Rnd3/RhoE Modulates HIF1α/VEGF Signaling by Stabilizing HIF1α and Regulates Responsive Cardiac Angiogenesis

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Short Title: Rnd3, Responsive Angiogenesis, HIF1α/VEGF Signal

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Supplemental Methods

Human failing heart tissues

Failing myocardial samples were obtained from 51 patients with end-stage heart failure at the time of transplantation. Normal heart tissues were obtained from 12 patients who died of non-cardiac causes as a normal heart group. Left ventricular ejection fraction was <20% in all heart failure patients. The procurement of human heart tissue usage for the study was obtained with written patient-informed consent and approval by the Institutional Review Board of Methodist Hospital and Texas A&M University Health Science Center.

Generation and verification of Rnd3 knockout (KO) and Rnd3 overexpression transgenic mouse lines

The establishment of the Rnd3 KO mouse line was described previously.[1] Human full-length Rnd3 cDNA was subcloned into the α-myosin heavy chain (α-MHC) promoter expression vector. The DNA fragment including α-MHC-V5-His-Rnd3-SV40p(A) was cut out by PacI and EcoRV for pronuclear microinjection with a C57/B6 background.[2] Genomic DNA isolated from the tails of the mice was used for genotyping purposes. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Texas A&M University Health Science Center-Houston.

Transverse aortic constriction (TAC) surgery and $CoCl_2$ treatment

TAC was conducted in 10-15 week-old adult male mice for 3 weeks as described previously followed by cardiac function analysis and molecular assessments.[3] For the $CoCl_2$ rescue experiments, mice were allowed access to drinking water containing 2 mM $CoCl_2$ (Alfa Aesar, 12303, MA, USA) for 3 weeks starting immediately after TAC surgery.

Assessments of cardiac function and coronary flow reserve (CFR)

Cardiac function was evaluated by Vevo770 High-Resolution Micro-Imaging System (VisualSonics, Toronto, ON, Canada) as described previously.[2] CFR was measured by Doppler with MM3 system (World Precision Instruments, FL, USA) and a Pulsed Doppler probe (Indus Instruments, TX, USA) as described previously.[4] Peak left main coronary flow velocity was recorded at baseline (1% isoflurane) and hyperemic conditions (2.5% isoflurane), respectively. The ratio of hyperemic/baseline coronary velocity was calculated as the index of CFR. Duplicate measurements were performed.

Immunoblotting, immunoprecipitation, ELISA, hematoxylin & eosin staining, fluorescence staining, Sirius Red staining, and hypoxyprobe-1 staining

Protein samples for Western blot analysis were extracted and separated as described previously,[5] and the immunoblotting densitometry was quantified by Gel Logic 6000 PRO Imaging System (Carestream Health, Inc.).[6] Antibodies were from the following sources: anti-Rnd3 (customer designed and made by Cocalico Biologicals, PA, USA); anti-VEGF (ab1316, Abcam, MA, USA); anti-HIF1α (H1alpha67, Novus Biologicals, CO, USA); anti-Tie2 (sc-7403), anti-c-Myc (sc-40) and anti-HA (sc-805) from Santa Cruz, TX, USA. Equal protein loading was verified by the intensity of the GAPDH blot (Santa Cruz, sc-20357, TX, USA). The recombinant GST-Rnd3 protein was synthesized[7] and incubated with the cell lysates with HA-HIF1α expression. The mutual co-immunoprecipitations were conducted in HeLa cells. VEGFA concentration was

assessed by the VEGF Mouse ELISA kit (ab100751, Abcam, MA, USA) using a microplate reader at the wavelength of 450 nm.
Paraffin sections of the whole heart were used for hematoxylin & eosin, wheat germ agglutinin (WGA) (W11262, Thermo Fisher Scientific, NY, USA), isolectin GS-IB4 (I21411, Invitrogen, NY, USA), and picrosirius red staining (26357-02; Electron Microscopy Sciences, Hatfield, PA, USA). The hypoxia regions in the heart were evaluated by Hypoxyprobe™-1 Kit (HP1-100Kit, Hypoxyprobe Inc, MA, USA). Pictures were taken under the 40x microscope objective. A total of 20 staining pictures from each group were quantified by Leica Application Suite Imaging Software (Version 4.0, Germany). Nuclei were visualized by DAPI staining. The images were acquired by fluorescence microscopy.

Cardiomyocyte isolation, cell culture, tube formation assay, and gene transient transfection
Mouse cardiomyocytes were isolated from 10-15 week-old adult male mice by enzymatic digestion with Langendorff perfusion system (120108, Radnoti, CA, USA) followed by calcium reintroduction. The collagenase cocktail isolation perfusion buffer contained 0.15 mg/mL Liberase TM (05401127001, Roche, IN, USA). The cells were cultured onto the laminin-coated dishes with 5% fetal bovine serum (FBS) for 16 hours, then the culture media were used for the tube formation experiments in human umbilical vein endothelial cells (HUVECs).[8] Briefly, HUVECs were seeded onto BME gel (3433-005-01, Trevigen, MD, USA) in precoated dishes with EBM culture media (CC-3156, Lonza, NJ, USA). After 10 min of cell attachment, the EBM media was replaced by the mouse cardiomyocytes conditional media. Images were acquired after 6 hours of cell culture under a 5x microscope objective. The tube formation images were analyzed by the software WimTube Image Analysis (ibidi GmbH, Germany). Hypoxia cell culture was performed in the hypoxia chamber (MIC-101, Billups-Rothenberg Inc, CA, USA) with 1% O_2.

The human HA-HIF1α-pcDNA3 construct was purchased from Addgene (plasmid #18949). The GST-Rnd3 construct was generated with pGEX-6P-1 backbone (GE Healthcare). The myc-Rnd3 expression vector and the siRNA specific for Rnd3 were described in our previous study.[1]

Quantitative PCR analysis
Transcripts were quantified by quantitative PCR (qPCR) analysis as described previously.[5] Total RNA was prepared by TRIzol extraction (Life Technologies). The forward and reverse PCR primers (5′ to 3′) were as follows: VEGFA (mouse): CTGTGCAGGCTGCTGTAACG/GTTCCCGAAACCCTGAGGAG; GAPDH (mouse): GAGTCAACGGATTTGGTCGT/TTGATTTTGGAGGGATCTCG. GAPDH expression levels were used for qPCR normalization. Expression levels were determined by the $2^{-\Delta\Delta Ct}$ threshold cycle method.

Luciferase assay
Luciferase reporter vector with the promoter containing three hypoxia response elements (24-mers) was purchased from Addgene (HRE-luciferase, plasmid #26731). The luciferase assay was conducted as described earlier.[9] Each sample was measured three times. All results were normalized to co-transfected *Renilla* luciferase enzyme *activity* (E1960, Promega).

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Supplemental Figures

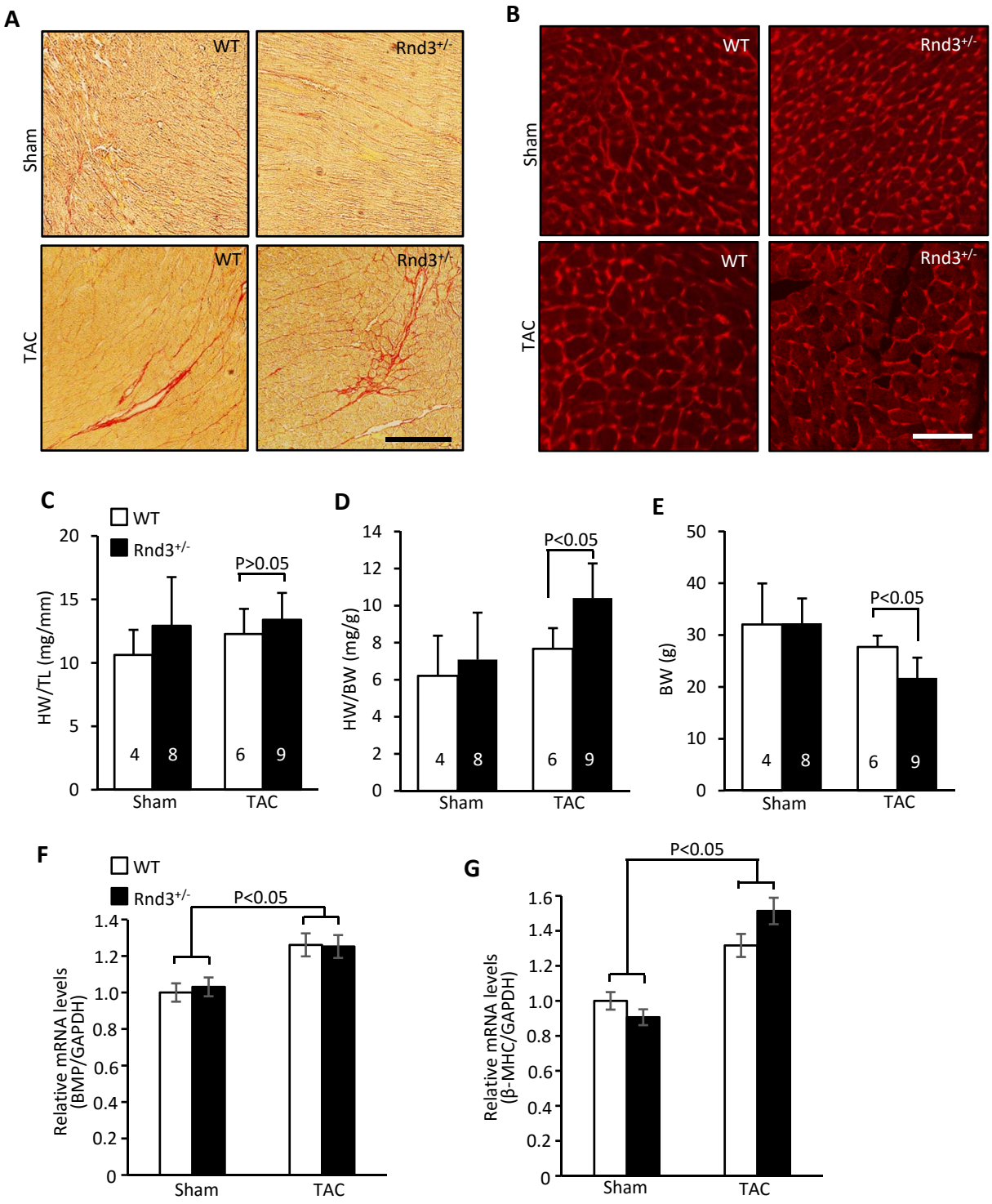

Figure S1. Cardiac fibrosis and hypertrophy were detected in both wild-type (WT) and Rnd3 haploinsufficient mice after 3-week TAC. Heart sections were stained with Sirius Red (**a**) for collagen deposition (red) and WGA (**b**) for cardiomyocyte size. Noticeable collagen deposition and enlarged cardiomyocytes were observed. No significant difference between the WT and mutant mice was observed. Scale bar represents 250 μm in **a** and 20 μm in **b**. Heart weight over tibial length (HW/TL) (**c**), heart weight over body weight (HW/BW) (**d**), and body weight (**e**) were summarized. The upregulation of hypertrophic markers were assessed by qPCR and presented in **f** and **g**. The qPCR data were pooled from 3 mice/group with analyses for each mouse in triplicates. WGA: wheat germ agglutinin.

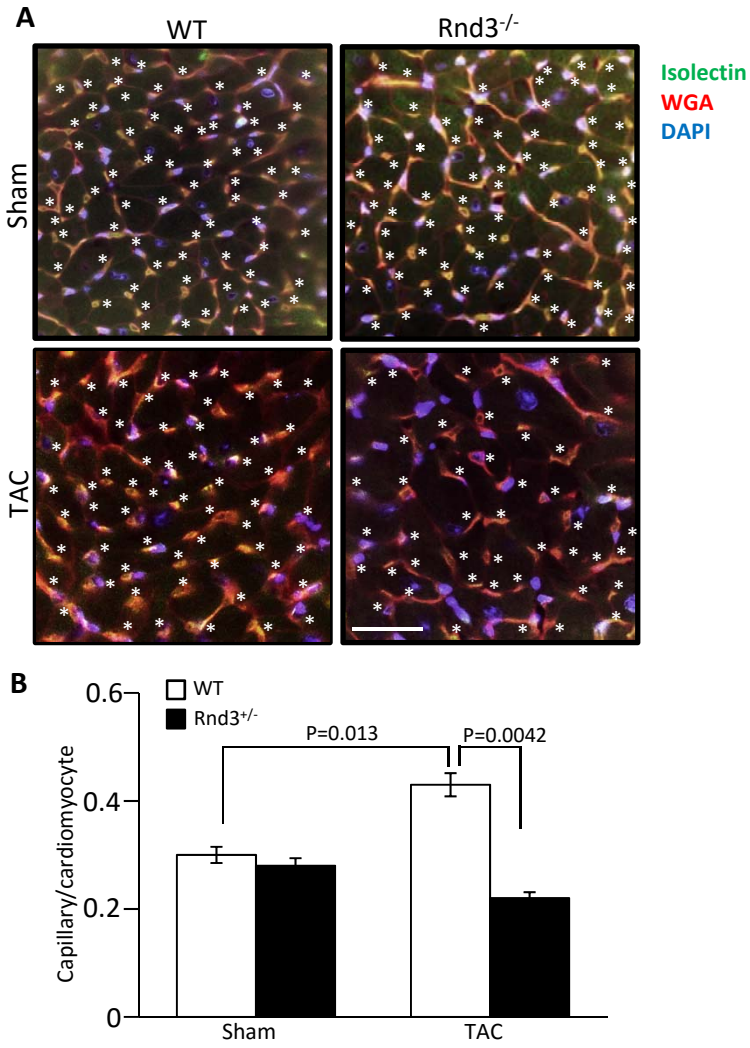

Figure S2. Angiogenesis was assessed by capillary/cardiomyocyte ratio. (**a**) Representative images showed capillaries (green, by isolectin), cardiomyocytes (red, by WGA), and nuclear counterstaining (blue, by DAPI). Scale bar represents 20 μm. * indicates the overlap of green and red staining. (**b**) The capillary/cardiomyocyte ratio in the left ventricle was quantified. A total of 20 staining pictures from 3 mouse hearts in each group were quantified for the capillary/cardiomyocyte ratio.

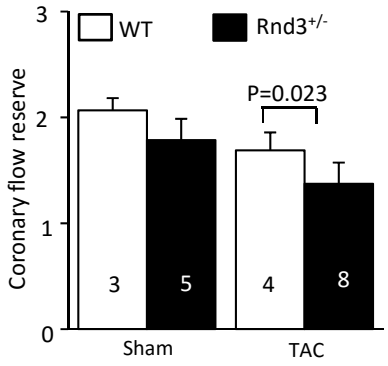

Figure S3. A reduction in coronary flow reserve was detected in WT mice and an even more profound reduction was measured in Rnd3$^{+/-}$ mice after TAC.

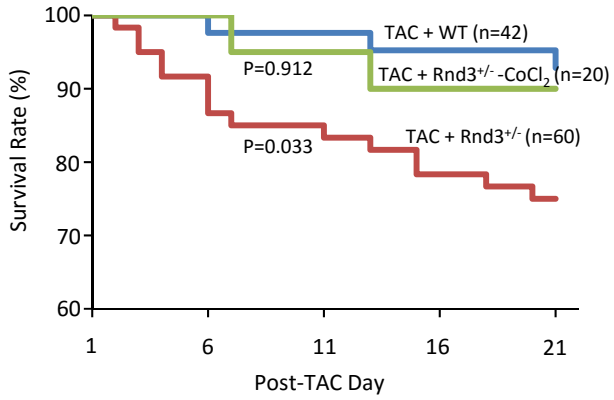

Figure S4. $CoCl_2$ treatment significantly increased the survival rate of $Rnd3^{+/-}$ mice after TAC. No significant difference was observed in the survival rate of the $Rnd3^{+/-}$ mice treated with $CoCl_2$ compared to the WT mice after TAC surgery. $Rnd3^{+/-}$ mice without $CoCl_2$ treatment had a lower survival rate compared to the WT mice post-TAC. P-values were calculated by two-tailed, chi-square Fisher's test.

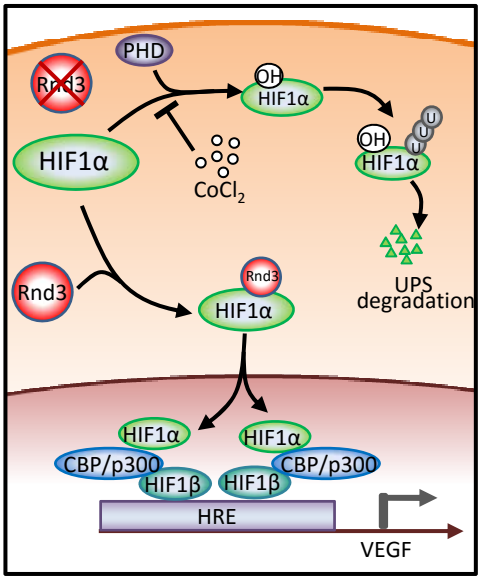

Figure S5. Proposed model outlining the molecular mechanism of the Rnd3 deficiency-mediated angiogenic defect. Rnd3 bound to and prevented HIFα protein from hydroxylation, and therefore enhanced its stability; enhancing VEGF secreted by cardiomyocytes. Downregulation of Rnd3 resulted in increased HIF1α UPS-mediated protein degradation, which in turn weakened the expression of its target genes, including VEGFA, and led to a myocardial angiogenesis defect and cardiac dysfunction. The impaired cardiac phenotype can be rescued by $CoCl_2$ treatment in animals. UPS: ubiquitin proteasome system.