Materials and Methods

Ventricular cardiomyocytes- Primary neonatal ventricular cardiomyocytes (VCM) were isolated from 2-3 day-old WT and RGS6$^{-/-}$ pups. Briefly, pups were sacrificed by de-capitation and their hearts were immediately surgically removed. Ventricles were excised, minced, and then digested for 5 min at 37°C with 0.5 ml of solution containing 0.5 mg/ml type IV collagenase (Sigma, St. Louis, MO), 0.075 mg/ml DNase I (Roche Applied Science, Indianapolis, IN), 1% BSA, 20 mM glucose, 137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4. After digestion, cells were dissociated from tissue by gently pipetting. The cell suspension was then collected and mixed with 5 ml of culture medium containing 20 mM Hepes, 1 mM sodium pyruvate, 1 X Pen/Strep, 5% FBS, and 10% horse serum in DMEM (Invitrogen, Grand Island, NY). The digestion/dissociation/collection steps were then repeated until ventricular tissue digestion was complete. Cell suspensions from each dissociation step were pooled in one centrifuge tube and ventricular cells harvested with a centrifugation at 200 g for 2 min. VCM were separated from rapidly attaching fibroblasts by pre-plating the initial ventricular cell isolate for 1 hr at 37^{o}C in culture medium. VCM were then plated in 48-well dishes coated with 1 μg/cm^2 of type IV collagen (Sigma, St. Louis, MO) and cultured in culture medium at 37^{o}C in a humidified 5% CO_2 atmosphere. Twenty four hrs later, the concentration of FBS in culture medium was reduced from 5% to 1% and cells were used for experiments 12-24 hrs later. VCM were treated with 0.5 μM Dox, a clinically relevant concentration (1), for the indicated periods of time in serum-free culture medium.

Immunoblotting for RGS6 and key proteins in heart and VCM- We interrogated effects of Dox on levels of RGS6 and key signaling proteins in ventricles and atria of WT and RGS6$^{-/-}$ mice and in cultured VCM derived from these mice. For studies *in vivo*, mice were sacrificed at 0 (Dox untreated), 4, 8 and 12 hrs following intraperitoneal administration of Dox (10 mg/kg) and hearts

were rapidly removed and dissected on ice before plunging into ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS). This treatment protocol, shown previously to induce pathological changes in hearts of mice (2) and rats (3), proved optimal for distinguishing the temporal relationship between Dox induction of RGS6 and changes in key signaling proteins in heart at these early times. Three mice were used for each time point for statistical analysis of changes in these proteins. Similarly, VCM were treated for various times with 0.5 μM Dox before removing medium, rinsing cells with ice cold DPBS (Invitrogen, Grand Island, NY), and adding ice cold RIPA buffer. Ventricles, atria and VCM were homogenized in ice-cold RIPA buffer, homogenates were centrifuged at 12,000 g for 10 min at 4^{o}C, and resulting supernatants were used for protein analyses. Western blotting analysis was performed as previously described (4). Briefly, approximately 20 μg of protein per sample were subjected to SDS PAGE electrophoresis and transferred to a nitrocellulose membrane (Bio-Rad laboratories, Hercules, CA). The resultant membrane was then sequentially incubated in blocking buffer (5% nonfat dried milk in 24.8 mM Tris, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20, pH 7.4) for 1 h at room temperature, in primary antibody solution (diluted in blocking buffer) overnight at 4°C, and in secondary antibody solution (diluted in blocking buffer) for 1 h at room temperature. The secondary antibodies were labeled with IRDye 800CW (Li-Cor Biosciences, Lincoln, NE). Immunoreactive proteins were detected with the Odyssey imaging system (Li-Cor Biosciences, Lincoln, NE). Primary antibodies used for blotting were as follows: RGS6 (developed in our laboratory, 1:2000), p53 (Santa Cruz, 1:2000), phosphorylated p53(S15) (Cell Signaling, 1:2000), phosphorylated H2AX(S139) (Millipore, 1:1000), total ATM (GeneTex, 1:500), phosphorylated ATM(S1981) (Cell Signaling, 1:500), Mdm2 monoclonal antibody 2A10 that does not detect the phospho-inactive form of Mdm2 (a gift from Dr Arnold Levine, 1:10 dilution of hybridoma medium), Bax (eBiosciences, 1:1000), Bcl-2 (BD Pharmingen,1:1000), PARP (Cell Signaling, 1:1000), caspase-3 (Cell Signaling, 1:1000), and actin (Sigma, 1:5000). Western blot

signals were recorded and measured with the Odyssey infrared imaging system (LI-COR Bioscience).

Measurement of RGS6 mRNA level using quantitative real time PCR- Mice were sacrificed at various times following intraperitoneal administration of Dox (10 mg/kg), hearts were rapidly removed and ventricles were dissected on ice before rinsing in ice-cold DPBS (Invitrogen, Grand Island, NY) and freezing in liquid nitrogen. Total RNA was isolated from these samples using a Qiagen RNeasy kit according to the manufacturer's recommendation. First strand cDNA synthesis and real time PCR was performed exactly as we described previously (5). Briefly, the first strand of cDNA was synthesized from 135 ng of total RNA using the SuperScriptIII First Strand Synthesis system (Invitrogen, Grand Island, NY). Real time PCR was carried out using iQ™ SYBR® Green Supermix (Bio-Rad laboratories, Hercules, CA), by following the manufacturer's recommendation. Plasmids containing cDNAs of RGS6 or 18S rRNA were diluted to individual concentration series of 50, 5, 5 X 10^{-1}, 5 X 10^{-2}, 5 X 10^{-3}, 5 X 10^{-4}, 5 X 10^{-5}, 5 X 10^{-6}, 5 X 10^{-7} pg/µl, and were used as quantification standards for determining corresponding mRNA levels. 18S rRNA level was used as internal control to normalize RGS6 mRNA level. PCR primers were the following: RGS6 forward, ATG GAG GGA GAT ACA CAT TTG AAG ATG CC; RGS6 reverse, CAG CGA CTT TCC CTT CTT CTT GGC C; 18S rRNA forward, CAA AGA TTA AGC CAT GCA TGT CTA AGT ACG C; 18S rRNA reverse, GGC ATG TAT TAG CTC TAG AAT TAC CAC AGT TAT CC.

Apoptosis and caspase-3 activity assays- The extent of Dox-induced apoptosis in WT and RGS6$^{-/-}$ VCM was quantified using the Roche Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN). This kit quantifies formation of cytoplasmic histone-associated DNA fragments (mono- and oligosomes) originating from apoptotic cell death. Results are expressed as a fold increase in enrichment factor (cytoplasmic nucleosomes). The activity of caspase-3 in

WT and RGS6$^{-/-}$ VCM cellular lysates were assayed using Biovision's caspase-3 activity kit according to the manufacturers protocol.

Statistical Analysis- Experimental data were expressed as means ± SE (standard error of the mean). Significance of differences was determined by the unpaired Student's t test using SigmaPlot software (Systat Software, CA); a $p < 0.05$ was considered as statistically significant.

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