Supplementary Online Materials

Materials and Methods

Plasmid constructs

The tandem affinity purification (TAP) tag was excised from pRC/CMV by digesting with Apa1 and BamH1.[1, 2] The PGK destination vector was digested with Xho1 and BamH1.[3, 4] The Apa1 and Xho1 sites were blunted and the two fragments were ligated to generate PGK-TAP. The Nkx2.5 sequence was excised by digesting with BamH1 and Not1 and ligated into PGK-TAP to generate PGK-Nkx2.5TAP, where the TAP tag was fused to the N-terminus of Nkx2.5. Flag-Nkx2.5, HA-PP1, myc-Mypt1 and myc-NIPP1 have been previously described.[5-7] PGK-Puro and B17 have also been previously described.[8, 9] Mypt1 mutants were generated using the QuikChangeII-XL kit, as per the manufacturer's instructions (Agilent).

Cell Culture and transfections

P19 cells (ATCC) were cultured and stable clones were generated as previously described.[10] P19 cells were transfected using Fugene (Roche), as per the manufacturer's instructions. Cells were treated with150 ng/ml Dkk1 (R&D Systems), 10 nmol/L LeptomycinB (Sigma-Aldrich) or 10 μmol/L y-27632 (EMD Chemicals) 3-5 hours after transfection and fixed for immunofluorescence one day later. For analysis of pMypt1Thr853 levels, total protein was harvested from P19 cells treated for 24 hours with Dkk1 or y-27632 and analyzed by western blot with antibodies specific to pMypt1Thr853 (Santa Cruz Biotechnology), total Mypt1 (Cell Signalling) and alpha-tubulin (Sigma-Aldrich). P19 cells were differentiated as previously described.[10] Briefly, cells were aggregated in non-adherent plates in the presence of 0.8% Dimethyl sulfoxide for 4 days. Aggregates were subsequently transferred to adherent plates for a further two days until Day 6. D3 mESCs (ATCC) were maintained as previously described.[11] Differentiation was induced by LIF withdrawal and cell aggregation using the hanging drop method (800cells/drop). Cells were aggregated in hanging drops for two days then transferred to non-adherent plates for three days. On day 5, aggregates were transferred to adherent plates for a further 2 days. Cells were treated with 200ng/ml Wnt3a (R&D Systems) or vehicle (0.1% BSA in PBS) from day 4-7. HEK-293 cells were maintained in α-MEM supplemented with 10% FBS and penicillin-streptomycin (100U/ml and 100ug/ml).

Protein Purification

P19[TAP] and P19[Nkx2.5TAP] cells were differentiated and protein was harvested for purification on Day 6. Cells were harvested in extraction buffer containing 20 mmol/L Hepes pH 7.4, 0.1% Tween20, 2mmol/L MgCl2 and 200 mmol/L NaCl, supplemented with Complete EDTA Free Protease Inhibitors (Roche), 1% aprotinin, and 0.1 mmol/L phenylmethylsulfonyl fluoride. Lysates were sonicated 4 times for 4 second bursts and centrifuged for 30 minutes at 55000rpm in a Beckman TLA120.2 rotor. 10mg of total protein was immunoprecipitated overnight at 4°C with rabbit IgG (Chemicon) coupled to magnetic Dynabeads, Tosylactivated as per the manufacturer's instructions (Life technologies). Beads were washed three times with extraction buffer and immunoprecipitated protein was eluted by heating in 25μl of protein sample buffer without beta-mercaptoethanol at 65°C for 10 minutes. Beads were collected

with a magnet and the eluate was removed, supplemented with 0.5 µl of 14.4mol/L beta-mercaptoethanol and boiled for 5 minutes for subsequent analysis by SDS-PAGE.

Mass Spectrometry analysis

Gel sections encompassing the whole sample lane were excised, reduced, alkylated, and digested as described previously.[12] The resulting peptide solutions were dried in a SpeedVac. LC-MS/MS was performed by dissolving the peptide samples in 5% formic acid and loading them into a 200µm x 5-cm precolumn packed in house with 5µm ReproSil-Pur C18-AQ beads (Dr. Maisch HPLC GmbH) using a micro Agilent 1100 HPLC system (Agilent Technologies). The peptides were desalted online with 95% water, 5% acetonitrile, 0.1% formic acid (v/v) for 10 min at 10 µl/min. The flow rate was then split before the precolumn to produce a flow rate of ~200 nl/min at the column. Following precolumn elution, the peptides were directed to a 75µm x 5cm analytical column packed with 5µm ReproSil-Pur C18-AQ beads. The peptides were eluted using a one hour gradient (5–80% acetonitrile with 0.1% formic acid) into an LTQ linear ion trap mass spectrometer (Thermo-Electron). MS/MS spectra were acquired in a data-dependant acquisition mode that automatically selected and fragmented the five most intense peaks from each MS spectrum generated. Peak lists were generated from the MS/MS .raw file using Mascot Distiller 2.0.0.0 (Matrix Science) to produce a .mgf file with default parameters except that for each MS/MS individual peak lists were generated assuming a +2 and a +3 charge. All .mgf files were analyzed and matched to the 56729 Mus musculus protein sequences in the International Protein Index (IPI) database (version 3.68) using the Mascot 2.1.04 database search engine (Matrix Science) with trypsin as the digestion enzyme, carbamidomethylation of cysteine as a fixed modification, and methionine oxidation as a variable modification. Peptide and MS/MS mass tolerances were set at +/- 3 and +/- 0.8 Da, respectively, with one miss-cleavage allowed and the significance threshold set to 0.01 ($p > 0.01$). Finally, an ion score cutoff of 30 was chosen to produce a false-positive rate of less than 1% in the MS data.[13] A protein hit required at least two "bold red peptides," i.e. the most logical assignment of the peptide in the database selected. Furthermore, when peptides matched to more than one database entry, only the highest scoring protein was considered

Co-immunoprecipitations

HEK 293 cells were transiently transfected with Flag-Nkx2.5, HA-PP1 and myc-Mypt1. Total protein was harvested using RIPA buffer supplemented with Complete-EDTA free protease inhibitors (Roche). Lysates were pre-cleared and immunoprecipitations were performed with anti-Flag M2 agarose (Sigma-Aldrich), anti-HA (Santa Cruz Biotechnology), anti-myc (Santa Cruz Biotechnology) or mouse IgG. Reactions were incubated overnight and antibody-antigen complexes were captured with Protein-A/G agarose beads (Life Technologies). Beads were washed with RIPA buffer and immunoprecipitated protein was eluted by boiling in sample buffer.

For endogenous Nkx2.5 immunoprecipitations, mESCs were differentiated for 10 days and protein was harvested in 50mmol/LTris pH7.5, 150mmol/L NaCl, 1mmol/L EDTA, 1% TritonX-100, 0.1% Sodium Deoxycholate, 2mmol/Lβ-glycerophosphate and 2mmol/L $MgCl_2$ supplemented with Complete- EDTA free Protease Inhibitors (Roche), Phospho-Stop (Roche) and 1mmol/L phenylmethanesulfonylfluoride. Lysates were further disrupted using a syringe then incubated on ice for 20 minutes and subsequently centrifuged at 14000rpm for 15 minutes. Lysates were precleared for two hours with Dynabeads Protein A (Life Technologies). Immunoprecipitations were performed using 750ug of protein and 5ug of anti-

Nkx2.5 antibody (Santa Cruz Biotechnology, H-114) or 5ug of normal rabbit IgG (Santa Cruz Biotechnology). Immunoprecipitations were supplemented with 90U/ml Benzonase and incubated overnight with rotation at 4°C. Antibody complexes were captured for 2 hours at 4°C with 50ul of Dynabeads Protein A that had previously been blocked overnight with 2mg/ml BSA. After capture, beads were washed twice for 10 minutes at 4°C with lysis buffer and immunoprecipitated proteins were subsequently eluted from the beads by incubation with 100mmol/L Glycine HCl pH3.5 at room temperature for 10 minutes with shaking. The pH and salt concentrations of the samples were adjused by adding 1/10 volume of buffer containing 500mmol/L Tris pH 7.5 and 1.5mol/L NaCl prior to loading on the gel for western blot analysis.

Luciferase assays

P19 cells were transiently transfected with Flag-Nkx2.5, HA-PP1 and myc-Mypt1or myc-NIPP1 along with ANF-luciferase[14] and SV40-Renilla as an internal reference control. Transfected cells were harvested after 24 hours and luciferase assays were conducted as per the manufacturer's instructions (Promega). Samples were analyzed on an LMax II 384 luminometer (Molecular Devices). Statistical significance was assessed using ANOVA followed by a Dunnett's post-hoc test.

Immunofluorescence

P19 cells were transfected with Flag-Nkx2.5 or GATA4 along with HA-PP1 and myc-Mypt1 or myc-NIPP1. Cells were fixed one day after transfection with ice-cold acetone, allowed to air dry, then rehydrated with PBS. Cells were blocked for 1 hour in PBS containing 10% donkey serum, 0.1% BSA and 0.1% Triton X-100. Cells were labelled with antibodies against Nkx2.5 or GATA4 (Santa Cruz Biotechnology) and myc (9E10 Hybridoma) or Nkx2.5 (Abcam) and p-Mypt1T853 (Santa Cruz Biotechnology). D3 mESCs were processed in the same way and labelled with antibodies against Nkx2.5 (Santa Cruz Biotechnology) and Mlc2v (Synaptic Systems) or pMypt1T853 (Millipore). Donkey-anti goat Alexa 488 (Life Technologies), donkey-anti-mouse Cy3 and Donkey-anti-rabbit Cy3 (Jackson ImmunoResearch) were used for detection as appropriate. All images were captured using a Zeiss LSM 510 microscope or a Zeiss LSM 710 Multiphoton microscope and were processed in Zen (Zeiss) and Canvas 11 softwares.

Quantification of Nkx2.5 localization was performed by capturing images of 10 independent fields per treatment in three biologically independent experiments (average of ~140 cells/treatment) and assessing the localization of Nkx2.5 in transfected cells. For co-transfection experiments, only $Nkx2.5^{+}/myc^{+}$ cells were quantified.

Quantification of Cy3-labelled Mlc2v or MF20-positive cells was performed with a Leica Microsystems DMI6000B microscope and Volocity (PerkinElmer Inc.) imaging software. High throughput quantification of cardiomyocyte cell number was performed by dividing the total surface area measured for Cy3 by the size of a single Mlc2v- or MF20-positive cell (μm^2). Quantification of total cell number was performed by dividing the total surface area measured for Hoechst signal by the size of a single nucleus (μm^2). Using the Volocity software, we determined the average size of a single nucleus (150 μm^2) or a single cardiomyocyte (415 μm^2) by measuring a minimum

of 100 Hoechst-stained nuclei or Cy-3-labeled, Mlc2v-positive cells, respectively. Between 8 and 20 random fields were captured for 4 biologically independent experiments (n=4). Using this method we counted an average of 7192 nuclei for the vehicle treatment and 4206 for the Wnt3a treatment. The total number of cardiomyocytes was divided by the total number of nuclei, and expressed as a percentage.

Gene Expression Analysis

Gene expression analysis was performed as described previously.[16] QPCR reactions were performed and analyzed on the Eppendorf Realplex2 using the Realplex software for analysis. β-actin was used as a reference for all QPCR reactions and expression changes were quantified relative to Day 0 levels. Statistical analysis was performed using Student's T Test.

Generation of shRNAmir knockdown cell lines

Expression Arrest GIPZ Lentiviral shRNAmir particles were obtained from Open Biosystems/Thermo Scientific. Source clones were designated as follows: Mypt1 V2LHS_152151 (catalogue number VGH5523-98817828, referred to herein as clone 152), Mypt1 V3LHS_324346 (catalogue number VGH5523-101516075, referred to herein as clone 346) and Mypt1 V3LHS_324348 (catalogue number VGH5523-101516681, referred to herein as clones 348). The non-target negative control clone was designated GV181119.

100 000 mESCs were infected in a 60mm tissue culture dish with each of the 4 shRNAmir lentiviruses at an MOI of 20. Infections were carried out as per the manufacturer's protocol with the exception that Polybrene was included in the infection medium at a final concentration of 6μg/mL. 48 hours after infection, cells were treated with and maintained in 2μg/mL puromycin. Cells that survived selection were pooled to generate the 4 stable cell lines. These cell lines were maintained as regular mESCs with the addition of puromycin to the medium. Cells were differentiated as described above.

1. Canton DA, Olsten ME, Kim K, Doherty-Kirby A, Lajoie G, Cooper JA, Litchfield DW. The pleckstrin homology domain-containing protein CKIP-1 is involved in regulation of cell morphology and the actin cytoskeleton and interaction with actin capping protein. *Mol Cell Biol.* 2005;25(9):3519-3534.

2. Yoon HJ, Feoktistova A, Wolfe BA, Jennings JL, Link AJ, Gould KL. Proteomics analysis identifies new components of the fission and budding yeast anaphase-promoting complexes. *Curr Biol.* 2002;12(23):2048-2054.

3. Adra CN, Boer PH, McBurney MW. Cloning and expression of the mouse pgk-1 gene and the nucleotide sequence of its promoter. *Gene.* 1987;60(1):65-74.

4. Skerjanc IS, Petropoulos H, Ridgeway AG, Wilton S. Myocyte enhancer factor 2C and Nkx2-5 up-regulate each other's expression and initiate cardiomyogenesis in P19 cells. *J Biol Chem.* 1998;273(52):34904-34910.

5. Eto M, Kirkbride JA, Brautigan DL. Assembly of MYPT1 with protein phosphatase-1 in fibroblasts redirects localization and reorganizes the actin cytoskeleton. *Cell Motil Cytoskeleton.* 2005;62(2):100-109.

6. Vincentz JW, Barnes RM, Firulli BA, Conway SJ, Firulli AB. Cooperative interaction of Nkx2.5 and Mef2c transcription factors during heart development. *Dev Dyn.* 2008;237(12):3809-3819.

7. Trinkle-Mulcahy L, Ajuh P, Prescott A, Claverie-Martin F, Cohen S, Lamond AI, Cohen P. Nuclear organisation of NIPP1, a regulatory subunit of protein phosphatase 1 that associates with pre-mRNA splicing factors. *J Cell Sci.* 1999;112 (Pt 2):157-168.

8. McBurney MW, Fournier S, Jardine K, Sutherland L. Intragenic regions of the murine Pgk-1 locus enhance integration of transfected DNAs into genomes of embryonal carcinoma cells. *Somat Cell Mol Genet.* 1994;20(6):515-528.

9. Skerjanc IS, Slack RS, McBurney MW. Cellular aggregation enhances MyoD-directed skeletal myogenesis in embryonal carcinoma cells. *Mol Cell Biol.* 1994;14(12):8451-8459.

10. Jamali M, Rogerson PJ, Wilton S, Skerjanc IS. Nkx2-5 activity is essential for cardiomyogenesis. *J Biol Chem.* 2001;276(45):42252-42258.

11. Kennedy KA, Porter T, Mehta V, Ryan SD, Price F, Peshdary V, Karamboulas C, Savage J, Drysdale TA, Li SC, Bennett SA, Skerjanc IS. Retinoic acid enhances skeletal muscle progenitor formation and bypasses inhibition by bone morphogenetic protein 4 but not dominant negative beta-catenin. *BMC Biol.* 2009;7:67.

12. Lambert JP, Mitchell L, Rudner A, Baetz K, Figeys D. A novel proteomics approach for the discovery of chromatin-associated protein networks. *Mol Cell Proteomics.* 2009;8(4):870-882.

13. Elias JE, Haas W, Faherty BK, Gygi SP. Comparative evaluation of mass spectrometry platforms used in large-scale proteomics investigations. *Nat Methods.* 2005;2(9):667-675.

14. Fan C, Liu M, Wang Q. Functional analysis of TBX5 missense mutations associated with Holt-Oram syndrome. *J Biol Chem.* 2003;278(10):8780-8785.

15. Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, Landegren U. Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods.* 2006;3(12):995-1000.

16. Savage J, Conley AJ, Blais A, Skerjanc IS. SOX15 and SOX7 differentially regulate the myogenic program in P19 cells. *Stem Cells.* 2009;27(6):1231-1243.

Online Table I. Mass spectrometry results of Nkx2.5 affinity purification

Entrez Gene ID	Protein Name	Mascot Score	% Coverage (total # of peptides)
18091	Nkx2-5	273	14% (5)
19046	Ppp1cb	804	38% (10)
17931	Ppp1r12a (Mypt1)	289	6% (5)