Coordinate β-adrenergic inhibition of mitochondrial activity and angiogenesis arrest tumor growth

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Mitochondrial metabolism has emerged as a promising target against the mechanisms of tumor growth. Herein, we have screened an FDA-approved library to identify drugs that inhibit mitochondrial respiration. The β1-blocker nebivolol specifically hinders oxidative phosphorylation in cancer cells by concertedly inhibiting Complex I and ATP synthase activities. Complex I inhibition is mediated by interfering the phosphorylation of NDUFS7. Inhibition of the ATP synthase is exerted by the overexpression and binding of the ATPase Inhibitory Factor 1 (IF1) to the enzyme. Remarkably, nebivolol also arrests tumor angiogenesis by arresting endothelial cell proliferation. Altogether, targeting mitochondria and angiogenesis triggers a metabolic and oxidative stress crisis that restricts the growth of colon and breast carcinomas. Nebivolol holds great promise to be repurposed for the treatment of cancer patients.
Cancer constitutes a major health problem worldwide. Despite the existence of standard protocols and therapies for colon\(^1\) and breast\(^2\) cancer, the establishment of new therapeutic approaches is imperative to minimize the social and economic burden caused by these diseases. An enhanced aerobic glycolysis is one of the hallmarks of cancer\(^3,4\) and glycolysis itself has been proposed as a potential chemotherapeutic target to combat the disease\(^5-7\). However, the complete understanding of the metabolic dependencies of tumors could provide additional strategies to restrain tumor growth\(^7\). In this regard, mitochondrial metabolism also affords a promising target to fight cancer progression\(^8-11\).

Mitochondria play essential cellular functions regulating the provision of metabolic energy by oxidative phosphorylation (OXPHOS), the execution of cell death, and intracellular signaling by Ca\(^{2+}\) and reactive oxygen species (ROS)\(^12-14\). In OXPHOS, the ATP synthase catalyzes the synthesis of ATP using as driving force the proton electrochemical gradient generated by the respiratory chain\(^12\). The activity of the ATP synthase is regulated by its physiological inhibitor, the ATPase inhibitory factor 1 (IF1), a small nuclear-encoded mitochondrial protein that is highly overexpressed in some human carcinomas\(^15-17\). The activity of IF1 as an inhibitor of the ATP synthase is regulated under normal physiological conditions by its expression and by the phosphorylation of S39 through the activity of a mitochondrial protein kinase A-like activity\(^18\). In human carcinomas, IF1 is found predominantly dephosphorylated and hence acting as an inhibitor of the ATP synthase\(^18\), contributing to metabolic reprogramming of the cells to an enhanced glycolytic phenotype\(^15,16,19\). Hence, the IF1/ATP synthase system offers a potential therapeutic target in cancer and other human disorders\(^20\), as recently stressed in aging and dementia\(^21\).

Cancer drug discovery and development is a costly and lengthy task that spans more than a decade before the drug is ready for treating patients\(^22\). The fact that only a few drugs are finally approved for use puts pressure on their price to compensate the economic burden caused by these diseases. An enhanced aerobic glycolysis is one of the hallmarks of cancer\(^3,4\) and glycolysis itself has been proposed as a potential chemotherapeutic target to combat the disease\(^5-7\). However, the complete understanding of the metabolic dependencies of tumors could provide additional strategies to restrain tumor growth\(^7\). In this regard, mitochondrial metabolism also affords a promising target to fight cancer progression\(^8-11\).

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Cancer drug discovery and development is a costly and lengthy task that spans more than a decade before the drug is ready for treating patients\(^22\). The fact that only a few drugs are finally approved for use puts pressure on their price to compensate the investment in drug discovery. Drug repurposing has emerged as an alternative strategy to overcome the costs and time invested in drug discovery\(^22\). The success of drug repurposing relies on that the compounds have already been introduced for another indication and tested in human therapy with acceptable known side effects that improve the quality of life of the patients\(^22\).

Herein, we screen an FDA-approved library of small compounds to find drugs that could inhibit the activity of the mitochondrial ATP synthase in cancer cells and consequently could prevent tumor growth. We find 13 compounds that inhibit mitochondrial respiration and the activity of the ATP synthase. We study in detail the mechanisms by which the third-generation \(\beta_1\)-blocker nebivolol halts colon and breast tumor growth in vivo. The results emphasize the role of blocking \(\beta_1\)-adrenergic signaling to inhibit cancer progression, supporting the repurposing of nebivolol as an anticancer drug to be used in combined chemotherapy of the oncoligic patient.

**Results**

**Nebivolol inhibits mitochondrial respiration.** To identify the inhibitors of OXPHOS that could interfere with cancer progression, we screened an FDA-approved library of 1018 small compounds that in short-term treatment of 3 h affected mitochondrial respiration of HCT116 colon cancer cells (Fig. 1a). The study was initially carried out in a Seahorse XF96 analyzer using the oligomycin-sensitive respiration (OSR) as a reporter of the drug’s effect because it represents an estimate of the activity of the ATP synthase. We identified compounds that enhanced or inhibited OSR by 40% when compared to cells treated with the vehicle (dimethyl sulfoxide (DMSO)) (Fig. 1a). Further in-depth investigation of the effect of the inhibitors on OXPHOS was carried out using the Seahorse XF24.

Thirteen FDA-approved drugs significantly inhibited basal, maximum mitochondrial respiration, and OSR of HCT116 cells (Fig. 1b and Table 1). Blocking of cardiac \(\beta\)-adrenoceptors by propranolol recently showed the relevance of the PKA/cAMP signaling pathway in preventing the phosphorylation of IF1 and the subsequent inhibition of OXPHOS in heart mitochondria\(^19\). Hence, of the 13 inhibitors of respiration identified (Table 1), we focused on nebivolol for further in-depth study because it is a \(\beta_1\)-adrenergic inhibitor whose mechanism of action is compatible with targeting OXPHOS, both at the level of the respiratory chain\(^23,24\) and at the level of the ATP synthase\(^15\).

Nebivolol inhibited mitochondrial respiration of both colon HCT116 (Fig. 1c) and breast MDA-MB-231 (Fig. 1d) cancer cells when glucose (Fig. 1c, d) or palmitate (Supplementary Fig. 1a) were used as respiratory substrates. Titration of the effect of increasing concentrations of nebivolol in OSR revealed an IC\(_{50}\) of 0.9 and ~2.1 µM in HCT116 and MDA-MB-231, respectively (Supplementary Fig. 1b). Similar results were obtained for the IC\(_{50}\) of nebivolol on the maximum respiratory rate (Supplementary Fig. 1b). Moreover, nebivolol also inhibited mitochondrial respiration of neuroblastoma (SH-SY5Y), lung (A549), and ovarian (OVCAR8) cancer cells (Fig. 1e). Remarkably, nebivolol did not affect mitochondrial respiration of the Hs 578T normal breast cell line (Fig. 1f) nor of mouse primary neuronal cultures and C2C12 myocytes (Fig. 1g). The lack of effect of the drug on mitochondrial respiration in isolated liver organelles (Supplementary Fig. 1c) excluded the possibility of a direct inhibitory effect of nebivolol in mitochondria.

The effect of four additional \(\beta_1\)-blockers, bisoprolol, metoprolol, betaxolol, and acetylbutolol, also significantly inhibited the mitochondrial respiration of HCT116 cancer cells (Fig. 1h). Interestingly, ICI 118,551 and SR 59230A, respectively, representing a \(\beta_2\)- and \(\beta_3\)-adrenergic receptor blockers, did not affect mitochondrial respiration in HCT116 cancer cells (Fig. 1i). These results suggest that the inhibition of mitochondrial respiration in cancer cells stems from \(\beta_1\)-adrenergic blockade. In fact, only cells that responded to nebivolol express \(\beta_1\)-adrenergic receptors (Fig. 1j).

**Nebivolol inhibits mitochondrial ATP synthesis.** Treatment of colon and breast cancer cells with nebivolol significantly diminished the synthesis of ATP by mitochondrial ATP synthase as assessed in permeabilized colon and breast cancer cells (Fig. 2a). In response to nebivolol, cancer cells partially induced aerobic glycolysis as a result of the inhibition of ATP supply by OXPHOS (Fig. 2b). In agreement with the inhibition of mitochondrial respiration by nebivolol, the drug triggered a slight but significant increase in mitochondrial membrane potential (ΔΨm) in cancer cells (Fig. 2c). Interestingly, and consistent with the inhibition of the ATP synthase by nebivolol, oligomycin, an inhibitor of the ATP synthase, exerted a similar increase in ΔΨm in both cancer cells (Fig. 2c). Moreover, we also observed a slight but significant increase in cellular ROS levels in nebivolol-treated cells when compared to controls (Fig. 2d). However, nebivolol-treated cells did not show significant differences in cellular proliferation (Supplementary Fig. 2a) and cell death responses to different death-inducing agents (Supplementary Fig. 2b).

**Nebivolol increases IF1 expression.** Interestingly, the effect of nebivolol in cellular respiration occurred in both cell lines in the absence of changes in the expression of subunits of respiratory complexes, albeit for the sharp increased expression of IF1...
(Fig. 2e). The nebivolol-mediated accumulation of IF1 is independent of significant changes in IF1-mRNA abundance (Supplementary Fig. 2c) and its accumulation could explain the inhibition of ATP synthesis observed in the cells (Fig. 2a).

The organization of OXPHOS complexes is not affected. Rapid changes in mitochondrial respiratory activity could also be related to a different organization of OXPHOS complexes.\textsuperscript{25,26} However, BN-PAGE analysis of the supramolecular organization of OXPHOS complexes after nebivolol treatment of HCT116 cells indicated no relevant changes in their supramolecular organization (Fig. 3a), supporting that the inhibition of maximum respiration triggered by nebivolol should be ascribed to another mechanism. Interestingly, IF1 was also found bound to the ATP
Fig. 1 Nebivolol inhibits mitochondrial respiration in cancer cells. a Effect of 1018 FDA-approved drugs on the oligomycin-sensitive respiration (OSR) of HCT116 colon cancer cells. Activators (≥1.4; in blue) and inhibitors (≤0.6; in red) that affected OSR by 40% when compared to control are highlighted. Compounds with no relevant effect (NRE) are shown in gray. b Box plots represent 25th to 75th percentiles with the median value in the middle line, with all data represented from minimal to maximal values indicated with whiskers of the inhibitors (red box) or the compounds with no relevant effect (NRE, black box). c Respiratory profile of HCT116 cells treated (red trace) or not (black trace) for 3 h with nebivolol (1 μM). OCR oxygen consumption rate, OL oligomycin, DNP 2,4-dinitrophenol, ROT rotenone, ANT antimycin A. d Respiratory profile of MDA-MB-231 cells treated (red trace) or not (black trace) for 3 h with nebivolol (1 μM). e Respiratory profile of neuroblastoma SH-SY5Y, lung A549 and ovarian OVCAR8 cells treated (red trace) or not (black trace) for 3 h with nebivolol (1 μM). f Respiratory profile of breast Hs 578T cells treated (red trace) or not (black trace) for 3 h with nebivolol (1 μM). g Respiratory profile of mouse primary neuronal cultures (left panel) and C2C12 myoblast (right panel) treated (red trace) or not (black trace) for 3 h with nebivolol (1 μM). h Basal and OSR of HCT116 cells treated for 3 h with nebivolol (red bar, *p = 0.01 and 0.01), bisoprolol (yellow bar, *p = 0.01 and 0.01), metoprolol (purple bar, *p = 0.04 and 0.03), betaxolol (blue bar, *p = 0.02 and 0.05), acebutolol (green bar, *p = 0.03 and 0.02) (1 μM) or left untreated (black bar). i Respiratory profile of HCT116 cells treated for 3 h (1 μM) with β1-antagonist nebivolol (NEB, red trace and bar); Basal: *p = 0.05; OSR: *p = 0.04; MAX: *p = 0.01). j β2-antagonist ICI 118,552 (orange trace and bar); β3-antagonist SR 59230A (gray trace and bar) or left untreated (CRL, black trace and closed bar). k Representative western blots of the expression of β1-adrenergic receptor (ADRB1) in cancer cells (HCT116, MDA-MB-231, A549, SH-SY5Y, and OVCAR8) and normal cells (Hs 578T, primary neurons and C2C12). Tubulin is shown as loading control. Bars indicate the mean ± SEM of three biological replicates. *p < 0.05 and b-e p < 0.05 when compared to CRL by two-sided Student’s t test. See also Supplementary Fig. 1. Source data are provided as a Source Data file.

Table 1 Potent inhibitors of mitochondrial respiration.

| Compound     | Indication | Target          | OSR     | Basal     | Pubmed hits | Clinical trials |
|--------------|------------|-----------------|---------|-----------|-------------|-----------------|
| Sorafenib    | Cancer     | VEGFR, PDGFR    | 0.33 ± 0.10 | 0.28 ± 0.09 | 1/1       | 3/1/25          |
| Regorafenib  | Cancer     | VEGFR           | 0.46 ± 0.01 | 0.67 ± 0.05 | 1/0       | 1/72           |
| Ponatinib    | Cancer     | Abl             | 0.46 ± 0.08 | 0.60 ± 0.04 | 0/0       | 0/0            |
| Itaconazole  | Cancer     | P450            | 0.27 ± 0.10 | 0.17 ± 0.10 | 1/0       | 3/0            |
| Telaprevir   | Infection  | HCV Protease    | 0.39 ± 0.10 | 0.58 ± 0.08 | 0/0       | 0/0            |
| Sulfameter   | Infection  | Dihydropteroate synthase | 0.39 ± 0.07 | 0.50 ± 0.05 | 0/0 | 0/0 |
| Crystal violet | Infection | Thioredoxin reductase 2 | 0.06 ± 0.09 | 0.26 ± 0.10 | 0/0 | 0/0 |
| Butaconazole | Infection  | Steroid synthesis | 0.47 ± 0.10 | 0.55 ± 0.09 | 0/0 | 0/0 |
| Nebivolol    | Cardiovascular | β1-adrenergic receptor | 0.28 ± 0.10 | 0.45 ± 0.08 | 0/0 | 0/0 |
| Pazopanib    | Cardiovascular | VEGFR | 0.40 ± 0.10 | 0.47 ± 0.05 | 0/0 | 17/4 |
| Lomerizine   | Cardiovascular | Ca2+ channel | 0.51 ± 0.01 | 0.51 ± 0.03 | 0/0 | 0/0 |
| Glyburide    | Endocrinology | β cells | 0.40 ± 0.09 | 0.50 ± 0.10 | 0/0 | 0/0 |
| Cicmetidine  | Inflammation | Histamine H2 receptor | 0.24 ± 0.02 | 0.41 ± 0.04 | 0/0 | 0/0 |

Compounds of the library that inhibit more than 40% mitochondrial respiration of HCT116 colon cancer cells. Name of the drug, clinical indication, target, OSR, and basal respiration. Values are mean ± SEM of five experiments referred to control values. *p < 0.05 when compared to CRL by two-sided Student’s t test. PubMed search results for searching each drug with either “breast cancer” (numerator) or “colon cancer” (denominator) AND mitochondria. Search results for the clinical trials (clinicaltrials.gov) for breast/colon cancer.

synthase and other oligomeric states of the enzyme in human cancer cells (Fig. 3a), in agreement with recent findings in mouse tissues. Moreover, we observed that the amount of IF1 co-fractionating with F1-ATPase and the monomeric ATP synthase (Complex V) in BN-PAGE increased significantly (Fig. 3b), mimicking the increase in IF1 observed in cellular extracts after nebivolol treatment (Fig. 2c).

Nebivolol affects phosphorylation of OXPHOS complexes. It is known that the phosphorylation of serine residues regulates the activity of proteins of OXPHOS. In this regard, the inhibition of maximum respiration induced by treatment with nebivolol could be due to a post-transcriptional β-adrenergic blockade of the respiratory chain (Fig. 1c–e) and of the ATP synthase (Fig. 2a). Hence, we investigated the existence of modifications in serine-phosphorylated proteins in OXPHOS complexes by BN-PAGE in response to nebivolol treatment (Fig. 4a). A ~50% decrease in phosphorylation of proteins contained in supercomplexes (SC) was observed when the cells were treated with nebivolol (Figs. 3a, 4a for comparison). Interestingly, protein phosphorylation in another complex migrating at ~600 kDa was unaltered in the same situation (Fig. 4a).

Since IF1 is also found in high molecular mass complexes co-migrating with SC (Fig. 3a), we initially studied if the reduction in protein phosphorylation by nebivolol at the level of SC (Fig. 4a) could be ascribed to deficient phosphorylation of IF1. Immunoprecipitation of IF1 and blotting with anti-phosphoserine antibody revealed that most of IF1 in HCT116 cells is in the dephosphorylated state (Fig. 4b) as compared to cells treated with the membrane permeable db-cAMP (Fig. 4b). To quantitate the relative amount of dephospho-IF1, we carried out 2D-gels to distinguish the phosphorylated forms of IF1 by differences in their pI (Fig. 4c) Consistent with immunoprecipitation experiments (Fig. 4b), we observed that HCT116 and MDA-MB-231 cells contained most of IF1 dephosphorylated (85–100%) focusing at pI 8 (Fig. 4c), which corresponds to the migration of the S39A phosphodefficient IF1 mutant protein (red dotted line in Fig. 4c). Only a small amount of phosphorylated IF1 (~pI 7.2) was found in HCT116 cells corresponding to the migration of the phosphomimetic S39E-IF1 mutant (blue dotted line in Fig. 4c). Overall, the results suggest that inhibition of ATP synthesis by nebivolol is not due to a relevant change in the phosphorylation of IF1 and most likely results from its increased expression in response to the 3-h treatment (Fig. 2e).

The upregulation of IF1 by nebivolol inhibits ATP synthase. To illustrate this latter possibility, we studied the ATP hydrolysis activity of the ATP synthase in isolated mitochondria from both HCT116 and MDA-MB-231 cancer cells (Fig. 4d). The results confirmed that the activity of the enzyme was significantly inhibited in nebivolol-treated cells (Fig. 4d). Moreover, we found
that the mitochondrial content of IF1 was significantly augmented in nebivolol-treated cells (Fig. 4e), in agreement with the increased IF1 found co-migrating with Complex V and F1-ATPase in BN gels (Fig. 3a, b). Consistently, silencing of IF1 expression in HCT116 (Fig. 4f) and MDA-MB-231 (Supplementary Fig. 3a) cells abolished the effect of nebivolol on basal and OSR of the cells. However, the effect of nebivolol on the maximum respiratory rate was maintained in IF1-silenced cells (Fig. 4f and Supplementary Fig. 3a), indicating that in addition to the effect of the drug on the ATP synthase (Figs. 2a, 4d, f), nebivolol was also affecting the activity of some of the respiratory complexes.

**Nebivolol prevents phosphorylation and inhibits complex I.** To verify this idea, we determined the activity of complexes I, II, and IV of the respiratory chain (Fig. 4g-i). Nebivolol significantly diminished the activity of complex I (Fig. 4g) without affecting complex II (Fig. 4h) and IV (Fig. 4i). The results suggest that nebivolol has a specific inhibitory effect on complex I, which may contribute to its anti-tumor activities.
Fig. 2 Nebivolol inhibits mitochondrial ATP synthase. HCT116 and MDA-MB-231 cells were treated during 3 h with 1 µM nebivolol (NEB; red dots and bars), oligomycin (OL; gray dots and bars) or left untreated (CRL; closed dots or bars). a Left panel, kinetic representation of the production of ATP in digitonin-permeabilized cells. The inhibition of ATP synthase activity was accomplished by the addition of 30 µM OL. Right panel, histograms show the ATP synthetic activity. Six replicates of six (HCT116, \( p = 0.001 \) and \( p = 0.00001 \)) and three (MDA-MB-231, \( p = 0.004 \) and \( p = 0.001 \)) different biological samples are shown. b Glycolytic flux measured by the initial rates of lactate production. Three replicates of three different biological HCT116 (\( p = 0.01 \) and 0.01) and MDA-MB-231 (\( p = 0.04 \) and 0.01) samples are shown. c Plots and histograms of TMRM\(^{+}\) stained cells to assess the mitochondria membrane potential (\( \Delta \Psi \text{m} \)). FCCP (orange) collapses \( \Delta \Psi \text{m} \). Three replicates of three different biological HCT116 (\( p = 0.01 \) and 0.01) and MDA-MB-231 (\( p = 0.05 \) and 0.02) samples are shown. See Supplementary Fig. 7a for gating strategy. d Histograms show ROS production in HCT116 (\( p = 0.02 \)) and MDA-MB-231 (\( p = 0.05 \)) cells treated (red) or not (black) for 3 h with 1 µM nebivolol. Three replicates of three different biological samples are shown. See Supplementary Fig. 7b for gating strategy. e Representative western blots of four different biological samples of the expression of mitochondrial proteins from complex I (NDUFA9), complex II (SDHB), complex III (Core 2), complex IV (COX IV), complex V (β-F1-ATPase) and IF1 (\( p = 0.011 \) and 0.02), Hsp60 and GAPDH and tubulin as loading controls. Two different samples of control (CRL; closed bars) and nebivolol-treated (NEB; red bars) cells are shown. Bars indicate the mean ± SEM of the indicated samples. *\( p < 0.05 \) and **\( p < 0.01 \) when compared to CRL by two-sided Student’s \( t \) test. See also Supplementary Fig. 2. Source data are provided as a Source Data file.

Fig. 3 Nebivolol increases the amount of IF1 bound to the ATP synthase. HCT116 cells were treated (NEB, red) or not (CRL, black) during 3 h with 1 µM nebivolol. Representative BN-immunoblots of mitochondrial membrane proteins blotted with the antibodies of the indicated subunits of the different OXPHOS complexes. The migration of supercomplexes (SC); complex I and I + III\( _2 \) (NDUFA9); complex II (SDHB); complex III (Core 2); complex IV (COX IV); oligomers (V\( _n \)), dimers (V\( _2 \)), monomers (V), F1-ATPase and β-F1-ATPase subunit of the ATP synthase and their co-migration with IF1 are indicated. The migration of molecular mass markers is indicated in the Coomassie-stained gel and applied for all blots. VDAC and Coomassie-stained gel are shown as loading controls. Two different samples of control (CRL; closed bars) and nebivolol-treated (NEB; red bars) cells are shown. Bars indicate the mean ± SEM of the indicated samples. *\( p < 0.05 \) and **\( p < 0.01 \) when compared to CRL by two-sided Student’s \( t \) test. Source data are provided as a Source Data file.

The activity of the other complexes (Fig. 4h, i). Changes in the activity of Complex I correlate with differences in the phosphorylation of subunits of the complex\(^{23} \). Nebivolol also significantly diminished the phosphorylation of SC (Fig. 4a), where complex I is usually present (Fig. 3a). Consistently, nebivolol specifically inhibited the phosphorylation of complex I immunocaptured proteins from HCT116 and MDA-MB-231 cells (Fig. 4). Phosphoproteomic analysis by MS-spectrometry of the peptides derived from the 15–20 kDa region of the gels (see red box in Fig. 4j) indicated that S117 contained in a tryptic peptide derived from NDUFS7 subunit was the only peptide from complex I that was not phosphorylated when the cells were treated.
with nebivolol (Fig. 4k). Interestingly, this subunit is located in the ubiquinone binding site of complex I (Fig. 4l).

**Nebivolol delays the in vivo growth of colon carcinomas.** Although nebivolol treatment does not affect the proliferation and death of cancer cells growing in culture (Supplementary Fig. 2a, b), we next tested whether restraining OXPHOS by inhibiting complex I and complex V activities with nebivolol could impede colon and breast cancer progression in vivo. For this purpose, nude mice were subcutaneously injected with HCT116-Luc cells into the right and left flanks. When the tumors had reached a volume of ~100 mm³, mice were treated with daily doses of nebivolol 5 days a week. A control NaCl-treated group was also included for comparison. Within 6 days after initiation of nebivolol treatment, mice revealed a significant reduction in tumor luminescence as compared to the controls (Fig. 5a) and...
Moreover, Kaplan–Meier survival analysis showed that nebivolol treatment increased the lifespan of mice as compared to NaCl-treated controls (Fig. 5c). The restraining of tumor growth in nebivolol-treated mice resulted from a significant inhibition of cellular proliferation, as revealed by Ki67 staining (Fig. 5d), and an enhanced cell death, as revealed by the activation of caspase-3 (Fig. 5e). Moreover, tumors of nebivolol-treated mice showed a sharp reduction of ATP content (Fig. 5f).

Since nebivolol treatment slightly increased ROS in breast and colon cancer cells (Fig. 2d), we next studied the potential influence of nebivolol in affecting the reduct status of the carcinomas. A significant increase in nitrotyrosine-modified proteins—a modification related to protein inactivation by reactive nitrogen species—was found in tumors of nebivolol-treated mice when compared to controls (Supplementary Fig. 3b). Moreover, the expression of mitochondrial proteins involved in the antioxidant response, such as the mitochondrial ROS scavenging enzymes superoxide dismutase 2 (SOD2), peroxiredoxine 3 (PRx3), and glutathione reductase (GR), was significantly increased in tumors of mice treated with nebivolol (Fig. 5g). In contrast, the expression of glucose-6-phosphate dehydrogenase (G6PDH), peroxiredoxine 6 (PRx6), and catalase (Cat) was either nonaffected or significantly diminished in the tumors of nebivolol-treated mice (Fig. 5g). Interestingly, and as shown previously in cells (Fig. 2e) and in mitochondria (Fig. 4e), tumors of nebivolol-treated mice showed a significant increase in IF1 expression (Fig. 5g). Altogether, the results indicate that inhibition of mitochondrial respiration in colon carcinomas of nebivolol-treated mice results in a metabolic and redox crisis as demonstrated by the diminished tumor ATP content (Fig. 5f), protein oxidative damage (Supplementary Fig. 3b), and increased mitochondrial antioxidant response (Fig. 5g) when compared to carcinomas of control mice.

To assess the effectiveness of nebivolol in combined therapy of colon cancer, we studied its effect in combination with 5-fluorouracil (5FU). Interestingly, the combined treatment (NEB + 5FU) significantly reduced tumor volume after 6 days of treatment (Fig. 5h). Moreover, Kaplan–Meier survival curves showed that NEB + 5FU significantly increased the lifespan of mice when compared to control or 5FU-treated mice (Fig. 5i).

β1-adrenergic blockade prevents tumor angiogenesis. In order to explain the different cytotoxic effect of nebivolol between in vitro and in vivo studies, we first address the possibility that a metabolite of the degradation pathway of nebivolol could be involved in cytotoxicity. 4OH-nebivolol, the major secondary metabolite of nebivolol degradation, has no effect on mitochondrial respiration (Supplementary Fig. 4a) and cellular proliferation (Supplementary Fig. 4b). Moreover, 4OH-nebivolol showed no cytotoxicity in colon (Supplementary Fig. 5a) and breast (Supplementary Fig. 5b) cancer cells. Likewise, the response of cancer cells to death induced by staurosporine (Supplementary Fig. 5a) or tamoxifen (Supplementary Fig. 5b) was not significantly affected by 4OH-nebivolol, emphasizing the role of blocking β1-receptors to arrest tumor growth in vivo.

β1-adrenergic signaling is involved in angiogenesis in vivo. Hence, we next assessed the potential implication of tumor angiogenesis as a contributing factor that could explain the different cytotoxic effect of nebivolol between in vitro and in vivo data. Analysis of the expression of the angiogenic marker isocitrate dehydrogenase 1 (IDH1) in endothelial cells of the carcinomas suggested that nebivolol arrested microvesSELs formation (Fig. 5j). The inhibition of angiogenesis in carcinomas of nebivolol-treated mice was additionally confirmed by the reduced expression of the endothelial cell CD31 marker (Fig. 5k), the basement membrane marker laminin (Fig. 5l) and of αSMA, a marker of pericytes (Fig. 5m). Altogether, this indicates that nebivolol triggers a significant inhibition of tumor angiogenesis (Fig. 5j–m).

Nebivolol arrests endothelial cells proliferation. Interestingly, whereas the expression of VEGF was not affected in nebivolol-treated carcinomas (Fig. 6a), nebivolol significantly diminished the expression of VEGF-receptor2 (VEGFR2) (Fig. 6a). Analysis of VEGFR2 expression in human umbilical vein endothelial cells (HUVEC) treated with nebivolol showed no relevant differences.
in VEGFR2 expression when compared to controls (Fig. 6b).
However, nebivolol significantly reduced HUVEC cell number (Fig. 6c) by inhibiting its proliferation (Fig. 6d). Thus, further indicating that the reduction of VEGFR2 expression (Fig. 6a) in nebivolol-treated carcinomas results from a limited angiogenesis.

It has been estimated that most ATP requirements in endothelial cells are provided by glycolysis29. Consistently, whereas nebivolol had no relevant effect on mitochondrial respiration of HUVEC cells (Supplementary Fig. 6a), despite expressing β1-adrenergic receptor (Fig. 6e), nebivolol significantly inhibited glycolysis in these cells (Fig. 6f). In fact, nebivolol significantly arrested cell cycle progression (Fig. 6g, see also Supplementary Fig. 6b) by preventing the activation of ERK (Fig. 6h), which is known to block cell cycle in S phase30, arresting HUVEC cells at G0/G1 (Fig. 6g, see also...
**Fig. 5 Nebivolol halts colon cancer growth in mice.** HCT116-Luc cells were injected into the flanks of nude mice. Mice were treated with saline (CRL; black trace and bar; n = 7), 10 mg kg−1 nebivolol (NEB; red trace and bars; n = 6), 0.2 mg kg−1 5-fluorouracil (5FU, green trace and bar; n = 6) or both compounds (NEB + 5FU, red trace and bar; n = 7) and sacrificed when tumor volume reached 2000 mm³. **a** Left panel shows representative images of the bioluminescence of HCT116-Luc tumors in mice after 6 days of initiation of the indicated treatment. Right panel, quantification of light emission of the cells (CRL, n = 10; NEB n = 11 tumors; p = 0.02). **b** Tumor volume (mm³) at day 0 and after 6 (p = 0.02), 14 (p = 0.002) and 30 (p = 0.02) days of treatment (CRL, n = 12; NEB n = 12 tumors). **c** Kaplan–Meier survival analysis. The log-rank test p value (0.015) is shown. **d, e** Immunofluorescence microscopy images of Ki67 (red) (**d** or cleaved active caspase-3 (c-caspase-3, red) (**e**) and DAPI (blue)-stained carcinomas treated as indicated. Scale bar, 50 µm. **f** ATP content of the carcinomas (CRL, n = 12; NEB n = 12 tumors; p = 0.05). **g** Representative western blots of two different samples and quantification of four different samples (Histograms) of the expression of SOD2 (**p = 0.0005), PRX3 (**p = 0.04), GR (**p = 0.03), G6PDH (**p = 0.01), PRX6 (**p = 0.0001), catalase (Cat) and IF1 (**p = 0.05) in tumors. Tubulin is shown as loading control. **h** Tumor volume (mm³) at day 6 of the indicated treatments (CRL, n = 12; NEB n = 10; NEB + 5FU, n = 10 tumors; p = 0.04 and **p = 0.004). **i** Kaplan–Meier survival analysis. The log-rank test p value is shown. **j** Immunofluorescence microscopy images of isolectin B4 (IB4, green; **p = 0.0002) and DAPI (blue)-stained carcinomas treated as indicated. Scale bar, 200 µm. **k–m** Immunofluorescence microscopy images of CD31 (**p = 0.007) (**k**), laminin (**p = 0.00001) (**l**) and αSMA (**p = 0.0004) (**m**) (red) and DAPI (blue)-stained carcinomas treated as indicated. Scale bar, 20 µm. Histograms represent the relative expression of CD31, laminin or αSMA-positive cells relative to cell nuclei of six different biological samples. Bars indicate the mean of indicated samples ± SEM. **p < 0.05 and **p < 0.01 when compared to CRL by two-sided Student’s t test. See also Supplementary Figs. 3–5. Source data are provided as a Source Data file.

Supplementary Fig. 6b). Overall, these findings support that nebivolol hinders tumor angiogenesis by inhibiting endothelial cell proliferation in vitro and in vivo.

**Nebivolol prevents the growth of orthotopic colon carcinomas.** Immunocompromised mice were injected with HCT116-luc cells in the cecum. After bioluminescence detection of a stable signal, mice were randomly allocated into the group of treated mice, with daily doses of nebivolol 5 days a week (NEB), and the control NaCl-treated group (CRL) (Fig. 7a, upper panel). Tumor growth was followed by the increase in bioluminescence signal every 2 days/week. Within 25 days after initiation of the treatment, mice revealed a significant reduction in tumor luminescence when compared to controls (Fig. 7a, lower panel). Nebivolol significantly decreased tumor growth by 15 days of treatment reaching a fivefold decrease at 34 days (Fig. 7b). Furthermore, nebivolol-treated mice significantly developed less tumors (Fig. 7c, left panel) and smaller tumors when compared to NaCl-treated mice. In addition, nebivolol treatment significantly diminished the number of micrometastases (Fig. 7d, left panel) and the macroscopic evidence of tumor angiogenesis (Fig. 7d, right panel) when compared to controls. Altogether, our results support that nebivolol also arrests tumor growth in vivo in the colon microenvironment.

**Nebivolol delays in vivo growth of breast carcinomas.** The effect of nebivolol was also assessed in breast cancer growth in vivo using an MDA-MB-231 xenograft mouse model (Fig. 8). Nebivolol treatment significantly reduced tumor volume when compared to control NaCl-treated mice (Fig. 8a). Kaplan–Meier survival curves showed that nebivolol significantly increased the lifespan of mice when compared to control (Fig. 8b). Restraining tumor growth by nebivolol treatment resulted in significant inhibition of cellular proliferation (Fig. 8c) and an enhanced cell death (Fig. 8d) in the carcinomas.

Tumors of nebivolol-treated mice showed a significant reduction in the total content of ATP (Fig. 8e). A significant increase in nitrotyrosine-modified proteins was also observed in breast carcinomas of nebivolol-treated mice (Fig. 8f). Likewise, nebivolol also increased the expression of proteins of the antioxidant response such as peroxiredoxin 3, glutathione reductase, and glucose-6 phosphate dehydrogenase, when compared to control NaCl-treated mice (Fig. 8g). In agreement with previous observations in breast cancer cells (Fig. 2e) and in isolated mitochondria (Fig. 4e), tumors of nebivolol-treated mice revealed an increased expression of IF1 when compared to tumors of control mice (Fig. 8g). Moreover, nebivolol significantly inhibited the expression of the angiogenic markers IB4 (Fig. 8h), CD31 (Fig. 8i), Laminin (Fig. 8j), αSMA (Fig. 8k), and VEGFR2 (Fig. 8l) in breast carcinomas, further supporting a relevant role for β-adrenergic signaling in favoring tumor angiogenesis. Overall, we show that nebivolol, by blocking β-adrenergic receptors, arrests the growth of carcinomas through the coordinate action of preventing vascularization of the tumor and inhibiting the bioenergetic function of cancer mitochondria (Fig. 9).

**Discussion**

Drug repurposing offers a valuable approach to reduce the socioeconomic burden of cancer. Although cancer cells reprogram their energy metabolism to an enhanced glycolysis, PGC-1-driven activation of mitochondrial OXPHOS is required for metastasis. Hence, mitochondrial metabolism provides a hopeful target to fight against cancer, when compared to tumors of control mice (Fig. 8g). Moreover, nebivolol significantly inhibited the expression of the angiogenic markers IB4 (Fig. 8h), CD31 (Fig. 8i), Laminin (Fig. 8j), αSMA (Fig. 8k), and VEGFR2 (Fig. 8l) in breast carcinomas, further supporting a relevant role for β-adrenergic signaling in favoring tumor angiogenesis.

We show that nebivolol inhibits mitochondrial respiration and the synthesis of ATP in a large number of cancer cells (Fig. 9). Remarkably, nebivolol has no effect in isolated mitochondria or in nontumor cells, which emphasizes its specificity and excludes any antimitotic toxicity. Recently, Gboxin, a small molecule that accumulates in cancer mitochondria driven by the membrane potential, impedes ATP synthesis and inhibits the growth of glioblastoma xenograft. The inhibition of respiration mediated by nebivolol is linked to a sharp decrease in the activity of Complex I of the respiratory chain (Fig. 9), in agreement with the role of the cAMP/PKA signaling pathway in controlling...
mitochondrial respiration. Metformin and its analogs, used to treat type 2 diabetes, also inhibit complex I of the respiratory chain and have anticancer properties. The exact mechanism by which metformin inhibits complex I remains unknown. However, it is interesting to note that biguanides inhibit ubiquinone reduction and stimulate ROS production by FMN at complex I. The likely mechanism of action of nebivolol is unrelated to the changes in ATP synthesis observed in this study.

Moreover, just as metformin inhibits the ATP synthase, so does nebivolol, which explains the sharp reduction of ATP observed in the tumors of nebivolol-treated mice (Fig. 9). The inhibition of complex I activity is by preventing the phosphorylation of IF1, which correlates with the rapid increase in the expression of IF1 and the reduction in ATP hydrolyase activity. However, this increase is not sufficient to suggest that ATP synthase is the sole target of nebivolol. The overexpression of IF1 is already known to inhibit ATP synthase in cancer cells and in different tissues of transgenic mice that overexpress the protein in vivo. Consistently, we show that nebivolol-treated cells have reduced ATP hydrolyase activity and increased dephosphorylated IF1 bound to the ATP synthase (Fig. 9).

**Fig. 6** Nebivolol inhibits endothelial cells metabolism and proliferation. a Representative western blots of two different samples and quantification of four different samples (histograms) of the expression of VEGF and VEGFR2 (*p = 0.01) in tumors of control (CRL) and nebivolol-treated (NEB) colon xenograft mice. Tubulin is shown as loading control. b−e HUVEC cells were treated (NEB, red dots and bars) or not (CRL, closed dots and bars) during 3 or 48 h with 10 µM nebivolol. b−c Immunofluorescence microscopy images of VEGFR2 (green) and DAPI (blue)-stained HUVEC treated as indicated. Scale bar, 50 µm. c Western blots were repeated two times with similar results. *p = 0.03. d The figure shows the proliferation after 48 h of CellTrace Red incorporation. The blue curve shows time 0. The plot shows the quantification of cellular proliferation of four different samples. **p = 3.1E−10. See Supplementary Fig. 7 for gating strategy. e Representative western blot of the expression of ERK and its phosphorylated form (pERK). Western blots were repeated two times with similar results. Bars indicate the mean of the indicated samples ± SEM. *p < 0.05 and **p < 0.01 when compared to CRL by two-sided Student’s t test. See also Supplementary Fig. 6. Source data are provided as a Source Data file.
Fig. 7 Nebivolol halts cancer growth in orthotopic colon mice. HCT116-Luc cells were injected into the cecum of nude mice. Mice were treated with saline (CRL; black trace and bars; \( n = 10 \)) or 10 mg kg\(^{-1} \) nebivolol (NEB; red trace and bars; \( n = 10 \)) and sacrificed after 35 days of treatment. Bars indicate the mean ± SEM. \( p < 0.05 \) and \( ^{*} p < 0.01 \) when compared to CRL by two-sided Student’s \( t \) test. a Representative images of the bioluminescence of HCT116-Luc tumors in mice at day 0 and after 25 days of initiation of the indicated treatment. Light emission of the cells was measured after injection of 150 mg kg\(^{-1} \) of \( \alpha \)-Luciferin. b The histograms show the fold change of the radiance increase after 4, 7, 11, 15 (\( ^{*} p = 0.03 \)), 22 (\( ^{*} p = 0.04 \)), 25 (\( ^{*} p = 0.02 \)) and 34 (\( ^{**} p = 0.0002 \)) days of treatment. c Left panel shows the histograms for the quantification of the number of tumors found in the mice (\( ^{*} p = 0.04 \)). Right panel shows representative images of the size and aspect of the tumors and the histograms for the quantification of tumor volume (\( ^{*} p = 0.04 \)). d Left panel shows the histograms for the quantification of the micrometastasis found in the mice. Right panel shows representative images of the micrometastasis. Black arrows point to the vessels around the tumors (\( ^{**} p = 0.003 \)). Source data are provided as a Source Data file.

Remarkably, the overexpression of IF1 in hepatocarcinomas\(^{48} \), gastric\(^{49} \), lung\(^{50} \) and bladder\(^{51} \) carcinomas and gliomas\(^{52} \) identifies patients with worst prognosis because IF1 favors proliferation and metastatic disease. In contrast, the overexpression of IF1 in colon and breast carcinomas correlates with better patients’ prognosis\(^{16,53} \), stressing the importance and tissue-specific relevance of IF1 as a biomarker and target of cancer chemotherapy. Interestingly, the expression of IF1 in normal human tissues\(^{57} \) and carcinomas\(^{16} \) occurs independently of changes in the tissue availability of IF1 mRNA. IF1 is a mitochondrial protein with a very short half-life (2–3 h) in differentiated osteocytes and in human stem and colon cancer cells\(^{16,54} \). Consistent with these observations, the nebivolol-promoted increase in IF1 expression observed in cancer cells is unrelated to changes in IF1 mRNA abundance, supporting the idea that the \( \beta \)-blocker is affecting the turnover rate of the protein. Altogether, these findings emphasize the need for future studies aimed at characterizing the tissue-specific mechanisms that control IF1 expression for the prominent role it plays in regulating the bioenergetics of cancer cells and the metastatic behavior of the carcinomas.

It is interesting to note the different cell-death behavior of cells growing in culture and in vivo towards nebivolol. Remarkably, the cancer cells expressing the \( \beta \)-adrenergic receptor respond to different \( \beta \)-agonists by inhibiting mitochondrial respiration while they do not respond to \( \beta \)- and \( \beta \)-adrenoreceptor inhibitors, supporting the role of \( \beta \)-adrenergic signaling in arresting tumor growth in vivo. Tumor angiogenesis and cancer progression requires \( \beta \)-adrenergic signaling\(^{28} \). In fact, the overexpression of PKA is considered a hallmark that correlates with bad clinical prognosis and pathological features of the carcinomas\(^{53} \). Moreover, PKA is also involved in uncontrolled proliferation, cytoskeleton remodeling, and the migration of cancer cells\(^{53,56} \) and, blocking PKA activation is known to halt cancer progression\(^{37,58} \). On the other hand, despite endothelial cells express \( \beta \)-adrenergic receptors, nebivolol significantly diminished their glycolytic flux—a main pathway in endothelial cells\(^{29} \)—further preventing their proliferation and tumor angiogenesis. Hence, we suggest that the differences in nebivolol cytotoxicity between in vitro and in vivo studies result from the restriction of oxygen and nutrients imposed by the deficient tumor angiogenesis in nebivolol-treated mice. Limiting vasculogenesis and mitochondrial function—the latter affecting both ATP production and the concurrent generation of mitochondrial oxidative stress at the level of complex I—are convergent pathways by which nebivolol arrests the
proliferation and enhances death of cancer cells growing in vivo (Fig. 9). Remarkably, in mice bearing colon carcinomas, life expectancy increased further when nebivolol was used in combination with 5FU, illustrating nebivolol’s ability to potentiate the activity of the classical anticancer colorectal drug.

Basket trials are defined as those including cancer patients with carcinomas from different tissue origins sharing a common mutation and/or biomarker. The expression of β1-receptors could be considered a biomarker of different cancer cells, as shown in this study and elsewhere. Interestingly, there are no clinical trials in which nebivolol has been used as an anticancer agent. Therefore, our findings point out that nebivolol is a promising drug to be repurposed to treat cancer patients in combined therapy because targeting β1-adrenergic signaling with
Fig. 8 Nebivolol halts breast cancer growth in mice. MDA-MB-231 cells were injected into the flanks of nude mice. Mice were treated with saline (CRL; black trace and bars; n = 5) or 10 mg kg⁻¹ nebivolol (NEB; red trace and bars; n = 5) and sacrificed when tumor volume reached 2000 mm³. a Tumor volume (mm³) at day 6 after treatment initiation (CRL, n = 7; NEB n = 6 tumors; *p = 0.048). b Kaplan–Meier survival analysis. The log-rank test p value (0.0018) is shown. c, d Immunofluorescence microscopy images of Ki67 (*p = 0.03) (red) or cleaved active caspase-3 (***p = 0.009) (c-caspase-3, red) (d) and DAPI (blue)-stained carcinomas treated as indicated. Scale bar, 50 µm. Histograms represent the ratio of Ki67- or c-caspase-3-positive cells relative to cell nuclei of six different biological samples. e ATP content of the carcinomas (CRL, n = 6; NEB, n = 6 tumors) (*p = 0.03). f Representative western blots of two different samples and quantification of n = 4 tumors (histograms) of nitrotyrosine modified tumor proteins. Tubulin is shown as loading control (*p = 0.04 and 0.03). g Representative western blots of two different samples and quantification of n = 4 tumors (histograms) of the expression of SOD2, PRx3 (*p = 0.04), GR (*p = 0.04), G6PDH (*p = 0.05), PRx6, catalase (Cat) and IF1 (*p = 0.04) in tumors. Tubulin is shown as loading control. h Immunofluorescence microscopy images of isoelectric B4 (IB4, green) and DAPI (blue)-stained carcinomas treated as indicated. Scale bar, 200 µm. Histograms represent the relative expression of IB4-positive cells relative to cell nuclei of six different biological samples (*p = 0.03). i–k Immunofluorescence microscopy images of CD31 (p = 0.01) (i); laminin (**p = 0.0008) (j) and αSMA (***p = 0.006) (k) (red) and DAPI (blue)-stained carcinomas treated as indicated. Scale bar, 20 µm. Histograms represent the relative expression of CD31, laminin or αSMA-positive cells relative to cell nuclei of six different biological samples. l Representative western blots of two different samples and quantification of four different samples of the expression of VEGF and VEGFR2. Tubulin is shown as loading control (*p = 0.04). Bars indicate the mean of the indicated samples ± SEM. *p < 0.05 and **p < 0.01 when compared to CRL by two-sided Student’s t test. Source data are provided as a Source Data file.

Fig. 9 Nebivolol induces a metabolic and redox crisis in carcinomas. The schematic summarizes the main events triggered by nebivolol to prevent the growth of colon and breast carcinomas. Nebivolol inhibits β-adrenergic signaling in cancer cells, preventing the phosphorylation of NDUFS7 (pNDUFS7, blue rectangle in CI) that limits the activity of Complex I (CI in orange) and mitochondrial respiration, favoring the generation of ROS (red star). Concurrently, nebivolol increases the mitochondrial content of the ATPase Inhibitory Factor 1 (IF1, yellow cylinder) that binds the ATP synthase (CV in light blue) and limits ATP production in the carcinomas. Moreover, nebivolol leads to diminished tumor angiogenesis by inhibiting proliferation in endothelial cells through β-adrenergic-mediated glycolysis inhibition and thus, cell cycle arrest. These events result in a metabolic (less ATP) and redox crisis (increased ROS) that limit cellular proliferation and enhanced death of cancer cells preventing the in vivo growth of the carcinomas.

Methods
Animal studies. For the in vivo studies, 6-week-old male or female nude mice with a body weight of 30–35 g were implanted with HCT116-Luc or MDA-MB-231, respectively. The Ethics Committee of Animal Experimentation (CSIC-UAM, CM PROEX 023/14) and the Institutional Review Board of UAM (CEI 75-1365) approved the project. Mice were housed in the Animal Facility of the CBMSO with a 12-h light/12-h dark cycle and temperatures of 18–23 °C with 40–60% humidity. Approximately, 4 × 10⁶ HCT116-luc or MDA-MB-231 cells were injected subcutaneously into the left and the right flanks of mice to develop the xenograft model. To develop the orthotopic model, 5 × 10⁵ HCT116-luc cells were injected between the mucosa and the muscularis externa layers of the cecal wall of anesthetized mice (isoflurane gas, 1.5%, Abbott) in 10 µl of saline solution using a 50-gauge needle. Tumor growth was monitored by bioluminescence acquisition on anesthetized mice (isoflurane gas, 1.5%, Abbott) using the IVIS Lumina II in vivo imaging system (Caliper Life Sciences) for the HCT116-Luc cells after intraarterial injection of 150 µg kg⁻¹ of body weight of α-Luciferin (Promega). Images
were analyzed using Perkin Elmer 3.2. In Vivo Imaging Software. Tumor size was also determined using a standard caliper and its volume calculated using the formula (width×length×length)/2 when width represents the shortest tumor dimension. In the xenograft models, when HCT116 tumors reached ~100 mm³ of volume, animals were randomly allocated into different groups and were treated 5 days a week with a single daily intraperitoneal injection of 10 mg kg⁻¹ neovibol, 0.2 mg kg⁻¹ 5-fluorouracil (5FU) or 10 mg kg⁻¹ neovibol combined with 0.2 mg kg⁻¹ 5FU. This same procedure was followed for mice using MDA MB-231 tumors that were treated with 10 mg kg⁻¹ neovibol. In both HCT116 and MDA-MB-231, a 0.9% NaCl-treated group was included as a control. Following the ethical criteria established by our Institutional Review Board, the animals were sacrificed when tumor volume reached ~2000 mm³ and the tumor removed for further analysis. In the orthotopic model, mice were allocated into the control and neovibol-treated groups when a stable luminescence signal of the implanted cells was attained and further treated as above indicated. Animals were euthanized after 35 days of treatment due to heavy tumor burden observed in the control group. Tumors formed and metastatic colonization were evaluated postmortem.

**Cell lines.** Cells were cultured in a humidified incubator at 37 °C with a controlled atmosphere of ambient air 10% CO₂. Human colorectal carcinoma HCT116, HCT116-Luc, and shiF1 HCT116 cells were grown in McCoy’s 5A media supplemented with 10% fetal bovine serum (FBS). The HCT116-Luc cells expressed luciferase and were used to analyze tumor growth in vivo. Human breast MDA-MB-231, SH-SY5Y, NRK, and ovarian OVCAR 8 carcinoma cells; normal rat kidney (NRK) and breast Hs 578T normal cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% FBS. Mouse myoblasts C2C12 cells were grown in α-MEM with 10% FBS and 2% N2 supplement. Mouse neuroblastoma SH-SY5Y cells were grown in DMEM-F12 media supplemented with 10% FBS. Primary cortical neuronal cultures were obtained from E15 to E16 mouse embryos and plated at a density of 5 × 10⁵ cells cm⁻² on poly-l-lysine and laminin-coated pretreated glass coverslips in Neurobasal medium supplemented with 2% B27, 1% glutamax (all from Invitrogen) and 100 mg ml⁻¹ penicillin-streptomycin. On the fifth day in vitro, half of the plating medium was removed from each well and replenished with BrainPhys medium (Stem cell Technologies) supplemented with 2% B27 and 100 mg ml⁻¹ penicillin-streptomycin. HUVEC cells were kindly provided by Dr. Jaime Millán (CRMUOS, Madrid) and grown on fibronectin-coated flasks (10 µg ml⁻¹) with EBM-2 medium supplemented with 2% FBS and endothelial cell growth factor (EGM-2) following the manufacturer’s instructions (Lonza, Walkersville). Cells were used at ~60% confluence. The NRK cell line was maintained in DMEM and transfected with pCMV-SPORT6-IF1, the phospho- deficient (S39A) or phosphomimetic (S39E) IF1 mutants18. Cells were harvested 24 h post-transfection and processed for 2D-gel electrophoresis.

**Isolation of mitochondria.** Cells were homogenized in a glass-Teflon homogenizer with several volumes of hypotonic buffer (83 mM MOPS pH 7.2). After homogenization, the same volume of hypotonic buffer (250 mM sucrose, 30 mM MOPS pH 7.2) was added and nuclei and unbroken cells were removed by centrifugation (10000 × g, 4 °C for 5 min) and washed in buffer A (320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris-HCl pH 7.4)18,19,65.

**Drug library screening.** The effects of compounds from a 2018 FDA-Approved Drug Library (Selleckchem) on mitochondrial respiratory parameters of HCT116 cells were determined in XF96 and XF24 Seahorse flux analyzers (Agilent Technologies) using 10 mM glucose, 1 mM pyruvate and 2 mM glutamine. For the respiration using palmitate as a substrate, cells were starved for 12 h in low-glucose DMEM. Culture medium was replaced by fresh medium supplemented with 1% FBS 1 h before the measurement. Samples (200 µl) of culture medium were taken at different time points (0, 30, 60 and 90 min) and precipitated with 800 µl of 0.1% cold perchloric acid, incubated on ice for 1 h and then centrifuged for 5 min, 11000 × g at 4 °C to obtain a protein-free supernatant. The supernatants were neutralized with 20% (w/v) KOH and centrifuged at 11000 × g for 5 min to sediment the KCIO₄ salt. Lactate levels were determined spectrophotometrically by following the reduction of NAD⁺ to NADH with 1 µM LDH.

**Determination of the mitochondrial membrane potential** was accomplished by the addition of 200 µM KCN. To determine the rates of glycolysis, the initial rates of lactate production were determined by the enzymatic quantification of lactate concentrations in the culture medium. Culture medium was replaced by fresh medium supplemented with 1% FBS 1 h before the measurement. Samples (200 µl) of culture medium were taken at different time points (0, 30, 60 and 90 min) and precipitated with 800 µl of 0.1% cold perchloric acid, incubated on ice for 1 h and then centrifuged for 5 min, 11000 × g at 4 °C to obtain a protein-free supernatant. The supernatants were neutralized with 20% (w/v) KOH and centrifuged at 11000 × g for 5 min to sediment the KCIO₄ salt. Lactate levels were determined spectrophotometrically by following the reduction of NAD⁺ to NADH with 1 µM LDH.

**Cellular proliferation, cell-death assays, and ROS production.** Cellular proliferation was determined by the incorporation of 5-ethyl-2′-deoxy-uridine (EdU) into cellular DNA using the Click-it EdU Flow Cytometry Assay Kit (Thermo Fisher Scientific) and using CellTrace™Far Red (Thermo Fisher Scientific), following the manufacturer’s instructions. Cell cycle was analyzed by flow cytometry. After treatment, cells were trypsinized, centrifuged at 1000 × g for 5 min, collected and washed with ice-cold PBS. Cellular pellets were resuspended and fixed with cold 70% ethanol overnight. After another wash with PBS, the cell pellets were resuspended in 1 ml of staining solution containing propidium iodide (PI, 50 µg ml⁻¹). Finally, the cells were incubated at 37 °C for 30 min in the dark before analysis. For cell death assays, 50,000 cells/well were seeded and treated with 1 µM staurosporine (STS), 120 µM hydrogen peroxide (H₂O₂) or 1 µM tamoxifen as indicated. Cell death was determined by flow cytometry after staining with annexin V and 7-AAD (Annexin V Apoptosis Detection Kit I, BD Pharmingen)18. The intracellular production of hydrogen peroxide was monitored by flow cytometry using 10 µM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCFH2-DA) (Thermo Fisher Scientific)19. Cells were analyzed in a BD FACScan. For each analysis, 10,000 events were recorded. Data were analyzed in FlowJo software v10.6.2.

**Protein extraction and western blot analysis.** Cells or isolated mitochondria were resuspended in lysis buffer containing 25 mM Hepes, 2.5 mM EDTA, 1% Triton X-100, supplemented with protease and phosphatase inhibitor cocktails. Tumor samples from humanly sacrificed mice were freeze-clamped in liquid nitrogen. Tumor proteins were extracted in a buffer containing 50 mM Tris-HCl pH 8.0, 1% NaCl, 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1% sodium dodecyl sulfate (SDS), 0.4 mM EDTA, supplemented with protease and phosphatase inhibitor cocktails. Lysates were clarified by centrifugation at 11000 × g for 15 min. The resulting supernatants were fractionated on SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) or nitrocellulose membranes for
immunoblot analysis. Protein concentrations were determined using Bradford reagent (Bio-Rad protein assay). The primary antibodies used were anti-human IF1 (clone E213-11000; Dako Cat. F1-AT-Pase (clone 11/21-7A5, 1-10000), anti-GAPDH (120,000, Abcam), anti-NDUFA9 (11000, Abcam), anti-NDUFVS3 (11000, Abcam) anti-SDF-H (1500, Invitrogen), anti-Core 2 (1500, Abcam), anti-COX4 (11000, Abcam), anti-Hsp60 (Stressgene SPA-807, 10000), anti-phosphopreactase (10000, Sigma), anti-nitrotyrosine (10000, Abcam), anti-glutathione reductase (11000, Santa Cruz, Biotechnology), anti-a-tubulin (11000, Sigma), anti-β-actin (120,000, Sigma), anti-SOD2 (11000, Abcam), anti-PRAX (11000, Abcam), anti-PR3X (11000, Invitrogen), anti-GpDPHD (11000, Cell signaling), anti-VDAC (1500, Abcam), anti-ADRB1 (15000, Abcam), anti-VEGF (11000, Abcam), anti-VEGFR2 (1500, Cell Signaling), anti-phosphoERK (1500, Cell Signaling) and anti-ERK (1500, Santa Cruz Biotechnologies). Peroxidase-conjugated anti-mouse or anti-rabbit IgG (Promega, 1/3000) were diluted in 5% non-fat-dried milk in Tris Buffered Saline (TBS) with 1% Tween 20 and used as secondary antibodies. The Novex® ECL (Thermo Fisher Scientific) system was used to visualize the bands. The intensity of the bands was quantified using a GS-5 Calibrated Densitometer (Bio-Rad) and Image software.

Immunofluorescence labeling. Human umbilical vein endothelial cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Merck Millipore) and labeled with anti-VEGF2 (11000, Cell Signaling). Nuclei were counter stained with 4,6-diamidino-2-phenylindole (DAPI) reagent. Cellular fluorescence was analyzed by confocal microscopy in a Zeiss LSM 710 inverted confocal microscope. The images were processed with Zeiss software.

RNA extraction and quantification. RNA was extracted and purified from cells with Trizol reagent (Thermo Fisher Scientific), according to the manufacturer’s instructions. Purified RNA was quantified with a Nanodrop Spectrophotometer (Thermo Fisher Scientific), and 1 μg was retrotranscribed into cDNA with the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). IF1 mRNA levels were analyzed by real-time PCR with SYBRGreen Master Mix (Thermo Fisher Scientific) and ABI Prism 7900HT sequence detection system (Thermo Fisher Scientific) at the Genomics and Massive Sequencing Facility (CBMSO-UAM). Primers used to amplify the target genes were as follows: human IF1 (forward 5′-GAGCTGCAGTTGTTC-3′, reverse 5′-TTCAAAGCTGCCAGTTGTTC-3′), human β-actin (forward 5′-CCAGCACCGCGAGCAGCTAG-3′, reverse 5′-CCAGCGGCTACAGCGGATAG-3′). Standard curves with serial dilutions of pooled cDNA were used to assess amplification efficiency of the primers and to establish the dynamic range of cDNA concentration for amplification. The relative expression of the mRNAs was determined with the comparative ΔΔCT method with β-actin as a control.

2D-gels and blue native gel electrophoresis. Isoelectrofocusing (IEF) was performed with 13-cm Immobiline DryStrips of 6-11L [linear] pH gradient using an Ettan LPHPhor3 IEF unit (GE Healthcare). In brief, 200 μg of cellular protein diluted in 250 μl of rehydration buffer (DeStreak Rehydration Solution, GE Healthcare) containing 0.05% of the corresponding IPG buffer (GE Healthcare) were loaded in the 13-cm strips. The equilibrated strips were transferred to the top of a 12.5% Novex Bis-Tris 12% Bis-Tris Gel (Invitrogen). The peptides in the supernatants were dried down and then desalted onto ZipTip C18 Pipette tips (Millipore) until the mass spectrometry analysis.

To perform the reverse phase-affinity chromatography-MS/MS analysis, the desalted protein digest was dried, resuspended in 10 μl of 0.1% formic acid and analyzed by RP-LC-MS/MS in an Easy-nLC II system coupled to an ion trap LTQ-Orbitrap-Velos-Pro hybrid mass spectrometer (Thermo Scientific). The peptides were concentrated (online) by reverse phase chromatography using a 0.1 mm × 20 mm C18 RP precolumn (Thermo Scientific) and then separated using a 0.075 mm × 250 mm C18 RP column (Thermo Scientific) operating at 0.3 μl min−1. Peptides were eluted using a 90-min dual gradient from 5% to 25% solvent B in 68 min followed by a gradient from 25 to 40% solvent B over 90 min (Solvent A: 0.1% formic acid in water, solvent B: 0.1% formic acid, 80% acetonitrile in water). ESI ionization was done using Nanospray emitters Stainless Steel ID 30 μm (Proxeon) interface. The Orbitrap resolution was set at 30,000. Peptide identification from raw data was carried out using PEAKS Studio 8.5 search engine (Bioinformatics Solutions Inc.). Database search was performed against uniprot-homo sapiens.fasta 12/03/2018 containing 71,790 sequences (decay-fusion database). False discovery rates for peptide-spectrum matches were limited to 0.01. Only those proteins with at least two distinct peptides being discovered from LC/MS/MS analyses were considered reliably identified.

Tumor analysis. Breast and colon tumors were fixed in 4% paraformaldehyde (Merck) and included in OCT blocks. Frozen 15-μm sections were incubated with fluorescein labeled GSI L-1 B4 (5 μg ml−1; Vector Laboratories), anti-CDC1 (1:200, BD Pharmingen), anti-ACT, a-Smooth Muscle-Cy3 (1:100, Sigma) and anti-Laminin (1:25, Sigma) to assess angiogenesis, anti-activated caspase-3 (1:200, Cell Signaling) to assess cell death and anti-Ki67 (1:250, Thermo Fisher Scientific) to assess proliferation. Nuclei were counter stained with DAPI (diamidino-2-fenilindol) reagent. Cellular fluorescence was analyzed by confocal microscopy in a Nikon A1R + microscope. The images were processed with ImageJ v1.46 software.

Aliquots of freeze-clamped tumor powder were used to measure total ATP content using the ATP Bioluminescence Assay Kit CLS II (Sigma-Aldrich) following the manufacturer’s instructions.

Immunofluorescence staining. Human umbilical vein endothelial cells were incubated with 10 μl 0.08% DAPI (Invitrogen) in 1 ml PBS for 10 min and fixed with 3% paraformaldehyde for 15 min. The cells were then washed with PBS and permeabilized with 0.1% Triton X-100 (Sigma) for 10 min. The cells were blocked with 10% goat serum in PBS for 30 min and then incubated with primary antibodies against human If1 (primary antibody 1:500, Abcam), anti-CD31 (1:100, Merck), anti-VEGF (1:200, Abcam), and anti-β-actin (1:200, Sigma). The cells were then washed with PBS and incubated with secondary antibodies (Jackson ImmunoResearch) for 30 min. Immunofluorescence was observed under a Zeiss LSM 710 inverted confocal microscope.

Statistical analyses were performed using Excel Microsoft 365 and GraphPad Prism 7. Survival curves were derived from Kaplan–Meier estimates and compared by log-rank test. Tests were calculated using the SPSS 24.0 software package (IBM).

Data availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD014970. All the other data supporting the findings of this study is available within the article and its supplementary information files and from the corresponding author upon reasonable request. A reporting summary for this article is available as a Supplementary Information file. Source data are provided with this paper.

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**Author contributions**

C.N.-T., F.S., K.S. and C.N.d.A. did the research and analyzed data; M.G.d.C. and L.F. contributed to the design of the study; C.N.-T. and J.M.C. designed research, analyzed data, and wrote the paper. All the authors read, contributed and approved the final manuscript.

**Competing interests**

The authors declare no competing interests.

**Additional information**

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