METHODS

Mouse alleles and transgenic lines. All animal protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine, Houston, Texas 77030, USA. All surgeries and echocardiographic studies were carried out blinded from genotype of the mice. Littermate controls were used whenever possible. Both male and female mice were used. The *MCK*^{cre}, *Myh6-cre/Esr1* (*Mhc*$^{cre\text{-}Ert}$) transgenic line, floxed alleles for *ww45*/salvador (*Salv*$^{f/f}$) and *Pitx2* (*Pitx2*$^{f/f}$), and Flag-tagged *Pitx2* allele (*Pitx2*^{flag}) have been described previously[7,25]. The *Pitx2*^{GoF} construct for overexpressing *Pitx2* was generated by introducing a 0.9 kb *Pitx2c* cDNA coding sequence into a CMV-CAG-loxP-eGFP-Stop-loxP-IRES-βGal expression vector[26], linearized construct was subjected to pronuclear injection. *MCK*^{cre};*Pitx2*$^{f/f}$ (*Pitx2* CKO), *Mhc*$^{cre\text{-}Ert}$;*Salv*$^{f/f}$ (*Salv* CKO), *Mhc*$^{cre\text{-}Ert}$;*Salv*$^{f/f}$;*Pitx2*$^{f/f}$ (DKO) and *Mhc*$^{cre\text{-}Ert}$;*Pitx2*^{GoF} (*Pitx2*-overexpressing) mice were generated by cross breeding. After Cre-mediated recombination, the *Pitx2* floxed allele removes all *Pitx2* isoform function. DNA was extracted from tail biopsies for genotyping. The primers for *Pitx2*^{GoF} are: forward, 5′-CACATGAAGCAGCACGACTT-3′; reverse, 5′-TGCTCAGGTAGTGGTTGTCG-3′. *Nrf2*$^{nu/nu}$ is available from the Jackson Laboratory (strain name B6.129X1-Nfe2l2^{tm1Ywk}/J, stock number 017009)[12]. *Yap*$^{f/f}$ strain has been described previously[27].

Cardiac apex resection. Surgical resection of the heart apex was performed on P1 and P8 mice as described previously[2,28]. For P8 surgery, tamoxifen was administrated daily from P7–P10, by subcutaneous injection at a dosage of 40 mg kg^{-1} (ref. 28). Vicryl sutures (6-0 absorbable) were used to close the thoracic cavity, and the entire procedure required approximately 12 minutes from the onset of hypothermia to recovery. Sham procedures excluded apex amputation. Mice were subjected to echocardiography and then euthanized at 21 DPR for P1 resection, or 28 DPR for P8 resection. Dissected hearts were processed for histology and immunohistochemistry. Fibrotic scar size was measured using ImageJ 1.43u (National Institutes of Health) and the *n* number for each group is as follows: Fig. 2, 10 for *Pitx2*$^{f/f}$; 8 for *Pitx2* CKO; Supplementary Fig. 2, 10 for *Mhc*$^{cre\text{-}Ert}$, and 7 for *Pitx2*-overexpressing; Supplementary Fig. 3, 5 for *Salv*$^{f/f}$;*Pitx2*$^{f/f}$; 7 for *Salv* CKO; 3 for DKO; Supplementary Fig. 8, 4 for each genotype.

LAD-O. LAD-O in P8 mice was performed according to previous descriptions; tamoxifen was administrated daily from P7–P10, by subcutaneous injection at a dosage of 40 mg kg^{-1} (ref. 28). Nylon sutures (8-0 non-absorbable) were used to occlude the LAD. Proper occlusion was noted by blanching of the myocardium and during dissection 3–4 weeks after occlusion via visual inspection. Vicryl sutures (6-0 absorbable) were used to close the thoracic cavity, and the entire procedure required approximately 12 minutes from the onset of hypothermia to recovery. Sham procedures excluded placement of a suture around the LAD. Mice were subjected to echocardiography, and then euthanized at 3–4 weeks after occlusion. Hearts were processed for histology and immunohistochemistry. Automated fibrotic scar size was measured using image segmentation MIQuant, open source code for Matlab[29]. The *n* number for each group is as follows: Fig. 3, 5 for *Salv*$^{f/f}$;*Pitx2*$^{f/f}$, 5 for *Salv* CKO, 4 for DKO. Alternatively, LAD-O was performed at P2, with minor modification from P1 apex resection and P8 LAD-O procedures described above, tamoxifen was administrated daily from P2–P3 when needed[30]. The *n* number for each group is as follows: Supplementary Fig. 1, 8 for *Pitx2*$^{f/f}$, 7 for *MCK*^{cre};*Pitx2*$^{f/f}$, 4 for *Mhc*$^{cre\text{-}Ert}$;*Pitx2*$^{f/f}$.

Adult LAD-O was performed as described for P8 with minor modifications[28]. For *Pitx2*-overexpressing and control (*Mhc*$^{cre\text{-}Ert}$) mice, surgery was performed in 8-week-old mice, and tamoxifen was administered by intraperitoneal injection at three time points: 7 and 6 days before LAD-O and within 2 h after LAD-O, at a dosage of 40 mg kg^{-1}. Echocardiography was performed at 2, 3 and 4 weeks after LAD-O. The mice were then euthanized and hearts were subjected to histology. Automated fibrotic scar size was measured as described for P8 LAD-O. The *n* number for each group is as follows: Fig. 1, 5 for control and 5 for *Salv* CKO, 5 sham controls for each group; Fig. 2, 5 for *Mhc*$^{cre\text{-}Ert}$ LAD-O, 8 for *Pitx2*-overexpressing LAD-O. 5 sham controls were used for each genotype.

Echocardiography. Echocardiography was performed in the Baylor College of Medicine Mouse Phenotyping Core using a VisualSonics 2100 system. Evaluation of ejection fraction and fractional shortening of apex resection model was performed as previously described[2,28]. The *n* number for each group is as follows: Fig. 2, *Pitx2*$^{f/+}$, 3 for sham, 6 for resection; *Pitx2*$^{f/f}$, 6 for sham, 5 for resection; *Pitx2* CKO, 5 for sham, 17 for resection; adult LAD-O, control (*Mhc*$^{cre\text{-}Ert}$), 4 for sham, 5 for LAD-O; *Pitx2*-overexpressing, 5 for sham, 8 for LAD-O. Fig. 3, control (*Salv*$^{f/f}$;*Pitx2*$^{f/f}$), 11 for sham, 12 for LAD-O; *Salv* CKO, 5 for sham, 4 for LAD-O; DKO, 6 for sham, 7 for LAD-O. Supplementary Fig. 1, *Pitx2*$^{f/f}$, 7 for sham, 8 for LAD-O; *MCK*^{cre};*Pitx2*$^{f/f}$, 5 for sham, 7 for LAD-O; *Mhc*$^{cre\text{-}Ert}$;*Pitx2*$^{f/f}$, 4 for sham, 4 for LAD-O. Supplementary Fig. 2, control (*Mhc*$^{cre\text{-}Ert}$), 6 for sham, 25 for resection; *Pitx2*-overexpressing, 5 for sham, 16 for resection. Supplementary Fig. 7, control (C57BL6), 3 for sham, 5 for LAD-O; *Nrf2*$^{nu/nu}$, 3 for sham, 5 for LAD-O.

EdU incorporation. For P6 and P16 mice, 0.25 mg of EdU was injected subcutaneously 7 h before collecting the hearts. After dissection, hearts were fixed with 10% neutral buffered formalin and processed for paraffin embedding. Seven-micrometre-thick tissue sections were prepared. EdU incorporation was detected using the Click-it EdU imaging kit (Life Technologies). Tissue slides were imaged with a Leica TCS SP5 confocal microscopy, and images were processed by Leica LAS AF software (Leica Microsystems).

Cardiomyocyte proliferation studies. To assess cardiomyocyte proliferation rates, 5 DPR *Pitx2* CKO and control (*Pitx2*$^{f/f}$) mice and 8 DPR *Pitx2*-overexpressing and control (*Mhc*$^{cre\text{-}Ert}$) mice (as described earlier) were used. EdU labelling and detection were performed as described above. Mouse monoclonal anti-cTnT (1:200) (Thermo Scientific) was used to label cardiomyocytes. Images were acquired as described earlier. The cardiomyocyte proliferation rate was calculated by dividing the number of EdU-positive cardiomyocytes by the total number of cardiomyocytes in the field. Three comparable sections (every third section) from each heart were used.

NAC administration. NAC (PharmaGrade, A5099 Sigma-Aldrich) was solved in sterile PBS at a concentration of 10 mg ml^{-1}. After P1 apex resection, mice were weighed daily, and NAC solution was injected subcutaneously from 1 to 10 DPR at a dosage of 75 mg kg^{-1}. Three comparable sections (every third section) from each heart were used, and five hearts were used in each group in Fig. 4j–n.

Cell culture. P19 cells (ATCC CRL-1825) were cultured in αMEM medium (Mediatech, Corning), supplemented with 10% FBS (Gibco, Life Technologies) and 1% penicillin/streptomycin (HyClone Laboratories, Thermo Scientific). 0.25% trypsin was used for dissociating and splitting cells. H_2O_2 (Sigma-Aldrich) and doxorubicin (D-4000, LC Laboratories) were diluted in αMEM with 1% FBS and 1% penicillin/streptomycin at final concentrations of 300 μM and 0.5 μM, respectively. After 8 h of treatment, cells were collected and subjected to mRNA or protein extraction. The ES cells used in this study have been described previously[6]. Mycoplasma detection kit (B39030, http://www.biotool.com) and MycoAlert kit (LT07-318, Lonza) were used, and no contamination was observed.

Transfection of siRNA in P19 cells. Lipofectamine RNAiMAX transfection reagent (ThermoFisher Scientific) was used to deliver siRNA targeting *Nrf2* into P19 cells following the manufacturer's guideline. The siRNA oligonucleotides were pre-designed DsiRNA Duplex from Integrated DNA Technologies. Oligonucleotide sequences: antisense, rGrArUrGrUrCrArArUrCrArArArUrCrCrArUrGrUrCrCrUrGrCrUrG; sense, rGrCrArGrGrArCrArUrGrGrArUrUrUrGrArUrUrGrArCrATC.

Generation of P19 *Pitx2* knockout cell line using CRISPR-Cas9 technique. Lentivirus expressing *Cas9* was used to transduce P19 cells for generating stable cell line expressing *Cas9* (J.F.M. *et al.*, unpublished data). Guides targeting exons 5 and 6 of the *Pitx2* locus were designed using Optimized CRISPR Design (http://crispr.mit.edu, F. Zhang laboratory, MIT 2015). Guides sequences used: upstream, 5′-CACCGAATGAGGATGTGGGCGCCG, 3′-AAACCGGCGCCCACATCCTCATTC; downstream, 5′-CACCGTGTCCCTATAAACGTACGG, 3′-AAACCCGTACGTTTATAGGGACAC. Guides were inserted into pSpCas9(BB)-2A-GFP (PX458) (F. Zhang, Addgene plasmid 48138)[31]. Cas9-expressing P19 cells were transfected with both guide plasmid simultaneously, using Lipofectamine 2000 Transfection Reagent (Thermo Scientific) according to the manufacturer's manual. GFP-positive cells were sorted using a BD FACSAria cell sorter. Single-cell clones were expanded and genotyped using the following primers: (1) 494 bp for wild type, undetectable for knockout: forward: 5′-GCACACACCCACACTTTCAC-3′, reverse: 5′-CTTCCACCCACCACTCCTAC-3′; (2) 272 bp for wild type, undetectable for knockout: forward: 5′-GAATGGGAAAAGAGGGGAAA-3′, reverse: 5′-CCAGCTTCTGGACTCAGCTT-3′; and (3) 558 bp for wild type, undetectable for knockout: forward: 5′-CCCCTTCTTCAACTCCATGA-3′, reverse: 5′-CTTGGGGACATTCCTTTGAA-3′. The forward primer of (1) and reverse primer of (3) were also combined as the fourth primer pair. The confirmed P19 *Pitx2* knockout cell line was used in this study.

Cytoplasmic and nuclear fraction. In brief, P19 cells were culture in 10-cm plates at 80% confluence. The cells were treated with vehicle (water) or H_2O_2 (300 μM) for 8 h before being collected for cell fraction assay. The NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) were used according to the manufacturer's manual.

Histology and immunofluorescence. Trichrome staining was performed as previously described[28]. Fixation, tissue processing, antigen retrieval and blocking for nonspecific staining have been described previously[25]. Samples were incubated in primary antibody at 4 °C overnight. After washing in PBS with Tween 20, sections were incubated in the appropriate fluorescent-labelled secondary antibodies, followed by counterstaining with DAPI (10 μg ml^{-1}) (Roche), and then mounted in VECTASHIELD hardset mounting medium (Vector Laboratories). P19 cells were fixed in formalin (VWR International) for 10 min, then permeabilized in

0.2% Triton X-100 (Bio-Rad Laboratories) in PBS. After blocking in 10% sheep serum (Sigma-Aldrich) for 30 min, cells were incubated with primary antibody for 2 h at room temperature, followed by a 1-h incubation in proper fluorescent-labelled secondary antibodies. Cells were counterstained with DAPI (Roche) then mounted in VECTASHIELD hardest mounting medium (Vector Laboratories). Primary antibodies used were as follows: mouse monoclonal anti-cTroponin T (1:200; Thermo Scientific), rabbit polyclonal anti-Pitx2 (1:400; Capra Science) and rabbit polyclonal anti-Nrf2 (1:200; Abcam). Secondary antibodies used were as follows: Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 546 donkey anti-mouse IgG (1:400–1:800; Life Technologies); biotinylated anti-mouse IgG (1:200; Vector Laboratories); streptavadin-Alexa Fluor 647 (1:200; Life Technologies). Immunofluorescent images were captured on (1) a Leica TCS SP5 confocal microscope (all functions controlled via Leica LAS AF software (Leica Microsystems)); (2) a Zeiss LSM 510 META laser scanning confocal microscope (all functions controlled via Zeiss LSM Image Browser software (Carl Zeiss Microimaging)); or (3) a Nikon Eclipse 80i upright microscope (all functions controlled by the NIS-Elements BR3.1 software program (Nikon Instruments)). All manuscript figures were prepared using Adobe Photoshop CS5 (Adobe Systems Inc.).

ROS detection. *Pitx2* CKO and control (*Pitx2*$^{f/f}$) 4 DPR hearts were cryo-embedded, and 10 μm tissue sections were prepared. CellROX green reagent (Life Technologies) was used to detect the presence of ROS according the manufacturer's manual with minor modifications. Tissue slides were warmed to room temperature (25 °C), and rinsed with PBS three times. CellROX substrate was added and incubated for 10 min at 37 °C. Slides were given three 5-min washes with PBS, and 10% neutral buffered formalin was added. After a 15 min fixation, mouse-anti-MF20 IgG (1:50; Developmental Studies Hybridoma Bank) and Alexa Fluor 546 donkey anti-mouse IgG (1:400; Life Technologies) were used to counterstain cardiomyocytes. Nuclei were highlighted by DAPI. Slides were mounted in VECTASHIELD hardest mounting medium (Vector Laboratories), and imaged using a Nikon Eclipse 80i upright microscope (Nikon Instruments) within 2 h of completion.

Co-immunoprecipitation. *Pitx2*^{flag} mice were subjected to P2 LAD-O, and ventricular tissue was collected at 4 DPMI. P19 cell fractions were obtained as described above. For Flag pull-down, anti-Flag M2 affinity gel (Sigma-Aldrich) was used. For Nrf2 pull-down, rabbit polyclonal anti-Nrf2 (Abcam) and protein A/G PLUS-Agarose (Santa Cruz Biotechnology) were used according to the manufacturer's manual.

Western blot. Ventricles of 6 DPR ($n = 3$) and sham ($n = 3$) *Pitx2*^{Flag} mice, P16 *Mhc*$^{cre\text{-}Ert}$ (control, $n = 3$) and *Pitx2*-overexpressing ($n = 3$), as well as P19 cells were collected and lysed in RIPA buffer, and the protein concentration was quantified using Pierce BCA protein assay kit (Pierce Biotechnology) as previously described[25], the P19 cell fraction were acquired as described earlier, with three biological replicates. Co-immunoprecipitation samples were acquired as described above. In brief, after separation via SDS–PAGE, proteins were transferred to PVDF membranes (EMD Millipore), blocked in 5% milk/TBS-Tween 20 and incubated with appropriate primary antibodies (all with 1:1,000 dilution in TBST) overnight at 4 °C (rabbit anti-Flag IgG and mouse anti-α-tubulin IgG, Sigma-Aldrich; rabbit anti-Pitx2 IgG, Capra Science; rabbit anti-Yap, Novus Biologicals; rabbit anti-TATA-binding protein, Cell Signaling Technology; rabbit polyclonal anti-Nrf2, Abcam). Membranes were then washed three times in TBST and incubated with goat-anti-rabbit and goat-anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5,000; Santa Cruz Biotechnology) for 1 h at room temperature. Protein detection was performed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). For quantification, Pitx2 and α-tubulin band densities for control (*Mhc*$^{cre\text{-}Ert}$) and *Pitx2*-overexpressing mice were determined using ImageJ software (National Institutes of Health).

ChIP. P1 apex resection or sham surgery was performed on *Pitx2*^{flag} neonates; P2 LAD-O was performed on C57BL6 neonates. At 5 days after surgery, whole ventricles were collected and subjected to ChIP assay using the EZ-ChIP kit (Millipore) according to the manufacturer's protocol. For Flag ChIP, anti-Flag M2 affinity gel (Sigma-Aldrich) was used; for Nrf2 ChIP, rabbit polyclonal anti-Nrf2 antibody (Abcam) was used; for Yap ChIP, rabbit polyclonal anti-Yap antibody (Novus Biologicals) was used.

ChIP-re-ChIP. P2 LAD-O was performed on *Pitx2*^{flag} neonates, and 4 days later whole ventricles were collected and subjected to the ChIP-re-ChIP assay as previously described[25]. Monoclonal anti-Flag BioM2 antibody (Sigma-Aldrich) was used for pulling down Flag; rabbit anti-YAP (Novus Biologicals) was used for pulling down Yap. For qPCR analysis of the peak and non-peak regions, the following primers were used. For peak region: *Oxnad1*, forward: 5′-GGGTTTTTAGTGGGCAACCTAT-3′, reverse: 5′-CTGGGCTTTAGAGACAGCTAGG-3′; *Ldha*, forward: 5′-CCAGAGATCTTGTCCAGTCCTT-3′, reverse: 5′-TTCAGTTCCAAAATGGGGATAC-3′; *Ndufb3*, forward: 5′-GTACCGGAACGTTTACCATCTC-3′, reverse: 5′-CACCAGCTCCCTAAATTACCTG-3′; *Ndufs8*, forward: 5′-CATAGTGCCCTTTCCTTCTTTG-3′, reverse: 5′-GGCGCATTAACCTCTCTGATAC-3′.

For non-peak-region: *Oxnad1*, forward: 5′-TAATGAGATCTGGTGCCCTCTT-3′, reverse: 5′-GACACACCCCATCTGTACTTCA-3′; *Ldha*, forward: 5′-GTATCTTCACAGGCCTTTCCTG-3′, reverse: 5′-GGCTGTGGGACAATGTTCTTAT-3′; *Ndufb3*, forward: 5′-TGCTACTCTTCCAGAGGACCTT-3′, reverse: 5′-GGTATGTGTGTGTGATGTGC-3′; *Ndufs8*, forward: 5′-TCTACTGCCTTTATGGCGTTTT-3′, reverse: 5′-CTCATGGGCTGTGACAATAGAA-3′.

qPCR. For Fig. 1h, i, mRNA was prepared from P19 cells, and control (C57BL6) and *Nrf2*$^{nu/nu}$ ventricles at P1. For Fig. 4d, DNA was prepared as described in the 'ChIP-re-ChIP' section. For Supplementary Fig. 9e, DNA was prepared according to the ChIP protocol as mentioned above. For Supplementary Fig. 2b, mRNA of *Mhc*$^{cre\text{-}Ert}$ (control) and *Pitx2*-overexpressing ventricles was prepared at P16, 8 days after the first tamoxifen injection (daily from P7–P10). For Supplementary Fig. 9f, g, mRNA was prepared from P19 cells and ES cells. Total mRNA was extracted using miRNeasy Mini Kit (Qiagen); cDNA was generated using qScript cDNA supermix (Quanta BioSciences); qPCR was performed on a StepOnePlue Real-Time PCR system (Applied Biosystems) with iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories), all according to the manufacturers' manuals. The primers used for ChIP-qPCR are as followed: *Gpx1* region1, forward: 5′-GCTTCATCCCTCCTAATGGA-3′, reverse: 5′-TGCCAGCATTAACTCAGAGC-3′; *Gpx1* region2, forward: 5′-TCTTCCTAGGCGGGACTCTA-3′, reverse: 5′-GGGTCTGGTCTAGCTCCTGT-3′; *Mt1*, forward: 5′-TTCTGCAGTCCAGTCTGACC-3′, reverse: 5′-ATAGGAGATGGCCTGGTGAC-3′; *Mt2* region1, forward: 5′-GCCCTCCCACCTACTCATTA-3′, reverse: 5′-GGTGACTGTCATCCCACTTG-3′; *Mt2* region2, forward: 5′-TTCACTAAGAGCTGCGAGGA-3′, reverse: 5′-ATCTGCAGAGCCAGGAAACT-3′; *Sod2* region1, forward: 5′-ACGTGGCTTCAGGAGAGTTT-3′, reverse: 5′-CAATATCGCTTGCTCTCAGC-3′; *Sod2* region2, forward: 5′-CTCTCATGCATGCAAATCCT-3′, reverse: 5′-CAGCTCTAAGGGACCCAGAC-3′; *Sod1* region1, forward: 5′-ACTGTGACCCTGCAAAAACA-3′, reverse: 5′-GTCCACCACTTCAGAGAGCA-3′; *Txn2* region1, forward: 5′-CCACACAGCTGAAGGAGAGA-3′, reverse: 5′-GGAGTGCTGGGAATGTAGGT-3′; *Txn2* region2, forward: 5′-CCAAAATACCCAAGCCTGTT-3′, reverse: 5′-TTTCCACATGCCTCTGTCTC-3′; *Ndufv1* region1, forward: 5′-GGCTGCGAGGAAGAAATAAC-3′, reverse: 5′-ACTAACGGTCCCAACTCCAG-3′; *Ndufv1* region2, forward: 5′-ACAAGATGCAGGTCATGGAA-3′, reverse: 5′-ATCAGAGCCACACTGTCTGC-3′; *Cox5a*, forward: 5′-GCTGTTCTGGGATTGGATCT-3′, reverse: 5′-AGAGCCTGTCTCTCCCAAAA-3′.

The primers used for other qPCR are as followed: *Gpx1*, forward: 5′-GTCCACCGTGTATGCCTTCT-3′, reverse: 5′-CTCCTGGTGTCCGAACTGAT-3′; *Mt2*, forward: 5′-CCGATCTCTCGTCGATCTTC-3′, reverse: 5′-AGGAGCAGCAGCTTTTCTTG-3′; *Mt1*, forward: 5′-GCTGTCCTCTAAGCGTCACC-3′, reverse: 5′-AGGAGCAGCAGCTCTTCTTG-3′; *Sod2*, forward: 5′-GGCCAAGGGAGATGTTACAA-3′, reverse: 5′-GCTTGATAGCCTCCAGCAAC-3′; *Txn2*, forward: 5′-CCCCTCAGTACAATGCTGGT-3′, reverse: 5′-TCCATCCTGGACGTTAAAGG-3′; *Pitx2*, forward: 5′-AGGGAGGGAGGCAAGAAAAG-3′, reverse: 5′-CTTGAAAGAGCCAGGGAACG-3′; *Nrf2*, forward: 5′-CCAGAAGCCACACTGACAGA-3′, reverse: 5′-GGAGAGGATGCTGCTGAAAG-3′.

RNA-seq and ChIP–seq. Total mRNA was extracted from the ventricles of *Pitx2* CKO and *Pitx2*$^{f/f}$ mice 5 days after P1 apex resection, using the miRNeasy Mini Kit (Qiagen); ChIP DNA was acquired as described above. RNA-seq and ChIP–seq were performed using the Ion Proton system for next-generation sequencing according to the manufacturer's direction. Sequenced reads were mapped to mm9 genome using Ion Torrent TMAP aligner with 'map4' option. We used HTSeq-Count (version 0.5.4) to quantify the aligned RNA-seq reads against exon regions of genes in RefSeq mm9 annotation. Differential expressed genes were detected using R package DESeq with threshold $P \leq 0.05$, fold change ≥ 1.5 and FDR $\leq 10\%$. ChIP–seq peaks were detected by Homer package 'findPeak' command using threshold FDR $\leq 10\%$. Only peaks detected from both biological replicates were annotated and overlaid with differential gene expression list. GO analysis was performed on DAVID online platform. Terms with $P \leq 0.05$ were included. Published data sets used in Fig. 1f were obtained from Gene Expression Omnibus (GEO) (series GSE52386, reviewed in ref. 32). Mapped human DHS-seq data were extracted from the GEO (GSE18927, GSE32970). In total, 1,002 genes were downregulated in *Pitx2* CKO 5 DPMI and were overlaid with Pitx2 ChIP–seq binding genes, which were further overlaid with Yap ChIP-seq from control 5 DPMI. We define genes that were co-regulated by both Pitx2 and Yap as expression level decreased in *Pitx2* CKO heart and bound by both factors on the promoter regions. Overrepresented GO analysis was performed using online tool DAVID 6.7 (https://david.ncifcrf.gov/).

GST pull-down assay. The mouse Yap, Pitx2a, Pitx2c and truncated proteins were prepared as previously described[33]. In brief, GST-tagged proteins were expressed in and purified from BL21 competent *Escherichia coli* (New England Biolabs), using Glutathione Sepharose 4B (GE Healthcare). YAP was cleaved from GST using PreScission Protease (GE Healthcare), and 1 μg was incubated with 15 μg of each truncated GST–Pitx2 protein for the pull-down assay. Incubation of corresponding truncated Pitx2 protein alone was used as controls. Purified YAP protein (2 μg) was loaded into the gel as a control. Rabbit-anti-YAP antibody (Cell Signaling Technology) was used for immunoblotting and the detection of YAP.

Coomassie blue staining. GST–Pitx2, GST–Yap and cleaved Yap protein were run on 10% SDS–PAGE gel. The gel was then stained for protein in Coomassie blue stain (2.5 $g l^{-1}$ in H_2O:methanol:glacial acetic acid at a ratio of 9:9:2) for 1 h with shaking, followed by destaining with Coomassie solvent (H_2O:methanol:glacial acetic acid = 9:9:2) for 2 h with shaking. The stained gel was scanned with EPSON Perfection 4490 Photo (Epson America).

Statistics. Each experimental group in the ChIP–seq and RNA–seq studies had $n = 2$. All quantitative experiments (for example, qPCR, western blot, cell count) have at least three independent biological repeats. For animal studies (neonatal and adult surgery), sample sizes were estimated based on our pilot studies. The n number for each experiment is summarized in relevant sections in the Methods. Differences between groups were examined for statistical significance using non-parametric test (Mann–Whitney test) (for two groups), or one-way ANOVA plus Bonferroni post-test (for more than two groups). Equal variances were assumed (no Welch's correction). Grubbs's test was used to determine outlier (GraphPad Prism, GraphPad Software). In the case of Fig. 4d, Wilcoxon signed-rank test (with a hypothetical value of 0) was used to compare anti-Yap plus anti-Flag group to anti-IgG group, as well as anti-Yap–anti-IgG group, since the latter two groups were undetected in qPCR assay, the same test (Wilcoxon signed-rank) was also applied to Fig. 1h, i (left panel) (with a hypothetical value of 1). All bar graphs represent mean ± s.e.m. $*P < 0.05$, $***P < 0.001$ were considered statistically significant.

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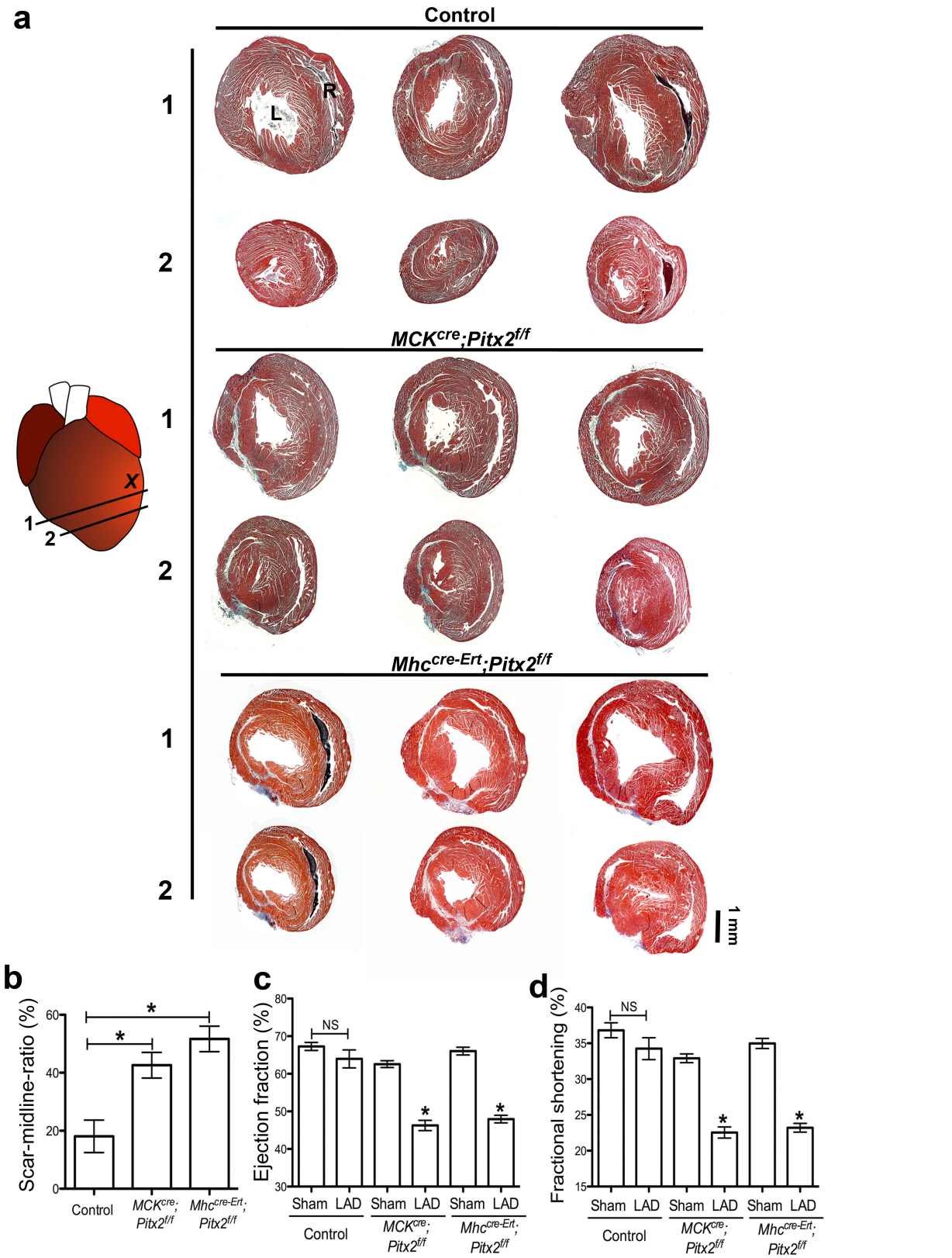
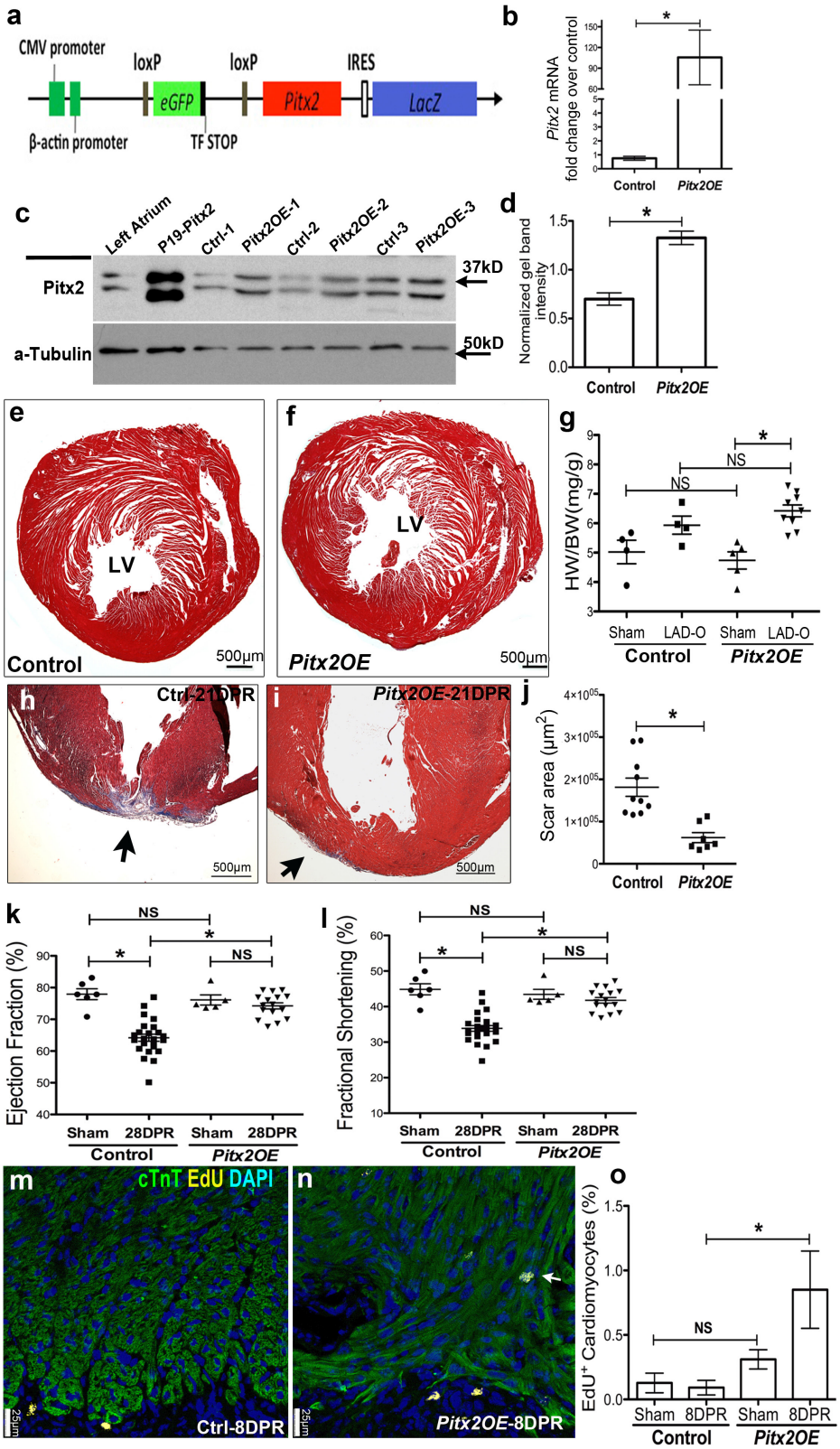
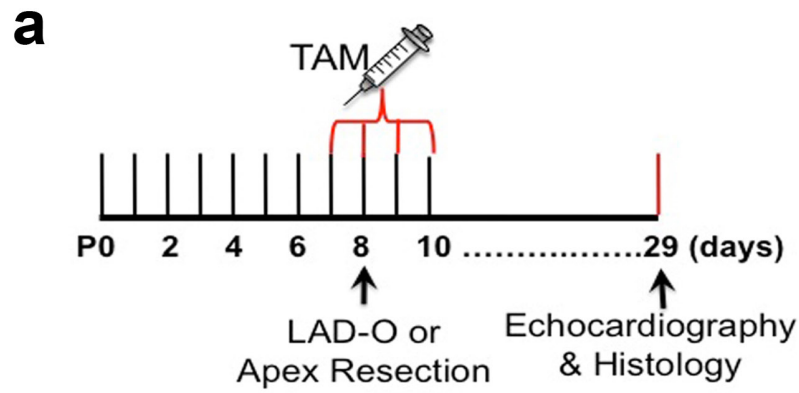
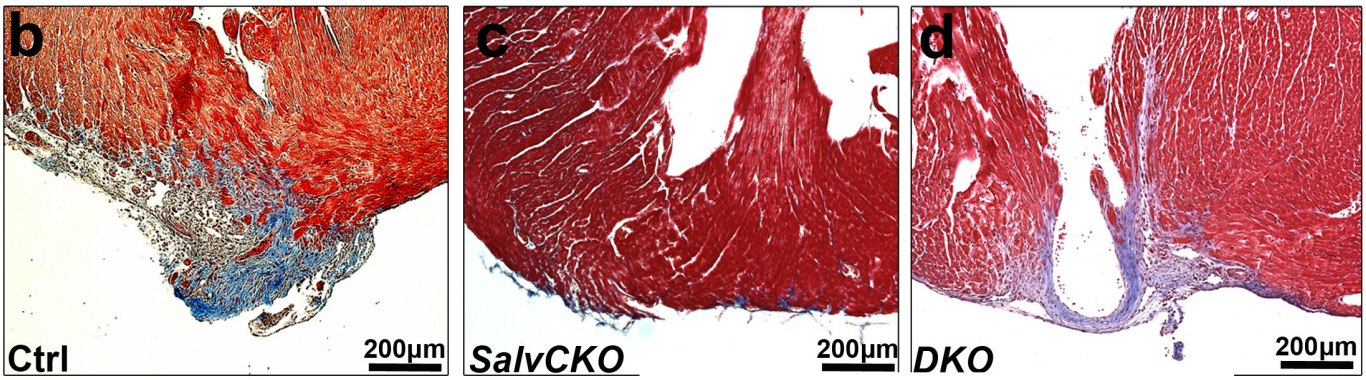


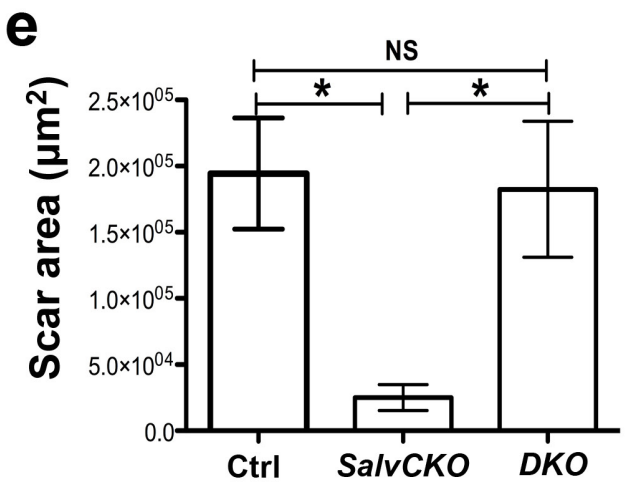
Extended Data Figure 1 | *Pitx2* is required in neonatal myocardial regeneration after LAD-O. a, Serial trichrome images of control (*Pitx2*$^{f/f}$), *MCK*^{cre};*Pitx2*$^{f/f}$, and *Mhc*$^{cre\text{-}Ert}$;*Pitx2*$^{f/f}$ 21 days after LAD-O performed in P2 mice. Three representative hearts of each genotype were shown. **b**, Percentage of fibrotic left ventricular myocardium quantified at 3 weeks after LAD-O; $n = 8$ for control (*Pitx2*$^{f/f}$), $n = 7$ for *MCK*^{cre};*Pitx2*$^{f/f}$, and $n = 4$ for *Mhc*$^{cre\text{-}Ert}$;*Pitx2*$^{f/f}$. **c**, **d**, Ejection fraction (**c**) and fractional shortening (**d**) of LAD-O and sham hearts (see Methods for *n*). L, left ventricle; R, right ventricle. Mean ± s.e.m. *$P < 0.05$ one-way ANOVA plus Bonferroni post-test (**c**, **d**) and Mann–Whitney test (**b**).

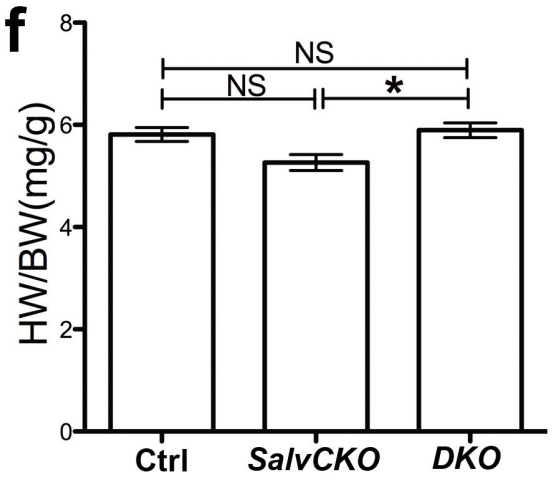


Extended Data Figure 2 | *Pitx2* promotes myocardial regeneration after apex resection at P8. a, Schematic of *Pitx2*-expressing construct (*Pitx2*^{Gof}). **b**–**d**, *Pitx2*^{Gof} was crossed with the *Mhc*$^{cre\text{-}Ert}$ strain to generate *Mhc*$^{cre\text{-}Ert/+}$;*Pitx2*^{Gof} (*Pitx2*-overexpressing) mice. After tamoxifen treatment from P7–P10, qPCR (**b**, $n=4$) and western blot (**c**, **d**, $n=3$) show the overexpression of *Pitx2* in the myocardium at P16. **e**, **f**, Trichrome-stained cross sections from 13-week-old sham hearts of control (**e**) and *Pitx2*-overexpressing (**f**) mice, with tamoxifen administrated at 7–8 weeks old. **g**, Heart weight over body weight ratio of adult sham and LAD-O hearts; $n=4$ (control sham), 4 (control LAD-O), 5 (*Pitx2*-overexpressing sham), 9 (*Pitx2*-overexpressing LAD-O). **h**–**j**, Apex resection of *Pitx2*-overexpressing (**i**) and control (*Mhc*$^{cre\text{-}Ert/+}$) (**h**) hearts at P8 followed by trichrome staining at 28 DPR; the scar area was quantified in **j**; $n=10$ (control mice), 7 (*Pitx2*-overexpressing mice). **k**, **l**, Echocardiography showed ejection fraction (**k**) and fractional shortening (**l**) at 28 DPR (see Methods for *n*). **m**–**o**, EdU labelling of *Pitx2*-overexpressing (**n**) and control (**m**) apical area, 8 days after P8 resection, sections were stained for cTnT (green), EdU (yellow), and DAPI (blue). Arrow indicates EdU-labelled cardiomyocytes, with quantification in **o**; $n=4$ mice per group. Mean ± s.e.m. $^*P<0.05$, one-way ANOVA plus Bonferroni post-test (**g**, **k**, **l**) and Mann–Whitney test (**b**, **d**, **j**, **o**).

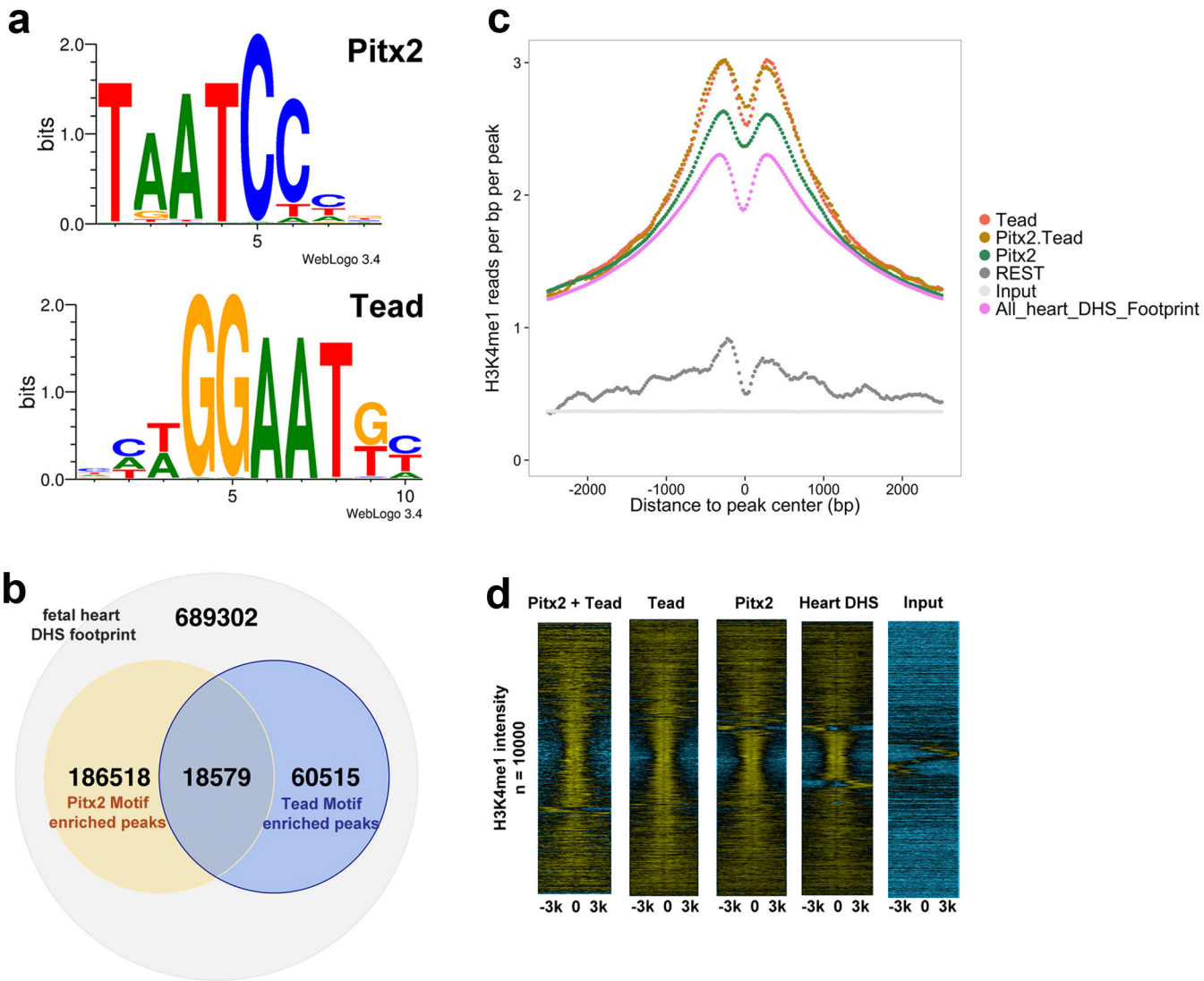




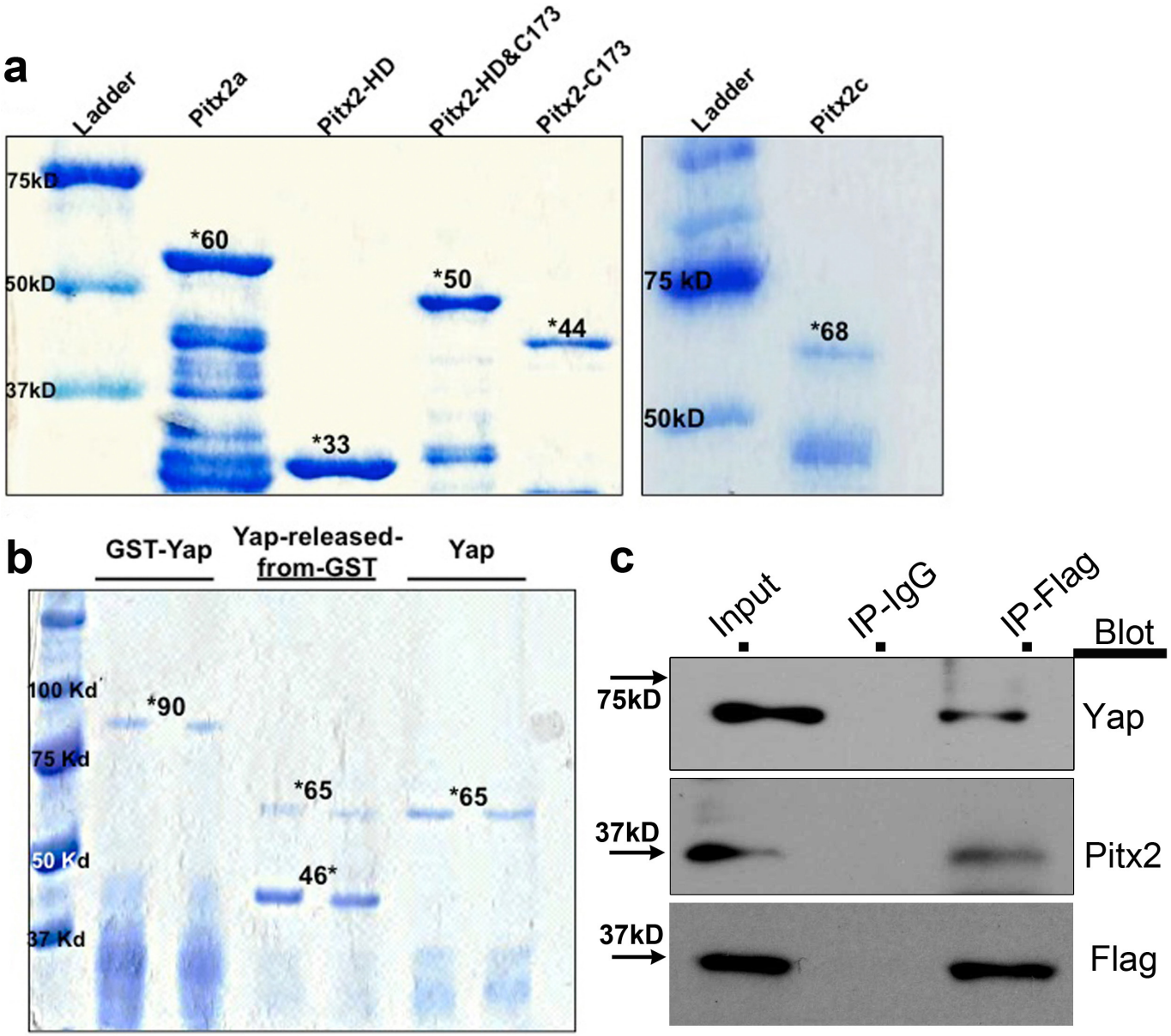




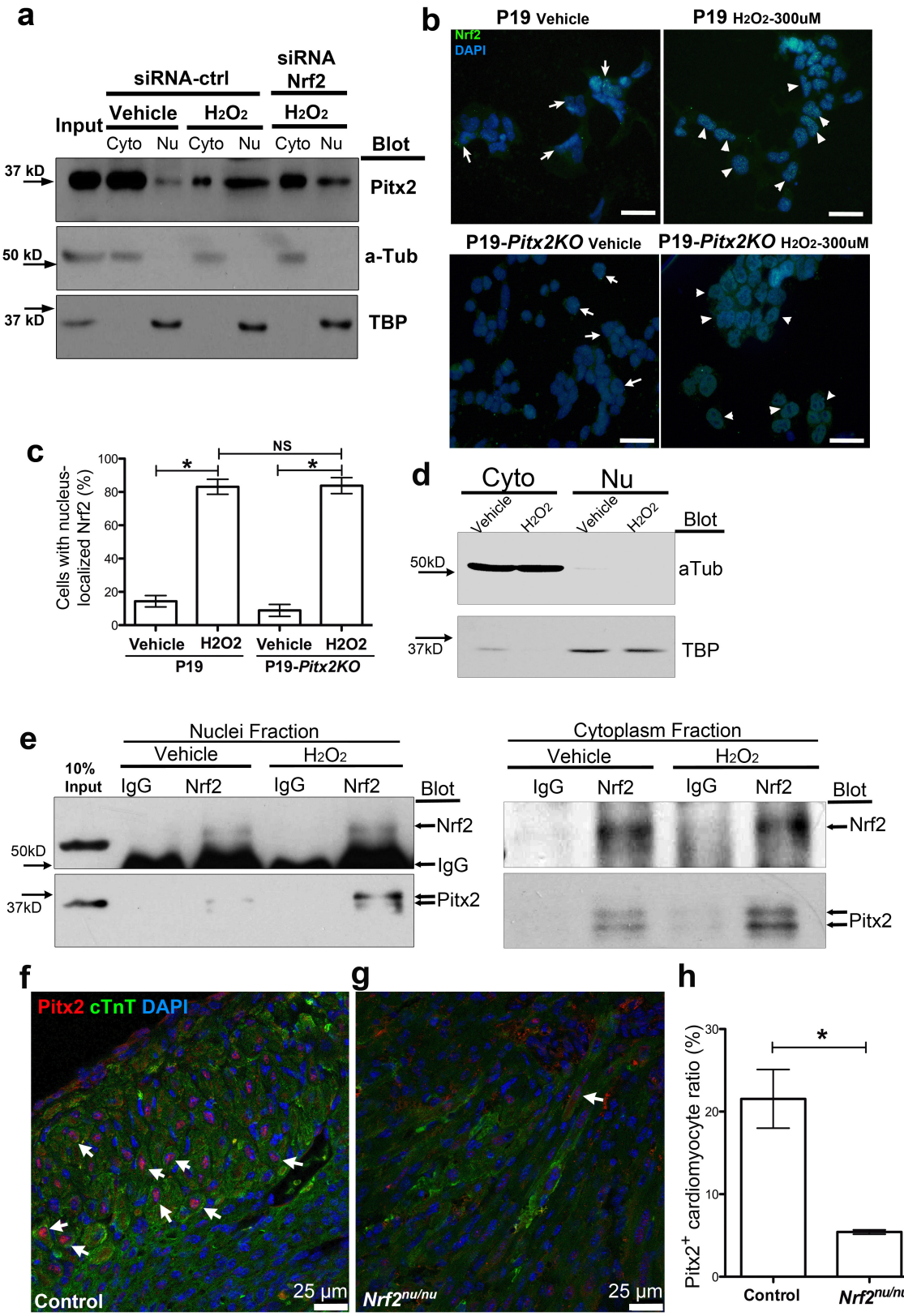
Extended Data Figure 3 | Pitx2 is required for Hippo-deficient heart regeneration. a, Schematic study plan for Fig. 3a–e. **b**–**e**, Trichrome-stained apical areas of control (**b**), *Salv* CKO (**c**) and double knockout (**d**) hearts 21 days after P8 apex resection. Scar area was quantified in **e**. **f**, Heart weight to body weight ratio of sham hearts at 28 days after tamoxifen administration. For *n* number, see Methods. Mean ± s.e.m. $*P < 0.05$, Mann–Whitney.



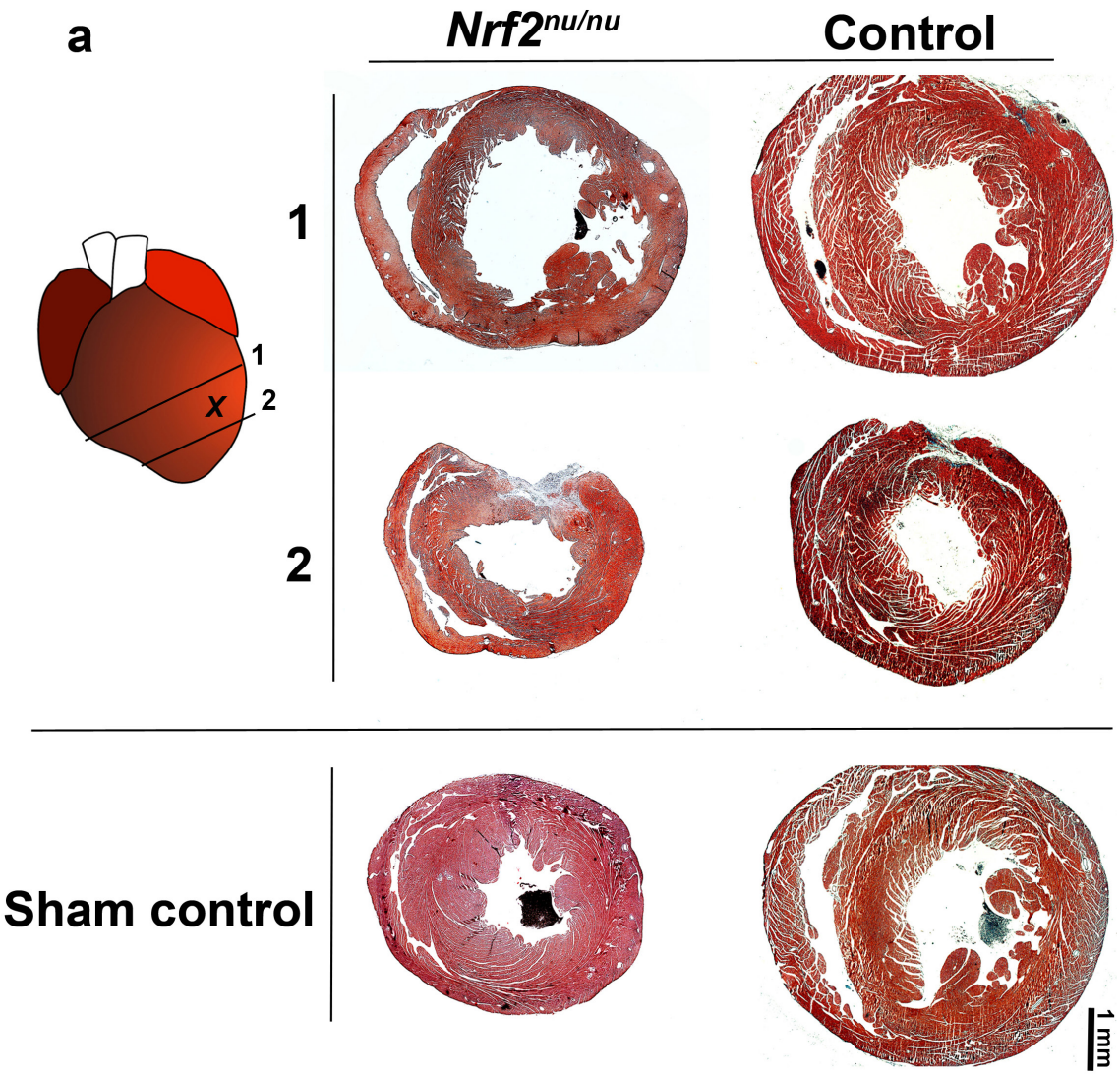
Extended Data Figure 4 | Co-occurrence of Pitx2 and Tead DNA-binding motifs in fetal heart enhancers. a, Consensus *Pitx2* and *Tead* motifs. **b**, *Pitx2* and *Tead* motif co-occurrence in fetal heart DHS peaks. **c**, Aggregate plot of H3K4me1 in fetal heart ChIP–seq reads within 6 kb range of DHS peaks. **d**, Heat map of fetal heart H3K4me1 ChIP–seq or input read density in 6-kb regions of DHS peaks. DHS peaks were centred on the *Pitx2* motif, *Tead* motif, *Pitx2-Tead* motifs, or randomly selected. The read density was in $\log_2$ scale. Blue, negative values; yellow, positive values.

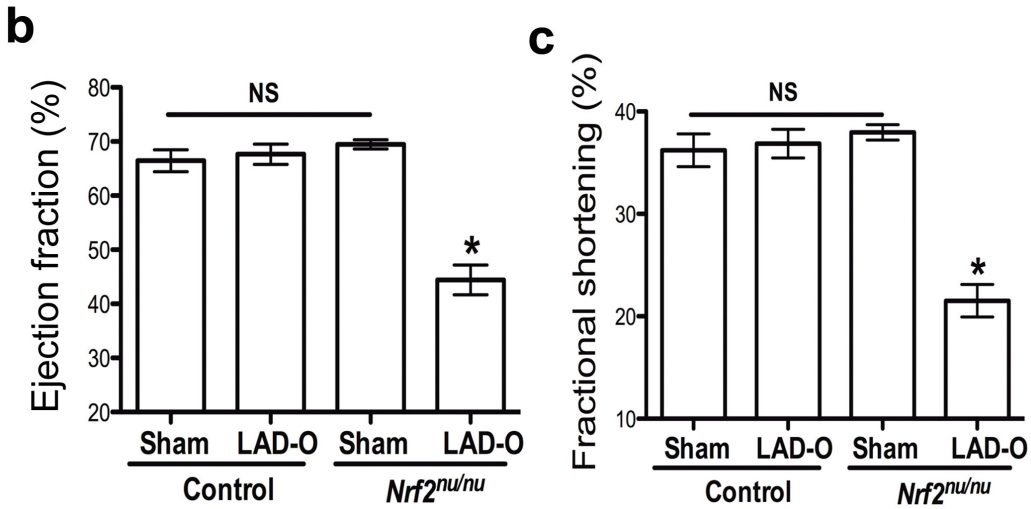


Extended Data Figure 5 | Generation of GST-tagged proteins and interaction between Pitx2 and Yap *in vivo*. **a**, The mouse Pitx2a, Pitx2c and truncated proteins were purified and run on a 10% SDS–PAGE gel, and Coomassie blue staining shows the GST fusion protein band with correct size (marked by asterisk). **b**, Coomassie blue staining of the purified GST–Yap, Yap cut by prescission protease and pure Yap protein. **c**, Co-immunoprecipitation of Flag in *Pitx2*^{flag} ventricles at 5 DPR, and blotting of Yap, Pitx2 and Flag.

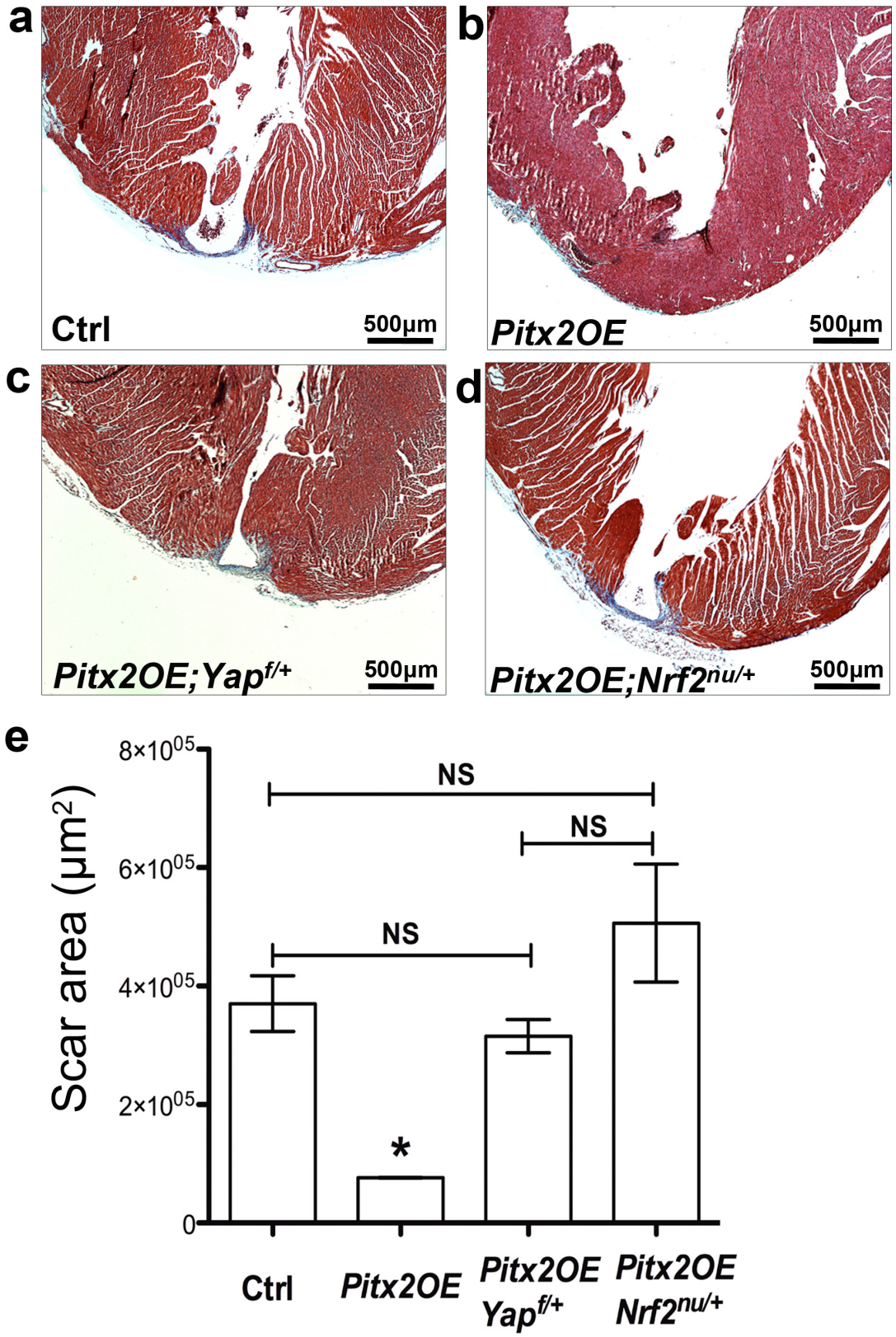


Extended Data Figure 6 | Nuclei-shuttling of Nrf2 is independent of *Pitx2*. **a**, Western blotting of Pitx2, α-tubulin, and TATA-binding protein (TBP) of P19 cell fraction after H_2O_2, with or without *Nrf2* siRNA treatment. **b**, Immunofluorescent staining of Nrf2 (green) in P19 control and *Pitx2* knockout cells after vehicle or H_2O_2 treatment. DAPI, blue. Scale bars, 50 μm. **c**, The ratio of cells with nuclear Nrf2 over total cell number; $n = 6$ biological repeats. **d**, Blotting of α-tubulin and TBP to show cell fraction of P19 cells used in **e**. **e**, Co-immunoprecipitation Nrf2 from nuclear and cytoplasmic fraction of P19 cells after vehicle or H_2O_2 treatment, blotting shows Nrf2 and Pitx2. **f**–**h**, 4 DPMI control (C57BL6) (**f**) and $Nrf2^{nu/nu}$ (**g**) cross-sections stained for Pitx2 (red), cTnT (green), and DAPI (blue), with the ratio of cardiomyocytes with nuclei-localized Pitx2 quantified in **h**; $n = 4$ mice per group. Arrows, $Pitx2^+$ cardiomyocytes. Mean ± s.e.m. $^*P < 0.05$, one-way ANOVA plus Bonferroni post-test (**c**) and Mann–Whitney test (**h**).

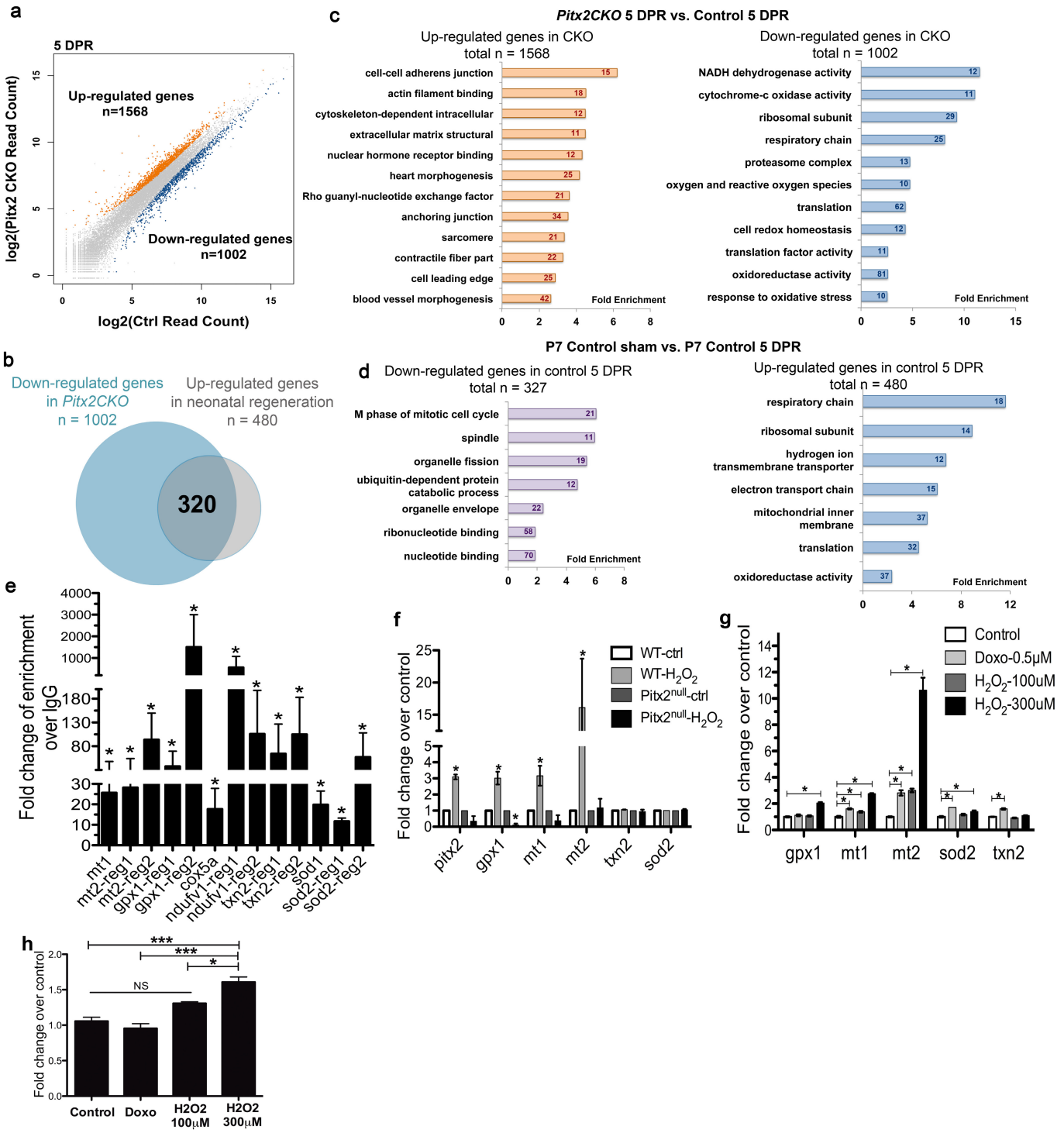




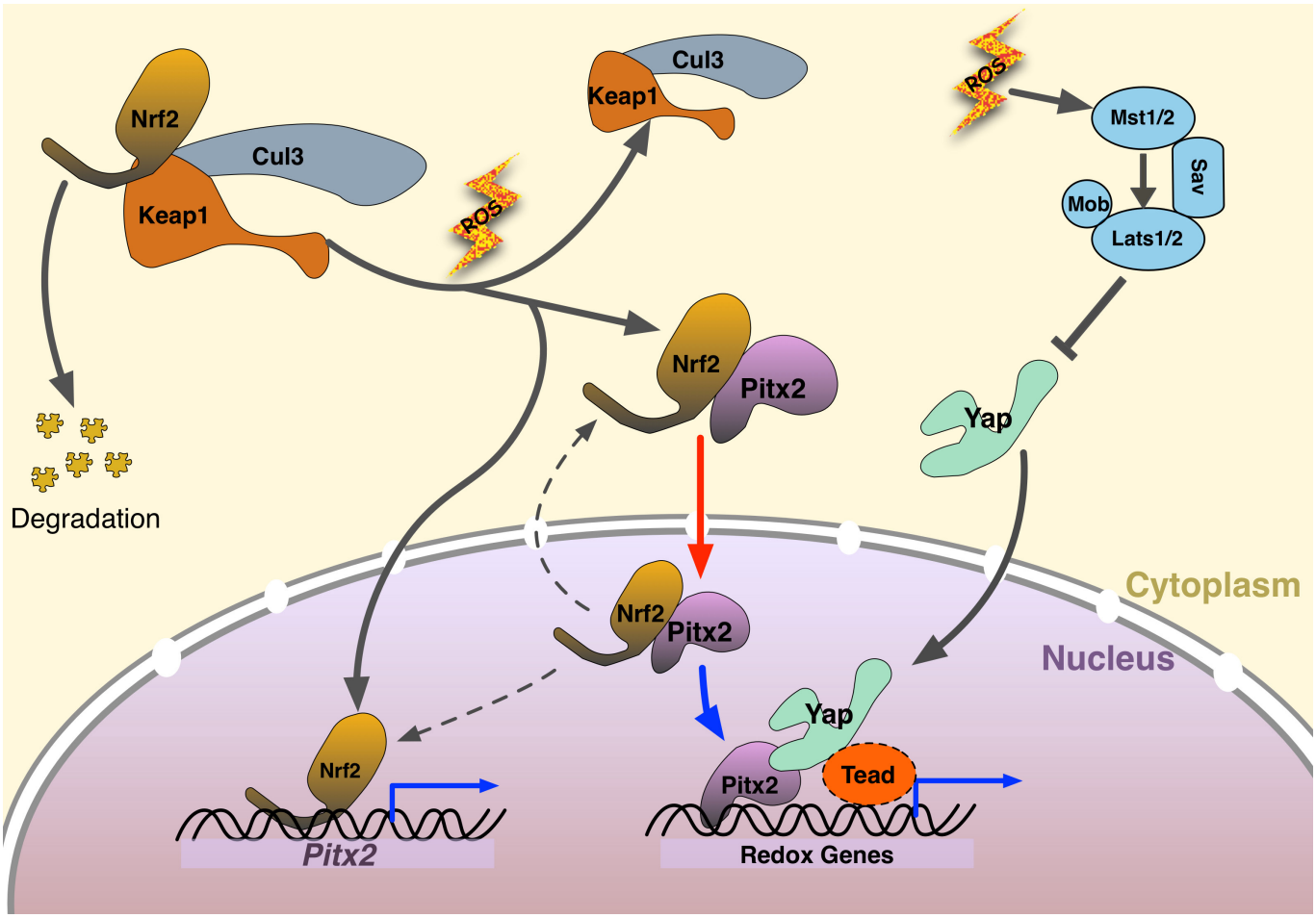
Extended Data Figure 7 | Nrf2 is required for neonatal myocardial regeneration. a, Trichrome images of *Nrf2*$^{nu/nu}$ and control heart (C57BL6) at 21 days after P2 LAD-O, along with sham controls. **b**, **c**, Ejection fraction (**b**) and fractional shortening (**c**) of LAD-O and sham hearts (see Methods for *n*). Mean ± s.e.m. *$P < 0.05$, Mann–Whitney test.



Extended Data Figure 8 | *Yap1* and *Nrf2* are essential for *Pitx2*-induced myocardial regeneration. a–d, Trichrome staining showing apical scarring of different groups at 28 DPR, apex resection was performed at P8. **e**, Quantification of scar area; $n = 4$ mice per group. Mean ± s.e.m. $^*P < 0.05$, Mann–Whitney test, control compared to the other three groups individually.



Extended Data Figure 9 | Pitx2 regulates antioxidant scavenger genes. **a**, Overall change of genes in *Pitx2* CKO mice compared to control. **b**, Upregulated genes in 5 DPR control over wild-type sham heart ($n = 480$) overlaid with downregulated genes in 5 DPR *Pitx2* CKO over 5 DPR control heart ($n = 1{,}002$). **c**, GO analysis of genes upregulated (left) and downregulated (right) in *Pitx2* CKO ventricles over controls at 5 DPR. **d**, GO analysis of genes upregulated (right) and downregulated (left) in 5 DPR control ventricles over age matching sham hearts. **e**, ChIP–qPCR confirming the binding of Pitx2 to the regulatory regions of target genes; $n = 4$ biological replicates. **f**, qPCR detecting *Pitx2* and antioxidant genes in wild-type and $Pitx2^{nu/nu}$ ES cells after vehicle or H_2O_2 treatment; $n = 4$ biological replicates. **g**, qPCR of antioxidant genes in P19 cells after doxorubicin or H_2O_2 treatment; $n = 5$ biological replicates. **h**, qPCR of *Pitx2* in P19 cells after doxorubicin or H_2O_2 treatment; $n = 5$ biological replicates. Mean ± s.e.m. $^*P < 0.05$; $^{***}P < 0.001$, Mann–Whitney test.



Extended Data Figure 10 | Mechanism model of *Pitx2*, *Nrf2* and *Yap1* responding to oxidative stress. When oxidative stress is low, Nrf2 is sequestered in cytoplasm by its degradation complex (Cul3, Keap1), and Pitx2 stays either in the cytoplasm or at low expression levels. When the redox balance is disturbed by ROS, Nrf2 breaks away from the degradation complex, and enters nuclei to upregulate *Pitx2* gene expression; Nrf2 also binds cytoplasmic Pitx2 and shuttles it to the nuclei, where Pitx2 and Yap co-regulate their common targets including critical antioxidant genes. In wild-type adult mouse heart, active Yap is maintained at a low level, even after ischaemic injury, and is thus not able to repair myocardium efficiently. When *Pitx2* is overexpressed in cardiomyocytes, sufficient amounts of Pitx2 will cooperate with low levels of resident active Yap to induce the expression of beneficial antioxidant scavengers in a synergetic pattern, rendering protection to injured myocardium. Red arrow, supported by *in vitro* evidence; Blue arrows, supported by *in vivo* evidence.