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Regan Esposito Date

Investigation of the Long Non Coding RNA Growth Arrest Specific 5 Control of Cell Fate

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# Investigation of the Long Non Coding RNA Growth Arrest Specific 5 Control of Cell Fate

By

Regan Esposito

B.A., Colgate University, 2014

Advisor: Eric Ortlund, PhD

An abstract of

A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University

in partial fulfillment of the requirements for the degree of
Masters of Science in Graduate Division of Biomedical Sciences, Biochemistry, Cell, and Developmental Biology.

2018

Abstract

Investigation of the Long Non Coding RNA Growth Arrest Specific 5 Control of Cell Fate

By Regan Esposito

Long non coding RNAs are a novel class of RNAs that are poorly understood and challenging to study. The long non coding RNA Growth Arrest Specific 5 (Gas5) is a known tumor suppressor. Gas5 has been shown to interact with two transcription factors: the Glucocorticoid Receptor (GR) and the Y-Box Binding protein 1 (YBX1) and potentially interacts with a wide variety of proteins. Gas5 structural interaction with the GR, its impact on transcription, and potential interactions with other proteins can explain how Gas5 controls apoptosis and proliferation as well as provide insight into the study of lncRNAs. This work examines the various activities of Gas5 and proposes a method to continue to study and characterize this lncRNA.

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## Table of Contents

# Chapter One: Investigating the Long Non Coding RNA Growth Arrest Specific 5

## Introduction:

*Long non-coding RNAs are regulators of gene expression.*

Long non-coding RNAs (lncRNAs) are non-protein coding RNAs with transcripts longer than 200 nucleotides, they are more abundant than protein coding RNAs and are just as complex.[1] LncRNAs contain intronic and extronic sequences, they can be differentially spliced, and are often capped and polyadenylated.[1] Many lncRNAs, such as HOTAIR and XIST, are involved in regulating gene expression through chromatin modifications and direct interaction with transcription factors.[1,2]

Dysregulation of lncRNAs results in many diseases, especially cancer. LncRNAs have diverse mechanisms, and dysregulation of lncRNAs often leads to cancerous outcomes.[1] For example, the lncRNA Steroid Receptor RNA Activator (SRA) levels are associated with increased nuclear receptor driven transactivation in breast cancer.[3] LncRNAs such as metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) control alternative splicing and translation; defects in MALAT1 are associated with tumor growth and metastasis.[4] The complexity and rapid evolution of lncRNAs can make them challenging to study. LncRNAs have high turnover rates,[5] their structure and function are challenging to predict,[6] and they are rarely conserved between species.[7] LncRNAs with some known biological functions and high conservation can be a useful tool to investigate lncRNA biology.

*LncRNA Growth Arrest Specific 5*

The long non-coding RNA Growth Arrest Specific 5 (Gas5) was initially discovered in a tumor suppressor screen and was thought to code for a protein important .[8] Gas5 is continuously transcribed with levels most likely regulated by nonsense mediated decay.[9] It was later discovered that the Gas5 transcript itself was important for function.[10] Gas5 has since then been established as a tumor suppressor[11] and can be used as a candidate to elucidate lncRNA cancer biology. Gas5 is conserved across many species reaching back to zebrafish and is one

of the most highly expressed genes in the human genome[12]. Gas5 has twelve exons, with the introns containing essential snoRNAs.[12] The majority of Gas5 studies examine Gas5 transcript levels in cancer cells lines and patient tumor samples, few studies have focused on Gas5's mechanism of action.

*LncRNA Growth Arrest Specific 5 is downregulated in multiple cancer types.*

Studies have shown decreased Gas5 steady-state transcript levels in patient and cancer cell models ranging from pancreatic cancer to renal cell carcinomas to T-lymphocytes.[3, 11, 14, 15, 16, 17] Increased levels of Gas5 in prostate cancer cell lines by transfection corresponds to an increase in basal apoptosis and a decrease in cell survival.[14] Gas5 transfection in renal cell carcinoma cells inhibited proliferation, induced apoptosis, and inhibited migration and invasion potential.[15] In breast cancer, transfection of a small stem-loop of RNA or DNA with the Gas5 sequence was sufficient to increase apoptosis and decrease proliferation.[17] Gas5 has been preliminarily biochemically characterized, but the mechanisms through which Gas5 controls apoptosis and tumorigenesis have not been sufficiently explored.

*The Glucocorticoid Receptor*

The Glucocorticoid Receptor (GR) is a steroid hormone receptor that functions as an essential transcription factor to control inflammation and metabolism following activation by cortisol (Figure 1.1).

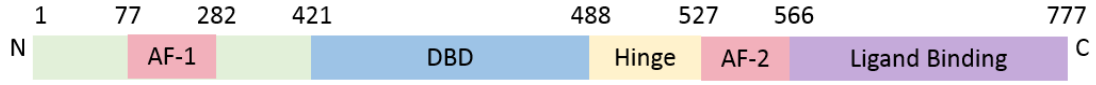


***Figure 1.1: Domain Map of GR.*** *The GR is comprised of an N-terminal transactivation domain, a DND Binding Domain (DBD) that has two highly conserved zinc fingers, a hinge region, and a C-terminal ligand binding domain (LBD) that also contains nuclear localization signals and a second activation domain, AF-2. Upon cortisol binding to the LBD, the nuclear localization signals are exposed.*

When chronically elevated, cortisol can have deleterious effects on weight, immune function, and chronic disease risk.[18] During acute stress, cortisol is released which floods the body with glucose while inhibiting insulin production in an attempt to prevent glucose from being stored,

favoring its immediate use. At the same time, cortisol narrows the arteries while epinephrine increases heart rate, both of which increase blood pressure. While these glucocorticoid-driven responses are beneficial to deal with short-term stress, they accelerate progression and symptoms in diabetes and heart disease.[19]

The GR Controls transcription through DNA binding dependent and independent mechanisms. The GR is responsible for the effects of cortisol through activation and repression of different transcriptional targets. Following cortisol binding in the cytosol, GR translocates to the nucleus and activates transcription by directly binding DNA.[20] In the case of GR mediated repression, GR tethers other proteins to impact their transcriptional activity in a non-DNA binding dependent manner. Nuclear factor kappa B (NFκB) and Activating Protein 1 (AP-1), both key co-inflammatory transcription factors, are potently suppressed by GR.[21]

*LncRNA Gas5 Interacts with Multiple Proteins*

Gas5 interacts with the Glucocorticoid Receptor (GR*) in vivo*[22] and *in vitro*[12] and with the Y box binding protein (YBX1) in stomach cancer cells,[23] and with PKR (unpublished data from the Conn lab). The GR binds to a Glucocorticoid Response Element Mimic (GREM) located in the twelfth exon of Gas5.[12] Gas5 studies do not identify the biochemical basis for the dysregulation or the resulting disease state. A deeper understanding for the pathways that Gas5 interacts with would broaden our knowledge of cancer lncRNA biology and allow for the generation of new therapeutic strategies.

The questions and hypothesis proposed in this work will examine the structural nature of the interaction between Gas5 and the GR, the effect of Gas5 on the transcriptional activity of the GR, and propose a method to identify additional endogenous Gas5 protein interactions (Figure 1.2).

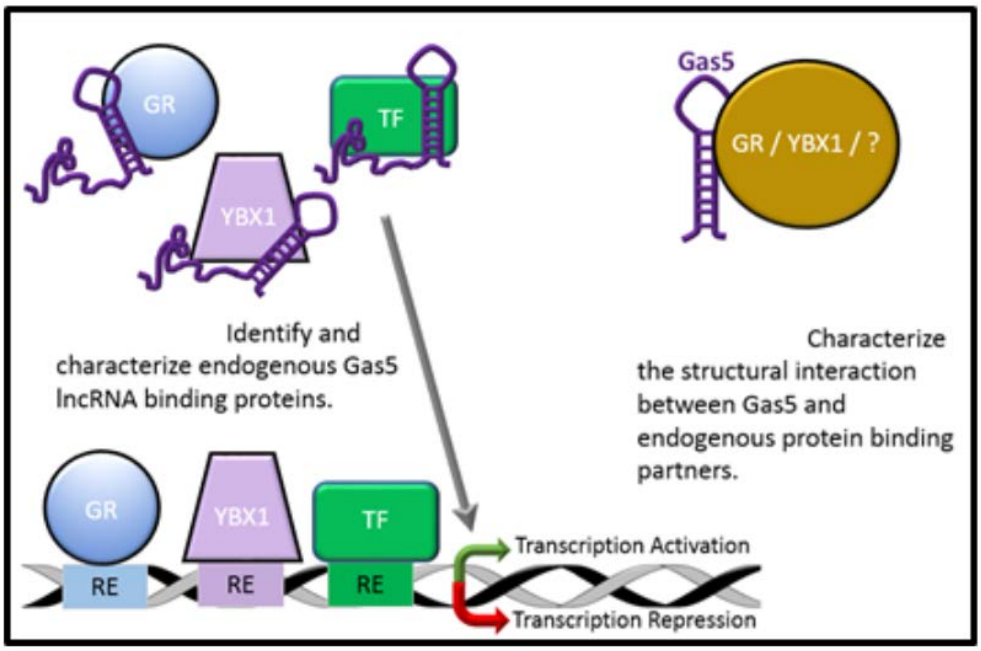


**Figure 1.2: Mechanisms of Transcriptional Regulation by Gas5.** Gas5 binds transcription factors and regulates their effect on gene expression at their Response Elements (RE). This project proposes to identify endogenous transcription factor binding partners, characterize the impact of Gas5 binding on transcriptional activity, and characterize the structural interaction between Gas5 and bound transcription factors.

In Chapter 2 we will explore *in vitro* Gas5 and the GR binding and pursue crystal structures with various Gas5 constructs. In Chapter 3 we will examine the impact of Gas5 transfection on GR mediated transcriptional activation and repression. In Chapter 4 we will propose a method to explore endogenous Gas5 and protein interactions. These projects elucidate how Gas5 potentially functions as a tumor suppressor and will expand our knowledge of lncRNA function.

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## Chapter 2: Structural Characterization of GR DBD and Gas5 as DNA

### Introduction

The lncRNA Growth Arrest Specific 5 (Gas5) is a known tumor suppressor that has been shown to interact with the Glucocorticoid Receptor (GR) *in vitro* and *in vivo.*[1] This interaction occurs at a Glucocorticoid Response Element Mimic (GREM) in the twelfth exon of Gas5, requiring a minimum 23 base stem loop to maintain binding.[1] A point mutant, G549A, ablates binding in some, but not all fluorescence polarization assays.[1] A crystal structure of this interaction will provide insight into how what aspects of Gas5 structure are necessary for binding to the GR and how the point mutation is affecting binding.

Previous studies in the lab pursued a structure of the GR DBD complexed with Gas5 GREM RNA (a schematic of GR is shown again in Figure 2.1). Recent data indicates the GREM as DNA (labelled HREM in Figure 2.2) is sufficient to induce apoptosis and limit proliferation to the same extent as full length Gas5 RNA[2] while the G549A mutation does not have any effect on apoptosis and proliferation. The GREM RNA only structure also adopts a similar structure to DNA at sites of GR mediated repression.[1] This indicates that DNA versions of the Gas5 GREM are a potential tool for solving a structure in the absence of a structure with the RNA GREM. The resulting DNA structure is informative for identifying binding register, optimizing RNA construct design, and may guide the generation of a more accurate model for molecular replacement structure solving if a GR DBD and Gas5 RNA structure is obtained.

## Results

*Gas5 as DNA GREM Binds with Lower Affinity than Gas5 as RNA GREM*

Various DNA constructs were designed based on the Gas5 RNA sequence that binds to GR (Figure 2.3). RNA loops were filled in to make canonical DNA, in some constructs non canonical base pairing is used to greater mimic the RNA. Fluorescence Polarization (FP) was used to confirm binding between DNA constructs and the DNA binding domain of GR (GR-DBD). SGK is a known GRE (GR response element) with strong binding was used as a positive control. DNA constructs show minimal binding to steroid hormone receptors with affinity in the low micro molar range (Figures 2.4, 2.5, 2.6). Full length Gas5 RNA binds with a $K_i$ in the 100 nM range.[1] Shorter RNA constructs bind with a $K_d$ from 500-800 nM.[1]

*Approaches to obtain a GR DBD and Gas5 RNA GREM Structure were Unsuccessful*

The GR DBD was expressed and purified[1] with Gas5 constructs in Table 2.1 made by *in vitro* transcription (IVT)[3] using T7 RNA polymerase. Gas5 is conserved across many species; Gas5 orthologues from alternate species (marmoset) that have higher binding affinity are also valid candidates for crystallization.[1] IVT and ordered constructs were mixed in varying RNA: protein ratios and set in crystallization screens. There were not any conditions that yielded single crystals or micro-crystals. Crystals were not observed in any trays when checked two weeks, six months, and one year later.

*Structure of GR DBD + Gas5 G549A DNA*

DNA constructs shown in Figure 2.3 were used in crystallization trials with the GR DBD. The DNA stem containing the G549A mutation complexed with the GR DBD was the first and only construct to yield crystals suitable for diffraction and structure solving shown in Figure 2.7. The structure was solved using molecular replacement. Statistics for data collection and model refinement are shown in Table 2.2. The mutation does not appear to interact with GR (Figure 2.8). GR DBD helix inserts into the major groove, similar to common steroid receptor DNA interactions and known GR DBD structures.

Other DNA stems show hits in 96 well screens (Figures 2.9, 2.10). Optimization was attempted, these hits were unable to be scaled up in hanging drop 24 well plates. Sitting drops were not attempted with these hits other than the initial 96 well screens.

## Discussion

*GR DBD Binds Gas5 GREM as DNA with a Lower Affinity as Gas5 GREM RNA*

The Gas5 GREM as DNA binds the GR DBD with a lower affinity as the Gas5 GREM as RNA, however crystals still can be grown from these interactions. These DNA constructs are a useful tool for alternative approaches to crystallization. DNA does not have the same structure as RNA and it lacks some of the flexibility – these DNA constructs are a useful tool, but they may not represent physiologically relevant interactions. *In vivo* studies need to be combined with *in vitro* experiments to confirm any observations made through Gas5 as DNA crystal structures.

*The G549A Mutation does not Interact with the GR DBD in the Crystal Structure*

Previous studies has shown that the G549A mutation abolishes the GR DBD binding to Gas5[1], however unpublished data from the same study indicates that full length G549A has a similar $K_i$ than Gas5 in FP competition assays with the GR DBD (Figure 2.11). There is no interaction between the G549A mutation and the GR DBD in the structure – this interaction could be entirely irrelevant. A Gas5 RNA and GR DBD structure is needed to further examine if this mutation is useful in any way. Unfortunately, repeated crystallization attempts with Gas5 RNA have been unsuccessful. This interaction is probably weaker and less significant than we expected.

## Figure and Tables

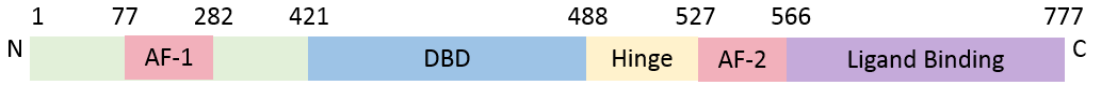



**Figure 2.1 Domain Map of GR.** The GR is comprised of an N-terminal transactivation domain, a DND Binding Domain (DBD) that has two highly conserved zinc fingers, a hinge region, and a C-terminal ligand binding domain (LBD) that also contains nuclear localization signals and a second activation domain, AF-2. Upon cortisol binding to the LBD, the nuclear localization signals are exposed.

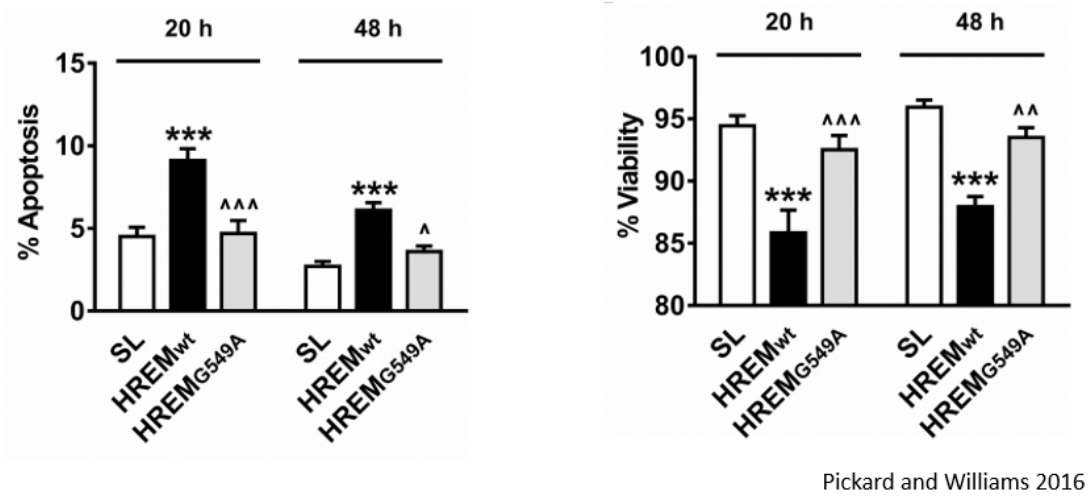



**Figure 2.2. Gas5 as DNA has the Same Effect as Full Length Gas5 RNA.** Gas5 hormone response element (HREM) sequence as DNA is as effective as full length RNA in increasing apoptosis and decreasing cell viability. Cells: MCF7s. G549A mutation does not bind GR and is included as a negative control.[9]

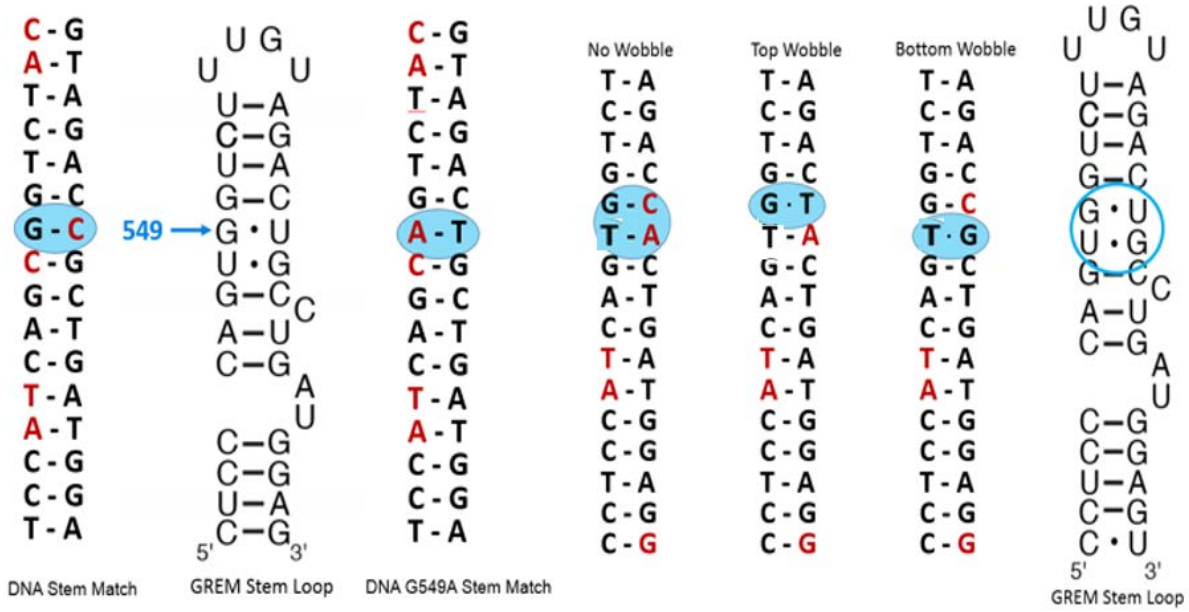



**Figure 2.3. Gas5 DNA Constructs.** DNA constructs designed to mimic Gas5 RNA. Red bases indicate changes from the original sequence. "Wobble" refers to non-canonical base pairing in the DNA construct. Blue circles indicate differences between each construct.

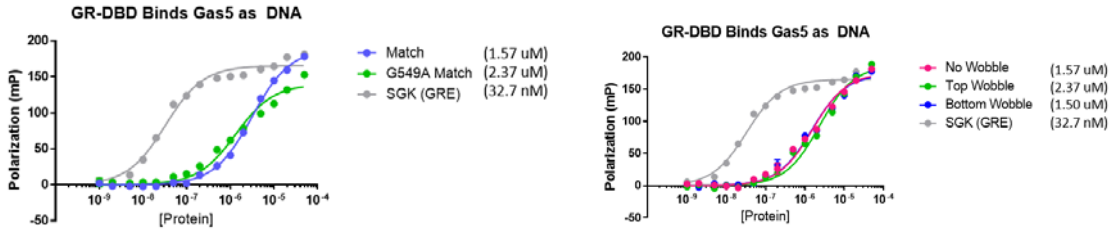
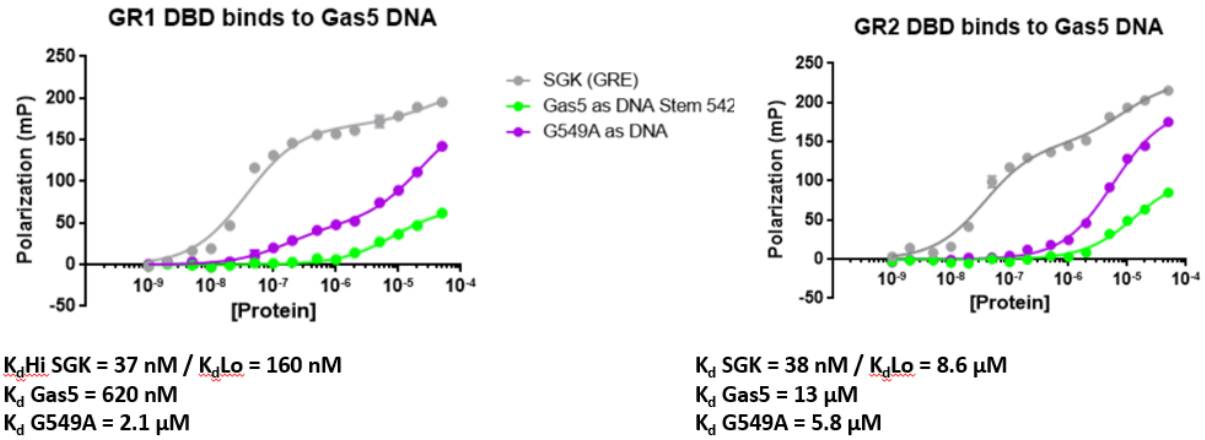



**Figure 2.4. FP with Gas5 as DNA and the GR DBD.** Fluorescence polarization was used to examine the binding affinity of the DNA constructs. $K_d$s are shown to the right of each graph. Full length Gas5 RNA binds with a $K_i$ in the 100 nM range. Shorter RNA constructs bind with a Kd from 500-800 nM. N=3.




$K_d$Hi SGK = 37 nM / $K_d$Lo = 160 nM
$K_d$ Gas5 = 620 nM
$K_d$ G549A = 2.1 µM

$K_d$ SGK = 38 nM / $K_d$Lo = 8.6 µM
$K_d$ Gas5 = 13 µM
$K_d$ G549A = 5.8 µM

**Figure 2.5. FP with Gas5 as DNA with ancestral GR proteins.** Fluorescence polarization binding experiments with ancestral versions of GR (GR1 and GR2) DBDs. SGK, a canonical GRE, showed expected binding affinities for the first binding event. Cooperative binding was lost with SGK, data was fit with a two-site equation. GR1 DBD bound Gas5 DNA stem as well as GR DBD bound Gas5 RNA stem (in the 600-800 nM range), however saturation was not necessarily reached. GR2 lost any binding specificity with Gas5 DNA. Both proteins did not show strong enough binding to warrant using them as alternate approaches to obtaining a crystal structure of the GR:Gas5 DNA complex. N=1.

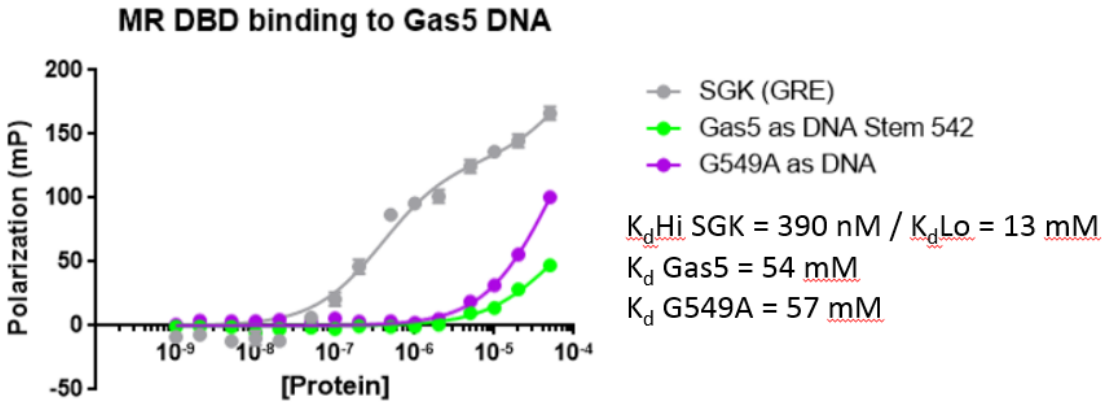



**Figure 2.6. Fluorescence polarization binding experiments with the MR DBD.** The MR DBD bound Gas5 RNA almost as well at the GR DBD, but it shows little affinity for the DNA versions of Gas5. SGK is fit with a two-site binding equation showing a loss of cooperativity in binding. Saturation was not reached for Gas5 and G549A, it is hard to accurately determine their binding values. N=1.

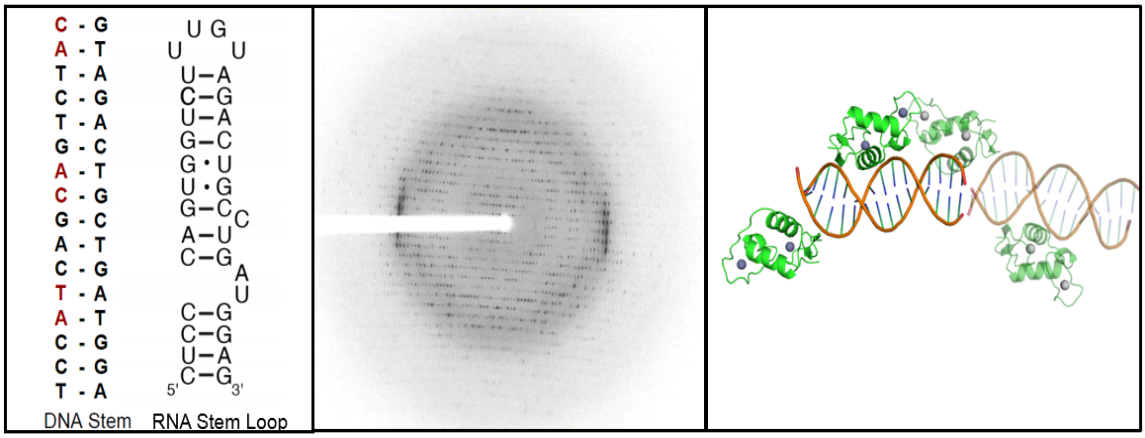



**Figure 2.7. Crystal Structure of the GR DBD and G549A DNA.** G549A DNA stem crystallized with the GR DBD. Crystals were grown in 0.1M Mes 6.5, 5% PEG 3K, and 5% glycerol. Data was collected to 3.1 Å. The asymmetric unit contained two copies of the GR DBD and one copy of the G549A DNA stem.

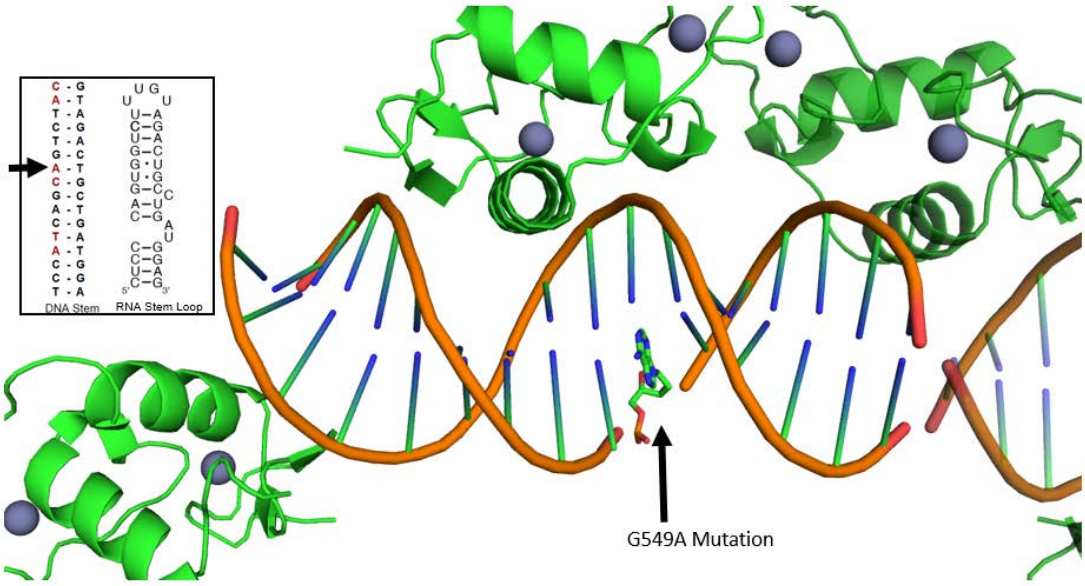



**Figure 2.8. Crystal structure of the GR DBD and G549A mutation as DNA**. Crystal diffracted to 3.1 angstroms. Molecular replacement and subsequent structure determination revealed that the G459A mutation does not interact with the GR DBD.

Gas5 DNA Stem
0.1M HEPES 7.5
25% PEG 3K

Gas5 DNA Stem
0.2M Calcium Acetate
20% PEG 3350

Gas5 Bottom Wobble
0.1M Sodium Acetate 4.6
25% PEG 550 MME

**Figure 2.9. Additional DNA Stem Constructs show Small Crystals in Screening Trays.** Other DNA constructs (the non-mutated stem and the bottom wobble) show hits in 96 well plates. Protein is at 250 uM, DNA at 333 uM.

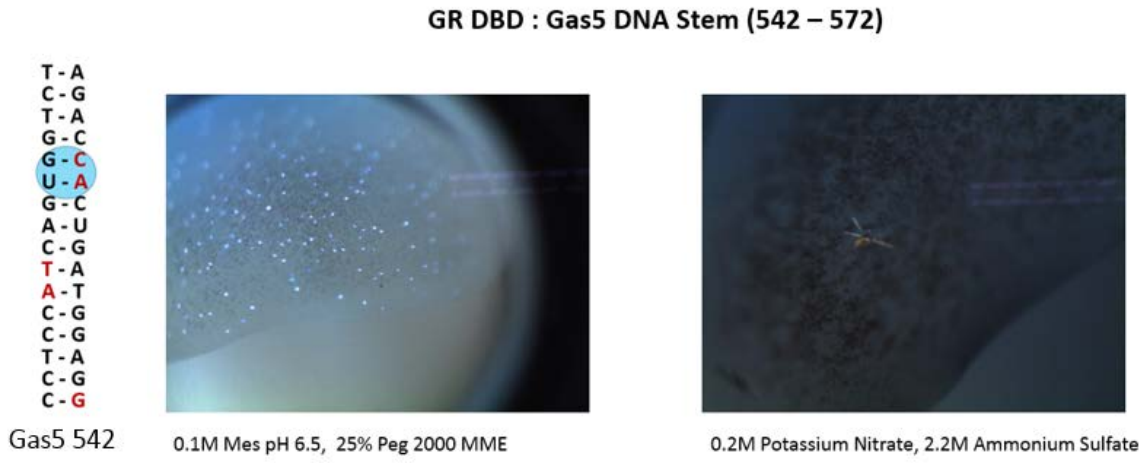



**Figure 2.10. GR DBD complexed with Gas5 stem 542.** The Peg Condition is more promising, the ammonium sulfate condition could very easily be salt.

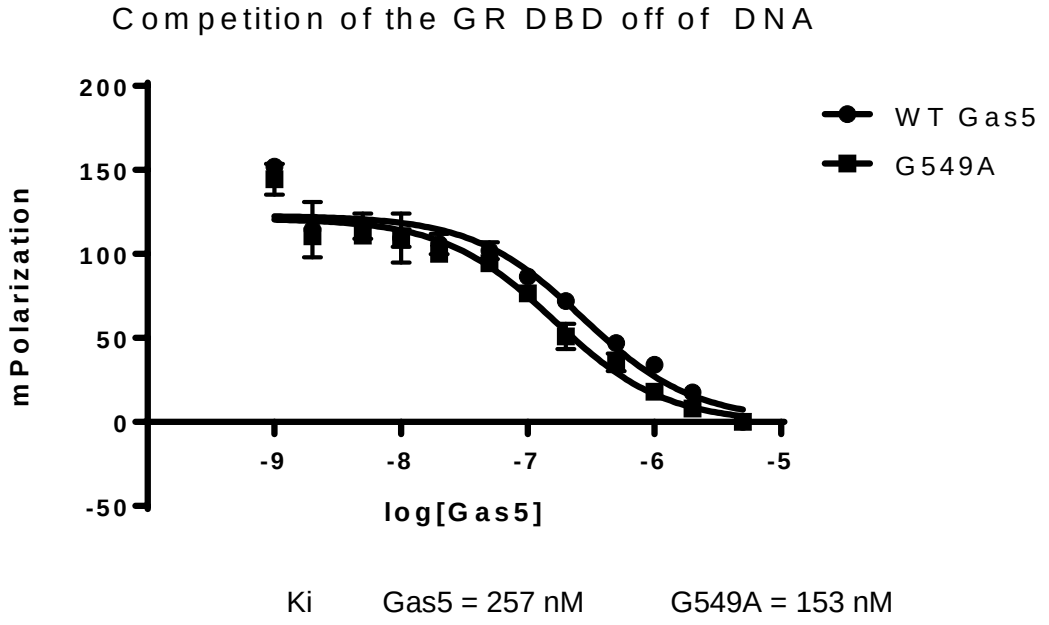



**Figure 2.11. Competition of the GR DBD off of DNA with full length Gas5 and G549A RNA.** Although G549A GREM stems do not bind to the GR DBD full length G549A is just as effective at competing the GR off of DNA. Data collected by Will Hudson, unpublished.

**Table 2.1.** Gas5 Stem-Loop Constructs from the GR-DBD Binding Region

| **IVT Constructs (DNA)** | 5’ Sequence 3’ |
|---|---|
| 50 nt | GATCCTCAGCCTCCCAGTGGTCTTTGTAGACTGCCTGATGGAGTCTCATG |
| 34 nt | GGCCTCCCAGTGGTCTTTGTAGACTGCCTGATGGA |
| **Ordered RNA** | |
| Human | CUCCCAGUGGUCUUUGUAGACUGCCUGAUGGAG |
| Marmoset | CUCCCAGUGGCCUUUGUAGACAGCCUGAUAUAGAA |

**Table 2.2. The GR DBD and G549A as DNA Crystal Structure Statistics.**

| | |
|---|---|
| **Data Collection** | |
| Resolution range | 37.15 - 3.123 (3.235 - 3.123) |
| Space group | P 21 21 21 |
| Unit cell | 39.7, 94.2, 105.0 |
| Cell Angles | 90.0, 90.0, 90.0 |
| Unique reflections | 7388 (674) |
| Completeness (%) | 99.8 (99.3) |
| Mean I/sigma(I) | 9.9 (2.1) |
| Wilson B-factor | 75.11 |
| Rpim | 0.12(0.41) |
| CC1/2 | 0.729 |
| **Refinement** | |
| R-work | 0.21 (0.30) |
| R-free | 0.25 (0.37) |
| RMS(bonds) | 0.003 |
| RMS(angles) | 0.49 |
| Ramachandran | |
| favored (%) | 96 |
| allowed (%) | 3.6 |
| outliers (%) | 0.72 |
| Rotamer outliers (%) | 0.83 |
| Average B-factor | 70.9 |
| Macromolecules | 71.0 |
| DNA | 47.4 |

## Materials and Methods

*Purification of the DNA Binding Domains of the Glucocorticoid Receptor (GR-DBD)*

GR-DBD (6x His tagged) was purified for crystallization as previously described[1] briefly: The GR-DBD containing pUC57 plasmid was provided and used to transform and plate pLysS cells. A 1 µl aliquot of DNA was rested on thawed cells for thirty minutes, cells were heat shocked at 42°C for one minute, and allowed to rest for five minutes on ice. All of the cells were plated on LB (Lysogeny Broth) plates and grown overnight at 37°C. Both 100 µg/mL ampicillin and 100 µg/mL chloramphenicol were used in all the growing steps for GR-DBD containing cells. Two 100 mL LB liquid cultures were inoculated with one colony each from the GR-DBD LB-agar plate and placed in a shaking incubator overnight at 37°C. These starter cultures were pooled and 15 mL was used to inoculate 1.3 L of Terrific Broth, 6 total flasks of TB. The cultures shook at 37°C until an OD600 of 0.6-0.8 was reached and then expression of GR was induced by addition of IPTG to a final concentration of 0.3 mM. The cultures grew for another four hours at 30°C, were centrifuged at 3000 xg for 15 minutes, and the pellet was collected and frozen at -80°C for later purification.

Cells were resuspended in Buffer A (1 M NaCl, 20 mM Tris pH 7.4, 25 mM Imidazole, 5% glycerol) with 1:1000 phenylmethanesulfonylfluoride (PMSF, protease inhibitor), a “sprinkle” of lysozyme, and DNase. Cells were lysed by sonication and then centrifuged at 18,000 x g for one hour. The supernatant was applied to a 5 mL His-Trap column with a 3-step protocol (5% B, 50% B, 100% B) (Buffer B :1 M NaCl, 20 mM Tris pH 7.4, 250 mM Imidazole, 5% glycerol). The GR protein containing fractions were pooled, concentrated, and applied to s200 superdex size exclusion column (GE Life Sciences). The buffer used contained 100 mM NaCl, 20 mM Tris 7.4, and 5% glycerol. SDS-PAGE was used to confirm the correct size for the GR-DBD. The GR-DBD is approximately 13 kDa. The protein was concentrated to 3-6 mg/mL and flash frozen for storage at -80°C for eventual use in crystallization.

*Fluorescence Polarization*

DNA and steroid hormone receptor binding was measured by fluorescence polarization as previously described[13]. Briefly: indicated amounts of steroid hormone receptor DBDs were added to wells containing 10 nM indicated 5' FAM labelled DNA ordered from IDT. Reactions were performed in 100mM NaCl, 20 mM Tris-HCl, pH 7.4, 5% glycerol and measured with a Biotek Synergy Plate Reader (BioTek) at an excitation/emission wavelength of 485/528. FP data was analyzed and graphed using GraphPad Prism 6 (Graphpad Software Inc). Sequences used in binding can be found in Figure 2.3. Each experiment is a minimum of three internal replicates with total number of experiments indicated within the figure.

*In Vitro Transcription*

In vitro transcription (IVT) was performed as described in Recombinant and In Vitro RNA Synthesis.[3] The most successful IVTs used 2 mM rNTPs in the transcription reaction. IVTs and order RNA was purified as follows: The RNA was diluted to 100 nM and mixed with an equal volume amount of a loading and denaturing dye (80% formamide, 10% glycerol, 10% TE buffer (10 mM TrisCl pH 8.0, 1 mM EDTA), bromophenol blue), denatured at 70 °C for five minutes, and then run on a 50% Urea 15% Acrylamide denaturing gel. The RNA was visualized by UV shadowing and cut from the gel. The gel was sliced into small pieces and left to tilt and soak in a 0.3 M NaAOc, 1% SDS, and 1 mM EDTA solution overnight at 4 °C. The solution was pipetted into a fresh tube and precipitated with three volumes of cold 100% 190 proof ethanol overnight and then spun at 3000 x g for 10 minutes. The pellet was washed with 70% cold Ethanol and spun again. The pellet was then left to dry before resuspension in TE buffer.

*Crystallization*

Ordered and synthesized nucleic acid constructs (IDT) were annealed by placing in a 90°C water bath and allowing the water to cool back to room temperature, approximately 5 hours. GR-DBD and nucleic acid constructs were mixed in a 2:1 molar ratio to ensure an excess of RNA and set in three sitting drop crystallization screens: JCSG+. PEGs. and Nucleix

(QIAGEN). Crystals were screened with conditions showing micro crystals optimized. Crystals were grown in 0.1M Mes 6.5, 5% PEG 3K, and 5% glycerol. Crystals were soaked in a cryoprotecting solution of 12.5% PEG3K and 40% Glycerol. Data were collected at the Advanced Photon Source BM-22 at a wavelength of 1.00 Å, and were integrated and indexed in HKL-2000. Structures were phased using molecular replacement based on previous GR: DNA structures (PDB 5CC1) as described in previous work[1] using COOT and Phenix.[4,5]

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## Chapter 3: Impact of Gas5 on the Glucocorticoid Receptor Transcriptional Activity

### Introduction

*The GR Could Impact Apoptosis through LncRNA Gas5 Interactions*

The Glucocorticoid Receptor (GR) has several transcriptional activities that are involved in apoptotic pathways. The GR can affect apoptosis by upregulating the transcription of cIAP2, an apoptosis inhibitor, and repressing NFκB pro-inflammatory cytokines.[1,2] The long non-coding RNA Growth Arrest Specific 5 (Gas5) functions as a tumor suppressor in a variety of cancer types, [3, 4, 5, 6, 7] and this could be through GR-mediated activities. Studying the effects of Gas5 on the GR's transcriptional activity will provide insight into how Gas5 controls apoptosis and proliferation.

*The GR Controls Transcription through DNA binding dependent and independent mechanisms.*

The GR affects apoptosis through activation and repression of different transcriptional targets. Following cortisol binding in the cytosol, GR translocates to the nucleus and activates transcription by directly binding DNA.[8] GR-mediated repression can be DNA binding dependent or GR can tether to other proteins to impact their transcriptional activity in a non-DNA binding dependent manner. [9] Nuclear factor kappa B (NFκB) and Activating Protein 1 (AP-1), both key co-inflammatory transcription factors, are potently suppressed by GR.[9]

*Gas5 is a Potential RNA Decoy*

The interaction between the GR and Gas5 is specific and can be ablated. The Glucocorticoid Response Element Mimic (GREM) is a non-canonical binding site on Gas5, however binding and activity can be ablated under certain conditions with a point mutant G549A.[10] Recent data indicates that DNA versions of the GREM (labelled HREM in Figure 3.1) is sufficient to induce apoptosis and limit proliferation to the same extent as full length Gas5 RNA.[4] These data indicate that Gas5 potentially functions as a decoy for canonical GR recognition sequences to impact the transcriptional activity of the GR. This project explores the

impact of Gas5 on transcriptional activation and repression by the GR in the presence of Gas5 and the G549A mutation.

## Results

*Overexpression of Gas5 is Inconsistant*

Gas5 was ovexpressed by Rapamycin, Dexamethasone treatment, serum starvation and by transfection (Figure 3.2). Overexpression is inconsistent by all methods with high error bars, however transfection appeared to be the most effective.

*Gas5 Overexpression does not Directly Impact GR Transcriptional Activation in HeLa Cells by qRT-PCR*

HeLa cells were transfected with Gas5 and G549A expressing plasmids and either treated with 100 nM dexamethasone or no drug treatment. Steady-state RNA levels were analyzed by qRT-PCR analysis of four known GR targets (Figure 3.3) with no significant impact of Gas5 or G549A to inhibit GR transcriptional activation.

*Gas5 Blocks Transcriptional Activation by GR in Luciferase Assays, but not all Repression*

Luciferase assays indicate that in HeLa cells, Gas5 represses the GR's ability to activate while G549A has no significant effect on GR transcriptional activation (Figure 3.4). Gas5 (G549A not tested) shows the same effect in A549 cells (Figure 3.5). Gas5 inhibits the ability of the GR to repress the IL-6 promoter, G549A has no significant impact on the GR transcriptional repression (Figure 3.6). Activation and repression was tested in CV1 cells (Figure 3.7) with inconclusive results.

## Discussion

### *Gas5 Can Act as a DNA Decoy*

Gas5's ability to directly block the GR's transcriptional activation and repression on the IL-6 promoter with effect ablation by the point mutation G549A indicate that Gas5 likely functions as a DNA decoy for the GR in luciferase assay conditions. Inconclusive results with repression and additional cell lines is potentially due to inconsistent transfection and expression efficiency. These experiments need to be repeated with qRT-PCR to control for variable Gas5/G549A transfection and expression efficiency. Real-time PCR analysis of common GR transcriptional activation targets indicate that Gas5 may not interact as strongly with GR as expected in actual biological environments. Transfection and expression efficiency of examined constructs was not examined, this experiment needs to be repeated and controlled for equal Gas5 and G549A expression across analyzed samples.

**Figures and Tables**

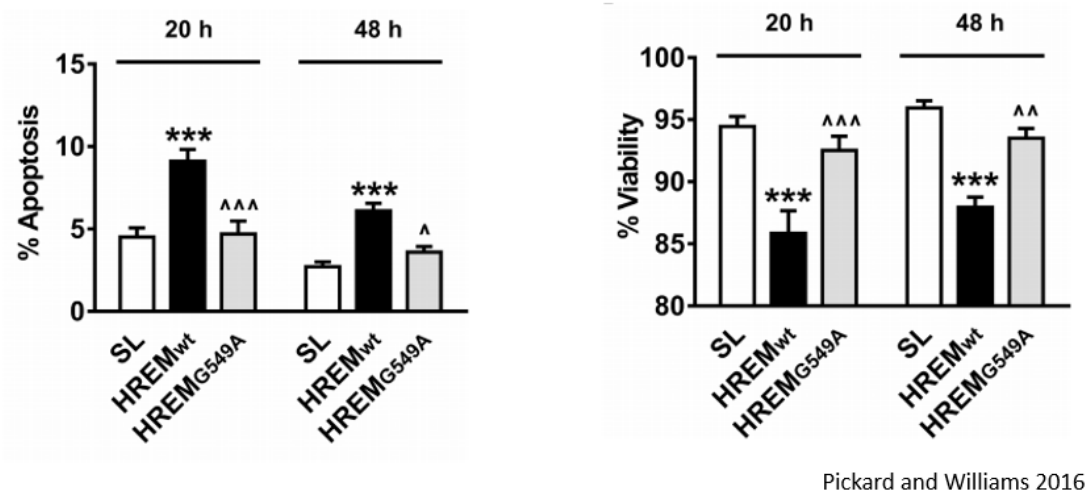


**Figure 3.1. Gas5 as DNA has the Same Effect as Full Length Gas5 RNA.** Gas5 hormone response element (HREM) sequence as DNA is as effective as full length RNA in increasing apoptosis and decreasing cell viability. Cells: MCF7s. G549A mutation does not bind GR and is included as a negative control.[11]

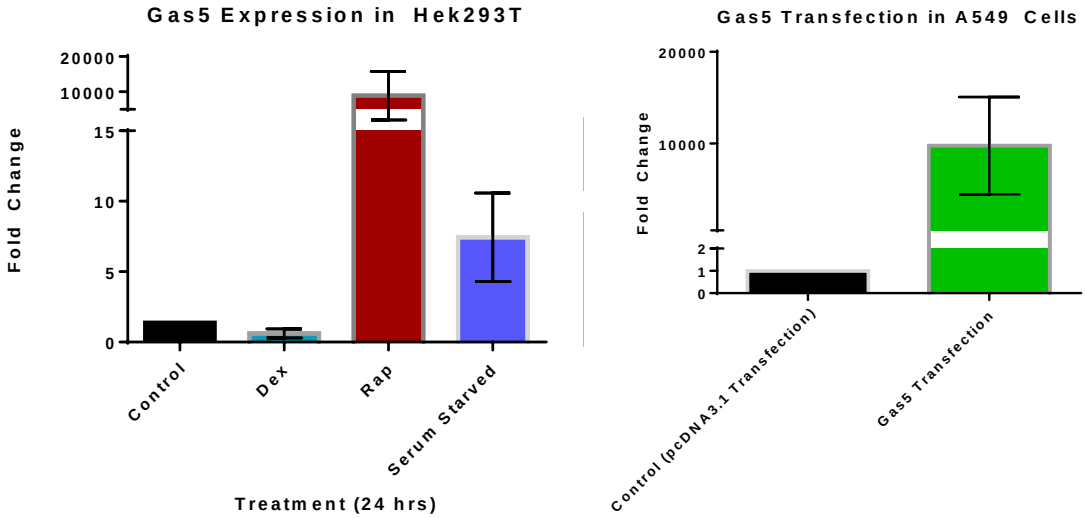


**Figure 3.2. Overexpression of Gas5 is inconsistent.** Cells were treated with dexamethasone, rapamycin, serum starvation, or Gas5 transfection. Dexamethasone, rapamycin, serum starvation, and transfection showed inconsistent results for increasing Gas5 expression. Cells were treated at 80-90% confluency. Overexpression may be easier to see if cells were treated in an earlier stage of growth.

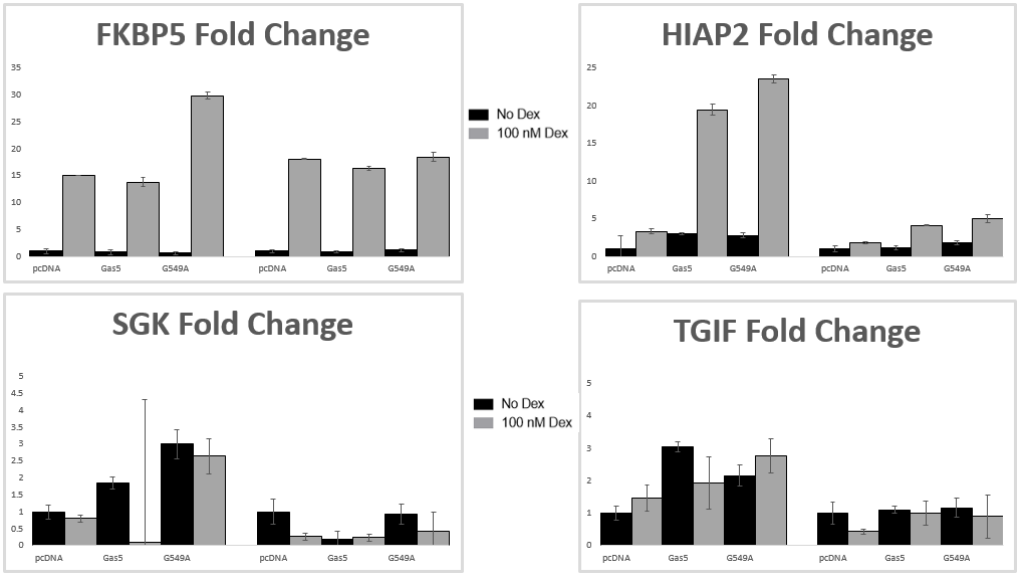


**Figure 3.3. Gas5 and G549A have no inhibitory effect of the GR transcriptional activation in HeLa cells.** RT qPCR of HeLa cells transfected with pcDNA, Gas5, or G549A and treated with no dex/100 nM dexamethasone. Data shown represents two experiments with two technical replicates each, preformed one week apart. The first week is the first set of bars on the left of the graph, the second week is shown in the bars on the right side of the graph.

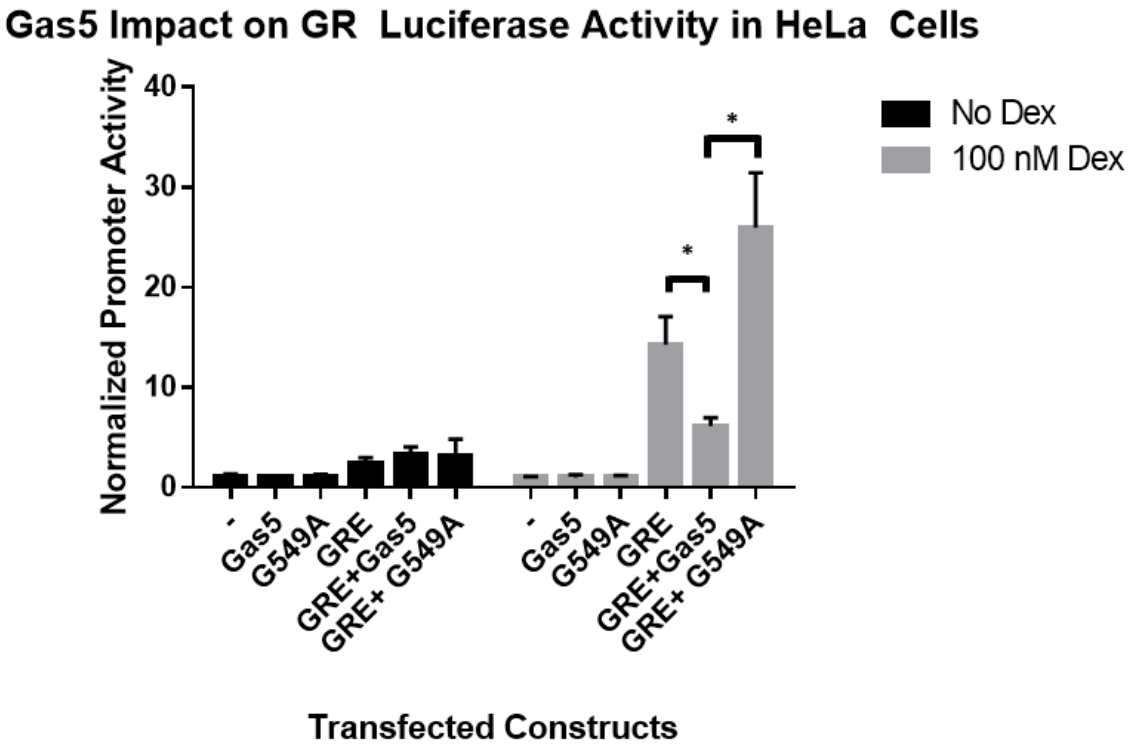


**Figure 3.4. Gas5 inhibits GR transcriptional activation in HeLa cells, G549A does not**. Luciferase assays in HeLa cells show that Gas5 inhibits GR activation at a canonical recognition site. The G549A mutation does not inhibit GR. GRE reporter plasmid was transfected at 10 ng/well. Gas5 and G549A plasmids were transfected at 50 ng/well.

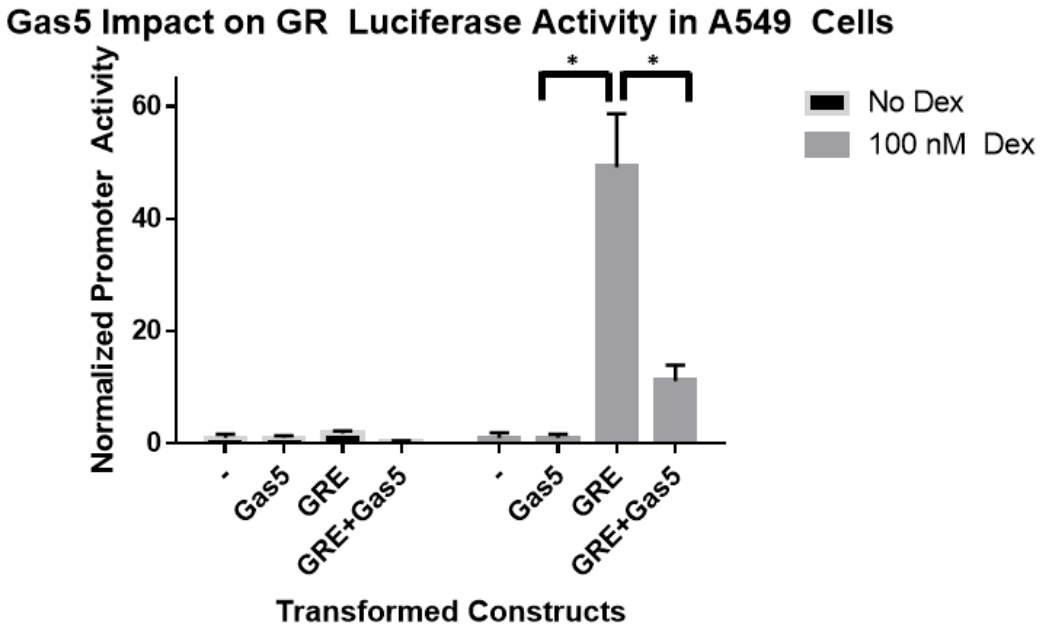


**Figure 3.5. Gas5 blocks transcriptional activation of the GR in A549 cells.** Gas5 can be successfully transfected into A549 cells. Gas5 inhibits GR activation at a canonical GR recognition element as observed in a luciferase assay. GRE reporter plasmid was transfected at 10 ng/well. Gas5 plasmid was transfected at 50 ng/well.

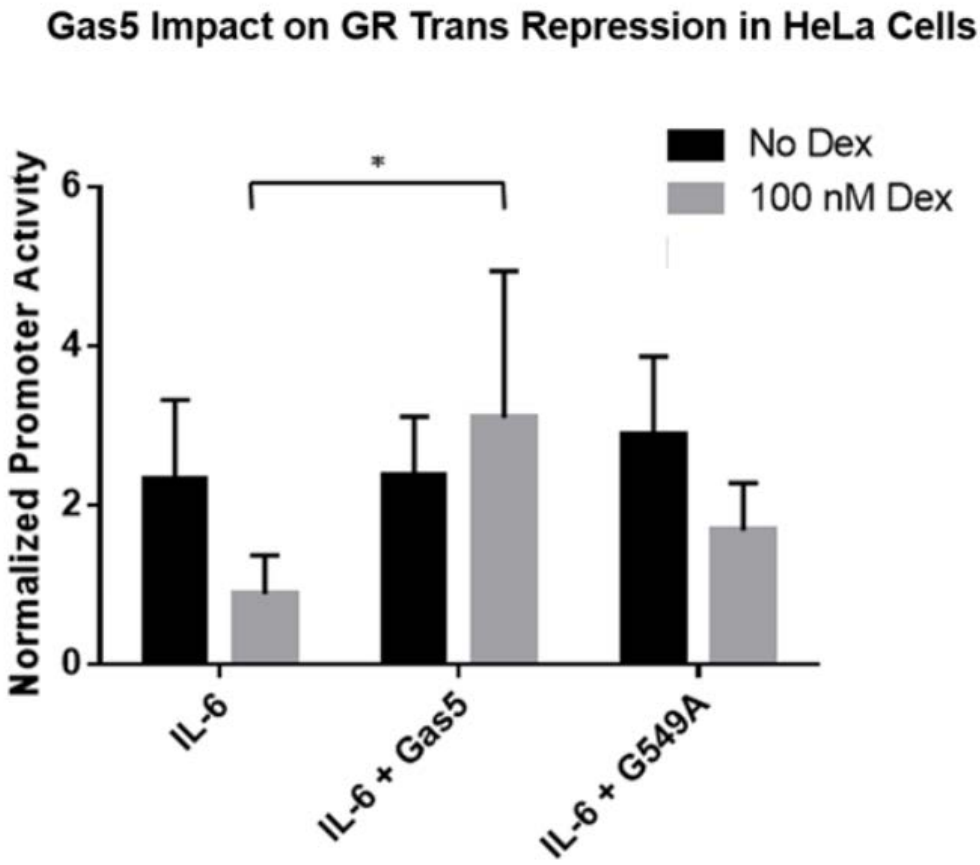


**Figure 3.6. Gas5 inhibits GR at the IL-6 promoter.** GR represses the IL-6 promoter, in the presence of Gas5, IL-6 is no longer repressed. There is no significant difference between promoter activity of either IL-6 alone or IL-6 and Gas5 in comparison to the G549A mutation.

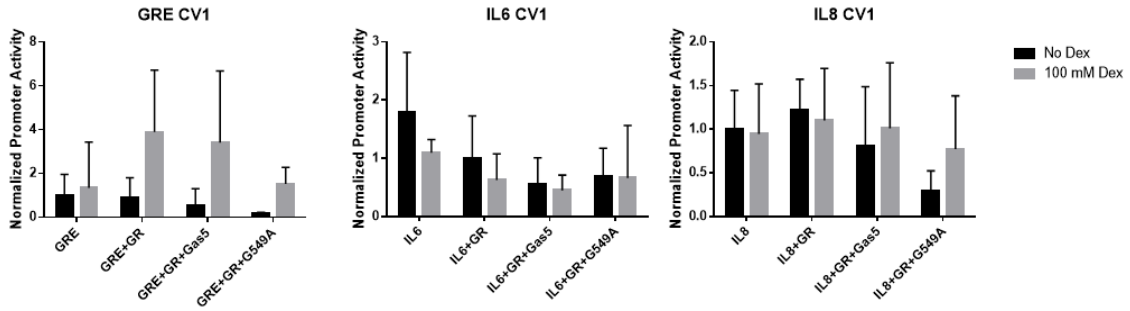


**Figure 3.7. Gas5 shows no significant effect on GR activity in CV1 cells.** Gas5 showed no effect on GR mediated activation. IL6 and IL8 have unreliable controls. The IL+GR experiment should show repression of signal. It is possible that more GR (or less IL) needs to be transfected in order to see an effect. Reporter plasmid was transfected at 50 ng/well. Gas5 and G549A plasmids were transfected at 50 ng/well. GR expressing plasmid was transfected at 20 ng/well.

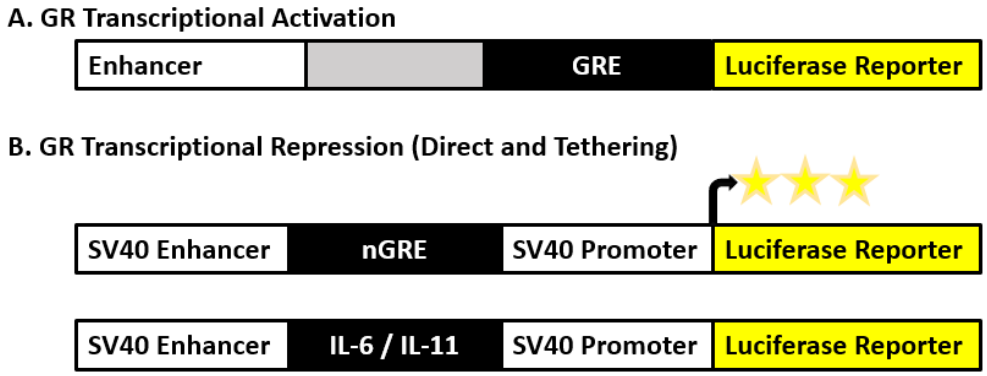


**Figure 3.8. Schematic of luciferase constructs available in the lab.** The GRE promoter is an artificial 6x repeat of a canonical Glucocorticoid Response Element. Repression constructs have a constitutively active promoter, loss of signal indicates GR repression.

**Table 3.1. qRT-PCR Primers**

| | |
|---|---|
| FKBP5 Forward | 5’ - AGGCTGCAAGACTGCAGAT |
| FKBP5 Reverse | 5’ - CTTGCCCATTGCTTTATTGG |
| HIAP2 Forward | 5’ - GACAGGAGTTCATCCGTCAAG |
| HIAP2 Reverse | 5’ - TTCCACGGCAGCATTAATC |
| SGK Forward | 5’ - CTATGCATGCAAACACCCTG |
| SGK Reverse | 5’ - GCCAAGGTTGATTTGCTGAG |
| TGIF Forward | 5’ - AGGATGAGGACAGCATGGAC |
| TGIF Reverse | 5’ - TCTGCACAGACTCCTTGGG |
| 18S Forward | 5’ - GTAACCCGTTGAACCCCATT |
| 18S Reverse | 5’ - CCATCCAATCGGTAGTAGCG |

## Materials and Methods

### *Cell Culture and Transfection*

All cells were cultured in DMEM supplemented with 1x Pen/Strep and 10% FBSS. Cells were passaged when they reached 85-95% confluence, approximately every 4-5 days. Cells were transfected in OPTI-MEM with FuGene with a 2:1 uL Fugene: uG DNA ratio. Renilla expressing plasmids were transfected at 5ng plasmid/well for all luciferase experiments. Total reporter and other plasmid amounts are indicated in figure legends. Total DNA across experiments was normalized to the same amount per well with empty pcDNA3.1+ plasmid (the destination vector for all constructs used). Drug treatment was delivered in indicated amounts in the above mentioned DMEM mixture.

### *qRT-PCR Assays*

Real time PCR analysis was performed as previously described[10] using primers shown in Table 3.1. Gas5 and GR responsive genes steady-state levels are expressed relative to 18S RNA steady-state levels.

### *Luciferase Assays*

Luciferase constructs are shown in Figure 3.8. Constructs were transfected as described above when cells reached 80% confluency. Transfection media was left for 24 hours, then removed and replaced with drug containing media for an additional 24 hours. Cells were then lysed and sssays were performed with the Duel-Glo kit and protocol by Promega and measured with a Biotek Synergy plate-reader. A549 cells required twice the lysis time as HeLa cells. Data was analyzed in GraphPad Prism 6 (Graphpad Software, Inc.).

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## Chapter 4: Investigating other lncRNA Gas5 Interaction Partners

### Introduction

*Gas5 Controls Apoptosis and Proliferation*

While the exact mechanisms of Gas5 are unknown, it has been shown to bind several proteins including the Glucocorticoid Receptor (GR),[1] YBX1 in stomach cancer cell lines[2], and PKR (unpublished data from the Conn lab). While Gas5 has been studied in various cancer types, there is no current study that examines the overall protein Interactome for Gas5.

*Identifying the Gas5 Interactome will Shed Light on Gas5*

Gas5 has multiple interaction partners that control tumorigenesis in various cell types. Preliminary pull downs from HEK293t whole cell lysates with biotinylated Gas5 reveal over 70 potential Gas5 binding proteins. A Gene Ontology (GO) analysis[3,4] implicates Gas5:protein interactions in pathways such as apoptosis, biological regulation, and metabolic processes (Figure 4.1). Several of the identified proteins are transcription factors.

A technique called RNA Antisense Purification coupled with Mass Spectrometry (RAP-MS) can be used to identify endogenous Gas5 interacting proteins. A representative schematic of RAP-MS is shown in Figure 4.2.[5] Briefly, cells will be treated to crosslink protein and nucleic acid interactions. Cell lysates are then denatured with single stranded probes complimentary to Gas5 used to pull down Gas5 and any crosslinked proteins. These will be identified with using mass spectrometry. Ultimately, the proteins identified in these experiments will help identify what pathways and interactions are potentially important for Gas5 function. This will further our understanding of Gas5 mechanisms and provide insight into new proteins for future study.

**Proposed Methodology**

This experiment is designed based on a previously established method[13]. Probe generation and cell culture and crosslinking have been completed. Mass spectrometry analysis (as described[6]) still needs to be performed.

Probe Generation

Two sets of probes were designed to cover the entire Gas5 sequence and one set was designed with random sequences. These probes are predicted to not interact with other RNAs, they are 120 nt long when fully synthesized. Each set of probes has their own unique tags: primers corresponding to these tags are used to amplify the specific probes from a general pool of oligos. After amplifying from the oligo pool, each set of probes is amplified with a T7 polymerase sequence added (Figure 4.3). RNA is *in vitro* transcribed from these sequences as previously described[7] (Figure 4.4). Biotinylated primers are used to reverse transcribe DNA from the RNA template. The RNA is digested and single stranded biotinylated probes remain.

Cell Culture and Crosslinking

JURKAT cells are being used in collaborator experiments with Gas5. These are suspension cells that can be easily grown in large quantities. Cells are maintained at $1x10^5$ – $3x10^6$ cells/mL in RPMI (with 10% FBS and 1X Pen/Strep) and diluted to $1x10^5$ cells every 3-4 days. Crosslinking was performed with 16% Formaldehyde and 2.5M Glycine stock solutions. Cells were spun down in 60 million cell aliquots at 125xg for 5 minutes. Pellets were washed twice with room temperature PBS and spun down at 125xg for 5 minutes after each wash. Cells were resuspended in 15 mL PBS with 1 mL of formaldehyde added (for a 1% concentration). Cells were left to crosslink for 5/10/15 minutes. Crosslinking was quenched by the addition of 0.8mL glycine for 5 minutes at room temperature. Pellets were washed twice with room temperature PBS and spun down at 125xg for 5 minutes after each wash. The cell pellets were resuspended in PBS to a total volume of 3 mL, flash frozen, and stored in 1 mL aliquots at -80 C until ready for use.

## Discussion

*The Gas5 Interactome will give Insight into the Pathways of Gas5 Involvement*

The successful completion of RAP-MS experiments will assist in understanding Gas5 mechanisms and provide insight into new interactions that can be examined with Gas5 and other proteins. Identified proteins will be potential targets for Gas5 and protein crystal structures, binding experiments, and transcription assays. These will elucidate lncRNA biology and the mechanisms through which Gas5 controls cell fate.

There are several potential issues with the proposed method. RAP-MS may fail to identify protein binding partners for Gas5. Biotinylated Gas5 will also be used as bait for JURKAT lysates as an alternative method to identify proteins that bind Gas5. Due to the denaturing conditions used by RAP-MS, only observe direct protein interactions will be observed; this method will not detect associated Gas5 complexes. The biotinylated Gas5 pulldowns are also a valid method for addressing the association of Gas5 with protein complexes. Although the ssDNA constructs are chosen to avoid off-target effects, Gas5 is retro transposed and these transcripts can also be detected. It is also possible that Gas5 copy number is too low to capture sufficient endogenous interactions for identification by MS. Gas5 copy number can be increased by transfection, although this will be expensive on a larger scale.

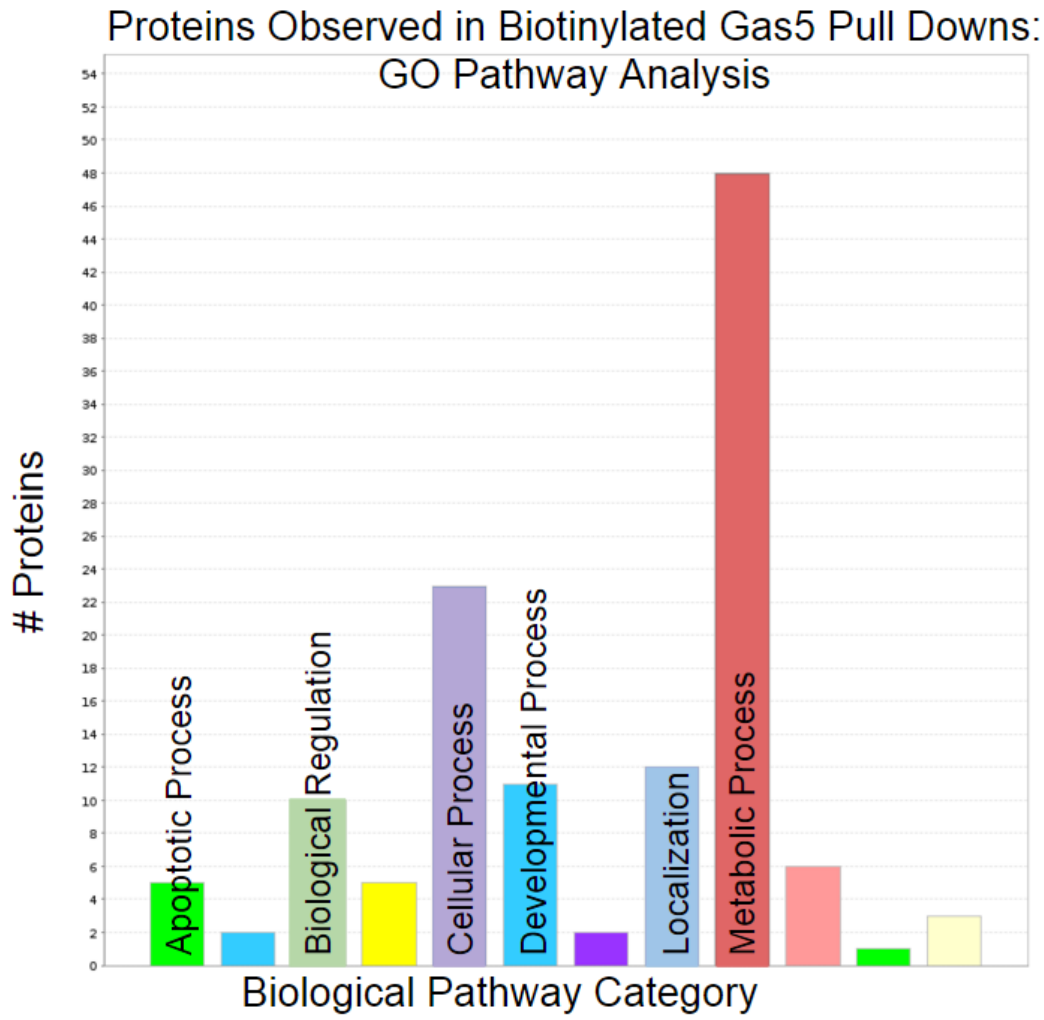


**Figure 4.1. Gene Ontology (GO) Analysis of Proteins Observed in a Biotinylated Gas5 Pull Down Experiment.** I performed a GO analysis[3,4] on 74 proteins from unpublished data in the lab generated by W. Hudson in a biotinylated Gas5 pull down from HEK293t cell lysate. Several pathways are implicated, including apoptosis, regulation, and metabolism.

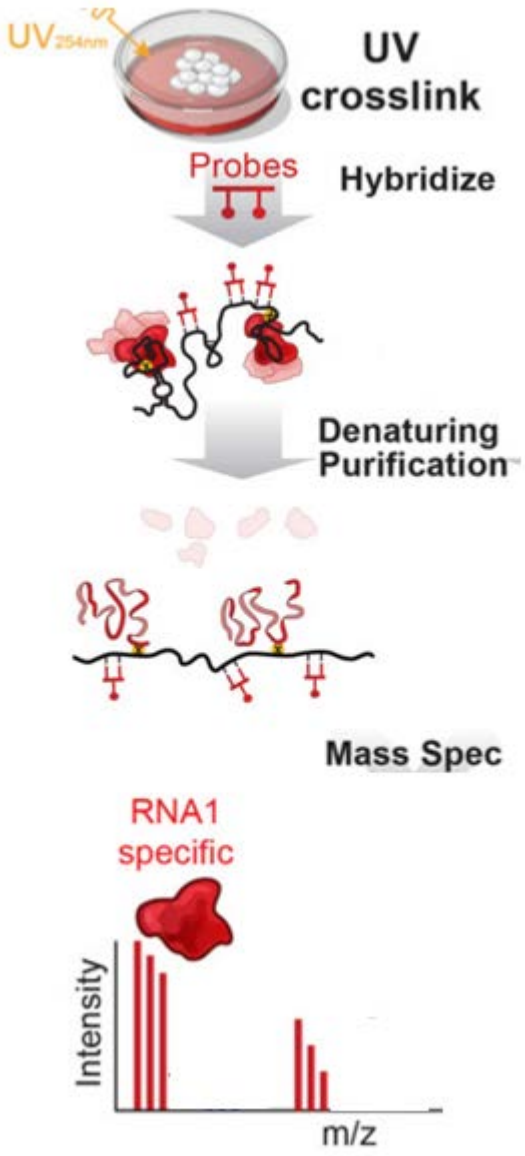


**Figure 4.2. RAP-MS Representative Schematic.** Figure adapted from an established method[5] depicting the RAP-MS experimental design. Cells are crosslinked. Lysates are denatured and single stranded complimentary probes pull out target RNA. Proteins are then identified by Mass Spectrometry.

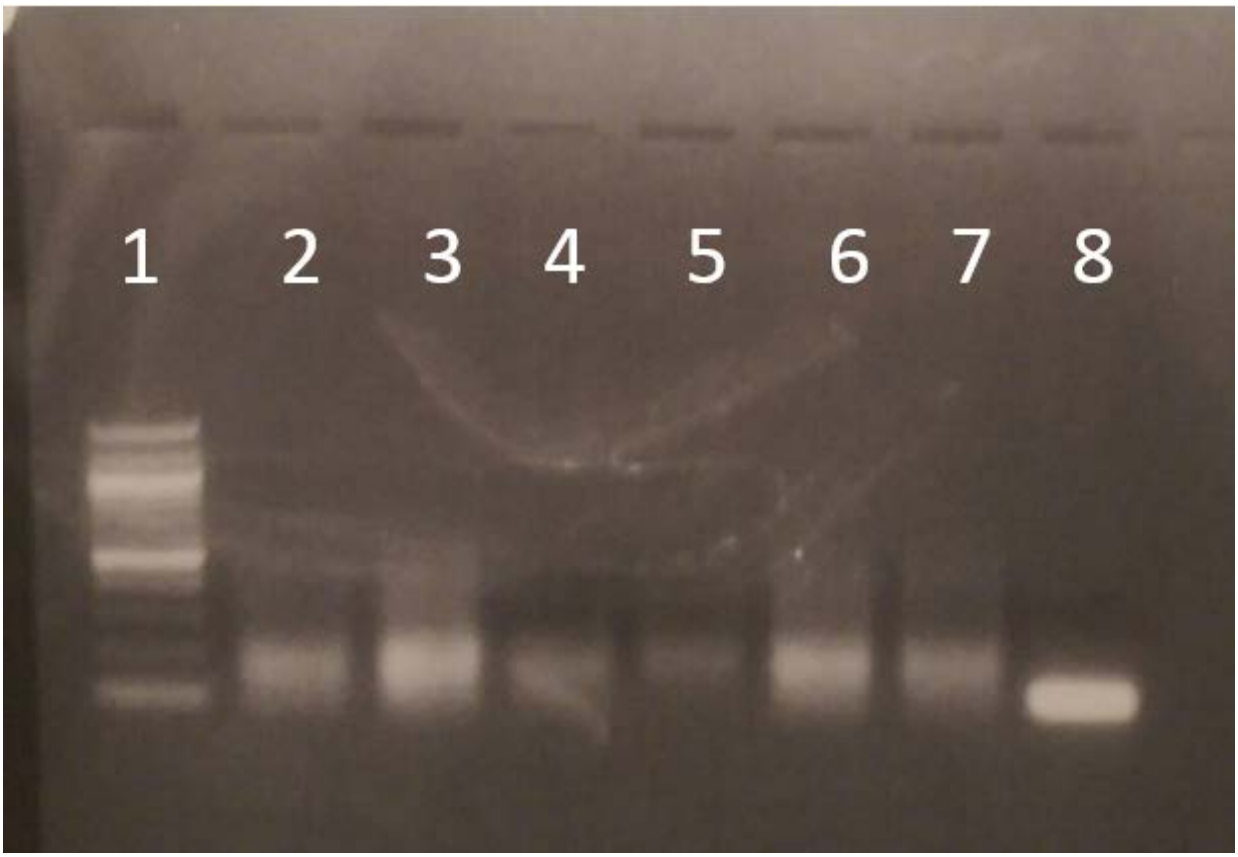


**Figure 4.3. Each probe set is amplified with a T7 polymerase sequence added.** Lane 1 is a 1000 bp ladder, probes for set A, set B, and random probes show up between the 100 and 200 markers as expected. Lanes 2-7 represent probe amplification. Lane 8 is a control PCR. Gel is 1% agarose visualized with ethidium bromide staining.


**Figure 4.4. Probe sets A and B were in vitro transcribed.** Both produced RNA, probe set A produced more RNA. Gel: 15% acrylamide 50% Urea visualized with ethidium bromide staining. F refers to forward, R to reverse.

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## Chapter 5: LncRNA Gas5 Could Control Cell Fate through the Glucocorticoid Receptor, but it isn't the Only Answer

The long non-coding RNA Gas5 represents a fascinating study into lncRNA biology, control of cell fate, and a new avenue for understanding transcriptional regulation. LncRNAs are just as complex and abundant as protein coding genes, but they are barely studied or understood. They have complex and hard to predict folding patterns, differential splicing, and incredibly varied function. Gas5's conservation is unusual for lncRNAs. Studying Gas5 was an opportunity to understand what function was so important that this ubiquitously expressed RNA was maintained throughout evolution.

The Gas5 project was initiated in the lab through interest in its involvement with the Glucocorticoid Receptor. My interest on the project grew after examining the current studies on Gas5. As discussed throughout this work, Gas5 is implicated in a wide variety of cancers. There are dozens of studies that show Gas5 downregulated in cancerous cells. The overexpression of Gas5 in these cells slowed growth, decreased cell migration, and increased cell sensitivity to apoptosis. Every cancer is varied and wildly different. While popular media loves to throw around the hope for the "cure for cancer", we know that there is no cure, not with something so complicated. Gas5 presented a potential uniting factor. It is an RNA present in all cells that shows some universal control over cell fate that could help us understand cell regulation on a new level. My projects only began to scratch the surface of that understanding, revealing that the role of this RNA is incredibly complicated.

My initial approach, as detailed in Chapter Two, was to look at the structural interaction between Gas5 and the GR. We wanted to understand how this non-canonical sequence was binding to GR – a transcription factor we expect to bind inverted repeat DNA sequences. Unfortunately, a GR and Gas5 RNA structure has yet to be obtained. I used DNA versions of Gas5 to pursue an initial structure. The idea was that these could be used for molecular

replacement of the eventual RNA structure, they were fast and cheap ways to identify a binding register, and could potentially be used as seed crystals. In an unexpected result, a point mutation that was meant to ablate binding (G549A) is the only construct that generated large enough single crystals for data collection. This structure showed zero interaction between the mutated residue and the bound protein. GR DBD also bound in an unexpected register as compared to 2D NMR data obtained with Gas5 RNA and the GR DBD. The entire structure could be an artifact. The construct is an altered Gas5 sequence as DNA and the GR DBD will bind just about anything. However, unpublished *in vitro* FP data indicates that the G549A mutant doesn't actually ablate binding to the GR. Unfortunately, there is also *in vivo* published data showing that G549A is nonfunctional while non mutated Gas5 retains function. This presents an interesting question for the future of this project. Do Gas5 and the GR interact how we expect? What factors determine if this mutation is relevant or not? And why did non mutated Gas5 as DNA versions never optimize or yield crystals?

I wanted to explore this question further in more relevant scenarios, so I moved into cells as the next part of my project (Chapter 3). Luciferase assays and qRT-PCR use full length RNA and full length GR. Luciferase assays are orchestrated interactions with high overexpression of both Gas5 and GR promoter constructs. We can see an effect of Gas5 on GR promoter activity, but that effect is lost in the qRT-PCR experiments. Variable Gas5 expression presents an issue to the validity of these studies, but it appeared that Gas5 just didn't have the strong effect on GR that we expected (hoped) for.

As with any project, we are left with more questions than answers. Ultimately, I believe Gas5 has a trivial interaction with the GR and should be studied on a macro level until the pathways and interactions are better understood. The implications of Gas5 and GR interactions are fascinating, but they are shots in the dark. My experiments forced interactions. They give no indication that Gas5 would have any actual impact on the GR in a biologically relevant scenario.

While I could have focused on the few results showing the interactions I wanted, repeat trials in the majority of my work saw little to no impact for Gas5 on the GR. Gas5 controls cell fate and the GR may be a part of that, but it is not the only answer. The proposed pull down in Chapter Four will give the first look at the global involvement of Gas5 and be a step in the right direction to understanding this novel lncRNA.

Gas5 clearly plays an important role in controlling cell fate, and that could involve the Glucocorticoid Receptor, but the GR isn't the only answer.