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HES1 Is a Master Regulator of Glucocorticoid Receptor-Dependent Gene Expression

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Abstract

Hairy and enhancer of split-1 (HES1) is a basic helix-loop-helix transcription factor that is a key regulator of development and organogenesis. However, little is known about the role of HES1 after birth. Glucocorticoids, primary stress hormones that are essential for life, regulate numerous homeostatic processes that permit vertebrates to cope with physiological challenges. The molecular actions of glucocorticoids are mediated by glucocorticoid receptor-dependent regulation of nearly 25% of the genome. We now establish a genome wide molecular link between HES1 and glucocorticoid receptors that controls the ability of cells and animals to respond to stress. Glucocorticoid signaling rapidly and robustly silenced *HES1* expression. This glucocorticoid-dependent repression of HES1 was necessary for the glucocorticoid receptor to regulate many of its target genes. Mice with conditional knockout of *HES1* in the liver exhibited an expanded glucocorticoid receptor signaling profile and aberrant metabolic phenotype. Our results indicate that HES1 acts as a master repressor, the silencing of which is required for proper glucocorticoid signaling.

Introduction

Hairy and enhancer of split-1 (HES1) is a highly conserved basic helix-loop-helix transcriptional repressor that mediates its biological effects by binding to N-boxes (CACNAG) throughout the genome and recruiting chromatin-modifying factors to these

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sites (1, 2). HES1 is required for organogenesis and development of several species as a component of the Notch signaling pathway (3–6). The molecular function of HES1 in adult tissues, however, is less clear.

Glucocorticoids are primary stress hormones necessary for life that are synthesized in the adrenal cortex and released into the bloodstream in response to environmental and physiological stress. Due to their hydrophobic nature, these hormones readily diffuse from the circulation into organs, tissues, and cells, where they orchestrate various physiological processes, including metabolism, energy production, immune system function, vascular tone, bone mineralization, and central nervous system function (7). Glucocorticoids are named for their vital role in glucose metabolism where they increase blood glucose concentrations by promoting liver gluconeogenesis and insulin insensitivity. Because of their potent antiinflammatory and immunosuppressive actions, synthetic glucocorticoids are widely used in the clinic to treat inflammatory and autoimmune diseases, as well as hematological cancers.

Intracellularly, glucocorticoids interact with the ubiquitously distributed glucocorticoid receptor (GR) and promote its translocation from the cytoplasm into the nucleus. Hormone-bound GR binds to glucocorticoid response elements (GREs) in the DNA or interacts with various transcription factors to either increase or decrease the expression of nearly a quarter of the mammalian genome (8). In this manner, glucocorticoids elicit changes in the transcriptional profile of cells and alter the physiology of the organism (9).

We have observed in genome-wide microarray studies that glucocorticoids repress *HES1* mRNA expression (10). The molecular mechanisms underlying the apparent mutual antagonism of HES1 and GR, however, remain unknown. Moreover, the genome wide impact of the interplay of these two transcription factors on the physiological actions of glucocorticoids has not been explored.

Here, we show that glucocorticoids silence *HES1* gene expression in multiple cell-types and tissues. Glucocorticoids rapidly decreased *HES1* mRNA abundance through a GR-dependent antagonism of nuclear factor κB (NFκB) at an NFκB regulatory element within the first exon of the *HES1* gene. This repression resulted in the concomitant decline of the HES1 protein within a few hours and to its disappearance from the promoters of genes regulated by glucocorticoids. Overexpression of *HES1* in human cells led to reduced glucocorticoid-mediated changes in gene expression, and knockdown of *HES1* enhanced sensitivity to glucocorticoids without altering GR association with DNA. Impairment of glucocorticoid signaling by HES1 was abolished when mutations rendering HES1 incapable of DNA-binding were introduced or when N-boxes are mutated. To evaluate the role of HES1 in vivo, we employed Albumin-Cre mice and created mice bearing loxP sites in the *HES1* gene to establish *HES1* liver knockout (HESKOL) animals. The absence of HES1 in hepatocytes resulted in no gross physiological or morphological defects in the adult liver. However, genome-wide microarray analysis revealed that HESKOL mice display abnormal glucocorticoid-dependent signaling profiles that affected genes associated with various biological functions including energy production, lipid metabolism, and carbohydrate metabolism. As a result of this dysregulation, HESKOL mice exhibited impaired glucose

tolerance. Removal of endogenous glucocorticoids by adrenalectomy corrected this phenotype, whereas injection of exogenous glucocorticoids restored it. These findings indicate that HES1 silencing is necessary to initiate GR-mediated changes in gene expression, and suggest that the dismissal of HES1 cooperates with the GR to regulate a large component of the transcriptional targets of glucocorticoids through a transcriptional derepression mechanism.

Results

Glucocorticoid Signaling Rapidly Silences *HES1*

Our laboratory identified *HES1* as a potential repressed target of glucocorticoid signaling from genome-wide microarray studies performed on human U2OS cells expressing the glucocorticoid receptor (GR) and treated with dexamethasone (10). RT-PCR analysis of U2OS cells engineered to express GR (U2OS-GR) revealed that dexamethasone treatment reduced *HES1* mRNA abundance in U2OS-GR cells but not in the parental line that lacks GR (Figure 1A). *HES1* was also silenced in dexamethasone-treated A549 cells, which have endogenous GR (Figure 1B).

Examination of the kinetics of HES1 silencing in U2OS-GR cells revealed that dexamethasone rapidly reduced *HES1* mRNA within 3 hours of treatment (Figure 1C). Maximal repression was reached at 6 hours and maintained thereafter until at least 24 hours (Figure 1C). Under the same conditions, Western blot analysis showed that HES1 protein abundance decreased as early as 6 hours, a reduction that was sustained for at least 24 hours (Figure 1D), indicating that HES1 is a short-lived protein. Comparing the newly transcribed “nascent” RNA to its “mature” form in the same samples at different time-points showed that the abundance of nascent *HES1* RNA decreased as early as 30 minutes after dexamethasone addition and remained low for the duration of the 3-hour experiment (Figure 1E), suggesting that glucocorticoids repress *HES1* gene expression at the level of transcription. In contrast, mature *HES1* mRNA started to significantly decline only after 1 hour of dexamethasone treatment (Figure 1E). These results establish that glucocorticoids can rapidly silence *HES1* RNA expression in GR containing cells.

The rapid repression of HES1 by glucocorticoids suggested that HES1 may be a primary rather than secondary target of the GR. To evaluate this prediction, U2OS-GR cells were pre-exposed to the protein synthesis inhibitor cycloheximide before dexamethasone treatment. Although *HES1* was induced after exposure to cycloheximide alone, dexamethasone still silenced *HES1* (Figure 1F), establishing HES1 as a primary target of glucocorticoid signaling because new protein synthesis was not required for its repression by GR.

The GR can alter expression patterns of target genes by either directly binding to GREs in the DNA or interacting with transcription factors without itself binding to DNA (9). We did not find any potential functional GREs up to two kbps upstream of the *HES1* gene transcriptional start site when using gene promoter analysis computer programs, suggesting that the GR can repress the *HES1* gene without needing to bind directly to the DNA. To address this question, we generated U2OS cells stably expressing a GR DNA-binding

mutant that interacts poorly with DNA (Dim4), but can still associate with transcription factors and inhibit their activity (11). After dexamethasone treatment, the Dim4 mutant GR decreased *HES1* abundance to a similar extent as wild-type GR (Figure S1), demonstrating that DNA binding by the GR is indeed largely dispensable for silencing *HES1* gene transcription.

Glucocorticoids Repress HES1 by Interfering with NFκB Mediated Transcriptional Activation of the *HES1* gene

Having established that HES1 is a primary target of glucocorticoid signaling, and that DNA binding by GR was not required for repression of *HES1* gene transcription, we reasoned that protein-protein interactions of the GR with transcription factors at the *HES1* promoter were responsible for the observed repression. To define the site where GR might be acting on the *HES1* promoter, luciferase reporter constructs carrying progressively smaller regions of the *HES1* promoter were created and stably expressed in U2OS-GR cells. In response to dexamethasone treatment, luciferase activity was reduced in all stable cell lines, except the one carrying the empty vector control (Figure 2A), suggesting that GR acts on the *HES1* gene near the transcriptional start site.

Chromatin-immunoprecipitation (ChIP) assays performed in U2OS-GR cells treated with dexamethasone also established that GR preferentially interacts with chromatin near the transcriptional start site of the *HES1* gene (Figure 2B). Gene promoter analysis computer programs identified a potential NFκB DNA binding site located at +26/+34 of the *HES1* gene [GGAATCCCC] within the 5′-untranslated region of the first exon (Figure S2). This sequence exhibited high homology to the consensus NFκB binding site and is conserved across multiple mammalian species (Figure S2). Because GR and NFκB signaling antagonize one another and basal NFκB activity promotes gene expression in these cells (12), we speculated that the GR-mediated silencing of *HES1* expression could be mediated by antagonism of basal NFκB activity on this site. Consistent with this idea, U2OS-GR cells stably expressing a luciferase construct in which this putative NFκB site was mutated exhibited lower basal expression and impaired glucocorticoid-dependent repression (Figure 2C).

To further determine whether GR regulatory interactions with NFκB could impact *HES1* gene expression, we knocked-down *RelA*, which encodes p65, a main component of the NFκB complex, in U2OS-GR cells (Figure 2D). *HES1* expression was lower in untreated cells depleted of *RelA* than in cells transfected with non-targeting controls, and exposure to dexamethasone did not further decrease the expression of *HES1* (Figure 2E). p65 ChIP assays probed at different regions of the *HES1* promoter revealed the presence of p65 near the transcriptional start site of the *HES1* gene (Figure 2F). These data indicate that basal NFκB activity at the conserved +26/+34 site of the *HES1* gene promotes its expression, and that glucocorticoids can silence *HES1* by inhibiting this activity.

Silencing of HES1 is Necessary for Glucocorticoid Signaling

Having elucidated a mechanism by which glucocorticoids repress *HES1*, we next sought to understand the molecular implications of this silencing. We generated U2OS-GR cells that

over- or under-expressed *HES1* to avert or mimic the effects of glucocorticoids on this gene (Figure 3A). Altering the expression of *HES1* did not obviously affect GR abundance (Figure 3A). However, genome-wide microarrays revealed that overexpression of *HES1* resulted in inhibition of GR-mediated changes in the glucocorticoid regulated transcriptome, as compared to non-overexpressing controls (Figure 3B). A comparison of the effects of *HES1* overexpression in these cells in the absence of glucocorticoids revealed that many of the gene changes that were repressed by glucocorticoid signaling were also repressed upon *HES1* overexpression, suggesting that the GR-signaling inhibitory effects of HES1 on these genes may be due to the overexpression of *HES1* alone (Figure 3B and Figure S3). In contrast, genes that were induced by glucocorticoid signaling were largely unaffected by overexpression of *HES1* in the absence of hormone, indicating that the removal of HES1 is necessary but not sufficient to elicit these GR-mediated changes in gene expression (Figure 3B).

From this genome-wide microarray analysis, we chose to focus on the following three genes that are targets of GR signaling (13–15) and were strongly induced by glucocorticoids in the control cell line, but not in the *HES1* overexpressors, and analyzed their expression by RT-PCR: nuclear receptor subfamily 0, group B, member 1 (*NRO-B1*), fibronectin leucine rich transmembrane protein 3 (*FLRT3*), and regulator of G-protein signaling 2 (*RGS2*). Overexpressing or knocking down *HES1* did not affect the basal expression of these genes in cells not treated with glucocorticoids (Figures 3C–E). However, the expression of these genes did not increase in a time dependent manner upon exposure to dexamethasone in *HES1*-overexpressing cells as compared to controls (Figures 3C–E). In contrast, in *HES1* knockdown cells, these genes exhibited a greater induction 24 hours after dexamethasone treatment (Figures 3C–E), a greater dexamethasone sensitivity (Figure S4), and faster induction kinetics (Figure S5). These results establish that repression of *HES1* is necessary for proper glucocorticoid signaling.

Because glucocorticoid-mediated changes in gene expression are largely responsible for promoting glucocorticoid-induced apoptosis (16), we also examined if *HES1* overexpressors would show an impaired response to glucocorticoid-induced apoptosis. Upon treatment with vehicle or dexamethasone, U2OS-GR overexpressing *HES1* were more resistant to glucocorticoid-induced apoptosis (Figure 3F). These data demonstrate that the silencing of HES1 is also essential for the functional actions of glucocorticoids in these cells, such as glucocorticoid-induced apoptosis.

HES1 plays a crucial role during development as one of the main targets of the NOTCH signaling pathway (17, 18), therefore we evaluated whether NOTCH could impair the actions of glucocorticoids through HES1. Overexpression of the NOTCH intracellular domain increased HES1 expression and decreased the dexamethasone-mediated gene induction of *FLRT3*, *RGS2*, and *NRO-B1* (Figure S6). These inhibitory effects were abolished when *HES1* was knocked down (Figure S6), suggesting that the NOTCH impairment of glucocorticoid signaling is dependent on HES1.

HES1 DNA Binding is Required to Inhibit Glucocorticoid Actions

To elucidate the molecular mechanism by which HES1 represses glucocorticoid signaling, we initially evaluated the ability of HES1 to physically associate with GR in a complex. Results from co-immunoprecipitation assays indicated that HES1 and GR do not interact directly with one another (Figure S7), and that HES1 does not impair GR from associating with other binding partners, such as the steroid receptor co-activator 1 (SRC1) (Figure S7). Because HES1 can directly bind to N-boxes (CACNAG) in the DNA, we next evaluated whether this binding activity could be responsible for the inhibitory effects of HES1 on glucocorticoid signaling. We transiently transfected luciferase reporter constructs containing wild type or mutant N-boxes, GREs, and a TATA box in their promoters into U2OS-GR cells (Figure 4A). Glucocorticoids induced expression of the luciferase construct carrying mutant N-boxes in a dose dependent manner, and co-transfection of *HES1* minimally repressed luciferase activity under the same conditions (Figure 4B). However, when a construct carrying wild type N-boxes was employed, luciferase activities at all dexamethasone concentrations tested were lower, and co-transfection of additional *HES1* further repressed these activities (Figure 4B). These data demonstrate that HES1 mediates its inhibitory actions on glucocorticoid signaling by binding to N-boxes in the DNA.

To investigate if the molecular basis for these inhibitory effects on GR signaling could be explained by HES1 promoting a conformational change in the DNA that renders it inaccessible to the GR, we performed ChIP assays on the luciferase constructs containing either wild-type or mutant N-boxes in dexamethasone-treated cells. We found that GR-immunoprecipitates were enriched on both of these constructs compared to IgG controls (Figure 4C). Moreover, neither the overexpression of *HES1* nor the presence of N-boxes nearby affected the ability of the GR to interact with these constructs, suggesting the GR can still bind GREs under these conditions (Figure 4C).

To further elucidate how HES1 inhibits GR signaling, we generated U2OS-GR cells overexpressing wild type *HES1* and a DNA-binding deficient mutant *HES1* (Figure S8), and examined the effects on endogenous gene expression upon exposure to dexamethasone. RT-PCR analysis revealed that the DNA-binding deficient *HES1*, unlike wild type *HES1*, did not inhibit the GR-mediated induction of *NRO-B1*, *FLRT3*, and *RGS2* (Figures 4D–F). Because these glucocorticoid responsive genes possess N-boxes in their proximal promoters, we performed ChIPs of endogenous HES1 to determine whether HES1 associated with these promoters. Compared to IgG controls, HES1 was greatly enriched at these gene promoters in untreated U2OS-GR cells, and dexamethasone exposure decreased these enrichments (Figure 4G). *PPIB* is a gene with robust expression that is unaffected by the expression of *HES1* or glucocorticoid signaling, and did not show significant HES1 enrichment in ChIP assays (Figure 4G), indicating that HES1 associates specifically with the promoters of the glucocorticoid-responsive genes *NRO-B1*, *FLRT3*, and *RGS2*. In summary, these data demonstrate that binding of HES1 to N-boxes in the DNA is required to inhibit the actions of glucocorticoids.

Glucocorticoids Silence *HES1* In Vivo

To examine how glucocorticoids affect the expression of *HES1* in vivo, time course experiments were performed in which dexamethasone was injected intraperitoneally to mice that had been adrenalectomized to remove endogenous glucocorticoids. Dexamethasone treatment rapidly reduced the abundance of liver *HES1* mRNA and protein by 3 hours (Figure 5A and 5B). By 24 hours, *HES1* mRNA and protein had returned to basal amounts. Glucocorticoid exposure also rapidly and robustly repressed *HES1* mRNA expression in heart, lung, and kidney (Figure S9). The transient nature of the dexamethasone effects on *HES1* expression is likely due to the metabolic breakdown of the steroid. We analyzed the effect of dexamethasone treatment on rat primary hepatocytes to determine whether glucocorticoids acted directly on liver cells. Compared to controls, dexamethasone treatment decreased *HES1* mRNA abundance within 3 hours, a repression that was sustained for at least 24 hours (Figure 5C). These data indicate that the glucocorticoid-dependent repression of *HES1* gene expression occurs in vivo in multiple tissues. In addition, consistent with the conservation across species of the NFκB binding site in the *HES1* 5′ untranslated region (Figure S2), silencing of *HES1* by glucocorticoids occurs in human, mouse, and rat cell types.

HES1 Liver Knockout (HESKOL) Mice Exhibit Abnormal Glucocorticoid Signaling

To evaluate the role of HES1 during glucocorticoid signaling in vivo, mice bearing loxP sites in the *HES1* gene were generated and crossed with Albumin-Cre mice (19) to specifically knock out *HES1* in hepatocytes (Figure 5D–F). This conditional approach avoids the lethal developmental defects observed in mice with global knockout of *HES1* (3). The HES1 liver knockout (HESKOL) mice developed normally, and their adult livers exhibited no gross morphological or physiological alterations, as compared to loxP/loxP control mice. We performed genome-wide microarrays on liver mRNA extracted from control and HESKOL males that had been adrenalectomized to remove endogenous glucocorticoids. In mice devoid of glucocorticoids, knockout of *HES1* in hepatocytes resulted in only 319 statistically different probes when compared to control mice (Figure 6A). However, dexamethasone treatment resulted in 5411 probe differences in the livers of control mice and 7288 probe differences in the livers from the HESKOL (Figure 6A). The proportion of induced compared to repressed probes differed between the dexamethasone-treated control and HESKOL mice (Figure 6B). In the control mice, only 393 probes were induced (7%), whereas 5018 were repressed (93%) by glucocorticoid treatment. In marked contrast, 3644 probes were induced (50%) and 3644 repressed (50%) in the HESKOL mice treated with glucocorticoids. The large increase in GR-induced genes that accompanies the loss of HES1 is consistent with the notion that HES1 acts primarily as a transcriptional repressor (20). A comparison between dexamethasone-treated control and dexamethasone-treated HESKOL livers revealed 5162 probes that were differentially regulated (Figure 6A). These data demonstrate that HES1 modulates the mode, extent, and diversity of glucocorticoid signaling in the liver, but the absence of HES1 by itself does not significantly alter basal gene expression patterns in hepatocytes.

To better understand the functional importance of HES1 during glucocorticoid signaling in the liver, we performed Ingenuity Pathway Analyses (IPA) on the set of genes regulated by

dexamethasone in control or HESKOL mice. Marked differences were observed in the molecular and cellular functions associated with the regulated genes, because only 3 of the top 10 functions were common to both gene lists (Table 1 and Table 2). Among the biological functions most affected by the absence of HES1 were energy production, lipid metabolism, and carbohydrate metabolism. Because glucocorticoids play a critical role in glucose homeostasis, we chose to further study carbohydrate metabolism. A large number of genes associated with carbohydrate metabolism were regulated by glucocorticoid signaling only in HESKOL livers, suggesting that disrupting HES1 in hepatocytes had expanded the number of carbohydrate metabolism genes that are targets of glucocorticoid signaling (Figure 6C).

To delineate how the absence of HES1 in the HESKOL mice could affect the GR gene expression profile, we focused our studies on insulin-like growth factor binding protein 1 (*IGFBP1*), a gene that encodes a key factor involved in carbohydrate metabolism (21, 22) and that is targeted by glucocorticoid signaling (23, 24). Compared to control animals, livers of HESKOL mice show higher basal expression of *IGFBP1* as well as a greater response to dexamethasone treatment (Figure 7A). Analysis of the *IGFBP1* promoter revealed a conserved N-box at approximately position −100 bp. In *IGFBP1*-promoter-driven luciferase assays performed in U2OS-GR cells, greater basal and dexamethasone-induced activities were observed when this N-box was mutated (Figure 7B), suggesting that HES1 can influence the expression of *IGFBP1* through this N-box. RT-PCR analysis of endogenous *IGFBP1* expression further showed that dexamethasone elicited a faster and stronger response in U2OS-GR cells when *HES1* had been knocked down (Figure 7C–D), suggesting that HES1 regulates both the timing and robustness of the glucocorticoid-mediated *IGFBP1* gene induction. In addition, glucose 6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK), genes that also encode factors that play a critical role in liver carbohydrate metabolism, exhibited a more rapid and greater induction in response to dexamethasone treatment in HESKOL animals (Figure S10). These results indicate that the absence of HES1 in the liver potentiates glucocorticoid signaling of carbohydrate metabolism-related genes.

HESKOL Mice Exhibit Glucocorticoid-Dependent Impaired Glucose Tolerance

Glucocorticoids are essential for carbohydrate metabolism because they increase blood glucose concentration during fasting, and thus we examined whether HESKOL mice had abnormal glucose homeostasis. Both male and female HESKOL animals exhibited normal fed and fasted blood glucose concentrations, consistent with the fact that many factors can regulate glucose concentrations. However, intraperitoneal glucose tolerance tests revealed significant impairment of glucose clearance in HESKOL mice, as compared to controls (Figure 8A–B). Removal of endogenous glucocorticoids by adrenalectomy completely abolished these phenotypes in both male and female HESKOL mice (Figure 8C–D), demonstrating that this phenotype was glucocorticoid-dependent. Indeed, treatment of the adrenalectomized mice with dexamethasone restored glucose tolerance in both male and female HESKOL mice (Figure 8E–F). Moreover, plasma insulin concentrations did not differ between HESKOL and control mice before or after glucose injection and throughout

these experiments (Figure S11), suggesting that insulin sensitivity, rather than insulin release, is likely responsible for these phenotypes.

Discussion

In this study, we provide new evidence demonstrating that HES1 operates as a master regulator of genome wide glucocorticoid signaling. First, glucocorticoids can directly and rapidly decrease *HES1* mRNA and protein abundance. A mechanism for this silencing involves GR mediated repression of *HES1* transcription through an NFκB binding site in the 5′-untranslated region of the *HES1* gene that is conserved among mammalian species. Second, overexpression of *HES1* in human cells leads to resistance to glucocorticoid-mediated changes in gene expression, whereas knockdown of *HES1* increases sensitivity to glucocorticoids. These inhibitory effects of HES1 on glucocorticoid signaling are mediated by its ability to bind to N-boxes in the DNA. Third, HESKOL mice exhibited abnormal changes in liver gene expression when exposed to glucocorticoids, despite the fact that the absence of HES1 by itself leads to few transcriptional changes. In particular, glucocorticoids affected more genes associated with carbohydrate metabolism in HESKOL mice than in controls, suggesting an expansion of glucocorticoid signaling. Finally, HESKOL mice are glucose intolerant when subject to intraperitoneal glucose tolerance tests. This phenotype disappears upon removal of the adrenal glands, the site of endogenous glucocorticoid synthesis, and is restored when exogenous glucocorticoids are injected. Taken together, these results reveal that glucocorticoid-mediated silencing of *HES1* derepresses large sections of the glucocorticoid responsive component of the genome.

The widespread actions of HES1 as an inhibitor of glucocorticoid signaling could be explained by the abundance of N-boxes throughout the genome, occurring about once every 1000 base pairs of DNA. Our data imply that HES1 inhibits glucocorticoid signaling by acting as a cellular brake that must be first removed if the GR is to elicit changes in gene expression. In this scenario, hormone-bound GR interacts with the chromatin in a gene promoter and attempts to activate gene transcription, but if HES1 were also present in this promoter, it would counteract the effects of the GR by promoting a transcriptionally silenced chromatin structure. Appropriately, *HES1* is silenced by the GR, and only after it has disappeared from the chromatin, the GR is able to induce expression of that gene. We also recognize that by acting in a similar manner, HES1 could also influence the magnitude, kinetics, and duration of glucocorticoid signaling. Additionally, the re-expression of *HES1* after having been silenced by glucocorticoids could act to reset the system in preparation for the next signaling event.

Consistent with glucocorticoids targeting NFκB for many of their repressive actions (25), our data identify a highly conserved NFκB element located near the transcriptional start site of *HES1*. Moreover, we show that this site is necessary for both the basal expression of *HES1* and for its silencing by glucocorticoids. The mutual antagonism of NFκB and GR likely silences *HES1* by GR directly blocking the positive transcriptional actions of NFκB on the *HES1* gene (9). Therefore, the transcriptional inhibition of the *HES1* gene by glucocorticoids could be sufficient to concomitantly reduce *HES1* mRNA and protein, because both *HES1* mRNA and protein have half-lives of only 30 minutes (26). However,

we cannot rule out that other mechanisms could also be involved. For instance, in the one other study on glucocorticoids and HES1, Lemke *et al.* (27) have suggested that glucocorticoids silence *HES1* in the liver through a GR binding site (TGTTCC) located at position −422 bp in the mouse *HES1* promoter. However, this potential GRE half site is not conserved in either the rat or human *HES1* promoters, organisms in which we also showed that glucocorticoids silence *HES1*. Moreover, deletion of this region in our human *HES1* promoter reporter constructs did not affect the ability of glucocorticoids to inhibit luciferase gene expression (Figure 2A).

We have begun to explore the effects of NOTCH signaling on glucocorticoid actions mediated through HES1. Our data suggest that NOTCH signaling, by promoting the expression of *HES1*, can inhibit glucocorticoid signaling. Although the functions of HES1 in tissues and homeostasis outside NOTCH signaling and development are unclear, the mice we have generated bearing loxP sites in the *HES1* gene for tissue and time specific knockout experiments should help shed new light on this matter. The deletion of *HES1* in the adult liver using Albumin-Cre mice revealed that, in the absence of glucocorticoids, disruption of *HES1* in the adult liver does not generate widespread changes in gene expression. However, HES1 alters the regulation of several thousand glucocorticoid-regulated genes, and among those, many are associated with carbohydrate metabolism, energy production and lipid metabolism. Physiologically, we discovered that disrupting *HES1* in hepatocytes impairs glucose tolerance, suggesting that HES1 plays an important role during glucose homeostasis in the adult animal. Ingenuity pathway analysis revealed that many other glucocorticoid-regulated processes are also likely to be altered in the HESKOL mice, suggesting an even broader role for HES1 in the modulation of GR responses. A previous study exploring the function of HES1 in the adult organism used overexpression of *HES1* by viral vectors in the liver of *db/db* mice, and suggested that HES1 prevents glucocorticoid-induced liver dyslipidemia (27).

In summary, we have discovered that HES1 is a master regulator of global GR signaling in both cells and in the whole animal. Glucocorticoid-dependent repression of *HES1* and its consequent dismissal from glucocorticoid-responsive genes is necessary for the full transcriptional complement of GR signaling. These findings reveal a role for HES1 in controlling the mode, timing, and magnitude of glucocorticoid-dependent stress responses and suggest that aberrant expression of *HES1* may contribute to acquired forms of glucocorticoid resistance frequently encountered in the clinic.

Materials and Methods

Reagents

Dexamethasone was purchased from Steraloids (RI). Cycloheximide (CHX) was purchased from Sigma (MO) and used as indicated. Rabbit anti-HES1 (AB15740) was purchased from Chemicon (now Millipore, MA). Rabbit anti-GR57 was produced as previously described (28). Rabbit anti-p65 (sc-109) was purchased from Santa Cruz Biotech (CA). Rabbit anti-β-Actin was purchased from Millipore (MA). Human *HES1* cDNA was a kind gift from Dr. T. Sudo, and it was cloned into the pcDNA3.1-zeocin, -hygro, or -puro (gift of Andrew Thorburn, University of Colorado Health Sciences Center) for expression in mammalian

cells and subsequent selection. Flag-HES1-WT and Flag-HES1-mut cDNAs were a kind gift from the Kadesch laboratory, and they were cloned into pcDNA3.1-puro. The mutant version contains three point mutations (E43A, K44A, and R47A) that render HES1 incapable of DNA binding (29). For gene silencing, we used the Mission shRNA system from Sigma (MO). After testing numerous constructs, we used the following: shRNA (SHC202); shHES1, CCG GTG GCC AGT TTG CTT TCC TCA TCT CGA GAT GAG GAA AGC AAA CTG GCC ATT TTT (TRCN0000018993) and shRelA, CCG GCA CCA TCA ACT ATG ATG AGT TCT CGA GAA CTC ATC ATA GTT GAT GGT GTT TTT (TRCN0000014686).

Cell Culture

A549 and U2OS cells (ATCC, VA) were maintained in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal calf serum, 100 IU of penicillin per ml, and 100 mg/ml of streptomycin. U2OS-GR cells (10) were maintained in the same media, except that geneticin (500 μg/ml; Invitrogen, CA) and hygromycin (200 μg/ml; Invitrogen, CA) were also included in the media. U2OS-GR cells bearing HES1 overexpressing constructs, shRNA, or shHES1 were generated and maintained by including puromycin (2 μg/ml) from Sigma (MO) in the media. Rat primary liver hepatocytes were isolated from adult male Sprague-Dawley rats, digested with the collagenase perfusion method, and treated with dexamethasone. All cells were transfected as recommended by the manufacturer with the Transit-LT1 transfection reagent (Mirus, WI). All dexamethasone treatments were performed in medium containing 10% charcoal-dextran-stripped fetal calf serum.

Luciferase Assays

For luciferase assays on the *HES1* promoter, the indicated sections of human *HES1* promoter were cloned into the reporter plasmid pGL4.20[luc2/Puro] (Promega, WI). For mutagenesis of the NFκB site, the sequence GGAATCCCCC on the *HES1* gene was altered to GGAATCCTTT. Constructs were transfected into U2OS-GR cells and, 48 hours after transfection, cells were selected with 2 μg/ml puromycin for two weeks. Stable cells were then plated onto 24-well plates at ~30,000 cells per well, allowed to rest for 24 hours in 10% charcoal-dextran-stripped fetal calf serum, and then treated with 100 nM dexamethasone or vehicle for 16 hours. For luciferase assays on the promoter containing GREs and N-boxes, the $pGRE_2$-luciferase construct (10) was modified by introducing sequences at the HindIII site upstream of the two GRE elements in the following manner: for 2x WT-N-Boxes, AAG CTT aca aca CTT GTG aca GAA TTC aca CTT GTG aca aca AAG CTT, for 2x mut-N-Boxes, AAG CTT aca aca tat ctc aca GAA TTC tat ctc aca aca AAG CTT, where AAG CTT is a HindIII site, CTT GTG is an N-box sequence, and GAA TTC is an EcoRI used for selection. For luciferase assays on the promoter containing the rat IGFBP1 promoter, the addgene plasmid 12146 (30) was left unchanged (WT) or modified (mut) by altering the N-box sequence CACAAG to TGTAAG by site-directed mutagenesis. U2OS-GR cells plated onto 24-well plates at ~30,000 cells per well, were transiently transfected with these constructs for one day, and allowed to rest for another day in media containing 10% charcoal-dextran-stripped fetal calf serum before treatment with dexamethasone at the indicted concentrations. Luciferase activities were measured as previously described (10).

Each experiment was repeated at least three times, and firefly luciferase activity was normalized to the protein concentration.

Primers and Real Time RT-PCR Analysis

RNA was isolated and treated with DNase by using a Qiagen (CA) RNeasy minikit as suggested by the manufacturer. Real-time PCR was measured on a 7900HT sequence detection system with custom made or predesigned primer-probe sets from Applied Biosystems (CA). The measurement from each primer-probe set was normalized to that of PPIB, an unregulated housekeeping gene. Values obtained from tissues isolated from mice injected for 3, 6, 12, or 24 hours with PBS- or dexamethasone were normalized to untreated mice. The following primer-probe sets from Applied Biosystems were utilized: LYPD1 (Hs00375992_m1), GLI1 (Hs01110766_m1), IL11 (Hs00174148_m1), NOX4 (Hs00276431_m1), NROB1 (Hs00230864_m1), PPIB (Hs00168719_m1), HES1 (Hs00172878_m1), FLRT3 (Hs00183798_m1), and RGS2 (Hs00180054_m1), mHES1 (Mm01342805_m1 and Mm00468601_m1), rHES1 (Rn00577566_m1), mPPIB (Mm00478295_m1), rPPIB (Rn03302274_m1), IGFBP1 (Hs00236877_m1), mIGFBP1 (Mm00515154_m1), mPEPCK(Mm01247058) and mG6P(Mm00839363). To detect nascent HES1 RNA, the following custom primers-probe was utilized: Probe: /56-FAM/ CCT GTA TCT CTT TGC AGC CCC TCA /3IABkQ/, Primers: CAG AAA GGT AAG GGC GGT AC and AAG AGT TCT GTG TTC CCA TGG. To detect nascent IGFBP1 RNA, the following custom primers-probe was utilized: Probe:/56-FAM/ AAT GCC TCT TTC TCT ACT CCA GCC C /3IABkFQ/; Primers: GCA AGC AGT CCA GAT GAG G and TGT TTG TAG CGG GAA GTG G. To detect nascent FLRT3 RNA, the following custom primers-probe was utilized: Probe: /56-FAM/AGG GTT CTG /ZEN/ AAG TAA CGG AAG CTA CCT/ 3IABkfFQ/, Primers: TTC AGT ATG CTG GCC TTA TTG T and GTC AGC AGT GTT GAG GTC TTT A. To detect nascent PPIB RNA (used to normalize nascent FLRT3 gene expression), the following custom primers-probe was utilized: Probe: /56-FAM/TT TGT GGC C/ZEN/T TAG CTA CAG GAG AGG T/3IABKFQ/, PRIMERS: TGA, ACT, CTG, CAG, GTC, AGT, TTG, CTG, and ATG AAG ATG TAG GCC GGG TGA TCT. To detect $pGRE_2$-luciferase construct bearing WT or mutant N-boxes by real-time RT-PCR from ChIP assays, the following custom primers-probe were utilized: Probe: /56-FAM/ TGG ACA AAC CAC AAC TAG AAT GCA GTG A /3IABkFQ/; Primers: AGC ATC ACA AAT TTC ACA AAT AAA GC and GGA TCC AGA CAT GAT AAG ATA CAT TG. To detect the promoter of NROB1 by real-time RT-PCR from ChIP assays, the following custom primers-probe were utilized: Probe: /56-FAM/ CC CGT AGC CCA GTT CTG CCC /3IABkFQ/, Primers: ATG TTG TAG AGG ATG CTG CC and CGC GCT AGG TAT AAA TAG GTC C. To detect the promoter of RGS2 by real-time RT-PCR from ChIP assays, the following custom primers-probe were utilized: Probe: /56-FAM/ TGC TGT AGG ACT CAT TCG ACA CCC /3IABkFQ/, Primers: ATT GCC TCA GTT CAC AGA CC and CGC GCC TCA TTT CTT GTT TG. To detect the promoter of FLRT3 by real-time RT-PCR from ChIP assays, the following custom primers-probe were utilized: Probe: /56-FAM/ CAT GTT GGT CAG GCT GGT CTC GAA /3IABkFQ/, Primers: TCT AAT TCC GGC ACT TTG GG and CAA GTG ATT CTC CTG CCT CAG. To detect the promoter of PPIB by real time RT-PCR from ChIP assays, the following custom primers-probe were utilized: Probe: /56-FAM/ TCT GAT ACC AAT CCC AAC GCT GCC TT /3IABkFQ/, Primers: AGT CTG AAA

GTT GGA TGG GCA GGT and TCT GAT TGG GTA TGT CAA GGC GGT. To detect the promoter of HES1 by PCR in agarose gels from ChIPs assays, the following custom primers were utilized: ~2kb upstream of TSS, TCT GGC GAA ATC AAT GAC AAC GT and CGT CTT GTT TGA TGT GGC CTC C; ~1kb upstream of TSS, GCA ATA AAA CAT CCT GGC ACG TG and TTT AAG AGC TAC ACC AGC CGA GC; and at TSS, GTT CGC GTG CAG TCC CAG ATA TAT and GTT CCA GGA CCA AGG AGA GAG G. To detect the promoter of HES1 by real-time RT-PCR from ChIP assays, the following custom primers-probe were utilized: ~2kb upstream of TSS, Probe: /56-FAM/ AAT TCC CCA CTC /3IABkFQ/, Primers: GCC AAG GTC AGC TCT TCC and AGT GAA AAC CCC AAG CCC; ~1kb upstream of TSS, Probe: /56-FAM/ CCA CCC CGT CTT /3IABkFQ/, Primers: GTA GTT CTT GAA TCC CAC CCC and TCT AAG GCC CCA AAT CCA AAC; and at TSS, Probe: /56-FAM/ CCA GAG GGA GAG /3IABkFQ/, Primers: 5-CGG AGG CTA CAA CGT CAA TC and GAC AAG ATC AAG ACC AAA GCG.

ChIP Assays

For ChIP assays, we utilized EZ ChIP (Catalog #17-408) or Magna ChIP (Catalog #17-610) kits from Millipore (MA) and followed the manufacturer's protocol. Briefly, 0.5×10^6 U2OS-GR cells were plated on 10-cm dishes and allowed to rest for two days or were transiently transfected with the indicated plasmids the following day. Cells were then exposed to 10% charcoal-stripped fetal calf serum for one day and subsequently treated with vehicle or dexamethasone (100 nM) as indicated. Cells were washed with ice-cold PBS, fixed for 15 min at room temperature by adding formaldehyde to the media to a final concentration of 1%. Crosslinking was then stopped by addition of glycine and incubating at room temperature for 10 minutes. Cells were harvested, and their DNA was sheared by sonication on ice (Branson Sonifier 150, five sets of 10-second pulses at setting 7). The antibodies for each immunoprecipitation reaction were 6 μg of anti-HES1, 4 μg of anti-GR, and 4 μg of anti-RNAP2 (clone 8WG16, Millipore, MA).

Western Blot Analysis

After the indicated treatments, cells were washed once with ice-cold PBS and lysed for 60 minutes in lysis buffer (50 mM Tris 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with inhibitor cocktail set II (Calbiochem, CA). The whole cell extract was later cleared of cellular debris by centrifugation and protein concentrations were quantitated by the Bradford assay (Biorad, CA). Tissue extracts were isolated, grinded with a tissue homogenizer in 1X Laemmli sample buffer (Biorad, CA), and debris was cleared by centrifugation. Cellular and tissue samples containing 20–30 μg/lane were resolved on 4–12% SDS-PAGE gels (Biorad, CA) and transferred onto PVDF (Invitrogen, CA). The dilutions for antibodies were as follow: anti-HES1, 1/1000; anti-GR57, 1/1000; anti-β-Actin, 1/10000; anti-p65, 1/1000.

Animal Studies

All experimental protocols were approved by the animal review committee of the National Institute of Environmental Health Sciences (NIEHS) and were performed in accordance with the guidelines set forth in the NIH Guide for the Care and Use of Laboratory Animals. C57BL/6 mice at 3–6 months of age, which were fed ad libitum, were sacrificed by carbon

dioxide asphyxiation. Organs were immediately collected, homogenized in 1× Laemmli's SDS sample buffer, and boiled for 5 minutes. Samples were then centrifuged to remove debris, and protein concentrations were measured by the Bradford assay (Biorad, CA).

Production and Characterization of HESKOL mice

Mice bearing two loxP sites for tissue specific deletion of the *HES1* gene were designed and generated by Xenogen on a C57BL/6 background. The loxP sites are located ~1800 bp upstream (A site) and ~1200 bp downstream (B site) of the *HES1* transcriptional start site. Cre-mediated recombination of these sites deletes a significant portion of the *HES1* promoter and exons 1, 2, and 3. These mice were crossed with Albumin-Cre mice from the Jackson Laboratories (Strain Name: B6.Cg-Tg(Alb-cre)21Mgn/J; Stock Number: 003574) to generate *HES1* liver knockout animals (Cre/+; HES1 loxP/HES1 loxP). For genotyping, we used a nested PCR to genotype the HESKOL mice with the following primers: A site external primers, CTT GCC CAA GGT CAT GCA GTC AAG GAA GCA and GTC ACG AGA TGC CTG ACC GCA CTT AGG AAG; A site internal primers, TCC GGG ACC AGA GCT GGA GAA ATC TTT CAC and AGG CTG CCG GCG GAC GGC TGG GAA GAG A; B site external primers, TCA GCT ACA TTT TAC TGC CTT GGC TCA CTC and ATA GCC ACA GCT CCC AAG TTG TTA CTG CTC; B site internal primers, GTT GGG AGG GTT GGG TAG GCT AAG AAC AG and CTC CAT CTA AAC CGA TCT CAG CTC CAG ATC; Cre external, GGA AGG TGT CCA ATT TAC TGA CCG TAC ACC and GGA TTA ACA TTC TCC CAC CGT CAG TAC GTG; and Cre internal, GCA TTA CCG GTC GAT GCA ACG AGT GAT GAG and GAG TGA ACG AAC CTG GTC GAA ATC AGT GCG.

Intraperitoneal glucose tolerance tests

After mice were injected with PBS or dexamethasone (1 mg/kg body weight) and fasted for 14 hrs, dextrose (2 g/kg body weight) was injected intraperitoneally, and blood glucose concentrations were measured at 0, 15, 30, 60, and 120 minutes after injection. Plasma was also collected at 0 and 15 minutes after glucose injection and used to measure insulin concentrations by ELISA (80-INSMS-E01, Alpco, NH).

Microarray analysis

For U2OS-GR cell lines, gene expression analysis was conducted using Agilent Whole Human arrays (Agilent Technologies, Palo Alto, CA). Two separate biological replicates of cytoplasmic RNA samples were purified from the U2OS-GR parental cells and the two independent hHES1 over-expressing cell lines by using RNeasy Midi kits (Invitrogen) after treatments with 100 nM dexamethasone or vehicle for 6 hours. Total RNA was amplified using the Agilent Low RNA Input Fluorescent Linear Amplification Kit protocol. Starting with 500 ng of total RNA, two cRNAs, one labeled with Cy3 and the other labeled with Cy5, were produced according to manufacturer's protocol. For each two-color comparison, 750 ng of each Cy3- and Cy5-labeled cRNAs were mixed and fragmented using the Agilent In Situ Hybridization Kit protocol. Hybridizations were performed for 17 hours in a rotating hybridization oven at 65 °C at 4 RPM. Slides were washed with 6X SSPE + 0.005% N-lauroyl sarcosine for 1 minute then 0.06X SSPE + 0.005% N-lauroyl sarcosine for 1 minute

at 37 °C. The slides were dried by slowly removing from second wash solution and then scanned with an Agilent G2565 Scanner (10 micron and with XDR) and processed with Agilent Feature Extraction v9.1. Twenty chips in total were used for these studies: 5 comparisons (Con compared to Dex for each of the three cell lines and parental clone compared to each of the two hHES1 over-expressing clones) X 2 biological replicates X 2 chips per replicate = 20. The resulting files were imported into the Rosetta Resolver system (Version 6.0, Rosetta Biosoftware, Kirkland, WA). Intensity ratios (treated/controls) were analyzed at the Entrez gene level in Resolver (Version 6.0, Rosetta Biosoftware, Kirkland, WA) and were considered differentially expressed if the p value was less than 0.0001. Clustering analysis was performed using the Rosetta Resolver analysis software. For mouse livers, gene expression analysis was conducted using Agilent Whole Mouse Genome 4×44 multiplex format oligo arrays (014868) (Agilent Technologies) following the Agilent 1-color microarray-based gene expression analysis protocol. Three separate biological replicates of cytoplasmic RNA samples were purified using RNeasy Midi kits (Invitrogen) from control or HESKOL mouse livers treated with 1 mg/kg Dex or vehicle for 6 hours. Starting with 500 ng of total RNA, Cy3 labeled cRNA was produced according to manufacturer's protocol. For each sample, 1.65ug of Cy3 labeled cRNAs were fragmented and hybridized for 17 hours in a rotating hybridization oven. Slides were washed and then scanned with an Agilent Scanner. Data were obtained using the Agilent Feature Extraction software (v9.5), using the 1-color defaults for all parameters. The Agilent Feature Extraction Software performed error modeling, adjusting for additive and multiplicative noise. To determine differentially expressed probes, an error-weighted ANOVA and Benjamini-Hochberg multiple test correction with a p value < 0.01 was performed using Rosetta Resolver System (version 7.0; Rosetta Biosoftware, Kirkland, WA). Significantly regulated genes were analyzed by Ingenuity Pathway Analysis software (Ingenuity Systems).

Statistical analysis

Student's t tests, one-way ANOVA with Tukey's post hoc analysis, or Mann-Whitney tests were used to evaluate whether differences were statistically significant using GraphPad Prism 6 software. Statistical significance was defined as $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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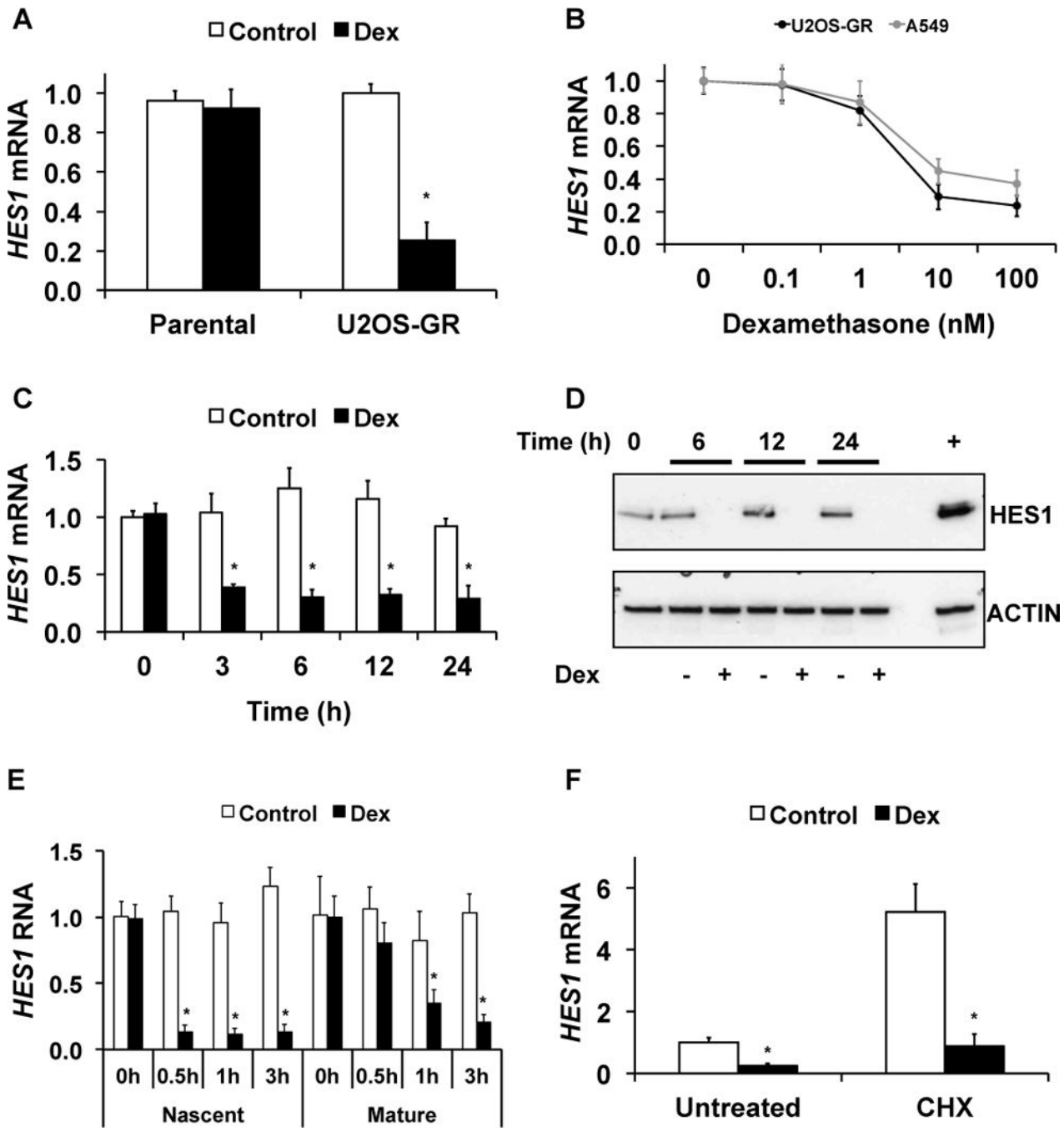

Figure 1. Glucocorticoid receptor (GR) signaling represses the expression of *HES1*

(**A**) *HES1* mRNA abundance in wild-type parental U2OS and U2OS cells expressing GR treated with dexamethasone. N=4 independent biological replicates. (**B**) Dose response of the repression of *HES1* by dexamethasone in U2OS-GR cells and A549. N=4 independent biological replicates. (**C** and **D**) Time course of the repression of *HES1* by dexamethasone in USOS-GR cells analyzed by RT-PCR (C) and Western blot (D). N=4 independent biological replicates for (C) and 3 independent biological replicates for (D). (E) Abundance of *HES1* nascent and mature RNA at early time points upon dexamethasone treatment. N=4 independent biological replicates. (F) Pre-treatment of U2OS-GR cells with cycloheximide (CHX) does not inhibit the

repression of *HES1* by dexamethasone. N=4 independent biological replicates. Asterisks (*) indicate $p<0.05$ as determined by a one-way ANOVA/Tukey analysis.

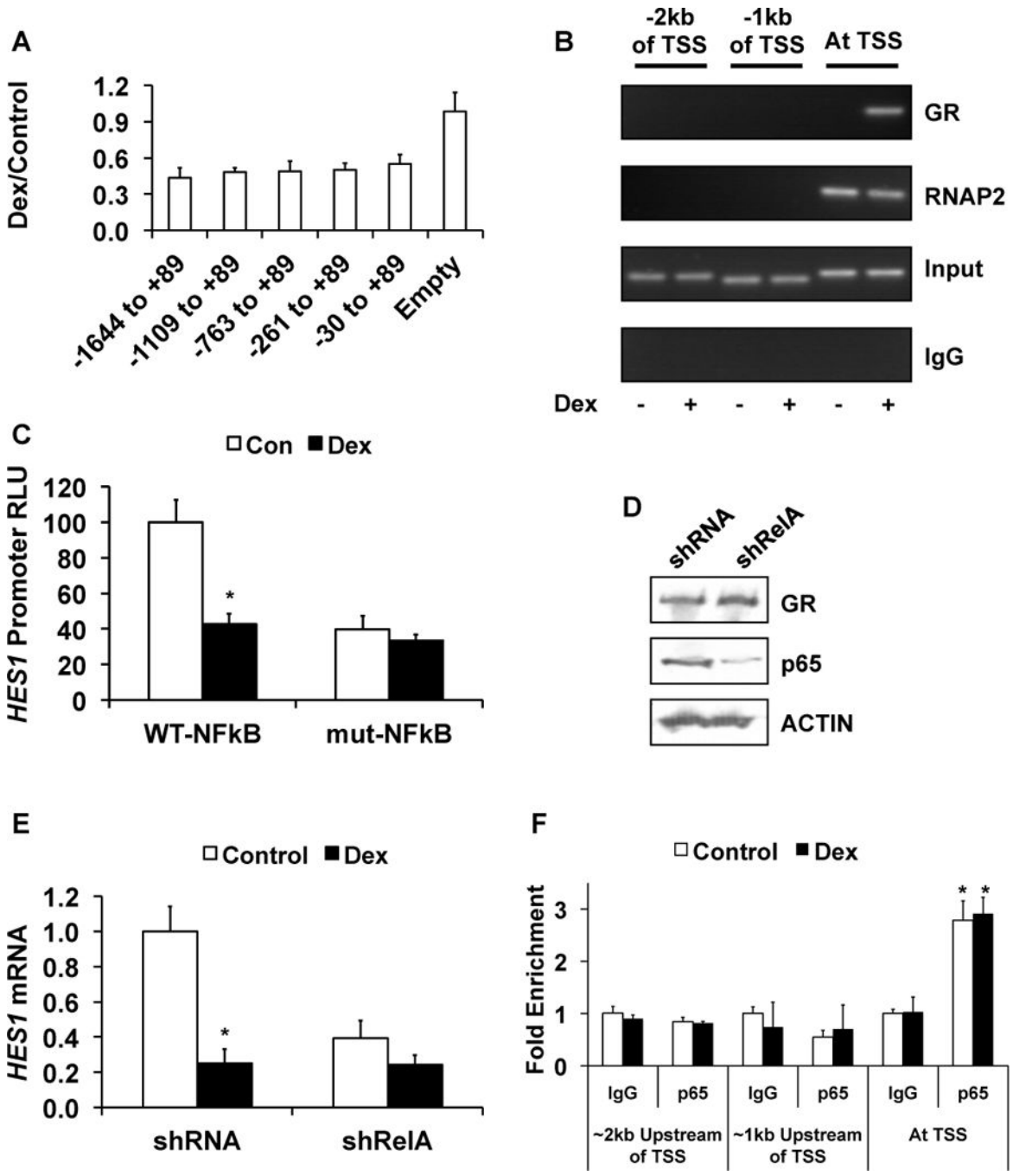

Figure 2. Glucocorticoids require RelA (p65) to repress HES1

(**A**) Activity of luciferase constructs bearing decreasing lengths of the *HES1* promoter in U2OS-GR cells. N=5 independent biological replicates. (**B**) ChIP results for anti-GR and anti-RNAP2 (RNA pol 2) of U2OS-GR cells treated with dexamethasone. N=3 independent biological replicates. (**C**) Activity of luciferase constructs with wild-type or NFκB-mutated *HES1* promoter upon dexamethasone treatment. N=5 independent biological replicates. (**D** and **E**) Knockdown of *RelA* in U2OS-GR cells (D), and its effect on *HES1* expression upon dexamethasone treatment (E). N=3 independent biological replicates for (D) and 4 independent biological replicates for (E). (**F**) ChIP assay for p65 with RT-PCR probes directed at three areas on the *HES1* promoter: −2 kb upstream of the transcriptional start site (TSS), −1 kb of TSS, and at the TSS. N=3 independent biological

replicates. Asterisks (*) indicate $p<0.05$ as determined by a one-way ANOVA/Tukey analysis (E and F) or the Mann-Whitney test (C).

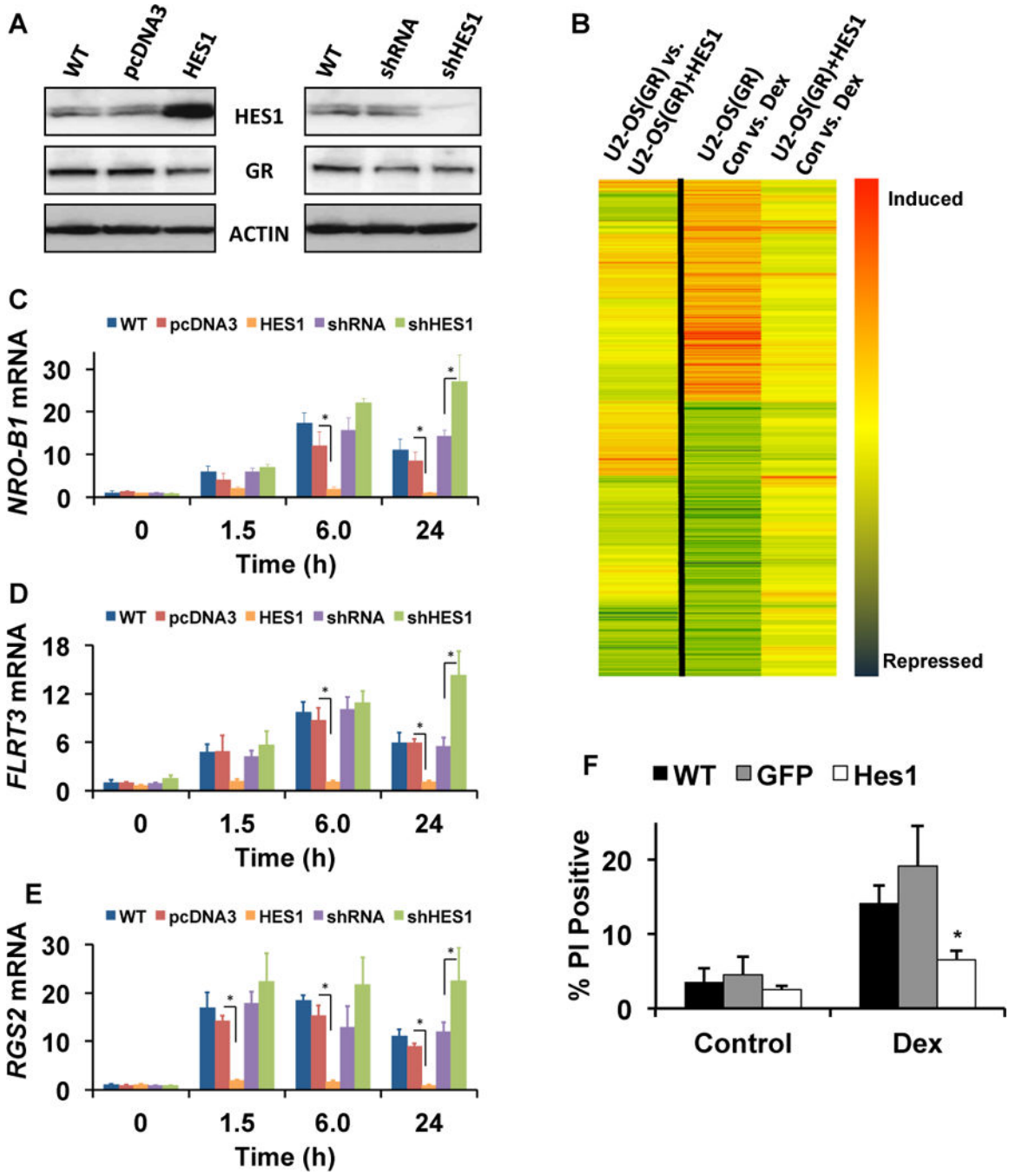

Figure 3. HES1 alters GR signaling

(**A**) U2OS-GR cells with different abundances of HES1, but similar amounts of GR. N=3 independent biological replicates. (**B**) *HES1* overexpression promotes the silencing of many genes and inhibits GR-induced changes in gene expression, as seen by microarray analysis. N=3 independent biological replicates. (**C** to **E**) *HES1* silencing was necessary, but not sufficient, to elicit GR-mediated changes in gene expression in *NRO-B1* (C), *FLRT3* (D), and *RGS2* (E). N=4 independent biological replicates for (C) to (E). (**F**) Percent of propidium iodide (PI) positive cells as determined by flow cytometry of WT, GFP-, and *HES1*-overexpressing cells untreated or treated with dexamethasone. N=5 independent biological replicates for (F). Asterisk (*) indicates $p<0.05$ as determined by a one-way ANOVA/Tukey analysis.

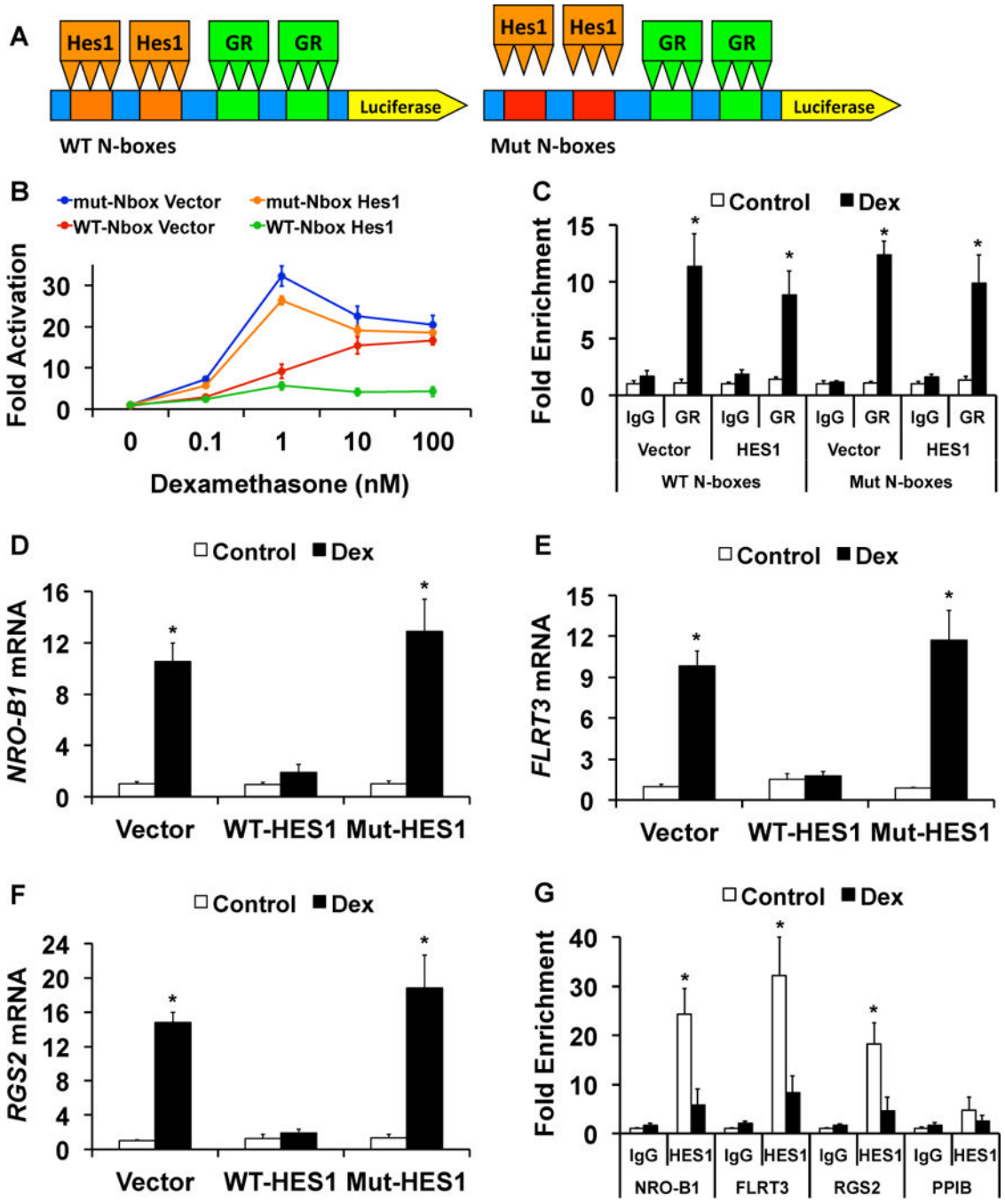

Figure 4. HES1 requires DNA binding to inhibit GR signaling

(**A** and **B**) Constructs bearing 2X N-boxes or 2X mutated N-boxes, and 2X GRE sites controlling the expression of a luciferase reporter (A) and their effects on luciferase expression by dexamethasone and *HES1* overexpression after dexamethasone treatment (B). N=5 independent biological replicates for (B). (**C**) Fold enrichment of luciferase constructs by chromatin immunoprecipitation of GR or IgG after dexamethasone treatment. N=3 independent biological replicates. (**D** to **F**) U2OS cells stably expressing GR and Flag-tagged WT HES1 or a mutant HES1 unable to bind to DNA and the effect of dexamethasone on *NRO-B1* (D), *FLRT3* (E), and *RGS2* (F) expression in these cells. N=4 independent biological replicates for (D) to (F). (**G**) Fold

enrichment of promoters of these genes by chromatin immunoprecipitation of HES1 or IgG after dexamethasone treatment. N=3 independent biological replicates. Asterisks (*) indicate $p<0.05$ as determined by a one-way ANOVA/Tukey analysis.

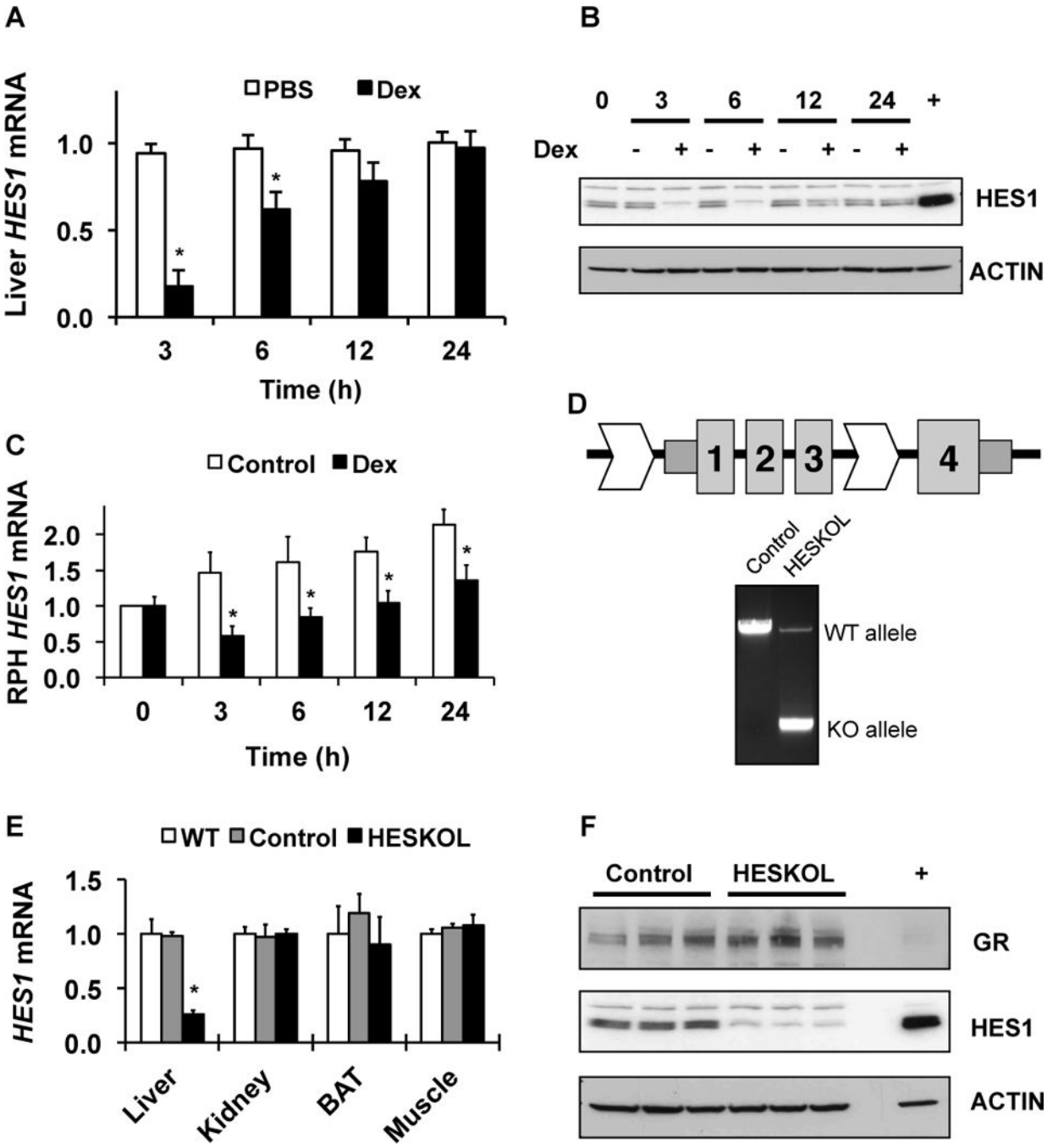

Figure 5. Glucocorticoids repress *HES1* expression in hepatocytes

(**A** and **B**) Time course of adrenalectomized male mice injected with dexamethasone or PBS and *HES1* mRNA abundance analyzed by RT-PCR (A), or HES1 protein abundance analyzed by Western blot (B). N=4 independent biological replicates for (A) and 3 independent biological replicates for (B). (**C**) The effect of dexamethasone treatment on the expression of *HES1* mRNA in rat primary hepatocytes (RPH). N=6 independent biological replicates. (**D**) Scheme showing the approximate location of the loxP sites in the *HES1* gene and PCR of liver DNA from control or HESKOL animals. (**E**) RT-PCR analysis of *HES1* mRNA in liver, kidney, brown adipose tissue, and muscle, in WT, control, and HESKOL animals. N=3 independent biological

replicates. (**F**) Western blot analysis of control and HESKOL liver extracts. N=3 independent biological replicates. Asterisks (*) indicate $p<0.05$ as determined by a one-way ANOVA/Tukey analysis.

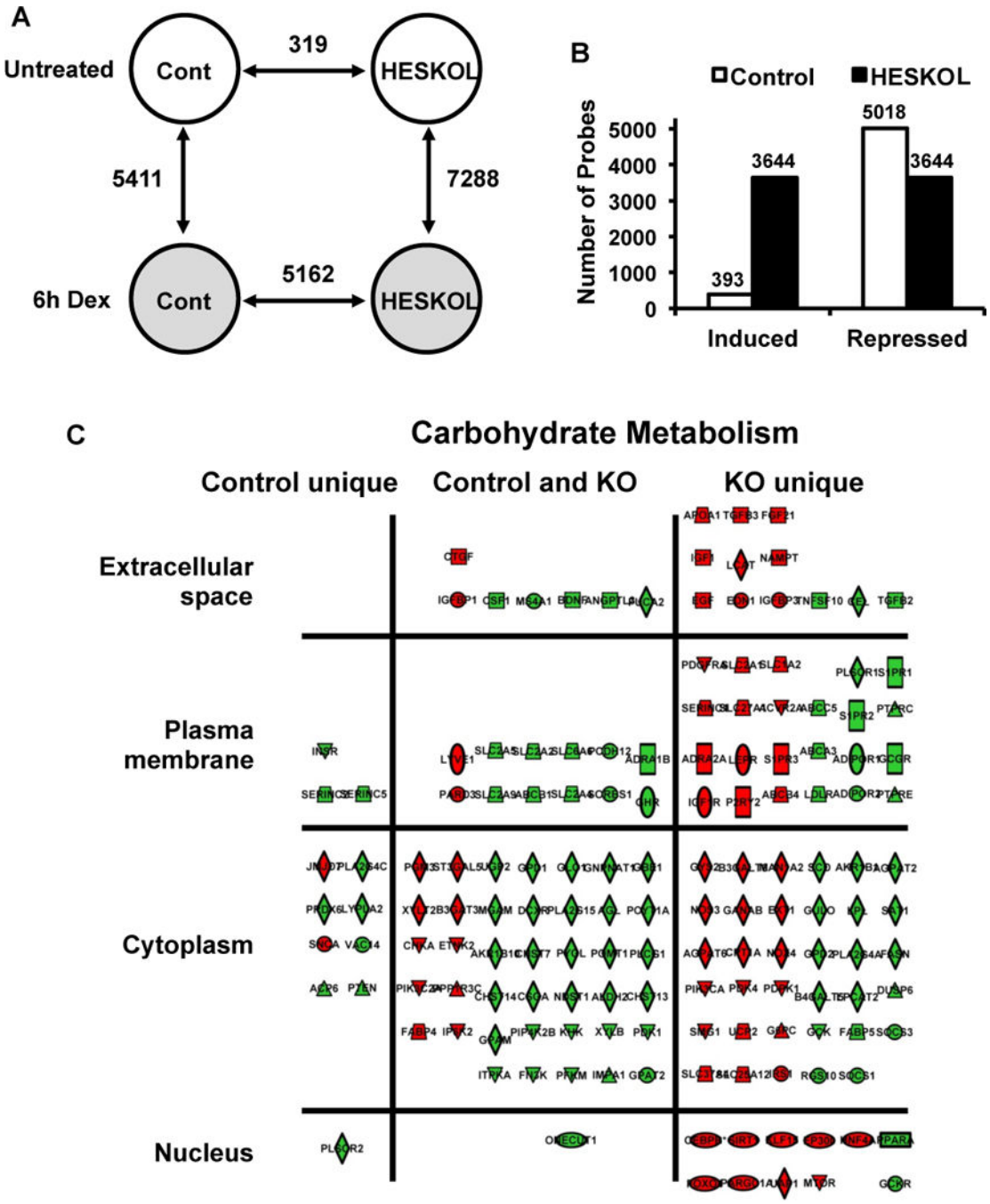

Figure 6. Ablation of liver *HES1* alters GR signaling

(**A**) Male control (*loxP/loxP*) or HESKOL mice were adrenalectomized and either injected with dexamethasone or left untreated. Number of probes statistically different ($p<0.01$, ANOVA) between the mRNA from livers of control and HESKOL, untreated or treated. N= 3 mice per condition. (**B**) The number of probes organized as either induced or repressed in control and HESKOL samples. (**C**) Ingenuity Pathway Analyses of carbohydrate metabolism pathway by using the gene lists of dexamethasone treated control and HESKOL mice. Red denotes gene induction and green denotes gene repression.

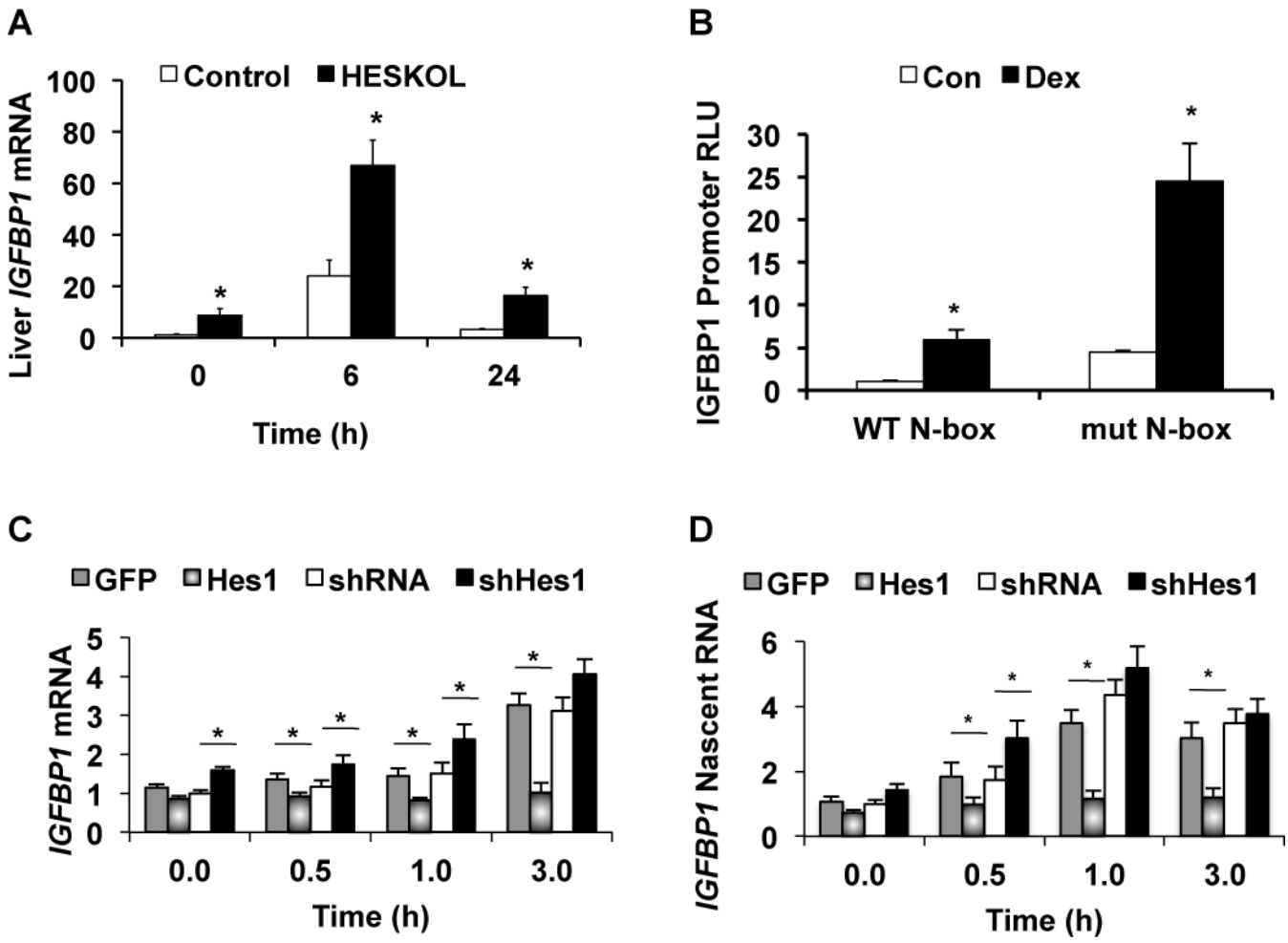

Figure 7. HES1 regulates glucocorticoid-mediated *IGFBP1* gene induction

(**A**) Male control (*loxP/loxP*) and HESKOL mice were adrenalectomized and injected with dexamethasone for the indicated time periods or left untreated. Liver RNA was extracted, and *IGFBP1* abundance was measured by RT-PCR assays. N=4 independent biological replicates. (**B**) Activity assays of luciferase in U2OS-GR cells under the control of the rat *IGFBP1* promoter with a wild type or mutated N-box in the presence of absence of dexamethasone. N=5 independent biological replicates. (**C** and **D**) Abundance of mature (C) and nascent (D) RNA of endogenous *IGFBP1* from U2OS-GR cells treated with dexamethasone for the indicated time periods. N=4 independent biological replicates for (C) and (D). Asterisks (*) indicate $p<0.05$ as determined by a one-way ANOVA/Tukey analysis (A, C, and D) or the Mann-Whitney test (B).

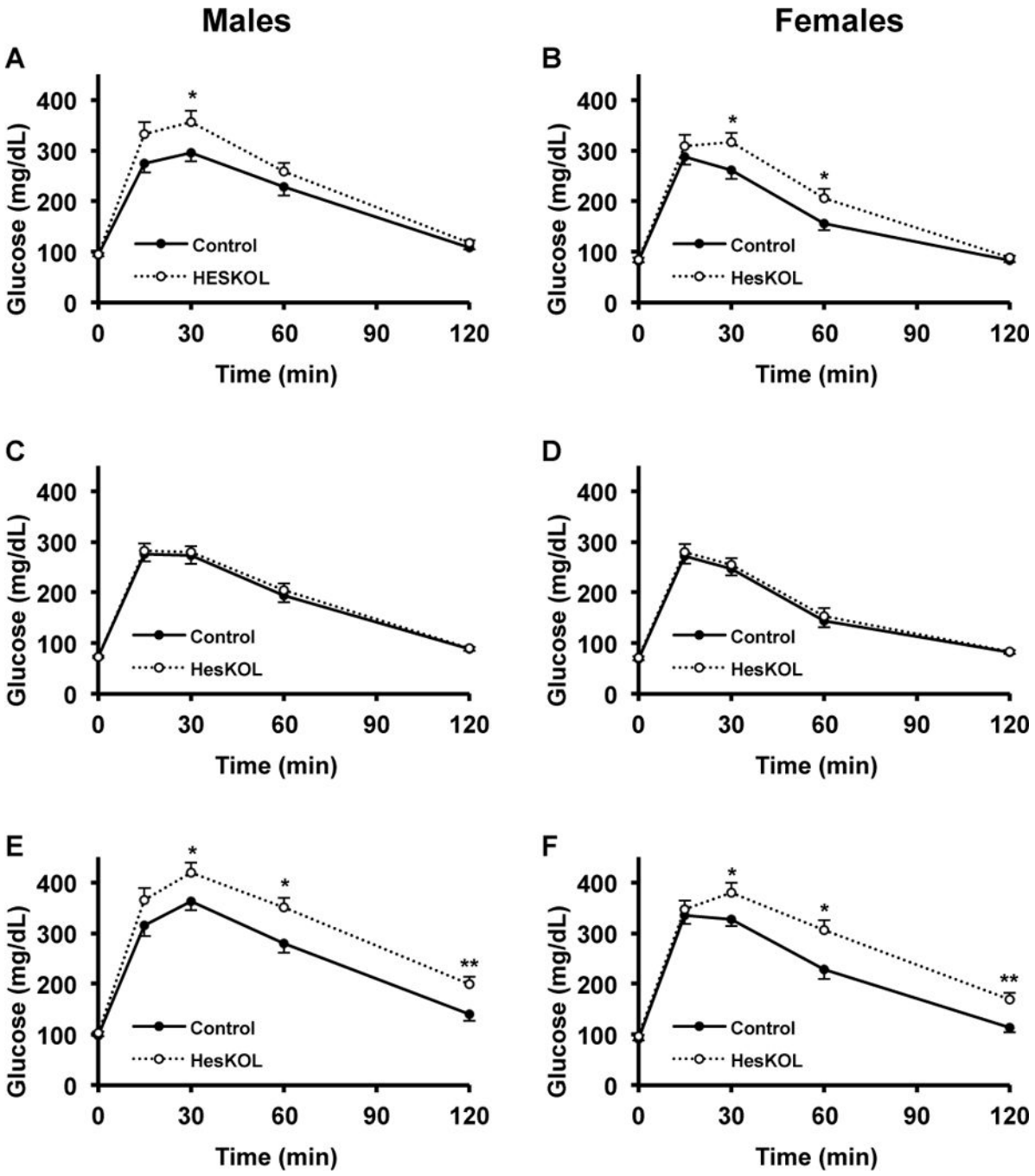

Figure 8. Glucose tolerance tests

(**A** to **F**) Mice were fasted overnight and injected with glucose, and their blood glucose concentrations were measured. Each group contained between 8 to 11 animals. Control and HESKOL intact males (A) and females (B). Adrenalectomized control and HESKOL males (C) and females (D). Dexamethasone was injected 14 hours before the test to adrenalectomized control and HESKOL males (E) and females (F). Single asterisks (*) indicate $p<0.05$, and double asterisks (**) indicates $p<0.01$, as determined by a two-tailed Student's t test.

Table 1

Biological functions regulated by glucocorticoids in control mice

Genes significantly regulated by glucocorticoids in the livers of control mice were analyzed by Ingenuity Pathway Analysis software. Shown are the top 10 Molecular and Cellular Functions that were most significantly associated with these genes.

Rank	Molecular & Cellular Function	P-value	# Molecules
1	Cellular Assembly and Organization	3.01E-05-4.25E-02	63
2	Molecular Transport	4.39E-05-4.75E-02	90
3	Protein Trafficking	4.39E-05-4.39E-05	54
4	DNA Replication, Recombination, and Repair	6.44E-05-4.25E-02	124
5	Cellular Compromise	2.32E-04-4.25E-02	44
6	Cellular Function and Maintenance	3.72E-04-4.25E-02	84
7	Cell Cycle	1.14E-03-4.75E-02	56
8	Cellular Development	1.14E-03-3.92E-02	41
9	Nucleic Acid Metabolism	1.14E-03-3.04E-02	18
10	Small Molecule Biochemistry	1.14E-03-4.25E-02	82

Table 2

Biological functions regulated by glucocorticoids in HESKOL Mice

Genes significantly regulated by glucocorticoids in the livers of HESKOL mice were analyzed by Ingenuity Pathway Analysis software. Shown are the top 10 Molecular and Cellular Functions that were most significantly associated with these genes.

Rank	Molecular & Cellular Function	P-value	# Molecules
1	Energy Production	1.23E-07-1.04E-02	83
2	Lipid Metabolism	1.23E-07-1.52E-02	319
3	Small Molecule Biochemistry	1.23E-07-1.52E-02	436
4	Cellular Growth and Proliferation	1.1E-06-1.18E-02	768
5	Carbohydrate Metabolism	1.31E-06-1.38E-02	196
6	Molecular Transport	4.49E-06-1.52E-02	310
7	Cellular Function and Maintenance	2.72E-05-1.23E-02	376
8	Cell Death and Survival	3E-05-1.41E-02	707
9	Post-Translational Modification	1.18E-04-9.08E-03	242
10	Cell Morphology	1.31E-04-1.47E-02	208