

# ERO1a fosters glioblastoma aggressiveness and metabolic flexibility by regulating mitochondria-associated membrane dynamics

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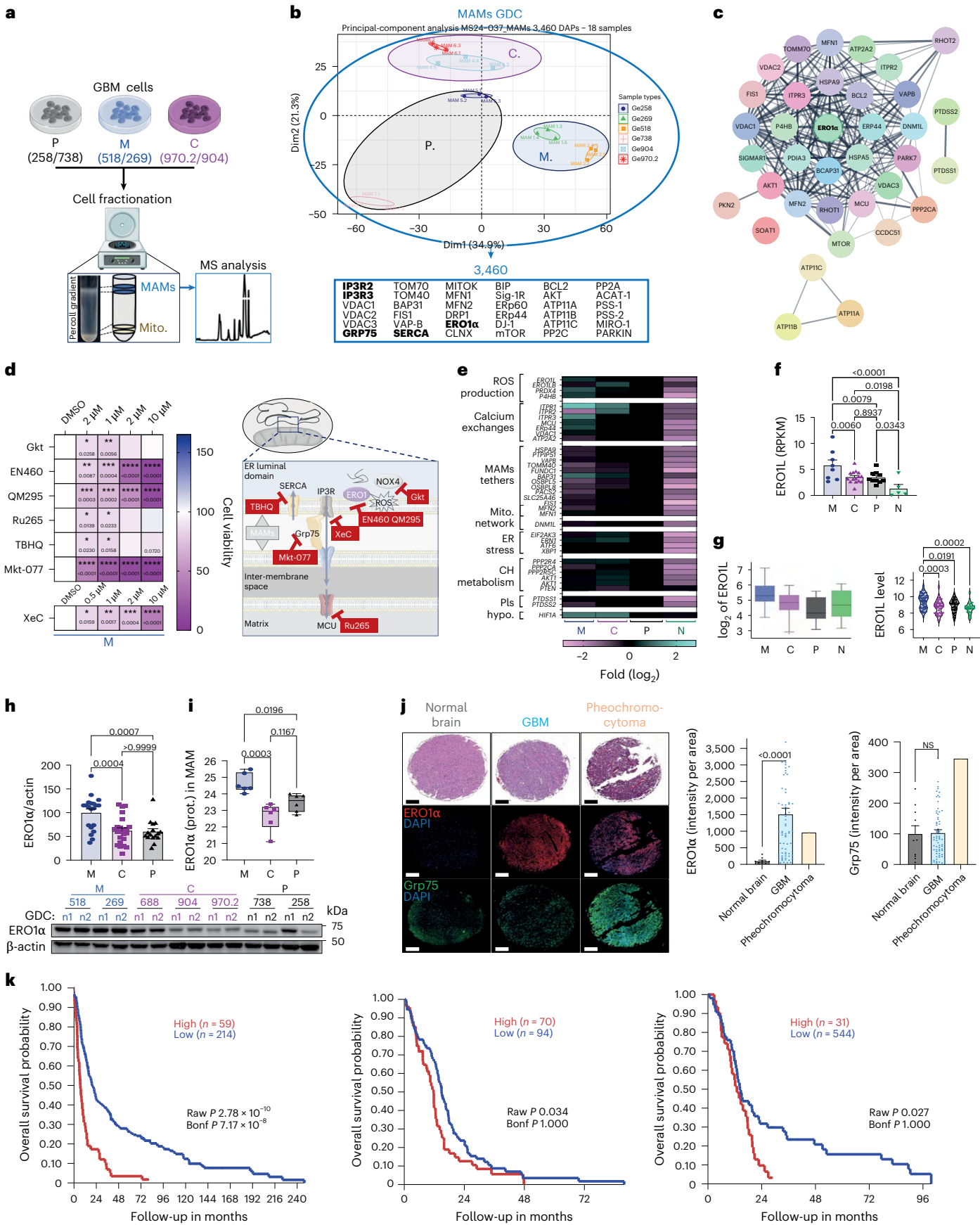
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Despite the wealth of data generated in the omics era to investigate molecular drivers, glioblastoma (GBM) remains one of the most incurable cancers with a poor median of survival. Here we unravelled the dynamic crosstalk between the endoplasmic reticulum and mitochondria, known as mitochondria-associated membranes (MAMs) and define how modulation of calcium fluxes and MAM structure influences GBM cell plasticity and metabolic flexibility. We identified ERO1 $\alpha$ , whose expression is significantly associated with poor GBM patient survival, as a critical MAM protein that regulates MAM structure, dynamics and calcium-mediated functions. Our data demonstrate that ERO1 $\alpha$  activity and expression promotes GBM aggressiveness in vitro and in vivo and enhances mitochondrial oxidative phosphorylation. By establishing a direct link between ERO1 $\alpha$ -mediated MAM modulation and the antitumour effects of ERO1 $\alpha$  inhibition, this work highlights a context-dependent, druggable vulnerability that can be exploited for GBM therapy.

Despite extensive efforts to develop new strategies against glioblastoma (GBM), classified as isocitrate dehydrogenase (IDH)-wild-type, patient outcome remains poor with a 5-year survival rate of only 5–10%. Treatment failure largely stems from the highly heterogeneous nature of GBM, prompting the use of integrative approaches to characterize its genetic and transcriptional heterogeneity<sup>2–6</sup> and the development of clinically informative classifications<sup>7</sup>, which suggest that molecular heterogeneity may reflect distinct metabolic requirements.

The tumour profiling described has revealed profound heterogeneity in GBM, which largely explains clinical trial failures and highlights the need to understanding its plasticity. GBM displays remarkable cellular diversity defined by hierarchical stratification encompassing glioma stem cells (GSCs), responsible for tumour progression, which harbour self-renewal properties, tumour-propagating abilities and intrinsic therapy-resistance mechanisms, and their differentiated descendants (GDCs), which adopt a more specialized phenotypes yet

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**Fig. 1 | The ERO1 $\alpha$  protein is found in MAM compartments in GBM patient-derived cells and its expression is correlated with poor survival.** **a**, Schematic diagram of the subcellular fractionation of two GDC models per GBM subtype: Ge258 and Ge738 for proneural (P), Ge518 and Ge269 for mesenchymal (M) and Ge970.2 and Ge904 for classical (C), allowing MAM isolation and characterization. Mito., mitochondria. **b**, Principal-component analysis (PCA) for all GDC models displaying 3,946 groups of proteins. Bold labels indicate proteins for which the corresponding activities are examined in **d**. DAPs, Differentially abundant proteins. **c**, String visualization of the main MAM proteins identified by MS. **d**, Left, viability of Ge518 cells under either control conditions (DMSO, vehicle) or treatment with inhibitors targeting MAMs, namely Gkt, EN460, QM295, Ru265, TBHQ, Mkt-077 and xestospongin C (XeC) at the indicated concentrations after 72 h, using a CellTiter-Glo kit ( $n = 6-9$  technical replicates in  $n = 3$  biological replicates). Right, schematic diagram of each inhibitor target within MAMs. **e**, Clustering of principal MAM genes according to their expression in each GBM subtype (Verhaak classification) and their cellular function in the Mayo Clinic Brain Tumour Patient-Derived Xenograft National Resource. MAM, mitochondria-associated membranes; Pls, phospholipids; Hypo., hypoxia. **f**, ERO1 $\alpha$  (ERO1L) mRNA expression was determined by RNA sequencing (normalized against RPKM) in Mayo Clinic resources ( $n = 5-15$  biological replicates). **g**, ERO1 $\alpha$  (ERO1L) mRNA expression extracted from Wellcome to R2 dataset (left) and extracted from Xenobrowser (Univ. California, Santa Cruz) (right) according to GBM subtype ( $n = 28-55$

patients and/or subtypes). **h**, Top, western blot showing the expression of ERO1 $\alpha$  in all GDC models in whole-cell lysate (WCL), normalized against  $\beta$ -actin expression. Left, histograms represent the fold change of protein (prot.) expression determined by densitometry analysis ( $n = 3-6$  biological replicates). **i**, Expression of ERO1 $\alpha$  in MAMs fractions. NS, not significant. **j**, Illustrative (left) and immunofluorescence (IF) protein expression quantifications (right) of tumour microarray (TMA) slices from normal brains ( $n = 10$ ), patients with GBM ( $n = 70$ ) and patients with pheochromocytoma ( $n = 1$ ); scale bar, 300  $\mu$ m. **k**, Left, Kaplan–Meier analysis of TCGA–Glioma dataset (French 284, MAS5.0, u133p2) for ERO1 $\alpha$  expression ( $n = 214$  ERO1 $\alpha^{\text{low}}$ ,  $n = 59$  ERO1 $\alpha^{\text{high}}$ ; expression cutoff of 341.7;  $P = 7.17 \times 10^{-8}$ ). Middle, Kaplan–Meier analysis of TCGA–GBM dataset (173, tpm, gene code 36, 2022-v32) for ERO1 $\alpha$  expression ( $n = 94$  ERO1 $\alpha^{\text{low}}$ ,  $n = 70$  ERO1 $\alpha^{\text{high}}$ ; expression cutoff of 22.69;  $P = 0.034$ ). Right, Kaplan–Meier analysis of TCGA–GBM dataset (540, MAS 5.0, u133a, based on identified subtypes) for ERO1 $\alpha$  expression ( $n = 54$  ERO1 $\alpha^{\text{low}}$ ,  $n = 31$  ERO1 $\alpha^{\text{high}}$ ; expression cutoff of 38.8;  $P = 0.027$ ). Survival differences ( $k$ ) were assessed using a two-sided log-rank test.  $P$  values were adjusted for multiple comparisons using the Bonferroni method, as implemented in the R2 platform. N, neural; HG, high glucose; LG, low glucose; EN, EN460. Histograms or heatmaps are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ; two-way (**d**) or one-way (**f**, **g** right, **h**) analysis of variance (ANOVA) followed by Fisher's (**d**, **f**), Tukey (**g**, right and **i**) or Bonferroni (**h**) multiple comparison tests. Two-tailed Mann–Whitney  $U$ -tests were used for **j**.

substantially contributing to tumour burden and invasive spread. In the brain, tumour cells cope with different types of stressors, giving rise to a fluctuating tumour microenvironment<sup>8</sup>, defined by limited delivery of cytotoxic chemotherapies, different gradients of oxygen tension and nutrients inducing spatial heterogeneity<sup>9–11</sup>. Given this dysfunctional microenvironment<sup>12</sup>, GBM cells are prime candidates for therapies targeting metabolic vulnerabilities/dependencies.

In cancer cells, glucose is directed to biosynthesis pathways and lactate production even in the presence of oxygen, a phenomenon known as the Warburg effect<sup>13,14</sup>. However, despite the prevalent glucose uptake in most GBM tumours, not all tumours exhibit this pattern, further emphasizing their metabolic heterogeneity and presenting challenges for dependency-based therapeutic strategies<sup>15–17</sup>. Upon metabolic stress, the unfolded protein response (UPR) is activated and the folding capacity of the endoplasmic reticulum (ER) is overwhelmed. Both transient and prolonged ER stress promote the formation of mitochondria-associated membranes (MAMs), specialized contact sites between the ER and mitochondria, thereby enabling physical and biochemical communication between the two organelles<sup>18–20</sup>. This crosstalk is involved in many fundamental metabolic processes, including calcium homeostasis, phospholipid and

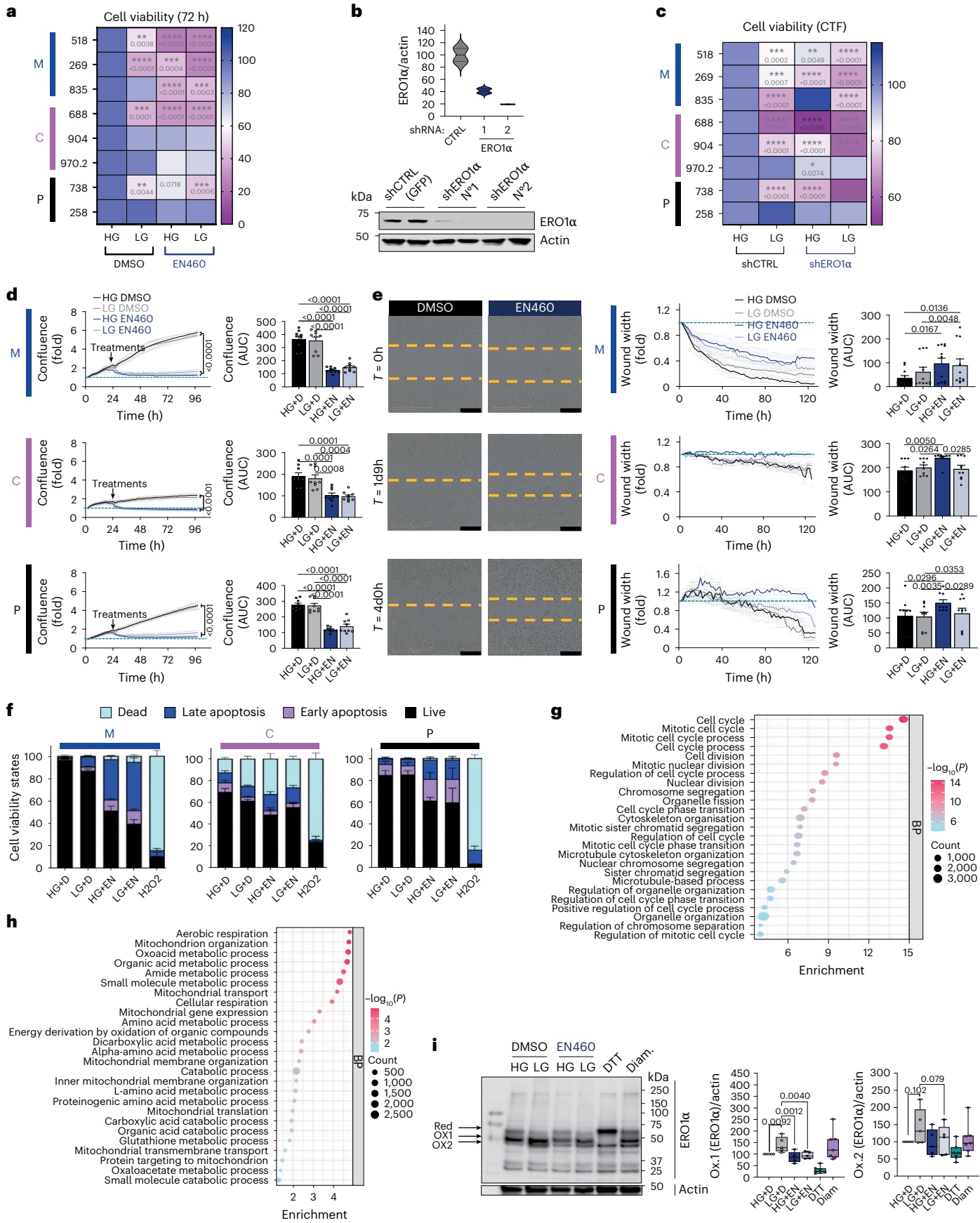
cholesterol metabolism, and the regulation of mitochondrial dynamics and bioenergetics<sup>21–27</sup>. In addition, multiple tethering protein complexes are enriched at MAMs to stabilize contact sites<sup>28</sup>. Several key enzymes mediating phospholipid synthesis are located and enriched at the MAMs, such as phosphatidylserine synthase 1 and 2 (PSS1/2)<sup>29,30</sup>. Similarly, several proteins involved in the redox regulation of proteins, such as thioredoxin-related transmembrane protein 1 (TMX1) and ER oxidoreductase 1 $\alpha$  (ERO1 $\alpha$ ), are also enriched in these contact sites<sup>31–34</sup>. The rapid transfer of Ca<sup>2+</sup> from the ER to mitochondria is also mediated via the calcium channelling complex composed of the inositol 1,4,5-trisphosphate receptors (IP3Rs), glucose-regulated protein 75 (Grp75) and the voltage-dependent anion-specific channels (VDACs)<sup>35,36</sup>. Ca<sup>2+</sup> kinetics are also modulated by the sarco/ER Ca<sup>2+</sup> ATPase (SERCA) pump localized at the ER membrane through several proteins residing at MAMs.

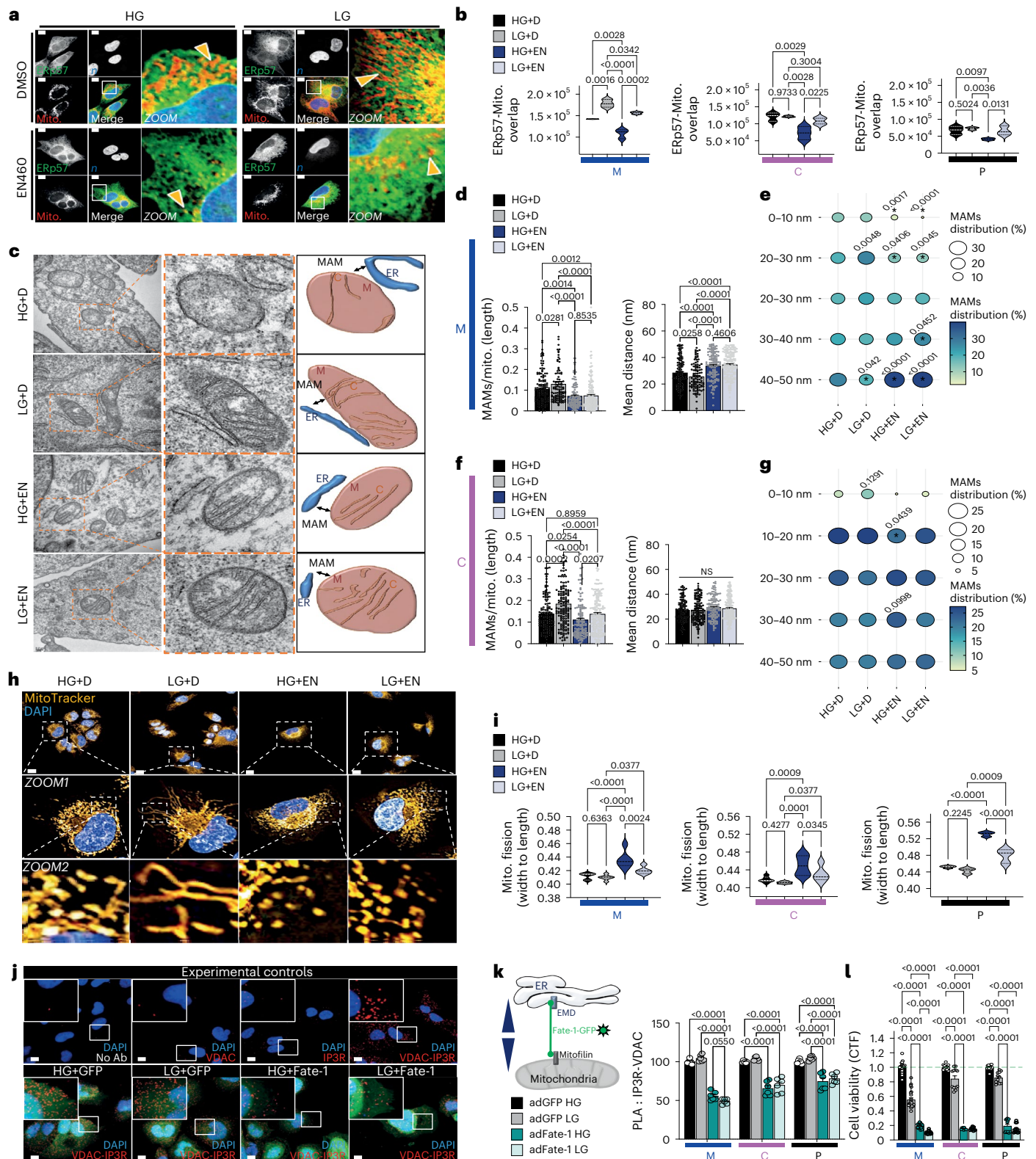
Here, we investigated and characterized MAM networks in several GBM subtypes, namely classical, proneural and mesenchymal GBM<sup>2</sup>. We identified ERO1 $\alpha$ , a MAM-localized protein, as a critical modulator of the UPR response and regulator of mitochondrial bioenergetics<sup>35</sup>. Analysis of clinical GBM samples and patient-derived differentiated and stem cells (GDCs and GSCs, respectively) revealed

## Fig. 2 | Targeting ERO1 $\alpha$ strongly inhibits GBM aggressiveness in vitro.

**a**, Effect of EN460 treatment on cell viability measured by CellTiter-Fluor in GDCs at 72 h ( $n = 8-9$  technical replicates in  $n = 3$  biological replicates). Ctrl, vehicle (DMSO) in HG versus LG concentrations with EN460 (HG/LG + EN460). Scale bar, 0–120 in percentage of viability. **b**, Immunoblots showing the expression of indicated proteins for Ge518 infected by shRNA control (CTRL) or shERO1 $\alpha$  (for 48 h). Histograms show the fold change of protein expression normalized against  $\beta$ -actin expression and determined by densitometry analysis ( $n = 2$  biological replicates). **c**, Cell viability of all indicated GDCs in shRNA control (Ctrl) versus shERO1 $\alpha$ , measured by CellTiter-Fluor at 72 h ( $n = 3$  biological replicates). **d**, Cell proliferation of GDC Ge518 (top), Ge970.2 (middle) and Ge258 (bottom) was quantified using Incucyte technology in the following conditions: HG or LG with EN460 (HG/LG + EN460). Histograms represent the cell confluence ( $n = 9$  technical replicates in  $n = 3$  biological replicates). **e**, Wound healing experiment showing migration of GDCs: Ge518 (top), Ge970.2 (middle) and Ge258 (bottom), measured using Incucyte technology in the following conditions: HG/LG + EN460. Histograms represent the wound width ( $n = 9$  technical replicates in  $n = 3$  biological replicates). Left, images at day 0 (top), day 1 (middle) and day 4 (bottom); middle graphs show the wound width in fold and, right shows the same data in histograms in area under the curve (AUC).

**f**, Histograms representing the percentage of live, dead, late and early apoptotic cells in the indicated GDCs and conditions by flow cytometry with annexin V/propidium iodide staining at 72 h ( $n = 6$  biological replicates for Ge518, Ge970.2;  $n = 3$  for Ge258). **g**, **h**, Downregulated (**g**) and upregulated (**h**) protein enrichment analysis of MAMs after EN460 treatment for 24 h ( $n = 4$  biological replicates) in GDCs, where dot size represents protein count and dot colour the  $-\log_{10}$   $P$  value. Gene Set Enrichment Analysis (**g**, **h**) was conducted using g:Profiler, applying a hypergeometric test (equivalent to a one-sided Fisher's exact test), with multiple testing correction applied using the g:SCS method as implemented in g:Profiler. **i**, Left, a representative immunoblot from non-reducing gels showing expression of indicated reduced or oxidized forms of ERO1 $\alpha$  in Ge518. Right, box plots show expression of the indicated proteins in the several specified conditions for 24 h ( $n = 5$  biological replicates). Box-and-whisker plots show median (centre line), interquartile range (25th–75th percentiles) and min–max (whiskers); all points are shown. M, mesenchymal (GDC Ge518); P, proneural (GDC Ge258); C, classical (GDC Ge970.2); EN, EN460. Histograms, curves, heatmaps and box plots are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ ; two-way (**a**, **c**) or one-way (**d**, **i**) ANOVA followed by Fisher's (**i**), Tukey (**d**) or Bonferroni (**a**, **c**) multiple comparison tests. Statistical significance was assessed using a two-tailed Student's  $t$ -test (**e**).





that the mesenchymal subtype particularly relies on ERO1 $\alpha$  and MAM flexibility to sustain cell metabolism under stress conditions. These two hallmarks strongly regulate cell metabolism in this GBM type, thereby allowing cell survival under stress conditions. Collectively, by defining this dependency pathway, our data provide a proof of principle for innovative therapeutic strategies and identify an Achilles heel that can be exploited in GBM therapy.

## Results

### ERO1 $\alpha$ is associated with MAM network and is a poor prognostic factor in GBM

To investigate the, as yet unknown, network of MAMs in GBM, we first isolated MAMs from GBM patient-derived cells of the three subtypes<sup>2</sup> (two per subtype) using Percoll fractionation. Comparative proteomics analysis was performed to profile the MAM-associated proteins (Fig. 1a,b



**Fig. 3 | Inhibition of ERO1 $\alpha$  decreases the contacts between the ER and the mitochondria.** **a**, A representative immunofluorescence of ERp57, an ER marker, and MitoTracker allowing the relative quantification of the ER–mitochondria distance; scale bar, 15  $\mu$ m. **b**, Violin plots showing ERp57 and MitoTracker overlap ( $n = 3$  biological replicates) in the designated conditions for 24 h. **c**, Representative EM images of Ge518 MAM (left) and MAM cartoon (right) in the indicated conditions; scale bar, 0.2  $\mu$ m. **d,e**, EM quantifications. Average measurements between ER–mitochondria (MAM): total MAM length (**d**, left), distance (**d**, middle) and MAM distance distribution (**e**).  $n = 105$ –138 MAMs in  $n = 3$  biological replicates analysed for the indicated conditions for 24 h in GDC Ge518, **f,g**, Same as **d,e**, in Ge970.2,  $n = 127$ –149 MAMs in  $n = 3$  biological replicates. **h**, A representative image of MitoTracker staining in Ge518 by IF. **i**, MitoTracker-CMXROS (100 nM) was used to evaluate mitochondrial network

architecture, in Ge518 (left), Ge970.2 (middle) and Ge258 (right) in the designated conditions for 24 h. Scale bar, 20  $\mu$ m, 100 ( $n = 3$ –6 biological replicates). **j**, A representative immunofluorescence images colocalization of endogenous IP3R3 and VDAC1 using of PLA in Ge518 (no Ab, no primary antibody; VDAC or IP3R, primary antibodies used); scale bar, 20  $\mu$ m. **k**, Left, schematic diagram depicting the Fate-1 molecular tool. Right, histograms showing the quantification of the dot number normalized by cell area ( $n = 6$  biological replicates) in the specified conditions for 24 h after 48 h of infection. **l**, Cell viability of all indicated GDCs in adenovirusFate-1 (adFate-1) versus control (adGFP) in the specified conditions for 72 h  $n = 12$ –24 technical replicates in  $n = 2$  biological replicates. Histograms and violin plots are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and \*\*\*\* $P < 0.0001$ ; two-way (**k,l**) or one-way (**b,d–g,i**) ANOVA followed by Fisher's (**b,i,k**) or Tukey (**d–g,l**) multiple comparison tests.

and Extended Data Fig. 1a). Proteins detected in all GBM patient-derived cells, including the well described MAM-associated proteins VDAC1/2/3, IP3R2/3, GRP75 and ERO1 $\alpha$  (Fig. 1b and Supplementary Table 1), were found to belong mostly to the same protein–protein interaction (PPI) network (Fig. 1c). The biological relevance was then addressed by assessing the viability of GDCs following specific pharmacologic knockdown of these major MAM proteins with specific inhibitors (Fig. 1d and Extended Data Fig. 1b). While all inhibitors induced a significant decrease in cell viability, Mkt-077, an inhibitor of Grp75, and QM295, inhibitor of ERO1 $\alpha$ , as well as EN460, which acts on ERO1 $\alpha$  active disulfide site and, among other mechanisms, requires covalent interaction with Cys397 (ref. 37). These inhibitors seemed to be the most effective drugs, suggesting the importance of both proteins in GDC survival (Fig. 1d, Extended Data Fig. 1b,c and Supplementary Table 2). Consistent with these findings, gene expression analysis of the Mayo Clinic Brain Tumour Patient-Derived Xenograft National Resource revealed a significant enrichment of ERO1 $\alpha$  messenger RNA (*ERO1L*) in the mesenchymal subtype (Fig. 1e,f and Extended Data Fig. 1d,e), aligning with the data found in the The Cancer Genome Atlas (TCGA) (Fig. 1g). Moreover, *ERO1L* displayed greater expression in GBM compared with normal brain (Extended Data Fig. 1f). Accordingly, histological analysis of a GBM tissue array confirms this pattern of expression at the protein level (Fig. 1j and Extended Data Fig. 2d). Notably, MCU and IP3R3 showed a similar pattern (Extended Data Fig. 1g). In line with this transcriptomic enrichment, we also found a significant enrichment of ERO1 $\alpha$  at the protein level in the whole-cell lysate (Fig. 1h) and MAM fraction (Fig. 1i) of the mesenchymal GDC models, further supporting its subtype-specific importance. However, no such enrichment was observed for other MAM-associated proteins, such as *HSPA9* (gene encoding GRP75), *ITPR1*, *ITPR3* (inositol 1,4,5-trisphosphate receptor type 1 and 3), *Erp44* (ER protein 44, involved in calcium exchange), *BAP31* (B cell receptor-associated protein 31, MAM tether) and *PPP2CA* (protein phosphatase 2 catalytic subunit  $\alpha$ , involved in cholesterol metabolism) (Fig. 1e and Extended Data Fig. 1e,g). Of note, ERO1 $\alpha$  was significantly more abundant in whole-cell lysate, MAMs and

cytoplasmic fractions compared with ERO1 $\beta$  (Extended Data Fig. 1h,i). Further interrogation of the TCGA dataset revealed that ERO1 $\alpha$  was the best MAM regulator among the different identified genes, whose mRNA expression was significantly correlated with poor survival in patients with GBM (Fig. 1k and Extended Data Fig. 2a–c), altogether guiding our study to focus on ERO1 $\alpha$ .

Collectively, our analysis identified ERO1 $\alpha$  as a central component of the MAM network in the mesenchymal GBM subtype, localized within the perinecrotic tumour regions,<sup>38</sup> a microenvironment characterized by severe cellular stress.

### ERO1 $\alpha$ fosters GDC aggressiveness in vitro

Given that ERO1 $\alpha$  expression was higher in perinecrotic GBM areas (Extended Data Fig. 1d), corresponding to the mesenchymal subtype, we speculated that this could represent a critical adaptation for tumour survival and progression. To test this hypothesis, we employed both genetic and pharmacological loss-of-function approaches to address the consequences of ERO1 $\alpha$  knockdown in several GBM patient-derived cells, subjecting them to glucose deprivation, a condition commonly encountered in these areas (Fig. 2a–c and Extended Data Fig. 3a–c). All mesenchymal GBM patient-derived cells (GDCs Ge518, Ge269 and Ge835) exhibited greater sensitivity to glucose deprivation compared with the classical or proneural subtypes, which displayed low or moderate sensitivity, respectively. A similar pattern, albeit more pronounced, was observed under EN460 treatment alone or in combination with glucose deprivation at 72 h (Fig. 2a and Extended Data Fig. 3a,b). Consistently, shRNA-mediated knockdown of ERO1 $\alpha$ , confirmed at the protein level (Fig. 2b), resulted in a significant and more pronounced decrease in cell viability in the mesenchymal subtype (Fig. 2c). To explore the mechanism underlying this decrease, we conducted a cell proliferation assay using Incucyte technology in GBM patient-derived cells from all subtypes upon EN460 treatment in high or low glucose conditions. We observed a significant and greater decrease in cell proliferation in the mesenchymal subtype (Fig. 2d and Extended Data Fig. 3c). Moreover, cell migration, assessed

**Fig. 4 | Inhibition of ERO1 $\alpha$  modulates Ca<sup>2+</sup> release in the several cellular compartments preferentially in mesenchymal GBM subtypes.** **a**, Schematic diagram representing Fluo-8, a cytoplasmic green fluorescent calcium binding dye and representative images of cells dyed with Fluo-8 (left) at  $t = 0$ ,  $t = 33$  and  $t = 100$  s; scale bar, 35  $\mu$ m. Middle graph displays Ca<sup>2+</sup> measurements upon ATP (600  $\mu$ M)-mediated release by IP3R in GDCs. Histograms show Fluo-8 peaks after addition of ATP to GDCs in the indicated conditions for 24 h ( $n = 490$ –721 cells in M;  $n = 403$ –612 cells in C;  $n = 371$ –532 cells in P; all in  $n = 4$  biological replicates). **b**, Representative images of cells expressing Mito-RGECO at  $t = 0$ ,  $t = 55$  and  $t = 400$  s, captured by live-cell confocal microscopy; scale bar, 50  $\mu$ m. The graph (middle) shows average peak heights relative to the average baseline, and the histograms (right) represent Mito-RGECO peaks after the addition of ATP to GDCs in the designated conditions for 24 h ( $n = 51$ –127 cells in M;  $n = 74$ –155 cells in C;  $n = 33$ –86 cells in P; all in  $n = 6$  biological replicates). **c**, Representative images of cells expressing ER-GAP1, an ER calcium sensor at  $t = 0$  and  $t = 500$  s; scale bar, 55  $\mu$ m. Graph displays the ER:GAP1 ratio following the addition of TBHQ (middle).

Plots show the quantitative analysis of the basal ER Ca<sup>2+</sup> stock in both specified conditions and GDCs (right) in the indicated conditions for 24 h ( $n = 63$ –92 cells in M;  $n = 29$ –53 cells in C;  $n = 48$ –73 cells in P; all in  $n = 6$  biological replicates). **d**, WCL lipid content was assessed in GDC Ge518 (top) and Ge970.2 (bottom) under EN460 treatment versus control (DMSO) in two independent experiments in the designated conditions for 24 h. **e**, Schematic diagram of phospholipid species and localization in MAM compartments (left). Pies display phospholipid percentage distribution in Ge518 (top) and Ge970.2 (bottom) in MAM fraction. **f**, Violin plots show the relative differences in PC, PE and PS content in Ge518 (left) and Ge970.2 (right), expressed in percentages ( $n = 4$  biological replicates) in the indicated conditions for 24 h in MAM fraction. **g**, Clusters display the relative proportion of PC, PE and PS content in Ge518 and Ge970.2 in MAM fraction. Histograms, violin plots and dot-clouds are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ; two (**e–g**) or one-way (**a–c**) ANOVA followed by Fisher (**b,c,f,g**), Tukey (**a**) or Bonferroni (**e**) multiple comparison tests.

Activation of ER stress was investigated and was found to correlate with both glucose deprivation and EN460 treatment (Extended Data Fig. 3f,g) and was associated with a slight increase in

**a**

ATP

Fluo-8

s: T = 0 T = 33 T = 100

Fluo-8 ( $F_1/F_0$ )

Time (s)

ATP

— HG+D  
— LG+D  
— HG+EN  
— LG+EN

Fluo-8 ( $F_1/F_0$ ) peak after ATP

M C P

**b**

ATP

Mito-R-GECO

s: T = 0 T = 55 T = 400

Mito-R-GECO ( $F_1/F_0$ )

Time (s)

ATP CCCP

— HG+D  
— LG+D  
— HG+EN  
— LG+EN

Mito-R-GECO ( $F_1/F_0$ ) peak after ATP

M C P

**c**

TBHQ

ER-GAP1

s: T = 0 T = 500

ER-GAP1 ratio ( $F_{470}/F_{403}$ )

Time (s)

ER-GAP1 ratio

M C P

**d**

M C

PC PE PS

DMSO EN460

WCL

**e**

ER luminal domain

MAMs

Inter-membrane space

Matrix

PS2 PS1

PA PS PE

PS PE

GDC 518

GDC 970.2

**f**

GDC

518 vs 970.2

PC PE PS

GDC 518

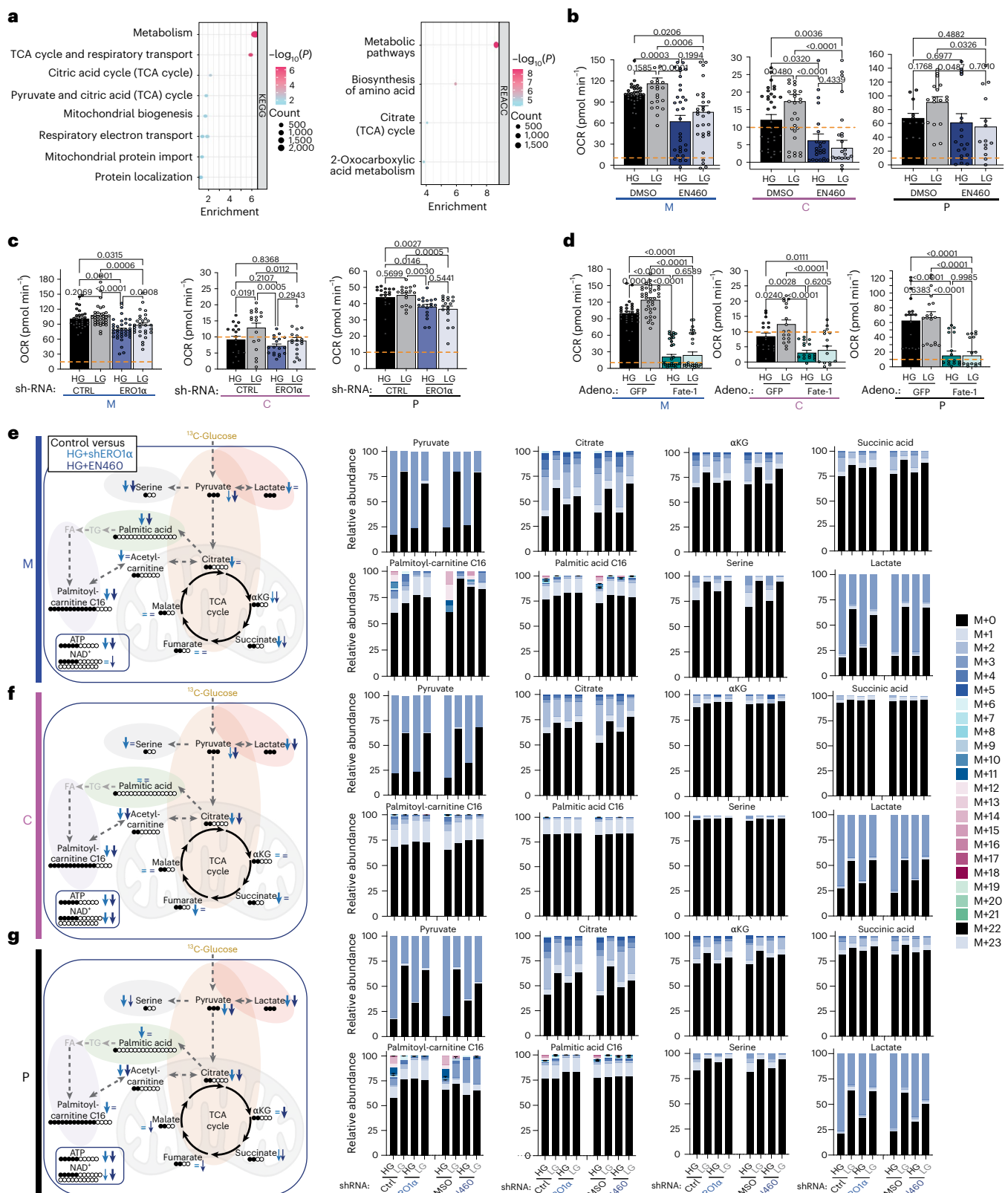
**g**

M C

PC PE PS

HG+D LG+D HG+EN LG+EN





occurs specifically under glucose deprivation conditions. Given the strong impact of ERO1α inhibition on the proliferation and survival of the mesenchymal subtype, we wondered whether EN460 treatment might reshape MAM protein composition. By analysing the significantly dysregulated proteins in MAMs under EN460 treatment using mass spectrometry (MS) data from the cell fractionation, we discovered a depletion of proteins involved in cell cycle regulation (Fig. 2g) and

an enrichment of proteins involved in mitochondrial organization as well as cell metabolism (Fig. 2h and Supplementary Table 3). These alterations reflect a shift in GBM cell behaviour under EN460 treatment, consistent with our previous findings.

ERO1α, along with PDI (protein disulfide-isomerase), is one of the two proteins of the pivotal pathway that catalyses oxidative protein folding in the ER. As the reduced state of the ER favours ERO1α activity,

**Fig. 5 | ERO1 $\alpha$  blockade induces a decrease in oxidative phosphorylation in GBM.** **a**, Dot plots represent protein enrichment analysis of the up regulated proteins of MAM content in GDCs mesenchymal treated with EN460 for 24 h, where dot size represents protein count and dot colour the  $-\log_{10} P$  value. Gene Set Enrichment Analysis was conducted using g:Profiler, applying a hypergeometric test (equivalent to a one-sided Fisher's exact test), with multiple testing correction applied using the g:SCS method as implemented in g:Profiler. KEGG, Kyoto Encyclopedia of Genes and Genomes. **b**, Bioenergetic responses of GDCs to metabolic inhibitors evaluated using Mito Stress kit. Histograms show OCR measurements in  $\text{pmol min}^{-1}$  in Ge518 (left), Ge970.2 (middle) and Ge258 (right) under the indicated conditions for 24 h ( $n = 12$ –31 technical replicates in  $n = 7$ –9 biological replicates). **c**, same as **b** following ERO1 $\alpha$  knockdown ( $n = 18$ –36 technical replicates in  $n = 3$  biological replicates). **d**, or following AdenoFate-1 infection ( $n = 18$ –36 technical replicates in  $n = 3$  biological replicates).

**e–g**, Diagrams and isotopologue distributions depicting  $^{13}\text{C}$ -glucose tracing in the indicated GDCs ( $n = 3$  biological replicates) under the specified conditions for 24 h. Left, schematic summary of the major metabolic effects induced by reduced expression (light blue) or activity (dark blue), highlighting pathway-specific changes (colour code: red, glycolysis; orange, OXPHOS; grey, glycine/serine metabolism; green, lipid storage; blue,  $\beta$ -oxidation). Large arrows indicate pronounced decreases; thin arrows, modest decreases; '=' indicates no detectable change. Black dots denote the principal labelled carbon positions for each metabolite. Right, distribution of  $^{13}\text{C}$ -glucose-derived isotopologues across metabolites (M + 0, unlabelled; M + 1 to M + 23,  $^{13}\text{C}$ -labelled).  $\alpha\text{KG}$ ,  $\alpha$ -ketoglutarate. Histograms and dot plots are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ; one-way (**b–d**) ANOVA followed by Fisher's (**b–d**) multiple comparison test.

we next measured the ER redox poise using ER-roGFP2, and observed a significant decrease in ER oxidation under both EN460 treatment and glucose deprivation (Extended Data Fig. 3j). As ERO1 $\alpha$  has two main redox isoforms with different electrophoretic mobilities (Ox1 and Ox2), representing the active and inactive states, respectively, we then assessed the reduced and oxidative states of ERO1 $\alpha$  by immunoblotting with non-reducing gels. Here, dithiothreitol (DTT) and diamide were used as fully reduced and oxidized controls, respectively (Fig. 2i). Under glucose deprivation, the oxidative form of ERO1 $\alpha$ , notably Ox1, which is associated with its activity, was increased, whereas EN460 prevented its oxidation, consistent with the fact that EN460 is well known to prevent electron transfer to ERO1 $\alpha$  and to freeze ERO1 $\alpha$  in its reduced form (Fig. 2i). Collectively, our results revealed that both glucose deprivation and EN460 treatment mainly affect the mesenchymal GBM subtype, altering its viability, proliferation and migration. The increase in ERO1 $\alpha$ 's oxidized forms, correlated with changes in ER redox state, is indicative of a metabolic response to energy stress.

### ERO1 $\alpha$ plays a critical role in ensuring MAM activity

To gain further insight into the interaction of ERO1 $\alpha$  with the ER and mitochondria, we assessed mitochondria morphology, including size and shape, together with the distance between the ER and mitochondria, following EN460 treatment, either by immunofluorescence (IF) (ERp57, ER resident protein 57 and MitoTracker staining) (Fig. 3a,b), electron microscopy (EM) (Fig. 3c–g and Extended Data Fig. 4a–c) or in situ proximity ligation assay (PLA) using IP3R and VDAC staining (Extended Data Fig. 4d). ERO1 $\alpha$  blockade led to a significant decrease in the length of contacts between the ER and mitochondria, in both GDC

Ge518 (M) and Ge970.2 (C), whereas it increased distance exclusively in Ge518 (Fig. 3a–g and Extended Data Fig. 4a–d). Notably, we observed an increased proximity in short distance (0–20 nm) under low glucose (LG) conditions (Fig. 3e and Extended Data Fig. 4a), known to favour  $\text{Ca}^{2+}$  transfer between the ER and mitochondria. In contrast, under EN460 treatment, a decrease in short distance (0–20 nm) associated with an increased in long distance (30–50 nm) was found (Fig. 3e and Extended Data Fig. 4a). In addition, a significant increase in mitochondrial fission (Fig. 3h,i), without inducing an increase in mitochondrial DNA (mtDNA) (Extended Data Fig. 4e,f), was observed in all subtypes. Notably, an increase in mtDNA, as evidenced by the level of *COX1* mt gene copy number in this subtype, a mitochondrial gene encoding a subunit of cytochrome C oxidase, was found in Ge970.2 compared with other subtypes (Extended Data Fig. 4e). Therefore, inhibition of the MAM protein ERO1 $\alpha$  triggers a decrease in cell viability together with an increase in MAM width and mitochondria fission. We then wondered whether increasing the distance between MAMs using the Fate-1 (fetal and adult testis expressed 1) spacer, as previously reported<sup>41</sup>, would copy the inhibition of ERO1 $\alpha$  and lead to a similar reduction in cell viability. As expected, following Fate-1 infection in GDC Ge518 (M), we observed a significant decrease in IP3R3 and VDAC1 interactions by PLA, reflecting looser ER–mitochondria contact sites (Fig. 3j,k). Although this decrease was less pronounced, we observed a similar effect in GDC Ge970.2 (C) and Ge258 (P) (Fig. 3k). As demonstrated in Fig. 3l, expression of the Fate-1 spacer led to a massive decrease in cell viability in all GBM patient-derived cells regardless of the experimental condition (Fig. 3l and Extended Data Fig. 4g). Of note, reducing the distance between MAMs using the L5 linker did not affect GDC Ge970.2

**Fig. 6 | GSC MAM network analysis highlights ERO1 $\alpha$  as fostering GSC aggressiveness, MAM interaction and metabolism.** **a**, Western blot showing the expression of ERO1 $\alpha$  in the WCL of all GSC models, normalized against  $\beta$ -actin expression. Histogram represents the fold change of protein expression determined by densitometry analysis ( $n = 1$ –8 biological replicates). **b**, Expression of ERO1 $\alpha$  in the MAMs fractions of Ge518 (M), Ge970.2 (C) and Ge258 (P) GSC cell lines. **c**, Left, representative images of spheroid area; scale bar, 200  $\mu\text{m}$ . Middle, Spheroid area (left,  $n = 12$ –24 spheres in  $n = 3$  biological replicates) and cell viability (right,  $n = 18$ –36 spheres in  $n = 3$  biological replicates) were measured by eVOS M5000 microscopy and CellTiter-Glo at the indicated conditions for 96 h. Right, histograms show the fold change of cell viability, quantified using CellTiter-Glo, following AdenoFate-1 infection (48 h) at the specified conditions for 96 h ( $n = 17$ –24 spheres in  $n = 3$  biological replicates). **d**, Top, schematic diagram depicting the Fate-1 molecular tool. Bottom, histograms show the fold change of cell viability, quantified using CellTiter-Glo, following AdenoFate-1 infection (48 h) at the specified conditions for 96 h ( $n = 17$ –24 spheres in  $n = 3$  biological replicates). **e**, Representative images of GSCs in Matrigel at day 0 and day 3 under described conditions; scale bar, 50  $\mu\text{m}$ . **f**, Box plots displaying relative 3D cell invasion of the indicated GDCs, under specified conditions ( $n = 14$ –16 spheres for M;  $n = 14$ –15 spheres for C;  $n = 11$ –13 spheres for P; all in  $n = 3$  biological replicates). Box-and-whisker plots show median (centre line), interquartile range (25th–75th percentiles) and

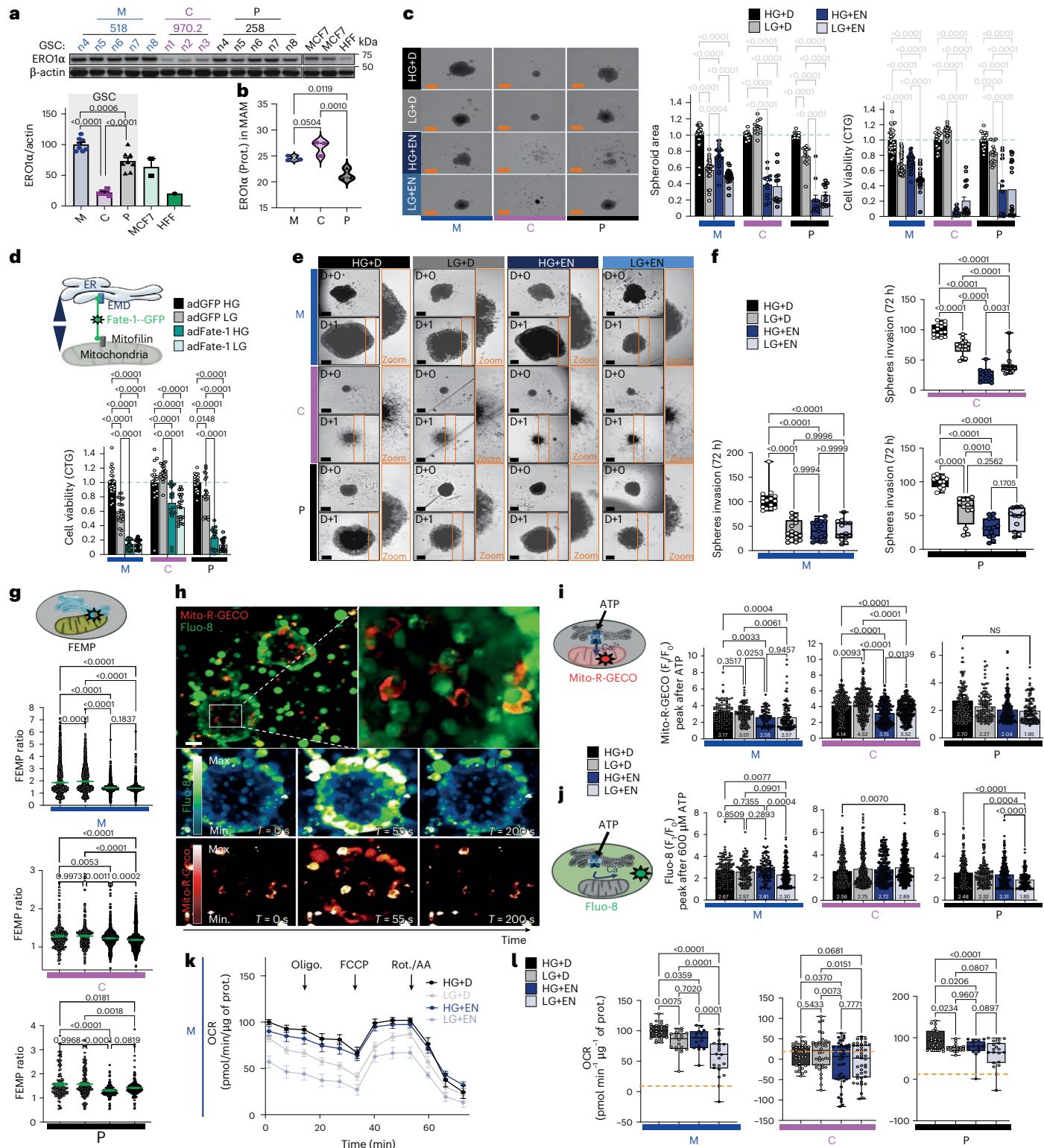
min–max (whiskers); all points are shown. **g**, Schematic diagram of the FEMP probe targeted to the mitochondrial outer membrane and ER (top). Violin plots showing the proximity between the ER and mitochondria in the indicated GSCs, under specified conditions for 24 h ( $n = 2,414$ –3,353 cells for M;  $n = 343$ –1,879 cells for C;  $n = 130$ –272 for P). **h**, Representative images of cells expressing Mito-RGECO (red) at  $t = 0$ ,  $t = 55$  and  $t = 200$  s, and Fluo-8 (green) captured by live-cell confocal microscopy; scale bar, 50  $\mu\text{m}$ . **i**, Schematic diagram representing Mito-RGECO expression (left) and histograms representing Mito-RGECO average peak heights relative to the average baseline after the addition of ATP to GSCs in the designated conditions for 24 h (right,  $n = 64$ –124 cells for M;  $n = 257$ –301 cells for C;  $n = 114$ –208 for P; all in  $n = 4$ –7 biological replicates). **j**, Same as **g** with Fluo-8 ( $n = 106$ –171 cells for M;  $n = 256$ –396 cells for C;  $n = 148$ –269 for P; all in  $n = 4$ –7 biological replicates). **k**, **l**, Bioenergetic responses of GSCs to metabolic inhibitors evaluated using the Mito Stress kit. Curves (**k**) and box plots (**l**) show OCR measurements in  $\text{pmol min}^{-1}$  normalized against protein concentration in Ge518 (left), Ge970.2 (middle) and Ge258 (right) under the indicated conditions for 24 h ( $n = 16$ –41 spheres in  $n = 4$  biological replicates). Box-and-whisker plots show median (centre line), interquartile range (25th–75th percentiles) and min–max (whiskers); all points are shown. Histograms, box plots and dot-clouds are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ ; two (c middle, c right, d) or one-way (a,b,f,g,i,j,l) ANOVA followed by Fisher's (b,l), Tukey (c right, d,g,i,j) or Bonferroni (a, c middle) multiple comparison tests.

(C) but induced a significant decrease in GDC Ge518 (M) and GDC Ge258 (P) viability under HG concentrations compared with the inactive L1 linker (Extended Data Fig. 3h–j). Altogether, our data highlight how ERO1 $\alpha$  blockade impacts MAM structure and the distance between ER and mitochondria, particularly in mesenchymal GBM models.

### ERO1 $\alpha$ modulates the ER/mitochondria Ca<sup>2+</sup> fluxes preferentially in the mesenchymal GBM subtypes

ERO1 $\alpha$  has been reported to shut down the activity of the SERCA calcium pump<sup>42</sup> and activate IP3R1 (refs. 43,44), whose activity depends on its

oxidative state<sup>45,46</sup>, and which is well known for its role in regulating Ca<sup>2+</sup> flux between the ER and mitochondria<sup>23,35,47</sup>. By measuring Ca<sup>2+</sup> with the Fluo-8 AM probe, we observed a decrease in the cytoplasmic calcium pool following ERO1 $\alpha$  inhibition using EN460 treatment in all models (Fig. 4a). ER-Mitochondrial Ca<sup>2+</sup> transfer and Ca<sup>2+</sup> mitochondrial content, assessed using the Mito-R-GECO and 4mtD3cpv probes, respectively, was also impacted by EN460 and glucose deprivation in GDCs Ge518 (M) and Ge970.2 (C), but not in Ge258 (P) (Fig. 4b and Extended Data Fig. 5a–c). These results suggest that, in contrast to stress conditions using glucose deprivation, ERO1 $\alpha$  inhibition reduces





$\text{Ca}^{2+}$  transfer to the mitochondria. In contrast, the ER calcium pool was increased following ERO1 $\alpha$  inhibition using EN460 treatment and decreased by glucose deprivation (Fig. 4c). Consistent with the measurements of ER–mitochondria interactions (Fig. 3a–g), calcium analyses revealed that inhibition of ERO1 $\alpha$  prevents calcium transfer from the ER to the mitochondrial compartment. In contrast, glucose deprivation increases MAM interactions and  $\text{Ca}^{2+}$  transfer between the ER and mitochondria. Considering the role of MAMs in phospholipid synthesis and dynamics, we also investigated the distribution of phosphatidylcholines (PCs), phosphatidylethanolamines (PEs), phosphatidylinositols (PIs), sphingomyelins (SMs) and free-fatty acids in MAMs and whole-cell lysates from mesenchymal and classical subtypes using lipidomic and metabolomic analyses. While no modulation of phospholipids was detected in Ge970.2 lysates under EN460 treatment, Ge518 lysates exhibited a decrease in PCs, PEs and PSs (Fig. 4d–g and Extended Data Fig. 5d,e). However, when focusing on the MAM phospholipid content, no significant modulation was detected in Ge518 under EN460 treatment, whereas Ge970.2 displayed a slight but significant increase in PSs (Fig. 4e and Extended Data Fig. 5d,e). Our data thus highlight that ERO1 $\alpha$  inhibition preferentially modulates whole phospholipid content in the mesenchymal subtype.

### ERO1 $\alpha$ favours mitochondrial oxidative phosphorylation in GBM

ER-mitochondrial contact sites are essential not only for calcium signalling, organelle dynamics, and lipid biosynthesis but also for mitochondrial oxidative phosphorylation. MAMs serve as ‘calcium hot spots’ enabling efficient ER-to-mitochondria calcium transfer. This calcium flux stimulates mitochondrial dehydrogenases (such as pyruvate dehydrogenase; PDH), thereby enhancing the tricarboxylic acid (TCA) cycle-derived reducing equivalents and promoting oxidative phosphorylation<sup>23,47,48</sup>. Accordingly, a significant enrichment of proteins involved in the TCA cycle was found in MAMs of the mesenchymal subtype under EN460 treatment (Fig. 5a). Then, to assess mitochondrial respiration and glycolysis in our GBM patient-derived models, we employed the Seahorse XFe96 technology. We observed strong metabolic heterogeneity between GBM cells, ranging from more aerobic (Ge904, C), quiescent (Ge970.2, C) or glycolytic (Ge835, M) metabolism, all the way to a more energetic type of metabolism (Ge518 and Ge269, both mesenchymal) (Extended Data Fig. 6a). Under glucose-deprived conditions, most GBM cells displayed a tendency toward elevated oxygen consumption rates (OCRs) (Fig. 5b–d and Extended Data Fig. 6a). Of note, ERO1 $\alpha$  inhibition caused either a significant decrease or a trend toward a reduction in basal and maximal respiration in all GBM subtypes (Fig. 5b and Extended Data Fig. 6a,b). Similar data were observed with ERO1 $\alpha$  knockdown (Fig. 5c) in all models. Likewise, the extracellular cellular acidification rate (ECAR), which is considered an indicator of glycolysis, was markedly and significantly attenuated following ERO1 $\alpha$  blockade in all GDC subtypes, except for the proneural

GDC Ge258, where a trend was observed (Extended Data Fig. 6c,d). As expected, Fate-1 induced a significant decrease in OCR in all subtypes (Fig. 5d). Concerning ECAR, Fate-1 led to a significant decrease in all subtypes, except for the proneural GDC Ge258, where a trend was also observed (Extended Data Fig. 6e). In line with these findings, metabolomic analysis highlighted several decreased metabolites, including  $\alpha$ -ketoglutarate and succinate, under EN460 treatment in mesenchymal GBM (Fig. 5e and Extended Data Fig. 6f), demonstrating that ERO1 $\alpha$  favours mitochondrial oxidative phosphorylation and glycolysis, particularly in the mesenchymal GBM. Of note, labelling from  $^{13}\text{C}$ -glucose revealed a decrease in glycolytic intermediates and glycolysis-derived metabolites (amino acids such as serine, glycine and cystine), as well as TCA intermediates, in all models following ERO1 $\alpha$  genetic and pharmacological inhibition (Fig. 5e–g and Extended Data Fig. 6g).

### ERO1 $\alpha$ is also present in the GSC MAM network and its blockade induces a decrease in their aggressiveness, affects their MAM structure and cell metabolism

GSCs are able to survive hypoxia and nutrient deprivation within the harsh brain microenvironment, thus associated with more aggressive tumour phenotypes<sup>49–51</sup>. We first evaluated ERO1 $\alpha$  expression in our GSC models and observed its enrichment in the mesenchymal subtype, consistent with findings in GDCs (Fig. 6a). Likewise, MAM content analyses revealed the presence of ERO1 $\alpha$  in all subtypes (Fig. 6b, Extended Data Fig. 7a and Supplementary Table 4). We then conducted experiments akin to those using GDCs to better understand the impact of ERO1 $\alpha$  blockade on GSCs. Glucose deprivation reduced cell viability in GDCs Ge518 and Ge258, but not Ge970.2, whereas EN460 treatment led to a decrease in cell viability in all GSCs (Fig. 6c).

Like GDC, where all subtypes were highly sensitive to the MAM spacer Fate-1, we also observed a decrease in cell viability for the three subtypes, although it was less pronounced for GSC Ge970.2 (C) (Fig. 6d and Extended Data Fig. 7b). In contrast, Ge970.2 showed a greater sensitivity to the MAM linker, whereas Ge518 and Ge258 displayed a slight but not significant decrease in their viability (Extended Data Fig. 7c,d). Three-dimensional (3D) cell invasion experiments in Matrigel then revealed a significant reduction in the invasive capacity of all GSC under both EN460 treatment and glucose deprivation even though glucose deprivation induced a significant and greater decrease in the invasive capacity of GSC Ge518 (M) (Fig. 6e,f). We then used a FEMP (FRET-based indicator of ER–mitochondria proximity) probe, which measures the proximity between the ER and mitochondria, and observed a greater proximity between both organelles under glucose-deprived conditions exclusively in GSC Ge518 (Fig. 6g), confirmed by EM (Extended Data Fig. 7e–h), a pattern seen in the GDC model (Fig. 3d). In contrast, EN460 treatment induced organelles distancing across all subtypes (Fig. 6g), similar from GDCs, where distancing between 0 and 20 nm was only observed upon EN460 treatment (Extended Data Fig. 4a,b). Regarding

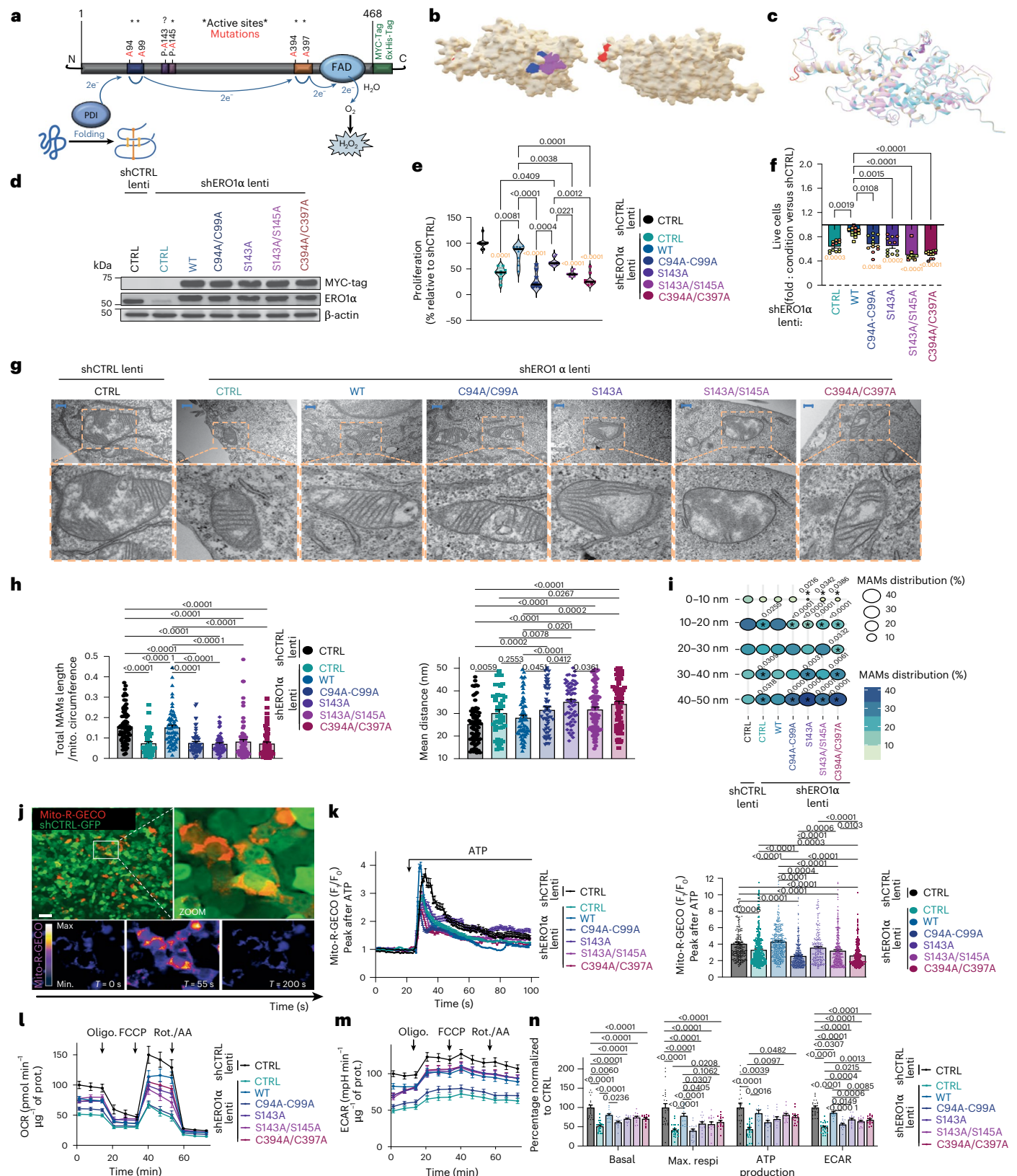
**Fig. 7 | ERO1 $\alpha$  mutations in the main activity sites alter cell metabolism by modulating MAM, causing repression of GBM aggressiveness.** **a**, ERO1 $\alpha$  structure diagram illustrating main sites involved in its activity mutated and used in our GBM models. **b**, ERO1 $\alpha$  3D structure showing at left the C94–C99 (CGRRDC, in magenta) and C394–C397 sites (CFKC, in blue) and at right, the S143–S145 sites (in red) by AlphaFold. **c**, Structure prediction of ERO1 $\alpha$  wild-type (WT) (beige) and ERO1 $\alpha$  mutant forms: C94–99A (blue), C394–397A (pink) by AlphaFold. **d**, Western blot showing the rescue expression of the described wt and mutant ERO1 $\alpha$  constructs in GDC Ge518, normalized against  $\beta$ -actin expression. **e**, Cell proliferation of all indicated ERO1 $\alpha$  constructs, evaluated using Incucyte technology in the following conditions ( $n = 9$  technical replicates in  $n = 3$  biological replicates). **f**, Viability of indicated ERO1 $\alpha$  constructs after 96 h, using a CellTiter-Glo kit ( $n = 12$  technical replicates in  $n = 3$  biological replicates). **g**, Representative EM images of GDC Ge518 of indicated ERO1 $\alpha$  constructs; scale bar, 0.2  $\mu\text{m}$ . **h**, **i**, EM quantifications. Average measurements between ER–mitochondria (MAM): total MAM length (**h**, left), distance (**h**, right) and MAM

distance distribution (**i**).  $n = 90$ –243 MAMs in  $n = 3$  biological replicates analysed for the indicated ERO1 $\alpha$  constructs in GDC Ge518. **j**, Representative images of cells expressing Mito-RGECO (red) in GDC Ge518 sh control (green) at  $t = 0$ ,  $t = 55$  and  $t = 200$  s, captured by live-cell confocal microscopy; scale bar, 100  $\mu\text{m}$ . **k**, Graph (left) shows average peak heights relative to the average baseline and histograms (right) represent Mito-RGECO peaks after the addition of ATP to GDCs in the designated ERO1 $\alpha$  constructs ( $n = 204$ –456 cells. All in  $n = 6$  biological replicates). **l**–**n**, Bioenergetic responses of the indicated ERO1 $\alpha$  constructs in GDC Ge518 evaluated using the Mito Stress kit. Curves (**l**, **m**) and histograms (**n**) show OCR measurements in  $\text{pmol min}^{-1}$  (**l**) and ECAR measurements in  $\text{pmol min}^{-1}$  (**m**) normalized against protein concentration in the indicated ERO1 $\alpha$  constructs in GDC Ge518 ( $n = 12$  technical replicates in  $n = 4$  biological replicates). Volin plots, histograms and curves are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  and  $P < 0.0001$ ; non-parametric, two-tailed test (Kruskal–Wallis, **e**, **f**) or one-way ANOVA (**h**, **i**, **k**, **n**) followed by Dunn’s (**e**, **f**), Tukey (**h** left, **k**, **n**) or Fisher’s (**h** right, **i**) multiple comparison tests.

ER-mitochondria  $\text{Ca}^{2+}$  transfer, the mesenchymal subtype displayed a reduction of  $\text{Ca}^{2+}$  flux under EN460 treatment, regardless of glucose concentration, whereas Ge970.2 (C) showed a higher rate of  $\text{Ca}^{2+}$  release under glucose deprivation and a reduction following EN460 treatment (Fig. 6h,i). No significant variation was observed in GSC Ge258 (P), irrespective of the experimental conditions (Fig. 6i).

Concerning cytoplasmic  $\text{Ca}^{2+}$ , we did not observe any modulation under EN460 treatment (Fig. 6j).

In parallel, the OCR in GSC Ge518 (M) and Ge258 (P) under both EN460 treatment and glucose deprivation was significantly decreased, whereas in Ge970.2 (C) we observed a significant decrease under EN460 treatment, but a trend toward an augmentation under





glucose deprivation (Fig. 6k,l). Of note, ECAR showed a similar pattern in GSCs Ge518 (M) with a significant decrease in LG and following EN460 treatment, whereas no modulation was found in GSC Ge970.2 regardless of the conditions and only glucose deprivation affected Ge258 (Extended Data Fig. 7i,j). Altogether, our data demonstrate how mesenchymal and proneural GSCs differently respond to stress and ERO1 $\alpha$  blockade compared with their respective GDCs, as exemplified by the decreased respiration at 24 h under glucose deprivation in GSC that is correlated with the ER–mitochondria Ca<sup>2+</sup> transfer.

### Active-site disruption of ERO1 $\alpha$ modulates MAM function and dampens GBM malignancy

As ERO1 $\alpha$  activity can be correlated with its phosphorylation<sup>52,53</sup>, we also aimed at evaluating the status of three putative phosphorylation sites identified at positions 106, 143 and 145 on serine residues in ERO1 $\alpha$ . Phospho-specific antibodies against p106, p143 and p145 generated for this purpose were validated by immunoblotting to the active form of ERO1 $\alpha$  (Extended Data Fig. 8b,c). No phosphorylation was detected at position 106 (no detected bands by immunoblotting). Of note, in non-reducing gel, we observed ERO1 $\alpha$  phosphorylation around 120 kDa, which could be explained by either the presence of a dimerized form or by its interaction with another protein partner (Extended Data Fig. 8d). Under EN460 treatment, ERO1 $\alpha$  activation was lower at positions 145 and 143, whereas glucose deprivation induced a significant increase in 145 phosphorylation (Extended Data Fig. 8d,e). These data showed that ERO1 $\alpha$  activity correlated with changes in its phosphorylation (Extended Data Fig. 8f). Then, to identify the important domains of ERO1 $\alpha$  involved in MAM interaction and function, the endogenous ERO1 $\alpha$  gene was silenced using lentiviral shRNA transduction. Regarding its primary catalytic activity, ERO1 $\alpha$  oxidizes PDI through sequential disulfide exchange involving its regulatory (C94–C99) and catalytic (C394–C397) cysteine pairs<sup>54,55</sup>, thereby maintaining PDI in its active state and promoting efficient oxidative protein folding in the ER<sup>53</sup>. As shown in Fig. 7a, several point mutation constructs unaffected by the shERO1 $\alpha$ , were generated, without affecting the protein's structure, as assessed by AlphaFold predictions (Fig. 7b,c), in the following regions of the ERO1 $\alpha$ : C94A–C97A (regulatory disulfide site; in magenta in Fig. 7b), S143A, S143A/S145A (both phosphorylation sites in red in Fig. 7b) and C394A/C397A (catalytic disulfide site; in blue in Fig. 7b). Efficient transduction of these mutants and ERO1 $\alpha$ -wt back into silenced cells was validated by western blotting (Fig. 7d). Notably, all mutants significantly decreased cell viability (Fig. 7e,f). EM analysis revealed a significant reduction in MAM length and an increase of MAM distance following ERO1 $\alpha$  knockdown and subsequent ectopic expression of ERO1 $\alpha$  mutants, whereas ectopic expression of ERO1 $\alpha$ -wt rescued the original phenotype (Fig. 7g–i and Extended Data Fig. 8a). Functionally, ER–mitochondria Ca<sup>2+</sup> transfer was significantly decreased (Fig. 7j,k), consistent with the observed reduction of OCR and ECAR (Fig. 7l–n),

in all mutants. Altogether, our data demonstrated the critical roles of the regulatory and catalytic disulfide sites, as well as the 145 and 143 phosphorylation sites, in ERO1 $\alpha$ 's role involved in MAM interaction and function.

### Targeting ERO1 $\alpha$ to halt GBM progression in vivo

Human brain organoids derived from pluripotent stem cells have emerged as powerful and relevant models for recapitulating the cytoarchitecture of the human brain and studying biological mechanisms and cellular processes<sup>56,57</sup>. Here, we generated brain organoids<sup>58</sup>, and co-cultured them with our GSC models. Analogous to glucose deprivation, EN460 significantly reduced the invasive and proliferative capacities of GSCs in the organoids (Fig. 8a–d), which was expected considering the effect of glucose deprivation and EN460 treatment on GSC Ge518 (Fig. 6c,f). Notably, we did not observe any impact of glucose deprivation or EN460 treatment on organoid size (Fig. 8d). Upon implementation of a doxycycline-regulated system enabling temporal repression of ERO1 $\alpha$ , validated in vitro at the protein level (Extended Data Fig. 9a,b) and by immunofluorescence (Extended Data Fig. 9c), we confirmed the decrease of Ge518 cell viability following doxycycline treatment. Moreover, we recapitulated the biological effects previously achieved with constitutive ERO1 $\alpha$  depletion in organoid models. (Extended Data Fig. 9c). Similar findings were likewise obtained when employing either Fate-1 or Linker (Extended Data Fig. 9d–f).

We next examined the therapeutic potential of EN460, predicted to cross the blood–brain barrier (Extended Data Fig. 10g). Its effects were evaluated in multiple in vivo systems, including zebrafish embryos and mice bearing intracranial tumours derived from our GSC models; as well as one GBM syngeneic model, SB28. Larval zebrafish xenograft models are a useful and suitable platform for early proof-of-principle studies and drug development. We generated xenografts by intracranially injecting Ad-mCherry-labelled GSCs into transgenic Tg(fli1:EGFP) zebrafish larvae. This approach allows for visualization of the vasculature and provides a neural context for the GBM tumours. Following GSCs injection into the optic tectum of zebrafish larvae, we found that EN460 treatment resulted in a significant reduction in both tumour cell invasion and growth (Fig. 8e–g), highlighting EN460 as a promising therapeutic agent for GBM. Then, to investigate the in vivo therapeutic impact of targeting ERO1 $\alpha$  and MAM interactions, we implanted immunodeficient nude mice with doxycycline-inducible ERO1 $\alpha$ -silencing or Fate-1-overexpressing Ge518 GSCs, along with their respective controls (Fig. 8h–l and Extended Data Fig. 9h). Administration of doxycycline in the drinking water significantly extended survival and slowed tumour growth, as assessed by bioluminescence imaging (Extended Data Fig. 9k,l). This effect was further enhanced in tumours bearing Fate-1-overexpressing cells compared with controls (Extended Data Fig. 9k,l). Consistently, mice bearing Ge518 GSCs Fate-1

### Fig. 8 | ERO1 $\alpha$ blockade delays GBM progression in several in vivo models.

**a**, Schematic diagram showing the brain organoid co-culture with mesenchymal GSCs, Ge518. **b**, Representative images of the co-culture (brain organoid + Ge518) in the indicated conditions for 1 week; scale bar, 100  $\mu$ m. **c**, Violin plots displaying tumour cell area per organoid in the designated conditions ( $n = 8$  biological replicates). **d**, Violin plots displaying organoid area in the designated conditions. **e**, Schematic diagram of brain cell injections in zebrafish larvae and image acquisition strategy. **f**, Representative images of zebrafish head in both shCTRL versus shERO1 $\alpha$ ; scale bar, 50  $\mu$ m. **g**, Box plots of tumour area in shCTRL versus shERO1 $\alpha$  zebrafish larvae ( $n = 5$ –7 zebrafish larvae) at  $t = 0$  (top) and  $t = 48$  h after GSC injection. Box-and-whisker plots show median (centre line), mean (dotted line), interquartile range (25th–75th percentiles) and min–max (whiskers). **h**, Experimental design to assess in vivo effects of doxycycline-inducible ERO1 $\alpha$ -silencing or Fate-1-overexpressing on xenograft GSC Ge518. **i**, Representative brain sections in the described conditions stained with HPS, showing the tumour boundary delineated by the yellow outline. HPS, hematoxylin phloxine saffron. **j**, Kaplan–Meier survival curve of female nu/nu nude immunocompromised

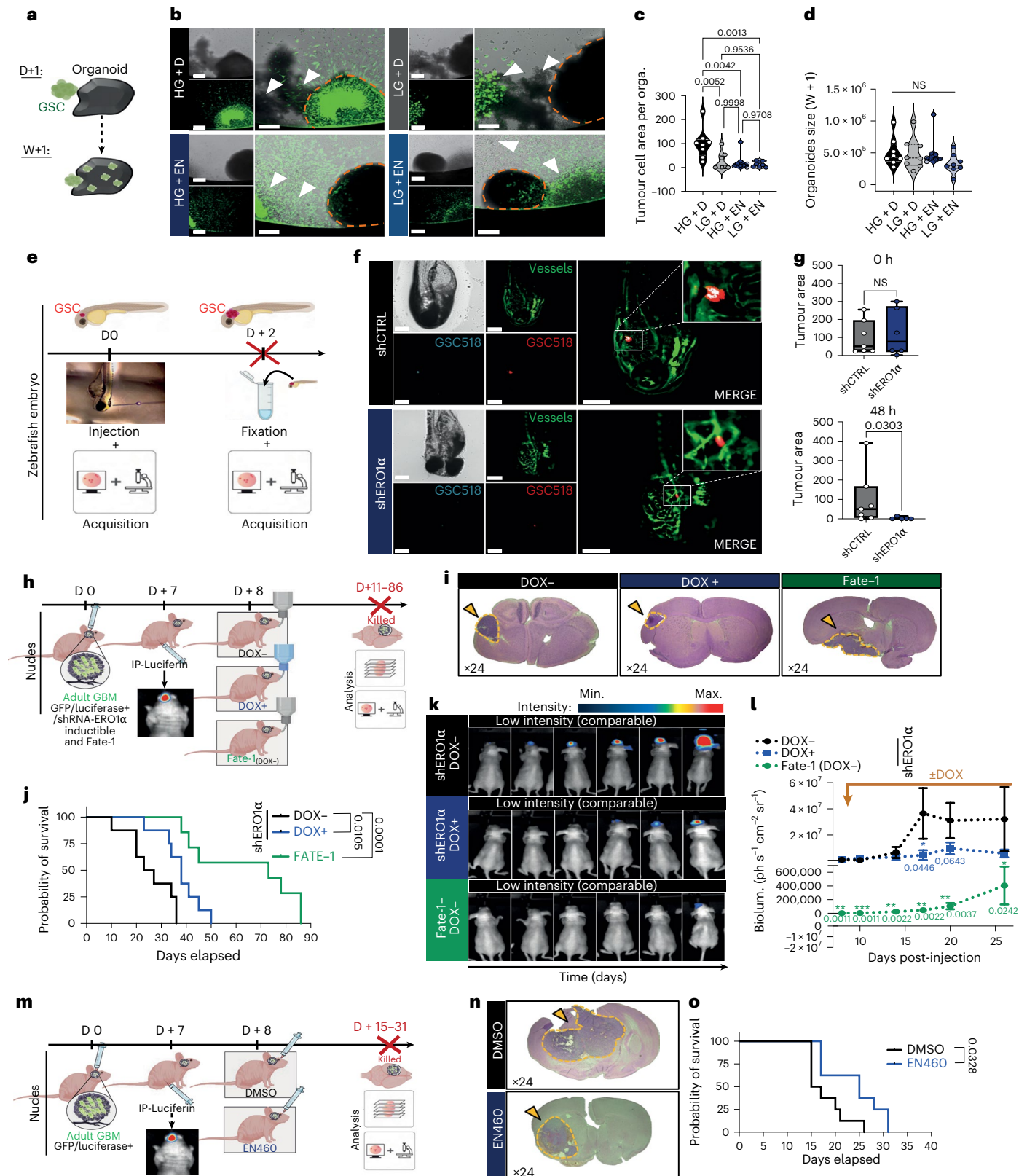
mice bearing GSC Ge 518 placed with drinking water containing doxycycline or not ( $n = 7$ –8 mice per group). **k**, Representative images illustrating comparable tumour bioluminescence intensities over time under the indicated conditions. **l**, Dot plots showing the quantification of the bioluminescence signal ( $\text{ph s}^{-1} \text{cm}^{-2} \text{sr}^{-1}$ ) over time under the indicated conditions ( $n = 8$  mice in shERO1 $\alpha$ -Dox- and shERO1 $\alpha$ -Dox-;  $n = 7$  mice in Fate-1-Dox- at  $t = 0$ ). **m**, Experimental design to assess in vivo effects of EN460 on xenograft GSC Ge518. **n**, Representative brain sections in the described conditions stained with HPS, showing the tumour boundary delineated by the yellow outline. **o**, Kaplan–Meier survival curve evaluating the effect of EN460 treatment on Ge518 tumour growth in vivo in female nu/nu nude immunocompromised mice ( $n = 8$  mice per group). Violin plots, box plots and curves are expressed with mean  $\pm$  s.e.m. \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ ; one-way ANOVA (**c,d,l**) followed by Tukey (**c,d**) or Fisher's (**l**) multiple comparison tests; Mann–Whitney non-parametric test (**g**) or log-rank (Mantel–Cox) test (**j,o**). Panels created in BioRender: **e**, Bassot, A. <https://biorender.com/q8cooyf> (2026); **h**, Bassot, A. <https://biorender.com/04gm28h> (2026); **m**, Bassot, A. <https://biorender.com/e1w5lcf> (2026).



tumours also exhibited a marked survival benefit relative to the control group (Fig. 8j). Histological analyses revealed increased ERO1 $\alpha$  expression, but not GRP75 within the tumour core (Extended Data Fig. 9i–l), underscoring the central role of ERO1 $\alpha$  in this phenotype.

To further evaluate the *in vivo* therapeutic effects of ERO1 $\alpha$  inhibition by EN460, we used both immunodeficient nude and immunocompetent mice by injecting Ge518 GSCs and the syngeneic SB28 model,

respectively (Fig. 8m–o and Extended Data Figs. 9a,b and 10c–h). *In vitro*, SB28 glioblastoma cells displayed a significant reduction in viability following either genetic or pharmacological inhibition of ERO1 $\alpha$  (Extended Data Fig. 10d). Notably, SB28 cells expressed lower baseline levels of ERO1 $\alpha$  than Ge518 cells, which likely explains their reduced sensitivity to EN460 compared with GSCs or GDCs (Extended Data Fig. 7c). Consistent with the ERO1 $\alpha$  knockdown *in vivo* experiment, mice bearing



Ge518 (Fig. 8o) or SB28 (Extended Data Fig. 10f) tumours exhibited significantly prolonged survival compared with control animals, with a more pronounced effect observed in nude mice injected with Ge518 GSCs. Tumours also displayed markedly higher ERO1 $\alpha$  expression relative to the surrounding brain tissue (Extended Data Fig. 10a,b,g–i). Taken together, these results demonstrate that systemic inhibition of ERO1 $\alpha$  using EN460 provides a therapeutically meaningful survival benefit in GBM models, establishing ERO1 $\alpha$  blockade as a promising treatment strategy.

## Discussion

GBM aggressiveness and treatment failure are largely attributed to its high level of intra- and intertumoral heterogeneity, which is partly influenced by the tumour microenvironment. Here, we focused on ERO1 $\alpha$  at MAMs and their pivotal function in GBM metabolic flexibility by analysing several patient-derived GDC and GSC models, enabling a comprehensive and in-depth investigation across all GBM subtypes.

By isolating and analysing MAM content, we identified ERO1 $\alpha$  as a central player of MAM function and dynamics. We used a focused library of potent, well-characterized inhibitors of MAM proteins and, with EN460, also identified Mkt-077, inhibitor of Grp75, as an interesting target. However, Grp75 would only be a viable therapeutic if selective delivery to GBM cells could be achieved while sparing Grp75-expressing normal brain parenchyma and systemic tissues. Moreover, its clinical relevance is limited, as its expression does not correlate with patient survival.

Our data showed an activation of the PERK signalling pathway following glucose deprivation, which was inhibited under EN460 treatment. Consistently, we observed inhibition of CHOP and ATF4 (activating transcription factor 4) expression, downstream to PERK, under the same conditions. As mentioned above, H<sub>2</sub>O<sub>2</sub> represents a byproduct of ERO1 $\alpha$  oxidase activity<sup>59,60</sup>, which can account for up to 25% of the cellular ROS produced during protein synthesis<sup>61</sup>. Consequently, ERO1 $\alpha$  oxidase activity has to be strictly regulated to avoid ROS accumulation at steady state and to be quickly activated once a burst of oxidative protein folding capacity is needed, for instance under stress conditions<sup>62</sup>. In line with this, an increase, but not significant, in intracellular ROS production was observed upon glucose deprivation in our models.

Notably, we demonstrated a different response between our differentiated and stem cell models, in which an increase in Ca<sup>2+</sup> transfer occurs and is associated with augmented respiration under glucose deprivation. No modulation (except for the classical model) was shown for the GSCs, which correlates with a decreased respiration in the mesenchymal and proneural models (but not classical). This discrepancy between subtypes may be due to the classical model's low OCR level. In addition, the mesenchymal model relies on glucose for survival, which is not the case for the classical model, as evidenced by our cell viability assays.

ERO1 $\alpha$  expression is higher in several cancer types and its expression levels correlate with cancer prognosis and progression<sup>63,64</sup>. Accumulating evidence suggests that ERO1 $\alpha$  impacts tumour aggressiveness. Nonetheless, no pharmacological inhibitor is currently available for clinical use. In this study we used two well-validated commercial inhibitors of ERO1 $\alpha$ , namely EN460 and QM295. EN460 binds ERO1 $\alpha$  in the FAD (flavin adenine dinucleotide) binding pocket and leads to the loss of FAD coenzyme, preventing ERO1 $\alpha$  reoxidation. EN460 binds the FAD binding pocket, which is conserved across other enzymes; thus giving rise to its potential off targets. Although EN460 has relatively low potency in vivo, targeting ERO1 $\alpha$  might represent a promising antitumour strategy, as highlighted by our mouse models' significant delay in tumour growth.

Collectively, by revealing a direct link between ERO1 $\alpha$  and Ca<sup>2+</sup> flux, MAM dynamics/structure and cell metabolism, this study identifies a relevant druggable vulnerability that can be exploited for GBM therapy.

## Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41556-026-01980-2>.

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## Methods

This research study complies with all relevant ethical regulations.

### Differentiated and stem cell patient-derived cell lines

Several patient-derived GBM models were used in this study. GDCs were maintained in DMEM supplemented with FBS 10% and antibiotics, whereas GSCs were cultured in DMEM/F12 with GlutaMAX supplemented with B27 supplement and b-FGF, EGF both at 10 ng ml<sup>-1</sup> with antibiotics (GSC medium). Ge269, 518, 688, 738, 835, 258, 904 and 970.2 were gifts from V. Dutoit and P.-Y. Dietrich to E. Cosset. Both GDCs and GSCs were confirmed to be *Mycoplasma* negative before experiments. Ge269, 738, 835, 904, 970.2 and 258 originated from male patients, whereas Ge518 and 688 originated from female patients. The patient-derived models used in the study were obtained through a material transfer agreement and were collected in a biobanking protocol, with no access to patient clinical data, except gender, approved by the ethics committee of Geneva. All patients provided written informed consent and did not receive any compensation. The molecular subtyping was evaluated by quantitative PCR with reverse transcription (qRT-PCR) and genomic DNA analysis (Ion Ampliseq Cancer Hotspot Panel v2) revealed a MET mutation in Ge518 (ref. 65).

### Chemicals

EN460 (HY-1283), DTT (441496P) and diamide (D3648) were purchased from MedChem Express, VWR and Sigma-Aldrich, respectively and were used at the respective concentrations of 25  $\mu$ M, 10 mM and 5 mM. Then, Gkt (Sigma, 5.34032), Ru265 (Sigma, 27056-01-05), TBHQ (tert-butylhydroquinone 97%, Sigma, 112941), Mkt-077 (MedChem Express, HY-15096) and xestospongine C (Cayman, 64950) were used at the concentrations indicated in the figures.

### Lentivectors

The lentiviral shRNAs ERO1 $\alpha$  (Horizon, RHS3979-201777540 and RHS3979-201777542) were used to knockdown ERO1 $\alpha$  expression. The firefly luciferase–GFP lentivirus (CAG, Puro) (Cellomics, PLV-10174-50) was used for the in vivo experiments.

### Lentivectors expressing ERO1A mutants

To perform rescue experiments in ERO1A-silenced cells, we first introduced by In-Fusion cloning (Takara Bio), silent point mutations in the region that is targeted by the shRNA in the ERO1A-myc complementary DNA<sup>66</sup>. This cDNA was then re-cloned into the CSII-puro lentivector with primers ERO1-CSII-F and ERO1-CSII-R and subsequently mutated on serine 143, serine 145, serines 153 and 145, cysteines C94 and 99, and cysteines 394 and 397 to generate the vectors expressing ERO1 $\alpha$ -wt, ERO1 $\alpha$ -S143A, ERO1 $\alpha$ -S145A, ERO1 $\alpha$ -S143A/S145A, ERO1 $\alpha$ -C94A/C99A and ERO1 $\alpha$ -C394A/C397A, respectively. All vectors were verified by Sanger sequencing.

### Primers used for ERO1A mutagenesis

The primers used for ERO1A mutagenesis were purchased from IDT-DNA Europe and are presented in Supplementary Table 5.

### Cell fractionation

Subcellular fractionation was conducted on GDCs and GSCs cells in accordance to the literature<sup>67</sup>. In brief, cells were grown to 80–90% confluency in ten 10-cm dishes for GDC cells and eight T75 flask for GSC cells per condition and resuspended in 5 ml of pre-cold homogenization buffer (0.25 M sucrose, 10 mM HEPES–NaOH, 1 mM EDTA and 1 mM EGTA, pH 7.4) supplemented with protease and phosphatase inhibitors. After centrifugation (400g, 10 min), the pellets were resuspended in 1 ml of homogenization buffer and were passaged at least 30 times through 26G<sup>5/8</sup>-gauge syringes during 10 min on ice. Broken cells states were evaluated with Trypan-blue staining for each cell line. Broken cells were centrifuged several times at 700g (1  $\times$  10 min and

2  $\times$  5 min at 4 °C) to remove the nucleus part (pellet). The supernatant was centrifuged for 10 min at 10,000g (4 °C) and separated into two parts (1) and (2).

Then, (1) the supernatant containing cytosol and light membranes was centrifuged twice for 10 min at 10,000g (4 °C) to remove heavy materials and for 1 h at 100,000g (4 °C) to separate light membranes contained in the pellet and cytosol contained in the supernatant. After one night at –20 °C with cold acetone, the cytosol precipitate part was centrifuged at 16,000g for 20 min (4 °C).

In parallel, (2) heavy membranes (mito. crude) containing mitochondria and MAMs were centrifuged twice for 10 min at 10,000g (4 °C) to remove light materials. The pellet was resuspended in 1 ml homogenization buffer, deposited on 8 ml of 18% Percoll (GE17-0891-02) homogenization buffer and centrifuged for 35 min at 100,000g (4 °C). Then, the MAM fractions (top band) were centrifuged at 100,000g for 1 h at 4 °C and the pellet was collected as the MAM fraction. The mitochondria fractions (bottom band) were centrifuged at 12,000g (4 °C) for 15 min and at 16,000g (4 °C) for 20 min and the pellet was collected as the pure mitochondria fraction<sup>67</sup>.

### Immunoblotting

RIPA lysis buffer (RIPA buffer; 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 1% NP-40 and 0.25% sodium deoxycholate), supplemented with protease and phosphatase inhibitors was used to prepare cell lysates, which were then quantified using the Pierce BCA kit (Thermo Fisher). Nuclei and unbroken cells were removed by 12,000g centrifugation for 15 min (4 °C). Then, 15–20  $\mu$ g of protein was prepared, mixed with Laemmli buffer containing DTT and boiled at 95 °C for 5 min. Samples were loaded onto a denaturing SDS–PAGE, transferred to PVDF membranes and blotted with anti-rabbit or anti-mouse HRP-conjugated secondary antibodies (Bio-Rad or Invitrogen). The following antibodies were used for immunoblotting: ERO1 $\alpha$  (GeneTex, GTX112589, 1:1,000 dilution), PERK (Abcam, ab65142, 1:800 dilution), p-PERK (R&D, AF3999, 1:800 dilution), EIF2 $\alpha$  (Cell Signalling, 5324S, 1:1,000 dilution), p-EIF2 $\alpha$  (Cell Signalling, 3398S, 1:1,000 dilution), ATF4 (Cell Signalling, 11815S, 1:1,000 dilution), CHOP (Cell Signalling, 2895T, 1:1,000 dilution), ERO1 $\beta$  (Abcam, 197290, 1:500 dilution),  $\alpha$ -tubulin (Thermo Scientific, MA1-850, 1:10,000 dilution), SERCA2 (Abcam, ab150435, 1:1,000 dilution), VDAC1/3 (Abcam, 14734, 1:1,000 dilution), MCU (Cell Signalling, 14997S, 1:1,000 dilution) and  $\beta$ -actin (Sigma, A3854, 1:10,000 dilution). For the non-reducing experiments, fresh samples were collected and prepared in Laemmli buffer free of DTT and loaded on SDS–PAGE (without a boiling step) the same day.

### Cell viability

Cell viability was assessed through two different readouts of CellTiter-Glo assay kit (CTG, Promega) and CellTiter-Fluor assay kit (CTF, Promega) for GDCs and CTG for GSCs following the manufacturer's recommendations. In brief, 5,000 to 20,000 cells per well in 96-well plates were seeded in triplicate in the respective control and deprived medium treated for 24 to 96 h with or without EN460 in LG (0.45 g l<sup>-1</sup>) or complete medium, HG (4.5 g l<sup>-1</sup>). Luminescence or fluorescence was assessed using a Spectramax ID3 (Molecular Devices) reader.

### Cell proliferation and migration

The Incucyte S3 (Sartorius), a live-cell imaging and analysis system was used to measure cell proliferation and migration (scratch wound automatic with the woundmaker 96-well plate), according to the manufacturer's recommendations. Cells were seeded on a 96-well plate at 5,000 cells and 20,000 cells per well for proliferation and migration assay, respectively, in the following conditions: complete medium (HG), deprived glucose condition (0.45 g l<sup>-1</sup>) with or without EN460. Plates were transferred to the Incucyte for incubation over 100 h and images were collected every 2 h.

### Annexin V/propidium iodide staining

Cell apoptosis and necrosis was evaluated with annexin V/propidium iodide staining following manufacturer recommendations (Thermo-Fisher, V13245) by flow cytometry analysis carried out using a FACS Fortessa (Flow Cytometry Core Facility of the Cancer Research Centre of Lyon; CRCL) and data were processed using BD FACSDiva software.

### 3D cell invasion in Matrigel

GSC were seeded into 96-well ultra-low attachment plates. After 24 h, 75 µl of Matrigel were loaded on each sphere, on ice. Then, 200 µl GSC medium was added on top of polymerized Matrigel and GSCs were incubated at 37 °C for 24 to 72 h to allow invasion assessment. Images were acquired using an EVOS microscope (Life Technologies) and analysed using the 'Analyze – Measure – Length' feature in Fiji software.

### In silico analysis

The R2 web application-based genomics analysis and visualization application (<http://r2.amc.nl>) was used to obtain survival curves and g:Profiler Bioinformatics resources to perform gene enrichment analyses<sup>68</sup>. The Ivy Glioblastoma Atlas Project was used to visualize ERO1α expression as well as the genes of interest, listed in the corresponding figure, in GBM tumours. Broad Institute tools were used to produce the single-cell diagram based on a glioblastoma study<sup>6</sup>.

### Fusion/fission measurements

The mitochondrial morphology and network were assessed using the MitoTracker Red CMXRos fluorescent probe (M7512, Thermo Fisher) at 100 nM following manufactory recommendations. Cells were treated for 30 min at 37 °C, then fixed, mounted and imaged using the Opera-Phenix (PerkinElmer). Mitochondrial length, width and area were quantified automatically using Harmony software.

### Cell immunofluorescence staining and imaging

Before fixation, mitochondria were stained with a MitoTracker Red CMXRos fluorescent probe (M7512, Thermo Fisher) at 100 nM (see above). Then, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 min at room temperature, followed by quenching with 50 mM NH<sub>4</sub>Cl for 10 min. After washing, cells were permeabilized with 0.3% Triton X-100 for 15 min and blocked in 5% BSA–0.1% Triton X-100 for 1 h. Cells were incubated with primary antibodies against ERp57 (1:50 dilution, Abcam 242367) overnight at room temperature in a humidified, light-protected chamber. Following washes (PBS with 0.3% Triton X-100 and 0.2% BSA, then PBS), cells were incubated with Alexa Fluor-conjugated secondary antibodies (1:400 dilution) for 1 h. After the final washes, coverslips were mounted using PBS:glycerol (1:1). Images were acquired using a confocal Zeiss 880 or OPERA-Phenix HCS inverted fluorescence microscope equipped with a ×63 objective.

### mtDNA quantification

The mitochondrial DNA content was assessed using RT–qPCR<sup>69</sup>. In brief, total RNA isolation was carried out using a NucleoSpin DNA kit from Macherey-Nagel (740952.50) according to the manufacturer's instructions. Here, mitochondrial DNA content (COX1) relative to nuclear DNA (PPIA) was evaluated with the primers sequences provided in Supplementary Table 5.

### ROS measurement

To measure cytoplasmic and mitochondrial ROS, CellROX Deep Red (C10422, Invitrogen) and MitoSOX RED Mitochondrial (M36008, Invitrogen) were used following manufacturer recommendations. Before Opera-Phenix (PerkinElmer) microscopy, cells were incubated with 5 mM CellROX or 5 mM MitoSOX for 30 min, at 37 °C. Then cells were washed (2× PBS) and fixed (PFA 4%) for 15 min before staining with DAPI (4,6-diamidino-2-phenylindole). Fixed cells were maintained

in PBS:glycerol (1:1) in the dark. The fluorescence signals of CellROX far-red and MitoSOX were quantified by Opera-Phenix (PerkinElmer). Signal intensities and cell number and area were automatically analysed using Harmony software.

To measure the ER redox poise, roGFP2 was overexpressed 48 h. Then, the measurements were performed by Opera-Phenix (PerkinElmer) in live imaging with an automatic injection of DTT (10 mM) after 60 s of acquisition. Signal ratio intensities were analysed with the time series analyser tool on Fiji software after the removal of background fluorescence.

### Immunofluorescence on tissue sections

Mouse brains were fixed in 4% PFA for 24 h at room temperature, dehydrated and embedded in paraffin. Formalin-fixed paraffin-embedded mouse brain sections (8 µm) and GBM tissue arrays were deparaffinized, rehydrated and subjected to antigen retrieval in citrate buffer (0.01 M, pH 6.0) using microwave heating (620 W, 3 × 5 min)<sup>69</sup>. Sections were incubated overnight at 4 °C with primary antibodies diluted in PBS containing 0.3% Triton X-100: rabbit anti-ERO1α (Abcam, 177156, 1:100 dilution), mouse anti-GRP75 (Abcam, 2799, 1:200 dilution) and rabbit anti-GFAP (Abcam or Dako, Z0334, 1:50 dilution). After washing in PBS, sections were incubated for 1 h at room temperature with Alexa Fluor 488- or 555-conjugated secondary antibodies raised in goat or donkey against mouse or rabbit IgG (Invitrogen, 1:1,000 dilution). Nuclei were counterstained with Hoechst (1:5,000 dilution) for 10 min before mounting in Fluoromount-G (Invitrogen, 00-4958-02). HPS staining (Hémalum, Phloxine, Safran) was used to visualize tumour regions in mouse brain sections. Images were acquired using a Thunder Leica microscope, and quantification of immunofluorescence-positive cells was performed using Fiji (National Institutes of Health; NIH).

### Electron microscopy

GDCs and GSCs were washed and fixed by adding an equal volume of 4% glutaraldehyde directly to the culture medium and incubated for 15 min at 4 °C. The solution was then replaced with a mixture of 4% glutaraldehyde and 0.2 M cacodylate buffer (1:1, pH 7.4). Cells were sectioned for transmission electronic microscopy (TEM)<sup>69</sup>. Fiji software (NIH) was used to quantify ER length, mitochondria circumference, distance between ER and mitochondria (up to 50 nm), as well as contact number and length of contacts. Here, a minimum of 60 pictures were taken per condition, and at least 100 cells were analysed per group.

### Proximity ligation assay

Cells were seeded on 96 wells plate (PerkinElmer). After 24 h of infection with FATE-1, cells were treated with LG (0.45 g l<sup>-1</sup>) or HG (4.5 g l<sup>-1</sup>) medium containing 10 mM glutamine or with or without EN460 (25 µM) for 24 h. Cells were fixed, permeabilized, blocked and incubated overnight at 4 °C with a mixture of anti-IP3R3 (Millipore AB9076, 1:400 dilution) and VDAC1 (Abcam 14734, 1:400 dilution) primary antibodies following the Naveniflex kit (NaveniFlex Cell MR Atto647N, NC.MR.100. ATTO) following the manufacturer's recommendations. Nuclei were stained using DAPI incubated for 10 min in pre-heated humidity chamber and maintained in PBS:glycerol (1:1) in the dark. Images were acquired in 0.25-mM stacks using Opera-Phenix (PerkinElmer). Dot numbers, dot intensities, cell number and cell area were automatically analysed using Harmony software.

### FRET imaging

The FRET imaging consisted of 5,000 cells seeded in 200 µl per well on a cell carrier 96-well plate (Greiner uClear bottom)<sup>70</sup>. At 24 h after seeding, the cells were transduced for 6 h. Then, 24 h later the medium was changed to HG or LG in the presence or absence of EN469. Following an additional 24 h of incubation, the cells were fixed imaged using an Operetta High Content imaging system (PerkinElmer). FEMP imaging using the Operetta System was carried out with the following



filters: CFP (excitation 410–430, emission 460–500), YFP (excitation 490–510, emission 520–560) and YFP-FRET (excitation 410–430, emission 520–560) and images were analysed using PerkinElmer Harmony 3.5 image analysis software. The YFP channel was selected to spot the region of interest (ROI) and around each ROI, a second boundary was drawn to quantify the background intensity. FRETbasal and FRETmax were calculated as  $(\text{FYFP-FRETcell} - \text{FYFP-FRETbg}) / (\text{FCFPcell} - \text{FCFPbg})$ . The FRET ratio was calculated as  $(\text{FRETmax} - \text{FRETbasal}) / \text{FRETbasal}$ .

### Seahorse assay

The OCR and ECAR were measured using Seahorse XFe96 Analyzer (Agilent). Cells were seeded at 10,000–20,000 cells per well in a Seahorse cell plate, at least in triplicate, and incubated in XF medium (DMEM-based medium) with additional HG ( $4.5 \text{ g l}^{-1}$ ) or LG ( $0.45 \text{ g l}^{-1}$ ) and 10 mM glutamine with or without EN460 or overexpression of Fate-1 or Linker proteins for 48 h. A Cell Mito Stress kit was used following the manufacturer's instructions, treating cells with  $1.5 \mu\text{M}$  oligomycin,  $1.5 \mu\text{M}$  FCCP and  $0.5 \mu\text{M}$  rotenone/antimycin A. All wells were normalized against protein concentration using the Pierce BCA protein quantification assay (Thermo Fisher).

### Proteomics

Before cell fractionation, cells were seeded and treated according to our conditions (HG, LG with or without EN460). Liquid chromatography (LC)–MS/MS data were normalized to the total protein amount. Fractions were first quantified and equal amounts of protein were processed for LC–MS/MS analysis ( $25 \mu\text{g}$  per sample for MS24-037 and  $20 \mu\text{g}$  per sample for MS24-055). Of note, the datasets used for the differential analyses were highly homogeneous, with a coefficient of variation calculated across all samples within each dataset (MS24-037 and MS24-055)  $<5\%$ . Samples were processed, subjected to short electrophoresis and trypsin in-gel digestion as reported<sup>71</sup>. For shotgun proteomics analysis, peptide batches were resolved by their hydrophobicity with a 90-min acetonitrile gradient by reverse-phase chromatography and subjected to high-resolution MS/MS with an Exploris 480 (Thermo)<sup>72</sup>, except that the data-independent analysis mode was activated. The raw data were processed with DIA-NN v1.8.1 software<sup>73</sup> with RT-dependent cross-run normalization and then  $\log_2$  transformed. For each protein, missing values were imputed the value 1,000 (background) with the in silico predicted spectral library was generated from the SwissProt\_Human database (20,550 sequences and 11,382,592 residues). PCA was performed using the PCA module from the FactoMineR package in R.

### Metabolomics

The MxP Quant 500XL assay (from Biocrates) was used to measure several endogenous metabolites including lipids and hexoses measured by flow injection analysis (FIA)–MS/MS using 5500 QTRAP instrument (AB Sciex) with an electrospray ionization (ESI) source, and small molecules were quantified by LC–MS/MS also using the same instrument. The metabolic peaks were normalized by the cell number collected for each sample.

### Lipidomics

Following treatments,  $100 \mu\text{g}$  of the MAM fraction sample was used for lipidomics. The lipids were extracted from each sample using the Folch method. Then,  $0.5 \text{ ml}$  NaCl  $0.73\%$  was added to each sample, which was then vortex-homogenized and transferred to a glass tube. Then,  $5 \text{ ml}$  of  $\text{CHCl}_3$ :methanol (2:1) was added to the glass tube and centrifuged ( $3,000 \text{ rpm}$  for  $3 \text{ min}$ ) following homogenization. The lower organic phase was collected then subjected to evaporation under nitrogen flux. The lipids were taken up in  $1 \text{ ml}$  of  $\text{CHCl}_3$ :methanol (2:1) and transferred to a vial. The latter was again subjected to evaporation under nitrogen flux. Then,  $200 \mu\text{l}$  of  $\text{CHCl}_3$ :methanol (2:1) was added to this vial, which was then homogenized before being placed in an insert. The vial was

stored at  $-30^\circ\text{C}$ . LC–HRMS analysis was performed using a Thermo Ultimate 3000 coupled with Orbitrap Fusion Tribrid Mass Spectrometer equipped with an EASY-MAX NG ion source (Thermo Scientific) and an Acquity UPLC ethylene-bridged HILIC column  $1.7 \mu\text{m}$ ,  $2.1 \times 100 \text{ mm}$  (Waters), allowing the separation of several polar lipid classes according to their polarity. Analysis were performed with Xcalibur software.

### <sup>13</sup>C tracing

Cells were seeded at 200,000 per  $9.6\text{-cm}^2$  dish. After 24 h, cells were treated with  $0.45 \text{ g l}^{-1}$  (LG) or  $4.5 \text{ g l}^{-1}$  (HG) of glucose-<sup>13</sup>C (D-glucose-13C6, Clinisciences, TRC-G595007) in DMEM containing 10% FBS, 1% penicillin–streptomycin and 10 mM glutamine with or without EN460 ( $25 \mu\text{M}$ ) or DMSO for 24 h. Cells were trypsinized, counted and stored as dry pellet at  $-80^\circ\text{C}$  before further LC–MS-based metabolomic analyses. Frozen pellets were used for metabolite extraction. The volume of the extraction solution was adjusted to the cell number. After addition of extraction solution, samples were vortexed for 5 min at  $4^\circ\text{C}$  and centrifuged at  $16,000g$  for 15 min at  $4^\circ\text{C}$ . LC–MS analyses were conducted on a QExactive Plus Orbitrap mass spectrometer equipped with an Ion Max source and a HESI II probe coupled to a Dionex UltiMate 3000 UPLC system (Thermo Fisher Scientific). The  $5\text{-}\mu\text{l}$  samples were injected onto a ZIC-pHILIC column (Millipore) for LC separation. Buffer A was 20 mM ammonium carbonate,  $0.1\%$  ammonium hydroxide (pH 9.2) and buffer B was ACN. The chromatographic gradient was run at a flow rate of  $0.200 \mu\text{l min}^{-1}$  as follows: 0 to 20 min, linear gradient from 80 to 20% of buffer B; 20 to 20.5 min, linear gradient from 20 to 80% of buffer B; and 20.5 to 28 min, 80% buffer B. The metabolites were detected across a mass range of 75–1,000 mass/charge ratio ( $m/z$ ) at a resolution of 35,000 (at  $200 m/z$ ). The peak areas of metabolites and isotopologues were determined using Thermo TraceFinder software (Thermo Fisher Scientific), identified by the exact mass of each singly charged ion and by the known retention time on the high-performance LC column<sup>74</sup>.

### Ca<sup>2+</sup> measurements

Calcium transfer from ER to mitochondria was performed with the non-ratiometric Mito-R-GECO probe. The ratiometric 4mtD3cpv (RRID: [Addgene\\_36324](#)) or erGAP1 biosensors were used to measure basal mitochondrial or ER calcium content, respectively<sup>75</sup>. Probes were overexpressed for 48 h. For cytoplasmic Ca<sup>2+</sup> measurements, Fluo-8 AM (AATBioquest/CliniSciences, AAT-21097) was used in accordance with the manufacturer's recommendations.

Ca<sup>2+</sup> measurements were performed in transparent DMEM (Thermo Fisher, A1443001) containing drugs or different glucose levels. Live-imaging curves of erGAP1 probes were performed in HBSS supplemented with  $1.75 \text{ mM}$   $\text{MgCl}_2$ ,  $410 \text{ mM}$   $\text{MgSO}_4$ ,  $100 \text{ mM}$  EGTA and live-imaging curves of 4mtD3cpv probes were performed in HBSS, Ca<sup>2+</sup> and Mg<sup>2+</sup>.

Cells were treated with  $600 \mu\text{M}$  final of ATP (Sigma-Aldrich, A2383) to stimulate Ca<sup>2+</sup> transfer from ER to mitochondria. Measurements were performed at  $37^\circ\text{C}$  in  $5\% \text{ CO}_2$  with an automatic injector ( $20 \mu\text{l}$  in  $180 \mu\text{l}$  per well of 96 wells) using an Opera-Phenix confocal (PerkinElmer) concerning adherent cells (GDCs) transfected with Mito-R-GECO or stained with Fluo-8 AM. Experiments performed on sphere cells (GSCs) or with the erGAP1 and 4mtD3cpv probes were performed in  $37^\circ\text{C}$  using a Zeiss 980 confocal microscope. TBHQ ( $60 \mu\text{M}$  final) or ATP ( $600 \mu\text{M}$  final) were injected manually ( $20 \mu\text{l}$  in  $180 \mu\text{l}$  per well of 96 wells). The fluorescence ratio of basal or post-stimulated Ca<sup>2+</sup> levels was quantified using the time series analyser tool on Fiji software after the removal of background fluorescence. The average curves of all analysed cells and histograms show basal or stimulated Ca<sup>2+</sup> levels by cell in at least three independent experiments.

### Generation of brain organoids and co-culture

The induced pluripotent cell line WTSii024-A (EBiSC, 66540094) was used to generate the brain organoids<sup>56</sup>. In brief, human induced

pluripotent stem cells were maintained on Matrigel-coated dishes in StemFlex medium supplemented with FGF2 (2 ng ml<sup>-1</sup>) and penicillin–streptomycin. Cells were passaged using a gentle dissociation solution and used for differentiation at ~70% confluence. For organoid generation, induced pluripotent stem cells were dissociated in the presence of ROCK inhibitor (Y-27632, 10 µM) and seeded at 200 µl per well onto 96-well ultra-low attachment plates to allow aggregation. Plates were centrifuged (290g for 2 min) to promote the formation of uniform embryoid bodies.

Differentiation was induced by sequential exposure to the defined medium, with medium changes every 2 days. The basal medium consisted of DMEM/F12 and Neurobasal (1:1), supplemented with B27 without vitamin A, non-essential amino acids (1%) and penicillin–streptomycin (1%).

From D0 to D4 (day 0–4), neural induction was achieved by dual SMAD inhibition using SB431542 (10 µM) and LDN193189 (0.5 µM). During this period, cells were also cultured in the presence of ROCK inhibitor (Y-27632, 10 µM; D0–D1) and WNT signalling was inhibited using XAV939 (1 µM; D2–D7).

From D5 to D6, SB431542 was withdrawn, while LDN193189 (0.5 µM) and XAV939 (1 µM) were maintained. From D7 to D9, FGF2 (10 ng ml<sup>-1</sup>) was added to promote neural progenitor expansion, together with cyclopamine (1 µM) to inhibit the Hedgehog pathway, while LDN193189 and XAV939 were maintained.

At D10, LDN193189 and XAV939 were removed, whereas FGF2 (10 ng ml<sup>-1</sup>) and cyclopamine (1 µM) were maintained. From D11 to D21, CHIR99021 (1 µM), a GSK3 inhibitor and WNT activator, was added in combination with FGF2 and cyclopamine.

From D22 to D30, neuronal and glial maturation was supported by supplementation with BDNF (20 ng ml<sup>-1</sup>) and GDNF (20 ng ml<sup>-1</sup>), while maintaining organoids in differentiation medium.

For co-culture experiments, organoids were transferred to 96-well plates (Phenoplate, PerkinElmer) and GSC spheres (40,000 cells per well) were deposited onto individual organoids and maintained for 1 week, with medium changes three times per week. Live co-culture imaging was performed at D1 and after 1 week using a high-resolution microscope (Thunder, Leica). Organoids were then collected at the indicated time points, fixed in 4% paraformaldehyde for 24 h at 4 °C and washed in PBS before embedding in paraffin.

## In vivo experiments

**Animals and housing conditions.** All procedures involving animals were conducted in the CRCL small animal facility (P-PAC, accredited under approval number D693880202) in accordance with European Directive 2010/63/EU and were approved by the local Institutional Animal Care (ACCeS ethical committee) and Use Committee and authorized by the French Ministry of Research (APAFIS no. 40391). Mice were housed in individually ventilated cages at a maximum density of five animals per cage, on poplar wood bedding (SAFE Select Fine). Environmental enrichment was provided with at least two dental cotton rolls per cage. Animals had ad libitum access to acidified drinking water (pH 3.0 ± 0.5).

Bedding was replaced every 2 weeks, and full cage changes were performed every 8 weeks. Animals were maintained under a 12-h light–dark cycle (lights on at 07:00 and off at 19:00), at an ambient temperature of 21 ± 2 °C. Relative humidity was approximately 45 ± 30% and was not actively regulated.

**Mouse experiments.** The 6–10-week-old female nu/nu nude immunocompromised mice weighing 20–25 g (Envigo) or C57BL/6J OlaHsd immunocompetent mice weighing 18–22 g (Envigo) were housed five per cage, with standard husbandry for specific-pathogen-free conditions provided by staff of the animal core facility (P-PAC, CRCL). Mice were allowed to acclimate for 2 weeks before experimentation. SB28 (10,000 cells) and GSC Ge518 (3 × 10<sup>5</sup> tumour cells) were injected

intracranially in 2 µl of PBS with a Hamilton syringe mounted on the frame into the C57BL/6J and nude mice putamen (through the pre-formed hole to a depth of 3.6 mm, a site far from the ventricles, and with little critical activity in the mouse), respectively. Twice a week (for the duration of the experiments) and before injection, body weight was measured and a 15% decrease from the pre-procedure body weight led to killing. To evaluate tumour progression, animals were monitored daily and bioluminescence was measured with the Biospace instrument. Those exhibiting signs of morbidity and/or development of neurological symptoms were killed immediately. Tumour presence was quantified using luciferase activity following intraperitoneal (i.p.) injection of luciferin. Mice were then randomly assigned to the different groups. Mice were treated 2–3 times a week by retro-orbital injection of 0.5–1 mg kg<sup>-1</sup> EN460 or DMSO; or given access to water containing 4% sucrose, with or without doxycycline (1 mg ml<sup>-1</sup>).

## Zebrafish model

Transgenic *Tg(fli1:EGFP)* zebrafish were maintained at 28.5 °C under a 14-h light–10-h dark cycle in the Zebrafish Facility at the Walther Straub Institute, Ludwig Maximilians University (LMU) Munich. The water was changed daily and larvae older than 7 days were fed twice a day with ground brine shrimp. All experimental procedures involving zebrafish were performed in accordance with the guidelines of the Regierung von Oberbayern, the European equivalent of the NIH's Guide for the Care and Use of Laboratory Animals (Directive 2010/63/EU) and the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>). Zebrafish younger than 5 days post-fertilization (dpf) are not classified as protected animals under regulations governing scientific procedures, adhering to the principles of the 3Rs. Three-day post-fertilized eggs were treated with 1 mg ml<sup>-1</sup> pronase to dechorionate the larvae. The larvae were then anaesthetized using tricaine (MS-222), and their heads were carefully exposed on the agarose before cancer cell injection. All injections were performed using a FemtoJet 4i microinjector (Eppendorf). Injected zebrafish larvae were maintained in six-well plates containing 5 ml of E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl<sub>2</sub> and 0.33 mM MgSO<sub>4</sub>, pH 6.8–6.9). The larvae were imaged immediately after injection (D0) using a confocal ZEISS 980 (Carl Zeiss) and again at the end of D4 using the Thunder 3D imager (Leica). Images were analysed using ImageJ.

## Statistics and reproducibility

Data shown, presented as mean ± s.e.m., are representative of results obtained for multiple experiments as noted in the figure legends. *P* values < 0.05 were considered significant. Following confirmation of data normality (using Shapiro–Wilk or Agostino and Pearson omnibus normality test) and homogeneity (using Bartlett's test), one-way or two-way analysis of variance (ANOVA) was performed to test the effects of the experimental conditions. Once a significant effect was noticed, a posteriori Bonferroni correction, Tukey correction or Fisher's test was used to analyse pairwise differences. For comparison between two groups, data were analysed with Kruskal–Wallis or a Student's *t*-test. Statistical analysis was performed using GraphPad Prism software. Concerning in vivo experiments (nu/nu nude immunocompromised and C57BL/6J OlaHsd immunocompetent), the minimum number of mice required by the group was determined with G\*Power 3 software. For in vitro and ex vivo data, no statistical method was used to predetermine sample sizes. During image analysis (IF, live imaging and EM), dead cells, identified by their round and small size, were excluded from the quantification data. No additional data points were excluded from the analyses. The investigators were not blinded to allocation, during experiments and outcome assessment. However, for live imaging and IF, automatic acquisition and injections were performed using Harmony software. Data collection and analysis were not performed blind to the conditions of the experiments.

## Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

## Data availability

MS data for glioblastoma differentiated and stem cell samples have been deposited in ProteomeXchange Consortium via the PRIDE<sup>76</sup> partner under the primary accession codes [PXD073353](https://doi.org/10.5281/zenodo.18340802) and [PXD073390](https://doi.org/10.5281/zenodo.18340802), respectively. The HPLC lipidomics data have been deposited at Zenodo at <https://doi.org/10.5281/zenodo.18340802> (ref. 77). The source data used for the conception and statistical analysis of the figure panels are available in the Source Data. All other data supporting the findings of this study are available from the corresponding author on reasonable request. Source data are provided with this paper.

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## Author contributions

A.B. and E. Cosset designed and supervised this work, carried out experiments, analysed and interpreted data, and wrote the paper. A.B., L.V., L.G., L.C., C.H., E.S.J., B.A.-B., L.M., E. Clement, H.D., M.C., O.B., C.V., M.G., A.C., L.L. and C.W., carried out some experiments and contributed to discussions. B.A.-B. and J.A. carried out the mass spectrometry experiments, and the analysis was performed by B.A.-B. and B.N. M.C. and O.B. performed the lipidomic analysis. I.N. performed the <sup>13</sup>C-glucose analysis. I.M. generated all mutants. R.G., I.M., J.N.S., E.Z., P.R.W., H.L., J.A., T.G., J.R., P.S.M., E.M.-B., P.S.M., P.-Y.D., T.S., D.M. and M.C. provided critical feedback on the study and the manuscript. P.R.W., H.L., D.M. and M.C. gave conceptual advice and contributed to discussions. All co-authors proofread the manuscript.

## Competing interests

The authors declare no competing interests.

## Additional information

**Extended data** is available for this paper at <https://doi.org/10.1038/s41556-026-01980-2>.

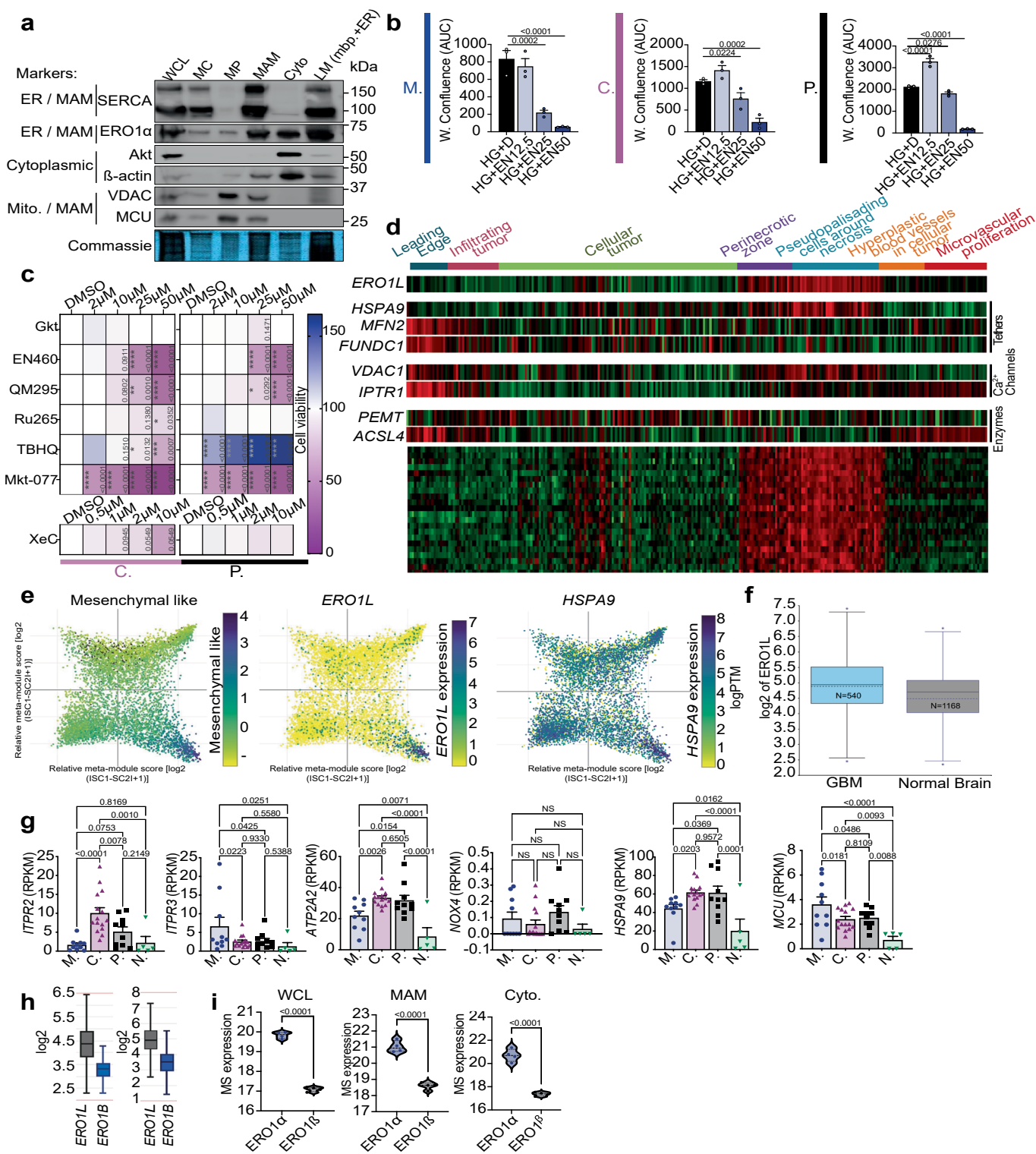
**Supplementary information** The online version contains supplementary material available at <https://doi.org/10.1038/s41556-026-01980-2>.

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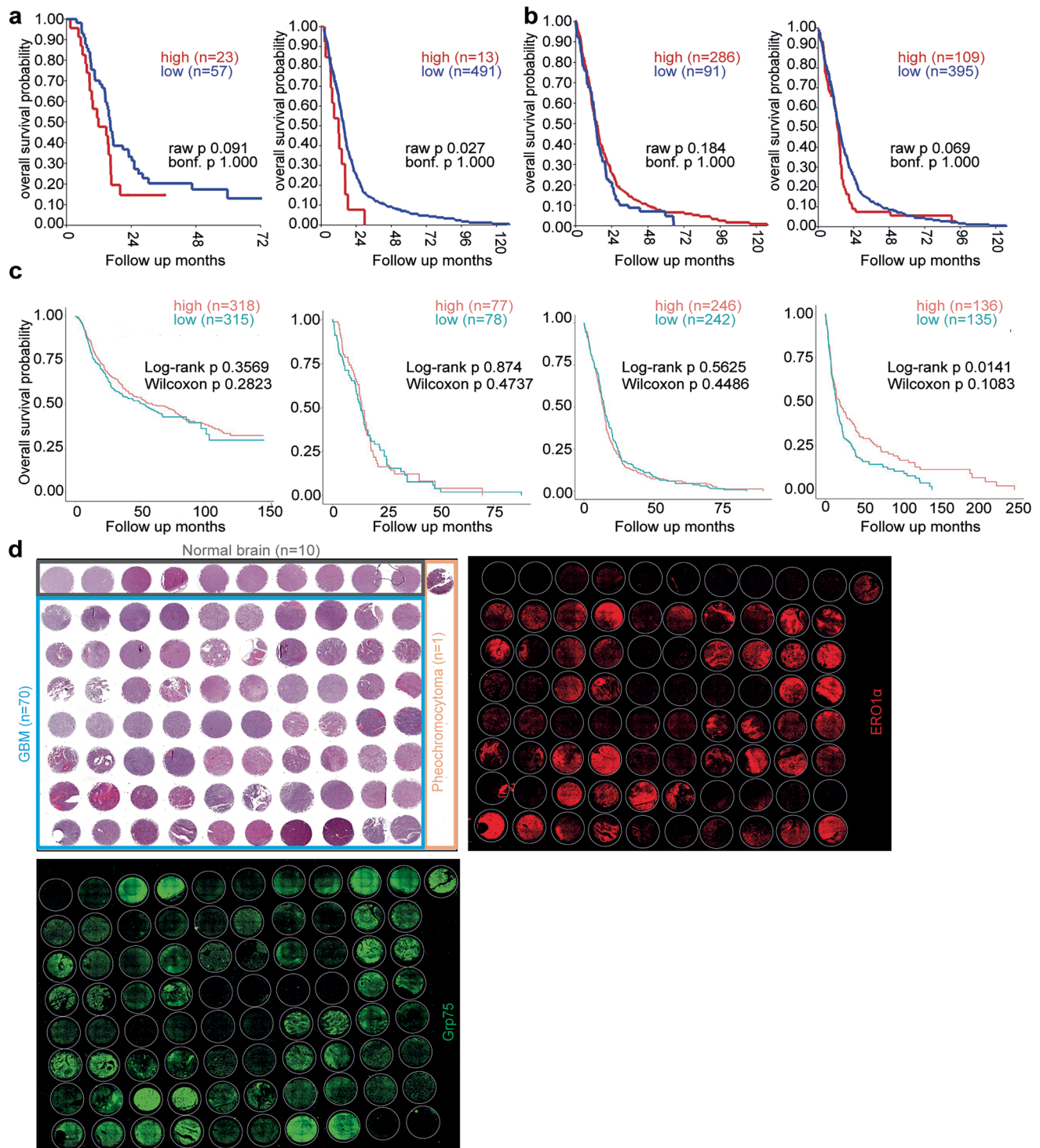




Extended Data Fig. 1 | See next page for caption.

**Extended Data Fig. 1 | The ERO1 $\alpha$  protein is enriched in the mesenchymal subtype.** **a**, Western blot showing the expression of the indicated proteins related to the cell compartments GDC Ge518. WCL = Whole Cell Lysate, MC = Mitochondria Crude, MP = Mitochondria Pure, Cyto = Cytoplasm, LM = Light Membranes (regrouping Endoplasmic Reticulum, ER and Plasma Membrane, mbp). **b**, Viability of indicated GDCs under EN460 treatment at 12, 5; 25 or 50  $\mu$ M after 72 h, using a CellTiter-Glo kit (n = 3 biological replicates). **c**, Viability of GDC cells under either control condition (DMSO, vehicle) or treatment with inhibitors targeting MAMs, namely Gkt, EN460, QM295, Ru265, TBHQ, Mkt-077 and XeC at the indicated concentrations after 72 h, using a CellTiter-fluor kit (n = 6–12 technical replicates in n = 3 biological replicates). **d**, Clustering of principal MAM genes according to their expression in GBM tumour extracted from the Ivy Glioblastoma Atlas Project (IvyGAP). **e**, Two dimensional representation of cellular states showing localization of mesenchymal cells (left), ERO1 $\alpha$  (middle) and Grp75 (right) where each quadrant matches to one cellular state, the precise position of malignant cells (dots) mirror their relative scores for the meta modules, and their colours reflect the density of cycling cells. **f**, ERO1 $\alpha$  mRNA expression in GBM vs Normal brain extracted

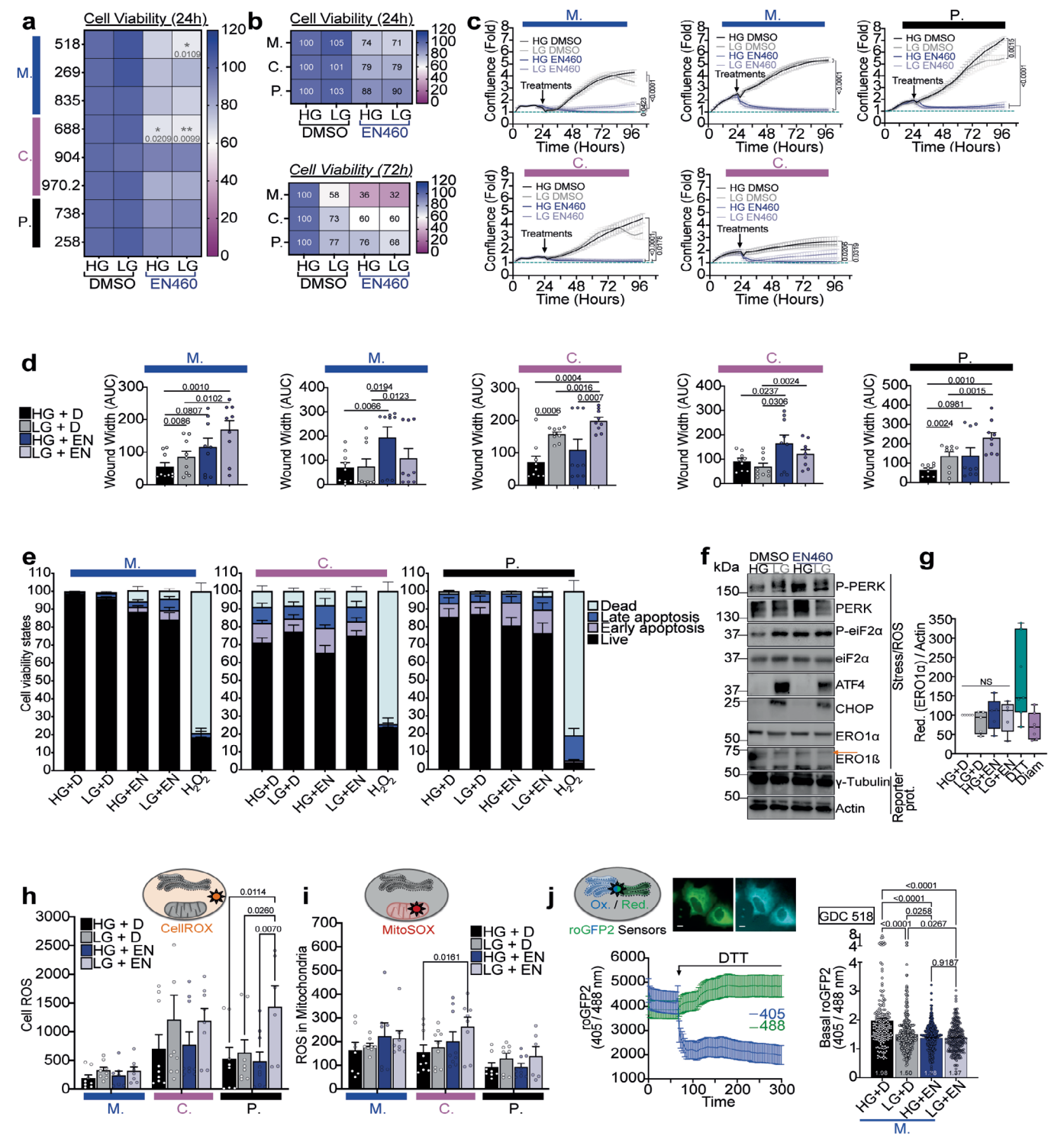
from Wellcome to R2 MAS5.0, TCGA 540 (n = 540) vs Li 1168 (n = 1168). Box-and-whisker plots show median (centre line), mean (dotted line), interquartile range (25th–75th percentiles) and min–max (whiskers). **g**, mRNA expression of the indicated gene was determined by RNA-seq (normalized against RPKM) in Mayo Clinic resources (n = 5–15 biological replicates). **h**, ERO1 $\alpha$  (ERO1A) and ERO1B mRNA expression extracted from Wellcome to R2 dataset MAS5.0, TCGA 540 (n = 540). Box-and-whisker plots show median (centre line), mean (dotted line), interquartile range (25th–75th percentiles) and min–max (whiskers). **i**, Expression of ERO1 $\alpha$  and ERO1 $\beta$  at the protein levels in the different cell compartments (WCL = whole cell lysate, MAM and Cyto = cytoplasmic fraction) using mass spectrometry data from the subcellular fractionation (n = 4 biological replicates). In all figures, M = mesenchymal, P = proneural, C = classical and N = neural; HG = high glucose, LG = low glucose and EN = EN460. In all figures, histograms and violins are expressed with mean  $\pm$  s.e.m. (\* p < 0.05, \*\* p < 0.01, \*\*\* p < 0.001, \*\*\*\* p < 0.0001). One-way (**b**, **c**, **g**) analysis of variance (ANOVA) followed by Fisher (**b**, **c**, **g**) multiple comparison tests. Parametric Unpaired t-test, two-tailed (**i**).



**Extended Data Fig. 2 | ERO1 $\alpha$  expression provides a stronger predictive marker of GBM aggressiveness than Grp75. a**, left, Kaplan-Meier analysis of Hegi-GBM dataset (84, MASS.0, u133p2) for ERO1 $\alpha$  expression (n = 57 ERO1 $\alpha$ low, n = 23 ERO1 $\alpha$ high; Expression Cut-off: 408.3; p = 0.091). Right, Kaplan-Meier analysis of TCGA-GBM dataset (540, MASS.0, u133a) for ERO1 $\alpha$  expression (n = 491 ERO1 $\alpha$ low, n = 13 ERO1 $\alpha$ high; Expression Cut-off: 98.3; p = 0.027). **b**, left, Kaplan-Meier analysis of TCGA-GBM dataset (395, MASS.0, u133a) for Grp75 expression (n = 91 Grp75low, n = 286 Grp75high; Expression Cut-off: 349.2; p = 0.184). Right, Kaplan-Meier analysis of TCGA-GBM dataset (540, MASS.0, u133a) for Grp75 expression (n = 395 Grp75low, n = 109 Grp75high; Expression Cut-off: 577.3; p = 0.069). **c**, left, Kaplan-Meier analysis of CGGA-Glioma dataset

for Grp75 expression (n = 315 Grp75low, n = 318 Grp75high; Expression Cut-off: median: 54.5; p = 0.2823). Middle left, Kaplan-Meier analysis of TCGA-GBM dataset for Grp75 expression (n = 78 Grp75low, n = 77 Grp75high; Expression Cut-off: median: 13.1; p = 0.4737). Middle right, Kaplan-Meier analysis of TCGA-GBM dataset for Grp75 expression (n = 242 Grp75low, n = 246 Grp75high; Expression Cut-off: median: 14.5; p = 0.4486). Right, Kaplan-Meier analysis of Gravendeel-GBM dataset for Grp75 expression (n = 135 Grp75low, n = 136 Grp75high; Expression Cut-off: median: 14.4; p = 0.1033). **d**, Images of whole-section staining and immunofluorescence (IF) corresponding protein-expression (ERO1 $\alpha$ , Grp75) quantification from tumour microarray (TMA) samples of normal brain (n = 10), glioblastoma (GBM; n = 70), and pheochromocytoma (n = 1) tissues.

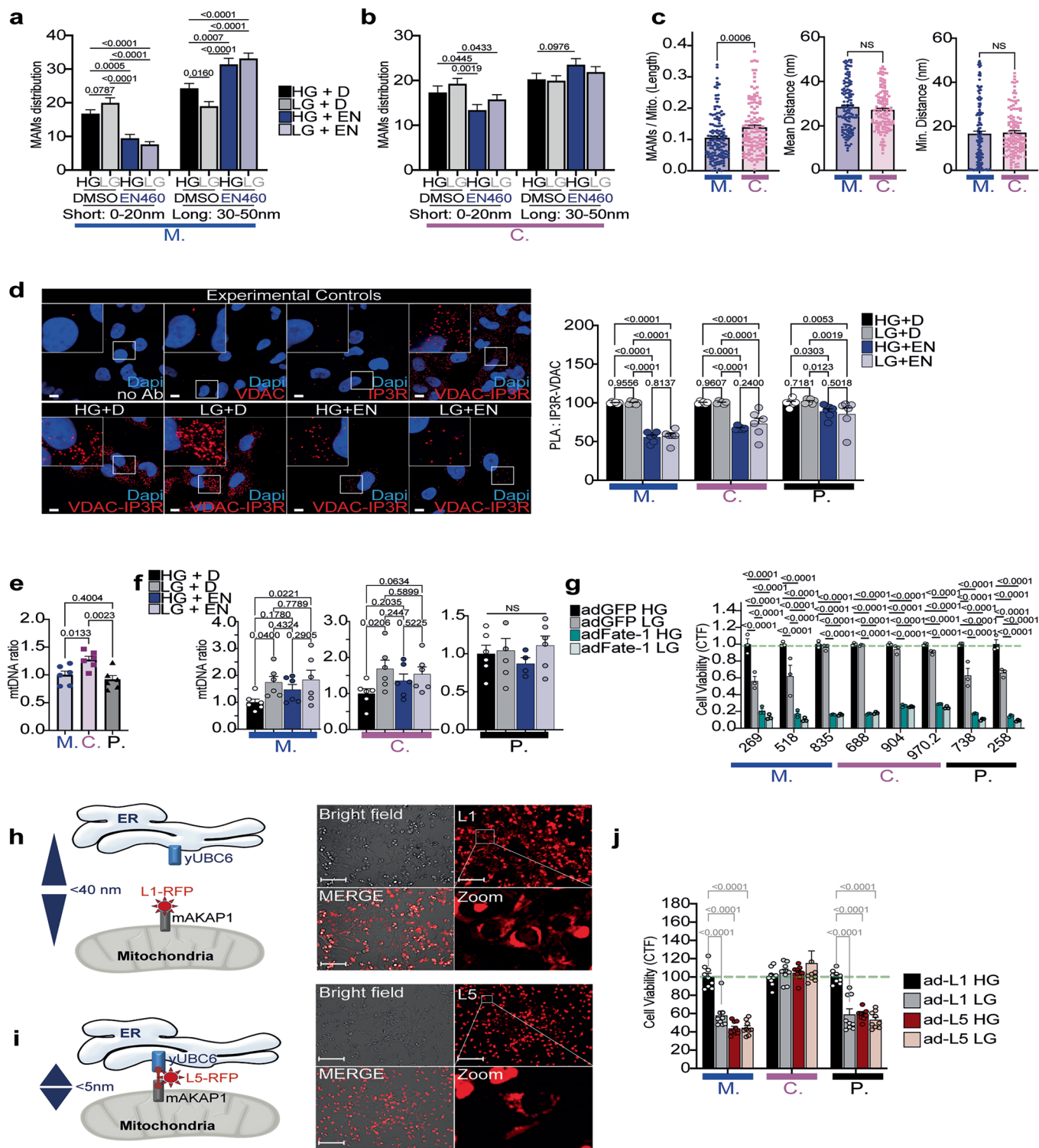




Extended Data Fig. 3 | See next page for caption.

**Extended Data Fig. 3 | ERO1 $\alpha$  blockade is associated with a decrease of GBM aggressiveness and in its activity. **a**,** Impact of EN460 treatment on cell viability measured by CellTiter-Glo kit in glioblastoma differentiated cells (GDCs) at 24h (n = 8–9 wells in n = 3 biological replicates). Ctrl = Vehicle (DMSO) in high (HG) vs low (LG) glucose concentrations with EN460 (HG/LG+EN460). Scale = 0 to 120 in percentage of viability. **b**, Cell viability of all indicated GDC subtype, measured by CellTiter-Glo kit at 24h (top) and 72h (bottom) (n = 8–9 wells in n = 3 biological replicates). **c**, Cell proliferation of all indicated GDCs, evaluated using Incucyte technology in the following conditions: high (HG) or low (LG) glucose with EN460 (HG/LG+EN460) (n = 9 wells in n = 3 biological replicates). **d**, Wound healing experiment showing migration of all indicated GDCs, measured using Incucyte technology in the following conditions: high (HG) or low (LG) glucose with EN460 (HG/LG+EN460) for three days (n = 9 wells in n = 3 biological replicates). **e**, Histograms representing the percentage of live, dead, late and early apoptotic cells in the indicated GDCs and conditions by flow cytometry with Annexin V/PI staining at 24h (n = 3–5 biological replicates). **f**, Representative Immunoblots showing the expression of indicated proteins for Ge518 in the following conditions: high (HG) or low (LG) glucose with EN460

(HG/LG+EN460) for 24h. **g**, Box plots show expression of reduced ERO1 $\alpha$  in the several specified conditions for 24h (n = 5 biological replicates). Box-and-whisker plots show median (centre line), interquartile range (25th–75th percentiles) and min–max (whiskers); all points are shown. **h**, Cytosolic (n = 6–9 biological replicates), **i**, and mitochondrial ROS (n = 6–9 biological replicates), measured using CellROX and MitoSOX, respectively in the designated GDCs in the designated conditions for 24h. **j**, Schematic diagram representing ER-roGFP2 ratiometric sensor allowing the measurement of ER redox state; **j**: scale bar = 20 $\mu$ m. Graph (middle) and histograms (right) show the average of the ER-roGFP2 ratiometric curve profiles following the addition of DTT (n = 206 – 426 cells in n = 5 – 6 biological replicates). In **a**, **e**, **h**, **i** figures, M = mesenchymal (GDC Ge518), P = proneural (GDC Ge258), and C = classical (GDC Ge970.2); HG = high glucose, LG = low glucose and EN = EN460. In **c** and **d** figures, M. left: 269, M. right: 835, P.: 738, C left: 688, C. right: 904. In all figures, histograms, heatmaps, box-dots and curves are expressed with mean  $\pm$  s.e.m. (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001). Two (**a**, **b**, **h**, **i**) or one-way (**c**, **j**, **g**) analysis of variance (ANOVA) followed by Fisher (**h**, **i**, **j**, **g**), Tukey (**c**) or Bonferroni (**a**, **b**) multiple comparison tests. Statistical significance was assessed using a two-tailed Student's t-test (**d**).

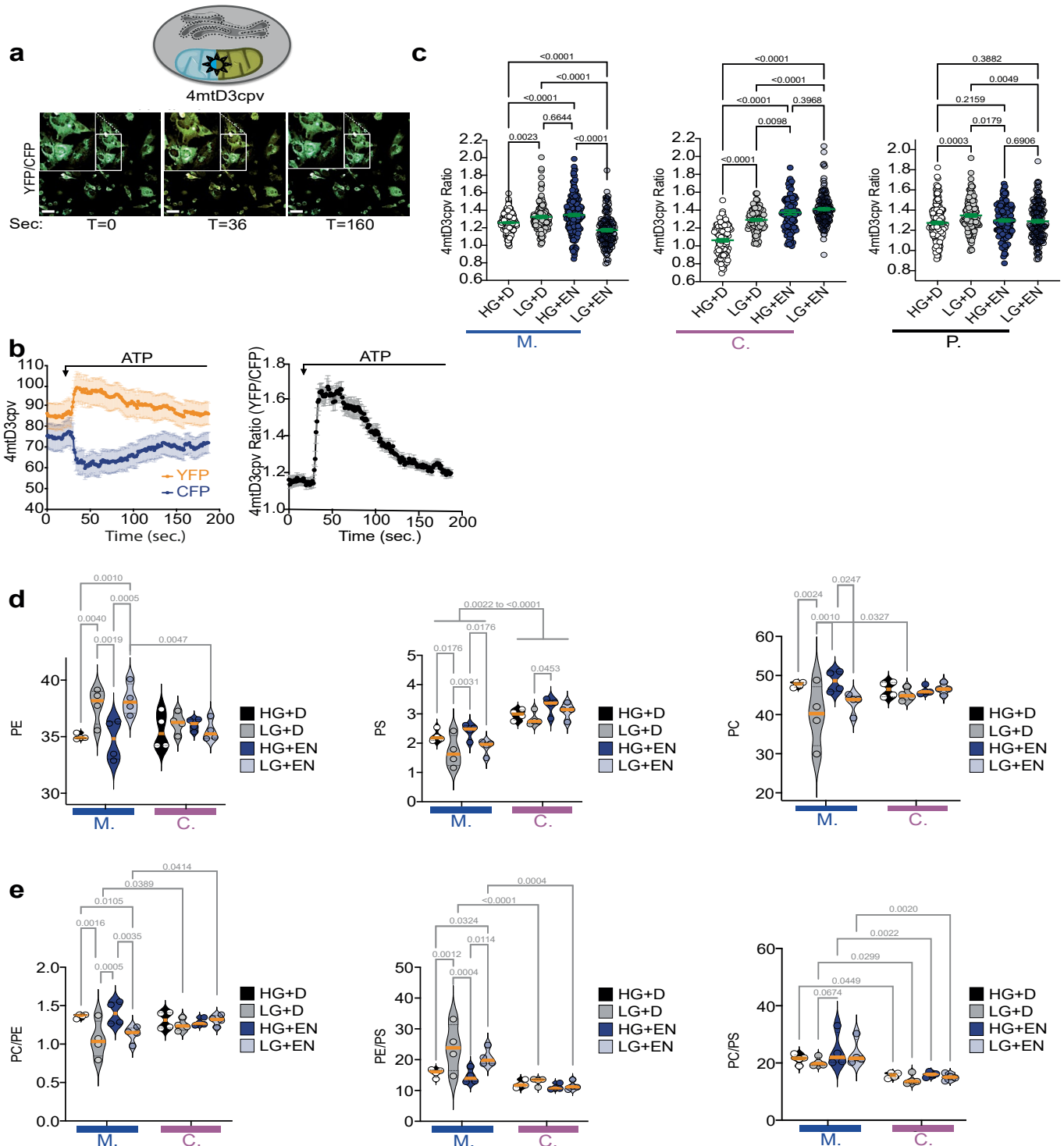


Extended Data Fig. 4 | See next page for caption.



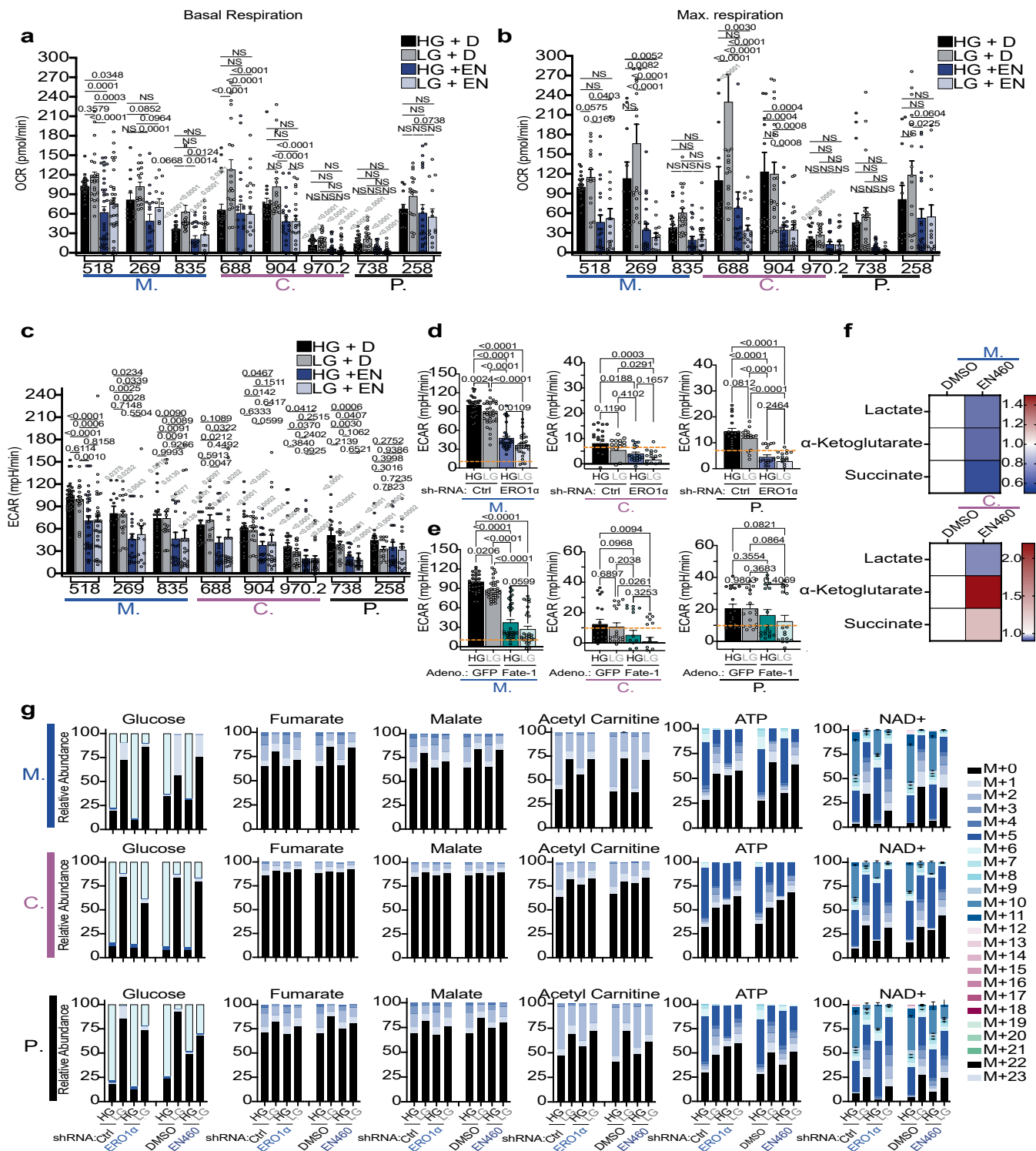
**Extended Data Fig. 4 | ERO1 $\alpha$  blockade is associated with an alteration of MAM and mitochondrial structures.** **a**, EM quantifications. Average measurements between ER–mitochondria (MAM) distribution for short (0–20 nm) and long (30–50 nm) distances.  $n = 105–138$  MAMs in  $n = 3$  biological replicates analysed for the indicated conditions for 24 h in glioblastoma differentiated cell (GDC) Ge518. **b**, Same as d–e, in Ge970.2,  $n = 127–149$  MAMs in  $n = 3$  biological replicates. **c**, EM quantifications. Average measurements between ER–mitochondria (MAM): total MAM length (left), mean (middle) and minimum (right) and MAM distance.  $n = 127–138$  MAMs in  $n = 3$  biological replicates analysed for the indicated conditions for 24 h in indicated GDCs. **d**, Illustrative pictures (left) of the co-localization of endogenous IP3R3 and VDAC1 using proximity ligation assay (PLA) in the indicated conditions in GDCs; scale bar = 20  $\mu\text{m}$ . Histograms showing the quantification of the dots number normalized by cell area ( $n = 6$  biological replicates) in the specified conditions (right). **e**, mitochondrial (COX1, Cytochrome c oxidase I) DNA content relative to nuclear (PPIA, Cyclophilin A)

DNA measured in indicated GDCs using RT-qPCR ( $n = 6$  wells in  $n = 2$  biological replicates). **f**, in designated conditions for 24 h ( $n = 6$  wells in  $n = 2$  biological replicates). **g**, Cell viability of all indicated GDCs in adenovirusFate-1 (adFate-1) vs. Control (adGFP) for 48 h in the specified conditions for 72 h  $n = 3$  biological replicates. **h**, Schematic diagram depicting the Linker, L5, molecular tool <40 nm, **i**, and <5 nm and representative images; scale bar = 100  $\mu\text{m}$ . **j**, Cell viability of all indicated GDCs in adenovirusLinker (ad-L5) vs. Control (ad-L1) for 48 h using CellTiter-Fluor in the specified conditions for 72 h  $n = 9$  wells in  $n = 3$  biological replicates. In all figures, M = mesenchymal (GDC 518), P = proneural (GDC 258), and C = classical (GDC 970.2); HG = high glucose, LG = low glucose and EN = EN460. In all figures, histograms are expressed with mean  $\pm$  s.e.m. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ ). Two (**d**, **g**, **j**) or one-way (**a**, **b**, **e**, **f**) analysis of variance (ANOVA) followed by Fisher (**a**, **b**, **d**, **e**, **f**) or Tukey (**g**, **j**) multiple comparison tests. Parametric Unpaired t-test, two-tailed (**c**).



**Extended Data Fig. 5 | ERO1 $\alpha$  blockade modulates total phospholipid abundance but does not affect their content in MAMs. a**, Schematic diagram representing 4mtD3cpv adenovirus ratiometric sensor allowing the measurement of basal Ca<sup>2+</sup> in the mitochondria. **b**, Graphs c, and histograms show the average of the 4mtD3cpv ratiometric curve profiles following the addition of ATP in the designated conditions for 24h (n = 139 – 201 cells for M.; n = 96 – 152 cells for c.; n = 122 – 169 cells for P. All in n = 3 biological replicates). **d**, Violin plots show the

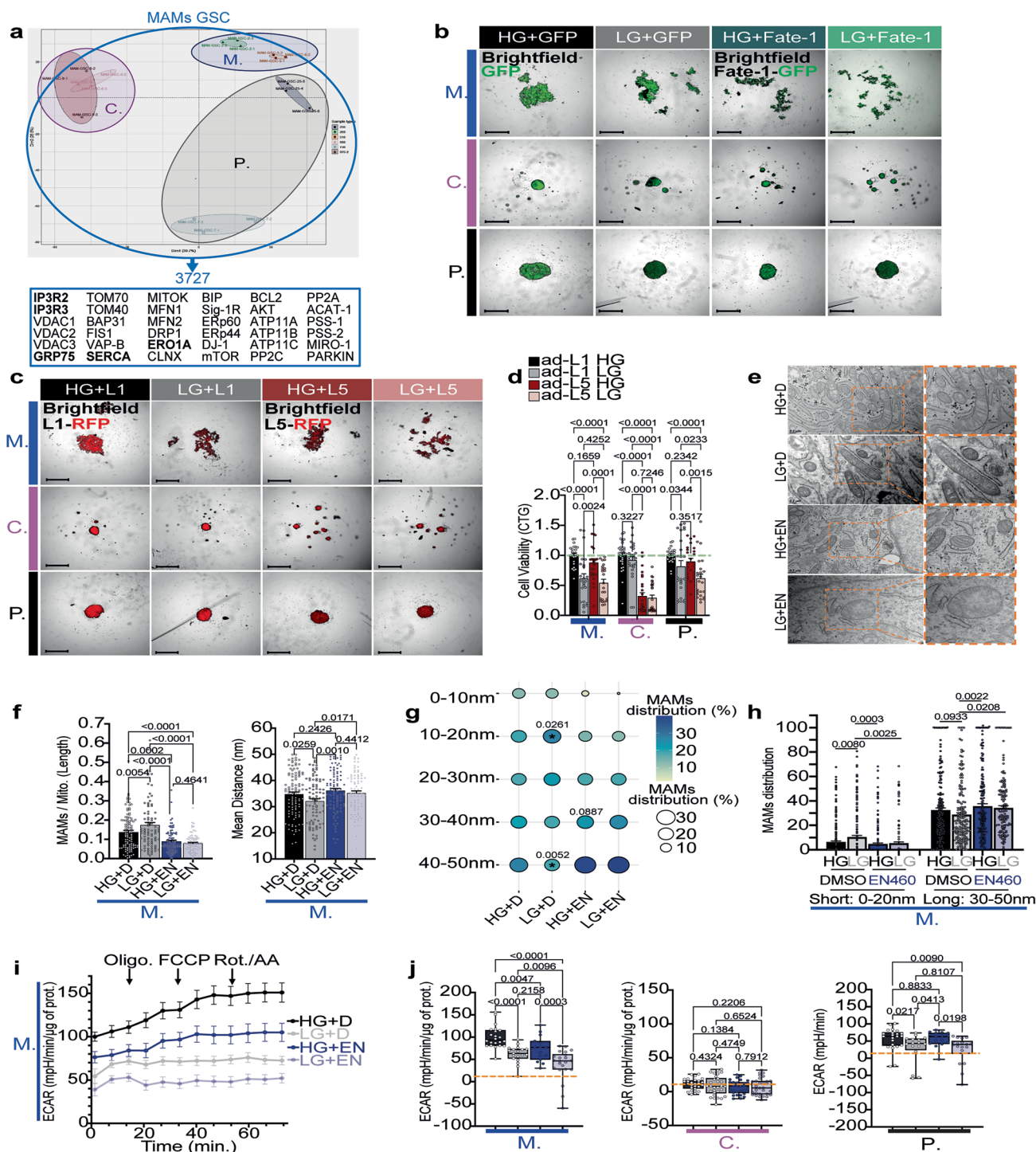
relative differences in PC, PE and PS content in M. (Ge518) and C. (Ge970.2) and ratio e, expressed in percentage (n = 4 biological replicates) in the designated conditions for 24h. In all figures, M = mesenchymal (GDC 518), P = proneural (GDC 258), and C = classical (GDC 970.2); HG = high glucose, LG = low glucose and EN = EN460. In all figures, violins or dot-clouds are expressed with mean  $\pm$  s.e.m. (\*p < 0.05, \*\*p < 0.01 and \*\*\*p < 0.001). Two (d, e) or one-way (c) analysis of variance (ANOVA) followed by Fisher (d, e) or Tukey (c) multiple comparison tests.



**Extended Data Fig. 6 | ERO1 $\alpha$  blockade and Fate-1 induces a decrease of metabolism in GBM. **a**, Bioenergetic responses including basal (n = 12 – 33 wells in n = 7–9 biological replicates), **b**, maximal respiration (n = 12 – 21 wells in n = 7 biological replicates) and **c**, extracellular acidification rate (n = 12 – 33 wells in n = 7 biological replicates) of all GDCs to metabolic inhibitors evaluated using Mito Stress kit. Histograms show OCR measurements in pmol/min. **d**, same as b following ERO1 $\alpha$  knockdown (n = 18 – 36 technical replicates in n = 3 biological replicates) **e**, or following AdenoFate-1 infection for 48h (n = 30 – 36 technical replicates in n = 3 biological replicates) in the designated conditions for 24h. **f**, Clusters displaying metabolomic analyses of the differences only in total lactate,  $\alpha$ -ketoglutarate and succinate of Ge518 (top), Ge970.2 (bottom)**

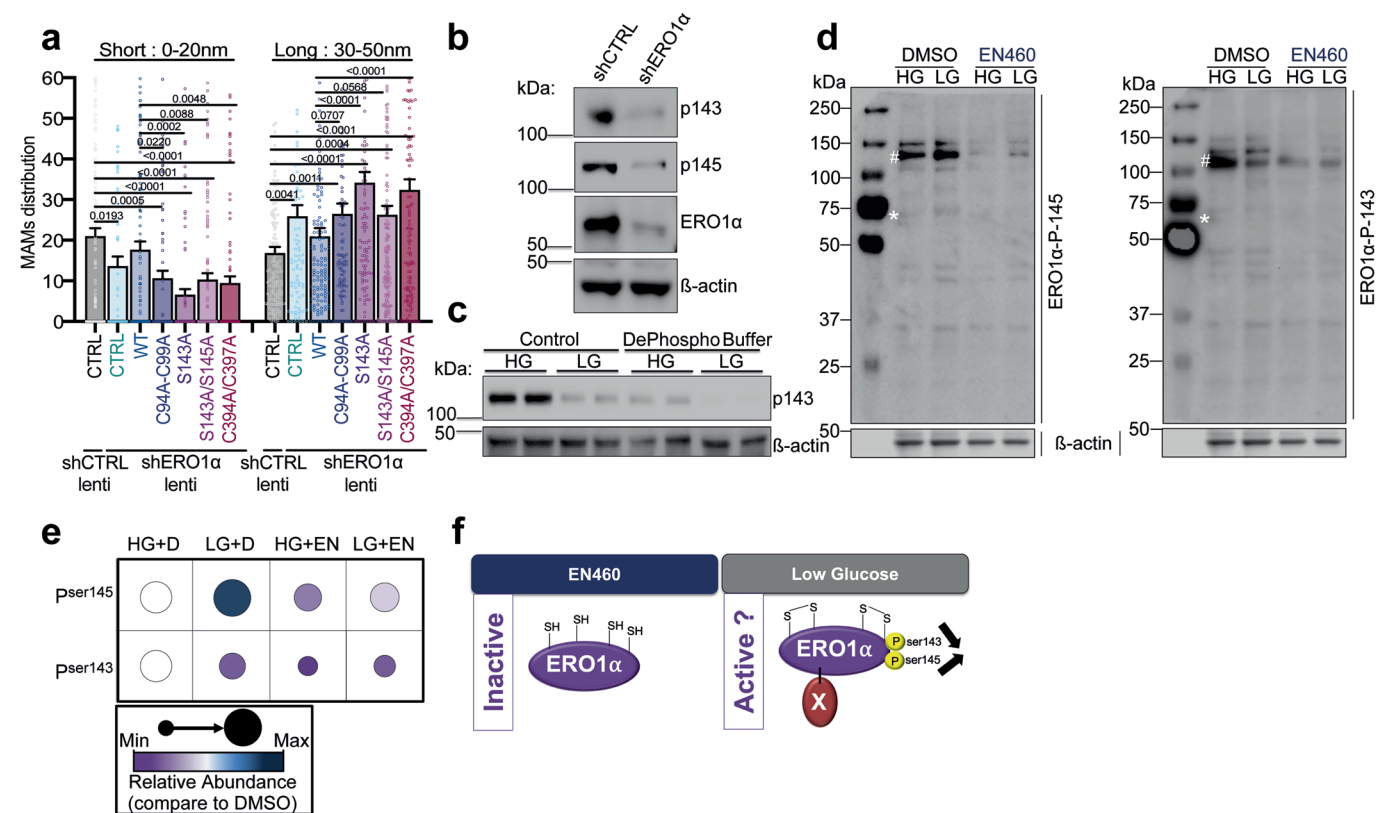
following ERO1 $\alpha$  inhibition for 24h (n = 2 biological replicates). **g**, Distribution of  $^{13}\text{C}$ -glucose derived isotopologues across metabolites (M+0, unlabelled; M+1 to M+23,  $^{13}\text{C}$ -labelled) in the indicated GDCs (n = 3 biological replicates) under the specified conditions for 24h. In all figures, M = mesenchymal (GDC 518), P = proneural (GDC 258), and C = classical (GDC 970.2); HG = high glucose, LG = low glucose and EN = EN460. In all figures, histograms are expressed with mean  $\pm$  s.e.m. (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001). One-way (**d**, **e**) or two-way (**a**, **b**, **c**) analysis of variance (ANOVA) followed by Fisher (**c**, **d**, **e**) or Tukey (**a**, **b**) multiple comparison tests. For panels **a** to **c**, grey p-values indicate comparisons of each condition with the corresponding condition in Ge518.





**Extended Data Fig. 7 | ERO1 $\alpha$  blockade is correlated with a decrease of GSC aggressiveness.** **a**, Principal-component analysis for all GSC models displaying 3,727 common proteins between subtypes. **b**, Representative images of spheroid in Fig. 6d. **c-d**, Representative images of spheroid; scale bar = 200 $\mu$ m (**c**). Histograms (**d**) show the fold change of cell viability, quantified using CellTiter-Glo, following AdenoLinker infection at the specified conditions for 96h in GSCs (n = 24–30 spheres in n = 4 biological replicates), measured by CellTiter-Glo. **e**, Representative Electron Microscopy (EM) images of GSCs Ge518 MAM; scale bar = 0.2 $\mu$ m. **f-h**, EM quantifications. Average measurements between ER-mitochondria (MAM): total MAM length (**f**, left), distance (**f**, right), all MAM distance distribution (**g**) and MAM distribution for short (0–20 nm) and long (30–50 nm) distances (**h**). n = 79–105 MAMs in n = 3 biological replicates analysed for the indicated conditions for 24h in glioblastoma stem cell (GSC) Ge518.

**i-j**, Bioenergetic responses of GSCs to metabolic inhibitors evaluated using the Mito Stress kit. Curves (**i**) and box plots (**j**) show ECAR measurements in pmol/min normalized against protein concentration in GSCs under the indicated conditions for 24h (n = 15–22 spheres for M.; n = 28–30 spheres for C.; n = 16–22 spheres for P. All in n = 4 biological replicates). Box-and-whisker plots show median (centre line), interquartile range (25th–75th percentiles) and min–max (whiskers); all points are shown. In all figures, M = mesenchymal (GSC 518), P = proneural (GSC 258), and C = classical (GSC 970.2); HG = high glucose, LG = low glucose and EN = EN460. In all figures, histograms or box-dots are expressed with mean  $\pm$  s.e.m. (\*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 and \*\*\*\*p < 0.0001). Two (**d, g**) or one-way (**f, h, j**) analysis of variance (ANOVA) followed by Fisher (**d, f, g, h, j**) multiple comparison tests.



**Extended Data Fig. 8 | Related to Fig. 7. a**, E.M. quantifications. Mean measurements of ER-mitochondria contact-site (MAM) distribution at short (0–20 nm) and long (30–50 nm) distances in GDC Ge518 cells expressing the indicated ERO1α constructs.  $n=90$ –243 MAMs in  $n=3$  biological replicates analysed for the indicated ERO1α constructs in GDC Ge518. **b**, Western blot showing the expression of ERO1α and its phosphorylation sites in shCTRL vs. sh ERO1α (for 48h). **c**, Western blot showing the phosphorylation of ERO1α at P143 using a dephosphorylation buffer, normalized to β-actin. **d**, Representative immunoblots showing expression of indicated phosphorylated sites (on the left,

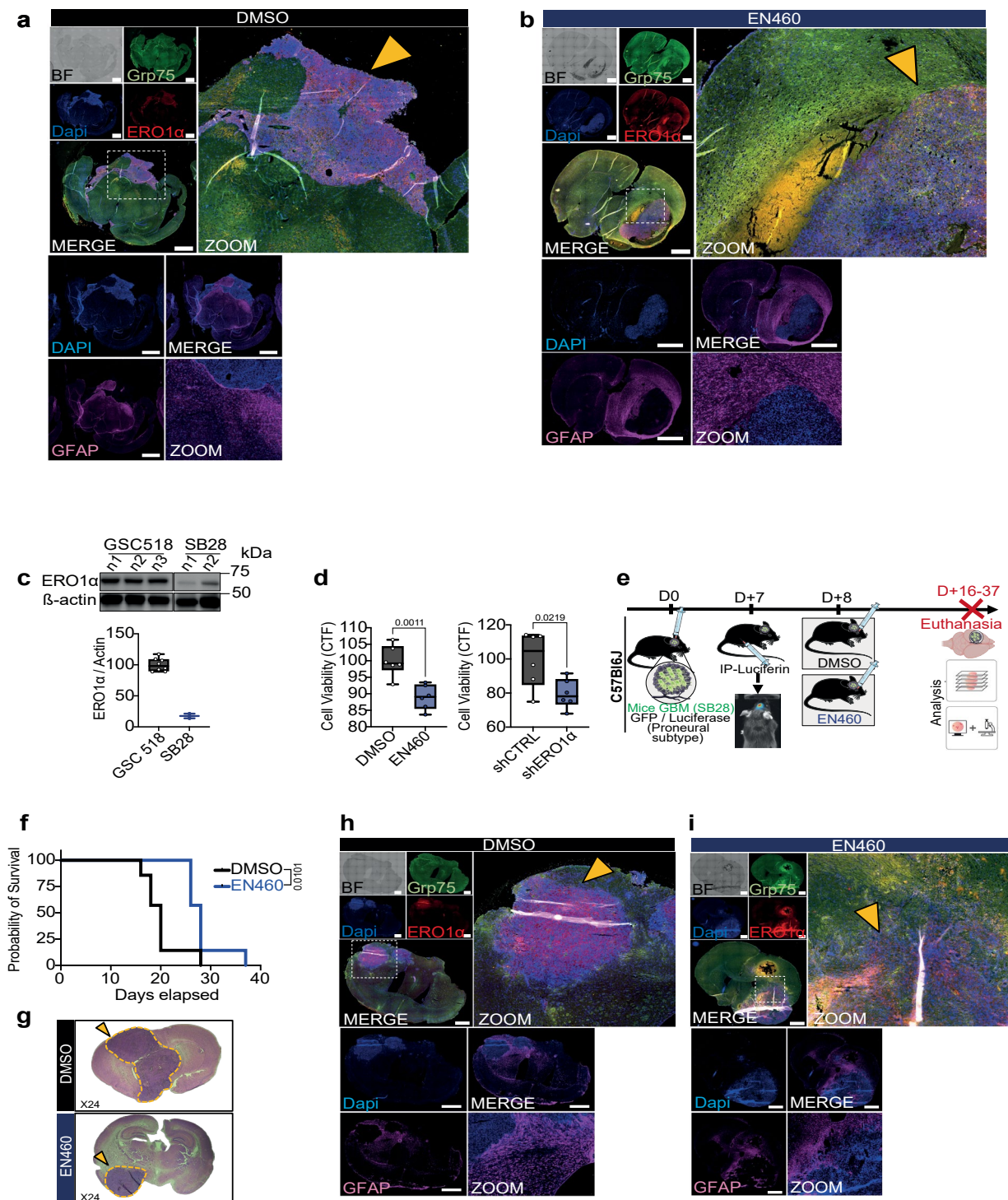
P-145 and P-143 on the right) of ERO1α, relative to β-actin expression in Ge518. **e**, Plots showing the relative abundance of each phosphorylation according to the indicated conditions for 24h ( $n=3$ –6 biological replicates). **f**, Schematic summary of the findings shown in Fig. 2i where glucose deprivation differentially affects P-145 and P-143, while EN460 inactivates ERO1α. In all figures, HG = high glucose, LG = low glucose and EN = EN460. In all figures, histograms or dot plots are expressed with mean  $\pm$  s.e.m. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$  and \*\*\*\*  $p < 0.0001$ ). One-way (a) analysis of variance (ANOVA) followed by Fisher (a) multiple comparison tests.





**Extended Data Fig. 9 | Related to Fig. 8. a**, Image (top) and quantification (bottom) of the western blot showing the expression of ERO1 $\alpha$  in GSC Ge518 stable for the inducible shERO1 $\alpha$  treated with (Dox-) or without (Dox+) doxycycline for 48h. **b**, Image (top) and cell viability quantification using CellTiter-Glo kit (bottom) of GSC Ge518 stable for the inducible shERO1 $\alpha$  treated with (Dox-) or without (Dox+, red-signal) doxycycline for 48h (n = 3 biological replicates per condition); scale bar = 100 $\mu$ m. **c** and **d**, Representative images of the co-culture (brain organoid + Ge518) in the indicated conditions; scale bar = 100 $\mu$ m. **c-right**, **e-f**, Violin plots displaying tumour cell area per organoid in Dox- or Dox+ conditions (**c**, right) (n = 3 biological replicates) or in adenovirusLinker (ad-L5) vs. Control (ad-L1) (**e**) (n = 3 biological replicates) and **f**, adenovirusFate-1 (adFate-1) vs. Control (adGFP) in the specified conditions for one week (n = 2–3 biological replicates). **g**, Table summarizing chemicals (or metabolites) predicted to cross the blood-brain barrier (BBB), as identified by an automated Tree2C script using the BBB Predictor platform. **h**, Representative

images showing high-intensity (non-comparable) tumour bioluminescence over time under the indicated conditions. **i-j**, Representative histological sections of nude mouse (Dox-) brain tumours: **i**, brightfield (BF) and immunofluorescence staining for Grp75 (green), DAPI (blue), and ERO1 $\alpha$  (red); **j**, DAPI (blue) and GFAP (magenta); scale bar = 1.5mm. **k**, Quantification of the indicated signal intensity within the region of interest (ROI; dotted circles in **i** and **j**) in tumour tissue relative to the corresponding ROI in normal brain (n = 3 replicates of brain samples per condition). **l**, Analysis showing signal-intensity profiles along the white lines indicated in panels **i** and **j**. In all figures, M = mesenchymal (GSC 518), HG = high glucose and LG = low glucose. In all figures, violins and histograms are expressed with mean  $\pm$  s.e.m. (\* p < 0.05, \*\* p < 0.01 and \*\*\*\* p < 0.0001). Two (**k**) or one-way (**e**, **f**) analysis of variance (ANOVA) followed by Fisher (**e**, **f**) or Sidak (**k**) multiple comparison tests. Parametric Unpaired t-test, two-tailed (**a**, **b**) or Mann-Whitney, one-tailed test (**c**).



**Extended Data Fig. 10 | Related to Fig. 8.** **a–b**, Representative histological sections of nude mice brain tumours treated with DMSO (**a**) or EN460 (**b**): top, brightfield (BF) and immunofluorescence staining for Grp75 (green), DAPI (blue), and ERO1α (red); bottom, DAPI (blue) and GFAP (magenta); scale bar = 1.5mm. **c**, immunoblots showing the expression of indicated proteins for GSC Ge518 and SB28. Box plots show the fold change of protein expression normalized against β-actin expression and determined by densitometry analysis (n = 2–8 biological replicates). **d**, left, Box plots show the fold change of cell viability, quantified using CellTiter-Glo kit, under EN460 treatment for 72h in SB28 GBM mice model (n = 6 wells in n = 2 biological replicates) or d-right, ERO1α knockdown (n = 6 wells in n = 2 biological replicates). Box-and-whisker plots (**c–d**) show median (centre line), interquartile range (25th–75th percentiles) and min–max (whiskers); all points are shown. **e**, Experimental design to assess in vivo effects

of EN460 on xenograft SB28 (left) partially generated using BioRender software. **f**, Kaplan-Meier survival curve of female C57BL/6J OlaHsd immunocompetent mice bearing SB28 under control (DMSO) or EN460 treatment (n = 7 mice per group). **g**, Representative brain sections in the described conditions stained with HPS, showing the tumour boundary delineated by the yellow outline. **h–i**, Representative histological sections of female C57BL/6J OlaHsd immunocompetent mice brain tumours treated with DMSO (**a**) or EN460 (**b**): top, brightfield (BF) and immunofluorescence staining for Grp75 (green), DAPI (blue), and ERO1α (red); bottom, DAPI (blue) and GFAP (magenta); scale bar = 1.5mm. In all figures, HG = high glucose, LG = low glucose and EN = EN460. In all figures, box-dots and violins are expressed with mean ± s.e.m. (\* p < 0.05, \*\* p < 0.01 and \*\*\* p < 0.001). Parametric Unpaired t-test (**d**) or Log-rank (Mantel-cox) test (**f**).

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|-----------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Data collection | <ul style="list-style-type: none"><li>- R2: Genomics Analysis and Visualization Platform : TGCA analysis (<a href="http://r2.amc.nl">http://r2.amc.nl</a>)</li><li>- Gliovis : Data Visualization tools for brain tumor datasets (<a href="https://gliovis.bioinfo.cnio.es/">https://gliovis.bioinfo.cnio.es/</a>)</li><li>- Xenabrowser (UoC, Santa-Cruz)</li><li>- Hegi-GBM dataset and CGGA-Glioma dataset)</li><li>- SRplot : dot plot diagrams (<a href="https://www.bioinformatics.com.cn/plot_basic_cluster_heatmap_plot_024_en">https://www.bioinformatics.com.cn/plot_basic_cluster_heatmap_plot_024_en</a>)</li><li>- G-profiler : performs functional enrichment analysis (<a href="https://biit.cs.ut.ee/gprofiler/gost">https://biit.cs.ut.ee/gprofiler/gost</a>)</li><li>- Broad-institute Single Cell portal : Single Cell analysis (<a href="https://singlecell.broadinstitute.org/single_cell/study/SCP393/single-cell-rna-seq-of-adult-and-pediatric-glioblastoma?cluster=Cell%20State%20Hierarchy%20Plot&amp;spatialGroups=-&amp;annotation=MESlike2--numeric--study&amp;subsample=all#study-visualize">https://singlecell.broadinstitute.org/single_cell/study/SCP393/single-cell-rna-seq-of-adult-and-pediatric-glioblastoma?cluster=Cell%20State%20Hierarchy%20Plot&amp;spatialGroups=-&amp;annotation=MESlike2--numeric--study&amp;subsample=all#study-visualize</a>).</li><li>- BBB predictor (<a href="https://www.ddl.unimi.it/vegaol/bbbp.htm">https://www.ddl.unimi.it/vegaol/bbbp.htm</a>)</li><li>- Mayo Clinic Brain Tumor Patient-Derived Xenograft National Resource : RNAseq on GBM (<a href="https://www.mayo.edu/research/labs/translational-neuro-oncology/mayo-clinic-brain-tumor-patient-derived-xenograft-national-resource/pdx-characteristics/pdx-phenotype">https://www.mayo.edu/research/labs/translational-neuro-oncology/mayo-clinic-brain-tumor-patient-derived-xenograft-national-resource/pdx-characteristics/pdx-phenotype</a>)</li><li>- The Ivy Glioblastoma Atlas Project (IvyGAP) was used to visualize gene expressions (<a href="https://glioblastoma.alleninstitute.org/static/home">https://glioblastoma.alleninstitute.org/static/home</a>)</li></ul> |
| Data analysis   | <ul style="list-style-type: none"><li>- Data analysis and statistical tests were done using PRISM (version 9 and 10).</li><li>- Mice sample sizes: G*Power3 software</li><li>- FACS data were processed BD FACSDiva software.</li><li>- Live imaging were processed with Fiji (NIH, USA) software (Time series analyzer).</li><li>- Electronic Microscopy were processed with Fiji (NIH, USA) software.</li><li>- IF data were processed with Harmony software (Revvity).</li><li>- Live imaging data performed with Incucyte were processed with Sartorius software.</li></ul>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |



- MS data were processed with DIA-NN v.1.8.1 software, R software and SwissProt Human database .
- 13C Glucose tracing were processed with Thermo TraceFinder software (Thermo Fisher Scientific).
- HPLC data were processed with : Xcalibur

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

## Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

- The mass spectrometry proteomics data for glioblastoma differentiated cell samples have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier <https://www.ebi.ac.uk/pride/archive/projects/PXD073353>.

- The mass spectrometry proteomics data for glioblastoma stem cell samples have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier <https://www.ebi.ac.uk/pride/archive/projects/PXD073390>.

- The HPLC Lipidomics data have been deposited to Zenodo. <https://doi.org/10.5281/zenodo.18340802>

- All raw data used for the conception and statistical analysis of the figure panels have been deposited in Excel files in Source Data section.

## Research involving human participants, their data, or biological material

Policy information about studies with [human participants or human data](#). See also policy information about [sex, gender \(identity/presentation\), and sexual orientation](#) and [race, ethnicity and racism](#).

Reporting on sex and gender

Ge269, 738, 835, 904, 970.2, and 258 originated from male patients, whereas Ge518 and 688 originated from female patients.

Reporting on race, ethnicity, or other socially relevant groupings

The patient-derived models used in the study were obtained through an MTA and were collected in a biobanking protocol approved by the ethics committee of Geneva. Each model, Ge269, Ge518, Ge688, Ge738, Ge835, Ge258, Ge904, correspond to the tumor of one patient that have been derived, biobanked and maintained in culture for performing the experiments. We have no access to patient clinical data, except gender, according these agreements. All patients provided written informed consent and did not received any compensation.

Population characteristics

N/A

Recruitment

N/A

Ethics oversight

N/A

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://nature.com/documents/nr-reporting-summary-flat.pdf)

## Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

Number of mice and Fish was determined performed experimentations with G\*Power3 software

Data exclusions

During image analysis (IF, live imaging, E.M.), dead cells, identified by their round and small size, were excluded from the quantification data.

Replication

All attempts at replication were successful. All experiments were repeated at least three times with exceptions for Extended data Fig. 4 e-f (n = 6 technical replicates in n = 2 biological replicates); Extended data Fig. 10 -d-left (n = 6 technical replicates in n = 2 biological replicates); Fig. 3 I (n = 12 -24 technicals replicates in n=2 biological replicates); Extended Fig. 6f.

Randomization

Group of mice (C57Bl6J and Nudes) were randomly realized before treatments injections.

## Blinding

The Investigators were not blinded to allocation during experiments and outcome assessment. However, for live imaging and IF, automatic acquisition and injections were performed using Harmony software.

## Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

### Materials & experimental systems

| n/a                                 | Involved in the study                                           |
|-------------------------------------|-----------------------------------------------------------------|
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Antibodies                  |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Eukaryotic cell lines       |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Palaeontology and archaeology          |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Animals and other organisms |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Clinical data                          |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Dual use research of concern           |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> Plants                                 |

### Methods

| n/a                                 | Involved in the study                              |
|-------------------------------------|----------------------------------------------------|
| <input checked="" type="checkbox"/> | <input type="checkbox"/> ChIP-seq                  |
| <input type="checkbox"/>            | <input checked="" type="checkbox"/> Flow cytometry |
| <input checked="" type="checkbox"/> | <input type="checkbox"/> MRI-based neuroimaging    |

## Antibodies

### Antibodies used

All antibodies details are included in the M&M section of the manuscript and listed below :  
 B-Actin-Peroxidase Sigma, A3854 (WB : 1/10 000); ERO1a: Clinisciences, GeneTex, GTX112589 (WB : 1/1000); ERO1a: Abcam, ab177156 (WB : 1/1000; IF: 1/100); SERCA2 : Abcam, ab150435 (WB : 1/1000); VDAC1/3 : Abcam, 14734 (WB : 1/1000, PLA: 1/400); IP3R1, MERCK-Millipore (WB : 1/1000), 250738; IP3R3 : MERCK-Millipore, AB9076 (WB : 1/1000, PLA : 1/400); MCU : Cell signaling/ 14997S (WB : 1/1000); HIF-1 $\alpha$  : Cell signaling/ 48085S (WB : 1/1000); p53 : Cell signaling : 48818S (WB : 1/1000); P-PERK : R&D, AF3999 (WB : 1/800); Perk : abcam : ab65142 (WB : 1/1000); P-eif2a : PhosphoPlus eIF2-alpha (Ser51) , Cell Signaling, 3398S (WB : 1/1000); eiF2a tot : Cell Signaling, 5324S (WB : 1/1000); PDI : Cell Signaling, 2446S (WB : 1/1000); ATF4 : Cell signaling, 11815S (WB : 1/1000); Akt: Cell Signaling, 4691S (WB : 1/1000); P-Akt : (T308), Cell Signaling, 9275S (WB : 1/1000); Chop : Ozyme, 2895T (WB : 1/1000); ERO1b : abcam, 197290 (WB : 1/1000); NOX4 : abcam : 154244 (WB : 1/1000); g-Tubulin : Thermo-Scientific, MA1-850 (WB : 1/10 000); Grp75 : abcam : 2799 (WB : 1/1000; IF: 1/50); Peroxiredoxin 4 : Abcam : 184167 (WB : 1/1000); GFP : Abcam 183734 (IF : 1/100); ERp57 : abcam : 242367 (WB : 1/1000, IF : 1/50); GFAP: Dako : Z0334 (IF: 1/50); ERO1a-P-ser106, P-ser143 and P-ser145 were made by Covalab factory (Lyon, France, WB : 1 / 400).

### Validation

- All antibodies were used according to the manufacturer's recommendations, data sheet and previous literature associated in the respective manufacturer's website.  
 - For Proximity Ligation Assay (PLA), Validation with single antibody were realized as shown in Figure 3J and Extended Figure 4J.  
 - ERO1a-P-ser106, P-ser143 and P-ser145 were made by Covalab factory (Lyon, France) and validated by Elisa Tests and Knock-down expression / Phospho-inhibitor buffer as illustrated in Extended Figure 4c.

## Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

### Cell line source(s)

Not applicable as we did not use cell lines.

### Authentication

Authentication was not applicable. The GBM cell lines used were cells derived from tumor of human patients. Ge269, 738, 835, 904, 970.2, and 258 originated from male patients, whereas Ge518 and 688 originated from female patients.

### Mycoplasma contamination

Mycoplasma tests were performed routinely

### Commonly misidentified lines (See [ICLAC](#) register)

Not applicable as we did not use cell lines.

## Animals and other research organisms

Policy information about [studies involving animals; ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

### Laboratory animals

Female C57BL/6J OlaHsd immunocompetent mice (envigo); female nu/nu nude immunocompromised mice (envigo) and Transgenic Tg(fli1:EGFP) zebrafish

### Wild animals

N/A

### Reporting on sex

Experiments were performed in C57bl6J and in nudes in female mice.

## Field-collected samples

- 6-10-week-old female nu/nu nude immunocompromised mice weighing 20-25 g (Envigo) or C57BL/6J0laHsd immunocompetent mice weighing 18-22g (Envigo) were housed five per cage, with standard husbandry for specific pathogen-free (SPF) conditions provided by staff of the animal core facility (P-PAC, CRCL). Mice were allowed to acclimate for two weeks prior to experimentation. All procedures involving animals were conducted in the CRCL small animal facility (P-PAC, accredited under approval number D693880202) in accordance with European Directive 2010/63/EU and were approved by the local Institutional Animal Care (ACCeS ethical committee) and Use Committee and authorized by the French Ministry of Research (APAFIS #40391). Mice were housed in individually ventilated cages at a maximum density of five animals per cage, on poplar wood bedding (SAFE Select Fine). Environmental enrichment was provided with at least two dental cotton rolls per cage. Animals had ad libitum access to acidified drinking water (pH  $3.0 \pm 0.5$ ). Bedding was replaced every two weeks, and full cage changes were performed every eight weeks. Animals were maintained under a 12 h light/12 h dark cycle (lights on at 07:00 and off at 19:00), at an ambient temperature of  $21 \pm 2$  °C. Relative humidity was approximately  $45\% \pm 30\%$  and was not actively regulated.

- Transgenic Tg(fli1:EGFP) zebrafish were maintained at 28.5°C under a 14-hour light/10-hour dark cycle in the Zebrafish Facility at the Walther Straub Institute, LMU Munich. The fish water was changed daily, and larvae older than 7 days were fed twice a day with ground brine shrimp.

## Ethics oversight

- All experimental procedures involving zebrafish were performed in accordance with the guidelines of the Regierung von Oberbayern, the European equivalent of the NIH's Guide for the Care and Use of Laboratory Animals (Directive 2010/63/EU), and the ARRIVE guidelines (<https://www.nc3rs.org.uk/arrive-guidelines>).

-All procedures involving mice were conducted in the CRCL small animal facility (P-PAC, accredited under approval number D693880202) in accordance with European Directive 2010/63/EU and were approved by the local Institutional Animal Care (ACCeS ethical committee) and Use Committee and authorized by the French Ministry of Research (APAFIS #40391).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

## Plants

## Seed stocks

N/A

## Novel plant genotypes

N/A

## Authentication

N/A

## Flow Cytometry

### Plots

Confirm that:

- ☐ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- ☐ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- ☐ All plots are contour plots with outliers or pseudocolor plots.
- ☒ A numerical value for number of cells or percentage (with statistics) is provided.

### Methodology

## Sample preparation

Sample preparation for Annexin V/PI staining was performed following the manufacturer's recommendations (Thermo Fisher Scientific, V13245)

## Instrument

FACS : BD Biosciences, Fortessa.

## Software

BD FACSDiva software.

## Cell population abundance

*Describe the abundance of the relevant cell populations within post-sort fractions, providing details on the purity of the samples and how it was determined.*

## Gating strategy

FSC/SSC gating was performed to include the entire population of dead and live cells in the sample. Analysis was conducted exclusively on single cells. For all cell lines and experimental replicates, gating was carried out using unstained cells, single PI staining, single Annexin V staining, and double staining with a positive control for cell death.

☐ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.