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This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1632984> since 2017-05-10T10:28:12Z

Published version:

DOI:10.1016/j.ygcen.2017.01.020

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1 **Mechanisms of modulation of the Egr gene family in mammary epithelial cells of different species**

2

3 **Santino P., Martignani E., Miretti S., Baratta M. and Accornero P.**

4

5 Dipartimento di Scienze Veterinarie, Largo Braccini 2, 10095 Grugliasco (TO), Italy

6 Email: paolo.santino@unito.it; silvia.miretti@unito.it; eugenio.martignani@unito.it; mario.baratta@unito.it;

7 paolo.accornero@unito.it.

8

9 **Corresponding author** Accornero P., Dipartimento di Scienze Veterinarie, Largo Braccini 2, 10095

10 Grugliasco (TO), Italy; tel: +39 011 6709326; email: paolo.accornero@unito.it

11

12

13 **Declaration of interest**

14 Conflicts of interest: none

15 **Abstract**

16

17 In the adult female, within the estrous cycle, the mammary gland undergoes multiple rounds of growth, with
18 increased cellular proliferation, and involution, with increased apoptosis. The increase in proliferation is
19 elicited by endocrine (Estrogen, Progesterone), as well as locally produced (Epidermal growth factor,
20 Insulin-like growth factor, etc) growth factors. Among the genes that are modulated during cellular
21 proliferation, immediate early genes play a fundamental role, being rapidly upregulated and then
22 downregulated within the G0/G1 phase of the cell cycle, allowing the progression to the subsequent phases.
23 Egrs (1 to 4) are immediate early genes that encode for trascription factors that promote, within different cell
24 types and depending on the strength and duration of the stimuli, several different responses like mitogenesis,
25 differentiation, apoptosis or even anti-apoptosis. In this work we have studied the mechanisms of modulation
26 of the Egr family, in mammary epithelial cells of different origin (bovine, canine, feline, murine). Following
27 stimulation with growth medium, Egr mRNA expression showed a strong upregulation reaching a peak at
28 45-60 minutes, that rapidly declined. Among several cytokines, particularly important for mammary
29 morphogenesis, that we have tested (EGF, IGF-I, insulin, estrogen, progesterone), only EGF upregulated
30 Egrs to levels close to those elicited by growth medium. In order to understand how the Egr trascription
31 factors were regulated, we have inhibited Erk 1/2 and PI3K, molecules that drive two major intracellular
32 signaling pathways. Inhibition of the Erk 1/2 pathway totally abolished Egr upregulation mediated by growth
33 medium or EGF. On the other hand, the PI3K-Akt pathway played a minor role on Egr levels, with a strong
34 inhibitory effect on cat GH2 cells only, that could be ascribed to reduced Erk phosphorylation following
35 PI3K inhibition. Finally we showed that addition of growth medium also upregulated that the mammary
36 luminal marker cytokeratin 18, but only in the murine NMuMG cell line. This is the first manuscript
37 describing how the Egr trascription factors are expressed in mammary epithelial cells of domestic animals
38 and which growth factors and signaling pathways modulate their expression.

39

40 **Keywords**

41

42 Egr; mammary gland; immediate early gene; proliferation; epidermal growth factor

43 1. Introduction

44

45 The mammary gland is one of few organs that develops almost exclusively postnatally. Before puberty this
46 organ grows isometrically with the animal, by developing rudimentary branches that are originated by
47 multipotent mammary epithelial cells that progressively invade the surrounding stroma, mainly composed of
48 adipose tissue and fibroblasts. In this phase, in mice, estrogen provides the endocrine signal that induces the
49 release of locally produced amphiregulin, one of the seven ligands able to activate the epidermal growth
50 factor receptor (EGFR). This endocrine-paracrine system is fundamental for prepubertal growth of the
51 mammary gland: estrogen receptor alpha (Mallepell, S. et al. 2006) or amphiregulin knock-out mice are
52 unable to develop this organ (Ciarloni, L. et al. 2007). Once adulthood is reached, the estrous cycle provides
53 the endocrine milieu that stimulates cycles of mammary growth and involution. Estrogen and progesterone
54 (Tanos, T. et al. 2012), acting through locally released growth factors, drive mammary cellular proliferation
55 that form newly developed lateral branches. The major locally produced factors that act during this phases
56 are Wnt-4 (Briskin, C. et al. 2000) and RANKL (Fernandez-Valdivia, R. et al. 2009). Once the estrous cycle
57 ends, lateral branches disappear, and are recreated the successive cycle. Within this system, control of cell
58 proliferation and death, is a major requisite.

59 Immediate early genes (IEGs) are a class of genes that encode for a variety of proteins involved in many
60 cellular functions, among which proliferation and differentiation. IEGs are expressed within minutes of
61 stimulation and thus, do not require de novo protein synthesis (Herschman, H.R. 1991). Within proliferation,
62 IEGs are normally induced in interphasic cells following stimulation by growth factors (e.g. insulin-like
63 growth factor I, epidermal growth factor, etc) as well as many mitogenic signals (Fowler, T. et al. 2011).
64 Following stimulation, IEGs mRNAs and proteins are rapidly expressed, but being unstable, rapidly
65 degraded as well (Healy, S. et al. 2013). Thus, the peak in expression lasts for a very short period of time,
66 usually less than an hour. Even if brief, this exposure drives the cell to turn on a set of secondary response
67 genes that allow the progression to the subsequent phases of the cycle.

68 A subclass of IEGs is represented by transcription factors like Egr (1, 2, 3 and 4), Myc, Fos, and Jun, key
69 players in cellular proliferation and well known proto-oncogenes, often found deregulated in cancers (Huang,
70 H. et al. 2014; Verde, P. et al. 2007; Zwang, Y. et al. 2012). Egrs are zinc finger transcription factors with

nuclear localization, that bind preferentially to GC-rich sequences (Thiel, G. and Cibelli, G. 2002). Upon DNA-binding they promote many biological functions that range from proliferation to differentiation to apoptosis and carcinogenesis, depending on the cell type, duration of the growth factor stimuli and intracellular context (Adamson, E.D. and Mercola, D. 2002; Fang, F. et al. 2013; Mayer, S.I. et al. 2009; Seiler, M.P. et al. 2012).

No detailed analysis has been made on Egrs in cells obtained by domestic animals of different species and no data is available on the timing of Egr modulation, which growth factors and which signaling pathways mediate their expression. Therefore we decided to use mammary epithelial cells from bovine, mice and cat (non tumor-derived) as well as dog (tumor-derived) in order to understand the basic mechanisms of modulation of Egrs in these species. We focused our study on different aspects of Egr biology: a) basal expression of Egrs, b) modulation of expression following growth factor addition, c) study of extrinsic stimuli able to modulate Egrs in mammary cells, d) examination of intracellular pathways able to modulate Egrs.

84

85

86 **2. Materials and methods**

87

88 *2.1 Materials and cell lines*

All reagents, unless specified, were from Sigma–Aldrich (St. Louis, MO, USA); recombinant Epidermal Growth Factor (EGF), Insulin-Like Growth Factor I (IGF-I) were from Immunotools (Friesoythe, Germany) and were resuspended in water + 0,1 % BSA; UO126, Selumetinib, Wortmannin and phorbol 12-myristate 13-acetate (PMA) were from LC Laboratories (Woburn, MA, USA) and were resuspended in DMSO; DC Protein Assay was from Bio-Rad Laboratories (Hercules, CA, USA); Hybond Enhanced Chemiluminescence (ECL) nitrocellulose membrane was from GE Healthcare Life Sciences (Little Chalfont, Buckinghamshire, UK); Clarity Western ECL Substrate was from Bio-Rad Laboratories (Hercules, CA, USA); CL-XPosure Film was from Thermo Scientific (Waltham, MA, USA).

Antibodies. Anti-Egr-1 (1:1000; Sc-189), anti-Erk 1 (1:1000; Sc-94) and anti-IGF-IR alpha (1:1000; Sc-712) rabbit polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti- α -tubulin

(1:5000) and anti-phospho-Erk 1/2 (1:5000; M 8159) mouse monoclonal antibodies were from Sigma–Aldrich; anti-phospho-Akt mouse monoclonal (1:1000; #4051; ser-473), anti-Akt (1:1000; #9272) and anti-EGFR (1:1000; #2232) rabbit polyclonal antibodies were from Cell Signaling Technologies (Danvers, MA, USA); rabbit polyclonal anti-Keratin 14 (1:500; Poly19053) was from BioLegend (Dedham, MA, USA); mouse monoclonal anti-Keratin 18 (1:200; RGE 53) was from Progen Biotechnik (Heidelberg, Germany).

NMuMG murine mammary epithelial cell line (ATCC number CRL-1636) was kindly provided by R. Montesano (University of Geneva Medical School, Switzerland) and cultured in DMEM supplemented with 10 % fetal bovine serum (FBS). BME-UV bovine mammary epithelial cell line kindly provided by I. Politis (Agricultural University of Athens, Athens, Greece; (Zavizion, B. et al. 1996)). BME-UV cells were grown in DMEM supplemented with 10 % FBS. *Felis catus* mammary cells (GH2) were obtained by an healthy mammary gland following mastectomy in a cat with a mammary tumor (the tumor was in the inguinal mammary gland while mammary cells were obtained from the thoracic mammary gland). Consensus for the surgery was given by the owner of the animal and approved by the Ethical and Welfare Committee of the Department of Veterinary Sciences. Fresh mammary tissue was dissected, minced, added to a 1:1 v/v mixture of DMEM/F12 medium supplemented with 2 % w/v bovine serum albumin (BSA, Fraction V), 300 U/ml collagenase, 100 U/ml hyaluronidase, 100 U/ml penicillin, 100 µg/ml streptomycin and placed in a shaking incubator at 37 °C for three h. Epithelial cell aggregates obtained by centrifugation at 80 g for 30 seconds were washed in fresh DMEM/F12 medium for 3 times. Aggregates were incubated with a 0.5 mg/ml trypsin solution supplemented with 0.2 mg/ml EDTA followed by vigorous pipetting for 4 minutes and subsequent washing in Hank's balanced salt solution (HBSS) supplemented with 2 % FBS. Cells were then treated for 3 min with 5 mg/ml dispase and 100 µg/ml DNaseI and passed through a 40 µm cell strainer (BD Biosciences, San Jose, CA, USA) to remove remaining cell aggregates. GH2 cells were then cultured in collagen coated wells with DMEM/F-12 media supplemented with cholera toxin (100 ng/ml), insulin (10 µg/ml), hydrocortisone (0.5 µg/ml), EGF (20 ng/ml) and 5 % horse serum. GH2, by immunostaining, were mostly keratin 14 positive, a unique marker for cells belonging to the basal compartment of mammary gland, with a small proportion of keratin 18 positive cells (luminal compartment; Supplementary Fig. 3). CF33 cells, a canine breast cancer cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). CF33 cells were grown in DMEM supplemented with 10 % heat-inactivated FBS. All cell lines

127 were maintained in a 5 % CO₂–water-saturated atmosphere and routinely passaged when confluent by
128 washing with PBS followed by trypsinization.

129

130 *2.2 RNA extraction and quantitative RT-PCR*

131 Total RNA was isolated from primary mammary cell subpopulations using Aurum™ Total RNA Mini Kit
132 (Bio-Rad Laboratories) following the manufacturer's instructions. The purification procedure included a
133 DNase I digest step to produce a DNA-free total RNA. One microgram of total RNA was reverse transcribed
134 with iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories) using a blend of oligo(dT) and random primers
135 following the manufacturer's instructions. Real-time PCR was used to measure the quantity of the genes of
136 interest relative to the quantity of HPRT-1 (hypoxanthine phosphoribosyl-transferase 1) and glyceraldehyde-
137 3-phosphate dehydrogenase (GAPDH) mRNAs. HPRT-1 and GAPDH were used to correct for sample
138 concentration and reverse transcription efficiency because their expression is stable in these cells under our
139 experimental conditions. Efficiencies for the different genes were calculated by standard dilution curves and
140 used to correct for amplification. The specificity of each amplification was confirmed by initial sequencing
141 of the reaction product and by subsequent verification of the dissociation curve consisting of a single peak.
142 Diluted cDNAs (1:20, 30 ng per reaction) were used for real-time PCR amplification (CFX Connect real-
143 time PCR system; Bio-Rad Laboratories) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad
144 Laboratories). Real-time PCR parameters were: cycle 1, 95 °C for 30 s; cycle 2, 95 °C 10 s, 60 °C 30 s for 40
145 cycles. Quantitative analysis and correction for efficiency was calculated by the CFX Manager Software
146 version 3.1 (Bio-Rad Laboratories) using the $\Delta(\Delta CT)$ method by Livak and Schmittgen (Livak, K.J. and
147 Schmittgen, T.D. 2001) and compared to a comparator sample (CTRL=1). Primers were used at a final
148 concentration of 250 nM (MWG-Biotech; Ebersberg, Germany). Primers, accession number, amplicon size,
149 melting temperature and efficiency (%) are indicated in Supplementary Table 1. N/D = Not Determined, due
150 to very low expression of the target mRNA (> than 30 Cq).

151

152 *2.3 Analysis of cell cytodieresis through time-lapse video microscopy*

153 Cells were plated and cultivated in their respective growth medium (20-30 % confluence) then either left in
154 their growth medium (GM) or starved in serum and growth factors deprived medium (ST) and photographed

155 at 4 min intervals for 24 h (360 frames) with a Leica AF6000 LX (Leica Microsystems, Wetzlar, Germany)
156 inverted microscope equipped with a Leica DFC350FX digital camera and a motorized stage controlled by
157 the LAS AF software (Leica Microsystems, Wetzlar, Germany). All cells within a 10x field were followed
158 from time 0 to time 24 h and the frames in which the cytodieresis event occurred were recorded. Finally, the
159 total amount of divisions occurring within every hour was calculated and divided by the mean number of
160 cells present at the same time interval (number of divisions / h / number of cells).

161

162 *2.4 Cell cycle analysis*

163 For cell cycle analysis cells were seeded in 6 cm plates in their respective growth medium then either left in
164 proliferation medium or starved in serum/growth factors deprived medium for 16 h. Cells were then
165 trypsinized, washed with PBS, fixed for 1 h in 50 % ethanol (in PBS), then centrifuged, resuspended in PBS
166 and stained with DAPI (1 µg/ml final concentration) for 2 h at 37 °C. Samples were run on a Attune
167 Acoustic Focusing Cytometer (Applied Biosystems) and the cell cycle distribution in G0/G1 and G2/M
168 phases was calculated using the Attune Cytometric Software (Applied Biosystems). Each experiment was
169 repeated four times independently, and within each experiment, each treatment was performed with two
170 replicate culture wells.

171

172 *2.5 Western-blot analysis*

173 For western-blot, all cell lines were seeded in six-well plates and allowed to grow to 50 % confluence. If
174 needed, cells were starved for 16 h in serum deprived medium. The indicated cytokines were added for 1 h.
175 UO126 (20 µM), Selumetinib (1 µM) and Wortmannin (100 nM) were resuspended in dimethyl sulfoxide
176 (DMSO) at 1000x concentration and added 1h before lysis or the indicated treatments. Control samples were
177 added with an equivalent amount of DMSO. Cells were washed with ice-cold PBS, lysed, and scraped in
178 lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X 100, 1mM h
179 glycerolphosphate) with Protease Inhibitor Cocktail (1:100) and 1 mM sodium orthovanadate. Protein lysates
180 (10 mg) were cleared of cellular debris by centrifugation at 4 °C for 10 min at 12 000 g, quantified using DC
181 Protein Assay, resolved in 10 % SDS-PAGE gels, and transferred to Hybond-C Extra nitrocellulose
182 membranes. After the transfer of proteins, the membranes were blocked at room temperature for 2 h with

183 Tris-buffered saline (TBS, 10 mM Tris and 150 mM NaCl, pH 7.4) containing 10 % BSA and then incubated
184 overnight at 4 °C with the appropriate primary antibodies. The membranes were washed six times for 5 min
185 each in TBS–Tween and then incubated for 1 h at room temperature with HRP-conjugated secondary
186 antibodies. The membranes were again washed six times in TBS–Tween and incubated for 5 min at room
187 temperature with Clarity Western ECL Substrate. The proteins were visualized by briefly exposing the
188 membrane to an autoradiographic CL-XPosure Film. Densitometric analysis for keratin-18 and p-Erk 1/2
189 levels was performed with ImageJ 1.50 (National Institutes of Health, USA; <https://imagej.nih.gov/ij/>) with
190 total Tubulin and Erk levels used as internal normalization controls (for Krt18 and p-Erk respectively).

191

192 *2.6 Statistics*

193 Experimental data are presented as mean \pm sem. Statistical differences between treatments were calculated
194 with one-way ANOVA using the Statgraphics package (STSC Inc., Rockville, MD, USA). When significant
195 differences were found, means were compared by an unpaired Student's t-test. For all tests, the level of
196 statistical significance was set at $p < 0.05$.

197

198

199 **3. Results**

200

201 *3.1 Egr-1, 2, 3 and 4 levels in mammary epithelial cells.*

202 In order to study the Egr behavior, we first analyzed its mRNA levels in mammary epithelial cells of
203 different origin under two different conditions: cells cultivated in their respective growth medium (GM, see
204 Materials and methods) or cultivated under starving conditions for 16 h (medium without any supplement:
205 ST). Basal expression rates, as indirectly monitored by Cq levels (in 30 ng of retrotranscribed RNA), were
206 higher for Egr-1 and progressively lower for Egr-2, 3 and 4 (Fig. 1A). Even though these transcription
207 factors are tightly connected to the cell cycle Egrs levels in GM and ST cells were similar. To understand
208 this result we used time lapse video microscopy (Supplementary Video 1) and counted all cytodieresis events
209 occurring simultaneously, e.g. every h (Fig. 1B). Under the GM condition cells showed a $3,2 \pm 0,8\%$
210 (NMuMG), $3,6 \pm 1,3\%$ (CF33), $4,4 \pm 1,2\%$ (BME-UV) and $5,8 \pm 0,8\%$ (GH2) cells undergoing cytodieresis

every h. Under the ST condition (after 14-18 h) cells showed a mild reduction of cytodieresis events occurring every h: $1,4 \pm 0,5\%$ (NMuMG), $1,9 \pm 0,5\%$ (CF33), $2,4 \pm 0,9\%$ (BME-UV) and $3,4 \pm 1,0\%$ (GH2). These data indicate that under both the GM or ST condition a very low proportion of cells are within the exact same phase of the cycle with more than 94% of the cells being unsynchronized. This effect produces a very high "background noise" that masks the behavior of Egrs. We thus decided to increase per percentage of cells within the same phase of the cycle in order to better study Egr modulation. For this purpose, cells were serum starved. Analysis by flow cytometry confirmed that, to the exception of BME-UV cells, starving increased the percentage of mammary cells in G0/G1 (Fig. 1C, D).

3.2 *Egr-1, 2 and 3 modulation in mammary epithelial cells*

Following starvation for 16 h cells were stimulated with their respective growth medium (see materials and methods) and RNA was extracted at multiple time points (from 15 to 120 min). Egr-1, 2 and 3 levels were measured by real-time PCR with time = 0 min being set arbitrarily to 1 (Fig. 2; CTRL). All cell lines reacted similarly reaching a peak in Egr levels at 45 to 60 min after growth medium addition (Fig. 2). Egr-1 and 2 returned to almost basal levels after 120 min of stimulation, while Egr-3 had a slightly delayed curve of increase and decrease in expression, in particular in CF-33 cells reaching a peak at 75-90 min. Egr-4 (data not shown) showed a similar, but much lower, trend of regulation with levels of expression that remained always very low (> 28 Cq). For this reason Egr-4 was not analyzed any further.

3.3 *Growth factors that affect Egr modulation in mammary epithelial cells*

We then verified the ability of multiple cytokines, important for mammary growth, to modulate Egr levels. Following 16 h of serum starvation several growth factors (EGF, IGF-I, insulin, 17β -estradiol, progesterone) were added for 1 h and Egr mRNAs were analyzed by real-time PCR. Among all the growth factors that we tested, epidermal growth factor (EGF) was unique in eliciting an Egr-1, -2 and -3 increase to levels close to growth medium (Fig. 3). In mouse NMuMG cells, insulin promoted some increase on Egr expression. Other cytokines (FGF β , PDGF α , VEGF, TNF α), with limited effect in mammary morphogenesis, were tested and found to have no effect on Egr expression (data not shown).

238 We thus assayed whether the cell lines expressed the receptors for the cytokines that we had tested
239 (Epidermal Growth Factor, Insulin, IGF-I, Estrogen or Progesterone receptors). EGFR and Erb-B2, activated
240 by EGF as homo- (EGFR/EGFR) or hetero- (EGFR/Erb-B2) dimers, and IGF-I receptors (activated by IGF-I
241 and insulin at the high concentrations that we used) were present in all cell lines (Fig. 3 A and B). Insulin,
242 Estrogen and Progesterone receptors were not expressed or expressed at very low levels.

243

244 *3.4 Identification of pathways that modulate expression of Egr-1, 2 and 3*

245 We analyzed whether two major intracellular pathways, the Erk-1/2 and the PI3K-Akt pathways, could
246 modulate Egr expression in mammary epithelial cells. To this end, starved mammary cells (16 h) were
247 pretreated for 1 h with UO126 (Erk-1/2 inhibitor, 20 μ M) or wortmannin (PI3K inhibitor, 100nM) and
248 stimulated with GM or EGF for 1 h in order to increase Egr-1, -2 and -3 expression. Inhibiting the Erk-1/2
249 pathway abolished the Egr mRNA increase downstream GM or EGF in all cell lines (Fig. 4A, GM vs
250 GM+UO and EGF vs EGF+UO). Inhibiting the PI3K pathway had no effect on bovine BME-UV and canine
251 CF33 cells, a mild effect on mouse NMuMG cells treated with GM (Fig. 4A, GM vs GM+W) and a strong
252 reducing effect on GH2 cells (Fig. 4A, GM vs GM+W and EGF vs EGF+W). Egr-1 protein levels, visualized
253 by western-blot in GH2 and NMuMG cells, reflected the results obtained by mRNA quantification (Fig. 4B).
254 We were unable to visualize Egr-1 in BME-UV and CF33 cells by western-blot, possibly because the
255 antibodies did not work in these species (2 different antibodies were tested). p-Erk and p-Akt levels, as
256 shown by western-blot, confirmed that UO126 and wortmannin inhibited their targets (Fig. 4B, p-Erk and p-
257 Akt) but showed also a reduction in p-Erk levels in GH2 cells treated with wortmannin. UO126 specificity
258 was confirmed with another more recently developed Erk inhibitor, Selumetinib (Zhou, Y. et al. 2016). Used
259 at 1 μ M, Selumetinib prevented Erk phosphorylation (supplementary Fig. 2A), and inhibited Egr-1, -2 and -3
260 upregulation following GM or EGF addition (supplementary Fig. 2B) in all cell lines.

261 To better clarify whether the strong inhibitory effect observed on GH2 cells treated with wortmannin could
262 depend on reduced of Erk rather than Akt activation, we performed time-course western-blot analysis of p-
263 Erk 1/2 and p-Akt following addition of GM, EGF or phorbol 12-myristate 13-acetate (PMA, a strong
264 activator of protein kinase C). PMA was included in this assay because it is a strong activator of Erk 1/2 with
265 different modulating activities on Akt phosphorylation (Han, S., and Meier, K.E. 2009; Liu, H. et al. 2006;

266 Moelling, K. et al. 2002). Western-blot analysis showed that wortmannin had a strong inhibitory effect on
267 Erk activation at 10 and 30 min following GM and EGF, but not PMA addition (Fig. 5A, 10' and 20'). At 40
268 and 60 min p-Erk was inhibited downstream all growth factors (Fig. 5A, 40' and 60'). p-Akt levels were
269 increased following GM or EGF treatment. On the contrary, PMA treatment reduced p-Akt below starved
270 (ST) levels. Egr-1, -2 and -3 levels under PMA stimulation, were minimally reduced by wortmannin (Fig.
271 5B: PMA+W), confirming that in GH2 Erk, but not Akt phosphorylation is the major inducer of these IEGs.

272

273 *3.5 Regulation of cytokeratin 18 following growth medium stimulation*

274 Recent evidence has shown that Egr-1 binds to the cytokeratin 18 promoter and modulates the expression its
275 mRNA and protein (Zhang, H. et al. 2014). In mammary cells, cytokeratin 18 (Krt18) is a marker of the
276 luminal lineage. Since Egr-1 is strongly upregulated by growth medium, we tested whether growth medium
277 could alter Krt18 expression in mammary cells that express this marker. We first identified Krt18 expressing
278 cells by immunohistochemistry (supplementary Fig. 3). BME-UV, GH2 and NMuMG cells express Krt18,
279 while CF33 do not. Real-time PCR confirmed this data (Cq levels: BME-UV 18,0±1,2, GH2 22,3±1,0,
280 NMuMG 17,5±1,3). To analyze whether Krt18 was modulated in these cell lines, serum starved BME-UV,
281 GH2 and NMuMG were stimulated with GM and Krt18 mRNA was quantified. Only NMuMG showed a
282 stable Krt18 mRNA upregulation following GM addition (Fig. 6A), that was confirmed by western-blot (Fig.
283 6B). Cytokeratin 14, expressed at high levels by GH2 cells only, did not show any modulation by GM (data
284 not shown).

285

286 **4. Discussion**

287 Egr-1 was first identified by screening cells for genes that are upregulated by the addition of serum
288 (Sukhatme, V.P. et al. 1987). The discovery of Egr-2, -3 and -4 followed briefly after (Chavrier, P. et al.
289 1988; Muller, H.J. et al. 1991; Patwardhan, S. et al. 1991). Egrs are zinc finger transcription factors that
290 regulate transcription, unusually, as monomers. They have found many roles in cellular physiology, most
291 importantly by regulating genes that allow the cell to proceed through the different phases of its cycle, but
292 also, in particular cell types and under well defined stimulatory conditions, promoting differentiation,
293 motility or even apoptosis (Oliveira, F.M. and Tourtellotte, W.G. 2015; Seiler, M.P. et al. 2012; Tao, X. et al.

2015; Tourtellotte, W.G. et al. 1999; Zwang, Y. et al. 2011). In pathology Egrs may act paradoxically, usually as a tumor suppressors by maintaining normal growth regulation, but also as a tumor progression agents, like in prostate cancer (Baron, V. et al. 2006; Baron, V.T. et al. 2015).

In this work we describe the biological behavior of Egr-1, -2 and -3 in three non tumorigenic mammary epithelial cell lines isolated from different species (bovine, mouse, cat) and in one tumorigenic mammary cell line isolated from the dog specie (Saito, T. et al. 2014). We first analyzed the levels of expression of the four Egrs under proliferating (GM) and serum starved (ST) culture conditions. We were expecting lower Egr levels under serum-starved conditions, but unexpectedly all cell lines showed similar levels (Fig. 1A). Since Egrs are Immediate Early Genes tightly connected to the cell cycle, we counted the number of cytodieresis events occurring every hour (a direct indicator of cells that are at the exact same point of the cell cycle). We found out that the maximum number of cytodieresis events occurring simultaneously in any 1 h in GM cells is about 5% (GH2 cells) that decreases in ST cells to about 3%. All other cells lines behave similarly with a small decrease in the percentage of cells undergoing cytodieresis when passing from the GM to the ST condition. This helped us to understand that, within a large population, only a minimal fraction of cells is at the exact same time point of the cell cycle. Since IEGs are highly upregulated in a narrow time interval that lasts less than one hour, having more than 95% of the cells unsynchronized during normal growth, explains why GM and ST treated cells have similar Egr levels.

Synchronization by serum starvation was a prerequisite that allowed us to analyze Egr modulation downstream growth factor stimulation. This treatment increased the percentage of cells in G0/G1 in three cell lines. BME-UV showed an opposite response probably due to the fact that, following serum starvation for 16 h, 30-40% of these cells undergo cell death and are visible as sub-G0 events (supplementary Fig. 1). After stimulation with growth medium all cell lines had a similar trend of Egr modulation, reaching a maximum level after 45-60 minutes that rapidly declined to near basal level after 120 min. Since starvation could not synchronize all cells we hypothesize that the real increase is much higher than the one we have observed. Egr-3 levels were maintained for up to 75-90 minutes before rapidly declining. This different mechanism of modulation has not been described before.

321 To the exception of CF33 cells (dog), which have been obtained from a mammary cancerous tissue, all other
322 cell lines come from healthy mammary glands. Grossly, the mammary epithelium is organized in two layers
323 of cells: luminal cells that face the lumen of the mammary ducts and express cytokeratin 18 and basal cells
324 that form the outer layer of the ducts and express cytokeratin 14. The cells that we have used in our work
325 express differentially markers of both compartments (data by real-time PCR, not shown, and
326 immunostaining, Supplementary Fig. 2): NMuMG cells are almost all Krt18 positive; GH2 are Krt14
327 positive with a small percentage of Krt18 positive cells, distributed in islands; BME-UV cells are mostly
328 Krt18 positive. CF33 canine tumoral cells are negative for both markers, therefore are probably of stromal
329 rather than epithelial origin, or have lost the keratin markers during selection. Our results show that the
330 behavior of Egrs is similar in all the cell lines that we examined, indicating that the cell of origin (basal or
331 luminal) or the cancerous state do not seem to modify the modulation of these transcription factors.

332 Our observation that only EGF can increase Egr expression close to the levels obtained by GM stimulation is
333 intriguing. In fact, we were expecting that other mammary "specific" growth factors or hormones (IGF-I,
334 estrogen, progesterone) could also modulate Egr expression. This result is probably a consequence of the
335 selection operated during the immortalization process that has stabilized these cell lines, in which the
336 majority of the multiple cellular lineages present within the mammary gland disappear. For example,
337 expression of estrogen or progesterone receptors is limited to a small percentage of terminally differentiated
338 cell population that is probably difficult to retain in culture and these receptor were absent in all the cell lines
339 examined. Even though all the cell lines express IGF-IR, and a low quantity of Insulin R, there was no Egr
340 modulation following treatment with IGF-I or insulin (that at the concentration used can activate the IGF-
341 IR). This effect can be explained by the low capacity of the IGF-IR to activate the Erk rather than Akt
342 (Moelling, K. et al. 2002), providing another confirmation that Erk is the major upregulator of Egrs.

343 EGF is a strong growth-promoting agent and, within the mammary gland, its receptor has long ago found an
344 important biological role (Fowler, K.J. et al. 1995). In depth analyses have then elucidated that among the
345 seven known EGF receptor ligands, only amphiregulin has a fundamental role during the pubertal mammary
346 development (Ciarloni, L. et al. 2007). Interestingly, this role is tied to the presence of the EGF receptor in
347 the stromal, but not the epithelial compartment of the mammary gland (Wiesen, J.F. et al. 1999). Our data
348 show that EGF, acting through its receptor, is the only growth factor able to elicit an increase in Egr

transcription factors in all the cell lines that we have tested. Therefore, a biological role for this growth factor and its receptor, mediated through Egrs, is possible even in the epithelial compartment. The NMuMG line depends on EGFR for its proliferation (Accornero, P. et al. 2012), therefore in this cell line Egr modulation downstream EGF might drive proliferation. Another possible function of Egrs could be to modulate the expression of some differentiation markers, like Krt14 or Krt18. A recent report has outlined a connection between Egr-1 levels and Krt18 (Zhang, H. et al. 2014). Krt18 expression is directly regulated by an Egr-1 binding site situated within its promoter. Among the cell lines examined, only NMuMG showed a mild upregulation of Krt18 expression following GM addiction. We think unlikely that the basal expression of this keratin is maintained by Egr levels for two reasons: a) BME-UV and GH2 Krt18 levels were unaffected following GM addition and, b) MCF-7, T47D and ZR-75D human mammary tumor cells that have high Krt18 basal expression (Cq levels around 16-18, data not shown) have basal Egr-1 levels lower than the cells that we have tested (Cq levels of 27-29; data not shown). Therefore in our models, Egr modulation downstream EGF or GM, most likely promotes a proliferative, antiapoptotic or migratory stimuli (Baron, V. et al. 2006; Tarcic, G. et al. 2012; Yu, J. et al. 2009). By using lipofectamine transfected siRNA (MISSION esiRNA; Sigma-Aldrich) we have tried to downregulate Egr expression but, unfortunately, we were unable to reduce their expression. For this reason, we cannot confirm what is the exact role of these transcription factors in these cell lines.

We have also analyzed which signaling pathway modulates Egr-1, -2 and -3 expression downstream GM or EGF. Our data show clearly that Erk 1/2 activation has a preeminent role in Egr modulation. All cell lines had all Egrs lowered to levels close or lower than the CTRL unstimulated samples, when the Erk 1/2 pathway was inhibited by UO126 or Selumetinib. Our data is in line with literature (Murphy, L.O. et al. 2002; O'Donnell, A. et al. 2012; Tarcic, G. et al. 2012; Whyte, J. et al. 2009), and extends the its significance to mammary cell lines of multiple origin. Inhibiting the PI3K pathway determined a strong reduction of Egrs in GH2 cells only. A possible explanation to this reduction is that inhibition on the PI3K-Akt pathway determined by wortmannin also induces a reduction on the Erk1/2 pathway. This action is apparent when observing the reduction in p-Erk in western-blot. This event could be due either by an off target effect of wortmannin (even at the low concentration used, 100nM, and only in this cell line) or by an Akt-Erk1/2 cross talk active exclusively in this cell line. We addressed this issue by examining p-Erk and p-Akt levels

377 downstream GM, EGF and PMA at multiple time points. We observed that wortmannin elicit a strong
378 reduction in p-Erk levels, but not under PMA treatment. The inability of wortmannin to reduce p-Erk levels
379 downstream PMA at 10' and 20' min was coupled with a minimal reduction of Egrs in PMA+wortmannin
380 treated samples. This data together with the observation that PMA reduces p-Akt levels in this cell line,
381 confirm that Egrs are modulated in an Erk-dependent but not Akt-dependent manner. Other authors have
382 observed that, in hemopoietic cells, Erk activation requires a PI3K generated signal (Sutor, S.L., et al. 1999).
383 In our assays we show, for the first time in mammary cells, that the PI3K pathway is not insulated from the
384 Erk signaling cascade, following GM or EGF treatment, thus extending previous observations to a different
385 cell type and stimulus. Future studies will address at what level in the signaling cascade PI3-K is required for
386 Erk activation.

387

388

389 *4.1 Conclusions*

390 In conclusion, in this study we have evaluated the mechanisms of modulation of Egr-1, -2 and -3 trascription
391 factors that belong to the immediate early genes group, in mammary epithelial cells of different species. We
392 have observed that transcription of Egr-1 and Egr-2 reach a maximum 45 to 60 min after a stimulatory event,
393 then rapidly decline. Egr-3 show a slightly delayed response. We have found that, among different growth
394 factors and hormones that we tested, only EGF is able to increase the Egrs to levels similar to those elicited
395 by growth medium. We have also identified the Erk 1/2 pathway as the major regulator of all three Egrs.
396 Finally, we have shown that NMuMG, but not BME-UV and GH2 cells, exhibit a Krt18 increase following
397 the growth medium acute stimulus.

398

399

400 **Acknowledgements**

401 This study was supported by Local Grants from the University of Torino (fondi ex 60%). We are grateful to
402 Cristina Cecere for technical assistance.

403 NMuMG cells were kindly provided by Roberto Montesano (University Medical Center of Geneva,
404 Switzerland).

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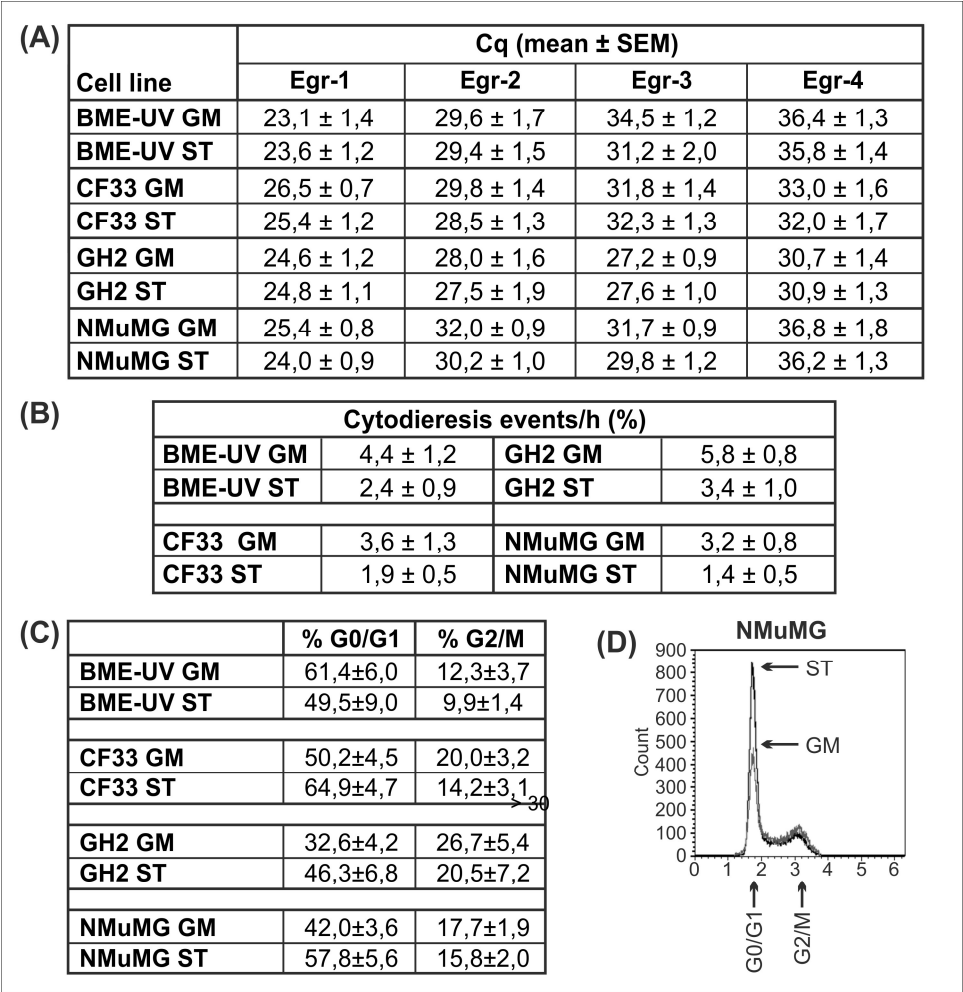
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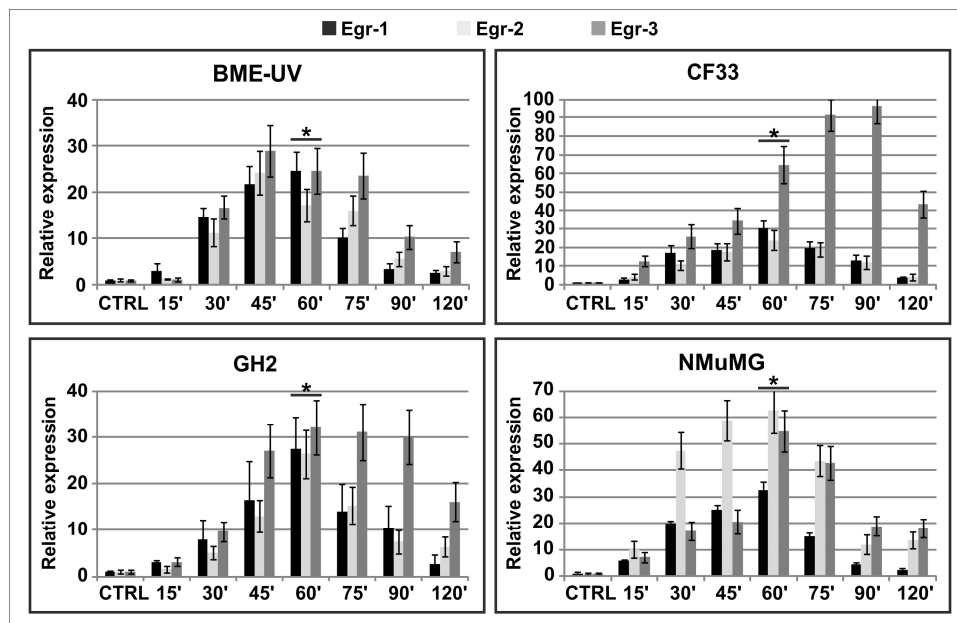
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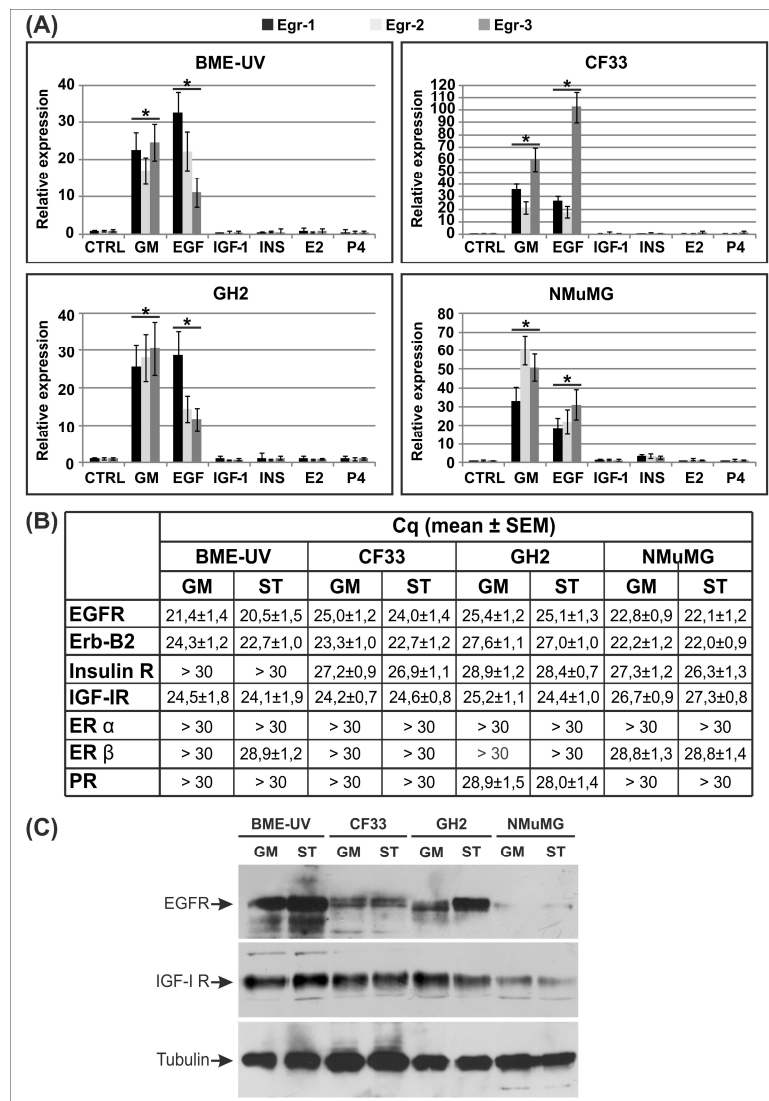
517 **Fig. 1.** Basal expression of Egr-1, 2 and 3 in mammary epithelial cells of different origin. **A:** Cq levels of
518 Egr-1, Egr-2, Egr-3 and Egr-4 in mammary epithelial cells cultivated in their respective growth medium
519 (GM) or under serum starved (ST) condition for 16 h. Each number represents the mean \pm standard error of
520 the mean of the four replicate experiments. **B:** Mean \pm standard error of the number of cytodieresis events
521 per h every 100 cells in mammary epithelial cells cultivated under GM or ST conditions (from the 14th to the
522 18th h if under serum starving condition). Time lapse video microscopy of multiple fields (n=4) were
523 acquired and cytodieresis events occurring every h were manually counted (see supplementary Video 1) and
524 divided by the number of cells in the field. **C:** Mean \pm standard error of the percentage of cells in G0/G1 and
525 G2/M in mammary cells cultivated under GM or ST conditions for 16 h (n = 4). **D:** Representative
526 cytofluorimetric plot of the NMuMG population (50.000 events), showing the variation in G0/G1 when cells
527 are cultivated for 16 h in ST condition.



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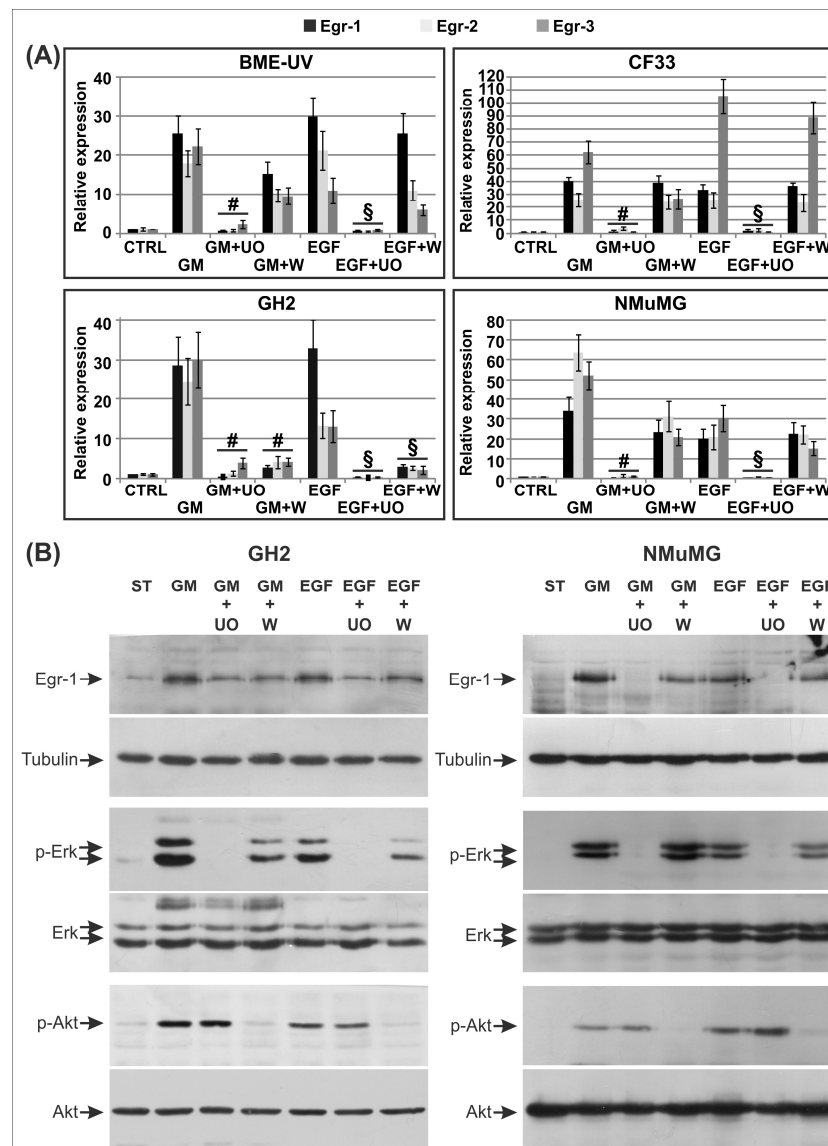
529 **Fig. 2.** Time course of Egr-1, Egr-2 and Egr-3 expression in mammary epithelial cell lines following
 530 stimulation with growth medium. Cells were synchronized by serum-starvation for 16 h then treated with
 531 their respective GM. RNA was extracted at the indicated time points and Egr expression was measured by
 532 real-time PCR and normalized with HPRT-1 and GAPDH. Egr expression in cells at the zero time point was
 533 set to 1. Each point represents the mean \pm standard error of the mean of the four replicate experiments.
 534 * $P < 0.001$ versus CTRL.

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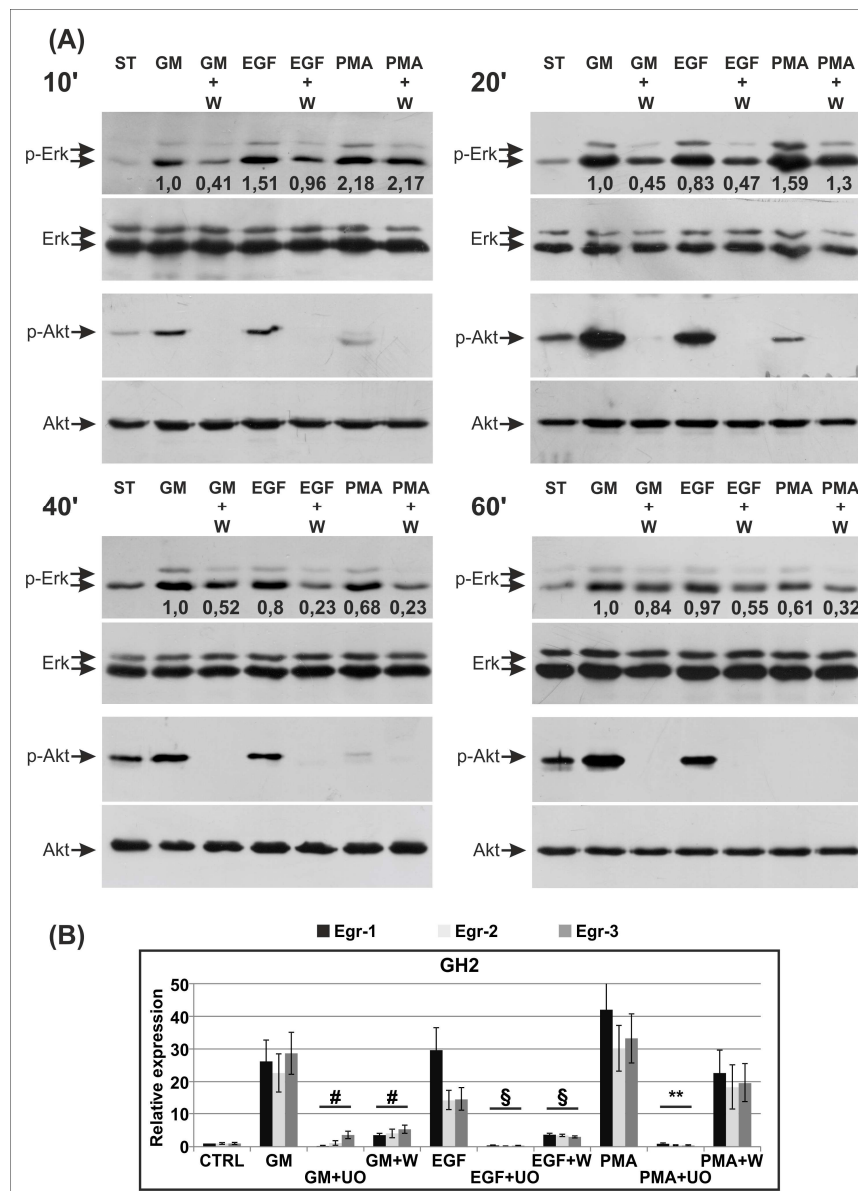
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537 **Fig. 3. A:** Egr modulation in mammary epithelial cell lines treated with different growth factors or
538 hormones. Cells were serum-starved for 16 h then treated with the indicated growths
539 factors/hormones/growth medium (EGF 20 ng/ml; IGF-I 10 ng/ml; Insulin 10 μ g/ml, β -estradiol 20 nM, 17 α -
540 hydroxyprogesterone 1 μ M). RNA was extracted after 1 h of treatment. Egrs expression was evaluated by
541 real-time PCR and normalized with HPRT-1 and GAPDH. The serum starved untreated control (CTRL) was
542 set to 1. Each point represents the mean \pm standard error of the mean of the four replicate experiments.
543 * P <0,001 versus CTRL. **B:** Cq levels of EGFR, Erb-B2, Insulin R, IGF-IR, ER α , ER β and PR in mammary
544 epithelial cells cultivated in their respective growth medium (GM) or under serum starved (ST) condition for
545 16 h. Each number represents the mean \pm standard error of the mean of the four replicate experiments. **C:**
546 GM or serum-starved (16 h) cultivated cells were analyzed by western-blot for EGFR and IGF-IR. Tubulin
547 was used as an internal control.



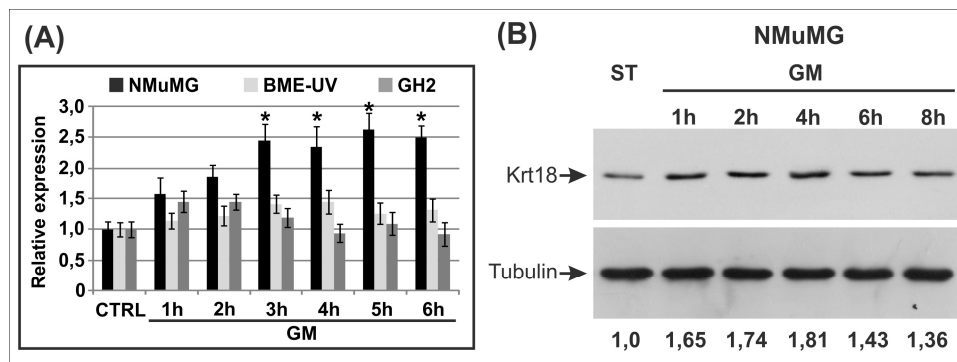
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549 **Fig. 4. A:** Egr-1, 2 and 3 mRNA and protein analysis in cells treated with growth medium or EGF in the
550 presence of an Erk1/2 or PI3K inhibitor. Cells were serum-starved for 16 h then pretreated for 1 h with
551 UO126 (20 μ M) or wortmannin (100 nM) and added with GM or EGF (10 ng/ml) always in presence of the
552 inhibitors. RNA was extracted after 1 h of treatment. Egr-1, 2 and 3 expression was measured by real-time
553 PCR and normalized with HPRT-1 and GADPH. The serum starved untreated control (CTRL) was set to 1.
554 Each point represents the mean \pm standard error of the mean of the four replicate experiments. # P <0,001
555 versus GM. § P <0,001 versus EGF. **B:** Western-blot analysis of the Egr-1 and tubulin normalization control
556 in GH2 and NMuMG cells treated like in A. Phospho-Erk 1/2 and phospho-Akt were used as internal
557 controls to evaluate the efficacy of the inhibitors (in this case proteins were extracted 10 min after the
558 treatment with GM or EGF). Total Erk 1/2 and total Akt are internal controls for phospho-Erk 1/2 and
559 phospho-Akt, respectively.



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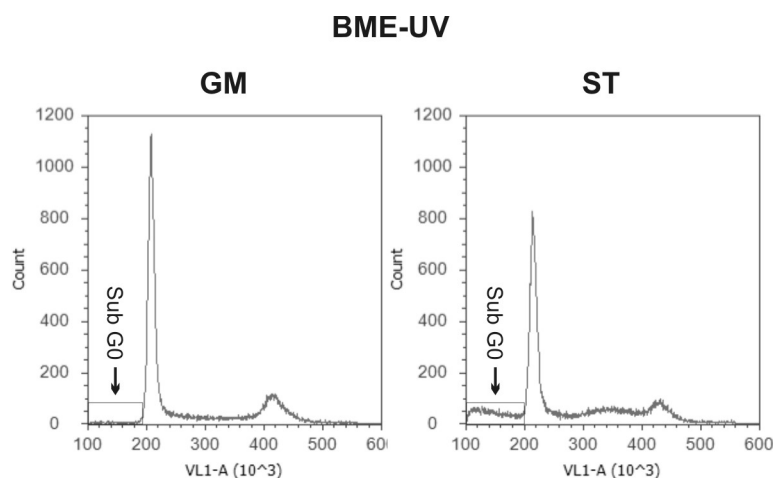
561 **Fig. 5. A:** Egr-1, 2 and 3 mRNA and protein analysis in cells treated with growth medium, EGF or PMA in
562 the presence of an Erk 1/2 or PI3K inhibitor. Cells were serum-starved for 16 h then pretreated for 1 h with
563 UO126 (20 μ M) or wortmannin (100 nM) and added with GM, EGF or PMA (10 ng/ml) always in presence
564 of the inhibitors. RNA was extracted after 1 h of treatment. Egr-1, 2 and 3 expression was measured by real-
565 time PCR and normalized with HPRT-1 and GADPH. The serum starved untreated control (CTRL) was set
566 to 1. Each point represents the mean \pm standard error of the mean of the four replicate experiments. # P <0,001
567 versus GM. § P <0,001 versus EGF. ** P <0,001 versus PMA. **B:** Time-course western-blot analysis of
568 phospho-Erk 1/2 (with densitometric quantification relative to Erk levels) and phospho-Akt in GH2 cells
569 treated with GM, EGF or PMA (10 ng/ml) in the absence or presence of Wortmannin (100 nM). Total Erk1/2
570 and total Akt are internal controls for phospho-Erk 1/2 and phospho-Akt, respectively.



571

572 **Fig. 6.** Cytokeratin 18 mRNA and protein levels following treatment with GM. **A:** Cells were serum-starved
 573 for 16 h then treated with their respective GM. RNA was extracted at the indicated time points and
 574 cytokeratin 18 expression was measured by real-time PCR and normalized with HPRT-1 and GADPH.
 575 * $P < 0.05$ versus CTRL. **B:** Serum-starved NMuMG cells (16 h) were treated with GM and protein were
 576 extracted at the indicated time points and analyzed by western-blot. Tubulin was used as an internal control
 577 for the densitometric analysis.

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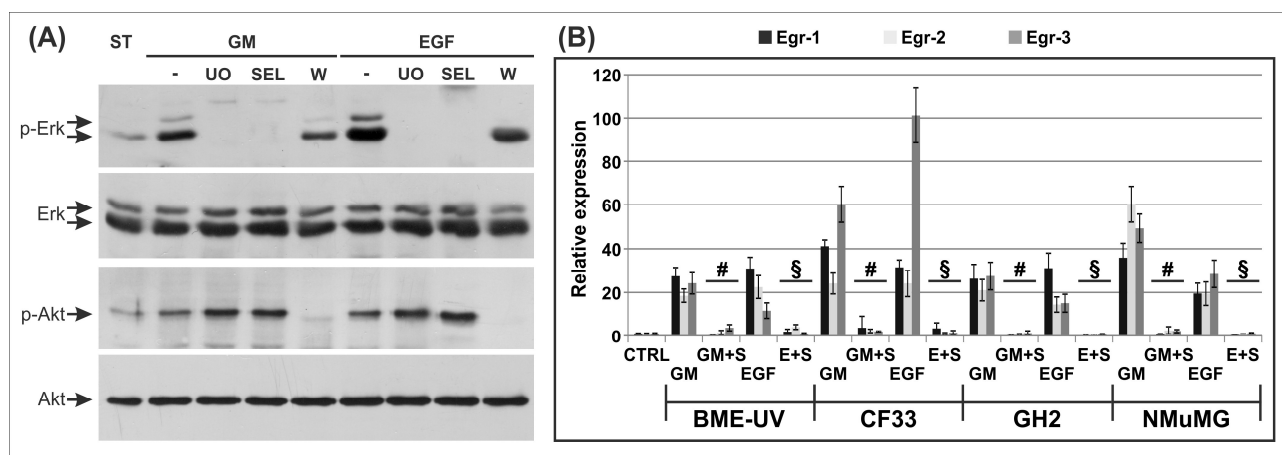


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580 **Supplementary Fig. 1.**

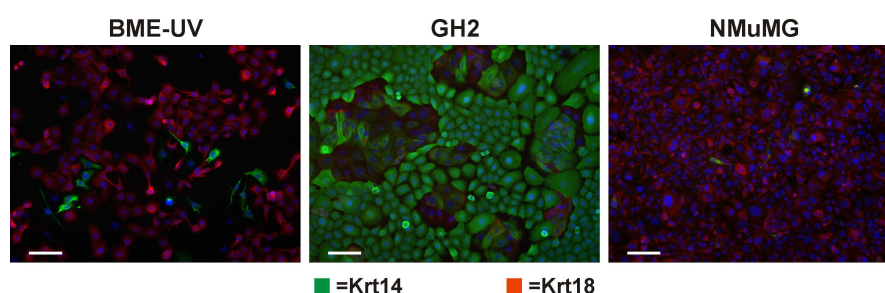
581 Cytofluorimetric plot of BME-UV cells cultivated in growth medium or under serum-starving conditions for
 582 16 h. Following serum-starvation for 16 h BME-UV cells show a decrease in the G0/G1 population and the
 583 appearance of a sub-G0 population that probably indicates cells undergoing apoptosis.

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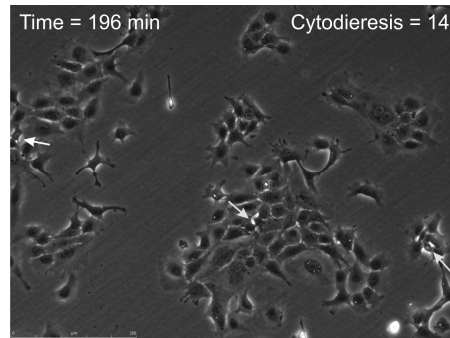
Supplementary Fig. 2.

A: Western-blot analysis of phospho-Erk 1/2 and phospho-Akt levels in GH2 cells treated for 10 min with GM or EGF and UO126 (20 μ M), Selumetinib (1 μ M) or Wortmannin (100 nM) showing the efficacy of the inhibitors. Total Erk 1/2 and total Akt are internal controls for phospho-Erk 1/2 and phospho-Akt, respectively. **B:** Egr-1, 2 and 3 mRNA levels in cells treated with growth medium or EGF in the presence of the Erk1/2 inhibitor Selumetinib. Cells were serum-starved for 16 h then pretreated for 1 h with Selumetinib (1 μ M) and added with GM or EGF (10 ng/ml) always in presence of the inhibitors. RNA was extracted after 1 h of treatment. Egr-1, 2 and 3 expression was measured by real-time PCR and normalized with HPRT-1 and GADPH. The serum starved untreated control (CTRL) was set to 1. Each point represents the mean \pm standard error of the mean of the four replicate experiments. # P <0,001 versus GM. § P <0,001 versus EGF.



Supplementary Fig. 3.

Immunohistochemistry analysis of non tumorigenic mammary epithelial cells for cytokeratin markers. BME-UV (bovine) and NMuMG (murine) cells are mainly Krt18 positive while GH2 (feline) cells are largely Krt14 positive with a small percentage of Krt18 positive cells that tend to group in islands. Bars = 100 μ m.



603

604 **Supplementary Video 1.**

605 Example of the technique used for cytodieresis quantitative analysis. Time lapse video (NMuMG cell line)
606 was acquired, with every frame corresponding to a 4 min interval, then manually analyzed to identify the
607 cytodieresis events (yellow arrow). All the events occurring every h were divided by the number of cells
608 present in the frame at that same time interval to obtain cytodieresis events per h %.

609

Supplementary Table 1.

Gene	Specie	Accession number	Primer Forward	Primer Reverse	Amplicon size	Melting temp. °C	Efficiency
Egr-1	Bos t.	NM_001045875.1	AGCCCTACGAGCACCTGAC	GGTTTGGCTGGGGTAACTC	92	82,5	102,8
	Canis f.	XM_846145.3	ACCTGACCGCAGAGTCTTTT	GTGGTTTGGCTGGGGTAACT	82	81,5	105,6
	Felis c.	XM_003980809.3	same as Canis f.	same as Canis f.	82	80,5	100,4
	Mus m.	NM_007913.5	AACCCTATGAGCACCTGAC	CGTTTGGCTGGGATAACTC	92	82,0	96,1
Egr-2	Bos t.	XM_003585156.4	TTTTCCCCAATGCCGAAGTG	GCCTGCACTCACAATGTTGA	245	86,5	103,1
	Canis f.	XM_536361.5	TCTTCCCCAATGCCGAAGTG	TGTTGATCATGCCATCTCCG	75	83,5	104,9
	Felis c.	XM_003993969.3	same as Canis f.	same as Canis f.	72	83,0	103,3
	Mus m.	NM_010118.3	CTCGTCGGTGACCATCTTCC	TTGATCATGCCATCTCCCGC	84	84,0	104,5
Egr-3	Bos t.	NM_001289818.1	CGCGCTCAACCTCTTTTCTG	TCCATCACATTCTCTGTAGCCA	75	81,5	104,3
	Canis f.	XM_543247.5	TGGCTACAGAGAATGTGATGGAC	CCCGAATAAGAGAGTTCCGGG	70	81,5	104,3
	Felis c.	XM_003984699.3	same as Canis f.	same as Canis f.	70	81,5	100,9
	Mus m.	NM_018781.3	ACAATCTGTACCCCGAGGAGA	TCACATTCTCTGTAGCCATCTGA	99	85,5	101,2
Egr-4	Bos t.	NM_001040497.1	CACCTTAGCGAGTTTTCCGG	GAGGCAGGAGTCGGTTAAGT	195	89,5	N/D
	Canis f.	XM_540228.5	ATCCAAGCAGTACCCGAACA	ATCCGGGGAGTAAAGGTCCG	183	90	N/D
	Felis c.	XM_006930186.2	same as Canis f.	CCAGATCCGGGGAGTAAAGG	187	90	N/D
	Mus m.	NM_020596.2	CTTCTTCATCCAGGCGGTTC	CTTCTTCATCCAGGCGGTTC	192	84,0	N/D
Krt-14	Bos t.	NM_001166575.1	TTGCAAAACCTGGAGATCGA	CACGCTGCTGATCAGCTCCT	129	N/D	N/D
	Canis f.	NM_001253741.1	GTGCAGAACCTGGAGATCGA	CACGTTGCCAATCAGGTCCT	129	N/D	N/D
	Felis c.	XM_003996860.3	same as Canis f.	CACGTTGCCGATCAGGTCCT	129	87	102,3
	Mus m.	NM_016958.2	ATGCAGAACCTGGAGATCGA	CACACTGCCGATCATCTCCT	129	N/D	N/D
Krt-18	Bos t.	NM_001192095.1	AAGGGTCTACAAAACCAGATTG	CTGCGAGGTGACCACTGTGG	192	87	104,8
	Canis f.	XM_534794.5	AAGGGTCTACAAAACCAAATCG	CTGTGTGGTGACCACTGTGG	192	86	104,4
	Felis c.	XM_003988746.2	AAGGGTCTACAAAACCAAATTG	CTGGGAGGTGATCACTGTGG	192	86,5	100,7
	Mus m.	NM_010664.2	AAGGTCTGGAAGCCCAGATT	CTTGGTGGTGACAACCTGTGG	191	86,5	99,8
IGF-IR	Bos t.	NM_001244612.1	AGCTGATGTGTACGTTCTGAC	CTCATCCTTGACCACGCCC	127	88,5	96,4
	Canis f.	XM_545828.5	GGACGCAGTATGCGGTTTAT	AGAGAGGGTGGGTTCCACTT	187	85	95,4
	Felis c.	XM_003986871.2	GACTAAAGCCTTGGACGCAGT	TGGAAGGAAGTGAAGCATTGG	129	84	103,4
	Mus m.	NM_010513.2	CTTTCCGAGGATCGTCGCCT	CGGGCCCACAGATTTCTCC	247	88	108
Insulin R	Bos t.	XM_015464089.1	ACGCTAGGCCTTCGAGGAAA	CACGGCTGCTGTCACATTC	62	84	N/D
	Canis f.	XM_005633222.1	GATCTAGCAGCCCGAAACTG	AGTGGTAAAGACCCCGTCCT	171	85,5	104,8
	Felis c.	XM_011288834.1	TCGGTTTCGAATTCCTCTCT	GGAAGGCAGCTTCAACCCTTT	159	85,5	N/D
	Mus m.	NM_010568.3	TCAGCCAGTCTTCGAGAACG	AAGAAGGCGGACCACATGAT	87	83	105,6
EGFR	Bos t.	XM_002696890.4	CTTCGGCTGCCTGCTGGACTA	CCAGGTAATTCATGCCCTTTGC	104	86,5	104,3

	Canis f.	XM_014120756.1	GGACTATGTCCGCGAGCACAA	CCAGGTAGTTCATGCCCTTTGC	89	84,5	96,7
	Felis c.	XM_006929086.2	same as Canis f.	CCAGGTAGTTCATGCCCTTCGC	89	83,5	92,4
	Mus m.	NM_207655.2	GGACTACGTCCGAGAACACAA	CCAGGTAGTTCATGCCCTTTGC	89	82,5	98,3
Erb-B2	Bos t.	NM_001303629.1	CTGAACTGGTGTGTGCAGATTGC	CATGGTTGGGACTCTTGACCA	177	87	N/D
	Canis f.	NM_001003217.2	CTGAACTGGTGTGTGCAGATTG	CATGGTTGGGACTCTTGACCA	109	86	103,4
	Felis c.	NM_001048163.1	same as Bos t.	CGTGGTTGGGACTCTTGACCA	109	86	N/D
	Mus m.	NM_001003817.1	CTCAACTGGTGTGTTTCAGATTGC	CGTGGTTGGGACTCTTGACTA	109	84,5	95,7
ERalfa	Bos t.	NM_001001443.1	AGGGAAGCTCCTATTTGCTCC	CGGTGGATGTGGTCCTTCTCT	234	N/D	N/D
	Canis f.	NM_001286958.1	AGGGAAGCTCTTCTTTGCTCC	CGGTGGATATGGTCCTTCTCT	234	N/D	N/D
	Felis c.	NM_001024231.1	same as Bos t.	CGATGGATATGGTCCTTCTCT	234	N/D	N/D
	Mus m.	NM_007956.5	GGGGAAGCTCCTGTTTGCTCC	same as Bos t.	234	N/D	N/D
ERbeta	Bos t.	NM_174051.3	GCTTCGTGGAGCTCAGCCTG	AGGATCATGGCCTTGACACAGA	262	87,5	N/D
	Canis f.	XM_005623489.2	GCTTTGTGGAGCTCAGCCTC	AGGACCATGGCCTTGACACAGA	262	N/D	N/D
	Felis c.	XM_006932907.2	GCTTTGTGGAGCTCAGCCTG	same as Canis f.	262	N/D	N/D
	Mus m.	NM_207707.1	same as Felis c.	AGAATCATGGCCTTCACACACA	316	85	N/D
PR	Bos t.	NM_001205356.1	GAGAGCTCATCAAGGCAATTGG	CACCATCCCTGCCAATATCTT	227	N/D	N/D
	Canis f.	NM_001003074.1	GAGAGCTCATCAAAGCAATTGG	CACCATCCCTGCCAAGATCTT	227	N/D	N/D
	Felis c.	XM_011286504.1	same as Bos t.	same as Canis f.	227	82,5	N/D
	Mus m.	NM_008829.2	GCGAATTGATCAAGGCAATTGG	CACCATGCCC GCCAGGATCTT	227	N/D	N/D
HPRT-1	Bos t.	NM_001034035.2	CACTGGGAAGACAATGCAGA	GGTCCTTTTCATCAGCAAGCT	92	81,5	99,4
	Canis f.	NM_001003357.2	CACTGGGAAAACAATGCAGA	ACAAAGTCAGGTTTATAGCCAACA	123	82,5	99,9
	Felis c.	XM_006944016.2	TGACACTGGCAAAACAATGCA	GGTCCTCTTCACCAGCAAGCT	94	82,5	97,7
	Mus m.	NM_013556.2	TGACACTGGtAAAACAATGCA	GGTCCTTTTCACCAGCAAGCT	94	8,5	101,3
GAPDH	Bos t.	NM_001034034.2	ACTTATGACCACTGTCCACGC	GATATTCTGGGCAGCCCCTC	100	87,5	100,8
	Canis f.	NM_001003142.2	TGAACGGATTTGGCCGTATTGG	TGAAGGGGTCATTGATGGCG	90	84,0	103,2
	Felis c.	NM_001009307.1	same as Canis f.	same as Canis f.	93	84,0	101,6
	Mus m.	NM_001289726.1	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG	87	84,0	95,5

Note: N/D = not determined due to too low abundance of target gene.