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An early requirement for *nkx*2.5 ensures the first and second heart field ventricular identity and cardiac function into adulthood

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ABSTRACT

Temporally controlled mechanisms that define the unique features of ventricular and atrial cardiomyocyte identities are essential for the construction of a coordinated, morphologically intact heart. We have previously demonstrated an important role for *nkx* genes in maintaining ventricular identity, however, the specific timing of *nkx*2.5 function in distinct cardiomyocyte populations has yet to be elucidated. Here, we show that heat-shock induction of a novel transgenic line, *Tg(hsp70l:nkx2.5-EGFP)*, during the initial stages of cardiomyocyte differentiation leads to rescue of chamber shape and identity in *nkx*2.5$^{-/-}$ embryos as chambers emerge. Intriguingly, our findings link an early role of this essential cardiac transcription factor with a later function. Moreover, these data reveal that *nkx*2.5 is also required in the second heart field as the heart tube forms, reflecting the temporal delay in differentiation of this population. Thus, our results support a model in which *nkx* genes induce downstream targets that are necessary to maintain chamber-specific identity in both early- and late-differentiating cardiomyocytes at discrete stages in cardiac morphogenesis. Furthermore, we show that overexpression of *nkx*2.5 during the first and second heart field development not only rescues the mutant phenotype, but also is sufficient for proper function of the adult heart. Taken together, these results shed new light on the stage-dependent mechanisms that sculpt chamber-specific cardiomyocytes and, therefore, have the potential to improve *in vitro* generation of ventricular cells to treat myocardial infarction and congenital heart disease.

Introduction

Mutations in *NKX*2-5 are associated with a myriad of congenital heart diseases (CHD) in humans (Benson et al., 1999; Elliott et al., 2003; Jay et al., 2003; McElhinney et al., 2003; Schott et al., 1998). Investigation of the underlying molecular and cellular basis of CHD in model systems has yielded insights into the functions of *Nkx*2-5 in progenitor specification in *Drosophila*, *Xenopus*, and mouse (Azpiazu and Frasch, 1993; Bodmer, 1993; Grow and Krieg, 1998; Prall et al., 2007) and in cardiac morphogenesis in mouse and zebrafish (Lyons et al., 1995; Prall et al., 2007; Tanaka et al., 1999a; Targoff et al., 2008; Tu et al., 2009). Through recent identification of null mutations in *nkx*2.5 and *nkx*2.7, two *Nkx*2-5 homologs expressed in zebrafish cardiomyocytes (Chen and Fishman, 1996; Lee et al., 1996), novel roles in maintaining cardiac chamber identity have also been revealed (Targoff et al., 2013). Furthermore, in postnatal hearts, transcriptional regulation by *Nkx*2-5 has been shown to be important in preserving highly differentiated cardiomyocyte properties and in controlling the cardiac gene program of the adult myocardium (Akazawa and Komuro, 2003, 2005; Takimoto et al., 2000). Despite an appreciation of these early and late roles of *Nkx* genes, their temporal requirement during cardiac development in safeguarding chamber-specific characteristics of differentiated cardiomyocytes has yet to be illuminated. Moreover, while the influence of specific signaling pathways during unique phases in cardiac morphogenesis has been uncovered (de Pater et al., 2009; Dohn and Waxman, 2012; Marques et al., 2008), rarely have the temporally coordinated functions of a cardiac transcription factor such as *nkx*2.5 been dissected with precision.

Innovative strategies for directing differentiation of pluripotent progenitors could benefit from insights regarding the timing of *nkx* genes in establishing specific ventricular and atrial cellular traits. Currently, protocols to convert ES and iPS cells into cardiomyocytes are being developed with improved rates of efficiency (Braam et al., 2009; Bu et al., 2009; Domian et al., 2009; Hansson et al., 2009; Lundy et al., 2013; Mercola et al., 2013; Murry and Keller, 2008; Yang et al., 2008). Yet, a central challenge for these techniques is the ability to favor differentiation of ventricular myocytes as opposed to

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mixed populations. Recently, novel approaches in regenerative medicine have enhanced the production of functional ventricular heart muscle through selection of progenitors expressing *Nkx*2-5 (Domian et al., 2009). Furthermore, there is evidence that Nkx2-5 participates in sub-type specific 'forward programming' of pluripotent stem cells towards a differentiated ventricular population (David et al., 2009). Given these recent advances, examination of the temporally controlled mechanisms mediated by *nkx* genes may help to generate improved protocols for *in vitro* production of ventricular cardiomyocytes for novel models of human cardiac disease and regeneration.

Timing is also relevant to our appreciation of the etiologies of congenital heart defects in humans given the importance of sequential differentiation of the first heart field (FHF) and the second heart field (SHF) (Bruneau, 2008; Nakano et al., 2008; Srivastava and Olson, 2000). *Nkx*2-5 is expressed in FHF and SHF of mouse and zebrafish embryos (Guner-Ataman et al., 2013; Stanley et al., 2002) and mutations in both lineages result in CHD (Lyons et al., 1995; Prall et al., 2007; Tanaka et al., 1999a). Different *Nkx*2-5 enhancer regions have also been shown to regulate gene expression in a temporally dynamic manner (Tanaka et al., 1999b). Furthermore, recent studies in mouse have highlighted the key roles of *Nkx*2-5 in orchestrating transitions between cardiac specification, proliferation, and morphogenesis in FHF and SHF populations (Prall et al., 2007). While *Nkx*2-5 expression begins in the cardiac progenitors of both heart fields and persists throughout embryogenesis into adulthood (Kasahara et al., 1998; Komuro and Izumo, 1993; Lints et al., 1993; Stanley et al., 2002), the specific temporally defined requirements of *Nkx*-dependent processes remain obscure. When are *Nkx* genes essential for developmental progression of cardiomyocyte fate and for insuring long-standing molecular signatures of the ventricle and atrium? Uncovering answers to these questions regarding the timing of *Nkx* gene function will enhance the improvement of therapeutic efforts *in vitro* and *in vivo*.

Our previous work in zebrafish revealed essential roles for *nkx*2.5 and *nkx*2.7 in limiting atrial cell number, promoting ventricular cell number, and preserving chamber-specific identity in differentiated myocardium (Targoff et al., 2013, 2008). From these studies, the initial manifestation of the *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ phenotype following heart tube formation suggests a late requirement for *nkx* genes in chamber identity maintenance. Therefore, to dissect the early (prior to heart tube formation) and late (after heart tube formation) functions of *nkx* genes, we systematically evaluated the influence of timing of *nkx* expression on cardiac chamber formation and preservation of identity using a novel transgenic line, *Tg*(*hsp*70*l:nkx*2.5-*EGFP*). Remarkably, we found that *nkx*2.5 activity is necessary early during cardiac progenitor differentiation to maintain ventricular and atrial chamber morphology and cellular traits later in development. This newly defined temporal relationship broadens our appreciation of the initial roles of *nkx* genes, coupling an early necessity with a later function of chamber identity maintenance. Furthermore, we demonstrate that the temporal requirement for *nkx* genes in SHF cardiomyocytes is shifted later in development, emphasizing the delayed specification and differentiation of this population (de Pater et al., 2009; Hami et al., 2011; Lazic and Scott, 2011; Zhou et al., 2011). Interestingly, our studies also reveal that early re-expression of *nkx*2.5 in *nkx*2.5$^{-/-}$ embryos is adequate to maintain a functional cardiac rescue through adulthood. In summary, our data provide insights into the mechanisms responsible for initiation and maintenance of chamber identity *in vivo* which have the potential to translate into discoveries of novel paradigms for directed differentiation of ventricular and atrial cardiomyocytes *in vitro*, ultimately facilitating a greater understanding of congenital heart disease and myocardial repair.

Methods

Zebrafish

We used zebrafish carrying the following previously described mutations and transgenes: *nkx*2.5^{vu179} (Targoff et al., 2013), *nkx*2.7^{vu413} (Targoff et al., 2013), and *Tg*(-5.1*myl*7:*nDsRed*2)^{f2} (Mably et al., 2003). To produce a novel transgene expressing the fusion protein Nkx2.5-EGFP driven by a heat-shock promoter, we employed the Gateway system (Kwan et al., 2007; Villefranc et al., 2007). Through Tol2 transposase-mediated transgenesis (Fisher et al., 2006), we generated stably integrated transgenic lines carrying *Tg*(*hsp*70*l:nkx*2.5-*EGFP*). We examined 2 independent integrants and found functional rescue of the *nkx*2.5$^{-/-}$ phenotype in each case. Propagation of one line, *Tg*(*hsp*70*l:nkx*2.5-*EGFP*)^{fcu1}, was performed for future work. In this study, experiments were implemented with one transgenic fish per cross unless otherwise specified. For analyses of adult zebrafish, hearts were collected, dissected, and morphometric analysis was performed as previously described (Singleman and Holtzman, 2012). All zebrafish work followed Institutional Animal Care and Use Committee (IACUC)-approved protocols.

In situ hybridization

We conducted whole-mount *in situ* hybridization as previously described (Yelon et al., 1999) with the following probes: *myl*7 (ZDB-GENE-991019-3), *vmhc* (ZDB-GENE-991123-5), *amhc* (*myh*6; ZDB-GENE-031112-1), and *nkx*2.5 (ZDB-GENE-980526-321).

Immunofluorescence

Whole-mount immunofluorescence was performed with variations of a published protocol (Alexander et al., 1998), using primary monoclonal antibodies against sarcomeric myosin heavy chain (MF20) and atrial myosin heavy chain (S46). MF20 and S46 were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Biological Sciences, University of Iowa, under contract NO1-HD-2–3144 from the NICHD. In embryos, the secondary reagents, goat anti-mouse IgG1 Alexa Fluor 488 and goat anti-mouse IgG2b Alexa Fluor 568 (Invitrogen), were used to recognize MF20 and S46, respectively. In adults, zebrafish hearts were incubated in 10 ug/ml Proteinase K (Roche) and blocked overnight before proceeding with the standard immunofluorescence protocol.

For the developmental timing assay, a modified version of a previously described protocol was employed using embryos carrying *Tg*(-5.1*myl*7:*nDsRed*2) (de Pater et al., 2009). Sequential immunostaining was performed with S46 and goat anti-mouse IgG1 Alexa Fluor 488, then with MF20 and goat anti-mouse IgG Cy5 (Invitrogen). Visualization of DsRed was performed without immunofluorescence to detect transgenic expression levels.

Genotyping

PCR genotyping was performed on genomic DNA extracted from individual embryos following *in situ* hybridization, immunofluorescence, or live imaging. Detection of *nkx*2.5^{vu179} was executed using primers 5′–TCACCTCCACACAGGTGAAGATCTG-3′ and 5′–CAGAAAGATGAATGCTGTCGGT-3′ to generate a 443 bp fragment. Primer placement in the 3′–UTR was chosen specifically to amplify the endogenous *nkx*2.5 allele as opposed to the transgene, *Tg*(*hsp*70*l:nkx*2.5-*EGFP*). Digestion of the mutant PCR product with Hinf1 creates 207 bp, 162 bp, 49 bp, and 25 bp fragments. Analysis of *nkx*2.7^{vu413} was performed using primers 5′–CTTTTTCAGG-CATGTGTCCA-3′ and 5′–AAAGCGTCTTTCCAGCTCAA-3′ to generate a 146 bp fragment. Digestion of the mutant PCR product with MseI

creates 111 bp and 35 bp fragments. Detection of EGFP in fish carrying *Tg(hsp70l:nkx2.5-EGFP)* was performed using primers 5′–TATATCATGGCCGACAAGCA-3′ and 5′–GAACTCCAGCAGGACCATGT-3′ to generate a 219 bp fragment.

Western blot

Embryos were dechorionated and homogenized manually in lysis buffer (20 mM Tris (pH 8.0), 50 mM NaCl, 2 mM EDTA, 1% NP-40) (Waxman and Yelon, 2011). Lysates were centrifuged and proteins from supernatants were quantified using the DC Protein Assay Kit (Bio-Rad). 30 ug of protein extracts were resolved using a precast polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). Immunoblots were probed overnight at 4 °C with rabbit anti-GFP (1:2500, Torrey Pines Biolab) or monoclonal mouse anti-Actin (1:1000, Sigma), as a loading control. Primary antibodies were labeled with anti-rabbit HRP (1:5000) or anti-mouse HRP (1:5000). Proteins were detected with chemiluminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific).

Imaging

Images were captured with Zeiss M2Bio and Axioplan microscopes and a Zeiss AxioCam digital camera. They were processed with Zeiss AxioVision and Adobe Creative Suite software. Confocal imaging was performed with a Nikon A1R MP and z-stacks were analyzed with Image J.

Cardiomyocyte counting

We counted cardiomyocytes as previously described (Targoff et al., 2008), using immunofluorescence to detect DsRed in cardiomyocyte nuclei of *Tg(-5.1myl7:nDsRed2)*-carrying embryos. Embryos were gently flattened using a coverslip in preparation for imaging. Student's t-test (homoscedastic, two-tailed distribution) determined statistical significance between the means of cell number data sets.

Heat shock conditions

Embryos from outcrosses of fish carrying *Tg(hsp70l:nkx2.5-EGFP)* were maintained at 28.5 °C and exposed to heat shock at desired stages. To implement heat shock, 50 embryos were placed in 2.5 mL of embryo medium in a Petri dish on top of a covered heat block for 1 h at 37 °C. Following this treatment, transgenic embryos were identified by genotyping for the *hsp70l:nkx2.5-EGFP* transgene or visualization of ubiquitous EGFP expression. Non-transgenic sibling embryos exposed to heat shock served as controls.

Results

Mutations of nkx2.5 and nkx2.7 result in ventricular-to-atrial fate transformation

Our previous results demonstrate that *nkx* genes are required for the maintenance of ventricular identity (Targoff et al., 2013). Yet, these studies predominantly utilized an anti-*nkx2.7* morpholino to deplete *nkx2.7* gene function. Here, we examine in greater detail the loss of *nkx* gene function in an allelic series to elucidate the effect of gene dosage in *nkx2.5*$^{-/-}$;*nkx2.7*$^{-/-}$ embryos. In the *nkx2.5*$^{-/-}$ embryo, following normal specification and chamber-specific differentiation during the early stages of cardiac development (Targoff et al., 2013), late ventricular-to-atrial fate transformation results in morphological abnormalities characterized by an enlarged, bulbous atrium and a minuscule ventricle (Fig. S1M). Furthermore, our recent data using chamber-specific markers, *ventricular myosin heavy chain* (*vmhc*) and *atrial myosin heavy chain* (*amhc*), highlight the fading of ventricular cardiomyocytes and the expansion of atrial cardiomyocytes (Fig. S1C and H). Interestingly, while there is no detectable phenotype in the *nkx2.7*$^{-/-}$ heart (Fig. S1B,G and L), a synergistic effect is visualized with progressive loss of *nkx2.7* alleles in the *nkx2.5*$^{-/-}$ embryo (Fig. S1D,E,I,J,N,O). Ultimately, the *vmhc*-expressing cells disappear in the *nkx2.5*$^{-/-}$;*nkx2.7*$^{-/-}$ embryo and the *amhc*-expressing cells extend into the outflow tract (OFT) (Fig. S1E and J). To substantiate the absence of *vmhc* expression, we conducted cell counting studies that indicate a complete loss of ventricular cardiomyocytes and a statistically significant increase in atrial cell number in the *nkx2.5*$^{-/-}$;*nkx2.7*$^{-/-}$ embryo (Fig. S1U). Taken together, our findings highlight the essential and synergistic roles of *nkx2.5* and *nkx2.7* in safeguarding ventricular characteristics. Moreover, these data complement and extend our previous studies by emphasizing the flexible nature of cardiomyocyte identity and by highlighting the importance of gene dosage in mediating the effects of *nkx* genes.

Despite these insights into the roles of *nkx* genes in chamber identity maintenance, the specific temporal requirements of *nkx2.5* in unique cardiomyocyte populations have yet to be elucidated. Given the initial manifestation of the loss of ventricular identity following heart tube elongation in the *nkx2.5*$^{-/-}$ embryos (Targoff et al., 2013), we postulated that *nkx2.5* is most likely essential between heart tube elongation and chamber emergence to preserve chamber-specific characteristics. Yet, the onset of *nkx2.5* expression in cardiac precursors suggests a potential early role, warranting investigation of the ability to imprint chamber-specific identity at the initial stages of cardiac differentiation. Thus, we aimed to dissect the effect of timing of *nkx2.5* expression in chamber identity maintenance by employing a novel heat-shock inducible transgene, *Tg(hsp70l:nkx2.5-EGFP)*.

A heat-shock inducible transgene, Tg(hsp70l:nkx2.5-EGFP), enables temporal control of nkx2.5 expression

Prior studies in fish examining the effects of *nkx2.5* overexpression have been limited to RNA injection at the one-cell stage (Chen and Fishman, 1996; Simoes et al., 2011). Therefore, we generated a transgene driven by the heat-shock promoter expressing Nkx2.5 tagged to EGFP at its C-terminus (Fig. 1A). To validate its function, stable transgenic embryos carrying *Tg(hsp70l:nkx2.5-EGFP)* were assessed for *nkx2.5* (Fig. 1B-E) and EGFP (Fig. 1F-I) expression following heat shock for one hour. Ubiquitous expression of *nkx2.5* is observed within one hour following initiation of heat shock in the transgenic embryos (Fig. 1C) while non-transgenic embryos from the same clutch show only endogenous cardiac expression of *nkx2.5* (Fig. 1B). By 8 h post-heat shock, ectopic *nkx2.5* expression from *Tg(hsp70l:nkx2.5-EGFP)* fades and only endogenous cardiac *nkx2.5* expression remains (Fig. 1E). These findings are mirrored by similar kinetics of EGFP expression; protein perdurance begins to diminish by 6 h and is significantly decreased by 8 h following heat shock (Fig. 1G-I; data not shown). Moreover, nuclear localization of EGFP confirms that the tagged Nkx2.5 protein is functional and translocates to the nucleus upon induction (Fig. 1G and H). Finally, protein stability was assessed by western blot analysis to confirm our assessment of the kinetics and perdurance of *Tg(hsp70l:nkx2.5-EGFP)* (Fig. 1J). Using an anti-GFP antibody, we determined that extracts from transgenic wild-type embryos demonstrate a dramatic reduction in protein levels at 8 h post-heat shock and complete absence of EGFP expression at 10 h post-heat shock. Taken together, our detailed analysis emphasizes the defined time course of heat-shock inducible *nkx2.5* expression as directed by the *hsp70l:nkx2.5-EGFP* transgene.

To evaluate the effects of overexpression of Nkx2.5-EGFP in wild-type embryos, we examined cardiac chamber proportionality

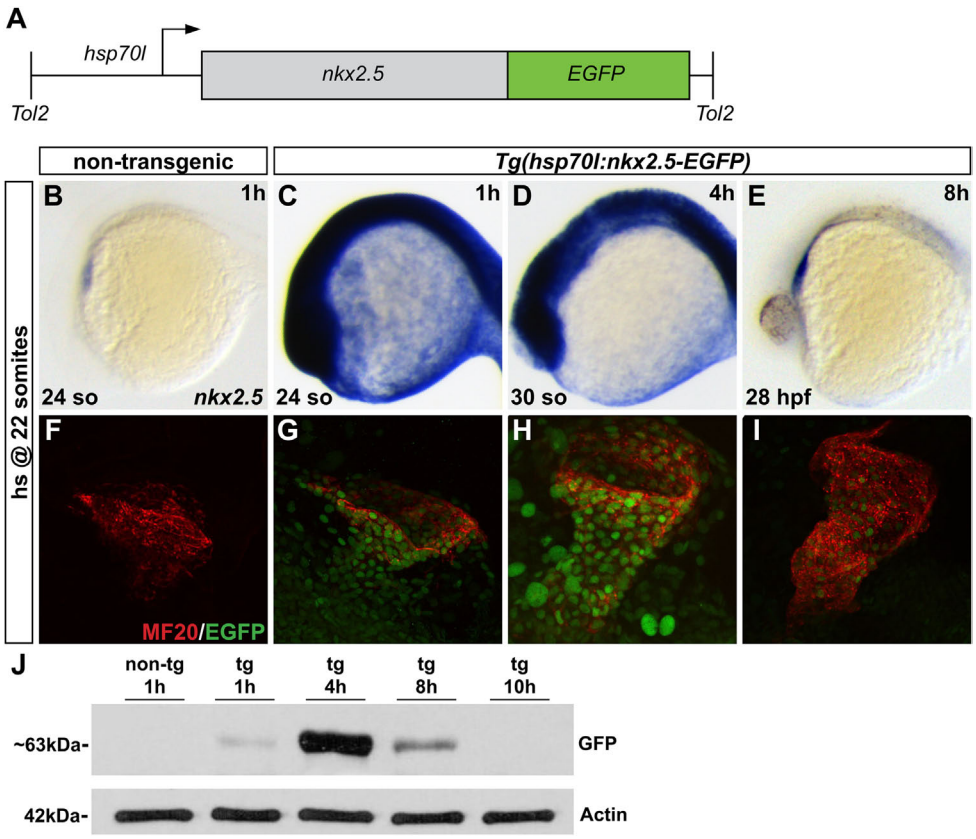


Fig. 1. Heat-shock inducible transgene, *Tg(hsp70l:nkx2.5-EGFP)*, allows for temporally controlled expression of *nkx2.5*. (A) Schematic representation of the heat-shock inducible transgene. (B-E) *In situ* hybridization depicts expression of *nkx2.5* in non-transgenic (B) and *Tg(hsp70l:nkx2.5-EGFP)* (C-E) embryos. Lateral views, anterior to the left. Following initiation of heat shock (hs) at 22 somites (20 hpf), non-transgenic embryos demonstrate endogenous *nkx2.5* expression in the heart tube at 24 somites (1 h post-hs) (B). In comparison, heat-shocked transgenic embryos reveal upregulation of *nkx2.5* ubiquitously in the embryo at 24 somites (1 h post-hs) (C) and at 30 somites (4 h post-hs) (D). By 28 hpf (8 h post-hs), global *nkx2.5* expression is significantly diminished and only cardiac specific expression remains (E). Time interval post-hs is indicated in the upper right corner of each panel. (F–I) MF20 immunofluorescence (red) indicates cardiac myosin heavy chain in all cardiomyocytes and EGFP (green) reflects transgenic expression following initiation of heat shock at 22 somites (20 hpf). Lateral views, anterior to the right. Confocal projections of fixed, dissected non-transgenic (F) and transgenic (G–I) hearts. EGFP can be visualized as early as 24 somites (1 h post-hs) (G) with strong perdurance through 30 somites (4 h post-hs) (H). Yet, only minimal residual expression is evident by 28 hpf (8 h post-hs) (I). (J) Western blots using anti-GFP and anti-Actin on protein extracts prepared from non-transgenic and transgenic embryos following heat shock. Samples were collected at 1 h, 4 h, 8 h, and 10 h post-hs, respectively. The full-length Nkx2.5-EGFP runs with apparent molecular weight of ~63 kDa. The same membrane was probed with anti-Actin, serving as a loading control.

and identity through assessment of MF20/S46 immunofluorescence and chamber-specific expression patterns (Fig. S2A–I). Following heat shock at 11 somites and 21 somites, specific developmental stages during somitogenesis associated with bilateral heart field and cardiac cone formation, non-transgenic and *Tg(hsp70l:nkx2.5-EGFP)* wild-type embryos exhibit normal ventricular and atrial morphology and identity (Fig. S2A–I). Furthermore, there is no statistically significant difference in atrial, ventricular, and total cardiomyocyte numbers between the non-transgenic and transgenic wild-type embryos (Fig. S2J). Thus, these results illustrate that, while *nkx2.5* is essential to maintain ventricular identity, it is insufficient to induce ventricular fate. Our findings differ from previous work demonstrating an enlarged heart following *nkx2.5* overexpression at the one-cell stage (Chen and Fishman, 1996; Tu et al., 2009). Yet, these results were reported at low frequencies and most likely reflect significant variability in mRNA stability when compared to expression of *Tg(hsp70l:nkx2.5-EGFP)*. Altogether, our data verify the use of *Tg(hsp70l:nkx2.5-EGFP)* to dissect the temporal roles of *nkx2.5* in discrete developmental windows without concerns of non-specific effects.

Early overexpression of nkx2.5 rescues late morphological defects in nkx2.5$^{-/-}$ embryos

To identify when *nkx2.5* is acting, we expressed *Tg(hsp70l:nkx2.5-EGFP)* in *nkx2.5*$^{-/-}$ embryos in a temporally controlled manner. Embryos from a cross of *nkx2.5*$^{+/-}$ fish with one transgenic parent were treated with heat shock prior to heart tube elongation (21 somites) (Fig. 2A–H). Representative examples of non-transgenic and transgenic wild-type (Fig. 2A and B) and *nkx2.5*$^{-/-}$*;Tg(hsp70l:nkx2.5-EGFP)* (Fig. 2D) embryos following heat shock at 21 somites demonstrate normal embryonic development without evidence of the gross pericardial edema present in the non-transgenic *nkx2.5*$^{-/-}$ embryo (Fig. 2C). Brightfield images of ventricular and atrial morphology highlight rescue of the diminutive, narrowed ventricular chamber and the bulbous, dilated atrial chamber in *nkx2.5*$^{-/-}$*;Tg(hsp70l:nkx2.5-EGFP)* embryos (Fig. 2E-H; compare F with H). Select embryos were genotyped to confirm transgenic carrier status and presence of the *nkx2.5*^{vu179} mutation. In summary, our data demonstrate the previously unappreciated function of *nkx2.5* at 21 somites to ensure normal ventricular and atrial proportions following chamber emergence.

Prior to embarking on larger scale studies, we further characterized the functionality of the *Tg(hsp70l:nkx2.5-EGFP)*. Initial assessment of non-transgenic and transgenic wild-type and *nkx2.5*$^{-/-}$ embryos without heat exposure yields no evidence of off-target effects or transgene activation in *Tg(hsp70l:nkx2.5-EGFP)* carriers (Fig. S3A,B,E,F). Moreover, uniform expression was observed in all transgene carriers and facilitated sorting of non-transgenic and transgenic embryos following heat shock, always comparing sibling wild-type (Fig. S3C and D) and *nkx2.5*$^{-/-}$ (Fig. S3G and H) embryos. Subsequent genotyping for *Tg(hsp70l:nkx2.5-EGFP)* confirmed reliable

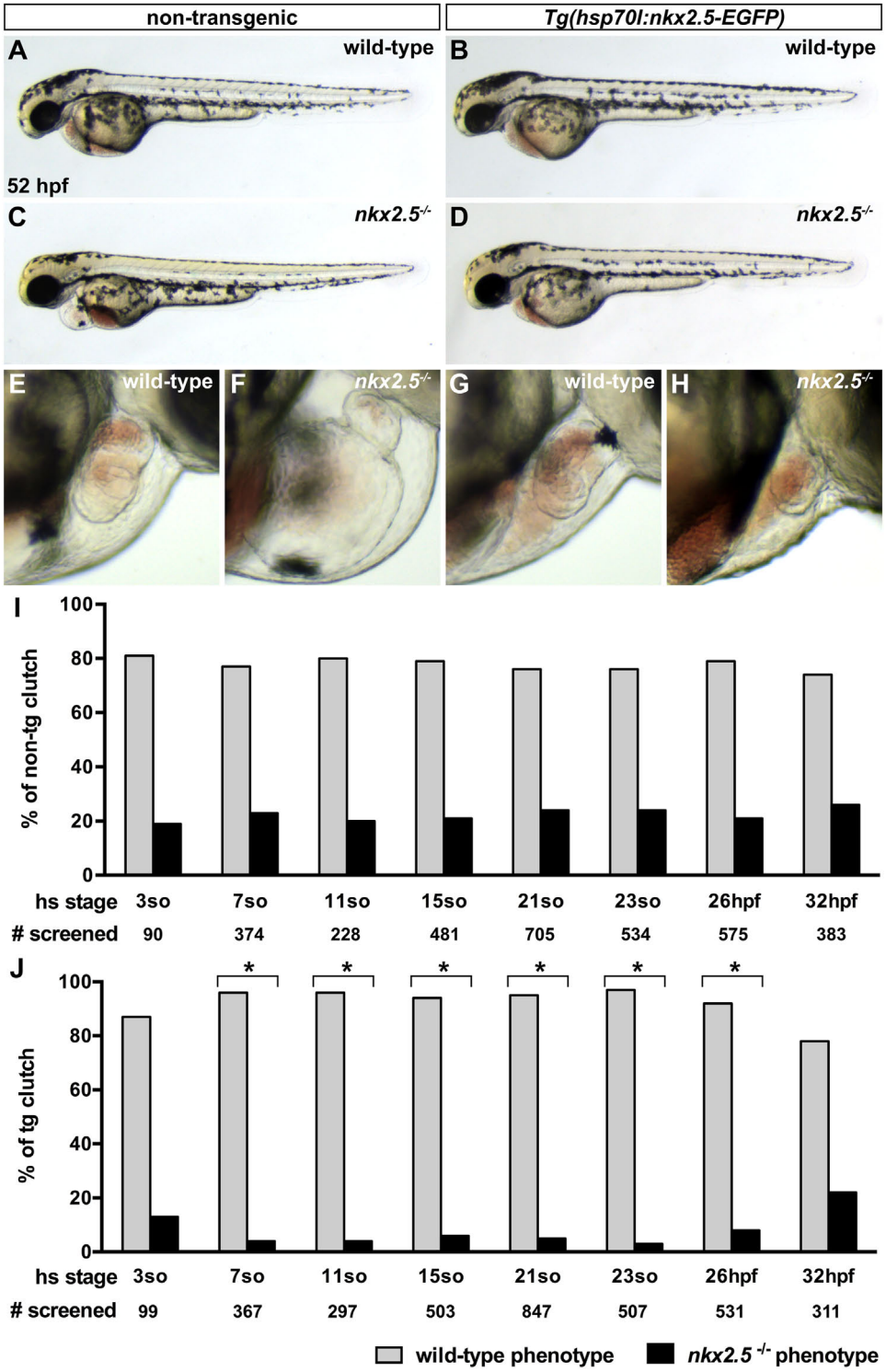


Fig. 2. Early overexpression of *nkx*2.5 rescues late morphological defects in *nkx*2.5$^{-/-}$ embryos. (A–D) Lateral views of live embryos, anterior to the left, at 52 hpf. All embryos were heat-shocked at 21 somites. Aside from cardiac defects and pericardial edema, the non- transgenic *nkx*2.5$^{-/-}$ embryos appear morphologically normal (C). While heat shock of wild-type embryos yields no evidence of toxicity (B), the cardiac defects and pericardial edema in the *nkx*2.5$^{-/-}$;*Tg(hsp70l:nkx2.5-EGFP)* embryos are dramatically improved (D). (E–H) Lateral views of live embryos, anterior to the right, at 52 hpf. Compared to non-transgenic (E) and transgenic (G) wild-type hearts, the non-transgenic *nkx*2.5$^{-/-}$ heart (F) is unlooped and has striking defects in both ventricular and atrial morphologies. In contrast, the transgenic *nkx*2.5$^{-/-}$ heart (H) resembles its wild-type sibling (G) indicating a rescue of chamber proportions. (I,J) Bar graphs illustrate the percentage of phenotypically wild-type (gray) and *nkx*2.5$^{-/-}$ (black) embryos in multiple clutches of non-transgenic (I) and transgenic (J) siblings following heat shock at specific developmental stages. Gross phenotypic assessment was performed assaying for morphological features noted in (A-H) at 52 hpf. The total number of embryos screened at each time point is noted. Fisher exact tests (one-sided; $p < 0.01$) were performed; asterisks indicate statistically significant differences between proportions of non-transgenic (I) and transgenic (J) wild-type and phenotypically *nkx*2.5$^{-/-}$ embryos. A notable decrement in the percentage of transgenic *nkx*2.5$^{-/-}$ embryos between 7 somites and 26 hpf denotes rescue of morphological ventricular and atrial defects.

phenotype-genotype correlation of carrier status, validating our ability to use EGFP fluorescence to identify *Tg(hsp70l:nkx2.5-EGFP)*-positive embryos consistently in future experiments (Fig. S3C,D,G,H).

Applying these insights, we sought to determine more precisely the pivotal developmental window when *nkx2.5* acts to preserve chamber-specific attributes. Thus, we induced heat shock at a series of time points from 3 somites to 32 hpf (Fig. 2I and J). These key stages were selected to span the onset of *nkx2.5* expression between 3 somites (Wu et al., 2011) and 5 somites (Lee et al., 1996) and the alteration in cell number that occurs in the *nkx2.5*$^{-/-}$ embryos between 26 hpf and 36 hpf (Targoff et al., 2013). Using live phenotypic assessment of chamber morphology at 52 hpf (as in Fig. 2E-H), we observed proportions approximating a Mendelian ratio of wild-type *versus* mutant embryos in the non-transgenic clutches at all time points evaluated (Fig. 2I). In these experiments, we detected slightly less than a quarter of the total embryos that displayed the *nkx2.5*$^{-/-}$ morphology. We concluded that this subtle discrepancy from expected ratios suggests mild variability in the phenotype as opposed to incomplete penetrance. Interestingly, we found that re-expression of Nkx2.5-EGFP from 7 somites through 23 somites rescues the expanded atrium and diminutive ventricle of the *nkx2.5*$^{-/-}$ embryos, yielding a statistically significant decrease in the proportion of mutant embryos phenotypically assessed (Fig. 2J). While only partial rescue of transgenic *nkx2.5*$^{-/-}$ embryos is achieved following heat shock at 26 hpf, the difference in proportion of mutant phenotypes between non-transgenic and transgenic siblings remains statistically significant. Earlier (3 somites) and later (32 hpf) transgenic expression results in an incomplete rescue with no statistically significant difference in the ratios of wild-type *versus* mutant embryos compared with non-transgenic siblings (Fig. 2I). These data are important because they demonstrate that the transgene is functional and define the developmental window when *nkx2.5* is required. Together, our findings support an intriguing model whereby *nkx2.5* plays an essential role during cardiomyocyte differentiation to establish proper ventricular and atrial proportionality when cardiac chambers expand.

Early expression of nkx2.5 actively maintains ventricular identity during chamber emergence

Next, we sought to determine if *nkx2.5* regulates chamber identity in addition to proportion and whether the temporal windows of genetic influence for each of these characteristics overlap. Using MF20/S46 immunofluorescence and subsequent genotyping, we first confirmed our findings from live phenotypic assessments (Fig. 2): transgenic *nkx2.5*$^{-/-}$ embryos demonstrate normal chamber size, morphology, and looping following induction of *Tg(hsp70l:nkx2.5-EGFP)* between 7 somites and 26 hpf (Fig. 3D,M,P compared to A,G,J; data not shown). Second, employing *in situ* hybridization of chamber-specific identity markers, we detected abrogation of

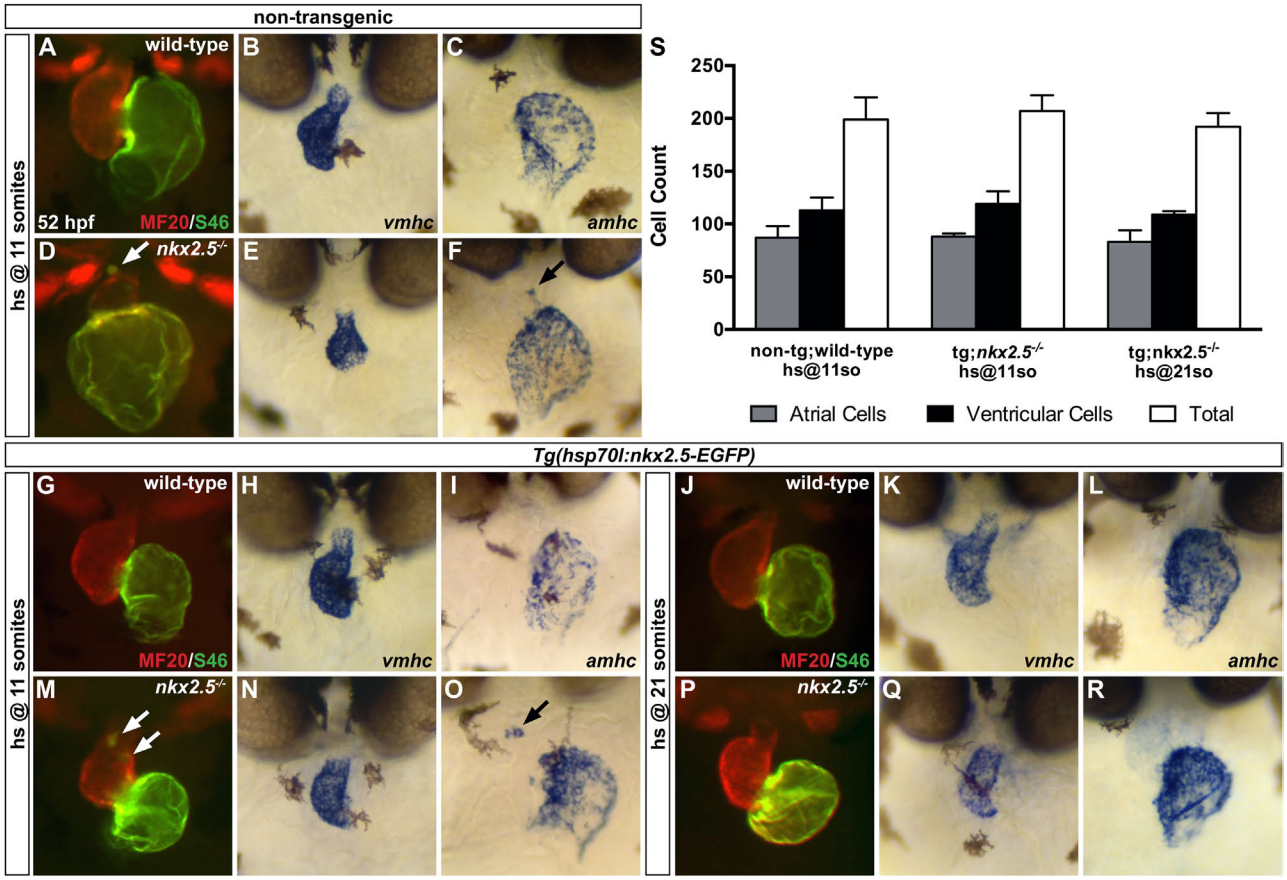


Fig. 3. Early expression of *nkx2.5* actively maintains ventricular identity during chamber emergence. (A-R) Ventral views, anterior to the top, at 52 hpf in non-transgenic (A-F) and *Tg(hsp70l:nkx2.5-EGFP)* (G-R) embryos. MF20/S46 immunofluorescence (A,D,G,J,M,P) distinguishes ventricular myocardium (red) from atrial myocardium (green) and *in situ* hybridization depicts expression of *vmhc* (B,E,H,K,N,Q) and *amhc* (C,F,I,L,O,R) in genotypically wild-type (A-C,G-I,J-L) and *nkx2.5*$^{-/-}$ (D-F,M-O,P-R) embryos. In comparison to non-transgenic *nkx2.5*$^{-/-}$ embryos (D-F), transgenic *nkx2.5*$^{-/-}$ embryos retain appropriate chamber-specific identity following heat shock at 11 somites (M-O) and 21 somites (P-R). However, ectopic S46^{+} cardiomyocytes near the OFT in the non-transgenic *nkx2.5*$^{-/-}$ embryo (D) are also present in the transgenic *nkx2.5*$^{-/-}$ embryo following heat shock at 11 somites (M), whereas complete rescue of ventricular chamber identity is achieved following heat shock at 21 somites (P). White arrows indicate ectopic S46^{+} cells (D,M). Black arrows highlight the corresponding ectopic *amhc*$^{+}$ population (F,O). (S) Bar graph indicates numbers of atrial, ventricular, and total cardiomyocyte nuclei; the transgene *Tg(-5.1myl7:nDsRed2)* in both cardiac chambers facilitates cell counting at 48 hpf. Mean and standard error of each data set are shown without detection of statistically significant differences between non-transgenic wild-type and transgenic *nkx2.5*$^{-/-}$ embryos following heat shock at 11 somites and 21 somites ($p > 0.01$), indicating rescue of chamber-specific cardiomyocyte cell numbers in transgenic *nkx2.5*$^{-/-}$ siblings. All embryos were genotyped following cardiomyocyte cell counting.

ventricular-to-atrial transdifferentiation in transgenic *nkx*2.5$^{-/-}$ embryos compared to non-transgenic *nkx*2.5$^{-/-}$ embryos that were heat shocked at the same time points (Fig. 3E,F,N,O,Q,R compared to B,C,H,I,K,L; data not shown). Specifically, the enlarged atrium and deficient ventricle of the non-transgenic *nkx*2.5$^{-/-}$ embryo is associated with extension of *amhc* into the ventricular chamber and OFT (Fig. 3E and F), a finding that is distinct from the discrete delineation of atrial and ventricular cardiomyocytes in the wild-type embryo (Fig. 3B and C). In contrast, in transgenic *nkx*2.5$^{-/-}$ sibling embryos, ventricular and atrial identity are maintained (Fig. 3N,O,Q,R) akin to transgenic wild-type embryos following heat shock at 11 somites (Fig. 3H and I) and 21 somites (Fig. 3K and L). Furthermore, by counting cardiomyocytes, we examined whether the numbers of cells in each chamber are similarly rescued, reflecting quantitatively the resolution of chamber proportionality and identity defects. Indeed, there are no statistically significant differences between the ventricular, atrial, and total cell numbers in the wild-type and transgenic *nkx*2.5$^{-/-}$ embryos following heat shock at 11 somites and 21 somites (Fig. 3S). Thus, normalization of the decrease in ventricular and the increase in atrial cells observed in *nkx*2.5$^{-/-}$ embryos (Fig. S1U) suggests that early expression of *nkx*2.5 is sufficient to restore chamber-specific cardiomyocyte populations. Taken together, we show that *nkx*2.5 is essential during initial cardiomyocyte differentiation to safeguard qualitative and quantitative ventricular characteristics as chamber emergence occurs, extending the window of influence of *nkx*2.5 earlier than previously appreciated (Targoff et al., 2013).

Nkx2.5 is necessary to maintain ventricular identity at discrete stages in distinct cardiomyocyte populations

Recent studies in zebrafish have established two distinct phases of cardiac differentiation (de Pater et al., 2009; Hami et al., 2011; Lazic and Scott, 2011; Zhou et al., 2011). FHF differentiation begins in the bilateral heart fields and continues through heart tube elongation. Subsequently, SHF differentiation leads to accretion of new myocardium at the poles of the heart, ultimately contributing to cardiac chamber emergence. Given the discretely defined stages of cardiomyocyte differentiation, we investigated whether a temporally distinct requirement for *nkx*2.5 exists in these separate populations using our novel heat-shock inducible transgene. We were intrigued to observe a residual cluster of ectopic *amhc*$^{+}$ cells near the OFT of the *nkx*2.5$^{-/-}$ embryos carrying *Tg(hsp70l:nkx2.5-EGFP)* following heat shock at 11 somites (Fig. 3O); these findings mirror the ectopic S46^{+} cells also present in the arterial pole of the rescued *nkx*2.5$^{-/-}$ embryos (Fig. 3M). Furthermore, while expression of ectopic *amhc* near the OFT is observed in the majority of embryos undergoing heat shock at 11 somites ($n=11/13$ genotyped *nkx*2.5$^{-/-}$ embryos), these cells were rarely detected in embryos undergoing heat shock at 21 somites ($n=1/12$ genotyped *nkx*2.5$^{-/-}$ embryos). Incorporating our appreciation of the 10-hour perdurance of the Nkx2.5-EGFP protein (Fig. 1), induction of *Tg(hsp70l:nkx2.5-EGFP)* at 11 somites (14.5 hpf) would result in early *nkx*2.5 expression during FHF differentiation, but would not extend to the later phase of SHF differentiation. In contrast, initiation of Nkx2.5-EGFP expression at 21 somites (19.5 hpf) would adequately span both phases of cardiomyocyte differentiation. Altogether, our findings suggest that *nkx*2.5 is required in the SHF progenitors following heart tube elongation to maintain cardiomyocyte identity during chamber formation.

In order to test this hypothesis, we employed a developmental timing assay that relies upon the delayed visualization of DsRed in embryos expressing *Tg(-5.1myl7:nDsRed2)* compared to detection of the pan-cardiac marker, MF20 (similar to the protocol described in (de Pater et al., 2009)). In non-transgenic wild-type embryos heat shocked at 11 somites, late-differentiating SHF-derived cells accumulate at the arterial pole as indicated by the MF20^{+} cardiomyocytes without nuclear localization of DsRed (Fig. 4A–C). Moreover, following heat induction at this same time point, non-transgenic *nkx*2.5$^{-/-}$ embryos also acquire SHF cells at the arterial pole, yet a small portion expresses S46 (Fig. 4D and F). Specifically, these S46^{+} cells are MF20^{+}DsRed^{-}, underscoring that accretion of this population occurs from late-differentiating SHF-derived cardiomyocytes (Fig. 4F). Thus, our data reveal the importance of *nkx* genes in maintaining ventricular identity not only in the FHF, but also in the SHF as cardiomyocytes are added to poles of the heart.

We used this strategy to investigate the developmental origin of the ectopic S46^{+} cardiomyocytes at the OFT in transgenic *nkx*2.5$^{-/-}$ embryos following heat shock at 11 somites (Fig. 3M). Strikingly, this developmental timing assay reveals MF20^{+}DsRed^{-}S46^{+} cardiomyocytes at the OFT of the rescued transgenic *nkx*2.5$^{-/-}$ embryos (Fig. 4J–L), validating our hypothesis that ectopic S46^{+} cells originate from late-differentiating SHF-derived progenitors and are sensitive to *nkx*2.5 depletion following heart tube elongation. Thus, in the absence of *nkx*2.5 gene function during this late phase of

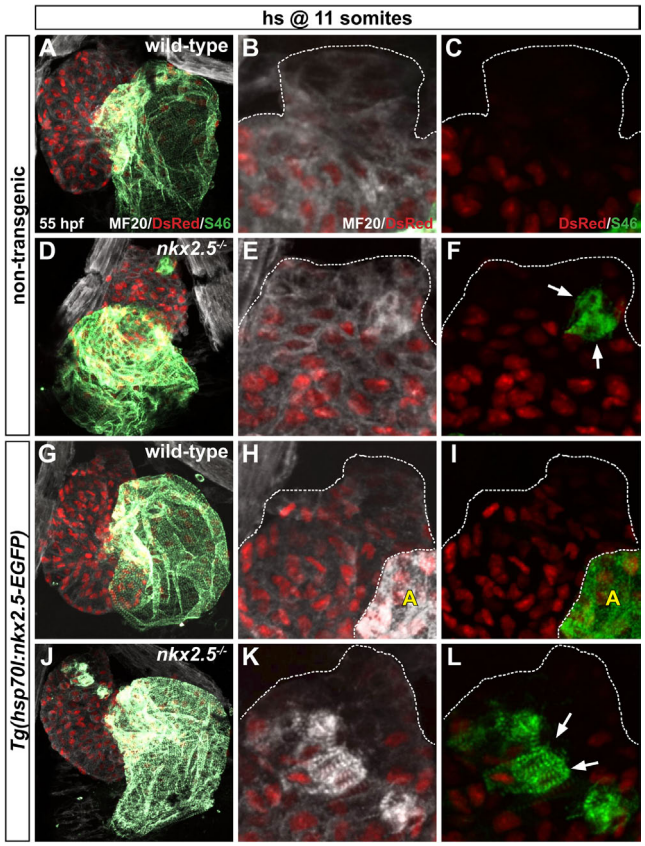


Fig. 4. *nkx*2.5 is necessary to maintain ventricular identity in late-differentiating cardiomyocytes derived from the SHF. MF20/S46 immunofluorescence distinguishes ventricular myocardium (white) from atrial myocardium (green) in non-transgenic (A-F) and *Tg(hsp70l:nkx2.5-EGFP)* (G-L) embryos. Confocal projections of wild-type (A-C, G-I) and *nkx*2.5$^{-/-}$ (D–F, J–L) hearts depict cardiomyocyte nuclei with *Tg(-5.1myl7:nDsRed2)* (red). Ventral views, arterial pole to the top, at 55 hpf. (B,C,E,F,H,I,K,L) White dots outline the MF20^{+} cardiomyocyte borders in the OFTs of hearts in (A,D,G,J), respectively. White arrows indicate ectopic S46^{+} cells; "A" denotes atrium. All embryos were heat-shocked at 11 somites. (A–C) In wild-type non-transgenic hearts, the late-differentiating cardiomyocyte population exhibits MF20, but not DsRed, fluorescence due to the delay in expression of *Tg(-5.1myl7:nDsRed2)* at the arterial pole. (D–F) Similarly, in non-transgenic *nkx*2.5$^{-/-}$ hearts, cardiomyocytes expressing MF20, but not DsRed, are present at the arterial pole. A few ectopic S46^{+} cardiomyocytes are also visualized in this region (DsRed^{-} nuclei). (G–I) In transgenic wild-type hearts, MF20, but not DsRed, fluorescence at the arterial pole designates the delayed differentiation of these SHF-derived cardiomyocytes. (J–L) Despite the rescue of the cardiac chamber morphology and identity, excess S46^{+} cardiomyocytes are visualized at the arterial pole of transgenic *nkx*2.5$^{-/-}$ hearts where the late-differentiating population accretes (DsRed^{-} nuclei).

differentiation, SHF-derived cardiomyocytes at the OFT take on an atrial identity. In summary, these data provide evidence for the essential roles of *nkx*2.5 genes in maintaining ventricular identity at discrete time points during development of specific myocardial lineages.

Resupplying nkx2.5 reveals specific and dose-dependent functions of nkx genes in nkx2.5$^{-/-}$;nkx2.7$^{-/-}$ embryos

Given previous studies in zebrafish and mouse demonstrating overlapping roles of co-expressed *nkx* genes (Tanaka et al., 2001; Targoff et al., 2008; Tu et al., 2009), we employed our novel transgenic line to dissect the shared and unique functions of *nkx*2.5 and *nkx*2.7 in specific cardiomyocyte populations. To this end, we performed heat shock at a range of developmental time points when *nkx*2.5 is known to play a vital role in maintaining chamber proportionality and cardiomyocyte identity in the FHF and SHF (Figs. 2,3). Remarkably, employing MF20/S46 immunofluorescence, we observed a complete rescue of chamber morphology and identity in transgenic *nkx*2.5$^{-/-}$;*nkx*2.7$^{+/-}$ embryos (Fig. 5G–J compared to F). It is also particularly interesting to note ectopic S46^{+} cardiomyocytes near the OFT of transgenic *nkx*2.5$^{-/-}$;*nkx*2.7$^{+/-}$ embryos following heat shock at 11 somites (Fig. 5G), reinforcing our conclusion that *nkx*2.5 is required following heart tube elongation to maintain ventricular identity in this SHF-derived population. In comparison, expression of Nkx2.5-EGFP at the same time points in *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryos yields only partial rescue of the characteristic ventricular deficiency and ventricular-to-atrial transdifferentiation (Fig. 5L–O compared to K). Specifically, the defect in ventricular chamber size is moderately rescued with residual S46 fluorescence extending into the OFT following heat shock at 11 somites and 15 somites (Fig. 5L and M) whereas minimal rescue of these phenotypic characteristics is achieved following heat shock at 21 somites and 23 somites (Fig. 5N and O). These results suggest two possible explanations for the inability of *nkx*2.5 to compensate efficiently for the loss of *nkx*2.7 gene function: either the requirement for *nkx*2.5 is dose-dependent or there is unique role of *nkx*2.7 during SHF development.

Given the potential sensitivity of the *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ phenotype to *nkx* gene dosage suggested by progressive ventricular-to-atrial identity exchange in the allelic series (Fig. S1), we hypothesized that the inability to achieve complete rescue in the *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryos could be overcome by increasing the levels of *nkx*2.5. Thus, we induced heat shock at two discrete time points, 11 somites and 21 somites, to enhance the Nkx2.5-EGFP expression at these crucial developmental stages (Fig. 6A). Indeed, incremental expression of *nkx*2.5 yields substantially improved ventricular chamber size, enhanced ventricular identity maintenance, and decreased ectopic S46^{+} cells (compare Figs. 6A with 5L and N). To extend these analysis further, we employed two *Tg(hsp70l:nkx2.5-EGFP)* carriers to induce overexpression of *nkx*2.5 in *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryos again at 11 somites and 21 somites separately (compare Figs. 6B and C with 5L and N). In embryos derived from two transgenic parents, the brightest transgenic fish were selected given the increased variability of Nkx2.5-EGFP expression in this population as compared to the relative uniformity of GFP fluorescence observed in embryos produced from single transgenic parent crosses. While amelioration of the ventricular dimensions is evident following double transgenic carrier expression at individual time points, complete rescue of *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryos is only achieved when heat shock is performed at two time points to deliver additional Nkx2.5-EGFP expression and to capture the specific windows of FHF and SHF differentiation (Fig. 6D). Thus, we conclude that *nkx* genes function in a dose-dependent manner to maintain

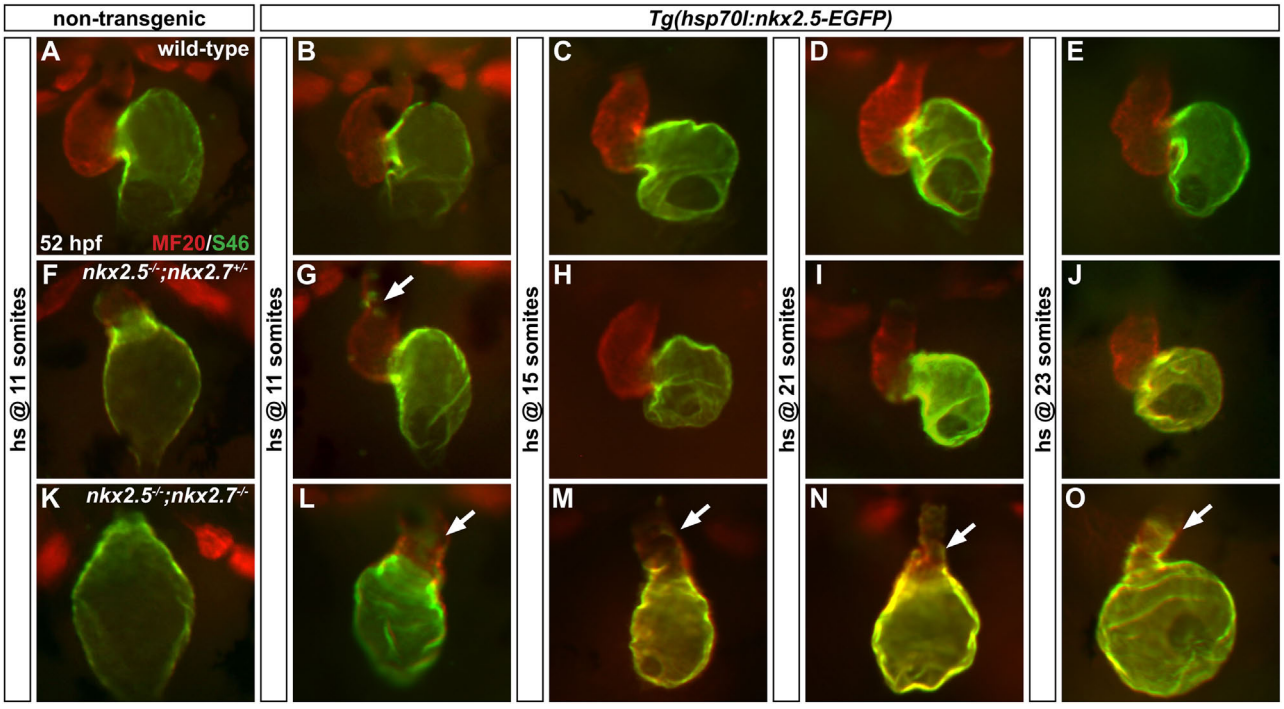


Fig. 5. Resupplying *nkx*2.5 in *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryos reveals dose-dependent functions of *nkx* genes. Frontal views, anterior to the top, of MF20/S46 immunofluorescence (as in Fig. 3) at 52 hpf. In non-transgenic wild-type (A) and *Tg(hsp70l:nkx2.5-EGFP)* embryos (B-E), cardiac morphology and chamber-specific identity are maintained following heat shock at 11 somites through 23 somites. In contrast, non-transgenic *nkx*2.5$^{-/-}$;*nkx*2.7$^{+/-}$ (F) and *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ (K) embryos have enlarged atrial chambers, underdeveloped or indiscernible ventricular chambers, and diminished outflow tracts. Following heat shock at the same time points as performed in the wild-type embryos, transgenic *nkx*2.5$^{-/-}$;*nkx*2.7$^{+/-}$ embryos (G-J) exhibit substantial improvement in ventricular chamber size with only a few residual ectopic S46^{+} cardiomyocytes remaining in embryos treated at 11 somites (G). However, only moderate rescue of abnormalities in morphology and identity is achieved in the transgenic *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryos following heat shock at 11 somites and 15 somites (L,M) and only minimal rescue is achieved following heat shock at 21 somites and 23 somites (N,O). White arrows indicate ectopic S46^{+} cardiomyocytes.

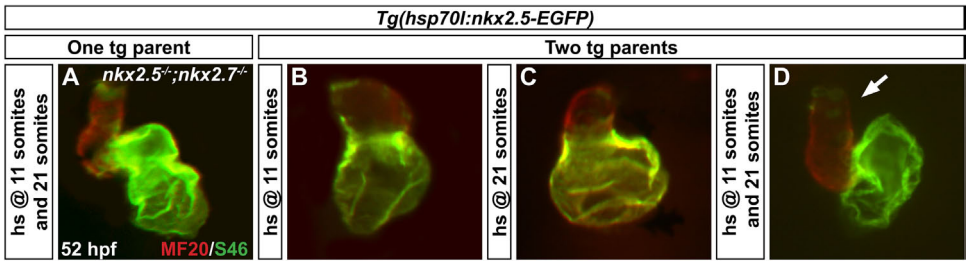
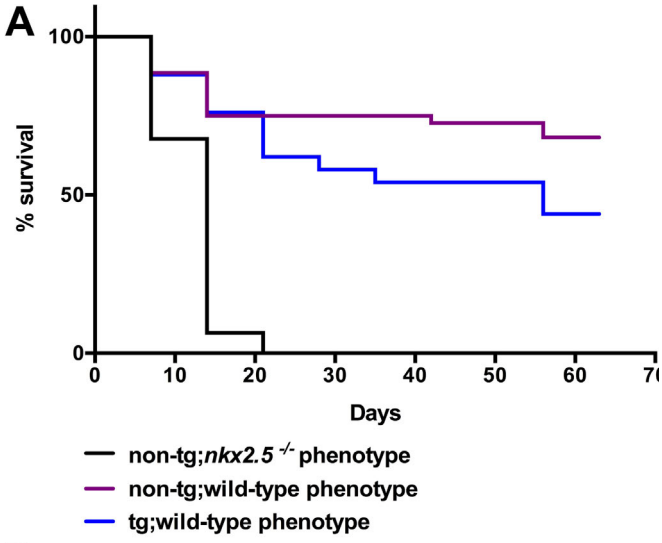


Fig. 6. Rescue of *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryos validates a dose-dependent role of *nkx*2.5 and suggests a unique function for *nkx*2.7. Frontal views, anterior to the top, of MF20/S46 immunofluorescence (as in Fig. 3) at 52 hpf. In contrast to *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ offspring of a single *Tg(hsp70l:nkx2.5-EGFP)* carrier following heat shock at one time point (Fig. 5L-O), a transgenic *nkx*2.5$^{-/-}$;*nkx*2.7$^{-/-}$ embryo subject to heat at 11 somites and 21 somites demonstrates enhanced rescue of ventricular and atrial size and identity defects (A). Despite only moderate improvement in offspring from a cross of two transgenic parents at 11 somites (B) and 21 somites (C), performing heat shock at two time points to augment *Tg(hsp70l:nkx2.5-EGFP)* expression further yields normalization of cardiac chamber morphology (D). Yet, residual, ectopic S46^{+} cardiomyocytes highlight chamber identity abnormalities in the late-differentiating SHF-derived population (D; arrow).

chamber-specific proportionality and identity. Intriguingly, while the chamber morphology and identity features are fully rescued following increased *nkx*2.5 gene dosage (Fig. 6D), ectopic S46^{+} cells are still present near the OFT. Although residual expression of this atrial identity marker in the SHF-derived cardiomyocytes at the arterial pole may represent additional *nkx* dosage requirements, our data also suggests a distinct role for *nkx*2.7 in this region. In summary, these results highlight the dose-dependent functions of *nkx* genes and reveal potential unique roles of *nkx*2.7 in the SHF given the inability of *nkx*2.5 to compensate in late-differentiating progenitors.

nkx2.5 expression prior to heart tube elongation Is sufficient for long-term survival

Given the importance of *nkx*2.5 transcriptional regulation in the adult myocardium (Akazawa and Komuro, 2003; Akazawa and Komuro, 2005; Takimoto et al., 2000), we explored the longevity of rescued *nkx*2.5$^{-/-}$ embryos in order to determine if survival into adulthood is achieved and whether there are functional consequences of *nkx*2.5 depletion following the embryonic period. Given our results highlighting the temporal and dose-dependent requirements of *nkx*2.5 during embryogenesis, we considered two potential hypotheses. First, resupplying *nkx*2.5 prior to heart tube elongation might be adequate to maintain chamber identity in the juvenile and adult *nkx*2.5$^{-/-}$ heart, leading to long-term survival and fertility. Alternatively, we hypothesized that repetitive doses of *nkx*2.5 overexpression would be required throughout embryonic and juveniles stages to preserve the chamber-specific characteristics necessary for adult cardiac health. To differentiate between these findings, we generated embryos from a cross of *nkx*2.5$^{+/-}$;*Tg(hsp70l:nkx2.5-EGFP)* and *nkx*2.5$^{+/-}$ fish, performed heat shock at 21 somites, and then separated non-transgenic from transgenic embryos according to EGFP expression at 24 hpf. Subsequently, based on live assessment at 52 hpf of all embryos, non-transgenic and transgenic animals were further sorted into subgroups according to phenotype (wild-type *versus* *nkx*2.5$^{-/-}$ morphology) and raised for 60 days with routine evaluation for survival. A Kaplan-Meier curve depicts the early death (<3 weeks) of all non-transgenic embryos displaying the *nkx*2.5$^{-/-}$ phenotype, while the non-transgenic and transgenic embryos with wild-type morphology demonstrate minimal attrition in the second and third week of life (Fig. 7A). After 60 days, all surviving embryos were genotyped for the *nkx*2.5^{vu179} mutation. As expected, 100% of the non-transgenic fish were wild-type or heterozygous for the *nkx*2.5^{vu179} allele confirming that all *nkx*2.5$^{-/-}$ embryos in this cohort perished during the embryonic and juvenile periods (Fig. 7B). In contrast, our genotyping results reveal a near-Mendelian distribution of the *nkx*2.5^{vu179} allele in the transgenic embryos with wild-type morphology (Fig. 7B). Excitingly, the increased proportion of genotyped *nkx*2.5$^{-/-}$ embryos in the transgenic group highlights our



B

Clutch	Genotype		
	% *nkx2.5*$^{+/+}$	% *nkx2.5*$^{+/-}$	% *nkx2.5*$^{-/-}$
non-tg;wild-type phenotype	38% (10)	62% (16)	0%
tg;wild-type phenotype	14% (3)	50% (11)	36% (8)

Fig. 7. Re-expression of *nkx*2.5 prior to heart tube elongation results in healthy *nkx*2.5$^{-/-}$ adults. (A) Kaplan-Meier survival curve depicts the pattern of larval death following phenotypic assessment of non-transgenic *nkx*2.5$^{-/-}$ embryos in addition to non-transgenic and *Tg(hsp70l:nkx2.5-EGFP)* wild-type embryos. All animals were exposed to heat shock at 21 somites, sorted according to EGFP fluorescence, and subsequently screened morphologically at 52 hpf. While non-transgenic *nkx*2.5$^{-/-}$ embryos perish within the first 3 weeks, 65% and 44% survival is observed in the non-transgenic and transgenic wild-type clutches, respectively. (B) Genotyping results for the non-transgenic phenotypically wild-type cohort and transgenic phenotypically wild-type cohort reveal *nkx*2.5$^{-/-}$ embryos in the transgenic rescued group only. Percentages represent the proportion of embryos with a particular genotype in each phenotypic cohort; the number of embryos in each group is noted in parentheses.

finding that early overexpression of *nkx*2.5 is sufficient to sustain cardiac function and ensure embryonic viability into adulthood.

To extend our analysis of the adult rescued *nkx*2.5$^{-/-}$ fish, we were interested to determine their degree of fertility and to assess their ability to produce maternal zygotic *nkx*2.5$^{-/-}$ offspring (*mznkx*2.5$^{-/-}$ embryos). Indeed, their fecundity is normal (Fig. S4A), yielding embryos with an unremarkable body axis (Fig. S4F). Although there is no evidence of *nkx*2.5 maternal expression detected with RT-PCR (data not shown), we proceeded to examine the

phenotype achieved in the *mznkx*2.5$^{-/-}$ embryos to confirm the absence of a subtle maternal effect due to low levels of early *nkx*2.5 expression. Similar to the zygotic *nkx*2.5$^{-/-}$ phenotype, *mznkx*2.5$^{-/-}$ embryos exhibit pericardial edema (Fig. S4B and F), abnormal looping, and diminished ventricular and bulbous atrial chambers (Fig. S4C and G). Given that the earliest manifestation of abnormal cardiac development in *nkx*2.5$^{-/-}$ embryos occurs during heart tube elongation (Targoff et al., 2013), we also examined cardiac chamber-specific markers immediately prior to this stage to inspect for evidence of maternal *nkx*2.5 loss-of-function effects. Corroborating previous results (Targoff et al., 2013), our data reveal normal *vmhc* (Fig. S4D and H) and *amhc* (Fig. S4E and I) expression patterns in the cardiac cones of both *nkx*2.5$^{-/-}$ and *mznkx*2.5$^{-/-}$ embryos. In summary, these findings validate the absence of a maternal role for *nkx*2.5 and highlight our ability to recapitulate the *nkx*2.5$^{-/-}$ phenotype with *mznkx*2.5$^{-/-}$ embryos.

In light of successful survival and productive fertility of the rescued *nkx*2.5$^{-/-}$ fish, we sought to probe deeper into the cardiac morphology of the *nkx*2.5-deficient adult heart following overexpression of Nkx2.5-EGFP at 21 somites. In order to ensure comparison of fish at similar stages of cardiac maturity (Singleman and Holtzman, 2012), we selected age- and size-matched non-transgenic wild-type and transgenic wild-type and *nkx*2.5$^{-/-}$ (rescued) fish that were originally heat-shocked at 21 somites. Following dissection, gross morphology (Fig. 8A,C,E) and cardiac chamber identity (Fig. 8B,D,F) were assessed with brightfield microscopy and MF20/S46 immunofluorescence, respectively. Applying previously established quantitative morphometrics to assess for normal postembryonic cardiac growth (Singleman and Holtzman, 2012), we observed no statistically significant difference in ventricle length (VL), ventricle width (VW) and bulbous arteriosus length (BAL) between non-transgenic and transgenic wild-type hearts (Fig. 8G). However, VL and VW were slightly increased in the adult rescued *nkx*2.5$^{-/-}$ fish when compared to non-transgenic wild-type fish, suggesting mild ventricular expansion. Thus, while subtle variation in ventricular chamber size of the adult rescued *nkx*2.5$^{-/-}$ fish warrants further investigation of the role of *nkx*2.5 during cardiac maturation, these findings highlight the early and limited requirement of *nkx*2.5 in establishing normal cardiac morphology and chamber identity in the adult heart.

Taken together, our data demonstrate the crucial, long-term benefits of *nkx*2.5 expression prior to heart tube formation in securing chamber-specific identity and in maintaining embryonic survival into adulthood. These intriguing results broaden our appreciation of the essential functions of early cardiac transcriptional regulation in establishing long-standing myocardial health. We anticipate that this innovative concept has the potential to shed light on novel mechanisms underlying cardiac disease in adult patients harboring *NKX*2-5 mutations and to enhance future therapeutic strategies aimed at regeneration of differentiated myocardium.

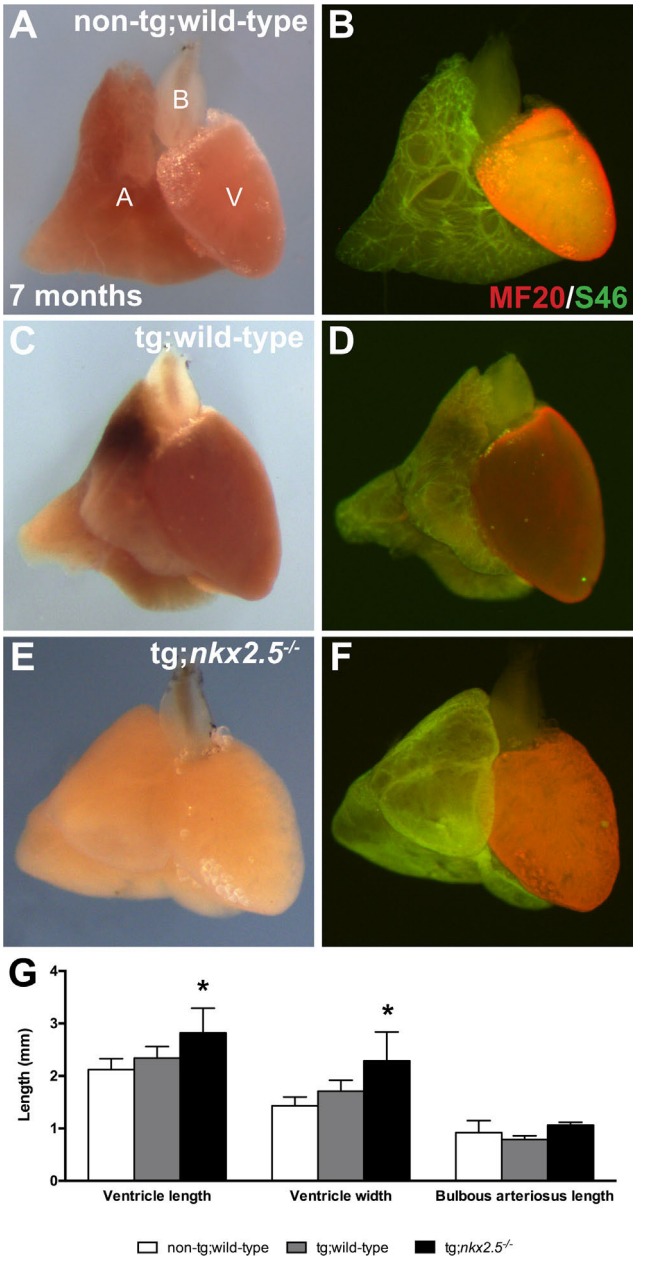


Fig. 8. Adult rescued *nkx*2.5$^{-/-}$ fish exhibit normal cardiac morphology and chamber identity. Whole mount images of 7-month-old non-transgenic (A,B) and *Tg(hsp70l:nkx2.5-EGFP)* (C-F) wild-type (A-D) and *nkx*2.5$^{-/-}$ (E,F) hearts. MF20/S46 immunofluorescence distinguishes ventricular (red) from atrial (green) myocardium. Dissected hearts are positioned ventrally with arterial poles to the top. "A" denotes atrium, "V" denotes ventricle, and "B" denotes bulbous arteriosus. All embryos were originally heat-shocked at 21 somites. All fish were genotyped prior to dissection for morphometric analyses. (A,C,E) Similar cardiac morphology is depicted in non-transgenic and transgenic wild-type and transgenic rescued adult *nkx*2.5$^{-/-}$ hearts. (B,D,F) Cardiac chamber identity is maintained following heat shock at 21 somites in non-transgenic and transgenic wild-type and transgenic rescued adult *nkx*2.5$^{-/-}$ hearts. (G) Bar graph indicates ventricle length (VL), ventricle width (VW) and bulbous arteriosus length (BAL) in non-transgenic wild-type ($n=8$), transgenic wild-type ($n=7$), and transgenic *nkx*2.5$^{-/-}$ ($n=2$) fish. Mean and standard error of each data set are shown with detection of statistically significant differences between VL and VW of transgenic rescued *nkx*2.5$^{-/-}$ and non-transgenic wild-type fish ($p < 0.01$).

Discussion

Our studies reveal the previously unrecognized early requirement for *nkx* genes in preserving cardiac chamber morphology and identity later in development. In addition to the established functions in patterning the lateral plate mesoderm, we show that *nkx*2.5 expression during cardiomyocyte differentiation is essential to maintain ventricular and atrial characteristics during chamber emergence. Specifically, we propose that aspiring ventricular progenitor cells require *nkx* transcriptional regulation in a dose-dependent manner to preserve molecular and cellular chamber-specific features. Furthermore, cardiomyocytes of the FHF and SHF are primed to receive these inputs at different times during development mirroring the distinct phases of specification and differentiation that occur in these unique populations. Finally, our data support a model in which *nkx* genes induce downstream targets to ensure chamber-specific identity within malleable ventricular cardiomyocytes and to sustain cardiac function into adulthood.

Although it is valuable to link early roles of *nkx*2.5 with later functions in both the FHF-derived and SHF-derived myocardium, the precise underlying mechanisms have yet to be fully uncovered. Recent studies imply potential direct and indirect pathways could be responsible for mediating the functions of *nkx*2.5 in chamber identity maintenance. Reciprocal direct regulation of *vmhc* in the ventricular and atrial chambers may enforce chamber-specific features in the FHF that endure in the adult myocardium (Jin et al., 2009; Zhang and Xu, 2009). Alternatively, the indirect functions of Bmp signals downstream of *Nkx*2-5 may be critical in preserving the identity of the late-differentiating SHF-derived progenitors (Prall et al., 2007). Moreover, recent analysis of embryos injected with anti-*nkx*2.5 morpholino hints at the possibility that *nkx*2.5 functions genetically upstream of *ltbp*3 to promote SHF contribution to the ventricular chamber (Guner-Ataman et al., 2013). Consistent with our previous data demonstrating downregulation of *ltbp*3 in *nkx*2.5$^{-/-}$ embryos (Targoff et al., 2013), we indeed observe a subtle decrease in the addition of late-differentiating cardiomyocytes to the OFT of wild-type *versus* *nkx*2.5$^{-/-}$ embryos (Fig. 4C compared with F). Yet, further investigation of this phenotypic discrepancy is required to confirm the specific nature of the role of *ltbp*3 downstream of *nkx* genes in arterial pole development. Regardless of the exact molecular mechanisms through which Nkx factors direct chamber identity, future studies will help to distinguish between distinct temporally controlled processes underlying the roles of *nkx* genes in the FHF- and SHF-derived progenitors. Finally, given the temporal overlap in rescue of chamber-specific proportions, identity, and cardiomyocyte number in the transgenic *nkx*2.5$^{-/-}$ embryo, it is intriguing to envisage that *nkx* genes act high in the transcriptional regulatory cascade. Ultimately, dissecting the timing of the impact of *nkx* genes in specific cardiomyocyte populations will enhance the application of insights from developmental models to innovative paradigms of ventricular differentiation.

Importantly, our understanding of the temporal regulation of *nkx* genes and their requirement prior to heart tube formation for adult cardiac function opens doors to a greater appreciation of congenital heart disease pathology and cardiomyopathy. While *Nkx*2-5 is expressed in the adult myocardium (Kasahara et al., 1998; Komuro and Izumo, 1993; Shiojima et al., 1996), its essential postnatal functions have yet to be fully elucidated. Ventricular-specific conditional deletion and mid-embryonic deletion of murine *Nkx*2-5 emphasize critical roles in maintaining the cardiac conduction system and normal contraction in the adult heart (Pashmforoush et al., 2004; Terada et al., 2011). Interestingly, our data exhibit successful, long-term rescue of *nkx*2.5$^{-/-}$ embryos following Nkx2.5-EGFP expression during somitogenesis. These findings are particularly exciting as they stress the ability for the cardiac genetic transcriptional program to be reset in the context of adequate *nkx* gene dosage during embryogenesis. Yet, it remains possible that *nkx*2.7 may compensate for the loss of *nkx*2.5 or that low-grade expression of *nkx*2.5 may result from leakiness of the *hsp*70*l* promoter (Hans et al., 2011, 2009), although this explanation is unlikely given our data highlighting the absence of EGFP expression by 10-hours post-heat shock. Alternatively, it is also fascinating to consider whether the rescued *nkx*2.5$^{-/-}$ hearts are instead compromised during juvenile or adult stages of cardiac growth. For example, a murine study suggests that perinatal loss of *Nkx*2-5 can lead to impaired conduction and contractility secondary to reduced expression of ion channel genes and defective Na^+ and Ca^{2+} handling (Briggs et al., 2008). Thus, despite the ability of rescued *nkx*2.5$^{-/-}$ embryos to preserve cardiac function and survival into adulthood, *nkx*2.5 may be required in the adult heart to sustain particular identity features necessary for efficient action potential transmission. These studies inspire further investigation of the patterning and function of postnatal ventricular, atrial and conduction system cardiomyocytes in the rescued *nkx*2.5$^{-/-}$*;Tg(hsp*70*l:nkx*2.5*-EGFP)* fish.

Through illustration of the early requirement of zebrafish *nkx*2.5 in maintenance of cardiac chamber identity, our studies complement and extend work in other model organisms. Previous studies demonstrate overlapping expression patterns of the multiple homologs of *Nkx* genes in various model systems and have pointed to precise functions at specific developmental windows in particular regions of the heart. *Nkx*2-5 and *Nkx*2-6 play essential roles in murine cardiac and pharyngeal morphogenesis (Tanaka et al., 2001), yet mice deficient for *Nkx*2.6 alone have no cardiac abnormalities (Tanaka et al., 2000). However, expression of *Nkx*2-6 is redundant with *Nkx*2-5 in the sinus venosus at E8.5 and in the OFT at E9.5 (Biben et al., 1998), thus intimating at potential roles of *Nkx*2-6 in developing poles of the heart. In *Xenopus*, *XNkx*2-10 expression resembles *XNkx*2-5 and *XNkx*2-3 cardiac mesodermal patterns at the outset (Newman et al., 2000). But, following initiation of cardiac differentiation markers, *XNkx*2-10 transcipts fade in the heart and remain abundant in the pharyngeal endoderm (Chambers et al., 1994; Drysdale et al., 1994). Interestingly, reduction of XNkx2-10 leads to anterior and cardiac defects during later stages of development, following looping and chamber emergence (Allen et al., 2006). Notably, the XNkx2-10 protein is more similar in length and its homeodomain is most identical to zebrafish Nkx2.7 than to the other Xenopus homologs, XNkx2-5 and XNkx2-3 (Newman et al., 2000). Synthesizing our findings with those from mouse and *Xenopus* studies yields a new possible explanation for these complex phenotyes: *Nkx* homologs function disparately in the early- and late-differentiating cardiomyocytes derived from the FHF and SHF. Specifically, in the *nkx*2.5$^{-/-}$*;nkx*2.7$^{-/-}$ embryos from two transgenic parents heat-shocked twice, the presence of residual S46$^+$ cells corroborates the notion that *nkx* gene dosage is crucial in establishing chamber identity. Yet, these findings also strongly suggest that the OFT is exquisitely sensitive to *nkx*2.7, highlighting its unique role in this region derived from SHF progenitors. The generation of temporally inducible transgenes to modulate *nkx*2.7 function would prove particularly beneficial to test this hypothesis. In summary, our data underscore the early redundant requirements of *nkx*2.5 and *nkx*2.7 in maintenance of ventricular identity of the FHF-derived myocardium while possible divergent functions become evident later in development; *nkx*2.7 may take on a separate role in the anterior SHF, mirroring expression patterns and functions of *Nkx*2-6 and *XNkx*2-10.

In conclusion, deciphering the precise temporal windows during development when *Nkx* transcriptional activity is required will improve our ability to direct differentiation of ventricular cardiomyocytes and to uncover the dynamics of morphogenetic errors in patients carrying *NKX*2-5 mutations. These studies shed light on the redundant, yet distinct roles of *nkx* genes by highlighting their ability to rescue cardiac morphology, chamber identity, and function during critical developmental periods in discrete FHF and SHF lineages. Altogether, our insights offer new directions to enhance rapid advances in novel cardiac tissue engineering and regenerative therapies to combat congenital heart defects and cardiomyopathies.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2014.12.019.

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