Jun Is Required in *Isl1*-Expressing Progenitor Cells for Cardiovascular Development

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Abstract

Jun is a highly conserved member of the multimeric activator protein 1 transcription factor complex and plays an important role in human cancer where it is known to be critical for proliferation, cell cycle regulation, differentiation, and cell death. All of these biological functions are also crucial for embryonic development. Although all Jun null mouse embryos die at midgestation with persistent truncus arteriosus, a severe cardiac outflow tract defect also seen in human congenital heart disease, the developmental mechanisms are poorly understood. Here we show that murine Jun is expressed in a restricted pattern in several cell populations important for cardiovascular development, including the second heart field, pharyngeal endoderm, outflow tract and atrioventricular endocardial cushions and post-migratory neural crest derivatives. Several genes, including Isl1, molecularly mark the second heart field. Isl1 lineages include myocardium, smooth muscle, neural crest, endocardium, and endothelium. We demonstrate that conditional knockout mouse embryos lacking Jun in Isl1-expressing progenitors display ventricular septal defects, double outlet right ventricle, semilunar valve hyperplasia and aortic arch artery patterning defects. In contrast, we show that conditional deletion of Jun in Tie2-expressing endothelial and endocardial precursors does not result in aortic arch artery patterning defects or embryonic death, but does result in ventricular septal defects and a low incidence of semilunar valve defects, atrioventricular valve defects and double outlet right ventricle. Our results demonstrate that Jun is required in Isl1-expressing progenitors and, to a lesser extent, in endothelial cells and endothelial-derived endocardium for cardiovascular development but is dispensable in both cell types for embryonic survival. These data provide a cellular framework for understanding the role of Jun in the pathogenesis of congenital heart disease.

Introduction

Jun (a.k.a. c-Jun) is a highly conserved member of the multimeric activator protein 1 (AP-1) transcription factor complex [1]. The AP-1 protein complexes are a heterogeneous group of transcriptionally active dimers and include members of the Jun family (Jun, JunD, JunB), Fos family (c-Fos, FosB, FosL1, FosL2) and other transcription factor families such as ATF and Maf [2]. Several Jun mutant mice have been generated to study AP-1 function. While Jun heterozygous mice are normal [3], all Jun null embryos die between E12.5 and E14.5 with persistent truncus arteriosus (PTA) [3,4,5]. PTA is a severe developmental cardiac abnormality seen in many patients as an isolated finding or as part of a syndrome such as DiGeorge/22q11 deletion syndrome. Jun proteins can form homo- or heterodimers to differentially regulate transcription [1]. Examination of the promiscuity of these dimer protein-protein interactions has revealed that as part of a DNA-binding complex, Jun is critical for multiple biological processes including cell proliferation, apoptosis, cell cycle progression and differentiation [6,7,8,9]. Although these cellular phenomena are critical for mammalian development and for diseases such as cancer, data regarding the role of Jun during embryogenesis is limited.

The cardiac outflow tract (OFT) incorporates the lineages of multiple cardiac progenitors and its development is dependent upon the complex interaction of several cell types. Neural crest (NC) cells migrate from the dorsal neural tube to the developing aortico pulmonary septation complex to mediate septation of the truncus arteriosus into the main pulmonary artery and aorta [10]. These NC cells contribute to the OFT endocardial cushion mesenchyme which is also comprised of endothelial-derived
Jun is Required in Isl1 Progenitors

Results

Jun is Detected during Mid-gestation in Restricted Cell Populations

Several cell populations are important for normal OFT development and septation. The 100% incidence of PTA in JUN null embryos indicates that Jun is clearly required in one or more of these cell populations. An overview of Jun’s spatial and temporal expression pattern during embryonic development in the mouse is lacking in the literature, particularly prior to E14.5. In limited expression analyses by in situ hybridization and Northern blot, it has been reported that JUN mRNA is expressed in the developing heart, cartilage, gut, central nervous system, lung, kidney, adrenal gland and placenta of the developing mouse [16,17,18,19,20]. To determine the specific cell populations in which Jun might be functioning to regulate cardiac morphogenesis, we examined the expression of JUN by in situ hybridization and immunohistochemistry at several stages of embryonic development between E8.5 and E15.5. Our Jun expression analysis revealed expression in multiple tissues important for heart development and aortic arch artery remodeling. At E8.5, Jun was expressed in the pharyngeal endoderm, dorsal aortic, common atrial chamber, endocardial cushions and in regions populated by SHF mesoderm (Fig. 1A). The anterior SHF expression was stronger than the posterior SHF (Fig. 1A). The expression of JUN in the SHF was also evident at E9.5 by whole mount in situ hybridization (Fig. 1B, C). This is consistent with our previous observation of Jun expression in SHF-derived OFT myocardium [21]. At E9.5, Jun was expressed in the otic vesicle, telencephalon, somites, and aortic arch arteries (Fig. 1B, C). The expression in the telencephalon, somites and pharyngeal arches is consistent with publicly available in situ hybridization data at E11 [http://goege/DeJero] [22]. At E10.5, Jun was highly expressed in the OFT endocardial cushions, AV endocardial cushions and cranial nerve IX (Fig. 1D). The high levels of Jun expression in the OFT endocardial cushions persists until E11.5 (Fig. 1E), where expression in pericardium (Fig. 1E) and dorsal root ganglia (data not shown) was also evident. At E15.5, Jun was broadly expressed in the myocardium and both the semilunar and AV valves (Fig. S1).

Conditional Deletion of Jun in Isl1-expressing Progenitors Results in Severe Cardiovascular Malformations

Jun is expressed in the SHF (Fig. 1) and SHF-derived myocardium (Fig. S1) [21]. The SHF comprises a specialized subset of cardiac progenitor cells derived from early splanchnic mesoderm [13]. These progenitors are marked by the expression of genes such as Isl1 [13,23,24] and play a critical role in the development of the right ventricle and OFT. To determine if Jun is expressed in Isl1-positive cells, we examined the expression of Jun and Isl1 at E8.5 using immunohistochemistry. Our analysis using confocal microscopy revealed co-localization of Jun and Isl1 in the anterior SHF mesoderm (Fig. 2A, C–F) and pharyngeal endoderm (Fig. 2A, B).

To determine if Jun is required in Isl1-expressing progenitors, we performed a conditional deletion of Jun using Isl1flox/flox knock-in mice [25,26]. There are several mutant mice expressing Cre recombinase in Isl1-expressing lineages [25,26,27,28]. To avoid the variable Cre activity observed with theIsl1-IRES-Cre-mouse (Isl1tm1(cre)Tmj),[25,27], we utilized the Isl1flox/flox knock-in mice [25] which have been extensively characterized and shown to drive Cre recombinase expression as early as E8.5 [25,26]. Jun conditional knockout mice (JUNflox/fox) [7] have been validated through tissue-specific deletion in liver [7,29], neuroepithelial cells [30], keratinocytes [31,32] and notochord and sclerotome [33]. To determine if Jun was deleted in the Isl1 lineage, we performed Jun immunostaining of E10.5 Isl1flox/+; Junflox/fox, mtT/mG and control embryos. Cre-mediated recombination in mtT/mG embryos [34] indelibly marks Isl1-derived progenitors with GFP expression. Confocal microscopy of Isl1flox/+; Junflox/fox and control embryonic sections revealed that Jun was efficiently deleted in derivatives of Isl1-expressing progenitors residing in the OFT (Fig. 3).

Isl1flox/+; JUNflox/fox embryos were present at the expected Mendelian ratios (E14.5-P0, n = 23, 100% of predicted; Table 1) and thus survived longer than Isl1 null embryos [3,4,5]. This suggests that Jun function in Isl1-expressing cells is not responsible for the embryonic mortality seen in Isl1 null embryos and supports the hypothesis of Hilberg and Eferl et al. that the embryonic death in Isl1 null is attributable to impaired hepatogenesis [3,5] rather to the cardiac defects. We examined E14.5 to P0 Isl1flox/+; JUNflox/fox and control embryos for cardiac defects. Upon careful examination, we found that 32% of Isl1flox/+; JUNflox/fox conditional mutant embryos (n = 22) showed aortic arch arch remodeling defects (Fig. 4B, B’; Table 2). These defects included interrupted aortic arch (IAA) type B, hypoplasia of the B segment of the aortic arch and aberrant retro-esophageal right subclavian artery. OFT defects were observed in 88% of conditional mutant embryos (n = 8) and included ventricular septal defect (VSD), double outlet right ventricle, and semilunar valve hyperplasia (Fig. 4F, K–N; Table 2). Although there is some evidence that Isl1 progenitors may contribute to the mature AV valves [35], we observed very few GFP-expressing cells derived from Isl1-expressing progenitors cells in the developing AV cushions (Fig. 3A, F). Consistent with this observation, we did not observe any AV valve defects in Isl1flox/+; JUNflox/fox embryos (Fig. 4G, H; Table 2).

Because Isl1flox/+ is a loss-of-function allele [25], we looked for evidence of a genetic interaction between Isl1 and Jun. Neither Isl1flox/flox; Isl1flox/+ nor Isl1flox/flox; Isl1flox/fox mice have discernible cardiac defects [23,25,36], thus heterozygosity of Isl1 from the Cre knock-in could not account for the phenotype in Isl1flox/+; JUNflox/fox mutants. We observed a single Isl1flox/+; JUNflox/fox embryo (1 of 22) with IAA type B. No other OFT or aortic arch abnormalities were noted among Isl1flox/+; JUNflox/fox embryos. While this suggests a subtle genetic interaction, it does not account for the significantly higher incidence of defects observed in Isl1flox/+; JUNflox/fox embryos.

Given Jun’s role in proliferation and apoptosis [6,7], we investigated whether an alteration in one or both of these cellular processes may contribute to the OFT defects observed in Jun mutant mice. Using phospho-histone H3 (pHH3) and TUNEL as markers of cell proliferation and apoptosis, we could not detect any...
Jun Is Required in Isl1 Progenitors

To determine if conditional deletion of Jun in Isl1 (a.k.a. AP-2, used NFATc1 as an endocardial marker [14,37] and Tfap2a important role in the development of the OFT [10,11,14,15]. We endocardial cushion mesenchyme, and cardiac NC cells play an

 Isl1 NFATc1 or Tfap2a expression in E10.5 NC cells. We could not detect any significant differences in expressing progenitors affected the developing endocardium or the phenotype.

These findings suggest that the observed OFT defects may not be solely the result of altered proliferation or apoptosis, but raises the possibility that in combination, these alterations could contribute to the phenotype.

Endothelial progenitors, contributing to the developing OFT endocardial cushion mesenchyme, and cardiac NC cells play an important role in the development of the OFT [10,11,14,15]. We used NFATc1 as an endocardial marker [14,37] and Tiąp2a (a.k.a. AP-2α) as a marker of migrating NC cells (and ectoderm) [36,38] to determine if conditional deletion of Jun in Isl1-expressing progenitors affecting the developing endocardium or NC cells. We could not detect any significant differences in NFATc1 or Tiąp2a expression in E10.5 Isl1Cre/+; Junfllox/− embryos compared with control littermates (Fig. 5F–I). These findings suggest that the defects observed in Isl1 conditional Jun mutant embryos are not attributable to a failure of endocardial formation or cardiac NC migration.

Jun is Required in the Endocardium and Endothelial Progenitors for Valve and Ventricular Septum Formation but not for Aortic Arch Artery Remodeling

Although Isl1-expressing progenitors populate the SHF mesoderm, Isl1 lineages also include other cell populations such as the endocardium and scattered endothelial cells in the aortic arch arteries [13,23,24,26,39]. Endothelial cells, giving rise to the endocardium, undergo an epithelial-to-mesenchymal transformation to contribute to the OFT cushion mesenchymal tissue during OFT septation [11]. The endocardial cushions subsequently give rise to the valves of the mature heart [14,13]. Our finding that Jun was strongly expressed in the OFT endocardial cushion mesenchyme (Fig. 1D, E) together with the prominent endocardial cushions noted in Jun null embryos [5] suggests that Jun may be required in the endothelial progenitors giving rise to endocardial cushion mesenchyme. Hence, we performed fate-mapping studies of endothelial cells in Jun mutant embryos. Endothelial cells and endothelial-derived endocardium were marked with β-galactosidase or GFP expression by crossing transgenic Tie2-Cre mice with R26R or mT/mG Cre reporter mice [34,40,41]. Tie2-Cre mice have been extensively characterized and shown to drive expression specifically in the embryonic endothelium and endocardium as early as E7.5 [41]. In global Jun nulls at E10.5 (Fig. 6D, E), and in endothelial-specific conditional mutants at E15.5 (Fig. 6F), we observed the contribution of Tie2-expressing endothelial progenitors to the endothelial cushion mesenchyme and endothelial lining of the truncus arteriosus and aortic sac and to the semilunar valves in a pattern similar to control embryos (Fig. 6A–C). Although these fate mapping experiments suggest that Jun is not required in Tie2-expressing endothelial progenitors, it remains possible that the loss of Jun in the endocardium results in a functional defect resulting in cardiac defects or aortic arch artery remodeling defects.

To determine if Jun was required for OFT development and aortic arch artery remodeling, we performed a conditional deletion of Jun in endothelium and endothelial-derived endocardium by crossing Junfllox/− donors with transgenic Tie2-Cre mice. To ensure that Tie2-Cre was efficiently deleting Jun in endothelial progenitors we performed co-immunostaining for Jun, the endothelial marker, CD31 (Pecam1) and the smooth muscle cell marker, α-smooth...
muscle actin (SMA). Confocal microscopy of Jun^floxed/floxed and Tie2-Cre; Jun^floxed/floxed embryonic sections revealed that Jun was efficiently deleted in endothelial cells (Fig. 7J) whereas expression in smooth muscle cells was unaffected (Fig. 7J, T).

We analyzed embryonic and neonatal Tie2-Cre; Jun^floxed/floxed mutants for a recapitulation of the cardiac phenotype observed in the Jun null and Isl1 conditional Jun mutant embryos. We quantified the incidence of embryonic death, aortic arch remodeling defects, and cardiac defects and found that, similar to the Isl1^Cre/+; Jun^floxed/floxed embryos, the endothelial-specific knockouts of Jun survive until late gestation or even until birth. Tie2-Cre; Jun^floxed/floxed embryos were present at the expected Mendelian ratios (E15.5-P0; Table 1). We examined E15.5 to P0 Tie2-Cre; Jun^floxed/floxed and control embryos for cardiac defects. Upon careful examination, we did not observe any Tie2-Cre; Jun^floxed/floxed conditional mutant embryos (n = 10) with aortic arch remodeling defects (Table 2). 43% of conditional mutant embryos had a perimembranous VSD and 14% had double outlet right ventricle, mitral or pulmonary valve hyperplasia (Fig. 8A, C; Table 2). We also noted thinning of the compact myocardium of the right ventricle (Fig. 8A) in 43% (n = 3/7) of Tie2-Cre; Jun^floxed/floxed embryos. This phenotype is similar to that previously described in the global Jun null embryos [5]. To determine if these defects were due to a cell non-autonomous effect on NC cell migration, we performed Tfap2a immunostaining of Tie2-Cre conditional Jun mutant and control embryos. We observed a similar pattern of Tfap2a expression in the developing OFT and pharyngeal arches in E10.5 Tie2-Cre; Jun^floxed/floxed embryos compared with controls (Fig. 6G–J) suggesting that defects observed in Tie2 conditional Jun mutant embryos are not attributable to a failure of cardiac NC cell migration. Thus, Jun is not required in endothelial cells and endothelial-derived endocardial cushions for aortic arch artery remodeling or embryonic survival, but is required for the formation of the ventricular septum, compact myocardium and to a lesser extent for formation of the OFT and valves.

Discussion
Congenital heart disease (CHD) is the most commonly occurring major birth defect in humans with an incidence of 6 per 1000 live births [42]. OFT malformations, including PTA, constitute the largest class of life-threatening CHD. It has been more than a decade since it was recognized that the global loss of Jun in mice uniformly results in PTA [5]. Information regarding Jun expression in cardiac progenitors during critical stages of heart development has been poorly described and thus it has remained unclear in what cell populations Jun may regulate transcription. We show that Jun is expressed in a restricted pattern in several cell populations including the SHF, pharyngeal endoderm, OFT and

Figure 2. Jun and Isl1 are co-expressed in a subset of cells in the developing outflow tract. (A, D) Transverse sections of an E8.5 wild-type embryo showing Jun (green) and Isl1 (pink) immunostaining in anterior SHF mesoderm and pharyngeal endoderm. Sections were co-stained with DAPI to illustrate nuclei. (B, C, E, F) Higher power confocal images of the areas indicated by white boxes in panels A and D. Cell populations co-expressing Jun and Isl1 are indicated by the open arrowheads. AoS, aortic sac; FG, foregut; H, heart; NT, neural tube; PC, pericardial cavity. doi:10.1371/journal.pone.0057032.g002
AV endocardial cushions and post-migratory NC derivatives such as cranial nerve IX and the dorsal root ganglia.

Although Jun null embryos die with PTA, we did not observe PTA in conditional jun mutants. There are at least two explanations for this finding. There may be functional redundancy among Jun proteins and/or Jun may be required in other cardiac progenitors not expressing Isl1. There is evidence to suggest some degree of functionally redundancy among Jun proteins, such as Jun, Junb and Jund, during heart development. Jund<sup>−/−</sup> mice have no cardiac defects [43] and Junb<sup>−/−</sup> null embryos die at E8.5-E10 due to multiple defects in extra-embryonic tissues [44]. Wagner et al. have generated mice in which either Junb or Jund is knocked-in to the Jun locus to test whether Junb or Jund, under the control of the endogenous Jun regulatory elements, can rescue the Jun null phenotype [20,45]. While both of these knock-in mice rescue the mortality at E13 seen in Jun<sup>−/−</sup> embryos [20,45], the rescue of the cardiac defects is not as straightforward. Jun<sup>amb/Jund</sup> embryos survive to E18.5 in Mendelian ratios but continue to have PTA (similar to Jun<sup>−/−</sup> embryos [20]) and VSDs. The same authors then tested whether overexpression of Junb, with Junb transgenic mice under the control of human ubiquitin C promoter [46] were able to rescue the Jun null phenotype. Junb-Tg; Jun<sup>−/−</sup> embryos are born with no cardiac defects demonstrating redundancy that is dependent on gene dosage [20]. Jun<sup>amb/Jund</sup> embryos survive to E18.5 in Mendelian ratios but also display PTA similar to Jun<sup>−/−</sup> embryos [45], suggesting that Jund is not functionally redundant for Jun during cardiac development. The rescue of embryonic lethality but not heart defects indicates that different developmental processes have different sensitivities to Jun dosage. It is unclear whether Jun overexpression can rescue the cardiac defects seen in Jun null embryos. Future studies to test whether decreasing the dosage of Jun or Jund may uncover functional redundancy during heart development may include the analysis of heart defects in Isl1<sup>Crea/+; Junb<sup>lox/lox</sup>; Junb<sup>−/−</sup> or Isl1<sup>Crea/+; Junb<sup>lox/lox</sup>; Jund<sup>−/−</sup> mutants.

Figure 3. Jun is efficiently deleted in derivatives of Isl1-expressing cells in the outflow tract. Saggital sections of E10.5 Isl1<sup>Crea/+; mT/mG; Jun<sup>Cre/+</sup> (A) and Isl1<sup>Crea/+; mT/mG; Jun<sup>lox/lox</sup> (F) embryos co-immunostained with anti-GFP (green) and anti-Jun (red) and analyzed by confocal microscopy showing nuclear Jun expression. Isl1<sup>Cre/+</sup>-expressing progenitors were marked with GFP expression using the mT/mG double-fluorescent Cre reporter mice. Sections were co-stained with DAPI to illustrate nuclei. (B–E, G–J) Higher power images of the areas indicated by white boxes in panels A, F. Jun is efficiently deleted in derivatives of Isl1-expressing progenitors located in the OFT (arrowheads) whereas AV cushion cells (arrows), not derived from Isl1-expressing progenitors, express Jun in both control (E) and mutant embryos (J). A, atrium; AoS, aortic sac; LV, left ventricle. Scale bar: 100 μm.

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Jun transcriptional activity is dependent on Jun N-terminal kinase (JNK) phosphorylation [47,48]. This JNK-dependent Jun phosphorylation is dispensable for embryonic development, including for cardiogenesis. Behrens et al. demonstrated this by generating mice in which the Jun locus was mutated to prevent JNK phosphorylation (Jun<sup>AA</sup>; [49]). Jun<sup>AA</sup> homozygous mice, unlike Jun<sup>−/−</sup> mice, are born in Mendelian ratios and are healthy and fertile, without heart defects, as adults. The notion that JNK-dependent phosphorylation is not essential for heart development is further supported by the observation that Jun<sup>mutant</sup> mice survive without heart defects [50].

An alternative explanation for the PTA seen in Jun null embryos but not in the conditional mutants is that Jun may have roles in multiple cell populations involved in regulating OFT septation. The proximal portion of the OFT cushions (conus) becomes the subpulmonary infundibulum and failure of this structure to form properly results in VSDs. The distal OFT cushions (truncus) gives rise to the semilunar valves and intrapericardial portions of the aorta and pulmonary artery. More distally, the dorsal wall of the aortic sac becomes the aorticopulmonary septum. In humans, defects in this structure result in an aorticopulmonary window [51,52]. A failure of septation in both the distal OFT and the aortic sac-derived portion of the great arteries results in PTA which usually, but not always [53], is associated with VSDs. The PTA in Jun null embryos [5] together with our observation of VSDs in the absence of both PTA and a common truncal valve, supports a model in which “conal” septation, regulated by Jun in Isl1-progenitors, is mechanistically separate from both “truncal” and aorticopulmonary septation. Jun may play a role in a non-Isl1-expressing domain to regulate “truncal” and aorticopulmonary septation.

Heart development is dependent upon the complex interaction and contribution of several cell types. There are multiple lines of evidence supporting the notion that within the early mesoderm, there is a common cardiovascular progenitor that gives rise to myocardial, smooth muscle and endothelial lineages [54]. Within
the splanchnic mesoderm, the SHF, a group of cardiovascular precursors destined to give rise to the right ventricle and OFT, is molecularly marked by the expression of Tbx1, a Mef2c regulatory module, and by Isl1 [13,23,55,56,57]. Data from both murine models and from humans suggest that ISL1 plays a role in heart development and possibly human CHD. There is a report of a diabetic patient who harbors an ISL1 mutation [58], but there have been none described in patients with CHD. Despite this, there is evidence to suggest that common ISL1 single nucleotide polymorphisms (SNPs) are associated with human CHD [59]. It remains to be determined if these SNPs are causative or if they are linked to an associated causative locus. The current study provides data to support a possible role for JUN in ISL1 progenitors and thus adds to the likelihood that this association may be causative.

Multipotent Isl1-positive progenitors, when isolated and cultured from either mouse or human embryonic hearts, are capable of differentiating into each of these three lineages [24,60]. This has also been demonstrated using a potentially overlapping population of Nkx2.5-positive progenitor cells [61]. This ex vivo data is consistent with in vivo fate-mapping studies, using an Isl1 inducible Cre, showing the contribution of Isl1 derivatives to the same three lineages [28]. Recent evidence suggests that some Isl1-expressing

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**Table 1. Summary of genotypes for Jun conditional mutants.**

| Genotype (E14.5-P0) | n (%) | Genotype (E15.5-P0) | n (%) |
|---------------------|-------|---------------------|-------|
| Isl1Cre/+;Junflox/+  | 23 (25%) | Tie2-Cre;Junflox/+  | 12 (24%) |
| Isl1Cre/+;Junflox/lox| 23 (25%) | Tie2-Cre;Junflox/lox| 10 (20%) |
| Junflox/+           | 26 (28%) | Junflox/+           | 15 (31%) |
| Junflox/lox         | 20 (22%) | Junflox/lox         | 12 (24%) |

χ² = 0.94, p = 0.34

χ² = 0.92, p = 0.34

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Figure 4. Isl1-specific deletion of Jun results in cardiovascular defects. Compared to control embryos (A, A’, C–E, I–L), the loss of Jun in Isl1-expressing precursors results in IAA (B, B’), VSD (arrowhead, E), and enlarged and hyperplastic pulmonary valve leaflets (arrowhead, O). The atrioventricular valves are unaffected (C, D, F, G). Ao, aorta; CA, carotid artery; DA, ductus arteriosus; DAO, descending aorta; LA, left atrium; lv, left ventricle; MV, mitral valve; PA, pulmonary artery; RA, right atrium; rv, right ventricle; SCA, subclavian artery; TV, tricuspid valve.

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X2 p = 0.94

X2 p = 0.92

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progenitor cells may also be of a NC lineage (discussed below) [62].

Table 2. Cardiovascular abnormalities in late gestation Jun conditional mutants.

|                     | Jun<sup>lox<sup>-<sup> or Jun<sup>lox<sup>-/lox<sup> | Isl1<sup>Cre<sup>-<sup>; Jun<sup>lox<sup>-/lox<sup> | Tie2-Cre; Jun<sup>lox<sup>-/lox<sup> |
|---------------------|-----------------------------------------------|-----------------------------------------------|----------------------------------|
| Aortic arch artery remodeling defects | 0% (0/46) | 32% (7/22) | 0% (0/10) |
| Ventricular septal defect | 0% (0/9) | 88% (7/8) | 43% (3/7) |
| Double outlet right ventricle | 0% (0/9) | 88% (7/8) | 14% (1/7) |
| Mitral valve defect | 0% (0/9) | 0% (0/8) | 14% (1/7) |
| Tricuspid valve defect | 0% (0/9) | 0% (0/8) | 0% (0/7) |
| Pulmonary valve defect | 0% (0/9) | 88% (7/8) | 14% (1/7) |
| Aortic valve defect | 0% (0/9) | 75% (6/8) | 0% (0/7) |

Our analysis of mutants lacking Jun in the endothelial lineage reveals that Jun is required for heart development in some, but not all, Tie2-derived endothelial cells and endothelial-derived endo-

Figure 5. Effect of the loss of Jun in Isl1-expressing progenitors on proliferation, apoptosis, cardiac neural crest cells and the endocardium. Sagittal sections of E10.5 mutant (B, D, G) and control (A, C, F) embryos analyzed by pH3 and NFATc1 immunostaining and by TUNEL assay. pH3 immunostaining (pink) reveals similar proliferation rates in mutant and control OFT cells (A, B). Dotted lines show representative areas used for cell counting with ImageJ software. TUNEL assay reveals similar numbers of TUNEL-positive (green) OFT cells in mutant and control embryos (C, D). (E) Quantitative analysis of the percentage of pH3- and TUNEL-positive OFT cells in serial sections showing a statistically insignificant trend toward less proliferation and more apoptosis in conditionally deleted embryos at E10.5. Results are expressed as mean ± SEM of the percent positive nuclei. The statistical significance of differences between groups was analyzed by the Student’s t-test. (F, G) Expression of the endocardial marker NFATc1 (pink), is not significantly different between conditional mutant and control embryos. Sagittal sections of anti-Tfap2a immunostained Jun<sup>lox<sup>-<sup> (H) and Isl1<sup>Cre<sup>-<sup>; Jun<sup>lox<sup>-/lox<sup> (I) embryos showing no significant difference in Tfap2a-expressing neural crest cells (arrowheads) at E10.5. Sections were co-stained with DAPI to illustrate nuclei. A, atrium; AVC, atrioventricular cushion; LV, left ventricle; OFT, outflow tract; OFTC, outflow tract cushion; SMA, smooth muscle actin.

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Figure 6. Loss of Jun does not alter the fate of Tie2-expressing endothelial derivatives or cardiac neural crest cells in the developing OFT. Transverse sections of X-gal stained Jun+/2; Tie2-Cre;R26R (A, B) and Jun−/−; Tie2-Cre; R26R (D, E) embryos showing no significant difference in endothelial derivatives (blue) populating the OFT endocardial cushion mesenchyme (arrowheads) at E10.5. Transverse sections of anti-GFP immunostained Tie2-Cre; Junflox/+ (C) and Tie2-Cre; Junflox/flox (F) embryos showing no significant difference in endothelial derivatives (green) populating the semilunar valves, heart and blood vessels at E15.5. Transverse sections of anti-Tfap2a immunostained Tie2-Cre; Junflox/+ (G, H) and Tie2-Cre; Junflox/− (I, J) embryos showing no significant difference in Tfap2a-expressing neural crest cells at E10.5. Sections were co-stained with DAPI to illustrate nuclei. Ao, aorta; AoS, aortic sac; LA, left atrium; PA, pulmonary artery; RA, right atrium; TA, truncus arteriosus.
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cardium. The thinning of the compact myocardium of the right ventricle observed in the Tie2 conditional Jun mutants suggests a model in which there is signaling from the endocardium to regulate the differentiation of primitive myocardial epithelium into compact myocardium. Pathways involved in reciprocal paracrine signaling between the endocardium and myocardium include Neuregulin-1, EphrinB2, Notch, Neurofibromin 1, VEGF, Angiopoietin-1, and Fgf [63,64,65,66,67]. The role of endocardial Jun in one of these pathways or in a parallel pathway remains to be determined. It is intriguing that there was no compact zone thinning of the left ventricle suggesting that endocardial Jun is not required for the differentiation of all primitive myocardial epithelium. Our findings of VSDs in both Isl1 and Tie2 conditional Jun mutants also support this paracrine model although we cannot conclude from our data whether or not the critical subset of Tie2-derived cells is descended from Isl1-positive progenitors. Currently available techniques do not allow us to conditionally delete Jun in Tie2/Isl1-positive progenitors in vivo while leaving Jun unaltered in Tie2-positive Isl1-negative progenitors. Thus, it remains to be tested if Jun is dispensable in the Isl1-negative endocardial lineage. Alternatively, the VSDs we observed may be a common phenotype resulting from independent requirement for Jun in

Figure 7. Jun is efficiently deleted in Tie2-expressing endothelial derivatives. Cross sections of E15.5 Junflx/flx (A, K) and Tie2-Cre; Junflx/flx (F, P) embryos co-immunostained with anti-CD31, anti-SMA and anti-Jun analyzed by confocal microscopy. Sections were co-stained with DAPI to illustrate nuclei. (B–E, G–J) Higher power images of the areas indicated by white boxes in panels A, F. Smooth muscle cells (arrows) express Jun (green) in control (E) and mutant embryos (J) whereas Jun is efficiently deleted in endothelial cells (arrowheads). (L–O, Q–T) Higher power images of the area indicated by white boxes in panels K, P. Jun (red) is expressed in SMA-positive smooth muscle cells (green; arrows) in control (K) and mutant embryos (P). Ao, aorta. Scale bar: 20 μm.
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both Isl1-negative endothelial/endocardial cells and Tie2-negative Isl1 progenitors. Our finding of aortic arch artery patterning defects in Isl1-specific Jun deleted embryos raises the possibility of a cell autonomous effect of Jun in an Isl1-derived smooth muscle lineage or alternatively a cell non-autonomous effect on another cell population such as NC. There are other examples of genetic alterations in the SHF affecting a tissue-tissue interaction with NC cells. We have shown that deletion of Notch in the SHF using Isl1Cre or Mef2c-AHF-Cre results in severe NC-related cardiac defects including PTA and IAA [36], defects identical to those seen in our Isl1Cre;Junfloxflox embryos and in Jun null embryos [5]. Further tissue-restricted deletion studies are required to determine the relative requirement for Jun specifically in the smooth muscle or myocardial lineages.

Our expression analysis indicates that Jun is expressed in post-migratory NC derivatives. NC cells are a specialized subset of neuroepithelial cells in the dorsal neural tube that migrate ventrally and contribute to a diverse array of tissues. Raivich et al. have previously reported that Jun is expressed in neuroepithelial cells [68] yet deletion of Jun in neuroepithelial cells with Nestin-Cre did not result in congenital heart defects [30,69]. It is unclear if Nestin-Cre is expressed in a cardiac NC subset of neuroepithelial cells. Cardiac NC cells, originating between the mid-otic placode and the third somite, invade the pharyngeal arches and encompass the aortic arch arteries around E10. By E10.5 they populate the cardiac OFT as two columns of cells subsequently forming a portion of the mesenchymal tissue in the OFT endocardial cushions. Notably, these cushions are abnormal in Jun null embryos [5]. Ultimately, descendants of this subset of NC cells contribute to the aorticopulmonary septum, dividing the truncus arteriosus into the aorta and pulmonary artery [10,70,71]. Although there is evidence that the complete loss of Jun does not globally affect the fate of NC cells, by E12.5 there are fewer

Figure 8. Tie2-specific deletion of Jun results in cardiovascular defects. Compared to control embryos (B, D), the loss of Jun in Tie2-expressing precursors results in VSD (open arrowhead, A), thinned RV (arrows, A) and thickened and hyperplastic mitral valve (closed arrowhead, A) and pulmonary valve leaflets (open arrowhead, C). Ao, aorta; PA, pulmonary artery; RV, right ventricle.

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connexin43-labeled cardiac NC cells populating the right ventricular OFT of Jun null embryos [5]. In contrast, our findings suggest that the defects observed in Isl1 and Tie2 conditional Jun mutant embryos are not attributable to a cell non-autonomous defect of cardiac NC migration. In the global Jun null embryo, it is unknown whether the fewer connexin43-labeled cells is due to a secondary cell non-autonomous mechanism affecting NC as the result of a loss of Jun function in another tissue such as SHF or pharyngeal endoderm. This is further complicated by the recent observation that some Isl1-expressing progenitor cells may be of a NC lineage [62]. This novel and surprising data raises the possibility that the defects observed in Isl1-conditioned Jun mutations may reflect a cell autonomous defect of NC. The effect of conditional deletion of Jun in Isl1-expressing progenitors on NC cell differentiation was not determined in the current study but is an important area for future investigation. Tissue-restricted deletion studies using Wnt1-Cre or Pax3CrePG mice [72,73] are required to determine the requirement for Jun specifically in NC derivatives.

Jun, as part of the AP-1 transcription factor complex, is a positive regulator of cell proliferation [7,8,32] and positively regulates cell cycle progression through p19, p53, cyclin D1 and cyclin A pathways [6,8,29,74,75,76,77]. It regulates the differentiation of varied cell populations such as hematopoietic cells and keratinocytes [6,9] and has also been shown to regulate apoptosis in such cells as fibroblasts, hepatocytes and neurons [6,8,78,79]. Although these pleiotropic cellular functions are likely to be important for all progenitors, our observation that Isl1- and Tie2-specific Jun knockouts survive embryogenesis in Mendelian ratios indicates that Jun is dispensable in these progenitors for overall embryonic survival. Though Jun is not required for formation or maintenance of the blood vessels (at least after the time of Tie2-Cre-mediated recombination), it is not completely dispensable in endothelial precursors as they display a low incidence of cardiac defects. Our data do not exclude the possibility of subtle defects in endothelial cell function in these Jun mutant embryos.

The molecular mechanisms by which Jun functions during heart development, in Isl1-expressing cells and other cells remains to be determined. In the embryonic human heart, ISL1 is strongly expressed in highly proliferative SHF-derived cell populations in the arterial and venous poles [80]. We observed a statistically insignificant trend toward a decrease in proliferating cells and an increase in apoptosis in the OFT of mutant embryos. These individual differences were modest when compared with controls, but in combination these two alterations may contribute to defects in OFT seption or complete formation of the ventricular septum. There are several transcriptional networks and signaling pathways known to regulate proliferation in the SHF. These include Nkx2.5, Wnt/β-catenin, Notch, Fgf, Shh, Isl1, and Tbx1 signaling pathways (reviewed in [13]). TBX1 is a molecular marker of the SHF and accruing evidence points to a causative role for this transcription factor in the pathogenesis of DiGeorge syndrome (DGS). It has been proposed that the loss of TBX1 in patients with DGS results in defective proliferation in the SHF [81,82], although little mechanistic data have yet been published to support this notion. Cardiac defects observed in patients with DGS and in Tbx1 mutant mice are strikingly similar to those seen in Jun mutant mouse embryos [83,84,85] and to mice in which NC has been disrupted [10]. Despite the similarity with NC mutants and reports of a disruption in the distribution of NC-derived cells in Tbx1 nulls, Tbx1 is not expressed in NC [86]. One hypothesis is that Jun and Tbx1 could be acting in concert to regulate the proliferation of SHF progenitors and subsequently affect interactions with other tissues such as NC. Future studies are required to determine if Jun may function in a Tbx1-dependent pathway and to further elucidate cell autonomous and cell non-autonomous mechanisms of Jun function during heart development.

Materials and Methods

Ethics Statement

Animal studies were conducted in accordance with the National Institutes of Health National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the Children’s Hospital of Philadelphia Research Institute Institutional Animal Care and Use Committee (No. 2008-6-840; Department of Health and Human Services Animal Welfare Assurance #A3442-01).

X-Gal Staining
Whole mount embryos were stained for β-galactosidase activity using previously described methods [87].

Confocal Microscopy
Confocal images were acquired with the Zeiss LSM 510/NLO META confocal microscope using 20x, 0.8 NA Plan-Apochromat air immersion and 63x, 1.4 NA Plan-Apochromat oil immersion objectives.

Mutant Mice and Genotyping
All mouse strains used are described and listed here: Jun<box>lox/lox</box> is Jun<box>Cre/+</box> [7], Isl1<box>Cre/+</box> is Isl1<box>Cre/+</box> [25], Tie2-Cre is Tg(Tek-cre)/Ywa [41], R26R is Gt(ROSA)26Sor<box>Cre</box> [40], mT/mG is Gt(ROSA)26Sor<box>Cre</box>/mG<box>Cre</box> [34], and Jun<box>Cre/+</box> is Jun<box>Cre/+</box> [4]. The last four lines were obtained from the Jackson Laboratory (Bar Harbor, ME). Mouse genotyping was performed using real-time quantitative polymerase chain reaction (qPCR) techniques. Genomic DNA was isolated with the Extract-N-Amp Tissue PCR kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer’s recommended protocol. PrimeTime qPCR assays (Integrated DNA Technologies, Coralville, IA) consisting of the primer and probe sequences listed in Table S1 were used. The mouse GAPD TaqMan Gene Expression Assay (Life Technologies, Carlsbad, CA) was used as the endogenous control.

Immunohistochemistry

Immunofluorescence (IF) and horseradish peroxidase (HRP) immunostaining were performed as described previously [88] using rabbit monoclonal anti-Jun 60A8 (1:100; IF, Cell Signaling Technology, Danvers, MA), rat monoclonal anti-CD31 MEC13.3 (Pecam1; 1:100; BD Pharmingen, Franklin Lakes, NJ), mouse monoclonal anti-Isl-1 39.4D5 (1:25 IF; Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit polyclonal anti-GFP (1:200 IF, Life Technologies), mouse monoclonal anti-SMA 1A4 (1:200 IF, Life Technologies), rabbit polyclonal phospho-histone H3 Ser10 (1:200 IF, Cell Signaling Technology), mouse monoclonal AP-2 alpha (Tfap2a) 3B5 (1:4 IF, Developmental Studies Hybridoma Bank) and mouse monoclonal NFATc1 7A6 (1:25 IF, Developmental Studies Hybridoma Bank). TUNEL assays were performed using the In Situ Cell Death Detection Kit (Roche, Indianapolis, IN) according to the manufacturer’s recommended protocol. Images were quantified by using the Image-based Tool for Counting Nuclei (ITCN) plug-in (http://goo.gl/QP2fB) for ImageJ (NIH, Bethesda, MD). The mean and SEM were calculated based on three independent sections per embryo.
Whole Mount in situ Hybridization

Whole mount in situ hybridization was performed as described [89] with modifications. Digoxigenin-UTP-labeled RNA probes were generated from plasmid template containing mouse Jun (IMAGE clone 3493246; GenBank accession number BC002681) by XmnI restriction digestion and transcription with T7 RNA polymerase. Embryos were fixed in 4% PFA overnight, then dehydrated in a graded series of methanol in PBS. The embryos were serially rehydrated to PBS. Embryos were permeabilized in RIPA buffer thrice for 5 minutes at room temperature. Embryos were refixed in 4% PFA/0.2% glutaraldehyde in PBS for minutes, washed and hybridized overnight at 70°C in hybridization solution containing 1 µg probe/mL. Embryos were washed and hybridized with alkaline phosphatase conjugated anti-digoxigenin antibody (Roche) overnight at 4°C, washed for 24 hours and developed in BM purple (Roche).

Supporting Information

Figure S1  Jun is broadly expressed in the late gestation mouse heart. (A, E) Transverse sections of an E15.5 wild-type mouse heart showing nuclear Jun immunostaining (green) in the myocardium and valves. (B–D) Higher power images of the areas shown in the white boxes in panel A showing Jun expression in the myocardium and atrioventricular valves. (F) Higher power image of the area shown in the white box in panel E showing Jun expression in the right ventricular outflow tract myocardium and pulmonary valve. Sections were co-stained with DAPI to illustrate nuclei. Ao, aorta; IVS, interventricular septum; LA, left atrium; LV, left ventricle; MV, mitral valve; PA, pulmonary artery; PV, pulmonary valve; RA, right atrium; RV, right ventricle; TV, tricuspid valve. (TIF)

Table S1 qPCR Genotyping Assays. Primer and probe sequences used for mouse genotyping. Mouse GAPDH was used as the endogenous control. (DOC)

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Author Contributions

Conceived and designed the experiments: FAI JZS. Performed the experiments: TZ JL JZ EBT TLM JZS. Analyzed the data: TZ JL JZ FAI FW JZS. Contributed reagents/materials/analysis tools: TZ EBT TLM FAI JZS. Wrote the paper: FAI JZS.

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Jun Is Required in Isl1 Progenitors