Supporting Information

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SI Materials and Methods

Materials. MAHMA NONOate and MG132 were purchased from Cayman Chemical and Sigma Aldrich, respectively. HA-tagged full-length DCC construct was kindly provided by Dr. Marc Tessier-Lavigne, Rockefeller University, New York. Myc-tagged human SIAH1 was a kind gift from Dr. Jing Zhao, Fudan University, Shanghai, China. Human SIAH2, obtained from M. Lienhard Schmitz, Justus Liebig University, Giessen, Germany, was subcloned into the plasmid of pCMV-Myc. Human ubiquitin, as a generous gift from Dr. Jian An, Mayo Clinic, Rochester, MN, was inserted into pCMV-Myc plasmid. Myc antibody and HA antibody were obtained from Cell Signaling Technology. DCC, SIAH1, and SIAH2 polyclonal antibodies were purchased from Santa Cruz Biotechnology. siRNA targeting both bovine and mouse SIAH1 (5′-GATAGGAACACGCAAGCAA-3′), SIAH2 (5′-CCAATGCCGCCAGAAGTTG-3′), and scrambled control siRNA were customized from Thermo Scientific.

Cell Culture and Transfection. BAECs (Cell Systems) were cultured in media 199 containing 10% (vol/vol) FBS as previously described (1–4). One day post confluent cells were starved in media containing 5% (vol/vol) FBS overnight before experiments. C2C12 and HEK293T cells (ATCC) were cultured in DMEM supplemented with 10% (vol/vol) FBS. Proliferating endothelial cells, C2C12, or HEK293T cells at 85% confluence were transfected with 100 nmol/L siRNA or indicated amount of plasmids using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h later for Western blot analysis of protein levels. HEK293T cells were used for all exogenous plasmid transfection experiments, whereas BAECs were used to detect changes in endogenous proteins. C2C12 cells were used for RNAi experiments in mouse cells.

Immunoprecipitation and Western Blotting. HEK293T cells in 35-mm dishes or BAECs in 100-mm dishes were lysed in 500 μL of IP buffer [50 mM Tris·HCl (pH 8.0); 150 mM NaCl; 1% Nonidet P-40; Protease Inhibitor Mixture 1:100) for 30 min at 4 °C on a rotating wheel (3). After centrifugation the supernatants were preclarified by incubating with protein A/G-Sepharose beads for 1 h. After the beads were discarded by centrifugation, the lysates were then immunoprecipitated with 1 μg HA or DCC antibody overnight at 4 °C. The protein A/G-agarose beads were added for an additional 2 h incubation at 4 °C. The beads were washed six times with 300 μL of cell lysis buffer and then boiled in 2× SDS sample buffer, and finally analyzed by Western blotting for protein expression. For Western blotting, ∼100 μg of protein was separated by 10% (wt/vol) SDS/PAGE, transferred to nitrocellulose membranes, and probed with DCC/SIAH1/SIAH2 (1:100) or Myc/HA (1:1,000) antibodies following standard Western blotting procedure.

In Vivo RNAi and Langendorff Perfusion. The siRNA were prepared in nanoparticle-based in vivo transfection reagent (Altogen Biosystems) for in vivo delivery as previously described (5). Male C57BL/6 mice (8-12 wk old) were obtained from Charles River Laboratories. The $DCC^{+/-}$ breeding colony was kindly provided by Dr. Marc Tessier-Lavigne from Rockefeller University. We performed tail vein injection of SIAH1/2 siRNAs (once every 24 h, ×2, 7.5 nmol each time) to induce in vivo silencing of SIAH1/2 protein expression. Forty eight hours later, the mice were anesthetized with i.p. pentobarbitone (60 mg/kg). Hearts were harvested immediately, and the aortas were cannulated with a 20-gauge stainless steel blunt needle and transferred to the Langendorff rig and perfused retrograde instantly with modified Krebs–Henseleit buffer for 30 min as previously described (6, 7). Then hearts were preperfused for 45 min with or without netrin-1 (100 ng/mL; R&D Systems) before being subjected to I/R injury (20-min global ischemia followed by 60-min reperfusion with or without netrin-1). Hearts were then harvested for analyses of infarct size.

Infarct Size Analysis. At the end of I/R protocol, hearts were sliced perpendicular to the long axis of the heart at 1-mm intervals and stained with 1% TTC in PBS for 10 min at room temperature. After washing with PBS once, sections of the hearts will be fixed in 10% (vol/vol) formalin overnight. The heart slices were then digitally photographed for planimetry using NIH Image 1.62. Infarct size is expressed as an infarct-to-risk zone ratio (the risk zone is the whole ventricular volume in this global ischemic model).

In Vivo Murine Model of Myocardial I/R Injury. After tail vain injection of siRNA (once every 24 h, ×2, 15 nmol each time), mice were premedicated with heparin (1,000 IU/kg, i.p.) and anesthetized 5 min later with sodium pentobarbital (60 mg/kg, i.p.). An additional dose of pentobarbital (50 μL; 20 mg/kg, i.p.) was given as needed to maintain anesthesia. After an adequate depth of anesthesia is attained, the mouse is fixed in a supine position with tape. Mice were then orally intubated and ventilated mechanically with a Harvard Apparatus Rodent Ventilator (model 845). A mix of oxygen and carbon dioxide (95:5%) was supplied, and body temperature was monitored using a rectal probe thermometer and controlled with a heating pad. Left thoracotomy was performed to reveal the LCA. Myocardial ischemia was achieved by tying a 7-0 Prolene thread around the LCA, which was then subsequently confirmed by the occurrence of regional cyanosis. The LCA was completely occluded for 30 min, and reperfusion was initiated by removal of the 7-0 suture. Reperfusion was confirmed by visualization of a hyperaemic response. The chest wound was then reapproximated, and mice were extubated and allowed to recover with supplemental oxygen until mobile. All mice received buprenorphine (0.1 mg/kg) s.c. to minimize pain. Twenty-four hours later, mice hearts were harvested for analyses of infarct size.

Echocardiography. Twenty-four hours after in vivo myocardial I/R injury, cardiac morphology and function were assessed on anesthetized (0.6–0.8% isoflurane in 95% oxygen; heart rate 430–450 beats per minute) mice by transthoraic echocardiography (Vevo2100 echocardiograph with MS-400 probe; Visualsonics). Two-dimensional images and M-mode tracing were recorded from the parasternal short axis view at the midpapillary level to determine the left ventricular internal diastole diameter (LVID:D) and left ventricular internal systolic diameter (LVID:S). Fractional shortening and ejection fraction were calculated directly from the short axis view of heart contraction.

Immunofluorescence Microscopy. In vivo RNAi-treated mouse hearts were frozen in optimum cutting temperature compound (OCT) and sectioned at 5 μm. After fixation in 4% (vol/vol) formaldehyde for 10 min, the slides with tissue were washed with PBS three times. Then slides were resolved by 0.2% Triton X-100 for 15 min at room temperature. Sections were blocked by block buffer [10% (vol/vol) donkey serum, 1% BSA in PBS] for 1 h at room temperature. The diluted antibody against SIAH1 or SIAH2 (1:50) was placed as a drop on the slides and incubated overnight at 4 °C in a humidified

chamber. The sections were then washed and covered with Alexa Fluor 488 donkey anti-goat IgG antibody (1:100; Invitrogen) for 1 h in the dark. After washing with PBS three times, coverslips were mounted on slides using ProLong antifade with DAPI (Invitrogen). The fluorescent images were captured using a Leica TCS-SP Confocal Microscope and analyzed with LCS Lite software.

Statistical Analysis. Densitometric data of Western blotting was obtained by ImageJ software. Grouped data were analyzed by Gradpad Prism 6 software. All values are expressed as mean ± SEM. Comparisons of more than two groups were performed using one-way ANOVA with the Newman-Keuls test as a post hoc test. Statistical significance was set as $P < 0.05$.

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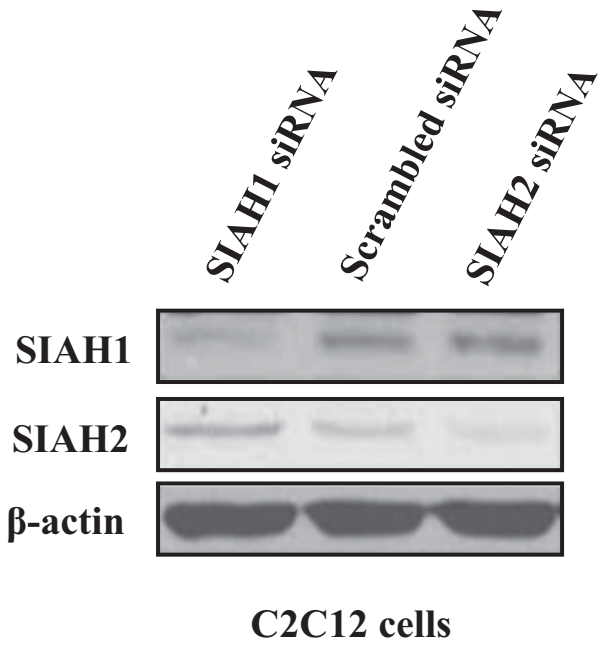

Fig. S1. SIAH1 and SIAH2 compensate for each other when expression of either is reduced. C2C12 cells were transfected with 100 nmol/L scrambled siRNA or siRNA for SIAH1, SIAH2 for 48 h before being harvested and subjected to Western blot analyses. SIAH1 and SIAH2 were detected using anti-SIAH1 antibody and anti-SIAH2 antibody, and actin was used as an internal control. RNAi silencing of SIAH1 or SIAH2 resulted in compensatory up-regulation of SIAH2 or SIAH1, respectively, in C2C12 cells.

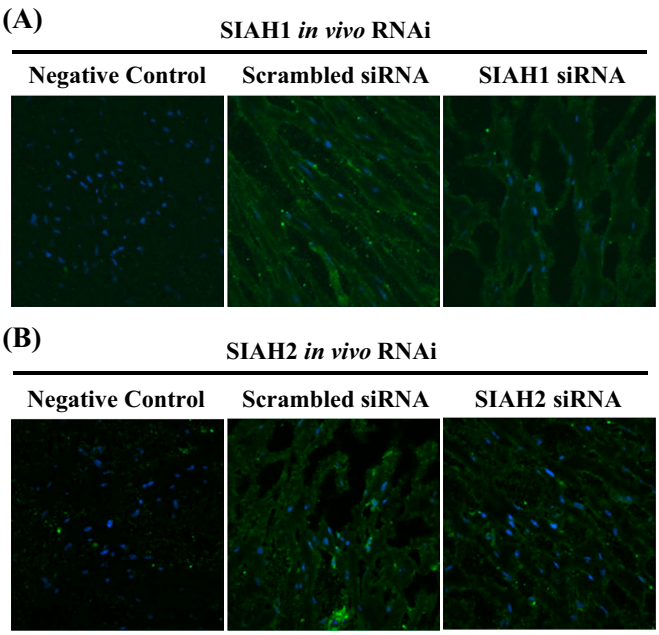

Fig. S2. In vivo RNAi silences SIAH expression in murine heart. Mice were transfected with SIAH1 siRNA, SIAH2 siRNA, or their corresponding scrambled siRNAs (every 24 h, 7.5 nmol each time) via tail vain injection. Sections of the hearts were incubated with or without SIAH1 or SIAH2 antibody (green). Representative micrographs are presented for SIAH1 in vivo RNAi (*A*) and SIAH2 in vivo RNAi (*B*).