Supplemental Material

Extended methods:

**Preparation of purified ferric sGC**
Human ferrous sGC was purified from Sf9 cells infected with baculoviruses expressing α1 and β1 sGC subunits, as described previously\(^1\). Purified ferrous sGC was stored at -80°C in 50 mM TEA pH 7.4 and 1 mM DTT, 1 mM EDTA and 1 mM EGTA until further use. To prepare ferric sGC, reducing thiols were removed from the buffer by passing the sample of ferrous sGC through a HiTrap desalting column equilibrated with 50 mM triethanolamine, pH 7.4. Then sGC was titrated with increasing concentrations of potassium ferricyanide (1-10 µM) until sGC heme moiety was fully converted into ferric form, as demonstrated by the conversion of the Soret band from 432 nm to 393 nm. The sample was then passed through the desalting column again to remove any traces of ferricyanide. This preparation of ferric sGC was used for spectroscopic studies and activity measurements.

**Generation of lentivirus**
The nt, Cyb5b and Cyb5R3 shRNA constructs in a pLKO.1 plasmid were purchased from Sigma (catalogue numbers: SHC016-1EA, SHCLNV-NM_025558, SHCLNG-NM_029787, respectively) and generated as previously described\(^2\).

**Cloning and adenovirus generation**
cDNA for rat cytochrome B5 reductase 3 (NM-138877.1) was purchased from Transomic Technologies. Standard PCR cloning was employed to add a 5’ BamHI site and a 3’ EcoRV site to the CDS using the following primers 5’ oligo ATGGATCCCCACCATGGGGCCAGCTGACG and 3’ oligo ATGGATATCTCAGAAGGTGAAGCATCGCTC. The PCR product was ligated into pENTR1A and subsequently recombined into pAd/CMV/V5-DEST for the generation of Adenovirus in 293A producer cells according to manufacturer directions (Life Tech Viral Power Adenoviral Expression System).

**Cyb5R3 knockdown and rescue cell lines**
For lentivirus transduction, 20 µl of concentrated virus was added to RASMCs cells at 50% confluency in 35 mm\(^2\) dish with 10 µg/mL polybrene. Twenty-four hours after transduction, cells were selected for 10 days with 1 µg/mL of puromycin to generate stable Cyb5R3 knockdown cells. Knockdown was confirmed via Western blot analysis.

**Western Blot**
RASMCs were lysed in ice-cold RIPA buffer containing protease and phosphatase inhibitors. Lysates were sonicated, boiled and subjected to electrophoresis on 4-12% Bis-Tris gels. Proteins were transferred to nitrocellulose membrane and blocked for 1 hour at room temp with either LI-COR Blocking Buffer or 1% BSA in PBS. Membranes were rocked overnight with primary antibodies (Online Table I) at 4°C. Membranes were washed and incubated with infrared dye secondary antibodies from LI-COR (Online
Table I) for 1 hour at room temp followed by washing with PBS and 0.05% tween 20. Visualization and analysis was completed utilizing a LI-COR Odyssey Imager.

**Co-Immunoprecipitation**

RASMCs were cultured in 10 cm dishes to 100% confluency and treated with 10 uM 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) for 15 min at 37°C 5% CO₂ followed by washing with ice cold PBS. Cells were lifted from the plate with ice cold PBS without Ca²⁺ and pelleted by centrifugation at 1,000 x g for 5 min at 4°C. Cell pellets were resuspended in lysis buffer containing 50 mM Tris-Cl pH 7.5, 0.5 mM EDTA, 1% SDS with 1 mM DTT, protease inhibitor (Sigma P8340) and phosphatase inhibitor added immediately before use. Lysates were boiled at 100°C for 10 min and debris removed by centrifugation at 12,000 x g for 10 min. 10% of lysate was preserved as input and remaining lysate diluted ten fold in RIPA buffer (25 mM Tris HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS and divided for pull down with sGC antibody and IgG control. 10 uL of Protein G Dynabeads conjugated to 1 ug of antibody (Online Table I) was added to lysates and rotated at room temperature for 1 hour. Beads were pelleted with a magnet and washed 3 times with RIPA. Proteins were eluted with 50 uL 2x Laemmli buffer, boiled for 10 min at 100°C and analyzed via western blot.

**Proximity Ligation Assay**

RASMCs were plated onto glass coverslips coated with 0.1% gelatin in a 12-well dish. At 50% confluency, cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with PBS + 0.25% Triton-X 100 for 15 min, and blocked with PBS + 0.25% Triton-X 100 + 10% horse serum + 1% fish skin gelatin (blocking buffer). All subsequent incubation steps were performed in a humidified chamber, reagents (40ml per coverslip) were distributed onto parafilm and coverslips placed cell side down on top. All washing steps occurred in 12-well dishes with coverslips facing cell side up. Coverslips were incubated with rabbit anti-sGC β and goat anti-Cyb5R3 primary antibodies (Online Table I) diluted in blocking buffer for 1hr at room temperature followed by 2 X 5 min washing in PBS + 0.1% Tween-20. Then the coverslips were incubated with Duolink In Situ PLA Probe anti-rabbit PLUS and anti-goat MINUS (Sigma) diluted 1:5 in blocking buffer for 1hr at 37°C followed by washing in Duolink Wash Buffer A 2 x 5 min. Next, ligation and amplification steps were carried out following manufacturer’s instructions for Duolink In Situ Detection Red (Sigma). After amplification, coverslips were stained with Alexa Fluor 488 Phalloidin for 45 min at room temperature followed by 2 X 5 min washing in PBS + 0.1% Tween-20. Coverslips were then mounted onto microscope slides using Duolink mounting medium containing DAPI. Cells were imaged using an Olympus FluoView 1000.

**cGMP-biosensor assay** – nt shRNA and Cyb5R3 shRNA RASMCs were transfected with 1 µg FlincG-cGMP biosensor (Addgene plasmid #49202) using a Nucleofector primary smooth muscle kit (Lonza) according to manufacturer directions. RASMCs were transoptimally plated on collagen coated MatTek coverglass bottom dishes. 70 kd TRITC dextran was used to define the necessary parameters for microinjection (90 hpa, 5 sec) for delivery of the NO donor, DEA NONOate. DEA NONOate was suspended using 0.01 M
NaOH at a range of concentrations and initially mixed with TRITC dextran at 5 mg/ml to further optimize delivery time relative to detectable cGMP flux. Ultimately 100 mM DEA NONOate was loaded without TRITC dextran. A continuous widefield time lapse sequence was collected with eGFP filter at 100 ms with pre and post differential interference contrast images. Prior to replicate experiments, we established microinjection parameters as described. All cells were selected based on relatively normal morphology coupled with moderate GFP expression (judged by eye) to allow detectable but not saturated flux upon stimulation. Further normalization of change in intensity was afforded by background subtraction of baseline intensity for each stimulated cell and then results were pooled for each treatment. Images were pseudocolored for presentation purposes only. Images were acquired on a Nikon TI live cell system, with 40X oil 1.25 NA objective and Retiga CCD camera; Nikon Elements software for used for data acquisition and analysis. Analysis involved background subtraction and measurement of peak amplitude of flux.

sGC reaction assay
Recombinant human Cyb5R3 and Cyb5b were prepared as previously described 3. All the reagents were placed on ice in an anaerobic chamber for 1 hr before starting the experiments. Human recombinant Cyb5R3 (10 µM), Cyb5b (10 µM) and human ferric sGC (500 ng) were combined in 250 mM Triethanolamine pH 7.4 buffer at 37°C. The reduction reaction of ferric sGC by Cyb5R3 was initiated by adding 100 µM NADH and the reaction was incubated for 1 hr. In order to block the activity of Cyb5R3, 50 µM Cyb5R3 inhibitor ZINC39395747 (as previously described 2) was added with 10 µM human recombinant Cyb5R3 and incubated in 250 mM triethanolamine buffer (pH 7.4) at 37°C for 1 hr before the sGC reduction reaction. The chemical reductant DTT (1 mM) was added as a positive control. All reactions were carried out in a final volume of 25 µL. Next, the sGC activity assay was carried out by mixing 5 µL of each sGC reaction assay condition above with 1 mM Mn^{2+} in 250 mM triethanolamine buffer (pH 7.4) at 37°C. Either 1 µM DEA NONOate or 1 µM BAY 58-2667 was added and activity was initiated by adding 1 mM GTP. The reaction was terminated after 5 min with 80 µL 125 mM Zn(CH_3COO)_2 and 100 µL 125 mM Na_2CO_3. Samples were then subjected to a cGMP ELISA assay (Cell Signaling 4360S).

Cyb5R3: NADH-dependent reduction of ferric sGC
To monitor the dynamics of ferric sGC reduction by Cyb5R3:NADH, 0.5 mL of 1 µM ferric sGC supplemented with 5 µM Cyb5R3 was placed in a gas-tight cuvette fitted with a resealable rubber septum. The sample was made anaerobic via 5 cycles of vacuum and argon replacement (30 s and 5 min, respectively) and was then placed inside an anaerobic chamber (Model 110V, Coy Laboratory Products, Inc.) filled with 5% H_2 in N_2 and fitted with a palladium-based O_2 scrubber. A gas analyzer (Model 10, Coy Laboratory) tracked both the hydrogen and oxygen level to ensure that the oxygen level was 0 ppm. The stock solutions of 5 mM non-cyclable GTP analog GpCpp and 2 mM NADH in 50 mM triethanolamine buffer (pH 7.4) were subjected to the same vacuum/argon cycles and placed in the anaerobic chamber. Two gas-tight syringes, each containing 5 µL of GpCpp or NADH were used to pierce the rubber septum of the cuvette adaptor without applying the nucleotide to the sGC/Cyb5R3 mixture. The entire cuvette-syringe assembly was
removed from the anaerobic chamber and placed into the Agilent 8453 UV-Vis spectrophotometer. After the spectra for the sGC/Cyb5R3 and sGC/Cyb5R3/GpCpp were recorded, NADH (10 µM final) was transferred from the syringe into the sample, mixed and the changes in the spectral properties of the mixture were recorded for 1 hr by the spectrophotometer in the kinetic mode. A similar procedure was performed for the sample without GpCpp. The difference spectra were generated by subtractions of the optical values for the initial sGC/Cyb5R3/GpCpp spectra from the optical values of the spectra collected at each time point.

**Superoxide measurements**
Quantification of superoxide (O$_2^-$) was accomplished by measuring the abundance of 2-OH-E$^+$; the O$_2^-$-specific product of hydroethidine oxidation via HPLC coupled to an electrochemical detector as previously described$^4,5$.

**Quantification of glutathione and glutathione disulfide in biospecimens**
The method for simultaneous determination of GSH and GSSG was adapted from Harwood et al.$^6$. Briefly, nt shRNA and Cyb5R3 shRNA cells were cultured in 6-well plates for 1-3 days until confluent. Media was aspirated and cells were washed 2x with sterile HBSS and incubated with PBS containing 100 mM N-ethylmaleimide (NEM) for 15 min at 37°C. The PBS/NEM solution was aspirated and 200 µL derivatizing solution (25 mM NEM, 40 mM HEPES/50 mM NaCl/1 mM EDTA; 10 µM $^{13}$C$_4^{15}$N GSH; 10 µM $^{13}$C$_4^{15}$N$_2$ GSSG) was added to each well and incubated for 15 min at room temperature. Cells were detached by scraping and transferred to Eppendorf tubes, followed by sonication in 4°C water bath 3x30 sec. Lysates were collected and cleared of precipitate by centrifugation at 15,000 RPM for 10 min at 4°C. A protein assay (BCA) was done in triplicate using 3x5 µL sample. A 100 µL aliquot of sample was taken and added to 1000 µL 200 proof molecular biology grade ethanol and cooled to -80°C overnight to precipitate proteins. Precipitates were centrifuged (15,000 rpm, 4°C, 15 min) and the supernatant was taken and dried under N$_2$ stream at room temperature. Samples were reconstituted in 100 µL dH$_2$O and 20 µL was injected for analysis by HPLC-MS/MS.

**Reversed phase isotope-dilution HPLC tandem mass spectrometry for GSH/GSSG quantification**
A Shimadzu HPLC (Columbia, MD) coupled to a Thermo Scientific CTC HTS PAL autosampler (Waltham, MA) and an AB Sciex (Framingham, MA) 5000 triple quadrupole mass spectrometer was used for the quantification of GS-NEM and GSSG. Sample (20 µL) was separated on a Phenomenex C18 (2.1 x 150 mm, 3.5 µm pore size) column. The solvent system employed aqueous 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B) with net flow rate of 0.6 mL/min, using a linear gradient of 2% B to 75% B from 0.1-6.2 min, followed by wash with 100% B for 2 min, and re-equilibration with 2%B for 6 min. Unlabeled and $[^{13}$C$_4^{15}$N$_2$] GSSG eluted at 2.0 min, while unlabeled and $[^{13}$C$_4^{15}$N] GS-NEM eluted at 2.7 min. API5000 mass spectrometer settings: CAD 4.00 arbitrary units, curtain gas 40.00 arbitrary units, GS1 45.00 arbitrary units, GS2 50.00 arbitrary units, IS 5500V, Temperature 550°C, EP 5.0V, CXP 10.0V. Multiple reaction monitoring was performed in positive ion mode. Transitions for respective species were as follows: GSH (Q1 308.3 → Q3 179.1; 65 msec, declustering
potential (DP) 60V, collision energy (CE) 18.5V). [\textsuperscript{13}C\textsubscript{2}\textsuperscript{15}N] GSH (Q1 311.3 $\rightarrow$ Q3 182.1; 65msec, DP 60V, CE 18.5V). GS-NEM (Q1 433.0 $\rightarrow$ Q3 304.2; 65msec, DP 65V, CE 38V); \textsuperscript{13}C\textsubscript{2}\textsuperscript{15}N GS-NEM (Q1 436.0 $\rightarrow$ Q3 307.2; 65msec, DP 65V, CE 38V); GSSG (Q1 613.2 $\rightarrow$ Q3 355.2; 65msec, DP 60V, CE 24V); [\textsuperscript{13}C\textsubscript{2}\textsuperscript{15}N\textsubscript{2}] GSSG(Q1 619.2 $\rightarrow$ Q3 361.2; 65msec, DP 60V, CE 24V). Calibration curves were generated using known GSH and GSSG standards and isotopic internal standards and showed linearity over 5 orders of magnitude and the limit of quantification (LOQ) for both GS-NEM and GSSG was 1 nM. Sample [GSH] and [GSSG] were determined from analyte: I.S. area ratios using general calibration curves, and intracellular GSH and GSSG were normalized to sample protein content, with results expressed as nmol GSH or GSSG per µg protein. Results are reported as mean +/- SEM of 6 replicates.
Online Figure Legends

Online Figure I. Knockdown or pharmacological inhibition of Cyb5R3 decreases sGC α protein expression and ODQ-induced oxidation combined with Cyb5R3 inhibition additively reduces sGC β protein expression. a, RASMCs were transduced with lentivirus to express nt shRNA or Cyb5R3 shRNA or b treated with the Cyb5R3 inhibitor ZINC39395747 (30 µM, 24 hrs) and were subjected to Western blot analysis for sGC α protein expression. c, RASMCs were treated with ODQ (10 µM, 24 hrs), ZINC39395747 (30 µM, 24 hrs) or both and analyzed for sGC β protein expression via Western blot analysis. Graphs show quantification of band density (n=3-6). * = p<0.05, ** = p<0.01*** = p<0.001, **** = p<0.0001 using a Student's t-test (a) or a 1-way ANOVA (b-c) and error bars are s.e.m.

Online Figure II. Measurements of GSH, GSSH and GSH/GSSH levels in nt-shRNA and Cyb5R3 shRNA RASMCs. RASMCs were transduced with lentivirus to express nt-shRNA or Cyb5R3 shRNA and measured for GSH/GSSG via mass spectrometry (n=6). Statistical testing was performed using a Student's t-test (GSH and GSSG) or a Mann-Whitney test (GSH/GSSG ratio) ** = p<0.01 and error and bars are s.e.m.

Online Figure III. Acute knockdown with Cyb5R3 siRNA causes loss of cGMP production following NO stimulation and IBMX-treated RASMCs loss of NO-stimulated cGMP. a, Western blot for Cyb5R3 and b sGC β from RASMCs transfected with nt shRNA or Cyb5R3 siRNA for 72 hrs. c, cGMP levels following stimulation with DEA NONOate (1 µM, 30 min) in the presence of sildenafil (10 µM) (n=4). d, cGMP measurements following stimulation with DEA NONOate (10 µM, 30 min) in the presence of IBMX (100 µM) (n=4). ** = p<0.01 and **** = p<0.0001 using a Student's t-test (a, b) or a 1-way ANOVA (c-d) and error bars are s.e.m.

Online Figure IV. Cyb5R3 inhibitor does not affect sGC activity. Purified sGC was incubated with ZINC39395747 (30 µM, 15 minute), followed by stimulation with DEA NONOate (1 µM, 5 min) and measurements of cGMP (n=3). Significance was tested for using a 1-way ANOVA and error bars are s.e.m.

Online Figure V. The sGC stimulator BAY 41-2272 does not fully rescue NO-stimulated cGMP signaling. RASMCs were transduced with lentivirus to express nt shRNA or Cyb5R3 shRNA. Cells were then pre incubated with BAY 41-2272 (1 µM, 15 min) followed by stimulation of with DEA NONOate (10 µM, 30 min) in the presence of sildenafil (10 µM) (n=3). **** = p<0.0001 using a 1-way ANOVA and error bars are s.e.m.

Online Figure VI. Acute Cyb5R3 inhibition does not increase cGMP following BAY 58-2667 stimulation but increases cGMP production in the presence of pro-oxidants. a, cGMP measurements from RASMCs treated with ZINC39395747 (30 µM, 0-3 hrs) followed by BAY 58-2667 (10 µM, 15 min). b, cGMP measurements from RASMCs
incubated with ZINC39395747 (30 µM, 3hrs) followed by antimycin A (1 µM) or H₂O₂ (25 µM) treatment in the presence of sildenafil (10 µM) and BAY 58-2667 (1 µM) for 1 hr (n=4). ** = p<0.01 and * = p<0.05 using a Student's t-test and error bars are s.e.m.

**Online Figure VII. Overexpression of GFP and Cyb5R3 with adenovirus in RASMCs.** Western blot of GFP, Cyb5R3, sGC β and α-tubulin from RASMCs transduced with GFP (2.4 x 10⁵ copies/mL) or rat Cyb5R3 (2.3 x 10⁷ copies/mL) for 48 hrs. Quantification of GFP and Cyb5R3, are shown in graphs on right (n=3). **** = p<0.0001 using a Student's t-test and error bars are s.e.m.
**Online Table I**

WB-Western blot, PLA-proximity ligation assay, IP-Immunoprecipitation, IHC-Immunohistochemistry

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Supplemental References


Online Figure IV
Online Figure VI

(a) Cyb5R3 inhibitor 0 hrs + + - - - -  
(b) Cyb5R3 inhibitor 1 hrs - - + + - -  
(c) Cyb5R3 inhibitor 3 hrs - - - - + +  

[Bar graphs showing cGMP levels (nM/mg protein) with vehicle and BAY 58-2667 treatments.]