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► **To cite this version:**

Razan Abou Ziki, Romain Teinturier, Yakun Luo, Catherine Cerutti, Jean-Marc Vanacker, et al.. MEN1 silencing triggers the dysregulation of mTORC1 and MYC pathways in ER+ breast cancer cells. *Endocrine-Related Cancer*, 2022, 29 (8), pp.451-465. 10.1530/ERC-21-0337 . hal-03757057

**HAL Id: hal-03757057**

**<https://hal.science/hal-03757057v1>**

Submitted on 25 Oct 2023

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***MEN1 silencing triggers the dysregulation of mTORC1 and MYC pathways  
in ER+ breast cancer cells***

**Running title** Menin regulates *mTORC1* and *MYC* in ER+ BC cells

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**Word count 5074**

## Abstract

8 Menin, encoded by the *MEN1* gene, has been identified as a critical factor regulating *ESR1* transcription,  
9 playing an oncogenic role in ER+ breast cancer (BC) cells. Here, we further dissected the consequences  
10 of menin inactivation in ER+ BC cells, by focusing on factors within two major pathways involved in BC,  
11 mTOR and MYC. *MEN1* silencing in MCF7 and T-47D resulted in an increase in phosphor-p70S6K1,  
12 phosphor-p85S6K1 and phosphor-4EBP1 expression. The use of an AKT inhibitor inhibited the  
13 activation of S6K1 and S6RP triggered by *MEN1* knockdown (KD). Moreover, *MEN1* silencing in ER+  
14 BC cells led to increased formation of the eIF4E and 4G complex. Clinical studies showed that patients  
15 with menin-low breast cancer receiving Tamoxifen plus Everolimus displayed a trend towards better  
16 overall survival. Importantly, *MEN1* KD in MCF7 and T-47D cells led to reduced MYC expression. ChIP  
17 analysis demonstrated that menin bound not only to the *MYC* promoter but also to its 5' enhancer.  
18 Furthermore, E2-treated *MEN1* KD MCF7 cells displayed a decrease in MYC activation, suggesting its  
19 role in estrogen-mediated *MYC* transcription. Finally, expression data mining in tumors revealed a  
20 correlation between the expression of *MEN1* mRNA and that of several mTORC1 components and  
21 targets, and a significant inverse correlation between *MEN1* and two MYC inhibitory factors, *MYCBP2*  
22 and *MYCT1*, in ER+ BC. The current work thus highlights altered mTORC1 and MYC pathways after  
23 menin inactivation in ER+ BC cells, providing insight into the crosstalk between menin, mTORC1 and  
24 MYC in ER+ BC.

## Key words

Menin, MYC, mTORC1, breast cancer, ER-positive subtypes

## 25 **Introduction**

26

27 Breast cancer (BC) is the most common cancer in women (Ghoncheh *et al.* 2016). A classification based  
28 on molecular markers and gene expression studies subdivides BC into four major subtypes, namely  
29 luminal A, luminal B, HER2-enriched and triple negative BC (TNBC). Luminal A and B subtypes,  
30 representing around 40% and 20% of BC, respectively, express ER $\alpha$ . The HER2-enriched subtype  
31 behaves more aggressively than luminal subtypes, but is sensitive to therapies targeting the HER2  
32 receptor. Lastly, the TNBC harbors more genomic instability and is highly heterogeneous; it also displays  
33 more proliferative features, and is the most aggressive BC with the worst prognosis (Lehmann *et al.* 2011,  
34 Dai *et al.* 2015). Despite the progress made in identifying factors responsible for BC development and  
35 progression, understanding the underlying mechanisms involved in the development of the different BC  
36 subtypes remains challenging.

37 ER $\alpha$ , a nuclear receptor, plays a crucial role not only in the development of luminal BC subtypes,  
38 but also in their treatment, since its presence is essential for the proliferation and survival of ER-positive  
39 (ER+) BC cells. Hormonotherapy using either selective estrogen receptor modulators (SERMs) or  
40 selective estrogen receptor downregulators (SERDs) has been developed over the last two decades, and  
41 has offered promising results in the treatment of luminal BC subtypes. However, resistance to these  
42 treatments ultimately occurs in a substantial proportion of cases. Consequently, a better understanding of  
43 the mechanisms and factors involved in the regulation of the ER $\alpha$  pathway is urgently needed to propose  
44 new strategies in BC management. As such, MYC, overexpressed in approximately 20-30% of BCs  
45 (Bièche *et al.* 1999), is not only a well-known estrogen-regulated oncogene (Shang *et al.* 2000), but has  
46 also been shown to interact with ER $\alpha$  to modulate estrogen-mediated signaling (Cheng *et al.* 2006).  
47 Identifying the factors influencing the expression and functions of MYC, in order to control its oncogenic  
48 effects, could thus be a promising approach. In addition, activation of the PI3K/PTEN/AKT/mTOR  
49 pathways occurs in 70% of BCs (Castaneda *et al.* 2010), and *PTEN* mutations and inactivation are  
50 negatively correlated with ER $\alpha$  and PR status in BC (Jones *et al.* 2013, Zhang *et al.* 2012), associated

51 with more aggressive behaviors and worse outcome (Li *et al.* 2017b). Importantly, the activation of the  
52 PI3K/AKT/mTOR pathways has been reported in the context of resistance to endocrine therapy, and their  
53 inhibition led to increased ER $\alpha$  expression and the restoration of hormone sensitivity (Bosch *et al.* 2015).  
54 Therefore, the combination of mTOR inhibitors and endocrine therapy has been proposed, in order to  
55 overcome resistance to endocrine therapy (Miller *et al.* 2010).

56 Menin, encoded by the *MEN1* gene, is a multifaceted cofactor, interacting physically or  
57 functionally with numerous transcription and epigenetic factors, including Smad3, JunD, AKT, MYC,  
58 KTM2A/B and Ezh2 (Dreijerink *et al.* 2017b). Interestingly, it has been identified as an ER $\alpha$  interacting  
59 partner (Dreijerink *et al.* 2006) and a crucial factor that binds to the regulatory sequences of the *ESR1*  
60 gene and participates in the positive regulation of its transcription in ER+ BC cells (Dreijerink *et al.*  
61 2017a). In recent years, increasing evidence has been published highlighting the complex and important  
62 role of menin in BC. For instance, our team observed that heterozygous *Men1* mutant mice develop  
63 mammary gland tumors in females and prostate cancers in males at an advanced age at low frequencies  
64 (Bertolino *et al.* 2003, Teinturier *et al.* 2021b). Moreover, conditional mammary-specific *Men1* knock-out  
65 female mice displayed higher amounts of early mammary intraepithelial neoplasia (MIN) (Seigne *et al.*  
66 2013). A comprehensive analysis of several cohorts of MEN1 patients revealed that female MEN1  
67 patients were at a higher risk of developing BC (Dreijerink *et al.* 2014). In human sporadic BCs, *MEN1*  
68 mutations, albeit at low frequency, were among driver mutations (Nik-Zainal *et al.* 2016), and reduced  
69 menin expression was reported (Seigne *et al.* 2013). Interestingly, Dreijerink *et al.* demonstrated that  
70 menin acts as a tumor suppressor in normal mammary cells, by regulating different sets of genes in  
71 primary luminal progenitor cells of normal individuals, compared to those found in ER+ BC cells, but  
72 plays an oncogenic role in the latter cells through its positive regulation of the *ESR1* gene (Dreijerink *et*  
73 *al.* 2017b). Moreover, Imachi *et al.* observed that, within 65 ER+ BC samples treated with tamoxifen for  
74 2-5 years as adjuvant therapies, menin-positive tumors (20 patients) had a worse clinical outcome and  
75 were more resistant to tamoxifen than menin-negative tumors (46 patients) (Imachi *et al.* 2010). Recently,

76 our analyses carried out in a panel of 354 breast cancer patients suggested that decreased menin  
77 expression is correlated with luminal B and ER- BC subtypes, and, among the ER+/HER2- cohort (294  
78 patients), it was associated with worse distant metastasis-free survival and with a trend for worse disease-  
79 free survival and over survival (Teinturier *et al* 2021a). Finally, menin is also known to interact with the  
80 PI3K/AKT/mTOR pathways. Indeed, Wang et al. demonstrated that menin interacts with AKT1, inhibits  
81 its kinase activity and AKT1-induced proliferation and anti-apoptosis in endocrine and non-endocrine  
82 cells (Wang *et al.* 2011b). Moreover, a recent study reported that menin is involved in milk protein  
83 synthesis through mTOR signaling in normal mammary epithelial cells (Li *et al.* 2017a), by suppressing  
84 the mTOR pathway components and milk protein  $\kappa$ -casein (CSNK). The above data suggest that menin  
85 may play important but complex, and likely opposite, roles in mammary cells and in different BC  
86 subtypes. In the current study, in order to clarify the role of menin in BC, we focused on MYC and  
87 mTOR pathways in human BC samples and derived cell lines.

88

## 89 **Materials and Methods**

90

### 91 *Patients*

92 We analyzed a total of 40 female patients with ER+/HER2- breast cancers who underwent either  
93 Tamoxifen or Tamoxifen + RAD001 treatment at the Centre Léon Bérard (CLB). 23 patients (10 with  
94 Tamoxifen and 13 with Tamoxifen + RAD001) with complete data and with adequate samples assessable  
95 for menin by IHC were included (See Supplementary Materials and Methods). The study was conducted  
96 in accordance with the guidelines in the Declaration of Helsinki and the use of all patient tissues was  
97 approved by local IRB and carried out according to French laws and regulations. Written informed  
98 consent has been obtained from each patient.

99

100 *Cell culture, estrogen treatment and siRNA transfections*

101 MCF7 (HTB-22), ZR-75-1 (CRL-1500™) and T-47D (HTB-133), derived from human invasive breast  
102 ductal carcinomas, are ER+ breast cancer cell lines. MCF7 and ZR-75-1 cells were cultured in DMEM  
103 (Gibco Thermofisher Scientific) containing 25 mM glucose supplemented with 10% (v/v) fetal calf serum  
104 (FCS), 2 mM L-Glutamine, 100 unit/mL Penicillin, 100 µg/mL streptomycin, 1X MEM non-essential  
105 amino acid solution (Gibco by life technologies), 100 mM β-mercaptoethanol, at 37°C with 5% CO<sub>2</sub>. T-  
106 47D cells cultured in RPMI medium (Gibco by life technologies) containing 25 mM glucose and  
107 supplemented with 10% (v/v) FCS, 2 mM L-Glutamine, 100 unit/mL Penicillin, 100 µg/mL streptomycin,  
108 10 mM HEPES, 1 mM sodium pyruvate, at 37°C with 5% CO<sub>2</sub>. MDA-MB-231 (HTB-26™) and MDA-  
109 MB-453 (HTB-131™) cell lines are human metastatic ER- breast cancer cell lines, which were cultured  
110 in the same medium as MCF7 and ZR-75-1, albeit devoid of non-essential amino acids. All cell lines  
111 were regularly tested for mycoplasma contamination, and their latest authentication was performed in  
112 2018.

113 MCF7 and T-47D cells were starved in serum-free medium 24 h before insulin stimulation. The  
114 serum-free medium was changed 3 - 4 h before stimulation. Firstly, the cells were treated with  
115 Everolimus (RAD001 SIGMA-ALDRICH) (20 nM) or vehicle (dimethylsulfoxide (DMSO), 0.01%) in  
116 serum-free medium for 1 h. The cells were then treated with 100 nM insulin and 20 nM Everolimus  
117 (RAD001) for 1 h (human insulin solution 10 mg/ml in 25 nM HEPES pH 8.2, SIGMA) in serum-free  
118 medium.

119 MCF7 and ZR-75-1 cells were seeded at 250,000 cells/well and T-47D cells were seeded at  
120 300,000 cells per well in 6-well plates and cultured for 24 h. Cells were then cultured in white complete  
121 phenol-red-free medium containing 10% charcoal-stripped serum (Biowest) and treated with 10 nM E2  
122 (β-estradiol, E2758-1G, SIGMA) for 30 min, 1 h, 2 h, 4 h, 8 h, 24 h (depending on the experiment) or  
123 transfected with 20 nM siRNA of either control siRNA (Eurogentec) or siRNA against the targeted gene  
124 using Jetprime® transfection reagent (polyplus) for 72 h.



125

126 *Protein extraction and Western blot*

127 Protein extracts were prepared by washing the plates twice with 1X PBS, then, 200-1000  $\mu$ L of complete  
128 RIPA buffer (50 mM Tris HCL pH 8.0; 150 mM NaCl; 0.1% SDS; 0.5% sodium deoxycholate, 1% NP40  
129 + Protease Inhibitors (Roche)) was added and left for at least 20 min. Cell lysates were then transferred to  
130 1.5 mL tubes and centrifuged at 13,000 rpm for 10 min at 4°C, in order to precipitate genomic DNA and  
131 other cellular debris. The supernatant was retained, and the proteins quantified by Bradford assay at 595  
132 nm.

133 20  $\mu$ g or 30  $\mu$ g (depending on experimental conditions) of cell lysate was boiled in 5  $\mu$ L Laemmli  
134 buffer and loaded onto 8%, 10% or 12% acrylamide gel depending on protein size. Proteins were then  
135 rapidly transferred onto a 0.2  $\mu$ m PVDF membrane (Biorad) by electrotransfer performed at 25 V for 10  
136 min. The membranes were saturated with 0.1% TBS-Tween, 5% milk for 30 min and incubated with the  
137 appropriate primary antibody overnight at 4°C. Membranes were washed three times for 10 min with 1X  
138 TBS-0.1% Tween solution and were incubated for 45 min with an appropriate secondary antibody  
139 coupled to HRP (horseradish peroxidase). Membranes were washed again and were visualized using the  
140 Roche® Lumi-light Plus Western blotting substrate and Chemiluminescence Film (GE Healthcare).  
141 GAPDH was used as an internal control for normalization of the results. The quantification of the level of  
142 protein was analyzed using ImageJ software (NIH). Briefly, after opening the gel image via FileOpen, the  
143 image was converted into an 8-bit grayscale image. Then, lanes were selected and numbered using  
144 Analyze /gels/, and the calculation was completed with the Wand tool. The calculated results were  
145 exported as an xls file. Finally, the area value of the target protein was divided by the area value of the  
146 reference protein to obtain the relative expression level. The detailed quantification method can be found  
147 in online technical suggestions at <https://www.plob.org/article/14865.html>.

148

149 *Real-time reverse transcriptase and qPCR analyses*

150 Total RNA from cells was extracted using RNeasy-Kits (Macherey-Nagel) according to the  
151 manufacturer's instructions. RNA was reverse transcribed to complementary DNA (cDNA) using  
152 SuperScript™ II Reverse Transcriptase (Life Technologies). cDNAs were then amplified and quantified  
153 in an ABI Prism 7500 Sequence Detection System (Applied Biosystems) using the SYBR Green I dye  
154 (SsoAdvanced Universal SYBR Green, Bio-Rad) according to manufacturer's instructions. RT-qPCR  
155 conditions were as follows: 1 activation step for 5 min at 94 °C, 35 cycles of denaturing at 94 °C for 15 s,  
156 primer annealing at 59 °C for 45 s and primer extension at 72 °C for 2 min and a final extension at 72 °C  
157 for 5 min. Data were normalized against the in-house control 28S and represented as fold change. Primers  
158 used are listed in the Supplementary Information.

159

160 *IHC analysis of human breast cancers*

161 Formalin-fixed paraffin-embedded invasive breast cancers were prepared and processed for  
162 immunostaining as previously described (Seigne *et al.* 2013). The blocks were sectioned at a thickness of  
163 4 µm. After deparaffinization and rehydration, endogenous peroxidases were blocked by incubating the  
164 slides in 5% hydrogen peroxide in sterile water. For heat induced antigen retrieval, tissue sections were  
165 boiled in 10 mM Citrate Buffer (Dako, Trappes, France) using a water bath at 98°C for 50 min. The slides  
166 were then incubated at room temperature for 60 min with antibodies against menin (Bethyl laboratories,  
167 A300 105A, 1:2000). After rinsing in Phosphate Buffer Saline (PBS), the slides were incubated with a  
168 biotinylated secondary antibody bound to a streptavidin peroxidase conjugate (LSAB+ Kit, Dako,  
169 Trappes, France) and bound antibody was revealed by adding the substrate 3, 3-diamino-benzidine.  
170 Sections were counterstained with hematoxylin. Blinded to the clinical data, the slides were analyzed by  
171 the pathologist. The percentage of stained cells was multiplied by the intensity of staining to obtain the H

172 score, and the whole cohort of patients was divided into high menin expression (H score > 100) and low  
173 menin expression (H score ≤ 100).

174

#### 175 *Proximity Ligation Assay (PLA)*

176 PLA assays were performed using Duolink In Situ PLA detection kit (orange) and probes: anti-goat or  
177 anti-mouse MINUS and anti-rabbit PLUS (#DUO92006, #DUO92004, #DUO92002, Sigma-Aldrich),  
178 according to the supplier's recommendations (All the reagents are part of the PLA kit). Briefly, cells were  
179 seeded in a 12-well plate containing cover slips for 72 h, on the second day cells were transfected with the  
180 desired siRNA as described below. When estrogen treatment was required, cells were treated with  
181 estrogen for 2 h before PLA. Cells were fixed with methanol for 2 min and washed 3 times with 1X PBS,  
182 blocked with Blocking Solution® and incubated at 37°C for 30 min. Each cover slip was mounted with a  
183 hydrophobic pen to prevent excessive evaporation. Then, 40 µL/slip of solution was added containing  
184 primary antibodies of interest at suitable concentrations in antibody diluents® and incubated for 1 h at  
185 37 °C. After three washes in Buffer A®, the secondary antibodies conjugated with complementary  
186 oligonucleotides (chosen, depending on the used primary antibodies), affinity purified donkey anti-rabbit  
187 IgG Duolink in Situ PLA probe PLUS® (1:5, Sigma Aldrich) and affinity purified donkey anti-mouse or  
188 anti-goat IgG Duolink in Situ PLA probe MINUS® (1:5, Sigma Aldrich) were added and incubated for  
189 1 h at 37 °C. Subsequently, cells were washed three times in Buffer A®, and incubated in 1 unit/µl of T4  
190 DNA ligase in diluted ligase buffer (1:5, Sigma Aldrich) for 30 min at 37 °C. After three washes in Buffer  
191 A®, cells were incubated in 5 units/µl of DNA polymerase in diluted polymerase buffer with red  
192 fluorescent-labeled oligonucleotides (1:5, Sigma Aldrich) for 100 min at 37 °C. A final round of washes  
193 was performed twice for 10 min in 1X Buffer B®, then 1 min in 0.01X Buffer B. The slides were then  
194 mounted using 5 µL of Duolink in situ mounting medium containing DAPI. After sealing the coverslip,  
195 imaging of the slides was performed under a Nikon Eclipse Ni microscope. Images were acquired under  
196 identical conditions at x60 magnification. Image acquisition was performed by imaging DAPI-stained

197 cells at a fixed Z-position, while analysis of Z-sections of  $\pm 5 \mu\text{m}$  at 1- $\mu\text{m}$  intervals was carried out.  
198 Images were acquired for at least 10 randomly chosen fields of view in an automated manner and at least  
199 500 cells were analyzed per condition. The final image was stacked to a single level before further  
200 quantification. Quantification of PLA dots was performed using the ImageJ software (NIH) combined  
201 with an automated Macro described in detail previously (Schneider *et al.* 2012, Poulard *et al.* 2020)

202

### 203 *Cell proliferation assays*

204 MCF7, T\_47D, MDA\_MB\_231 and MDA\_MB\_453 cells were seeded in white complete medium at 15,000  
205 cells per well for MCF7 and MDA\_MB\_453 and 20,000 cells per well for T\_47D in a 48-well plate. 24 h  
206 after seeding, cells were transfected with siRNA (same as described before), or treated with 10058-F4  
207 (also called F4) (F3680, Sigma, St. Louis, Missouri, USA), at the concentration of 20  $\mu\text{M}$  to inhibit MYC  
208 expression. The plate was then left for 144 h in the IncuCyte™ device (Essen Bioscience), to monitor cell  
209 growth kinetics. This device was connected to the IncuCyte™ software to follow cell confluence in real-  
210 time.

211

### 212 *ChIP-qPCR assay*

213 Chromatin immunoprecipitation (ChIP) was performed with the Millipore ChIP Assay Kit (17-295)  
214 according to the manufacturer's recommendations (also see Supplementary Materials and Methods).

215

### 216 *Mining of public gene expression data*

217 RNA-seq data (Illumina HiSeq 2000, version 2 analysis) obtained in human BC samples in The Cancer  
218 Genome Atlas (TCGA) project were previously collected from the Genomic Data Commons data portal  
219 (<https://portal.gdc.cancer.gov>). In addition, using the cBioPortal (<https://www.cbioportal.org/>), a

220 recognized and widely used online portal for cancer genomics, other BC datasets could be investigated for  
221 *MEN1* co-expression. Among the most recent ones, we selected the SMC 2018 study focusing on young  
222 Asian patients (Z *et al.* 2018) (See Supplementary Materials and Methods).

223

#### 224 Statistical analyses

225 Each biological experiment was independently repeated at least three times to ensure accuracy, with each  
226 containing at least three technical replicates for both RT-qPCR, ChIP-qPCR and cell proliferation assays.  
227 Each variable was analyzed with the unpaired Student's *t*-test to compare two groups of independent  
228 samples, using the Prism5 software (GraphPad Software, San Diego, USA). Results are expressed as  
229 means +/- standard errors of the means (SEM), *P* values of < 0.05 were considered to be significant.  
230 Statistical significance is displayed as ns, non-significant versus control, \* *P* < 0.05, \*\* *P* < 0.01, \*\*\* *P* <  
231 0.001, \*\*\*\* *P* < 0.0001 for all analyses.

232

### 233 **Results:**

#### 234 ***MEN1* knockdown decreases cell proliferation in ER+ BC cell lines**

235 It is well-known that menin regulates ER $\alpha$  expression in ER+ BC cells and is critical for the growth of  
236 ER+ BC cell lines (Dreijerink *et al.* 2014, Teinturier *et al.* 2021a). To extend our knowledge on the role  
237 of menin in BC cells, we studied the effects of *MEN1* KD on the proliferation of two ER+ BC cell lines,  
238 MCF7 and T-47D, and two ER- BC cell lines, MDA-MB-231 and MDA-MB-453. As shown in Fig 1a  
239 (upper panels), the proliferation of MCF7 and T-47D cells started to decrease 72 h after *MEN1* KD and  
240 continued to decrease even after 120 h of transfection, compared to untreated and siCtrl cells. Moreover,  
241 *MEN1* KD had similar effects on the proliferation of MDA-MB-231 cells but did not affect that of MDA-  
242 MB-453 cells (Fig 1a, lower panels). We noticed that the levels of menin protein expression were

243 independent of the ER status among the BC cell lines tested (Fig S1a). Hence, data confirmed that *MEN1*  
244 is critical for the proliferation of ER+ MCF7 and T<sub>47D</sub> BC cells, and revealed that it plays  
245 heterogeneous roles in ER- MDA-MB-231 and MDA-MB-453 cell lines, suggesting that the role of  
246 menin in the proliferation of mammary cancer cells could be context-specific. Therefore, we hereafter  
247 mainly focused on the role of menin in ER+ BC cells.

248

### 249 ***MEN1* knockdown activates the mTORC1 pathway in ER+ BC cell lines**

250 To investigate the molecular mechanisms triggered by *MEN1* silencing in ER+ cells, we first studied its  
251 effects on the mTORC1 pathway, which is critical for cell growth and breast cancer development, and is  
252 involved in resistance to endocrine therapy (Paplomata and O'Regan 2014). We knocked down menin  
253 expression in MCF7 and T<sub>47D</sub> cells treated with insulin (to activate the mTORC1 pathway) and/or the  
254 mTOR inhibitor RAD001. Surprisingly, we observed that *MEN1* KD in these cells increased the  
255 expression of the active form of mTORC1 components, namely phosphorylated p70, and p85 (T389) of  
256 S6K1 and S6RP Ser 235/236, after insulin treatment, which was inhibited by RAD001 treatment (Fig 1b).  
257 The analysis was further carried out in MCF7 cells treated with essential amino acids (EAA), another  
258 mTOR stimulator, and similar results were obtained for phosphorylated S6K1 expression (Fig S1b).  
259 *MEN1* silencing also led to higher levels of phosphorylated 4EBP1 (ser 65), which is an inhibitor of  
260 4EBP1 in MCF7 and T<sub>47D</sub> cells (Fig 1c). Indeed, both phosphorylation of S6K1 and S6RP, and that of  
261 4EBP1 have an activating effect on the mTORC1 pathway. To further confirm these unexpected results,  
262 we used a reverse approach by overexpressing menin in MCF7 cell line. The results showed that menin  
263 overexpression led to the decrease in the expression of phosphorylated S6K1, S6RP and 4EBP1,  
264 conversely to the effects seen by *MEN1* KD (Fig 1d). We also noticed that there was an increase in the  
265 expression of total 4EBP1 protein in MCF7 cells upon menin overexpression (Fig 1d).

266 Previous studies have reported a link between menin and AKT in other types of cancers (Wang *et*  
267 *al.* 2011b), such as sporadic pancreatic endocrine tumors in which AKT is dysregulated and in which

268 menin is frequently inactivated (Jiao *et al.* 2011). We thus wondered whether the role played by menin on  
269 the mTORC1 pathway was AKT-dependent or -independent in our breast cancer cell lines. We analyzed  
270 AKT expression after menin knockdown in MCF7 cells and observed that *MENI* KD slightly increased  
271 phosphorylated AKT without altering the total protein level (Fig 1e), and the use of AKT inhibitor  
272 inhibited the activation of S6K1 and S6RP triggered by *MENI* KD (Fig S1c), suggesting that the effect of  
273 menin knockdown on mTORC1 activation is AKT-dependent.

274

### 275 ***MENI* KD increases the activity of the translation initiation complex**

276 To further dissect the downstream effects of mTORC1 activation triggered by reduced menin expression,  
277 we studied the consequences of *MENI* silencing on the translation initiation complex eIF4F (one of the  
278 downstream targets of the mTORC1 pathway) composed of 3 factors: eIF4A, the enzymatic core, eIF4E,  
279 responsible for cap-dependent translation of all nuclear encoded mRNA, and eIF4G, the scaffold protein  
280 that holds the complex together (Shveygert *et al.* 2010). We first verified whether the expression of these  
281 factors was altered after *MENI* KD. The data showed a reduced expression of eIF4A, eIF4E and eIF4G  
282 on mRNA levels, but no significant change in all 3 factors at the protein level (Fig 2a). We then analyzed  
283 the formation of eIF4F by assessing the interactions between eIF4G/eIF4E and eIF4G/eIF4A using  
284 proximity ligation assay (PLA). We found that *MENI* silencing resulted in an increase in the interaction  
285 between eIF4G and eIF4E, but not that of eIF4G and eIF4A in *MENI* KD MCF7 and T\_47D cells (Fig  
286 2b), compared to the menin-negative ZR\_75\_1 cell line under similar treatment (Fig S2a).

287 Interestingly, a clinical study in patients with menin-low breast cancer showed that patients  
288 receiving Tamoxifen plus RAD001 (13 patients) treatment had a trend towards a better overall survival  
289 than patients receiving Tamoxifen alone (10 patients), whereas no such effect was seen in patients with  
290 menin-high breast cancers,  $P = 0.0768$  (Fig 2c, Fig S2b). The data may suggest the restoration of

291 hormone sensitivity due to lower menin expression and support the above results showing that *MEN1* KD  
292 activated the mTORC1 pathway in ER+ breast cancer cells.

293 In addition, mining *MEN1* gene co-expression in BC TCGA data provided a correlation coefficient  
294 for each of the genes available in the dataset. Gene set enrichment analysis identified the GO term  
295 “translational initiation” as enriched in genes positively correlated with *MEN1* in ER+ tumors (Fig 2d).  
296 As shown in Fig 2e and S2b, among the so-annotated genes, *EIF4EBP1* encoding 4EBP1, as well as  
297 *EIF4BP3* were positively correlated with *MEN1*, consistent with the increased expression of 4EBP1  
298 protein observed in MCF7 cells upon menin overexpression. Inversely, four other genes encoding  
299 components related to the eIF4F complex displayed an inverse correlation with *MEN1*: *EIF4E3*, *EIF4G2*,  
300 *EIF4G3*, and *EIF4E* that was slightly below the correlation threshold. These results corroborate at the  
301 mRNA level the increased formation of the translation initiation complex eIF4G/eIF4E after *MEN1* KD  
302 seen above. A similar inverse relationship was observed for *MTOR* (Fig 2e). These data mining results  
303 thus substantiated the experimental data obtained above in ER+ BC cell lines.

304

### 305 **Menin regulates MYC expression and binds to the MYC promoter and enhancer in ER+** 306 **breast cancer cells**

307 Having found an interesting and intriguing link between our results showing the activation of the  
308 mTORC1 pathway and the reduced cell proliferation observed in *MEN1* KD ER+ breast cancer cells, we  
309 then focused our investigations on the effects of *MEN1* silencing on MYC. Indeed, this is an important  
310 estrogen-regulated oncogene, known to play a major role in proliferation, metabolism, and apoptosis of  
311 different cancer cells, including BC cells. Menin inactivation decreased MYC expression at both the  
312 mRNA (Fig 3a, left panels) and protein levels (Fig 3a, right panels) in *MEN1* KD MCF7 and T\_47D cells.  
313 However, our data showed heterogeneous results in the two ER- cell lines tested, with reduced MYC  
314 transcription but increased MYC protein levels in MDA\_MB\_231 cells, and no effect in MDA\_MB\_453



315 cells (Fig 3b). To better understand how menin regulates MYC expression at the transcriptional level in  
316 ER+ breast cancer cells, we investigated the binding of menin to both the *MYC* proximal promoter and  
317 the previously reported *MYC* 5' enhancer (Wang *et al.* 2011a) by ChIP analysis. Interestingly, menin  
318 bound to the *MYC* 5' enhancer and to the proximal *MYC* promoter, including areas involving the  
319 transcription start site (TSS) and +300 bp sites of the *MYC* promoter in MCF7 cells (Fig 3c). We also  
320 analyzed the influence of MYC expression on the expression of ER $\alpha$  and menin. Our data demonstrated  
321 that *MYC* KD had no effect on ER $\alpha$  expression, but decreased MEN1 expression at the transcriptional and  
322 protein levels in MCF7 and T<sub>47D</sub> cells (Fig S3a, b).

323 We observed that *MEN1* knockdown also altered protein and mRNA expression of several known  
324 MYC target genes, which are either involved in cell cycle control, including cyclins, cyclin-dependent  
325 kinases (CDKs), p53, and cyclin-dependent kinase inhibitors (CKI) such as p21 and p27, or in apoptosis,  
326 including cleavage of PARP and caspase 3, in MCF7 cells (Fig 3d). These data support the reduced MYC  
327 expression detected in *MEN1* KD MCF7 and T<sub>47D</sub> cells.

328

### 329 **Menin is needed for estrogen-mediated MYC activation and physically interacts with MYC** 330 **in BC cells**

331 Since MYC is an estrogen-regulated oncogene and our findings were obtained in the absence of estrogen  
332 (charcoal-stripped medium), we wondered whether the regulation of MYC expression is influenced by  
333 estrogen stimulation. We thus first treated MCF7 cells with  $\beta$ -estradiol (E2) at different time points (Fig  
334 S4). Though the levels of ER $\alpha$  protein decreased after E2 stimulation, the expression of MYC  
335 significantly increased at both protein and mRNA levels 1 h after treatment, whereas menin expression  
336 did not change (Fig 4a). Interestingly, *MEN1* silencing resulted in decreased MYC expression under E2  
337 stimulation in MCF7 cells, suggesting that menin may impact estrogen-mediated MYC activation (Fig  
338 4b).

339 In order to gain further insight into the interaction between menin and MYC in BC cells, following  
340 previous reports in HeLa cells (Brès *et al.* 2009), we analyzed whether there was a physical interaction  
341 between menin and MYC in ER+ BC cells used in the current work, and if so whether this interaction was  
342 estrogen-dependent. Our data showed that menin interacts with MYC in MCF7 cells, but their interaction  
343 was reduced under E2 stimulation (Fig 4c left panel), We also noticed that E2 treatment in MCF7 cells  
344 led to increased interaction between menin and ER $\alpha$  (Fig 4c right panel), suggesting a role for menin in  
345 estrogen-mediated MYC activation functions, likely through its physical interaction with ER $\alpha$ .

346

### 347 **Co-expression of menin and MYC in some breast cancers**

348 Data mining analyses were then carried out using different publicly available BC mRNA expression  
349 datasets to further investigate co-expression between menin and MYC in BC. Although no positive  
350 correlation was detected between *MEN1* and *MYC* mRNA expression in TCGA BC samples in all tumors  
351 and in ER+ tumors, an interesting significant positive correlation was seen in all tumors from the SMC  
352 2018 dataset, as well as in ER+ tumors only (Fig 5a). With regards to the TCGA data, a positive  
353 correlation was observed for ER- tumors (Fig 5b).

354 Next, we focused on the relationship between *MEN1* and MYC-related genes. Interestingly, we  
355 found that two genes, *MYCBP2* and *MYCT1* described as negative regulating factors in the MYC  
356 pathway, displayed a significant negative correlation with *MEN1* in ER+ tumors (Fig 5c). Knowing that  
357 both of these MYC-related proteins play an inhibitory role in the MYC pathway, the data seem to support  
358 the relationship between *MEN1* and *MYC* uncovered above.

359 Based on these findings, we tested whether *MEN1* KD could provide or not synergistic effects with  
360 MYC inhibition to reduce cell proliferation. Although separate inhibition of MYC and menin using  
361 siRNA or 10058-F4 (MYC/MAX inhibitor) decreased cell proliferation in MCF7 cells, the combined use

362 of siMEN1 and siMYC did not further decrease cell proliferation in MCF7 line, whereas the combined  
363 use of siMEN1 and MYC inhibitor 10058-F4 had synergistic inhibitory effects (Fig 5d).

364

## 365 **Discussion**

366 We noticed that *MEN1* silencing led to reduced cell proliferation in ER<sup>+</sup> BC cell lines as previously  
367 reported (Dreijerink *et al.* 2017b), whereas it triggered a distinct cell growth reaction in an ER<sup>-</sup> BC cell  
368 line, indicating the effects exerted by the menin protein are dependent on cellular and molecular context.  
369 Since it is known that ER<sup>-</sup> BC is a particularly heterogeneous subtype, which has recently been suggested  
370 to be further divided into at least 6 subgroups (Lehmann *et al.* 2011), it is not surprising that menin could  
371 play different roles in ER<sup>-</sup> BC cell lines. In fact, Dreijerink *et al.* reported that menin did not regulate  
372 ER $\alpha$  target genes in ER<sup>-</sup> BC cell lines. It would, therefore, be interesting to further dissect the role of  
373 menin in responsive or non-responsive ER<sup>-</sup> BC cells, beyond the ER $\alpha$  pathways, in order to gain insight  
374 into the factors and/or pathways participating in the growth and survival of these cells.

375 Menin is known to physically and functionally interact with the AKT/mTOR pathway in pancreatic  
376 endocrine cells, mainly via the retention of AKT in the cytoplasm (Wang *et al.* 2011). However, its role in  
377 the mTOR pathway, in particular on the downstream effectors remains elusive. Our data showed that  
378 menin expression is crucial for mTORC1 activity in BC ER<sup>+</sup> cells. Moreover, the latter seemed to mainly  
379 result in the activation of translation initiation, since 4EBP1 expression decreased and its phosphorylated  
380 form increased after *MEN1* KD, which should allow the components of eIF4F complex to interact and  
381 thus initiate mRNA translation. Indeed, our data showed that menin inactivation in ER<sup>+</sup> BC cells  
382 specifically favored the formation of eIF4E and eIF4G complex, which is considered to be the rate-  
383 limiting determinant of cap-dependent protein synthesis (Shveygert *et al.* 2010) and one of the  
384 characteristic activities of cancer cells, including BC cells (Chu *et al.* 2016). Data mining of gene mRNA  
385 expression in ER<sup>+</sup> human tumors corroborate the effects of menin inactivation on 4EBP1 and on the

386 formation of the eIF4F complex observed in ER+ BC cells. Notably, our clinical data analysis on the  
387 TamRad trial also supports the possible activation of the mTOR pathway in menin-low BC ER+ patients,  
388 even though the trend observed in the trial needs to be further confirmed by other studies with more cases.  
389 We noticed that a recent study reported the reduced ER $\alpha$  signaling pathway upon PI3K/AKT activation  
390 (Bosch *et al.* 2015). It is, therefore, plausible that the activation of AKT/mTORC1 triggered by *MEN1*  
391 silencing contributes to the reduced ER $\alpha$  expression and cell proliferation observed in ER+ BC cells in  
392 our works, along with the inhibition of the MYC pathway. Another possibility would be that the  
393 activation of the mTORC1 pathway triggered by menin inactivation could lead to oncogenic stress in  
394 these cells, as formerly reported (Dermitt *et al.* 2017).

395 Interestingly, *MEN1* knockdown led to reduced MYC expression in ER+ cells. Our analyses  
396 demonstrated, for the first time, that menin is a critical factor regulating *MYC* transcription in these cells,  
397 through its binding not only to the *MYC* promoter, but also to the *MYC* 5' enhancer. Furthermore, we  
398 observed that *MEN1* silencing in these cells triggered altered expression of factors known to be MYC  
399 targets and responsible for cell growth, cell cycle and cell death. The novel role of menin uncovered in the  
400 current work in regulating *MYC* transcription and the MYC pathway is, therefore, consistent with  
401 molecular alterations observed in these cells. Our finding is also in agreement with the recently emerged  
402 notion that menin could play a dual role in the tumorigenesis of BC cells. The fact that menin is essential  
403 for *ESR1* and *MYC* transcription revealed in the previous studies (Dreijerink *et al.* 2014, Luo *et al.* 2021)  
404 and the current work on ER+ BC cells makes it a powerful oncogenic factor in these cells, by regulating  
405 two main actors. Imachi *et al* previously reported that menin expression in BC patients is clinically  
406 correlated with resistance to tamoxifen (Imachi *et al.* 2010). It would be relevant to verify whether the  
407 role of menin in regulating MYC expression could be one of the molecular bases conferring such  
408 resistance. In parallel, we observed that, in ER- BC cells, the role of menin in regulating MYC and cell  
409 proliferation varies among different cell lines tested, suggesting more diverse molecular elements may  
410 participate in the carcinogenesis of these cancers. In *MEN1* KD ER- BC cells showing reduced *MYC*

411 transcription and reduced cell proliferation without altered MYC protein expression, as in MDA-MB-231,  
412 menin may also be relevant to its post-transcriptional regulation via factors, such as MYCBP1 and  
413 MYCT1, and/or to the expression of other genes that are critical for the growth of these cells. Future  
414 works on ER- BC cells could provide useful answers to explain these findings. We observed the positive  
415 correlation between menin and MYC expression in some BC samples. Moreover, even in the absence of  
416 correlation in *MYC-MEN1* co-expression in other BC datasets, a clear inverse relationship was detected  
417 between *MEN1* and *MYCT1* or *MYCBP2* expression in ER+ as well as ER- tumors, suggesting the  
418 involvement of menin in post-transcriptional regulation of MYC. Considering the heterogeneous nature of  
419 BC (Jackson *et al.* 2020), it would be relevant to further dissect and validate the molecular crosstalk  
420 unveiled in the current work between menin and its related pathways, including MYC and mTORC1, in  
421 different BC subtypes.

422 Our current work revealed that *MEN1* silencing dysregulates mTORC1 and MYC pathways,  
423 specifically in ER+ BC cell lines, providing useful clues for understanding the molecular mechanisms  
424 underlying the oncogenic effects of menin observed in these cells.

425

426

427

## 428 **DECLARATIONS SECTION**

### 429 *Ethics approval and consent to participate*

430 The study was conducted in accordance with the guidelines in the Declaration of Helsinki and the use of  
431 all specimens of patients with breast cancer was approved by our local IRB, biological resource center at  
432 the Léon Bérard Centre (code: n°BB-0033-00050) and carried out according to French laws and  
433 regulations.

### 434 *Consent for publication*

435 All of the authors approved the contents and the data in this manuscript and agreed for its submission for  
436 publication.

#### 437 *Availability of data and material*

438 The authors confirm that the data supporting the findings of this study are available within the article  
439 [and/or] its supplementary materials.

#### 440 *Competing of Interests*

441 The authors declare that they have no competing interest.

#### 442 *Funding*

443 This study was supported by the Ligue Inter-régionale contre le Cancer (R19040CC), Institut National du  
444 Cancer (PRTK2017) and the Association: « Le Cancer du sein, parlons-en ». R.A.Z was the recipient of a  
445 PhD-fellowship from the Association "G04MEDIA S.A.R.L", Lebanon. R.T was the recipients of a PhD-  
446 fellowship from Ministère de l'Enseignement Supérieur et de la Recherche, France. Y.L was the recipient  
447 of a PhD-fellowship from the China Scholarship Council.

#### 448 *Author Contributions*

449 R.A.Z, R.T and Y.L conducted the experiments, analyzed and interpreted the data, prepared figures and  
450 manuscript; C.C and J.M.V performed bioinformatics analyses and data interpretation, as well as  
451 participated in manuscript preparation; C.P provided technical and material support and participated in  
452 data interpretation; T.B and I.T conducted clinical study design, collection and interpretation of data;  
453 M.L.R and C.X.Z conceived and supervised the study and manuscript preparation, and obtained funding.

#### 454 *Acknowledgements*

455 We are grateful to Brigitte MANSIP for her assistance in editing and proofreading the manuscript,  
456 Sarah Ensenlaz for her help in experiments.

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583 **Legends:**

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585

586 **Fig 1. Menin downregulation leads to mTORC1 activation in ER+ cell lines. a** Cell proliferation

587 curves of ER+ BC cell lines MCF7, T<sub>47D</sub> (2 upper panels), and that of MDA-MB-231 and MDA-MB-

588 453 ER- BC cell lines (2 lower panels) transfected with siCtrl or siMEN1 hs1 24 h after cell culture.

589 Significance was calculated in comparison with siCtrl at the same time point. Red arrowheads showing

590 the timing of siRNA transfection. Western blot showing menin expression in the above cells treated with

591 different siRNAs as indicated, with the quantification of the band intensity of siMEN1- against siCtrl-

592 treated samples, after normalization against Tubulin (a), Actin (b, d, e) or GAPDH (c), shown under each

593 corresponding graph. **b** Western blot analysis showing the protein expression of phosphorylated and total

594 S6K1 and S6RP in MCF7 (left panel) and T<sub>47D</sub> (right panel) cells transfected with siCtrl or siMEN1 hs1

595 and treated with 100 nM of the mTOR activator insulin and/or 20 nM of the mTOR inhibitor RAD001 as

596 indicated. **c** Western blot analysis detecting the phosphorylated and total 4EBP1 in MCF7 and T<sub>47D</sub>

597 cells transfected with siCtrl or siMEN1 hs1 or siMEN1 hs3, and treated with insulin and/or RAD001 as

598 indicated. **d** Western blot analysis showing the protein expression of S6K1, S6RP, and 4EBP1 in MCF7

599 cells transfected with the control vector PCI-Neo or the vector PCI-MEN1 overexpressing menin and

600 treated with insulin and/or RAD001 as indicated. **e** Western blot analysis showing the protein expression

601 of phosphorylated (Ser 473) and total AKT in MCF7 cells transfected with siCtrl or siMEN1 hs1 and

602 treated with insulin and/or the AKT inhibitor MK2206 as indicated.

603 **Fig 2. MEN1 silencing results in activation of eIF4F formation. a** Quantitative RT-PCR (left panel)

604 and Western blot (right panel) showing the mRNA and protein expression of eIF4E, eIF4A, eIF4G in

605 MCF7 cells transfected with siCtrl or two different siMEN1 hs1 or hs3. The quantification of the band

606 intensity of siMEN1- against siCtrl-treated samples, after normalization against GAPDH (for menin) or  
607 Tubulin (for others), is shown under each corresponding Western blot. **b** Proximity ligation assay (PLA)  
608 showing the interaction between either eIF4G/eIF4E or eIF4G/eIF4A in MCF7 (Upper panel) and T<sub>47D</sub>  
609 (lower panel) cell lines transfected with siCtrl, siMEN1 hs1, siEIF4G and siEIF4G+MEN1 hs1.  
610 Quantification of PLA signals, represented by dots/cell, for each condition is shown in the right-hand  
611 panels. Scale bar = 10  $\mu$ m. **c** Comparison of Kaplan-Meier curves estimating the survival rate (in  
612 months): Upper panel, in 23 breast cancer patients with low menin expression, with 10 patients being  
613 treated with Tamoxifen only (Tam) and 13 patients being treated with Tamoxifen + mTOR inhibitor  
614 RAD001 (TamRad). Lower panel: in 17 breast cancer patients with high menin expression, with 8  
615 patients being treated with Tam and 9 patients being treated with TamRad. Blue curve: Tamoxifen alone,  
616 Dashed red curve: Tamoxifen + RAD001. TTP: time to progression. **d** GO term “translational initiation”  
617 gene set identified with Gene Set Enrichment Analysis among *MEN1* positively correlated genes in ER+  
618 BC from TCGA. **e** Expression correlation from TCGA data between *MEN1* and *mTOR*, *MEN1* and  
619 *4EBP1*, *MEN1* and *EIF4E*, *MEN1* and *EIF4G2* in ER+ BC. Quantitative RT-PCR data shown,  
620 normalized against GAPDH, are presented as means ( $\pm$  SEM) of 3 independent experiments. \*  $P < 0.05$ ,  
621 \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , ns, not significant vs Ctrl.

622 **Fig 3. Menin regulates MYC expression and binds to the MYC promoter in ER+ BC cells.**

623 Quantitative RT-PCR (left panels) and Western blot (right panels) showing MYC expression in ER+  
624 MCF7 and T<sub>47D</sub> cell lines (**a**) and ER- MDA<sub>MB</sub>-231 and MDA<sub>MB</sub>-453 cell lines (**b**) transfected with  
625 siCtrl or two different siMEN1 hs1 or hs3. Quantification of the band intensity of siMEN1- against siCtrl-  
626 treated samples, after normalization against GAPDH, is shown under each corresponding Western blot. **c**  
627 Schematic representation of the primers used for ChIP-quantitative PCR analysis (left panel). ChIP-qPCR  
628 analysis showing menin binding (normalized with input) on different regions of the *MYC* promoter (right  
629 panel). Results were compared to IgG and an unrelated region on chromosome 1 (Chr1) used as controls.  
630 Cells were also transfected with siMEN1 hs1 to confirm the binding specificity. **d** Quantitative RT-PCR  
631 (left panel) and Western blot (2 right panels) showing the mRNA and protein expression of different

632 MYC target genes in MCF7 cells transfected with siCtrl or two different siMEN1 hs1 or hs3. Quantitative  
633 RT-PCR data shown, normalized against GAPDH, are presented as means ( $\pm$  SEM) of 3 independent  
634 experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , \*\*\*\*  $P < 0.0001$ , ns, not significant vs Ctrl.

635 **Fig 4. Menin is needed for estrogen-mediated MYC activation and physically interacts with MYC**  
636 **in BC cells. a** Quantitative RT-PCR (left panel) and Western blot (right panel) showing the protein and  
637 mRNA expression of menin, ER $\alpha$ , and MYC in MCF7 cells after estrogen treatment (10 nM E2) for 1 h  
638 or 2 h. Quantification of the band intensity of treated samples against the control, after normalization  
639 against GAPDH, is shown under each corresponding Western blot. **b** Left panel: Quantitative RT-PCR  
640 showing the expression of *MEN1*, *ESR1*, and *MYC* in MCF7 cells transfected with siCtrl or siMEN1 hs1  
641 or hs3 in the absence or presence of estrogen (10 nM E2) for 1 h; Right panel: Western blot showing the  
642 expression of menin, ER $\alpha$ , and MYC in MCF7 cells transfected with siCtrl or siMEN1 hs1 or hs3 in the  
643 absence or presence of estrogen for 2 h. **c** Upper panel: PLA highlighting the physical interaction between  
644 menin and MYC (left panel) or menin and ER $\alpha$  (right panel) in MCF7 cells before and after estrogen  
645 treatment. siMEN1 hs1 and siMYC were used as controls. Lower panel: Quantification of PLA signals  
646 measured in terms of numbers of dots/cell. Scale bar = 10  $\mu$ m. Quantitative RT-PCR data shown,  
647 normalized against GAPDH, and quantification of PLA analyses are presented as means ( $\pm$  SEM) of 3  
648 independent experiments. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ , ns, not significant vs ethanol treatment.

649 **Fig 5. Correlation between *MEN1* and *MYC* mRNA expression in breast cancer and effects of cell**  
650 **proliferation inhibition with combined inactivation of *MEN1* and *MYC*.** **a** Left panel: data mining  
651 analysis of the SMC 2018 dataset showing the positive correlation between *MEN1* and *MYC* mRNA  
652 expression in BC including all subtypes. Right panel: same analysis of the SMC 2018 dataset in ER+ BC  
653 subtypes. **b** Correlation of mRNA expression between *MEN1* and *MYC* observed in ER+ (left panel) and  
654 ER- (right panel) tumors from TCGA dataset. **c** Inverse correlation between the expression of *MEN1* and  
655 2 inhibitory factors in the MYC pathway, *MYCB2* and *MYCT1*, in ER+ BC from the TCGA dataset. **d**  
656 Proliferation curve of MCF7 cells transfected with either siMEN1 hs1 or siMYC or both (left panel), and

657 transfected with siMEN1 hsl or treated with 20  $\mu$ M 10058 F4 (MYC/MAX inhibitor) or both (middle  
658 panel). Western blot showing MYC protein expression in MCF7 cells treated with DMSO or 20 and 50  
659  $\mu$ M of 10058 F4 inhibitor (right panel).

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