factors in a *Prox1*-deficient background (E), as was previously determined for *Actn2* (Fig. 3O). In (D,E) data are presented as mean +/- SEM; * $p<0.05$; ** $p<0.001$; *** $p<0.003$; **** $p<9E-07$.

SUPPLEMENTARY MATERIAL

Figure S1. Prox1 is expressed in the nuclei of cardiomyocytes within the developing heart and coexpressed with sarcomeric markers. Confocal sections of immunostained whole mount control E13.5 hearts using antibodies against Prox1 (green; A-D) and sarcomeric α-actinin (red; A, B) and phalloidin staining to visualise F-actin (blue; C, D). Prox1 is coexpressed with sarcomeric α-actinin and F-actin in cardiomyocytes of both atrial and ventricular myocardium. Furthermore, as predicted for a transcription factor, Prox1 localises to the nucleus. Scale bars: A-D, 10 μm.

Figure S2. *Prox1*-null mice have cardiac defects. *Prox1* heterozygote (+/-) P5 hearts are smaller than wildtype (+/+; A) and have hypoplastic ventricular walls, a thickened interventricular septum (black lines in B, C), cytoplasmic hyalinisaton (asterisks in B, D) and persistent muscle surrounding the aorta (black arrowhead in B). Frontal sections through E14.5 wildtype (E, G) and *Prox1*-null (-/-; F, H) embryos. The myocardium, in particular the interventricular septum (black arrowhead in H), in *Prox1*-null embryonic hearts appears to lack appropriate myofibrillar organisation into striated muscle even at low resolution. Abbreviations: lv, left ventricle; rv, right ventricle; ivs, interventricular septum. Scale bars: A and B-D, 500 μm; E-F, 50 μm; G-H, 10 μm

Figure S3. *Prox1* knockdown does not affect the developing coronary vasculature. PECAM (green) and SMαA (red) immunofluoresence on E14.5 sections through control (A,B) and *Prox1*^{Nkx} mutant hearts reveals extensive endothelial cell (PECAM+) and smooth muscle cell (SMαA+) differentiation throughout the myocardium of both control and *Prox1*^{Nkx} mutant hearts. Note the PECAM+ cells forming the endocardium are lining the trabeculae (B, D), which are disorganised in the *Prox1*^{Nkx} mutant hearts (D). Scale bars: A and C, 50 μm; B and D, 10 μm.

Figure S4. Localisation of sarcomere components is disrupted in *Prox1*-conditional myocardium. (A) The sarcomere is the basic unit of a myofibril and consists of the region between two Z-discs (Z); the centre of the sarcomere is distinguished by the M-band (M). The sarcomere is composed of interlinked myosin thick filaments and actin thin filaments. Titin is considered to be a third 'elastic' filament system and spans half the width of a sarcomere from the Z-disc, where it interacts with α-actinin, to the M-band where it interacts with myomesin. α-actinin is the major component of the Z-disc where it cross-links actin filaments and participates in signal transduction pathways. Similarly, myomesin localises to the M-band where it provides stability and elasticity between myosin filaments. Many other proteins associate with each filament system including myosin binding protein C (MyBP-C), CapZ, tropomodulin, troponins, α-tropomyosin and telethonin/T-Cap. Several proteins found at the Z-disc are essential for connections to adjacent myofibrils and to the sarcolemma at costameres and adherens junctions, such as the intermediate filament protein, desmin (A). All sarcomere components shown to be disrupted in *Prox1*-conditional myocardium are highlighted in B.

Figure S5. Cell-cell contact is not affected in *Prox1*^{Nkx} mutant hearts. Confocal sections through E13.5 control (co; A-C) and *Prox1*^{Nkx} (D-F) whole mount hearts immunostained for phalloidin (red; A, D) and β-catenin (green; B, E). β-catenin staining in *Prox1*^{Nkx} hearts reveals intact cell-cell contact and presence of adherens type junctions (white arrowheads), which was confirmed by TEM on E13.5 sections through control (G) and *Prox1*^{Nkx} mutant (H) hearts; adherens junctions (highlighted by green circles). Intact desmosomes were also revealed by TEM on sections through control (I) and *Prox1*^{Nkx} (J) E13.5 hearts (highlighted by red circles). Scale bars: A-F, 10 μm; G, I and J, 0.5 μm and H, 1 μm.

Figure S6. Severity of phenotype correlates directly with degree of Prox1 knockdown. Confocal sections through E13.5 control (co; A, D) and *Prox1*^{Nkx} (B, C, E, F) whole mount (half) hearts immunostained for phalloidin (green; A-C) and sarcomeric α-actinin (red; D-F). Panels B/E and C/F relate to the heart samples in lanes 4 and 6 of the western blot shown in Fig. 3P. There is a direct correlation between the level of Prox1 reduction, sarcomeric α-actinin reduction and severity of myofibril disarray. Scale bar: A-E, 10 μm.

Figure S7. Prox1 is not required for the initial stages of myofibrillogenesis in the developing heart. E12.5 whole mount control (co; A, B) and *Prox1*^{Nkx} (C, D) hearts stained with phalloidin (green; A, C) or immunostained with an antibody against sarcomeric α-actinin (red; B, D). *Prox1*^{Nkx} cardiomyocytes develop normally until E12.5

at which point the myofibril ultrastucture begins to break down (C, D) suggesting that Prox1 is required to maintain appropriate myofibril organisation but is not required in the initial stages of myofibrillogenesis. Western analysis on a time course of wild type hearts (E9.5-E12.5) for sarcomeric α-actinin (E) and quantification of protein levels normalised to GAPDH using scanning densitometry, reveals increasing sarcomeric α-actinin expression levels from E9.5 consistent with the onset of myofibrillar-dependent hypertrophic growth. Scale bar 10 μm.

Figure S8. Proliferation and apoptosis are unchanged in *Prox1*^{Nkx} myocardium. Immunofluoresence (A-D) and immunohistochemistry (E-F) with an antibody against phospho-histone H3 (P-HH3) and TUNEL assays (G-H) on frontal sections through E10.5 (A-D) and E13.5 (E-H) control (co; A, C, E, G) and *Prox1*^{Nkx} (B, D, F, H) embryos. Levels of proliferation and apoptosis (arrows in A, B and G, H) were normal in *Prox1*^{Nkx} myocardium between E10.5 and E13.5. Abbreviations: ec, endocardial cushion; oft, outflow tract; v, ventricle. Scale bars: A-D, 20 μm; E-F and G-H, 50 μm

Figure S9. Prox1 binds ChIP-identified elements from the structural proteins α-actinin, N-RAP and Zyxin. PCR to confirm that the chromatin preparation contained the *Actn2*, *Nrap* and *Zyxin* elements (A). Chromatin input bands are shown for each of the three elements (A, *Actn2*; N, *Nrap* and Z, *Zyxin*). No bands are identified in the negative control chromatin that underwent immunoprecipitation (IP) without addition of Prox1 antibody (-) and specific bands for each element are identified in duplicate IP samples in the presence of antibody (+); highlighted by white arrowheads. EMSAs with in vitro

translated Prox1 and overlapping, 60 bp ^{32}P-labelled oligonucleotides covering the entire *Actn2* (B), *Nrap* (C), *Zyxin* (D) putative Prox1-binding elements, isolated via the ChIP-on-chip shown in Fig. 6A. IVT Prox1 bound a single oligonucleotide (red arrowhead and highlighted in red in the accompanying sequence) within each of the three elements (B-D). Elements are annotated with respect to gene accession number, chromosome and position relative to start site. Lanes labelled 1-6 represent binding reactions of IVT Prox1 with each of the overlapping 60 bp oligonucleotides per binding element. Non-specific (ns) bands for the *Nrap* EMSA are highlighted by white arrowheads. Text highlighted in blue under each EMSA represents flanking sequence incorporated to facilitate binding that may have occurred at the most distal 5'- or 3'-end of each element; primer sequences for cloning the elements into luciferase reporter constructs (Fig. 6C) are underlined.

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