodihydrofluorescein diacetate (DCF, D6883) or 5 μ M dihydroethidium (DHE, 37291). The level of fluorescence was assessed by flow cytometry as described [4].

Assessment of glucose uptake, lactate production, and glucose content in culture media

Glucose uptake was evaluated after 15 min incubation with 50 μ M 2-nitrobenzodeoxyglucose (2-NBDG; ThermoFisher, N13195) by flow cytometry as described [5], using BD FacsCalibur or BD LSRFortessa. In some experiments, cells were pre-treated for 6 hours by 0.5 or 5 μ M MitoVES or 7.5 μ M myxothiazol. Lactate concentration in the culture medium was assessed after 19 h of cell cultivation using lactate detection kit (TrinityBiotech, Bray, Ireland, 735-10), following manufacturer's instructions with all volumes scaled down 10 times. Glucose content in the cultured media was determined after 6, 16, 24 and 38 h of cultivation under normal experimental conditions (proliferating cells in 6-well format, quiescent cells in 24-well format, both in 1 ml of media) using low and high glucose media and Amplex™ Red Glucose/Glucose oxidase assay kit (ThermoFisher, A22189) following manufacturer's instructions. Fluorescence was measured by Tecan Infinite M200 instrument (Tecan Group Ltd., Switzerland). Data were normalised to glucose content in fresh media.

Evaluation of intracellular ATP

Intracellular ATP levels were assessed by the CellTiter-Glo kit (Promega, Madison, WI, USA, G7570) following manufacturer's instructions in 96 well format. The resulting luminescence was evaluated using the Tecan Infinite M200 instrument (Tecan Group Ltd.). Data were normalized to the cell number measured by crystal violet in a parallel 96-well plate, and are shown as relative to non-treated controls.

Measurements of surface GLUT1 exposure

Cells were exposed to the indicated agents and collected by trypsinization, incubated for 30 min in 100 μ l PBS with 1:200 Anti-GLUT1 antibody (Abcam, ab15309), sedimented by centrifugation at 300g/4min and gently resuspended in PBS with 1:100 secondary Cy3-stained antibody (Sigma-Aldridge, C2306). Both incubations were performed at 37°C, 5%CO₂ incubator. Fluorescence intensity was determined by flow cytometry using BDFortessa.

In-gel assessment of antioxidant protein activity

In-gel activity of antioxidant proteins was determined as described [25], with minor changes. Samples were prepared on the Misonix Sonicator 3000 instrument (QSonica, Newtown, CT, USA) at 30% power using microtip ~3 W, 3x15 s with 25 s pause performed on ice. The incubation time in the SOD2 activity assay was reduced to 7 min.

NADPH/NADP and GSH/GSSG measurements

Cells were seeded in a 96 well plate and ratios of GSH/GSSG and NADPH/NADP⁺ were determined using luminescence assays (GSH/GSSG-Glo™, Promega, V6612 and NADP/NADPH-Glo™, Promega, G9081) according to manufacturer's instructions. The resulting luminescence was measured using the Tecan Infinite M200 instrument.

Cell cycle evaluation and visualization of adherence junctions

For cell cycle assay, cells were harvested by trypsin, stained by Hoechst 33342 (10 μ g/ml, B2261) for 35 min at 37°C, 5% CO₂ in normal culture media. After that pyronin Y (P9172) was added to the final

concentration of 0.5 $\mu\text{g/ml}$ for 15 min. 2×10^5 of stained cells were examined by flow cytometry at a flow rate of 400 cells/s. Hoechst-low/pyronin Y-low cells were assigned to G_0 phase of the cell cycle. For detection of adherence junctions, cells grown on coverslips were permeabilized and blocked with 5% fetal bovine serum, 0.075% Tween-20, 0.075% Triton-X100, 100 mM glycine (Serva, Heidelberg, Germany) in PBS for 1 h, incubated with anti-VE cadherin (Santa Cruz, sc-9989) or anti-E-cadherin (Santa Cruz, sc-21791) primary antibodies (1:100) for 1 h at room temperature, followed by 1 h incubation with anti-mouse Alexa Fluor 647-conjugated antibody (Thermo Fisher, A21236, 1:1000). Samples were embedded in DAPI-containing mowiol and imaged by the Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a white light laser and pulsed UV laser using a 63x lens and sequential scanning at 400 Hz. Excitation was at 405 and 640 nm and detection at 420-490 and 655-730 nm using HyD detectors. Images were deconvolved in Huygens Professional software and processed in the Fiji software.

Statistical analysis

Data were analyzed using the GraphPad Prism 5.04 software, and are presented as mean values $\pm$ S.E.M. of at least three independent experiments, unless indicated otherwise. Statistical significance was determined using unpaired Student's *t*-test or ANOVA when suitable; $p < 0.05$ was considered statistically significant.

RESULTS

Electron transport chain activity is increased in quiescent cells.

Increased ETC assembly in cancer cells has been linked to the susceptibility to ETC-dependent cell death [5]. To understand if this relationship is maintained in non-transformed quiescent cells, we first examined the ETC utilization and assembly in proliferating and quiescent endothelial-derived Ea.hy926 and breast epithelia-derived MCF10A cells. To investigate the effect of nutrient availability (which can be scarce for example in poorly-perfused areas of tumors), the cells were grown in high (4.5 g/l) and low (1 g/l) glucose media that are expected to represent situations of ample and limited

glucose supply. Both tested cell types undergo contact growth inhibition upon reaching confluence, as evidenced by the increase of the G₀ cell cycle subpopulation (Fig 1 A,B) and assembly of adherence junctions (Fig. 1 C,D), and represent therefore a plausible experimental model of quiescence induced by contact inhibition. Respiration measurements revealed that oxygen consumption was increased in quiescent cells in both cell types (Fig. 2A, B), suggesting that ETC activity is elevated when cells become quiescent. On the other hand, lactate production and glucose uptake were reduced in quiescent cells (Fig. 2C,D), indicating lower rate of glycolysis, in agreement with previously-reported data [26-28]. We then performed a more detailed analysis of the ETC in Ea.hy926 cells. Blue native electrophoresis (BNE) of mitochondria isolated from proliferating and quiescent cells revealed more SCs in quiescent cells (Fig. 2E), and in-gel activity measurements using high resolution clear native electrophoresis (hrCNE) demonstrated that these SCs were enzymatically active (Fig. 2F). This suggests that the ETC in quiescent cells is primed for increased performance, consistent with the proposed role of respiratory SC [29, 30]. Finally, *in situ* examination by two photon microscopy documented that mitochondrial NADH was significantly elevated upon addition of the CI inhibitor rotenone in quiescent but not proliferating cells (Fig. 3 A,B). This points to increased mitochondrial NADH production in quiescent cells, which is at baseline avidly consumed by the ETC, but is substantially elevated when CI activity is suppressed by rotenone. In summary, these results demonstrate that ETC gains prominence in quiescence. Consequently, ETC blockade should have more profound consequences in quiescent cells than in proliferating (*i.e.* cancer) cells.

Quiescent cells are protected from cell death induced by the ETC blockade when glucose is not limiting.

While in cancer the dependence on oxidative phosphorylation and higher ETC SC assembly correlate with sensitivity to ETC inhibition, in non-transformed cells this relationship has not been rigorously tested. Importantly, ETC blockade may result in both the suppression of mitochondrial ATP generation due to an impaired proton-motive force, but also in the stimulation of ROS production, due to accumulating electrons at the ETC complexes. However, the relative importance of elevated ROS

or ATP depletion in cell death induction may vary depending on the cellular state (proliferating or quiescent), and on the nutritional status (availability of glucose). We therefore exposed proliferating and quiescent Ea.hy926 and MCF10A cells to three distinct classes of agents. The first group consisted of compounds that generate ROS without acute inhibition of the ETC, hydrogen peroxide and phenethyl-isothiocyanate (PEITC) [31], (Fig. S1 A,B), expected to induce cell death solely in an ROS-dependent manner. The second group comprised agents that block the ETC (Fig. S1 C-F), the CI inhibitors rotenone, piericidin A and MitoTam [5], the CII inhibitor MitoVES [4, 6], and the CIII inhibitor myxothiazol. These compounds may either induce ROS via the ETC, but may also suppress mitochondrial ATP synthesis. The third group contained agents that specifically block mitochondrial ATP generation, the ATP synthase inhibitor oligomycin, and mitochondrial uncoupler carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Fig. S1 G,H).

When compared to quiescent cells, proliferating cells under high glucose conditions were more susceptible to ROS inducers (Fig. 4A,G) ETC inhibitors (Fig. 4B, H), as well as to oligomycin and CCCP (Fig 4 C,I). Under low glucose, this sensitivity pattern was maintained only for pure ROS inducers (Fig. 4 D,J). Importantly, however, upon treatment with ETC inhibitors (Fig. 4 E,K), oligomycin and CCCP (Fig. 4F,L), quiescent cells became highly susceptible to cell death induction. This suggests that the sensitivity of quiescent cells to ETC and ATP synthase inhibitors, but not to pure ROS inducers, is significantly altered based on the availability of glucose. Moreover, under conditions of ample glucose supply, quiescent cells are much less sensitive to cell death induction by ROS inducers and inhibitors of the ETC.

ETC inhibition in quiescent cells leads to ATP depletion when ambient glucose is below the physiological range.

To properly interpret the above results, we first assessed glucose levels at various time points in the cell-conditioned media. This revealed that in low glucose media, the glucose concentration is less than 20% of the initial 5.5 mM concentration (corresponding to less than 1 mM) at the time of the

treatment (16 h after the media change), while in high glucose media it was about half of the initial 25 mM concentration (10-15 mM) (Fig. 5 A). Thus, in low glucose cultures the glucose concentration is significantly below physiological levels of well perfused tissues (about 5 mM) [32], but in high glucose media such conditions of glucose limitation are not reached. ATP production by glycolysis requires glucose, therefore generation of ATP by oxidative phosphorylation may gain prominence when glucose is limiting. For this reason, we assessed the consequences of ETC blockade on intracellular ATP levels. To this end we exposed proliferating and quiescent cells to ETC inhibitors and disruptors of mitochondrial ATP production described above, and assessed total cellular ATP by a luminescence-based assay. While this treatment did not affect ATP levels in proliferating or quiescent cells in high glucose media (Fig 5 B,C), it resulted in a severe depletion of ATP in quiescent cells cultured in low glucose media (Fig. 5 D,E). This ATP depletion correlated with enhanced cell death (cf. Fig. 4 E,F,K,L), suggesting that the shortage of intracellular ATP is the defining factor for cell death in quiescent cells when glucose is below the normal physiological range, but not when glucose is in ample supply. In support of this notion, when quiescent cells were exposed to ETC inhibitors in media containing the physiological glucose level (5.5 mM), depletion of intracellular ATP was not observed (Fig. 5 F).

Quiescent cells cannot compensate for the loss of mitochondrial ATP synthesis when glucose is limiting.

ATP depletion in quiescent cells after inhibition of the ETC at sub-physiological glucose levels suggests that quiescent cells may be unable to compensate for the defect in mitochondrial ATP production when glucose is a limiting factor. As the observed ATP depletion could result from the failure of quiescent cells to switch to glycolysis, we assessed changes in the rate of lactate production and glucose uptake upon ETC inhibition in Ea.hy926 cells to characterize the possible compensatory regulation. Indeed, we found that upon ETC blockade the proliferating cells increased their glucose uptake much more efficiently than quiescent cells (Fig. 5 G). Glucose uptake is mediated by glucose transporters [33], therefore we next assessed the level of GLUT1 using flow cytometry after

immunostaining of non-permeabilized cells. This analysis revealed that whereas ETC suppression significantly increased the level of GLUT1 in proliferating cells, little or no increase was observed in quiescent cells (Fig. 5H). Hence, the reduced flexibility of glucose uptake in quiescent cells renders these cells vulnerable to ETC blockade in glucose concentrations significantly below the physiological range.

Generation of ROS upon ETC inhibition corresponds to cell death when glucose is not limiting.

Having established that factors other than ATP depletion determine sensitivity to cell death in quiescent cells at physiological glucose concentrations, we next assessed the relevance of increased ROS as another major consequence of ETC blockade [4, 11, 12]. We used flow cytometry and 2',7'-dichlorofluorescein diacetate (DCF) to assess the amount of ROS produced upon the administration of tested compounds to proliferating and quiescent Ea.hy926 and MCF10A cells and compared these results to cell death levels presented in Fig. 4. For ROS inducers hydrogen peroxide and PEITC, the magnitude of ROS generation correlated with the cells' sensitivity to cell death both in high (Fig. 6 A,G) and low (Fig. 6 D,J) glucose conditions. In contrast, for ETC inhibitors oligomycin and CCCP the correlation between ROS generation and cell death was found only under high glucose media (Fig. 6 B,C,H,I), while no clear correlation between cell death and ROS was apparent under low glucose conditions (Fig. 6 E,F,K,L). These results support the notion that the level of formed ROS determines the level of cell death induced by ETC inhibition when glucose is not limiting. In addition, lower ROS in quiescent cells, despite their more active ETC, suggests that these cells can neutralize ROS more efficiently.

Antioxidant defense is upregulated in quiescent cells.

To further elaborate why quiescent cells produce less ROS upon ETC inhibition and why they are protected from cell death despite their more active ETC, we investigated the status of relevant antioxidant defense components. In mitochondria, this includes SOD2 that detoxifies superoxide, and

glutathione and thioredoxin systems that inactivate hydrogen peroxide [14, 15]. In Ea.hy926 cells, the expression of mitochondrial antioxidant defense proteins SOD2 and glutathione peroxidase-1 (GPX1) was elevated in quiescent cells, while peroxiredoxin-3 (PRX3), thioredoxin-2 (TRX2) were not significantly changed, irrespective of glucose concentration (Fig. 7 A). Cytoplasmic catalase (CAT) and superoxide dismutase 1 (SOD1) were slightly increased in proliferating cells (Fig. 7 A). This was corroborated by in-gel activity assays for superoxide dismutase, glutathione peroxidase and catalase (Fig. 7 C, quantified in Fig. 7 E), showing that the enzymatic activity of SOD2 and glutathione peroxidase was significantly increased in quiescent cells. In MCF10A cells, the protein expression of antioxidant defense components was similar in proliferating and quiescent cells both in high and low glucose (Fig. 7 B), but the in-gel activity assays revealed that the enzymatic activity of GPX and SOD2, but not that of SOD1 or catalase, is increased in quiescent cells (Fig. 7 D, quantified in Fig. 7 E). This was again independent of glucose concentration. Furthermore, the ratio of reduced to oxidized glutathione was reduced and NADPH/NADP ratios were increased in quiescent cells (Fig. 7 E,F). Collectively, this suggests that the mitochondrial antioxidant defense is more active in quiescent cells and these cells are well equipped to deal with the oxidative insult induced by ETC inhibition.

Antioxidant defense is functionally relevant in protection of quiescent cells from ETC blockade-induced cell death.

To evaluate the functional relevance of the elevated antioxidant defense for the protection of quiescent cells from ETC-induced cell death under the condition where glucose is not limiting, we used lentiviral delivery of shRNA to suppress its individual protein components. We performed these experiments in Ea.hy926 cells, as the downregulation of antioxidant defense components was only poorly tolerated by MCF10A cells. SOD2 controls mitochondrial superoxide, which is the major ROS species produced when ETC is blocked [11]. Downregulation of SOD2 by a specific shRNA resulted in a significantly increased cell death upon treatment of quiescent cells with ETC inhibitors, while little or no effect of SOD2 knockdown was observed in proliferating cells (Fig. 8 A). This was accompanied by increased ROS production in quiescent cells, as assessed both by dihydroethidium

(DHE), a fluorescent probe that preferentially detects superoxide, and by DCF, a more general oxidative stress indicator (Fig. 8 B,C). We then knocked down GPX1, which inactivates hydrogen peroxide in mitochondria in a glutathione-dependent manner. GPX1 knock-down had much less effect on cell death upon application of ETC inhibitors in quiescent cells than SOD2 knockdown (Fig. 8 D), but resulted in an increased ROS signal (Fig. 8 E). This indicates that in GPX1 knock-down cells, ROS is uncoupled from cell death. Similar to GPX1, knock-down of glutathione reductase (GSR), a glutathione recycling enzyme, did not significantly increase cell death in quiescent cells (Fig. 8 F), and the effect on ROS was moderate (Fig. 8 G). Finally, interference with thioredoxin reductase 2 (TXNRD2), a mitochondrial thioredoxin recycling enzyme [34], significantly increased cell death (Fig. 8 H), and also increased ROS generation (Fig. 8 I). In line with the shRNA data, thioredoxin reductase inhibitor auranofin-dependent cell death was increased in quiescent cells treated with ETC inhibitors more than in proliferating cells (Fig. 8 J).

To complement the above results, we also took an inverse approach and boosted the available antioxidant capacity by administration of the antioxidant N-acetyl cysteine (NAC). While little or no effect of this treatment was observed in quiescent cells, a significant reduction in cell death was detected in proliferating cells (Fig. 8 K). Importantly, in low glucose media the knock downs of antioxidant enzymes described above did not affect cell death in quiescent cells (Fig. S2 A-D), in line with the notion that ROS are not the causal agents of cell death in that situation. Collectively, these results demonstrate that antioxidant defense is functionally relevant in protecting quiescent cells from cell death, and determines to a large degree their sensitivity to ETC inhibition in a situation when cells are not affected by nutrient limitation.

DISCUSSION

Targeting of the ETC holds promise for cancer therapy and has demonstrated its efficacy and low toxicity in multiple pre-clinical cancer models [5, 6, 8]. Considerable effort has been invested in uncovering the rules that govern the susceptibility of cancer cells to ETC inhibition-induced cell death

and a direct correlation has been found between oxygen consumption, assembly of ETC SCs and sensitivity to ETC disruption [5]. On the other hand, the question why these ETC disrupting agents are well tolerated on the organismal level and in non-cancerous cells has received much less attention, even though it is of high importance for efficient and save cancer therapy. For this reason, the key finding of the present study is that the elevated antioxidant defense in non-proliferating cells ensures resistance to ETC inhibition in a situation when glucose is not limiting.

Most somatic cells in an organisms are in a quiescent, post-mitotic state, and features associated with quiescence may play an important role in resistance to ETC inhibition, as suggested by our previous work [20, 21]. In the present study we found that oxygen consumption was upregulated in quiescent cells, these cells featured elevated SC assembly and their mitochondria were primed to produce more NADH consistent with fully active TCA cycle [26]. This indicates that the ETC gains prominence under the state of quiescence. Consequently, quiescent non-transformed cells would be expected to be susceptible to ETC inhibition. Rather unexpectedly, when glucose was not limiting, this was not the case. Exposure to several ETC inhibitors revealed that quiescent cells were resistant to cell death and produced less ROS, despite increased mitochondrial NADH accumulation expected to stimulate ROS generation from the ETC [11]. Furthermore, ROS inducers that do not inhibit ETC and agents that interfere with mitochondrial ATP production also induced lower levels of ROS and less cell death in quiescent cells, indicating that these cells may be able to detoxify ROS more efficiently, as previously suggested [35-37]. This points to ROS, but not to ATP depletion, as the dominant factor in cell death induction upon ETC inhibition in quiescent cells when glucose is not limiting, and suggests that ROS detoxification, but not the ETC status and increased ETC-mediated ATP production, may determine sensitivity to cell death. This is consistent with reports showing that redox modulation may be an effective anti-cancer strategy [31, 38].

The activity of antioxidant defense was elevated in quiescent cells, and functional interference by shRNA with multiple antioxidant defense components increased cell death and ROS prominently, while little or no effect was observed in proliferating cells. In other words, when glucose is not limiting, effective ROS detoxification protects quiescent non-transformed cells from cell death

induced by the ETC blockade. This clearly differentiates these non-proliferating cells from cancer cells, where direct correlation between ETC activity/assembly and the efficacy of ETC inhibitors to induce cell death has been established in multiple studies [4, 5, 17, 18, 39, 40].

Our data indicate that quiescent cells are protected by the SOD2 - thioredoxin axis, as knocking down SOD2 or TXNRD2, the mitochondrial thioredoxin reductase [34] produced large increases in cell death. On the other hand, while interference with the components of the glutathione system (GPX1 and GSR) led to some elevation of ROS upon ETC blockade, it did not increase cell death, and supplementation with NAC did not ameliorate cell death in quiescent cells. This is consistent with superoxide being the primary agent of ROS-induced damage in quiescent cells, possibly leading to direct oxidation of various mitochondrial components [41-43]. The thioredoxin system may counter this oxidative damage, consistent with its role in the regulation of cell death [14, 44-46]. In agreement, deletion of SOD2, TRX2 or TXNRD2 in mice is incompatible with life [47-49], while genetic inactivation of GSR or GPX1 is not associated with lethality [50, 51].

The sensitivities of proliferating vs. quiescent cells to ETC inhibition change dramatically upon conditions of glucose limitation. Here, a substantial decrease of cellular ATP upon ETC blockade in quiescent cells was observed, which correlates with increased cell death. Downregulation of antioxidant defense components did not increase cell death any further, suggesting that cell death in this situation is largely ROS-independent, instead relating to acute bioenergetic stress. In contrast to quiescent cells, proliferating cells strongly upregulate glucose uptake upon ETC suppression by increasing the level of glucose transporters, which likely compensates for the loss of ATP production in mitochondria. This effect may be particularly important for scavenging glucose when its availability is low, and may explain why quiescent cells are more sensitive to ETC inhibition when glucose is below its normal physiological range. A similar mechanism has been reported in cancer cells, where the failure to upregulate glucose transporters and glucose uptake sensitized to ETC inhibition only when glucose availability was limiting [52].

To interpret the results of this study, it is important to realize that the high and low glucose media represent situations of ample and limited glucose supply. In low glucose media, the glucose

concentrations found in our system within the time frame of the experiment are much lower than those that are regularly encountered *in vivo* in well perfused tissues [32, 53]. In fact, when ETC inhibitors were applied in fresh low (1 g/l) glucose media corresponding to the physiological 5 mM glucose concentration, no ATP depletion was observed. This means that in normal healthy tissues where perfusion is intact, glucose does not present a limiting factor, and it is expected that effective inactivation of ETC blockade-induced ROS by antioxidant defense will play the primary role in governing sensitivity. On the other hand, in poorly perfused regions, such as hypoxic areas of tumors where the availability of nutrients is low, the application of ETC inhibitors may significantly accelerate cell death in a ROS-independent manner. This is consistent with reports of low glucose availability in tumors [53-55], and previous findings showing that ETC inhibition was highly effective in non-perfused hypoxic regions [56]. We therefore propose that the mechanism for the observed selectivity of ETC inhibition in cancer is two-fold. First, elevated antioxidant defense limits ROS production upon ETC inhibition in somatic quiescent cells that do not suffer from glucose limitation, the large majority of cells in an organism. Second, in poorly perfused tumors where glucose is limiting, the application of ETC inhibitors will lead to bioenergetic catastrophe, amplifying the ROS-related effects, and broadening effectivity to cancer cells that might have a 'quiescent'-like phenotype [17].

The mechanism discussed above is not exhaustive, particularly because not all somatic cells in the body are quiescent. In addition, factors specific to each ETC inhibitor, such as its pharmacological properties, blood-brain barrier penetrance, specific accumulation in tumor tissues and off-target effects will also be important, and so will be the rate of ROS production induced by the inhibitor for the given level of ETC suppression. Consistent with this, we propose that ETC inhibitors that induce maximal ROS will kill proliferating cancer cells most efficiently. Yet, this study introduces a general concept that is relevant to all ETC-targeted agents that produce ROS, and describes the molecular mechanisms with the potential to contribute to the specificity observed [5, 19]. To conclude, ETC targeting represent a new, promising approach for treatment of multiple cancer types, including neoplasias that are difficult-to-treat, and resistant populations of cancer cells within tumors [5, 16, 18,

39]. As such, this study is relevant for future development and optimization of very interesting, novel anti-cancer agents that target the ETC and present potential broad-spectrum therapeutics for a range of malignancies.

CONCLUSION

In summary, we propose that specificity of ETC inhibition in cancer is in part conferred by the increased antioxidant defense in non-proliferating non-transformed cells. ROS production from the ETC is the dominant factor that determines susceptibility to cell death induced by ETC disruption in a situation when glucose is not limiting. Hence, ETC inhibitors that induce maximal ROS will kill proliferating cancer cells most efficiently while maintaining specificity. On the other hand, in a situation of glucose limitation characteristic for tumors, ATP depletion upon ETC disruption gains prominence, and non-proliferating cells become highly susceptible to cell death because they are unable to upregulate glucose uptake. This will supplement the ROS-related effects and optimize impact towards cancer, including cancer cells with a dormant-like phenotype.

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AUTHOR'S DISCLOSURE STATEMENT

J.N. is an inventor of a patent ‘Tamoxifen analogues for treatment of neoplastic diseases, especially with high Her2 protein level’. The authors declare no additional competing financial interests.

LIST OF ABBREVIATIONS

BNE	blue native electrophoresis
CAT	catalase
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
DCF	2',7'-dichlorofluorescein diacetate
DHE	dihydroethidium
ETC	electron transport chain
FCCP	carbonyl cyanide p-(trifluoromethoxyl)-phenyl-hydrozone
GPX1	glutathione peroxidase-1
GSR	glutathione reductase
hrCNE	high-resolution clear native electrophoresis
NAC	N-acetyl cysteine
PEITC	phenethyl-isothiocyanate
PRX3	peroxiredoxin-3
ROS	reactive oxygen species
SCs	supercomplexes
SOD1	superoxide dismutase 1
SOD2	manganese superoxide dismutase
TRX2	thioredoxin-2

TXNRD2 thioredoxin reductase 2

WB western blot

REFERENCES

- [1] A.S. Tan, J.W. Baty, L.F. Dong, A. Bezawork-Geleta, B. Endaya, J. Goodwin, M. Bajzikova, J. Kovarova, M. Peterka, B. Yan, E.A. Pesdar, M. Sobol, A. Filimonenko, S. Stuart, M. Vondrusova, K. Kluckova, K. Sachaphibulkij, J. Rohlena, P. Hozak, J. Truksa, D. Eccles, L.M. Haupt, L.R. Griffiths, J. Neuzil, M.V. Berridge, Mitochondrial genome acquisition restores respiratory function and tumorigenic potential of cancer cells without mitochondrial DNA, *Cell Metab.* 21(1) (2015) 81-94.
- [2] F. Weinberg, R. Hamanaka, W.W. Wheaton, S. Weinberg, J. Joseph, M. Lopez, B. Kalyanaraman, G.M. Mutlu, G.R. Budinger, N.S. Chandel, Mitochondrial metabolism and ROS generation are essential for Kras-mediated tumorigenicity, *Proc. Natl. Acad. Sci. U. S. A.* 107(19) (2010) 8788-93.
- [3] L.F. Dong, J. Kovarova, M. Bajzikova, A. Bezawork-Geleta, D. Svec, B. Endaya, K. Sachaphibulkij, A.R. Coelho, N. Sebkova, A. Ruzickova, A.S. Tan, K. Kluckova, K. Judasova, K. Zamecnikova, Z. Rychtarcikova, V. Gopalan, L. Andera, M. Sobol, B. Yan, B. Pattnaik, N. Bhatraju, J. Truksa, P. Stopka, P. Hozak, A.K. Lam, R. Sedlacek, P.J. Oliveira, M. Kubista, A. Agrawal, K. Dvorakova-Hortova, J. Rohlena, M.V. Berridge, J. Neuzil, Horizontal transfer of whole mitochondria restores tumorigenic potential in mitochondrial DNA-deficient cancer cells, *Elife* 6 (2017).
- [4] K. Kluckova, M. Sticha, J. Cerny, T. Mracek, L. Dong, Z. Drahota, E. Gottlieb, J. Neuzil, J. Rohlena, Ubiquinone-binding site mutagenesis reveals the role of mitochondrial complex II in cell death initiation, *Cell Death Dis.* 6 (2015) e1749.
- [5] K. Rohlenova, K. Sachaphibulkij, J. Stursa, A. Bezawork-Geleta, J. Blecha, B. Endaya, L. Werner, J. Cerny, R. Zabalova, J. Goodwin, T. Spacek, E. Alizadeh Pesdar, B. Yan, M.N. Nguyen, M. Vondrusova, M. Sobol, P. Jezek, P. Hozak, J. Truksa, J. Rohlena, L.F. Dong, J. Neuzil, Selective Disruption of Respiratory Supercomplexes as a New Strategy to Suppress Her2high Breast Cancer, *Antioxid. Redox. Signal.* 26(2) (2017) 84-103.
- [6] L.F. Dong, V.J. Jameson, D. Tilly, J. Cerny, E. Mahdavian, A. Marin-Hernandez, L. Hernandez-Esquivel, S. Rodriguez-Enriquez, J. Stursa, P.K. Witting, B. Stantic, J. Rohlena, J. Truksa, K. Kluckova, J.C. Dyason, M. Ledvina, B.A. Salvatore, R. Moreno-Sanchez, M.J. Coster, S.J. Ralph, R.A. Smith, J. Neuzil, Mitochondrial targeting of vitamin E succinate enhances its pro-apoptotic and anti-cancer activity via mitochondrial complex II, *J. Biol. Chem.* 286(5) (2011) 3717-28.
- [7] J. Rohlena, L.F. Dong, S.J. Ralph, J. Neuzil, Anticancer drugs targeting the mitochondrial electron transport chain, *Antioxid. Redox. Signal.* 15(12) (2011) 2951-74.
- [8] G. Cheng, J. Zielonka, O. Ouari, M. Lopez, D. McAllister, K. Boyle, C.S. Barrios, J.J. Weber, B.D. Johnson, M. Hardy, M.B. Dwinell, B. Kalyanaraman, Mitochondria-Targeted Analogues of Metformin Exhibit Enhanced Antiproliferative and Radiosensitizing Effects in Pancreatic Cancer Cells, *Cancer Res.* 76(13) (2016) 3904-15.

- [9] H. Schagger, K. Pfeiffer, Supercomplexes in the respiratory chains of yeast and mammalian mitochondria, *EMBO J.* 19(8) (2000) 1777-83.
- [10] R. Acin-Perez, P. Fernandez-Silva, M.L. Peleato, A. Perez-Martos, J.A. Enriquez, Respiratory active mitochondrial supercomplexes, *Mol. Cell* 32(4) (2008) 529-39.
- [11] M.P. Murphy, How mitochondria produce reactive oxygen species, *Biochem. J.* 417(1) (2009) 1-13.
- [12] M.D. Brand, Mitochondrial generation of superoxide and hydrogen peroxide as the source of mitochondrial redox signaling, *Free Radic. Biol. Med.* 100 (2016) 14-31.
- [13] T. Rabilloud, M. Heller, M.P. Rigobello, A. Bindoli, R. Aebersold, J. Lunardi, The mitochondrial antioxidant defence system and its response to oxidative stress, *Proteomics* 1(9) (2001) 1105-10.
- [14] A.G. Cox, C.C. Winterbourn, M.B. Hampton, Mitochondrial peroxiredoxin involvement in antioxidant defence and redox signalling, *Biochem. J.* 425(2) (2009) 313-25.
- [15] M. Mari, A. Morales, A. Colell, C. Garcia-Ruiz, J.C. Fernandez-Checa, Mitochondrial glutathione, a key survival antioxidant, *Antioxid. Redox. Signal.* 11(11) (2009) 2685-700.
- [16] R. Haq, J. Shoag, P. Andreu-Perez, S. Yokoyama, H. Edelman, G.C. Rowe, D.T. Frederick, A.D. Hurley, A. Nellore, A.L. Kung, J.A. Wargo, J.S. Song, D.E. Fisher, Z. Arany, H.R. Widlund, Oncogenic BRAF regulates oxidative metabolism via PGC1 α and MITF, *Cancer Cell* 23(3) (2013) 302-15.
- [17] A. Viale, P. Pettazoni, C.A. Lyssiotis, H. Ying, N. Sanchez, M. Marchesini, A. Carugo, T. Green, S. Seth, V. Giuliani, M. Kost-Alimova, F. Muller, S. Colla, L. Nezi, G. Genovese, A.K. Deem, A. Kapoor, W. Yao, E. Brunetto, Y. Kang, M. Yuan, J.M. Asara, Y.A. Wang, T.P. Heffernan, A.C. Kimmelman, H. Wang, J.B. Fleming, L.C. Cantley, R.A. DePinho, G.F. Draetta, Oncogene ablation-resistant pancreatic cancer cells depend on mitochondrial function, *Nature* 514(7524) (2014) 628-32.
- [18] A. Roesch, A. Vultur, I. Bogeski, H. Wang, K.M. Zimmermann, D. Speicher, C. Korbel, M.W. Laschke, P.A. Gimotty, S.E. Philipp, E. Krause, S. Patzold, J. Villanueva, C. Krepler, M. Fukunaga-Kalabis, M. Hoth, B.C. Bastian, T. Vogt, M. Herlyn, Overcoming intrinsic multidrug resistance in melanoma by blocking the mitochondrial respiratory chain of slow-cycling JARID1B(high) cells, *Cancer Cell* 23(6) (2013) 811-25.
- [19] L.F. Dong, V.J. Jameson, D. Tilly, L. Prochazka, J. Rohlena, K. Valis, J. Truksa, R. Zabalova, E. Mahdavian, K. Kluckova, M. Stantic, J. Stursa, R. Freeman, P.K. Witting, E. Norberg, J. Goodwin, B.A. Salvatore, J. Novotna, J. Turanek, M. Ledvina, P. Hozak, B. Zhivotovsky, M.J. Coster, S.J. Ralph, R.A. Smith, J. Neuzil, Mitochondrial targeting of α -tocopheryl succinate enhances its pro-apoptotic efficacy: a new paradigm for effective cancer therapy, *Free Radic. Biol. Med.* 50(11) (2011) 1546-55.
- [20] J. Rohlena, L.F. Dong, K. Kluckova, R. Zabalova, J. Goodwin, D. Tilly, J. Stursa, A. Pecinova, A. Philimonenko, P. Hozak, J. Banerjee, M. Ledvina, C.K. Sen, J. Houstek, M.J. Coster, J. Neuzil, Mitochondrially targeted α -tocopheryl succinate is antiangiogenic: potential benefit against tumor angiogenesis but caution against wound healing, *Antioxid. Redox. Signal.* 15(12) (2011) 2923-35.
- [21] L.F. Dong, E. Swettenham, J. Eliasson, X.F. Wang, M. Gold, Y. Medunic, M. Stantic, P. Low, L. Prochazka, P.K. Witting, J. Turanek, E.T. Akporiaye, S.J. Ralph, J. Neuzil, Vitamin E analogues inhibit

angiogenesis by selective induction of apoptosis in proliferating endothelial cells: the role of oxidative stress, *Cancer Res.* 67(24) (2007) 11906-13.

[22] J. Truksa, L.F. Dong, J. Rohlena, J. Stursa, M. Vondrusova, J. Goodwin, M. Nguyen, K. Kluckova, Z. Rychtarcikova, S. Lettlova, J. Spacilova, M. Stapelberg, M. Zoratti, J. Neuzil, Mitochondrially targeted vitamin E succinate modulates expression of mitochondrial DNA transcripts and mitochondrial biogenesis, *Antioxid. Redox. Signal.* 22(11) (2015) 883-900.

[23] S. Schmitt, F. Saathoff, L. Meissner, E.M. Schropp, J. Lichtmanegger, S. Schulz, C. Eberhagen, S. Borchard, M. Aichler, J. Adamski, N. Plesnila, S. Rothenfusser, G. Kroemer, H. Zischka, A semi-automated method for isolating functionally intact mitochondria from cultured cells and tissue biopsies, *Anal. Biochem.* 443(1) (2013) 66-74.

[24] M. Vondrusova, A. Bezawork-Geleta, K. Sachaphibulkij, J. Truksa, J. Neuzil, The effect of mitochondrially targeted anticancer agents on mitochondrial (super)complexes, *Methods Mol. Biol.* 1265 (2015) 195-208.

[25] C.J. Weydert, J.J. Cullen, Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue, *Nat. Protoc.* 5(1) (2010) 51-66.

[26] J.M. Lemons, X.J. Feng, B.D. Bennett, A. Legesse-Miller, E.L. Johnson, I. Raitman, E.A. Pollina, H.A. Rabitz, J.D. Rabinowitz, H.A. Collier, Quiescent fibroblasts exhibit high metabolic activity, *PLoS Biol.* 8(10) (2010) e1000514.

[27] S. Schoors, K. De Bock, A.R. Cantelmo, M. Georgiadou, B. Ghesquiere, S. Cauwenberghs, A. Kuchnio, B.W. Wong, A. Quaegebeur, J. Goveia, F. Bifari, X. Wang, R. Blanco, B. Tembuyser, I. Cornelissen, A. Bouche, S. Vinckier, S. Diaz-Moralli, H. Gerhardt, S. Telang, M. Cascante, J. Chesney, M. Dewerchin, P. Carmeliet, Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis, *Cell Metab.* 19(1) (2014) 37-48.

[28] I. Diamond, A. Legg, J.A. Schneider, E. Rozengurt, Glycolysis in quiescent cultures of 3T3 cells. Stimulation by serum, epidermal growth factor, and insulin in intact cells and persistence of the stimulation after cell homogenization, *J. Biol. Chem.* 253(3) (1978) 866-71.

[29] E. Maranzana, G. Barbero, A.I. Falasca, G. Lenaz, M.L. Genova, Mitochondrial respiratory supercomplex association limits production of reactive oxygen species from complex I, *Antioxid. Redox. Signal.* 19(13) (2013) 1469-80.

[30] J.A. Letts, K. Fiedorczuk, L.A. Sazanov, The architecture of respiratory supercomplexes, *Nature* 537(7622) (2016) 644-648.

[31] D. Trachootham, Y. Zhou, H. Zhang, Y. Demizu, Z. Chen, H. Pelicano, P.J. Chiao, G. Achanta, R.B. Arlinghaus, J. Liu, P. Huang, Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by beta-phenylethyl isothiocyanate, *Cancer Cell* 10(3) (2006) 241-52.

[32] M.Z. Shrayyef, J.E. Gerich, Normal Glucose Homeostasis, in: L. Poretsky (Ed.), *Principles of Diabetes Mellitus*, Springer US, Boston, MA, 2010, pp. 19-35.

[33] M. Mueckler, B. Thorens, The SLC2 (GLUT) family of membrane transporters, *Mol. Aspects Med.* 34(2-3) (2013) 121-38.

- [34] S.R. Lee, J.R. Kim, K.S. Kwon, H.W. Yoon, R.L. Levine, A. Ginsburg, S.G. Rhee, Molecular cloning and characterization of a mitochondrial selenocysteine-containing thioredoxin reductase from rat liver, *J. Biol. Chem.* 274(8) (1999) 4722-34.
- [35] G.J. Kops, T.B. Dansen, P.E. Polderman, I. Saarloos, K.W. Wirtz, P.J. Coffey, T.T. Huang, J.L. Bos, R.H. Medema, B.M. Burgering, Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress, *Nature* 419(6904) (2002) 316-21.
- [36] E.H. Sarsour, S. Venkataraman, A.L. Kalen, L.W. Oberley, P.C. Goswami, Manganese superoxide dismutase activity regulates transitions between quiescent and proliferative growth, *Aging Cell* 7(3) (2008) 405-17.
- [37] J. Naderi, M. Hung, S. Pandey, Oxidative stress-induced apoptosis in dividing fibroblasts involves activation of p38 MAP kinase and over-expression of Bax: resistance of quiescent cells to oxidative stress, *Apoptosis* 8(1) (2003) 91-100.
- [38] D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach?, *Nat. Rev. Drug Discov.* 8(7) (2009) 579-91.
- [39] B. Yan, M. Stantic, R. Zabalova, A. Bezawork-Geleta, M. Stapelberg, J. Stursa, K. Prokopova, L. Dong, J. Neuzil, Mitochondrially targeted vitamin E succinate efficiently kills breast tumour-initiating cells in a complex II-dependent manner, *BMC Cancer* 15 (2015) 401.
- [40] S. Boukalova, J. Stursa, L. Werner, Z. Ezrova, J. Cerny, A. Bezawork-Geleta, A. Pecinova, L. Dong, Z. Drahota, J. Neuzil, Mitochondrial Targeting of Metformin Enhances Its Activity against Pancreatic Cancer, *Mol. Cancer Ther.* 15(12) (2016) 2875-2886.
- [41] Z. Dong, S. Shanmughapriya, D. Tomar, N. Siddiqui, S. Lynch, N. Nemani, S.L. Breves, X. Zhang, A. Tripathi, P. Palaniappan, M.F. Riitano, A.M. Worth, A. Seelam, E. Carvalho, R. Subbiah, F. Jana, J. Soboloff, Y. Peng, J.Y. Cheung, S.K. Joseph, J. Caplan, S. Rajan, P.B. Stathopoulos, M. Madesh, Mitochondrial Ca²⁺ Uniporter Is a Mitochondrial Luminal Redox Sensor that Augments MCU Channel Activity, *Mol. Cell* 65(6) (2017) 1014-1028 e7.
- [42] V.E. Kagan, V.A. Tyurin, J. Jiang, Y.Y. Tyurina, V.B. Ritov, A.A. Amoscato, A.N. Osipov, N.A. Belikova, A.A. Kapralov, V. Kini, Vlasova, II, Q. Zhao, M. Zou, P. Di, D.A. Svistunenko, I.V. Kurnikov, G.G. Borisenko, Cytochrome c acts as a cardiolipin oxygenase required for release of proapoptotic factors, *Nat. Chem. Biol.* 1(4) (2005) 223-32.
- [43] M. Ott, J.D. Robertson, V. Gogvadze, B. Zhivotovsky, S. Orrenius, Cytochrome c release from mitochondria proceeds by a two-step process, *Proc. Natl. Acad. Sci. U. S. A.* 99(3) (2002) 1259-63.
- [44] K.K. Brown, S.E. Eriksson, E.S. Arner, M.B. Hampton, Mitochondrial peroxiredoxin 3 is rapidly oxidized in cells treated with isothiocyanates, *Free Radic. Biol. Med.* 45(4) (2008) 494-502.
- [45] A.G. Cox, K.K. Brown, E.S. Arner, M.B. Hampton, The thioredoxin reductase inhibitor auranofin triggers apoptosis through a Bax/Bak-dependent process that involves peroxiredoxin 3 oxidation, *Biochem. Pharmacol.* 76(9) (2008) 1097-109.
- [46] Y. Chen, J. Cai, D.P. Jones, Mitochondrial thioredoxin in regulation of oxidant-induced cell death, *FEBS Lett.* 580(28-29) (2006) 6596-602.

- [47] M. Conrad, C. Jakupoglu, S.G. Moreno, S. Lippl, A. Banjac, M. Schneider, H. Beck, A.K. Hatzopoulos, U. Just, F. Sinowatz, W. Schmahl, K.R. Chien, W. Wurst, G.W. Bornkamm, M. Brielmeier, Essential role for mitochondrial thioredoxin reductase in hematopoiesis, heart development, and heart function, *Mol. Cell. Biol.* 24(21) (2004) 9414-23.
- [48] L. Nonn, R.R. Williams, R.P. Erickson, G. Powis, The absence of mitochondrial thioredoxin 2 causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice, *Mol. Cell. Biol.* 23(3) (2003) 916-22.
- [49] Y. Li, T.T. Huang, E.J. Carlson, S. Melov, P.C. Ursell, J.L. Olson, L.J. Noble, M.P. Yoshimura, C. Berger, P.H. Chan, D.C. Wallace, C.J. Epstein, Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase, *Nat. Genet.* 11(4) (1995) 376-81.
- [50] L.K. Rogers, T. Tamura, B.J. Rogers, S.E. Welty, T.N. Hansen, C.V. Smith, Analyses of glutathione reductase hypomorphic mice indicate a genetic knockout, *Toxicol. Sci.* 82(2) (2004) 367-73.
- [51] Y.S. Ho, J.L. Magnenat, R.T. Bronson, J. Cao, M. Gargano, M. Sugawara, C.D. Funk, Mice deficient in cellular glutathione peroxidase develop normally and show no increased sensitivity to hyperoxia, *J. Biol. Chem.* 272(26) (1997) 16644-51.
- [52] K. Birsoy, R. Possemato, F.K. Lorbeer, E.C. Bayraktar, P. Thiru, B. Yucel, T. Wang, W.W. Chen, C.B. Clish, D.M. Sabatini, Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides, *Nature* 508(7494) (2014) 108-12.
- [53] T.B. Rodrigues, E.M. Serrao, B.W. Kennedy, D.E. Hu, M.I. Kettunen, K.M. Brindle, Magnetic resonance imaging of tumor glycolysis using hyperpolarized ¹³C-labeled glucose, *Nat. Med.* 20(1) (2014) 93-7.
- [54] A. Hirayama, K. Kami, M. Sugimoto, M. Sugawara, N. Toki, H. Onozuka, T. Kinoshita, N. Saito, A. Ochiai, M. Tomita, H. Esumi, T. Soga, Quantitative metabolome profiling of colon and stomach cancer microenvironment by capillary electrophoresis time-of-flight mass spectrometry, *Cancer Res.* 69(11) (2009) 4918-25.
- [55] J. Goveia, A. Pircher, L.C. Conradi, J. Kalucka, V. Lagani, M. Dewerchin, G. Eelen, R.J. DeBerardinis, I.D. Wilson, P. Carmeliet, Meta-analysis of clinical metabolic profiling studies in cancer: challenges and opportunities, *EMBO Mol. Med.* 8(10) (2016) 1134-1142.
- [56] X. Zhang, M. Fryknas, E. Hernlund, W. Fayad, A. De Mito, M.H. Olofsson, V. Gogvadze, L. Dang, S. Pahlman, L.A. Schughart, L. Rickardson, P. D'Arcy, J. Gullbo, P. Nygren, R. Larsson, S. Linder, Induction of mitochondrial dysfunction as a strategy for targeting tumour cells in metabolically compromised microenvironments, *Nat. Commun.* 5 (2014) 3295.

Fig. 1. Confluent cells undergo cell cycle arrest and form adherence junctions. (A, B) Analysis of cell cycle distribution of (A) Ea.hy926 and (B) MCF10A cells was performed by double staining for Hoechst 33342 and Pyronin Y. Flow cytometry was used to assign cells to G₀/G₁/S/G₂M phase of the cell cycle based on the level of Hoechst 33342 and Pyronin Y staining. Data are shown as means $\pm$ S.E.M. of 5 independent experiments. (C, D) Adherence junctions in (C) Ea.hy926 and (D) MCF10A cells were detected by immunostaining for VE-cadherin and E-cadherin, respectively, and analyzed by confocal microscopy. Representative images are shown.

Fig. 2. Electron transport chain activity and assembly is enhanced in quiescent cells. (A, B) Oxygen consumption rate (OCR, pMoles/min) profiles in (A) Ea.hy926 (B) and MCF10A cells in both low glucose (1 g/l) and high glucose (4.5 g/l) was measured using Seahorse Bioscience XF24 extracellular flux analyzer. Data correlated to DNA content are present as means $\pm$ S.E.M. from at least three independent experiments. (C) Excreted lactate was measured in the culture medium after 19 hours cultivation. Data represent means $\pm$ S.E.M. of 5 independent experiments, and are correlated to cell number. The symbol * indicates values significantly different between proliferating and quiescent cells. (D) Glucose consumption of Ea.hy926 and MCF10A cells was measured upon 24 h pre-incubation in low glucose media (1 g/l glucose) by flow cytometry using 2-NBDG. Data are correlated to quiescent Ea.hy926 cells in high glucose condition. Data represent means $\pm$ S.E.M., $n \geq 4$. The symbol * indicates values significantly different between proliferating and quiescent cells. (E) Isolated mitochondrial fraction were probed for the presence of SCs by WB following BNE with the following antibodies: CI, NDUFB8; CII, SDHA; CIII, MTCO2; CIV, UQCRCF1; and CV, ATP5A; mtHSP70 was used as loading control. A representative experiment is shown. (F) In-gel assessment of enzymatic activity of respiratory SCs was performed in isolated mitochondrial fraction following hrCNE as follows: CI - NADH:NTB reductase, CII - succinate:NTB reductase, CIII - DAB oxidase, CIV - DAB:cytochrome c reductase, CV - ATP hydrolase. A representative experiment is shown.

Fig. 3. In-situ measurements of mitochondrial NAD(P)H indicate increased NADH utilization by the ETC in quiescent cells. (A) Mitochondrial NAD(P)H signal was measured by two-photon microscopy in control, CCCP (5 μ M) or rotenone (1.25 μ M)-treated cells. Representative images are shown. (B) Average values of NAD(P)H signal quantified in Fiji as described in ‘Materials and methods’. Data represent the means $\pm$ S.E.M. of 4 independent experiments. The symbol * indicates values significantly different between control and rotenone-treated cells.

Fig. 4. Glucose availability affects sensitivity of quiescent cells to cell death induced by inhibitors of the ETC and ATP synthesis, and not by oxidant agents. (A-F) Ea.hy926 and (G-H) MCF10A cells cultivated in high glucose (4.5 g/l glucose, A-C, G-I) or low glucose (1 g/l glucose, D-F, J-L) media, respectively, were treated with H₂O₂ (800 mM), PEITC (10 μ M), rotenone (Rot, 2 μ M), piericidin (Pier, 80 μ M high glucose, 0.05 μ M low glucose), MitoTam (mTAM, 4 μ M high glucose and 2 μ M low glucose), MitoVES (mVES, 10 μ M), myxothiazol (Myxo, 15 μ M high glucose and 0.1 μ M low glucose), CCCP (75 μ M high glucose and 10 μ M low glucose), oligomycin (Omy, 25 μ M high glucose and 0.5 μ M low glucose) for 22 h. The cells were stained with Annexin V-FITC/PI, and evaluated by flow cytometry as described in ‘Materials and methods’. Data represent the means $\pm$ S.E.M., $n \geq 4$. The symbol * indicates values significantly different between proliferating and quiescent cells.

Fig. 5. Cellular ATP is depleted in quiescent cells upon ETC inhibition in sub-physiological glucose levels. (A) Glucose concentration in cell-conditioned media was determined after 6, 16, 24 and 38 h of cultivation using amplex red-based assay. Data are correlated to fresh media and presented as means $\pm$ S.E.M., $n=3$. (B, D) Ea.hy926 and (C, E) MCF10A cells in (B, C) high and (D, E) low glucose media were treated for 0.5 h with rotenone (2 μ M), piericidin (5 μ M), mVES (10 μ M), myxothiazol (15 μ M), oligomycin (25 μ M), CCCP (75 μ M) and ATP level was assessed using luminescent assay. Results were corrected to cell number measured by crystal violet in a parallel

plate. Data represent means $\pm$ S.E.M. of 3 independent experiments, the symbol * indicates significant differences to untreated control. **(F)** Quiescent Ea.hy926 cells in fresh low glucose (5.5 mM) media or low glucose media cell-conditioned for 18 hours were exposed to the indicated agents as described above, and the ATP content was determined. Data represent means $\pm$ S.E.M., n=3. **(G)** Glucose uptake was assessed using 2-NBDG staining and flow cytometry after 6 h treatment with MitoVES (0.5 μ M low glucose or 5 μ M high glucose) and myxothiazol (7.5 μ M). Data shown represent means $\pm$ S.E.M. of at least 3 independent experiments, and are expressed relative to the untreated control. The symbol * indicates values significantly different between proliferating and quiescent cells. **(H)** Surface GLUT1 was determined by immunostaining of non-permeabilized cells using primary anti-GLUT1 IgG and secondary Cy3 labelled anti-Rabbit IgG and flow cytometry. Cells were pretreated for 2 h by MitoVES (5 μ M) and myxothiazol (7.5 μ M). Data shown represent means $\pm$ S.E.M. of 4-7 independent experiments, and are expressed relative to the untreated control. The symbol * indicates values significantly different from untreated control.

Fig. 6. ROS production upon treatment by oxidant agents and oxidative phosphorylation

inhibitors is reduced in quiescent cells. (A-F) Ea.hy926 cells cultivated in high glucose (4.5 g/l glucose, **A-C**) or in low glucose (1 g/l glucose, **D-F**) media were treated with H₂O₂ (800 μ M), PEITC (3 μ M), rotenone (rot, 20 μ M), piericidin (pier, 50 μ M high glucose, 10 μ M low glucose), MitoTAM (mTAM, 7.5 μ M) MitoVES (mVES, 10 μ M), myxothiazol (myxo, 15 μ M), CCCP (75 μ M), and oligomycin (omy, 2.5 μ M) for 1 h, followed by staining with the DCF fluorescent probe. **(G-L)** MCF10A cells cultivated in high glucose (4.5 g/l glucose, **G-I**) or in low glucose (1 g/l glucose, **J-L**) media were treated with H₂O₂ (800 μ M), PEITC (10 μ M), rotenone (2 μ M), piericidin (50 μ M), mVES (10 μ M), myxothiazol (25 μ M), CCCP (75 μ M), oligomycin (2.5 μ M) for 1 h and then stained using the DCF fluorescent probe. Data are expressed relative to the non-treated controls and represent the mean $\pm$ S.E.M. of more than 4 independent experiments. The symbol * indicates values significantly different in proliferating and quiescent cells.

Fig. 7. Activity of antioxidant defense is increased in quiescent cells. Whole cell lysates from (A) Ea.hy926 and (B) MCF10A cultured in high and low glucose media were assessed by WB for expression of the indicated antioxidant proteins. Quantification is done relative to actin. Data represent 3 independent experiments. (C-E) In-gel activities of indicated antioxidant enzymes were assessed in (C) Ea.hy926 and (D) MCF10A cells in high and low glucose media. (E) Data were quantified by densitometry, and correlated to quiescent cells in high glucose media. Data shown are means $\pm$ S.E.M. of at least 4 independent experiments. (F, G) GSH/GSSG ratio (F) and NADPH/NADP⁺ ratio (G) was determined by luminescence assay in whole cell extracts. Data shown are means $\pm$ S.E.M., n=4. The symbol * indicates significantly different values between proliferating and quiescent cells.

Fig. 8. Antioxidant defense protects quiescent cells from cell death when glucose is not limiting. (A-C) Ea.hy926 cells were silenced for SOD2 and assessed for (A) cell death by the Annexin V-FITC/ PI method, (B) ROS production by DHE and (C) DCF. (D,E) GPX1-silenced cells were assessed for (D) cell death and (E) ROS production by DCF. (F,G) GSR silenced cells were assessed (F) for cell death and (G) ROS production by DCF. (H,I) TXNRD2-silenced cells were assessed for (H) cell death and (I) ROS production by DCF. Data represent means $\pm$ S.E.M., n $\geq$ 4., are given relative to cells expressing non-silencing shRNA treated in the identical manner, and measured as described in the legends to figures 4 and 6. The symbol * indicates values significantly different between cells expressing specific shRNA and non-silencing shRNA. (J) Cell were pre-treated by auranofin (0.75 μ M) for 2 h, followed by MitoVES (mVES, 10 μ M) and myxothiazol (myxo, 15 μ M) treatment. After 22 h, cell death was assessed using Annexin V-FITC/ PI staining. Data are shown as means $\pm$ S.E.M. of at least 5 independent experiments. (K) Cells were pre-treated with NAC (1 mM) for 1 h, followed by rotenone (5 μ M), MitoVES (mVES, 10 μ M) and myxothiazol (myxo, 15 μ M) treatment. After 22 h, cell death was assessed using Annexin V-FITC/PI staining. Data shown are

means $\pm$ S.E.M., $n \geq 5$. The symbol * indicates values significantly different with or without (J) auranofin and (K) NAC pre-treatment.

Fig. S1. The effect of investigated agents on cellular respiration. Oxygen consumption by proliferating Ea.hy926 cultured in both low glucose (1 g/l) and high glucose (4.5 g/l) media was monitored by the Oroboros Oxygraph instrument. Basal respiration was established after adding succinate (10 mM) and ADP (3 mM), and was followed by titration by pharmacological agents indicated in A-J.

Fig. S2. Antioxidant defense does not protect quiescent cells from cell death when glucose is limiting. Ea.hy926 cells cultured in low glucose media were silenced for SOD2 (A), GPX1 (B), GSR (C) and TRDXN2 (D). The cells were treated with indicated compounds as describe in figure 4 for 22 h and cell death was assessed by the Annexin V/PI method. Data shown are means $\pm$ S.E.M. of 4 independent experiments, and are shown relative to non-silencing control.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Fig. S1. The effect of investigated agents on cellular respiration. Oxygen consumption by proliferating *Ea.hy926* cultured in both low glucose (1 g/l) and high glucose (4.5 g/l) media was monitored by the Oroboros Oxygraph instrument. Basal respiration was established after adding succinate (10 mM) and ADP (3 mM), and was followed by titration by pharmacological agents indicated in **A-J**.

Fig. S2. Antioxidant defence does not protect quiescent cells from cell death when glucose is limiting. Ea.hy926 cells cultured in low glucose media were silenced for SOD2 (A), GPX1 (B), GSR (C) and TRDXN2 (D). The cells were treated with indicated compounds as describe in figure 4 for 22 h and cell death was assessed by the Annexin V/PI method. Data shown are means $\pm$ SEM of 4 independent experiments, and are shown relative to non-silencing control.