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Identification of an AP1-ZFP36 Regulatory Network Associated with Breast Cancer Prognosis

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Abstract

It has been established that ZFP36 (also known as Tristetraprolin or TTP) promotes mRNA degradation of proteins involved in inflammation, proliferation and tumor invasiveness. In mammary epithelial cells *ZFP36* expression is induced by STAT5 activation during lactogenesis, while in breast cancer *ZFP36* expression is associated with lower grade and better prognosis. Here, we show that the AP-1 transcription factor components, i.e. *JUN, JUNB, FOS, FOSB*, in addition to *DUSP1, EGR1, NR4A1*, *IER2* and *BTG2*, behave as a conserved co-regulated group of genes whose expression is associated to *ZFP36* in cancer cells. In fact, a significant down-modulation of this gene network is observed in breast, liver, lung, kidney, and thyroid carcinomas compared to their normal counterparts. In breast cancer, the normal-like and Luminal A, show the highest expression of the *ZFP36* gene network among the other intrinsic subtypes and patients with low expression of these genes display poor prognosis. It is also proposed that AP-1 regulates *ZFP36* expression through responsive elements detected in the promoter region of this gene. Culture assays show that AP-1 activity induces *ZFP36* expression in mammary cells in response to prolactin (PRL) treatment thorough ERK1/2 activation. These results suggest that *JUN, JUNB, FOS and FOSB* are not only co-expressed, but would also play a relevant role in regulating *ZFP36 expression* in mammary epithelial cells.

Keywords ZFP36 · TTP · AP-1 · Breast cancer · Mammary gland

Introduction

The *ZFP36* gene encodes a protein with zinc finger domains (CCCH), which targets AU-rich sequences (AREs) located at the mRNA 3' UTRs [1]. It is known that ZFP36 negatively regulates the stability and translational efficiency of mRNAs involved in inflammation [2] and its deficiency has been associated to the development of several immunological diseases through up-regulation of multiple cytokines [3–5]. On the other hand, the number of evidences supporting the tumor suppressor role of *ZFP36* has significantly grown [6, 7]. Low *ZFP36* expression levels have been detected in tumors of the brain, prostate, thyroid, ovary, uterus and breast, among others [8, 9]. Besides, it has been reported that mRNA stability of numerous genes involved in tumor progression, such as *TNFα, CMYC, COX2, HIF1, IL6, MMP1, VEGF* and *uPA/uPAR,* is increased in response to ZFP36 loss or down-regulation [7, 10], and that eventually leads to a poor prognosis for the patient [8]. Notably, restoring *ZFP36* expression in tumor cells suppresses cell proliferation, resistance to pro-apoptotic stimuli, and angiogenesis [8, 11]. Besides, it was determined that *Zfp36* expression is up-regulated in the lactating mammary gland, compared to virgin or pregnant, of healthy female mice, and in non-neoplastic HC11 mammary cells treated with the lactogenic hormones, prolactin and glucocorticoids. In addition, analysis of human breast samples showed moderate to strong ZFP36 protein expression in normal breast tissues and

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differentiated carcinomas, while ZFP36 expression was mostly negative in undifferentiated carcinomas [9].

Gene co-expression network analysis can be used for various purposes, including identification of gene-disease associations, gene regulatory networks, and functional gene annotation. Co-expression networks indicate correlations between genes that are simultaneously active, probably participating in the same biological processes [12]. Besides, it has been shown that detection of evolutionary conserved co-expressed genes allows effective predictions of regulatory networks [13]. Therefore, by analyzing the *ZFP36* regulatory network in different scenarios, we aimed to shed light on the mechanisms that regulate this gene expression in mammary cells.

Materials and Methods

Bioinformatics Analysis

Interspecific comparative analysis of *ZFP36* gene co-expression was performed using *Homo sapiens* and *Mus musculus* gene expression profiling data. Positive correlation analysis was carried out at the transcriptomic level on the *Multi Experiment Matrix* resource (http://biit.cs.ut.ee/mem/index.cgi) [14]. Using as template pattern the *ZFP36* gene expression, the following oligo-microarrays studies were analyzed: 100 datasets from *Homo sapiens* (representing 5391 normal samples) and 100 datasets from *M. musculus* (representing 3271 normal samples). The top 100 co-expressed genes from each species were detected and compared, for the identification of the commonly co-expressed genes among species. The cellular and biological processes associated with the commonly co-expressed genes were explored by gene enrichment analysis using *Enrichr* (http://amp.pharm.mssm.edu/Enrichr/enrich) [15] and *STRING 11.0* (https://string-db.org/) [16] was used to explore gene/proteins networks.

Co-expression correlation analyses, relevance networks and normal vs. tumor comparisons were conducted using human samples from TCGA Pan-Cancer dataset (7800 normal and tumor samples) obtained from UCSC Xena resource (http://xena.ucsc.edu/) [17] (cohort: TCGA Pan-Cancer - PANCAN; dataset: batch effects normalized mRNA data). Pearson's correlation scores and the corresponding *p*-values were computed using *R* to evaluate co-expression association among genes. Furthermore, a breast cancer dataset of 1211 cases (normal and tumor samples) from the TCGA-BRCA project was analyzed considering the breast cancer subtypes according to the RNAseq PAM50 call. Also, recurrence-free survival (RFS) analysis was conducted using the *Kaplan-Meier Plotter* resource (http://kmplot.com) over an independent breast cancer dataset [18].

Correlation analysis of ZFP36 mRNA expression levels with 1387 constituent integrated pathway activities predicted by PARADIGM was performed in Luminal A and normal-like tumors ($n = 433$) with data retrieved from UCSC Xena browser (cohort: TCGA Breast Cancer - BRCA; dataset: pathway activity - z score of 1387 constituent PARADIGM pathways). Top Pearson's correlation coefficients ($r > 0.6$; $p < 0.01$) were determined and visualized with R and MeV software, respectively.

Breast Tissue Samples

Breast tissue specimens were obtained from Dr. Leonidas Lucero Hospital (Bahia Blanca, Buenos Aires) after approval of the Institutional Bioethics Committee. Fifty-nine human mammary tissue samples: 19 normal and 40 intraductal carcinomas were collected from female patients 28–86 years old. Clinico-pathological data were obtained from the patient's clinical history. Tissue samples were processed and stored for further gene expression analysis. All studies involving human samples complied with the 1964 Helsinki Declaration. Informed consent was obtained from all the patients included in the study.

HC11 Cell Culture and Differentiation Protocol

HC11 cells were grown in RPMI 1640 (GIBCO laboratory, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) and 5 μg/ml insulin (Sigma, St. Louis, MO) (*Proliferating cells*). Then, confluent HC11 cells were maintained in RPMI with 2% FCS and 5 μg/ml insulin for 3 days (*Competent cells*), after which 5 μg/ml ovine PRL (Sigma) and 10^{-7} M Dexamethasone (Sigma) were added (*Differentiated cells*) [9].

Assessment of ERK/pERK Pathway Activation by Prolactin and the Impact of its Inhibition on *ZFP36* Gene Network Expression

The HC11 cells were cultured to confluence and submitted to 1 and 8 h of serum starvation previous to PRL (5 mg/ml) treatment. Cells were harvested with TRI Reagent® (Thermo Fisher Scientific, Waltham, MA, USA) 5, 15 and 30 min after PRL stimulus. EGF treatment was used as a positive control for ERK 1/2 [19]. ERK1/2 and pERK1/2 levels were evaluated by western blot. To inhibit ERK1/2 phospholylation the MEK1 inhibitor PD98059 (CAS 167869–21-8, Calbiochem®, Merck KGaA, Darmstadt, Germany) was added, 5 min after PRL stimuli, to the culture medium at a 10 μM final concentration. After 15 min, cells were harvested and total RNA was isolated with TRI Reagent®. The *Zfp36* gene network expression was evaluated by quantitative RT-PCR (RT-qPCR).

Evaluation of AP-1-Responsive Elements on the Promoter of the *ZFP36* Gene

The presence of AP-1 putative binding sites, known as TPA-responsive element (TRE element), in the promoter region of the human and mouse *ZFP36* genes (from −500 to +50 relative to the TSS) was explored using the *LASAGNA-Search 2.0* web tool [20]. The activity of PRL-responsive elements at the *ZFP36* promoter region was evaluated using the pGL3-mTTP Luc-reporter vector previously described [9] and the jAP1-luc reporter as control, since it contains an AP-1 binding site from murine *c-jun* promoter [21]. The pcDNAIII-βgal vector, expressing the enzyme β-galactosidase (β-gal), was used to evaluate transfection efficiency. HC11 cells were grown in a 6-well plate to 80% confluence and then transfected with one of the Luc-reporters and the βgal construct, using polyethyleneimine (PEI, 23966–1, Polysciences, Warrington, PA, USA). Forty-eight hours after transfection, cells grown in fresh media containing 2% FCS were treated or not (control) with PRL during 24 for further luciferase activity measure by cells resuspension in passive lysis buffer 5X (E194A, Promega). Cell lysates were analized by the addition of luciferin (Luciferase Reporter System, Promega). Data were normalized for the efficiency of DNA transfection by measuring β-galactosidase activity.

RT-qPCR Analysis of ZFP36 and Associated Genes

Zfp36, Fos, Jun, Junb, Dusp1, Btg2 and *Csn2* expression was assessed by RT-qPCR on human normal and tumor breast tissue samples, as well on non-tumorigenic (HC11) mouse mammary cell lines. Total RNA was isolated using TRI Reagent®. The SuperScript™ III Reverse Transcriptase kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for cDNA synthesis. Gene expression was measured using the StepOne™ Real-Time PCR System and associated Software v2.3 (Thermo Fisher Scientific, Waltham, MA, USA). The SYBR™ Select Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) was used for RT-qPCR reaction solution. The thermal profile was as follows: one cycle of 5 min at 95 °C; 40 cycles of 30 s at 95 °C, 30 s at 50–65 °C - according to primer pair- and 30 s at 72 °C; a final cycle of 95 °C for 1 min, 55 °C for 30 s and 96 °C for 30 s. Relative mRNA levels were calculated by the $2^{\wedge(-dCt)}$ method using as reference the *rRNA18S* [22]. Fifty-nine analyzed samples were classified into low or high *ZFP36*, *FOS*, or *JUN* expression levels based on their median expression values. Correlation analyses among breast tissue samples were performed based on their discretized relative expression values (0: low expression and 1: high expression) by Kendall's test. Human and mouse primers pair sequences are listed in Supplementary Table 1.

Results

Interspecific Co-Expression Analysis of the *ZFP36* and their Associated Gene Network

The in silico interspecific co-expression analysis allowed the identification of 42 genes commonly co-expressed with *ZFP36* in *H. sapiens* and *M. musculus* (Fig. 1a). Functional enrichment analysis of the commonly co-expressed genes revealed bioprocess association with IL6-mediated signaling events (adj. *p* value = 5.49e-16), AP-1 transcription factor network (adj. p value = 1.82e-10), ATF-2 transcription factor network (adj. p value = 1.78e-7), among others (Fig. 1b). In addition, *ZFP36* and 9 out of the 42 co-expressing genes belong to a network of strong protein-protein interactions, which we called the *ZFP36* gene network/module, in which the members of the AP-1 transcription factor family i.e. *JUN, JUNB, FOS* and *FOSB,* stand out (Fig. 1c).

Regulation of the *ZFP36* Gene Network in HC11 Mammary Cells

We have tested whether expression of at least some members of the *Zfp36* gene network were co-regulated on the successive differentiation stages of HC11 mammary cells to validate the relationship among those genes in experimental conditions. Figure 2a shows that *Zfp36, Jun, Junb, Btg2* and *Dusp1* mRNA levels, as *Csn2, which encodes the milk protein b-casein,* showed a statistically significant up-regulation in the differentiated stage ($p < 0.05$).

As JUN and JUNB proteins are subunits of the AP-1 transcription factor we hypothesized that induction of these proteins by PRL addition might result into AP-1 activation, which would bind to TRE sequences and activate transcription of target genes. One of which might be *ZFP36* as in silico analysis revealed four putative TRE elements in the promoter region of the human gene ($p < 0.032$), and three in mouse ($p < 0.05$). Besides, PRL induced a statistically significant increment of luciferase activation in HC11 cells transfected with a vector carrying either the *ZFP36* promoter region or the AP1 binding site, upstream the LUC (luciferase) gene ($p = 0.019$) (Fig. 2b).

To analyze the signaling pathway mediating the *Zfp36* gene network induction upon PRL treatment, phosphorylation of ERK1/2 MAP kinase was determined at 5, 15 and 30 min after the stimulation. A statistically significant increment of pERK1/2 was detected upon PRL addition respect to vehicle treatment at all time points ($p < 0.0005$). As positive control for ERK1/2 activation, cells were stimulated with EGF, which triggered pERK1/2 levels ($p < 0.0001$) respect to control (Fig. 2c). Then, expression of the *Zfp36* gene network and *Csn2* were evaluated after PRL stimulus in the presence of an ERK1/2 phosphorylation inhibitor PD98059 (PD). Under these conditions, down-modulation of *Zfp36* ($p = 0.017$),

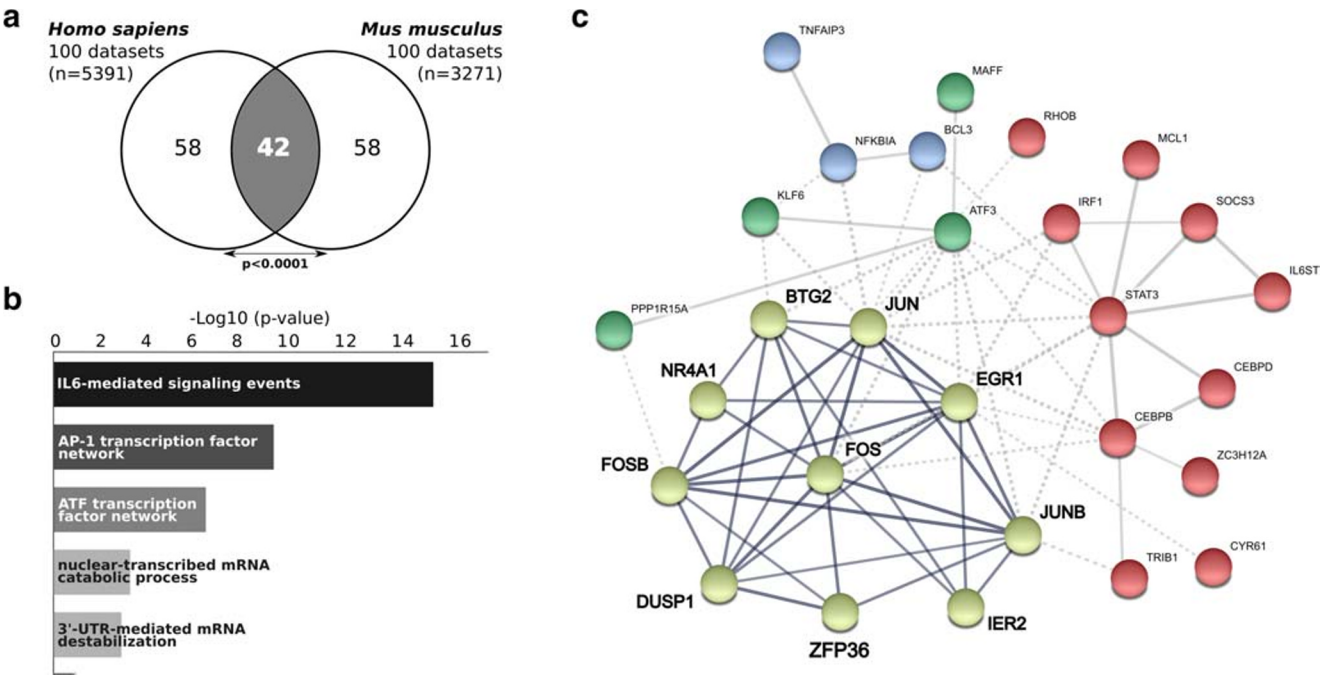

Fig. 1 Interspecific *ZFP36* co-expression analysis in *Homo sapiens and Mus musculus* gene expression datasets. a. Venn's diagram for the top 100 *ZFP36* co-expressed genes in human and mouse, outstanding the 42 commonly co-expressed genes among the analyzed species ($p < 0.0001$). **b.** Functional enrichment analysis for the 42 commonly co-expressed genes. Most of the genes are involved in bioprocess associated with transcription regulation and inflammation. **c.** Interaction network indicating the relations between the commonly co-expressed genes. It is possible to identify 10 strongly related genes composed by *ZFP36*, *JUN, JUNB, FOS, FOSB, DUSP1, EGR1, NR4A1, IER2, and BTG2* (the *ZFP36* gene network). The intensity of the lines indicates confidence in the interaction

Fos ($p < 0.001$) and *Csn2* ($p < 0.002$) expression, in respect to PRL treatment without PD, were detected (Fig. 2d). Surprisingly PD pre-treatment caused an enhanced induction of c-*Jun* expression ($p = 0.021$, Fig. 2d). To further corroborate the impact of blocking ERK1/2 over the *Zfp36* modulation in the HC11 cells upon PRL treatment, luciferase assay using the *ZFP36* promoter vector was re-evaluated (Supplementary Fig. 1). These results showed a significant decrease in the *ZFP36* promoter activity of HC11 cells treated with prolactin + PD, compared to HC11 cells under prolactin stimulation ($p < 0.01$).

Finally, *Zfp36* mRNA expression levels from HC11 cells in proliferating and differentiated states were compared with luminal-like (EO771, J110) and basal-like (SCg6) murine mammary cancer cell lines and mammary tumors. These results corroborate that normal mammary cells express more *Zfp36* than murine cancer cell lines and tumor cells ($p < 0.05$). Interestingly, luminal-like cancer cell lines showed the highest expression levels compared to the basal-like cell line ($p < 0.05$) (Supplementary Fig. 2).

ZFP36, FOS and JUN Expression and AP-1 Activity in Human Breast Tissues

In agreement to what we observed in different stages of HC11 mouse mammary cells, expression of *ZFP36, FOS* and *JUN* was analyzed in human normal mammary tissue and intraductal breast carcinomas (IDC) by RT-qPCR (Fig. 3 a). Significant positive correlations were detected between *ZFP36* and *FOS* ($r = 0.69$, $p < 0.01$), *ZFP36* and *JUN* ($r = 0.66$, $p < 0.01$) and *FOS* and *JUN* ($r = 0.93$, $p < 0.01$) by Kendall's test.

Expression analysis of *ZFP36* gene network among normal and tumor breast samples from the TCGA BRCA project ($n = 1211$) showed consistent down-regulation in the tumor samples compared to normal samples (Fig. 3b). Furthermore, average expression of the *ZFP36* gene network also showed significant differential expression among breast cancer subtypes, the normal-like and Luminal A showed higher levels than any of the others subtypes (Fig. 3c). Similar results were obtained when considering only *ZFP36* expression. It was found down-modulated in breast cancer respect to normal tissue ($p < 0.0005$) and in the normal-like breast cancer subtype compared to the others ($p < 0.0005$) (data not shown). Interestingly, *ZFP36* co-expression analysis according to breast cancer intrinsic subtypes demonstrated that the highest correlations coefficients between *ZFP36* and each of their associated gene network were detected in Luminal A and normal-like tumors (see Supplementary Table 2).

Survival analysis of breast cancer patients grouped according to low or high *ZFP36* gene network expression was performed in an independent dataset ($n = 3951$ primary breast

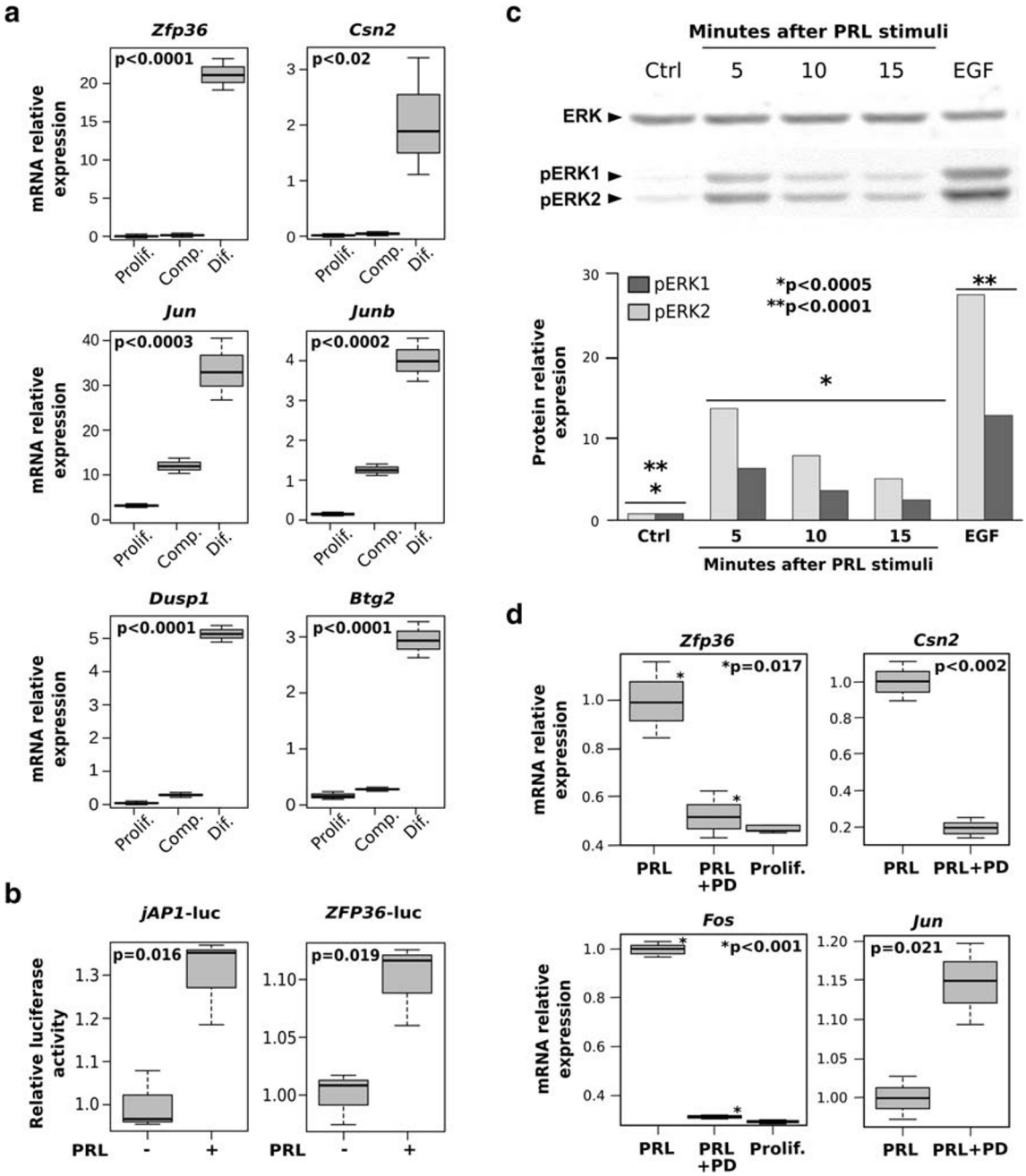

Fig. 2 Use of the HC11 cell model for evaluation of the *ZFP36* gene network expression and regulation. a. Relative mRNA expression levels of *Zfp36* and the associated gene network: *Jun, Junb, Btg2* and *Dusp1*. A statistically significant up-regulation of the *Zfp36* gene network was detected on the differentiated (Dif.) cell stage ($p \leq 0.0003$), as well as the cell differentiation marker *Csn2* ($p < 0.02$) with respect to the proliferative (Prolif.) and competent (Comp.) cell stages. **b.** Gene expression regulation measured with the aid of gene promoter reporter constructs intended to evaluate the response of gene promoters containing TRE elements. Namely, jAP-1 has been engineered to contain several AP-1 binndign (TRE) motifs. Alternatively, the other construct responds to the promoter region of the ZFP36 human gene. Both constructs display luciferase expression which is measured as luciferase activity. HC11 cells stimulated with PRL exhibited a statistically significant increment of luciferase expression by AP-1 activity on the ZFP36 human gene promoter ($p = 0.019$), and in the known AP-1 responsive jAP1 promoter ($p = 0.016$), respect to non-stimulated cells (Ctrl) **c.** ERK1/2 activation was evaluated in response to PRL stimuli. A statistically significant increase of the phosphorylated forms of ERK1/2 was detected in response to PRL ($p < 0.0005$), and to EGF stimuli ($p < 0.0001$, positive control) with respect to non-stimulated cells (negative control), at different time points after stimulation. **d.** Evaluation of the PRL-ERK pathway in the regulation of *ZFP36* gene network expression. Inhibition of the ERK pathway by addition of the PD98059 inhibitor to HC11 cells after PRL stimuli (PRL + PD) induced a significant down-modulation of *Zfp36* ($p = 0.017$), *Fos* ($p < 0.001$) and *Csn2* ($p < 0.002$), with respect to cells, solely stimulated with PRL (PRL). Instead, *Jun* expression was up-modulated ($p = 0.021$) on PRL + PD cells

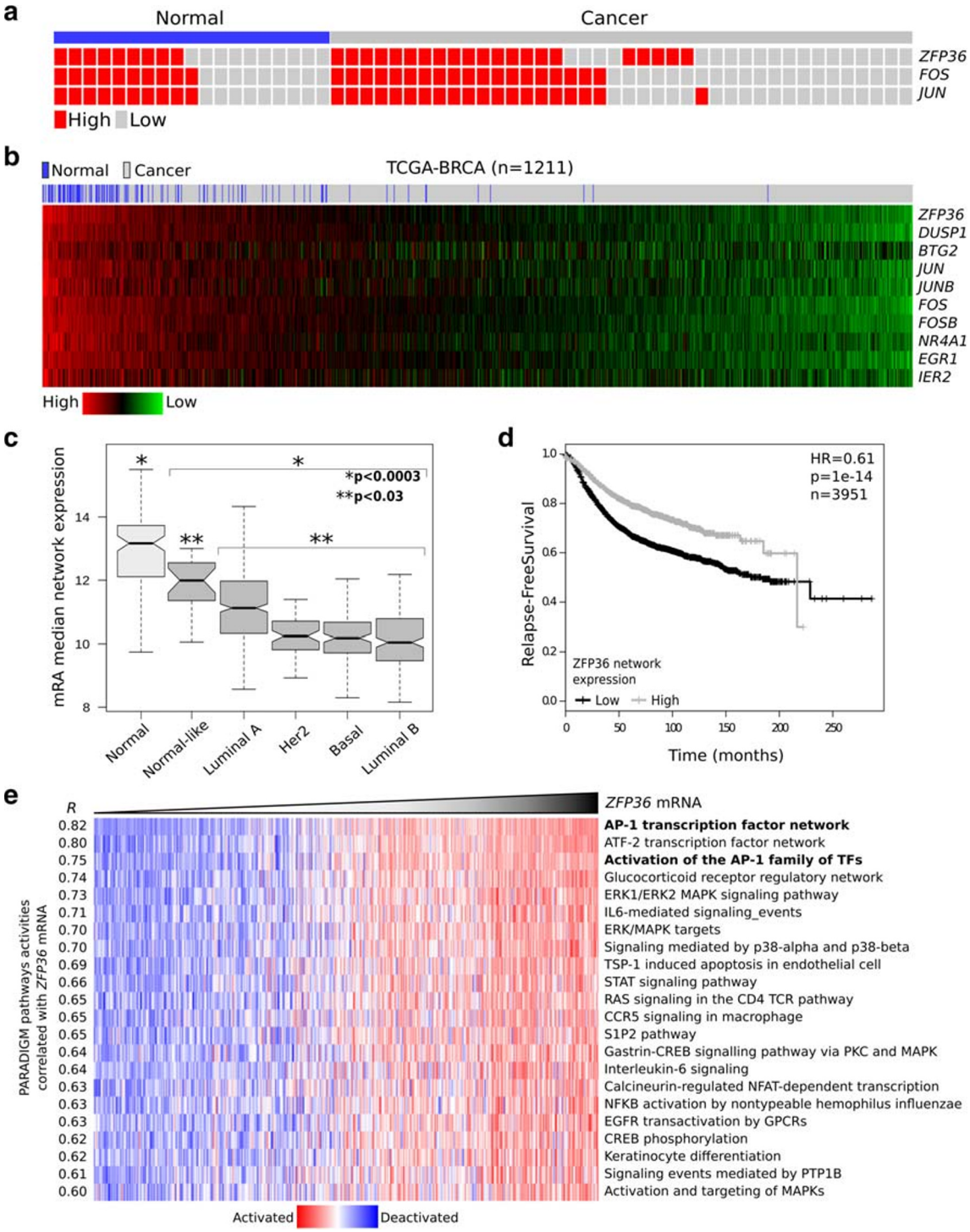

Fig. 3 ***ZFP36*** **gene network expression in human breast tissues. a.** Evaluation of *ZFP36*, *FOS* and *JUN* by RT-qPCR on breast normal and tumor samples. **b.** Heatmap of the *ZFP36* gene network in breast normal and tumor samples obtained from the TCGA-BRCA Project. A loss of the *ZFP36* gene network expression is observed in the tumor samples compared with normal samples. **c.** A statistically significant down-modulation of *ZFP36* gene network expression was detected in tumor samples respect to normal ($p < 0.0005$). This down-modulation was significantly noticeable in the Luminal B, Her2 and Basal breast cancer subtypes than in the normal-like and Luminal A subtypes ($p < 0.001$). **d.** Patients with low *ZFP36* gene network expression showed reduced RFS ($p < 0.0001$) with respect to patients with higher expression levels of *ZFP36* gene network. **e.** Top *ZFP36* correlated pathways ($r > 0.6$; $p < 0.001$) among normal-like and Luminal A primary breast carcinoma

carcinomas) using *Kaplan-Meier Plotter* resource. Low *ZFP36* gene network expression was statistically significant associated with shorter RFS ($p < 0.0001$, Fig. 3d). Similar results were obtained when the survival analysis was performed considering only the *ZFP36* mRNA expression ($p < 0.0001$). Interestingly, low *ZFP36* and *ZFP36* gene network expression levels were associated with shorted RFS in patients with Luminal A and Luminal B breast cancer subtypes ($p < 0.0001$) (Supplementary Fig. 3). There were no statistically significant differences among patients with low or high *ZFP36* or *ZFP36* gene network expression for Basal-like breast cancer subtype ($p > 0.05$) (Supplementary Fig. 3). These results suggest that down-modulation of *ZFP36* gene network is associated with poor prognosis of breast cancer patients, particularly for those carrying Luminal subtypes.

To further evaluate the relevance of AP-1 activity over *ZFP36* modulation, *ZFP36* mRNA expression was correlated with 1387 constituent integrated pathway activities predicted by PARADIGM algorithm among 433 luminal A and normal-like tumors derived from TCGA-BRCA project. The PARADIGM algorithm integrates pathway, expression and copy number data to infer activation of pathway features within a superimposed pathway network structure extracted from NCI-PID, BioCarta, and Reactome. Figure 3e showed the top correlation coefficients between *ZFP36* expression and their associated pathways activities ($r > 0.6$; $p < 0.001$). These results remarkably corroborate that the AP-1 predicted activity is associated with *ZFP36* mRNA with the highest correlation coefficient ($r = 0.82$) as well as the ERK1/2 signaling followed by other important pathways previously described as modulators or targets of ZFP36 (e.g., Glucocorticoid receptor, IL6). In addition, this analysis suggests novel signaling pathways that could be associated with the transcriptional regulation of *ZFP36* expressions (e.g.: *RAS*, *CREB*, and *CCR5* signaling pathways).

Pan-Cancer *ZFP36* Co-Expression Correlation Analysis

Co-expression and relevance network analyses on the TCGA Pan-Cancer dataset ($n = 7800$) corroborated the *ZFP36* gene network across different tumor sites beyond breast carcinomas (Fig. 4a, b). The expression level of the *ZFP36* gene network was also compared between normal and tumor samples among 17 tumor types. Interestingly, down-modulation of the *ZFP36* gene network was observed in tumor tissues compared with their normal counterparts for most anatomical sites (Fig. 4c). Particularly, breast, liver, lung, kidney, thyroid and head and neck tumors showed a statistically significant down-modulation of the *ZFP36* gene network with respect to normal tissues ($p < 1.0e\text{-}11$). On the other hand, no expression differences were detected on rectum adenocarcinomas ($p > 0.05$, Fig. 4c).

Discussion

ZFP36 is a well-known AU-rich element mRNA binding protein that regulates the mRNA stability of several cytokines and chemokines such as TNFα, IL3, GM-CSF, and CXCL2 [1]. It is considered as a global post-transcriptional regulator of inflammation and a critical regulator of dendritic cell maturation [2, 23]. Under normal physiological conditions, ZFP36 is involved on mammary gland differentiation, while under

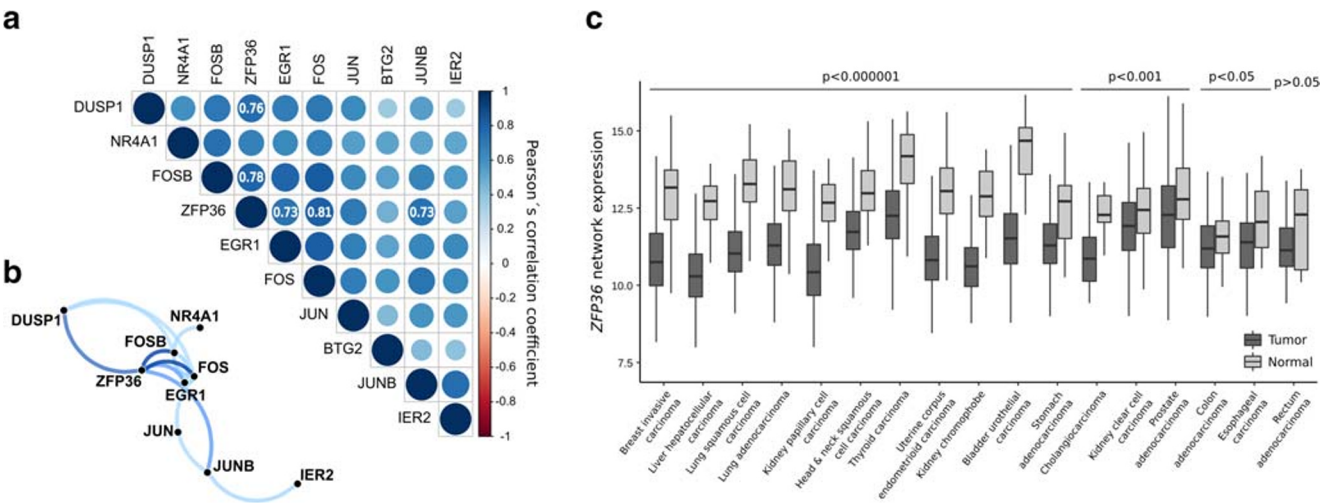

Fig. 4 In silico analysis of the *ZFP36* gene network expression among TCGA Pan-Cancer dataset of tumor and normal human tissues ($n = 7800$). a. Co-expression correlation matrix for the *ZFP36* gene network indicates that the 5 strongly correlated genes are *FOS, FOSB, JUNB, EGR1 and DUSP1* ($R > 0.70$, $p < 0.001$). **b.** Relevance network analysis of ZFP36 correlated genes. **c.** Boxplot of the *ZFP36* gene network on normal and tumor samples from differente primary cancer sites. Most locations showed a significant loss of *ZFP36* gene network in tumors respect to normal tissues ($p < 0.05$). Tumors from breast, liver, lung, kidney and thyroid showed the most significant loss of ZFP36 gene network expression ($p < 0.00001$)

pathological conditions the loss of its expression has been associated with breast cancer progression [8–10, 24]. ZFP36 behaves as a tumor suppressor through two different mechanisms. In the cytoplasm ZFP36 promotes the decay of hundreds of mRNAs encoding cell factors involved in inflammation, tissue invasion, and metastasis, while in the nucleus it is involved in the transcriptional co-repression of the estrogen receptor alpha, and the modulation of the transactivation of the progesterone, glucocorticoid and androgen receptors [25].

Many studies have focused on describing the ZFP36 mechanism of action, its target mRNAs and the pathways that induce its expression in an inflammatory context. Several stimuli such as growth factors (insulin, IGF1, EGF), cytokines (TNF, IFNγ, GM-CSF), lipopolysaccharides, glucocorticoids, and MAPKs, have been proved to induce it [26]. However, in the mammary gland physiology, little is known about the mechanisms and pathways regulating *ZFP36* expression.

Gene expression similarity search has been used in pathway reconstruction studies. It has been shown that the co-expression of a group of genes, which are evolutionarily conserved between species, can be used in a more effective prediction and prioritization of functional networks of genes associated with specific biological processes [12, 27, 28]. Based on this principle, an in silico comparative co-expression analysis was conducted between the species *H. sapiens* and *M. musculus* to identify the most significant and phylogenetically conserved genes associated to *ZFP36* expression. The interspecific analysis allowed the identification of 42 transcripts co-expressed with *ZFP36* and evolutionary conserved. A subsequent gene enrichment analysis revealed that these genes are related to inflammatory process (IL-6 signaling pathway) and transcription regulation mechanisms (AP-1 transcription factor network).

To understand the relationship between these genes, an interaction network reconstruction analysis allowed the identification of 10 strongly interactive genes which were considered as part of the "*ZFP36* gene network". To validate these in silico data, the expression of the *ZFP36* gene network was assessed in a murine mammary cell model. HC11 cells in the differentiated stage showed significantly elevated expression levels of *Zfp36, Jun, JunB, Btg2, Dusp1, and β-casein*, while proliferating and competent HC11 cells showed reduced *Zfp36* gene network expression. These results corroborate the co-expression of the *ZFP36* gene network identified by in silico analysis, and confirmed the previous observations that *Zfp36* expression is associated with cell differentiation stages [9].

The core of the *ZFP36* gene network is represented by the transcription factors: *JUN, JUNB, FOS,* and *FOSB.* These genes belong to the AP-1 transcription factor family and can associate in homo/heterodimers complexes for gene expression regulation [29, 30]. Interestingly, an in silico analysis allowed the identification of several putative consensus binding sites for the AP-1 transcription factor family in the human and mouse *ZFP36* gene promoters. As of today, AP-1 involvement in *ZFP36* expression regulation had not been reported. As it has been reported that PRL, rapidly triggers AP-1 activity [31], we propose that the AP-1 associated genes that belong to the network, *i. e. JUN, JUNB*, *FOS,* and *FOSB* might regulate *ZFP36* expression in the mammary gland in response to PRL.

It is known that PRL signals through a complex web of kinases including Janus kinase 2 (Jak2), Src kinase, phosphatidylinositol 3′-kinase (PI3K), protein kinase C (PKC), and MAPKs (mainly ERK1/2) [31]. To further explore the PRL pathway regulating the *Zfp36* gene network expression, the impact of blocking ERK1/2 with the PD98059 inhibitor was evaluated in the HC11 cell model. The experiments showed that the inhibition of ERK1/2 elicited a significant down-modulation of *Zfp36*, *Fos* and *Csn2* expression. On the contrary, *Jun* expression was up-modulated. The down-modulation of *Csn2*, *Zfp36*, and *Fos* in response to ERK1/2 inhibition is in concordance with previous studies where was proved that PRL can activate AP-1 through the Ras/Raf/MEK/ERK signaling pathway inducing the expression of *Csn2, Fos* and *Jun* [31, 32].

The transcription factor AP-1 is involved in cellular proliferation, transformation and death. Using mice and cells lacking AP-1 components, the target-genes and molecular mechanisms mediating these processes were recently identified. Interestingly, the growth-promoting activity of c-Jun is also mediated by repression of tumour suppressors, as well as up-regulation of positive cell cycle regulators. Mostly, c-Jun is a positive regulator of cell proliferation, whereas JunB has the converse effect [33].

Regarding the opposite behavior of *Jun with respect to Csn2*, *Zfp36*, and *Fos*, we understand its expression could be regulated by other factors, as previously proved in several studies [33, 34]. Two AP-1 binding sites are located in the proximal region of the c-Jun gene promoter. They can bind any of the dozens of combinations of heterodimers that constitute the AP-1 family of proteins that have reported bound to a TRE element. c-jun, in addition, can heterodimerize with members of the ATF-2 and CREB families leading to a complexity of factors that exert what has been called 'combinatorial control'. This heterogeneity of molecular components is the basis of the strong dependence between the cellular context and the outcome of responses in the expression of c-jun. Moreover, effects exerted by MAPKs different than ERK1/2 might have a strong influence on MEF-2 transcription factors which account for 70% of the response at the c-jun promoter level [34]. In view of this information, we understand that the explanation for the observed up-regulation of c-jun awaits further analysis that goes beyond the scope of this study.

Therefore, the evidences presented here suggest that the PRL-ERK-AP1 axis participates in *ZFP36* expression regulation in association with mammary gland differentiation

pathways. Loss of *ZFP36* expression or activity has been described in a variety of human malignancies (brain, breast, cervix, colon, liver, lung, ovary, prostate, and thyroid). It was shown that its re-expression could induce cell type-specific growth inhibitory effects, implicating ZFP36 as a tumor suppressor [35]. Besides, previous studies found *ZFP36* as well as *JUN, JUNB, FOSB*, and *IER2,* as down-modulated in breast cancer [9, 36]. Therefore, the *ZFP36* gene network expression was evaluated on TCGA Breast Cancer and Pan-Cancer datasets.

Analysis of the TCGA Breast Cancer dataset showed a significant down-modulation of *ZFP36* and the associated gene network on all the breast cancer subtypes with respect to normal breast tissue. However, the normal-like subtype showed higher expression levels than the others. This may be explained by the similar gene profile of the normal-like subtype with the normal breast tissues, and the more proliferative and malignant phenotypes of the other intrinsic subtypes [37]. It is important to note that the low/negative ZFP36 protein expression has been previously associated with undifferentiated carcinomas and high tumor grade [8, 9].

This association between malignancy and loss of *ZFP36* gene network expression was reinforced when we evaluated the prognosis value of *ZFP36* and the gene network in an independent data set of breast cancer patients using the *Kaplan-Meier Plotter* resource. The analysis indicated poor prognosis for patients with low expression of *ZFP36* and its gene network. At this respect, down-modulation of ZFP36 was associated in previous studies with reduced patients overall survival and high tumor grade [8, 9].

Considering the breast cancer intrinsic subtypes, we detected an association between the loss of the *ZFP36* and its gene network expression with shorted RFS for Luminal subtypes, but not for HER2 and Basal-like subtypes. Therefore, down-modulation of *ZFP36* as well as *ZFP36* gene network could be considered as a gene expression signature with prognosis value for patients with luminal breast cancer. Moreover, the signaling pathways activity prediction analysis in luminal A/B breast cancer subtypes remarkably supports the role of ERK1/2 signaling and AP-1 transcription factor activity as modulator of *ZFP36* gene expression.

Finally, the expression of the *ZFP36* gene network was compared between normal and tumor samples from several primary sites. A significant down-modulation of the *ZFP36* gene network was detected for most of the tumor locations included in the analysis. The most significant differences among tumor and normal samples were detected for breast, liver, lung and kidney carcinomas, among others indicating that the loss of *ZFP36* gene network is associated with cancer progression at different tumor sites.

In conclusion, we propose that the pathway PRL-ERK-AP1 might regulate *ZFP36* expression during the differentiation process of the mammary gland. Also, we show that loss of *ZFP36* as well as it associated gene network would have a significant role in cancer progression, probably associated with the acquisition of an undifferentiated tumor cell phenotype.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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