Supplemental Material

Data S1.

SUPPLEMENTAL MATERIALS AND METHODS

Cell Culture

IRB approval was obtained for the use of animal cells in this study. All the procedures followed were in accordance with guidelines set by The Chinese University of Hong Kong. mESC line D3 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and was cultured as we previously described [19-22]. mESCs were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 15% FBS, 2 mM L-glutamine, 0.1 mM NEAA, 0.1 mM β-mercaptoethanol, 1,000 U/mL LIF, 50 U/mL penicillin and 50 μg/mL streptomycin. To maintain the undifferentiated state and prevent spontaneous differentiation, mESCs were seeded onto irradiated CD-1 Mouse Embryonic Fibroblast (MEF). Cells were cultured in incubator at 37°C with 5% CO_2.

Cardiac differentiation of mESCs

To induce cardiac differentiation, hanging drops method was employed. Hanging drops were made at 800 cells per 20 μL hanging drops. The hanging drops were

placed onto the lid of 100mm petri dish and incubated at 37°C with 5% CO_2. The differentiation medium consisted of DMEM supplemented with 15% FBS, 2 mM L-glutamine, 0.1 mM NEAA, 0.1 mM β-mercaptoethanol, 50 U/mL penicillin and 50 μg/mL streptomycin. On day 2 of differentiation, embryoid bodies (EB) were formed and were washed into another 100mm petri dish and incubated at 37°C with 5% CO_2 for another 5 days. At differentiation day 7, EBs were transferred to 100mm culture dish coated with 0.1% gelatin and cultured at 37°C with 5% CO_2. Differentiation media was changed every 4 days.

PCR, subcloning and sequencing for the first identification of mPinX1t

Forward primer franking 23bp upstream of mPinX1 (5'-TAAGGGAATTCAT***CAGCGTTCGACAAACTTGAG***-3'; italic and bold sequence indicates sequence that is complementary to mPinX1 gene) and reverse primer franking 26bp downstream of mPinX1 (5'-TAAGGGCGGCCGC***ACAGTTGAGTGGTTGGAGGC***-3'; italic and bold sequence indicates sequence that is complementary to mPinX1 gene) coding sequence (accession number: NM_028228) were designed for PCR using mESCs and their differentiation derivatives as the template. The amplified PCR products were separated by DNA gel electrophoresis in 1% TAE agarose (Invitrogen, Waltham, MA,

USA) gel with 0.7 μg/mL ethidium bromide (Invitrogen). The PCR product was visualized using 2UV™ Transilluminator and excised out and cut. The DNA was purified using QIAquick DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions and were digested with restriction enzymes EcoRI and NotI and further subcloned into the pTriEx4-neo vector. DNA sequencing was performed by commercial sequencing service conducted by the TechDragon Company.

Polysome fractionation assay

100 µg cycloheximide (CHX) was added to mESCs and incubated at 37°C with 5% CO_2 for 15 mins. MESCs were further washed twice with PBS supplemented with 100 µg/mL CHX. Cells were pelleted and lysed on ice with hypotonic lysis buffer [50 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$, 1.5 mM KCl, DEPC water] supplemented with 0.59 mM PMSF, 0.12 mg/mL CHX, 2.35 mM DTT and 0.09 U/μL RNasin for 15 mins. 0.59% Triton X-100 and 0.59% Na deoxycholate were added to the lysate and vortexed briefly. The lysate was put on ice for 15 mins and centrifuged at 16,000 g for 7 mins at 4°C. A sucrose gradient density was set up by layering high density sucrose gradient buffer [20 mM Tris HCl (pH 7.5), 140 mM KCl, 5 mM $MgCl_2$, 50% sucrose, 0.5 mM DTT, 100 μg/mL CHX, DEPC water] with low density sucrose gradient

buffer [20 mM Tris HCl (pH 7.5), 140 mM KCl, 5 mM $MgCl_2$, 7% sucrose, 0.5 mM DTT, 100 μg/mL CHX, DEPC water] in 1:1 ratio. 800 ng cell lysate was loaded on top of the sucrose gradient density and subjected to ultracentrifugation at 217,874 g (35,000 rpm) for 3 hours. Fractions were collected to microcentrifuge tubes and Trizol LS solution (Life Technologies, Waltham, MA, USA) was added to the fractions for RNA extraction. The extracted RNA was reverse transcribed and mPinX1t expression was determined by qPCR using mPinX1t specific primers. mPinX1t forward primer: 5'-AAAGGGAAGGATCTGTCCTC-3' mPinX1t reverse primer: 5'-CAATTTAATCCGAGGAGCCTGAG-3'.

Full length RNA ligase-mediated rapid amplification of 5' cDNA ends assay (RLM 5'RACE)

The 5' RACE assay was carried according to the manufacturer's instructions (L1502-01, Life Technologies). Briefly, total RNA was extracted from mESCs using Trizol solution (Life Technologies). The extracted RNA was dephosphorylated and de-capped using reagents provided in the kit. The initial dephosphorylation reaction specifically removed the phosphate group at the 5' end of the truncated mRNA or the non-coding mRNA but not 5' capped intact mRNAs. Subsequently, after the decapping reaction, only RNA with phosphate group at the 5' end (i.e. previously 5'

capped intact mRNAs) will be ligated to the GeneRacer RNA oligo supplied in the kit. The resultant RNA was reverse transcribed using the oligo-dT primer provided in the kit. The 5’ UTR sequence was amplified using 5’ GeneRacer-specific forward primer: 5’-CGACTGGAGCACGAGGACACTGA-3’ and mPinX1t-specific reverse primer: 5’-CACAATTTAATCCGAGGAGCCTGAGCAA-3’ with thermocycling condition as follows: initial denaturation at 94°C for 2 mins, followed by 34 amplification cycles of denaturation at 94°C for 30 secs, annealing and extension at 71°C for 90 secs and final extension at 72°C for 5 mins. A second round PCR utilizing 1 μL of PCR product from the first round PCR was performed by changing the forward primer to 5’ GeneRacer nested primer: 5’-GGACACTGACATGGACTGAAGGAGTA-3’. Thermocycling condition was the same as the first round PCR.

Full length RNA ligase-mediated rapid amplification of 3’ cDNA ends assay (RLM 3’RACE)

The 3’ RACE assay was carried according to the manufacturer’s instructions (L1502-01, Life Technologies). Briefly, total RNA was extracted from mESCs using Trizol solution (Life Technologies). The resultant RNA was reverse transcribed using the oligo-dT primer provided in the kit. The 3’ UTR sequence was amplified using 3’ GeneRacer-specific reverse primer: 5’-GCTGTCAACGATACGCTACGTAACG-3’

and mPinX1t-specific forward primer: 5’-TTGCTCAGGCTCCTCGGATTAAATTGTG-3’ with thermocycling condition as follows: initial denaturation at 94°C for 2 mins, followed by 34 amplification cycles of denaturation at 94°C for 30 secs, annealing and extension at 71°C for 90 secs and final extension at 72°C for 5 mins. A second round PCR utilizing 1 μL of PCR product from the first round PCR was performed by changing the forward primer to 5’ nested primer: 5’-GTGAAGGCAGAGCAACTTTGCCAGA-3’ and reverse primer to 3’ GeneRacer nested primer: 5’-CGCTACGTAACGGCATGACAGTG–3’. Thermocycling condition adopted a touchdown approach: 1) 94°C for 2 mins, 2) 94°C for 30 secs, 3) 68°C for 1 min, 4) repeat steps 2 and 3 for 5 cycles, 5) 94°C for 30 secs, 6) 67°C for 30 secs, 7) 68°C for 1 min, 8) repeat steps 5 to 7 for 5 cycles, 9) 94°C for 30 secs, 10) 65°C for 30 secs, 11) 68°C for 1 min, 12) repeat steps 9 to 11 for 20 cycles, 13) final extension 68°C for 10 mins.

SDS PAGE and Western Blotting

Cells were washed with ice cold PBS and lysed by ice cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) freshly supplemented with protease inhibitor cocktail [1 μg/mL Leupeptin, 5 μg/mL Aprotinin, 0.59 mM PMSF, 1 mM sodium orthovanadate, 1 mM EGTA (pH 8.0) and 1 mM EDTA (pH 8.0)] and

phosphatase inhibitor cocktail [2 mM NaF, 4 mg/mL 2-glycerolphosphate and 0.4 mM HEPES (pH 7.3)]. The lysate was put on ice for 10 mins and centrifuged at 16,000g for 20 mins at 4°C. The supernatant was collected and protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). 50 to 100 µg proteins were mixed with 2x sample loading dye [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.06% bromophenol blue] with 10% β-mercaptoethanol added. The samples were boiled at 100°C for 10 mins before loading onto 10% SDS polyacrylamide gel for polyacrylamide gel electrophoresis (PAGE). Proteins were separated by SDS-PAGE and transferred onto 0.22 µm PVDF membrane. The membrane was blocked with 5% non-fat dry milk in TBST buffer for 1 hr at room temperature. The membrane was then incubated with primary antibody anti-PinX1 (NBP1-83643, Novus Biologicals, Littleton, FL, USA) targeting the N-terminal of PinX1 or anti-PinX1 (ORB47163, Biorbyt, Cambridge, Cambridgeshire, UK) targeting the C-terminal of PinX1 at dilution ratio of 1:1000 and 1:1000 overnight, respectively. The membrane then probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies at dilution ratio of 1:3000 for 1 hr at room temperature and developed using chemiluminescent substrate (Pierce, Waltham, MA, USA).

Lentiviral vector subcloning and packaging

cDNA encoding myc-tag fused with the full coding sequence of mPinX1 and cDNA encoding myc-tag fused with the full coding sequence of mPinX1t were subcloned into pWPI (Addgene Cambridge, MA, USA) to make the lentiviral backbone DNA plasmids pWPI-myc-mPinX1 and pWPI-myc-mPinX1t respectively. shRNA against mPinX1 (sense sequence: 5'-AAGAAGAAAGTTTCCAGATAA-3') and scrambled shRNA sequence (sense sequence: 5'-GAAACAGATAATAGACAGTTA-3') were subcloned into pLVTHM (Addgene) to make the lentiviral backbone DNA plasmids pLVTHM-shmPinX1 and pLVTHM-shscrambled. To package lentivirus, $2X10^{6}$ HEK293FT at early passage were plated on T25 flask a day before transfection. Lentiviral backbone DNA constructs and the packaging plasmids (psPAX2 and pMD2.G) were co-transfected into the HEK293FT cells in OPTI-MEM I (Invitrogen) using Lipofectamine 2000 (Invitrogen). OPTI-MEM I medium with the complexes was changed back to normal medium 6 hrs after transfection and lentiviruses were collected at 24, 48 and 72 hrs post-transfection.

Lentiviral vector-mediated gene transfer to mESCs

On day 0, $1x10^{4}$ mESCs at passage 8 were seeded on 12-well plates with MEFs. On day 1, 200 μL of lentivirus, 200 μL of medium and 6 μg/mL polybrene were added

to each well. The transduction was repeated two more times at 12 hrs and 24 hrs after the first round of transduction. 12 hrs after the final round of transduction, all lentiviruses containing medium was removed and 500 μL of fresh medium was added to each well for maintenance. Clones with brightest green fluorescent signal and morphology at undifferentiated state were selected. For pWPI-mPinX1 stable cell line, 4 clones were selected. For pWPI-mPinX1t, pWPI only, pLVTHM-shmPinX1 and pLVTHM-shscrambed stable cell lines, 3 clones were selected.

Telomere Repeat Amplification Protocol (TRAP) Assay

The TRAP assay was done with Telo*TAGGG* telomerase PCR ELISA (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, the cells were harvested and counted using a hemocytometer. 2 x 10^5 cells were transferred to a new Eppendorf tube and centrifuged at 3,000 g for 10 mins at 4 ^{o}C. Supernatant was removed and the cell pellet was resuspended with 200 μL lysis reagent and incubated on ice for 30 mins. After that, the lysate was centrifuged at 16,000 g for 20 mins at 4 ^{o}C. 3 μL of the supernatant (cell extract) was mixed with 25 μL of reaction mixture and topped to 50 μL with autoclaved double distilled water. The tubes were then transferred to a thermo cycler and a combined primer elongation/ amplification reaction was performed by the following protocol: Primer elongation at 25 ^{o}C for 30

mins, then telomerase inactivation at 94 °C for 5 mins, followed by 30 cycles of denaturation at 94 °C for 30 secs, annealing at 50 °C for 30 secs and polymerization at 72 °C for 90 secs, and at last a final extension at 72 °C for 10 mins.

After elongation and amplification, the PCR products were hybridized to a digoxigenin (DIG)-labeled, telomeric repeat-specific detection probe and immobilized to a streptavidin-coated microplate. Firstly, 20 μL of the denaturation reagent was transferred to each new reaction tube, and then 5 μL of the PCR product was added and incubated at room temperature for 10 mins. Afterwards 225 μL of hybridization buffer was added per tube and mixed thoroughly by vortexing. 100 μL of the mixture was transferred per well of the precoated MP modules supplied, the wells were then covered with the self-adhesive cover foil and incubated at 37 °C for 2 hrs with shaking at 300 rpm. After incubation, the solution was removed completely and the wells were washed 3 times with 250 μL washing buffer. After removing all washing buffer, 100 μL of anti-DIG-POD working solution was added per well and incubated at room temperature for 30 mins in dark with shaking at 300 rpm. Again all the solution was removed completely and the wells were washed with 250 μL of washing buffer for 5 times. Then 100 μL of TMB substrate solution was added per well and incubated at room temperature for 20 mins with shaking at 300 rpm. Without removing the reacted substrate, 100 μL stop reagent was added to stop color development. By using a

microplate reader, the absorbance of the samples at 450 nm and 690 nm (as a reference wavelength) was measured.

Quantitative polymerase chain reaction

Total RNA was extracted by Trizol reagent according to the manufacturer's instruction. RNA was DNase I (Life Technologies) treated and reverse transcribed using SuperScript III reverse transcriptase (Life Technologies). SYBR GREEN PCR Master Mix (Applied BioSystems, Foster City, CA, USA) was used and the qPCR reaction was performed using ABI 7500 Fast Real Time PCR System. The reactions were performed in triplicate in the following conditions: 95°C for 30 secs, 40 cycles of 95°C for 5 secs and 60°C for 30 secs. Relative quantification was performed by using the $2^{-\Delta\Delta Ct}$ method where β-actin or Rpl13a were used as the housekeeping genes and the relative expressions of each gene was compared to that of the control cell line. Sequences of qPCR primers for each gene were listed below. mPinX1 forward primer: 5'-CCACGGTCAGGAAACAGCAG-3'; mPinX1 reverse primer: 5'-AGCCATCCTGAGCAAGCTTC-3'; mPinX1t forward primer: 5'-AAAGGGAAGGATCTGTCCTC-3'; mPinX1t reverse primer: 5'-CAATTTAATCCGAGGAGCCTGAG-3'; MHC forward primer: 5'-ACATACTCGTTCCCCACCTTC-3'; MHC reverse primer: 5'-AGCTGACAGGGGCCATCA-3'; cTnI forward primer:

5'-AGGGCCCACCTCAAGCA-3'; cTnI reverse primer: 5'-GGCCTTCCATGCCACTCA-3'; cTnT forward primer: 5'-TTCATGCCCAACTTGGTGCC-3'; cTnT reverse primer: 5'-CTCTCTTCAGCCAGGCGGTTC-3'; Cardiac actin forward primer: 5'-CCATTGTCACACACCAAAGC-3'; Cardiac actin reverse primer: 5'-CCAGCCCAGCTGAATCC-3'; Gata4 forward primer: 5'-CCCCAATCTCGATATGTTTGA-3'; Gata4 reverse primer: 5'-GGCATTGCACAGGTAGTGT-3'; Tbx5 forward primer: 5'-TCGGATGCAAAGTAAAGAGT-3'; Tbx5 reverse primer: 5'-GGTACTGGGACCCTAAATTG-3'; β-actin forward primer: 5'-AGAGGGAAATCGTGCGTGAC-3'; β-actin reverse primer: 5'-CAATAGTGATGACCTGGCCGT-3'; Rpl13a forward primer: 5'-GAGGTCGGGTGGAAGTACCA-3'; Rpl13a reverse primer: 5'-TGCATCTTGGCCTTTTCCTT-3'

Immunocytochemistry

Single mESC-derived cardiomyocytes were isolated as we have previously described [44] and were seeded onto 0.1% gelatin coated coverslips overnight. The cells were then fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA) in PBS for

25 mins in the dark and permeabilized with 0.1% Triton X 100 in PBS for 15 mins. The cells were blocked with 0.5% milk/ 5% normal goat serum (NGS) for 1 hr at room temperature. The coverslips were incubated with primary antibody anti-α-actinin at a dilution ratio of 1:800 in 0.5% milk/ 5% NGS overnight at 4°C. The coverslips were washed with PBST 4 times and incubated with AlexaFluor 594-conjugated goat anti-mouse secondary antibodies at a dilution ratio of 1:100 for 1 hr at room temperature. The coverslips were then washed 4 times PBST and incubated with AlexaFluor 488-conjugated phallodin stain (Life Technologies) at dilution ratio of 1:40 for 30 mins. The coverslips were incubated with DAPI in PBST at dilution ratio of 1:5000 for 10 mins at room temperature. The stain coverslips were put on glass slides with mounting solution (DAKO, Santa Clara, CA, USA) added and viewed using Olympus Fluoview FV1000 confocal system. Line analysis was performed using ImageJ software.

Confocal calcium imaging

Single spontaneously beating mESC-CMs were obtained as described above. Tyrode's solution with the following components (in mM): $MgCl_2$ 1, $CaCl_2$ 1.8, KCl 5.4, glucose 10, HEPES 10 and NaCl, 140, pH 7.2 (adjusted by NaOH) was used to wash the cells before imaging. After incubation with 5 μM Fluo-4 (Invitrogen) and

0.02% pluronic acid F-127 (Sigma) in dark for 12 min at 37 °C, mESC-CMs were gently washed with Tyrode's solution again. For measurement of calcium transients (CaTs), images were obtained using Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Japan) equipped with an argon laser of 488 nm at a frequency of 59 Hz. Raw traces of CaTs were shown as fluorescence intensity. Data analysis was performed with FV1000 (Olympus) and Origin 6.1 software (OriginLab, Northampton, MA, USA).

RNA immunoprecipitation (RNA-IP) assay

HEK293FT cells were grown on T75 format to 70% confluency. Pwpi-myc-mPinX1 or Pwpi-myc-mPinX1t plasmids together with plasmids harboring the cDNAs of cardiac transcription factors, including pCI-Gata4 (kindly provided by Prof. Qiangrong Liang in College of Osteopathic Medicine in New York Institute of Technology) or pAC-CMV-Tbx5 (kindly provided by Prof. Katherine Yutzey in Division of Molecular Cardiovascular Biology in The Heart Institute Cincinnati Children's Hospital Medical Center) were transfected into HEK293FT using Lipofectamine 2000 (Invitrogen). Transfection was performed according to the manufacturer's protocol.

Transfected HEK293FT cells were washed twice with ice-cold PBS and

subjected to 1% paraformaldehyde fixation for 10 mins at room temperature. The fixing reaction was quenched by adding 125 mM glycine solution for 5 mins at room temperature. The transfected cell pellet was collected and resuspended in 100 μL plasma membrane lysis buffer [5 mM PIPES, 85 mM KCl, 0.5% NP40, 1x Roche protease inhibitors cocktail, RNase inhibitor (50 U/mL)]. The lysate was centrifuged at 2300 g for 5 mins at 4°C. The supernatant (cytosolic fraction) was transferred to a new micro-centrifuge tube and the remaining pellet was further resuspended in 300 μL nuclear membrane lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris HCl pH 8.1, 1x Roche protease inhibitors cocktail, RNase inhibitor (50 U/mL)]. The lysate was put on ice for 10 mins and mixed with the cytosolic fraction. The lysate was sonicated 3 times on ice for 10 to 15 secs each. The lysate was centrifuged at 16,000 g for 10 mins at 4°C. The supernatant was collected and 50 μL was kept as control. The remaining lysate was diluted to 1 mL by IP Buffer [0.01% SDS, 1.1% Triton X 100, 1.2 mM EDTA, 16.7 mM Tris pH 8.1, 167 mM NaCl, 1x Roche protease inhibitors cocktail, RNase inhibitor (50 U/mL)] and aliquoted into two tubes. Anti-myc antibody (9B11; Cell signaling, Danvers, MA, USA) at dilution ratio of 1:250 and isotype antibody IgG2a (Abcam, Cambridge, UK) were added to each tube and incubated overnight at 4°C with gentle rotation. 50 μL Protein A/Protein G agarose beads (sc-2003; Santa Cruz Biotechnology, Dallas, TX, USA) were added to each tube and incubated with

gentle rotation for 3 hrs at 4°C. Immunoprecipitates were centrifuged down at 400 g for 2 mins at 4°C.

The immunoprecipitates were washed sequentially with the following buffers: a) low salt buffer (0.1% SDS, 1% Triton X 100, 2 mM EDTA, 20 mM Tris HCl pH 8.1, 150 mM NaCl), b) high salt buffer (0.1% SDS, 1% Triton X 100, 2 mM EDTA, 20 mM Tris HCl pH 8.1, 500 mM NaCl), c) LiCl buffer (0.25 M LiCl, 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris HCl pH 8.1), d) TE buffer (pH 8.0) (10 mM Tris HCl pH 8.1, 1 mM EDTA) each for two times. After washing, the immunoprecipitates were centrifuged at 400 g for 2 mins at 4°C. The immunoprecipitates were eluted out by adding 350 μL elution buffer [1% SDS, 0.1 M $NaHCO_3$, RNase inhibitor (50 U/mL)] and rotated at room temperature for 30 mins. NaCl was added to a final concentration of 200 mM. The immunoprecipitates were reverse cross-linked by incubating at 65°C for 2 hrs. RNA of the pull-down lysate was extracted by Trizol LS. RNA was reverse transcribed into cDNA and subjected to qPCR analysis.

Yeast-two hybrid (Y2H)

The Y2H assay was performed using Matchmaker Gold Yeast Two-Hybrid System (Clontech, Mountain View, CA, USA) according to the instruction manual

from the manufacturer. Briefly, sequence encoding mPinX1t was subcloned into pGBKT7 vector as the bait plasmid and transformed into yeast strain Y2H Gold. To screen out potential interacting partners of mPinX1t in mESC lysates, the bait transformed yeast strain Y2H Gold was mated with another yeast strain Y187 pre-transformed with normalized mESC library (Clontech, catalog no.: 630484). The mated yeasts were allowed to grow on selective media in which only those with interactions could grow. The potential screened out library plasmids were rescued by transforming into E. coli DH5α, and the normalized mESCs library cDNA inserts were sequenced. The screened out interacting partners was classified using Gene Ontology (GO) classification system by Panther Classification System

Co-immunoprecipitation (Co-IP) assay

pWPI-myc-mPinX1t or pWPI-myc-mPinX1 together with CMV-HA-Nup133 were transfected into one T25 flask of HEK293FT cells for 48 hrs. After 48 hrs transfection, cells were washed with PBS and lysed with Co IP lysis buffer (20 mM Tris at pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$ and 0.5% NP-40) supplemented with protease inhibitor cocktail [1 μg/mL Leupeptin, 5 μg/mL Aprotinin, 0.59 mM PMSF, 1 mM sodium orthovanadate, 1 mM EGTA and 1 mM EDTA (200 mM)] and phosphatase inhibitor cocktail [2 mM NaF, 4 mg/mL 2-glycerolphosphate and 0.4 mM

HEPES (pH 7.3)] on ice for 10 mins. The lysate was sonicated 2 times on ice and centrifuged down at 16,000 g for 20 mins. Supernatant was collected and 100 µL was kept as input control. The remaining lysate was diluted to 1mL by Co IP lysis buffer and aliquoted into two tubes. Anti-myc tagged antibody (9B11, Cell Signaling) at dilution ratio 1:1000 and same amount of isotype control anti-IgG2a (ab18413, Abcam) were added to the tubes respectively and rotated at 4°C overnight. In the next day, 40 µL Protein A/Protein G agarose beads (sc2003, Santa Cruz Biotechnology) were added to each tube and rotated at 4°C for 3 hrs. Immunoprecipitate was washed 3 times with PBS at 4°C. The immunoprecipitate was eluted by 50 µL 1X sample loading dye and boiled at 100°C for 10 mins. The samples were loaded onto 10% SDS PAGE.

Transient transfection

The Transient transfection was performed by using DharmaFECT Transfection Reagent (Dharmacon) according to manufacturer instructions. In brief, 30µg of Pwpi empty vector, Pwpi-mPinX1 or Pwpi-mPinX1t were mixed with pCI-Gata4 in Opti-MEM I reduced serum medium. For siNup133 knockdown experiments, 25nM siRNA targeting hNup133 (Dharmacon) was additionally added to the mixture. 75µL DharmaFECT reagent was diluted in Opti-MEM I reduced serum medium in another

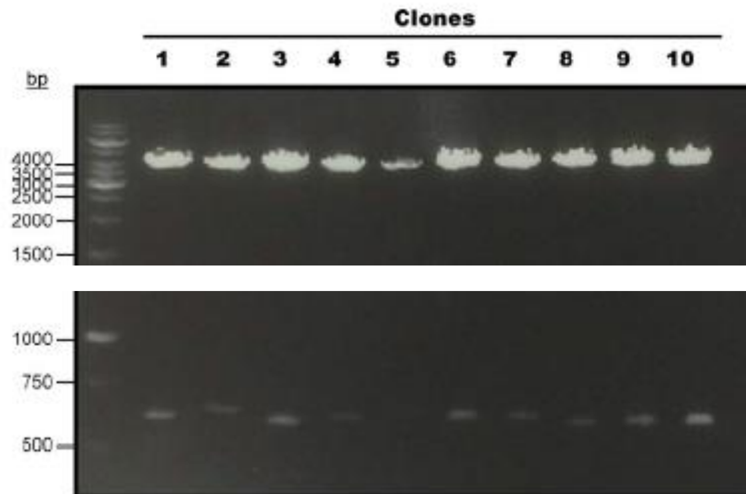
tube. Both tubes were incubated at room temperature for 5 mins. The diluted plasmid DNA and the DharmaFECT reagent were mixed and incubated for another 20 mins at room temperature. The mixture was then added to the HEK293FT cell and incubated at 37 °C with 5% CO_2 for 48 hours.

Fractionation to separate cytosolic and nuclear RNAs

The RNA fractionation protocol was modified from two publications [23, 24]. 1 x 10^7 transfected HEK293FT cells was washed with 1X PBS. Cells were collected and resuspended in ice-cold HLB buffer [10 mM Tris (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 0.3 % NP40 and 10 % glycerol] supplemented with 100 U SUPERase In (ThermoFisher, Waltham, MA, USA). The resultant lysates were incubated on ice for 10 mins and centrifuged for 1,000 g at 4 °C for 5 mins. The supernatant corresponded to the cytosolic fraction and the pellet corresponded to the nuclear fraction. Supernatant was transferred to a new conical tube and 1 mL RNA precipitation buffer (9.5 mL 100 % ethanol with 0.5 mL of 3 M sodium acetate) was added. The supernatant was stored at -20 °C for 1 hr. The supernatant was washed with 75 % ethanol twice and centrifuged for 16,000 g at 4 °C for 5 mins. The pellet was allowed to partially air-dry and 1 mL Trizol solution was added for RNA extraction. For the nuclear pellet, 1 ml ice-cold HLB buffer was added to wash the pellet for 3 times. The

nuclear pellet was resuspended in 0.88 M sucrose solution and overlaid onto 2.2 M sucrose solution. The nuclear lysate was centrifuged at 16,000 g for 20 mins. 1 mL Trizol solution was added to extract RNA of the nuclear fraction. For both the cytosolic and nuclear RNAs resuspended in Trizol solution, 2 μL of spike in RNA (TATAA Universal RNA Spike I, TATAA Biocenter, Göteborg, Sweden) was added. 10 μL of 0.5 M EDTA was added to both cytosolic and nuclear RNAs resuspended in Trizol solution and warmed at 65 °C for 20 mins. RNAs were extracted according to the manufacturer's instruction. The RNAs extracted were reverse transcribed into cDNAs and subjected to qPCR analysis. Spike in RNA was used as loading control for normalization [25].

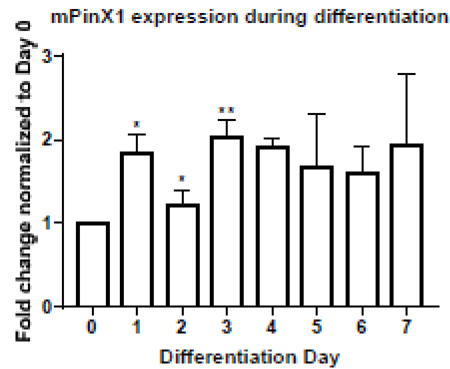
Figure S1. Restriction digestion pattern of ligation products from RNA ligase-mediated rapid amplification of cDNA ends (RLM 5' RACE).



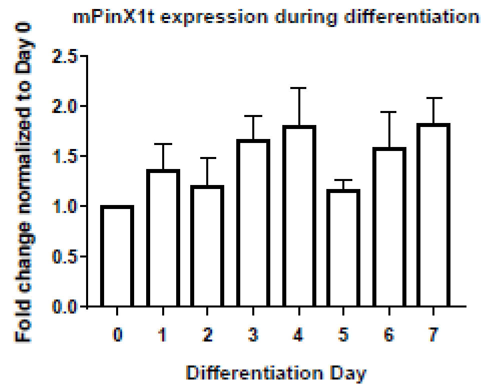
Three different sizes of digested products were observed between 500 and 750 bp apart from the 4,000bp TOPO T vector backbone. Clones 2, 3 and 6 were selected for sequencing.

Figure S2. Expressions of mPinX1 and mPinX1t at mRNA level during early stage of mESC differentiation.

A



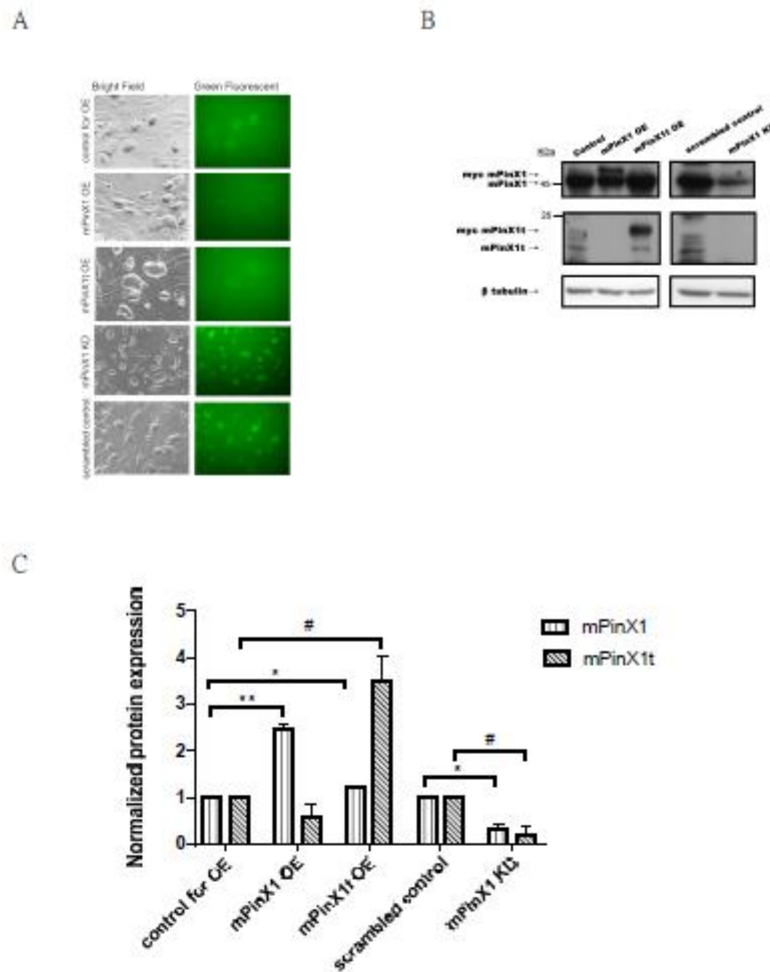
B



A) – B) Expression profile of (A) mPinX1 and (B) mPinX1t from undifferentiated day 0 to differentiation day 7. All data are normalized to that of undifferentiated day 0. Increase in mPinX1 expression was observed during early stage of differentiation with the expression of mPinX1 peaked at differentiation day 3 – 4.

Similarly, trend of increase in mPinX1t expression was observed during early stage of differentiation with expression of mPinX1t peaked at differentiation day 3 - 4. Data were presented as mean ± SEM (n = 4; where n represents data from independent differentiations). * $P < 0.05$. ** $P < 0.01$

Figure S3. Establishment of mPinX1 overexpression (mPinX1 OE), mPinX1t overexpression (mPinX1t OE) and mPinX1 knockdown (mPinX1 KD) cell lines.



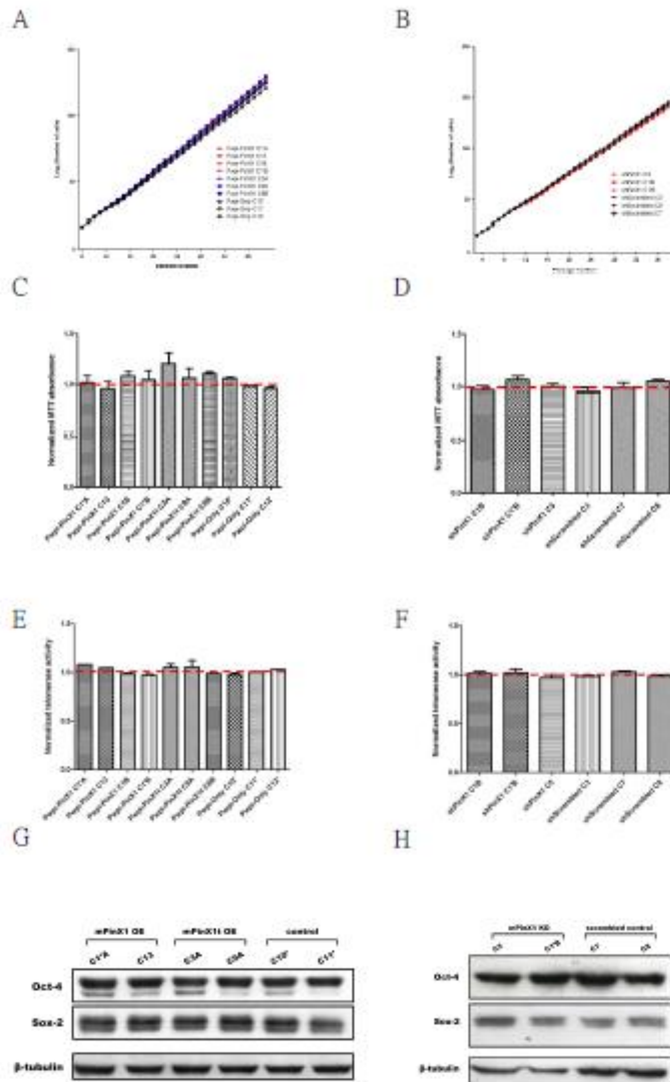
A) Morphology of undifferentiated mESCs in the established overexpression and knockdown cell lines. Morphology of mESCs in (left panel) bright field and (right panel) green fluorescent field. No obvious change in morphology for these established cell lines when compared to their respective control cell lines. Presence of green

fluorescent GFP signal indicated the success of the establishment of the cell lines.

B) Representative Western Blot showing the overexpression of mPinX1, overexpression of mPinX1t and knockdown of mPinX1 in the respective undifferentiated mESC lysates. In mPinX1 overexpression line, an additional myc-mPinX1 band could be detected above the endogenous mPinX1 band. In mPinX1t overexpression line, an additional myc-mPinX1t band could be detected above the endogenous mPinX1t band. In mPinX1 knockdown line, mPinX1 expression was greatly diminished compared to the scrambled control line. β-tubulin was used as the loading control.

C) Bar chart showing the expression of mPinX1 and mPinX1t proteins in different mESC lines. Data were presented as mean ± SEM (n = 3; where n represents western blot data from mESCs at different passages). * $P < 0.05$ vs control line.

Figure S4. Lack of effects of mPinX1/mPinX1t overexpression and knockdown on the proliferation, pluripotency and telomerase activity of mESCs.



A) Proliferation curve of different clones of mPinX1 overexpression (Pwpi-PinX1), mPinX1t overexpression (Pwpi-PinX1t) and control (Pwpi only) cell lines. The graph was plotted with Log2 (total number of cells) against the passage number. There was no significant difference in the rate of proliferation between these cell lines.

B) Proliferation curve of different clones of mPinX1 knockdown (shPinX1) and control (shScrambled) cell lines. The graph was plotted with Log2 (total number of cells) against the passage number. There was no significant difference in the rate of proliferation between these cell lines.

C) MTT assay showing the proliferation of different clones of mPinX1 overexpressed mESCs and mPinX1t overexpressed mESCs compared to the control mESCs. No obvious difference was observed between these clones.

D) MTT assay showing the proliferation of different clones of mPinX1 knocked down mESCs compared to the control mESCs. No obvious difference was observed between these clones.

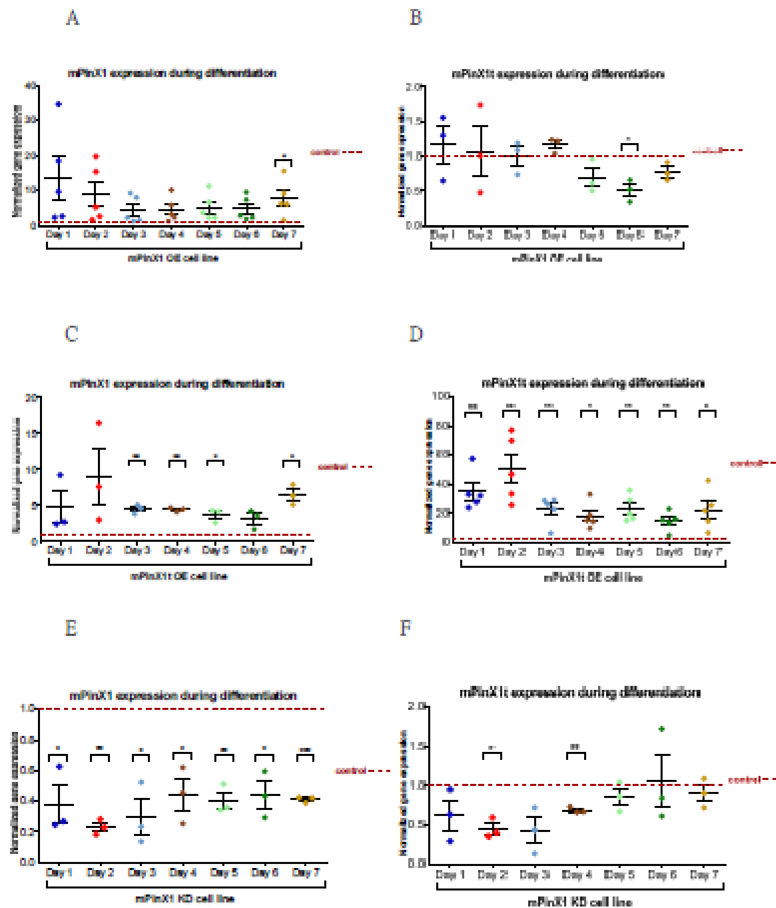
E) TRAP assay results of different clones of mPinX1 overexpressed mESCs, mPinX1t overexpressed mESCs and the control mESCs. No obvious difference was observed between these clones.

F) TRAP assay results of different clones of mPinX1 knocked down mESCs and the control mESCs. No obvious difference was observed between these clones.

G) Western blot showing the expression of pluripotent markers Sox-2 and Klf-4 in different clones of mPinX1 overexpressed mESCs, mPinX1t overexpressed mESCs and the control mESCs. β-tubulin was used as the loading control. No obvious difference was observed between these clones.

H) Western blot showing the expression of pluripotent markers Sox-2, Klf-4 and Oct-4 in different clones of mPinX1 knocked down mESCs and the control mESCs. β-tubulin was used as the loading control. No obvious difference was observed between these clones.

Figure S5. Expression profile of mPinX1 and mPinX1t during early differentiation in different mPinX1/mPinX1t overexpression and knockdown cell lines.



A) mPinX1 expression profile at different differentiation time points in mPinX1 overexpression cell line. mPinX1 was up-regulated at different time points. Red dotted line indicated the normalized expression level in control cell line. Data were

presented as mean ± SEM (n = 5; where n represents data from independent differentiations). * $P < 0.05$ vs control line.

B) mPinX1t expression profile at different differentiation time points in mPinX1 overexpression cell line. No significant change was observed at most time points except a slight decrease was observed at later differentiation time points. Red dotted line indicated the normalized expression level in control cell line. Data were presented as mean ± SEM (n = 3; where n represents data from independent differentiations). * $P < 0.05$ vs control line.

C) mPinX1 expression profile at different differentiation time points in mPinX1t overexpression cell line. mPinX1 was slightly up-regulated at different time points. Red dotted line indicated the normalized expression level in control cell line. Data were presented as mean ± SEM (n = 3; where n represents data from independent differentiations). * $P < 0.05$, ** $P < 0.01$ vs control line.

D) mPinX1t expression profile at different differentiation time points in mPinX1t overexpression cell line. mPinX1t was largely up-regulated at different time points. Red dotted line indicated the normalized expression level in control cell line. Data were presented as mean ± SEM (n = 5; where n represents data from independent differentiations). * $P < 0.05$, ** $P < 0.01$ vs control line.

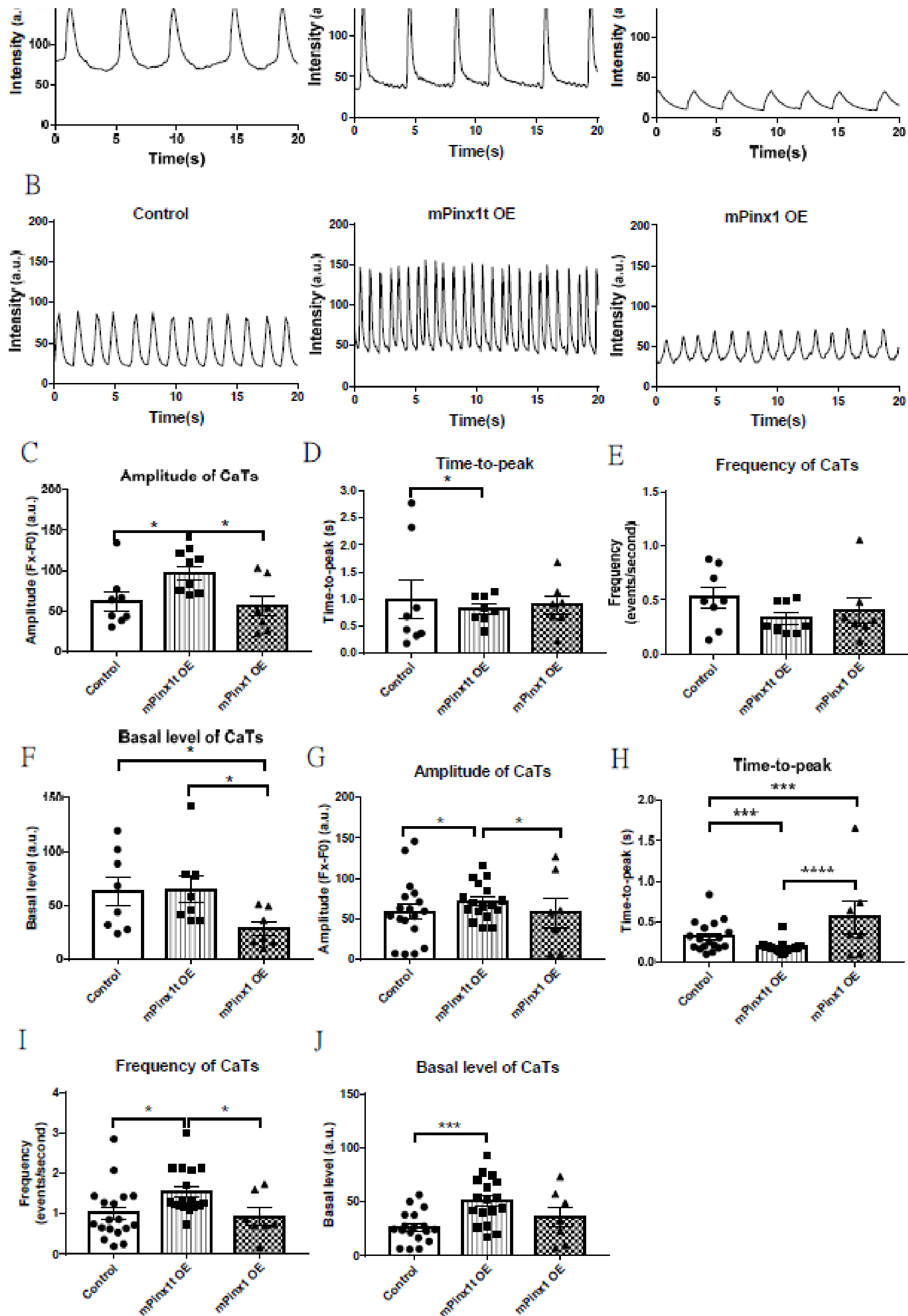
E) mPinX1 expression profile at different differentiation time points in mPinX1

knockdown cell line. mPinX1 was down-regulated at different time points. Red dotted line indicated the normalized expression level in control cell line. Data were presented as mean ± SEM (n = 3; where n represents data from independent differentiations). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs control line.

F) mPinX1t expression profile at different differentiation time points in mPinX1 knockdown cell line. mPinX1t was down-regulated at some early differentiation time points. Red dotted line indicated the normalized expression level in control cell line.

Data were presented as mean ± SEM (n = 3; where n represents data from independent differentiations). * $P < 0.05$, ** $P < 0.01$ vs control line.

Figure S6. Effects of overexpression of mPinX1 or mPinX1t on the calcium transients (CaTs) of mESC-CMs.



A) - B) Representative fluorescence intensity plots of global CaTs of mESC-CMs in different experimental groups at (A) early and (B) late

differentiation stages.

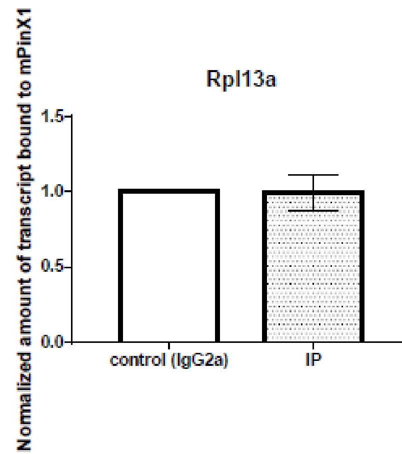
C) – F) Summarized plots showing the (C) amplitude, (D) time-to-peak, (E) frequency and (F) basal calcium level of global CaTs in mESC-CMs in different groups at early differentiation stage. Results were expressed as means ± S.E.M. (control group, n = 8; mPinX1t OE group, n = 8; mPinX1 OE group, n = 7; where n represents number of cardiomyocytes). Cell were obtained from 4 independent differentiations. The unit of statistical analysis was the number of cardiomyocytes (n numbers).

* $P < 0.05$.

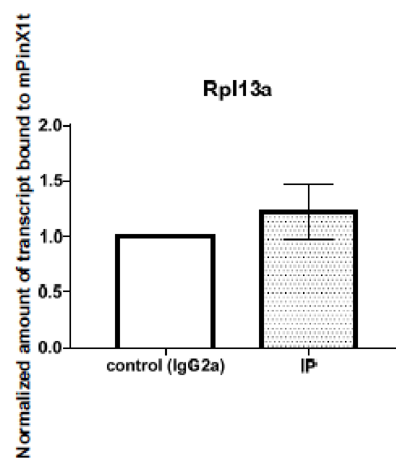
G) – J) Summarized plots showing the (G) amplitude, (H) time-to-peak, (I) frequency and (J) basal calcium level of global CaTs in mESC-CMs in different groups at late differentiation stage. Results were expressed as means ± S.E.M. (control group, n = 18; mPinX1t OE group, n = 17; mPinX1 OE group, n = 7; where n represents number of cardiomyocytes). Cell were obtained from 4 independent differentiations. The unit of statistical analysis was the number of cardiomyocytes (n numbers). * $P < 0.05$, *** $P < 0.001$.

Figure S7. mPinX1 and mPinX1t proteins did not universally bind to all mRNAs.

A



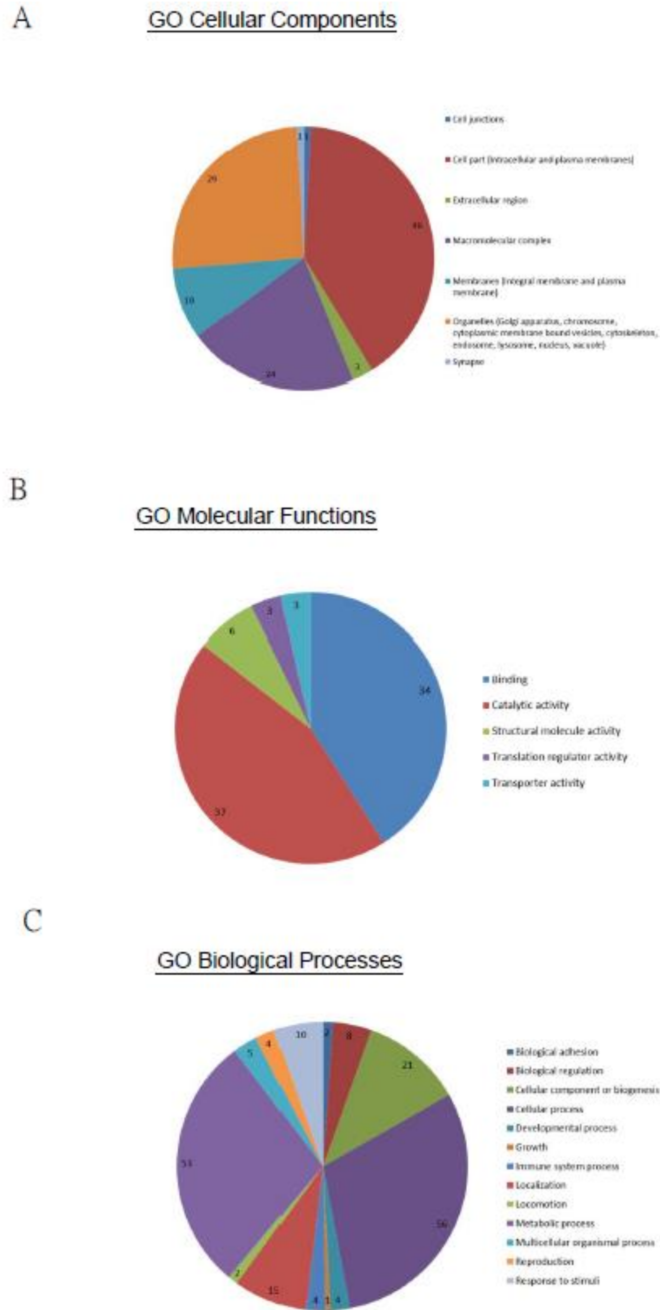
B



A) – B) RNA immunoprecipitation of HEK293FT cells overexpressed with (A) myc-mPinX1 or (B) myc-mPinX1t. Anti-myc was used to perform the immunoprecipitation. IgG2a was used in isotype control experiment. The presence of Rpl13a mRNA in the immunoprecipitant was quantitated by subsequent qPCR. Both

mPinX1 and mPinX1t proteins were not found to bind to Rpl13a mRNA. Data were presented as mean ± SEM (n = 4; where n represents independent RNA immunoprecipitation experiments).

Figure S8. Gene Ontology (GO) analysis of mPinX1t interacting partners screened by Yeast two Hybrid assay.



A) GO Cellular Components

B) GO Molecular Functions

C) GO Biological Processes