Molecular Cell, Volume *54*

Supplemental Information

Mst1 Promotes Cardiac Myocyte Apoptosis through Phosphorylation and Inhibition of Bcl-xL

Dominic P. Del Re, Takahisa Matsuda, Peiyong Zhai, Yasuhiro Maejima, Mohit Raja Jain, Tong Liu, Hong Li, Chiao-Po Hsu, and Junichi Sadoshima

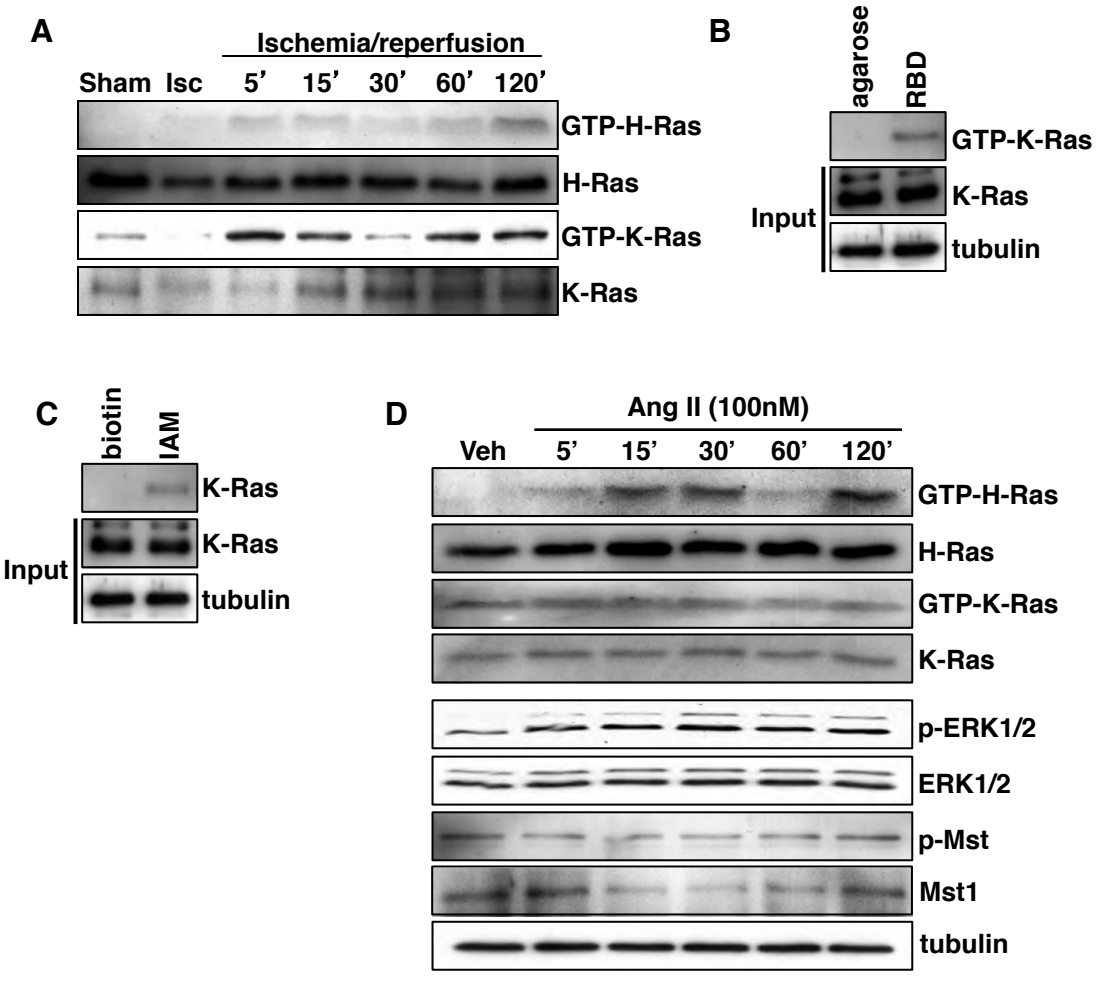


Figure S1. Characterization of Ras isoform activation, related to Figure 1.
(A) Timecourse of Ras isoform activation in wild-type mouse hearts subjected to ischemia/reperfusion.
(B) Representative control experiment for RBD pulldown assay.
(C) Representative control experiment for iodoacetamide (IAM)-biotin pulldown assay.
(D) Timecourse of Ang II-induced Ras and ERK1/2 activation in neonatal rat cardiac myocytes.

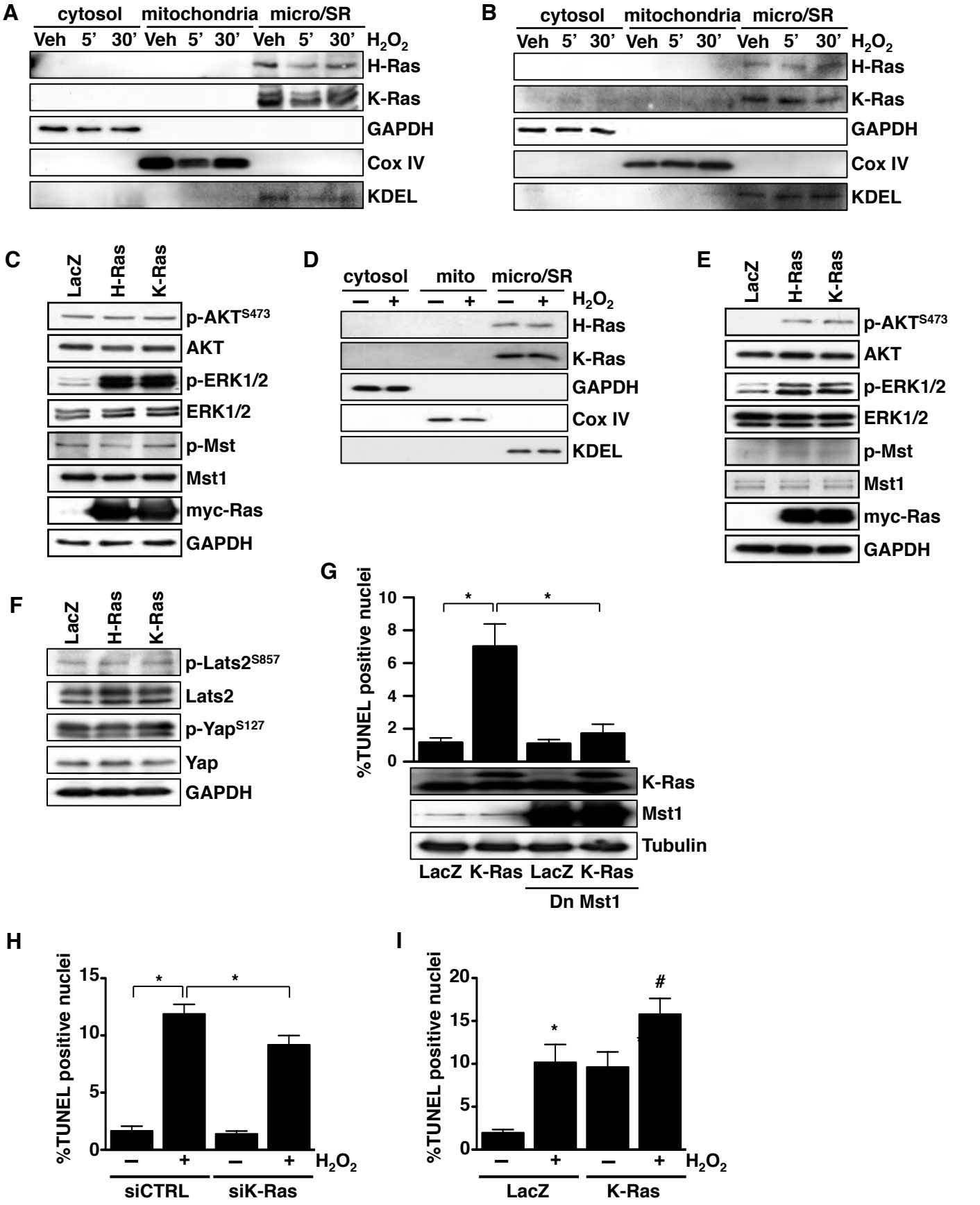

A
cytosol
mitochondria
micro/SR
Veh 5' 30' Veh 5' 30' Veh 5' 30'
H2O2
H-Ras
K-Ras
GAPDH
Cox IV
KDEL
B
cytosol
mitochondria
micro/SR
Veh 5' 30' Veh 5' 30' Veh 5' 30'
H2O2
H-Ras
K-Ras
GAPDH
Cox IV
KDEL
C
LacZ
H-Ras
K-Ras
p-AKTS473
AKT
p-ERK1/2
ERK1/2
p-Mst
Mst1
myc-Ras
GAPDH
D
cytosol
mito
micro/SR
– + – + – +
H2O2
H-Ras
K-Ras
GAPDH
Cox IV
KDEL
E
LacZ
H-Ras
K-Ras
p-AKTS473
AKT
p-ERK1/2
ERK1/2
p-Mst
Mst1
myc-Ras
GAPDH
F
LacZ
H-Ras
K-Ras
p-Lats2S857
Lats2
p-YapS127
Yap
GAPDH
G
%TUNEL positive nuclei
10
8
6
4
2
0
*
*
K-Ras
Mst1
Tubulin
LacZ K-Ras LacZ K-Ras
Dn Mst1
H
%TUNEL positive nuclei
15
10
5
0
*
*
– + – + H2O2
siCTRL
siK-Ras
I
%TUNEL positive nuclei
20
15
10
5
0
*
#
– + – + H2O2
LacZ
K-Ras

Figure S2. Ras isoform localization and signaling in non-cardiac myocytes, related to Figure 2.
(A and B) H-Ras and K-Ras localization in HEK293 (A) and COS7 (B) cells under basal and oxidative stress conditions. GAPDH, Cox IV and KDEL served as markers of cytosol, mitochondria and microsome/SR respectively.
(C) HEK293 cells were transfected with activated H-Ras or K-Ras and activation of downstream signaling pathways was examined.
(D) C2C12 cells were treated with vehicle or H_2O_2 (100 μM; 30 min) and subcellular fractions prepared.
(E) C2C12 cells were transduced with H- or K-Ras and downstream signaling examined.
(F) Cardiac myocytes were transduced with LacZ, H-Ras12V or K-Ras12V adenovirus. Phosphorylation of Lats2 and Yap was determined by immunoblotting.
(G) Cardiac myocytes were transduced with control LacZ or K-Ras12V adenovirus in combination with LacZ or DN-Mst1(K59) adenovirus. Apoptosis was determined by TUNEL assay after 48hrs. *,$p<0.05$.
(H) Cardiac myocytes were transfected with siCTRL or siK-Ras prior to H_2O_2 (100 μM) or vehicle and apoptosis determined. *,$p<0.05$.
(I) Cardiac myocytes were transduced with LacZ or K-Ras12V adenovirus and treated with vehicle or H_2O_2 (100 μM). Apoptosis was determined by TUNEL assay. *,$p<0.05$ vs. LacZ + Veh. #,$p<0.05$ vs. LacZ + H_2O_2, K-Ras + Veh. Data are represented as mean ± SEM.

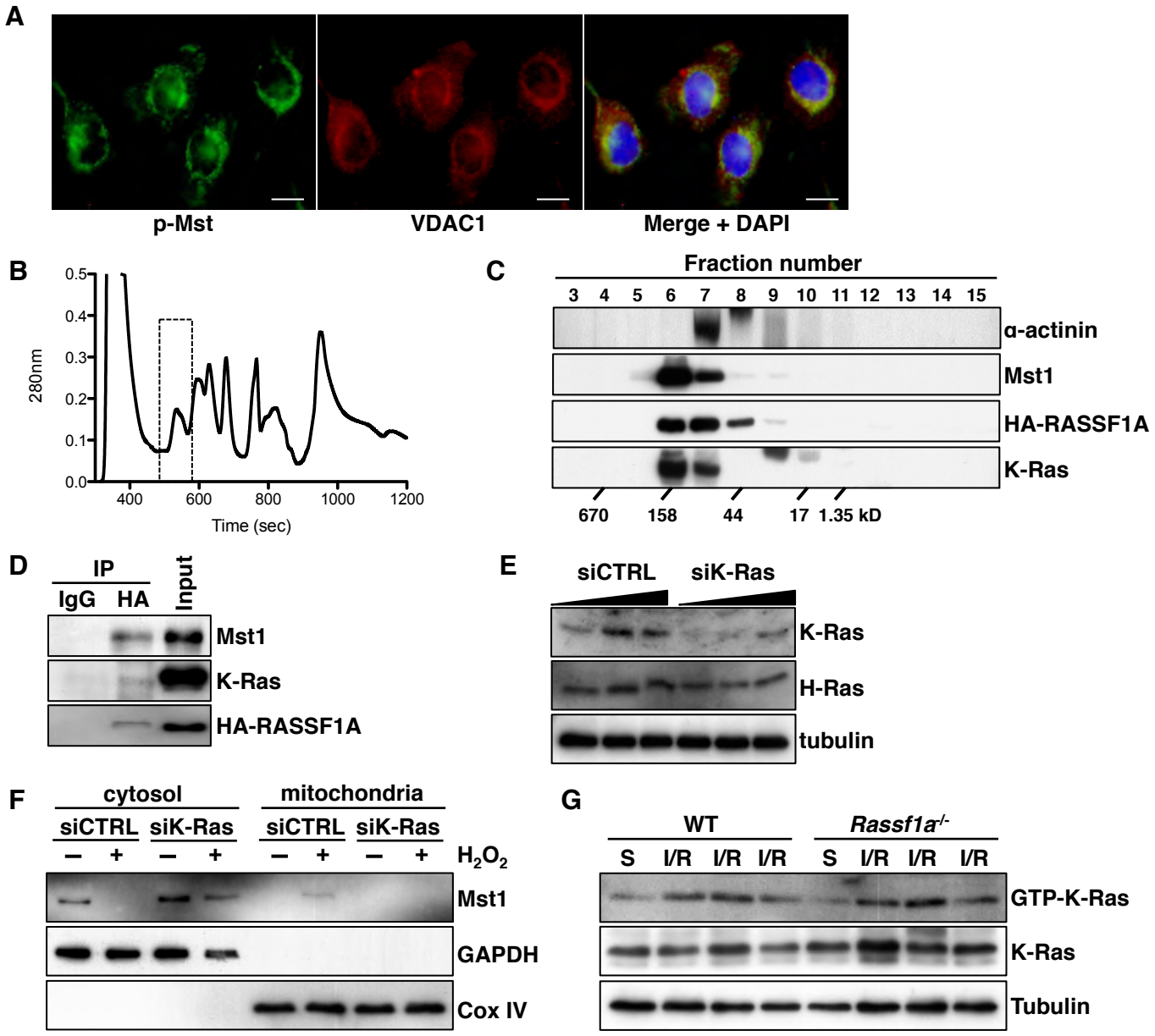


Figure S3. Activated Mst1 localizes to mitochondria in cardiac myocytes, related to Figure 3.
(A) Cardiac myocytes were treated with H_2O_2 (100 μM; 30 min). Immunofluorescence was used to detect p-Mst (green) and VDAC1 (red), a marker of the outer mitochondrial membrane. DAPI was used to visualize nuclei. Scale bar, 20 μm.
(B-D) Ventricular lysate from RASSF1A TG mice subjected to I/R (30'/30') was assayed using high pressure liquid chromatography and a size exclusion column.
(B) A representative UV trace is shown.
(C) Fractions were subjected to immunoblot for Mst1, RASSF1A, K-Ras and α-actinin as a size control. Fractions 6 and 7 (box, dotted line) were enriched for Mst1, RASSF1A and K-Ras. Molecular weight standards are indicated below the blot.
(D) Fraction 6 was subjected to immunoprecipitation using anti-HA or control IgG antibody.
(E) Depletion of endogenous K-Ras by increasing dose of siRNA pool (10, 30, and 100 nM).
(F) Cardiac myocytes were transfected with siCTRL or siK-Ras (30nM) and treated with H_2O_2 (100 μM; 30 min) or vehicle control. Cytosolic and mitochondria-enriched fractions were probed for Mst1. siK-Ras attenuated Mst1 translocation to the mitochondria-enriched fraction.
(G) Immunoblot of K-Ras activation by I/R in WT and *Rassf1a*-/- mouse heart tissue.

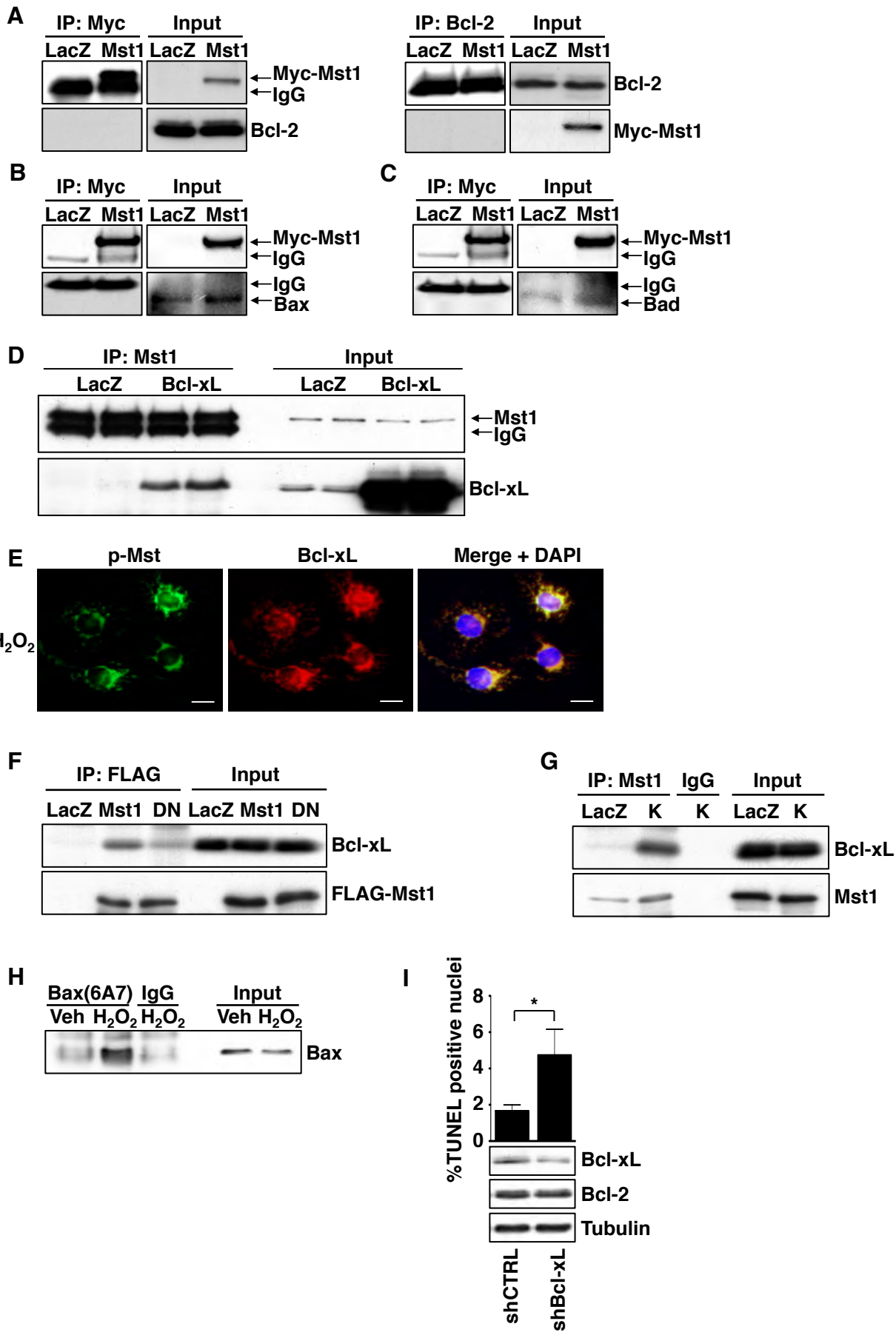
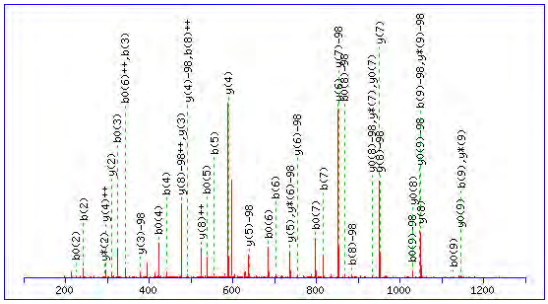

A
IP: Myc
Input
LacZ Mst1 LacZ Mst1
Myc-Mst1
IgG
Bcl-2
IP: Bcl-2
Input
LacZ Mst1 LacZ Mst1
Bcl-2
Myc-Mst1
B
IP: Myc
Input
LacZ Mst1 LacZ Mst1
Myc-Mst1
IgG
IgG
Bax
C
IP: Myc
Input
LacZ Mst1 LacZ Mst1
Myc-Mst1
IgG
IgG
Bad
D
IP: Mst1
Input
LacZ Bcl-xL LacZ Bcl-xL
Mst1
IgG
Bcl-xL
E
p-Mst
Bcl-xL
Merge + DAPI
H2O2
F
IP: FLAG
Input
LacZ Mst1 DN LacZ Mst1 DN
Bcl-xL
FLAG-Mst1
G
IP: Mst1
IgG
Input
LacZ K K LacZ K
Bcl-xL
Mst1
H
Bax(6A7) IgG
Input
Veh H2O2 H2O2 Veh H2O2
Bax
I
%TUNEL positive nuclei
8
6
4
2
0
*
Bcl-xL
Bcl-2
Tubulin
shCTRL
shBcl-xL

Figure S4. Mst1 associates with Bcl-xL, related to Figure 4.
(A-C) No interaction was detected between ectopically expressed Mst1 and Bcl-2 (A), Bax (B) or Bad (C) following immunoprecipitation in cardiac myocytes.
(D) Endogenous Mst1 interacts with ectopically expressed Bcl-xL in cardiac myocytes.
(E) Staining of endogenous p-Mst (green) and Bcl-xL (red) was performed in cardiac myocytes treated with H_2O_2 (100 μM; 30 min). Scale bar, 20 μm.
(F) Cardiac myocytes were transduced with Mst1, DN-Mst1 (K59) or LacZ adenovirus and complexes were immunoprecipitated by anti-FLAG. A stronger association was observed between Bcl-xL and active Mst1 compared to the kinase-inactive Mst1 mutant.
(G) Cardiac myocytes were transduced with K-Ras12V (K) or LacZ. Interaction between endogenous Mst1 and Bcl-xL was determined by co-immunoprecipitation.
(H) Treatment of cardiac myocytes with H_2O_2 (100 μM; 30 min) caused increased Bax activation as determined by 6A7 co-IP.
(I) Cardiac myocytes were transduced with shCTRL or shBcl-xL adenovirus and apoptosis was determined by TUNEL. *,$p<0.05$ vs. shCTRL.

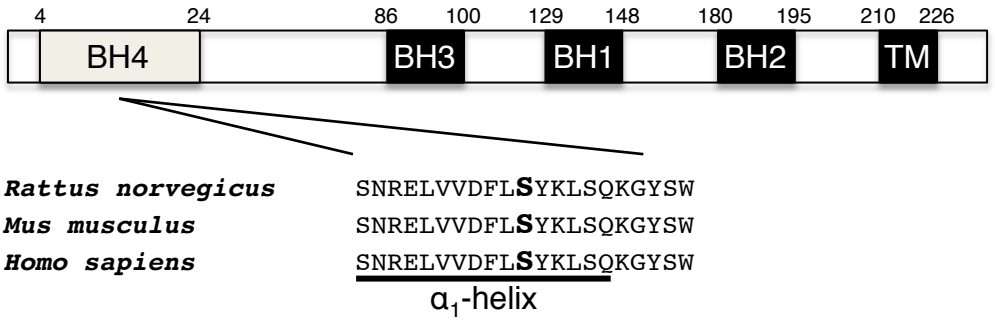
A



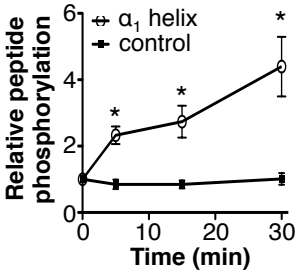
B

```
1   MSQSNRELVV DFLSYKLSQK GYSWSQFSDV EENRTEAPEG TESEMETPSA
51  INGNPSWHLA DSPAVNGATG HSSSLDAREV IPMAAVKQAL REAGDEFELR
101 YRRAFSDLTS QLHITPGTAY QSFEQVVNEL FRDGVNWGRI VAFFSFGGAL
151 CVESVDKEMQ VLVSRIAAWM ATYLNDHLEP WIQENGGWDT FVELYGNNAA
201 AESRKGQERF NRWFLTGMTV AGVVLLGSLF SRK
```

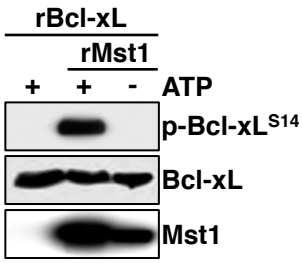
C



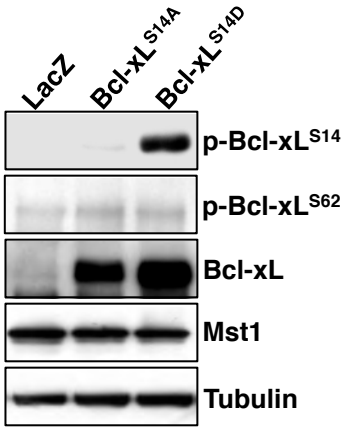
D



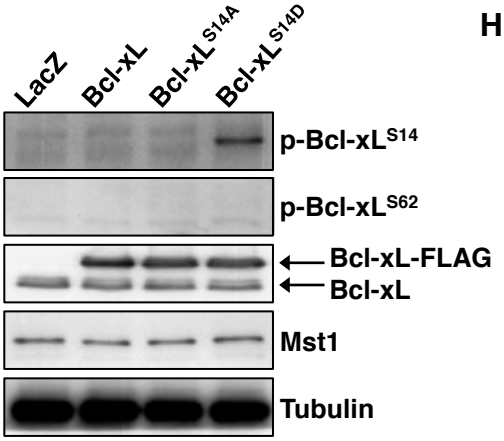
E



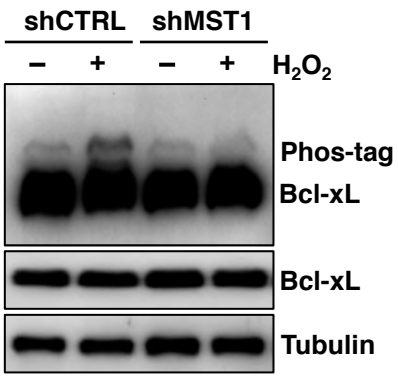
F



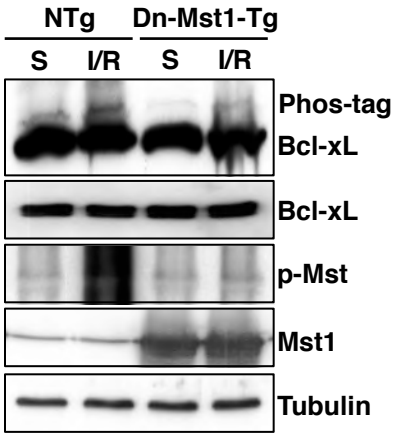
G



H



I



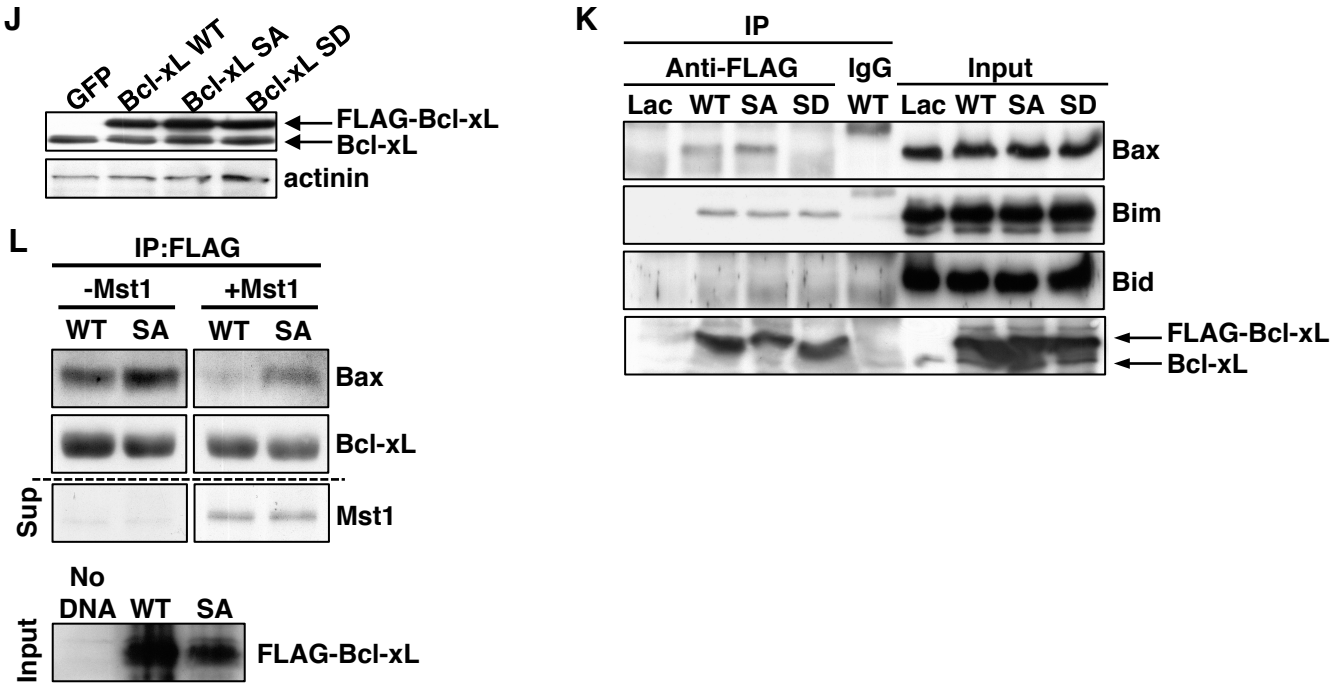


Figure S5. Mst1 phosphorylates serine 14 of Bcl-xL, related to Figure 5.
(A and B) Recombinant human Bcl-xL protein was subjected to MS/MS analysis to determine sties of phosphorylation following in vitro kinase assay with recombinant Mst1.
(A) MS/MS spectrum of a doubly-charged ion (*m/z* 646.81) corresponding to the peptide sequence of ^{7}**ELVVDFL*pS*YK**^{16} with a phosphorylation modification at **S**^{14}. The observed ***y-*** and ***b-***ion series confirmed the peptide sequence and modification.
(B) Approximately 70% sequence coverage was attained (red) and serine 14 (green) was positively identified.
(C) Structure of Bcl-xL with BH4 domain comparison between rat, mouse and human. Serine 14 is located in the first alpha helix and is highlighted in bold.
(D) [γ-^{32}P]ATP incorporation demonstrates selective Bcl-xL peptide phosphorylation by Mst1 *in vitro*. Recombinant Mst1 was incubated with control or α_1-helix peptide in kinase buffer for times shown and beta emission counted, n = 3. *$p<0.05$ compared to control peptide at respective time points. Data are presented as mean ± SEM.
(E) Mst1 directly phosphorylates serine 14 *in vitro*. Following kinase reaction and SDS-PAGE, the membrane was probed with phospho-specific antibody raised against Ser14 of Bcl-xL.
(F and G) A band corresponding to the observed molecular weight of FLAG-Bcl-xL was detected in Bcl-xL Ser14➔Asp (S14D) mutant-expressing HEK293 cells (F) and cardiac myocytes (G), but not Bcl-xL Ser14➔Ala (S14A) mutant-expressing HEK293 cells (F) or cardiac myocytes (G).
(H) Treatment of cardiac myocytes with H_2O_2 (100 μM; 30 min) increased Bcl-xL phosphorylation, as assessed by phos-tag SDS-PAGE, and was inhibited by Mst1 depletion (shMST1).
(I) Non-transgenic (NTg) control and Dn-Mst1-Tg mice were subjected to sham or I/R (30'/30'). Bcl-xL phosphorylation, as assessed by phos-tag SDS-PAGE, was increased in NTg I/R heart tissue and was attenuated in Dn-Mst1-Tg heart tissue.
(J) Expression of Bcl-xL constructs *in vivo*. GFP, Bcl-xL wild-type, S14A or S14D mutant adenovirus was injected into the myocardium and expression was determined by immunoblot 48 hours later.
(K) HEK293 cells were transfected with LacZ control or Bcl-xL wild-type (WT), S14A or S14D mutant constructs. Bcl-xL association with Bax, Bim and Bid was determined by co-immunoprecipitation of Bcl-xL using anti-FLAG antibody followed by immunoblot.
(L) In vitro transcription/translation reactions were performed using WT and S14A Bcl-xL plasmids. Resultant proteins were captured, eluted and subjected to kinase reaction with recombinant Mst1. Following kinase reaction, samples were incubated with recombinant Bax protein. Pulldown assays were performed using anti-FLAG and complexes were probed for Bcl-xL and Bax. The resultant supernatants were also collected and assayed for Mst1 (Sup). Lower panel, inputs for WT, S14A Bcl-xL and negative control (no DNA) are shown.

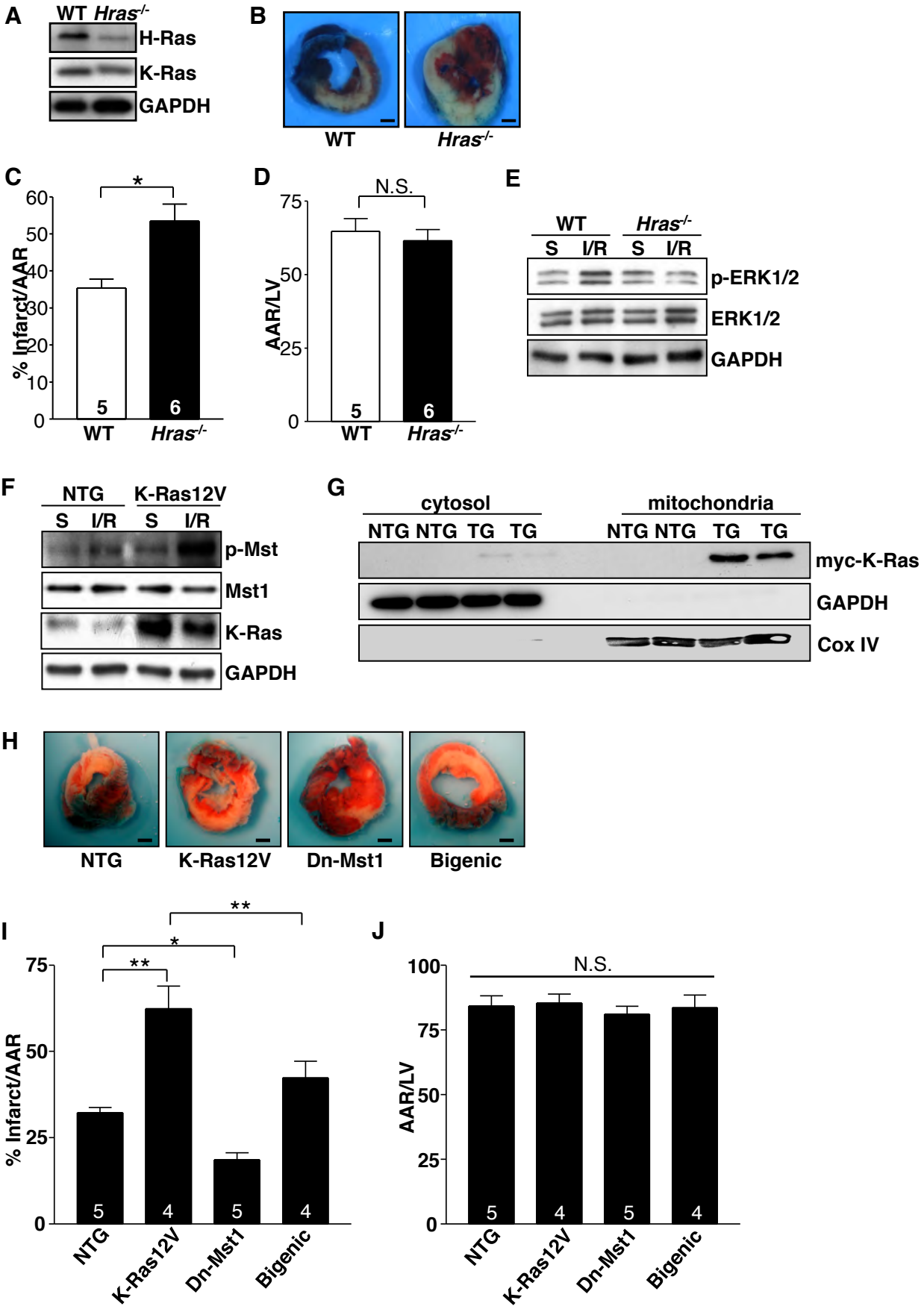

A
WT Hras-/-
H-Ras
K-Ras
GAPDH
B
WT
Hras-/-
C
% Infarct/AAR
60
50
40
30
20
10
0
*
5
6
WT
Hras-/-
D
AAR/LV
75
50
25
0
N.S.
5
6
WT
Hras-/-
E
WT
Hras-/-
S
I/R
S
I/R
p-ERK1/2
ERK1/2
GAPDH
F
NTG
K-Ras12V
S
I/R
S
I/R
p-Mst
Mst1
K-Ras
GAPDH
G
cytosol
mitochondria
NTG NTG TG TG
NTG NTG TG TG
myc-K-Ras
GAPDH
Cox IV
H
NTG
K-Ras12V
Dn-Mst1
Bigenic
I
% Infarct/AAR
75
50
25
0
**
*
**
5
4
5
4
NTG
K-Ras12V
Dn-Mst1
Bigenic
J
AAR/LV
100
75
50
25
0
N.S.
5
4
5
4
NTG
K-Ras12V
Dn-Mst1
Bigenic

Figure S6. Deletion of H-Ras, or transgenic expression of K-Ras12V, promotes myocardial injury, related to Figure 6.

(A) Expression of K-Ras was unaltered in *Hras*-/- hearts.

(B) Representative images showing increased infarct size in *Hras*-/- hearts. Scale bar, 1mm.

(C) Quantitation of infarct size in WT and *Hras*-/- hearts following I/R (30'/24hr). *$p<0.05$.

(D) No difference in AAR/LV was observed between WT and *Hras*-/- hearts. N.S. = not significant.

(E) Activation of ERK1/2 was attenuated in *Hras*-/- hearts in response to I/R (30'/60').

(F) Mst1 activation was increased in K-Ras12V transgenic mice following I/R (30'/60').

(G) Myc-tagged K-Ras12V protein localized primarily to mitochondria-enriched fractions from transgenic mouse hearts. GAPDH and Cox IV served as markers of cytosol and mitochondria respectively.

(H) Representative images of heart sections from non-transgenic (NTg), K-Ras12V, Dn-Mst1 and Bigenic (K-Ras12V x Dn-Mst1) mice. Scale bar, 1mm.

(I and J) Infarct size and area at risk (AAR) following I/R (30'/24hr). *$p<0.05$, **$p<0.01$. N.S. = not significant. Data are represented as mean ± SEM.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Ischemia/reperfusion injury. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc.) was used with 65% oxygen. The animals were kept warm with heat lamps. Rectal temperature was monitored and maintained between 36°C and 37°C. The chest was opened by a horizontal incision at the third intercostal space. I/R was achieved by ligating the anterior descending branch of the left coronary artery with an 8-0 Prolene suture, with silicon tubing (1 mm outer diameter) placed on top of the left anterior descending coronary artery, 2 mm below the border between the left atrium and left ventricle (LV). Ischemia was confirmed by ECG change (ST elevation). After occlusion for 30 minutes, the silicon tubing was removed to achieve reperfusion, and the rib space and overlying muscles were closed. When recovered from anesthesia, the mice were extubated and returned to their cages. They were housed in a climate-controlled environment.

Measurement of infarct size. Twenty-four hours after reperfusion, mice were reanesthetized and intubated, and the chest was opened. After the heart was arrested at the diastolic phase by KCl injection, the ascending aorta was cannulated and perfused with saline to wash out blood. The left anterior descending coronary artery was occluded with the same suture, which had been left at the site of the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts

were excised, and LVs were sliced into 1-mm-thick cross sections, and incubated with a 1% triphenyltetrazolium chloride (TTC) solution at 37°C for 15 minutes. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured with the use of Adobe Photoshop (Adobe Systems Inc.), and the values obtained were averaged. The percentages of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct area/AAR were expressed as percentages (Sadoshima and Izumo, 1993)

Adenovirus constructs. Recombinant adenovirus vectors were constructed as described previously (Matsui et al., 2008). Briefly, pBHGloxΔE1,3Cre (Microbix), including the ΔE1 adenoviral genome, was co-transfected with the pDC316 shuttle vector containing the gene of interest into HEK293 cells. We made replication-defective human adenovirus type 5 (devoid of E1) harboring activated myc-K-Ras12V, activated myc-H-Ras12V, 3xFlag-Bcl-xL, 3xFlag-Bcl-xL(S14A) and 3xFlag-Bcl-xL(S14D). Mutation of serine at position 14 was accomplished by PCR using wild-type Bcl-xL (Addgene plasmid 8790) as a template and the following primers: (WT sense) 5'-ATCGAGATCTATGTCTCAGAGCAACCGGGAGCTGGTGGTTGACTTTCTCTCC TACAAGCTTTCCCAGAAA-3', (SA sense) 5'-ATCGAGATCTATGTCTCAGAGCAACCGGGAGCTGGTGGTTGACTTTCTCGC CTACAAGCTTTCCCAGAAA-3', (SD sense) 5'-ATCGAGATCTATGTCTCAGAGCAACCGGGAGCTGGTGGTTGACTTTCTCGAC

TACAAGCTTTCCCAGAAA-3' and (common antisense) 5'-ATCGGAGCTCTCATTTCCGACTGAAGAGTGAG-3'. The mutation site is underlined. Plasmids were sequenced to confirm fidelity. Adenoviruses harboring HA-RASSF1A, mutant (L308P) HA-RASSF1A, Mst1, Dn-Mst1(K59R), and short hairpin RNA targeted to RASSF1A (shRASSF1A), Mst1 (shMST1) and Bcl-xL (shBcl-xL) were generated as described previously (Del Re et al., 2010; Maejima et al., 2013). Adenovirus harboring beta-galactosidase (LacZ) or scrambled shRNA (shCTRL) were used as controls.

Subcellular fractionation. Isolated mouse ventricles or cardiac myocytes were homogenized as described previously (Ago et al., 2010). Briefly, homogenates were prepared in ice-cold Buffer A [200 mmol/L mannitol, 50 mmol/L sucrose, 10 mmol/L KCl, 1 mmol/L EDTA, 10 mmol/L Hepes-KOH (pH 7.4), 0.1% BSA, 0.1 mmol/L Na_3VO_4, 5 μg/ml aprotinin, and 5 μg/ml leupeptin] and centrifuged at 600 × *g* for 5 min at 4°C. Supernatants were then centrifuged at 3,500 × *g* for 15 min at 4°C. The resultant supernatant was centrifuged at 100,000 × *g* for 2 hours to separate cytosol and microsome/SR fractions. The pellet was resuspended in Buffer A and centrifuged at 1,500 × *g* for 5 min. The resulting supernatant was centrifuged at 10,000 × *g* for 20 min at 4 °C, and then the pellet was resuspended in Buffer A as the mitochondria-enriched fraction.

Immunoprecipitation and immunoblotting. For immunoprecipitation assays, left ventricular homogenates or cardiac myocyte extracts were prepared in lysis buffer containing 50 mmol/L Tris·HCl (pH 7.5), 150 mmol/L NaCl, 0.5%

IGEPAL CA-630, 0.1% SDS, 0.5% deoxycholic acid, 1 mmol/L EDTA, 0.1 mmol/L Na_3VO_4, 1 mmol/L NaF, 50 µmol/L phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml aprotinin, and 5 µg/ml leupeptin. Active Bax precipitation was carried out in 1% CHAPS buffer (150 mmol/L NaCl, 10 mmol/L HEPES, pH 7.4) to prevent conformational activation of Bax (Hsu and Youle, 1997). Samples were incubated with primary antibody overnight at 4°C, and immunocomplexes were precipitated following 1 hour of incubation with sepharose A/G beads (Santa Cruz). For immunoblot analysis, the antibodies used were H-Ras (Epitomics), K-Ras (Sigma), GAPDH (Cell Signaling), Cox IV (Cell Signaling), KDEL (Enzo Life Sciences), Mst1 (BD Transduction Labs), p-Mst1/2(T183/180) (Cell Signaling), HA-tag (Santa Cruz), myc-tag (Sigma), ERK1/2 (Cell Signaling), phospho-ERK1/2 (Cell Signaling), Raf-1 (Cell Signaling), p110α (Cell Signaling), AKT (Cell Signaling), p-AKT(S473) (Cell Signaling), RASSF1A (eBiosciences), Bcl-xL (Cell Signaling), p-Bcl-xL(S62) (GenScript), Bcl-2 (BD Transduction Labs), Bax (Cell Signaling), active Bax (6A7 epitope) (Calbiochem), caspase-3 (Cell Signaling), caspase-9 (Cell Signaling), cytochrome *c* (Cell Signaling), Bad (Cell Signaling), Bak (Cell Signaling), Bim (Cell Signaling), Bid (Santa Cruz), Lats2 (Bethyl), p-Lats2 (kind gift of Dr Hiroshi Nojima, Osaka University), Yap (Cell Signaling), p-Yap (S127)(Cell Signaling), actinin (Sigma), tubulin (Sigma), FLAG (Cell Signaling) and His-tag (Cell Signaling). Antisera were raised against p-Bcl-xL(S14) by immunizing rabbits with the phospho-peptide FL{pSER}YKLSQKGYSWSC (GenScript). To detect Bcl-xL(S14)

phosphorylation, lysis buffer was supplemented with PhosSTOP (Roche) and membranes were blocked in 5% PhosphoBLOCKER (Cell Biolabs). Recombinant human proteins were purchased from Thermo Scientific and Millipore. For the Phos-tag experiments, the Phos-tag reagents were purchased from Wako Chemicals, and gels containing Phos-tag were prepared according to the manufacturer's instructions. Densitometry was performed using ImageJ software.

Detection of thiolated cysteines. Lysates were prepared using lysis buffer containing 200 µmol/L biotinylated iodoacetamide (Sigma) and incubated at room temperature for 30 minutes. Lysates were cleared by centrifugation and the supernatant incubated with streptavidin-agarose beads (Sigma) for 2 hours at 4°C. Beads were washed in lysis buffer containing 200 µmol/L biotinylated-IAM and subjected to SDS-PAGE.

Immunostaining. Cardiac myocytes were plated on gelatin-coated glass coverslips. MitoTracker Red CMXRos (Molecular Probes) was used according to manufacturer's instructions. Cells were fixed in PBS containing 4% paraformaldehyde, permeabilized in PBS containing 0.3% Triton-X, and blocked with 5% normal goat serum. Immunostaining was performed using anti-phospho-Mst rabbit polyclonal antibody (Cell Signaling), anti-VDAC1 mouse monoclonal antibody (Abcam), anti-Bcl-xL mouse monoclonal antibody (BD Transduction Labs), Alexa-fluor 488 goat anti-rabbit IgG (Molecular Probes), Alexa-fluor 594 goat anti-mouse IgG (Molecular Probes) and Vectashield mounting medium with

DAPI (Vector Laboratories). Microscopic analyses were performed using fluorescence microscopy (Zeiss).

In vitro kinase assay. For the in vitro kinase assay, human recombinant Mst1 (10 ng) was incubated with human recombinant full-length Bcl-xL (1 µg) or Bcl-xL peptides (1 µg) in kinase buffer (25 mmol/L HEPES pH 7.4, 50 mmol/L NaCl, 5 mmol/L $MgCl_2$, 5 mmol/L $MnCl_2$, 5 mmol/L β-glycerophosphate, 1 mmol/L dithiothreitol, 10 µmol/L ATP and 2 µCi [γ-^{32}P]ATP [Amersham]) and reactions were carried out at 30°C for 30 min. Reactions were stopped by adding SDS sample buffer and heating at 95°C for 5 min. Reaction products were visualized by SDS-PAGE followed by autoradiography. For peptide phosphorylation analysis, samples were transferred to P81 filter paper, washed 3X with 0.75% phosphoric acid and once with acetone. CPM was determined in 5 mL of scintillation cocktail (Fisher) using a scintillation counter. Results were normalized to background CPM present in Mst1-only samples.

In vitro transcription/translation. Wild type and S14A mutant Bcl-xL protein was generated in vitro using 3x FLAG-tagged constructs and the TnT Quick Coupled Transcription/Translation System according to manufacturer's instructions (Promega). Following the reaction, Bcl-xL proteins were captured using M2 anti-FLAG (Sigma) and subsequently eluted with 3x FLAG peptide (Sigma) prior to kinase reaction with recombinant Mst1.

siRNA knockdown. siRNA-mediated knockdown of endogenous K-Ras was performed in neonatal rat cardiac myocytes. Cells were transfected with

Lipofectamine 2000 Transfection Reagent (Life Technologies) using the described concentrations of pre-designed siRNAs (IDT) diluted in OPTIMEM (Gibco). siRNA duplexes used were: siK-Ras1 (RNC.RNAI.N031515.12.1), siK-Ras2 (RNC.RNAI.N031515.12.2), siK-Ras3 (RNC.RNAI.N031515.12.3), siRNA CTRL (NC1, Negative Control Sequence).

Size exclusion chromatography. Using a BioCAD SPRINT Perfusion chromatography system (PerSeptive BioSystems), clarified heart tissue lysate was injected (200 µl per injection) onto a TSKgel G3000SWxl column (Tosoh Bioscience) equilibrated with 50 mM Tris, 150 mM NaCl, pH 7.5. The protein elution profile was detected by UV absorbance at 280 nm. All fractions were immediately lyophilized and stored at -80 C for further analysis. To assess the size of the protein complex of interest, the column was calibrated with protein standards (Bio-Rad Laboratories) consisting of thyroglobulin (bovine) (670,000 Da), γ-globulin (bovine) (158,000 Da), ovalbumin (chicken) (44,000 Da), myoglobin (horse) (17,000 Da), and vitamin B12 (1,350 Da).

Mass spectrometry. Following the kinase reaction, proteins were separated by SDS-PAGE. After staining with Coomassie Brilliant Blue (CBB), the gel band corresponding to Bcl-xL was excised for in-gel trypsin digestion. Half of the resulting peptides were subjected to LC-MS/MS analysis directly on an Ultimate 3000 LC system coupled with an Orbitrap Velos tandem mass spectrometry instrument (Thermo Fisher Scientific), whereas the other half were TiO_2 enriched for phosphopeptides followed by LC-MS/MS analysis. The MS

spectra were acquired in a positive mode with a nano-spray voltage of 2kV and a capillary temperature of 275 ºC. The automatic gain control (AGC) target was set to 1.0E+6 for full scan in the Orbitrap mass analyzer and 5.0E+4 for MS/MS scans in the ion trap mass analyzer. The top 10 intensity ions in each full MS scan were selected for MS/MS analysis in the collision-induced dissociation (CID) fragmentation mode. The lock mass feature was used for accurate mass measurement. The MS/MS spectra were searched against a Swissprot human database with an MS error window of 10 ppm and an MS/MS window of 0.5 Da. Methionine oxidation, cysteine carbamidomethyl modification, and serine or threonine phosphorylation were set as variable modifications. Phosphopeptides with a confidence interval value greater than 95% were identified. Assignment of the phosphorylation site was manually evaluated.

Human heart samples. The samples from explanted hearts used in this study were obtained from 4 patients who had received heart transplants and 4 age-matched donors at the Taipei Veterans General Hospital. The study was approved by the institutional Ethics Committee (VGHIRB No.:2012-06-028D), and all patients or their family expressed their willingness to participate through an informed consent form. Myocardial posterior wall samples were collected during preparation of donor hearts for transplantation in the hospital. Myocardial samples from near the mitral annulus were obtained from recipients at the time of therapeutic transplantation. Immediately after tissue procurement, the samples were stored in liquid nitrogen and kept at -80°C.

SUPPLEMENTAL REFERENCES

Ago, T., Kuroda, J., Pain, J., Fu, C., Li, H., and Sadoshima, J. (2010). Upregulation of Nox4 by hypertrophic stimuli promotes apoptosis and mitochondrial dysfunction in cardiac myocytes. Circ Res *106*, 1253-1264.

Del Re, D.P., Matsuda, T., Zhai, P., Gao, S., Clark, G.J., Van Der Weyden, L., and Sadoshima, J. (2010). Proapoptotic Rassf1A/Mst1 signaling in cardiac fibroblasts is protective against pressure overload in mice. J Clin Invest *120*, 3555-3567.

Hsu, Y.T., and Youle, R.J. (1997). Nonionic detergents induce dimerization among members of the Bcl-2 family. J Biol Chem *272*, 13829-13834.

Maejima, Y., Kyoi, S., Zhai, P., Liu, T., Li, H., Ivessa, A., Sciarretta, S., Del Re, D.P., Zablocki, D.K., Hsu, C.P.*, et al.* (2013). Mst1 inhibits autophagy by promoting the interaction between Beclin1 and Bcl-2. Nat Med *19*, 1478-1488.

Matsui, Y., Nakano, N., Shao, D., Gao, S., Luo, W., Hong, C., Zhai, P., Holle, E., Yu, X., Yabuta, N.*, et al.* (2008). Lats2 Is a Negative Regulator of Myocyte Size in the Heart. Circ Res *103*, 1309-1318.

Sadoshima, J., and Izumo, S. (1993). Molecular characterization of angiotensin II-induced hypertrophy of cardiac myocytes and hyperplasia of cardiac fibroblasts: a critical role of the AT1 receptor subtype. Circ. Res. *73*, 413-423.