

## **SUPPLEMENTAL MATERIAL**

**Auxilin is a novel susceptibility gene for congenital heart block which directly impacts fetal heart function**

**Meisgen S et al.**

## **CONTENTS**

Supplemental Patients and Methods

Supplemental figures S1-S3

Supplemental tables S1-S4

Legends, Supplemental movies S1-S6

## SUPPLEMENTAL PATIENTS AND METHODS

### Study populations, genotyping, and analysis

The cohort of patients diagnosed with congenital heart block and their families has been previously described,<sup>(1, 2)</sup> and clinical information is summarised in supplemental table 1. Confirmed maternal Ro/SSA autoantibodies were a prerequisite for inclusion and patients with major cardiac structural abnormalities, or postoperative or infection-induced block were excluded. DNA preparation and genotyping were performed on the Illumina 660W-Quad Beadchip. For family-based analysis, samples with an individual genotype call rate >95%, Mendelian errors per family <2%, SNP genotype call rate >95%, SNP Mendelian error <4%, minor allele frequency (MAF) >0.01 and Hardy-Weinberg equilibrium (HWE)  $p > 1 \times 10^{-8}$  were included. The genomic inflation factor was  $\lambda = 1.01$  (supplemental figure 1). Association was calculated using the family-based association for disease trait (DFAM) analysis on the 534,192 SNPs included after quality control (QC) (NCBI build 36.3) in the 92 CHB cases and 256 non-affected family members using PLINK.  $P_{DFAM}$  values  $\leq 1 \times 10^{-4}$  were considered for further analysis. Risk allele and transmission frequencies, odds ratios, and confidence interval calculations were performed using PLINK. Genotyping data from population-based controls for the case-control analysis were obtained from the Swedish Multiple Sclerosis cohort,<sup>(3)</sup>  $n = 527$ , and the Swedish section of the PROCARDIS study,<sup>(4)</sup>  $n = 678$ . After QC with individual call rate >98%, SNP call rate >98%, MAF >0.05, HWE  $p > 0.001$ , average Mendelian error rate 0.06% (CHB cohort) and IBD removal of out-of study controls with pHAT >0.12, 465,202 overlapping markers were included for further analysis. The genomic inflation factor was  $\lambda = 1.006$  (supplemental figure 1). After principal component analysis (PCA, EIGENSTRAT smartPCA<sup>(5)</sup>) we removed 13 population outliers (10 controls, 3 cases). In total, 1195 out-of-study controls and 89 cases were left for logistic regression analysis to confirm family-based SNP associations with CHB using the additive model and correcting for the only significant PC.

The studies were approved by the Regional Ethical Committee, Stockholm, Sweden. Participants or guardians gave informed written consent.

### Study material

Samples for genotyping were collected as described above. Cardiac, mammary artery and aortic

biopsy specimens were obtained from patients enrolled in the Advanced Study of Aortic Pathology (ASAP), described in,<sup>(6)</sup> and undergoing aortic valve surgery at the Karolinska University Hospital, Stockholm, Sweden. Fetal tissues from electively terminated normal pregnancies were collected at the Women's and Children's Health Department, Karolinska University Hospital, Stockholm, Sweden and the Hospital for Sick Children, Toronto, Canada. The studies were approved by the Regional Ethical Committee, Karolinska Institutet, Stockholm, Sweden, and The Hospital for Sick Children, Toronto, Canada respectively. Participants gave informed written consent.

### **Expression quantitative trait loci analysis**

Expression quantitative trait loci (eQTL) analysis was performed for disease-associated SNPs (dbSNP 130) and RNA expression data from the ASAP study population.<sup>(6)</sup> Microarray (Affymetrix GeneChip Human Exon Array 1.0) data were analyzed by R (version 2.14.2) using the RMA algorithm<sup>(7)</sup> in the Affymetrix Power Tools-1.12.0 package. Tests for association between genotype and log-transformed gene expression were performed using the additive model in R. Information on genes within the 1 Mb interval was retrieved from ENSEMBL release 58 (GRCh37).

### **Cardiac gene expression profiling**

RNA sequencing was performed on an Illumina HiSeq 2500 System at 2x101 bp length and a depth of 64 to 115 million read pairs. The reads were aligned using STAR<sup>(8)</sup> to the hg38 assembly and gencode v21 exon-exon junctions, and FPKM (fragments per kilo base and million mapped reads) expression values were calculated using `rpkmforgenes.py` (<http://sandberg.cmb.ki.se/rnaseq>)<sup>(9)</sup> version 13, with settings `-minqual 255 -rmnameoverlap -midread -fulltranscript` and with RefSeq annotation. After QC and testing of cardiac-tissue specificity (FPKM *TNNT2* > 0), 32 samples remained.

### **Fetal cardiac microdissection and expression analysis**

Total RNA of dissected human fetal heart AV junctional and apical myocardium tissue was extracted using the Qiagen RNeasy kit, quantified by Nanodrop and Bioanalyzer, labeled and

applied onto the Affymetrix Human U133 plus 2 array. The results were normalized and analyzed by Partek® Genomics Suite™.

### **Experimental animals**

Animals (auxilin<sup>-/-</sup> mice<sup>(10)</sup> and C57/BL6 (Jackson Laboratory, USA)) were kept and bred at the AKM animal facility at the Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden. All experimental protocols were approved by the Ethics Committee Stockholm North.

### **Quantitative PCR and transcript alignment**

Auxilin transcript-specific quantitative PCR analysis was performed using cDNA generated from total mRNA prepared from cardiac tissue biopsies of individuals enrolled in the ASAP study (n=224), from human fetal tissue and cultured iCell cardiomyocytes<sup>2</sup> (Cellular Dynamics International, Madison, WI, US) using the RNAeasy kit (Quiagen) and the SuperScript® III First-Strand Synthesis SuperMix (LifeTechnologies). For auxilin protein transcript alignment and the design of specific exon-exon spanning primers for the protein coding transcript variants auxilin-001, 002, 201, and 008, and for overall auxilin expression the ENSEMBL release 75 (GRCh37 assembly) was used. Primers used are listed in supplemental figure S2. For fetal tissue and iPS induced cardiomyocyte expression analysis, ABI gene expression assays were used. Quantitative PCR with 5ng template cDNA was performed using the iQ SYBR Green Supermix (Biorad, Sweden) or Taqman Universal Mastermix II NO UNG and the CFX384 Touch™ Real-Time PCR Detection System (Biorad, Sweden) using a two-step protocol (95°C for 10 minutes, followed by 95°C for 15 seconds and 60°C for 1 minute for 40 cycles). The amplification was performed in 384-well plates in duplicates and for SYBR PCR a standard curve was constructed using two-fold dilutions of pooled cDNA from five samples. Expression of genes of interest was normalized to β2-microglobulin or Taf8 using the delta Ct method.

### **Cloning of recombinant EYFP-auxilin**

The auxilin-201 transcript was per amplified using a cdna library generated from human fetal heart (gestational week 12) and cloned into the peyfp-c1 vector (clontech). The construct was verified by sequencing. Primer sequences available upon request.

### HeLa cell culturing and transfection

HeLa cells were plated in culture medium (DMEM supplemented with 10% FBS, 2 mM L-glutamine and 100 µg/mL Penicillin/Streptomycin) at a density of 250,000 cells/ml. Two hours after plating, transfection was performed by adding 100 µl DMEM, supplemented with 3µl XtremeGene 9 DNA transfection agent (Roche) and 1 µg plasmid DNA (pEYFP-C1, pEYFP-auxilin) to each well. Cells were harvested 22 hours after transfection.

### Western blot

Total cellular protein extracts were prepared by lysing human fetal and neonatal mouse organs, cultured differentiated human cardiomyocytes (iCell Cardiomyocytes<sup>2</sup>, Cellular Dynamics International, Madison, WI, US), HeLa or Daudi cells in T-PER Tissue Protein Lysis buffer Reagent (Thermo Scientific) or CellLytic M Reagent (Sigma) supplemented with proteinase inhibitor cocktail (Thermo Scientific) using the Qiagen TissueRuptor for 2 minutes at 40 Hz. Total protein extracts were denatured by boiling for 5 minutes in 5% SDS sample buffer and loaded on a Mini-PROTEAN® TGX™ 4–15% precast linear gradient polyacrylamide gel (BioRad, Sweden). Size-separated proteins were blotted onto a nitrocellulose membrane. The membrane was blocked with 5% (w/v) fat-free milk 0.1% Tween 20-phosphate-buffered saline (TPBS) over night at 4°C. Membranes were washed with TPBS and incubated with a polyclonal anti-auxilin antibody (HPA031182 (Sigma) 1:250 for 1 hour at room temperature (RT), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, DakoCytomation, Glostrup, Denmark) for 30 minutes at RT. Pre-incubation of the anti-auxilin antibody with 10 µg of the HPRR2550352 peptide (HPA project, Sweden) was performed for 1 hour at RT. Membranes were stripped by incubating in a 0.1 M NaOH solution for 15 minutes at RT to remove bound antibodies, subsequently washed with di-ionized water, and further incubated with anti-β-actin-HRP (1:50,000, Sigma) or anti-GAPDH-HRP (1:1000, Cell Signaling) for 1 hour at RT. All antibodies were diluted in 1% (w/v) fat-free milk in TPBS, and the membranes were washed in TPBS between incubations. Blots were developed with the ECL system (Amersham Biosciences, Little Chalfont, UK). Protein size was determined using the Precision Plus Protein™ Kaleidoscope Standards (BioRad, Sweden).

### **Immunohistochemistry staining**

Tissue was fixed in 4% paraformaldehyde, paraffin-embedded, and sectioned with a microtome and placed on positively charged glass slides. The 8 µm sections were de-paraffinized by heating at 60°C for 18 hours, incubated in xylene twice for 5 minutes and rehydrated by immersing for 5 minutes into gradually diluted ethanol solutions (99, 95, and 70%), and thereafter washed in PBS. Antigen retrieval was performed by heating the slides in citrate solution (pH 6) at 98°C for 40 minutes, thereafter allowing slides to cool, and washing in water for 10 minutes. Slides were then consecutively treated at room temperature with 1% hydrogen peroxide for 30 minutes in the dark, avidin and biotin blocking solutions (VECTASTAIN) and 2% normal horse or goat serum for 20 minutes. Washing in PBS was performed between each step. Primary antibodies (2 µg/ml anti-auxilin (HPA031182, Sigma) or corresponding amount of isotype control (Negative control Rabbit Ig Fraction Ab, DakoCytomation) were added to the sections and incubated at RT for 60 minutes in a humidified chamber. After rinsing and washing in PBS, biotinylated goat-anti-rabbit or horse-anti-mouse IgG (1:750, DakoCytomation) diluted in PBS with 2% normal horse or goat serum was added to the sections and incubated for 60 minutes. The slides were then washed and treated with a pre-formed complex of biotin and peroxidase-labeled avidin (VECTASTAIN) for 45 minutes and then developed with the diaminobenzidine (DAB) kit (Vector) shielded from light for 10 minutes. Slides were counterstained with Mayer's hematoxylin and mounted with coverslips using Mountex (HistoLab, Gothenburg, Sweden). Sections were scanned with the Hamamatsu Nano Zoomer Slide Scanner and analyzed in the NPD View software.

### **Immunofluorescence staining of human fetal cardiac cells**

After preparation, cells were fixed in 4% paraformaldehyde (PFA) for 10 minutes at 4°C and permeabilized in 0.1% (v/v) Triton-PBS buffer for 10 minutes at 4°C. Blocking was carried out using the permeabilization buffer supplemented with 2% (w/v) DSA and 1% (w/v) BSA for 1 hour at RT. Primary antibodies (goat anti-troponin I (Pierce), dilution 1:200; rabbit anti-auxilin (Labome), dilution 1:300; mouse anti-clathrin (Thermo Scientific), dilution 1:1,200) were diluted in blocking buffer and incubated for 2 hours at RT and shaking at 150 rpm. Cells were washed twice for 15 minutes with permeabilization buffer. Secondary antibodies (anti-goat-Alexa647 1:500 (Jackson Immunolabs), anti-rabbit-DyLight488 1:1,000 (Jackson Immunolabs), anti-mouse-DyLight549 1:1,000 (Jackson Immunolabs) were diluted in blocking buffer and applied

for 1 hour at RT and 150 rpm in the dark. In the last 10 minutes, DAPI (1:10 000) was added for nuclear counterstaining. Cells were washed three times for 15 minutes with PBS. All centrifugation steps within the staining protocol were carried out at 900 rpm for 3 minutes at 4°C. After staining, cells were transferred to glasses coated with 0.3% (w/v) gelatin-deionized water for microscopically imaging using Quorum Spinning Disc Confocal 2 (Olympus) equipped with a Hamamatsu C9100-13 back-thinned EM-CCD camera (Hamamatsu).

### **Cardiomyocyte preparation and culturing**

Hearts from auxilin knockout or wild-type neonatal mice were isolated and incubated in HBSS buffer supplemented with 5 mM BDM and 1 mg/ml collagenase type 2 overnight at 4°C with gentle shaking. Hearts were then transferred to mincing buffer (HBSS supplemented with 10 mM taurine, 10 mM BDM, 1 mg/ml BSA and 10 mg/ml collagenase type 2) and gently stirred for 7 minutes at 37°C in presence of a magnetic bar. Eluted cells were collected and filtered using a 100 µm strainer. The elution process was performed three times. Obtained cells were then washed in HBSS and spun down for 5 minutes at 4°C at 150 rcf (Sigma 3-18K centrifuge, rotor 11180, swing-out). Primary cardiomyocytes were plated at a density of  $2.5 \times 10^5$  cells/ml on 18 mm (time lapse recordings),  $1.5 \times 10^4$  cells/ml on 12 mm (Immunostaining) glasses coated with fibronectin supplemented with 0.02% gelatine solution and cultured over night or 48h at 37°C, 5% CO<sub>2</sub> in Claycomb medium supplemented with 10% FBS, 0.1 mM Norepinephrine, 2 mM L-glutamine and 100 µg/mL Penicillin/Streptomycin.

Human differentiated cardiomyocytes (iCell Cardiomyocytes<sup>2</sup>, Cellular Dynamics International, Madison, WI, US) were cultured and harvested according to the manufacturer's protocol.

### **Immunofluorescence staining of mouse primary cardiomyocytes**

After preparation and culturing overnight, glasses with primary cardiomyocytes were washed twice in pre-warmed PBS and cells were fixed in 2% PFA/PBS for 10 minutes at RT. Quenching was carried out in 10 mM glycine/PBS for 10 minutes at RT followed by permeabilization of the cells in 0.1% (v/v) Triton for 10 minutes at RT. Blocking was performed with 5% BSA/PBS for 15 minutes at RT followed by primary antibody staining in 2.5% BSA/PBS for 1h at RT in a wet-chamber rabbit anti-auxilin (Sigma, HPA031182) 1:200, mouse anti-clathrin (Thermo Scientific,

MA1-065), dilution 1:1200. Secondary antibody incubation was performed in 2.5% BSA/PBS for 20 minutes at RT in the dark (anti-mouse-Alexa546, anti-rabbit-Alexa488, (Thermo Fisher, Molecular Probes, US), dilutions 1:400). Nuclear staining with DAPI 1:20,000, was added to one of the secondary antibody staining solutions. One to three washing steps were performed in PBS or 2.5% BSA/PBS after each of the treatment steps. Glasses were washed in ddH<sub>2</sub>O prior to mounting using Fluoromount G (Southern Biotechnology) and thereafter stored at 4°C in the dark.

### **Time lapse Ca<sup>2+</sup> recordings**

Cardiomyocytes were loaded with the Ca<sup>2+</sup>-fluorescence indicator Fluo-4 AM (10 µM) (ref F14201, Molecular Probes, Life Technologies, Stockholm, Sweden) dissolved in DMSO (Invitrogen, UK), 0.2‰ pluronic acid (F-127, Life Technologies, Stockholm, Sweden) in 500 µl Claycomb supplemented culture medium without norepinephrine for 30 minutes at 37°C and 5% CO<sub>2</sub>. Subsequently, de-esterification was performed in culture medium for 10 minutes (37°C, 5% CO<sub>2</sub>). Cover slips (18 mm) were mounted in a chamber and cytosolic Ca<sup>2+</sup> measurements were carried out in complete medium supplemented with norepinephrine (final concentration 10 µM) at 37°C using a ZeissAxio Examiner D1. AX10 microscope (Zeiss 20x, water immersion objective, N.A. 1.0) equipped with a photometrics eVolve EMCCD-camera at a 0.1-2.0 s interval and filter set 38HE (Zeiss). Cardiomyocytes were perfused with complete medium (2.5 ml/min) using a peristaltic pump and temperature was controlled using a Chamlide Inline Heater (IL-H-10, Life Cell Instruments, Seoul, Korea) and Chamlide AC-PU perfusion chamber. Time-lapse calcium imaging time traces were normalized through  $\Delta F/F_0$ , where  $\Delta F = F_1 - F_0$ .  $F_1$  is the specific fluorescence intensity at a specific time point, and  $F_0$  is the average intensity of 10 s before and after  $F_1$ .

### **Flow cytometry staining and analysis**

Primary cardiocytes from mouse neonatal pups were prepared as described under primary cardiomyocyte preparation and culturing. For staining 0.1 mM EGTA was added to the mincing buffer. Obtained cells were stained with live/dead fixable violet dead stain 1:1,100 (Life Technologies, Sweden) for 10 minutes at RT. Cells were then pelleted and washed in HBSS supplemented with 0.1mM EGTA and 2% BSA. FcR blockade (anti-CD32/16 (eBioscience, US),

anti-CD64 (R&D Systems, UK) 1:500 and anti-CD89 1:200 (Santa Cruz, US)) was performed for 10 minutes at 4°C and gentle shaking. Cells were resuspended in 2% BSA/HBSS staining buffer containing anti-Ca<sub>v</sub>1.3 primary antibody, 1:900 (Alomone Labs, Israel), and stained for 30 minutes at 4°C with gentle shaking. After washing, cells were stained with Alexa633 anti-rabbit 1:10,000 (Life Technologies, Sweden) and directly labelled anti-Sirpa-PE, 1:100 (BD Pharmingen, Europe) for 30 minutes at 4°C with gentle shaking. Cells were washed and transferred to HBSS buffer for flow cytometry analysis. Cardiac cell surface molecule expression was analyzed using a Gallios Flow Cytometer (Beckman Coulter, Sweden), and the data was analyzed with the FlowJo software (version 7.6.4, Ashland, US).

### **Doppler recordings**

Pregnant mice were anesthetized with isoflurane (5% induction, 2% maintenance) and ultrasound examinations of the unborn mouse fetuses were performed with a Siemens S2000 ultrasound machine (Siemens Medical Solutions), equipped with a linear 18L6 HD transducer. Guided by color Doppler, pulsed Doppler recordings were obtained with a sample volume encompassing the whole heart in an angle showing inflow through the atrioventricular valves in a different direction from the outflow in the great arteries. Analysis and measurements on digitally stored Doppler tracings were made offline using a Siemens syngo US Workplace. Normal heart rate was defined as the 95% CI of that observed in wild-type fetal mice (100-225 bpm). The inflow a-wave, caused by atrial contraction, was used as marker of atrial activation and the outflow wave as marker of ventricular activation. Atrioventricular (AV)-time intervals used as a surrogate for the PR interval on the ECG were measured from the peak of the a-wave to the start of outflow profile. The isovolumetric contraction time (ICT) was measured from the end of AV inflow to the start of the outflow profile and the isovolumetric relaxation time from the end of the outflow profile to the beginning of ventricular filling. Measurements were made on three consecutive profiles and averaged.

The case presented in figure 7 was examined using the same ultrasound system with a 6C2 transducer. Moving 2D images, M-mode and pulsed Doppler recordings from the mitral valve and aortic outflow were used to diagnose cardiac rhythm and function.

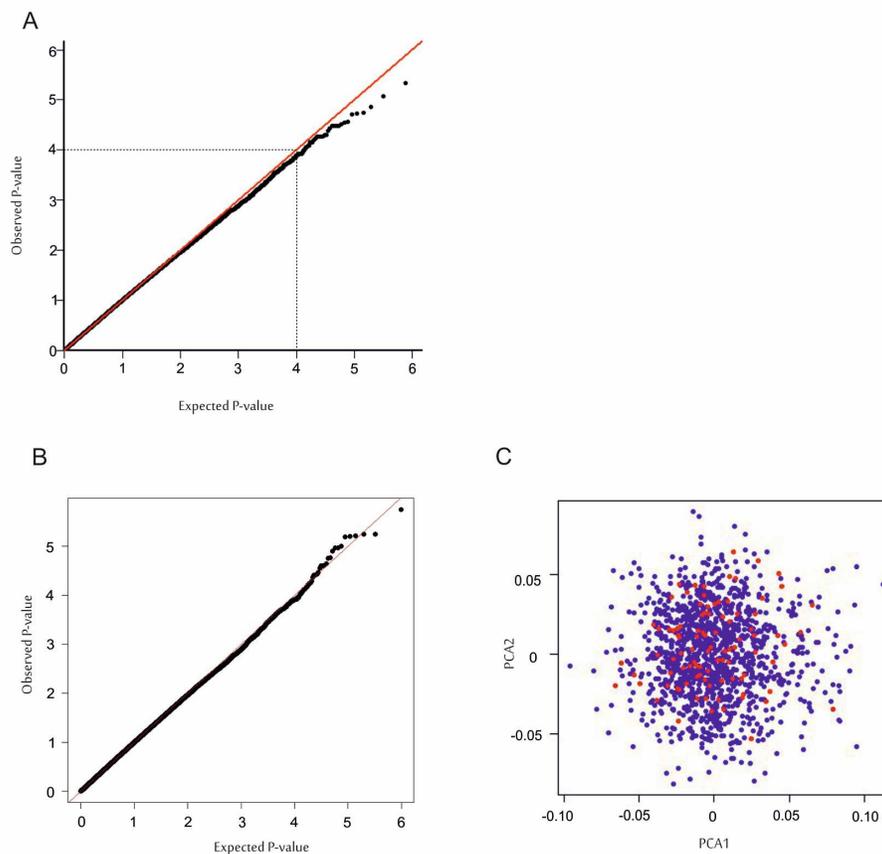
### Programs and statistical analysis

GWAS data was analyzed in the PLINK program (<http://pngu.mgh.harvard.edu/~purcell/plink/>). The R program (version 2.14.2) was used for eQTL analysis (lm package), probability and cardiac expression value plotting. All data processing time lapse  $[Ca^{2+}]_i$  transients were performed with Image J 1.48 (Image J NIH, Bethesda, USA) and MatLab data system (Mat Works inc). Correlation analysis was performed with MatLab data system according to previous reports.<sup>(11, 12)</sup> This identifies intercell-synchronized  $Ca^{2+}$  peaks, visualizing cells that are connected to each other. Utilizing the MatLab BGL-library, network properties such as connectivity, mean shortest path-length and clustering coefficient were calculated to evaluate the organization of cardiac cell activity. Statistica and SigmaPlot were used to analyze the Doppler data and Graphpad Prism 5 was used for all other statistical tests. Statistical tests used for the individual experiments are stated in the respective figure legends. Schematic illustrations are from Servier Medical Art.

### REFERENCES

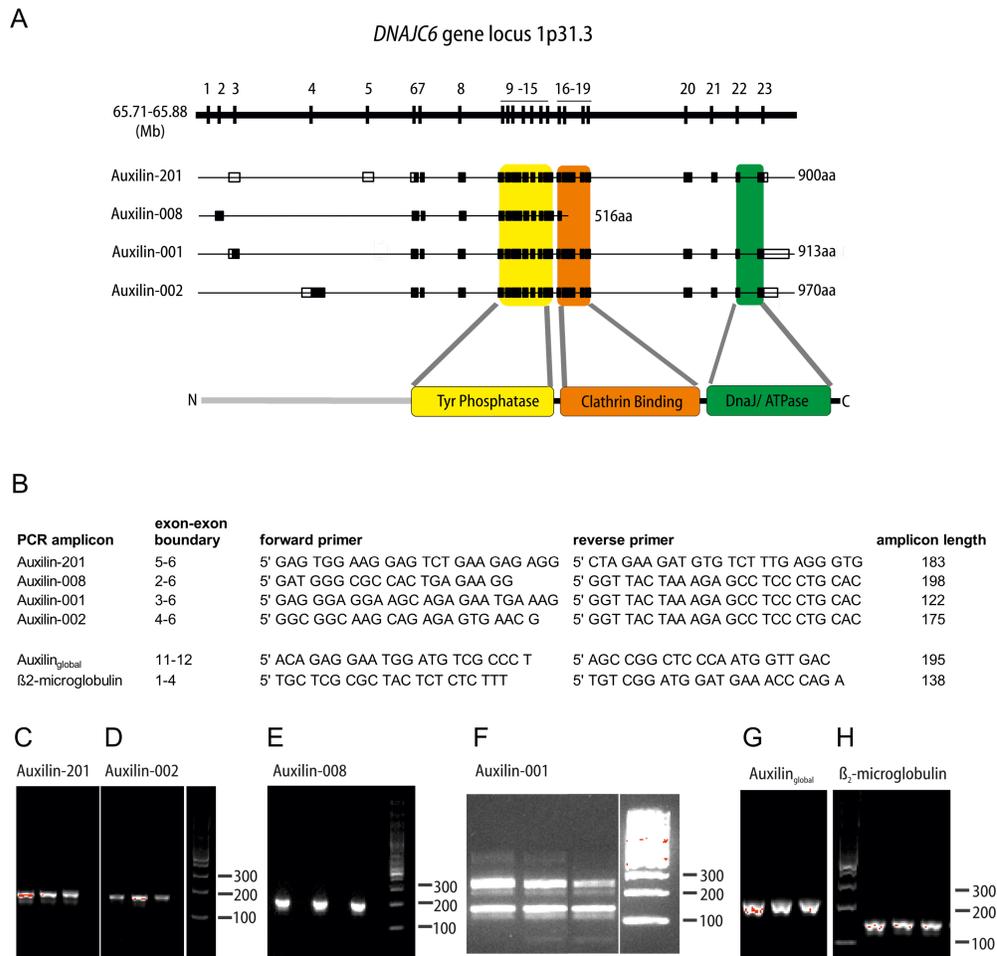
1. A. Ambrosi *et al.*, Development of heart block in children of SSA/SSB-autoantibody-positive women is associated with maternal age and displays a season-of-birth pattern. *Annals of the rheumatic diseases* **71**, 334-340 (2012).
2. S. Salomonsson *et al.*, A population-based investigation of the autoantibody profile in mothers of children with atrioventricular block. *Scandinavian journal of immunology* **74**, 511-517 (2011).
3. C. International Multiple Sclerosis Genetics *et al.*, Genetic risk and a primary role for cell-mediated immune mechanisms in multiple sclerosis. *Nature* **476**, 214-219 (2011).
4. C. Coronary Artery Disease Genetics, A genome-wide association study in Europeans and South Asians identifies five new loci for coronary artery disease. *Nature genetics* **43**, 339-344 (2011).
5. A. L. Price *et al.*, Principal components analysis corrects for stratification in genome-wide association studies. *Nature genetics* **38**, 904-909 (2006).
6. L. Folkersen *et al.*, Association of genetic risk variants with expression of proximal genes identifies novel susceptibility genes for cardiovascular disease. *Circulation. Cardiovascular genetics* **3**, 365-373 (2010).

7. B. M. Bolstad, R. A. Irizarry, M. Astrand, T. P. Speed, A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* **19**, 185-193 (2003).
8. A. Dobin *et al.*, STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**, 15-21 (2013).
9. D. Ramskold, E. T. Wang, C. B. Burge, R. Sandberg, An abundance of ubiquitously expressed genes revealed by tissue transcriptome sequence data. *PLoS computational biology* **5**, e1000598 (2009).
10. Y. I. Yim *et al.*, Endocytosis and clathrin-uncoating defects at synapses of auxilin knockout mice. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 4412-4417 (2010).
11. E. Smedler, S. Malmersjo, P. Uhlen, Network analysis of time-lapse microscopy recordings. *Frontiers in neural circuits* **8**, 111 (2014).
12. E. Smedler, P. Uhlen, Frequency decoding of calcium oscillations. *Biochimica et biophysica acta* **1840**, 964-969 (2014).



### Supplemental Figure S1. Probability distributions and principal component analysis.

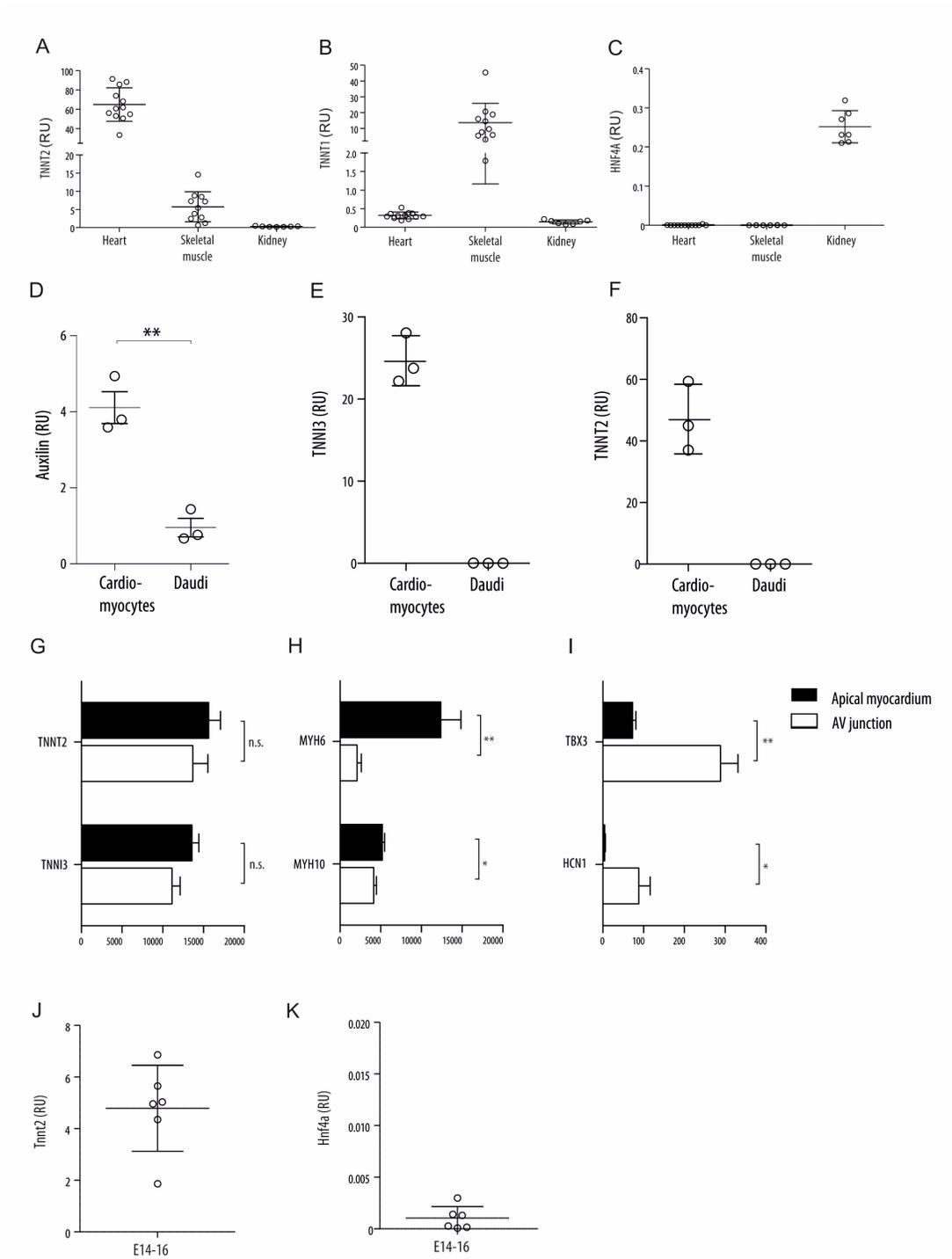
(A) Quantile-Quantile (Q-Q) plot for observed versus expected SNP associations in the CHB family analysis. Plot shows genome-wide associations (DFAM analysis) with CHB. The observed association significances are plotted against the expected association significance for included SNPs. The genomic control inflation factor was  $\lambda=1.01$ . Dashed lines indicate the cut-off for significances at  $P < 10^{-4}$ . (B) Q-Q-plot shows observed versus expected genome-wide SNP associations with CHB in the case-control analysis (logistic regression analysis after correction with one PCA). Plot excludes the extended MHC region chromosome 6:25-33Mb. The genomic inflation factor was  $\lambda=1.006$  (A-B) Scales  $-\log_{10}$ . (C) Principal component analysis (PCA) plot of PCA2 versus PCA1 distribution among cases (red) and controls (blue).



**Supplemental Figure S2. PCR primer design for auxilin protein transcript variant expression analysis in cardiac tissue.**

(A) *DNAJC6* gene locus (1p31.1) at 65.77-65.88 Mb including protein transcript variants and conserved protein domains. Red arrows indicate approximate location of forward and reverse primer annealing for amplification of each transcript variant. (B) Summary of qPCR primer design. The exon-exon boundary column lists exon junctions amplified according to genomic exon counting. Sequences for forward and reverse primers and PCR amplicon length are listed for each transcript variant. No primer design for unique amplification of auxilin-001 was possible. (C-H) PCR product size confirmation after amplification with the indicated primer pair on a 2% TBE agarose gel. GeneRuler<sup>TM</sup> DNA 100 bp ladder was used to determine PCR

amplicon size. (C) Auxilin-201. (D) Auxilin-002. (E) Auxilin-008. (f) Auxilin-001. (G) Auxilin<sub>global</sub>. (H)  $\beta$ 2-microglobulin.



**Supplemental Figure S3. Confirmation of fetal and embryonic mouse tissue identity, cardiomyocyte auxilin RNA expression and cell-type identity.**

(A-C) Identity of fetal tissue (week 10-12 of gestation) was confirmed by qPCR of tissue-specific genes (expression level relative to  $\beta$ 2-microglobulin). (A) Cardiac tissue identity was verified by high *TNNT2* expression ( $>30$  AU) in  $n=12$  samples. Samples with low expression were excluded from further analysis. *TNNT2* expression was used as a tissue negative marker for skeletal muscle and kidney samples. (B) Skeletal muscle identity was verified via *TNNT1* expression ( $>1.5$  AU) and  $n=11$  samples were included. *TNNT1* expression was not observed in cardiac or renal tissue. (C) Kidney tissue identity was tested by *HNF4A* expression ( $>0.2$  AU) analysis and  $n=7$  samples were included. The renal tissue marker was not expressed in the other tested tissues. Bars represent mean  $\pm$  s.d. (D) Auxilin RNA expression in human cardiomyocytes from induced iPS cells compared to Daudi cells. (D-F) Identity of cardiac tissue was confirmed by qPCR of tissue-specific genes (expression level relative to  $\beta$ 2-microglobulin). Cardiac tissue identity was verified by high *TNNT3* (E) and *TNNT2* (F) expression ( $>20$  RU) in iCell cardiomyocyte cultures ( $n=3$ ). Daudi cells ( $n=3$ ) were used as a negative control for tissue specific gene expression. Bars represent mean  $\pm$  s.e.m. (G-I) Identity of apical myocardial and AV junctional tissue after microdissection of human fetal hearts ( $n=6$ ; gestational age 20-22 weeks) was confirmed by gene expression analysis. Expression levels are arbitrary units relative to all genes expressed on the Affymetrix Human U133 plus 2 Array. (G) Cardiac tissue identity was verified by high *TNNT2* and *TNNT3* expression among all samples taken. (H) Apical myocardial identity was confirmed by *MYH6* and *MHY10* expression and (I) AV junctional identity by *TBX3* and *HCN1* specific gene expression. (J, K) Quantitative PCR for cardiac cell-type identity in embryonic wildtype mice relative to *Taf8* expression. (J) Cardiac tissue specificity was verified by *Tnnt2* expression ( $>0.5$  AU) in samples from mouse fetuses (E14-16,  $n=6$ ). (K) *Hnf4a* (kidney marker) expression levels were used as tissue negative markers. Bars represent mean  $\pm$  s.d. Bars represent mean  $\pm$  s.e.m. \*  $p < 0.05$ , \*\*  $p < 0.01$

**Supplemental Table S1. Clinical characteristics of the mothers and their children with congenital heart block.**

---

**Maternal age at blood sampling** 53.8 (50.8-56.8)  
mean (95% CI)

**Maternal diagnosis<sup>1</sup> n (%)** (n=88)

pSS	14 (16%)
SLE	12 (14%)
SLE with sSS	18 (20%)
RA	1 (1%)
RA with sSS	1 (1%)
No rheumatic diagnosis	39 (44%)
Not available <sup>2</sup>	2 (2%)

**Autoantibodies<sup>3</sup> n (%)** (n=80)

anti-Ro52	77 (96%)
anti-Ro60	49 (61%)
anti-La	46 (58%)
anti-Histone	6 (8%)
anti-SmB	1 (1%)
anti-SmD	3 (4%)
anti-RNP	2 (2%)
anti-Cenp-B	2 (2%)
anti-Ribosomal P	1 (1%)

**Diagnosis in the children<sup>4</sup>** (n=92)<sup>5</sup>

AVB II-III	92
AVB I	0

---

<sup>1</sup>Clinical diagnosis registered at the time of sampling of the family.

<sup>2</sup>Deceased/lost to follow-up

<sup>3</sup>Sera available from 80 mothers

<sup>4</sup>Highest degree of AVB observed

<sup>5</sup>Four mothers each gave birth to two children with AVB II-III.

Supplemental Table S2. Genetic regions associated with CHB at  $P \leq 1 \times 10^{-4}$ .<sup>1</sup>

Chr	SNP marker	Position	RA	RAF <sub>[founders]</sub>	P-value	Trans[%]	OR(CI)	Gene	Region
1	rs1570868	65603196	A	0.461	3.27E-05	75.00	3.00 (1.73-5.19)	<i>DNAJC6</i>	intronic
1	rs6588138	65610954	A	0.471	7.06E-05	75.38	3.06 (1.74-5.38)	<i>DNAJC6</i>	intronic
1	rs3818513	65646625	A	0.441	7.06E-05	25.00	0.33 (0.18-0.58)	<i>DNAJC6</i>	intronic
1	rs7552323	167369947	A	0.449	8.22E-05	71.05	2.45 (1.49-4.03)	<i>NME7</i>	intronic
2	rs1477511	220330082	C	0.144	2.78E-05	86.67	6.50 (2.26-18.62)	<i>SLC4A3</i>	intergenic
3	rs12633887	15564595	A	0.274	5.69E-05	77.97	3.53 (1.91-6.54)	<i>PHYH2</i>	intergenic
3	rs1993331	16047351	G	0.240	5.47E-05	78.26	3.60 (1.78-7.25)	<i>GALNTL2</i>	intergenic
3	rs2730367	16048270	G	0.240	5.47E-05	78.26	3.60 (1.78-7.25)	<i>GALNTL2</i>	intergenic
3	rs2730335	16052851	A	0.240	5.47E-05	78.26	3.60 (1.78-7.25)	<i>GALNTL2</i>	intergenic
7	rs11983987	75495786	G	0.174	3.66E-05	84.62	5.50 (2.30-13.13)	<i>STYXL1</i>	intronic
7	rs1639609	75521517	G	0.341	3.36E-05	75.41	3.06 (1.71-5.49)	<i>MDH2</i>	intronic
7	rs4732595	75593075	G	0.341	9.57E-05	73.33	2.75 (1.55-4.87)	<i>MDH2</i>	intergenic
7	rs10085567	75572142	C	0.341	3.36E-05	75.41	3.06 (1.71-5.49)	<i>MDH2</i>	intergenic
7	rs6953665	75606985	A	0.329	5.01E-05	75.00	3.00 (1.67-5.38)	<i>MDH2</i>	intergenic
9	rs4540481	29980455	A	0.368	8.32E-05	72.58	2.64 (1.51-4.62)	<i>LRRN6C</i>	intergenic
9	rs12552164	30007230	A	0.380	7.18E-05	72.31	2.61 (1.51-4.49)	<i>LRRN6C</i>	intergenic
9	rs12375503	30028860	A	0.387	1.36E-05	75.00	3.00 (1.70-5.28)	<i>LRRN6C</i>	intergenic
9	rs4745225	75030376	C	0.203	5.18E-05	18.42	0.22 (0.09-0.51)	<i>ANXA1</i>	intergenic
12	rs2030130	24165338	G	0.169	8.50E-06	84.38	5.40 (2.08-14.02)	<i>SOX5</i>	intronic
12	rs10878353	64668799	G	0.201	8.93E-05	79.55	3.88 (1.86-8.09)	<i>HMG A2</i>	intergenic
12	rs10878354	64671152	A	0.201	8.93E-05	79.55	3.88 (1.86-8.09)	<i>HMG A2</i>	intergenic
12	rs719450	119438416	A	0.160	6.25E-05	79.41	3.85 (1.68-8.85)	<i>COQ5</i>	intronic
15	rs17521464	94384822	G	0.169	6.02E-05	80.00	4.00 (1.84-8.68)	<i>NR2F2</i>	intergenic
18	rs981738	63980419	C	0.157	2.88E-05	83.78	5.16 (2.15-12.38)	<i>TXNDC10</i>	intergenic
18	rs641672	63980432	G	0.158	1.81E-05	84.21	5.33 (2.23-12.75)	<i>TXNDC10</i>	intergenic
20	rs2148218	54324150	G	0.246	5.50E-05	22.92	0.29 (0.15-0.58)	<i>C20orf108</i>	intergenic

20	rs6024799	54338231	C	0.287	1.87E-05	19.61	0.24 (0.12-0.48)	<i>C20orf108</i>	intergenic
20	rs988166	54354265	G	0.246	8.38E-05	23.40	0.30 (0.15-0.60)	<i>C20orf108</i>	intergenic
20	rs8118732	54356605	G	0.190	1.92E-05	16.22	0.19 (0.08-0.46)	<i>C20orf108</i>	intergenic
20	rs6099095	54357169	A	0.194	3.05E-05	16.67	0.20 (0.08-0.48)	<i>C20orf108</i>	intergenic
20	rs6024830	54371614	A	0.269	5.50E-05	22.92	0.29 (0.15-0.58)	<i>C20orf108</i>	intronic
21	rs1394369	23690630	G	0.364	4.67E-06	20.31	0.25 (0.13-0.46)	<i>C21orf74</i>	intergenic

<sup>1</sup>The analysis of association was performed using the family-based association for disease trait (DFAM) analysis. CHB cases (n=92), first degree relatives (n=256). Associations with  $P \leq 1 \times 10^{-4}$  are included.

Chr, chromosome; RA, risk allele; RAF, risk allele frequency; Trans, parental transmission frequency to index cases; OR, odds ratio; CI, 95% confidence interval.

**Supplemental Table S3. Validation analysis of CHB-associated SNPs at  $PDFAM \leq 1 \times 10^{-4}$  using a case-control design.<sup>1</sup>**

Chr	SNP marker	Position	RA	RAF <sub>cases/ controls</sub>	P-value	OR(CI)	Gene	Region
1	rs1570868	65603196	A	0.588/ 0.421	6.22E-06	2.01 (1.50-2.81)	<i>DNAJC6</i>	intronic
1	rs6588138	65610954	A	0.537/ 0.416	1.36E-03	1.66 (1.22-2.27)	<i>DNAJC6</i>	intronic
1	rs3818513	65646625	A	0.375/ 0.481	5.84E-03	0.64 (0.47-0.88)	<i>DNAJC6</i>	intronic
1	rs7552323	167369947	A	0.557/ 0.408	2.20E-04	1.82 (1.33-2.49)	<i>NME7</i>	intronic
2	rs1477511	220330082	C	0.182/ 0.105	2.73E-03	1.92 (1.25-2.94)	<i>SLC4A3</i>	intergenic
3	rs12633887	15564595	A	0.365/ 0.298	1.51E-01	1.27 (0.92-1.75)	<i>PHYH2</i>	intergenic
3	rs1993331	16047351	G	0.302/ 0.175	3.10E-04	1.86 (1.33-2.61)	<i>GALNTL2</i>	intergenic
3	rs2730367	16048270	G	0.304/ 0.174	2.20E-04	1.90 (1.35-2.67)	<i>GALNTL2</i>	intergenic
3	rs2730335	16052851	A	0.297/ 0.173	5.20E-04	1.82 (1.30-2.56)	<i>GALNTL2</i>	intergenic
7	rs11983987	75495786	G	0.255/ 0.206	1.17E-01	1.33 (0.93-1.90)	<i>STYXL1</i>	intronic
7	rs1639609	75521517	G	0.417/ 0.324	4.61E-02	1.40 (1.01-1.94)	<i>MDH2</i>	intronic
7	rs10085567	75572142	C	0.417/ 0.323	4.50E-02	1.40 (1.01-1.95)	<i>MDH2</i>	intergenic
7	rs4732595	75593075	G	0.417/ 0.323	4.40E-02	1.40 (1.01-1.94)	<i>MDH2</i>	intergenic
7	rs6953665	75606985	A	0.412/ 0.321	6.04E-02	1.37 (0.99-1.90)	<i>MDH2</i>	intergenic
9	rs4540481	29980455	A	0.438/ 0.328	1.13E-02	1.50 (1.10-2.06)	<i>LRRN6C</i>	intergenic
9	rs12552164	30007230	A	0.443/ 0.336	1.39E-02	1.49 (1.08-2.04)	<i>LRRN6C</i>	intergenic
9	rs12375503	30028860	A	0.458/ 0.341	5.16E-03	1.56 (1.14-2.14)	<i>LRRN6C</i>	intergenic
9	rs4745225	75030376	C	0.147/ 0.198	1.06E-01	0.70 (0.46-1.08)	<i>ANXA1</i>	intergenic
12	rs2030130	24165338	G	0.243/ 0.149	1.75E-03	1.63 (1.13-2.35)	<i>SOX5</i>	intronic
12	rs10878353	64668799	G	0.276/ 0.213	3.72E-02	1.47 (1.02-2.11)	<i>HMGA2</i>	intergenic
12	rs10878354	64671152	A	0.276/ 0.219	5.34E-02	1.43 (1.00-2.06)	<i>HMGA2</i>	intergenic
12	rs719450	119438416	A	0.214/ 0.139	6.81E-03	1.76 (1.17-2.65)	<i>COQ5</i>	intronic
15	rs17521464	94384822	G	0.229/ 0.184	2.84E-01	1.22 (0.85-1.76)	<i>NR2F2</i>	intergenic
18	rs981738	63980419	C	0.211/ 0.207	9.34E-01	1.02 (0.70-1.48)	<i>TXNDC10</i>	intergenic
18	rs641672	63980432	G	0.214/ 0.190	8.95E-01	1.03 (0.70-1.50)	<i>TXNDC10</i>	intergenic
20	rs2148218	54324150	G	0.177/ 0.253	3.52E-02	0.65 (0.43-0.97)	<i>C20orf108</i>	intergenic

20	rs6024799	54338231	C	0.186/ 0.280	1.93E-02	0.63 (0.42-0.93)	<i>C20orf108</i>	intergenic
20	rs988166	54354265	G	0.172/ 0.252	4.43E-02	0.65 (0.43-0.99)	<i>C20orf108</i>	intergenic
20	rs8118732	54356605	G	0.104/ 0.204	6.14E-03	0.50 (0.30-0.82)	<i>C20orf108</i>	intergenic
20	rs6099095	54357169	A	0.104/ 0.204	6.14E-03	0.50 (0.30-0.82)	<i>C20orf108</i>	intergenic
20	rs6024830	54371614	A	0.177/ 0.276	1.60E-02	0.61 (0.41-0.91)	<i>C20orf108</i>	intronic
21	rs1394369	23690630	G	0.266/ 0.352	1.77E-02	0.66 (0.46-0.93)	<i>C21orf74</i>	intergenic

<sup>1</sup>Logistic regression analysis after PCA correction (one significant PCA vector) of SNPs associated with CHB ( $P \leq 1 \times 10^{-4}$ ) in the DFAM analysis between cases (n=89) and controls (n=1112).  $\lambda=1.006$ .

Chr, chromosome; RA, risk allele; RAF, risk allele frequency; Trans, parental transmission frequency to index cases; OR, odds ratio; CI, 95% confidence interval.

**Supplemental Table S4. Cardiac abnormalities in auxilin-deficient mouse fetuses.<sup>1</sup>**

	Auxilin <sup>+/+</sup> (n=147)	Auxilin <sup>+/-</sup> (n=89)	Auxilin <sup>-/-</sup> (n=131)	P-values <sup>2</sup>
Gestational age (days)	14.6±2.2	14.7±2.0	14.2±2.1	0.15 (0.24)
Intrauterine death	2	1	1	
Heart rate (bpm)	162±32	160±30	160±42	0.89 (0.89)
Mechanical time intervals				
AV-time (ms)	69.8±13.1	72.7±15.0	78.9±22.4	<b>0.014 (0.010)</b>
ICT (ms)	32.5±9.9	36.3±12.4	41.1±14.2	<b>&lt;0.001 (&lt;0.001)</b>
IRT (ms)	54.2±9.4	52.6±9.6	50.1±12.4	0.29 (0.35)
ET (ms)	136.7±29.6	133.5±28.5	137.5±31.9	0.72 (0.98)
Abnormal heart rhythm				
Atrial pause	2			
Atrial arrest with VES	1	2		
SVES (> 1:5)	1	2	16	
VES (> 1:5)	1	4		
AV-block II (Mobitz II)		2		
Abnormal heart rate (HR) and rhythm				
Tachycardia (HR>225 bpm)	6	1	9	
Tachycardia with SVES			1	
Bradycardia (HR<100 bpm)	2	3	2	
Bradycardia with VES		2	3	
Bradycardia with atrial pause			1	
Ectopic beats with normal heart rate	1/137 (1%)	4/82 (5%)	22/114 (19%)	<b>&lt;0.001 (&lt;0.001)</b>
Ectopic beats	1/145 (1%)	6/88 (7%)	26/130 (20%)	<b>&lt;0.001 (&lt;0.001)</b>
Abnormal rate or rhythm	11/145 (9%)	12/88 (15%)	38/130 (30%)	<b>&lt;0.001 (&lt;0.001)</b>

<sup>1</sup>Doppler echocardiographic measurements in fetuses *in utero*. Values represent number of affected mouse fetuses or mean  $\pm$  1 SD. AV, atrioventricular; AV-time, mechanical estimate of the PR-interval on ECG; ICT, isovolumetric contraction time; IRT, isovolumetric relaxation time; ET, ejection time; SVES, supraventricular ectopic beats, VES, ventricular ectopic beats. <sup>2</sup>*P*-values denote statistics comparing all three groups of genotypes (One-way ANOVA (Tukey HSD), Kruskal-Wallis (Dunn's post hoc), Chi square 2x3 contingency table). *P*-values comparing *auxilin*<sup>+/+</sup> versus *auxilin*<sup>-/-</sup> are given within parentheses.

## SUPPLEMENTAL MOVIE LEGENDS

**Supplemental Movie S1. Parallel display of  $[Ca^{2+}]_i$  oscillations in cultured primary neonatal auxilin wild-type and knockout cardiomyocytes.** Time-lapse imaging showing  $[Ca^{2+}]_i$  oscillations and connectivity in primary neonatal cardiomyocytes from auxilin wild-type ( $Aux^{+/+}$ , left) and knockout ( $Aux^{-/-}$ , right) mice. Cells from littermates were pooled and cultured on collagen at a density of  $2.5 \times 10^5$  cells/mL for 48h before visualization by Fluo4-AM. 1 second corresponds to 15 seconds real time (30 frames per second).

**Supplemental Movie S2. Parallel phase-contrast display of cultured primary neonatal auxilin wild-type and knockout cardiomyocytes.** Phase-contrast time-lapse imaging sequence showing connectivity and physical contractions in primary neonatal cardiomyocytes from auxilin wild-type ( $Aux^{+/+}$ , left) and knockout ( $Aux^{-/-}$ , right) mice. Cells from littermates were pooled and cultured on collagen at a density of  $2.5 \times 10^5$  cells/mL for 48h before time-lapse imaging. 1 second corresponds to 15 seconds real time (30 frames per second).

### **Supplemental Movies S3 and S4. Doppler echocardiographic recordings of mouse fetuses *in utero*.**

Supplemental Movie S3. 2D moving image of a pregnant mouse showing three of her fetuses with their placentas *in utero*. The heart activity is clearly seen.

Supplemental Movie S4. 2D moving image with color Doppler showing three mouse fetuses *in utero*. Note the typical flow pattern in the heart with inflows through the AV-valves and outflows in the great arteries in opposite directions. Flow towards the transducer is coded red and flow from the transducer is coded blue.

### **Supplemental Movies S5 and S6. A case of junctional ectopic tachycardia progressing to CHB in a human fetus.**

Supplemental Movie S5. 2D moving image corresponding to Figure 7l,m (gestation age 21 weeks). Transverse thoracic cut showing the heart in a 4 chamber view. The heart has a normal

size and structure. Heart rhythm and rate are normal; however those are not generated in the atria but from an ectopic focus.

Supplemental Movie S6. 2D moving image of the same case as in Supplementary Movie S5, but 3 weeks later (gestational age 24 weeks), corresponding to Figure 7n,o. Transverse thoracic cut showing the heart in a 4 chamber view. The heart is dilated with patchy echogenic changes, diagnosed as cardiomyopathy and endocardial fibroelastosis. The rhythm is regular but slow at 46 beats per minute.