CORRIGENDUM

Prox1 maintains muscle structure and growth in the developing heart

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The authors apologise to readers for this mistake.

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Prox1 maintains muscle structure and growth in the developing heart

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Impaired cardiac muscle growth and aberrant myocyte arrangement underlie congenital heart disease and cardiomyopathy. We show that cardiac-specific inactivation of the murine homeobox transcription factor Prox1 results in the disruption of expression and localisation of sarcomeric proteins, gross myofibril disarray and growth-retarded hearts. Furthermore, we demonstrate that Prox1 is required for direct transcriptional regulation of the genes encoding the structural proteins α-actinin, N-RAP and zyxin, which collectively function to maintain an actin–α-actinin interaction as the fundamental association of the sarcomere. Aspects of abnormal heart development and the manifestation of a subset of muscular-based disease have previously been attributed to mutations in key structural proteins. Our study reveals an essential requirement for direct transcriptional regulation of sarcomere integrity, in the context of enabling foetal cardiomyocyte hypertrophy, maintenance of contractile function and progression towards inherited or acquired myopathic disease.

KEY WORDS: Prox1, Mouse, Heart development, Myocardium, Sarcomere, Hypertrophy, Myopathy, N-RAP (Nrap), Zyxin

INTRODUCTION

Cardiac muscle integrity and function is dependent upon the maintenance of a rhythmically contracting meshwork of myofibrils, the basic structural and functional unit of which is known as the sarcomere. Sarcomeres are multi-protein complexes comprising interlacing myosin (thick) and actin (thin) filaments bordered by Z-discs. Several proteins important for the stability of sarcomeric structure are found in the Z-disc, which not only has a structural role for cross-linking thin filaments and transmitting contractile force, but also provides a vital interface for signal transduction and biomechanical sensing (Frank et al., 2006; Pyle and Solaro, 2004).

There is an absolute requirement for cardiac function during embryogenesis in mammals and as such the sarcomeric components are expressed very early in development and are correctly localised in the myofibrils by the time the linear heart tube begins to contract (Ehler et al., 1999). As development progresses, the heart increases in mass not only by cardiomyocyte hyperplasia, but also through a recently recognised foetal phase of physiological hypertrophy. These results, therefore, provide important insight into the molecular mechanisms that govern the ultrastructure and growth of cardiac muscle during development and highlight how transcriptional misregulation of myofibril assembly may underlie cardiac hypotrophy and myopathic disease.

Despite insight into the morphogenetic events that accompany myofibrillogenesis and hypertrophic growth, very little is known about the precise regulation of these processes during development. Furthermore, the importance of appropriate assembly and maintenance of the myofibrillar apparatus is underscored by the fact that defects in the terminal differentiation and arrangement of contractile protein filaments are associated with a number of cardiac myopathies (Engel, 1999; Gregorio and Antin, 2000; Seidman and Seidman, 2001).

The homeobox transcription factor Prox1 is essential for murine lymphatic, hepatocyte, retinal and pancreatic development (Dyer et al., 2003; Harvey et al., 2005; Sosa-Pineda et al., 2000; Wigle and Oliver, 1999). Multiple lines of evidence suggest a role for Prox1 during cardiac morphogenesis. Prox1 is expressed in the developing heart (Oliver et al., 1993; Rodriguez-Niedenfuhr et al., 2001; Tomarev et al., 1996; Wigle and Oliver, 1999) and embryos deficient in Prox1 die at –E14.5, a critical time point when lethality often results from grossly reduced cardiac performance (Dyson et al., 1995). Moreover, in a specific genetic background, a proportion of Prox1-heterozygote mice fail to survive and become cyanotic soon after birth (Harvey et al., 2005), a phenotype that is consistent with impaired blood circulation and heart defects.

Here we reveal how Prox1 functions transcriptionally upstream of sarcomere assembly, myofibril organisation and foetal cardiomyocyte growth. We provide evidence that Prox1 activity is required for the normal expression and localisation of multiple sarcomeric components and that it directly regulates the genes encoding α-actinin, N-RAP (nebulin-related anchoring protein, Nrap) and zyxin, which are essential structural proteins required for stabilising actin within the thin filaments and ultimately for establishing cardiomyocyte elongation and coordinated muscle contraction. These results, therefore, provide important insight into the molecular mechanisms that govern the ultrastructure and growth of cardiac muscle during development and highlight how transcriptional misregulation of myofibril assembly may underlie cardiac hypotrophy and myopathic disease.

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MATERIALS AND METHODS

Mouse strains and histology

The mouse strains used have been described previously: Prox1-null (Wigle et al., 1999), Prox1loxP (Harvey et al., 2005), Nkx2.5CreKI (Moses et al., 2001) and MLC2vCreKI (Chen et al., 1998). Prox1loxPloxP mice were crossed with Nkx2.5CreKI;Prox1loxP or MLC2vCreKI;Prox1loxP to generate Nkx2.5CreKI;Prox1loxPloxP (Prox1Nkx) or MLC2vCreKI;Prox1loxPloxP (Prox1MLC) embryos, respectively. Embryos and hearts were dissected in PBS, fixed in 4% paraformaldehyde (PFA), dehydrated, embedded in paraffin and sectioned at 15 μm. Sections were stained with Haematoxylin and Eosin.

Immunohistochemistry and TUNEL assays

Embryos were treated as above and sectioned at 10 μm. Immunohistochemistry was performed using anti-Prox1 (Reliatech) and anti-phosphohistone H3 (Upstate) antibodies, developed using a standard streptavidin-HRP method and counterstained with Haematoxylin. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling (TUNEL) assays were performed according to the manufacturer’s protocols (Promega).

Immunofluorescence and confocal microscopy

E10.5-14.5 whole-mount hearts were dissected in PBS and fixed overnight in 4% PFA. E18.5 hearts were dissected, embedded in paraffin and sectioned as above. Immunofluorescence and confocal microscopy were performed as described previously (Ehler et al., 1999). Samples were analysed on a Zeiss LSM 510 confocal microscope equipped with argon and helium neon lasers with a 63×1.4 lens. Image processing was performed using Zeiss software and Photoshop (Adobe). Cell outlines were traced on sections (blinded to genotype), using ImageJ (http://rsb.info.nih.gov/ij) to assist in assessing cell shape and calculating cell area as indicators of foetal hypertrophy.

Antibodies

The following antibodies were used: sarcomeric α-actinin (clone EA-53; Sigma), β-catenin, vinculin and smooth muscle α-actin (clone 1A4; Sigma), cardiac α-actin (Progen), titin (T12; gift of Prof. D. Fürst, Institut für Zellbiologie, Universität Bonn, Germany), desmin (clone D33; Dako), MHC (A4.1025; DSHB), cardiac MyBP-C (gift of Prof. T. Ohinata, Department of Biology, Chiba University, Japan), CD31 (Pecam1; BD Pharmingen), Prox1 (Reliatech) and Gapdh (Chemicon). For western blots, rabbit anti-sarcomeric α-actinin antibody was used (gift of Prof. D. Fürst).

Transmission electron microscopy

E13.5/E18.5 hearts were dissected and fixed in 3% glutaraldehyde (EM grade, Agar Scientific), 0.1 M sodium cacodylate, 5 mM CaCl₂ (pH 7.4). Hearts were processed in a Lynx automated tissue processor (Australian Biomedical) and embedded in resin. All sectioning was performed on a Reichert Ultracut S ultramicrotome. Sections were imaged using a Philips CM 10 transmission electron microscope and images collected using Kodak Megaview II and SIS Kineview digital imaging systems and SIS software. Four Prox1Nkx and four control hearts were examined for each stage.

Western blotting

E13.5 heart lysates were prepared in RIPA buffer. Western blotting was performed using standard methods. Scanning densitometry was performed and signal quantified using Scion Image (Scion Corporation) and ImageJ.

Quantitative real-time (qRT) PCR analysis

mRNA was isolated from E12.5 hearts using the Micro FastTrack 2.0 Kit (Invitrogen) according to the manufacturer’s instructions. Reverse transcription was performed using Superscript III reverse transcriptase (Invitrogen) according to the manufacturer’s instructions. qRT-PCR analysis was performed on an ABI 7000 Sequence Detector (Applied Biosystems) using SYBR Green (Quantitect SYBR Green PCR Kit, Qiagen). Data were normalised to Hprt1 expression and analysed using DART-PCR (Peirson et al., 2003). P-values were obtained using Student’s t-test (n=9). Primers for qRT-PCR (Table 1) were obtained from Primer Bank (http://pga.mgh.harvard.edu/primerbank) or designed using Primer Express (version 2.0, Applied Biosystems).

RNA in situ hybridisation on embryonic sections

E13.5 embryos were fixed in 4% PFA, embedded in paraffin and sectioned at 15 μm. RNA in situ hybridisation on sections was performed as previously described (Moorman et al., 2001), using a digoxigenin-labelled antisense riboprobe specific for Nppa (Kuo et al., 1997).

ChIP-on-chip

E12.5 hearts were dissected in PBS containing 0.3% Triton X-100 and cross-linked for 3 hours at room temperature in 1.8% formaldehyde, homogenised in lysis buffer and sonicated. Sixty micrograms of chromatin lysate was used per immunoprecipitation with 10 μg anti-Prox1 antibody (Reliatech) in ChIP dilution buffer at 4°C overnight. A no-antibody ‘immunoprecipitation’ was performed as a control. Immune complexes were pulled down with Protein A/G beads, washed, resuspended in TE (10 mM Tris, 5 mM EDTA, pH 8.0), the cross-links reversed overnight at 65°C and the DNA purified. DNA (10 ng) was blunt-ended and unidirectional adapters were ligated overnight at 16°C. Adapter-ligated DNA was amplified by PCR. Experimental conditions, buffer composition, adapter sequences and PCR conditions are available on request. ChIP and no-antibody samples were checked by qRT-PCR for enrichment of a positive control, Fgrf3 (a previously identified in vitro target of Prox1) (Shin et al., 2006), and against a negative control, Cyp7a1 (Qin et al., 2004). Amplified DNA (7.5 μg) was fragmented and end labelled using the GeneChip WT Double-Stranded DNA Terminal Labelling Kit, hybridised to GeneChip Mouse Promoter 1.0R Arrays, and then stained using the GeneChip Hybridisation, Wash and Stain Kit (all Affymetrix). ChIP data were analysed

Table 1. Primer sequences (5′ to 3′) for qRT-PCR

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<th>Gene</th>
<th>Forward</th>
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<tr>
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PCR to confirm ChIP

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<tr>
<td>Zyx</td>
<td>CTCTAGGGCACAGCGCTG</td>
<td>AGATATGGAGAAGGGCAC</td>
<td>273</td>
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using CisGenome (v1.0. beta; http://www.biostat.jhsph.edu/~hji/cisgenome/ index.htm). Three independent ChIP and no-antibody control reactions were performed. Standard PCR to confirm ChIP of the enhancer elements was carried out using the primers listed in Table 1.

**EMSA**s
Electrophoretic mobility shift assays (EMSA) were carried out as described previously (Angelo et al., 2000). Briefly, in vitro translated Prox1, or nuclear extracts from mouse P19Cl6 cells (Hanaba-Ohtkubo, 1996) transfected with Flag-Prox1, were incubated with ³²P-labelled oligonucleotide in binding buffer at room temperature for 30 minutes. Overlapping 60 bp oligonucleotides, which spanned the entire Prox1-binding elements of Actn2, Nrap or Zyx, and a chick α-cardiac actin minimal promoter (Hill and Riley, 2004), either with or without pcDNA3-Prox1 (250 ng) and a β-actin–β-galactosidase (β-gal) expressing plasmid to normalise luciferase activity for transfection efficiency. Luciferase and β-gal activity were assayed 48 hours post-transfection as described (Hill and Riley, 2004).

**Results**
**Prox1 is expressed in the embryonic heart**
In the first instance we characterised Prox1 expression during murine embryonic heart development. Immunostaining revealed that Prox1 is expressed throughout the entire myocardium of the atra, ventricles and outflow tract from ~E10.5 (Fig. 1). At E12.5 and E14.5, Prox1 expression was notable in the interventricular septum and myocardium (Fig. 1B-E), but was excluded from the smooth muscle of the outflow tract (not shown). Furthermore, Prox1 was expressed in the outer endocardial layer of the atrioventricular endocardial cushion leaflets (Fig. 1F,G) and the mitral valve leaflets (Fig. 1F,H), but was absent from cushion mesenchyme (Fig. 1F,G). Confocal analysis confirmed the predicted nuclear localisation of Prox1 in atrial and ventricular myocytes (see Fig. S1A-D in the supplementary material).

**Heart development is abnormal in Prox1-null mice**
Mice that are homozygous null for Prox1 die between E14.5 and E15 (Wigle and Oliver, 1999). The cause of lethality in Prox1-null embryos has not been determined. To address whether loss of cardiac expression of Prox1 is a potential contributing factor, we initially examined surviving post-natal day 5 (P5) Prox1-heterozygous mice and observed, at a gross anatomical level, that the hearts were reduced in size (by an average of 30%) as compared with those of wild-type littermate controls (see Fig. S2A in the supplementary material). Histological analysis revealed a range of cardiac anomalies including hypoplastic ventricular walls, loss of muscle striation, a disorganised interventricular septum and abnormally persistent muscle surrounding the aorta (see Fig. S2B,D in the supplementary material). Analysis of Prox1-null embryos at E13.5 and E14.5 revealed that mutant hearts were significantly smaller (up to 50%) than those of wild-type littermates (see Fig. S2E,F in the supplementary material) and, consistent with the phenotype in the P5 heterozygotes, we observed myocardial disarray, including a disrupted interventricular septum (see Fig. S2G,H in the supplementary material).

**Prox1 is an essential regulator of heart development**
Conventional Prox1-null embryos have a lymphatic phenotype arising from defects in endothelial cell budding as early as E11.5 (Wigle and Oliver, 1999), which may adversely affect cardiovascular development. To confirm a primary role for Prox1 in the developing heart, we generated a cardiac-specific knockout of Prox1 by crossing a conditional homozygous floxed Prox1 strain (Prox1^loxP/loxP) (Harvey et al., 2005) with two cardiac-expressing Cre strains: Nkx2.5-CreKI (designated Prox1^KI), which directs expression of Cre recombinase throughout the majority of cardiomyocytes from E7.5 (Moses et al., 2001); and MLC2v-CreKI (designated Prox1^MLC^KI), in which Cre is expressed specifically in ventricular myocardium from E8.75 (Chen et al., 1998). The two Cre strains were employed, therefore, to target Prox1 globally throughout the entire myocardium and in a subpopulation of ventricular myocardium, thus acting as respective internal controls for a cardiomyocyte-specific loss of Prox1 function.

Prox1 protein levels in Prox1^KI and Prox1^MLC E13.5 hearts were analysed by western blot on whole heart lysates, followed by densitometry. This confirmed a ~3-fold reduction in Prox1 protein levels in the cardiac-specific knockout hearts as compared with littermate control hearts (Prox1^loxP or Cre allele only) (Fig. 2A).
Whole-mount and histological sections of Prox1\textsuperscript{Nkx} and Prox1\textsuperscript{MLC} embryos were examined between E10.5 and E18.5. No phenotype was evident between E10.5 and E12.5 (not shown), despite expression of Prox1 in the heart at these stages. At E13.5 and E14.5, the Prox1\textsuperscript{Nkx} - and Prox1\textsuperscript{MLC} -conditional mutants recapitulated the cardiac phenotype of the conventional knockout embryos described above. Prox1\textsuperscript{Nkx} embryos were slightly growth restricted (on average 5% smaller) compared with control littersmates, with cranial haemorrhaging. Both Prox1\textsuperscript{Nkx} and Prox1\textsuperscript{MLC} embryos had oedema of varying severity (Fig. 2B), indicative of inadequate cardiac function. The hearts of both Prox1-conditional mutants were up to 30% smaller than controls hearts (Fig. 2C-E). Furthermore, in Prox1\textsuperscript{Nkx} hearts, the right atrium was reproducibly expanded (up to 2-fold) and blood-filled, never appearing to empty appropriately, irrespective of fixation stage during the cardiac cycle, suggesting that there might be impaired blood flow through the heart (Fig. 2D). Moreover, in the Prox1\textsuperscript{MLC} hearts, there was reduced expansion of the left ventricle (25% reduction in chamber size) consistent with haemodynamic obstruction (Fig. 2E). Haematoxylin and Eosin-stained frontal sections through E13.5 and E14.5 hearts (Prox1\textsuperscript{Nkx}, Fig. 2F-K; Prox1\textsuperscript{MLC}, not shown) revealed small thin-walled ventricles and disrupted myocardium with a highly disorganised interventricular septum (Fig. 2H,I). Additionally, membranous ventricular septal defects (VSDs) were observed in Prox1\textsuperscript{MLC} embryos (Fig. 2J,K) that were unlikely to be due to myocardial disruption, but possibly related to additional endocardial cushion defects (not shown). By E18.5, the overall area of ventricular myocardium was significantly smaller in Prox1\textsuperscript{Nkx} than control hearts, in particular of the right ventricle. Moreover, the myocardium was disorganised and less compacted (reduced by up to 50%) and the surface of the ventricles was irregular, although both compact and trabecular layers did appear to form (Fig. 2L-N). Consistent with the predominantly right-sided defects in Prox1\textsuperscript{Nkx} embryos, the pulmonary trunk was reduced in diameter, often being half the size of the aorta, suggesting impaired outflow tract remodelling. At E18.5, the membranous VSD was still evident in Prox1\textsuperscript{Nkx} embryos, and we also observed muscular VSDs (Fig. 2N) that were likely to result from the reduced level of myocardial compaction in the ventricles.

We observed variable and incomplete levels of Prox1 deletion in the developing heart (Fig. 2A), which may be attributed to the mosaic nature of Cre expression as previously described for the Nkx2.5\textsuperscript{CreKI} and MLC\textsuperscript{CreKI} strains (Smart et al., 2007). Analysis of both Prox1\textsuperscript{Nkx} and Prox1\textsuperscript{MLC} mutant hearts confirmed specificity of phenotype at the level of the myocardium, as supported by immunostaining for markers of vascular endothelial and smooth muscle cells (Pecam1 and smooth muscle \(\alpha\)-actin, respectively) that revealed normal coronary vessel development (see Fig. S3A-D in the supplementary material). Owing to the similarities in myocardial phenotype observed in both models, the term Prox1-conditional will be used to describe both the Prox1\textsuperscript{Nkx} and Prox1\textsuperscript{MLC} hearts unless specifically stated otherwise.

**Sarcomeric integrity is disrupted in Prox1-conditional hearts**

At a gross level, as determined by low-resolution histological analyses, loss of Prox1 function in the heart appeared to lead to myocardial disarray that was manifested in disorganisation of the...
myofibrils. To investigate this in more detail, the expression and localisation of sarcomeric markers was analysed by high-resolution confocal microscopy following immunostaining of whole-mount embryonic hearts. The relative distribution of the major components of the sarcomere is illustrated in Fig. S4A (see Fig. S4A in the supplementary material). In the first instance, colocalisation of Prox1 and selected sarcomeric markers (actin and sarcomeric α-actinin) was confirmed in E13.5 control hearts (see Fig. S1A-D in the supplementary material). Prox1-conditional E13.5 and E14.5 whole-mount hearts were stained with phalloidin to visualise F-actin and the arrangement of the thin filaments, and immunostained with antibodies to sarcomeric myosin heavy chain (MHC) and cardiac myosin binding protein C (MyBP-C) to visualise the thick filament architecture. Additionally, hearts were immunostained with antibodies to sarcomeric α-actinin, a crucial Z-disc protein that cross-links sarcomeric actin and is involved in many signalling transduction pathways, to myomesin, which localises to the M-band where it interacts with myosin and titin providing elasticity and stability to the sarcomere, and to β-catenin, which localises to the adherens junctions and demarcates cell-cell contacts (Fig. 3; see Fig.
S4A and Fig. S5A-F in the supplementary material). In Prox1-conditional hearts at E13.5, we consistently observed severe global disruption of thin filament, thick filament, Z-disc and M-band organisation in the ventricular myocardium as compared with littermate controls (Fig. 3A-I,L). Variation in the severity of disruption of actin filaments and α-actinin was evident (see Fig. S6 in the supplementary material), consistent with mosaicism in the level of Prox1 knockdown (Fig. 2A; a western analysis for Prox1 expression with scanning densitometry normalised to Gapdh). We classified overall phenotype severity based on the following three criteria as revealed by the confocal ultrastructure analysis: (1) cardiomyocyte cell shape (using ImageI, see Materials and methods), (2) sarcomeric striation and (3) myofibrillar organisation/cell alignment. Defects in one of these categories were classed as mild, of which we recorded 85.7% of Prox1-conditional mutants; defects in all three criteria were classed as severe, of which we recorded 14.3% of Prox1-conditional mutants to post-natal Prox1-conditional and control individual hearts were examined by quantitative real-time (qRT)-PCR or western blotting, respectively. qRT-PCR was carried out for genes encoding the sarcomere components that were shown to be mislocalised in Prox1-conditional myocardium. α-cardiac actin (Actc1), sarcomeric α-actinin (Actn2) and β-MHC (Myl7) were significantly downregulated and MyBP-C (Myhpc3) was significantly upregulated (Fig. 3O). Titin (Ttn) and desmin (Des) were consistently downregulated, but to varying levels, which led to the changes not being statistically significant (Fig. 3O). Nonetheless, these results confirm the extensive sarcomere dysgenesis in the Prox1-conditional mutant hearts both in terms of appropriate myofibril organisation and gene expression. Additionally, we analysed sarcomere component protein levels in E13.5 individual hearts. In accordance with the changes in gene expression, sarcomeric α-actinin and sarcomeric MHC were downregulated in Prox1-conditional hearts (Fig. 3P). Levels of desmin, β-catenin and vinculin were unchanged (not shown), which, alongside confocal immunofluorescence for β-catenin (see Fig. S5A-F in the supplementary material), confirmed that cell-cell contacts through adherens-type junctions were unaltered between neighbouring Prox1-conditional cardiomyocytes.

Subsequently, we examined the relationship between the level of Prox1 reduction and α-actinin expression further. Individual heart samples were analysed by both western blotting and immunostaining and we observed a direct correlation between the degree of Prox1 knockdown and the reduction in sarcomeric α-actinin levels (Fig. 3O,P). This suggests that Prox1 activity might regulate the expression of sarcomeric α-actinin and, moreover, that a modest yet significant reduction (0.7-fold) in α-actinin is sufficient to contribute to the myofibrillar disarray in the conditional mutant hearts (Fig. 3L).
Furthermore, this analysis demonstrated a definitive association between the level of Prox1 expression and the severity of the myocardial phenotype (Fig. 3O,P; see Fig. S6 in the supplementary material).

Sarcomere ultrastructure disruption was examined at higher resolution by transmission electron microscopy (TEM) in E13.5 and E18.5 Prox1\textsuperscript{Nkx} myocardium. At both developmental stages we observed variation in the severity of sarcomere ultrastructure defects in the Prox1\textsuperscript{Nkx} ventricular myocardium (Fig. 4A-F). Z-disc material was either entirely absent (Fig. 4B) or relatively dense but inappropriately organised (Fig. 4D). There was also an associated misalignment of the M-bands, with thick and thin filament disarray, suggesting that reduced Prox1 function impacts globally on sarcomeric organisation (Fig. 4D; see Discussion). Further examination of the cell-cell contacts, as a possible source of disruption to the muscle ultrastructure, revealed both intact adherens-type junctions (see Fig. S5G,H in the supplementary material), consistent with the \( \beta \)-catenin immunofluorescence data (see Fig. S5A-F in the supplementary material), and intact desmosomes (see Fig. S5I,J in the supplementary material) in the Prox1-conditional mutant hearts. Despite phenotypic variation, in the most severely affected cases we consistently observed a total lack of sarcomeric organisation at both E13.5 and E18.5 (Fig. 4E,F). Therefore, in combination, the confocal and TEM studies implicate Prox1 in organising components of the sarcomere and, furthermore, suggest that Prox1 is involved in maintaining myofibrillar ultrastructure throughout cardiogenesis.

**Prox1 is essential for physiological foetal hypertrophy**

We next investigated whether Prox1 plays a role in either the initial stages of myofibrillogenesis or in the maintenance of appropriate myofibrillar structure throughout later stages of cardiac development. To this end, we immunostained whole-mount Prox1-conditional hearts at E10.5, E11.5 and E12.5 for sarcomeric \( \alpha \)-actinin and phalloidin to pinpoint exactly when the myocardial defects first arise. In the Prox1\textsuperscript{Nkx} mutants, Prox1 levels were reduced from early cardiac crescent stages (E7.5) (Moses et al., 2001) onwards, and yet at E10.5 and E11.5 there was no sarcomeric disruption and the myofibrils appeared to have assembled correctly (Fig. 5A,D), whereas by E12.5 the first signs of myofibrillar disorganisation were observed (see Fig. S7A-D in the supplementary material). The fact that sarcomere defects do not manifest until E13.5 and then persist throughout the rest of development suggests that Prox1 is not required for the initiation of myofibrillogenesis but for the subsequent maintenance of appropriate sarcomeric structure and stability. In addition, we observed that in E13.5 Prox1-conditional hearts cardiomyocyte morphology and distribution were noticeably altered: the cardiomyocytes were more rounded in shape, disorganised and distributed in an apparently random pattern as compared with the control hearts, in which the cardiomyocytes had begun to elongate and align in parallel (Fig. 3A-N). Consistent with this, we observed a significant increase in \( \alpha \)-actinin expression in wild-type hearts between E9.5 and E12.5, indicative of a requirement for sarcomeric-dependent cell growth after the recruitment of cells and at the onset of physiological hypertrophy (see Fig. S7E in the supplementary material). When sections of E18.5 Prox1-conditional and control hearts were examined by phalloidin staining and immunostaining for sarcomeric \( \alpha \)-actinin we observed that a large proportion of cardiomyocytes were still rounded at E18.5, having failed to undergo hypertrophic growth and acquire the characteristic rod shape of mature cardiomyocytes (Fig. 5C,F).

![Fig. 5. Prox1 is required for foetal cardiomyocyte hypertrophy.](image)

(A-F) Phalloidin staining on E10.5 (A,D), E13.5 (B,E) and E18.5 (C,F) control (co) and Prox1\textsuperscript{Nkx} (D-F) whole-mount (A,B,D,E) and sections through (C,F) isolated mouse hearts. At E10.5, Prox1\textsuperscript{Nkx} cardiomyocytes are developing normally and the appropriate ultrastructure is laid down (A,D). From E13.5 onwards, Prox1\textsuperscript{Nkx} cardiomyocytes remain as small rounded cells that do not acquire the characteristic rod shape (arrowheads; E,F). (G-J) In situ hybridisation for Nppa transcripts on frontal sections of E13.5 control (G,I) and Prox1\textsuperscript{Nkx} (H,J) embryos. There is greatly reduced Nppa expression in Prox1\textsuperscript{Nkx} myocardium (H,J). lv, left ventricle; rv, right ventricle; ra, right atrium. (K) Nppa expression in Prox1\textsuperscript{Nkx} myocardium is confirmed by qRT-PCR on E12.5 isolated hearts. \( \beta \)-MHC (Myh7) was also found to be downregulated. (L) Morphometric analysis of cell shape (using ImageJ) confirmed a lack of increase in cell size because of impaired elongation and hypertrophic growth in Prox1\textsuperscript{Nkx} cardiomyocytes during development, excluding the possibility that the rounded cells simply reflect an alteration in cell shape. In K, \( \mu \text{m} \), mean ± s.e.m.; *P<0.001, **P<0.003, ***P<9×10^{-7} (K), ****P<7×10^{-8} (L), *****P<3×10^{-12}. (M) Foetal cardiomyocyte hypertrophic growth through normal development and in the absence of Prox1, where sarcomere striation is lost, myofibrils do not align and cardiomyocytes do not grow by hypertrophy. Green dotted lines, striated myofibrils; solid green lines, failed striation; blue ovals, nuclei. Scale bars: 10 \( \mu \text{m} \) in A-F; 50 \( \mu \text{m} \) in G,H; 20 \( \mu \text{m} \) in I,J.
Hypertrophy in adult hearts is induced by a large variety of stimuli, but the consistent end point is re-expression of a foetal gene programme, including Nppa (Anf), Myh7 (β-MHC) and Nppb (BNP) (Molkentin et al., 1998). During development, Nppa, Myh7 and Nppb are markers of myocardial differentiation and chamber expansion, but because differentiation per se is accompanied by a concomitant increase in cardiomyocyte cell growth, we examined the expression levels of Nppa and Myh7 as surrogate markers of foetal hypertrophy. qRT-PCR on E12.5 hearts and in situ hybridisation on E13.5 embryo sections revealed that both genes were significantly downregulated in ProxlNks hearts (Fig. 5G-K), not only indicating that was there markedly reduced foetal hypertrophic growth in the absence of Prox1, but also suggesting that Proxl-conditioned hearts are not hypoplastic, but hypertrophic. Morphometric measurements of cell size revealed that cardiomyocytes in ProxlNks hearts failed to enlarge during development, confirming a defect in hypertrophic growth as opposed to an alteration in cell shape (Fig. 5L). Immunostaining for phosphohistone H3, a marker of cells in mitosis, and TUNEL assays confirmed that there was no decrease in proliferation or increase in apoptosis between E10.5 and E13.5 (see Fig. S8 in the supplementary material). Therefore, in the absence of Prox1, although laid down appropriately in early heart development, the sarcomere structure is not maintained, resulting in myofibril disruption, loss of striation and a failure of cardiomyocyte hypertrophic growth and maturation (Fig. 5M).

Prox1 directly regulates fundamental components of the sarcomere

To gain specific insight into the molecular mechanism(s) by which Prox1 regulates myofibril organisation we sought to identify direct downstream targets of Prox1 in the developing heart. Until now, no in vivo target of Prox1 has been identified, nor has a definitive consensus Prox1 DNA-binding site been determined. Therefore, we combined chromatin immunoprecipitation (ChIP) against endogenous Prox1 with microarray analysis (ChIP-on-chip). ChIP-on-chip revealed putative Prox1-bound enhancer regions of genes encoding the Z-disc protein α-actinin (Actn2) and the myofibrillar and adherens junction proteins N-RAP (Nrap) and zyxin (Zyx), both of which directly interact with α-actinin in the Z-disc (Fig. 6A). ChIP of the three enhancer elements was confirmed by PCR (see Fig. S9A in the supplementary material). We analysed the Actn2, Nrap and Zyx target sequences for predicted transcription factor binding sites using MatInspector Professional (http://www.genomatix.de): 40-50 transcription factor binding sites were predicted per sequence but no putative Prox1 or Prospero (the Drosophila homologue of Prox1) binding sites were identified, although the Prox1 sites described to date are highly degenerate and predicted based on a Prospero consensus (Cook et al., 2003; Lengler et al., 2005; Shin et al., 2006). The lack of a core consensus motif within the three Prox1-bound enhancer elements identified for Actn2, Nrap and Zyx, despite the conservation of each element across species, is consistent with results obtained from unbiased screens of genome-wide conserved regulatory sequence variants (Pennacchio et al., 2006).

The ChIP data were subsequently validated by EMSAs with overlapping probes from each of the three enhancer regions and in vitro translated Prox1 or lysates from mouse P19C16 cells overexpressing Prox1 (see Fig. S9B-D in the supplementary material). Competition gel shifts with labelled probe (Fig. 6B) and antibody supershifts (Fig. 6C) of the refined oligonucleotide sequences (60 bp) confirmed specific Prox1 binding to the enhancers within Actn2, Nrap and Zyx. Prox1-induced transcription via all three enhancer elements was demonstrated by cotransfection and reporter gene activation assays (Actn2, 15-fold activation; Nrap, 32-fold; Zyx, 9-fold) (Fig. 6D). qRT-PCR for Nrap and Zyx (Fig. 6E) confirmed reduced expression of these factors in a Proxl-deficient background, as was previously determined for Actn2 (Fig. 3O).

DISCUSSION

The failure of Proxl-conditioned cardiomyocytes to grow and to maintain sarcomere organisation throughout development results in hypotrophic hearts that are insufficient to sustain life beyond birth. We observed disruption of the sarcomere at the level of both gene expression and protein localisation and have identified Proxl-bound enhancer elements for key sarcomere-associated genes, indicating that Prox1 regulates myofibrillar organisation both directly in terms of protein expression, and indirectly via intermediate factors that control sarcomere protein localisation and integration.

Prox1 deficiency impacts directly on sarcomeric components that facilitate Z-disc and thin filament interaction. Reduced expression of Nrap and Zyx in Proxl-conditioned hearts, two genes that are direct transcriptional targets of Prox1, is highly significant in terms of maintaining Z-disc stability. N-RAP has been proposed to act as a catalytic scaffold for the association of thin filament actin and Z-disc α-actinin during myofibrillogenesis (Dhume et al., 2006), and zyxin also interacts with α-actinin to facilitate actin assembly and organisation (Crawford et al., 1992; Frank et al., 2006). During myofibrillogenesis, N-RAP and zyxin are associated with cell-cell contacts that make up the developing intercalated discs, which ultimately mature during post-natal stages (Perriard et al., 2003). Our confocal and TEM observations that cell junctions (β-catenin-positive adherens-type junctions and desmosomes) are appropriately established and remain intact between neighbouring cardiomyocytes in Proxl-conditional mutants, suggest that the role of Prox1 is primarily to regulate Nrap and Zyx to facilitate cross-linking between actin and α-actinin in the Z-disc as one of the fundamental associations of the sarcomere (see Fig. S4A in the supplementary material). Actn2 is also implicated in this study as a direct target of Prox1, and the modest, yet significant, reduction in the expression levels of Actn2 in a Proxl mutant background, correlating with the Z-disc disruption, underlines the crucial role of α-actinin in maintaining sarcomere integrity. Moreover, as Zyx-null mice are viable (Hoffman et al., 2003), although their hearts have not been examined in detail for histological defects, the phenotype we describe represents a cumulative effect on the actin–α-actinin interaction: directly, via α-actinin expression and localisation, and through interactions with the co-factors N-RAP and zyxin.

Perturbation of the actin–α-actinin association directly explains the Z-disc anomalies in Proxl-null hearts. Although we cannot entirely exclude additional effects of loss of Prox1 function, normal levels of cardiomyocyte proliferation and apoptosis and the specificity of phenotype at the level of sarcomeric maintenance suggest that the latter is the primary defect in Proxl-conditional mutants. Moreover, thin filament–Z-disc disruption will feedback directly onto the thin and thick filament arrangement of the sarcomere, as observed at the level of TEM, resulting in a more global disorganisation of myofibrils (see Fig. S4B in the supplementary material). The latter might also explain the observed M-band defects, but equally these might relate more directly to misregulation of Nrap in the Proxl-mutant background, as N-RAP associates with the M-bands of maturing myofibrils where it acts as a catalytic scaffold (Lu et al., 2005).

Prox1 is not required for the initial stages of myofibrillogenesis because the phenotypic defects do not begin to manifest until E12.5. Sarcomere proteins are expressed immediately prior to the onset of
Fig. 6. Prox1 directly regulates the genes encoding the structural proteins α-actinin, N-RAP and zyxin. (A) The sarcomere and sarcomere-related protein genes Actn2 (sarcomeric α-actinin), Nrap and Zyx were identified as potential downstream targets of Prox1 by ChIP-on-chip. For each locus, the genomic region immunoprecipitated by anti-Prox1 is indicated by a yellow box, the closest gene is labelled and the degree of conservation shown. Conservation patterns are based on phastCons scores (http:// genome.ucsc.edu). (B) EMSAs with in vitro translated (IVT) Prox1 and 32P-labelled oligonucleotides (60 bp) identified from each of the Actn2, Nrap and Zyx putative Prox1-bound elements (see Fig. S9 in the supplementary material) isolated via the ChIP-on-chip shown in A. A 10-fold excess of unlabelled oligonucleotide was used in competitive assays as evidence of specific binding (lanes C). (C) EMSAs with nuclear extracts from mouse P19Cl6 cell lysates either untransfected (lanes 1-3) or transfected with Flag-Prox1 (lanes 4-6) and 32P-labelled elements as in B. Lanes 1 and 4 are lysate alone, lanes 2 and 5 are lysate plus an anti-Flag antibody, and lanes 3 and 6 are anti-Flag-alone controls. Note the evidence of a supershift in lane 5 compared with lane 4 for each of the Actn2, Nrap and Zyx elements (arrowheads), which is indicative of specific binding by Flag-Prox1. The presence of a comparatively weak band in lanes 1 and 2 in each case represents binding by endogenous Prox1, which is expressed in P19Cl6 cells (data not shown). (D) In vitro transcription assays demonstrate Prox1 transactivation of a luciferase reporter downstream of the Actn2, Nrap and Zyx putative Prox1-binding elements and minimal reporter. Note the significant activation by Prox1 of the Actn2, Nrap and Zyx reporters. (E) qRT-PCR for Nrap and Zyx confirms reduced expression of these factors in a Prox1-deficient background, as was previously determined for Actn2 (see Fig. 3O). In D,E, data are presented as mean ± s.e.m.; *P<0.05, **P<0.001, ***P<0.003, ****P<9×10^{-7}. 
beating and, once integrated into mature myofibrils, they tend to have a relatively long half-life that varies between 3 and 10 days (Martin, 1981). Moreover, between E8.25 and E10.5, the developing heart increases in mass primarily through the addition of cells from the second cardiac lineage (Zaffran et al., 2004). Therefore, there may be little requirement for newly synthesised structural proteins during these early stages of heart development. The initial activation of the genes encoding sarcomeric proteins is clearly carried out by alternate, as yet unidentified, transcriptional regulation pathways, with the role of Prox1 confined to regulating sarcomere maintenance and stability from E10.5 onwards, when all populations of cardiac cells have been acquired and the developing heart continues to grow by a combination of both cardiomyocyte hyperplasia and hypertrophy. The fact that we observed significantly impaired hypertrophic growth following loss of Prox1 is secondary to the primary defect of disrupted assembly of sarcomere proteins. Developing cardiomyocytes elongate in a unidirectional manner by addition of sarcomeres to the existing myofibrils, the timing of which corresponds precisely with the onset of myocardial disruption and failure of the cells to elongate in Prox1-dependent signalling pathways (Frey et al., 2004; Molkentin et al., 2005), or perturbations in the associated calcium-dependent signalling pathways (Bourajjaj et al., 2006; Nelson et al., 2005), Gata4, Nkx2.5 and Desplanc (2003). Distinction between color photoreceptor cell fates is controlled by Prospero in Drosophila. Cell 4, 853-864.


References


Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/3/495/DC1


