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PIASy Represses CCAAT/Enhancer Binding Protein δ (C/EBPδ) Transcriptional Activity by Sequestering C/EBPδ to the Nuclear Periphery

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CCAAT/Enhancer Binding Protein δ (C/EBPδ) plays a key role in mammary epithelial cell G_0 growth arrest and "loss of function" alterations in C/EBPδ have been reported in breast cancer and acute myeloid leukemia (AML). C/EBPδ is regulated at the transcriptional, post-transcriptional and post-translational levels, suggesting tight control of C/EBPδ content and function. Protein inhibitors of activated STATs (PIASs) regulate a growing number of transcription factors, including C/EBPs. HC11 nontransformed mammary epithelial cells express PIAS3, PIASxβ and PIASy and all three PIAS family members repress C/EBPδ transcriptional activity. PIASy is the most potent however, repressing C/EBPδ transcriptional activity by >80%.

PIASy repression of C/EBPδ transcriptional activity is dependent upon interaction between the highly conserved PIASy N-terminal nuclear matrix binding domain (SAPD) and the C/EBPδ transactivation domain (TAD). PIASy repression of C/EBPδ transcriptional activity is independent of histone deacetylase activity, PIASy E3 SUMO ligase activity and C/EBPδ sumoylation status. PIASy expression is associated with C/EBPδ translocation from nuclear foci, where C/EBPδ co-localizes with p300, to the nuclear periphery. PIASy-mediated translocation of C/EBPδ is dependent upon the PIASy SAPD and C/EBPδ TAD. PIASy reduces the expression of C/EBPδ adhesion-related target genes and enhances repopulation of open areas within a cell monolayer in the *in vitro* "scratch" assay. These results demonstrate that PIASy represses C/EBPδ by a mechanism that requires interaction between the PIASy SAPD and C/EBPδ TAD and does not require PIASy SUMO ligase activity or C/EBPδ sumoylation. PIASy alters C/EBPδ nuclear localization, reduces C/EBPδ transcriptional activity and enhances cell proliferation/migration.

INTRODUCTION

C/EBPδ is a member of the highly conserved CCAAT/Enhancer Binding Protein (C/EBP) family of nuclear proteins (1). Six mammalian C/EBP family members have been identified including C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε and C/EBPζ (CHOP10)(1). C/EBPs are highly conserved in evolution, with homologues identified in the sea slug (*Aplysia californica,* (ApC/EBP)), zebrafish (*Danio rerio,* (cebpd)), frog (*xenopus laevis, (C/EBPδ-1 and -2))* and fruit fly (*Drosophila melangaster* (DmC/EBP)) (2-5). C/EBPs are characterized by conserved structural domains including a transactivation domain (TAD), a regulatory domain (RD), and highly conserved DNA binding (DB) and leucine zipper domains (LZ) (1). Although primarily recognized as transcriptional activators, C/EBP family members, including C/EBPδ, also function in protein-protein interactions with key cell cycle regulatory proteins such as Rb, p21, CDK2 and CDK4 (6-9).

Reports from our laboratory and others demonstrate that C/EBPδ is regulated at the transcriptional, post-transcriptional and post-translational levels (10-17). At the

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transcriptional level, the C/EBPδ gene promoter is induced by activated (phosphorylated) STAT3 (pSTAT3), Sp1, pCREB and the transcriptional co-activator NcoA/SRC-1 (10,16). Using nuclear run-on assays we made the unexpected observation that C/EBPδ gene transcription rates are markedly elevated in G_0 growth arrested cells even though overall biosynthetic activity is reduced during G_0 growth arrest (11). Although C/EBPδ gene transcription is highly induced, C/EBPδ gene products, ie, C/EBPδ mRNA and protein, exhibit relatively short half lives in G_0 growth arrested cells (13,17). The rapid turnover of C/EBPδ gene products suggests that cells maintain tight control of C/EBPδ content and functional activity.

Published reports support a role for C/EBPδ in cell cycle arrest, differentiation and cell fate determination. C/EBPδ gene expression is highly induced in G_0 growth arrested, nontransformed, human and mouse mammary epithelial cells and anti-sense mediated reduction of C/EBPδ delays entry into G_0 growth arrest (11,12,14,15,18-21). In vivo, virgin female C/EBPδ knockout mice exhibit increased mammary epithelial cell proliferation and mammary gland ductal hyperplasia (22). In adipocyte differentiation models, C/EBPδ is expressed in pre-adipocytes prior to commitment to differentiation and adipocyte differentiation is defective in C/EBPδ knockout MEFs (23-25). Recent work indicates that C/EBPδ inhibits growth and promotes self-renewal in human limbic stem cells, suggesting a role for C/EBPδ in the maintenance of stem cell pluripotency (26). In addition, “loss of function” alterations in C/EBPδ have been reported in human breast cancer and acute myeloid leukemia (27-30). Collectively, these results demonstrate that C/EBPδ functions in growth control and cell fate determination and alterations in C/EBPδ are associated with tumorigenesis.

PIAS gene family members (PIAS1, PIAS3, PIASy and PIASx) regulate transcription factor function by sumoylation, inhibition of DNA binding, HDAC recruitment and sequestration in nuclear foci or in the nuclear periphery (31,32). PIAS proteins are characterized by a highly conserved domain structure that includes a SAP domain (SAPD) that contains an LXXLL motif and a conserved Ring Finger-like domain (RFD) that is required for PIAS SUMO E3 ligase activity (31,32). PIAS proteins regulate C/EBPα and C/EBPε sumoylation status and function (33-35), however, it is unknown if PIAS proteins regulate C/EBPs exclusively by sumoylation or if alternate mechanisms, ie, HDAC recruitment or subnuclear sequestration are also utilized. In this report we demonstrate that PIASy represses C/EBPδ transcriptional activity by sequestering C/EBPδ in the nuclear periphery. PIASy-mediated sequestration of C/EBPδ is associated with reduced expression of adhesion related C/EBPδ target genes and enhanced mammary epithelial cell proliferation/migration. These results suggest a potential role for PIASy-C/EBPδ interactions in the control of mammary epithelial cell growth or migration.

EXPERIMENTAL PROCEDURES

Plasmid construction --- C/EBPδ constructs (C/EBPδ full length cDNA, C/EBPδ ΔTAD (amino acids 102-268) and K120R mutant) were cloned into the PCDNA3.1/V5-His-TOPO TA expression vector (Invitrogen, Carlsbad, CA). The Flag-PIASy expression plasmid was constructed by PCR amplification of the PIASy coding sequence using a forward primer bearing a Flag tag coding sequence, and the PCR product was inserted into the PCDNA3 expression vector. The ΔSAP and ΔRFD PIASy mutants were generated by site-directed mutagenesis, where amino acids 12-46 were deleted in ΔSAP and amino acids 319-363 were deleted in ΔRFD. The GST-C/EBPδ expression vector was generated by cloning the full length C/EBPδ cDNA into pGEX-4T-1 vector (Amersham Biosciences, Piscataway, NJ) for expression in the E.coli strain DE3 (BL21). ΔTAD (102-268), DBLZ (171-268), ΔLZ (1-233) and TAD (1-102) GST fusion protein expression plasmids were

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constructed by PCR amplification of the corresponding cDNA sequences and cloned into the same vector. All expression vectors and inserts were verified by DNA sequencing.

Cell culture and transient transfections--- HC11 cells (mouse immortalized mammary epithelial cell line) were grown in complete growth media (CGM) containing RPMI 1640 medium (Invitrogen) containing 10% FBS and supplemented with 10 ng/ml epidermal growth factor, 10μg/ml insulin, 50 units/ml penicillin, 50μg/ml streptomycin and 500ng/ml fungizone in a humidified incubator at 37°C and 5% CO_2. Transient transfections were performed using Lipofectamine transfection reagent and plus reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Lipofectamine™ Reagent is a 3:1 (w/w) liposome formulation of the polycationic lipid 2,3-dioleyloxy-N-[2(sperminecarboxamido)ethyl]-N,Ndimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane-filtered water. Selected transient transfection experiments were performed using the Nucleofector device (Amaxa, Gaithersburg, MD) according to manufacturer's instructions and utilizing proprietary reagents (Amaxa, Gaithersburg, MD). Following Lipofectamine and nucleofection protocols HC11 cells were cultured in CGM.

Luciferase reporter assay--- HC11 cells were cultured in 12 well plates. Experiments were performed by co-transfecting each well with 100ng wild type C/EBPδ (FL) or C/EBPδ K120R mutant plus 100ng C/EBP consensus site promoter driven luciferase reporter vector (pC/EBP-Luc, $(ATTGCGCAAT)_3$, (Stratagene, catalog # 240112, La Jolla, CA), 200ng PIAS protein and/or 100ng SENP1 expression vectors (Open Biosystems, Huntsville, AL). Co-transfection experiments were also performed with increasing amounts of SUMO-1, SUMO-2 and SUMO-3 (0, 50, 100ng). A renilla luciferase expression plasmid was used as the transfection efficiency control (1ng/well). Luciferase activity was analyzed 24 hrs after transfection using a dual-luciferase reporter assay system (Promega, Madison, WI), and the luciferase results were normalized to renilla luciferase activity. The normalized luciferase data for each independent experiment was derived triplicate wells and experiments were replicated 2-3 times. Statistical analysis was performed with pooled data using one way analysis of variance (ANOVA) and post test analysis by Dunnet's test with each PIAS treatment compared to the no PIAS control (CON). Statistical significance was set at $\alpha=0.05$. Results from transfection experiments in which Trichostatin A was used are representative of 3 independent experiments with at least two replicates/experiment. Pooled data were analyzed with two-way ANOVA with statistical significance was set at $\alpha=0.05$.

In vivo sumoylation assay--- HC11 cells (cultured in 10cm dishes) were co-transfected with HA-SUMO (SUMO-1, SUMO-2 or SUMO-3) expression constructs (2μg), C/EBPδ-V5-His (wild type or K120R mutant) (2μg), Flag-PIASy (2μg) and SENP1 expression vectors (2μg). The total amount of DNA was equalized for each treatment by the addition of PCDNA3 vector DNA. Cells were harvested 24 hours after transfection. Cell pellets were lysed in buffer A (8M urea, 0.1M Na_2HPO_4/NaH_2PO_4, 10mM imidazole, PH 8.0) and lysate protein levels were quantitated by the Micro BCA Protein assay (Pierce, Rockford, IL). Equal amounts of lysate protein were incubated with Ni-NTA agarose beads (Qiagen, Valencia, CA), and the beads were sequentially washed with buffer A, buffer A/TI (1 volume buffer A, 3 volume buffer TI), and buffer TI (25mM Tris-HCl, 20mM imidazole, PH 6.3). Bound proteins were eluted in 40μl 2× Laemmli sample buffer by boiling for 5 min. Samples were analyzed by SDS-PAGE/western blotting with anti-HA antibody (Cell Signaling, Danvers, MA).

GST pull-down assay--- E. coli expressing GST-C/EBPδ fusion proteins were lysed with

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lysis buffer (50mM Tris-HCl, 150mM NaCl, 5mM DTT, 1% Triton X-100, 1mM EDTA, PH7.5) supplemented with protease inhibitors including 1mM PMSF, 1μg/ml Aprotinin and 1μg/ml Pepstatin. GST-C/EBPδ fusion proteins were immobilized on glutathione sepharose 4B beads (Amersham Biosciences). HC11 cells expressing wild type or mutant Flag-PIASy were lysed in the same lysis buffer, and lysates were incubated overnight with glutathione sepharose beads bound with GST-C/EBPδ fusion proteins at 4°C. Glutathione sepharose beads were then collected by centrifugation, washed with lysis buffer and boiled in 40μl 2× Laemmli sample buffer for 5 min. Samples were analyzed by SDS-PAGE/western blotting with anti-Flag antibody (Cell Signaling).

Immunofluorescent staining and confocal microscope imaging--- HC11 cells were transfected with C/EBPδ-V5-His vector constructs (wild type or mutant) using the Lipofectamine Plus protocol and cultured on coverslips with or without Flag-PIASy (wild type or mutant) expression vector. Approximately 24 hrs after transfection, cells were fixed with 2% paraformaldehyde/PBS, and permeabilized with 1% Triton X-100/PBS. Cells were blocked in 10% normal goat serum in 0.1% Triton X-100/PBS at room temperature for 1 hr, and then incubated with mouse anti-V5 and rabbit anti-p300 or rabbit anti-Flag antibody at 4°C overnight in a humidified chamber. Cells were then incubated with alexa fluor 633 goat anti-mouse and alexa fluor 488 goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA) at room temperature for 1 hr. Coverslips were mounted with a drop of Vectashield mounting media with DAPI (Vector Laboratories, Burlingame, CA). Protein localization was visualized using Leica DM IRE2 confocal microscope system (Wetzlar, Germany).

HC11 cell proliferation and migration: the "scratch" assay--- The *in vitro* "scratch" assay was performed essentially as described by Liang, et al (36). Briefly, an open area was produced in ~90% HC11 cell monolayers using a 200 μl pipet tip and repopulation of the open area was assessed by crystal violet staining at 0, 24 and 48 hours. Staining was performed with 0.25% crystal violet in 40% methanol at room temperature for 5 minutes followed by extensive washing and air-drying.

To investigate the influence of C/EBPδ on the capacity of cells to repopulate the open area produced by the scratch protocol, transfections were performed using the Lipofectamine transfection reagent and plus reagent according to manufacturer's instructions (Invitrogen, Carlsbad, CA). C/EBPδ levels were reduced using the p*Silencer*™ neo Kit (Ambion, Inc., Austin TX). Briefly, HC11 cells were subjected to the Lipofectamine transfection protocol in which cells received either the pSilencer 2.1 vector (vector control) or a C/EBPδ siRNA-pSilencer 2.1 construct (C/EBPδ siRNA). Following transfection, the vector control and C/EBPδ siRNA treated HC11 cells were plated in complete growth media (CGM) plus G418 (400 μg/mL). Stable vector control and C/EBPδ siRNA cell lines were selected and maintained in G418 (200 μg/mL). To investigate the influence of PIASy expression on repopulation of the open area created in the monolayer by the scratch protocol, HC11 cells were subjected to transient transfection with the C/EBPδ or C/EBPδ + PIASy expression constructs using the Lipofectamine transfection reagent and plus reagent protocol according to manufacturer's instructions (Invitrogen, Carlsbad, CA). Following transfection HC11 cells were cultured in CGM, grown to a monolayer and the scratch assay was performed as described above.

mRNA isolation and Real Time PCR---Total mRNA was isolated using RNAzol B (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instruction. Total mRNA (1μg) was used for reverse transcription using the reverse transcriptase kit (Invitrogen, Carlsbad, CA). The reverse transcription products were amplified by Real-time PCR using the LightCycler® 480 Real-Time PCR System

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(Roche, Indianapolis, IN). Amplification was performed in a total volume of 20 µL containing 10 µL of a 2×SYBR Green PCR master mix, 0.2 µL of forward and reverse primers and 1 µL cDNA in each reaction. PCR specificity was verified by assessing the melting curves of each amplification product. Real-time PCR data were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA control. The following primers were used to assess C/EBPδ cell adhesion related target gene mRNA levels: glycoprotein V (GP5) sense:5' CGCCAGCCTGTCGTTCT, GP5 antisense: 5'GCCTGTTATT GGGACTTTCA C; integrin beta 8 (ITGB8) sense: 5' TTCTCCTGTCCCTATCTCCA, ITGB8 antisense: 5' TGAGACAGAT TGTGAGGGTG and protocadherin 9 (PCDH9) sense: 5' ACAGCCACCACGGTCCTCTA, PCDH9 antisense: 5' CCCTTGTTGT TCCCGCTCAC; GAPDH: sense:5'CTCACTGGCATGGCCTTCCG GAPDH antisense: (37) 5'ACCACCCTGTTGCTGTAGCC. The fold change in specific mRNA levels was calculated using the comparative CT (ΔΔCT) method (38). Results presented as mean ± SEM of the fold changes derived from three experiments with triplicate analyses performed for each treatment. The Student's *t* test was used to analyze the Real Time PCR results, the fold change in individual C/EBPδ target gene mRNA levels was considered significant at $P<0.05$.

RESULTS

PIAS family members repress C/EBPδ transcriptional activity. Previous reports have demonstrated that PIAS mediated sumoylation of the conserved regulatory domain motif (RDM) of C/EBPε increases C/EBPε transcriptional activity (33,34,39). These results suggest that PIAS proteins can regulate the function of C/EBPs in cells that co-express PIAS and C/EBP family members. Kim and coworkers reported that C/EBPδ contains an RDM and that C/EBPδ can be sumoylated, however, the role of specific PIAS proteins in C/EBPδ sumoylation was not defined (39). C/EBPδ gene expression is highly induced in G_0 growth arrested human and mouse mammary epithelial cells and C/EBPδ protein levels are essentially undetectable in growing cells (11,12,14,15,18-21). We hypothesized that PIAS family members expressed in G_0 growth arrested cells could influence C/EBPδ transcriptional activity and alter cell function. Since the influence of growth status on the expression of PIAS family members in mammary epithelial cells had not been previously reported, we assessed the expression of three PIAS family members (PIAS3, PIASxβ and PIASy) in exponentially growing and G_0 growth arrested HC11 mouse mammary epithelial cells. The results demonstrated that the mRNA levels of all three PIAS family members were present at relatively constitutive levels in growing and G_0 growth arrested HC11 cells (Fig. 1A). The constitutive expression of PIAS family members suggests that PIAS proteins function in a broad range of cellular contexts.

Having determined that PIAS family members were expressed in the HC11 mammary epithelial cell line, we next investigated the influence of PIAS proteins on C/EBPδ transcriptional activity. Full length PIAS3, PIASxβ and PIASy cDNAs cloned into the pcDNA3 expression vector were co-transfected into HC11 cells with a pC/EBP-Luciferase (pC/EBP-Luc) reporter construct and luciferase activity assessed. The results demonstrated that each of the three PIAS family members significantly repressed C/EBPδ transcriptional activity, however, PIASy was the most potent, inhibiting C/EBPδ transcriptional activity by ~80% (Fig. 1B). In addition, PIASy inhibited C/EBPδ transcriptional activity in a dose-dependent manner, demonstrating a direct correlation between PIASy levels and inhibition of C/EBPδ transcriptional activity (Fig. 1C).

PIASy inhibits transcriptional activity by a multiple mechanisms, including histone

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deacetylase (HDAC) recruitment (37,40). To investigate HDAC recruitment as the mechanism underlying PIASy-mediated inhibition of C/EBPδ transcriptional activity HC11 cells were co-transfected with PIASy plus the pC/EBP-Luc reporter construct in the presence or absence of trichostatin A (TSA), an HDAC inhibitor (37). PIASy expression significantly reduced C/EBPδ transcriptional activation of the pC/EBP-Luc reporter construct (Fig. 1D). However, treatment with increasing doses of TSA (0-330nM) had no effect on PIASy mediated inhibition of C/EBPδ transcriptional activation of the pC/EBP-Luc reporter construct as assessed by luciferase activity (Fig. 1D). These results suggest that HDAC recruitment does not play a major role in PIASy mediated inhibition of C/EBPδ transcriptional activity.

The K120 residue within the C/EBPδ RDM [(I/VL)KXEP] is the site for post translational SUMO modification, however, sumoylation does not influence C/EBPδ transcriptional activity. Having determined that PIASy significantly inhibits C/EBPδ transcriptional activity, we sought to determine if PIASy expression increased C/EBPδ sumoylation. Using transient transfection and co-expression/pull-down assays we found that C/EBPδ is sumoylated by SUMO-1 (Fig. 2A), SUMO-2 and SUMO-3 (data not shown). PIASy co-expression, however, did not enhance C/EBPδ sumoylation (Fig. 2A, lane 2 vs lane 3). Co-expression of SENP1, an isopeptidase that cleaves the SUMO moiety from protein substrates, significantly reduced the detectable level of C/EBPδ/SUMO conjugates (Fig. 2A, lanes 2, 3 vs lane 4). These results demonstrate that C/EBPδ is a sumoylation target, but PIASy expression does not significantly enhance C/EBPδ sumoylation.

C/EBP family members, including C/EBPδ, contain a conserved transcriptional inhibitory domain [(I/V/L)KXEP], or regulatory domain motif (RDM) that is a target for sumoylation (33,39). We produced a C/EBPδ K120R RDM mutant construct and found that the C/EBPδ K120R mutant was not sumoylated (Fig. 2A, lane 5). These results demonstrate that the conserved K120 consensus site within the C/EBPδ RDM [(I/V/L)KXEP] is the site for post translational SUMO modification.

Williams and coworkers demonstrated that conjugation of SUMO-1 to the conserved lysine residue within the RDM in C/EBPε releases the transcriptional inhibition mediated by the unmodified RDM, indicating that sumoylation is associated with increased transcriptional activation of C/EBPε (33). In contrast, Wang, et, al reported that sumoylation of NF-IL6β (C/EBPδ) reduced transcriptional activation (41). To gain new insights into the influence of sumoylation on C/EBPδ transcriptional activity HC11 cells were co –transfected with C/EBPδ or the C/EBPδ K120R mutant with increasing amounts of SUMO-1, SUMO-2 and SUMO-3. Consistent with previous reports we found that the transcriptional activity of the C/EBPδ K120R mutant was ~30% higher than the C/EBPδ wild type construct (Fig. 2B) (39). However, increasing SUMO-1 levels by transient transfection had no effect on the transcriptional activity of either the wild type C/EBPδ or the C/EBPδ K120R mutant (Fig. 2B). Similarly, co-expression of SUMO-2 or SUMO-3 had no effect on C/EBPδ transcriptional activity (data not shown). These results confirm the inhibitory role of the C/EBPδ RDM on C/EBPδ transcriptional activity and demonstrate that expression of SUMO family members does not influence C/EBPδ transcriptional activity.

PIASy repression of C/EBPδ transcriptional activity is independent of C/EBPδ sumoylation status. We next investigated the effect of PIASy on the transcriptional activity of wild type C/EBPδ and the sumoylation defective C/EBPδ K120R mutant by transfecting HC11 cells with PIAS and C/EBPδ expression constructs plus the pC/EBP-Luc reporter construct. The results indicate that PIASy significantly inhibits the transcriptional activity of both wild type C/EBPδ

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(WT) and the C/EBPδ K120R mutant (Fig. 3A). In addition, the results confirm the finding that the C/EBPδ K120R mutant transcriptional activity is ~30% higher than that of wild type C/EBPδ (WT) (see Fig. 2B, Fig. 3A). Co-expression of PIASy plus the SENP1 isopeptidase, which cleaves SUMO-1 from substrate proteins, slightly increased C/EBPδ wild type and C/EBPδ K120R transcriptional activity; however, both remained at ~50% of the control (CON) levels (Fig. 3A). Western blot analysis demonstrated that the protein levels of the expressed constructs, i.e., C/EBPδ WT/ C/EBPδ K120R mutant and PIASy, were relatively even. This indicates that PIASy mediated inhibition of wild type C/EBPδ and C/EBPδ K120R transcriptional activity was not due to artefactual differences in protein levels (Fig. 3B). The combined data from the PIASy, C/EBPδ wild type and C/EBPδ K120R mutant co-expression experiments and the SENP1 isopeptidase treatments indicate that PIASy inhibition of C/EBPδ transcriptional activity is sumoylation independent.

The PIASy SAP nuclear matrix binding domain (SAPD) interacts with the C/EBPδ transactivation domain (TAD). To gain a better understanding of the mechanism by which PIASy inhibits C/EBPδ transcriptional activity, we investigated the structural domains that mediate PIASy-C/EBPδ protein-protein interactions. The following GST tagged C/EBPδ constructs (Fig. 4A) were used in pull down assays with full length Flag-PIASy: C/EBPδ full length (FL), C/EBPδ ΔTAD (transactivation domain, (TAD) deleted), C/EBPδ DBLZ (TAD and regulatory domain (RD) deleted) and C/EBPδ ΔLZ (leucine zipper (LZ) deleted). The initial results demonstrated that the GST-C/EBPδ full length (FL) construct pulled down the full length Flag-tagged PIASy protein from HC11 cell lysates (Fig. 4B). To identify the C/EBPδ domains that interact with PIASy additional pull down experiments were performed with GST-C/EBPδ domain constructs. The results demonstrated that GST-C/EBPδ constructs that contain the TAD, ie, C/EBPδ FL and C/EBPδ ΔLZ pulled down the full length Flag-tagged PIASy protein (Fig. 4C). In contrast, GST-C/EBPδ constructs lacking the TAD (ΔTAD, DBLZ) were ineffective in pulling down PIASy (Fig. 4C). We next performed the pull down assays with the C/EBPδ TAD (TAD alone, RD, DB and LZ deleted) and demonstrated that the C/EBPδ TAD alone is capable of pulling down PIASy (Fig. 4C, right panel). These results demonstrate that the C/EBPδ TAD, a region that encompasses amino acids 1-102, is required for C/EBPδ-PIASy interaction.

To investigate the domains of the PIASy protein that directly or indirectly interact with C/EBPδ, we performed pull down experiments with GST-full length C/EBPδ (FL) and PIASy ΔSAP (lacking the SAPD) and PIASy ΔRFD (lacking the RFD) constructs (Fig. 4A). The results demonstrated that C/EBPδ FL pulled down the PIASy ΔRFD (SAPD present) construct but was ineffective in pulling down the PIASy ΔSAP (SAPD deleted) construct (Fig. 4D). These results are consistent with a direct or indirect interaction between the C/EBPδ TAD and the PIASy SAPD. These results also indicate that the RFD domain, which has been shown in previous reports to mediate substrate recognition and to catalyze SUMO conjugation (42,43), does not play a major role in the interaction between PIASy and C/EBPδ.

PIASy inhibits C/EBPδ transcriptional activity. Having demonstrated that full length PIASy inhibits C/EBPδ transcriptional activity (Fig. 1B-D) and that full length PIASy and PIASy ΔRFD (SAPD intact) interact with C/EBPδ (Fig. 4B-D); we next investigated the influence of PIASy ΔRFD and ΔSAP mutant constructs on C/EBPδ transcriptional activity. As expected, the full length, wild type PIASy (WT) significantly (~70%) inhibited C/EBPδ transcriptional activity (compare Fig. 1A and Fig. 5A). The PIASy ΔSAP deletion mutant, which

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did not interact with C/EBPδ in pull down assays (Fig. 4D), also did not inhibit C/EBPδ transcriptional activity (Fig. 5A). However, the PIASy ΔRFD deletion mutant, which did interact with C/EBPδ in *in vitro* pull down assays (Fig. 4D), did not inhibit C/EBPδ transcriptional activity (Fig. 5A). Western blot analysis of whole cell lysates demonstrated that C/EBPδ protein levels were relatively even across all treatments (Fig. 5B, upper panel). However, the PIASy wild type (WT) and PIASy ΔSAP protein levels were ~5 fold higher than the PIASy ΔRFD level (Fig. 5B, middle panel). The rationale for the reduced PIASy ΔRFD levels was initially unclear, however, subsequent experiments demonstrated that PIASy ΔRFD does not localize to the nucleus (see below, Fig. 6F and 6K) despite the presence of a nuclear localization signal within the PIASy ΔRFD construct. The inability of PIASy ΔRFD to localize to the nucleus is consistent with the lack of effect of PIASy ΔRFD on C/EBPδ transcriptional activity (Fig. 5A) and the persistent presence of PIASy ΔRFD in the cytoplasm could result in reduced PIASy ΔRFD protein stability resulting in reduced levels of the PIASy ΔRFD protein (Fig. 5B).

PIASy translocates C/EBPδ from discrete nuclear foci to the nuclear periphery. We next used confocal microscopy to investigate the intracellular interaction between PIASy and C/EBPδ and the influence of this interaction on C/EBPδ subnuclear localization in HC11 mammary epithelial cells. We previously reported that C/EBPδ is almost exclusively localized to the nucleus in mammary epithelial cells using cell lysis protocols that separate nuclear and cytoplasmic compartments with C/EBPδ detection by western blot (13,18). In this report we used confocal microscopy to extend these findings and the results indicated that full length C/EBPδ, C/EBPδ ΔTAD and the C/EBPδ K120R (sumoylation defective) mutant are all localized to discrete foci within the nucleus (Fig. 6A-C). In addition, confocal image merging demonstrated that all three C/EBPδ constructs co-localize with p300, a key co-activator of RNA Polymerase II-mediated transcription (Fig. 6A-C). As a critical transcriptional co-activator, p300 is localized in subnuclear regions associated with active gene transcription (44,45). These results demonstrate that nuclear localized C/EBPδ constructs co-localize with p300 in transcriptionally active nuclear foci (Fig. 6A-C). We next used confocal analyses to demonstrate that PIASy (full length) is localized to the nuclear periphery (Fig. 6D), PIASy ΔSAP is diffusely dispersed within the nucleus (Fig. 6E) and PIASy ΔRFD does not enter the nucleus at all, localizing in the cytoplasm (Fig. 6F).

Having determined that nuclear localized C/EBPδ constructs are concentrated in nuclear foci in association with p300, we next investigated the influence of full length PIASy, and the PIASy ΔSAP and ΔRFD constructs on C/EBPδ nuclear localization. Co-expression of full length C/EBPδ plus full length PIASy resulted in the dramatic translocation of C/EBPδ from discrete nuclear foci to the nuclear periphery (Fig. 6G). Confocal image merging demonstrated that C/EBPδ and PIASy co-localized to the nuclear periphery (Fig. 6G). Co-expression of the C/EBPδ ΔTAD mutant plus full length PIASy resulted in differential localization of both proteins within the nucleus with the C/EBPδ ΔTAD mutant present in nuclear foci and the full length PIASy localized to the nuclear periphery (Fig. 6H). Co-expression of the sumoylation defective C/EBPδ K120R mutant plus full length PIASy resulted in the co-localization of both proteins to the nuclear periphery (Fig. 6I). However, co-expression of full length C/EBPδ plus the PIASy ΔSAP (SAP domain deleted) mutant did not alter C/EBPδ subnuclear localization from nuclear foci (Fig. 6J). Co-expression of full length C/EBPδ plus the PIASy ΔRFD (RFD domain deleted) mutant resulted in a complex localization pattern in which C/EBPδ remained within nuclear foci and the PIASy ΔRFD mutant

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localized to the cytoplasm, exhibiting an apparent defect in nuclear import despite the presence of a nuclear localization signal (Fig. 6K).

Previous reports have shown that C/EBPβ and C/EBPδ interact with CBP/p300 and this interaction is associated with increased transcriptional activity (46,47). To determine if PIASy expression induced p300 subnuclear localization in a manner similar to C/EBPδ, we expressed PIASy and assessed endogenous p300 nuclear localization. The results demonstrated that p300 remained localized in discrete nuclear foci and did not co-localize to the nuclear periphery with PIASy (Fig. 6L). This indicates that PIASy does not translocate intact C/EBPδ/p300 transcription complexes and suggests that PIASy may interact with and translocate "free" or uncomplexed C/EBPδ prior to or after interaction with p300 on target gene promoters. Finally, the specificity of the primary and secondary antibodies used in these experiments was assessed by confocal microscopy and the results were negative, indicating that no antibody binding occurs in the absence of specific target proteins (data not shown).

PIASy decreases the expression of C/EBPδ cell-adhesion related target genes and enhances HC11 cell proliferation/migration. Having demonstrated that PIASy sequesters C/EBPδ in the nuclear periphery and reduces C/EBPδ transcriptional activity, we hypothesized that PIASy expression would influence biological responses associated with C/EBPδ, such as growth control. We implemented the *in vitro* "scratch" assay to investigate the biological effects of PIASy-C/EBPδ interactions (36). The scratch assay assesses the capacity of cells to repopulate an open area created in a confluent cell monolayer by the combined effects of cell proliferation and migration (36,48). We hypothesized that PIASy mediated sequestration of C/EBPδ would mimic C/EBPδ siRNA treatment as both reduce C/EBPδ function. To determine if C/EBPδ siRNA treatment influenced mammary epithelial cell proliferation/migration, we performed "scratch" assays using parental HC11 cells (nontransfected), HC11 cells stably transfected with the *pSilencer* vector (vector control) and HC11 cells stably transfected with C/EBPδ siRNA-*pSilencer* vector (C/EBPδ siRNA) (Fig. 7A). The results demonstrated that repopulation of the open area created by the "scratch" was markedly enhanced in C/EBPδ siRNA treated HC11 cells compared to nontransfected parental and vector control transfected HC11 cells (Fig. 7A). The efficiency of reduction in C/EBPδ protein levels by the siRNA treatment was confirmed by western blot analysis of HC11 cells at ~90% confluence (determined by visual inspection, t = 0) and 24, 48 and 72 hours after reaching confluence (Fig. 7B). The results demonstrated that C/EBPδ expression was induced in parental HC11 cells and vector control HC11 cells within 24 hours of reaching confluence and that C/EBPδ protein levels remained elevated 48 and 72 hours after reaching confluence (Fig. 7B). In contrast, C/EBPδ protein levels were minimally detectable in C/EBPδ siRNA treated HC11 cells at all time points, indicating that the siRNA treatment was effective in reducing C/EBPδ protein levels (Fig. 7B).

C/EBPs are well-established transcriptional activators (1), therefore, we hypothesized that reducing C/EBPδ content by specific siRNA, or repression of C/EBPδ transcriptional activity by PIASy expression, would reduce C/EBPδ target gene expression. In experiments using "ChIP-chip" assays we have identified C/EBPδ target genes that function in transcriptional regulation, cell adhesion, signal transduction, apoptosis, DNA repair and intermediary metabolism[2]. Having demonstrated in this study that cellular confluence is associated with the persistent induction of C/EBPδ protein levels (Fig. 7B), we assessed the influence of cellular confluence on the expression of a subset of C/EBPδ target genes that function in cell adhesion, ie, glycoprotein V (platelet) (GP5),

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protocadherin 9 (PCDH9) and integrin beta 8 (ITGB8)[1]. The rationale for analyzing these three adhesion-related C/EBPδ target genes is that contact mediated growth inhibition plays a major role in cell growth control and tissue homeostasis and C/EBPδ has been identified as a regulatory gene that is highly induced in contact inhibition-mediated growth arrest (49). The results indicated that cellular confluence is associated with a dramatic (~15-30 fold) increase in the mRNA levels of all three adhesion-related C/EBPδ target genes (GP5, PCDH9 and ITGB8) compared to growing cells (Fig. 7C). To determine if reducing C/EBPδ levels also reduced C/EBPδ cell adhesion-related target gene expression, HC11 cells were transiently transfected with C/EBPδ siRNA and a scrambled siRNA control. The results demonstrated that transient C/EBPδ siRNA treatment reduced GP5, PCDH9 and ITGB8 mRNA levels, but only ITGB8 reduction was statistically significant (Fig. 7D).

To test the hypothesis that PIASy mediated sequestration of C/EBPδ would mimic the effects of C/EBPδ siRNA treatment, the scratch assay was performed using HC11 cells transiently transfected with C/EBPδ and C/EBPδ + PIASy expression constructs. The results demonstrated that PIASy + C/EBPδ expressing HC11 cells exhibited enhanced repopulation of the open area created by the scratch at 24 and 48 hours compared to C/EBPδ expressing controls (Fig. 7E). We next investigated the hypothesis that PIASy expression would reduce the expression of C/EBPδ cell adhesion related target genes. The results indicated that PIASy expression significantly reduced PCDH9 and ITGB8 mRNA levels (Fig. 7F). These results indicate that PIASy expression reduces C/EBPδ target gene expression and increases cell proliferation/migration in the *in vitro* scratch assay.

DISCUSSION

This report demonstrates that PIASy is a potent inhibitor of C/EBPδ transcriptional activity. Mechanistic studies indicate that PIASy translocates C/EBPδ from discrete transcriptionally active nuclear foci to the nuclear periphery, a region generally associated with reduced transcriptional activity (50). PIASy mediated inhibition of C/EBPδ transcriptional activity is independent of HDAC recruitment, C/EBPδ sumoylation status and PIASy E3 SUMO ligase activity.

PIAS proteins alter transcription by a number of mechanisms including post translational modification with SUMO family members, SUMO-1, SUMO-2 and SUMO-3 (32,51). In this study we found that co-expression of SUMO-1, SUMO-2 or SUMO-3 resulted in sumoylation of wild type C/EBPδ, however, expression of SUMOs at levels up to ~4 times those used in previous studies did not affect C/EBPδ transcriptional activity (Fig. 2B and data not shown). Although co-expression of SUMO family members had little effect on C/EBPδ transcriptional activity, PIASy significantly inhibited the transcriptional activity of both the C/EBPδ wild type and the sumoylation defective C/EBPδ K120R mutant constructs. This inhibition was mediated by interaction between the PIASy N-terminal SAP domain and the C/EBPδ N-terminal TAD domain. These findings are consistent with accumulating evidence indicating that the PIASy SAP domain interacts with transcription factors and represses transcription factor activity by multiple mechanisms including interaction with the nuclear matrix and AT rich DNA (32,51).

The essential role of the PIASy SAP domain in PIASy mediated inhibition of C/EBPδ transcriptional activity contrasts with the apparently limited role of the Ring-Finger domain. The PIASy SP-Ring-Finger domain (RFD) is homologous to the Ring-Finger domain of ubiquitin E3 ligases and functions in substrate protein recognition and SUMO conjugation (32). The PIASy ΔRFD construct interacts with C/EBPδ in *in vitro* GST pull down experiments (Fig. 4D), but the PIASy ΔRFD construct does not inhibit C/EBPδ transcriptional activity in

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cell-based luciferase assays (Fig. 5A). Confocal microscopy demonstrated that PIASy ΔRFD does not enter the nucleus, localizing in the cytoplasm (Fig. 6F, 6K). This indicates that nuclear import or nuclear retention of the PIASy ΔRFD is defective, despite the presence of the PIASy NLS. These results suggest that PIASy SUMO ligase activity may play a role in PIASy nucleocytoplasmic transport. Sumoylation has been shown to regulate nuclear pore function and nucleocytoplasmic trafficking (52). In addition, our results demonstrate that the PIASy N terminal SAP domain, which is highly conserved among PIAS proteins, plays a major role in PIAS-mediated C/EBPδ repression by subnuclear translocation and sequestration.

The mechanism underlying PIASy sequestration of C/EBPδ within the nuclear periphery is under investigation. The nuclear periphery is associated with inner nuclear membrane proteins that have been linked to transcriptional repression including lamin B receptor (LBR), lamina-associated polypeptide 2β (LAP2β) and emerin (50). Sachdev et al reported that PIASy represses LEF1 transcriptional activity by sumoylation independent sequestration of LEF1 in PML nuclear bodies (43). PIASy also represses the transcriptional activation of Nurr1 and Ets-1 by targeting to the nuclear matrix and this repression is also independent of sumoylation status (53,54). The present results are similar to these observations in that we also found that PIASy mediated repression of C/EBPδ transcriptional activity is independent of sumoylation. One area in which our results differ from previous studies is that even though PIASy repression and sequestration of LEF1, Nurr1 and ETS is sumoylation independent, PIASy enhances LEF1, Nurr1 and ETS sumoylation (43,53,54). Our results demonstrate that PIASy does not significantly enhance sumoylation of C/EBPδ. These collective findings highlight the complexity of the role of PIASy and sumoylation in the regulation of individual transcription factors. In addition, it has recently been reported that C/EBPα transcriptional activity may be regulated by sequestration in transcriptionally inactive pericentromeric heterochromatin (55). Since the PIASy SAP domain binds AT-rich DNA present in scaffold attachment regions (SARS), also called matrix attachment regions, it is possible that subnuclear sequestration could include the binding of C/EBPδ to AT-rich or repetitive DNA present at the nuclear periphery (31,56). C/EBPδ and C/EBPβ have been reported to bind to satellite DNA and this binding was associated with reduced C/EBPδ and C/EBPβ transcriptional activity (57).

In studies investigating the biological significance of PIASy subnuclear sequestration of C/EBPδ we found that PIASy expressing HC11 cells exhibited reduced expression of C/EBPδ cell adhesion related target genes (GP5, PCDH9 and ITGB) and an increased capacity to repopulate open areas in the cell monolayer induced by the “scratch”. These responses paralleled those observed in C/EBPδ siRNA treated HC11 cells, suggesting that PIASy could alter mammary epithelial cell growth and migration through interactions with C/EBPδ. However, PIASy interacts with a broad range of transcriptional control proteins and these interactions may also impact mammary epithelial cell growth control and migration (32). In addition, C/EBPδ activates a broad range of target genes[1] that may be altered by PIASy expression. Therefore, these findings both identify new regulatory interactions and raise new questions regarding the broad biological significance of these interactions.

From the perspective of mammary gland biology, these results suggest that PIASy-C/EBPδ interactions could influence mammary gland development, gestation, differentiation or involution by regulating C/EBPδ transcriptional activity and target gene expression. From a breast cancer perspective, adhesion molecules function in epithelial cell contact inhibition and disruption of this critical function by aberrant PIASy expression could

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promote tumorigenesis (58). The inhibitory effects of PIASy on ITGB8 may be particularly relevant to mammary gland biology and breast cancer. ITGB8 functions in epithelial cell growth inhibition by a unique mechanism involving activation of the latent form of TGF-β and reduced ITGB8 levels are associated with increased mammary epithelial cell proliferation *in vivo* and *in vitro* (59-62).

Finally, the results from this work indicate that the expression of PIAS gene family members, including PIASy, is independent of mammary epithelial cell growth status. This suggests that biological responses that are influenced by PIAS proteins may be determined by the cellular content of individual PIAS interacting proteins, such as C/EBPδ. Recent results indicate that PIASy induces cellular senescence, or apoptosis, depending on the cellular content of p53 and Rb (63). The results from the present study suggest that the role of PIASy in cellular senescence and apoptosis may also be influenced by interactions with C/EBPδ. In addition, it has recently been shown that C/EBPδ induces G_0 growth arrest of chronic myelogenous leukemia (CML) cell lines and this arrest is associated with increased detection of C/EBPδ/Rb complexes (64). These findings raise the possibility that interactions between Rb, C/EBPδ and PIASy could play a major role in cell fate determination. Future work will investigate the functional interactions between Rb, C/EBPδ and PIASy as a potential convergence point in cell fate determination.

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FOOTNOTES

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[1] J. Si and T. Liu contributed equally to this manuscript.

[2] Y. Zhang, T. Liu, P. Yan, T. Huang and J. DeWille, unpublished.

The abbreviations used are: C/EBP, CCAAT/enhancer binding protein; PIAS: protein inhibitor of activated STAT; TAD, transactivation domain; RDM, regulatory domain motif; SUMO, small ubiquitin-like modifier; SAP, scaffold-attachment factor A (SAFA) and SAFB, apoptotic chromatic condensation inducer in the nucleus (ACINUS) and PIAS domain; RFD, ring-finger domain; SENP, SUMO/sentrin-specific protease; HDAC, histone deacetylase.

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FIGURE LEGENDS

FIGURE 1. **PIASy represses C/EBPδ transcriptional activity.** (A) Total RNA was isolated from subconfluent, growing HC11 cells (Grow) and 48 hour confluent, G_0 growth arrested HC11 cells (GA), reverse transcribed (RT) and the RT products amplified with primers specific for PIASy, PIASxβ, PIAS3 and GAPDH (constitutively expressed mRNA control). Amplified products were separated by agarose gel electrophoresis and stained with ethidium bromide (EtBr). (B) HC11 cells were co-transfected with C/EBPδ, pC/EBP-Luciferase and individual PIAS family members. Cells were harvested 24 hours post transfection and luciferase activity was determined and normalized to renilla luciferase activity (transfection efficiency control). Transfection of HC11 cells with the empty expression vector was used as the negative control for C/EBPδ and PIAS expression and is designated as "His" (pcDNA3.1/V5-His-TOPO TA). "CON" lane denotes transfection with C/EBPδ plus an empty expression vector as a control for luciferase activity in the absence of PIAS family gene expression. C/EBPδ expression levels plus the β-actin protein loading control are shown in the bottom panels. (C) HC11 cells were co-transfected with C/EBPδ, increasing amounts of PIASy (0, 25, 50, 100, 200ng) plus pC/EBP-Luc. Cells were harvested 24 hours post transfection, luciferase activity was determined and normalized to the renilla transfection efficiency control. Flag-PIASy and C/EBPδ-V5 expression levels plus the β-actin protein loading control are shown in the bottom panels. (D) HC11 cells were transfected with or without PIASy and treated with increasing amount of Trichostatin A (TSA) (0,165, 330nM) and luciferase activity determined. Representative C/EBPδ levels plus the β-actin protein loading control are shown in the bottom panels.

FIGURE 2. **C/EBPδ sumoylation does not influence C/EBPδ transcriptional activity.** (A) HC11 cells were co-transfected with C/EBPδ-His wild type, C/EBPδ-His K120R mutant, PIASy-FLag or SENP1 expression constructs plus HA-SUMO-1. Cell lysates were incubated with Ni-NTA agarose beads and bound proteins were eluted and separated by SDS/PAGE and detected with anti-HA tag antibody. Protein expression levels of C/EBPδ and PIASy in crude cell lysates was detected with anti-His and anti-Flag antibodies respectively. Lanes: 1: HA-SUMO-1 + empty vector (negative control), 2. HA-SUMO-1 + C/EBPδ, 3. HA-SUMO-1 + C/EBPδ + PIASy, 4. HA-SUMO-1 + C/EBPδ +PIASy + SENP1, 5. HA-SUMO-1 + C/EBPδ K120R mutant + PIASy. (B) HC11 cells were co-transfected with C/EBPδwild type (WT) or C/EBPδK120R. pC/EBP-Luc + increasing amounts of HA-SUMO-1 (0, 50, 100ng). Luciferase activity was assessed and normalized to renilla luciferase activity. C/EBPδ, SUMO-1 and β-actin (protein loading control) were detected by western blot and are presented in the lower panels. Western blot results shown are representative of 3 independent experiments.

FIGURE 3. **PIASy represses C/EBPδ transcriptional activity independent of C/EBPδ sumoylation status.** (A) HC11 cells were co-transfected with C/EBPδ wild type (WT) or C/EBPδ K120 mutant (K120R) plus pC/EBP-luc plus empty expression vector (pcDNA3.1/V5-his) (His) (CON), PIASy, or PIASy + SENP1. Luciferase activity was assessed and normalized to renilla luciferase control activity. ANOVA analysis with pooled data demonstrated a significant effect of PIASy on C/EBPδ transcriptional activity compared to control (CON) (α=0.05). (B) Western blot analysis of C/EBPδ wild type (WT), or C/EBPδ K120 mutant, PIASy and β-actin (loading control) protein levels. Western blot results shown are representative of 3 independent experiments.

FIGURE 4. **PIASy N-terminal SAP domain interacts with C/EBPδ N-terminal TAD domain.**

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(A) Schematic representation of C/EBPδ (C/EBPδ full length (FL), C/EBPδ ΔTAD, C/EBPδ DBLZ, C/EBPδ TAD) and PIASy (PIASy, PIASy ΔSAP, PAISy ΔRFD) constructs. (B) C/EBPδ and PIASy interact. GST-C/EBPδ full length (FL) was expressed in E. coli, immobilized on glutathione sepharose beads and incubated with lysates from HC11 cells transfected with a Flag-PIASy expression construct. Protein complexes formed in pull down reactions were analyzed by SDS/PAGE and detected with an anti-Flag antibody. The 5% input lane is derived from the crude cell lysate (positive control). Lysates incubated with glutathione sepharose beads alone are presented in the "GST" lane. (C) Analysis of C/EBPδ and PIASy interacting domains. Bacterial expressed GST-tagged C/EBPδ full length (FL), C/EBPδ ΔTAD, C/EBPδ DBLZ and C/EBPδ ΔLZ deletion construct-encoded proteins were immobilized on glutathione sepharose beads and incubated with lysates from HC11 cells transfected with Flag-PIASy constructs (PIASy, PIASy ΔSAP, PAISy ΔRFD). Protein complexes formed in pull down reactions were analyzed by SDS/PAGE and detected with an anti-Flag antibody. GST-C/EBPδ proteins present in pull down assays were detected by coomassie blue staining (lower panel). (D) Bacterial expressed GST-C/EBPδ FL was immobilized on glutathione sepharose beads and incubated with lysates from HC11 cells expressing Flag-PIASy ΔSAP or Flag-PIASy ΔRFD constructs. Protein complexes were analyzed by SDS/PAGE and detected with an anti-Flag antibody. "E" designates an empty lane. The "5% input" lane contains 5% of the crude HC11 cell lysate used for pull-down assay (positive control). GST-C/EBPδ proteins present in pull down assays were detected by coomassie blue staining (lower panel).

FIGURE 5. **The PIASy SAP domain is required for inhibition of C/EBPδ activity.** HC11 cells were co-transfected with C/EBPδ + pC/EBP-Luc + PIASy (WT), PIASy ΔSAP or PIASy ΔRFD constructs. Luciferase activity was assessed and normalized to renilla luciferase activity. HC11 cells co-transfected with C/EBPδ + pC/EBP-Luc without PIASy expression vectors were used as the positive control for the luciferase activity in the absence of exogenous PIASy ("Con"). Statistical analysis was performed with pooled data using ANOVA with post test analysis by Dunnet's test with each PIAS treatment compared to the no PIAS control (Con). Statistical significance was set at $\alpha=0.05$. (B) Western blot analysis of C/EBPδ, PIASy WT, PIASy ΔSAP, PIASy ΔRFD and β-actin (loading control) protein levels. Results shown are representatives of 3 independent experiments.

FIGURE 6. **PIASy sequesters C/EBPδ from discrete nuclear foci to the nuclear periphery.** HC11 cells were transfected with designated expression constructs (A-L): (A) C/EBPδ wild type (WT), (B) C/EBPδ ΔTAD, (C) C/EBPδ K120R, (D) PIASy (WT), (E) PIASy ΔSAP, (F) PIASy ΔRFD (G) C/EBPδ (WT) + PIASy (WT), (H) C/EBPδ ΔTAD + PIASy (WT), (I) C/EBPδ K120R + PIASy (WT), (J) C/EBPδ (WT) + PIASy ΔSAP, (K) C/EBPδ (WT) + PIASy ΔRFD, (L) PIASy (WT) constructs. C/EBPδ full length, C/EBPδ ΔTAD and K120R constructs were detected with a mouse anti-V5 antibody and visualized with alexa fluor 633 goat anti-mouse antibody (red). PIASy full length, PIASy ΔSAP and PIASy ΔRFD constructs were detected with a rabbit anti-Flag antibody and visualized with alexa fluor 488 goat anti-rabbit antibody (green). Nuclei were stained with DAPI. In panel (L), endogenous p300 was detected with a rabbit anti-p300 antibody and visualized with alexa fluor 488 goat anti-rabbit antibody (green). PIASy was detected with a mouse anti-Flag antibody and visualized with alexa fluor 488 goat anti-mouse antibody (red). The results presented are representative of 3-5 independent experiments.

FIGURE 7. **PIASy expression increases HC11 cell repopulation of open "scratch" areas in cell monolayers and decreases C/EBPδ cell adhesion-related target gene expression.** (A) A 200μl

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pipet tip was used to produce an open area or “scratch” in confluent monolayers of HC11 parental, HC11 stably transfected vector (*pSilencer* ™ 2.1 neo) control and HC11 C/EBPδ siRNA treated cells (0 hour, 0 h). Repopulation of the open area was assessed at 0h, 24h and 48h by crystal violet staining. (B) Western blot analysis of C/EBPδ protein levels from whole cell lysates isolated from cell lines used in Panel A at 0, 24, 48 and 72 hours post confluence. Blots were probed with a β-actin antibody as the protein loading control. (C) Total RNA was isolated from exponentially growing and 48 hour confluent HC11 cells. RNA was reverse transcribed (RT) and the RT products amplified using primers specific for C/EBPδ target genes: GP5, PCDH9 and ITGB8 using the Roche LightCycler® 480 Real-Time PCR System. C/EBPδ target gene mRNA levels were quantified using the relative quantification method following normalization to the GAPDH housekeeping gene mRNA control. (D) Total RNA was isolated from 48 hour confluent HC11 cells transiently transfected with scrambled siRNA or C/EBPδ specific siRNA. Real Time PCR analysis was performed as described in section “C” above. (E) HC11 cells were transiently transfected with C/EBPδ and C/EBPδ + PIASy expression constructs, grown to confluence (90%) and an open area was created in the monolayer using a 200µl pipet tip (0 hour (h)). Repopulation of the open area was monitored at 0h, 24h and 48h by crystal violet staining. (F) Total RNA was isolated from confluent monolayers of HC11 cells transiently transfected with C/EBPδ and C/EBPδ + PIASy expression constructs. Real Time PCR analysis was performed as described in section “C” above. Student’s *t* tests were used for statistical evaluation of the data in Panels C, D and F. The fold change in individual C/EBPδ target gene mRNA levels was considered significant at $\alpha = 0.05$.

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Figure 1

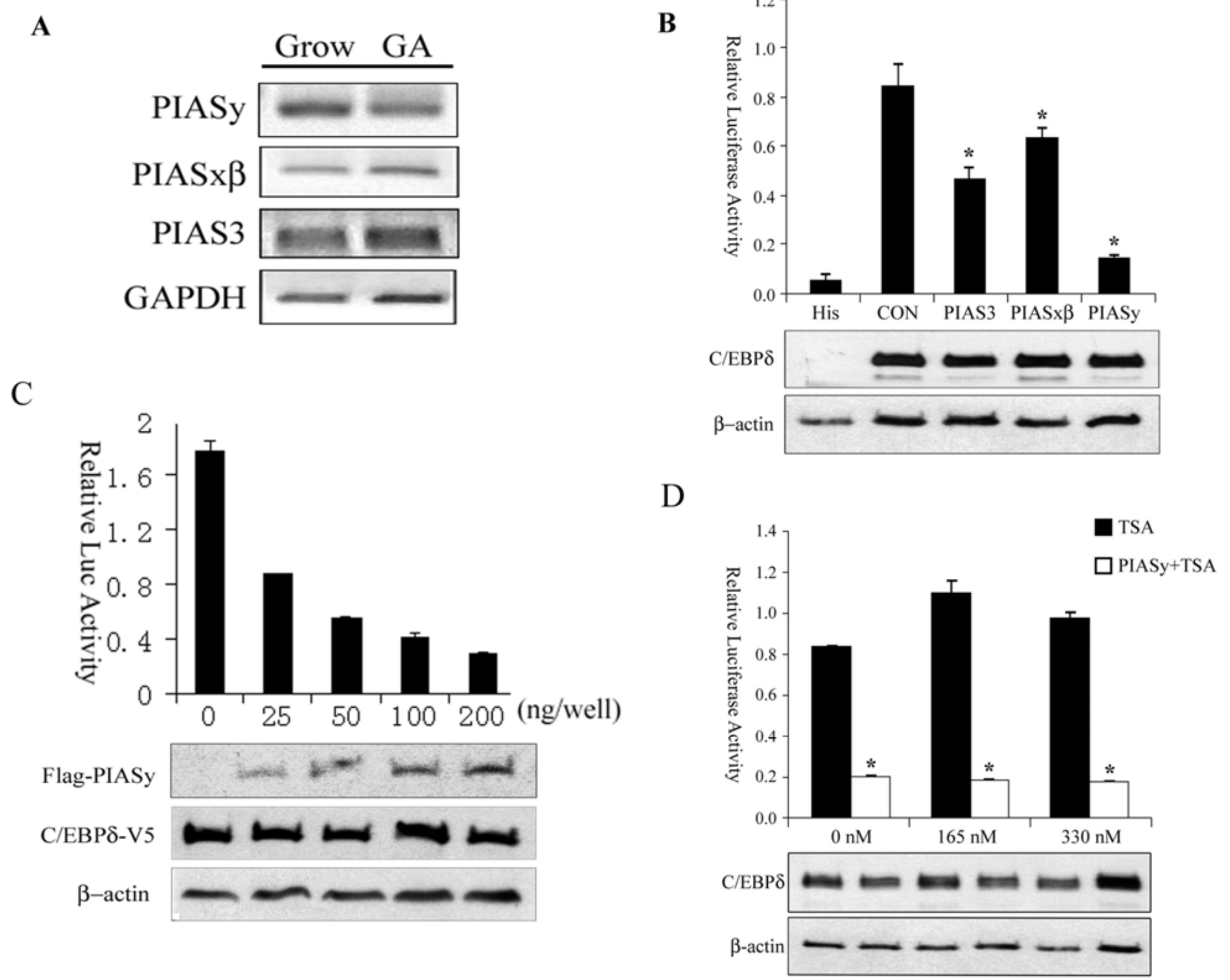

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Figure 2

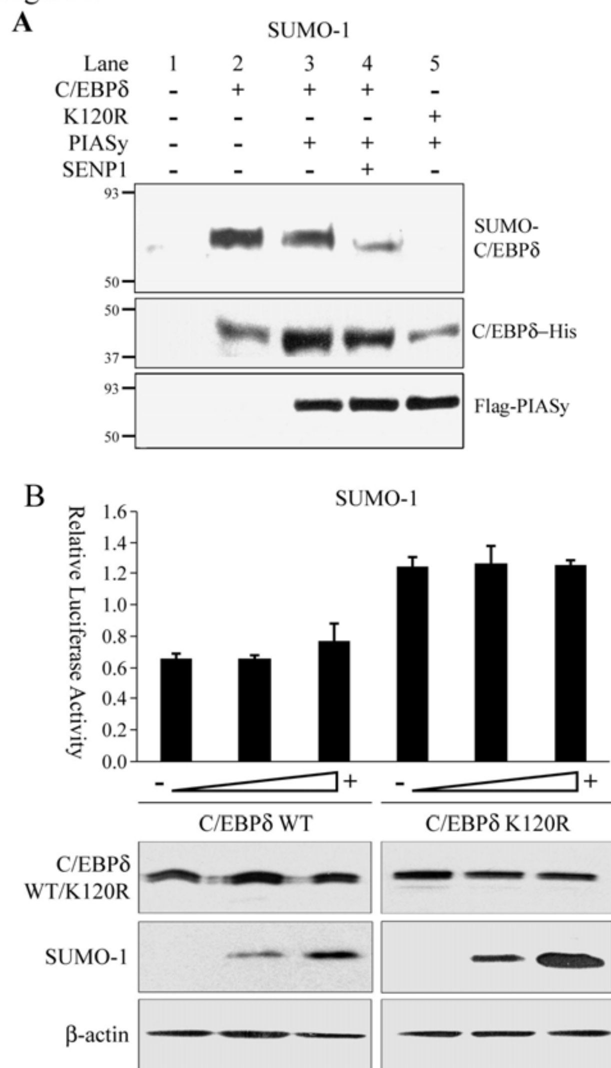

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Figure 3

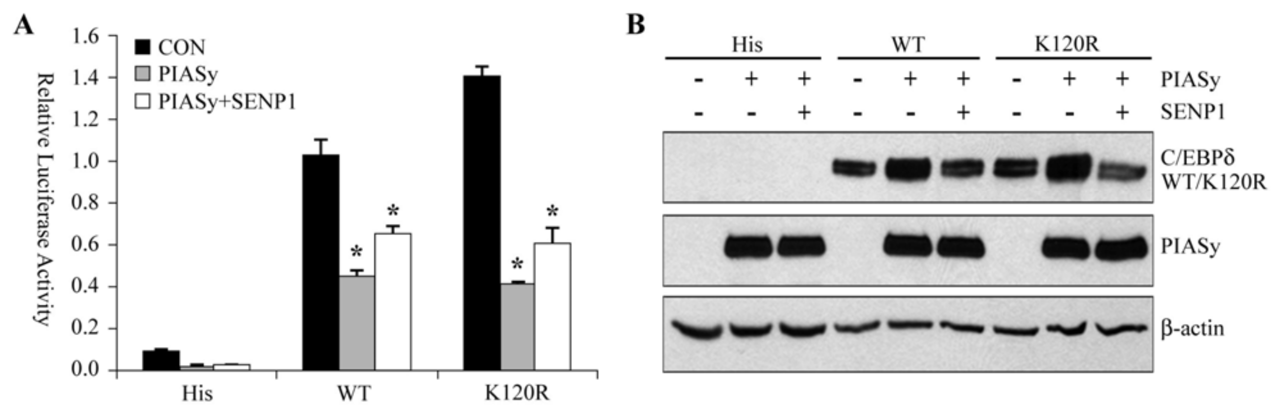

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Figure 4

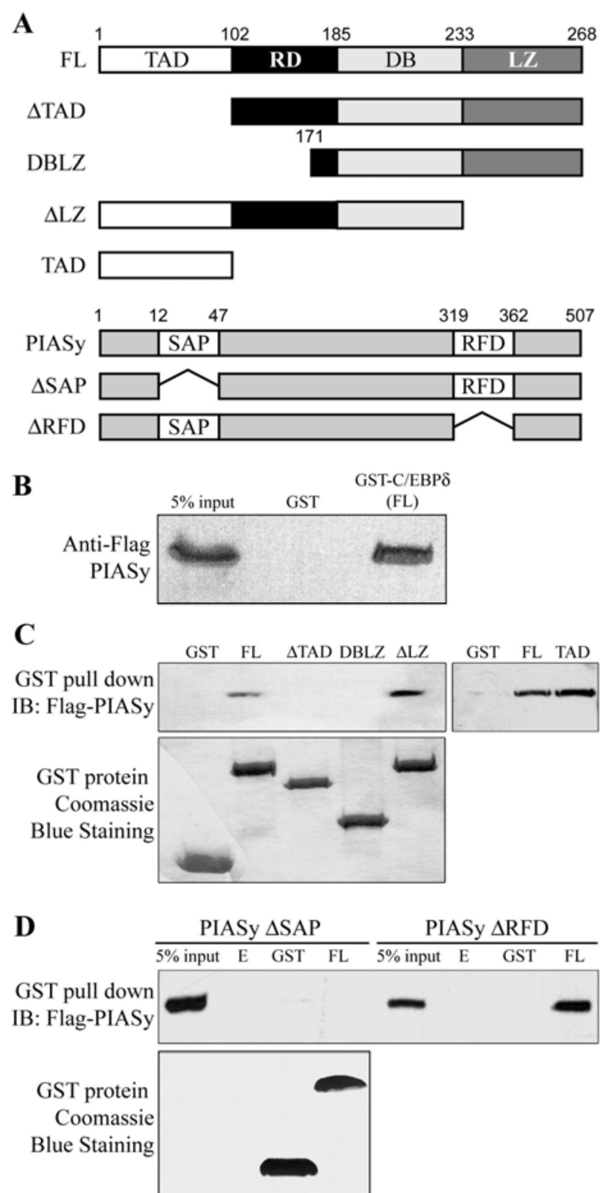

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Figure 5

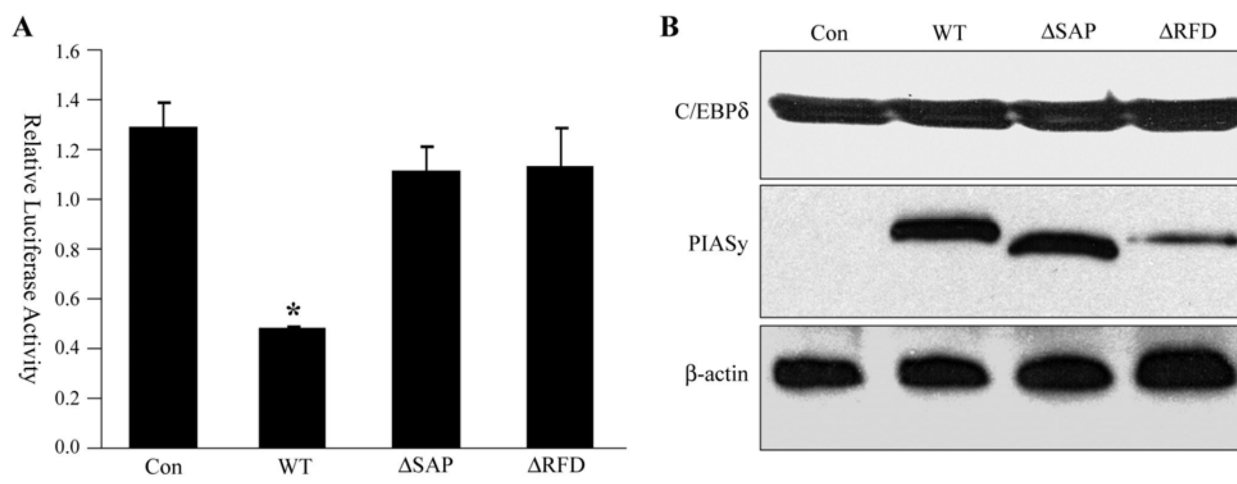

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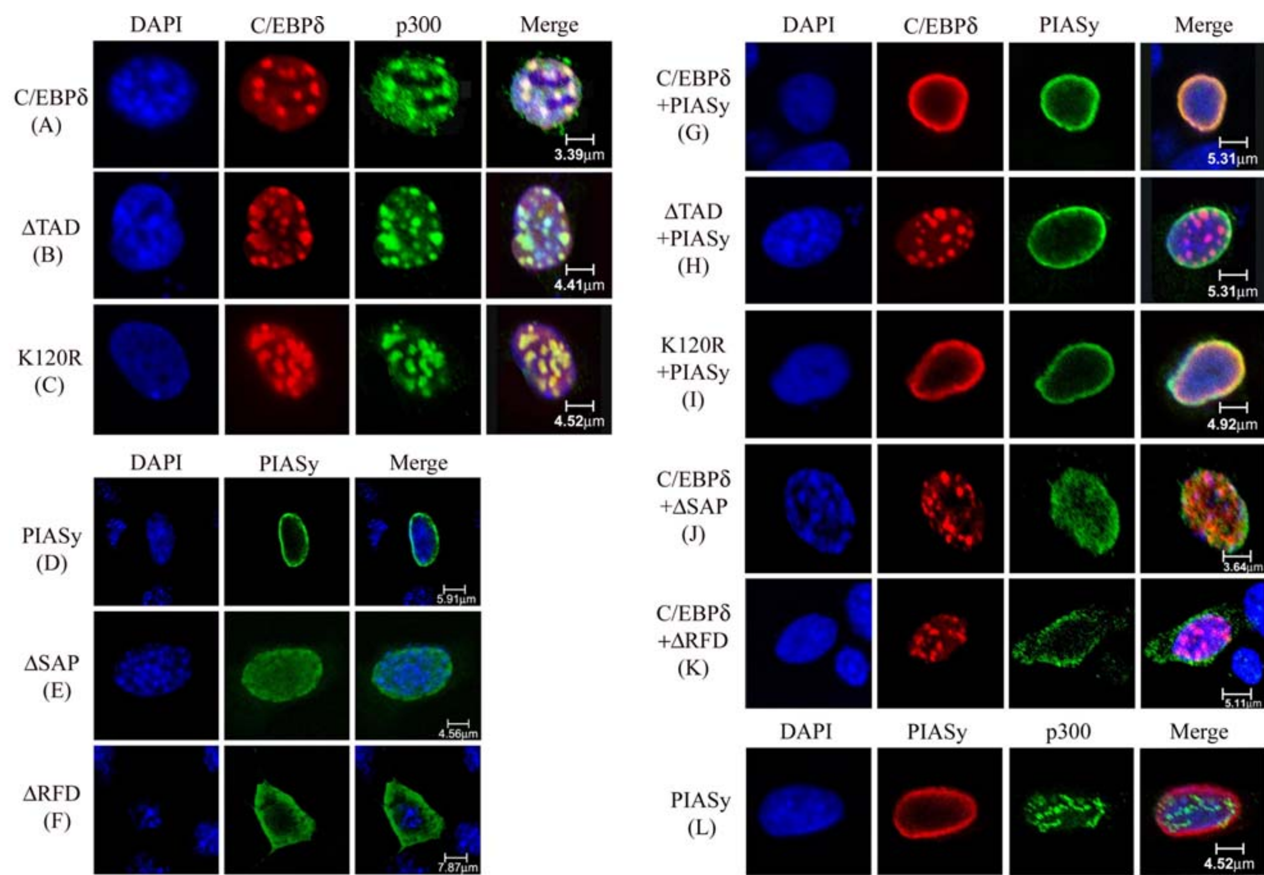
Figure 6



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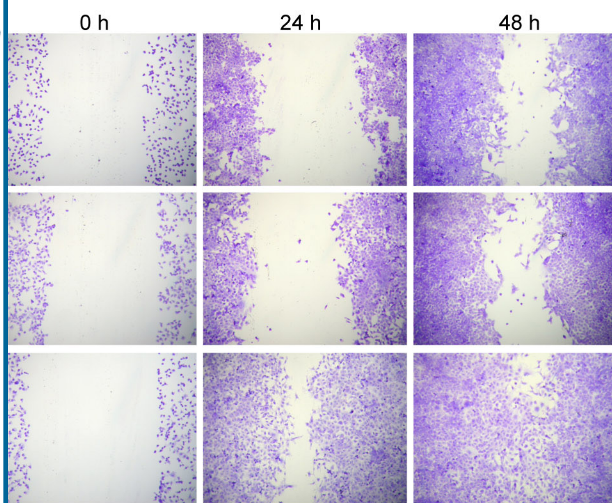

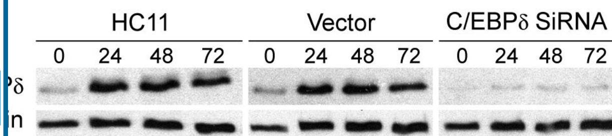

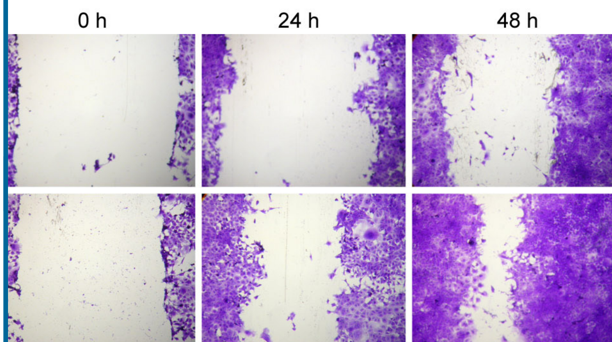

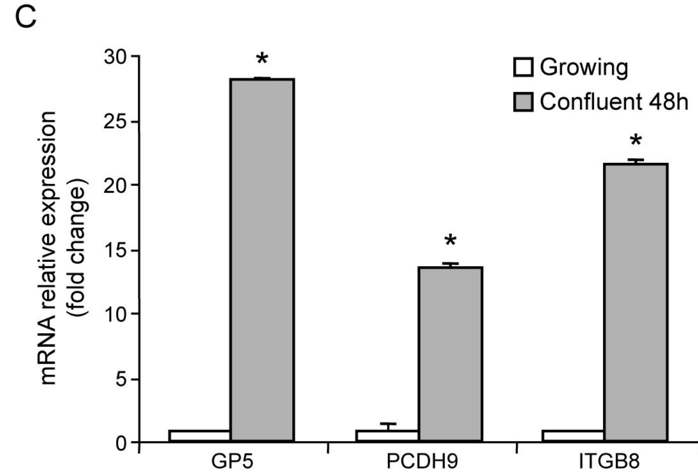

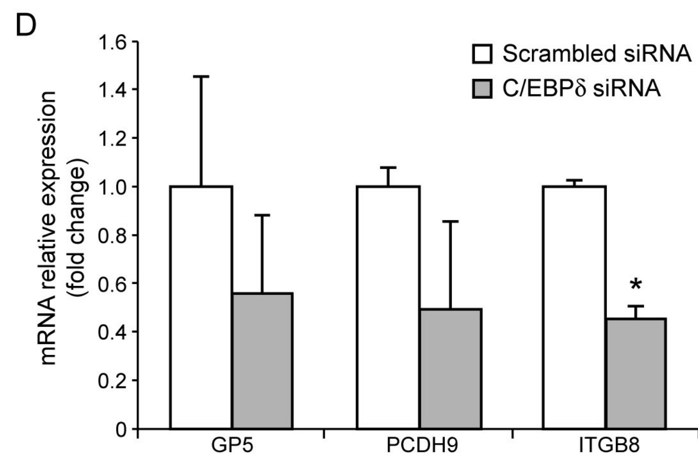

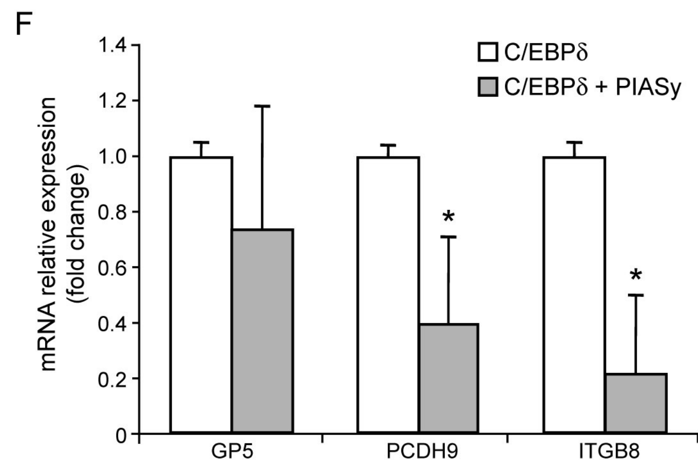

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