Cross-talk between the glucocorticoid receptor and MyoD family inhibitor domain-containing protein provides a new mechanism for generating tissue-specific responses to glucocorticoids

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Glucocorticoids are primary stress hormones that regulate many physiological processes, and synthetic derivatives of these molecules are widely used in the clinic. The molecular factors that govern tissue specificity of glucocorticoids, however, are poorly understood. The actions of glucocorticoids are mediated by the glucocorticoid receptor (GR). To discover new proteins that interact with GR and regulate its function, we performed a yeast two-hybrid assay. The MyoD family inhibitor domain-containing protein (MDFIC) was identified as a binding partner for GR. MDFIC associated with GR in the cytoplasm of cells, and treatment with glucocorticoids resulted in the dissociation of the GR-MDFIC complex. To investigate the function of the GR-MDFIC interaction, we performed a genome-wide microarray in intact and MDFIC-deficient A549 cells that were treated with glucocorticoids. A large cohort of genes was differentially regulated by GR depending on the presence or absence of MDFIC. These gene changes were strongly associated with inflammation, and glucocorticoid regulation of the inflammatory response was altered in MDFIC-deficient cells. At a molecular level, the interaction of MDFIC with GR altered the phosphorylation status of the receptor. We demonstrate in COS-1 cells that changes in receptor phosphorylation underlie the ability of MDFIC to modulate the transcriptional activity of GR. Finally, we show that GR directly represses the MDFIC gene, revealing a negative feedback loop by which glucocorticoids limit MDFIC activity. These findings identify a new binding partner for cytoplasmic GR that modulates the receptor transcriptome and contributes to the tissue-specific actions of glucocorticoids.

Glucocorticoids are released by the hypothalamic-pituitary-adrenal axis in a circadian manner and in response to stress (1). They act on nearly every tissue and organ of the body and function to maintain homeostasis. Biological processes regulated by these hormones include intermediary metabolism, cellular proliferation and differentiation, apoptosis, skeletal growth, cognition, cardiac function, development, reproduction, and immune function (2). The ability of glucocorticoids to inhibit inflammation and suppress the immune system has made them one of the most prescribed drugs in the world today. Synthetic glucocorticoids are used to treat inflammatory and autoimmune diseases, prevent organ transplant rejection, and combat cancers of the lymphoid system. In addition, glucocorticoids are routinely given to preterm babies to improve survival for their effects on lung maturation (3, 4). The therapeutic benefit of glucocorticoids, however, is limited by severe side effects that develop in patients chronically treated with these steroids. Adverse responses include hypertension, osteoporosis, glaucoma, abdominal obesity, diabetes, growth retardation in children, and depression. Many of these symptoms are also observed in patients with excessive glucocorticoid production due to chronic stress or Cushing’s disease.

The physiological and pharmacological actions of glucocorticoids are mediated by the glucocorticoid receptor (GR; NR3C1), a member of the nuclear receptor superfamily of ligand-dependent transcription factors (5). GR is a modular protein composed of an amino-terminal transactivation domain (NTD), a central DNA binding domain (DBD), and a carboxy-terminal ligand binding domain (LBD). Separating the DBD and LBD is a flexible linker called the hinge region. In the absence of glucocorticoids, GR is found predominantly in the cytoplasm of cells in a complex with various chaperone proteins that maintain the receptor in a conformation that binds gluco-

2 The abbreviations used are: GR, glucocorticoid receptor; MDFIC, MyoD family inhibitor domain-containing protein; MDFI, MyoD family inhibitor isoform 1; NTD, amino-terminal transactivation domain; DBD, DNA binding domain; LBD, carboxy-terminal ligand binding domain; GRE, glucocorticoid-responsive element; Dex, dexamethasone; MMTV, mouse mammary tumor virus; NTC, non-targeting control; KD, knockdown; Cc12, C-C motif chemokine ligand 2; Bmp6, bone morphogenetic protein 6; Kcnj2, potassium channel, inwardly rectifying subfamily J, member 2; Ank3, ankryin-3; Traf1, TNF receptor-associated factor 1; Csf1, colony stimulating factor 1; ANOVA, analysis of variance.
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corticoids with high affinity (6, 7). Upon binding glucocorticoids, GR undergoes a conformational change that results in the dissociation of the chaperone proteins, the exposure of nuclear localization sequences, and receptor translocation into the nucleus (8). GR in the nucleus interacts with an assortment of co-activators and co-repressors to induce or repress the expression of thousands of genes. GR alters gene transcription by directly binding to specific sequences of DNA termed glucocorticoid-responsive elements (GREs) and/or by physically interacting with other DNA-bound transcription factors.

The cellular response to glucocorticoids is remarkably diverse across tissues and cell types (9–12). Glucocorticoids induce apoptosis in thymocytes and osteoblasts but promote the survival of hepatocytes and cardiomyocytes (13–15). Sensitivity to glucocorticoids varies among individuals, among tissues from the same individual, and even within the same cell depending on the phase of the cell cycle. The development of tissue-specific glucocorticoid resistance is a major limitation to effective long-term glucocorticoid therapy (16, 17). Various factors have been shown to modulate the type and/or magnitude of the glucocorticoid response including ligand availability, the cellular composition of GR isoforms, post-translational modifications of GR, the availability of specific co-activators and co-repressors, epigenetic regulators, and the chromatin landscape (1, 18, 19). Understanding the molecular mechanisms that contribute to the heterogeneity and tissue specificity of glucocorticoid signaling will facilitate the development of novel glucocorticoids and treatment strategies with improved benefit/risk ratios.

In the following study we performed a yeast two-hybrid assay to discover new proteins that interact with GR and modulate its function. We show that the MyoD family inhibitor domain-containing protein (MDFIC) interacts with the hinge region of unliganded GR in the cytoplasm of cells. Binding of glucocorticoids promotes the dissociation of the GR-MDFIC complex. The interaction of MDFIC with the receptor alters the GR transcriptional activity and leads to unique cellular responses after glucocorticoid treatment. At a molecular level the presence of MDFIC modulates both the basal and glucocorticoid-induced phosphorylation status of GR. We also demonstrate that glucocorticoids directly repress MDFIC gene expression revealing a negative feedback loop by which GR can curb the modulatory actions of MDFIC. These findings suggest that alterations in the expression level of MDFIC provide a novel mechanism for generating tissue- and cell type-specific responses to glucocorticoids.

Results
MDFIC associates with GR in the cytoplasm of cells
To discover new proteins that interact with GR, we performed a yeast two-hybrid screen using as bait a region of human GR spanning the hinge domain (Fig. 1A). The hinge domain was chosen because relatively little is known about how this region modulates GR activity compared with the well-studied NTD, DBD, and LBD. cDNA libraries derived from human brain, spleen, breast cancer/prostate cancer, and liver/small intestine/adipose tissues were used as prey. One clone obtained from screening the breast cancer/prostate cancer cDNA library contained an open reading frame with 100% identity to the carboxyl-terminal half (amino acids 126–246) of MDFIC (Fig. 1B). MDFIC belongs to a small family of gene expression regulators characterized by having a unique cysteine-rich carboxyl-terminal domain (20, 21). The other member of this family is MyoD family inhibitor isoform 1 (MDFI) (Fig. 1B). The cysteine-rich carboxyl-terminal domain of MDFIC is composed of 81 amino acids (166–246) and shares 77% identity with the corresponding region of MDFI. A total of 24 and 26 cysteine residues are located within the cysteine-rich carboxyl-terminal domain for MDFIC and MDFI, respectively.

To determine whether the full-length GR and MDFIC interact in mammalian cells, we expressed GR and FLAG-MDFIC in COS-1 cells (which lack detectable levels of endogenous GR) and performed a co-immunoprecipitation assay. In the absence of glucocorticoids, MDFIC co-immunoprecipitated with GR (Fig. 2A). Treatment of cells for 1 h with the synthetic glucocorticoid dexamethasone (Dex) (100 nm) resulted in the dissociation of the complex. Reversing the order of the antibodies in the co-immunoprecipitation experiment yielded identical results; GR co-immunoprecipitated with MDFIC and the complex dissociated after Dex treatment (Fig. 2B). Dissociation of the GR-MDFIC complex was also observed with the natural glucocorticoid cortisol and the partial agonist/antagonist RU486 (Fig. 2C). A time-course was performed with 100 nm Dex to evaluate the kinetics of the GR-MDFIC dissociation. As shown in Fig. 2D, a reduction in the GR-MDFIC interaction was detected as early as 5 min after glucocorticoid addition. These data demonstrate that GR and MDFC associate in a complex in the absence of glucocorticoids and that glucocorticoid binding to GR promotes the dissociation of the complex.

The cysteine-rich carboxyl-terminal domain of MDFIC is embedded in the region of the protein retrieved from the yeast two-hybrid screen (Fig. 1B). Therefore, we examined whether this domain was necessary for the interaction of MDFIC with GR. Co-immunoprecipitation experiments were performed in COS-1 cells expressing GR and either full-length MDFIC or a truncated version missing the entire cysteine-rich carboxyl-terminal domain (MDFIC(1–164)). As shown in Fig. 2E, only the full-length MDFIC co-immunoprecipitated with GR, indicating the cysteine-rich carboxyl-terminal domain of MDFIC is necessary for its interaction with the receptor. Because the related family member MDFI also contains a homologous cysteine-rich carboxyl-terminal domain (Fig. 1B), we investigated whether MDFI interacted with GR. Similar to our findings for MDFIC, MDFI also co-immunoprecipitated with GR in untreated cells, and the addition of 100 nm Dex promoted the dissociation of the complex (Fig. 2F). These data indicate that the cysteine-rich carboxyl-terminal domain of MDFIC and MDFI mediates their interaction with GR.

GR is distributed primarily in the cytoplasm of cells in the absence of glucocorticoids and translocates into the nucleus following glucocorticoid binding. Translocation of GR is directed by two nuclear localization signals, one of which is located at the junction of the DBD and hinge region (22, 23). To determine whether MDFIC co-localizes with GR in the cytoplasm and whether its interaction with GR in the hinge region alters receptor movement into the nucleus, we performed immunocytochemistry on COS-1 cells expressing GR and
MDFIC resided predominantly in the cytoplasm of control cells (Fig. 3A, upper panels). The strong overlay in their distribution, indicated by the yellow color in the merged image, is consistent with the GR-MDFIC interaction occurring in the cytoplasm of cells. Treatment with 100 nM Dex resulted in the robust translocation of GR into the nucleus and no change in the distribution of MDFIC (Fig. 3A, lower panels). Colocalization analysis revealed a 77.0% overlap of GR with MDFIC in control cells that was reduced to 14.3% in cells treated with Dex (Fig. 3B). Similarly, MDFIC exhibited a 69.0% overlap with GR in control cells that was decreased to 3.7% in response to Dex (Fig. 3B). The reduction in overlap of the two proteins after glucocorticoid treatment suggests that ligand binding triggers the dissociation of the GR-MDFIC complex. These findings are in agreement with our co-immunoprecipitation data. In addition, they suggest that the interaction of MDFIC with cytoplasmic GR does not impede its subsequent translocation into the nucleus after binding glucocorticoids. To detect endogenous MDFIC, we generated an anti-MDFIC antibody against amino acids 126–246 of MDFIC. The anti-MDFIC antibody detected MDFIC endogenously expressed in human A549 lung adenocarcinoma cells that are used classically to study glucocorticoid signaling (Fig. 4, B and C). The interaction was specific, as detection of MDFIC in A549 cells was prevented by preincubating the antibody with the immunizing peptide and by siRNA-mediated gene silencing of MDFIC (Fig. 4, B and C). We utilized the anti-MDFIC antibody in co-immunoprecipitation experiments to examine whether the endogenous GR and MDFIC associate in a complex. A549 cells were treated with or without 100 nM Dex for 1 h, and protein lysates were immunoprecipitated with the anti-MDFIC antibody. In the absence of glucocorticoids, GR co-immunoprecipitated with MDFIC, indicating the two endogenous proteins associate in a complex (Fig. 4D). Dex treatment resulted in the dissociation of the complex as the amount of co-immunoprecipitated GR was reduced in the glucocorticoid-treated cells (Fig. 4D). Consistent with the
interaction of these two proteins occurring in the cytoplasm of cells, we found by cell fractionation studies that endogenous MDFIC and GR reside in the cytoplasm of A549 cells not exposed to glucocorticoids (Fig. 4E). After glucocorticoid treatment, GR translocated into the nucleus, whereas MDFIC remained in the cytoplasm (Fig. 4E). These data indicate that the endogenous GR and MDFIC form a complex in the cytoplasm of cells that is dissociated upon glucocorticoid binding to GR.

**MDFIC alters the gene regulatory profile of GR**

MDFIC has been characterized as a transcriptional regulator (20, 24–28); therefore, we initially investigated whether the association of MDFIC with GR altered the transcriptional activity of the receptor on glucocorticoid-responsive reporter genes. A549 cells were transfected with a luciferase reporter driven by the glucocorticoid-responsive mouse mammary tumor virus promoter (MMTV-LUC) and either empty vec-
tor or MDFIC. As shown in the left panel of Fig. 5A, an 18-h treatment with 100 nM Dex resulted in 4.7-fold induction of luciferase expression in cells receiving the empty vector. In cells transfected with MDFIC, however, glucocorticoid treatment resulted in a greater 14.8-fold induction in luciferase expression. The MMTV promoter is a complex promoter that contains binding sites not only for GR and but also for other transcription factors such as octamer binding factor 1 and nuclear factor 1. Therefore, we next tested the ability of MDFIC to alter the transcriptional activity of GR on a simple glucocorticoid-responsive promoter composed solely of 2 tandem GREs and a TATA box. A549 cells were transfected with a luciferase reporter driven by this simple glucocorticoid-responsive promoter (GRE2-LUC) and either empty vector or MDFIC. Glucocorticoid treatment resulted in a 15.6-fold increase in luciferase expression in cells receiving the empty vector (Fig. 5A, right panel). Cells transfected with MDFIC displayed an even greater 28.1-fold increase in luciferase expression in response to glucocorticoids. To determine if the observed increase in GR transactivation of these two reporter genes depended on its association with MDFIC, we utilized the MDFIC(1–164) truncation mutant, which does not interact with GR due to the absence of the cysteine-rich carboxyl-terminal domain (Fig. 2E). The enhanced transcriptional activity of GR on both these promoters was largely abolished in cells transfected with the MDFIC(1–164) truncation mutant (Fig. 5, A and B). We next examined GR transactivation of the MMTV-LUC and GRE2-LUC reporters in A549 cells depleted of endogenous MDFIC by siRNA-mediated gene silencing (Fig. 5, C and D). Loss of MDFIC resulted in a significant reduction in the ability of GR to transactivate both reporter genes after glucocorticoid treatment. These data suggest that the interaction of MDFIC with GR can alter its transcriptional activity on both complex and simple glucocorticoid-responsive promoters.

To evaluate whether the interaction of MDFIC with GR alters its regulation of endogenous genes, we performed a genome-wide microarray in A549 cells that were transfected with non-targeting control (NTC) siRNA or MDFIC siRNA (MDFIC-KD). Knockdown of MDFIC was efficient and had no effect on the expression of GR (Fig. 6, A and B). In addition, the subcellular distribution of GR was not altered by depletion of MDFIC (Fig. 6C). Treatment of cells for 6 h with 100 nM Dex resulted in the regulation of 3281 and 3598 genes in the NTC and MDFIC-KD cells, respectively (Fig. 6D). The percentage of...
genes induced (42.9% in NTC cells; 41.9% in MDFIC-KD cells) and repressed (57.1% in NTC cells; 58.1% in MDFIC-KD cells) by Dex was unaffected by depletion of MDFIC (Fig. 6D). A comparison of the glucocorticoid-regulated genes in the NTC and MDFIC-KD cells revealed three major groups: a group of 2473 genes (common) that were regulated by Dex independent of MDFIC, a group of 808 genes (NTC unique) that were regulated by Dex only in the presence of MDFIC, and a group of 1125 genes (MDFIC-KD unique) that were regulated by Dex only in the absence of MDFIC (Fig. 6E).

For validation of the microarray, we performed RT-PCR on an independent set of NTC and MDFIC-KD samples and evaluated the expression of genes belonging to each of the three groups. Members of the common group include zinc finger protein 36 (Zfp36) and C-C motif chemokine ligand 2 (Ccl2), and these two genes were induced and repressed, respectively, to a similar extent by Dex in the NTC and MDFIC-KD cells (Fig. 7A, upper panels). Other members of the common group exhibited differences in the magnitude of the observed glucocorticoid regulation (Fig. 7A, lower panels). Dex treatment resulted in a 79.3% repression of the DAX-1 nuclear receptor (Nr0b1) in NTC cells but a smaller, 58.9%, repression in the MDFIC-KD cells. On the other hand, administration of Dex resulted in a 43.7% repression of interleukin-8 (IL-8; Cxcl8 gene) in the NTC cells but a greater, 68.8%, repression in the MDFIC-KD cells. Members of the NTC unique group include the bone morphogenetic protein 6 (Bmp6) and potassium channel, inwardly rectifying subfamily J, member 2 (Kcnj2) genes (Fig. 7B). In the presence of MDFIC, glucocorticoid treatment resulted in the induction of Bmp6 and the repression of Kcnj2. However, in cells lacking MDFIC, the glucocorticoid-dependent regulation of these two genes was largely abolished.
Figure 5. MDFIC modulates glucocorticoid receptor signaling

A. MDFIC alters the transcriptional activity of GR on glucocorticoid-responsive reporter genes in A549 cells. A549 cells were transfected with the pMMTV-LUC reporter (left panel) or the pGRE2-LUC reporter (right panel) and either empty vector, FLAG-MDFIC, or FLAG-MDFIC(1–164). After an 18-h treatment with vehicle or 100 nM Dex, the cells were harvested, and luciferase activity was measured. Data represent the mean ± S.D. from four independent experiments performed in quadruplicate. A one-way ANOVA followed by Tukey’s post hoc test was performed to determine significance. ***, p < 0.001 for Dex versus con (vehicle). ###, p < 0.001 for MDFIC Dex versus vector Dex and for MDFIC(1–164) Dex versus MDFIC Dex.

B. Protein lysates from A549 cells transfected as above with FLAG-MDFIC or FLAG-MDFIC(1–164) were immunoblotted with the anti-FLAG antibody (upper blot) or anti-actin antibody (lower blot). Shown is a representative immunoblot from three independent experiments.

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D. RT-PCR analysis of MDFIC mRNA expression in A549 cells transfected as above with reporter gene and either NTC siRNA or MDFIC siRNA (Student’s t test, mean ± S.E. *** , p < 0.001).
Members of the MDFIC-KD unique group include the ankyrin-3 (Ank3) and tumor necrosis factor (TNF) receptor-associated factor 1 (Traf1) genes (Fig. 7C). These two genes were not regulated by glucocorticoids in cells expressing MDFIC. However, in the MDFIC-KD cells, GR gained the ability to induce Ank3 and to repress Traf1 after glucocorticoid exposure. Collectively, these data demonstrate that the interaction of GR with MDFIC in the cytoplasm of cells alters the global gene regulatory profile of GR such that GR gains the ability to regulate some genes but loses the ability to regulate others.

We analyzed the common, NTC unique, and MDFIC-KD unique gene sets using literature-based Ingenuity Pathway Analysis software to gain insight into the diseases and biological functions most significantly associated with the glucocorticoid regulated genes. Shown in Fig. 8 are the top 10 annotations for each gene group. Remarkably, only one annotation, Cell Death and Survival, was shared across all 3 sets of genes. Among the annotations displaying the greatest divergence between the NTC unique and MDFIC-KD unique gene sets were Immune Cell Trafficking and Inflammatory Response.
ular interest given the widespread clinical use of glucocorticoids to suppress the immune system and inhibit inflammation (29). Immune Cell Trafficking and Inflammatory Response were strongly associated with the MDFIC-KD unique gene set (rank = 5 and 6, respectively) but very poorly associated with the NTC unique gene set (rank = 50 and 51, respectively). Loss of MDFIC not only resulted in different genes becoming regulated by glucocorticoids but also an expansion (~2.5-fold) in the total number of regulated genes associated with these two annotations. For example, a total of 44 genes associated with Immune Cell Trafficking were regulated by Dex only in the presence of MDFIC, whereas 121 genes were regulated by Dex only in the absence of MDFIC (supplemental Fig. S1). Similarly, a total of 79 genes associated with Inflammatory Response were

Figure 7. GR differentially regulates genes in A549 cells depending on the presence or absence of MDFIC. Total RNA was isolated from A549 cells transfected with NTC siRNA or MDFIC siRNA (MDFIC-KD) and treated with vehicle or 100 nM Dex for 6 h. A, genes commonly regulated by GR in the NTC and MDFIC-KD cells. The expression of zinc finger protein 36 (Zfp36), Ccl2, Nr0b1, and IL-8 was measured by RT-PCR. B, genes uniquely regulated by GR in the NTC cells. The expression of Bmp6 and Kcnj2 was measured by RT-PCR. C, genes uniquely regulated by GR in the MDFIC-KD cells. The expression of Ank3 and Traf1 was measured by RT-PCR. Data are plotted as -fold change and represent the mean ± S.E. from 3–6 independent experiments. A one-way ANOVA followed by Tukey’s post hoc test was performed to determine significance. ***: p < 0.001 for Dex versus vehicle (con). #: p < 0.05; ##: p < 0.01; ###: p < 0.001 for MDFIC-KD Dex versus NTC Dex.
**MDFIC modulates glucocorticoid receptor signaling**

A. **Common**

| Rank | Diseases and Biological Functions | P-value       | Genes |
|------|-----------------------------------|---------------|-------|
| 1    | Cellular Development              | 1.51E-28-1.19E-05 | 856   |
| 2    | Cellular Movement                 | 5.57E-26-9.32E-06 | 556   |
| 3    | Cellular Growth and Proliferation | 1.16E-25-1.19E-05 | 864   |
| 4    | Digestive System Development and Function | 1.69E-22-5.4E-06 | 275   |
| 5    | Cell Death and Survival           | 4.49E-22-1.02E-05 | 785   |
| 6    | Tissue Development                | 1.96E-21-1.19E-05 | 782   |
| 7    | Cancer                            | 5.02E-20-8.98E-06 | 2004  |
| 8    | Organismal Development            | 2.31E-19-1.09E-05 | 779   |
| 9    | Embryonic Development             | 8.02E-19-1.02E-05 | 607   |
| 10   | Tissue Morphology                 | 1.46E-18-1.16E-05 | 582   |

B. **NTC Unique**

| Rank | Diseases and Biological Functions | P-value       | Genes |
|------|-----------------------------------|---------------|-------|
| 1    | Cancer                            | 4.82E-07-1.13E-02 | 631   |
| 2    | Organismal Injury and Abnormalities | 1.2E-06-9.93E-03 | 387   |
| 3    | Cell Death and Survival           | 4.61E-06-1.08E-02 | 237   |
| 4    | Hematological Disease             | 9.51E-06-5.1E-03  | 97    |
| 5    | Renal and Urological Disease      | 1.36E-05-2.36E-03 | 131   |
| 6    | Reproductive System Development and Function | 1.39E-05-9.66E-03 | 76    |
| 7    | Tissue Morphology                 | 1.39E-05-9.93E-03 | 65    |
| 8    | Cell-To-Cell Signaling and Interaction | 1.83E-05-9.93E-03 | 54    |
| 9    | Cellular Assembly and Organization | 1.83E-05-1.11E-02 | 89    |
| 10   | Cellular Function and Maintenance | 1.83E-05-9.93E-03 | 46    |

C. **MDFIC-KD Unique**

| Rank | Diseases and Biological Functions | P-value       | Genes |
|------|-----------------------------------|---------------|-------|
| 1    | Cellular Development              | 2.43E-05-1.95E-02 | 168   |
| 2    | Cellular Growth and Proliferation | 2.43E-05-1.91E-02 | 307   |
| 3    | Hematological System Development and Function | 2.43E-05-2.19E-02 | 179   |
| 4    | Cell-To-Cell Signaling and Interaction | 7.78E-05-2.17E-02 | 128   |
| 5    | Immune Cell Trafficking           | 7.94E-05-2.19E-02 | 121   |
| 6    | Inflammatory Response             | 7.94E-05-2.19E-02 | 112   |
| 7    | Cardiovascular System Development and Function | 2.17E-04-2.11E-02 | 114   |
| 8    | Cell Death and Survival           | 2.17E-04-2.02E-02 | 281   |
| 9    | Cellular Compromise               | 2.22E-04-1.97E-02 | 34    |
| 10   | Cellular Assembly and Organization | 3.05E-04-1.99E-02 | 141   |

Figure 8. Distinct biological functions are associated with the MDFIC-dependent alteration in the GR transcriptome in A549 cells. The set of genes commonly regulated by glucocorticoids in the NTC and MDFIC-KD cells (2473 total genes), uniquely regulated by glucocorticoids in the NTC cells (808 total genes), and uniquely regulated by glucocorticoids in the MDFIC-KD cells (1125 total genes) were analyzed using literature-based Ingenuity Pathway Analysis software. Shown are the top 10 diseases and biological functions most significantly associated with the common (A), NTC unique (B), and MDFIC-KD unique (C) genes.

regulated by Dex only in the presence of MDFIC, whereas 191 genes were regulated by Dex only in the absence of MDFIC (supplemental Fig. S2).

The gene enrichment predictions suggest that glucocorticoids differentially affect the inflammatory response depending on the association of GR with MDFIC. We tested this directly by evaluating glucocorticoid-mediated antagonism of TNFα-induced pro-inflammatory gene changes in A549 cells transfected with NTC siRNA or MDFIC siRNA. For this experiment we used nanostring technology, which provides direct measurement of mRNA expression without the synthesis of cDNA or amplification of transcripts. Cells were treated with vehicle, 100 nM Dex, 10 ng/ml TNFα, or both 100 nM Dex and 10 ng/ml TNFα for 6 h. Many of the inflammatory genes induced by TNFα, such as Cc2 and nuclear factor κB subunit 1 (Nfkb1), were antagonized by co-administration of glucocorticoids, and the extent of this inhibition was unaffected by MDFIC (Fig. 9B). However, the ability of glucocorticoids to antagonize the TNFα-mediated up-regulation of other genes, including C-C motif chemokine ligand 20 (Ccl20) and colony stimulating factor 1 (Csf1), was abolished in cells depleted of MDFIC (Fig. 9B). In fact, co-treatment of MDFIC-KD cells with Dex and TNFα resulted in a significant up-regulation of Csf1 mRNA compared with TNFα treatment alone. Although glucocorticoids and TNFα are generally considered to act in an opposing manner, recent reports have identified genes that are co-regulated by these molecules (30). We observed a similar phenomenon as co-administration of Dex and TNFα robustly stimulated the expression of C-C motif chemokine 19 (Ccl19) and prostaglandin I2 receptor (Ptgir) mRNA in NTC cells (Fig. 9C). Strikingly, the up-regulation of these two genes was eliminated in cells lacking MDFIC. These data provide evidence that the interaction of MDFIC with GR may play an important functional role fine-tuning glucocorticoid regulation of the inflammatory response.

**MDFIC alters the phosphorylation status of GR**

GR is phosphorylated on multiple serine residues (see the Fig. 1A schematic) in both an agonist-independent and agonist-dependent manner, and these phosphorylation events have been shown to influence the transcriptional activity of GR in a
Figure 9. MDFIC alters glucocorticoid regulation of the inflammatory response in A549 cells. Nanostring analysis was performed on RNA isolated from A549 cells transfected with NTC siRNA or MDFIC siRNA (MDFIC-KD) and treated for 6 h with vehicle, 100 nM Dex, 10 ng/ml TNF-α, or both 100 nM Dex and 10 ng/ml TNF-α. A, RNA counts for the Ccl2 and nuclear factor κB subunit 1 (Nfkb1) genes. B, RNA counts for the Ccl20 and Csf1 genes. C, RNA counts for the C-C motif chemokine 19 (Ccl19) and prostaglandin I2 receptor (Ptgir) genes. Data shown are the raw RNA counts normalized to six housekeeping genes as described under “Experimental Procedures” and represent the mean ± S.E. from three independent experiments. A one-way ANOVA followed by Tukey’s post hoc test was performed to determine significance. ***, p < 0.001 for TNF-α versus con (vehicle). &, p < 0.05; &&& p < 0.001 for Dex+TNF-α versus TNF-α. ##, p < 0.01. ###, p < 0.001 for MDFIC-KD Dex+TNF-α versus NTC Dex+TNF-α.
gene-specific fashion (31, 32). Among the well studied GR phosphorylation sites are serine 203 (Ser-203) and serine 211 (Ser-211). Ser-203 and Ser-211 exhibit a basal level of phosphorylation and become hyper-phosphorylated after glucocorticoid treatment (31, 32). We hypothesized that the interaction of MDFIC with GR in the cytoplasm of A549 cells might modulate the phosphorylation status of the receptor and thereby contribute to the observed diversity in GR signaling. To explore this possibility, we examined GR phosphorylation at Ser-203 and Ser-211 in NTC and MDFIC-KD cells using phospho-specific antibodies. Specificity of the antibodies for phosphorylated GR was confirmed by siRNA-mediated knockdown of the receptor (Fig. 10A). In untreated cells, knockdown of MDFIC did not alter the basal level of phosphorylation measured for Ser-203 but did lead to a significant 1.5-fold increase in the basal phosphorylation of Ser-211 (Fig. 10B). Treatment of cells for 1 h with 100 nM...
results were found, however, when we compared the 497 new genes uniquely regulated by GR only in the presence of MDFIC with the 887 genes regulated by Dex in the S211A+MDFIC cells (Fig. 11H). Only 26.6% of these genes (132/497) were regulated by the S211A mutant. These findings demonstrate a major loss in the ability of MDFIC to modulate the GR transcriptome when Ser-211 phosphorylation is prevented, suggesting that MDFIC-mediated changes in Ser-211 phosphorylation underlie many of its effects on GR signaling.

**Glucocorticoids negatively regulate MDFIC gene expression**

The interaction of MDFIC with GR has a profound influence on the receptor transcriptome. Therefore, factors that control MDFIC expression will shape the cellular response to glucocorticoids. A search of the genes identified in the A549 microarray to be regulated by glucocorticoids in both the presence and absence of MDFIC uncovered the MDFIC gene itself as a target for glucocorticoid-dependent repression. We confirmed the negative regulation of MDFIC by glucocorticoids in an independent set of NTC and MDFIC-KD samples (Fig. 12A). The magnitude of the repression was time-dependent: a 25.7%, 34.2%, and 43.7% decrease in MDFIC mRNA was observed after a 3-, 6-, and 12-h treatment of A549 cells with 100 nM Dex, respectively (Fig. 12B). The glucocorticoid-mediated decrease in MDFIC mRNA also led to a 39.9% reduction in MDFIC protein levels after a 12-h Dex treatment (Fig. 12C). To define the molecular mechanism underlying the repression, we first examined whether GR was required for the regulatory event. The Dex-dependent decrease in MDFIC gene expression was completely abolished in A549 cells depleted of GR by siRNA (Fig. 12D). We next investigated whether MDFIC was a primary or secondary target of glucocorticoid regulation by using the protein synthesis inhibitor cycloheximide. In the presence of cycloheximide, Dex administration still resulted in a significant repression of MDFIC, suggesting MDFIC is a direct target of GR regulation (Fig. 12E). Furthermore, we used nascent RNA primers targeting intronic sequences of MDFIC to examine whether GR altered the transcription of the MDFIC gene. After a 3-h exposure to glucocorticoids, a 34.1% reduction in MDFIC nascent RNA was observed (Fig. 12F). Finally, we examined whether the glucocorticoid-dependent regulation of MDFIC occurred in other human cell types. As shown in Fig. 12, G and H, Dex treatment resulted in a time-dependent reduction of MDFIC in both human THP-1 monocytes and human U2OS osteosarcoma cells. Collectively, these data indicate that glucocorticoids act through GR to directly repress the expression of MDFIC in multiple cell types.

**Discussion**

Elucidating the mechanisms by which glucocorticoids generate cell type- and tissue-specific effects is an area of intense investigation because of the widespread clinical use of these steroids, the adverse effects resulting from sustained elevations in glucocorticoids, and the development of glucocorticoid resistance which limits the therapeutic benefit. Many factors appear to shape the cellular response to glucocorticoids, including the nature and concentration of the GR agonist, the expression level of GR, the repertoire of various splicing and translational GR isoforms, post-translational modifications of the receptor,
Figure 11. MDFIC modulation of the GR transcriptome is impaired in COS-1 cells expressing a phosphorylation-defective receptor. A, COS-1 cells were transfected with GR alone, GR and MDFIC, S211A alone, or S211A and MDFIC. GR, S211A, and MDFIC levels were evaluated by immunoblot analysis. Shown is a representative immunoblot from three independent experiments. B, COS-1 cells were transfected with GR alone or S211A alone. Basal and Dex-dependent phosphorylation of GR and the S211A receptor mutant were evaluated by immunoblot analysis using the anti-GR(Ser-211) antibody. Shown is representative immunoblot from three independent experiments. C, COS-1 cells were transfected with GR alone or GR and MDFIC. After treatment with vehicle or 100 nM Dex for the indicated times, the cells were harvested, and GR phosphorylation was evaluated by immunoblot using the anti-GR(Ser-211) antibody. Shown are representative immunoblots and quantitation of phosphorylated GR to total GR. Data represent the mean ± S.E. from four independent experiments. A one-way ANOVA followed by Tukey’s post hoc test was performed to determine significance. *, p < 0.05; ***, p < 0.001 for Dex versus Con (vehicle). ##, p < 0.01 for GR + MDFIC Dex versus GR Dex. D, microarray analysis was performed on RNA isolated from COS-1 cells transfected with GR alone, GR and MDFIC, S211A alone, or S211A and MDFIC. Cells were treated with vehicle or 100 nM Dex for 6 h. Shown are the total number of genes regulated by Dex and the number of genes with increased or decreased expression. Differentially expressed genes were determined using an error-weighted ANOVA and Benjamini-Hochberg False Discovery Rate multiple test correction with a p value of p < 0.01. E, genes regulated by Dex in GR cells were compared with genes regulated by Dex in the S211A cells using a Venn diagram. F, genes regulated by Dex in GR cells were compared with genes regulated by Dex in the GR + MDFIC cells using a Venn diagram. G, genes regulated by Dex-activated GR independent of MDFIC (GR and GR + MDFIC common genes shown in panel F) were compared with genes regulated by Dex in S211A + MDFIC cells using a Venn diagram. H, genes regulated by Dex-activated GR only in the presence of MDFIC (GR + MDFIC unique genes shown in panel F) were compared with genes regulated by Dex in S211A + MDFIC cells using a Venn diagram.
Figure 12. GR directly represses MDFIC gene expression in multiple human cell types. A, total RNA was isolated from A549 cells transfected with NTC siRNA or MDFIC siRNA (MDFIC-KD) and treated with vehicle or 100 nM Dex for 6 h. The left panel shows MDFIC knockdown as measured by RT-PCR. The right panel shows glucocorticoid-dependent regulation of MDFIC mRNA in NTC and MDFIC-KD cells as measured by RT-PCR and plotted as -fold change. B, A549 cells were treated with 100 nM Dex for the indicated times, and MDFIC mRNA was measured by RT-PCR. C, A549 cells were treated with 100 nM Dex for 12 h, and MDFIC levels were evaluated by immunoblot. The left panel shows quantitation of MDFIC normalized to actin (mean ± S.E. from three independent experiments). The right panel shows a representative immunoblot. Protein lysates from A549 cells transfected with NTC siRNA or MDFIC siRNA were included as controls. D, total RNA was isolated from A549 cells transfected with NTC siRNA or GR siRNA (GR-KD) and treated with vehicle or 100 nM Dex for 6 h. The left panel shows GR knockdown as measured by RT-PCR. The right panel shows glucocorticoid-dependent regulation of MDFIC mRNA in NTC and GR-KD cells as measured by RT-PCR and plotted as -fold change. E, A549 cells pretreated for 1 h with vehicle or 10 μg/ml of cycloheximide (CHX) were exposed to 100 nM Dex for 6 h, MDFIC mRNA levels were analyzed by RT-PCR. F, A549 cells were treated with 100 nM Dex for 3 h, and the level of MDFIC nascent RNA was analyzed by RT-PCR. G, THP-1 monocytes were treated with 100 nM Dex for indicated times, and MDFIC mRNA was measured by RT-PCR. H, U2OS osteosarcoma cells stably expressing GR were treated with 100 nM Dex for the indicated times, and MDFIC mRNA was measured by RT-PCR. Data represent the mean ± S.E. from 3–7 independent experiments. Student’s t test or a one-way ANOVA followed by Tukey’s post hoc test was performed to determine significance. *, p < 0.05; **, p < 0.01; ***, p < 0.001 for Dex versus Con.
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the availability of specific co-regulators, and the chromatin landscape. In this report we have identified a novel protein-protein interaction between unliganded GR and MDFIC that occurs in the cytoplasm of cells. This interaction is dissociated in the presence of glucocorticoids but has a profound impact on the ensuing transcriptional and cellular responses elicited by activated GR. The association of MDFIC with GR alters both the pattern and magnitude of receptor phosphorylation, which contributes to the modulated GR transcriptome. Cross-talk between GR and MDFIC is bi-directional, as we show that glucocorticoids operate in a negative feedback loop to directly repress MDFIC gene expression. These findings identify MDFIC as a new binding partner for cytoplasmic GR that contributes to the heterogeneity and tissue specificity of glucocorticoid action.

MDFIC belongs to a small family of proteins that possess a unique cysteine rich carboxyl-terminal domain. The physiological function of MDFIC remains incompletely understood in large measure because mice with a disrupted MDFIC gene have not yet been described. In loss of function studies performed in *Xenopus*, MDFIC was found to be necessary for development (34). Embryos depleted of MDFIC were missing head structures, neural tube, notochord, and paraxial mesoderm, and these effects were attributed to the loss of MDFIC-dependent repression of the transcription factor T cell factor 3 (TCF-3). In mammalian cells MDFIC has been shown to interact with a variety of different proteins that directly or indirectly modulate transcription, including axin, cyclin T1, cyclin T2, lymphocyte enhancer factor 1 (LEF-1), and the viral transactivators human immunodeficiency virus type 1 (HIV-1) Tat and human T-cell leukemia virus type 1 (HTLV-1) Tax (24, 26, 27, 35). The association of MDFIC with these proteins is mediated by the cysteine-rich carboxyl-terminal domain and results in an altered transcriptional activity. For this reason, MDFIC is generally described as a transcriptional regulator. Although these early studies laid a foundation for our understanding of MDFIC, they were limited by their heavy reliance on reporter genes and overexpression of MDFIC. Our current work, performed on endogenous MDFIC and on a genome-wide scale, reveals an expanded role for MDFIC as a gene expression regulator. We find that MDFIC is required for GR to regulate the expression of 808 unique genes in A549 cells. Unexpectedly, we also discovered that GR gains the ability to regulate 1125 unique genes in the absence of MDFIC. These findings suggest that the interaction of MDFIC with GR is required not only for the transcriptional activity of GR on one set of genes but also for the silencing of GR activity on a completely different set of genes.

Transcriptional regulators of GR typically associate with the receptor in the nucleus of cells after ligand activation. The GR-MDFIC interaction is unique because it occurs in the cytoplasm of cells and is dissociated after binding of glucocorticoids. MDFIC has been shown to inhibit the activity of other nuclear proteins by sequestering them in the cytoplasm, preventing their DNA binding, and/or promoting their degradation (24, 35, 36). In A549 cells depleted of MDFIC, we did not detect alterations in the expression level or cellular distribution of GR. However, we did observe changes in both the pattern and magnitude of GR phosphorylation. Knockdown of MDFIC increased the basal level of Ser-211 phosphorylation, and the glucocorticoid-dependent phosphorylation of Ser-203 and Ser-211 was impaired. In COS-1 cells, overexpression of MDFIC augmented GR phosphorylation at Ser-211 after glucocorticoid treatment. Microarray data from COS-1 cells expressing wild-type GR or the phosphorylation-defective mutant S211A revealed an important role for Ser-211 phosphorylation in the ability of MDFIC to modulate the GR transcriptome. Not all MDFIC-sensitive gene changes depended on Ser-211 phosphorylation, however, suggesting Ser-203 phosphorylation may also contribute to the regulatory activity of MDFIC on receptor signaling.

These findings suggest that the interaction of MDFIC with GR can influence receptor conformation, making it more or less favored as a substrate for kinase and/or phosphatase activity. The resultant perturbations in GR phosphorylation would then lead to distinct gene transcription programs and cellular responses to glucocorticoids. One of the primary functions of the site-specific phosphorylation is to regulate the transcriptional activity of GR. Via changes in cofactor recruitment and chromatin occupancy, alterations in phosphorylation have been shown to dramatically change the genomic gene regulatory profile of GR and result in the activation of distinct signaling pathways (33, 37–39). GR is also subject to a variety of other post-translational modifications, including sumoylation and acetylation (1). Sumoylation of GR has been shown recently to modulate the chromatin occupancy of GR and the ensuing transcriptional and cellular response to glucocorticoids (40). Whether the association of MDFIC with GR can influence other receptor post-translational modifications is currently unknown but is an important question for future studies.

The ability of MDFIC to alter the GR transcriptome suggests that the expression level of MDFIC will have a major impact on cellular responsiveness to glucocorticoids. Multiple studies have reported that MDFIC is expressed in a tissue- and cell type-specific manner. MDFIC is expressed in human spleen, thymus, prostate, uterus, and small intestine but is not detected in testis and colon (20). MDFIC is also found in primary human immune cells, but its expression varies considerably among immune cell subsets with NK cells (CD56+) and monocytes (CD14+) having high levels, CD4+ T-cells and CD8+ T-cells displaying intermediate levels, and B-cells (CD19+) expressing low levels (41). Many transformed cell lines also exhibit differential expression of MDFIC (27, 41). These cell type-specific expression patterns of MDFIC may contribute to the diverse actions of glucocorticoids that are observed in different tissues.

Factors that induce or repress MDFIC gene expression within a given cell type will also have an important influence on GR signaling. In the current study we made the novel discovery that glucocorticoids themselves directly repress MDFIC gene expression in a negative feedback loop. This repression requires GR, is direct, occurs at the level of transcription, and takes place in multiple cell types. By feeding back on MDFIC, glucocorticoids can limit the changes in GR signaling mediated by MDFIC. The only previously described regulator of MDFIC gene expression is the pro-inflammatory cytokine interleukin 2 (IL-2), which has been shown to induce the expression of MDFIC in a variety of hematopoietic cell lines and in primary human immune cells (41). That anti-inflammatory glucocorticoids and pro-inflammatory IL-2 regulate MDFIC gene expres-
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Experimental procedures

Reagents

Dexamethasone, RU486, and cortisol were purchased from Steraloids (Newport, RI). The rabbit anti-MDFIC antibody 2075 was produced using a peptide (CIHHGAKHGSADNRK) synthesized by AnaSpec (San Jose, CA), and the antisera was produced by Covance (Denver, PA).

Yeast two-hybrid assay

ProNet technologies automated yeast two-hybrid screening was performed by Myriad Genetics as previously described (49). A DNA sequence encoding amino acids 466–546 of human GR was used as bait. Human brain, spleen, pooled breast cancer/prostate cancer, and pooled liver/small intestine/ adipose cDNA libraries were used to screen for prey proteins. Isolated bait and prey plasmids were co-transformed into yeast for confirmation of interactions by liquid β-galactosidase assays. DNA sequencing was used to determine the identity of the prey.

Cell culture

A549, COS-1, and U2OS cells (ATCC) were maintained in Dulbecco’s modified Eagle’s medium F-12 (DMEM) supplemented with 10% heat-inactivated FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. U2OS cells stably expressing the human GR have been previously described (50). THP-1 monocytes were maintained in RPMI medium supplemented with 10% heat-inactivated FBS, 50 μM β-mercaptoethanol, 25 mM HEPES (pH 7.0), and 100 units/ml penicillin/streptomycin. Before glucocorticoid treatment, cells were cultured overnight in medium supplemented with 10% charcoal-stripped FBS.

Plasmids

MDFIC was cloned from a human spleen cDNA library (Ambion) into the cloning vector pCR2.1-TOPO (Invitrogen) using the primers 5’-GCCACCATGTTCCGGCCGGCGCGAAGC-3’ and 5’-TTATGAAGGAAAACAAATTCCACAGC-3’. The FLAG epitope was added to the amino terminus of MDFIC using the primers 5’-GGTAAAGCTTGCCACATGGACTACAAGGAGATGACGACAAGTCGCGCCGGGGGCGAAGC-3’ and 5’-GGTCTCTAGATTGAAGGAAACAAATTCCACAGC-3’ and subcloned into the expression vector pcDNA3.1zeo+ (Invitrogen) using HindIII/XbaI. The human GR was used as bait. Human brain, spleen, pooled breast cancer/prostate cancer, and pooled liver/small intestine/adipose cDNA libraries were used to screen for prey proteins. Isolated bait and prey plasmids were co-transformed into yeast for confirmation of interactions by liquid β-galactosidase assays. DNA sequencing was used to determine the identity of the prey.

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RNA isolation and quantitative RT-PCR analysis

Total RNA was harvested from A549 cells using the RNeasy Mini kit and RNase-Free DNase kit (Qiagen). Individual mRNA abundance was determined using a TaqMan one-step RT-PCR procedure on the 7900HT sequence detection system (Applied Biosystems), and all primer/probe sets were from Applied Biosystems. For analysis of MDFIC nascent RNA, primer sequences were designed to amplify a region spanning an exon-intron boundary and thereby detect only unprocessed, newly expressed transcripts. The primer sequences were as follows: forward primer, 5’-ACAGCCAGGGTGATGTG-3’ (exon 2/intron2); probe, 5’-TCTGTGCACTTTGAGCAC-3’ (intron 2). Relative expression values for each gene were calculated using the ΔΔCt analysis method and the house-keeping gene peptidylprolyl isomerase B, which was unaffected by glucocorticoid treatment.

Co-immunoprecipitation assay

COS-1 cells were transfected with GR alone, FLAG-MDFIC alone, FLAG-MDFIC(1–164) alone, FLAG-MDFI alone, both GR and FLAG-MDFIC, both GR and FLAG-MDFIC(1–164), or both GR and FLAG MDFI. Forty-eight hours after transfection the cells were treated with vehicle or Dex for 1 h. Cells were harvested in cold PBS and resuspended in TENT lysis buffer (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, 0.5% Triton X-100) containing protease inhibitors. After incubating with rotation for 1 h at 4°C, the samples were centrifuged, and supernatant was removed for protein quantification. Equivalent amounts of protein were immunoprecipitated overnight with anti-FLAG antibody (Sigma, #F3165), anti-GR antibody 41 (BD Biosciences, #611227), or anti-GR antibody 57 (51). The following day protein A/G-agarose beads were added for a 90-min incubation at 4°C. The beads were washed with cold TENT lysis buffer, resuspended in sample buffer containing 5% β-mercaptoethanol, and heated for 6 min at 95°C. For co-immunoprecipitation of endogenous GR-MDFIC complexes, A549 cells were treated with vehicle or Dex for 1 h. Cells were washed with cold PBS, harvested in TENT lysis buffer containing protease inhibitors, Dounce-homogenized on ice, and processed as above. Immunoprecipitations were performed overnight with the anti-MDFIC 2075 antibody. Recovered proteins were resolved on Tris-glycine gels, transferred to nitrocellulose, and immunoblotted as described below.

Immunoblot analysis

Cells were washed once with cold PBS, lysed in SDS sample buffer (Invitrogen) supplemented with β-mercaptoethanol, sonicated on ice, and boiled for 6 min. Total protein was determined using the Pierce 660-nm protein assay with an ionic detergent compatibility reagent (Thermo Scientific). Cell fractionation experiments were performed using the Nuclear/Cytosol Fractionation kit (Biovision). For analysis of glucocorticoid regulation of MDFIC expression, A549 cells were harvested using radioimmuno precipitation assay buffer. Equivalent amounts of protein were separated on 4–20% Tris-glycine gels. Proteins were then transferred to nitrocellulose membranes and probed overnight with the following antibodies:

- rabbit anti-GR antibody 57 or mouse anti-GR antibody 59 (51), anti-GR antibody D8H2 (Cell Signaling, #3660S), anti-FLAG antibody (Sigma, #F3165), anti-FLAG antibody (Sigma, #F7425), anti-MDFIC antibody 2075, anti-GR(Ser-203) antibody (Abcam, #ab195703), anti-GR(Ser-211) antibody (Cell Signaling, #4161S), and/or anti-actin antibody (Millipore, #MAB1501). After washing, blot were incubated with goat anti-rabbit Alexa Fluor 680-conjugated secondary antibody (Life Technologies, #A21109) and/or goat anti-mouse IRDye800-conjugated secondary antibody (LI-COR Biosciences, #926–32210) and developed in the linear dynamic range using the LI-COR Odyssey imaging system. For some experiments, blots were incubated with HRP-linked secondary antibodies and developed using enhanced chemiluminescence (GE Healthcare).

Immunocytochemistry

COS-1 cells were transfected with GR and FLAG-MDFIC, and A549 cells were transfected with NTC siRNA or MDFIC siRNA. The cells were plated in 35-mm glass-bottom dishes (MatTek, Ashland, MA). The next day, cells were treated with vehicle or 100 nM Dex for 1 h. Cells were then washed with cold PBS, fixed for 30 min at room temperature with 4% paraformaldehyde, and processed as previously described (52). The anti-GR antibody 57 (51), anti-GR antibody D8H2 (Cell Signaling, #3660S), and/or the anti-FLAG antibody (Sigma, #F3165) were incubated with cells overnight at 4°C. The next day, goat anti-mouse Alexa Fluor 594 and/or goat anti-rabbit Alexa Fluor 488 secondary antibodies were incubated with the cells for 1 h at room temperature. A Zeiss laser-scanning confocal microscope (LSM 510 or LSM710; Carl Zeiss, Thornwood, NY) with a Plan-Apochromat 63×/1.4 oil objective was used to analyze the cells. Images were collected sequentially using dual excitation (488 nm from argon laser, 543 nm from HeNe laser) and emission filter sets (band pass, 500–530; long pass, 560 nm). Colocalization analysis in COS-1 cells was performed with ImageJ (National Institutes of Health) using the JAcoP Plugin (Institut Curie, France) (53). The Manders colocalization coefficient was computed from each image using a pixel intensity threshold of 75 for both channels.

Luciferase assays

A549 cells were transfected in six-well plates using TransIT-LT1 (Mirrus) with a firefly luciferase reporter (pMMTV-LUC or pGRE2-LUC), Renilla luciferase reporter pGL3-hRL (50), and either empty vector, MDFIC, or MDFIC(1–164). For knockdown experiments, A549 cells were transfected with the reporters above and either NTC siRNA or MDFIC siRNA using Dharmafect Duo (Thermo Scientific). The glucocorticoid-responsive firefly luciferase reporters, pMMTV-LUC and pGRE2-LUC, have been described previously (52). Cells were harvested the day after transfection and re-plated at equal densities in a 48-well plate. Vehicle or 100 nM Dex was added to the cells for an overnight (~18 h) incubation. For cells transfected with siRNA, vehicle or 100 nM Dex was added for a 6-h incubation. Cells were then lysed in passive lysis buffer, and luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega). Firefly luciferase values divided by Renilla luciferase values are reported as luciferase activity.
RNA interference assays

NTC siRNA, MDFIC SMARTpool siRNA, and GR SMARTpool siRNA were purchased from Thermo Scientific (Lafayette, CO). A549 cells were transfected with 60 nm concentrations of each siRNA using Dharmatect transfection reagent (Thermo Scientific) according to the manufacturer’s instructions. Cells were harvested the day after transfection and re-plated at appropriate tissue culture densities. All experiments were performed 72 h post transfection.

Microarray analysis

A549 cells were transfected with NTC siRNA or MDFIC siRNA. Seventy-two hours post-transfection, cells were stimulated with either vehicle or 100 nm Dex for 6 h. Total RNA from three biological replicates of NTC and MDFIC siRNA-treated cells was harvested as described above. COS-1 cells were transfected with GR alone, GR and MDFIC, S211A alone, or S211A and MDFIC. Forty-eight hours post-transfection, cells were stimulated with either vehicle or 100 nm Dex for 6 h. Total RNA from three biological replicates of the transfected COS-1 cells was harvested as described above. Gene expression profiles were analyzed using Agilent Human Whole Genome 4-by-44 multiplex format oligonucleotide arrays (catalogue number 014850, Agilent technologies, Santa Clara, CA) according to the Agilent one-color microarray-based gene expression analysis protocol. To identify differentially expressed probes, an analysis of variance (ANOVA) was used to determine if there was a statistical difference between the means of the different groups. In addition, an error-weighted ANOVA and Benjamini-Hochberg False Discovery Rate multiple test correction, with a p value of p < 0.01, was performed using Rosetta Resolver (A549 data) or OmicSoft Array Studio software (COS-1 data) to reduce the numbers of false positives. Finally, the statistically significant probes were analyzed by Ingenuity Pathway Analysis software (Ingenuity Systems) for functional analysis. Gene enrichment p values (p < 0.05) for biological functions were determined using Fischer’s exact test. The microarray data presented in this publication were deposited in the NCBI Gene Expression Omnibus (www.ncbi.nlm.nih.gov) (54) and are accessible through Gene Expression Omnibus series accession numbers GSE86115, GSE93899, and GSE93900.

Nanostring analysis

A549 cells were transfected with NTC siRNA or MDFIC siRNA. Seventy-two hours post-transfection, cells were stimulated with either vehicle, 100 nm Dex, 10 ng/ml TNFα, or 100 nm Dex and 10 ng/ml TNFα for 6 h. Total RNA from three biological replicates of NTC and MDFIC knockdown cells was harvested as described above. Gene expression was examined utilizing the human inflammation code set (Nanostring Technologies) that measures 249 endogenous genes and 6 housekeeping genes. RNA expression was quantified on the nCounter Digital Analyzer according to the manufacturer’s protocol (Nanostring Technologies). Raw and normalized counts were generated with nSolver (v3.0)™ software. Data were normalized utilizing the manufacturer’s positive and negative experimental control probes as well as all six housekeeping genes. To identify significant differences in RNA expression, an ANOVA was performed with post-hoc Benjamini-Hochberg FDR (false discovery rate)-corrected p values (p < 0.01).

Statistical analysis

Student’s t test or one-way ANOVA with Tukey’s post hoc analysis were used to determine whether differences between groups were statistically significant. The statistical analyses were performed using GraphPad Prism software.

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References

1. Oakley, R. H., and Cidlowski, J. A. (2013) The biology of the glucocorticoid receptor: new signaling mechanisms in health and disease. J. Allergy Clin. Immunol. 132, 1033–1044
2. Sapolsky, R. M., Romero, L. M., and Muncy, A. U. (2000) How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrinol. Rev. 21, 55–89
3. Liggins, G. C. (1994) The role of cortisol in preparing the fetus for birth. Reprod. Fertil. Dev. 6, 141–150
4. Miracle, X., Di Renzo, G. C., Stark, A., Fanaroff, A., Carbonell-Estrany, X., Saling, E., and Coordinators Of World Association of Perinatal Medicine Prematurity Working Group (2008) Guide for use of antenatal corticosteroids for fetal maturation. J. Perinat. Med. 36, 191–196
5. Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. Science 240, 889–895
6. Grad, I., and Picard, D. (2007) The glucocorticoid responses are shaped by molecular chaperones. Mol. Cell. Endocrinol. 275, 2–12
7. Pratt, W. B., and Toft, D. O. (1997) Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr. Rev. 18, 306–360
8. Freedman, N. D., and Yamamoto, K. R. (2004) Importin 7 and importin α/importin β are nuclear import receptors for the glucocorticoid receptor. Mol. Biol. Cell 15, 2276–2286
9. Gorovits, R., Ben-Dror, I., Fox, L. E., Westphal, H. M., and Vardimon, L. (1994) Developmental changes in the expression and compartmentalization of the glucocorticoid receptor in embryonic retina. Proc. Natl. Acad. Sci. U.S.A. 91, 4786–4790
10. Hsu, S. C., and DeFranco, D. B. (1995) Selectivity of cell cycle regulation of glucocorticoid receptor function. J. Biol. Chem. 270, 3359–3364
11. Kino, T., De Martino, M. U., Charmandari, E., Mirani, M., and Chrousos, G. P. (2003) Tissue glucocorticoid resistance/hypersensitivity syndromes. J. Steroid Biochem. Mol. Biol. 85, 457–467
12. Lamberts, S. W., Huizenga, A. T., de Lange, P., de Jong, F. H., and Koper, J. W. (1996) Clinical aspects of glucocorticoid sensitivity. Steroids 61, 157–160
13. Lu, N. Z., Collins, J. B., Grissom, S. F., and Cidlowski, J. A. (2007) Selective regulation of bone cell apoptosis by translational isoforms of the glucocorticoid receptor. Mol. Cell. Biol. 27, 7143–7160
14. Ren, R., Oakley, R. H., Cruz-Topete, D., and Cidlowski, J. A. (2012) Dual Role for Glucocorticoids in Cardiomyocyte Hypertrophy and Apoptosis. Endocrinology 153, 5346–5360
15. Scolltock, A. B., Heimlich, G., and Cidlowski, J. A. (2007) Glucocorticoids inhibit the apoptotic actions of UV-C but not Fas ligand in hepatoma cells: direct evidence for a critical role of Bcl-xL. Cell Death Differ. 14, 840–850
MDFIC modulates glucocorticoid receptor signaling

16. Barnes, P. J. (2013) Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. J. Allergy Clin. Immunol. 131, 636–645
17. Yang, N., Ray, D. W., and Matthews, L. C. (2012) Current concepts in glucocorticoid resistance. Steroids 77, 1041–1049
18. John, S., Sabo, P. J., Thurman, R. E., Sung, M. H., Biddle, S. C., Johnson, T. A., Hager, G. L., and Stamatoyannopoulos, J. A. (2011) Chromatin accessibility pre-determines glucocorticoid receptor binding patterns. Nat. Genet. 43, 264–268
19. Uhlenhaut, N. H., Barish, G. D., Yu, R. T., Downes, M., Karunasiri, M., Liddle, C., Schwale, P., Hübner, N., and Evans, R. M. (2013) Insights into negative regulation by the glucocorticoid receptor from genome-wide profiling of inflammatory cistromes. Mol. Cell 49, 158–171
20. Thébault, S., Gachon, F., Lemasson, I., Devaux, C., and Mesnard, J. M. (2000) Molecular cloning of a novel human I-mfa domain-containing protein that differently regulates human T-cell leukemia virus type 1 and HIV-1 expression. J. Biol. Chem. 275, 4848–4857
21. Thébault, S., and Mesnard, J. M. (2001) How the sequestration of a protein interferes with its mechanism of action: example of a new family of proteins characterized by a particular cysteine-rich carboxyl-terminal domain involved in gene expression regulation. Curr. Protein Pept. Sci. 2, 155–167
22. Picard, D., and Yamamoto, K. R. (1987) Two signals mediate hormone-dependent nuclear localization of the glucocorticoid receptor. EMBO J. 6, 3333–3340
23. Savory, J. G., Hsu, B., Laquian, I. R., Giffin, W., Reich, T., Haché, R. J., and Lefebvre, Y. A. (1999) Discrimination between NL1- and NL2-mediated nuclear localization of the glucocorticoid receptor. Mol. Cell. Biol. 19, 1025–1037
24. Gautier, V. W., Sheehy, N., Duffy, M., Hashimoto, K., and Hall, W. W. (2005) Direct interaction of the human I-mfa domain-containing protein, HIC, with HIV-1 Tat results in cytoplasmic sequestration and control of Tat activity. Proc. Natl. Acad. Sci. U.S.A. 102, 16362–16367
25. Kusano, S., and Eizuru, Y. (2010) Human I-mfa domain proteins specifically interact with HTLV-1 Tax and repress its transactivating functions. Mol. Cell. Biol. 30, 630–646
26. Kusano, S., and Raab-Traub, N. (2002) I-mfa domain proteins interact with cyclin T1 and modulate transcription. Proc. Natl. Acad. Sci. U.S.A. 99, 731–735
27. Wang, Q., Young, T. M., Mathews, M. B., and Pe’ery, T. (2007) Developmental regulators containing the I-mfa domain interact with T cyclins and Tat and modulate transcription. J. Mol. Biol. 367, 630–646
28. Young, T. M., Wang, Q., Pe’ery, T., and Mathews, M. B. (2003) The human I-mfa domain-containing protein, HIC, interacts with cyclin T1 and modulates P-TEFb-dependent transcription. Mol. Cell. Biol. 23, 6373–6384
29. Rhen, T., and Cidlowski, J. A. (2005) Antinflammatory action of glucocorticoids: new mechanisms for old drugs. N. Engl. J. Med. 353, 1711–1723
30. Liddle, C., and De Bosscher, K. (2009) Crosstalk in inflammation: the interaction of the human I-mfa domain-containing protein MDFIC modulates glucocorticoid receptor signaling. Mol. Cell. Biol. 29, 7309–7322
31. Galliher-Beckley, A. J., Williams, J. G., and Cidlowski, J. A. (2011) Ligand-independent phosphorylation of the glucocorticoid receptor integrates cellular stress pathways with nuclear receptor signaling. Mol. Cell. Biol. 31, 4663–4675
32. Galliher-Beckley, A. J., Williams, J. G., Collins, J. B., and Cidlowski, J. A. (2008) Glycogen synthase kinase 3β-mediated serine phosphorylation of the human glucocorticoid receptor redirects gene expression profiles. Mol. Cell. Biol. 28, 7309–7322
33. Peffer, M. E., Chandran, U. R., Luthra, S., Volonte, D., Galbiati, F., Garabedian, M. I., Monaghan, A. P., and DeFranco, D. B. (2014) Cavelolin-1 regulates genomic action of the glucocorticoid receptor in neural stem cells. Mol. Cell. Biol. 34, 2611–2623
34. Snider, L., and Tasscott, S. J. (2005) XIC is required for Sia Mossays and dorsal-ossenoid development. Mol. Cell. Biol. 25, 5061–5072
35. Kusano, S., Yoshimitsu, M., Hachiman, M., and Ikeda, M. (2015) I-mfa domain proteins specifically interact with HTLV-1 Tax and repress its transcriptional activating functions. Virology 486, 219–227
36. Snider, L., Thirlwell, H., Miller, J. R., Moon, R. T., Groudine, M., and Tasscott, S. J. (2001) Inhibition of Tcf3 binding by I-mfa domain proteins. Mol. Cell. Biol. 21, 1866–1873
37. Edgar, R., Domrachev, M., and Lash, A. E. (2002) Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30, 207–210