PinX1t, a Novel PinX1 Transcript Variant, Positively Regulates Cardiogenesis of Embryonic Stem Cells

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Background—Pin2/TRF1-interacting protein, PinX1, was previously identified as a tumor suppressor. Here, we discovered a novel transcript variant of mPinX1 (mouse PinX1), mPinX1t (mouse PinX1t), in embryonic stem cells (ESCs). The aims of this investigation were (1) to detect the presence of mPinX1 and mPinX1t in ESCs and their differentiation derivatives; (2) to investigate the role of mPinX1 and mPinX1t on regulating the characteristics of undifferentiated ESCs and the cardiac differentiation of ESCs; (3) to elucidate the molecular mechanisms of how mPinX1 and mPinX1t regulate the cardiac differentiation of ESCs.

Methods and Results—By 5′ rapid amplification of cDNA ends, 3′ rapid amplification of cDNA ends, and polysome fractionation followed by reverse transcription–polymerase chain reaction, mPinX1t transcript was confirmed to be an intact mRNA that is actively translated. Western blot confirmed the existence of mPinX1t protein. Overexpression or knockdown of mPinX1 (both decreased mPinX1t expression) both decreased while overexpression of mPinX1t increased the cardiac differentiation of ESCs. Although both mPinX1 and mPinX1t proteins were found to bind to cardiac transcription factor mRNAs, only mPinX1t protein but not mPinX1 protein was found to bind to nucleoporin 133 protein, a nuclear pore complex component. In addition, mPinX1t-containing cells were found to have a higher cytosol-to-nucleus ratio of cardiac transcription factor mRNAs when compared with that in the control cells. Our data suggested that mPinX1t may positively regulate cardiac differentiation by enhancing export of cardiac transcription factor mRNAs through interacting with nucleoporin 133.

Conclusions—We discovered a novel transcript variant of mPinX1, the mPinX1t, which positively regulates the cardiac differentiation of ESCs. (*J Am Heart Assoc*. 2020;9:e010240. DOI: 10.1161/JAHA.118.010240.)

Key Words: cardiac development • cardiac differentiation • embryonic stem cell • PinX1 • transcript variants

Myocardial infarction has become the leading cause of death around the globe and is characterized by the loss of cardiomyocytes.[1] Cardiomyocytes cannot be regenerated after myocardial infarction; therefore, embryonic stem cells (ESCs), which have unlimited self-renewal potential and can differentiate into a variety of cells, including cardiomyocytes, have become a promising cell source for generating cardiomyocytes for transplantation therapy.[2]

Several crucial transcription factors, including Nkx2.5, Mef2C, Gata4, and Tbx5, have been identified to be important for ESCs to differentiate into cardiac lineage.[3–6] Knockdown of Gata4 leads to the decrease in expressions of cardiac structural genes and transcription factors including cardiac actin, myosin heavy chain, Nkx2.5 and Mef2C.[7] Gata4 has also been reported to regulate Nkx2.5 transcription and interact with Tbx5 for cardiac differentiation.[8–11] Tbx5 overexpression promotes cardiac differentiation and increases the beating frequency of cardiomyocytes.[12] Similarly, overexpression of Gata4 promotes cardiac differentiation, increases the cardiac-specific gene expression, and increases the number of beating clusters.[13] After the differentiation of ESCs into cardiomyocytes, actin and myosin filaments are arranged into a highly organized structure known as sarcomere.[14,15] Sarcomere structure is the basic unit of cardiomyocytes for force generation. A mature cardiomyocyte expresses different cardiac structural genes, including cardiac actin, myosin heavy chain, cardiac troponin T, and cardiac

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Clinical Perspective

What Is New?

- A new transcript variant (PinX1t) of a previously known tumor suppressor Pin2/TRF1-interacting protein (PinX1) has been discovered.
- Expression of PinX1t was found to be positively correlated to the cardiac differentiation of embryonic stem cells.
- PinX1 protein was found to bind to nucleoporin 133 protein, a component of nuclear pore complex; PinX1t-containing cells were found to have a higher cytosol-to-nucleus ratio of cardiac transcription factor mRNAs when compared with control cells.
- Our data suggested that PinX1t may positively regulate cardiac differentiation by enhancing export of cardiac transcription factor mRNAs through interacting with Nucleoporin 133.

What Are the Clinical Implications?

- Altering PinX1t expression/activity may be used as a way to enhance cardiac differentiation of embryonic stem cells (or pluripotent stem cells) so as to increase the generation of cardiomyocytes for future transplantation/regenerative medicine, drug screening, etc.

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troponin I[4]; these cardiac structural genes together form the major constituents of sarcomere.

Pin2/TRF1 binding protein, PinX1, was first identified as a Pin2 binding protein by yeast-2 hybrid screening using the cDNA library from human HeLa cells.[16] PinX1 is characterized by having 2 special domains at its N- and C-termini. At the N-terminal, it contains a glycine-rich region known as G-Patch.[16] Many proteins have been reported to be RNA-binding proteins by having the G-Patch domain,[17] suggesting that PinX1 may be a potential RNA-binding protein. At the C-terminal, PinX1 contains a telomerase inhibitory domain, which allows PinX1 to directly bind to the catalytic subunit of telomerase, telomeric reverse transcriptase, and downregulate its activity.[16] As such, PinX1 was reported to be tumor suppressor gene in various cancer cells through downregulating the activity of telomerase.[18]

In our present study, we discovered that there is a new transcript variant of PinX1 existed in mouse ESCs (mESCs). This novel transcript variant encodes a protein containing only the N-terminal G-Patch domain but not the C-terminal telomerase inhibitory domain. As a result, this novel transcript variant is named as PinX1t, where "t" stands for "truncated."

How mPinX1 and mPinX1t regulate pluripotency and differentiation in mESCs is unknown. On the other hand, the naturally occurring mPinX1t allowed us to have an in-depth investigation on the cellular functions of the N-terminal of mPinX1. This current study has investigated the expressions of mPinX1 and mPinX1t in undifferentiated and differentiated mESCs and the roles of mPinX1 and mPinX1t in maintaining the pluripotency of mESCs and in regulating the cardiac differentiation. This study has also elucidated the molecular mechanisms of how mPinX1/mPinX1t regulate the cardiac differentiation.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Detailed experimental procedures can be found in Data S1.

Cell Culture

Institutional review board 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 cultured and cardiac differentiation was induced as we previously described.[19–22]

Polymerase Chain Reaction, Subcloning, and Sequencing for the First Identification of mPinX1t

Forward primer franking 23 bp upstream of mPinX1 (5′-TAAGGGAATTCAT**CAGCGTTCGACAAACTTGAG**-3′; italic and bold sequence indicates sequence that is complementary to mPinX1 gene) and reverse primer franking 26 bp downstream of mPinX1 (5′-TAAGGGCGGCCGC**ACAGTTGAGTGGTTGGAGG C**-3′; italic and bold sequence indicates sequence that is complementary to mPinX1 gene) coding sequence (accession number: NM_028228) were used in polymerase chain reaction (PCR) using mESCs and their differentiation derivatives as the template.

Polysome Fractionation Assay

Cycloheximide was added to mESCs and cells were pelleted and lysed on ice with hypotonic lysis buffer (50 mmol/L Tris-HCl [pH 7.5], 2.5 mmol/L $MgCl_2$, 1.5 mmol/L KCl, diethylpyrocarbonate water) supplemented with 0.59 mmol/L phenylmethylsulfonyl fluoride, 0.12 mg/mL cycloheximide, 2.35 mmol/L dithiothreitol and 0.09 U/μL ribonuclease inhibitor for 15 minutes. Then 0.59% Triton X-100 and 0.59% Na deoxycholate were added to the lysate and vortexed. The lysate was centrifuged at 16 000*g* for 7 minutes at 4°C. A sucrose gradient density was set up by layering high-density sucrose gradient buffer (20 mmol/L Tris HCl [pH 7.5], 140 mmol/L KCl, 5 mmol/L $MgCl_2$, 50% sucrose, 0.5 mmol/L dithiothreitol, 100 μg/mL cycloheximide, diethylpyrocarbonate water) with low-density

sucrose gradient buffer (20 mmol/L Tris HCl [pH 7.5], 140 mmol/L KCl, 5 mmol/L $MgCl_2$, 7% sucrose, 0.5 mmol/L dithiothreitol, 100 µg/mL cycloheximide, diethylpyrocarbonate water) in 1:1 ratio; 800 ng cell lysate was loaded on top of the sucrose gradient density and subjected to ultracentrifugation at 217 874g (35 000 rpm) for 3 hours. Fractions were collected to microcentrifuge tubes and Trizol LS solution (Life Technologies, Waltham, MA) was used for RNA extraction. The extracted RNA was reverse transcribed and mPinX1t expression was determined by quantitative PCR (qPCR) using mPinX1t specific primers. mPinX1t forward primer: 5′-AAAGGGAAGGATCTGTC CTC-3′ mPinX1t reverse primer: 5′-CAATTTAATCCGAGGAGCCT GAG-3′.

Full-Length RNA Ligase–Mediated Rapid Amplification of 5′ cDNA Ends Assay and RNA Ligase–Mediated Rapid Amplification of 3′ cDNA Ends Assay

The rapid amplification of 5′ cDNA ends and rapid amplification of 5′ cDNA ends assays were carried according to the manufacturer's instructions (L1502-01, Life Technologies, Rockville, MD). The 5′ untranslated region (UTR) sequence was amplified using 5′ GeneRacer-specific forward primer: 5′-CGACTGGAGCACGAGGACACTGA-3′ and mPinX1t-specific reverse primer: 5′-CACAATTTAATCCGAGGAGCCTGAGCAA-3′. A second-round PCR using 1 µL of PCR product from the first-round PCR was performed by changing the forward primer to 5′ GeneRacer nested primer: 5′-GGACACTGACATGGACT-GAAGGAGTA-3′. For rapid amplification of 3′ cDNA ends (3′ RACE), the 3′ UTR sequence was amplified using 3′ GeneRacer-specific reverse primer: 5′-GCTGTCAACGATACGC-TACGTAACG-3′ and mPinX1t-specific forward primer: 5′-TTGCTCAGGCTCCTCGGATTAAATTGTG-3′. A second-round PCR using 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′-CGCTACGTAA CGGCATGACAGTG–3′.

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) to make the lentiviral backbone DNA plasmids pWPI-myc-mPinX1 and pWPI-myc-mPinX1t, respectively. Small hairpin RNA against mPinX1 (sense sequence: 5′-AAGAAGAAAGTTTCCAGATAA-3′) and scrambled small hairpin RNA sequence (sense sequence: 5′-GAAACA GATAATAGACAGTTA-3′) were subcloned into pLVTHM (Addgene) to make the lentiviral backbone DNA plasmids

pLVTHM-shmPinX1 and pLVTHM-shscrambled. Lentiviral backbone DNA constructs and the packaging plasmids (psPAX2 and pMD2.G) were cotransfected into the human embryonic kidney (HEK)293FT cells in OPTI-MEM I (Invitrogen, Waltham, MA) using Lipofectamine 2000 (Invitrogen). OPTI-MEM I medium with the complexes was changed back to normal medium 6 hours after transfection and lentiviruses were collected at 24, 48, and 72 hours after transfection.

Lentiviral Vector–Mediated Gene Transfer to mESCs

Stable mESC lines were made using lentiviral vector-mediated gene transfer. Detailed experimental procedures can be found in Data S1.

Telomere Repeat Amplification Protocol Assay

The telomere repeat amplification protocol assay was done with Telo*TAGGG* telomerase PCR ELISA (Roche, Basel, Switzerland) according to the manufacturer's instructions.

Quantitative PCR

SYBR GREEN PCR Master Mix (Applied BioSystems, Foster City, CA) was used for qPCR, and the qPCR reaction was performed using ABI 7500 Fast Real Time PCR System. Detailed experimental procedures can be found in Data S1.

SDS PAGE and Western Blotting and Immunocytochemistry

Detailed experimental procedures can be found in Data S1.

RNA Immunoprecipitation Assay

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. Qian-grong Liang in College of Osteopathic Medicine in New York Institute of Technology) or pAC-CMV-Tbx5 (kindly provided by Prof. Katherine Yutzey in the Division of Molecular Cardio-vascular Biology at the Heart Institute, Cincinnati Children's Hospital Medical Center) were transfected into HEK293FT using Lipofectamine 2000 (Invitrogen). The details of RNA immunoprecipitation can be found in Data S1. Briefly, anti-myc antibody (9B11; Cell Signaling Technology, Inc, Danvers, MA) at a dilution ratio of 1:250 and isotype antibody IgG2a (Abcam, Cambridge, UK) were used for the immunoprecipitation. RNA of the immunoprecipitates was extracted by Trizol LS. RNA was reverse transcribed into cDNA and subjected to qPCR analysis.

Yeast-2 Hybrid

The yeast-2 hybrid assay was performed using Matchmaker Gold Yeast Two-Hybrid System (Clontech, Mountain View, CA) according to the instruction manual from the manufacturer.

Coimmunoprecipitation Assay

pWPI-myc-mPinX1t or pWPI-myc-mPinX1 together with CMV-HA-Nup133 were transfected into HEK293FT cells using Lipofectamine 2000. Detailed experimental procedures of coimmunoprecipitation can be found in Data S1.

Fractionation to Separate Cytosolic and Nuclear RNAs

The RNA fractionation protocol was modified from 2 publications[23,24] and detailed experimental procedures can be found in Data S1. For both the cytosolic and nuclear fractions resuspended in Trizol solution, spike in RNA (TATAA Universal RNA Spike I, TATAA Biocenter, Göteborg, Sweden) was added as loading control for normalization in later qPCR.[25]

Statistical Analysis

Data were presented as mean±SEM. Data between 2 groups were compared by unpaired Student t test. Data among 3 or more groups were compared by ANOVA followed by Tukey's multiple comparison tests. $P<0.05$ was considered to be statistically significant.

Results

mPinX1 and its Novel Transcript Variant Were Expressed in mESCs and Their Differentiation Derivatives

To investigate the expression of mPinX1 in mESCs and their differentiated derivatives, a pair of primers flanking the coding region of mPinX1 was designed and a routine PCR reaction was performed. As expected, a distinct band at 1074 bp, which corresponded to mPinX1, was observed in both the undifferentiated and differentiation derivatives of mESCs (Figure 1A). Interestingly, an additional band at 1185 bp was also observed in both the undifferentiated and differentiation derivatives (Figure 1A). This additional band was excised and sent for DNA sequencing. The result showed that the additional band contained an extra 111 nucleotides in between exon 6 and exon 7 of mPinX1 gene (Figure 1B). This additional 111 bp was blast-searched and was found to be located in Locus NT_039606.7 of chromosome 14 of mouse genome where the mPinX1 gene is located. The position and the sequence of

the extra sequence was annotated in Figure 1B. The result suggested a novel transcript variant of mPinX1 existed in both the undifferentiated and differentiated derivatives.

mPinX1t Transcript Contained Intact 3′ Poly(A) Tail and 5′ Cap

Aforementioned PCR was conducted using cDNA reverse-transcribed from whole-cell RNA using oligo-dT as primers. Therefore, those cDNAs should represent reverse-transcribed product from RNA with poly(A) tail. To further confirm that the mPinX1t transcript was a functional mRNA (ie, with 5′ cap and 3′ poly(A) tail), RNA ligase–mediated rapid amplification (RLM) 5′ rapid amplification of cDNA ends assay was carried out using total RNA from undifferentiated mESC lysate. Calf intestine alkaline phosphatase was used to selectively eliminate all 5′ truncated RNAs and non-mRNAs that lack an intact 5′ cap. The RNA was then treated with tobacco acid pyrophosphatase to remove the cap structure from full-length mRNAs. RNA oligo of known sequence was ligated to the 5′ end of the mRNA population. The resultant mRNA was reverse transcribed into cDNA by oligo-dT primer and amplified using specific 5′ primer targeting the RNA oligo sequence and 3′ primer targeting mPinX1t-specific sequence. A product between 500 and 750 bp was obtained after PCR (Figure 1C). The band was excised and subcloned into TOPO T-vector. After restriction digestion, 3 different clones of the recombinant plasmids, representing 3 different 5′ UTR sequences of mPinX1t, were found, as revealed by 3 slightly different digestion patterns (eg, clone 2, clone 3, and clone 6 were 3 different clones; Figure S1). The 3 different 5′ UTRs were further analyzed by DNA sequencing and were found to be of different sequences, yet correspond to the known 5′ UTR of mPinX1 (data not shown).

In addition, RLM 3′ rapid amplification of 5′ cDNA ends assay was carried out using total RNA from undifferentiated mESC lysate. Our results indicated that mPinX1t transcript contains 3′ poly(A) tail; subsequent sequencing of the 3′ UTR confirmed the existence of an extra exon and the connection of this extra exon to the exon 7 of mPinX1. Altogether, our results indicated that mPinX1t exists as an intact mRNA with 5′ cap and 3′ poly(A) tail.

Sequence analysis revealed that the additional exon in mPinX1t contained a stop codon, making mPinX1t encodes for a protein containing only the N-terminal but not the C-terminal of mPinX1 (Figure 1D). Therefore, we named this novel transcript variant mPinX1t where "t" stands for "truncated."

mPinX1t mRNA Was Actively Translating Into Protein

High-sucrose density gradient polysome fractionation assay was performed and was found to successfully fractionate different RNAs into free ribonucleoprotein fractions, 40S and
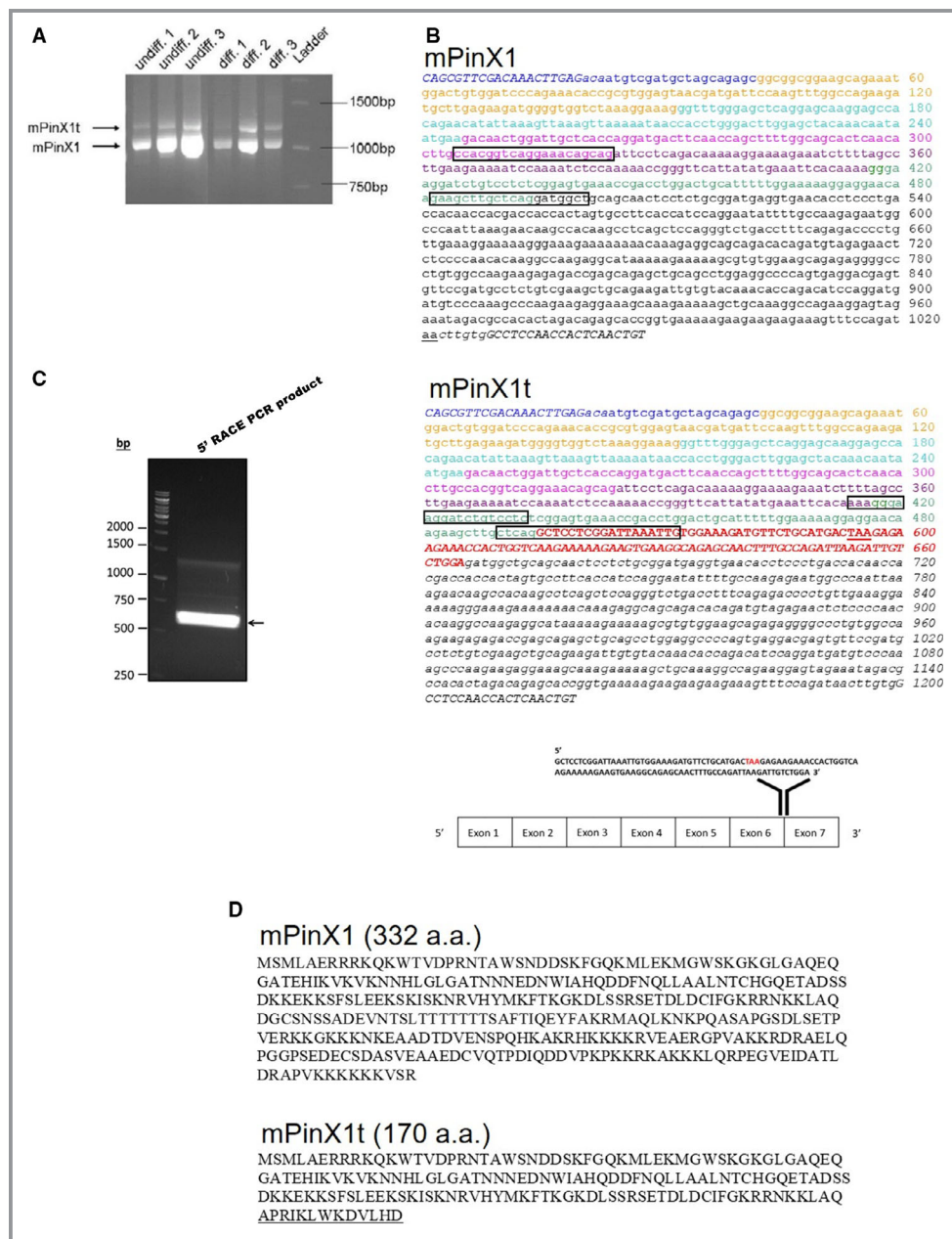
Figure 1. Discovery of mPinX1t, the novel transcript variant of mPinX1. **A**, Presence of 2 splice variants, mPinX1 and mPinX1t, as revealed by RT-PCR. In the PCR, primers flanking the coding sequence of mPinX1 were used. Two splice variants were detected in both undifferentiated mESCs (undiff) and their differentiation derivatives (diff). **B**, cDNA sequences of (upper panel) mPinX1 and (middle panel) mPinX1t. Different colors represent different exons. Note that there is an extra exon in mPinX1t as represented by red and bold nucleotides. Start codon and stop codon are underlined. Italic nucleotides represent nucleotides at the untranslated regions. Italic and bold nucleotides located at the beginning and the end of the sequences represent PCR primer sequences used in (**A**). Boxed regions indicate the location of primers used in qPCR reactions. (Lower panel) Extra 111 nucleotides of mPinX1t was found to locate between exon 6 and exon 7 of mPinX1 sequence. Stop codon "TAA" is highlighted in red. **C**, Result of 5′RACE PCR reaction using primer specific for the 5′ added sequence and primer complementary to the mPinX1t sequence. The result indicated the presence of mPinX1t mRNA with intact 5′UTR. **D**, Amino acid sequence of (upper panel) mPinX1 and (lower panel) mPinX1t. mPinX1t contains the N-terminal of mPinX1 (1−157 amino acids) but lacks the C-terminal of mPinX1. Amino acids that are underlined represent the sequence that is present only in mPinX1t. 5′RACE indicates 5′ rapid amplification of cDNA ends; 5′UTR, 5′ untranslated region; mESCs, mouse embryonic stem cells; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

60S (which contain 18S rRNA and 28S rRNA, respectively) fractions, monosome fractions, and polysome fractions (Figure 2A). Different RNA fractions (fractions 1, 2, 5, 6, 10, 11, 16, and 17; Figure 2A) were selected, reverse transcribed into cDNAs and amplified using mPinX1t-specific primers by PCR reaction. Both the monosome and polysome fractions, but not the free ribonucleoprotein fractions, gave rise to mPinX1t-specific band at 200 bp. These results suggested that mPinX1t mRNA was incorporated into ribosomes and actively translated into proteins (Figure 2B).

To validate the expression of mPinX1t at protein level, western blot analysis was performed using an anti-PinX1, which has an epitope at the N-terminal of PinX1. mPinX1 was detected at its expected size at ≈45 kDa, while mPinX1t was detected between 15 and 25 kDa (Figure 2C). On the other hand, using an antibody having an epitope at the C-terminal of PinX1, mPinX1 but not mPinX1t (which lacks the C-terminal of mPinX1) was detected (Figure 2D).

Establishment of Stable mESC Lines With Altered Expression of mPinX1 and mPinX1t

The expressions of mRNAs of mPinX1 and mPinX1t for mESCs at the undifferentiated stage and mESCs during the early stage of differentiation were plotted (Figure S2). Our results showed that the expression of mPinX1 and mPinX1t increased at the beginning of differentiation and peaked at around differentiation day 3 to 4, a time point where cardiac differentiation starts.[26] The expression of mPinX1 and mPinX1t started to fall after the peak. These data hinted at the potential role of mPinX1 and mPinX1t and their interaction in regulating cardiogenesis. To study the effects of mPinX1 and mPinX1t on the cardiac commitment of mESCs, cell lines that were stably overexpressed or knocked down with mPinX1 or mPinX1t were established (Figure S3). While an attempt was made to knock down mPinX1t, we found that the unique 111 nucleotides in the mPinX1t transcript was too short to allow us to design effective small hairpin RNA (note that as revealed by RLM rapid amplification of 3′ cDNA ends, for mPinX1t transcript, exon 7 sequence of mPinX1 immediately followed the unique 111 nucleotide exon of mPinX1t. Therefore, the 111 nucleotides were the only unique sequence of mPinX1t that distinguished itself from mPinX1). As such, there was no specific small hairpin RNA to effectively knock down mPinX1t.

Altered Expression of mPinX1 and/or mPinX1t Did Not Affect the Characteristics of mESCs at the Undifferentiated State

First, whether altering the expression of mPinX1 and/or mPinX1t would affect the characteristics of mESCs at the undifferentiated state was examined. To study the long-term effect on cell proliferation, different stable cell line clones were kept on being passaged for 38 to 39 passages starting from the day of their establishment. The number of cells was counted by trypan blue exclusion assays every time when the cells were passaged. Both overexpression of mPinX1 or mPinX1t or the knockdown of mPinX1 did not cause a significant change in cell proliferation in the long run as determined by trypan blue exclusion assays (Figure S4A and S4B). MTT assay also revealed that there was no significant difference in the proliferation of these stable overexpression/knockdown cell line clones when compared with their respective control cell lines (Figure S4C and S4D). In addition, the telomere repeat amplification protocol assay was done to examine the telomerase activity of the stable cell line clones. Our results showed that there was no significant change of the telomerase activity in these cell lines (Figure S4E and S4F).

Western blot was also done to examine the expression of pluripotent markers in these stable cell line clones. The expression of pluripotent markers, including octamer-binding transcription factor 4 and SRY-box transcription factor 2, were not consistently altered among these clones (Figure S4G and S4H).

Altogether, altering the expression of mPinX1 and/or mPinX1t did not affect the proliferation, telomerase activity, and pluripotency of mESCs.

Overexpression and Knockdown of mPinX1 Inhibited Cardiac Differentiation While Overexpression of mPinX1t Promoted Cardiac Differentiation

Upon differentiation, the overexpression and/or the knockdown of mPinX1/mPinX1t of different stable cell line persisted as expected (Figure S5). qPCR analysis showed that both overexpression and knockdown of mPinX1 decreased the expressions of cardiac structural markers including cardiac actin, cardiac troponin I, cardiac troponin T, and myosin heavy chain (Figure 3A and 3B). In contrast, overexpression of mPinX1t increased the expressions of these cardiac structural markers (Figure 3A).

Sarcomere assembly allows actin and myosin filaments to align in proper orientations for force contraction of cardiomyocytes and set the basis for heart contraction. α-Actinin is one of the components of Z-disc in sarcomere. α-Actinin crosslinks with F-actin to maintain the integrity of the Z-disc structure.[27] To study the role of mPinX1 and mPinX1t on the sarcomere assembly of cardiomyocytes, immunocytochemical staining on 2 sarcomere assembly-related markers α-actinin and F-actin was done at different differentiation time points (differentiation day [7+4] and differentiation day [7+8]) among all the overexpression and knockdown cell lines of mPinX1 and mPinX1t. Proper
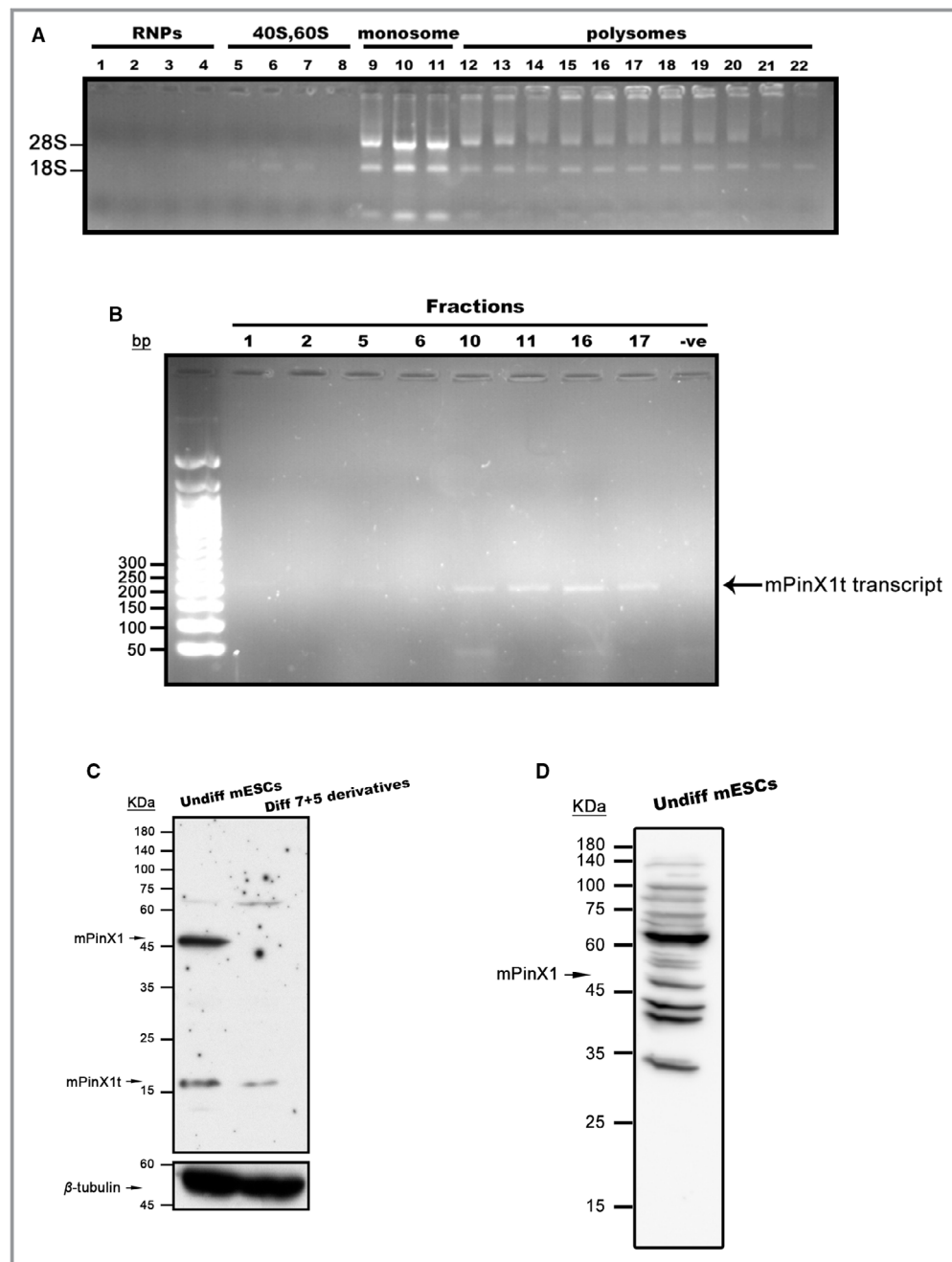
Figure 2. mPinX1t is a protein encoding gene. **A** and **B**, High-density sucrose gradient polysome fractionation was performed using mESC lysate. **A**, Nondenaturing RNA agarose gel showing the presence and the integrity of 28S and 18S rRNAs in different fractions. Fractions 1 to 4 represent free ribonucleoprotein fractions; fractions 5 to 8 represent 40S, 60S fractions; fractions 9 to 11 represent monosome fractions; fractions 12 to 22 represent polysome fractions. **B**, PCR reactions of different fractions showing the presence of mPinX1t transcripts in monosome and polysome fractions. Equal volume of PCR product was loaded onto each lane. "–ve" represents negative control. **C**, Representative western blot showing the expressions of mPinX1 and mPinX1t proteins in undifferentiated mESCs and mESC differentiation derivatives at day 7+5. β–Tubulin was used as the loading control. mPinX1 was observed at around 45 kDa and mPinX1t was observed between 15 and 25 kDa. **D**, Representative western blot showing the expression of mPinX1 proteins in undifferentiated mESCs using an antibody targeting the C-terminal of mPinX1 (Orb47163). mPinX1 was observed at around 45 kDa; mPinX1t, which lacks the C-terminal of mPinX1, was not detected. mESC indicates mouse embryonic stem cell; RNPs, ribonucleoproteins; PCR, polymerase chain reaction.
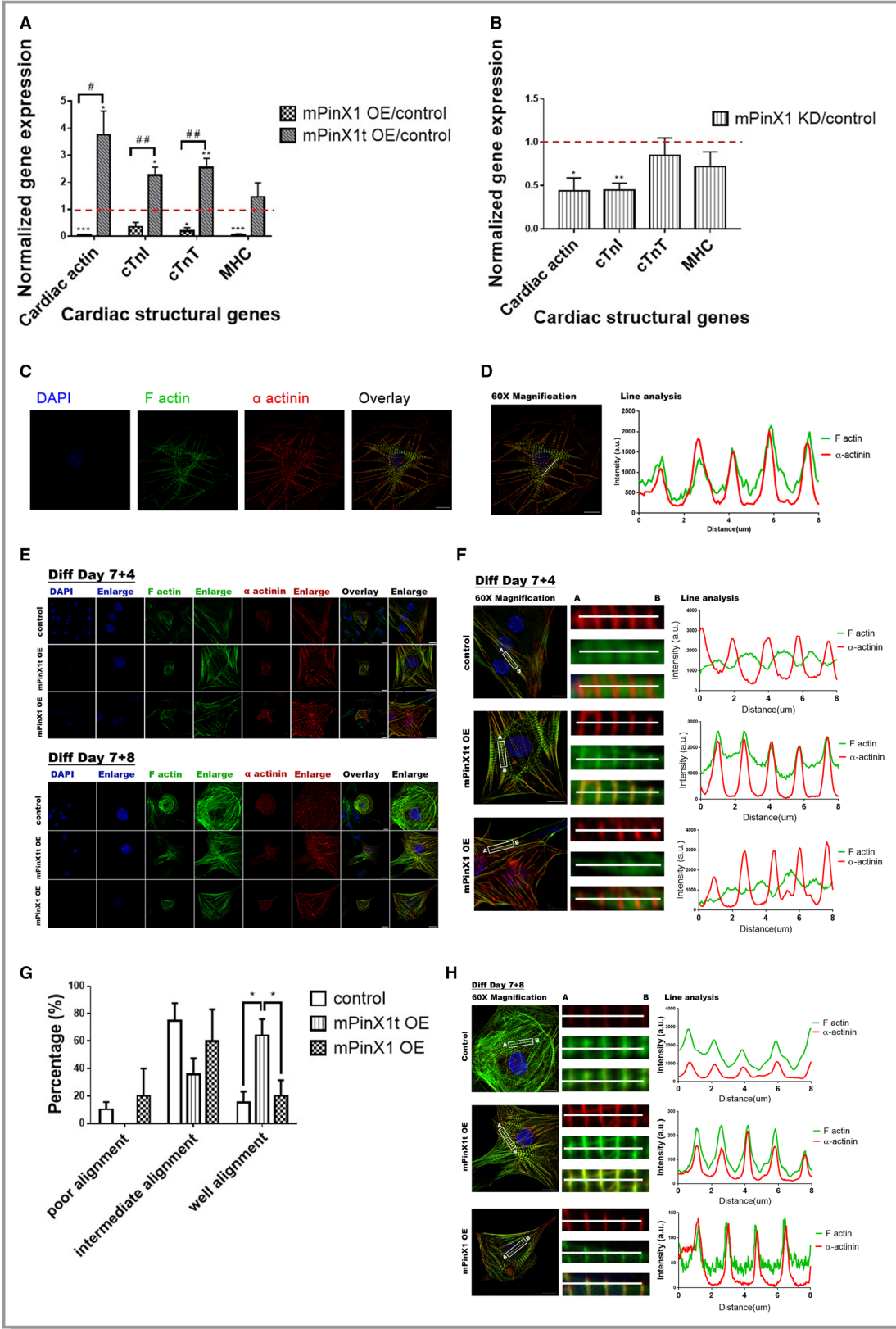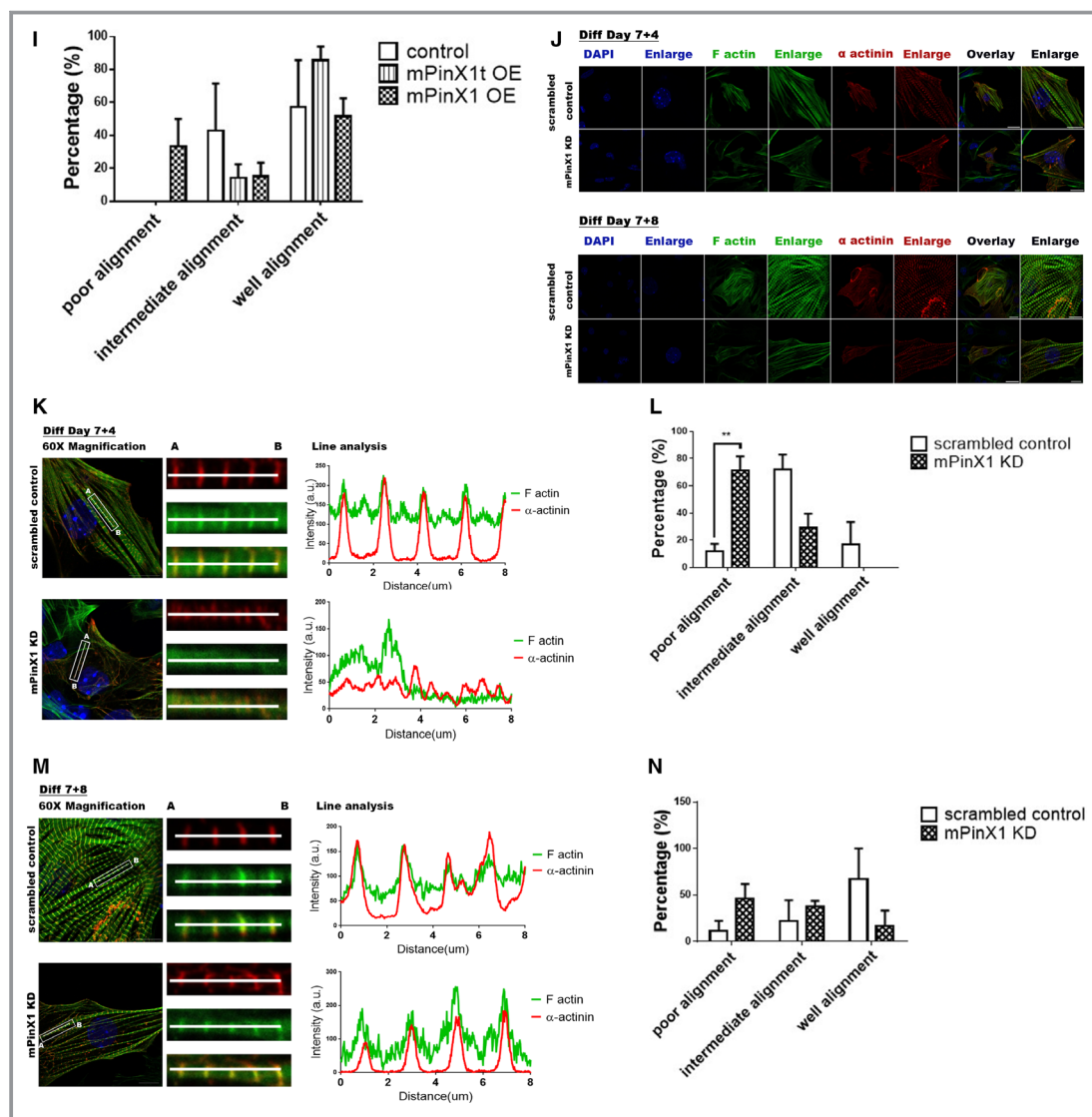
Figure 3. Continued

Figure 3. Continued

alignment of α-actinin and F-actin in terminally differentiated cardiomyocytes would appear as a clear striated pattern of α-actinin and F-actin in which α-actinin signal would lie within the peak of F-actin signal when a line scan is performed (Figure 3C and 3D). For most cardiomyocytes in mPinX1 overexpression group, α-actinin and F-actin were not well aligned at the early differentiation time point (Figure 3E through 3G). On the other hand, a higher percentage of cardiomyocytes in the mPinX1t overexpression group had a well-aligned α-actinin and F-actin pattern, and no cardiomyocyte in this group had a poorly aligned pattern (Figure 3G). The trend of difference among mPinX1 overexpression, mPinX1t overexpression, and control lines persisted at the immediate differentiation time point but was less apparent (Figure 3H and 3I).

A similar phenotype as that in the mPinX1 overexpression group could be observed in the mPinX1 knockdown cell line at

the early differentiation time point (Figure 3J through 3L). Knockdown of mPinX1 led to an irregular α-actinin and F-actin pattern. A higher percentage of cardiomyocytes in the mPinX1 knockdown group had a poorly aligned α-actinin and F-actin pattern, and no cardiomyocyte in this group had a well-aligned pattern (Figure 3L). The trend of difference between the mPinX1 knockdown line and control line persisted at the immediate differentiation time point but was less apparent (Figure 3M and 3N).

To further confirm the difference in the cardiomyocytes when mPinX1 and mPinX1t were differentially expressed, imaging on calcium transients (CaTs) was performed. When mPinX1t was overexpressed, the amplitude of the CaTs increased while the time to peak of CaTs decreased at both the early and late differentiation stages when compared with control (Figure S6). In addition, mPinX1t overexpression led to

Figure 3. Effects of the overexpression and knockdown of mPinX1/mPinX1t on cardiac differentiation and sarcomere assembly. **A**, Bar chart showing the change in expressions of cardiac actin, cTnI, cTnT), and myosin heavy chain (MHC) of cells on differentiation day 7+25 in mPinX1 and mPinX1t overexpression lines normalized to that of control. Dotted line indicates the expression level of the control line. Overexpression of mPinX1 (mPinX1 OE) decreased while overexpression of mPinX1t (mPinX1t OE) increased the expressions of cardiac structural genes. Data were presented as mean±SEM (mPinX1 OE group, n=3; mPinX1t OE group, n=5; control group, n=5; where n represents data from each independent differentiations). $*P<0.05$, $***P<0.001$ vs control line. $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs mPinX1 overexpression line. **B**, Bar chart showing the change in expressions of cardiac actin, cTnI, cTnT, and MHC of cells on differentiated day 7+25 in mPinX1 knockdown line normalized to that of control. Dotted line indicates the expression level of the control line. Knockdown of mPinX1 (mPinX1 KD) decreased the expression of cardiac structural genes. Data were presented as mean±SEM (n=3; where n represents data from each independent differentiations). $*P<0.05$, $**P<0.01$ vs control line. **C**, Immunostaining of α-actinin and F-actin of differentiation day 7+12 cardiomyocyte reveals proper sarcomere formation when cardiomyocytes are mature. Scale bar: 10 μm. **D**, Line analysis showing α-actinin and F-actin patterns of differentiation day 7+12 cardiomyocyte in (**C**). Mature cardiomyocytes have overlapping pattern of α-actinin and F-actin. Scale bar: 10 μm. **E**, Representative immunostaining results of α-actinin and F-actin of cardiomyocytes at early differentiation time point (day 7+4) and intermediate differentiation time point (day 7+8) in control line, mPinX1t overexpression line and mPinX1 overexpression line. Scale bar: 20 μm for photos of overlay views and 10 μm for photos with enlarged views. **F**, Line analysis of cardiomyocytes at early differentiation time point (day 7+4) in control line, mPinX1t overexpression line and mPinX1 overexpression line shown in (**E**). Scale bar: 10 μm. **G**, Bar graph summarizing the effects of mPinX1 and mPinX1t overexpression on sarcomere assembly at early differentiation time point (day 7+4). mPinX1t overexpression cell line showed a higher percentage of cardiomyocytes with a well-aligned α-actinin and F-actin pattern, while it did not have cardiomyocytes with poor alignment. Data were presented as mean±SEM (15 cardiomyocytes from mPinX1 OE group, 19 cardiomyocytes from mPinX1t OE group, and 19 cardiomyocytes from control group were analyzed. Cells were obtained from 3 independent differentiations. The unit of statistical analysis was the number of independent differentiations). $*P<0.05$. **H**, Line analysis of cardiomyocytes at intermediate differentiation time point (day 7+8) in control line, mPinX1t overexpression line and mPinX1 overexpression line shown in (**E**). Scale bar: 10 μm. **I**, Bar graph summarizing the effects of mPinX1 and mPinX1t overexpression on sarcomere assembly at intermediate differentiation time point (day 7+8). Control, mPinX1 overexpression and mPinX1t overexpression cell lines all had >50% cardiomyocytes showing a well-aligned α-actinin and F-actin pattern. The mPinX1t overexpression cell line had a trend of having higher percentage of cardiomyocytes with a well-aligned α-actinin and F-actin pattern. The mPinX1 overexpression cell line had cardiomyocytes with poorly aligned α-actinin and F-actin pattern, while mPinX1t overexpression cell line or the control line did not have cardiomyocyte with poor alignment. Data were presented as mean±SEM (19 cardiomyocytes from mPinX1 OE group, 20 cardiomyocytes from mPinX1t OE group, and 19 cardiomyocytes from control group were analyzed. Cell were obtained from 3 independent differentiations. The unit of statistical analysis was the number of independent differentiations.). **J**, Representative immunostaining results of α-actinin and F-actin of cardiomyocytes at early differentiation time point (day 7+4) and intermediate differentiation time point (day 7+8) in scrambled control line and mPinX1 knockdown line. Scale bar: 20 μm for photos of overlay views and 10 μm for photos with enlarged views. **K**, Line analysis of cardiomyocytes at early differentiation time point (day 7+4) in scrambled control line and mPinX1 knockdown line shown in (**J**). Scale bar: 10 μm. **L**, Bar graph summarizing the effects of mPinX1 knockdown on sarcomere assembly at early differentiation time point (day 7+4). mPinX1 knockdown cell line showed a higher percentage of cardiomyocytes with poorly aligned α-actinin and F-actin pattern, while it did not have cardiomyocytes with good alignment. Data were presented as mean±SEM (17 cardiomyocytes from the mPinX1 KD group and 18 cardiomyocytes from the scrambled control group were analyzed. Cells were obtained from 3 independent differentiations. The unit of statistical analysis was the number of independent differentiations.). $**P<0.01$. **M**, Line analysis of cardiomyocytes at intermediate differentiation time point (day 7+8) in scrambled control line and mPinX1 knockdown line shown in (**J**). Scale bar: 10 μm. **N**, Bar graph summarizing the effects of mPinX1 knockdown on sarcomere assembly at intermediate differentiation time point (day 7+8). mPinX1 knockdown cell line had a trend of having higher percentage of cardiomyocytes with poorly aligned α-actinin and F-actin pattern and had a trend of having lower percentage of cardiomyocytes with a well-aligned α-actinin and F-actin pattern. Data were presented as mean±SEM (17 cardiomyocytes from the mPinX1 KD group and 15 cardiomyocytes from the scrambled control group were analyzed. Cells were obtained from 3 independent differentiations. The unit of statistical analysis was the number of independent differentiations.) cTnI indicates cardiac troponin I; cTnT, cardiac troponin T; DAPI 4′,6-diamidino-2-phenylindole.

an increase in CaT frequency and basal calcium level in late differentiation stage. On the other hand, when mPinX1 was overexpressed, the basal calcium level decreased in early differentiation stage, while the time to peak of CaTs increased in late differentiation stage (Figure S6). In summary, CaT data support the notion that mPinX1t overexpression positively regulates cardiac differentiation while mPinX1 overexpression negatively regulates cardiac differentiation.

Taken together, overexpression and knockdown of mPinX1 decreased cardiac differentiation and led to a delay in proper sarcomere formation, while overexpression of mPinX1t increased cardiac differentiation and led to an earlier sarcomere formation.

mPinX1 Protein and mPinX1t Protein Bound to the mRNAs of Cardiac Transcription Factors Gata4 and Tbx5 While Only mPinX1t Protein but Not mPinX1 Protein Bound to the Nucleoporin 133

Sequence analysis revealed that both mPinX1 and mPinX1t contain the putative RNA-binding domain G-Patch at the N-terminal. We hypothesized that mPinX1 and mPinX1t regulate cardiac differentiation through binding to the mRNAs of key cardiac transcription factors. By RNA immunoprecipitation assay, both mPinX1 and mPinX1t were found to bind to the mRNAs of the core cardiac transcription factors Gata4 and Tbx5 (Figure 4A through 4D). In HEK cells coexpressed with myc-mPinX1 and mRNAs of cardiac transcription factors, when compared with the isotype control group, the pulldown fraction by anti-myc was found to contain 7.47 ± 2.02-fold of Gata4 mRNA and 4.91 ± 0.96-fold of Tbx5 mRNA (Figure 4A and 4C). In HEK cells coexpressed with myc-mPinX1t and mRNAs of cardiac transcription factors, when compared with the isotype
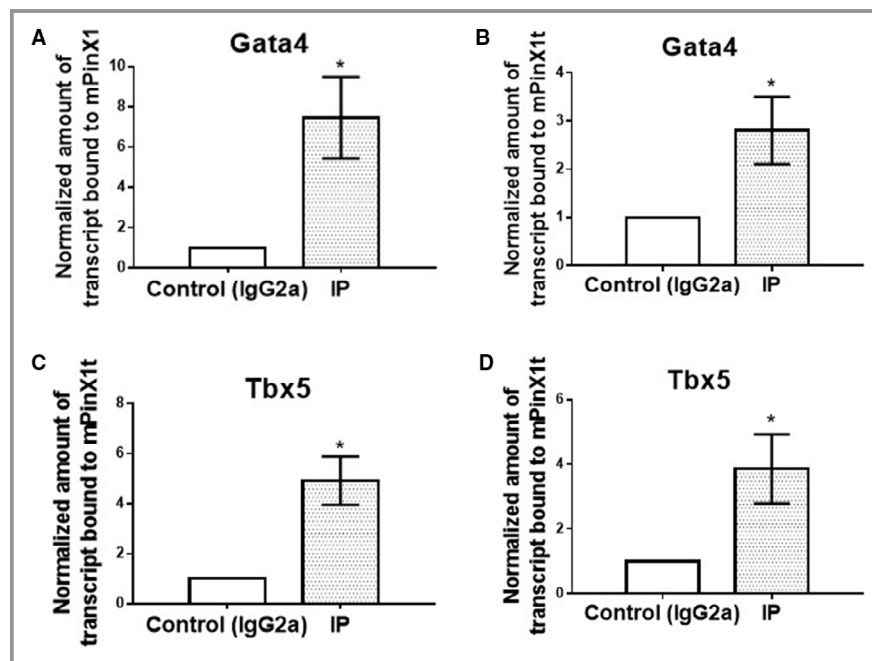
Figure 4. Both mPinX1 and mPinX1t proteins bound cardiac transcription factor mRNAs, while only mPinX1t protein but not mPinX1 protein-bound Nucleoporin 133 (Nup133). **A** through **D**, RNA immunoprecipitation of HEK293FT cells overexpressed with (**A** and **C**) myc-mPinX1 or (**B** and **D**) myc-mPinX1t and (**A** and **B**) Gata4 or (**C** and **D**) Tbx5. Anti-myc was used to perform the immunoprecipitation. IgG2a was used in isotype control experiment. The presence of (**A** and **B**) Gata4 mRNA or (**C** and **D**) Tbx5 mRNA in the immunoprecipitant was quantitated by subsequent quantitative polymerase chain reaction. The results showed that both mPinX1 and mPinX1t proteins could bind to Gata4 mRNA and Tbx5 mRNA. Data were presented as mean$\pm$SEM (n=3; where n represents independent RNA immunoprecipitation experiments). *$P<0.05$ vs control. **E**, Coimmunoprecipitation assay of HEK293FT cells overexpressed with myc-mPinX1t (left panel) or myc-mPinX1 and HA-Nup133 (right panel). (Left panel) In the immunoprecipitant of anti-myc (which contained myc-mPinX1t), HA-Nup133 was detected. No HA-Nup133 was detected in the isotype control group. (Right panel) In the immunoprecipitant of anti-myc (which contained myc-mPinX1), HA-Nup133 was not enhanced when compared with isotype control group. Another control experiment was also performed in which myc only and HA-Nup133 were overexpressed in HE293FT cells. Using anti-myc for immunoprecipitation, as expected, no HA-Nup133 was detected in the immunoprecipitant. **F**, Coimmunoprecipitation assay of HEK293FT cells overexpressed with myc-mPinX1t. As shown in the input lane, HEK293FT expressed endogenous Nup133. In the immunoprecipitant of anti-myc (which contained myc-mPinX1t), endogenous Nup133 was detected. No Nup133 was detected in the isotype control group. DAPI indicates 4′,6-diamidino-2-phenylindole.
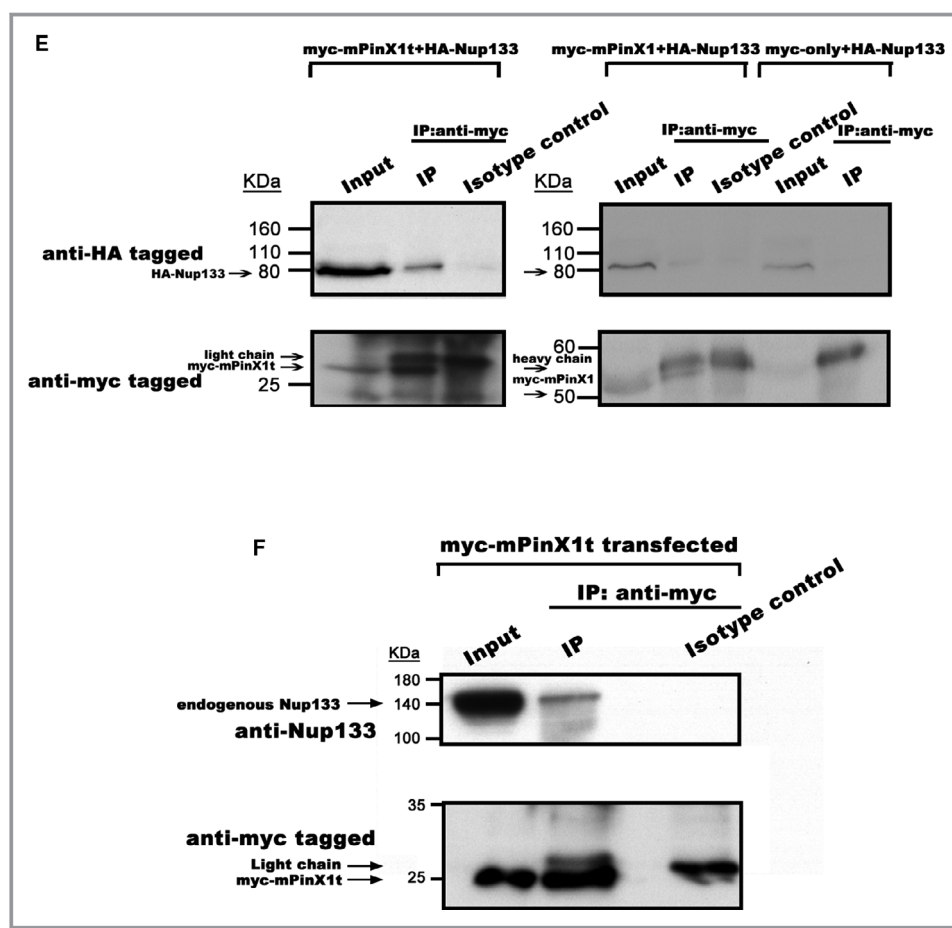
Figure 4. Continued

control group, the pulldown fraction by anti-myc was found to contain 2.80±0.70-fold of Gata4 mRNA and 3.86±1.07-fold of Tbx5 mRNA (Figure 4B and 4D). On the other hand, myc-mPinX1t and myc-mPinX1 were not found to bind to Rpl13A mRNA (Figure S7).

A yeast–2 hybrid assay was carried out to screen out the mPinX1t-interacting partners in mESCs. More than 100 interacting partners were identified and classified according to Gene Ontology terms classification (Figure S8). One of the interacting proteins was known as Nucleoporin 133 (Nup133). Nup133 is one of the components of nuclear pore complex, which is responsible for transport of macromolecules across the nuclear envelope from nucleus to cytoplasm.[28,29] We hypothesized that mPinX1t promotes cardiac differentiation through facilitating the transport of Gata4 and Tbx5 mRNAs from the nucleus to the cytoplasm for translation. To further validate the interaction between mPinX1t and Nup133, a coimmunoprecipitation assay was performed using HEK293FT cells coexpressed with myc-mPinX1t and HA-Nup133. Our result showed that myc-mPinX1t could successfully pull down HA-Nup133 (Figure 4E). Similar coimmunoprecipitation assay was performed in myc-mPinX1 and HA-Nup133

overexpressed HEK293FT. The result showed that myc-mPinX1 failed to pull down HA-Nup133 (Figure 4E).

To test if mPinX1t would interact with endogenous Nup133, a further coimmunoprecipitation assay was performed. In HEK293FT overexpressed with myc-mPinX1t, endogenous Nup133 was present in the pulldown fraction by anti-myc but not in its isotype control (Figure 4F). The result indicated that mPinX1t is able to interact with endogenous Nup133.

mPinX1t Increased the Ratio of Cytosolic Gata4 mRNAs to Nuclear Gata4 mRNAs; Knockdown of Nup133 Abolished the Effect

To investigate whether mPinX1t facilitates the export of cardiac transcription factor mRNAs from the nucleus to the cytoplasm, Gata4 and mPinX1/mPinX1t were coexpressed in HEK293FT, followed by fractionation of cytosolic and nuclear mRNAs. Reverse transcriptase qPCR on the Gata4 mRNAs was then performed using the cytosolic and nuclear fractions. In the mPinX1t and Gata4 cotransfected group, a higher ratio of cytosolic Gata4 mRNAs to nuclear Gata4 mRNAs was

detected when compared with that in the control group (empty vector and Gata4 cotransfected group) (Figure 5A). On the other hand, the mPinX1 and Gata4 cotransfected group tended to have a lower ratio of cytosolic Gata4 mRNAs to nuclear Gata4 mRNAs (Figure 5A). Since interaction between mPinX1t and Nup133 was observed, to determine the importance of this interaction in the export of mRNA, the same experiment as above but with knockdown of Nup133 by small interfering RNA was performed. Interestingly, knockdown of Nup133 abolished the differences between different groups in term of the ratio of cytosolic Gata4 mRNAs to nuclear Gata4 mRNAs (Figure 5B), suggesting the importance of interaction of mPinX1t and Nup133 in helping with the export of Gata4 mRNAs.
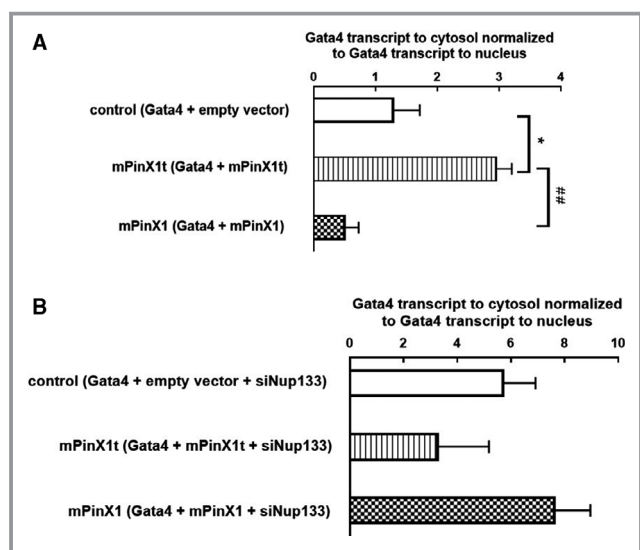
Figure 5. mPinX1t increased the ratio of cytosolic Gata4 mRNAs to nuclear Gata4 mRNAs; knockdown of Nup133 abolished the effect. **A**, Gata4 and empty vector/mPinX1/mPinX1t were overexpressed in HEK293FT, followed by fractionation of cytosolic and nuclear mRNAs. RT-qPCR on the Gata4 mRNAs was then performed using the cytosolic and the nuclear fractions. The amount of Gata4 transcripts in cytosol was normalized to that in nucleus. Our results showed that, in mPinX1t and Gata4 co-transfected group, a higher ratio of cytosolic Gata4 mRNAs to nuclear Gata4 mRNAs was detected when compared with that in control group (empty vector and Gata4 co-transfected group). On the other hand, mPinX1 and Gata4 co-transfected group tended to have a lower ratio of cytosolic Gata4 mRNAs to nuclear Gata4 mRNAs. Data were presented as mean±SEM (n=3; where n represents independent experiments). *$P<0.05$ vs control group. ##$P<0.01$ vs mPinX1 group. **B**, Gata4, empty vector/mPinX1/mPinX1t and siRNA against Nup133 (siNup133) were overexpressed in HEK293FT, followed by fractionation of cytosolic and nuclear mRNAs. RT-qPCR on the Gata4 mRNAs was then performed using the cytosolic and the nuclear fractions. The amount of Gata4 transcripts in cytosol was normalized to that in nucleus. Our results showed that there was no statistical difference in the ratio of cytosolic Gata4 mRNAs to nuclear Gata4 mRNAs between different groups. Data were presented as mean±SEM (n=3; where n represents independent experiments).

Discussion

The Discovery of a Novel Transcript Variant of mPinX1, mPinX1t

A new transcript variant of mPinX1, the mPinX1t, was detected in mESCs and their differentiated derivatives in a PCR reaction trying to detect the endogenous mPinX1 expression. Sequencing results revealed that this new transcript variant harbors an additional exon of 111 nucleotides located between exon 6 and exon 7 of mPinX1. This additional exon was found to be located in Locus NT_039606.7 of chromosome 14 of the mouse genome where the mPinX1 gene is located. Intriguingly, there is a stop codon within this extra 111 nucleotide sequence; rendering this new transcript encodes a protein that has the N-terminal but lacks the C-terminal of mPinX1. There is also an extra sequence that exists only in mPinX1t but not mPinX1 (see Figure 1D). RLM rapid amplification of 5′ cDNA ends followed by subsequent sequencing of the 5′ UTR revealed that there are at least 3 different sequences in the 5′ UTR of the mRNA of mPinX1t. These 3 different 5′ UTR sequences fall within the same locus of mPinX1 gene and completely aligned with the known 5′ UTR sequence of mPinX1. The result provided further evidence that mPinX1t is a transcript variant of mPinX1.

In fact, a search in the National Center for Biotechnology Information database indicated that human tissues also express a transcript variant similar to the transcript of mPinX1t; that is, this human PinX1 transcript variant is also predicted to encode a protein that lacks the C-terminal of human PinX1. The result indicated that both mouse and human express at least 2 isoforms of PinX1 protein, and that the N-terminal of PinX1 may have a unique function independent of the function of the C-terminal of PinX1.

PinX1 is a well-known tumor suppressor protein that works by negatively regulating the telomerase activity at its C-terminal telomerase inhibitory domain.[16] Many recent publications focused on how PinX1 regulates tumor progression and metastasis,[18,30,31] while little has been done on the functions of the N-terminal of mammalian PinX1. The newly discovered transcript variant mPinX1t provides a good opportunity for us to investigate the role and the function of the N-terminal of mPinX1 in mESCs and their differentiated derivatives.

mPinX1t Encodes for a Functional Intact mRNA That Is Actively Translated Into Protein

Our RLM rapid amplification of 5′ cDNA ends assay and RLM rapid amplification of 3′ cDNA ends assay confirmed that mPinX1t encodes for a functional mRNA with intact 5′ cap and poly(A) tail. Consistently, polysome fractionation followed by PCR showed that mPinX1t mRNA was present in the monosome and polysome fractions, clearly suggesting that mPinX1t
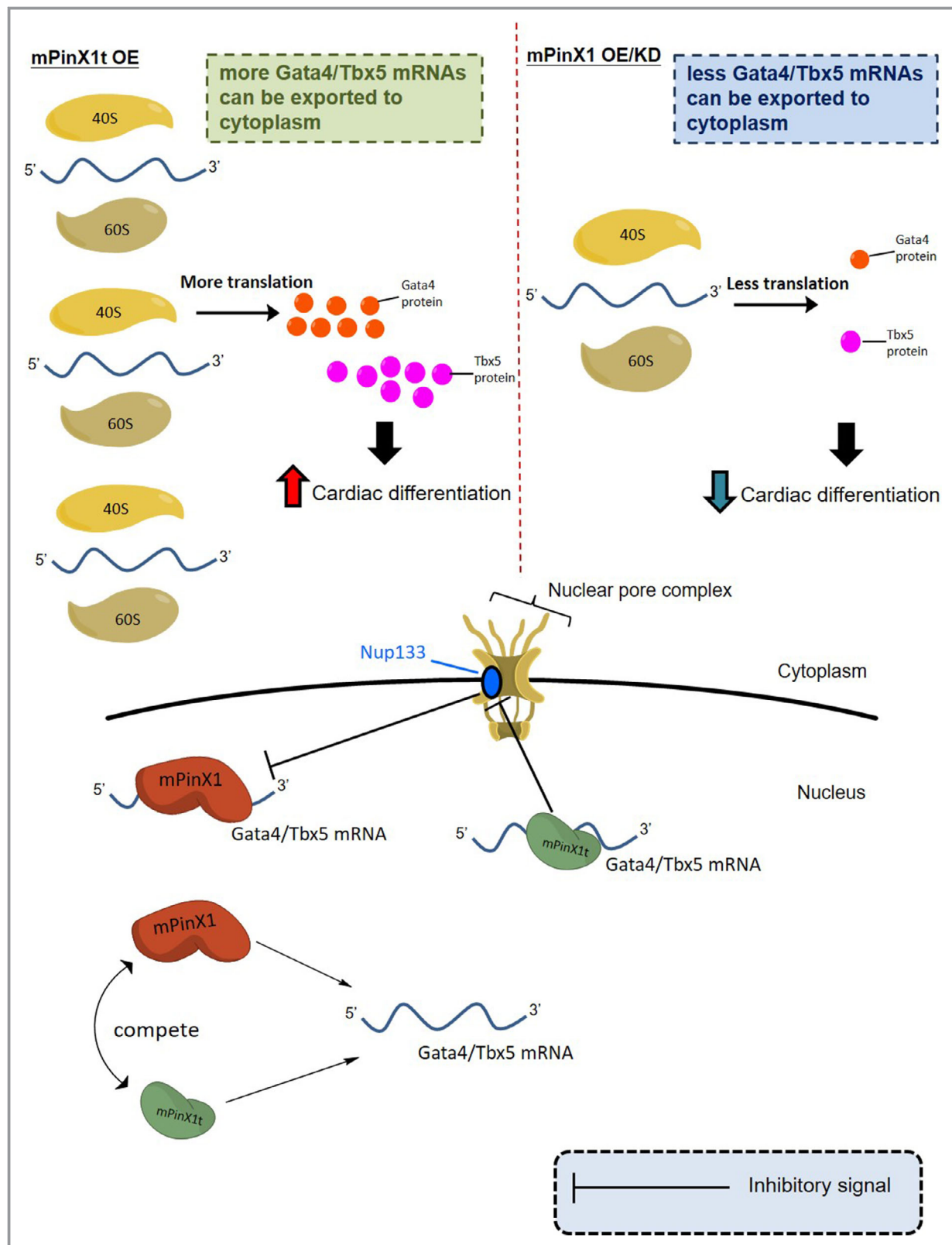
transcript encodes for an actively translating mRNA. Using an antibody having an epitope at the N-terminal of PinX1 also revealed the existence of mPinX1 and mPinX1t proteins in both undifferentiated mESCs and differentiated derivatives. To our knowledge, our study is the first study to document the existence of a truncated isoform of PinX1 protein.

Figure 6. Schematic diagram showing a model of how mPinX1t regulates cardiac differentiation. mPinX1 and mPinX1t contain the RNA-binding domain G-Patch. This domain allows mPinX1 and mPinX1t to compete with each other for binding to the mRNAs of the cardiac transcription factors Gata4 and Tbx5. Only mPinX1t would interact with Nucleoporin 133 (Nup133), a component of the nuclear pore complex. We speculate that mammalian Nup133 negatively mediates the transport of mRNAs from nucleus to cytoplasm; mPinX1t interacts with Nup133 and relieves its inhibitory effect on the transport of Gata4 and Tbx5 mRNAs from nucleus to cytoplasm. Therefore, in the presence of mPinX1t, more Gata4 and Tbx5 mRNAs can be exported to the cytoplasm for later translation. This would result in larger amount of cardiac transcription factor proteins produced for cardiac differentiation. In the upper part of the figure, what happens in the cytoplasm under different scenarios are indicated. When mPinX1t is overexpressed, mPinX1t protein would bind to the mRNAs of Gata4 and Tbx5; these mPinX1t proteins would also interact with Nup133 to relieve its inhibitor effect on the transport the mRNAs, so more mRNAs are exported to the cytoplasm for translation. The translated Gata4 and Tbx5 proteins would then function as cardiac transcription factors for cardiac differentiation. When mPinX1 is overexpressed, it competes with mPinX1t and binds to Gata4 and Tbx5 mRNAs. Therefore, less mPinX1t protein binds to Gata4 and Tbx5 mRNAs. On the other hand, when mPinX1 is knocked down, mPinX1t is also knocked down; therefore, there is less mPinX1t protein to interact with Nup133. In both cases, mRNA transport inhibitor effect of Nup133 still exists; export of mRNAs of Gata4 and Tbx5 to the cytoplasm would decrease, leading to a decrease in cardiac differentiation.

mPinX1 and mPinX1t Affect mESC Differentiation Into Cardiac Lineage

Various mESC lines with stable overexpression or knockdown of mPinX1 and/or mPinX1t were established to study the effect of mPinX1 and mPinX1t in determining the self-renewal and pluripotent characteristics of mESCs and in regulating the cardiac differentiation of mESCs. Altering the expression level of neither mPinX1 nor mPinX1t could affect the proliferation, telomerase activity, and the pluripotent characteristics of mESCs.

ESCs have high levels of telomerase expression and activity, both of which are rapidly downregulated upon differentiation.[32,33] This high telomerase activity in ESCs is involved in maintaining the indefinite proliferation ability of ESCs.[32] While PinX1 was first identified as a tumor suppressor protein and overexpression of it inhibited telomerase activity and shortened telomere,[16] later studies also reported that PinX1 was associated with telomeres primarily at mitosis and that knockdown of PinX1 caused delayed mitotic entry.[34] Therefore, knockdown of PinX1 may not necessarily lead to a change in proliferation at the cellular level because of its dual functions. In addition, many pathways are known to regulate telomerase activity in mESCs,[35,36] and mPinX1 might not be the rate-limiting physiological regulator of telomerase at the undifferentiated stage; therefore, its effect on telomerase activity and/or telomere length would be minor. Moreover, previous studies have shown that murine telomerase reverse transcriptase and RNA subunit of telomerase are not immediately essential for normal development and cell viability but is required for telomere-length maintenance in vivo.[37,38] However, interestingly, mESCs can be obtained from telomeric reverse transcriptase knockout mice, and these cells can be maintained in culture.[39] In addition, mice are known to have unusually long telomeres, and this may not be generalized to other species. This suggests that even if there is a change in telomerase activity/telomere length, mESCs may not be affected for many passages. All these may help explain why an alteration in mPinX1 expression level may not affect mESCs at undifferentiated state.

On the other hand, upon differentiation of mESCs, the effect of mPinX1 and mPinX1t could be observed. Stable overexpression and knockdown of mPinX1, which were also found to decrease mPinX1t expression, decreased cardiac differentiation has revealed the expression of cardiac structural markers and the sarcomere assembly. In contrast, stable overexpression of mPinX1t was found to promote cardiac differentiation. The result suggested that mPinX1t might be a potential positive regulator for cardiac differentiation. Previous studies reported that switching expression level of splice variants of some genes could be associated with stem cell differentiation.[40,41] In our study, the expression of mPinX1 was found to decrease to a low level during mESC differentiation, while the expression of mPinX1t was not found to decrease during mESC differentiation. This switch in the relative expression level of mPinX1 and mPinX1t is in line with the positive role of mPinX1t in the differentiation process of mESCs.

Both mPinX1 and mPinX1t Could Bind to mRNAs of Cardiac Transcription Factors While Only mPinX1t but Not mPinX1 Could Bind to Nup133

The amino acid sequence of mPinX1 and mPinX1t suggest that they both contain a G-patch domain at their N-terminal. Many previous reports showed that proteins containing a G-patch domain are associated with RNA-binding proteins or are able to interact with RNA themselves.[17] We speculated that mPinX1 and mPinX1t proteins may regulate cardiac differentiation by interacting with the mRNAs of cardiac transcription factors. In fact, our RNA immunoprecipitation assay results indicated that both mPinX1 and mPinX1t proteins could interact with the mRNAs of cardiac transcription factors Gata4 and Tbx5.

A previous study has shown that human PinX1 could bind to the RNA-binding domain of the human telomeric reverse transcriptase and the RNA subunit of human telomerase in vitro.[42] In vivo study revealed that human PinX1 binds to the assembled human telomeric reverse transcriptase – human telomerase complex.[41] This study suggested that mammalian PinX1 is capable of binding to RNA. Interestingly, the yeast homolog of mammalian PinX1, which bears the G-patch, was found to be involved in preribosomal RNA processing and in the final 3′-end trimming of U18 and U24 small nucleolar RNAs.[43] A mutational analysis showed that the G-patch is essential for these functions. Importantly, human PinX1 can complement the mutation of the G-patch in yeast PinX1, suggesting that human PinX1 has a dual function in telomere length regulation and ribosomal RNA maturation. Our current study revealed a novel function of the mPinX1 and mPinX1t, which bear the G-patch: They can bind to the mRNAs of cardiac transcription factors.

In our yeast-2 hybrid assay, mPinX1t protein was found to interact with Nup133, a subunit of a protein complex that forms the nuclear pore at the nuclear membrane.[28,29] Our further coimmunoprecipitation assay validated the interaction between mPinX1t and Nup133. Interestingly, this interaction was not found between mPinX1 and Nup133.

mPinX1t Positively Regulates Cardiac Differentiation Possibly by Facilitating the Export of Cardiac Transcription Factor mRNAs From the Nucleus to the Cytosol

From the result of the RNA fractionation assay on Gata4 and mPinX1/mPinX1t cotransfected HEK293FT cells, cytosolic Gata4 mRNA to nuclear Gata4 mRNA ratio was found to be higher in mPinX1t-containing cells. Interestingly, when Nup133 was knocked down, the difference between the effect of mPinX1t and mPinX1 in affecting the Gata4 mRNA transport was abolished. Previous study showed that yeast Nup133 assisted in mRNA export, as revealed by accumulation of poly(A)-containing RNA in nucleus in yeast Nup133 negative cells.[44] While previous studies showed the importance of mammalian Nup133 in the assembly of the nuclear pore complex and in anchoring the nuclear pore complex to the nuclear envelope,[28,29] the role of mammalian Nup133 in mediating the mRNA transport from nucleus to cytosol is unclear. Since we observed an increase in Gata4 mRNA transport from nucleus to cytosol when Nup133 was knocked down, we speculate that mammalian Nup133 negatively regulates mRNA export in this cellular context. Combined with our molecular data showing the interaction between mPinX1t and Nup133, we speculate that mPinX1t binds to Nup133 and relieves Nup133's inhibitory effect on mRNA transport; therefore, when mPinX1t is overexpressed, Gata4 mRNA transport from nucleus to cytosol increases. The cardiac transcription factor mRNAs in the cytoplasm can then be later translated to facilitate cardiac differentiation. However, when Nup133 is knocked down, the inhibitory effect of Nup133 no longer exists, and therefore the differences between mPinX1t-overexpressed cells and mPinX1-overexpressed cells is abolished.

Proposed Model on How mPinX1 and mPinX1t Regulated Cardiogenesis

In this study, mPinX1t was found to promote cardiac differentiation in early differentiation. A model is proposed to explain the observed phenotypes on how mPinX1t regulates cardiogenesis (Figure 6).

From the result of RNA immunoprecipitation assay, both mPinX1 and mPinX1t were found to interact with the mRNAs of the key cardiac transcription factors Gata4 and Tbx5. In the yeast-2 hybrid assay, mPinX1t but not mPinX1 was found to interact with Nup133. We speculate that mammalian Nup133 negatively mediates the transport of mRNAs from nucleus to cytoplasm; mPinX1t interacts with Nup133 and relieves its inhibitory effect on the transport of Gata4 and Tbx5 mRNAs from nucleus to cytoplasm for later translation.

Because of the high similarity between the N-terminal of mPinX1 protein and mPinX1t protein, it is believed that mPinX1 and mPinX1t proteins might act as competitors to each other for binding to the mRNAs of Gata4 and Tbx5. When mPinX1 is overexpressed, mPinX1 competes with mPinX1t and binds to Gata4 and Tbx5 mRNAs. Therefore, less mPinX1t protein binds to Gata4 and Tbx5 mRNAs. On the other hand, when mPinX1 is knocked down, mPinX1t is also knocked down; therefore, there is less mPinX1t protein to interact with Nup133. In both cases, the mRNA transport inhibitor effect of Nup133 still exists; export of mRNAs of Gata4 and Tbx5 to the cytoplasm would decrease, leading to a decrease in cardiac differentiation. In contrast, when mPinX1t is overexpressed, mPinX1t protein binds to the mRNAs of Gata4 and Tbx5; these mPinX1t proteins also interact with Nup133 to relieve its inhibitor effect on the transport the mRNAs, so more mRNAs are exported to the cytoplasm for translation. The translated Gata4 and Tbx5 proteins would then function as cardiac transcription factors for cardiac differentiation.

In conclusion, we discovered a novel transcript variant of mPinX1, the mPinX1t, to be present in both the undifferentiated and differentiated mESCs. This mPinX1t exists as an intact mRNA and protein. Importantly, mPinX1t positively regulates cardiac differentiation of mESCs. While both mPinX1 protein and mPinX1t protein interact with the mRNAs of cardiac transcription factors, only mPinX1t protein but not mPinX1 protein interacts with Nup133, a component of the nuclear pore complex. Interestingly, mPinX1t-

containing cells was found to have a higher cytosol to nucleus ratio of cardiac transcription factor mRNAs when compared with control cells. The present study reports the presence of this novel mPinX1t protein and reveals its novel role in promoting cardiogenesis. This study provides important information for developmental biology and also provides novel insights on the possibility of increasing the yield of cardiomyocytes from ESCs, a hurdle that has to be overcome if ESC-derived cardiomyocytes are applied for drug screening or for future therapeutic uses.

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Disclosures

None.

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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. 2×10^5 cells were transferred to a

new Eppendorf tube and centrifuged at 3,000 g for 10 mins at $4\,^{\circ}C$. 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

$^{\circ}C$. 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 $25\,^{\circ}C$ 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).
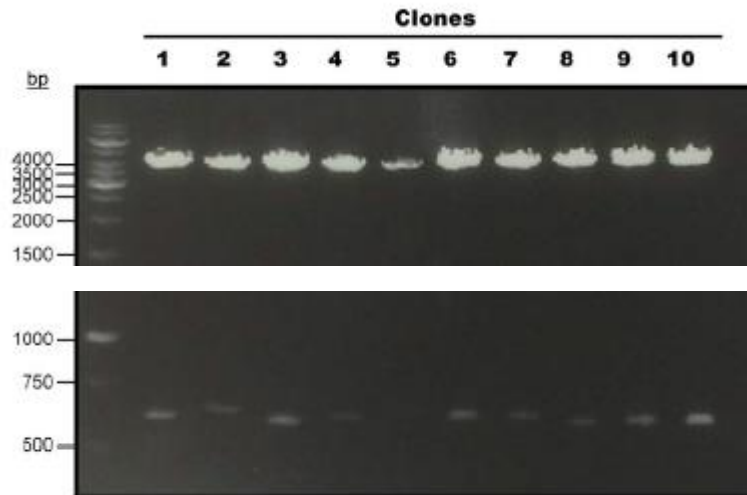
Three different sizes of digested products were observed between 500 and 750 bp apart from the 4,000bp TOPO T vector backbone. Clones 2, 3 and 6 were selected for sequencing.

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

A
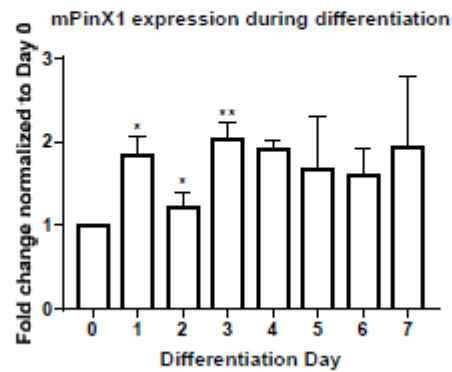
B
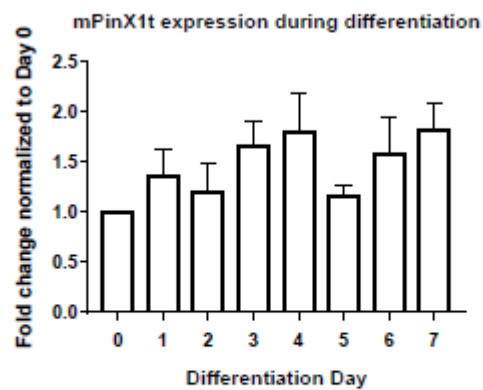
A) – B) Expression profile of (A) mPinX1 and (B) mPinX1t from undifferentiated day 0 to differentiation day 7. All data are normalized to that of undifferentiated day 0. Increase in mPinX1 expression was observed during early stage of differentiation with the expression of mPinX1 peaked at differentiation 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.
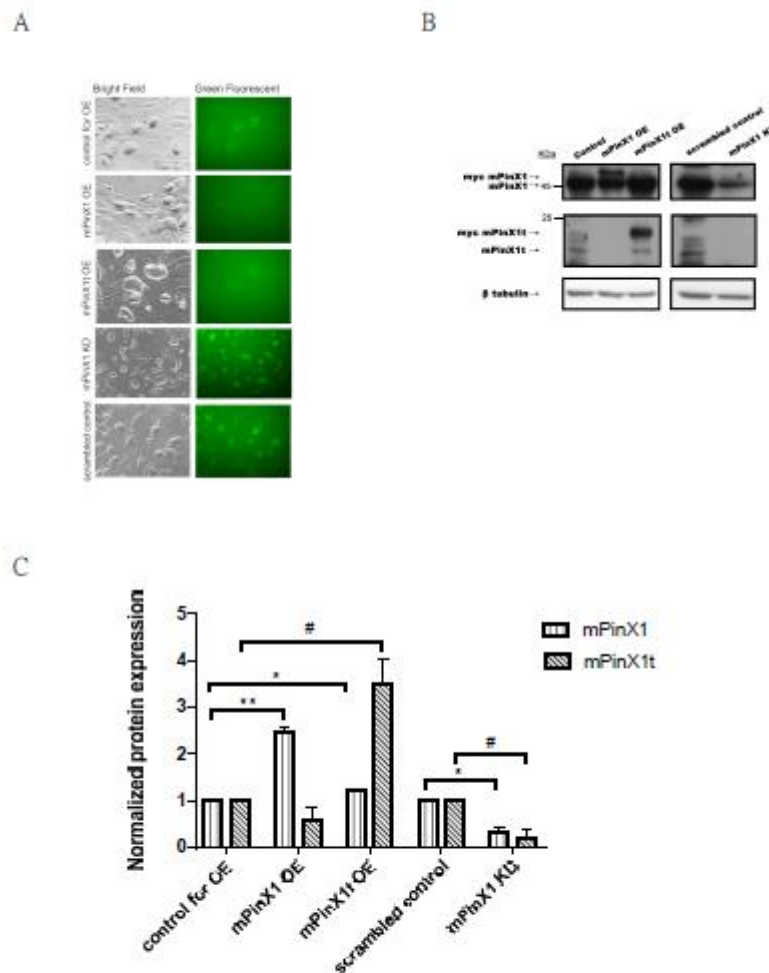
A) Morphology of undifferentiated mESCs in the established overexpression and knockdown cell lines. Morphology of mESCs in (left panel) bright field and (right panel) green fluorescent field. No obvious change in morphology for these established cell lines when compared to their respective control cell 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.
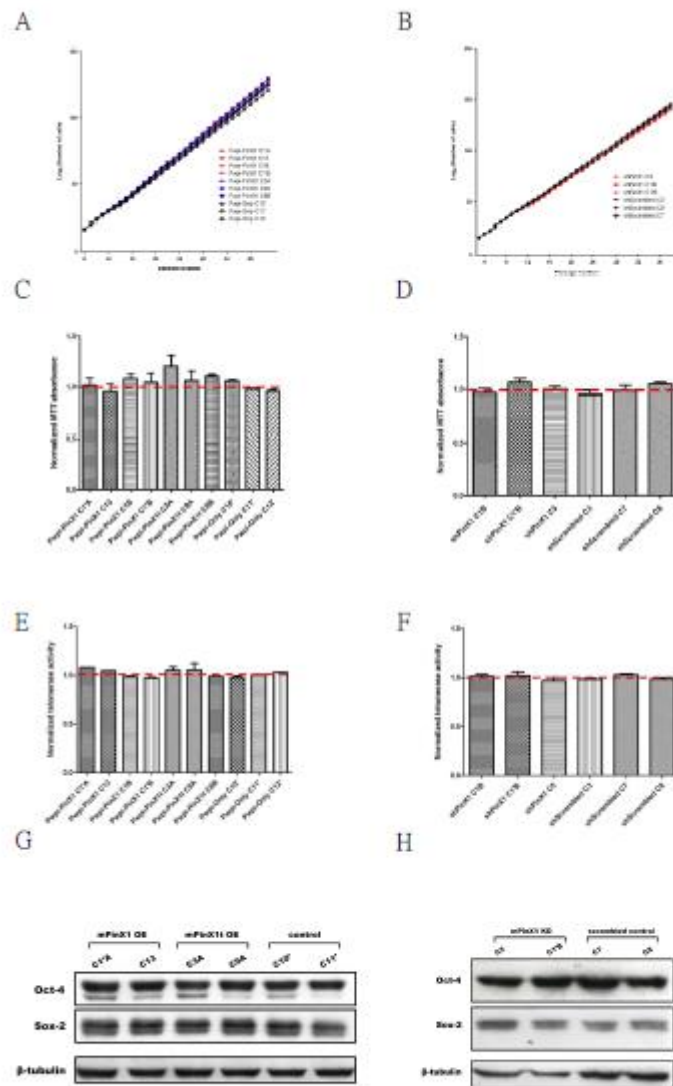
A) Proliferation curve of different clones of mPinX1 overexpression (Pwpi-PinX1), mPinX1t overexpression (Pwpi-PinX1t) and control (Pwpi only) cell lines. The graph was plotted with Log2 (total number of cells) against the passage number. There was no significant difference in the rate of proliferation 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.
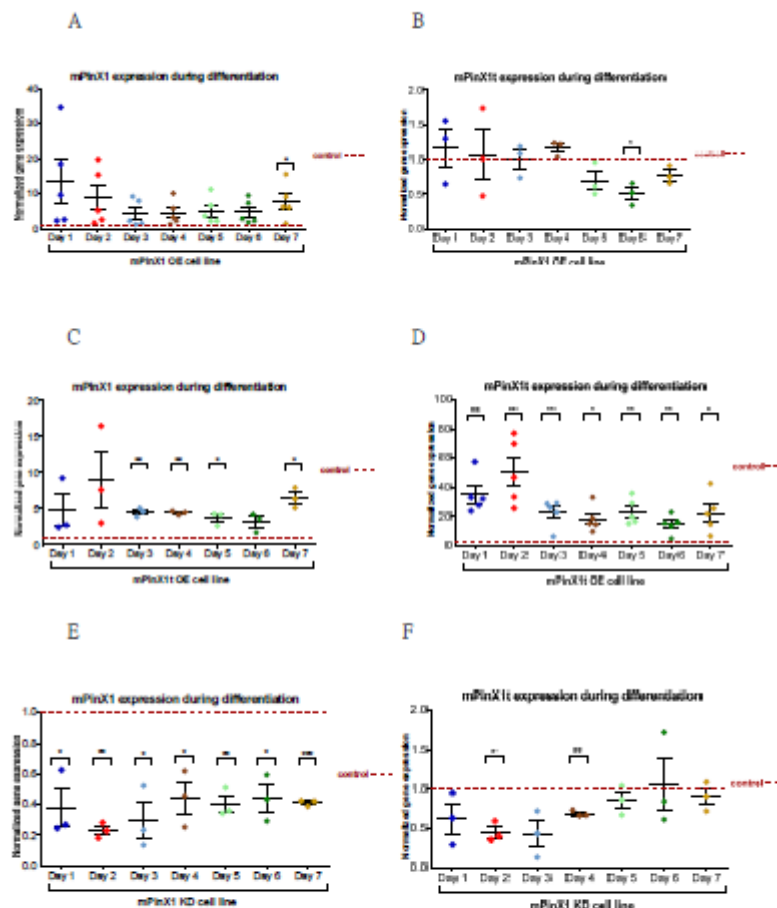
A) mPinX1 expression profile at different differentiation time points in mPinX1 overexpression cell line. mPinX1 was up-regulated at different time points. Red dotted line indicated the normalized expression level in control cell line. Data were

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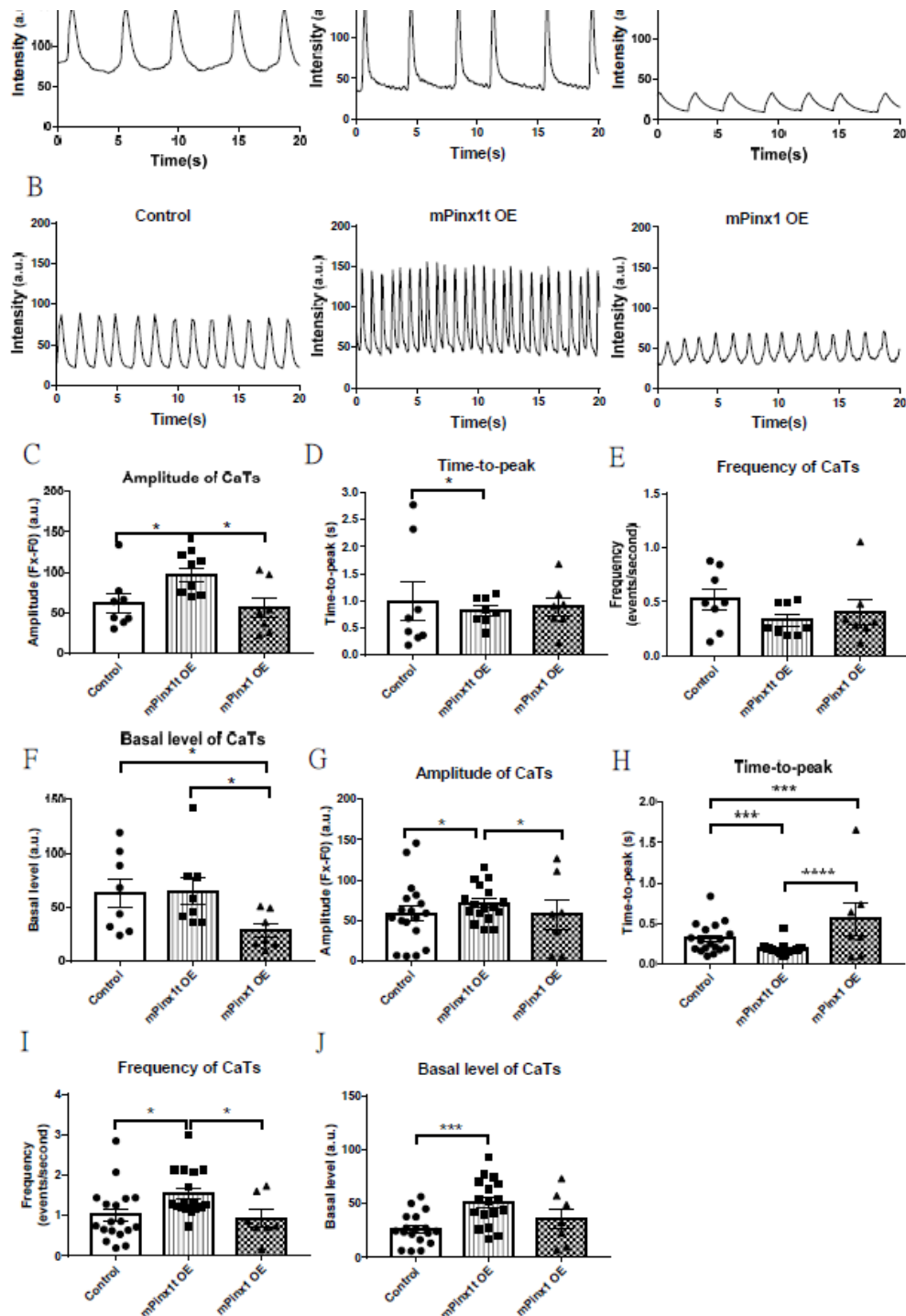
A) - B) Representative fluorescence intensity plots of global CaTs of mESC-CMs in different experimental groups at (A) early and (B) late

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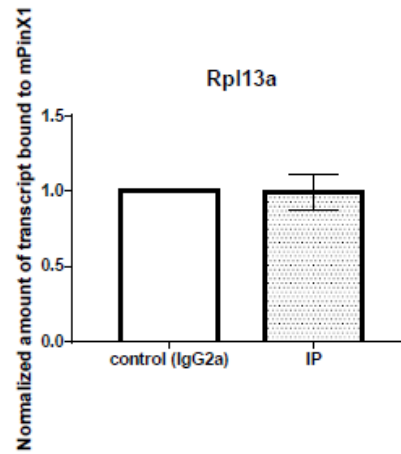
B
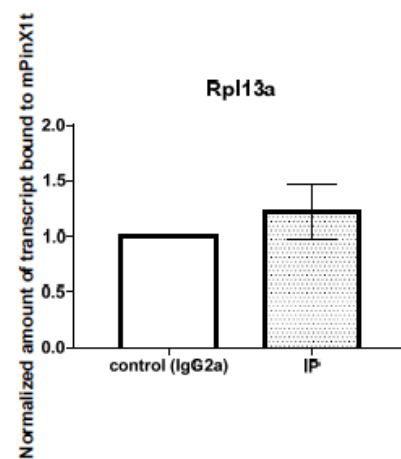
A) – B) RNA immunoprecipitation of HEK293FT cells overexpressed with (A) myc-mPinX1 or (B) myc-mPinX1t. Anti-myc was used to perform the immunoprecipitation. IgG2a was used in isotype control experiment. The presence of Rpl13a mRNA in the immunoprecipitant was quantitated by subsequent qPCR. Both

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.
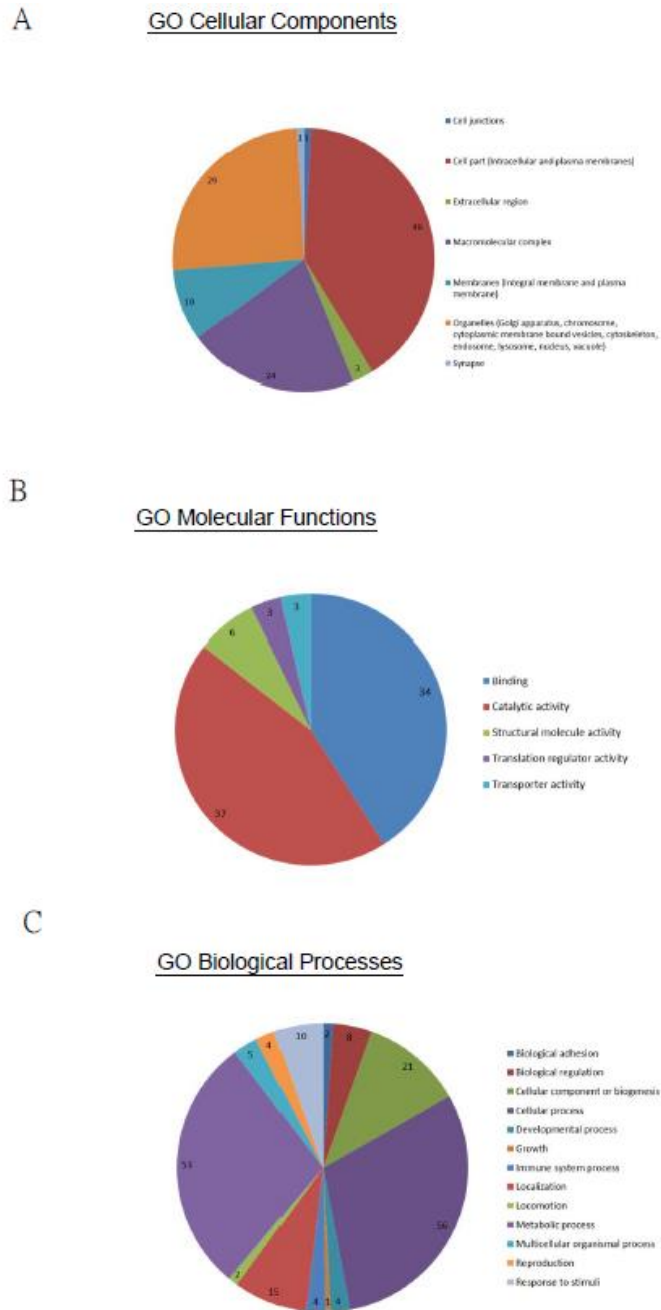
A) GO Cellular Components

B) GO Molecular Functions

C) GO Biological Processes