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Modulation of PLAGL2 transactivation by positive cofactor 2 (PC2), a component of the ARC/Mediator complex

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ABSTRACT

The pleomorphic adenoma gene (PLAG) family of transcription factors regulates a wide range of physiological processes, including cell proliferation, tissue-specific gene regulation, and embryonic development, although little is known regarding the mechanisms that regulate PLAG protein activity. In this study, a yeast two-hybrid screen identified PC2, a component of the Mediator complex, as a PLAGL2-binding protein. We show that PC2 cooperates with PLAGL2 and PU.1 to enhance the activity of a known PLAGL2 target promoter (*NCF2*). The PLAGL2-binding element in the *NCF2* promoter consisted of the core sequence of the bipartite PLAG1 consensus site, but lacked the G-cluster motif, and was recognized by PLAGL2 zinc fingers 5 and 6. Promoter and PLAGL2 mutants showed that PLAGL2 and PU.1 were required to bind to their respective sites in the promoter, and PC2 knockdown demonstrated that PC2 was essential for enhanced promoter activity. Co-immunoprecipitation and promoter-reporter studies reveal that the effect of PC2 on PLAGL2 target promoter activity was conferred via the C-terminus of PLAGL2, the region that is required for PC2 binding and contains the PLAGL2 activation domain. Importantly, chromatin immunoprecipitation analysis and PC2 knockdown studies confirmed that endogenous PC2 protein associated with the *NCF2* promoter in MM1 cells in the region occupied by PLAGL2, and was required for PLAGL2 target promoter activity in TNF-α-treated MM1 cells, respectively. Lastly, the expression of another known PLAGL2 target gene, *insulin-like growth factor II* (IGF-II), was greatly diminished in the presence of PC2 siRNA. Together, the data identify PC2 as a novel PLAGL2-binding protein and important mediator of PLAGL2 transactivation.

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1. Introduction

PLAGL2 is a member of the recently identified PLAG family of transcription factors. The additional members include PLAG1 and PLAGL1. PLAG proteins are highly homologous in the N-terminal zinc finger domain (PLAGL1 and PLAGL2 are 73% and 79% identical to PLAG1, respectively) with the C-terminal region being more divergent (Kas et al., 1998). Although they have been implicated in a range of important physiological processes, including cancer initiation and progression, little is known regarding the mechanisms whereby PLAG proteins regulate these processes (reviewed by Abdollahi, 2007; Van et al., 2007). To date, few PLAG target genes or regulatory cofactors have been reported.

PLAG1 and PLAGL2 are considered oncogenic, while PLAGL1 appears to function as a tumor suppressor. PLAG1 was the initial member identified due to its involvement in the t(3:8)(p21;q12) chromosomal translocation associated with about 25% of all human pleomorphic adenomas of the salivary glands (Kas et al., 1997). Upregulation of PLAG1 has also been identified as the primary genetic factor behind lipoblastomas and hepatoblastomas (Astrom et al., 2000; Hibbard et al., 2000; Zatkova et al., 2004). Activation of both PLAG1 and PLAGL2 has been demonstrated in leukaemogenesis in retroviral promoter insertion studies with *Cbfb-MYH11* knock-in chimeric mice (Castilla et al., 2004). In addition, PLAG1 and PLAGL2 show increased expression in 20% of human acute myeloid leukemia (AML) samples, with PLAGL2 expression preferentially induced in human AML samples with inv(16) (Landrette et al., 2005). Interestingly, PLAGL2

Abbreviations: PC2, positive cofactor 2; NCF2, neutrophil cytosolic factor 2; PLAG, pleomorphic adenoma gene; PLAG1, pleomorphic adenoma gene 1; PLAGL1, pleomorphic adenoma gene-like 1; PLAGL2, pleomorphic adenoma gene-like 2; IGF-II, insulin-like growth factor II; AML, acute myeloid leukemia; CASTing, cyclic amplification and selection of target sequences; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; Co-IP, co-immunoprecipitation; HEK293, human embryonic kidney 293 cells; MM1, MonoMac1 cells; RPMI, Roswell Park Memorial Institute; 3AT, 3-amino-1,2,4-triazole; X-α-Gal, 5-bromo-4-chloro-3-indolyl α-D-galactopyranoside; BLAST, Basic Local Alignment Search Tool; bp, base pair; NT, N-terminus; CT, C-terminus; GFP, green fluorescent protein; TRR, TNF-α-responsive region; EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcription polymerase chain reaction; TNF-α, tumor necrosis factor α; ChIP, chromatin immunoprecipitation; PCR, polymerase chain reaction; SREBP, sterol regulatory element binding protein; NLS, nuclear localization signal.

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has also been shown to regulate the pro-apoptotic factor, NIP-3, demonstrating that PLAGL2 may also function as a tumor suppressor (Mizutani et al., 2002). In contrast, PLAGL1 inhibits tumor cell growth by controlling apoptosis and cell cycle progression (Spengler et al., 1997), and the loss of PLAGL1 expression during spontaneous transformation of ovary surface epithelial cells and transcriptional silencing in a variety of human cancers strongly suggests PLAGL1 functions as a tumor suppressor (Abdollahi et al., 1997; Kamikihara et al., 2005). Elucidating the mechanisms of PLAG activation will provide important insight into the role of these proteins in tumorigenesis.

The similarity in DNA-binding specificity of PLAG proteins suggests that there may be some functional redundancy in the family, as implied by the normal expression of PLAG1 target genes in PLAG1-deficient mice (Hensen et al., 2004; Declercq et al., 2003). CASTing identified a bipartite consensus sequence for PLAG1 containing a core sequence (GGRGGCC), recognized by PLAG1 zinc fingers 6 and 7, and a G-cluster (GGG) located six to eight nucleotides downstream, recognized by zinc finger 3 (Voz et al., 2000). While PLAGL2 was also shown to bind to the PLAG1 consensus sequence with analogous zinc fingers (5, 6 and 2), PLAGL1 recognized a sequence that was also GC rich (GGGGGGCCCC) but lacked the G-cluster. With relatively few PLAG target genes identified, the full range of *in vivo* PLAG DNA-binding sites, and the degree of functional redundancy or competition for target sites between family members, remains unclear.

Post-translational modification has been shown to play an important role in the activity of PLAG1 and PLAGL2 proteins. While SUMOylation of PLAG1 and PLAGL2 reduces the transcriptional activity of these proteins, acetylation at the same lysine residues targeted for SUMOylation increases their transcriptional activity (Van et al., 2004; Zheng and Yang, 2005). On the other hand, PLAGL1 activity appears to be regulated more through epigenetic means affecting the expression of PLAGL1, including CpG methylation and histone deacetylation (Arima et al., 2006; Abdollahi et al., 2003).

In recent studies, we observed that PLAGL2 plays a role in the regulation of *NCF2*, the gene encoding the NADPH oxidase cytosolic protein known as $p67^{phox}$ (Ammons et al., 2007). Although we observed direct binding of PLAGL2 to the *NCF2* promoter sequence, overexpression of PLAGL2 alone was not capable of activating an *NCF2* promoter-reporter plasmid (unpublished data), suggesting that other factors were required. To further elucidate the mechanisms whereby PLAGL2 regulates gene expression, a yeast two-hybrid screen, baited with full-length human PLAGL2, was carried out to identify potential PLAGL2 co-activators. PC2, a component of the Mediator complex, was identified as a putative PLAGL2-binding protein, and *in vivo* co-immunoprecipitation (Co-IP) studies confirmed this interaction. We demonstrate that PC2 enhances the activity of a known PLAGL2 target promoter (*NCF2*) in cooperation with PLAGL2 and PU.1, is required for PLAGL2 regulation of *NCF2* expression in TNF-α-treated MonoMac1 cells, and is important in PLAGL2-induced expression of *IGF-II*. The data presented here identify PC2 as a PLAGL2-binding protein and an important regulator of PLAGL2 transactivation.

2. Materials and methods

2.1. Reagents

Oligonucleotide primers were synthesized by Integrated DNA Technologies, and PLAGL2 and PC2 antibodies were from Proteintech Group, Inc. Alexa Fluor 546 goat anti-rabbit antibodies were from Invitrogen.

2.2. Cell culture

HEK293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Human MonoMac1 (MM1) macrophage cells were grown in RPM1 supplemented with 10% fetal bovine serum, penicillin, and streptomycin.

2.3. Yeast two-hybrid screen

The Matchmaker yeast two-hybrid system 2 (Clontech, Mountain View, CA) was used according to the manufacturer's protocol with some modifications. The yeast reporter vector pAS2-1, containing full-length PLAGL2 cDNA, was used to transform the competent yeast strain Y187 to generate the bait strain Y187[pAS2-1(PLAGL2)]. No autoactivity of the pAS2-1(PLAGL2) reporter was detected and 45 mM 3AT was sufficient to inhibit leaky *HIS3* expression of the bait strain. The AH109 [pACT2 (human leukocyte cDNA)] strain was mated with the bait strain Y187 [pAS2-1 (PLAGL2)] generating 1.3×10^8 library clones on medium lacking tryptophan and leucine, sufficiently covering the library. Diploid clones were replica plated onto plates lacking histidine, tryptophan, and leucine to allow leaky *HIS3* background for a histidine jump-start assay. Plates were incubated for 6 days at 30 °C and colonies were replated on medium plates lacking histidine, adenine, tryptophan, and leucine and supplemented with 45 mM 3AT to screen for reporter activation. One hundred ninety two diploid reporter-positive clones were streaked onto fresh selection plates and assayed for β-galactosidase activity using a filter lift assay and for α-galactosidase activity using X-α-Gal medium plates. Prey plasmids recovered from diploid clones positive for nutritional and colorimetric reporter gene activity were amplified, sequenced, and analyzed using the BLAST algorithm.

2.4. Plasmid constructs

The pGL3-*NCF2*(500) promoter-reporter plasmid contains 500 bp of sequence proximal to the ATG translational start site of the *NCF2* gene and was described previously (Gauss et al., 2002). PLAGL2, PLAGL2 N-terminus (NT), and PLAGL2 C-terminus (CT) PCR amplified cDNA was cloned into the pAcGFP1 vector (Clontech) or pcDNA3.1 vector (PLAGL2-NT and PLAGL2-CT) using pcDNA3.1-PLAGL2 as template. $PC2_{434–784}$ cDNA was subcloned from pACT2-$PC2_{434–784}$ into the pProLabel-C vector using *EcoRI* and *XhoI* sites. Full-length PC2 was subcloned from pOTB7-PC2 into the pProLabel-C vector and the pcDNA3.1 vector using *EcoRI* and *XhoI* sites. pcDNA3.1-PLAGL2 and pAcGFP1-PLAGL2 zinc finger mutants were generated using a site directed mutagenesis kit (Stratagene) with pcDNA3.1-PLAGL2 or pAcGFP1-PLAGL2 as template. Zinc finger mutagenesis primers were designed to replace the first histidine of the C2H2 motif with an alanine. All recombinant plasmids were sequenced to confirm valid nucleotide sequence and expressed in HEK293 cells, followed by immunoblot analysis with the appropriate antibodies when available, or expressed in rabbit reticulocytes, to confirm expression of proteins. All recombinant proteins migrated to their predicted molecular mass as follows: PLAGL2 (54.6 kD), PC2 (86.8 kD), GFP-PLAGL2 (81.5 kD), GFP-PLAGL2-NT (56.0 kD), and GFP-PLAGL2-CT (52.6 kD).

2.5. Immunocytochemistry

HEK293 cells (2×10^4 per well) were cultured on Permanox chamber slides (Nalge Nunc International Corp.) and transfected with 100 ng of expression plasmid using Lipofectamine 2000. Immunostaining for PC2 was performed using the BD Cytofix/Cytoperm Kit (BD Biosciences) as follows. Cells were fixed for 20 min at room temperature (RT) with 250 μL fixation/permeabilization solution per well, followed by two 10-min washes at RT in 250 μL 1× perm/wash solution. Cells were then incubated with anti-PC2 antibodies (1:100) in 1× perm/wash solution for 30 min at RT, followed by 2 washes. Cells were then incubate for 1 h at RT, in 125 μL of Alexa Fluor

546 goat anti-rabbit antibodies (Invitrogen) diluted 1:1000 in 3% powdered milk/1× PBS, followed by 4 washes.

2.6. In vitro translation of protein in rabbit reticulocytes

Recombinant expression plasmids were transcribed and translated *in vitro* using the TNT Quick Coupled Transcription/Translation Systems (Promega). [^{35}S]Methionine labeled protein was run on a 10% SDS-PAGE gel, and the gel was subjected to autoradiography to confirm molecular mass and similar expression levels.

2.7. Electrophoretic mobility shift assay (EMSA)

EMSAs were carried out essentially as previously described (Ammons et al., 2007; Gauss et al., 2002). Briefly, double-stranded wild-type or mutant *NCF2* TRR (TNF-α-responsive region) or PLAGL2 consensus site DNA was incubated with wild-type or mutant PLAGL2 protein generated in rabbit reticulocyte lysate in 1× binding buffer (Roche) for 20 min at 5 °C. Samples were run on a 5% non-denaturing polyacrylamide gel (19:1) in 0.5× TBE electrophoresed at 170 V for 3.5 h. Gels were fixed, dried and subjected to autoradiography.

2.8. In vivo co-immunoprecipitation (Co-IP) assay

In vivo Co-IP was carried out using the Matchmaker Chemiluminescent Co-IP System according to the manufacturer's protocol (Clontech). This system utilizes a fluorescent AcGFP1 tag and the enzymatic ProLabel reporter for chemiluminescent detection of physical interactions between proteins that are expressed in mammalian cells. Following co-transfection of GFP-tagged bait and ProLabel-tagged prey plasmids, cell lysates are subjected to Co-IP using anti-GFP antibodies, and protein-protein interactions are detected using the ProLabel enzyme complementation assay. Initially, the functional reporter is split into two non-functional fragments: the small ProLabel and the larger Enzyme Acceptor. In the presence of the Enzyme Acceptor, the ProLabel and Enzyme Acceptor combine and become an active enzyme that cleaves the chemiluminescent substrate. This system has been demonstrated to be highly sensitive and quantitative (Eglen and Singh, 2003; Eglen, 2002).

Prior to experimentation, all pAcGFP1 recombinant plasmids were tested for expression of correct size protein by anti-GFP immunoblot, and all ProLabel-tagged recombinant plasmids were tested to verify activity as follows. Cells were seeded the day before transfection at 5×10^4 cells in 24-well tissue culture plates. Cells were transfected with 800 ng of DNA using Lipofectamine 2000. After 6 h, medium was replaced with fresh complete medium. Twenty-four hours post-transfection, cells were harvested for immunoblot analysis or ProLabel verification.

For *in vivo* Co-IP, HEK293 cells were seeded at 1.5×10^6 cells per 100-mm tissue culture plates 24 h prior to transfection. DNA (8 μg) was transiently transfected using Lipofectamine 2000. Twenty-four hours post-transfection, cells were harvested for Co-IP. Protein concentration was determined by BCA and 250 μg of protein cell lysate was added to each co-immunoprecipitation reaction and incubated with anti-GFP antibodies (Clontech) in 500 μL cell lysis buffer for 2 h at 5 °C. Protein G Plus/A agarose beads (Clontech) were added, and the reactions were incubated overnight at 5 °C. Beads were washed, and samples were assayed for ProLabel activity in triplicate at 10-min intervals over 180 min using a microplate luminometer (Fluroscan Ascent FL, Thermo Electron, Waltham, MA).

For *in vivo* Co-IP of endogenous PC2 and GFP-PLAGL2, HEK293 cells were transfected as above and lysates were immunoprecipitated with anti-GFP or anti-PC2 antibodies and protein G Plus/A agarose beads (Clontech) overnight. Beads were washed and samples were subjected to immunoblot analysis using anti-PC2 or anti-GFP antibodies, respectively.

2.9. Transfections and luciferase assay

HEK293 and HeLa cells were transiently transfected as above using Lipofectamine 2000. Briefly, cells were seeded 1 day prior to transfections at 5×10^4 per well in 24-well tissue culture plates. On the day of the transfection, 50 ng of pGL3 reporter plasmid, 150 ng of expression plasmids, and 50 ng of pRL-TK were co-transfected. Empty pcDNA3.1 plasmid was added to appropriate samples to adjust the total amount of DNA to 800 ng per transfection. Each transfection was done in duplicate. At 24 h post-transfection, cells were assayed for promoter-reporter activity (firefly luciferase) and *Renilla* luciferase activity using the dual-luciferase reporter assay according to the manufacture's protocol (Promega) and read on a Lumat LB 9507 luminometer (EG&G Berthold, Germany).

For PC2 siRNA studies using the *NCF2* promoter-reporter plasmid, HEK293 cells were mock transfected or transfected with 5 pmol of PC2 siGENOME SMARTpool siRNA (CCAAGACCCGGGACGAAUA, CGACAAGAACGAAGACAGA, CGUCAGUGAUCCUAUGAAU, GGUCAGUCAAAUCGAGGAU) (Dharmacon) or control siGLO RNA using Lipofectamine 2000. At 24 h post-transfection, cells were replated and allowed to grow 24 h. Cells were then transfected with appropriate promoter-reporter and expression plasmids, followed by luciferase detection as above at 72 h post-siRNA transfections. To determine the effect of PC2 knockdown on *insulin-like growth factor II* (*IGF-II*) mRNA levels, HEK293 cells were transfected as above, and RNA was isolated 72 h post-siRNA transfection using an RNeasy kit (Qiagen). RNA (100 ng) was subjected to RT-PCR using *IGF-II*-specific (sense, 5′-GCTGTTTCCGCAGCTGTGA-3′; antisense, 5′-CTGCTTCCAGGTGTCATATTGG-3′) and ribosomal 28S-specific (sense, 5′-TTGAAAATCCGGGGGAGAG-3′; antisense, 5′-ACATTGTTCCAACATGCCAG-3′) primers and a One-Step RT-PCR kit (Invitrogen). PCR products were visualized, at increasing cycle numbers to ensure analysis during the linear phase of PCR amplification, on an agarose gel stained with ethidium bromide.

The effect of PC2 knockdown on PC2 protein expression was determined by immunoblot analysis as follows. HEK293 cells were transfected as above, cells were harvested at 24 and 48 h post-transfection, and cell lysate was subjected to SDS-PAGE analysis. Proteins were transferred to nylon membrane and PC2 protein was detected using anti-PC2 antibodies and chemiluminescent detection.

2.10. Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed essentially as described (Ammons et al., 2007). MM1 cells were treated with TNF-α (20 ng/mL) for 24 h to induce endogenous PLAGL2 binding to the endogenous *NCF2* TRR, followed by chromatin isolation. Briefly, cells were treated with formaldehyde and processed for ChIP experiments using a ChIP-IT kit (Active Motif), according to the manufacturer's protocol. A sample of precleared chromatin was reserved for the input in PCR analysis. Protein/DNA complexes were immunoprecipitated overnight with the appropriate antibodies (negative control antibodies provided with the ChIP-IT kit or anti-PLAGL2 or anti-PC2 antibodies) and processed for PCR amplification. PCR amplification of each ChIP sample was performed with primers flanking the *NCF2* TRR in the endogenous *NCF2* promoter (sense, 5′-CATCTGGCCCAGAAAGTGA-3′; antisense, 5′-CTTCATTCCAGAGGCTGATGG-3′) and GAPDH promoter-specific primers (sense, 5-'TACTAGCGGTTTTACGGGCG-3′; antisense, 5′-TCGAACAGGAGGAGCAGAGAGCG-3′). PCR products were assayed at increasing cycle numbers to ensure analysis during the linear phase of PCR amplification. PCR products were subjected to agarose gel electrophoresis and stained with ethidium bromide.

2.11. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

RNA was isolated using the RNeasy Kit (Qiagen). The qRT-PCR amplification was performed in an ABI PRISM 7500 Fast Real-Time PCR System, using 12.5 mL SYBR Green PCR Master Mix (Qiagen), 1 μM gene-specific primer mix (QuantiTect Primer Assays), 0.25 μL QuantiFast RT mix, 100 ng template RNA, and water to 25 μL total volume. PCR amplification was carried out as follows: 10 min at 50 °C, 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 30 s at 60 °C. Samples were analyzed in triplicate and were normalized to beta-actin expression levels.

2.12. Statistical analysis

One-way ANOVA was performed on the indicated sets of data. Post-test analysis used Tukey's pair-wise comparisons (GraphPad Prism Software). Pair-wise comparisons with differences at $P<0.05$ were considered to be statistically significant.

3. Results

3.1. Yeast two-hybrid screen identifies PC2 as a putative PLAGL2-binding protein

To identify potential PLAGL2 cofactors, we utilized a yeast two-hybrid screen baited with full-length human PLAGL2. The reporter strain (AH109), carrying a human leukocyte cDNA library, and the bait strain (Y187) were mated yielding 1.3×10^8 library clones for nutritional and colorimetric screening.

Ubiquitin conjugating enzyme E2I, the human homolog to the yeast enzyme UBC9, was one of the putative PLAGL2-binding proteins identified in the screen (data not shown). UBC9 has been previously identified in a yeast two-hybrid screen using a partial murine PLAGL2 as bait (Van et al., 2004), demonstrating that the above screen successfully identified a known PLAGL2 protein-protein interaction. Another interesting clone retrieved from this screen was a partial cDNA corresponding to positive cofactor 2 (PC2), also known as ARC105, MED15, CTG7A, PCQAP, TIG1, and TNRC7 and a component of Mediator, a multiprotein complex that is recruited to promoters by direct interaction with gene-specific regulatory proteins (Casamassimi and Napoli, 2007; Yang et al., 2006). The partial PC2 clone contained amino acid residues 434–784, a region which includes a number of phosphorylation sites, a nuclear localization signal, and part of a proline rich region (Fig. 1A).

To confirm the *in vivo* interaction between PLAGL2 and PC2 in yeast, the bait and prey plasmids were recovered from their respective yeast strains and transformed into the opposite strain. The bait and prey strains were mated, and screening for both nutritional and colorimetric gene reporter activity confirmed the interaction between PLAGL2 and $PC2_{434–784}$ in yeast (data not shown).

3.2. PC2 interacts with the C-terminal domain of PLAGL2

Full-length PLAGL2, as well as the N-terminus and C-terminus of PLAGL2 (Fig. 1B), were expressed as GFP-tagged proteins from the pAcGFP1 vector, while full-length PC2 and $PC2_{434–784}$ were expressed as ProLabel-tagged proteins from the pProLabel-C vector. Transfection of HEK293 cells with the pcDNA3.1-PLAGL2, pAcGFP1-PLAGL2, and the ProLabel-PC2 expression plasmids, and subsequent immunoblot analysis, showed expression of exogenous proteins migrating to their predicted molecular weights (Fig. 2A). In addition, Fig. 2A showed barely detectable levels of endogenous PC2 protein, consistent with a previous report demonstrating endogenous PC2 expression in HEK293 cells (Ishikawa et al., 2006). Fluorescence microscopy showed nuclear localization of full-length GFP-PLAGL2 (Fig. 2B and C), as previously demonstrated in HEK293 cells (Zheng and Yang, 2005), relative to the diffuse expression of GFP throughout the cell. GFP-PLAGL2-NT (N-terminus) also localized to the nucleus (Fig. 2C), suggesting that, like PLAG1 (Braem et al., 2002), the nuclear localization signal (NLS) is located in the N-terminal domain of PLAGL2. GFP-PLAGL2-CT (C-terminus) was diffusely expressed throughout the cell, similar to GFP expression (Fig. 2C), which is consistent with nuclear localization being conferred via the N-terminus of PLAGL2.

Co-localization of GFP-PLAGL2 and PC2 was observed in HEK293 cells transiently transfected with pAcGFP1-PLAGL2 and pProLabel-PC2 (full-length) expression plasmids and subsequently immunostained for PC2 protein (Fig. 3D). Mock transfected cells (Fig. 3D, Mock panels) demonstrated low levels of endogenous PC2 protein in all the cells, while cells transfected with pProLabel-PC2 (Fig. 3D, PC2 panels) showed much greater levels of exogenous PC2 protein in transfected versus non-transfected cells in the same panel. When pAcGFP1-PLAGL2 and pProLabel-PC2 were co-transfected (Fig. 3D, GFP-PLAGL2/PC2 panels), co-localization of pAcGFP1-PLAGL2 and pProLabel-PC2 was apparent in the merged panels. The data demonstrate that both PLAGL2 and PC2 localize to the nucleus in HEK293 cells, and are consistent with the role of both proteins as transcriptional regulators and a previous report demonstrating nuclear localization of endogenous and exogenous PC2 protein in HEK293 cells (Ishikawa et al., 2006).

To confirm the interaction between PLAGL2 and PC2 *in vivo*, HEK293 cells were transiently transfected with pAcGFP1-PLAGL2 and

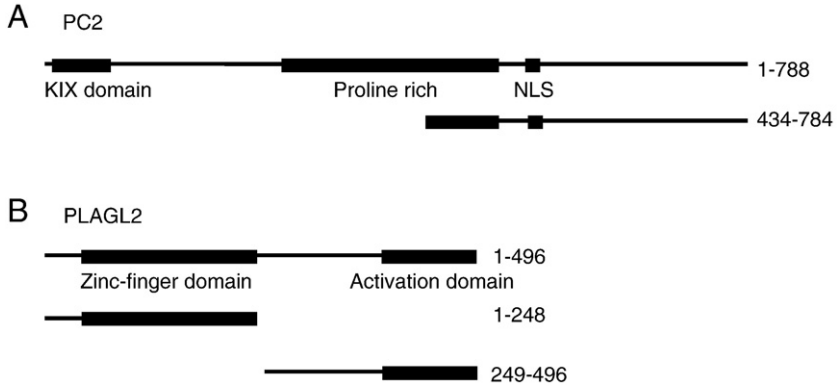

Fig. 1. Diagram of PC2 and PLAGL2 proteins indicating functional domains and partial protein regions used for Co-IP and promoter-reporter studies. (A) PC2 full-length protein (upper diagram) indicating the KIX domain, the proline rich domain and the nuclear localization signal (NLS). Lower diagram represents the partial PC2 yeast two-hybrid clone. (B) PLAGL2 full-length protein (upper diagram) with the zinc finger and activation domains indicated. Middle and lower diagrams represent the N- and C-terminal domains of PLAGL2 used for experiments. Numbers on the right designate amino acids.

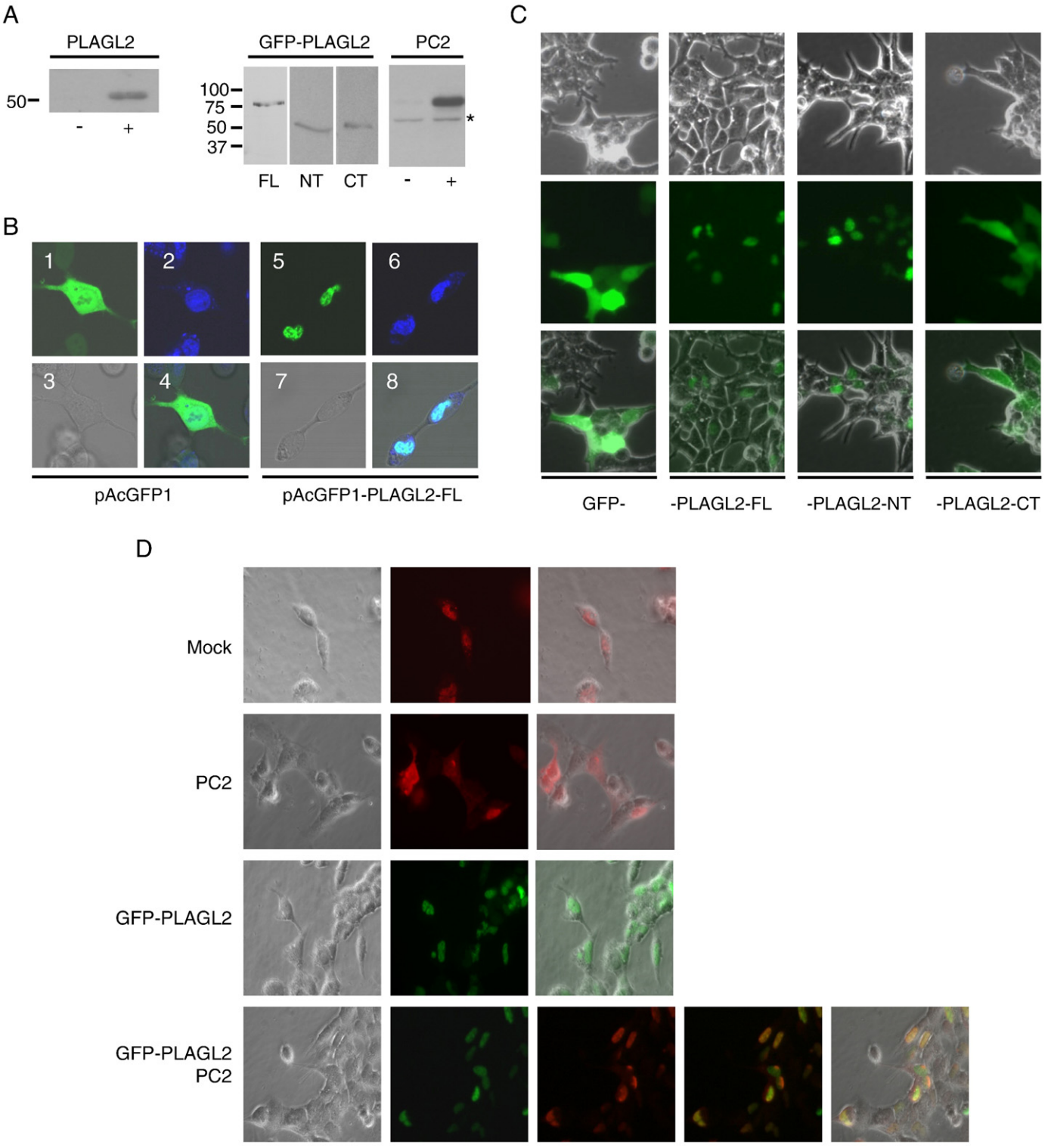

Fig. 2. Nuclear localization of PLAGL2 is supported via the N-terminal domain. (A) Immunoblot of PLAGL2, GFP-PLAGL2 full-length (FL), GFP-PLAGL2 N-terminal (NT), GFP-PLAGL2 C-terminal (CT) and Prolabel-PC2 full-length. HEK293 cells were mock transfected (−) or transfected with pcDNA-PLAGL2 (+), pAcGFP1-PLAGL2-FL (FL), pAcGFP1-PLAGL2-NT (NT), pAcGFP1-PLAGL2-CT (CT) or pProLabel-PC2 (+) plasmid DNA. Lysates were subjected to anti-PLAGL2, anti-GFP, or anti-PC2 immunoblot analysis as indicated above the blot. Molecular mass is indicated on the left. (B and C) Cellular localization of full-length GFP-PLAGL2, GFP-PLAGL2-NT and GFP-PLAGL2-CT. HEK293 cells were transfected with pAcGFP1, pAcGFP1-PLAGL2-FL, pAcGFP1-PLAGL2-NT, or pAcGFP1-PLAGL2-CT as indicated and cells were analyzed by phase or fluorescence microscopy. For nuclear staining, cells were treated with DRAQ5 (B, panels 2 and 6). Phase contrast: B, panels 3 and 7, and C, upper panel. FITC filter: B, panels 1 and 5, and C, middle panel. Merged images: B, panels 4 and 8, and C, lower panel. (D) Co-localization of GFP-PLAGL2 and PC2. HEK293 cells were transfected with pAcGFP1-PLAGL2-FL or pProLabel-PC2 plasmids, both plasmids, or mock transfected as indicated. Cells were immunostained for PC2 and analyzed by microscopy. Mock, PC2, and GFP-PLAGL2 transfections: first panel, phase; second panel, fluorescence; third panel, merge of phase and fluorescence. PC2 and GFP-PLAGL2 co-transfection: first panel, phase; second and third panels, fluorescence; fourth panel, merge of green and red panels; fifth panel, merge of green, red and phase panels.

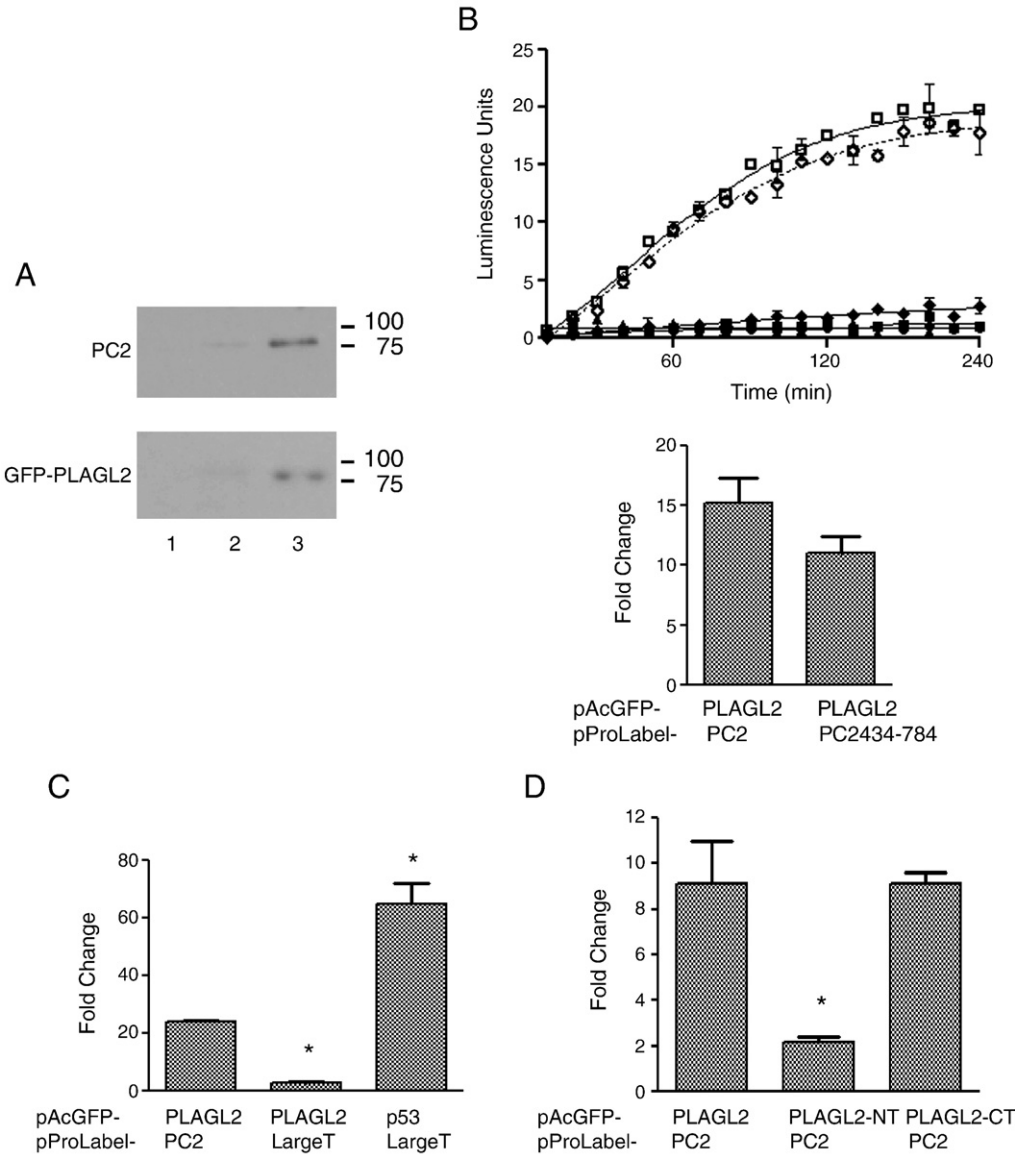

Fig. 3. PC2 interacts with the C-terminal domain of PLAGL2. (A) Co-IP of endogenous PC2 and GFP-PLAGL2. pAcGFP (lane 1), pAcGFP1-PLAGL2-FL (lane 2), or pAcGFP1-PLAGL2-FL and pProLabel-PC2 full-length (lane 3) transfected HEK293 cells were immunoprecipitated with anti-GFP antibodies (upper panel) or anti-PC2 antibodies (lower panel) and subjected to immunoblot analysis using anti-PC2 antibodies (upper panel) or anti-GFP antibodies (lower panel). (B) HEK293 cells were transfected with negative controls pAcGFP1 and pProLabel vectors (solid circle), pAcGFP1 and pProLabel-PC2 full-length (solid square) or pProLabel-$PC2_{434–784}$ (solid diamond), pAcGFP1-PLAGL2-FL and pProLabel (solid triangle), or samples pAcGFP1-PLAGL2-FL and pProLabel-PC2 full-length (open square) or pProLabel-$PC2_{434–784}$ (open diamond). Lysates were immunoprecipitated with anti-GFP antibodies and ProLabel detection was performed in triplicate. Upper graph demonstrates kinetic response over 180 min, and lower graph shows fold change relative to corresponding controls at 100 min. (B–D) Cells were transfected with pAcGFP1 and pProLabel plasmids, as indicated below the graphs. Data are presented as fold change relative to corresponding controls (empty pAcGFP1 and pProLabel plasmids). Results are representative of at least three separate experiments and samples that are statistically significantly different ($P>0.05$) from the PLAGL2/PC2 sample are indicated (*).

pProLabel-PC2 (full-length) for co-immunoprecipitation studies. As seen in Fig. 3A (upper panel, lanes 2 and 3), both endogenous and exogenous PC2 protein was co-immunoprecipitated from cells expressing GFP-PLAGL2 and immunoprecipitated with anti-GFP antibodies. Similarly, when analogous samples were immunoprecipitated with anti-PC2 antibodies, GFP-PLAGL2 was co-immunoprecipitated in the presence and absence of exogenously expressed PC2 (Fig. 3A, lower panel, lanes 2 and 3). The data demonstrate that GFP-PLAGL2 interacts with endogenous PC2 protein, as well as exogenous PC2, and provide additional evidence for a functional interaction between PLAGL2 and PC2.

The Matchmaker Chemiluminescent Co-IP system (Clontech) was used to further characterize the interaction between PLAGL2 and PC2 *in vivo*. As more fully explained in section 2.8, this system incorporates the highly sensitive ProLabel enzyme complementation assay that allows a quantitative measure of physical interactions between bait and prey proteins in mammalian cells (Eglen and Singh, 2003; Eglen, 2002). HEK293 cells were transiently transfected with pAcGFP1-PLAGL2 and pProLabel-PC2 (full-length) or pProLabel-$PC2_{434–784}$ for co-immunoprecipitation studies. PLAGL2 protein-protein complexes were immunoprecipitated with anti-GFP antibodies followed by detection of ProLabel activity over 180 min (Fig. 3B, upper panel). Both full-length PC2 and $PC2_{434–784}$ interacted with PLAGL2, as demonstrated by the significant ProLabel activity detected relative to Co-IPs performed with the pAcGFP1 and pProLabel-PC2 (full-length) or pProLabel-$PC2_{434–784}$ controls. The lower panel of Fig. 3B shows the fold change at 100 min. These results confirm the interaction between PLAGL2 and PC2 and demonstrate that the C-terminus of PC2 is capable of supporting this interaction.

To demonstrate the specificity of the PLAGL2/PC2 interaction, HEK293 cells were transfected with pAcGFP1-PLAGL2 and pProLabel-T, a plasmid expressing a ProLabel-tagged protein (SV40 large T antigen) that was not predicted to interact with PLAGL2. As expected, there was no significant ProLabel activity detected with the pAcGFP1-PLAGL2 and pProLabel-T sample relative to the pAcGFP-PLAGL2 and pProLabel-PC2 sample (Fig. 3C). SV40 large T antigen (pProLabel-T) was, however, efficiently co-immunoprecipitated with GFP-p53 (positive bait for SV40 large T), as seen by the ~55-fold change relative to the control (pAcGFP1 and pProLabel-T).

PLAGL2 contains six C2H2 zinc fingers in the N-terminus and an activation domain in the C-terminus (Fig. 1B). To determine if PC2 bound to the N- or C-terminus of PLAGL2, HEK293 cells were transfected with pAcGFP1-PLAGL2, pAcGFP1-PLAGL2-NT or pAcGFP1-PLAGL2-CT and pProLabel-PC2, followed by immunoprecipitation and detection of ProLabel activity. Figure 3D shows that the interaction between PC2 and PLAGL2 is via the C-terminus of PLAGL2. This result is consistent with the finding that mutagenesis of any single PLAGL2 zinc finger in the N-terminus showed no significant difference in ProLabel activity when compared to the full-length PLAGL2/PC2 sample (data not shown).

3.3. PLAGL2, PC2 and PU.1 cooperate to enhance activation of the NCF2 promoter-reporter

In a previous study, we determined that PLAGL2 plays a role in regulation of the *NCF2* gene (Ammons et al., 2007); however, overexpression of PLAGL2 alone was not sufficient to activate an *NCF2* promoter-reporter plasmid, suggesting the requirement for other factors. To determine if PC2 expression could modulate PLAGL2 transactivation, HEK293 cells were transfected with an *NCF2* promoter-reporter plasmid along with pcDNA3.1 expression plasmids for PLAGL2 and PC2. As seen in Fig. 4 (upper graph), there was no significant increase in promoter-reporter activity in the sample co-expressing PLAGL2 and PC2, as compared to the control (promoter-reporter and empty expression plasmid DNA). Transfection of PU.1, another factor previously shown to be required for *NCF2* basal activity (Li et al., 2001; Gauss et al., 2002), consistently showed a slight increase in promoter activity that was not enhanced by co-transfection of either PLAGL2 or PC2. However, when PLAGL2 and PC2 plasmids were co-transfected with PU.1, there was enhanced activity (~2 fold) from the *NCF2* promoter-reporter plasmid relative to samples transfected with any single or double combination of expression plasmids. Similar results were observed with HeLa cells (Fig. 4, lower graph), although the small increase in promoter activity, consistently observed in HEK293 cells in the presence of PU.1, was not detected. These results are consistent with a previous report demonstrating that, although PC2 is a component of larger protein complex, overexpression of PC2 alone is capable of stimulating a promoter-reporter plasmid containing a binding element for Smad3, a factor demonstrated to directly interact with PC2 (Kato et al., 2002). This effect was not a result of PC2 affectively increasing the expression of PLAGL2 or PU.1, as observed by Western blot analysis during the characterization of these expression plasmids and respective antibodies (data not shown). The requirement for PU.1, and the close juxtaposition of PU.1 and PLAGL2 on the promoter, raised the possibility of an interaction between PU.1 and PLAGL2 or PC2 or both; however, Co-IP studies supported no such interactions (data not shown). Taken together, the data show that PLAGL2, PC2, and PU.1 cooperate to enhance *NCF2* promoter-reporter activity.

3.4. PLAGL2 recognizes a partial PLAG1 consensus site in the NCF2 promoter via C2H2 zinc fingers 5 and 6

The data suggest a model for PLAGL2 transactivation whereby PLAGL2 and PU.1 bind directly to the *NCF2* promoter, via their

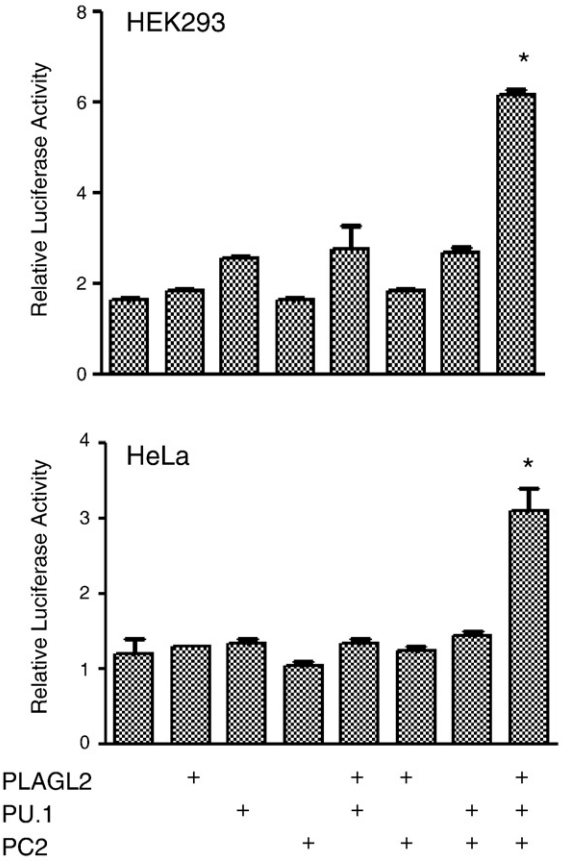

Fig. 4. PC2 cooperates with PLAGL2 and PU.1 to enhance activity from the *NCF2* promoter-reporter. HEK293 (upper graph) and HeLa (lower graph) cells were transfected with the pGL3-*NCF2*(500) promoter-reporter plasmid and pcDNA3.1-PLAGL2, pcDNA3.1-PU.1, and pcDNA3.1-PC2 expression plasmids as indicated and lysates were assayed for luciferase activity. pRL-TK plasmid was used to control for transfection efficiency. Activity is shown as fold change relative to empty pGL3 vector transfected with similar plasmids for each sample. Transfections were done in duplicate and results are representative of at least three separate experiments. The difference between the PLAGL2, PU.1, and PC2 sample and other samples was statistically significant ($P<0.05$) as indicated (*).

respective DNA recognition sites, and that PC2 associates with the promoter through PLAGL2 interactions, resulting in cooperative activation of the promoter. In previous studies, we and others identified functional PU.1 sites in the *NCF2* promoter (Li et al., 2001; Gauss et al., 2002), and although the exact PLAGL2-binding site was not determined, we did determine that PLAGL2 binds to the TNF-α-responsive region (TRR) of the *NCF2* promoter, although with approximately 50-fold less affinity relative to the PLAG consensus site (Ammons et al., 2007). To further test the proposed model, we needed to first characterize the PLAGL2 recognition sequence in the *NCF2* TRR.

The nucleotide sequence required for PLAGL2 binding to the *NCF2* TRR was determined by subjecting a series of mutant *NCF2* TRR double-strand oligonucleotides to EMSA. The sequence of the *NCF2* TRR is shown in Fig. 5A along with the altered nucleotides of the various TRR mutants. Figure 5B shows that TRR mutants 10 and 11 both prevent PLAGL2 binding to the TRR relative to the wild-type TRR and TRR mutants 1 through 9, thus identifying the PLAGL2-binding site. The sequence altered by mutants 10 and 11 (GG**A**GGCC), when read 5′ to 3′ on the complementary strand, matched the core sequence (GGRGGCC) of the bipartite PLAG1 consensus site (Voz et al., 2000). Although there is no obvious G-cluster six to eight nucleotides downstream from this core sequence, the remaining TRR sequence is relatively GC rich. Since previous studies have shown that PLAGL2 zinc fingers 5 and 6 bind to the core sequence of the PLAG1 consensus site, and zinc finger 2 binds the G-cluster (Hensen et al., 2002), the

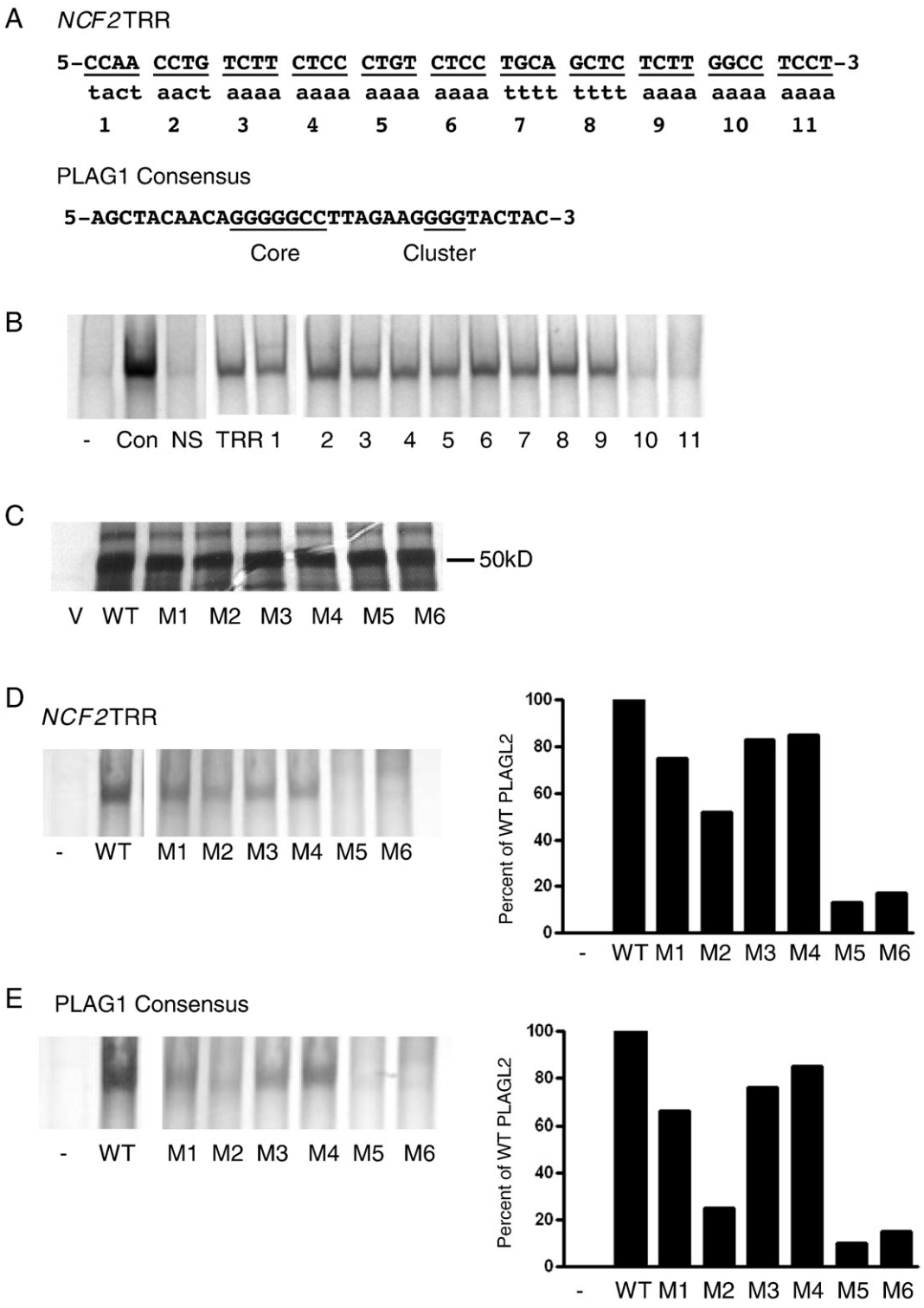

Fig. 5. PLAGL2 binds the *NCF2* promoter via a GC-rich sequence. (A) Sequence (uppercase) of wild-type *NCF2* TRR and PLAG1 consensus binding site are indicated. Sequential mutations are designated 1 through 11, and substituted nucleotides are shown (lower case). The core and cluster sequences of the PLAG1 consensus binding site are underlined. (B) EMSA of PLAGL2 protein binding to the wild-type and mutant *NCF2* TRR. PLAGL2 protein was incubated with no DNA (−), or DNA containing the PLAG1 consensus (Con), non-specific sequence (NS), wild-type *NCF2* TRR (TRR), or *NCF2* TRR mutants 1 through 11, as indicated and analyzed by EMSA. (C) PLAGL2 wild-type (WT) or zinc finger mutants (M1-M6) were generated in rabbit reticulocytes and subjected to SDS-PAGE analysis. V represents sample generated with empty pcDNA3.1 vector. Molecular mass is indicated on the right. (D and E) EMSA using wild-type PLAGL2 protein (WT) and zinc finger mutants (M1-M6) and *NCF2* TRR (D) or PLAG1 consensus DNA (E). Densitometry of EMSA is to the right with wild-type PLAGL2 set to 100%.

differences in relative binding of PLAGL2 to the PLAG1 consensus binding site and TRR shown here (Fig. 5B, Con versus TRR), and in our previous report (Ammons et al., 2007), were most likely due to the lack of the G-cluster motif of the bipartite PLAG1 consensus site in the TRR.

Absence of the G-cluster in the *NCF2* TRR suggested that PLAGL2 zinc finger 2 would not play an important role in PLAGL2 recognition of the TRR sequence. To determine which zinc fingers were required for PLAGL2 binding to the *NCF2* TRR relative to the PLAG1 consensus

sequence, each PLAGL2 zinc finger was mutated individually by replacing the first histidine of the C2H2 motif with an alanine to prevent formation of a functional zinc finger (Ikeda and Kawakami, 1995). The PLAGL2 zinc finger mutant proteins were produced in rabbit reticulocytes to demonstrate that each mutant plasmid generated approximately equal amounts of protein of the correct molecular weight (Fig. 5C). EMSA was used to test the ability of the zinc finger mutants to bind to the *NCF2* TRR, as well as to the PLAG1 consensus site (Fig. 5D and E). As reported previously (Hensen et al., 2002), we also found that zinc fingers 2, 5 and 6 were important for PLAGL2 binding to the PLAG1 consensus sequence (Fig. 5E) which is shown by the appreciable loss of binding with these specific zinc finger mutants. Figure 5D shows the importance of zinc fingers 5 and 6 in binding to the TRR with less of a role for zinc finger 2. This was consistent with results from the TRR mutagenesis analysis showing that the PLAGL2-binding site in the TRR consisted of only the core sequence of the PLAG1 consensus site, which is recognized by PLAGL2 zinc fingers 5 and 6.

3.5. Direct binding of PLAGL2 and PU.1 to their respective DNA recognition sites, and the C-terminus of PLAGL2 are necessary for enhanced NCF2 promoter-reporter activity

The data suggest that PLAGL2 and PU.1 are required at the *NCF2* promoter, presumably through direct binding to their respective recognition sites, and that PC2 is targeted to the promoter via the C-terminal domain of PLAGL2 for increased promoter activity. To further test this model, *NCF2* promoter-reporter assays were performed using a promoter containing a mutation in the PLAGL2-binding site in the TRR analogous to mutant 10 in Fig. 5A. While there was still a slight increase in promoter activity observed in the presence of PU.1, the loss of the PLAGL2-binding site in the TRR completely abrogated the enhanced activation of the promoter-reporter (Fig. 6A) relative to the PU.1 expressing samples. Because the samples expressing PLAGL2, or PC2, or PLAGL2 and PC2 consistently did not show any promoter-reporter activity over basal levels, these controls were not included in the remaining promoter-reporter studies. When the zinc fingers (5 or 6) required for TRR binding were mutated, there was, again, no enhanced activation of the *NCF2* promoter-reporter observed (Fig. 6B). Finally, when the plasmid (PLAGL2-NT) expressing only the N-terminal zinc finger DNA-binding domain of PLAGL2 and lacking the C-terminus required for PC2 binding was substituted for wild-type PLAGL2, there was also no increase in promoter activity observed (Fig. 6C). The data show that activation of the *NCF2* promoter-reporter by PLAGL2, PU.1 and PC2 requires binding of PLAGL2 to the identified PLAGL2-binding site in the *NCF2* TRR via zinc fingers 5 and 6 and the C-terminal domain of PLAGL2, which is involved in PC2 binding.

To determine the role of PU.1 in the enhanced *NCF2* promoter activity, a promoter-reporter construct with mutated PU.1 sites (Gauss et al., 2002) was tested. As seen in Fig. 6D (middle bar), the

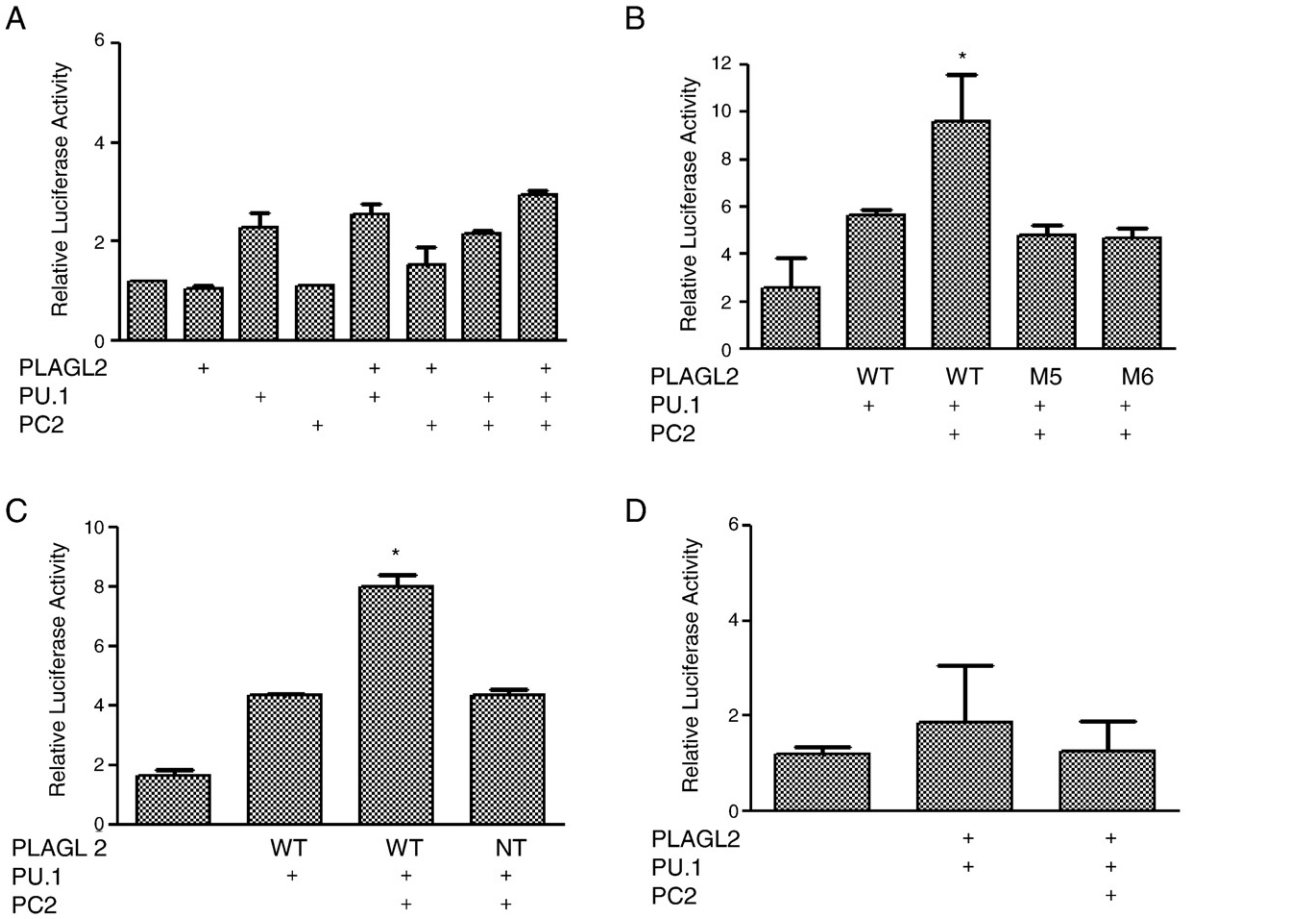

Fig. 6. PLAGL2 and PU.1 binding to the *NCF2* promoter is required for the PLAGL2/PU.1/PC2 enhanced *NCF2* promoter-reporter activity. HEK293 cells were transfected with pGL3-*NCF2*(500) promoter-reporter plasmid along with the indicated pcDNA3.1 expression plasmids and lysates were assayed for luciferase activity. Activity is shown as fold change relative to controls (empty pGL3 vector transfected with similar plasmids). (A) pGL3-*NCF2*(500) containing the *NCF2* TRR mutation #10 (Fig. 5A) was transfected along with the indicated expression plasmids. (B and C) Wild-type pGL3-*NCF2*(500) was transfected with either wild-type PLAGL2 or PLAGL2 zinc finger mutants M5 or M6 (B) or PLAGL2-NT (C) and PU.1 and PC2 expression plasmids, as indicated. (D) pGL3-*NCF2*(500) containing three mutated PU.1 sites (Gauss et al., 2002) was transfected along with the indicated expression plasmids. Transfections were done in duplicate and results are representative of at least three separate experiments. Samples that were statistically significantly different ($P>0.05$) from the WT PLAGL2/PU.1 sample are indicated (*).

slight increase in promoter activity usually observed with PU.1 expression (Fig. 6A–C) was not detected in the absence of PU.1 binding sites. In addition, there was no enhanced promoter activity with PLAGL2, PU.1 and PC2 expression, demonstrating the importance of PU.1 in the cooperative activation of *NCF2* promoter-reporter. Together, the data demonstrate that PLAGL2 and PU.1 must bind their respective sites in the promoter and that the C-terminal domain of PLAGL2, which is essential for PC2 binding, is required for enhanced promoter activity.

3.6. PC2 knockdown completely abrogates the PLAGL2, PU.1, and PC2 cooperative activation of the NCF2 promoter-reporter plasmid

To establish the requirement for PC2 in enhanced activation of the *NCF2* promoter, promoter-reporter assays were repeated in the presence of PC2 siRNA. HEK293 cells were mock transfected, or transfected with control siRNA (siGLO) or PC2 siRNA, and 48 h post-siRNA transfection, cells were transfected with expression plasmids. As seen in Fig. 7A, there was a significant difference in promoter-reporter activity when comparing the PLAGL2, PU.1, and PC2 sample and the analogous sample transfected in the presence of PC2 siRNA, demonstrating that PC2 is essential for this cooperative promoter activation. These results were consistent with levels of PC2 observed at 48 h post-siRNA transfection (Fig. 7C), where there was little to no detectable levels of PC2 in the PC2 siRNA sample compared to samples expressing exogenous PC2 in the absence or presence of control siRNA. Importantly, there was no significant reduction in promoter activity of a similar transfection in the presence of control siRNA (siGLO), and consistent with previous findings (Yang et al., 2006), PC2 knockdown did not globally inhibit transcription, as the activity from the thymidine kinase promoter of the pRL-TK plasmid was virtually the same between samples (Fig. 7B). These data demonstrate that PC2 is required for the cooperative promoter-reporter activity.

3.7. Endogenous PC2 associates with the endogenous NCF2 promoter at the NCF2 TRR and is required for PLAGL2 regulation of NCF2 in TNF-α-treated MonoMac1 cells

Together, the data strongly suggest that the effects of PC2 on the enhanced promoter activity are due to a physical association of PC2 with the promoter via an interaction with PLAGL2 at the *NCF2* TRR. The lack of activation of the endogenous *NCF2* promoter in PLAGL2, PU.1 and PC2 transfected HEK293 cells (data not shown), possibly due to negative epigenetic regulation (Fuks, 2005; Chen et al., 2002), prompted us to use MM1 cells, a model we used previously demonstrating PLAGL2 association with the *NCF2* TRR in response to TNF-α (Ammons et al., 2007), to determine if endogenous PC2 localized to the endogenous *NCF2* promoter in the same region recognized by PLAGL2 (*NCF2* TRR). ChIP analysis was performed using chromatin isolated from TNF-α-treated MM1 cells. Figure 8A (upper panel) shows that, relative to the no antibody and negative control antibodies, the *NCF2* TRR was specifically immunoprecipitated by either PLAGL2 or PC2 antibodies, demonstrating that PC2 associates with the endogenous *NCF2* promoter in a similar region occupied by PLAGL2. Importantly, no significant association of PLAGL2 or PC2 was detected with the control GAPDH gene (Fig. 8A, lower panel). Sequential ChIP analysis performed to further demonstrate co-occupancy of PLAGL2 and PC2 on the *NCF2* TRR was inconclusive, possibly due to the low levels of these endogenous transcriptional regulators.

In a previous report (Ammons et al., 2007), we showed that PLAGL2 bound to the *NCF2* TRR and was required for increased expression of *NCF2* in response to TNF-α in MM1 cells. To determine if PC2 was also necessary for PLAGL2 regulation of *NCF2* in response to TNF-α, MM1 cells were mock transfected, or transfected with control siRNA (siGLO) or PC2 siRNA 48 h prior to TNF-α treatment. RNA was isolated 24 h post TNF-α treatment and subjected to *PC2*- and *NCF2*-specific qRT-PCR. As seen if Fig. 8B (upper graph), the ~5 fold increase in *NCF2* mRNA with TNF-α treatment is affectively and significantly reduced in the presence of PC2 siRNA, but not with control siRNA. The lower graph shows that *PC2* mRNA levels were lower in the presence of PC2 siRNA, and although this change was not statically significant,

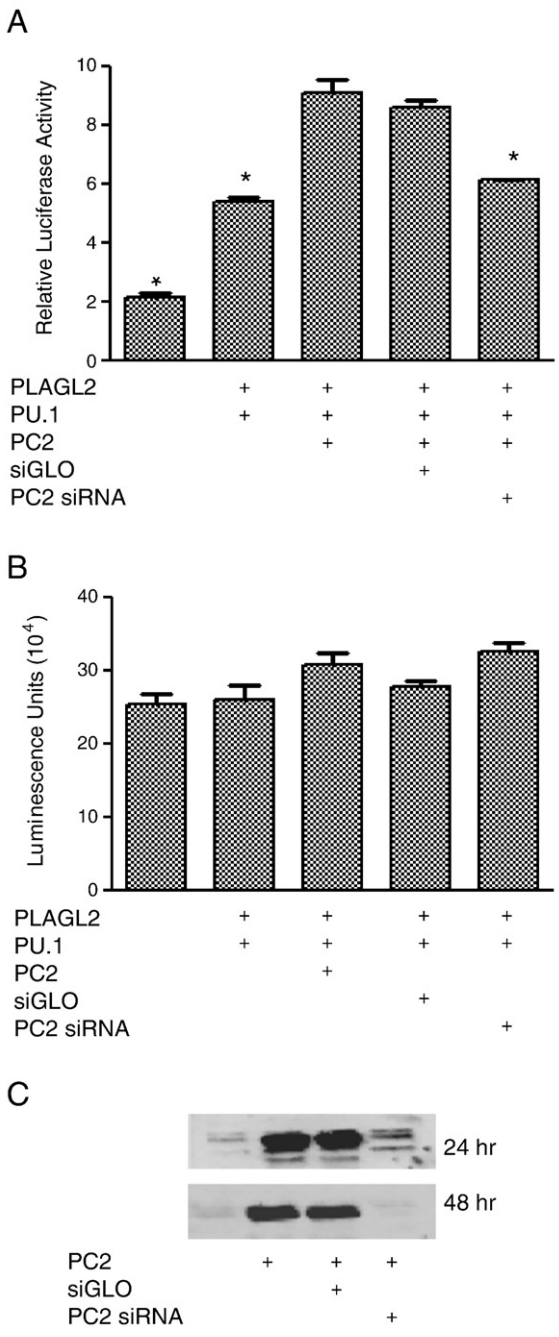

Fig. 7. PC2 is required for enhanced *NCF2* promoter-reporter activity. HEK293 cells were mock transfected or transfected with control siRNA (siGLO) or PC2 siRNA as indicated, followed by transfection with pGL2-*NCF2*(500) and the indicated expression plasmids. Lysates were assayed for luciferase activity. (A) Luciferase activity from the *NCF2* promoter-reporter is presented as fold change relative to controls as in Figs. 4 and 6. (B) Luciferase activity from the pRL-TK plasmid is presented as luminescent units. Transfections were done in duplicate and results are representative of three separate experiments. Samples that were statistically significantly different ($P>0.05$) from the PLAGL2/PU.1/PC2 sample are indicated (*). (C) HEK293 cells were transfected as above with PC2 expression plasmid, control siRNA (siGLO) or PC2 siRNA as indicated. Cell lysates were harvested at 24 h (upper panel) and 48 h (lower panel) and subjected to anti-PC2 immunoblot analysis.

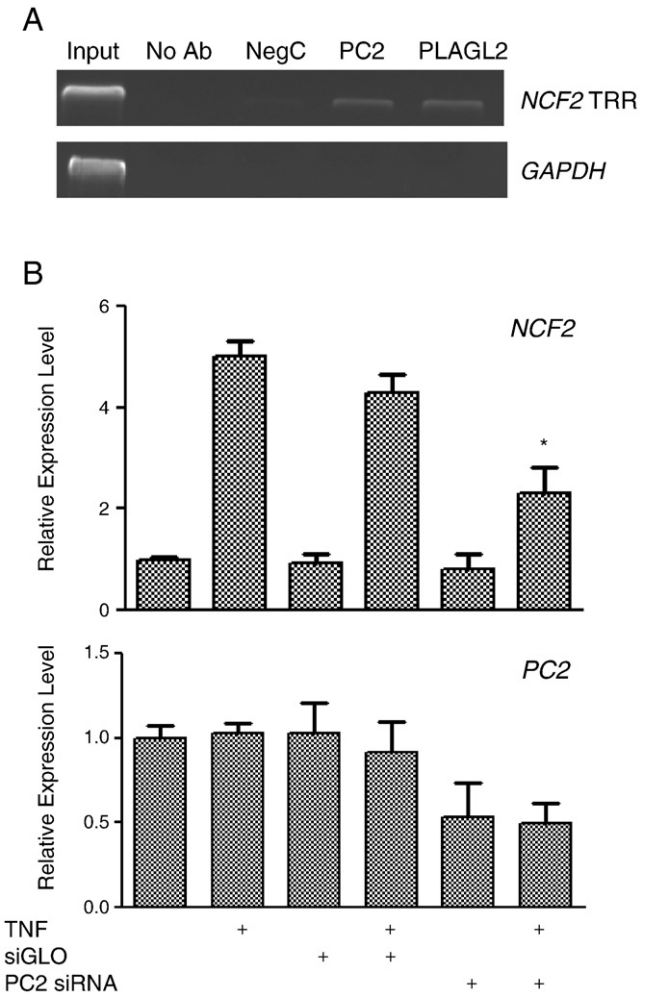

Fig. 8. Endogenous PC2 associates with the *NCF2* promoter at the *NCF2* TRR and is required for PLAGL2 regulation of *NCF2* in TNF-α-treated MM1 cells. (A) MM1 cells were treated with TNF-α (20 ng/mL) prior to chromatin isolation (Ammons et al., 2007). ChIP analysis was performed with no antibody (No Ab), negative control antibodies (NegC), anti-PLAGL2, or anti-PC2 antibodies, as indicated. ChIP samples and the input sample were subjected to *NCF2* TRR-specific (upper panel) or GAPDH-specific (lower panel) PCR amplification. Results are representative of three individual experiments. (B) MM1 cells were mock transfected or transfected with control siRNA (siGLO) or PC2 siRNA as indicated. Cells were treated with TNF-α (20 ng/mL) and RNA was isolated and subjected to *NCF2*-specific (upper graph) and *PC2*-specific (lower graph) qRT-PCR in triplicate. Data are presented as fold expression with mock transfected, non-TNF-α-treated sample set to one. Results are representative of three individual experiments. The PC2 siRNA, TNF-α-treated *NCF2* sample was statistically significantly different ($P>0.05$) from the mock transfected, TNF-α-treated sample and is indicated (*).

the reduction in *PC2* mRNA levels was consistent between experiments. The inability to completely knockdown *PC2* mRNA was likely due to the relative inefficiency of MM1 cell transfection compared to HEK293 cells. This also explains that, while the reduction in *NCF2* mRNA was significant with PC2 knockdown, it was not down at basal levels. Together, the data show that endogenous PLAGL2 and PC2 associate with the *NCF2* TRR and that PC2 is required for PLAGL2 regulated expression of *NCF2* in TNF-α-treated MM1 cells, thus demonstrating the functional relevance of PC2 as a modulator of PLAGL2 transactivation.

3.8. PC2 knockdown inhibits PLAGL2 induced expression of IGF-II

To determine if PC2 played a role in the regulation of other PLAGL2 target genes, we evaluated the effect of PC2 knockdown on the expression of *IGF-II*, a known PLAGL2 target gene in PLAGL2 expressing HEK293 cells (Hensen et al., 2002). HEK293 cells were transfected with PLAGL2 expression plasmid in the presence or absence of PC2 siRNA, followed by RT-PCR analysis of *IGF-II* mRNA levels. As seen in Fig. 9, the increase in *IGF-II* mRNA levels with PLAGL2 expression was greatly diminished in the presence of PC2 siRNA (Fig. 9, lane 6), relative to expression in the mock and negative control siRNA (siGLO) transfections (Fig. 9, lanes 2 and 4). The data demonstrate that PC2 is also important in the regulation of a second PLAGL2 target gene, *IGF-II*.

4. Discussion

All three members of the PLAG family have been shown to be involved in tumorigenesis (reviewed by Van et al., 2007; Abdollahi, 2007); however, little is known about the mechanisms that regulate their activity. In the present study, we identified PC2, a component of the ARC/Mediator complex, as a novel PLAGL2-binding protein and an important modulator of PLAGL2 transactivation.

The partial $PC2_{434–784}$ clone isolated from the yeast two-hybrid screen demonstrated that the C-terminal domain of PC2 was sufficient for PLAGL2 binding. *In vivo* Co-IP studies in HEK293 cells confirmed the yeast two-hybrid results and show that PC2 binds to PLAGL2 via the C-terminus of PLAGL2. This is consistent with previous reports showing a direct interaction of the Mediator subunit, PC2, with other transcriptional regulators, including the sterol regulatory element binding protein (SREBP), and Smad factors 2/3 and 4 (Yang et al., 2006; Kato et al., 2002). The ability of PC2 to interact with various gene-specific transcription factors suggests that, as a component of the ARC/Mediator complex, PC2 may play a key role in targeting Mediator to a distinct set of promoters.

Full-length PLAGL2 expressed in the Co-IP studies was shown to be nuclear, as previously reported (Zheng and Yang, 2005). We further ascertained that, like PLAG1 (Braem et al., 2002), nuclear localization was determined by the N-terminal domain of PLAGL2. Although we did not attempt to specifically identify a nuclear localization signal, PLAGL2 does contain a similar sequence (PRPR) at the same amino acid location corresponding to the putative PLAG1 nuclear localization

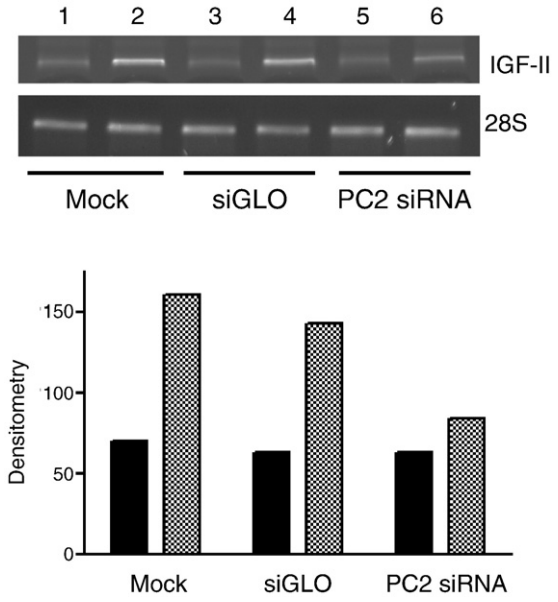

Fig. 9. PC2 is important in PLAGL2 regulation of *IGF-II*. HEK293 cells were mock transfected or transfected with control siRNA (siGLO) or PC2 siRNA as indicated, followed by transfection with empty pcDNA3.1 vector (lanes 1, 3, and 5) or pcDNA3.1-PLAGL2 expression plasmid (lanes 2, 4, and 6). RNA was isolated and subjected to RT-PCR using *IGF-II*- and 28S-specific primers. Samples were separated on an agarose gel (upper panel), and the *IGF-II* specific bands were subjected to densitometry (lower panel). Results are representative of three individual experiments.

sequence (KRKR). Thus, it will be of interest to determine if the PRPR sequence of PLAGL2 serves a similar function.

The binding of PLAGL2 to the *NCF2* TRR in previous studies was somewhat surprising, as there appeared to be no obvious binding site for PLAGL2 within this sequence. In this report, mutagenesis of the TRR showed that the nucleotide sequence required for PLAGL2 binding contained the PLAG1 consensus core sequence (GGRGGCC) but lacked the G-cluster motif. Although this binding site is located on the reverse strand, this arrangement is similar to the functional PLAG1 binding site characterized in the *IGF-II* promoter (Voz et al., 2000). Mutagenesis of PLAGL2 zinc fingers demonstrated that zinc fingers 5 and 6 were important for TRR binding, with zinc finger 2 playing much less of a role. These data are consistent with a previous report demonstrating the importance of PLAGL2 zinc fingers 5 and 6 in binding to the PLAG1 core sequence and of zinc finger 2 in binding to the G-cluster (Hensen et al., 2002). The ability of PLAGL2 to bind relatively efficiently to the core sequence alone demonstrates that, like PLAGL1, PLAGL2 can also bind GC-rich DNA in the absence of the G-cluster. These data support the idea that these family members can recognize DNA-binding sites that are, in general, GC rich with some variability in sequence, possibly leading to functional redundancy between proteins and/or competition for similar target genes. Elucidation of the complete set of PLAG target genes and DNA recognition sites in their respective target promoters will be important in addressing this issue.

As stated earlier, overexpression of PLAGL2 alone had little to no affect on the activity of the *NCF2* promoter-reporter plasmid in HEK293 cells, although we demonstrated binding of endogenous PLAGL2 to the *NCF2* promoter in TNF-α-treated MM1 cells in a recent study (Ammons et al., 2007). The lack of *NCF2* promoter-reporter activity by PLAGL2 alone, however, was not unprecedented, as similar results were reported for PLAGL2 transactivation of an *IGF-II* promoter-reporter (Ning et al., 2008). In those studies, overexpression of PLAGL2 had no significant effect on the activity of the *IGF-II* promoter-reporter; however, when PLAGL2 was overexpressed with Tip60, a PLAGL2-binding protein that modulates PLAGL2 transactivation through acetylation, the activity of the *IGF-II* promoter-reporter was stimulated ~2 fold. In contrast, another PLAGL2 target promoter, SP-C, showed no enhanced activity in the presence of Tip60, demonstrating that the effect of Tip60 on PLAGL2 transactivation was promoter-specific. In this study, we show that PC2 enhanced PLAGL2 transactivation of the *NCF2* promoter 2- to 3-fold in cooperation with PU.1. We also show the importance of endogenous PC2 in PLAGL2-induced *IGF-II* expression, implying that the effect of PC2 on PLAGL2 target promoter activity may be a general mechanism of PLAGL2 transactivation. Additional studies, however, are required to determine if PC2 is indeed being targeted to the *IGF-II* promoter via PLAGL2 binding. Together, the findings presented here demonstrate that PC2 is a modulator of PLAGL2 transactivation, and it will be of interest to determine if PC2 plays a role in the regulation of all PLAGL2 responsive genes or, as suggested for Tip60 regulated PLAGL2 activation, if the effect is limited to a subset of PLAGL2 target promoters.

The data presented here support a model whereby PLAGL2, PU.1, and PC2 physically associate with the *NCF2* promoter directly, or indirectly through protein-protein interactions, and that they cooperate to enhance the activity of the *NCF2* promoter-reporter. The data suggest that PLAGL2 and PU.1 are required to bind to their respective sites in the promoter, and that PC2 is targeted to the promoter via the C-terminus of PLAGL2, the region required for transactivation. We demonstrate that GFP-PLAGL2 interacts with endogenous PC2 and that endogenous PC2 associates with a PLAGL2 target promoter in TNF-α-treated MM1 cells in a similar region occupied by PLAGL2. In addition, we show that PLAGL2 regulation of *NCF2* in response to TNF-α is inhibited with PC2 knockdown, thus demonstrating the physiological relevance of PC2 as an effector of PLAGL2 transactivation. We cannot, however, rule out the possibility that the interaction between PLAGL2 and PC2 is indirect, and that there may be additional proteins acting as linkers between PLAGL2 and PC2, as suggested by the inconclusiveness of the sequential ChIP studies (data not shown). The role of PC2 as a subcomponent of the large multiprotein Mediator complex implies that the PC2-enhanced *NCF2* promoter activity is likely the result of Mediator recruitment to the promoter via PLAGL2, allowing formation of a stable pre-initiation complex. Additional studies are necessary to further characterize this model and to determine if the effect of PC2 is indeed through targeting Mediator to PLAGL2 target promoters.

Although there are many reports alluding to the role of the PLAG proteins in key physiological processes, including oncogenesis, understanding the mechanisms whereby PLAG proteins regulate these processes, including the identification of the complete set of target genes and regulatory cofactors, is far from complete. This study further contributes to our understanding of PLAG gene regulation by establishing PC2 as a novel PLAGL2-binding protein and modulator of PLAGL2 transactivation. Considering the oncogenic potential of PLAG proteins, elucidating their mechanisms of activation by identifying gene targets, signaling pathways, and the functional relationship between family members will be important in identifying avenues for early diagnosis and novel therapeutic targets for treatment of diseases and/or disorders associated with the aberrant expression of these proteins.

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