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PPAR-independent event involved in the  
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J2 and Thiazolidinediones in breast cancer cells**

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Authors: Sarra Chbicheb, Xiao Yao, Jean-Luc Rodeau, Stéphane Salamone, Michel Boisbrun, Gerald Thiel, Daniel Spohn, Isabelle Grillier-Vuissoz, Yves Chapleur, Stéphane Flament, Sabine Mazerbourg

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**EGR1 EXPRESSION : A CALCIUM AND ERK1/2 MEDIATED PPAR $\gamma$  -  
INDEPENDENT EVENT INVOLVED IN THE ANTIPROLIFERATIVE EFFECT OF  
15-DEOXY- $\Delta$ 12,14-PROSTAGLANDIN J2 AND THIAZOLIDINEDIONES IN  
BREAST CANCER CELLS**

**Sarra Chbicheb<sup>a</sup>, Xiao Yao<sup>a</sup>, Jean-Luc Rodeau<sup>b</sup>, Stéphane Salamone<sup>c1</sup>, Michel Boisbrun<sup>c</sup>,  
Gerald Thiel<sup>d</sup>, Daniel Spohn<sup>d</sup>, Isabelle Grillier-Vuissoz<sup>a</sup>, Yves Chapleur<sup>c</sup>, Stéphane Flament<sup>a</sup>,  
and Sabine Mazerbourg<sup>a</sup>**

<sup>a</sup>EA 4421 Signalisation, Génomique et Recherche Translationnelle en Oncologie (SIGRETO) Nancy-  
Université, 54506 Vandœuvre-lès-Nancy, France  
SC: sarra.chbicheb@sbiol.uhp-nancy.fr; SF: stephane.Flament@sbiol.uhp-nancy.fr; IGV:  
isabelle.grillier-vuissoz@sbiol.uhp-nancy.fr; SM:sabine.mazerbourg@sbiol.uhp-nancy.fr;  
XY: dawnnyao@yahoo.com

<sup>b</sup>Institut des Neurosciences Cellulaires et Intégratives (INCI) CNRS UPR-3212 67084 Strasbourg,  
France  
JLR : jlr@inci-cnrs.unistra.fr

<sup>c</sup>Groupe S.U.C.R.E.S., UMR 7565 CNRS, Nancy-Université, 54506 Vandœuvre-lès-Nancy, France  
MB : Michel.Boisbrun@pharma.uhp-nancy.fr; YC: yves.chapleur@suces.uhp-nancy.fr; SS:  
stephane.salamone@pasteur.fr

<sup>d</sup>Department of Medical Biochemistry and Molecular Biology, University of Saarland Medical Center,  
D-66421 Homburg, Germany (G.T., D.S.)  
GT: gerald.thiel@uniklinik-saarland.de; DS: daniel.spohn@uniklinik-saarland.de

Corresponding author: Sabine Mazerbourg, EA 4421 Signalisation, Génomique et Recherche  
Translationnelle en Oncologie (SIGRETO), Université Henri Poincaré, Nancy-Université, Faculté des  
Sciences, BP 70239, 54506 Vandœuvre-lès-Nancy; Cedex France. Tél : 011 33(0) 3 83 68 42 57, Fax :  
011 33(0) 3 83 68 40 01; E-mail: sabine.mazerbourg@sbiol.uhp-nancy.fr

**Abstract**

1  
2 Our aim was to get new information about the Peroxisome Proliferator Activated  
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4 Receptor gamma (PPAR $\gamma$ )–independent pathway involved in the antiproliferative action of  
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6 PPAR $\gamma$  ligands in breast cancer cells. We investigated the effects of Troglitazone (TGZ),  
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8 Ciglitazone (CGZ), Rosiglitazone (RGZ) and, 15-deoxy- $\Delta$ 12,14-prostaglandin J2 (15d-PGJ<sub>2</sub>)  
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10 on the hormone-dependent breast cancer cell line MCF7. The early transcription factor *EGR1*  
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12 (Early Growth Response gene 1) mRNA and protein levels peaked after 3 hours of incubation  
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14 with 25  $\mu$ M TGZ, CGZ or 15d-PGJ<sub>2</sub> and then gradually decreased. RGZ, the most potent  
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16 activator of PPAR $\gamma$ , did not show this effect. The PPAR $\gamma$  antagonist GW 9662 did not block  
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18 *EGR1* mRNA induction which also still occurred in case of PPAR $\gamma$  silencing as well as in  
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20 case of treatment with the PPAR $\gamma$ -inactive compound  $\Delta$ 2-TGZ. *EGR1* mRNA induction  
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22 required ERK1/2 phosphorylation which was not blocked by EGF Receptor (EGFR)  
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24 inhibition. The ERK1/2 pathway was also involved in  $\Delta$ 2-TGZ-induced *EGR1* mRNA  
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26 expression in the hormone-independent breast cancer cell line MDA-MB-231. Using the  
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28 fluorescent dye Fura2, we showed in MCF7 that TGZ or  $\Delta$ 2-TGZ induced an immediate  
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30 increase in cytosolic calcium which was required for ERK1/2 phosphorylation and *EGR1*  
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32 mRNA induction as demonstrated by calcium chelation experiments. Furthermore, in MCF7  
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34 transfected with siRNA targeting *EGR1*,  $\Delta$ 2-TGZ inhibited less efficiently cell proliferation.  
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45 **Graphical abstract** : Troglitazone (TGZ) and  $\Delta$ 2-TGZ induce an increase in cytosolic  
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47 calcium leading to ERK1/2 phosphorylation and EGR1 expression in breast cancer cells,  
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49 leading to growth inhibition. This event is independent of PPAR $\gamma$  and EGFR activation.  
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55 **Keywords** : Thiazolidinedione, Troglitazone, EGR1, calcium signaling, breast cancer  
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**Abbreviations list:**

1  
2 PPAR : peroxisome proliferator-activated receptor  
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4 EGR1 : Early Growth Response gene 1  
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7 15d-PGJ<sub>2</sub> : 15-deoxy- $\Delta$ 12,14-prostaglandin J2  
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9 TGZ : Troglitazone  
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11 CGZ : Ciglitazone  
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13 RGZ : Rosiglitazone  
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15 EGFR : EGF Receptor  
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17 NAB : NGFI-A-binding protein  
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19 FCS : fetal calf serum  
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21 DMSO : dimethylsulfoxide  
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23 DMEM : Dulbecco's modified Eagle's medium  
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25 dNTP : desoxynucleotide triphosphate  
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27 DTT : dithiothreitol  
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29 Ig : Immunoglobulin  
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31 MAPK : mitogen activated protein kinase  
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33 RPLPO : acidic ribosomal phosphoprotein PO  
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## 1. Introduction

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4 The peroxisome proliferator-activated receptors (PPARs) are a family of ligand-  
5 activated transcription factors belonging to the nuclear receptor superfamily [1]. Three PPAR  
6 subtypes encoded by separate genes have been identified:  $\alpha$ ,  $\beta/\delta$  and  $\gamma$ . Ligands of PPAR $\gamma$   
7 include 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>), oxidative metabolites of  
8 polyunsaturated fatty acids and synthetic compounds like thiazolidinediones (troglitazone  
9 TGZ, ciglitazone CGZ, rosiglitazone RGZ...), a class of drugs used in the treatment of type II  
10 diabetes [2]. Various PPAR $\gamma$  agonists have anti-cancer activities [3, 4]. For instance, TGZ and  
11 15d-PGJ<sub>2</sub> inhibit tumor growth of human prostate, colorectal or breast cancer cells in  
12 immunodeficient mice [4]. Anticancer effects of such compounds have also been observed *in*  
13 *vitro*. For example, in breast cancer cell lines, they inhibit proliferation, induce differentiation  
14 or trigger apoptosis [5, 6]. Recently, a nonapoptotic and nonautophagic cytoplasmic  
15 vacuolation death was observed in response to 15d-PGJ<sub>2</sub> whereas autophagy was described in  
16 response to thiazolidinediones in the same breast cancer cell line MDA-MB-231 [7, 8].  
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38 Several data suggest that the anticancer effects of PPAR $\gamma$  ligands could be the result of  
39 PPAR $\gamma$ -independent events [9]. This was deduced from studies using PPAR $\gamma$  antagonists,  
40 transfection of dominant negative PPAR $\gamma$  isoforms, PPAR $\gamma$ -targeted RNA interference as well  
41 as thiazolidinedione derivatives like  $\Delta^2$ -TGZ that could not activate PPAR $\gamma$ . The proteasomal  
42 degradation of estrogen receptor alpha and cyclin D1 is an example of such a PPAR $\gamma$ -  
43 independent effect triggered by several PPAR $\gamma$  ligands which has been well described in  
44 breast cancer cells [10-12]. However, in these cells, few data are available on the identity of  
45 mediators involved in the PPAR $\gamma$ -independent pathway activated by thiazolidinediones.  
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58 Interestingly, in human colon cancer cells, Early growth response gene 1 (EGR1) was  
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1 identified as an early transcription factor induced after a three hour treatment with TGZ in a  
2 PPAR $\gamma$ -independent manner [13].  
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7 EGR is a family of zinc-finger transcription factors (EGR1-4) with an aminoterminal  
8 activation domain, a central domain that interacts with the co-repressors NGFI-A-binding  
9 proteins 1 and 2 (NAB1 and NAB2) (except for EGR4), and a DNA binding domain  
10 consisting of three Cys2-His2 zinc fingers near the carboxy-terminal end of the protein  
11 sequence. They are described as early response genes because they are rapidly and transiently  
12 induced [14]. EGR proteins bind the consensus nucleotide sequence GCGGGGGCG to  
13 regulate expression of target genes [15]. EGR1, (also known as NGF-1A, KROX24, ZIF268,  
14 and TIS8) is a nuclear phosphoprotein that has been reported to be rapidly induced by serum,  
15 growth factors, ultraviolet light, ionizing radiations, mechanical injury, and stress. Thus,  
16 EGR1 is involved in a variety of cell processes including growth, differentiation, angiogenesis,  
17 wound healing and apoptosis. In prostate cancer cells and few other models, EGR1 is  
18 described as a protumoral factor contributing to regulation of genes which are critical for cell  
19 cycle progression and counteracting apoptosis [16]. In contrast, EGR1 expression is absent or  
20 very weak not only in several human breast cancer cell lines compared to immortalized  
21 normal human mammary cell lines (MCF10A and 184A1N4), but also in breast cancer tissues  
22 compared to normal breast tissue [17, 18]. Moreover, induced-overexpression of EGR1  
23 negatively regulates the growth of the breast cancer cell line ZR75 [19]. These findings  
24 suggest that in breast cancer cells and tissues, reduced expression of EGR1 could enhance  
25 tumorigenesis while EGR1 overexpression could be linked to growth inhibition.  
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56 In an effort to better understand the intracellular mode of action of thiazolidinediones,  
57 the aim of our study was 1) to determine if the potential growth inhibitor EGR1 could be an  
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element of their signaling in breast cancer cells, 2) to evaluate the role of the nuclear receptors PPAR $\gamma$  in this process and 3) to characterize the cellular and molecular events leading to this response. Our studies performed in the hormone-dependent cell line MCF7 show that TGZ, CGZ and 15d-PGJ<sub>2</sub> induce the early expression of EGR1 in a PPAR $\gamma$ -independent manner. In contrast, RGZ, the most potent PPAR $\gamma$  agonist, did not stimulate EGR1 expression. EGR1 induction was associated with ERK activation but the transactivation of EGFR was not involved. Similar results were observed in the hormone-independent breast cancer cell line MDA-MB-231. Furthermore, an early increase in cytosolic calcium appeared to be a key actor in the signaling pathway leading to *EGR1* expression. Finally, siRNA experiments demonstrated that EGR1 was as a mediator of the inhibition of proliferation triggered by  $\Delta$ 2-TGZ in MCF7 cells.

## 2. Materials and Methods

### 2.1. Cell culture and reagents

MCF7 and MDA-MB-231 human breast cancer cell lines were obtained from American Type Culture Collection (Rockville, MD, USA). Both cell lines were grown at 37°C under 5% CO<sub>2</sub> in phenol red Dulbecco's modified Eagle medium (DMEM, Invitrogen, Cergy Pontoise, France) for MCF-7 and in L-15 medium (Invitrogen) for MDA-MB-231. These media were supplemented with 10% fetal calf serum (FCS) (Sigma-Aldrich, Lyon, France) and 2 mM L-glutamine (Invitrogen).

TGZ, AG1478, A23187 ionophore (Sigma-Aldrich), CGZ, RGZ, GW9662, U0126, PD153035, SP600125, SB203580, BAPTA-AM (Calbiochem Merck, Darmstadt, Germany), 15d-PGJ<sub>2</sub> (Interchim, Montluçon, France) were dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich). Recombinant human Epidermal Growth Factor (EGF) (Invitrogen) was resuspended in PBS. The synthesis of  $\Delta$ 2-TGZ was achieved according to slight modifications of known procedures [17, 20]. Experimental details will be published elsewhere. The identity and purity of this synthetic derivative were verified by proton nuclear magnetic resonance and elemental analysis. This compound was also dissolved in DMSO.

### 2.2. Cell treatment

After seeding and 24 hours of incubation to allow for cell attachment, cells were washed with PBS and the medium was replaced with phenol red-free DMEM (MCF7) or L-15 medium (MDA-MB-231) supplemented with 1% FCS and 2 mM L-glutamine. After 24 hours, cells were washed with PBS and treated with either DMSO, 15d-PGJ<sub>2</sub>, thiazolidinediones, EGF, or the calcium ionophore A23187 at the indicated concentrations and times in phenol red-free, FCS-free DMEM for MCF7 or 1% FCS L-15 medium for MDA-MB-231 supplemented with 2 mM L-glutamine. To test the involvement of the MAPK and calcium pathways or the PPAR $\gamma$  and EGF receptors, cells were treated with different inhibitors for 30

minutes prior to PPAR $\gamma$  ligands exposure. Cells were then collected for RNA or protein extraction.

### 2.3. Semi-quantitative RT-PCR

Cells were seeded in 6-well plates at a density of  $5 \times 10^5$  cells/well (MCF7 cells) or  $4 \times 10^5$  cells/well (MDA-MB-231 cells) in 2 mL of medium with 10% FCS and 2 mM L-glutamine. Cells were treated as previously described. Total RNA was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized in 25  $\mu$ L volume containing 1  $\mu$ g RNA, 12 ng random hexamer primer, 1X Invitrogen Buffer, 4 mM DTT, 0.1 mM of each dNTP, 4 U RNase inhibitor and 75 U of MMLV reverse transcriptase (Invitrogen). The mixture was incubated for 10 minutes at 25°C, for 110 minutes at 37°C and subsequently for 10 minutes at 70°C in order to stop the reaction. The cDNA were further amplified by PCR. The specific primer sequences are as follows:

*EGRI* (NM\_001964) forward 5'-GGCCACCTCCTCTCTCTCTT-3', reverse 5'-TGTTGGCCAATAGACCTTCC-3'; *PPAR $\gamma$*  (NM\_138712) forward 5'-GACCACTCCCCTCTCTTT-3', reverse 5'-CGACATTCAATTGCCATGAG-3'; *EGFR* (NM\_005228) forward 5'-AAGCTCACGCAGTTGGGCAC-3', reverse 5'-GGTTCTGGAAGTCCATCGAC-3'; *NAB2* (NM\_005967) forward 5'-GACCCTGCAGCCCAGACTC-3', reverse 5'-CTTCGATAGTGGTGACGGACC-3'; *RPLPO* (NM\_053275) forward 5'-ACAACCCAGCTCTGGAGAAA-3', reverse 5'-TCGTTTGTACCCGTTGATGA-3'. PCR was carried out in a thermal cycler (Eppendorf, Le Pecq, France) in a final volume of 25  $\mu$ L containing 2  $\mu$ L cDNA, 1X EconoTaq™ Reaction Buffer (-Mg), 1.5 mM MgCl<sub>2</sub>, 0.2 mM of dNTP mix, 0.2  $\mu$ M of each primer and 2.5 U EconoTaq. Negative controls were always included. Amplification was carried out under the following conditions: (1) initial denaturation 94°C for 2 minutes; (2) 94°C for 30 s, 58°C for

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30s and 72°C for 45s. 30 PCR cycles were performed for gene amplification, with the exception of RPLPO amplified with 25 cycles. (3) 10 minutes extension step at 72°C. The number of cycles was chosen to realize the analysis in the linear phase of the PCR reaction. 20 µL of the PCR products were mixed with loading buffer (5 µl) and submitted to electrophoresis in a 1.2 % agarose gel at 90 V for 35 minutes at room temperature. The gel was stained with ethidium bromide, viewed and photographed on a UV-transilluminator (GelDoc 2000, Bio-Rad Laboratories, Marnes-La-Coquette, France). A software package (Quantity One v.4.3.1, Bio-Rad Laboratories) was used to quantify the PCR products.

#### 2.4. RNA interference

The small-interfering RNA (siRNA) duplexes for targeting PPAR $\gamma$ , EGR1 and EGFR as well as a scrambled sequence (control siRNA duplex, negative control) were purchased from Eurogentec (Angers, France). The sequences are shown in Table 1. MCF7 cells (1.45 x 10<sup>5</sup>) were plated overnight in twelve-well culture plates in DMEM supplemented with 10% FCS and 2 mM L-glutamine. Cells were transiently transfected with either target gene siRNA (200 nM of the mix duplex 1 and duplex 2 or 200nM of one duplex) or negative control siRNA-sequence (Table 1) using the Oligofectamine™ Reagent (Invitrogen) as described by the manufacturer. The concentration of siRNA used was standardized to get maximum knockdown without affecting the viability of the cells. After 24 hours, cells were washed with PBS and the medium was replaced with phenol red-free DMEM supplemented with 1% FCS and 2 mM L-glutamine. 24 hours later, cells were treated in phenol red-free and FCS-free DMEM with 2 mM L-glutamine and harvested for further analyses.

## 2.5. Nuclear protein extracts

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2 At the end of the treatment, cells were washed twice with cold PBS and collected in 1  
3 mL PBS. Nuclear and cytoplasmic extracts were prepared by detergent lysis. Briefly, after  
4 pelleting, the cells were incubated in hypotonic solution (10 mM Hepes-KOH, pH 7.9, 1.5  
5 mM MgCl<sub>2</sub>, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.5% Nonidet P40, 2.5X Protease  
6 inhibitor cocktail tablet, Roche) for 15 minutes on ice and vortexed each 5 minutes. Nuclei  
7 were collected by centrifugation at 13 000 rpm for 5 minutes at 4°C. The nuclei pellet was  
8 then resuspended in a hypertonic buffer (10 mM Hepes-KOH pH 7.9, 25% Glycerol, 420 nM  
9 NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.5% Triton, 2.5X Protease inhibitor  
10 cocktail tablet, Roche, Fontenay sous Bois, France). Nuclei were incubated for 15 minutes at  
11 4°C with shaking. After centrifugation (13 000 rpm, 5 minutes, 4°C), the supernatant  
12 containing nuclear proteins was stored at -80°C. Protein concentrations were determined by  
13 the Bradford method (Bio-Rad Protein Assay, Bio-Rad Laboratories).  
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## 2.6. Western immunoblotting

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32 For EGR1 detection, 30 µg of nuclear proteins were mixed with 2X Laemmli buffer,  
33 boiled (100°C, 5 minutes) and separated by 10% SDS-PAGE. For other protein analyses, cells  
34 were harvested by addition of 150 µL 2X Laemmli buffer and boiled. For each sample, 30 µL  
35 of total proteins were separated by 10% SDS-PAGE. After electrophoresis, proteins were  
36 transferred onto nitrocellulose membrane. The homogeneity of loaded amounts and transfer  
37 efficiency were confirmed by Ponceau S staining of nitrocellulose membranes for each  
38 experiment. Non specific binding sites were blocked in TNT buffer (5 mM Tris-HCl, 15 mM  
39 NaCl, 0.1% Tween 20) with 5% non-fat powder milk and incubated with the primary  
40 antibodies diluted in blocking solution overnight at 4°C. The rabbit monoclonal antibodies  
41 anti-EGR1 (SC-189, Santa Cruz Biotechnology, USA) and anti-phospho-p44/42 MAPK  
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1 (ERK1/2) (Thr 202/Tyr 204) (Cell Signaling Technology/Ozyme, Saint-Quentin en Yvelines,  
2 France) were diluted at 1:1000. Total ERKs were detected using a rabbit monoclonal anti-  
3 p44/42 MAPK (ERK1/2) antibody (Cell Signaling Technology) at 1:5000 in blocking  
4 solution. The rabbit polyclonal antibody anti-actin was used diluted at 1:3000.  
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9 Next, the membranes were washed 3 times with TNT and incubated for 2 hours with a  
10 peroxidase-linked secondary antibody (Santa Cruz Technology) at room temperature. After 3  
11 washings with TNT, the membranes were developed with ECL detection reagent using  
12 chemiluminescence (Amersham, Orsay, France). After Phospho-p44/42 MAPK analysis, the  
13 blots were stripped and reprobed with anti-p44/42 MAPK antibody.  
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## 24 **2.7. Immunocytochemistry**

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26 MCF7 cells ( $1.45 \times 10^5$ ) were seeded on glass coverslips and grown in their medium  
27 as described before. Cells were treated with TGZ or DMSO for 3 hours in phenol red-free  
28 DMEM containing 1% FCS. At the end of treatment, coverslips were washed with PBS and  
29 fixed in 4% paraformaldehyde (Sigma-Aldrich) for 15 minutes. After washing in PBS,  
30 preparations were permeabilized for 10 minutes in 0.5% Triton X100. After washing in PBS,  
31 non specific binding was blocked by incubation for 30 minutes in a blocking solution (4%  
32 bovine serum albumin, 0.1% Triton X100 in PBS Buffer). Samples were incubated with the  
33 anti-EGR1 antibody (SC-189, Santa Cruz Biotechnology) diluted at 1:400 in the blocking  
34 solution for 1 hour. They were then washed 3 times in the blocking solution and exposed for 1  
35 hour to Alexa Fluor 555 goat anti-mouse IgG antibody (Invitrogen) diluted 1:1000 in the  
36 blocking solution. Finally, cells were rinsed with PBS 0.1% Triton X100 for 15 minutes and  
37 cells were counterstained with Hoechst dye to visualize nuclei. Fluorescence labelling was  
38 observed under an Eclipse 80i microscope (Nikon, Champigny sur Marne, France). Images  
39 were collected using LuciaG software 4.81 (Laboratory imaging/Nikon).  
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## 2.8. Intracellular calcium imaging

MCF7 cells were seeded on glass coverslips as described above. The cytoplasmic  $\text{Ca}^{2+}$  concentration was detected by the ratiometric fluorescent probe Fura-2. Cells were loaded with 5  $\mu\text{M}$  of the cell-permeant precursor Fura-2 acetoxymethyl ester (Fura-2/AM; Molecular Probes Invitrogen) in presence of pluronic F-127. After 1 hour incubation, they were washed three times with the recording saline solution containing (in mM): NaCl, 120; KCl, 5;  $\text{CaCl}_2$ , 2;  $\text{MgCl}_2$ , 2; D-glucose, 10; and Hepes, 20; pH 7.3. Fluorescence measurements were performed on an inverted microscope (Axiovert 35; Zeiss, Germany) with an oil-immersion  $\times 40$  Nikon objective (Fluor 40, NA 1.30) and a cooled CCD camera (CoolSnap HQ; Photometrics, USA). The Imaging Workbench 4.0 software (Axon Instruments, USA) was used for image acquisition and analysis. Fluorescence was excited alternately at 350 and 380 nm with a Lambda-10 filter wheel (Sutter Instrument, USA), and emitted light was collected above 520 nm. Pairs of images were acquired every 2 seconds. Intracellular calcium was expressed throughout as the fluorescence ratio  $F_{350} / F_{380}$ , calculated after background subtraction. Experiments were performed at room temperature (25-30°C). During calcium measurements, the cells were continuously superfused with saline solution: the whole dish by a bath perfusion of control medium and the recorded field by a single-tip multichannel gravity-fed system, allowing switching between various solutions. All drugs were prepared at the indicated concentrations in saline solution.

## 2.9. Cell proliferation assay

48 h after siRNA transfection, the medium was replaced by DMEM supplemented with 1% FCS and 2 mM L-glutamine. After 4h, cells were treated for 24 h with 15 $\mu\text{M}$   $\Delta 2$ -

1 TGZ in DMEM supplemented with 0% FCS and 2 mM L-glutamine. Control wells received  
2 0.05% DMSO. At the end of the treatment, cells were washed with PBS, trypsinized and  
3  
4 counted with the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Charbonnières,  
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6 France). Each treatment was performed five times.  
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### 3. Results

#### 3.1. Troglitazone induces early EGR1 expression in MCF7 breast cancer cells

Exposure of the estrogen-dependent human breast cancer epithelial cell line MCF7 to 25  $\mu$ M TGZ induced a transient expression of *EGR1* mRNA which appeared at 30 minutes, peaked at 3 hours and then decreased (Fig. 1A). The vehicle DMSO (0.05%) did not induce a significant increase in *EGR1* mRNA expression compared to the non-treated sample (Fig. 1A). Furthermore, TGZ stimulated *EGR1* mRNA expression in a dose-dependent manner with a better response observed at 25  $\mu$ M than 5  $\mu$ M (Fig. 1B). At the protein level, EGR1 was also stimulated with a maximum in nuclear extracts of cells that had been exposed to 25  $\mu$ M TGZ for 3 hours (Fig. 1C). Fetal calf serum (FCS) used as a positive control induced a higher and earlier stimulation of EGR1 (Fig. 1C). Ponceau S protein staining of membranes after transfer confirmed equivalence of loaded samples (not shown). In immunocytochemistry experiments, we observed a nuclear staining with the anti-EGR1 antibody in cells exposed for 3 hours to 25  $\mu$ M TGZ. However, all the cells did not display the same staining intensity which varies from faint to intense (Fig 1D). In MCF7 cells exposed for 3 hours to 20% FCS, the nuclear staining intensities were higher, that was in accordance with the results of western blot analyses. Staining intensity was not homogeneous between cells, as previously described.

In order to confirm the activation of the EGR1 pathway in cells exposed to TGZ, we analyzed the EGR1 target gene *NAB2*. *NAB2* mRNA was constitutively expressed in MCF7 cells (Fig. 1E). 25  $\mu$ M TGZ induced a transient expression of *NAB2* mRNA which appeared at 6 hours, peaked at 9 hours and then decreased (Fig. 1E). The vehicle DMSO (0.05%) did not induce a significant increase in *NAB2* mRNA expression compared to the non-treated sample (Fig. 1E).

### 3.2. EGR1 induction by troglitazone, ciglitazone and 15d-PGJ<sub>2</sub> is PPAR $\gamma$ -independent

Other PPAR $\gamma$  ligands were tested. Both CGZ and 15d-PGJ<sub>2</sub> (25  $\mu$ M) were able to induce a transient expression of *EGR1* mRNA with a peak of expression observed after 3 hours of treatment (Fig. 2A). The transcription of *EGR1* was followed by translation of EGR1 and by *NAB2* stimulation (Fig. 2B and C). In contrast, these events were not observed after 25  $\mu$ M RGZ treatment (Fig. 2A-B). Since RGZ is a more potent PPAR $\gamma$  agonist than TGZ, CGZ or 15d-PGJ<sub>2</sub>, it suggested that the induction of *EGR1* expression required a PPAR $\gamma$ -independent mechanism.

It was demonstrated by three different complementary approaches. In MCF7 cells co-treated for 3 hours with 25  $\mu$ M TGZ, CGZ or 15d-PGJ<sub>2</sub> and the irreversible PPAR $\gamma$  antagonist GW9662 (5 or 25  $\mu$ M) *EGR1* mRNA expression was still stimulated (Fig. 3A). When TGZ (25  $\mu$ M) was tested on *PPAR* $\gamma$  siRNA transfected MCF7 cells (in which PPAR $\gamma$  mRNA level was decreased by more than 80%), the induction of *EGR1* mRNA expression was still induced after 3 hours of treatment (Fig. 3B). Besides, MCF7 cells that were treated for 3 hours with 25  $\mu$ M  $\Delta$ 2-TGZ, a TGZ derivative devoid of PPAR $\gamma$  agonist activity, displayed an increase in *EGR1* and *NAB2* mRNA levels (Fig. 3C). Such a  $\Delta$ 2-TGZ treatment was also able to induce the expression of EGR1 protein (Fig. 3D).

### 3.3. Troglitazone, ciglitazone and 15d-PGJ<sub>2</sub> rapidly activate ERK1/2 pathway

We studied if the extracellular signal-regulated kinase (ERK) 1/2 pathway could be involved in EGR1 expression stimulation. TGZ, CGZ and 15d-PGJ<sub>2</sub> (25  $\mu$ M) induced ERK1/2 phosphorylation that could be detected as soon as 5 minutes after stimulation (Fig. 4A). The signal intensity increased between 15 and 45 minutes following treatment and decreased thereafter (Fig. 4A). In case of  $\Delta$ 2-TGZ (25  $\mu$ M) treatment, ERK1/2

1 phosphorylation occurred more slowly: it was detected 15 minutes after stimulation and  
2 increased up to 75 minutes (Fig. 4B). In contrast to these compounds, RGZ induced no  
3 change in ERK1/2 phosphorylation (data not shown).  
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7 In MCF7 cells that were pretreated for 30 minutes with the MEK1/2 inhibitor U0126 (5  $\mu$ M)  
8 and subsequently treated with vehicle or 25  $\mu$ M TGZ, CGZ, 15d-PGJ<sub>2</sub> (15 minutes) and  $\Delta$ 2-  
9 TGZ (30 minutes), no activated ERK1/2 was found (Fig. 4C). At a concentration as low as 5  
10  $\mu$ M, U0126 was efficient to inhibit the *EGR1* expression usually induced by TGZ, CGZ, 15d-  
11 PGJ<sub>2</sub> (Fig. 4D). In case of  $\Delta$ 2-TGZ treatment, U0126 inhibited *EGR1* mRNA induction  
12 partially at 5  $\mu$ M whereas a complete inhibition was observed at 25  $\mu$ M (Fig. 4D). The  
13 MAPK inhibitor was also able to inhibit the TGZ-induced increase in EGR1 protein (Fig. 4E).  
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15 To determine the role of the other MAP Kinases on the regulation of *EGR1* expression, cells  
16 were treated with  $\Delta$ 2-TGZ in the presence of 10  $\mu$ M of a specific inhibitor of p38 MAPK  
17 (SB203580) (Fig. 5A) and JNK (SP600125) (Fig. 5B). In contrast to U0126 that completely  
18 abolished *EGR1* expression, neither SB20358 nor SP600125 could antagonize this response to  
19  $\Delta$ 2-TGZ (Fig. 5A and B).  
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#### 40 **3.4. PPAR $\gamma$ -independent expression of EGR1 does not require EGFR activation**

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42 We tested the possibility for EGFR to be involved in the PPAR $\gamma$ -independent  
43 activation of *EGR1* in MCF7 cells. For this purpose, we used the EGFR tyrosine kinase  
44 inhibitors PD153035 or AG1478. At 5  $\mu$ M, these compounds totally inhibited ERK1/2  
45 phosphorylation in MCF7 cells exposed to EGF (15 nM for 5 minutes) (Fig. 6A). We also  
46 observed that the exposure to EGF (15 nM) for 1 hour could increase *EGR1* mRNA level in  
47 MCF7 cells and that this effect did not occur in the presence of the EGFR antagonists (Fig.  
48 6B). However, in our conditions,  $\Delta$ 2-TGZ-induced *EGR1* mRNA expression was not blocked  
49 by the two EGFR tyrosine kinase inhibitors AG1478 and PD153035 (Fig. 6C). Our study  
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1 performed in MCF7 cells transfected with siRNA targeting EGFR confirmed the absence of a  
2 role of EGFR in the induction of *EGR1* mRNA expression and ERK phosphorylation after  
3  $\Delta 2$ -TGZ treatment (Fig. 6D).  
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### 10 **3.5. PPAR $\gamma$ -independent expression of EGR1 in MDA-MB-231 cells**

11 In order to determine whether the PPAR $\gamma$ -independent expression of EGR1 expression  
12 could be observed in other breast cancer cells, we studied its expression in the hormone-  
13 independent cell line MDA-MB-231. After 3 hours of treatment,  $\Delta 2$ -TGZ was able to induce  
14 *EGR1* mRNA expression (Fig. 7). Then, we investigated whether the ERK1/2 pathway was  
15 involved in this event. A pretreatment with the ERK1/2 inhibitor U0126 (5  $\mu$ M) abolished  
16 *EGR1* mRNA expression induced by  $\Delta 2$ -TGZ in MDA-MB-231 cells (Fig. 7). In contrast, the  
17 EGFR inhibitor AG1478 had no effect (Fig. 7). Thus, the PPAR $\gamma$ -independent expression of  
18 EGR1 is not restricted to MCF7 cells.  
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### 35 **3.6. PPAR $\gamma$ -independent expression of EGR1 is mediated by Ca<sup>2+</sup>**

36 In order to determine whether calcium signaling could be involved in the PPAR $\gamma$ -  
37 independent ERK1/2-mediated induction of *EGR1* expression, MCF7 cells were pretreated  
38 for 30 minutes with the Ca<sup>2+</sup> chelator BAPTA-AM before their exposure to PPAR $\gamma$  ligands  
39 (25  $\mu$ M). This treatment abolished the usual increase in *EGR1* mRNA level (Fig. 8A).  
40 ERK1/2 could not be activated in these conditions (Fig. 8A). Interestingly, we observed that  
41 in MCF7 cells, the Ca<sup>2+</sup> ionophore A23187 also induced *EGR1* mRNA expression but with an  
42 earlier response than those usually observed with  $\Delta 2$ -TGZ (Fig. 8B). Moreover, A23187  
43 treatment also induced ERK1/2 activation before *EGR1* mRNA expression (2 minutes versus  
44 15 minutes respectively) (Fig. 8C). This A23187-triggered *EGR1* induction was prevented in  
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1 the presence of the antagonist U0126 which also inhibited ERK1/2 phosphorylation (data not  
2 shown).

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4 Then, we studied intracellular  $\text{Ca}^{2+}$  in MCF7 cells (Fig. 8D). In control cells exposed to  
5 DMSO no change in the basal level of  $\text{Ca}^{2+}$  was observed. In contrast, MCF7 cells exposed  
6 for 2 minutes to 25  $\mu\text{M}$  TGZ showed a fast increase in intracellular calcium,  $[\text{Ca}^{2+}]_i$ , which  
7 disappeared as soon as cells were back in TGZ-free medium (Fig. 8D, upper panel). After  
8 recovery to basal level, a new exposure to TGZ induced again a rise in  $[\text{Ca}^{2+}]_i$ . An increase in  
9  $[\text{Ca}^{2+}]_i$  was also observed in response to 25  $\mu\text{M}$   $\Delta 2$ -TGZ, showing that the calcium increase  
10 observed with TGZ was a PPAR $\gamma$ -independent event (Fig. 8D, middle panel). However, after  
11 elimination of  $\Delta 2$ -TGZ, cytosolic calcium decreased more slowly than after TGZ treatment.  
12 This rise in  $[\text{Ca}^{2+}]_i$  did not occur in the presence of BAPTA-AM but it was still observed  
13 when  $\Delta 2$ -TGZ was applied in a  $\text{Ca}^{2+}$ -free solution suggesting that a release from intracellular  
14 stores was involved, rather than an influx of external  $\text{Ca}^{2+}$  (Fig. 8D, middle and lower panels).  
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### 32 33 34 **3.7. EGR1 is a mediator of $\Delta 2$ -TGZ-induced growth arrest**

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36 To test whether EGR1 is critical for the antiproliferative action of  $\Delta 2$ -TGZ, we  
37 performed gene silencing in MCF7 cells. The suppression of EGR1 expression was confirmed  
38 by RT-PCR (Fig. 9A). In each experiment, at least 50% of *EGR1* mRNA expression was  
39 observed. In MCF7 cells transfected with Ctrl siRNA, after 24h treatment with 15 $\mu\text{M}$   $\Delta 2$ -  
40 TGZ, the number of viable cells was 47% of those exposed to DMSO. When the cells were  
41 transfected with the *EGR1* siRNA,  $\Delta 2$ -TGZ affected less efficiently MCF7 cells since viable  
42 cells were 61% of those exposed to DMSO (Fig. 9B).  
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#### 4. Discussion

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2 In this study, we show that *EGR1* is an early target gene of TGZ in MCF7 cells with a  
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4 peak of expression after 3 hours of treatment. Such a transient induction of EGR1 expression  
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6 was also observed in response to CGZ and 15d-PGJ<sub>2</sub>. The expression of EGR1 protein was  
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8 confirmed by Western blotting and by the delayed induction of *NAB2*, a well known EGR1  
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10 target gene [21]. EGR1 could be a mediator of PPAR $\gamma$  ligand action since a transient  
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12 stimulation of its expression has also been reported in other cell lines like human colorectal  
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14 carcinoma cells HCT116 or activated T cells in response to TGZ or 15d-PGJ<sub>2</sub> respectively [10,  
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24 Our results demonstrate that EGR1 expression is stimulated in breast cancer cell lines  
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26 in a PPAR $\gamma$ -independent manner. This was first suggested by the fact that RGZ was not able  
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28 to induce EGR1 expression although it was the most potent PPAR $\gamma$  agonist tested [10]. We  
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30 confirmed the PPAR $\gamma$ -independent mechanism by the use of the PPAR $\gamma$  antagonist GW9662  
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32 and PPAR $\gamma$  silencing. Furthermore, the TGZ inactive derivative  $\Delta$ 2-TGZ was also able to  
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34 stimulate EGR1 expression after 3 hours of treatment in both hormone-dependent (MCF7)  
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36 and hormone-independent (MDA-MB-231) breast cancer cells. These results are consistent  
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38 with data obtained in human colorectal carcinoma cells HCT116 [13]. Thus, EGR1 expression  
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40 can be added to the increasing list of PPAR $\gamma$ -independent effects of thiazolidinediones  
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42 observed in cancer cell lines [9].  
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52 The activation of the MEK/ERK/ELK cascade is the classical pathway essential for  
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54 enhanced EGR1 biosynthesis [23]. In MCF7 cells, we clearly demonstrated that a transient  
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56 phosphorylation of ERK1/2 occurred early after TGZ, CGZ and 15d-PGJ<sub>2</sub> treatment whereas  
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58 no change in ERK phosphorylation was observed in response to RGZ. A similar effect of  
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1 TGZ was previously described in MCF7 cells [24, 25], in porcine endothelial cells [26], and  
2 in the rat liver epithelial cell line GN4 [27, 28]. In GN4 cells, the phosphorylation of ERK  
3 was also stimulated by CGZ [27]. Consistent with our data, in astrocytes, CGZ and 15d-PGJ<sub>2</sub>  
4 stimulated ERK phosphorylation whereas RGZ was inefficient [29]. Interestingly, we found  
5 that ERK1/2 phosphorylation was also stimulated by  $\Delta^2$ -TGZ, demonstrating that it was a  
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7 PPAR $\gamma$ -independent event. This was previously reported in astrocytes not only with  $\Delta^2$ -TGZ  
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9 but also with  $\Delta^2$ -CGZ [27]. We further show that this early activation is essential for EGR1  
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11 induction in MCF7 and MDA-MB-231 cells by the use of the MEK inhibitor U0126, like in  
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13 HCT116 cells [13].  
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22 In parallel to ERK activation, thiazolidinediones are able to induce various MAPKs in  
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24 different cell models [30]. For instance, in MCF7 cells, TGZ and TZD18 also activate p38  
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26 and c-jun N-terminal kinase (JNK) pathways [25, 31]. However, concerning the regulation of  
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28 *EGR1* expression in MCF7 cells exposed to  $\Delta^2$ -TGZ, p38 and JNK pathways are likely not  
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30 involved. Indeed, ERK1/2 phosphorylation inhibition is sufficient to completely block *EGR1*  
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32 synthesis whereas JNK and p38 inhibitors have no effect. Consistent with our data, the p38  
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34 MAPK inhibitor PD169316 did not prevent *EGR1* induction by the retinoid AHPN in human  
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36 lung carcinoma cells [32]. Similarly, in intestinal epithelial cells U0126 was able to block  
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38 *EGR1* expression induced by sulindac sulfide whereas SP600125 and SB203580 were  
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40 inefficient [33].  
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49 Previous studies suggested a connection between the EGFR and MAPK signaling by  
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51 PPAR $\alpha$  and PPAR $\gamma$  ligands [24, 34-36]. MCF7 and MDA-MB-231 cells expressed significant  
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53 levels of EGFR protein [37]. However, in our study, although the EGFR antagonists AG1478  
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55 and PD153035 inhibited efficiently EGF-stimulated ERK phosphorylation and *EGR1*  
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57 expression, they did not affect the stimulation of *EGR1* expression in case of  $\Delta^2$ -TGZ  
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1 treatment. Similarly, TGZ did not induce EGFR activation in rat liver epithelial cells GN4  
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7 MAPK phosphorylation is also often associated with an increase in  $[Ca^{2+}]_i$ . The  $Ca^{2+}$ -  
8 regulated protein kinase C can activate ERK through a RAF-dependent mechanism.  
9 Interestingly, TGZ and CGZ were shown to increase  $[Ca^{2+}]_i$  in mouse ES cells, in rat liver  
10 epithelial cells GN4 as well as in normal urothelial cells [28, 38, 39]. In MCF7 cells, we show  
11 that TGZ triggers an immediate increase in cytosolic  $Ca^{2+}$  and that this event is also obtained  
12 in response to the PPAR $\gamma$ -inactive compound  $\Delta 2$ -TGZ. This result is in accordance with the  
13 fact that both TGZ and CGZ trigger an increase in  $[Ca^{2+}]_i$  not only in PPAR $\gamma^{+/+}$  but also in  
14 PPAR $\gamma^{-/-}$  mouse ES cells [38]. Moreover, we show for the first time that  $Ca^{2+}$  is essential for  
15 the activation of ERK1/2 and the expression of EGR1 by  $\Delta 2$ -TGZ, TGZ, CGZ, or 15d-PGJ<sub>2</sub> in  
16 MCF7 cells and MDA-MB-231. Interestingly, biosynthesis of EGR1 in MIN6 insulinoma  
17 cells treated with glucose, tolbutamide or KCl requires an elevation of  $Ca^{2+}$  [40].  
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20 We further observed that the increase in cytosolic  $Ca^{2+}$  occurred even when MCF7 cells were  
21 superfused in a  $Ca^{2+}$ -free solution containing EGTA, demonstrating liberation from  
22 intracellular stores rather than an influx from extracellular medium. This is in accordance with  
23 the effects described previously for TGZ and CGZ in mouse ES cells [38].  
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46 The roles of EGR1 in cancer development and response to therapies are ambiguous  
47 since EGR1 may either inhibit or stimulate cell growth depending on the stimulus and the  
48 cellular context. Many observations support the notion that EGR1 contributes to prostate  
49 cancer progression. The *EGR1* mRNA is highly expressed in prostate adenocarcinoma,  
50 compared with normal tissues [41]. In contrast, *EGR1* expression is absent or very weak in  
51 several human breast cancer cell lines and tissues compared to normal cells or tissues [17, 18].  
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1 We confirmed these data in the two breast cancer cell lines MCF7 and MDA-MB-231. We  
2 further show that induction of *EGR1* expression is partly involved in the anti-proliferative  
3 action of the thiazolidinedione  $\Delta 2$ -TGZ. Indeed, *EGR1* depletion reduced by 14% the  
4 antiproliferative activity of  $\Delta 2$ -TGZ on MCF7 cells. Similarly to our results, cellular  
5 sensibility to different therapeutic agents is modified after *EGR1* interference. For example,  
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Concerning the events downstream of EGR1, it might be interesting to address the involvement of HSP70 and NF- $\kappa$ B. Indeed, in lung fibroblasts, cigarette smoke water extracts induce an EGR1-dependent expression of HSP70 [44]. The latter can block the activation of the transcription factor NF $\kappa$ B which is antiproliferative [45]. Moreover, the suppression of NF- $\kappa$ B is involved in TGZ-induced inhibition of colon cancer cell growth [46].

Besides, EGR1 induction was reported to occur in mouse embryonic fibroblasts exposed to the endoplasmic reticulum (ER)-stress inducer thapsigargin [47]. EGR1 was also shown to play a role in thapsigargin-induced apoptosis in the melanoma cell line A375-C6 [48]. Since some PPAR $\gamma$  ligands are known to trigger ER stress in breast cancer cell lines, the involvement of EGR1 in the ER stress in breast cancer cells could be considered [31].

In summary, we provide evidence that TGZ, CGZ and 15d-PGJ<sub>2</sub> stimulate an early expression of EGR1 in breast cancer cells in a PPAR $\gamma$ -independent manner. This event requires a rise in  $[Ca^{2+}]_i$ , and the subsequent activation of ERK1/2 signaling pathway. We also show that EGR1 participates in the PPAR $\gamma$ -independent antiproliferative action. It will be

1 interesting to determine if thiazolidinediones also activate the Ca<sup>2+</sup>/ERK/EGR1 pathway in  
2 non cancerous mammary epithelial cells or if this event is restricted to cancer cells.  
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11  
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14 François Dupire for technical assistance.  
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### 31 Footnotes

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<sup>1</sup> Stephane Salamone present address: Unité de Chimie des Biomolécules (URA CNRS 2128), Institut Pasteur, 75724 Paris, France. stephane.salamone@pasteur.fr

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2 **Figure legends**  
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4 **Fig. 1. Troglitazone induces *EGR1* expression.** A, Time-dependent induction of *EGR1*  
5 mRNA in MCF7 cells exposed to 25  $\mu$ M TGZ (T) for the indicated times. Control cells were  
6 treated with 0.05% DMSO (D) or non-treated (NT). *EGR1* and the housekeeping gene  
7 *RPLPO* mRNA expression were analyzed by RT-PCR. The amplified products were analyzed  
8 by agarose gel electrophoresis. B, Dose-dependent induction of *EGR1* mRNA in MCF7 cells  
9 treated with decreasing doses of TGZ (T) for 3 hours. Control cells were treated with 0.05%  
10 DMSO (D) or non-treated (NT). *EGR1* mRNA was analyzed by RT-PCR and compared to the  
11 housekeeping gene *RPLPO*. The amplified PCR products were detected after agarose gel  
12 electrophoresis. C, Time-dependent induction of EGR1 protein (80 kDa) in MCF7 cells  
13 exposed to 25  $\mu$ M TGZ (T), 0.05% DMSO (D) or 20% fetal calf serum (FCS) for the  
14 indicated times. 20% FCS treatment was used as positive control. Nuclear extracts were  
15 prepared as described under “experimental procedures” and subjected to western blot analysis  
16 with anti-EGR1 antibody. D, Immunodetection of EGR1 (EGR1, middle panels) in MCF7  
17 cells exposed to 25  $\mu$ M TGZ (T) or 20% FCS for 3 hours. Control cells were treated with  
18 0.05% DMSO (D). The results of the counterstaining with Hoechst dye (left panels) as well as  
19 the merged pictures (right panels) are shown for each treatment. Bar represents 50  $\mu$ m and the  
20 magnification is identical for all pictures. E, Time-dependent induction of *EGR1* target gene  
21 *NAB2* in MCF7 cells exposed to 25  $\mu$ M TGZ (T) for the indicated times. Control cells were  
22 treated with 0.05% DMSO (D). *NAB2* and the housekeeping gene *RPLPO* expressions were  
23 analyzed at the mRNA level by RT-PCR. The amplified products were analyzed by agarose  
24 gel electrophoresis.  
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**Fig. 2. Ciglitazone and 15d-PGJ<sub>2</sub> also stimulate the expression of *EGR1* and its target gene *NAB2*.** A, Time-dependent induction of *EGR1* mRNA in MCF7 cells exposed to 25  $\mu$ M CGZ (C), 15d-PGJ<sub>2</sub> (P) and RGZ (R) for the indicated times. Control cells were treated with 0.05% DMSO (D, vehicle) or non-treated (NT). B, Induction of EGR1 protein (80kDa) in MCF7 cells exposed to 25  $\mu$ M CGZ (C), 15d-PGJ<sub>2</sub> (P), RGZ (R) or 0.05% DMSO (D) for 3 hours. Nuclear extracts were prepared as described under “experimental procedures” and subjected to western blot analysis using anti-EGR1 antibody. C, RT-PCR analysis of the expression of *NAB2* mRNA in MCF7 cells exposed for 6 and 9 hours to 25  $\mu$ M CGZ (C) and 15d-PGJ<sub>2</sub> (P), or 0.05% DMSO (D). The amplified products were detected after agarose gel electrophoresis and expressed relatively to the housekeeping gene *RPLPO*.

**Fig. 3. *EGR1* expression is independent of PPAR $\gamma$  activation.** A, Absence of inhibition of *EGR1* induction in the presence of the PPAR $\gamma$  antagonist GW9662. MCF7 cells were pretreated with 5 or 25  $\mu$ M GW9662 (GW) for 30 minutes before treatment with 25  $\mu$ M TGZ (T), CGZ (C) or 15d-PGJ<sub>2</sub> (P) for 3 hours. Control cells were treated with 0.05% DMSO (vehicle, D) or non-treated (NT). mRNA expression was analyzed by RT-PCR. B, TGZ-induced expression of *EGR1* mRNA after silencing of PPAR $\gamma$  receptors by RNA interference. MCF7 cells were transiently transfected with double-strand siRNA targeting PPAR $\gamma$  or control (Ctrl) siRNA. 48 hours after transfection, cells were exposed to 25  $\mu$ M TGZ (T) or 0.05% DMSO (D) for 3 hours. C, Expression of *EGR1* and its target gene *NAB2* following treatment with the PPAR $\gamma$  inactive analogue of TGZ,  $\Delta$ 2-TGZ. Cells were treated with 25  $\mu$ M  $\Delta$ 2-TGZ ( $\Delta$ 2T) or 0.05% DMSO (D) for the indicated times. mRNA expression was analyzed by RT-PCR. The amplified products were detected after agarose gel electrophoresis and expressed relatively to the housekeeping gene *RPLPO*. D, Induction of EGR1 protein (80kDa) in MCF7 cells exposed to 25  $\mu$ M TGZ (T), its inactive analogue,  $\Delta$ 2-TGZ, ( $\Delta$ 2T) or 0.05%

1 DMSO (D) for 3 hours. Nuclear extracts were prepared as described under “experimental  
2 procedures” and subjected to western blot analysis using anti-EGR1 antibody.  
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7 **Fig. 4. The induction of *EGR1* expression depends on the activation of the ERK1/2**  
8 **pathway.** A, Changes in ERK1/2 phosphorylation were assessed in MCF7 cells after 25  $\mu$ M  
9 TGZ (T), CGZ (C), and 15d-PGJ<sub>2</sub> (P) treatment for the indicated times. Cells were harvested  
10 in 2X Laemmli buffer and total proteins were subjected to western blot analysis using anti-  
11 phospho-ERK1/2 antibody (P-ERK1/2). The blots were stripped and reprobed using antibody  
12 directed against total ERK1/2 (ERK1/2). B, A similar experiment was done in MCF7 cells  
13 after  $\Delta$ 2-TGZ ( $\Delta$ 2T) treatment for the indicated times. C, MCF7 cells were pretreated with 5  
14  $\mu$ M U0126, a specific inhibitor of MEK1/2, for 30 minutes before treatment with 25  $\mu$ M TGZ  
15 (T), CGZ (C), 15d-PGJ<sub>2</sub> (P) for 15 minutes and 25  $\mu$ M  $\Delta$ 2-TGZ ( $\Delta$ 2T) for 30 minutes.  
16 Control cells were treated with 0.05% DMSO (vehicle). Phospho-ERK1/2 and total ERK1/2  
17 were analyzed by western blot. D, MCF7 cells were pretreated with 5 or 25  $\mu$ M U0126, a  
18 specific inhibitor of MEK1/2, for 30 minutes before treatment with 25  $\mu$ M TGZ (T), CGZ (C),  
19 15d-PGJ<sub>2</sub> (P) and  $\Delta$ 2-TGZ ( $\Delta$ 2T) for 3 hours. Control cells were treated with 0.05% DMSO  
20 (vehicle) or non-treated (NT). *EGR1* mRNA expression was analyzed by RT-PCR. The  
21 amplified products were detected after agarose gel electrophoresis and expressed relatively to  
22 the housekeeping gene *RPLPO*. E, Cells were pretreated with 5  $\mu$ M U0126 for 30 minutes  
23 before treatment with 25  $\mu$ M TGZ (T) for 3 hours. Nuclear extracts were prepared as  
24 described under “experimental procedures” and subjected to western blot analysis to detect  
25 the 80 kDa EGR1 protein. Control cells were treated with 0.05% DMSO (D, vehicle) or non-  
26 treated (NT).  
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**Fig. 5. The induction of *EGR1* expression is independent on the activation of the p38 and JNK pathways.** MCF7 cells were pretreated for 30 minutes with 10  $\mu$ M SB203580 (A) or SP600125 (B), specific inhibitors of p38 and JNK MAPK respectively, before treatment with 25  $\mu$ M  $\Delta$ 2-TGZ ( $\Delta$ 2T) for 3 hours. Control cells were treated with 0.05% DMSO (vehicle). *EGR1* mRNA expression was analyzed by RT-PCR. The amplified products were detected after agarose gel electrophoresis and expressed relatively to the housekeeping gene *RPLPO*.

**Fig. 6. EGFR is not involved in the PPAR $\gamma$ -independent expression of *EGR1*.** A, Phospho-ERK1/2 and total ERK1/2 were analyzed by western blot. MCF7 cells were pretreated for 30 minutes with 5  $\mu$ M of the EGFR tyrosine kinase inhibitors PD153035 (PD) or AG1478 (AG) before treatment with 15 nM EGF for 5 minutes. Control cells were treated with 0.05% DMSO (D). B, *EGR1* mRNA expression induced by EGF and effects of different EGFR inhibitors. MCF7 cells were pretreated for 30 minutes with 5  $\mu$ M of the EGFR tyrosine kinase inhibitors PD153035 (PD) or AG1478 (AG) before treatment with 15 nM EGF for 1 hour. Control cells were treated with 0.05% DMSO (D). mRNA expression was analyzed by RT-PCR. The amplified products were detected after agarose gel electrophoresis and expressed relatively to the housekeeping gene *RPLPO*. C, *EGR1* mRNA expression in presence of different EGFR inhibitors. MCF7 cells were pretreated for 30 minutes with 5  $\mu$ M of the EGFR tyrosine kinase inhibitors PD153035 (PD) or AG1478 (AG) before treatment with 25  $\mu$ M  $\Delta$ 2-TGZ ( $\Delta$ 2T) for 3 hours. Control cells were treated with 0.05% DMSO (D). mRNA expression was analyzed by RT-PCR as described in B. D,  $\Delta$ 2-TGZ-induced expression of *EGR1* mRNA and ERK phosphorylation after silencing of EGFR receptors by RNA interference. MCF7 cells were transiently transfected with double-strand siRNA targeting EGFR or control (Ctrl) siRNA. 48 hours after transfection, cells were exposed to 25  $\mu$ M  $\Delta$ 2-TGZ ( $\Delta$ 2T) or 0.05% DMSO (D). After 3 hours treatment, the expressions of *EGFR*,

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*EGR1* and *RPLPO* were analyzed at the mRNA level by RT-PCR. The amplified products were detected after agarose gel electrophoresis. After 30 minutes treatment, phospho-ERK1/2 and total ERK1/2 were analyzed by western blot.

**Fig. 7. The PPAR $\gamma$ -independent activation of *EGR1* expression is also observed in the hormone-independent breast cancer cell line MDA-MB-231.** MDA-MB-231 cells were pretreated with 5  $\mu$ M U0126 (specific inhibitor of MEK1/2) or 5  $\mu$ M AG1478 (AG; EGFR tyrosine kinase inhibitor) for 30 minutes before treatment with 25  $\mu$ M  $\Delta$ 2-TGZ ( $\Delta$ 2T) for 3 hours. Control cells were treated with 0.05% DMSO (D, vehicle) or non-treated (NT). *EGR1* mRNA expression was analyzed by RT-PCR. The amplified products were detected after agarose gel electrophoresis. The housekeeping gene *RPLPO* was used as an internal control. The band intensities of *EGR1* were evaluated in terms of optical density arbitrary units and normalized with the *RPLPO* signal intensity. Data are presented in the histogram.

**Fig. 8. *EGR1* expression and ERK1/2 phosphorylation depend on calcium mobilization.**

A, Inhibition of *EGR1* mRNA expression and ERK1/2 phosphorylation in presence of the calcium chelator BAPTA-AM. MCF7 cells were pretreated with 50  $\mu$ M BAPTA-AM for 30 minutes before treatment with 25  $\mu$ M  $\Delta$ 2-TGZ ( $\Delta$ 2T), TGZ (T), CGZ (C) or 15d-PGJ2 (P) for 3 hours for *EGR1* expression detection. For ERK1/2 phosphorylation experiments, cells were treated with TGZ (T), CGZ (C) or 15d-PGJ2 (P) for 15 minutes and 30 minutes with  $\Delta$ 2-TGZ ( $\Delta$ 2T). Control cells were treated with 0.05% DMSO (D). *EGR1* mRNA expression was analyzed by RT-PCR. The amplified products were detected after agarose gel electrophoresis and expressed relatively to the housekeeping gene *RPLPO*. To detect changes in ERK1/2 phosphorylation status, cells were harvested in 2X Laemmli buffer and total proteins were subjected to western blot analysis using anti-phospho-ERK1/2 antibody (P-ERK1/2). The

1 blots were stripped and reprobed using antibody directed against total ERK1/2 (ERK1/2). B,  
2 Comparative study of *EGR1* mRNA expression induced by  $\Delta 2$ -TGZ and the A23187  
3 ionophore in MCF7 cells. Cells were treated with 25  $\mu\text{M}$   $\Delta 2$ -TGZ ( $\Delta 2\text{T}$ ) and 1 or 5  $\mu\text{M}$   
4 A23187 ionophore for the indicated times. Control cells were treated with 0.05% DMSO (D).  
5 *EGR1* mRNA expression was analyzed by RT-PCR. The amplified products were detected  
6 after agarose gel electrophoresis and expressed relatively to the housekeeping gene *RPLPO*. C,  
7 Kinetic of ERK1/2 phosphorylation induced by the A23187 ionophore in MCF7 cells. Cells  
8 were treated with 1  $\mu\text{M}$  A23187 ionophore (A) for the indicated time and total proteins were  
9 subjected to western blot analysis as previously described. Control cells were treated with  
10 0.05% DMSO (D). D, Cytoplasmic  $\text{Ca}^{2+}$  concentration was detected by the ratiometric  
11 fluorescent probe Fura-2. MCF7 cells were superfused with a saline solution and exposed  
12 during the indicated time to 0.05 % DMSO (upper panel), 25  $\mu\text{M}$  TGZ (upper panel) or 25  
13  $\mu\text{M}$   $\Delta 2$ -TGZ (middle and lower panels). The effect of  $\Delta 2$ -TGZ was also studied in MCF7  
14 cells pretreated with 50  $\mu\text{M}$  of the calcium chelator BAPTA-AM (middle panel) as well as in  
15 calcium-free conditions (lower panel). The results are expressed as mean (black lines)  $\pm$  s.e.m.  
16 (grey lines) of several measurements.

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42 **Fig. 9. EGR1 plays a part in the antiproliferative action of  $\Delta 2$ -TGZ.** A, MCF7 cells were  
43 transiently transfected with double-strand siRNA targeting *EGR1* or control (Ctrl) siRNA. 48  
44 hours after transfection, cells were exposed to 15  $\mu\text{M}$   $\Delta 2$ -TGZ ( $\Delta 2\text{T}$ ) or 0.05% DMSO (D) for  
45 3 hours. mRNA expression for *EGR1* and *RPLPO* was analyzed by RT-PCR. The amplified  
46 products were detected after agarose gel electrophoresis. B, 28 hours after control (Ctrl) and  
47 *EGR1* siRNA transfection, cells were exposed to 15  $\mu\text{M}$   $\Delta 2$ -TGZ ( $\Delta 2\text{T}$ ) or 0.05% DMSO (D)  
48 for 24 hours. The number of viable cells was measured using the CellTiter-Glo™  
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1 Luminescent Cell Viability Assay. Results are given as mean  $\pm$  s.e.m. of cell number  
2 (arbitrary units) (n=5).  
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4 \* Significantly different from Ctrl siRNA (p<0.01) using the unpaired t-Test.  
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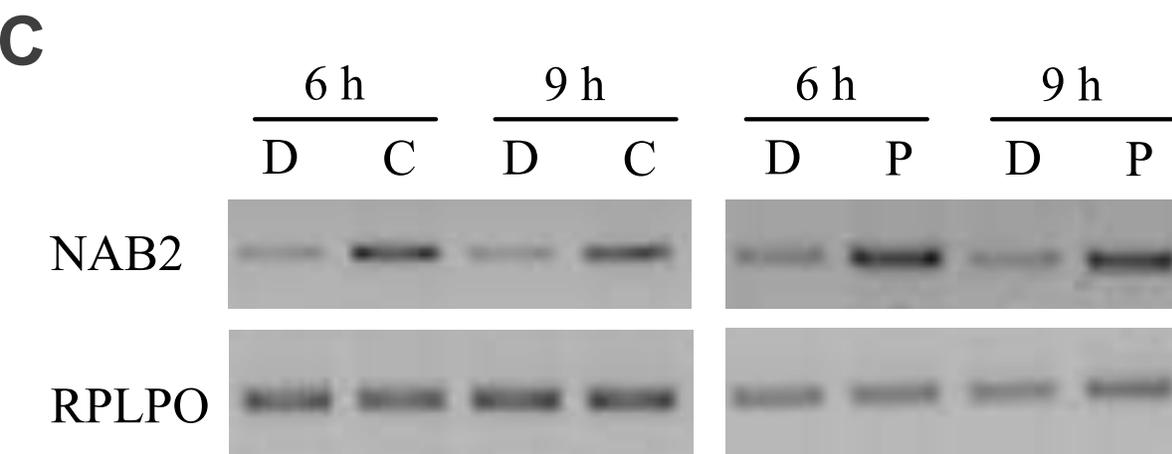
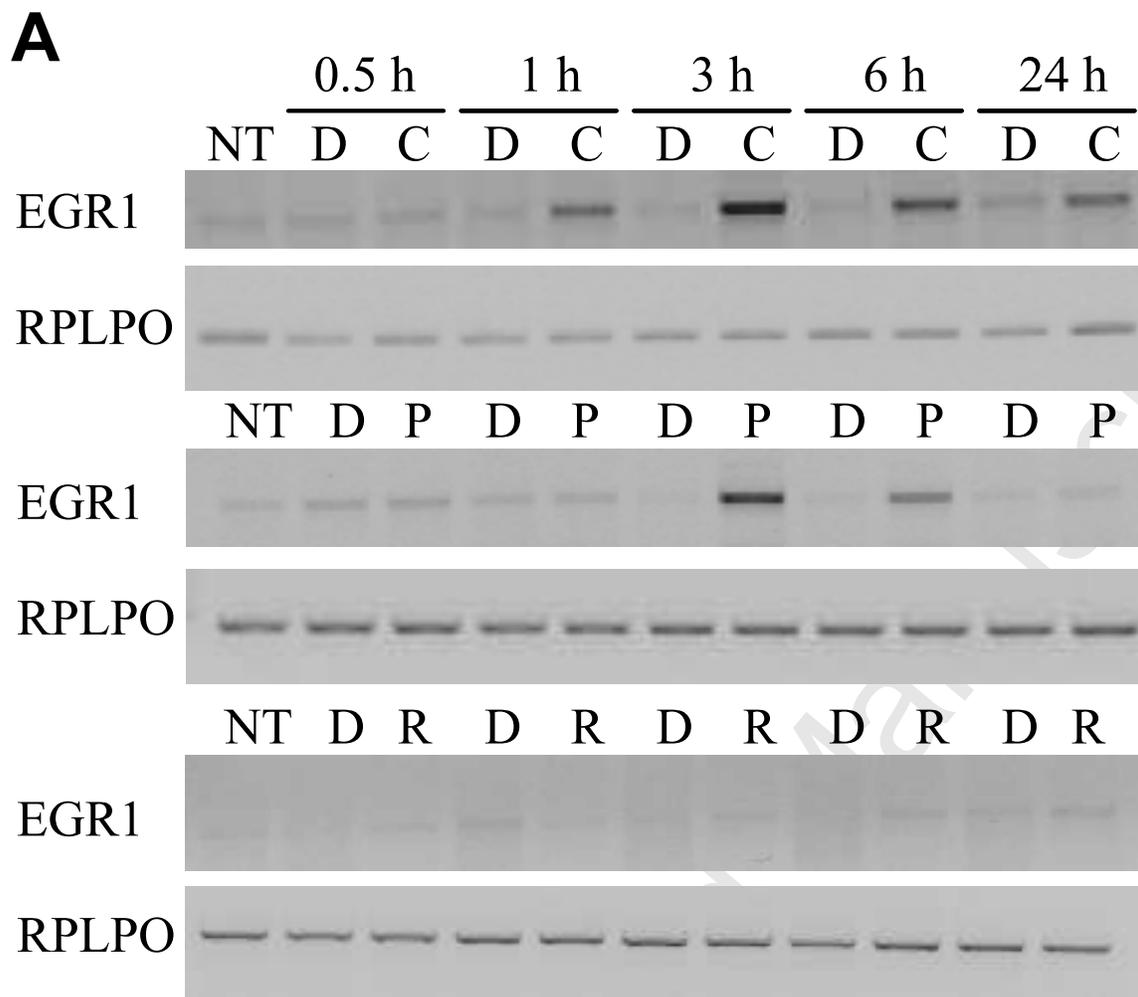
TABLE 1. **siRNA sequences for PPAR $\gamma$ , EGF receptors, and EGR1.** Sense and antisense oligonucleotides for each receptor gene were designed by Eurogentec (Angers, France). The sense (S) and antisense (AS) sequences for the siRNA duplexes are shown in the right column. *TT* did not belong to the target sequences and were added as 3' DNA over-hang. Control siRNA encodes random sequences with no homology to any known human genes.

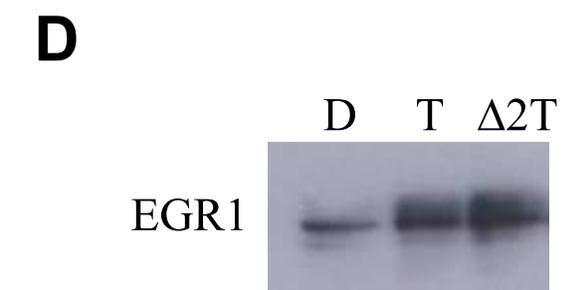
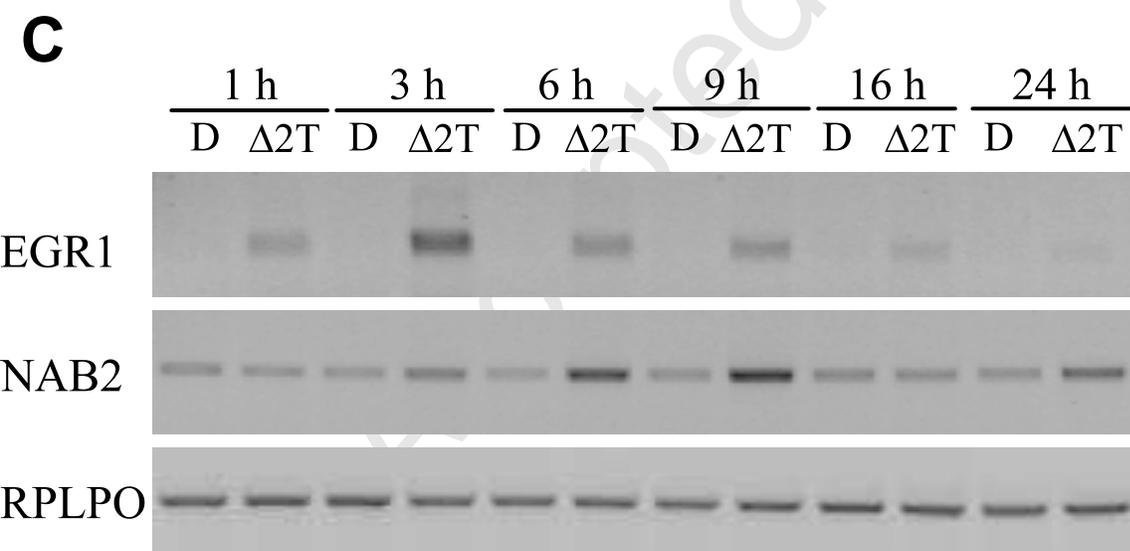
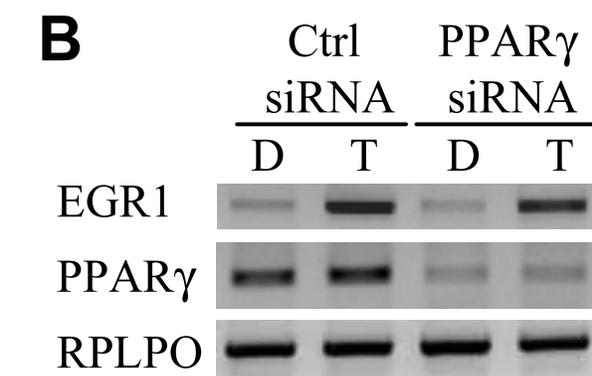
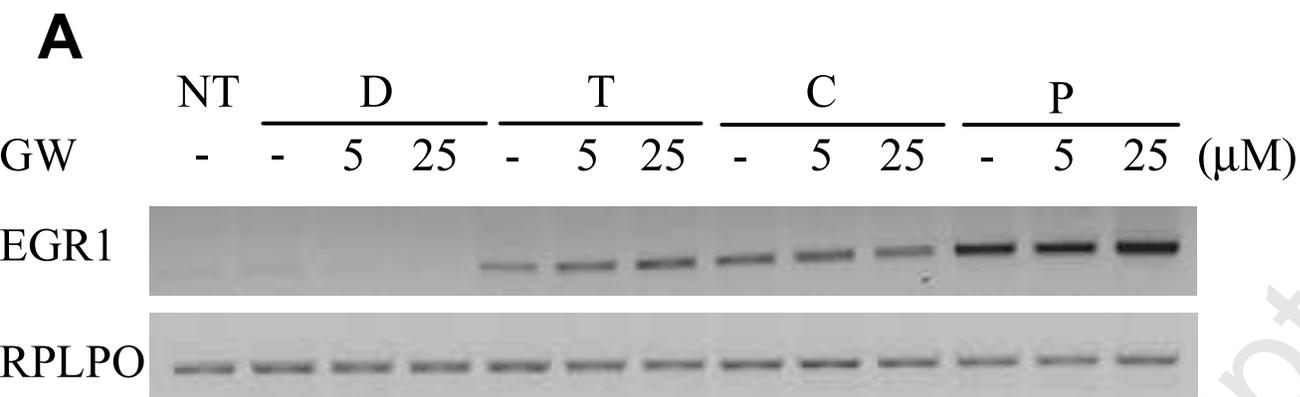
Gene	Accession number		Target sequence (5'-3')	siRNA sequence (5'-3')
PPAR $\gamma$	NM_138712	Duplex 1	GTACCAAAGTGCAATCAAA	S:GUACCAAAGUGCAAUCAAAATT AS:UUUGAUUGCACUUUGGUACTT
		Duplex 2	CAATCAGATTGAAGCTTAT	S:CAAUCAGAUUGAAGCUUAUTT AS:AUAAGCUUCAUUCUGAUUGTT
EGFR	NM_201283	Duplex 1	GCAGTGACTTTCTCAGCAA	S:GCAGUGACUUUCUCAGCAAATT AS:UUGCUGAGAAAGUCACUGCTT
		Duplex 2	GAGGAAATATGTACTACGA	S:GAGGAAAUAUGUACUACGATT AS:UCGUAGUACAUAUUUCCUCTT
EGR1	NM_001964.2		GTGACTGTTTGGCTTATA	S:GUGACUGUUUGGCUUAUATT AS:UUAUAAGCCAAACAGUCACTT
Negative control	Eurogentec SR-CL000-005		none	non available

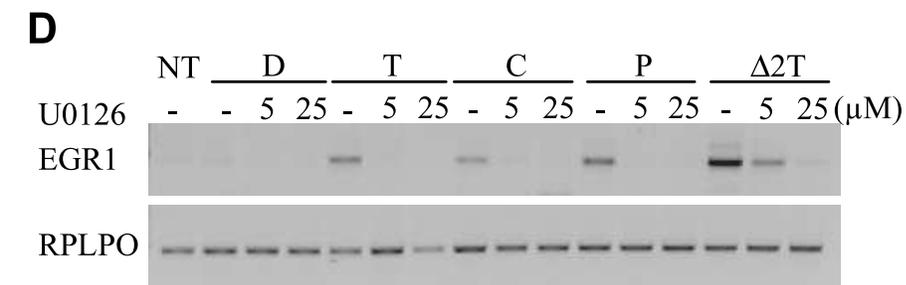
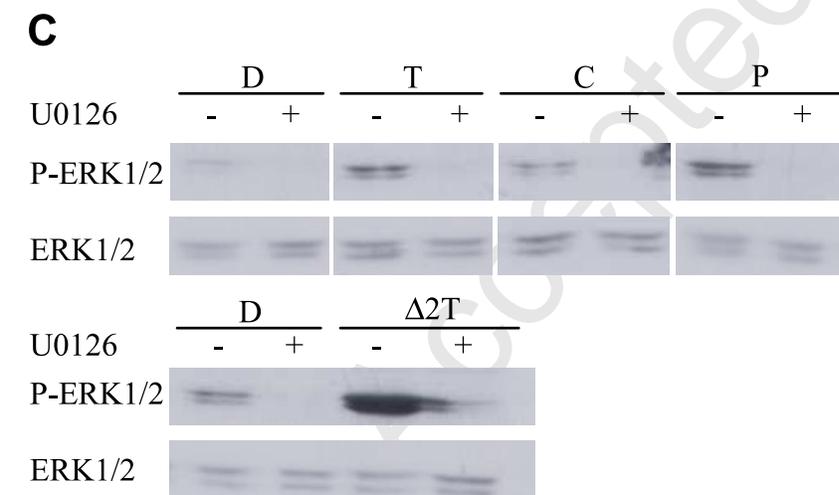
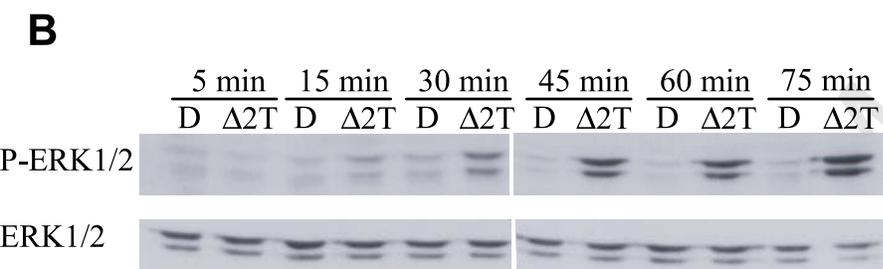
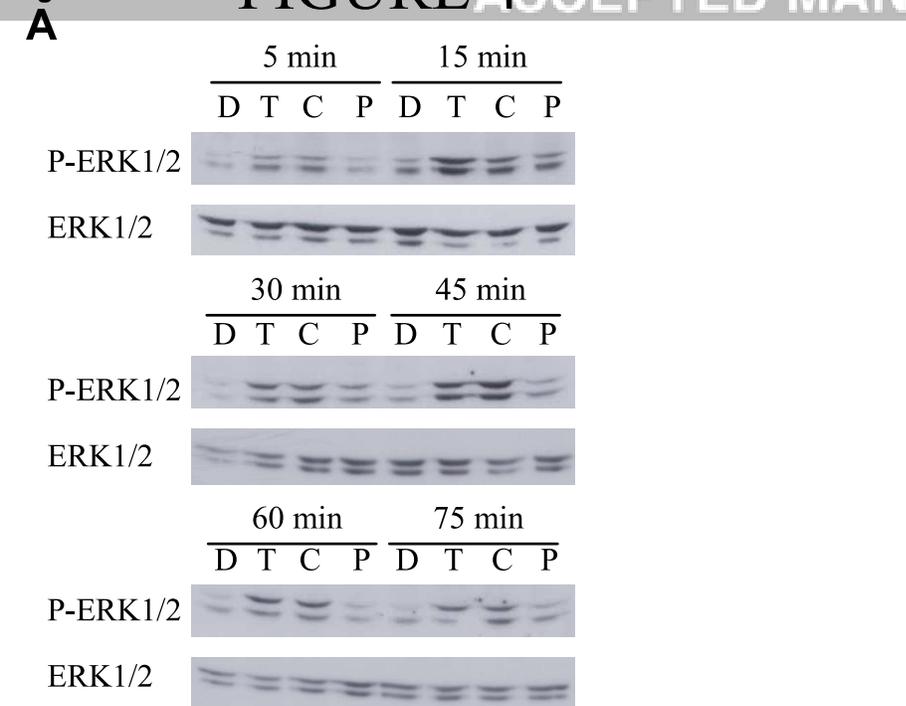
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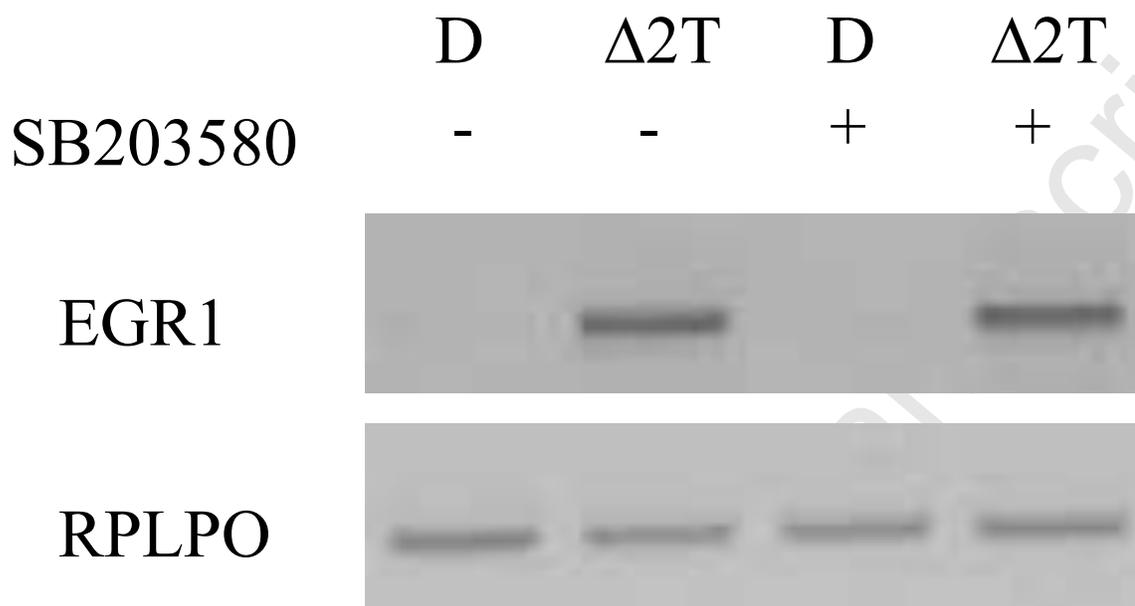
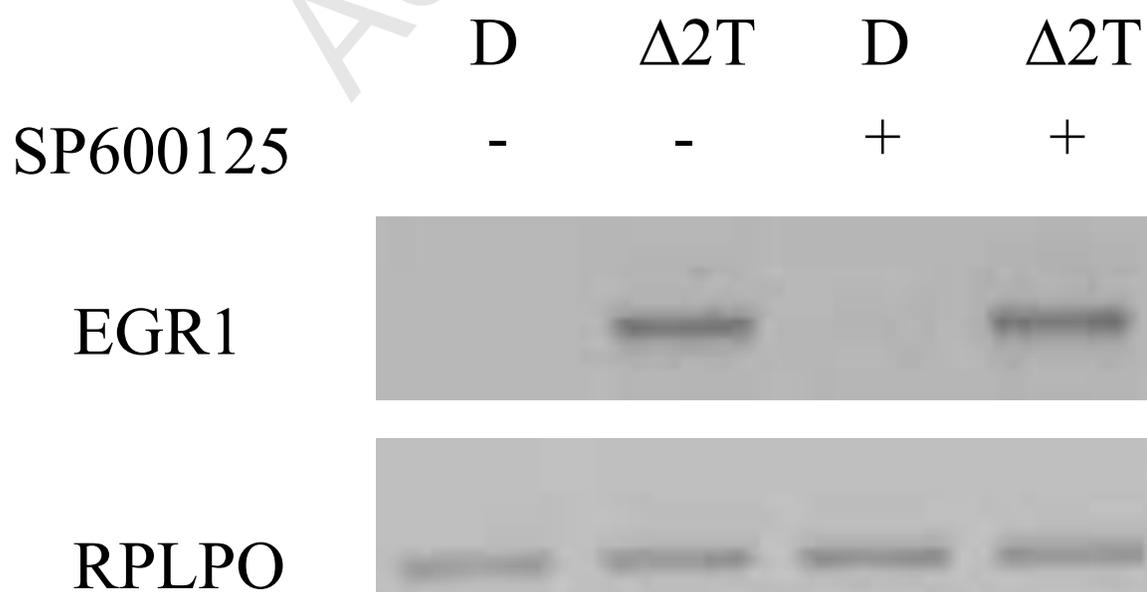


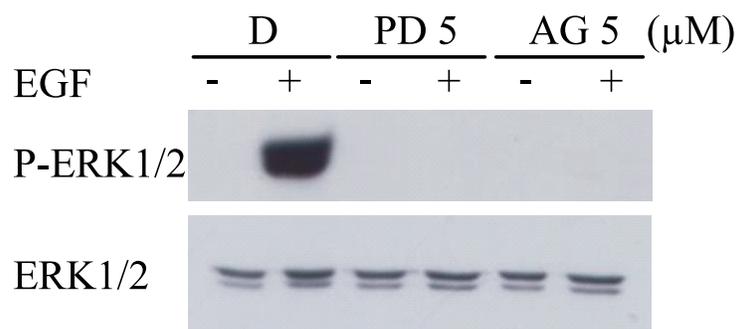
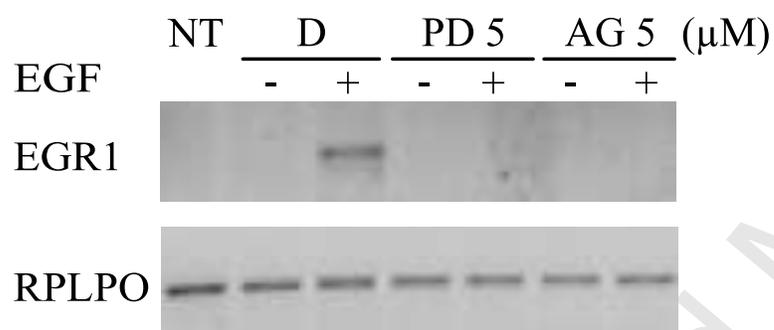
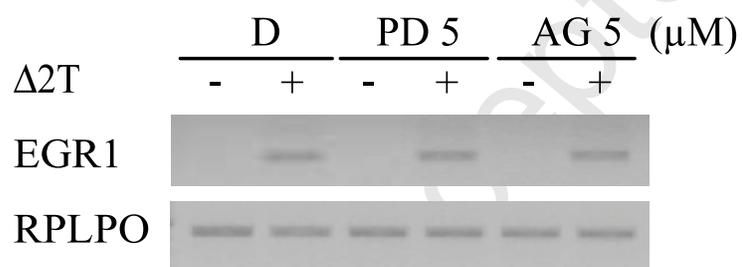
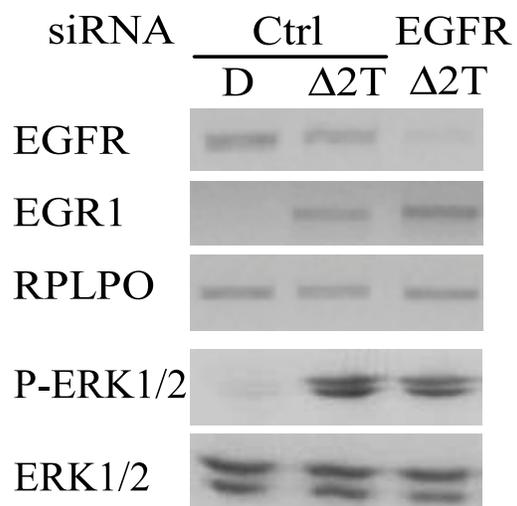
## FIGURE 2



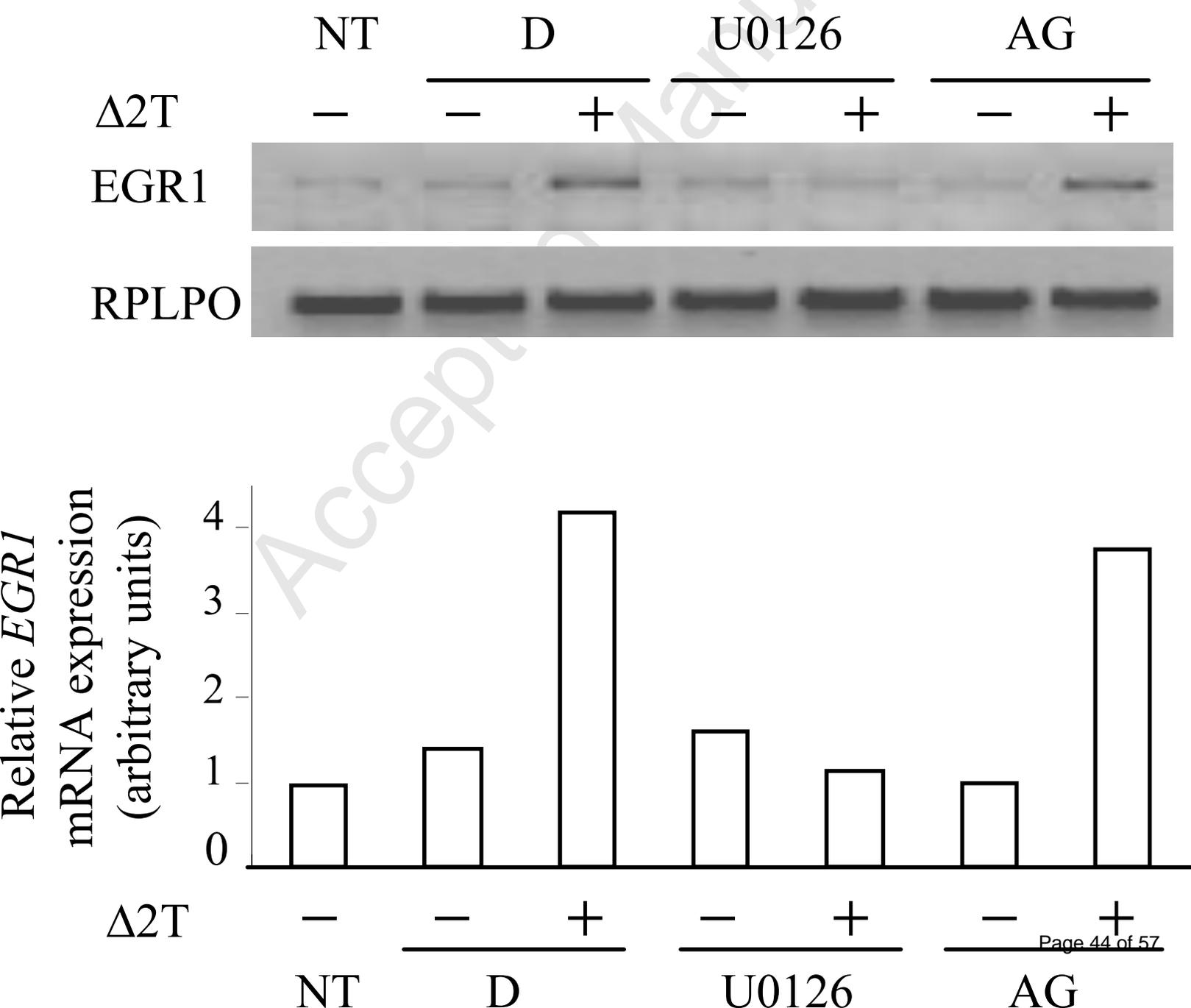




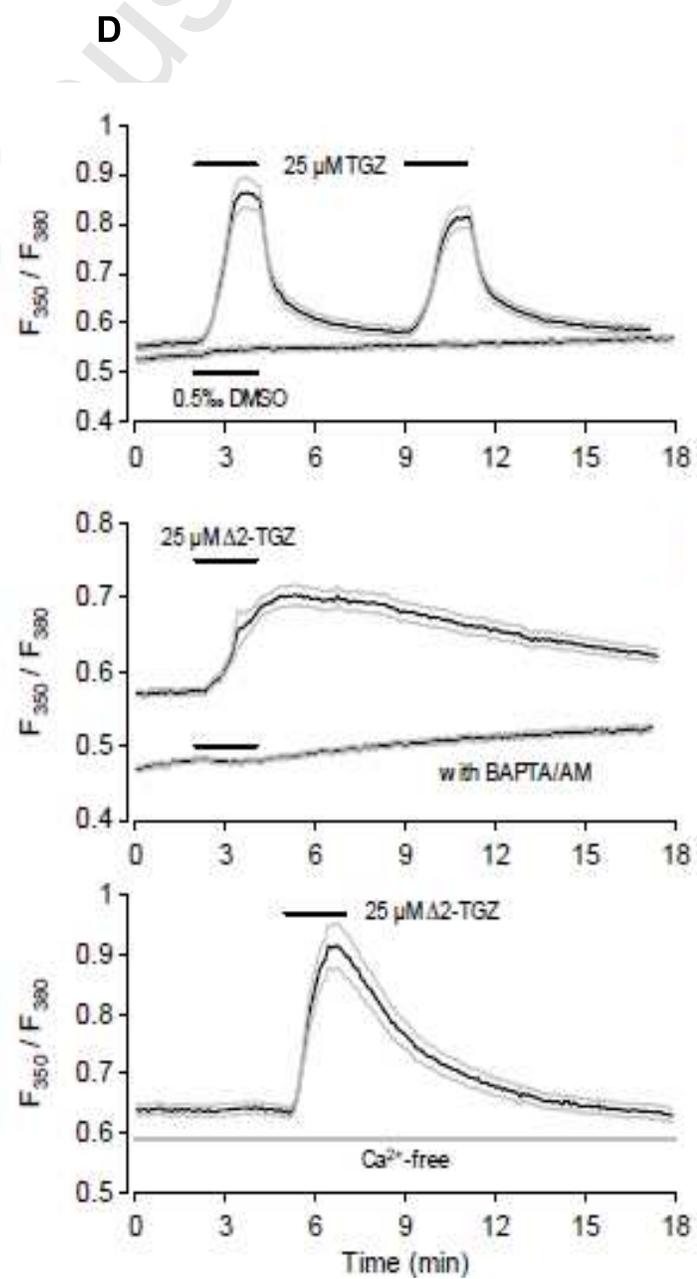
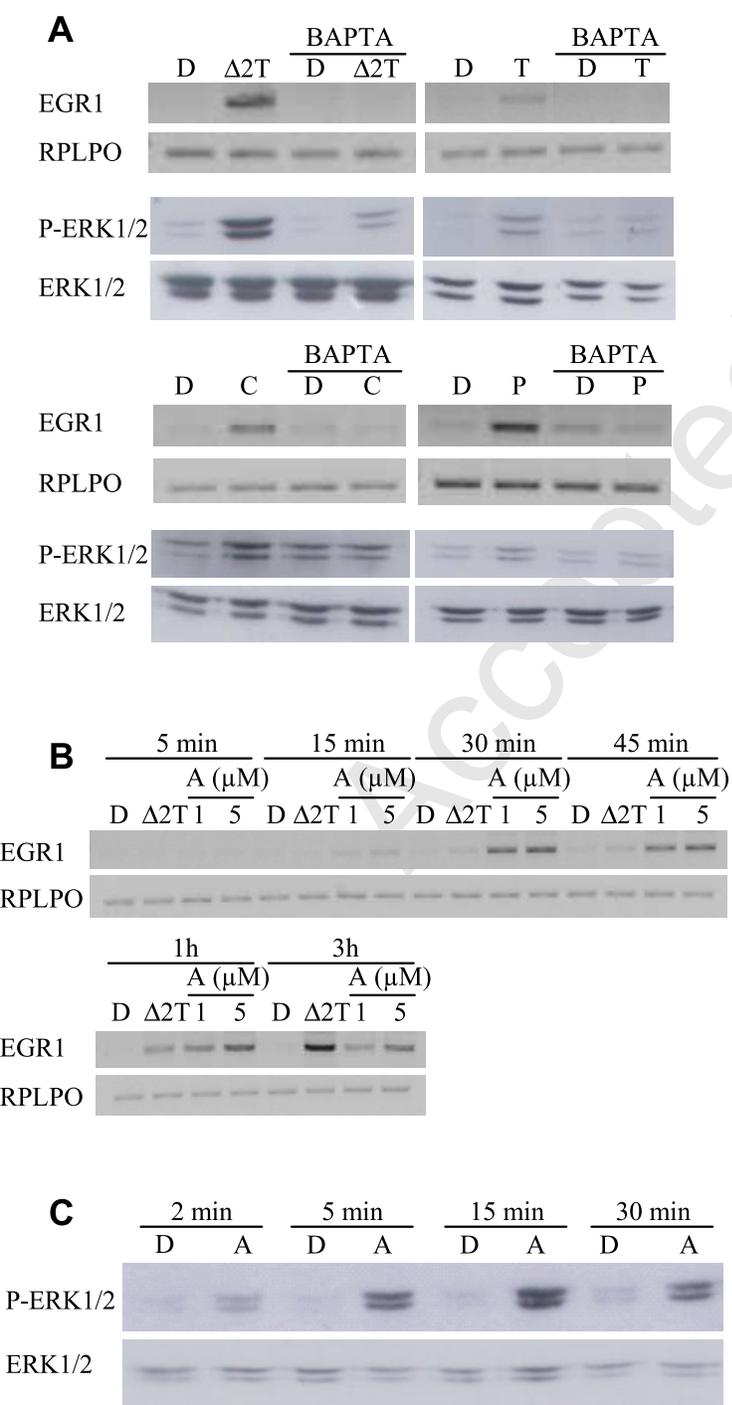
**A****B**

**A****B****C****D**

## FIGURE 7



## FIGURE 8



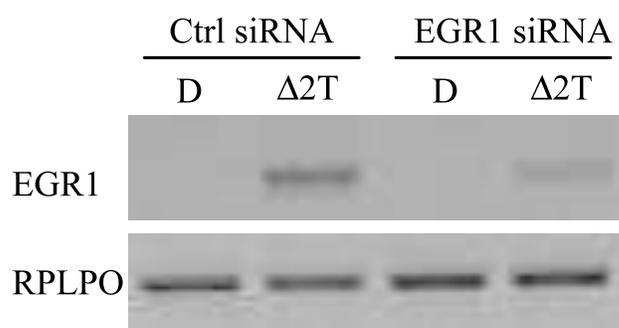
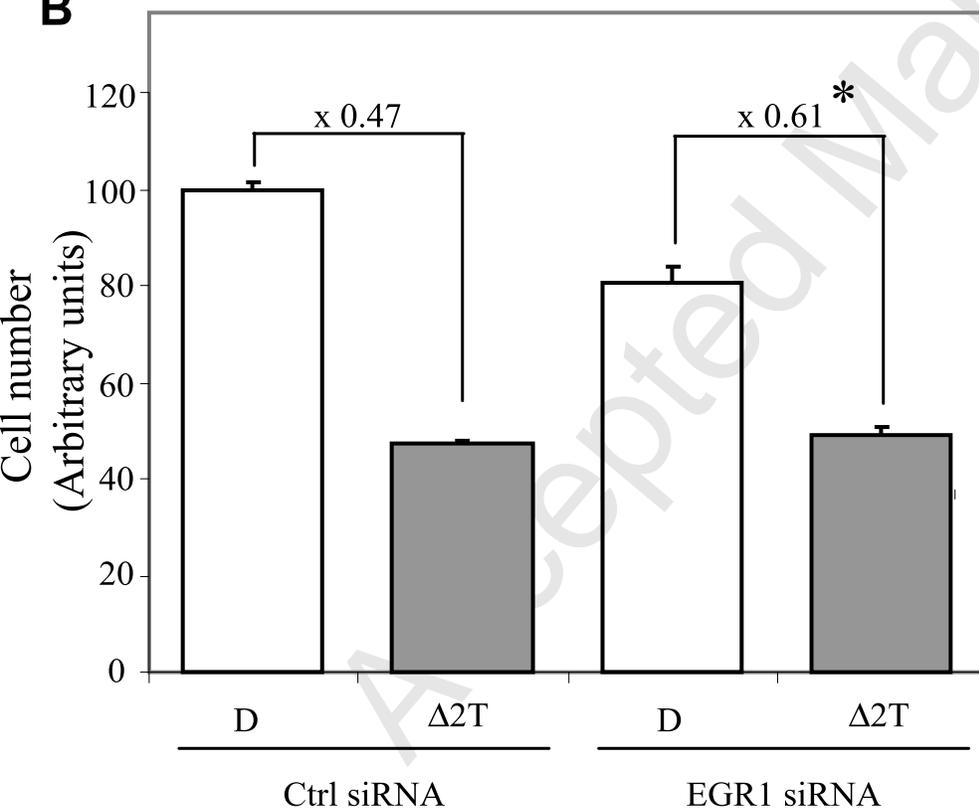
**A****B**

FIGURE 1

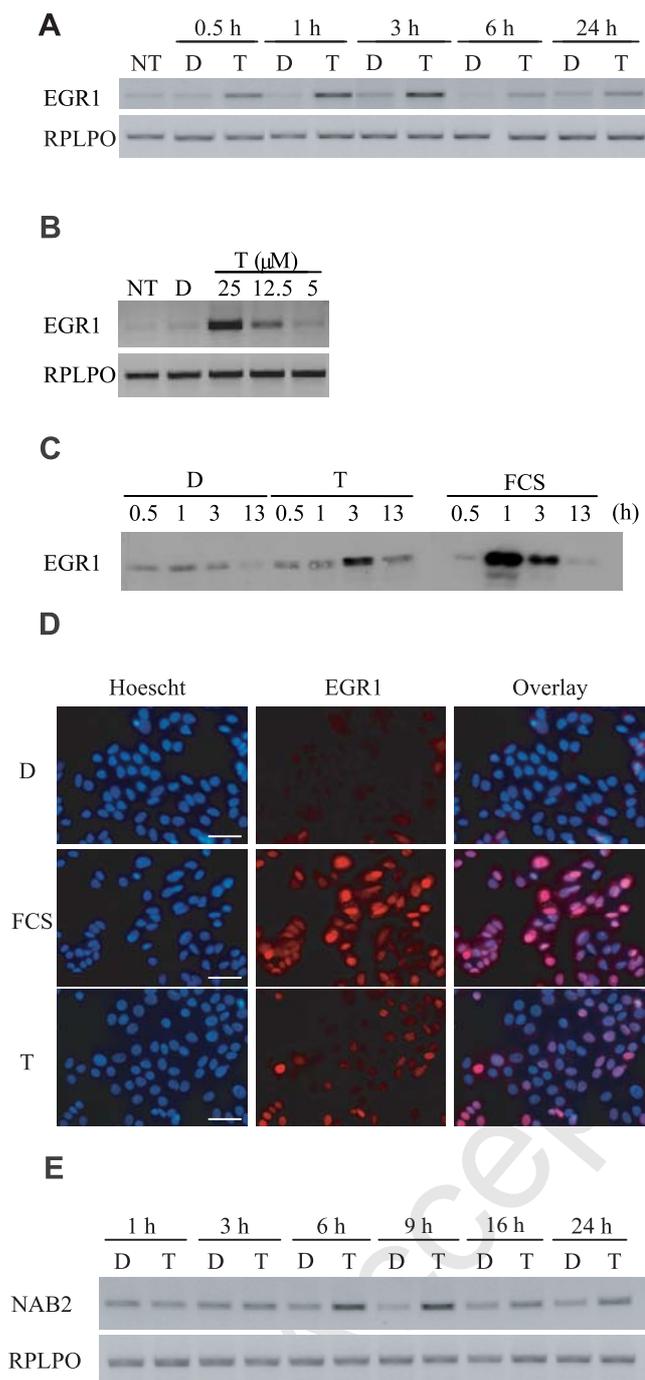


FIGURE 2

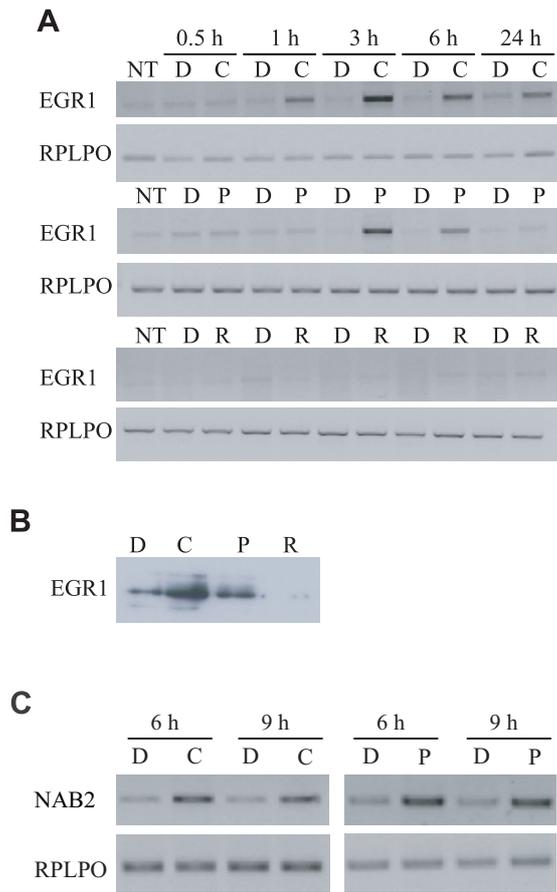


FIGURE 3

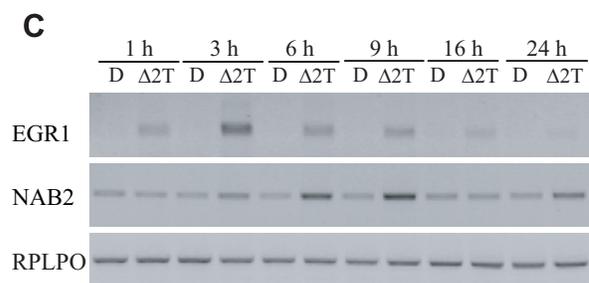
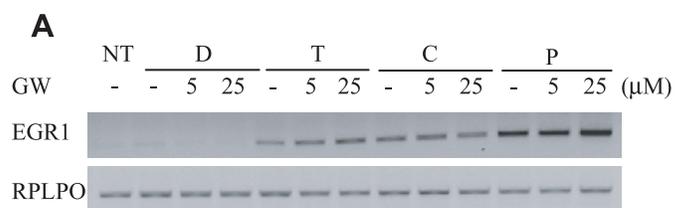


FIGURE 4

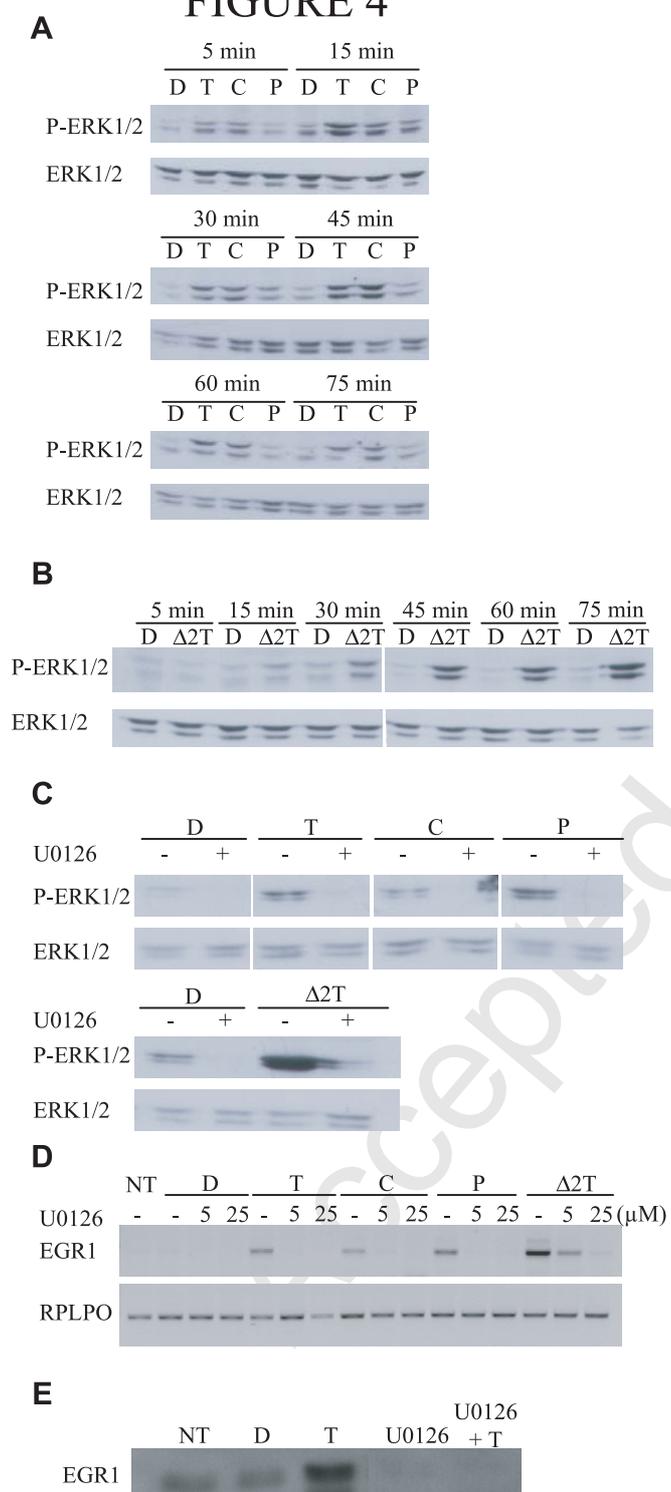


FIGURE 5

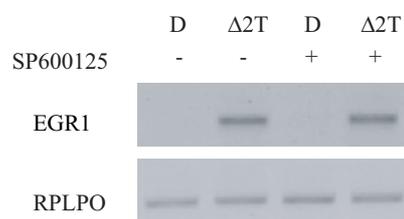
**A****B**

FIGURE 6

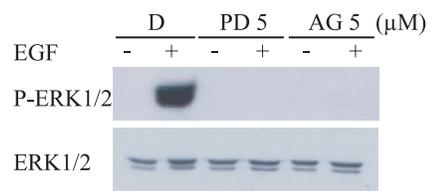
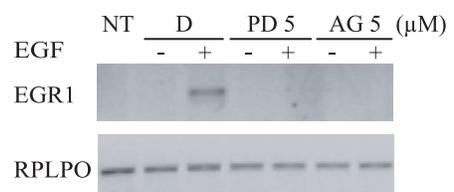
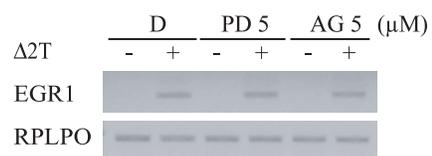
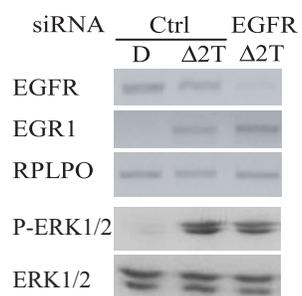
**A****B****C****D**

FIGURE 7

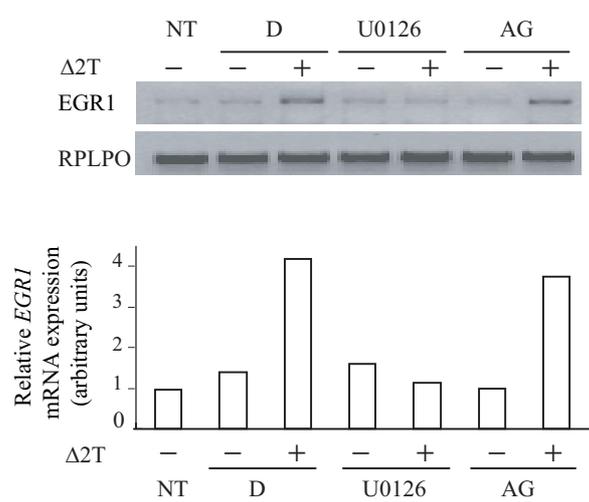


FIGURE 8

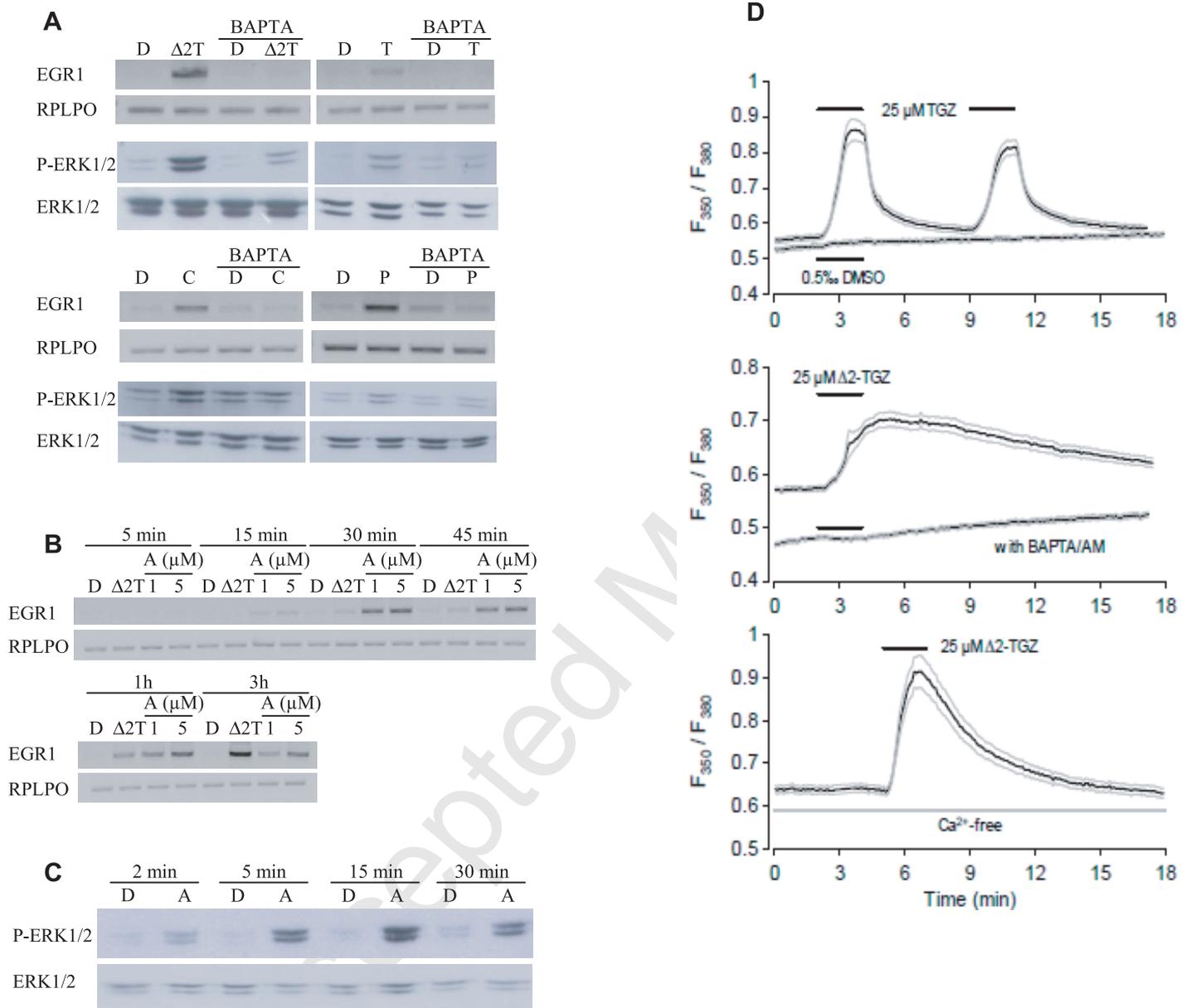
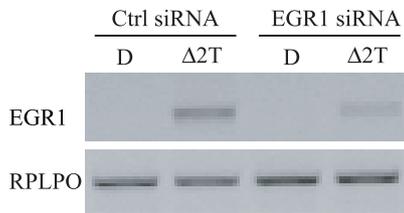
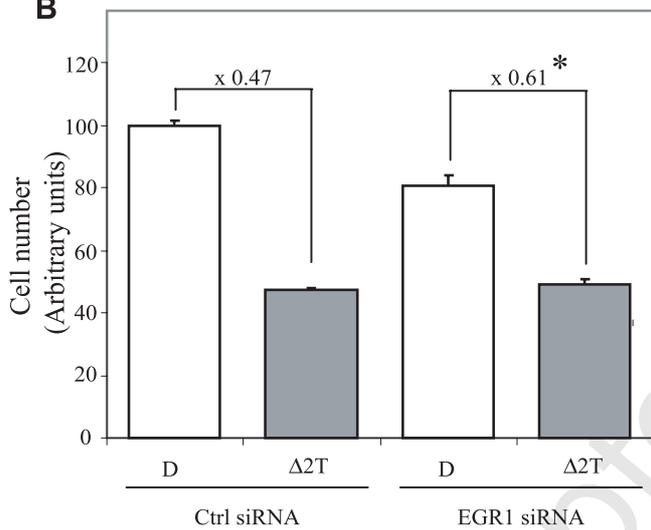


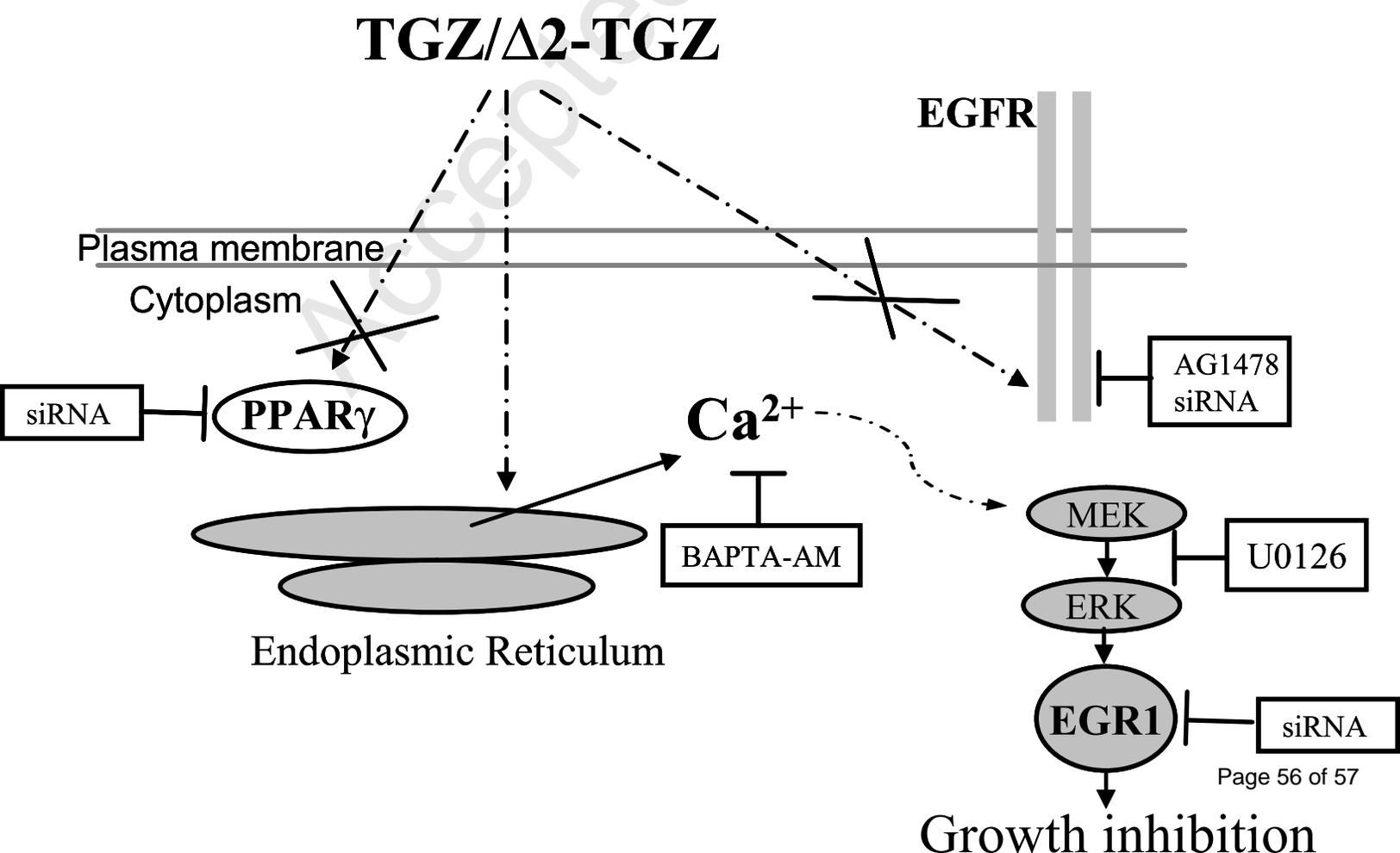
FIGURE 9

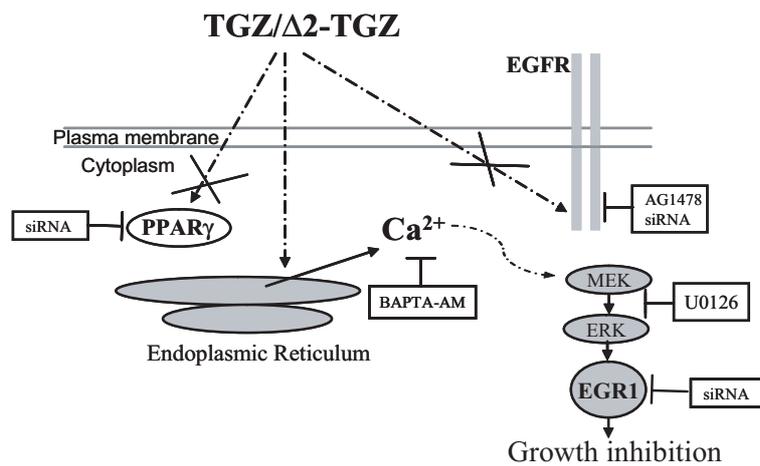
A



B







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