## SUPPLEMENTAL MATERIALS AND METHODS

### Cell culture

NECs were isolated and cultured as previously described (1). For low pH experiments 5mM HEPES and MES were added to the media and pH adjusted with HCl.

### Animals

The UTSW IACUC approved all animal protocols. Adult ICR wild type or TNR transgenic male mice were injected ip once daily for seven days with the indicated small-molecule (16mg/kg) formulated in 20% w/v 2-hydroxypropyl-β-cyclodextrin (Sigma). Animals were observed daily and sacrificed 7 days post-injury unless otherwise noted.

### Surgical procedures

The UTSW IACUC approved all animal protocols. Adult ICR wild type or TNR transgenic male mice were subjected to LAD-MI or sham surgery. Mice were anesthetized with 2.4% isoflurane and intubated. For MI, the LAD was visualized and ligated with a (6-0) prolene-suture. Animals were observed daily and sacrificed at day 7 post-injury.

### RNA extraction/QPCR

Total RNA was isolated with Trizol (Invitrogen) and reverse transcription performed using the Superscript First-Stand Synthesis System (Invitrogen). QPCR was performed using SYBR Green QPCR or Taqman Mastermix (ABI) and relative quantification of gene expression was calculated using the comparative method (ΔΔCT) with Gapdh as the endogenous control and specific control calibrator as described (1). Neonatal heart was used as positive control. Primer sequences are available upon request.

### Immuno-cyto/histochemistry

Cells plated on coverslips were fixed with 10% neutral buffered formalin for 10 minutes at room temperature and stained with mouse anti-sarcomeric α-actinin (EA-53) (Sigma). Paraffin-embedded sections from 10% neutral buffered formalin fixed adult mouse hearts were deparaffinized and treated with Pronase for antigen retrieval. Slides were stained with rabbit anti-GPR68 (3E6) (Novus) using the Tyramide Signal Amplification Kit (Molecular Probes) and co-stained with mouse anti-sarcomeric α-actinin (EA-53) (Sigma).

**Flow cytometry**

Antibodies: PE anti-CD45 (30-F11), PE anti-CD31 (MEC 13.3) (BD). All samples were stained as recommended by the manufacturer. After dissociation, cells were sorted and collected with the MoFlo sorter (Beckman-Coulter).

**Stable cell lines**

HeLa cells were co-transfected using FuGENE 6 (Roche) with pCMV-puromycin and GPR68-EGFP in a 1:10 ratio, respectively. After 48 hours, cells were subjected to positive selection using 1 μg/ml of puromycin (Sigma). Positive clones were validated with microscopy, flow cytometry and RT-PCR.

**Transfections/luciferase assays**

NECs were transfected with GPR68 or control siRNA (SCBT) using Lipofectamine 2000 (Invitrogen). After transfection overnight, cells were treated with the indicated small molecule. After 24 hours of treatment, cells were harvested in Trizol (Invitrogen) for RNA or Cell Lysis Buffer (Cell Signaling Technology) for luciferase assays. Luciferase activity of the lysates was assayed using BrightGlo (Promega) and normalized to protein concentration determined by the Pierce BCA protein assay kit (Thermo-scientific).

**Immunoblot analysis**

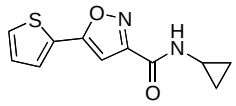
Protein extracts were prepared in RIPA buffer containing 1μM PMSF in the presence of a phosphatase inhibitor cocktail (Roche). Blots were probed with an anti-GFP rabbit polyclonal antibody (Invitrogen), anti-CTGF goat polyclonal (L-20) (Santa Cruz) and anti-alpha tubulin mouse monoclonal (B-5-1-2) (Sigma).

**$Ca^{2+}$ imaging**

Cells were plated on gelatin-coated glass coverslips (Deckgläser, Germany) and $Ca^{2+}$ imaging was performed using a PTI (Photon Technology International) Calcium Imaging System (Birmingham, NJ) with a 40X objective on an automated fluorescence microscope with CCD camera. NECs were loaded with 5 μM fura-2-acetoxymethyl ester (Fura-2 AM) in extracellular buffer (in mM, 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 Hepes) (EB) for 30 min at 37°C while shielded from light, then cells were washed twice with EB. The glass coverslips were fitted into the bottom of a perfusion chamber and the cells were perfused with EB at room temperature, and agonists/antagonists were delivered with the perfusate as described (3).

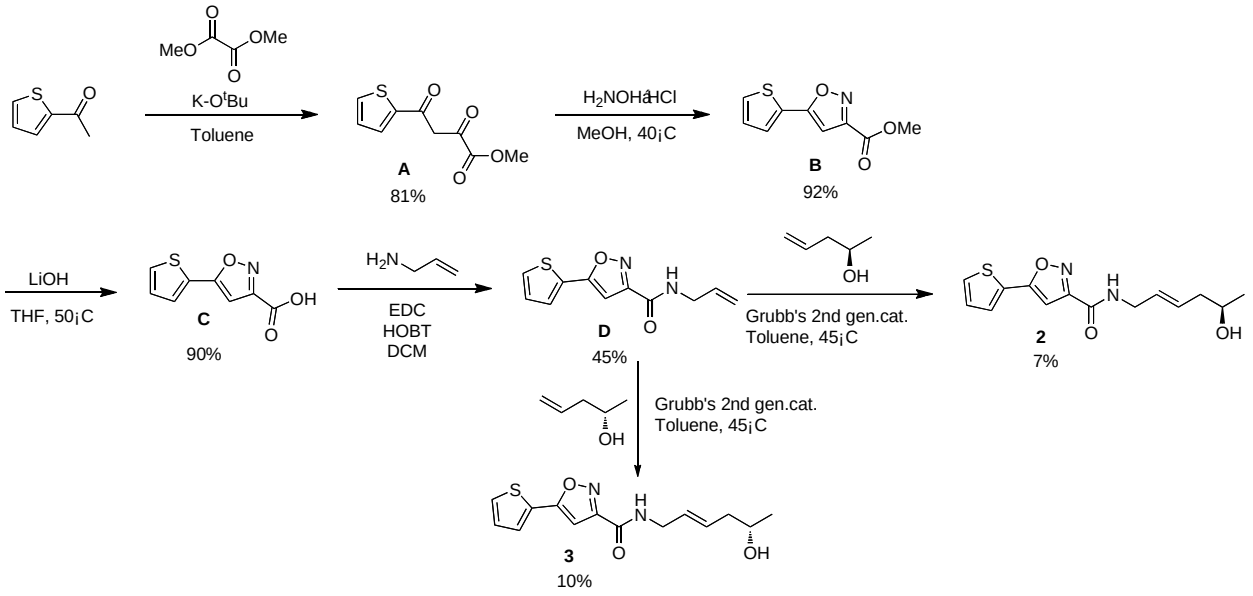
Compounds (Isx-1 or DMSO) were added and recording was started (time 0). For $Ca^{2+}$ free medium (in mM, 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 glucose and 10 HEPES, 2 EGTA) experiments and Isx-1 plus inhibitor experiments, cells were pre-treated with $Ca^{2+}$ free medium, calcium influx "cocktail" inhibitors (in µM, 10 Nifedipine, 100 $La^{3+}$, 10 SKF 96365) or calcium release "cocktail" inhibitors (in µM, 5 U73122, 30 2-APB, 100 Ryanodine) for 5 minutes before Isx-1 treatment. We measured Fura-2 fluorescence by illuminating the cells every 2 seconds with an alternating 340/380 nm light source and fluorescent images were captured at 510 nm. Changes in $[Ca^{2+}]_i$ were derived from changes in the ratio of fluorescence intensity at 340 and 380 nm. Data were analyzed and images captured using of PTI ImageMaster software.

**Synthetic procedures for Isx 1-4**



***N*-cyclopropyl-5-(thiophen-2-yl)isoxazole-3-carboxamide (Isx-1)** was synthesized as previously reported (*4*).

**General synthetic scheme for Isx-2 and -3.**




**Methyl 2,4-dioxo-4-(thiophen-2-yl)butanoate** (**A**)

50 mL of potassium tert-butoxide (1.0 M in THF) was added to a stirred solution of 2-acetylthiophene (40 mmol) and dimethyloxalate (52 mmol) in toluene (200 mL) at room temperature. The reaction was monitored by TLC and quenched with 1N HCl (100 mL) upon completion. The solution was transferred to a separatory funnel. The organic layer was washed

with deionized water (100 mL), followed by brine (100 mL), and dried with anhydrous sodium sulfate. The solution was concentrated to yield an orange solid, which was re-crystallized with hot hexanes to yield pure product in 81% yield.

**Methyl 5-(thiophen-2-yl)isoxazole-3-carboxylate** (**B**)

Hydroxylamine hydrochloride (36 mmol) was added to a stirred solution of **A** (30 mmol) in methanol (150 mL) and the reaction heated to 60°C under nitrogen atmosphere, and reaction completion monitored by TLC. When completed, the reaction was concentrated, yielding a light-brown solid, and water was added drop wise with stirring. After 1 hr, the solids were vacuum filtered and left to dry overnight. Product was isolated in 92% yield.

**5-(thiophen-2-yl)isoxazole-3-carboxylic acid** (**C**)

1.0 M LiOH (28 mL) was added to a stirred solution of **B** (16 mmol) in THF (18 mL) and the reaction heated to 50°C, and completion monitored by TLC. When completed, the reaction was extracted with toluene and the aqueous layer acidified to pH 1 with 3 M HCl. The aqueous layer was washed with ethyl acetate and the organic layer was washed with brine, dried with anhydrous sodium sulfate, and then concentrated to produce a brown solid in 90% yield.

**N-allyl-5-(thiophen-2-yl)isoxazole-3-carboxamide** (**D**)

EDC (9.3 mmol) and HOBT (9.3 mmol) were added to a stirred solution of **C** (7.7 mmol) in DCM (77 mL) at room temperature for 15 minutes. Allylamine (9.3 mmol) was added and the reaction completion monitored by TLC. When completed, the reaction was concentrated and resulting residue diluted with ethyl acetate. The solution was washed with water, brine, and dried with anhydrous sodium sulfate. After concentration, the crude product was an orange solid, which was purified by silica gel column chromatography using a 5% methanol 95% dichloromethane solution. The final product was a white crystalline solid in 45% yield.

**(*R*,*E*)-N-(5-hydroxyhex-2-enyl)-5-(thiophen-2-yl)isoxazole-3-carboxamide (Isx-2)**

**D** (1 mmol) was added to Grubb's 2$^{nd}$ generation catalyst (10 mol%) in a round bottom flask, which was fitted with a rubber septum and flushed with nitrogen. Toluene (10 mL) and R-(-)-4-Pentene-2-ol (10 mmol) were added via syringe and the reaction heated at 40°C overnight. The reaction did not reach completion and the product was isolated via PTLC as a white solid in 7% yield.

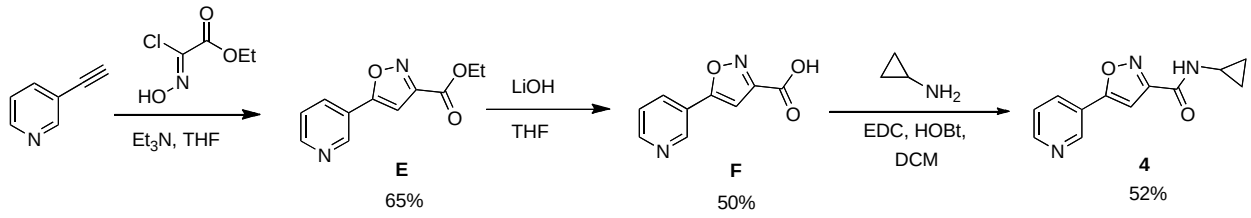
$^1$H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, *J*= 3.6 Hz,1H), 7.48 (d, *J*=5.1 Hz, 1H), 7.14 (t, *J*= 4.5 Hz, 1H), 6.87 (bs, 1H), 6.83 (s, 1H), 5.61-5.77 (m, 2H), 4.05 (t, *J*=5.7, 2H), 3.83-3.88 (m, 1H), 2.16-2.26 (m, 2H), 1.23 (d, *J*= 3.9 Hz,3H). MS (ESI) 293.1 (M+H), 315.0 (M+Na).

**(*S,E*)-N-(5-hydroxyhex-2-enyl)-5-(thiophen-2-yl)isoxazole-3-carboxamide (Isx-3)**

**D** (1 mmol) was added to Grubb's 2$^{nd}$ generation catalyst (10 mol%) in a round bottom flask, which was fitted with a rubber septum and flushed with nitrogen. Toluene (10 mL) and S-(+)-4-Pentene-2-ol (10 mmol) were added via syringe and the reaction heated at 40°C overnight. The reaction did not reach completion and the product was isolated via PTLC as a white solid in 10% yield.

$^1$H NMR (500 MHz, $CDCl_3$) δ 7.54 (d, *J*= 3.6 Hz,1H), 7.48 (d, *J*=5.1 Hz, 1H), 7.14 (t, *J*= 4.5 Hz, 1H), 6.87 (bs, 1H), 6.83 (s, 1H), 5.61-5.77 (m, 2H), 4.05 (t, *J*=5.7, 2H), 3.83-3.88 (m, 1H), 2.16-2.26 (m, 2H), 1.23 (d, *J*= 3.9 Hz,3H). MS (ESI) 293.1 (M+H), 315.0 (M+Na).

**General synthetic scheme for Isx-4.**




**Ethyl 5-(pyridin-3-yl)isoxazole-3-carboxylate (E)**

3-ethynylpyridine (5 mmol) was added to ethyl 2-chloro-2(hydroxyimino)acetate (15 mmol) in THF (30 mL) in a round bottom flask. Triethylamine (20 mmol) was slowly added and the reaction stirred at room temperature. When completed, saturated $NH_4Cl$ was added to the reaction and the aqueous layer washed with ether. The organic layers were combined and washed with water, brine, and then dried with anhydrous sodium sulfate. Following concentration, the residue was purified by silica gel column chromatography using 30% ethyl acetate/hexanes, and the product was a pale yellow solid in 65% yield.

**5-(pyridin-3-yl)isoxazole-3-carboxylic acid (F)**

1.0 M LiOH (3.5mL) was added to a stirred solution of **E** (2 mmol) in THF (5 mL). When completed, the reaction was partitioned between ethyl acetate and water, and the aqueous layer acidified to pH 1 with 3 M HCl. The white precipitate was rinsed with hexanes and isolated as a white solid product in 50% yield.

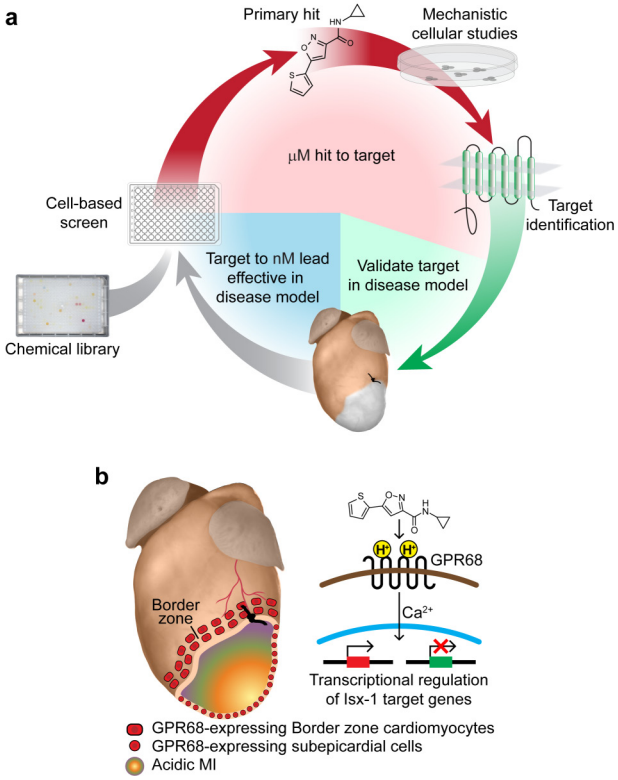
***N*-cyclopropyl-5-(pyridin-3-yl)isoxazole-3-carboxamide (4)**

EDC (0.39 mmol) and HOBT (0.32 mmol) were added to a stirred solution of **F** (0.26 mmol) in DCM (2 mL), and the reaction was stirred at room temperature for 15 minutes. Cyclopropylamine (0.32 mmol) was added and reaction completion monitored by TLC. When completed, the reaction residue was diluted with ethyl acetate, washed with water, brine, and then dried with anhydrous sodium sulfate. After concentration, the crude product was a yellow solid, which was purified via silica gel column chromatography using 50:50 ethyl acetate/hexanes solution, producing a white solid in 52% yield.

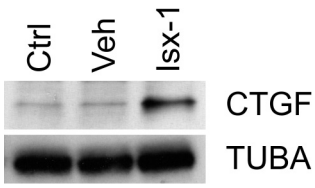
## REFERENCES


1. Russell, J. L., Goetsch, S. C., Gaiano, N. R., Hill, J. A., Olson, E. N., and Schneider, J. W. (2011) A dynamic notch injury response activates epicardium and contributes to fibrosis repair, *Circulation research 108*, 51-59.
2. Luo, X., Shin, D. M., Wang, X., Konieczny, S. F., and Muallem, S. (2005) Aberrant localization of intracellular organelles, Ca2+ signaling, and exocytosis in Mist1 null mice, *The Journal of biological chemistry 280*, 12668-12675.

## SUPPLEMENTAL FIGURES




**Supplemental Figure 1.** (**a**) Model of chemical biology discovery cycle. Cell-based screen of a chemical library generates a 1° hit that is effective in µM concentration and is useful for mechanistic cellular studies that lead to identification of a candidate target, which is validated in disease models. This target is then employed in target-based screening assays of focused chemical libraries to generate small-molecule compounds effective in nM concentration and suitable as starting points for drug discovery and development. (**b**) Model depicting unique GPR68-expressing cell subpopulations (red) in the injury-activated subepicardium and the infarct border zone, establishing a buffer zone that normally monitors ischemic myocardial pH through proton sensing GPCRs, but can also respond to Isx-1 to regulate downstream pro-survival and cardiomyogenic transcriptional circuits.




**Supplemental Figure 2.** Protein blotting for CTGF in NECS cultured for 4 days in media alone (Ctrl) or in the presence of DMSO (Veh) or 20µM Isx-1 with TUBA as a loading control.