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The Mouse C/EBPδ Gene Promoter is Regulated by STAT3 and Sp1 Transcriptional Activators, Chromatin Remodeling and c-Myc Repression

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Abstract CCAAT/enhancer binding proteinδ (C/EBPδ) gene transcription is highly induced in G_0 growth arrested mammary epithelial cells and ''loss of function'' alterations in C/EBPδ have been reported in human breast cancer. To gain a better understanding of the positive and negative factors that control C/EBPδ gene expression we investigated the role of transcriptional activators, coactivators, repressors, histone modifications, chromatin remodeling and basal transcriptional machinery components in growing and growth arrested HC11 mouse mammary epithelial cells. Growth arrest treatments result in increased STAT3 activation (pSTAT3) and increased C/EBPδ expression. Co-immunoprecipitation and chromatin immunoprecipitation (ChIP) assays demonstrated that pSTAT3 and Sp1 interact and bind to the transcriptionally active C/EBPδ promoter. ChIP assays performed under exponentially growing (C/EBPδ non-expressing) conditions demonstrated that the C/EBPδ promoter is preloaded with transcriptional activators (Sp1 and CREB) and transcriptional machinery components (TBP and RNA Pol II). In contrast, under G_0 growth arrest (C/EBPδ expressing) conditions ChIP analysis detected pSTAT3, Sp1, NCoA/SRC1, CBP/p300, pCREB, TBP, and serine 2 phosphorylated Pol II (pPol II) in association with the C/EBPδ proximal promoter. C/EBPδ promoter-associated histone post-translational modification analysis revealed histone H3 and H4 acetylation and methylation patterns consistent with a constitutively ''open'' chromatin conformation. Chromatin remodeling experiments demonstrated that BRG1, the ATPase component of the SWI/SNF chromatin remodeling complex, is required for C/EBPδ transcription. Finally, C/EBPδ expression is repressed in proliferating mammary epithelial cells by c-Myc via a mechanism that involves the binding of c-Myc:Max dimers to C/EBPδ promoter-bound Miz-1. These results provide a molecular model of C/EBPδ transcriptional regulation under G_0 growth arrest conditions. J. Cell. Biochem. 102: 1256–1270, 2007.

Key words: C/EBPδ; growth arrest; gene transcription; transcription factors; coactivators; chromatin remodeling; Histone modifications; ChIP

CCAAT/enhancer binding proteinδ (C/EBPδ) is a member of the highly conserved C/EBP family of leucine zipper DNA binding proteins [Ramji and Foka, 2002; Vinson et al., 2002]. C/EBPs are expressed in a highly tissue and cell type-specific manner in response to a wide range of extracellular ligands including growth factors, cytokines, eicosanoids and a variety of cellular stress conditions [Belmonte et al., 2001; Johnson, 2005; Ramji and Foka, 2002]. C/EBPs function in the control of cell growth, differentiation and apoptosis and alterations in the structure or expression of C/EBP family members have been reported in a wide range of human cancers [Johnson, 2005]. We previously reported that C/EBPδ gene expression is rapidly

Abbreviations used: C/EBP, CCAAT, enhancer binding protein; STAT, signal transducers and activators of transcription; OSM, oncostatin M; GA, growth arrest; IP, immunoprecipitation; Co-IP, co-immunoprecipitation; ChIP, chromatin immunoprecipitation; Pol II, RNA polymerase II; CREB, cAMP response element-binding; PBS, phosphate buffered saline; RT, reverse transcriptase.

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and persistently induced in human and mouse mammary epithelial cells in response to G_0 growth arrest treatments such as serum and growth factor withdrawal, contact inhibition and IL-6 family cytokine treatment [O'Rourke et al., 1997, 1999a,b; Hutt et al., 2000; Hutt and DeWille, 2002; Gigliotti et al., 2003; Sivko and DeWille, 2004; Sivko et al., 2004; Tang et al., 2005]. C/EBPδ gene expression is reduced in experimental mammary tumors and in primary human breast cancer, however, mutations in the intronless C/EBPδ gene are relatively uncommon in solid tumors, including breast cancer [Vegesna et al., 2002; Tang and DeWille, 2003]. This suggests that alternate mechanisms, such as aberrant intracellular signaling, defective transcriptional activation, epigenetic promoter methylation, or reduced mRNA or protein stability may influence C/EBPδ gene expression in breast cancer cells. Although the role of transcriptional repressors has not been previously reported for C/EBPδ we recently reported that C/EBPδ gene expression is silenced in the SUM-52PE human breast cancer cell line and primary human breast tumors due to promoter hyper- and site-specific methylation [Tang et al., 2005].

The goal of the present study was to investigate the molecular mechanisms underlying C/EBPδ transcriptional regulation in mammary epithelial cells. The mechanisms controlling C/EBPδ transcriptional activation are important, as nuclear run-on assays indicate that C/EBPδ gene transcription is induced ~sixfold in G_0 growth arrested mammary epithelial cells [O'Rourke et al., 1999a]. Previous studies have correlated STAT3 and Sp1 *cis*-acting elements in the C/EBPδ promoter with increased activation using promoter-luciferase assays [Cantwell et al., 1998]. The present investigation investigated the role of pSTAT3 and Sp1 transcriptional activators, coactivators, chromatin remodeling complex components, histone modifications, basal transcription machinery and repressors in the regulation of C/EBPδ transcription. The results describe, for the first time, the biochemical mechanisms that activate and repress C/EBPδ gene transcription.

MATERIALS AND METHODS

Antibodies and Molecular Biology Reagents

Polyclonal antibodies against C/EBPδ (C-22), NcoA/SRC1 (M-341), P300/CBP, TBP, RNA polymerase II and Miz-1 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies against Sp1, CREB, pCREB, acetyl-histone H3 (Cat# 06-599) (detects histone H3 acetyl-lysine 9 and 14), acetyl-histone H4 (Cat# 06-598), dimethyl-histone H3 (Lys4) (Cat# 07-030, Lot#26335), monomethyl-histone H3 (Lys9, clone RR103 Cat# 07-713, Lot#24484) and methylated histone H3 (K4 or K9) were purchased from Upstate Biotechnology, Charlottesville, VA. Antibodies against phospho-STAT3 (Tyr^{705}), Max, c-Myc, and β-actin were purchased from Cell Signaling, Danvers, MA. RNA polymerase II H5 monoclonal antibody (specific for phosphoserine 2 of the CTD of Pol II) was purchased from Covance, Princeton, NJ. All restriction enzymes were obtained from New England Biolabs, Ipswich, MA. Recombinant mouse Oncostatin M was purchased from R&D Systems, Minneapolis, MN. The mouse C/EBPδ proximal promoter P-1.2k, P-322, and P-127 luciferase reporter constructs were described previously [Hutt et al., 2000]. Wild type STAT3 expression construct (WTSTAT3) and constitutive STAT3 construct (STAT3C) were generous gifts from Dr. James Darnell (Rockefeller University, New York, NY). Dominant negative STAT3 construct (DNStat3, mutated at tyrosine 705 residue) was a generous gift from Dr. Richard. Jove (H. Lee Moffitt Cancer Center & Research Institute, Tampa, FL). BRG1 and mutant BRG1 (mBRG1) constructs have been previously described [Sif et al., 2001].

Cell Culture

The nontransformed HC11 mouse mammary epithelial cell line was cultured in complete growth medium (CGM) containing RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 10 ng/ml epidermal growth factor, and 10 μg/ml bovine insulin. The Mm5MT (ATCC CRL 1673) and MMT 060562 (ATCC CCL51) mouse mammary-derived cell lines were cultured in Dulbecco's modified Eagle's medium (4.5 g/ml glucose) supplemented with 10% FBS. The human primary small cell carcinoma cell line SW-13 (ATCC CCL-105) was cultured in Dulbecco's modified Eagle's medium (4.5 g/ml glucose) supplemented with 10% FBS. All media contained 5 units/ml penicillin and 5 μg/ml streptomycin. Growth arrest plus Oncostatin M (GA + OSM) experiments were performed by using 70% confluent cell cultures washed with PBS and switched to

growth arrest media (GAM, 0.1% FBS) plus mouse Oncostatin M (OSM, 25 ng/ml final concentration) for up to 48 h.

Transient Transfections

Twenty-four hours after HC11 cells were plated in six-well plates (2×10^5 cells/well) cotransfection assays were performed using 1 μg C/EBPδ-promoter luciferase reporter construct, 100 ng of Renilla reporter construct (Promega, Madison, WI) and 1 μg of selected expression constructs, i.e., DNSTAT3, STAT3C, WTSTAT3 and c-Myc, or pcDNA3 vector control. Transfections were performed using the enhanced Lipofectamine transfection protocol (Invitrogen, Carlsbad, CA) as previously described [Hutt et al., 2000]. Twenty-four hours after transfection, HC11 cells were harvested with or without OSM treatment and assayed using the Dual-Luciferase1 Reporter Assay System (Promega) with luminescence detection performed using a Hewlett-Packard Lumicount microplate luminometer. All cotransfection assays were performed in triplicate and repeated at least three times. Lumicount data was normalized to the Renilla transfection control and analyzed as previously described [Hutt et al., 2000]. c-Myc siRNA transfection assays were performed using the Amaxa Nucleofector (Amaxa, Inc., Cologne, Germany). Briefly, 2×10^6 cells were suspended in Amaxa Proprietory Nucleofector Solution V, mixed with 50 pmol c-Myc Smartpool siRNAs (Dharmacon, Inc., Lafayette, CO) and the nucleofection performed using cell-type specific protocols. Following nucleofection, cells were plated in CGM and treated with OSM. Nucleofection control ("mock" treated) HC11 cells undergo the nucleofection protocol in the absence of siRNA. siRNA control HC11 cells were nucleofected with junk or "scrambled" siRNAs. Transient nucleofections with BRG1 and mBRG1 expression plasmids were performed in SW-13 cells as described for HC11 cells. All transfection and nucelofection experiments were performed in triplicate and repeated at least three times.

Biotinylated DNA Precipitation Assays

Biotinylated DNA precipitation assays were performed using the ProFound Pull-Down Biotinylated Protein:Protein Interaction Kit (Pierce Biotechnology, Rockford, IL). Region −226 to −24 of the mouse C/EBPδ promoter (designated P200) containing the STAT3 and Sp1 binding sites was labeled with biotinyl-dATP (Invitrogen) by PCR amplification. About 50 μl of immobilized streptavidin gel slurry was transferred to Handee Mini-Spin Columns and washed with TE buffer (pH 8.0). About 5 μg of biotin-labeled P200 C/EBPδ promoter fragments were solubilized in 200 μl TE buffer, added to the strepavidin containing mini-spin columns and incubated at 4°C on a rotating platform for 2 h. Streptavidin–biotin column complexes were treated with 250 μl of biotin blocking solution (5 min), washed with Tris buffered saline (TBS) and 200 μl (300 μg) of whole cell lysates was added to the columns and incubated on a rotating platform for an additional 4 h at 4°C. The gel slurry was then washed three times with TBS and eluted with 100 μl elution buffer. Eluates were analyzed by Western blot. The upstream −1856 to −1676 region of the C/EBPδ promoter (designated P1.8k) was used as a promoter binding specificity control. Non-biotin-labeled P200 C/EBPδ promoter fragments were used a negative control.

Western Blot Analyses and Immunoprecipitation Assays

Whole cell protein lysates were prepared by standard protocols as described previously [Hutt et al., 2000]. Protein concentrations were determined by BCA microprotein assay (Pierce Biotechnology, Rockford, IL). Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Charlottesville, VA) by electroblotting. Membranes were blocked in 10% non-fat milk, probed with primary and secondary antibodies in 5% non-fat milk and specific protein bands detected using ECL Plus detection systems (GE Healthcare, Life Sciences, Piscataway, NJ). For immunoprecipitation assays, whole cell protein lysates (300 μg in 1 ml RIPA buffer) were precleared with 60 μl of protein A-Agarose beads (50% slurry in PBS, Upstate) for 2 h on a rotating platform at 4°C. Cleared lysates were immunoprecipitated with primary antibodies on a rotating platform at 4°C overnight. Immunocomplexes were isolated by incubation with protein A-Agarose beads (60 μl of protein A-Agarose beads, 50% slurry in PBS, 2 h). Agarose bead/immunocomplexes were isolated by gentle centrifugation, washed with RIPA buffer and

PBS and solubilized in Laemmli buffer. Primary antibodies derived from mouse, protein G-Agarose beads were used to enhance immunocomplex precipitation. For c-Myc co-IP experiments, proteins were crosslinked with 1% formaldehyde prior to cell lysis.

Chromatin Immunoprecipitation (ChIP) Assays

ChIP assays were performed using the Chromatin Immunoprecipitation (ChIP) Assay Kit and protocols (Upstate, Biotechnology, Charlottesville, VA). Briefly, 1×10^6 HC11 cells were cross-linked with 1% formaldehyde (10 min, 37°C), washed two times with PBS (4°C), pelleted by centrifugation and resuspended in 200 μl SDS lysis buffer supplemented with protease inhibitors (as described in the kit protocol). HC11 cell lysates were sonicated to shear DNA to <1 kb (verified by agarose gel analysis). Sonicated lysates were centrifuged to remove debris, diluted 1:10 in dilution buffer and used for IP with desired antibodies. After immunoprecipitation, pellets were washed with 1 ml Low Salt Immune Complex Wash Buffer, High Salt Immune Complex Wash Buffer and LiCl Immune Complex Wash Buffer and TE buffer. Bead precipitates were eluted twice with fresh elution buffer (1% SDS, 0.1 M $NaHCO_3$) and eluates were pooled and heated at 65°C for 4 h to reverse protein–DNA crosslinks. DNA was purified by phenol extraction and ethanol precipitation. PCR analysis (35 cycles) was performed with 5 μl of the 50 μl DNA preparation plus the following primers: P200 (region −226 to −24 of the mouse C/EBPδ promoter containing STAT3 and Sp1 binding sites), 5′-GCGTGTCGGGGCCAAATCCA-3′ (forward primer), 5′-TTTCTAGCCCCAGCTGACGCGC-3′ (reverse primer); P1.8k (region −1856 to −1676 of the promoter) as control, 5′-TGCTTCTATGGCATCCAG-3′ (forward primer), 5′-GAGGGGCTGTGGAATATT-3′ (reverse primer). PCR amplification products were analyzed by agarose gel electrophoresis with ethidium bromide staining and photographed using AlphaImager (Alpha Innotech, San Leandro, CA). ChIP assays performed using RNA polymerase II H5 Monoclonal Antibody (MMS-129R) (Covance, Princeton, NJ), an IgM isotype antibody specific for the RNA pol II phosphoserine 2 required goat anti-mouse IgM-Agarose (A 4540) (Sigma, St. Louis, MO) in the pull down step as a substitute for the Agarose A beads from Upstate Biotechnology. All ChIP assays were performed three to four times with representative results presented.

RNA Extraction and Reverse Transcriptase (RT)-PCR Analysis

Total RNA was isolated using RNABee (TelTest, Inc., Friendswood, TX). RNA samples (1 μg) were treated with amplification grade DNase I and reverse transcribed with an oligo [Reisman et al.] primer in 20 μl using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen LifeTechnologies). One microliter cDNA aliquots were amplified by 25 cycles of PCR using primer pairs for the C/EBPδ gene, 5′-CTCCAGGCTTGGACGGCTAAGTAGG-3′ (forward primer) and 5′-AAGTTGGCTGTCACCTCGCC-3′ (reverse primer), or for the house keeping gene ALAS, 5′-GGAGGACGTGCAGGAAATG-3′ (forward primer) and 5′-GCCTCTGCTTTCGCATGATG-3′ (reverse primer). The expression of the 5-aminolaevulinic acid synthase (ALAS) housekeeping gene was used as a gene expression positive control. PCR amplification products were analyzed by agarose gel electrophoresis with ethidium bromide staining and photographed using Alphaimager (Alpha Innotech).

Densitometry and Statistical Analyses

Quantification of Western blot bands and DNA gel bands of PCR products were performed with AlphaEaseFC™ software version 4.0.0 (Alpha Innotech) before comparison of the differential mean intensities among treatments. Statistical analyses were performed using GraphPad PRISM software (GraphPad Software, Inc.). Student's *t*-test or one-way analysis of variance (ANOVA) was used to compare means for different treatment groups. Calculated *P* values of <0.05 ($P < 0.05$) were considered statistically significant.

RESULTS

Activated (Phosphorylated) STAT3 (pSTAT3) Functions in C/EBPδ Transcriptional Activation in Growth Arrest Plus Oncostatin M (GA + OSM) Treated Mammary Epithelial Cell Lines

Previous reports have demonstrated that transcriptional activation of mouse and human C/EBPδ promoter-luciferase constructs in response to growth arrest or cytokine treatment required intact STAT3 and Sp1 consensus binding sites [Cantwell et al., 1998; Hutt

et al., 2000; Sivko et al., 2004]. To characterize the expression and functional interaction between activated (phosphorylated) STAT3 (pSTAT3) and Sp1 under C/EBPδ nonexpressing and C/EBPδ expressing conditions we assessed pSTAT3, Sp1 and C/EBPδ protein levels in HC11, Mm5mt and MT060562 cell lines. HC11, Mm5MT and MMT 060562 cell lines all exhibit epithelial morphology in culture and all grow in an anchorage dependent manner. The HC11 cell line is an immortalized, nontransformed cell line, the Mn5MT and MMT060562 cell lines were derived from mouse mammary tumors. pSTAT3 and Sp1 levels were assessed under exponentially growing (C/EBPδ nonexpressing) and growth arrest (GA) plus Oncostatin M (OSM) (C/EBPδ expressing) conditions. The GA + OSM treatment was used in this study as we have previously demonstrated that C/EBPδ expression is maximal in mammary epithelial cells exposed to GA + OSM [Hutt et al., 2000; Hutt and DeWille, 2002; Sivko and DeWille, 2004]. pSTAT3 levels were relatively low in all three mouse mammary epithelial cell lines under exponentially growing conditions (GROW); pSTAT3 levels increased dramatically following exposure to growth arrest plus OSM (GA + OSM) treatments (Fig. 1A, pSTAT3). In contrast, Sp1 levels were not significantly influenced by growth conditions or OSM treatment (Fig. 1A, Sp1). C/EBPδ protein levels were low in all three cell lines under exponentially growing conditions; C/EBPδ protein levels increased in parallel with pSTAT3 levels in response to GA + OSM treatment (Fig. 1A, C/EBPδ). STAT3 levels were unaffected by growth status or cytokine treatment. β-Actin was used as the loading control (Fig. 1A, β-actin).

The acute phase response element (APRE) or STAT3 binding site located at −101 to −107 in the mouse C/EBPδ promoter and at −282 to −290 in the human C/EBPδ promoter plays a key role in C/EBPδ promoter activation [Cantwell et al., 1998; O'Rourke et al., 1999a; Sivko et al., 2004; Sanford and DeWille, 2005]. Since alteration of the extracellular environment by complex treatments such as GA + OSM alters multiple intracellular signaling pathways and transcriptional control networks, we used a constitutively activated STAT3 construct (STAT3C) developed by Darnell and co-workers [Bromberg et al., 1999] to specifically investigate the role of pSTAT3 in the transcriptional induction of the mouse proximal C/EBPδ promoter p-127. Transient transfection of STAT3C induced C/EBPδ core promoter luciferase activity ∼three-fold compared to the wild type STAT3 (WTSTAT3) or the pcDNA3 vector control (Fig. 1B). These results demonstrate that activated STAT3 plays a primary role in the transcriptional activation of the C/EBPδ promoter in mammary epithelial cells.

We next investigated the binding of Sp1 and pSTAT3 to the mouse C/EBPδ proximal promoter using DNA precipitation and ChIP assays. Sp1, which is constitutively present in HC11 cells (Fig. 1A, Sp1), also exhibits constitutive binding to the C/EBPδ proximal promoter by both assays (Fig. 1C, lane 3, Sp1 and 1D, lane 4). In contrast to Sp1, pSTAT3 levels are extremely low in exponentially growing mammary epithelial cells (Fig. 1A, pSTAT3) and as a result, virtually no pSTAT3 is detectable in the "Input" lysate control lane (Fig. 1C, lane 1, pSTAT, GROW) and no pSTAT3 binding to the C/EBPδ proximal promoter is detected under exponentially growing (C/EBPδ nonexpressing) conditions (Fig. 1C and D, lane 3, pSTAT3 GROW). In contrast to exponentially growing cells, pSTAT3 levels are detectable in the "Input" lane in response to GA + OSM treatment (Fig. 1C, lane 1, pSTAT, GA + OSM) and pSTAT3 binding to the C/EBPδ proximal promoter was significantly enhanced in response to OSM treatment (Fig. 1C and D, lane 3, pSTAT, GA + OSM). The upstream promoter (P1.8k) ChIP results were negative, demonstrating the specificity of the ChIP assay to detect pSTAT3 and Sp1 binding to the C/EBPδ proximal promoter region. These results provide direct evidence that Sp1 is constitutively bound to the C/EBPδ proximal promoter whereas GA + OSM treatment is required to induce activation of STAT3 and increased pSTAT3 binding to the C/EBPδ proximal promoter.

The mouse C/EBPδ proximal promoter pSTAT3 and Sp1 consensus binding sites are adjacent to one another at −101 to −107 (STAT3) and −112 to −117 (Sp1) [Cantwell et al., 1998; O'Rourke et al., 1999a]. We used co-immunoprecipitation (Co-IP) assays to assess the interaction between Sp1 and pSTAT3 transcriptional activators in GA + OSM treated HC11 cells. Co-IP assays performed using anti-pSTAT3 antibody with Western blot detection

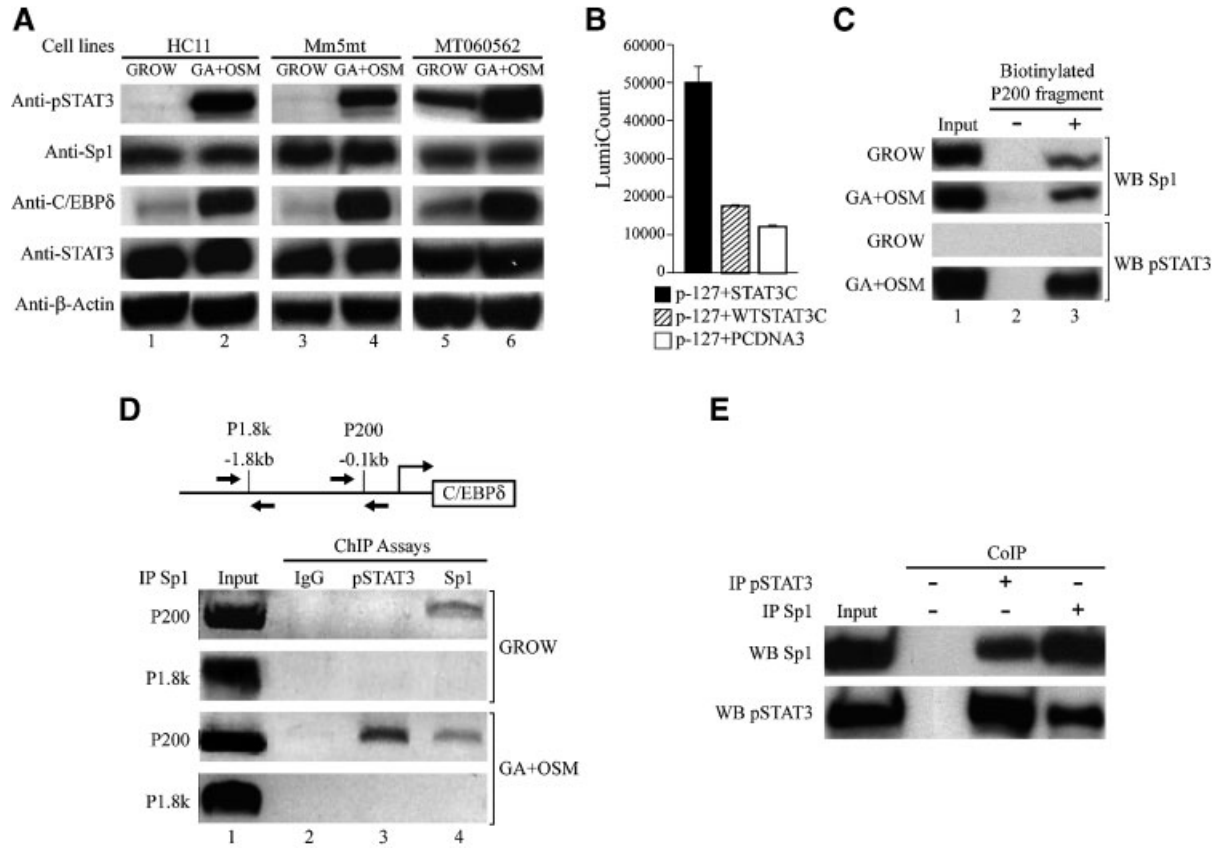

Fig. 1. pSTAT3 and Sp1 activate C/EBPδ gene expression under GA + OSM conditions through direct binding to its proximal promoter as an activator complex. (**A**) Western blot analysis of pSTAT3, Sp1, C/EBPδ and STAT3 in whole cell lysates from HC11, Mm5mt and MT060562 mammary epithelial cell lines cultured under exponentially growing (GROW) or growth arrest + Oncostatin M (GA + OSM) conditions. (**B**) C/EBPδ promoter luciferase construct (p-127) containing STAT3 and Sp1 consensus sites was cotransfected with STAT3C (constitutively active STAT3), WTSTAT3 (wild type STAT3) and pcDNA3 (vector control). Luciferase activities were measured at 24 h and normalized to Renilla luciferase transfection control activities. The LumiCount data is presented as mean ± SEM ($P < 0.05$). (**C**) Biotin-labeled P200 C/EBPδ proximal promoter/DNA precipitation assays. Lysates were derived from HC11 cells cultured under C/EBPδ nonexpressing (GROW) and C/EBPδ expressing (GA + OSM) conditions. Biotin-labeled promoter precipitated Sp1 and pSTAT3 were detected by Western blot (**lane 3**). Unlabeled DNA was used as negative control (**lane 2**). (**D**) ChIP assays were performed with formaldehyde crosslinked lysates from GA + OSM treated HC11 cells using anti-pSTAT3 and Sp1 antibodies (**lanes 3 and 4**). ChIP isolated DNA was PCR amplified using primers that flank the C/EBPδ proximal promoter region (P200) and the C/EBPδ upstream promoter region (P1.8k). 'Input' results were obtained from genomic DNA isolated from treated HC11 cells (positive control). 'IgG' results were obtained from ChIP assays performed with nonspecific rabbit IgG (negative control). (**E**) Co-IP reactions were performed with lysates from GA + OSM treated HC11 cells using anti-pSTAT3 and Sp1 antibodies. Reciprocal Western blots were performed on the Co-IP complexes using the same antibodies (**lanes 3 and 4**). 'Input' results were obtained from HC11 cell lysates (positive control). The results shown are representative of three independent experiments.

of Sp1 demonstrated that the anti-pSTAT3 complex contained Sp1 (Fig. 1E, lane 3, Sp1). Similarly, the anti-Sp1 complex contained pSTAT3 (Fig. 1E, lane 4, pSTAT3). These results demonstrate that pSTAT3 and Sp1 interact to form a transcriptional activation complex on the C/EBPδ promoter. The interaction between Sp1 and pSTAT3 may facilitate the binding of pSTAT3 to the C/EBPδ promoter or enhance the recruitment of co-activators to the C/EBPδ promoter.

The C/EBPδ Proximal Promoter Is Associated With pSTAT3, the Transcriptional Coactivator NcoA/SRC1 and Is "Preloaded" With CREB and Transcriptional Machinery Components

Transcriptional coactivators carry out essential functions in transcriptional regulation by functioning as adaptors between DNA bound transcriptional activators and the basal transcriptional machinery and as modifiers of histones and chromatin remodeling complex

components [Naar et al., 2001; Kalkhoven, 2004]. We hypothesized that the transcriptional co-activator NcoA/SRC1 may play a role in the activation of C/EBPδ during growth arrest as NcoA/SCR1 had previously been shown to interact with pSTAT3 in the transcriptional activation of the cyclin dependent kinase inhibitor $p21^{waf1/Cip1}$ gene [Giraud et al., 2002]. The transcriptional co-activator NcoA/SRC1 is present at detectable levels in both exponentially growing (GROW) HC11 cells and in GA + OSM treated HC11 cells (Fig. 2A, NcoA/SRC1). Using Co-IP assays we showed that pSTAT3 and NcoA/SRC1 interact in GA + OSM-treated HC11 cells (Fig. 2B). ChIP assays further demonstrated that NcoA/SRC1 is bound to the C/EBPδ proximal promoter in GA + OSM treated HC11 cells (Fig. 2C, lane 3). Since NcoA/SRC1 does not bind directly to DNA, the association of NcoA/SRC1 with the C/EBPδ proximal promoter is most likely through interaction with C/EBPδ promoter-bound pSTAT3.

To facilitate rapid transcriptional activation, the promoters of cytokine inducible genes, such as the TNFα-inducible A20 gene, are constitutively bound with selected transcription factors, such as Sp1, and components of the general transcription machinery [Ainbinder et al., 2002]. Because Sp1 is constitutively bound to the C/EBPδ proximal promoter (Fig. 1D) and its binding sites are in close proximity to the TATA box, we hypothesized that the C/EBPδ proximal promoter may be "preloaded" with additional general transcription factors and transcriptional machinery components in mammary epithelial cells. In addition to the well-documented STAT3 and Sp1 consensus binding sites, the mouse C/EBPδ proximal promoter also contains a cyclic AMP response element (CRE) at −40 [Cantwell et al., 1998; O'Rourke et al., 1999a]. Although the C/EBPδ proximal promoter CRE is insufficient to activate the C/EBPδ promoter in response to IL-6 [Cantwell et al., 1998] or GA + OSM treatments [Hutt et al., 2000], an intact CRE is required for full activation of the C/EBPδ promoter (data not shown). We used ChIP assays to assess the presence of CREB, phospho-CREB (pCREB),

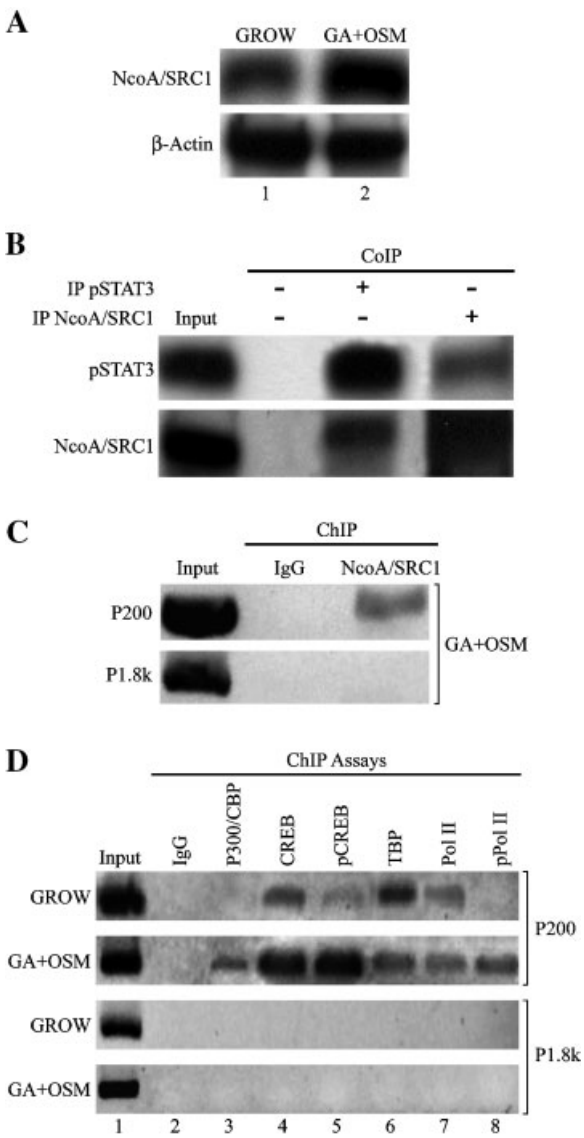

Fig. 2. pSTAT3 interacts with the transcriptional co-activator NcoA/SRC1 and recruits it to the C/EBPδ proximal promoter. (**A**) Western blot analysis of NcoA/SRC1 in whole cell lysates from HC11 cells cultured under C/EBPδ nonexpressing (**lane 1**) and C/EBPδ expressing (**lane 2**) conditions using β-actin as loading control. (**B**) Co-IP assays were performed with lysates from GA + OSM treated HC11 cells using anti-pSTAT3 and NcoA/SRC1 antibodies. Reciprocal Western blots were performed on the Co-IP complexes using the same antibodies (**lanes 3 and 4**). (**C**) ChIP assays were performed with GA + OSM treated HC11 cells using anti-NcoA/SRC1 antibodies (**lane 3**). ChIP isolated DNA was PCR amplified using primers that flank the C/EBPδ proximal promoter region (P200) and the C/EBPδ upstream promoter region (P1.8k). (**D**) ChIP assays were performed with HC11 cell lysates from C/EBPδ nonexpressing (GROW) and C/EBPδ expressing (GA + OSM) conditions using anti-P300/CBP, anti-CREB, anti-pCREB, anti-TBP, anti-Pol II and anti-pPol II ser 2 antibodies (**lanes 3–8**). ChIP isolated DNA was PCR amplified using primers that flank the C/EBPδ proximal promoter region (P200) and the C/EBPδ upstream promoter region (P1.8k). 'Input' results were obtained from genomic DNA isolated from treated HC11 cells (positive control). 'IgG' results were obtained from ChIP assays performed with nonspecific rabbit IgG (negative control). The results shown are representative of three independent experiments.

p300/CBP, TBP, RNA Polymerase II (Pol II) and pPol II (Pol II phosphorylated at serine 2) on the C/EBPδ proximal promoter under exponentially growing (C/EBPδ nonexpressing) and GA + OSM (C/EBPδ expressing) conditions in HC11 cells. The ChIP assays results indicated that CREB, TBP and Pol II were bound to, or "preloaded" onto, the C/EBPδ proximal promoter under exponentially growing (C/EBPδ nonexpressing) conditions (Fig. 2D, lanes 4, 6, 7, GROW, P200). When HC11 mammary epithelial cells were switched to GA + OSM (C/EBPδ expressing) conditions, ChIP assays detected p300/CBP, CREB, pCREB, TBP, Pol II and pPol II ser2 in association with the C/EBPδ proximal promoter (Fig. 2D, lanes 3–8, GA + OSM, P200). The binding of transcriptional machinery components was restricted to the C/EBPδ proximal promoter region as these proteins components were not associated with the C/EBPδ distal (P1.8k) promoter fragment. These results demonstrate that the C/EBPδ proximal promoter in mammary epithelial cells is preloaded with Sp1, CREB and transcriptional machinery components under exponentially growing (C/EBPδ nonexpressing) conditions. Exposure to GA + OSM (C/EBPδ expressing) conditions increases the binding of pSTAT3, NcoA/SRC1, p300/CBP to the C/EBPδ proximal promoter and also induces the posttranslational modification (phosphorylation) of two preloaded C/EBPδ proximal promoter-bound components CREB and Pol II in association with active transcription of the C/EBPδ gene.

The C/EBPδ Proximal Promoter Is Associated With an "Open" Chromatin Conformation

C/EBPδ gene is physically located less than 5 mega bases from the centromere of chromosome 8 in the human genome, and chromosome 16 in the mouse genome [Jenkins et al., 1995] (http://genome.ucsc.edu). Centromeric regions are generally associated with a transcriptionally repressive chromatin environment that is inaccessible to transcriptional machinery [Perrod and Gasser, 2003; Lam et al., 2006]. To investigate the C/EBPδ promoter chromatin structure we focused on two general mechanisms that underlie the dynamic regulation of chromatin structure, gene promoter accessibility and functional activation: covalent modification of histones and ATP-driven nucleosome remodeling [Narlikar et al., 2002; Gibbons, 2005; Roeder, 2005]. Histones exhibit a number of covalent modifications that correlate with specific gene activation or repression, including N-terminal acetylation, methylation, phosphorylation and ubiquitinylation [Narlikar et al., 2002; Gibbons, 2005; Roeder, 2005]. Using ChIP assays we demonstrate that the C/EBPδ proximal promoter is associated with acetylated histones H3 and H4 under exponentially growing (C/EBPδ nonexpressing) and GA + OSM (C/EBPδ expressing) conditions (Fig. 3A, lanes 5 and 6). ChIP assays assessing histone H3-K4 methylation, a marker of active gene transcription, are also strongly positive irrespective of growth or treatment conditions. In contrast, ChIP assays assessing histone H3-K9 methylation, a marker of gene silencing, are negative (Fig. 3A, lanes 3 and 4). Collectively, these results indicate that the C/EBPδ gene proximal promoter is in a constitutively "open" chromatin conformation.

ATP-dependent chromatin remodeling plays a key role in the dynamic biochemical and structural alterations of nucleosomes that control the accessibility and transcriptional activation of a gene promoter [Sif, 2004]. Initial ChIP assays indicated that BRG1, a critical SWI/SNF complex ATPase component, is associated with the CEBPδ proximal promoter under exponentially growing (C/EBPδ nonexpressing) and GA + OSM (C/EBPδ expressing) conditions (Fig. 3B). To investigate the role of BRG1 in C/EBPδ gene expression, we used the BRG1 deficient SW13 cell line [Reisman et al., 2002]. Western blot analysis confirmed that SW13 cells lack BRG1 (Fig. 3C, lane 1, BRG1). BRG1 deficient SW13 cells were transiently transfected with transfection vehicle (mock transfected), pcDNA3 (control vector) or a BRG1 expression construct and treated with GA + OSM (Fig. 3C, lanes 2–4, top panel). GA + OSM treated mock and vector transfected BRG1 deficient SW13 cells exhibited increased pSTAT3 levels, but no detectable C/EBPδ (Fig. 3C, lanes 2 and 3, middle panel). In contrast, SW13 cells transfected with the BRG1 expression construct exhibited detectable levels of BRG1, pSTAT3 and C/EBPδ in response to the GA + OSM treatment (Fig. 3C, lane 4, lower panel). To further investigate the role of BRG1 in C/EBPδ expression we reduced endogenous BRG1 activity by transfecting HC11 cells with a catalytically inactive form of BRG1 (mBRG1). The results demonstrate that C/EBPδ expression is reduced in mBRG1

transfected HC11 cells (Fig. 3D, lane 1 vs. 2), further supporting a key role for BRG1 in the transcriptional activation of C/EBPδ gene expression.

The C/EBPδ Promoter Is Repressed by C-Myc Under Exponentially Growing Conditions

The histone modification analyses suggested that the C/EBPδ proximal promoter is in an open chromatin conformation, however, C/EBPδ gene transcription is silent, or repressed, under exponentially growing conditions [O'Rourke et al., 1999a]. We hypothesized that a repressive complex may be associated with the C/EBPδ promoter preventing activation of transcription from the C/EBPδ promoter. Aberrant over expression of Myc gene family members is a common characteristic of cancer cells and c-Myc regulates cell proliferation by the combined effects of transcriptional activation of growth promoting genes and transcriptional repression of growth inhibitory genes including gas1, p15, p21, p27, GADD45α and GADD153 (C/EBPζ) [Lee et al., 1997; Gartel et al., 2001; Staller et al., 2001; Yang et al., 2001; Barsyte-Lovejoy et al., 2004; Brenner et al., 2005]. In addition, c-Myc has been reported to repress the transcription of the C/EBPα core promoter [Antonson et al., 1995]. The mechanisms of c-Myc repression of growth arrest genes include: (1) formation of c-Myc-Sp1/Sp3 dimers resulting in repression of Sp1 or Sp3 mediated transcription [Gartel et al., 2001; Adhikary and Eilers, 2005]; (2) binding of c-Myc-Max dimers to promoter-bound Miz-1 resulting in c-Myc interference with Miz-1-P300 co-activator recruitment [Gartel and Shchors, 2003; Adhikary and Eilers, 2005]; (3) binding of c-Myc-Max dimers to DNA-bound Miz-1 with recruitment of the co-repressor Dnmt3a [Adhikary and Eilers, 2005; Brenner et al., 2005].

We investigated the correlation between c-Myc levels and proliferation in HC11 cells and found that c-Myc levels are elevated in

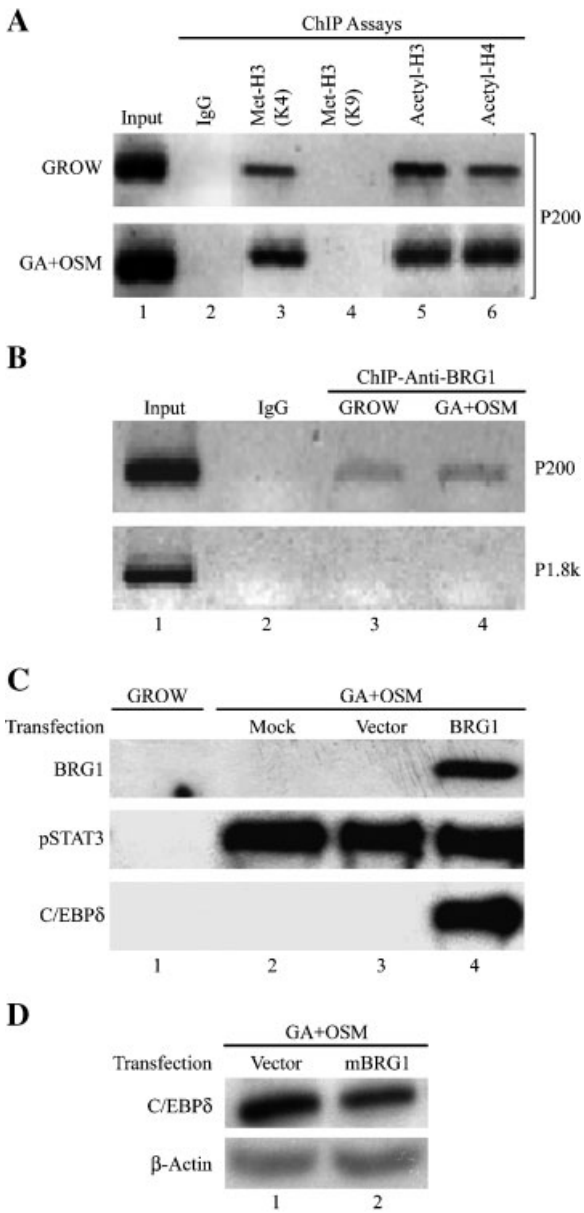

Fig. 3. The C/EBPδ proximal promoter exhibits a complex chromatin state. (**A**) ChIP assays were performed with HC11 cell lysates from C/EBPδ nonexpressing (GROW) and C/EBPδ expressing (GA + OSM) conditions using anti-Met-H3 (K4), anti-Met-H3 (K9) and anti-acetyl-H3 and anti-acetyl-H4 antibodies (**lanes 3–6**). ChIP isolated DNA was PCR amplified using primers that flank the C/EBPδ proximal promoter region (P200) and the C/EBPδ upstream promoter region (P1.8k). 'Input' results were obtained from genomic DNA isolated from treated HC11 cells (positive control). 'IgG' results were obtained from ChIP assays performed with nonspecific rabbit IgG (negative control). (**B**) ChIP assays were performed using anti-BRG1 antibodies with GROW and GA + OSM treated HC11 cells (**lanes 3 and 4**). ChIP isolated DNA was PCR amplified using primers that flank the C/EBPδ proximal promoter region (P200) and the C/EBPδ upstream promoter region (P1.8k). 'Input' results were obtained from genomic DNA isolated from treated HC11 cells (positive control). 'IgG' results were obtained from ChIP assays performed with nonspecific rabbit IgG (negative control). (**C**) BRG1 deficient SW-13 cells were transfected with BRG1 expression and vector control constructs and cultured under GA + OSM conditions. Whole cell lysates were isolated and analyzed by Western blot using anti-BRG1, pSTAT3 and anti-C/EBPδ antibodies (**lanes 2–4**). (**D**) HC11 cells were transfected with an expression vector control (pcDNA3, **lane 1**) or a mutant, inactive BRG1 construct (mBRG1, **lane 2**) and cultured under GA + OSM culture conditions. Whole cell lysates were isolated and Western blot performed using anti-C/EBPδ antibody. The results shown are representative of three independent experiments.

exponentially growing (GROW) and exponentially growing plus OSM (GROW + OSM) treated HC11 cells (Fig. 4A, c-Myc). In contrast, serum and growth factor withdrawal-induced growth arrest (GA) or GA + OSM treatment dramatically reduced c-Myc levels (Fig. 4A, c-Myc). In contrast to c-Myc, HC11 Miz-1 protein levels are not significantly altered by changes in cell growth status or cytokine treatment (Fig. 4A, Miz-1). It is noteworthy that pSTAT3 levels are similar in HC11 cells under GROW + OSM, GA and GA + OSM conditions, but C/EBPδ is only significantly induced under GA and GA + OSM conditions (Fig. 4A). We hypothesized that the uncoupling of pSTAT3 and C/EBPδ expression may be due to growth regulated differences in c-Myc expression and possible c-Myc repression of the C/EBPδ promoter. To more closely examine the temporal expression of C/EBPδ gene expression in response to OSM, we compared the induction of C/EBPδ mRNA levels in HC11 cells under GROW + OSM and GA + OSM conditions. The RT-PCR results indicated that the C/EBPδ mRNA content was consistently higher in the GA + OSM treated HC11 cells compared to the GROW + OSM treated HC11 cells at all time points from 15 min to 24 h (Fig. 4B, C/EBPδ). These results suggest that C/EBPδ gene expression is repressed under exponentially growing (GROW) conditions, and that this repression is released under growth arrest plus OSM (GA + OSM) conditions. The mechanism underlying this repression does not appear to involve pSTAT3, the principal transcriptional activator of C/EBPδ, as pSTAT3 levels are comparable between exponentially growing plus OSM (GROW + OSM) and growth arrest (GA, GA + OSM) treated HC11 cells (Fig. 4A, pSTAT3, lanes 2–4).

We next directly assessed the repressive role of c-Myc on C/EBPδ promoter activity by

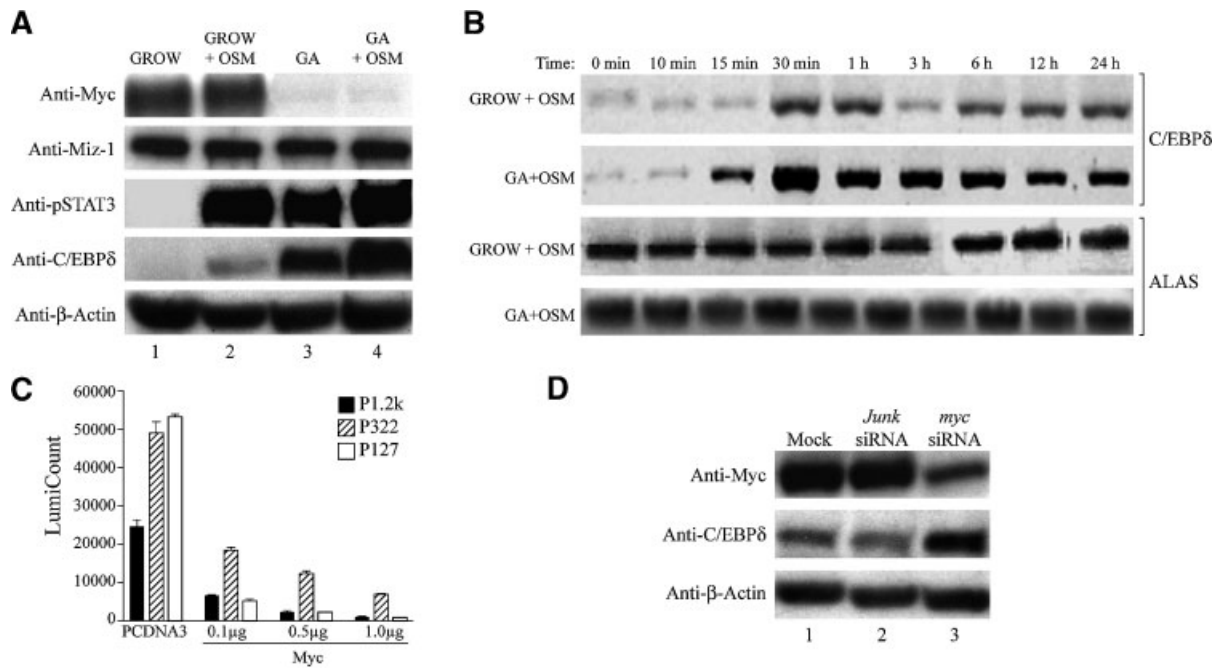

Fig. 4. c-Myc represses the C/EBPδ promoter. (**A**) Western blots were performed on HC11 whole cell lysates using anti-c-Myc, anti-Miz-1, anti-pSTAT3 and anti-C/EBPδ antibodies under GROW, GROW + OSM, GA, GA + OSM conditions (**lanes 1–4**). β-Actin was the loading control. (**B**) Reverse transcription PCR analysis of the 0–24 h time course of OSM-induced C/EBPδ mRNA accumulation in HC11 cells cultured under GROW + OSM and GA + OSM conditions. Aminolevulinate synthase (ALAS) mRNA detection was used as loading control. (**C**) c-Myc represses C/EBPδ promoter transcriptional activation. HC11 cells were co-transfected with pcDNA3 (vector control) or P-1.2k, P-322 and P-127 C/EBPδ promoter-luciferase constructs plus 0.1, 0.5 and 1 μg of a c-Myc expression construct under growth arrest (GA + OSM) culture conditions. Luciferase activities measured at 24 h were normalized to Renilla luciferase activities and the mean ± SEM are presented. (**D**) HC11 cells were nucleofected with a 'junk' (scrambled) siRNA construct and a c-Myc siRNA construct using the Amaxa nucleofector protocol. Nucleofected HC11 cells were cultured under GROW + OSM conditions. Mock transfected cells were nucleofected in the absence of any siRNA construct. Whole cell lysates were isolated and analyzed by Western blot using anti-c-Myc and anti-C/EBPδ antibodies (**lanes 1–3**). β-Actin was the loading control. The results shown are representative of three independent experiments.

co-transfection of a c-Myc expression construct with C/EBPδ promoter-luciferase constructs ranging in size from 1.2 kb to 127 bp. The results demonstrated a dose dependent c-Myc-mediated repression of all C/EBPδ promoter constructs (Fig. 4C). c-Myc effectively repressed activation of even the minimal 127 bp (P-127) C/EBPδ promoter construct which is limited to essential promoter elements such as the Sp1, pSTAT3 and CREB consensus sites and the TATA box [Cantwell et al., 1998; O'Rourke et al., 1999a]. We next reduced endogenous c-Myc levels using siRNA and assessed C/EBPδ expression under GROW + OSM conditions. Western blot analysis demonstrated that c-Myc siRNA treatment reduced endogenous c-Myc levels by ~four fold compared to mock transfected and junk or "scrambled" siRNA transfected controls (Fig. 4D, lanes 1 and 2 vs. lane 3). siRNA-mediated c-Myc knockdown resulted in ~four fold increase in C/EBPδ protein levels under GROW + OSM conditions (Fig. 4D, lanes 1 and 2 vs. lane 3). These results are consistent with c-Myc repression of C/EBPδ gene expression.

C-Myc Represses the C/EBPδ Proximal Promoter via the Formation of a Protein Complex Composed of C-Myc, Max, and DNA-Bound Miz -1

To investigate the mechanism of c-Myc repression of the C/EBPδ proximal promoter we used Co-IP assays to investigate candidate c-Myc interacting proteins in exponentially growing (C/EBPδ nonexpressing) HC11 cells. Co-IP results indicated that c-Myc interacts with Max and Miz-1, but not Sp1 (Fig. 5A). We next used ChIP assays to determine if the increased levels of c-Myc present in GROW and GROW + OSM treated HC11 cells are associated with detectable c-Myc binding to the C/EBPδ proximal promoter. The ChIP results demonstrate that c-Myc is physically associated with the C/EBPδ proximal promoter under GROW and GROW + OSM conditions; i.e., conditions associated with repression of C/EBPδ expression (Fig. 5B, lanes 3 and 4, P200). Importantly, the ChIP results also demonstrate that c-Myc dissociates from the C/EBPδ proximal promoter under GA (alone) or GA + OSM conditions (Fig. 5B, lanes 5 and 6, P200), i.e., conditions associated with activation of C/EBPδ expression (Fig. 4A, C/EBPδ Panel). ChIP assays demonstrated that Miz-1 remains constitutively associated with the C/EBPδ proximal promoter under C/EBPδ expressing and C/EBPδ nonexpressing conditions (Fig. 5C, lanes 3–6, P200). These results are consistent with a model in which a complex composed of c-Myc, Max and DNA-bound Miz-1 repress the C/EBPδ proximal promoter in exponentially growing cells [Gartel

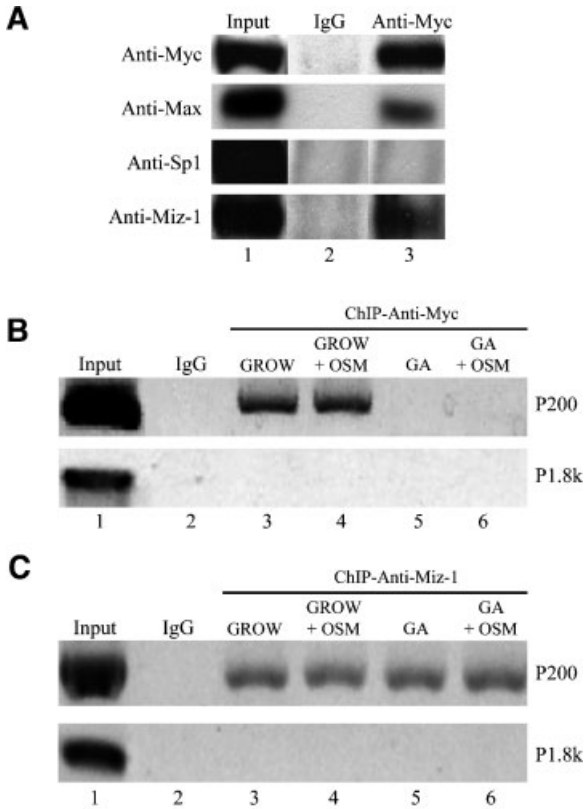

Fig. 5. c-Myc represses the C/EBPδ proximal promoter by interacting with DNA-bound Miz-1. (**A**) Co-IP reactions were performed to determine c-Myc interacting proteins in lysates from C/EBPδ nonexpressing HC11 cells (GROW). Anti-c-Myc antibody was used in pull down reactions and the IP complexes were analyzed by Western blot using anti-c-Myc (positive control), anti-Sp1, anti-Miz1 and anti-Max antibodies (**lane 3**). (**B**) ChIP assays were performed with lysates from GROW, GROW + OSM, GA and GA + OSM treated HC11 cells using anti-c-Myc antibody (**lanes 3–6**). ChIP isolated DNA was PCR amplified using primers that flank the C/EBPδ proximal promoter region (P200) and the C/EBPδ upstream promoter region (P1.8k). 'Input' results were obtained from genomic DNA isolated from treated HC11 cells (positive control). 'IgG' results were obtained from ChIP assays performed with nonspecific rabbit IgG (negative control). (C) ChIP assays were performed with lysates from GROW, GROW + OSM, GA and GA + OSM treated HC11 cells using anti-Miz-1 antibodies (**lanes 3–6**). ChIP isolated DNA was PCR amplified using primers that flank the C/EBPδ proximal promoter region (P200) and the C/EBPδ upstream promoter region (P1.8k). 'Input' results were obtained from genomic DNA isolated from treated HC11 cells (positive control). 'IgG' results were obtained from ChIP assays performed with nonspecific rabbit IgG (negative control). The results shown are representative of three independent experiments.

and Shchors, 2003; Wanzel et al., 2003; Adhikary and Eilers, 2005].

DISCUSSION

The present report provides a molecular model of the transcriptional regulation of C/EBPδ in mammary epithelial cells. The results establish that the cellular content of pSTAT3 and C/EBPδ increase in parallel in response to growth arrest plus cytokine treatment and that C/EBPδ is a direct pSTAT3 downstream target gene. The induction of C/EBPδ promoter activity by STAT3C, a constitutively active STAT3 construct that mimics the transcriptional activity of pSTAT3, is consistent with a recent report in which STAT3C induced genes were identified using the Affymetrix human genome U133A GeneChip. Affymetrix GeneChip analysis identified C/EBPδ among the top 20 genes induced by STAT3C, and, in addition, ranked C/EBPδ as the highest STAT3C inducible gene in the "Cell Cycle" and "Transcription factors" gene categories [Dauer et al., 2005]. Our results demonstrating the direct binding of pSTAT3 and Sp1 to C/EBPδ proximal promoter by ChIP analysis and DNA precipitation assays suggests that these specific protein–DNA interactions are independent of analytical technique or chromatin structure. These results demonstrate that C/EBPδ is a direct downstream target of activated STAT3 and that growth arrest is the primary STAT3 mediated physiological response in nontransformed mammary epithelial cells.

We further show that Sp1 and pSTAT3 interact and initiate the formation of a transcriptional activation complex on the C/EBPδ promoter that includes the transcriptional coactivator NcoA/SRC1. Loeffler et al. recently demonstrated that IL-6 induces the formation of STAT3/Sp1 complexes on the vascular endothelial growth factor (VEGF) promoter and increases VEGF transcriptional activation in astrocytes [Loeffler et al., 2005]. NcoA/SRC1 has previously been shown to bind to DNA-bound pSTAT3 in the transcriptional activation of $p21^{waf1/cip1}$, a cyclin-dependent kinase inhibitor that functions in cell cycle (growth) arrest [Giraud et al., 2002; Barre et al., 2003]. These results indicate that IL-6 family cytokines activate STAT3 which can then interact with Sp1 and NcoA/SRC1 to activate growth regulating genes, such as C/EBPδ, VEGF and $p21^{waf1/cip1}$ [Giraud et al., 2002; Barre et al., 2003; Loeffler et al., 2005].

The promoters of cytokine inducible genes, such as the TNFα-inducible A20 gene, are constitutively bound with selected transcription factors, such as Sp1, and components of the general transcription machinery [Ainbinder et al., 2002]. In this study, we show that transcriptional activators Sp1 and CREB and transcriptional machinery components TBP and RNA polymerase II are pre-loaded on the C/EBPδ promoter. GA + OSM treatment induces an increase in pSTAT3 levels and the assembly of a transcriptional activation complex composed of pSTAT3, Sp1 and NcoA/SRC1 on the C/EBPδ proximal promoter. This complex may facilitate the subsequent recruitment of p300/CBP to the C/EBPδ promoter. Alternatively, p300/CBP binding to the C/EBPδ promoter may be mediated by pCREB as C/EBPδ promoter associated pCREB also increases in response to GA + OSM treatment. ChIP assays indicated that p300 was associated with the transcriptionally active C/EBPδ gene promoter (Fig. 3), but CBP is not (data not shown). These results differ from those reported by Coqueret and coworkers in which STAT3 mediated transcriptional activation of $p21^{waf1/cip1}$ and c-myc was associated with the recruitment of CBP [Giraud et al., 2002; Barre et al., 2003]. The pSTAT3 recruitment of CBP was cell type specific however, suggesting that the recruitment of transcriptional coactivators is regulated in a cell type specific manner [Barre et al., 2003]. Accumulating evidence indicates that despite significant structural homology and functional redundancy, p300 and CBP exhibit unique protein–protein interactions, histone acetylation specificities and tissue-specific functions [Kalkhoven, 2004]. The specific recruitment of p300 to the C/EBPδ suggests a specific role for p300 in the activation of C/EBPδ gene expression in mammary epithelial cells in response to G_0 growth arrest and cytokine treatment.

In addition to the specific recruitment of p300, GA + OSM treatment is also associated with RNA Pol II CTD domain Ser2 phosphorylation in association with the C/EBPδ gene promoter. The Ser2 phosphorylated form of RNA Pol II is the transcriptional elongating form of RNA Pol II [Sims et al., 2004]. This observation is of interest as cellular gene transcription is dramatically reduced in growth arrested cells [Johnson et al., 1975, 1976]. The regulation of the RNA Pol II "transcription cycle", i.e., RNA

Pol II preinitiation, initiation, promoter clearance, elongation and termination is emerging as a complex integration of multiple biochemical processes [Sims et al., 2004]. The coordinated sequence of events that result in the loading and site-specific modifications of transcription machinery components on the C/EBPδ promoter under G_0 growth arrest conditions are currently under investigation.

Alterations in histone modifications and chromatin remodeling complex components play a major role in eukaryotic gene transcriptional regulation by influencing the accessibility of chromatin DNA targets to transcriptional proteins [Sif et al., 2001; Narlikar et al., 2002; Gibbons, 2005; Roeder, 2005]. It has been speculated that a subset of gene promoters may exist in an "open" chromatin conformation to facilitate rapid transcriptional activation in response to external conditions or cellular stresses [Michelotti et al., 1997; Xing et al., 2005]. We observed histone post-translational modifications that are consistent with an open conformation for the C/EBPδ proximal promoter. The specific histone acetyltransferase (HAT) responsible for acetylating histone H3 and H4 in association with the C/EBPδ proximal promoter is unknown. One intriguing possibility is that the "open" or uncompacted structure of the C/EBPδ proximal promoter in exponentially growing cells is an indicator of "gene bookmarking"; and the C/EBPδ gene is "bookmarked" for expression following emergence from mitosis [Sarge and Park-Sarge, 2005; Xing et al., 2005]. Genes that are "bookmarked" are characterized by an open or "hypersensitive" gene promoter structure during mitosis [Sarge and Park-Sarge, 2005]. This has led to the hypothesis that bookmarking is a mechanism to mark genes for expression upon emergence from mitosis [Sarge and Park-Sarge, 2005; Xing et al., 2005]. For example, bookmarked genes such as c-myc have been linked to functions in early G_1, suggesting that bookmarking facilitates cell cycle re-entry following completion of mitosis [Sarge and Park-Sarge, 2005]. Alternatively, bookmarking may identify a subset of genes required for protection from cellular stress (i.e. *hsp70i*) [Xing, 2005]. C/EBPδ may be "bookmarked" to facilitate entry into a transient G_0 state prior to cell cycle re-entry in actively mitotic (exponentially growing) cells, or, C/EBPδ may be "bookmarked" to facilitate entry into a more prolonged G_0 state in quiescent cells. Alternatively, the C/EBPδ promoter may be bookmarked to facilitate C/EBPδ expression in response to stress or to maintain an essential function in mammary epithelial cells. In addition to histone modifications, our data showed that "loss of function" alterations in BRG1 reduce C/EBPδ expression, suggesting a mechanism by which BRG1 may contribute to loss of growth control and tumorigenesis [Gibbons, 2005].

The present work also demonstrated that pSTAT3 activation of C/EBPδ expression is repressed in exponentially growing, nontransformed mammary epithelial cells exposed to Oncostatin M (OSM), a growth inhibitory cytokine. This observation led to the discovery of c-Myc mediated repression of C/EBPδ expression and the detection of a c-Myc containing protein complex that blocked pSTAT3 activation of C/EBPδ transcriptional activation. Early studies had shown that c-Myc repressed C/EBPα transcription through interaction with the C/EBPα core promoter [Antonson et al., 1995]. It is of interest that both C/EBPα and C/EBPδ have been extensively linked to tissue and cell-type specific growth arrest and differentiation [Johnson, 2005]. The current results indicate that c-Myc is associated with a dynamic repression of C/EBPδ promoter activation during cell proliferation and this c-Myc mediated repression is lost during growth arrest. Co-IP results suggest that a c-Myc-Max repressive complex interacts with C/EBPδ proximal promoter bound Miz-1. Ongoing studies are investigating the protein-protein interactions that mediate c-Myc repression of C/EBPδ promoter activation as well as the mechanisms underlying the dissociation of c-Myc in response to growth arrest conditions.

The results in this study advance our understanding of C/EBPδ transcriptional regulation by providing new insights into the molecular mechanisms underlying the coordinated interaction of transcriptional activators (pSTAT3 and Sp1, pCREB), co-activators (NcoA/SRC1 and p300), chromatin remodeling complex and the transcriptional machinery components. In addition, the C/EBPδ proximal promoter is maintained in an active chromatin conformation allowing constitutively association with the transcriptional machinery components. This novel "pre-loaded" state is believed to facilitate rapid induction of C/EBPδ transcription in response to growth arrest conditions and cytokine

treatment. We also show that C/EBPδ transcription is subject to c-Myc repression and this repression cannot be "overridden" by activation signals (increased pSTAT3). This suggests that c-Myc repression plays a dominant role in the regulation of C/EBPδ expression under growing conditions. These results provide new insights into the regulation of C/EBPδ, a novel gene that is expressed in response to growth arrest and cytokine treatment and a gene that is localized in close proximity to the centromere, a chromosomal location traditionally associated with reduced transcriptional activation. These insights provide a new understanding of the comprehensive regulation of C/EBPδ gene expression and will be useful in identifying alterations that reduce C/EBPδ expression in tumorigenesis. In addition, a better understanding of the regulation of C/EBPδ may lead to therapeutic strategies to re-activate C/EBPδ transcription and inhibit cancer cell growth.

REFERENCES

Adhikary S, Eilers M. 2005. Transcriptional regulation and transformation by Myc proteins. Nat Rev Mol Cell Biol 6:635–645.

Ainbinder E, Revach M, Wolstein O, Moshonov S, Diamant N, Dikstein R. 2002. Mechanism of rapid transcriptional induction of tumor necrosis factor alpha-responsive genes by NF-kappaB. Mol Cell Biol 22:6354–6362.

Antonson P, Pray MG, Jacobsson A, Xanthopoulos KG. 1995. Myc inhibits CCAAT/enhancer-binding protein alpha-gene expression in HIB-1B hibernoma cells through interactions with the core promoter region. Eur J Biochem 232:397–403.

Barre B, Avril S, Coqueret O. 2003. Opposite regulation of myc and p21waf1 transcription by STAT3 proteins. J Biol Chem 278:2990–2996.

Barsyte-Lovejoy D, Mao DY, Penn LZ. 2004. c-Myc represses the proximal promoters of GADD45a and GADD153 by a post-RNA polymerase II recruitment mechanism. Oncogene 23:3481–3486.

Belmonte N, Phillips BW, Massiera F, Villageois P, Wdziekonski B, Saint-Marc P, Nichols J, Aubert J, Saeki K, Yuo A, Narumiya S, Ailhaud G, Dani C. 2001. Activation of extracellular signal-regulated kinases and CREB/ATF-1 mediate the expression of CCAAT/enhancer binding proteins beta and -delta in preadipocytes. Mol Endocrinol 15:2037–2049.

Brenner C, Deplus R, Didelot C, Loriot A, Vire E, De Smet C, Gutierrez A, Danovi D, Bernard D, Boon T, Pelicci PG, Amati B, Kouzarides T, de Launoit Y, Di Croce L, Fuks F. 2005. Myc represses transcription through recruitment of DNA methyltransferase corepressor. EMBO J 24:336–346.

Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE Jr. 1999. Stat3 as an oncogene. Cell 98:295–303.

Cantwell CA, Sterneck E, Johnson PF. 1998. Interleukin-6-specific activation of the C/EBPdelta gene in hepatocytes is mediated by Stat3 and Sp1. Mol Cell Biol 18:2108–2117.

Dauer DJ, Ferraro B, Song L, Yu B, Mora L, Buettner R, Enkemann S, Jove R, Haura EB. 2005. Stat3 regulates genes common to both wound healing and cancer. Oncogene 24:3397–3408.

Gartel AL, Shchors K. 2003. Mechanisms of c-myc-mediated transcriptional repression of growth arrest genes. Exp Cell Res 283:17–21.

Gartel AL, Ye X, Goufman E, Shianov P, Hay N, Najmabadi F, Tyner AL. 2001. Myc represses the p21 (WAF1/CIP1) promoter and interacts with Sp1/Sp3. Proc Natl Acad Sci USA 98:4510–4515.

Gibbons RJ. 2005. Histone modifying and chromatin remodelling enzymes in cancer and dysplastic syndromes. Hum Mol Genet 14 Spec No 1:R85–92.

Gigliotti AP, Johnson PF, Sterneck E, DeWille JW. 2003. Nulliparous CCAAT/enhancer binding proteindelta (C/EBPdelta) knockout mice exhibit mammary gland ductal hyperlasia. Exp Biol Med (Maywood) 228:278–285.

Giraud S, Bienvenu F, Avril S, Gascan H, Heery DM, Coqueret O. 2002. Functional interaction of STAT3 transcription factor with the coactivator NcoA/SRC1a. J Biol Chem 277:8004–8011.

Hutt JA, DeWille JW. 2002. Oncostatin M induces growth arrest of mammary epithelium via a CCAAT/enhancer-binding protein delta-dependent pathway. Mol Cancer Ther 1:601–610.

Hutt JA, O'Rourke JP, DeWille J. 2000. Signal transducer and activator of transcription 3 activates CCAAT enhancer-binding protein delta gene transcription in G0 growth-arrested mouse mammary epithelial cells and in involuting mouse mammary gland. J Biol Chem 275:29123–29131.

Jenkins NA, Gilbert DJ, Cho BC, Strobel MC, Williams SC, Copeland NG, Johnson PF. 1995. Mouse chromosomal location of the CCAAT/enhancer binding proteins C/EBP beta (Cebpb), C/EBP delta (Cebpd), and CRP1 (Cebpe). Genomics 28:333–336.

Johnson PF. 2005. Molecular stop signs: regulation of cell-cycle arrest by C/EBP transcription factors. J Cell Sci 118:2545–2555.

Johnson LF, Williams JG, Abelson HT, Green H, Penman S. 1975. Changes in RNA in relation to growth of the fibroblast. III. Posttranscriptional regulation of mRNA formation in resting and growing cells. Cell 4:69–75.

Johnson LF, Levis R, Abelson HT, Green H, Penman S. 1976. Changes in RNA in relation to growth of the fibroblast. IV. Alterations in theproduction and processing of mRNA and rRNA in resting and growing cells. J Cell Biol 71:933–938.

Kalkhoven E. 2004. CBP and p300: HATs for different occasions. Biochem Pharmacol 68:1145–1155.

Lam AL, Boivin CD, Bonney CF, Rudd MK, Sullivan BA. 2006. Human centromeric chromatin is a dynamic chromosomal domain that can spread over noncentromeric DNA. Proc Natl Acad Sci USA 103:4186–4191.

Lee TC, Li L, Philipson L, Ziff EB. 1997. Myc represses transcription of the growth arrest gene gas1. Proc Natl Acad Sci USA 94:12886–12891.

Loeffler S, Fayard B, Weis J, Weissenberger J. 2005. Interleukin-6 induces transcriptional activation of vascular endothelial growth factor (VEGF) in astrocytes in

vivo and regulates VEGF promoter activity in glioblastoma cells via direct interaction between STAT3 and Sp1. Int J Cancer 115:202–213.

Michelotti EF, Sanford S, Levens D. 1997. Marking of active genes on mitotic chromosomes. Nature 388:895–899.

Naar AM, Lemon BD, Tjian R. 2001. Transcriptional coactivator complexes. Annu Rev Biochem 70:475–501.

Narlikar GJ, Fan HY, Kingston RE. 2002. Cooperation between complexes that regulate chromatin structure and transcription. Cell 108:475–487.

O'Rourke J, Yuan R, DeWille J. 1997. CCAAT/enhancer-binding protein-delta (C/EBP-delta) is induced in growth-arrested mouse mammary epithelial cells. J Biol Chem 272:6291–6296.

O'Rourke JP, Hutt JA, DeWille J. 1999a. Transcriptional regulation of C/EBPdelta in G(0) growth-arrested mouse mammary epithelial cells. Biochem Biophys Res Commun 262:696–701.

O'Rourke JP, Newbound GC, Hutt JA, DeWille J. 1999b. CCAAT/enhancer-binding protein delta regulates mammary epithelial cell G0 growth arrest and apoptosis. J Biol Chem 274:16582–16589.

Perrod S, Gasser SM. 2003. Long-range silencing and position effects at telomeres and centromeres: parallels and differences. Cell Mol Life Sci 60:2303–2318.

Ramji DP, Foka P. 2002. CCAAT/enhancer-binding proteins: structure, function and regulation. Biochem J 365:561–575.

Reisman DN, Strobeck MW, Betz BL, Sciariotta J, Funkhouser W Jr, Murchardt C, Yaniv M, Sherman LS, Knudsen ES, Weissman BE. 2002. Concomitant down-regulation of BRM and BRG1 in human tumor cell lines: differential effects on RB-mediated growth arrest vs CD44 expression. Oncogene 21:1196–1207.

Roeder RG. 2005. Transcriptional regulation and the role of diverse coactivators in animal cells. FEBS Lett 579:909–915.

Sanford DC, DeWille JW. 2005. C/EBPdelta is a downstream mediator of IL-6 induced growth inhibition of prostate cancer cells. Prostate 63:143–154.

Sarge KD, Park-Sarge OK. 2005. Gene bookmarking: keeping the pages open. Trends Biochem Sci 30:605–610.

Sif S. 2004. ATP-dependent nucleosome remodeling complexes: enzymes tailored to deal with chromatin. J Cell Biochem 91:1087–1098.

Sif S, Saurin AJ, Imbalzano AN, Kingston RE. 2001. Purification and characterization of mSin3A-containing Brg1 and hBrm chromatin remodeling complexes. Genes Dev 15:603–618.

Sims RJ 3rd, Belotserkovskaya R, Reinberg D. 2004. Elongation by RNA polymerase. II. the short and long of it. Genes Dev 18:2437–2468.

Sivko GS, DeWille JW. 2004. CCAAT/Enhancer binding protein delta (C/EBPdelta) regulation and expression in human mammary epithelial cells. I. "Loss of function" alterations in the c/EBPdelta growth inhibitory pathway in breast cancer cell lines. J Cell Biochem 93:830–843.

Sivko GS, Sanford DC, Dearth LD, Tang D, DeWille JW. 2004. CCAAT/Enhancer binding protein delta (C/EBP-delta) regulation and expression in human mammary epithelial cells. II. Analysis of activating signal transduction pathways, transcriptional, post-transcriptional, and post-translational control. J Cell Biochem 93:844–856.

Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Moroy T, Bartek J, Massague J, Hanel F, Eilers M. 2001. Repression of p15INK4b expression by Myc through association with Miz-1. Nat Cell Biol 3:392–399.

Tang D, DeWille J. 2003. Detection of base sequence changes in the CEBPD gene in human breast cancer cell lines and primary breast cancer isolates. Mol Cell Probes 17:11–14.

Tang D, Sivko GS, Dewille JW. 2005. Promoter methylation reduces C/EBPdelta (CEBPD) gene expression in the SUM-52PE human breast cancer cell line and in primary breast tumors. Breast Cancer Res Treat 1–10.

Vegesna V, Takeuchi S, Hofmann WK, Ikezoe T, Tavor S, Krug U, Fermin AC, Heaney A, Miller CW, Koeffler HP. 2002. C/EBP-beta, C/EBP-delta, PU.1, AML1 genes: mutational analysis in 381 samples of hematopoietic and solid malignancies. Leuk Res 26:451–457.

Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M. 2002. Classification of human B-ZIP proteins based on dimerization properties. Mol Cell Biol 22:6321–6335.

Wanzel M, Herold S, Eilers M. 2003. Transcriptional repression by Myc. Trends Cell Biol 13:146–150.

Xing H, Wilkerson DC, Mayhew CN, Lubert EJ, Skaggs HS, Goodson ML, Hong Y, Park-Sarge OK, Sarge KD. 2005. Mechanism of hsp70i gene bookmarking. Science 307:421–423.

Yang W, Shen J, Wu M, Arsura M, FitzGerald M, Suldan Z, Kim DW, Hofmann CS, Pianetti S, Romieu-Mourez R, Freedman LP, Sonenshein GE. 2001. Repression of transcription of the p27(Kip1) cyclin-dependent kinase inhibitor gene by c-Myc. Oncogene 20:1688–1702.