Supporting Information

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SI Materials and Methods

Mice. C57BL/6J mice were obtained from the Jackson Laboratory. γβC93, βC93A, and γβC93A mice were kindly provided by Tim Townes (University of Alabama at Birmingham, Birmingham, AL). Genotypes of γβC93, βC93A, and γβC93A mice were confirmed by PCR using the following primers: human γ-chain 5′-GTTTAGC-CAGGGACCGTTTCAG/5′-TTGAGCAATGTGGACAGAGA-AGG (identifies γβC93 mice) and 5′-AATTCTGGCTTATCG-GAGGCAAG/5′-TTGAGCAATGTGGACAGAGAAGG (identifies γβC93A mice). For positive identification of βC93A mice, the presence of the inserted promoter was verified with two additional primers: 5′-AAACTAGAGACCTCTTAATGCAGTC/5′-TTG-AGCAATGTGGACAGAGAAAGG (1).

Hb Concentration and Blood O_2 Content. Blood Hb and O_2 contents were quantified using a co-oximeter (GEM OPL; Instrumentation Laboratories). Thoracotomies were conducted on deeply anesthetized mice with ventilator support under room air, and a needle was inserted into the left ventricle under direct visualization. Arterial blood was drawn from the ventricle into a heparinized syringe and was analyzed immediately.

Assessment of Hb S-Nitrosylation.

Mercury-coupled photolysis-chemiluminescence. Hb S-nitrosylation was quantified by mercury-coupled photolysis-chemiluminescence as described (2). In this method, nitric oxide released from Hb by photolytic cleavage is quantified by ozone chemiluminescence, and the difference between signals obtained with and without treatment by inorganic mercury (to remove NO groups selectively from thiol versus heme iron) represents SNO-Hb. Standard curves are generated with GSNO. Blood was drawn from the inferior vena cava of male C57BL/6J, γβC93, βC93A, and γβC93A mice (8–12 wk of age) under pentobarbital anesthesia. Oxygenated RBCs were collected by centrifugation at $1,000 \times g$ and were rinsed three times in PBS containing 0.1 mM EDTA at 4 °C and were lysed in water containing 0.1 mM EDTA at 4 °C. Lysates were spun through Sephadex G-25 to remove low-molecular-weight components. Then Hb concentration was quantified by spectrophotometry, and all samples were adjusted to contain 50 μM heme before analysis by photolysis-chemiluminescence (NITROLITE, Thermedics; Anuva Innovations).

SNO-RAC. Analysis of the S-nitrosylation status of individual Hb subunits was carried out with the SNO-RAC method essentially as described (3), with modifications noted below. Blood was drawn from the left ventricle of Avertin-anesthetized mice ventilated mechanically with room air and was processed immediately. Samples obtained from individual mice were not pooled. RBCs were collected by centrifugation at $2,000 \times g$. The buffy coat was removed, followed by three washes with 50 mM phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 0.1 mM diethylene triamine pentaacetic acid (DTPA). RBCs were resuspended in lysis buffer containing 100 mM Hepes, 1 mM EDTA, 1 mM DTPA, and 100 mM neocuproine (HEN buffer), as well as 0.1% (vol/vol) Nonidet P-40 and protease inhibitor mixture (Roche), pH 8. The lysis buffer also contained, as a thiol-blocking agent, 0.2% (wt/vol) methyl methanethiosulfonate (MMTS). RBCs were lysed by probe sonication, and SDS [final concentration 2% (wt/vol)], and additional MMTS (0.2% final concentration of MMTS) were added to the lysate, followed by incubation for 25 min at 55 °C. The blocking reaction was stopped by adding 2.5 volumes of 100% acetone (−20 °C). After incubation for 5 min at −80 °C, precipitated protein was collected by centrifugation for 5 min at $5,000 \times g$. The resultant pellet was washed four times with 70% (vol/vol) acetone (−20 °C); then proteins were resuspended by sonication in HEN buffer containing 1% SDS followed by reprecipitation with 100% acetone and then by four rinses with 70% acetone. The final pellet was resuspended by probe sonication in HEN buffer containing 1% SDS, and the Hb concentration was measured spectrophotometrically. Samples containing equal amounts of protein were incubated with freshly prepared ascorbate (150 mM) and thiopropyl-Sepharose for 3 h in the dark with rotation, and beads were collected and washed three times with HEN/1% SDS and three times with 0.1× HEN/1% SDS. Beads were eluted in 0.1× HEN buffer containing 1% SDS and 10% β-mercaptoethanol at room temperature for 30 min. Eluates were analyzed by SDS/PAGE followed by Western blotting for individual Hb subunits. Antibodies used were monoclonal antibodies raised to human chain-specific peptide epitopes: rabbit monoclonal ab92492 (Abcam) for Hbα, mouse monoclonal ab55081 (Abcam) for Hbβ, and mouse monoclonal clone 70050 (MyBioSource) for Hbγ.

Measurement of Tissue Perfusion and pO_2 in Vivo. Local blood flow and tissue oxygenation were measured with a dual-use OxyLite needle probe outfitted with a laser Doppler and ruthenium crystal tip (Oxford Optronix Ltd.). Mice were anesthetized with Avertin (0.25 mg/g i.p.) and were secured in the supine position on a temperature-controlled small animal surgical table. The probe was inserted into the hindlimb gastrocnemius muscle. After a stabilization period, blood flow and tissue pO_2 were recorded under normoxia ($FiO_2 = 0.21$) and during sequential 5-min exposures to mild, moderate, and severe hypoxia (FiO_2 of 0.15, 0.10, and 0.05, respectively), with gas delivered through a face mask. Flow-mediated perfusion (hyperemia) was assessed after manual compression of the femoral artery for 5 min.

Transfer of NO from βC93 to Extracellular Thiol at Low pO_2. RBCs prepared from freshly drawn blood were deoxygenated by sparging with N_2 for 30 min in the absence or presence of 1 mM GSH, and SNO was quantified in the supernatant (after the removal of RBCs by centrifugation) using mercury-coupled photolysis-chemiluminescence.

Bioassay of RBC-Induced Vasodilation and Measurement of Plasma Nitrite.

RBCs. Blood was drawn from the inferior vena cava of male 8- to 12-wk-old C57BL/6J, γβC93, βC93A, and γβC93A mice under pentobarbital anesthesia, and RBCs, collected by centrifugation at $1,000 \times g$, were rinsed three times in PBS at 4 °C, stored in PBS on ice at 50% hematocrit, and used within 1 h. The plasma was retained for measurement of nitrite using a Sievers Nitric Oxide Analyzer (GE Analytical Instruments).

Preparation of aortic ring segments. Male endothelial NO synthase-null ($eNOS^{-/-}$) 8- to 12-wk-old mice (Jackson Laboratory) were killed by CO_2 inhalation. A segment of thoracic aorta was excised, placed in Krebs solution, and cleaned of adventitial tissue and adherent blood cells before division into rings 2–3 mm in length.

Bioassay. Aortic rings were attached to isometric force transducers (Grass) and suspended in jacketed organ chambers containing Krebs-bicarbonate buffer (pH 7.4, 37 °C) sparged with 21% O_2/5% CO_2/74% N_2. Resting tension was maintained at 0.5 g. Active tension was evoked by phenylephrine (1 μM), and the absence of vasorelaxation evoked by acetylcholine (1 μM) was verified. Rings then were washed three times and allowed to rest at 21% O_2/5%

CO_2/74% N_2 for ∼15 min before the sparging gas was switched to 1% O_2/5% CO_2/94% N_2. After 20 min, phenylephrine (1 μM) was administered again to evoke active tension, and RBCs were added to produce a bath hematocrit of 0.4% (higher hematocrit resulted in excessive hemolysis). Tension was recorded with a PowerLab data acquisition system and analyzed with LabChart software (ADInstruments).

Invasive Hemodynamic Measurements. The instrumentation procedure followed Pacher et al. (4). Mice were anesthetized with Avertin (0.25 mg/g i.p.) and were secured in the supine position on a temperature-controlled small animal surgical table; respiratory support was supplied with a rodent ventilator (MiniVent 845; Harvard Apparatus). If needed, mice received supplemental doses of Avertin during the procedure (∼0.08 mg/g i.p.). The right internal jugular vein was visualized and catheterized for fluid infusion (normal saline at 2 μL/min). A Millar SPR-1000 catheter was inserted into the right common carotid artery to measure blood pressure, and a Millar SPR-839 pressure–volume catheter was inserted through the apex into the left ventricle (Millar Instruments). Pressure and volume data were obtained with an MPVS-300 system (Millar Instruments) coupled to a PowerLab data acquisition system (ADInstruments). After a stabilization period, hemodynamic parameters were recorded under normoxia ($FiO_2 = 0.21$) and then sequentially after 5 min of moderate and severe hypoxia (FiO_2 of 0.10 and 0.05, respectively) delivered through the ventilator. After the final measurements, mice were euthanized humanely by an American Veterinary Medical Association-approved method. Calculated cardiovascular factors were determined off-line using LabChart (ADInstruments).

Telemetered Heart Rate and Blood Pressure. Continuous hemodynamic telemetry in awake mice was carried out with a PA-C10 pressure transmitter (Data Science International). Under isoflurane anesthesia, a midline incision was made from the sternum to the jaw, and the left carotid artery was revealed by blunt dissection. The catheter tip was inserted into the carotid artery, and the catheter sensor was positioned in the aortic arch. The catheter was secured in place, and the transmitter was placed s.c. along the flank between the forelimb and the hind limb. The incision site was closed in layers, and mice were allowed a surgical recovery period before hemodynamic recording. Data were recorded for at least 3 d before analysis.

Echocardiography. Mice were anesthetized with Avertin (0.25 mg/g i.p.), and transthoracic echocardiography was performed using a Vevo 770 High-Resolution Imaging System equipped with an RMV-707B 30-MHz probe (VisualSonics). Standard M-mode sampling was used through the left ventricular short axis at the midpapillary level. Images were recorded under normoxia ($FiO_2 = 0.21$) and then after 5 min of mild, moderate, and severe hypoxia (FiO_2 of 0.15, 0.10, and 0.05, sequentially). Ejection fraction, fractional shortening, and other parameters were determined using the system's software. For both echocardiographic and electrocardiographic measurements (described below), gas was delivered through a face mask.

Electrocardiography. The ECG (lead II) was recorded with s.c. bipolar limb leads. T-wave and ST areas, defined as shown in Fig. S4, were calculated digitally using LabChart software. Hyperacute T-waves were defined by increases in T-wave area (broadening and/or peak-heightening) caused immediately by hypoxia. Data were obtained from γβC93, βC93A, and γβC93A mice (13–21 mice per strain), and at least three heartbeat cycles were analyzed for each mouse at each FiO_2 examined (0.21, 0.15, 0.10, and 0.05) at the end of each cycle.

Statistical Analysis. Data are presented as means ± SEM or means ± SD except where noted. Student's *t* test, ANOVA, and Fisher's exact test, as appropriate, were used to test for strain and treatment effects. Tukey's and Dunnett's tests were used post hoc to identify group differences when the F statistic was significant. Under all conditions, values of $P < 0.05$ were taken to indicate a statistically significant difference between groups.

1. Isbell TS, et al. (2008) SNO-hemoglobin is not essential for red blood cell-dependent hypoxic vasodilation. *Nat Med* 14(7):773–777.
2. Stamler JS, et al. (1992) Nitric oxide circulates in mammalian plasma primarily as an S-nitroso adduct of serum albumin. *Proc Natl Acad Sci USA* 89(16):7674–7677.
3. Forrester MT, et al. (2009) Proteomic analysis of S-nitrosylation and denitrosylation by resin-assisted capture. *Nat Biotechnol* 27(6):557–559.
4. Pacher P, Nagayama T, Mukhopadhyay P, Bátkai S, Kass DA (2008) Measurement of cardiac function using pressure-volume conductance catheter technique in mice and rats. *Nat Protoc* 3(9):1422–1434.

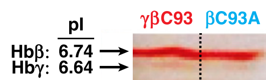

Fig. S1. Isoelectric focusing (IEF) to separate Hbβ and Hbγ indicates that Hbγ remains a minor species in mutant mice expressing the human Hbγ subunit. An unstained IEF gel segment containing two adjacent lanes is shown, 40 μg total Hb protein per lane. Hbα resolves poorly on these gels and is not shown.

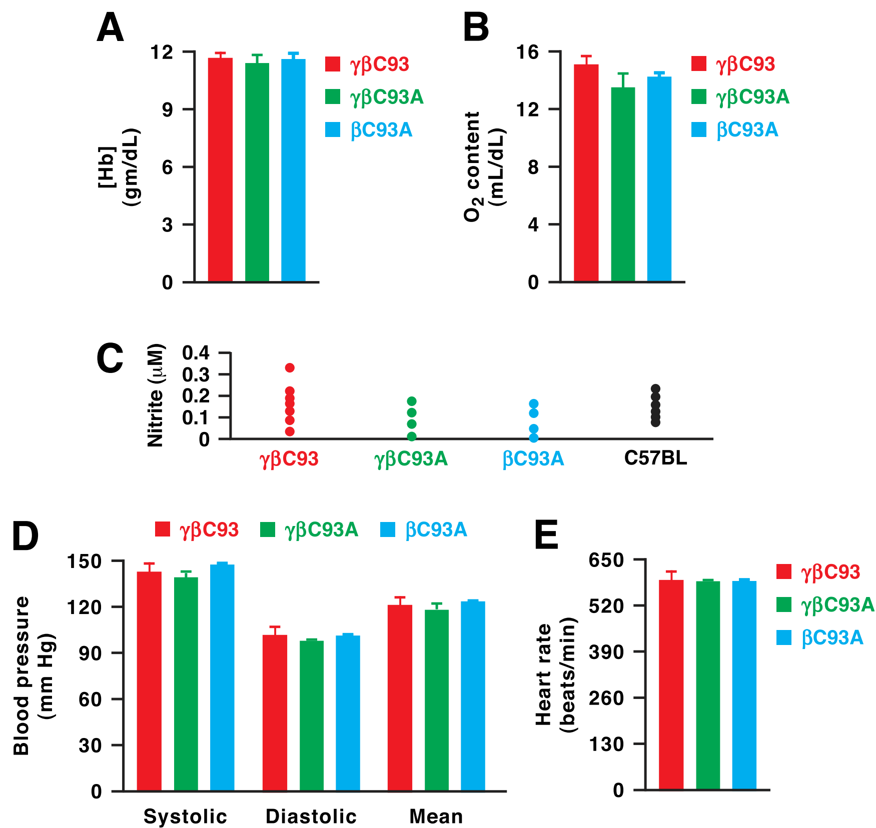

Fig. S2. Absence of significant differences among humanized strains in Hb concentration, blood O_2 content, plasma nitrite content, blood pressure, and heart rate. (*A*–*C*) Hb concentration (*A*), blood O_2 concentration (*B*), and plasma nitrite concentration (a signature of NO production by eNOS) (*C*) do not differ among γβC93, βC93A, and γβC93A mice. (Plasma nitrite also is comparable in mutant strains and wild-type C57BL/J6 mice.) Blood was drawn from the left ventricle at normoxia. (*D* and *E*) Telemetry in conscious, freely moving mice demonstrates no differences in blood pressure (systolic, diastolic, mean) (*D*) or average heart rate (*E*) in γβC93, βC93A, and γβC93A mice. Telemetry was monitored for 3–5 d. Data are shown as mean ± SEM; n = 4–14 mice per strain; $P > 0.05$ by one-way ANOVA.

Fig. S3. Impaired cardiac responses to hypoxia in βCys93-deficient mice (see Fig. 4). Assessment of principal measures of left ventricular function by invasive hemodynamic monitoring (*A*) and echocardiography (*B*–*D*) demonstrates that cardiac function is significantly compromised during exposure to progressive global hypoxia in βC93A and γβC93A mice versus γβC93 mice. Measures include dP/dT max (*A*) left ventricular ejection fraction (*B*), fractional shortening (*C*), and end systolic diameter (*D*) (see also Fig. 4*F*). Data are presented as mean ± SEM; $n = 9–12$; $^{*}P < 0.05$ by one-way ANOVA for differences between βC93A and γβC93A mice versus γβC93 mice; $^{\dagger}P < 0.05$ by one-way ANOVA for differences between βC93A mice and γβC93 mice in *A* and between γβC93A mice and γβC93 mice in *B*–*D*.

Fig. S4. Representative raw electrocardiographic recordings from three mice during respiration at $FiO_2 = 0.21$ and subsequently at $FiO_2 = 0.05$. Characteristic ischemia-related impairment in cardiac function is seen in mice lacking βCys93 under hypoxia, illustrating the method used in calculating T-wave and ST-wave area.