Myocardial *Notch-Rbpj* deletion does not affect heart development or function

Short title: NOTCH-RBPJ is dispensable in the myocardium

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26 Abstract

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

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45 Introduction

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47 The heart is the first organ to form and function during vertebrate development. At embryonic day 7.0 (E7.0) in
 48 the mouse, cardiac progenitor cells, migrating from the primitive streak, reach the head folds on either side of the
 49 midline (1) and by E8.0, fuse and form the primitive heart tube (2). The heart tube consists internally of the
 50 endocardium, that is separated from the primitive myocardium by an extracellular matrix termed cardiac jelly (3).
 51 The NOTCH signaling pathway is crucial for the endocardial-myocardial interactions that regulate the patterning,
 52 growth and differentiation of chamber and non-chamber tissues that will develop from E8.5 onwards (4-8). The
 53 main components of the pathway are the single-pass transmembrane NOTCH receptors (NOTCH1-4 in mammals)
 54 that interact with membrane-bound ligands of the JAGGED (JAG1 and JAG2) and DELTA families (DELTA
 55 LIKE1, 3 and 4), expressed in neighboring cells (9, 10). Ligand-receptor interactions leads to three consecutive
 56 cleavage events that generate the NOTCH intracellular domain (NICD), which can translocate to the nucleus of
 57 the signaling-receiving cell (11). In the nucleus, NICD binds directly to the DNA-binding protein CSL
 58 (CBF1/RBPJ/Su(H)/Lag1) (12) and recruits the co-activator Mastermind-like (13, 14). In the absence of NICD,
 59 ubiquitously expressed RBPJ (recombination signal binding protein for immunoglobulin kappa J region) may act
 60 as a transcriptional repressor (15). The best characterized NOTCH targets in the heart are the HEY family of basic
 61 helix-loop-helix (bHLH) transcriptional repressors (16), although various other cardiac-specific targets have
 62 been described (4, 17-19). Functional studies in *Xenopus* or in *Rbpj*-targeted mouse embryonic stem cells have
 63 shown that NOTCH suppresses cardiomiogenesis (20, 21), although studies with targeted mutant mice have
 64 demonstrated an essential requirement for NOTCH in cardiac development only after heart tube formation (around
 65 E8.5) (22, 23).

66 One of the first sign of cardiac chamber development is the appearance of trabeculae at E9.0-9.5 (24).
 67 Trabeculae are myocardial protrusions covered by endocardium that grow towards the ventricular lumen, and
 68 serve to facilitate oxygen exchange and nourishment between the blood and the developing heart. The ligand
 69 DLL4 and the active NOTCH1 receptor are expressed in the endocardium prior to the onset of trabeculation (4,
 70 25). DLL4-NOTCH1 signaling is reflected by the endocardial expression of the CBF:H2B-Venus transgenic
 71 NOTCH reporter in mice (17). Conditional inactivation of *Dll4*, *Notch1* or *Rbpj* in the endocardium, results in
 72 very similar phenotypes (more severe in *Rbpj* mutants) consisting of ventricular hypoplasia and impaired

73 trabeculation (4, 17, 26), while myocardial deletion of *Jag1* does not affect trabeculation (17). Later, NOTCH1
 74 signaling in the endocardium is activated in a temporal sequence from the myocardium by the JAGGED1/2
 75 ligands in a MIB1-dependent manner, to sustain ventricular compaction and maturation (17, 27). Conditional
 76 myocardial deletion of *Mib1* or combined inactivation of *Jag1* and *Jag2* abrogates endocardial NOTCH activity,
 77 and leads to abnormally thin compact myocardium and large and non-compacted trabeculae, a phenotype strongly
 78 reminiscent of a cardiomyopathy termed left ventricular non-compaction (LVNC) (17, 27). Thus, endocardial
 79 NOTCH signaling and its downstream effectors are essential for the endocardium-to-myocardium signaling that
 80 regulates chamber patterning and growth (28-30). Endocardial NOTCH activity is also crucial for development
 81 and morphogenesis of the cardiac valves (5, 18, 29, 31-37). NOTCH receptors or transgenic reporter lines are
 82 expressed in the endocardium, coronary vessels endothelium and smooth muscle cells (17, 25, 38, 39). There is
 83 not clear evidence of endogenous NOTCH expression or activity in the embryonic myocardium, and despite
 84 elegant ectopic expression experiments have reported a function for NOTCH in cardiomyocytes (40, 41), a
 85 physiological role for NOTCH in the developing myocardium has not been clearly demonstrated *in vivo*.

86 To address this question, we have conditionally inactivated the NOTCH effector RBPJ in the myocardium
 87 using two early-acting myocardial drivers, and examined its consequences in heart development. We find that
 88 *cTnT-Cre* mediated myocardial-specific deletion of *Rbpj* does not affect NOTCH signaling in the endocardium,
 89 heart development or adult heart function. In contrast, while *Nkx2.5-Cre* mediated *Rbpj* inactivation in the
 90 myocardium does not affect cardiac development and structure, *Rbpj* inactivation in valve endocardial cells
 91 disrupts valve morphogenesis. Our data demonstrate that myocardial NOTCH-RBPJ is not required for cardiac
 92 development or function, reinforcing the notion that physiological NOTCH-RBPJ signaling occurs in the
 93 endocardium, endothelium and smooth muscle cells of the developing heart.

94 Results and Discussion

95 We first compared expression of the NOTCH effector RBPJ to the pattern of NOTCH activation in the
96 E12.5 heart. RBPJ was widely expressed in the nucleus of endocardial, myocardial and epicardial cells (Fig 1a-
97 a'), while NOTCH1 activity was restricted to the endocardium (Fig 1b-b'). Expression of the NOTCH transgenic
98 reporter *CBF:H2B-Venus* in endocardial cells indicated that NOTCH activity was restricted to the endocardium
99 (Fig 1c-c').

100

101 **Fig 1. RBPJ is ubiquitously expressed in the nucleus of cardiac cells, while NOTCH activity is restricted to**
102 **the endocardium during cardiac development.** (a-a') RBPJ (red) and (b-b') N1ICD (red) nuclear
103 immunostaining in wild type (WT) E12.5 cardiac sections. (c-c') *CBF:H2B-Venus* reporter line expression (green)
104 in E12.5 cardiac sections. The myocardium is cTnT-counterstained (green in a-b', red in c-c'). White arrows
105 indicate cardiomyocytes, white arrowheads point to endocardial cells, and the thick arrow in (a') indicates
106 epicardial RBPJ expression. Note that cardiomyocytes do not express CBF:H2B-Venus (c,c'). Scale bars: a-c,
107 200µm, a'-c', 50µm.

108 We then generated myocardial-specific conditional mutants by breeding *Rbpj^{fllox/fllox}* mice (42) with *cTnT-*
109 *Cre^{tg/+}* mice, which express the CRE recombinase specifically in cardiomyocytes from E8.0 onwards (43). At
110 E16.5, the heart of *Rbpj^{fllox};cTnT-Cre* (*Rbpj^{fllox/fllox};cTnT^{Cre/+}*) embryos was indistinguishable from control
111 (*Rbpj^{fllox/fllox};cTnT^{+/+}*) littermates (Fig 2a-b'), and compact and trabecular myocardium thickness was similar in
112 both genotypes (Fig 2c). Immunostaining confirmed full myocardial RBPJ deletion in E16.5 *Rbpj^{fllox};cTnT-Cre*
113 embryos (Fig 2d-e''). Thus, while in control embryos RBPJ was found in the nucleus of both endocardium and
114 myocardium (Fig 2d-d''), it was not detected in cardiomyocytes of *Rbpj^{fllox};cTnT-Cre* embryos while endocardial
115 RBPJ expression was normal (Fig 2e-e'').

116

117 **Fig 2. Myocardial *Rbpj* deletion does not affect ventricular development and structure.** (a-b') Hematoxylin
118 and eosin (H&E) staining of heart sections from control and *Rbpj^{fllox};cTnT-Cre* E16.5 embryos. (c) Quantification
119 of compact myocardium (CM) and trabecular myocardium (TM) thickness in E16.5 control and *Rbpj^{fllox};cTnT-*
120 *Cre* embryos. LV CM control = 146.4 ± 2.1 µm, LV CM mutant = 143.4 ± 8.4 µm, RV CM control = 89.6 ± 9.8

121 μm , RV CM mutant = $84.7 \pm 5.5 \mu\text{m}$, LV TM control = $142.2 \pm 3.5 \mu\text{m}$, LV TM mutant = $142.5 \pm 5.7 \mu\text{m}$, RV
122 TM control = $117.0 \pm 5.5 \mu\text{m}$, RV TM mutant = $115.5 \pm 7.6 \mu\text{m}$ (Data are mean $\pm$ s.e.m; $n = 5$ control embryos
123 and $n = 7$ mutant embryos; $P < 0.05$ by Student's t -test; n.s., not significant). (d-e'') RBPJ (red) immunostaining
124 of control and *Rbpj^{fllox};cTnT-Cre* E16.5 cardiac sections, myosin heavy chain (MF20, green), and isolectin B4
125 (IB4, white). White arrows indicate cardiomyocytes; white arrowheads point to endocardial cells. Scale bars:
126 200 μm in a,a',d; 100 μm in d'; 25 μm in d''.

127

128 Genetic manipulation of NOTCH elements leading to signal inactivation in the endocardium, disrupts
129 myocardial patterning and chamber maturation (17, 27). We analyzed if ventricular patterning was affected after
130 deletion of RBPJ in the myocardium. E16.5 *Rbpj^{fllox};cTnT-Cre* embryos showed normal expression of both
131 compact (*Hey2*) (44-46) and trabecular myocardial markers (*Bmp10* (47), *Cx40* (48)) (Fig 3a-f), indicating that
132 myocardial patterning did not require myocardial RBPJ.

133

134 **Fig 3. Expression pattern of compact and trabecular myocardium markers, NOTCH target genes, and**
135 **fibrosis marker staining is normal in *Rbpj^{fllox};cTnT-Cre* embryos, while *Vegf* is increased.** *In situ*
136 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)
137 in E16.5 *Rbpj^{fllox};cTnT-Cre* and control hearts. (o) *Vegfa* expression quantification from *Vegfa* ISH. E16.5
138 *Rbpj^{fllox};cTnT-Cre* and control cardiac sections stained with Masson's Trichrome (MT), (p-q); Periodic acid-Schiff
139 (PAS), (r-s), and Sirius Red (SR), (t-u). Scale bar is 200 μm .

140

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

148 A previous report in which *Rbpj* was inactivated in the myocardium using the *α Mhc-Cre* driver (49)
149 showed that myocardial RBPJ represses hypoxia-inducible factors (HIFs) to negatively regulate *Vegfa* expression
150 in a NOTCH-independent manner (50). *In situ* hybridization of *Vegfa* in E16.5 *Rbpj^{fllox};cTnT-Cre* embryos showed
151 a small but significant increase of *Vegfa* transcription in the ventricular wall of mutant embryos (Fig 3k-l, o),
152 supporting previous observations (50). VEGFA positively regulates the formation of blood vessels in the
153 ventricles (51). Thus, we analyzed the expression of the coronary vessels marker *Fabp4* (52) and observed a
154 similar pattern and intensity in E16.5 *Rbpj^{fllox};cTnT-Cre* and control embryos (Fig 3m-n) suggesting that coronaries
155 development was normal. Thus, in agreement with Díaz-Trelles et al., these results revealed a NOTCH-
156 independent role for RBPJ in the negative regulation of *Vegfa* in the myocardium.

157 Cardiomyopathies may result in the appearance of fibrosis and accumulation of collagen fibers in the
158 myocardium, due to defective vascularization or loss of metabolic homeostasis (53). We performed Masson's
159 Trichrome and Sirius Red staining in E16.5 *Rbpj^{fllox};cTnT-Cre* mutant hearts, and found no signs of fibrosis (Fig.
160 3p-s). Periodic Acid-Schiff (PAS) staining detects glycogen accumulation that could be induced by inflammation,
161 but PAS staining was relatively normal in E16.5 *Rbpj^{fllox};cTnT-Cre* hearts (Fig 3t-u). These results indicated that
162 *Rbpj* deletion in the embryonic myocardium does not affect myocardial fetal development.

163 *Rbpj^{fllox};cTnT-Cre* mutant mice reached adulthood in similar proportions than control littermates.
164 Genotyping of neonatal and adult litters showed that all genotypes appeared at the expected Mendelian
165 proportions (Table 1), indicating that RBPJ loss in the myocardium did not compromise postnatal viability.
166 Morphological analysis of 6-month old *Rbpj^{fllox};cTnT-Cre* adults revealed normal heart structure compared to
167 control animals (Fig 4a,b). In order to detect potential physiological impairments in the heart, we analyzed cardiac
168 function by echocardiography (Fig 4e). Ejection fraction (EF%) and fractional shortening (FS%) were similar in
169 wild type and mutant mice (Fig 4e). The diastolic function, indicated by the E/A ratio (ratio of early diastolic
170 velocity to atrial velocity) (54), was also normal. Physiological measurements indicate that ventricular volumetric
171 and mass parameters were normal compared to control mice. Overall, the echocardiography study indicates that
172 myocardial *Rbpj* inactivation does not affect postnatal heart growth and adult myocardial function. We confirmed
173 these results by inactivating *Notch1* with the *cTnT-Cre* driver. Six-month old *Notch1^{fllox};cTnT-Cre* adult hearts
174 did not show any morphological phenotypes compared to control animal (Fig. 4c,d). In terms of heart function,
175 *Notch1^{fllox};cTnT-Cre* mice exhibited a slightly better cardiac performance with a minor but significantly increased

176 EF% and FS% compared to control mice (Fig 4f). The diastolic function was normal, as it was in *Rbpj^{fllox};cTnT-*
177 *Cre*.

178

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

Age	Litters	<i>RBPJk^{fllox/fllox};</i> <i>cTnT^{Cre/+}</i>	<i>RBPJk^{fllox/fllox};</i> <i>+/+</i>	<i>RBPJk^{fllox/+};</i> <i>cTnT^{Cre/+}</i>	<i>RBPJk^{fllox/+};</i> <i>+/+</i>
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%

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183

184 **Fig 4. Cardiac structure and function are preserved in both *Rbpj^{fllox};cTnT-Cre* and *Notch1^{fllox};cTnT-Cre***
185 **mice.** (a-d) H&E staining of cardiac sections from six months-old *Rbpj^{fllox};cTnT-Cre* and *Notch1^{fllox};cTnT-Cre* mice
186 and their control littermates. (e,f) Echocardiography analysis of six months-old *Rbpj^{fllox};cTnT-Cre* and
187 *Notch1^{fllox};cTnT-Cre* mice. For *Rbpj^{fllox}; cTnT-Cre*: EF% control = 54.5 ± 2.4 , EF% mutant = 59.6 ± 1.7 , FS%
188 control = 28.3 ± 1.6 , FS% mutant = 31.4 ± 1.1 , E/A control = 1.7 ± 0.1 , E/A mutant = 1.7 ± 0.1 , Cardiac Output/TL
189 control = 1.5 ± 0.2 , Cardiac Output/TL mutant = 1.4 ± 0.2 , Stroke volume/TL control = 3.2 ± 0.3 , Stroke
190 volume/TL mutant = 2.8 ± 0.2 , LV mass/TL control = 5.7 ± 0.3 , LV mass/TL mutant = 4.9 ± 0.4 . *Notch1^{fllox};cTnT-*
191 *Cre*: EF% control = 40.8 ± 1.6 , EF% mutant = 48.21 ± 2.0 , FS% control = 19.7 ± 0.9 , FS% mutant = 23.8 ± 1.2 ,
192 E/A control = 1.9 ± 0.2 , E/A mutant = 1.8 ± 0.4 , Cardiac Output/TL control = 0.9 ± 0.1 , Cardiac Output/TL mutant
193 = 0.9 ± 0.1 , Stroke Volume/TL control = 2.0 ± 0.1 , Stroke Volume/TL mutant = 1.8 ± 0.1 , LV mass/TL control =
194 4.9 ± 0.1 , LV mass/TL mutant = 4.1 ± 0.2 . (g) Electrocardiogram analysis of control and *Rbpj^{fllox}; cTnT-Cre* 6
195 months old mice. PR control = 44.8 ± 1.9 , PR mutant = 46.7 ± 1.4 , QRS control = 13.9 ± 0.6 , QRS mutant = 14.7
196 ± 0.4 , QT control = 47.1 ± 2.0 , QT mutant = 43.3 ± 2.5 . Data are mean $\pm$ s.e.m. For echo, $n = 15$ *Rbpj^{fllox}* control
197 mice, $n = 16$ *Rbpj^{fllox};cTnT-Cre* mutant mice, $n = 8$ *Notch1^{fllox}* control mice, $n = 8$ *Notch1^{fllox};cTnT-Cre* mutant mice.
198 For ECG, $n = 6$ control mice and $n = 6$ mutant mice. $P < 0.05$ by Student's *t*-test; n.s., not significant; * $P < 0.05$;
199 ** $P < 0.01$. EF, ejection fractions; FS, fractional shortening; TL, tibial length; LV, left ventricle. Scale bars is
200 $600\mu\text{m}$.

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

207 To further confirm that myocardial RBPJ is dispensable for cardiac development and function, we used a
208 second early myocardial driver *Nkx2.5-Cre*, active in the myocardium and in a subset of endocardial cells from
209 E7.5 onwards (55). Morphological analysis of E16.5 *Rbpj^{fllox/flox};Nkx2.5^{Cre/+}* (*Rbpj^{fllox};Nkx2.5-Cre*) embryos
210 revealed the presence of a membranous ventricular septal defect (VSD, Fig 5a-b) and dysmorphic valves (Fig 5c-
211 d). Among thirteen *Rbpj^{fllox};Nkx2.5-Cre* mutants examined, twelve (92%) showed membranous VSD, twelve
212 (92%) showed double outlet right ventricle (DORV), in which the aorta is connected to the right ventricle instead
213 of to the left one (Fig 5e-f). Seven mutants (54%) had bicuspid aortic valve (BAV), characterized by either right
214 to non-coronary (75% of cases) or right to left (25% of cases) morphology and resulting in a two-leaflet valve
215 instead of the normal three-leaflet valve (Fig 5c-d). *Rbpj^{fllox};Nkx2.5-Cre* mutant embryos developed a normal
216 compact and trabecular myocardium layers, with a thickness similar to controls (Fig 5g). *Rbpj^{fllox};Nkx2.5-Cre*
217 mutants showed perinatal lethality and died around postnatal day 0 (P0; Table 2).

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

221

Age	Litters	<i>Rbpj^{fllox/flox}; Nkx2.5^{Cre/+}</i>	<i>Rbpj^{fllox/flox}; +/+</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%

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223

Figure 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^{lox};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^{lox};Nkx2.5-Cre* embryos. LV CM control = $142.8 \pm 4.5 \mu\text{m}$; LV CM mutant = $167.0 \pm 16.8 \mu\text{m}$; RV CM control = $86.2 \pm 11.4 \mu\text{m}$, RV CM mutant = $99.0 \pm 19.0 \mu\text{m}$; LV TM control = $144.3 \pm 3.7 \mu\text{m}$, LV TM mutant = $151.1 \pm 12.2 \mu\text{m}$; RV TM control = $116.2 \pm 55.5 \mu\text{m}$, RV TM mutant = $131.9 \pm 6.5 \mu\text{m}$ (Data are mean $\pm$ s.e.m, $n=5$ control embryos and $n=3$ mutant embryos; $P<0.05$ by Student's *t*-test; n.s, not significant). (h-j) *Nkx2.5-Cre* lineage tracing analysis using *mTmG* mice show recombination in the entire heart at E9.5 (h), including the outflow track (OFT) endocardium (i). At E16.5, *mTmG;Nkx2.5-Cre* shows 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^{lox};Nkx2.5-Cre* embryos. White arrows indicate cardiomyocytes; white arrowheads point to endocardial cells. Scale bar: 200 μm in a-f, k,l; 100 μm in h,j,k',m,n; 50 μm in i; 25 μm in m'.

To determine the precise contribution of *Nkx2.5*-expressing cells to the developing heart, we took advantage of the *mTmG* 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 *mTmG;Nkx2.5-Cre* mice revealed both myocardial and endocardial contribution of CRE-expressing cells (Fig 5h-j), including partial 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^{lox};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-l'). 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-n'). 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

251 Notch signaling for valve morphogenesis, and suggesting that the lethality observed in *Rbpj^{fllox};Nkx2.5-Cre* mutant
252 mice was very likely due to *Rbpj* inactivation in valve endocardium.

253 These results demonstrate that myocardial inactivation of *Rbpj* in *cTnT-Cre;Rbpj^{fllox}* mice does not affect
254 heart development and structure, nor does impair adult heart function, as it occurs with NOTCH signaling
255 inactivation in the endocardium (4, 17, 18, 26, 30, 57). In contrast, *Rbpj* deletion driven by the *Nkx2.5-Cre* driver
256 leads to VSD, DORV and BAV, phenotypes due to *Nkx2.5*-mediated CRE activity in valve endocardial cells in
257 which RBPJ mediates NOTCH signaling, with VSD being the likely cause of perinatal lethality of these mutants.

258

259 Conclusions

260 Our data indicate that: 1) Targeted inactivation of *Notch-Rbpj* in the myocardium does not affect cardiac
261 development or function; 2) myocardial RBPJ does not mediate NOTCH signaling; 3) myocardial *Rbpj*
262 inactivation does not affect endocardial NOTCH activity; 4) NOTCH does not play a direct role in the
263 myocardium. Thus, the physiological role of NOTCH-RBPJ signaling in cardiac development is restricted to the
264 endocardium, coronary endothelium and epicardium. Our data also suggest that the previously described roles for
265 myocardial NOTCH in which the pathway was experimentally overactivated in the myocardium with various
266 drivers (*αMhc-Cre*, *Nkx2.5-Cre*, *Mef2c-Cre*) (40, 58, 59) do not represent physiological roles for NOTCH
267 signaling, but may offer experimental options for the manipulation of cardiac progenitors and/or lineages in the
268 diseased heart.

269

270 **Materials and Methods**

271 **Mouse strains and genotyping**

272 Animal studies were approved by the CNIC Animal Experimentation Ethics Committee and by the Community
273 of Madrid (Ref. PROEX 118/15). All animal procedures conformed to EU Directive 2010/63EU and
274 Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific
275 purposes, enforces in Spanish law under Real Decreto 1201/2005. Mouse strains were *CBF:H2B-Venus* (60),
276 *Rbpj^{fllox}* (42), *cTnT-Cre* (43), *Nkx2.5-Cre* (55), *Notch1^{fllox}* (61), mTmG (56). Details of genotyping will be provided
277 on request.

278 **Tissue processing, histology and *in situ* hybridization**

279 Embryos were fixed in 4% paraformaldehyde (PFA) at 4°C overnight. Adult hearts were perfused with Heparin
280 (5U/ml in PBS) and fixed during 48 hours in PFA 4%. Both embryos and adult samples were embedded in paraffin
281 following standard protocols. Hematoxylin-eosin (H&E) staining and *in situ* hybridization (ISH) on paraffin
282 sections were performed as described previously(62) . Masson's trichrome, Sirius Red and PAS (periodic acid-
283 Schiff) were performed using standard procedures (CNIC Histology Facility). *mTmG;Nkx2.5-Cre* embryos were
284 fixed in 4% PFA for an hour at room temperature, washed in PBS followed by 1 hour incubation in 30% sucrose
285 in PBS and embedded in OCT.

286 **Immunohistochemistry**

287 Paraffin sections (10 µm) were incubated overnight with primary antibodies, followed by 1h incubation with a
288 fluorescent-dye-conjugated secondary antibody. RBPJ and N1ICD staining was performed using tyramide signal
289 amplification (PerkinElmer NEL744B001KT). *CBF:H2B-Venus* expression was detected using anti-GFP
290 antibody. Antibodies used in this study are: anti-RBPJ (CosmoBio 2ZRB2, 1:50), anti-Troponin T (DSHB CT3,
291 1:20) anti-Cleaved Notch1 ICD (Cell Signaling Technology 2421S, 1:100), anti-GFP (Aves Labs GFP-1010,
292 1:400), and anti-Myosin Heavy Chain MF-20 (DHSB, 1:20). DAPI (Sigma-Aldrich D9542, 1:1000) and Isolectin
293 B4 glycoprotein (ThermoFisher I32450, 1:100). Confocal images were obtained using Leica SP5 confocal
294 fluorescence microscope.

295 **Quantification of *Rbpj* deletion**

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

299 **Quantification of compact and trabecular myocardium thickness**

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

304 **Quantification of *Vegfa* expression**

305 *Vegfa* expression was assessed by ISH in paraffin sections. Images of heart sections were obtained with an
306 Olympus BX51 microscope and analyzed with ImageJ software. Using an inverted gray scale, pixel intensity was
307 measured throughout the myocardium and a mean pixel intensity was obtained for every heart sections. 7 sections
308 of each heart at different levels were analyzed and a mean pixel intensity was obtained for each heart.

309 **Ultrasound**

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

318 **Electrocardiograms**

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

322 **Statistical analysis**

323 Statistical analysis was carried out using Prism 7 (GraphPad). All statistical test were performed using a two-
324 sided, unpaired Student's *t*-test. Data are represented as mean $\pm$ s.e.m. All experiments were carried out with at
325 least three biological replicates. In the case of adult image analysis by echo and electrocardiogram analysis, the
326 experimental groups were balanced in terms of age and sex. Animals were genotyped before the experiment and
327 were caged together and treated in the same way. The experiments were not randomized. For adult image analysis,
328 the investigators were blinded to allocation during experiments and outcome assessment. All quantifications are
329 included in Supplementary Table 1.

330

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Figure 1_Salguero-Jimenez et al.

Figure 3_Salguero-Jimenez et al.

Figure 4_Salguero-Jimenez et al.

Figure 5_Salguero-Jimenez et al.