

Glucose deprivation induces cancer cell death through oncogene overdose

Igor Vivanco

`igor.vivanco@kcl.ac.uk`

Institute of Cancer Research, UK <https://orcid.org/0000-0001-6347-9416>

Khine Nyein Myint

Alice Harbery

Institute of Cancer Research

Eleftherios Kostaras

Institute of Cancer Research

Sara Heuss

Institute of Cancer Research

Glorianne Lazaro

Institute of Cancer Research

Smriti Rajesh

King's College London

Natalia Senakh

King's College London

Courtney Savage

Institute of Cancer Research

Nikos Koundouros

Weill Cornell Medicine

Pedro Casado

Institute of Cancer, Queen Mary University of London <https://orcid.org/0000-0002-4207-9349>

Nicolaos Palaskas

University of California Los Angeles

Pedro Cutillas

Consejo Superior de Investigaciones Científicas (CSIC) <https://orcid.org/0000-0002-3426-2274>

George Poulogiannis

The Institute of Cancer Research <https://orcid.org/0000-0002-0529-8614>

Aurelien Tripp

The Institute of Cancer Research, London

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2 Khine Nyein Myint*¹, Alice Harbery*², Eleftherios Kostaras², Sara Farrah Heuss², Aurelien
3 Tripp³, Glorianne Lazaro², Smriti Rajesh¹, Natalia Senakh¹, Courtney Savage², Nikos
4 Koundouros³, Pedro Casado⁴, Nicolaos Palaskas⁵, Pedro R. Cutillas⁴, George Poulgiannis³,
5 Igor Vivanco^{1§}

6
7 ¹ Institute of Pharmaceutical Science, King's College London, 150 Stamford Street, SE1
8 9NH, London, UK. ²Division of Cancer Therapeutics, The Institute of Cancer Research, 15
9 Cotswold Rd., SM2 5NG, London, UK. ³ Division of Cell and Molecular Biology, The Institute
10 of Cancer Research, 237 Fulham Road, SW3 6JB, London, UK. ⁴Centre for Haemato-
11 Oncology, Barts Cancer Institute, Queen Mary University of London, Charterhouse Square,
12 EC1M 6BQ, London, UK. ⁵Division of Hematology and Oncology, David Geffen School of
13 Medicine, UCLA, Los Angeles, USA.

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23
24
25
26
27
28 *Contributed equally to this work

29 § Correspondence: igor.vivanco@kcl.ac.uk
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Abstract

Metabolic rewiring is a hallmark of cancer, commonly exemplified by the Warburg effect and increased glucose dependence. The cytotoxic effects of glucose withdrawal are typically attributed to energy depletion or impaired anabolic processes. While oncogenic drivers shape metabolic reprogramming during tumorigenesis, and therapy resistance induces further metabolic alterations, it remains unclear whether metabolic perturbations can directly modulate oncogenic signalling. Here, we show that glucose withdrawal induces cell death in EGFR-, BRAF-, or PI3K-driven cancer cells through oncogene overdose rather than metabolic failure. Transcriptomic and phosphoproteomic analyses reveal that glucose depletion sensitizes specific transcripts and phosphopeptides to oncogene inhibition, indicating that metabolic stress rewires oncogenic function. Mechanistically, pyruvate depletion promotes the accumulation of reactive oxygen species (ROS), triggering oncogene superactivation and JNK-dependent cell death, accompanied by dysregulation of mitotic and cytokinesis regulators. Together, these findings have uncovered a critical function of the Warburg effect in preventing toxic levels of oncogene activation, highlighting the importance of metabolic context when targeting oncogenic signalling in cancer.

Reprogramming of energy metabolism is an important feature of cancer cells that allows them to adjust to the demands of enhanced and ectopic proliferation¹. When oxygen is available, normal cells metabolise glucose through glycolysis to generate pyruvate, which is subsequently oxidised through mitochondrial respiration. Under hypoxic conditions, however, cells limit the shuttling of pyruvate to oxygen-consuming mitochondria and instead convert it to lactate. The Warburg effect refers to the predilection of cancer cells to continue converting pyruvate to lactate even when oxygen is present² and mitochondrial function is intact^{3,4}. This metabolic switch allows cancer cells to accumulate and divert glycolytic intermediates to critical biosynthetic pathways that generate nucleotides, non-essential amino acids, lipids and hexosamines, rendering them unusually dependent on glucose for energy and biomass production and potentially creating a cancer-selective metabolic vulnerability^{5,6}. Consistently, glucose deprivation can elicit cytotoxic effects in tumour cells both in vitro and in vivo^{5,7,8}. To exploit this vulnerability, therapeutic agents targeting various aspects of cancer metabolism have been under development for several years⁶. Additionally, dietary manipulation to reduce glucose levels in tumours has also been pursued as a therapeutic approach⁹. The basic rationale for such treatment strategies is that interference with cancer-specific energy and/or biosynthetic pathways will induce a profound metabolic imbalance that selectively kills cancer cells¹⁰.

Oncogenic drivers such as the epidermal growth factor receptor (EGFR) or the serine/threonine kinase BRAF promote metabolic reprogramming, and tumours that respond to inhibitors of these proteins undergo significant metabolic changes upon treatment¹¹⁻¹⁴. Further changes in cellular metabolism have also been found to coincide with the emergence of drug resistance¹⁵, and pharmacological modulators of these altered metabolic routes can overcome resistance, suggesting that these pathways are indeed important determinants of response to targeted therapies. However, whether simultaneous targeting of metabolic and oncogene dependence in treatment naïve tumours can delay or prevent the emergence of acquired drug resistance remains unclear⁷. Altogether, these data point to a clear functional link between activation of oncogenic drivers and the rewiring of metabolic programmes that support oncogenic growth. This interaction may be particularly meaningful in the context of glioblastoma (GBM), the most aggressive form of primary brain cancer, where de novo drug resistance has restricted therapeutic benefit to a handful of anecdotal responses despite the presence of likely oncogenic drivers, most notably EGFR¹⁶. In these patients, the metabolic features of the tumour have been proposed to be the culprit of the limited therapeutic activity of targeted agents¹⁷⁻¹⁹.

The dependence of tumours on the activity of oncogenic drivers, a phenomenon known as oncogene addiction, is the basis for the majority of targeted therapies in oncology. Interestingly, similar to inhibition, overstimulation of driver oncoproteins (e.g. EGFR), can also result in cell death²⁰⁻²³, suggesting that, in order to survive, cancer cells with aberrantly activated oncogenes must carefully restrict the activation

level of these molecules to a defined permissive range (Figure 1a). Despite growing evidence that oncogene addiction is linked to the altered energetics programmes of cancer cells²⁴, and although the ability of various nutrients to engage signal transduction is well documented²⁵, the cellular and molecular consequences of metabolic perturbations on oncogene-driven signalling outputs that support tumour growth are less well characterised.

Here, we demonstrate that glucose deprivation in cancer cells carrying various oncogenic drivers (including mutant EGFR, mutant BRAF, mutant PIK3CA or MYC amplification) causes oncogene activity overload secondary to a rise in intracellular reactive oxygen species (ROS) ultimately resulting in cancer cell death. Importantly, we find that inhibitors that normalise the activity of these oncogenic drivers, or pyruvate supplementation, protect against glucose-deprivation-induced cell death. Furthermore, pyruvate supplementation prevents both the accumulation of ROS and the superactivation of oncogenic drivers. We also find that a number of gene expression and phosphorylation changes caused by glucose withdrawal in EGFR-mutant glioma cells are reversed by EGFR inhibitor treatment, suggesting that some signal transduction events elicited by glycolytic stress are in fact oncogene-driven. Taken together, these data provide evidence that the cytotoxic effects of glucose deprivation are largely a consequence of oncogene overdose rather than solely the result of metabolic imbalance.

RESULTS

Glucose deprivation in oncogene-driven cancer cell lines kills through oncogene overdose.

Functional blockade of either driver oncoproteins or glucose uptake in oncogene-addicted cancer cells has been shown to have significant anti-tumour effects in multiple cancer types. In GBM, where de novo resistance to most targeted therapies is nearly universal, EGFR-activating lesions are found in nearly 60% of cases²⁶ and BRAF-V600E, while not a frequent mutation, (3% incidence²⁷), predicts clinical response to BRAF inhibitors²⁸. Currently, therapeutic options for GBM are largely limited to surgery and radiochemotherapy, and prognosis remains dismal. Therefore, there is an urgent need for new treatments. Given the potential role of cellular metabolism in de novo resistance to targeted therapies, we sought to test whether simultaneous targeting of oncogene and glucose dependence would exert a therapeutically synergistic effect in GBM cells. We therefore treated EGFR-mutant SKMG3²⁹ and BRAF-mutant DBTRG³⁰ glioma cells with either the EGFR inhibitors lapatinib or afatinib, or the BRAF inhibitors vemurafenib or dabrafenib or the MEK inhibitor trametinib, respectively, in media containing high (10mM) or low (1mM) glucose. As expected, glucose depletion had significant anti-tumour effects in both cell lines (Figure 1b, c and d, Supplemental Figure 1). However, treatment with sub-

therapeutic doses of inhibitors of the driver oncogene (i.e. EGFR or BRAF) almost completely abolished the cytotoxic (but not the cytostatic) effects of glucose withdrawal (Figure 1b, c and d). Treatment of glucose-starved mutant-BRAF DBTRG cells with an inhibitor (trametinib) of MEK, an obligate effector of BRAF signalling, also rescued cells from cell death (Figure 1d), suggesting that over-stimulation of signalling downstream of the driver oncogene is necessary to induce glucose-deprivation-induced (GDI) toxicity. Importantly, lapatinib was unable to rescue the effects of glucose depletion in BRAF-mutant cells (Figure 1e), and similarly, dabrafenib could not protect EGFR-mutant cells from GDI toxicity (Figure 1f). Based on these findings, we next explored whether glucose-deprivation-induced death in cancer cells driven by oncogenic lesions other than protein kinases could also be blocked by inhibitors of the relevant oncoproteins. We selected cell lines with a driver lesion in PI3K (a lipid kinase) or focal amplification of MYC (a transcription factor). In both cases, glucose-deprivation-induced death was prevented by inhibitors of PI3K α ³¹ (Figure 1g) or MYC function³² (Supplemental Figure 2), respectively. We also confirmed that GDI toxicity can be blocked by BRAF inhibitors in additional BRAF-mutant cancer cell lines (Supplemental Figure 3a and b), and by EGFR inhibitors (lapatinib or erlotinib, respectively) in 170-MG-BA glioblastoma cells and the epidermoid carcinoma cell line A431, both of which carry high-level amplification of the wild type EGFR locus³³ (Supplemental Figure 3c and d).

Next, we analysed the effects of glucose withdrawal on the activation state of the oncogenic drivers (i.e. EGFR and BRAF) in the SKMG3 and DBTRG models. We found that the activity of the already mutationally activated oncoproteins (EGFR-A289D and BRAF-V600E) was further increased by glucose withdrawal (Figure 1h, i and j). Similar effects were observed in EGFR-amplified 170-MG-BA (Supplemental Figure 4a) and BRAF-V600E A2058 cells (Supplemental Figure 4b). Interestingly, while MAPK signalling was increased by glucose deprivation in SKMG3 cells (consistent with an increase in EGFR activity; Figure 1h), EGFR phosphorylation was modestly inhibited by glucose depletion in DBTRG cells (Figure 1j), suggesting that the effects on driver oncoproteins are at least partly selective. Similarly, glucose depletion led to superactivation of the PI3K pathway (as judged by increased AKT phosphorylation) in PIK3CA-mutant MCF7 cells (Supplemental Figure 4c), further demonstrating the link between glycolytic stress and activation of driver oncogenes. To further demonstrate the specific role of oncogene over-activation in GDI toxicity, we engineered an inducible EGFR knockout SKMG3 cell line through stable lentiviral co-transduction of a doxycycline-regulated Cas9 and an EGFR-targeting gRNA. Similar to EGFR inhibitor treatment, CRISPR-mediated EGFR depletion (Supplemental Figure 5a), while growth inhibitory on its own (Supplemental Figure 5b), protected cells from GDI toxicity (Supplemental Figure 5c). These data demonstrate that superactivation of a driver oncogene may be necessary to induce GDI toxicity.

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190 **Oncogene superactivation is sufficient to induce cell death in cancer cells**

191 We next asked whether direct superactivation of driver oncogenes was sufficient to
192 induce cancer cell death in our models. First, to drive superactivation of the
193 constitutively active BRAF-V600E mutant, we stably transduced DBTRG cells that
194 already express the mutant protein endogenously, with a doxycycline-inducible
195 overexpression construct (Figure 1l, right panel). As a control, we also generated a
196 separate DBTRG cell line with inducible overexpression of wild type BRAF (Figure
197 1l, left panel), as the wild-type protein would not be able to super-activate the
198 pathway in the absence of growth factor stimulation. Indeed, we found that acute
199 overexpression of mutant (but not wild-type) BRAF caused significant cell death
200 (Figure 1m), which could be rescued by treatment with sub-therapeutic doses of
201 dabrafenib (Figure 1n). Additionally, we used EGF stimulation to test whether direct
202 EGFR superactivation could induce cell death in 170-MG-BA cells that carry high
203 level EGFR amplification. Indeed, EGFR stimulation with different EGF
204 concentrations (50-200mg/mL) was sufficient to induce significant cell killing (Figure
205 1o). These findings show that acute superactivation of driver oncogenes alone is
206 enough to induce cancer cell death, highlighting oncogene overdrive as a lethal
207 vulnerability in tumour cells.

208

209 **Pyruvate supplementation is sufficient to overcome GDI oncogene over-**
210 **activation and cell death.**

211 Next, we wanted to determine whether glucose withdrawal causes cell death and
212 oncogene over-activation through depletion of glycolytic intermediates, as these
213 metabolites are thought to be critical in the glycolytic dependence of cancer cells⁵.
214 We started by assessing the effects of adding back exogenous pyruvate, the end-
215 product of glycolysis. First, we confirmed that in SKMG3 cells, glucose withdrawal
216 caused a significant decrease in cellular pyruvate levels which was not rescued by
217 lapatinib treatment (Figure 2a). We then tested the effects of pyruvate
218 supplementation on GDI EGFR super-activation and cell death. Surprisingly, we
219 found that addition of methyl-pyruvate completely prevented both in SKMG3 cells
220 (Figure 2b and g), suggesting that the signals driving these phenotypes are not
221 generated through depletion of specific glycolytic intermediates, but rather by
222 perturbation of pyruvate levels or metabolic routes that use pyruvate as a substrate.
223 Similarly, in the BRAF-mutant DBTRG line, pyruvate supplementation fully protected
224 cells from GDI cell death and MAPK superactivation (Figure 2c and f). We also
225 assessed the effects of glucose depletion, with or without pyruvate supplementation
226 or lapatinib treatment, on cellular glyceraldehyde-3-phosphate (G3P) levels to rule
227 out the possibility that these treatments might reactivate glycolysis. As expected,
228 G3P levels plummeted following glucose depletion, and remained low with pyruvate

or lapatinib treatment (Figure 2e). Under aerobic conditions, glycolytic pyruvate is oxidised in mitochondria to generate acetyl-coA, which is later converted to citrate in the TCA cycle. We therefore asked whether supplementation with either citrate or acetate (a major Acetyl-CoA precursor) could protect cells from GDI cell death³⁴. We found that neither of these metabolites was able to fully rescue cells from GDI toxicity (Figure 2b and c). In tumours, pyruvate is rapidly converted to lactate, leading to lactate concentrations of 10-30 mM³⁵. Because the reactions converting pyruvate to lactate and lactate to pyruvate are both catalysed by lactate dehydrogenase (LDH) depending on substrate availability, we reasoned that in the absence of pyruvate, excess lactate could drive its conversion to pyruvate. Indeed, supplementation with 20 mM (but not 2 mM) lactate significantly, although not fully, protected cells from GDI cell death, and this effect was blocked by treatment with the LDH inhibitor methylmalonate³⁴ (Figure 2d). Interestingly, unlike inhibitors of EGFR and BRAF, which do not prevent GDI proliferation arrest, pyruvate supplementation also provided some protection from the cytostatic effects of glucose depletion (Figure 2b and c, left panels). This suggests that pyruvate alone is sufficient to support some level of growth and proliferation, but that GDI toxicity and cytostasis may result from disruption of distinct pyruvate-sensitive processes, only the former of which results from oncogene overdose. Finally, while pyruvate supplementation could protect cancer cells from GDI toxicity, it could not prevent cell death caused by induced overexpression of mutant BRAF in DBTRG cells or wild type EGFR in SKMG3 cells, suggesting that oncogene superactivation-induced cancer cell death occurs downstream of pyruvate depletion (Figure 2h and i).

In addition to its central role in anabolic and catabolic processes, pyruvate is a strong reactive oxygen species (ROS) scavenger³⁶. Consequently, we suspected that the plummeting levels of pyruvate after glucose starvation would result in intracellular ROS accumulation. Indeed, ROS levels, as measured by DCF-DA staining, were increased in SKMG3 and DBTRG cells when switched to media containing 1 mM glucose (Figure 2K). Furthermore, this increase in ROS could be completely reversed by pyruvate supplementation. We also observed that treatment of SKMG3 cells with lapatinib (1µM) or of DBTRG cells with dabrafenib (10nM) partially reversed the observed GDI ROS accumulation, suggesting that GDI EGFR or BRAF superactivation could lead to a feedforward loop of ROS generation (Figure 2k). Mitochondrial superoxide levels were examined using mitoSOX, since mitochondria may become a source of oxidative stress by producing excess ROS upon glucose withdrawal³⁷. We found that elevated mitochondrial ROS levels were rescued by pyruvate supplementation, but not by lapatinib treatment (Supplemental Figure 6a), indicating that the potential ROS amplification loop triggered by elevated EGFR activity may not affect the mitochondrial superoxide pool. Mitochondrial activity, as indirectly measured by MTS assay, was affected by glucose deprivation but could not be rescued by supplementation with glycolytic intermediates, pyruvate, or lapatinib treatment (Supplemental figure 6b), further suggesting that the observed GDI cell death was unlikely caused by mitochondrial functional impairment. Various

key cofactors and intermediates of central carbon metabolism (such as AMP, ADP, ATP, NAD, NADH, NADP, NADPH and succinic acid) were measured via LC-MS-based analysis of cellular extracts. None of these analytes were significantly altered under glucose-deprived conditions, indicating that GDI cytotoxicity is unlikely to be caused solely by impaired cellular energetics (Supplemental Figure 6c and d). Importantly, ROS have been shown to induce activation of both EGFR and MAPK^{38,39}. Consistently, treatment of SKMG3 cells with hydrogen peroxide, one of the predominant ROS species, was sufficient to induce EGFR superactivation (Figure 2j).

In recent years, inhibitors of GLUT transporters have been developed as a strategy to target the Warburg-driven glucose dependence of cancer cells⁴⁰. However, the therapeutic window of these compounds may be limited by glucose requirements in normal tissues, particularly in the brain. We reasoned that if GDI toxicity is primarily driven by ROS-dependent sulfenylation of driver oncoproteins, the effects of GLUT inhibition on oxidative stress could be amplified by addition of antioxidant inhibitors. Such an approach would avoid the need for potentially toxic doses of GLUT inhibitors. Peroxiredoxins (or PRDXs) are the first-line of defence against oxidative damage⁴¹, and their inhibition is associated with enhanced protein S-sulfenylation^{42,43}. We therefore assessed the potential therapeutic effect of combining the PRDX2 inhibitor conoidin A with non-cytotoxic doses of the pan-GLUT inhibitor KL11743 in SKMG3 and DBTRG cells. We observed that this drug combination, but not KL11743 alone, significantly induced cell death (Supplementary Figure 7a,b) as well as superactivation of MAPK in DBTRG cells (Supplementary Figure 7c) and of EGFR in SKMG3 cells (Supplementary Figure 7d). Furthermore, the effect of this combination could be significantly rescued by either dabrafenib or lapatinib treatment, respectively (Supplementary Figure 7a and b). Altogether, these data suggest that concurrent glucose uptake and peroxideroxin inhibition could be a potential strategy to harness oxidative stress to drive oncogene superactivation-induced cancer cell killing.

Sulfenylation of EGFR at C797, which can be induced by hydrogen peroxide treatment, has been shown to increase kinase activity^{39,44}. We, therefore, hypothesised that ROS accumulation caused by glucose starvation could result in EGFR sulfenylation. Using DYn-2-based labelling of sulfenylated proteins and click chemistry⁴⁵, we found that sulfenylated EGFR levels were indeed increased in cells cultured in 1 mM glucose compared to the 10 mM glucose group, indicating that GDI EGFR hyperactivation could result from ROS-induced sulfenylation of EGFR (Figure 2i). Additionally, our data show that treatment of SKMG3 cells with afatinib, an EGFR inhibitor that covalently binds to C797⁴⁶, significantly impairs GDI EGFR sulfenylation, suggesting that this residue is indeed the primary site of this ROS-driven modification (Supplementary figure 8).

Glucose depletion does not cause oncogene superactivation in cells with acquired resistance to hypoglycaemic growth conditions.

Our data suggest that oncogene superactivation following glycolytic stress may represent a Warburg-effect-enabled fail-safe mechanism in oncogene-driven cells to boost their primary survival pathway in the face of metabolic insult. We therefore hypothesised that glucose depletion might not affect oncogene activity in cells where glucose availability no longer influences cell survival. To test this, we generated SKMG3 and DBTRG cells capable of growing under low glucose conditions. Cells were cultured in 1 mM glucose for a period of 4 months and populations that survived and were able to proliferate were expanded for further analysis. First, we assessed whether the ability to survive glucose withdrawal in these cells was transient. Glucose deprivation had no effect on the growth of these cells even after they were switched back to higher glucose conditions for 3 weeks, suggesting that adaptation to low glucose was stable (Figure 3a and b). Consistent with our hypothesis, glucose depletion had no effect on EGFR phosphorylation in 1 mM glucose-adapted SKMG3 cells, and only modest effects on ERK phosphorylation in DBTRG cells compared to parental controls (Figure 3c). We then assessed the metabolic profile of these cells using Seahorse technology and found, as expected, that glycolytic capacity and glycolysis were significantly reduced (Figure 3d). This glycolytic impairment was accompanied by an increased capacity to oxidise glutamine, likely providing an alternative substrate for the TCA^{47,48}, as suggested by the increase in basal and maximal respiration (Figure 3e and f).

Similarly, we reasoned that if cells became resistant to inhibition of a driver oncogene (and therefore less dependent on its activity), the impact of glucose depletion on their viability might also be altered. We therefore generated lapatinib-resistant SKMG3 cells through chronic drug exposure (Supplemental Figure 9). We found that GDI toxicity was significantly blunted in these cells compared to parental controls (Figure 3g). Furthermore, in lapatinib-resistant cells, EGFR phosphorylation was unaffected by glucose deprivation, further supporting the notion that super-activation of a driver oncogene by glycolytic stress is a major driver of GDI toxicity (Figure 3h).

GDI oncogene superactivation induces the activation of several early response genes and engages conflicting cell cycle and mitotic signals.

To understand the molecular basis of GDI oncogene super-activation and cell death, we carried out transcriptomic and phosphoproteomic profiling in SKMG3 cells following glucose depletion in the presence or absence of lapatinib. First, we analysed the effects of these perturbations on transcription via RNAseq. We focused on genes whose expression was altered by glucose depletion but not when lapatinib was also present. We identified 62 genes whose response to glucose deprivation was EGFR-dependent. Of these, 7 genes were downregulated by glucose depletion, while the remaining 55 were upregulated (Figure 4a and b). Gene set enrichment analysis comparing lapatinib-treated and control cells, under both

normal and glucose-deprived conditions, identified several critical pathways whose regulation depends on both EGFR activity and glucose levels. Notably, these include TNF α –NF- κ B signalling, the reactive oxygen species (ROS) pathway, and the PI3K/AKT/mTOR signalling cascade (Figure 4c). Importantly immediate early genes – including AP-1 and EGR transcription factors – were overrepresented among the 62 genes, consistent with the activation of growth factor signalling. AP-1 transcription factors are activated through phosphorylation by members of the JNK family of MAPKs, which are known to promote cell death in various contexts, including glucose depletion^{34,49}. We asked whether pharmacological blockade of JNK could prevent GDI- cell death. Indeed, treatment of SKMG3 cells with a selective JNK inhibitor (JNK-IN-8) significantly protected cells from GDI-cell death (Figure 4e), consistent with a previous report showing protection from hypoglycaemic cell death through expression of a dominant negative JNK variant in a breast cancer cell line⁵⁰. Furthermore, GDI-induced upregulation of AP-1 transcription factors (such as c-Jun and JunB), along with increased JNK activity (measured by c-Jun phosphorylation), was reduced upon EGFR inhibition using lapatinib (Figure 4f). In contrast, JNK-IN-8 treatment did not alter EGFR phosphorylation (Figure 4f). Collectively, these data suggest that in these cells, JNK and AP-1 activation play a significant role in GDI-cell death downstream of oncogene superactivation.

Next, we examined the effects of glucose depletion and EGFR inhibition on the phosphoproteome using LC-MS/MS. Focusing on phosphorylation sites known to regulate protein function, we found that 22 functionally annotated phosphopeptides were significantly altered by glucose depletion and restored to baseline by lapatinib treatment, providing additional evidence that superactive EGFR-driven signal transduction is qualitatively distinct (Figure 4d). One of the phosphosites identified through this analysis was Thr384 on STK3/MST2, a site that has been shown to inhibit MST2 activity when phosphorylated⁵¹. We confirmed the phosphorylation pattern at this site by immunoblot (Figure 5c). MST2 is a kinase in the Hippo pathway that inhibits YAP activation, suggesting that the latter may be one of the consequences of glucose deprivation. Interestingly, while YAP activation has been linked to the inhibition of apoptosis, it is also associated with context-dependent engagement of alternative forms of cell death, including ferroptosis⁵², which has previously been shown to be induced by glucose depletion⁵³.

To explore the mechanism of GDI cell death, we first looked for evidence of apoptosis using an annexin V (AV) assay. We found that a significant proportion of SKMG3 cells exposed to low glucose (1mM) conditions stained positive for both AV and propidium iodide (PI), consistent with cell death (Figure 5a). However, we did not detect singly AV-stained cells, suggesting that cell death did not occur through apoptosis. Cell cycle analysis demonstrated that glucose depletion in SKMG3 cells resulted in G2/M arrest, coinciding with increased Histone 3 Ser10 phosphorylation and decreased in Cdc2 Tyr15 phosphorylation, both markers of chromatin condensation (Figure 5b and c). However, EGF-induced centrosome separation has

been reported to require active MST2⁵⁴, which is likely inhibited under glucose-deprived condition, as suggested by increased Ser384 phosphorylation. In addition, our phosphoproteomic data showed that glucose depletion increased phosphorylation of CEP55 at Ser428, which is required for complete cytokinesis (Figure 4d). We reasoned that a potential consequence of these conflicting signals is that cells are pushed towards G2 but encounter mitotic defects, ultimately leading to mitotic catastrophe. Since mitotic catastrophe can result in necrotic cell death in the absence of p53 function⁵⁵, we explored necrosis as a potential mechanism of GDI cell death in SKMG3 cells, which carry an endogenous p53 mutation⁵⁶. Indeed, we found that treatment with the necrosis inhibitor IM-54 led to a significant and dose-dependent protection from GDI toxicity (Figure 5d). Collectively, our results suggest that GDI oncogene superactivation drives aberrant mitotic signalling programs that are incompatible with successful cell division, ultimately leading to cell death.

Discussion

Metabolic rewiring is a hallmark of cancer¹. The high energetic and biosynthetic demands of rapidly proliferating cancer cells can only be met through efficient and strategic utilisation of cellular and nutritional resources. Importantly, these changes are generally orchestrated by perturbation in signal transduction pathways under the influence of oncogenes and tumour suppressor genes⁵⁷. The Warburg effect, a preference to metabolise glucose to lactate even in the presence of oxygen, is a prominent feature of cancer cell metabolism, and has been linked to enhanced sensitivity to glucose deprivation⁵⁸. Although the basis of this glucose dependence remains a matter of debate, one prevailing view is that glycolytic intermediates provide substrates for multiple biosynthetic pathways, including hexosamine, fatty acid, and serine biosynthesis, as well as the pentose phosphate pathway, all of which are important for tumour maintenance^{5,6}. However, our data provide evidence that the main driver of GDI cell death across a variety of oncogene-driven cancer cell line models is not metabolic imbalance but rather oncogene overdose. Critically, although many commonly used cell lines are routinely cultured in super-physiological levels of glucose concentrations (as high as 25mM in standard DMEM formulations), the effects we report here were also clearly evident when cells were switched from 4 mM glucose (normoglycaemic conditions) to 3 mM glucose (hypoglycaemic conditions) (Supplemental Figure 1b).

We show that the GDI decrease in cellular pyruvate levels results in ROS accumulation and toxic superactivation of driver oncoproteins. Our observation that pyruvate supplementation is sufficient to completely protect cells from GDI toxicity suggests that other glycolytic intermediates may, in fact, be dispensable for cancer cell survival. We provide evidence for this across cancer cell lines driven by diverse classes of oncogenic drivers, including protein and lipid kinases, as well as a

transcription factor. Furthermore, we demonstrate that direct superactivation of a driver oncoprotein (e.g. BRAF or EGFR) is sufficient to induce cell death. To our knowledge, this represents the first direct demonstration of oncogene-overdose-driven cell death. Our study also suggests that GDI toxicity may result from engagement of clashing cell cycle regulatory signals that trigger a non-apoptotic form of cell death.

The concept of oncogene overdose has been investigated by several groups previously²⁰⁻²³. The Goldilocks principle provides a unifying framework for understanding how oncogenic signalling can impair cancer cell viability both when inhibited and when present in excess²⁰. Given that the magnitude of oncogenic signalling appears to be critical for cancer cell survival, it is reasonable to expect that cancer cells have evolved mechanisms to prevent signalling from exceeding a permissive range. Our data suggest that the Warburg effect may represent one such mechanism. Since pyruvate depletion drives ROS-dependent superactivation of driver oncogenes, perhaps the Warburg effect may serve not only to support the energetic demands of proliferation but also to maintain pyruvate levels that constrain oncogene activity to a viable range. The growth inhibitory effects of combining pan-GLUT and PRDX2 inhibitors provides an example of how mechanistic understanding of oncogene overdose toxicity can be harnessed to design rational therapeutic strategies to selectively kill cancer cells.

Finally, our transcriptomic and phosphoproteomic data in the EGFR-driven SKMG3 model suggest that oncogene superactivation does not simply produce an incremental increase in canonical signalling output but instead induces a qualitative change in the nature of the signal. This observation also has important translational implications, as it raises the possibility that oncogene overdose toxicity is mediated by a distinct superactivation-specific signalling signature. If such a signature can be defined, pharmacological mimicry of this effect could represent a novel therapeutic strategy.

Materials and methods

Cell lines and cell culture

Cell lines (Table 1) were maintained, unless otherwise stated, in high-glucose DMEM medium (4.5 g/l glucose, 110 mM pyruvate; Sigma) supplemented with 10% fetal bovine serum (Life technologies) and antibiotics (primocin and normocin; InvivoGen) at 37°C in a humidified 5% CO₂ incubator. Cells were detached from flasks using 0.25% Trypsin-EDTA (Sigma). Glucose-free and sodium pyruvate-free conditioned media were supplemented with 10% fetal bovine serum and supplemented with high-glucose medium as indicated.

Low glucose-adapted cell lines were generated by culturing the cells in low glucose media (4 mM and 1 mM glucose, no sodium pyruvate) and subcultured weekly for up to four months.

Drug resistant cell lines were generated by culturing the cells in media containing TKIs (Lapatinib or Dabrafenib/Trametinib), passaged weekly for up to six months until resistant clones emerged. These lines were maintained in TKIs and transitioned to drug-free media one week before experiments.

Table 1. Sources and characteristics of cell lines

Cell line	Source	Growth Medium	Cancer	Driver Oncogene	Mutations
SKMG3	Michael Burger (Goethe University)	DMEM	Glioblastoma	EGFR	EGFR p.A289D, PTEN null
A431	ATCC	DMEM	Epidermoid carcinoma	EGFR	EGFR WT
170-MG- BA	DSMZ	DMEM	Glioblastoma	EGFR	EGFR WT, PTEN null
DBTRG	DSMZ	RPMI	Glioblastoma	BRAF	BRAF p.V600E
A2058	ATCC	DMEM	Melanoma	BRAF	BRAF p.V600E, PTEN null,
DMS-273	ECACC	Waymouth's MB 752	Small cell lung cancer	c-MYC	Amp.
BT474	ATCC	RPMI	Breast	HER2	PIK3CA p.K111N
MCF7	ATCC	EMEM	Breast	PIK3CA	PIK3CA p.E545K
H358	ATCC	RPMI	Lung	KRAS	KRAS p.G12C
HEK293T/17	ATCC	DMEM	N/A	N/A	
UACC62	AddexBio	RPMI	Melanoma	BRAF	BRAF p.V600E

Lentiviral transduction

For lentiviral or retroviral production, HEK293T cells were seeded at a density of 2 x 10⁶ cells/10 cm dish. The following day, cells were transfected with plasmid DNA using

the calcium phosphate method. Briefly, lentivirus was generated using psPAX2 and pMD2.G packaging plasmids (Addgene) and the vector of interest in a 3:1:5 ratio with a total of 30 µg DNA. Virus was collected 36 hrs and 60 hrs post-transfection, filtered (0.45microns) and cell lines were infected on consecutive days for 3 hours with the viral soup. For retroviral production, cell lines were infected on consecutive days for 12 hrs. One week after transfection, stable expressers were generated by antibiotic selection.

BRAF and EGFR inducible overexpression cell line models

Stable cell lines for the inducible expression of EGFR and BRAF were generated using the ViraPower™HiPerform™T-REx™ Gateway Expression System (Thermo Fisher Scientific, Cat# A11141). This system allows genes of interest to be shuttled into the tetracycline-inducible pLenti6.3/TO/V5 vector via recombination-based (Gateway) cloning. The cDNAs of interest were cloned from pDONR223_EGFR_WT, pDONR223_BRAF_WT pDONR223_BRAF_V600E using Gateway LR Clonase II mix (Thermo Fisher Scientific, Cat# 11791020). Target cells were first transduced with a lentivirus carrying the regulatory vector (pLenti3.3/TR). This was followed by antibiotic selection (500µg/mL G418). Stable lines were then subjected to a second round of lentiviral infection with viruses carrying pLenti6.3/TO/V5 vectors with the appropriate cDNA. After infection, cells were selected with 10 µg/mL blasticidin to generate stable lines. Once stable lines were established, cells were subjected to single-cell cloning, and clones with maximum induction were selected. 293FT were used to generate all lentiviruses. Doxycycline (10 µg/mL) was added to induce expression of the cDNA of interest.

CRISPR/Cas9 cell line generation

To generate doxycycline inducible EGFR KO lines, cells were first transduced with a doxycycline-inducible Cas9 plasmid (Edit-R-Inducible Lentiviral Cas9 Nuclease vector, Horizon Discovery, CAS11229). A stable cell line was generated through Blasticidin selection and cells were subsequently transduced with lentiviral guide RNAs targeting EGFR. Sequences of EGFR gRNAs were GTGGAGCCTCTTACACCCAG, TCTTGCCGGAATGTCAGCCG and GTCTGCGTACTTCCAGACCA. Stable lines were subjected to puromycin selection at 1 ug/ml for one week. Cloning was performed using single-cell seeding with a MoFlo Astrios instrument (Beckman Coulter). 0.45 micron filtered 50% conditioned media was used to initiate single-cell cultures in a 96-well (Corning) plate. To induce EGFR knockout, 1µg/mL doxycycline was added for 7 days prior to assays.

Cell viability assay

Cell viability was measured by trypan blue exclusion using a Vi-Cell XR cell viability analyser (Beckman Coulter, USA) with optimised, cell type-specific imaging parameters to distinguish live and dead cells. Cells were seeded in triplicate at optimal densities in 6 cm dishes on day 0. On day 1, one triplicate was counted to establish

baseline cell number, and cells were treated with conditioned media. In glucose deprivation experiments, cells were seeded in high-glucose DMEM and washed twice with PBS prior to addition of conditioned media. Cell growth and viability were assessed between 72-120 hrs after treatment, at which time both attached and unattached cells were harvested and counted. Cell death was expressed as the percentage of trypan-blue positive cells over the total number of cells. Statistical analysis was performed using GraphPad Prism v10.

Cell lysis and western blotting

Cells were seeded at appropriate densities in 10 cm dishes, allowed to adhere for 24 hours and treated for the desired periods as described in the Results section. After removal of the medium, cells were washed twice with ice-cold PBS and lysed on ice with 1% Triton lysis buffer (Cell Signalling technologies, Product No. 9803) supplemented with protease and phosphatase inhibitors (Millipore). The ThermoFisher NE-PER Nuclear and Cytoplasmic Extraction kit was used to prepare nuclear and cytoplasmic lysates according to the manufacturer's instructions. Lysates were sonicated, cleared by centrifugation at 13,000 rpm at 4°C and total protein was normalised using the DC protein assay (Bio-Rad). Equal quantities of protein (10-50 µg, as required) were diluted in lysis buffer, and 6X Laemmli buffer was added. Samples were subsequently denatured at 95°C for 5 minutes. Proteins were separated by SDS-PAGE on precast Tris-glycine gels (4-20% or 10%) in TGS buffer at 0.05 A for 60 minutes. Proteins were transferred onto nitrocellulose membranes at 30V overnight with a wet transfer method with 1x transfer buffer (Thermo Scientific Pierce). Membranes were incubated in blocking buffer for 1 hour at room temperature and then incubated overnight at 4°C with primary antibodies (Table 2). Membranes were washed with TBS-T, incubated in HRP-conjugated secondary antibodies for 1 hour and washed with TBS-T. Proteins were visualised using enhanced chemiluminescence (GE Healthcare) and hyper film.

Table 2. Details of antibodies used for experiments

Antibody	Supplier	Catalogue number	Dilution	Source
EGFR (D38B1)	Cell Signalling Technology	4267	1:10000	Rabbit
p-EGFR (Y1068)	Cell Signalling Technology	3777	1:5000	Rabbit
p-EGFR (Y1045)	Cell Signalling Technology	2237	1:3000	Rabbit
Acetyl-CoA Carboxylase	Cell Signalling Technology	3662	1:1000	Rabbit
p-Acetyl-CoA Carboxylase (S79)	Cell Signalling Technology	3661	1:1000	Rabbit
P44/42 MAPK (ERK1/2) 137F5	Cell Signalling Technology	4695	1:1000	Rabbit
p-P44/42 MAPK (ERK1/2)	Cell Signalling Technology	4696	1:1000	Rabbit
p53	Cell Signalling Technology	30313	1:1000	Rabbit
H3	Cell Signalling Technology	4499	1:1000	Rabbit
p-H3 (S10)	Cell Signalling Technology	3377	1:1000	Rabbit
RB1	Cell Signalling Technology	9313	1:1000	Rabbit
p-RB1 (S807/811)	Cell Signalling Technology	8516	1:1000	Rabbit

CDC6	Cell Signalling Technology	3387	1:1000	Rabbit
p-CDC6 (S54)	Abcam	AB75809	1:1000	Rabbit
c-Jun (60A8)	Cell Signalling Technology	9165	1:1000	Rabbit
p-c-JUN (S63) II	Cell Signalling Technology	9261	1:1000	Rabbit
JunB	Cell Signalling Technology	3753	1:1000	Rabbit
MST2	Cell Signalling Technology	3952	1:1000	Rabbit
p-MST2 (T384)	Invitrogen	PA5-110159	1:1000	Rabbit
CEP55	Cell Signalling Technology	81693	1:1000	Rabbit
p-CDC2 (Y15)	Cell Signalling Technology	4539	1:1000	Rabbit
Cyclin B1	Cell Signalling Technology	4138	1:1000	Rabbit
Cyclin E1	Cell Signalling Technology	20808	1:1000	Rabbit
Cyclin A2	Cell Signalling Technology	67955	1:1000	Rabbit
CDK2	Cell Signalling Technology	18048	1:1000	Rabbit
CDT1	Cell Signalling Technology	8064	1:1000	Rabbit
Geminin	Cell Signalling Technology	52508	1:1000	Rabbit
Thymidine Kinase	Cell Signalling Technology	28755	1:1000	Rabbit
Vinculin (E1E9V)	Cell Signalling Technology	13901	1:4000	Rabbit
GAPDH	Cell Signalling Technology	5174	1:4000	Rabbit
Beta-actin	Sigma Aldrich	A5441	1:10000	Mouse
Anti-rabbit HRP	Jackson ImmunoResearch	1035144	1:5000	-
Anti-mouse HRP	Jackson ImmunoResearch	5035003	1:5000	-
Anti-Streptavidin HRP	Cell Signalling Technology	3999	1:2000	-

564

565 LC-MS/MS Phosphoproteomics

566 Treatments were carried out for 24 hours in 10% DMEM in the following conditions:
567 10 mM glucose vehicle, 1 mM glucose vehicle, 10 mM glucose with 1 μ M lapatinib, 1
568 mM glucose with 1 μ M lapatinib. For each condition, five independent biological
569 replicates were performed. Plates were washed three times with 5 mL ice-cold PBS
570 containing protease and phosphatase inhibitors (2% (v/v) NaF and 10% (v/v) Na_3VO_4).
571 Cells were harvested on ice, directly from tissue culture plates with 300 μ L lysis buffer
572 (250 mM $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$, 1 M β -glycerol phosphate, 500 mM NaF, 100 mM Na_3VO_4 , 8 M
573 Urea in 20 mM HEPES pH 8.0). Cells were sonicated on ice three times at 20%
574 intensity for 10 seconds, with a 10 second rest, using a Diagenode Bioruptor® Plus.
575 Cell suspensions were centrifuged at 20,000xg for 10 minutes at 5°C. Protein was
576 quantified by DC assay. Supernatant was recovered in 1.5 mL Eppendorf Protein Lo-
577 bind tubes. Lysates were snap-frozen on dry ice before being sent to the
578 phosphoproteomics facility for further processing. Processing and analysis were
579 carried out by Dr Cutillas's team at Queen Mary University of London.

580 Phosphopeptides were resuspended in 12 μ L of reconstitution buffer (20 fmol/ μ L
581 enolase in 3% ACN, 0.1% TFA) and 5.0 μ L were loaded onto an LC-MS/MS system
582 consisting of a Dionex UltiMate 3000 RSLC directly coupled to an Orbitrap Q-Exactive
583 Plus mass spectrometer (Thermo Fisher Scientific) through an EasySpray system. LC-

MS/MS was performed as previously described⁵⁹. Mascot Daemon 2.5.0 was used to automate peptide identification from MS data as described previously⁵⁹. Label-free peptide quantification was performed using Pescal, an in-house developed software, that constructs XICs for all identified peptides across all samples (± 7 ppm mass and ± 2 min retention time windows) and calculates the peak areas of the generated XICs⁶⁰. Normalized peak areas of phosphopeptides were used to calculate fold change and statistical significance between conditions. Kinase activity was estimated from phosphoproteomics data using a kinase substrate enrichment analysis (KSEA) approach^{59,61}. Briefly, phosphorylated peptides were grouped into substrate groups associated to particular kinases as annotated in the PhosphoSite database. Z-scores for each kinase were calculated as $(mS - mP) * m^{1/2} / d$, where mS is the log2 mean abundance of each kinase group, mP is the log2 mean abundance of the entire data set, m is the size of each substrate group, and d is the SD of the mean abundances of the entire dataset. Excel software was used to transform Z-scores into P-values. For statistical analysis only P-values < 0.05 were considered, along with a fold change threshold of 0.75.

LC-MS measurement of metabolites

Glyceraldehyde-3P and the metabolites AMP, ADP, ATP, NAD, NADH, NADP, NADPH and succinic acid were measured using LC-MS as follows.

Each cell pellet was resuspended in 250 μ L of 75% methanol in an Eppendorf tube. After cell lysis on an MM 400 mill mixer for 1 min, repeated three times at a shaking frequency of 30 Hz with the aid of two metal balls, samples were centrifuged at 21,000 g for 10 min at 5°C. The supernatants were collected and used for subsequent LC-MS analyses. The protein pellets were used to measure protein content using a standardized Bradford procedure.

For assaying the TCA cycle, a standard stock solution of all the targeted carboxylic acids was prepared in 75% methanol at concentration of 200-1000 μ M. This solution was serially diluted 1 to 4 (v/v) to generate working standard solutions. 20 μ L of each standard solution or sample supernatant was mixed with 20 μ L of an internal standard solution containing 10 deuterium-labeled carboxylic acids, 20 μ L of 200-mM 3-NPH solution and 20 μ L of 150- mM EDC-6% pyridine solution. The mixture was allowed to react at 30°C for 30 min. After the reaction, 240 μ L of water was added, and 10 μ L of the resulting solution was injected onto a C18 UPLC column (2.1 x 100 mm, 1.8 μ m) for quantitation of carboxylic acids by UPLC-MRM/MS with negative-ion detection on an Agilent 1290 UHPLC coupled to a Sciex 4000 QTRAP MS instrument.

For assaying glucose and selected sugar phosphates, 20 μ L of each sample supernatant or serially diluted standard solutions of glucose, glucose-6P and mannose-6P, etc., was mixed sequentially with 20 μ L of a ¹³C6-glucose/¹³C6-

glucose-6P internal standard solution, 40 μ L of 25 mM AEC solution, 20 μ L of 50 mM NaCBH₃ solution and 8 μ L of LC/MS grade acetic acid, in turn. The mixtures were allowed to react at 60°C for 70 min. After the reaction, 120 μ L of water and 120 μ L of chloroform were added. After vortex-mixing for 15 s and centrifugation for 5 min. 50 μ L of each supernatant was mixed with 450 μ L of water. 10 μ L was injected onto a PFP LC column (2.1 x 150 mm, 1.7 μ m) for UPLC-MRM/MS analysis on an Agilent 1290 UHPLC coupled to an Agilent 6495B QQQ instrument with positive-ion detection for quantitation of glucose and glucose-6P.

For assaying other metabolites, serially diluted standard solutions of all targeted metabolites were prepared at concentrations ranging from 0.0002 to 10 μ M in an internal standard solution containing isotope-labelled NAD, NADH, AMP, GMP, UMP, UTP, ATP, GTP, glucose-6P, fructose-1,6-bisP and glycerol-3P. 10 μ L of each sample supernatant was mixed with 90 μ L of the same internal standard solution. 10 μ L of each standard or sample solution was injected onto a C18 UPLC column (2.1 x 100 mm, 2.5 μ m) for UPLC-MRM/MS runs with negative-ion detection on a Waters Acquity UPLC system coupled to a Sciex 6500 Plus MS instrument, using a tributylamine acetate buffer - acetonitrile mobile phase for gradient elution (5%-50% over 22 min) at a flow rate of 0.25 mL/min and 60°C.

For data analysis, concentrations of detected analytes were calculated using internal standard calibration by interpolating linear-regression curves constructed for individual compounds, based on analyte-to-internal standard peak area ratios measured in each assay

Intracellular Pyruvate measurements

Intracellular pyruvate levels were measured from whole cell lysates using the fluorometric Pyruvate Assay Kit (Sigma Aldrich, Cat No. MAK332) according to the manufacturer's specifications.

Seahorse Real-Time Cell Metabolic Analysis

Glycolytic and mitochondrial activity measurement of parental and low-glucose-adapted (4mM and 1mM) SKMG3 were conducted on a Seahorse XFe96 Extracellular Flux Analyzer (Agilent) following recommended protocols. Briefly, SKMG3 cells were seeded the day prior to measurement at a density of 2×10^4 cells/well in a Seahorse XFe96 cell culture microplate (101085-004, Seahorse) in 100 μ L DMEM containing the corresponding glucose concentration and supplemented with 10 % FBS. Cells were then incubated overnight at 37°C in a 5% CO₂ incubator.

On the day of the assay, each test was conducted as follows:

For the Glyco Stress test, cells were washed twice with 200 μ L of DMEM supplemented with 2 mM glutamine (no glucose, no sodium pyruvate) and then

transferred to a non-CO₂ incubator for 1 hour at 37°C. Extracellular acidification rate (ECAR) was measured under basal conditions and following sequential injections of glucose (10mM), oligomycin (1µM) and 2-deoxyglucose (100mM) (final concentrations in the well) to measure glycolysis rate, glycolytic capacity and non-glycolytic acidification, respectively. Data were normalised using CyQUANT Cell Proliferation Assays (C7026, ThermoFisher).

For the Mito Stress test, cells were washed twice with 200 µL of DMEM supplemented with 2mM glutamine, 10mM glucose and 1mM sodium pyruvate and then transferred to a non-CO₂ incubator for 1 hour at 37°C. Oxygen consumption rate (OCR) was measured under basal conditions and following sequential injections of oligomycin (1µM), FCCP (0.5µM) and rotenone/antimycin A (0.5µM) (final concentrations in the well) to measure ATP-linked respiration, maximal respiration and non-mitochondrial oxygen consumption, respectively. Data were normalised using Cyquant Cell Proliferation Assays (C7026, ThermoFisher).

For the Mito Fuel Flex test, cells were washed twice with 200 µL of DMEM supplemented with 2 mM glutamine, 10 mM glucose and 1 mM sodium pyruvate and then transferred to a non-CO₂ incubator for 1 hour at 37°C. Fuel oxidation capacity was determined by measuring OCR at baseline, followed by combined inhibition of alternative pathways and subsequent inhibition of the target pathway. Glutamine oxidation capacity was assessed by blocking glucose and fatty acid utilization with UK5099 (2 µM) and etomoxir (4 µM), followed by inhibition of glutamine oxidation with BPTES (3 µM). Glutamine capacity was calculated using the following equation:

Capacity (%) = {1 - [(Baseline OCR - Alternative pathway inhibitor OCR)/ (Baseline OCR - All combined inhibitors)]} x 100

MTS assay

MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Cell titre-96, G3580, Promega) was performed according to the manufacturer's instructions. MTS assay was used to measure mitochondrial metabolic activity using the soluble form of MTT, a tetrazolium compound that is reduced to formazan in mitochondria. This reduction depends on NADPH, making MTT a marker of mitochondrial reducing power.

Propidium iodide staining and flow cytometry-based cell cycle analysis

For propidium iodide cell cycle analyses, after the indicated treatments, cells were resuspended and fixed in ice-cold 75% ethanol in PBS overnight at 4°C. BrdU was added at 50 µM for 30 min before harvesting. Cells were incubated with Alexa Fluor® 488 anti-BrdU and stained with 50 mg/mL propidium iodide in PBS for 20 min before analysis using a BeckmanCoulter MoFlo Astrios flow cytometer.

Flow cytometry-based ROS measurements

Cells were incubated with either 5 μ M CM-H₂DCFDA or 2.5 μ M mitoSOX (Invitrogen) for 45 min or 30 min respectively before analysis using a Beckman Coulter Cytoflex or BeckmanCoulter MoFlo Astrios flow cytometer. Cells were gated using forward and side scatter to remove debris and dead cells, and 10,000 live cell events were recorded. To quantify changes in DCF-DA or mitoSOX signal, the average of median fluorescence intensity-area after gating was used.

RNA sequencing

Cells were incubated in the appropriate conditions for 24 hours and RNA was extracted. In brief, cells were washed once with PBS and collected manually in RLT plus buffer. RNA was extracted using the QIAGEN RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. RNA was eluted in 40 μ l RNase-free water. RNA quality was assessed using the Agilent 2100 Bioanalyzer, and next-generation sequencing was performed on the BGISEQ-500 platform (BGI technologies, Hong Kong). Sequencing was carried out on Illumina NovaSeq 6000 and generated 30 million paired-end reads per sample using single-cell RNAseq.

RNAseq analysis

Unaligned reads went through QA/QC. Data was aligned with STAR 2.5.3a and post-alignment QA/QC was performed to assess alignment quality. A target human reference transcriptome was downloaded from Ensembl (http://ftp.ensembl.org/pub/release-92/fasta/homo_sapiens/cdna/Homo_sapiens.GRCh38.cdna.all.fa.gz) and a transcriptome index was built with Salmon⁶². Transcripts were quantified using Salmon and summarized to the gene level using the R package tximeta⁶³. Differential expression was using DESeq2⁶⁴, with default multiple-hypothesis testing correction applied. An adjusted p-value threshold of <0.05 was used to identify genes of interest and an additional cut-off of 1.5-fold change was applied to focus on biologically relevant expression differences. GSEA was performed on the Partek Flow platform.

Sulfenic acid labelling, click chemistry and detection of sulfenylated EGFR using immunoblot

SKMG3 cells culturing in different treatment conditions (10 mM or 1 mM glucose or 500 μ M hydrogen peroxide for 24h) were labelled with DYn-2 (MedChemExpress, Cat# HY-116424) and then subjected for lysate preparation, EGFR immunoprecipitation, click chemistry reaction, and sulfenylated EGFR detection as previously described with modifications^{44,45}. SKMG3 cells were lifted with 0.25% trypsin-EDTA, harvested by centrifugation at 1,500 RPM for 5 min, washed with PBS and resuspended in serum-free DMEM containing DMSO or 5 mM DYn-2 at a density of 5×10^6 cells/ml. Cell suspensions were incubated at 37°C in a 5% CO₂ humidified

atmosphere for 2 h with gentle agitation every 10 mins. After incubation, cells were collected by centrifugation at 3,000 RPM for 5 min and washed with ice-cold PBS twice. Cell pellets were stored at -80°C until lysis. Cell pellets were lysed on ice in HEPES lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Igepal, pH 7.5) freshly supplemented with 200 U/ml catalase (Sigma) to prevent oxidative reactions during lysis. Cell lysate (2 mg) was incubated with 10 µl of Dynabeads M280 Streptavidin (ThermoFisher) for 1 h at 4°C. Precleared lysates were incubated with 5 µg of EGFR antibody overnight at 4°C, followed by incubation with 50 µl of protein A agarose beads for 4 h at 4°C. The reaction mixtures were then washed five times with lysis buffer with each wash for 2 min incubation on ice followed by centrifuging at 6,000 RPM for 2 min at 4°C. The washed beads with immunoprecipitated proteins were then subjected to click chemistry by incubating the beads with 45 µl of CuAAC mixture containing 100 µM azido-biotin, 100 µM Tris((1-benzyl-4-triazolyl)methyl)amine (TBTA) ligand, 1 mM CuSO₄ and 1mM sodium ascorbate for 1 h at 25 °C with gentle rocking. The reactions were quenched by boiling with 15 µl Laemmli sample buffer for 10 min. The collected proteins were resolved by SDS-PAGE and analysed by immunoblotting as described above.

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FIGURE LEGENDS

Figure 1. Glucose deprivation causes cancer cell death through superactivation of driver oncogenes.

(A) A schematic diagram of the Goldilocks principle that outlines the relationship between the level of oncogene activity level and cancer cell viability.

(B) EGFR-mutant SKMG3 cells were cultured under 10mM or 1mM glucose conditions and in the presence or absence of 1 μ M of the EGFR inhibitors lapatinib or afatinib. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer. Proliferation data is presented as the fold change in viable cell number (trypan-blue-negative cells) between day 1 and day 6 of drug treatment (left). Cell death is plotted as the percent of dead cells (% trypan-blue-positive cells) on day 6 of treatment. BRAF-mutant DBTRG cells were cultured under 10mM or 1mM glucose conditions and in the presence or absence of 10nM of the BRAF inhibitor dabrafenib (C), and also in the presence or absence of 1nM of the MEK inhibitor trametinib or 1 μ M of the BRAF inhibitor vemurafenib (D). Effects of treatment on proliferation (left) and cell death (right) were assessed as in (B). DBTRG and SKMG3 cells were also grown under 10mM or 1mM glucose conditions in the presence or absence of either 10 μ M vemurafenib or 1 μ M lapatinib (E), or 1 μ M lapatinib or 5nM dabrafenib (F), respectively. Effects of treatment on proliferation (left) and cell death (right) were assessed as in (B). (G) PIK3CA-mutant MCF7 cells were grown under 10mM or 50 μ M glucose conditions and in the presence or absence of the indicated doses of the PI3K α -selective inhibitor alpelisib. Effects of treatment on proliferation (left) and cell death (right) were assessed as in (B). (H) SKMG3 cells were grown under the indicated conditions for 24hrs and lysed. Whole cell lysates subjected to immunoblot analysis with the indicated antibodies. (I) SKMG3 cells were grown under 10mM or 1mM glucose for the indicated times. Cells were lysed and subjected to immunoblot analysis with the indicated antibodies. (J) DBTRG cells were grown under 10mM or 1mM glucose conditions and in the presence or absence of either 1nM trametinib (Tram) or 1 μ M vemurafenib (Vem) for 24hrs. At the end of treatment, cells were lysed and subjected to immunoblot analysis with the indicated antibodies. (K) MCF7 cells were grown under 10mM or 50 μ M glucose conditions and in the presence or absence of the indicated doses of alpelisib for 24hrs. Cells were then lysed and subjected to immunoblot analysis with the indicated antibodies. (L) DBTRG cells stably expressing tetracycline-regulated wild type BRAF (left panel, WT) or BRAF-V600E (right panel, V600E) cDNAs (DBTRG-iBRAF) were treated with 1 μ g/mL doxycycline for 72 hours and lysed. Cell lysates were then subjected to immunoblot analysis with the indicated antibodies. (M) Cells from (L) were cultured for 72hrs in the presence or absence of 1 μ g/mL doxycycline to induce transgene expression. Effects of treatment on proliferation (left) and cell death (right) were assessed as in (B). (N) DBTRG cells stably overexpressing a tetracycline-regulated BRAF-V600E cDNA (DBTRG-iBRAF-V600E) were cultured in the presence or absence of 1 μ g/mL doxycycline and in the presence or absence of 1nM dabrafenib for 6 days. Effects of treatment on proliferation (left) and cell death (right) were assessed as in (B). (O) EGFR-amplified 170-MG-BA cells were cultured in the

presence of increasing concentrations of EGF (as indicated) for 4 days. Effects of treatment on proliferation (left) and cell death (right) were assessed as in (B). A parallel set of plates was treated lysed 24 hours after EGF treatment and lysates subjected to immunoblot analysis with the indicated antibodies (inset).

Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Figure 2. Glucose-deprivation-induced oncogene superactivation and cell death can be reversed by pyruvate supplementation.

(A) Pyruvate levels in SKMG3 cells cultured under the indicated conditions for 24hrs as measured with a fluorimetric assay.

SKMG3 (B) and DBTRG (C) cells were grown under 10mM or 1mM glucose conditions in the presence or absence of the indicated metabolites at the doses shown. Effects of treatment on proliferation (left panels) and cell death (right panels) were assessed using a ViCell Cell Viability Analyzer as in Figure 1. (D) SKMG3 cells were grown under 10mM or 1mM glucose conditions and in the presence or absence of 2mM or 20mM lactate, 25mM of the lactate dehydrogenase inhibitor methylmalonate (MMA), or a combination of 20mM lactate and 25mM MMA. Effects of treatment on proliferation (left panels) and cell death (right panels) were assessed using a ViCell Cell Viability Analyzer as in Figure 1. (E) Glyceraldehyde-3-Phosphate levels in SKMG3 cells grown under the indicated conditions as measured by LC-MS/MS. DBTRG (F) and SKMG3 (G) cells were grown under the indicated conditions and lysed. Lysates were subjected to immunoblot analysis with the indicated antibodies.

(H) SKMG3 cells stably expressing a tetracycline-regulated wild type EGFR cDNA (SKMG3-i-WT-EGFR) were cultured in the presence or absence of 1 μ g/mL doxycycline + 50ng/mL EGF (Dox) and in the presence or absence of 2mM methyl-pyruvate (pyruvate). The effects of treatment on proliferation (left panels) and cell death (right panels) assessed using a ViCell Cell Viability Analyzer are shown. Overexpression of EGFR in SKMG3-i-WT-EGFR cells following doxycycline treatment is shown in the inset. Cells were treated with 1 μ g/mL doxycycline for 72 hours and lysed. Lysates were subjected to immunoblot with antibodies for EGFR and β -Actin as a loading control, as shown. (I) DBTRG-i-BRAF-V600E cells were cultured in the presence or absence of 1 μ g/mL doxycycline (Dox) and in the presence or absence of 2mM methyl-pyruvate (pyruvate). The effects of treatment on proliferation (left panels) and cell death (right panels) assessed using a ViCell Cell Viability Analyzer are shown. (J) SKMG3 cells were treated with the indicated doses for H₂O₂ for 24hrs and lysed. Lysates were subjected to immunoblot analysis with the indicated antibodies.

(K) SKMG3 (top) and DBTRG (bottom) cells were probed with DCF-DA and analysed by flow cytometry after 24hrs of growth in 10 mM or 1 mM Glucose with and without

supplementation of 2 mM methyl-pyruvate or 1 μ M lapatinib. Mean ROS levels in the fraction of cells with the highest DCF-DA signal were quantified (right panels).

(L) SKMG3 cells were cultured under the indicated conditions for 24hrs followed by Dyn-2 labelling and biotinylation of labelled peptides using azido-biotin click chemistry. EGFR was then immunoprecipitated and blotted. Sulfenylated EGFR was detected using straptavidin-HRP (top). An aliquot of the whole cell lysates (prior to immunoprecipitation) was also analysed by immunoblot with the indicated antibodies (bottom).

Statistical analyses of viability assays were done in GraphPad Prism v10. n.s., not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.

Figure 3. Sensitivity to glucose depletion and sensitivity to inhibition of oncogenic drivers are functionally coupled.

SKMG3 (A) and DBTRG (B) cells were adapted to growth in low glucose conditions through chronic passaging in 1mM or 4mM glucose for 4 months when stable growth was reached. Proliferation (left panels) and viability (right panels) of low-glucose-adapted cells was assessed using the ViCell Cell Viability Analyzer as described before. (C) Parental and low-glucose-adapted SKMG3 (top) and DBTRG (bottom) cells were seeded in fresh media containing 10mM, 4mM or 1mM glucose for 24 hrs and lysed. Lysates were analysed by immunoblot with the indicated antibodies. (D) Glycolysis Stress Test carried out in a Seahorse XFe96 Extracellular Flux Analyzer. The plot on the left panel displays extracellular acidification rates (ECAR) for parental (control) and low-glucose-adapted (4mM and 1mM) SKMG3 cells with dotted lines indicating when reagents (GLUC: glucose); OLIGO: oligomycin; and 2DG: 2-deoxyglucose) were injected. Glycolysis (middle panel) was calculated by subtracting the final rate measurement before 2-DG injection from then maximum rate measurement before oligomycin injection. Glycolytic capacity (right panel) was calculated by subtracting the final rate measurement before glucose injection from the maximum rate measurement after oligomycin injection. (E) Mito Stress Test carried out in a Seahorse XFe96 Extracellular Flux Analyzer. Shown is the oxygen consumption rate (OCR) in parental and low-glucose-adapted SKMG3 cells (left panel) with dotted lines indicating when reagents (OLIGO: oligomycin; FCCP: Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone; AA+ROT: antimycin A + rotenone) were injected. Basal respiration (middle panel) was calculated by subtracting the final OCR value after AA+ROT from the final OCR value before oligomycin injection). Maximal respiration (right panel) was calculated by subtracting the final OCR value after oligomycin injection from the final OCR value after FCCP injection. (F) XF Mito Fuel Flex Test carried out in a Seahorse XFe96 Extracellular Flux Analyzer. Shown is the mitochondrial capacity to oxidise glutamine (Glutamine capacity) in parental and low-glucose-adapted SKMG3 cells. Capacity (%) = [(baseline OCR – Etomoxir and UK5099 treatment OCR)/(Baseline OCR – All 3 inhibitors OCR)] $\times$ 100. (G) The growth (left panels) and viability (right panels) of parental and lapatinib-resistant (1.75 μ M-Lapatinib-Res.) SKMG3 cells grown in either 10mM or 1mM glucose were assessed with a Vicell Cell Viability analyzer as before. (H) Western blots of parental and lapatinib-resistant SKMG3 cells grown with 1mM or 10mM glucose in the presence or absence of 1 μ M lapatinib probed with the indicated antibodies.

Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Figure 4. GDI oncogene hyperstimulation generates a distinct oncogene-dependent molecular signature.

Venn diagrams of RNA sequencing data from SKMG3 cells showing genes downregulated (A, left) or upregulated (B, left) when cells were grown in 10mM glucose (veh_10), 1mM glucose with 1 μ M lapatinib (lap_1), and 10mM glucose with 1 μ M lapatinib (lap_10) each relative to 1mM glucose (veh_01) growth conditions, with accompanying dot plots of normalised counts for genes whose expression is either downregulated (A, right) or upregulated (B, right) by switching from 10mM to 1mM glucose conditions but restored to basal levels by lapatinib treatment. (C) GSEA analysis plot of Normalised Enrichment Scores (NES) for gene sets that were significantly enriched (NES < -1.5) by lapatinib treatment only in 1mM glucose but not 10mM glucose conditions. (D) GSEA analysis plot of False Discovery Rates (FDR) for gene sets that were significantly enriched (FDR < 0.25) by lapatinib treatment, but only in 1mM glucose and not 10mM glucose conditions. (E) Heatmap representation of all significant phosphosites whose abundance is changed by glucose depletion (1mM vs 10mM) and normalised by lapatinib (1 μ M) treatment in SKMG3 cells. Red indicates upregulated phosphosites and blue indicates downregulated phosphosites. (F) Effects of JNK inhibitor treatment (3 μ M JNK-IN-8) on the proliferation (left panel) and viability (right panel) of SKMG3 cells grown in 10mM or 1mM glucose. (G) Western blot analysis of SKMG3 cells after 24 h in 10 mM or 1 mM glucose in the presence or absence of 1 μ M Lapatinib or 3 μ M JNK inhibitor (JNK-IN-8) with the indicated antibodies. Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Figure 5. GDI oncogene hyperactivation elicits conflicting cell-cycle and mitotic signals.

(A) Flow cytometry analysis of Annexin V and propidium iodide (PI) staining of SKMG3 cells in response to 10 mM and 1 mM Glucose with or without EGFR inhibitor (1 μ M Lapatinib) treatment or supplementation with methyl-pyruvate (2mM) for 24 hours. (B) Flow cytometric cell cycle analysis using propidium iodide staining in SKMG3 cells grown in either 10mM (left) or 1mM (right) glucose. Quantification of the effects of 2mM methyl-pyruvate and 1 μ M lapatinib on the cell cycle distributions of SKMG3 cells grown in 1mM or 10mM glucose (bottom). (C) Immunoblot analysis of SKMG3 cells cultured in 10mM or 1mM glucose in the presence or absence of 1 μ M lapatinib or 2mM methyl-pyruvate with the indicated antibodies. (D) Effects of the indicated doses of the necrosis inhibitor IM-54 on the proliferation (left) and viability (right) of SKMG3 cells grown in 10 mM versus 1 mM Glucose as measured with a ViCell Cell Viability Analyzer. Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Figure 1

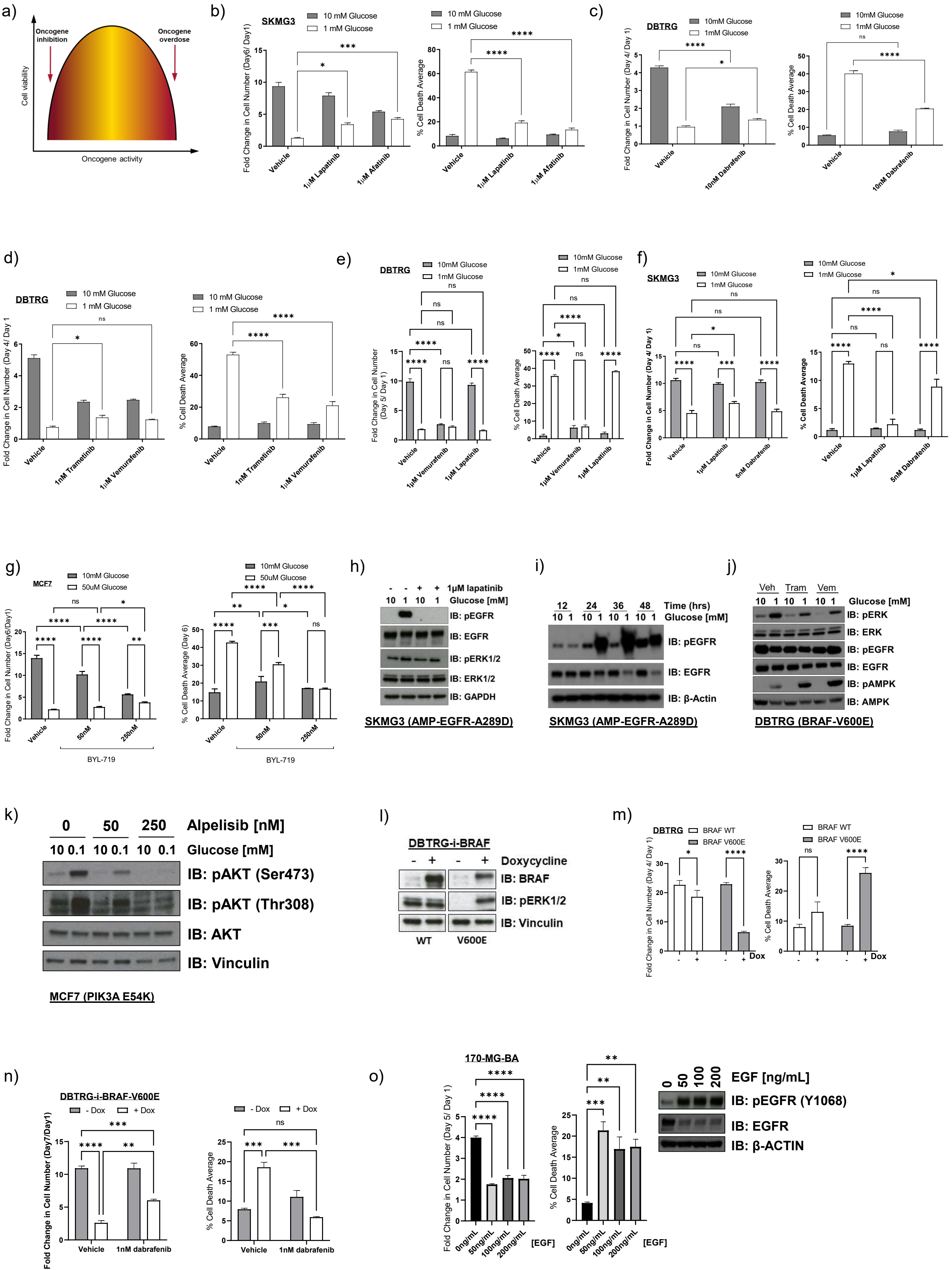


Figure 2

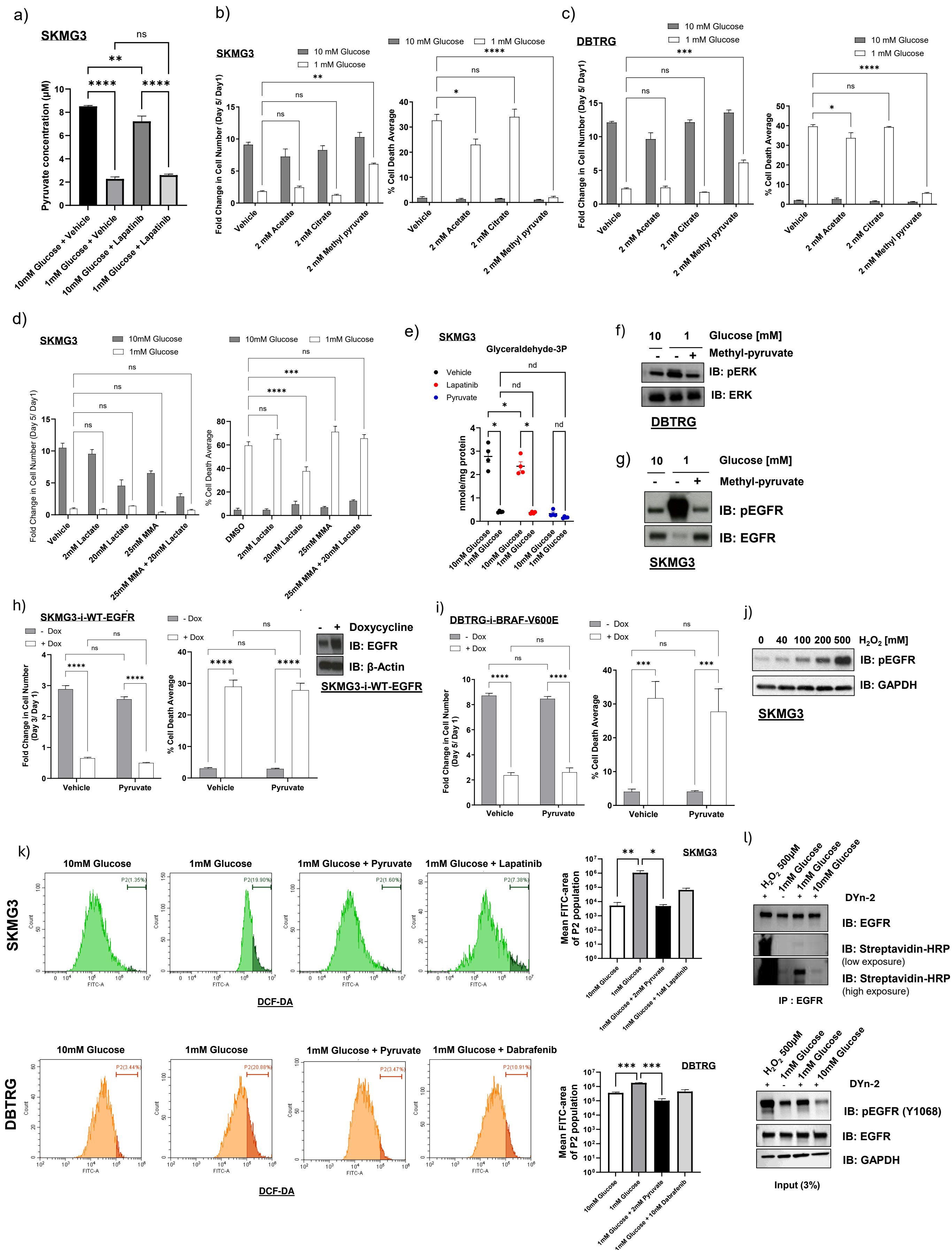


Figure 3

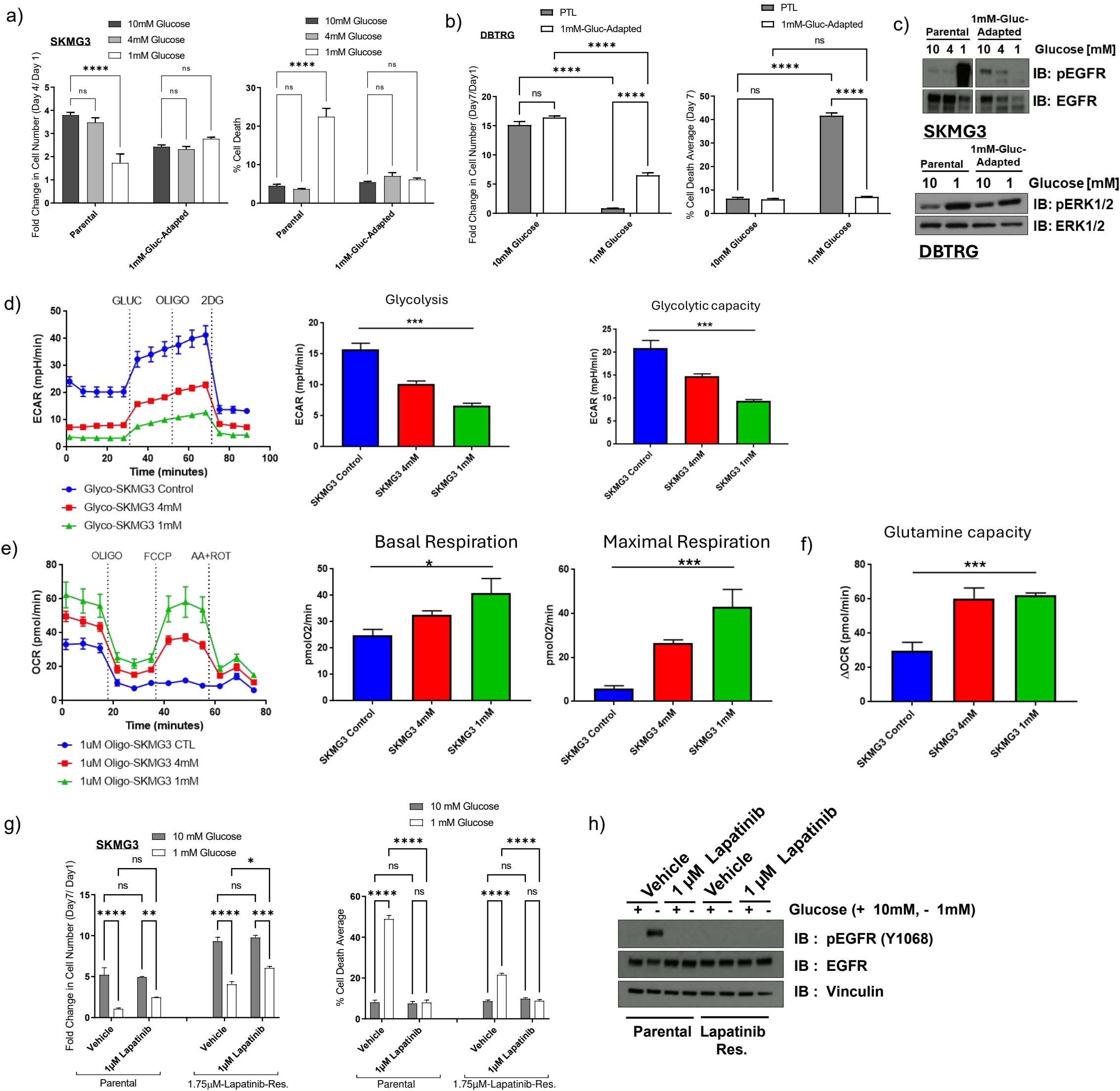
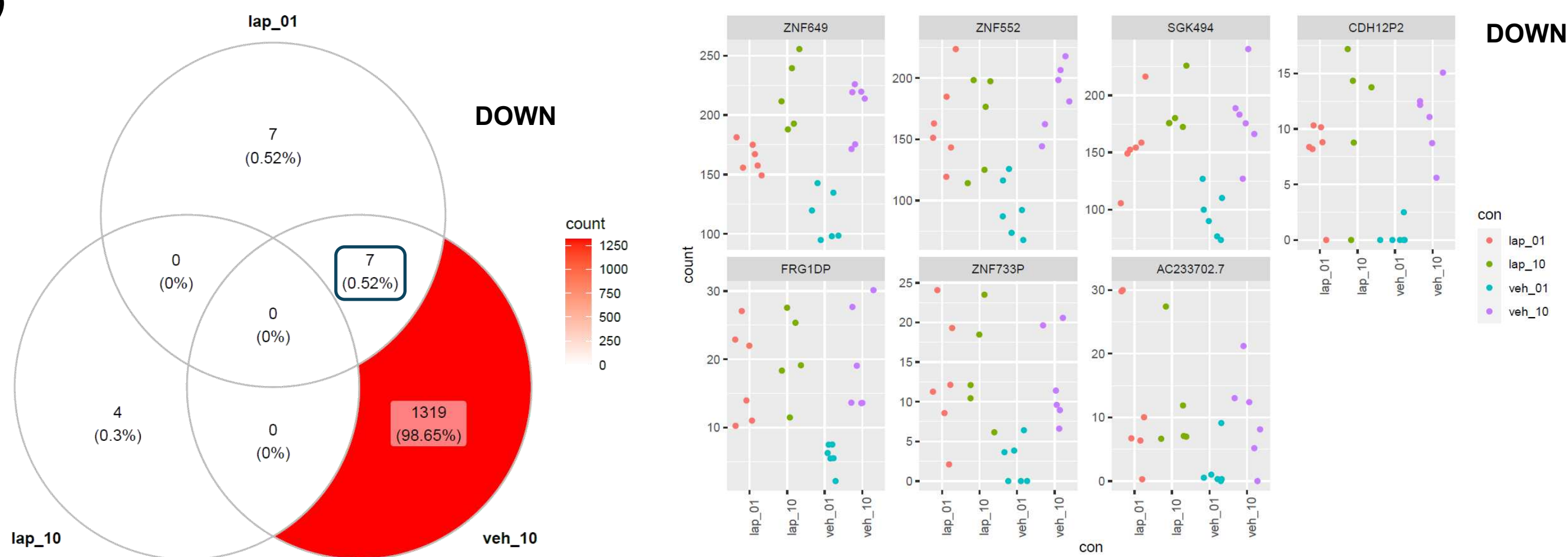
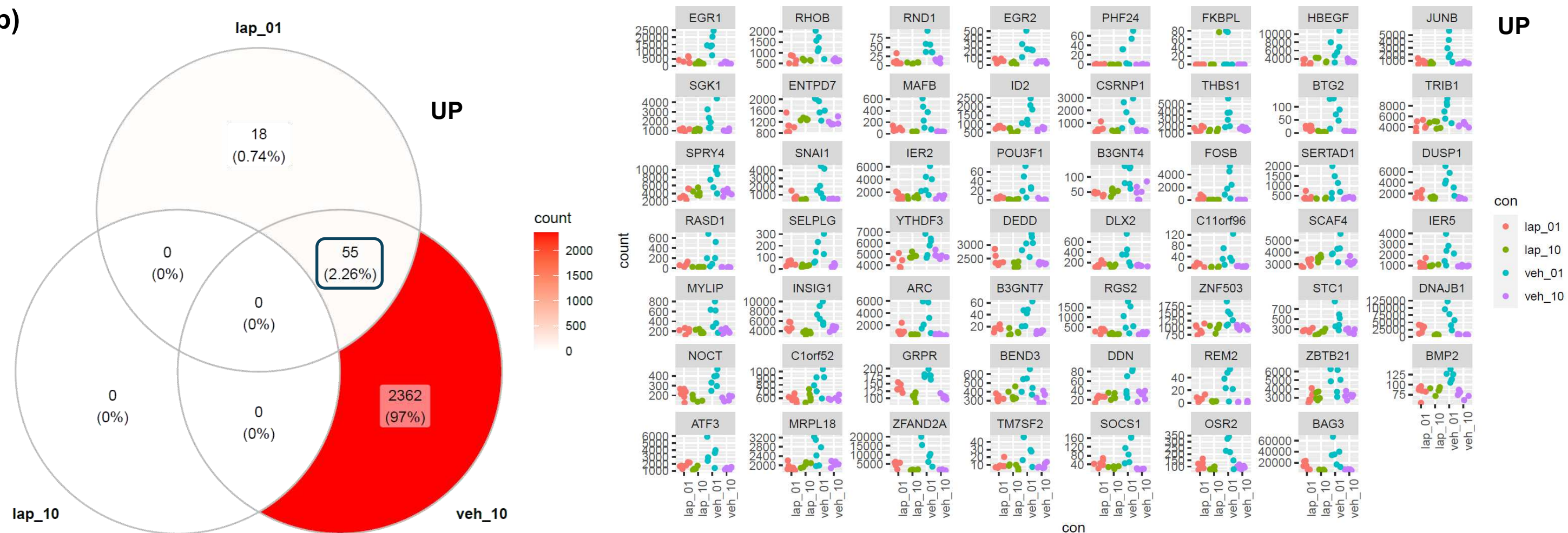


Figure 4

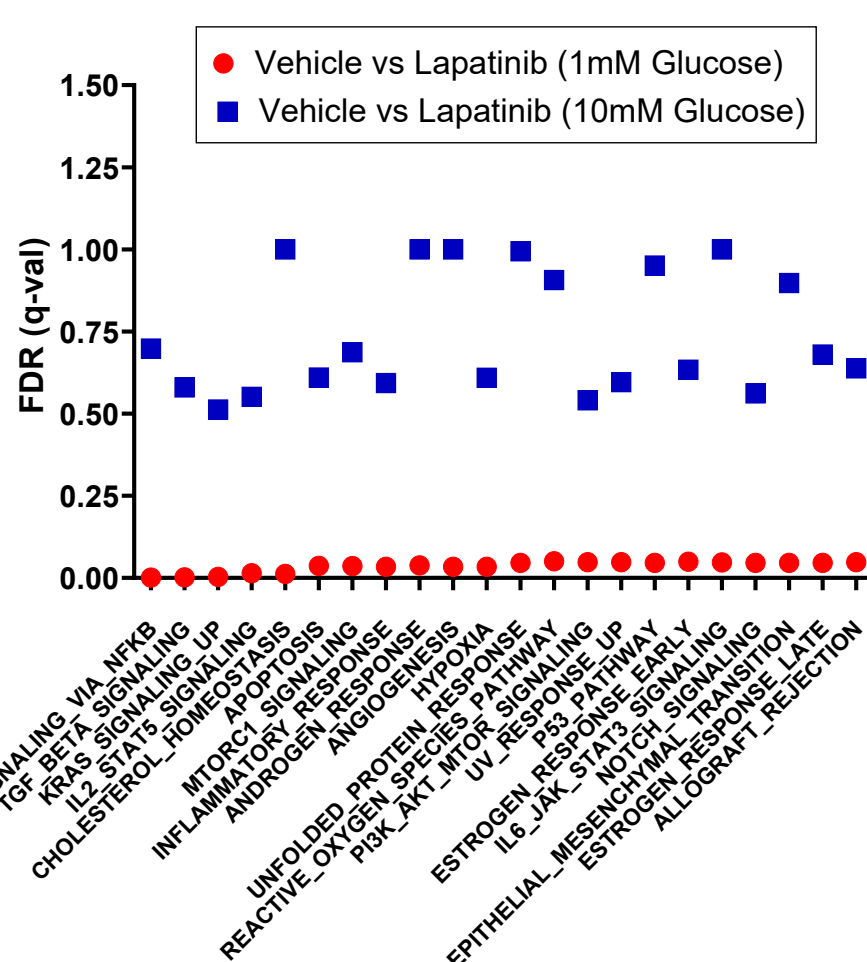
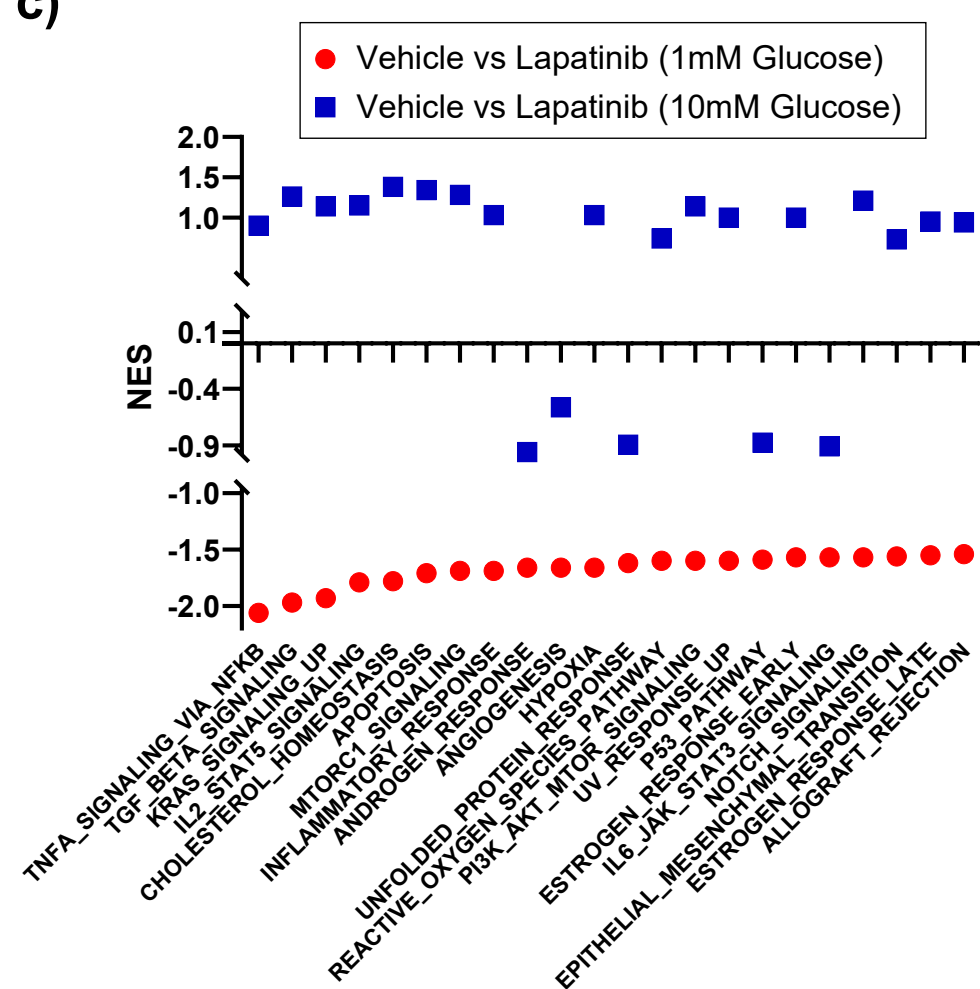
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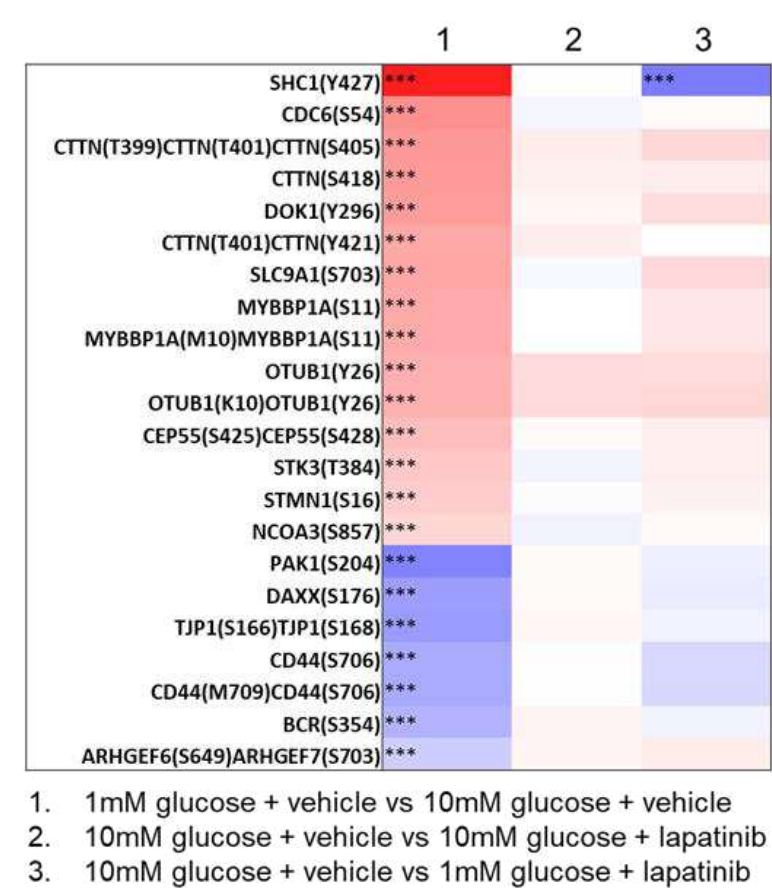
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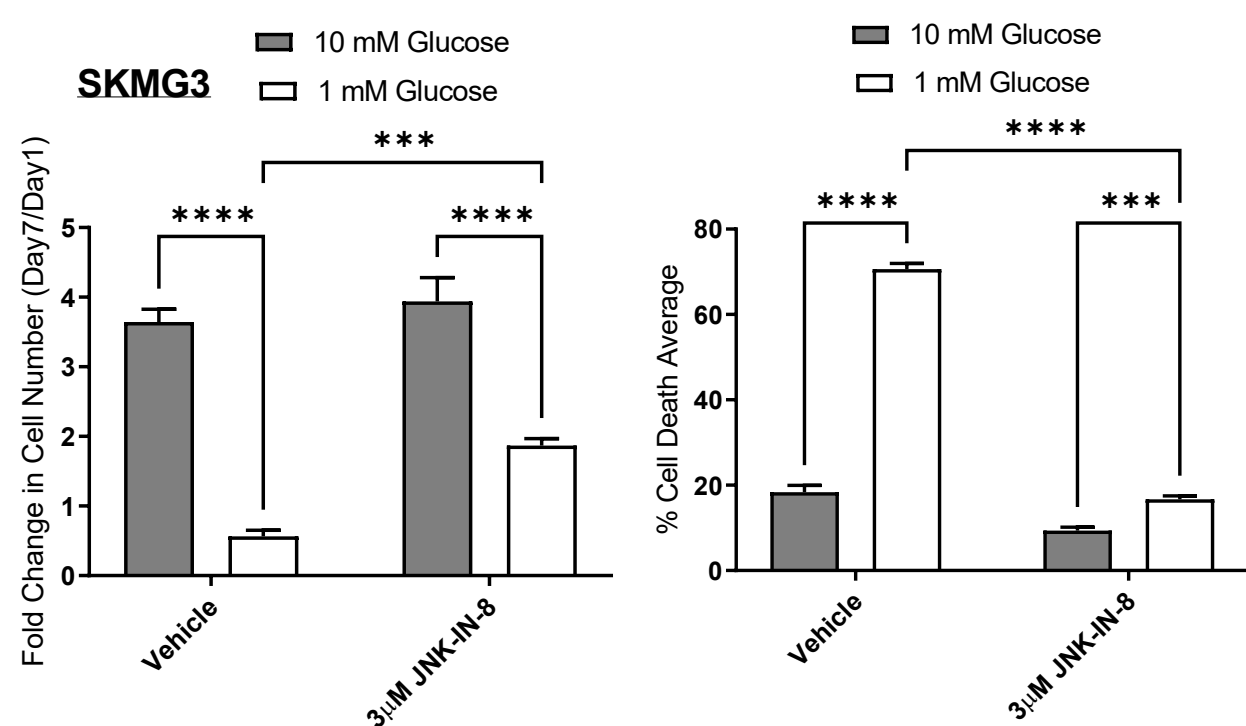
c)



d)



e)



f)

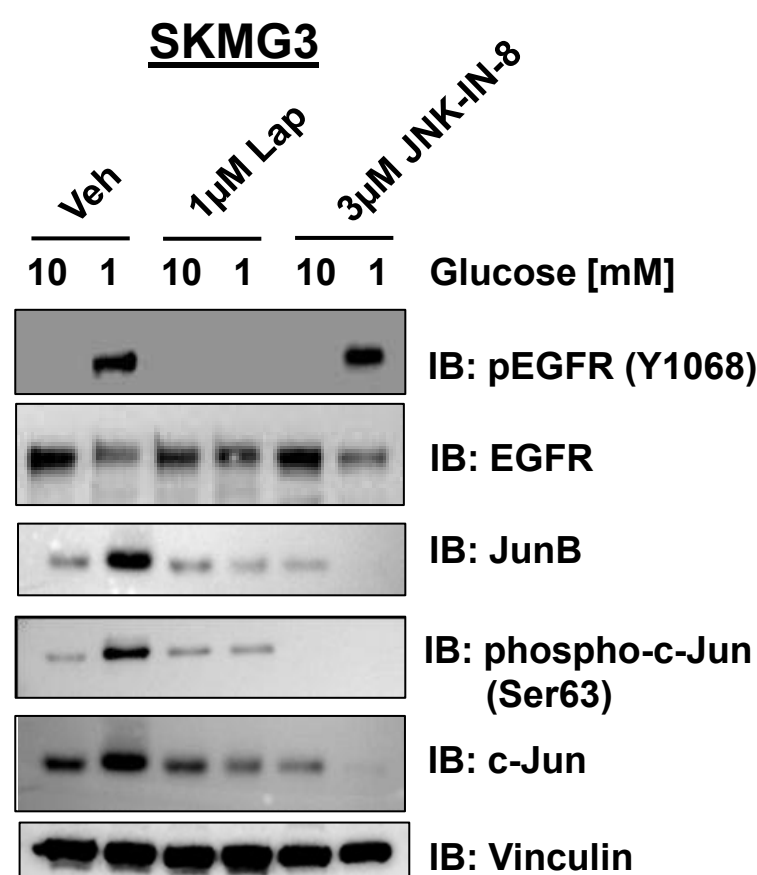
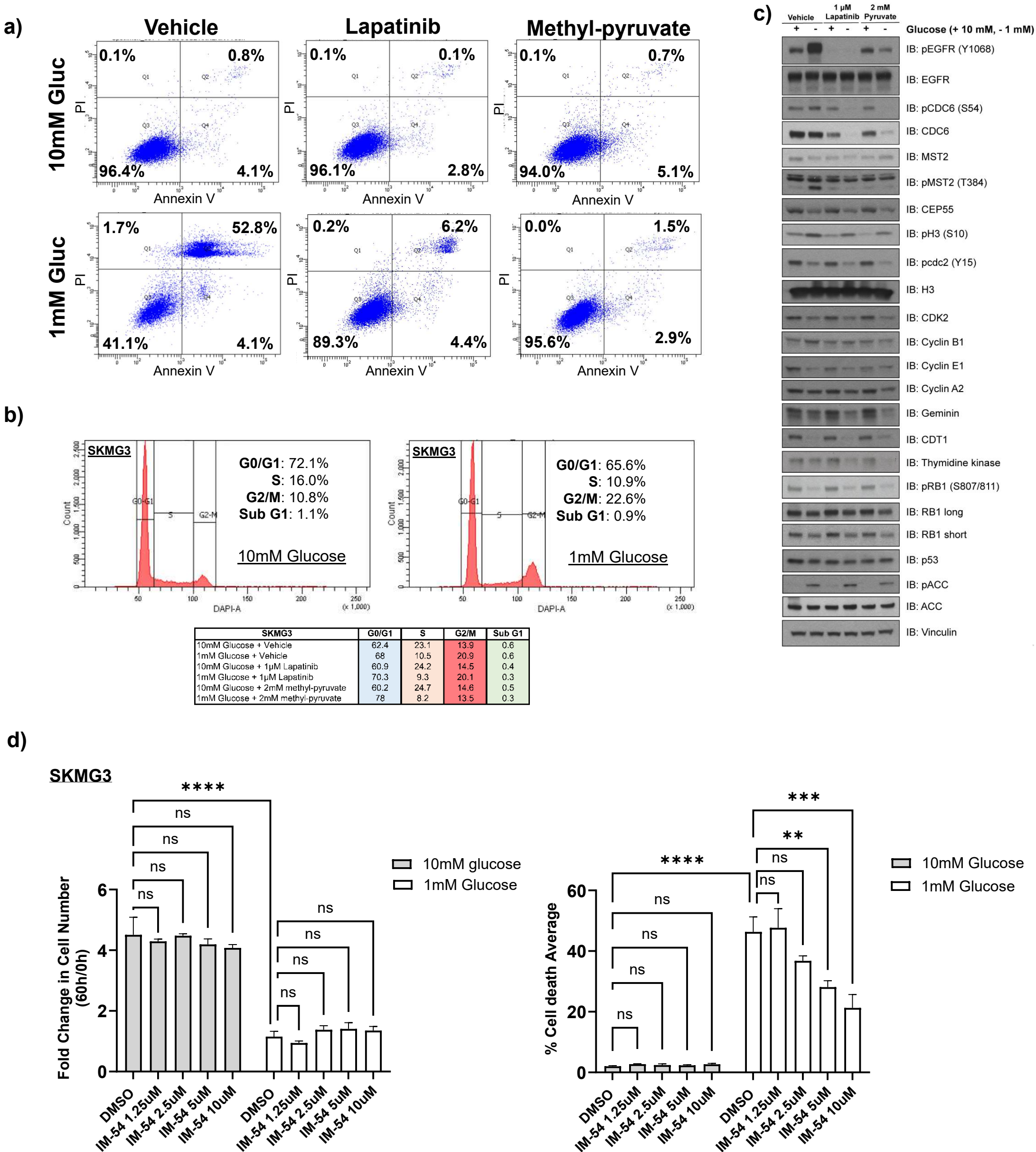
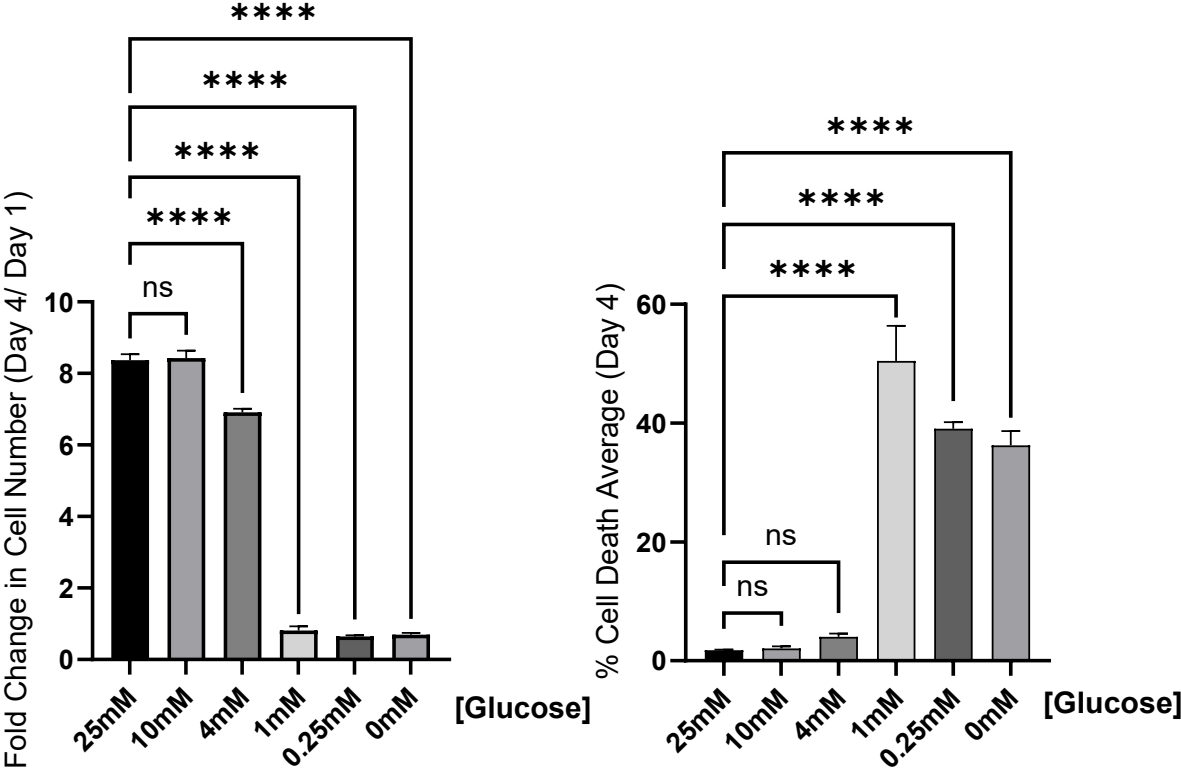


Figure 5

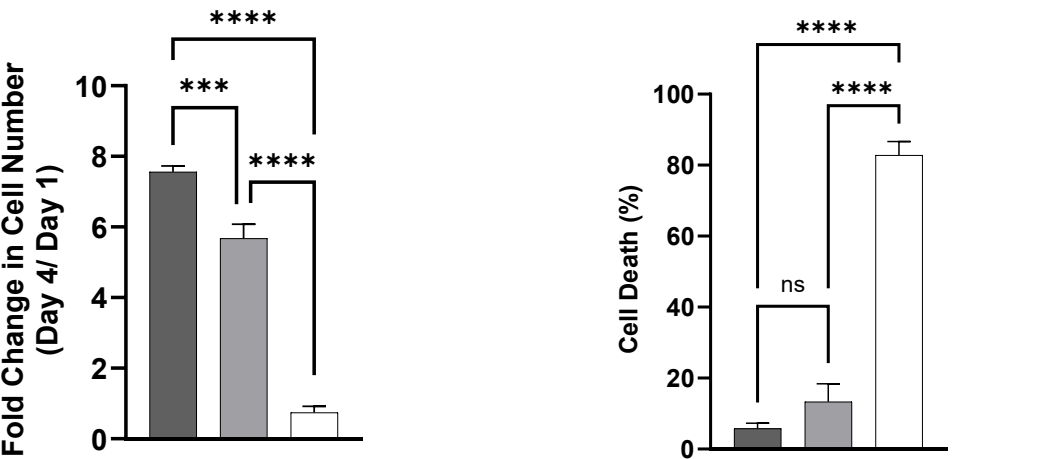


Supplemental Figure 1

a) SKMG3



b) SKMG3 10mM glucose adapted cell line

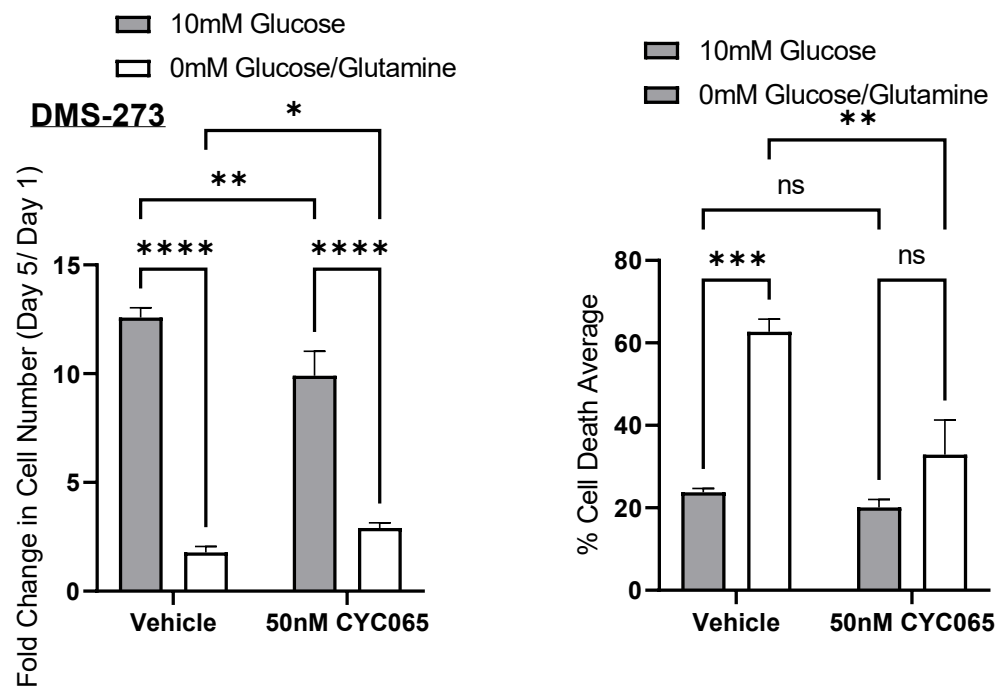


Supplemental Figure 1. SKMG3 cells undergo significant cell death when grown in hypoglycaemic conditions.

(A) EGFR-mutant SKMG3 cells were cultured in varying concentrations of glucose as indicated. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer. Proliferation data (A) is presented as the fold change in viable cell number (trypan-blue-negative cells) between day 1 and day 4 under the indicated glucose conditions (left). Cell death is plotted as the percent of dead cells (% trypan-blue-positive cells) on day 4 under the indicated glucose conditions. (B) SKMG3 cells were first grown in media containing 10mM glucose for 6 weeks. Afterwards, cells were cultured in media containing the indicated glucose concentrations. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer as in (A).

Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Supplemental Figure 2

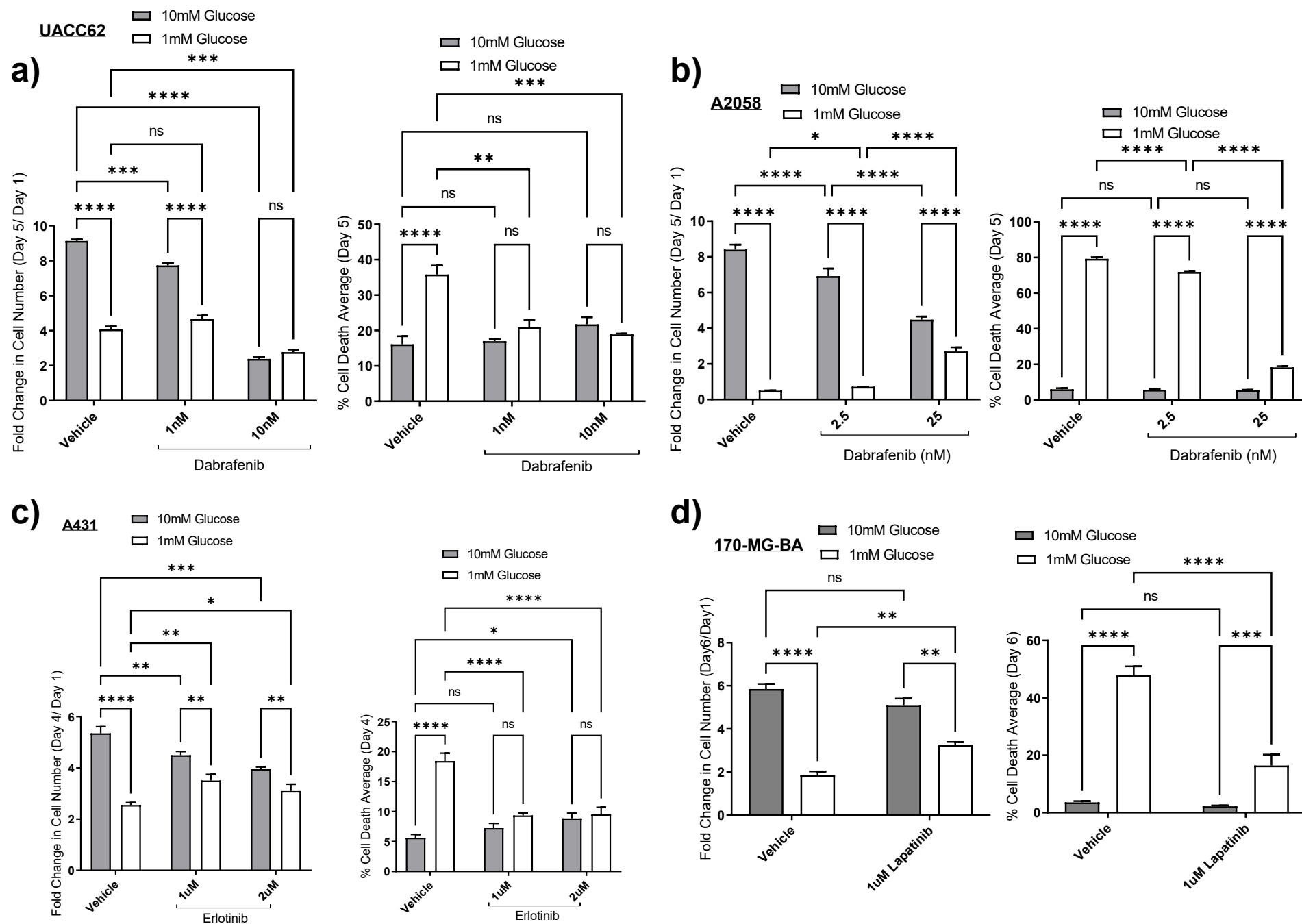


Supplemental Figure 2. Glucose depletion induced cell death in MYC-amplified cancer cell line.

C-MYC-amplified DMS-273 small-cell lung cancer cells were cultured in full media (10mM glucose/4mM glutamine) or media devoid of glucose and glutamine in the presence or absence of 50nM of the CDK2/9 inhibitor CYC065. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer.

Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Supplemental Figure 3



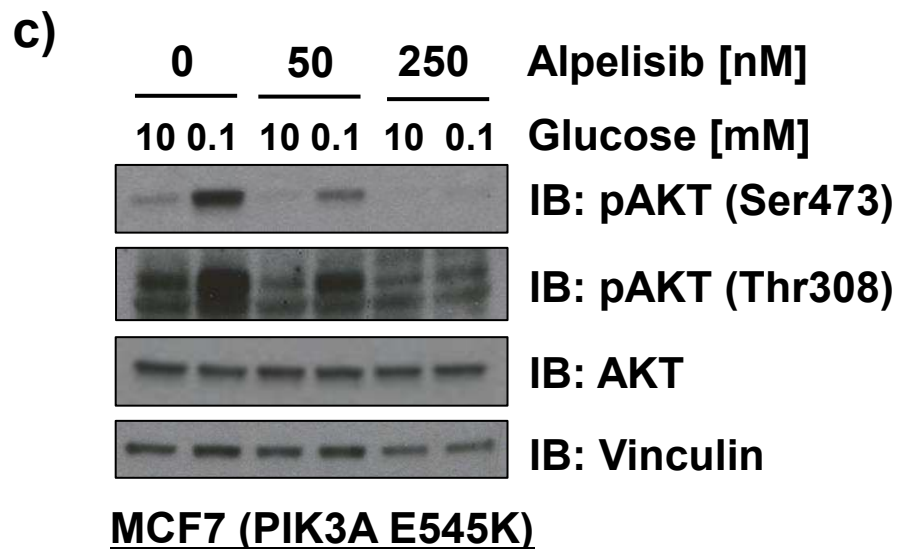
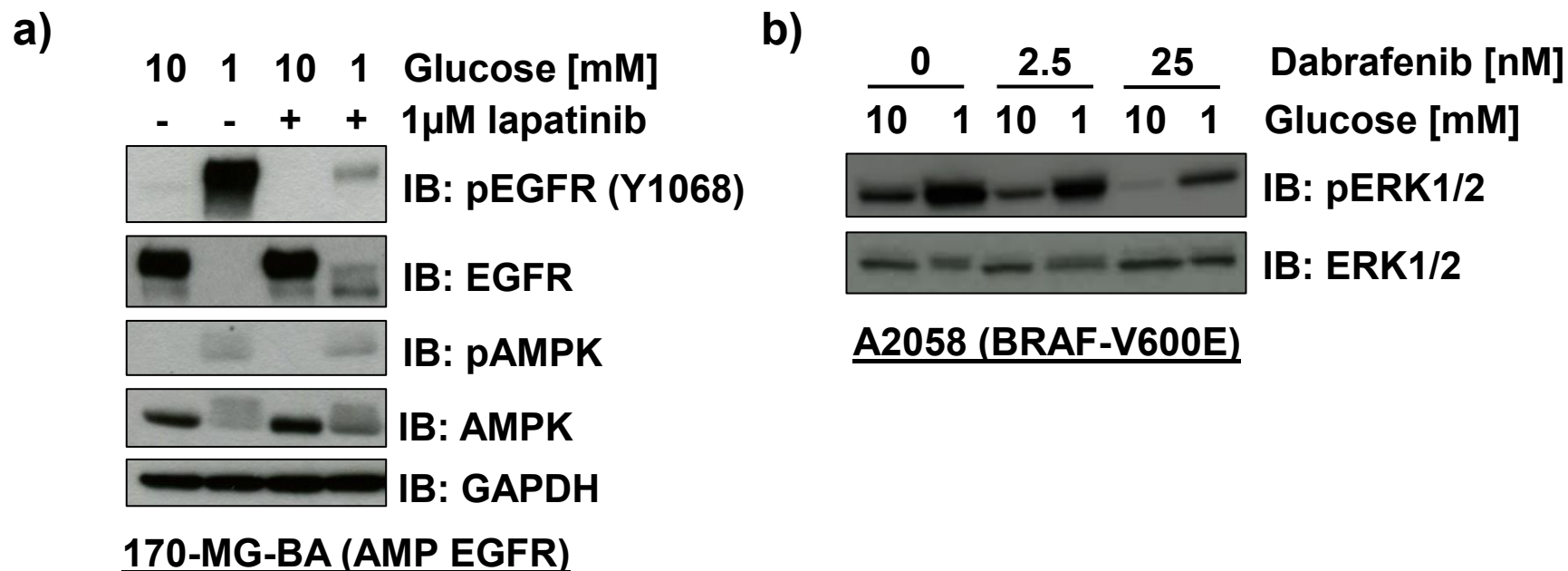
Supplemental Figure 3. Glucose depletion induced cell death in cancer cell lines driven by BRAF or EGFR.

UACC62 (A) and A2058 (B) melanoma cell lines carrying an endogenous BRAF-V600E mutation were cultured in media containing either 1mM or 10mM glucose and in the presence or absence of the indicated doses of the BRAF inhibitor dabrafenib. Viable cell proliferation (left panels) and cell death (right panels) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer.

A431 epidermoid carcinoma (C) or 170-MG-BA glioblastoma (D) cells that harbour high-level EGFR amplification were cultured in media containing either 1mM or 10mM glucose and in the presence or absence of the indicated doses of the EGFR inhibitors erlotinib or lapatinib, respectively.

Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Supplemental Figure 4



Supplemental Figure 4. Glucose depletion induced superactivation of driver oncogenes in different cancer cell lines.

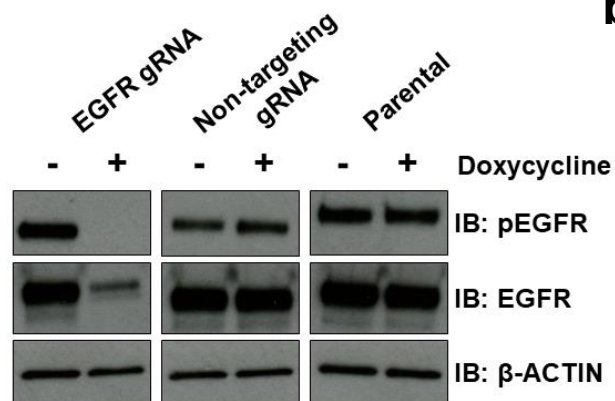
(A) Immunoblot analysis of EGFR-amplified 170-MG-BA cell lines grown in 10mM or 1 mM glucose in the presence or absence of 1 μ M lapatinib. Whole cell lysates were generated, blotted, and probed with the indicated antibodies. AMPK was included as a control for the known effects of glucose depletion.

(B) Immunoblot analysis of BRAF-V600E-mutant A2058 cells cultured in media containing either 1mM or 10mM glucose and in the presence or absence of the indicated doses of the BRAF inhibitor dabrafenib. Whole cell lysates were generated, blotted, and probed with the indicated antibodies.

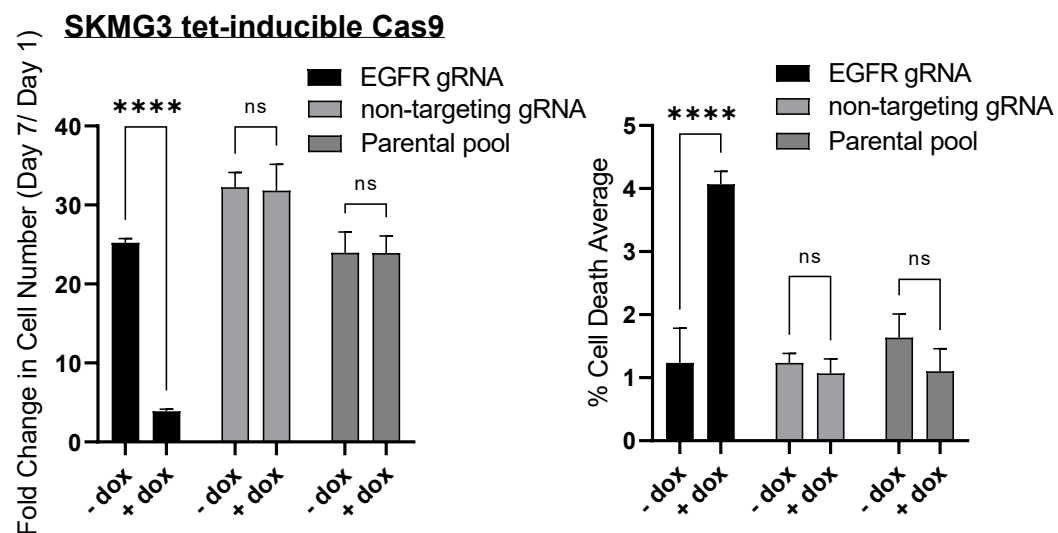
(C) Immunoblot analysis of PIK3CA-mutant MCF7 breast cancer cells grown under the indicated glucose concentrations and in the presence or absence of the indicated doses of the selective PI3K α inhibitor alpelisb.

Supplemental Figure 5

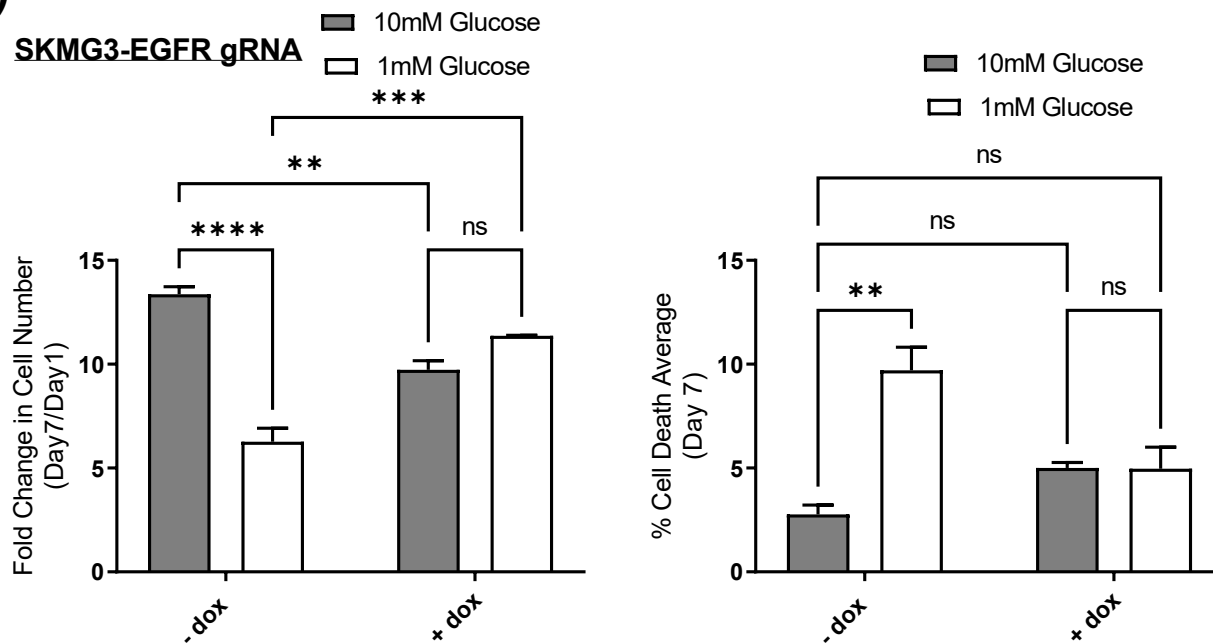
a)



b)



c)



Supplemental Figure 5. CRISPR-based EGFR knockout conferred protection against GDI toxicity in SKMG3 cells.

(A) Immunoblot analysis of SKMG3 cells stably expressing a doxycycline-inducible Cas9 cDNA together with either a constitutive EGFR gRNA (SKMG3-EGFR gRNA) or a non-targeting gRNA control following 7 days of doxycycline treatment (1µg/ml). Cell lysates were analysed using the indicated antibodies.

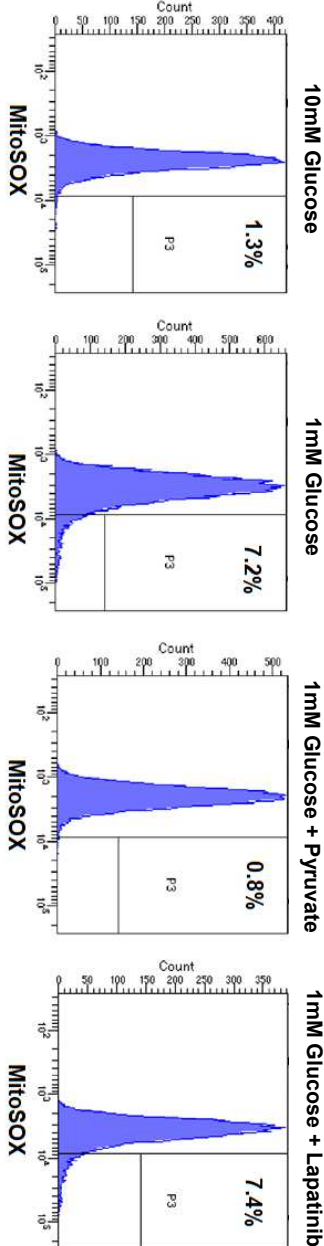
(B) Cells from (A) were cultured in the presence or absence of 1µg/ml doxycycline for 6 days. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer.

(C) SKMG3-EGFR gRNA cells were cultured in 1mM or 10mM glucose and in the presence or absence of 1µg/ml doxycycline for 6 days. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer.

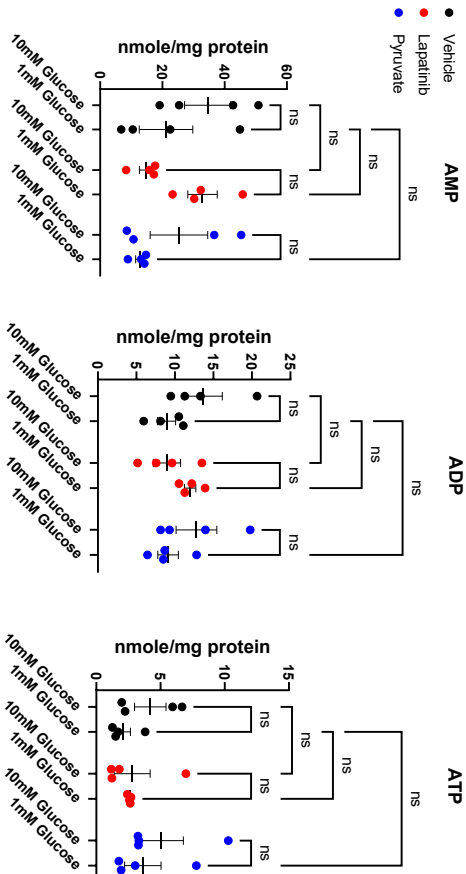
Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

Supplemental Figure 6

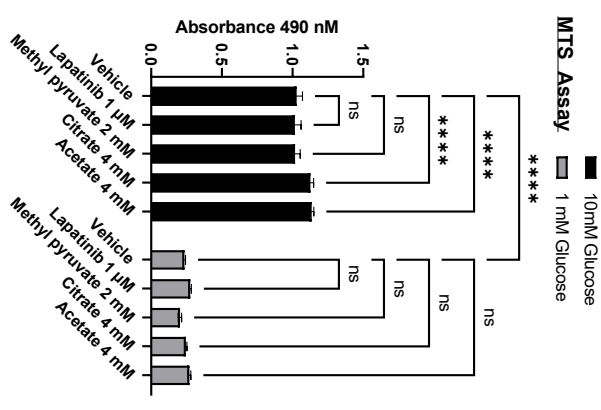
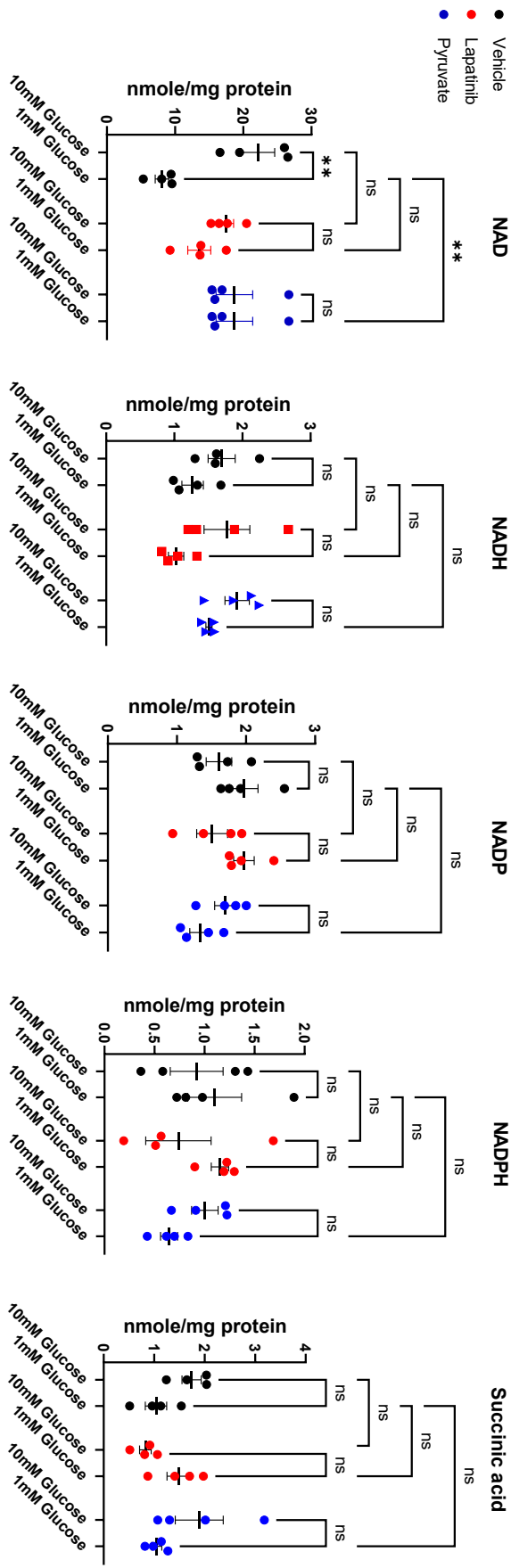
a) b)



c)



d)



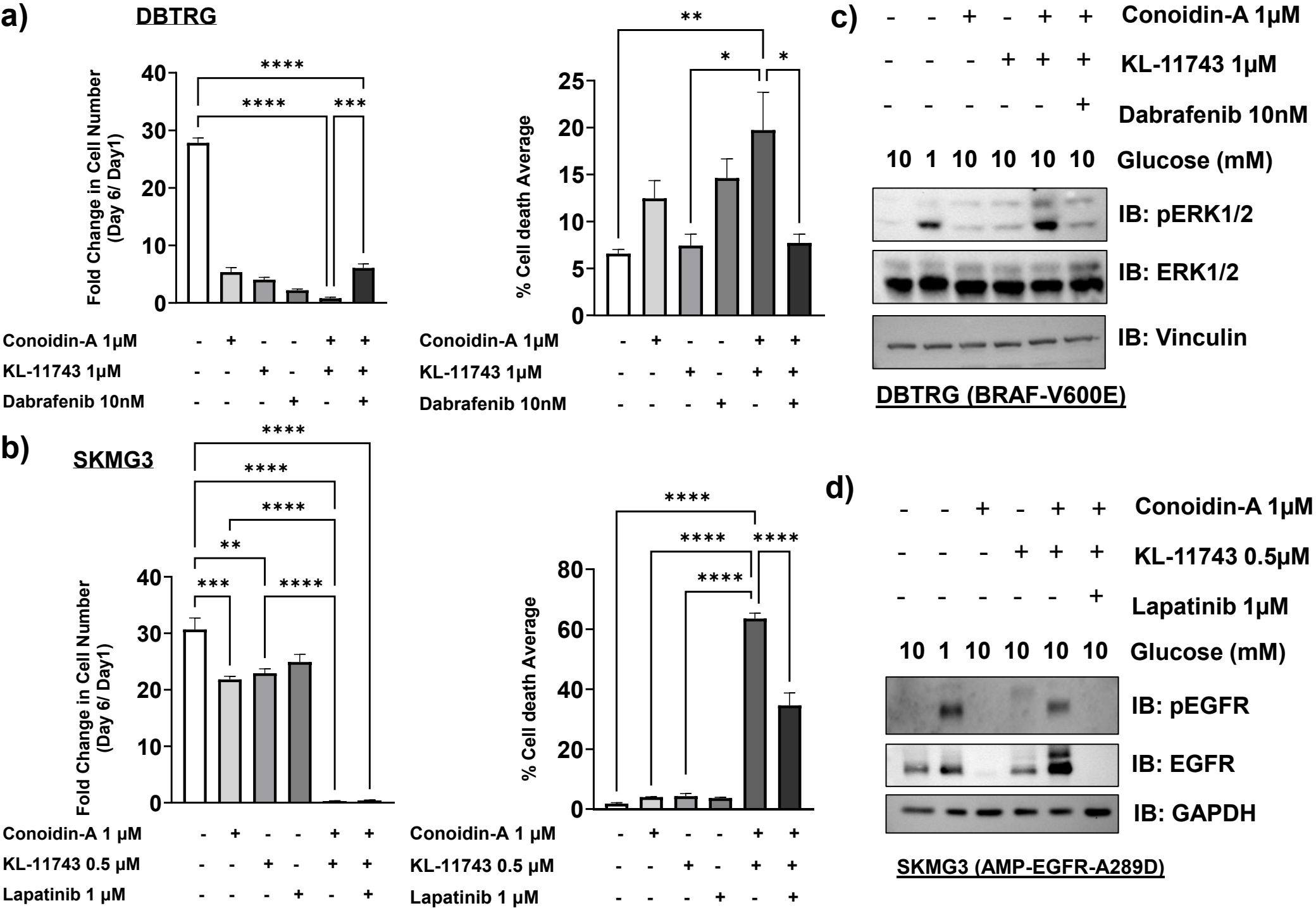
Supplemental Figure 6. Pyruvate lowered mitochondrial ROS levels, with no change in mitochondrial function during glucose depletion.

(A) Relative mitochondrial reactive oxygen species (ROS) levels were measured by flow cytometry using the mitochondrial superoxide indicator MitoSOX in SKMG3 cells following 24 hours of growth under the indicated conditions (1mM glucose, 10mM glucose, 2mM methylpyruvate, and 1 μ M lapatinib).

(B) MTS assay in SKMG3 cells grown in 1mM of 10mM glucose and supplemented with vehicle (DMSO) of the indicated glycolytic intermediates (4mM citrate and 4mM acetate), 2mM methylpyruvate, or 1 μ M lapatinib.

LC-MS measurements of AMP/ADP/ATP (C) and NAD/NADH/NADP/NADPH/succinate (D) in SKMG3 cells grown under the indicated conditions.

Supplemental Figure 7



Supplemental Figure 7. Combined pan-GLUT and PRDX2 inhibition kills cancer cells in an oncogene-dependent manner.

(A) DBTRG cells were treated with the shown combinations of the PRDX2 inhibitor Conoidin A, the pan-GLUT inhibitor KL-11743, and the BRAF inhibitor dabrafenib at the indicated doses. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer.

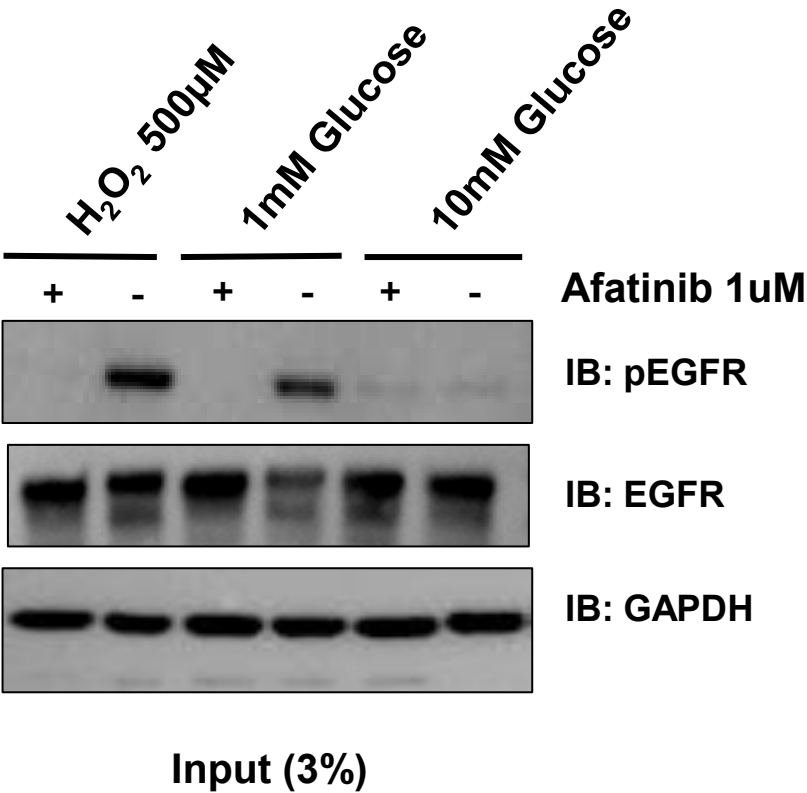
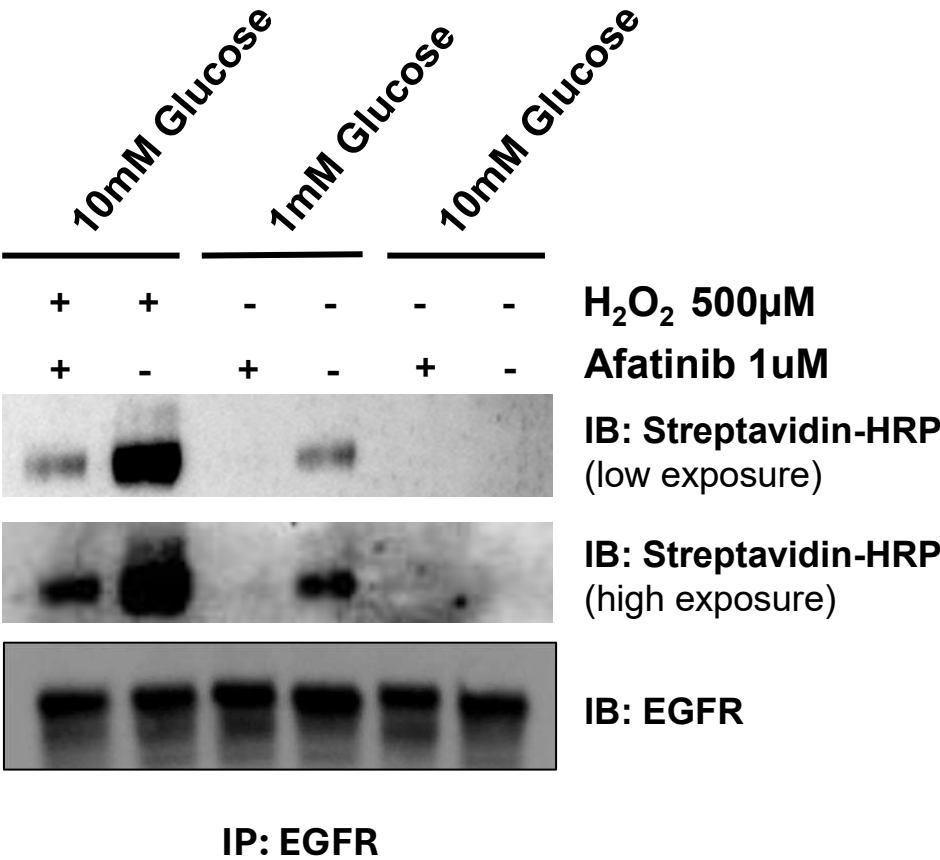
(B) SKMG3 cells were treated with the shown combinations of the PRDX2 inhibitor Conoidin A, the pan-GLUT inhibitor KL-11743, and the EGFR inhibitor lapatinib at the indicated doses. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer.

(C) Immunoblot analysis of DBTRG cells treated for 24 hours as in (A). Cell lysates were probed with the indicated antibodies.

(D) Immunoblot analysis of SKMG3 cells treated for 24 hours as in (B). Cell lysates were probed with the indicated antibodies.

Statistical analyses were done in GraphPad Prism v10. n.s., not significant, $*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$ and $****P \leq 0.0001$.

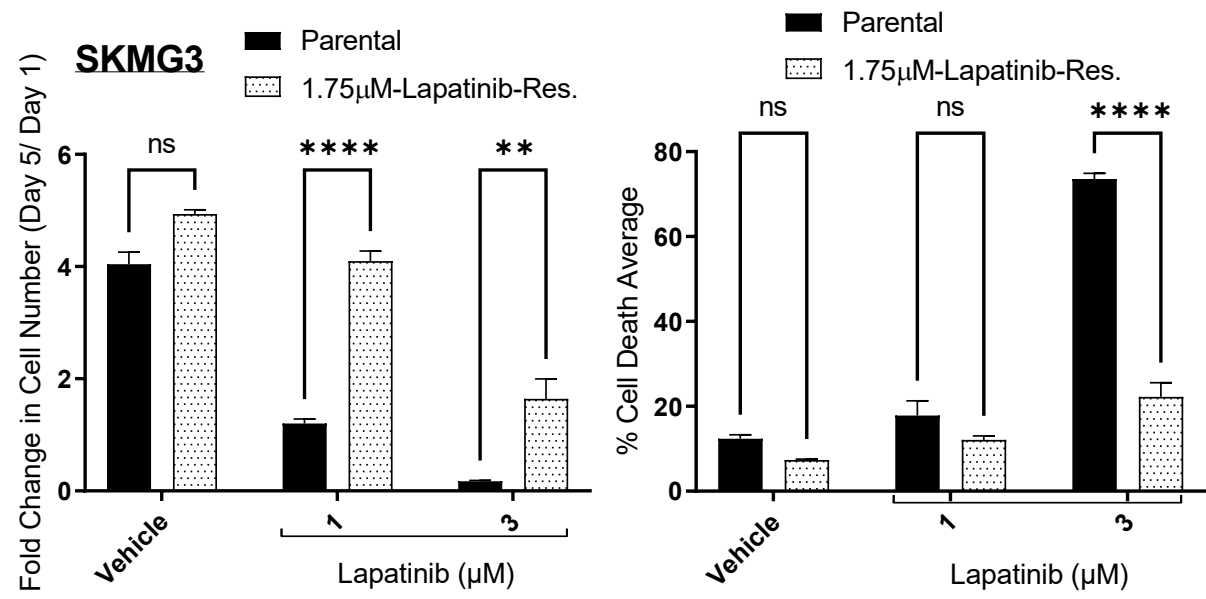
Supplemental Figure 8



Supplemental Figure 8. Glucose deprivation or H₂O₂ exposure leads to afatinib-sensitive sulfenylation of EGFR in SKMG3 cells.

SKMG3 cells were cultured under the indicated glucose conditions in the presence or absence of afatinib, a covalent EGFR inhibitor that targets Cys797, for 24 hours. Cells were then labelled with the sulfenylation probe Dyn-2 and subjected to immunoprecipitation with an EGFR antibody. Immunoprecipitates were subsequently treated with azido-biotin reagent to allow detection of labelled receptor. Sulfenylated EGFR was detected by chemiluminescence using streptavidin-HRP (left). Whole lysates were also analysed by immunoblot prior to immunoprecipitation with the indicated antibodies (right).

Supplemental Figure 9



Supplemental Figure 9. Validation of lapatinib-resistant SKMG3 cell line by growth assay.

Parental and lapatinib-resistant SKMG3 cells were treated with 1 μ M or 3 μ M lapatinib for 4 days. Lapatinib-resistant cells were generated by chronic exposure of parental SKMG3 cells to 1.75 μ M lapatinib. Viable cell proliferation (left) and cell death (right) were assessed by trypan blue exclusion using the ViCell Cell Viability analyzer.

Statistical analyses were done in GraphPad Prism v10. n.s., not significant, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ and **** $P \leq 0.0001$.