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SUPPLEMENTARY MATERIAL

corresponding to:

Expression patterns and immunohistochemical localization of PITX2B transcription factor in the developing mouse heart

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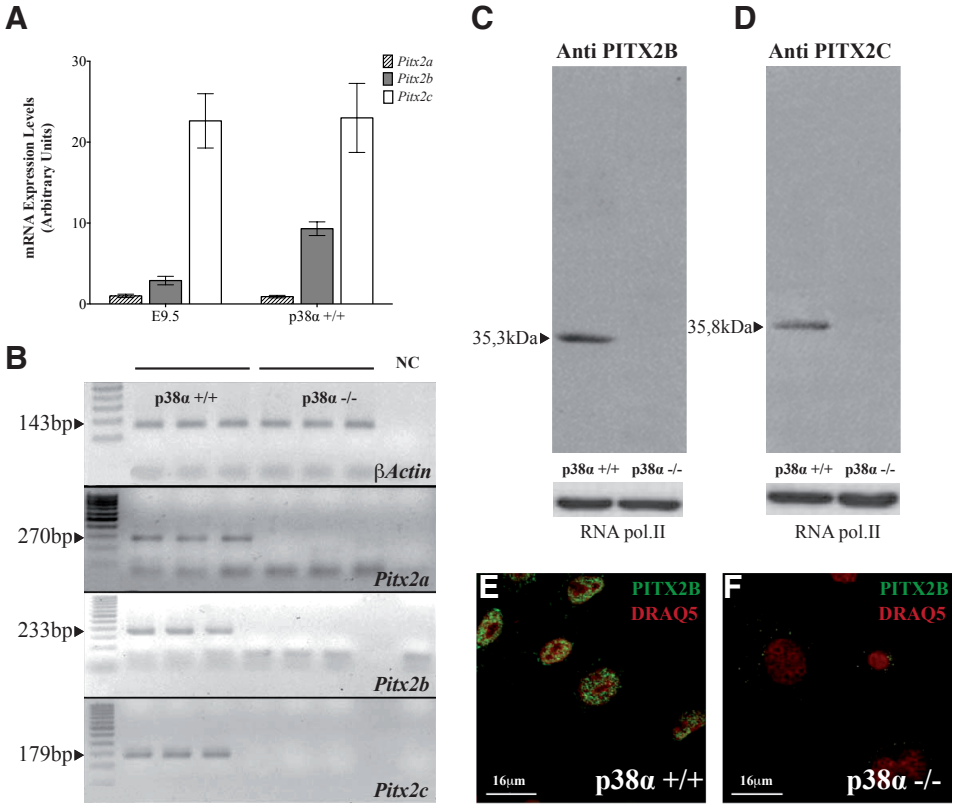

Supplementary Fig. S1. Experimental validation of the specific antibodies antiPITX2B. (A) *Quantitative RT-PCR experiments show that* Pitx2a, Pitx2b, *and* Pitx2c *mRNA expression levels are similar in mouse heart at stage E9.5 and in immortalized cardiomyocytes isolated from mice at stage E9.5 (p38α+/+). Data are expressed as ratio of each* Pitx2 *isoform mRNA to β-*actin *mRNA* **(B)** *RT-PCR experiments show that* Pitx2a, Pitx2b *and* Pitx2c *mRNA expression is depleted in immortalized cardiomyocites that do not express MAP-Kinase p38α protein (p38α-/-). β-*actin *mRNA is used as internal control for retrotranscription.* **(C,D)** *Western blot analysis of nuclear extract proteins from E9.5 wild-type (p38α +/+) and p38α -/- embryonic cardiomyocytes. PITX2B (35.3 kDa) and PITX2C (35,8 kDa) proteins are detected in wild-type but not in p38α -/- embryonic cardiomyocytes by using the antiPITX2B and antiPITX2C antibodies* **(E,F).** *Immunocytofluorescence experiments by using the antiPITX2B antibodies showed a clear nuclear stain in wild type (p38α+/+), but not in (p38α-/-) cells.*

Supplementary Materials and Methods

Cell lines and culture conditions

p38α -/- and wild-type (p38α +/+) embryonic cardiomyocytes were isolated from E 9.5 embryos obtained by intercrossing mice heterozygous for p38α and those carrying large T antigen under the control of a temperature interferon-γ (IFN-γ)-inducible H-2K promoter (immorto transgene) (Jat *et al.*, 1991). Cardiomyocytes were isolated as previously described (Adams *et al.*, 2000) and were grown on collagen-coated tissue-culture plates in DMEM containing 10% foetal bovine serum (Invitrogen), IFN-γ (10 U/ml; SIG- MA), and cardiotrophin-1 (0.2 ng/ml; R&D Systems, Minneapolis, MN, USA) at 33°C in a humidified atmosphere of 5% CO2.

Western blot

For western-blot analysis, nuclear extract proteins were isolated (Nuclear Extract Kit – Active Motif), separated by SDS–PAGE and transferred onto nitrocellulose membranes. Then, antiPITX2B antibodies were probed 1:100. Membranes were washed four times, 5 min each time, with 2% dry milk in TBST. After incubating with an antirabbit IgG-horse- radish peroxidase-conjugated secondary antibody (diluted 1:10.000) at room temperature for 1 h, and washing four times, 5 min each time, with TBST, the blots were visualized by an ECL kit following the manufacturer's instructions. To normalize a monoclonal anti-RNApol.II antibody (8wg16, sc-56767; Santa Cruz) was used.

Immunocytochemistry

Cells were washed in PBS (0.01 M, pH 7.4) twice for 5 min, fixed in a 4% paraformaldehyde fix solution in PBS for 20 min at room temperature and hydrated through graded ethanol steps. Cells were briefly rinsed in PBS and unspecific bindings were blocked using TBSA-BSAT (10 mM Tris, 0.9% NaCl, 0.02% sodium azide, 2% bovine serum albumin and 0.1% Triton X-100 detergent) at room temperature. Rehydrated cells were incubated overnight at room temperature with antiPITX2B antibodies diluted in TBSA-BSAT (1:50). Thereafter cells were washed with PBS and subsequently incubated with antirabbit Cy3 secondary antibodies (Jackson Labs, USA) diluted in TBSA-BSAT (1:100) for 5 h. Nuclear staining was performed using DRAQ-5 (Red Fluorescence Cell-Permeable DNA Probe, Biostatus Limited, United Kingdom). Immunofluorescence analysis was performed by confocal laser microscopy with a Leica TCS SL microscope (Leica LCS Version 2.0).

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