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Phenformin-Induced Mitochondrial Dysfunction Sensitizes Hepatocellular Carcinoma for Dual Inhibition of mTOR.

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STATEMENT OF TRANSLATIONAL SIGNIFICANCE: Hepatocellular Carcinoma is the second cause of cancer mortality worldwide, with available therapeutic options having poor outcomes. Allosteric mTOR inhibitors were predicted to be effective in HCC, but failed in showing efficacy in a phase 3 clinical trial. However, subsequent trial sample analyses suggested that genotypic stratification would have benefited a large percentage of patients, particularly those with loss of TSC2. Here we examined the use of the combined allosteric and ATP-binding site mTOR inhibitors in HCC, together with the mitochondrial Complex1 inhibitor Phenformin, as recent data suggested that the mTOR inhibitors may promote cancer cell survival by maintaining mitochondrial oxidation and/or autophagy. We show that treatment of HCC cells *in vitro* with Phenformin causes a metabolic shift to glycolysis, mitochondrial dysfunction and fragmentation, with Phenformin pretreatment sensitizing orthotopic liver tumors to dual inhibition of mTOR, leading to a striking increase in overall survival

ABSTRACT (max 250 words):

Purpose: Hepatocellular carcinoma (HCC) ranks second in cancer mortality and has limited therapeutic options. We recently described the synergistic effect of allosteric and ATP-site competitive inhibitors against the mammalian target of rapamycin (mTOR) for the treatment of HCC. However, such inhibitors induce glycemia and increase mitochondrial efficiency. Here we determined whether the mitochondrial complex I inhibitor Phenformin could reverse both side effects, impose an energetic-stress on cancer cells and suppress the growth of HCC.

Experimental Design: Human HCC cell lines were used *in vitro* to access the signaling and energetic impact of mTOR inhibitors and Phenformin, either alone or in combination. Next, the therapeutic utility of these drugs alone or in combination was investigated pre-clinically in human orthotopic tumors implanted in mice, by analyzing their impact on the tumor burden and overall survival.

Results: We found Phenformin caused mitochondrial dysfunction and fragmentation, inducing a compensatory shift to glycolysis. In contrast, dual inhibition of mTOR impaired cell growth and glycolysis, while increasing mitochondrial fusion and efficiency. In a mouse model of human HCC, dual inhibition of mTOR, together with Phenformin, was highly efficacious in controlling tumor burden. However, more striking, pretreatment with Phenformin sensitized tumors to dual inhibition of mTOR, leading to a dramatic improvement in survival.

Conclusion: Treatment of HCC cells *in vitro* with the biguanide Phenformin causes a metabolic shift to glycolysis, mitochondrial dysfunction and fragmentation, and

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dramatically sensitizes orthotopic liver tumors to dual inhibition of mTOR. We therefore

propose this therapeutic approach should be tested clinically in HCC.

INTRODUCTION

Worldwide, liver cancer is the second cause of cancer death, second in most years of lost life, and the most rapidly expanding malignant tumor in Western countries. Accounting for >70% of the total liver cancer burden, hepatocellular carcinoma (HCC) is its major histological subtype. The most common liver insults leading to HCC are hepatitis B or C virus (HBV or HCV) and alcohol (1); however, in Western societies, the abrupt increase in HCC has been linked to non-alcoholic steatohepatitis (NASH) and the recent epidemic in obesity (2,3). Despite being a very heterogeneous tumor type without clear genetic drivers, the most aggressive forms of HCC are associated with the TGF-β1, PI3K/Akt/mTOR, Wnt/β-catenin and NOTCH proliferative pathways (4,5). The Cancer Genome Atlas (TCGA) consortium has recently performed the first large-scale multi-platform analysis of HCC, which showed a prevalence of alterations in WNT and p53 signaling as well as TERT upregulation (6). Clinically, surgical resection leads to ~70% disease-free survival over 5 years in HCC patients (7,8), although it is only an option for a small portion of early-stage patients with solitary tumors and normal liver function. For advanced stage HCC the standard of care is Sorafenib (9), followed upon-progression by the recently approved Regorafenib, both multi-protein kinase inhibitors, which render modest life-extending benefits (10,11). Recently, striking effects have been reported with Nivolumab, an immunotherapeutic agent (12). Of note, such treatments may benefit from combination therapy with targeting agents (13).

The mammalian Target of Rapamycin (mTOR) emerged several years ago as an attractive target for HCC, as it is hyperactivated in 50-60% of HCC and is associated with high grade tumors and worst case prognosis (14-17). Blocking tumor growth by

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inhibiting mTOR has led to the approval of the rapamycin family of allosteric drugs (rapalogs) in the treatment of a number of cancers (18,19). Nevertheless, the rapalog Everolimus (RAD001) did not improve overall survival in the phase 3 EVOLVE-1 trial for advanced HCC after Sorafenib failure (20). However, a recent retrospective analysis of the EVOLVE-1 data showed that patients exhibiting mutations in the Tuberous Sclerosis 2 (TSC2) protein, a tumor suppressor which results in constitutive activation of mTOR Complex 1 (mTORC1), tended to benefit from second-line treatment with RAD001 (21). Importantly, the same authors reported that loss of TSC2 is observed in approximately 20% of the Asian HBV(+) HCC patients (21). These findings have further underscored the necessity of stratifying patients as a function of sub-populations in liver cancer clinical trial designs (22).

The failure of rapalogs in a number of clinical trials may also be attributed in part to their incomplete inhibition of mTORC1 and mTORC2 (23), as well as the loss of the negative feedback loop from ribosomal protein S6K1 kinase to Akt (24,25). We recently showed that these two shortcomings could be overcome by combining an allosteric (RAD001) and an ATP-site competitive (BEZ235) mTOR inhibitor (26). These observations have led to the proposal that the rapalogs should be reconsidered in the treatment of HCC. However, in the clinic mTOR inhibitors hinder glycolysis, inducing hyperglycemia (27), which may independently drive tumor growth (28,29). Moreover, it was recently demonstrated that mTOR inhibitors decrease mitochondrial biogenesis and activity, but counter-intuitively increase mitochondrial efficiency (30). This overall increase in mitochondrial efficiency may provide cancer cells the potential to survive the catabolic state induced by mTOR inhibitors, particularly a small population of stem-like

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cells. In the clinic, hyperglycemia induced by rapalogs is treated with the biguanide Metformin (31,32). Given that biguanides inhibit CI and mTOR inhibitors suppress glycolysis, we reasoned that their combination or their sequential use might place a further energetic-stress on HCCs. However, Metformin requires the OCT1 transporter to enter tumor cells, which is largely downregulated in HCC (33), making the hydrophobic analogue Phenformin an attractive alternative, as it does not require a transporter and is readily taken up by all tissues (34). Phenformin was initially withdrawn from clinical use due to deleterious lactic acidosis in a subset of renal patients (35). However, arguments that Phenformin could be considered as an anticancer agent, given distinct dosing and usage (36), have aided in the recent approval of a Phase 1 clinical trial for its use in melanoma (37).

Here we set out to determine the effects of RAD001/BEZ235 and Phenformin treatment in human HCC cell lines, tumoroids and mouse orthoxenografts. In cells we found that both treatments inhibited mTOR signaling, but that mTOR inhibition was not augmented by the triple combination. In parallel, Phenformin induced mitochondrial dysfunction and fragmentation, consistent with the activation of AMP-activated protein kinase (AMPK) (38), and led to a metabolic shift towards glycolysis, increased ROS production and cell death. Unexpectedly, a number of the Phenformin responses appeared to be mitigated by the energy sparing effects of RAD001/BEZ235 treatment. The growth-inhibition of orthotopically implanted tumors was more dramatic when using mTOR inhibitors, as compared to Phenformin alone, but more penetrant with the triple drug combination, an effect also observed in overall survival. Given that the mTOR inhibitors suppressed the effects of Phenformin on mitochondria homeostasis *in vivo*,

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HCC bearing mice were first treated with the biguanide, followed by the mTOR inhibitors. Such a treatment schedule dramatically sensitized tumors to mTOR inhibitors, profoundly increasing overall survival. Given the recent approval of Phenformin IND in melanoma (37), this regimen should be explored clinically in HCC.

MATERIALS AND METHODS

Cell culture and proliferation assay

Huh7 cells, purchased from the JCRB Cell Bank, were maintained in Dulbecco's modified Eagle's culture medium (Gibco) containing 10% heat-inactivated fetal bovine serum (Sigma-Aldrich) and 1% Penicillin/Streptomycin (10,000 units/ml and 10 mg/ml respectively) (Sigma-Aldrich). Cell number was measured by crystal violet staining (Sigma-Aldrich).

Animal studies

Four week-old male Athymic Nude-$Foxn1^{nu}$ mice were purchased from Harlan Laboratories (Envigo, Barcelona). Six to eight week-old mice were injected intra-hepatically with 1-2x10^6 Huh7 cells in an ice-cold phosphate buffered saline (PBS) (Gibco) / Matrigel Growth Factor Reduced phenol free (Corning - BD Biosciences) solution. After ~ four weeks, tumors were collected and equal size tumor pieces (~0.05) were implanted surgically in the liver of experimental athymic mice cohorts. Two weeks after tumor implantation mice were treated *per os* daily for 15 or 21 days with RAD001 (2.5 mg/kg) and/or BEZ235 (6 mg/kg) and/or INK128 (0.3mg/kg), and *per ip* with the specified doses of Phenformin (70 or 100 mg/kg). The mice were starved for 2 hours before sacrifice and received their last dose of drug 1 hour before sacrifice. Liver and tumor tissues were snap-frozen in liquid nitrogen for protein extraction or fixed in 10% formalin for IHC analysis. For survival experiments, mice were weaned from drug and monitored every-other day, or daily when signs of stress emerged (weight-shedding >10% and blood glucose <90 mg/dL). Glucose levels <60mg/dl, visible ascites, weight-shedding >15%, and loss of body temperature were end-point criteria. All *in vivo* studies

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were approved by the IDIBELL's Animal Care and Use Committee and the Generalitat de Catalunya.

Statistical analyses

Data are presented as mean ± SD. All the statistical analyses were performed using GraphPad Prism 6. For one-group variables, statistical significance was determined by Unpaired two-tailed t test; whenever variances were different, we applied the Unpaired two-tailed t test with Welch's correction. For two-grouping variables, two-way analysis of variance (ANOVA) was performed. Kaplan-Meier Survival curves differences were analyzed with the Log-rank (Mantel-Cox) Test. All statistical analyses were made in a 95% confidence interval, with P values of <0.05, <0.01, <0.001 considered to be significant and represented with *, **, and *** respectively.

Tumoroid Cultures; Tumorigenic Capacity (Limiting Dilution) Assay; Immunoblot analysis; GST-BHMT assay; Oxygen consumption rate (OCR) and Extracellular Acificicacion rate (ECAR); Mitochondrial Probes for FACS analyzes; High performance liquid chromatography (HPLC) analysis of nucleotides; Newly-synthesized RNA and Protein synthesis rate; Oxygen consumption of tumor mitochondria: Refer to Supplementary Materials and Methods.

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RESULTS

Effect of combined mTOR inhibitors on HCC orthoxenografts and tumoroids growth

We initially set out to determine whether mTOR inhibition was effective in blocking the proliferation of a panel of six HCC cell lines both sensitive and resistant to Sorafenib, the current first line treatment of HCC (39). The RAD001/BEZ235 combination was more effective than either agent alone in all HCC cell types (Supplementary Fig. S1A), including two we have previously analyzed (26). To test the sensitivity of HCC cells to mTOR inhibitors *in vivo*, we established orthotopic models from Huh7 and Hep3B cell lines. After 14 days of tumor engraftment, cohorts of tumor-bearing mice were treated with mTOR inhibitors alone or in combination. In all cases, suppression of tumor growth was observed within 15 days, with the strongest effect elicited by the RAD001/BEZ235 combination (Fig. 1A and Supplementary Fig. S1B), consistent with our earlier findings in DEN-induced tumors in C57BL/6 mice (26). Likewise, the combined mTOR inhibitor treatment had a small impact on the weight of tumor bearing mice (Supplementary Fig. S1C) and induced the dephosphorylation of mTORC1 kinase target RPS6 (Fig. 1B). Strikingly the on-target effects were more pronounced in tumor tissue (Fig. 1B), possibly due to their higher vascularization. Moreover, in combination with RAD001, the effects of BEZ235 in Huh7 tumors were recapitulated by a second-generation ATP-site specific mTOR inhibitor INK128 (Supplementary Fig S1D), with similar effects on body weight (Supplementary Fig. S1E). In addition, as we previously showed for RAD001/BEZ235 (26), we find the RAD001/INK128 combination is more effective than either agent alone in inhibiting

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mTORC1 signaling and the proliferation of Huh7 cells (Supplementary Figs. S1F and S1G). To further test this response, we generated three-dimensional tumoroids (41) from Huh7 orthotopic tumors, and treated them *in vitro* for six days with the RAD001/BEZ235 combination. The combined mTOR inhibitor treatment *in vitro* was as effective in inhibiting the growth of tumoroids (Fig. 1E), as it was inhibiting orthotopic tumors (Fig. 1A). These studies suggest that the combination of an mTOR allosteric and an ATP site competitive inhibitor is efficacious in treating tumors derived from human HCC cell lines.

Effect of combined mTOR inhibitors on the tumorigenic capacity of Huh7 tumors

Others have reported that mTOR inhibition by rapalogues protects a small population of stem-like cells (42,43), potentially due to the maintenance of high mitochondrial oxidation capacity and/or the induction of autophagy (43,44). To determine whether RAD001/BEZ235 would impact cell's tumorigenic capacity, we first generated tumoroids from Huh7 orthotopic tumors treated with or without RAD001/BEZ235 *in vivo*. The results showed that cells derived from RAD001/BEZ235 treated tumors were impaired in their ability to grow as tumoroids in 3D-culture, as compared to controls (Fig. 1D). To further validate this finding, we conducted tumor initiation assays *in vivo* under conditions of limiting dilution with fixed numbers of cells. Huh7 tumor-bearing mice were treated for 15 days, tumor cells were isolated as for tumoroids, but instead were subcutaneously implanted in nude mice and monitored for tumor growth (Figs. 1E). In contrast to the effects reported for the rapalogs as single agents (42), the time of tumor appearance is reduced by the dual inhibition of mTOR, as compared to the vehicle (Fig. 1E). Together, our results demonstrate that combined

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mTOR inhibition *in vivo* diminishes, rather than enhances, the tumorigenic potential of Huh7 cells.

Impact of Phenformin on glycemia, proliferation and signaling

Clinically, persistent hyperglycemia is most commonly treated with the biguanide Metformin (45), including those patients whose hyperglycemia is induced by rapalogs (31,32). This raised the possibility of adding a biguanide together, or potentially sequentially, with the RAD001/BEZ235 combination, which would effectively inhibit both glycolysis and mitochondrial oxidative phosphorylation (OXPHOS), directing tumors towards energetic stress. As previously reported for mTOR inhibition (27), treatment of Huh7 tumor bearing animals with the RAD001/BEZ235 combination (RB) leads to hyperglycemia due to lower peripheral glucose uptake, an effect blunted by the addition of Phenformin, which is known to decrease gluconeogenesis (Fig. 2A). To assess the mechanism of Phenformin action, we examined a number of proliferative and signaling parameters in Huh7 cells *in vitro*. We found that cell proliferation is inhibited by either Phenformin or RAD001/BEZ235 alone and that the inhibitory response is further enhanced when all three drugs are used in combination (Fig. 2B). Nevertheless, the kinetics of inhibition of mTORC1 signaling were much slower with Phenformin than RAD001/BEZ235, as measured by S6K1, RPS6 and 4EBP1 phosphorylation (Fig. 2C). Moreover, Phenformin in combination with RAD001/BEZ235 appeared to have no further effect on mTORC1 signaling (Fig. 2C), suggesting that the effects of Phenformin on cell proliferation are mediated through mTORC1-dependent and -independent pathways. Consistent with this observation, in contrast to treatment with RAD001/BEZ235, Phenformin strongly induced AMPK activation, as measured by

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increased AMPK T172 phosphorylation, with concomitant increases in the phosphorylation of AMPK substrate ACC S79 (Fig. 2C). Unexpectedly, these responses are blunted in the presence of RAD001/BEZ235, even at later times (Fig. 2C). Despite the latter findings, the combined effect of the three drugs in impeding proliferation may be explained by the inhibition of mTORC1 signaling and the activation of AMPK.

Impact of Phenformin on glycolysis

The induction of AMPK activation and ACC phosphorylation by Phenformin (Fig. 2C), supports a change in cellular energy usage. Since Phenformin inhibits OXPHOS, we hypothesized that it would induce a metabolic shift towards glycolysis. To test this possibility we followed extracellular acidification rate (ECAR), a surrogate for lactate production and glucose utilization. The results show that glucose addition to culture media in the presence of Phenformin led to sharp increase in ECAR, whereas the combined mTOR inhibitors had a small inhibitory effect alone and blunted the effect of Phenformin (Fig. 2D and Supplementary Fig. S2A). We predicted that, if Phenformin treated cells rely on glycolysis for survival, they should be sensitive to external glucose levels. In agreement with this hypothesis, lowering glucose levels in the culture media from 25 mM to either 12.5 mM or 6.25 mM for 72 hrs led to the death of all the Huh7 cells subjected to 1mM Phenformin alone, but had no impact on the survival of RAD001/BEZ235 treated cells (compare Figs. 2B and 2E). The effect of Phenformin on cell growth is blunted by the RAD001/BEZ235 combination at the higher concentrations of glucose, an effect that is almost completely lost at the lowest concentration of glucose (compare Figs. 2B and 2E). Phenformin-induced cell death appears to occur through apoptosis, as measured by the induction of caspase 3/7 (Supplementary Fig.

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S2B) and the inhibition of cell death by pan-caspase inhibitors (Fig. 2F). More striking, in tumoroids derived from Huh7-tumors we found that when we lowered glucose levels in culture medium from 17.5 mM to physiological levels of 5 and 1 mM, we achieved inhibition of proliferation at Phenformin concentrations as low as 30 µM (Fig. 2G). The results demonstrate that in the presence of Phenformin, cells become more sensitive to the glycolytic input.

Mitochondrial Activity and Energy Status

Recent studies have shown that mTORC1 inhibition also induces a reduction in the mitochondrial O_2 consumption rate (OCR) and mass, leading to an ~30% decrease in cellular ATP levels, responses attributed to inhibition of translation of nucleus-encoded mitochondrial gene transcripts (30). Given this observation, it was important to distinguish the impact of RAD001/BEZ235 versus Phenformin on mitochondrial activity. We found that both Phenformin and RAD001/BEZ235 reduced mitochondrial OCR (Fig. 3A, Supplementary Fig. S3A and Supplementary Table S1). By 3 hrs of treatment, the combined mTOR inhibitors caused a 25% reduction in OCR, while Phenformin led to a striking 75% reduction in OCR (Fig. 3A, Supplementary Figs. S3A-C), an effect that is established within minutes of treatment, consistent with Phenformin directly inhibiting CI (Fig. S3B). When combined with mTOR inhibitors, Phenformin induced a further decrease in OCR (Fig. 3A). Furthermore, Phenformin caused a dramatic reduction in mitochondrial mass measured by MitoTracker Green FM probe (Fig. 3B); and, although ATP levels do not drop (Fig. 3C), the AMP/ATP and ADP/ATP ratios were markedly increased (Fig. 3D, Supplementary Fig. S3D-F), consistent with Phenformin inducing AMPK activation (Fig. 2C). Taken together, these results suggest that the Phenformin-

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induced shift to glycolysis is not sufficient to relieve the energetic stress, with cells striving to maintain ATP levels.

In agreement with recent findings (30) we observe a small effect of RAD001/BEZ235 treatment on mitochondrial mass (Fig. 3B), but in contrast we observe no effect on ATP levels (Fig. 3C), consistent with no changes in the AMP/ATP or ADP/ATP ratios (Fig. 3D and Supplementary Figs. S3D-E). The latter findings agree with the lack of AMPK activation by RAD001/BEZ235 treatment (Fig. 2C). Given that RAD001/BEZ235 treatment reduces OCR, but does not alter AMP/ATP or ADP/ATP ratios, we asked whether this could result from lower energy needs. Inhibition of mTOR negatively affects ribosome biogenesis and protein synthesis, two of the most energy consuming catabolic cellular processes (46). We first analyzed steady-state levels of 18S and 28S rRNA by ethidium bromide (EB) staining, and nascent rRNA levels by pulse labeling with ^{3}H-uridine. The EB-stained agarose gel shows that that there is no selective impact on the ratio of mature 18S to 28S rRNA (Fig. 3E). However, ^{3}H-Uridine labeling of 47S precursor rRNA and the newly synthesized 18S and 28S mature rRNA species were strongly impaired by the RAD001/BEZ235 combination, with no apparent damage to processing, but an overall reduction in rRNA biogenesis (Fig. 3E). These effects are paralleled by a strong reduction of ^{3}H-Leucine incorporation into nascent protein by RAD001/BEZ235 treatment (Fig. 3F). In contrast, the effects of Phenformin on either response are not as profound (Fig. 3E and 3F). Taken together, the results suggest that mTOR inhibitors sustain a healthy cellular energetic state where energy production is balanced by controlling the rate of major energy-consuming biochemical processes, and such effects suppress AMPK activation by Phenformin.

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Mitochondrial Dysfunction and Fragmentation

The inability of cells to maintain the AMP/ATP ratio in the presence of Phenformin raised the possibility that, beyond lowering mitochondrial activity, the biguanide may also cause mitochondrial dysfunction and fragmentation, similar to other mitochondrial electron transport chain inhibitors and AMPK activators (38). Although treatment with RAD001/BEZ235 decreases mitochondrial activity (Fig. 3A, Supplementary Fig. S3A), it did not perturb mitochondrial efficiency, as measured by O_2 consumption in the presence of oligomycin, a CV ATPase inhibitor (Fig. 4A). In contrast, the dramatic drop in mitochondrial activity induced by Phenformin (Fig. 3A) is accompanied by a decrease in mitochondrial O_2 consumption coupled to ATP production (Fig. 4A), indicative of mitochondrial dysfunction (47). Although RAD001/BEZ235 does not negatively affect mitochondrial efficiency, it slightly augments the effects of Phenformin (Fig. 4A). Consistent with the biguanide inducing mitochondrial damage, by confocal imaging of a MitoTracker probe, we could observe that the fusion/fission equilibrium of the mitochondrial network was progressing towards fragmentation in the presence of Phenformin, consistent with the activation of AMPK (38). This was not the case for RAD001/BEZ235 alone, where mitochondria appeared to be more fused (Fig. 4B), as recently reported for mTOR inhibitors (48). In agreement with these findings, Phenformin, but not RAD001/BEZ235, induced cleavage of optic atrophy 1 (OPA1) (Fig. 4C), an inner mitochondrial membrane GTPase required for fusion, whose cleavage is associated with extensive mitochondrial damage (38). Phenformin-induced mitochondrial damage has been shown to stimulate mitophagy and ROS production,

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leading to apoptosis (49). Unexpectedly, we found that Phenformin treatment alone did not induce autophagy, and even blocked autophagy initiated by RAD001/BEZ235 treatment, as measured by the cargo based endpoint GST-BHMT assay (Fig. 4D) (50). This would argue that in the presence of Phenformin damaged mitochondria are not being cleared by autophagic mechanisms. In parallel, Phenformin induced mitochondrial ROS as early as 3h post-treatment, at levels significantly higher than that of RAD001/BEZ235 treatment, with no further effect of the triple combination (Fig. 4E). Critically, by applying the mitochondrial ROS scavenger MitoTempo together with pan-caspase inhibitors, we rescued Phenformin-induced cell death at drug doses as high as 1mM (Fig. 4F). Thus Phenformin, either alone or in combination with RAD001/BEZ235, inhibits mitochondrial efficiency, evoking mitochondrial network fragmentation, ROS production and apoptosis.

Tumor growth suppression by RAD001/BEZ235 and Phenformin

To determine the tumor growth-suppressing efficacy of Phenformin alone, or in combination with RAD001/BEZ235, we used the orthotopic Huh7 tumor-bearing mouse model (Fig. 1A). Establishing the highest tolerated dose of Phenformin in combination with RAD001/BEZ235 required lowering its dosage as a single agent in tumor-bearing nude mice from 100mg/kg/day to 70mg/kg/day. Although not as effective as the RAD001/BEZ235 combination, Phenformin alone at 70mg/kg had a significant inhibitory effect on tumor weight (Fig. 5A) and minimal effect on body weight (Supplementary Fig. S4A). Phenformin treatment in combination with RAD001/BEZ235, appeared to improve tumor-suppression beyond that of the RAD001/BEZ235 combination (Fig. 5A). In addition, Phenfomin combined with either mTOR inhibitor alone, did not reach the same

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extent of tumor-suppression as the triple combination (Supplementary Fig. S4B). In four independent experiments, the tumor suppressor effect exerted by RBP led to more homogeneously small tumors when compared to the RAD001/BEZ235 combination alone. Using the Bartlett test of homogeneity of variances, we determined that the variance within the RBP group versus the RAD001/BEZ235 combination had a p value 0.76 +/- 0.033, statically different in a 90% confidence interval. Analyses of treated tumor tissues revealed strong inhibition of phosphorylation of mTORC1 targets RPS6 and 4E-BP1 by RAD001/BEZ235 alone, or in combination with Phenformin, with a less pronounced effect of Phenformin alone (Supplementary Fig. S4C). In parallel RAD001/BEZ235, in the absence or presence of Phenformin, had little effect on mTORC2 signaling, as measured by Akt phosphorylation (Supplementary Fig. S4C). This was most likely due to the low concentration of RAD001/BEZ235 administered to nude mice versus the higher doses, which we employed earlier in C57BL/6 mice (26). Consistent with the inhibition of mTORC1 signaling, RAD001/BEZ235, alone or in combination with Phenformin, causes a sharp drop in cyclin D1 levels, indicative of G_1 cell cycle arrest (Supplementary Fig. S4D). As described above, RAD001/BEZ235 treated mice showed a significant increase in glycemia (Fig. 2A), whereas Phenformin treatment suppressed the glycemic effect of the mTOR inhibitors (Fig. 5B). Although, RAD001/BEZ235 treated mice did not display a significant inhibition of lactate production, the combined mTOR inhibitors blunted the strong pro-glycolitic effect of Phenformin on lactate production (Fig. 5C). These results show that RAD001/BEZ235 and Phenformin treatment is highly efficacious in suppressing tumor growth (Fig. 5A Supplementary Fig. S4B). Moreover, Phenformin decreases circulating glucose and

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increases lactic acid production, suggesting that, as in cells, it is acting to impede mitochondrial activity and favoring glycolysis.

Survival benefit of Phenformin treatment followed by RAD001/BEZ235

Given the findings above we asked whether the extent of tumor suppression induced by Phenformin when added to RAD001/BEZ235 translated into a greater overall survival benefit after drug exposure for three weeks. RAD001/BEZ235 treatment alone significantly increased median survival by 26% over vehicle treated control as shown by Kaplan-Meier analysis (Fig. 5D), with little effect on body weight (Supplementary Fig. S5A). In combination with RAD001/BEZ235, Phenformin treatment further increased the median overall survival by 33% (Fig. 5D), with similar tumor weights at sacrifice (Supplementary Fig. S5B), suggesting that mice eventually perish due to primary tumor burden. Likewise, in a second experiment, the addition of Phenformin to RAD001/BEZ235, increased median survival from 26% to 37%, with animals dying with similar tumor weights (Supplementary Figs. S5C and S5D, respectively). *In vivo*, lactic acid production indicated a systemic inhibition of mitochondrial oxidation and a shift towards glycolysis under Phenformin administration. To ascertain the extent to which the treatments affected tumor mitochondrial function *in vivo*, we isolated mitochondria from treated tumors and measured their ability to consume O_2 employing a succession of OXPHOS substrates. In brief, the addition of glutamate, malate and ADP (GM_P) shows that RAD001/BEZ235 treatment improves the usage of CI substrates, as compared to Phenformin treatment alone or in the triple combination (Fig. 5E). However, the addition of succinate led to higher Substrate Control Ratio after treatment with Phenformin alone (Fig. 5F and Supplementary Fig. S5E); suggesting that, unlike

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under the RAD001/BEZ235 combination, such mitochondria are relying more on CII versus CI to maintain mitochondrial oxidation. When combined with Phenformin, mTOR inhibition appears to decrease CII usage forced by Phenformin inhibition of CI. These mitochondrial alterations did not appear due to altered OXPHOS complex expression (Supplementary Fig. S5F). The data support the notion that, in tumors treated with Phenformin, CI function is hindered, evoking a compensatory mechanism by which mitochondrial electron transport is channeled through CII.

Although we observed benefit from adding Phenformin to the RAD001/BEZ combination, the results above suggested that the Phenformin concentrations we were achieving *in vivo* were not sufficient to attain a more dramatic effect on tumor mitochondrial activity. Moreover, we find that mTOR inhibitors appear to increase mitochondria efficiency and fusion, in agreement with others (30,48), events that could potentially counteract Phenformin-induced mitochondrial dysfunction and fragmentation. Therefore we reasoned that if we first treated tumors with Phenformin alone, we could potentially drive mitochondrial dysfunction, such that they would be more sensitive to the subsequent addition of mTOR inhibitors. In a sequential design, we treated tumor-bearing mice with Phenformin 100mg/kg for 8 days, and then either maintained them on Phenformin or switched them to the RAD001/BEZ235 combination for 14 days. After 8 days treatment, Phenformin at 100mg/kg, had to be administered every other day (QOD), to be tolerated. Treatment with Phenformin alone showed no enhancement in survival, whereas sequential addition of RAD001/BEZ235 had a dramatic effect (Fig. 5G). The combination of Phenformin followed by mTOR inhibitors increased median overall survival by 70% from 28 days to 48 days (Fig. 5G), without effecting body weight

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(Supplementary Fig. S5G), effectively doubling the response observed by adding all three agents together (Fig. 5D). Critically, at the time of sacrifice, the tumor size was similar in each treatment group, suggesting that the mice succumb to primary tumor burden (Fig. 5H). Although we cannot discount a systemic effect, the results are consistent with the recent findings in cell culture suggesting that mTOR inhibitors are hindered by their capacity to promote mitochondrial fusion and increase mitochondrial efficiency (30,48). Here we propose that in vivo pretreatment of HCC bearing mice with Phenformin compromises mTOR inhibitors undesirable effects, by inducing mitochondrial dysfunction and fragmentation while generating high ROS levels, such that subsequent mTOR inhibitor treatment results in a striking increase in overall mouse survival (See model Fig. 6).

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DISCUSSION

Sorafenib has shown an unprecedented clinical activity in advanced HCC and is presently the standard of care (51). However its effects are not durable, with patients eventually succumbing to disease (52). Given these observations and that the mTOR pathway is hyperactivated in 50-60% of HCC patients, the mTOR inhibitor Everolimus phase 3 trial EVOLVE was initiated. Although no benefits were observed (20), retrospective stratification of patients suggested those with loss of TSC2 benefited from RAD001 treatment (21). Interestingly, conditional depletion of TSC1 in the liver leads to HCC in the mouse (53). Such mice were cleared of disease if treated with rapamycin before the onset of tumorigenesis (53). In contrast, others have found that chronic treatment with rapamycin accelerates HCC in the DEN-induced mouse model, potentially due to IL6 production (54), or the protection of a specific subpopulation of HCC cells (43,44). In apparent agreement with these findings, using carcinogen-induced (26) or orthotopically implanted HCC human cell line mouse models, we found little protection against tumor progression with RAD001 alone. However, in combination with the mTOR ATP-site inhibitor BEZ235, we observed strong tumor-suppression with no apparent overall enhancement of tumorigenic capacity. Despite the fact that the development of BEZ235 in cancer has been discontinued, the successor of INK128, TAK228, has had encouraging results in phase 1/2 clinical trials (40); and, in our preliminary studies, when combined with RAD001, is as effective as BEZ235 in repressing mTORC1 signaling and tumor growth. In support of this approach, others have developed a dual inhibitor which can bind to both the allosteric- and ATP- binding sites of mTOR, which is as effective as targeting the two sites in combination (55,56).

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The approach of combining allosteric and catalytic site inhibitors to increase efficacy is not confined to mTOR, as such an approach has been recently reported for Abl/Bcr positive chronic myeloid leukemia (57). Based on the synergistic activity of the new allosteric inhibitor ABL001 with nilotinib, imatinib or dasatinib, this combination is being tested in clinical trials (58).

The utility of targeting mitochondrial metabolism in cancer has been recognized for some time (59). In addition, clinically, biguanides have been used to resolve hyperglycemic states associated with mTOR inhibitors (31,32). We chose Phenformin over Metformin because the hydrophobic biguanide does not require cell surface organic cation transporter 1 (OCT1) (60), and since OCT1 appears downregulated in advanced HCC (33). Both *in vitro* and *in vivo* studies show that Phenformin acts mainly through inhibition of the mitochondrial OXPHOS CI (34,49), and we find that Phenformin recapitulates the effects of other mitochondrial electron transport chain inhibitors by inducing acute activation of AMPK (38), as a consequence of the increased AMP/ATP ratio. Moreover, even though Phenformin is a potent inhibitor of mTORC1 through AMPK dependent and independent mechanisms (61,62), it seems to have little additional effect on this pathway in presence of RAD001 and BEZ235. Therefore, it appears to contribute to tumor suppression by distinct mechanisms. This may be through the activation of AMPK, whose role as tumor suppressor is complex, but which is largely thought to stem from its function as a suppressor of major anabolic process and the activation of catabolic responses (63,64). It is clear from our study that activation of AMPK by Phenformin suppresses both ribosome biogenesis and protein synthesis, two of the most major anabolic processes in the cell. However, we find that

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the activation of AMPK by Phenformin is strongly suppressed in the presence of RAD001/BEZ235, potentially due to the energy-sparing effects of mTOR inhibition, underscoring the antagonist effects that such therapies may exert.

The rational for pursuing Phenformin in combination with RAD001/BEZ235 was that together they would significantly impact cell metabolism by attacking both mitochondrial and glycolytic capacity, eventually leading to energetic catastrophe. Cellular low energy-state and mitochondrial dysfunction are known to further stimulate the generation of mitochondrial ROS (65), consistent with our findings that the Phenformin-induced apoptotic effects appear to be ROS-mediated. Moreover, the effects on ROS may be further exacerbated by a lack of autophagic clearance of damaged-mitochondria in the presence of Phenformin. The mitochondrial impact of Phenformin is highlighted by the apparent effort of treated cells to use alternative ATP-generating processes to survive, including glycolysis and potentially fatty acid oxidation (66). Nevertheless, the observed glycolitic shift does not seem to resolve the energetic stress imposed by Phenformin *in vitro*, since the AMP/ATP ratios are not normalized, and cells become strikingly reliant on glucose levels.

Although energetic catastrophe, leading to apoptosis, appears to be ultimately dictated by ROS levels, the managing of energetic stress seems to be largely mediated by the regulation of mitochondrial OXPHOS capacity, efficiency and dynamics (67). Here we show that Phenformin induces a low energy-state characterized by an increase in AMPK activation, mitochondrial dysfunction and mitochondrial fragmentation, all potentially contributing to Phenformin's tumor suppressive effects. Recent studies have shown that the electron transport inhibitors rotenone and antimycin A induce

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mitochondrial fragmentation by abrogating the mitochondrial localization of the fission protein DRP1, in an AMPK dependent manner (38). However, these agents do not induce OPA1 cleavage, a fragmentation-related event which is only triggered after more extensive mitochondrial damage (38,68), such as we observe here with Phenfomin. This more extensive damage may also be a consequence of Phenformin' inhibitory effect on autophagy. From a bioenergetics point of view, one would have predicted that the initiation of autophagy and mitochondrial biosynthetic pathways would be activated in order to reestablish an efficient mitochondrial network and decrease ROS levels (69,70). However, it has been reported that autophagy initiates at mitochondria-associated ER-membranes (MAM) (71), such that Phenformin-induced fragmentation may structurally compromise this contact sites, blocking the initiation of autophagy.

Consistent with our data, mTOR inhibitors were previously found to decrease cell respiration and mitochondrial mass, while maintaining coupled ATP-production, although we did not observe a drop in ATP levels, as reported by Morita et al. (30). This finding was also consistent with RAD001/BEZ235 treatment having no effects on the AMP/ATP and ADP/ATP ratios or on AMPK activation. Instead, the maintenance of ATP levels appears to be the result of increased mitochondrial efficiency and lower cellular ATP demand. In agreement, we observed an increased mitochondrial network fusion phenotype following RAD001/BEZ235 treatment, which was recently reported for mTOR inhibitors (48), and that may be responsible for the increase in OXPHOS efficiency (67). The data suggest that mTOR control of mitochondrial dynamics may partially be responsible for the cytostatic effects of mTOR inhibitors; inducing a hyperfusion state that protects mitochondria against apoptosis, reminiscent of nutrient deprivation (72).

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Indeed, Morita and co-workers have demonstrated that inducing mitochondrial fragmentation could render mTOR inhibitors more cytotoxic (48). However, *in vivo*, the potential impact of Phenformin on mitochondrial inhibition of CI is apparently much less than that achieved *in vitro*, suggesting that it might be insufficient to overcome the protective effects of the mTOR inhibitors when added in combination.

Given the findings above, our goal was to maximize each drugs effect in suppressing tumor growth, leading to the hypothesis that their sequential use may be more efficacious. This reasoning suggested that an initial treatment with Phenformin followed by mTOR inhibitors would favor tumor-growth suppression by limiting the capacity of cells to respond to mTOR inhibition by hyperfusion of mitochondria and increased mitochondrial efficiency. Concomitantly, Phenformin-induced mitochondrial dysfunction forces tumor cells to use glycolysis, which might render them more prone to the mTOR inhibitors, which impair this response. Such a strategy of metabolic synthetic lethality was recently reported in a breast cancer model using a tyrosine kinase receptor inhibitor and either Phenformin or ME344, a CI inhibitor in clinical trials (73). The administration of a Phenformin prior the use of mTOR inhibitors dramatically increased the overall survival of tumor-bearing mice, supporting the notion that there is a metabolic synthetic lethality that could be exploited when combining these agents. The paradigm of combining Phenformin with the mTOR inhibitors needs to be further developed, in terms of dose, frequency and time of drug exposure. In this context, FDG-PET could be used to ascertain the effects of Phenformin on the induction of glucose uptake when cells shift to glycolysis, which should recede when mTOR inhibitors are administered, as recently proposed by Navarro et al in a similar context (73). This will be of interest given

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the recent preclinical studies showing the benefit of combining Phenformin with B-RAF inhibitors in melanoma (74,75), which have led to the initiation of a Phase I clinical trial with Phenformin in melanoma (37). It will be of interest to determine its efficacy in such a setting and whether Phenformin with the dual mTOR inhibitors can be expanded to HCC and other cancers.

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FIGURE LEGENDS

Figure 1. RAD001 and BEZ235 suppress HCC orthoxenograft growth without promoting tumorigenic capacity. Huh7 orthotopically-implanted tumor pieces were allowed to graft for 15 days (t0) in athymic mice. Mouse cohorts were treated with either vehicle, RAD001 (2.5 mg /kg) or BEZ235 (6 mg/kg), or the latter two in combination. **A,** Tumor weight before (t0) and after 15 days of treatment (n=5-6). **B,** Amount of phosphorylated and total proteins in tumor lysates; β-actin was used as loading control. **C,** Tumoroid cultures established from Huh7 tumors (scale bar 200 μM) treated for 6 days with RAD001 (5 nM) and BEZ23 (20 nM) (n=4). **D,** Tumoroid cultures established from Huh7 tumors (n=2-3) pretreated in vivo for 7 days with RAD001 (2.5 mg/kg) and BEZ235 (6 mg/kg). **E,** Tumor incidence over time following a limiting-dilution assay performed with the indicated numbers of cells obtained from tumors pretreated *in vivo* for 15 days as in D (representative experiment, n=3). Veh (vehicle); RAD (RAD001); BEZ (BEZ235); RB (RAD001/BEZ235).

Figure 2. Phenformin activates AMPK, induces a shift towards glycolysis and increases susceptibility to cell death by decreased glucose levels. **A,** Circulating glucose levels in mice fed *ad libitum* and treated for 14 days with RAD001 (2.5 mg/kg) and BEZ235 (6 mg/kg) together, or in combination with Phenformin (70 mg/kg) (n=5). **B,** Relative number of Huh7 cells after 72 hrs of exposure to RAD001 (5 nM) and BEZ235 (20 nM) together, or in combination with Phenformin (1 mM) (n=3). **C,** Extent of phosphorylation of indicated proteins in Huh7 cell lysates after treatment for the stated times with

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RAD001 (5 nM), BEZ235 (20 nM) together, or in combination with Phenformin (1 mM); β-actin was used as loading control. **D,** Extra-cellular Acidification Rate (ECAR) after addition of 25 mM glucose (n=3) to Huh7 cells pre-treated with RAD001 (5nM) and BEZ235 (20 nM) together, or in combination with Phenformin (1 mM) for 16 hrs. **E,** Relative cell number of Huh7 after 72 hrs of exposure to RAD001 (5 nM) and BEZ235 (20 nM) together, or in combination with Phenformin (1 mM), in culture medium containing the stated glucose concentrations (n=3). **F,** Relative cell number of Huh7 after 24 hrs of exposure to Phenformin (0.5 and 1 mM) in culture medium containing 5 mM glucose, with or without pan-caspase inhibitor Q-VDOPH (20 µM) (n=3). **G,** Tumoroid cultures established from Huh7 tumors treated for 6 days with RAD001 (5 nM) and BEZ235 (20 nM) alone, or in combination with Phenformin (30 µM), at the stated glucose concentrations (scale bar 200 µM) (representative experiment, n=4). Ctrl (Control); RB (RAD001/BEZ235); Phf (Phenformin); RBP (RAD001/BEZ235/Phenformin).

Figure 3. Phenformin, but not mTOR inhibition, reduces the energy state of Huh7 cells. Huh7 cells were treated with RAD001 (5 nM) and BEZ235 (20 nM) together, or in combination with Phenformn (1 mM). **A,** Routine Oxygen Consumption Rate (OCR) of Huh7 cells treated for the stated times, relative to control cells (n=3-5); *t-student* Test versus control. **B,** Mitochondrial mass measured by flow cytometry analyses of Huh7 cells stained with MitoTracker Green FM, after 16 hrs of the indicated treatments (n=6). **C,** ATP levels analyzed by HPLC after 16 hrs of indicated treatment relative to control and **D,** AMP/ATP ratio from the same samples (n=3). **E,** Newly synthesized RNA, after

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[3]H-Uridine pulse for 30 mins or 3.5 hrs, following 16 hrs of the indicated treatment (representative experiment, n=2). Upper panel: loading controls in agarose gel stained with ethidium bromide; Lower panel: Analysis of nascent rRNA synthesis by pulse labeling with ^{3}H-Uridine. **F,** Rate of global protein synthesis as determined by pulse labeling cells with ^{3}H-Leucine, following 16 hrs of the indicated treatments; Cycloheximide for 50 mins (100 μg/ml) as a positive control (representative experiment, n=2).

Figure 4. Phenfomin, but not mTOR inhibitors, induces mitochondrial fragmentation and high levels of ROS. Huh7 cells were treated with RAD001 (5 nM), BEZ235 (20 nM) and Phenformin (1 mM). **A,** ATP-coupled mitochondrial oxygen consumption of Huh7 cells after 3 and 16 hrs of treatment (n=3-5). **B,** Representative confocal microscopy images of the mitochondria network of 16 hrs-treated Huh7 cells visualized by Mitotracker Red CMXROS probe (scale bar 10 μm). **C,** OPA1 protein isoforms in total extracts from 16 hrs-treated Huh7 cells; TIM44 as mitochondrial loading control. **D,** Autophagy cargo BHMT-assay in Huh7 cells treated for 6 hrs; GFP-myc as a transfection control. **E,** Mitochondrial superoxide analyzed by flow cytometry of Huh7 with MitoSox Red probe, after 3 hrs of treatment; Antimycin A (AA) (50 μM) and Rotenone (Rot) (25 μM). for 45 mins as positive controls (n=4). **F,** Relative cell number of Huh7 after 24 hrs of exposure to Phenformin (0.5 and 1 mM) in culture medium containing 5 mM glucose, with or without pan-caspase inhibitor Q-VD-OPH (20 μM) and mitochondrial ROS scavenger MitoTempo (MT) (20μM) (n= 3). Ctrl (Control); RB (RAD001/BEZ235); Phf (Phenformin); RBP (RAD001/BEZ235/Phenformin).

Figure 5. Combining RAD001/BEZ235 and Phenformin *in vivo* efficiently halts Huh7-tumor growth, and the sequential administration of Phenformin followed by RAD/BEZ235 dramatically improves Huh7-tumor-bearing mouse survival. **A,** Huh7-tumor weight before (t0) and after 15 days of treatment with RAD001 (2.5 mg/kg) and BEZ235 (6 mg/kg) together, or in combination with Phenformin (70 mg/kg), (n= 8-9). **B,** Circulating levels of glucose and **C,** Lactate in mice treated for 15 days, while fed *ad libitum* (n=6-8). **D,** Kaplan-Meier survival curve of Huh7-tumor bearing mice after 22 days of treatment with RAD001 (2.5 mg/kg) and BEZ235 (6 mg/kg) together, or in combination with Phenformin (70 mg/kg), (n=6-9). **E,** Oxygen consumption of mitochondria from 7-day treated tumors in the presence of glutamate, malate and ADP (GMP) (n=3). **F,** Succinate substrate control ratio: calculated as the ratio between oxygen consumption in the presence of glutamate, malate and ADP with (GMSP) or without succinate (GMP) (n=3). **G,** Kaplan-Meier survival curve of Huh7 tumor-bearing mice after 8-days of treatment with Phenformin (100 mg/kg), followed by 15 days treatment with RAD/BEZ235 (2.5 mg/kg and 6 mg/kg) or Phenformin (100mg/kg.) QOD and **H,** tumor weight at ethical time of sacrifice (n=8-10). Veh (vehicle); RB (RAD001/BEZ235); Phf (Phenformin); RBP (RAD001/BEZ235/Phenformin); P_{100} (Phenformin 100mg/kg); P_{100}+RB (Phenformin 100mg/kg followed by RAD001 plus BEZ235).

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Figure 6. Model of RAD001/BEZ235 and Phenformin effects on mitochondrial dynamics, OXPHOS and glycolysis; and their impact on overall survival of tumor-bearing mice.
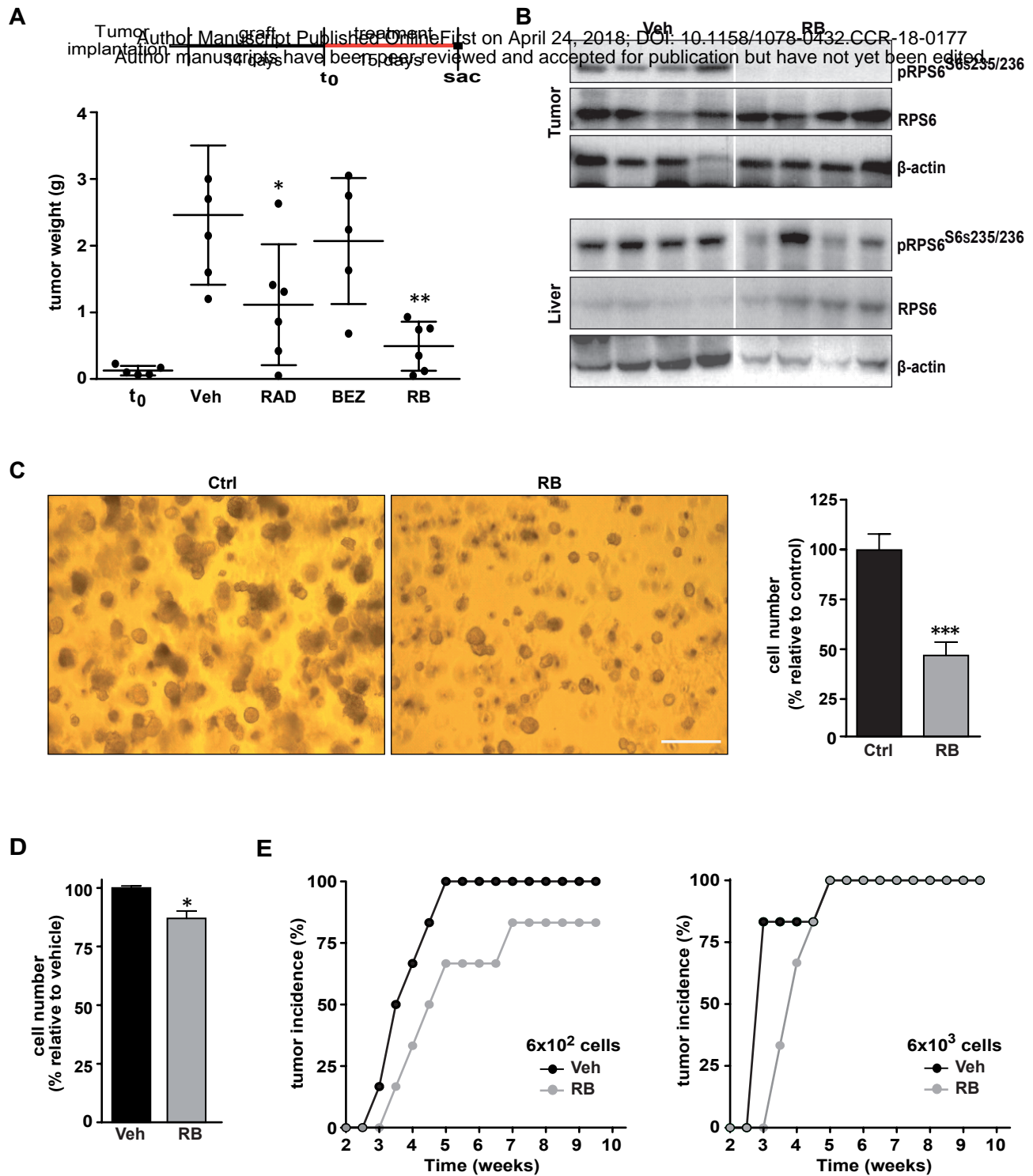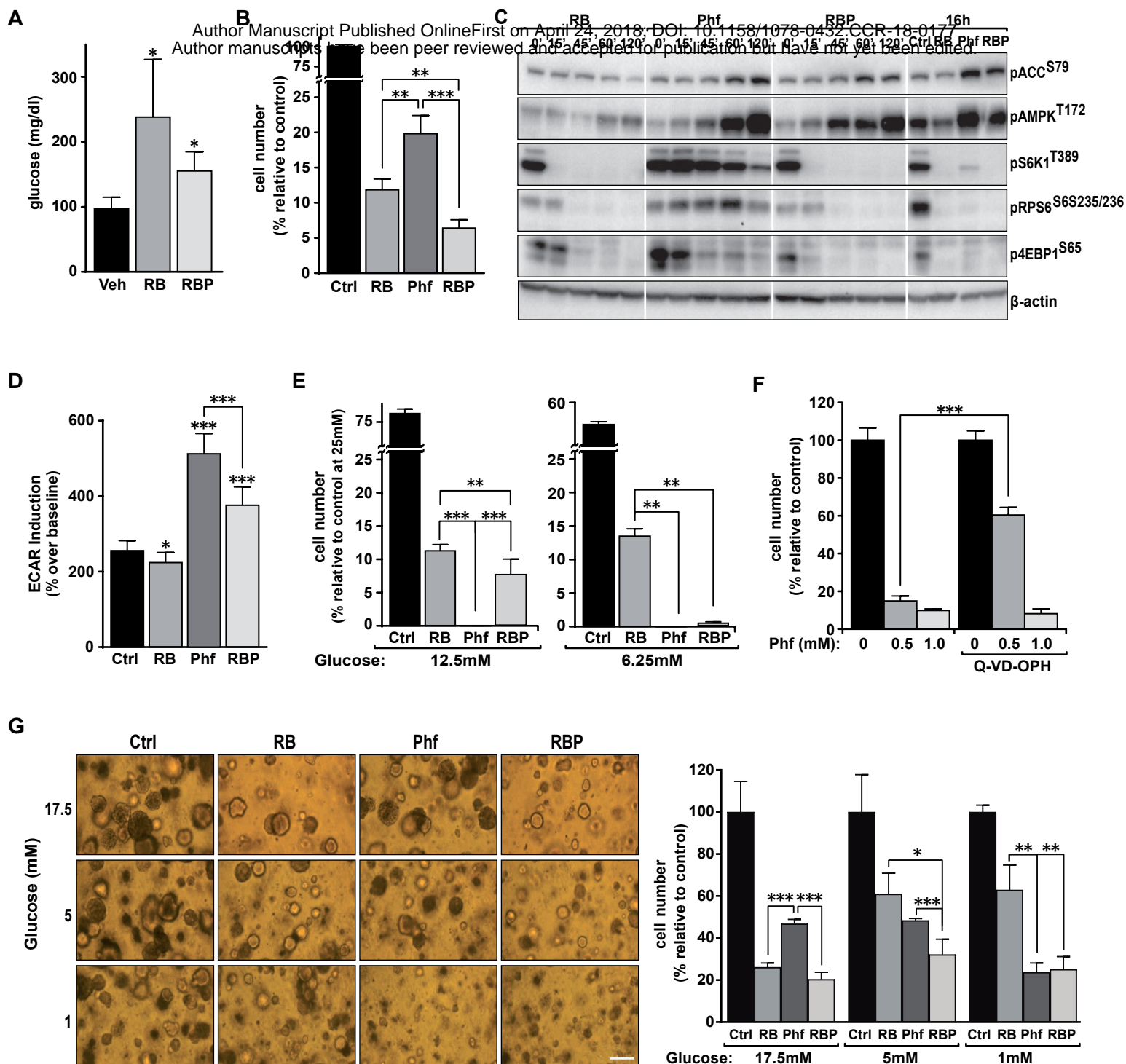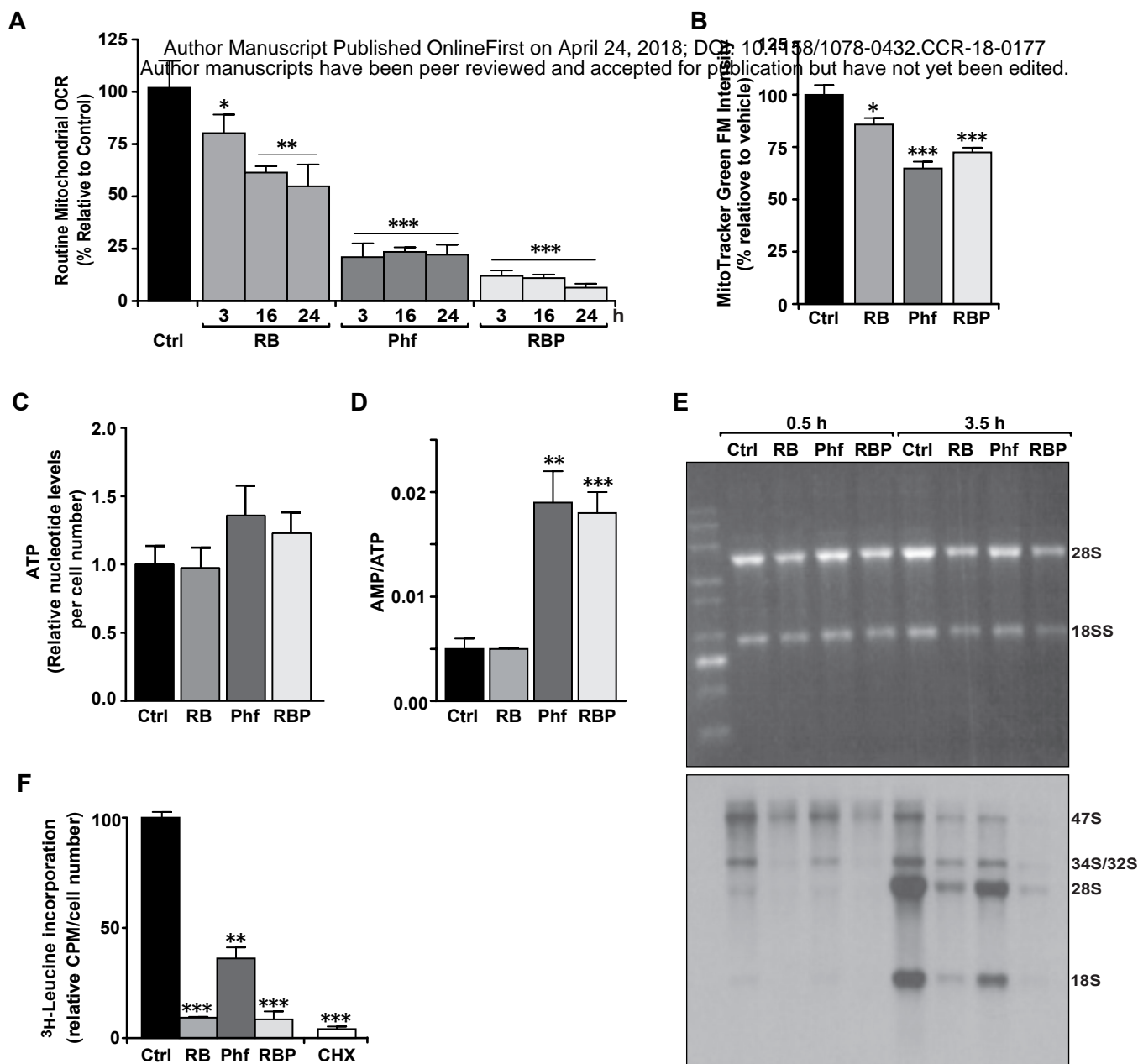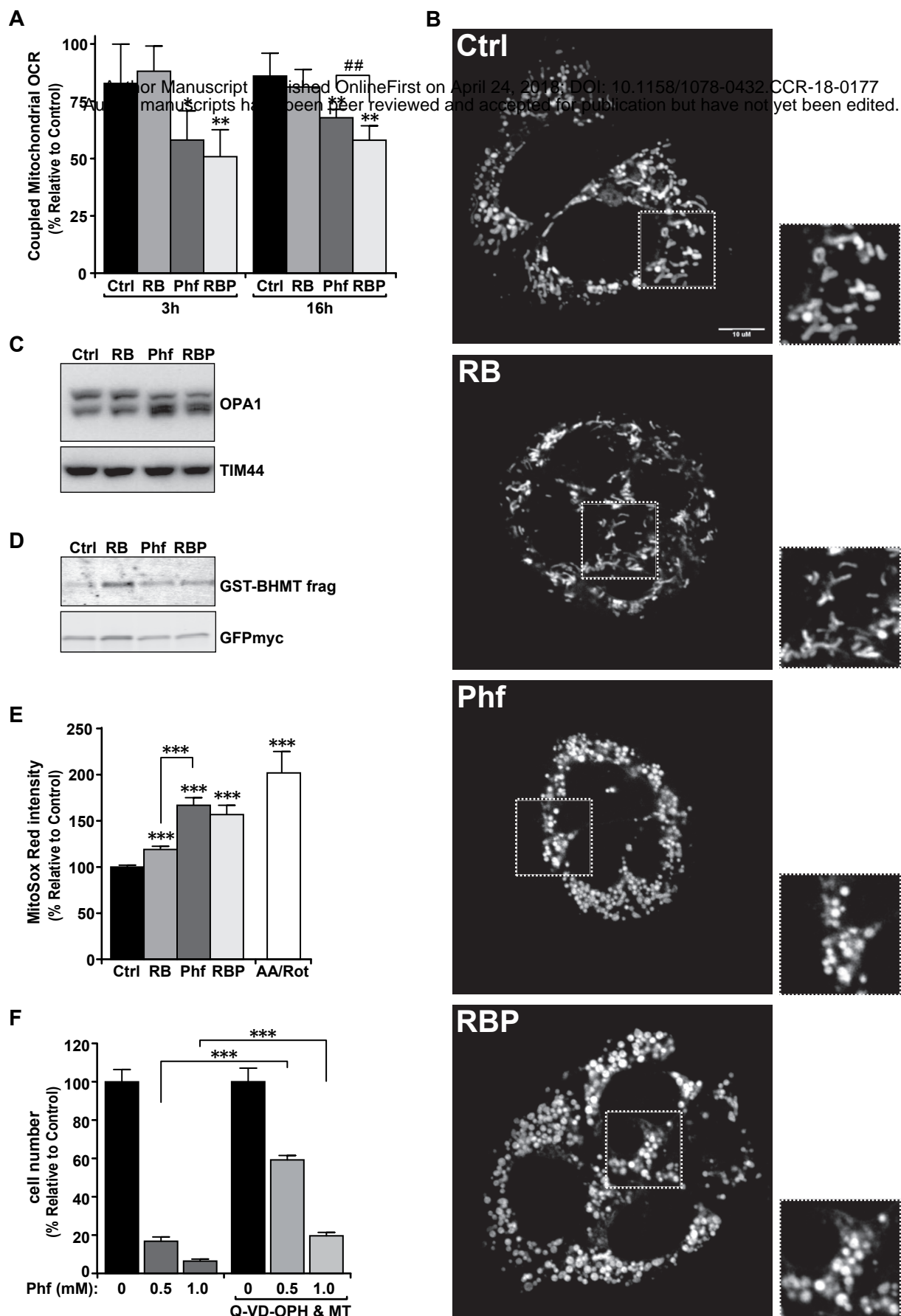
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Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

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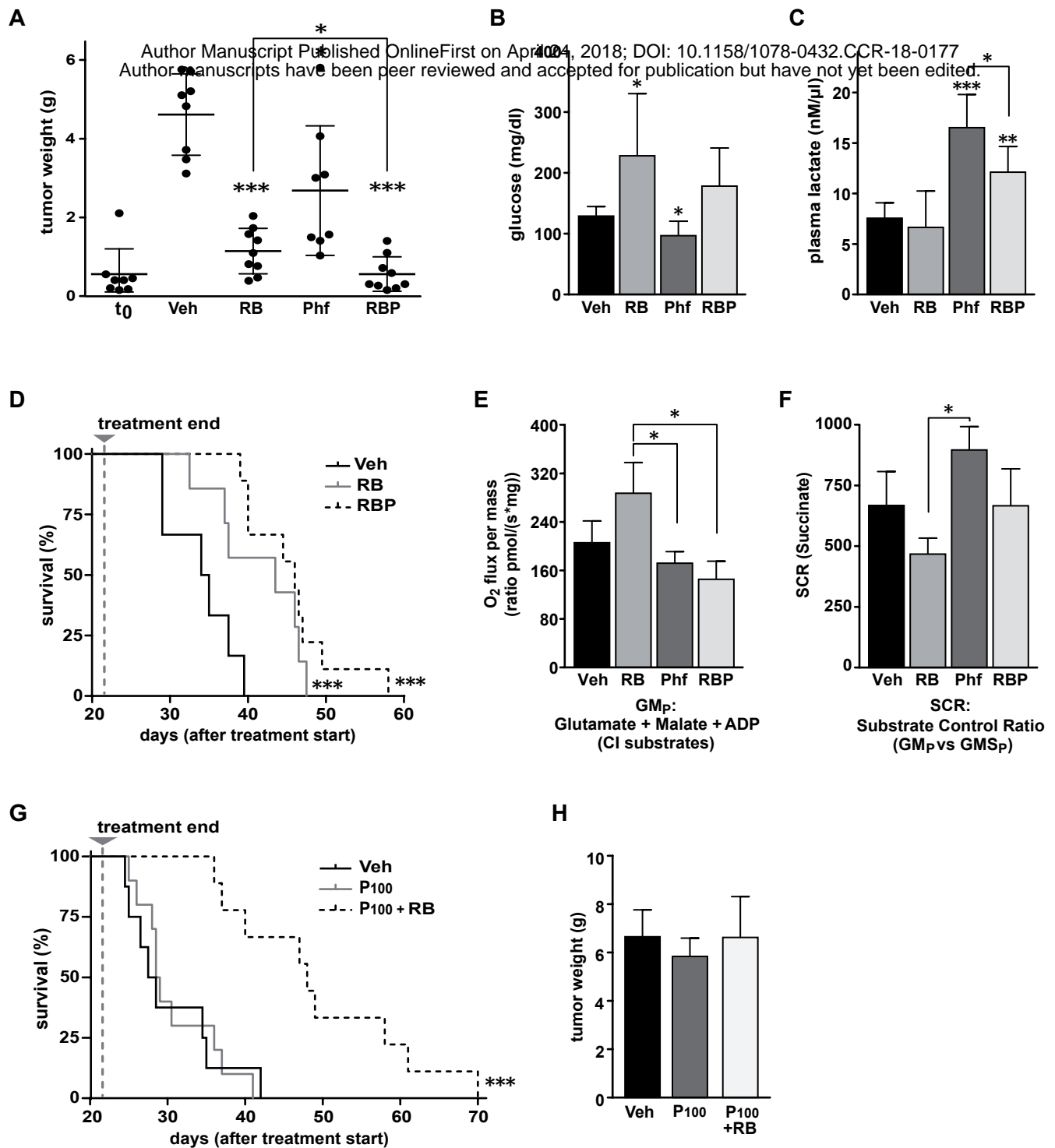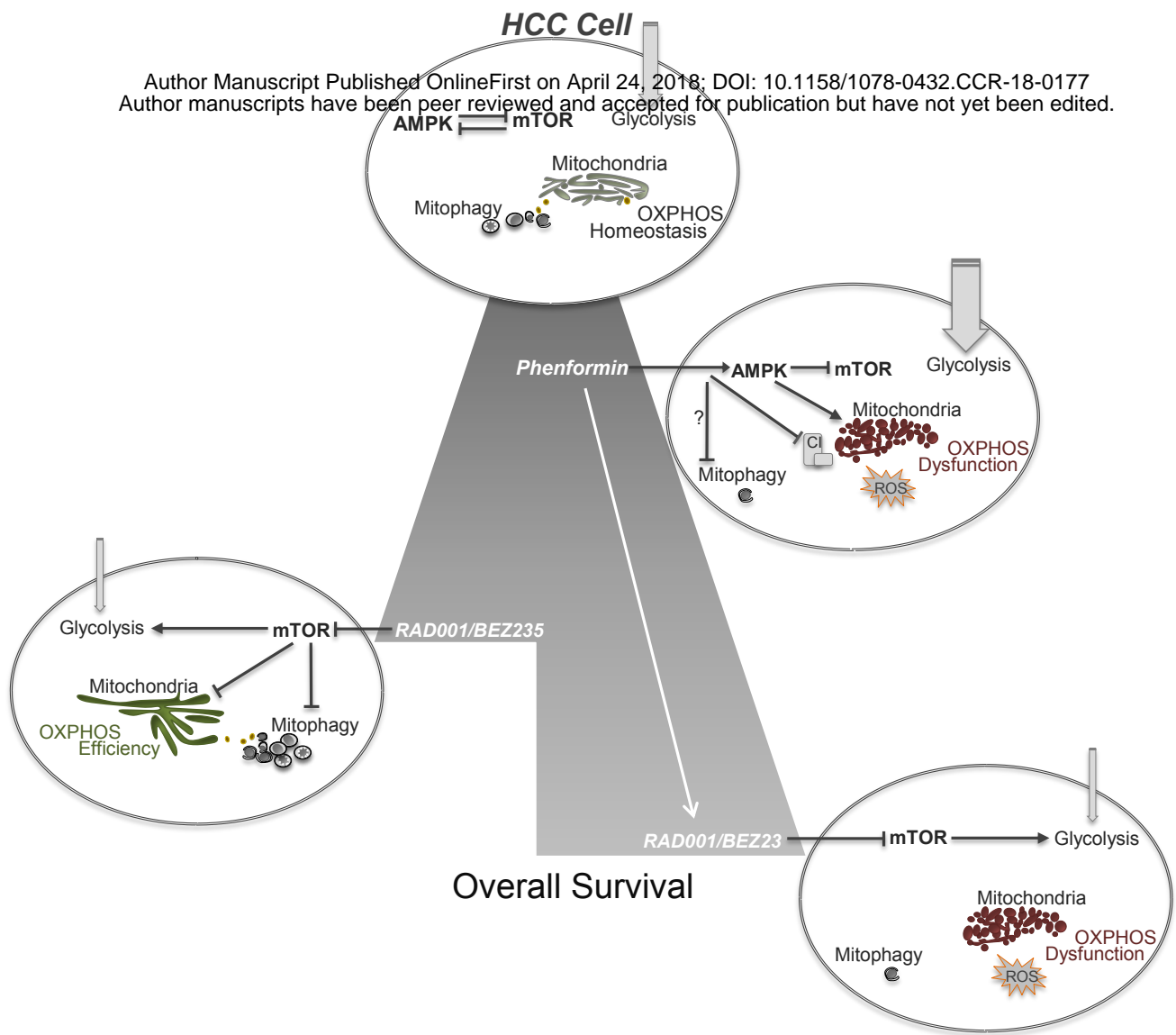
Clinical Cancer Research

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Sonia Rosa Veiga, Xuemei Ge, Carol A Mercer, et al.

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