Chapter 2

Repression of DNA-Binding-Dependent Glucocorticoid Receptor-Mediated Gene Expression

The text of this chapter was taken in part from a manuscript co-authored with Nicholas G. Nickols and Peter B. Dervan (California Institute of Technology)

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Abstract

The glucocorticoid receptor (GR) affects the transcription of genes involved in diverse processes including energy metabolism and the immune response through DNA-binding-dependent and -independent mechanisms. The DNA-binding-dependent mechanism occurs by direct binding of GR to glucocorticoid response elements (GREs) at regulatory regions of target genes; the DNA-binding-independent mechanism involves binding of GR to transcription factors and co-activators that in turn contact DNA. A small molecule that competes with GR for binding to GREs could be expected to selectively affect the DNA-binding-dependent pathway by interfering with the protein–DNA interface. We show that a DNA-binding polyamide that targets the consensus GRE sequence binds the glucocorticoid induced zipper (*GILZ*) GRE, inhibits expression of *GILZ* and several other known GR target genes, and reduces GR occupancy at the *GILZ* promoter. Genome-wide expression analysis of the effects of this polyamide on a set of glucocorticoid induced and repressed genes could help elucidate the mechanism of GR regulation for these genes.

2.1 Introduction

Glucocorticoids are a major subclass of steroid hormones known to modulate a large number of metabolic, cardiovascular, immune, and behavioral functions and play diverse roles in growth, development, and maintenance of basal and stress-related homeostasis. Glucocorticoids exert a major influence on inflammatory processes and approximately 20% of the genes expressed in human leukocytes are regulated positively or negatively by glucocorticoids.[1] Glucocorticoids are among the most widely prescribed drugs worldwide, primarily used as anti-inflammatory and immunosuppressive agents.

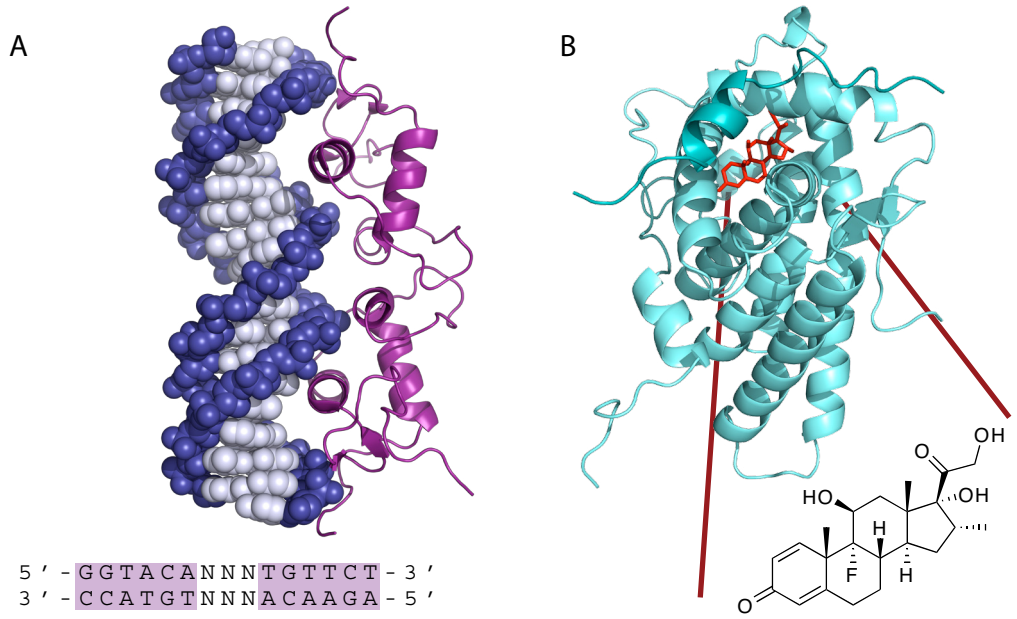

Figure 2.1 Crystallographic structures of the glucocorticoid receptor

A)DNA binding domain bound to DNA (PDB 1R4O) with consensus DNA binding site depicted below. B) Ligand binding domain bound to dexamethasone (PDB 1M2Z)

At the cellular level, the actions of glucocorticoids are mediated by a 94-kDa intracellular receptor protein, the glucocorticoid receptor (GR). The GR belongs to the superfamily of steroid/thyroid/retinoic acid receptor proteins that function as ligand-dependent transcription factors and binds with high affinity to glucocorticoids (GCs) such as cortisol

and dexamethasone. As a member of the same class of transcription factors, GR is structurally similar to the androgen and progesterone receptors, containing a zinc-finger motif DNA binding domain, a dimerization domain, and a ligand binding domain.[2] In the absence of ligand, GR resides in the cytoplasm of cells as part of a complex of proteins including chaperon heat shock proteins (hsps) 90, 70, and 50. Hsp90 regulates ligand binding, as well as cytoplasmic retention of GR by exposing the ligand binding site and masking the two nuclear localization sequences contained within the protein.[3-5] Ligand binding releases GR from sequestration by cytoplasmic heat shock proteins[5] and activates a series of cellular activities, which leads to nuclear localization and homodimerization (**Figure 2.2**).

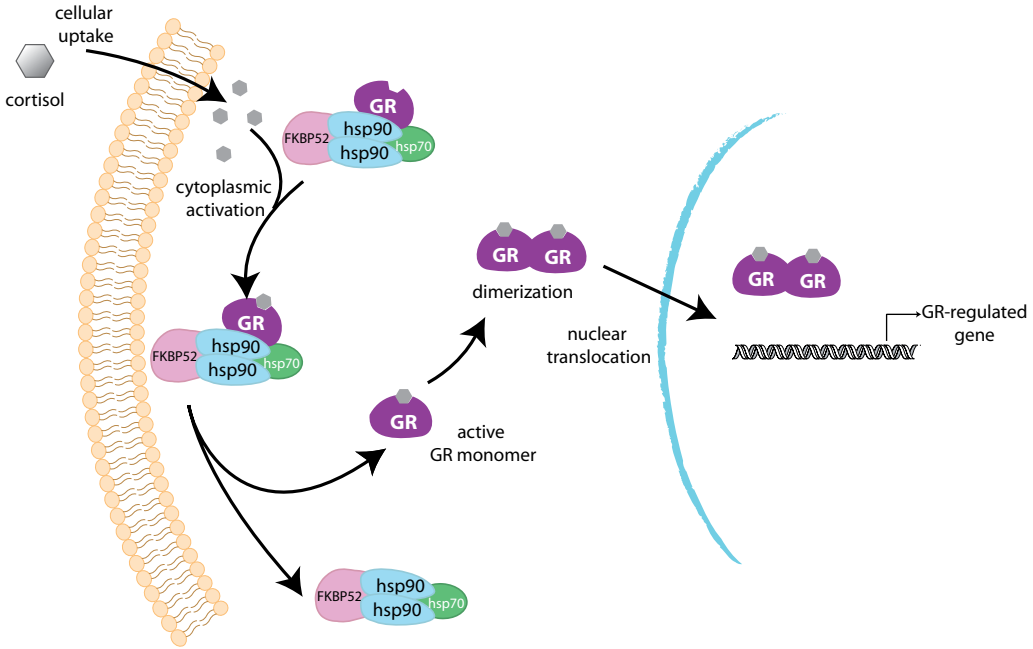

Figure 2.2 Simplified model for the response of glucocorticoid receptor to steroid hormone stimulus

The glucocorticoid hormone, cortisol, passes through the plasma membrane into the cytoplasm where it binds to the specific, high-affinity glucocorticoid receptor (GR), releasing it from sequestration by heat shock proteins. The activated GR forms a homodimer and is translocated to the nucleus where the receptor complex binds to specific DNA-responsive elements to activate gene transcription.

Like other steroid hormone receptors, GR is known to modulate gene transcription via the binding of receptor dimers to specific palindromic sequences called glucocorticoid response elements (GREs), usually located in the *cis*-regulatory region of target genes—this mode of action is termed transactivation. Additionally, the GR has been shown to exert its actions through an indirect, non-DNA-binding mechanism, termed transrepression, in which transcriptional modulation is achieved through crosstalk between GR and other transcription factors such as nuclear factor-κB (NF-κB),[6] activator protein-1 (AP-1),[7,8] Sma and Mad-related protein (Smad), and signal transduction and activator of transcription (STAT).[9] This protein-protein cross talk does not require the DNA-binding activity of the GR, as GR mutants that are deficient in dimerization function have been shown to lose DNA binding ability as well as simple GRE-mediated transcription function but retain their transrepression activity.[7,10]

Because many GR target genes are immune modulators, synthetic GR agonists such as dexamethasone are among the most effective anti-inflammatory drugs available for the treatment of a variety of chronic and acute inflammatory diseases. Unfortunately, because of the functions of other GR target genes, long term treatment with corticosteroids results in metabolic and behavioral derangements that can be treatment limiting. While the GR targets involved in inflammatory and immune regulation have not been comprehensively defined, there is a great deal of evidence suggesting that transrepression—that is, GR interaction with NF-kB and/or AP-1 and the subsequent suppression of their target genes—is the major mechanism by which glucocorticoids achieve their desired anti-inflamatory effect.[8,11,12]

An understanding of the mechanisms of GR activity on target genes has been explored using a variety of approaches including microarray analysis,[13,14] ChIP-scanning,[15] and modulation of GR activity using siRNA,[16] genetic mutants,[7] and ligands with modified

structures.[17] However, these methods would not be expected to explicitly differentiate between the direct and indirect mechanisms of GR action. A small molecule that competes with GR for binding to the consensus GRE could be expected to specifically disrupt GR-DNA binding and be used as a tool to identify GR target genes whose regulation mechanism depends on a direct protein–DNA interface. This differentiation of mechanisms of GR target gene regulation could contribute to efforts to develop more specific GR modulators that retain immunosuppressant functions while minimizing side effects resulting from other GR targets.[18] Py-Im polyamides have previously been used to modulate gene expression in cell culture via inhibition of the transcription factor–DNA interface of both hypoxia inducible factor[19,20] and androgen receptor[21] to their respective DNA response elements. Because Py-Im polyamides can be selectively programmed to recognize the known DNA-binding sequence of the GR, interruption of the GR–DNA binding interaction by polyamides represents a unique opportunity to inhibit the DNA-binding-dependent activity of endogenous GR while leaving the protein–protein- mediated activity unaffected (**Figure 2.3**).

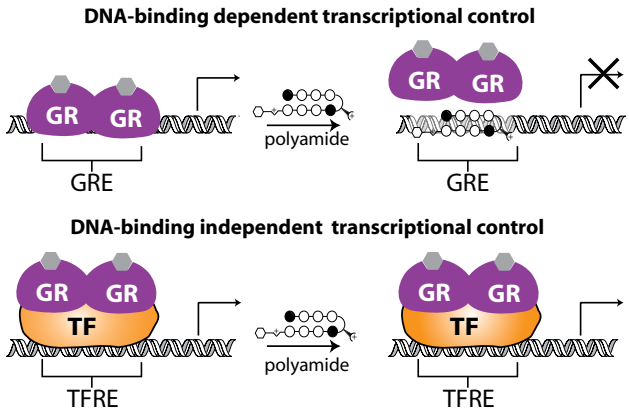

Figure 2.3 Effect of polyamide-DNA binding on GR gene regulation

Top: Direct mechanism is dependent upon GR-DNA binding at the GR response element (GRE). Bottom: Indirect mechanism is dependent upon GR binding to another protein, indicated here as a general transcription factor TF (with binding site TF response element TFRE). A sequence-specific polyamide designed to bind to the GRE but not to the TFRE would alter gene expression controlled by the DNA-binding-dependent but not the DNA-binding-independent mechanism.

In this study we designed a polyamide targeted to the sequence 5'-WGWWCW-3' (where W represents either a T•A or an A•T base pair), found in the consensus GRE, with the goal of disrupting GR-GRE binding (**Figure 2.3**A, polyamide **1**). This polyamide binds the two known GREs found in the promoter of the well-characterized glucocorticoid-induced leucine zipper (GILZ) gene, inhibits expression of GILZ and 17% of transcripts induced by dexamethasone in cultured alveolar epithelial cells (A549), and reduces GR occupancy at the GILZ promoter *in vivo*. A "mismatch" polyamide that targets the sequence 5'-WGWCGW-3' (**Figure 2.3**A, polyamide **2**) is used as a control for non-GRE binding polyamide effects. The subset of GR-regulated genes that are uniquely affected by the GRE-targeted polyamide may represent a set of genes that are regulated by GR through direct GR-GRE binding.

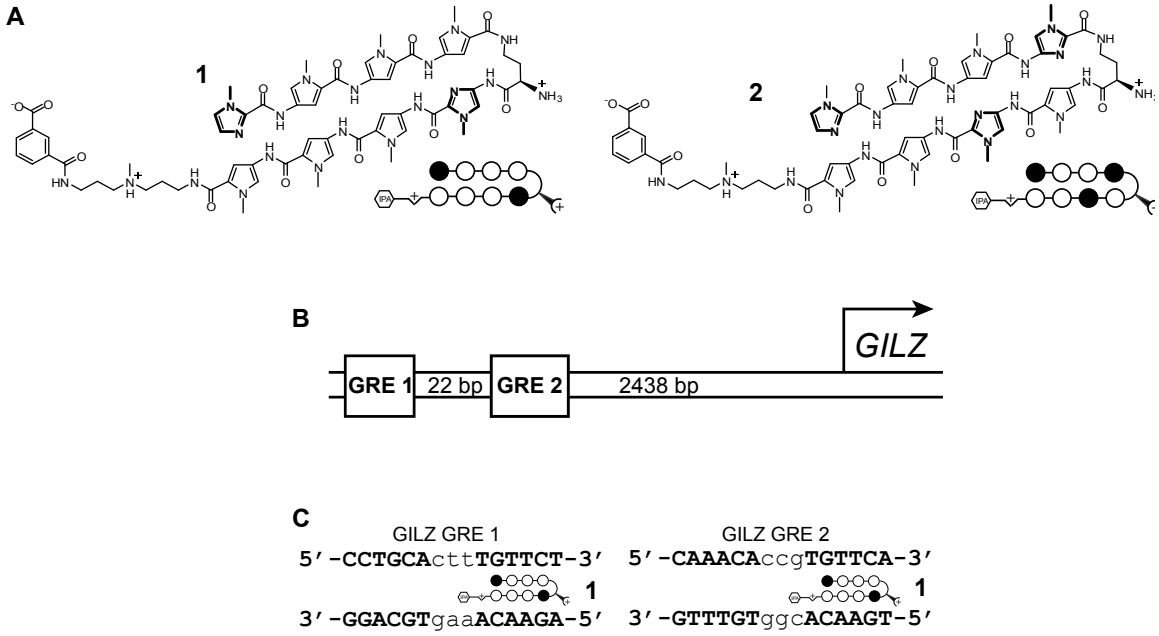

Figure 2.4 Polyamide design and *GILZ* promoter structure

*A) Structure of match polyamide **1** designed to bind 5'-WGWWCW-3' and mismatch control polyamide **2** designed to bind 5'-WGWCGW-3', where W represents a T•A or an A•T basepair. Ball-and-stick models of polyamides represent the structure shown, imidazole and pyrrole monomer units are represented by filled and open circles, respectively. The isophthalic acid tail moiety is represented by a hexagon. B) Representation of the GILZ promoter region with its two functional GRE sites indicated. C) Sequences of the two GILZ GREs shown with polyamide **1** bound to its target site.*

2.2 Results

I. Binding affinities of Py-Im polyamides to GRE1 and GRE2 of the GILZ promoter

The proximal GILZ promoter contains two functional GREs[15] (GRE1: 5'-CCTGCActtTGTTCT-3' and GRE2: 5'-AAACAccgTGTTCA-3' spaced 22 base pairs apart approximately 2500 base pairs upstream of the transcription start site (**Figure 2.4** B,C). The DNA binding affinity of polyamides **1** and **2** on this sequence was measured by quantitative DNase I footprint titrations using a 5' ^{32}P-labeled PCR fragment of pGR_GILZ, which contains a 78 base pair sequence from the promoter encompassing both functional GILZ GREs (**Figure 2.5**). Polyamide **1** has $K_a = 1.9 \pm 0.8 \times 10^{10}\ M^{-1}$ for the GRE1 consensus half-site 5'-TGTTCT-3' and $K_a = 8.8 \pm 1.8 \times 10^{9}\ M^{-1}$ for the GRE2 consensus half-site 5'-TGTTCA-3'. Binding of polyamide **2**, which targets the sequence 5'-WGWCGW-3', to the GREs is not measurable by these methods ($K_a \leq 1 \times 10^{7}\ M^{-1}$). Binding of polyamide **2** with a K_a of $6.3 \pm 0.4 \times 10^{9}\ M^{-1}$ is observed at the site 5'-TGTCTT-3' located between the GREs. This is a single base pair mismatch site for polyamide **2**, and thus **2** can be expected to demonstrate some binding to this site.[22]

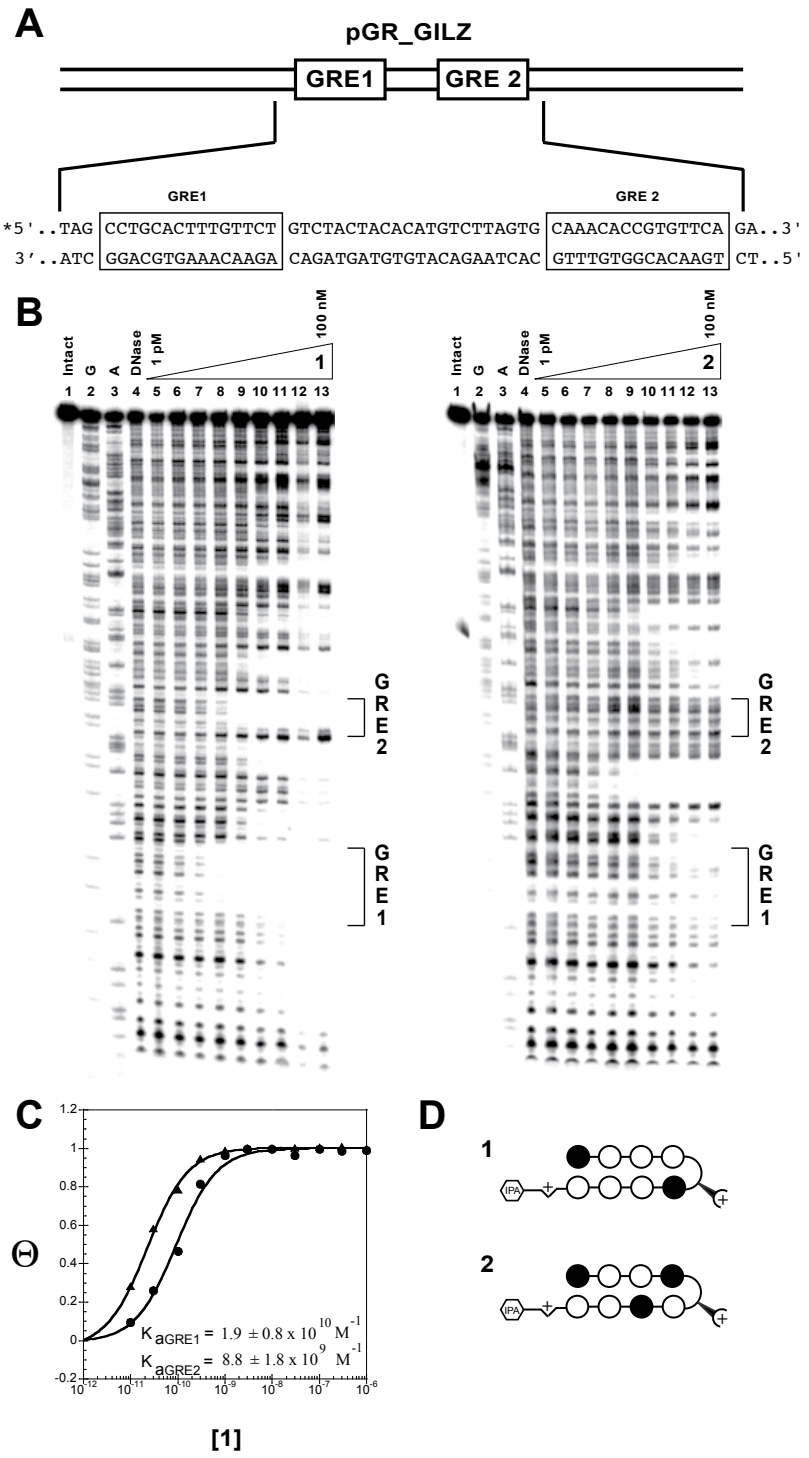

Figure 2.5 DNase I footprinting of *GILZ* promoter region

*A) Sequence of the pGR_GILZ plasmid insert. B) Storage phosphor autoradiograms from quantitative DNase I footprint titrations of polyamides **1** and **2**. Lane 1: intact DNA, lane 2: G reaction, lane 3: A reaction, lane 4: DNase control, lanes 5-13: DNase I digestion products in the presence of 1 pM, 3 pM 10 pM, 30 pM, 100 pM, 300 pM, 1 nM 3 nM, 10 nM , 30 nM, 100 nM polyamide, respectively. C) binding isotherms for polyamides **1** and **2** bound to GRE 1 D) Ball-and-stick models of polyamides **1** and **2**.*

II. Electrophoretic mobility shift assay (EMSA)

The effects of polyamides **1** and **2** on the binding of recombinant human glucocorticoid receptor to an oligo containing the GRE1 site of the *GILZ* promoter were measured by an electrophoretic mobility shift assay (**Figure 2.6**). Incubation of the ^{32}P-labeled GRE DNA with recombinant human GR produces a gel shift that is reduced in the presence of polyamide **1** at concentrations as low as 10 nM. Polyamide **2** has minimal effect at the same concentrations. The shift is abolished by incubation with a 100-fold excess of unlabeled GRE DNA but is unaffected by similar treatment with a scrambled control DNA, indicating a specific shift resulting from a GR-GRE binding event.

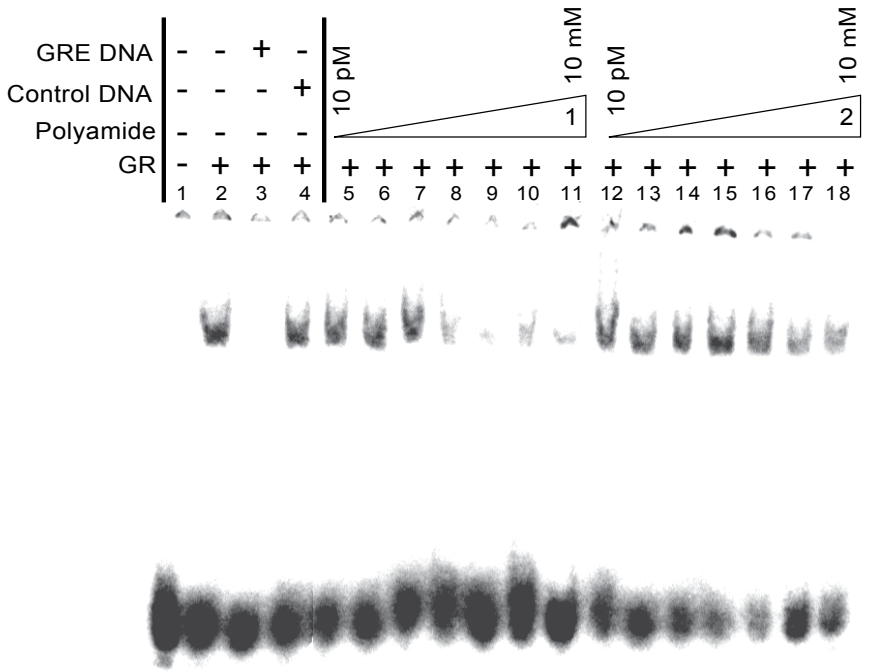

Figure 2.6 *In vitro* displacement of GR binding by polyamide **1**

*Storage phosphor autoradiogram from EMSA of recombinant human glucocorticoid receptor binding to a 27-bp oligonucleotide duplex containing the GILZ GRE1. Lanes represent the following conditions, where * represents ^{32}P labeling: 1) Free *GRE DNA 2) *GRE DNA + GR 3) *GRE DNA+ GR + GRE DNA 4) *GRE DNA + GR + scrambled DNA 5-11) *GRE DNA + GR + polyamide* **1** *(lanes 5-11) or* **2** *(lanes 12-18) in concentrations increasing from 10 pM, 100 pM, 1 nM, 10 nM, 100 nM, 1 μM to 10 μM, respectively.*

III. Inhibition of glucocorticoid-induced GILZ expression

Induction of *GILZ* mRNA by dexamethasone in the presence of polyamides **1** and **2** in A549 cells was measured by quantitative real-time RT-PCR. Polyamide **1** inhibits the expression of dexamethasone-induced *GILZ* mRNA up to 65% at 5 and 10 μM, as measured in this assay (**Figure 2.7***A*). Polyamide **2** does not show a measurable effect on *GILZ* expression at these concentrations. The GR antagonist mifepristone was used as a control and inhibits the expression of *GILZ* up to 79% at 3 μM. GR occupancy at the *GILZ* promoter was assessed by chromatin immunoprecipitation (**Figure 2.7***B*). Six hour dexamethasone treatment results in a 15-fold increase in GR occupancy at the *GILZ* promoter, treatment of the cells with polyamide **1** for 48 hours prior to harvest reduces this occupancy, while treatment with the mismatch polyamide **2** shows a more modest effect. While polyamide **2** does not bind the GRE sites we cannot exclude the possibility that it may bind to other regions of the *GILZ* promoter. While polyamide **2** affects *GILZ* promoter occupancy, albeit significantly less so that **1**, it is somewhat surprising that **2** only minimally affects *GILZ* mRNA. However, it is not known what degree of occupancy is necessary for maximal induction of *GILZ* under these conditions. Treatment of cells by both polyamides **1** and **2** modestly affects cell proliferation and viability in a concentration and time dependent manner (**Figure 2.7**C).

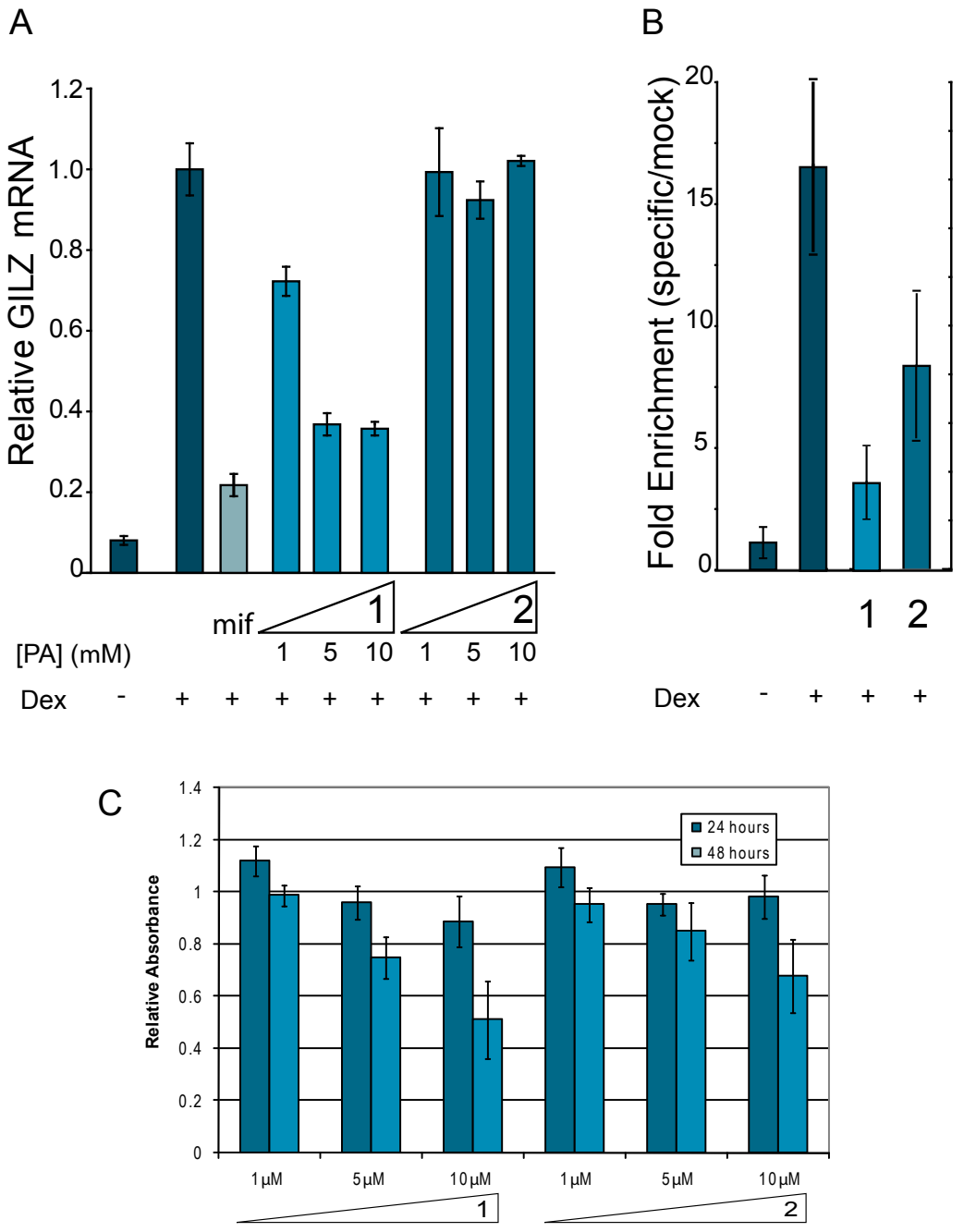

Figure 2.7 Inhibition of dexamethasone-induced *GILZ* expression by **1** and **2**

*A) Induction of GILZ mRNA in the presence of mifepristone (mif), **1** and **2**, as measured by quantitative real-time PCR. Polyamide **1** inhibits expression of GILZ mRNA up to 65% at 5 and 10 µM while polyamide **2** shows no effect. 3 µM mifepristone control inhibits expression up to 79%. Error bars represent SD. B) Chromatin immunoprecipitation assays with anti-GR or mock antibody treatment expressed as fold-enrichment (specific/mock) of DNA sequences at the GILZ promoter. GR occupancy at the GILZ promoter is decreased in the presence of **1** (10 µM) and to a lesser extent by **2**. Error bars represent SD. C) WST-1 colorimetric assay of cell viability and proliferation. Cells were treated for 24 and 48 hours with either polyamide **1** or **2** and subsequently assayed for cell viability and proliferation by measuring the absorbance of the dye at A_{max} of 450 nm on a multiwell plate reader.*

IV. Genome-wide microarray analysis

Global effects of polyamides **1** and **2** and the GR antagonist mifepristone on gene expression in dexamethasone-stimulated A549 cells were monitored with Affymetrix high-density Human Genome U133 Plus 2.0 arrays, which interrogate >50,000 transcripts. Of these transcripts, 431 were affected greater than 2-fold by dexamethasone compared to non-induced control, with 323 transcripts induced while 108 were repressed. An agglomerative clustering analysis of the transcripts affected by dexamethasone demonstrates distinctive patterns of genes that are GC-responsive and GR-mediated but have differential response to treatment by polyamide **1** and **2** (**Figure 2.8***A*) at 5 μM concentration.

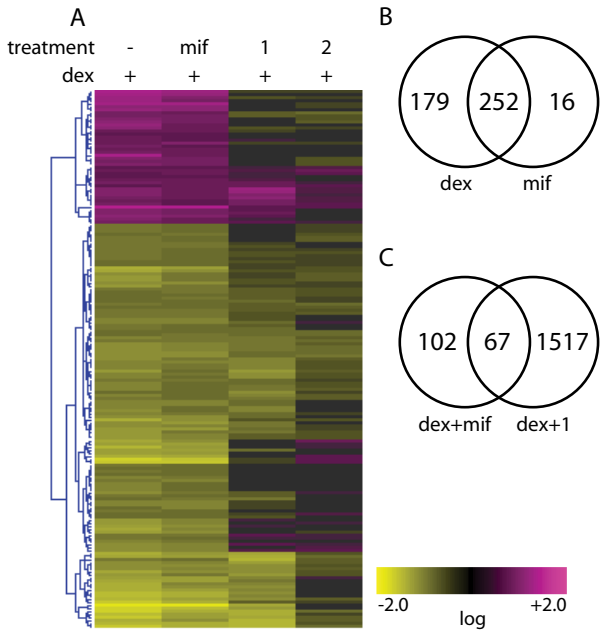

Figure 2.8 Global effects of polyamides on GR-regulated genes

Interrogation by Affymetrix high-density Human Genome U133 Plus 2.0 arrays. A) Agglomerative clustering of transcripts induced or repressed by dexamethasone (dex) by at least 2-fold under the four specified conditions: no treatment control, mifepristone (mif, 3 μM), 1 (5 μM) and 2 (5 μM). Clustering was based on an error-weighted Euclidean correlation of intensity ratios for each treatment as compared with dex-induced controls. (B) Venn diagram representing genes affected (|fold change| >2.0, P< 0.01) by dex and mif and (C) Venn diagram representing genes affected (|fold change| > 2.0, P< 0.01) by dex and mif as well as by dex and 1. Numbers inside the intersections represent transcripts affected by both treatments.

The GR antagonist mifepristone abolishes the activity of GR and therefore was used as a control to identify those transcripts that were affected by a GR-related mechanism. 431 transcripts were either induced or repressed by 2-fold or greater ($p < 0.01$) by dexamethasone treatment. Of these, the effects of dexamethasone were abolished by simultaneous treatment by 3 μM mifepristone for 252 transcripts, indicating that these 252 were a result of GR activity (**Figure 2.8***B*). For 76 transcripts of this set of 252, the effects of dexamethasone treatment were abolished by polyamide **1** to a greater extent than by polyamide **2**, indicating a sequence-specific polyamide effect on those transcripts (**Figure 2.8***C*). This set of 76 transcripts corresponds to 67 genes, listed in **Table 2.1**.

Table 2.1 Genes affected >2-fold by dexamethasone and mifepristone whose activity is modulated by polyamide **1** and not by polyamide **2**

Gene	Fold Induction	Gene	Fold Induction	Gene	Fold Induction	Gene	Fold Repression
CDKN1C (N33167)	20	IGFBP1 (NM_000596)	4	49111_at (N80935)	3	NR4A2 (NM_006186)	6
FKBP5 (NM_004117)	14	CDC42EP3 (AI754416)	4	TIPARP (AL556438)	3	IER2 (NM_004907)	3
DNAJC15 (NM_013238)	14	PLEKHA7 (AA758861)	4	EPB41L4A (AU144565)	3	EREG (NM_001432)	3
FGD4 (AI949549)	11	CEP3 (AI801777)	4	CEBPD (NM_005195)	3	IER3 (NM_003897)	3
CIDEC (NM_022094)	8	LOC153346 (AU157049)	4	EMP1 (NM_001423)	3	NR0B1 (NM_000475)	3
EDN3 (NM_000114)	8	IL6R (NM_000565)	4	LOC54492 (AK026748)	3	MAFK (BG231691)	3
PTGER4 (AA897516)	8	ARRB1 (BE207758)	4	ARRB1 (BC003636)	3	CYP24A1 (NM_000782)	3
METTL7A (NM_014033)	7	CDC42EP3 (AL136842)	4	SOCS1 (AB005043)	3	EDN1 (NM_001955)	2
FAM105A (NM_019018)	7	AKAP13 (NM_006738)	3	SCNN1G (AI985987)	3	NEIL3 (NM_018248)	2
ATAD4 (NM_024320)	6	244650_at (AA581439)	3	EPB41L4B (NM_019114)	3	PTGS2 (AY151286)	2
GOLSYN (NM_017786)	6	PKP2 (NM_004572)	3	ARRB1 (NM_004041)	3	RND1 (U69563)	2
CORO2A (AL515381)	5	FBXL16 (AI613010)	3	IL6R (NM_000565)	3		
TFCP2L1 (NM_014553)	5	CEBPD (AV655640)	3	MT1F (BF246115)	2		
RASSF4 (N49935)	5	FOXO3 (AV725666)	3	JPH2 (AA716165)	2		
CDH16 (NM_004062)	4	FLVCR2 (NM_017791)	3	SLC22A5 (NM_003060)	2		
ACSL1 (NM_021122)	4	MT2A (NM_005953)	3	RHOU(AL096776)	2		
MT1X (NM_002450)	4	KIAA0146 (AI363213)	3	MAN1C1 (NM_020379)	2		
AKAP13 (NM_006738)	4	43511_s_at (AI201594)	3	KIAA0232 (D86985)	2		
ACSL1 (NM_001995	4	ETNK2 (NM_018208	3				

These genes may represent glucocorticoid-responsive genes that are controlled via direct GR-binding mechanisms.

We anticipate that the regulation of these genes by GR involves a direct GR-GRE interaction. In order to analyze the potential GRE sequence-specific role of polyamide treatment within this subset of GR-regulated genes, those genes that showed similar effect from both polyamides **1** and **2** were considered to be non-GRE-specific effects and were

not included among those analyzed. Many of the genes identified by the microarray as dexamethasone-responsive are consistent with those previously identified in this cell line.[15] A list of the effect of all treatment conditions on the GR-regulated genes is available in the Materials and Methods section of this chapter.

2.3 Discussion

A mechanistic understanding of cell signaling is fundamental to the development of improved medicines and diagnostics. Towards this end, the functions of individual biomolecules and their involved pathways have been explored using both biological and chemical methods. Genetic approaches include gene knockouts, dominant negative proteins, and siRNA to affect the activity of selected genes. Chemical approaches have utilized pre-existing, designed, or discovered small molecules that perturb a particular protein or specific protein-protein interaction, and have been employed successfully to characterize cell signaling pathways.[23-25]

The elucidation of transcription factor, coactivator, and corepressor activation, their gene targets, and mechanisms of activity is a focus of intense research interest. Genome-wide, high-throughput approaches including mRNA microarray analysis,[26] ChIP-chip,[27-29] and more recently ChIP-sequencing[30] have been used to define the targets of transcription factors. Specific protein-DNA interactions are the interfaces where information from protein signaling is converted into programs of gene expression. Used in the context of genome-wide analysis, small molecules that perturb this interface in a predictable manner could become useful tools for understanding gene expression. Programmable, DNA-binding Py-Im polyamides offer a chemical approach to perturbing protein-DNA interactions that could be used for characterization of transcription factor-DNA interactions. We have reported previously that Py-Im polyamide **1**, which targets

5'-WGWWCW-3', inhibits androgen receptor (AR) binding to its consensus sequence 5'-GGTACAnnnTGTTCT-3'.[22] As members of the same class of transcription factors, AR and GR share highly conserved DNA-binding domains, similar consensus sequences and transactivation mode of action. However, each acts within their individual biological contexts and responds to distinct chemical stimuli. The use of polyamide **1** in the context of disrupting the GR-GRE interface offers additional perspective in the mechanistic study of GR gene-regulatory action and its dual transactivation/transrepression mechanisms. Because of the central role of GR in multiple inflammatory response pathways and its role as a drug target, mechanistic studies of GR-modulated gene expression are a growing field of study. However, the multiple mechanisms by which GR mediates gene expression makes identification of GR target genes a challenge.[9,18,31]

The complex transcriptional activity of the GR involves DNA-binding-dependent as well as DNA-binding-independent mechanisms that result in both gene activation as well as repression. In general the most well-understood mechanism is that by which genes are activated by direct DNA binding of GR to a GRE in the regulatory region of target genes followed by recruitment of coactivators and the general transcriptional machinery. Another well-documented, though less understood mechanism is the transrepression mechanism, in which GR exerts its influence indirectly by binding to other proteins and transcription factors leading to the repression of genes controlled by those proteins. Intriguingly, an increasing body of evidence indicates that much of the anti-inflammatory activity of GCs is mediated by the transrepression mechanism, a finding that has been attributed largely to the repression of key inflammatory transcription factors including AP-1 and NF-κB.[6-8] Meanwhile, many of the reported side effects of GC treatment have been attributed to transactivation mechanism.[32] Efforts to develop new GC-based drugs that can dissociate anti-inflammatory and immunosuppressive effects from side effects have focused largely on establishing a method of dissociating the transrepression from the transactivation activity

of the GR. Much of the work in this area has centered about the development of new GR-binding ligands that display a more selective gene regulation pattern than classical GCs. The end result of this body of effort has demonstrated that the problem is complex, as slight alterations in ligand structure and chemical reactivity can have a pronounced influence on the transcriptional regulatory activity of GR.[17]

An additional complication to mechanistic study of the GR is the fact that examples have also been noted in which genes are repressed through DNA-binding-dependent GR action, as well as those in which genes are activated through DNA-binding-independent action. Techniques using immunoprecipitated chromatin fragments reveal a great deal of useful information about GR target sites, but are not expected to distinguish between sites of direct GR-GRE interaction and sites of indirect GR-protein-DNA interaction. Our approach utilizes a DNA binding polyamide targeting the consensus GRE that would be expected to dissociate the direct DNA-binding from the indirect, DNA-binding-independent GR gene-regulatory mechanisms.

In this study, a sequence-specific polyamide targeted towards the consensus DNA-binding sequence of the glucocorticoid receptor was tested on a well-known glucocorticoid-induced gene, *GILZ*, in order to establish its ability to disrupt GR-DNA binding and thereby regulate gene transcription. Polyamide **1**, designed to target the sequence 5'- WGWWCW-3', has been shown by DNase I footprinting to bind at subnanomolar concentrations to the right half-site of each of two different functional GREs located in the promoter region of the *GILZ* gene. Quantitative real-time PCR analysis of RNA isolated from A549 cells treated with polyamide **1** demonstrates a 60% reduction in dexamethasone-induced *GILZ* mRNA levels as compared with vehicle control. Chromatin immunoprecipitation indicates that in the presence of polyamide **1**, the dexamethasone-induced GR occupancy of the *GILZ* promoter is reduced, suggesting that it is polyamide

occupancy at this site that is responsible for the lowered mRNA expression levels. A control polyamide **2** targeted at a different sequence, 5'-WGWCGW-3', shows reduced affect on GR promoter occupancy and no effect on *GILZ* gene transcription.

The established ability of polyamide **1** to disrupt the GR-GRE interaction for *GILZ* led us to a genome-wide search for other transcriptional events that are interrupted sequence-specifically. Affymetrix microarrays interrogating >50,000 transcripts were chosen to examine the global effect of polyamides **1** and **2** at 5 μM concentration on dexamethasone-treated cells. The GR antagonist mifepristone was used as a control in order to isolate which of the dexamethasone-responsive transcripts result from GR activity. These conditions isolated 252 genes which were considered genuine GR-modulated GC-affected transcripts. Both polyamides had a similar and modest effect on cell proliferation under these experimental conditions.

In order to tease out which of these effects is due to a binding event at a 5'-WGWWCW-3' sequence corresponding to a GRE, we have eliminated genes from the list that were affected by polyamide **2**, as both the footprinting and ChIP data shows that it is possible that this compound may have an effect on genes whose promoters contain a 5'-WGWCGW-3' site. Of the list of 252 GR-modulated transcripts, treatment with polyamide **1** had a unique effect on 170 transcripts over treatment with polyamide **2**. This left us with a final list of 170 transcripts whose expression is confidently affected both by GR as well as our sequence-specific small molecule. Of this final list of confidently interrogated transcripts we find 76 transcripts, corresponding to 67 genes, which are identified as genes whose expression was modulated by polyamide **1**. We believe this list of genes to represent 67 genes that are regulated by direct GR-DNA interactions. While little information exists in the literature linking glucocorticoid response with these genes, we note that several genes with known functional GRE binding sites are included in the list (**Table 2.2**). For genes

in which the binding site has been identified, the sequence is listed, with the predicted polyamide **1** target binding site underlined. The GREs for the genes listed in this table have been determined to have GC-induced GR occupation at the indicated location relative to the given gene. For some of these genes, functionality has been confirmed via point mutation or a luciferase-driven reporter assay. The fact that these genes contain polyamide **1** binding sites and demonstrate responsiveness to polyamide **1** treatment gives us confidence that the subset of genes identified here represents a new list of DNA-binding dependent genes to be explored by the GR field.

Table 2.2 Genes identified in our study that have previously been shown to have GR-occupied and/or functional GREs

Gene	GRE (5`→3')	Location	Ref
CDKN1C	AGAACAgccTGTCCT	Promoter	(32)
FKBP5	AGAACAgggTGTTCT	Intron	(31, 33)
ACSL1	AGCACAtcgAGTTCA	Intron	(31)
AKAP13	--	Exon	(31)
ETNK2	--	Intron	(31)
IGFBP1	--	Intron, Upstream	(31)
MT2A	AGGACAgccTGTCCT AGAACAggaTGTTTA	Upstream	(31)

There is still very little known about many of the genes that are affected by glucocorticoids, including which genes are activated as a result of transrepressed mechanisms or transactivated mechanisms. The lack of identified GREs to be found in the literature complicates the search for genes that are regulated by the transactivation mechanism. DNA-binding polyamides represent a unique approach to differentiating the transrepression from transactivation activity of the GR—the potential to selectively block the protein-DNA interactions of endogenous GR while leaving the protein-protein interactions unaffected. This approach is limited by the target site degeneracy of both the polyamide as well as GR, effects of specific polyamide-DNA binding on programs of other

transcription factors, and non-specific effects of polyamides that are independent of sequencespecific polyamide-DNA binding. Small molecules that affect specific GR-protein interactions represent a complementary approach that is as yet unexplored. It is our hope that the list of genes provided by this study serves as a guide towards genes that necessitate further exploration in the search to selectively regulate the transactivation mechanism of glucocorticoid response.

2.4 Materials and Methods

Materials

Unless otherwise stated, DNA oligonucleotides were ordered PAGE-purified from Integrated DNA Technologies. Unless otherwise stated, reagents were purchased from Sigma-Aldrich.. All solvents were purchased from Aldrich or EMD Biosciences. Trifluoroacetic acid (TFA) was purchased from Halocarbon. rac-Dithiothreitol (DTT) was purchased from ICN. RNase-free DEPC water was purchased from US Biochemicals. Water (18.2 MΩ) was purified using a Millipore water purification system.

Methods

UV spectra were recorded using an Agilent 8453 UV-Vis spectrophotometer. Polyamide concentrations were measured as a solution in water at λ=310 nm using an estimated extinction coefficient of ε=69,500 M^{-1} cm^{-1} for 8-ring polyamides.[39] LASER desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed using an Applied Biosystems Voyager DE Pro spectrometer. Analytical and preparative high-performance liquid chromatography (HPLC) were performed with a Beckman Gold system equipped with a diode array (analytical) or single-wavelength (preparative) detector.

i. Polyamide synthesis

Pyrole and imidazole monomer units [Boc-Py-OBt (1,2,3-Benzotriazol-1-yl 4-[(tert-Butoxycarbonyl)amino]-1-methylpyrrole-2-carboxylate) and Boc-Im-OH (4-[(tert-Butoxycarbonyl)amino]-1-methylimidazole-2-carboxylic acid) respectively] are synthesized according to established protocols[37] and maintained as general group stock. Fmoc-D(Dab)-Boc-OH turn monomer unit and Boc_2O were purchased from Peptides International.

Polyamides **1** and **2** were synthesized by solid-phase methods on Kaiser oxime resin (Nova Biochem, Darmstadt, Germany) following established protocols.[33] In a typical synthesis, 4 molar equivalents (relative to resin loading for the lot of oxime resin used) of monomer unit were activated with 4 equivalents of PyBOP (NovaBiochem) and 4 equivalents of diisopropylethylamine (DIEA) in a solution of dimethylformamide (DMF) (solution was approximately 0.3-0.5 M in monomer unit). In a glass peptide synthesis vessel, the resin was shaken with this solution for 2-4 hours at room temperature until the reaction was complete, as assessed by analytical HPLC of a cleaved sample of resin. Resin was deprotected by shaking in a solution of 20% trifluoroacetic acid (TFA) in methylene chloride for 20 minutes at room temperature. After completion of synthesis of the oligomer the Fmoc protecting group was removed by 10 minute incubation with 20% piperidine in DMF at room temperature. The free amine was subsequently protected by incubation of the resin for 1 hour in a solution of Boc_2O in DMF. The polyamide was subsequently cleaved from resin by incubation at 55 °C with 3,3-diamino-N-methyl-dipropylamine overnight (8-14 hours). The cleavage product was purified by reverse-phase HPLC, frozen in LN_2 and lyophilyzed to dryness.

Isophthalic acid (IPA) was activated with PyBOP (Nova Biochem) and conjugated to the polyamides as previously described.[34] Briefly: a 1 M solution of IPA in DMF and DIEA (50eq) was mixed with a 0.75 M solution of PyBOP in DMF. This solution was added to a lyophilized aliquot of cleaved polyamide and allowed to sit at room temperature for 1 hour. Following conjugation the polyamide was precipitated from solution by addition of cold diethyl ether and the precipitate was pelleted by centrifugation, the supernatant was removed and the pellet was allowed to air-dry. The Boc protecting group was removed by the addition of 100 μL of neat TFA to the pellet. The deprotection proceeded for 10 min at room temperature before being diluted with 20% acetonitrile/water (0.1% TFA) followed by purification by reverse-phase HPLC. Purities and identities of the polyamides were assessed by HPLC, UV-visible spectroscopy, and MALDI-TOF MS.

1: (MALDI-TOF) $[M+H]^+$ calcd for $C_{65}H_{77}N_{22}O_{12}^+$ 1357.6, observed 1357.5

2: (MALDI-TOF) $[M+H]^+$ calcd for $C_{64}H_{77}N_{23}O_{12}^+$ 1358.6, observed 1358.6

ii. Plasmid preparation

Plasmid pGR_GILZ was constructed by ligating the following hybridized inserts (a 78-bp sequence from the GILZ promoter containing GRE1 and GRE2) into the BamHI / HindIII polycloning site in pUC19:

5’-GATC CCATA AGTATAGC CTGCACTTTG TTCTGTCT ACTACACATGT CTTAGTGC AAACACCGTGT TCAGAGAG GTTGTG-3’

5’-AGCT CACAA CCTCTCTG AACACGGTGT TTGCACTA AGACATGTGTA GTAGACAG AACAAAGTGCA GGCTATAC TTATGG-3’

The ligated plasmid was then transformed into JM109 subcompetent cells (Promega) by standard methods (30 minute incubation on ice followed by 45 second heat shock (42 ºC) followed by 1 hour incubation at 37 ºC). Colonies were selected for α-complementation on agar plates containing 50 mg/L ampicillin, 120 mg/L IPTG, and 40 mg/L X-gal after overnight growth at 37 ºC. Cells were harvested after 16 h growth at 37 ºC in LB medium containing 50 mg/L ampicillin. Plasmids were then purified by mini-prep kits. The presence of the desired inserts was determined by capillary electrophoresis dideoxy sequencing methods (Largen).

iii. Preparation of 5'-labeled DNA for DNase I footprinting

Two primer oligonucleotides, 5'-AATTCGAGCTCGGTACCCGGG-3' (forward, corresponding to EcoRI restriction enzyme cut site) and 5'-CTGGCACGACAGGTTTCCCGA-3' (reverse, corresponding to PvuII restiction enzyme cut) were constructed for PCR amplification. The forward primer was radiolabeled using [γ-^{32}P]-dATP (MP Biomedicals) and polynucleotide kinase (Roche) according to the manufacturer's protocol, followed by purification using ProbeQuant G-50 spin columns. The desired PCR product was generated from the plasmid pGR_GILZ using the primer pair and Expand High Fidelity PCR Core Kit (Roche) following the manufacturer's protocol. The labeled fragment was loaded onto a 7% nondenaturing preparatory polyacrylamide gel (5% cross-link), and the desired 283 base pair band was visualized by autoradiography and isolated. Chemical sequencing reactions were performed according to published protocols.[38]

iv. Quantitative DNase I footprint titrations

All reactions were carried out in a volume of 400 mL according to published protocols. Polyamides were equilibrated with the radiolabeled DNA for 14 h prior to DNase I cleavage at 23 ºC. Quantitation by storage phosphor autoradiography and determination of equilibrium association constants were as previously described.[35]

v. Electrophoretic mobility shift assay (EMSA)

The oligonucleotide 5'-GTATAGCCTGCACTTTGTTCTGTCTAC-3' representing a 27-bp section of the *GILZ* promoter containing GRE1 (underlined) was annealed to its complement and end-labeled with [γ-^{32}P]dATP and polynucleotide kinase (Roche) following the manufacturer's instructions. Aqueous solutions of polyamides **1** and **2** at the indicated concentrations were incubated with the duplex at room temperature for 2 h in 5 μL 5x buffer containing 100 mM HEPES, pH 7.9, 300 mM KCl, 25 mM $MgCl_2$, 10 mM dithiothreitol (DTT), and 50% glycerol. Recombinant human glucocorticoid receptor (Affinity Bioreagents, 3.5 mg/ml) was diluted 1:20 with 50 μg/μl bovine serum albumin (BSA) and stored as a frozen stock solution. Fresh aliquots of this solution were used for each experiment and discarded after use. 2 μL of the diluted GR protein, 1 μL of 200 ng/μL poly dI-dC, 1 μL of unlabeled DNA (for control lanes) were taken up in water to a final volume of 15 μL and incubated at 4 °C for 30 min. This solution was added to the polyamide-DNA complex and incubated at room temperature for 1 h before being separated on a 6% polyacrylamide gel in 1x TBE. Gels were visualized on a phosphorimager.

vi. Measurement of dexamethasone-induced mRNA.

RNA isolation:

A549 cells (ATCC) were plated in 24-well plates at a density of 20-25 x 10^3 cells per well (40-50 x10^3 cells per mL) in F12-K medium (ATCC) supplemented with 10% FBS (Irvine Scientific, Santa Ana, CA) and 4 mM penicillin/streptomycin. After 24 h, the medium was replaced with F12-K containing 10% charcoal-stripped FBS, 4 mM penicillin/streptomycin, and polyamides or mifepristone at the designated concentrations. Cells were grown for an additional 48 h and then treated with 100 nM dexamethasone for 6 h. (**Figure 2.9**). The medium was removed, cells were washed with ice-cold PBS and immediately lysed with RLT buffer from an RNeasy kit (Qiagen). Further RNA isolation was carried out with the RNeasy kit as described in the manufacturer's manual. The isolated total RNA was quantified. The yields were 12-15 μg per well.

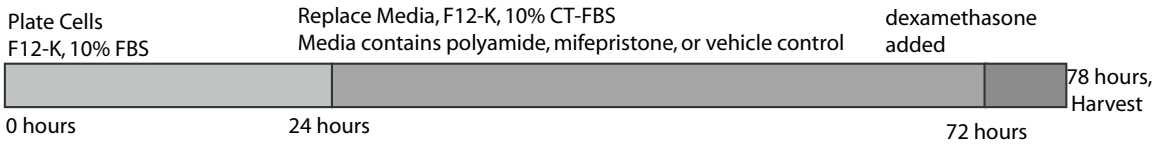

Figure 2.9 Timeline of cell treatment protocol, used for RT-PCR, ChIP and microarray assays

Reverse transcription:

A 2.5 μg sample of total RNA was used to reverse transcribe cDNA using Superscript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Random hexamers were used as primers. The total volume for each RT reaction was 20 μl.

Real-time quantitative RT-PCR:

Analysis was performed using the GILZ gene primers described below, purchased from Integrated DNA Technologies Quantitative real-time RT-PCR was performed with SYBR

Green PCR Master Mix (Applied Biosystems, Foster City, CA) following the manufacturer's suggested protocol. Temperature cycling and detection of the SYBR Green emission were performed with an ABI 7300 real-time instrument using Applied Biosystems Sequence Detection System version 1.2. Statistical analysis was performed on three independent experiments. mRNA of the genes of interest were measured relative to β-glucuronidase as an endogenous control. Primer sequences were designed using Primer3.[36]

To amplify the 97-bp fragment from the 3'-translated region of GILZ:

Forward primer: 5'- CTCCCCGTTTGTTTTTCTCA -3'
Reverse primer: 5'- TGCTCCTTCAGGATCTCCAC -3'

To amplify the β-glucuronidase gene as an endogenous control:

Forward primer: 5'- CTCATT TGGAATTTTGCCGATT -3'
Reverse primer: 5'- CCGAGTGAAGATCCCCTTTTTA -3'

vii. Measurement of Cell Proliferation and Viability.

A549 cells were plated in 96-well plates, 100 µL at 20 x 10^3 cells/mL in F12-K medium containing 10% charcoal-stripped FBS and 4 mM penicillin/streptomycin. After the indicated time period, 10 µL of Cell Proliferation Reagent WST-1 (Roche, Cat. No. 05 015 944 001) was added to each well. Reagent was allowed to incubate at 37 °C for 30 minutes and absorbance was read at 450 nm on a Perkin-Elmer Victor 3 multiwell plate reader.

viii. Chromatin Immunoprecipitation.

A549 cells were plated in 15 cm diameter plates at a density of 10 x 10^5 cells per plate. Media conditions, polyamide treatment, time course, and dexamethasone stimulation were identical to the conditions described above for qPCR. Upon completion of the 6 h dexamethasone treatment, cells were cross-linked by treatment with 1% formaldehyde for 10 minutes. Chromatin was isolated using a ChIP-IT kit (Amersham) following the manufacturer's protocol. Chromatin was sheared and immunoprecipitated by overnight incubation at 4 °C with anti-GR antibody N499 (A gift from Keith Yamamoto, UCSF). A 1:1 mixture of Protein G and Protein A Agarose beads (Upstate) was used to isolate the immunoprecipitated material via centrifugation. Cross-links were reversed and the DNA was isolated via phenol/chloroform extraction followed by ethanol precipitation. qPCR using primers targeted to the GILZ promoter were used to assess enrichment of bound fragments as compared with mock-precipitated (no antibody) controls. PCRs were monitored with SYBR Green PCR Master Mix (Applied Biosystems) on an ABI 7300 qPCR instrument. Primer sequences, described below, were designed using Primer3.[36] Detailed procedure can be found in Nicholas Nickols' PhD dissertation.[40]

To amplify the 88-bp fragment from the 5'-promoter region of GILZ:

Forward primer: 5'- GCACTGATTCATGGGTACTGG -3'

Reverse primer: 5'- ACCAACTCAGGACCAAAGGAG -3'

ix. Analysis of Gene Expression with Oligonucleotide Microarrays.

A549 cells were plated in 12-well plates at a density of 40-50 x 10^3 cells per well. Media conditions, polyamide treatment, time course, and dexamethasone stimulation were identical to the conditions described above for qPCR and ChIP. Mifepristone was added

at the same time as polyamide was added. RNA was isolated as previously described. The RNA was submitted to the Millard and Muriel Jacobs Gene Expression Facility at the California Institute of Technology where labeled mRNA was hybridized to Affymetrix high-density Human Genome U133 Plus 2.0 arrays according to established protocols. Gene expression was analyzed by using Resolver (Rosetta Biosoftware, Seattle, WA). Data were uploaded to the Gene Expression Omnibus repository (accession no. GSE17307). **Table 2.3** lists the effect of all treatment conditions on the GR-regulated genes.

2.5 Acknowledgements

We thank Keith Yamamoto for his kind gift of GR antibody. This work was supported by National Institutes of Health Grant GM051747. Mass spectrometry analyses were performed in the Spectrometry Laboratory of the Division of Chemistry and Chemical Engineering at the California Institute of Technology, supported in part by the National Science Foundation Materials Research Science and Engineering program. Oligonucleotide microarray experiments were performed in the Millard and Muriel Jacobs Genetics and Genomics Laboratory at the California Institute of Technology.

Table 2.3 Microarray fold changes under all treatment conditions of sequences affected ≥2-fold by dexamethasone and mifepristone

Sequence	Accession #	Dex	Mif	1	2	Sequence	Accession #	Dex	Mif	1	2
CDKN1C	N33167	20.3	-18.9	-5.0	-1.1	STOM	M81635	3.5	-2.8	-1.8	-1.5
FKBP5	NM_004117	14.3	-5.0	-5.9	-2.9	AKAP13	NM_006738	3.5	-2.6	-4.1	-3.1
RRAD	NM_004165	13.8	-9.6	-1.4	2.0	NEXN	AF114264	3.5	-2.9	-4.4	-3.2
DNAJC15	NM_013238	13.7	-12.1	-15.4	-2.4	244650_at	AA581439	3.5	-3.1	-2.2	-1.1
TFCP2L1	AI928242	12.9	-5.5	-5.5	-2.0	PKP2	NM_004572	3.4	-3.3	-3.4	-1.5
CDKN1C	NM_000076	12.6	-7.8	-3.6	1.0	THBD	NM_000361	3.4	-2.3	-1.3	-1.9
FKBP5	W86302	12.1	-5.2	-5.8	-2.1	ANKRD44	AU157224	3.4	-3.1	-5.8	-5.5
RRAD	NM_004165	11.4	-11.4	-1.3	1.8	FOXO3	NM_001455	3.4	-2.5	-2.4	-2.1
FKBP5	AI753747	11.2	-4.5	-4.9	-1.8	FGD4	AA296351	3.4	-3.1	-2.6	-2.1
TSC22D3	AL110191	10.9	-6.4	-1.8	-1.3	FOXO3	N25732	3.4	-2.8	-3.3	-2.1
FGD4	AI949549	10.8	-6.1	-7.5	-2.0	LOC283278	AI690465	3.4	-2.6	-5.9	-2.5
CIDEC	NM_022094	8.4	-4.7	-5.4	-4.0	KIAA1462	AL553774	3.4	-3.4	-1.9	-2.4
CDKN1C	N95363	8.3	-5.8	-2.6	1.3	FBXL16	AI613010	3.4	-3.7	-2.3	-1.2
EDN3	NM_000114	8.2	-4.3	-5.8	-1.3	CEBPD	AV655640	3.3	-3.1	-4.4	-2.3
CDKN1C	R78668	7.9	-5.7	-3.3	1.1	FOXO3	AV725666	3.3	-2.9	-3.4	-1.9
PTGER4	AA897516	7.6	-4.3	-3.1	-1.9	SNAI2	AI572079	3.3	-3.1	-2.0	-2.0
FGD4	AI277617	7.6	-5.8	-6.9	-1.6	BIRC3	U37546	3.2	-2.8	1.9	1.4
FAM43A	AW264102	7.2	-3.9	-1.2	1.5	CALD1	AL577531	3.2	-2.5	-3.0	-2.3
METTL7A	NM_014033	6.6	-3.2	-7.2	-1.8	TSC22D3	NM_004089	3.2	-3.0	-1.6	-1.3
UNC5B	AK022859	6.6	-4.8	1.1	2.0	227082_at	AI760356	3.2	-2.1	-4.1	-3.7
FAM105A	NM_019018	6.5	-4.6	-2.8	-1.2	ALPP	NM_001632	3.2	-2.7	-1.2	1.7
ANGPTL4	NM_016109	6.3	-3.4	-1.6	-1.5	ANPEP	NM_001150	3.2	-2.6	-1.9	1.4
ATAD4	NM_024320	6.2	-2.8	-2.9	-1.0	ZBTB20	AW499525	3.2	-2.1	-8.1	-4.5
GOLSYN	NM_017786	5.9	-3.6	-5.1	-1.6	CALD1	NM_018495	3.2	-2.8	-3.1	-2.0
RGS2	NM_002923	5.5	-4.2	-1.4	-1.6	RBM20	AI539118	3.2	-3.1	-4.5	-3.8
CORO2A	AL515381	5.4	-3.4	-3.1	1.2	MT1P2	AF333388	3.2	-2.8	-2.4	-2.0
TFCP2L1	NM_014553	5.2	-3.5	-3.2	-1.5	MT1F	M10943	3.2	-2.8	-2.5	-1.9
ANGPTL4	AF169312	5.1	-3.0	-1.5	-1.6	FLVCR2	NM_017791	3.1	-2.9	-2.3	-1.4
CDKN1C	D64137	5.1	-4.2	-2.5	1.2	SERPINE1	NM_000602	3.1	-2.3	-7.7	-6.2
GMPR	NM_006877	5.0	-3.1	-1.1	2.1	213158_at	AA045174	3.1	-2.2	-21.5	-5.7
RASSF4	N49935	4.9	-4.4	-6.0	-2.0	MT1H	NM_005951	3.1	-2.9	-2.4	-1.9
PER1	NM_002616	4.9	-2.6	1.3	1.1	MT2A	NM_005953	3.1	-2.9	-2.3	-2.0
LOC286167	AV721528	4.5	-3.9	-1.2	1.0	KIAA0146	AI363213	3.1	-2.7	-4.1	-1.8
ZFP36	NM_003407	4.5	-3.2	1.0	-1.3	ABHD2	AI557319	3.1	-2.2	-3.6	-3.1
CDH16	NM_004062	4.4	-4.3	-2.6	1.4	GPR115	W67511	3.0	-2.5	-4.6	-4.3
ACSL1	NM_021122	4.3	-2.8	-4.5	-2.0	FOXO3	BE888885	3.0	-2.5	-2.7	-1.8

Accession numbers given are Affymetrix accession numbers.

Table continued on page 70

Table 2.3 Microarray fold changes under all treatment conditions of sequences affected ≥2-fold by dexamethasone and mifepristone (*continued from page 69)*

Sequence	Accession #	Dex	Mif	1	2	Sequence	Accession #	Dex	Mif	1	2
C13orf15	NM_014059	4.3	-4.4	-2.0	1.2	CHST7	NM_019886	3.0	-2.7	1.7	1.9
MT1X	NM_002450	4.3	-3.9	-3.3	-2.2	43511_s_at	AI201594	3.0	-2.2	-2.9	-1.1
GRAMD4	AB018310	4.2	-4.3	-1.1	1.4	213156_at	BG251521	3.0	-2.0	-10.6	-4.8
SDPR	BF982174	4.2	-3.4	1.4	1.3	227121_at	BF476076	3.0	-2.2	-6.8	-4.0
AKAP13	M90360	4.2	-2.5	-5.9	-3.3	EPB41L4A	NM_022140	3.0	-2.1	-3.2	-2.2
ACSL1	NM_001995	4.1	-2.9	-4.2	-2.0	AKAP13	AW575773	2.9	-2.4	-4.4	-2.4
KCNB1	L02840	4.0	-4.0	-4.5	-4.3	EPB41L4B	AB032179	2.9	-2.4	-2.2	-2.1
AKAP13	AK022014	4.0	-2.3	-4.8	-3.1	SLC4A11	AF336127	2.9	-2.8	-1.4	1.1
DUSP1	AA530892	3.9	-2.2	-1.2	-1.4	SH3TC1	NM_018986	2.9	-2.7	-1.6	-1.0
SDPR	NM_004657	3.9	-2.9	1.4	1.2	ERRFI1	AL034417	2.9	-2.0	1.2	1.0
MT1X	NM_005952	3.9	-3.5	-2.8	-2.2	CALD1	NM_004342	2.9	-2.4	-3.6	-2.2
IGFBP1	NM_000596	3.8	-2.6	-2.5	-1.6	ETNK2	NM_018208	2.9	-2.6	-2.6	-1.5
ABHD2	AI832249	3.8	-2.3	-4.1	-3.7	PALM2-AKAP2	NM_007203	2.9	-2.2	-2.0	-2.5
CDC42EP3	AI754416	3.7	-2.2	-3.6	-1.9	MOBKL2B	AI692878	2.9	-2.7	-6.0	-5.1
PLEKHA7	AA758861	3.7	-3.0	-5.8	-2.1	49111_at	N80935	2.9	-2.1	-2.7	1.0
STOM	AI537887	3.7	-2.7	-1.8	-1.5	BAIAP2	BC002495	2.8	-2.5	-1.2	1.1
CEP3	AI801777	3.7	-2.3	-3.7	-1.6	GADD45A	NM_001924	2.8	-2.6	1.2	1.3
REEP1	BE535746	3.6	-3.0	-6.6	-3.8	TIPARP	AL556438	2.8	-2.2	-2.3	-1.6
LOC153346	AU157049	3.6	-3.6	-2.2	-1.3	EPB41L4A	AU144565	2.8	-2.4	-3.2	-1.5
THBD	NM_000361	3.6	-2.3	-1.3	-1.8	CEBPD	NM_005195	2.8	-2.3	-2.1	-1.4
IL6R	NM_000565	3.6	-2.6	-2.9	-2.1	PRKCD	NM_006254	2.8	-2.1	-1.1	1.2
RAB11FIP1	NM_025151	3.6	-2.9	-1.2	1.3	MAOA	AA923354	2.8	-2.0	-3.3	-2.0
THBD	AW119113	3.6	-2.6	-1.5	-1.9	EMP1	NM_001423	2.8	-2.4	-2.7	-1.5
ABHD2	BE671816	3.6	-2.3	-4.2	-3.3	LOC54492	AK026748	2.8	-2.7	-2.2	-1.0
ARRB1	BE207758	3.5	-2.6	-4.5	-2.2	ARRB1	BC003636	2.7	-2.2	-3.3	-1.7
CDC42EP3	AL136842	3.5	-2.2	-3.3	-2.0	SOCS1	AB005043	2.7	-2.7	-2.0	1.1
BAIAP2	AB017120	2.7	-2.7	-1.4	-1.0	RHOU	AB051826	2.2	-2.1	-1.1	-1.0
EPB41L4B	NM_019114	2.7	-2.3	-2.3	-1.9	DNER	BF059512	2.1	-2.3	-3.4	-2.6
EMP1	BF445047	2.7	-2.7	-2.7	-2.3	COBLL1	NM_014900	2.1	-2.1	-3.0	-2.1
KIAA1462	AL050154	2.7	-3.3	-2.3	-2.5	BAIAP2	NM_017450	2.1	-2.6	-1.3	1.2
ARRB1	NM_004041	2.7	-2.0	-2.7	-1.7	HSD11B2	NM_000196	2.1	-2.0	-1.3	1.6
EMP1	NM_001423	2.7	-2.3	-2.7	-1.5	KIAA1545	AA527531	2.1	-2.2	-1.8	-1.8
B3GNT5	BE672260	2.7	-2.1	1.1	1.1	MAN1C1	NM_020379	2.1	-2.1	-2.0	-1.4
TFCP2L1	AW195353	2.7	-2.2	-2.3	-1.8	PPL	NM_002705	2.1	-2.3	-1.9	-1.4
SERPINE1	AL574210	2.6	-2.1	-3.9	-6.0	FLVCR2	AW001026	2.1	-2.1	-1.6	-1.3
NEXN	NM_144573	2.6	-2.4	-3.8	-3.0	KIAA0232	D86985	2.1	-2.1	-3.1	-1.4

Accession numbers given are Affymetrix accession numbers.

Table continued on page 71

Table 2.3 Microarray fold changes under all treatment conditions of sequences affected ≥2-fold by dexamethasone and mifepristone (*continued from page 70)*

Sequence	Accession #	Dex	Mif	1	2	Sequence	Accession #	Dex	Mif	1	2
IL6R	AV700030	2.6	-2.2	-2.2	-1.5	AKAP2	BE879367	2.1	-2.1	-1.9	-2.2
RAB11FIP1	AA143793	2.6	-2.8	-1.1	1.3	GPR37	U87460	-2.0	2.3	1.5	1.8
TNS4	AA158731	2.6	-2.2	-1.1	-1.2	FOXA2	AB028021	-2.0	2.0	1.5	1.3
NDRG1	NM_006096	2.6	-2.4	-1.6	1.2	FZD7	AI333651	-2.0	2.1	3.3	2.1
PLEKHA2	BF347859	2.5	-2.1	-2.8	-2.6	LHX8	BC040321	-2.0	2.0	-1.1	-1.2
ITGB4	AF011375	2.5	-2.1	-2.1	-2.0	VASH2	AI961235	-2.1	2.1	1.5	1.6
STARD13	AA128023	2.5	-2.4	-2.9	-2.9	EDN1	NM_001955	-2.1	2.1	2.3	1.8
KIAA1462	AL553774	2.5	-2.4	-1.6	-1.9	KCNK3	NM_002246	-2.1	2.9	-1.4	-1.3
LIFR	AA701657	2.5	-2.2	-2.7	-3.0	SLC40A1	AL136944	-2.2	2.1	-1.4	1.1
RHOB	BI668074	2.5	-2.1	-1.1	-1.7	FAM113B	BF056901	-2.2	2.0	1.4	-1.1
CORO2A	NM_003389	2.5	-2.1	-1.8	1.0	TGFA	NM_003236	-2.2	2.2	1.8	2.1
CKB	NM_001823	2.5	-2.5	-1.4	-1.1	MYOCD	AI093327	-2.3	2.0	-1.0	-1.0
SEC14L2	NM_012429	2.5	-2.4	-2.1	-2.1	TGFB2	NM_003238	-2.3	2.3	-1.1	-1.1
CTGF	M92934	2.5	-2.9	-2.9	-2.2	LOC285513	AK026379	-2.3	2.3	1.8	1.3
FOS	BC004490	2.5	-2.1	1.1	-1.7	NEIL3	NM_018248	-2.4	2.0	2.7	1.6
MT1F	BF246115	2.4	-2.4	-2.2	-1.4	PTGS2	AY151286	-2.4	2.2	3.3	1.8
MT1G	NM_005950	2.4	-2.2	-1.8	-1.6	EDN1	J05008	-2.5	2.1	2.7	2.0
SRGN	J03223	2.4	-2.0	-1.3	-1.3	RND1	U69563	-2.5	2.3	2.3	1.5
PER2	NM_022817	2.4	-2.1	1.4	1.4	240173_at	AI732969	-2.5	2.3	-1.1	-1.2
THBS1	NM_003246	2.4	-2.2	-4.4	-2.7	SLITRK6	AI680986	-2.6	2.7	-1.7	-1.7
KIAA0232	AF143884	2.4	-2.2	-7.4	-1.9	GREM1	AF154054	-2.6	2.3	-1.9	-2.0
JPH2	AA716165	2.4	-2.5	-2.5	-1.9	RBM24	AI677701	-2.6	2.1	1.8	1.4
PACSIN2	BC008037	2.4	-2.1	-2.0	-2.3	PTGS2	NM_000963	-2.7	2.5	3.7	2.0
THBS1	AV726673	2.4	-2.2	-3.8	-2.5	HMOX1	NM_002133	-2.7	2.4	1.2	-1.6
ZBTB20	AW974823	2.4	-2.4	-1.5	-1.1	FAM84B	AL039862	-2.7	2.0	8.6	3.6
ARRB1	BF446943	2.4	-2.1	-2.4	-1.8	LONRF2	AV709727	-2.8	2.0	1.3	-1.1
PACSIN2	NM_007229	2.4	-2.0	-2.1	-2.1	IER2	NM_004907	-2.8	2.2	3.1	1.5
AHNAK	BG287862	2.3	-2.1	-1.5	-1.4	HLCS	AI682088	-2.8	2.1	1.4	1.1
HPCAL1	NM_002149	2.3	-2.2	-3.2	-3.0	SLC7A11	AB040875	-2.9	2.4	1.2	-1.6
213817_at	AL049435	2.3	-2.2	-1.9	1.3	EREG	NM_001432	-2.9	2.3	4.8	1.6
KLF6	BU683415	2.3	-2.1	-1.2	-1.4	IER3	NM_003897	-3.0	2.3	5.4	3.5
KLF6	BG250721	2.3	-2.0	-1.2	-1.3	GREM1	NM_013372	-3.0	2.5	-1.0	-1.5
LIFR	AI680541	2.3	-2.3	-2.8	-2.4	SLC7A11	AA488687	-3.1	2.7	1.2	-1.5
EMP2	NM_001424	2.3	-2.2	-1.2	1.3	NR0B1	NM_000475	-3.2	2.6	2.2	1.2
LRRC8A	AK024649	2.3	-2.1	-1.8	-2.1	LOC644943	AA876179	-3.2	2.8	1.8	1.2
SLC19A2	AF153330	2.3	-2.1	-1.2	-1.1	MAFK	BG231691	-3.2	2.2	4.0	1.7

Accession numbers given are Affymetrix accession numbers.

Table continued on page 72

Table 2.3 Microarray fold changes under all treatment conditions of sequences affected ≥2-fold by dexamethasone and mifepristone (*continued from page 71)*

Sequence	Accession #	Dex	Mif	1	2	Sequence	Accession #	Dex	Mif	1	2
SLC22A5	NM_003060	2.3	-2.2	-2.1	1.0	CYP24A1	NM_000782	-3.3	3.0	2.1	1.0
KLF4	BF514079	2.3	-2.6	1.9	1.4	DIO2	AI038059	-3.3	2.7	1.2	-1.7
S100P	NM_005980	2.3	-2.1	1.1	-1.0	ID4	AL022726	-3.4	2.4	-1.5	-1.4
MOBKL2B	AI375115	2.3	-2.2	-3.3	-3.5	TGFB2	M19154	-3.6	3.3	1.0	-1.3
TMEM43	W74580	2.3	-2.2	-1.4	-1.3	NAV3	NM_014903	-3.8	2.1	-1.0	-1.2
MT1E	AL031602	2.2	-2.3	-1.8	-1.6	NR4A2	AI935096	-3.9	2.5	40.9	5.8
ITGB4	NM_000213	2.2	-2.1	-1.5	-1.5	TM4SF20	NM_024795	-4.2	3.6	-1.0	-1.2
EPB41L4A	NM_022140	2.2	-2.1	-1.9	-1.6	SOX2	AI669815	-4.3	2.7	-1.2	-1.1
KLHL29	BE465475	2.2	-2.3	-4.9	-4.1	MYOCD	AI452798	-4.6	3.1	-1.5	-1.3
GPR115	NM_153838	2.2	-2.0	-3.1	-2.8	ST8SIA4	AA552969	-5.3	3.8	-1.2	-1.2
RHOU	AL096776	2.2	-2.4	-2.5	1.3	ST8SIA4	AA352113	-5.6	5.1	-1.2	-1.4
RHOB	AI263909	2.2	-2.4	-1.1	-1.5	NR4A2	NM_006186	-5.7	3.2	53.5	7.0
AKAP2	BG540494	2.2	-2.3	-2.1	-2.0	ST8SIA4	AI422986	-5.9	4.6	-1.3	-1.3
ENTPD2	AW134837	2.2	-2.3	-1.7	-1.5	IL11	NM_000641	-5.9	2.3	-1.1	1.1
KCNG1	AI332979	2.2	-2.1	-1.5	1.1	NR4A2	S77154	-6.0	3.1	63.6	7.0
TMEM43	AA115485	2.2	-2.1	-1.4	-1.3	GDF15	AF003934	-8.4	4.9	16.2	5.7
ST3GAL1	NM_003033	2.2	-2.2	1.0	1.2	229242_at	BF439063	-9.2	7.6	2.1	1.9

Accession numbers given are Affymetrix accession numbers.

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