Supporting Information

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SI Materials and Methods

Rosa26^{fsTRAP} Gene Targeting. The Rosa26^{fsTRAP} targeting construct is summarized in Fig. 1. Briefly, a loxP-flanked neo-stop cassette preventing transcription of the downstream EGFP-L10a follows a ubiquitously active CAG promoter. EGFP-L10a cDNA (1) was a kind gift from N. Heintz (Rockefeller University, New York). The CAG:fs:EGFP-L10a construct was tested by transient transfection with or without a Cre expression plasmid in 293T cells. Following verification of GFP-L10a fusion protein expression in a Cre-activated manner, the construct was cut with PacI and AscI and subcloned into the Rosa26 targeting vector (2).

Rosa26^{fsTRAP} was linearized by SwaI and electroporated into J1 ES cells. After selection by G418, surviving colonies were screened by PCR primer pairs 5′-CCACTGACCGCACGGGGATTC-3′ and 5′-TCAATGGGCGGGGGTCGTT-3′ (1.5-kb PCR product). Positive colonies were additionally confirmed by Southern blotting.

The Rosa26^{fsTRAP} ES cells were karyotyped, and one clone was injected into the blastocysts to generate chimeric mice. Germline transmission was determined by PCR. The mice are available through The Jackson Laboratory (stock number for Rosa26^{fsTRAP}: 022367; Rosa26$^{CAG\text{-}TRAP}$: 022386).

Generation of Rosa26$^{CAG\text{-}TRAP}$ ES Cells. ES cells were grown on feeder cells or gelatin-coated dishes and cultured using high-glucose DMEM supplemented with 15% (vol/vol) FBS (HyClone), 1 mM sodium pyruvate, 1 mM nonessential amino acids, 2 mM glutamine, 0.1 mM 2-mercaptoethanol, and 1,000 U/mL leukemia inhibitory factor (LIF). Rosa26^{fsTRAP} J1 ES cells were electroporated with a CMV-Cre plasmid and plated out with 500 cells in each 10-cm dish. Colonies with activated translating ribosome affinity purification (TRAP) were picked under a fluorescence microscope and then expanded for TRAP or polysome fractionation.

Sucrose Gradient Polysome Fractionation. Sucrose gradient polysome fractionation was performed as described (3), with minor modifications. For Rosa26$^{CAG\text{-}TRAP}$ ES cells, ~5×10^7 cells were incubated with 100 μg/mL cycloheximide (Sigma) for 15 min to arrest translation before harvesting the cells. The harvested cell pellet was suspended in lysis buffer (10 mM Hepes, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 100 μg/mL cycloheximide, protease inhibitors, and recombinant RNase inhibitors) and homogenized with 10 strokes in a glass homogenizer (small-clearance). Homogenates were centrifuged for 10 min at 2,000 × *g*, 4 °C, to pellet nuclei and large cell debris, and Igepal CA-630 (Sigma) and 1,2-diheptanoyl-*sn*-glycero-3-phosphocholine (DHPC; Avanti Polar Lipids) were added to the supernatant at final concentrations of 1% (vol/vol) and 30 mM, respectively. After incubation on ice for 5 min, the lysate was centrifuged for 10 min at 13,000 × *g* to pellet insolubilized material. Then 1 mL of clear lysate was loaded onto 10-mL linear 7–47% (mass/vol) sucrose gradients in 50 mM Tris·HCl (pH 7.5), 0.8 M KCl, 15 mM $MgCl_2$, and 100 μg/mL cycloheximide and centrifuged at 130,000 × *g* in an SW40-Ti swinging-bucket rotor (Beckman) for 2 h at 4 °C. Twenty-two fractions were collected from the top of the gradients into cold microfuge tubes and immediately placed on dry ice. Twenty microliters from each fraction was mixed with 2× formamide loading buffer and loaded onto a 1.5% (mass/vol) agarose gel for analysis. RNA was precipitated by isopropanol and further purified using the RNeasy Kit (Qiagen).

DMDA-PatA Inhibition of Translational Initiation in ES Cells. Des-methyl, des-amino-pateamine A (DMDA-PatA) is a nearly equipotent derivative of the marine sponge-derived natural product pateamine A (4). DMDA-PatA was synthesized as described (5). DMDA-PatA was dissolved in DMSO to obtain a 100 μM stock solution. Fifty percent confluent Rosa26$^{CAG\text{-}TRAP}$ ES cells were treated with DMDA-PatA for 60 min, and then cycloheximide was added to the medium to a final concentration of 100 μg/mL and incubated for 15 min. Cells were then harvested for TRAP and polysome fractionation.

Mice. All animal procedures were approved by the Institutional Animal Care and Use Committee of Boston Children's Hospital. TNT-Cre (6), Tie2-Cre (7), and EIIa-Cre (8) alleles were described previously. Constitutively activated TRAP mice (CAG-TRAP) were obtained with two rounds of mating. First, EIIa-Cre males were mated with Rosa26^{fsTRAP} females to yield progeny harboring the recombined Rosa26$^{CAG\text{-}TRAP}$ allele in the germ line. These females were then mated with wild-type C57BL6 males to obtain germ line-activated TRAP mice. Mice were a mixed C57BL6/J and 129 genetic background. For embryo collection, noon of the day of vaginal plug was defined as embryonic day (E)0.5.

Aortic Banding. Ascending aortic banding was performed as described (9). Male mice (25–30 g) were anesthetized with isoflurane blended with oxygen. The chest was shaved and cleaned with alcohol. Before the incision, 0.1 mL of 0.1% lidocaine was introduced under the skin. The chest cavity was opened by an incision of the left second intercostal space. The pericardial sac was opened and dissected apart, the ascending portion of the aorta was dissected from the surrounding tissues, and a silk suture was passed underneath the aorta and ligated against a 25-gauge needle. The needle was then removed, resulting in a ligature with a fixed diameter tied around and constricting the aorta. The sham procedure was identical except that the aorta was not ligated.

Echocardiography. Echocardiography was performed to measure heart function 2 wk after surgery. Echocardiography was performed on awake mice with a Vevo2100 (VisualSonics). The chest was cleared of hair with hair remover. Mice were held by standard handhold to obtain the echocardiogram. The transducer was placed on the chest and short-axis M-mode images were acquired.

Tissue Collection for RNA Analysis. Mice were euthanized by CO_2 and the tissue was quickly put into ice-cold PBS with 100 μg/mL cycloheximide (Sigma). Heart tissue was quickly weighed, and the atria were dissected away and the heart ventricles were used for RNA analysis. The tissue was minced into a cell suspension by a motor-driven homogenizer (IKA; T10 basic ULTRA-TURRAX). The cell pellet was suspended in lysis buffer (10 mM Hepes, pH 7.4, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 100 μg/mL cycloheximide, protease inhibitors, and recombinant RNase inhibitors). The cell suspension was then processed for either sucrose gradient sedimentation or TRAP.

Translating Ribosome Affinity Purification. TRAP was performed as described (1) and at www.bactrap.org/downloads/Polysome_IP_Protocol.pdf. Cell suspension from tissue obtained as described in the preceding section was further homogenized with 10 strokes in a glass homogenizer (small-clearance). Homogenates were centrifuged for 10 min at 2,000 × *g*, 4 °C, to pellet nuclei and large cell debris, and Igepal CA-630 (Sigma) and DHPC (Avanti Polar Lipids) were added to the supernatant at final concentrations of

1% (vol/vol) and 30 mM, respectively. After incubation on ice for 5 min, the lysate was centrifuged for 10 min at 13,000 × *g* to pellet insoluble material. Fifteen percent of the clear lysate was kept as input. Two anti-GFP antibodies, Htz-GFP19C8 and Htz-GFP19F7 (Memorial Sloan-Kettering Cancer Center, New York), were bound to protein G Dynal magnetic beads (Invitrogen). The beads were then added to the cell-lysate supernatant, and the mixture was incubated at 4 °C with end-over-end rotation for 30 min. Beads were subsequently collected on a magnetic rack, washed five times with high-salt polysome wash buffer (10 mM Hepes, pH 7.4, 350 mM KCl, 5 mM $MgCl_2$, 1% Igepal CA-630, 0.5 mM DTT, and 100 μg/mL cycloheximide), and immediately placed in the RLT buffer of the RNeasy Mini Kit (Qiagen). RNA was purified with in-column DNase digestion.

In a typical TRAP on an adult heart ventricle, 100 μL protein G Dynal magnetic beads coated with 30 μg anti-GFP antibodies (15 μg Htz-GFP19C8 and 15 μg Htz-GFP19F7) was applied for pull down of tagged ribosomes.

Histology. Embryos were collected in PBS on ice and fixed in 4% (mass/vol) paraformaldehyde at 4 °C for 4 h. After washing in PBS, tissues were treated with 30% (mass/vol) sucrose until tissue was fully penetrated. Then they were embedded in OCT and snap frozen. Six- to 10-μm cryosections were collected on positively charged slides. Tissues were blocked with PBS supplemented with 0.1% Triton X-100 and 5% (vol/vol) normal donkey serum for 1 h at room temperature, followed by primary antibody incubation at 4 °C overnight. Primary antibodies used were: GFP (Rockland; 600-101-215); TNNI3 (Abcam; ab56357); PECAM1 (BD Biosciences; 553371). Signals were developed with Alexa-conjugated secondary antibodies (Invitrogen). Fluorescent images were acquired on an FV1000 confocal microscope (Olympus).

Western Blotting. Western blotting was performed on whole-cell lysates by standard methods. Primary antibodies were GFP (Rockland; 600-101-215) and RPL10A (Sigma-Aldrich; SAB1101200).

Quantitative RT-PCR. Input or TRAP RNA was purified by RNeasy Kit with on-column DNaseI digestion. First-strand cDNA was generated using a SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and Oligo dT primer with 500 ng of total RNA. Quantitative PCR (qPCR) was performed using ABI Power SYBR Green PCR Master Mix (Applied Biosystems). Gene expression was normalized to *Gapdh* unless otherwise indicated. Primers for qPCR were designed using PrimerBank (10). Primer sequences are provided in Dataset S6. For fold enrichment in TRAP, the target gene was normalized to *Gapdh* in both input and TRAP fractions [i.e., fold TRAP enrichment = $2^{(\Delta Ct\_target\ -\ \Delta Ct\_Gapdh)}$].

RNA-Seq. RNA from three sham or band-operated heart ventricles was used for RNA-seq as described previously (11), with modifications. Briefly, 2 μg of pooled input or TRAP RNA was used for two rounds of poly(A) mRNA purification by Dynabeads Oligo $(dT)_{25}$ (Invitrogen). RNA was reverse-transcribed by SuperScript III (Invitrogen) and random hexamer primers. After RNaseH treatment and PolI-catalyzed second-strand cDNA synthesis, DNA end repair was achieved by End-It Kit (Epicentre). DNA was then A-tailed with Exo$^-$ Klenow (New England BioLabs), and adaptors were ligated using Quick T4 DNA Ligase (New England BioLabs). Fragments of 150–300 bp were size-selected by agarose gel electrophoresis. Recovered DNA was amplified using Phusion DNA polysome (New England BioLabs), multiplexing PCR primer 1.0, and one indexed primer. The amplified libraries were purified with Agencourt AMPure XP beads (Beckman Coulter). The libraries were quantitated using the Quant-iT DNA quantitation kit (Invitrogen). The DNA size distribution of the library was measured by an Agilent Bioanalyzer. Six indexed libraries were pooled for one lane. Fifty-nucleotide paired-end reads were obtained using an Illumina HiSeq 2000.

Sequencing data including raw reads and visualization tracks are available on the Cardiovascular Development Consortium server at https://b2b.hci.utah.edu/gnomex. Read alignment was performed using TopHat (12), default parameters, and iGenomes (http://cufflinks.cbcb.umd.edu/igenomes.html) UCSC mm9 or Ensembl NCBIM37.67 transcriptome annotations. Read counting against the same annotation files was performed using HTSeq-count (www-huber.embl.de/users/anders/HTSeq) using uniquely mapped reads with unambiguous annotations. Differential expression analysis was performed using DESeq (13) or edgeR (14) as indicated.

For annotation of transcript type, we used Ensembl NCBIM37.67 annotations. There were several categories that contained few transcripts, so categories were aggregated as follows: "pseudogene" contains "processed_pseudogene," "transcribed_processed_pseudogene," "pseudogene," and "transcribed_unprocessed_pseudogene"; "lincRNA" contains "lincRNA" plus "non_coding"; "nonsense-mediated_decay," "processed_transcript," and "protein_coding" retained their original labels; and "other" contains all other miscellaneous transcript types. For analysis of noncoding RNAs, we excluded transcripts with low expression (fewer than three reads per million).

Gene enrichment analysis was performed using DAVID (15). Additional statistical analyses and plotting were performed using Java Treeview (16) and JMP 10 (SAS Institute).

Analysis and Statistics. We calculated TRAP:input ratio as a relative measure of enrichment in the TRAP fraction compared with the input. Because RNA-seq measurements reflect the position of a transcript with respect to all other transcripts, the TRAP:input ratio can be greater than 1. For example, assume endothelial transcript X has 50 fragments per kb per million input reads, and that endothelial transcripts constitute 10% of input reads [reads per kilobase per million (RPKM) =50]. Then, in an ideal TRAP experiment, X will have 50 fragments per kb per 0.1 million reads (RPKM 500). Therefore, TRAP:input = 500/50 = 10.

Unless otherwise noted, results were expressed as mean ± SD. Intergroup comparisons were performed by *t* test with $P = 0.05$ used as the significance threshold.

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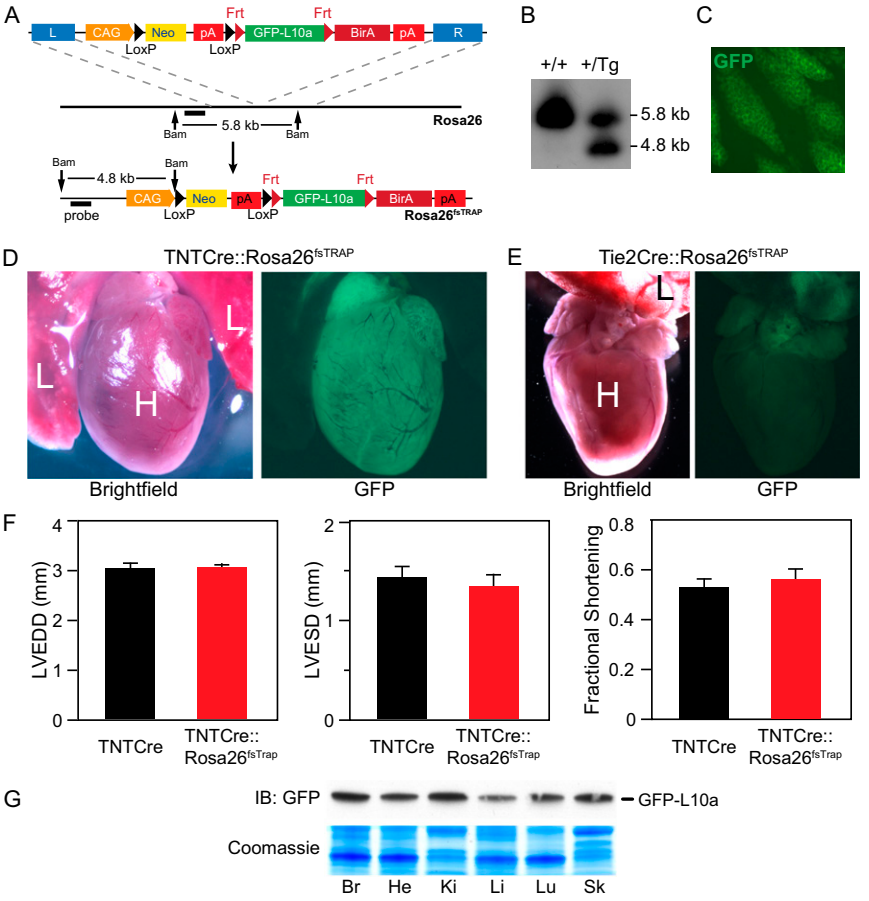

Fig. S1. Generation and characterization of a Cre-activated Rosa26^{fsTRAP} allele. (*A*) Gene targeting strategy. (*B*) Southern blot analysis of targeted ES clone and wild-type DNA digested with BamHI. (*C*) EGFP-L10a expression following Cre recombination in ES cells. (*D* and *E*) Cardiomyocyte [cardiac troponin T (TNT)-Cre] or endothelial cell (Tie2-Cre) or selective activation of Rosa26^{fsTRAP}. L, lung; H, heart. (*F*) TNT-Cre activation of Rosa26^{fsTRAP} did not alter heart size or function as assessed by echocardiography. Results are mean ± SEM. LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end dystolic diameter. (*G*) Expression of GFP-L10a in various organs of Rosa26$^{CAG\text{-}TRAP}$ adult mice. Br, brain; He, heart; Ki, kidney; Li, liver; Lu, lung; Sk, skeletal muscle.

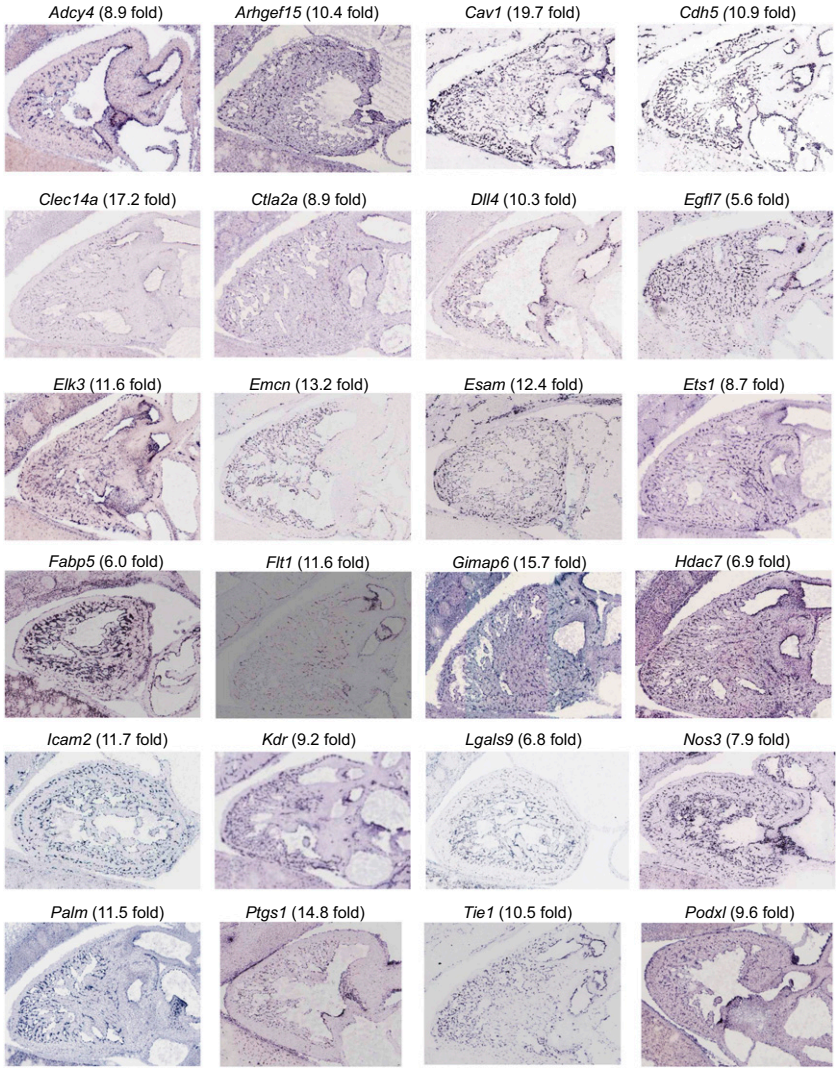

Fig. S2. Genes with high Tie2-TRAP:input ratio and endothelial expression pattern in the Eurexpress in situ database. Eurexpress in situ images at E14.5. Tie2-TRAP:input ratios from RNA-seq data are indicated in parentheses.

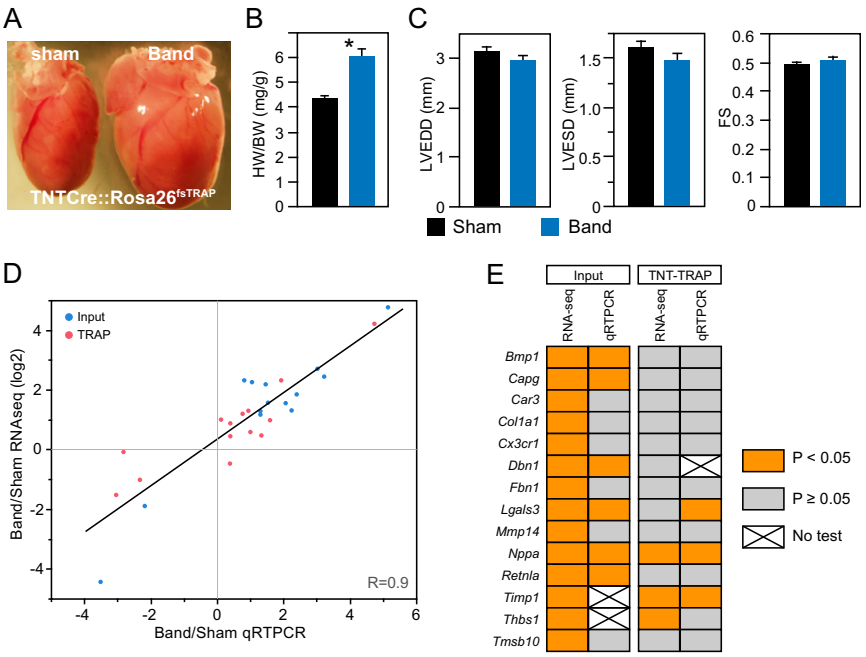

Fig. S3. Pressure overload model of cardiac hypertrophy. (*A*) TNT-Cre::Rosa26^{fsTRAP} mice underwent aortic banding or sham operation. Banding induced visible cardiac hypertrophy. (*B*) Gravimetric analysis 2 wk postbanding demonstrated banding-induced cardiac hypertrophy. *$P < 0.05$. (*C*) Banding induced compensated cardiac hypertrophy, as revealed by echocardiography 2 wk postbanding. Echocardiographic parameters did not differ significantly between groups. (*D*) Comparison of fold change measured by RNA-seq and qRT-PCR. Correlation coefficient was $R = 0.9$. (*E*) Comparison of RNA-seq and qRT-PCR measurements of differential expression in band versus sham. Overall, the two methods agreed in 18 of 25 comparisons (72%). Results in *B* and *C* are mean ± SEM.

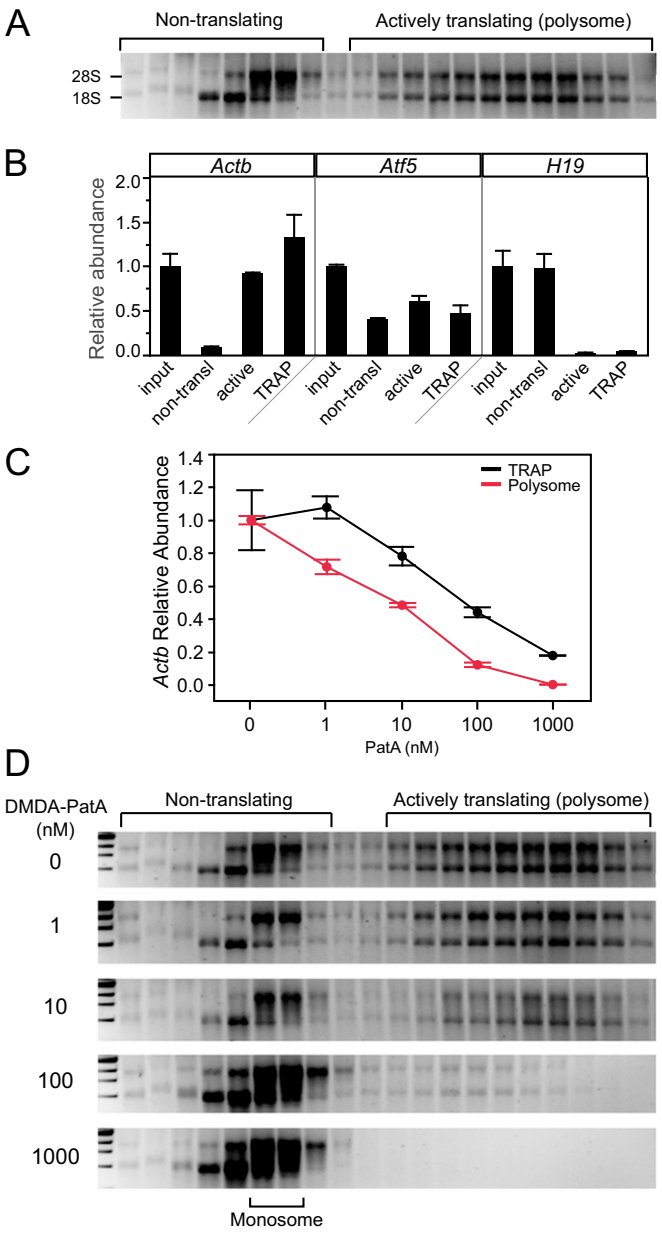

Fig. S4. TRAP measures RNA translational efficiency in ES cells. (*A*) Polysome fractionation on a sucrose gradient. ES cell cytoplasmic lysates were subject to sucrose gradient centrifugation, and aliquots of collected fractions were run on an agarose gel. Fractions pooled as nontranslating and actively translating samples are indicated. (*B*) Comparison of sucrose gradient and TRAP measurements of active translation. Nontranslating or actively translating RNA from sucrose gradient fractionation was pooled as indicated. Relative transcript abundance was measured by qRT-PCR. (*C*) Inhibition of translational initiation reduced actively translating RNA as measured by either TRAP or polysome fractionation. Translational initiation of ES cells was inhibited by DMDA-PatA (Fig. S1). (*D*) DMDA-PatA effect on mRNA translation as assessed by sucrose density gradient fractionation. RNA from ES cells cultured in the indicated concentrations of PatA was analyzed by sucrose density gradient centrifugation. Fractions from sucrose gradients were run on agarose gels to visualize the distribution of ribosomal RNAs. Increasing doses of PatA blocked assembly of polysomes but not monosomes. Results in *B* and *C* are mean ± SEM.

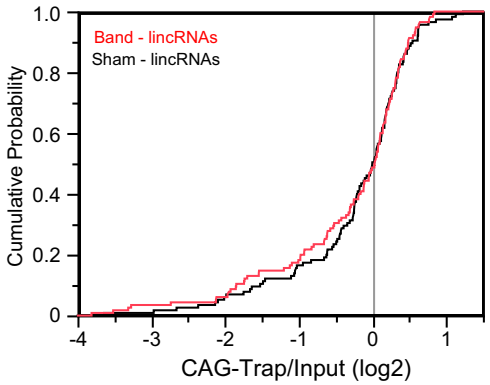

Fig. S5. TRAP evaluation of long intergenic noncoding RNAs (lincRNAs). Cumulative probability plot of the strength of lincRNA binding to ribosomes in sham and band-operated Rosa26$^{CAG\text{-}TRAP}$ heart 2 wk after the operation.

Dataset S1. Summary of RNA-seq datasets

Dataset S1

Dataset S2. Comparison of TNT-TRAP, Tie2-TRAP, and input RNA expression profiles

Dataset S2

Dataset S3. RNA-seq analysis of gene expression in TRAP and input RNA and sham versus band-operated heart ventricles

Dataset S3

Dataset S4. RNA-seq analysis of CAG-TRAP and input from sham or band-operated ventricles

Dataset S4

Dataset S5. lincRNAs differentially expressed in pressure overload

Dataset S5

Dataset S6. Primers used in this study

Dataset S6