RESEARCH ARTICLE

Arid3b is essential for second heart field cell deployment and heart patterning

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ABSTRACT

Arid3b, a member of the conserved ARID family of transcription factors, is essential for mouse embryonic development but its precise roles are poorly understood. Here, we show that Arid3b is expressed in the myocardium of the tubular heart and in second heart field progenitors. Arid3b-deficient embryos show cardiac abnormalities, including a notable shortening of the poles, absence of myocardial differentiation and altered patterning of the atrioventricular canal, which also lacks epithelial-to-mesenchymal transition. Proliferation and death of progenitors as well as early patterning of the heart appear normal. However, Dil labelling of second heart field progenitors revealed a defect in the addition of cells to the heart. RNA microarray analysis uncovered a set of differentially expressed genes in Arid3b-deficient tissues, including Bhlhb2, a regulator of cardiomyocyte differentiation, and Lims2, a gene involved in cell migration. Arid3b is thus required for heart development by regulating the motility and differentiation of heart progenitors. These findings identify Arid3b as a candidate gene involved in the aetiology of human congenital malformations.

KEY WORDS: Heart development, Arid3b, Second heart field, Transcription factor, Dil labelling, AEC explant, Cell motility, Mouse

INTRODUCTION

Understanding heart development (Vincent and Buckingham, 2010; Bruneau, 2013; Miguérol and Kelly, 2013; Rana et al., 2013) is important for identifying the origin of congenital heart malformations, the most common birth defects (Fahed et al., 2013). The heart develops from two populations of cardiac progenitors (Buckingham et al., 2005). The initial heart tube (which will form the left ventricle) originates from cardiogenic mesodermal cells, which fuse at the ventral midline of the embryo. Elongation of the heart is driven by the addition at the poles of a second pool of Isl1+ (Isl1 – Mouse Genome Informatics) progenitor cells located in the pharyngeal mesoderm beneath the heart tube and called the second heart field (SHF) (Kelly, 2012). As these progenitors get incorporated into the heart poles, they differentiate and start to express myocardium markers, such as smooth muscle actin (SMA) and TroponinT (TnT). Cells from the SHF contribute to the outflow tract (OFT), right ventricle, atrioventricular canal (AVC), atria and the inflow tract (IFT). Multiple signalling pathways are implicated in regulating the proliferation and differentiation of the SHF population (Black, 2007; Rochais et al., 2009); however, little is known about the cellular mechanisms that drive the deployment of SHF cells to the heart poles. Chick dye labelling showed that cells from a caudal proliferative centre are able to move to the arterial pole (van den Berg et al., 2009). Cardiogenic mesoderm in this region in the chick is reported to be a cohesive epithelial sheet possessing a columnar pseudostratified structure (Waldo et al., 2005). Recently, Wnt5a-activated PCP signalling in the mouse has been proposed to induce a mesenchymal-to-epithelial conversion of splanchnic mesoderm, promoting SHF contribution to the OFT through a process of cell intercalation (Sinha et al., 2012).

The AVC separates the ventricle from the atria, and cells in this area are distinguished from atrial and ventricular neighbours by a distinct molecular code. The AVC is also characterized by the expression of Bmp2, which signals to the endocardium and promotes epithelial-to-mesenchymal transition (EMT) (Sugi et al., 2004; Ma et al., 2005), generating the AVC cushions that will subsequently form the valves that separate the chambers (von Gise and Pu, 2012). Bmp2 directly regulates myocardial expression of Tbx2, which, together with Tbx3, is expressed in the AVC and represses the expression of chamber-specific genes, including Anf (Nppa – Mouse Genome Informatics) and Cx40 (Gja5 – Mouse Genome Informatics) (Singh et al., 2009, 2012).

The Arid family is a conserved group of genes characterized by the presence of a distinctive DNA-binding domain, the AT-rich interacting domain ARID (Kortschak et al., 2000; Wilsker et al., 2005). Members of this family play important roles in the regulation of cell cycle, gene expression, development and differentiation (Shandala et al., 2002; Wilsker et al., 2002). ARID proteins seem to function as transcriptional regulators in multiprotein complexes, acting as context-dependent activators or repressors (Valentine et al., 1998) and also participate in chromatin-remodelling activities as part of the SWI/SNF and polycomb repressor complexes (Nagl et al., 2007; Peng et al., 2009).

Arid3b (also known as Bdp or Dri2) was first cloned in a two-hybrid screen as a binding partner of retinoblastoma protein (Numata et al., 1999). Arid3b is expressed in different types of cancers, including malignant neuroblastomas. It can transform mouse embryonic fibroblasts and, together with Mycn, forms a combination with strong oncogenic activity (Kobayashi et al., 2006); in mouse embryonic stem cells (ESCs) these two genes are involved in cell proliferation (Kobayashi et al., 2013). Arid3b is overexpressed in malignant ovarian cancers and is involved in the mesenchymal transformation of ovarian epithelial cells (Cowan Dahl et al., 2009). Transient silencing of Arid3b in breast cancer cells decreases cell migration, implicating Arid3b as a miR-125b target with roles in cell motility (Akhavantabasi et al., 2012). Arid3b...
is also essential for embryonic development. Arid3b mutants die at mid-gestation, showing severe craniofacial and cardiovascular defects (Takebe et al., 2006), although a role for Arid3b in heart development has been dismissed. We have shown that Arid3b contributes to the proper maturation of the limb apical ectodermal ridge in both chick and mouse embryos by controlling cell rearrangements, as well as being necessary for proper motility of mouse embryonic fibroblasts (MEFs) in vitro (Casanova et al., 2011). However, the specific roles of this gene remain elusive.

Here, we show that Arid3b is dynamically expressed in the developing embryonic heart. Absence of Arid3b leads to failed deployment of cells from the SHF to both heart poles and to defective patterning of the AVC and disruption of EMT in the endocardial cushions. Microarray analysis reveals upregulation of Lims2 and Bhlhb2 (Bhlhe40 – Mouse Genome Informatics) as candidate mediators of Arid3b function in regulating cell movements and differentiation of SHF cardiac progenitors.

RESULTS

Arid3b is expressed in the heart from early stages of development

Arid3b is essential during embryonic development and mouse embryos lacking Arid3b die at mid-gestation (Takebe et al., 2006; Casanova et al., 2011). As the possible role of Arid3b in heart development has not been fully investigated, we first analysed its expression pattern by RNA in situ hybridisation. Arid3b is expressed from early stages in the heart crescent (Fig. 1A). At E8.0, expression is observed throughout the primary heart tube myocardium and also strongly in the SHF (Fig. 1B-D; supplementary material Fig. S1). At E8.5, expression in the heart tube is fainter but remains strong in the SHF and the heart poles (Fig. 1E; supplementary material Fig. S1). At E9.0, high-level expression persists in the heart poles (IFT and OFT) and in the SHF (Fig. 1F-J; supplementary material Fig. S1). However, expression gradually decreases in the heart chambers, whereas it is clearly observed in the AVC (Fig. 1I). At E9.5, expression is still strong in the sinus venosus, dorsal mesocardium and OFT (Fig. 1K-O). At E10.5, expression in the heart myocardium is much fainter, but a clear signal is still seen in the OFT (Fig. 1P,Q). From E11 onwards, expression in the myocardium is no longer detected. Arid3b is also expressed from E10.5 in the endocardium and between E11.5 and E12.5 there is an especially strong signal in the endocardium of the AVC and OFT (Fig. 1R-U). At E14.5, strong expression is detected in the arterial trunk (Fig. 1V). Expression is also present in the epicardium at these stages (Fig. 1R,T). In blood vessels, Arid3b is not expressed in the endothelium at early stages, although expression is high in the yolk sac endoderm (supplementary material Fig. S2). However, from E9.5, expression is readily seen in the vessel endothelium (Fig. 1X). This expression is first seen in the aorta and large vessels, including veins, and later extends to smaller vessels (Fig. 1V-X).

Arid3b null embryos show cardiac defects

To analyse the role of Arid3b in heart development we have used a mouse gene trap line in which Arid3b lacks its functional domains (Casanova et al., 2011). Embryos deficient for Arid3b (Arid3b<sup>gt/gt</sup>) die at E10.5 and show reduced size, arrest in somitogenesis and severe craniofacial and cardiovascular malformations, including an abnormally dilated heart with altered looping and pericardial effusion (Fig. 2A,B). Moreover, the yolk sac lacks large mature blood vessels, suggesting that appropriate vascular remodelling is impaired (Fig. 2C). However, mutant hearts beat and Indian Ink distributes to the body after injection into the heart at E9.25, confirming blood circulation (Fig. 2J,O). Closer examination of the mutant hearts reveals a shortening of the OFT and IFT at E8.5 (Fig. 2D,E) and E9.0 (Fig. 2F,G,K,L), which becomes more evident at E9.5 (Fig. 2H,I,M,N; supplementary material Fig. S3 shows a quantitative comparison of the lengths). In histological sections at E9.5, the mutant heart shows disorganized sinus horns, thinner myocardium walls in atria and the ventricle, and reduced trabeculation compared with the wild-type (WT) heart (Fig. 2P-S,U-X). Moreover, the mutant OFT is short and the AVC is narrow and does not show the EMT of the endocardium that is already evident in WT hearts (Fig. 2R,W). Transformed mesenchymal cells cannot be detected in the OFT cushion either, suggesting a general defect in EMT (supplementary material Fig. S4).

The expression pattern of Arid3b and the early heart defects in the mutant embryos suggest that Arid3b is directly required for proper heart morphogenesis.

Arid3b is required for AVC patterning and EMT

To evaluate whether Arid3b deletion affects genes known to be important for heart formation, we performed in situ hybridisation to detect mRNA expression of cardiac transcription factors, signalling molecules and myofiber genes. Wild-type and mutant hearts showed similar patterns and levels of expression at E9.0 for Nkx2-5 and Gata4 (two early cardiac master genes). Normal expression was also detected for the key cardiac transcription factors and chamber-specific markers Mef2c, Tbx5, Mlc2v (Myl2 – Mouse Genome Informatics), Hand1, Hand2, Hey1 and Hey2 (supplementary material Fig. S5), suggesting that early patterning of the heart is not affected in Arid3b<sup>gt/gt</sup> mutants.

An important heart region affected in the mutant is the AVC. Expression of Bmp2, an upstream regulator of AVC patterning (Rutenberg et al., 2006), was the same in mutant and WT hearts at E9.0 (Fig. 3A). However, Arid3b null hearts showed significantly below-normal expression of Tbx2 and Tbx3, targets of BMP signalling and determinants of AVC specification through the repression of chamber-specific genes (Fig. 3A). Notably, expression of the chamber myocardium marker Anf was expanded to the AVC region (Fig. 3A), suggesting the loss of AVC patterning. These altered expression patterns persisted at E9.5 (Fig. 3B), but were now accompanied by a significant drop in the expression of Bmp2, as detected by in situ hybridisation (Fig. 3B) and quantitative PCR (supplementary material Fig. S6A). This indicates disruption of the regulatory-feedback loop between Bmp2, Tbx2 and Tbx3, thus impairing the maintenance of AVC identity. This result was confirmed by analysis of the chamber markers Cx40 and Cx43 (Gja1 – Mouse Genome Informatics), which were expanded into the mutant AVC (supplementary material Fig. S7). To characterize the origin of the AVC alterations we analysed nuclear localization of pSmad, which mediates BMP signalling. In the AVC myocardium, similar nuclear localization of pSmad, indicative of pathway activation, was observed in WT and Arid3b null hearts at E9.0 (Fig. 3Cc,d) and E9.5 (Fig. 3Cg,h), despite the lower Bmp2 expression in mutants at E9.5 (compare Fig. 3Ca,b with e,f). Moreover, nuclear pSmad localization was present in mutant AVC endocardium at E9.0 (Fig. 3Cc,d), whereas at E9.5 the number of pSmad-positive endocardial cells was significantly lower in mutant than in WT AVC (30% reduction, n=3; P<0.01 in Student’s t-test) (Fig. 3Cg,h).

These results suggest that Arid3b acts in the myocardium downstream of pSmad nuclear translocation and that Bmp2 signalling from the AVC myocardium is not transduced properly to the endocardium. To gain further insight into Arid3b function in the AVC, we analysed the expression of EMT markers (Snail, 5

Tgfβ2, Twist1, Has1 and Tbx20), some of which are known targets of BMP signalling. At E9.5, EMT is already detected in the WT AVC, and transformed cells expressing mesenchymal markers are observed in the cardiac jelly (Fig. 4A). By contrast, these cells are not present in Arid3b-deficient AVC (Fig. 4A). Moreover, although Tgfβ2 expression was detected in WT and Arid3b-null AVC myocardium (Fig. 4A), expression of Snai1, Tgfβ2, Twist1 and Tbx20 were undetectable in mutant AVC endocardium (Fig. 4A).

These data show that EMT is impaired in Arid3b mutant hearts and suggest that the primary defect in these mutants is incorrect signalling from myocardial Bmp2 to the endocardium. To test this possibility, we cultured AVC explants on collagen gels. In these conditions, endocardial cells undergo EMT and invade the collagen gel, which can be measured and quantified as the transformation index [% invading cells/total number of transformed cells, see Materials and Methods (Luna-Zurita et al., 2010)]. Compared with WT AVC explants, mutant explants generated significantly fewer transformed cells and, more importantly, a significantly lower transformation index (Fig. 4B-D). Addition of Bmp2 to the culture medium rescued these defects, restoring the total number of transformed cells and the

**Fig. 1. Arid3b is expressed in the heart from early stages of development.** RNA in situ hybridization showing Arid3b expression at different stages of mouse heart development in whole-mount embryos (A,B,E-G,K,L) and on paraffin sections (C,D,H-J,M-X). From early stages expression is observed throughout the heart tube and in the SHF (A-D, arrowheads). In the looping heart expression diminishes in the developing chambers (E) but is maintained strongly in the myocardium of the heart poles (olf in F,K and ift in G) and in the SHF (F-O, arrowheads in F,H,J,M,O). Expression can also be observed in the AVC myocardium at E9.0 (I, arrow), but not at E9.5. From E10.5, expression is observed in the endocardium, especially in the AVC cushions (P,P,R,R′,S,S′,S″) and later in the OFT cushions (T,U, arrows). We also observed expression in the epicardium at E12.5 (S″, arrowheads). In the vessels Arid3b expression starts in the dorsal aorta at E9.5 (V,W,X, white arrowheads) and later also appears in small vessels (arrows) and in veins (black arrowheads) (W,X). Scale bars: 150 μm in C,D,H-J,M-O,V-X; 250 μm in A,B,E,F,G,K,L,P-S″; 500 μm in T,U. Stages are indicated as embryonic day (e.g. E10.5) or pairs of somites, ps. a, atrium; ba, branchial arches; cc, cardiac crescent; fg, foregut; fhf, first heart field; ht, heart tube; ift, inflow tract; np, neural plate; nt, neural tube; oft, outflow tract; ph, pharynx; shf, second heart field; v, ventricle. Lines in B,F,G,K,L mark plane of sections showed in the indicated panels.
transformation index to values similar to those of WT explants (Fig. 4B-D). This result confirms that the EMT deficiency in Arid3b mutant AVC is caused by inefficient Bmp2 signalling.

Microarray analysis identifies candidate genes and processes mediating Arid3b effects on heart development

To identify the genes mediating Arid3b function in heart development, we performed a microarray comparison of mRNA expression in WT and mutant embryos. In two separate experiments, RNA was extracted from whole E9.0 embryos or from E9.5 hearts, heads and trunks, and expression was compared between the samples using Agilent chips (Fig. 5Aa-c; supplementary material Tables S1-S4). Of the genes showing differential expression in Arid3b-null embryos, 93 were altered in both E9.0 whole embryos and E9.5 hearts, and 453 genes were common to the three tissues analysed in E9.5 embryos (Fig. 5Ad,e; supplementary material Tables S5, S6). Ingenuity functional analysis of the sets of common genes revealed cell morphology and cellular movement as the most highly overrepresented functions (Fig. 5B; supplementary material Table S7). These results support a role for Arid3b in the control of cell motility and cellular rearrangements, in agreement with a previous analysis (Casanova et al., 2011).

To validate the microarray data, we conducted mRNA in situ hybridisation analysis of genes showing high differential expression and which have established roles in embryonic development (Fig. 5C; supplementary material Fig. S8). Selected genes included members of important signalling pathways and genes involved in vessel or muscle differentiation. Clear up- or downregulation of these genes was observed, in agreement with the microarray result. For more in-depth analysis, we focused on two upregulated genes that could provide
mechanistic insight into Arid3b function. One of them, Bhlhb2, is a transcription factor of the basic helix-loop-helix family that has been shown to regulate cardiac progenitor cell fate determination downstream of Wnt/β-catenin signalling (Kwon et al., 2009). Bhlhb2 was one of the most strongly upregulated genes in the microarray analysis, both in E9.0 whole embryos and in E9.5 hearts. In situ hybridisation revealed much higher expression in Arid3b-null embryos, both in whole-mount specimens (Fig. 5Ca,b,g,h) and in sections (Fig. 5Cc-f,i-l). Strong upregulation was also confirmed by qPCR (supplementary material Fig. S6B,C). Expression was particularly strong in the heart poles, especially in the IFT (Fig. 5Cj,l, arrowheads; compare with d,f).

Bhlhb2 has been shown to negatively regulate Smyd1, a histone methyltransferase essential for cardiomyocyte differentiation (Kwon et al., 2009). Smyd1 expression was reduced in mutant hearts, notably in the IFT, the same area in which Bhlhb2 was highly expressed (as detected by in situ hybridisation, see Fig. 5C, and confirmed by qPCR, supplementary material Fig. S6B,C). This region, which corresponds to a site of high Arid3b expression in WT hearts and to the pole-shortening defect in the Arid3b mutants, also lacked SMA and TnT expression. Expression of SMA is observed at the heart poles in WT and mutant embryos (Fig. 6Aa-d). However, mutant embryos lack expression in the distal regions closer to the SHF, both in the IFT (at E9.5, Fig. 6Af) and in the OFT (at E9.0, Fig. 6Ad, and at E9.5, Fig. 6Ah), suggesting defective differentiation or delayed maturation of heart progenitors. A similar pattern was observed for TnT, the expression of which was absent in the distal part of the heart poles in the mutant (supplementary material Fig. S9). These results suggest that Arid3b regulates cardiac progenitor differentiation at the heart poles indirectly through repression of Bhlhb2.

The other upregulated gene selected from the microarray analysis was Lims2 (also known as Pinch2), a focal adhesion protein that binds to integrin-linked kinase and modulates its activity. Lims2 is an adaptor protein linking integrins to the actin cytoskeleton and has been shown to regulate cell shape and migration (Zhang et al., 2002; Boudoukha et al., 2010). In situ hybridisation of Lims2 confirmed clear upregulation in Arid3b mutants, again very conspicuous at the heart poles (Fig. 5D, also confirmed by qPCR, see supplementary material Fig. S6B,C). Overexpression of Lims2 in vitro impairs cell adhesion and motility (Zhang et al., 2002), similar to observations in Arid3b-deficient cells (Casanova et al., 2011), suggesting that its upregulation also contributes to the heart pole defects observed in Arid3b mutants.
Arid3b deficiency does not affect cell proliferation or cell death of SHF progenitors

To further investigate the cause of the heart pole defects in the absence of Arid3b, we analysed cell proliferation and cell death in Islet1+ progenitors in the SHF (and also in the heart tube and the forming heart chambers) between stages E8.0 and E9.5. Wild-type and mutant hearts contained similar total numbers of Islet1+ progenitors and there were no significant differences in proliferation in any region at the stages analysed, as measured by pH3 staining and 5-bromo-2'-deoxyuridine (BrdU) incorporation (SHF analysis in supplementary material Fig. S10A and heart analysis in supplementary material Fig. S11A). Apoptotic cell death assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) labelling was also similar, except for a significantly higher level of TUNEL in the SHF and atria of mutant hearts at E9.5, which was the only stage at which we detected a reduction in the total number of Islet1+ cells in Arid3b mutants (supplementary material Fig. S10B and Fig. S11B).

The level of Isl1 expression in heart progenitors appeared normal in mutant embryos (supplementary material Fig. S10C), as was the expression of other genes reported to regulate SHF cardiac progenitors and normal OFT formation, such as Fgf8 and Tbx1 (supplementary material Fig. S10D). Moreover, expression of several genes (Fgf8, Tbx1, Shh) in the pharyngeal endoderm, a tissue important for heart formation where Arid3b is also expressed, was similar in mutant as compared with WT embryos (supplementary material Fig. S10E). Notably, accumulation of Islet1+ progenitors was seen in the SHF in the OFT region at E9.5 (Fig. 6Ah, asterisk). Additionally, the actin

Fig. 4. EMT is defective in Arid3b mutant hearts. (A) In situ hybridization of molecules involved in EMT in the AVC. Expression levels of Snai1 (a,b), Tgfb2 (c,d), Twist1 (e,f) and Tbx20 (g,h) are significantly lower in the mutant AVC endocardium, whereas Has2 (i,j) is unaltered; note also the almost total absence of mesenchymal cells invading the cushions in the mutant. (B) AVC explants on collagen gels stained for SMA (red) and phalloidin (green). Both the total number of mesenchymal cells (scattered cells on top panels) and the proportion of cells invading the gel (arrowheads in bottom panels, z-axis) were lower in mutant (b) than in WT (a) explants. After addition of Bmp2, the total number of mesenchymal cells and the transformation index (TI) in mutant explants (d) were restored to the levels in WT explants (c). (C,D) Quantification of the results (TI, in %). Scale bar: 150 µm. Results are mean±s.d. *P<0.05, ***P<0.001; Student’s t-test.
The cytoskeleton of Islet1+ cells in the SHF and the overall tissue architecture of this region were abnormal in mutants. SHF cells in WT hearts have a wide band of actin on the luminal side (Fig. 6B, WT), which is thicker at the IFT region (Fig. 6Bc), suggestive of a polarized epithelium. By contrast, mutant SHF cells do not show such a clear actin band (Fig. 6Bd). Moreover, cells dorsal to the wall epithelium, which appear loose and mesenchyme-like in the WT (Fig. 6Bg), appear more compact and epithelial-like in the mutant (Fig. 6Bh).
Taken together, these results suggest that a difference in cell number is not the primary cause of the observed defects in mutant hearts and that Arid3b deletion appears not to alter the specification of heart progenitors in the SHF, which nonetheless has abnormal tissue architecture.

**Addition of SHF progenitors to the heart poles is impaired in Arid3b mutant embryos**

The results presented so far show that Islet1+ progenitors are correctly specified in the mutant SHF, that proliferation and cell death are unaltered and that the early patterning of the heart is normal. However, the cellular architecture and tissue organization in the SHF are disrupted and eventually the poles of the heart are malformed. Moreover, Lims2, which regulates cell motility, is upregulated in the heart poles of Arid3b mutants. This suggests abnormal incorporation of Islet1+ progenitors into the heart, probably due to defective cell movements or cell rearrangements. To directly analyse the fate of mutant SHF cells, we DiI-labelled Islet1+ progenitors, both in ventro-caudal positions at E8.0 (6 ps), which will contribute to the IFT (Fig. 7), and in rostro-lateral positions at E8.5 (10 ps), which will contribute to the OFT and right ventricle (Fig. 8). Cells were labelled, embryos cultured in vitro for 24 h and the position of DiI-positive cells was recorded. Labelling of IFT progenitors led to the presence of DiI-positive cells in the IFT of most WT embryos (66%), with a significant proportion showing cells in the right ventricle (13%). By contrast, in most mutant embryos (55%), DiI-positive cells were found in the distal OFT close to the SHF, and only reached the right ventricle in one embryo (5%) (Fig. 8N). This reduced contribution of cells to the IFT and OFT in mutant as compared with WT embryos was statistically significant. Taken together, these results suggest that Islet1+ progenitors, although present in Arid3b mutant SHF, have an impaired capacity to contribute to the heart poles, which eventually causes the observed cardiac defects. The results from our microarray analysis offer a possible mechanistic explanation for the defects observed in the mutant embryos.
DISCUSSION

Arid3b is essential for mouse embryo development, but its precise functions are not well characterised. Our results show that Arid3b is required for heart development during three stages: pole formation, myocardium differentiation and AVC patterning and maturation.

A previous report (Takebe et al., 2006) dismissed a direct role for Arid3b in cardiac development on the basis that Arid3b expression was barely detected in the developing WT heart and that cardiac muscle and heartbeat were present in Arid3b null mutants. The observed cardiovascular defects were attributed to death of neural crest cells and thus a failure in their contribution to the OFT. Here, we show a clear expression of Arid3b in cardiac progenitors from early stages of heart development in both the cardiac crescent and in the SHF. Although expression in the chamber myocardium soon regresses, high levels of expression are maintained at the heart poles, areas that do not form properly in Arid3b null embryos. The expression pattern and the early appearance of the defects (shortening of the poles, detectable from E9.0 on) strongly suggest a cell-autonomous defect in SHF cardiac progenitors, contrary to what was previously reported.

Although we observed heart beating and circulation in mutant embryos, their vasculature was abnormal: remodelling of the

Fig. 7. Addition of Islet1-positive progenitors to the IFT is impaired in Arid3b<sup>gt/gt</sup> embryos. (A) Ventral view of a 6-somite embryo, as used in Dil fate-mapping experiments, showing the left-right and cranial-caudal axes for orientation. (B) Example Dil labelling of the posterior SHF (pSHF) to study the contribution of Islet1+ cells to the IFT. A grid was used to map the position of the injection; for the analysis shown here, positions 3 and 4 (caudo-lateral) were considered. Dil (red fluorescence, B,C) was injected into the region containing Islet1+ cells, later detected by whole-mount immunohistochemistry (D, green). (E-H) Arid3b<sup>gt/gt</sup> and WT embryos labelled with Dil at similar positions (t=0) and (t=24 h) of in vitro culture. Images show WT (E,F,I,J) and Arid3b<sup>gt/gt</sup> (G,H,K,L) embryos with Dil-labelled cells in the heart IFT (L,K) recorded as 'IN' and those with labelled cells outside the heart (J,L) recorded as 'OUT'. (M-P) Additional examples of Dil labelling in the pSHF at t=0; in this case, 24 h after labelling embryos were cryosectioned and Dil staining observed on longitudinal sections (Q-T). Dil-labelled cells could be detected in the heart IFT in some wild-type (Q) and Arid3b<sup>gt/gt</sup> (S) embryos. In other cases, Dil-labelled cells appear outside the IFT (R, wild type; T, Arid3b<sup>gt/gt</sup>). (U) Summary of the contributions of the pSHF to the IFT in normal and Arid3b<sup>gt/gt</sup> embryos expressed as the percentage of embryos with labelling inside or outside of the IFT. A two-sample test for equality of proportions was used to compare the two groups. The percentage of embryos with labelling in the IFT is significantly higher in the WT than the Arid3b<sup>gt/gt</sup> group (***P=0.001903). Scale bars: 250 µm.
primitive network in the yolk sac was not accomplished and vessels in the embryo were smaller than in WT embryos. As Arid3b is not expressed in the endothelium at early stages, this suggests that defects in vessel maturation are non-autonomous. In fact, Arid3b is expressed in the endoderm of the yolk sac, pointing to an indirect role for Arid3b in vessel maturation. Later on, Arid3b is indeed expressed in the endothelium, suggesting that it has additional roles in the maintenance or physiology of this tissue; however, the early death of mutant embryos prevents studying this possibility. Noteworthy, a genome-wide association study has recently identified eight loci associated with elevated blood pressure and death of mutant embryos prevents studying this possibility. Eventually (at E9.5), BMP signal transduction appears to be normal in the myocardium, as pSmad is localised in the nucleus of myocardial cells. This suggests that Arid3b functions downstream of pSmad in this tissue and that the defect in Arid3b mutants lies in the function of transcriptional complexes that drive the expression of Bmp2, a direct target of Bmp2/pSmad signalling (Singh et al., 2009). It is tempting to speculate that Arid3b acts as a coactivator in one of those complexes, perhaps competing with Tbx20, which acts as a repressor of Tbx2 expression in this context (Singh and Kispert, 2010). Moreover, nuclear localization of pSmad in the mutant endocardium, although detected at E9.0, is greatly reduced at E9.5, coinciding with the decrease in myocardial Bmp2 expression. Myocardium-derived Bmp2 induces EMT in the endocardium, and some EMT markers, such as Twist1 or Snai1, are direct Bmp2 targets (Shelton and Yutzey, 2008; Luna-Zurita et al., 2010). The low expression of Bmp2 in the Arid3b mutants could explain the defective EMT observed, a proposition strongly supported by the rescue of EMT in AVC explants in vitro by application of Bmp2; this result shows that the defective response of

**Fig. 8. Contribution of progenitors to the OFT is altered in Arid3b<sup>gt/gt</sup>.** (A) Ventral view of Dil labelling in a representative embryo at t=0. (B) Lateral view after 24 h in vitro culture. (C,D) Dil labelling in an Islet1+ region (D, immunostaining with anti-Islet1 antibody). (E) Scheme illustrating the three regions in which the heart was subdivided for data analysis. (F,G) Cryosections of WT and mutant embryos 24 h after labelling; note the accumulation of Dil-labelled cells at the entrance of the heart in the mutant (arrowhead). (H-M) Examples of the three locations of the Dil staining 24 h after labelling in WT and mutant embryos. (N) Summary of the contributions of the AHF to the heart in Arid3b<sup>gt/gt</sup> and control embryos expressed as the percentage of embryos with labelled cells in each of the regions. Fisher’s exact test was used to compare the two groups. The difference in the distribution between WT and Arid3b<sup>gt/gt</sup> embryos is statistically significant (*P=0.01149). Scale bars: 250 µm.
endocardial cells is not caused directly by the lack of Arid3b but is secondary to the patterning defect in the myocardium. This is consistent with the expression of Arid3b in the myocardium and its absence in endocardial cells at E9.5, when EMT takes place. However, an autonomous role for Arid3b in the endocardium cannot be completely ruled out, as Arid3b could be expressed at a functional level that is below the level of detection by in situ hybridization. Moreover, we cannot exclude a later role for Arid3b in the endocardium either, as Arid3b is robustly expressed in this tissue from E10.5.

A major defect in Arid3b mutant embryos is the shortening and abnormal morphology of the heart poles. We could not detect alterations in cell proliferation or cell death of cardiac progenitors in the SHF (increased apoptosis is seen only at E9.5, particularly in the IFT, where the phenotype is usually more severe than in the OFT). These results suggest that the cause of the malformation is not the number of heart progenitors. Moreover, expression of several genes in the pharyngeal endoderm that have been shown to be important for heart formation (Fgf8, Tbx1, Shh) was also normal in Arid3b mutants, suggesting that they are not responsible for the cardiac defects. The abnormal morphology and tissue organization of SHF cells in Arid3b null embryos is particularly interesting. It is reminiscent of the defects seen in dishevelled (Sinha et al., 2012) and other planar cell polarity mutants, such as Wnt11 knockout mice (Zhou et al., 2007; Cohen et al., 2012), which also show cardiac malformations, especially in OFT development. Moreover, the altered actin cytoskeleton in Arid3b mutant cells is consistent with the regulation by Arid3b of cell motility in vitro and in vivo (Casanova et al., 2011). Together, these data suggest that Arid3b is required for proper deployment of cardiac progenitors to the heart, which fits with the observed accumulation of Islet1+ cells in the SHF next to the OFT. The DiI fate-mapping experiments confirm a defect in the contribution of SHF progenitors to the heart in Arid3b mutant embryos. Although the precise cellular mechanisms by which cells are incorporated into the heart (e.g. active migration, cell intercalation, oriented cell division) are not completely understood, regulation of these processes appear to require Arid3b. Interestingly, the functional analysis of differentially expressed genes in mutant and WT embryos shows that cell morphology and cellular movement are the most overrepresented pathways. Moreover, one of the most upregulated genes in the mutant hearts is Lim5, a component of integrin-linked kinase complex. Overexpression of this gene has been shown to alter cell adhesion and cell motility (Zhang et al., 2002; Boudoukh et al., 2010), making it a likely candidate mediator of Arid3b effects.

Our results show that Arid3b has a previously unnoticed, direct role in cardiac development, regulating the shape, movements and differentiation of heart progenitors. This establishes Arid3b as a candidate regulator of human heart development and cardiovascular physiology, and it would be important to establish the relevance of human ARID3B mutations in congenital heart disease and hypertrophy.

MATERIALS AND METHODS

Ethics statement

All animal work has been performed according to the guidelines of the Committee on Ethics and Animal Welfare established at CNIC, in accordance to Spanish and European laws. The CNIC Committee on Ethics and Animal Welfare has approved this study.

Embryos

Mouse embryos were collected at the indicated gestational stages, with E0.5 defined as noon on the day on which a vaginal plug was detected. For a more precise staging, pairs of somites (ps) were counted and groups were assigned as follows: E8.5 (8-12 ps), E9.0 (13-16 ps), E9.25 (17-23 ps), E9.5 (24-26 ps). Embryos were dissected in cold PBS and fixed overnight in 4% PFA at 4°C.

Mouse gene-trap model and Arid3b mutants

A mouse line (CD1;129P2-Arid3b<sup>Cre</sup> in vivo) was generated as previously described (Casanova et al., 2011) from a Bayogenics gene-trap ESC line (http://www.genetrap.org/cgi-bin/annotation.py?cellline=RR0j28), which has an insertion of the vector pGT0lfx in the intron after the first coding exon of Arid3b. This produces a fusion protein containing the β-galactosidase (β-Gal) enzyme but lacking all the functional domains of Arid3b. Adult mice were genotyped by PCR based on amplification of the first Arid3b exon (WT and heterozygous) and the presence of the β-Geo cassette in the gene-trap vector (heterozygous). Mutant (referred to as Arid3b<sup>−/−</sup> or Arid3b-null) embryos were obtained by mating heterozygous animals. Embryos were genotyped according to β-Gal activity, measured as the different intensity of staining due to different lacZ expression levels. Heterozygous mice were viable and fertile, and both wild-type and heterozygous embryos were used as controls; for simplicity, we always use the term wild type for the control.

β-galactosidase staining

Embryos were dissected and fixed with 0.125% glutaraldehyde. β-Galactosidase staining was performed in whole embryos or tails with X-Gal as substrate, as previously described (Stoykova et al., 1998).

Histology and immunohistochemistry

Embryos were collected and fixed in 4% PFA overnight at 4°C, dehydrated and embedded in paraffin. Alternatively, embryos were included in gelatine-sucrose and frozen for cryostat sectioning. For histological analysis, 5 µm paraffin sections were stained with haematoxylin and eosin (H&E) using a standard protocol. For immunohistochemistry on 5 µm paraffin sections, slides were hydrated and antigens retrieved by microwave heating for 15 minutes in 10 mM citrate buffer, pH 6.0. Sections were incubated overnight at 4°C with primary antibodies against: Islet1 (Hybridoma Research, 115-066-071; 1:500), H-Ras anti-rabbit (Dako, P0448; 1:100). Slides were then incubated with either streptavidin-peroxidase (Dako, P0448; 1:100) or streptavidin-biotin (Jackson ImmunoResearch, 115-066-071; 1:500), HRP-goat anti-rabbit (Dako, P0448; 1:100). Slides were then incubated with secondary antibodies for 1 h at room temperature: anti-mouse biotin (Jackson ImmunoResearch, 115-066-071; 1:500), H-Ras goat anti-rabbit (Dako, P0448; 1:100). Slides were then incubated either with streptavidin-peroxidase (Dako, P0448; 1:100) or streptavidin-biotin (Jackson ImmunoResearch, 115-066-071; 1:500), or revealed with the TSA Fluorescence System (PerkinElmer, NEL744001KT). Alternatively, 8 µm cryostat sections were used with primary anti-CD31 antibodies (BD Biosciences, 553370; 1:50) or β-galactosidase (Hybridoma Bank, clone 40-1a; 1:10). For actin staining Alexa 488-conjugated phalloidin (Molecular Probes, A12397; 1:200) was used on frozen sections. Slides were mounted with Vectashield containing DAPI. Images were acquired on a Leica TCS SP5 confocal microscope.

RNA in situ hybridisation in whole-mount embryos and on paraffin sections

Whole-mount in situ hybridisation was performed as previously described (Wilkinson and Nieto, 1993). In situ hybridisation on sections (7 µm) was performed in paraffin-embedded embryos according to previously described protocols with some modifications (Kanazawa et al., 1998). Images were acquired with a Nikon Eclipse 90i microscope and in a Leica MZ-12 dissecting microscope. Details of the probes used are provided in supplementary material Table S8.

Proliferation and cell death

Cell death was detected using a TUNEL kit (Roche, In Situ Apoptosis Detection Kit – Fluorescein). Cell proliferation was measured by anti-phospho-Histone3 labelling (Millipore, 06-570; 1:200) on paraffin sections.
as described above. For BrdU staining, embryos at E8.5 were dissected and incubated in culture medium (DMEM, 10% FBS) with 10 μg/ml BrdU for 1 h at 37°C and 5% CO2. Embryos were quickly transferred to ice-cold PBS to quench BrdU incorporation and were fixed overnight in 4% PFA at 4°C. Embryos were embedded in paraffin and immunostaining was performed as described above with anti-BrdU antibody (BD Biosciences, 7580; 1:30). The total number of nuclei (DAPI staining) and BrdU-positive, PH3-positive and TUNEL-positive cells were counted on 9-20 sections from at least three different embryos per condition. Islet1 was used as a marker of the SHF population. Statistical analysis was performed using a two-tailed unpaired Student’s t-test.

**Ink injection**

Indian Ink was injected following a modified version of a previously described protocol (Winnier et al., 1999). Embryos at stage E9.25 were dissected in warm culture medium (DMEM, 10% FBS) and Pelikan Indian Ink diluted 1:40 in PBS was injected into the left ventricle. Embryos were fixed in 4% PFA overnight and dehydrated with methanol. To visualise the ink, embryos were cleared for several hours in benzyl benzoate/benzyl alcohol (vol: 2:1).

**Length measurement in OFT and IFT regions**

For the quantification of the length of the poles of the heart, pictures of the right side (for OFT) or left side (for IFT) of embryos at E9 and E9.5 were analysed using ImageJ software. Lines were manually draw marking the contour of the inner curvature of the OFT up to the start of the right ventricle or the outer curvature of the IFT up to the start of the left ventricle and the length was measured.

For measuring the IFT length we used 12 wild-type and 15 mutant embryos at stage E9.0, and 10 wild-type and 13 Arid3b-null embryos at stage E9.5. For measuring the length of OFT, 12 wild-type and 17 Arid3b<sup>-/-</sup> embryos were used at stage E9.0, and 12 wild-type and 12 mutant embryos at stage E9.5.

**AVC explants**

AVC explants were prepared as described previously (Luna-Zurita et al., 2010). A 1.5 mg/ml solution of rat-tail collagen type I (BD Bioscience) was prepared and allowed to solidify on 4-well micro culture dishes at 37°C and 5% CO2. Gels were washed several times with DMEM containing 10% FBS, antibiotics, anticytotoxic (Fumigzone, Gibco) and non-essential amino acids, and were left for at least 4 h in the same medium supplemented with 1% insulin-transferrin-selenium (ITS; Gibco). Hearts from E9.5 embryos were dissected in the same medium and AVCs were isolated, cut and placed with the endocardium facing the collagen gel. Explants were left to attach overnight at 37°C and 5% CO2. Complete medium was added and explants were left to grow for three days. Explants were fixed with 4% PFA for 30 min at room temperature and stained with phalloidin-Alexa 488 (1:100, Molecular Probes) to reveal the actin cytoskeleton and with anti-SMA-Cy3 (1:80, Sigma) to detect mesenchymal cells. Explants were mounted in Vectashield medium containing DAPI to stain the nuclei. For treatment of AVC explants with Bmp2, collagen gels were incubated 30 min before explant culture in medium containing 500 ng/ml recombinant human Bmp2 (R&D Biosciences) and the medium added to attached explants was supplemented with the same concentration of Bmp2. Two-dimensional migration (number of scattered cells) was counted with Imaris software. Three-dimensional migration (number of cells invading the gel) was determined by counting DAPI stained cells in the z-stack using ImageJ (NIH). At least 17 explants were considered per condition.

**Real-time quantitative PCR**

To perform qRT-PCR, total RNA was extracted from hearts. Embryonic hearts at stage E9.5 were dissected in cold PBS in two regions: the first containing the OFT with the pharyngeal endoderm and mesoderm located beneath (OFT), and the second consisting of atria, AVC, ventricle and IFT (chambers). The dissected hearts were stored at –80°C until RNA extraction. Four pools of five mutant or five wild-type embryos were used. RNA was isolated with a Direct-zol RNA MiniPrep Kit (Zymo Research, R2052).

cDNA was synthesised with a High Capacity cDNA Transcription Kit (Applied Biosystems, 4368814). qRT-PCR was performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems, 4329001), using β-actin and GAPDH as normalising genes. Details of the primers used are provided in supplementary material Table S9.

**Dil labelling and embryo culture**

Dil labelling and embryo culture were performed as described previously (Franco et al., 2001; Dominguez et al., 2012). Embryos ranging from 4 to 11 somites were dissected in Hank’s solution without damaging the yolk sac. A long-chain carboxyamine dye (Dil, Molecular Probes) was dissolved in pure ethanol and diluted 1:9 in 0.3 M sucrose containing 0.1% Nile Blue Sulfate after heating at 42°C for 10 min. To track the contribution to the inflow region embryos with 4-7 pairs of somites were injected in a caudal position within the left lateral mesenchyme positive for Islet1 (posterior SHF). The position was determined by using a grid centred on the midline of the embryo. To track the contribution to the OFT embryos from 8 to 11 somites were injected beneath the heart tube in a rostral Islet1+ region (AHF). The time of injection was considered t=0. Labelled embryos were photographed under a Leica MZ12F dissecting microscope fitted with a fluorescent lamp and cultured in rolling bottles for 24 h in 75% rat serum (Harlan, SR-0100D) and 25% DMEM. For the first 12 h, a 5% CO2, 5% O2, 90% N2 mixture was used; from then on, a 5% CO2, 20% O2, 75% N2 mixture was employed. Two embryos were cultured per bottle, and were distinguished by Dil injection into the head (embryo 2) or without dye (embryo 1). After 24 h in culture embryos were collected in PBS, the yolk sac was removed and embryos were fixed in 4% PFA overnight at 4°C. From the total number of labelled embryos, 51 were excluded due to abnormal development. We also excluded 70 embryos due to inappropriate labelling (cases that presented leakage of Dil into the pericardial cavity or the heart lumen during injection, and cases when labelling was too deep and Dil appeared mostly in the pharyngeal endoderm but not in the heart OFT). A total of 43 wild-type embryos and 23 Arid3b-null embryos were considered for the analysis of embryos marked for IFT contribution, and a total of 82 wild-type embryos and 20 Arid3b-null embryos were analysed in the case of embryos labelled for OFT contribution. The contribution to the IFT was measured by determining whether Dil-labelled cells were observed inside or outside of the heart tube (binomial distribution). The contribution to the OFT was classified into three regions: the length of the OFT was measured by ImageJ and divided into an upper half (distal OFT) and a lower half (proximal OFT). The third region was the right ventricle, defined by using the outer curvature of the heart tube. Employing R, a two-sample test for equality of proportions with continuity correction was used to analyse the results of pSHF labelling, and Fisher’s exact test was used to analyse the contribution to the OFT.

**Whole-mount immunoochemistry**

Embryos were dissected in warm Hank’s medium and marked with Dil, and images were acquired as described above. Embryos were fixed immediately in 4% PFA overnight at 4°C. The next day they were dehydrated with methanol and left overnight at –20°C. Whole-mount immunoochemistry was performed according to standard protocols. Embryos were rehydrated, permeabilised with 0.5% Triton X-100 (Sigma), left 1 h in blocking solution and incubated overnight at 4°C with anti-Islet1 antibody (Hybridoma Bank, clone 39.4D5; 1:100). Over the next two days embryos were washed in PBT four times and incubated overnight, first with biotin Sp anti-mouse IgG (Jackson ImmunoResearch; 1:500) and then with streptavidin-Alexa 488 (Invitrogen; 1:500). Embryos were stored in Vectashield and images were acquired under a Leica fluorescent microscope.

**DNA microarray**

E9.5 embryos were dissected in cold PBS. Three different regions were separated: head (including head and first branchial arch); heart (including the heart tube and the pharyngeal mesoderm and endoderm located beneath); and trunk (including the body from the otic vesicle to somite 15). Four wild-type and four mutant embryos were collected for each of the microarray triplicates. For the E9.0 whole-embryo microarray, four wild-type and four mutant samples, consisting of 3-4 embryos each, were used. RNA was...
extracted with a Qiagen extraction kit and RNA quality was checked using a Bioanalyzer. The data for the microarrays have been deposited in NCBI’s Gene Expression Omnibus and are accessible through GEO Series accession number GSE62072.

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Competing interests

The authors declare no competing financial interests.

Author contributions

V.U. and J.J.S.-E. designed the study, analysed the data, discussed the results and wrote the manuscript. V.U. performed most experiments, with the help of C.B.-C. (histology and in situ hybridisation) and J.C.C. (AVC explants and in situ hybridisation). J.N.D. advised on Dil labelling. J.L.d.l.P. provided probes, reagents and advised on AVC explants. J.J.S.-E. supervised the whole project.

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