Central role of SIAH inhibition in DCC-dependent cardioprotection provoked by netrin-1/NO

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Deleted in colorectal cancer (DCC), a large transmembrane receptor of netrin-1, is critical for mediating netrin-1’s cardioprotective function. In the present study we investigated novel mechanisms underlying netrin-1–induced, rapid, and feed-forward up-regulation of DCC, which is believed to sustain nitric oxide (NO) production to potentiate cardioprotection. Intriguingly, NO markedly reduced expression of the E3 ubiquitin ligase seven in absentia homolog (SIAH) that is specific for regulation of proteosome-dependent DCC degradation, resulting in accumulation of DCC. The two SIAH isoforms compensate for each other when one is repressed; inhibition of both SIAH1 and SIAH2 using combined siRNAs significantly reduced infarct size while improving cardiac function after ischemia/reperfusion injury of the heart. This effect was absent in DCC-deficient mice. Moreover, in vivo RNAi inhibition of SIAH1/2 further augmented netrin-1’s cardioprotective function. In summary, these data identify a novel therapeutic target of SIAH in facilitating NO/netrin-1–dependent cardioprotection, using the DCC receptor. Combination of netrin-1 and SIAH RNAi may prove to be a substantially effective therapy for myocardial infarction.

SIAH | netrin-1 | nitric oxide | DCC | cardioprotection

D eleted in colorectal cancer (DCC), a single transmembrane receptor of netrin-1, was first discovered in 1990 during the search for candidate tumor suppressor genes in chromosome 18q (1). At present DCC is known to play important roles in the following biological processes: guidance of developing axons (2, 3), conditional control of apoptosis (4), tumorigenesis (5), and angiogenesis (6, 7). DCC-dependent production of nitric oxide (NO) mediates angiogenic responses of endothelial cells (6). We have recently identified a novel and robust effect of netrin-1 in cardioprotection (8, 9). Netrin-1 induces cardioprotection against ischemia/reperfusion (I/R) injury both ex vivo and in vivo, which is mediated by DCC/ERK1/2/eNOS/NO-dependent protection of cardiomyocytes from necrosis and apoptosis, mediated by NO-dependent attenuation of oxidative stress and preservation of mitochondrial function (6–10). A deficiency of DCC abolished the protective effects of netrin-1 against cardiac I/R injury, implying a crucial role of DCC in mediating netrin-1–induced cardioprotection (8, 10).

Although it is a large transmembrane protein, DCC protein abundance was significantly up-regulated by netrin-1 in I/R-injured heart within a very short time of 2 h (8). Intriguingly, the DCC accumulation induced by netrin-1 was attenuated by the NOS inhibitor L-NAME, implicating an NO-dependent mechanism (8). This NO-DCC feed-forward regulation, in addition to the initial DCC/ERK1/2/eNOS/NO pathway, may be extremely beneficial in potentiating netrin-1–dependent cardioprotection.

It is possible that a proteosome dependent mechanism was involved in this quick action of protein regulation. Protein ubiquitination plays a key role in determining the half-life of proteins in mammalian cells. In ubiquitin-proteosome systems, E3 ubiquitin ligases recognize target substrates to induce transfer of ubiquitin from an E2 ubiquitin-conjugating enzyme to the substrates (11). Several specific E3 ligases play important roles in cardiac physiology and pathophysiology (12). We hypothesize that some specific E3 ligases might be involved in quickly regulating DCC in response to an increase in NO production, contributing to NO-mediated cardioprotection.

In the present study we fully characterized a novel role of an E3 ubiquitin ligase, SIAH (seven in absentia homolog), in mediating NO up-regulation of DCC to induce cardioprotection. Here we found that NO donor rapidly increased protein abundance of DCC by decreasing its level of ubiquitination, consequent to NO inhibition of SIAH. Inhibition of SIAH by in vivo RNAi significantly reduced infarct size to improve cardiac function, measured by echocardiography. Combined with netrin-1, SIAH inhibition further increased cardioprotective potency of netrin-1. Therefore, through accumulating DCC, SIAH inhibition can induce cardioprotection alone, or enhance netrin-1/NO–induced cardioprotection. Without doubt, SIAH can be developed as a novel target for the treatment of acute myocardial infarction, particularly for cardiac I/R injury induced by angioplasty.

Results

NO Donor Increases Protein Abundance of DCC in Endothelial Cells.

Our previous results have shown that DCC protein abundance was rapidly increased in netrin-1 perfused hearts during I/R injury, which is mediated by NO (8). Evidence from previous studies has also shown that endothelial cells can promote cardiomyocytes survival (13, 14). Indeed, activation of eNOS by netrin-1/DCC/ERK1/2 pathway increases NO production to result in cardioprotection. We hypothesize that NO-DCC feed-forward regulation occurs in endothelial cells to facilitate more NO production that is diffused to underneath cardiomyocytes to promote survival and preserve function. To test this hypothesis, we first examined the effect of NO donor on DCC protein abundance in

Significance

Therapies for myocardial infarction consequent to ischemia/reperfusion injury (I/R) have been lacking. The netrin-1 receptor deleted in colorectal cancer (DCC) mediates netrin-1–dependent cardioprotective signaling. It is anticipated that any means of augmenting DCC signaling may induce cardioprotection on its own and enhance netrin-1/NO–dependent cardioprotection. The present study identifies a novel mechanism by which NO upregulates DCC abundance. NO inhibits seven in absentia homolog (SIAH), an E3 ubiquitin ligase that suppresses DCC. Inhibition of SIAH by in vivo RNA interference markedly reduces infarct size to improve cardiac function that was determined by echocardiography. Combined with netrin-1 perfusion, it further potentiated netrin-1’s cardioprotective effect. SIAH may therefore serve as a novel therapeutic target for myocardial infarction, particularly those suffering from cardiac I/R injury.

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endothelial cells. As shown in Fig. 1, exposure of bovine aortic endothelial cells (BAECs) to NO donor dimethylamine NONOate (MAHMA NONOate) resulted in a time-dependent increase in DCC protein expression, which occurred as quickly as 5 min after treatment (1.65 ± 0.16-fold vs. 0 min, \( P < 0.01 \)). The response maximized at 30 min (2.15 ± 0.20-fold vs. 0 min, \( P < 0.001 \)). These data implicate that NO can rapidly up-regulate protein abundance of DCC in endothelial cells.

**SIAH1 and SIAH2 Regulate DCC Stability via a Ubiquitin-Proteasome Pathway.** Remarkably, netrin-1 is able to feed-forwardly up-regulate its large transmembrane receptor DCC, which is required for NO production to mediate cardioprotection, as shown above and previously (8). NO donor increased protein abundance of DCC in endothelial cells within a very short time, which suggests a potential involvement of a proteasome-dependent degradation pathway. We then treated BAECs with the proteasome inhibitor MG132 for 0, 5, 15, 30, and 60 min and determined the protein levels of endogenous DCC by Western blots. Of note, MG132 treatment rapidly elevated DCC protein levels (Fig. 2A), suggesting that DCC stability is regulated by a proteasomal pathway.

The E3 ubiquitin ligases SIAH1 and SIAH2 have been implicated in the regulation of DCC stability in mammalian cells. SIAH1 shares 77% amino acid identity with SIAH2 in humans (15–17). To examine a regulatory role of SIAH1 and SIAH2 in DCC degradation, we generated SIAH1 and SIAH2 dominant-negative (DN) mutants (18). Cotransfection with SIAH1 or SIAH2, but not their DN mutants, dose-dependently diminished DCC protein abundance in HEK293T cells (Fig. 2B). Moreover, coimmunoprecipitation experiments indicated that both SIAH1 and SIAH2 can interact with DCC (Fig. 2C). To determine whether SIAH1 and SIAH2 affect DCC ubiquitination, we performed an in vivo ubiquitination assay. DCC was seen as a strong smear of bands when it was coexpressed with WT SIAH1 or SIAH2, but a much weaker smear of bands in the presence of SIAH1-DN or SIAH2-DN, which was similar to the control lane (Fig. 2D). Taken together, these data strongly suggest that SIAH1 and SIAH2 regulate DCC stability via a ubiquitin-proteasome pathway.

**SIAH Inhibition Mediates NO Regulation of DCC.** Our data above confirmed a role of SIAH1 and SIAH2 in regulating DCC degradation. We next aimed to determine whether this pathway is involved in NO up-regulation of DCC. We examined the effect of NO donor on the ubiquitination level of endogenous DCC. After treatment with NO donor MAHMA NONOate, the polyubiquitinated forms of DCC were immunoprecipitated and detected by Western blots. Of note, NO donor markedly reduced ubiquitination of endogenous DCC (Fig. 3A). Furthermore, NO donor time-dependently abrogated SIAH1 expression, which corresponded well with an elevation in DCC abundance (Fig. 1 and Fig. 3B and D). These data indicate that SIAH1-dependent ubiquitination of DCC was attenuated by NO donor to result in DCC up-regulation.

However, SIAH2 protein level was not affected by NO donor stimulation (Fig. 3C and D). It was puzzling at first, but our data showed that the SIAH isoforms compensate for each other (see below). To determine whether overexpression of SIAH1 can block NO up-regulation of DCC, we transfected Myc-SIAH1 or Myc-SIAH2 constructs into endothelial cells. Overexpression of either SIAH1 or SIAH2 prevented DCC up-regulation by NO donor (Fig. 3E and F). These data reveal a central role of SIAH inhibition in mediating NO-dependent up-regulation of DCC.

**SIAH1 and SIAH2 Compensate for Each Other.** To explore whether genetic inhibition of SIAH1 can promote DCC accumulation, we performed RNA interference experiments. SIAH1 and SIAH2 targeting siRNAs markedly decreased endothelial expression of SIAH1 and SIAH2 proteins to 39.3 ± 9.6% and 58.6 ± 3.0%
of control, respectively (Fig. 4 A, B, D, and E), similar to what was shown in other cell lines (19, 20). Intriguingly, SIAH1 or SIAH2 were able to compensate for each other when one was suppressed, because RNAi for SIAH1 led to up-regulated SIAH2 by 1.37 ± 0.11-fold (Fig. 4C), and vice versa (1.34 ± 0.10-fold for SIAH1 when SIAH2 RNAi was applied; Fig. 4F). This observation was also confirmed in a mouse myoblast cell line C2C12 (Fig. S1). Cotransfection of both SIAH1 and SIAH2 siRNAs into endothelial cells increased protein abundance of DCC by 1.57 ± 0.06-fold (Fig. 5A). These data showed that SIAH1 and SIAH2 compensate for each other to synergistically regulate DCC protein abundance.

**Inhibition of SIAH Induces and Potentiates Cardioprotection ex Vivo.**

In view of the potent effect of SIAH in mediating NO regulation of DCC, we examined whether attenuation of SIAH leads to cardioprotection during I/R injury with or without nitren-1 perfusion. A combination of SIAH1 and SIAH2 siRNAs were delivered in vivo via tail vein injection, following our published protocol that demonstrates efficacy of in vivo RNAi (21). As shown in Fig. S2 A and B, results of immunofluorescent assay confirmed successful knockdown of corresponding SIAH isoforms in vivo in mouse heart. Intriguingly, in vivo RNAi of SIAH1/2 resulted in up-regulation of DCC protein abundance in I/R-injured hearts by 2.92 ± 0.39-fold (Fig. S2B). These data further demonstrate that SIAH1 and SIAH2 regulate DCC in mouse hearts.

I/R injury was performed in mouse hearts using a Langendorff system, as illustrated in Fig. 6A. In vivo RNAi inhibition of SIAH induced a significant reduction in infarct size compared with controls (40.6 ± 4.5% vs. 55.6 ± 1.4% for SIAH1/2 siRNA treated I/R vs. scrambled siRNA treated I/R, P < 0.01; Fig. 6 B and C). In additional experiments, a combinatory effect of nitren-1 and SIAH in vivo RNAi was examined. Our previous studies have established nitren-1’s cardioprotective role against I/R injury both ex vivo and in vivo (8–10). In scrambled siRNA treated group, nitren-1 reduced infarct size from 55.6 ± 1.4% to 27.7 ± 3.6% (P < 0.001). In vivo RNAi inhibition of SIAH further reduced infarct size of the nitren-1 treated group to 15.2 ± 1.2% (P < 0.01). These data demonstrate a clear additive effect of SIAH inhibition in affording augmented cardioprotection against I/R injury.

**SIAH Inhibition Attenuates I/R-Induced Myocardial Infarction and Improves Cardiac Function in Vivo.**

To explore whether lowered SIAH levels have any effect on I/R damage in vivo, RNAi-treated mice were subjected to a 30 min of ischemia by coronary occlusion, followed by a 24-h reperfusion (Fig. 7A) (9). As shown in Fig. 7 B–E, in agreement with our ex vivo data, SIAH1/2 siRNA alone significantly decreased infarct size compared with the scrambled siRNA group. Mice receiving scrambled siRNA displayed a 35.7 ± 4.5% infarct size per area at risk (Inf/AAR) and a 19.6 ± 3.2% infarct size per left ventricle (LV) size (Inf/LV). The SIAH1/2 siRNA-treated group, however, developed infarct of 17.4 ± 2.3% for Inf/AAR and 9.5 ± 1.1% for Inf/LV. The percentage AAR per LV (AAR/LV) was similar among different groups, indicating similar severities of myocardial ischemia.

To further validate cardioprotective properties of SIAH inhibition in vivo, we measured cardiac function via echocardiography on animals that underwent I/R injury. The results, shown in Fig. 7 F–H, illustrated that both fractional shortening and ejection fraction were significantly increased in SIAH1/2 siRNA-treated mice treated with 100 nmol/L scrambled siRNA or siRNA for SIAH1 (A–C), SIAH2 (D–F) 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. Grouped densitometric data of SIAH1 or SIAH2 protein levels are presented as mean ± SEM (n = 3). **P < 0.01, *P < 0.05.
were significantly increased in SIAH1/2 siRNA plus netrin-1-treated hearts at 1 d after I/R, compared with scrambled siRNA treated hearts, suggesting improvement in cardiac function.

**Inhibition of SIAH Augments Netrin-1 Induced Cardioprotection in Vivo.** To further examine whether SIAH inhibition is effective in potentiating cardioprotection induced by netrin-1 in vivo, the same coronary artery occlusion assay was performed. Netrin-1 was injected into the LV lumen at the onset of reperfusion at a dosage as small as 5 μg/kg. Our recent findings have established netrin-1’s cardioprotective function in this model (9). Data in Fig. 8 demonstrated that inhibition of SIAH markedly augmented the cardioprotective effect of netrin-1 (Fig. 8 A–D). The scrambled siRNA plus netrin-1 group displayed an 18.5 ± 2.5% Inf/AAR and a 9.8 ± 0.9% Inf/LV. The SIAH1/2 siRNA plus netrin-1 group showed a 12.1 ± 1.0% Inf/AAR and a 6.7 ± 0.9% Inf/LV. These data indicate robust additive effects of cardioprotection induced by in vivo RNAi of SIAH and netrin-1 perfusion. Of note, AAR/LV was similar for both groups, indicating similar degree of ischemic injury.

Moreover, cardiac function was measured by echocardiography. There was no obvious difference between the two groups at 1 d after I/R. However, fractional shortening and ejection fraction were significantly increased in SIAH1/2 siRNA plus netrin-1-treated hearts 3 d after I/R, compared with scrambled siRNA plus netrin-1-treated hearts (Fig. 8 E–G).

**SIAH Inhibition-Induced Cardioprotection Is Absent in DCC-Deficient Mice.** To further confirm whether SIAH inhibition-induced cardioprotection is mediated by DCC, hearts isolated from DCC+/− mice and WT littersmates were subjected to I/R injury using a Langendorff system. DCC deficiency on its own increased infarct size after I/R injury (52.4 ± 3.2% vs. 39.2 ± 2.5% for DCC+/− mice vs. WT mice, P < 0.05; Fig. 9 A and B), implicating an important role of endogenous DCC in cardioprotection. Moreover, as shown in Fig. 9 C and D, in vivo RNAi inhibition of SIAH1/2 had no additional effect on infarct size in DCC+/− mice (SIAH1/2 siRNA group, 55.4 ± 5.3% vs. scrambled siRNA group, 54.4 ± 7.5%). These data indicate that cardioprotection afforded by SIAH inhibition is mediated by DCC.

**Discussion**

The most significant finding of the study is the innovative identification of an NO/SIAH/DCC pathway in DCC-dependent cardioprotection, and its role in potentiating netrin-1/NO–provoked cardioprotection. NO down-regulates total SIAH protein abundance, leading to decreased degradation of DCC via a ubiquitin–proteasome pathway. This feed-forward loop is believed to augment cardioprotective signaling of netrin-1/DCC/ERK1/2/eNOS/NO by accumulation of DCC receptor. SIAH inhibition not only effectively reduced infarct size after I/R injury, but also markedly improved cardiac function. More importantly, it also markedly potentiated netrin-1’s cardioprotective effects. These findings define a central mediator role of SIAH in DCC-dependent cardioprotection provoked by netrin-1/NO.

As we described earlier, DCC protein level is critical for netrin-1 signaling. Several previous studies have provided evidence for mechanisms of regulation for DCC. For example, Kuo et al. (22) have shown that a zinc finger transcription factor, Bcl11A/CTIP1, regulates expression of DCC to control axon branching and dendrite outgrowth. In addition, Fu and colleagues identified that Drosophila Sina and its mammalian homologs SIAH can regulate DCC protein level via proteasome-dependent mechanisms (15, 16). In the present study, endothelial DCC protein accumulated in response to NO donor within a very short time. Because DCC is a large protein, we hypothesized that this quick response is at the posttranslational level, and likely through a proteasome signaling pathway. Indeed, reduced ubiquitination of endogenous DCC after NO donor treatment was observed. A proteasome signaling pathway has been considered as an important mechanism in regulating protein degradation. Three enzymes, known as E1, E2, and E3, act in series to catalyze ubiquitination (23). Among them, E3 ligase is thought to play a role in recognizing the specific substrate protein (24). We therefore next examined a role of SIAH E3 ubiquitin ligase in NO modulation of DCC protein stability.
Our findings showed that both SIAH family members SIAH1 and SIAH2 regulate DCC protein abundance through direct interaction, resulting in increased ubiquitination of DCC in HEK293T cells, which suggests functional redundancy among the members of the family. It is not surprising that the same substrate can be degraded by one or more E3 ubiquitin ligase family members, because they share a similar motif for substrate binding. For example, TGF-β receptor can be degraded by four members of the Nedd4-like family, including Smurf1, Smurf2, WWP1, and Nedd4-2 (25). House et al. (26) identified a peptide motif (RPVAxVxPxxR) that mediates the interaction of SIAH protein with an array of protein partners. The relative contribution of E3 members might be dependent on the cellular context and distribution of each member in different cell types or tissues. More importantly, we have further identified the involvement of SIAH in NO up-regulation of DCC, to our knowledge for the first time. In our study, NO donor diminished SIAH protein abundance, whereas overexpression of SIAH blocked NO up-regulation of DCC. These data establish a novel rapidly responsive NO/SIAH/DCC signaling pathway in endothelial cells that is important for induction of cardioprotection.

Another interesting finding is that SIAH1 and SIAH2 compensate for each other in both BAECs and C2C12 cells. The inhibition of one isoform increases protein abundance of the other, in which the organism ensures enough functional proteins at work. This seems to share some similarities with other E3 systems. For example, loss of Pellino 1 E3 ubiquitin ligase in peripheral innate

**Fig. 7.** SIAH inhibition attenuates I/R-induced myocardial infarction and improves cardiac function in vivo. (A) SIAH1/2 siRNAs were delivered by tail vain injection. I/R injury of the heart was induced by 30 min of left coronary artery (LCA) ligation, followed by a 24 h of reperfusion in WT C57BL6 mice in vivo. Evans blue was used to visualize the nonischemic area. Sections of hearts were stained with 2,3,5-TTC. (B) Representative TTC staining of I/R-injured hearts. The white area indicates infarct zone, whereas the blue area indicates noninfarcted area. The red and white areas represent area at risk. (C–E) Infarct size analyzed by (C) AAR/LV, (D) Inf/LV, and (E) Inf/AAR. The results are represented as means ± SEM (n = 4). *P < 0.05. (F–H) Echocardiography was performed on in vivo RNAi-ed WT mice reperfused for 24 h after 30 min of LCA ligation. Ejection fraction and fractional shortening were measured. (F) Representative echocardiography data of scrambled siRNA group and SIAH1/2 siRNA group. (G) Grouped ejection fraction (EF) and (H) grouped fractional shortening (FS) data (n = 6). *P < 0.05.

**Fig. 8.** Inhibition of SIAH augments netrin-1–induced cardioprotection in vivo. SIAH1/2 siRNAs were delivered by tail vain injection. I/R injury of the heart was induced by 30 min of LCA ligation followed by 24 h of reperfusion in WT C57BL6 mice in vivo. Netrin-1 was injected into the LV lumen at the onset of reperfusion at doses of 5 μg/kg. Evans blue was used to visualize the nonischemic area. Sections of hearts were stained with 2,3,5-TTC. (A) Representative TTC staining of I/R-injured hearts. The white area indicates infarct zone, whereas the blue area indicates noninfarcted area. The red and white areas represent area at risk. (B–D) Infarct size analyzed by (B) AAR/LV, (C) Inf/LV, and (D) Inf/AAR. The results are represented as Means ± SEM, n = 5. *P < 0.05. (E–G) Echocardiography was performed on in vivo RNAi-ed WT mice reperfused for 72 h after 30 min of LCA ligation. Ejection fraction and fractional shortening were measured. (E) Representative echocardiography data of scrambled siRNA with netrin-1 group and SIAH1/2 siRNA with netrin-1 group. (F) Grouped EF and (G) grouped FS data (n = 4). **P < 0.01.
The cardioprotective role of SIAH1/2 inhibition alone was mediated by endogenous SIAH/DCC pathway, because DCC−/− mice displayed no cardioprotection in response to SIAH inhibition. In addition, in vivo SIAH1/2 RNAi, combined with netrin-1 perfusion, markedly augmented cardioprotective effects of netrin-1. The effects and mechanisms of netrin-1 alone have been established by our previous work (8–10, 28). Taken together, reduced SIAH1/2 can work very well either alone or in combination with netrin-1 to induce robust cardioprotection.

In summary, the present study characterized a novel signaling mechanism whereby NO up-regulates DCC by inhibition of its E3 ubiquitin ligase SIAH, leading to sustained netrin-1/NO signaling to provoke cardioprotection. SIAH can quickly regulate DCC protein abundance through a ubiquitin-proteasome pathway. RNAi inhibition of SIAH proved to be beneficial in reducing infarct size and improving cardiac function by accumulation of DCC. In addition to our previously established netrin-1/DCC/ERK1/2/eNOS/NO signaling, the feed-forward NO/SIAH/DCC signaling also augments cardioprotection induced by netrin-1/NO. This study may facilitate development of novel therapeutics targeting SIAH, for the treatment of myocardial infarction, particularly in patients suffering from cardiac I/R injury.

**Materials and Methods**

Detailed information of materials, cell culture and transfection, immunoprecipitation and Western blotting, in vivo RNAi and Langendorff perfusion, infarct size analysis, in vivo murine model of myocardial I/R injury, echocardiography, immunofluorescence microscopy, and statistical analysis are provided in **SI Materials and Methods**. The use of animals and experimental procedures were approved by the Institutional Animal Care and Usage Committee at the University of California, Los Angeles (UCLA).

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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′-GATAGGAAACACGCAAGCAA-3′), SIAH2 (5′-CCATGTCCGCGAAGTGTG-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 cell lines (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 nl/mL 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 100 μl/mL 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.

In Vivo RNAi and Langendorff Perfusion. The siRNAs 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, x2, 7.5 nmol each time) to induce in vivo silencing of SIAH1/2 for 24 h, and perfused retrograde 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 vein injection of siRNA (once every 24 h, x2, 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 reaproximated, 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.

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 transthoracic 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 diastolic 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 Graphpad 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 \( P < 0.05. \)


**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 vein 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).