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UCP2 inhibition induces ROS/Akt/mTOR axis: Role of GAPDH nuclear translocation in genipin/everolimus anticancer synergism

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Abstract: Several studies indicate that mitochondrial uncoupling protein 2 (UCP2) plays a pivotal role in cancer development by decreasing reactive oxygen species (ROS) produced by mitochondrial metabolism and by sustaining chemoresistance to a plethora of anticancer drugs. Here, we demonstrate that inhibition of UCP2 triggers Akt/mTOR pathway in a ROS-dependent mechanism in pancreatic adenocarcinoma cells. This event reduces the antiproliferative outcome of UCP2 inhibition by genipin, creating the conditions for the synergistic counteraction of cancer cell growth with the mTOR inhibitor everolimus. Inhibition of pancreatic adenocarcinoma cell growth and induction of apoptosis by genipin and everolimus treatment are functionally related to nuclear translocation of the cytosolic glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The synthetic compound (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-dihydroisoxazol-5-yl-acetate (AXP3009), which binds GAPDH at its redox-sensitive Cys152, restores cell viability affected by the combined treatment with genipin and everolimus, suggesting a role for ROS production in the nuclear translocation of GAPDH. Caspase-mediated apoptosis by genipin and everolimus is further potentiated by the autophagy inhibitor 3-methyladenine revealing a protective role for Beclin1-mediated autophagy induced by the treatment. Mice xenograft of pancreatic adenocarcinoma further confirmed the antiproliferative outcome of drug combination without toxic effects for animals. Tumor masses from mice injected with UCP2 and mTOR inhibitors revealed a strong reduction in tumor volume and number of mitosis associated with a marked GAPDH nuclear positivity. Altogether, these results reveal novel mechanisms through which UCP2 promotes cancer cell proliferation and support the combined inhibition of UCP2 and of Akt/mTOR pathway as a novel therapeutic strategy in the treatment of pancreatic adenocarcinoma.

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BIOMEDICINA E MOVIMENTO

Free Radical Biology & Medicine

Verona, July 28th 2017

Editorial Office

Dear Professor Vina,

we wish to thank you for your efforts in handling our manuscript (FRBM-D-17-00405) entitled “*UCP2 inhibition induces ROS/Akt/mTOR axis: role of GAPDH nuclear translocation in genipin/everolimus anticancer synergism*” and for giving us the opportunity to revise and improve it. We greatly appreciated your thoughts and those from the Reviewers. We revised the manuscript accordingly. As you proposed in your Editorial decision letter on June 25th 2017 reported below, we kindly ask you to use the same Referees to assure consistency in the review process of our manuscript.

Dear Dr. Donadelli,

Thank you for resubmitting the above-mentioned article to Free Radical Biology & Medicine.

We have completed the review of your manuscript and a summary is enclosed below. Although some merit was found in your manuscript (see below), the stated priority for publication (to the Editor) was quite low. Free Radical Biology & Medicine can now accept about 30% of submitted manuscripts. Therefore, as is the case with your manuscript, the decision reflects the relative priority assigned by the reviewer as much as the assessment of scientific merit.

It appears from the enclosed comments that some portions of the study were considered interesting but not sufficiently well documented. Those comments suggest that an entirely rewritten manuscript addressing each of the issues raised may receive a more positive response from these reviewers. That manuscript may have to delete some parts and include new experiments. Such a substantially altered manuscript would then be considered as a new submission. This, of course, does not guarantee acceptance of the new submission, which would still undergo a rigorous review. If you desire to follow this path, please refer to this letter and manuscript number, as well as provide responses to the reviewer comments, and I will use these same referees to assure consistency in the review process.

Yours sincerely,

Jose Vina

Associate Editor

Free Radical Biology & Medicine

As required by Reviewers, we have revised the manuscript in order to highlight the novelty of the study and to help the readers to keep the overall aim of the study. As required by Reviewers, we

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have also deleted some parts of the manuscript and performed additional experiments, which straighten the conclusions of the manuscript. The new results have been inserted in the main figures and properly discussed in the text. Overall, we have point-by-point answered to all the criticisms of the Reviewers and we hope our manuscript might be suitable for publication in your journal.

Yours sincerely,

Ilaria Dando and Massimo Donadelli

University of Verona, Italy



Free Radical Biology & Medicine
Editorial Office

Verona, July 28th 2017

Dear Professor Vina,
please find below the detailed, itemized list of our responses to the Reviewers' suggestions/comments and the changes we have made in the resubmitted Manuscript No.: FRBM-D-17-00405. Title: *UCP2 inhibition induces ROS/Akt/mTOR axis: role of GAPDH nuclear translocation in genipin/everolimus anticancer synergism.*

Reviewers' comments:

Reviewer #1:

The goal of the authors is to demonstrate that the combined inhibition of UCP2 and of Akt/mTOR pathway is a novel therapeutic strategy in the treatment of PDAC. The paper is well carried out and examines with a lot of suitable methods each step of experimental protocol, but there are some contradictions. Therefore, I have these remarks for the authors:

Introduction

Pag 5, lines 89-92. The authors previously demonstrated that UCP2 inhibition triggers PDAC cell death by ROS-dependent nuclear translocation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and stimulation of autophagy in PDAC cells [19]. In the present study, they showed that UCP2 inhibition triggers the oncogenic Akt/mTOR pathway, which has an established role in cancer proliferation. Therefore the authors must also give to the PDAC cells an inhibitor of this pathway.

Therefore, UCP2 inhibition has two conflicting effects. What is the explanation for these conflicting effects? Inhibition of UCP2 causes death of PDAC cells or induces cell proliferation?

Response: As reported by the Reviewer, our previous studies have shown that ROS stimulation after UCP2 inhibition with specific siRNA or genipin results in cell death of PDAC cells caused by oxidative stress that promotes GAPDH nuclear translocation (Dando et al., BBA 2013), gemcitabine chemotherapy sensitivity (Dalla Pozza et al., BBA 2012), and cellular energy metabolism alterations (Brandi et al., FRBM 2016). As shown in our previous studies, cell death of chemoresistant PDAC cells is strictly dependent on the production of ROS since the addition of the NAC scavenger allows the recovery of cell death induced by UCP2 inhibition. In the same context of cancer cell death by oxidative stress (also demonstrated by other researchers in other types of cancer), we here demonstrate that ROS produced by UCP2 inhibition also stimulate the Akt/mTOR oncogenic pathway in vitro and in vivo. This event may be interpreted as an extreme attempt of the tumor cells to survive in the hostile and cytotoxic condition, such as that generated by UCP2 inhibition. This behavior is not surprising because many pro-survival molecular mechanisms are known to be adopted by tumor cells in critical conditions. The consequence of our discovery implies that an Akt/mTOR pathway inhibitor, such as everolimus, which is a molecule of high clinical interest in oncology, has to be associated to UCP2 inhibition and oxidative stress in order to induce a full cell death of PDAC cells. In this study, we show for the first time that the association of genipin and everolimus can determine a synergistic cell death effect on PDAC cells both in vitro and in a murine model. We have modified the Introduction of the manuscript to better highlight this crucial issue.



Is the reduction of cell growth by genipin due to another mechanism different from UCP2 inhibition?

Response: The possibility that cell growth reduction by genipin could be due to another mechanism different from UCP2 inhibition is very unlikely. Indeed, we have previously demonstrated and published that the knockdown by siRNA of the specific target of genipin, i.e. UCP2, significantly reduces cell death by genipin in PDAC cells (Brandi et al., FRBM 2016).

Results

Pag 18, line 402: "drug combination strongly enhances ROS production as compared with single drugs (Fig. 2B) and the ROS-mediated antiproliferative effect in PDAC cells (Fig. 2C)." Previously the authors affirm that ROS trigger Akt/mTOR pathway (Figure 1D shows that Akt and P70S6K phosphorylation induced by genipin is at least partially rescued by NAC demonstrating the involvement of ROS production in this signaling regulation driven by UCP2 blockage). It needs to explain this.

Response: We thank the Reviewer for this suggestion because this crucial consideration might need further explanations for clarity to the readers. As the Reviewer reports, we show that inhibition of UCP2 causes the stimulation of the Akt/mTOR oncogenic pathway in a ROS-dependent manner (Figure 1). Then, we show that combined genipin and everolimus treatment results in a strong increase in ROS (Figure 2B) and cell death (Figure 2C). This apparent contradiction can be explained by the observation that in the combined treatment the Akt/mTOR pathway is blocked by the pharmacological effect of everolimus even in the presence of high ROS levels (Figure 2A). In addition, the strong ROS stimulation and the concomitant Akt/mTOR inhibition observed after the combined treatment are mechanisms known to lead to GAPDH nuclear positivity, which we observe both in vitro and in vivo (Figure 3). Therefore, the GAPDH nuclear translocation is likely due to its oxidation and its failure to be phosphorylated by Akt/mTOR. These observations explain why the combined treatment results in a strong cell death effect despite ROS increase may potentially strongly stimulate the oncogenic Akt/mTOR pathway. An explanation of the above mentioned issue has been added in the appropriate Results section indicated by the Reviewer.

Reviewer #2:

1. Sedlak et al reported that the GAPDH mediates a novel cell death cascade (JAMA. 2006;295(1):81-9.). Cytotoxic stimuli, via nitric oxide generation, lead to the binding of GAPDH to the protein Siah1, translocation of GAPDH-Siah1 to the nucleus, and ultimately cell death. Xing et al. Identified GAPDH as a protein target of the saframycin antiproliferative agents (Proc Natl Acad Sci USA. 2004;101(16):5862-6.). Drugs targeting on GAPDH hence plays important role for their action on cancer cells.

Your previous reports disclosed that UCP2 inhibition triggers ROS-dependent nuclear translocation of GAPDH and autophagic cell death in pancreatic adenocarcinoma cells (REF-18,19). Rapamycin and genipin enhanced antitumor activity in lung cancer (Su et al., Journal of Clinical Oncology. 2015;33(15, Suppl.):e13595.).

Fiorini et al. (Onconase induces autophagy sensitizing pancreatic cancer cells to gemcitabine and activates Akt/mTOR pathway in a ROS-dependent manner. Biochim Biophys Acta. 2015;1853(3):549-60.) studied the precise mechanisms determining cytotoxicity in pancreatic cancer cells of onconase (ONC). ONC inhibited the expression of UCP2 and of MnSOD triggering



mitochondrial superoxide ion production. ONC-induced reactive oxygen species (ROS) were responsible for Akt/mTOR pathway stimulation determining the sensitivity of cancer cells to mTOR inhibitors and lessening autophagic stimulation. The conclusion made in this manuscript is not extended far from that of previous studies, thus reveals limited novelty for this manuscript.

Response: The Reviewer mentions some studies that constitute the scientific bases and, thus, the prerequisites of the present manuscript. Indeed, in the Discussion section, we have described our results referring to those previous studies mentioned by the Reviewer. For the Reviewer point of view, the present study reveals limited novelty. However, it should be stressed that we demonstrate here for the first time the strong synergistic antiproliferative effect on PDAC cells after UCP2 and mTOR inhibition. The Reviewer mentions the study “Rapamycin and genipin enhanced antitumor activity in lung cancer (Su et al., *Journal of Clinical Oncology*. 2015;33 (15, Suppl.):e13595.)”. However, this is an abstract meeting and, to our best knowledge, no peer-reviewed articles related to this statement have yet been published.

In addition, we demonstrate that GAPDH nuclear translocation observed in vitro and in vivo is not a side effect in our system, but it is functionally involved in growth inhibition and apoptosis as shown by using the AXP3009 compound. Indeed, the addition of AXP3009 significantly counteracts GAPDH nuclear translocation (Fig. 4D) and cell growth inhibition (Fig. 4E) and apoptosis (Fig. 5B) induced by combined treatment.

Furthermore, in the present study, we demonstrate for the first time that UCP2 can regulate the critical pathway Akt/mTOR in a ROS-dependent way in vitro and in vivo. Since UCP2 is a mitochondrial antioxidant protein also involved in some key regulations of the energetic metabolism, the aberrant alteration of Akt/mTOR by UCP2 can be considered a relevant crossroad of the two distinct aspects in cancer, regulation of oxidative stress and energetic metabolism. Although several years of studies have highlighted the critical role of the antioxidant mitochondrial uncoupling protein UCP2 in driving tumor progression, the downstream effects and the mechanisms by which UCP2 intersects and alters the activity of other signaling pathways remains largely unknown. Our manuscript provides novel mechanistic insights into the oncogenic signaling regulation by UCP2 and unveils the combined inhibition of the antioxidant UCP2 and mTOR as a strategic therapeutic approach for cancer. Moreover, beyond cancer UCP2 is involved in a plethora of physiologic and pathologic events, including regulation of atherosclerotic plaque formation, immune response, food intake, and metabolic and oxidant diseases (Donadelli et al. *Cell. Mol. Life Sci.* 2014, 71:1171–1190). Thus, we believe that the consequences of our findings might be very relevant not only for cancer research.

The Reviewer mentions our previous study by Fiorini et al. describing the effect of onconase in PDAC cells. Although the involvement of some antioxidant proteins/enzymes as UCP2 and MnSOD has been described and the Akt/mTOR pathway has been demonstrated to be regulated in a ROS-mediated manner, the overall context is completely different because onconase is a ribonuclease enzyme secreted in oocytes and early embryos of *Rana pipiens*. Onconase may determine a plethora of unknown events into the cells altering the overall conclusions, thus confining the results of that article to the specific experimental conditions used. Instead, in the present manuscript, we used specific target drugs, everolimus and genipin, to specifically study the regulation of UCP2 and Akt/mTOR signaling and their therapeutic potential in mice xenograft.



2. Everolimus is a currently approved drug for the treatment of pancreatic tumours. However, genipin has not been developed for clinical use yet. Genipin and everolimus combination for pancreatic tumor therapy seems not feasible in clinical application.

Response: We agree with the Reviewer about the clinical relevance of everolimus. Concerning genipin, we are fully aware that this compound has not been developed for clinical use, however, in our opinion, it remains a valuable tool for the preclinical investigation of UCP2 roles and for the study of the potential clinical usage of new generation UCP2 inhibitors for therapy. We have modified the Discussion section by adding these observations.

3. There are several main themes in this manuscript that authors may need to figure out which is the major one. 1. UCP2 inhibition induces ROS/Akt/mTOR axis. 2. Role of GAPDH nuclear translocation. 3. Combination of UCP2 inhibitor genipin and mTOR inhibitor everolimus for pancreatic adenocarcinoma cell treatment. If the genipin/everolimus combination for anticancer synergism is the main theme of this manuscript, more experimental designs should address the synergistic effects. UCP2 inhibition induces ROS/Akt/mTOR axis and the GAPDH nuclear translocation could merely be the markers for the effect evaluation.

Response: As the Reviewer underlines, the synergistic antiproliferative effect on pancreatic cancer cells of the combined treatment genipin+everolimus is a crucial finding of our manuscript. We have now extended the analysis of synergism on five PDAC cell lines having different origins (primary tumor or metastasis); different mutational status; different resistance/sensitivity to the standard chemotherapy (i.e. gemcitabine). The curves of PDAC cell growth after increasing concentrations of single drugs and their combinations are reported in the new Figure 3A. The analysis of synergism obtained by using the dedicated software CalcuSyn is reported in the new Figure 3B. To further describe the level of synergism observed, we report in the new Table 1 the relative parameters of synergism obtained with CalcuSyn, i. e. CI_{50} , CI_{75} , the percentage of synergism, and the DRI_{50} values for each drug (genipin and everolimus) and for all five PDAC cell lines tested. Further, we report in the new Supplementary Figure 3 the isobolograms of IC_{50} values for all cell lines tested. Material and Methods, Results, Figures, and Discussion Section have been modified accordingly. Overall, these new data strongly reinforce our statement that genipin and everolimus can synergistically inhibit growth of PDAC cells.

4. In the Fig.4, AXP3009 counteracts cell growth inhibition by genipin/everolimus treatment. The G alone, G + AXP3009, E alone, E + AXP3009 should be included in the experimental design.

Response: We agree with the Reviewer's comment and we have now performed new experiments. Data are reported in the new Figure 5E of the resubmitted manuscript. Intriguingly, concerning single drug treatments, AXP3009 is able to counteract cell growth inhibition by genipin in accordance with our previous article that demonstrated that UCP2 inhibition triggers ROS-dependent nuclear translocation of GAPDH and PDAC cell death (Dando et al. BBA-Molecular Cell Research 2013). On the contrary, AXP3009 is not able to counteract cell growth inhibition by everolimus in accordance with the observation that everolimus alone fails to regulate GAPDH nuclear translocation (Figure 4B). We thank the Reviewer for this suggestion, which gave us the opportunity to improve our manuscript.

5. The synergistic effect of genipin/everolimus combination in cancer therapy should be based on the effect of genipin/everolimus \gg sum of (genipin alone + everolimus alone). Additive effect is different to synergistic effect.

Response: In the present manuscript, we refer to synergistic effects (and not additive) on the basis of the computational elaboration of our data reporting inhibition of cell growth of PDAC cells



treated with increasing concentrations of the two drugs in both single and combined settings with a fixed molar ratio. CalcuSyn software is specifically dedicated to the analysis of synergism by calculating the Combination Index (CI) values with the Chou-Talalay equation, which takes into account both the potency (IC_{50}) and the shape of the dose-effect curves. All synergism parameters, i.e. CI, DRI, percentage of synergism, isobolograms, are provided for all the five PDAC cell lines tested.

6. The 1st paragraph in the Discussion displays only introductory information that lacks specific discussing issue. You may omit it or combine with the 2nd paragraph.

Response: We agree with the Reviewer. The mentioned paragraph has been removed.

1 **UCP2 inhibition induces ROS/Akt/mTOR axis: role of GAPDH nuclear**
2 **translocation in genipin/everolimus anticancer synergism**

3
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24
25 *Running title:* Everolimus and genipin induce anticancer synergism

26 *Keywords:* pancreas cancer, uncoupling proteins, UCP2, mTOR, everolimus, GAPDH, cell death.

28 **Abstract**

29 Several studies indicate that mitochondrial uncoupling protein 2 (UCP2) plays a pivotal role
30 in cancer development by decreasing reactive oxygen species (ROS) produced by mitochondrial
31 metabolism and by sustaining chemoresistance to a plethora of anticancer drugs. Here, we
32 demonstrate that inhibition of UCP2 triggers Akt/mTOR pathway in a ROS-dependent mechanism
33 in pancreatic adenocarcinoma cells. This event reduces the antiproliferative outcome of UCP2
34 inhibition by genipin, creating the conditions for the synergistic counteraction of cancer cell growth
35 with the mTOR inhibitor everolimus. Inhibition of pancreatic adenocarcinoma cell growth and
36 induction of apoptosis by genipin and everolimus treatment are functionally related to nuclear
37 translocation of the cytosolic glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase
38 (GAPDH). The synthetic compound (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-dihydroisoxazol-5-yl-
39 acetate (AXP3009), which binds GAPDH at its redox-sensitive Cys152, restores cell viability
40 affected by the combined treatment with genipin and everolimus, suggesting a role for ROS
41 production in the nuclear translocation of GAPDH. Caspase-mediated apoptosis by genipin and
42 everolimus is further potentiated by the autophagy inhibitor 3-methyladenine revealing a protective
43 role for Beclin1-mediated autophagy induced by the treatment. Mice xenograft of pancreatic
44 adenocarcinoma further confirmed the antiproliferative outcome of drug combination without toxic
45 effects for animals. Tumor masses from mice injected with UCP2 and mTOR inhibitors revealed a
46 strong reduction in tumor volume and number of mitosis associated with a marked GAPDH nuclear
47 positivity. Altogether, these results reveal novel mechanisms through which UCP2 promotes cancer
48 cell proliferation and support the combined inhibition of UCP2 and of Akt/mTOR pathway as a
49 novel therapeutic strategy in the treatment of pancreatic adenocarcinoma.

50

51 **1. Introduction**

52 Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease that remains the fourth
53 leading cause of cancer-associated deaths [1, 2]. Despite advances in multimodal therapy [3, 4],
54 PDAC remains extraordinarily lethal, with a 5-year overall survival rate of approximately 5% [1, 5].
55 Delayed diagnosis, early metastasis, and resistance to almost all the classes of cytotoxic drugs are
56 considered the main reasons for the extremely poor prognosis of pancreas cancer patients [6]. For
57 these reasons, research is now focused on the identification of more efficient therapeutic targets as
58 well as new prognostic and diagnostic biomarkers in order to improve the clinical management of
59 PDAC.

60 Everolimus, a well-tolerated analogue of rapamycin with improved pharmacokinetic
61 properties and reduced immunosuppressive effects [7], has been approved by the Food and Drug
62 Administration (FDA) in 2012 for the treatment of advanced/metastatic renal cell carcinoma,
63 subependymal giant cell astrocytoma and progressive neuroendocrine tumors of pancreatic origin
64 [8]. Recently, Peng *et al.* demonstrated that everolimus, by inhibiting mTOR complex, can
65 overcome the resistance to gemcitabine-based treatment of PDAC cell lines inducing caspase-
66 dependent apoptosis and cell cycle arrest in the G2 phase [9].

67 The uncoupling proteins (UCPs) belong to the superfamily of mitochondrial carrier proteins
68 located in the mitochondrial inner membrane [10]. Among them, UCP2 has been found
69 overexpressed in several tumor types triggering both tumorigenesis and cancer progression [11]. An
70 extensive study on Oncomine data sets revealed that UCP2 gene is overexpressed in most of the
71 cancer types examined, i.e. ovarian, bladder, esophageal, testicular, kidney, colorectal, lung, breast,
72 leukemia, prostate, as well as pancreas cancers [12]. Concerning PDAC, some studies have shown
73 that also the protein level of UCP2 is significantly higher in cancer samples than in the adjacent
74 normal tissues, suggesting that UCP2 may sustain pancreatic tumor growth [13]. Along this line of
75 evidence, our research group previously demonstrated that UCP2 is involved in PDAC

76 chemoresistance to gemcitabine treatment and that this treatment further induces the expression of
77 UCP2 gene [14]. Furthermore, we recently demonstrated that UCP2 promotes the metabolic shift
78 from mitochondrial oxidative phosphorylation (mtOXPHOS) to the glycolytic pathway sustaining
79 the Warburg effect and PDAC cell proliferation [15]. All these properties are mainly ascribable to
80 the diminution of mitochondrial reactive oxygen species (ROS) driven by UCP2, whose antioxidant
81 property is due to the transport of protons from the intermembrane space to the mitochondrial
82 matrix bypassing ATP synthase. This results in a decrease of several consequential events,
83 including mitochondrial inner membrane potential, electron leakage from respiratory electron
84 transport chain and superoxide ion generation into the mitochondrial matrix [16]. Therefore, mild
85 uncoupling of mtOXPHOS may be considered the first line of defense against oxidative stress in
86 cancer cells [17]. Overall, UCP2 overexpression may be considered a strategy adopted by cancer
87 cells to protect themselves from excessive ROS production but can also be a potential target for
88 therapy for different cancers particularly resistant to standard chemotherapies, such as PDAC [18].
89 We previously demonstrated that UCP2 inhibition triggers PDAC cell death by ROS-dependent
90 nuclear translocation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase
91 (GAPDH) and stimulation of autophagy in PDAC cells [19]. In the present study, we show for the
92 first time that although UCP2 inhibition determines PDAC cell growth inhibition, it can also
93 stimulate the oncogenic Akt/mTOR pathway in a ROS-dependent manner, *in vitro* and *in vivo*. This
94 is likely due to an extreme attempt of cancer cells to survive in these stressful conditions and
95 provides the rationale for a combined therapeutic setting with the mTOR inhibitor everolimus in
96 PDAC.

97

98 **2. Material and Methods**

99 *2.1 Drugs and chemicals*

100 Genipin (methyl-2-hydroxy-9-hydroxymethyl-3-oxabicyclonona-4,8-diene-5-carboxylate),
101 everolimus (RAD001), *N*-acetyl-l-cysteine (NAC), and 3-methyladenine (3-MA) were obtained
102 from Sigma-Aldrich. Genipin and everolimus were solubilized in DMSO, NAC was solubilized in
103 bi-distilled sterile water, and 3-MA was freshly prepared and solubilized in cell culture medium.
104 The GAPDH inhibitor (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-dihydroisoxazol-5-yl-acetate
105 (AXP3009) has been designed and synthesized in the laboratory of Dr. Paola Conti at the
106 Department of Pharmaceutical Sciences (University of Milan, Italy) and solubilized in methanol.
107 The chemical structure and the synthesis of the AXP3009 compound have been previously
108 described in Bruno and colleagues [20]. All chemicals were stored at -80°C.

109

110 *2.2 Cell culture*

111 Pancreatic adenocarcinoma PaCa44, PaCa3, Panc1, MiaPaCa2, and T3M4 cell lines were
112 grown in RPMI 1640 medium (Gibco, Thermo Fisher), supplemented with 2 mM glutamine, 10%
113 FBS, and 50 µg/ml gentamicin sulfate (BioWhittaker, Lonza), and incubated at 37°C with 5% CO₂.

114

115 *2.3 Immunoblot analysis*

116 *Whole cell lysates.* Cells were harvested, washed in PBS, and re-suspended in lysis buffer in
117 the presence of phosphatase and protease inhibitors (50 mM Tris-HCl pH 8, 150 mM NaCl, 1%
118 Igepal CA-630, 0.5% Na-Doc, 0.1% SDS, 1 mM Na₃VO₄, 1 mM NaF, 2.5 mM EDTA, 1 mM
119 PMSF, and 1× protease inhibitor cocktail). After incubation on ice for 30 min, the lysates were
120 centrifuged at 5,000 × g for 10 min at 4 °C and the supernatant fractions were used for Western blot
121 analysis.

122 *Cytoplasmic and nuclear lysates.* Cells were harvested, washed in PBS, and re-suspended in
123 lysis buffer A (10 mM Hepes, 0.1% Igepal CA-630, 1.5 mM MgCl₂, 10 mM KCl) in the presence of

124 phosphatase and protease inhibitors (1 mM Na₃VO₄, 1 mM NaF, 0.5 mM DTT, 1 mM PMSF, and
125 1× protease inhibitor cocktail). After incubation on ice for 10 min, the lysates were centrifuged at
126 3,000 × g for 10 min at 4 °C and the supernatant fractions, containing cytoplasmic proteins, were
127 used for Western blot analysis, whereas the pellets, containing nuclei, were re-suspended in lysis
128 buffer C (20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA) in the
129 presence of phosphatase and protease inhibitors. After incubation on ice for 15 min, the lysates
130 were centrifuged at 15,000 × g for 10 min at 4 °C and the supernatant fractions were used for
131 Western blot analysis.

132 Protein concentration was measured by Bradford reagent (Pierce) using bovine serum
133 albumin as a standard. Protein extracts (50 µg/lane for whole cell lysate immunoblots and 6 µg/lane
134 for cytoplasmic and nuclear cell lysate immunoblots) were resolved on a 12% SDS-polyacrylamide
135 gel and electro-blotted onto PVDF membranes (Millipore). Membranes were blocked in 5% low-fat
136 milk in TBST (50 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature (RT)
137 and probed overnight at 4 °C with anti-phospho(Ser473)-Akt (1:2,000; Cell Signalling #4060), anti-
138 Akt (1:1,000; Cell Signalling #9272), anti- α -tubulin (1:2,500; Oncogene #CP06-100UG), anti-
139 phospho(Thr389)-P70S6K (1:1,000; Cell Signalling #9206), anti-P70S6K (1:1,000; Cell Signalling
140 #2708), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000; Cell Signaling,
141 #5174S), anti-Lamin B1 (1:1,000; Millipore #05-1531), anti- α -tubulin (1:2,500; Oncogene #CP06-
142 100UG), anti-Bcl2 (1:1,000; Cell Signalling #2872), anti-caspase 3 full length (1:1,000; Cell
143 Signalling #9662), anti-caspase 9 full length (1:1,000; Enzo Life Sciences #ALX-210-815-C100),
144 anti-Beclin1 (1:1,000; Cell Signalling Technology #4122), anti-Hsp90 (1:1,000; Cell Signalling
145 #4874) antibodies. Horseradish peroxidase conjugated anti-mouse (1:10,000; KPL #074-1806) or
146 anti-rabbit IgGs (1:2,000; Cell Signalling #7074) were used as secondary antibodies. The
147 immunocomplexes were visualized by chemiluminescence using the ChemidocMP imaging system
148 (Bio-Rad Laboratories) and the intensity of the chemiluminescence response was measured by

149 processing the image with NIH Image J software (<http://rsb.info.nih.gov/nih-image/>). Amido Black
150 staining was used to confirm equal cytoplasmic and nuclear protein loading in different lanes.

151

152 *2.4 Transient transfection assays*

153 Exponentially growing cells were seeded at 5×10^3 cells/well in 96-well plates for
154 monodansylcadaverine (MDC) assay and at 2.5×10^5 cells/plate in 60 mm cell culture plates for
155 protein extractions. UCP2 and Beclin-1 silencing experiments were carried out with specific small
156 interfering RNA (siRNA): siRNA UCP2 (5'-GCUAAAGUCCGGUUACAGATT-3'), siRNA
157 Beclin-1 (5'-ACAGUGAAUUUAAACGACAGCAGCU-3'), siRNA GAPDH (Ambion,
158 #4390850), and a non-targeting siRNA (5'-CAGUCGCGUUUGCGACUGG-3') used as negative
159 control were purchased by Ambion, Thermo Fisher. Cells were transfected with siRNA at a final
160 concentration of 50 nM Lipofectamine 3000 transfection reagent (Thermo Fisher), according to the
161 manufacturer's instructions. Transfection efficiency of cells was about 40% as previously assessed
162 by cytofluorimetric analysis using a pGFP plasmid vector [14].

163

164 *2.5 Analysis of reactive oxygen species (ROS) and mitochondrial superoxide ion production*

165 *ROS production.* The non-fluorescent diacetylated 2',7'-dichlorofluorescein (DCF-DA)
166 probe (Sigma-Aldrich), which becomes highly fluorescent upon oxidation, was used to evaluate
167 intracellular ROS production. Briefly, cells were plated in 96-well plates (5×10^3 cells/well) and, the
168 day after, were treated with the various compounds at the indicated concentrations for 24 h. At the
169 end of the various treatments, the cells were incubated in culture medium with 10 μ M DCF-DA for
170 15 min at 37 °C. The cells were washed with Hanks' buffer (20 mM HEPES, pH 7.2, 10 mM
171 glucose, 118 mM NaCl, 4.6 mM KCl, and 1 mM CaCl_2) and the DCF fluorescence was measured
172 by using a multimode plate reader (EX_{485 nm} and Em_{535 nm}) (GENios Pro, Tecan). The values were
173 normalized on cell proliferation by crystal violet assay.

174 *Mitochondrial superoxide ion production.* The nonfluorescent MitoSOX red probe
175 (Molecular Probes) was used to evaluate mitochondrial O₂•⁻ production. The probe is live-cell
176 permeative and is rapidly and selectively targeted to the mitochondria where it becomes fluorescent
177 after oxidation by O₂•⁻, but not by other ROS or reactive nitrogen species. Briefly, cells were plated
178 in 96-well plates (5x10³ cells/well) and, the day after, were treated with the various compounds at
179 the indicated concentrations for 24 h. At the end of the various treatments, cells were incubated in
180 culture medium with 0.5 μM MitoSOX at 37 °C for 15 min. The cells were then washed with
181 Hanks' buffer and fluorescence was measured by using a multimode plate reader (EX_{535 nm} and
182 Em_{590 nm}) (GENios Pro, Tecan). The values were normalized on cell proliferation by crystal violet
183 assay. Three independent experiments were performed for each assay condition.

184

185 *2.6 Cell proliferation assay*

186 Cells were seeded in 96-well plates (5x10³ cells/well) and the day after were incubated with
187 various compounds at the indicated conditions (see figure legends). At the end of the treatments,
188 cell growth was evaluated by Crystal Violet (Sigma-Aldrich) staining. The dye was solubilized in
189 1% SDS in PBS and measured photometrically (A_{595 nm}) to determine cell viability (GENios Pro,
190 Tecan). Three independent experiments were performed for each assay condition.

191

192 *2.7 Drug combination studies*

193 The compounds were added for 48 h at the following concentration ranges: 500 nM–200 μM
194 for genipin and 25 nM–10 μM for everolimus, maintaining the ratio genipin:everolimus 20:1. The
195 CI was calculated by the Chou-Talalay equation, which takes into account both the potency (IC₅₀)
196 and the shape of the dose-effect curve [21] by using the CalcuSyn software. The general equation
197 for the classic isobologram is given by $CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} + \frac{[(D)1 * (D)2]}{[(Dx)1 * (Dx)2]}$,
198 where (Dx)1 and (Dx)2 in the denominators are the doses (or concentrations) for D1 (drug 1) and
199 D2 (drug 2) alone that give x% growth inhibition, whereas (D)1 and (D)2 in the numerators are the

200 doses of drug 1 and drug 2 in combination that also inhibited x% cell viability (i.e., isoeffective).
201 $CI < 1$ indicates synergism, whereas $CI = 1$ and $CI > 1$ indicates additive effect and antagonism,
202 respectively. CI/effect curve represents the CI versus the fraction (0–1) of cells killed by drug
203 combination. The synergism percentage was obtained analyzing CI/effect curve and measuring the
204 CI values at each 0.05 fraction, that is, 5% growth inhibition, of the antiproliferative effect. Dose
205 reduction index (DRI) represents the measure of how much the dose of each drug in a synergistic
206 combination may be reduced at a given effect level compared with the doses of each drug alone.

207

208 *2.8 Immunofluorescence analysis of GAPDH nuclear translocation*

209 PaCa44 cells treated or untreated with genipin and everolimus were fixed in 4%
210 paraformaldehyde for 15 min and, after 4 changes (10 min each) of PBS, were permeabilized in
211 0.1% Triton X-100 for 5 min in PBS. To saturate unspecific binding sites, the cells were incubated
212 for 45 min at RT with a blocking solution containing 5% BSA and 0.05% Triton X-100 in PBS.
213 Samples were then incubated overnight at 4 °C with anti-GAPDH (1:100; Cell Signalling #5174S)
214 primary antibody diluted in blocking solution. After 3 washes with PBS (10 min each), cells were
215 incubated for 1 h at RT in the dark with specific secondary antibodies (1 µg/mL) conjugated with
216 Alexa Fluor-488 (Molecular Probes). The incubation with secondary antibody was followed by 10
217 min incubation at RT with Hoechst dye (10 µg/ml). Samples were mounted in anti-bleaching
218 medium (Dako Fluorescent Mounting Medium). The negative control procedure omitted the
219 primary antibody. Slices were examined by fluorescence microscope (DM6000B, Leica
220 Microsystem).

221

222 *2.9 Structural characterization of AXP3009-bound recombinant GAPDH*

223 His-tagged human GAPDH was recombinantly expressed in *E. coli*. Circular dichroism
224 spectra of GAPDH at 10 µM concentration were collected at 25°C with a JASCO J-715
225 spectropolarimeter in a buffered solution containing 10 mM sodium phosphate at pH 7.6.

226 Measurements were made either in the 250-345 nm range, using a path length of 1 cm, or in the
227 190-250 nm range, using a path length of 0.1 cm, in both cases with a 0.5 nm data pitch, a
228 50 nm/min scanning speed and with 3-5 accumulations per spectrum. GAPDH spectra were
229 collected in the absence of AXP3009 and upon overnight incubation with 30-100 μ M AXP3009, to
230 allow for the completion of the time-dependent binding [20]. The thermal stability of AXP3009-
231 bound and unbound GAPDH was monitored with the spectropolarimeter set at 222 nm using a
232 temperature ramp in the 30-80°C range, generated with a JASCO PTC-348 WI temperature
233 controller. Each spectral observation and each denaturation kinetics was confirmed by at least two
234 independent experiments. The elution times of AXP3009-bound and unbound GAPDH were
235 measured on a superdex 200 increase 3.2/300 column (GE Healthcare) mounted on a Prominence
236 HPLC system (Shimadzu) with the detector set at 280 nm. The column was pre-equilibrated and
237 then developed with a solution containing 10 mM sodium phosphate, 50 mM sodium chloride pH
238 7.6. The flow rate was 0.15 mL/min. The column was calibrated with commercially available gel-
239 filtration standards (Sigma-Aldrich), including carbonic anhydrase (29 kDa), conalbumin (75 kDa),
240 ferritin (440 kDa) and blue dextrane (2000 kDa).

241

242 *2.10 Apoptosis assay*

243 Cells were seeded in 96-well plates (5×10^3 cells/well) and, the day after, treated with the
244 various compounds at the indicated conditions (see figure legends). At the end of the treatment,
245 cells were fixed with 2% paraformaldehyde in PBS at RT for 30 min, then washed twice with PBS
246 and stained with annexinV/FITC (Bender MedSystem) in binding buffer (10 mM HEPES/NaOH
247 pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2) for 10 min at RT in the dark. Finally, cells were washed
248 with binding buffer solution and fluorescence was measured by using a multimode plate reader
249 ($\text{Ex}_{485 \text{ nm}}$ and $\text{Em}_{535 \text{ nm}}$) (GENios Pro, Tecan). The values were normalized on cell proliferation by
250 Crystal Violet assay. Three independent experiments were performed for each assay condition.

251

252 *2.11 RNA extraction and qPCR*

253 Total RNA was extracted from 10⁶ cells using TRIzol Reagent (Thermo Fisher) and 1 µg of
254 RNA was reverse transcribed using first-strand cDNA synthesis. Real-time quantification was
255 performed in triplicate samples by SYBR Green detection chemistry with Power SYBR Green PCR
256 Master Mix (Applied Biosystems) on a 7900 HT Fast Real-Time PCR System (Thermo Fisher).
257 Normalization was performed analysing the ribosomal protein large P0 (RPLP0) mRNA expression
258 level. The primers used were: CHOP For, 5'-GCAGCCCATGAAGGAGAAAG-3'; CHOP Rev, 5'-
259 CGGTCGATTTTCCTGCTTGAG-3'; UCP2 For, 5'-CTCCTGAAAGCCAACCTCAT-3'; UCP2
260 Rev, 5'-CCCAAAGGCAGAAGTGAAGT-3'; GAPDH For, 5'-ATCAGCAATGCCTCCTGCAC-
261 3'; GAPDH Rev, 5'-TGGTCATGAGTCCTTCCACG-3'; Beclin1 For, 5'-
262 GTAGACCGGACTTGGGTGAC-3'; Beclin1 Rev, 5'-CTGCATGGTGCTGTTGTTG-3'; RPLP0
263 For, 5'-ACATGTTGCTGGCCAATAAGGT-3' and RPLP0 Rev, 5'-
264 CCTAAAGCCTGGAAAAGGAGG-3'. The thermal cycle reaction was performed as follows: 95
265 °C for 10 minutes followed by 40 cycles at 95 °C for 15 seconds and 60°C for 1 minute. The
266 average of cycle threshold of each triplicate was analyzed according to the 2^(-ΔΔCt) method. Three
267 independent experiments were performed for each assay condition.

268

269 *2.12 Autophagosome formation assay*

270 To quantify the induction of autophagy, cells were treated with the various compounds (see
271 figure legends) and then were incubated with the fluorescent probe monodansylcadaverine (MDC;
272 Sigma-Aldrich). MDC is a selective marker for acidic vesicular organelles, such as autophagic
273 vacuoles and especially autolysosomes.

274 *Flow cytometry:* briefly, following the treatments, cells were incubated with 50 µM MDC in
275 PBS at 37 °C for 15 min. After incubation, cells were washed with PBS, trypsinized, and
276 immediately analyzed by flow cytometry. All fluorescences were analyzed with a FACScalibur
277 flow cytometer (Becton Dickinson). The fluorescent emissions were collected through a 530 nm

278 band pass filter (FL-1 channel). At least 10,000 events were acquired in log mode. For the
279 quantitative evaluation of MDC, CellQuest software (Becton Dickinson) was used to calculate
280 mean fluorescence intensities (MFIs) by the formula $MFI\text{-treated}/MFI\text{-control}$, where MFI-treated
281 is the fluorescence intensity of cells treated with the various compounds and MFI-control is the
282 fluorescence intensity of cells untreated. Figures show the means of fluorescence intensity \pm SD
283 from three independent experiments.

284 *Multimode plate reader:* briefly, cells were seeded in 96-well plates (5×10^3 cells/well) and
285 treated with the various compounds at the indicated conditions (see figure legends). At the end of
286 the treatments, cells were incubated in culture medium with 50 μ M MDC at 37 °C for 15 min. After
287 incubation, cells were washed with Hanks' buffer and fluorescence was measured by using a
288 multimode plate reader (Ex_{340 nm} and Em_{535 nm}) (GENios Pro, Tecan). The values were normalized
289 on cell proliferation by Crystal Violet assay. Three independent experiments were performed for
290 each assay condition.

291

292 *2.13 Detection of acidic vesicular organelles with acridine orange*

293 In acridine orange (AO) stained cells, the cytoplasm and nucleus are bright green and dim
294 red, whereas acidic compartments are bright red. The intensity of the red fluorescence is
295 proportional to the degree of acidity. Following the treatments (see figure legend), cells were
296 incubated with AO solution (1 μ g/ml) at 37 °C for 15 min, then were washed with PBS, and
297 observed with a fluorescence microscope (Nikon Eclipse TE300 Inverted microscope - Nikon
298 Instruments).

299

300 *2.14 Immunoprecipitation assay*

301 Following the treatments (see figure legend), cells were washed twice with ice-cold
302 PBS/BSA, and lysed for 30 min at 4 °C in lysis buffer (50 mM Tris, pH 7.4, 150 mM sodium
303 chloride, 1% Nonidet P-40, 1 mM EDTA, 1 mM sodium orthovanate, 1 mM sodium fluoride, 1

304 $\mu\text{g/ml}$ leupeptin, $1 \mu\text{g/ml}$ aprotinin, $1 \mu\text{g/ml}$ pepstatin A, 1 mM PMSF). Lysates were centrifuged at
305 $10,000 \times g$ for 10 min and supernatants were collected. Protein concentration was determined by
306 Lowry method using bovine serum albumin for the standard curve. Protein lysates ($120 \mu\text{g}$) were
307 incubated with anti-Beclin1 (Cell Signalling Technology #4122) and anti-Hsp90 (Cell Signalling
308 #4874) primary antibodies overnight at 4°C . Immune complexes were collected with $50 \mu\text{l}$ of
309 protein A-agarose and incubated for 16 h at 4°C . The protein A-agarose/ immune complex was
310 washed twice with cold PBS, resuspended in $20 \mu\text{l}$ of SDS-loading buffer, heated to 95°C for 5 min
311 and used for protein gel blotting analysis. Proteins were separated by 12.5% polyacrylamide gel
312 SDS-PAGE (Mini-PROTEAN[®] TGX Stain-Free[™] Precast Gels, BIORAD). After electrophoretic
313 separation the proteins were transferred on nitrocellulose filter (Trans-Blot Turbo Mini, BIORAD)
314 by Western Blotting, using Trans-Blot Turbo[®]Transfer System (BIORAD) and the filter was
315 reacted using either anti-Beclin1 or anti-Hsp90 antibodies. After incubation with secondary
316 antibodies, the signal was detected using enhanced chemiluminescence detection reagents Clarity[™]
317 Western ECL Blotting Substrate (BIORAD). The bands derived from western blotting were
318 analyzed and quantified by Image Lab 5.2.1 software obtained by ChemiDoc[™]MXRS⁺ (BIORAD)
319 and the expression of total protein was used for normalization. The error bars shown in the
320 histograms represent the standard deviation from the mean of different densitometric scanning in at
321 least 3 different experiments.

322

323 *2.15 In vivo studies*

324 All procedures involving mice were performed in compliance with our institutional animal
325 care guidelines and following national and international directives (D.L. 4 March 2014, no. 26;
326 directive 2010/63/EU of the European parliament and of the council). PaCa44 cells (1×10^6
327 cells/mice) were s.c. injected into the dorsal flank of six nude female mice for each condition (5
328 weeks of age; Charles River Laboratories, Inc.). For the control groups, mice received $200 \mu\text{l}$
329 injections of 1X PBS. One week after cell inoculation, experimental group received 30 mg/kg

330 genipin based on previous published data [22], or/and 4.5 mg/m² everolimus based on its clinical
331 use following US Food and Drug Administration directives, and vehicle solution (PBS) for control
332 group, by intraperitoneal injection biweekly for 4 weeks. Body mass was recorded weekly for each
333 animal. Tumor size was monitored weekly in two perpendicular dimensions parallel to the surface
334 of the mouse using a caliper. Tumor volume was calculated using the formula of $V = \pi/6 \times [(w \times$
335 $L)^{3/2}]$. Animals were sacrificed at the volume of 2 cm³. After euthanizing the mice, the
336 neoplasms along with the visceral organs (heart, kidney, liver, lung, and pancreas) were retrieved
337 for each mouse. All the samples were formalin fixed [in 10% (v/v) neutral-buffered formalin for 24-
338 48 h] and paraffin embedded for further evaluations. Serial histological sections (4-6 μm thick)
339 were obtained from each paraffin block and stained for histology assessment.

340 *Hematoxylin and eosin (H&E) stain:* One 4μm H&E slide was obtained for each sample
341 (neoplasm and normal tissue) for each mouse to perform histopathological evaluation, including
342 mitotic rate, grade of tumour necrosis and presence of lympho-granulocytic infiltrate in parenchyma
343 of visceral organs and skin as sign of potential drug-related cytotoxicity. Mitotic count was assessed
344 as absolute number of mitosis on 10 consecutive HPFs (high power field, 400X magnification) for
345 each neoplastic sample.

346 *Immunohistochemistry:* 4 μm slides were obtained for each sample for
347 immunohistochemical evaluation for anti-GAPDH (clone D16H11; 1:900; Cell Signalling) and
348 phospho-Akt (clone EP2109Y; 1:100; Abcam). Human breast carcinoma was considered as positive
349 control for GAPDH, while prostatic carcinoma for p-Akt. All the samples were analysed by a
350 pathologist, blind for the treatment and control groups distribution. GAPDH-positivity was defined
351 by the identification of a more intense nuclear stain compared to cytoplasmic and/or background
352 stain. A count of viable GAPDH-positive neoplastic cells was assessed on 20 consecutive HPFs,
353 away from necrotic area, in order to avoid potential artefacts due to necrosis. The apoptotic cells
354 were excluded from the count because it was not always possible to discriminate between nuclear
355 and cytoplasmic positivity due to the alterations occurring in the late apoptotic phases (nuclear

356 shrinkage and blebbing). Qualitative and quantitative p-Akt cytoplasmic and membranous
357 expression was evaluated on neoplastic viable cells on 20 consecutive HPFs, away from necrotic
358 area, in all the samples analysed. The quantitative analysis was performed through an evaluation of
359 the percentage of p-Akt positive cells on each slide.

360

361 *2.16 Statistical analysis*

362 ANOVA (post hoc Bonferroni) analysis was performed by GraphPad Prism 5 (GraphPad
363 Software, Inc., La Jolla, CA, USA). P-values < 0.05, 0.01, or 0.001 were indicated as *, **, or ***,
364 respectively.

365 **3. Results**

366 *3.1 UCP2 inhibition stimulates Akt/mTOR signaling pathway in a ROS-dependent manner*

367 Previously, we demonstrated that ROS production by UCP2 inhibition triggers PDAC cell
368 death [19]. Here, we show that this production concurrently induces the oncogenic Akt/mTOR
369 pathway. **Figure 1A** shows that the activated form of Akt (phospho-Ser473Akt) is significantly
370 increased after treatment with the UCP2 inhibitor genipin both in tumor masses derived from
371 PDAC xenografts and in PDAC PaCa44 cells. Following the knockdown of UCP2 expression, the
372 observed increase of the Thr389-phosphorylated level of P70S6K, a direct target of mTOR kinase
373 complex, indicates that the Akt/mTOR pathway is functionally stimulated by UCP2 inhibition and
374 also provides a valuable control of UCP2 inhibition (**Fig. 1B**). Accordingly, **Supplementary**
375 **Figure 1** demonstrates the effective knockdown of UCP2 gene expression by siRNA-UCP2 as
376 compared with a scramble siRNA used as negative control. In addition, the increase of Thr389-
377 phosphorylation of P70S6K is revealed in three PDAC cell lines treated with genipin, thus
378 indicating that Akt/mTOR induction is a common event in PDAC cells after UCP2 blockage
379 (**Suppl. Fig. 2**). **Figure 1C** shows that genipin strongly induces ROS production and the radical
380 scavenger *N*-acetyl-L-cysteine (NAC) counteracts this event. To investigate whether Akt/mTOR
381 pathway regulation is functionally related to the antioxidant effect of UCP2, we analyzed Akt and

382 P70S6K phosphorylation levels in genipin-treated PDAC cells after NAC pre-treatment. **Figure 1D**
383 shows that Akt and P70S6K phosphorylation induced by genipin is at least partially rescued by
384 NAC demonstrating the involvement of ROS production in this signaling regulation driven by
385 UCP2 blockage.

386

387 *3.2 Genipin and everolimus synergistically inhibit PDAC cell proliferation through GAPDH* 388 *nuclear translocation*

389 Since stimulation of Akt/mTOR pathway has an established role in cancer proliferation and
390 everolimus, an oral anticancer mTOR inhibitor used in clinics, has shown particularly promising
391 results in cancer patients by inhibiting tumour growth and displaying anti-angiogenic effects also in
392 combined therapies [23], we investigated whether genipin and everolimus combination might
393 determine an enhanced antiproliferative effect in PDAC cells. First, we demonstrated that the
394 addition of everolimus counteracts both Akt and P70S6K phosphorylation induced by genipin (**Fig.**
395 **2A**), establishing the rational basis for the combined therapeutic strategy. In addition, drug
396 combination strongly enhances ROS production as compared with single drugs (**Fig. 2B**) and the
397 ROS-mediated antiproliferative effect in PDAC cells (**Fig. 2C**). By a therapeutic point of view, the
398 addition of everolimus in the combined setting can assure the blockage of the Akt/mTOR pathway,
399 avoiding a further ROS-mediated Akt/mTOR signaling stimulation even in the presence of
400 enhanced ROS production.

401 In order to evaluate whether the antiproliferative effect of genipin+everolimus treatment was
402 synergistic, we analyzed cell growth inhibition curves by using the dedicated software CalcuSyn
403 (see Material and Methods). Dose-dependent analyses performed with increasing concentrations of
404 genipin and/or everolimus showed that the combined setting induces a strong antiproliferative
405 synergism in all five PDAC cell lines tested, as revealed by the curve of the combination index (CI)
406 values \pm sd *versus* the fraction (0 \rightarrow 1) of cells killed by drug combination (**Fig. 3A**). CI values
407 lying below 1 are widely distributed in the x axis, indicating a synergistic effect occurring at both

408 low and high drug concentrations in all PDAC cell lines tested (**Fig. 3B**). In particular, CI_{50} values
409 of all PDAC cell lines are markedly below 1 indicating a strong synergism, and the reduction folds
410 (dose reduction index; DRI) of drug concentration to obtain 50% cell growth inhibition in
411 combination setting as compared to each drug administered alone (DRI_{50}) are reported in **Table 1**.
412 Moreover, isobolograms at the IC_{50} of each drug for each PDAC cell line are reported in
413 **Supplementary Figure 3**, further supporting the synergistic effect observed in these conditions.
414 Taken together, these data demonstrate that the addition of everolimus to genipin treatment
415 produces a synergistic PDAC cell growth inhibition which is likely due to both enhanced ROS
416 production in combined setting and to the counteraction of genipin-induced Akt/mTOR oncogenic
417 signaling by everolimus. DRI_{50} values obtained *in vitro* suggest that this synergism might allow
418 decreasing the concentrations of the two compounds reducing eventual side effects.

419 Since we previously demonstrated that UCP2 inhibition determines a ROS-dependent
420 nuclear translocation of GAPDH [19] and since GAPDH nuclear translocation is counteracted by
421 Akt/mTOR pathway through direct phosphorylation of the enzyme [24], we explored if nuclear
422 GAPDH positivity is further enhanced by the drug combination treatment. Importantly, we proved
423 that tumor masses derived from mice treated with the combined therapy show a considerable
424 increase in the number of GAPDH-positive nuclei (**Fig. 4A**). Also *in vitro*, we observed that the
425 combined treatment strongly increases the amount of GAPDH positive nuclei, as compared to
426 genipin-treated PDAC cells (**Fig. 4B**). Representative images of nuclear GAPDH positivity after
427 PDAC cell treatment with the drug combination are reported in **Figure 4C**. The total amount of
428 GAPDH mRNA remains unchanged after treatments (**Fig. 4D**), indicating that genipin and
429 everolimus regulate the subcellular distribution of the enzyme rather than its expression level. To
430 investigate the functional role of GAPDH nuclear translocation in cell growth inhibition by drug
431 combination, we used the 3-bromo-isoxazoline derivative (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-
432 dihydroisoxazol-5-yl-acetate (AXP3009) (see Material and Methods), previously reported to
433 alkylate the catalytic cysteine residue of tetrameric *Plasmodium falciparum* GAPDH (*PfGAPDH*)

434 without affecting other solvent-exposed cysteine residues [20]. In consideration of the negative
435 cooperativity of NAD⁺ binding [25] and structural observations [26], it was concluded that the
436 alkylation of one subunit of the GAPDH tetramer produces a conformational rearrangement that
437 severely limits the accessibility of the remaining active sites [20]. Since 3-bromo-isoxazoline
438 derivatives were also shown to bind to the redox sensitive catalytic cysteine of *human* GAPDH
439 (*hGAPDH*) [27], we hypothesized that AXP3009 can alter *hGAPDH* conformation, prevent
440 intracellular GAPDH oxidation by ROS produced after cell treatment with genipin and everolimus,
441 and counteract GAPDH nuclear translocation. To test this hypothesis, we first investigated
442 AXP3009-induced conformational rearrangements of recombinant *hGAPDH*. Circular dichroism
443 spectra of *hGAPDH* pre-incubated with AXP3009 show significant differences in the 250-345 nm
444 range, suggesting significant tertiary alterations in comparison to the control sample (**Figure 5A**).
445 The melting temperature, as determined by the fitting of the thermic denaturation kinetics, is 2.2°C
446 lower when *hGAPDH* is pre-incubated with AXP3009 (61.4±0.1 *versus* 63.6±0.1), suggesting a
447 difference in stability of the two forms (**Figure 5B**). Finally, gel filtration experiments of
448 AXP3009-bound and unbound *hGAPDH* show a small but significant difference in the apparent
449 molecular weight (**Figure 5C**). Both forms fall within the range of the tetramer (expected weight
450 148000 Da), but with a 12% difference that might reflect a difference in shape or a different
451 stability of the quaternary structure. Furthermore, we tested the specificity of AXP3009 on GAPDH
452 interaction by knocking-down intracellular GAPDH expression with siRNA-GAPDH (**Suppl. Fig.**
453 **4A**), revealing that after target deprivation the compound loses its cytotoxic effect on PDAC cells
454 (**Suppl. Fig. 4B**). Importantly, **Figure 5D** shows that AXP3009 stabilizes the cytosolic localization
455 of the GAPDH enzyme by diminishing its nuclear transport, thus providing a valuable tool to
456 investigate the role of nuclear GAPDH in cell death induced by genipin and everolimus treatment.
457 According to the cell death-related role assumed by nuclear GAPDH translocation [28], we
458 observed that AXP3009 is able to completely restore PDAC cell proliferation affected by genipin
459 alone or by genipin and everolimus combined treatment (**Fig. 5E**).

460

461 *3.3 Genipin and everolimus strongly induce apoptosis in PDAC cells*

462 We further investigated whether apoptosis is involved in PDAC cell growth inhibition by
463 drug combination. As shown in **Figure 6A**, annexin V assay revealed that PDAC cells treated with
464 the drug combination expose a strongly higher amount of phosphatidylserine as compared to single
465 treatments or untreated control. Notably, apoptotic cell death induced by the combined treatment is
466 markedly reduced by the addition of AXP3009 or of the antioxidant compound NAC (**Fig. 6B**),
467 further supporting the antiproliferative role of GAPDH nuclear translocation mediated by
468 intracellular oxidant conditions [19, 29]. Moreover, we observed a strong increase of CHOP
469 expression by genipin+everolimus within the first eight hours after cell treatment (**Fig. 6C**), while
470 the expression of the Bcl 2 protein dramatically decreases in the combined setting (**Fig. 6D**),
471 according to the common assumption that the main mechanism of CHOP-induced apoptosis is the
472 suppression of the anti-apoptotic protein Bcl-2 [30]. In addition, the expression of the full length
473 caspase 3 and 9 is significantly reduced by the combined treatment as compared to single
474 treatments, suggesting the involvement of downstream effector caspases in the apoptotic cell death
475 induced by the concomitant inhibition of UCP2 and mTOR complex (Fig. 6D). The regulation of
476 Bcl 2 and of caspase 9, which in turn cleaves the downstream pro-caspase 3 in its truncated active
477 form, suggests that the mitochondrial death pathway is implicated in genipin/everolimus induced
478 apoptosis.

479

480 *3.4 Anti-apoptotic effect of Beclin1-dependent autophagy induced by genipin and everolimus*

481 Since GAPDH nuclear translocation can induce autophagy-related genes [31], we
482 investigated whether the combined treatment stimulates the formation of autophagic vesicles in
483 PDAC cells by using staining with the fluorescent probe monodansylcadaverine (MDC). FACS
484 analysis revealed that PDAC cells treated with the drug combination incorporate a higher amount of
485 MDC probe as compared with single treatments, suggesting an autophagy stimulation (**Fig. 7A**).

486 These data have been confirmed analyzing MDC incorporation using a microplate fluorescence
487 reader (data not shown). To demonstrate that autophagy stimulation by drug combination results in
488 the fusion of autophagy vesicles with lysosomes, we stained cells with the fluorescent probe
489 acridine orange (AO), which changes its fluorescence emission from green to red upon
490 accumulation into lysosomal acidic compartments revealing the typical pH acidification of
491 lysosomes during autophagic stimulation. Fluorescence microscopy experiments confirmed the
492 presence of wide red areas (acidic lysosomes containing accumulation of AO probe) into the
493 cytoplasm of PDAC cells treated with drug combination (**Fig. 7B**), supporting the statement that the
494 concomitant inhibition of UCP2 and mTOR can contribute to the formation of autophagic vesicles
495 and their fusion with lysosomes in cancer cells.

496 The investigation of the mechanism involved in the stimulation of autophagy revealed that
497 Beclin 1 (Atg 6), a well-known key regulator of autophagy, is strongly induced by the combined
498 drug setting (**Fig. 7C**). In addition, since heat shock protein 90 (Hsp 90) is a molecular chaperone
499 that regulates the stability of signaling proteins, including the maintenance of Beclin 1 protein
500 stability through an evolutionarily conserved domain [32], we investigated whether genipin and
501 everolimus can affect Beclin1:Hsp90 complex. Intriguingly, we observed by immunoprecipitation
502 assay that everolimus promotes the interaction between Beclin 1 and Hsp 90 and that the formation
503 of this complex is further enhanced by drug combination (**Fig. 7D**), strongly suggesting a role for
504 Hsp 90 in Beclin 1 induction by the combined treatment. To test the functional role of Beclin 1
505 enhancement in autophagy stimulation, we knocked-down its expression by using a siRNA-Beclin1
506 (see control experiment in **Suppl. Fig. 5**). **Figure 7E** indicates that autophagosome vesicle
507 formation by drug combination is completely abolished by siRNA-Beclin1, highlighting the key
508 role of this autophagy mediator protein in the overall process. Furthermore, since the role of
509 autophagy in modulating cell growth is highly dependent on the metabolic context and on the
510 microenvironmental conditions in which the cells lie [33], we investigated the impact of this
511 process on the overall antiproliferative effect induced by drug combination. **Figure 7F** shows that

512 the percentage of PDAC cell growth is further decreased when a non-toxic concentration of 3-
513 methyladenine (3-MA), a known inhibitor of the autophagosome formation process, is added to
514 increasing concentrations of genipin+everolimus combination. Notably, the apoptotic effect of the
515 drugs is strongly potentiated by 3-MA (**Fig. 7G**), demonstrating that Beclin1-dependent autophagy
516 retains a protective and pro-survival role counteracting apoptotic cell death induced by the drugs.

517

518 *3.5 Genipin and everolimus strongly inhibit growth of PDAC cells in mice xenografts*

519 The effect of the intraperitoneal delivery of genipin and/or everolimus on growth inhibition
520 of PaCa44 cells subcutaneously xenografted in nude mice was also investigated. **Figure 8A** shows
521 that the volume of tumors in mice treated with the drug combination remains essentially unchanged
522 during the observation time, whereas it strongly increases in the control group and, at a lower
523 extent, in either genipin- or everolimus-treated groups. Tumor masses after mice sacrifice were
524 recorded and shown in **Figure 8B** for control and combined treatment, further pointing out the high
525 efficiency in the reduction of PDAC masses *in vivo*. To confirm this antiproliferative effect by
526 genipin+everolimus treatment and the synergistic outcome observed in PDAC cell cultures, we
527 demonstrated that the number of mitoses dramatically decreases only in tumor masses derived from
528 mice injected with the drug combination (**Fig. 8C**). To investigate whether treatments might
529 determine toxic effects, we analysed mice body weights during all the experiment period and, at the
530 sacrifice of mice, we collected various organs (heart, kidney, liver, lung and pancreas) for
531 haematoxylin and eosin (H&E) staining. **Figure 8D** shows that all the treatments do not change
532 mice body masses during the experiment, suggesting the absence of any apparent toxicity. In
533 addition, H&E histopathologic evaluation of the organs examined revealed the absence of any
534 significant side effects for the animals (**Suppl. Fig. 6**).

535

536

537 **4. Discussion**

538 In this study, we demonstrate *in vitro* and in mice xenograft that UCP2 inhibition strongly
539 triggers the oncogenic pathway Akt/mTOR, providing the rationale for the antiproliferative
540 synergistic effect mediated by the UCP2 inhibitor genipin when used in combination with the
541 mTOR inhibitor everolimus. Beyond the Akt/mTOR/P70S6K signaling inhibition, we demonstrate
542 that everolimus strongly potentiates ROS production and PDAC cell death induced by genipin. This
543 anti-proliferative effect by drug combination setting is due to a massive GAPDH nuclear
544 translocation observed both *in vitro* and in mice xenograft. This determines caspase-mediated
545 apoptotic cancer cell death as well as a protective autophagic process mediated by Beclin1 in PDAC
546 cells (**Figure 9**). This latter event can be likely considered as an extreme tentative of cancer cells to
547 survive in drug-induced stressing conditions. Thus, the full anticancer effect by genipin and
548 everolimus combination can be achieved when the autophagic process is blocked.

549 Beyond glycolysis, several reports have demonstrated that GAPDH has a variety of other
550 functions, including DNA repair [34], transcriptional regulation [35], membrane fusion and
551 transport [36, 37], autophagy [31], and cell death [38, 39]. Indeed, in accord with its multiple
552 activities, the intracellular localization of GAPDH is not restricted to the cytosol for energy
553 production but is detected in the plasma and nuclear membrane, in the endoplasmic reticulum, in
554 the Golgi apparatus, and in the nucleus [28]. GAPDH nuclear translocation is a particularly critical
555 event in the light that, although the enzyme contains a nuclear export signal [40], it does not contain
556 an equivalent nuclear localization signal (NLS). Thus, it is likely that other mechanisms need to be
557 utilized for GAPDH translocation probably by binding with other molecules after post-translational
558 modifications of the enzyme. For instance, upon exposure to oxidative stress, GAPDH nuclear
559 translocation involves S-nitrosyl post-translational modification at its active site Cys152 [29].
560 Oxidized GAPDH binds to Siah1, an ubiquitin ligase, which contains its own NLS sequence
561 thereby facilitating the intracellular transfer of this protein:protein complex and the subsequent
562 transcriptional induction of apoptotic and cell death-related genes [29, 41]. Many other post-

563 translational modifications of GAPDH have been described, including phosphorylation, acetylation,
564 glycosylation, poly ADP-ribosylation, and pyruvylation [42]. For these reasons GAPDH has
565 received considerable attention as an appealing drug target for anticancer therapy [43].

566 Here, we show that the strong increase in GAPDH nuclear translocation and subsequent
567 PDAC cell death by genipin and everolimus combination is due to both: *i*) oxidation of the enzyme
568 by ROS increase, and *ii*) the everolimus-mediated counteraction of the Akt/mTOR pathway
569 stimulated by genipin. Indeed, this oncogenic signaling is described to stabilize GAPDH in the
570 cytosol through phosphorylation in Thr-237 of the enzyme decreasing its nuclear translocation and
571 GAPDH-mediated apoptosis [24, 44]. The functional involvement of GAPDH nuclear translocation
572 has been confirmed by the capability of AXP3009 to counteract PDAC cell death and apoptosis
573 induced by drug combination. AXP3009 is a novel synthetic compound addressed to interact with
574 the *Pf*GAPDH catalytic cysteine [20]. Here, we demonstrate that AXP3009 can prevent the nuclear
575 translocation of GAPDH in PDAC cells, likely through conformational changes observed in
576 recombinant *h*GAPDH that can elude the oxidation of the redox-sensitive catalytic cysteine.
577 Notably, we observe a strong increase in the percentage of GAPDH nuclear positivity in tumor
578 masses in nude mice injected with the drug combination. Concomitantly, tumor volume in mice
579 treated with the two drugs remains essentially unchanged during the observation time without any
580 signs of toxic effects for animals.

581 The synergistic PDAC cell growth inhibition obtained with everolimus and genipin
582 treatment can allow to markedly reduce the concentrations of the two drug when used in
583 combination. Optimal dosing is a crucial factor in order to achieve a maximum therapeutic efficacy
584 with appropriate drug exposure and to maintain patient quality of life. CalcuSyn software analysis
585 shows that the concentration of everolimus and that of genipin can be strongly reduced to obtain
586 50% PDAC cell growth inhibition in combined setting as compared to the concentrations needed to
587 obtain the same effect in single treatment (Table 1). Notably, mTOR inhibitors-induced side effects,
588 which can potentially compromise their therapeutic effectiveness, are well characterized and

589 include epithelial-cutaneous adverse events, pulmonary dysfunctions, metabolic alterations, and
590 fatigue [45]. Although everolimus is a currently approved drug for the treatment of pancreatic
591 cancer, genipin has not been developed for clinical use yet but it represents a valuable tool for the
592 preclinical investigation of UCP2 roles and for the study of the potential clinical usage of next
593 generation UCP2 inhibitors for therapy. In conclusion, our results and the statement that UCP2
594 overexpression promotes chemoresistance in cancers support the combined inhibition of UCP2 and
595 of Akt/mTOR pathway as a novel therapeutic strategy in the treatment of PDAC.

596

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602

603 **Conflict of interest**

604 The authors declare that they have no conflicts of interest.

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- 733
- 734

735 **Figure Legends**736 **Figure 1. Akt/mTOR pathway regulation by UCP2 in pancreatic ductal adenocarcinoma cells.**

737 (A) Qualitative and quantitative analysis of p-Akt expression on PaCa44 derived xenograft tumors
738 (*in vivo*) and on PaCa44 cell line (*in vitro*) untreated/treated with genipin. P-Akt expression
739 representative images are reported for tumors grown in control mice and in mice treated with
740 genipin (for genipin subadministration see Materials and Methods) at 20-fold magnification, scale bar
741 50 μm , and for *in vitro* a representative immunoblot image is reported for p-Akt expression in
742 PaCa44 cells untreated/treated with 200 μM Genpin for 24h. (B) Western blot analysis of the total
743 and phosphorylated form of the mTOR target P70S6K in PaCa44 cells transfected with 200 nM
744 siRNA control or siRNA UCP2 for 72 h. (C) Analysis of DCF fluorescence intensity,
745 corresponding to the level of ROS production, by a multimode plate reader in PaCa44 cells treated
746 with 200 μM genipin and/or 10 mM NAC for 24 h. (D) Western blot analysis of the total and
747 phosphorylated form of Akt or of the mTOR target P70S6K in PaCa44 cells treated with 200 μM
748 genipin and/or 10 mM NAC for 24 h. The value of α -tubulin was used as a normalizing factor and
749 protein fold change is relative to each control. Values are the means ($\pm\text{SD}$) of three independent
750 experiments. Statistical analysis: (*) $P < 0.05$ and (***) $P < 0.001$ for treated cells vs each control or
751 genipin vs genipin+NAC. Legend: (ctrl) control and (G) genipin.

752

753 **Figure 2. The combined treatment genipin/everolimus impairs Akt/mTOR signaling and**

754 **increases ROS production and anti-proliferative effects.** (A) Western blot analysis of the total
755 and phosphorylated form of Akt and P70S6K in PaCa44 cells treated with 200 μM genipin and/or
756 10 μM everolimus for 24 h. (B) Analysis of MitoSox fluorescence intensity, corresponding to the
757 level of mitochondrial superoxide production, by a multimode plate reader in PaCa44 cells treated
758 with 140 μM genipin and/or 7 μM everolimus for 24 h. (C) Cell growth analysis through the
759 Crystal Violet colorimetric assay of PaCa44 cells treated with 100 μM genipin and/or 5 μM
760 everolimus and/or 10 mM NAC for 48h. Values are the means of three independent experiments
761 each performed in triplicate. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ for
762 treated cells vs control or genipin+everolimus vs single treatments. Legend: (ctrl) control, (G)
763 genipin, and (E) everolimus.

764

765 **Figure 3. The combined treatment genipin/everolimus has a synergistic anti-proliferative**
766 **effect on five PDAC cell lines.** (A) Cell growth analysis through the Crystal Violet colorimetric

767 assay of five PDAC cell lines (PaCa44, Panc1, MiaPaCa2, PaCa3, and T3M4) treated with
 768 increasing doses of the drugs [genipin from 500 nM to 200 μ M; everolimus from 25 nM to 10 μ M]
 769 maintaining the ratio genipin:everolimus 20:1 for 48 h. Black squares, black rhombus and white
 770 circles correspond to genipin, everolimus and the combined treatment, respectively. Values are the
 771 means of three independent experiments each performed in triplicate. **(B)** Analysis of the
 772 synergistic cell growth inhibition of the five PDAC cell lines (PaCa44, Panc1, MiaPaCa2, PaCa3,
 773 and T3M4) treated with increasing doses of the drugs [genipin from 500 nM to 200 μ M; everolimus
 774 from 25 nM to 10 μ M] maintaining the ratio genipin:everolimus 20:1 for 48 h. The values on the x
 775 axis correspond to the fraction of growth inhibition (0 \rightarrow 1) given by increasing concentrations of
 776 drug combinations. The values on the y axis correspond to the measurement of CI (see Materials
 777 and Methods).

778

779 **Figure 4. mTOR inhibition enhances GAPDH nuclear translocation in genipin-treated cells.**

780 **(A)** Count of GAPDH positive nuclei in vital neoplastic cells on 20 consecutive HPFs of PaCa44
 781 derived xenograft tumors. Mice have been untreated/treated with genipin and/or everolimus, as
 782 reported in Materials and Methods. Representative images are reported for control mice and for
 783 mice treated with genipin+everolimus at 40-fold magnification, scale bar 20 μ m. **(B)** PaCa44 cells
 784 untreated or treated with 200 μ M genipin and/or 10 μ M everolimus for 24 h. GAPDH positive
 785 nuclei have been counted on several different fields for each condition and the amount of positive
 786 nuclei has been normalized on cell viability. **(C)** Representative immunofluorescence images of
 787 GAPDH (green) translocation into the nuclei (labelled with Hoechst dye) of PaCa44 cells untreated
 788 or treated with 200 μ M genipin and 10 μ M everolimus for 24 h, at 40-fold magnification, scale bar
 789 10 μ m. **(D)** Quantitative analysis of GAPDH mRNA through real-time PCR (qPCR) of PaCa44 cells
 790 treated with 200 μ M genipin and/or 10 μ M everolimus for 24 h. Total RNA was extracted from
 791 cells and qPCR was performed as described in Materials and Methods. Statistical analysis: (*)
 792 $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ for treated cells vs control or genipin+everolimus vs single
 793 treatments. Legend: (ctrl) control, (G) genipin, and (E) everolimus.

794

795 **Figure 5. AXP3009 alters GAPDH conformation and counteracts cell growth inhibition by**

796 **genipin/everolimus treatment.** **(A)** Comparison of the circular dichroism spectra of AXP3009-
 797 bound (dashed line) and unbound *h*GAPDH (solid line) in the 190-250 nm range. Circular
 798 dichroism spectra of GAPDH at 10 μ M in a buffered solution containing 10 mM sodium phosphate

799 at pH 7.6 in the 250-345 nm range were collected in the absence of AXP3009 (solid line) and upon
800 overnight incubation with 100 μ M AXP3009 (dashed line). **(B)** Thermal denaturation kinetics of
801 AXP3009-bound (open symbols) and unbound *h*GAPDH (closed symbols) from two independent
802 experiments each. The solid lines are the fitting to a sigmoidal equation, indicative of a two-state
803 model. **(C)** Apparent molecular weights of AXP3009-bound and unbound GAPDH on a superdex
804 200 increase 3.2/300 column as estimated by comparison with calibrants in the range 29 -440 kDa.
805 The reported values are the means \pm s.e.m. of five independent measurements. **(D)** Western blot
806 analysis and quantification of GAPDH expression into the cytosol and nuclei of PaCa44 cells
807 untreated/treated with 50 μ M AXP3009 for 48 h. α -tubulin and Lamin B1 have been used as
808 controls of the quality of the cytoplasmic and nuclear protein fractions, respectively. Amido Black
809 staining was used to confirm equal cytoplasmic and nuclear protein loading in the different lanes.
810 **(E)** Cell growth analysis through the Crystal Violet colorimetric assay of PaCa44 cells treated with
811 50 μ M of AXP3009 and/or with 100 μ M genipin and/or 5 μ M everolimus for 48h. Values are the
812 means (\pm SD) of three independent experiments. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and
813 (***) $P < 0.001$ for treated cells vs control or single treatment vs the relative single
814 treatment+AXP3009 or genipin+everolimus vs genipin+everolimus+AXP3009. Legend: (ctrl)
815 control, (G) genipin, (E) everolimus, (C) cytoplasmic fraction, and (N) nuclear fraction.
816

817 **Figure 6. mTOR inhibition increases apoptosis in genipin-treated cells.** Apoptosis analysis
818 through the Annexin V binding assay of PaCa44 cells treated with **(A)** 100 μ M genipin and/or 5 μ M
819 everolimus for 48 h and/or with **(B)** 1 μ M AXP3009 and 10 mM NAC. **(C)** Quantitative analysis of
820 CHOP mRNA through real-time PCR (qPCR) of PaCa44 cells treated with 200 μ M genipin and/or
821 10 μ M everolimus for 8 h. Total RNA was extracted from cells and qPCR was performed as
822 described in Materials and Methods. **(D)** Western blot analysis of three apoptosis-related proteins
823 (Bcl2, caspase 3 full length, and caspase 9 full length) in PaCa44 cells treated with 100 μ M genipin
824 and/or 5 μ M everolimus for 48 h. The bands of Western blot analyses were scanned as digital peaks
825 and the areas of the peaks were calculated in arbitrary units. The value of α -tubulin was used as a
826 normalizing factor and protein fold change is relative to control cells. Values are the means (\pm SD)
827 of three independent experiments. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$
828 for treated cells vs control, or single treatments vs genipin+everolimus, or genipin+everolimus vs
829 genipin+everolimus+AXP3009 or vs genipin+everolimus+NAC. Legend: (ctrl) control, (G)
830 genipin, and (E) everolimus.

831

832 **Figure 7. mTOR inhibition induces autophagy and related-mechanisms in genipin-treated**
833 **cells.** (A) Autophagosome formation assay through the analysis of the incorporation of
834 monodansylcadaverine (MDC) probe in PaCa44 cells treated with 100 μ M genipin and/or 5 μ M
835 everolimus for 48 h through flow cytometry. (B) Representative immunofluorescence images of
836 autophagosome formation through acridine orange staining of PaCa44 cells treated with 100 μ M
837 genipin and 5 μ M everolimus for 24 h, at 40-fold magnification, scale bar 10 μ m. (C) Western blot
838 analysis of Beclin1 in PaCa44 cells treated with 100 μ M genipin and/or 5 μ M everolimus for 24 h.
839 Amido black staining was used to confirm equal protein loading in the different lanes. (D)
840 Immunoprecipitation analysis and relative quantification of Beclin-1/Hsp90 in PaCa44 cells treated
841 with 200 μ M genipin and/or 10 μ M everolimus for 24 h. (E) Autophagosome formation assay
842 through the incorporation of the MDC probe in PaCa44 cells transfected with 50 nM siRNA
843 Beclin1 for 48 h and/or treated with 200 μ M genipin and 10 μ M everolimus for 48h. (F) Cell
844 growth analysis through the Crystal Violet colorimetric assay of PaCa44 cells pre-treated for 1 h
845 with 5 mM 3-MA and/or treated with increasing doses of the drugs [genipin from 500 nM to 140
846 μ M; everolimus from 25 nM to 7 μ M] maintaining the ratio genipin:everolimus 20:1 for 48 h. (G)
847 Apoptosis analysis through the Annexin V binding assay of PaCa44 cells pre-treated for 1 h with 5
848 mM 3-MA and/or treated with 100 μ M genipin and 5 μ M everolimus for 48 h. Values are the
849 means (\pm SD) of three independent experiments. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and
850 (***) $P < 0.001$ for treated cells vs control, or single treatments vs genipin+everolimus, or
851 genipin+everolimus vs genipin+everolimus+3-MA, or genipin+everolimus vs
852 genipin+everolimus+siRNA Beclin1. Legend: (ctrl) control, (G) genipin, and (E) everolimus.

853

854 **Figure 8. *In vivo* effect of mTOR inhibition in genipin-treated nude mice.** PaCa44 cells were
855 subcutaneously injected into female nude mice. After 1 week, i.p. injections with PBS (solution
856 vehicle), genipin, and/or everolimus were administered twice a week for 4 weeks, as described in
857 Materials and Methods. (A) Tumor volumes were measured at 3 days after each injection and
858 values are the mean of mice tumor volumes in each group. Statistical analysis is referred to the days
859 at which the tumor volume of the mice treated with genipin+everolimus is significantly lower than
860 the other three groups, such as the control mice and the mice treated with the drugs alone. (*)
861 $P < 0.05$. (B) Four representative images of tumor masses grown in control mice and in mice treated
862 with genipin+everolimus. (C) Count of mitotic figures on 10 consecutive HPFs (high power field,
863 40-fold magnification, scale bar 20 μ m) for each neoplastic sample. Representative images are
864 reported for control mice and for mice treated with genipin+everolimus. Statistical analysis is

865 referred to mice treated with the drug combination genipin+everolimus *vs* control mice. (***)
866 $P < 0.001$. **(D)** Mice body mass was measured at 3 days after each injection and values are the means
867 of mice body in each group. Legend: (ctrl) control, (G) genipin, and (E) everolimus.

868

869 **Figure 9.** Schematic representation of the molecular mechanisms regulated by UCP2 and
870 Akt/mTOR inhibition identified in this study.

871

872 **Supplementary Figure 1.** Quantitative analysis of UCP2 mRNA through real-time PCR (qPCR) of
873 PaCa44 cells transfected with 200 nM siRNA control or siRNA UCP2 for 72 h. Total RNA was
874 extracted from cells and qPCR was performed as described in Materials and Methods.

875 **Supplementary Figure 2.** Western blot analysis of the total and phosphorylated form of the mTOR
876 target P70S6K in three pancreatic ductal cell lines (PaCa3, PaCa44, and Panc1) treated with 400
877 μ M genipin for 24 h. Legend: (ctrl) control and (G) genipin.

878 **Supplementary Figure 3.** Isobolograms obtained from the CalcuSyn output at 0.5 of fractional
879 effect (IC_{50}) for five PDAC cell lines (PaCa44, Panc1, MiaPaCa2, PaCa3, and T3M4) treated for 48
880 h. IC_{50} of genipin and everolimus alone are reported as cross on the x and y axis, respectively,
881 whereas the black dot represents the IC_{50} of the combined treatment. The combination data point
882 indicates an additive effect when it is plotted on the diagonal, a synergistic effect when it is on the
883 lower left, and an antagonistic effect when it is on the upper right.

884 **Supplementary Figure 4.** **(A)** Quantitative analysis of GAPDH mRNA through real-time PCR
885 (qPCR) of PaCa44 cells transfected with 200 nM siRNA control or siRNA GAPDH for 72 h. Total
886 RNA was extracted from cells and qPCR was performed as described in Materials and Methods. **(B)**
887 Cell growth analysis through the Crystal Violet colorimetric assay of PaCa44 cells treated with 50
888 μ M AXP3009 and/or with GAPDH siRNA for 48h.

889 **Supplementary Figure 5.** Quantitative analysis of Beclin-1 mRNA through real-time PCR (qPCR)
890 of PaCa44 cells transfected with 50 nM siRNA Beclin1 (siBECN1) for 48 h. Total RNA was
891 extracted from cells and qPCR was performed as described in Materials and Methods.

892 **Supplementary Figure 6.** Haematoxylin and eosin (H&E) tissue staining of nude mice injected
893 subcutaneously with PaCa44 cells. Representative images of heart, kidney, liver, lung and pancreas

894 in control mice and in mice treated with the drug combination genipin+everolimus taken at 10-fold
895 magnification, scale bar 100 μm .

1 **UCP2 inhibition induces ROS/Akt/mTOR axis: role of GAPDH nuclear**
2 **translocation in genipin/everolimus anticancer synergism**

3
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24
25 *Running title:* Everolimus and genipin induce anticancer synergism

26 *Keywords:* pancreas cancer, uncoupling proteins, UCP2, mTOR, everolimus, GAPDH, cell death.

27

28 **Abstract**

29 Several studies indicate that mitochondrial uncoupling protein 2 (UCP2) plays a pivotal role
30 in cancer development by decreasing reactive oxygen species (ROS) produced by mitochondrial
31 metabolism and by sustaining chemoresistance to a plethora of anticancer drugs. Here, we
32 demonstrate that inhibition of UCP2 triggers Akt/mTOR pathway in a ROS-dependent mechanism
33 in pancreatic adenocarcinoma cells. This event reduces the antiproliferative outcome of UCP2
34 inhibition by genipin, creating the conditions for the synergistic counteraction of cancer cell growth
35 with the mTOR inhibitor everolimus. Inhibition of pancreatic adenocarcinoma cell growth and
36 induction of apoptosis by genipin and everolimus treatment are functionally related to nuclear
37 translocation of the cytosolic glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase
38 (GAPDH). The synthetic compound (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-dihydroisoxazol-5-yl-
39 acetate (AXP3009), which binds GAPDH at its redox-sensitive Cys152, restores cell viability
40 affected by the combined treatment with genipin and everolimus, suggesting a role for ROS
41 production in the nuclear translocation of GAPDH. Caspase-mediated apoptosis by genipin and
42 everolimus is further potentiated by the autophagy inhibitor 3-methyladenine revealing a protective
43 role for Beclin1-mediated autophagy induced by the treatment. Mice xenograft of pancreatic
44 adenocarcinoma further confirmed the antiproliferative outcome of drug combination without toxic
45 effects for animals. Tumor masses from mice injected with UCP2 and mTOR inhibitors revealed a
46 strong reduction in tumor volume and number of mitosis associated with a marked GAPDH nuclear
47 positivity. Altogether, these results reveal novel mechanisms through which UCP2 promotes cancer
48 cell proliferation and support the combined inhibition of UCP2 and of Akt/mTOR pathway as a
49 novel therapeutic strategy in the treatment of pancreatic adenocarcinoma.

50

51 1. Introduction

52 Pancreatic ductal adenocarcinoma (PDAC) is a devastating disease that remains the fourth
53 leading cause of cancer-associated deaths [1, 2]. Despite advances in multimodal therapy [3, 4],
54 PDAC remains extraordinarily lethal, with a 5-year overall survival rate of approximately 5% [1, 5].
55 Delayed diagnosis, early metastasis, and resistance to almost all the classes of cytotoxic drugs are
56 considered the main reasons for the extremely poor prognosis of pancreas cancer patients [6]. For
57 these reasons, research is now focused on the identification of more efficient therapeutic targets as
58 well as new prognostic and diagnostic biomarkers in order to improve the clinical management of
59 PDAC.

60 Everolimus, a well-tolerated analogue of rapamycin with improved pharmacokinetic
61 properties and reduced immunosuppressive effects [7], has been approved by the Food and Drug
62 Administration (FDA) in 2012 for the treatment of advanced/metastatic renal cell carcinoma,
63 subependymal giant cell astrocytoma and progressive neuroendocrine tumors of pancreatic origin
64 [8]. Recently, Peng *et al.* demonstrated that everolimus, by inhibiting mTOR complex, can
65 overcome the resistance to gemcitabine-based treatment of PDAC cell lines inducing caspase-
66 dependent apoptosis and cell cycle arrest in the G2 phase [9].

67 The uncoupling proteins (UCPs) belong to the superfamily of mitochondrial carrier proteins
68 located in the mitochondrial inner membrane [10]. Among them, UCP2 has been found
69 overexpressed in several tumor types triggering both tumorigenesis and cancer progression [11]. An
70 extensive study on Oncomine data sets revealed that UCP2 gene is overexpressed in most of the
71 cancer types examined, i.e. ovarian, bladder, esophageal, testicular, kidney, colorectal, lung, breast,
72 leukemia, prostate, as well as pancreas cancers [12]. Concerning PDAC, some studies have shown
73 that also the protein level of UCP2 is significantly higher in cancer samples than in the adjacent
74 normal tissues, suggesting that UCP2 may sustain pancreatic tumor growth [13]. Along this line of
75 evidence, our research group previously demonstrated that UCP2 is involved in PDAC

76 chemoresistance to gemcitabine treatment and that this treatment further induces the expression of
 77 UCP2 gene [14]. Furthermore, we recently demonstrated that UCP2 promotes the metabolic shift
 78 from mitochondrial oxidative phosphorylation (mtOXPHOS) to the glycolytic pathway sustaining
 79 the Warburg effect and PDAC cell proliferation [15]. All these properties are mainly ascribable to
 80 the diminution of mitochondrial reactive oxygen species (ROS) driven by UCP2, whose antioxidant
 81 property is due to the transport of protons from the intermembrane space to the mitochondrial
 82 matrix bypassing ATP synthase. This results in a decrease of several consequential events,
 83 including mitochondrial inner membrane potential, electron leakage from respiratory electron
 84 transport chain and superoxide ion generation into the mitochondrial matrix [16]. Therefore, mild
 85 uncoupling of mtOXPHOS may be considered the first line of defense against oxidative stress in
 86 cancer cells [17]. Overall, UCP2 overexpression may be considered a strategy adopted by cancer
 87 cells to protect themselves from excessive ROS production but can also be a potential target for
 88 therapy for different cancers particularly resistant to standard chemotherapies, such as PDAC [18].
 89 We previously demonstrated that UCP2 inhibition triggers PDAC cell death by ROS-dependent
 90 nuclear translocation of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase
 91 (GAPDH) and stimulation of autophagy in PDAC cells [19]. In the present study, we show *for the*
 92 *first time* that *although* UCP2 inhibition *determines PDAC cell growth inhibition, it can also*
 93 *stimulate triggers* the oncogenic Akt/mTOR pathway *in a ROS-dependent manner, in vitro and in*
 94 *vivo.* *This is likely due to an extreme attempt of cancer cells to survive in these stressful conditions*
 95 *and provides ing* the rationale for a combined therapeutic setting with the mTOR inhibitor
 96 everolimus in PDAC.

97

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98 2. Material and Methods

99 2.1 Drugs and chemicals

100 Genipin (methyl-2-hydroxy-9-hydroxymethyl-3-oxabicyclonona-4,8-diene-5-carboxylate),
101 everolimus (RAD001), *N*-acetyl-L-cysteine (NAC), and 3-methyladenine (3-MA) were obtained
102 from Sigma-Aldrich. Genipin and everolimus were solubilized in DMSO, NAC was solubilized in
103 bi-distilled sterile water, and 3-MA was freshly prepared and solubilized in cell culture medium.
104 The GAPDH inhibitor (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-dihydroisoxazol-5-yl-acetate
105 (AXP3009) has been designed and synthesized in the laboratory of Dr. Paola Conti at the
106 Department of Pharmaceutical Sciences (University of Milan, Italy) and solubilized in methanol.
107 The chemical structure and the synthesis of the AXP3009 compound have been previously
108 described in Bruno and colleagues [20]. All chemicals were stored at -80°C.

109

110 2.2 Cell culture

111 Pancreatic adenocarcinoma PaCa44, PaCa3, ~~and~~ Panc1, MiaPaCa2, and T3M4 cell lines
112 were grown in RPMI 1640 medium (Gibco, Thermo Fisher), supplemented with 2 mM glutamine,
113 10% FBS, and 50 µg/ml gentamicin sulfate (BioWhittaker, Lonza), and incubated at 37°C with 5%
114 CO₂.

115

116 2.3 Immunoblot analysis

117 *Whole cell lysates.* Cells were harvested, washed in PBS, and re-suspended in lysis buffer in
118 the presence of phosphatase and protease inhibitors (50 mM Tris-HCl pH 8, 150 mM NaCl, 1%
119 Igepal CA-630, 0.5% Na-Doc, 0.1% SDS, 1 mM Na₃VO₄, 1 mM NaF, 2.5 mM EDTA, 1 mM
120 PMSF, and 1× protease inhibitor cocktail). After incubation on ice for 30 min, the lysates were
121 centrifuged at 5,000 × g for 10 min at 4 °C and the supernatant fractions were used for Western blot
122 analysis.

123 *Cytoplasmic and nuclear lysates.* Cells were harvested, washed in PBS, and re-suspended in
124 lysis buffer A (10 mM Hepes, 0.1% Igepal CA-630, 1.5 mM MgCl₂, 10 mM KCl) in the presence of
125 phosphatase and protease inhibitors (1 mM Na₃VO₄, 1 mM NaF, 0.5 mM DTT, 1 mM PMSF, and
126 1× protease inhibitor cocktail). After incubation on ice for 10 min, the lysates were centrifuged at
127 3,000 × g for 10 min at 4 °C and the supernatant fractions, containing cytoplasmic proteins, were
128 used for Western blot analysis, whereas the pellets, containing nuclei, were re-suspended in lysis
129 buffer C (20 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA) in the
130 presence of phosphatase and protease inhibitors. After incubation on ice for 15 min, the lysates
131 were centrifuged at 15,000 × g for 10 min at 4 °C and the supernatant fractions were used for
132 Western blot analysis.

133 Protein concentration was measured by Bradford reagent (Pierce) using bovine serum
134 albumin as a standard. Protein extracts (50 µg/lane for whole cell lysate immunoblots and 6 µg/lane
135 for cytoplasmic and nuclear cell lysate immunoblots) were resolved on a 12% SDS-polyacrylamide
136 gel and electro-blotted onto PVDF membranes (Millipore). Membranes were blocked in 5% low-fat
137 milk in TBST (50 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature (RT)
138 and probed overnight at 4 °C with anti-phospho(Ser473)-Akt (1:2,000; Cell Signalling #4060), anti-
139 Akt (1:1,000; Cell Signalling #9272), anti-α-tubulin (1:2,500; Oncogene #CP06-100UG), anti-
140 phospho(Thr389)-P70S6K (1:1,000; Cell Signalling #9206), anti-P70S6K (1:1,000; Cell Signalling
141 #2708), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:1,000; Cell Signalling,
142 #5174S), anti-Lamin B1 (1:1,000; Millipore #05-1531), anti-α-tubulin (1:2,500; Oncogene #CP06-
143 100UG), anti-Bcl2 (1:1,000; Cell Signalling #2872), anti-caspase 3 full length (1:1,000; Cell
144 Signalling #9662), anti-caspase 9 full length (1:1,000; Enzo Life Sciences #ALX-210-815-C100),
145 anti-Becclin1 (1:1,000; Cell Signalling Technology #4122), anti-Hsp90 (1:1,000; Cell Signalling
146 #4874) antibodies. Horseradish peroxidase conjugated anti-mouse (1:10,000; KPL #074-1806) or
147 anti-rabbit IgGs (1:2,000; Cell Signalling #7074) were used as secondary antibodies. The
148 immunocomplexes were visualized by chemiluminescence using the ChemidocMP imaging system

149 (Bio-Rad Laboratories) and the intensity of the chemiluminescence response was measured by
150 processing the image with NIH Image J software (<http://rsb.info.nih.gov/nih-image/>). Amido Black
151 staining was used to confirm equal cytoplasmic and nuclear protein loading in different lanes.

152

153 *2.4 Transient transfection assays*

154 Exponentially growing cells were seeded at 5×10^3 cells/well in 96-well plates for
155 monodansylcadaverine (MDC) assay and at 2.5×10^5 cells/plate in 60 mm cell culture plates for
156 protein extractions. UCP2 and Beclin-1 silencing experiments were carried out with specific small
157 interfering RNA (siRNA): siRNA UCP2 (5'-GCUAAAGUCCGGUUACAGATT-3'), siRNA
158 Beclin-1 (5'-ACAGUGAAUUUAAACGACAGCAGCU-3'), siRNA GAPDH (Ambion,
159 #4390850), and a non-targeting siRNA (5'-CAGUCGCGUUUGCGACUGG-3') used as negative
160 control were purchased by Ambion, Thermo Fisher. Cells were transfected with siRNA at a final
161 concentration of 50 nM Lipofectamine 3000 transfection reagent (Thermo Fisher), according to the
162 manufacturer's instructions. Transfection efficiency of cells was about 40% as previously assessed
163 by cytofluorimetric analysis using a pGFP plasmid vector [14].

164

165 *2.5 Analysis of reactive oxygen species (ROS) and mitochondrial superoxide ion production*

166 *ROS production.* The non-fluorescent diacetylated 2',7'-dichlorofluorescein (DCF-DA)
167 probe (Sigma-Aldrich), which becomes highly fluorescent upon oxidation, was used to evaluate
168 intracellular ROS production. Briefly, cells were plated in 96-well plates (5×10^3 cells/well) and, the
169 day after, were treated with the various compounds at the indicated concentrations for 24 h. At the
170 end of the various treatments, the cells were incubated in culture medium with 10 μ M DCF-DA for
171 15 min at 37 °C. The cells were washed with Hanks' buffer (20 mM Hepes, pH 7.2, 10 mM
172 glucose, 118 mM NaCl, 4.6 mM KCl, and 1 mM CaCl_2) and the DCF fluorescence was measured
173 by using a multimode plate reader (Ex_{485 nm} and Em_{535 nm}) (GENios Pro, Tecan). The values were
174 normalized on cell proliferation by crystal violet assay.

175 *Mitochondrial superoxide ion production.* The nonfluorescent MitoSOX red probe
176 (Molecular Probes) was used to evaluate mitochondrial $O_2^{\bullet-}$ production. The probe is live-cell
177 permeative and is rapidly and selectively targeted to the mitochondria where it becomes fluorescent
178 after oxidation by $O_2^{\bullet-}$, but not by other ROS or reactive nitrogen species. Briefly, cells were plated
179 in 96-well plates (5×10^3 cells/well) and, the day after, were treated with the various compounds at
180 the indicated concentrations for 24 h. At the end of the various treatments, cells were incubated in
181 culture medium with 0.5 μ M MitoSOX at 37 °C for 15 min. The cells were then washed with
182 Hanks' buffer and fluorescence was measured by using a multimode plate reader (Ex_{535 nm} and
183 Em_{590 nm}) (GENios Pro, Tecan). The values were normalized on cell proliferation by crystal violet
184 assay. Three independent experiments were performed for each assay condition.

185

186 *2.6 Cell proliferation assay*

187 Cells were seeded in 96-well plates (5×10^3 cells/well) and the day after were incubated with
188 various compounds at the indicated conditions (see figure legends). At the end of the treatments,
189 cell growth was evaluated by Crystal Violet (Sigma-Aldrich) staining. The dye was solubilized in
190 1% SDS in PBS and measured photometrically (A_{595 nm}) to determine cell viability (GENios Pro,
191 Tecan). Three independent experiments were performed for each assay condition.

192

193 *2.7 Drug combination studies*

194 The compounds were added for 48 h at the following concentration ranges: 500 nM–200 μ M
195 for genipin and 25 nM–10 μ M for everolimus, maintaining the ratio genipin:everolimus 20:1. The
196 CI was calculated by the Chou–Talalay equation, which takes into account both the potency (IC₅₀)
197 and the shape of the dose–effect curve [21] by using the CalcuSyn software. The general equation
198 for the classic isobologram is given by $CI = \frac{(D)1}{(Dx)1} + \frac{(D)2}{(Dx)2} + \frac{[(D)1 * (D)2]}{[(Dx)1 * (Dx)2]}$,
199 where (Dx)1 and (Dx)2 in the denominators are the doses (or concentrations) for D1 (drug 1) and
200 D2 (drug 2) alone that give x% growth inhibition, whereas (D)1 and (D)2 in the numerators are the

201 doses of drug 1 and drug 2 in combination that also inhibited x% cell viability (i.e., isoeffective).
202 $CI < 1$ indicates synergism, whereas $CI = 1$ and $CI > 1$ indicates additive effect and antagonism,
203 respectively. CI/effect curve represents the CI versus the fraction (0–1) of cells killed by drug
204 combination. The synergism percentage was obtained analyzing CI/effect curve and measuring the
205 CI values at each 0.05 fraction, that is, 5% growth inhibition, of the antiproliferative effect. Dose
206 reduction index (DRI) represents the measure of how much the dose of each drug in a synergistic
207 combination may be reduced at a given effect level compared with the doses of each drug alone.

208

209 *2.8 Immunofluorescence analysis of GAPDH nuclear translocation*

210 PaCa44 cells treated or untreated with genipin and everolimus were fixed in 4%
211 paraformaldehyde for 15 min and, after 4 changes (10 min each) of PBS, were permeabilized in
212 0.1% Triton X-100 for 5 min in PBS. To saturate unspecific binding sites, the cells were incubated
213 for 45 min at RT with a blocking solution containing 5% BSA and 0.05% Triton X-100 in PBS.
214 Samples were then incubated overnight at 4 °C with anti-GAPDH (1:100; Cell Signalling #5174S)
215 primary antibody diluted in blocking solution. After 3 washes with PBS (10 min each), cells were
216 incubated for 1 h at RT in the dark with specific secondary antibodies (1 µg/mL) conjugated with
217 Alexa Fluor-488 (Molecular Probes). The incubation with secondary antibody was followed by 10
218 min incubation at RT with Hoechst dye (10 µg/ml). Samples were mounted in anti-bleaching
219 medium (Dako Fluorescent Mounting Medium). The negative control procedure omitted the
220 primary antibody. Slices were examined by fluorescence microscope (DM6000B, Leica
221 Microsystem).

222

223 *2.9 Structural characterization of AXP3009-bound recombinant GAPDH*

224 His-tagged human GAPDH was recombinantly expressed in *E. coli*. Circular dichroism
225 spectra of GAPDH at 10 µM concentration were collected at 25°C with a JASCO J-715
226 spectropolarimeter in a buffered solution containing 10 mM sodium phosphate at pH 7.6.

227 Measurements were made either in the 250-345 nm range, using a path length of 1 cm, or in the
228 190-250 nm range, using a path length of 0.1 cm, in both cases with a 0.5 nm data pitch, a
229 50 nm/min scanning speed and with 3-5 accumulations per spectrum. GAPDH spectra were
230 collected in the absence of AXP3009 and upon overnight incubation with 30-100 μ M AXP3009, to
231 allow for the completion of the time-dependent binding [20]. The thermal stability of AXP3009-
232 bound and unbound GAPDH was monitored with the spectropolarimeter set at 222 nm using a
233 temperature ramp in the 30-80°C range, generated with a JASCO PTC-348 WI temperature
234 controller. Each spectral observation and each denaturation kinetics was confirmed by at least two
235 independent experiments. The elution times of AXP3009-bound and unbound GAPDH were
236 measured on a superdex 200 increase 3.2/300 column (GE Healthcare) mounted on a Prominence
237 HPLC system (Shimadzu) with the detector set at 280 nm. The column was pre-equilibrated and
238 then developed with a solution containing 10 mM sodium phosphate, 50 mM sodium chloride pH
239 7.6. The flow rate was 0.15 mL/min. The column was calibrated with commercially available gel-
240 filtration standards (Sigma-Aldrich), including carbonic anhydrase (29 kDa), conalbumin (75 kDa),
241 ferritin (440 kDa) and blue dextrane (2000 kDa).

242

243 *2.10 Apoptosis assay*

244 Cells were seeded in 96-well plates (5×10^3 cells/well) and, the day after, treated with the
245 various compounds at the indicated conditions (see figure legends). At the end of the treatment,
246 cells were fixed with 2% paraformaldehyde in PBS at RT for 30 min, then washed twice with PBS
247 and stained with annexinV/FITC (Bender MedSystem) in binding buffer (10 mM HEPES/NaOH
248 pH 7.4, 140 mM NaCl and 2.5 mM CaCl_2) for 10 min at RT in the dark. Finally, cells were washed
249 with binding buffer solution and fluorescence was measured by using a multimode plate reader
250 ($\text{EX}_{485 \text{ nm}}$ and $\text{Em}_{535 \text{ nm}}$) (GENios Pro, Tecan). The values were normalized on cell proliferation by
251 Crystal Violet assay. Three independent experiments were performed for each assay condition.

252

253 *2.11 RNA extraction and qPCR*

254 Total RNA was extracted from 10⁶ cells using TRIzol Reagent (Thermo Fisher) and 1 µg of
 255 RNA was reverse transcribed using first-strand cDNA synthesis. Real-time quantification was
 256 performed in triplicate samples by SYBR Green detection chemistry with Power SYBR Green PCR
 257 Master Mix (Applied Biosystems) on a 7900 HT Fast Real-Time PCR System (Thermo Fisher).
 258 Normalization was performed analysing the ribosomal protein large P0 (RPLP0) mRNA expression
 259 level. The primers used were: CHOP For, 5'-GCAGCCCATGAAGGAGAAAG-3'; CHOP Rev, 5'-
 260 CCGTTCGATTTCTGCTTGAG-3'; UCP2 For, 5'-CTCCTGAAAGCCAACCTCAT-3'; UCP2
 261 Rev, 5'-CCCAAAGGCAGAAGTGAAGT-3'; GAPDH For, 5'-ATCAGCAATGCCTCCTGCAC-
 262 3'; GAPDH Rev, 5'-TGGTCATGAGTCCTTCCACG-3'; Beclin1 For, 5'-
 263 GTAGACCGGACTTGGGTGAC-3'; Beclin1 Rev, 5'-CTGCATGGTGCTGTTGTTG-3'; RPLP0
 264 For, 5'-ACATGTTGCTGGCCAATAAGGT-3' and RPLP0 Rev, 5'-
 265 CCTAAAGCCTGGAAAAAGGAGG-3'. The thermal cycle reaction was performed as follows: 95
 266 °C for 10 minutes followed by 40 cycles at 95 °C for 15 seconds and 60°C for 1 minute. The
 267 average of cycle threshold of each triplicate was analyzed according to the 2^(-ΔΔCt) method. Three
 268 independent experiments were performed for each assay condition.

269

270 *2.12 Autophagosome formation assay*

271 To quantify the induction of autophagy, cells were treated with the various compounds (see
 272 figure legends) and then were incubated with the fluorescent probe monodansylcadaverine (MDC;
 273 Sigma-Aldrich). MDC is a selective marker for acidic vesicular organelles, such as autophagic
 274 vacuoles and especially autolysosomes.

275 *Flow cytometry*: briefly, following the treatments, cells were incubated with 50 µM MDC in
 276 PBS at 37 °C for 15 min. After incubation, cells were washed with PBS, trypsinized, and
 277 immediately analyzed by flow cytometry. All fluorescences were analyzed with a FACScalibur
 278 flow cytometer (Becton Dickinson). The fluorescent emissions were collected through a 530 nm

279 band pass filter (FL-1 channel). At least 10,000 events were acquired in log mode. For the
280 quantitative evaluation of MDC, CellQuest software (Becton Dickinson) was used to calculate
281 mean fluorescence intensities (MFIs) by the formula $MFI_{\text{treated}}/MFI_{\text{control}}$, where MFI-treated
282 is the fluorescence intensity of cells treated with the various compounds and MFI-control is the
283 fluorescence intensity of cells untreated. Figures show the means of fluorescence intensity \pm SD
284 from three independent experiments.

285 *Multimode plate reader:* briefly, cells were seeded in 96-well plates (5×10^3 cells/well) and
286 treated with the various compounds at the indicated conditions (see figure legends). At the end of
287 the treatments, cells were incubated in culture medium with 50 μ M MDC at 37 °C for 15 min. After
288 incubation, cells were washed with Hanks' buffer and fluorescence was measured by using a
289 multimode plate reader (Ex_{340 nm} and Em_{535 nm}) (GENios Pro, Tecan). The values were normalized
290 on cell proliferation by Crystal Violet assay. Three independent experiments were performed for
291 each assay condition.

292

293 *2.13 Detection of acidic vesicular organelles with acridine orange*

294 In acridine orange (AO) stained cells, the cytoplasm and nucleus are bright green and dim
295 red, whereas acidic compartments are bright red. The intensity of the red fluorescence is
296 proportional to the degree of acidity. Following the treatments (see figure legend), cells were
297 incubated with AO solution (1 μ g/ml) at 37 °C for 15 min, then were washed with PBS, and
298 observed with a fluorescence microscope (Nikon Eclipse TE300 Inverted microscope - Nikon
299 Instruments).

300

301 *2.14 Immunoprecipitation assay*

302 Following the treatments (see figure legend), cells were washed twice with ice-cold
303 PBS/BSA, and lysed for 30 min at 4 °C in lysis buffer (50 mM Tris, pH 7.4, 150 mM sodium
304 chloride, 1% Nonidet P-40, 1 mM EDTA, 1 mM sodium orthovanate, 1 mM sodium fluoride, 1

305 $\mu\text{g/ml}$ leupeptin, $1 \mu\text{g/ml}$ aprotinin, $1 \mu\text{g/ml}$ pepstatin A, 1 mM PMSF). Lysates were centrifuged at
306 $10,000 \times g$ for 10 min and supernatants were collected. Protein concentration was determined by
307 Lowry method using bovine serum albumin for the standard curve. Protein lysates ($120 \mu\text{g}$) were
308 incubated with anti-Becn1 (Cell Signalling Technology #4122) and anti-Hsp90 (Cell Signalling
309 #4874) primary antibodies overnight at 4°C . Immune complexes were collected with $50 \mu\text{l}$ of
310 protein A-agarose and incubated for 16 h at 4°C . The protein A-agarose/ immune complex was
311 washed twice with cold PBS, resuspended in $20 \mu\text{l}$ of SDS-loading buffer, heated to 95°C for 5 min
312 and used for protein gel blotting analysis. Proteins were separated by 12.5% polyacrylamide gel
313 SDS-PAGE (Mini-PROTEAN[®] TGX Stain-Free[™] Precast Gels, BIORAD). After electrophoretic
314 separation the proteins were transferred on nitrocellulose filter (Trans-Blot Turbo Mini, BIORAD)
315 by Western Blotting, using Trans-Blot Turbo[®] Transfer System (BIORAD) and the filter was
316 reacted using either anti-Becn1 or anti-Hsp90 antibodies. After incubation with secondary
317 antibodies, the signal was detected using enhanced chemiluminescence detection reagents Clarity[™]
318 Western ECL Blotting Substrate (BIORAD). The bands derived from western blotting were
319 analyzed and quantified by Image Lab 5.2.1 software obtained by ChemiDoc[™]MXRS⁺ (BIORAD)
320 and the expression of total protein was used for normalization. The error bars shown in the
321 histograms represent the standard deviation from the mean of different densitometric scanning in at
322 least 3 different experiments.

323

324 *2.15 In vivo studies*

325 All procedures involving mice were performed in compliance with our institutional animal
326 care guidelines and following national and international directives (D.L. 4 March 2014, no. 26;
327 directive 2010/63/EU of the European parliament and of the council). PaCa44 cells (1×10^6
328 cells/mice) were s.c. injected into the dorsal flank of six nude female mice for each condition (5
329 weeks of age; Charles River Laboratories, Inc.). For the control groups, mice received $200 \mu\text{l}$
330 injections of 1X PBS. One week after cell inoculation, experimental group received 30 mg/kg

331 genipin based on previous published data [22], or/and 4.5 mg/m² everolimus based on its clinical
332 use following US Food and Drug Administration directives, and vehicle solution (PBS) for control
333 group, by intraperitoneal injection biweekly for 4 weeks. Body mass was recorded weekly for each
334 animal. Tumor size was monitored weekly in two perpendicular dimensions parallel to the surface
335 of the mouse using a caliper. Tumor volume was calculated using the formula of $V = \pi/6 \times [(w \times$
336 $L)^{(3/2)}]$. Animals were sacrificed at the volume of 2 cm³. After euthanizing the mice, the
337 neoplasms along with the visceral organs (heart, kidney, liver, lung, and pancreas) were retrieved
338 for each mouse. All the samples were formalin fixed [in 10% (v/v) neutral-buffered formalin for 24-
339 48 h] and paraffin embedded for further evaluations. Serial histological sections (4-6 μm thick)
340 were obtained from each paraffin block and stained for histology assessment.

341 *Hematoxylin and eosin (H&E) stain:* One 4μm H&E slide was obtained for each sample
342 (neoplasm and normal tissue) for each mouse to perform histopathological evaluation, including
343 mitotic rate, grade of tumour necrosis and presence of lympho-granulocytic infiltrate in parenchyma
344 of visceral organs and skin as sign of potential drug-related cytotoxicity. Mitotic count was assessed
345 as absolute number of mitosis on 10 consecutive HPFs (high power field, 400X magnification) for
346 each neoplastic sample.

347 *Immunohistochemistry:* 4 μm slides were obtained for each sample for
348 immunohistochemical evaluation for anti-GAPDH (clone D16H11; 1:900; Cell Signalling) and
349 phospho-Akt (clone EP2109Y; 1:100; Abcam). Human breast carcinoma was considered as positive
350 control for GAPDH, while prostatic carcinoma for p-Akt. All the samples were analysed by a
351 pathologist, blind for the treatment and control groups distribution. GAPDH-positivity was defined
352 by the identification of a more intense nuclear stain compared to cytoplasmic and/or background
353 stain. A count of viable GAPDH-positive neoplastic cells was assessed on 20 consecutive HPFs,
354 away from necrotic area, in order to avoid potential artefacts due to necrosis. The apoptotic cells
355 were excluded from the count because it was not always possible to discriminate between nuclear
356 and cytoplasmic positivity due to the alterations occurring in the late apoptotic phases (nuclear

357 shrinkage and blebbing). Qualitative and quantitative p-Akt cytoplasmic and membranous
358 expression was evaluated on neoplastic viable cells on 20 consecutive HPFs, away from necrotic
359 area, in all the samples analysed. The quantitative analysis was performed through an evaluation of
360 the percentage of p-Akt positive cells on each slide.

361

362 *2.16 Statistical analysis*

363 ANOVA (post hoc Bonferroni) analysis was performed by GraphPad Prism 5 (GraphPad
364 Software, Inc., La Jolla, CA, USA). P-values < 0.05, 0.01, or 0.001 were indicated as *, **, or ***,
365 respectively.

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375 **3. Results**

376 *3.1 UCP2 inhibition stimulates Akt/mTOR signaling pathway in a ROS-dependent manner*

377 Previously, we demonstrated that ROS production by UCP2 inhibition triggers PDAC cell
378 death [19]. Here, we show that this production concurrently induces the oncogenic Akt/mTOR
379 pathway. **Figure 1A** shows that the activated form of Akt (phospho-Ser473Akt) is significantly
380 increased after treatment with the UCP2 inhibitor genipin both in tumor masses derived from
381 PDAC xenografts and in PDAC PaCa44 cells. Following the knockdown of UCP2 expression, the
382 observed increase of the Thr389-phosphorylated level of P70S6K, a direct target of mTOR kinase
383 complex, indicates that the Akt/mTOR pathway is functionally stimulated by UCP2 inhibition and
384 also provides a valuable control of UCP2 inhibition (**Fig. 1B**). Accordingly, **Supplementary**
385 **Figure 1** demonstrates the effective knockdown of UCP2 gene expression by siRNA-UCP2 as
386 compared with a scramble siRNA used as negative control. In addition, the increase of Thr389-
387 phosphorylation of P70S6K is revealed in three PDAC cell lines treated with genipin, thus
388 indicating that Akt/mTOR induction is a common event in PDAC cells after UCP2 blockage
389 (**Suppl. Fig. 2**). **Figure 1C** shows that genipin strongly induces ROS production and the radical
390 scavenger *N*-acetyl-L-cysteine (NAC) counteracts this event. To investigate whether Akt/mTOR
391 pathway regulation is functionally related to the antioxidant effect of UCP2, we analyzed Akt and
392 P70S6K phosphorylation levels in genipin-treated PDAC cells after NAC pre-treatment. **Figure 1D**
393 shows that Akt and P70S6K phosphorylation induced by genipin is at least partially rescued by
394 NAC demonstrating the involvement of ROS production in this signaling regulation driven by
395 UCP2 blockage.

396

397 *3.2 Genipin and everolimus synergistically inhibit PDAC cell proliferation through GAPDH* 398 *nuclear translocation*

399 Since stimulation of Akt/mTOR pathway has an established role in cancer proliferation and
400 everolimus, an oral anticancer mTOR inhibitor used in clinics, has shown particularly promising

401 results in cancer patients by inhibiting tumour growth and displaying anti-angiogenic effects also in
 402 combined therapies [23], we investigated whether genipin and everolimus combination might
 403 determine an enhanced antiproliferative effect in PDAC cells. First, we demonstrated that the
 404 addition of everolimus counteracts both Akt and P70S6K phosphorylation induced by genipin (**Fig.**
 405 **2A**), establishing the rational basis for the combined therapeutic strategy. In addition, drug
 406 combination strongly enhances ROS production as compared with single drugs (**Fig. 2B**) and the
 407 ROS-mediated antiproliferative effect in PDAC cells (**Fig. 2C**). By a therapeutic point of view, the
 408 addition of everolimus in the combined setting can assure the blockage of the Akt/mTOR pathway,
 409 avoiding a further ROS-mediated Akt/mTOR signaling stimulation even in the presence of
 410 enhanced ROS production.

411 In order to evaluate whether the antiproliferative effect of genipin+everolimus treatment was
 412 synergistic, we analyzed cell growth inhibition curves by using the dedicated software CalcuSyn
 413 (see Material and Methods). Dose-dependent analyses performed with increasing concentrations of
 414 genipin and/or everolimus showed that the combined setting induces a strong antiproliferative
 415 synergism in all five PDAC cell lines tested PaCa44 cells, as revealed by the curve of the
 416 combination index (CI) values \pm sd *versus* the fraction (0 \rightarrow 1) of cells killed by drug combination
 417 (**Fig. 3A2D**). CI values lying below 1 are widely distributed in the x axis, indicating a synergistic
 418 effect occurring at both low and high drug concentrations in all PDAC cell lines tested (Fig. 3B). In
 419 particular, CI₅₀ values of all PDAC cell lines are markedly below 1 indicating a strong synergism,
 420 and the is 0.129 and the reduction folds (dose reduction index; DRI) of drug concentration to obtain
 421 50% cell growth inhibition in combination setting as compared to each drug administered alone
 422 (DRI₅₀) is 24.16 for everolimus and 11.39 for genipin are reported in Table 1 (Table 1). Moreover,
 423 isobolograms at the IC₅₀ of each drug for each PDAC cell line are reported in Supplementary
 424 Figure 3, further supporting the synergistic effect observed in these conditions. Taken together,
 425 these data demonstrate that the addition of everolimus to genipin treatment produces a synergistic
 426 PDAC cell growth inhibition which is likely due to both enhanced ROS production in combined

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427 setting and to the counteraction of genipin-induced Akt/mTOR oncogenic signaling by everolimus.
428 DRI₅₀ values obtained *in vitro* suggest that this synergism might allow decreasing the
429 concentrations of the two compounds reducing eventual side effects.

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430 Since we previously demonstrated that UCP2 inhibition determines a ROS-dependent
431 nuclear translocation of GAPDH [19] and since GAPDH nuclear translocation is counteracted by
432 Akt/mTOR pathway through direct phosphorylation of the enzyme [24], we explored if nuclear
433 GAPDH positivity is further enhanced by the drug combination treatment. Importantly, we proved
434 that tumor masses derived from mice treated with the combined therapy show a considerable
435 increase in the number of GAPDH-positive nuclei (**Fig. 43A**). Also *in vitro*, we observed that the
436 combined treatment strongly increases the amount of GAPDH positive nuclei, as compared to
437 genipin-treated PDAC cells (**Fig. 43B**). Representative images of nuclear GAPDH positivity after
438 PDAC cell treatment with the drug combination are reported in **Figure 43C**. The total amount of
439 GAPDH mRNA remains unchanged after treatments (**Fig. 43D**), indicating that genipin and
440 everolimus regulate the subcellular distribution of the enzyme rather than its expression level. To
441 investigate the functional role of GAPDH nuclear translocation in cell growth inhibition by drug
442 combination, we used the 3-bromo-isoxazoline derivative (S)-benzyl-2-amino-2-(S)-3-bromo-4,5-
443 dihydroisoxazol-5-yl-acetate (AXP3009) (see Material and Methods), previously reported to
444 alkylate the catalytic cysteine residue of tetrameric *Plasmodium falciparum* GAPDH (*Pf*GAPDH)
445 without affecting other solvent-exposed cysteine residues [20]. In consideration of the negative
446 cooperativity of NAD⁺ binding [25] and structural observations [26], it was concluded that the
447 alkylation of one subunit of the GAPDH tetramer produces a conformational rearrangement that
448 severely limits the accessibility of the remaining active sites [20]. Since 3-bromo-isoxazoline
449 derivatives were also shown to bind to the redox sensitive catalytic cysteine of *human* GAPDH
450 (*h*GAPDH) [27], we hypothesized that AXP3009 can alter *h*GAPDH conformation, prevent
451 intracellular GAPDH oxidation by ROS produced after cell treatment with genipin and everolimus,
452 and counteract GAPDH nuclear translocation. To test this hypothesis, we first investigated

453 AXP3009-induced conformational rearrangements of recombinant *h*GAPDH. Circular dichroism
454 spectra of *h*GAPDH pre-incubated with AXP3009 show significant differences in the 250-345 nm
455 range, suggesting significant tertiary alterations in comparison to the control sample (**Figure 54A**).
456 The melting temperature, as determined by the fitting of the thermic denaturation kinetics, is 2.2°C
457 lower when *h*GAPDH is pre-incubated with AXP3009 (61.4±0.1 *versus* 63.6±0.1), suggesting a
458 difference in stability of the two forms (**Figure 54B**). Finally, gel filtration experiments of
459 AXP3009-bound and unbound *h*GAPDH show a small but significant difference in the apparent
460 molecular weight (**Figure 54C**). Both forms fall within the range of the tetramer (expected weight
461 148000 Da), but with a 12% difference that might reflect a difference in shape or a different
462 stability of the quaternary structure. Furthermore, we tested the specificity of AXP3009 on GAPDH
463 interaction by knocking-down intracellular GAPDH expression with siRNA-GAPDH (**Suppl. Fig.**
464 **43A**), revealing that after target deprivation the compound loses its cytotoxic effect on PDAC cells
465 (**Suppl. Fig. 43B**). Importantly, **Figure 54D** shows that AXP3009 stabilizes the cytosolic
466 localization of the GAPDH enzyme by diminishing its nuclear transport, thus providing a valuable
467 tool to investigate the role of nuclear GAPDH in cell death induced by genipin and everolimus
468 treatment. According to the cell death-related role assumed by nuclear GAPDH translocation [28],
469 we observed that AXP3009 is able to completely restore PDAC cell proliferation affected by
470 genipin alone, or by genipin and everolimus combined treatment ~~in a concentration dependent~~
471 ~~manner~~ (**Fig. 54E**).

472

473 *3.3 Genipin and everolimus strongly induce apoptosis in PDAC cells*

474 We further investigated whether apoptosis is involved in PDAC cell growth inhibition by
475 drug combination. As shown in **Figure 65A**, annexin V assay revealed that PDAC cells treated with
476 the drug combination expose a strongly higher amount of phosphatidylserine as compared to single
477 treatments or untreated control. Notably, apoptotic cell death induced by the combined treatment is
478 markedly reduced by the addition of AXP3009 or of the antioxidant compound NAC (**Fig. 65B**),

479 further supporting the antiproliferative role of GAPDH nuclear translocation mediated by
480 intracellular oxidant conditions [19, 29]. Moreover, we observed a strong increase of CHOP
481 expression by genipin+everolimus within the first eight hours after cell treatment (**Fig. 65C**), while
482 the expression of the Bcl 2 protein dramatically decreases in the combined setting (**Fig. 65D**),
483 according to the common assumption that the main mechanism of CHOP-induced apoptosis is the
484 suppression of the anti-apoptotic protein Bcl-2 [30]. In addition, the expression of the full length
485 caspase 3 and 9 is significantly reduced by the combined treatment as compared to single
486 treatments, suggesting the involvement of downstream effector caspases in the apoptotic cell death
487 induced by the concomitant inhibition of UCP2 and mTOR complex (**Fig. 65D**). The regulation of
488 Bcl 2 and of caspase 9, which in turn cleaves the downstream pro-caspase 3 in its truncated active
489 form, suggests that the mitochondrial death pathway is implicated in genipin/everolimus induced
490 apoptosis.

491

492 *3.4 Anti-apoptotic effect of Beclin1-dependent autophagy induced by genipin and everolimus*

493 Since GAPDH nuclear translocation can induce autophagy-related genes [31], we
494 investigated whether the combined treatment stimulates the formation of autophagic vesicles in
495 PDAC cells by using staining with the fluorescent probe monodansylcadaverine (MDC). FACS
496 analysis revealed that PDAC cells treated with the drug combination incorporate a higher amount of
497 MDC probe as compared with single treatments, suggesting an autophagy stimulation (**Fig. 76A**).
498 These data have been confirmed analyzing MDC incorporation using a microplate fluorescence
499 reader (data not shown). To demonstrate that autophagy stimulation by drug combination results in
500 the fusion of autophagy vesicles with lysosomes, we stained cells with the fluorescent probe
501 acridine orange (AO), which changes its fluorescence emission from green to red upon
502 accumulation into lysosomal acidic compartments revealing the typical pH acidification of
503 lysosomes during autophagic stimulation. Fluorescence microscopy experiments confirmed the
504 presence of wide red areas (acidic lysosomes containing accumulation of AO probe) into the

505 | cytoplasm of PDAC cells treated with drug combination (**Fig. 76B**), supporting the statement that
506 | the concomitant inhibition of UCP2 and mTOR can contribute to the formation of autophagic
507 | vesicles and their fusion with lysosomes in cancer cells.

508 | The investigation of the mechanism involved in the stimulation of autophagy revealed that
509 | Beclin 1 (Atg 6), a well-known key regulator of autophagy, is strongly induced by the combined
510 | drug setting (**Fig. 76C**). In addition, since heat shock protein 90 (Hsp 90) is a molecular chaperone
511 | that regulates the stability of signaling proteins, including the maintenance of Beclin 1 protein
512 | stability through an evolutionarily conserved domain [32], we investigated whether genipin and
513 | everolimus can affect Beclin1:Hsp90 complex. Intriguingly, we observed by immunoprecipitation
514 | assay that everolimus promotes the interaction between Beclin 1 and Hsp 90 and that the formation
515 | of this complex is further enhanced by drug combination (**Fig. 76D**), strongly suggesting a role for
516 | Hsp 90 in Beclin 1 induction by the combined treatment. To test the functional role of Beclin 1
517 | enhancement in autophagy stimulation, we knocked-down its expression by using a siRNA-Beclin1
518 | (see control experiment in **Suppl. Fig. 54**). **Figure 76E** indicates that autophagosome vesicle
519 | formation by drug combination is completely abolished by siRNA-Beclin1, highlighting the key
520 | role of this autophagy mediator protein in the overall process. Furthermore, since the role of
521 | autophagy in modulating cell growth is highly dependent on the metabolic context and on the
522 | microenvironmental conditions in which the cells lie [33], we investigated the impact of this
523 | process on the overall antiproliferative effect induced by drug combination. **Figure 76F** shows that
524 | the percentage of PDAC cell growth is further decreased when a non-toxic concentration of 3-
525 | methyladenine (3-MA), a known inhibitor of the autophagosome formation process, is added to
526 | increasing concentrations of genipin+everolimus combination. Notably, the apoptotic effect of the
527 | drugs is strongly potentiated by 3-MA (**Fig. 76G**), demonstrating that Beclin1-dependent autophagy
528 | retains a protective and pro-survival role counteracting apoptotic cell death induced by the drugs.

529

530 | *3.5 Genipin and everolimus strongly inhibit growth of PDAC cells in mice xenografts*

531 The effect of the intraperitoneal delivery of genipin and/or everolimus on growth inhibition
532 of PaCa44 cells subcutaneously xenografted in nude mice was also investigated. **Figure 87A** shows
533 that the volume of tumors in mice treated with the drug combination remains essentially unchanged
534 during the observation time, whereas it strongly increases in the control group and, at a lower
535 extent, in either genipin- or everolimus-treated groups. Tumor masses after mice sacrifice were
536 recorded and shown in **Figure 87B** for control and combined treatment, further pointing out the
537 high efficiency in the reduction of PDAC masses *in vivo*. To confirm this antiproliferative effect by
538 genipin+everolimus treatment and the synergistic outcome observed in PDAC cell cultures, we
539 demonstrated that the number of mitoses dramatically decreases only in tumor masses derived from
540 mice injected with the drug combination (**Fig. 87C**). To investigate whether treatments might
541 determine toxic effects, we analysed mice body weights during all the experiment period and, at the
542 sacrifice of mice, we collected various organs (heart, kidney, liver, lung and pancreas) for
543 haematoxylin and eosin (H&E) staining. **Figure 87D** shows that all the treatments do not change
544 mice body masses during the experiment, suggesting the absence of any apparent toxicity. In
545 addition, H&E histopathologic evaluation of the organs examined revealed the absence of any
546 significant side effects for the animals (**Suppl. Fig. 65**).

547

548

549 **4. Discussion**

550 ~~UCP2 over expression is a general phenomenon correlated with the progression of human~~
551 ~~cancers, including PDAC, and can be thus considered a valuable potential target for therapy [12,~~
552 ~~18]. UCP2 has been reported to promote chemoresistance [34], metabolic aberrant alterations in~~
553 ~~cancer cells [15, 35], and to overcome oxidant stressful stimuli of various origins [36] enhancing~~
554 ~~the development of the neoplastic disease.~~

555 In this study, we demonstrate *in vitro* and in mice xenograft that UCP2 inhibition strongly
556 triggers the oncogenic pathway Akt/mTOR, providing the rationale for the antiproliferative
557 synergistic effect mediated by the UCP2 inhibitor genipin when used in combination with the
558 mTOR inhibitor everolimus. Beyond the Akt/mTOR/P70S6K signaling inhibition, we demonstrate
559 that everolimus strongly potentiates ROS production and PDAC cell death induced by genipin. This
560 anti-proliferative effect by drug combination setting is due to a massive GAPDH nuclear
561 translocation observed both *in vitro* and in mice xenograft. This determines caspase-mediated
562 apoptotic cancer cell death as well as a protective autophagic process mediated by Beclin1 in PDAC
563 cells (**Figure 98**). This latter event can be likely considered as an extreme tentative of cancer cells
564 to survive in drug-induced stressing conditions. Thus, the full anticancer effect by genipin and
565 everolimus combination can be achieved when the autophagic process is blocked.

566 Beyond glycolysis, several reports have demonstrated that GAPDH has a variety of other
567 functions, including DNA repair [37], transcriptional regulation [38], membrane fusion and
568 transport [39, 40], autophagy [31], and cell death [41, 42]. Indeed, in accord with its multiple
569 activities, the intracellular localization of GAPDH is not restricted to the cytosol for energy
570 production but is detected in the plasma and nuclear membrane, in the endoplasmic reticulum, in
571 the Golgi apparatus, and in the nucleus [28]. GAPDH nuclear translocation is a particularly critical
572 event in the light that, although the enzyme contains a nuclear export signal [43], it does not contain
573 an equivalent nuclear localization signal (NLS). Thus, it is likely that other mechanisms need to be
574 utilized for GAPDH translocation probably by binding with other molecules after post-translational

575 modifications of the enzyme. For instance, upon exposure to oxidative stress, GAPDH nuclear
576 translocation involves S-nitrosyl post-translational modification at its active site Cys152 [29].
577 Oxidized GAPDH binds to Siah1, an ubiquitin ligase, which contains its own NLS sequence
578 thereby facilitating the intracellular transfer of this protein:protein complex and the subsequent
579 transcriptional induction of apoptotic and cell death-related genes [29, 44]. Many other post-
580 translational modifications of GAPDH have been described, including phosphorylation, acetylation,
581 glycosylation, poly ADP-ribosylation, and pyruvylation [45]. For these reasons GAPDH has
582 received considerable attention as an appealing drug target for anticancer therapy [46].

583 Here, we show that the strong increase in GAPDH nuclear translocation and subsequent
584 PDAC cell death by genipin and everolimus combination is due to both: *i*) oxidation of the enzyme
585 by ROS increase, and *ii*) the everolimus-mediated counteraction of the Akt/mTOR pathway
586 stimulated by genipin. Indeed, this oncogenic signaling is described to stabilize GAPDH in the
587 cytosol through phosphorylation in Thr-237 of the enzyme decreasing its nuclear translocation and
588 GAPDH-mediated apoptosis [24, 47]. The functional involvement of GAPDH nuclear translocation
589 has been confirmed by the capability of AXP3009 to counteract PDAC cell death and apoptosis
590 induced by drug combination. AXP3009 is a novel synthetic compound addressed to interact with
591 the *Pf*GAPDH catalytic cysteine [20]. Here, we demonstrate that AXP3009 can prevent the nuclear
592 translocation of GAPDH in PDAC cells, likely through conformational changes observed in
593 recombinant *h*GAPDH that can elude the oxidation of the redox-sensitive catalytic cysteine.
594 Notably, we observe a strong increase in the percentage of GAPDH nuclear positivity in tumor
595 masses in nude mice injected with the drug combination. Concomitantly, tumor volume in mice
596 treated with the two drugs remains essentially unchanged during the observation time without any
597 signs of toxic effects for animals.

598 The synergistic PDAC cell growth inhibition obtained with everolimus and genipin
599 treatment can allow to markedly reduce the concentrations of the two drug when used in
600 combination. Optimal dosing is a crucial factor in order to achieve a maximum therapeutic efficacy

601 with appropriate drug exposure and to maintain patient quality of life. CalcuSyn software analysis
602 shows that the concentration of everolimus ~~can be reduced of about 24 folds~~ and that of genipin ~~of~~
603 ~~about 11 folds~~ can be strongly reduced to obtain 50% PDAC cell growth inhibition in combined
604 setting as compared to the concentrations needed to obtain the same effect in single treatment
605 (Table 1). Notably, mTOR inhibitors-induced side effects, which can potentially compromise their
606 therapeutic effectiveness, are well characterized and include epithelial-cutaneous adverse events,
607 pulmonary dysfunctions, metabolic alterations, and fatigue [48]. Although everolimus is a currently
608 approved drug for the treatment of pancreatic cancer, genipin has not been developed for clinical
609 use yet but it represents a valuable tool for the preclinical investigation of UCP2 roles and for the
610 study of the potential clinical usage of next generation UCP2 inhibitors for therapy. Overall
611 in conclusion, our results and the statement that UCP2 overexpression promotes chemoresistance in
612 cancers support the combined inhibition of UCP2 and of Akt/mTOR pathway as a novel therapeutic
613 strategy in the treatment of PDAC.

614

615

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621

622 **Conflict of interest**

623 The authors declare that they have no conflicts of interest.

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761

762

763 **Figure Legends**764 **Figure 1. Akt/mTOR pathway regulation by UCP2 in pancreatic ductal adenocarcinoma cells.**

765 (A) Qualitative and quantitative analysis of p-Akt expression on PaCa44 derived xenograft tumors
 766 (*in vivo*) and on PaCa44 cell line (*in vitro*) untreated/treated with genipin. P-Akt expression
 767 representative images are reported for tumors grown in control mice and in mice treated with
 768 genipin (for genipin subadministration see Materials and Methods) at 20-fold magnification, scale bar
 769 50 μm , and for *in vitro* a representative immunoblot image is reported for p-Akt expression in
 770 PaCa44 cells untreated/treated with 200 μM Genpin for 24h. (B) Western blot analysis of the total
 771 and phosphorylated form of the mTOR target P70S6K in PaCa44 cells transfected with 200 nM
 772 siRNA control or siRNA UCP2 for 72 h. (C) Analysis of DCF fluorescence intensity,
 773 corresponding to the level of ROS production, by a multimode plate reader in PaCa44 cells treated
 774 with 200 μM genipin and/or 10 mM NAC for 24 h. (D) Western blot analysis of the total and
 775 phosphorylated form of Akt or of the mTOR target P70S6K in PaCa44 cells treated with 200 μM
 776 genipin and/or 10 mM NAC for 24 h. The value of α -tubulin was used as a normalizing factor and
 777 protein fold change is relative to each control. Values are the means ($\pm\text{SD}$) of three independent
 778 experiments. Statistical analysis: (*) $P < 0.05$ and (***) $P < 0.001$ for treated cells vs each control or
 779 genipin vs genipin+NAC. Legend: (ctrl) control and (G) genipin.

780

781 **Figure 2. The combined treatment genipin/everolimus impairs Akt/mTOR signaling and**
 782 **increases ROS production and anti-proliferative effects.**

783 (A) Western blot analysis of the total and phosphorylated form of Akt and P70S6K in PaCa44 cells treated with 200 μM genipin and/or
 784 10 μM everolimus for 24 h. (B) Analysis of MitoSox fluorescence intensity, corresponding to the
 785 level of mitochondrial superoxide production, by a multimode plate reader in PaCa44 cells treated
 786 with 140 μM genipin and/or 7 μM everolimus for 24 h. (C) Cell growth analysis through the
 787 Crystal Violet colorimetric assay of PaCa44 cells treated with 100 μM genipin and/or 5 μM
 788 everolimus and/or 10 mM NAC for 48h. Values are the means of three independent experiments
 789 each performed in triplicate. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ for
 790 treated cells vs control or genipin+everolimus vs single treatments. Legend: (ctrl) control, (G)
 791 genipin, and (E) everolimus.

792

793 **Figure 3. The combined treatment genipin/everolimus has a synergistic anti-proliferative**
 794 **effect on five PDAC cell lines.** (A) Cell growth analysis through the Crystal Violet colorimetric

795 assay of five PDAC cell lines (PaCa44, Panc1, MiaPaCa2, PaCa3, and T3M4) treated with
 796 increasing doses of the drugs [genipin from 500 nM to 200 μ M; everolimus from 25 nM to 10 μ M]
 797 maintaining the ratio genipin:everolimus 20:1 for 48 h. Black squares, black rhombus and white
 798 circles correspond to genipin, everolimus and the combined treatment, respectively. Values are the
 799 means of three independent experiments each performed in triplicate. (B) Analysis of the
 800 synergistic cell growth inhibition of the five PDAC cell lines (PaCa44, Panc1, MiaPaCa2, PaCa3,
 801 and T3M4) treated with increasing doses of the drugs [genipin from 500 nM to 200 μ M; everolimus
 802 from 25 nM to 10 μ M] maintaining the ratio genipin:everolimus 20:1 for 48 h. The values on the x
 803 axis correspond to the fraction of growth inhibition (0 \rightarrow 1) given by increasing concentrations of
 804 drug combinations. The values on the y axis correspond to the measurement of CI (see Materials
 805 and Methods).

806

807 **Figure 4. mTOR inhibition enhances GAPDH nuclear translocation in genipin-treated cells.**
 808 (A) Count of GAPDH positive nuclei in vital neoplastic cells on 20 consecutive HPFs of PaCa44
 809 derived xenograft tumors. Mice have been untreated/treated with genipin and/or everolimus, as
 810 reported in Materials and Methods. Representative images are reported for control mice and for
 811 mice treated with genipin+everolimus at 40-fold magnification, scale bar 20 μ m. (B) PaCa44 cells
 812 untreated or treated with 200 μ M genipin and/or 10 μ M everolimus for 24 h. GAPDH positive
 813 nuclei have been counted on several different fields for each condition and the amount of positive
 814 nuclei has been normalized on cell viability. (C) Representative immunofluorescence images of
 815 GAPDH (green) translocation into the nuclei (labelled with Hoechst dye) of PaCa44 cells untreated
 816 or treated with 200 μ M genipin and 10 μ M everolimus for 24 h, at 40-fold magnification, scale bar
 817 10 μ m. (D) Quantitative analysis of GAPDH mRNA through real-time PCR (qPCR) of PaCa44 cells
 818 treated with 200 μ M genipin and/or 10 μ M everolimus for 24 h. Total RNA was extracted from
 819 cells and qPCR was performed as described in Materials and Methods. Statistical analysis: (*)
 820 $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ for treated cells vs control or genipin+everolimus vs single
 821 treatments. Legend: (ctrl) control, (G) genipin, and (E) everolimus.

822

823 **Figure 5. AXP3009 alters GAPDH conformation and counteracts cell growth inhibition by**
 824 **genipin/everolimus treatment.** (A) Comparison of the circular dichroism spectra of AXP3009-
 825 bound (dashed line) and unbound *h*GAPDH (solid line) in the 190-250 nm range. Circular
 826 dichroism spectra of GAPDH at 10 μ M in a buffered solution containing 10 mM sodium phosphate

827 at pH 7.6 in the 250-345 nm range were collected in the absence of AXP3009 (solid line) and upon
 828 overnight incubation with 100 μ M AXP3009 (dashed line). **(B)** Thermal denaturation kinetics of
 829 AXP3009-bound (open symbols) and unbound *h*GAPDH (closed symbols) from two independent
 830 experiments each. The solid lines are the fitting to a sigmoidal equation, indicative of a two-state
 831 model. **(C)** Apparent molecular weights of AXP3009-bound and unbound GAPDH on a superdex
 832 200 increase 3.2/300 column as estimated by comparison with calibrants in the range 29 -440 kDa.
 833 The reported values are the means \pm s.e.m. of five independent measurements. **(D)** Western blot
 834 analysis and quantification of GAPDH expression into the cytosol and nuclei of PaCa44 cells
 835 untreated/treated with 50 μ M AXP3009 for 48 h. α -tubulin and Lamin B1 have been used as
 836 controls of the quality of the cytoplasmic and nuclear protein fractions, respectively. Amido Black
 837 staining was used to confirm equal cytoplasmic and nuclear protein loading in the different lanes.
 838 **(E) Cell growth analysis through the Crystal Violet colorimetric assay of PaCa44 cells treated with**
 839 **50 μ M of AXP3009 and/or with 100 μ M genipin and/or 5 μ M everolimus for 48h. Values are the**
 840 **means (\pm SD) of three independent experiments. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and**
 841 **(***) $P < 0.001$ for treated cells vs control or single treatment vs the relative single**
 842 **treatment+AXP3009 or genipin+everolimus vs genipin+everolimus+AXP3009. Legend: (ctrl)**
 843 **control, (G) genipin, (E) everolimus, (C) cytoplasmic fraction, and (N) nuclear fraction.**

844

845 **Figure 6. mTOR inhibition increases apoptosis in genipin-treated cells.** Apoptosis analysis
 846 through the Annexin V binding assay of PaCa44 cells treated with **(A)** 100 μ M genipin and/or 5 μ M
 847 everolimus for 48 h and/or with **(B)** 1 μ M AXP3009 and 10 mM NAC. **(C)** Quantitative analysis of
 848 CHOP mRNA through real-time PCR (qPCR) of PaCa44 cells treated with 200 μ M genipin and/or
 849 10 μ M everolimus for 8 h. Total RNA was extracted from cells and qPCR was performed as
 850 described in Materials and Methods. **(D)** Western blot analysis of three apoptosis-related proteins
 851 (Bcl2, caspase 3 full length, and caspase 9 full length) in PaCa44 cells treated with 100 μ M genipin
 852 and/or 5 μ M everolimus for 48 h. The bands of Western blot analyses were scanned as digital peaks
 853 and the areas of the peaks were calculated in arbitrary units. The value of α -tubulin was used as a
 854 normalizing factor and protein fold change is relative to control cells. Values are the means (\pm SD)
 855 of three independent experiments. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$
 856 for treated cells vs control, or single treatments vs genipin+everolimus, or genipin+everolimus vs
 857 genipin+everolimus+AXP3009 or vs genipin+everolimus+NAC. Legend: (ctrl) control, (G)
 858 genipin, and (E) everolimus.

859

860 **Figure 7. mTOR inhibition induces autophagy and related-mechanisms in genipin-treated**
 861 **cells.** (A) Autophagosome formation assay through the analysis of the incorporation of
 862 monodansylcadaverine (MDC) probe in PaCa44 cells treated with 100 μ M genipin and/or 5 μ M
 863 everolimus for 48 h through flow cytometry. (B) Representative immunofluorescence images of
 864 autophagosome formation through acridine orange staining of PaCa44 cells treated with 100 μ M
 865 genipin and 5 μ M everolimus for 24 h, at 40-fold magnification, scale bar 10 μ m. (C) Western blot
 866 analysis of Beclin1 in PaCa44 cells treated with 100 μ M genipin and/or 5 μ M everolimus for 24 h.
 867 Amido black staining was used to confirm equal protein loading in the different lanes. (D)
 868 Immunoprecipitation analysis and relative quantification of Beclin-1/Hsp90 in PaCa44 cells treated
 869 with 200 μ M genipin and/or 10 μ M everolimus for 24 h. (E) Autophagosome formation assay
 870 through the incorporation of the MDC probe in PaCa44 cells transfected with 50 nM siRNA
 871 Beclin1 for 48 h and/or treated with 200 μ M genipin and 10 μ M everolimus for 48h. (F) Cell
 872 growth analysis through the Crystal Violet colorimetric assay of PaCa44 cells pre-treated for 1 h
 873 with 5 mM 3-MA and/or treated with increasing doses of the drugs [genipin from 500 nM to 140
 874 μ M; everolimus from 25 nM to 7 μ M] maintaining the ratio genipin:everolimus 20:1 for 48 h. (G)
 875 Apoptosis analysis through the Annexin V binding assay of PaCa44 cells pre-treated for 1 h with 5
 876 mM 3-MA and/or treated with 100 μ M genipin and 5 μ M everolimus for 48 h. Values are the
 877 means (\pm SD) of three independent experiments. Statistical analysis: (*) $P < 0.05$, (**) $P < 0.01$, and
 878 (***) $P < 0.001$ for treated cells vs control, or single treatments vs genipin+everolimus, or
 879 genipin+everolimus vs genipin+everolimus+3-MA, or genipin+everolimus vs
 880 genipin+everolimus+siRNA Beclin1. Legend: (ctrl) control, (G) genipin, and (E) everolimus.

881

882 **Figure 8. *In vivo* effect of mTOR inhibition in genipin-treated nude mice.** PaCa44 cells were
 883 subcutaneously injected into female nude mice. After 1 week, i.p. injections with PBS (solution
 884 vehicle), genipin, and/or everolimus were administered twice a week for 4 weeks, as described in
 885 Materials and Methods. (A) Tumor volumes were measured at 3 days after each injection and
 886 values are the mean of mice tumor volumes in each group. Statistical analysis is referred to the days
 887 at which the tumor volume of the mice treated with genipin+everolimus is significantly lower than
 888 the other three groups, such as the control mice and the mice treated with the drugs alone. (*)
 889 $P < 0.05$. (B) Four representative images of tumor masses grown in control mice and in mice treated
 890 with genipin+everolimus. (C) Count of mitotic figures on 10 consecutive HPFs (high power field,
 891 40-fold magnification, scale bar 20 μ m) for each neoplastic sample. Representative images are
 892 reported for control mice and for mice treated with genipin+everolimus. Statistical analysis is

893 referred to mice treated with the drug combination genipin+everolimus vs control mice. (***)
894 $P < 0.001$. **(D)** Mice body mass was measured at 3 days after each injection and values are the means
895 of mice body in each group. Legend: (ctrl) control, (G) genipin, and (E) everolimus.

896

897 **Figure 9.** Schematic representation of the molecular mechanisms regulated by UCP2 and
898 Akt/mTOR inhibition identified in this study.

899

900 **Supplementary Figure 1.** Quantitative analysis of UCP2 mRNA through real-time PCR (qPCR) of
901 PaCa44 cells transfected with 200 nM siRNA control or siRNA UCP2 for 72 h. Total RNA was
902 extracted from cells and qPCR was performed as described in Materials and Methods.

903 **Supplementary Figure 2.** Western blot analysis of the total and phosphorylated form of the mTOR
904 target P70S6K in three pancreatic ductal cell lines (PaCa3, PaCa44, and Panc1) treated with 400
905 μM genipin for 24 h. Legend: (ctrl) control and (G) genipin.

906 **Supplementary Figure 3.** Isobolograms obtained from the CalcuSyn output at 0.5 of fractional
907 effect (IC_{50}) for five PDAC cell lines (PaCa44, Panc1, MiaPaCa2, PaCa3, and T3M4) treated for 48
908 h. IC_{50} of genipin and everolimus alone are reported as cross on the x and y axis, respectively,
909 whereas the black dot represents the IC_{50} of the combined treatment. The combination data point
910 indicates an additive effect when it is plotted on the diagonal, a synergistic effect when it is on the
911 lower left, and an antagonistic effect when it is on the upper right.

912 **Supplementary Figure 4.** **(A)** Quantitative analysis of GAPDH mRNA through real-time PCR
913 (qPCR) of PaCa44 cells transfected with 200 nM siRNA control or siRNA GAPDH for 72 h. Total
914 RNA was extracted from cells and qPCR was performed as described in Materials and Methods. **(B)**
915 Cell growth analysis through the Crystal Violet colorimetric assay of PaCa44 cells treated with 50
916 μM AXP3009 and/or with GAPDH siRNA for 48h.

917 **Supplementary Figure 5.** Quantitative analysis of Beclin-1 mRNA through real-time PCR (qPCR)
918 of PaCa44 cells transfected with 50 nM siRNA Beclin1 (siBECN1) for 48 h. Total RNA was
919 extracted from cells and qPCR was performed as described in Materials and Methods.

920 **Supplementary Figure 6.** Haematoxylin and eosin (H&E) tissue staining of nude mice injected
921 subcutaneously with PaCa44 cells. Representative images of heart, kidney, liver, lung and pancreas

922 in control mice and in mice treated with the drug combination genipin+everolimus taken at 10-fold
923 magnification, scale bar 100 μm .

Table 1. Combination Index (CI) and Dose Reduction Index (DRI) values for genipin (G) and everolimus (E) combination.

Cell lines	CI ₅₀	CI ₇₅	CI<1	DRI ₅₀	<i>r</i>
PaCa44	0.129	0.561	75%	E: 24.16 G: 11.39	0.84
Panc1	0.085	0.823	100%	E: 88.6 G: 13.6	0.91
MiaPaCa2	0.112	1.37	100%	E: 5929.7 G: 8.9	0.88
PaCa3	7.1×10^{-5}	6.9×10^{-5}	90%	E: 5×10^{14} G: 1.4×10^4	0.88
T3M4	0.09	1.45	80%	E: 548.9 G: 11.2	0.90

CI₅₀ was calculated for 50% cell growth inhibition in the combined treatment by isobologram analyses performed with the CalcuSyn software. CI value < 1 indicates the percentage of experimental conditions with increasing drug concentrations determining a synergistic effect. DRI₅₀ represents the folds of dose reduction to obtain 50% cell growth inhibition in combination setting as compared to each drug alone.

Figure 1
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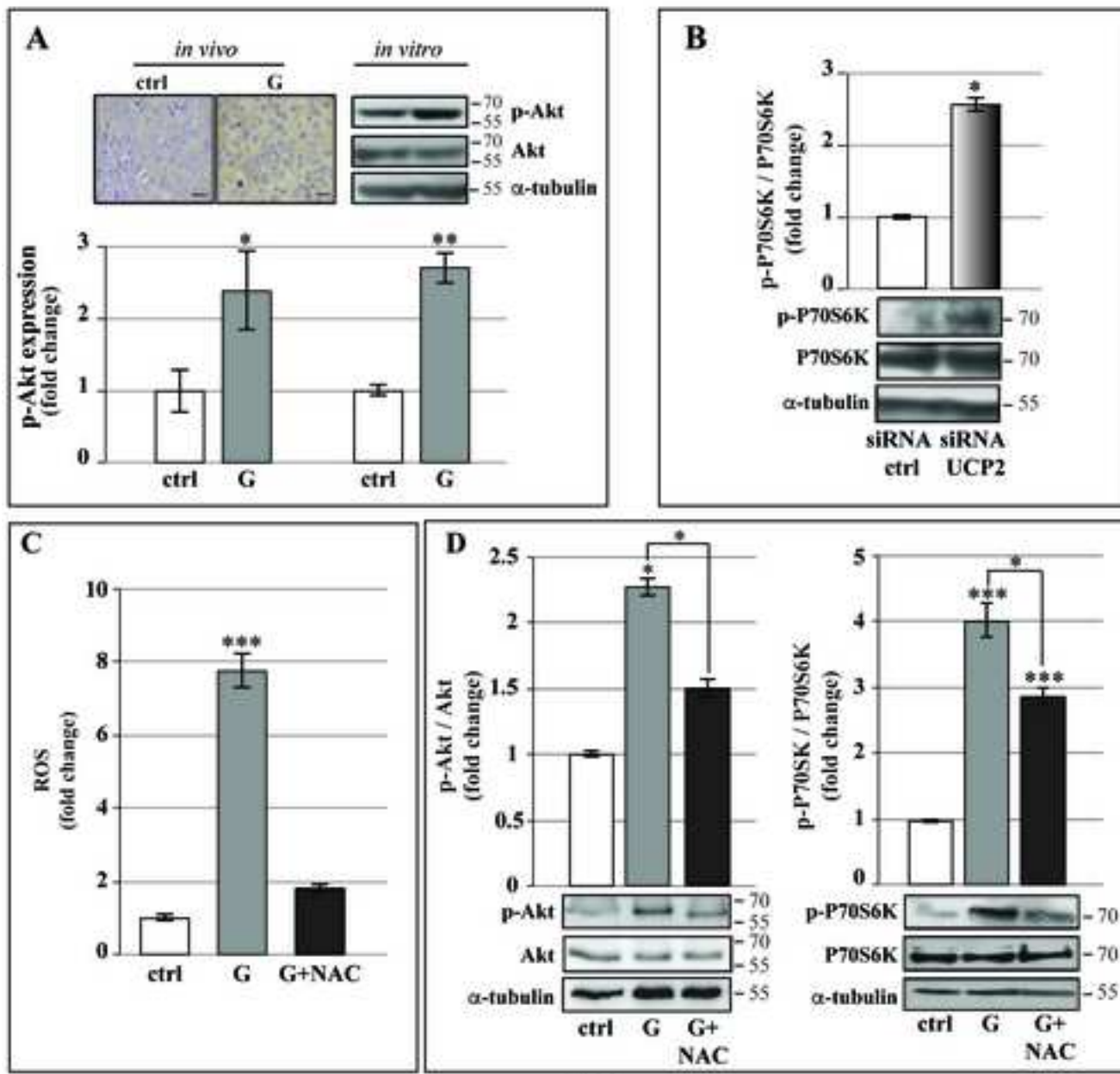


Fig.1

Figure 2
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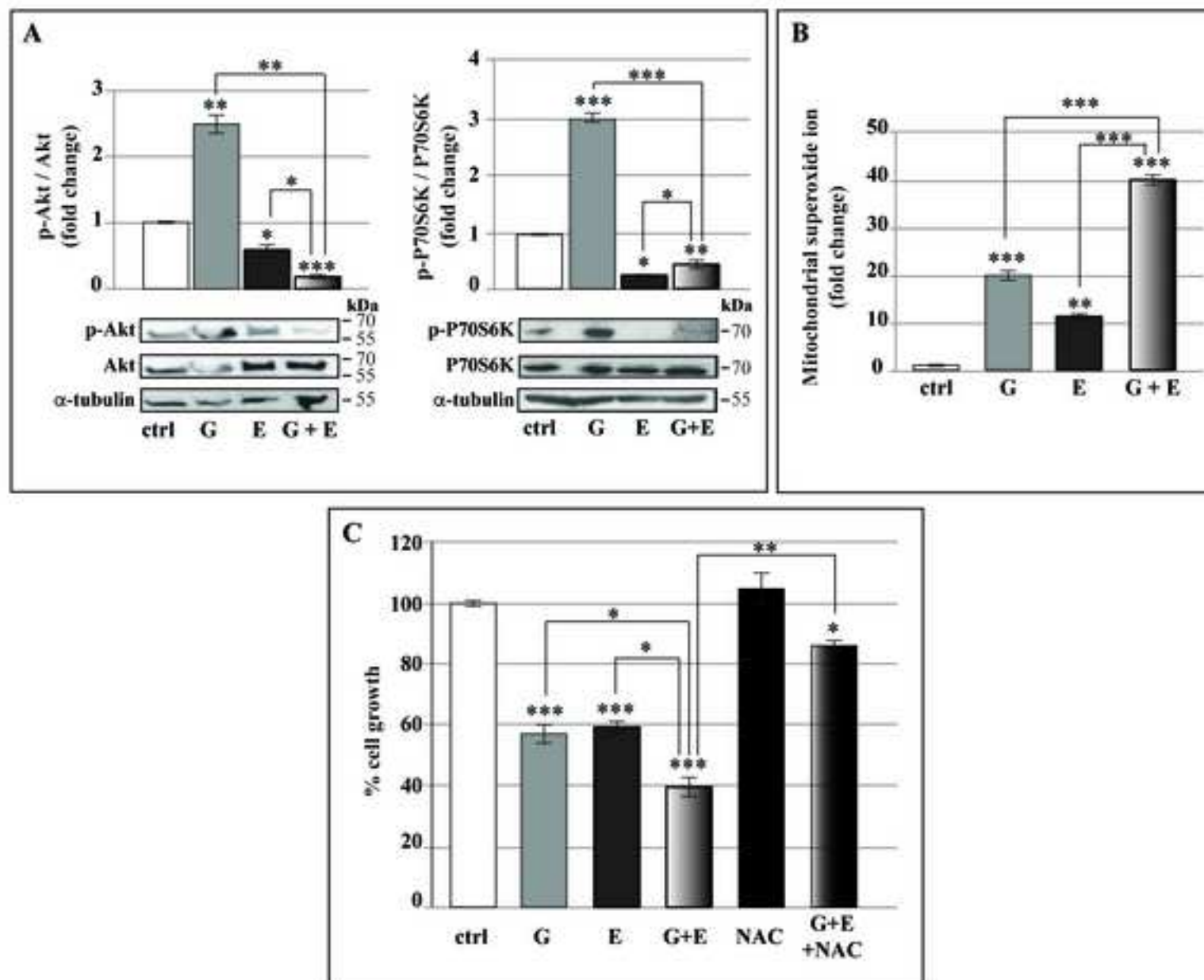
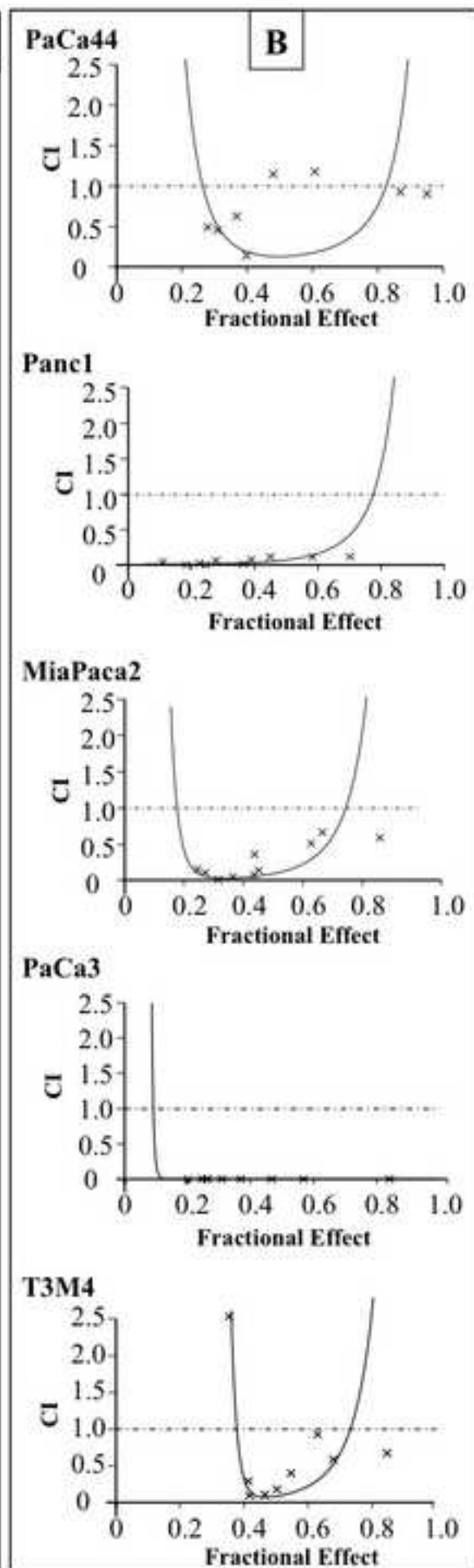
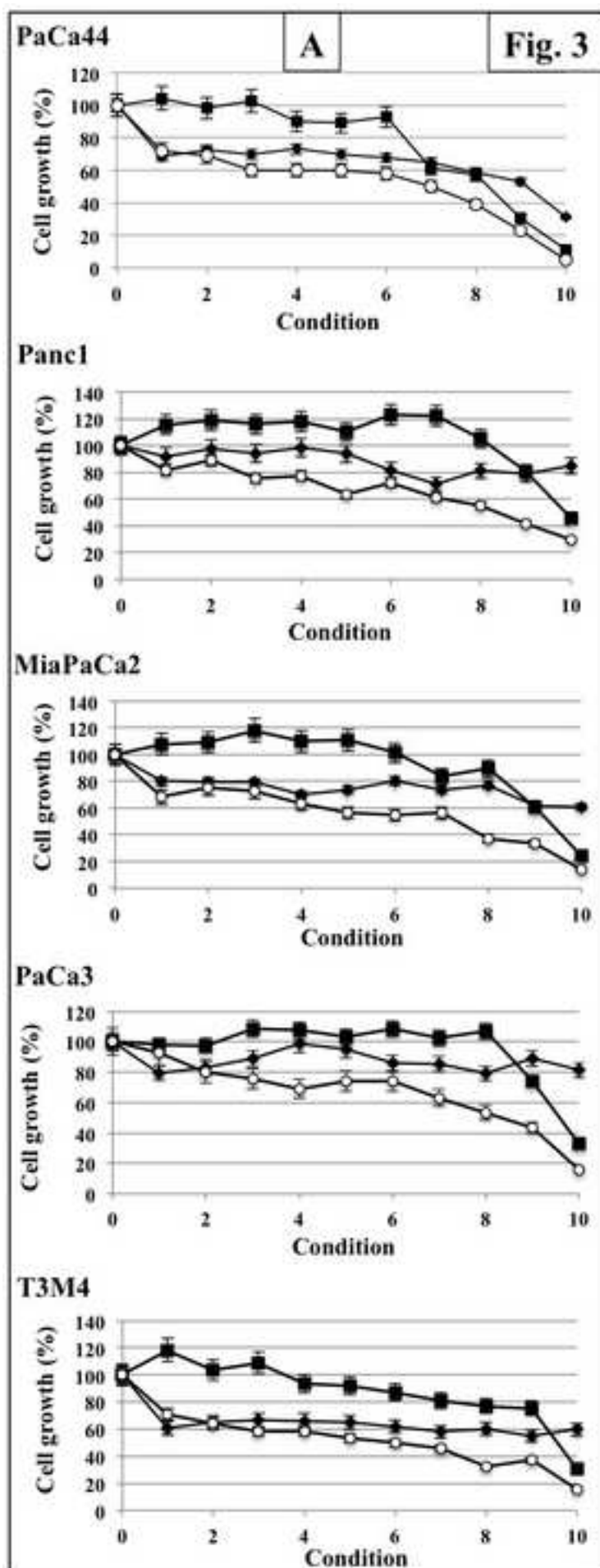


Fig. 2

Figure 3
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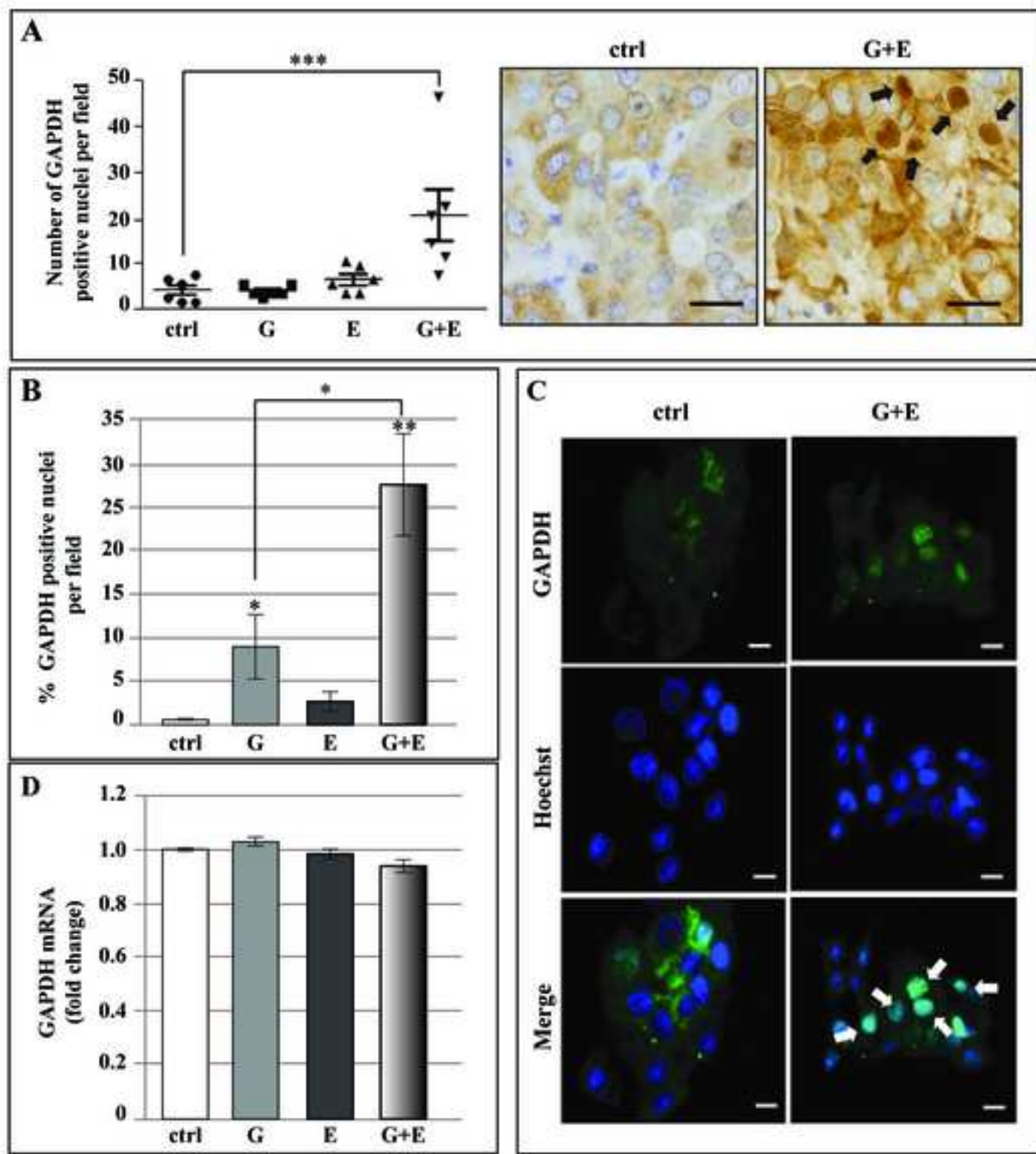


Fig. 4

Figure 5
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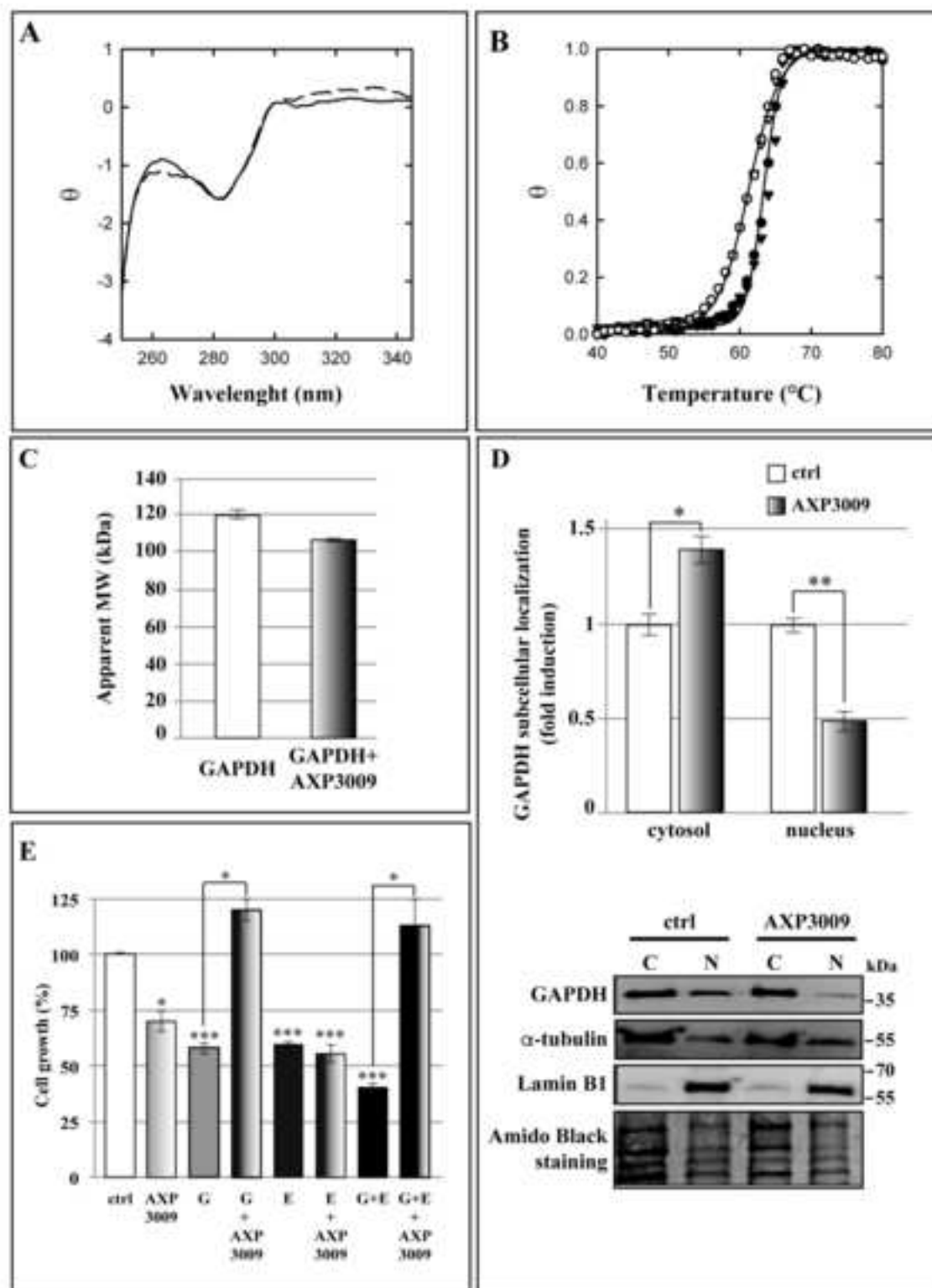


Fig. 5

Figure 6
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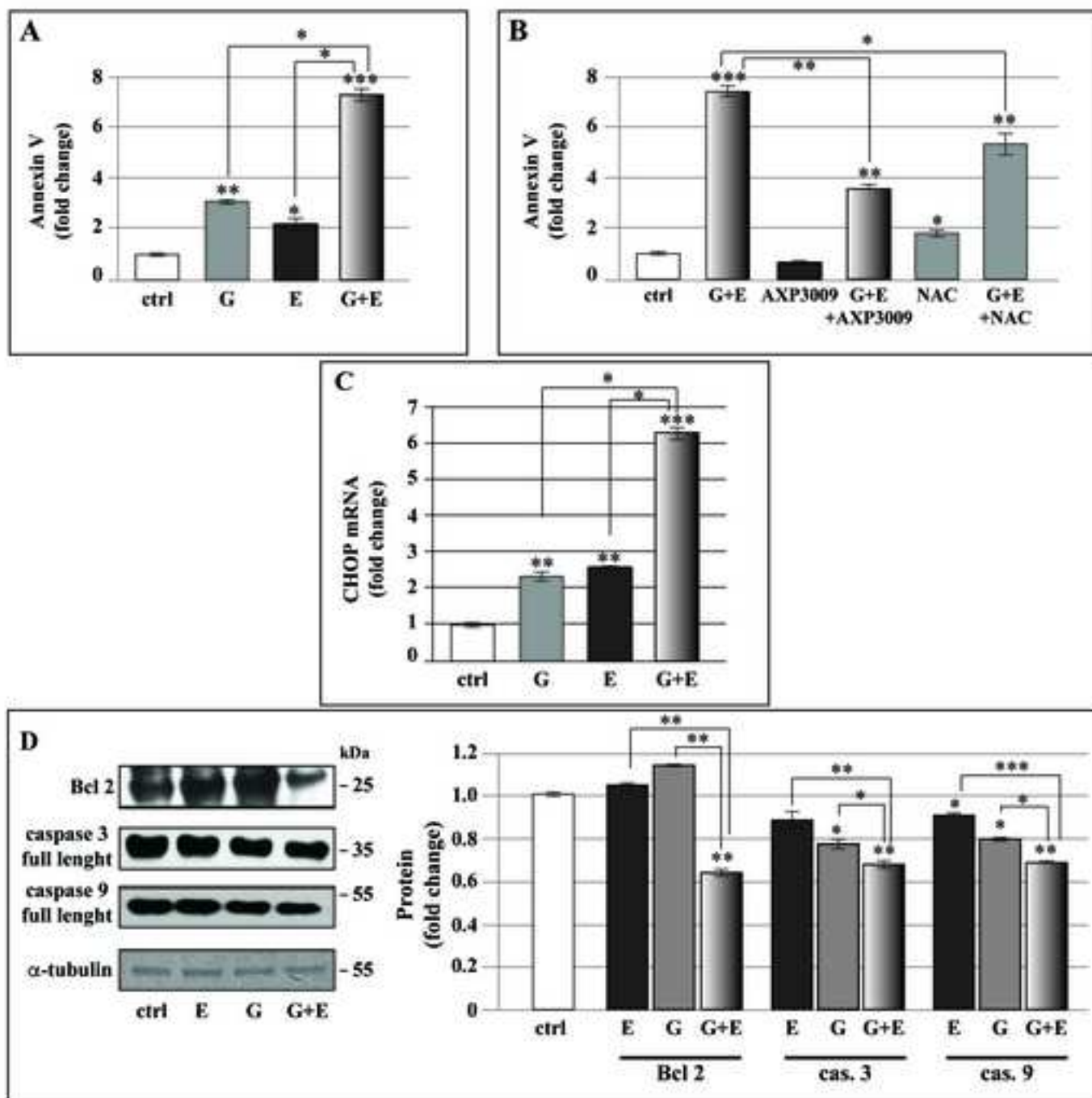


Fig. 6

Figure 7
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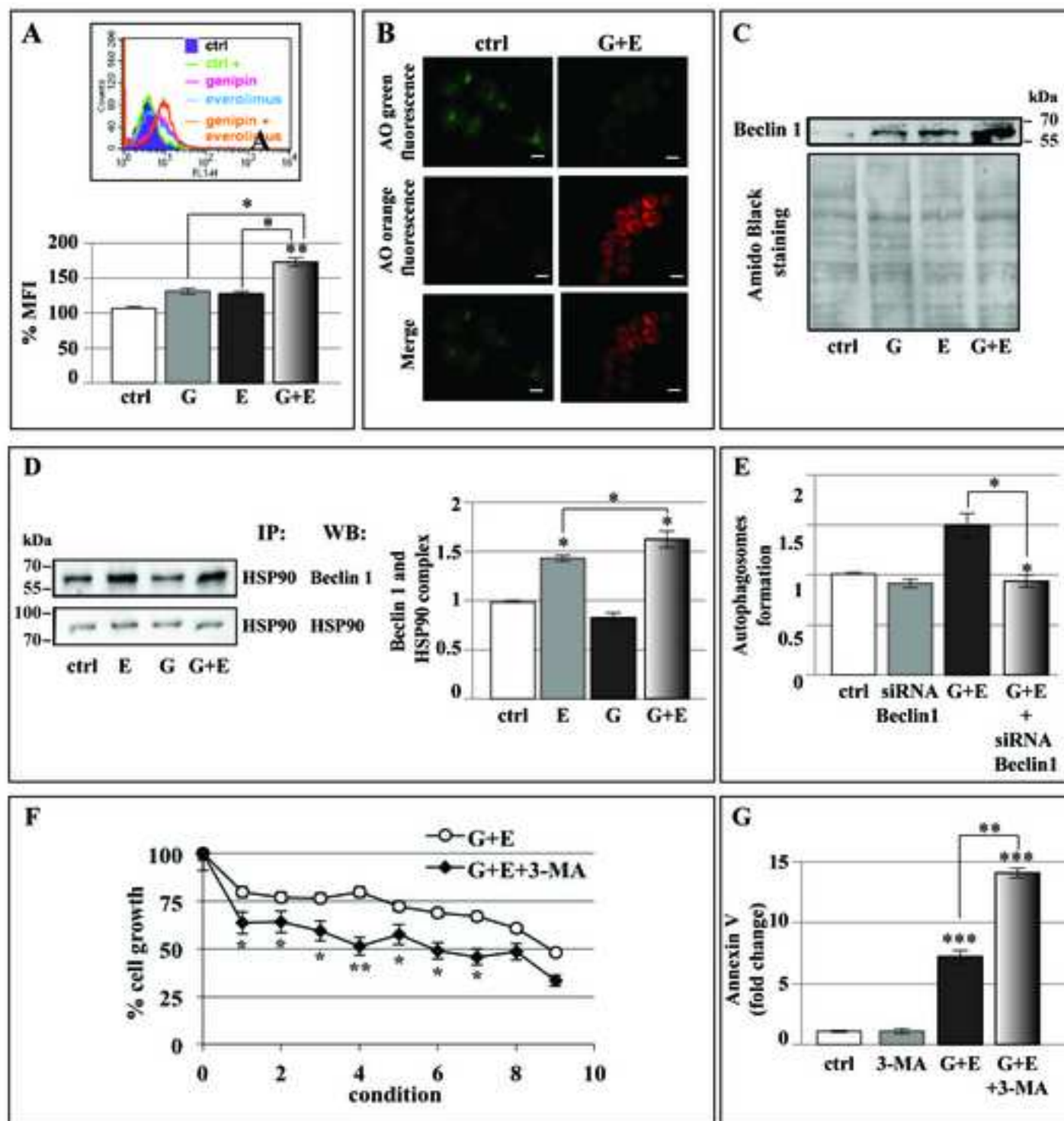


Fig.7

Figure 8
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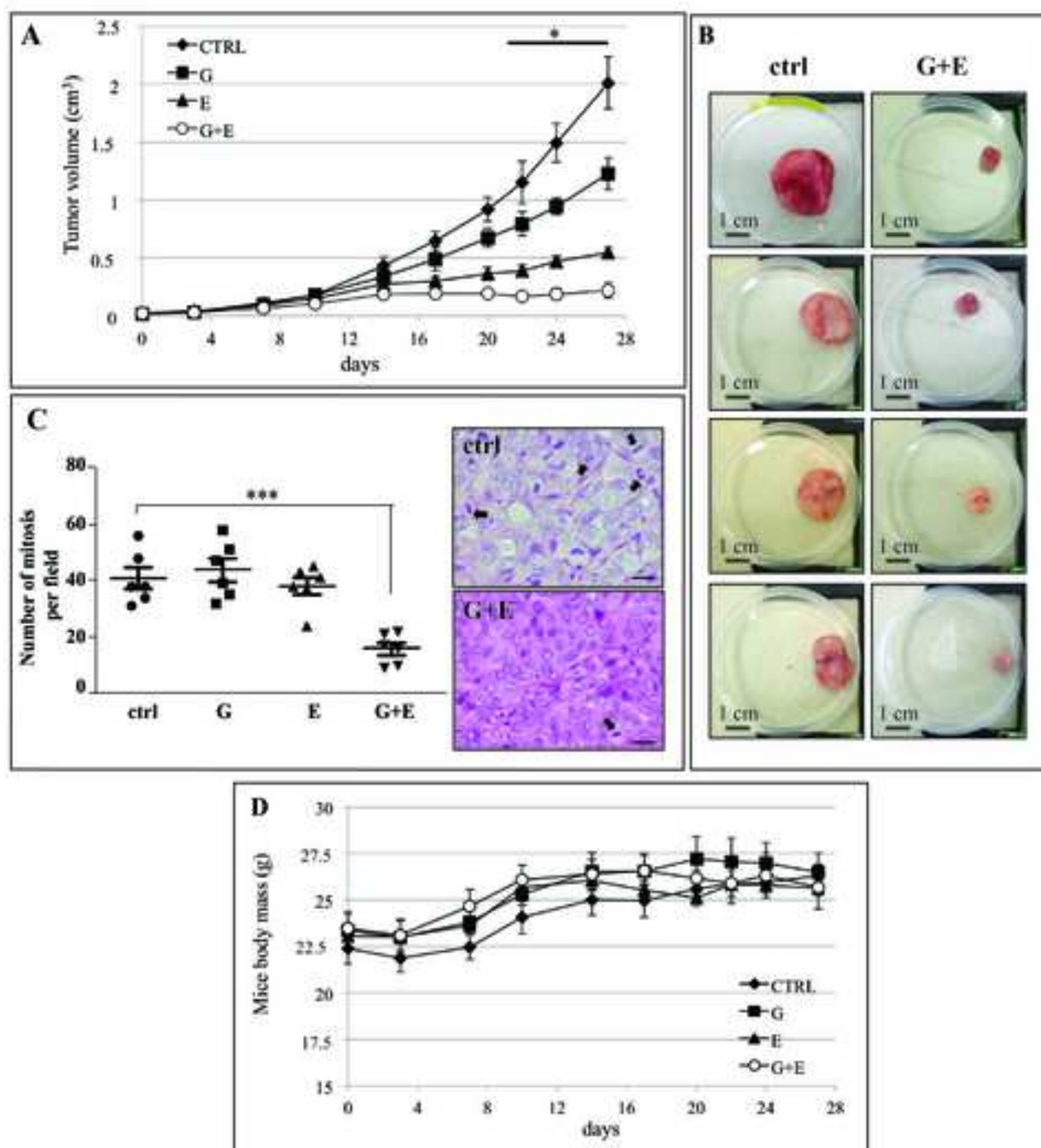


Fig.8

Figure 9
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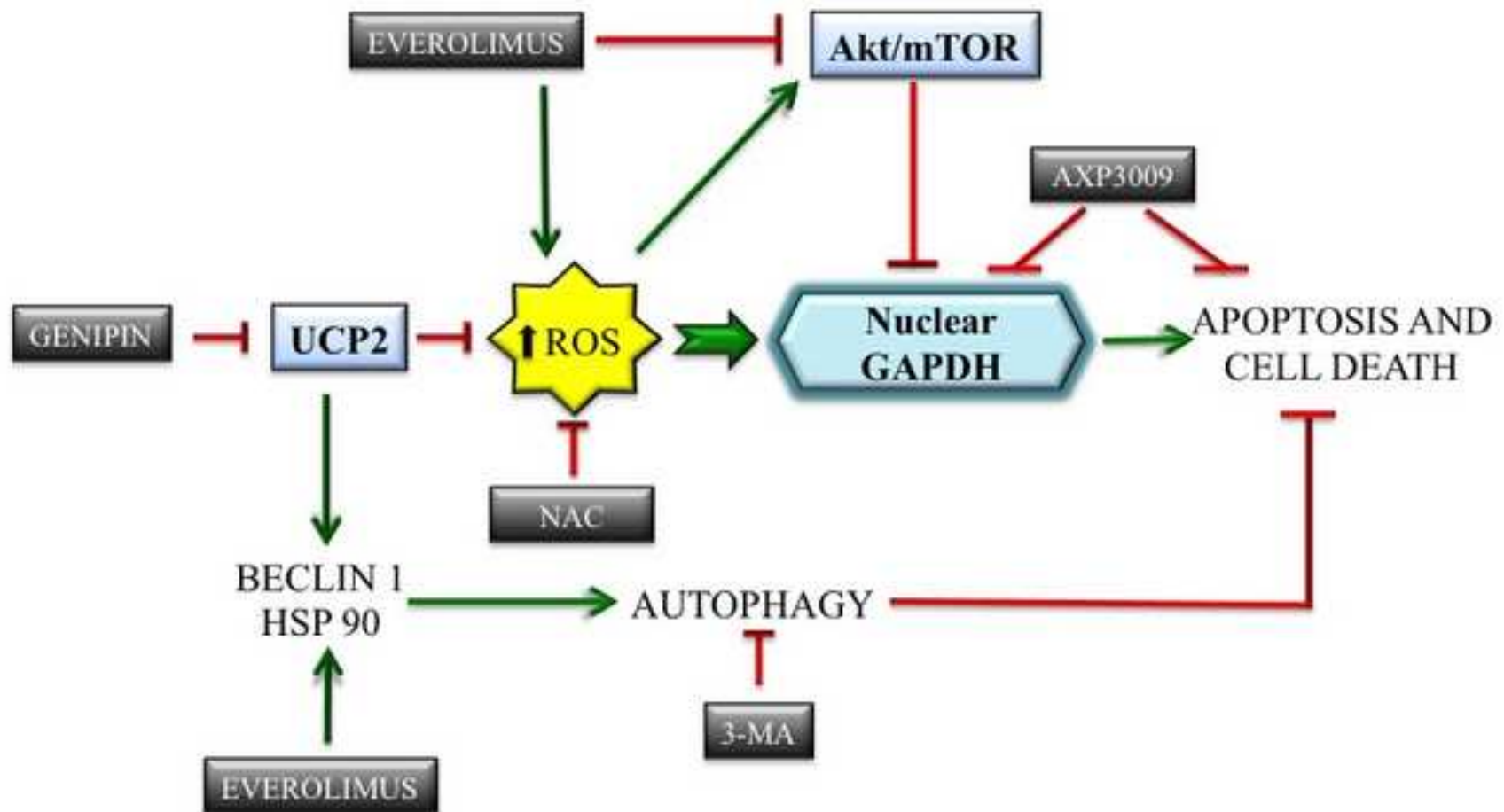


Fig. 9

Supplementary Figure 1

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Supplementary Figure 2

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Supplementary Figure 3

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Supplementary Figure 4

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Supplementary Figure 5

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Supplementary Figure 6

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