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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

Razan Abou Ziki^{1#}, Romain Teinturier^{1#}, Yakun Luo^{1#}, Catherine Cerutti⁴, Jean-Marc Vanacker⁴,
Coralie Poulard¹, Thomas Bachelot², Mona Diab-Assaf⁵, Isabelle Treilleux^{3*}, Chang Xian
Zhang^{1*} and Muriel Le Romancer¹

¹ Univ Lyon, Université Claude Bernard Lyon 1, INSERM 1052, CNRS 5286, Centre Léon
Bérard, Centre de recherche en cancérologie de Lyon, Lyon, 69008, France

² Department of Medical Oncology, Centre Léon Bérard, Lyon, France

³ Département de Biopathologie, Centre Léon Bérard, Lyon, France

⁴ Institut de Génomique Fonctionnelle de Lyon, Université de Lyon, Université Claude Bernard
Lyon 1, CNRS UMR5242, Ecole Normale Supérieure de Lyon, 69007 Lyon, France.

⁵ Faculty of Sciences II, Lebanese University Fanar, Beirut, Lebanon

RAZ, RT and YL share the first authorship

Correspondence and requests for reprints:

1 *IT and CXZ are co-corresponding authors of this study.

- 2 Isabelle Treilleux, for clinical TamRad trial study
- 3 Centre Léon Bérard, Lyon, 69008, France
- 4 Email: isabelle.treilleux@lyon.unicancer.fr
- 5 Chang Xian Zhang,
- 6 Cancer Research Centre of Lyon, France, F-69008, France
- 7 Email: chang.zhang@lyon.unicancer.fr

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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 α . 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 α , 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 α 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 α 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 α 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 α 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 α 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 κ -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% CO₂. 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% CO₂. 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 μ 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 μ g or 30 μ g (depending on experimental conditions) of cell lysate was boiled in 5 μ 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 μ 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- μm 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 μM 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 α 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_{47D} 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_{47D} 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_{47D} 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 α and menin. Our data demonstrated
321 that *MYC* KD had no effect on ER α expression, but decreased MEN1 expression at the transcriptional and
322 protein levels in MCF7 and T_{47D} 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_{47D} 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 β -estradiol (E2) at different time points (Fig
334 S4). Though the levels of ER α 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 α (Fig 4c right panel), suggesting a role for menin in
345 estrogen-mediated MYC activation functions, likely through its physical interaction with ER α .

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⁺ BC cell lines as previously
367 reported (Dreijerink *et al.* 2017b), whereas it triggered a distinct cell growth reaction in an ER⁻ 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⁻ 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⁻ BC cell lines. In fact, Dreijerink *et al.* reported that menin did not regulate
372 ER α target genes in ER⁻ BC cell lines. It would, therefore, be interesting to further dissect the role of
373 menin in responsive or non-responsive ER⁻ BC cells, beyond the ER α 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⁺ 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⁺ 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⁺ 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 α 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 α 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.

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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.

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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_47D (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_47D (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_47D

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_{47D}
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 μ 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_{47D} cell lines (**a**) and ER- MDA_{MB}-231 and MDA_{MB}-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 α , 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 α , 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 α (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 μ 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 μ 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 μ M of 10058 F4 inhibitor (right panel).

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