



HAL
open science

PGC-1 alpha induced browning promotes involution and inhibits lactation in mammary glands

Elena Piccinin, Annalisa Morgano, Claudia Peres, Annalisa Contursi, Justine Bertrand-Michel, Maria Arconzo, Hervé Guillou, Gaetano Villani, Antonio Moschetta

► To cite this version:

Elena Piccinin, Annalisa Morgano, Claudia Peres, Annalisa Contursi, Justine Bertrand-Michel, et al.. PGC-1 alpha induced browning promotes involution and inhibits lactation in mammary glands. Cellular and Molecular Life Sciences, 2019, 76 (24), pp.5011-5025. 10.1007/s00018-019-03160-y . hal-02622923

HAL Id: hal-02622923

<https://hal.inrae.fr/hal-02622923>

Submitted on 8 Dec 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**PGC-1 α induced browning promotes involution
and inhibits lactation in mammary glands**

Elena Piccinin^{1f}, Annalisa Morgano^{1f}, Claudia Peres^{1,2}, Annalisa Contursi³,

Justine Bertrand-Michel⁴, Maria Arconzo², Hervé Guillou⁵, Gaetano Villani⁶, Antonio Moschetta^{1,7*}

¹Department of Interdisciplinary Medicine, "Aldo Moro" University of Bari, 70124 Bari, Italy;

²INBB, National Institute for Biostuctures and Biosystems, 00136 Rome, Italy;

³Department of Neuroscience, Imaging and Clinical Sciences and Center for Research on Aging and Translational Medicine (CeSI-MeT), "G. d'Annunzio" University of Chieti, 66100 Chieti, Italy;

⁴Plateforme Lipidomique Inserm/UPS UMR 1048 – I2MC, Institut des Maladies Métaboliques et Cardiovasculaires, Toulouse, France;

⁵Toxalim (Research Centre in Food Toxicology), Université de Toulouse, INRA, ENVT, INP-Purpan, Université Paul Sabatier, Toulouse, France;

⁶Department of Basic Medical Sciences, Neurosciences and Sense Organs, "Aldo Moro" University of Bari, 70124 Bari, Italy;

⁷National Cancer Research Center, IRCCS Istituto Oncologico "Giovanni Paolo II", 70124 Bari, Italy.

Notes: f these authors contributed equally.

Correspondence: *Antonio Moschetta, MD PhD, Clinica Medica "Cesare Frugoni", Department of

Interdisciplinary Medicine, University of Bari "Aldo Moro", Piazza Giulio Cesare 11, 70124 Bari;

Phone: +39 0805593262; Fax: +39 0805555388; E-mail: antonio.moschetta@uniba.it

Abbreviations: PGC-1, peroxisome proliferator-activated receptor gamma coactivator 1; UCP1, uncoupling protein 1; TFAM, mitochondrial transcription factor A; mmtv, mouse mammary tumor virus.

Abstract

1
2 The PPAR γ coactivator 1 α (PGC-1 α) is a transcriptional regulator of mitochondrial biogenesis and
3
4 oxidative metabolism. Recent studies have highlighted a fundamental role of PGC-1 α in promoting
5
6 breast cancer progression and metastasis, but the physiological role of this coactivator in the
7
8 development of mammary glands is still unknown. First, we show that PGC-1 α is highly expressed
9
10 during puberty and involution, but nearly disappeared in pregnancy and lactation. Then, taking
11
12 advantage of a newly generated transgenic mouse model with a stable and specific overexpression
13
14 of PGC-1 α in mammary glands, we demonstrate that the re-expression of this coactivator during
15
16 the lactation stage leads to a precocious regression of the mammary glands. Thus, we propose that
17
18 PGC-1 α action is non-essential during pregnancy and lactation, whereas it is indispensable during
19
20 involution. The rapid preadipocyte-adipocyte transition, together with an increased rate of
21
22 apoptosis promotes a premature mammary glands involution that cause lactation defects and pup
23
24 growth retardation. Overall, we provide new insights in the comprehension of female reproductive
25
26 cycles and lactation deficiency, thus opening new roads for mothers that cannot breastfeed.
27
28
29
30
31
32
33
34
35
36
37
38

39 **Keywords:** Mammary glands, Nuclear Receptor, Coactivator, Development, Adipocytes, Involution.
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1. Introduction

1
2 Development of the mammary gland is initiated during embryogenesis, although it's not until the
3
4 onset of puberty that ductal elongation takes place and fat becomes filled with a system of epithelial
5
6 ducts. The mammary glands undergo substantial changes in morphology during reproduction, being
7
8 one of the most active metabolic tissues. In response to pregnancy, mammary epithelial cells
9
10 develop into mature alveoli in concert with de-differentiation of the adipocytes of the fat-pad [1].
11
12 Parturition induces copious milk production in response to diminished level of progesterone
13
14 resulting from placenta loss and increased secretion of prolactin by pituitary gland [2]. During
15
16 lactation, the mammary glands display a high synthesis of triglycerides, utilizing both fatty acids
17
18 obtained from bloodstream, as well as by *de novo* lipogenesis [3], thus providing sufficient energy
19
20 to fulfil the developmental demands of the offspring. In humans, the lactating mammary glands
21
22 secrete 800mL of milk per day containing almost 32g of fat [4]. The mammary glands of a lactating
23
24 mouse secrete 5mL of milk per day containing 30% of lipids, accounting for approximately 30g of
25
26 milk lipids over the course of 20 days, equivalent to the entire body weight of the mother [5].
27
28
29
30
31
32
33
34
35

36 Upon weaning, milk accumulation induces apoptosis of the secretory epithelial cells and
37
38 their shedding in the lumen. This first phase of involution is reversible and lactation can
39
40 recommence if suckling is resumed within 48 hours. If the gland remains unemptied for an extended
41
42 period of time, the mammary gland undergoes changes to regenerate the fat pad, characterized by
43
44 tissue remodelling and re-differentiation of adipocytes [1;6].
45
46
47
48

49 Until recently, the leading hypothesis of mammary gland development during pregnancy
50
51 involved transdifferentiating of subcutaneous white adipocytes into "pink adipocytes", the
52
53 mammary gland alveolar epithelial cells responsible for the milk production and secretion [7]. Upon
54
55 interruption of lactation, pink adipocytes transdifferentiate into brown adipocytes, establishing a
56
57 pregnancy-lactation adipocyte-to-epithelium-to-adipocyte circle [7;8]. Notably, this hypothesis has
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

been recently challenged by the new evidence showing that over multiple pregnancies, a cycle of adipocytes de-differentiation into preadipocyte and fibroblast-like cells during pregnancy and lactation occurred, and de-differentiated fibroblasts proliferated and re-differentiated into adipocytes upon weaning [9].

The peroxisome proliferator-activated receptor- γ coactivator 1- α (PGC-1 α) is a transcriptional coactivator, playing a central role in metabolism. Together with the other family members, PGC-1 β and PRC, PGC-1 α promotes mitochondrial biogenesis and respiration [10;11]. Typically expressed at low levels under normal conditions, PGC-1 α is highly expressed in tissues with high oxidative capacity, such as brown adipose tissue during thermogenesis [12], skeletal muscle in fiber type switching [13] and in liver during fasting to promote fatty acids β -oxidation and gluconeogenesis [14;15]. Despite recent progress in understanding PGC-1 α contribution to mammalian development [13;16-18], the role of this coactivator in mammary glands is still not well defined. Indeed, although different studies have pointed out a crucial role for PGC-1 α in promoting breast cancer tumor growth and metastasis [19-21], its physiological role in mammary glands development remains fairly unexplored.

In this study, we investigate the contribution of PGC-1 α coactivator in mammary gland development, taking advantage of a newly generated *in vivo* mouse model in which PGC-1 α is selectively and specifically overexpressed in mammary tissue. The constitutive PGC-1 α overexpression throughout all the stages of mammary glands evolution resulted in a precocious involution, characterized by re-differentiation into adipocytes and promotion of apoptosis, which ultimately mediated lactation and growth retardation defects.

2. Material and Methods

2.1 Animals

1
2 The mmtvPGC1 α transgenic mice were generated by injecting the mmtv-SV40-PGC1 α plasmid
3
4 digested with HpaI into the pronuclei of the fertilized eggs of the FVB/N mice. To generate the
5
6 mmtv-SV40-PGC1 α , first hPGC1 α (2.4 kb) fragment was generated by PCR from pcDNA4-His-PGC1 α
7
8 plasmid (Addgene, USA). Then the fragment was subcloned at the HindIII and EcoRI restriction sites
9
10 downstream from the mmtv promoter region of the mmtv-SV40-Bssk plasmid (Addgene, USA). Mice
11
12 carrying the transgene were identified by PCR of genomic DNA to confirm the presence of an
13
14 hPGC1 α coding sequence. Kidney, uterus, ovary, salivary glands, hypophysis and heart of transgenic
15
16 mice were dissected to evaluate the specific mammary glands expression of transgene under the
17
18 mmtv promoter control. For mammary gland analyses during pregnancy, lactation and involution,
19
20 8- to 16-wk-old nulliparous female mmtvPGC-1 α and littermates' mice were bred to wild-type
21
22 FVB/N male mice. For weight gaining-pups experiments, on day 1 from birth litter size was
23
24 normalized to seven pups. For foster mother experiments, two pairs of mmtvPGC1 α and control
25
26 wild-type female mice were bred with wild-type FVB/N male mice and delivered pups on the same
27
28 day were used. On P1, the litter size was normalized to seven pups. Five pups were switched
29
30 between the mmtvPGC1 α mother and the wild-type mother, and two pups were left with the
31
32 original mother as controls. The fostered pups and original pups were distinguished by ear punching.
33
34 For milk analysis, milk was isolated from mammary tissue at day 10 of lactation after cervical
35
36 dislocation of the mice. While pressure was applied to the tissue, the milk was harvested using a
37
38 Pasteur pipette put onto the nipple. Milk was then transferred into a microcentrifuge tube and
39
40 stored at -80°C .
41
42
43
44
45
46
47
48
49
50
51
52
53

54 All mice were housed with a standard diet provided *ad libitum* and examined daily.
55
56 Genotyping was performed using DNA extracted from tail biopsies of 4 weeks-old pups, and new
57
58 breeding harems of 8 weeks-old mice were established to expand the population. The ethics
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
committee of the University of Bari approved this experimental setup, which was also certified by the Italian Ministry of Health in accordance with internationally accepted guidelines for animal care.

2.2 Whole Mount and histological analysis

For whole mount analysis the first inguinal glands were dissected at the indicated ages and were spread on a glass slide. After fixation with Carnoy's fixative for 2-4 hours, the tissues were hydrated and stained in Carmine alum overnight as previously described [22]. Samples were then dehydrated, cleared with xylene and mounted.

For histology and immunohistochemistry, inguinal mammary glands were dissected and fixed in 10% (vol/vol) formalin for 24 h, dehydrated and paraffin embedded and then 5µm-thick sections were obtained. Standard histology (H&E staining) and immunohistochemical procedures were performed. Briefly, samples were treated with 3% (vol/vol) hydrogen peroxide for 5 min to quench endogenous peroxidase and were subjected to antigen retrieval by boiling the slides in an antigen-unmasking solution (Vector Laboratories, California, USA) for 20 min according to the manufacturer's instructions. Sections were sequentially incubated for 1 h at room temperature in 50% (vol/vol) non immune serum (from the host animal in which the secondary antibody was raised) in PBS (to avoid unspecific signal) and overnight at 4 °C with primary antibodies rabbit peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) (in-house antibody), uncoupling protein 1 (UCP1, ab10983, Abcam, Cambridge, UK) and cytochrome c oxidase 1 (COX1, LS-C343872, LifeSpan Biosciences, Washington, USA). Sections were washed for 10 min in PBS and incubated for 30 min at room temperature with the secondary biotinylated antibody (Vector Laboratories, California, USA). After three 5-min washing steps with PBS, sections were incubated with the avidin-biotin complex (Vector Laboratories, California, USA) for 30 min at room temperature. After washing in PBS, the peroxidase reaction was developed by incubation with 3,3-diaminobenzidine (Sigma-

1 Aldrich, Missouri, USA). Counterstaining was carried out with methylene blue (Sigma-Aldrich,
2 Missouri, USA). For negative controls, the primary antibodies were replaced by 1% non-immune
3 serum in PBS.
4
5

6
7 For immunofluorescence, 4µm-thick mammary gland sections were double stained with
8 primary antibody for Wheat Germ Agglutinin, Alexa Fluor 488 Conjugate (WGA, W11261, Thermo
9 Fisher Scientific, Massachusetts, USA) and Adipophilin, Alexa Fluor 594 conjugate (ADFP, ab206356,
10 Abcam, Cambridge, UK). Specifically, the slides were incubated with 10% goat serum for 1 hour at
11 room temperature. Slides were incubated overnight at 4°C with WGA 5µl/ml and ADFP 1:50 mix. All
12 sections were counterstained with TO-PRO-3 (Thermo Fisher Scientific, Massachusetts, USA).
13 Negative controls were prepared with irrelevant antibody. The sections were analysed using the
14 Leica TCS SP2 (Leica, Wetzlar, Germany) confocal laser-scanning microscope.
15
16
17
18
19
20
21
22
23
24
25
26
27

28 Image processing was performed using ImageScope software (Leica, Wetzlar, Germany). For
29 each sample, 15 representative images were taken and the percentage of stained area/total area
30 was measured. Values from all consecutive images for each sample were averaged and displayed as
31 mean±SEM.
32
33
34
35
36
37
38
39
40

41 *2.3 Apoptotic Assay*

42 For the TUNEL assay in vivo, tissue specimens were fixed in 10% formalin for 12–24 h, dehydrated,
43 and paraffin embedded. Detection of apoptosis at the single-cell level based on the labelling of DNA
44 strand breaks was performed using the In-Situ Cell Detection kit (Roche, Basel, Switzerland)
45 following the manufacturer's instructions.
46
47
48
49
50
51
52
53
54
55
56

57 *2.4 RNA Extraction and Quantitative Real-Time PCR Analysis*

1 Inguinal mammary glands tissues were snap frozen in liquid nitrogen and stored at -80°C . Total RNA
2 was isolated by RNeasy lipid tissue kit (QIAGEN, Hilden, Germany) following the manufacturer's
3 instructions. To avoid possible DNA contamination, RNA was treated with DNase (Thermo Fisher
4 Scientific, Massachusetts, USA). RNA purity also was checked by spectrophotometer, and RNA
5 integrity was checked by examination on agarose gel electrophoresis. For RT-qPCR analysis of
6 pregnancy and lactation stages, cDNA was synthesized by retro-transcribing $1\mu\text{g}$ of total RNA in a
7 total volume of $100\mu\text{L}$ utilizing the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher
8 Scientific, Massachusetts, USA) according to the manufacturer's instructions. PCR assays were
9 performed in 96-well optical reaction plates using a Quantum5 machine (Thermo Fisher Scientific,
10 Massachusetts, USA). PCR assays were conducted in triplicate wells for each sample. The following
11 reaction mixture was used in each well: $10\mu\text{L}$ Power SYBR Green (Thermo Fisher Scientific,
12 Massachusetts, USA), $2.4\mu\text{L}$ of primers at a final concentration of 150nM each, $1.6\mu\text{L}$ RNase-free
13 water, and $3\mu\text{L}$ cDNA. The following PCR conditions were used: denaturation at 95°C for 10 min,
14 followed by 40 cycles at 95°C for 15 s and then at 60°C for 60 s. For RT-qPCR analysis of different
15 developmental stages, cDNA was synthesized by utilizing the High-Capacity RNA to cDNA Kit
16 (Thermo Fisher Scientific, Massachusetts, USA) according to the manufacturer's instructions. PCR
17 assays were performed in triplicate wells for each sample, in 96-well optical reaction plates using a
18 Quantum5 machine (Thermo Fisher Scientific, Massachusetts, USA). PCR assays were conducted
19 utilizing TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix with UNG following
20 the manufacturer's instructions. The following PCR conditions were used: incubation at 50°C for 2
21 min, denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, and then at 60°C for 60
22 s. For all the experiments, quantitative normalization of cDNA in each sample was performed using
23 TBP mRNA as an internal control. Relative quantification was done using the $\Delta\Delta\text{CT}$ method
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

2.5 Gel Electrophoresis

1
2 For immunoblot analysis, tissue samples were homogenized in RIPA buffer supplemented with
3
4 protease and phosphatase inhibitors. Equal amount of protein lysates (50µg), quantified using
5
6 Bradford-based assay (BioRad Laboratories, Hercules, CA), were denaturated with Laemmli sample
7
8 buffer, separated by 10% or 12.5% SDS-PAGE and then transferred onto a nitrocellulose membrane
9
10 (Protran, Whatman). Membranes were blocked with 5% bovine serum albumin in Tris-buffered
11
12 saline/0.01% Tween 20 and probed with specific antibodies against PGC-1α (in-house antibody) or
13
14 UCP1 (ab10983, Abcam, Cambridge, UK). Nuclear encoded β-actin (ab82229, Abcam, Cambridge, UK)
15
16 were used as loading control where possible. Membranes were finally incubated with horseradish
17
18 peroxidase–conjugated secondary antibodies and developed with a chemiluminescent reagent (Bio-
19
20 Rad Laboratories, Hercules, CA).
21
22

23
24 For milk quality analyses, equivalent amounts of protein from milk collected on day 10 of lactation
25
26 from each mouse genotype were analysed by 12% SDS-PAGE. Post electrophoresis, the gel was
27
28 fixed, stained with Coomassie Brilliant Blue, destained and kept in preservation solution.
29
30
31
32
33
34
35
36
37

2.6 Fatty acids analysis

38
39 Fatty acids were analysed as fatty acid methyl esters (FAMES). The milk was transmethylated with
40
41 1 mL of BF3 in methanol (1:20, v/v) for 60 min at 80 °C, evaporated to dryness, and the FAMES
42
43 extracted with hexane/water (3:1). The organic phase was evaporated to dryness and dissolved in
44
45 50 µL ethyl acetate. One microliter of FAMES was analysed by gas-liquid chromatography on a 5890
46
47 Hewlett-Packard system (Hewlett-Packard, Palo Alto, CA) using a Famewax fused-silica capillary
48
49 column (30 m, 0.32 mm internal diameter, 0.25 mm film thickness; Restek, Belfast, UK). The oven
50
51 temperature was programmed from 110 to 220 °C at a rate of 2 °C/min, with hydrogen as the carrier
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 gas (0.5 bar). The injector and detector were at 225 and 245 °C, respectively. Heptadecanoic acid
2 (Sigma) was used as an external standard for fatty acid methyl esters.
3
4
5
6

7 *2.7 Statistical analysis*

8
9
10 All results are expressed as mean \pm SEM. Data distribution and gene expression statistical analysis
11 were performed with GraphPad Prism software (v5.0; GraphPad Software Inc., La Jolla, CA).
12
13 Comparisons of two groups were performed using Mann-Whitney U test. Comparison of four groups
14
15 were performed using Kruskal-Wallis test. A value of $P < 0.05$ was considered as statistically
16
17 significant.
18
19
20
21
22
23
24

25 **3. Results**

26 *3.1 PGC-1 α is highly expressed in mammary gland during puberty and involution.*

27
28 As the expression and localization of coactivator PGC-1 α in the mammary gland is still unknown, we
29 first investigated the expression of endogenous PGC-1 α throughout the stages of mammary glands
30
31 development. Immunohistochemistry analysis on wild-type female mice revealed that PGC-1 α is
32
33 expressed in normal mammary epithelium, being upregulated during puberty and within the first
34
35 12 days of pregnancy. By contrast, PGC-1 α expression starts to decrease by the 18th day of
36
37 pregnancy and is wholly absent during lactation (Figure 1A).
38
39
40
41
42
43
44
45

46 At weaning, involution of mammary gland occurs. This process takes place just after suckling
47 interruption and it is associated with programmed cell death of secretory epithelium cells,
48
49 redevelopment of mammary adipose tissue together with a remodelling of the lobular-alveolar
50
51 structure [23]. Histological analysis on mammary gland on the first days after weaning displayed
52
53 ductal structure surrounded by adipocytes. Moreover, as involution proceeded, we observed an
54
55 increase in PGC-1 α expression, which becomes extremely evident on the 3rd day (Figure 1B). The
56
57
58
59
60
61
62
63
64
65

1 involuted mammary glands displayed PGC-1 α expression similar to that of virgin mammary glands
2 (i.e. puberty), with prominent nuclear PGC-1 α staining, surrounded by increasing accumulation of
3 adipocytes. Concomitantly to the PGC-1 α gain expression, TUNEL analysis showed a high apoptosis
4 rate on day 3, thus suggesting that the high levels of PGC-1 α actively correlates with the induction
5 of apoptosis (Figure 1B).
6
7
8
9
10
11

12 Intriguingly, the re-expression of PGC-1 α during the involution stage is associated with
13 browning markers induction. In particular, the expression of Ucp1 and PR domain containing 16
14 (Prdm16) nearly disappeared during pregnancy and lactation, but they are promptly induced during
15 involution (Figures 1A-C). Differently, cell death-inducing DNA fragmentation factor alpha-like
16 effector A (Cidea) levels are low during puberty and pregnancy, but importantly increased
17 throughout the lactation stage. At involution, Cidea expression is turned down, but still significantly
18 induced compare to puberty and pregnancy (Figure 2C). Indeed, beside its expression in brown and
19 beige adipocytes in mouse, Cidea is also an essential transcriptional coactivator capable of
20 regulating mammary gland secretion of milk lipids [24-26].
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35

36 The dynamic changes observed in PGC-1 α expression during the mammary gland
37 development suggest that this coactivator is dispensable during lactation, but its induction is crucial
38 during involution.
39
40
41
42
43
44
45

46 *3.2 Maternal PGC-1 α overexpression in mammary glands results in growth retardation defects*

47 To dissect the role of PGC-1 α in mammary glands development, we generated a transgenic mouse
48 model with mammary specific gain of function of PGC-1 α (mmtvPGC-1 α). To this end, the sequence
49 coding for human PGC-1 α was subcloned to the mmtv promoter, thereby allowing a tissue-specific
50 expression of hPGC-1 α in the mammary gland epithelium (Figure 3A). Successful overexpression
51 was confirmed by mRNA and protein analysis of 8 weeks-old virgin mice mammary glands (Figure
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

3B-3C). The exclusive overexpression of hPGC-1 α in mammary glands was subsequently confirmed by comparison with other tissues examined by real time qPCR (Supplementary Figure 1A).

Despite similar body weight between wild type and mmtvPGC-1 α mice (Figure 3D), mammary glands harvested from 8 weeks-old transgenic virgin mice were significantly smaller compared to wild type ones as indicated by inguinal mammary glands weight to body weight ratio (MGW/BW) (Figure 3E), thus suggesting that high levels of PGC-1 α may disrupt a proper mammary gland development.

Examination of wild type and mmtvPGC-1 α mammary glands from 8 weeks-old virgin mice by paraffin section (Figure 4A) and whole mount (Figure 4B) revealed a difference in terminal end bud formation, ductal growth and ductal branching. Indeed, transgenic-derived mammary glands showed thinner ductal branches, lower alveolar bud-like structures and a less branched ductal network than WT mice (Figure 4B).

Then, we crossed mmtvPGC-1 α female with male of same genotype and we did the same for WT mice. We monitored pups' weight from birth until the time of weaning (21 days). On the 2nd day, the litter size was normalized to seven pups. Daily measurements of pup body weight from female mmtv-PGC-1 α revealed growth retardation (Figure 4C-4E, red closed square) and, in rare cases (5%), hair cycling defects and gout, compared to WT littermates (blue closed circle). At the time of weaning (postnatal day 21), these pups exhibited low dimensions and body weights (Supplementary Figure 1B). Interestingly, weight gain resumed immediately when the pups were placed on a standard chow diet (data not shown). Afterwards, pups maintained a normal phenotype that was completely dependent on maternal genotype (mmtv-PGC-1 α).

To further confirm that PGC-1 α expression in lactating mammary glands are indeed responsible for the observed pups' phenotype, we subjected the mice to a cross-fostering experiment. Fostering is the movement of pups from the birth (donor) dam to a recipient (foster)

1 dam. We fostered pups within 48 hours of birth to recipient dams that had delivered age matched
2 litters. We completely replaced the recipient dam's litter with fostered neonates, in order to
3 maintain a controlled number of mice (7 mice per litter) and to fully characterize the mutant
4 newborns. As expected, mmtv-PGC-1 α pups nursed by WT female completely lost their phenotype
5 (Figure 4D-4E, red open circle) and did not show any growth retardation. On postnatal day 19, WT-
6 fostered mmtv-PGC-1 α pups reached a weight gain similar to the one observed for WT pups nursed
7 by WT mother (8.4g \pm 0.13 versus 8.2g \pm 0.38) (Figure 4D-4E). On the other side, WT pups nursed by
8 mmtv-PGC-1 α dam (blue open square) showed a considerable delay on body growth, that became
9 less consistent around weaning time when switched from maternal milk to cage food. notably, on
10 postnatal day 19th, WT-fostered mmtv-PGC-1 α pups weighted over one gram more than the WT
11 pups nursed by mmtv-PGC-1 α foster mother (8.4g \pm 0.13 versus 7.2g \pm 0.16) (Figure 4D-4E –
12 Supplementary Figure 1C).

13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31 Collectively, these data suggest that PGC-1 α is physiologically turned off during lactation in
32 order to totally fulfil the metabolic needs for offspring development and its re-expression during
33 lactation – as we observed with our transgenic model – may interfere o delay a complete
34 development of newborns. However, as the transgenic phenotype was rescued at weaning and
35 during cross-fostering experiments, it is not improbable to hypothesize that nutritional defects due
36 to milk quality or quantity from mmtvPGC-1 α mothers may mediate the growth retardation
37 observed in offspring.

3.3 PGC-1 α overexpression during lactation does not affect milk quality

38
39
40
41
42
43
44
45
46
47
48
49
50
51
52 To explore whether pup's growth defects could be ascribable to the ectopic PGC-1 α overexpression,
53
54
55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
70
71
72
73
74
75
76
77
78
79
80
81
82
83
84
85
86
87
88
89
90
91
92
93
94
95
96
97
98
99
100
101
102
103
104
105
106
107
108
109
110
111
112
113
114
115
116
117
118
119
120
121
122
123
124
125
126
127
128
129
130
131
132
133
134
135
136
137
138
139
140
141
142
143
144
145
146
147
148
149
150
151
152
153
154
155
156
157
158
159
160
161
162
163
164
165
166
167
168
169
170
171
172
173
174
175
176
177
178
179
180
181
182
183
184
185
186
187
188
189
190
191
192
193
194
195
196
197
198
199
200
201
202
203
204
205
206
207
208
209
210
211
212
213
214
215
216
217
218
219
220
221
222
223
224
225
226
227
228
229
230
231
232
233
234
235
236
237
238
239
240
241
242
243
244
245
246
247
248
249
250
251
252
253
254
255
256
257
258
259
260
261
262
263
264
265
266
267
268
269
270
271
272
273
274
275
276
277
278
279
280
281
282
283
284
285
286
287
288
289
290
291
292
293
294
295
296
297
298
299
300
301
302
303
304
305
306
307
308
309
310
311
312
313
314
315
316
317
318
319
320
321
322
323
324
325
326
327
328
329
330
331
332
333
334
335
336
337
338
339
340
341
342
343
344
345
346
347
348
349
350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374
375
376
377
378
379
380
381
382
383
384
385
386
387
388
389
390
391
392
393
394
395
396
397
398
399
400
401
402
403
404
405
406
407
408
409
410
411
412
413
414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447
448
449
450
451
452
453
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478
479
480
481
482
483
484
485
486
487
488
489
490
491
492
493
494
495
496
497
498
499
500
501
502
503
504
505
506
507
508
509
510
511
512
513
514
515
516
517
518
519
520
521
522
523
524
525
526
527
528
529
530
531
532
533
534
535
536
537
538
539
540
541
542
543
544
545
546
547
548
549
550
551
552
553
554
555
556
557
558
559
560
561
562
563
564
565
566
567
568
569
570
571
572
573
574
575
576
577
578
579
580
581
582
583
584
585
586
587
588
589
590
591
592
593
594
595
596
597
598
599
600
601
602
603
604
605
606
607
608
609
610
611
612
613
614
615
616
617
618
619
620
621
622
623
624
625
626
627
628
629
630
631
632
633
634
635
636
637
638
639
640
641
642
643
644
645
646
647
648
649
650
651
652
653
654
655
656
657
658
659
660
661
662
663
664
665
666
667
668
669
670
671
672
673
674
675
676
677
678
679
680
681
682
683
684
685
686
687
688
689
690
691
692
693
694
695
696
697
698
699
700
701
702
703
704
705
706
707
708
709
710
711
712
713
714
715
716
717
718
719
720
721
722
723
724
725
726
727
728
729
730
731
732
733
734
735
736
737
738
739
740
741
742
743
744
745
746
747
748
749
750
751
752
753
754
755
756
757
758
759
760
761
762
763
764
765
766
767
768
769
770
771
772
773
774
775
776
777
778
779
780
781
782
783
784
785
786
787
788
789
790
791
792
793
794
795
796
797
798
799
800
801
802
803
804
805
806
807
808
809
810
811
812
813
814
815
816
817
818
819
820
821
822
823
824
825
826
827
828
829
830
831
832
833
834
835
836
837
838
839
840
841
842
843
844
845
846
847
848
849
850
851
852
853
854
855
856
857
858
859
860
861
862
863
864
865
866
867
868
869
870
871
872
873
874
875
876
877
878
879
880
881
882
883
884
885
886
887
888
889
890
891
892
893
894
895
896
897
898
899
900
901
902
903
904
905
906
907
908
909
910
911
912
913
914
915
916
917
918
919
920
921
922
923
924
925
926
927
928
929
930
931
932
933
934
935
936
937
938
939
940
941
942
943
944
945
946
947
948
949
950
951
952
953
954
955
956
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973
974
975
976
977
978
979
980
981
982
983
984
985
986
987
988
989
990
991
992
993
994
995
996
997
998
999
1000

1 overexpression of ectopic hPGC-1 α remained unchanged during lactation. Moreover, mmtvPGC-1 α
2 displayed a net increase in COX-1 staining compared to WT mice, indicating that the overexpression
3 of PGC-1 α is closely related with the activation of mitochondria metabolism (Figure 5C). In line with
4 this data, mice overexpressing PGC-1 α in the mammary glands exhibited the induction of
5 mitochondrial transcription factor A (Tfam) and phosphoenolpyruvate carboxykinase (Pepck), two
6 PGC-1 α target genes involved in mitochondrial biogenesis and gluconeogenesis, respectively (Figure
7 5D). To further determine the consequences of PGC-1 α expression during lactation stage, we
8 quantified the milk composition to find out if any anomalies occurred. The proteins (Figure 5E) and
9 fatty acids (Figure 5F) profiles were comparable in the milk harvested from both wild type and
10 mmtvPGC-1 α mice. Overall, these data indicate that the stable PGC-1 α expression during lactation
11 stage is able to boost mitochondria respiration without overt effects on milk quality.
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30

3.4 Mammary PGC-1 α overexpression during lactation leads to a rapid mammary glands involution

31 Since we did not observe any substantial differences between WT and PGC-1 α mice regarding milk
32 composition, we wondered if the ectopic expression of PGC-1 α could have any consequences on
33 milk quantity. At 10 days of lactation, mammary glands of transgenic mice displayed smaller
34 cytoplasmic lipids droplets and, consequently, reduced lumina, as compared with WT (Figure 6A).
35 However, the analysis of lipid droplets accumulation using a specific antibody against adipophilin
36 (ADPH), a protein found in cytoplasmic and secreted-milk lipid droplets coat [27;28], did not reveal
37 eminent differences between the two groups (Figure 6A-6B). Surprisingly, H&E staining of mammary
38 glands sections revealed the presence of small cells, containing multilocular cytoplasmic lipid
39 droplets, resembling brown adipocytes (Figure 6A). To confirm our hypothesis, we tested mammary
40 glands immunoreactivity for uncoupling protein 1 (UCP1), a mitochondrial thermogenic protein
41 uniquely expressed in brown adipose tissue [29] and we found marked areas of UCP1 staining in
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1 mmtvPGC-1 α mammary glands compared to wild type (Figure 6A-6B). At the same time, gene
2 expression analysis revealed a trend toward induction of fat-browning related genes, such as Ucp1,
3
4 Cidea and Prdm16 (Figure 6C). Interestingly, we observed high concentration of UCP1 also in
5
6 alveolar epithelial cells, thus pointing out that the expression of PGC-1 α during lactation promotes
7
8 an early changing in mammary gland architecture, becoming repopulated with adipocytes that
9
10 rapidly differentiate into brown ones. Brown adipose cells are usually densely packed with small
11
12 lipid droplets together and abundant mitochondria. Indeed, we observed that mmtvPGC-1 α
13
14 mammary glands displayed a higher induction of mitochondrial metabolism compared to wild type
15
16 ones (Figure 5C-5D). Since fatty acid β -oxidation is critically required for the thermogenic function
17
18 of BAT, to further confirm our results, we examined the expression of genes involved in fatty acid
19
20 β -oxidation (Peroxisome proliferator-activated receptor α , Pyruvate dehydrogenase kinase 4, and
21
22 Carnitine Palmitoyltransferase 1B) and we found a trend towards increase of those genes in
23
24 mammary gland harvested from transgenic mice compared to wild type ones (Figure 6D).
25
26
27
28
29
30
31
32

33 To understand whether the overexpression of PGC-1 α is involved in the modulation of signals
34
35 mediating the regression of mammary glands development, we determined the ratio between
36
37 inguinal mammary gland and total mice weight on day 10 of lactation, revealing a marked decrease
38
39 in mmtvPGC-1 α mice compared to WT mice (Figure 6E). Concurrently, analysis on paraffin section
40
41 and whole mount confirmed that the overexpression of PGC-1 α in mammary glands was highly
42
43 correlated with a hypoplastic phenotype and reduced alveolar structures (Figure 6A-6F). Moreover,
44
45 we observed a marked increment of apoptosis rate, as indicated by TUNEL assay and the shedding
46
47 of epithelial cells into the alveolar lumen (Figure 6A).
48
49
50
51
52
53

54 Taken together, our results depict a unique scenario in which high levels of PGC-1 α during
55
56 lactation prevent mammary glands development and maturation by both inducing an early
57
58 apoptosis and promoting a rapid modification of mammary gland architecture, characterized by
59
60
61
62
63
64
65

1 differentiation into brown adipocytes. Taken together, all these transformations collectively
2 accelerate the involution of tissue, and finally lead to a decrease of milk production, hence affecting
3
4 normal pups' growth and development.
5
6
7
8
9

10 **4. Discussion**

11
12 The fundamental function of the mammary gland is to provide nourishment to newborns. Milk
13 represents the food for young mammals, being one essential constituents of mammalian life.
14
15 Human breastfeeding conveys established benefits for both maternal and child health, as maternal
16 milk supplies calories from lipids and essential fatty acids together with signalling molecules.
17
18 However, one out of two mothers stop breastfeeding before the recommended timespan, mostly
19 due to the perception that milk quantity is insufficient to address the need of infants [30;31].
20
21
22
23
24
25
26

27
28 In our *in vivo* study, we used integrated approaches of molecular genetics, biochemistry and
29 metabolomic to demonstrate that PGC-1 α is dispensable during pregnancy and lactation, but
30 specifically required during involution, where the coactivator causes regression and apoptosis of
31 mammary epithelial cells. The timing of the observed phenotype is clearly related to the temporal
32 expression of PGC-1 α , as highlighted by immunohistochemical analysis (Figure 1A).
33
34
35
36
37
38
39
40

41 By using a transgenic mouse model, we showed that the stable and specific expression of
42 PGC-1 α in mammary gland is associated with lactation defects and retarded pups' growth. Indeed,
43 the specific overexpression of PGC-1 α resulted in an altered mammary gland morphogenesis, as
44 well as a precocious mammary gland regression. Surprisingly, this regression occurred during the
45 lactation stage, through the development of abnormal alveolar structures that contributed to
46 lactation deficiency. In line with our experiments, it has been reported that several transgenic and
47 knockout mouse models exhibit lactation defects as a result of insufficient development of the
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

alveolar structures during pregnancy [32-34] and it would be interesting if those genes could be related to the PGC-1 α coactivator activity.

Normally, mammary gland involution is a two-step process which encompasses the apoptosis of secretory epithelial cells followed by re-differentiation of the adipocytes [6;9]. In the present study, we show that PGC-1 α is physiologically involved in the promotion of apoptosis during involution, and its expression throughout the lactation stage contributes to the regression of mammary glands, thus limiting the availability of milk.

Recently, it has been observed that mammary adipocytes fully de-differentiate into preadipocytes during lactation and promptly re-differentiate during involution [7;9]. Interestingly, several lines of evidence indicate that white adipocytes may transdifferentiate into brown adipocytes (a process known as “browning” or “brightening” of WAT), characterized by UCP1 expression coupled with a multilocular lipid droplets morphology [35;36]. Whether this process occurs as a transdifferentiation of pre-existing white adipocytes or by *de novo* adipogenesis from a subgroup of precursor cells is still unknown [35;37;38]. Our study revealed that re-expression of PGC-1 α during lactation promotes an early adipocytes transdifferentiation of mammary glands, consequently affecting milk production. Indeed, mmtvPGC-1 α lactating mammary gland display large area of resembling brown adipocytes, with increased mitochondrial UCP1 expression associated with the reduction of cytoplasmic lipid droplets. It is not unfair to hypothesize that the uncoupling of the oxidative phosphorylation would release the feedback control exerted by the mitochondrial membrane potential on the respiratory NADH oxidation, thus allowing the cells to reach maximum capacity of fatty acids β -oxidation. The indication of that the re-expression of PGC-1 α during lactation is sufficient to induce UCP1 expression in the mammary gland is consistent with the described regulation of the brown fat in the interscapular fat pad, as well as the induction of brown-fat-like regions within white adipose depots [39;40]. Interestingly, it has been recently

1 described that during mammary gland involution some milk secreting epithelial cells in the anterior
2 subcutaneous depot (pink adipocytes) may transdifferentiate to brown adipocytes [8;41]. Although
3 this data may contradict the “cycle of adipocyte-pre-adipocyte transition” postulated by Wang and
4 colleagues [9], our results depict the possibility that re-expression of PGC-1 α during mammary gland
5 involution may contribute to browning of de-differentiated mammary adipocytes.
6
7
8
9
10
11

12 Intriguingly, several studies have indicated that brown adipocytes are a component of the
13 mammary fatty stroma during postnatal development or in virgin mice exposed to cold, and that
14 PGC-1 α and genes involved in fatty acids β -oxidation are coordinately upregulated in this tissue
15 [42;43]. Moreover, multilocular brown adipocytes have been detected in the adult mammary gland
16 of Brca1 mutant mice, which develop high grade undifferentiated adenocarcinoma in a similar way
17 to human BRCA-1 mutated breast cancer [44]. Noteworthy, a high expression of PGC-1 α in cell
18 culture and in tissue specimens isolated from established human breast cancer correlates with low
19 survival and metastasis [45-47]. It would therefore, be intriguing to explore whether the expression
20 of this coactivator is essential to promote tumor onset and progression. Recent observations
21 regarding the increased risk of breast cancer in women who do not breastfeed [48-50] highlight
22 once more the importance of PGC-1 α in the pathophysiology of mammary glands, suggesting that
23 turning off the expression of this coactivator during lactation could exert protective actions against
24 cancer development.
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44

45 All together, these data infer that PGC-1 α is indeed necessary in the post-lactation stage, as
46 it is involved in both steps of mammary glands involution by promoting apoptosis and remodelling
47 of mammary gland architecture. Therefore, its expression is physiologically turned off during late
48 pregnancy and lactation in order to allow the full development of secretory epithelial cells and milk
49 production. The overall effects exerted by enhanced PGC-1 α expression, including the precocious
50 regression of the glands suggest a possible role of PGC-1 α in mastitis and, consequently, milk loss.
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Premature mammary glands involution promoted by PGC-1 α could be mediated through NF- κ B, a transcription factor that has been involved in mastitis, where it sustains the rapid loss of milk and secretory structures [51]. However, due to its fundamental role in host defence, the therapeutic intervention to block NF- κ B is not recommended. Alternatively, a strategy aimed to inhibit PGC-1 α could be designed to provide beneficial effects for nursing mothers.

The limitation of the present study is the lack of a loss of function model. However, it is rather possible that the effects of specific PGC-1 α ablation from the mammary glands would not be easy to identify, since different coping mechanisms may occur in order to counteract the loss of this coactivator. PGC-1 β expression could represent one of the possible compensatory strategies. Indeed, PGC-1 β and PGC-1 α exert similar functions in many tissues, and are both considered as master regulators of oxidative metabolism due to their capacity to promote mitochondrial biogenesis [52]. In mammary glands, both coactivators are significantly downregulated during pregnancy and lactation, and are target of nutri-regulated miRNA [53;54]. At the same time, oestrogens are able to induce only PGC-1 β expression in the breast, thereby supporting mitochondrial biogenesis and oxidative phosphorylation in this tissue [55]. On the other hand, at a variance of PGC-1 α that do not affect milk composition, PGC-1 β modulation interferes with lipogenic process, thus altering the quantity and the quality of fatty acids incorporated into milk. Precisely, the negative regulation exerted by miR-25 on PGC-1 β activity results in a reduced triglyceride synthesis and lipid droplets accumulation [56]. Moreover, fat-dietary type and physiological stimuli, such as the increased amount of polyunsaturated fatty acids (PUFAs) in lactating mammary glands, diminish PGC-1 β expression and promotion of lipogenesis in mammary glands [57]. Thereby, it is plausible that a compensatory adaptive increase of PGC-1 β under suitable stimuli would result in aberrant milk production which may impact in normal growth and development of the newborns.

1 Overall, taking advantage of this newly generated transgenic mouse model, we depicted a
2 direct involvement of PGC-1 α in the physiological homeostasis of mammary glands, where it actively
3 promotes involution. Our model provides a hypothetical underlying mechanism controlling the
4 process of adipocyte de-differentiation and re-differentiation during a female reproductive cycle.
5 However, high coactivator levels during lactation lead to a precocious re-differentiation into
6 adipocytes together with increased apoptosis rates consequently hasten tissue involution, thus
7 preventing a complete development of mammary glands. Our results provide new insights in the
8 comprehension of lactation deficiency, opening new possibilities for mothers that, for several
9 reasons, have to face an early stop in breastfeeding and offering future perspective for prevention
10 of breast cancer onset.
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27

28 *Acknowledgments:* We thank L. Salvatore and G. Di Tullio, for their invaluable help during the study,
29 and J-M. Lobaccaro and J. Hardfeldth, for their help with the manuscript. A. Moschetta is funded by
30 Italian Association for Cancer Research (AIRC, IG 18987), NR-NET FP7 Marie Curie People ITN and
31 EU-JPI FATMAL 2017.
32
33
34
35
36
37
38
39
40

41 *Author contributions:* E.P. contributed to study design, performed experiments, analysed data and
42 wrote the paper; A.Mor. contributed to study design, performed experiments and data analysis;
43 C.P., A.C., J.B.M, M.A. and H.G. performed experiments; G.V. contributed to paper writing; A.Mos.
44 designed the study, supervised the project and paper writing.
45
46
47
48
49
50
51
52
53

54 *Competing Financial Interest:* The authors declare no competing financial interests.
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Ethics Statement: The Ethical Committee of the Consorzio Mario Negri Sud and the University of Bari approved this experimental set-up, which also was certified by the Italian Ministry of Health in accordance with internationally accepted guidelines for animal care.

References

- [1] Watson CJ & Kreuzaler PA (2011) Remodeling mechanisms of the mammary gland during involution. *Int. J. Dev. Biol.*, **55**, 757-762.
- [2] Neville MC (1999) Physiology of lactation. *Clin. Perinatol.*, **26**, 251-79, v.
- [3] Neville MC & Picciano MF (1997) Regulation of milk lipid secretion and composition. *Annu. Rev. Nutr.*, **17**, 159-183.
- [4] Allen JC, Keller RP, Archer P, & Neville MC (1991) Studies in human lactation: milk composition and daily secretion rates of macronutrients in the first year of lactation. *Am. J. Clin. Nutr.*, **54**, 69-80.
- [5] Schwertfeger KL, McManaman JL, Palmer CA, Neville MC, & Anderson SM (2003) Expression of constitutively activated Akt in the mammary gland leads to excess lipid synthesis during pregnancy and lactation. *J. Lipid Res.*, **44**, 1100-1112.
- [6] Watson CJ (2006) Involution: apoptosis and tissue remodelling that convert the mammary gland from milk factory to a quiescent organ. *Breast Cancer Res.*, **8**, 203.
- [7] Morroni M, Giordano A, Zingaretti MC, Boiani R, De MR, Kahn BB, Nisoli E, Tonello C, Pisoschi C, Luchetti MM, Marelli M, & Cinti S (2004) Reversible transdifferentiation of secretory epithelial cells into adipocytes in the mammary gland. *Proc. Natl. Acad. Sci. U. S. A*, **101**, 16801-16806.
- [8] Giordano A, Perugini J, Kristensen DM, Sartini L, Frontini A, Kajimura S, Kristiansen K, & Cinti S (2017) Mammary alveolar epithelial cells convert to brown adipocytes in post-lactating mice. *J. Cell Physiol*, **232**, 2923-2928.
- [9] Wang QA, Song A, Chen W, Schwalie PC, Zhang F, Vishvanath L, Jiang L, Ye R, Shao M, Tao C, Gupta RK, Deplancke B, & Scherer PE (2018) Reversible De-differentiation of Mature White Adipocytes into Preadipocyte-like Precursors during Lactation. *Cell Metab*, **28**, 282-288.
- [10] Puigserver P & Spiegelman BM (2003) Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (PGC-1 alpha): transcriptional coactivator and metabolic regulator. *Endocr. Rev.*, **24**, 78-90.
- [11] Scarpulla RC (2002) Nuclear activators and coactivators in mammalian mitochondrial biogenesis. *Biochim. Biophys. Acta*, **1576**, 1-14.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [12] Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, & Spiegelman BM (1999) Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. *Cell*, **98**, 115-124.
 - [13] Lin J, Wu H, Tarr PT, Zhang CY, Wu Z, Boss O, Michael LF, Puigserver P, Isotani E, Olson EN, Lowell BB, Bassel-Duby R, & Spiegelman BM (2002) Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. *Nature*, **418**, 797-801.
 - [14] Yoon JC, Puigserver P, Chen G, Donovan J, Wu Z, Rhee J, Adelmant G, Stafford J, Kahn CR, Granner DK, Newgard CB, & Spiegelman BM (2001) Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature*, **413**, 131-138.
 - [15] Puigserver P, Rhee J, Donovan J, Walkey CJ, Yoon JC, Oriente F, Kitamura Y, Altomonte J, Dong H, Accili D, & Spiegelman BM (2003) Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature*, **423**, 550-555.
 - [16] Handschin C, Chin S, Li P, Liu F, Maratos-Flier E, Lebrasseur NK, Yan Z, & Spiegelman BM (2007) Skeletal muscle fiber-type switching, exercise intolerance, and myopathy in PGC-1alpha muscle-specific knock-out animals. *J. Biol. Chem.*, **282**, 30014-30021.
 - [17] Herzig S, Long F, Jhala US, Hedrick S, Quinn R, Bauer A, Rudolph D, Schutz G, Yoon C, Puigserver P, Spiegelman B, & Montminy M (2001) CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature*, **413**, 179-183.
 - [18] Puigserver P, Wu Z, Park CW, Graves R, Wright M, & Spiegelman BM (1998) A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell*, **92**, 829-839.
 - [19] McGuirk S, Gravel SP, Deblois G, Papadopoli DJ, Faubert B, Wegner A, Hiller K, Avizonis D, Akavia UD, Jones RG, Giguere V, & St-Pierre J (2013) PGC-1alpha supports glutamine metabolism in breast cancer. *Cancer Metab*, **1**, 22.
 - [20] Audet-Walsh E, Papadopoli DJ, Gravel SP, Yee T, Bridon G, Caron M, Bourque G, Giguere V, & St-Pierre J (2016) The PGC-1alpha/ERRalpha Axis Represses One-Carbon Metabolism and Promotes Sensitivity to Anti-folate Therapy in Breast Cancer. *Cell Rep.*, **14**, 920-931.
 - [21] LeBleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, de Carvalho FM, Damascena A, Domingos Chinen LT, Rocha RM, Asara JM, & Kalluri R (2014) PGC-1alpha mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat. Cell Biol.*, **16**, 992-15.
 - [22] Kordon EC, McKnight RA, Jhappan C, Hennighausen L, Merlino G, & Smith GH (1995) Ectopic TGF beta 1 expression in the secretory mammary epithelium induces early senescence of the epithelial stem cell population. *Dev. Biol.*, **168**, 47-61.
 - [23] Stein T, Salomonis N, & Gusterson BA (2007) Mammary gland involution as a multi-step process. *J. Mammary. Gland. Biol. Neoplasia.*, **12**, 25-35.
 - [24] Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, Giacobino JP, De MR, & Cinti S (2010) The emergence of cold-induced brown adipocytes in mouse white fat depots

- is determined predominantly by white to brown adipocyte transdifferentiation. *Am. J. Physiol Endocrinol. Metab*, **298**, E1244-E1253.
- [25] Wang W, Lv N, Zhang S, Shui G, Qian H, Zhang J, Chen Y, Ye J, Xie Y, Shen Y, Wenk MR, & Li P (2012) Cidea is an essential transcriptional coactivator regulating mammary gland secretion of milk lipids. *Nat. Med.*, **18**, 235-243.
- [26] Zhou Z, Yon TS, Chen Z, Guo K, Ng CP, Ponniah S, Lin SC, Hong W, & Li P (2003) Cidea-deficient mice have lean phenotype and are resistant to obesity. *Nat. Genet.*, **35**, 49-56.
- [27] Russell TD, Palmer CA, Orlicky DJ, Fischer A, Rudolph MC, Neville MC, & McManaman JL (2007) Cytoplasmic lipid droplet accumulation in developing mammary epithelial cells: roles of adipophilin and lipid metabolism. *J. Lipid Res.*, **48**, 1463-1475.
- [28] Straub BK, Gyoengyoesi B, Koenig M, Hashani M, Pawella LM, Herpel E, Mueller W, Macher-Goeppinger S, Heid H, & Schirmacher P (2013) Adipophilin/perilipin-2 as a lipid droplet-specific marker for metabolically active cells and diseases associated with metabolic dysregulation. *Histopathology*, **62**, 617-631.
- [29] Cannon B & Nedergaard J (2004) Brown adipose tissue: function and physiological significance. *Physiol Rev.*, **84**, 277-359.
- [30] Ahluwalia IB, Morrow B, & Hsia J (2005) Why do women stop breastfeeding? Findings from the Pregnancy Risk Assessment and Monitoring System. *Pediatrics*, **116**, 1408-1412.
- [31] Lewallen LP, Dick MJ, Flowers J, Powell W, Zickefoose KT, Wall YG, & Price ZM (2006) Breastfeeding support and early cessation. *J. Obstet. Gynecol. Neonatal Nurs.*, **35**, 166-172.
- [32] Seagroves TN, Hadsell D, McManaman J, Palmer C, Liao D, McNulty W, Welm B, Wagner KU, Neville M, & Johnson RS (2003) HIF1alpha is a critical regulator of secretory differentiation and activation, but not vascular expansion, in the mouse mammary gland. *Development*, **130**, 1713-1724.
- [33] Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, & Hennighausen L (1997) Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev.*, **11**, 179-186.
- [34] Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Edery M, Brousse N, Babinet C, Binart N, & Kelly PA (1997) Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev.*, **11**, 167-178.
- [35] Wang QA, Tao C, Gupta RK, & Scherer PE (2013) Tracking adipogenesis during white adipose tissue development, expansion and regeneration. *Nat. Med.*, **19**, 1338-1344.
- [36] Wu J, Cohen P, & Spiegelman BM (2013) Adaptive thermogenesis in adipocytes: is beige the new brown? *Genes Dev.*, **27**, 234-250.
- [37] Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, & Cinti S (2000) Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am. J. Physiol Cell Physiol*, **279**, C670-C681.

- 1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65
- [38] Cinti S (2009) Transdifferentiation properties of adipocytes in the adipose organ. *Am. J. Physiol Endocrinol. Metab*, **297**, E977-E986.
- [39] Tsukiyama-Kohara K, Poulin F, Kohara M, DeMaria CT, Cheng A, Wu Z, Gingras AC, Katsume A, Elchebly M, Spiegelman BM, Harper ME, Tremblay ML, & Sonenberg N (2001) Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. *Nat. Med.*, **7**, 1128-1132.
- [40] Bostrom P, Wu J, Jedrychowski MP, Korde A, Ye L, Lo JC, Rasbach KA, Bostrom EA, Choi JH, Long JZ, Kajimura S, Zingaretti MC, Vind BF, Tu H, Cinti S, Hojlund K, Gygi SP, & Spiegelman BM (2012) A PGC1-alpha-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. *Nature*, **481**, 463-468.
- [41] Giordano A, Smorlesi A, Frontini A, Barbatelli G, & Cinti S (2014) White, brown and pink adipocytes: the extraordinary plasticity of the adipose organ. *Eur. J. Endocrinol.*, **170**, R159-R171.
- [42] Gouon-Evans V & Pollard JW (2002) Unexpected deposition of brown fat in mammary gland during postnatal development. *Mol. Endocrinol.*, **16**, 2618-2627.
- [43] Master SR, Hartman JL, D'Cruz CM, Moody SE, Keiper EA, Ha SI, Cox JD, Belka GK, & Chodosh LA (2002) Functional microarray analysis of mammary organogenesis reveals a developmental role in adaptive thermogenesis. *Mol. Endocrinol.*, **16**, 1185-1203.
- [44] Jones LP, Buelto D, Tago E, & Owusu-Boaitey KE (2011) Abnormal Mammary Adipose Tissue Environment of Brca1 Mutant Mice Show a Persistent Deposition of Highly Vascularized Multilocular Adipocytes. *J. Cancer Sci. Ther.*
- [45] Klimcakova E, Chenard V, McGuirk S, Germain D, Avizonis D, Muller WJ, & St-Pierre J (2012) PGC-1alpha promotes the growth of ErbB2/Neu-induced mammary tumors by regulating nutrient supply. *Cancer Res.*, **72**, 1538-1546.
- [46] LeBleu VS, O'Connell JT, Gonzalez Herrera KN, Wikman H, Pantel K, Haigis MC, de Carvalho FM, Damascena A, Domingos Chinen LT, Rocha RM, Asara JM, & Kalluri R (2014) PGC-1alpha mediates mitochondrial biogenesis and oxidative phosphorylation in cancer cells to promote metastasis. *Nat. Cell Biol.*, **16**, 992-15.
- [47] McGuirk S, Gravel SP, Deblois G, Papadopoli DJ, Faubert B, Wegner A, Hiller K, Avizonis D, Akavia UD, Jones RG, Giguere V, & St-Pierre J (2013) PGC-1alpha supports glutamine metabolism in breast cancer. *Cancer Metab*, **1**, 22.
- [48] Millikan RC, Newman B, Tse CK, Moorman PG, Conway K, Dressler LG, Smith LV, Labbok MH, Geradts J, Bensen JT, Jackson S, Nyante S, Livasy C, Carey L, Earp HS, & Perou CM (2008) Epidemiology of basal-like breast cancer. *Breast Cancer Res. Treat.*, **109**, 123-139.
- [49] Phipps AI, Malone KE, Porter PL, Daling JR, & Li CI (2008) Reproductive and hormonal risk factors for postmenopausal luminal, HER-2-overexpressing, and triple-negative breast cancer. *Cancer*, **113**, 1521-1526.
- [50] Lord SJ, Bernstein L, Johnson KA, Malone KE, McDonald JA, Marchbanks PA, Simon MS, Strom BL, Press MF, Folger SG, Burkman RT, Deapen D, Spirtas R, & Ursin G (2008) Breast cancer

1 risk and hormone receptor status in older women by parity, age of first birth, and
2 breastfeeding: a case-control study. *Cancer Epidemiol. Biomarkers Prev.*, **17**, 1723-1730.

- 3
4 [51] Connelly L, Barham W, Pigg R, Saint-Jean L, Sherrill T, Cheng DS, Chodosh LA, Blackwell TS, &
5 Yull FE (2010) Activation of nuclear factor kappa B in mammary epithelium promotes milk
6 loss during mammary development and infection. *J. Cell Physiol*, **222**, 73-81.
7
8 [52] Lin J, Handschin C, & Spiegelman BM (2005) Metabolic control through the PGC-1 family of
9 transcription coactivators. *Cell Metab*, **1**, 361-370.
10
11 [53] Mobuchon L, Marthey S, Le GS, Laloe D, Le PF, & Leroux C (2015) Food Deprivation Affects
12 the miRNome in the Lactating Goat Mammary Gland. *PLoS. One.*, **10**, e0140111.
13
14 [54] Han LQ, Li HJ, Wang YY, Zhu HS, Wang LF, Guo YJ, Lu WF, Wang YL, & Yang GY (2010) mRNA
15 abundance and expression of SLC27A, ACC, SCD, FADS, LPIN, INSIG, and PPARGC1 gene
16 isoforms in mouse mammary glands during the lactation cycle. *Genet. Mol. Res.*, **9**, 1250-
17 1257.
18
19 [55] Ivanova MM, Radde BN, Son J, Mehta FF, Chung SH, & Klinge CM (2013) Estradiol and
20 tamoxifen regulate NRF-1 and mitochondrial function in mouse mammary gland and uterus.
21 *J. Mol. Endocrinol.*, **51**, 233-246.
22
23 [56] Ma L, Qiu H, Chen Z, Li L, Zeng Y, Luo J, & Gou D (2018) miR-25 modulates triacylglycerol and
24 lipid accumulation in goat mammary epithelial cells by repressing PGC-1beta. *J. Anim Sci.*
25 *Biotechnol.*, **9**, 48.
26
27 [57] Rodriguez-Cruz M, Tovar AR, Palacios-Gonzalez B, Del PM, & Torres N (2006) Synthesis of
28 long-chain polyunsaturated fatty acids in lactating mammary gland: role of Delta5 and Delta6
29 desaturases, SREBP-1, PPARalpha, and PGC-1. *J. Lipid Res.*, **47**, 553-560.
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2 **Figures Legend**
3

4
5 **Figure 1**
6

7 *Dynamic expression of PGC-1 α protein levels during mammary glands development and involution.*

8
9
10 Highly expressed during puberty and in the first 12 days of pregnancy, the PGC-1 α levels slightly
11
12 disappear in the last days of pregnancy and during lactation. On the first days after cessation of
13
14 lactation, PGC-1 α starts to be re-expressed, reaching its steady state on day 3 of involution. (A)
15
16 Staining of mammary glands sections from wild type mice at different development stages with H&E
17
18 (upper panel) and PGC-1 α immunohistochemistry (lower panel) (n=3 mice per stage; Magnification
19
20 200X). (B) Mammary glands prepared from wild type mice on the indicated days of involution and
21
22 stained for H&E, PGC-1 α immunohistochemistry, and TUNEL (n=3 mice per stage; Magnification
23
24 100X)
25
26
27
28
29
30
31
32

33 **Figure 2**
34

35
36 *PGC-1 α expression in mammary glands is associated with UCP1 induction.*
37

38 (A) Staining of mammary glands sections from wild type mice at different development stages with
39
40 H&E (upper panel) and PGC-1 α (middle panel) and UCP1 immunohistochemistry (lower panel) (n=5
41
42 mice per stage; Magnification 200X). (B) Western blot analysis of PGC-1 α and UCP1 on mammary
43
44 glands samples throughout different developmental stages. Ponceau stain was used as loading
45
46 control. (C) Relative expression of PGC-1 α and fat-browning related genes, Ucp1, Cidea and Prdm16,
47
48 at different stages of mammary glands development evaluated by Real Time qPCR. TBP was used as
49
50 housekeeping gene. Comparison of different groups (n=6,7) was performed using Kruskal-Wallis
51
52 test. Data are expressed as mean \pm SEM (*p<0.05; **p<0.01).
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2 **Figure 3**
3

4
5 *Generation of mouse model with stable specific overexpression of human PGC-1 α in mammary*
6
7 *glands.* A transgenic mouse model, mmtvPGC-1 α , was generated by cloning the human sequence
8
9 of PGC-1 α downstream the MMTV promoter. Analysis of 8 weeks-old wild type and mmtvPGC-1 α
10
11 virgin mice shows a specific PGC-1 α overexpression in the mammary glands of transgenic mice. (A)
12
13 Scheme of the mmtvPGC-1 α transgenic mouse model generated: the hPGC-1 α coding sequence was
14
15 cloned downstream of the MMTV promoter of MMTV-SV40-Bssk plasmid and then injected into the
16
17 pronuclei of the fertilized eggs of the FVB/N mice. (B) Relative mRNA expression of hPGC-1 α in
18
19 mammary glands isolated from mmtvPGC-1 α and WT control mice measured by real time qPCR,
20
21 using TBP as housekeeping gene and WT mice as calibrator. (C) Western blot analysis of PGC-1 α on
22
23 mammary glands samples isolated from transgenic and WT mice. (D) Body weight and (E) inguinal
24
25 mammary glands weight to body weight ratio (MGW/BW) of wild type and mmtvPGC-1 α mice.
26
27 Results are expressed as mean \pm SEM (**p<0.001, *p<0.05). Comparison of wild type and
28
29 transgenic mice (n=6,7) was performed using Mann-Whitney U test.
30
31
32
33
34
35
36
37
38
39
40

41 **Figure 4**
42

43 *Maternal PGC-1 α overexpression decreases lobulogenesis in mammary glands during early*
44
45 *development and results in growth retardation in the nursing neonates.* Examination of wild type
46
47 and mmtvPGC-1 α mammary glands from 8 weeks-old virgin mice reveals that PGC-1 α
48
49 overexpression in transgenic mice is associated with an impairment of ductal growth and ductal
50
51 branching. (A) H&E staining and PGC-1 α immunohistochemistry of mammary glands sections from
52
53 wild type and mmtvPGC-1 α mice (Magnification 200X). (B) Whole mount of the inguinal mammary
54
55 glands in a virgin wild type and a mmtvPGC-1 α mouse. Developmental time line is depicted as the
56
57
58
59
60
61
62
63
64
65

1 average body weight (\pm SEM) of pups from each of 4 independent litters on each postnatal day,
2 nursed by the (C) biological mother and the (D) foster one. (E) Comparison of the four different
3 group depicted as percentage of body weight increase. Blue color indicates WT pups, whereas red
4 one specifies mmtvPGC-1 α newborns. Circle symbol is used for WT breastfeeding mother and
5 square one for mmtvPGC-1 α breastfeeding mother. Closed circle indicates that pups are fed by
6 biological mother, while open circle is used to denote pups fed by foster mother.
7
8
9
10
11
12
13
14
15
16
17

18 **Figure 5**

19 *PGC-1 α overexpression during lactation does not affect milk quality.*

20
21
22
23 Analysis of wild type and mmtvPGC-1 α mammary glands from lactating females on day 10 of
24 lactation shows that PGC-1 α overexpression is preserved in transgenic mice during lactation. (A)
25 H&E staining (left) and PGC-1 α immunohistochemistry (right) of mammary glands sections from wild
26 type and mmtvPGC-1 α female mice on 10th day of lactation. (B) Relative expression of human PGC-
27 1 α in lactating mammary glands evaluated by Real Time qPCR demonstrating that the transgene is
28 not lost during lactation. GAPDH was used as housekeeping gene to normalize data and wild type
29 mice was used as calibrators (C) COX1 immunohistochemistry of mammary glands sections from
30 wild type and mmtvPGC-1 α female mice on 10th day of lactation. (D) Relative expression of PGC-1 α
31 target genes, Tfam and Pepck, in lactating mammary glands evaluated by Real Time qPCR. TBP was
32 used as housekeeping gene. (E) A 12% SDS-polyacrylamide gel analysis of different types of casein
33 in milk derived from lactating wild type and mmtvPGC-1 α mice. The sizes of the protein molecular
34 weight markers are indicated in lane 1. (F) Fatty acids composition of milk harvested from lactating
35 wild type and mmtvPGC-1 α females. Fatty acids were analyzed as fatty acid methyl esters (FAMES)
36 by gas-liquid chromatography. Comparison of wild type and transgenic mice (n=6,7) was performed
37 using Mann-Whitney U test. Results are expressed as mean \pm SEM (*p<0.05; ***p<0.001).
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure 6

Stable overexpression of PGC-1 α during lactation promotes apoptosis and mammary glands regression.

(A) Mammary tissue sections from 10 days lactating wild type and mmtvPGC-1 α females mice stained with H&E (different magnification), with UCP1 immunohistochemistry, and ADPH immunofluorescence. In 100X H&E, white arrows indicate the shedding of epithelial cells into the alveolar lumen; and black arrows represents brown adipocytes. Immunolocalization of ADPH was performed using Alexa 594-conjugated antibodies against the N-terminus of mouse ADPH (red, arrowed). Luminal borders of mammary alveoli were identified by staining with Alexa 488-conjugated WGA (green). Nuclei were stained with TO-PRO-3 (Blue). (B) Quantification of UCP1 and ADPH immunostaining. Relative expression of (C) fat-browning related genes, Ucp1, Cidea and Prdm16, and (D) β -oxidation genes, Ppar α , Pdk4 and Cpt1b, in lactating mammary glands evaluated by Real Time qPCR. TBP was used as housekeeping gene. (E) Inguinal mammary glands weight to body weight ratio (MGW/BW) of wild type and mmtvPGC-1 α lactating mice. (F) Low and high magnification of whole mount staining together with TUNEL staining of mammary glands isolated from lactating mmtvPGC-1 α and WT mice. Comparison of wild type and transgenic mice (n=6,7) was performed using Mann-Whitney U test. Data are expressed as mean \pm SEM (*p<0.05; **p<0.01).

Supplementary Figure 1

PGC-1 α overexpression affects pups development.

(A) hPGC-1 α (white bars) and mPGC-1 α (black bar) relative mRNA expression in different tissue specimens isolated from transgenic and wild type mice (n=6) by real time qPCR. TBP was used as housekeeping gene to normalize data and wild type mice was used as calibrators. Results are

expressed as mean \pm SEM. (B) Picture of wild type and mmtvPGC-1 α newborns at 21 days after birth.

(C) Picture of wild type and mmtvPGC-1 α newborns at 19 days after birth fostered by mmtvPGC-1 α and wild type mother respectively.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

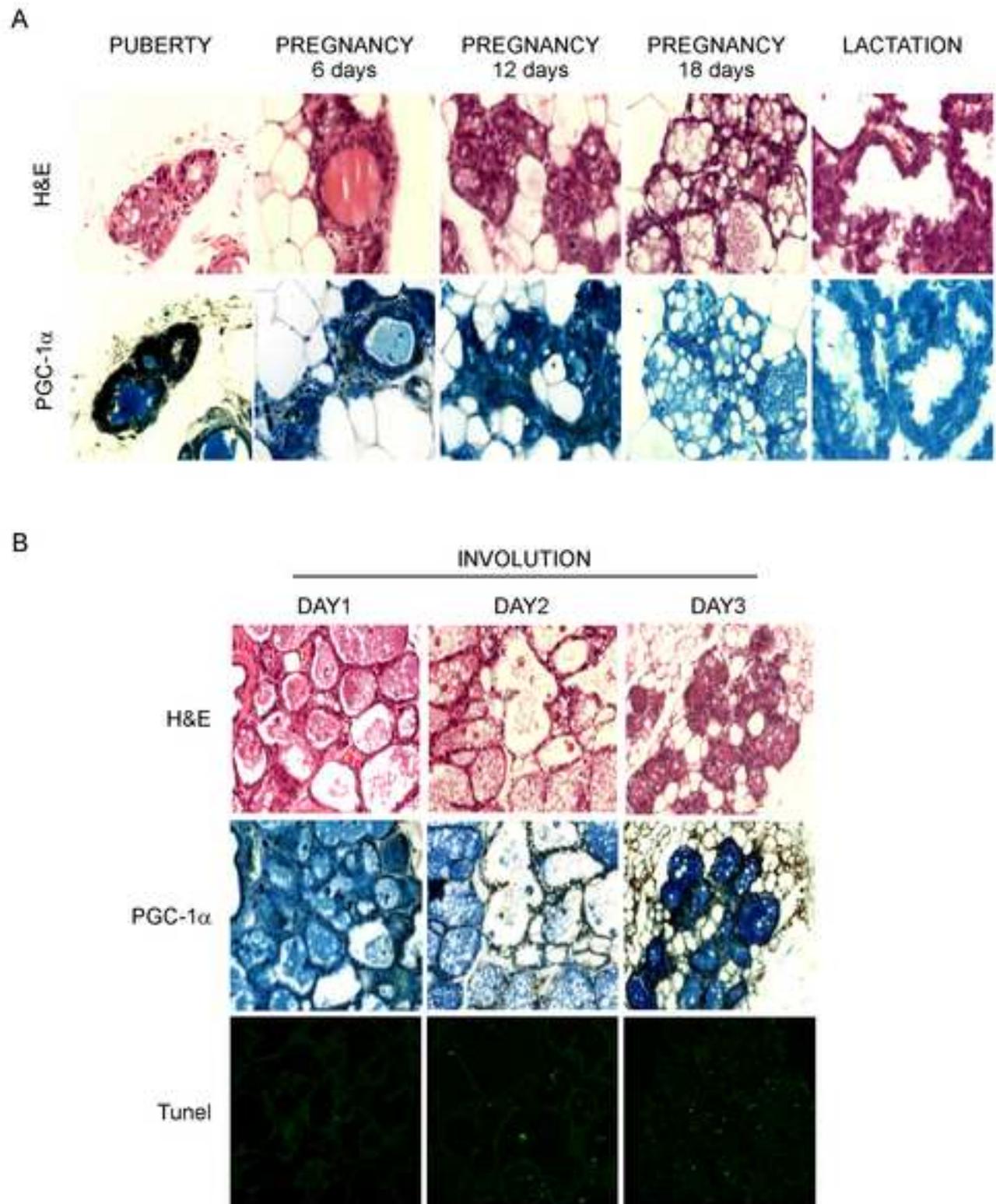


Figure 1

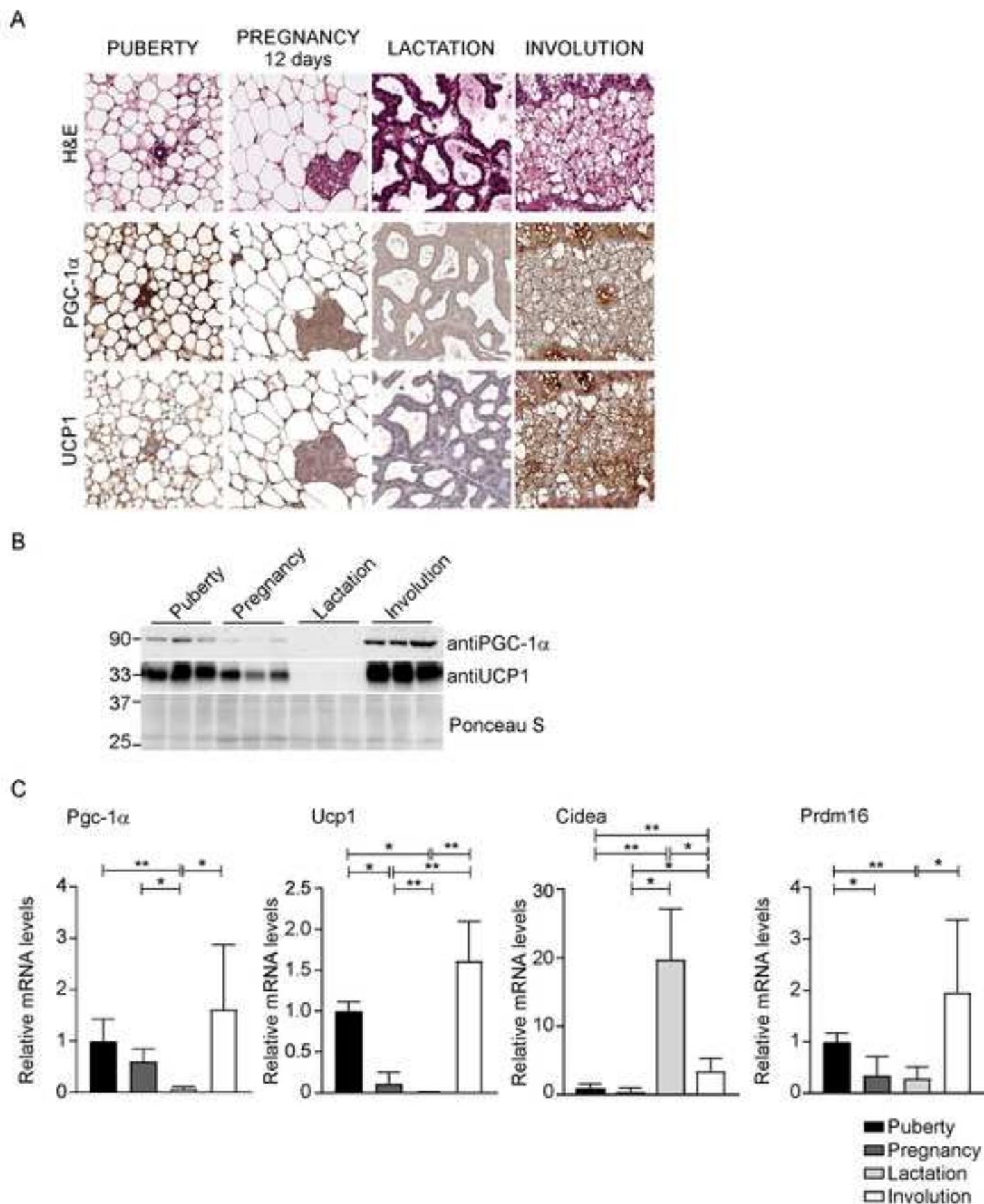


Figure 2

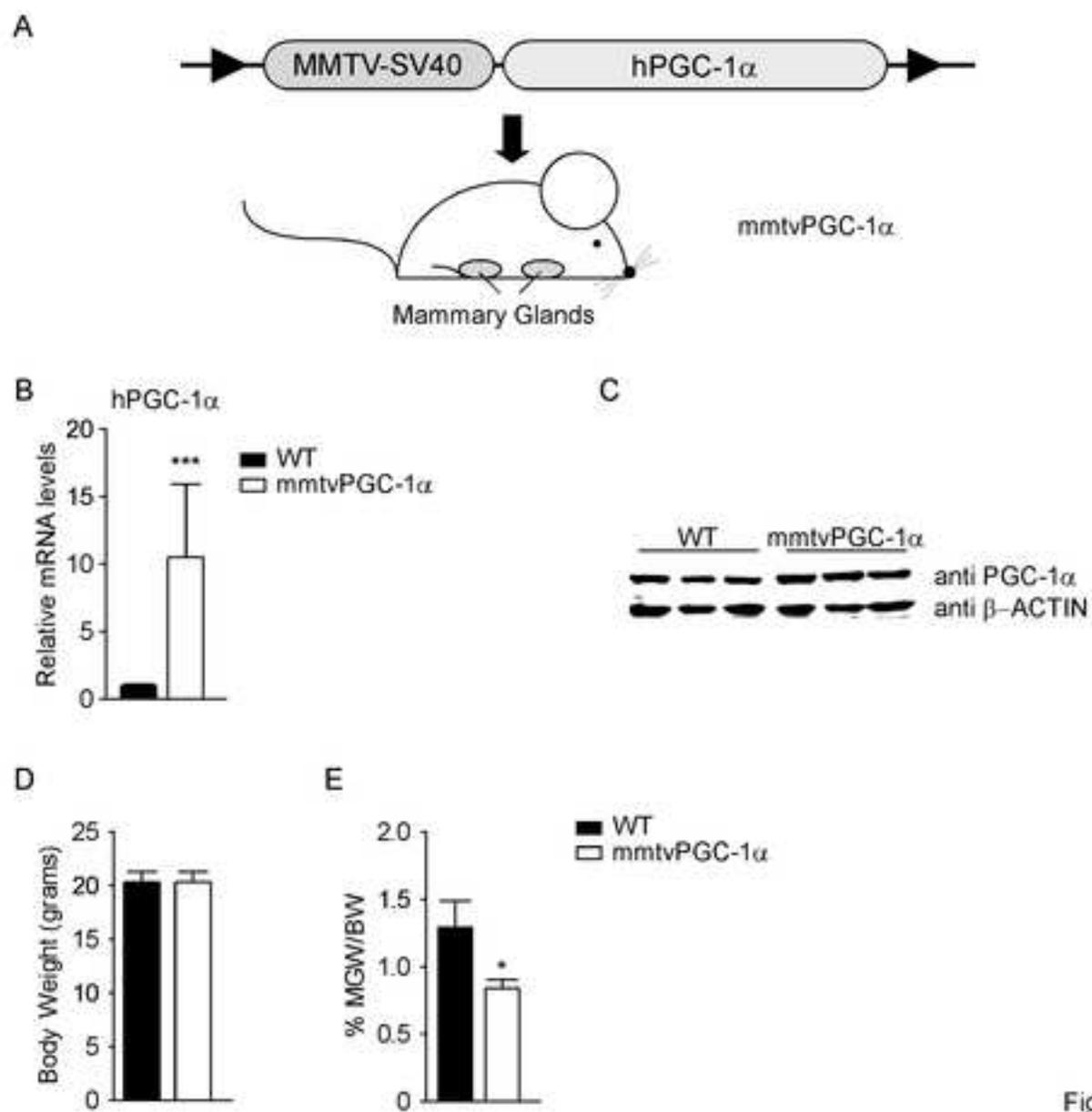


Figure 3

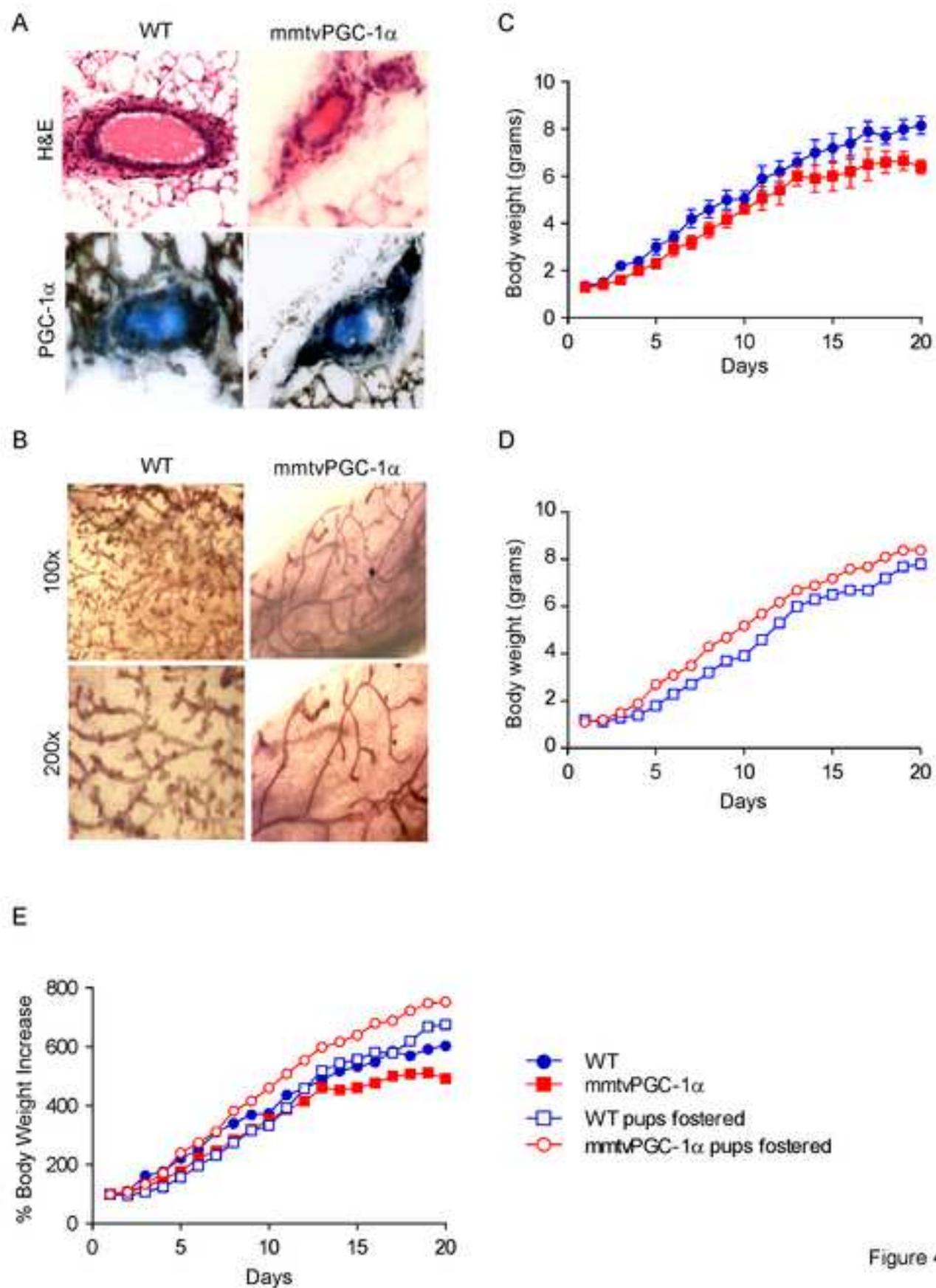
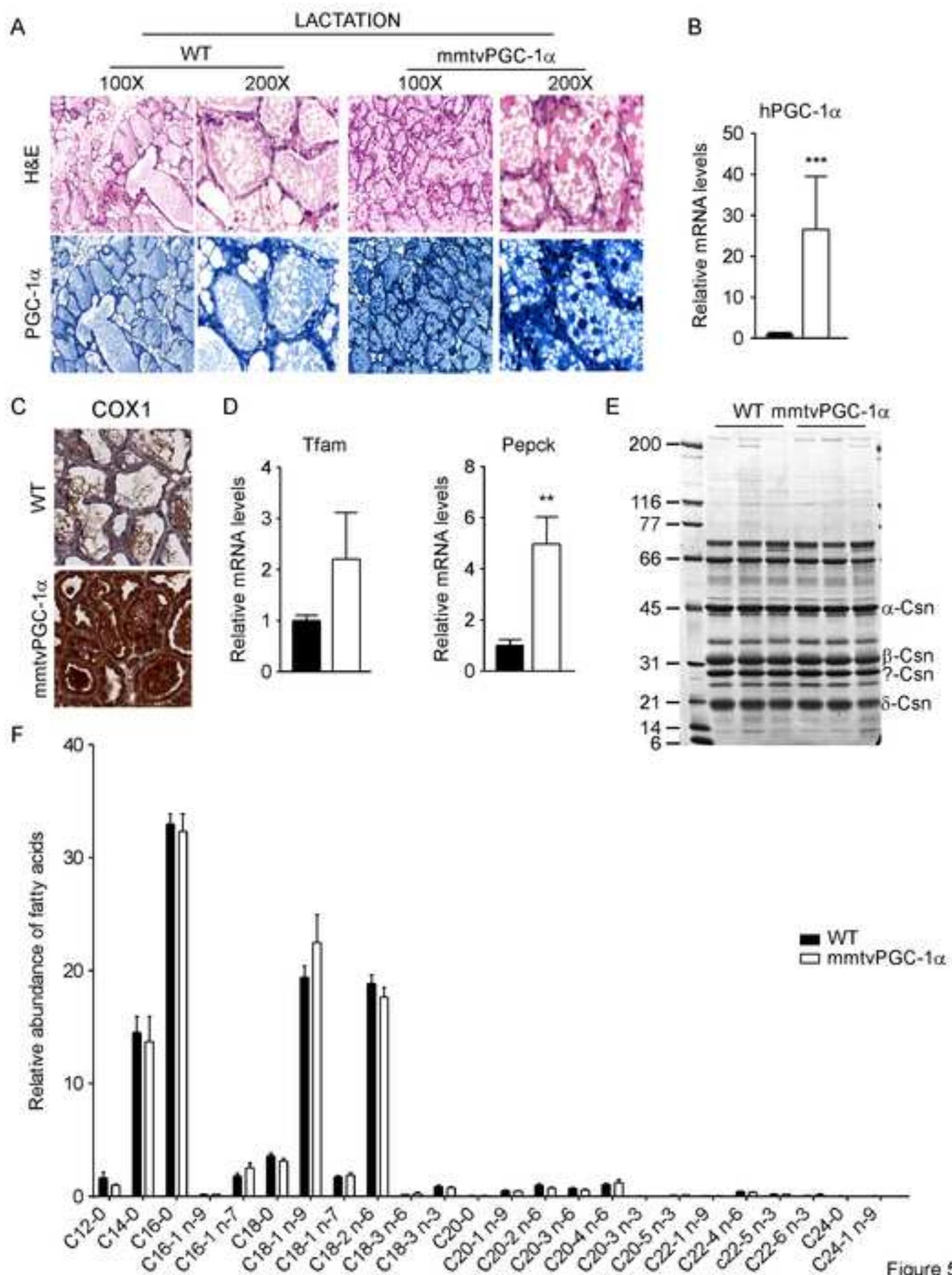


Figure 4



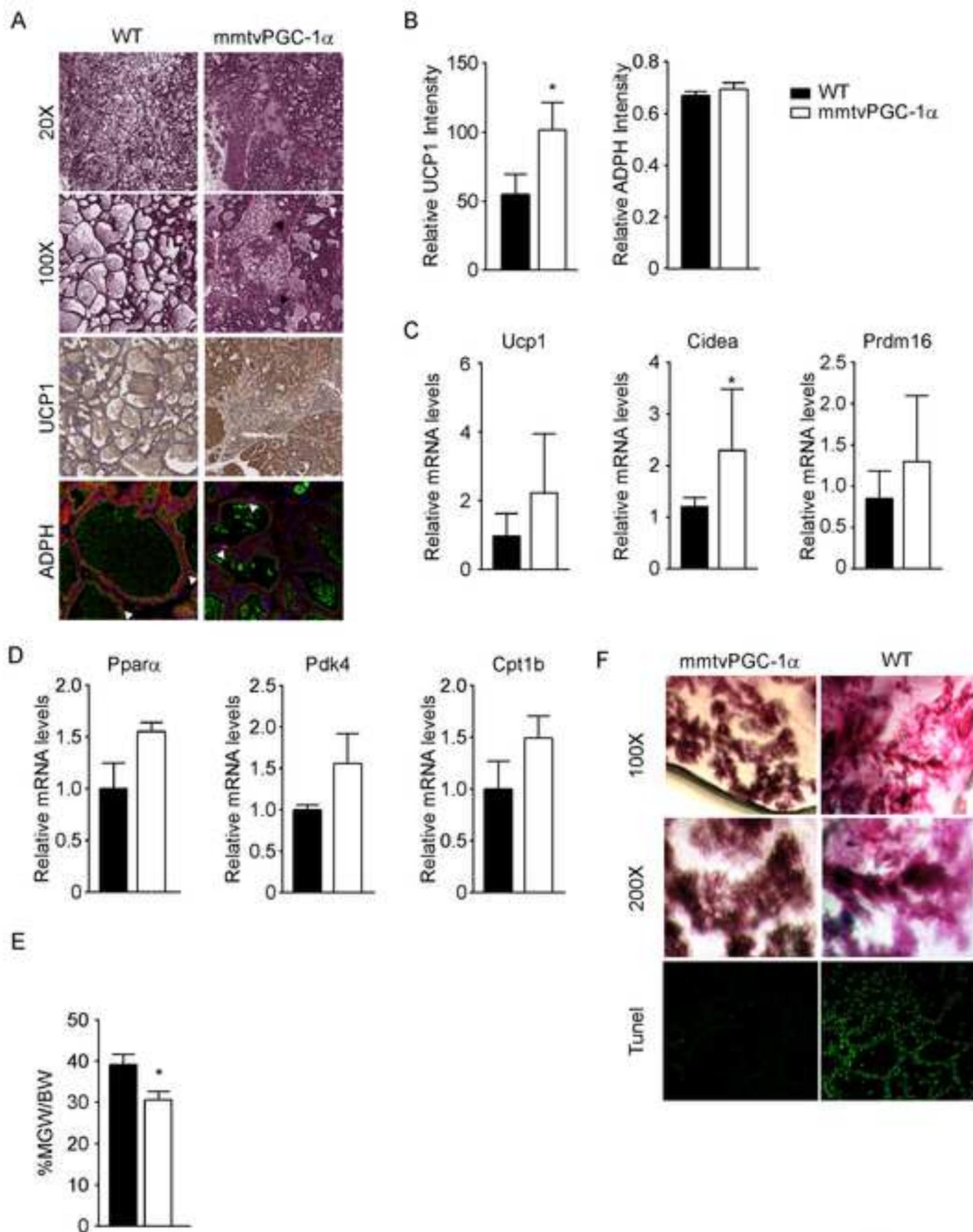


Figure 6



Click here to access/download
Supplementary Material
SupFig1R.tif

