β-Arrestin-1 inhibits glucocorticoid receptor turnover and alters glucocorticoid signaling

Received for publication, December 13, 2018, and in revised form, May 30, 2019. Published, Papers in Press, June 5, 2019, DOI 10.1074/jbc.RA118.007150

Maria G. Petrillo, Robert H. Oakley, and John A. Cidlowski

From the Signal Transduction Laboratory, NIEHS, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina 27709

Edited by Henrik G. Dohlman

Glucocorticoids are among the most widely used drugs to treat many autoimmune and inflammatory diseases. Although much research has been focused on investigating glucocorticoid activity, it remains unclear how glucocorticoids regulate distinct processes in different cells. Glucocorticoids exert their effects through the glucocorticoid receptor (GR), which, upon glucocorticoid binding, interacts with regulatory proteins, affecting its activity and function. These protein–protein interactions are necessary for the resolution of glucocorticoid-dependent physiological and pharmacological processes. In this study, we discovered a novel protein interaction between the glucocorticoid receptor and β-arrestin-1, a scaffold protein with a well-established role in G protein–coupled receptor signaling. Using co-immunoprecipitation and in situ proximity ligation assays in A549 cells, we observed that β-arrestin-1 and unliganded GR interact in the cytoplasm and that, following glucocorticoid binding, the protein complex is found in the nucleus. We show that siRNA-mediated β-arrestin-1 knockdown alters GR protein turnover by up-regulating the E3 ubiquitin ligase Pellino-1, which catalyzes GR ubiquitination and thereby marks the receptor for proteasomal degradation. The enhanced GR turnover observed in β-arrestin-1–deficient cells limits the duration of the glucocorticoid response on GR target genes. These results demonstrate that β-arrestin-1 is a crucial player for the stability of the glucocorticoid receptor. The GR/β-arrestin-1 interaction uncovered here may help unravel mechanisms that contribute to the cell type–specific activities of glucocorticoids.

Glucocorticoids (GCs) are lifesaving drugs that are widely prescribed in the treatment of inflammatory diseases and other conditions requiring suppression of the immune system (1). These steroids are physiologically synthesized in the zona fasciculata of the adrenal cortex as end products of the hypothalamic-pituitary-adrenal axis in response to a variety of stress-producing stimuli. Both physiological and pharmacological actions of glucocorticoids occur through the binding to the glucocorticoid receptor (GR; gene ID: NR3C1, nuclear receptor subfamily 3, group C, member 1), a transcription factor that, like other members of the nuclear receptor family, functions as a ligand-activated transcriptional gene expression regulator (2). Unliganded GR is located in the cytoplasm associated in a heterocomplex with chaperone proteins that favor GR maturation and protect the receptor from its degradation (3, 4). Upon steroid binding, GR undergoes activation, dissociates from the chaperone complex, and exerts its effects via nongenomic and genomic mechanisms (5). The latter requires the translocation of the receptor to the nucleus, where it can bind either to specific nucleotide sequences on DNA called glucocorticoid-responsive elements (GREs), or it can involve the tethering of GR with other transcription factors (1). Throughout its intracellular journey, liganded GR creates physical contacts with a multitude of regulatory proteins, thus affecting its activity and function (6).

β-Arrestin-1 (gene ID: ARRB1) and its related family member β-arrestin-2 (gene ID: ARRB2) were first described as negative regulators of G protein–coupled receptors (GPCRs) (7). However, β-arrestin proteins are now known to exert other actions that go beyond their conventional role of GPCR terminators. Indeed, β-arrestins have been reported to take part in a variety of functions, including protein trafficking, protein subcellular redistribution, transcriptional regulation, and protein post-translational modifications, thus affecting cell proliferation, differentiation, and apoptosis signaling (8).

We have previously shown that both β-arrestin-1 and β-arrestin-2 are glucocorticoid-responsive genes (9). Glucocorticoid treatment induced β-arrestin-1 and repressed β-arrestin-2 expression, suggesting that a cross-talk between the glucocorticoid receptor and the β-arrestin proteins may occur. β-Arrestin proteins have been reported to interact with other members of the nuclear receptor superfamily, such as the androgen receptor (AR) (10). Furthermore, β-arrestin-1 contributes to internalizing the membrane-bound estradiol receptor; DKD, double knockdown; FAIRE, formaldehyde-assisted isolation of regulatory elements; AR, androgen receptor; PMSF, phenylmethylsulfonfyl fluoride; DAPI, 4′,6-diamidino-2-phenylindole.
**β-Arrestin-1 modulates the glucocorticoid receptor turnover**

![Diagram](image)

**Figure 1. The glucocorticoid receptor associates with β-arrestin-1.** A, A549 cells were untreated or treated with Dex (100 nM) for 1 and 3 h. Subsequently, whole-cell lysates were immunoprecipitated (IP) with an anti-β-arrestin-1 antibody, and recovered proteins were immunoblotted for GR. Levels of GR, β-arrestin-1 (β-arr-1), tubulin, and lamin A are shown below the immunoprecipitation blot. Lysates were also immunoprecipitated with goat anti-rabbit IgG as negative control. B, protein lysates from cells untreated or treated with Dex (100 nM) for 1 and 3 h were subjected to subcellular fractionation. Cytosolic and nuclear extracts fractions were then immunoprecipitated with an anti-β-arrestin-1 antibody, and recovered proteins were immunoblotted for GR. Cytosolic and nuclear lysates were immunoblotted to detect GR and β-arrestin-1 subcellular distribution along with tubulin (cytosolic marker) and lamin A (nuclear marker). C, representative confocal microscopic images of the in situ PLA showing the interaction between GR and β-arrestin-1 in A549 cells. Each red dot represents the detection of protein–protein interaction complexes in cells treated with Dex (100 nM) for 0, 1, and 3 h. Specificity of the assay was shown by lack of red signal in the negative control, displayed using mouse GR antibody alone with the PLA minus and plus probes. Scale bar, 20 μm. Data are for a representative experiment from three independent experiments.

In the absence of its ligand, the GR co-immunoprecipitated with β-arrestin-1, and the association persisted upon 1- and 3-h treatment with a 100 nM concentration of the synthetic glucocorticoid dexamethasone (Dex) (Fig. 1A). To examine the subcellular distribution of GR/β-arrestin-1 complexes, we next immunoprecipitated β-arrestin-1 in both cytoplasmic and nuclear fractions from A549 cells that were treated with or without Dex for 1 and 3 h. The results shown in Fig. 1B demonstrate that, in the absence of glucocorticoids, GR/β-arrestin-1 complexes were detected in the cytoplasm, whereas in the presence of Dex, the GR/β-arrestin-1 complexes were detected in the nucleus. To further analyze the existence of GR/β-arrestin-1 association, we performed an in situ proximity ligation assay (PLA) (17). In the absence of glucocorticoids, the GR/β-arrestin-1 dimerization signals were observed in the cytoplasm, and, upon Dex treatment, we observed a loss of cytoplasmic signals that was accompanied by detection of fluorescent signals in the nuclear compartment (Fig. 1C). These data establish an association between GR and β-arrestin-1 that takes place in the cytoplasm and persists even after hormone-induced GR nuclear translocation.

### Results

**β-Arrestin-1 associates in a complex with the glucocorticoid receptor**

β-Arrestin-1 has been shown to be a scaffolding protein that brings numerous proteins together to promote their concerted interactions (12–14). Moreover, existing evidence suggests that β-arrestin-1 acts as a modulator of a variety of cellular processes, such as proliferation, differentiation, and apoptosis, that are also widely known to be regulated by the glucocorticoid receptor (15, 16). Therefore, we queried whether β-arrestin-1 could associate with GR by performing a co-immunoprecipitation assay in A549 lung adenocarcinoma cells, which were chosen as a classic glucocorticoid-responsive cell line. In the absence of its ligand, the GR co-immunoprecipitated with β-arrestin-1, and the association persisted upon 1- and 3-h treatment with a 100 nM concentration of the synthetic glucocorticoid dexamethasone (Dex) (Fig. 1A). To examine the subcellular distribution of GR/β-arrestin-1 complexes, we

To evaluate the potential impact of β-arrestin-1 interaction on the GR function, we transfected A549 cells with β-arrestin-1 siRNA to silence β-arrestin-1 expression. Forty-eight hours after siRNA transfection, β-arrestin-1 mRNA expression was reduced by ~80% compared with cells transfected with the nontargeting control (NTC) siRNA (Fig. 2A). β-Arrestin-1 knockdown was also efficient at the protein levels. Indeed, Fig. 2B shows that the well-characterized Dex-induced up-regulation of β-arrestin-1 (9) was abolished in β-arrestin-1 knockdown (ARRB1-KD) cells up to 48 h after steroid exposure. Dexamethasone treatment led to a decrease of GR levels in a

| IP: IgG | β-arrestin-1 |
|---------|-------------|
| Dex (h) |             |
| 0       | 1           |
| 3       |             |

| IP: β-arrestin-1 |
|------------------|

Cytosol | Nucleus |
|--------|---------|
| 0      | 1       |
| 3      | 0       |
| 1      | 1       |

**Silencing of endogenous β-arrestin-1 alters the glucocorticoid receptor stability**

To evaluate the potential impact of β-arrestin-1 interaction on the GR function, we transfected A549 cells with β-arrestin-1 siRNA to silence β-arrestin-1 expression. Forty-eight hours after siRNA transfection, β-arrestin-1 mRNA expression was reduced by ~80% compared with cells transfected with the nontargeting control (NTC) siRNA (Fig. 2A). β-Arrestin-1 knockdown was also efficient at the protein levels. Indeed, Fig. 2B shows that the well-characterized Dex-induced up-regulation of β-arrestin-1 (9) was abolished in β-arrestin-1 knockdown (ARRB1-KD) cells up to 48 h after steroid exposure. Dexamethasone treatment led to a decrease of GR levels in a
time-dependent fashion in control cells (NTC) (18). Interestingly, the process of GR protein down-regulation was significantly enhanced when β-arrestin-1 was knocked down (Fig. 2, B and C). In NTC cells, Dex repressed the expression of GR protein by ~50% after 6 h and remained relatively stable over the 48-h treatment. In ARRB1-KD cells, GR was also down-regulated by dexamethasone in a time-dependent manner but to a greater extent that reached ~75% protein reduction over a 48-h period (Fig. 2C). Glucocorticoid-induced GR down-regulation was also enhanced in mouse embryonic fibroblasts (MEFs) with targeted deletion of β-arrestin-1 (Fig. 2, D and E) (19), suggesting that GR turnover is affected by β-arrestin-1 expression in multiple cell types. Because the difference in GR down-regulation observed in control and β-arrestin-1 KD cells occurred in the late phase of Dex exposure, we speculated that β-arrestin-1 may affect GR protein stability. To evaluate the GR protein
**β-Arrestin-1 modulates the glucocorticoid receptor turnover**

turnover over time, cells transfected with NTC and ARRB1 siRNAs were treated with dexamethasone for 2 h prior to the addition of the protein synthesis inhibitor cycloheximide (CHX; 50 μM). Compared with control, we observed that GR half-life was reduced from 12.7 to 6.9 h in β-arrestin-1 KD cells (Fig. 2, F and G).

Regulation of GR expression is controlled at both transcriptional and post-transcriptional levels, two key mechanisms involved in the regulation of the amount of active GR protein in cells (20, 21). The glucocorticoid receptor is primarily degraded via activation of the ubiquitin-proteasome machinery (22). Based on the effects that β-arrestin-1 exerts on GR turnover, we investigated whether β-arrestin-1 affects GR degradation via engaging ubiquitin and proteasome pathways. A549 cells transfected with NTC and β-arrestin-1 siRNAs were then treated with the proteasome inhibitor MG132 (10 μM) in the presence or absence of Dex (100 nM) for 6 h, and ubiquitination of GR was evaluated by using the proximity ligation assay. We carried out PLA using an antibody directed to GR and a second antibody directed to ubiquitin, and we examined the PLA signals per cell to determine the amount of endogenous ubiquitinated GR. In untreated cells, knockdown of β-arrestin-1 did not affect the basal level of GR ubiquitination. However, silencing of β-arrestin-1 increased the content of GR that undergoes ubiquitination following GR activation by Dex (Fig. 2, H and I). It is well-characterized that monoubiquitination regulates protein trafficking, whereas polyubiquitination drives the target protein to proteasomal degradation (23). To assess the type of ubiquitination that marks GR, we performed an in vivo ubiquitination assay using control and β-arrestin-1 KD cells. Twenty-four hours after NTC and ARRB1 siRNA transfection, the experimental groups were transfected with HA-ubiquitin for 24 h and then treated for 6 h with or without Dex under condition of proteasome inhibition. Protein lysates were subjected to GR immunoprecipitation followed by immunoblotting with anti-HA antibodies. MG132 treatment alone did not show GR polyubiquitination either in NTC or ARRB1-KD cells. However, GR activation by Dex induced GR polyubiquitination in NTC cells, which was enhanced in the β-arrestin-1 KD precipitates (Fig. 2F). These data indicate that β-arrestin-1 stabilizes the glucocorticoid receptor by protecting it against an excessive degradation induced by glucocorticoid binding.

**β-Arrestin-1 influences the duration of the GR transcriptional response**

GR activation by glucocorticoids results in changes in gene expression levels, with some genes being induced and others repressed (24, 25). We hypothesized that the enhanced GR turnover occurring in β-arrestin-1–deficient cells might affect the glucocorticoid responsiveness by limiting GR-mediated transcriptional activity. To explore this possibility, we evaluated the effects of endogenous β-arrestin-1 on GR-mediated transcription. We examined the expression of three known GR target genes TSC22D3 (26), IL-1β (27), and CXCL5 (28) in NTC and β-arrestin-1 KD A549 cells treated with dexamethasone at different time points over 48 h. As shown in Fig. 3, the duration of Dex-induced activation of TSC22D3 and Dex-induced inhibition of IL-1β and CXCL5 genes was attenuated. These data indicate that β-arrestin-1, by preventing enhanced GR turnover, plays a regulatory role in the duration of the glucocorticoid gene regulation.

**PELI1 as a candidate for β-arrestin-1–dependent GR turnover**

To identify the specific E3 ubiquitin ligase required for ubiquitin-dependent GR degradation in β-arrestin-1 KD cells, we screened some of the HECT (homologous to the E6-AP C terminus) and RING-finger E3 ligases that have been implicated in ubiquitination of nuclear steroid receptors (29–32). We evaluated gene transcription in both control and β-arrestin-1 KD cells treated with or without Dex for 3 h. It is noteworthy that knockdown of endogenous β-arrestin-1 was efficient at both mRNA and protein levels (Fig. 4A), and we chose the 3-h treatment because at this time point knockdown of endogenous β-arrestin-1 did not affect GR mRNA, protein levels (Fig. 4B), or its subcellular localization (Fig. 4C). Our data showed that PELI1 is the only E3 ubiquitin ligase gene induced by Dex exclusively when β-arrestin-1 expression is abolished in A549 cells (Fig. 4D). PELI1 (Entrez Gene: 57162) encodes for the protein E3 ubiquitin-protein ligase Pellino homolog-1 (shorter name: Pellino-1), a 47-kDa protein that, along with its two isoforms Pellino-2 and Pellino-3 (33, 34), contains a conserved RING-like domain at the C terminus that confers ubiquitin E3 ligase activity (35). Pellino-1 is emerging as an important component in inflammation, autoimmunity, and tumorigenesis by modulating signaling pathways that elicit inflammatory responses. Indeed, there is a positive correlation between its expression and grade of inflammation (36–41). For this reason,
we decided to investigate whether Pellino-1 could play a role in the glucocorticoid receptor degradation enhanced when β-arrestin-1 is knocked down. Expression of PELLI1 mRNA did not change in NTC cells, either in the presence or absence of glucocorticoids. Interestingly, cells that were suppressed for β-arrestin-1 expression had an induction of PELLI1 expression ∼2.3-fold after Dex treatment, and PELLI1 up-regulation persisted up to 6 h upon Dex exposure, thereafter gradually returning to baseline levels (Fig. 5A). We next evaluated whether the alteration in PELLI1 mRNA led to corresponding changes in Pellino-1 protein expression. Pellino-1 was expressed at basal levels in NTC cells, and Dex exposure induced its down-regulation in a time-dependent fashion, reaching ∼50% reduction in 24 h. However, glucocorticoid treatment in β-arrestin-1 KD cells sustained Pellino-1 expression (Fig. 5, B and C). These results suggest that the PELLI1 gene is a strong candidate for mediating the β-arrestin-1 regulation of GR turnover in GC-treated cells.

**PELI1 is a novel GR-responsive gene up-regulated in β-arrestin-1 KD cells**

Fig. 5A shows that PELLI1 mRNA levels were induced within 3 h after Dex treatment in ARRB1-KD cells, suggesting that PELLI1 might undergo transcriptional regulation by GR. We analyzed PELLI1 nascent RNA following glucocorticoid exposure. Using a primer/probe set spanning an exon–intron boundary, we observed that, compared with NTC cells, Dex promoted an increase in PELLI1 nascent RNA transcription (∼3- and 4-fold induction after 30 and 60 min, respectively) in β-arrestin-1 KD cells, suggesting that only in β-arrestin-1 KD cells PELLI1 behaves as a primary target gene (Fig. 6A). Moreover, treatment with the glucocorticoid receptor antagonist RU-486 (1 μM) prevented the glucocorticoid-mediated induction of PELLI1 in ARRB1-KD cells at both mRNA and protein levels (Fig. 6, B and C), indicating that PELLI1 is regulated by glucocorticoids. Hormone-bound GR can bind to DNA via palindromic GREs to modulate gene transcription (42–46). Therefore, we examined the PELLI1 gene for putative GRE-binding sites. Sequence analysis disclosed two putative GREs (GRE#1 and GRE#2) that displayed high homology with the consensus GRE sequence (Fig. 6D). Both GRE#1 and GRE#2 are located upstream of the transcription start site of the human PELLI1 locus (∼30 and ∼8 kb, respectively; Fig. 6D).

Moreover, the GRE#1 sequence appeared to be functional and highly conserved in the mouse genome, and GR recruitment to this regulatory site was associated with induction of Peli1 transcription (47). ChIP assays showed that both GRE putative sites were recognized by GR in a ligand-dependent manner only in A549 cells knocked down for β-arrestin-1 (Fig. 6E). To investigate whether there is a differential specificity of GR binding to the GREs in the PELLI1 upstream region of the transcription start site, we analyzed the recruitment of activated GR to the GRE located in the proximal promoter of the primary glucocorticoid target gene TSC22D3 (48). We detected a significant GR occupancy to the regulatory sequence of TSC22D3 promoter in both NTC and β-arrestin-1 KD cells following Dex treatment (Fig. 6E). These data suggest that PELLI1 is a novel glucocorticoid-responsive gene regulated by glucocorticoids extensively following the knockdown of β-arrestin-1. These findings demonstrate that the differential regulation of PELLI1 depends on β-arrestin-1 expression, leading us to hypothesize that β-arrestin-1 might regulate the chromosome architecture by allowing, or inhibiting, transcription factors to bind to DNA (49, 50). To assess whether β-arrestin-1 maintains the GR-responsive region of PELLI1 promoter inaccessible to GR binding, we performed a formaldehyde-assisted isolation of regulatory elements (FAIRE) assay. On control and
β-Arrestin-1 modulates the glucocorticoid receptor turnover

β-arrestin-1 KD cells, FAIRE-enriched DNA was analyzed by quantitative real-time PCR using the same primer/probe sets designed to detect PELI1 GRE#1 and GRE#2 elements by ChIP. The FAIRE induction seen at the two responsive GR sites in β-arrestin-1 KD cells suggests that there is an accessible chromatin environment that may facilitate the recruitment of GR to DNA only in the absence of β-arrestin-1 (Fig. 6F). As a control, no difference was found in DNA accessibility at the GRE element in the TSC22D3 locus between NTC and β-arrestin-1 KD cells (Fig. 6F).

Knockdown of β-arrestin-1 allows Pellino-1 to interact with GR and promotes GR ubiquitination

To evaluate the functional consequence of PELI1 transcriptional regulation in a system lacking β-arrestin-1, we performed co-immunoprecipitation experiments to verify whether Pellino-1 associates with GR. A549 cells, transfected with siRNAs for NTC and β-arrestin-1 (ARRB1-KD) and co-transfected with siRNAs against β-arrestin-1 and PELI1 double knockdown (DKD), were treated with or without Dex for 6 h in the presence of MG132, which inhibits protein proteasomal degradation (Fig. 6F).
β-Arrestin-1 modulates the glucocorticoid receptor turnover

7A, Total lysates). Western blot analysis of endogenous Pellino-1 immunoprecipitation revealed that endogenous GR was readily detected in Pellino-1–associated immunoprecipitates only in cell extracts from ARRB1-KD group treated with dexamethasone (Fig. 7A). Importantly, GR was immunoprecipitated with the anti-Pellino-1 antibody but not with the control IgG antibody (Fig. 7A). These findings raised the possibility that the E3 ubiquitin ligase Pellino-1 can bind to GR only in a context of β-arrestin-1 deficiency, suggesting that Pellino-1 could be the “executor” of GR down-regulation. To analyze this question, we evaluated Dex-induced GR down-regulation in control cells (NTC) and cells that lack β-arrestin-1 (ARRB1-KD), Pellino-1 (PELI1-KD), or both (DKD) at different time points after glucocorticoid treatment. Fig. 7B shows a representative Western blotting, where GR undergoes down-regulation in the four different experimental groups. In NTC cells, Dex-induced GR down-regulation resulted in a GR reduction of ~70% after 48-h treatment. Interestingly, PELI1-KD cells revealed a GR degradation kinetics comparable with that in NTC cells (Fig. 7, B (lanes 13–18 versus lanes 1–6) and C). Reduction in PELI1 expression in cells that are also knocked down for β-arrestin-1 (DKD) attenuated the augmented GR down-regulation that occurred in β-arrestin-1 KD cells (Fig. 7, B (lanes 19–24 versus lanes 7–12) and C). These findings demonstrate that, when β-arrestin-1 expression is reduced, up-regulation of Pellino-1 is crucial for GR protein stability.

Pellino-1 has an intrinsic E3 ubiquitin ligase activity that mediates Lys-48– and Lys-63–linked polyubiquitination, which marks target proteins to proteasomal degradation or stabilization, respectively (34, 52–54). To add support to the aspect of Pellino-1 in GR ubiquitination, we analyzed its possible involvement in GR degradation by measuring the amount of Lys-48–linked ubiquitin chains on GR. Using the in situ proximity ligation assay with antibodies recognizing GR and the Lys-48 linkage–specific polyubiquitin chain, we observed an increased ubiquitination signal in Dex-treated ARRB1-KD cells that was ~2-fold higher than NTC and PELI1-KD cells, suggesting that the Pellino-1 bound to GR induced the ubiquitination and degradation of the receptor (Fig. 7D). Conversely, the enhanced Lys-48–linked polyubiquitination of GR decreased in cells where the expression of PELI1 and β-arrestin-1 was silenced (Fig. 7D). Collectively, these data indicate that in the absence of β-arrestin-1, Pellino-1 expression is sustained; thereby, it binds to activated GR and is responsible for its enhanced polyubiquitination and further proteasomal degradation.

Discussion

The glucocorticoid receptor was described for the very first time in the 1960s (55). Since then, it has been found in almost all cells and tissues and mediates the biological and physiological actions of glucocorticoids either under normal or challenging circumstances. Belonging to the nuclear receptor superfamily, GR is a ligand-dependent transcription factor that acts mainly in the nucleus, where it regulates transcription of a multitude of target genes. Indeed, hormone-bound GR, by regulating cellular mechanisms including cell proliferation, differentiation, and apoptosis, acts as a “bodyguard” ensuring continuous systemic immune surveillance and body homeostasis. However, impairments at any level in the receptor regulation might lead to GR dysfunction. An impaired GR response is linked to polymorphisms of the gene, expression of a specific GR isoform over another, GR reduced expression, altered binding affinity, abnormal nuclear translocation, chromatin reshaping, and GR post-translational modifications. These processes are all key components of the glucocorticoid resistance (56). Moreover, dysfunctions, as well as actions, of the glucocorticoid receptor may be the result of the cross-talk between the receptor and an array of elements, such as co-modulators, co-activators, corepressors, and DNA-remodeling factors (6). For example, some molecules, such as MDFIC (MyoD inhibitor domain–
containing), can interact with and influence GR conformation by altering the phosphorylation status of the receptor, which in turn reshapes its gene transcription program (57, 58). Other co-modulators affect unliganded GR, working to stabilize (59) or reduce (60) the fraction of ligand-bound GR.

Here, we report the identification of the versatile adapter β-arrestin-1 protein as a novel GR regulator. We demonstrate for the first time that β-arrestin-1 associates in the cytoplasm with unliganded GR, and, upon hormone binding, the complex is detected in the nucleus, where GR can regulate gene transcription. Surprisingly, deletion of endogenous β-arrestin-1 via siRNA or gene knockout enhances GR turnover, suggesting that the association of β-arrestin-1 with GR protects the receptor from degradation.

The glucocorticoid receptor is subjected to numerous post-translational modifications, which represent a crucial mechanism in the regulation of GR signaling. The best-characterized GR post-translational modification is phosphorylation, mostly because GR has been shown to be a phosphoprotein (57, 61–68). Conversely, less is known about GR ubiquitination.
Glucocorticoid-induced GR down-regulation is a classic response occurring in most cells, that is necessary to limit the duration of glucocorticoid action. An excess of this response contributes to glucocorticoid resistance (69). The first observation of ubiquitin-mediated degradation of GR occurred in COS-1 cells when treatment with the proteasome inhibitor MG132 inhibited Dex-induced down-regulation of GR (24).

Indeed, mutation of lysine 419 of the PEST motif (which is associated with protein ubiquitination and proteasomal degradation (70)) mimicked the actions of MG132 (24, 71, 72). Seeking the biological meaning of the GR/β-arrestin-1 protein complex, we found that loss of endogenous β-arrestin-1 expression affected GR protein turnover. In A549 cells knocked down for β-arrestin-1, GR half-life was shorter along with an

Figure 7. Pellino-1 binds to GR and marks GR for Lys-48–linked polyubiquitination. A, A549 cells, transfected with NTC siRNA or ARRB1 siRNA or co-transfected with β-arrestin-1 and PEL1 siRNAs (DKD), were treated with or without Dex for 6 h in the presence of MG132 (10 μM). Whole-protein extracts were immunoprecipitated (IP) with an anti-Pellino-1 or the IgG isotype control antibodies, and recovered proteins were immunoblotted for GR. Before immunoprecipitation, protein total lysates were immunoblotted to detect the expression of GR, β-arrestin-1, Pellino-1, and actin in the above mentioned experimental groups. B, A549 cells, transfected with NTC siRNA, ARRB1 siRNA, or PEL1 siRNA (PEL1-KD) or co-transfected with β-arrestin-1 siRNA and PEL1 siRNA (DKD) were treated with 100 nM Dex for the indicated times. A representative immunoblot to measure the level of GR, β-arrestin-1, Pellino-1, and actin. C, densitometry analysis of GR down-regulation normalized to actin. A base-2 log scale is used for the y-axis. *, p < 0.05; **, p < 0.01; ***, p < 0.001 for ARRB1-KD versus NTC and DKD versus NTC. #, p < 0.05 for ARRB1-KD versus NTC. Data represent mean ± S.D. (error bars) from three independent experiments.

β-Arrestin-1 modulates the glucocorticoid receptor turnover

J. Biol. Chem. (2019) 294(29) 11225–11239

11233
β-Arrestin-1 modulates the glucocorticoid receptor turnover

increase in receptor ubiquitination and degradation via proteasome. The resultant changes in GR turnover limit the duration of GR transcription activity on target genes, suggesting that alteration in β-arrestin-1 expression may influence cell type-specific responsiveness to glucocorticoids.

Beyond the very conventional role of G protein–coupled receptor negative modulators, β-arrestin-1, as well as β-arrestin-2, also participates in the ubiquitination process orchestrating binding specificity and defining timing and dynamics. β-Arrestins can act either as adaptors that link a wide array of proteins (GPCRs and non-GPCRs) with their cognate E3 ubiquitin ligases, or they can interfere with the E3 ligase/substrate binding, preventing protein ubiquitination (73). For example, β-arrestin-1 brings the tyrosine kinase receptor IGF-1 close to the E3 ubiquitin ligase MDM2 for its ubiquitination and protein degradation (74). A similar process happens to another member of the nuclear receptor superfamily; the AR has β-arrestin-2 as one of its co-repressors, which impacts AR-dependent gene expression because it scaffolds MDM2 for androgen receptor ubiquitination and proteasomal degradation (10). Conversely, β-arrestin-1 inhibits degradation of ubiquitinated CXCR4 by interacting with signal-transducing adaptor molecule (STAM)-1 (75) but also protects the insulin-dependent IRS-1 from ubiquitination and degradation by inhibiting the formation of IRS-1/MDM2 complexes (76). Our findings suggest that β-arrestin-1 protects GR from an enhanced glucocorticoid-induced protein down-regulation, allowing the receptor to remain activated and accomplish its gene regulation task. Our data suggest that the protective role of β-arrestin-1 is due to the differential transcriptional regulation of PELI1 in control and β-arrestin-1 KD cells. PELI1 is expressed in control cells, and its transcription is independent of GR activation. However, loss of β-arrestin-1 expression makes PELI1 become a glucocorticoid-responsive gene. Indeed, we found two functional GR regulatory regions that in β-arrestin-1 KD cells are accessible and involved in the binding with activated GR. As a result, there is an induction of PELI1 mRNA levels which sustains the levels of Pellino-1 protein in a context of β-arrestin-1 deficiency. An alternative hypothesis could be that, similarly to IRS-1, β-arrestin-1, by binding to GR, competes with Pellino-1 and/or other components and impedes GR from becoming a Pellino-1 substrate. Indeed, we provide that in a context of reduced β-arrestin-1 expression, Pellino-1 associates with GR causing an increase in ubiquitination of the receptor (Fig. 7, A and D). In conclusion, whether GR degradation is limited due to a prevention of GR-induced PELI1 transcription, or due to a competitive binding between β-arrestin-1 and Pellino-1 to GR is currently unknown but is an important question to be addressed in future studies.

Because of the divergent roles exerted by β-arrestins, it is complicated to extrapolate how they can contribute to the physiology, and pathophysiology, of the immune system. Loss of β-arrestin-1 function favors decreased migration in lymphocytes but promotes the opposite phenotype in neutrophils (77). In a situation involving viral infection, β-arrestins hamper the inflammatory axis and virus removal; in the setting of autoimmune disease, β-arrestins protect against rheumatoid arthritis exacerbation because they negatively modulate Th1 (T helper 1)-associated cytokine secretion (78).

The GPCR-dependent and -independent roles of β-arrestins are essential for multiple aspects of immune cell function. Being regulators of β2-adrenergic receptors, β-arrestins modulate leukocyte and lymphocyte response by altering the sensitivity and the expression of critical cell surface receptors. In the context of immune function, many studies have shown that β-arrestins promote desensitization and internalization of chemokine receptors (CXCR family) and CCR5 (C-C chemokine receptor type 5), hence modulating neutrophil, monocyte, and T lymphocyte response. Despite the fact that β-arrestins play a pivotal role in several immune cell processes, it is still early to draw conclusions about the role of β-arrestins in inflammation. Our discovery suggests that β-arrestin-1 acts as a modulator of the anti-inflammatory effects of glucocorticoids, because it exerts a protective role in stabilizing the glucocorticoid receptor protein. In a recent study by Zhang et al. (79), there is evidence that β-arrestin-1 and β-arrestin-2 double KO mice die soon after birth due to pulmonary hypoplasia and hepatic impairment. They found that GR protein levels were significantly decreased compared with WT controls, concluding that GR might be a potential downstream effector involved in β-arrestin-mediated signaling pathway during development (79).

In conclusion, we define β-arrestin-1 as a regulator of the glucocorticoid receptor. β-Arrestin-1 is a novel GR-binding partner that stabilizes GR protein expression. Loss of β-arrestin-1 compromises GR protein turnover by allowing liganded GR to induce PELI1 transcription, the gene encoding for the E3 ubiquitin ligase Pellino-1 responsible for enhanced GR degradation. These findings reveal a new mechanism by which the actions of glucocorticoids can be regulated in a cell type–specific manner.

Experimental procedures

Cell culture

Mycoplasma-free human lung adenocarcinoma A549 cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Gibco) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Mycoplasma-free mouse embryonic fibroblasts, kindly provided by Dr. Robert J. Lefkowitz (Duke University), were maintained in Dulbecco’s modified Eagle’s medium/high glucose (Gibco) with 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. To evaluate response to dexamethasone, cell lines were grown overnight in their respective media supplemented with 10% charcoal-stripped fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine, to deplete steroids, and then treated with vehicle (H2O) or 100 nM dexamethasone for the indicated time periods. Cell cultures were kept in an incubator at 37 °C in a humidified atmosphere with 5% CO2.

Reagents

Dexamethasone (chemical name: 1,4-pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione) and mifepr...
tistone (RU-486) were purchased from Steraloids (Newport, RI). CHX and MG132 were purchased from MilliporeSigma (Burlington, MA).

**siRNA experiments**

A549 cells were reseeded in complete growth medium for 24 h prior to transfection (50% confluence). Then cells were cultured in serum-free medium and transfected with 30 nM NTC siRNA or siRNAs targeting β-arrestin-1 and/or PELI1, using Dharmafect-1 transfection reagent (Dharmacon-Horizon Discovery, Lafayette, CO) according to the manufacturer’s instructions. Twenty-four hours after transfection, transfection medium was replaced, and cells were reseeded with complete medium in tissue culture dishes appropriated for the type of assay performed.

**RNA isolation and quantitative real-time PCR (qRT-PCR)**

Total RNA was harvested using the Rneasy mini kit (Qiagen, Hilden, Germany) as described in the manufacturer’s protocol. Then, 100 ng of total RNA were used as template to directly perform the one-step qRT-PCR using the Bio-Rad CFX96 detection system. The qRT-PCR mix comprised MuLV reverse transcriptase, RNase inhibitor, Taqman universal PCR master mix, and validated primer/probe sets for ARRB1 (Hs00244527_m1), NR3C1 (Hs00230813), PELI1 (Hs00900505_m1), TSC22D3 (Hs00608272_m1), IL-1β (Hs01555410_m1), CXCL5 (Hs00199660_g1), FBXW7 (Hs00217794_m1), MDM2 (Hs01066930_m1), UBR1 (Hs00233292_m1), NEDD4 (Hs004 06454_m1), NEDD4L (Hs00969321_m1), SMURF1 (Hs00410929_m1), SMURF2 (Hs00224203_m1), SIAH2 (Hs00192581_m1), STUB1 (Hs01071598_g1), and PPIB (Hs00168719_m1) genes. All reagents and primer/probe sets were purchased from Life Technologies, Inc. (Carlsbad, CA). RNA messenger levels were quantified relative to the housekeeping gene PPIB using the ΔCt method, and where indicated, the ΔΔCt method has been calculated.

To determine expression of PELI1 nascent RNA, we designed a primer/probe set that anneals and amplifies a region spanning an intron–exon boundary, thus detecting unprocessed RNA precursors. Primer/probe sequences were the following: forward primer, 5’-AAGTTGGAAGCTCAGCTCATTAT-3’ (intron 5); probe, 5’/36-FAM/AT GGA CAG A/Zen/T GGA TGG CTG AAC CA/31ABkQ/-3’ (exon 6); reverse primer, 5’-CTATGCGTGTCTCTGTGATGCATC-3’ (intron 6).

To assess the amount of DNA contamination present in the RNA preparation, a no reverse transcriptase control was included as a negative control. PELI1 nascent RNA levels were quantified relative to the housekeeping gene PPIB using the ΔΔCt method.

**Western blotting**

For protein expression experiments, the cycloheximide chase assay, and proteasome inhibition experiments, A549 cells were lysed in radioimmune precipitation assay buffer containing protease inhibitor mixture (MilliporeSigma), phosphatase inhibitor mixture (MilliporeSigma), and 1 mM PMSF (MilliporeSigma). To separate nuclear from cytoplasmic proteins, cells were lysed using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific), following the manufacturer’s instructions. The primary antibodies used were anti-GR (DH2H, Cell Signaling Technology), anti-β-arrestin-1 (D7Z3W, Cell Signaling Technology), anti-Pellino-1 (D224F, Cell Signaling Technology), anti-actin (clone C4, EMD Millipore), anti-lamin A (133A2, Abcam), and anti-tubulin (DM1A, Invitrogen). The secondary antibodies used were IRDye 800CW goat anti-mouse (LI-COR Biosciences, Lincoln, NE) and AF680 goat anti-rabbit (Invitrogen). The immune blots were developed in the linear dynamic range using the LI-COR Odyssey imaging system from LI-COR Biosciences. The specificity of the antibody for β-arrestin-1 was confirmed by siRNA-mediated knockdown of β-arrestin-1 and by using ARRB1-KO MEF cells. The specificity of the antibody for Pellino-1 was confirmed by siRNA-mediated knockdown of Pellino-1 protein. The specificity of the antibody for GR was already confirmed by siRNA-mediated knockdown of GR in cells (9) and in mouse tissue (80).

**Immunoprecipitation assay**

For analysis of complex formation in nuclear and cytoplasmic extracts, the above-mentioned NE-PER reagents were used. For analysis of complex formation from whole-cell extracts, a buffer suited for immunoprecipitation was used (81). In both cases, 1000 μg of lysates were incubated overnight at 4 °C with rotation with anti-β-arrestin-1 (D7Z3W, Cell Signaling Technology) or anti-Pellino-1 (D224F, Cell Signaling Technology) antibodies. As control of the specificity of antibody binding, lysates were immunoprecipitated with rabbit IgG antibody (MilliporeSigma). The following day, antibody-bound proteins were immunoprecipitated with protein A/G-agarose (Thermo Fisher Scientific) for 2 h 30 min at 4 °C with rotation. Co-immunoprecipitated proteins were assessed by Western blot analysis using anti-GR antibody.

**In vivo ubiquitination assay**

The in vivo ubiquitination assay was performed as described by Choo and Zhang (82) with a few modifications. Briefly, cells were transfected with NTC and ARRB1 siRNAs to knock down β-arrestin-1 expression, and 24 h after siRNA transfection, cells were transfected with expression plasmid HA-ubiquitin, which was a gift from Edward Yeh (Addgene plasmid number 18712) (83). Twenty-four hours after HA-ubiquitin transfection, cells were treated with or without dexamethasone (100 nM) in the presence of MG132 (10 μM). Cells were lysed with complete cell lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl, pH 8.0) with 1 mM PMSF, 5 mM N-ethylmaleimide, and protease inhibitor mixture following the paper’s instructions. Then cells were diluted using dilution buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton, 1 mM PMSF, and protease inhibitor mixture) and incubated at 4 °C for 60 min with rotation. After measuring protein concentration, 1000 μg of lysates were incubated overnight at 4 °C with rotation with anti-GR antibody. The following day, antibody-bound proteins were immunoprecipitated with protein A/G-agarose (Thermo Fisher Scientific) for 2 h 30 min at 4 °C with rotation, and then washed
with the washing buffer (10 mM Tris-HCl, pH 8.0, 1 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, and protease inhibitor mixture) twice and with PBS once. A/G-agarose resin was spun down at maximum speed to aspirate the residual washing buffer. After adding 25 μl of SDS Laemmli buffer and 1 μl of 14.3 mM β-mercaptoethanol, agarose resin was boiled for 5 min, and samples were loaded onto SDS-polyacrylamide gels for immunoblotting. GR ubiquitination was detected by using anti-HA tag antibody (EMD Millipore).

**ChIP and FAIRE assays**

ChIP experiments were performed in A549 NTC and ARRB1-KD cells that were plated on 150-mm dishes at 90% confluence. After 2-h dexamethasone or vehicle treatment, 1% formaldehyde was added to the dish to cross-link proteins to DNA. After formaldehyde inactivation with glycine, cells were scraped and resuspended in cell lysis buffer provided by the MagnaChIP kit (EMD Millipore) to isolate nuclei. The crude nuclei were collected, resuspended in nuclear lysis buffer (EMD Millipore), subjected to sonication using a Diagenode Bioruptor (15 cycles on high setting, 30 s on, 30 s off, twice). DNA extracts were first run onto 1% agarose gel to evaluate chromatin shearing range from 200 to 1000 bp and then precleared and immunoprecipitated with anti-GR antibody or IgG control antibody. The GR/DNA complex was pulled down using the MagnaChIP kit (EMD Millipore).

Immunoprecipitated DNA was purified using a PCR purification kit (Qiagen), and quantitative real-time PCR was performed. We designed the following primer/probe sets for GREs in the PELI1 and TSC22D3 loci: PELI1 –30Kb: forward primer, 5′-CAGTGGGTGAGATGTTGTGT-3′; probe, 5′/-56-FAM/ATGTCTCTCT/Thermo Scientific); forward primer, 5′-TACAGGAGATGGCTTCTAAGT-3′; reverse primer, 5′-TTGCTCGACCATGTAGTTT-3′; probe, 5′/-56-FAM/CACGTGCAGA/Zen/Thermo Scientific); reverse primer, 5′-ATTGCCACATAGGGACAG-3′; TSC22D3–1500bp: forward primer, 5′-TTCATGGTGATCTCCTTAC-3′; probe, 5′/-56-FAM/TGTGGTGGA/Zen/Thermo Scientific); reverse primer, 5′-GTTGATGCAACCGGGAATA-3′.

For the FAIRE procedure, we used the same sonicated DNA extracts that were used for the ChIP assay. We followed the protocol described by Simon et al. (84). Quantitative real-time PCR was performed using the primers designed for the two GREs of the PELI1 locus and the GRE –1500 bp of the TSC22D3 locus.

**In situ PLA and immunofluorescence staining**

In situ PLA detection was performed using the Duolink PLA kit (Sigma-Aldrich) according to the manufacturer’s instructions. Briefly, A549 cells were grown in glass bottom culture dishes (MatTek Corp.) for the time and with the treatment required by the experiment. Then cells were washed with warm PBS, fixed with warm 4% paraformaldehyde for 20 min at room temperature, and blocked in 1× PBS containing 2% BSA and 0.1% Triton X-100 for 30 min at room temperature. Cells were then incubated for 1 h with blocking buffer (1× PBS, 5% normal goat serum, and 0.1% Triton X-100) at room temperature prior to incubating the samples at 4 °C overnight with rabbit anti-β-arrestin-1 antibody (D8H2, Cell Signaling Technology) diluted in 1× PBS containing 2% BSA. The following morning, samples were washed with 1× PBS containing 0.1% Tween and incubated with the secondary antibody goat anti-rabbit AF594 for 1 h at room temperature. Samples were then washed, air-dried, and mounted with ProLong gold antifade mountant with DAPI (Thermo Scientific). A Zeiss laser-scanning confocal microscope (LSM 780 and LSM 880; Carl Zeiss) was used to analyze protein–protein interactions. To quantify the PLA signals (also known as puncta), maximum intensity projections of the raw images were split into single-channel images by MetaMorph software (Molecular Devices). A minimum fluorescent threshold of PLA puncta was used for all samples within one experiment. The number of puncta measured was divided by the number of cells obtained by thresholding the DAPI channel (used as nuclear stain). For quantification, each bar (mean ± S.D.) represents the mean obtained from three independent experiments. The specificity of the antibodies for ubiquitin and anti-Lys-48-ubiquitin was confirmed, performing ubiquitination assays in the presence or absence of the proteasome inhibitor (data not shown).

For immunofluorescence staining, NTC and ARRB1-KD cells were grown in glass bottom culture dishes (MatTek Corp.) with or without Dex for 3 h. Then, cells were washed twice with warm PBS, fixed with warm 4% paraformaldehyde for 20 min at room temperature, and blocked in 1× PBS containing 2% BSA and 0.1% Triton X-100 for 30 min at room temperature. Cells were then incubated for 1 h with blocking buffer (1× PBS, 5% normal goat serum, and 0.1% Triton X-100) at room temperature prior to incubating the samples at 4 °C overnight with rabbit anti-GR antibody (D8H2, Cell Signaling Technology) diluted in 1× PBS containing 2% BSA. The following morning, samples were washed with 1× PBS containing 0.1% Tween and incubated with the secondary antibody goat anti-rabbit AF594 for 1 h at room temperature. Samples were then washed, air-dried, and mounted with ProLong gold antifade mountant with DAPI (Thermo Scientific). A Zeiss laser-scanning confocal microscope (LSM 780 and LSM 880; Carl Zeiss) was used to analyze GR cellular distribution. A technical negative control was performed omitting the GR antibody.

**Statistical analysis**

All data are presented as means ± S.D. Student’s t test and one- and two-way analysis of variance with Sidak’s and Dunnnett’s multiple-comparison tests, according to the number of groups, were used to determine whether differences between experimental groups were statistically significant. All analyses were analyzed using the GraphPad Prism 7 software package (GraphPad Software).
References

1. Cain, D. W., and Cidlowski, J. A. (2017) Immune regulation by glucocorticoids. *Nat. Rev. Immunol.* 17, 233–247 CrossRef Medline

2. Robinson-Rechavi, M., Carpenter, A. S., Dufraisse, M., and Laudet, V. (2001) How many nuclear hormone receptors are there in the human genome? *Trends Genet.* 17, 554–556 CrossRef Medline

3. Kirschke, E., Goswami, D., Southworth, D., Griffin, P. R., and Agard, D. A. (2014) Glucocorticoid receptor function regulated by coordinated action of the Hsp90 and Hsp70 chaperone cycles. *Cell* 157, 1685–1697 CrossRef Medline

4. Pratt, W. B., and Toft, D. O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocrin. Rev.*, 18, 306–360 CrossRef Medline

5. Kadmiel, M., and Cidlowski, J. A. (2013) Glucocorticoid receptor signaling in health and disease. *Trends Pharmacol. Sci.* 34, 518–530 CrossRef Medline

6. Petta, I., Dejager, L., Ballester, M., Lievens, S., Tavernier, J., De Bosscher, K., and Libert, C. (2016) The interchangeability of the glucocorticoid receptor and its influence on the actions of glucocorticoids in combattling inflammatory and infectious diseases. *Microbiol. Mol. Biol. Rev.* 80, 495–522 CrossRef Medline

7. Palczewski, K. (1994) Structure and functions of arrestins. *Protein Sci.* 3, 1355–1361 CrossRef Medline

8. Smith, J. S., and Rajagopal, S. (2016) The β-arrestins: multifunctional regulators of G protein-coupled receptors. *J. Biol. Chem.* 291, 8969–8977 CrossRef Medline

9. Oakley, R. H., Revollo, J., and Cidlowski, J. A. (2012) Glucocorticoid regulate arrestin gene expression and redirect the signaling profile of G protein-coupled receptors. *Proc. Natl. Acad. Sci. U.S.A.* 109, 17591–17596 CrossRef Medline

10. Lakshminathan, V., Zou, L., Kim, J. I., Michal, A., Nie, Z., Messias, N. C., Benovic, J. L., and Daaka, Y. (2009) Identification of β-arrestin2 as a corepressor of androgen receptor signaling in prostate cancer. *Proc. Natl. Acad. Sci. U.S.A.* 106, 9379–9384 CrossRef Medline

11. Wong, A. M., Abrams, M. C., and Mickey, P. E. (2015) β-Arrestin regulates estradiol membrane-initiated signaling in hypothalamic neurons. *PLoS One* 10, e0120530 CrossRef Medline

12. Tohgo, A., Pierce, K. L., Choy, E. W., Lefkowitz, R. J., and Luttrell, L. M. (2002) β-Arrestin scaffolding of the ERK cascade enhances cytosolic ERK activity but inhibits ERK-mediated transcription following angiotensin AT1a receptor stimulation. *J. Biol. Chem.* 277, 9429–9436 CrossRef Medline

13. Peterson, Y. K., and Luttrell, L. M. (2017) The diverse roles of arrestin scaffolds in G protein-coupled receptor signaling. *Pharmacol. Rev.* 69, 256–297 CrossRef Medline

14. Miller, W. E., and Lefkowitz, R. J. (2001) Expanding roles for β-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr. Opin. Cell Biol.* 13, 139–145 CrossRef Medline

15. Sapolsky, R. M., Romero, L. M., and Munck, A. U. (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrin. Rev.* 21, 55–89 CrossRef Medline

16. Song, Q., Ji, Q., and Li, Q. (2018) The role and mechanism of β-arrestins in cancer invasion and metastasis. *Int. J. Mol. Med.* 41, 631–639 CrossRef Medline

17. Söderberg, O., Gullberg, M., Jarvis, M., Riddershåk, L., Leuchouw, K. J., Jarvis, J., Wester, K., Hydbring, P., Brahram, F., Larsson, L. G., and Landegren, U. (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat. Methods* 3, 955–1000 CrossRef Medline

18. Silva, C. M., Powell-Oliver, F. E., Jewell, C. M., Sar, M., Allgood, V. E., and Cidlowski, J. A. (1994) Regulation of the human glucocorticoid receptor by long-term and chronic treatment with glucocorticoid. *Steroids* 59, 436–442 CrossRef Medline

19. Kohout, T. A., Lin, F. S., Perry, S. J., Conner, D. A., and Lefkowitz, R. J. (2001) β-Arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. *Proc. Natl. Acad. Sci. U.S.A.* 98, 1601–1606 CrossRef Medline

20. Wallace, A. D., and Cidlowski, J. A. (2001) Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. *J. Biol. Chem.* 276, 42714–42721 CrossRef Medline

21. Okret, S., Dong, Y., Brönnegård, M., and Gustafsson, J. A. (1991) Regulation of glucocorticoid receptor expression. *Biochemistry (Paris)* 73, 51–59 CrossRef Medline

22. D’Adamo, F., Zollo, O., Moraca, R., Ayroldi, E., Bruscoli, S., Bartoli, A., Cannarile, L., Migliorati, G., and Riccardi, C. (1997) A new deoxymetha-sone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* 7, 803–812 CrossRef Medline

23. Park, C. W., and Ryu, K. Y. (2014) Cellular ubiquitin pool dynamics and homeostasis. *BMB Rep.* 47, 475–482 CrossRef Medline

24. Wallace, A. D., and Cidlowski, J. A. (2001) Proteasome-mediated glucocorticoid receptor degradation restricts transcriptional signaling by glucocorticoids. *J. Biol. Chem.* 276, 42714–42721 CrossRef Medline

25. Davies, L., Karthekeyan, N., Lynch, J. T., Sial, E. A., Gkourtsa, A., Demonacos, C., and Krstic-Demonacos, M. (2008) Cross talk of signaling pathways in the regulation of the glucocorticoid receptor function. *Mol. Endocrinol.* 22, 1331–1344 CrossRef Medline

26. D’Adamo, F., Zollo, O., Moraca, R., Ayroldi, E., Bruscoli, S., Bartoli, A., Cannarile, L., Migliorati, G., and Riccardi, C. (1997) A new deoxymetha-sone-induced gene of the leucine zipper family protects T lymphocytes from TCR/CD3-activated cell death. *Immunity* 7, 803–812 CrossRef Medline

27. Lee, S. W., Tsou, A. P., Chan, H., Thomas, J., Petrie, K., Eugui, E. M., and Allison, A. C. (1998) Glucocorticoids selectively inhibit the transcription of the interleukin 1 β gene and decrease the stability of interleukin 1 β mRNA. *Proc. Natl. Acad. Sci. U.S.A.* 85, 1204–1208 CrossRef Medline

28. Gibbs, J., Ince, L., Matthews, L., Mei, J., Bell, T., Yang, N., Saer, B., Begley, N., Poolman, T., Parilloaud, M., Farrow, S., DeMayo, F., Russell, T., Worthen, G. S., Ray, D., and Loudon, A. (2014) An epithelial circadian clock controls pulmonary inflammation and glucocorticoid action. *Nat. Med.* 20, 919–926 CrossRef Medline

29. Helzer, K. T., Hooper, C., Miyamoto, S., and Alarid, E. T. (2015) Ubiquitylation of nuclear receptors: new linkages and therapeutic implications. *J. Mol. Endocrinol.* 54, R151–R167 CrossRef Medline

30. Tsou, S. M., Smits, M. M., Ng, B. H., Lee, J., and Wright, M. E. (2016) Discovery proteomics identifies a molecular link between the coatomer protein complex I and androgen receptor-dependent transcription. *J. Biol. Chem.* 291, 18818–18842 CrossRef Medline

31. Kinyamu, H. K., and Archer, T. K. (2003) Estrogen receptor-dependent proteosomal degradation of the glucocorticoid receptor is coupled to an increase in mdm2 protein expression. *Mol. Cell. Biol.* 23, 5867–5881 CrossRef Medline

32. Wang, X., and DeFranco, D. B. (2005) Alternative effects of the ubiquitin-proteasome pathway on glucocorticoid receptor down-regulation and transactivation are mediated by CHIP, an E3 ligase. *Mol. Endocrinol.* 19, 1474–1482 CrossRef Medline

33. Jin, W., Chang, M., and Sun, S. C. (2012) Peli: a family of signal-responsive E3 ubiquitin ligases mediates TLR signaling and T-cell tolerance. *Cell Mol. Immunol.* 9, 113–122 CrossRef Medline
34. Moynagh, P. N. (2014) The roles of Pellino E3 ubiquitin ligases in immunity. Nat. Rev. Immunol. 14, 122–131 CrossRef Medline
35. Humphries, F., and Moynagh, P. N. (2015) Molecular and physiological roles of Pellino E3 ubiquitin ligases in immunity. Immunol. Rev. 266, 93–108 CrossRef Medline
36. Chang, M., Jin, W., Chang, J. H., Xiao, Y., Brittain, G. C., Yu, J., Zhou, X., Wang, Y. H., Cheng, X., Li, P., Rabinovich, B. A., Hwu, P., and Sun, S. C. (2011) The ubiquitin ligase Pell1 negatively regulates T cell activation and prevents autoimmunity. Nat. Immunol. 12, 1002–1009 CrossRef Medline
37. Chang, M., Jin, W., and Sun, S. C. (2009) Pell1 facilitates TRIF-dependent Toll-like receptor signaling and proinflammatory cytokine production. Nat. Immunol. 10, 1089–1095 CrossRef Medline
38. Li, D., Tavana, O., Sun, S. C., and Gu, W. (2018) Pell1 modulates the subcellular localization and activity of Mdmx. Cancer Res. 78, 2897–2910 CrossRef Medline
39. Wang, H., Meng, H., Li, X., Zhu, K., Dong, K., Mookhtiar, A. K., Wei, H., Li, Y., Sun, S. C., and Yuan, J. (2017) Pell1 functions as a dual modulator of necroptosis and apoptosis by regulating ubiquitination of RIPK1 and mRNA levels of c-FLIP. Proc. Natl. Acad. Sci. U.S.A. 114, 11944–11949 CrossRef Medline
40. Xiao, Y., Jin, J., Chang, M., Chang, J. H., Hu, H., Zhou, X., Brittain, G. C., Stansberg, C., Torkildsen, Ø., Wang, X., Brink, R., Cheng, X., and Sun, S. C. (2013) Pell1 promotes microglia-mediated CNS inflammation by regulating Traf3 degradation. Nat. Med. 19, 595–602 CrossRef Medline
41. Ordureau, A., Smith, H., Windheim, M., Peggie, M., Carrick, E., Morrice, N., and Cohen, P. (2008) The IRAK-catalysed activation of the E3 ligase function of Pellino isoforms induces the Lys63-linked polyubiquitination of IRAK1. Biochem. J. 409, 43–52 CrossRef Medline
42. Reddy, E. T., Pauli, F., Sprouse, R. O., Neff, N. F., Newberry, K. M., Garabedian, M. J., and Myers, R. M. (2009) Genomic determination of the glucocorticoid receptor complex. Mol. Endocrinol. 23, 1719–1726 CrossRef Medline
43. Yudt, M. R., and Cidlowski, J. A. (2002) The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. Mol. Endocrinol. 16, 1719–1726 CrossRef Medline
44. Phuc Le, P., Friedman, J. R., Schug, J., Brestelli, J. E., Parker, J. B., Bochkis, J. M., and Kaestner, K. H. (2005) Glucocorticoid receptor-dependent gene regulatory networks. PLoS Genet. 1, e16 CrossRef Medline
45. So, A. Y., Cooper, S. B., Feldman, B. J., Manuchehr, M., and Yamamoto, K. R. (2008) Conservation analysis predicts in vivo occupancy of glucocorticoid receptor-binding sequences at glucocorticoid-induced genes. Proc. Natl. Acad. Sci. U.S.A. 105, 5745–5749 CrossRef Medline
46. Wang, J. C., Derynck, M. K., Nonaka, D. F., Khodabakhsh, D. B., Haqq, C., and Yamamoto, K. R. (2004) Chromatin immunoprecipitation (ChIP) scanning identifies primary glucocorticoid receptor target genes. Proc. Natl. Acad. Sci. U.S.A. 101, 15603–15608 CrossRef Medline
47. Cremer, T., and Cremer, C. (2001) Chromosome territories, nuclear architecture and gene regulation in mammalian cells. Nat. Rev. Genet. 2, 292–302 CrossRef Medline
48. Dilworth, F. J., and Champon, P. (2001) Nuclear receptors coordinate the activities of chromatin remodeling complexes and coactivators to facilitate initiation of transcription. Oncogene 20, 3047–3054 CrossRef Medline
49. Cidlowski, J. A., Bellingham, D. L., Powell-Oliver, F. E., Lubahn, D. B., and Sar, M. (1990) Novel antipeptide antibodies to the human glucocorticoid receptor: recognition of multiple receptor forms in vitro and distinct localization of cytoplasmic and nuclear receptors. Mol. Endocrinol. 4, 1427–1437 CrossRef Medline
50. Jeon, Y. K., Kim, C. K., Hwang, K. R., Park, H.-Y., Koh, J., Chung, D. H., Lee, C.-W., and Ha, G.-H. (2017) Pellino-1 promotes lung carcinogenesis via the stabilization ofSlug and Snail through K63-mediated polyubiquitination. Cell Death Differ. 24, 469–480 Medline
51. Butler, M. P., Hanly, J. A., and Moynagh, P. N. (2007) Kinase-active interleukin-1 receptor-associated kinases promote polyubiquitination and degradation of the Pellino family: direct evidence for PELLINO proteins being ubiquitin-protein isopeptide ligases. J. Biol. Chem. 282, 29729–29737 CrossRef Medline
52. Ohtake, F., Saeki, Y., Ishido, S., Kanno, J., and Tanaka, K. (2016) The K48–K63 branched ubiquitin chain regulates NF-κB signaling. Mol. Cell 64, 251–266 CrossRef Medline
53. Wira, C., and Munck, A. (1970) Specific glucocorticoid receptors in thymus cells: localization in the nucleus and extraction of the cortisol-receptor complex. J. Biol. Chem. 245, 3436–3438 Medline
54. Silverman, M. N., and Sternberg, E. M. (2012) Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. Ann. N.Y. Acad. Sci. 1261, 55–63 CrossRef Medline
55. Oakley, R. H., Busillo, J. M., and Cidlowski, J. A. (2017) Cross-talk between the glucocorticoid receptor and MyoD family inhibitor domain-containing protein provides a new mechanism for generating tissue-specific responses to glucocorticoids. J. Biol. Chem. 292, 5825–5844 CrossRef Medline
56. Alam, M., and Cidlowski, J. A. (2013) Exploring the molecular implications of FKBP51 in glucocorticoid receptor signaling. Mol. Cell. Biol. 33, 3700–3714 CrossRef Medline
57. Kim, Y. S., Jang, S. W., Sung, H. J., Lee, H. J., Kim, I. S., Na, D. S., and Ko, J. (2005) Role of 14–3–3 η as a positive regulator of the glucocorticoid receptor transcriptional activation. Endocrinology 146, 3133–3140 CrossRef Medline
58. Murphy, P. J., Morishima, Y., Kovacs, J. I., Yao, T. P., and Pratt, W. B. (2005) Regulation of the dynamics of hsp90 action on the glucocorticoid receptor by acetylation/deacetylation of the chaperone. J. Biol. Chem. 280, 33792–33799 CrossRef Medline
59. Bouazza, B., Debba-Pavard, M., Amrani, Y., Issacs, L. O’Connell, D., Ahamed, S., Formella, D., and Tilba, O. (2014) Basal p38 mitogen-activated protein kinase regulates unliganded glucocorticoid receptor function in airway smooth muscle cells. Am. J. Respir. Cell Mol. Biol. 50, 301–315 CrossRef Medline
60. Ismaili, N., and Garabedian, M. J. (2004) Modulation of glucocorticoid receptor function via phosphorylation. Ann. N.Y. Acad. Sci. 1024, 86–101 CrossRef Medline
61. Stansberg, C., Torkildsen, Ø., Wang, X., Brink, R., Cheng, X., and Sun, S. C. (2013) Peli1 promotes microglia-mediated CNS inflammation by regulating Traf3 degradation. Nat. Med. 19, 595–602 CrossRef Medline
71. Wallace, A. D., Cao, Y., Chandramouleeswaran, S., and Cidlowski, J. A. (2010) Lysine 419 targets human glucocorticoid receptor for proteasomal degradation. *Steroids* **75**, 1016–1023 CrossRef Medline

72. Deroo, B. J., Rentsch, C., Sampath, S., Young, J., DeFranco, D. B., and Archer, T. K. (2002) Proteasomal inhibition enhances glucocorticoid receptor transactivation and alters its subnuclear trafficking. *Mol. Cell. Biol.* **22**, 4113–4123 CrossRef Medline

73. Kommaddi, R. P., and Shenoy, S. K. (2013) Arrestins and protein ubiquitination. *Prog. Mol. Biol. Transl. Sci.* **118**, 175–204 CrossRef Medline

74. Girnita, L., Shenoy, S. K., Sehat, B., Vasilcanu, R., Girnita, A., Lefkowitz, R. J., and Larsson, O. (2005) β-Arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth factor-1 receptor by acting as adaptor for the MDM2 E3 ligase. *J. Biol. Chem.* **280**, 24412–24419 CrossRef Medline

75. Malik, R., Soh, U. J., Trejo, J., and Marchese, A. (2012) Novel roles for the E3 ubiquitin ligase atrophin-interacting protein 4 and signal transduction adaptor molecule 1 in G protein-coupled receptor signaling. *J. Biol. Chem.* **287**, 9013–9027 CrossRef Medline

76. Usui, I., Imamura, T., Huang, J., Satoh, H., Shenoy, S. K., Lefkowitz, R. J., Hupfeld, C. J., and Olefsky, J. M. (2004) β-Arrestin-1 competitively inhibits insulin-induced ubiquitination and degradation of insulin receptor substrate 1. *Mol. Cell. Biol.* **24**, 8929–8937 CrossRef Medline

77. Fong, A. M., Premont, R. T., Richardson, R. M., Yu, Y. R., Lefkowitz, R. J., and Patel, D. D. (2002) Defective lymphocyte chemotaxis in β-arrestin2- and GRK6-deficient mice. *Proc. Natl. Acad. Sci. U.S.A.* **99**, 7478–7483 CrossRef Medline

78. Ahmadzai, M. M., Broadbent, D., Occhiuto, C., Yang, C., Das, R., and Subramanian, H. (2017) Canonical and noncanonical signaling roles of β-arrestins in inflammation and immunity. *Adv. Immunol.* **136**, 279–313 CrossRef Medline

79. Zhang, M., Teng, H., Shi, J., and Zhang, Y. (2011) Disruption of β-arrestins blocks glucocorticoid receptor and severely retards lung and liver development in mice. *Mech. Dev.* **128**, 368–375 CrossRef Medline

80. Quinn, M. A., Xu, X., Ronfani, M., and Cidlowski, J. A. (2018) Estrogen deficiency promotes hepatic steatosis via a glucocorticoid receptor-dependent mechanism in mice. *Cell Rep.* **22**, 2690–2701 CrossRef Medline

81. Ayroldi, E., Petrillo, M. G., Bastianelli, A., Marchetti, M. C., Ronchetti, S., Nocentini, G., Ricciotti, L., Cannarile, L., and Riccardi, C. (2015) L-GILZ binds p53 and MDM2 and suppresses tumor growth through p53 activation in human cancer cells. *Cell Death Differ.* **22**, 118–130 CrossRef Medline

82. Choo, Y. S., and Zhang, Z. (2009) Detection of protein ubiquitination. *J. Vis. Exp.* **10.3791/1293** CrossRef Medline

83. Kamitani, T., Kito, K., Nguyen, H. P., and Yeh, E. T. (1997) Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. *J. Biol. Chem.* **272**, 28557–28562 CrossRef Medline

84. Simon, J. M., Giuresi, P. G., Davis, I. J., and Lieb, J. D. (2013) A detailed protocol for formaldehyde-assisted isolation of regulatory elements (FAIRE). *Curr. Protoc. Mol. Biol.*, Chapter 21, Unit 21.26 CrossRef Medline