NR4A1 Antagonists Inhibit β1-Integrin-Dependent Breast Cancer Cell Migration

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Running Title: β1-Integrin can be targeted by NR4A1 antagonists

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Abstract:

Overexpression of the nuclear receptor 4A1 (NR4A1) in breast cancer patients is a prognostic factor for decreased survival and increased metastasis and this has been linked to NR4A1-dependent regulation of TGFβ signaling. Results of RNA interference studies demonstrate that basal migration of aggressive SKBR3 and MDA-MB-231 breast cancer cells is TGFβ-independent and -dependent on regulation of β1-integrin gene expression by NR4A1 which can be inhibited by the NR4A1 antagonists 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH) and a related *p*-carboxymethylphenyl (DIM-C-pPhCO$_2$Me) analog. The NR4A1 antagonists also inhibited TGFβ-induced migration of MDA-MB-231 cells by blocking nuclear export of NR4A1 which is an essential step in TGFβ-induced cell migration. We also observed that NR4A1 regulates expression of both β1- and β3-integrins and unlike other β1-integrin inhibitors which induce prometastatic β3-integrin, NR4A1 antagonists inhibit expression of both β1- and β3-integrin demonstrating a novel mechanism-based approach for targeting integrins and integrin-dependent breast cancer metastasis.

Introduction

Cell adhesion and attachment are essential for tissue integrity and cellular homeostasis, and the heterodimeric integrin cell surface receptors play a critical role in these processes (1-3). There are 18 different α and 8 different β subunits that form 24 αβ-integrin receptor heterodimers, and the large 12-member β1-integrin sub-group bind multiple extracellular matrix (ECM) molecules to activate multiple intracellular pathways and also induce crosstalk with other signaling systems (1-3). The functions of integrin heterodimers are highly tissue-specific, and many human pathologies also involve integrin signaling (reviewed in 4, 5). β1-Integrin is highly expressed in most tumors and is associated with a negative prognostic significance such as overall and disease-free survival, recurrence, and metastasis for head and neck and squamous cell carcinoma, melanoma, lung, breast, prostate, laryngeal and pancreatic cancers (6-17). A recent immunostaining study of 225 breast invasive ductal carcinomas (IDCs) showed that β1-integrin was overexpressed in 32.8% of patients with IDCs (13). Numerous studies show that focal adhesion kinase (FAK) which is downstream from β1-integrin is also a negative prognostic factor for breast cancer patients (18-20). The important functional role of β1-integrin has been demonstrated in mouse models expressing *erbB2* under the control of the mouse mammary tumor virus and crossed with mammary tissue-specific β1-integrin-deficient mice. These mice exhibit a decrease in tumor volume, increased apoptosis and decreased lung metastasis compared to animals expressing wild-type β1-integrin (21-23). Although small molecules, peptides and antibodies that inhibit β1-integrin signaling have been developed, clinical application of agents that target β1-integrin for cancer chemotherapy are not currently available.

The orphan nuclear receptor 4A1 (NR4A1, TR3, Nur77) is overexpressed in breast cancer and other tumors, and functional studies show that NR4A1 exhibits pro-oncogenic

activity (reviewed in 24). Studies in this laboratory have characterized a series of 1,1-bis(3'-indolyl)-1-(*p*-substituted phenyl)methane (C-DIM) analogs that bind NR4A1 and act as receptor antagonists to inhibit growth and induce apoptosis in several cancer cell lines and in tumors from mouse xenografts (25-30). A recent study demonstrated functional interactions between NR4A1 and TGFβ and in estrogen receptor (ER)-negative MDA-MB-231 cells, knockdown of NR4A1 decreased migration and also inhibited TGFβ-induced migration of this cell line (31). Results of gene array studies in pancreatic cancer cells identified *β1-integrin* as a potential NR4A1-regulated gene (27). In this study, we demonstrate that NR4A1 regulates β1-integrin expression and β1-integrin-dependent migration of breast cancer cells and this is accompanied by decreased expression of β3-inegrin. In MDA-MB-231 cells, results of our studies show that both constitutive and TGFβ-induced migration are dependent on nuclear and extranuclear NR4A1-regulated pathways, respectively. C-DIM/NR4A1 antagonists inhibit NR4A1-dependent expression of β1- and β3-integrins and other pro-oncogenic NR4A1-regulated genes and pathways and represent a novel class of mechanism-based anticancer agents.

Materials and Methods

Cell lines and antibodies

SKBR3, MDA-MB-231 and MCF-7 breast cancer cells were purchased from American Type Culture Collection (Manassas, VA). Cells were maintained 37°C in the presence of 5% CO_2 in Dulbecco's modified Eagle's medium/Ham's F-12 medium with 10% fetal bovine serum with antibiotic. NR4A1 antibody was purchased from Novus Biologicals (Littleton, CO). TGFβ was purchased from BD Biosystems (Bedford, MA). β-Actin antibody, Dulbecco's Modified Eagle's Medium, PMI-1640 Medium, and 36% formaldehyde were purchased from Sigma-

Aldrich (St. Louis, MO). Hematoxylin was purchased from Vector Laboratories (Burlingame, CA). β3-Integrin, p-FAK, FAK, Axin2, leptomycin B, and NR4A1 immunofluorescent antibody were purchased from Cell Signaling Technologies (Manassas, VA). β1-Integrin antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA), p84 antibody from GeneTex (Irvine, CA), and GAPDH antibody from Biotium (Hayward, CA).

Cell adhesion assay

SKBR3, MDA-MB-231, and MCF-7 cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hours. Cells were seeded and subsequently treated with varying concentrations of DIM-C-pPhOH or DIM-C-pPhCO_2Me for 24 hours or 1 hour prior (± TGFβ [5 ng/ml], 4 hours cotreatment) or with 100 nm of siβ1-integrin or siNR4A1 for 48 hours. Cells were trypsinized, counted, and then placed for 90 min on BD BioCoat Human Fibronectin Cellware 24-well plates (Bedford , MA); medium was then aspirated, wells gently washed with PBS, and stained with 0.5% Crystal Violet Stain. Cells were then counted for adhesion to fibronectin. Wells coated with BSA and poly-L-lysine were used as negative controls.

Boyden chamber assay

SKBR3, MDA-MB-231, and MCF-7 cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hours. Cells were seeded and subsequently treated with varying concentrations of DIM-C-pPhOH or DIM-C-pPhCO_2Me for

24 hours or 1 hour prior (± TGFβ [5 ng/ml], 4 hours cotreatment) or with 100 nM of siβ1-integrin, siNR4A1, siSp1 or sip300 for 48 hours. Cells were trypsinized, counted, then placed in 24-well 8.0 µm pore ThinCerts from BD Biosciences (Bedford, MA), allowed to migrate for 24 hours, fixed with formaldehyde, and then stained with hematoxylin. Cells that migrated through the pores were then counted.

Real time PCR

RNA was isolated using Zymo Research *Quick-RNA* MiniPrep kit (Irvine, CA). Quantification of mRNA (β1-integrin, β3-integrin) was performed using Bio-Rad iTaq Universal SYBER Green 1-Step Kit (Richmond, CA) using the manufacturer's protocol with real-time PCR. TATA Binding Protein (TBP) mRNA was used as a control to determine relative mRNA expression.

Immunoprecipitation

MDA-MB-231 cancer cells (3.0×10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium containing 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGFβ (5 ng/ml) was added for 4 hours (after ± pretreatment with leptomycin B (20 nM) for 24 hours or ± pretreatment with 20 µM DIM-C-pPhOH or DIM-C-pPhCO_2Me. Protein A Dynabeads were prepared and binding of antibody with protein and protein-protein interactions were isolated by Life technologies Immunoprecipitation Kit using Dynabeads coated with Protein A (Grand

Island, NY) following manufacturer's protocol. Protein-protein interactions of interest were determined by western blot analysis.

Chromatin immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP-IT Express magnetic chromatin immunoprecipitation kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. SKBR3 and MDA-MB-231 cells were treated with DMSO, DIM-C-pPhOH, or DIM-C-pPhCO_2Me (15 or 20 μM) for 24 hours. Cells were then fixed with 1% formaldehyde, and the cross-linking reaction was stopped by addition of 0.125 M glycine. After washing twice with phosphate-buffered saline, cells were scraped and pelleted. Collected cells were hypotonically lysed, and nuclei were collected. Nuclei were then sonicated to the desired chromatin length (~200 to 1,500 bp). The sonicated chromatin was immunoprecipitated with normal IgG, p300 (Santa Cruz), siSp1 (Abcam), NR4A1 (Novus Biologicals), or RNA polymerase II (pol II; Active Motif) antibodies and protein A-conjugated magnetic beads at 4°C for overnight. After the magnetic beads were extensively washed, protein-DNA cross-links were reversed and eluted. DNA was prepared by proteinase K digestion followed by PCR amplification. The primers for detection of the β1-integrin promoter region were 5'–TCACCACCCTTCGTGACAC–3' (sense) and 5'–GAGATCCTGCATCTCGGAAG–3' (antisense), the primers for detection of the β3-integrin promoter region were 5'–TCTCAGGCGCAGGGTCTAGAGAA–3' (sense) and 5'–TCGCGGCGCCCACCGCCTGCTCTACGCT–3' (antisense). PCR products were resolved on a 2% agarose gel in the presence of RGB-4103 GelRed Nucleic Acid Stain.

Nuclear/cytosolic extraction

MDA-MB-231 cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGFβ (5 ng/ml) was added for 4 hours (after ± pretreatment with 20 nM leptomycin B for 24 hours or ± pretreatment with 20 μM DIM-C-pPhOH or DIM-C-pPhCO_2Me. Nuclear and cytosolic fractions were then isolated using Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Kit (Rockford, IL) according to manufacturer's protocol. Fractions were then analyzed by western blot. GAPDH and p84 were used as cytoplasmic and nuclear positive controls respectively.

Immunofluorescence

MDA-MB-231 (1.0 x 10^5 per well) were seeded in 2-well Nunc Lab-Tek chambered B#1.0 Borosilicate coverglass slides from Thermo Scientific and were allowed to attach for 24 hours. The medium was then changed to DMEM/Ham F-12 medium contained 2.5% charcoal-stripped fetal bovine serum, and either DMSO or TGFβ (5 ng/ml) was added for 4 hours (after ± pretreatment with leptomycin B (20 nM) for 24 hours or ± pretreatment with 20 μM DIM-C-pPhOH or DIM-C-pPhCO_2Me. Cells were then treated with fluorescent NR4A1 primary antibody (Nur77 (D63C5) XP®) and immunofluorescence was observed according to Cell Signaling Technology's immunofluorescence protocol. DAPI staining was observed using Hoechst staining according to Biotium's Apoptotic and Necrotic assay kit following the manufacturer's protocol. Cells were visualized by microscopy (Advanced Microscopy) and

NR4A1 localization was determined by green fluorescence. DAPI was used to stain the nucleus and images were taken sequentially of NR4A1, DAPI, and then merged (28-30).

Western blot analysis

SKBR3, MDA-MB-231, and MCF-7 cancer cells (3.0 x 10^5 per well) were seeded in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 2.5% charcoal-stripped fetal bovine serum and were allowed to attach for 24 hours. Cells were transfected with 100 nm of siβ1-integrin, siNR4A1, siSp1, or sip300 for 72 hours or treated with various C-DIM compounds. Cell lysates were analyzed by western blot as described previously (28-30).

Small interfering RNA interference assay

SiRNA experiments were conducted as described previously (28-30). The siRNA complexes used in the study are as follows: siGL2-5': CGU ACG CGG AAU ACU UCG A; siNR4A1: SASI_Hs02_00333289[1], SASI_Hs02_00333290[2]; siβ1-integrin: SASI_Hs02_00333437[1], SASI_Hs01_00159474; siSp1:SASI_Hs02_003; sip300: SASI_Hs01_00052818.

TNBC orthotopic xenograft studies

Female BALB/c nude mice (6–8 weeks old) were obtained (Charles River Laboratory, Wilmington, MA, USA) maintained and treated as previously described (30). Tumor volumes and tumor weights were determined as previously described (30). Tumor lysates were obtained and analyzed by western blots.

Statistical analysis

206 Statistical significance of differences between the treatment groups was determined by
207 Student's *t*-test. The results are expressed as means with error bars representing 95% CIs for at
208 least three experiments for each group unless otherwise indicated. A P value < 0.05 was
209 considered statistically significant. All statistical tests were two sided.
210
211 **Results**
212 *NR4A1 regulates β1-integrin expression*
213 β1-Integrin is expressed in ER-positive MCF-7, ER-negative MDA-MB-231, and erbB2-
214 overexpressing SKBR3 breast cancer cells, and knockdown of NR4A1 (siNR4A1) by RNAi
215 (RNA interference) decreased expression of β1-integrin protein and mRNA (Fig. 1A). Previous
216 studies identified 1,1-bis(3'-indolyl)-1-(*p*-hydroxyphenyl)methane (DIM-C-pPhOH; C-DIM8)
217 and 1,1-bis(3'-indolyl)-1-(*p*-carboxymethylphenyl)methane (DIM-C-pPhCO_2Me; C-DIM14) as
218 NR4A1 ligands that act as antagonists in breast and other cancer cell lines (25-30), and both
219 compounds also decreased expression of β1-integrin protein (Fig. 1B) and mRNA (Fig. 1C) in
220 MCF-7, MDA-MB-231 and SKBR3 cells. Moreover, western blot analysis of tumor lysates
221 from mice bearing MDA-MB-231 cells (orthotopic) (30) showed that DIM-C-pPhCO_2Me
222 significantly decreases β1-integrin protein expression (Fig. 1D). β1-Integrin regulates
223 phosphorylation of FAK (p-FAK), and transfection of MCF-7, MDA-MB-231 and SKBR3 cells
224 with siNR4A1 (Fig. 2A) or treatment with DIM-CpPhOH (Fig. 2B) or DIM-C-pPhCO_2Me (Fig.
225 2C) decreased phosphorylation of FAK. In addition, results from the *in vivo* orthotopic study
226 (30) showed that p-FAK is decreased in tumors from mice bearing MDA-MB-231 cells and
227 treated with DIM-C-pPhCO_2Me (Fig. 2D). Fibronectin-induced cell adhesion is also a
228 prototypical β1-integrin-regulated response, and cell adhesion was significantly decreased in

MCF-7, MDA-MB-231 and SKBR3 after transfection with siNR4A1 (Fig. 2E) or after treatment with DIM-C-pPhOH or DIM-C-pPhCO$_2$Me (Fig. 2F). As a positive control, we showed that knockdown of β1-integrin (siβ1-integrin) by RNA also decreased cell adhesion (Fig. 2E) (see Suppl. Fig. S1).

Mechanisms of NR4A1-regulation of β1-integrin and β3-integrin

NR4A1 regulates gene expression through direct interactions with genomic NGFBα response elements (NBRE) and Nur response elements (NuRE) or by interactions with specificity protein 1 (Sp1) bound to GC-rich promoter elements (32, 33). NBRE and NuRE were not identified in the β1-integrin promoter, whereas two GC-rich sequences were located at -760 and -676 in the proximal region of the β1-integrin promoter (Fig. 3A). Previous studies show that NR4A1, Sp1 and the nuclear coregulatory gene p300 interact with the GC-rich region of the survivin promoter to regulate survivin gene expression (25). Using the more aggressive SKBR3 and MDA-MB-231 cells as models, cells were treated with dimethyl sulfoxide (DMSO), DIM-C-pPhOH or DIM-C-pPhCO$_2$Me and analyzed in a chromatin immunoprecipitation (ChIP) assay using primers targeted to the GC-rich region of the β1-integrin promoter. The results show that pol II, NR4A1, Sp1 and p300 interact with the GC-rich promoter regions and after treatment with DIM-C-pPhOH or DIM-C-pPhCO$_2$Me for 24 hr, the band for pol II was decreased in both cell lines (Fig. 3A) and this was consistent with decreased β1-integrin expression. Ligand-induced inactivation of NR4A1 also decreased NR4A1 binding to the promoter; however, changes in the Sp1 and p300 bands were somewhat variable and dependent on cell context and ligand. For example, the loss of p300 was observed in SKBR3 but not MDA-MB-231 cells, and it is possible that p300 may be interacting with the *trans*-acting factors in the proximal region of

the β1-integrin promoter. We further investigated the role of Sp1 and p300 in regulating β1-integrin expression in SKBR3 and MDA-MB-231 cells by RNAi, and knockdown of Sp1 (siSp1) and p300 (sip300) also decreased β1-integrin expression (Fig. 3B), suggesting that like survivin (25), NR4A1 regulates β1-integrin expression through a NR4A1/p300/Sp1 complex. P300 knockdown also decreases Sp1 expression, suggesting that p300 plays a role in regulating expression of this gene. These results do not exclude a role for other factors in NR4A1 regulation of β1-integrin and this is currently being investigated.

Previous reports show that inhibition of β1-integrin by RNAi or other β1-integrin inhibitors increases expression of β3-integrin resulting in enhanced metastasis (34-36). The β3-integrin promoter is also GC-rich (37) and therefore we investigated the possible regulation of β3-integrin by NR4A1. Western blot analysis showed that constitutive β3-integrin protein levels were barely detectable and remained low after treatment with C-DIM/NR4A1 antagonists or siNR4A1 (Fig. 3C), whereas knockdown of β1-integrin by RNAi increased β3-integrin protein as previously reported (36). There was a more robust expression of β3-integrin mRNA in MDA-MB-231 and SKBR3 cells and transfection of siNR4A1 or treatment with C-DIM/NR4A1 antagonists significantly decreased β3-integrin mRNA levels (Fig. 3D). ChIP assays showed that NR4A1, Sp1 and p300 bound the proximal GC-rich region of the β3-integrin gene and treatment with DIM-C-pPhOH or DIM-C-pPhCO_2Me decreased binding of pol II, NR4A1 and Sp1 but differentially affected p300 binding to the promoter. In addition, we also observed that knockdown of Sp1 (siSp1) or p300 (sip300) in MDA-MB-231 and SKBR3 cells decreased β3-integrin mRNA levels (Fig. 3E). These results demonstrate that NR4A1 regulates both β1- and β3-integrin expression and in contrast to β1-inegrin-specific inhibitors, NR4A1 antagonists downregulate expression of both β1- and β3-integrin.

Migration of MDA-MB-231 and SKBR3 cells: role of NR4A1 and β1-integrin

Both MDA-MB-231 and SKBR3 cells undergo migration (constitutive) in a Boyden chamber assay in the absence of a stimulus. Transfection of these cells with siNR4A1 (Fig. 4A) or siβ1-integrin (Fig. 4B) decreased migration of both cell lines and similar results were observed with two oligonucleotides targeting NR4A1 and β1-integrin. Treatment of SKBR3 and MDA-MB-231 cells with DIM-C-pPhOH (CDIM8) or DIM-C-pPhCO_2Me (CDIM14) also decreased migration (Fig. 4C), and the effects of DIM-C-pPhOH as an inhibitor of cell migration was not affected by cotreatment with leptomycin B (LMB), confirming that the inhibitory effects of this NR4A1 antagonist did not require nuclear export (25). We also investigated the role of NR4A1 in mediating DIM-C-pPhCO_2Me-dependent inhibition of migration of MDA-MB-231 and SKBR3 cells by knocking down NR4A1 and then treating with the NR4A1 antagonist DIM-C-pPhcO_2Me (Fig. 4E). Treatment of the NR4A1-depleted cells with DIM-C-pPhCO_2Me resulted in minimal inhibition of cell migration. Similar results were observed after treatment of β1-integrin-depleted cells with DIM-C-pPhCO_2Me and we also observed that DIM-C-pPhOH did not inhibit invasion in cells depleted of NR4A1 or β1-integrin (Suppl. Fig. S2). This would suggest that induction of β3-integrin after knockdown of β1-integrin (Fig. 3B) does not play a very significant role in cell migration using the Boyden chamber assay. Thus, inhibition of breast cancer cell migration by C-DIMs/NR4A1 antagonists is both NR4A1- and β1-integrin-dependent and consistent with regulation of β1-integrin by NR4A1. Overexpression of β1-integrin in SKBR3 and MDA-MB-231 cells slightly increases cell migration and in NR4A1-depleted cells which exhibit decreased migration, overexpression of β1-integrin significantly reverses this response (Fig. 4F). In addition, NR4A1 ligand-mediated inhibition of breast cancer

cell migration was also rescued by β1-integrin overexpression (Fig. 4G), further confirming that β1-integrin-mediated migration is NR4A1-dependent. Thus, the constitutive or basal migration of SKBR3 and MDA-MB-231 cells in the absence of endogenous stimuli is linked to nuclear NR4A1 regulation of β1-integrin.

TGFβ-induced migration of MDA-MB-231 cells: role of extranuclear NR4A1

A recent study reported that TGFβ-induced migration of MDA-MB-231 cells was also NR4A1-dependent and involved a pathway associated with SMAD7 degradation resulting in activation of TGFβR1 (31). Treatment of MDA-MB-231 cells with 5 ng/ml TGFβ significantly induced cell migration (Fig. 5A) as previously described (31), and knockdown of NR4A1 or treatment with DIM-C-pPhOH or DIM-C-pPhCO_2Me blocked TGFβ-induced migration and significantly decreased overall migration, similar to that observed after knockdown of NR4A1 or treatment with the NR4A1 antagonists alone (Figs. 4A-4C). TGFβ-induced migration was inhibited after cotreatment with the TGFβR1 inhibitor ALK5i and also the nuclear export inhibitor LMB and ALK5i had no effect on endogenous cell migration (data not shown). Analysis of cytosolic and nuclear extracts show that TGFβ induced expression and nuclear export of NR4A1 which was blocked by LMB (Fig. 5B) indicating that TGFβ-induced migration requires cytosolic NR4A1, whereas constitutive migration which is not inhibited by ALK5i is due to nuclear NR4A1-dependent regulation of β1-integrin. We also examined SMAD7 expression and observed minimal endogenous expression in MDA-MB-231 and SKBR3 cells and TGFβ increased SMAD7 only in SKBR3 cells (Fig. 5C). In contrast, cotreatment with TGFβ plus LMB, CDIM8 or CDIM14 dramatically increased SMAD7 protein expression of both cell lines, suggesting nuclear localization of NR4A1 inhibits degradation of SMAD7 which is a

cytosolic protein. These results are consistent with previous studies, suggesting that NR4A1 (cytosolic) plays a role in proteasome-dependent degradation of SMAD7 (31). Immunostaining of NR4A1 in MDA-MB-231 cells confirms that NR4A1 is nuclear, and treatment with TGFβ induces nuclear export of this receptor and this is blocked by LMB (Fig. 5C).

Since C-DIM/NR4A1 antagonists act through binding nuclear NR4A1, we examined the effects of short term (4 hr) treatment of MDA-MB-231 with DIM-C-pPhOH or DIM-C-pPhCO_2Me on TGFβ-induced migration. Like LMB, both compounds blocked TGFβ-induced migration (Fig. 6A) and this was accompanied by inhibition of TGFβ-induced nuclear export of NR4A1 (Fig. 6B) and paralleled results observed for LMB (Figs. 5A and 5B). The inhibitory effects observed after treatment with the C-DIM/NR4A1 ligands for 4 hr was not due to decreased β1-integrin expression (Fig. 6C), suggesting that bound NR4A1 was resistant to TGFβ-induced nuclear export and the factors that regulate nuclear export are currently being investigated. A previous report showed that TGFβ-induced NR4A1 interacts with axin 2 and other factors (e.g. E3 ligases Arkadia and RNF12) to form a polyubiquitination complex (31), and after treatment of MDA-MB-231 cells with TGFβ, LMB, C-DIMs and their combinations, western blot analysis of the cytosolic fraction immunoprecipitated with axin 2 antibodies gave a strong band for NR4A1 only in cells treated with TGFβ alone (Fig. 6D). In contrast, treatment with DIM-C-pPhOH, DIM-C-pPhCO_2Me or LMB which inhibit TGFβ-induced nuclear export of NR4A1 resulted in decreased intensities of cytosolic NR4A1 bands associated with the axin 2 antibody immunoprecipitates. The results demonstrate that NR4A1plays an important role in breast cancer cell migration by regulation of β1-integrin (endogenous activity) and TGFβ-induced migration which is dependent on NR4A1 nuclear export (Fig. 6E).

344 **Discussion**

345 The NR4A family of orphan nuclear receptors NR4A1, NR4A2 and NR4A3 were
346 initially identified as stress-induced immediate early genes with a characteristic domain structure
347 observed for nuclear receptors. NR4A receptors have both unique and overlapping functions and
348 there is increasing evidence that they play an important role in cellular homeostasis and diseases
349 associated with metabolism, cardiovascular and neurological functions, inflammation and the
350 immune system (38-40). Endogenous ligands for NR4A1 have not been identified; however,
351 synthetic ligands that are structurally related to cytosporone B have been developed (41-43) and
352 have potential clinical applications. For example, ethyl[2,3,4-trimethoxy-6-(*i*-
353 octanoyl)phenyl]acetate is an NR4A1 ligand that acts as a receptor antagonist to decrease
354 NR4A1-dependent hepatic gluconeogenesis and lower blood glucose levels in a rodent model for
355 type 2 diabetes (43). NR4A1 is also overexpressed in solid tumors including both ER-positive
356 and ER-negative breast tumors and is a negative prognostic factor for lung, colon, breast cancer
357 patients (26, 31, 44).

358 Initial studies targeting NR4A1 for cancer chemotherapy showed that cell death observed
359 in some cancer cell lines treated with several apoptosis agents was due to nuclear export of
360 NR4A1 and the subsequent interactions of NR4A1 with bcl-2 to form a pro-apoptotic complex
361 that disrupted mitochondria (45, 46). The pro-apoptotic effects were also observed using
362 peptides and paclitaxel that mimic NR4A1 interactions with bcl-2 (42, 47). Studies in this
363 laboratory have identified C-DIMs as NR4A1 ligands that act as antagonists in cancer cell lines,
364 and previous studies have demonstrated that C-DIM/NR4A1 antagonists inhibit growth and
365 induce cell death through inactivation of nuclear NR4A1-dependent pro-oncogenic pathways in
366 pancreatic, lung, colon, kidney and breast cancer cells lines (24-30).

A recent report showed that high expression of NR4A1 in breast tumors correlated with decreased relapse-free survival and this was linked to the role of NR4A1 in TGFβ and TGFβ/cytokine-induced migration/invasion and metastasis (31). Results of ongoing genomic and functional studies in several cancer cell lines identified *β-integrin* as a possible NR4A1-regulated pro-migration/invasion gene and this correlated with previous *in vivo* studies showing that β1-integrin was important for metastasis of mammary tumors overexpressing the *erbB2* oncogene (9, 21-23). Results in Figures 1, 2, and 4 demonstrate that knockdown of NR4A1 or treatment with the NR4A1 antagonists DIM-C-pPhOH and DIM-C-pPhCO_2Me decreased expression of β1-integrin protein and mRNA and β1-integrin-dependent responses in MCF7, MDA-MB-231 and SKBR3 cells and also inhibited migration of the latter two cell lines.

The mechanism of NR4A1 regulation of β-integrin in SKBR3 and MDA-MB-231 cells did not involve direct binding to *cis*-acting genomic sequences but through an indirect mechanism in which NR4A1/p300 act as a coregulatory complex to activate Sp1-regulated genes. The ChIP assays show that NR4A1, Sp1 and p300 interacted at the GC-rich region of the *β1-integrin* gene promoter (Fig. 3), and knockdown of anyone of these factors or treatment with C-DIM/NR4A1 antagonists resulted in decreased β1-integrin expression. These results are similar to that previously observed for NR4A1/p300/Sp1-mediated regulation of survivin in pancreatic cancer cells (25) and are consistent with other reports showing that other nuclear receptors also regulate expression of other Sp-dependent genes through NR4A1/Sp1 complexes (48-50). Previous studies show that knockdown or inhibition of β1-integrin in breast cancer cells results in the expression of β3-integrin and this "integrin-switching" enhances TGFβ-induced metastasis (34-36) and this presents a problem for applications of β1-integrin inhibitors in treatment of breast cancer. Like β1-integrin, the 5'-promoter region of the β3-integrin gene

390 contains GC-rich sequences (37) and our results demonstrates that NR4A1 also regulates β3-
391 integrin expression, and NR4A1 antagonists or NR4A1 knockdown decreases expression of both
392 genes (Fig. 3). Thus, coregulation of β1- and β3-integrin by NR4A1 negates the "integrin-
393 switching" phenomena (34-36) and further demonstrates that the C-DIM/NR4A1 antagonists
394 represent a novel therapeutic approach for inhibiting β1/β3-integrin-induced signaling and
395 metastasis in breast cancer cells.

396 MDA-MB-231 and SKBR3 cells readily migrate in the absence of TGFβ or cytokine
397 stimulus, and results of RNAi studies show that inhibition of cell migration by C-DIM/NR4A1
398 antagonists was observed only in cells expressing NR4A1 or β1-integrin (Fig. 4). Moreover,
399 since the inhibitory effects of C-DIMs were similar in the presence or absence of the nuclear
400 export inhibitor LMB (Fig. 4F), our results indicate that constitutive migration of these cells was
401 due to nuclear NR4A1-dependent regulation of β1-integrin. This is also supported by the
402 observation that the TGFβ receptor inhibitor ALK5i inhibits TGFβ-induced migration but does
403 not affect the high rate of constitutive migration of MDA-MB-231 cells (Fig. 5A). A recent
404 study showed that NR4A1 was also required for TGFβ-induced migration of MDA-MB-231 and
405 other cell lines and this was due to interactions of NR4A1, axin 2 and E3 ligases which enhanced
406 SMAD7 degradation, resulting in activation of the TGFβR1 pathway (31). We also observed
407 that TGFβ induced NR4A1 expression and migration of MDA-MB-231 cells; however, the key
408 essential element in this pathway was that TGFβ induced nuclear export of NR4A1 (Figs. 5B,
409 5D and 6C). Moreover, inhibition of nuclear export by the NR4A1 ligands (DIM-C-pPhOH or
410 DIM-C-pPhcO_2Me) or LMB also blocked TGFβ-induced migration and enhanced SMAD7
411 expression (Fig. 5C). Previous studies on SMAD7 degradation in MDA-MB-231 cells used
412 transfected FLAG-SMAD7 (31), whereas in this study, we observed low to non-detectable

SMAD7 expression in MDA-MBA-231 and SKBR3 cells. However, LMB, DIM-C-pPhOH and DIM-C-pPhCO_2Me which prevent NR4A1 export also increased SMAD7 expression in cells cotreated with these compounds plus TGFβ (Fig. 5C) and this is consistent with a role for cytosolic NR4A1 in SMAD7 degradation as previously reported (31). Thus, TGFβ-induced migration of MDA-MB-231 cells is due to nuclear export of NR4A1 and the C-DIM/NR4A1 antagonist block this pathway presumably by inhibiting factors/pathways required for nuclear export and these are currently being investigated.

In summary, results of this study show that nuclear NR4A1 regulates β1-integrin expression in breast cancer cells, and C-DIM/NR4A1 antagonists inhibit expression of β1-integrin and β1-integrin-mediated responses including cell migration and the antagonists also inhibit NR4A1-regulated expression of β3-integrin. In contrast, TGFβ-induced migration of MDA-MB-231 cells requires nuclear export of NR4A1 which is inhibited not only by LMB but also by C-DIM/NR4A1 antagonists. Thus, constitutive and TGFβ-induced migration are dependent on nuclear and extranuclear NR4A1, respectively, and the C-DIM/NR4A1 antagonists inhibit both pathways by decreasing NR4A1-dependent expression of β1-integrin and by inhibition of TGFβ-induced nuclear export of NR4A1 (Fig. 6E). This study expands on the pro-oncogenic functions of NR4A1 and indicates that C-DIM compounds and other NR4A1 antagonists represent an important new class of mechanism-based anticancer drugs for treating patients with tumors overexpressing this receptor.

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Figures

Fig. 1. NR4A1 regulates β1-integrin expression in breast cancer cells and tumors. (**A**) Breast cancer cells were transfected with siNR4A1 and cell extracts were analyzed for protein and mRNA expression by western blots or real time PCR, respectively, as outlined in the Materials and Methods. Breast cancer cells were treated with DMSO, DIM-C-pPhOH or DIM-C-pPhCO$_2$Me for 24 hr and extracts were analyzed for protein (**B**) or mRNA (**C**) levels by western blots and real time PCR, respectively, as outlined in the Materials and Methods. (**D**) Cell lysates from tumors (MDA-MB-231 orthotopic) (30) derived from animals treated with corn oil (control) or DIM-C-pPhCO$_2$Me (C-DIM-14; 40 mg/kg/d) were analyzed by western blots and decreased protein expression was determined and normalized the β-actin protein loading control. Quantified data are presented as means ± SE (at least 3 replicates) and significant (P<0.05) decreases are indicated (*).

Fig. 2. NR4A1 regulates β1-integrin-dependent responses. Breast cancer cells were transfected with siNR4A1 (**A**), treated with DMSO and DIM-C-pPhOH (**B**) or DIM-C-pPhCO$_2$Me (**C**) for 24 hr, and whole cell lysates were analyzed by western blots as outlined in the Materials and Methods. (**D**) Tumor lysates from mice (MDA-MB-231 orthotopic-derived (30) treated with corn oil or DIM-C-pPhCO$_2$Me (40 mg/kg/d) were analyzed by western blots and quantitated as outlined in Figure 1D. The effects of siNR4A1 and siβ1-integrin (**E**) or DIM-C-pPhOH and DIM-C-pPhCO$_2$Me (**F**) on fibronectin-induced adhesion of breast cancer cells was determined as outlined in the Materials and Methods. Results (D-F) are means ± SE (at least 3 replicates) and a significant (P<0.05) decrease indicated (*). Western blots in Figures 1 and 2 were derived from the same experiment showing effects on β1-integrin (Fig. 1) and β1-integrin-regulated responses (Fig. 2).

620 **Fig. 3.** Role of NR4A1/p300/Sp1 in regulation of β1- and β3-integrin. (**A**) Analysis of polII,
621 NR4A1, Sp1 and p300 binding to the β1-integrin promoter was determined in a ChIP
622 assay using primers as indicated. (**B**) Cells were treated with oligonucleotides that
623 knockdown Sp1 (siSp1) and p300 (sip300), and whole cell lysates were analyzed by
624 western blots as outlined in the Materials and Methods. (**C**) Cells were transfected with
625 siNR4A1 or treated with DIM-C-pPhOH (C-DIM8) or DIM-C-pPhCO_2Me (C-DIM14),
626 and whole cell lysates were analyzed by western blots as outlined in the Materials and
627 Methods. (**D**) Cells were transfected with siNR4A1 or treated with C-DIM8 or C-DIM14
628 and effects on β3-integrin (ITGB3) mRNA levels were determined. The treatments
629 significantly (P<0.05) decrease mRNA levels. (**E**) Analysis of polII, NR4A1, Sp1 and
630 p300 binding to the proximal GC-rich region of the β3-integrin promoter was determined
631 in a ChIP assay as outlined in the Materials and Methods. (**F**) Cells were transfected with
632 siSp1 and sip300 and analyzed by real time PCR for β3-integrin mRNA levels. Both
633 oligonucleotides significantly (P<0.05) decreased β3-integrin mRNA levels.
634 **Fig. 4.** NR4A1-regulates β1-integrin-dependent breast cancer cell migration. Cells were
635 transfected with siNR4A1 (**A**), siβ1-integrin (**B**), or treated with DIM-C-pPhOH and
636 DIM-C-pPhCO_2Me (**C**), and DIM-C-pPhOH ± LMB (**D**), and breast cancer cell
637 migration was determined in a Boyden chamber assay as outlined in the Materials and
638 Methods. (**E**) Cells were transfected with a non-specific oligonucleotide (siCtl),
639 siNR4A1, siβ1-integrin and treated with DIM-C-pPhCO_2Me, and cell migration was
640 determined in a Boyden chamber assay as outlined in the Materials and Methods. Cells
641 were transfected with siNR4A1 alone (**F**) or treated with DIM-C-pPhOH/DIM-C-
642 pPhCO_2Me (**G**) in combination with β1-integrin (ITGB1) expression plasmid, and effects

643 on cell migration were determined in a Boyden chamber assay as outlined in the
644 Materials and Methods. Results are expressed as means ± SE for at least 3 replicates for
645 each treatment group and significantly (P<0.05) decreased migration (*) or rescue by β1-
646 integrin overexpression (**) are indicated.

647 **Fig. 5.** Role of NR4A1 on TGFβ-induced migration of MDA-MB-231 cells. (**A**) MDA-MB-231
648 cells were treated with TGFβ alone for 5 hr or in combination with siNR4A1, DIM-C-
649 pPhOH and DIM-C-pPhCO_2Me (24 hr treatment), LMB and ALK5i, and LMB (alone).
650 Cell migration was determined in a Boyden chamber assay. (**B**) MDA-MB-231 cells
651 were treated with DMSO, TGFβ and LMB (alone) and in combination for 5 hr. Nuclear
652 and cytosolic extracts were analyzed by western blots using nuclear (p84) and cytosolic
653 (GADPH) loading controls. (**C**) Cells were treated with DMSO, TGFβ, LMB alone and
654 TGFβ in combination with LMB, DIM-C-pPhOH (CDIM8) or DIM-C-pPhCO_2Me
655 (CDIM14) for ___ hr, and whole cell lysates were analyzed for SMAD7 expression by
656 western blot analysis. (**D**) Cells were treated with DMSO, 5 ng/ml TGFβ, LMB and
657 LMB plus TGFβ for 5 hr and immunostained with both NR4A1 antibodies and DAPI as
658 outlined in the Materials and Methods.

659 **Fig. 6.** (**A**) MDA-MB-231 cells were treated with TGFβ, DIM-C-pPhOH and DIM-C-
660 pPhCO_2Me alone and TGFβ plus C-DIMs for 4 hr, and cell migration was determined in
661 a Boyden Chamber assay and immunostaining (NR4A1) and DAPI staining was
662 determined as outlined in Figure 5C. (**B**) Cells were treated as described in (Fig. 5B) and
663 the cytosolic and nuclear extracts were further examined by western blot analyses. (**C**)
664 MDA-MB-231 cells were treated with DIM-C-pPhCO_2Me or DIM-C-pPhOH for
665 different times and whole cell lysates were analyzed by western blots for β1-integrin

expression. (**D**) Cells were treated as outlined in Figure 6B and whole cell lysates were immunoprecipitated with axin 2 antibodies and analyzed by western blots. (**E**) Schematic outline of the role of NR4A1 in constitutive and TGFβ-induced migration in breast cancer cells.

Figure 1

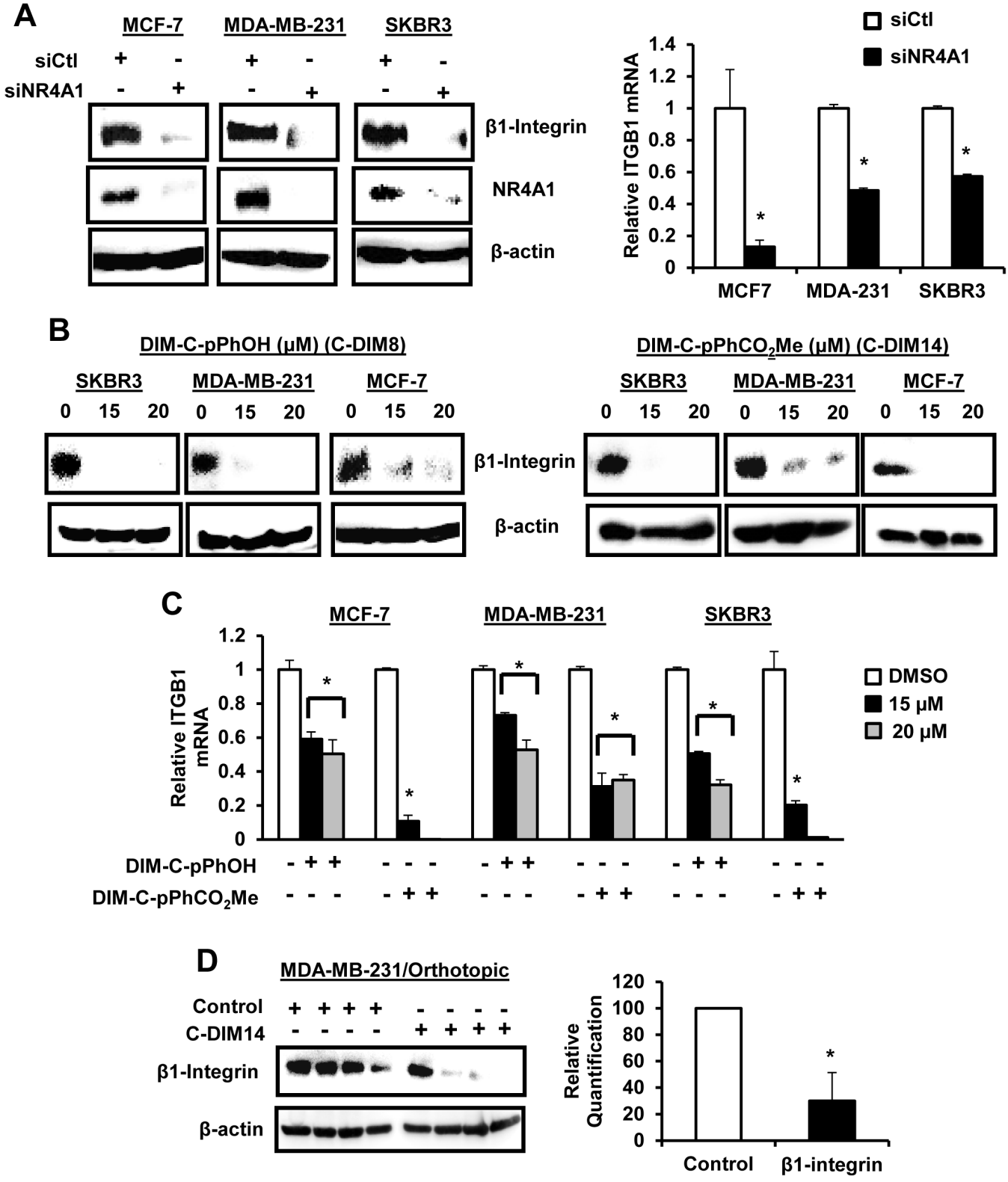


Figure 2

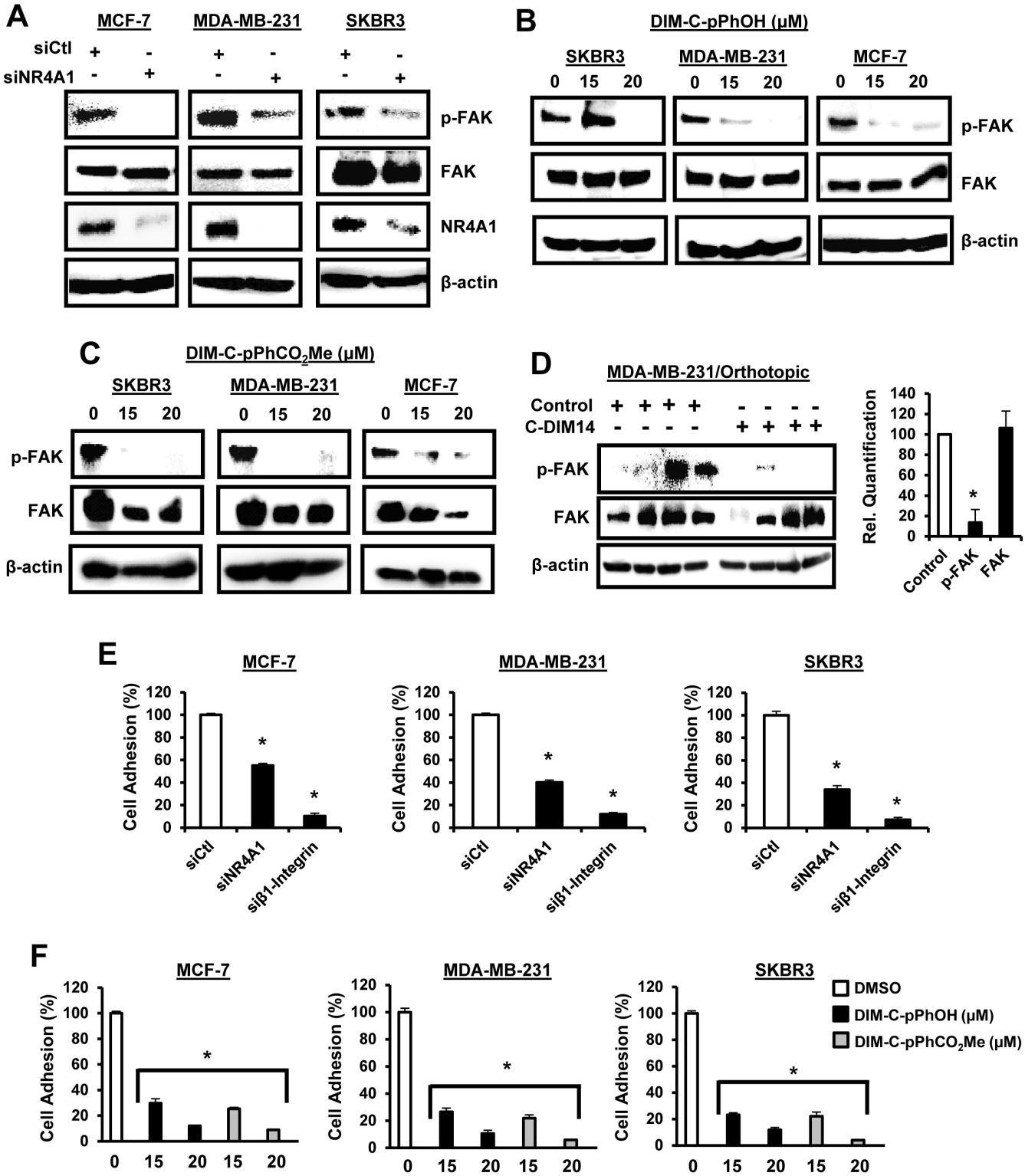


Figure 3

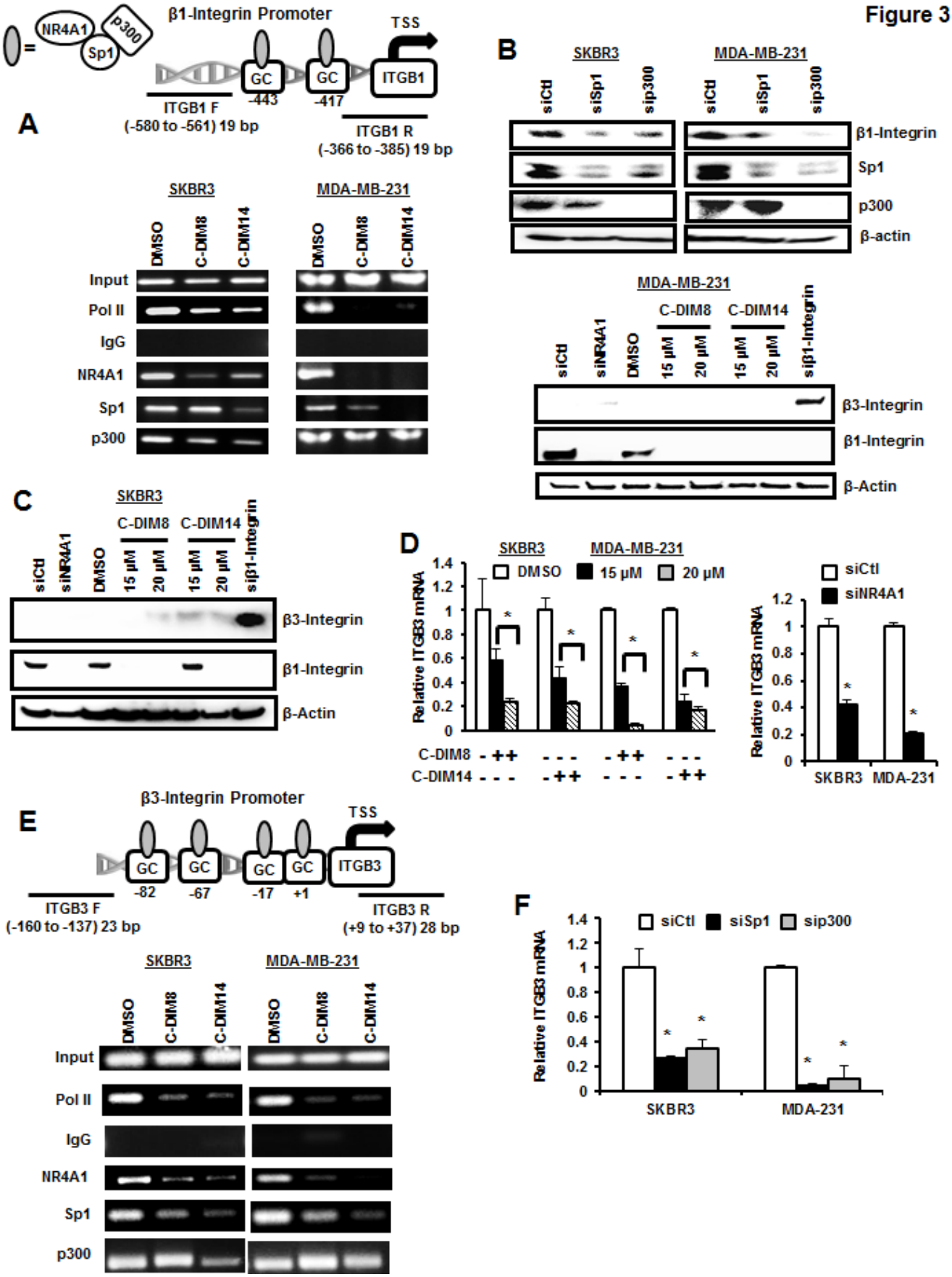


Figure 4

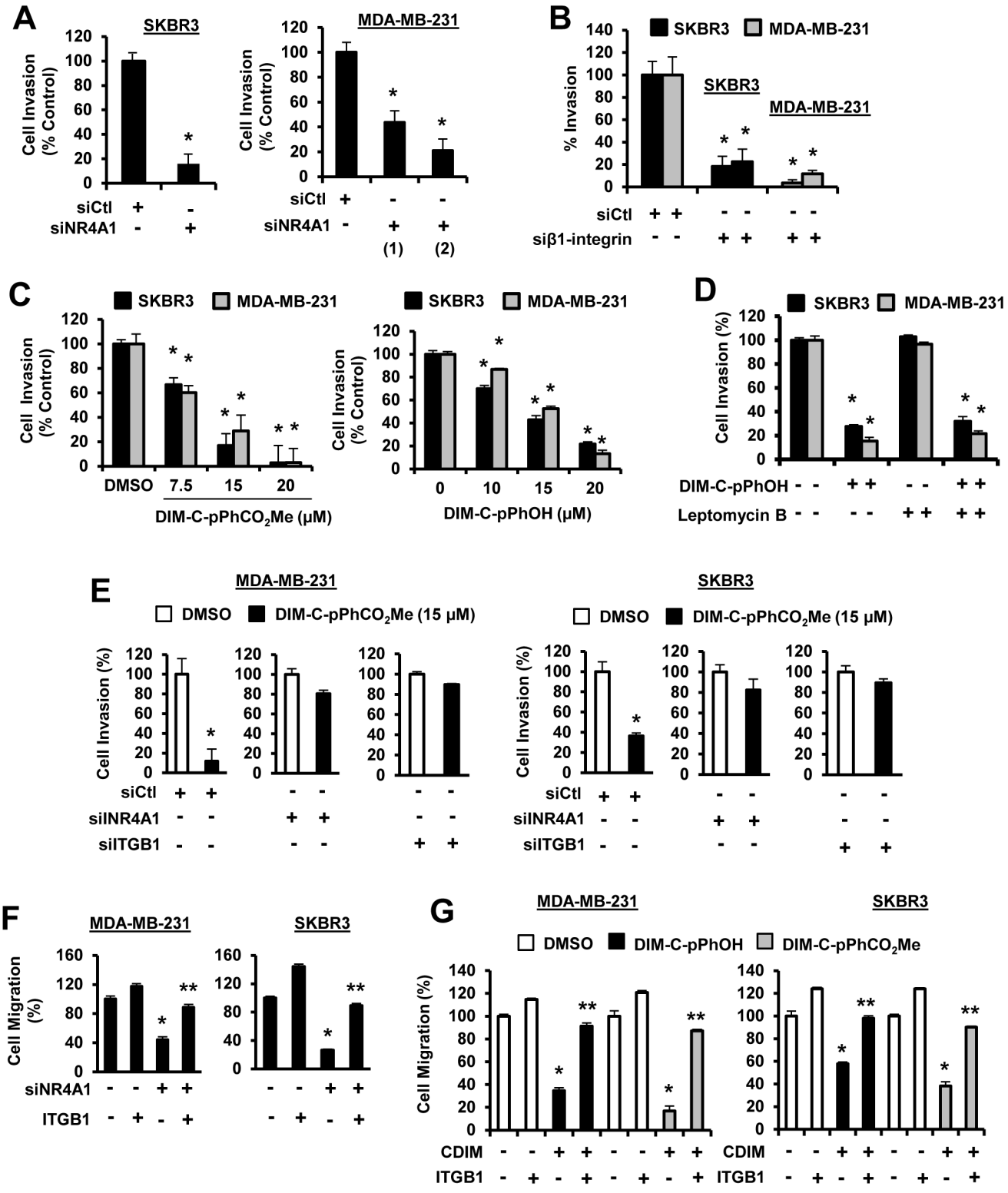


Figure 5

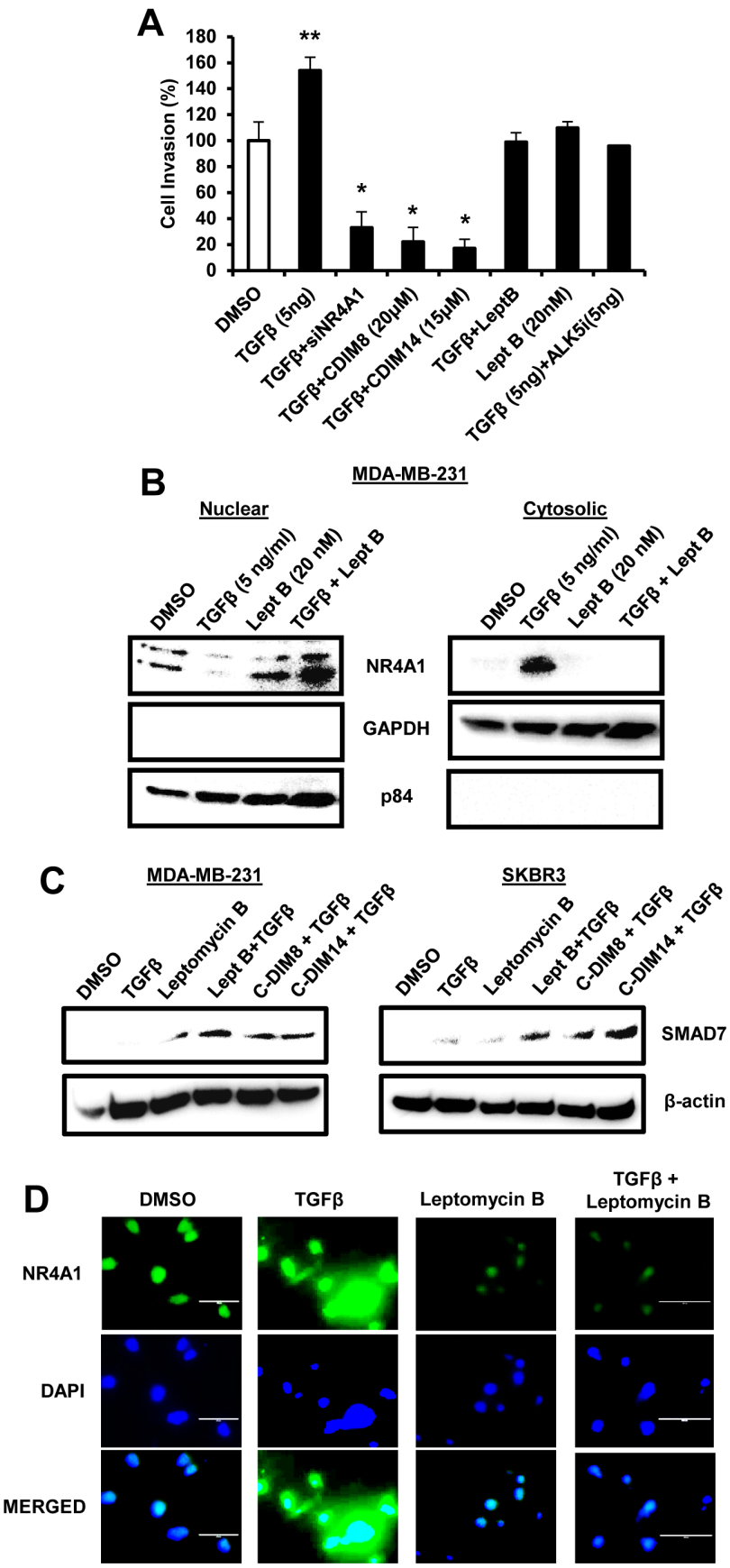
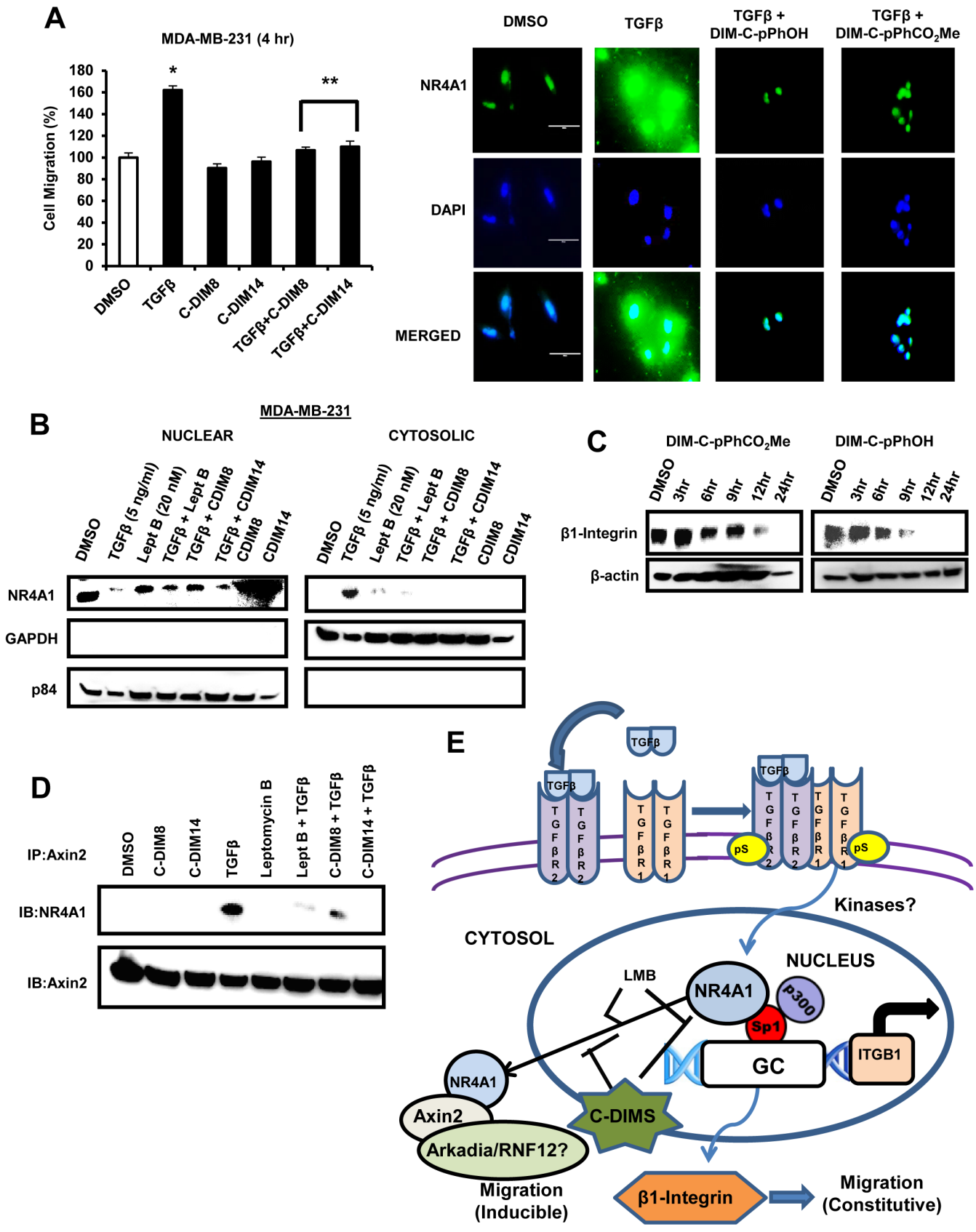


Figure 6



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AUTHOR CORRECTION

Correction for Hedrick et al., "NR4A1 Antagonists Inhibit β1-Integrin-Dependent Breast Cancer Cell Migration"

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Volume 36, no. 9, p. 1383–1394, 2016, https://doi.org/10.1128/MCB.00912-15. Page 1385, Fig. 1: The β-actin controls for the MCF-7 cell lysate analysis for panels A and B were run on the same gel. We inadvertently used in panel A the last two β-actin lanes of panel B, left. The corrected MCF-7 β-actin bands for panel A should appear as shown below.

Page 1386, Fig. 2: The same lysates and β-actin bands from Fig. 1 were used in panels A and B, with the same problem. Therefore, the corrected MCF-7 β-actin bands for panel A should appear as shown above.

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