**Supplemental Methods and Figures**

*Murine 25 minute bilateral renal ischemia/reperfusion surgery*
All research research involving the use of mice were performed in strict accordance with protocols approved by the Animal Studies Committee of Harvard Medical School. Mouse strains include B6.Cg-Tg(Rgs4-EGFP)4Lvt/J (Jackson Laboratory Inc.), B6;129P2-Rgs4tm1Dgen/J (Jackson Laboratory Inc.), SMMHC-Cre *rgs4*$^{fl/fl}$. Adult male C57/B6 congenic controls and RGS4 transgenic (R4Tg), or SMMHC-Cre and SMMHC-Cre *rgs4*$^{fl/fl}$ mice were anesthetized with a mixture of xylazine (16 mg/ kg) and ketamine (80 mg/kg). Core temperature was continuously monitored by rectal probe and maintained between 36.5 – 37.5 C. Simultaneous bilateral renal pedicle clamping was employed using a 60G microaneurysm clip for each renal artery. First, a left flank incision was made and the kidney was exposed. A pedicle clamp was placed on the left main renal artery under direct visualization using a Zeiss OPMI 6 Surgical Microscope (12.5x optic, f=175mm) After 25 minutes of ischemia each clamp was released and removed. The incisions were closed and the animal was allowed to recover.

*10 minute unilateral ischemia/reperfusion injury*
Animals were anesthetized and maintained between 36.5 – 37.5 C. Under direct visualization hilar fascia was dissected to separate the ureter from the renal blood supply. A 60G microaneurysm clip was applied to the left renal artery for 10 minutes and released. The incisions were closed and the animal was allowed to recover. Imaging analysis proceeded 18 hours after clamp release.

*Tail-cuff blood pressure measurement*
A volume pressure recording (VPR) device (Kent Scientific Corp., Torrington, CT) was used for tail cuff blood pressure measurements of mice. Animals were acclimated to the apparatus and measured 2 days after acclimation. 10 cycles were completed per animal and averaged.

*Multiphoton Intra-vital microscopy*
Animals underwent ischemia/reperfusion injury then were secured and interrogated on a multiphoton microscope platform (Bruker Corp.). Kidney was exposed and secured and afterward imaged using a 20x objective lens positioned near the hilum where cortical and medullary macrostructures could be visualized. 100microliters of Lycopersicon esculentum lectin was injected intravenously to outline microvascular structures in the kidney. Images were generated at 1 micron intervals, compiled and aligned using Prarie View software (v4.0). Compiled images were analyzed using Volocity software v6.3 (Perkin Elmer, Inc.). A microvascular cross section was defined as an image that maintained an x-axis dimension (r1) no greater or less than 0.33 the length of the y-axis (r2) for a distance of 10 microns. Ellipse area was then calculated using the formula: $A=\pi \cdot r1 \cdot r2$

*Magnetic microbead-assisted Cytokine Immunoassay*
Adult vascular smooth muscle cells (Sciencell) were grown to 95% confluency and stimulated with Angiotensin II (0.1μM) for 12hrs. Cells were washed and lysed with NP40 lysis buffer containing pepstatin (1μg/mL), leupeptin (5μg/mL). Multi-analyte microsphere-based fluorometric assay was performed using Luminex technology (Luminex Inc.) under the

manufacturer's specifications comparing a population of Angiotensin II-exposed cells (n=8) compared to saline (vehicle)(n=8) exposed cells.

*CCL5/RANTES Immunoassay*
Samples were collected from cells or cell culture supernatants and processed according to the manufacturer's recommendations (R&D Systems Inc.). Standard curves were generated using 40, 20, 10, 5, 2.5, 1.25 pg/mL dilutions. VSMC were transfected (Lipofectamine) with human RGS4 siRNA (Life Technologies) and monitored for deletion of human RGS4 mRNA (**Suppl. Figure 3**).

*Human vascular smooth muscle cell culture*: Cells were obtained (Sciencell, Inc.) and cultured in vascular smooth muscle cell (VSMC) media (Sciencell, Inc.)

*Pre-glomerular vascular smooth muscle cell culture*: Animals were sedated and $Fe_2O_3$ nanoparticles (20nm) were injected in the arterial system. Kidney was procured and capsule removed. Tissue was minced, digested with collagenase II (Life Technologies, Inc.) at 37C, and then passed through a 70 micron filter. Dissociated arterioles were then placed in an electromagnetic field, and selected by charge. Arterioles were plated in VSMC media (Sciencell, Inc.). Monolayer cell colonies were isolated and grown to confluency. Cell type was confirmed by positive staining for a monoclonal anti-mouse alpha smooth muscle actin antibody (Abcam, Inc.). Differential expression of RGS4 protein in cells derived from transgenic RGS4 overexpressor animals and congenic controls was confirmed by immunoblot probe for monoclonal antibody against mouse-specific RGS4 (Abcam, Inc.) (**Suppl. Figure 4**).

*Pre-glomerular arteriole interrogation*: Above technique was used for arteriole isolation. Arterioles were placed in Krebs-Henseleit buffer and supplemented with 2mM calcium chloride. AngII was added directly to each condition. Short axis, perpendicular cross sections were photographed prior to, and five minutes after AngII exposure. All image capture was performed using a Nikon ECLIPSE Ti Inverted Research Microscope and processed with NIS-Elements Imaging/analysis Software.

*Angiotensin II Immunoassay:* Angiotensin II Immunoassay (Cayman Chemical Co., Ann Arbor, Michigan) was used to determine tissue angiotensin-II concentrations. Samples were snap frozen in liquid nitrogen until processed. Mouse kidneys were homogenized and prepared according to the manufacturer's recommendations. In vitro generation of Ang I and Ang II from angiotensinogen and Ang I by renin and angiotensin converting enzyme and in vitro degradation of Ang II to Ang III by angiotensinases was avoided by using the following additives at time of homogenization: o-phenanthroline 0.44 mM, EDTA 25 mM, p-hydroxy-mercuribenzoic acid 1mM and pepstatin A 0.12 mM. Sample was then passed through a phenyl column containing silica gel (60Å pore size) pre-washed with methanol. Samples were eluted with methanol, vaccum dried, and resuspended in ELISA-plate compatible buffer and exposed to immobilized monoclonal anti-Angiotensin II antibody.

*Dynamic Magnetic Resonance Imaging*
All dynamic MR images were generated in the Beth Israel Deaconess Medical Center Small Animal Imaging Facility. High relaxivity gadolinium chelate (Multihance, gadobenate dimeglumine, 529mg/mL, Bracco Diagnostics) was administered in a standard weight-based protocol throughout the study. Mice were sedated using 1.5% inhaled isoflurane throughout the imaging procedures. Contrast agent was injected in mice through a 28g flexible venous jugular

catheter (SAI Inc.) placed prior to the initiation of the scanning protocol. Mice were secured in a prone position and fitted in a bird cage-designed body coil. The 9.4-T MRI scanner (Bruker Biospin GmbH) employs an actively shielded assembly a 20-cm warm bore diameter. Data acquisition was processed using TopSpin 5.1 software (Bruker-Instruments). Anatomic imaging confirmed animal position and was immediately followed by the dynamic imaging protocol. Imaging protocol parameters included echo time, 1085ms; repetition time 2170ms; excitation pulse angle, 60; flip angle, 30; FOV 5x4 cm; matrix size 128x96; slice thickness, 1.5mm. All images were obtained in the coronal plane. When the procedure ended the mice were returned to a clean containment cage to recover. Blood flow was calculated using a local (25) rather than a global arterial input function (45).

Bayesian Analysis Software is open software available through the Washington University in St. Louis Biomedical Magnetic Resonance Laboratory. It was used to process all dynamic MR imaging. It employs Random Walk Monte Carlo methods to sample the distributed intensity vs. time plots defining the local rate change of gadolinium transit through the kidney and therefore local blood flow. Blood flow calculations relied on assumptions of gadolinium contrast transit through the vascular space as described below.

Renal blood flow is a practical calculation to understand the hemodynamic state of the kidney before, during, and after an ischemic event. It is made of component parts which contribute to the final calculation of RBF including arterial contrast in-flow rate (α), arterial contrast out-flow rate (β). Each variable was measured in arbitrary units and therefore a matched control was compulsory to interpret results.

Local arterial input function ($C_A(t)$) was based on the temporal concentration change of contrast agent inflow ($(t-t_0)^{\alpha}$) and outflow ($\exp[-\beta(t-t_0)]$) summarized as the gamma variate (GV)

$$GV(t) = (t-t_0)^{\alpha} \exp[-\beta(t-t_0)]$$

Distributed over a time interval and accompanied by a normalization constant the arterial input function can be represented in terms of the gamma variate (GV):

$$C_A(t) = aGV(t,t_0) + c$$

This multi-dimensional integral was constructed to account for the properties of contrast in-flow, out-flow, and recirculation, within single voxels of space. Posterior probabilities were derived from conditional probabilities by applying Bayesian evidential probability theory (34). Parameters were constructed based on the known properties of gadolinium contrast in the kidney (35-38). The parameters of each conditional prior probability were fit to a Gaussian distribution accounting for the properties of a bolused solution in a vascular compartment (39). 50 Markov chain Monte Carlo simulations were repeated 50 times fitting signal intensity to integral kernels over a time period required for 100 image acquisitions. Each signal intensity kernel was correlated with kernels acquired prior and afterward in time sequence to best identify a logical equilibrium distribution. This approach prevented illogical scenarios that chronologically transpose α and β or disobey known dispersion properties of gadolinium contrast in a vascular space.

Measure of contrast transit time from renal artery to renal vein in shunted renal blood flow **(Suppl. Movie 2)** was used as an internal control to characterize gadolinium contrast through the vasculature compared to urinary excretion. We then captured data from cortical and medullary regions of interest to determine intravascular contrast transit during the first 50s post-bolus interval avoid the component of urinary filtration.
If distinct change in signal intensity could not be seen between the cortex and medulla prior to contrast injection the corticomedullary junction was defined as pixel distance from outermost cortex to hilum x 0.5. Single pixel regions of interest were averaged in the cortex of the superior, mid and inferior kidney pole, and in the medulla of the superior, mid-, and inferior kidney pole. Blood flow was calculated as previously described (26) using a local arterial input function rather than a global arterial input function (40).

*Isolated kidney perfusion:* Our ex vivo kidney perfusion technique is an adaptation of the original protocol described by Stegbauer and colleagues (46) and described by our group previously (2). We modified the technique for unilateral isolation of the left kidney to reduce warm ischemia time. A luer-lock infusion port was placed in-line directly proximate to the arterial cannula. Once extracted, the kidney was maintained at a constant flow rate using a programmable peristaltic pump. Pressure was recorded digitally (2 kHz) by an in-line recording system composed of an ETH-256C amplifier and PB-100 probe (iWorx, Dover, NH), and the resulting pressure recordings were interfaced with a computer using an A/D converter (ADinstruments, Dunedin, New Zealand) and LabChart 7.1 software (ADinstruments, Dunedin, New Zealand) to control signal acquisition, data saving and off-line analysis. The kidney was maintained in a thermo-controlled chamber and infused with Krebs-Henseleit buffer at 37℃.

*PD123319 injection*
Compound PD123319 (R&D Systems, Inc.) was injected by tail vein approach using isoflurane anesthetic, 10mg/kg, prior to unilateral kidney ischemia.

*In vivo imaging of monocrystalline nanoparticles*
MION 47 (monocrystalline iron oxide nanoparticle) (Center for Molecular Imaging Research, Massachusetts General Hospital, Harvard Medical School) was injected in mice through a 28g flexible venous jugular catheter (SAI Inc.) immediately before kidney ischemia. An MR imaging protocol (Rapid Acquisition with Relaxation Enhancement with variable repetition time [RAREVTR]) was performed 18 hours after injury and incorporated the following parameters: echo time 12.6, 37.7, 62.9, 88.1, 113.2, 138.4, 163.5, 188.7ms; FOV 5x4cm; matrix size, 256 x 192 pixels; slice thickness, 1mm; contiguous images, 3. MRI Mapper software (Beth Israel Deaconess Medical Center) was used to calculate T2 using the least squares method.

*Protein analysis*
Cytosolic protein lysates from dissected kidney or cell lyaste were prepared for immunoblot analysis using Triton X lysis buffer including pepstatin (1µg/mL), leupeptin (5µg/mL), and MG-132 (10µM) as preservatives. PVDF membranes were extensively washed in TBS/T then incubated with primary antibody, monoclonal anti-RGS4 antibody (1:400; Abcam ), Calponin-1 (1:200, Millipore), Thrombomodulin (1:200, Abcam). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Amersham, a GE Healthcare). Bands were visualized

by use of the ECL system (Amersham). Scanned blot densitometry was performed with Image J (v1.24) software.

*Tissue preparation histology, and analyses*

For renal histology 8 micron thick paraffin kidney sections were routinely stained with hematoxylin and eosin. X-gal staining involved sectioning tissue blocks frozen in Optimal Cutting Tissue compound (Torrance, CA). Tubular injury was assessed in at least 10 nonoverlapping fields using a 20x objective. Frozen sections were stained with monoclonal anti-RGS4 antibody (1:400; Abcam ), SMMHC (1:150,Abcam), VCAM (1:100,Abcam), ED-1 (1:100 Abcam), Calponin-1 (1:200, Millipore), Thrombomodulin (1:150, Abcam). Vascular smooth muscle cell-endothelial cell co-culture was prepared for immunohistochemistry with 8% paraformaldehyde (PFA) fixation. Cells were then blocked with 5% bovine serum albumin (BSA) in PBS for one hour. Monoclonal anti-RGS4 antibody (1:400; Abcam) and anti-cleaved caspase 3 (1:200) primary antibody were diluted in 5% BSA and applied to cells for 6 hours at 4℃. After washing the primary antibody, cells were incubated in α-hamster Cy3 (1:100; Abcam) and α-rabbit FITC (1:200) diluted in 5% BSA for one hour on ice. Cells were washed once more and then mounted with Vectashield and 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). The procedure was also done separately with PECAM (1:100; SantaCruz) and SMA (1:75) as primary antibody and α-Goat Cy3 (1:200; Jackson ImmunoResearch) with reapplied SMA (1:75) as secondary antibody.

To calculate a tubular injury score kidney sections were processed after 24hrs of reperfusion and were evaluated for tubule flattening, necrosis, apoptosis, or presence of casts as a percentage of total number of tubular epithelial cells per high power field.

RGS4 signal intensity was quantified in histologic sections using ImageJ software to outline non-overlapping pixel areas surrounding the peritubular capillaries in cross-section and excluding the apical membrane and urinary space of the tubular epithelial cells. Thrombomodulin and VCAM expression were quantified by singal intensity per pixel area. Signal intensity was normalized to baseline values in wild type control mice undergoing sham surgeries, (519nm λ signal – background signal)/(pixel area). Calponin-1 + cells in the peritubular capillary (PTC) space were identified if >75% of the capillary lumen stained positive for Cy3 fluorescence in the presence a DAPI+ cell embedded in the wall of the PTC. 570nm λ signal intensity was normalized to pixel area as above.

*Flow Cytometry*

Cells were collected and digested in collagenase in a 37˚C water bath for 30 minutes. 2.5 ml of cold EC medium was added for every 5 ml of collagenase solution in the tissue suspension. Cells were then centrifuged and resuspended in 0.01% paraformaldehyde for 15 minutes. Following fixation, cells were centrifuged and resuspended in 1% Triton-X, 0.5% Tween 20, and 0.5% BSA in PBS for 10 minutes. Primary antibodies were then added and cells were incubated for 30 minutes in room temperature, followed by an additional 30 minutes on ice. After primary antibody incubation, cells were rinsed and resuspended in 1% Triton-X, 0.5% Tween 20, and 0.5% BSA in PBS. Secondary antibodies were added and incubated at room temperature for 30 minutes. Following secondary antibody incubation, cells were rinsed by centrifugation and resuspended in PBS for analysis on a BD Accuri C6 flow cytometer (BD Biosciences).

*Prussian Blue Staining*

Slides were first deparaffinized and rehydrated. Equal parts of 20% hydrochloric acid and 10% potassium ferrocyanide were mixed immediately before use. Slides were then immersed in this solution for 20 minutes, followed by three rinses with distilled water. Nuclear fast red was used as a counterstain and slides were rinsed twice in distilled water. Slides were then dehydrated with 95% ethanol and twice with 100% ethanol, followed by two changes of xylene for three minutes each. Permount was used as a mounting medium to coverslip the slides.

*SMMHC x RGS4 flox design*

The gene targeting vector included a neomycin resistance cassette to select for screened embryonic stem cells incorporating the target sequence into the genome by recombination. Embryonic stem cells of interest were then injected into an E3.5 blastocyst and transferred into a foster mother at the Washington University in St. Louis Mouse Genetics Core. Heterozygous offspring were then bred and crossed with other heterozygotes until both floxed RGS4 alleles were consistently expressed through germline transmission. RGS4 deletion was induced by daily administration of 5mg/500μL of tamoxifen (Sigma Aldrich) intraperitoneally for five days. Floxed RGS4 genotyping included the following primer sequences, GCA GCA CTT CCT ACG GAC TCT C (sense) and CTG GAC CAC ATT CCT TCA TTC A (antisense).

SMMHC Cre mice were a gift, first generated by Dr. Stefan Offermanns and maintained by Dr. Kenneth J. Blumer. SMMHC Cre animals were genotyped with the following primer sequences TGA CCC CAT CTC TTC ACT CC (sense), AAC TCC ACG ACC ACC TCA TC (antisense). Genotyping was performed as a service of Transnetyx, Inc.

**Supplemental Figures**

**Suppl. Figure 1**

Tail cuff blood pressure measurement. Systolic blood pressure in congenic control (n=6) (109.4±4.9 mmHg) vs RGS4 Transgenic animals (n=7) (110.1±6.1)

**Suppl. Figure 2**

Dynamic MR image processing. Contrast in-flow rate (α) from a single pixel area in the kidney is plotted against the arterial contrast out-flow rate (β) for sham animals (A) and animals undergoing UMIRI (B). Gaussian distribution of the calculated renal blood flow under each condition corresponds with the same data used to generate α vs. β plots.

**Suppl. Figure 3**

RGS4 siRNA transfection of human vascular smooth muscle cells. Following transfection of RGS4 siRNA, RGS4 mRNA levels were reduced by 96.8% compared to cells transfected with scrambled siRNA (*, $p<0.001$). Experiments were completed in triplicate and repeated four times.

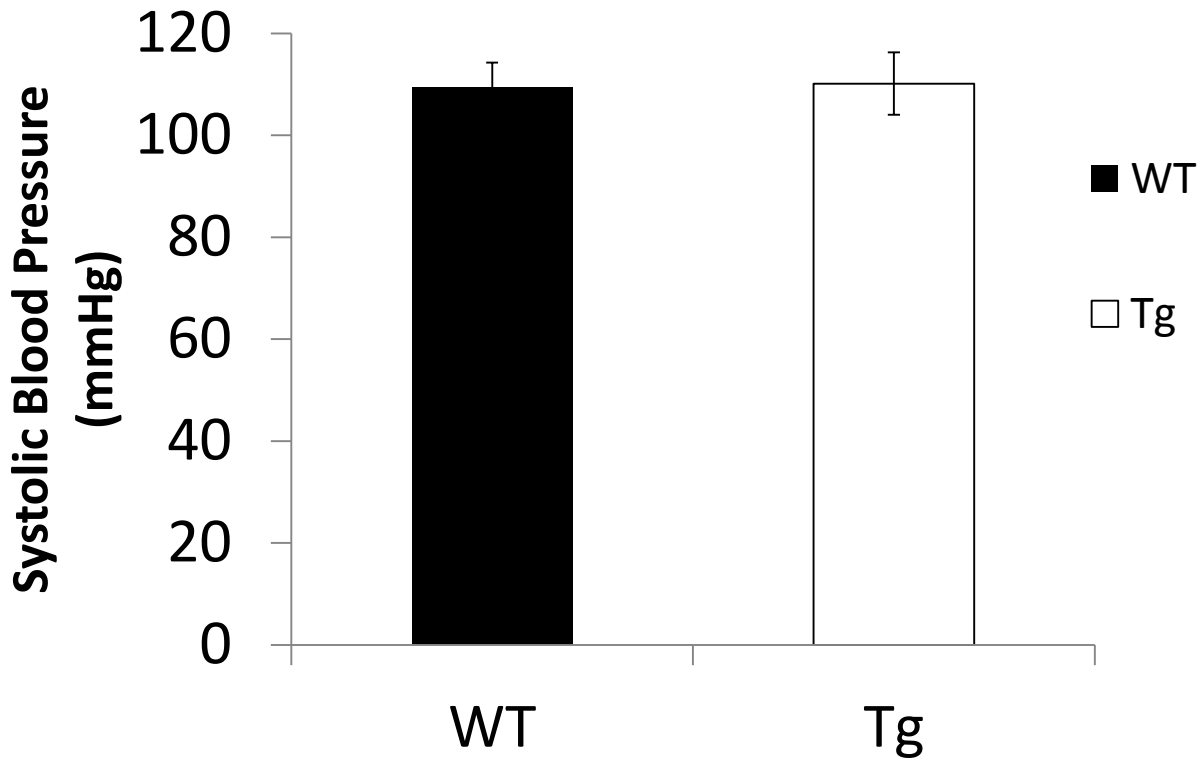
**Suppl. Figure 4**

RGS4 protein levels in pre-glomerular vascular smooth muscle cells. RGS4 protein levels in PGVSMC procured from RGS4 transgenic animals (Tg) was increased 2.3-fold compared to congenic controls (WT) (*, $p<0.001$). Experiments were completed in triplicate and repeated four times.

**Suppl. Movies 1,2,3**

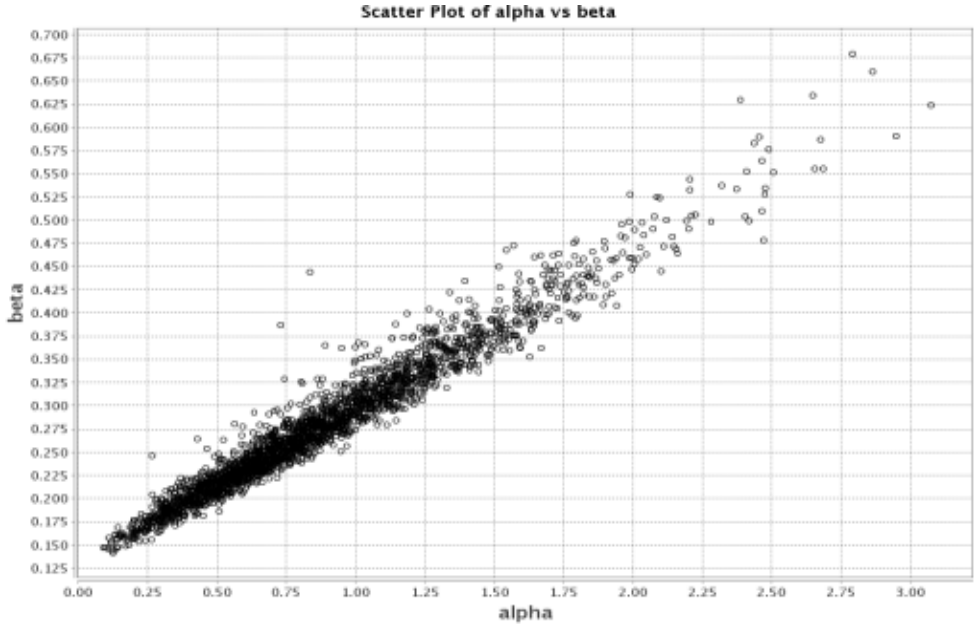
Note: Cine images are best viewed with Image J software. Movie 1, sham renal injury followed by gadolinium contrast injection. Movie 2, congenic control undergoing renal injury followed by gadolinium contrast injection. Movie 3, RGS4 transgenic undergoing renal injury followed by gadolinium contrast injection.

Supplemental Figure 1



# Supplemental Figure 2

A

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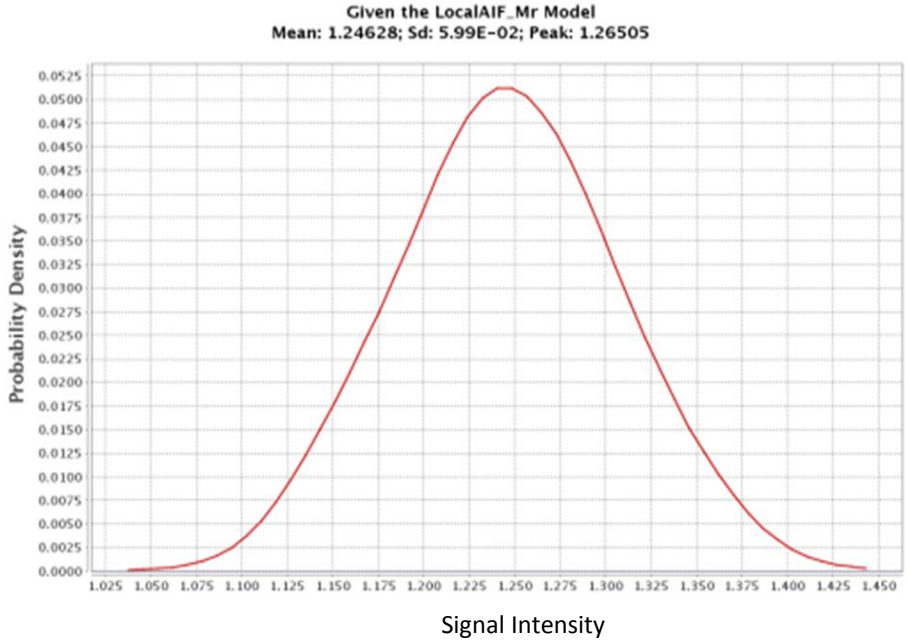
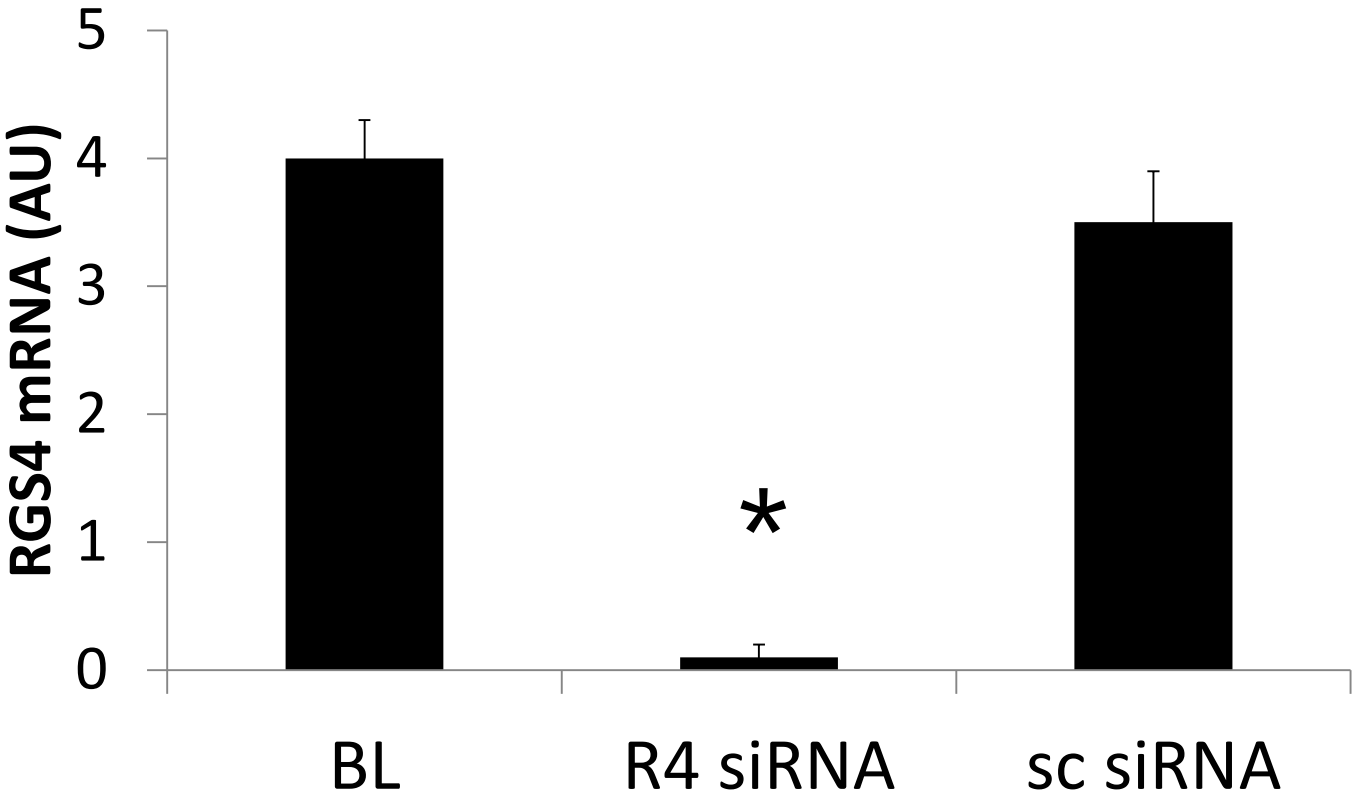
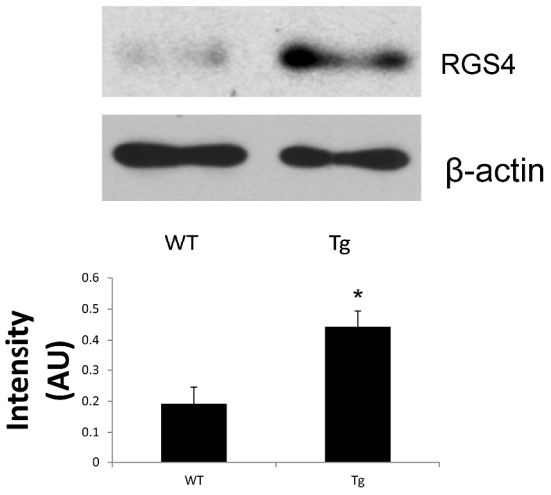


Supplemental Figure 3



Supplemental Figure 4

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