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14-3-3epsilon Controls Multiple Developmental Processes in the Mouse Heart

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Abstract

Background—14-3-3ε plays an important role in the maturation of the compact ventricular myocardium by modulating the cardiomyocyte cell cycle *via* p27^{kip1}. However, additional cardiac defects are possible given the ubiquitous expression pattern of this protein.

Results—Germline deletion of 14-3-3ε led to malalignment of both the outflow tract (OFT) and atrioventricular (AV) cushions, with resulting tricuspid stenosis and atresia, mitral valve abnormalities, and perimembranous ventricular septal defects (VSDs). We confirmed myocardial non-compaction, and detected a spongy septum with muscular VSDs and blebbing of the epicardium. These defects were associated with abnormal patterning of p27^{kip1} expression in the subendocardial and possibly the epicardial cell populations. In addition to abnormal pharyngeal arch artery patterning, we found deep endocardial recesses and paucity of intramyocardial coronary vasculature as a result of defective coronary plexus remodeling.

Conclusions—The malalignment of both endocardial cushions provides a new explanation for tricuspid and mitral valve defects, while myocardial non-compaction provides the basis for the abnormal coronary vasculature patterning. These abnormalities might arise from p27^{kip1} dysregulation and a resulting defect in epithelial to mesenchymal transformation. These data suggest that 14-3-3ε, in addition to left ventricular non-compaction (LVNC), might be linked to different forms of congenital heart disease (CHD).

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Keywords

14-3-3; mouse; heart development; cardiac outflow tract; ventricular septal defect; endocardial cushion; myocardial hypoplasia; coronary artery hypoplasia

Introduction

The 14-3-3 proteins are involved in several signal transduction cascades both *in vitro* and *in vivo* (Xing *et al.*, 2000; Du *et al.*, 2005; Brunelli *et al.*, 2007) and bind to phosphoserine/threonine containing-motifs (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). The 14-3-3 family consist of at least seven isoforms (gamma, beta, epsilon, zeta, eta, sigma, and tau/theta), all with slightly different functions. Although all isoforms are involved in apoptosis (Gardino and Yaffe, 2011), only zeta and eta have been shown to inhibit cardiomyocyte apoptosis (Xing *et al.*, 2000). In addition to being expressed in cardiomyocytes (Kosaka *et al.*, 2012), and cardiac fibroblasts (Du *et al.*, 2005), 14-3-3 ϵ (*Ywhae* gene, but henceforth 14-3-3 ϵ , protein; *14-3-3 ϵ* , gene) plays a role in endothelial differentiation (Brunelli *et al.*, 2007). Moreover, 14-3-3 ϵ is expressed in neural crest cells (NCCs) (McConnell *et al.*, 1995) and vascular smooth muscle cells (VSMCs) (Umahara *et al.*, 2012), and it is linked to several cardiac developmental gene pathways, including TGF β signaling (McGonigle *et al.*, 2001).

In mice, *14-3-3 ϵ* is essential for the formation of the compact ventricular myocardium through the regulation of the cardiomyocyte cell cycle (Kosaka *et al.*, 2012). Deletion of *14-3-3 ϵ* can increase the levels of p15^{Ink4b}, p57^{Kip2}, and p27^{Kip1} (*Cdkn1b* gene, but henceforth p27^{Kip1}, protein; *p27^{Kip1}*, gene) while decreasing cyclin E, thus impairing the function of the cdk2-cyclin E complex and leading to G₁ arrest (Kosaka *et al.*, 2012). Since p57^{Kip2} has a limited spatio-temporal expression in cardiac development (Burton *et al.*, 1999; Kochilas *et al.*, 1999) and p15^{Ink4b} can regulate p27^{Kip1} (Reynisdottir and Massague, 1997), p27^{Kip1} has been proposed as the key mediator of G₁ arrest in *14-3-3 ϵ ^{-/-}* mice (Kosaka *et al.*, 2012). Moreover, p27^{Kip1} is found in many tissues, including VSMCs (Neukamm *et al.*, 2008), and endothelial cells (Sakai *et al.*, 2010); and both cardio-specific overexpression (Lavine *et al.*, 2008) and germline deletion of *p27^{Kip1}* (Merchant *et al.*, 2016) leads to ventricular non-compaction in mice. Although the study by Kosaka *et al.* has identified ventricular septal defects (VSDs) in *14-3-3 ϵ ^{-/-}* mice (Kosaka *et al.*, 2012), this work primarily focused on the non-compaction phenotype. However, other cardiac defects are possible given the ubiquitous cardiac expression of 14-3-3 ϵ .

The aim of this study was to comprehensively analyze the cardiac defects of *14-3-3 ϵ ^{-/-}* mice by visualizing the atrial and ventricular myocardium, second heart field-derived cells, epicardium and epicardium-derived cells (EPDCs), endocardium, and NCCs. We found a large number of different cardiac malformations from subtle to severe. These data suggest that *14-3-3 ϵ* is required for normal cardiac development and that, consistent with the recent findings in patients with left ventricular non-compaction (LVNC) (Chang *et al.*, 2013), mutations of the *14-3-3 ϵ* gene or its impaired expression might be involved in congenital heart disease (CHD) in humans.

Results

Among all 33 *14-3-3e*^{-/-} embryonic hearts (E12.5 to E18.5) that were analyzed only one (E15.5) was found without a phenotype. Comparing the *14-3-3e*^{-/-} mouse hearts with the normal wild type mouse hearts, the defects were categorized according to the age group (Table 1). The E11.5 hearts (n. 3) were only used to analyze the abnormalities at the tissue level as these samples are too young to determine a true phenotype, and the E18.5 hearts (n. 10) were analyzed for both cardiac malformations and coronary vascular problems (Table 2a,b). The aortic arch could not be studied at E18.5 since hearts were isolated from the embryo without the aortic arch system. The main abnormalities were identified in the endocardial cushion structures, the ventricular myocardium, the epicardium, and the vascular structures, including the intramyocardial coronary arteries.

Endocardial cushions

In wild type embryos, we distinguished outflow tract (OFT) endocardial cushions that extend into the myocardial OFT, being the septal and parietal extension, and at the orifice level two intercalated cushions that in concert contribute to the formation of the semilunar valve leaflets. In the atrioventricular (AV) canal we distinguished the AV endocardial cushions (superior, inferior and two lateral cushions). In the stages studied, the superior and inferior AV cushions had already fused so we will refer to an AV cushion mass with an inferior and a superior part. Also the OFT cushions have fused at the orifice level at these stages, leaving recognizable only the proximal extensions into the myocardial OFT (Figure 1a–e). There was no significant difference in volume of the cushions between the *14-3-3e*^{+/+} and the *14-3-3e*^{-/-} embryos, although there were differences in architecture as described below. Immunohistochemistry relevant to endocardial cushion development was performed with the endothelial marker CD31 (PECAM), which was specifically useful for distinguishing the described OFT cushion structures (Figure 1e,j).

Malalignment of OFT and AV cushions in *14-3-3e*^{-/-} embryos—In *+/+* embryos, the parietal OFT cushion connects to the superior part of the AV cushion mass and the septal cushion does so at a more proximal level (Figure 1a,b,d; Figure 2a–d; and interactive pdf in Supplemental Figures 1 (E12.5 *+/+*), 2 (E12.5 *-/-*), 3 (E13.5 *+/+*), and 4 (E13.5 *-/-*)). An abnormal alignment was evident in 1/3 of E12.5 and 3/5 of E13.5 *14-3-3e*^{-/-} embryos (Table 2). In two E13.5 embryos, solely the parietal OFT cushion contacted the right lateral AV cushion instead of the superior AV cushion mass (Figure 2f–j; and Supplemental Figure 4). In the one E12.5 (Figure 1f–j; and Supplemental Figure 2) and in one E13.5 heart both the parietal and septal OFT cushions have fused with the lateral AV cushion, instead of the superior AV cushion mass. These malalignment defects were accompanied by perimembranous VSDs (Figure 2g–j). In one E13.5 *14-3-3e*^{-/-} embryo there was an AV septal defect (AVSD) with a common AV valve with left ventricular (LV) dominance. The one E12.5 *14-3-3e*^{-/-} heart with an abnormal fusion of both parietal and septal endocardial cushions to the lateral AV cushion and an interventricular communication (Figure 1i,j) was too young to designate a VSD as normally an interventricular communication is still present at this stage.

Normally the aortic intercalated cushion merges with the parietal OFT cushion before merging with the superior part of the AV cushion complex. In one E12.5 and one E13.5 *14-3-3e*^{-/-} mouse, the aortic intercalated cushion (belonging to the future non-coronary aortic semilunar valve cusp) could be found as a separate structure next to the parietal cushion extending deeply into the ventricular muscular OFT (Table 2a and Figure 1g,h). At older stages (E14.5 and E15.5), the fusion of the cushion tissue masses did not allow us to separately distinguish the components of the various cushions.

Abnormal p27^{kip1} patterning but intact TGFβ signaling in 14-3-3e^{-/-} hearts—

The p27^{kip1} expression showed some interesting characteristics in the *14-3-3e*^{+/+} mouse. The protein was expressed in most endocardial cells, most markedly in the cuboid cells, which were variably seen, lining the developing semilunar and AV valves (Figure 3a–c). There were few positive cells in the cushion mesenchyme. This was different in the p27^{kip1}-stained knock-out embryos where again the cuboid endocardial cells were positive but also clusters of subendocardial cushion mesenchymal cells could be identified. This could be seen in both leaflets of the mitral (Figure 3d,e) and tricuspid valves as well as the semilunar valves (Figure 3f). The distribution of migrating NCCs ascertained by AP2α staining was also investigated, but we did not identify a marked difference between wild type and knock-out embryos (data not shown). Moreover, since *14-3-3e* is a regulator of TGFβ signaling (McGonigle et al., 2001) and defects in TGFβ2 can cause abnormal epithelial-to-mesenchymal and endothelial-to-mesenchymal transformation (Zeng *et al.*, 2013), we tested the involvement of TGFβ signaling. We did not detect differences in the expression pattern of TGFβ receptor I (TGFβRI) (data not shown), and results for TGFβ2 were inconclusive due to a weak signal. We also evaluated the downstream effector phospho-Smad2, but again we did not identify any differential expression pattern between wild type and knock-out embryos (data not shown).

Defective mitral and tricuspid valves, and perimembranous VSDs—At all examined time points we identified mitral and tricuspid valve abnormalities (Table 2a,b). Four cases showed an abnormal mitral valve morphology mostly characterized by thickened leaflets and stenosis (Table 2a,b, Figure 4c compare to b). Abnormalities of the tricuspid valve, including severe tricuspid valve stenosis (Figure 4g,h), were found in five cases and correlated with RV hypoplasia (Figure 4d compare to a,b) and a long and narrow RV OFT (Table 2a). Multiple perimembranous VSDs (Figure 4c; Figure 2 h–j), correlating with the malaligned endocardial cushions, were found in *14-3-3e*^{-/-} hearts. The knock-out hearts at both E13.5 (Fig 4g compare to e) and E14.5 (Figure 4h, compare to f) showed a hypoplastic myocardium as well as one case with a bicuspid aortic valve (Table 2a). The observed phenotypes persist at E18.5. At this time point, we uncovered tricuspid atresia ending as a blind dimple in the myocardium of the right ventricle without evidence of tricuspid valve formation (Figure 5c). In cases of tricuspid hypoplasia, small valve remnants were observed. Furthermore, we found subaortic (Figure 5d,f) and perimembranous VSDs. One case, in combination with a perimembranous VSD and mitral valve deformities, presented with a Gerbode defect, positioned between the left ventricular outflow tract and the right atrium (Figure 5b).

Thin compact myocardium, deep intertrabecular recesses, and muscular VSDs

We observed severe thinning of the compact ventricular myocardium in the three *14-3-3e*^{-/-} embryos at E11.5 (Figure 6a,b), where the trabeculae also appeared to be diminished. In 4/5 embryos at E13.5 the thinning of both the RV and LV free wall was clear (Figure 6d compare to c). In the 3-D reconstruction of the *14-3-3e*^{-/-} mouse the abnormal trabecular patterning compared to the *14-3-3e*^{+/+} mouse can be clearly distinguished as well as the thinner compact myocardium (Figure 6e,f). At E14.5 and E15.5 the phenomenon became less prominent with, however, deep intertrabecular recesses in the compact myocardium of the LV (Figure 4f, 5e,f). The thin compact myocardium was often found in combination with a spongy interventricular septum (Figure 2h-j, 4h, 5e,f, 6g). In the spongy septum at several places small muscular VSDs were found (Figure 4h, 6g). Some cases showed a large centrally located muscular VSD (Table 2a,b, Figure 6g) that resembled the central muscular VSD observed in human CHD. This phenomenon was maintained in E18.5 *-/-* embryos (Table 2b, Figure 5e,f) where we also showed an apparent paucity of myocardial coronary arteries (see below).

Epicardial Defects

The epicardium lines the cardiac wall of both the atria and the ventricles. “Blebbing” of the epicardium over the right and left ventricles was found in several *14-3-3e*^{-/-} embryos (Table 2, Figure 7a-d). Almost all hearts presenting with epicardial abnormalities also showed a thin compact myocardium and a spongy ventricular septum. The epicardium lining the ventricles was positive for WT-1, with no difference between *14-3-3e* *+/+* and *-/-* embryos (Figure 7a,b). With regard to p27^{kip1} expression, there appeared to be some patchy upregulation in the epicardium in the mutant embryos as compared to the controls at E12.5 and E13.5 (Figure 7c,d). This difference was not observed at E14.5 and E15.5. For technical reasons we were unable to perform double staining of p27^{kip1} and WT-1 so we could not distinguish the intramyocardial EPDC population which is considered to be the main source of intracardiac fibroblasts and smooth muscle cells. Therefore, it remains unclear whether fibroblasts have an increased expression of p27^{kip1} in *14-3-3e*^{-/-} embryos.

Vascular malformations

Side by side aorta and pulmonary trunk—Three E15.5 hearts showed a side by side ascending aorta and pulmonary trunk. This was always found in combination with an abnormal vertical orientation of the ductus arteriosus. The pulmonary trunk turned to its normal orientation once it connected to the ventricle, and the aorta was properly connected to the LV.

Pulmonary hypoplasia—Pulmonary trunk hypoplasia was detected in two E15.5 specimens. It was always found in combination with an abnormal orientation of the ductus arteriosus, and in combination with a side by side aorta and pulmonary trunk (Table 2a).

Retro-esophageal subclavian artery and aortic sling—Two specimens with a right retro-esophageal subclavian and two specimens with an aortic sling were observed. From E14.5, both aortic arch malformations were found in combination mainly with an abnormal orientation of the ductus arteriosus (Table 2a). The E18.5 embryos could not be evaluated for

their possible aortic arch phenotype as these hearts had been isolated from the embryo without arch connections.

Myocardial coronary vessel anomalies—In wildtype embryos, at later stages, the formation of a compact myocardium is accompanied by the development of coronary vessels to replace the intertrabecular endocardial finger-like projections, allowing oxygen and blood supply to the ventricular myocardium (Sedmera et al., 2000). We carried out immunofluorescence staining using endocardial and endothelial specific antibodies on E18.5 hearts to examine coronary vessel development. In wild type hearts, VEGFR2 was expressed in the intramyocardial coronary vessels of the compact myocardium but not in the endocardium of the trabecular zone, while endoglin was strongly expressed in the endocardium, with a lower expression in the coronary vessels (Figure 8a,e). In *14-3-3e*^{-/-} hearts, deep intertrabecular endocardial projections positive for endoglin and negative for VEGFR2 had invaded the compact myocardium, with some of them reaching the epicardial layer (asterisks in Figure 8b,f). Next, we analysed Cx40 expression because it is specifically expressed at this stage in the ventricular conduction system and in endothelial cells of coronary arteries (Miquerol et al., 2004). Similar to wild type hearts, Cx40 was detected in the atrioventricular bundle (AVB) and Purkinje fibers (PF) of mutant hearts (Figure 8c,d). However, we observed a drastic reduction in the number and size of Cx40-positive coronary arteries in mutants (arrows in Figure 8c–f). The phenotype of coronary artery hypoplasia was obvious especially at the apex of the heart and highly penetrant in E18.5 mutants (Table 2b).

To better understand the mechanisms underlying coronary vessel development, we performed whole mount immunofluorescence analysis on E18.5 hearts using anti-PECAM and VEGFR2 antibodies. Although the main coronary artery orifices and proximal main stems were not decreased in number/hypoplastic in either ventricle, the abnormal coronary vasculature phenotype was more severe in right ventricle, which is consistent with the frozen section results (Figure 8). In the external view of right ventricle apex (RVA), in which we analysed the sub- and epicardial vasculature, we identified a lack of well-organized and remodelled coronary plexus, with numerous vessels ending directly into the epicardium as well as no obvious subepicardial coronary vessels (Figure 9b–d compare to f–h). Although we did not observe a defect of the coronary plexus in the (sub-)epicardial vasculature of the left ventricular apex (Figure 9i,m), we still observed a defect in coronary artery remodeling in the internal views where deeper vessels can be investigated. In wild types, we detected arteries of varying sizes as the normal arterial tree remodels in a well-organized branching pattern (Figure 9j–l). Interestingly, we found that VEGFR2 detected only large coronary arteries and ceased at the branching points from large to small arteries (asterisks), while PECAM could still detect smaller coronary arteries beyond the branching points. In knock-outs, coronary vessels were formed by a small- and same-diameter caliber network visible with PECAM but not VEGFR2 (Figure 9n–p). We also could not detect VEGFR2-positive large coronary arteries in the internal views of the RVA. Moreover, we performed immunofluorescence with SM22, a marker of smooth muscle cells (SMC), and vimentin to stain fibroblasts. Using these markers, we showed that in *14-3-3e*^{-/-} hearts SMC is expressed at the subepicardial surface of the ventricles (Figure 10, arrowheads) and coronary

arteries (Figure 10, arrows) in both the right and left ventricles similarly to controls, although the development of coronary arteries appeared decreased in mutants. Moreover, vimentin-positive cells were detected in both control and knock-out hearts (Figure 10b,d,f,h). Altogether these data suggest that, while the conduction system and mesenchymal cells appear to differentiate normally, the defect in the development of the coronary vasculature is likely linked to a defect in coronary artery remodeling related to the non-compaction phenotype.

Discussion

Our phenotype analysis shows that germline deletion of the *14-3-3e* gene leads to a profound spectrum of cardiac malformations in all parts of the heart, with the exception of the inflow tract at the atrial level. Although a complete molecular mechanism for this wide range of defects is currently unknown, our findings suggest a defect in the p27^{Kip1} pathway, and the remodeling of the primitive coronary plexus, possibly related to compact myocardial thinning. Finally, the findings reported here represent the basis for new insight into the development of cardiac malformations in general.

p27^{Kip1} is upregulated in *14-3-3e*^{-/-} (Kosaka et al., 2012), and can play a crucial role at several levels because of its ubiquitous expression (Neukamm et al., 2008; Sakai et al., 2010; Merchant et al., 2016). In this study, we identified abnormal patterning of p27^{Kip1} in the myocardial wall as well as the endocardial and possibly the epicardial populations. This is consistent with studies showing that the epicardium, EPDCs, and, in zebrafish, the endocardium can contribute to myocardial compaction (Brutsaert, 2003; Gittenberger-de Groot et al., 2012; Kikuchi and Poss, 2012). In addition, *14-3-3e* is highly expressed in cardiac fibroblasts (Du et al., 2005), which can be formed by differentiated EPDCs and can play important roles in cardiac remodeling. Although we were unable to investigate p27^{Kip1} in fibroblasts, abnormal proliferation of these cells could contribute to the wide range of abnormalities uncovered in this study. A combination of disturbed proliferation in both cardiomyocytes and cardiac fibroblasts might also support the finding of deep intertrabecular recesses that connect to the subepicardial layer where epicardial blebbing is observed. However, as p27^{Kip1} can also regulate cell migration (Besson et al., 2004), future studies will need to test whether the migration of fibroblasts from the subepicardial layer might be involved in the generation of the deep intertrabecular recesses. These studies will help understand the mechanisms for the abnormal coronary vascularization with fistulae formation, as was reported in other models of abnormal myocardial to sub- and epicardial interaction (Lie-Venema et al., 2005; Red-Horse et al., 2010; Gittenberger-de Groot et al., 2012; Tian et al., 2013). Based on our findings with endocardial, endothelial and SMC markers, the role of the interaction of the endocardium/endothelium with both the myocardium and epicardium appears important and deserves further investigation.

The *14-3-3e*^{-/-} mouse shows the rare phenomenon of abnormal alignment of both OFT and AV endocardial cushions. At the tissue level, we observed a different patterning of the p27^{Kip1}-positive endocardium and the endocardial cushion mesenchyme. p27^{Kip1} was markedly expressed in the cuboid endocardial cells on the ventricular surface of the AV valves and the arterial surface of the developing semilunar valves. In the mutant embryos we

observed marked clusters of p27^{Kip1}-positive cells in the endocardial cushions, in which the mesenchymal cells did not seem to have dispersed. These findings suggest the possibility of an abnormal endocardial to mesenchymal transformation. Although the size of the endocardial cushions between wild type and *14-3-3e*^{-/-} hearts could not be compared due to a lack of appropriate tools to accurately assess cushion structures, the malalignment in the mutant embryos was very obvious, with the parietal and the septal OFT cushion not connecting normally to the AV cushion complex. The main problem appeared to be a deficient rightward expansion of the AV canal, which would explain the often observed tricuspid stenosis, and provide a new developmental etiology for this malformation. Hitherto tricuspid stenosis and atresia have been attributed to deficient looping and subsequent rightward expansion of the AV canal, as in the trisomy 16 mice with tricuspid malformations (Webb et al., 1999). Another new finding that we could not however link to a clear semilunar valve or OFT malformation was the extremely deep extension of the parietal cushion related to the intercalated aortic cushion. The frequent observation of a perimembranous VSD could be understood from the malalignment of outflow cushions and/or septal components (Gittenberger-de Groot et al., 2014; Poelmann et al., 2014). The finding of only a single case of AVSD supports the conclusion that these malformations are mostly restricted to the ventricular and OFT part of the heart. Finally, the multiple small muscular VSDs and the occasional larger muscular VSD could be the consequences of the defective development of the compact and trabecular myocardium (Gittenberger-de Groot et al., 2014).

From E15.5 onwards there was a clear increase in vascular OFT malformations, varying from a right aortic arch and an aortic sling to a retro-esophageal subclavian artery and an abnormal upright course of the ductus arteriosus. The latter has been described in the Ptx1 mutant mouse (Bergwerff et al., 1998), which however cannot yet be conclusively linked to the findings in *14-3-3e*^{-/-} hearts or p27^{Kip1} expression. Nevertheless, all differentiated cellular contributions to the arterial pole vasculature express 14-3-3e (McConnell et al., 1995) and p27^{Kip1} (Neukamm et al., 2008; Sakai et al., 2010). Remodelling of the aortic (pharyngeal) arch arteries is known to be related to NCC and the second heart field (Bartram et al., 2001; Molin et al., 2002; Molin et al., 2003; Poelmann et al., 2004; Ward et al., 2005; High and Epstein, 2008; Keyte and Hutson, 2012) as well as Notch (High and Epstein, 2008) and TGFβ signaling (Bartram et al., 2001; Molin et al., 2002; Molin et al., 2003). 14-3-3e binds to TGFβRI to enhance TGFβ signaling (McGonigle et al., 2001), and defects in TGFβ2, which also binds TGFβRI, can cause numerous cardiac anomalies, including abnormal epithelial-to-mesenchymal and endothelial-to-mesenchymal transformation (Zeng et al., 2013) as well as interruption of the aortic arch, double outlet right ventricle (DORV) and AV septal defects (Sanford et al., 1997; Bartram et al., 2001; Molin et al., 2002). However, we could not identify any abnormality in TGFβ signaling. Although 14-3-3e has not been implicated in the second heart field, it is highly expressed in NCCs (McConnell et al., 1995). As seen in ablation models, NCCs are very important in remodeling the aortic arch arteries as well as in myocardialization and fusion of the endocardial cushions during OFT septation (Poelmann et al., 1998; High and Epstein, 2008; Keyte and Hutson, 2012). As we did not identify any abnormality in the distribution of migrating NCCs as assessed by AP2α staining, the effect of knocking down *14-3-3e* in NCCs remains to be fully evaluated.

This report has a number of limitations. First, different backgrounds were used, including congenic 129S6, mixed 129S6/Black6, and congenic Black6. In contrast to our original description (Kosaka et al., 2012), this prevents a clear assessment of the specific influence of each individual background on disease severity and/or midgestation/perinatal lethality. Although we analyzed only a few congenic Black6 mice, the number of mutants did not depart from the expected mendelian distribution, suggesting no significant difference in midgestation lethality among genetic backgrounds. Although the presence of significant cardiac malformations in the majority of *14-3-3e*^{-/-} mice suggests no substantial difference in all three backgrounds, including cell proliferation, an in depth analysis will require future investigations. Finally, this study was not set up to quantify the results but aimed at describing the morphological variations to show qualitatively the spectrum of abnormalities which can be linked to the unique finding of outflow tract cushion malalignment, the novel finding of tricuspid valve problems, and coronary vessel abnormalities.

The identified defect in coronary vasculature remodeling, associated with an apparent reduction in the number and size of coronary vessels, suggests that *14-3-3e* is a regulator of coronary vessel development, and the remodeling of the primitive coronary sinus in particular. This finding highlights the link between non-compaction and coronary vessel development, as was recently reported in the endocardial overexpression of *manic fringe* (MFng) (D'Amato et al., 2015). Moreover, these findings suggest that *14-3-3e* might play important roles in the patterning of subepicardial endothelial cells derived from the sinus venosus (Red-Horse et al., 2010; Tian et al., 2013) or in the arterial development from the endocardium and epicardium (Wu et al., 2012), consistent with the known role of *14-3-3e* in endothelial cell differentiation (Brunelli et al., 2007).

In conclusion, the *14-3-3e*^{-/-} mouse represents a new model for understanding cardiac development. The unique combination of abnormal alignment of both the OFT and AV cushions leading to tricuspid and mitral valve abnormalities have not been reported before, and might be related to the abnormal patterning of p27^{Kip1}. These data also indicate that *14-3-3e* might be involved in the development of myocardial coronary arteries in humans, consistent with the reported role of *14-3-3e* in LVNC (Chang et al., 2013). Future studies should concentrate on the molecular pathways that link *14-3-3e* to known mutations in the human population that underlie these defects.

Experimental Procedures

Mouse strains

A complete developmental series of *14-3-3e*^{-/-} mice ranging from E11.5 to E15.5, and E18.5 were investigated, using wild type embryos of comparable age as control (Table 1). We analyzed *14-3-3e*^{-/-} embryos in different backgrounds, i.e. congenic 129S6 (aka 129SVE) as reported previously (Kosaka et al., 2012), mixed 129S6/Black6, and congenic Black6 background. Genotyping was performed on the yolk sac DNA (e10.5 – e12.5) and the tail DNA (e13.5 and older) by PCR (Kosaka et al., 2012).

Immunohistochemistry

The embryos were fixed in 4% PFA and afterwards embedded in paraffin. A selection of the wild type embryos and all the available *14-3-3e*^{-/-} embryos were sectioned at 5µm in a transverse plane with a Leica RM2255. The sections were mounted serially (1:5), so five different immunohistochemical stainings could be compared from each embryo. For the evaluation of the myocardial architecture a series of E18.5 embryos was used, subjected to similar procedures as indicated above.

Immunohistochemistry was performed on tissue fixed in PFA. The slides were rehydrated and heated in 0.01M citric buffer (pH=6.0) to 97 °C for 12 minutes to expose the antigen, except for the slides stained for MLC2a. For inhibition of endogenous peroxidase, slides were treated with 0.3% H₂O₂ diluted in PBS for 20 minutes. Antibodies were diluted in 0.05% Tween 20 (PBS-T)/1% BSA for blocking and applied on the slides. The antibodies used were MLC2a (1:2000; Kubalak), WT-1 (1:500; Calbiochem, VWR), PECAM (1:4000; Sanquin Amsterdam), AP2α (1:500; GeneTex Inc), TGFβRI (1:100; Santa Cruz), TGFβ2 (1:100; Santa Cruz), Phospho-Smad2 (Ser465/467) (1:100; Cell Signaling Technology), and Nkx2.5 (1:4000; Santa Cruz, bio-connect). The slides were incubated overnight at room temperature. Secondary antibody goat anti-rabbit-biotin (1:200; Vector labs, Brunschwig chemie) with normal goat serum (1:66; Vector labs, Brunschwig chemie) diluted in PBS-T was applied on the slides for one hour, except for the Nkx2.5 slides, on which horse anti-goat (1:200; Vector labs, Brunschwig chemie) with normal horse serum (1:66; Vector labs, Brunschwig chemie) diluted in PBS-T was applied. For visualization slides were incubated with avidin-biotin horseradish peroxidase complex (ABC) with DAB (3,3'-diaminobenzidine) and counterstained with haematoxylin. They were dehydrated and mounted with permount.

Immunofluorescence

For immunofluorescence for VEGFR2 (1:50; R&D SYSTEMS), endoglin (1:50; DSHB), and Cx40 (4µg/ml) (Gros et al., 1994), SM22 (1:500; Abcam), and Vimentin (1:500; Covalab), the dissected E18.5 hearts were fixed with 4% PFA for 2-3 hours at room temperature and washed them in PBS. They were subsequently incubated overnight in two successive baths of increasing concentrations of sucrose (15% and 30% sucrose in PBS). Finally, samples were embedded in Optimal Cutting Temperature (OCT, Euromedex) solution and stored at -80 °C before sectioning (20 µm) using a cryostat (Leica CM3050S). Frozen sections were incubated with a 0.2% permeabilization solution (0.2% triton X-100 in PBS) for 20 minutes and blocked for 1 hour in saturation buffer (2% BSA, 0.05% saponin in PBS). Samples were subsequently incubated overnight at 4°C in the presence of primary antibodies. The next day, sections were washed and incubated with secondary antibodies conjugated with different fluorophores for 1 hour at room temperature. Finally, sections were washed and counterstained with Hoechst (Sigma) to detect nuclei.

For whole-mount immunofluorescence with VEGFR2 and PECAM (1:500; BD Pharmingen), fixed embryonic hearts were washed in PBS, incubated in 0.5% permeabilization solution for 1 hour and in saturation buffer (3% BSA, 0.1% Triton X100 in PBS) for 3 hours. The primary antibodies were incubated in saturation buffer for two days at

4°C. Hearts were incubated with secondary antibodies in saturation buffer for one day at 4°C and washed for a several hours. They were embedded in 4%LMP agarose, and observed under a Zeiss LSM780 confocal microscope. Analysis and 3D visualization of confocal data were performed with Volocity Imaging software.

Imaging

The bright field images were taken with an Olympus Provis AC70 microscope fitted with Olympus UPlanApo-objectives, using an Olympus XC50 camera. Fluorescent images were taken with an Olympus AX70 microscope fitted with Olympus UPlanApo-objectives, using a Spot Diagnostic camera. Confocal images for VEGFR, endoclin, and Cx40 were acquired with Zeiss Apotome axioplan or confocal (LSM 780) microscopes. Images were edited with Adobe Photoshop CS6 extended and/or ImageJ.

Amira 3D reconstruction

3D reconstructions were made of an E12.5 and E13.5 knock-out embryo and age compared wild type embryos. Every fifth section was used for the reconstruction. The distance between each section was 25 µm. The pictures were optimized for Amira with Adobe Photoshop CS6 Extended. Amira software package version 5.4.2 was used to create a 3D model. The sections were in part automatically and in part manually aligned and labels were added to the different structures, based on morphology and stains. Surface views were executed to PDF formats by using Adobe Acrobat 9.5 Extended.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Key Findings

- Germline deletion of 14-3-3e in the mouse leads to a very high incidence of multiple cardiac malformations.
- Malalignment of both the outflow tract (OFT) and the atrioventricular (AV) endocardial cushions, leading to tricuspid stenosis/atresia and mitral valve abnormalities.
- In addition to muscular VSDs and myocardial non-compaction, we identified epicardial blebbing and deep endocardial recesses.
- Abnormal pharyngeal arch artery patterning and defects in coronary vasculature remodeling.
- Abnormal patterning of p27^{kip1} in the endocardium might trigger some of the described developmental malformations through the disturbance of epithelial to mesenchymal transformation.

Figure 1.

Malalignment of the OFT and AV endocardial cushions in E12.5 *14-3-3e*^{-/-} embryos. 3-D reconstructions (a,f) and transverse sections (b–e and g–j) of embryonic hearts E12.5 of *14-3-3e* wild type (+/+) and *14-3-3e* knock-out (-/-). The colorcode for the endocardial outflow tract cushions is blue where it contacts the surrounding myocardium and purple where it is in contact with the lumen of the OFT. The atrioventricular cushion (AVC) mass of the already fused on superior and inferior AV cushion is green where it is contact with the myocardium and moss green where it contacts the lumen. The lateral AV cushions (LC) are golden. The aortic arch system is red and the pulmonary trunk (small part seen in a) and the arterial duct are blue green. In the sections of the +/+ embryos (b–e) it can be seen that the parietal outflow tract cushion (PC) and the septal cushion (SC) merge in the OFT. The extension of the SC is somewhat more proximal. At the level of the tricuspid orifice (T)

there is no connection with the LC. In the Nkx2.5 staining, the nuclei of the myocardium are brown, and there are no Nkx2.5 positive cells in the endocardial cushion tissue (see dash line and arrows in c). In the PECAM staining (e) the endocardium (EC) and the endothelial cells of the coronary vasculature (arrows) can be observed. In the 14-3-3e $-/-$ heart the relative alignment of the OFT and the AVC mass is different as compared to the $+/+$ situation (compare a with f). The AV canal (moss green) has not moved appropriately to the right and the OFT cushions connect to the right LC. In sections g and h, the intercalated cushion (IC) of the aortic semilunar valves is found at a more proximal level. More proximal in the heart the combined PC and SC merge with the right LC combined with a more right-sided T (f), and a small interventricular foramen that cannot be designated as a VSD at this stage (arrows in i and j.). Abbreviations: LA: left atrium, LV: left ventricle, LVOT: left ventricular outflow tract, M: mitral orifice, RA: right atrium, RV: right ventricle, RVOT: right ventricular outflow tract. MLC2a: Staining myosin light chain a. Magnification bars: 100 μm .

Figure 2.

Malalignment of the OFT and AV endocardial cushions in E13.5 14-3-3e knock-out embryos. The OFT and AV endocardial cushions are malaligned in E13.5 14-3-3e $-/-$ hearts. 3-D reconstructions (a,b,f,g) and transverse sections (c–e and h–j) of 14-3-3e wild type (+/+) and 14-3-3e knock-out ($-/-$) embryonic hearts. The colorcode for the endocardial outflow tract cushions is blue where it contacts the surrounding myocardium and purple where it is in contact with the lumen of the outflow tract (OFT). The atrioventricular cushion (AVC) mass, with fused superior and inferior AV cushions, is green where it is contact with the myocardium and moss green where it contacts the lumen. The lateral AV cushions (LC) are golden. The aortic arch system is red and the pulmonary trunk (small part seen in f) and the arterial duct are blue green. In the reconstruction without myocardium (b) and sections

of the +/+ embryos (c) it can be seen that the parietal outflow tract cushion (PC) and the septal cushion (SC) merge in the OFT. The PC merges with the already partly myocardialized AVC (d). At the level of the tricuspid orifice (T) there is no connection between PC and LC (e). In the 14-3-3e knock out (-/-) the AV canal (moss green) has not moved appropriately to the right (f) and the PC of the fused OFT cushions connects to the right LC (g). The T remains more left-sided (f,j) and a small perimembranous VSD is present (arrows in h-j). Abbreviations: LV: left ventricle, LVOT: left ventricular outflow tract, M mitral orifice, RV: right ventricle, RVOT: right ventricular outflow tract, VS: ventricular septum. MLC2a: Staining myosin light chain a. Magnification bars: 100 μ m.

Figure 3.

Increased expression of p27^{kip1} in the subendocardial cushion cells of 14-3-3 ϵ knock-out hearts. Transverse sections stained for p27^{kip1} (a–f). In the 14-3-3 ϵ wild type embryo (+/+) at E13.5 (a–c), expression is apparent in the squamous endocardial cells (EC) and increased if they have a cuboidal shape (arrows) and in the myocardial cells (a). In the cells of the 14-3-3 ϵ knock-out (–/–) embryo p27^{kip1} positive cells appear to cluster subendocardially (d, arrowhead in e). In the –/– embryo the primary foramen in between the right (RA) and left atrium (LA) is not completely closed (asterisk). Abbreviations: AVC: atrioventricular cushion complex, Cu: endocardial outflow tract cushion, LV: left ventricle, M: mitral orifice, Pu: pulmonary trunk, RVOT; right ventricular outflow tract, VS ventricular septum. Magnification bars in a,d: 100 μ m; in b,c,e,f: 20 μ m.

Figure 4.

Defects in the mitral and tricuspid valves, and perimembranous VSDs in 14-3-3e knock-out hearts. Transverse sections through 14-3-3e $+/+$ (a,b,e,f) and $-/-$ (c,d,g,h) hearts. At ages E13.5 (c,g) $-/-$ specimen (Table 2, specimen 3 at E13.5) showing (c) a large perimembranous VSD, (g) an abnormal thickened mitral valve (MV), an apical spongy (arrow) interventricular septum (VS) and tricuspid valve (TV) stenosis. At ages E14.5 (d,h) $-/-$ specimen (Table 2 specimen 1 at E14.5) showing (d) a hypoplastic RV (arrow), (f) TV stenosis, a hypoplastic RV, and a spongy VS with muscular VSD (arrow). For an estimation of the level of the sections, see Figure 2 which has 3-D images. All embryos (E11.5 – E15.5) have been sectioned in a standard fashion. Abbreviations: LA: left atrium, LV: left ventricle,

LVOT: left ventricular outflow tract, RA: right atrium, RV: right ventricle. Magnification bars: 200 μm .

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Figure 5.

Defects in E18.5 14-3-3e knock-out hearts. Sections are made frontally, mostly resembling a four chamber view. Wild type (+/+) (a), and knock-out (-/-) (b-f) embryos (Table 2b). The section shows the location of the Gerbode defect (b, arrow), located between the ventricular septum (VS) and an additional flap (asterisk) of the mitral valve (MV). The tricuspid valve (TV) is hypoplastic as well as the RV (c,d). In (c) the tricuspid atresia with a dimple ending blindly into the myocardium (arrow) above the hypoplastic RV. In (d) there is a small subaortic VSD (arrow) between the LVOT and the RV. In (e,f) there is marked myocardial (M) hypoplasia with deep intertrabecular spaces (IS) and a large muscular VSD (black arrow). The muscle band within the RV is the septal band, and the tricuspid valve is atretic (arrow) (e). Abbreviations: LA: left atrium, LV: left ventricle, LVOT: left ventricular outflow tract, RA: right atrium, RV: right ventricle. Magnification bar: 500 μ m for all sections.

Figure 6.

Defects in the mitral and tricuspid valves, and perimembranous VSDs in 14-3-3e knock-out hearts. Transverse sections of 14-3-3e wild type (+/+) and knock out (-/-) hearts with a thin myocardial phenotype. (a) +/+ heart of E11.5. (b) -/- embryo at E11.5 shows very thin myocardial wall (arrow) with a highly deficient formation of the interventricular septum (VS). (c) +/+ heart (E13.5) with well-developed left ventricular (LV) myocardium. (d) Thin compact LV myocardium in a -/- heart (Table 2, specimen 5 at E13.5), which is often seen in combination with deep intertrabecular recesses depicted in the 3-D reconstructions (e,f) (see also Supplemental Figures 3,4). (g) In a -/- heart (Table 2, specimen 7 at E15.5) a muscular VSD is seen (arrow). Abbreviations: Ao: aorta, AVC: atrioventricular cushion

complex, Ep: epicardium, LV: left ventricle, RA: right atrium, RV: right ventricle.
Magnification bars: a,b,e-g: 100 μm ; c,d: 200 μm .

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Figure 7. Blebbing and apparent upregulation of p27^{kip1} in the epicardium. Sections of the epicardium (Ep). (a, b) There is no difference in the WT-1 staining between 14-3-3 ϵ wild type (+/+) and knock out (-/-) embryos. In the -/- embryo the Ep is less adhered to the myocardial wall (Myo) which is referred to as blebbing (arrows). (c, d) In the p27^{kip1}-stained sections there seem to be less positive Ep cells in the +/+ embryo as compared to the -/- embryo that shows within the blebbing subepicardial area epicardium derived cells (EPDC). Magnification bars: 20 μ m.

Figure 8.

Coronary artery hypoplasia and deep intertrabecular recesses in *14-3-3ε*^{-/-} hearts.

Immunofluorescence with VEGFR2 (a–f), endoglin (a, b, e, f) and Cx40 (c–f) antibodies on sagittal sections of E18.5 wild type (+/+) (a, c, e), and knock-out (–/–) (b, d, f) hearts. In mutants, endoglin positive intertrabecular projections, indicated by arrows, invade the compact layer of both ventricles as delineated by dotted lines. The number of Cx40-positive coronary arteries developing in the ventricular myocardium is more abundant in wild type heart compared to mutant heart (arrows in c and d). Higher magnification of serial sections around insets (e and f). Asterisks show that endoglin positive intertrabecular endocardium (red) deeply recesses into compact myocardium (green). Abbreviations: AVB: atrio-ventricular bundle, LPF: left Purkinje fibers, LV: left ventricle, RPF: right Purkinje fibers, RV: right ventricle.

Figure 9.

Defects of coronary plexus remodelling in *14-3-3e*^{-/-} hearts. 3D reconstruction of E18.5 whole mount immunofluorescence with VEGFR2 (a,b,e,f and i,j,m,n), PECAM (c,g and k,o), and merged images (d,h and l,p). External view of right ventricle apex (RVA) (a–h), with higher magnification (b–d and f–h). Wild type (+/+) (a–d), and knockout (–/–) (e–h). Note that sub- and epicardial vasculature are disorganized in mutant hearts compared to wild type in which they are well connected and aligned along the ventricular wall (arrowheads in b–d). External view of left ventricle apex (LVA) (i,m). Internal view of LVA with higher magnification (j–l and n–p). Wild type (+/+) (i–l), and knock-out (–/–) (m–p). Large coronary arteries are both VEGFR2 and PECAM positive (arrows), however, smaller coronary arteries are limited to PECAM (arrowheads). Asterisk (*) indicates the boundary between large and small coronary arteries. Note that there are no large coronary arteries (VEGFR2) in knock-outs (n–p). Magnification bars: 200μm.

Figure 10.

No significant difference in smooth muscle cell and mesenchyme cell lineages between wild type and *14-3-3e*^{-/-} hearts. In wild type (+/+), right ventricle (a,b), and left ventricle (c,d) with SM22 (a,c) and Vimentin (b,d). In knock-out (-/-), right ventricle (e,f) and left ventricle (g,h) with SM22 (e,g) and Vimentin (f,h). Arrows show coronary arteries, and arrowheads show subepicardial surface immunofluorescence stained with SM22. Magnification bars: 100μm.

Table 1

Overview of the developmental age (E), number (N), knock-out (KO) and wildtype (WT) embryos studied.

Age	N of WT	N of KO
E11.5	2	3
E12.5	3	3
E13.5	3	5
E14.5	4	5
E15.5	3	10
E18.5	5	10

Table 2a

List of cardiac malformations in *14-3-3e*^{-/-} embryos. The tissue abnormalities, cardiac and vascular malformations in each *14-3-3e*^{-/-} embryo at E12.5 – E15.5 with a phenotype. Blank box indicates no phenotype or not identifiable.

Type of abnormality	E12.5			E13.5			E14.5			E15.5						
	1	2	3	1	2	3	4	5	1	2	3	4	5	6	7	
Tissue abnormalities																
Endocardial cushions																
Malaligned outflow and AV cushions				x	x											
Deep intercalated cushion	x		x													
Ventricular myocardium																
Spongy septum	x					x	x		x						x	
Thin compact myocardium	x			x	x	x	x		x	x					x	
Deep intertrabecular spaces															x	
Epicardium																
“blebbing” epicardium	x		x	x	x		x								x	
Cardiac malformations																
Arterioventricular canal																
Abnormal mitral valve								x			x					
Tricuspid stenosis						x			x						x	
VSD						x	x		x						x	
AVSD				x												
Ventricular myocardium																
Muscular VSD	x					x	x		x		x				x	
Right ventricular hypoplasia						x			x							
Cardiac outflow																
Long subpulmonary infundibulum				x	x	x			x						x	
DORV							x									
Vascular malformations																
Side by side aorta and pulmonary trunk											x	x			x	
Bicuspid aortic valve																x

Type of abnormality	E12.5			E13.5					E14.5					E15.5						
	1	2	3	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5	6	7
<i>Pulmonary arteries</i>																				
Common pulmonary artery							x	x	x											
Large pulmonary arteries										x										
Abnormal orientation ductus arteriosus						x			x	x	x			x	x	x	x	x		
Pulmonary trunk hypoplasia														x	x					
<i>Aortic arch</i>																				
Ascending aorta hypoplasia						x											x			
Right aortic arch																				x
Right retro-esophageal artery								x												x
Aortic sling																x				

CHD phenotypes in E18.5 knock-outs; HE for cases 1–4, fluorescent evaluation for cases 5–8, and whole mount for cases 9–10. Blank box indicates no phenotype or not identifiable.

Table 2b

Type of abnormality	1	2	3	4	5	6	7	8	9	10
E18.5										
<u>Cardiac malformations</u>										
Tricuspid hypoplasia	x	x						x		
Tricuspid atresia			x	x						
Right ventricular hypoplasia		x	x					x		
Mitral valve abnormality		x								
Spongy septum				x		x				
Muscular VSD				x						
Myocardial hypoplasia				x		x	x			
Deep intertrabecular spaces				x	x	x	x	x		
Coronary artery hypoplasia					x	x	x	x	x	x
<i>Cardiac outflow</i>										
Double Outlet Right Ventricle	x									
Subaortic VSD	x	x	x			x				
Perimembranous/inlet VSD		x					x	x		
Gerboden defect		x								