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SHORT COMMUNICATION

Chromatin-associated CSF-1R binds to the promoter of proliferation-related genes in breast cancer cells

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The colony-stimulating factor-1 (CSF-1) and its receptor CSF-1R physiologically regulate the monocyte/macrophage system, trophoblast implantation and breast development. An abnormal CSF-1R expression has been documented in several human epithelial tumors, including breast carcinomas. We recently demonstrated that CSF-1/CSF-1R signaling drives proliferation of breast cancer cells via 'classical' receptor tyrosine kinase signaling, including activation of the extracellular signal-regulated kinase 1/2. In this paper, we show that CSF-1R can also localize within the nucleus of breast cancer cells, either cell lines or tissue specimens, irrespectively of their intrinsic molecular subtype. We found that the majority of nuclear CSF-1R is located in the chromatin-bound subcellular compartment. Chromatin immunoprecipitation revealed that CSF-1R, once in the nucleus, binds to the promoters of the proliferation-related genes *CCND1*, *c-JUN* and *c-MYC*. CSF-1R also binds the promoter of its ligand CSF-1 and positively regulates CSF-1 expression. The existence of such a receptor/ligand regulatory loop is a novel aspect of CSF-1R signaling. Moreover, our results provided the first evidence of a novel localization site of CSF-1R in breast cancer cells, suggesting that CSF-1R could act as a transcriptional regulator on proliferation-related genes.

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INTRODUCTION

The colony-stimulating factor-1 (CSF-1) was first described as a hematopoietic growth factor regulating key functions of monocytes and macrophages through the activation of the class III receptor tyrosine kinase (RTK) CSF-1R.[1,2] It is well established that the CSF-1/CSF-1R pathway has regulatory roles also outside the hematopoietic system.[3–5] The abnormal expression of CSF-1R, with or without that of CSF-1, has been reported in several cancers and cancer-derived cell lines.[3,6,7] In particular, CSF-1/CSF-1R overexpression is associated with poor prognosis[8,9] and is predictive of ipsilateral recurrence in breast cancer patients.[10]

CSF-1-induced activation of CSF-1R promotes receptor dimerization and tyrosine transphosphorylation in the intracellular kinase domain, resulting in the activation of downstream signaling pathways.[11] In addition to this 'classical' RTK signaling, increasing evidences support the translocation of RTK from the plasma membrane to the nucleus.[12] In particular, epidermal growth factor receptor family members,[13–15] fibroblast growth factor receptors 1 and 3,[16–18] insulin and insulin-like growth factor-1 receptors[19,20] and the vascular endothelial growth factor receptor 2,[21,22] localize within the nucleus, as either full-length receptors or cleaved fragments, with or without their ligands. Once in the nucleus, RTK can regulate the expression of target genes, such as *CCND1*,[13,23] *FGF2*,[24] *COX2*[15] and *c-Jun*.[23]

We, along with others, have previously showed that CSF-1R is expressed in breast cancer cell lines and tissues.[7,25,26] Importantly, we demonstrated that CSF-1/CSF-1R signaling can drive cell proliferation of breast cancer cells via the activation of extracellular signal-regulated kinase 1/2 and the subsequent regulation of c-Jun, cyclin D1 and c-Myc expression.[7] Here we demonstrated that in breast cancer cells (i) CSF-1R localizes in the nucleus, (ii) nuclear CSF-1R binds to the promoter region of proliferation-related genes and (iii) CSF-1R regulates the transcription of its ligand CSF-1.

RESULTS AND DISCUSSION

CSF-1R localizes in the nucleus of breast cancer cells

We recently reported that CSF-1R mRNA is expressed in breast cancer cells and this is matched by protein expression at the cell surface.[7] Among the breast cancer cell lines analyzed, SKBR3 cells express the highest levels of membrane-bound CSF-1R and produce CSF-1 that sustains an autocrine proliferative loop.[7] While assessing CSF-1R expression in SKBR3 cells by western blotting, we found that the composition of lysis buffer dramatically affected CSF-1R protein yield (Figure 1a). In particular, Laemmli buffer allowed the recovery of the highest amount of CSF-1R, while either radioimmunoprecipitation assay buffer or Frackelton buffers resulted in incomplete CSF-1R solubilization. In this respect, it should be noted that Frackelton buffer, which contains the non-ionic Triton X-100 as the only detergent, mainly extracts integral plasma membrane proteins and is normally used

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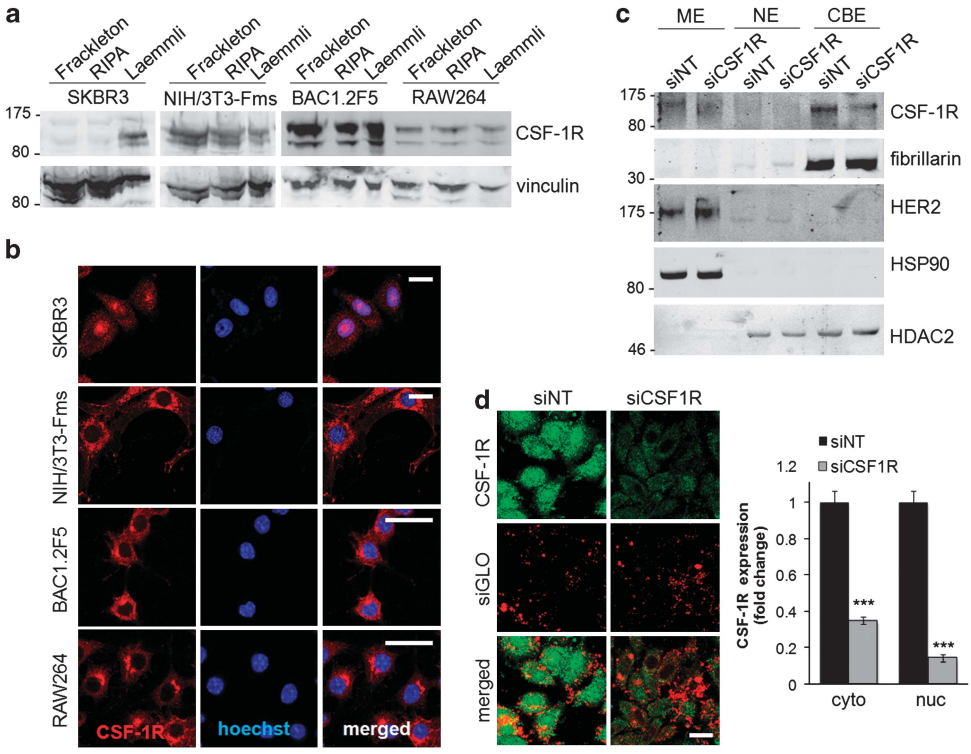

Figure 1. CSF-1R localizes in the nucleus of SKBR3 breast cancer cells. (**a**) Effects of the composition of lysis buffer on CSF-1R protein yield. BAC1.2F5 and RAW264.7 (RAW264) murine macrophages,[41] NIH/3T3 murine fibroblasts expressing or not ectopic human Fms (NIH/3T3-Fms; kind gift of MF Roussel, St. Jude Children's Research Hospital, Memphis, TN, USA)[42] and the breast cancer cell lines SKBR3 (HER2 positive), MDAMB468 (triple negative, basal-like 1; see below) and MDAMB231 (triple negative, mesenchymal stem like; see below)[43,44] were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. L-cell conditioned medium was added (15%) to BAC1.2F5 cells as a source of CSF-1.[45] Cells were lysed in Laemmli,[7] complete radioimmunoprecipitation assay buffer (RIPA)[46] or Frackelton[47] lysis buffer. Proteins were subjected to immunoblotting using rabbit α-CSF-1R (C-20, raised against the C terminus of CSF-1R, Santa Cruz, Santa Cruz Biotechnology, Inc., Heidelberg, Germany, sc-692) or mouse α-vinculin (Sigma, Sigma-Aldrich, S.r.l. Milano, Italy, V9131) antibodies. (**b**) Determination of CSF-1R intracellular localization by immunofluorescence (IF). Routinely cultured cells were subjected to IF using a rabbit α-CSF-1R antibody[48] and nuclei stained with the Hoechst dye. Scale bars: 20 μm. (**c**) CSF-1R localization in SKBR3 cells by cell fractionation. Gene silencing was performed with 50 nM SMART-pool small interfering RNA (siRNA) targeting CSF-1R mRNA (NM_005211 mRNA, Dharmacon, Thermo Fisher Scientific Inc., Rockford, IL, USA, number M-003109-03) or 50 nM siCONTROL non-targeting pool (siNT, Dharmacon, number D-001206-13) as previously described.[48] Three days after transfection, cell fractionation was performed according to the manufacturer's instructions using the Subcellular Protein Fractionation Kit for Cultured Cells (Pierce, Thermo Fisher Scientific Inc., Rockford, IL, USA, number 78840), which allows the subsequent separation of the cytosol, membrane, soluble and chromatin-bound nuclear fractions and cytoskeleton. Extracted proteins were subjected to immunoblotting with a rabbit α-CSF-1R antibody. Cell fractionation accuracy for membrane (ME), nuclear soluble (NE) and chromatin-bound (CBE) extracts was assessed by immunoblotting with α-fibrillarin (chromatin-bound marker; Santa Cruz, D-14, number sc-11336), α-HER2 (membrane marker; Cell Signaling, Technology, Danvers, MA, USA, number 2242), α-HSP90 (membrane/cytosol marker; Santa Cruz, number sc-13199) or α-HDAC2 (nuclear marker; Santa Cruz C-19, number sc-6296) antibodies. Immunoblotting with a marker for cytoskeleton (vimentin) did not produce any significant signal within nuclear compartments (not shown). (**d**) Effects of CSF-1R silencing in SKBR3 cells on nuclear *CSF-1R*. Gene silencing was performed as reported above using siGLO red (Cy3-labeled siGLO RISC-free siRNA, Dharmacon, number D-001600-01) as a transfection efficiency read-out following the manufacturer's instructions. Three days after transfection, IF was performed with a rabbit α-CSF-1R antibody; fluorescence in the cytosol (cyto) or the nucleus (nuc) was quantified using ImageJ (graph) (http://rsb.info.nih.gov/ij/). Histograms represent means ± s.e.m.; Student's *t*-test: $^{***}P<0.001$ ($n=2$, 20 cells were quantified). Scale bars: 20 μm. Migration of molecular weight markers is indicated on the left.

for CSF-1R signaling studies. By contrast, the type of buffer did not significantly affect CSF-1R recovery from lysates of RAW264.7 or BAC1.2F5 murine macrophages that express high levels of CSF-1R, or NIH/3T3 fibroblasts stably transfected (NIH/3T3-Fms cells) in order to express human CSF-1R (Figure 1a). These results suggested that CSF-1R solubility in SKBR3 breast cancer cells is different from that in macrophages or fibroblasts ectopically expressing CSF-1R.

To test whether differences of solubility were due to a different distribution of CSF-1R among cell compartments, we performed immunofluorescence in confocal microscopy, observing that CSF-1R localizes in the plasma membrane, cytoplasm and nucleus of SKBR3 cells (Figure 1b). In contrast, in keeping with a previous work,[27] CSF-1R localized in the plasma membrane, cytosol and Golgi, but not in the nucleus of fibroblasts (NIH3T3-Fms) or macrophages (BAC1.2F5 and RAW264.7) (Figure 1b and Supplementary Figure 1).[28] We then carried out subcellular fractionation of SKBR3 cells using a protocol that allows to discriminate between the soluble and chromatin-bound nuclear fractions as well as the plasma membrane (see figure legends for further details). The full-length mature form of CSF-1R was the prevailing protein recovered from the nucleus and in particular from the chromatin-bound nuclear fraction (Figure 1c, see also Supplementary Figure 2e). The cross-contamination between subcellular compartments was excluded using established controls.

A possible nuclear localization of CSF-1R was suggested by immunohistochemical studies of cervical pre-neoplastic tissues.[29] Our paper is the first to report the localization of CSF-1R within the nucleus in the chromatin-bound compartment, a phenomenon occurring in breast cancer cells selectively (see also above). Nuclear localization of CSF-1R was indeed undetectable in other CSF-1R-expressing cells, such as macrophages, where, by contrast, CSF-1R localization at the nuclear envelope has been reported.[30]

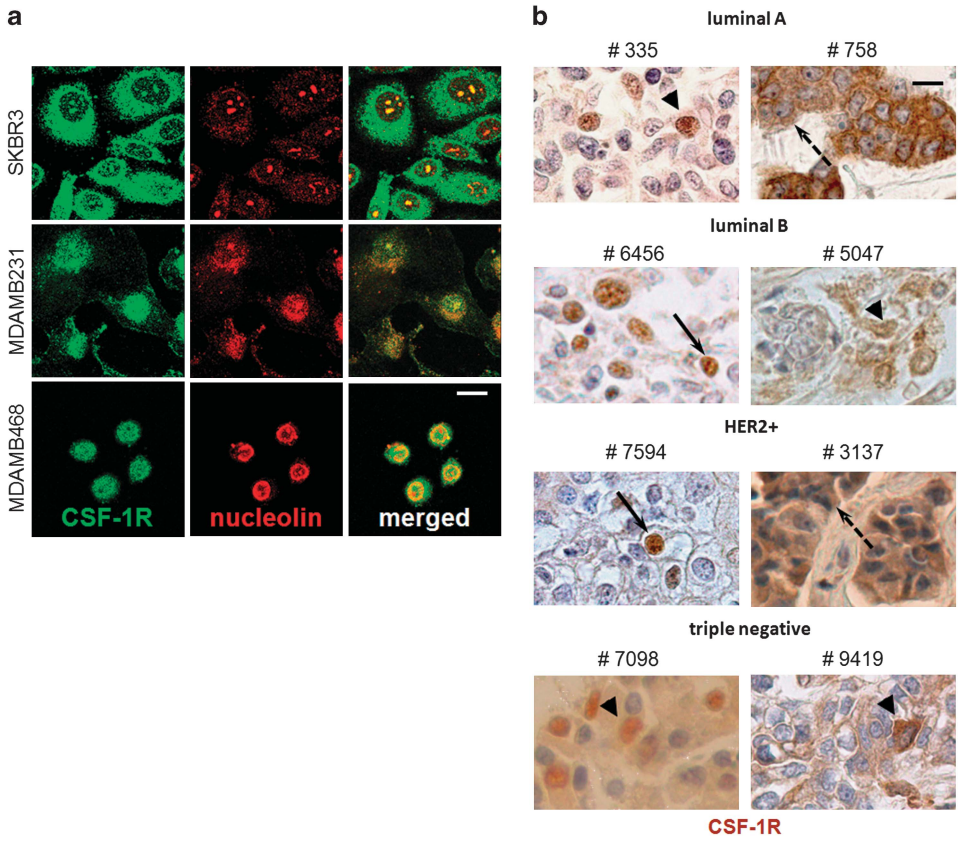

Figure 2. Nuclear localization of CSF-1R in breast cancer cell lines and tissue samples. (**a**) Immunofluorescence (IF) of CSF-1R in breast cancer cell lines. SKBR3, MDAMB231 and MDAMB468 cells were subjected to IF using rabbit α-CSF-1R and α-nucleolin (Santa Cruz, number sc-8031) antibodies. (**b**) Immunohistochemistry of CSF-1R in invasive breast cancers. Antigen retrieval was induced with sodium citrate buffer (pH 6.0) for 20 min at 97°C, rabbit α-CSF-1R antibody applied at 1:500 dilution and detection performed with labeled polymer (EnVision, DAKO Italia S.p.A., Milano, Italy). Examples of nuclear (arrowheads), cytosolic (dashed-line arrows) or cytosolic and nuclear (arrows) are indicated. Scale bars: 20 μm. Case (#) details are reported in Supplementary Table 1. The Research Ethics Committee of the Prato Hospital approved the use of breast cancer samples for this study (Protocol number 7741, 10 April 2012).

CSF-1R silencing using small interfering RNA in SKBR3 cells (Figures 1c and d) reduced CSF-1R protein levels in the nuclear fractions, as assessed by western blotting or immunofluorescence. CSF-1R silencing efficiency shown in Figures 1c and d was at least 50%, as previously reported.[7] The fact that the data obtained by western blotting were confirmed by immunofluorescence (Figure 1d) highlights the reliability of confocal imaging in studies of CSF-1R subcellular localization.

The mechanism of RTK trafficking to the nucleus has not been fully clarified yet. A mechanism involving cognate ligand binding and signaling proteins such as PI3K and Rab5 has been described[31] and could explain CSF-1R translocation to the nuclear envelope.[30] However, the steps required for CSF-1R to enter into the nucleus are still unknown. Nuclear localization signals (NLS) have been identified in RTK.[32] To determine whether CSF-1R contains putative NLS, we used the online database NucPred (http://www.sbc.su.se/~maccallr/nucpred).[33] NucPred assigns a score equal to 1 to an amino-acid sequence that is recognized as a *bona fide* NLS. When the sequence of c-erb3, which localizes within the nucleus,[34] was run as a control, a score of 0.58 was obtained. When CSF-1R was run, no NLS was identified (score 0.15). By contrast, when CSF-1 (Gene ID: 1435; isoform 1) was run, the NLS score obtained (0.63) was similar to that found for c-erb3. In CSF-1, indeed, a cluster of three basic amino acids (arginine residues 521–524) showed NLS properties. This cluster was also present in CSF-1 isoforms 2 (438 amino acids) and 3 (256 amino acids), alternative splicing variants of the 554 amino acid-long proteoglycan precursor. No NLS was predicted (score 0.22) in interleukin-34, a recently described CSF-1R ligand.[35] Previous studies indicated that CSF-1 is produced by breast cancer cell lines[7] and may be located in the nucleus in breast cancer tissues.[9] Thus, the high NLS score of CSF-1 led us to speculate that CSF-1R could translocate into the nucleus together with CSF-1. This hypothesis was strengthened by confocal immunofluorescence showing nuclear colocalization of CSF-1 and CSF-1R (Supplementary Figures 2a–c). Interestingly, biochemical fractionation revealed that an ~32 kDa CSF-1 form, which may well correspond to membrane CSF-1, was the prevailing form in the cytosol/membrane fraction. In contrast, the ~45 kDa form enriched in the nuclear fraction may represent different CSF-1 forms[36] (Supplementary Figure 2d). Therefore, it is difficult to predict which form of CSF-1 is involved in CSF-1R nuclear trafficking. It should be noted that the cluster of arginine residues responsible for NLS is in the C-terminal domain of CSF-1 so that it would be relevant only in membrane-spanning CSF-1 precursor but not in the secreted mature forms.[37] Nevertheless, CSF-1R nuclear translocation seems to be CSF-1 dependent, as nuclear CSF-1R increased following CSF-1 administration to serum-starved SKBR3 cells (Supplementary Figure 2e). The CSF-1-dependent, NLS-mediated nuclear translocation of CSF-1R may be only one of the mechanisms driving CSF-1R nuclear trafficking. For instance, Fms-interacting protein, which includes a NLS, binds transiently to the cytoplasmic domain of, and is phosphorylated on tyrosine by, activated CSF-1R.[38] This may result in CSF-1R nuclear translocation. Further experiments are needed to address the mechanism of CSF-1R entry into the nucleus.

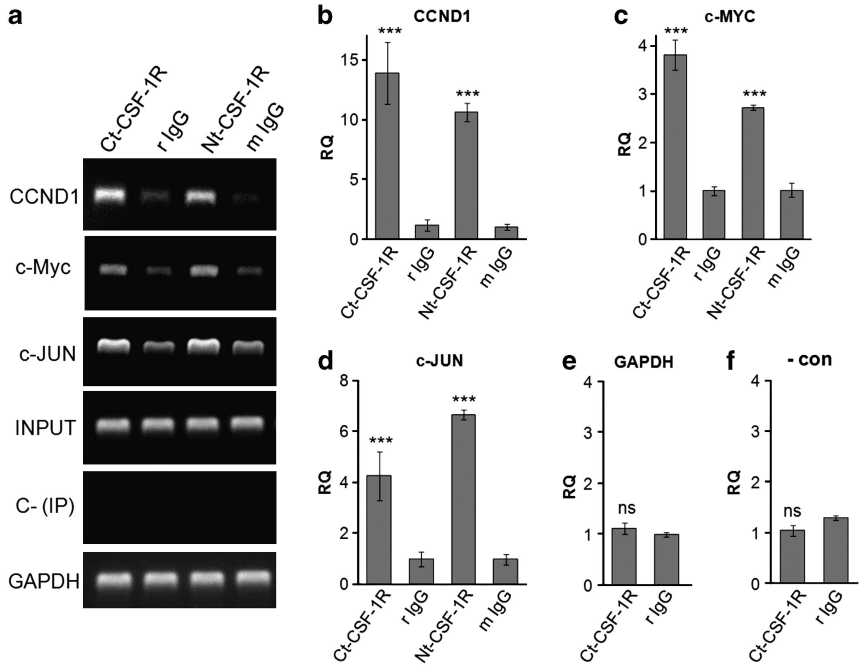

Figure 3. CSF-1R binds to the promoters of proliferation-related genes in SKBR3 cells. Chromatin immunoprecipitation (ChIP) was performed as previously described.[49–51] Immunoprecipitation (IP) was performed with the indicated antibodies: rabbit α-C-terminal-CSF-1R (Ct-CSF-1R; 0.4 μg per sample), mouse α-N-terminal-CSF-1R (Nt-CSF-1R; B-8, Santa Cruz, number sc-46662, 4 μg per sample), control rabbit IgG (rIgG; 0.4 μg per sample, Sigma number G5518), control mouse IgG (mIgG; 4 μg per sample, number M7023). Input and a negative control for the IP procedure (that is, without antibody; C-(IP)) are shown. The primers used were as follows: CCND1prom fw 5′-GAGGGGACTAATATTTCCAGCAA-3′, rev 5′-TAAAGGGATTTCAGCTTAGCA-3′; c-Myc prom fw 5′-agggcttctcagaggcttg-3′, rev 5′-cctattcgctccggatctc-3′; c-JUN prom fw 5′-AAAGCTAT GTATGTATGTGCTGCAT-3′, rev 5′-AACCGAGAGAACCTTCCTTTTTAT-3′; GAPDH prom fw 5′-TACTAGCGGTTTTACGGGCG-3′, rev 5′-TCGAACAGGAGG AGCAGAGAGCGA-3′; negative ChIP control fw 5′-ATGGTTGCCACTGGGGATCT-3′, rev 5′-TGCCAAAGCCTAGGGGAAGA-3′. Reverse transcriptase–PCR (**a**) and quantitative PCR (**b**–**f**) for the promoter regions of the indicated genes were performed as previously described.[7,51] Histograms represent the relative quantification of DNA recovered from IP with the indicated antibodies. Values were intra-experimentally normalized for input DNA and expressed as fold-change with respect to control IgG. Values are means ± s.e.m. of data from three independent experiments. Student's *t*-test comparing CSF-1R IP with the relative IgG: ***$P < 0.001$.

CSF-1R localizes in the nucleus of breast cancer cell lines and tissue irrespective of the intrinsic molecular subtypes

The nuclear localization of CSF-1R is not restricted to SKBR3 cells, as it was confirmed in other breast cancer cell lines, such as MDAMB231 and MDAMB468 (Figure 2a). Moreover, the colocalization of CSF-1R with nucleolin seems to indicate that, once in the nucleus, CSF-1R may localize also within the nucleolus (Figure 2a). Notably, the same subcellular distribution of CSF-1R was observed using two different antibodies raised against the C- or N terminus of CSF-1R (Supplementary Figure 3). These results are also in keeping with the presence of full-length CSF-1R within the nucleus (Figure 1c and Supplementary Figure 2c) and with what was observed for CSF-1R at the nuclear envelope.[30] However, we sequenced *CSF1R* mRNA in SKBR3 cells in order to exclude nucleotide changes that could justify different properties of CSF-1R protein, including acquisition of NLS. *CSF1R* mRNA in SKBR3 cells, when compared with wild-type *CSF1R* sequence (ENSG00000182578.9), exhibited two single nucleotide changes (83A > G; 726G > A) that are conservative (Supplementary Figure 4). Next, the rabbit α-CSF-1R antibody was optimized for immunohistochemical staining (Supplementary Figure 1) and used to stain samples of invasive breast cancers. Figure 2b shows representative images of the nuclear and/or cytoplasmic immunohistochemical staining for CSF-1R in tissue specimens derived from breast cancer patients with different intrinsic molecular subtypes. We found that 40 out of 42 samples of breast cancers expressed CSF-1R; of these, 9 out of 40 expressed CSF-1R in both the nucleus and the cytosol, 3 out of 40 in the nucleus only and 28 out of 40 in the cytosol only (Supplementary Table 1). Notably, despite the limited number and heterogeneity of tissue specimens studied, we found that the nuclear expression of CSF-1R negatively correlates with progesterone receptor expression ($r_s = -0.37$, $P = 0.018$). The size of our cohort of patients could not provide the statistical power to establish the prognostic and/or predictive value of nuclear CSF-1R in breast cancer. However, as progesterone receptor expression correlates with a favorable prognosis in breast cancer, our data support previous reports where CSF-1R expression was related to poor prognosis.[8–10]

Feedback regulation of CSF-1/CSF-1R signaling in SKBR3 cells: CSF-1R regulates the transcription of CSF-1 and proliferation genes

We previously showed that 'classical' RTK signaling exists in breast cancer cells where CSF-1R is activated by its ligand CSF-1, leading to CSF-1-induced proliferation and expression of genes involved in cell growth such as *CCND1*, *c-MYC* and c-*JUN*.[7] Previous reports demonstrated that *CCND1* and c-*JUN* promoters are targets of other nuclear RTK.[31] Consequently, we investigated whether CSF-1R could bind the promoter of *CCND1*, c-*JUN* and c-*MYC* by chromatin immunoprecipitation. Chromatin immunoprecipitation was performed in SKBR3 cells using two different α-CSF-1R antibodies. As revealed by reverse transcriptase–PCR (Figure 3a) and quantified by quantitative PCR (Figures 3b and d), CSF-1R bound the promoter regions of *CCND1*, *c-MYC* and c-*JUN*. The reliability of chromatin immunoprecipitation procedure was supported by the absence of CSF-1R binding to *GADPH* promoter region (which is constitutively open and prone to transcription) or to an inaccessible region used as a negative control (Figures 3e and f). CSF-1R binding to gene promoters is a novel finding and may represent an additional mechanism of regulation of

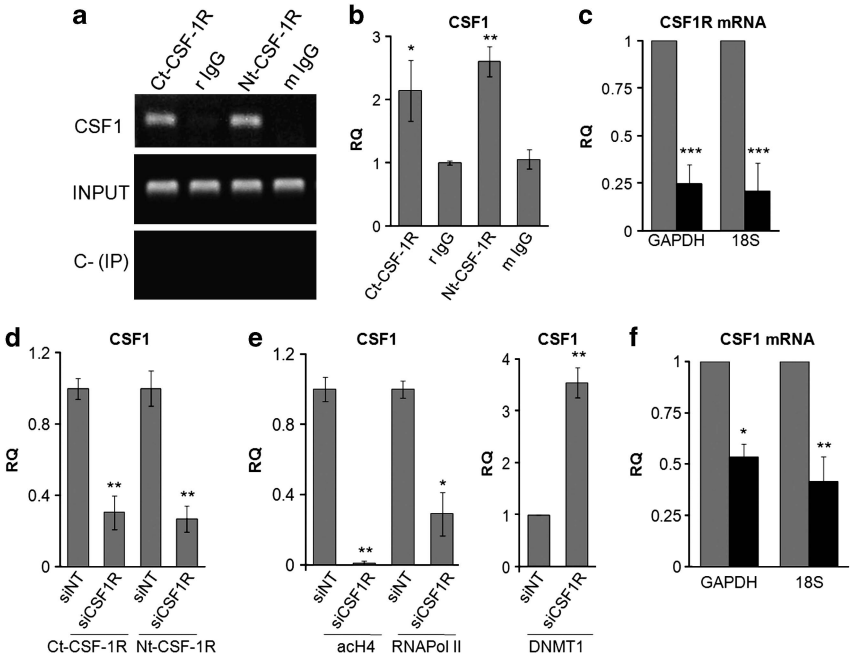

Figure 4. CSF-1R binds to the promoter of *CSF1* and regulates its transcription in SKBR3 cells. Chromatin immunoprecipitation was performed (**a**, **c**) using the antibodies indicated in Figure 3 or rabbit α-pan-acetylated H4 (acH4; Millipore, S.p.A., Milano, Italy, number 06-598), mouse α-RNA-polymerase-II (Abcam, Cambridge, UK, number ab5408) or rabbit α-DNA-methyltransferase-1 (Abcam, number ab5208). *CSF1* promoter region was amplified by PCR using the following primers: CSF1prom fw 5′-CACGAGGGAGCAAGTAACAC-3′; rev 5′-AGCCTTCAGCAAACGAG-3′. (**a**) Reverse transcriptase (RT)–PCR for *CSF1* promoter region. INPUT and a negative control for the IP procedure (that is, without antibody; C-(IP)) are shown. (**b**, **d**, **e**) Quantitative (Q)-PCR for *CSF1* promoter region. Histograms represent the relative quantification (RQ) of DNA recovered from IP with the indicated antibodies from routinely cultured cells (**b**) or 72 h after transfection with the indicated small interfering RNA (**d**, **e**). Values were normalized for input DNA in each experiment and expressed as fold-change with respect to control IgG. (**c**, **f**) Effects of *CSF1R* silencing on *CSF1* expression. Q-PCR from DNA obtained 72 h after transfection with siNT (light gray) or siCSF1R (dark grey). RNA extraction, complementary DNA synthesis, quantitative and RT–PCR were performed as previously described.[7,49–51] The primers used were the following: CSF1 mRNA fw 5′-ATGACAGACAGGTGGAACTGCCAG-3′, rev 5′-TCACACAACTTCAGTAGGTTCAGG-3′; CSF1R mRNA (N-term) fw 5′-GGAGGCTGCCCAGATCGT-3′, rev 5′-GCGAGCTTGGTGTTGTTGTG-3′; and CSF1R mRNA (C-term) fw 5′-CCTCGCTTCCAAGAATTGCA-3′, rev 5′-CCCAATCTTGGCCACATGA-3′; histograms represent the RQ of mRNA using GAPDH or 18S to normalize data and siNT as calibrator. Values are means ± s.e.m. of data from three independent experiments. Student's *t*-test: $*P<0.05$; $**P<0.01$; $***P<0.001$.

well-known targets downstream CSF-1R.[39] Of note, the increased nuclear localization of epidermal growth factor receptor in tumors is associated with treatment resistance and poor prognosis.[40]

The expression of CSF-1R together with CSF-1 has been reported in several cancers and cancer-derived cell lines.[3,6,7] On the other hand, nuclear RTK may regulate the expression of cognate ligands by binding to their promoters.[31] As revealed by reverse transcriptase–PCR (Figure 4a) and quantified by quantitative PCR (Figure 4b) in chromatin immunoprecipitation experiments, CSF-1R bound the promoter region of *CSF1* in SKBR3 cells. CSF-1R silencing (Figure 4c) reduced significantly the amount of CSF-1R bound to the *CSF1* promoter region (Figure 4d), supporting our finding. Furthermore, CSF-1R silencing resulted in a 'more closed' conformation of *CSF1* promoter, as indicated by the reduction of acetylated H4 and RNA-polymerase-II bound to the *CSF1* promoter region and the increase of bound DNA-methyltransferase-1 (Figure 4e). Accordingly, *CSF1* expression decreased after CSF-1R silencing (Figure 4f). These data indicated that CSF-1R drives a 'self-sustaining' loop of CSF-1R signaling in breast cancer cells by regulating CSF-1 expression. Further experiments, however, need to address how CSF-1R work as a transcriptional regulator. In this respect, it should be noted that transcriptional activity has been found for other RTK.[31]

In conclusion, our data highlighted a novel aspect of CSF-1R function. Nuclear CSF-1R could work in parallel, and synergizes with, the classical RTK activity of CSF-1R. Further investigations have to be directed to determine whether nuclear CSF-1R is a druggable target and/or is suitable as a prognostic or predictive factor in breast cancer.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

ER conceived and designed the experiments. ER, VB, AM, MR, SB, AG, IT, GD, IM and MGC performed the experiments. ER, VB, AM, AG, SB and ADL analyzed the data. ER, AM and PDS wrote the paper.

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