BCL(X)L and BCL2 increase the metabolic fitness of breast cancer cells: a single cell imaging study

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Abbreviations: BCL2 (B-cell lymphoma), FRET (Förster resonance energy transfer), ATP (adenosine triphosphate), OXPHOS (oxidative phosphorylation), VDAC (voltage-dependent anion channel), $\Delta\Psi_m$ (mitochondrial membrane potential), ER^+ (estrogen receptor positive), STS (staurosporine), FCCP (Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone), CFP (Cyan fluorescent protein), TMRM (tetramethylrhodamine methyl ester), IMM (inner mitochondrial membrane), OMM (outer mitochondrial membrane), SILAC (stable isotope labelling with amino acids in cell culture).

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ABSTRACT

The BCL2 family of proteins regulate apoptosis by controlling mitochondrial outer membrane permeability. However, effects on mitochondrial structure and bioenergetics have also been reported. Here we comprehensively characterized the effects of BCL2 and BCL(X)L on cellular energetics in MCF7 breast cancer cells using time-lapse confocal single cell imaging and mitochondrial and cytosolic FRET reporters. We found that BCL2 and BCL(X)L increase the metabolic robustness of MCF7 cells, and that this was associated with increased mitochondrial NAD(P)H and ATP levels. Experiments with the F_1F_0 synthase inhibitor oligomycin demonstrated that BCL2 and in particular BCL(X)L, while not affecting ATP synthase activity, more efficiently coupled the mitochondrial proton motive force with ATP production. This metabolic advantage was associated with an increased resistance to nutrient deprivation and enhanced clonogenic survival in response to metabolic stress, in the absence of profound effects on cell death. Our data suggest that a primary function of BCL(X)L and BCL2 overexpression in tumor cells is to increase their resistance to metabolic stress in the tumor microenvironment, independent of cell death signaling.

INTRODUCTION

BCL2 family members regulate the intrinsic apoptosis pathway by controlling the process of mitochondrial outer membrane permeabilisation (MOMP) (1). Apart from this function, BCL2 proteins also regulate mitochondrial fusion and fission (2-5). This process is important for mitochondrial quality control, but also regulates mitochondrial bioenergetics (6). BCL2 proteins may also control mitochondrial metabolism directly. BCL2 and BAX regulate the activity of the mitochondrial adenine nucleotide translocator (7). BCL(X)L preserves the physiological conformation of the voltage-dependent anion channel (VDAC) and promotes exchange of metabolites (8). In neurons, a pool of BCL(X)L has been found to localise in the inner mitochondrial membrane (IMM) and to interact with F_1F_0 ATP synthase, increasing its enzymatic activity and stabilizing mitochondrial membrane potential ($\Delta\Psi_m$) (9, 10). A similar function was attributed to a truncated form of MCL1 that localizes to the matrix (11).

A comprehensive characterization of the effects of BCL2 and BCL(X)L on mitochondrial bioenergetics at the single cell level and its relation to the process of MOMP has rarely been performed. It is important to dissect these events at this level, as cell death signalling may affect metabolism and *vice versa* (12, 13). Effects of BCL2 family proteins on bioenergetics may be particularly important in cancer cells, which undergo periods of metabolic stress, and indeed often accumulate extraordinary high levels of BCL2 and BCL(X)L as seen in breast cancer (14-16). Here, we aimed to comprehensively characterize the effects of BCL2 and BCL(X)L on cellular energetics in breast cancer cells using time-lapse single cell imaging and mitochondrial/cytosolic ATP FRET reporters.

RESULTS

Characterisation of BCL2 and BCL(X)L overexpressing ER^+ MCF7 breast cancer cells

We assessed absolute BCL2 protein levels in previously characterised MCF7 cells stably overexpressing BCL2 or BCL(X)L (17-19), using BCL2 quantitative western blotting (20)

(Fig. 1A and B). MCF7-pSFFV (control) cells showed a higher BCL2 concentration over BCL(X)L and MCL1 (Fig. 1A and B). In MCF7-BCL2 cells, BCL2 was present at a concentration of 5.59 μM, while MCF7-BCL(X)L had levels comparable to MCF7-pSFFV cells (0.35 μM). BCL(X)L reached a concentration of 3.5 μM in MCF7-BCL(X)L (Fig. 1B). MCL1, BAX and BAK levels were similar in the three cell lines (Supplementary Fig. 1A and B). Thus, high BCL2 or BCL(X)L levels were observed in the respective overexpressing cell lines, with little compensatory alterations in other BCL2 proteins.

Next, we analysed the subcellular localization of BCL2 and BCL(X)L in the respective overexpressing cells, through immunofluorescence. Both BCL2 and BCL(X)L antibodies co-localised with MitoTracker Red CMXRos, while no signal was detected in the nucleus (Fig. 1C). BCL(X)L cells displayed slightly different sub-localisation pattern when compared to BCL2 cells, as previously reported in the same cell lines, by Antonietti *et al* (17, 21).

We also tested whether BCL2 and BCL(X)L overexpressing MCF7 possessed enhanced survival rates following exposure to staurosporine (STS; 2 μM) or cisplatin (40 μM), through flow cytometry. MCF7-pSSFV were susceptible to both treatments, while BCL2 and BCL(X)L overexpressing cell lines were less sensitive (Fig. 1D and E).

BCL2 and BCL(X)L expressing cells produce similar amounts of mitochondrial ATP by employing less mitochondrial NAD(P)H

We transfected cells with a plasmid encoding a mitochondrial targeted ATP FRET probe (22), which was multiplexed with the $\Delta\Psi_m$ sensitive dye TMRM, to measure alterations in mitochondrial ATP levels simultaneously with $\Delta\Psi_m$ depolarisation or hyperpolarisation (23, 24). To investigate the effects of BCL2 and BCL(X)L on mitochondrial bioenergetics without contribution from glycolysis, cells were starved for 3 hours and then fuelled with the mitochondrial substrate pyruvate. Cells were imaged with a laser scanning confocal microscope every minute (Supplementary Fig. 2A). Baseline values during starvation were

recorded for the first 20 minutes, then 2 mM pyruvate was added and single cell kinetics were followed for another 20 minutes. Finally, ATP synthase inhibitor oligomycin was used to block the F_0 subunit of the ATP synthase.

Pyruvate addition activated mitochondrial ATP production, observed through an increase in the normalized FRET/CFP ratio in all three cell lines (Fig. 2A). Following oligomycin, mitochondrial ATP dropped as a result of ATP synthase inhibition (Fig. 2A). $\Delta\Psi_m$ increased during pyruvate exposure due to electron transport chain (ETC) activity and further increased after oligomycin addition, due to proton motive force not being used for ATP production (Fig. 2A). We compared the absolute FRET/CFP ratio of the 3 different MCF7 clones, which is more indicative of actual mitochondrial ATP (Fig. 2B). Baseline and post-pyruvate addition mitochondrial ATP levels were lower in BCL2 cells when compared to MCF7-pSFFV and BCL(X)L cells. Following oligomycin, ATP levels were similar in all cell lines (Fig. 2B). Interestingly, a lower absolute TMRM fluorescence was recorded for BCL2 and BCL(X)L overexpressing cells at baseline (Fig 2D). Furthermore, BCL(X)L cells showed a significantly higher $\Delta\Psi_m$ following oligomycin addition, when compared to the other cells (Fig 2A and C).

Using similar treatment protocol, we next measured NAD(P)H kinetics (Supplementary Fig. 2B). An increase in NAD(P)H fluorescence was measured when pyruvate was added to the experimental buffer (Fig. 2E). Quantification of NAD(P)H levels in the different cells revealed that during starvation and after pyruvate addition, BCL2 and BCL(X)L cells accumulated higher NAD(P)H levels (Fig. 2F). Despite lower NAD(P)H levels, MCF7-pSFFV cells exhibited higher NAD(P)H production kinetics following pyruvate (Fig. 2E). Slope analyses for pyruvate addition revealed that both MCF7-BCL2 and BCL(X)L displayed slower NAD(P)H production rates when compared to MCF7-pSFFV (Fig. 2G). Next, we calculated the NAD(P)H/ATP ratio, by dividing NAD(P)H absolute fluorescence to

FRET/CFP absolute ratio. We found that in all conditions MCF7-BCL2 and BCL(X)L cells accumulated a higher ratio when compared to MCF7-pSFFV cells (Fig. 2H). This suggested that BCL2 and BCL(X)L overexpressing clones produce similar amount of mitochondrial ATP, by employing significantly less mitochondrial NAD(P)H.

BCL2 and BCL(X)L overexpressing cell lines couple the mitochondrial proton motive force with ATP production in a more efficient way

We next explored whether BCL2 and BCL(X)L were acting on F_1F_0 ATP synthase to enhance mitochondrial energetics of breast cancer cells, by performing an oligomycin titration. Cells, transiently transfected with mitochondrial ATeam probe, were placed in KB with 2 mM pyruvate; after 20 minutes of baseline, we applied 1 nM, 10 nM and 100 nM oligomycin, every 20 minutes (Supplementary Fig. 3). These concentrations were chosen in order to avoid saturation of ATP synthase. ATP production decreased after addition of 1 nM of oligomycin in all cell lines, while TMRM signal indicated increased $\Delta\Psi_m$ (Fig. 3A). Analysis of FRET/CFP ratios revealed that both MCF7-BCL2 and MCF7-BCL(X)L possessed higher ATP production following 1 nM oligomycin treatment compared to control cells. No changes were observed after 10 nM and 100 nM of oligomycin addition, as the enzyme was likely saturated (Fig. 3B). Of note, an increased TMRM intensity was recorded in response to oligomycin for MCF7-BCL(X)L cells, compared to control cells (Fig. 3A and D). Kinetics analysis revealed higher slope values (see Materials and methods, time lapse imaging section) for BCL2 and BCL(X)L clones after 1 nM oligomycin treatment (Fig. 3C), indicating slower ATP consumption (since larger negative slope values indicate a faster descent of the kinetic curve).

Next, we determined ATP synthase β subunit protein levels by WB, as it was previously shown that BCL(X)L can establish an interaction with this subunit (9). As shown in Fig. 3F no significant difference was detected. We determined the enzymatic activity of

immunocaptured ATP synthase from isolated mitochondria by monitoring the oxidation reaction of NAD(P)H to NAD+. Again, no difference was observed (Fig. 3G). Moreover, we analysed the effect of the proton uncoupler FCCP on cytosolic ATP levels in pyruvate. We observed that BCL2 and BCL(X)L overexpressing cells maintained higher cytosolic ATP levels after FCCP treatment (Supplementary Fig. 4). Collectively, these data suggested that BCL2 and BCL(X)L overexpressing cancer cells possessed higher rates of proton motive force coupling, with little alterations in ATP synthase activity.

BCL2 and BCL(X)L overexpressing cells maintain higher cytosolic ATP levels during starvation and exhibit higher clonogenic potential during nutrient deprivation

To determine whether these mitochondrial alterations in BCL2 and BCL(X)L cells precipitated in survival/growth advantage during metabolic stress, we subjected MCF7 clones to nutrient depletion. As starvation has an impact on the cytosolic ATP kinetics, and we wanted to analyse the ability of BCL2 and BCL(X)L to rescue cell functions by maintaining cytosolic ATP levels, we here used the cytoplasmic version of the ATeam construct (22). Cells were placed in KB with no nutrient, and cytosolic ATP levels measured over 24 hours; (Supplementary Fig. 5A). As shown in Fig. 4A, ATP kinetics decreased during the time-course. Five mM glucose partially rescued ATP production due to glycolysis activation (Fig. 4A). $\Delta\Psi_m$ measured at the same time showed a small initial increase during the first 2-4 hours, followed by a small decrease (Supplementary Fig. 5B). Measurement of the FRET/CFP ratio revealed lower baseline values for MCF7-BCL(X)L compared to pSFFV and BCL2 cells (Fig. 4B). From 12 hours until the end of the experiment, both BCL2 and BCL(X)L overexpressing cells maintained higher ATP levels compared to control cells (Fig. 4B). Kinetic analysis of ATP consumption highlighted higher slope values for MCF7-BCL2 and BCL(X)L compared to pSFFV cells, from 6 to 20 hours after nutrients removal (Fig. 4C), suggesting slower ATP loss. TMRM levels were higher for BCL(X)L cells after 2, 6 and 18

hours starvation compared to the other cells (Supplementary Fig. 5B).

We next performed clonogenic assay under identical conditions as above. When cells were cultured in full RPMI medium (NT), BCL(X)L and BCL2 cells revealed a higher capacity to grow colonies over 7 days compared to MCF7-pSFFV (Fig. 4D and E). Similarly, in the starvation group [cells placed in SILAC (stable isotope labelling with amino acids in cell culture) medium with the addition of dialysed fetal bovine serum, without glutamine or glucose] for 24 hours and then switched to RPMI medium for 7 days, both BCL2 and BCL(X)L overexpressing cells showed an increased clonogenic potential (Fig. 4D and E). Similar results were observed when the cells were grown in medium with 5 mM glucose (SILAC medium without glutamine and supplemented with 5 mM glucose). We also measured levels of Annexin V/PI negative cells by flow cytometry. A survival of 90 to100% was observed in all conditions and cell lines (Fig. 4F). Therefore BCL2 and BCL(X)L overexpressing cells possessed higher clonogenic potential during metabolic stress, both during nutrient starvation and in low glucose media, and this effect was not accompanied by significant cell death induction (Fig. 4E and F).

BCL2 and BCL(X)L cells possess increased mitochondrial respiration during hypoxia

Next, we investigated the potential role for BCL2 proteins in ATP consumption during hypoxia. First, we employed sodium azide and measured the levels of cytosolic ATP (using the cytosolic version of the ATeam construct, as we again aimed to analyse the ability of BCL2 and BCL(X)L to rescue cell functions by maintaining cytosolic ATP levels) when cells were fuelled with pyruvate (Supplementary Fig. 6). After sodium azide treatment a decrease in ATP production was observed in all cell lines (Fig 5A), as this prevents cytochrome *c* oxidase from using O_2. In parallel, the TMRM fluorescence intensity increased in all cells, since O_2 is no longer reduced to H_2O and protons are not pumped to the IMS (Fig. 5A and C). ATP production was partially restored after glucose addition since glycolysis was activated

and compensated for OXPHOS malfunction (Fig. 5A). FRET analysis showed that both BCL2 and BCL(X)L cells maintained a higher FRET/CFP ratio compared to MCF7-pSFFV (Fig. 5B). Slope analysis for sodium azide highlighted higher values for BCL2 and BCL(X)L overexpressing cells compared to control cell line, suggesting a slower ATP consumption (Fig. 5D).

We performed intracellular oxygen measurement using the MitoImage^{TM} MM2 probe (Supplementary Fig. 6B) (25, 26). Cells treated as described in materials and methods, were placed in KB supplemented with 2 mM pyruvate. After 30 min of baseline recording, oxygen concentration in the atmosphere was reduced to 2% (Fig 5E). Under 2% O2, cells were treated with 10 μM oligomycin and 5 mM glucose to block ATP synthase related O2 consumption. The increase of intracellular O_2 availability recorded was higher in the MCF7-BCL2 and BCL(X)L (Fig 5E and F). 10 μM FCCP was added to observe maximum respiration caused by permeabilisation of the inner $\Delta\Psi_m$ to protons, unrelated to ATP synthase activity. Finally, O_2 was increased back to ambient concentration showing that 21% O_2 was saturating the MM2 probe again. We also employed MitoXpress®-Intra (O_2-sensitive cell-penetrating nanoparticle probe) to measure intracellular oxygen levels (icO_2) during hypoxia (1% O_2 concentration), together with pH (via phenol red absorbance), under the influence of different substrates. Cells were incubated in SILAC medium plus dialysed FBS with either 2 mM of glucose, pyruvate or lactate, without glutamine (see Materials and Methods for details). As shown in Supplementary Fig. 7A we recorded a decrease in icO_2 after switching to hypoxia. icO_2 reached different plateau among the clones, with BCL(X)L highlighting higher values in glucose (Supplementary Fig. 7A). We analysed the slope of oxygen consumption kinetics and found that in glucose, MCF7-BCL(X)L overexpressing MCF7 possessed slower O_2 consumption rates, when compared to MCF7-pSFFV and BCL2. Both BCL2 and BCL(X)L cells showed slower consumption kinetics in 2 mM lactate

(Supplementary Fig. 7C), while in 2 mM pyruvate, only BCL2 cells showed slower oxygen consumption when compared to the other clones (Supplementary Fig. 7C). pH simultaneously decreased with different kinetics from 7.2 to 7.1, in 2 mM lactate or pyruvate (Supplementary Fig. 7B), while in glucose, pH reached 7.0 in all clones after 10 hours hypoxia (Supplementary Fig. 7B). We analysed slopes for the pH decrease and found that in 2 mM glucose, MCF7-BCL2 and BCL(X)L overexpressing cells exhibited decreased slope values compared to MCF7-pSFFV cells, highlighting a faster acidification of the medium (Supplementary Fig. 7D). On the other hand, in lactate and pyruvate, only MCF7-BCL(X)L, showed higher pH slope values compared to MCF7-pSFFV and BCL2, highlighting a slower medium acidification (Supplementary Fig. 7D). We also measured clonogenic potential of MCF7 clones growth in hypoxia and medium containing 2 mM or 5 mM glucose to simulate tumour microenvironment (Supplementary Fig. 7E). We observed that MCF7-BCL(X)L cells grew a higher number of colonies in all conditions, while MCF7-BCL2 showed similar effect in 5mM glucose, when compared to pSFFV cells (Fig. 5H).

BCL2 and BCL(X)L silencing reduces mitochondrial ATP production following pyruvate addition and reduces clonogenic potential of MCF7 cells

To confirm our results, we employed siRNA to selectively silence BCL2 or BCL(X)L in MCF7-pSFFV cells. As shown in Supplementary Fig. 8A and B, BCL2 and BCL(X)L protein levels were reduced by respective siRNAs. Subsequently, we co-transfected the cells with control (ctrl), BCL2 or BCL(X)L siRNA together with the mitoATeam FRET probe, and performed the pyruvate experiment of Figure 2 (Supplementary Fig. 9). As shown in Fig. 6A, we observed an increase in mitochondrial ATP production. However, BCL2 or BCL(X)L silenced cells registered decreased ATP production kinetics when compared to MCF7-pSFFV (Fig. 6A). We analysed the absolute FRET/CFP ratio and observed that BCL(X)L siRNA treated cells possessed lower ATP level, when compared to control siRNA. A trend was seen

when comparing BCL2 to control siRNA (Fig. 6B). Of note we found that BCL2 siRNA treated cells accumulated lower TMRM fluorescence after oligomycin (Fig. 6C) but higher baseline absolute TMRM (Fig. 6D) when compared to control or BCL(X)L siRNA. To confirm these results, we repeated similar experiment in the MDA-231 breast cancer cell line, since these accumulate high levels of BCL2 and BCL(X)L (27). Both BCL2 and BCL(X)L silenced cells recorded lower mitochondrial ATP levels following pyruvate addition, when compared to ctrl siRNA transfected cells (Supplementary Fig. 8F). Moreover, we analysed the clonogenic potential of cells transfected with the different siRNA and found that both BCL2 and BCL(X)L siRNA treated cells highlighted a lower number of colonies after 7 days when compared to control siRNA (Fig. 6E and F). No cell death was recorded under same conditions (Fig. 6G), suggesting a potential block in proliferation upon reduction of BCL2 and BCL(X)L protein levels.

BCL2 and BCL(X)L overexpression improve cell growth and migration in low nutrients conditions

We tested whether overexpression of BCL2 and BCL(X)L could also enhance growth and migration of breast cancer cells. MCF7 clones were placed in full RPMI or SILAC medium plus dialysed FBS with 2 mM glucose (without glutamine), and an acid phosphatase assay used to infer the number of cells. No change in growth was recorded in MCF7 cells placed in full RPMI (Fig. 7A). However, in 2mM glucose, we found that BCL2 and BCL(X)L overexpressing cells showed higher number of cells after 4 to 6 days when compared to MCF7-pSFFV (Fig. 7B). We then performed a wound scratch assay to measure changes in migration after 24, 48 and 72 hours (Supplementary Fig. 10). We found that both BCL2 and BCL(X)L overexpressing MCF7 showed a reduced wound area in RPMI, when compared to control cells (Fig. 7C). Similar results were obtained for cells in 2mM glucose medium at 48 and 72 h timepoints (Fig. 7D), suggesting that BCL2 and BCL(X)L overexpressing cell lines possess higher proliferation rates and improved migratory capacity during metabolic stress.

Gene expression analysis reveals different profiles for respiratory chain complexes

Finally, in order to elucidate potential gene expression changes and mechanisms responsible for the effects of BCL2 and BCL(X)L in modulating mitochondrial bioenergetics, we performed RNA-seq experiments of the three MCF7 cell lines. Moreover, we compared these data with genes expression profiles from publically available breast cancer cell lines and patient samples. For our in-house dataset, we found that pSFFV and BCL2 clones showed similar expression patterns. Higher expression in pSFFV and BCL2 overexpressing cell lines compared to BCL(X)L cells have been found for the majority of respiratory chain complexes. Nonetheless, COX1, COX2 and COX3 (Complex IV) displayed lowest and highest expression in BCL(X)L and BCL2 clones, respectively. Additionally, NDUFA11 (Complex I) expression was lowest in the MCF7-pSFFV cells and increased in the overexpressing clones. UQCRC1 (Complex II) expression was found altered only in the BCL(X)L overexpressing cells (Fig. 8). We investigated the association between the expression of BCL2 and BCL(X)L (BCL2L1) with the expression of the 40 genes found to be statistically significant different in the analysis of the MCF7 clones in publicly available datasets derived from cell lines (CCLE, n=50), patient-derived breast cancer xenografts (n=38 PDXs, Supplementary Figure 11) (28) and tumour resections from the METABRIC (n=1904) collection. We found that COX1, COX2 and COX3 genes expression is negatively correlated with BCL2 (small blue dots), consistent with our in house data, showing a lower expression of these genes in the BCL2 overexpressing clones than in the pSFFV controls. Furthermore, COX1, COX2, COX3 were found to be positively correlated with BCL(X)L gene expression, also consistent with findings in our cell lines.

DISCUSSION

In this work we described the role of BCL2 and BCL(X)L in regulating breast cancer bioenergetics. Absolute concentrations of the overexpressed proteins were found to be similar

to levels seen in breast cancer patients and other cancer patients (16, 20), or breast tumour xenografts (29, 30). We found that overexpression of either of these proteins improved ATP consumption (ability to consume metabolites)/production (efficiency to synthesize ATP) after various metabolic or toxic challenges.

We highlighted that the addition of low pyruvate concentrations increased mitochondrial ATP synthesis, in line with other studies showing that cancer cells employ OXPHOS (31-33). Overexpression of BCL2 or BCL(X)L did not increase mitochondrial ATP production after starvation. Interestingly, after pyruvate addition, NAD(P)H production rates were slower in BCL2 and BCL(X)L cells when compared to MCF7-pSFFV, suggesting that these cells produced similar amounts of mitochondrial ATP by consuming/producing less NAD(P)H. Moreover, BCL2 and BCL(X)L overexpressing cells accumulated higher levels of NAD(P)H, pointing to an improved bioenergetics status of these cells. Interestingly, we found that levels of NDUFA11 (Complex I) were higher in overexpressing cell lines.

FCCP (and sodium azide), will impair mitochondrial function and elucidate if ATP is still synthesized under conditions where cells can only employ pyruvate as source of energy, and glycolysis is not occurring. Oligomycin will specifically and directly impair ATP synthesis and highlight if ATP synthase is more efficiently working, even when $\Delta\Psi_m$ is hyper-polarised. Indeed, these treatments highlighted an increased respiratory capacity of BCL2 and BCL(X)L overexpressing cells. In fact, FCCP and oligomycin appeared to be more 'effective' in MCF7-pSFFV cells, when compared to cells overexpressing BCL2 and BCL(X)L, suggesting that both proteins improve coupled respiration and reduce the proton leak. We also found that BCL(X)L cells accumulated higher $\Delta\Psi_m$ after oligomycin titration. This reduced proton leak may be potentially linked to VDAC activity regulation by BCL2 proteins (34, 35), or the ability of BCL2 proteins to form ion channels in lipid bilayers (36). In our study, we did not observe differences in levels of the ATP synthase subunit β, or in

ATP synthase activity between MCF7 control and BCL2 or BCL(X)L overexpressing cells. A previous study conducted in neurons using patch clamp techniques, showed that BCL(X)L increased ATP synthase activity through direct binding to the β and α subunit (9). This could suggest tissue specific modalities of BCL2 proteins in regulating cell bioenergetics. Nonetheless, in line with our data, it has been previously shown that BCL(X)L regulates mitochondrial energetics through the stabilisation of the inner $\Delta\Psi_m$ in neurons (10). Deletion or inhibition of BCL(X)L led to large fluctuation of membrane potential by increasing futile ion flux across the inner mitochondrial membrane, enhancing the probability of an energetic crisis during stress (10). A different study revealed that recombinant BCL(X)L was able to restore metabolite exchange across the outer membrane, without inducing the loss of cytochrome *c* from the intermembrane space through the inhibition of VDAC closure (8). Together these data suggest that BCL2 proteins improve cellular bioenergetics potentially through the reduction of the proton leak or through ion exchange through the inner and outer mitochondrial membrane (8, 37).

When cells were starved, both BCL2 and BCL(X)L cells maintained higher ATP concentration and slower consumption. This was linked to higher NAD(P)H levels as such levels maintain OXPHOS for longer times (38), and high NAD(P)H autofluorescence is a marker for increased OXPHOS capacity (39). Cardiac-specific BCL2 overexpression in mice led to increased NAD(P)H and pyruvate oxidation (40) and reduced the levels of ATP consumption post-FCCP treatment (41). The slower ATP consumption recorded in overexpressing cells likely improves their ability to grow under unfavourable conditions. Indeed, we observed increased clonogenic potential in BCL2 and BCL(X)L cells starved or grown in reduced glucose. Similar results were observed in normal conditions, suggesting that BCL2 and BCL(X)L sustained slower ATP consumption/production therefore consuming less nutrients. This could also explain why we did not observe similar

317 mitochondrial or cytosolic ATP levels, at baseline, across the different clones. Furthermore,
318 BCL2 and BCL(X)L cells showed increased growth rates and migration in glucose limiting
319 conditions. The increased nutrient availability might sustain surrounding cell growth
320 increasing tumorigenic potential of BCL2 and BCL(X)L overexpressing tumours. Moreover,
321 stabilization of the $\Delta\Psi_m$ by BCL2 and BCL(X)L might be important for clonogenic growth of
322 cancer cells, in addition to the observed effects on bioenergetics. Indeed, $\Delta\Psi_m$ has a role in
323 the regulation of other processes inside the cells (42-44) and cancer cells usually accumulate
324 higher $\Delta\Psi_m$ (45-47).
325 Our results also showed that both BCL2 and BCL(X)L cells maintain higher ATP levels
326 during chemical hypoxia. Another study linked BCL2 overexpression in human leukaemia
327 cells to increased oxygen consumption and higher mitochondrial respiration rates (48).
328 Indeed, our intracellular oxygen imaging approach showed that when ATP synthesis was
329 blocked, BCL2 and BCL(X)L overexpressing cells showed decreased O_2 consumption,
330 indicating a close link of O_2 consumption to the activity of the ATP synthase. These effects
331 could depend on altered levels of cytochrome c oxidase found in our gene expression
332 analysis. When oxygen concentration was decreased to 1%, under different substrate
333 availability, BCL2 and BCL(X)L cells registered slower oxygen depletion, indicating a
334 potential improved adaptation to hypoxia. Indeed, when cells were cultured in glucose
335 limiting conditions both BCL2 and BCL(X)L cells showed a higher clonogenic potential.
336 In conclusion, we found that BCL2 or BCL(X)L overexpression can increase the resistance of
337 tumour cells to metabolic stress independent of apoptosis inhibition, by more efficiently
338 coupling the mitochondrial proton motive force with ATP production. The effects reported in
339 this manuscript may be important contributors to the oncogenic activity of BCL2 family
340 proteins and the survival of cancer cells in a metabolically stressed tumour
341 microenvironment.

MATERIALS AND METHODS

Materials and reagent

Fetal bovine serum, tetramethylrhodamine methyl ester (TMRM) and Lipofectamine® 2000 were from Invitrogen (Bio Sciences). RPMI 1640 medium, Oligomycin, Carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), staurosporine, crystal violet, phenol red, sodium pyruvate, D-glucose, 2-deoxy-D-glucose, L-glutamine, L-arginine, L-lysine and 4-Nitrophenyl phosphate disodium salt hexahydrate came from Sigma-Aldrich. Cisplatin (CDDP) was purchased from Selleckchem. Glass bottom dish (35x10) 12mm aperture used for time lapse imaging were from WillCo Wells BV. SILAC™ RPMI 1640 Flex Media and dialysed fetal bovine serum were purchased from Thermo Scientific.

Cell lines

MCF7-pSFFV, MCF7-BCL2 and MCF7-BCL(X)L were cultured in RPMI 1640 supplemented with 100 U/mL of penicillin/streptomycin, 10% fetal bovine serum and incubated at 37˚C in humidified atmosphere with 5% of CO_2. Where specified, MCF7 clones were placed under SILAC with dialysed FBS, 0.2 g/L arginine, 0.04 g/L lysine and 0.0053 g/L phenol red, without glutamine and with/without the presence of glucose. Cell lines were free of mycoplasma.

Plasmids and transfection

The ATeam1.03-nD/nA/pcDNA3 and MitoATeam1.03 probes (22) were kindly provided by Prof. Hiroyuki Noji (Osaka University) and Prof. Hiromi Imamura (Kyoto University). Cells were seeded at a density of $2*10^3$ onto prewashed, poly-D-lysine (5µg/ml)-coated glass Will-Co dishes (WillCo Wells BV) and incubated overnight at 37 °C with 5% CO_2. Twenty-four hours following seeding, a transfection mix was prepared which consisted of 70 µL of Opti-MEM, 0.7 µL of Lipofectamine 2000 and 0.2 µg/µL of DNA. After 20 minutes incubation at

RT, the medium from each dish was removed, and the transfection mix pipetted onto the cells. Plates were then incubated for 4 hours at 37 °C. Finally, the media containing this transfection mix was removed and 2 mL of fresh medium added to each dish.

Generation of cytosolic ATeam stable clones

MCF7-pSFFV, MCF7-BCL2 and MCF7-BCL(X)L were seeded in 6 well plates at a density of $1*10^6$ cells per well. Cells were transfected with 200 ng of ATeam plasmid using Lipofectamine for 4 hours in Opti-MEM medium. After 24 hours transfection was assessed on a fluorescent microscope and cells cultivated to reach confluency. Subsequently, cells were tripsinized, diluted 1 in 100 and seeded in new 6 well plates. After 7 days cells were observed for the presence of fluorescent colonies. Positive cells carrying ATeam plasmid were then picked up with a p200 pipette, placed in 2 ml medium, gently separated and moved to a new 6 well plate. The same procedure was then repeated 2-3 times to insure 90-100% of positive-fluorescent colonies. Cells were then left to reach confluency and moved to a 25 flask and regularly growth.

Western blotting and BCL2 profiling

Cells were scraped, collected and lysed in RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, protease and phosphatase inhibitors mix 1:100). Protein concentration was determined with micro BCA (bicinchoninic acid) assay (Pierce, Rockford, IL) and a total of 30 µg of protein loaded into a SDS-gel after complete denaturation at 90° C for 10 minutes in Laemmli buffer. The samples were then transferred to nitrocellulose membrane and blocked in TBS-T/5 % milk for 1 h. Primary antibodies to MCL1 (1:250; BD Biosciences), BCL2 (1:100; Santa Cruz Biotechnology), BCL(X)L (1:250; Santa Cruz Biotechnology, Inc.), ATP synthase sub. β (1:1000; Invitrogen) and tubulin (1:5,000; Sigma) were mouse monoclonal. Antibodies to BAK (1:250; Santa Cruz Biotechnology, Inc.) and BAX (1:1,000; Upstate Biotechnology) were rabbit

polyclonal. The horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch (1:5000). Detection of protein bands was carried out using chemiluminescence (EMD Millipore) on a LAS-3000 Imager (FUJIFILM UK Ltd). BCL2 profiling and absolute protein concentration was carried on as previously described (20) and all densitometry analysis was performed with ImageJ. Briefly, standard calibration curves were constructed with varying concentrations (0.1–10.0 ng) of recombinant BCL2 proteins and HeLa extract, by plotting blot intensity against mass of loading. Cellular concentrations for BCL2 proteins in cellular lysates were calculated from calibration curves, considering HeLa cell volume of 3.1 pL (20) and the appropriate molecular weights for BAK, BAX, BCL2, BCL(X)L, and MCL1.

Immunofluorescence of BCL2 and BCL(X)L

MCF7 cell lines were cultured on 13-mm cover slips for 24 hours. On the next day cells were stained for 30 min at 37° C with MitoTracker Red CMXRos (Thermo Fisher Scientific) to stain mitochondria, subsequently fixed with 4% paraformaldehyde (PFA, Affymetrix) for 10 min and permeabilized with 95% ethanol, 5% glacial acetic acid. For detection of BCL2, a mouse monoclonal anti-BCL2 (clone 8E12, Thermo Fisher Scientific) and an Alexa Fluor® 488-conjugated secondary anti-mouse (Thermo Fisher Scientific) were used. For immunostaining of BCL(X)L a rabbit monoclonal anti-BCL(X)L (clone 54H6, Cell Signaling) and an Alexa Fluor® 488-conjugated secondary anti-rabbit (Thermo Fisher Scientific) were used. Coverslip were mounted on a microscope slide with ProLong® Gold Antifade Mountant (Thermo Fisher Scientific) with DAPI to visualize nuclei. Imaging was performed with a LSM 710 confocal microscope (Carl Zeiss, Germany) using 405, 488, and 561 nm for DAPI, Alexa Fluor 488 and MitoTracker Red laser excitation, respectively, and using the proper emission bands of the detection unit. Each field of view was acquired with a stack covering the whole cell body with an optical slice thickness of 1 μm (FWHM) and steps

of 0.20 mm with a 100 × 1.4 NA oil immersion plan apochromat objective. Subsequently, the stacks were de-convolved using Autoquant X (version 2.1.0, Media Cybernetics, UK) and image further processed with ImageJ 1.45s (National Institutes of Health, Bethesda, MD, USA).

Flow Cytometry

Cells were treated with 40 μM Cisplatin and 2 μM STS for 24 hours at 37° C. After incubation time cells were collected by tripsinization and stained with Annexin V-FITC and propidium iodide (Biovision) for 20 minutes at room temperature in dark condition and analyzed using a CyFlow ML (Partec) flow cytometer and FloMax software. A minimum of 10,000 events were recorded for each sample. Surviving cells were defined as the fraction of Annexin V and PI negative cells. The percentage of apoptotic cells was defined as Annexin V positive/ PI negative plus Annexin V positive/ PI positive.

Clonogenic Assay

MCF7 cell lines were seeded at a density of 1000 cells per well in 6 well plate. Starvation conditions were mimicked by using SILAC medium (Thermo Scientific) with dialysed FBS (Thermo Scientific), 0.2 g/L arginine, 0.04 g/L lysine and 0.0053 g/L phenol red (Sigma-Aldrich). No glucose or glutamine was added to the medium. After 24 hours treatment cells medium was replaced with fresh RPMI 1640. 5 mM glucose medium were achieved by using above described SILAC medium with the addition of 5 mM D-glucose. After a week incubation cells were washed and fixed with 4% PFA (Affymetrix) and stained with 0.5% crystal violet. Plates were scanned on a CanoScan LiDE 80 (Canon) at a resolution of 1200 ppi. Images were then cropped with ImageJ and analyzed with OpenCFU software (49).

Time lapse imaging

Cells were seeded at a concentration of 2×10^3 in sterile Willco dishes and let to adhere over-night. Then, ATeam constructs were transfected according to the protocol described above.

442 On the day of the experiment, adherent cells were washed twice with krebs-hepes buffer (KB,
443 140 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl2, 15 mM HEPES) and the medium replaced with
444 1 mL of KB containing 30 nM TMRM, 2 mM sodium pyruvate and 2.5 mM $CaCl_2$; an
445 equilibration time of 40 minutes was applied before starting the imaging procedure. For
446 experiments in Fig. 2, KB with $CaCl_2$ without pyruvate was first used for 3 hours, followed
447 by replacement to KB with TMRM, $CaCl_2$ and 2mM pyruvate. Mineral oil was added on top
448 of the KB to prevent evaporation and the dishes transferred to a heated stage (37°C) in a 5%
449 CO_2 environment above a 63x/1.4 NA Plan-Apochromat oil immersion objective lens on an
450 inverted confocal laser-scanning microscopes (LSM 710, Carl Zeiss). Mitochondrial ATP
451 kinetics measurements were carried out using lasers of 405, 488 and 561 nm for excitation of
452 FRET/CFP, YFP and TMRM respectively with a pixel dwell time of 2.55 µs and images
453 taken every minute. Detection ranges were set to 445-513 nm and 513-562 nm for CFP and
454 FRET/YFP, while 562-710 nm was used for TMRM with pinholes set to 2 µm optical
455 sectioning (FWHM). For NAD(P)H kinetics measurement MCF7 clones were prepared as
456 before and the medium replaced with 1 mL of KB with 2 mM pyruvate and 2.5 mM CaCl2.
457 Mineral oil was added on top of KB to prevent evaporation and the dishes transferred to a
458 heated stage (37°C) in a 5% CO_2 environment above a 40x/1.3 Numerical Aperture (NA)
459 Plan-Neofluar of an inverted epifluorescence microscope (Axiovert 200M, Carl Zeiss).
460 NAD(P)H was measured through autofluorescence as the reduced form of these molecules is
461 different from the oxidized form as they absorb and emit light at 340 nm and 445 nm,
462 respectively. These experiments were carried out using 25% of a HBO 100 mercury short-arc
463 lamp for excitation with illumination wavelength of 340/20 nm for NAD(P)H excitation with
464 an exposure time of 100 ms, and a binning of 4x4. Emission was collected at 447/60 nm and
465 images taken every minute. All kinetics were measured for twenty minutes without treatment
466 in order to obtain a baseline signal. Images were processed using ImageJ2 (National Institutes

of Health, Bethesda, MD, USA) and Metamorph 7.5 (Universal Imaging Co., Westchester, PA, USA). Time-lapse sequences were imported into ImageJ and background was first subtracted from each image. After creating combined images of the three fields of views for each channel sequence, a median filter with a radius of one pixel was applied. The combined images were then processed using Metamorph. Mitochondria within cells were segmented from background using the YFP time lapse images. The segmented mitochondrial areas were converted into a mask used to remove background values from any further analysis of the FRET/CFP stack. To this end the FRET image stack was first multiplied by the YFP-mask and divided by CFP image stack, and regions of interest were then selected for analysis.

For cytosolic ATP measurement, stable expressing ATeam MCF7 clones were seeded in sterile Willco dishes. For the FCCP and sodium azide treatment, on the day of the experiment, KB with 30 nM TMRM, 2 mM sodium pyruvate and 2.5 mM $CaCl_2$ was added on top of the cells, with mineral oil to prevent evaporation; an equilibration time of 40 minutes was applied before starting the imaging procedure. For the starvation experiment, KB with 30 nM TMRM and 2.5 mM $CaCl_2$ was used instead. The dishes were transferred to a heated stage (37°C) in a 5% CO_2 environment above a 40×/1.3 Numerical Aperture (NA) Plan-Neofluar oil immersion objective lenses on an inverted epifluorescence microscope (Axiovert 200M, Carl Zeiss), used with selected polychroic mirror and filter wheel settings. Experiments were carried out using 0.09% of a HBO 100 mercury short-arc lamp for excitation with a band pass of 438/24 nm (center wavelength and band width) for FRET/CFP (cyan fluorescent protein) and a band pass filter with 500/24 nm YFP (yellow fluorescent protein) with exposure time of 20 ms. CFP emission was measured in the range of 483/32 nm and FRET/YFP emission in the range of 542/27 nm. TMRM was exposed to 531/40 nm for of 10 ms and detected in the emission range of 593/40 nm (all filters from Semrock). For all experiments, a custom made Metamorph journal was used to obtain the

492 average intensity signal from all regions, and an excel macro was then applied to sort the
493 values and to converted them to percentage normalised to the baseline (each cell line was
494 normalized to its own baseline). All experiments were performed at least three times
495 independently of each other. For mito ATeam and cytosolic ATeam experiments, slope
496 values were assessed from normalised traces: ΔFRET was calculated by subtracting the
497 minimal fluorescence value obtained following treatment (offset) to the fluorescence value at
498 the onset. Δtime was calculated accordingly by subtracting the time when the probe reached
499 the minimal value to the time at the onset. The slope over time was finally obtained by
500 dividing ΔFRET to Δtime. For NADH experiment, a non linear fit function
501 (Y=Autofluorescence max*X/(Km+X)) in GraphPhad Prism was employed to obtain Km
502 values.

503 **Mitochondrial isolation and ATP synthase activity assay**

504 ATP synthase activity measurement was carried on with a commercially available kit from
505 Abcam, following mitochondrial isolation. Mitochondria were isolated accordingly to Frezza
506 *et al* (50). Briefly, MCF7 clones were seeded in t175 flasks and allowed to reach 80-90%
507 confluency. Then, cells were detached, collected with fresh medium in 50 mL falcon tubes
508 and centrifuged at 600 x *g* for 10 min at 4 ˚C. The supernatant was discarded and 3 mL of
509 ice-cold isolation buffer (0.1 M Tris–MOPS, 0.1 M EGTA/Tris, 1 M sucrose and pH at 7.4)
510 were added. Cells were homogenised using a tissue homogenizer at 708 x *g* per 6 times, and
511 centrifuged again at 600 x *g* for 10 min at 4 ˚C. Then, the supernatant was collected in a 15
512 mL falcon tube and centrifuged at 7,000g for 10 min at 4 °C. The supernatant was discarded
513 and the pellet washed with 200 µL of ice cold isolation buffer, resuspended in 200 µL and
514 transferred to a 1.5 mL Eppendorf tube. The homogenate was then centrifuged at 7,000g for
515 10 min at 4 °C, supernatant discarded and pellet resuspended in 50 µL of ice-cold isolation
516 buffer. The homogenate sample underwent 2 cycles of freeze-thaw, and then centrifuged at

16,100 x *g* for 10 min at 4 ˚C. The sample was then diluted with 100 µL of solution 1 from the kit, and the protein concentration assessed with BCA assay. Protein concentration was adjusted to 5.5 mg/ml and 1/10 volume of detergent solution added. After 30 min incubation on ice the samples were centrifuged at 16,100 x *g* for 20 min at 4 ˚C and the pellet discarded. The microplate from the kit was then loaded with 50 µL of solution 1, and 2.5 µg of each sample. Empty wells with the solution 1 alone were added as background control. The microplate was then stored at 4 ºC overnight to let the ATP synthase enzymes bound the antibody-coated surface of each well. The next day, wells were emptied and washed with 300 µL of solution 1. Subsequently, 40 µL of lipid mix were added to each well and the microplate incubated at RT for 45 min. After incubation time, 200 µL of reagent mix were pipetted into each well and the plate inserted into the Clariostar reader at 30 °C. Absorbance was recorded at 340 nm, with a kinetic program every minute for 2 hours. An excel template was used to calculate the activity (after background subtraction and according to manufacturer protocol), and normalized to ATP synthase sub. β levels from Western Blotting (WB) experiments.

Intracellular O_2 and pH measurement using MitoXpress®-Intra

Intracellular oxygen assay was performed using commercially available MitoXpress®-Intra (NanO2) from Luxcel Bioscences (Cork, Ireland). This fluorophore is an O_2-sensitive cell-penetrating nanoparticle probe, whose phosphorence is quenched by oxygen such that measured signal is proportional to intracellular oxygen concentration. MCF7 clones were seeded at a concentration of $1.5*10^3$ in a Nunc Micro Well 96 well optical bottom plate. After overnight incubation, the medium was removed and cells were stained with 100 µL of fresh medium containing 5 µg/mL of MitoXpress®-Intra and 1 µg/mL Hoechst 33588, for 24 hours. Before the measurements, the staining medium was replaced with SILAC medium with dialysed FBS, 0.2 g/L arginine, 0.04 g/L lysine, 0.0053 g/L phenol red and 2 mM

glucose, pyruvate or lactate as indicated (without glutamine). The plate was placed in a plate reader at 37 ºC, 5% CO_2, and 21% O_2 (ClarioStar, BMG Labtech, Germany). After 3 hours in normoxia (21% O_2), oxygen was decreased to 1% through the plate reader atmospheric regulator and time-resolved fluorescence measured every 5 minutes for 24 hours. A custom-made scripting function for the assay was employed. Fluorescent intensities were measured at delay times of 30 μs and 70 μs, with 30 μs window time, through the bottom optic. The probe was excited at 340-400 nm and excitation was collected at 635-655 nm, using a bandpass filter. A gain of 3000 was used for all experiments. To account for changes in proliferation rates, nuclei were stained with Hoechst and the nanoprobe fluorescence values were normalized to absolute Hoechst fluorescence. We only considered data from the start of the experiment until 600 minutes, as no significant change was observed after 10 hours, and to exclude excessive cell death due to limited nutrient and oxygen availability. Hoechst was excited 355/20 nm and emission collected at 455/30 nm with a gain at 1500. Values were analysed with a custom excel template to obtain intracellular oxygen concentration (IcO_2) based on the calibration in Fercher *et al* (51) after subtraction of blank (medium without cells). Then, both IcO_2 and pH signals were divided by the Hoechst signal in order to accounts for proliferation. pH was recorded in parallel through the measurement of phenol red absorbance spectra. The wavelength range was 350-650 nm with a step width of 5 nm and a bidirectional mode was employed for the reading. An excel template was utilised to calculate the 560/440 nm ratio and the formula $\log\left[\frac{\frac{560nm}{460nm}}{0.0002}\right]/1.18$ was employed to obtain pH values. icO_2 slopes were measured using a one phase exponential decay function in GraphPad Prism, while pH slopes, obtained from measurement of phenol red absorbance spectra, were measured using a linear regression Line function in the same software.

Monitoring changes in O_2 concentration using the ratiometric MM2 probe

MCF7-pSFFV, MCF7-BCL2 or MCF7-BCL(X)L cells were grown in 4 chamber glass bottom dishes (CellView, Greiner, Germany). The nanoparticle-based phosphorescent probe, MitoImage^{TM}-MM2, which consists of the O_2-sensitive phosphorescent reporter dye (PtTFPP), O2- insensitive component (PFO) embedded in a cationic polymer, was purchased from (Luxcel now Agilent, Ireland) (26, 52). Cells were loaded with 10 μg/ml of MM2 in RPMI medium supplemented with 1% FBS medium for 16 h at 37°C. MM2 probe intensity ratio was recorded on an LSM 7live Duoscan confocal microscope (Carl Zeiss, Germany) equipped with a 40×, 1.3 NA Plan-Neofluar oil-immersion objective and a thermostatically regulated chamber at 37°C in a humidified atmosphere of 5% CO_2 and the O_2 concentration as given in the figures (Pecon, Germany). The MM2 probe was excited using 5% of the 30 mW 405 nm DPSS Laser, and the emission was collected through a 415–480 nm band pass and a 570 nm long pass filter using a 565 nm secondary dichroic to split the emission between the 2 detectors. Cells were placed in KB with the addition of 2 mM pyruvate. After baseline measurement, oxygen concentration was reduced to 2%; after 90 minutes 10 μM Oligomycin and 5 mM glucose were added to stop ATP synthase related O_2 consumption. Signal was measured for 60 min and 10 μM FCCP added for maximum respiration for 1h; for the last hour of the experiment O_2 was increased to ambient concentration. All images were processed using ImageJ (version 1.52r, Wayne Rasband, NIH, USA). The intensity ratio images between the PFO and the PtTFPP (FPFO/FPtTFPP) were calculated for all areas of the image with PFO and PtTFPP fluorescence above background noise and after background subtraction multiplied by 1000 to display on a 16bit scale (25). Measurements where then normalized to the baseline and processed in excel (Version 2016, Microscoft, USA) and Prism (version 5, GraphPad, USA).

Acid phosphatase assay

590 Acid phosphatase assay was used to measure cell number based on the conversion of pNPP to
591 p-nitrophenol by cytosolic acid phosphatase (53). MCF7 clones were grown in a 24 well plate
592 at a density of 3×10^4 cells per well in either RPMI 1640 or SILAC medium (with dialysed
593 FBS, 0.2 g/L arginine, 0.04 g/L lysine and 0.0053 g/L phenol red) with 2 mM glucose and no
594 glutamine. After each time point (1, 2, 4, 6 days), medium was removed and each well was
595 washed once with 200 µL of 1X PBS. To each well, 100 µL of assay buffer (0.1 M sodium
596 acetate at pH 5.0, 0.1% Triton X-100, and 7.25 mM p-nitrophenyl phosphate) was added. The
597 plates were then incubated at 37 °C for 2 h. The reaction was finally stopped with the addition
598 of 50 µL and color development was assayed at 405 nm using a Multiskan® EX plate reader.
599 The non-enzymatic hydrolysis of the pNPP substrate was also determined by including wells
600 with the assay buffer and without any cells. A standard curve, performed with the same assay
601 and fixed number of cells (1000, 5000, 10000, 50000, 100000, 500000) was used to obtain
602 the cell number from the different wells, using excel.

603 **BCL2 silencing**

604 For BCL2 proteins silencing experiments 100 ng of siRNA targeting BCL2 (Santa Cruz
605 Biotechnology, sc-61899) or BCL(X)L (Santa Cruz Biotechnology, sc-43630) was
606 transfected employing Lipofectamine. MCF7-pSFFV and MDA-231 cells were seeded at a
607 density of $2*10^5$ in six-well plates to assess silencing efficiency by WB. After 24 hours
608 incubation at 37 °C, cells were transfected as previously discussed. A siRNA consisting of a
609 scrambled sequence was used a negative control (ctrl, Santa Cruz Biotechnology, sc-7007).
610 One day following transfection, protein lysates were prepared from each well and stored at -
611 80°C for further analysis. To assess the effect of BCL2 or BCL(X)L silencing on ATP
612 production kinetics, cells were seeded on a glass wilco at a density of 2×10^3 cells per dish,
613 and allowed to adhere for 24 hours. Cells were then co-transfected with 100 ng of MitoAteam
614 plasmid and 100ng of BCL2, BCL(X)L or control siRNA, as previously described. After 24

hours (48h for MDA-231), cells were prepared for time-lapse live cell imaging. To assess clonogenic ability and viability after BCL2 or BCL(X)L silencing, MCF7-pSFFV cells were seeded in a 6-well plate at a density of thousand cells per well and at a density of $6*10^4$ in 24-well plate for Annexin V/PI staining. Cells were incubated for 24 hours and transfected with 100 ng of BCL2, BCL(X)L or control siRNA; in both cases wells treated with Lipofectamine 2000 and no DNA were added to exclude any effect that transfection reagents might have on cell viability. Following 24 hours incubation, 2 mL of fresh medium was added to each 6-well plates and cells let to grow colonies for 7 days, while 24 well plates were to detect apoptosis rates with flow cytometry.

Wound healing assay

Cells were seeded at a concentration of 3×10^4 in sterile 24 well plates and let to adhere over-night. A 2 well insert from Ibidi was placed into each well, before seeding the cells. The following day the insert was carefully removed in order to have a clear separation to follow. All clones were imaged at selected timepoints (0, 24, 48 and 72 hours) on a Nikon TE2000 microscope. Images were imported in ImageJ2; a Find Edges function followed by Sharpen was used to increase the contrast of the cell area compared to the wound. The LUT (lookup table) was inverted and the analyze particle function was used (size between 100000 and 50000000, Circularity: 0.00-1.00) with Outlines showed to obtain the area of the wound. Data were then processed with excel.

RNA-seq experiments and gene expression analysis of publically available datasets

MCF7 clones were seeded in t25 flasks and let adhere overnight. On the following day, cells were trypsinised, collected in eppendorf tubes and centrifuged. RNA was extracted with TRIzol reagent (ThermoFisher Scientific) and samples mechanically disrupted using a homogeniser. Subsequently, chloroform was added to separate an upper acqueous phase (containing RNA) from the rest. This was mixed with isoprophanol and centrifuged at 12,000

x *g* at 4°C; the resulting pellet was put in 75% ethanol and centrifuged again at 7,500 x *g* at 4°C. Pellet was let to air dry and resuspended in RNase free water for further quantification with Nanodrop 2000. Next, whole transcript RNA-seq was performed on the samples. Specifically, poly-A containing mRNA was purified from the RNA samples and RNA sequencing libraries were created using the KAPA stranded mRNAseq Kit (Illumina), according to the manufacturer's instructions. Briefly, sequencing libraries were prepared by converting the RNA to cDNA followed by adapter ligation and enrichment of exon coding sequences by PCR using sequence-specific probes. The resulted cDNA libraries were sequenced on a HiSeq 4000 using a flow-cell generating 1 X 50 bp reads. Transcriptome profiles were created using an existing in-house bioinformatic pipeline. More specifically, after removing the optical duplicates with Clumpify and removing sequencing adapters, the reads were mapped to the human reference genome using TopHat-Bowtie and HTSeq-count was used to create gene-count matrices. Raw counts from RNA-seq experiments were normalized using DESeq2 (R library, version 1.26.0) (54) using 'rlog' as method and '~ cell_line' as design formula. ENSEMBL ids were mapped to symbols with mygene (python package, version 3.1.0). Genes involved in the respiratory chain complexes I to V were retrieved from the "gene names" database curated by the HGNC - HUGO Gene Nomenclature Committee (https://www.genenames.org/data/genegroup/#!/group/639). Of the 97 genes of interest, 96 were present in the dataset.

Table 1. Breakdown of genes included for each respiratory chain complex.

Complex	# genes
Complex I	44
Complex II	4
Complex III	10
Complex IV	19

Complex V	20

Transcriptomic data from the Cancer Cell Line Encyclopedia (CCLE) were downloaded on 2019-06-29 from the CCLE website (55). In downstream analyses, we focused on breast cancer cell lines (set to "BRCA", n=50) and on genes (n=40) from the respiratory chain complexes found to be altered in the MCF7 clones. Transcriptomic data (RNASeq, FPKM) from patient-derived xenografts (PDXs) were downloaded from the supplementary materials of Gao *et al*., (28). We restricted the analysis to untreated PDXs from breast cancer patients (n=38) and on genes from the respiratory chain complexes found to be altered in the MCF7 clones (n=37 out of 40 were found with COX1, COX2 and COX3 missing). Transcriptomic data (mRNA z-Scores, Illumina Human v3 microarray) for n=1904 primary tumour samples from breast cancer patients from the "Molecular Taxonomy of Breast Cancer International Consortium" (METABRIC) (56) study were downloaded on 2020-08-07 from the cPiortal website (https://www.cbioportal.org/study/summary?id=brca_metabric). Downstream analyses included genes from the respiratory chain complexes found to be altered in the MCF7 clones (n=36 out of 40 were found with NDUFS1, COX1, COX2 and COX3 missing). We investigated the dependency of the respiratory chain complexes by BCL2 family members by comparing the expression of the mitochondrial genes listed above in parental (pSFFV) and BCL2 or BCL(X)L overexpressing MCF7 clones. We fitted univariate regression models and we evaluated statistical significance with overall ANOVA p-values. We did not adjust p-values for multiple comparisons as these analyses were considered exploratory. We reported pairwise cell line comparisons by TukeyHSD Post-Hoc tests and mean expression difference, 95% confidence intervals (CI, lower and upper) and p-values (Supplementary Table 1). Next, we examined the association between BCL2 genes (BCL2 or BCL2L1) and those mitochondrial genes found to be differential expressed in the MCF7

clones to examine whether these findings are generalizable to other cell lines, PDX models and patient tumour samples. We reported Spearman correlation as m effect size metric and corresponding p-values in Supplementary Table 2.

Data and Code availability

The raw and processed RNA sequencing data for the MCF7 cell lines generated in this study are publicly available in GEO (reference number GSE158808). Data, processing and analysis code for the transcriptomic-based analysis is publicly available and archived at Zenodo (https://doi.org/10.5281/zenodo.4058036).

Statistical analysis

Data are given as means ± S.D. (standard deviation). The number of independent experiments performed is indicated in the figure legends (from 3 to 5). The variance was assumed to be similar between the compared groups with a normal distribution. For statistical comparison, two-way analysis of variance (ANOVA) or one-way analysis followed by Tukey's post hoc test were employed. p-values < 0.05 were considered to be statistically significant

Supplementary information is available at Cell Death and Differentiation's website.

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850 **Figure Legend**

851 **Figure 1. Characterisation of MCF7 clones.**

852 **(A)** Representative WB for MCF7 cell lines. Cells were lysed and analysed by
853 immunoblotting to detect levels of two pro-apoptotic protein, BAX and BAK, and three anti-
854 apoptotic protein, BCL2, BCL(X)L and MCL1. Actin was used as loading control to
855 normalize protein concentration values post densitometry and HeLa cell line lysates were
856 used for absolute quantification. **(B)** Expression levels for BCL2 proteins as determined by
857 comparing densitometric signals and considering absolute quantification levels in HeLa cells.
858 Data represent mean ± SD from n=3 independent experiments and quantifications were

analysed by two-way ANOVA with Tukey post-test (* indicates a p-value < 0.5, ** indicates a p-value < 0.01, *** indicates a p-value < 0.001). **(C)** Immunofluorescence staining showing localization of BCL2 and BCL(X)L, respectively. MCF7-BCL2 and BCL(X)L overexpressing cells were fixed with paraformaldehyde (4% PFA) and permeabilised with a solution of 95% ethanol and 5% glacial acetic acid. DAPI was used to visualize nuclei while MitoTracker Red CMXRos was used to stain mitochondria. Images are representative of immunofluorescence performed on n=3 independent cultures. **(D)** Percentages of surviving cells (Annexin V-/PI- fraction) following 24 hours control, STS (2 μM) or Cisplatin (40 μM) treatment. **(E)** Percentages of apoptotic cells (sum of Annexin V+/PI- and Annexin V+/PI+ fractions) post treatments. Significance was assessed with a two-way ANOVA and Tukey post-test (* indicates a p-value < 0.05, ** indicates a p-value < 0.01, *** indicates a p-value < 0.001). Column represents mean ± SD for n = 3 experiments.

Figure 2. MCF7-BCL2 and BCL(X)L clones produces similar amounts of mitochondrial ATP by producing/consuming less NAD(P)H, when compared to MCF7-pSFFV cells.

(A) Representative traces of mitochondrial ATP signal and mitochondrial membrane potential. FRET/CFP ratio kinetics and TMRM fluorescence were monitored simultaneously in all MCF7 clones. Cells were starved for three hours; after that, the baseline was recorded for 20 minutes and 2 mM sodium pyruvate was added into the medium. Following 20 minutes treatment, 10 μM of oligomycin was supplemented for the last 20 minutes. All data represent mean ± SD from n=4-5 independent experiments and both signals are normalized to the baseline levels (each cell line normalised to its own baseline). **(B)** The absolute FRET/CFP ratio were analysed by taking into account the maximal value reached by the probe after pyruvate addition and minimal value after oligomycin treatment. Box and whiskers represents single cell values from all experiments. **(C)** TMRM intensity values, normalised to the baseline levels from TMRM kinetics, were analysed by taking into account

884 the maximal value reached following each treatment. **(D)** TMRM fluorescence absolute
885 values obtained from baseline. Box and whiskers represents single cell values from all
886 experiments. All values were analysed by two-way ANOVA with Tukey post-test (*
887 indicates a p-value < 0.05, ** indicates a pvalue < 0.01 *** indicates a p-value < 0.001). **(E)**
888 Representative traces of mitochondrial NAD(P)H signal. NAD(P)H autofluorescence was
889 monitored in all MCF7 clones, by following similar experimental protocol as above. All data
890 represent mean ± SD from n=3 independent experiments and signals are normalized to the
891 baseline levels (each cell line normalised to its own baseline). **(F)** The absolute NAD(P)H
892 fluorescence was analysed by taking into account the maximal autofluorescence value
893 reached after treatments. Box and whiskers represents single cell values from all experiments.
894 **(G)** NAD(P)H curve fitting for pyruvate addition was calculated using a fit function in
895 GraphPad Prism. **(H)** NAD(P)H/ATP ratio, calculated by dividing NAD(P)H absolute
896 fluorescence to absolute mitoAteam FRET/CFP ratio. All values were analysed by two-way
897 ANOVA with Tukey post-test (* indicates a pvalue < 0.05, ** indicates a p-value < 0.01 ***
898 indicates a p-value < 0.001).

899 **Figure 3. BCL2 and BCL(X)L cells maintain higher mitochondrial ATP after**
900 **oligomycin titration.**

901 **(A)** Representative traces of mitochondrial ATP signal and mitochondrial membrane
902 potential. FRET/CFP ratio kinetics and TMRM fluorescence were monitored simultaneously
903 in all MCF7 clones. Cells were placed in KB with 2 mM pyruvate; baseline was recorded for
904 20 minutes and oligomycin titration started by adding 1 nM, 10 nM and 100 nM of the drug
905 with an interval of 20 minutes for each concentration. All data represent mean ± SD from n=3
906 independent experiments and both signals are normalized to the baseline levels (each cell line
907 normalised to its own baseline). **(B)** The absolute FRET/CFP ratio were analysed by taking
908 into account the minimal value reached by the probe after each addition. **(C)** Slope values of

oligomycin titration were assessed by dividing the ΔFRET/CFP ratio to the Δtime (time offset – time onset). Box and whiskers represents single cell values from all experiments. **(D)** TMRM intensity values normalised to the baseline levels from TMRM kinetics were analysed by taking into account the maximal value reached after each treatment. All values were analysed by two-way ANOVA with Tukey post-test (* indicates a pvalue < 0.05, ** indicates a p-value < 0.01 *** indicates a p-value < 0.001). **(E)** TMRM fluorescence absolute values obtained from baseline. **(F)** Protein levels for ATP synthase subunit β in MCF7 clones were obtained from cell lysates and normalized to actin. **(G)** A commercially available kit from Abcam was used after isolation of mitochondrial fraction from MCF7 cell lines, to calculate the activity of ATP synthase. In order to account for protein concentration, the activity was normalized to ATP synthase subunit β. All data represent mean ± SD from 3 independent experiments. All values were analysed by one-way ANOVA with Tukey post-test.

Figure 4. BCL2 and BCL(X)L cells maintain higher cytosolic ATP during starvation.

(A) Representative traces of cytosolic ATP signal and mitochondrial membrane potential. FRET/CFP ratio from ATeam probe and TMRM fluorescence were monitored simultaneously in all MCF7-ATeam clones. Cells were placed in KB with no nutrients and signal recorded every 5 minutes. After 23 hours, 5 mM glucose was supplemented for the remaining hour. All data represent mean ± SD from n=3 independent experiments and both signals are normalized to the baseline levels (each cell line normalised to its own baseline). **(B)** The absolute FRET/CFP ratio were analysed by taking into account the minimal value reached by the probe at each time-point selected. **(C)** Slope values of the decrease in cytosolic ATP, during starvation, were assessed by dividing the ΔFRET/CFP ratio to the Δtime (time offset – time onset), at the indicated time-points. All values were analysed by two-way ANOVA with Tukey post-test (* indicates a p-value < 0.05, ** indicates a p-value <

0.01 *** indicates a p-value < 0.001). **(D)** Clonogenic assay of MCF7 clones in full medium (NT), starvation medium (SILAC medium plus dialysed FBS with no glucose and no glutamine) and reduced glucose medium (SILAC medium plus dialysed FBS with 5mM glucose and no glutamine). In the case of starvation, the medium was replaced with full RPMI after 24 h. Clonogenic potential was assayed after 7 days in culture. **(E)** Colonies were counted using OpenCFU software and the change in colony growth was normalized to non-treated (NT) MCF7-pSFFV cells. **(F)** Percentages of surviving cells (Annexin V-/PI- fraction) in non-treated (NT), starved and reduced glucose conditions. Bars represent means ± SD from three independent experiments. One-way ANOVA with Tukey post-test was used to assess significance (* indicated a p-value < 0.05, ** indicates a p-value < 0.01, *** indicates a p-value < 0.001).

Figure 5. BCL2 and BCL(X)L maintain higher cytosolic ATP after sodium azide treatment and regulate oxygen consumption during hypoxia.

(A) Representative traces of cytosolic ATP signal and mitochondrial membrane potential. FRET/CFP ratio kinetics from ATeam probe and TMRM fluorescence were monitored, in all MCF7-ATeam clones. Cells were placed in KB with pyruvate; baseline was recorded for 20 minutes and 0.1 mM NaAzide was added into the medium. After 30 minutes treatment, 5 mM glucose was supplemented for the last 20 minutes. All data represent mean ± SD from n=3 independent experiments and both signals are normalized to the baseline levels (each cell line normalised to its own baseline). **(B)** The absolute FRET/CFP ratio were analysed by taking into account the minimal value reached by the probe after NaAzide addition and maximal value after glucose addition. **(C)** TMRM intensity values normalised to the baseline levels from TMRM kinetics were analysed by taking into account the maximal value reached after each treatment. **(D)** Slope values of sodium azide addition were assessed by dividing the ΔFRET/CFP ratio to the Δtime (time offset – time onset). All values were analysed by two-

959 way ANOVA with Tukey post-test (* indicates a p-value < 0.05, ** indicates a p-value <
960 0.01 *** indicates a p-value < 0.001). **(E)** Kinetics of intracellular oxygen levels were
961 measured with MitoImageTM (MM2) and normalised to baseline at 21% O_2. After reduction
962 of the atmospheric O_2 from 21 to 2% cells were treated with 10 μM oligomycin and 5 mM
963 glucose followed by 10 μM FCCP and the atmospheric O_2 was set back to 21% before the
964 end of the experiment in all MCF7 clones, as indicated on top of the left graph for MCF7-
965 pSFFV. All data represent mean ± SD from a minimum of 3 independent experiments. **(F)**
966 Area above baseline (at 2% oxygen) for oligomycin + glucose and FCCP treatments.
967 Addition of oligomycin and glucose inhibit mitochondrial ATP synthesis; with the increase of
968 the intracellular O_2, the O_2 consumption in BCL2 and BCL(X)L overexpressing cells was
969 decreased, due to the activity of the ATP synthase, than in the pSFFV clone. One-way
970 ANOVA with Tukey post-test was used to assess significance (* indicated a p-value < 0.05,
971 ** indicates a p-value < 0.01, *** indicates a p-value < 0.001). **(G)** Clonogenic assay of
972 MCF7 clones in full RPMI medium (11 mM glucose) and SILAC medium plus dialysed FBS
973 and 2 mM or 5 mM glucose, respectively, in hypoxic conditions (1% oxygen). Colonies were
974 counted using OpenCFU software and the change in colony growth was normalized to non-
975 treated (NT) MCF7-pSFFV cells. All values were analysed by two-way ANOVA with Tukey
976 post-test (* indicates a p-value < 0.05, ** indicates a p-value < 0.01 *** indicates a p-value <
977 0.001). Bars represent means ± SD from three independent experiments

978 **Figure 6. BCL2 and BCL(X)L silencing decreases ATP production following pyruvate**
979 **addition and decrease clonogenic potential.**

980 **(A)** Representative traces of mitochondrial ATP signal and mitochondrial membrane
981 potential in siRNA co-transfected cells. FRET/CFP ratio kinetics from mitoATeam probe and
982 TMRM fluorescence were monitored simultaneously in MCF7-pSFFV. Cells were starved
983 for three hours; after that a baseline was recorded for 20 minutes and 2 mM sodium pyruvate

was added into the medium. After 20 minutes treatment, 10 μM of oligomycin was supplemented for the last 20 minutes. All data represent mean ± SD from n=3 independent experiments and both signals are normalized to the baseline levels (each cell line normalised to its own baseline). **(B)** The absolute FRET/CFP ratio were analysed by taking into account the maximal value reached by the probe after pyruvate addition and minimal value after oligomycin. **(C)** TMRM intensity values normalised to the baseline levels from TMRM kinetics were analysed by taking into account the maximal value reached after each treatment. **(D)** TMRM fluorescence absolute values obtained from baseline. All values were analysed by two-way ANOVA with Tukey post-test (* indicates a p-value < 0.05, ** indicates a p-value < 0.01 *** indicates a p-value < 0.001). **(E)** Clonogenic assay of MCF7 cells treated with control, BCL2 or BCL(X)L siRNA and Lipofectamine in full medium. Clonogenic potential was assessed after 7 days in culture. **(F)** Colonies were counted automatically with OpenCFU and the change in colony growth was normalized to control siRNA treated cells. (**G**) Percentages of surviving cells (Annexin V-/PI- fraction) in same conditions. Bars represent means ± SD from n=3 independent experiments. One-way ANOVA with Tukey post-test was used to assess significance (* indicated a pvalue < 0.05, ** indicates a p-value < 0.01, *** indicates a p-value < 0.001).

Figure 7. BCL2 and BCL(X)L overexpression increase proliferation and migration properties in glucose limiting conditions.

(A and B) Cell numbers obtained from acid phosphatase assay for MCF7 clones in full RPMI (11 mM glucose) and SILAC medium plus dialysed FBS with 2 mM glucose, respectively. **(C and D)** Wound healing assay for MCF7 clones in full RPMI (11 mM glucose) and SILAC medium plus dialysed FBS with 2 mM glucose, respectively. Bars represent means ± SD from n=3 independent experiments. All values were analysed by two-way ANOVA with

Tukey post-test (* indicates a p-value < 0.05, ** indicates a p-value < 0.01 *** indicates a p-value < 0.001).

Figure 8. Gene expression analysis of respiratory chain in relation to BCL2 and BCL(X)L in MCF7 clones, CCLE and METABRIC datasets.

Comparison between expression of the genes from the respiratory chain, grouped by complex. For the in-house dataset, the relative gene levels, expressed as rlog normalized and z-scored values, for the control, BCL2 and BCL(X)L overexpressing clones (in duplicates, R1 and R2) are size- and color-coded (lower in blue, higher in red). Only genes that were statistically significantly different (non-adjusted pvalues <0.05; n=40 out of n=96 tested genes) are included in the figure. The statistical output including the effect size and comparison between individual cell lines for the complete set of genes that mapped to the complexes (n=96) are reported in the Supplementary Table 1. For the other datasets, we used the expression at the transcript level (BCL2 and BCL2L1). The association effect size expressed as Spearman correlation between BCL2 or BCL(X)L and each gene was size- and color-coded similarly to the data from the MCF7 clones to facilitate comparison. Genes with statistically significant correlation with BCL2 or BCL(X)L expression were annotated in the figure. The statistical output including effect size and p-values (unadjusted) for all the tested genes are included in the Supplementary table 2. For the CCLE, METABRIC data sets (Fig. 8) and Gao PDXs (Supplementary Fig. 11) positive correlation is indicated in a bigger red dot and negative correlation with a smaller blue dot.

Figure 1

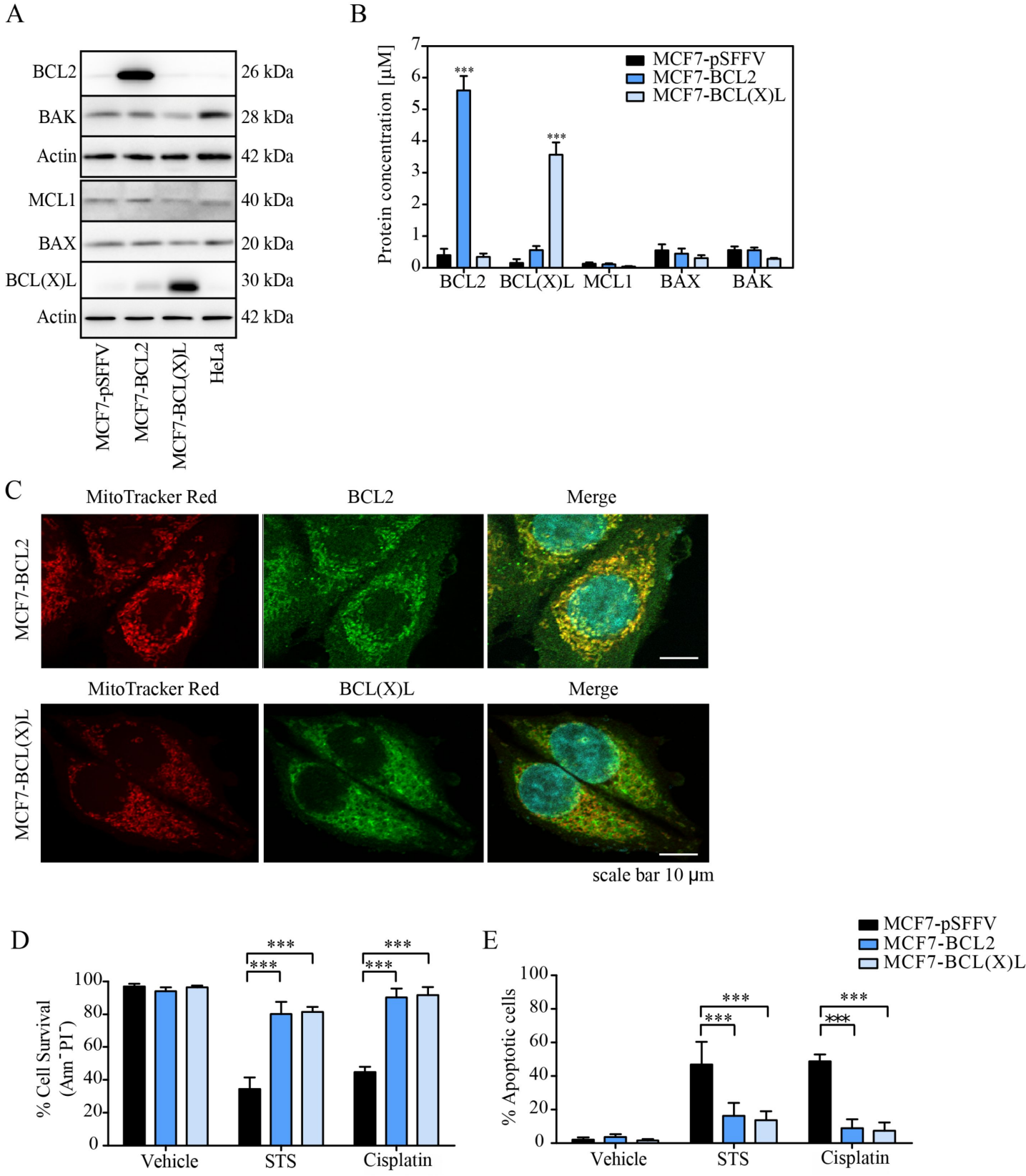

A
BCL2
BAK
Actin
MCL1
BAX
BCL(X)L
Actin
26 kDa
28 kDa
42 kDa
40 kDa
20 kDa
30 kDa
42 kDa
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
HeLa
B
Protein concentration [µM]
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
***
***
BCL2
BCL(X)L
MCL1
BAX
BAK
C
MitoTracker Red
BCL2
Merge
MCF7-BCL2
MitoTracker Red
BCL(X)L
Merge
MCF7-BCL(X)L
scale bar 10 µm
D
% Cell Survival (Ann⁻PI⁻)
***
***
***
***
Vehicle
STS
Cisplatin
E
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
% Apoptotic cells
***
***
***
***
Vehicle
STS
Cisplatin

Figure 2

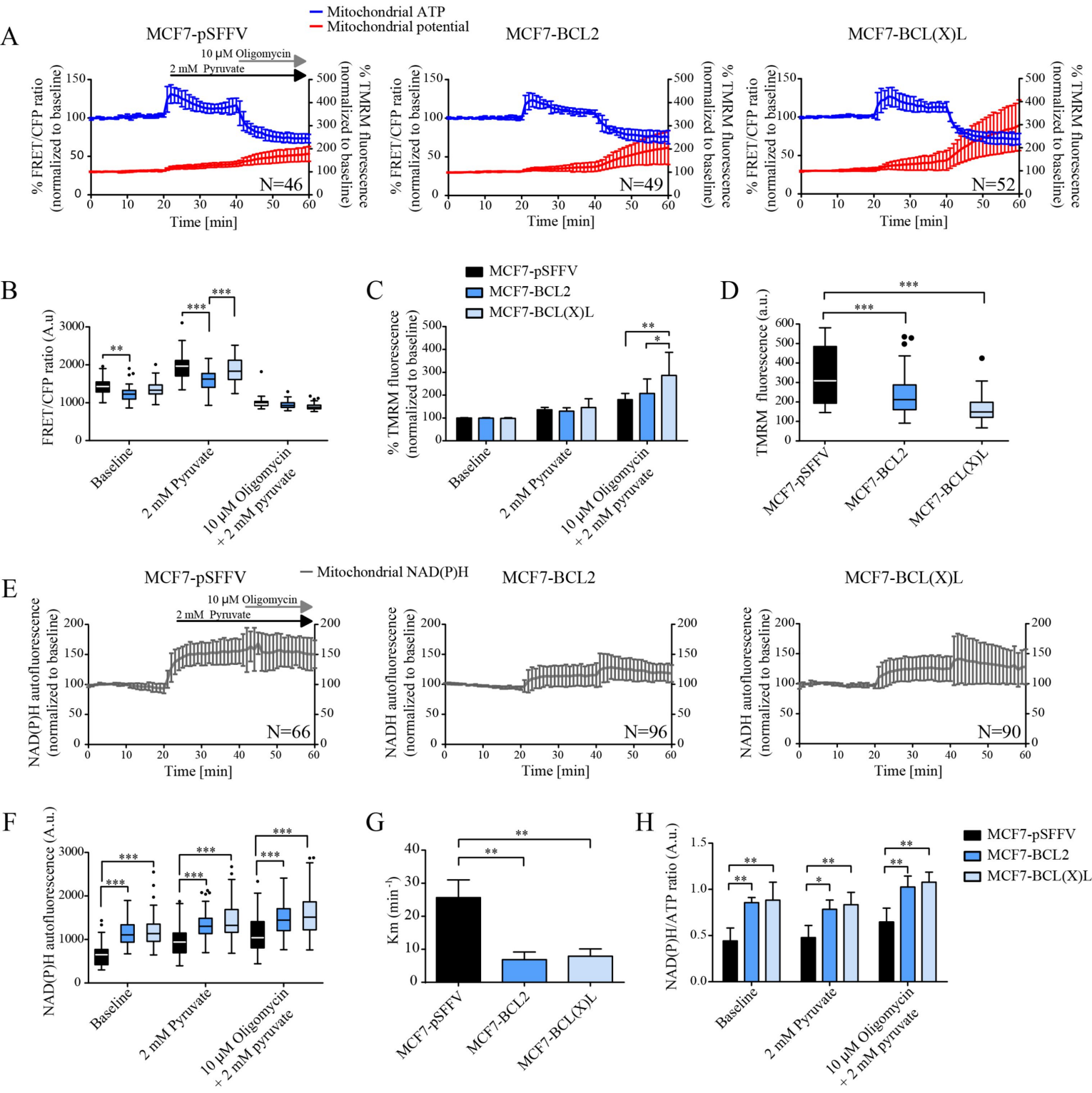

A
Mitochondrial ATP
Mitochondrial potential
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
10 µM Oligomycin
2 mM Pyruvate
% FRET/CFP ratio (normalized to baseline)
% TMRM fluorescence (normalized to baseline)
Time [min]
N=46
N=49
N=52
B
FRET/CFP ratio (A.u)
Baseline
2 mM Pyruvate
10 µM Oligomycin + 2 mM pyruvate
C
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
% TMRM fluorescence (normalized to baseline)
D
TMRM fluorescence (a.u.)
E
Mitochondrial NAD(P)H
NAD(P)H autofluorescence (normalized to baseline)
NADH autofluorescence (normalized to baseline)
N=66
N=96
N=90
F
NAD(P)H autofluorescence (A.u.)
G
Km (min-1)
H
NAD(P)H/ATP ratio (A.u.)

Figure 3

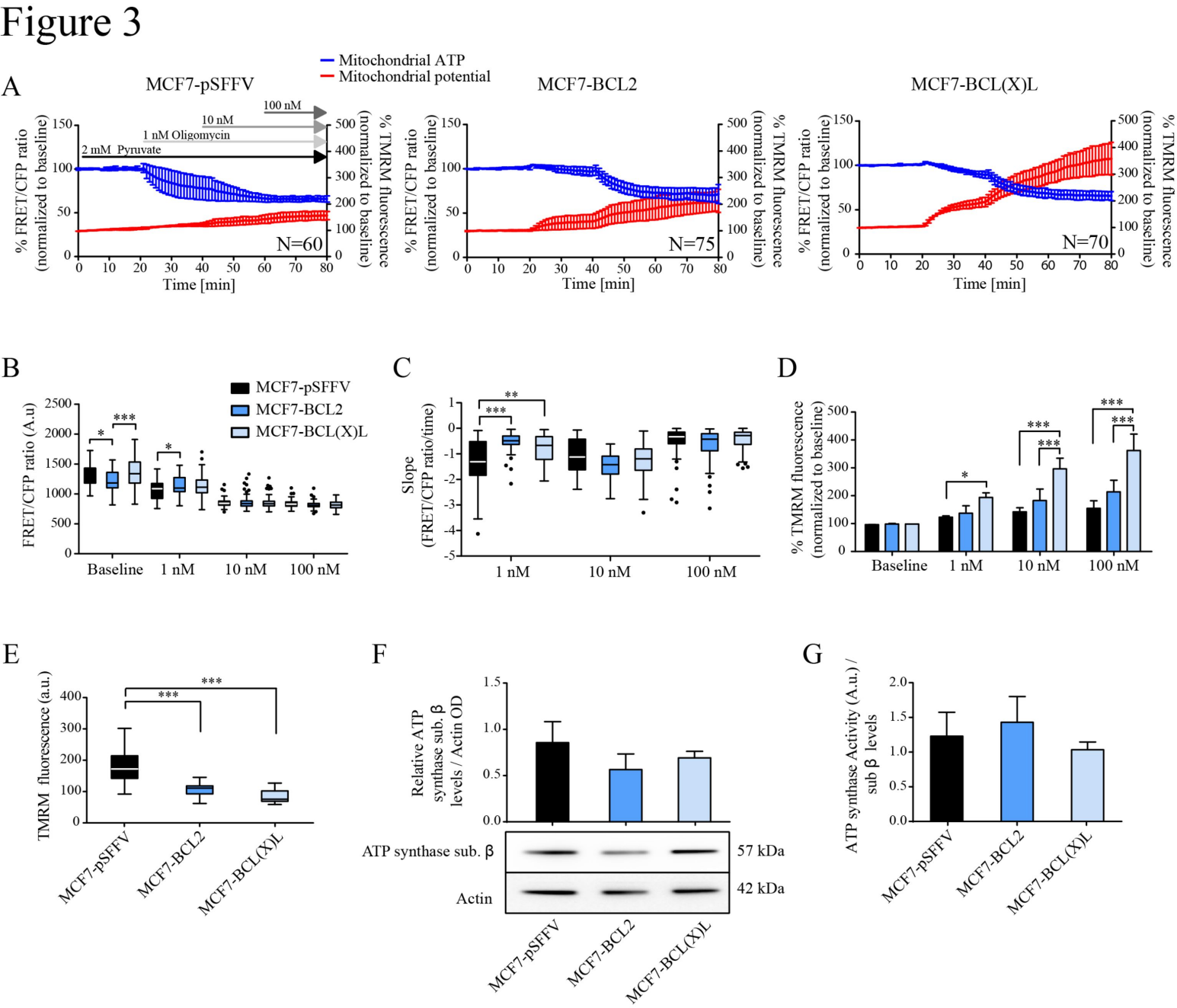

A
Mitochondrial ATP
Mitochondrial potential
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
100 nM
10 nM
1 nM Oligomycin
2 mM Pyruvate
% FRET/CFP ratio (normalized to baseline)
% TMRM fluorescence (normalized to baseline)
Time [min]
N=60
N=75
N=70
B
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
FRET/CFP ratio (A.u)
Baseline
1 nM
10 nM
100 nM
C
Slope (FRET/CFP ratio/time)
D
% TMRM fluorescence (normalized to baseline)
E
TMRM fluorescence (a.u.)
F
Relative ATP synthase sub. β levels / Actin OD
ATP synthase sub. β
Actin
57 kDa
42 kDa
G
ATP synthase Activity (A.u.) / sub β levels

Figure 4

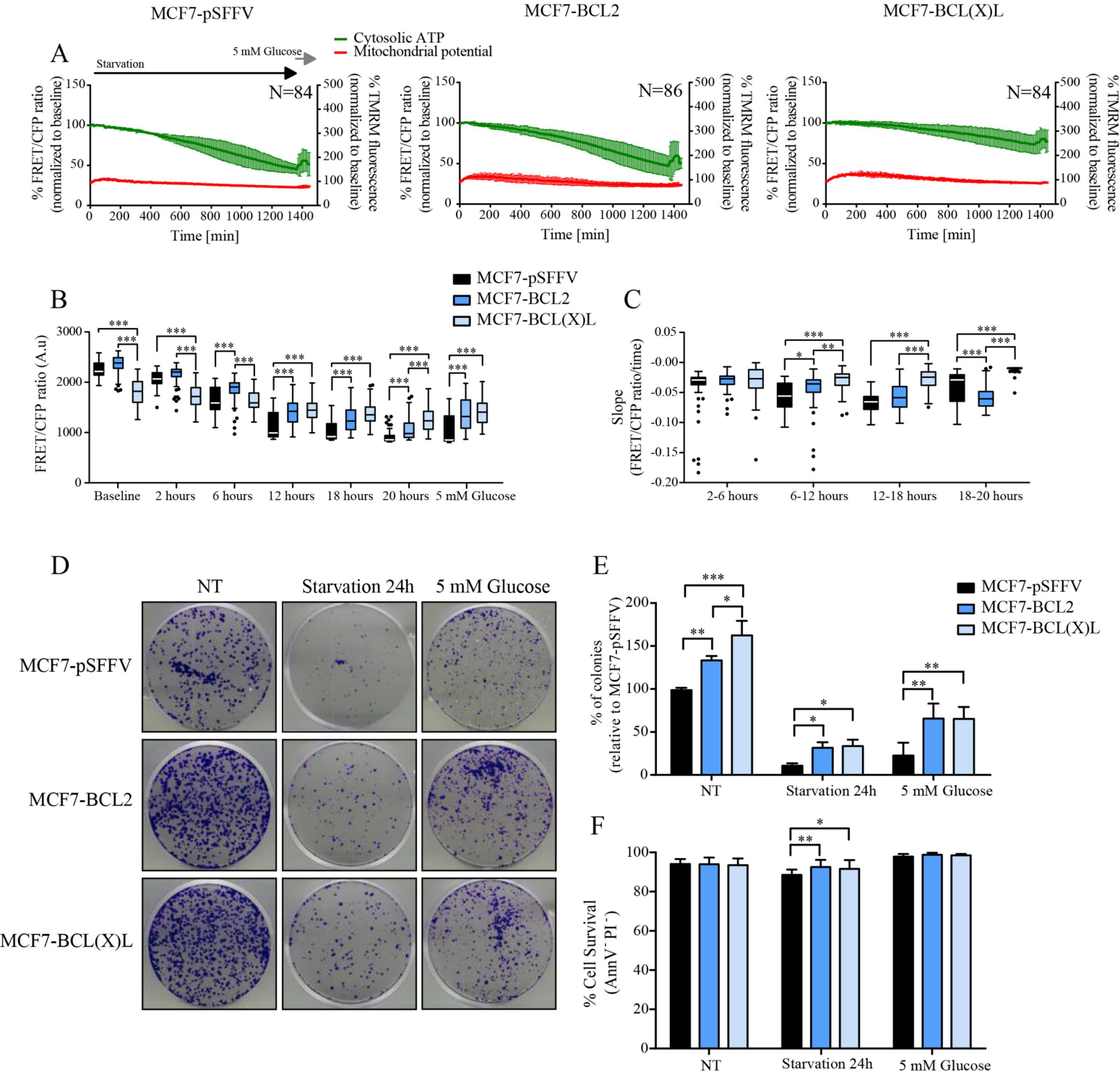

MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
A
Starvation
5 mM Glucose
Cytosolic ATP
Mitochondrial potential
N=84
N=86
N=84
% FRET/CFP ratio (normalized to baseline)
% TMRM fluorescence (normalized to baseline)
Time [min]
B
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
FRET/CFP ratio (A.u)
Baseline
2 hours
6 hours
12 hours
18 hours
20 hours
5 mM Glucose
C
Slope (FRET/CFP ratio/time)
2-6 hours
6-12 hours
12-18 hours
18-20 hours
D
NT
Starvation 24h
5 mM Glucose
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
E
% of colonies (relative to MCF7-pSFFV)
NT
Starvation 24h
5 mM Glucose
F
% Cell Survival (AnnV- PI-)
NT
Starvation 24h
5 mM Glucose

Figure 5

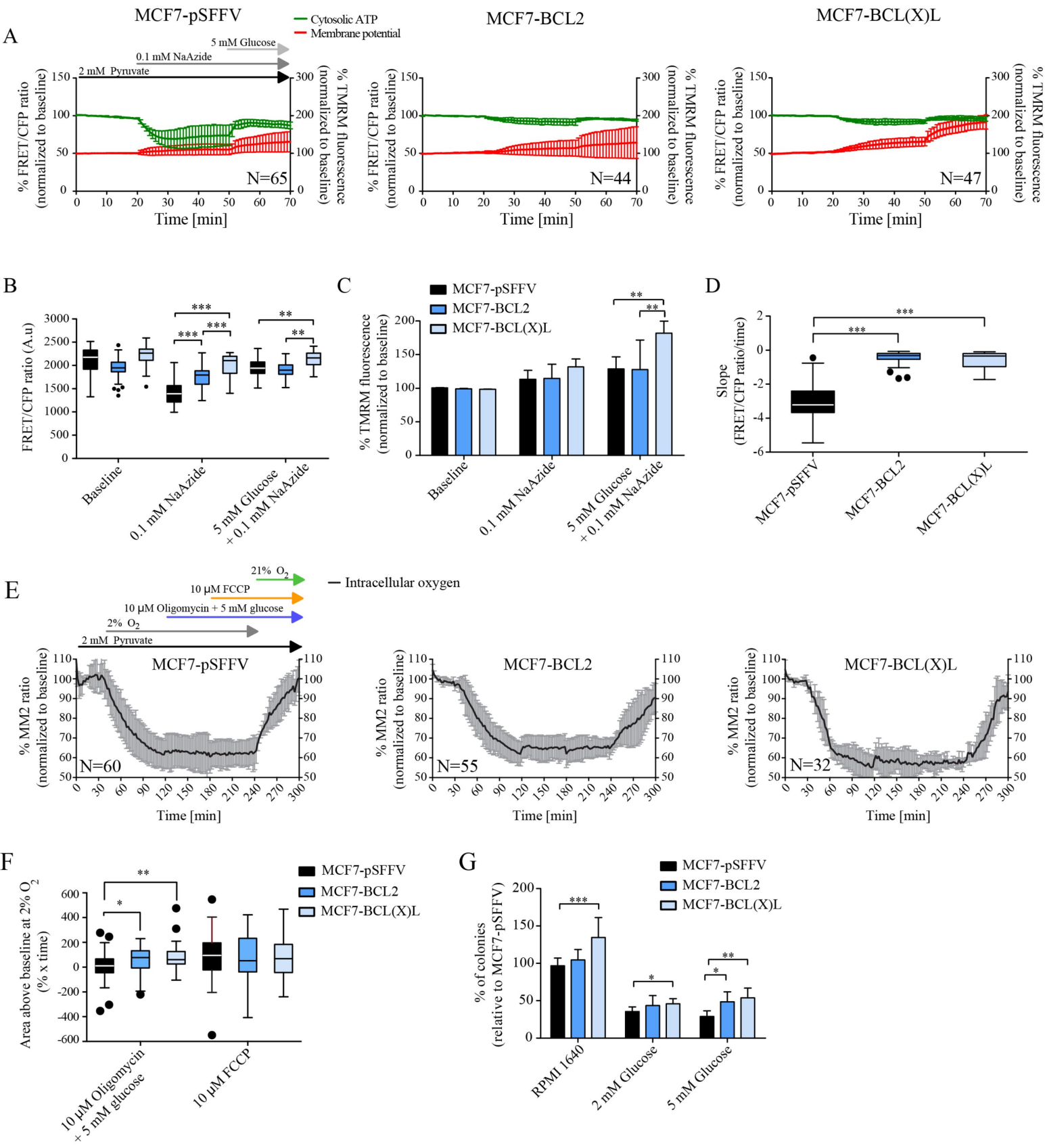

A
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
Cytosolic ATP
Membrane potential
5 mM Glucose
0.1 mM NaAzide
2 mM Pyruvate
% FRET/CFP ratio (normalized to baseline)
% TMRM fluorescence (normalized to baseline)
Time [min]
N=65
N=44
N=47
B
FRET/CFP ratio (A.u)
Baseline
0.1 mM NaAzide
5 mM Glucose + 0.1 mM NaAzide
C
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
% TMRM fluorescence (normalized to baseline)
D
Slope (FRET/CFP ratio/time)
E
21% O2
10 µM FCCP
10 µM Oligomycin + 5 mM glucose
2% O2
2 mM Pyruvate
Intracellular oxygen
% MM2 ratio (normalized to baseline)
N=60
N=55
N=32
F
Area above baseline at 2% O2 (% x time)
10 µM Oligomycin + 5 mM glucose
10 µM FCCP
G
% of colonies (relative to MCF7-pSFFV)
RPMI 1640
2 mM Glucose
5 mM Glucose

Figure 6

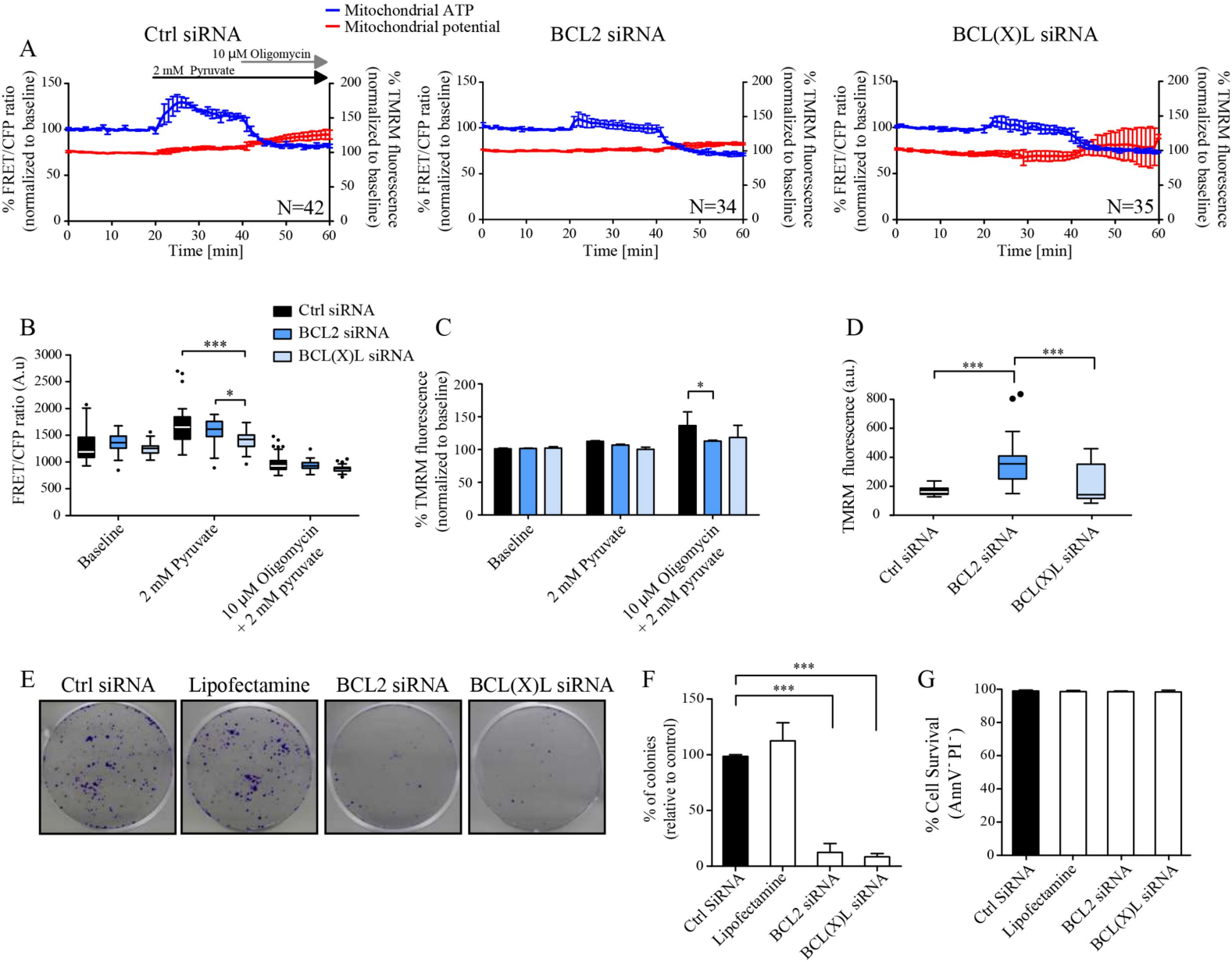

A
Mitochondrial ATP
Mitochondrial potential
Ctrl siRNA
BCL2 siRNA
BCL(X)L siRNA
10 µM Oligomycin
2 mM Pyruvate
% FRET/CFP ratio (normalized to baseline)
% TMRM fluorescence (normalized to baseline)
Time [min]
N=42
N=34
N=35
B
Ctrl siRNA
BCL2 siRNA
BCL(X)L siRNA
FRET/CFP ratio (A.u)
Baseline
2 mM Pyruvate
10 µM Oligomycin + 2 mM pyruvate
C
% TMRM fluorescence (normalized to baseline)
D
TMRM fluorescence (a.u.)
E
Ctrl siRNA
Lipofectamine
BCL2 siRNA
BCL(X)L siRNA
F
% of colonies (relative to control)
G
% Cell Survival (AnnV⁻ PI⁻)

Figure 7

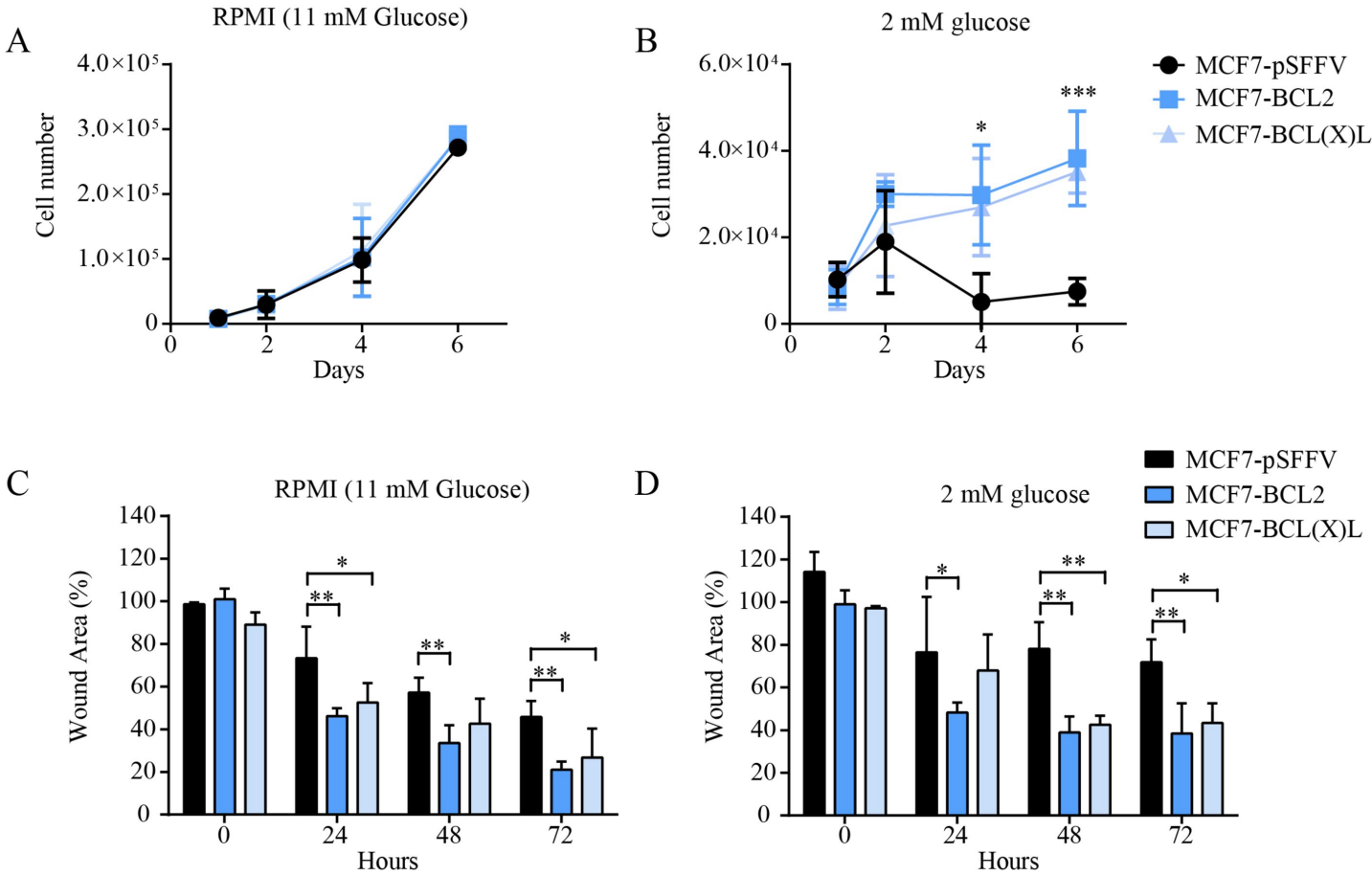
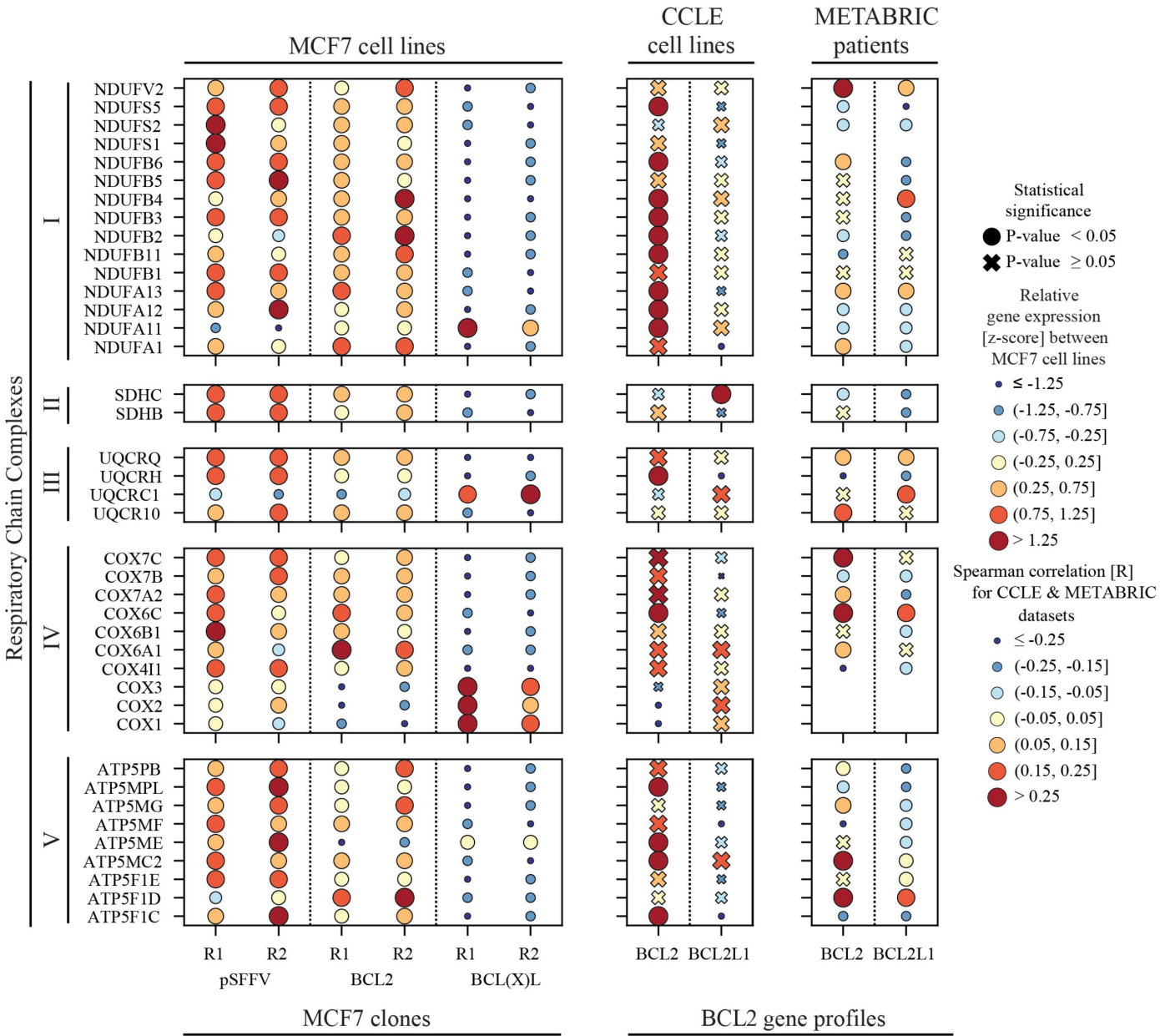

A
RPMI (11 mM Glucose)
Cell number
4.0×10⁵
3.0×10⁵
2.0×10⁵
1.0×10⁵
0
0
2
4
6
Days
B
2 mM glucose
Cell number
6.0×10⁴
4.0×10⁴
2.0×10⁴
0
*
***
0
2
4
6
Days
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L
C
RPMI (11 mM Glucose)
Wound Area (%)
140
120
100
80
60
40
20
0
*
**
**
**
*
**
0
24
48
72
Hours
D
2 mM glucose
Wound Area (%)
140
120
100
80
60
40
20
0
*
**
**
**
*
**
0
24
48
72
Hours
MCF7-pSFFV
MCF7-BCL2
MCF7-BCL(X)L

Figure 8


MCF7 cell lines
CCLE cell lines
METABRIC patients
Respiratory Chain Complexes
I
II
III
IV
V
NDUFV2
NDUFS5
NDUFS2
NDUFS1
NDUFB6
NDUFB5
NDUFB4
NDUFB3
NDUFB2
NDUFB11
NDUFB1
NDUFA13
NDUFA12
NDUFA11
NDUFA1
SDHC
SDHB
UQCRQ
UQCRH
UQCRC1
UQCR10
COX7C
COX7B
COX7A2
COX6C
COX6B1
COX6A1
COX4I1
COX3
COX2
COX1
ATP5PB
ATP5MPL
ATP5MG
ATP5MF
ATP5ME
ATP5MC2
ATP5F1E
ATP5F1D
ATP5F1C
R1
R2
R1
R2
R1
R2
pSFFV
BCL2
BCL(X)L
MCF7 clones
BCL2
BCL2L1
BCL2
BCL2L1
BCL2 gene profiles
Statistical significance
P-value < 0.05
P-value ≥ 0.05
Relative gene expression [z-score] between MCF7 cell lines
≤ -1.25
(-1.25, -0.75]
(-0.75, -0.25]
(-0.25, 0.25]
(0.25, 0.75]
(0.75, 1.25]
> 1.25
Spearman correlation [R] for CCLE & METABRIC datasets
≤ -0.25
(-0.25, -0.15]
(-0.15, -0.05]
(-0.05, 0.05]
(0.05, 0.15]
(0.15, 0.25]
> 0.25