

Thiazolidinediones Mimic Glucose Starvation in Facilitating Sp1 Degradation through the Up-Regulation of β -Transducin Repeat-Containing Protein

Shuo Wei, Hsiao-Ching Chuang, Wan-Chi Tsai, Hsiao-Ching Yang, Shiuh-Rong Ho, Andrew J. Paterson, Samuel K. Kulp, and Ching-Shih Chen

Division of Medicinal Chemistry, College of Pharmacy, the Ohio State University, Columbus, Ohio (S.W., H.C.C., S.K.K., C.S.C.); School of Medical Laboratory and Biotechnology, Chung Shan Medical University, Taichung, Taiwan (W.C.T.); Department of Chemistry, Fu-Jen Catholic University, Taipei, Taiwan (H.C.Y.); and Department of Cell Biology (S.R.H.) and Division of Endocrinology, Diabetes and Metabolism, Department of Medicine (A.J.P.), University of Alabama at Birmingham, Birmingham, Alabama

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ABSTRACT

This study investigated the mechanism by which the transcription factor Sp1 is degraded in prostate cancer cells. We recently developed a thiazolidinedione derivative, (Z)-5-(4-hydroxy-3-trifluoromethylbenzylidene)-3-(1-methylcyclohexyl)-thiazolidine-2,4-dione (OSU-CG12), that induces Sp1 degradation in a manner paralleling that of glucose starvation. Based on our finding that thiazolidinediones suppress β -catenin and cyclin D1 by up-regulating the E3 ligase SCF $^{\beta$ -TrCP, we hypothesized that β -transducin repeat-containing protein (β -TrCP) targets Sp1 for proteasomal degradation in response to glucose starvation or OSU-CG12. Here we show that either treatment of LNCaP cells increased specific binding of Sp1 with β -TrCP. This direct binding was confirmed by in vitro pull-down analysis with bacterially expressed β -TrCP. Although ectopic expression of β -TrCP enhanced the ability of OSU-CG12 to

facilitate Sp1 degradation, suppression of endogenous β -TrCP function by a dominant-negative mutant or small interfering RNA-mediated knockdown blocked OSU-CG12-facilitated Sp1 ubiquitination and/or degradation. Sp1 contains a C-terminal conventional DSG destruction box (⁷²⁷DSGAGS⁷³²) that mediates β -TrCP recognition and encompasses a glycogen synthase kinase 3 β (GSK3 β) phosphorylation motif (SXXXS). Pharmacological and molecular genetic approaches and mutational analyses indicate that extracellular signal-regulated kinase-mediated phosphorylation of Thr739 and GSK3 β -mediated phosphorylation of Ser728 and Ser732 were critical for Sp1 degradation. The ability of OSU-CG12 to mimic glucose starvation to activate β -TrCP-mediated Sp1 degradation has translational potential to foster novel strategies for cancer therapy.

In addition to maintaining the basal transcription of housekeeping genes, increasing evidence indicates that the

transcription factor Sp1 also plays an important role in regulating the expression of a host of key effectors of signaling pathways governing cell cycle progression, cell proliferation, angiogenesis, apoptosis, and metastasis (Wierstra, 2008). These target proteins include receptor tyrosine kinases and their growth factor ligands, cyclin-dependent kinase inhibitors, c-Myc, Mdm2, Mcl-1, survivin, XIAP, Fas ligand, PUMA, and death receptor 5 (Wierstra, 2008). Moreover, Sp1

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ABBREVIATIONS: PPAR γ , peroxisome proliferator-activated receptor- γ ; AR, androgen receptor; β -TrCP, β -transducin repeat-containing protein; ER, estrogen receptor; ERK, extracellular signal-regulated kinase; GSK3 β , glycogen synthase kinase 3 β ; GST, glutathione transferase; HA, hemagglutinin; JNK, Jun NH₂-terminal kinase; MEK, mitogen-activated protein kinase kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WT, wild-type; Δ F- β -TrCP, F-box-deleted β -transducin repeat-containing protein; IP, immunoprecipitation; IB, immunoblotting; HA-UB, hemagglutinin-ubiquitin; Hsp90, heat shock protein 90; MAP, mitogen-activated protein; STE buffer, Tris/NaCl/EDTA/dithiothreitol/phenylmethylsulfonyl fluoride; M-PER, mammalian protein extraction reagent; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; IKK α , I κ B kinase α ; siRNA, small interfering RNA; FBS, fetal bovine serum; MG132, N-benzoyloxycarbonyl (Z)-Leu-Leu-leucinal; PD98059, 2'-amino-3'-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; SB216763, 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione; SP600125, anthra[1,9-c,d]pyrazol-6(2H)-one; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)1H-imidazole; OSU-CG12, (Z)-5-(4-hydroxy-3-trifluoromethylbenzylidene)-3-(1-methylcyclohexyl)thiazolidine-2,4-dione.

interacts with a diversity of transcription factors, oncogenes, and tumor suppressors as part of the mechanism regulating its transcriptional activity, which underscores the complexity of its biological activities and its implications for tumorigenesis. Consequently, Sp1 overexpression has been linked to tumor progression and poor prognosis in many human cancers, including those of stomach, liver, thyroid, and pancreas (Kitadai et al., 1992; Liétard et al., 1997; Shi et al., 2001; Chiefari et al., 2002; Wang et al., 2003; Jiang et al., 2004, 2008; Yao et al., 2004). In addition, siRNA-mediated Sp1 knockdown decreased tumor growth and/or metastasis of gastric and pancreatic cancers in animal model studies (Jiang et al., 2004; Yuan et al., 2007). Together, these findings underscore the translational value of targeting dysregulated Sp1 expression in cancer therapy. However, despite advances in understanding Sp1's biological functions, the mechanism that controls the turnover of this transcriptional factor remains unclear.

Under physiological conditions, metabolic stress, such as glucose starvation, or stimulation with cAMP promotes the degradation of Sp1 through a proteasome-dependent pathway (Han and Kudlow, 1997; Su et al., 1999, 2000). We recently demonstrated that members of the thiazolidinedione family of peroxisome proliferator-activated receptor- γ (PPAR γ) agonists were able to promote proteasomal degradation of Sp1 in prostate cancer cells, leading to PPAR γ -independent transcriptional repression of androgen receptor (AR) (Yang et al., 2007). This mechanistic finding provides a molecular basis for the pharmacological exploitation of thiazolidinediones to develop potent Sp1-targeted agents, among which the ciglitazone-derived analog OSU-CG12 represents an optimal agent (Yang et al., 2008). Although devoid of PPAR γ activity, OSU-CG12 facilitated Sp1 degradation at low micromolar concentrations in a manner qualitatively similar to that of glucose starvation. In light of the therapeutic potential of targeting Sp1 in cancer therapy, we embarked on characterizing the mechanism by which OSU-CG12 and glucose starvation facilitate the proteasomal degradation of Sp1. It is noteworthy that OSU-CG12 and related thiazolidinediones promote the degradation of β -catenin, cyclin D1, and other cell-cycle regulatory proteins in LNCaP cells by up-regulating the expression of β -transducin repeat-containing protein (β -TrCP), an F-box component of the Skp1-Cul1-F-box protein E3 ubiquitin ligase (Wei et al., 2007, 2008). In this study, we obtained several lines of evidence to support the mechanistic link between β -TrCP and Sp1 degradation in response to OSU-CG12 and glucose deprivation. Furthermore, mutational and modeling analyses indicate that β -TrCP recognizes Sp1 through its ⁷²⁷DpSGAGpS⁷³² motif, in which extracellular signal-regulated kinases (ERKs) and GSK3 β play an obligatory role.

Materials and Methods

Cell Line, Culture, and Reagents. LNCaP androgen-responsive and PC-3 androgen-unresponsive prostate cancer cells and MCF-7 estrogen receptor-positive breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA) and were maintained in 10% fetal bovine serum (FBS)-supplemented RPMI 1640 or F-12/Dulbecco's modified Eagle's medium at 37°C in a humidified incubator containing 5% CO₂. In experiments assessing the effects of glucose deprivation, cells were cultured in glucose-free RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% FBS. Ciglitazone

and its PPAR γ -inactive derivative Δ 2CG and OSU-CG12 were synthesized according to a published procedure (Yang et al., 2008). The following pharmacological agents were purchased from various sources: MG132, PD98059, U0126, lithium chloride, SB216763, and SP600125 were from Sigma-Aldrich (St. Louis, MO); and PD169316 and cycloheximide were from Calbiochem (San Diego, CA). Stock solutions of these agents were made in DMSO and added to medium with a final DMSO concentration of 0.1%. Antibodies against various proteins were obtained from the following sources: mouse monoclonal antibodies: β -catenin, Wee1, and p53 were from Santa Cruz Biotechnology (Santa Cruz, CA); hemagglutinin (HA) and Myc were from Roche (Indianapolis, IN); β -TrCP and Skp2 were from Invitrogen; Flag was from Sigma; β -actin was from MP Biomedicals (Irvine, CA); rabbit antibodies: Sp1, AR, estrogen receptor (ER) α , p-Ser9-GSK3 β , GSK3 β , p-Ser473-Akt, Akt, p-Thr202/Tyr204-ERK, ERK, p-Thr183/Tyr185-Jun NH2-terminal kinase (JNK), JNK, p-Ser63-c-Jun, c-Jun, p-Thr180/Tyr182-p38 and p38, p-Thr334-MAPKAPK-2, MAPKAPK-2, c-Raf, I κ B kinase α (IKK α), and Stat3 were from Cell Signaling Technology (Danvers, MA); I κ B α was from Santa Cruz Biotechnology; glutathione transferase (GST) was from Sigma; and Fbx4 was from Rockland (Gilbertsville, PA). The HA-tagged GSK3 β K85A plasmid was purchased from Addgene (Cambridge, MA). Dominant-negative (kinase-defective) mitogen-activated protein kinase kinase (MEK) 1 (K97A) and constitutively active MEK1 (S218/222D) subcloned into pCMVHA were prepared as described previously (Slack et al., 1999).

Plasmid Construction and Site-Directed Mutagenesis. To achieve the expression of Flag-tagged wild-type (WT) Sp1 protein, the pCMVSp1 plasmid (Yang et al., 2007) encoding full-length Sp1 was subcloned into EcoRI/XbaI sites of the p3XFLAG-CMV26 expression vector. Plasmids encoding various Sp1 mutations were generated from pWT Sp1-Flag by site-directed mutagenesis using the QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). Primers used to generate Sp1 mutations were as follows: S728A, 5'-GTGGGCACTTTGCCCTGGACGCTGGGGCAGGT-3' and 5'-ACCTGCCAGCGTCCAGGGGCAAAGTGCCAC-3'; S732A, 5'-CTGGACAGTGGGGCAGGTGCAGAAGGCAGTGGCACTGCC-3' and 5'-GGCAGTGCCACTGCCTTCTGCACCTGCCCACTGTCCAG-3'; and T739A, 5'-AGGCAGTGGCACTGCCGCTCCTCAGCCCTTATTA-3' and 5'-TAATAAGGGCTGAAGGAGCGGCAGTGGCCACTGCCT-3'.

To generate the construct for expression of F-box (residues 180–226)-deleted β -TrCP (Δ F- β -TrCP), the DNA sequence encoding amino acid residues 1 to 179 of β -TrCP was PCR-amplified from the β -TrCP-Myc plasmid (Wei et al., 2007) with the primers 5'-CCGATATAAGCTTATGGACCCGCGCAG-3' (forward) and 5'-GCGCGGAAGCTTATCTCTCTGCAACAT-3' (reverse), which were flanked by HindIII restriction sites. The sequence encoding β -TrCP amino residues 227 to 605 was obtained by digestion of β -TrCP-Myc plasmid with HindIII/XbaI. The resulting fragments were incubated with those corresponding to residues 1 to 179 followed by the ligation into the pMyc4-CMV14 expression vector (Yang et al., 2005) to generate the Δ F- β -TrCP-Myc plasmid. All constructs were verified by DNA sequencing.

RNA Isolation and Semiquantitative PCR Analysis. Total RNA was isolated from drug-treated LNCaP cells using the RNeasy mini kit (Qiagen, Valencia, CA) and then reverse-transcribed to cDNA using the Omniscript RT Kit (Qiagen) according to the manufacturer's instructions. Primers used for PCR were as follows: Sp1, 5'-GGCGAGAGGCCATTATGTGT-3' and 5'-AGTGGCATCAACGTCATGCA-3'; AR, 5'-ACACATTGAAGGCTATGAATGTC-3' and 5'-TCACTGGGTGTGGAAATAGATGGG-3'; ER α , 5'-ACTGCATCAGATCCAAGGGAACG-3' and 5'-GGCAGCTCATGTCTCAGCAGA-3'; and β -actin, 5'-TCTACAATGAGCTGCGTGTG-3' and 5'-GGTCAGGATCTTCATGAGGT-3'. PCR products were separated electrophoretically in 1% agarose gels and visualized by ethidium bromide staining.

Transient Transfection, RNA Interference, and Luciferase Assay. Cells were transfected with various plasmids using Nucleo-

factor kit R of the Amaxa Nucleofector system (Amaxa Biosystems, Gaithersburg, MD) according to the manufacturer's instructions. Cells were then seeded into six-well plates (5×10^5 cells/well) and incubated in 10% FBS-containing medium for 24 h before drug treatment. Transfection efficiency was >75% as determined by co-transfection with pmaxGFP plasmids and visualization of green fluorescent protein-positive cells by fluorescence microscopy. For siRNA experiments, cells were nucleofected with scrambled or β-TrCP siRNA (Santa Cruz Biotechnology) and seeded into six-well plates (5×10^5 cells/well) for drug treatments and subsequent analyses. For the luciferase assay, LNCaP cells expressing AR promoter-luciferase and herpes simplex virus thymidine kinase promoter-*Renilla reniformis* luciferase were prepared as we described previously (Yang et al., 2007) and transfected with WT or mutant forms of Sp1. Cells were cultured in 24-well plates (1×10^5 cells/well) in 10% FBS-containing RPMI 1640 medium for 24 h and treated with OSU-CG12 for the indicated intervals. Reporter gene assays were then performed as we reported previously (Yang et al., 2007).

Immunoprecipitation and Immunoblotting. Ectopically expressed and endogenous proteins were immunoprecipitated from cell lysates after various treatments. These proteins included Flag-tagged WT and mutant Sp1, endogenous Sp1, and β-TrCP-Myc. Immunoprecipitation with anti-Flag, anti-Myc, and anti-Sp1 antibodies followed by immunoblotting for various target proteins were performed as described previously (Wei et al., 2007).

In Vivo Ubiquitination Assay. Cells were transfected with the expression vector for HA-tagged ubiquitin in combination with the plasmid for WT or mutant (S732A) Flag-tagged Sp1, the plasmid for WT or ΔF-β-TrCP-Myc, or scrambled or β-TrCP siRNA. After nucleofection with plasmids or siRNA, cells were cultured in six-well plates for 24 h and then treated with 5 μM OSU-CG12 for 12 or 36 h, followed by 12-h cotreatment with the proteasome inhibitor MG132. Cells were harvested into M-PER buffer containing 1% protease inhibitor cocktail and centrifuged at 13,000g for 20 min. The supernatants were collected, preincubated with protein A-agarose for 15 min, and centrifuged at 1000g for 5 min. One-tenth volume of each supernatant was stored at 4°C for use as the input sample for immunoblotting, and the remainder was incubated with anti-Sp1 or anti-Flag affinity gels overnight at 4°C. Immunoprecipitates were centrifuged, collected, washed, suspended in 2× SDS sample buffer, and subjected to Western blot analysis with antibodies against HA, Flag, or Sp1.

GST Pull-Down Assay. The expression and purification of GST-fusion proteins and the pull-down of cellular proteins were performed as described previously (Wei et al., 2007). In brief, GST, and the GST-β-TrCP and GST-Skp2 fusion proteins were expressed in *Escherichia coli* strain BL21 (DE3) by isopropyl-1-thio-β-D-galactopyranoside induction for 3 h at 37°C. After centrifugation at 7,000g for 10 min, bacterial pellets were suspended in 10 ml of STE buffer (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride), and lysed by sonication on ice. The lysates were centrifuged for 20 min at 30,000g, and the pellets were dissolved in 10 ml of 1.5% *N*-laurylsarcosine (sarkosyl)-containing STE buffer at 4°C for 1 h. After centrifugation at 30,000g for 20 min, supernatants were collected and mixed with 2.5 ml of 2% Triton X-100. Bacterial lysates were subjected to Western blot analysis with GST antibody for assessment of fusion protein expression. Recombinant GST and GST-fusion proteins were purified from supernatants by incubation with glutathione-Sepharose beads, which were used for the pull-down of Sp1 variants from LNCaP cells.

LNCaP cells were transfected by nucleofection with plasmids encoding Flag-tagged WT or mutant (S728A or S732A) Sp1 protein. Cell lysates were incubated with GST or GST fusion proteins immobilized onto glutathione beads at 4°C for 2 h. The immunocomplexes were washed three times with M-PER buffer, and the resulting precipitates were immunoblotted with antibodies against Flag and GST.

Molecular Modeling Procedures. The interface structure between the β-TrCP1 WD40 domain and doubly phosphorylated ³²DS-GIHSG³⁸ motif of β-catenin was retrieved from the published crystal structure (Protein Data Bank entry code 1P22) (Wu et al., 2003), whereas the interface between the WD40 domain and Sp1 is a representative model structure calculated by energy minimization by using a reported modeler program (Fiser et al., 2000) with the CHARMM force field (Mackerell, 2004). The β-catenin-bound β-TrCP WD40 domain structure was subjected to the addition of hydrogens and the assignment of atomic charges. The doubly phosphorylated ⁷²⁷DSGAGSE⁷³³ motif in Sp1 was superimposed onto the crystal structure of the ³²DSGIHSG³⁸ motif of β-catenin by using

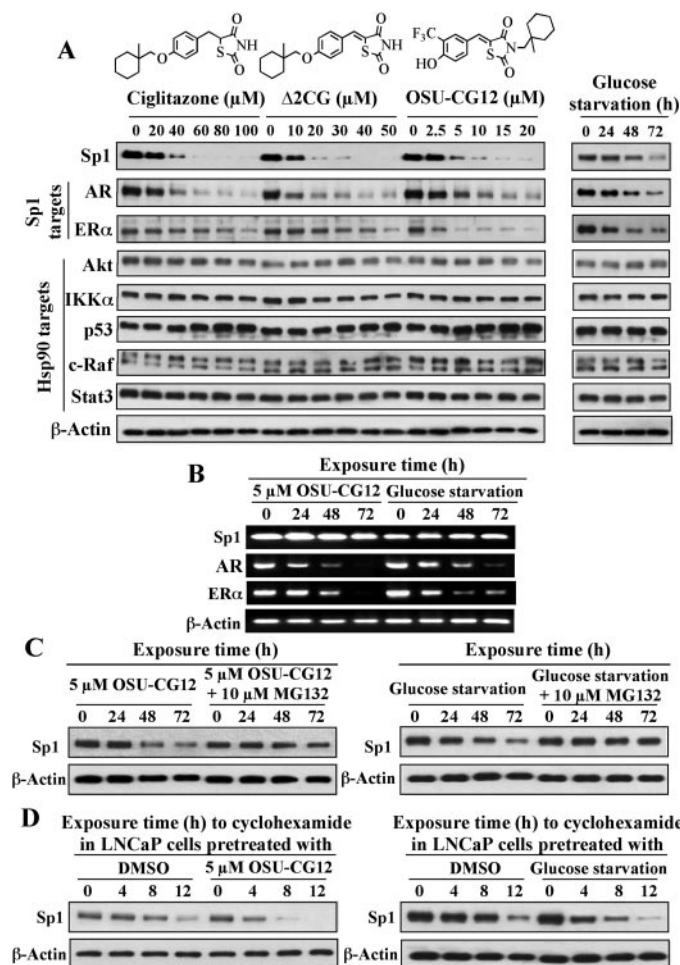


Fig. 1. Evidence that the ability of ciglitazone, Δ2CG, and OSU-CG12 to facilitate the proteasomal degradation of Sp1 in LNCaP cells parallels that of glucose starvation. A, chemical structures of ciglitazone, Δ2CG, and OSU-CG12, and Western blot analysis of the effects of individual thiazolidinediones and glucose starvation on the expression of Sp1 and its downstream targets, AR and ERα, and the Hsp90 client proteins Akt, IKKα, p53, c-Raf, and Stat3. Cells were treated with thiazolidinediones for 72 h or with glucose-free medium. Cell lysates were immunoblotted for the indicated proteins. B, reverse transcription-polymerase chain reaction analysis of the time-dependent effect of 5 μM OSU-CG12 versus glucose starvation on the mRNA levels of Sp1, AR, and ERα. C, the proteasome inhibitor MG132 (10 μM) rescued Sp1 from degradation after treatment of LNCaP cells with 5 μM OSU-CG12 (left) or glucose starvation (right). Cells were treated for the indicated times with OSU-CG12 or glucose starvation alone and in combination with MG132. Cell lysates were immunoblotted for the indicated proteins. D, OSU-CG12 (left) and glucose starvation (right) shortened the half-life of Sp1 in LNCaP cells. Cells were treated with DMSO, 5 μM OSU-CG12, or glucose-deprived RPMI 1640 medium for 12 h, followed by exposure to 100 μg/ml cycloheximide for the indicated time intervals. Cell lysates were immunoblotted with anti-Sp1 and anti-β-actin antibodies.

their sequence alignments and under the controlling of steric and electrostatic effects. Subsequently, molecular mechanical and dynamics simulations were carried out to envisage the interactions between Sp1 and β -TrCP WD40 domain.

Cell Viability Assay. Cell viability was carried out by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. LNCaP cells ectopically expressing the WT, S732A, or T739A forms of Sp1-Flag were seeded in 96-well plates at a density of 5000 cells/well and incubated in 10% FBS-supplemental RPMI 1640 medium for 24 h, followed by drug treatment for an additional 72 h. Spent medium in each well was then replaced with 1 \times MTT (0.5 mg/ml in RPMI 1640) and incubated at 37°C for 2 h. Medium was removed, the reduced MTT dye was solubilized in 200 μ l/well DMSO, and absorbances were measured at 570 nm.

Flow Cytometric Analysis. LNCaP cells transfected with the WT, S732A, or T739A form of Sp1-Flag were seeded in six-well plates (2.5×10^5 cells/well) and treated with DMSO or 5 μ M OSU-CG12 for 48 h. Cells were washed with phosphate-buffered saline, fixed in ice-cold 80% ethanol at 4°C overnight, and stained with propidium iodide (50 μ g/ml) in the presence of RNase A (100 U/ml) in phosphate-buffered saline. Cell-cycle phase distributions were determined on a FACSsort flow cytometer and analyzed by the ModFitLT V3.0 program (BD Biosciences, San Jose, CA).

Results

Mechanistic Link between β -TrCP Up-Regulation and Sp1 Degradation in Thiazolidinedione-Treated and Glucose-Deprived LNCaP Cells. We have previously shown that thiazolidinediones can induce the PPAR γ -independent proteolysis of Sp1, resulting in the suppression of AR expression in LNCaP cells (Yang et al., 2007). Δ 2CG and OSU-CG12 are PPAR γ -inactive ciglitazone derivatives with improved potency over that of the parental compound in repressing AR expression (Yang et al., 2008). As shown in Fig. 1A, these thiazolidinediones, like glucose starvation, reduced the expression of Sp1 and its downstream target gene products, AR and ER α , with varying potency in LNCaP cells. Several lines of evidence suggest that proteasomal degradation of Sp1 underlies the suppression of Sp1 level in these drug-treated cells, as it does in cells deprived of glu-

cose. First, reverse transcription-polymerase chain reaction analysis indicates that OSU-CG12 did not alter the mRNA level of Sp1 while causing time-dependent transcriptional suppression of AR and ER α mRNA expression levels (Fig. 1B). Second, the proteasome inhibitor MG132 rescued Sp1 from drug-induced ablation (Fig. 1C). Third, the ability of OSU-CG12 to modulate Sp1 protein stability was confirmed by the significantly shortened half-life of Sp1 in drug-treated LNCaP cells relative to the DMSO control (4 versus >8 h) (Fig. 1D). Similar findings were seen in glucose-starved LNCaP cells (Fig. 1, B–D). Moreover, none of the above treatments caused changes in the expression level of Hsp90's client proteins, including Akt, IKK α , p53, c-Raf, and Stat3, thereby refuting the involvement of Hsp90 inhibition in thiazolidinedione- or glucose starvation-induced Sp1 degradation (Fig. 1B).

We have reported that the ability of troglitazone and its thiazolidinedione derivatives to facilitate the degradation of β -catenin was, in part, attributable to the up-regulation of the expression of β -TrCP (Wei et al., 2007). Despite differences in structure, ciglitazone, Δ 2CG, and OSU-CG12 were also able to increase the cellular level of β -TrCP, which was associated with corresponding decreases in the expression of β -catenin and other β -TrCP-target proteins, such as I κ B α and Wee1 (Fig. 2A). Equally important, the effect of glucose deprivation on expression levels of β -TrCP and its target proteins paralleled that of thiazolidinediones. Together, these findings suggested that β -TrCP might play a role in thiazolidinedione- and glucose deprivation-induced Sp1 degradation.

To substantiate this mechanistic link, LNCaP cells expressing Flag-tagged Sp1 were used to verify the targeting of Sp1 by β -TrCP for ubiquitin-dependent degradation in response to OSU-CG12 and glucose deprivation. After exposing the transfected cells to 5 μ M OSU-CG12 or glucose-deficient medium for 12 or 24 h, the cell lysates were immunoblotted with various antibodies (input) or were immunoprecipitated with anti-Flag antibody-agarose conjugates. Equivalent

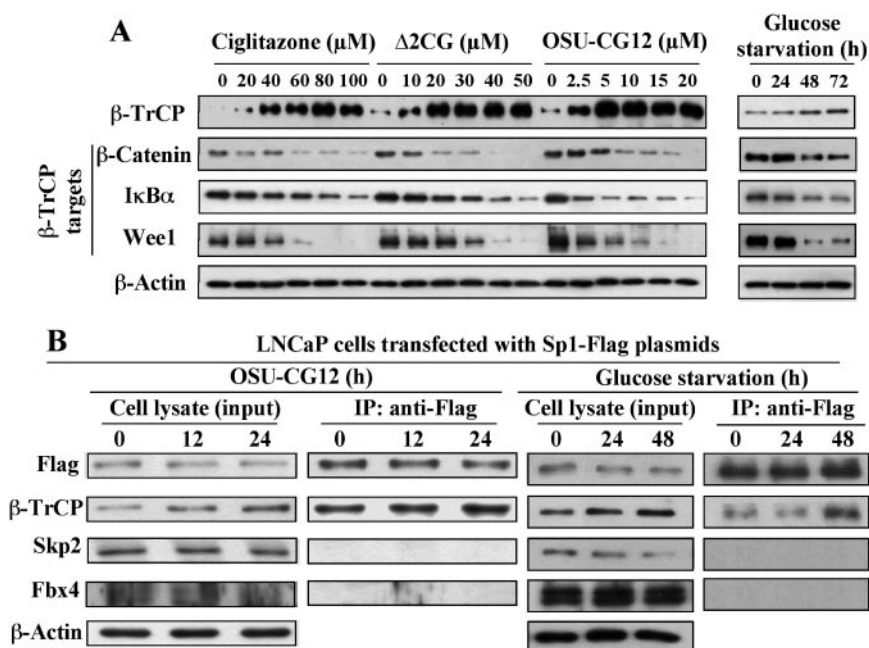


Fig. 2. A mechanistic link between β -TrCP up-regulation and Sp1 degradation in response to thiazolidinedione treatment or glucose starvation. A, Western blot analysis of the dose-dependent effect of ciglitazone, Δ 2CG, and OSU-CG12 vis-à-vis the time-dependent effect of glucose starvation on modulating the expression levels of β -TrCP and its substrates including β -catenin, I κ B α , and Wee1 in LNCaP cells. Cells were treated with thiazolidinediones for 72 h or with glucose-free medium. Cell lysates were immunoblotted for the indicated proteins. B, OSU-CG12 and glucose starvation promote the association of Sp1 with the F-box protein β -TrCP. LNCaP cells transiently transfected with the Sp1-Flag plasmid were exposed to 5 μ M OSU-CG12 for 6 or 18 h or glucose starvation for 18 or 42 h followed by cotreatment with the proteasome inhibitor MG132 for an additional 6 h. The cell lysates were immunoblotted with antibodies against Flag or various F-box proteins (input), or immunoprecipitated with anti-Flag antibody-agarose conjugates. Equal amounts of the immunoprecipitated proteins were immunoblotted for the indicated proteins.

amounts of the immunoprecipitated proteins were subjected to Western blotting with antibodies against Flag and the F-box proteins β -TrCP, Skp2, and Fbx4. As shown in the input columns (Fig. 2B), both treatments, as expected, led to a time-dependent decrease in the expression level of Sp1-Flag, accompanied by a substantial increase in β -TrCP expression. In contrast, cellular levels of the other two F-box proteins, Skp2 and Fbx4, decreased or remained unaltered after treatment. Moreover, immunoprecipitation indicates a time-dependent increase in the association of Flag-Sp1 with β -TrCP but not with Skp2 or Fbx4 in response to either treatment.

The involvement of β -TrCP in Sp1 degradation was further borne out by two lines of evidence. First, ectopically expressed β -TrCP enhanced the ability of OSU-CG12 to facilitate the degradation of Sp1, whereas the expression of Δ F- β -TrCP, which acts as a dominant-negative mutant (Latres et al., 1999), increased the stability of Sp1 in drug-treated cells (Fig. 3A). Moreover, this modulation of Sp1 stability was

associated with corresponding changes in Sp1 ubiquitination. Enforced expression of Δ F- β -TrCP in LNCaP cells cotransfected with HA-tagged ubiquitin and Sp1-Flag eliminated the OSU-CG12-induced increase in Sp1-Flag ubiquitination that was observed in cells expressing the WT- β -TrCP (Fig. 3B). Because Δ F- β -TrCP lacks the F-box motif for association with the Cul1/Skp1 complex, it could compete with endogenous β -TrCP for Sp1 binding in a nonproductive manner (Fig. 3B), thereby resulting in the accumulation of higher levels of Sp1 relative to the WT- β -TrCP control. Second, siRNA-mediated knockdown of β -TrCP provided a complete protection against OSU-CG12-facilitated Sp1 ablation (Fig. 3C).

β -TrCP Up-Regulation and Sp1 Degradation in PC-3 and MCF-7 Cells. Pursuant to the findings described above, we examined this mechanistic link in two other cell lines, the androgen-unresponsive PC-3 and estrogen receptor-positive MCF-7 cells. Exposure of PC-3 (left) or MCF-7 (right) cells to ciglitazone (60 μ M), OSU-CG12 (5 μ M), or glucose-deprived

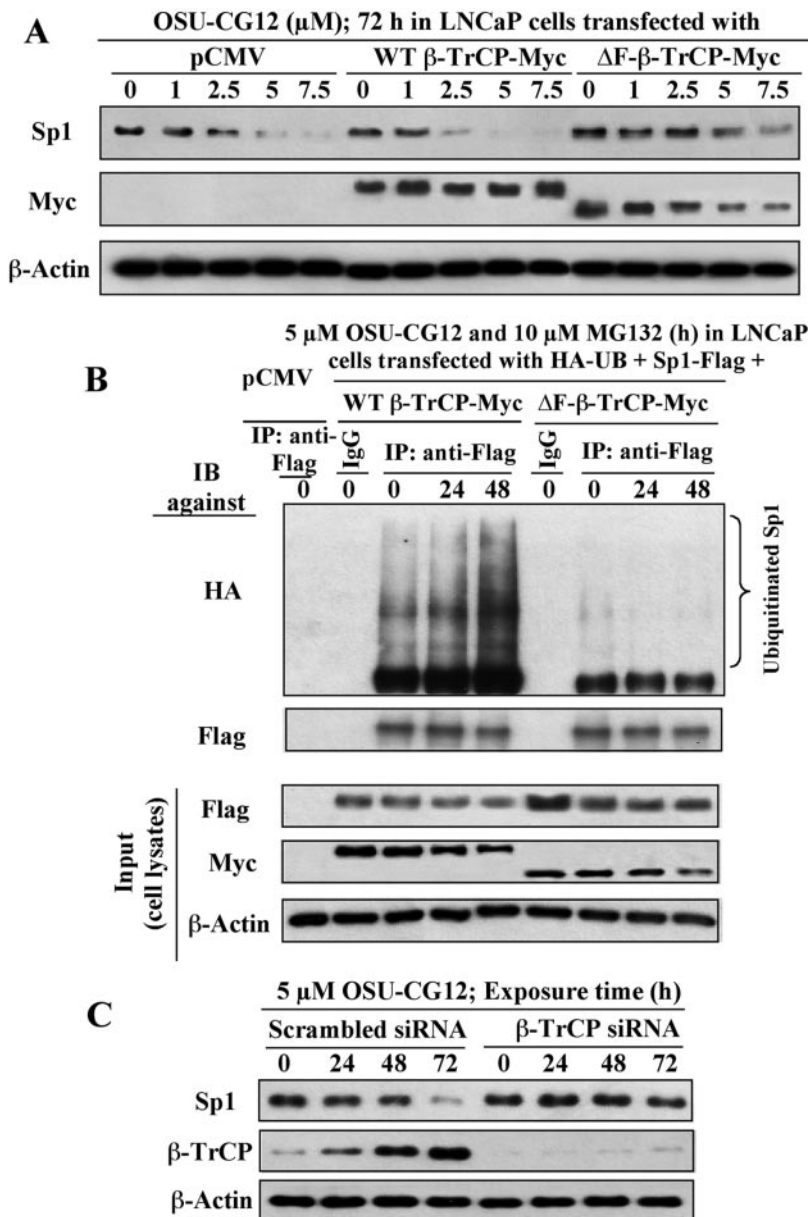


Fig. 3. Evidence that β -TrCP is involved in OSU-CG12-mediated Sp1 degradation in LNCaP cells. **A**, effects of ectopically expressed WT β -TrCP-Myc and Δ F- β -TrCP-Myc on OSU-CG12-mediated Sp1 proteolysis. LNCaP cells transiently transfected with plasmids encoding the Myc-tagged WT β -TrCP or Δ F- β -TrCP proteins, or the empty vector as control, were treated with OSU-CG12 as indicated. Cell lysates were immunoblotted with anti-Sp1 and anti-Myc antibodies. **B**, ectopic expression of Δ F- β -TrCP-Myc blocked OSU-CG12-mediated Sp1 ubiquitination. LNCaP cells cotransfected with plasmids encoding HA-ubiquitin, Sp1-Flag, and WT- or Δ F- β -TrCP-Myc were treated with 5 μ M OSU-CG12 for 12 or 36 h, followed by cotreatment with proteasome inhibitor MG132 (10 μ M) for an additional 12 h. Equal amounts of cell lysates were probed with anti-Flag and anti-Myc antibodies (input) or immunoprecipitated with anti-Flag affinity gels followed by immunoblotting with anti-HA and anti-Flag antibodies. **C**, siRNA-mediated suppression of β -TrCP expression protects Sp1 from OSU-CG12-mediated degradation. LNCaP cells transfected with siRNA for β -TrCP or scrambled siRNA were treated with OSU-CG12 as indicated, and cell lysates were immunoblotted with anti-Sp1 and anti- β -TrCP antibodies.

medium led to time-dependent increases in β -TrCP levels, accompanied by concomitant decreases in Sp1 expression (Fig. 4A), paralleling that observed in LNCaP cells. As was observed in LNCaP cells, MG132 rescued Sp1 from this OSU-CG12-induced degradation in PC-3 or MCF-7 cells (Fig. 4B). Equally important, in both cell lines, siRNA-mediated knockdown of β -TrCP blocked OSU-CG12-induced Sp1 ubiquitination (Fig. 4C, top), thereby inhibiting the subsequent Sp1 ablation (bottom, input).

Kinase Dependence of OSU-CG12-Induced Sp1 Degradation in LNCaP Cells. Because phosphorylation is a prerequisite for β -TrCP recognition of target proteins, the effect of glucose starvation and OSU-CG12 on Sp1 phosphorylation was examined. Western blot analysis indicates that both treatments increased Sp1 phosphorylation levels in LNCaP cells (Fig. 5A). Although JNK and ERK1/2 have been reported to regulate Sp1 transcriptional activity through phosphorylation (Benasciutti et al., 2004; Bonello and Khachigian, 2004; Chu and Ferro, 2006), the involvement of individual kinases in modulating Sp1 degradation remains unclear. To shed light on this issue, the effect of OSU-CG12 and glucose starvation on the phosphorylation status of various kinases was determined by Western blot analysis. It is noteworthy that the profile of kinase phosphorylation after OSU-CG12 treatment was virtually identical with that in glucose-starved cells (Fig. 5B). Treatment of LNCaP cells

with OSU-CG12 or glucose-deprived medium led to increased phosphorylation of all kinases examined except for Akt, which included GSK3 β , MEK1, ERK1/2, p38, and JNK.

To discern the role of individual kinases in mediating Sp1 degradation, the effects of various pharmacological inhibitors on rescuing OSU-CG12-mediated Sp1 degradation in LNCaP cells were assessed. These inhibitors included the MEK/MAP kinase inhibitors PD98059 and U0126, the GSK3 β inhibitors LiCl and SB216763, the JNK inhibitor SP600125, and the p38 inhibitor PD169316. Our data indicate that inhibition of MEK/MAP kinase and GSK3 β resulted in a dose-dependent protection against OSU-CG12-facilitated Sp1 repression (Fig. 5C, top). In contrast, pharmacological inhibition of JNK and p38, as evidenced by the reduced phosphorylation levels of their respective downstream targets Ser63-c-Jun and Thr334-MAPKAPK-2, had no significant effect on rescuing OSU-CG12-induced Sp1 degradation (bottom). To validate these findings, MEK1 and GSK3 β activities were suppressed by transfection of LNCaP cells with plasmids encoding either the dominant-negative mutant of MEK1 (MEK K97A) or the kinase-dead mutant of GSK3 β (GSK3 β K85A), and their effects on drug-induced Sp1 degradation were assessed. As shown, both mutants conferred resistance to OSU-CG12-induced Sp1 proteolysis (Fig. 5D, left and right). Moreover, we found that ectopic expression of constitutively active MEK1 (MEK1 DD) could facilitate Sp1 degradation in a

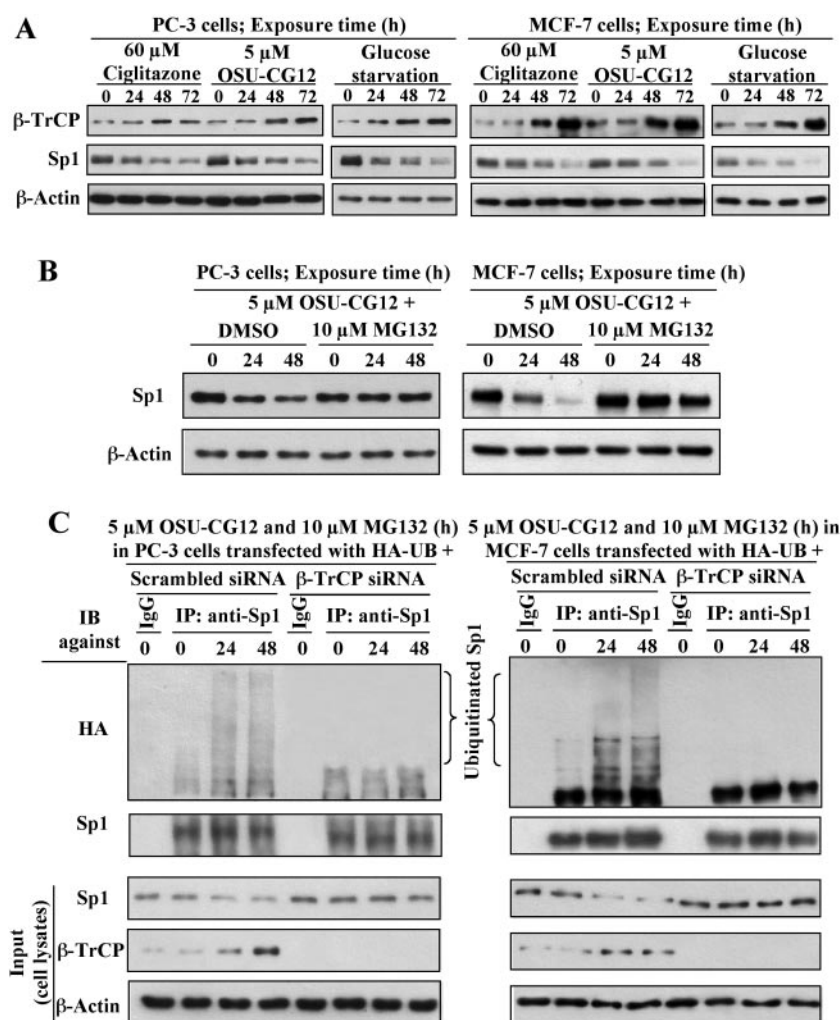


Fig. 4. Involvement of β -TrCP in drug- or glucose deprivation-induced proteasomal degradation of Sp1 in PC-3 and MCF-7 cells. **A**, time-dependent effects of ciglitazone (60 μ M), OSU-CG12 (5 μ M), and glucose deprivation on β -TrCP and Sp1 expression in PC-3 (left) and MCF-7 (right) cells. **B**, the proteasomal inhibitor MG132 (10 μ M) prevented OSU-CG12-mediated Sp1 degradation in PC-3 (left) and MCF-7 (right) cells. **C**, siRNA-mediated suppression of β -TrCP expression hindered OSU-CG12-induced Sp1 ubiquitination in PC-3 (left) and MCF-7 (right) cells. Cells transfected with the plasmid expressing HA-ubiquitin, and siRNA for β -TrCP or scrambled siRNA was treated with 5 μ M OSU-CG12 for 12 or 36 h, followed by cotreatment with proteasome inhibitor MG132 (10 μ M) for an additional 12 h. Equal amounts of cell lysates were probed with antibodies against β -TrCP and Sp1 (input) or were immunoprecipitated with Sp1-conjugated agarose followed by immunoblotting with anti-HA or anti-Sp1 antibodies.

manner qualitatively similar to that of OSU-CG12 or glucose starvation (Fig. 5D, left). Together, these findings demonstrate that ERKs and GSK3β played a key role in regulating Sp1 degradation.

The β-TrCP Recognition Site in Sp1. β-TrCP recognizes the consensus sequence of DSGX_nS (X, any amino acid; *n* = 2–4) in many of its target proteins, including IκB, β-catenin, Cdc25A, and Emi1, after phosphorylation of both serine residues (Nakayama and Nakayama, 2005). Analysis of the Sp1 sequence revealed a putative β-TrCP recognition motif, ⁷²⁷DSGAGS⁷³², close to the C terminus. This DSG motif encompasses a well characterized GSK3β phosphorylation motif (SXXXS) and is adjacent to the ERK phosphorylation site at Thr739. The involvement of this β-TrCP recognition motif in controlling Sp1 degradation was corroborated by

mutational analyses. As shown in Fig. 6A, replacement of Ser728 or Ser732 with alanine (S728A and S732A, respectively) greatly enhanced the stability of Sp1 protein. Although the half-life of ectopically expressed Flag-tagged WT Sp1 was approximately 10 h, the levels of the S728A and S732A mutants in the presence of cycloheximide remained unaltered after 12 h. Moreover, these Sp1 mutants, along with the T739A mutant, exhibited different degrees of resistance to OSU-CG12-mediated degradation, in the order of S732A > T739A > S728A (Fig. 6B). Evidence indicates that these mutations blocked β-TrCP-mediated Sp1 ubiquitination, thereby inhibiting subsequent proteasomal degradation (Fig. 6C). LNCaP cells doubly transfected with plasmids for HA-ubiquitin and the WT or S732A mutant form of Sp1 were exposed to 5 μM OSU-CG12 alone for 12 or 36 h, followed by

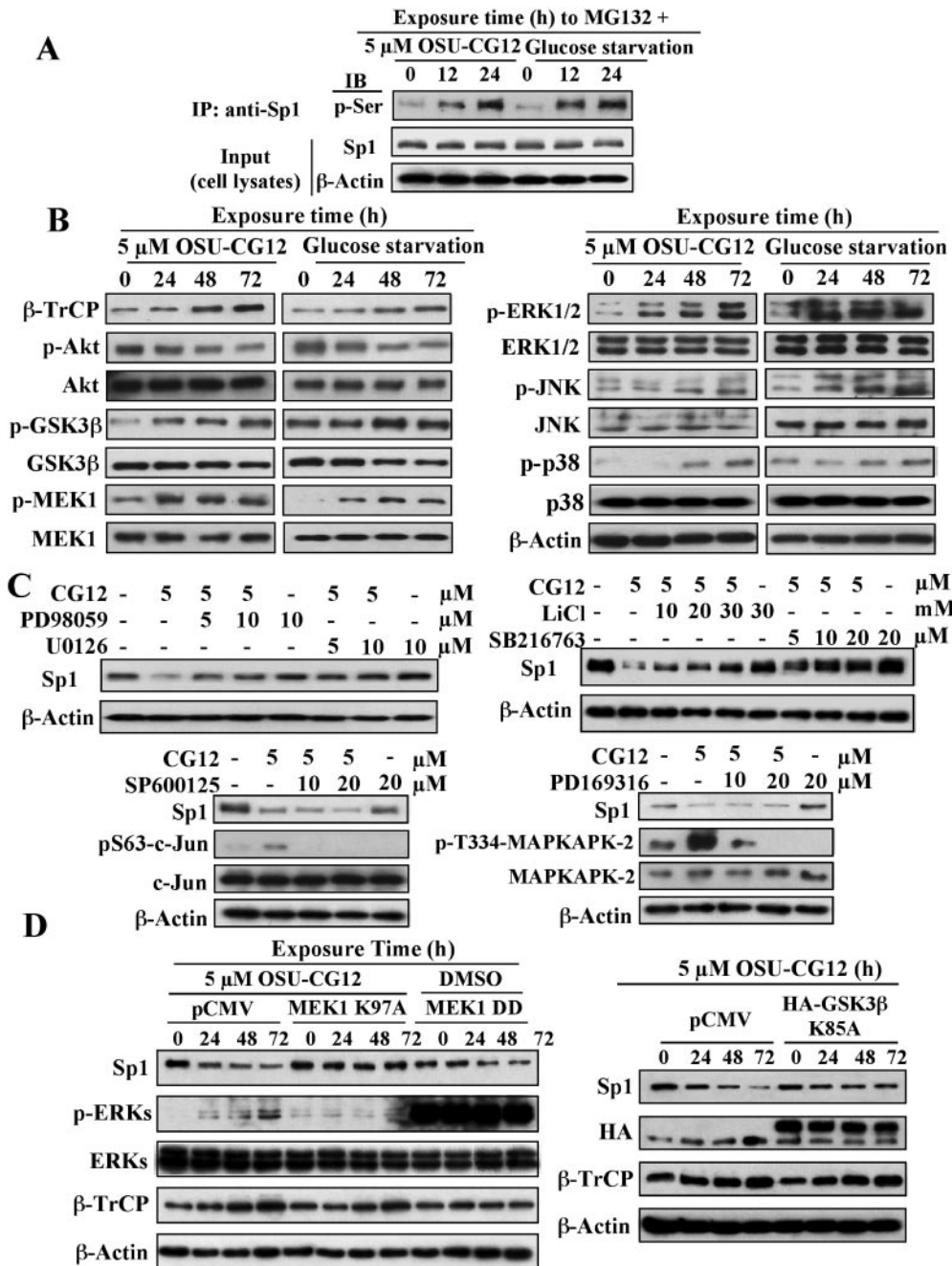


Fig. 5. ERK1/2 and GSK3β play a pivotal role in OSU-CG12-facilitated Sp1 degradation. A, time-dependent effect of 5 μM OSU-CG12 versus glucose starvation on serine phosphorylation of Sp1. LNCaP cells were exposed to 5 μM OSU-CG12 or glucose-free medium in the presence of 10 μM MG132 for the indicated time intervals. Cell lysates were immunoprecipitated with anti-Sp1 antibodies followed by immunoblotting with anti-phosphoserine antibodies. B, effect of OSU-CG12 and glucose starvation on the phosphorylation status of various kinases including Akt, GSK3β, MEK1, ERK1/2, JNK and p38. C, effects of kinase-specific inhibitors (PD98059 and U0126, ERK1/2; LiCl and SB216763, GSK3β; SP600125, JNK; PD169316, p38) on OSU-CG12-mediated Sp1 degradation and/or the phosphorylation of c-Jun and MAPKAPK-2, the kinase substrates of JNK and p38, respectively. LNCaP cells were treated with OSU-CG12 in combination with individual kinase inhibitors at the indicated concentrations for 72 h followed by immunoblotting of cell lysates with anti-Sp1 antibodies. D, changes in ERK or GSK3β kinase activity affect Sp1 degradation in LNCaP cells. Left, ectopic expression of the dominant-negative MEK1 mutant (MEK1 K97A) rescued Sp1 from OSU-CG12-induced degradation, whereas overexpression of the constitutively active MEK1 mutant (MEK1 DD) mimicked the effect of OSU-CG12 on Sp1 degradation. Right, inhibition of GSK3β kinase activity by ectopic expression of the kinase-dead GSK3β mutant (HA-GSK3β K85A) protected Sp1 from OSU-CG12-facilitated degradation. LNCaP cells transfected with plasmids encoding the indicated MEK1 and GSK3β mutants were treated with OSU-CG12 or DMSO as indicated, followed by immunoblotting of cell lysates for the indicated proteins. CG12, OSU-CG12.

cotreatment with 10 μ M MG132 for an additional 12 h. Cell lysates were then immunoblotted with Flag (input) or were immunoprecipitated by anti-Flag antibody-agarose conjugates followed by immunoblotting with anti-HA or -Flag antibodies. As shown, relative to WT Sp1, no ubiquitination was noted with S732A-Sp1.

Although the findings described above establish the importance of Ser732 and Ser728 for Sp1 ubiquitination and proteolysis, we rationalized that the differential resistance of the S728A and S732A Sp1 mutants to degradation was attributable to their respective effects on abrogating Sp1 binding to

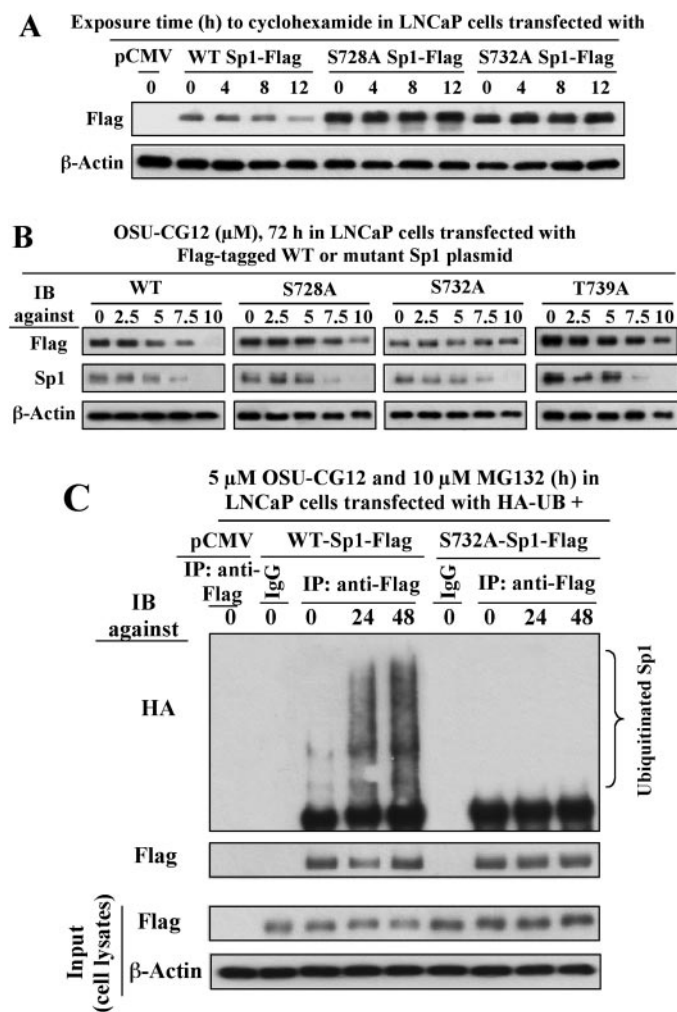


Fig. 6. Mutational analyses demonstrating the crucial role of Ser728 and Ser732 in regulating OSU-CG12-mediated Sp1 degradation and ubiquitination in LNCaP cells. A, the S728A and S732A mutants of Sp1 were more stable than WT Sp1 in LNCaP cells. LNCaP cells ectopically expressing the WT, S728A, or S732A form of Sp1-Flag were exposed to 100 μ g/ml cycloheximide for the indicated time intervals, followed by immunoblotting of cell lysates with anti-Flag antibodies. B, dose-dependent effects of OSU-CG12 on the degradation of WT Sp1-Flag versus various Sp1-Flag mutants. LNCaP cells ectopically expressing WT Sp1-Flag or the S728A, S732A, or T739A mutants were treated with OSU-CG12 at the indicated concentrations for 72 h and immunoblotted for Flag and Sp1. Endogenous Sp1 degradation was used as an internal control for OSU-CG12 activity. C, the S732A mutant of Sp1 blocked OSU-CG12-induced Sp1 ubiquitination. LNCaP cells ectopically expressing hemagglutinin-ubiquitin (HA-UB) and WT or S732A-Sp1-Flag were treated with 5 μ M OSU-CG12 for 12 or 36 h, followed by cotreatment with 10 μ M MG132 for an additional 12 h. Cell lysates were immunoprecipitated with anti-Flag affinity gels, followed by immunoblotting with antibodies against HA and Flag.

β -TrCP. To corroborate this premise, coimmunoprecipitation was carried out in LNCaP cells doubly transfected with β -TrCP-Myc and the WT, S728A, or S732A form of Sp1-Flag, and cotreated with OSU-CG12 and MG132. Figure 7A indicates that, relative to the WT Sp1, only trace amounts of β -TrCP binding with the S728A mutant occurs, whereas no interaction was noted with the S732A mutant. This differential recognition and interaction with β -TrCP was confirmed by an in vitro pull-down analysis (Fig. 7B). GST, GST- β -TrCP, and GST-Skp2 proteins were expressed in *E. coli* and then purified on glutathione-Sepharose beads. Lysates from LNCaP cells ectopically expressing the WT, S728A, or S732A form of Sp1-Flag were incubated with the immobilized GST, GST- β -TrCP, and GST-Skp2 proteins. The resulting immunocomplexes were subjected to immunoblotting with Flag antibodies. Relative to WT Sp1, the S728A mutant exhibited low binding affinity for β -TrCP, whereas the S732A mutant showed no detectable binding. In contrast, neither WT Sp1 nor the mutants displayed appreciable binding to GST or GST-Skp2, indicating the specificity of Sp1 binding to β -TrCP.

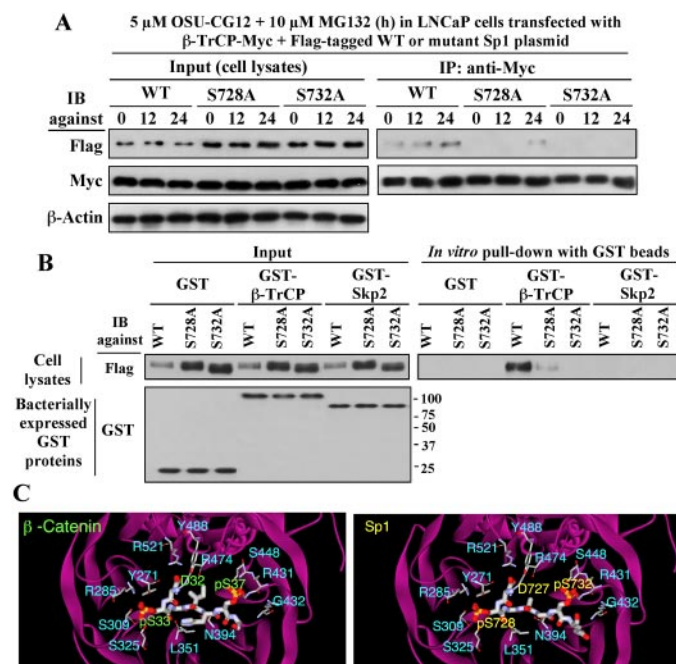


Fig. 7. Evidence that β -TrCP recognizes the ⁷²⁷DSGAGS⁷³² motif in Sp1. A, Ser728 and Ser732 are important for β -TrCP recognition and binding of Sp1. LNCaP cells ectopically expressing both Myc-tagged β -TrCP (β -TrCP-Myc) and Flag-tagged Sp1 variants (WT, S728A, or S732A) were treated with OSU-CG12 for 6 or 18 h followed by cotreatment with 10 μ M MG132 for an additional 6 h. Immunoprecipitation with anti-Myc-agarose conjugates and immunoblotting for Flag and Myc were performed. B, in vitro pull-down of Flag-tagged Sp1 variants (WT, S728A, or S732A) with bacterially expressed GST- β -TrCP fusion protein confirm the importance of Ser728 and Ser732 for β -TrCP recognition and binding of Sp1. Lysates from LNCaP cells ectopically expressing WT, S728A, or S732A-Sp1-Flag were incubated with recombinant GST, GST- β -TrCP, or GST-Skp2 proteins immobilized onto glutathione beads. The resulting complexes were immunoblotted with Flag antibodies (right). One-tenth volume of each LNCaP cell lysate was collected as input and immunoblotted with Flag antibodies to determine Sp1 variant expression (left). Purity and integrity of recombinant GST-fusion proteins were confirmed by immunoblotting of bacterial lysates with GST antibody (left). C, docking of the doubly phosphorylated β -catenin DSGIHS peptide (left) and the doubly phosphorylated Sp1 ⁷²⁷DSGAGS⁷³³ peptide (right) into the β -TrCP1 WD40 domain. Green text, β -catenin residues; yellow text, Sp1 residues; blue text, WD40 domain residues.

To envision the mode of interaction between this Sp1 recognition sequence and β-TrCP, molecular modeling analysis was performed by docking the phosphorylated binding motif (⁷²⁷DSGAGSE⁷³³) of Sp1 into the top face of the β-TrCP WD40 domain in a manner similar to that described for the doubly phosphorylated β-catenin destruction motif (³²DSGIHSG³⁸) (Wu et al., 2003). As shown in Fig. 7C, these two phosphorylated peptides displayed a virtually identical mode of binding into the WD40 channel. Residues Asp727, pSer728, and pSer732 in Sp1, and Asp32, pSer33, and pSer37 in β-catenin, form a negatively charged triad that interacts with surrounding hydrophilic/basic side chains and backbone amide functions in the WD40 channel through a network of hydrogen bonding and electrostatic interactions.

Ectopic Expression of Wild-Type or Mutant Sp1 Confers Resistance to OSU-CG12-Mediated AR Transcriptional Repression. Considering the pivotal role of Sp1 in AR transcriptional regulation, we rationalized that disruption of β-TrCP-facilitated Sp1 degradation would cause dysregulated AR expression in LNCaP cells. To corroborate this premise, we examined the effect of ectopic expression of the WT, S732A, or T739A form of Sp1-Flag on OSU-CG12-mediated AR degradation. As shown, these cells exhibited different degrees of resistance to drug-induced AR ablation, in the order of S732A > T739A > WT (Fig. 8A). This differential

protection against AR repression paralleled the abilities of these Sp1 mutants to rescue AR promoter-luciferase activity (Fig. 8B) and LNCaP cell viability (Fig. 8C) from OSU-CG12-mediated suppression. As shown, ectopic expression of either a wild-type or mutant form of Sp1 significantly protected OSU-CG12-mediated suppression of cell growth compared with that of pCMV ($P < 0.005$), and the mutant forms, especially S732A, provided a significant protection relative to the wild-type form ($P < 0.05$). Moreover, the antiproliferative effect of OSU-CG12 in pCMV- or WT Sp1-expression LNCaP cells was, at least in part, due to apoptosis, as reflected by increases in the sub-G₁ population by flow cytometric analysis. Together, these findings indicate the functional role of β-TrCP in regulating AR expression in LNCaP cells.

Discussion

In this study, we report a novel β-TrCP-mediated pathway for Sp1 degradation in LNCaP cells. Because the aberrant expression of many Sp1 target genes underlies tumorigenesis and progression of human cancers, targeting Sp1 degradation by small-molecule agents like OSU-CG12 represents a therapeutically relevant strategy for the treatment of Sp1-overexpressing tumors. Moreover, from a mechanistic perspective, OSU-CG12 yields insights into the mechanism gov-

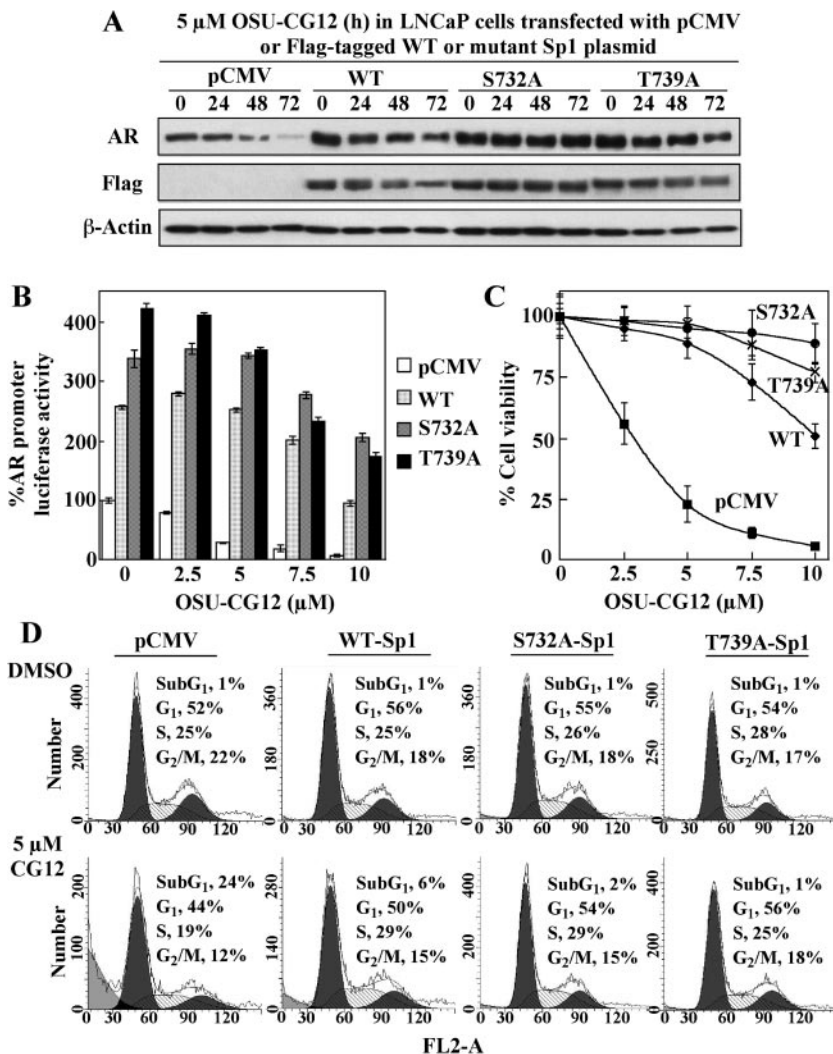


Fig. 8. Functional relevance of β-TrCP-mediated Sp1 degradation for the transcriptional regulation of AR in LNCaP cells. A, mutated Sp1 protects AR from OSU-CG12-induced suppression. LNCaP cells ectopically expressing WT Sp1-Flag or the S732A or T739A mutants were treated with 5 μM OSU-CG12 for the indicated time periods, and cell lysates were immunoblotted for AR and Flag. Cells were also transfected with the empty vector as controls. B, mutated Sp1 reversed OSU-CG12-mediated suppression of AR promoter transcriptional activity. Cells coexpressing an AR promoter-luciferase reporter construct and the WT, S732A, or T739A form of Sp1-Flag were exposed to different concentrations of OSU-CG12 for 48 h, followed by a luciferase assay to assess AR promoter activity. Columns, means ($n = 3$); bars, S.D. C, mutated Sp1 rescues LNCaP cell viability from OSU-CG12-induced inhibition. LNCaP cells ectopically expressing the WT, S732A, or T739A form of Sp1-Flag were treated with OSU-CG12 at the indicated concentrations for 72 h. Cell viability was analyzed by MTT assay. Data points, means ($n = 6$); bars, S.D. D, flow cytometric analysis of LNCaP cells ectopically expressing pCMV, WT Sp1-Flag, or the S732A or T739A mutants after treatment with DMSO or 5 μM OSU-CG12 for 48 h.

erning Sp1 degradation in response to glucose deprivation in cancer cells. When cancer cells are starved of glucose, an obvious consequence is the reorganization of protein turnover and gene expression programs, in which Sp1 might represent a prime target considering its role in controlling the transcription of housekeeping genes pertinent to cell growth and cell cycle.

Based on our previous report describing thiazolidinedione-induced proteasomal degradation of β -catenin, cyclin D1, and other cell-cycle regulatory proteins through the up-regulation of β -TrCP (Wei et al., 2007, 2008), we hypothesized that β -TrCP played a crucial role in regulating Sp1 degradation in response to OSU-CG12 or glucose starvation. This mechanistic link was borne out by several lines of evidence. First, exposure of LNCaP cells to either treatment led to an increased association of Sp1 with β -TrCP but not with other F-box proteins examined, including Skp2 and Fbx4. Second, the in vitro pull-down analysis with bacterially expressed β -TrCP indicates direct binding with Sp1. Third, ectopic expression of β -TrCP enhanced the ability of OSU-CG12 to facilitate Sp1 degradation. Fourth, suppression of endogenous β -TrCP function by a dominant-negative mutant (i.e., Δ F- β -TrCP) or siRNA-mediated knockdown blocked OSU-CG12-facilitated Sp1 ubiquitination and/or degradation. Moreover, this mechanistic link was also demonstrated in PC-3 prostate cancer and MCF-7 breast cancer cells.

Because phosphorylation is a prerequisite for β -TrCP recognition of target proteins, we examined the effects of OSU-CG12 and glucose starvation on the activation status of a series of kinases that are potentially implicated in Sp1 phosphorylation. Although the involvement of various kinases in modulating the transcriptional activity of Sp1 has been well characterized (Samson and Wong, 2002), their role in regulating Sp1 degradation remains unclear. OSU-CG12 modulated the phosphorylation levels of these kinases in a pattern virtually identical with that noted in response to glucose deprivation, suggesting a high degree of similarity among the respective signaling networks. Inhibition of GSK3 β and ERKs through both pharmacological and molecular genetic approaches protected Sp1 from OSU-CG12-facilitated degradation. In contrast, although OSU-CG12 and glucose starvation each increased the phosphorylation levels of JNK and p38, pharmacological inhibition of either kinase conferred no protection on Sp1 against proteolysis, refuting roles for these two kinases in regulating Sp1 protein stability. This finding contrasts with an earlier report that JNK exerted a protective effect on the ubiquitin-dependent degradation of Sp1 (Chuang et al., 2008).

From a structural perspective, the C terminus of Sp1 contains a conventional DSG destruction box (⁷²⁷DSGAGS⁷³²) for β -TrCP recognition. Replacement of Ser728 or Ser732 with an alanine residue resulted in the loss of β -TrCP binding and inhibition of subsequent ubiquitination and degradation. This recognition motif encompasses a well characterized GSK3 β phosphorylation motif (SXXXS), which underlies the involvement of GSK3 β in regulating Sp1 degradation. Although OSU-CG12 and glucose deprivation caused phosphorylative inactivation of GSK3 β , there was sufficient residual activity in the cells to phosphorylate Sp1 to facilitate the subsequent degradation. In addition, our data indicate that replacement of Thr739 with alanine in the nearby ERK phosphorylation site resulted in resistance to OSU-CG12-

facilitated Sp1 degradation, suggesting a role for ERK-mediated Thr739 phosphorylation in regulating Sp1 stability.

This study presents the first evidence that β -TrCP up-regulation is part of the cellular response to glucose deprivation in cancer cells. In response to this metabolic stress, β -TrCP facilitates the proteasomal degradation of a wide range of target proteins involved in different aspects of cellular functions (Frescas and Pagano, 2008). The complexity of this response is underscored by the host of transcription factors that are targeted by β -TrCP. In addition to Sp1, β -TrCP-targeted transcription factors include Snail (Zhou et al., 2004), Gli2 (Bhatia et al., 2006), Gli3 (Tempé et al., 2006), STAT1 (Soond et al., 2008), and REST (Westbrook et al., 2008), all of which are involved in the transcriptional regulation of a multitude of genes governing cell proliferation, differentiation, and apoptosis. Consequently, the concerted action of these β -TrCP target proteins might, in part, contribute to growth arrest and apoptosis in glucose-starved cancer cells. From a clinical perspective, the ability of OSU-CG12 to mimic glucose starvation to activate β -TrCP-mediated proteasomal degradation has translational potential to foster novel strategies for cancer therapy and prevention.

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Address correspondence to: Dr. Ching-Shih Chen, Division of Medicinal Chemistry, College of Pharmacy, The Ohio State University, 500 W. 12th Avenue, Columbus, OH 43210. E-mail: chen.844@osu.edu