

Inhibition of Notch2 by Numb/Numblike controls myocardial compaction in the heart

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Abstract

Aims: The ventricular wall of the heart is composed of trabeculated and compact layers, which are separated by yet unknown processes during embryonic development. Here, we wanted to explore the role of Notch2 and Numb/Numblike for myocardial trabeculation and compaction.

Methods and Results: We found that Notch2 activity is specifically downregulated in the compact layer during cardiac development of the mouse. The biological role of Notch2 downregulation was investigated by expression of constitutively active Notch2 in the myocardium of transgenic mice resulting in hypertrabeculation, reduced compaction, and ventricular septum defects. To disclose the mechanism that inhibited Notch2 activity during formation of myocardial layers, we analyzed potential suppressors of Notch signaling. We unveiled that concomitant but not separate ablation of Numb and Numbl like in the developing heart leads to increased Notch2 activity along with hypertrabeculation, reduced compaction, and ventricular septum defects phenocopying effects gained by overexpression of constitutively active Notch2. Expression profiling revealed a strong upregulation of Bmp10 in Numb/Numbl like mutant hearts, which might also interfere with trabeculation and compaction.

Conclusions: This study identified potential novel roles of Numb/Numbl like in regulating trabeculation and compaction by inhibiting Notch2 and Bmp10 signaling.

Introduction

Myocardial trabeculation and compaction are two highly related and critical processes for cardiac ventricular morphogenesis ¹. During embryonic development, the heart is initially established as a tubular structure composed of a one-cell layer of myocardium and an inner endocardium lining. Trabeculation starts after cardiac looping by outgrowth of finger-like myocardial projections from discrete points of the inner surface of the ventricle. The appearance of trabeculae essentially separates the myocardium into a trabeculated and a compact layer ². At early stages of cardiac development, when the compact myocardium is only a few cells thick, trabeculae represent the predominant form of the myocardium. During further development the compact layer thickens and the trabeculated myocardium becomes integrated into the ventricular wall ³. Trabeculation and compaction are tightly controlled during ventricular development, and dysregulation of these processes correlates with congenital heart diseases, such as ventricular non-compaction ⁴. The regulation of trabeculation and compaction in a temporal and spatial manner is still only partially understood.

Notch signaling plays important roles in cardiac development. Mutations in Notch signaling components are related to human congenital heart diseases ⁵. Mammalian Notch receptors (Notch1, 2, 3, 4) and ligands (delta-like1, 3, 4 and jagged1, 2) are both single-pass transmembrane proteins containing one large extracellular domain, which mediates binding of ligands to receptors. Ligand binding results in proteolytic cleavage of the Notch receptor and release of the Notch intracellular domain (NICD) into the cytoplasm. Subsequently, NICD

translocates to the nucleus where it forms a complex with the transcriptional factor RBPJK/CBF1/Su(H) and additional co-activators to activate target genes ⁶. Analysis of constitutive and cell-type specific Notch1 mutations revealed that the activity of Notch1 during heart development is mainly restricted to the endocardium ⁷. It was found that endocardial-specific deletion of Notch1 phenocopies the constitutive inactivation of Notch1, which severely impairs ventricular morphogenesis ⁷, while myocardium-specific deletion of Notch1 using MLC2v-Cre did not affect heart development ⁸. In contrast, Notch2 is expressed in the myocardium ⁹, and hypomorphic mutations of Notch2 result in cardiac defects characterized by reduced trabeculation and thin compact layer ¹⁰, indicating Notch2 may play an important role in this process although mechanistic insights are missing.

Genetic analysis in *Drosophila* revealed that cell fate determinant Numb inhibits Notch signaling and is a critical factor in binary cell fate specification during the development of the nervous system ¹¹. Two homologues, Numb and Numlike (Numbl), are expressed in mammals which also act as inhibitors of Notch signaling ¹². Mechanistically, this is achieved by interaction of Numb with the E3 ligase Itch, which promotes ubiquitination of Notch1 and degradation of Notch1 intracellular domain ¹³. Numb also serves as an endocytic adaptor and binds to the endocytic machinery components α -adaptin ¹⁴ and Eps15 ¹⁵.

In this study, we investigated the molecular mechanisms regulating trabeculation and compaction using different model systems and identified a novel role of Numb/Numbl in the regulation of myocardial trabeculation and compaction by repression of Notch2 and Bmp10 signaling.

Methods

Please see also the detailed methods description in the supplemental material.

Mice

Nkx2.5-Cre¹⁶, α MHC-Cre¹⁷, Flk1-Cre¹⁸, RosaN2ICD¹⁹, XMLC-Cre²⁰, Numb^{flox/flox}²¹, and Numbl ^{Δ/Δ} ²² mice have been described previously. All animal manipulations were carried out in accordance with the guidelines for animal experiments at Max-Planck-Institute for Heart and Lung Research, which conforms with the guide for the care and use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Approval of animal experimentation was granted by the local authorities (Regierungspräsidium Darmstadt, V54-19c20/15-B2/158).

Zebrafish work

Activation of the Notch signaling pathway in embryonic hearts was monitored in Notch reporter fish Tg(Tp1bglob:eGFP) crossed to the Tg(myI7:HHRASmCherry) strain (kindly supplied by Didier Stainier, UCSF, USA), in which cardiomyocytes are labeled by expression of membrane-bound mCherry²³. Knockdown of Numb and Numlike was accomplished by injection of morpholinos directed against the AUG translation start site of both zebrafish Numb and Numlike as described previously²⁴. Changes in the activity of the Notch reporter were monitored using a confocal laser scanning microscope (Zeiss, Germany).

Morphological analysis, Immunohistochemistry, and In situ hybridization

To isolate embryonic hearts, pregnant mice were sacrificed by cervical dislocation. Embryos

were removed from uteri, decapitated, and embedded in OCT or paraffin before H&E (hematoxylin and eosin) staining to visualize tissue structures. For immunohistochemistry, 10µm cryosections were stained using the VECTASTAIN Elite ABC kit (PK-7200, Vector laboratories) according to the manufacturer's instructions. In situ hybridization on paraffin sections was performed as described²⁵.

Western blotting

Embryonic hearts were isolated, snap frozen in liquid nitrogen, and subjected to protein extraction. Equal amounts of protein from control and mutant hearts were loaded on 4-12% SDS-PAGE gradient gels, separated, and transferred onto nitrocellulose membranes and analyzed as described previously²⁶.

Antibodies

The following antibodies were used in immunostaining and/or western blotting (see also detailed information in the supplemental material): activated Notch1 (#2421, Cell Signaling), activated Notch2 (ab72803, Abcam), phospho-Smad1/5/8 (#9511, Cell Signaling), Numb (C44B4, Cell Signaling), phospho-histone H3 (#9701, Cell Signaling).

RT-PCR, Real-time PCR and DNA microarray analysis

Total RNA was isolated from embryonic hearts using RNeasy Mini Kit (Qiagen). cDNA was generated from 1µg RNA with SuperScript II Reverse Transcriptase (Invitrogen) and used for RT-PCR and Real-time PCR. Absolute Blue QPCR SYBR Green Low ROX Mix (Thermo Fisher Scientific Inc.) was used to prepare real-time PCR master mixes. Amplifications were done using an iCycler iQ qPCR machine (Bio-Rad). The $\Delta\Delta C_t$ method was used to calculate

relative quantities employing GAPDH as internal standard ²⁷. For microarray, the quality of RNA was checked using the Agilent 2100 Bioanalyzer. Labeled probes were hybridized to Affymetrix GeneChip Mouse Gene 1.0 ST arrays according to the manufacturer's instructions. Data analysis was performed using the Affymetrix Expression Console and the RMA algorithm ²⁷.

Statistics

Two-tailed student's t test was used to assess the statistical significance of differences between samples. $P < 0.05$ was considered significant.

Results

Notch2 activity becomes restricted to the trabecular layer of the myocardium during embryonic heart development

Based on previous observations that led to identification of several growth factors involved in regulation of trabecular growth we reasoned that separation of the different myocardial layers will go along with spatially restricted gene expression patterns. Therefore, we analyzed the activity of components of developmentally relevant pathways, such as the Wnt-pathway, the Shh-pathway and the Notch-pathway. Immunostaining using antibodies specific for activated Notch1 (N1ICD) and Notch2 (N2ICD) revealed dynamic changes in the activity pattern of Notch signaling, which caught our attention. We found that Notch1 activity within the heart was restricted to the endocardium (Fig.1 A, B), consistent with previous observations ⁷. In contrast, Notch2 activity was exclusively localized to the myocardium (Fig.1 C-F). At E9.5

and E10.5, Notch2 was active throughout the myocardium including trabeculae and compact layer (Fig.1 C and D). Interestingly, however, we observed that Notch2 activity became confined to the trabecular layer at E11.5 (Fig.1 E) although residual signals were still present in the compact layer at this stage. The layer-specific activity of Notch2 was even more obvious at E13.5 when virtually no Notch2 activity was left in the compact layer (Fig.1 F).

To determine whether the layer-specific activity of Notch2 was caused by differential accumulation of Notch2 transcripts, we performed *in situ* hybridization experiments on paraffin sections of E11.5 and E13.5 embryonic hearts. No signals were present in the endocardium indicating that lack of Notch2 mRNA expression is responsible for the absence of Notch2 activity in the endocardium observed by immunostaining. In contrast, we found that Notch2 mRNA was expressed throughout the whole myocardium (Fig.1 G and H) suggesting a regulation of Notch2 activity at the post-transcriptional level in the compact layer. This observation corresponds well to previous reports demonstrating multiple layers of complexity of Notch signaling *in vivo*²⁸.

Myocardium-specific overexpression of N2ICD results in hypertrabeculation and reduced compaction.

Since Notch2 activity persisted in trabeculae and decreased in the compact layer during ventricular development, we hypothesized that layer-specific regulation of Notch2 might be an important factor for regulating trabeculation and compaction. To test this hypothesis, we overexpressed N2ICD specifically in the myocardium by crossing α MHC (alpha-myosin heavy chain) -Cre mice with RosaN2ICD mice, which carry a modified *Rosa26* locus targeted

with a constitutively active form of Notch2 preceded by a floxed STOP cassette¹⁹. Embryos derived from overexpression of N2ICD by α MHC-Cre, hereafter referred to as N2ICD^{MHC}, died between E12.5 and E15.5. The successful initiation of N2ICD overexpression by Cr recombinase mediated recombination in N2ICD^{MHC} hearts was analyzed by RT-PCR. We found a robust increase of Notch2 transcript levels compared to WT controls confirming that our approach worked (Fig. S6). Histological analysis revealed several cardiac defects in N2ICD^{MHC} hearts including hypertrabeculation, reduced compaction and ventricular septum defects (Fig. 2A-D). Overproduction of trabeculae and reduced compaction was further confirmed by quantifying the thickness of trabecular and compact layers (Fig. 2E, F). These data suggested that Notch2 might promote trabeculation and repress compaction. We further examined the expression level of two Notch target genes, Hey1 and Hey2, in N2ICD^{MHC} hearts. We observed a 3.3 fold upregulation of Hey1 after N2ICD overexpression while expression of Hey2 expression was not affected (Fig.5D). These results suggest that Hey1 is a specific Notch target gene in mouse embryonic hearts, consistent with a previous report demonstrating that Hey1 but not Hey2 is upregulated after overexpression of active Notch1 ICD in the mesoderm²⁹.

Nkx2.5-Cre-mediated deletion of Numb/Numbl in the heart causes hypertrabeculation, compromised myocardial compaction, and ectopic Notch2 activity

The confinement of Notch2 activity to the trabecular layer despite the broad expression of Notch2 mRNA throughout the myocardium suggested a posttranscriptional mode of

regulation. Potential negative regulators of Notch2 activity are Numb and Numbl, which are involved in the regulation of endocytotic processing and proteolytic degradation of Notch. Numb-deficient mice die before embryonic day E11.5 due to multiple defects²¹ while the constitutive knockout of Numbl does not result in any obvious phenotypes except that mutant females show a low fertility²². Concomitant deletion of both Numb and Numbl results in early lethality (E9.0) and a generally more severe phenotype compared to Numb mutants suggesting (partial) redundancy of Numb and Numbl³⁰. To analyze whether either Numb or Numbl are involved in inhibition of Notch activity in the developing heart we performed an *in situ* hybridization analysis. We found that Numb and Numbl are both expressed throughout the whole myocardium, but not in the endocardium (Fig. 3A, B). No preferential expression in the compact or the trabecular layer was seen using Bmp10 as marker for the trabecular layer (Fig. 3C). Next, we generated a heart-specific knockout of Numb by crossing Nkx2.5-Cre with floxed Numb mice. We did not observe obvious cardiac defects in the resulting homozygous mutant offspring, which reached adulthood and were fully viable (data not shown). Similarly, re-examination of homozygous Numbl constitutive mutants did not reveal any cardiac abnormalities (data not shown). In contrast, concomitant inactivation of Numb and Numbl in the heart by breeding Nkx2.5-Cre/floxed Numb to Numbl mutant mice caused embryonic lethality between E14.5-18.5 in double homozygous mutants (NbNbI^{NK}). We observed severe cardiac defects including hypertrabeculation, reduced compaction and ventricular septum defects (Fig. 3D-I, supplemental Fig. S1), which strongly resembled the phenotype of N2ICD^{MHC} hearts. Quantitative analysis of the thickness of trabecular and compact layer confirmed overproduction of trabeculae and reduced compaction defects (Fig.

3J, K). To exclude the possibility that multiple cell lineages contribute to the phenotype of NbNbl^{NK} mutant hearts, we used the XMLC-Cre strain to delete Numb/Numbl specifically in the myocardium. The resultant mutants exhibited similar defects as NbNbl^{NK} mutant hearts and also died between E14.5-E18.5 (Fig. S2). In contrast, Flk1-Cre-mediated deletion of Numb/Numbl in the endocardium did not result in an obvious phenotype (data not shown).

To corroborate the loss of Numb and Numbl in NbNbl^{NK} mutants we analyzed the expression of Numbl mRNA in the heart. RT-PCR of Numbl revealed a complete absence of full length Numbl mRNA in NbNbl^{NK} mutant hearts (Fig. 3M). Similarly, we found a major reduction of Numb protein levels in NbNbl^{NK} mutant hearts. We reasoned that the residual expression of Numb protein, which was detected by Western blot analysis, was most likely derived from non-cardiomyocytes (Fig. 3L). Next, we examined changes of Notch1 and Notch2 activities in NbNbl^{NK} mutant hearts. Immunostaining of N1ICD in control and mutant hearts revealed no significant differences in the activity pattern of Notch1, which remained confined to the endocardium (Fig. 4C, D). Strikingly, however, ectopic Notch2 activity was seen by immunohistochemistry in the compact layer of mutant hearts (Fig. 4E, F) suggesting that Numb/Numbl repress Notch2 activity in the compact layer. This finding was further supported by western blot analysis, which revealed a modest but significant upregulation of N2ICD in NbNbl^{NK} mutant hearts (Fig. 4B) while the expression level of N1ICD did not change (Fig. 4A). For western blot analysis we used total hearts, since it was not possible to separate compact and trabecular layers mechanically, which might explain the N2ICD signals in wildtype hearts. To further corroborate the upregulation of Notch2 activity we examined the expression of Hey1 and Hey2 in NbNbl^{NK} mutant hearts by real-time PCR. We found that

Hey1 but not Hey2 was upregulated in mutant hearts, which is consistent with the increase of N2ICD detected by western blot analysis.

Nkx2.5-Cre-mediated deletion of Numb/Numbl causes upregulation of Bmp10 in the heart

To further analyze the molecular mechanisms that caused hypertrabeculation and reduced compaction in NbNbl^{NK} mutant hearts, we performed an Affymetrix DNA microarray analysis using total RNA isolated at E14.5 (n=3). 68 genes (cut-off=1.6-fold change) showed a significant upregulation and 45 (cut-off=0.6-fold change) a significant downregulation (supplementary table S1) in mutant hearts when compared to age- and tissue-matched wildtype controls. Bmp10 (2.25-fold on DNA microarrays) was among the most strongly up-regulated genes in NbNbl^{NK} mutant hearts (supplementary table S1). Real-time PCR analysis confirmed the upregulation of Bmp10 (2.9-fold) (Fig. 5A). Bmp10 mediates its cellular effects by phosphorylation of Smad1/5/8 via Alk3 and BmprII³¹. To analyze whether upregulation of BMP-10 in NbNbl^{NK} mutant hearts resulted in activation of the Smad-pathway we used antibodies against phospho-Smad 1/5/8. We detected a moderate increase of phospho-Smad 1/5/8 in NbNbl^{NK} mutant hearts compared to wild-type controls by western blot analysis (Fig. 5C). Again, the impossibility to separate the compact and the trabecular layer might account for the relatively high levels of phospho-Smad 1/5/8 in wildtype hearts (Fig. 5C). Real-time PCR analysis of N2ICD^{MHC} mutant hearts revealed that BMP10 expression was not significantly changed by overexpression of N2ICD (Fig. 5D) indicating

that Numb/Numbl might inhibit BMP10 signaling in a Notch2-independent manner, which questions the function of BMP10 as a direct Notch2 target gene. This hypothesis was further supported by in situ hybridization experiments showing the absence of ectopic BMP10 expression in NbNbl^{NK} mutant hearts (Fig. S3). To rule out that the upregulation of BMP10 was solely due to an increase in trabecular cell numbers, we counted the number of trabecular cells using DAPI as nucleus marker. We found only a two-fold increase in the number of trabecular cells compared to the nearly three fold-increase of BMP-10 expression in NbNbl^{NK} mutants indicating a relative increase of BMP-10 expression in individual cells. Interestingly, BMP10 expression remained unchanged in N2ICD^{MHC} mutant hearts, which showed a 1.8-fold increase in trabecular cell numbers suggesting a relative decline of BMP-10 expression (Fig. 5B, D).

Nkx2.5-Cre-mediated inactivation of Numb and Numbl did not only result in a significant upregulation of N2ICD in trabecula but also to a strong increase of N2ICD in the compact layer (Fig. 4) suggesting a widespread deregulation of growth factor signaling pathways and cell proliferation in N2ICD^{MHC} and NbNbl^{NK} mutant hearts. To determine the distribution of proliferation cells in N2ICD^{MHC} and NbNbl^{NK} mutant hearts we stained for phospho-histone H3 positive cells. As expected we monitored a massive increase of phospho-histone H3 positive cells in trabecula of the right and left ventricle both in NbNbl^{NK} and in N2ICD^{MHC} mice (Fig. S4). Interestingly, however, we also detected a dramatic increase of proliferating cells in the outer part of ventricles, which form the compact layer of the heart. Apparently, the enhanced trabecular growth is not solely caused by a local increase of the proliferation rate within trabeculae but also to a contribution from proliferating cells of the compact layer,

which might be recruited into the trabecular layer by a process of directed delamination leaving behind a thinned compact layer. This view is also supported by the fact the compact layer of the left ventricle contains more proliferating cells than the trabecular layer (Fig. S4) and that death of Bmp10 mutant mice (around E9.5) and failure to form trabeculae occurs at a time point of very low proliferation in the heart tube ³².

The up-regulation of N2ICD in the myocardium of NbNbl^{NK} mice and the similarities of the phenotypes of N2ICD^{MHC} and NbNbl^{NK} mutants suggested that inactivation of Numb/Numbl and directed expression of N2ICD might result in activation of an overlapping set of downstream target genes. Therefore, we performed an Affymetrix DNA microarray analysis based expression analysis of E14.5 hearts of N2ICD^{MHC} mutants and compared the results to the data from the Affymetrix DNA microarray analysis of NbNbl^{NK} mutants, which was described above. We observed a major overlap of genes that were dysregulated in either mutant (Table S1-S3). To validate the DNA microarray data we selected two genes Tnnt3 and Tnni2. Both genes were up-regulated to a similar extent in N2ICD^{MHC} and NbNbl^{NK} mutants (Fig. S7). It should be pointed out, however, that we also identified significant differences in the expression profiles, which might be due to additional functions of Numb/Numbl in the myocardium, different expression levels of NICD in N2ICD^{MHC} and NbNbl^{NK} hearts, or other reasons.

Knockdown of Numb and Numbl in zebrafish leads to ectopic Notch reporter gene activity in the myocardium

We demonstrated that directed expression of Notch2 ICD in the myocardium phenocopies inactivation of Numb and Numbl in developing mouse hearts, which suggests, together with the increased presence of Notch2 ICD in NbNbl^{NK} mutant hearts, that augmented Notch signaling is responsible for hypertrabeculation and reduced compaction. To further confirm this assumption and to evaluate the evolutionary conservation of Numb/Numbl-mediated inhibition of Notch signaling in the myocardium we turned to the zebrafish system taking advantage of a Notch reporter strain ²³. Analysis of double transgenic zebrafish embryos carrying a Notch-responsive EGFP gene and a mCherry reporter gene expressed in the myocardium revealed that Notch signaling is mostly confined to the endocardial layer of the fish heart under normal conditions (Fig. 6A). Knockdown of Numb/Numbl using morpholino injections ²⁴, however, resulted in a major upregulation of Notch-reporter gene activity in the myocardium. All analyzed embryos (n=5 randomly selected of a group of >100) that were injected with anti-sense morpholinos directed against zebrafish Numb and Numbl but not with control morpholinos showed EGFP fluorescence in myocardial cells. Interestingly, this ectopic Notch-reporter gene activity, which mirrors the increased presence of N2ICD in NbNbl^{NK} mutant mouse myocardium, was present both in atrial and in ventricular cardiomyocytes (Fig. 6B).

Discussion

In our study we have uncovered the role of regional restriction of Notch2 activity for

myocardium trabeculation and compaction. In our search for inhibitors that limited Notch2 activity we identified a functional requirement of Numb and Numbl for ventricular morphogenesis. Deletion of Numb/Numbl resulted in increased expression of N2ICD and the growth factor Bmp10, which might be instrumental for augmented recruitment of cardiomyocytes into trabeculae at the expense of the compact layer.

Cardiac trabeculation is an important morphogenetic process during cardiogenesis. It comprises several steps including: (i) formation of sheet-like protrusions into the lumen by cardiomyocytes along the inner wall of the thickening myocardium. (ii) compaction of the outer trabecular myocardium to form a thicker, compact ventricular wall. Normally, this process is completed after E14.5 when the majority of trabeculae has become compacted. During trabeculation the myocardium undergoes extensive expansion by recruiting cardiomyocytes from the myocardial wall into the trabecular ridges or via cellular proliferation of trabecular cardiomyocytes ¹. A reduction of trabeculation is commonly associated with ventricular compact zone deficiencies and a hypoplastic wall, which can be readily explained by a lack of pro-proliferative cues or a failure of directed migration. In fact, inactivation of different growth factors (Vegf, Bmp10, Nrg1) or their receptors (ErbB2, ErbB4) results in absence of trabecular formation in mouse. Conversely, increased proliferation of trabecular cardiomyocytes, which went along with increased Notch2 activity and profound upregulation of Bmp10 growth factor in Nbnbl^{Nk} mutants, may account for the hypertrabeculation/reduced compaction defects (this study). In addition, adenovirus-mediated overexpression of N2ICD in embryonic stem cell -derived and early neonatal cardiomyocytes induces cyclin D expression and stimulates cell cycle entry ³³. Taken together, these results

clearly suggest an important role of Notch2-mediated increased cardiomyocyte proliferation in the pathogenesis of hypertrabeculation/reduced compaction.

Yet, it is difficult to explain hypertrabeculation/reduced compaction solely by excessive cardiomyocyte proliferation in the trabecular layer, since the proliferative activity is normally consistently higher within the compact myocardium compared to trabeculae ³⁴. In fact, we also found increased rates of dividing cells both in the trabecular and compact layers of N2ICD^{MHC} and NbNbl^{NK} mutant hearts despite a severe thinning of the compact layer suggesting that a significant number of cells from the compact layer are recruited to trabeculae. Therefore, we postulate that Notch2 also actively prevents compaction, thereby leading to increased trabeculation at the expense of the compact layer (see model in Fig. S8). The ability of Notch2 and its downstream effects to suppress compaction seems to be stage-dependent since Notch2 is ubiquitously active in the whole myocardium at early developmental stages. Similarly, Bmp10 is initially also found throughout the myocardium and only becomes confined to the trabecular layer at later stages of development ³². Regional inhibition of Notch2 signaling within the myocardium might be crucial to release the block on compaction. In our study we found that regional inhibition of Notch2 in the compact layer does not occur on the transcriptional level, since Notch2 mRNA is found throughout the myocardium, but on the posttranscriptional level and that Numb and Numbl are critical for mediating Notch2 inhibition in the heart. So far, the role of Numb/Numbl in the developing myocardium was enigmatic although Numb/Numbl have been shown to be involved in regulating spindle orientation in epicardial epithelial-to-mesenchymal transition ³⁵. Numb/Numbl-mediated inhibition of Notch2 might be achieved by promoting N2ICD

ubiquitination and proteasome-mediated degradation¹³. In fact, it has been demonstrated recently that Numb binds to N2ICD but not N3ICD and inhibits its transactivation capacity³⁶ albeit effects of Numb on Notch2 were less consistent compared to Notch1. The use of a zebrafish reporter strain to monitor activation of Notch signaling provided us with additional evidence for Numb/Numbl-mediated inhibition of Notch activity in the myocardium independent of antibodies that detect processing of the intracellular domain of Notch2. Furthermore, the zebrafish experiments revealed that Numb/Numbl suppression of Notch signaling in the myocardium is conserved during evolution.

Although we identified the mechanism by which Notch2 is inhibited in the myocardium we currently cannot explain why Notch2 activity is preferentially repressed in compact but not in the trabecular layer given that Numb/Numbl genes are expressed in the whole myocardium. Several explanations seem feasible: (i) differential expression of Numb isoforms in trabeculae and compact layers. In mouse, four Numb isoforms (Numb65, Numb66, Numb71, and Numb72) are generated by alternative splicing of exon3 and exon9, which correspond to the amino-terminal phosphotyrosine-binding (PTB) domain and the proline rich carboxyl-terminal region (PRR), respectively³⁷. All four Numb isoforms are expressed in mouse embryonic heart (Fig. S5). Only Numb66, Numb71, and Numb72 but not Numb65 inhibit N2ICD activity³⁶. It is possible that Numb65 is exclusively present in trabeculae while Numb66, 71 and 72 are preferentially expressed in the compact layer resulting in a compact-layer-specific inhibition of Notch2 activity. Unfortunately, the available *in situ* probes do not resolve this issue. (ii) Numb/Numbl depend on layer-specific posttranslational activation or require additional co-factors, which might be directed by one or more

layer-specific transcription factors. N-myc, a member of the MYC gene family, is preferentially expressed in the compact layer during heart development and its inactivation causes non-compaction defects ³⁸, which is compatible with a role in the regulation of Numb-cofactors or modulators.

We found that Bmp10 was upregulated in NbNbl^{NK} mutant hearts indicating that Bmp10 is genetically downstream of Numb/Numbl during compaction (Fig. S8). Bmp10 is a peptide growth factor of the TGF- β superfamily, transiently expressed in trabeculae during ventricular development ³². Bmp10 upregulation was also observed in other hypertrabeculation/reduced compaction mouse models such as ventricular-restricted Nkx2.5 knockout mouse ³⁹ and FKBP12 constitutive knockout mouse ³². Overexpression of Bmp10 during embryonic development results in hypertrabeculation and reduced compaction phenotypes ³⁹ suggesting that Bmp10 plays important roles in the recruitment of cardiomyocytes into trabeculae at the expense of the compact layer.

In conclusion, our study gives new insights into the genetic pathway that regulates trabeculation and compaction during mouse heart development. Additional experiments are necessary to further define the process that prevents compaction of trabecular cardiomyocytes and to identify the mechanisms that enable layer-specific regulation of Numb/Numbl activities. It seems also promising to evaluate activities of the various components of the Notch pathway in human patients suffering from left ventricular non-compaction (LVNC), which is a relatively common medical condition that has been considered a fifth form of cardiomyopathy in addition to dilated, hypertrophic, restrictive, and arrhythmogenic cardiomyopathies ⁴⁰.

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Conflict of Interest: none declared

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Figure Legends

Figure 1. Differential activity pattern of Notch1 and Notch2 during heart development.

(A, B) Immunostaining of activated Notch1 (N1ICD) as readout of Notch1 activity on frozen sections of E9.5 (A) and E13.5 (B) hearts. Notch1 activity is seen in the endocardium (black arrows) but not in the myocardium (red arrows). (C-F) Immunostaining of activated Notch2 (N2ICD) in E9.5 (C), E10.5 (D), E11.5 (E) and E13.5 (F) hearts. Red arrows indicate Notch2 activity in the myocardium. Black arrows indicate the endocardium. Green arrows in (E) and (F) indicate the position of compact layer. (G, H) In situ hybridization of Notch2 in E11.5 (G) and E13.5 (H) hearts. Notch2 expression is seen throughout the myocardium (red arrows) but not in the endocardium (yellow arrows). BW, body wall. Scale bars: 5µm in (A), (C) and (D), and 10µm in (B) and (E-H).

Figure 2. Hypertrabeculation, non-compaction, and septum defects in mice with myocardium-specific overexpression of Notch2 intracellular domain (N2ICD^{MHC}).

(A-D) H&E (Hematoxylin & eosin) staining of paraffin sections of E12.5 (A, B) and E14.5 (C, D) control (A, C) and N2ICD^{MHC} mutant (B, D) hearts. Hypertrabeculation (yellow arrows), and ventricular septum defects (black arrow) are apparent in N2ICD^{MHC} hearts. (A'-D') and (A'' - D'') show higher magnification of boxed areas in (A-D), indicating non-compaction defects in N2ICD^{MHC} hearts. (E, F) Quantification of thickness of trabecular (E) and compact layer (F) in control and N2ICD^{MHC} hearts. NS, not significant. *, P<0.05. Scale bars: 10µm.

Figure 3. Multiple cardiac defects in mice with Nkx2.5-Cre-mediated deletion of Numb and Numbl (NbNbl^{NK}).

(A-C) In situ hybridization for Numb (A), Numbl (B) and Bmp10 (C). Numb and Numbl are expressed in the whole myocardium (red arrows), but not in endocardium (green arrows). Bmp10 was used as a marker for trabeculae. (D- I) Hematoxylin & eosin (H&E) staining on paraffin sections of E14.5 (D-F) and E16.5 (G-I) control (D, G) and mutant (E, F, H, I) hearts. Black and yellow arrows indicate ventricular septum defects and hypertrabeculation, respectively. (D'-H') and (D''-H'') show higher magnification of

boxed areas in (D-H). **(J, K)** Quantification of thickness of trabecular (J) and compact layer (K) in control and mutant hearts. *, $P < 0.05$ **(L)** Western blot analysis of Numb expression in E14.5 control and mutant hearts. Actin was used as a loading control. **(M)** Numbl mutants lack exons 3, 4 and 5. Numbl RT-PCR was performed using primers flanking Exon 2 and Exon 6 to examine the expression level of full length Numbl mRNA (FL) from E14.5 hearts of wildtype Nbl(+/+), heterozygous Nbl(+/-) and homozygous Nbl(-/-). MT, mutant Numbl mRNA. Com, compact layer; Tr, trabeculae. Scale bars: 10 μ m.

Figure 4. Increased Notch2 but not Notch1 activity in the compact layer of NbNbl^{NK} mutant hearts. **(A, B)** Western blot analysis of the levels of N1ICD (A) and N2ICD (B) in control and NbNbl^{NK} mutant hearts (*, $P < 0.05$). **(C, D)** Immunostaining of N1ICD on frozen sections of E13.5 control (C) and NbNbl^{NK} mutant hearts (D). Black arrows indicate Notch1 activity in the endocardium. **(E, F)** Immunostaining of N2ICD of E13.5 control (E) and mutant (F) hearts. **(E', F')** show higher magnification of boxed areas in (E, F) Green arrows show Notch2 activity in trabeculae. The red arrows indicate ectopic Notch2 activity in the compact layer of NbNbl^{NK} mutant hearts. Scale bars: 5 μ m in (C-F), and 2.5 μ m in (E', F').

Figure 5. Analysis of BMP10, Hey1 and Hey2 expression in NbNbl^{NK} and N2ICD^{MHC} mutant hearts. **(A)** Real-time PCR analysis of BMP10, Hey1 and Hey2 expression using E14.5 control and NbNbl^{NK} mutant hearts (* $P < 0.01$). **(B)** Fold change of trabeculae cell numbers in NbNbl^{NK} and N2ICD^{MHC}. (* $P < 0.05$) **(C)** Western blot analysis of phospho-Smad1/5/8 levels in E14.5 control and NbNbl^{NK} mutant hearts. Actin was used as a loading control. (* $P < 0.05$) **(D)** Real-time PCR analysis of BMP10, Hey1 and Hey2 expression in E11.5 control and N2ICD^{MHC} mutant hearts. (* $P < 0.05$).

Figure 6. Knockdown of NbNbl resulted in ectopic Notch activity in myocardium. **(A, B)** Zebra fish injected with standard control morpholino (A) and NbNbl morpholino (B) were examined by confocal microscopy at 55 hpf. Cardiomyocytes were labeled by membrane-bound DsRed (red), and cells with Notch activity by EGFP (green). Arrow heads

indicate Notch activity in endocardium. (**A'**, **A''**) and (**B'**, **B''**) show higher magnification of boxed areas in (A, B). Arrows show ectopic Notch activity in the myocardium. Scale bars: 50µm in (A, B), and 15µm in (A', A'', B' , B'').

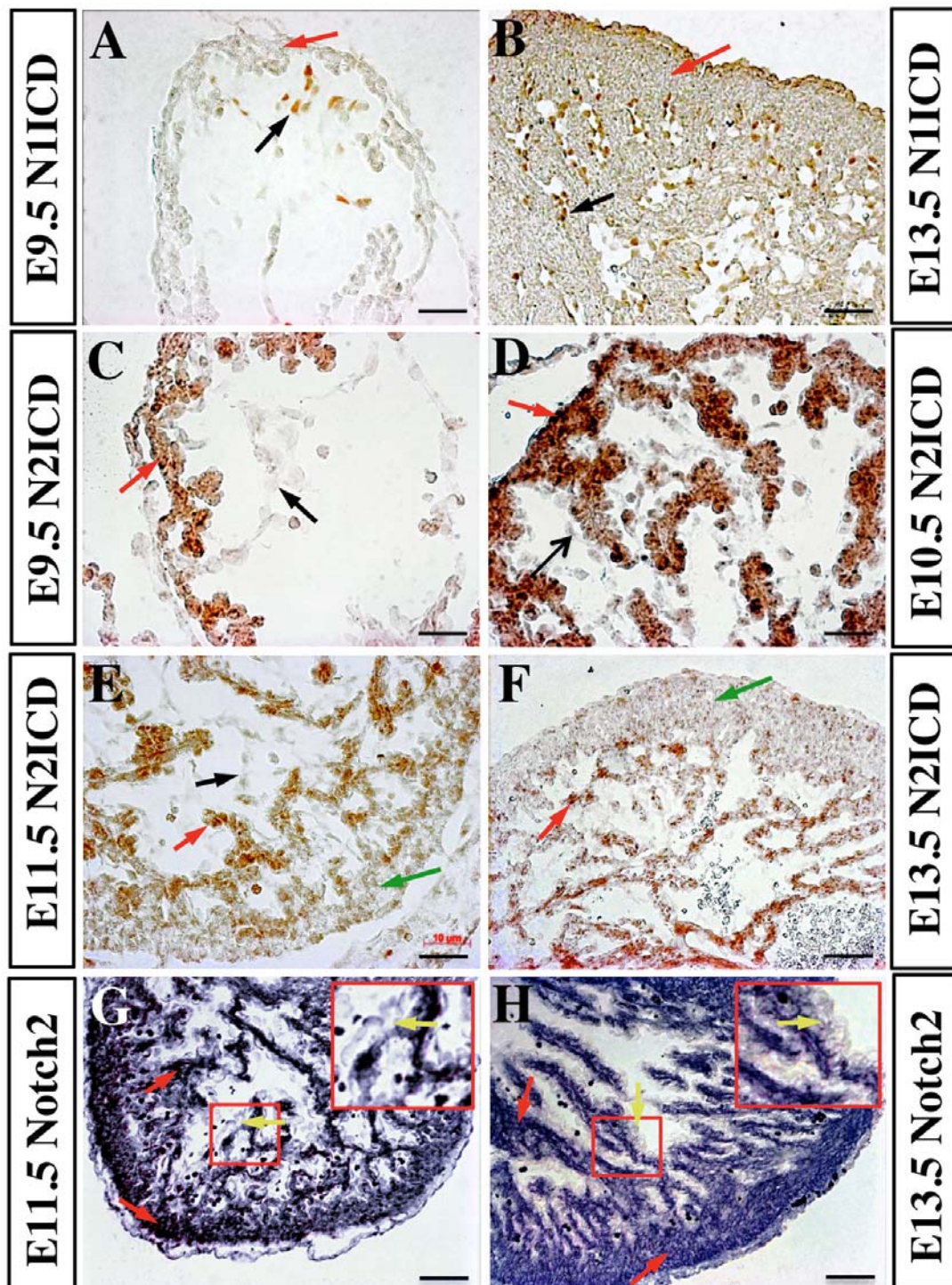


Fig. 1

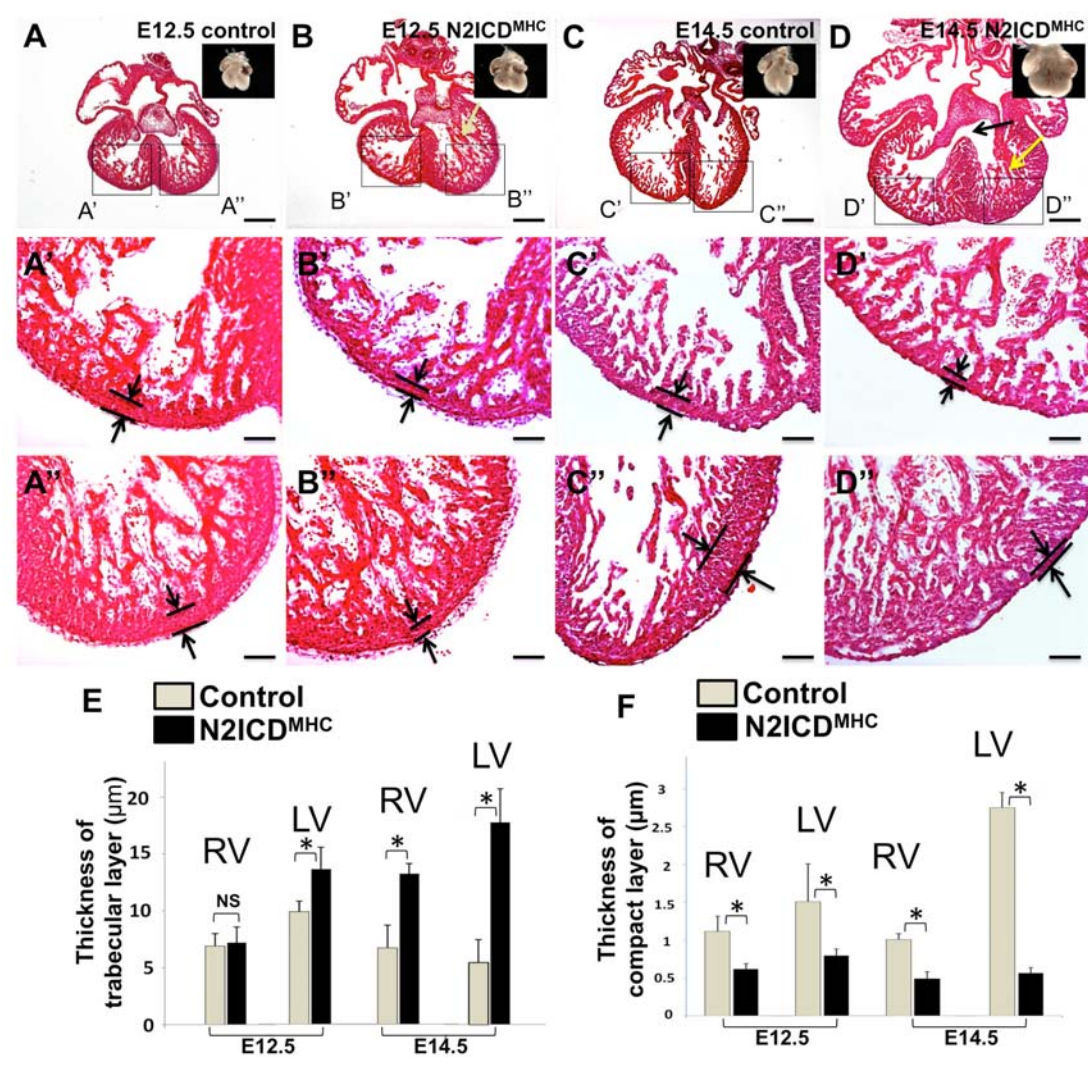


Fig. 2

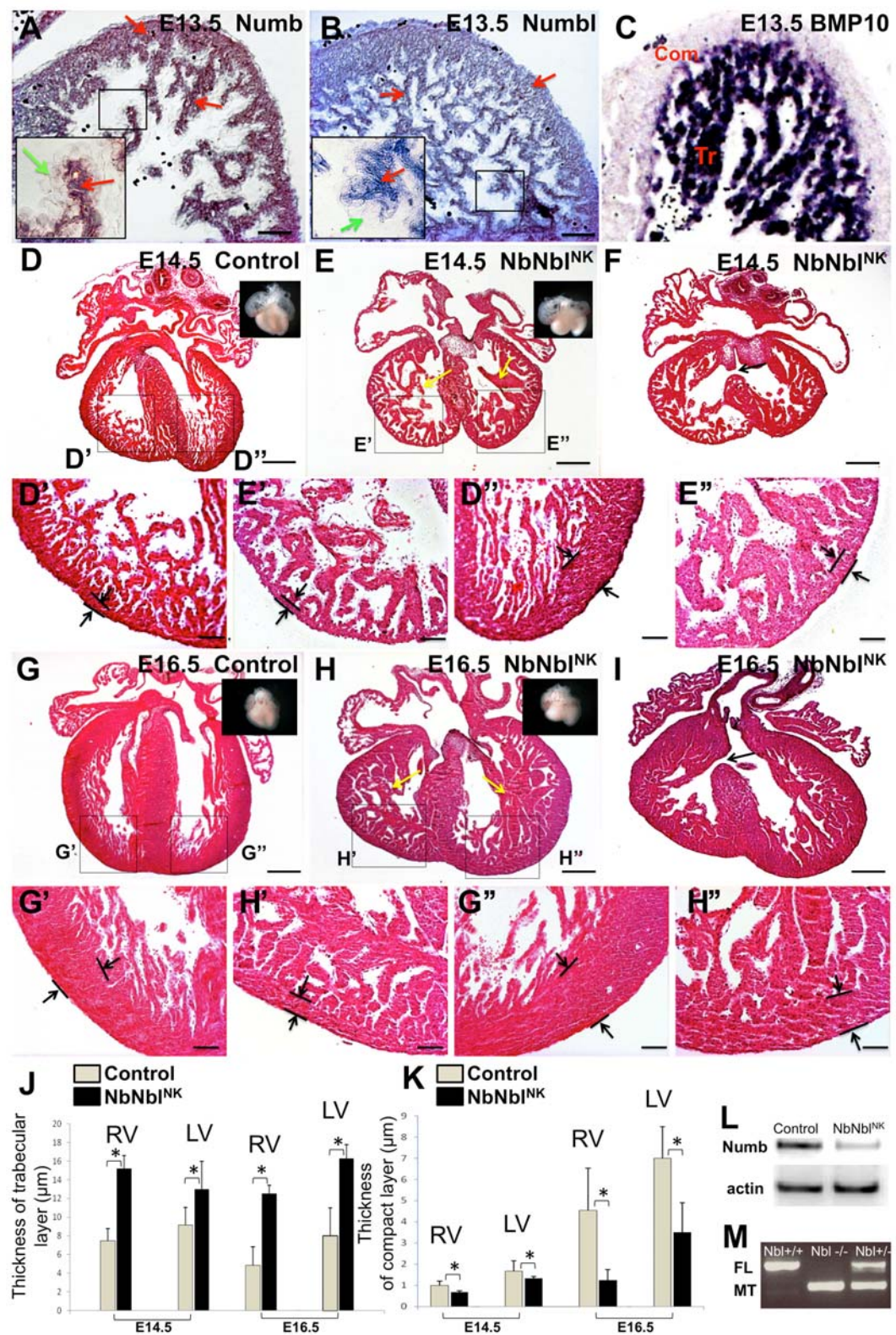


Fig. 3

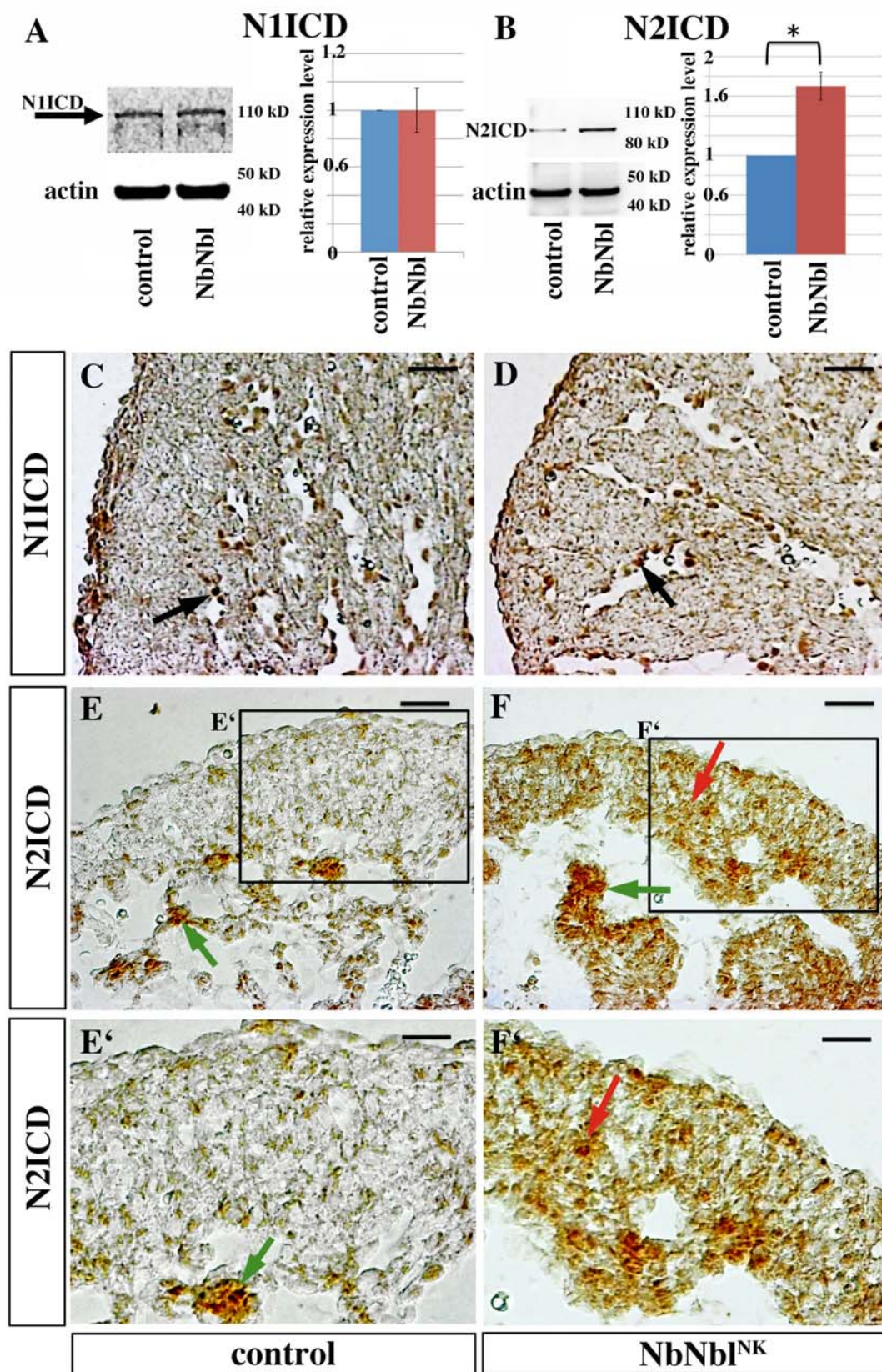


Fig. 4

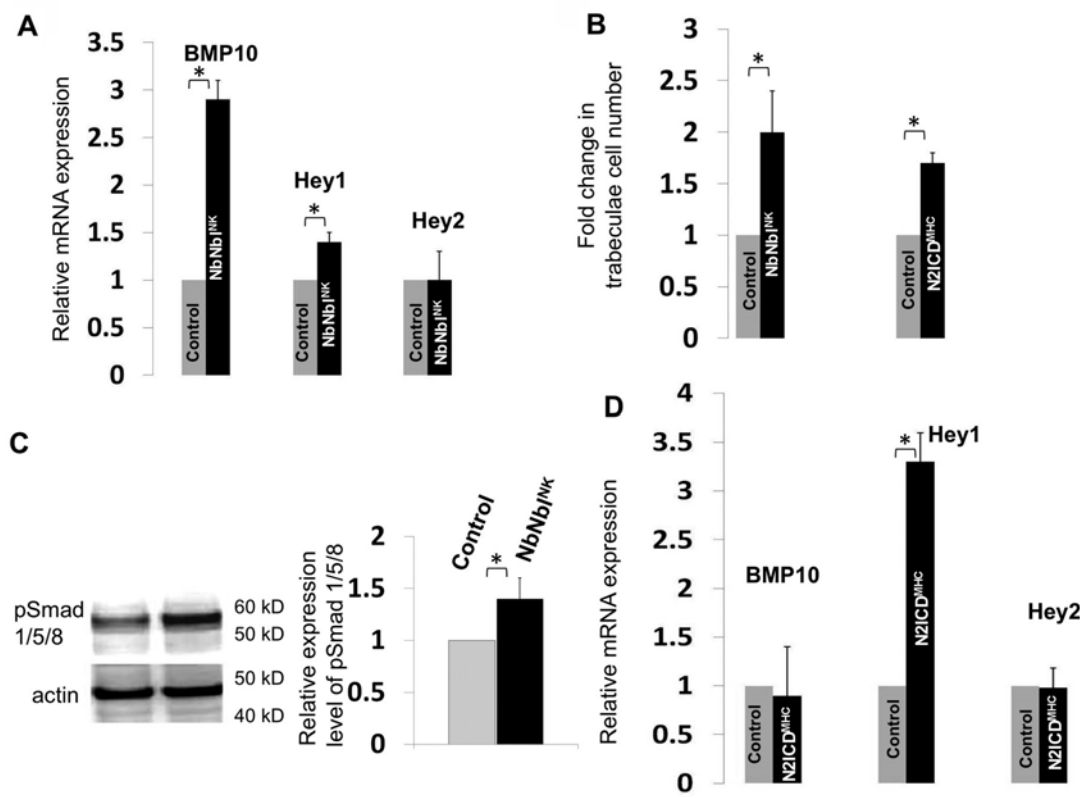


Fig. 5

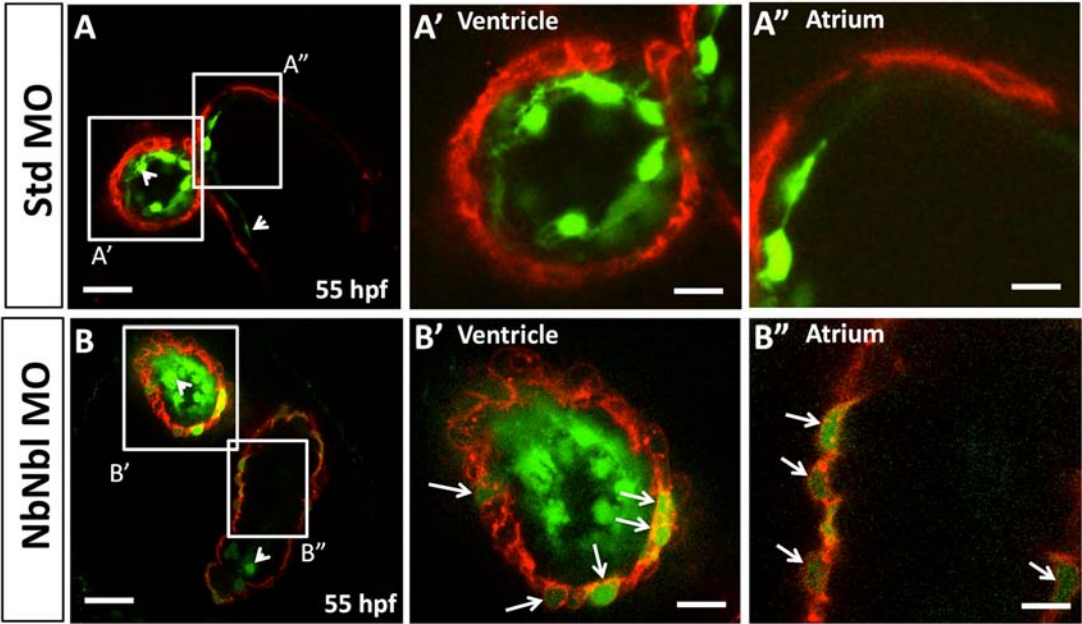


Fig. 6