

RESEARCH ARTICLE

Myocardial *Notch1-Rbpj* deletion does not affect NOTCH signaling, heart development or function

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OPEN ACCESS

Citation: Salguero-Jiménez A, Grego-Bessa J, D'Amato G, Jiménez-Borreguero LJ, de la Pompa JL (2018) Myocardial *Notch1-Rbpj* deletion does not affect NOTCH signaling, heart development or function. PLoS ONE 13(12): e0203100. <https://doi.org/10.1371/journal.pone.0203100>

Editor: Thomas Brand, Imperial College London, UNITED KINGDOM

Received: August 10, 2018

Accepted: December 11, 2018

Published: December 31, 2018

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Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This study was funded by grants SAF2016-78370-R, CB16/11/00399 (CIBER CV) and RD16/0011/0021 (TERCEL) from the the Spanish Ministry of Science, Innovation and Universities (MCIU) and grants from the Fundación BBVA (Ref.: BIO14_298) and Fundación La Marató (Ref.: 20153431) to JLDLP. AS-J holds a FPU fellowship from the Ministerio de Educación,

Abstract

During vertebrate cardiac development NOTCH signaling activity in the endocardium is essential for the crosstalk between endocardium and myocardium that initiates ventricular trabeculation and valve primordium formation. This crosstalk leads later to the maturation and compaction of the ventricular chambers and the morphogenesis of the cardiac valves, and its alteration may lead to disease. Although endocardial NOTCH signaling has been shown to be crucial for heart development, its physiological role in the myocardium has not been clearly established. Here we have used mouse genetics to evaluate the role of NOTCH in myocardial development. We have inactivated the unique and ubiquitous NOTCH effector RBPJ in early cardiomyocytes progenitors, and examined its consequences in cardiac development and function. Our results show that mice with *Tnnt2-Cre*-mediated myocardial-specific deletion of *Rbpj* develop to term, with homozygous mutant animals showing normal expression of cardiac development markers, and normal adult heart function. Similar observations have been obtained after *Notch1* deletion with *Tnnt2-Cre*. We have also deleted *Rbpj* in both myocardial and endocardial progenitor cells, using the *Nkx2.5-Cre* driver, resulting in ventricular septal defect (VSD), double outlet right ventricle (DORV), and bicuspid aortic valve (BAV), due to NOTCH signaling abrogation in the endocardium of cardiac valves. Our data demonstrate that NOTCH-RBPJ inactivation in the myocardium does not affect heart development or adult cardiac function.

Introduction

The heart is the first organ to form and function during vertebrate development. At embryonic day 7.0 (E7.0) in the mouse, cardiac progenitor cells, migrating from the primitive streak, reach the head folds on either side of the midline [1] and by E8.0, fuse and form the primitive heart tube [2]. The heart tube consists internally of the endocardium, that is separated from the primitive myocardium by an extracellular matrix termed cardiac jelly [3]. The NOTCH

Cultura y Deporte of Spain. JG-B is funded by the Programa de Atracción de Talento of the Comunidad de Madrid (2016T1/BMD1540). The cost of this publication was supported in part with FEDER funds. The CNIC (<https://www.cnic.es/en>) is supported by the MEIC and the Pro-CNIC Foundation, and is a Severo Ochoa Center of Excellence (SEV-2015-0505).

Competing interests: The authors have declared that no competing interests exist.

signaling pathway is crucial for the endocardial-myocardial interactions that regulate the patterning, growth and differentiation of chamber and non-chamber tissues that will develop from E8.5 onwards [4–8]. The main components of the pathway are the single-pass transmembrane NOTCH receptors (NOTCH1–4 in mammals) that interact with membrane-bound ligands of the JAGGED (JAG1 and JAG2) and DELTA families (DELTA LIKE1, 3 and 4), expressed in neighboring cells [9, 10]. Ligand-receptor interactions leads to three consecutive cleavage events that generate the NOTCH intracellular domain (NICD), which can translocate to the nucleus of the signaling-receiving cell [11]. In the nucleus, NICD binds directly to the DNA-binding protein CSL (CBF1/RBPJ/Su(H)/Lag1) [12] and recruits the co-activator Mastermind-like [13, 14]. In the absence of NICD, ubiquitously expressed RBPJ (recombination signal binding protein for immunoglobulin kappa J region) may act as a transcriptional repressor [15]. The best characterized NOTCH targets in the heart are the HEY family of basic helix–loop–helix (bHLH) transcriptional repressors [16], although various other cardiac-specific targets have been described [4, 17–19]. Functional studies in *Xenopus* or in *Rbpj*-targeted mouse embryonic stem cells have shown that NOTCH suppresses cardiomyogenesis [20, 21], although studies with targeted mutant mice have demonstrated an essential requirement for NOTCH in cardiac development only after heart tube formation (around E8.5) [22, 23].

One of the first signs of cardiac chamber development is the appearance of trabeculae at E9.0–9.5 [24]. Trabeculae are myocardial protrusions covered by endocardium that grow towards the ventricular lumen, and serve to facilitate oxygen exchange and nourishment between the blood and the developing heart. The ligand DLL4 and the active NOTCH1 receptor are expressed in the endocardium prior to the onset of trabeculation [4, 25]. DLL4–NOTCH1 signaling is reflected by the endocardial expression of the CBF:H2B–Venus transgenic NOTCH reporter in mice [17]. Conditional inactivation of *Dll4*, *Notch1* or *Rbpj* in the endocardium, results in very similar phenotypes (more severe in *Rbpj* mutants) consisting of ventricular hypoplasia and impaired trabeculation [4, 17, 26], while myocardial deletion of *Jag1* does not affect trabeculation [17]. Later, the ligands JAGGED1 and JAGGED2 expressed in the myocardium, activate NOTCH1 signaling in the endocardium in a MIB1-dependent manner, to sustain ventricular compaction and maturation [17, 27]. Conditional myocardial deletion of *Mib1* or combined inactivation of *Jag1* and *Jag2* abrogates endocardial NOTCH activity, and leads to abnormally thin compact myocardium and large and non-compacted trabeculae, a phenotype strongly reminiscent of a cardiomyopathy termed left ventricular non-compaction (LVNC) [17, 27]. Thus, endocardial NOTCH signaling and its downstream effectors are essential for the endocardium-to-myocardium signaling that regulates chamber patterning and growth [28–30]. Endocardial NOTCH activity is also crucial for development and morphogenesis of the cardiac valves [5, 18, 29, 31–37]. NOTCH receptors or transgenic reporter lines are expressed in the endocardium, coronary vessels endothelium and smooth muscle cells [17, 25, 38, 39]. There is no evidence of endogenous NOTCH expression or activity in the embryonic myocardium, and despite elegant ectopic expression experiments have reported a function for NOTCH in cardiomyocytes [40, 41], a physiological role for NOTCH in the developing myocardium has not been clearly demonstrated *in vivo*.

To address this question, we have conditionally inactivated the NOTCH effector RBPJ in the myocardium using two early-acting myocardial drivers, and examined its consequences in heart development. We find that *Tnnt2-Cre* mediated myocardial-specific deletion of *Rbpj* does not affect NOTCH signaling in the endocardium, heart development or adult heart function. Similar findings were obtained upon *Notch1* inactivation in the myocardium. In contrast, while *Nkx2.5-Cre* mediated *Rbpj* inactivation in the myocardium does not affect cardiac development and structure, *Rbpj* inactivation in valve endocardial cells disrupts valve morphogenesis. Our data demonstrate that myocardial NOTCH–RBPJ is not required for cardiac muscle

development or function, reinforcing the notion that physiological NOTCH-RBPJ signaling occurs in the endocardium, endothelium and smooth muscle cells of the developing heart.

Results and discussion

We first compared expression of the NOTCH effector RBPJ to the pattern of NOTCH activation in the E12.5 heart. RBPJ was widely expressed in the nucleus of endocardial, myocardial and epicardial cells (Fig 1A–1A'), while NOTCH1 activity was restricted to the endocardium (Fig 1B–1B'). Expression of the NOTCH transgenic reporter *CBF:H2B-Venus* [17] was restricted to endocardial cells at E12.5, indicating that NOTCH activity was restricted to the endocardium (Fig 1C–1C').

We then generated myocardial-specific conditional mutants by breeding *Rbpj^{fllox/fllox}* mice [42] with *Tnnt2-Cre^{tg/+}* mice, which express the CRE recombinase specifically in cardiomyocytes from E8.0 onwards [43]. At E16.5, the heart of *Rbpj^{fllox};Tnnt2-Cre* (*Rbpj^{fllox/fllox};Tnnt2^{Cre/+}*) embryos was indistinguishable from control (*Rbpj^{fllox/fllox};Tnnt2^{+/+}*) littermates (Fig 2A–2B'), and compact and trabecular myocardium thickness was similar in both genotypes (Fig 2C). Immunostaining confirmed full myocardial RBPJ deletion in E16.5 *Rbpj^{fllox};Tnnt2-Cre* embryos (Fig 2D and 2E"). Thus, while in control embryos RBPJ was found in the nucleus of both endocardium and myocardium (Fig 2D–2D"), it was not detected in cardiomyocytes of *Rbpj^{fllox};Tnnt2-Cre* embryos while endocardial RBPJ expression was normal (Fig 2E–2E").

Genetic manipulation of NOTCH elements leading to signal inactivation in the endocardium, disrupts myocardial patterning and chamber maturation [17, 27]. We analyzed if ventricular patterning was affected after deletion of RBPJ in the myocardium. E16.5 *Rbpj^{fllox};Tnnt2-Cre* embryos showed normal expression of both compact (*Hey2*) [44–46] and trabecular myocardial markers (*Bmp10* [47], *Cx40* [48]) (Fig 3A–3F), indicating that myocardial patterning was not affected after deletion of RBPJ in cardiomyocytes.

Canonical NOTCH signaling requires NICD binding to RBPJ in the nucleus to activate target genes expression [11]. The Notch target genes *Hey1* and *HeyL* are expressed in both endocardium and coronaries endothelium of E16.5 wild type embryos (Fig 3D–3J). The endothelial-endocardial pattern of *Hey1* and *HeyL* expression was maintained E16.5 *Rbpj^{fllox};Tnnt2-Cre* embryos, indicating that *Rbpj* deletion in the myocardium did not affect NOTCH targets expression in the heart. These results are consistent with the data showing that NOTCH activity in the heart is restricted to endocardium (Fig 1C–1C') and coronaries endothelium, and that physiological NOTCH activity does not occur in the embryonic myocardium [4, 17, 25].

A previous report in which *Rbpj* was inactivated in the myocardium using the α Mhc-Cre driver [49] showed that myocardial RBPJ represses hypoxia-inducible factors (HIFs) to negatively regulate *Vegfa* expression in a NOTCH-independent manner [50]. *In situ* hybridization of *Vegfa* in E16.5 *Rbpj^{fllox};Tnnt2-Cre* embryos showed an apparent increase in *Vegfa* transcription in the ventricular wall of mutant embryos (Fig 3K and 3L). Nevertheless, the expression of both *Hif1 α* and *Vegfa* expression was not significantly altered by quantitative RT-PCR analysis (Fig 3O). VEGFA positively regulates the formation of blood vessels in the ventricles [51]. Thus, we analyzed the expression of the coronary vessels marker *Fabp4* [52] and observed a similar pattern and intensity in E16.5 *Rbpj^{fllox};Tnnt2-Cre* and control embryos (Fig 3M and 3N) suggesting that coronaries development was normal. Thus, unlike the previous report by Díaz-Trelles et al. [49] we did not observed a RBPJ negative regulation of *Vegf* expression in the myocardium.

Cardiomyopathies may result in the appearance of fibrosis and accumulation of collagen fibers in the myocardium, due to defective vascularization or loss of metabolic homeostasis [53]. We performed Masson's Trichrome and Sirius Red staining in E16.5 *Rbpj^{fllox};Tnnt2-Cre* mutant hearts, and found no signs of fibrosis (Fig 3P–3S). Periodic Acid-Schiff (PAS) staining

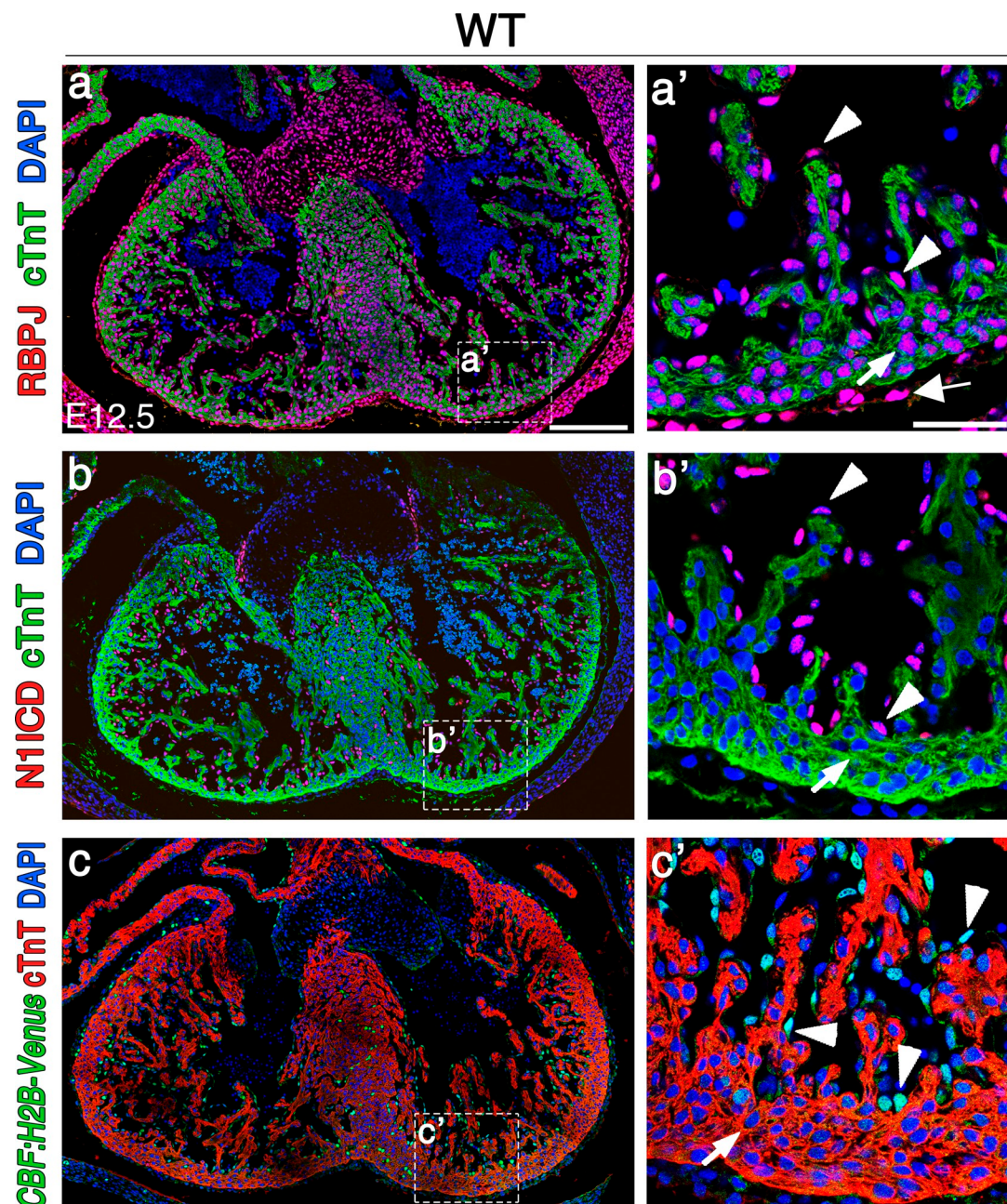


Fig 1. RBPJ is ubiquitously expressed in the nucleus of cardiac cells, while NOTCH activity is restricted to the endocardium during cardiac development. (a-a') RBPJ (red) and (b-b') N1ICD (red) nuclear immunostaining in wild type (WT) E12.5 cardiac sections. (c-c') CBF:H2B-Venus reporter line expression (green) in E12.5 cardiac sections. The myocardium is cTnT-counterstained (green in a-b', red in c-c'). White arrows indicate cardiomyocytes, white arrowheads point to endocardial cells, and the thick arrow in (a') indicates epicardial RBPJ expression. Note that cardiomyocytes do not express CBF:H2B-Venus (c,c'). Scale bars: a-c, 200μm, a'-c', 50μm.

<https://doi.org/10.1371/journal.pone.0203100.g001>

detects glycogen accumulation that could be induced by inflammation, but PAS staining was relatively normal in E16.5 *Rbpj*^{fllox}; *Tnnt2*-Cre hearts (Fig 3T and 3U). These results indicated that *Rbpj* deletion in the embryonic myocardium does not affect myocardial fetal development.

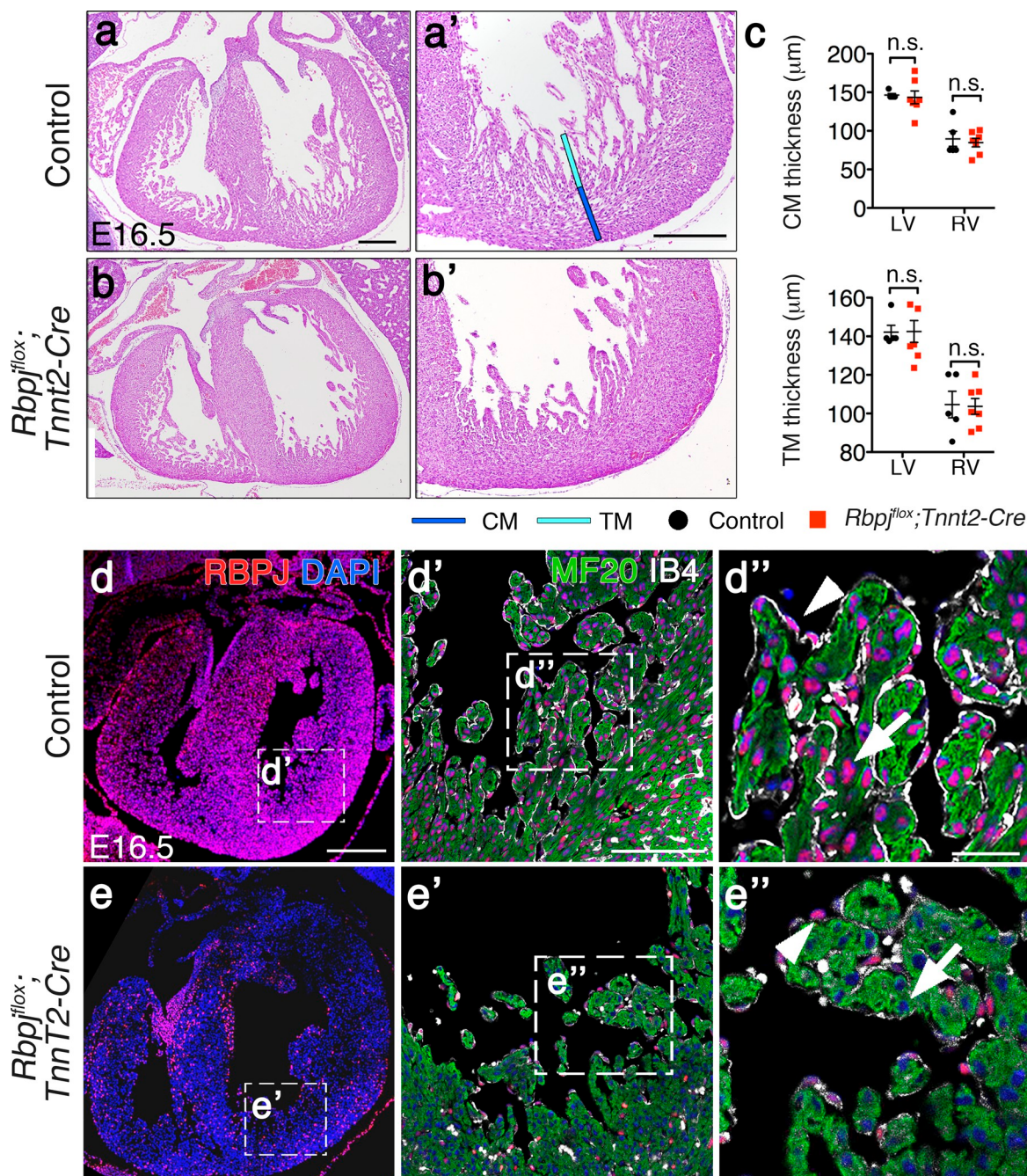


Fig 2. Myocardial *Rbpj* deletion does not affect ventricular development and structure. (a–b') Hematoxylin and eosin (H&E) staining of heart sections from control and *Rbpj^{lox};Tnnt2-Cre* E16.5 embryos. (c) Quantification of compact myocardium (CM) and trabecular myocardium (TM) thickness in E16.5 control and *Rbpj^{lox};Tnnt2-Cre* embryos. (Data are mean ± s.e.m; $P < 0.05$ by Student's *t*-test; n.s., not significant. Quantitative data shown in S1 Table). (d–e'') RBPJ (red) immunostaining of control and *Rbpj^{lox};Tnnt2-Cre* E16.5 cardiac sections, myosin heavy chain (MF20, green), and isolectin B4 (IB4, white). White arrows indicate cardiomyocytes; white arrowheads point to endocardial cells. Scale bars: 200 μm in a,a',d; 100 μm in d'; 25 μm in d''.

<https://doi.org/10.1371/journal.pone.0203100.g002>

Rbpj^{lox};Tnnt2-Cre mutant mice reached adulthood in similar proportions than control littermates. Genotyping of neonatal and adult litters showed that all genotypes appeared at the expected Mendelian proportions (Table 1), indicating that RBPJ loss in the myocardium did

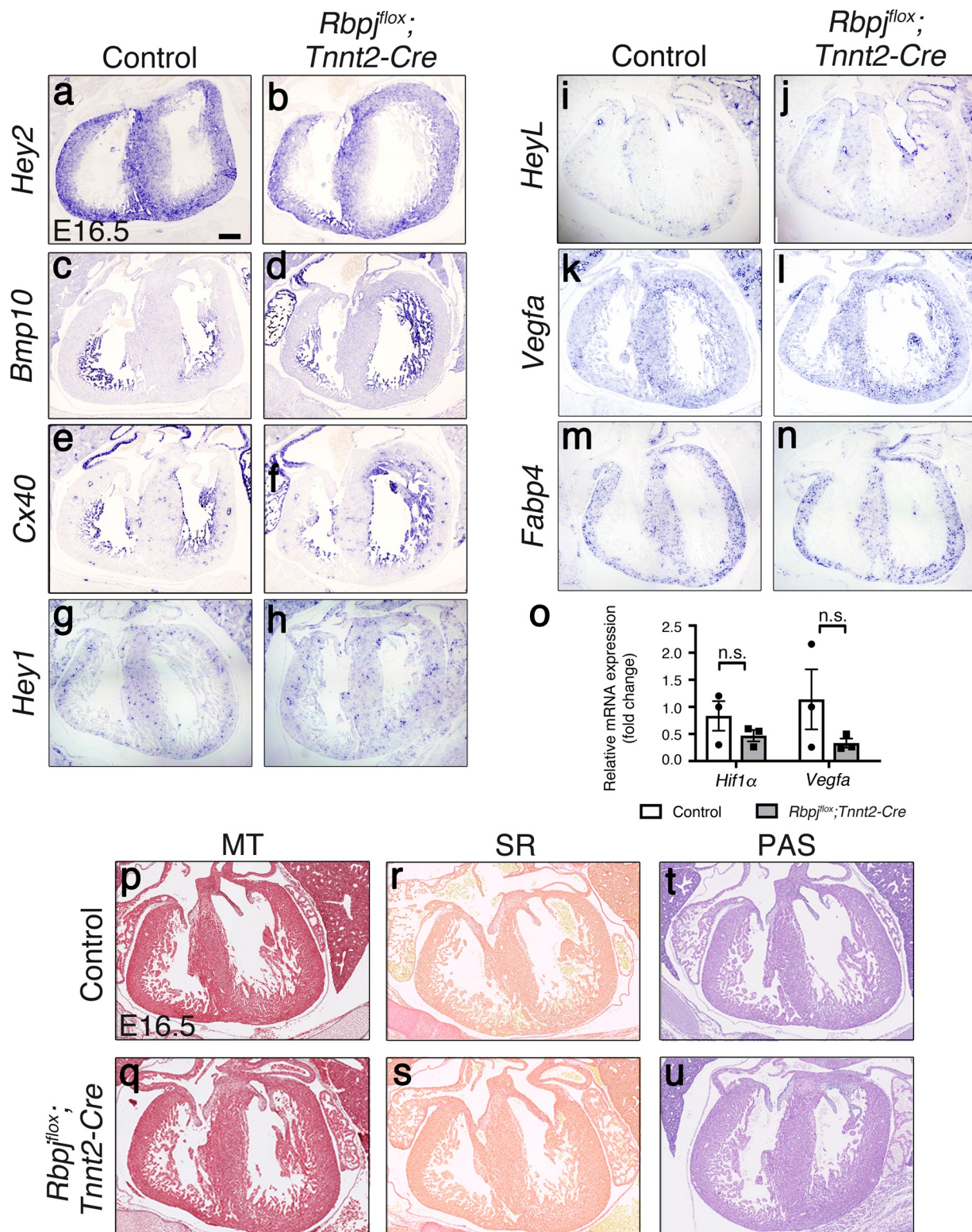


Fig 3. Expression pattern of compact and trabecular myocardium markers, NOTCH target genes, and fibrosis marker staining is normal in *Rbpj^{flox};Tnnt2-Cre* embryos, while *Vegf* is not significantly increased. In situ hybridization (ISH) of *Hey2* (a-b), *Bmp10* (c-d), *Cx40* (e-f), *Hey1* (g-h), *HeyL* (i-j), *Vegfa* (k-l) and *Fabp4* (m-n) in E16.5 *Rbpj^{flox};Tnnt2-Cre* and control hearts. (o) qRT-PCR showing *Hif1α* and *Vegfa* expression levels in E15.5 WT and *Rbpj^{flox};Tnnt2-Cre* hearts. (p-u) E16.5 *Rbpj^{flox};Tnnt2-Cre* and control cardiac sections stained with Masson's Trichrome (MT), (p-q); Periodic acid-Schiff (PAS), (r-s), and Sirius Red (SR), (t-u). Scale bar is 200 μm.

<https://doi.org/10.1371/journal.pone.0203100.g003>

Table 1. Myocardium-specific *Rbpj*^{flox} mutants are viable and reach adulthood. Distribution of the different genetic combinations resulted from the intercross of *Rbpj*^{flox/+}; *Tnnt2*^{Cre/+} males with *Rbpj*^{flox/flox} females compared to the expected Mendelian proportions.

Age	Litters	<i>RBPJk</i> ^{flox/flox} ; <i>Tnnt2</i> ^{Cre/+}	<i>RBPJk</i> ^{flox/flox} ; +/+	<i>RBPJk</i> ^{flox/+} ; <i>Tnnt2</i> ^{Cre/+}	<i>RBPJk</i> ^{flox/+} ; +/+
E16.5	6	13 (29,5%)	8 (18,2%)	13 (29,5%)	10 (22,7%)
P0	3	7 (25,9%)	4 (14,8%)	9 (33,3%)	7 (25,9%)
6 months	18	38 (26,5%)	37 (25,8%)	35 (24,5%)	33 (23,2%)
Expected		25%	25%	25%	25%

<https://doi.org/10.1371/journal.pone.0203100.t001>

not compromise postnatal viability. Morphological analysis of 6-month old *Rbpj*^{flox}; *Tnnt2*-*Cre* adults revealed normal heart structure compared to control animals (Fig 4A and 4B). In order to detect potential physiological impairments in the heart, we analyzed cardiac function by echocardiography. Ejection fraction (EF%) and fractional shortening (FS%) were similar in wild type and mutant mice (Fig 4E). The diastolic function, indicated by the E/A ratio (ratio of early diastolic velocity to atrial velocity) [54], was also normal. Physiological measurements indicate that ventricular volumetric and mass parameters were normal compared to control mice. Overall, the echocardiography study indicates that myocardial *Rbpj* inactivation does not affect postnatal heart growth and adult myocardial function. We confirmed these results by inactivating *Notch1* with the *Tnnt2*-*Cre* driver. Six-month old *Notch1*^{flox}; *Tnnt2*-*Cre* adult hearts did not show any morphological phenotypes compared to control animal (Fig 4C and 4D). In terms of heart function, *Notch1*^{flox}; *Tnnt2*-*Cre* mice exhibited a slightly better cardiac performance with a minor but significantly increased EF% and FS% compared to control mice (Fig 4F). The diastolic function was normal, as it was in *Rbpj*^{flox}; *Tnnt2*-*Cre* mice.

Previous reports suggested that ectopic myocardial Notch signaling directs the differentiation of cardiomyocytes towards specialized conduction cells *in vitro* [41]. Although the expression of the ventricular conduction system marker *Cx40* was normal in E16.5 *Rbpj*^{flox}; *Tnnt2*-*Cre* mutant embryos (Fig 3E and 3F), we further analyzed cardiac conduction system activity of *Rbpj*^{flox}; *Tnnt2*-*Cre* adult mice. Electrocardiogram showed no significant differences neither in the main intervals PR and QT nor in the QRS complex duration compared to control mice, suggesting that the conduction system is fully functional in *Rbpj*^{flox}; *Tnnt2*-*Cre* adult mice (Fig 4G).

To study the effect of RBPJ inactivation in the embryonic heart we used another early cardiac driver, *Nkx2.5*-*Cre*, active in the myocardium and in a subset of endocardial cells from E7.5 onwards [55]. Morphological analysis of E16.5 *Rbpj*^{flox/flox}; *Nkx2.5*^{Cre/+} (*Rbpj*^{flox}; *Nkx2.5*-*Cre*) embryos revealed the presence of a membranous ventricular septal defect (VSD, Fig 5A and 5B) and dysmorphic valves (Fig 5C and 5D). Among thirteen *Rbpj*^{flox}; *Nkx2.5*-*Cre* mutants examined, twelve (92%) showed membranous VSD, twelve (92%) showed double outlet right ventricle (DORV), in which the aorta is connected to the right ventricle instead of to the left one (Fig 5E–5F). Seven mutants (54%) had bicuspid aortic valve (BAV), characterized by either right to non-coronary (75% of cases) or right to left (25% of cases) morphology and resulting in a two-leaflet valve instead of the normal three-leaflet valve (Fig 5C and 5D). *Rbpj*^{flox}; *Nkx2.5*-*Cre* mutant embryos developed a normal compact and trabecular myocardium layers, with a thickness similar to controls (Fig 5G). *Rbpj*^{flox}; *Nkx2.5*-*Cre* mutants showed perinatal lethality and died around postnatal day 0 (P0; Table 2).

To determine the precise contribution of *Nkx2.5*-expressing cells to the developing heart, we took advantage of the *mT/mG* system in which following CRE-mediated excision, the *mTomato* transgene is removed so that the CAG promoter drives the expression of membrane localized EGFP [56]. Lineage tracing analysis of *mT/mG*; *Nkx2.5*-*Cre* mice revealed both myocardial and endocardial contribution of CRE-expressing cells (Fig 5H–5J), including partial

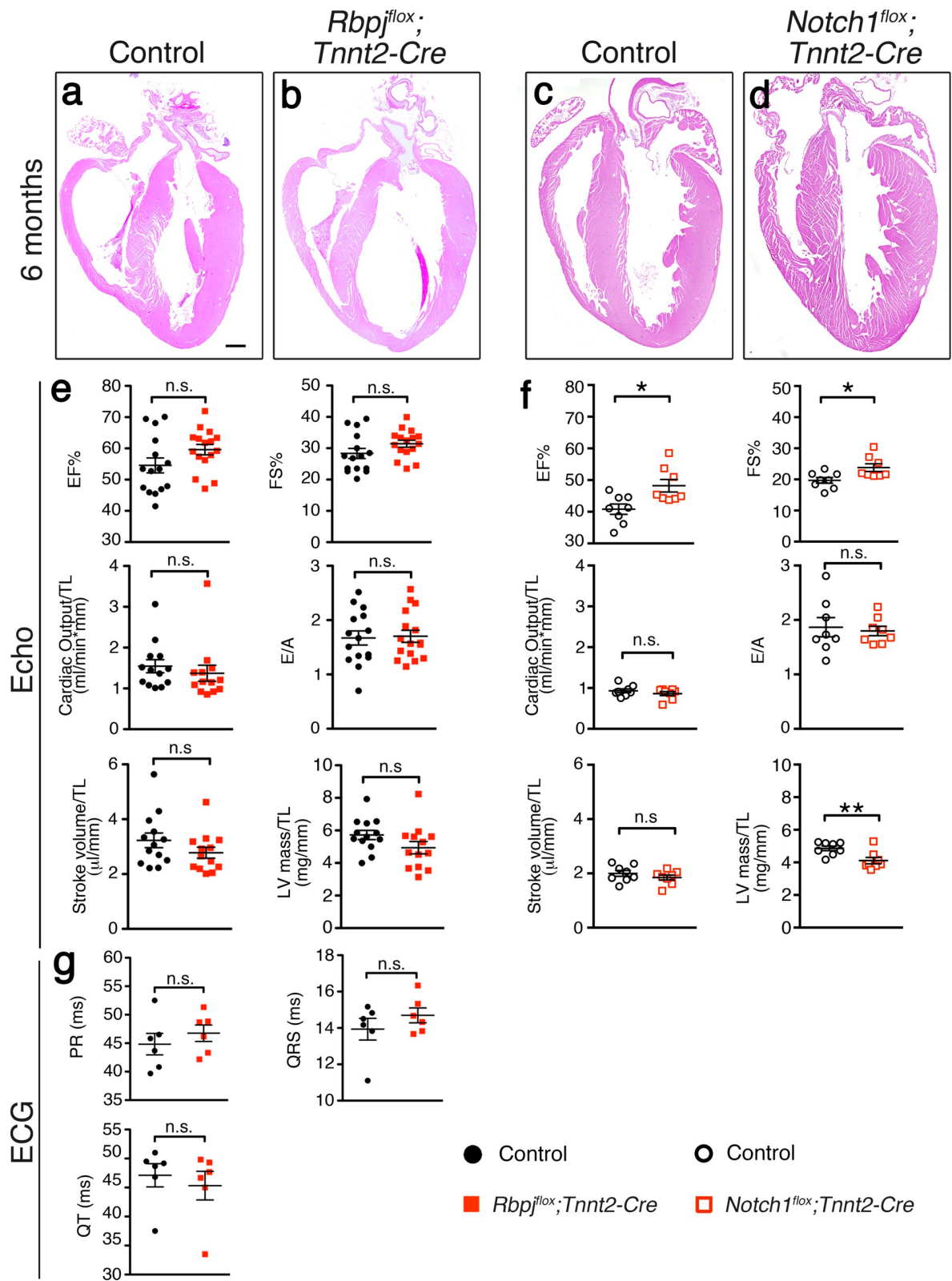


Fig 4. Cardiac structure and function are preserved in both *Rbpj^{fllox}; Tnnt2-Cre* and *Notch1^{fllox}; Tnnt2-Cre* mice. (a-d) H&E staining of cardiac sections from six months-old *Rbpj^{fllox}; Tnnt2-Cre* and *Notch1^{fllox}; Tnnt2-Cre* mice and their control littermates. (e,f) Echocardiography analysis of six months-old *Rbpj^{fllox}; Tnnt2-Cre* and *Notch1^{fllox}; Tnnt2-Cre* mice (Data are mean $\pm$ s.e.m; $P < 0.05$ by

Student's *t*-test; n.s., not significant; **P*<0.05; ***P*<0.01. Quantitative data are shown in S2 Table. (g) Electrocardiogram analysis of control and *Rbpj*^{fllox}; *Tnnt2-Cre* 6 months old mice (Data are mean ± s.e.m; *P*<0.05 by Student's *t*-test; n.s., not significant. Quantitative data are shown in S3 Table). EF, ejection fractions; FS, fractional shortening; TL, tibial length; LV, left ventricle. Scale bars is 600μm.

<https://doi.org/10.1371/journal.pone.0203100.g004>

recombination in the E9.5 outflow track (OFT) endocardium (Fig 5I) and in the mitral valve endocardium at E16.5 (Fig 5J). RBPJ immunostaining in E16.5 control and *Rbpj*^{fllox}; *Nkx2.5-Cre* embryos showed efficient RBPJ abrogation throughout the ventricular myocardium (99.98 ± 0.02 of cardiomyocytes recombined), while RBPJ was preserved in the majority of ventricular endocardial cells ($29.06 \pm 9.67\%$ of endocardial cells recombined; Fig 5K and 5L). In contrast, in endocardial cells overlying the valves, RBPJ depletion was significantly more efficient ($76.91 \pm 6.48\%$; *P*<0.01 by Student's *t*-test) (Fig 5M and 5N). Our results are in agreement with previous reports showing that NOTCH signaling abrogation by deletion of *Notch1* or *Jagged1*, using the *Nkx2.5-Cre* driver leads to VSD, DORV and BAV [18], demonstrating the requirement of endocardial NOTCH signaling for valve morphogenesis, and suggesting that the lethality observed in *Rbpj*^{fllox}; *Nkx2.5-Cre* mutant mice was very likely due to *Rbpj* inactivation in valve endocardium. A recent report has shown that genetic inactivation of the ubiquitin ligase MIB1, or combined deletion of the ligands JAG1 and JAG2, using the *Tnnt2-Cre* driver disrupts formation of the intercalary cushion in the aortic valve [57]. We have not observed any defect in intercalary cushion formation in *Rbpj*^{fllox}; *Tnnt2-Cre* or *Notch1*^{fllox}; *Tnnt2-Cre* mice, suggesting that neither RBPJ nor NOTCH1 are required in the cardiac cell population that expresses *Tnnt2-Cre*.

Our results demonstrate that myocardial inactivation of *Rbpj* in *Tnnt2-Cre*; *Rbpj*^{fllox} mice does not affect heart development and structure, nor does impair adult heart function, as occurs with NOTCH signaling inactivation in the endocardium [4, 17, 18, 26, 30, 58]. In contrast, *Nkx2.5-Cre*-mediated *Rbpj* deletion in valve cushion endocardial cells, in which RBPJ mediates NOTCH signaling, leads to VSD, DORV and BAV, with VSD being the likely cause of perinatal lethality observed in these mutants. Our data also imply that previous reports in which *Notch1* was inactivated in cardiac progenitor cells (including endocardium and myocardium progenitors) using early-acting drivers like *Isl1-Cre* [59], may likely demonstrate the requirement of NOTCH1 signaling in second heart field endocardium, where *Isl1* is also expressed, thus disrupting the development of the right ventricle. Phenotypes resulting from a constitutively activated NOTCH receptor (NICD), using various drivers (*αMhc-Cre*, *Nkx2.5-Cre*, *Mef2c-Cre*, *Tnnt2-Cre*) [32, 40, 60, 61] do not reflect a physiological role for NOTCH signaling in the myocardium. Rather, because NICD is a very potent transactivator, these phenotypes are likely the result of ectopic expression of critical target genes. Therefore, these gain-of-function approaches are useful to complement loss-of-function studies, which are more likely to reveal the true physiological function of NOTCH in heart development.

Conclusions

Our data indicate that: 1) Targeted inactivation of *Notch1* or *Rbpj* in the embryonic myocardium does not affect cardiac development or function; 2) myocardial RBPJ does not mediate NOTCH signaling; 3) NOTCH does not play a direct role in the myocardium. Thus, we propose that the effects on myocardial development that result from NOTCH signaling inactivation in the endocardium can be explained by a non-cell autonomous mechanism, whereby secreted factors dependent on NOTCH endocardial signaling affect myocardial development [4, 17, 18, 62].

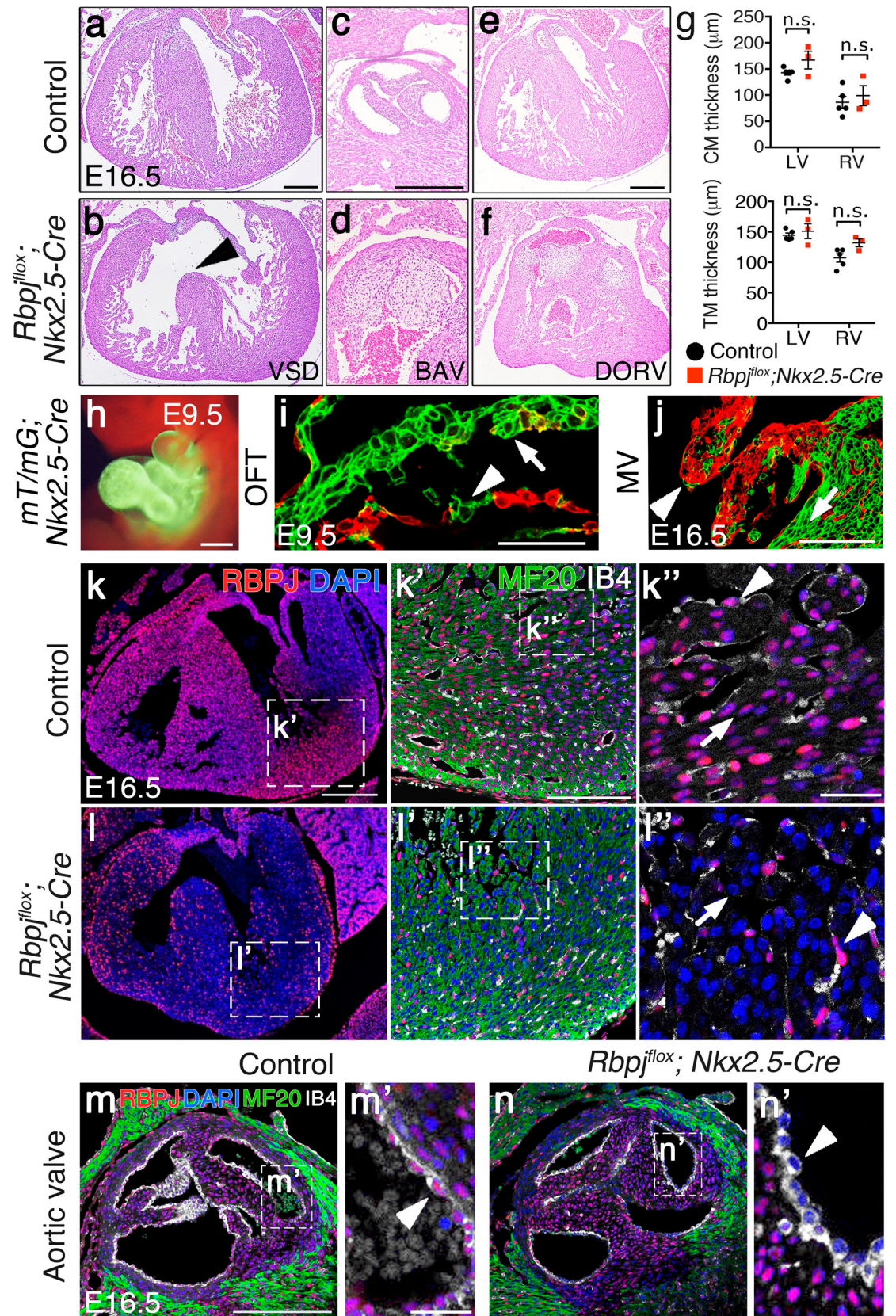


Fig 5. *Nkx2.5-Cre*-mediated *Rbpj* deletion results in ventricular septal defect, bicuspid aortic valve and double outlet right ventricle. (a-f) H&E staining of cardiac sections from E16.5 control and *Rbpj^{fllox};Nkx2.5-Cre* embryos showing ventricular septal defect (a,b), bicuspid aortic valve (c,d), and double outlet right ventricle (e,f). (g) Quantification of compact myocardium (CM) and trabecular myocardium (TM) thickness in E16.5 control and *Rbpj^{fllox};Nkx2.5-Cre* embryos (Data are mean $\pm$ s.e.m; $P < 0.05$ by Student's *t*-test; n.s., not significant. Quantitative data are shown in S4 Table). (h-j) *Nkx2.5-Cre* lineage tracing analysis using *mT/mG* mice show recombination in the entire heart at E9.5 (h), including the outflow tract (OFT) endocardium (i). At E16.5, *mT/mG;Nkx2.5-Cre* hearts show partial recombination both at the mitral valve endocardium and in endocardium-derived mesenchyme (j). (k-n') RBPJ (red), myosin heavy chain (MF20, green) and isolectin B4 (IB4, white) immunostaining in E16.5 control and *Rbpj^{fllox};Nkx2.5-Cre* embryos. White arrows indicate cardiomyocytes; white arrowheads point to endocardial cells. Scale bar: 200 μ m in a-f, k,l; 100 μ m in h,j,k',m,n; 50 μ m in i; 25 μ m in m'.

<https://doi.org/10.1371/journal.pone.0203100.g005>

Materials and methods

Mouse strains and genotyping

Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and by the Community of Madrid (Ref. PROEX 118/15). All animal procedures conformed to EU Directive 2010/63EU and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enforces in Spanish law under Real Decreto 1201/2005. Mouse strains were *CBF:H2B-Venus* [63], *Rbpj^{fllox}* [42], *Tnnt2-Cre* [43], *Nkx2.5-Cre* [55], *Notch1^{fllox}* [64], and *mT/mG* [56]. Genotyping primers are listed in S5 Table.

Tissue processing, histology and *in situ* hybridization

Embryos were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Adult hearts were perfused with Heparin (5U/ml in PBS) and fixed during 48 hours in PFA 4%. Both embryos and adult samples were embedded in paraffin following standard protocols. Hematoxylin-eosin (H&E) staining and *in situ* hybridization (ISH) on paraffin sections were performed as described previously [65]. Masson's trichrome, Sirius Red and PAS (periodic acid-Schiff) were performed using standard procedures (CNIC Histology Facility). *mTmG;Nkx2.5-Cre* embryos were fixed in 4% PFA for an hour at room temperature, washed in PBS followed by 1 hour incubation in 30% sucrose in PBS and embedded in OCT.

Immunohistochemistry

Paraffin sections (10 μ m) were incubated overnight with primary antibodies, followed by 1h incubation with a fluorescent-dye-conjugated secondary antibody. RBPJ and N1ICD staining was performed using tyramide signal amplification (PerkinElmer NEL744B001KT). *CBF:H2B-Venus* expression was detected using anti-GFP antibody. Antibodies used in this study are: anti-RBPJ (CosmoBio 2ZRB2, 1:50), anti-Troponin T (DSHB CT3, 1:20) anti-Cleaved Notch1 ICD (Cell Signaling Technology 2421S, 1:100), anti-GFP (Aves Labs GFP-1010, 1:400), and anti-Myosin Heavy Chain MF-20 (DHSB, 1:20). DAPI (Sigma-Aldrich D9542, 1:1000)

Table 2. *Rbpj^{fllox};Nkx2.5-Cre* embryos show perinatal lethality. Distribution of the different genetic combinations resulted from the intercross of *Rbpj^{fllox/+};Nkx2.5^{Cre}* males with *Rbpj^{fllox/fllox}* females compared to the expected Mendelian proportions.

Age	Litters	<i>Rbpj^{fllox/fllox}; Nkx2.5^{Cre} /+</i>	<i>Rbpj^{fllox/fllox}; +/+</i>	<i>Rbpj^{fllox/+}; Nkx2.5^{Cre} /+</i>	<i>Rbpj^{fllox/+}; +/+</i>
E14.5	5	9 (27,3%)	8 (24,2%)	9 (27,3%)	7 (21,2%)
E16.5	7	11 (22%)	10 (20%)	17 (34%)	12 (24%)
P0	5	4 (11,1%)	14 (42,2%)	9 (27,3%)	6 (18,2%)
P1	5	0 (0%)	14 (46,7%)	8 (26,7%)	8 (26,7%)
Expected		25%	25%	25%	25%

<https://doi.org/10.1371/journal.pone.0203100.t002>

and Isolectin B4 glycoprotein (ThermoFisher I32450, 1:100). Confocal images were obtained using Leica SP5 confocal fluorescence microscope.

Quantification of *Rbpj* deletion

Rbpj immunostaining was analyzed using ImageJ software. *Rbpj*-positive nuclei were divided by the total number of nuclei (counterstained with DAPI) counted on sections both in the myocardium and endocardium of the valve and ventricles of 4 different E16.5 *Rbpj^{flox}*; *Nkx2.5-Cre* embryos.

Quantification of compact and trabecular myocardium thickness

H&E and images were obtained with an Olympus BX51 microscope. ImageJ software was used for the measurements, drawing a 10-pixel wide straight line along the width of compact and trabecular myocardium. Three measurements (in μm) of both compact and trabecular myocardium were taken, from both the apex and the basal region of the ventricle. Left and right ventricles were analyzed separately.

Quantitative RT-PCR

Total RNA from E15.5 ventricles was extracted with RNeasy Mini Kit (QIAGEN). cDNA was synthesized from 1 μg of total RNA using the SuperScript III First Strand kit (Invitrogen). qPCR was performed with the Power SYBR Green Master Mix (Applied Biosystems) and IDT primers. Oligonucleotide sequences for real-time PCR analysis performed in this study are listed in S5 Table. Data are presented as mean $\pm$ s.e.m. Differences were considered statistically significant at $P < 0.05$ (Student's *t*-test).

Ultrasound

Left ventricle (LV) function and mass were analyzed by transthoracic echocardiography in 6 months of age mice. Mice were mildly anaesthetized by inhalation of isoflurane/oxygen (1–2%/98.75%) adjusted to obtain a target heart rate of 450 ± 50 beats/min and examined using a 30MHz transthoracic echocardiography probe. Images were obtained with Vevo 2100 (Visual-Sonics). From these images, cardiac output, stroke volume and LV mass were calculated. These measurements were normalized by the tibial length of each mice. Ventricular systolic function was assessed by estimating LV shortening fraction and the ejection fraction. Diastolic function was assessed by the E/A ratio. We performed a second echocardiography analysis two weeks after the first one and calculate the mean for each parameter and each mouse.

Electrocardiograms

Electrocardiograms were recorded with an MP36 system and analyzed using the Acknowledge 4 software. 6 months old mice were anesthetized by inhalation of isoflurane/oxygen (1–2%/98.75%) adjusted to obtain a target heart rate of 450 ± 50 beats/min.

Statistical analysis

Statistical analysis was carried out using Prism 7 (GraphPad). All statistical tests were performed using a two-sided, unpaired Student's *t*-test. Data are represented as mean $\pm$ s.e.m. All experiments were carried out with at least three biological replicates. In the case of adult image analysis by echo and electrocardiogram analysis, the experimental groups were balanced in terms of age and sex. Animals were genotyped before the experiment and were caged together.

and treated in the same way. The experiments were not randomized. For adult image analysis, the investigators were blinded to allocation during experiments and outcome assessment.

Supporting information

S1 Table. [Fig 2C](#) quantitative data.

(PDF)

S2 Table. [Fig 4E and 4F](#) quantitative data.

(PDF)

S3 Table. [Fig 4G](#) quantitative data.

(PDF)

S4 Table. [Fig 5G](#) quantitative data.

(PDF)

S5 Table. Genotyping primers list.

(PDF)

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