NCoA3 upregulation in breast cancer-associated adipocytes elicits an inflammatory profile

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Abstract. Nuclear receptor coactivator 3 (NCoA3) is a transcriptional coactivator of NF-κB and other factors, which is expressed at relatively low levels in normal cells and is amplified or overexpressed in several types of cancer, including breast tumors. NCoA3 levels have been shown to be decreased during adipogenesis; however, its role in tumor-surrounding adipose tissue (AT) remains unknown. Therefore, the present study assessed the modulation of NCoA3 in breast cancer-associated adipocytes and evaluated its association with the expression of inflammatory markers. 3T3-L1 adipocytes were stimulated with conditioned medium from human breast cancer cell lines and the expression levels of NCoA3 were evaluated by reverse transcription-quantitative (q)PCR. NF-κB activation was measured by immunofluorescence, and tumor necrosis factor and monocyte chemoattractant protein 1 levels were analyzed by qPCR and dot blot assays. The results obtained from the *in vitro* model were supported using mammary AT (MAT) from female mice, MAT adjacent to tumors from patients with breast cancer and bioinformatics analysis. The results revealed that adipocytes expressing high levels of NCoA3 were mainly associated with a pro-inflammatory profile. In 3T3-L1 adipocytes, NCoA3 downregulation or NF-κB inhibition reversed the expression of inflammatory molecules. In addition, MAT from patients with a worse prognosis exhibited high levels of this coactivator. Notably, adipocyte NCoA3 levels could be modulated by inflammatory signals from tumors. The modulation of NCoA3 levels in synergy with NF-κB activity in MAT in a tumor context could be factors required to establish breast cancer-associated inflammation. As adipocytes are involved in the development and progression of breast cancer, this signaling network deserves to be further investigated to improve future tumor treatments.

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Introduction

Adipose tissue (AT) is an active endocrine organ with a number of secretory products and is also a part of the innate immune system. The phenotype of AT is modified in diseases such as obesity, metabolic syndrome and cancer (1,2). Notably, AT is the most abundant tissue surrounding breast cancer cells, and the importance of the microenvironment during tumorigenesis, for example via its effects on proliferation, invasion and chemoresistance, has been realized (3). Even though autocrine signals regulate tumor behavior, effects exerted by adjacent stromal cells also serve a role in this modulation. These signals could be soluble secreted factors, extracellular matrix components and also cell-cell contact. This signaling involves a constant crosstalk between normal and transformed cells (4).

It has been demonstrated that mammary AT (MAT) adjacent to breast tumors serves as a source of pro-inflammatory cytokines and chemokines, such as interleukin-6 (IL-6), tumor necrosis factor (TNF) and monocyte chemoattractant protein 1 (MCP1, also known as CCL2) (5). One of the key transcription factors that leads to the expression of

inflammation-associated molecules is NF-κB (6). However, there is still a lack of knowledge regarding how adipocytes induce this pro-inflammatory profile.

Positive and negative coregulators are master genes that respectively enhance or suppress the transcription of several genes by binding transcription factors. Nuclear coactivator 3/receptor associated-coactivator 3 (NCoA3, also known as RAC3) is a NF-κB coactivator and is a member of the p160 family of steroid receptor coactivators. Our previous studies demonstrated that NCoA3 expression levels are upregulated in response to inflammatory injury (7) and are downregulated during adipocyte differentiation (8). Notably, NCoA3 is highly expressed in several types of human cancer, including breast cancer (9,10). In addition, NCoA3 overexpression has been reported to serve an anti-apoptotic, anti-autophagic and pro-proliferative role (11-13). Moreover, NCoA3 expression levels are correlated with higher chemoresistance (14). However, to the best of our knowledge, its levels in the AT adjacent to breast cancer remain uncharacterized.

The present study aimed to evaluate whether NCoA3 levels in AT were modulated by breast cancer cells, and the role of this coactivator in this context. The results obtained in this work bring new insights into the behavior of breast cancer-associated adipocytes and how these cells contribute to tumor-promoting inflammation.

Materials and methods

Cell culture and reagents. The murine pre-adipocyte cell line, 3T3-L1 [American Type Culture Collection (ATCC)® CL-173™]; the non-tumorigenic mammary epithelial cell line, MCF10A (ATCC CRL-10317™); and the human breast cancer cell lines, T-47D (ATCC HTB133™), MCF7 (ATCC HTB22™) and MDA-MB-231 (ATCC CRMHTB26™), were purchased from ATCC. The 3T3-L1 cell line was grown in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc); MCF10A cells were grown in DMEM/F12 (1:1) containing hydrocortisone (0.5 μg/ml; Sigma-Aldrich; Merck KGaA), epidermal growth factor (20 ng/ml; Sigma-Aldrich; Merck KGaA) and insulin (10 μg/ml; Sigma-Aldrich; Merck KGaA); and MDA-MB-231, MCF7 and T-47D cell lines were grown in DMEM/F12 (1:1). All media were supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 mg/ml). Cells were maintained at 37˚C in a humidified atmosphere containing 5% CO_2. Mycoplasma testing was performed for the cell lines used.

Conditioned medium (CM) from breast cell lines (BrCM) was collected after 24 h of cell culture in serum-free medium and cell debris was removed by centrifugation at 800 RCF for 5 min at room temperature. BrCM aliquots were conserved at -20˚C prior to adipocyte stimulation. Before adipocyte stimulation, 3T3-L1 cells were plated and differentiated using induction differentiation medium (1 μg/ml insulin, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine and 0.1 mM indomethacin; Sigma-Aldrich; Merck KGaA), as described previously (8). Insulin (1 μg/ml) was added every 3 days until 1 week was completed. After 3T3-L1 cell differentiation, medium was replaced with BrCM or fresh free-serum medium (basal condition) for 24 h, and then protein or RNA extraction was performed. To study the role of NF-κB activation, 3T3-L1-derived adipocytes were pre-incubated with NF-κB inhibitor sulfasalazine (SSZ; 0.2 mM; Santa Cruz Biotechnology, Inc.) for 30 min before stimulation. 3T3-L1 adipocyte CM was collected after being cultivated in fresh DMEM/F12 serum-free medium for 24 h for the dot blot assay.

Plasmid construction and transfection. The murine short hairpin (sh)RNA for NCoA3 (shNCoA3; 5'-CAGTCGCCA TCTTCCTATCAGAACAGCAG-3') and the scrambled sequence (control; 5'-GCCTAAGTCCCTCCTATGCAGACG TAACA-3') were prepared using the pGFP-V-RS shRNA vector system (OriGene Technologies, Inc.) according to the manufacturer's protocol, as previously reported (8). Briefly, 3T3-L1 cells were transfected with 0.5 μg shRNAs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 5 h of incubation at 37˚C in a humidified atmosphere, the transfection medium was replaced with culture medium and 3 days after transfection, the cells were incubated in medium containing 0.5 μg/ml puromycin (Thermo Fisher Scientific, Inc.) as a selection agent. After 14 days of selection, protein and mRNA expression levels were analyzed by reverse transcription-quantitative PCR (RT-qPCR) and western blotting, as reported in a previous study (8).

Patients and tissue specimens. MAT specimens (n=36 patients) were obtained from the Institute of Medical Research Alfredo Lanari, School of Medicine, University of Buenos Aires (Ciudad Autónoma de Buenos Aires, Argentina). All samples corresponded to MAT around breast tumors (n=32) or non-malignant mammary lesions (n=4) obtained during tumor extirpation or exploratory biopsies (0.5-2 cm away from the invasive front or lesion), respectively. Eligible patients were postmenopausal women aged ≥45 years with a diagnosis of breast cancer undergoing surgery. Non-tumoral MAT samples were obtained from patients who did not present with breast cancer at the time of surgery and had not been previously diagnosed with breast cancer. According to pathological examination, the molecular status of breast tumors was determined by immunohistochemistry (IHC) following the guidelines of the Allred score (15) [estrogen receptor (ER), progesterone receptor and human epidermal growth factor 2]. Staging was performed according to the National Comprehensive Cancer Network (Guidelines Version 4.2017, Breast Cancer Staging) (16). Meanwhile, patients with non-malignant lesions (mastopathy, mastitis or usual epithelial hyperplasia) were monitored for ≥6 years and, to date, none have presented with tumors. All patients were aged 53-94 years (median, 75 years). According to NCoA3 mRNA expression, samples were classified as 'low' or 'high'; the cut-off value was the mean of NCoA3 level obtained by RT-qPCR in non-malignant mammary lesions.

All procedures were performed in accordance with ethical standards, including National Laws and The 1964 Declaration of Helsinki and its later amendments, and were approved by the Institutional Research Ethic Committee of Institute of Medical Research Alfredo Lanari (approval no. #312). Written informed consent was obtained from all patients.

For the collection of CM, human MAT explants (0.5 g) were incubated in PBS containing 5 mg/ml bovine serum albumin (3 ml/g of tissue; Sigma-Aldrich; Merck KGaA) for 30 min,

and centrifuged for 30 sec at 400 x g at room temperature in order to reduce contamination with blood cells, soluble factors and tissue containing insufficient adipocytes to float (17). Subsequently, explants were cultivated in fresh DMEM/F12 serum-free medium for 24 h and CM was collected for the dot blot assay.

Animals. Animal experimental protocols performed in the present study were approved by the National University of Quilmes Institutional Animal Care and Use Committee (Quilmes, Argentina), in accordance with the National Institutes of Health guide and the ARRIVE guides for the care and use of laboratory animals (18).

A total of 15 adult female C57-BL/6J mice, (6-8 months-old; weight, 25-30 g) were divided into three groups (n=5 per group), were housed in the animal facility of the National University of Quilmes, were given *ad libitum* access to food and water, under a 12-h light/dark cycle, with room temperature set at 20±2˚C. To isolate the mammary glands, animals were sacrificed by 5% isoflurane inhalation (Piramal Healthcare) using gas anesthesia equipment (SurgiVet) and cervical dislocation. The mammary glands (#2-4) were surgically removed and explants were cultivated.

Murine explants (0.2 g) of MAT were prepared in the same manner as human explants. Subsequently, explants were stimulated with BrCM or fresh serum-free medium (basal condition) and incubated at 37˚C in humidified atmosphere for 24 h and processed for RNA extraction or IHC. For the collection of CM, explants were cultivated in fresh DMEM/F12 serum-free medium for 24 h, the obtained CM was centrifuged at 800 RCF for 5 min at room temperature and the dot blot assay was performed.

Western blot analysis. Western blotting was performed as previously described (14). For experiments where NCoA3 expression was determined, 3T3-L1-derived adipocytes were stimulated with BrCM, or not, and were then harvested and treated with RIPA lysis buffer (50 mM Hepes, 250 mM NaCl, 1 mM EDTA and 1% NP-40, pH 7.4; Sigma-Aldrich; Merck KGaA) plus protease inhibitors (1 mM PMSF, 5 μg/ml Pepstatin A and 1 mM DTT; Sigma-Aldrich; Merck KGaA). To validate the knockdown efficiency of shNCoA3, protein extracts were obtained from 3T3-L1 undifferentiated cells, as aforementioned. The protein concentrations were quantified using the Bradford assay (Bio-Rad Laboratories, Inc.).

In both cases, samples (100 μg total proteins) were separated by SDS-PAGE on 7% gels and transferred to PVDF membranes, which were blocked with 10% skim milk for 1 h at room temperature. The membranes were then incubated with rabbit polyclonal anti-NCoA3 (1:4,000; cat. no. sc-25742) or mouse monoclonal anti-tubulin (1:5,000; cat. no. sc-58666) antibodies (Santa Cruz Biotechnology, Inc.) overnight at 4˚C in TBS-0.02%Tween containing 0.5% bovine serum albumin (Sigma-Aldrich; Merck KGaA). Subsequently, washed membranes were incubated for 1h at room temperature with HRP-conjugated secondary antibodies (1:5,000; cat. nos. PI-1000 and PI-2000; Vector Laboratories, Inc.). Proteins were then detected using luminol reagent (Santa Cruz Biotechnology, Inc.). When bands were detected in all experimental conditions, densitometry units for each band were obtained using ImageJ software v1.52a (National Institutes of Health) and were normalized to anti-tubulin densitometry values.

IHC and immunofluorescence staining. Human and murine blocks were fixed in 4% formaldehyde for 24 h at room temperature. Tissue blocks were then embedded in paraffin, sectioned (3 μm), then deparaffinized in xylene and rehydrated for IHC staining (14). Antigen retrieval was performed using sodium citrate for 25 min at 100˚C in a water bath. The sections were then incubated in H_2O_2 (3%) for 10 min, blocked in horse serum (cat. no. PK-6200; Vector Laboratories, Inc.) for 60 min at room temperature and incubated with rabbit anti-NCoA3 (1:250; cat. no. sc-25742) or mouse anti-phosphorylated (p)-p65 (Ser536; 1:500; cat. no. sc-136548) antibodies (Santa Cruz Biotechnology, Inc.) at 4˚C overnight. Following incubation with a universal biotinylated secondary antibody for 60 min at room temperature (1:250; cat. no. PK-6200; Vector Laboratories, Inc.), the specimens were incubated with H2O2-diaminobenzidine until the desired staining intensity was observed. The sections were counterstained with hematoxylin, dehydrated, mounted and observed under a light Olympus BX51 microscope (Olympus Corporation). The area of positive staining was calculated using ImageJ software with the plugin ColourDeconvolution2 (19).

MAT paraffin-embedded sections obtained from patients were prepared as aforementioned and stained with pure hematoxylin for 3 min at room temperature. Crown-like structures (CLS) were identified as adipocytes with at least two infiltrating immune cells (with macrophage morphology) around the cell using light microscopy; the number of CLS per field was manually counted.

For immunofluorescence assays, 3T3-L1-derived adipocytes were seeded in 24-well plates on 12-mm glass coverslips. After 24 h of culturing, the cells were stimulated with BrCM or serum-free medium (basal condition) for 1 h at 37˚C in a humidified atmosphere, and were then fixed with 3% formaldehyde and 0.02% glutaraldehyde for 15 min. The cells were incubated with a primary antibody against p-p65 at the aforementioned dilution for 2 h at room temperature. Subsequently, the cells were washed and incubated with a TRITC-labeled secondary antibody (1:250; cat. no. #115-025-003; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature, washed with PBS, counterstained with Hoechst 33342 (Santa Cruz -Biotechnology, Inc.) and mounted on glass slides with PBS/Glycerol 1:1 solution. Cells were analyzed using an Olympus BX51 fluorescence microscope (Olympus Corporation). Images were captured using a digital camera in 10 randomly-chosen fields; ≥10 cells/field were analyzed with ImageJ software. The experiment was performed in triplicate.

RT-qPCR. Total RNA was isolated from human and murine MAT using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol and adapted for AT. Briefly, ~0.5 g tissue was homogenized using a Tissumizer homogenizer (Teledyne Tekmar Company) with 1 ml TRIzol reagent. The extracts were centrifuged at 12,000 x g for 10 min at 4˚C, after which the top layer of excess lipid was removed and the solution containing RNA was transferred to a fresh

tube (20). For RNA extraction from 3T3-L1 adipocytes, the standard TRIzol protocol was used (8).

RT was carried out using SuperScript II kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed using *Homo sapiens* (hs) and *Mus musculus* (mm) sequence-specific primers for: hs NCoA3, forward (F) 5'-AAGTGAAGAGGGATCTGGA-3', reverse (R) 5'-CAGATGACTACCATTTGAGG-3'; hs cyclophilin A (CyA), F 5'-ATGCTGGACCCAACACAAAT-3', R 5'-TCTTTCACTTTGCCAAACACC-3'; mm NCoA3, F 5'-ACATGGTGCATATGAACAGC-3', R 5'-GATGTCAGCAGTATTTCTGATCG-3'; mm MCP1, F 5'-CGGCTGGAGCATCCACGT-3', R 5'-TGGGGTCAGCACAGACCT-3'; mm CyA, F 5'-CCACCGTGTTCTTCGACATC-3', R 5'-GCTCGAAAGTTTTCTGCTGT-3'.

For gene expression quantitative analysis, CyA mRNA expression was used as a reference gene for normalization. According to the literature, CyA has been confirmed as an appropriate housekeeping gene (21). In our previous study, CyA expression was constitutively high, and there was no variation between conditions in both human and murine models (8). The $2^{-\Delta\Delta Cq}$ method was used to quantify mRNA expression (8,22).

Dot blot assay. CM from adipocytes and AT was spotted onto nitrocellulose membrane with Bio-Dot Microfiltration Apparatus (Bio-Rad Laboratories, Inc.). The membranes were blocked in 10% skim milk for 1 h at room temperature, incubated with mouse monoclonal anti-TNF (1:5,000; cat. no. sc-133192; Santa Cruz Biotechnology, Inc.) or mouse monoclonal anti-human MCP1 (1:1,000, cat. no. sc-32771; Santa Cruz Biotechnology, Inc.) overnight at 4˚C, and then incubated for 1 h at room temperature with HRP-conjugated secondary antibodies at the aforementioned dilution. Proteins were visualized by autoradiography using luminol reagent (Santa Cruz Biotechnology, Inc.). The densitometry values for each band were determined using ImageJ and their intensity was normalized to the amount of total proteins in the conditioned medium quantified by Bradford reaction. All samples were performed in triplicate for mice explants and 3T3-L1 adipocytes. For patient explants, each patient was considered an independent sample.

Analysis of gene expression values from data repositories. In order to compare the expression levels of NCoA3 and different adipokines between MAT surrounding normal mammary gland or breast cancer tissue, values retrieved from the E-MTAB-8638 dataset (18 MAT samples) using the A-MEXP-2072 platform (Illumina HumanHT12 V4.0; Illumina, Inc.) were obtained from the EBI databank, ArrayExpress (https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8638/) (23) were analyzed. Quantile normalized expression values were retrieved. Heatmap generation was performed using the heatmap function in limma R package (24). Cytokine expression was studied in samples from The Cancer Genome Atlas (TCGA) (https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga); TCGA Breast Cancer dataset containing 394 breast tumor samples was analyzed using UCSC Xena platform (https://xenabrowser.net/heatmap/).

Statistical analysis. All the data are presented as the mean ± SEM of at least three independent experiments in 3T3-L1 adipocytes or murine MAT; in patients, each tissue sample was considered an independent sample. For two group comparison, the significant differences between means were determined using unpaired Student's t-test. For multiple comparisons, when data were normally distributed, one-way ANOVA and Tukey's post-hoc tests were performed, otherwise the non-parametric Kruskal-Wallis test and Dunn's multiple comparison post-hoc test were performed. Differences in a given variable between groups of patient samples were assessed using Welch's t-test and Mantel-Haenszel's test was performed to assess the distribution of patients. For bioinformatics analysis, Student's t-test for paired samples was performed. Pearson's r correlation coefficient was calculated to assess correlation. Two-tailed $P<0.05$ was considered to indicate a statistically significant difference.

Results

NCoA3 levels in adipocytes are modulated by secreted factors from certain breast tumor cell lines. AT is considered relevant in the study of tumor development (3,25-27); however, it remains unknown as to which molecules are involved in the changes that tumor-associated adipocytes undergo. Among the transcription factors studied in these cells, NF-κB is known to exert several effects on the tumor microenvironment (28).

The present study first investigated NF-κB activation in BrCM-stimulated 3T3-L1 adipocytes, using immunodetection of the phosphorylated form of the NF-κB subunit p65 (p-p65) (29). It was revealed that BrCM from all breast cancer cell lines enhanced the presence of p-p65 in the nucleus when compared with that in cells under basal conditions and non-tumoral MCF10A BrCM (Fig. 1A-C). The average maximum fluorescence intensity for each experimental condition was calculated, as it is inaccurate to average the profiles given the differences in size and rounded shape of the adipocytes. The fluorescence profiles shown in Fig. 1B are representative of the cell population in each condition observed in Fig. 1A.

Since our previous studies demonstrated that NCoA3 is a NF-κB transcriptional coactivator, which is overexpressed in several types of cancer and is associated with pro-tumoral effects mediated by NF-κB (11,13,14,30), the present study evaluated whether NCoA3 levels in 3T3-L1 adipocytes were modulated upon BrCM treatment. Notably, it was observed that only BrCM from the T-47D cell line induced a marked increase in the mRNA and protein expression levels of NCoA3 (Fig. 1D and E).

Subsequently, the levels of TNF and MCP1 were detected, which are both regulated by NF-κB, involved in pro-inflammatory processes and have been reported to be secreted by adipocytes adjacent to tumors (6,26,31). Notably, only T-47D BrCM was capable of inducing a significant increase in the expression of both molecules (Figs. 1F and G, and S1A).

These findings indicated that signals released by tumor cells have the ability to regulate the activation of the transcription factor NF-κB. However, NCoA3 expression in adipocytes seems to depend on the phenotypic characteristics of the breast cancer cell line. Notably, only when NCoA3 levels were

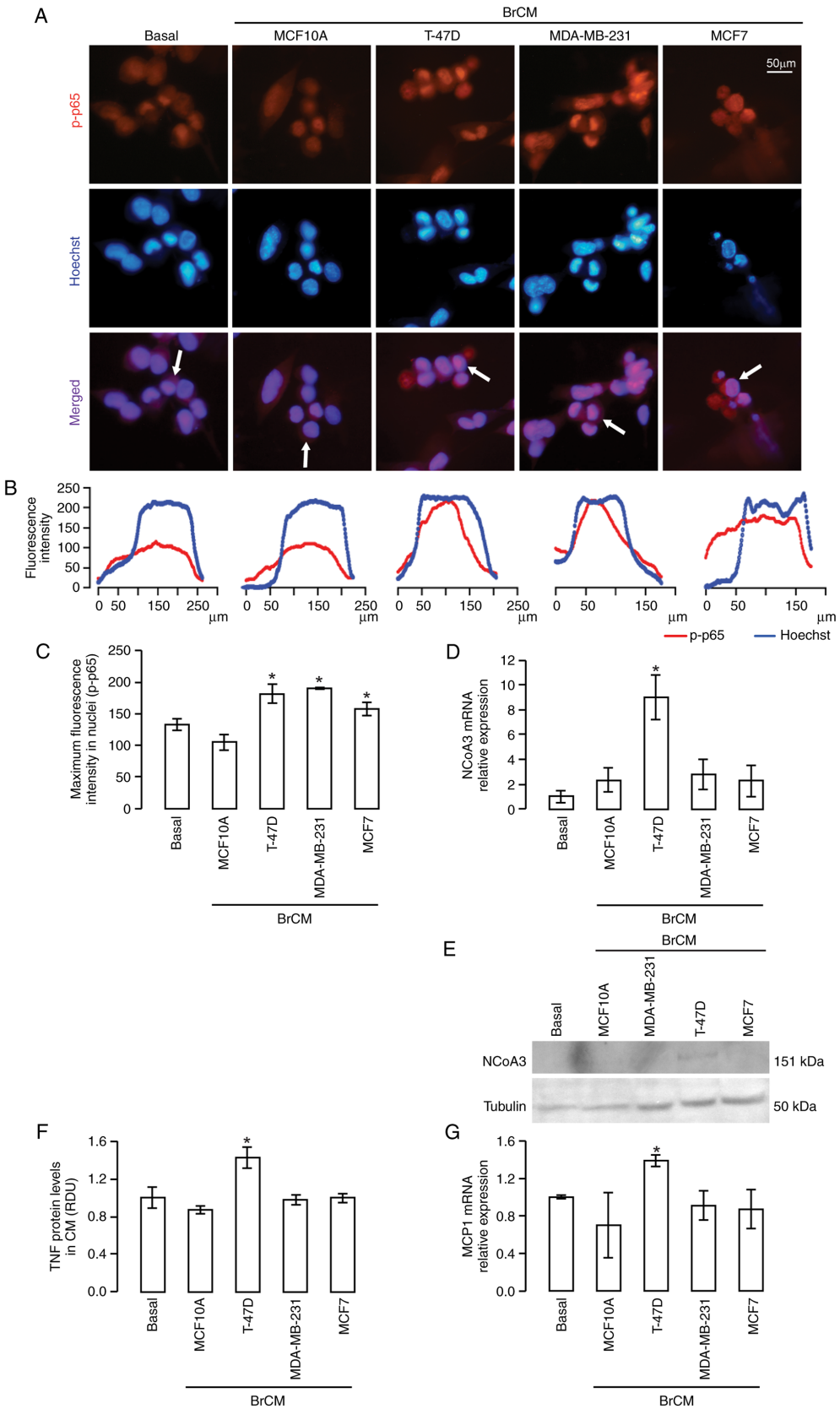

Figure 1. NCoA3 expression in 3T3-L1 adipocytes stimulated with BrCM. p-p65 was detected by immunofluorescence. (A) Representative microphotographs are shown; upper panels, p-p65 staining (red); middle panels, Hoechst staining (blue); lower panels, merged. (B) Plots showing the fluorescence intensity profile of the indicated cells in (A) (white arrows). (C) Maximum fluorescence intensity quantification was performed in ≥10 individual cell profiles in 10 fields for each condition. *P<0.05 vs. basal or MCF10A, one-way ANOVA and Tukey's post hoc test. (D) NCoA3 expression was evaluated in 3T3-L1 adipocytes by RT-qPCR. *P<0.05 vs. basal or MCF10A. (E) NCoA3 and tubulin protein expression levels were assessed by western blotting in extracts from 3T3-L1 adipocytes stimulated with or without BrCM. (F) TNF secretion was studied by dot blot assay. (G) mRNA expression levels of MCP1 were determined by RT-qPCR and normalized to CyA. *P<0.05 vs. MCF10A, one-way ANOVA and Tukey's post hoc test. Data are presented as the mean ± SEM. BrCM, conditioned medium from breast cell lines; CyA, cyclophilin A; MCP1, monocyte chemoattractant protein 1; NCoA3, nuclear receptor coactivator 3; p-p, phosphorylated; RDU, relative densitometry units; RT-qPCR, reverse transcription-quantitative PCR; TNF, tumor necrosis factor.

upregulated, were the expression levels of TNF and MCP1 significantly increased.

NCoA3 knockdown or NF-κB inhibition in 3T3-L1 adipocytes reverses pro-inflammatory adipokine expression. The aforementioned results revealed a relationship between the expression levels of NCoA3 and inflammatory markers. In order to further study this association, 3T3-L1-derived adipocytes transfected with shNCoA3 or control plasmid under T-47D BrCM stimulation were assessed. RT-qPCR and western blot analysis were performed to validate the knockdown efficiency (Fig. 2A).

In order to confirm the role of NF-κB activation in the upregulation of TNF and MCP1 induced by T-47D BrCM, 3T3-L1 adipocytes were pre-incubated with SSZ before stimulation with T-47D BrCM. As shown in Fig. 2B-D, pretreatment with SSZ inhibited T-47D BrCM-induced p65 phosphorylation. In agreement with this result, the expression levels of TNF and MCP1 were diminished in the presence of SSZ, confirming that the expression of these genes in adipocytes is regulated by NF-κB (Figs. 2E and F, and S1B). Notably, NCoA3 knockdown also reversed the effects induced by BrCM from T-47D cells on TNF and MCP1 expression, in the same way as NF-κB inhibition (Figs. 2E and F, and S1B).

These results indicated that NCoA3 expression may be required to regulate NF-κB-dependent inflammatory mediators, such as TNF and MCP1. As NF-κB is a key transcription factor of a number of pro-inflammatory genes, the role of NCoA3 in their expression should be considered. In response to T-47D BrCM, the levels of TNF and MCP1 were increased in adipocytes; this is relevant since both molecules are associated with inflammation.

NCoA3 expression levels in MAT are modulated by T-47D BrCM. The characteristics of AT depend on its location; notably, MAT shows totally distinctive features compared with visceral and subcutaneous AT (32). Accordingly, the present study analyzed murine MAT following BrCM treatment. Taking into account the results obtained in 3T3-L1 adipocytes, T-47D and MDA-MB-231 BrCM were used, as T-47D BrCM was able to upregulate NCoA3 expression, whereas MDA-MB-231 BrCM was not. Since MCF7 cells lack a secretory pro-inflammatory profile, CM from this cell line was excluded from these experiments (33).

The results revealed that murine MAT exhibited a significant increase in the mRNA expression levels of NCoA3 following stimulation with T-47D BrCM (Fig. 3A), which is consistent with the results obtained in 3T3-L1 adipocytes. In addition, similar results were obtained regarding the protein expression levels of NCoA3 measured by IHC staining, and the protein localization was mainly nuclear (Fig. 3B and C).

The present study also analyzed NF-κB activation; it was observed that murine MAT stimulated with T-47D BrCM exhibited enhanced staining of p-p65 determined by IHC analysis, as compared with in MAT under basal conditions and that stimulated with MDA-MB-231 BrCM (Fig. 3D and E). These findings demonstrated that the transcription factor NF-κB was induced by T-47D-secreted molecules.

Alongside the increase in NF-κB activation and NCoA3 expression, it was revealed that T-47D BrCM increased the levels of TNF secreted by MAT (Figs. 3F and S1C). Subsequently, MCP1 expression was detected and it was observed that, as with TNF, only the BrCM from T-47D cells induced MCP1 expression in murine MAT (Fig. 3G).

In conclusion, these results mimic what was observed in 3T3-L1 adipocytes and suggested that breast cancer cells take advantage of adipocyte plasticity, stimulating them in different ways. Furthermore, in MAT when NCoA3 is upregulated, pro-inflammatory molecules are also increased.

Table I. Association between NCoA3 expression in mammary adipose tissue and clinicopathological features of breast cancer.

Variable	Number of patients	NCoA3 expression (RT-qPCR)	
		Low (%)	High (%)
Sample type			
Non-tumoral lesion	4	3 (75)	1 (25)
Malignant breast cancer	32	13 (41)	19 (59)
Clinical stage			
I	7	4 (57)	3 (43)
II-III	24	9 (37)	15 (63)
Molecular status			
Luminal	19	10 (53)	9 (47)
Triple negative	9	2 (22)	7 (78)

NCoA3, nuclear receptor coactivator 3; RT-qPCR, reverse transcription-quantitative PCR.

NCoA3 expression is associated with a pro-inflammatory profile in tumors in MAT from patients with breast cancer. Hybrid human/mouse models have been widely used to study the interaction between tumors and AT (34,35). However, it is important to study whether the phenomena observed in these models actually occur in patients. Therefore, MAT samples were collected from patients with breast cancer or mammary non-tumoral -lesions. Adjacent to breast tumors, a total of 59% of MAT samples exhibited high NCoA3 mRNA expression levels, as determined by RT-qPCR (Table I; Fig. 4A). In addition, similar findings were determined regarding the protein expression levels of NCoA3, which were detected by IHC staining, and as in mice, the subcellular location of NCoA3 was nuclear (Fig. 4B and C).

The association between NCoA3 expression in MAT surrounding tumors and molecular tumor markers or clinical stage was also assessed. Regarding molecular tumor markers, when AT samples adjacent to lesions were classified into low or high NCoA3 expression, the distribution of patients significantly differed between the groups. Surrounding AT presented high NCoA3 expression in 25% of patients with non-tumoral lesions, in 47% of patients with luminal tumors and 78% of patients with triple negative (TN) tumors (Fig. 4D). According to clinical stages, differences were also detected in the distribution of patients between groups. AT adjacent to lesions presented high NCoA3 expression in 43% of patients with

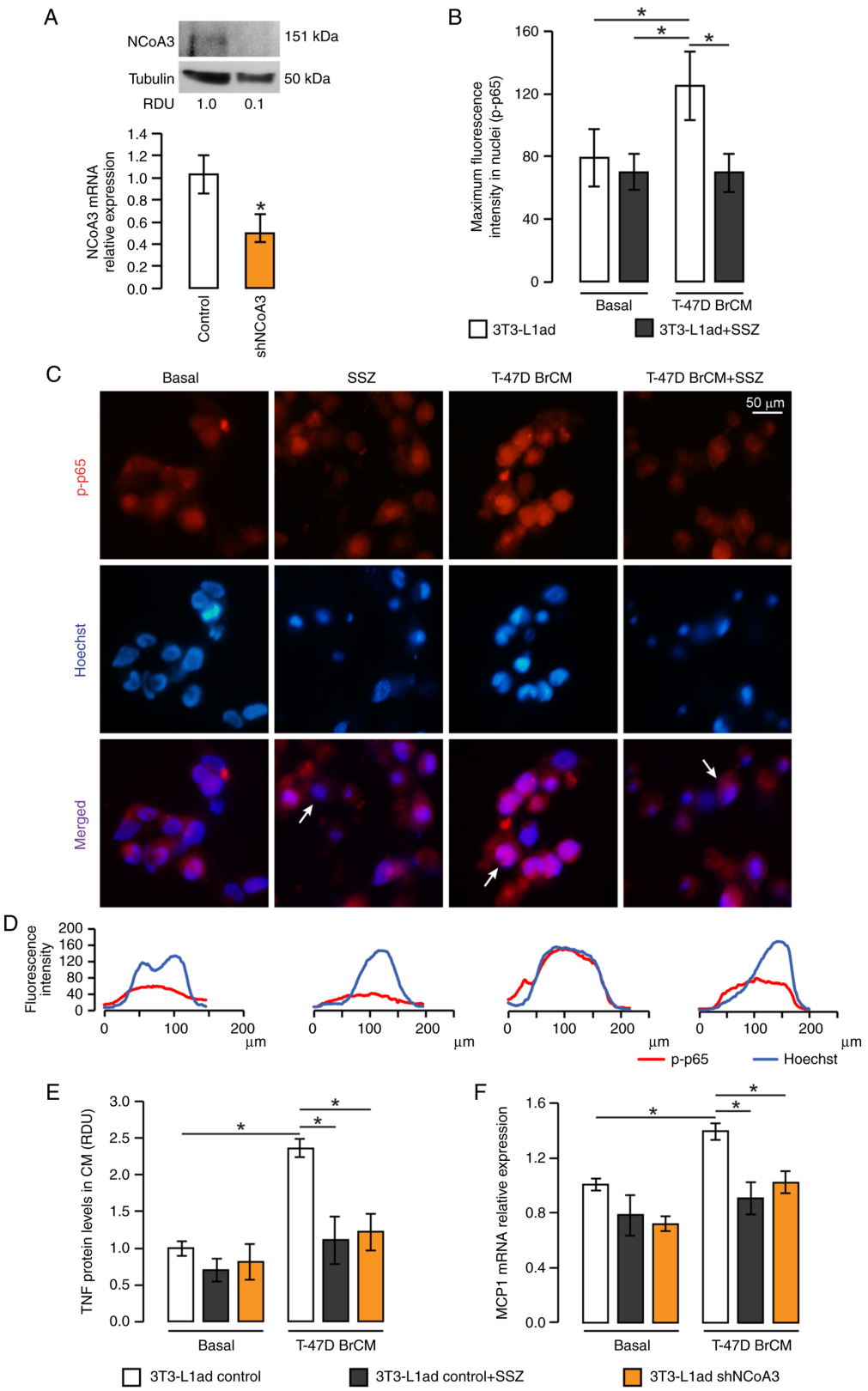

Figure 2. NCoA3 knockdown inhibits the expression of pro-inflammatory markers. (A) Knockdown efficiency of shNCoA3 in 3T3-L1 cells. NCoA3 mRNA expression was determined by RT-qPCR and was normalized to CyA mRNA expression, and NCoA3 protein expression was determined by western blotting. *P<0.05 vs. control, unpaired Student's t-test. RDU corresponds to the densitometry unit with respect to tubulin expression. p-p65 location was studied by immunofluorescence in 3T3-L1 adipocytes stimulated with BrCM from T-47D tumoral cells or with serum-free medium (basal) in the presence or absence of SSZ (0.2 mM). (B) Maximum fluorescence intensity quantification for each condition. *P<0.05, two-way ANOVA and Tukey's post hoc test (C) Representative microphotographs are shown. (D) Plots show the fluorescence intensity profile of the indicated cells in (C) (white arrows). Control or shNCoA3-transfected 3T3-L1 adipocytes were incubated with T-47D BrCM or serum-free medium in the presence or absence of SSZ. (E) TNF secretion was studied by dot blot assay, and membranes were incubated with anti-TNF antibody. (F) mRNA MCP1 expression was determined by RT-qPCR and normalized to CyA mRNA. *P<0.05, two-way ANOVA and Tukey's post hoc test. Data are presented as the mean ± SEM. BrCM, conditioned medium from breast cell lines; CyA, cyclophilin A; MCP1, monocyte chemoattractant protein 1; NCoA3, nuclear receptor coactivator 3; p-p, phosphorylated; RDU, relative densitometry units; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; SSZ, sulfasalazine; TNF, tumor necrosis factor.

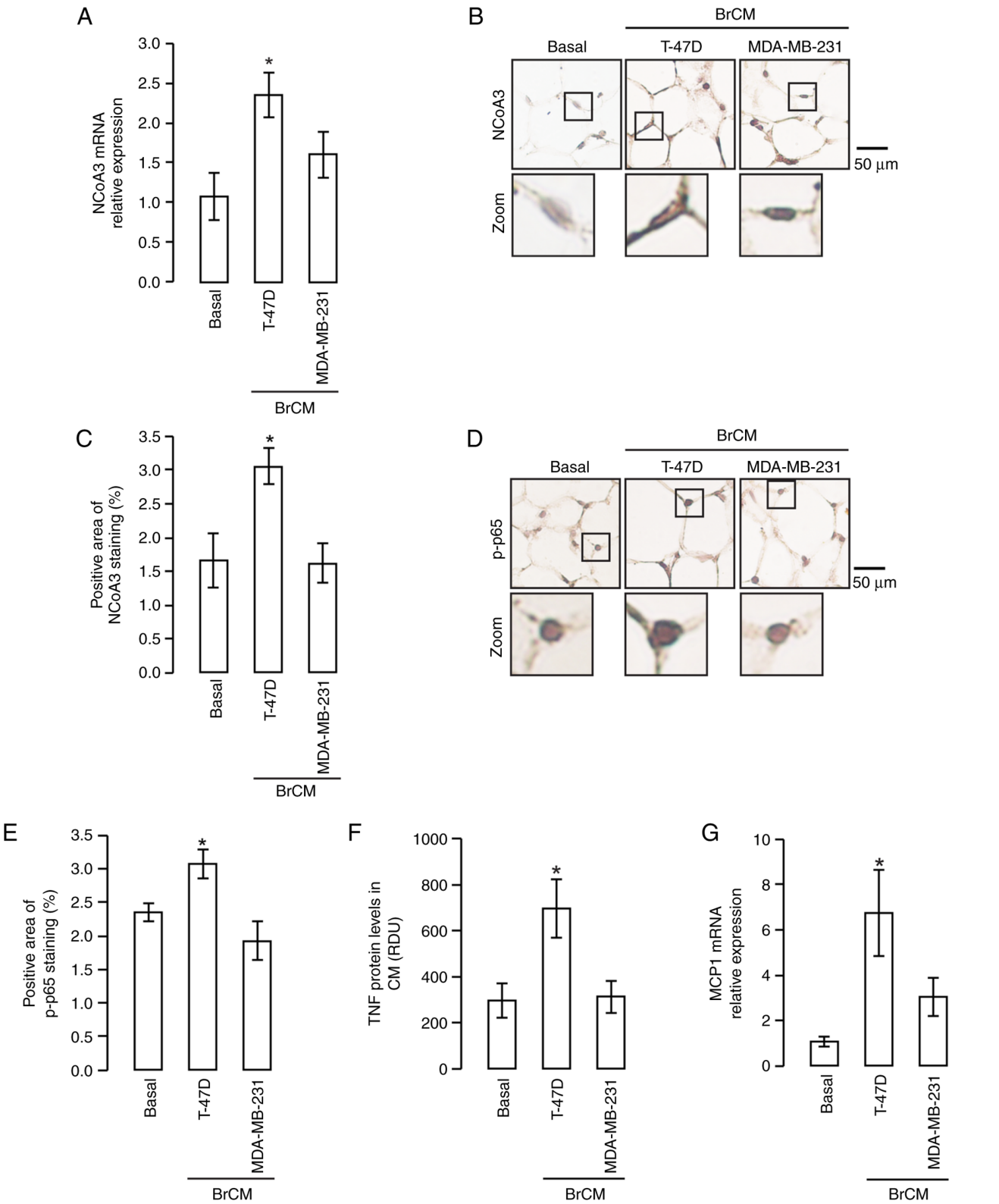

Figure 3. NCoA3 expression in MAT stimulated with BrCM NCoA3 expression levels were detected in murine MAT cultured with or without BrCM from T-47D or MDA-MB-231 cell lines. (A) NCoA3 mRNA expression was analyzed by RT-qPCR. *P<0.05 vs. basal, one-way ANOVA and Tukey's post hoc test. Immunostaining of NCoA3 protein was performed. (B) Representative microphotographs (magnification, x400) are shown, lower panels are amplifications of the black boxes in the upper panels. (C) Semi-quantification of positive NCoA3 staining area. Immunostaining of p-p65 protein was performed in murine MAT cultured with or without BrCM from T-47D or MDA-MB-231 cell lines. *P<0.05 vs. basal, one-way ANOVA and Tukey's post hoc test. (D) Representative microphotographs (magnification, x400) are shown, lower panels are amplifications of the black boxes in the upper panels. (E) Semi-quantification of positive p-p65 staining area. *P<0.05 vs. basal, one-way ANOVA and Tukey's post hoc test. (F) TNF secretion was studied by dot blot assay in CM from murine MAT after being incubated with BrCM for 24 h followed by incubation in serum-free medium for another 24 h. *P<0.05 vs. basal, one-way ANOVA and Tukey's post hoc test. (G) MCP1 expression was analyzed by RT-qPCR in murine explants cultured in the presence or absence of BrCM. *P<0.05 vs. basal, one-way ANOVA and Tukey's post hoc test. Data are presented as the mean ± SEM. BrCM, conditioned medium from breast cell lines; CyA, cyclophilin A; MAT, mammary adipose tissue; MCP1, monocyte chemoattractant protein 1; NCoA3, nuclear receptor coactivator 3; p-p, phosphorylated; RDU, relative densitometry units; RT-qPCR, reverse transcription-quantitative PCR; TNF, tumor necrosis factor.

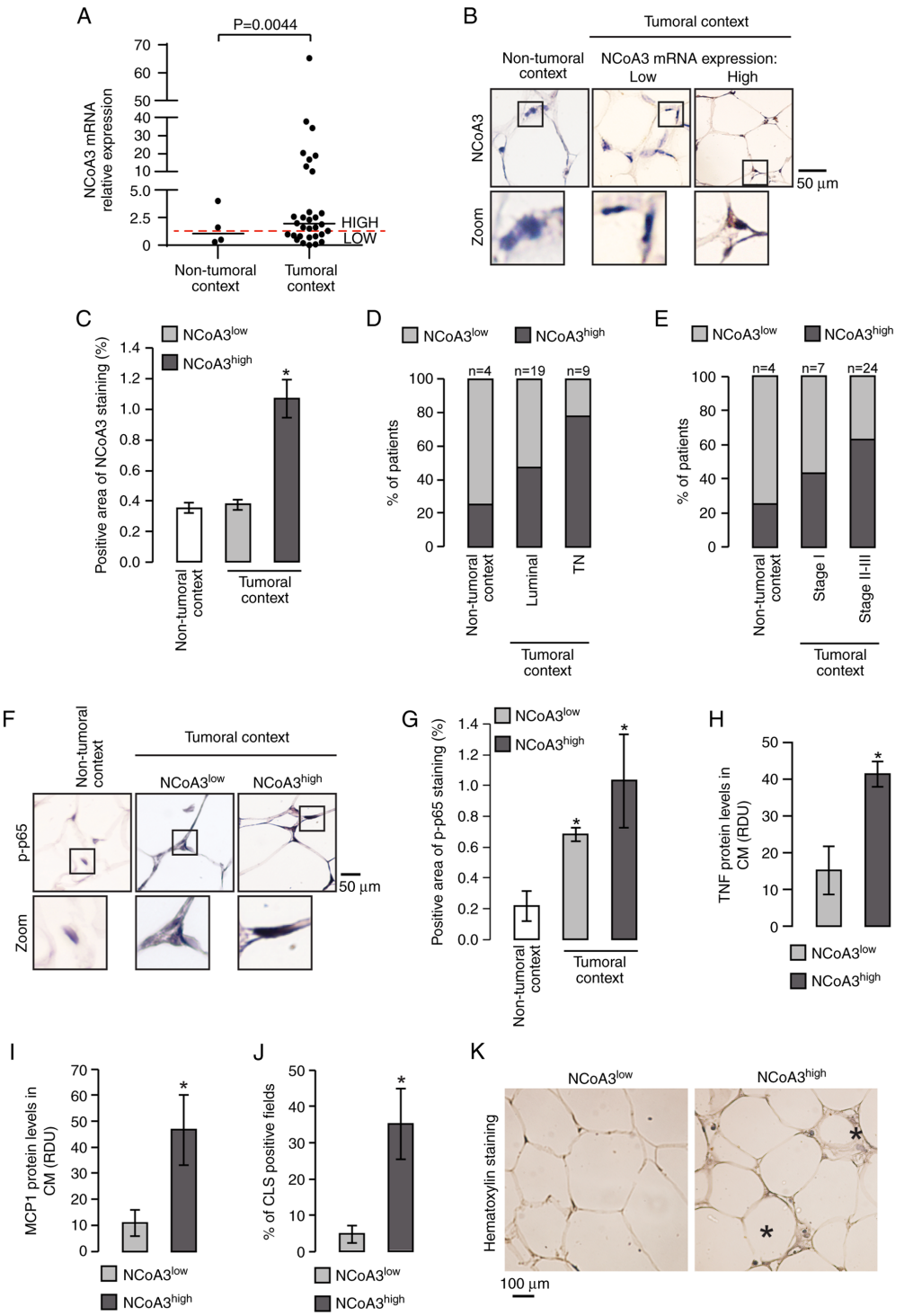

Figure 4. Association between NCoA3 levels and NF-κB activation in MAT from the tumor microenvironment: (A) NCoA3 mRNA expression levels were measured by reverse transcription-quantitative PCR in MAT from patients with non-tumoral or tumoral lesions. Horizontal lines indicate the median of NCoA3 expression and each dot corresponds to one patient. P<0.005, Welch's test. The red dotted line corresponds to the average of NCoA3 levels obtained in MAT from a non-tumoral context. Immunostaining of NCoA3 protein was performed in AT adjacent to mammary glands with non-tumoral lesions or breast cancer. (B) Representative microphotographs (magnification, x400) are shown, lower panels are amplifications of the black boxes in the upper panels. (C) Graph corresponds to positive NCoA3 staining area. Data are presented as the mean ± SEM. *P<0.05 vs. $NCoA3^{low}$ in tumoral context and non-tumoral context, one-way ANOVA and Tukey's post hoc test. Bar graphs show the distribution of patients (expressed as a percentage) with high or low NCoA3 expression in the AT adjacent to their breast tumors classified by (D) molecular status (TN and luminal: Estrogen receptor-positive and/or progesterone receptor-positive) and (E) breast cancer stage. P<0.05, Mantel-Haenszel's test. p-p65 was detected by immunohistochemistry in MAT adjacent to breast tumors or non-tumoral lesions. (F) Representative microphotographs are shown; lower panels are amplifications of the black boxes in the upper panels. (G) Graph shows positive p-p65 staining area. Data are presented as the mean ± SEM. *P<0.05 vs. non-tumoral context, one-way ANOVA and Tukey's post hoc test. (H) TNF and (I) MCP1 secretion was determined by dot blot assay in the CM from MAT explants obtained from patients with breast cancer. Data are presented as the mean ± SEM. *P<0.05 vs. low NCoA3 expression, unpaired Student's t-test. (J) Analysis of CLS in hematoxylin-stained MAT from patients with breast cancer expressing low or high NCoA3, bars indicate the percentage of fields containing at least one CLS per patient sample. Data are presented as the mean ± SEM. *P<0.05 vs. low NCoA3 expression, unpaired Student's t-test. (K) Representative microphotographs are shown, asterisks indicate CLS. CLS, crown-like structures; H&E, hematoxylin and eosin; MAT, mammary adipose tissue; MCP1, monocyte chemoattractant protein 1; NCoA3, nuclear receptor coactivator 3; p-p, phosphorylated; RDU, relative densitometry units; TN, triple negative; TNF, tumor necrosis factor.

stage I cancer and in 63% of patients with stages II-III cancer, in comparison to 25% observed in patients with non-tumoral lesions (Fig. 4E).

Furthermore, human MAT in the tumoral context showed an increase in the nuclear staining of p-p65, which was independent of NCoA3 expression levels (Fig. 4F and G). However, MAT expressing high levels of NCoA3 secreted more TNF (Figs. 4H and S1D).

The measurement of MCP1 levels revealed a positive association with NCoA3 expression levels (Figs. 4I and S1E). MCP1 is a chemokine responsible for recruiting macrophages, and during local white AT inflammation, the presence of dead adipocytes surrounded by macrophages, which form CLS, have been observed (27). Therefore, the present study analyzed the number of CLS in hematoxylin-stained sections. An increased percentage of CLS was observed per field in MAT expressing high levels of NCoA3 (Fig. 4J and K).

The present study aimed to validate the results obtained in the cohort of patients with breast cancer; therefore, the records from the E-MTAB-8638 dataset were analyzed (23). In accordance with the results of the present study, a significant increase in NCoA3 mRNA expression was detected in MAT adjacent to human breast tumors compared with that in distant MAT (Fig. 5A). Notably, high expression levels of NCoA3 were associated with increased expression of several inflammatory molecules in adMAT, including TNF and CCL2 (Fig. 5B). There was also a positive correlation between NCoA3 expression and CD68, a macrophage marker (Fig. 5C).

Our previous study reported that NCoA3 expression is positively modulated by inflammation (7); therefore, the profile of inflammatory cytokines in breast tumors was evaluated according to their molecular characteristics. A bioinformatics analysis of 394 human breast cancer samples from TCGA was performed using the USCS Xena platform (36). It was revealed that most of the TN tumors presented a pro-inflammatory profile, whereas a small subgroup of luminal tumors exhibited these features (Fig. 5D). However, the statistical analysis showed significant differences in cytokine expression between the luminal and TN breast cancer groups (Fig. 5E). In addition, when tumors were classified by clinical stage, there were no differences between patients with stage I cancer and those with cancer in more advanced stages (II-III) (data not shown).

Consistent with the results obtained in murine MAT and 3T3-L1 adipocytes, the patients that exhibited upregulation of NCoA3 expression in cancer-associated AT also presented active nuclear NF-κB, and high levels of TNF and MCP1. Notably, CLS were identified in AT expressing high NCoA3 levels, which is a strong indicator of AT inflammation. Furthermore, most TN tumors exhibited NCoA3 upregulation in adjacent AT (7/9 patients). Moreover, this type of tumor was revealed to express pro-inflammatory cytokines, which could be involved in NCoA3 modulation.

Discussion

It has been demonstrated that stromal cells and their secreted factors can exert different and diverse effects on tumor development, influencing proliferation, invasion and metastasis (25). Experiments performed with CM revealed that secreted proteins from adipocytes have effects more pronounced on breast cancer proliferation than factors released by other cell types (27). However, it is unknown which transcription factors and cofactors regulate adipokine expression in the tumor microenvironment.

In breast cancer, constitutive activation of the transcription factor NF-κB is associated with poor prognosis and chemoresistance, as well as with hormone-independent breast cancer progression (37). Since NF-κB has an important role in breast cancer development, the present study assessed the implication of this transcription factor in several models. The results revealed that adipocytes in a tumoral context had greater NF-κB activation, even though this is not enough to modulate the expression of the studied target genes. This transcription factor depends on the presence of cofactors, such as NCoA3 (30). To the best of our knowledge, the present study demonstrated for the first time that NCoA3 expression in breast tumor-associated adipocytes was upregulated by certain types of tumors. This result was confirmed by the data obtained in all of the models, including MAT adjacent to human breast tumors, murine MAT, cell line-derived adipocytes stimulated with BrCM and bioinformatics analysis of public datasets.

Previous studies have described a role for NCoA3 in AT under normal physiological conditions (38,39) and our previous study demonstrated that NCoA3 levels diminished during adipogenesis (8), revealing the importance of this coactivator in adipocyte behavior.

In breast cancer, it has been reported that the NCoA3 gene is amplified in 5-10% of human tumors and its protein is overexpressed in 60% of tumors (9,40). In the breast cancer context, the present results showed that 59% of MAT adjacent to tumors exhibited upregulation of NCoA3, suggesting that the expression of this coactivator gives a growth advantage to mammary tumors and the surrounding AT.

In breast tumors, the association between tumor NCoA3 expression levels and breast cancer molecular markers is contradictory, however, patients with breast cancer expressing high levels of tumoral NCoA3 have shown poor prognosis and chemoresistance (41-44). Despite the size of the present cohort, which represents a limitation of the present study, the percentage of patients expressing high levels of NCoA3 in AT increased in groups with worse prognosis (TN tumors and advanced stages). In general, TN tumors exhibit an inflammatory phenotype that, taking into account our previous results, could lead to the modulation of NCoA3 levels in AT (7).

In the present hybrid models, it was observed that T-47D BrCM upregulated NCoA3 expression in adipocytes more so than BrCM from non-tumoral MCF10A cells, and also tumoral MDA-MB-231 and MCF7 cell lines. The differential expression of NCoA3 in adipocytes could be due to differences in tumoral secretomes. Notably, it has been evidenced that these mammary tumor cell lines secrete distinct cytokine patterns; for example, T-47D cells release more pro-inflammatory cytokines, including TNF, than MDA-MB-231, MCF7 and MCF10A cell lines (33). Moreover, our previous study reported that TNF is able to induce NCoA3 expression via NF-κB activation (7) and this could explain high expression of this coactivator in adipocytes stimulated by T-47D BrCM. However, due to the heterogeneity of BrCM, other molecules

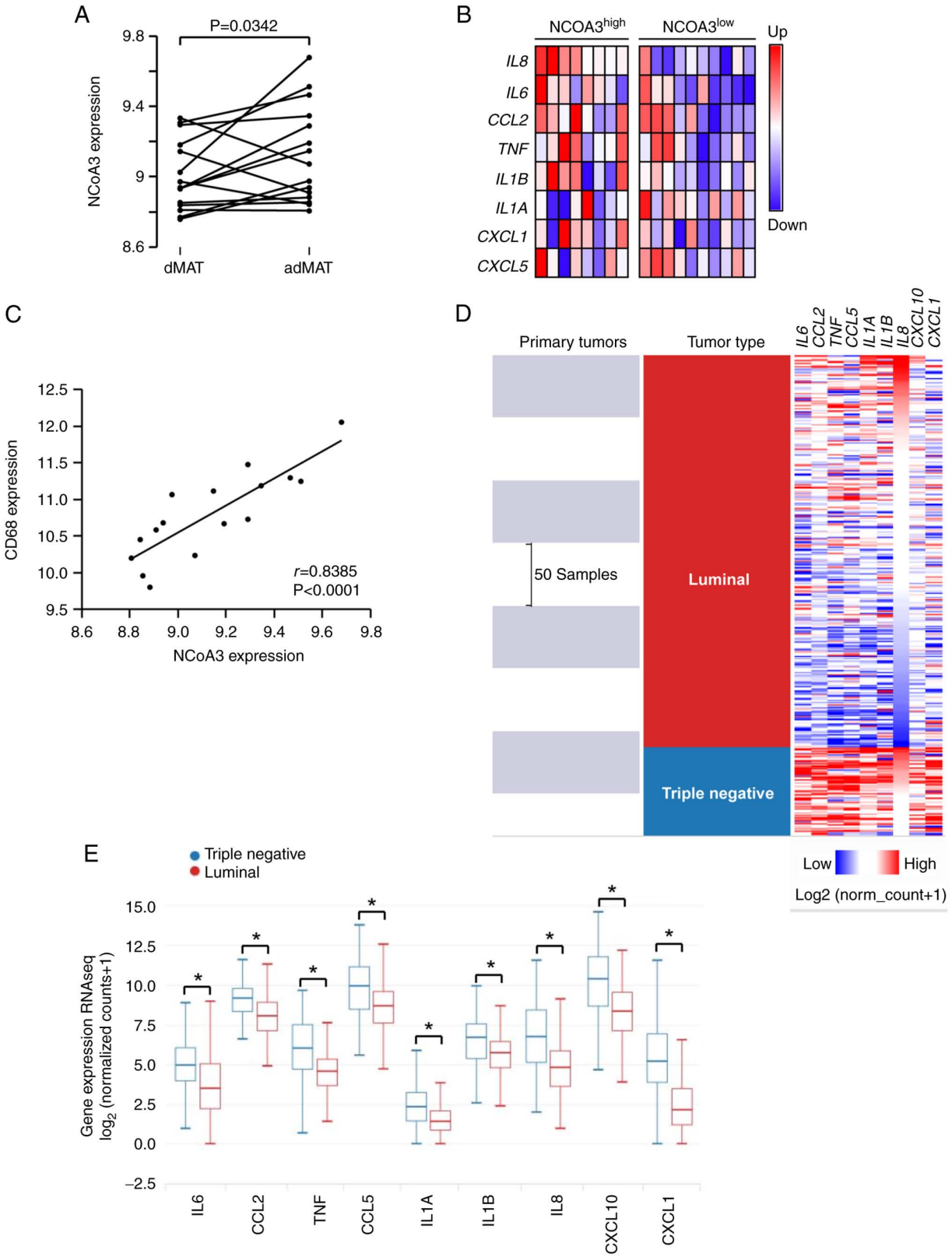

Figure 5. Association between NCoA3 expression and adipokines in MAT surrounding breast cancer. mRNA expression data in adMAT (0.5-1 cm from tumors) and dMAT (>5 cm from tumors) were retrieved from the E-MTAB-8638 dataset. (A) NCoA3 mRNA expression levels in dMAT and adMAT from patients with breast cancer. Each value obtained in each adMAT sample was paired with the corresponding value in dMAT sample from the same patient. P=0.0342 (n=16, paired Student's t-test). (B) Heatmap illustrates the differences in expression of each pro-inflammatory biomarker in adMAT regarding NCoA3 expression levels, each column represents one patient (n=18). (C) Scatter plot shows the correlation between CD68 expression levels and NCoA3 expression levels in adMAT (n=16, Pearson r=0.8385; P<0.001). Bioinformatics analysis was performed using the Xena platform. (D) Human breast cancer primary tumor samples (The Cancer Genome Atlas Breast Cancer) were classified according to molecular features and their cytokine profile was analyzed. Samples undetermined for the selected variable were excluded (394 valid datapoints). Red represents upregulated expression whereas blue corresponds to downregulated expression. (E) Box plot of cytokine expression (IL6, CCL2, TNF, CCL5, IL1A, IL1B, IL8, CXCL10 and CXCL1) in triple-negative and luminal breast tumor samples. *P<0.00005, Welch's test. adMAT, MAT adjacent to human breast tumors; dMAT, distant MAT; MAT, mammary adipose tissue; NCoA3, nuclear receptor coactivator 3.

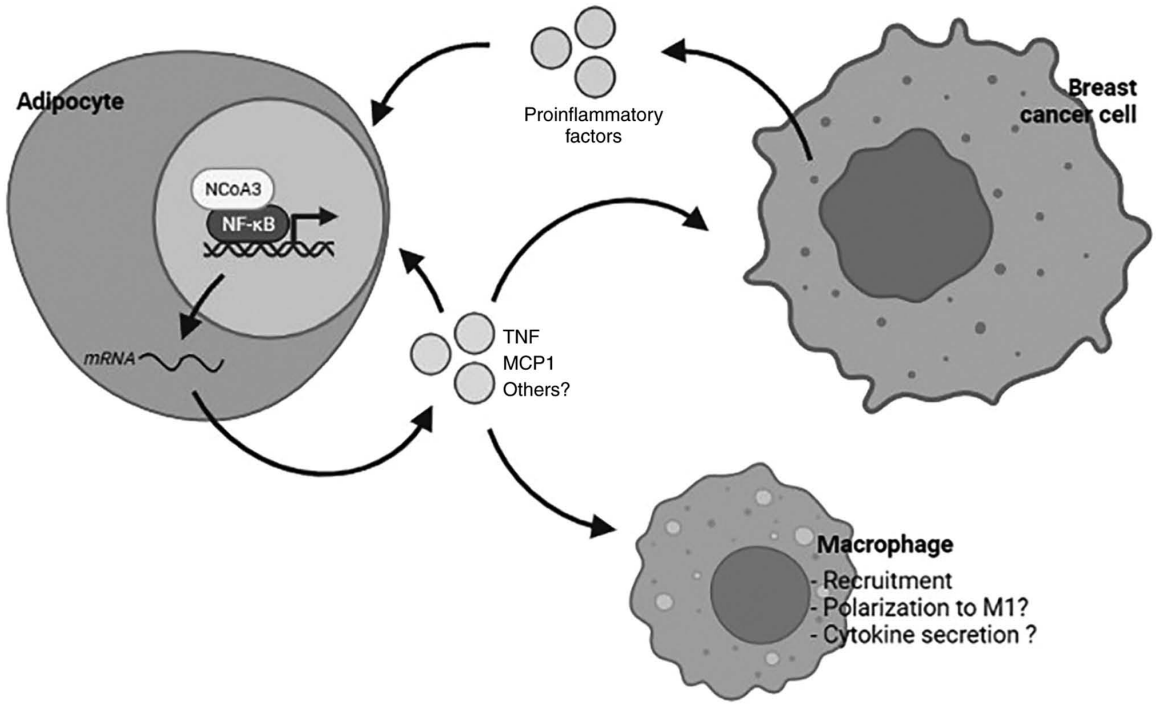

Figure 6. Role of NCoA3 in breast tumor-surrounding adipose tissue. Tumors secrete factors that trigger the upregulation of NCoA3 expression and NF-κB activation in adjacent adipocytes. The modulation of these molecules induce pro-inflammatory cytokines and chemokines that promote cancer-associated inflammation. This original figure was created using BioRender.com. MCP1, monocyte chemoattractant protein 1; NCoA3, nuclear receptor coactivator 3; TNF, tumor necrosis factor.

should not be excluded. Furthermore, elucidation of the pathways leading to the regulation of NCoA3 expression is the aim of our future studies.

Luminal A cell lines show differences in biological behavior and protein expression. In this regard, proteomic data have suggested that T-47D cells express a higher number of proteins than MCF7 cells (33,45,46). According to their biological functions, proteins expressed in T-47D cells are involved in cell proliferation stimulation, anti-apoptosis mechanisms and tumorigenesis, and only a few biological processes are shared by both cell lines (33,45,46). Notably, our previous study demonstrated that the pro-inflammatory cytokine TNF, via NF-κB, ER and NCoA3 complex, can stimulate T-47D cell proliferation, but this effect was not observed in MCF7 cells (13), demonstrating that this tumoral cell line takes advantage of inflammation. Furthermore, the MCF7 cell line has been shown to be sensitive to TNF-induced cell death (47).

Although the present study used BrCM from human cell lines to stimulate murine adipocytes and the cross-reaction should not be guaranteed, the capability of human molecules to bind and stimulate signaling cascades in mice has been reported by other groups (34,35,48).

In the present results obtained from patient samples, it was observed that TN tumors exhibited NCoA3 upregulation in AT. However, the TN tumor cell line (MDA-MB-231) used in the *in vitro* study did not elicit this effect, which could be explained by its non-proinflammatory profile (33). Furthermore, there was a small subgroup of patients with TN breast cancer from TCGA database that showed non-proinflammatory features. In addition, several patients with luminal tumors expressed high levels of pro-inflammatory cytokines, revealing that this inflammatory phenotype does not exclusively depend on the molecular markers. Notably, although one of the non-tumoral samples was revealed to express high NCoA3 levels, it was from a patient diagnosed with mastitis, which by clinical definition is characterized by localized inflammation. These findings suggested the relevance of the inflammatory context to NCoA3 expression.

NCoA3 enhances NF-κB activity (30) and this transcription factor is activated by a wide range of stimuli, such as cytokines and mitogens, and regulates the expression of these molecules and others like adhesion molecules, metalloproteinases and apoptotic genes (6). Two of the NF-κB target genes that are crucial to the inflammatory process are TNF and MCP1. Particularly, TNF exerts several effects not only on tumor cells but also on other stromal cells, including adipocytes, in an autocrine manner, whereas MCP1 is responsible for monocyte infiltration and differentiation into macrophages (1,13,27,31,49). Previous studies have shown that adipocytes can produce cytokines, such as TNF and MCP1, in the inflammatory context (5,26). Moreover, the present study revealed that fat cells in a tumoral context not only expressed more NCoA3 but also presented high levels of TNF and MCP1.

The dysregulated secretion of pro-inflammatory cytokines and chemokines, such as TNF and MCP1, by adipocytes can lead to recruitment of macrophages and the appearance of CLS (5,49), as observed in the present study. CLS are a hallmark of chronic AT inflammation, and increased numbers of CLS in the breast have been associated with increased risk and poor prognosis of patients with breast cancer in both obese

individuals and those with a normal body mass index (49). CLS formation has been shown to enhance the expression of inflammatory cytokines/chemokines, further activating macrophages for clearing apoptotic adipocytes, generating a positive feedback.

Given the association between NCoA3 and the inflammatory profile of breast cancer-associated AT, it would be of great interest in the future to evaluate a panel of inflammatory cytokines on the CM from samples obtained from responders and non-responders to current treatments according to immune profile. This analysis could be correlated with NCoA3 expression in order to determine whether this coactivator influences antitumor immunity. However, the informed consent of the patients for the present study only involved the study of mammary AT and clinical history; therefore, we were not able to obtain the corresponding tumor samples for this approach.

The present study also demonstrated that NCoA3 silencing or NF-κB inhibition resulted in a similar reversal in cytokine expression, suggesting that these molecules are co-dependent in this process. These results are relevant considering that the evaluated cytokines favor tumor progression (13,50), and contribute to the comprehension of the interaction between adipocytes and breast cancer.

In conclusion, to the best of our knowledge, this is the first time that the expression of NCoA3 has been reported to be increased in MAT adjacent to breast cancer. It was hypothesized that breast cancer-secreted factors may induce the activation of NF-κB and upregulation of NCoA3 expression, which in turn could induce the transcription of TNF and MCP1. Once these inflammatory cytokines are released, they could have effects on adipocytes themselves, breast cancer cells and also macrophages, propitiating an inflammatory context that promotes tumor progression and worse prognosis (Fig. 6).

As adipocytes are involved in the development and progression of breast cancer, the modulation of NCoA3 levels in MAT and its possible role in inflammatory processes related to cancer deserve to be further investigated to improve future cancer treatments.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the EBI databank, https://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-8638/. The other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MCL, FDR, IA, AGP and MSM were involved in investigation, methodology, data curation and formal analysis. MCL performed the experiments using the 3T3-L1 adipocyte model. MCL, FDR, IA and NP were in charge of animal handling, and obtained murine MAT and performed related experiments. MCL, AGP, MSM, MCSG, LP, SB, PJA were involved in obtaining and processing human AT, and performed the related experiments and corresponding validation. AGP, MAC and MFR were in charge of data curation and validation. MAC was involved in funding acquisition, supervision, conceptualization, reviewing and editing. MFR was involved in conceptualization, project administration, supervision and writing the original draft. MAC and AGP confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures involving human participants were approved by the Institutional Research Ethic Committee of Institute of Medical Research Alfredo Lanari (approval no. #312), in accordance with the ethical standards of the National Law No 25326 'Protección de datos personales', Res. 1480/11, Art 58 and 59 from Commercial and Civil Code of Argentina The 1964 Declaration of Helsinki and its later amendments, or comparable ethical standards. Written informed consent was obtained from all individual participants included in the study. Animal experimental protocols performed in the present study were approved by the National University of Quilmes Institutional Animal Care and Use Committee, in accordance with the National Institutes of Health guide and the ARRIVE guide for the care and use of laboratory animals.

Patient consent for publication

Participants provided written informed consent for publication.

Competing interests

The authors declare that they have no competing interests.

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