Supporting Information

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SI Materials and Methods

Plasmids and Antibodies. Flag-tagged hydrogen peroxide-inducible clone-5 (Hic-5) expression vector was obtained from Origene, and the mammalian expression vector pCDNA-hGR was obtained from Michael Garabedian (New York University, New York). Antibodies used were as follows: Flag M2 and β-actin (Sigma-Aldrich), glucocorticoid receptor (GR), CREB-binding protein (CBP), p300, Mediator complex subunit 1 (MED1) (Santa Cruz Biotechnology), and Hic-5 (BD Biosciences) for immunoblot analysis and GR (H300), CBP, p300, MED1 (Santa Cruz Biotechnology), RNA polymerase II (RNA Pol II) (Millipore), and Hic-5 (Pierce) for immunoprecipitation.

Cell Culture. A clonal line of U2OS cells that stably express rat GRα was grown as previously described (1). Cos-7 cells were maintained in DMEM supplemented with 10% (vol/vol) FBS. DNA plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After transfection, the cells were incubated at 37 °C for 48 h. Dexamethasone (Dex) (Sigma) was added at a final concentration of 100 nM.

RNA Isolation and RT-Quantitative PCR Analysis. Total RNA was isolated using QIAshredder and RNeasy mini columns (Qiagen) and was subjected to reverse transcription using random primers with the iScript cDNA synthesis kit (Bio-Rad). Quantitative PCR (qPCR) amplification was performed with the SensiFAST SYBR Green master mix (Bioline) on the LightCycler 480 system (Roche). Pre-mRNA amplification was performed by using primers spanning the first exon/intron boundary to amplify nascent transcripts.

Microarray Analysis. Total RNA was isolated using QIAshredder and RNeasy mini columns (Qiagen). The quality of RNA samples was evaluated by the A260/A280 ratio, which was at least 1.9, and the integrity was analyzed by electrophoresis on an Agilent Bioanalyzer using the Experion RNA StdSens analysis kit (Bio-Rad). For each experimental condition, 5 μg of high-quality total RNA was submitted to the Southern California Genotyping Consortium at the University of California, Los Angeles. Four biological replicates of each experimental sample from independent experiments that were collected on different days were placed randomly on the Illumina HT12v4 BeadChips and were processed following standard Illumina procedures.

Bioinformatics Analysis. Illumina's Genome Studio software was used for output of summarized probe expression values with minimal preprocessing. These data then were imported into Bioconductor (2) using limma, and background correction was performed using a normal-exponential convolution model. Further quantile normalization and $\log_2$ transformation was carried out (3), and probes identified as nonspecific in previous studies were removed (4). Except for a few cases in which multiple microarray probes represented one gene, each gene generally was represented by a single probe on the microarray; therefore we refer to "genes" rather than "probes" in this paper. Differentially expressed genes were identified using a moderated two-sample modified *t* test from eBayes in the limma package (5), and multiple testing was controlled for by computing *q* values (6). Comparison of the no siRNA treatment group and the siNS group found no significantly different probes, indicating no off-target effects of the siRNA; therefore these two groups were combined and used as the control group. Genes with significant changes in expression upon Dex treatment in the intact cells where Hic-5 was present (Hic-5$^+$) were identified by comparing the average intensity values for each gene at 0 h versus 2, 4, or 24 h of Dex treatment (Fig S1*E*, *b* versus *a*). Most of the Dex-regulated genes at 2 h also were regulated at 4 h, although with greater fold-changes at 4 h (Gene Expression Omnibus accession no. GSE46448). Our main objective was to study primary effects of GR signaling, so we focused on the 4-h time point for further analysis, because it was a relatively early time point at which we could robustly measure changes in mRNA accumulation. The statistical cutoffs for the Dex-regulated subset included a *q*-value cutoff of 0.02 and a fold-change cutoff of 2.0. To define Hic-5–regulated genes, we compared the hormone-treated control gene set with the hormone-treated siHic-5 gene set and applied a *q*-value cutoff of 0.02 with no fold-change cutoff. The Gene Ontology (GO) tool was used for functional annotation of different gene subsets (7).

ChIP. Cells were transfected with siRNA, and after 3 d Dex treatment was carried out for the indicated times. Before harvesting, cells were washed with PBS and crosslinked with ethylene glycol bis[succinimydyl succinate] for 20 min followed by 1% formaldehyde for 10 min. Crosslinking was quenched by adding 125 mM glycine. Cells were removed from the dishes by scraping, pelleted by centrifugation, and resuspended in hypotonic buffer containing 10 mM Hepes-KOH (pH 7.8), 10 mM KCl, and 1.5 mM $MgCl_2$ for 10 min. Nuclei pellets were collected by centrifugation and resuspended in lysis buffer containing 1% SDS, 50 mM Tris-HCl (pH 8.0), and 10 mM EDTA. The chromatin extracts were sonicated for 15–30 min (in repeated cycles of 30-s on/30-s off) using a Bioruptor (Diagenode) kept at 4 °C with a circulating water bath to achieve a DNA fragment size of 400–600 bp. Immunoprecipitation of sonicated chromatin samples was conducted by end-over-end rotation at 4 °C using anti-GR (H300), anti-CBP, anti-p300, anti-MED1 (Santa Cruz Biotechnology), RNA Pol II (Millipore), and anti–Hic-5 (Pierce Biotechnology). Immune complexes were isolated using either Protein G Sepharose magnetic beads (GE Healthcare) or Protein A/G beads (Santa Cruz Biotechnology). Crosslinking was reversed by heating, and immunoprecipitated DNA was purified using MinElute PCR purification columns (Qiagen). Purified DNA was analyzed by qPCR amplification using the SensiFAST SYBR Green master mix (Bioline) on the LightCycler 480 system (Roche). Primer sequences are described in Dataset S3. Results are expressed as percentage of input, which was calculated by normalizing to input DNA (preimmunoprecipitation).

1. Rogatsky I, Trowbridge JM, Garabedian MJ (1997) Glucocorticoid receptor-mediated cell cycle arrest is achieved through distinct cell-specific transcriptional regulatory mechanisms. *Mol Cell Biol* 17(6):3181–3193.
2. Gentleman RC, et al. (2004) Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol* 5(10):R80.
3. Shi W, Oshlack A, Smyth GK (2010) Optimizing the noise versus bias trade-off for Illumina whole genome expression BeadChips. *Nucleic Acids Res* 38(22):e204.
4. Barbosa-Morais NL, et al. (2010) A re-annotation pipeline for Illumina BeadArrays: Improving the interpretation of gene expression data. *Nucleic Acids Res* 38(3):e17.
5. Smyth GK (2004) Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 3:Article3.
6. JD S (2003) The positive false discovery rate: A Bayesian interpretation and the q-value. *Ann Stat* 31:2013–2035.
7. Dennis G, Jr., et al. (2003) DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* 4(5):3.

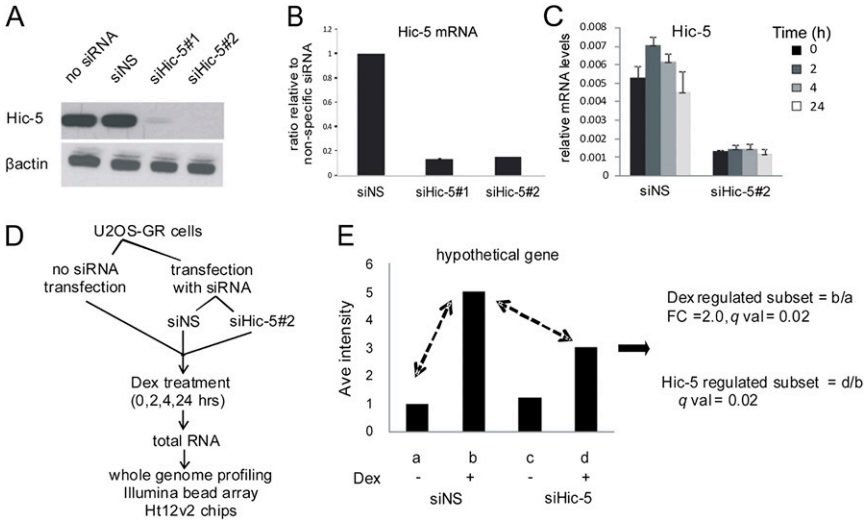

Fig. S1. Experimental strategy and outline for analyzing genome-wide effects of Hic-5 depletion on GR signaling. (*A*) Immunoblot showing the efficiency of two different siRNAs for depleting Hic-5 protein (*Upper*) 3 d after transfection of U2OS-GR cells with the indicated siRNA. β-Actin protein levels were used as internal controls. (*B*) Hic-5 mRNA levels were determined by RT-qPCR after transfection of cells with the indicated siRNA. Values were calculated relative to *Gapdh* mRNA and then were normalized to mRNA levels in cells transfected with nonspecific siRNA (siNS). Values shown are the mean ± SEM of three independent biological replicates. (*C*) Hic-5 mRNA levels in U2OS-GR cells transfected with the indicated siRNA and then treated with Dex for the indicated time period. Cells labeled as 0 h were treated with an equivalent volume of ethanol for 4 h. Levels of mRNA are expressed relative to *Gapdh* mRNA as mean ± SE for three independent biological replicates. (*D*) Schematic illustration of the experimental strategy used for global gene-expression profiling in U2OS-GR cells transfected with the indicated siRNA (or no siRNA) and then treated with Dex for the indicated time period before mRNA was harvested for analysis by microarray. (*E*) Illustration of the bioinformatics analysis performed on microarray data for a hypothetical gene from cells transfected with siNS or siHic-5 and then treated with ethanol or Dex for 4 h. The Dex-regulated subset of genes was defined by comparing *b* and *a* using a fold-change (FC) cutoff of 2.0 and a *q*-value cutoff of 0.02. The Hic-5–regulated subset was obtained by comparing *b* and *d* using a *q*-value cutoff of 0.02.

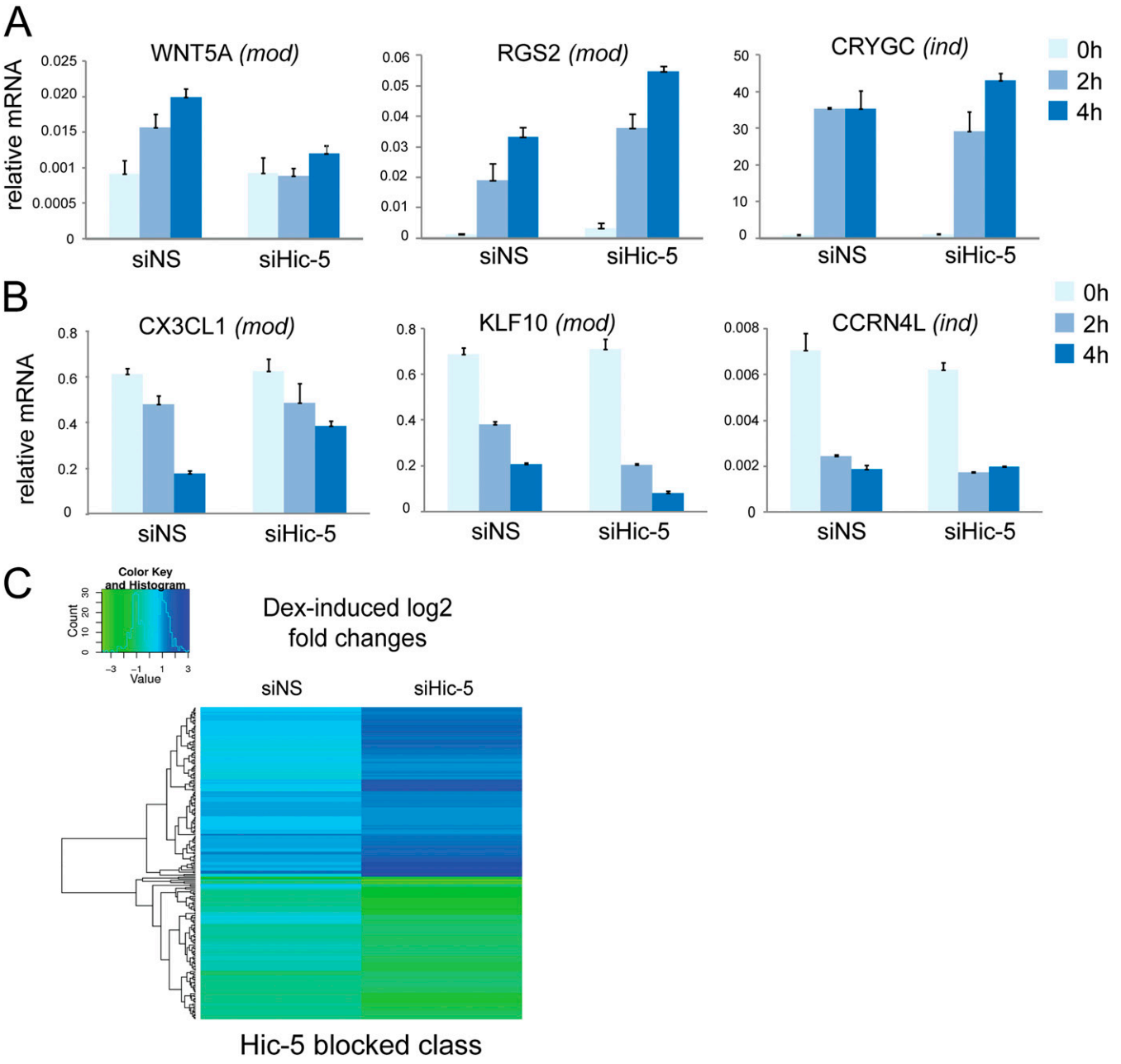

Fig. S2. Effects of Hic-5 depletion on gene expression. RT-qPCR validation showing relative mRNA levels of representative genes [Hic-5–modulated (*mod*) and Hic-5–independent (*ind*)] that are induced (*A*) or repressed (*B*) by Dex. mRNA expression values were normalized to *Gapdh* mRNA levels for each sample and are represented as mean ± SEM of three biological replicates. (*C*) Heatmap showing a comparison of genes that are excluded from the Dex-regulated subset in siNS-treated cells but induced significantly by Dex at 4 h in the siHic-5 group (twofold or more with $q < 0.02$). The corresponding log-fold regulation by Dex is represented by color intensity (*Inset*) for siNS-treated cells (*Left*) and for siHic-5–treated cells (*Right*).

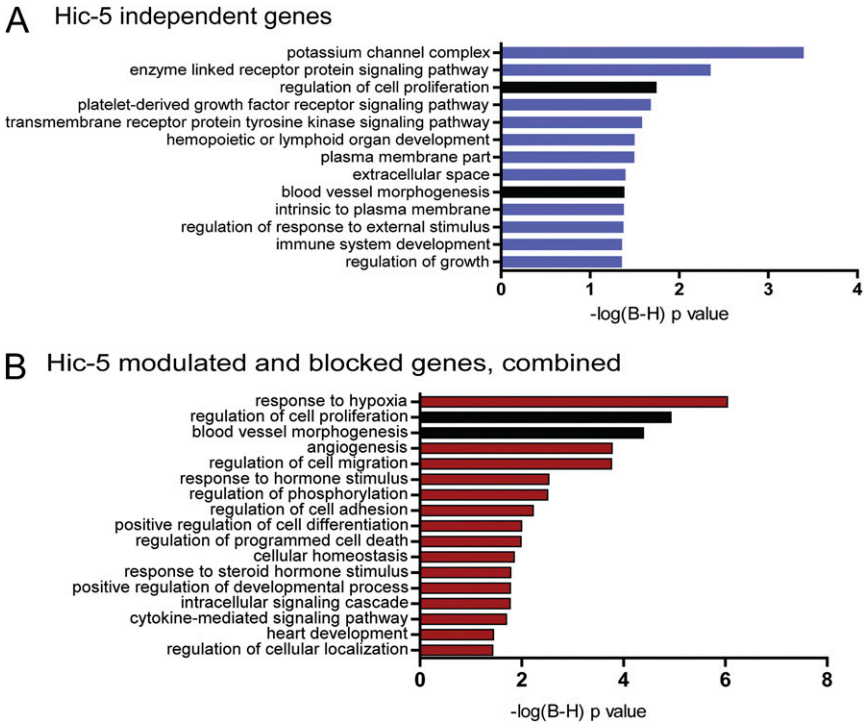

Fig. S3. Functional categorization of Dex-responsive genes that are regulated by Hic-5. Diagrammatic representation of GO analysis for two different sets of Dex-responsive genes: Hic-5–independent (*ind*) genes (*A*) and combined Hic-5–modulated (*mod*) and Hic-5–blocked (*block*) genes (*B*). Only subgroups with a Benjamini-adjusted *P* value <0.05 were selected, and the −log(*P* value) for the process was plotted. To simplify visualization of the data, only one GO term from a group of terms with similar functions is shown in *A* and *B*. Black bars indicate terms that are common to *A* and *B*. The colored bars (blue for *ind* and crimson for combined *mod* and *block*) indicate unique terms in each category. Complete lists of significantly enriched GO terms are given in Dataset S2, sheets 1 and 2.

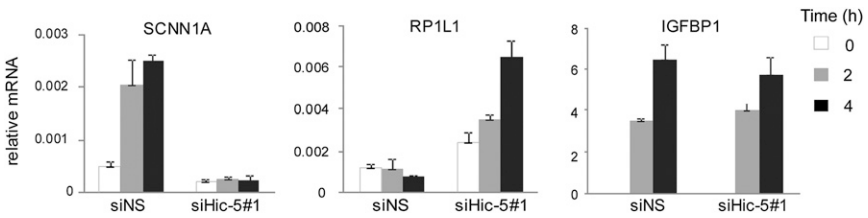

Fig. S4. Validation of effects of Hic-5 depletion using a second siRNA. Differential effects of Hic-5 depletion on candidate genes in the Hic-5–modulated (*SCNN1A*), Hic-5–blocked (*RP1L1*) and Hic-5–independent (*IGFBP1*) classes of Dex-regulated genes were validated with an additional siRNA (siHic-5#1) using RT-qPCR. Cells were treated with Dex for 2 or 4 h or with an equivalent volume of ethanol for 4 h (0 h). Data shown are mean ± SEM for three biological replicates.

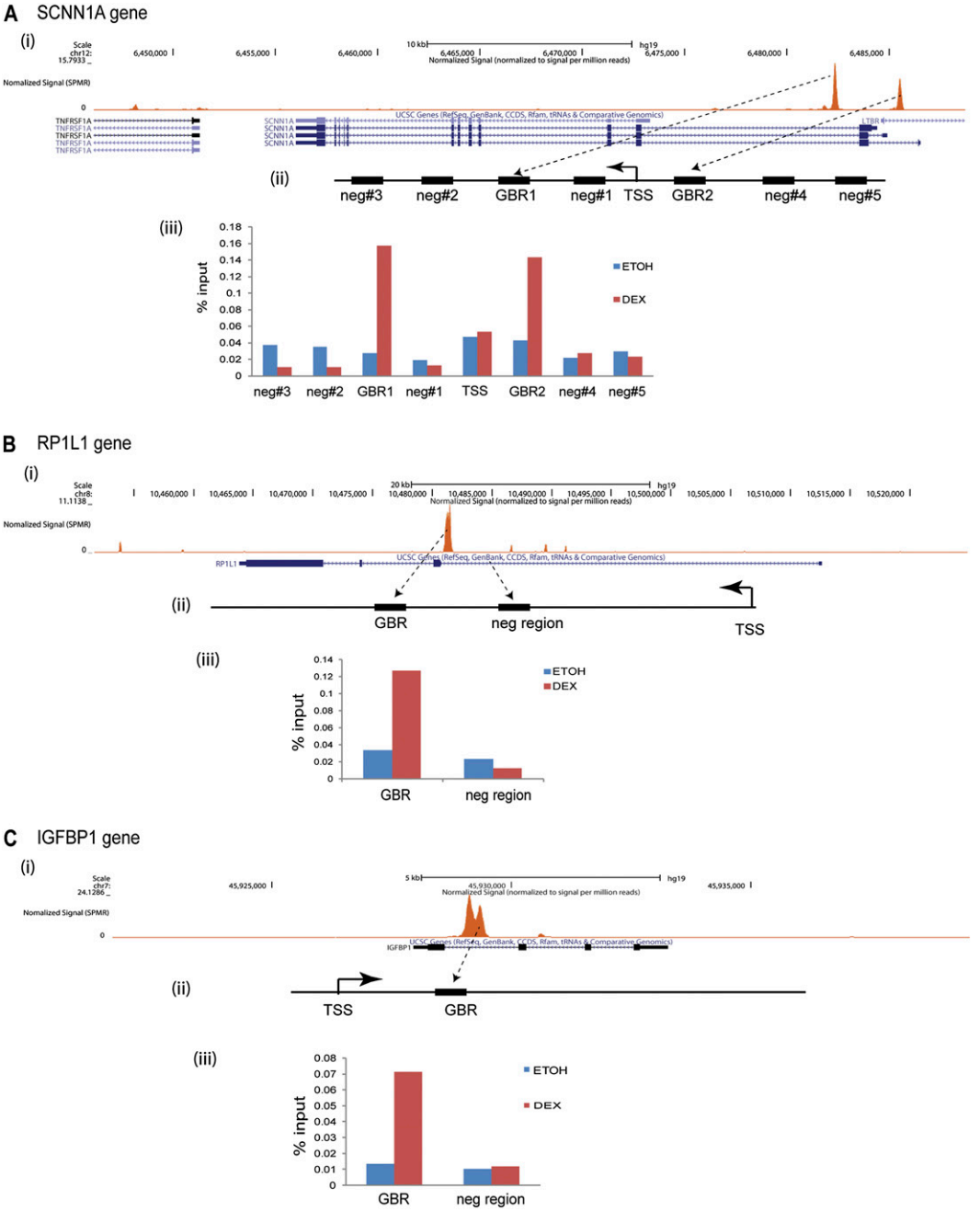

Fig. S5. Validation of GR-binding sites (obtained from ChIP-seq analysis in U2OS-GRα cells) on selected GR target genes. (*A*) *SCNN1A*, (*B*) *RP1L1*, (*C*) *IGFBP1*. Shown from top to bottom in each panel are (*i*) screen shots of selected GR target genes from the UCSC Gene Browser, (*ii*) amplicons used for ChIP-qPCR validation of GR binding, and (*iii*) ChIP-qPCR data. GR binding was measured after 1 h of Dex treatment, and values obtained were normalized to input chromatin. neg region, negative control region in which GR does not bind.

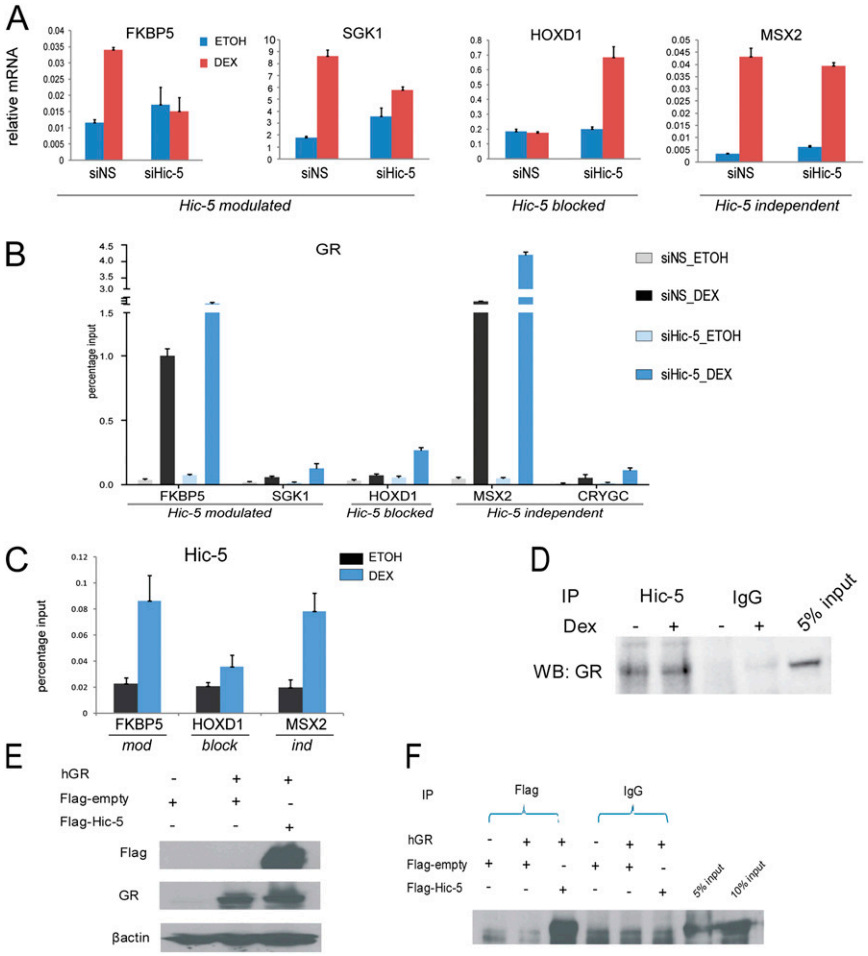

Fig. S6. GR and Hic-5 occupy GR-binding regions of selected GR target genes and interact in vivo. (*A*) RT-qPCR showing effects of Hic-5 depletion on mRNA levels for selected GR target genes. Cells transfected with the indicated siRNA were treated with ethanol or 100 nM Dex for 4 h. Relative mRNA values were normalized to *Gapdh* mRNA levels and are mean ± SEM of three biological replicates. (*B*) Effect of Hic-5 depletion on GR occupancy. Cells were transfected with the indicated siRNA and treated with Dex or an equivalent amount of ethanol for 1 h. DNA from chromatin immunoprecipitated with GR antibody was analyzed by qPCR using the primer sets for the GR-binding region associated with the indicated gene and normalized to input chromatin. (*C*) Dex-induced Hic-5 recruitment. ChIP was performed in cells treated with Dex or ethanol for 1 h, and Hic-5 occupancy at the indicated GR-binding sites was measured by qPCR. The data shown are mean values and range of variation for two technical replicates from one experiment and are representative of two independent experiments. (*D*) Endogenous coimmunoprecipitation of GR and Hic-5. Extracts from U2OS-GR cells treated with Dex for 1.5 h were subjected to immunoprecipitation (IP) with nonspecific IgG or with an antibody against Hic-5, and GR was detected by immunoblot (WB). (*E*) Immunoblots showing input expression levels of Hic-5 and GR in Cos-7 cells that were transfected with plasmids encoding full-length human GR (pCDNA.hGR) and FLAG-tagged full-length Hic-5 (pCMV6-FLAG-Hic-5) and treated with Dex for 1.5 h. β-Actin protein levels were used as internal loading control. (*F*) Immunoprecipitation with anti-FLAG or anti-IgG antibodies was performed on extracts from cells transfected with plasmids encoding the indicated proteins. GR was detected by immunoblot. Flag-empty, empty Flag-encoding plasmid.

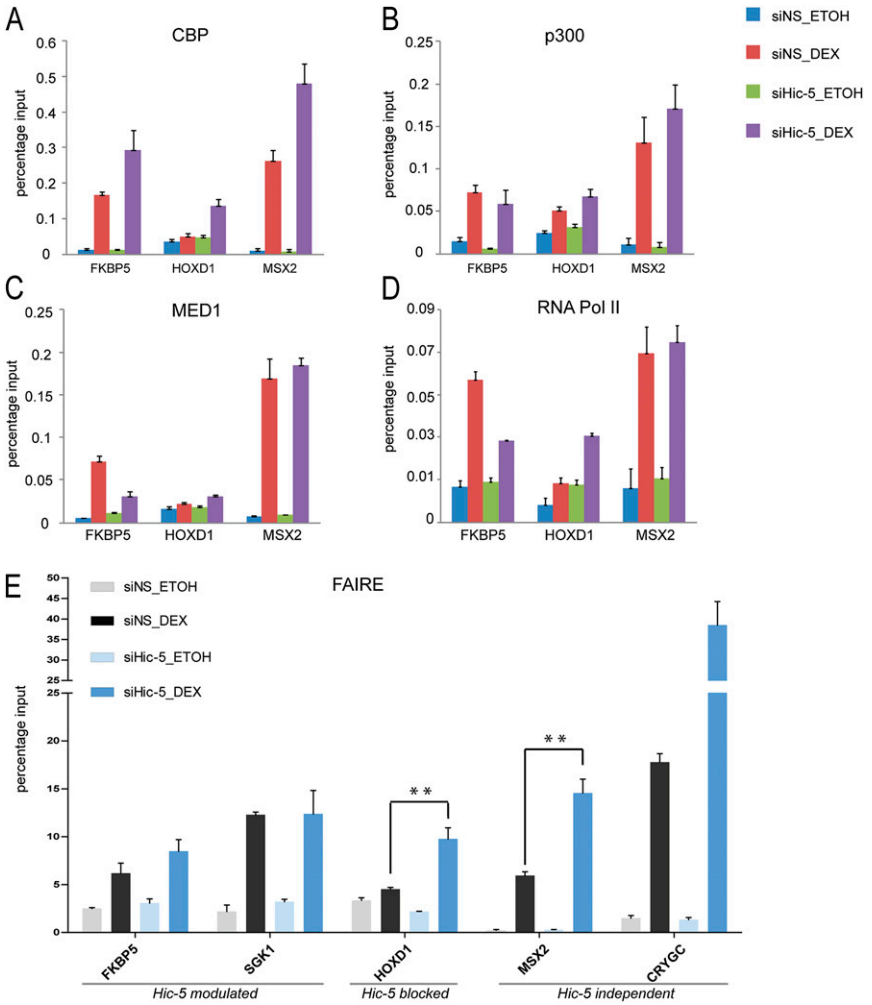

Fig. S7. Effect of Hic-5 depletion on recruitment of coregulators and RNA Pol II and on chromatin remodeling of selected GR target genes. Cells were transfected with the indicated siRNA and treated 3 d later with vehicle ethanol (ETOH) or Dex for 1 h. A ChIP assay was used to evaluate occupancy by CBP (*A*), p300 (*B*), MED1 (*C*), and RNA Pol II (*D*). Immunoprecipitated DNA was analyzed by qPCR using primers specific for glucocorticoid receptor-binding regions (*A*–*C*) or *FKBP5* transcription start site (TSS), *HOXD1* GBR, and *MSX2* TSS (*D*) of the indicated genes and normalized to input chromatin. The data shown are the mean and range of variation of two technical replicates from one experiment and are representative of three independent experiments. (*E*) Chromatin remodeling was measured by Formaldehyde Assisted Isolation of Regulatory Elements (FAIRE) coupled with qPCR for GBRs of the indicated genes in cells transfected with siNS or siHic-5 and then treated with Dex or ethanol for 1 h. The graph depicts the percentage of the input DNA that was recovered in the soluble fraction after chromatin sonication, expressed as the mean ± SD of the FAIRE signal for three technical replicates from a single experiment and are representative of at least three independent experiments. $^{**}P < 0.01$ calculated using a paired *t*-test for three independent experiments.

Dataset S1, sheet 1. Genes regulated by Dex in cells containing endogenous Hic-5 (Dex-regulated subset)

Dataset S1

This microarray dataset includes all genes from the control group (no siRNA + siNS samples) that were significantly different ($q < 0.02$) and changed 2.0-fold or more upon Dex treatment for 4 h as compared with the ethanol-treated samples. Column E represents $\log_2$ fold change in expression (b/a in Fig. S1*E*); positive values indicate that gene expression was up-regulated by Dex; negative values indicate that the gene expression was down-regulated by Dex. Column F shows the *q* values for the Dex-induced changes. Column G represents the corresponding $\log_2$ fold change in the 4-h Dex-induced mRNA level caused by Hic-5 depletion (d/b in Fig. S1*E*). Column H shows the corresponding *q* values.

Dataset S1, sheet 2. Genes belonging to the Hic-5–modulated class

Dataset S1

This microarray dataset lists all Hic-5–modulated genes that belong to the 4-h Dex-regulated subset (Dataset S1) and whose expression was significantly different ($q < 0.02$) at the 4-h Dex time point upon Hic-5 depletion. Column G represents $\log_2$ fold change in the 4-h Dex-induced mRNA levels caused by Hic-5 depletion (d/b in Fig. S1*E*). Column H indicates the *q* values for the changes. Positive values in Column G indicate that gene expression was up-regulated upon Hic-5 depletion; negative values indicate that gene expression was down-regulated upon Hic-5 depletion. Columns E and F show Dex-induced fold changes and *q* values, respectively, for the Hic-5–modulated genes in the control group (no siRNA + siNS samples).

Dataset S1, sheet 3. Genes belonging to the Hic-5–independent class

Dataset S1

This microarray dataset lists all Hic-5–independent genes that belong to the 4-h Dex-regulated subset (Dataset S1) and whose expression was not significantly different ($q < 0.02$) upon Hic-5 depletion. Column G represents the $\log_2$ fold change in the 4-h Dex-induced mRNA level caused by Hic-5 depletion (d/b in Fig. S1*E*). Column H indicates the *q* values for the changes.

Dataset S1, sheet 4. Genes belonging to the Hic-5–blocked class

Dataset S1

This microarray dataset lists Hic-5–blocked genes that were significantly regulated by 4-h Dex treatment with a fold change cutoff of 2.0 and *q* value <0.02 upon Hic-5 depletion and are excluded from the Dex-regulated subset (Dataset S1). Column E represents $\log_2$ fold changes in the 4-h Dex-induced mRNA level caused by Hic-5 depletion (d/c in Fig. S1*E*). Column F indicates the *q* values for the changes. Positive values in Column E indicate that gene expression was up-regulated upon Hic-5 depletion; negative values indicate that gene expression was down-regulated upon Hic-5 depletion. Columns G and H show Dex-induced fold changes and *q* values, respectively (b/a in Fig S1*E*), for the Hic-5–blocked genes in the control group (no siRNA + siNS samples).

Dataset S2, sheet 1. Gene Ontology terms for Dex-regulated, Hic-5–independent gene set

Dataset S2

This table shows enriched GO analysis of the Dex-regulated and Hic-5–independent gene set performed using the functional annotation tool in the Database for Annotation, Visualization and Integrated Discovery (DAVID). Top GO terms that showed increased statistical significance represented by a Benjamini Hochberg (B–H) corrected *P* value of <0.05 were selected.

Dataset S2, sheet 2. Gene Ontology terms for the Dex-regulated, Hic-5–dependent gene sets

Dataset S2

This table shows enriched GO analysis of the Dex-regulated, Hic-5–dependent gene sets (combined Hic-5–modulated and Hic-5–blocked gene sets) performed using the functional annotation tool in DAVID. Top GO terms that showed increased statistical significance represented by a Benjamini–Hochberg (B–H) corrected *P* value of <0.05 were selected.

Dataset S3. Oligonucleotide sequences of qPCR primers and siRNAs

Dataset S3