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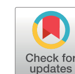
Protein phosphatase 1 alpha enhances glucocorticoid receptor activity by a mechanism involving phosphorylation of serine-211



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ABSTRACT

By acting as a ligand-dependent transcription factor the glucocorticoid receptor (GR) mediates the actions of glucocorticoids and regulates many physiological processes. An impaired regulation of glucocorticoid action has been associated with numerous disorders. Thus, the elucidation of underlying signaling pathways is essential to understand mechanisms of disrupted glucocorticoid function and contribution to diseases. This study found increased GR transcriptional activity upon overexpression of protein phosphatase 1 alpha (PP1α) in HEK-293 cells and decreased expression levels of GR-responsive genes following PP1α knockdown in the endogenous A549 cell model. Mechanistic investigations revealed reduced phosphorylation of GR-Ser211 following PP1α silencing and provided a first indication for an involvement of glycogen synthase kinase 3 (GSK-3). Thus, the present study identified PP1α as a novel post-translational activator of GR signaling, suggesting that disruption of PP1α function could lead to impaired glucocorticoid action and thereby contribute to diseases.

1. Introduction

Glucocorticoids (GC) are steroid hormones naturally produced and secreted by the adrenal gland in order to maintain body homeostasis. They act highly tissue-specifically and affect almost every organ in the human body by regulating energy homeostasis (carbohydrates, lipids, proteins), bone metabolism, cell growth and differentiation, apoptosis, stress and immune responses, as well as brain function (Ahmad et al., 2019; Blum and Maser, 2003; Buckingham, 2006; Garabedian et al., 2017; Gross and Cidlowski, 2008; Quax et al., 2013). GC are essential for life and dysregulation of their signaling is involved in numerous disorders including metabolic and cardiovascular diseases, asthma and chronic obstructive pulmonary disease (COPD), mood and cognitive disorders, immune diseases and cancer. Because of their anti-inflammatory, immunosuppressive and pro-apoptotic effects, synthetic GC are amongst the most widely prescribed drugs for the treatment of inflammation, autoimmune disorders and cancer.

GC exert their effects by binding to the glucocorticoid receptor (GR), a ligand-dependent transcription factor belonging to the nuclear receptor superfamily. The GR consists of an N-terminal transactivation domain (NTD), a DNA binding domain (DBD) and a C-terminal ligand-binding domain (LBD) (Zhou and Cidlowski, 2005). The NTD contains a transcriptional activation function domain (AF-1) responsible for post-translational modifications and protein-protein interactions. In the absence of ligand, the GR is sequestered in the cytoplasm in a heat-shock protein 90 (HSP-90)-chaperone complex. Ligand binding induces conformational changes that allow the release of the cytosolic GR from the HSP-90-chaperone complex, followed by receptor dimerization and translocation into the nucleus. The hormone-activated GR binds then to specific palindromic DNA sequences in the promoter regions of target genes (glucocorticoid response elements, GREs) and regulates their transcription. Induction or suppression of target gene transcription can occur, depending on the cell type, promoter context and cofactor recruitment. Upon prolonged exposure to GC, the GR is subjected to degradation by the ubiquitin-proteasome pathway, resulting in termination of the transcriptional response (Connell et al., 2001; Galigniana et al., 2004; Kinyamu and Archer, 2003; Sengupta and Wasylyk, 2001; Wallace and Cidlowski, 2001; Wang and DeFranco, 2005; Webster et al., 1997).

In addition to ligand binding, cross-talk with other signaling

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pathways can modulate GR activity by post-translational modifications, including phosphorylation, ubiquitination, and SUMOylation (Faus and Haendler, 2006; Garza et al., 2010; Ismaili and Garabedian, 2004; Le Drean et al., 2002; Wallace and Cidlowski, 2001). GR phosphorylation affects its interaction with co-regulators, subcellular localization, DNA binding and protein stability. Phosphorylation is paramount in receptor regulation and highly cell-, tissue-, species- and promoter-specific and the vast diversity of kinases and phosphatases mediates the high variety of cellular responses to GC (Galliher-Beckley and Cidlowski, 2009; Zhou and Cidlowski, 2005). Various kinases, including cyclin-dependent kinases (CDKs), p38 mitogen activated protein kinases (MAPKs), c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), protein kinase B (Akt), and glycogen synthase kinase 3 (GSK-3) were shown to modulate GR activity by phosphorylation at several sites, including serine residues 134, 203, 211, 226 and 404 (DeFranco et al., 1991; Galliher-Beckley et al., 2008, 2011; Habib et al., 2017; Itoh et al., 2002; Kino et al., 2007; Krstic et al., 1997; Miller et al., 2005; Rogatsky et al., 1998a,b; Webster et al., 1997).

GR function is regulated by numerous phosphatases. Recent studies emphasized the importance of protein phosphatases in regulating GR and contributing to the cytokine-induced GC insensitivity seen in patients with severe asthma (Bouazza et al., 2012; Kobayashi et al., 2011; Pazdrak et al., 2016). Protein phosphatase 2A (PP2A) was found to dephosphorylate JNK, resulting in decreased GR-Ser226 phosphorylation and enhanced GR nuclear translocation, while protein phosphatase 5 (PP5) was proposed to negatively regulate GR activity by direct dephosphorylation of GR-Ser211. Besides, PP2A was suggested to form a trimeric complex with GR and the scaffold protein striatin-3 (Petta et al., 2017). In the presence of striatin-3, PP2A might be recruited to facilitate dephosphorylation of GR-Ser211, thereby resulting in diminished GR transcriptional activity.

Protein phosphatase 1 alpha (PP1α) has been shown to control the expression and activity of several steroid receptors, including the mineralocorticoid receptor (MR), the androgen receptor (AR) and the estrogen receptor-α (ER) (Bollig et al., 2007; Liu et al., 2016; Nagarajan et al., 2017). PP1α was found to bind to the LBD of these steroid receptors, thereby stabilizing their expression by dephosphorylation and inactivation of the E3 ubiquitin-ligase mouse double minute 2 (MDM2). In contrast to PP2A and PP5, it remained unclear whether PP1α also regulates GR function. In rat fibroblasts, the subcellular distribution of the GR was influenced by inhibiting activities of PP1 and PP2A using okadaic acid, a potent but unspecific inhibitor of PP1 (IC_{50} = 15–20 nM) and PP2A (IC_{50} = 0.1 nM) (Bialojan and Takai, 1988; Cohen et al., 1989; DeFranco et al., 1991; Somers and DeFranco, 1992). The PP1 holoenzyme is a serine/threonine-specific phosphoprotein phosphatase consisting of a catalytic subunit (PP1c) and multiple regulatory subunits. PP1c has four isoforms PP1α, PP1β, and the splice variants PP1γ_1 and PP1γ_2, encoded by three different genes PPP1CA, PPP1CB and PPP1CC. These isoforms share a high level of sequence homology and are ubiquitously expressed, except of PP1γ_2, which is restricted to the testis (Ceulemans and Bollen, 2004; Cohen, 2002; Moorhead et al., 2007).

The present study evaluated the impact of PP1α on GR function by performing a reporter gene assay in HEK-293 cells expressing recombinant human GRα and PP1α. To investigate the effect of PP1α on endogenously expressed GR activity, the transcriptional expression of GC-induced genes was followed after knockdown of PP1α in A549 human lung carcinoma cells. Furthermore, to assess whether PP1α regulates GR through dephosphorylation of MDM2, as shown for the related steroid receptors, or by a direct mechanism similarly to PP2A and PP5, the pathways underlying the PP1α-mediated effect on GR stimulation were assessed in A549 cells after silencing of PP1α.

2. Materials and methods

2.1. Chemicals and reagents

Cortisol (CAS Nr. 50-23-7) was purchased from Steraloids (Newport, RI). Inhibitors of JNK (SP600125, CAS Nr. 129-56-6), p38 MAPK (SB203580, CAS Nr. 152121-47-6), Akt1/2 kinase (CAS Nr. 612847-09-3), MEK1/2 (PD98059, CAS Nr. 167869-21-8), and GSK-3 (CHIR99021, CAS Nr. 252917-06-9), as well as the GR antagonist mifepristone (RU-486, CAS Nr. 84371-65-3) were obtained from Sigma-Aldrich (Buchs SG, Switzerland). Stock solutions (10 mM) were prepared in dimethyl sulfoxide (DMSO, CAS Nr. 67-68-5; AppliChem, Darmstadt, Germany).

2.2. Cell culture and treatments

Human embryonic kidney-293 (HEK-293) cells and A549 human alveolar carcinoma cells were obtained from ATCC (Manassa, VA, USA). Cell culture media were purchased from Sigma-Aldrich. HEK-293 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 4.5 g/L glucose, 100 U/mL penicillin/streptomycin, 2 mM L-glutamine, 10 mM HEPES, pH 7.4, and 10% MEM non-essential amino acid solution. A549 cells were grown in Kaighn's Modification of Ham's F-12 medium (F-12K medium) completed with 10% fetal bovine serum and 100 U/mL penicillin/streptomycin.

Lipofectamine RNAiMax (Thermo Fisher Scientific, Waltham, MA, USA) was used for small interfering RNA (siRNA) delivery. Cells (150′000/6-well) were reverse transfected with 100 pmol siRNA against PP1α and/or 50 pmol siRNA against GSK-3 and 3.75 μL lipofectamine reagent for 64–66 h. The target sequences recognized by the siRNAs are: mock (negative control siRNA with a randomly selected non-targeting sequence) 5′-UGGUUUACAUGUUUUCUGA-3′, PPP1CA (PP1α) 5′-CAAGAGACGCUACAACAUC-3' (both from Microsynth AG, Balgach, Switzerland); GSK3A (L-003009-00-0005) 5′-UCACAAGCUU UAACUGAGA-3′, 5′-GAAGGUGACCACAGUCGUA-3′, 5′-GAGUUCAAG UUCCCUCAGA-3′, 5′-CUGGACCACUGCAAUAUUG-3′ and GSK3B (L-003010-00-0005) 5′-GAUCAUUUGGUGUGGUAUA-3′, 5′-GCUAGAUCA CUGUAACAUA-3′, 5′-GUUCCGAAGUUUAGCCUAU-3′, 5′-GCACCAGAG UUGAUCUUUG-3' (all from Dharmacon ON-TARGET plus SMART POOL; Dharmacon, Lafayette, CO, USA).

To determine effects of PP1α knockdown on endogenous GC-induced transcripts, A549 cells were cultured in steroid-free medium for 16–18 h prior to incubation with increasing concentrations of cortisol (18.75 nM–1200 nM) or vehicle (DMSO) for 4 h. To study effects of kinase inhibitors on PP1α-dependent downregulation of GC-induced genes, A549 cells were treated with indicated inhibitors (10 μM, except for GSK-3 inhibitor (5 μM)) in serum-free medium for 16–18 h prior to incubation with cortisol (500 nM) or vehicle (DMSO) for another 4 h. Following treatments, total RNA was isolated and quantitative polymerase chain reaction performed to quantify *GILZ* (glucocorticoid-induced leucine zipper), *IGFBP1* (insulin-like growth factor binding protein 1) and *SDPR* (serum deprivation-response protein).

Cellular fractionation and phosphorylation of GR was assessed by pre-incubating the cells with steroid-free medium overnight following treatment of cortisol for another 1 h prior to cell lysis. Cellular fractionation experiments in HEK-293 and A549 cells were performed using 50 nM and 500 nM cortisol, respectively. GR phosphorylation in A549 cells was analyzed in the presence of 10 nM and 50 nM cortisol. In all cell treatments, the final concentration of DMSO did not exceed 0.05%.

2.3. GR-dependent reporter gene assay

HEK-293 cells (100'000 cells/well) were seeded in poly-L-lysine coated 24-well plates, incubated for 24 h and co-transfected by calcium phosphate precipitation with the reporter gene TAT3-TATA luciferase

(0.375 μg/well), pCMV-Renilla constitutive luciferase transfection control (0.03 μg/well) and the indicated plasmids coding for human GRα, Flag-PP1α and Myc-MDM2 (at a ratio of 1:4:4). Empty vector pcDNA3.1 was supplemented to equalize the total amount of DNA in the transfection. After 4 h, cells were washed with phosphate-buffered saline (PBS) and incubated in DMEM for another 18 h. Cells were then adapted to charcoal-treated DMEM (cDMEM) for 2 h. Cells were exposed 24 h post-transfection to DMSO control, cortisol (0.1–100 nM) or mifepristone (RU-486; 1 μM), followed by incubation for another 24 h. Cells were then lysed in passive lysis buffer (Promega, Madison, WI, USA) and fire fly and Renilla luciferase activity were determined according to the Dual-Luciferase® reporter assay kit (Promega, Madison, WI, USA) using a SpectraMax-L luminometer (Molecular Devices, Devon, UK).

2.4. RNA isolation and quantitative polymerase chain reaction (RT-qPCR)

For isolation of total RNA, the RNeasy Mini Kit (QIAGEN, Hilden, Germany) was applied on a QIAcube extraction robot (QIAGEN) according to the manufacturer. The extracted RNA was treated with the RapidOut DNA Removal Kit (Thermo Fisher Scientific) and complementary DNA (cDNA) was subsequently synthesized using the GoScript Reverse Transcription System (Promega) following the manufacturer's protocol. RT-qPCR comprising 40 cycles of 95 °C for 10 s, 60 °C for 15 s, followed by a final extension at 72 °C for 20 s, and a dissociation curve (72 °C–95 °C) was performed in technical triplicate for each sample in the Rotor-Gene Q (QIAGEN) by using KAPA SYBR Fast qPCR Master Mix (2X) Kit (KAPA Biosystems, Woburn, MA, USA). For relative quantification, expression levels were normalized to those of the endogenous control gene cyclophilin A (*CYPA*) according to the comparative $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001). *CYPA* was chosen as housekeeping gene since its expression did not alter between experimental conditions and complied with the quality criteria for reference genes (Taylor et al., 2010). Table 1 shows sequences of oligonucleotide primers.

2.5. Western blot analysis

Upon treatment, cells were washed in PBS and lysed for 30 min on ice in RIPA buffer (Sigma-Aldrich) containing protease inhibitor cocktail (Roche, Rotkreuz, Switzerland). All steps of the lysis process were performed at 4 °C, which was sufficient to drastically reduce the activity of phosphatases. An initial experiment showed no effect of phosphatase inhibitor cocktail (data not shown), therefore it was not added to the lysis buffer. After centrifugation (16′000 × g, 15 min, 4 °C), the supernatant was analyzed using a Pierce® biocinchonic acid protein assay kit (Thermo Fisher Scientific). Laemmli solubilization buffer (LSB; 60 mM Tris–HCl, 10% glycerol, 2% (w/v) sodium dodecyl sulfate, 0.01% bromophenol blue, pH 6.8) supplemented with 5% β-mercaptoethanol (Promega) was added and samples were boiled for 3 min. Total protein (20 μg) of whole-cell extracts was resolved on 8% or 12% bis-acrylamide gels by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). All proteins were transferred to Immun-Blot® polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA), which were subsequently blocked with 5% defatted milk in Tris-buffered saline (20 mM Tris buffer (pH 7.6), 150 mM NaCl) containing 0.1% Tween-20 (TBS-T) for 1 h. Membranes were incubated with the indicated primary antibodies in blocking solution overnight at 4 °C. After washing with TBS-T, the membranes were probed with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (A0168) or goat anti-rabbit secondary antibody (A0545; both 1:10′000, from Sigma-Aldrich) for 1 h at room temperature. Visualization and detection of the protein bands was performed on a Fujifilm ImageQuant™ LAS-4000 (GE Healthcare, Glattbrugg, Switzerland) using the Immobilon Western Chemiluminescent HRP substrate kit (Merck, Kenilworth, NJ, USA). Quantitation of at least two independent experiments was done by using NIH ImageJ software and densitometry values were corrected for loading differences by normalization to those of β-actin or CYPA housekeeping control.

Table 1
Sequences of gene-specific primers used for RT-qPCR.

Gene name	Forward primers (5′-3′)	Reverse primers (5′-3′)
CYPA	CATCTGCACTGCCAAGACTGA	TGCAATCCAGCTAGGCATG
GILZ	ACAAGATCGAACAGGCCATG	TTGCCAGGGTCTTCAACAG
IGFBP1	CCATGTCACCAACATCAAAAA	TCGTAGAGAGTTTAGCCAAGGC
SDPR	AAGAGCGCATGGATAGGCAG	GTTTCACAAACACGCTGGCA

The following primary antibodies were used: Anti-GR antibody (1:2′000; sc-1003; validated earlier (Mani et al., 2016)), anti-PP1α antibody (1:5′000; sc-271762), and anti-β-actin antibody (1:10′000; sc-47778; all from Santa Cruz Biotechnology, Dallas, TX, USA); anti-pSer134 GR antibody (1:2′000; 85060), anti-pSer211 GR antibody (1:2′000; 4161; verified earlier (Oakley et al., 2017)), anti-pSer226 GR antibody (1:10′000; 97285), and anti-GSK-3 antibody (1:5′000; 5676; all from Cell Signaling Technology, Danvers, MA, USA); anti-pSer203 GR antibody (1:2′000; orb127112; Biorbyt Ltd., Cambridge, United Kingdom); anti-Flag antibody (1:2′000; MA1-91878; Thermo Fisher Scientific), anti-pSer404 GR antibody (1:1′000; described earlier (Galliher-Beckley et al., 2008)), anti-CYPA antibody (1:10′000, ab41684; Abcam Inc, Cambridge, United Kingdom).

2.6. Cellular fractionation

Treated cells were washed with PBS, scraped from culture dishes on ice and centrifuged at 500 × g for 5 min at 4 °C. Cell pellets were resuspended in ice-cold lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, pH 7.6) supplemented with protease inhibitor cocktail (Roche) and 1 mM dithiothreitol (DTT, AppliChem). After incubation for 15 min, cells were permeabilized with 0.57% IGEPAL CA-630 (Sigma-Aldrich), vortexed vigorously for 10 s and centrifuged at 8′000 × g for 5 min at 4 °C. The supernatant was collected as cytosolic fraction and clarified by centrifugation (16′000 × g, 5 min, 4 °C) following protein quantification. After removing the cytosolic fraction, the pellet was washed twice with PBS, centrifuged (800 × g, 5 min, 4 °C) and the supernatant discarded. The pellet was resuspended in extraction buffer (20 mM HEPES, 1.5 mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 25% (v/v) glycerol, pH 7.9) supplemented with protease inhibitor cocktail (Roche) and 1 mM DTT for 20 min at 4 °C and 1′400 rotations/min on an orbital shaker (Thermomixer, Vaudaux-Eppendorf, Buchs, Switzerland). The homogenate was centrifuged at 21′000 × g for 5 min at 4 °C and the supernatant removed as nuclear fraction, followed by protein quantification. Nuclear and cytoplasmic fractions were electrophoretically separated and blotted to PVDF membranes. Membranes were probed with anti-β-actin or anti-α-tubulin (1:10′000; GTX628802; GeneTex, Irvine, CA, USA) antibodies as cytoplasmic markers and anti-HDAC1 antibody (1:5′000; 5356; Cell Signaling Technology) as nuclear marker.

2.7. Co-immunoprecipitation

For co-immunoprecipitation experiments using Pierce™ NHS-Activated Agarose Slurry (Thermo Scientific), anti-GR antibody (sc-1003, Santa Cruz Biotechnology) was immobilized on beads according to the manufacturer. Cells suspended in lysis buffer (Atanasov et al., 2008) and proteins (1000 μg) were immunoprecipitated with antibody-coupled beads overnight at 4 °C. As a control of antigen-antibody binding specificity, lysates were incubated with rabbit IgG antibody (sc-2027, Santa Cruz Biotechnology). After elution of the precipitated protein, Western blot analysis was performed.

Co-immunoprecipitation with Pierce™ Protein A/G Plus Agarose (Thermo Scientific) was performed as described previously (Petrillo et al., 2019). Briefly, 1000 μg of whole-cell extract proteins were incubated with anti-GR antibody (3660, Cell Signaling Technology) or anti-PP1α antibody (sc-271762, Santa Cruz Biotechnology) overnight at 4 °C. Rabbit IgG antibody (12–370, Merck, Darmstadt, Germany) or mouse IgG antibody (sc-2025, Santa Cruz Biotechnology) was used as a control of non-antigen specific binding. Beads were added to each sample for another 3 h at 4 °C. The beads were washed twice with Co-IP buffer (50 mM Tris buffer, pH 7.6, 150 mM NaCl, 1% NP-40, 10% glycerol, 5 mM $MgCl_2$) containing protease inhibitor cocktail and 1 mM phenylmethanesulfonyl fluoride (PMSF; AppliChem) and twice with PBS. After eluting the proteins from the beads by adding LSB supplemented with 5% β-mercaptoethanol and heating for 5 min at 99 °C, they were subjected to Western blot analysis.

2.8. Statistical analysis

Statistical evaluation was conducted in GraphPad Prism version 5.0. Statistical significance of differences between treatments was calculated using an unpaired two-tailed Student's *t*-test, one-way ANOVA followed by Tukey's multiple comparisons test or two-way ANOVA followed by Bonferroni's multiple comparisons test to adjust the false discovery rate. Values represent mean ± SD and levels of significance are: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$.

3. Results

3.1. PP1α stimulates GR activity independently of MDM2

Although PP1α was found to enhance the activity of AR and MR through a mechanism involving MDM2-dependent control of receptor protein stability (Liu et al., 2016; Nagarajan et al., 2017), it remained unclear whether PP1α would stimulate GR activity and if so by which mechanism. To investigate the effect of PP1α on GR activity, HEK-293 cells were transiently transfected with human GR, PP1α and a GR-dependent luciferase reporter gene. An excess of PP1α over GR expression plasmid of 4:1 optimally stimulated GR-dependent luciferase reporter activity. Co-transfection with PP1α significantly increased basal and cortisol-dependent GR activity, by a cortisol concentration-dependent manner (Fig. 1A). In PP1α-overexpressing cells, the maximal GR transcriptional activity was observed when cells were treated with 3.7 nM cortisol, resulting in a 7-fold higher GR-mediated luciferase activity compared with cells expressing GR alone. In the absence of PP1α, maximal GR activity was detected in the presence of 30 nM cortisol. PP1α overexpression significantly affected the affinity of GR for cortisol ($EC_{50\ GR} = 5.9 \pm 2.8$ nM; $EC_{50\ GR+PP1\alpha} = 1.4 \pm 0.1$ nM; P value = 0.01). To exclude non-specific effects of PP1α on the promoter, HEK-293 cells transiently expressing GR and PP1α were treated with the GR antagonist mifepristone (RU-486) in the absence or presence of cortisol (Suppl. Fig. 1A). As mifepristone is known to exhibit also some weak agonistic effects (Jewell et al., 1995; Qi et al., 1990; Zhang et al., 2007), co-expression with PP1α slightly increased the activity of GR upon incubation with mifepristone. However, in the presence of cortisol, the pronounced PP1α-dependent activation was almost completely reversed by co-treatment with mifepristone. Furthermore, in the absence of GR, *i.e.* in HEK-293 cells transfected with the GR-dependent luciferase reporter gene and PP1α alone, no significant induction of the reporter by PP1α was observed in both the absence and presence of cortisol (Suppl. Fig. 1B). This indicates that the PP1α-dependent stimulation is mediated via GR.

Since the effects of PP1α on MR and AR were assumed to be mediated by inhibition of MDM2-dependent receptor degradation (Liu et al., 2016; Nagarajan et al., 2017), the impact of MDM2 on GR activity was assessed in HEK-293 cells expressing the respective recombinant proteins. Although MDM2 overexpression led to decreased GR-mediated reporter gene activity, the PP1α-dependent stimulation of GR transactivation could not be prevented, neither under basal (Fig. 1B) nor under cortisol-stimulated (Fig. 1C) conditions. In the basal state, PP1α enhanced the GR transactivation in the absence of MDM2 4.5-fold and in its presence 4-fold. Similarly, in the presence of 100 nM cortisol PP1α stimulated GR activity about 2-fold.

Furthermore, to test whether PP1α alters the translocation of cytosolic GR into the nucleus, nuclear and cytosolic fractions of HEK-293 cells transfected with GR in the absence or presence of PP1α were analyzed by Western blotting (Fig. 1D and E). As expected, cortisol treatment induced nuclear translocation of GR; however, PP1α overexpression significantly enhanced GR translocation by about 3 times when the cells were treated with cortisol (Fig. 1E).

3.2. PP1α knockdown decreases GR-dependent gene transcription in A549 cells

In order to support the stimulatory influence of PP1α on GR activity that was observed in the HEK-293 overexpression model, A549 human alveolar carcinoma cells endogenously expressing PP1α and GR were applied. PP1α was downregulated using siRNA, followed by detection of GR protein by Western blot analysis. PP1α protein was efficiently down regulated (Fig. 2A and B), but without significantly affecting GR protein expression (Fig. 2A and C). Next, cytosolic and nuclear fractions of A549 cells transfected with PP1α siRNA were analyzed by Western blotting (Fig. 2D and E), whereby no significant alterations of cytosolic and nuclear GR protein amounts could be detected. PP1α was found to be mainly located in the nucleus. In contrast, a homogenous distribution of PP1α between the cytoplasm and the nucleus along with an increased GR translocation was observed in HEK-293 cells upon overexpression of PP1α and GR (Fig. 1D and E). This difference to the A549 cells might be a result of limited availability of GR and PP1α associated regulatory proteins in HEK-293 cells. Nevertheless, these data suggest that the PP1α-dependent GR activation is not a cause of altered receptor protein stability and unlikely mediated through MDM2.

Furthermore, co-immunoprecipitation assays using A549 cell lysates were performed according to two different protocols using two antibodies against different epitopes on GR in order to determine whether PP1α and the GR interact directly. Moreover, pull-down experiments were conducted once with an antibody against GR (Suppl. Fig. 2A and 2B) and once with an antibody against PP1α (Suppl. Fig. 2C and 2D). Nevertheless, there was no evidence for a physical interaction between PP1α and GR, both in the absence (Suppl. Fig. 2A and 2C) and presence (Suppl. Fig. 2B and 2D) of cortisol.

Importantly, the cortisol-induced expression of the endogenous GR-responsive genes *IGFBP1* and *GILZ* was markedly downregulated, in a concentration-dependent manner, in PP1α siRNA transfected cells compared to mock siRNA transfected cells (Fig. 2F and G). In addition, using a different PP1α-specific siRNA confirmed the effect of PP1α silencing on the expression of *GILZ* and *IGFBP1* (Suppl. Fig. 3).

3.3. PP1α knockdown results in reduced GR-Ser211 phosphorylation

Since PP1α stimulated the activity of the GR but did not seem to influence its stability nor directly interact with the receptor, it was next assessed whether altered phosphorylation at four well-characterized sites on GR, namely serine residues 134, 203, 211 and 226, may affect the transcriptional response to GC. For this purpose, A549 cells were transfected with siRNA against PP1α, treated with 10 nM or 50 nM cortisol for 1 h, prior to cell lysis, and Western blot analysis using phospho-specific and total anti-GR antibodies was performed to determine the serine residues influenced (Fig. 3A). Silencing of PP1α did not affect GR phosphorylation of serine residues 134, 203 and 226 (Suppl. Fig. 4A–C), but reduced the phosphorylation status of Ser211 in a GC-independent manner (Fig. 3B). Influences on the phosphorylation

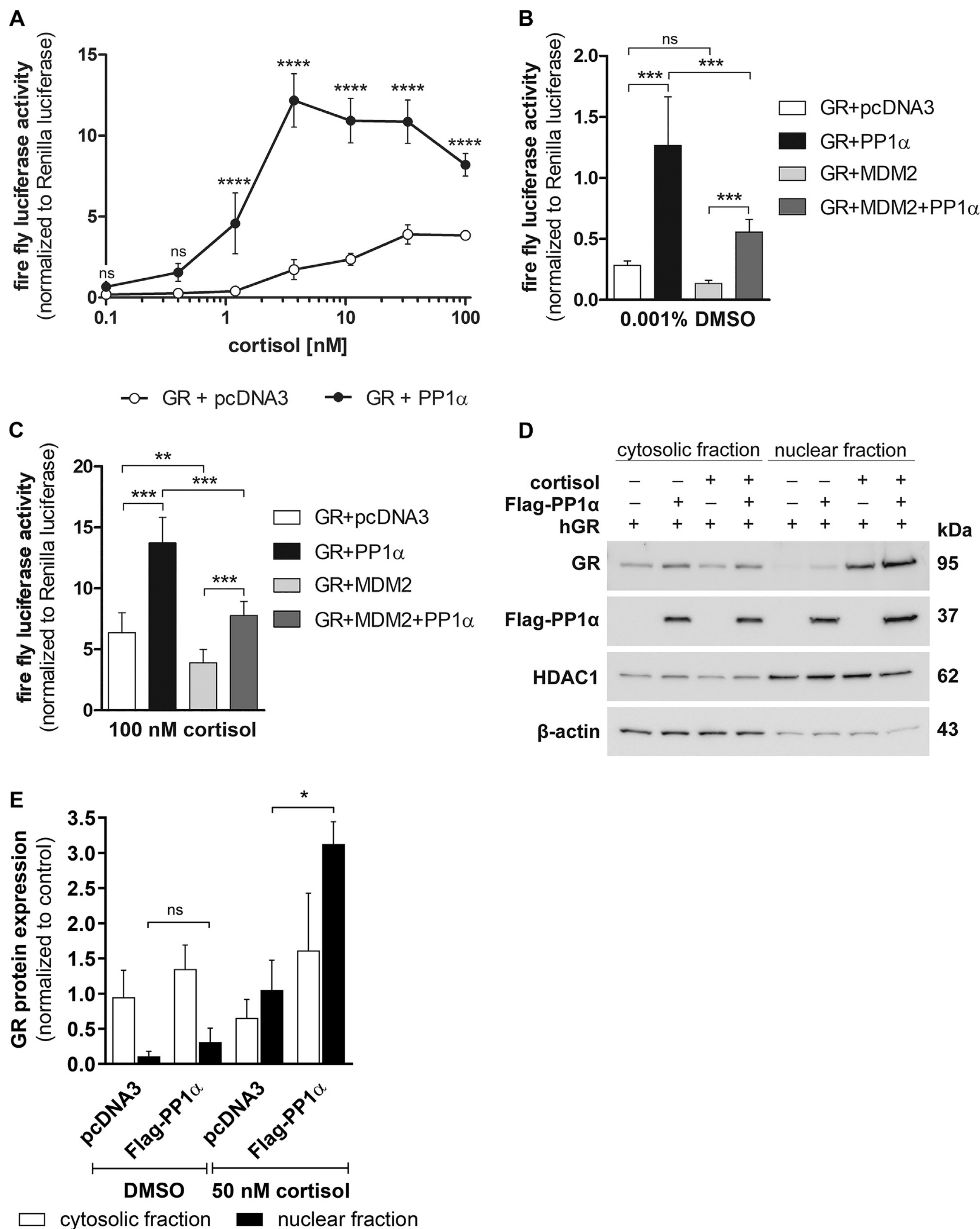

Fig. 1. Effect of PP1α overexpression on GR activity and nuclear translocation. (A, B and C) HEK-293 cells were transiently transfected with plasmids coding for GR with or without PP1α, a luciferase reporter gene and a Renilla luciferase transfection control. Empty vector pcDNA3.1 was supplemented to equalize the total amount of DNA in the transfection. After 24 h of transfection, cells were incubated with vehicle or increasing concentrations of cortisol (A) for another 24 h. The luciferase reporter activity was normalized to the internal Renilla control. (B), under basal conditions, and (C) under cortisol-stimulated conditions as in (A) but with co-transfection of MDM2. Data represent mean ± SD from at least two independent experiments, each performed in triplicate, $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, *ns* not significant. (D and E) HEK-293 cells were transfected with GR, with and without PP1α for 24 h, incubated overnight in charcoal-treated medium and then exposed to vehicle or 50 nM cortisol for 1 h. Cytosolic and nuclear fractions were analyzed by Western blot using antibodies against GR and the Flag-tag on PP1α. As controls, the fractions were reprobed with anti-β-actin (cytosolic) and anti-HDAC1 (nuclear) antibodies. A representative blot (D) and analysis of band density (E) from two independent experiments are shown. Values are depicted as mean ± SD, $^{*}P < 0.05$, *ns* not significant.

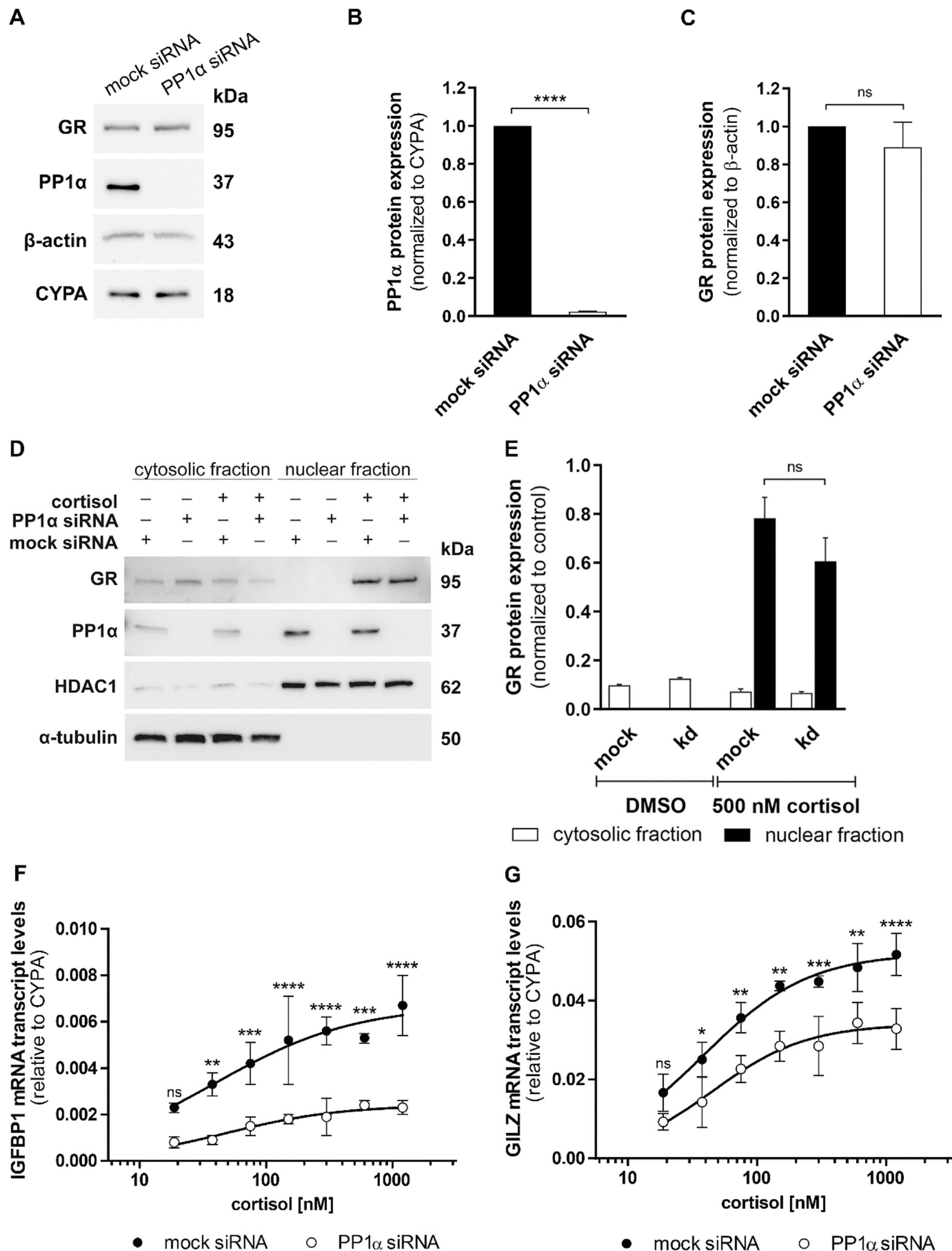

Fig. 2. Impact of PP1α knockdown on endogenous GR protein expression, translocation and GC-induced transcripts. (A, B and C) Western blot analysis for protein expression of GR and PP1α was performed after mock or anti-PP1α siRNA treatment in A549 cells. A representative blot (A) and densitometry analysis of PP1α (B) and GR (C) from three independent experiments are shown. Data are normalized to mock siRNA samples (mean ± SD, $^{****}P < 0.0001$, *ns* not significant). (D and E) A549 cells were transfected with mock or anti-PP1α siRNA for 48 h, incubated overnight in serum-free medium and treated with vehicle or 500 nM cortisol for 1 h. Western blot analysis for cytosolic and nuclear fractions was performed using antibodies against GR and PP1α. As controls, the fractions were reprobed with anti-α-tubulin (cytosolic) and anti-HDAC1 (nuclear) antibodies. A representative blot (D) and analysis of band density (E) from two independent experiments are shown. Values are presented as mean ± SD, *ns* not significant. (F and G) At 48 h after knockdown of PP1α, A549 cells were cultured in steroid-free medium overnight prior to incubation with vehicle or increasing concentrations of cortisol (18.75 nM–1200 nM) for 4 h. Cortisol-induced transcription of the GR-responsive genes *IGFBP1* (F) and *GILZ* (G) was measured by RT-qPCR in technical triplicate for each sample. Data are represented as $2^{-\Delta Ct}$ mean ± SD from three independent experiments, $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$, $^{*}P < 0.05$, *ns* not significant.

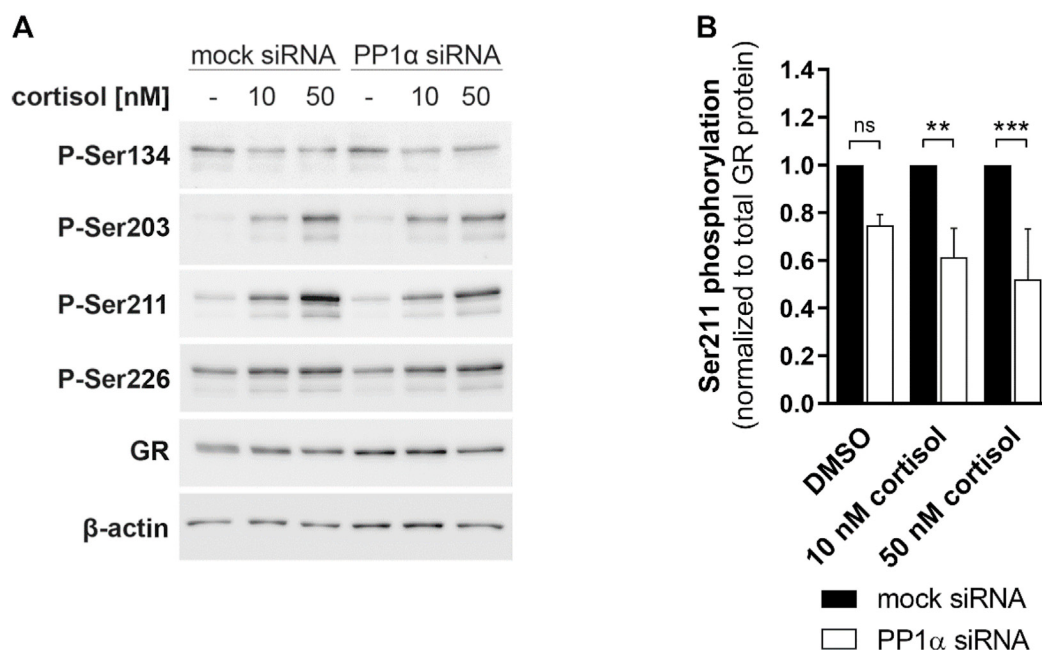

Fig. 3. Effect of PP1α knockdown on GR phosphorylation. A549 cells were transfected with mock or anti-PP1α siRNA for 48 h, incubated in serum-free medium for 16–18 h, treated with vehicle or cortisol (10 nM and 50 nM cortisol) for 1 h, followed by cell lysis and Western blot analysis. A representative Western blot image (A) and densitometry values (B) are presented. Levels of phosphorylated Ser211 from three independent experiments were normalized to total GR levels and are depicted as values normalized to mock siRNA control (mean ± SD, $^{***}P < 0.001$, $^{**}P < 0.01$, *ns* not significant).

of Ser404 were also tested. The antibody signal for GR-Ser404 was very weak and several other bands were detected, not allowing densitometry analysis; however, there was no evidence supporting altered phosphorylation upon PP1α knockdown (data not shown).

3.4. Impact of various kinase inhibitors on the PP1α-mediated effect on GR-induced transcripts

Next, in an attempt to start to understand the mechanism underlying the influence of PP1α on GR-Ser211 phosphorylation, A549 cells transfected with mock or anti-PP1α siRNA were exposed to inhibitors of several known kinases. The mRNA expression levels of the two GR-responsive genes *IGFBP1* and *GILZ* were then measured in order to identify the kinase(s) involved in the PP1α-dependent GR stimulation. In both, the basal and cortisol-activated state, a significant decrease in the *GILZ* and *IGFBP1* mRNA expression was observed in cells transfected with siRNA against PP1α compared to mock siRNA treated cells (Fig. 4A–D). This effect of PP1α knockdown was retained when the cells were exposed to inhibitors of p38, MEK1/2, JNK and Akt1/2. In contrast, the PP1α-mediated effect on both genes was reversed by treatment with the GSK-3 inhibitor, *i.e.* expression levels in mock treated cells were reduced to those of cells transfected with anti-PP1α siRNA, indicating an involvement of GSK-3 in the PP1α-dependent GR stimulation. However, *IGFBP1* expression was abrogated in the presence of GSK-3 inhibitor. *IGFBP1* carries a thymine-rich insulin response element (TIRE) in its promoter region (Finlay et al., 2004), implying that GSK-3 has to be active for efficient gene transcription. Therefore, *IGFBP1* is not appropriate to demonstrate the impact of GSK-3 on the PP1α-mediated GR activation, and another GR-dependent gene, *SDPR*, was analyzed under both basal and cortisol-stimulated conditions (Fig. 4E and F). The results support an involvement of GSK-3 in the PP1α-related GR modulation (see Fig. 6 for an overview of the proposed mechanism). It needs to be noted that, when comparing mock transfected cells incubated with DMSO or cortisol vs inhibitors, p38 and MEK1/2 inhibitors did not exert a pronounced effect on *GILZ* and *IGFBP1* gene expression levels, whereas JNK and Akt1/2 inhibitors markedly increased the expression levels of these genes in both basal and cortisol-stimulated state. This suggests that JNK and Akt1/2 pathways are active in A549 cells and their inhibitors influenced *GILZ* and *IGFBP1* expression independent of PP1α.

3.5. Inhibition or knockdown of GSK-3 abrogates the PP1α-dependent effect on GR-Ser211 phosphorylation

To assess whether pharmacological inhibition of GSK-3 can diminish the PP1α-dependent effect on GR-Ser211 phosphorylation, A549 cells were treated with GSK-3 inhibitor, followed by Western blot analysis. Upon cortisol stimulation in mock siRNA transfected cells, GSK-3 inhibition significantly decreased GR-Ser211 phosphorylation, reducing the phosphorylation level to that observed in the absence of PP1α, *i.e.* in anti-PP1α siRNA treated cells (Fig. 5A and B), suggesting that GSK-3 mediates the PP1α-dependent activation of GR. To further support a role for GSK-3 and exclude an off-target effect of the GSK-3 kinase inhibitor as well as to examine the involved GSK-3 isoform, isoform-specific anti-GSK-3 siRNAs were applied under cortisol-stimulated conditions (Fig. 5C and D). Whereas silencing of either GSK-3α or GSK-3β both only partially reversed the effect of PP1α knockdown on Ser211 phosphorylation, the combination of both siRNAs fully prevented it. These results imply that GSK-3 inhibition could prevent the effect of PP1α on Ser211 phosphorylation (see Fig. 6 for an overview of the proposed mechanism).

4. Discussion

The cell- and tissue-specific sensitivity towards GC plays a pivotal role in the fine-tuned regulation of physiological functions as well as for the response to pharmacological doses in the treatment of pathological conditions. Phosphorylation at multiple sites belongs to the most important mechanisms allowing a rapid modulation of the sensitivity of the GR towards GC. The basal and ligand-stimulated phosphorylation of the GR is regulated by various kinases and phosphatases, and an impaired phosphorylation can result in hypersensitivity or resistance to GC, thereby contributing to diseases (Bouazza et al., 2012; DeFranco et al., 1991; Galliher-Beckley et al., 2008, 2011; Habib et al., 2017; Itoh

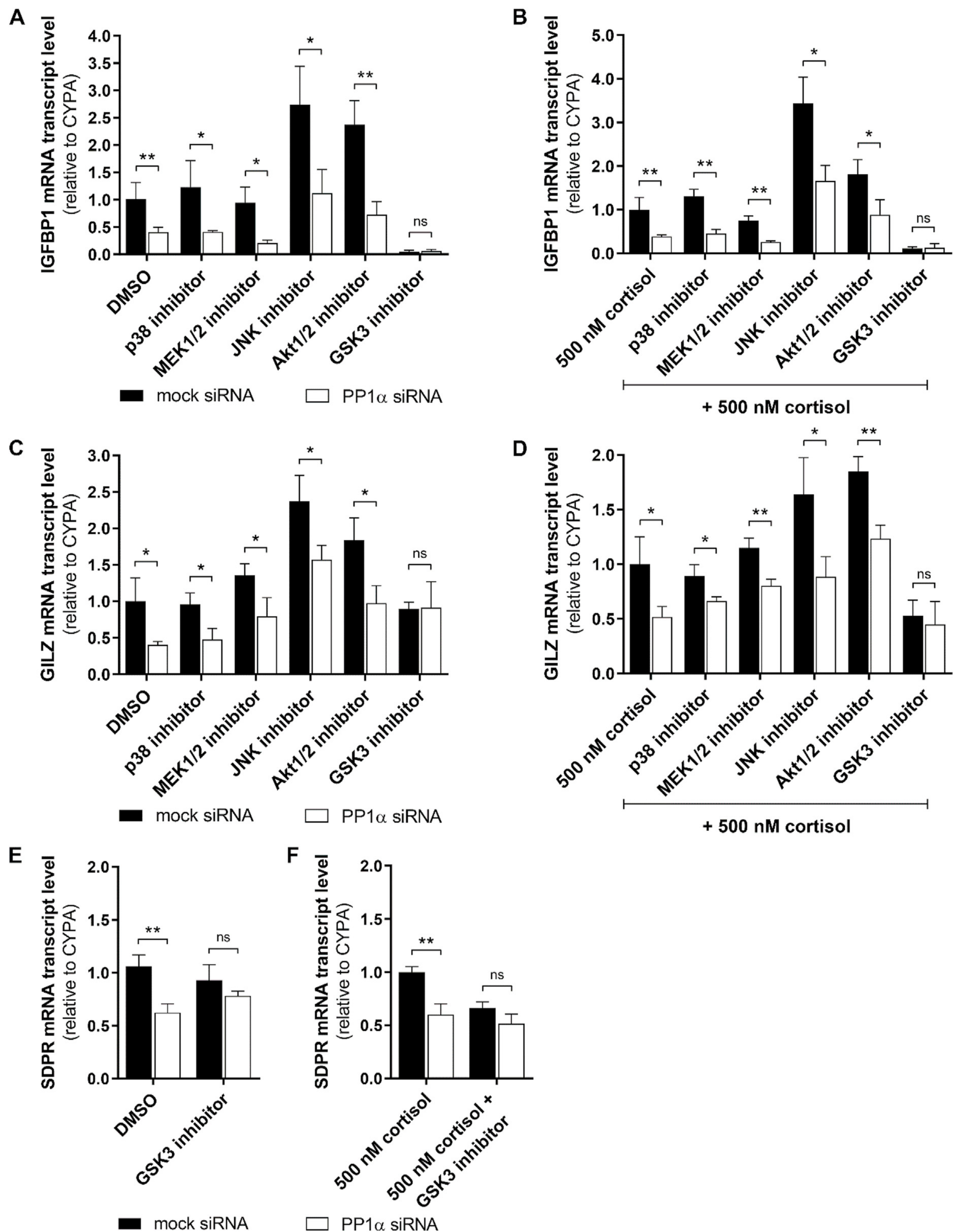

Fig. 4. Effect of various kinase inhibitors on the PP1α-dependent effect on GR-induced transcripts. A549 cells were transfected with mock or anti-PP1α siRNA for 48 h, incubated overnight with indicated inhibitors (10 μM; except for GSK-3 inhibitor, 5 μM) in serum-free medium prior to further incubation with vehicle (A, C and E) or 500 nM cortisol (B, D and F) for another 4 h. Expression levels of GR-responsive genes *IGFBP1* (A and B), *GILZ* (C and D) and *SDPR* (E and F) were analyzed by RT-qPCR. Values are relative to those of the endogenous control gene using the comparative $2^{-\Delta Ct}$ method, followed by normalization to mock siRNA samples. Data are presented as mean ± SD from three independent experiments, each performed in technical triplicate. $^{**}P < 0.01$, $^{*}P < 0.05$, *ns* not significant.

et al., 2002; Kino et al., 2007; Kobayashi et al., 2011; Krstic et al., 1997; Miller et al., 2005; Pazdrak et al., 2016; Rogatsky et al., 1998a; Rogatsky et al., 1998; Webster et al., 1997;).

PP1α is a ubiquitously expressed protein phosphatase that regulates a vast variety of cellular processes by dephosphorylation of serine/threonine-phosphorylated protein substrates, including cell cycle progression, protein synthesis, mRNA splicing and transcription, calcium signaling and carbohydrate metabolism (Ceulemans and Bollen, 2004;

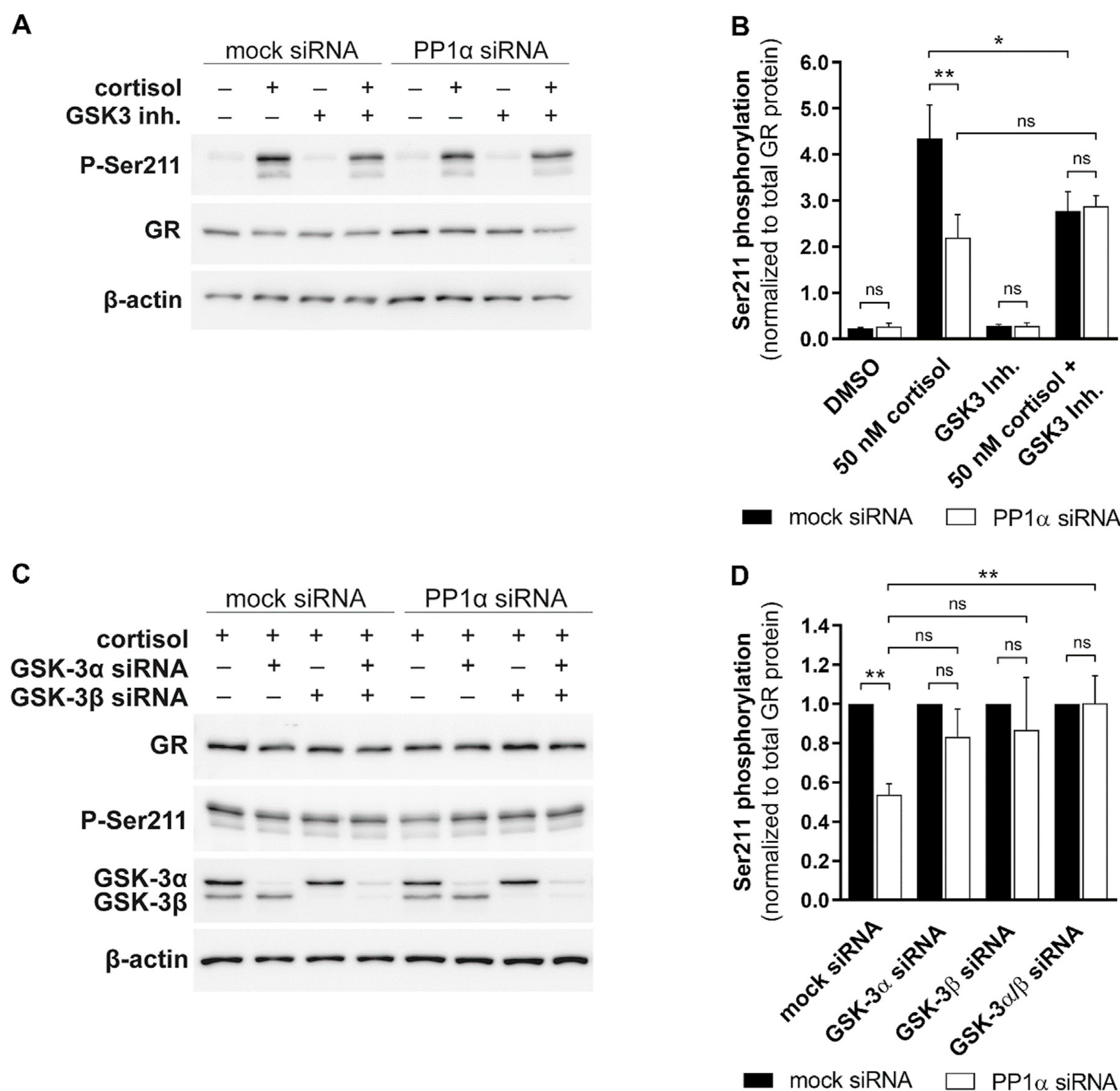

Fig. 5. Effect of GSK-3 on the PP1α-mediated impact on GR-Ser211 phosphorylation. (A and B) After treatment with mock or anti-PP1α siRNA, A549 cells were incubated overnight with 5 μM GSK-3 inhibitor in serum-free medium prior to further incubation with vehicle or 50 nM cortisol for another 1 h. Western blot analysis for expression of total GR protein and Ser211 phosphorylation was performed. A representative blot (A) and densitometry values of Ser211 phosphorylation (B) from two independent experiments normalized to total GR are shown. (C and D) A549 cells were co-transfected with siRNA against PP1α and/or GSK-3α and/or GSK-3β for 48 h, incubated overnight in serum-free medium prior to treatment with 50 nM cortisol for another 1 h. Western blotting for expression of total GR protein and Ser211 phosphorylation was conducted. A representative blot (C) and densitometry analysis of Ser211 phosphorylation (D) from three independent experiments normalized to total GR is presented as values normalized to mock siRNA control. Data represent mean ± SD, $^{**}P < 0.01$, $^{*}P < 0.05$, *ns* not significant.

Cohen, 2002). Despite its multiple functions, the impact of PP1α on the modulation of GR activity has not yet been investigated. The current study assessed whether PP1α can stimulate GR function and tested two different hypotheses: First, that PP1α regulates GR activity through suppression of MDM2 activity by dephosphorylating it at Ser166, thereby reducing the MDM2-mediated ubiquitination of GR and the subsequent proteasomal degradation of the receptor, as shown for the MR and AR (Liu et al., 2016; Nagarajan et al., 2017); and second, that PP1α directly dephosphorylates the GR at a particular site to relieve functional repression as demonstrated for PP2A and PP5 (Bouazza et al., 2012; Kobayashi et al., 2011; Pazdrak et al., 2016).

The results revealed a stimulating effect of PP1α on GR activity in HEK-293 cells overexpressing recombinant proteins and a GR-dependent luciferase reporter (Fig. 1A), as well as in A549 cells expressing endogenous GR and PP1α levels by PP1α silencing and assessment of the expression of the GC-target genes *IGFBP1* and *GILZ* (Fig. 2F and G). In addition, PP1α overexpression seems to enhance the affinity of GR to cortisol (Fig. 1A). In the absence of ligand, only few receptor molecules exist in the active conformation. The GR might adopt a low affinity antagonist state wherein helix 12 occupies a position covering the coactivator binding site and in which the steroid binding pocket remains closed (Frego and Davidson, 2006; Hu et al., 2011; Kauppi et al., 2003; Quax et al., 2013). Upon ligand binding, the number of receptors in the active conformation increases, indicating that the position of helix 12 enables interaction with coactivator proteins and consists of an open ligand binding pocket. Co-expression with PP1α might further augment the number of receptors in the active conformation, indirectly by promoting phosphorylation of Ser211, leading to increased ligand binding and a shift to higher affinity.

MDM2 has been shown to control the expression of several steroid receptors including the GR (Sengupta and Wasylyk, 2001). In line with previous observations, co-transfection with MDM2 in HEK-293 cells was found to decrease GR transcriptional activity, both in the basal and cortisol-activated state. However, MDM2 overexpression could not

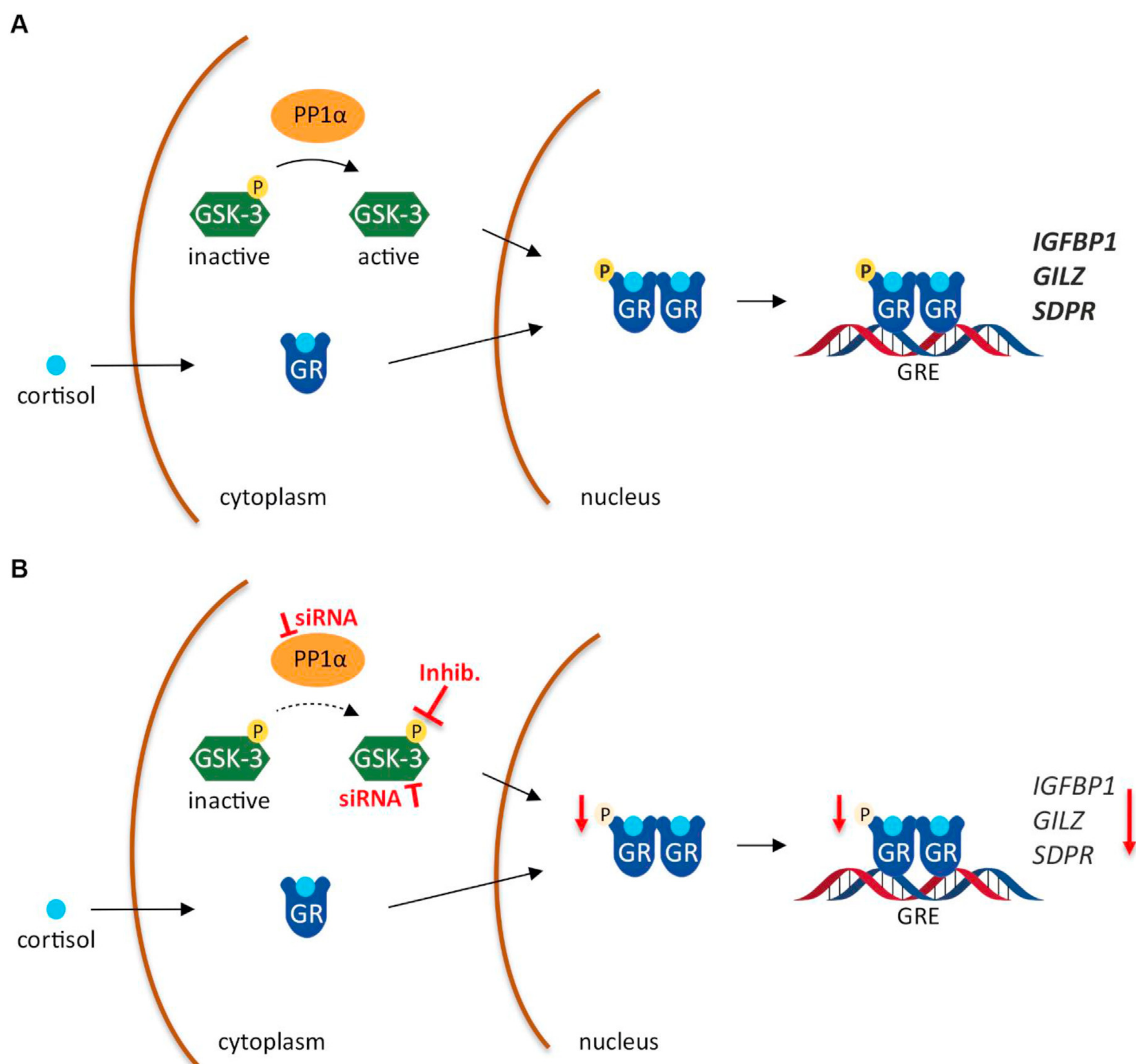

Fig. 6. Schematic representation of the modulation of GR activity by PP1α and GSK-3. (A) Upon entering the cell, cortisol binds to GR and leads to receptor dimerization. For gaining full activity, GR is phosphorylated at Ser211 to activate the transcription of target genes such as *IGFBP1*, *GILZ* and *SDPR*. Phosphorylation of Ser211 is mediated, at least in part, by a mechanism involving active GSK-3. PP1α dephosphorylates and activates GSK-3. (B) Knockdown of PP1α prevents GSK-3 activation, and inhibition as well as knockdown of GSK-3 diminishes GR-Ser211 phosphorylation, its activation and stimulation of target gene transcription.

prevent the PP1α-mediated GR activation, with similar relative increases of GR activity in the absence or presence of MDM2. This indicates that PP1α stimulates GR activity by an MDM2-independent mechanism. Moreover, knockdown of PP1α in A549 cells revealed reduced GR-dependent gene expression but did not result in decreased GR protein expression levels, further supporting that PP1α-dependent GR stimulation is independent of MDM2 modulated receptor stability and differs from the mechanism observed for MR and AR (Liu et al., 2016; Nagarajan et al., 2017).

Furthermore, proximity ligation assays suggested an interaction between PP1α and MR, and immunoprecipitation analysis indicated that PP1α interacts with the LBD of the AR (Liu et al., 2016; Nagarajan et al., 2017). In this study, co-immunoprecipitation assays failed to detect a direct interaction between GR and PP1α, further favoring a different mechanism compared to MR and AR. Nevertheless, methods to detect weak and transient interactions such as proximity ligation assays should be performed in follow-on experiments.

To achieve its transcriptional activity, the GR has to translocate from the cytoplasm into the nucleus. Analysis of nuclear and cytosolic fractions of HEK-293 cells overexpressing recombinant GR and PP1α revealed a slight but significantly enhanced cortisol-induced GR translocation from the cytoplasm to the nucleus in the presence of PP1α (Fig. 1D and E). In contrast, the amount of GR in the nuclear fraction was not affected by PP1α silencing in A549 cells expressing endogenous receptor levels (Fig. 2D and E). This difference in the nuclear import of the GR between the two cell lines might derive from the presence or absence of receptor-associated co-regulators. Furthermore, it should be noted that while the overexpressed PP1α in HEK-293 cells shows an equal distribution between cytoplasm and nucleus, endogenous PP1α in A549 cells is mainly located in the nucleus. This suggests that subcellular localization less likely is a major factor contributing to the PP1α-dependent GR activation.

The site-specific phosphorylation of GR regulates its transcriptional activity (Galliher-Beckley and Cidlowski, 2009; Zhou and Cidlowski, 2005). Earlier studies reported that phosphorylation of GR-Ser211 enhances the activity of the receptor, whereas phosphorylation of serine residues 134, 203, 226 and 404 is associated with reduced GR function (Chen et al., 2008; Galliher-Beckley et al., 2008, 2011; Habib et al., 2017; Kino et al., 2007; Krstic et al., 1997; Miller et al., 2005, 2007; Rogatsky et al., 1998a,b; Takabe et al., 2008; Wang et al., 2002). Thus, this study explored whether PP1α-dependent GR stimulation was due to an altered phosphorylation of GR at specific serine residues known to be

critical for its activity. The data obtained demonstrated that the level of GR-Ser211 phosphorylation significantly decreased after PP1α silencing (Fig. 3A and B) whereas all other phosphorylation sites tested were unaffected (Suppl. Fig. 4). Since phosphorylation of GR-Ser211 is essential for receptor activity, a reduced phosphorylation of this specific residue implicates a decreased transcriptional activity, which was supported by the diminished expression of GC-inducible genes in A549 cells upon PP1α knockdown (Fig. 2F and G). The involvement of GR-Ser211 phosphorylation supports the assumption that altered subcellular trafficking is a mechanism less likely contributing to the PP1α-dependent GR activation. Earlier findings have shown that mutation of murine GR at Ser220, the phosphorylation site corresponding to human Ser211, did not affect hormone-dependent nuclear translocation (Webster et al., 1997). The fact that PP1α altered the phosphorylation state of GR-Ser211, and bearing in mind that PP1α is a protein phosphatase, indicates an indirect effect of PP1α on GR, involving another protein.

The present study addressed whether PP1α might dephosphorylate a specific kinase, which in turn is activated, thereby phosphorylating GR at Ser211. Treatment of A549 cells with different inhibitors of known kinases indicated an involvement of GSK-3 in the PP1α-dependent regulation of GC-responsive genes (Fig. 4). Interestingly, inhibitors of JNK and Akt1/2 markedly enhanced mRNA expression levels of *GILZ* and *IGFBP1*. JNK was postulated to promote the nuclear export of GR accompanied by termination of GR-dependent transcription through phosphorylation of GR-Ser226 (Itoh et al., 2002), whereas Akt1 was found to delay GR nuclear translocation and reduce GR transcriptional activity by phosphorylation of GR at Ser134 (Habib et al., 2017). Consequently, inhibition of these kinases may result in a decrease in the inhibitory phosphorylation of GR-Ser226 and GR-Ser134, respectively, ultimately leading to increased GR-dependent transcription, as observed in Fig. 4A–D.

Inhibition of GSK-3 prevented the PP1α-mediated difference in the expression levels of *GILZ* and *SDPR* as well as completely abolished the effects on *IGFBP1*. *IGFBP1* contains a thymine-rich insulin response element (TIRE) in its promoter region which requires GSK-3 to be active for gene transcription (Finlay et al., 2004), providing an explanation for the repression of *IGFBP1* gene expression upon treatment of cells with GSK-3 inhibitor. To assess the role of GSK-3 in the PP1α-dependent effect on GR function, Western blot analysis was performed to determine GR-Ser211 phosphorylation levels. Treatment of A549 cells with GSK-3 inhibitor (Fig. 5A and B) and siRNA against GSK-3α/β (Fig. 5C and D) resulted in a diminished GR-Ser211 phosphorylation and an abrogated PP1α-mediated effect. Specific knockdown of either GSK-3α or GSK-3β partially abolished the PP1α-dependent effect, suggesting that the isoforms might compensate for each other and both are involved in the stimulatory effects of PP1α on GR activity.

GSK-3 is a serine/threonine protein kinase encoded by two genes GSK3A and GSK3B. The closely related isoforms α and β are highly homologous in their catalytic domains but differ significantly in the N- and C-terminal regions (Woodgett, 1990, 1991), suggesting that they could be differentially regulated. However, both proteins are similarly modified at the N-terminal region: phosphorylation of Ser21 on GSK-3α and Ser9 on GSK-3β (Sutherland et al., 1993; Sutherland and Cohen, 1994). PP1α is a known activator of GSK-3β by dephosphorylation of Ser9 (Morfini et al., 2004; Szatmari et al., 2005), and was shown to be engaged in a positive feedback loop with its inhibitor-2 and GSK-3β. GSK-3β can phosphorylate inhibitor-2 of PP1α at Thr72, resulting in PP1α activation, which in turn dephosphorylates and further stimulates GSK-3β (Szatmari et al., 2005; Zhang et al., 2003). It is also assumed that PP1α can dephosphorylate the inhibitory Ser21 on GSK-3α (Spokoini et al., 2010; Zhang et al., 2003). GSK-3 was originally identified as a protein capable of regulating glycogen synthase in order to inhibit glycogen synthesis (Embi et al., 1980) and since then has been described to phosphorylate a wide variety of substrates that are involved in many cellular processes, including glucose metabolism, cell differentiation and apoptosis (Ali et al., 2001; Forde and Dale, 2007; Soutar et al., 2010). In addition, GSK-3 was found to interact with the GR. In the absence of a ligand, GSK-3α is supposed to be sequestered to the GR and dissociates from it upon exposure to GC (Spokoini et al., 2010). So far, most studies focused on the β-isoform of GSK-3. Inhibition of GSK-3β was reported to suppress GR reporter gene activity and reduce mRNA expression levels of *GILZ*, implying that GSK-3β has a positive role in GR stimulation (Rubio-Patiño et al., 2012). However, GSK-3β was also proposed to phosphorylate GR-Ser404 and thereby decreasing its function (Galliher-Beckley et al., 2008). Further studies are required to clarify the role of GSK-3 in GR regulation, considering cell type-specific differences. Furthermore, most of the GSK-3 substrates need to be primed by pre-phosphorylation at a specific serine-proline site prior to recognition by GSK-3 (Harwood, 2001). Up to now, such a priming site for GSK-3 within the GR has not been described. Signaling pathways are complex involving a number of components that can cross-talk with other signal transduction pathways. Further research needs to address whether GSK-3 is directly responsible for the PP1α-dependent phosphorylation of GR-Ser211 or whether the observed effect is mediated indirectly through another coregulator. Additionally, it cannot be excluded that GSK-3 dephosphorylation by PP1α is indirect and mediated by a PP1α regulatory protein.

Moreover, the physiological relevance of PP1α-mediated GR signaling needs to be examined. PP1α has been reported to be involved in the progression of several disease states, including memory loss, type II diabetes and cancer (Figueiredo et al., 2014; Ladha et al., 2010; Shastry et al., 2016). PP1α is assumed to suppress learning and memory and acts as a potential mediator of cognitive decline during aging (Genoux et al., 2002). Excessive GSK-3 activity has been associated with neurodegenerative and psychiatric disorders, and GSK-3 inhibitors as well as GR antagonists are discussed as new potential treatments of Alzheimer's disease (Canet et al., 2019; Martinez and Perez, 2008; Muyllaert et al., 2008). In addition, aberrant GC action and excessive activity of GSK-3 have been linked with obesity and type II diabetes (Henriksen and Dokken, 2006; Rose et al., 2010), and regulatory subunits of PP1 have been associated with insulin resistance (Ragolia and Begum, 1998; Xia et al., 1998). Finally, chronic GC treatment can lead to reduced sensitivity or even resistance in specific cell-types or tissues and underlying mechanisms have been mainly attributed to the impaired GR signaling pathway (Barnes, 2010; Yang et al., 2012). Due to the ability of PP1α to enhance GR activity, one could speculate that cells with impaired PP1α function may have an altered response to GC. Further elucidation of post-translational modification of GR is essential to understand mechanisms of aberrant GC action potentially leading to GC insensitivity.

In this study, PP1α was identified as novel regulator of GR, enhancing its activity but not affecting the expression of the receptor, both under basal and ligand-dependent conditions. Furthermore, the results provided a first insight into the molecular mechanism, proposing GR-Ser211 to be modulated through involvement of GSK-3 (Fig. 6). Thus, PP1α constitutes a new component of the GR signaling pathway, suggesting that impaired activity of PP1α in specific situations could alter GC action and contribute to diseases.

CRediT authorship contribution statement

Melanie Patt: Conceptualization, Visualization. **Joël Gysi:** Investigation, Formal analysis. **Nourdine Faresse:** Resources, Writing - review & editing. **John A. Cidlowski:** Resources, Writing - review & editing. **Alex Odermatt:** Conceptualization, Project administration, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors have nothing to disclose.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110873.

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