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## TRANSLATIONAL SCIENCE

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

Sabrina Meisgen,<sup>1</sup> Malin Hedlund,<sup>1</sup> Aurelie Ambrosi,<sup>1</sup> Lasse Folkersen,<sup>1,2</sup> Vijole Ottosson,<sup>1</sup> David Forsberg,<sup>3</sup> Gudny Ella Thorlacius,<sup>1</sup> Luca Biavati,<sup>4</sup> Linn Strandberg,<sup>1</sup> Johannes Mofors,<sup>1</sup> Daniel Ramskold,<sup>1</sup> Sabrina Ruhrmann,<sup>5</sup> Lauro Meneghel,<sup>1</sup> William Nyberg,<sup>1</sup> Alexander Espinosa,<sup>1</sup> Robert Murray Hamilton,<sup>6</sup> Anders Franco-Cereceda,<sup>7</sup> Anders Hamsten,<sup>1</sup> Tomas Olsson,<sup>5</sup> Lois Greene,<sup>8</sup> Per Eriksson,<sup>1</sup> Kristina Gemzell-Danielsson,<sup>3</sup> Stina Salomonsson,<sup>1</sup> Vijay K Kuchroo,<sup>9</sup> Eric Herlenius,<sup>3</sup> Ingrid Kockum,<sup>5</sup> Sven-Erik Sonesson,<sup>3</sup> Marie Wahren-Herlenius <sup>1,10</sup>

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For numbered affiliations see end of article.

**Correspondence to**

Professor Marie Wahren-Herlenius, Department of Medicine, Karolinska Institute, Stockholm, Stockholm, Sweden; [marie.wahren@ki.se](mailto:marie.wahren@ki.se)

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**ABSTRACT**

**Objective** Neonatal lupus erythematosus (NLE) may develop after transplacental transfer of maternal autoantibodies with cardiac manifestations (congenital heart block, CHB) including atrioventricular block, atrial and ventricular arrhythmias, and cardiomyopathies. The association with anti-Ro/SSA antibodies is well established, but a recurrence rate of only 12%–16% despite persisting maternal autoantibodies suggests that additional factors are required for CHB development. Here, we identify fetal genetic variants conferring risk of CHB and elucidate their effects on cardiac function.

**Methods** A genome-wide association study was performed in families with at least one case of CHB. Gene expression was analysed by microarrays, RNA sequencing and PCR and protein expression by western blot, immunohistochemistry, immunofluorescence and flow cytometry. Calcium regulation and connectivity were analysed in primary cardiomyocytes and cells induced from pluripotent stem cells. Fetal heart performance was analysed by Doppler/echocardiography.

**Results** We identified *DNAJC6* as a novel fetal susceptibility gene, with decreased cardiac expression of *DNAJC6* associated with the disease risk genotype. We further demonstrate that fetal cardiomyocytes deficient in auxilin, the protein encoded by *DNAJC6*, have abnormal connectivity and Ca<sup>2+</sup> homeostasis in culture, as well as decreased cell surface expression of the Ca<sub>v</sub>1.3 calcium channel. Doppler echocardiography of auxilin-deficient fetal mice revealed cardiac NLE abnormalities in utero, including abnormal heart rhythm with atrial and ventricular ectopias, as well as a prolonged atrioventricular time intervals.

**Conclusions** Our study identifies auxilin as the first genetic susceptibility factor in NLE modulating cardiac function, opening new avenues for the development of screening and therapeutic strategies in CHB.

**INTRODUCTION**

Neonatal lupus erythematosus (NLE) may develop in children of rheumatic women with autoantibodies to the Ro/SSA and La/SSB antigens.<sup>1–4</sup> The most common manifestations of NLE are skin rash

**Key messages****What is already known about this subject?**

- ⇒ Congenital heart block may develop after transplacental transfer of maternal autoantibodies.
- ⇒ A recurrence rate of only 12%–16% despite persisting maternal autoantibodies suggests that additional factors are required for congenital heart block (CHB) development.

**What does this study add?**

- ⇒ We here identify fetal genetic variants conferring risk of CHB and elucidate their effects on cardiac function.

**How might this impact on clinical practice or future developments?**

- ⇒ The findings open new avenues for the development of screening and therapeutic strategies in CHB.

and congenital heart block (CHB). While the former is most often benign and resolves as maternal autoantibodies are cleared from the child's circulation, the latter is characterised by an irreversible disruption of electric signal conduction at the atrioventricular (AV) node (third-degree AV block) and has a high mortality rate around 20% if left untreated,<sup>5</sup> with survivors often requiring pacemaker implants for the remainder of their life.<sup>6,7</sup>

CHB typically develops between weeks 18–24 of pregnancy and is often detected when the fetus presents with signs of bradycardia and complete AV block. The bradycardia is preceded and paralleled by other cardiac pathologies leading up to the end-stage third-degree AV block caused by fibrosis and calcification of the AV node.<sup>8,9</sup> Sinus node dysfunction, lower-degree AV block and a prolonged isovolumetric contraction time have thus been observed in early stages of CHB.<sup>10–12</sup> Up to 15%–20% of fetuses affected by CHB have also been shown to develop more diverse myocardial manifestations



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before birth,<sup>13 14</sup> and signs of junctional ectopic tachycardia or ventricular tachycardia have been reported in nearly one third of fetuses with CHB.<sup>15 16</sup> CHB thus collectively refers to the spectrum of fetal cardiac manifestations occurring in neonatal lupus.

An association between CHB and the presence of maternal autoantibodies to the Ro/SSA autoantigen has long been established, and, when the diagnosis of fetal third-degree AV block without major malformations is established in utero, more than 95% of the mothers test positive for anti-Ro/SSA antibodies.<sup>17</sup> However, a recurrence rate of only approximately 12%–16% for second/third-degree AV block despite persisting maternal autoantibodies indicates that fetal susceptibility, governed by genetic factors, may contribute to disease development.<sup>18–21</sup> Fetal MHC alleles have been linked to the susceptibility, but no other genes thus far.<sup>22–24</sup> In this study, we, therefore, aimed at identifying genetic variants related to CHB by performing a genome-wide association study in families with at least one case of CHB, and sought to define the biological and functional relevance of identified candidate gene(s) for CHB.

## PATIENTS AND METHODS

A detailed Patients and Methods section is available in online supplemental materials.

### Study population and genotyping

The cohort of patients diagnosed with CHB (n=92) and their families has been previously described.<sup>17 18</sup> Briefly, AVB II–III in the index case and confirmed maternal Ro/SSA autoantibodies constituted inclusion criteria for a family, and families in which the index case had major cardiac structural abnormalities, post-operative or infection-induced block were excluded. Maternal diagnoses at the time of blood sampling were primary Sjögren's syndrome (n=14), SLE (n=12), SLE with secondary Sjögren's syndrome (n=18), rheumatoid arthritis (n=1), rheumatoid arthritis with secondary Sjögren's syndrome (n=1), while 39 mothers had no rheumatic diagnosis. Information was not available for two mothers. Anti-Ro52 autoantibodies were present in 96% of the mothers, anti-Ro60 in 61% and anti-La antibodies in 58%. Other analysed autoantibodies (anti-Histone, and-SmB, anti-SmD, anti-RNP, anti-Cenp-B and Ribosomal P) were present in less than 10% of the mothers (online supplemental table 1).

Genotyping was performed on the Illumina 660W-Quad Beadchip.

### Statistical analysis

Genome-wide associations were analysed using PLINK. Statistica and SigmaPlot were used for analysing Doppler-recorded data. Graphpad Prism V.5 was used for all other statistical tests. The statistical tests used for analysis of data from individual experiments are stated in respective figure legend.

## RESULTS

### Identification of Auxilin/*DNAJC6* as a susceptibility gene for CHB

To identify genes that influence fetal susceptibility to CHB, we performed a genome-wide association study of >5 000 000 single-nucleotide polymorphisms (SNP) in a population-based cohort of families with children diagnosed with CHB. To segregate CHB-unique disease traits from potential inherited maternal traits reflecting the maternal rheumatic autoimmune status, we used a family-based study strategy and included SNP genotype data from index cases and their parents and unaffected siblings.

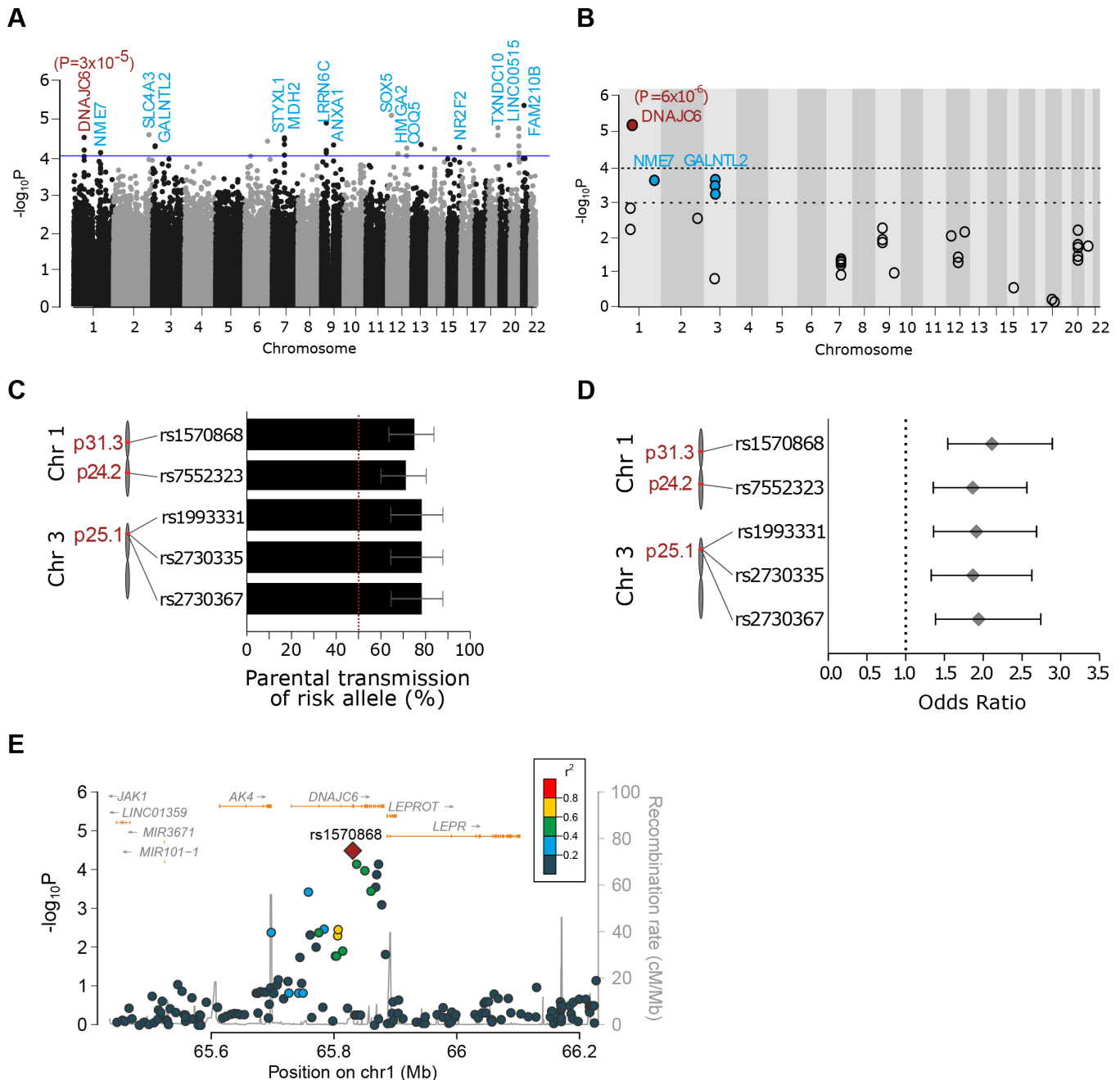
Analysing transmission of SNPs based on genotypes of index cases (n=92) and first-degree relatives (n=256) using the family-based association for disease trait (DFAM) method, we identified 32 polymorphisms associated with CHB at  $p \leq 1 \times 10^{-4}$  (figure 1A, online supplemental figure 1 and online supplemental table 2). Subsequent validation analysis of these 32 CHB-associated polymorphisms in a population-based case-control (C-C) set-up confirmed the association of the locus on chromosome 1p31.3 at a higher level of significance (rs1570868,  $P_{DFAM} = 3 \times 10^{-5}$  and  $P_{C-C} = 6 \times 10^{-6}$ ), and verified suggestive associations in two other genomic regions 1q24.2 (rs7552323,  $P_{DFAM} = 3 \times 10^{-5}$ ,  $P_{C-C} = 2 \times 10^{-4}$ ) and 3p25.1 (rs1993331,  $P_{DFAM} = 5 \times 10^{-5}$  and  $P_{C-C} = 3 \times 10^{-4}$ ; rs2730335,  $P_{DFAM} = 5 \times 10^{-5}$  and  $P_{C-C} = 5 \times 10^{-4}$ ; and rs2730367,  $P_{DFAM} = 5 \times 10^{-5}$  and  $P_{C-C} = 2 \times 10^{-4}$ ) (figure 1B and online supplemental table 3). Parental transmission of the risk alleles to the affected individuals was 75% (95% CI 63.6% to 83.8%) for rs1570868, 80% (95% CI 60% to 71%) for rs7552323 and 78% (95% CI 64.4% to 87.3%) for rs1993331, rs2730335, and rs2730367, respectively (figure 1C and online supplemental table 2). ORs for the same SNPs in the validation analysis were 2.01 (95% CI 1.50 to 2.81) for rs1570868, 1.82 (95% CI 1.33 to 2.49) for rs7552323 and ranged between 1.82 and 1.90 (95% CI 1.30 to 2.67) for rs1993331, rs2730335 and rs2730367 (figure 1D and online supplemental table 3). Closer examination of the associated locus on chr 1p31.3 revealed the highest association with intronic variants in *DNAJC6* (figure 1E, online supplemental table 2).

Expression quantitative trait loci (eQTL) analysis in cardiac tissue of the genes present in the regions surrounding the top replicating SNPs ( $\pm 500$  kb) revealed a significant effect of rs1570868 on the expression of the *DNAJC6* gene, but not on the expression of other genes in the chromosomal interval (figure 2A). Interestingly, individuals carrying the risk allele at this position had a lower cardiac *DNAJC6* expression compared with carriers of the non-risk allele (figure 2B). Notably, *DNAJC6* expression in other tested tissues was not affected by the rs1570868 polymorphism (figure 2C and data not depicted).

*DNAJC6* encodes the putative tyrosine-protein phosphatase auxilin, which is involved in clathrin-mediated endocytosis. Four protein-coding transcripts have been predicted for auxilin (figure 2D), and we could confirm expression of all four variants in cardiac tissue by qPCR (figure 2E, online supplemental figure 2). Auxilin-201 is the only transcript conserved between human and mouse, suggesting that it may be important functionally. Interestingly, analysis of transcript-specific auxilin expression according to the rs1570868 genotypes revealed that carriers of the CHB risk allele have a lower expression of auxilin-201 compared with carriers of the non-risk allele ( $p < 7 \times 10^{-4}$ ) (figure 2F). In contrast, cardiac expression levels of the three other transcript variants are not affected by the rs1570868 SNP.

### Auxilin is highly expressed in the fetal heart and colocalised with clathrin in vesicular structures in primary cardiomyocytes

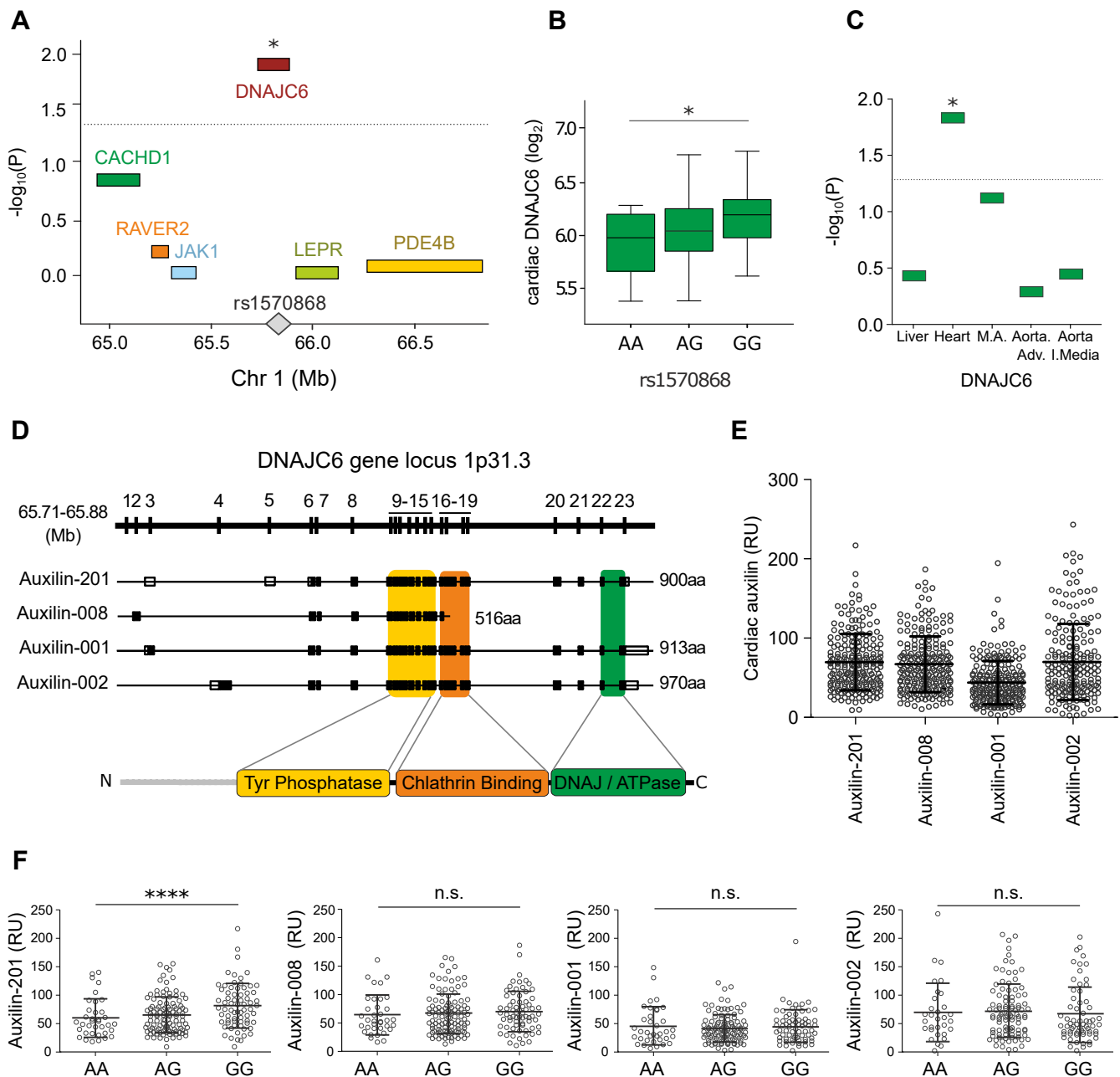
To address the functional basis for auxilin deficiency involvement in CHB, we first investigated whether auxilin is expressed in the heart during fetal development. We found that auxilin is indeed expressed in human fetal cardiac tissue both before and during the risk period for CHB development (figure 3A and online supplemental figure 3A–C). Interestingly, auxilin expression is remarkably higher in the fetal heart compared with the adult heart (figure 3A), as well as in comparison with other fetal tissues (figure 3B). Of note, cardiac tissue



**Figure 1** Identification of novel genomic loci associated with CHB. (A) Manhattan plot of genome-wide family-based association for disease trait (DFAM) transmission statistics based on SNP genotyping of CHB cases ( $n=92$ ) and first-degree relatives ( $n=256$ ). (B) Logistic regression analysis of association statistics for SNPs with  $P_{DFAM} \leq 1 \times 10^{-4}$  comparing CHB cases ( $n=89$ ) vs 1195 population-based out-of-study controls. Replicating polymorphisms with  $p \leq 1 \times 10^{-4}$  (red) and  $p \leq 1 \times 10^{-3}$  (blue). Approximate chromosomal positions are indicated. Dashed lines indicates  $p=1 \times 10^{-4}$  and  $p \leq 1 \times 10^{-3}$  (A, B). (C) Percentage of parental transmission to CHB cases (95% CI) for replicating risk variants (DFAM analysis). Dashed line indicates 50% transmission. (D) OR (95% CI) for replicating risk variants (logistic regression). Dashed line indicates OR=1.0. (E) LocusZoom (<http://locuszoom.org/>) plot of the associated *DNAJC6* region on chromosome 1. CHB, congenital heart block; SNP, single-nucleotide polymorphisms;

expression profiling not only confirmed high expression of auxilin in fetal heart but also revealed that the homologous cyclin-G associated kinase (GAK), also denoted auxilin-2, is expressed only at low levels in the fetal heart (figure 3C). This relation is reversed in adult cardiac tissue, where auxilin is expressed at lower levels than GAK (figure 3D), suggesting that lack of auxilin may specifically affect the fetal rather than the adult heart. Auxilin expression was confirmed at RNA expression level (online supplemental figure 3D-F) and the protein level by immunoblotting of human fetal cardiac tissue

and in cardiomyocytes (figure 3E-I). Ubiquitous expression of auxilin was observed throughout the fetal heart by immunohistochemistry (figure 3J), and these data were confirmed by similar auxilin expression levels in human fetal cardiac tissue surgically dissected from the apical myocardium and from the AV node (figure 3K and online supplemental figure 3G-I). Immunofluorescence staining of single cell preparations of human fetal cardiomyocytes demonstrated subcellular localisation of auxilin, which is present in the cytoplasm in a vesicular pattern and partly colocalises with clathrin (figure 3L).



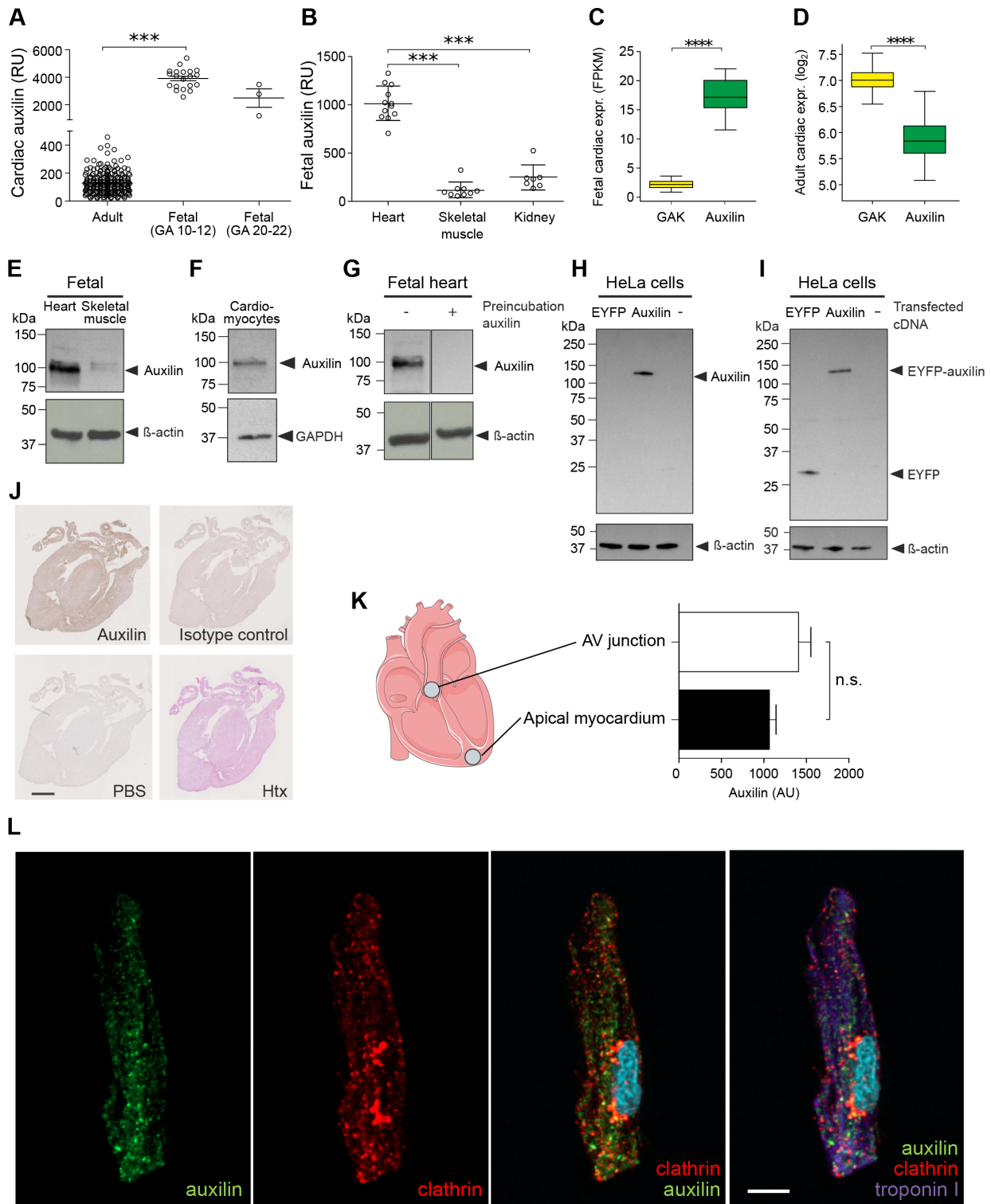
**Figure 2** Identification of *DNAJC6* (*auxilin*) as a novel fetal genetic risk factor for CHB. (A) Effect of rs1570868 genotype on cardiac expression of genes located within a 1 Mb interval centred on rs1570868 (chromosomal position 1p31.3). (B) Cardiac *DNAJC6* expression stratified by rs1570868 genotypes. Allele frequency: 0.416 (A) and 0.584 (G) (n=101),  $\beta=0.066$ . (C) Effect of rs1570868 genotype on *DNAJC6* expression in different tissues. Liver (n=151), heart (n=101), m.a.; mammary artery (n=88), aorta Adv.; aorta adventitia (n=90), aorta I. media; aorta intima media (n=104). An additive linear regression model was applied (A–C). Dashed line indicates  $p=0.05$ . (D) *DNAJC6* gene locus and predicted protein-coding auxilin transcript variants and functional domains. (E, F) Cardiac expression of auxilin transcript variants (E), stratified according to rs1570868 genotype (F), relative to cardiac  $\beta 2$ -microglobulin expression (n=217). Results are shown as mean $\pm$ SD. Linear regression analysis was applied. \* $P<0.05$ , \*\*\*\* $p<0.0001$ . n.s., not significant.

**Auxilin deficiency impairs cardiac cell connectivity and disturbs calcium homeostasis**

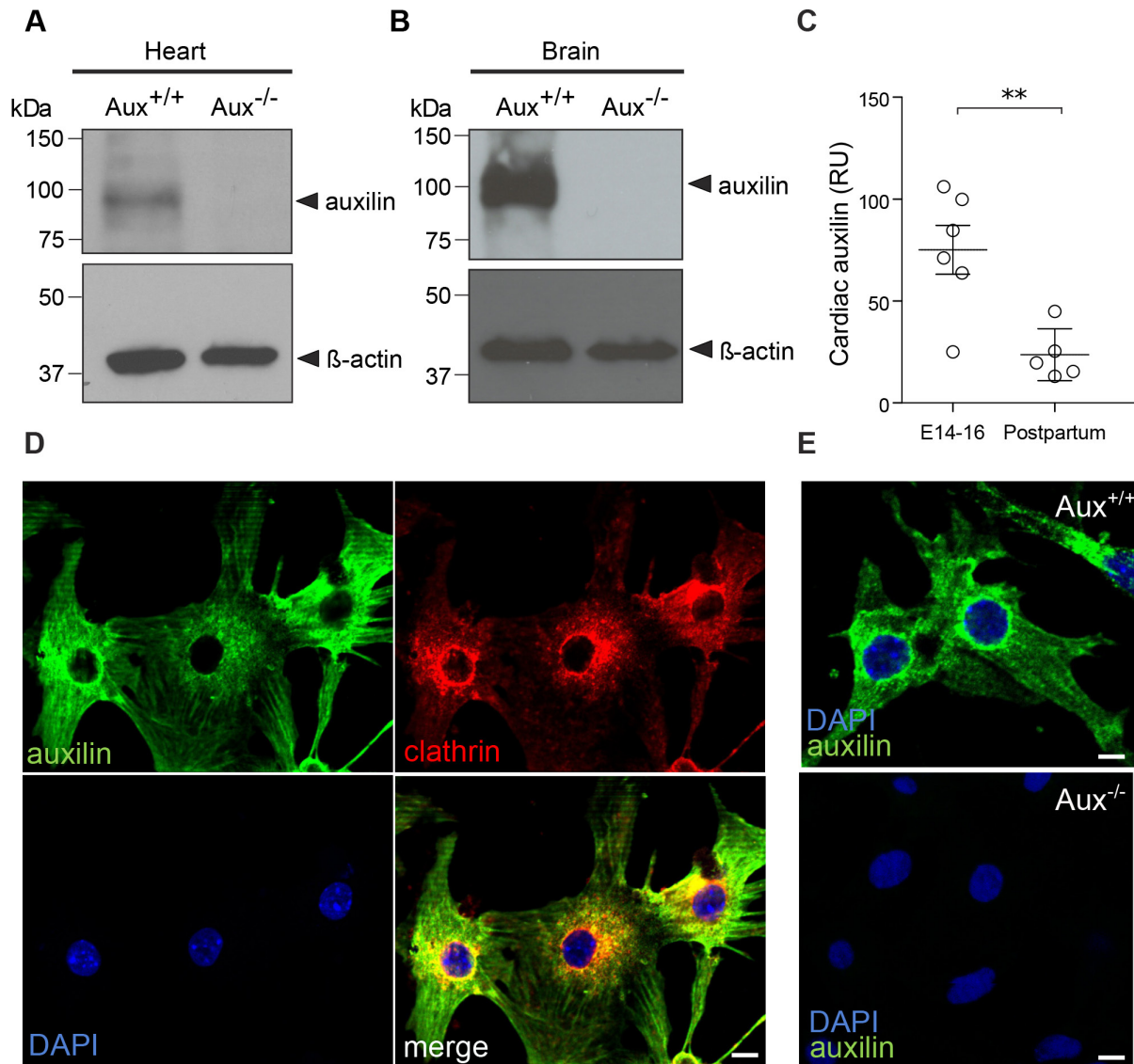
We next set out to investigate the role of auxilin in cardiac function using auxilin-deficient mice. We first confirmed that auxilin is indeed expressed in wild-type mouse neonatal heart (figure 4A), and also verified its described, high expression in mouse brain (figure 4B). In line with our findings of high fetal cardiac auxilin expression in human tissue, we observed that cardiac auxilin expression in the mouse also peaks in fetal heart

tissue (figure 4C and online supplemental figure 3J,K), and verified that auxilin localises to vesicular structures partly colocalising with clathrin also in cultured neonatal mouse cardiomyocytes (figure 4D,E).

$Ca^{2+}$  is one of the main regulators of cardiomyocyte function, and to evaluate the impact of auxilin deficiency on cardiomyocyte performance we analysed spontaneous ( $Ca^{2+}$ )<sub>i</sub> oscillations in primary cultures of wild-type and auxilin knockout neonatal cardiomyocytes using the calcium sensitive fluorescent dye



**Figure 3** Auxilin is expressed in the human fetal heart and co-localises with clathrin in primary cardiomyocytes. (A) Auxilin cardiac expression in human adult (n=217) and fetal tissue, gestational age (GA) 10–12 weeks (n=20) and 20–22 weeks (n=3). (B) Auxilin expression in human fetal heart (n=12), skeletal muscle (n=9), and kidney (n=7), GA 10–12 weeks. auxilin expression relative to β<sub>2</sub>-microglobulin expression (A, B). (C) Human cardiac expression of GAK and auxilin in fetal tissue, GA 10–12 weeks (n=32). (D) Human cardiac expression of GAK and auxilin in adult tissue (n=127). (E) Auxilin protein expression in human fetal heart and skeletal muscle. (F) Auxilin protein expression in human cardiomyocytes differentiated from iPS cells. (G) Antibody specificity verified by preincubation with recombinant auxilin before Western blot of human fetal heart. (H, I) Immunoblotting with anti-auxilin (H) and anti-EYFP (I) of HeLa cell lysates transfected with recombinant EYFP (EYFP) or EYFP-auxilin (auxilin) or untransfected (-). (J) ubiquitous expression of auxilin detected by immunohistochemistry in sections of paraformaldehyde-fixed, paraffin-embedded human fetal cardiac tissue, GA 12 weeks. Scale bar represents 1 mm. (K) Auxilin mRNA expression within the apical myocardial and AV junctional tissue after microdissection of human fetal hearts (n=6; gestational age 20–22 weeks). (L) Subcellular localisation of auxilin and clathrin in cultured primary human fetal cardiomyocytes, GA 22 weeks. Counterstain by DAPI to visualise the nucleus (cyan). Scale bar represents 7.9 μm. Results are shown as mean±SE; Mann-Whitney U test. \*\*\*p<0.001, \*\*\*\*p<0.0001. AV, atrioventricular;

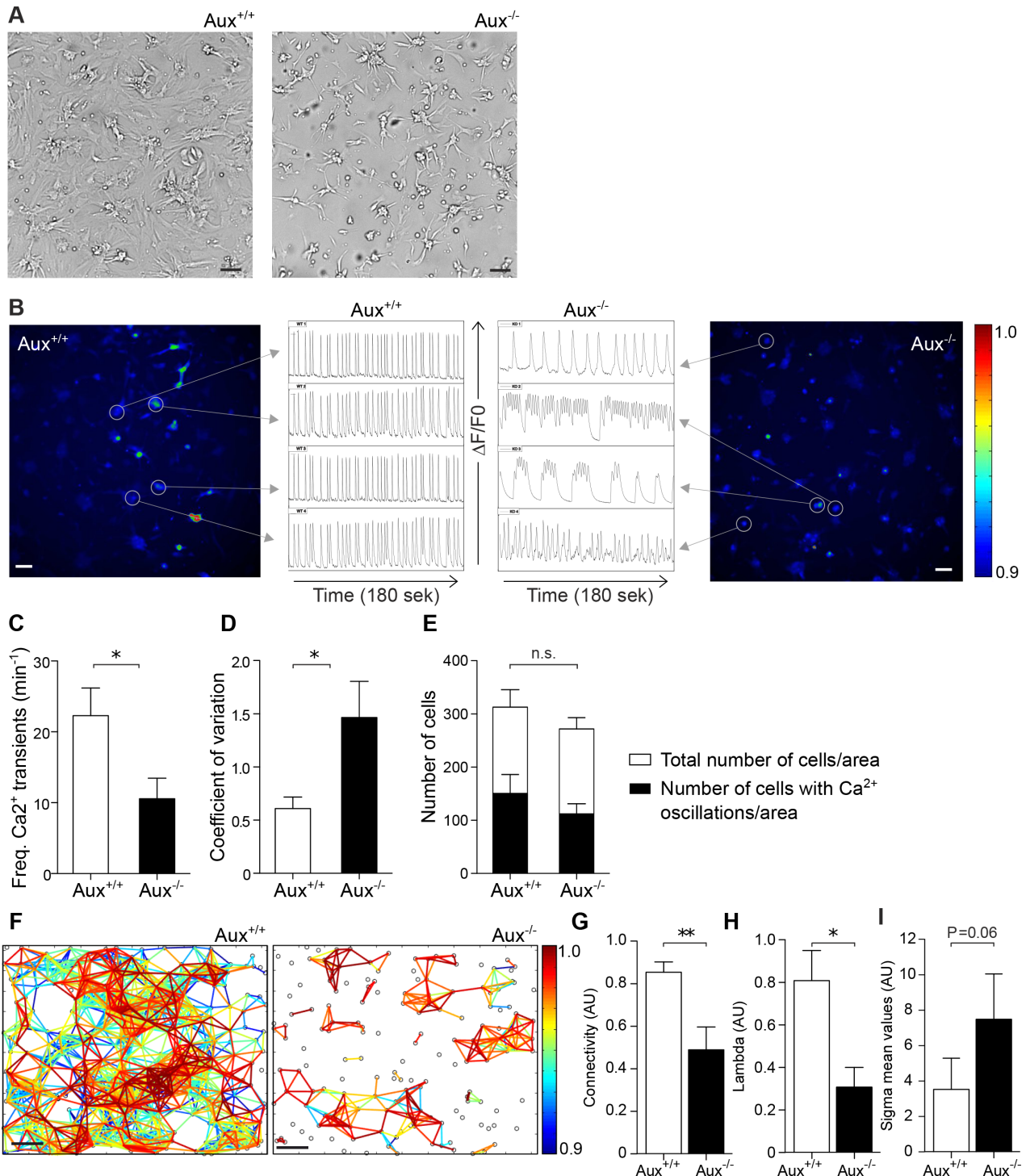


**Figure 4** Auxilin is expressed in the mouse neonatal and fetal heart and colocalises with clathrin in primary cardiomyocytes. (A, B) Auxilin protein expression in the heart (A) and brain (B) of neonatal mice. Upper panel: auxilin; lower panel:  $\beta$ -actin. (C) Cardiac RNA expression of auxilin in mouse fetuses E14-E16 (n=6) and pups post partum (n=5). Expression levels are relative to TAF8 expression. (D) Subcellular localisation of auxilin and clathrin in cultured primary neonatal mouse cardiomyocytes. Scale bar represents 20  $\mu$ m. (E) Immunofluorescence staining of cultured wild-type or auxilin knockout primary neonatal mouse cardiomyocytes using anti-auxilin antibody HPA031182, 1:200. Results are shown as mean $\pm$ SE; two-tailed Student's t-test, \*\*p<0.01.

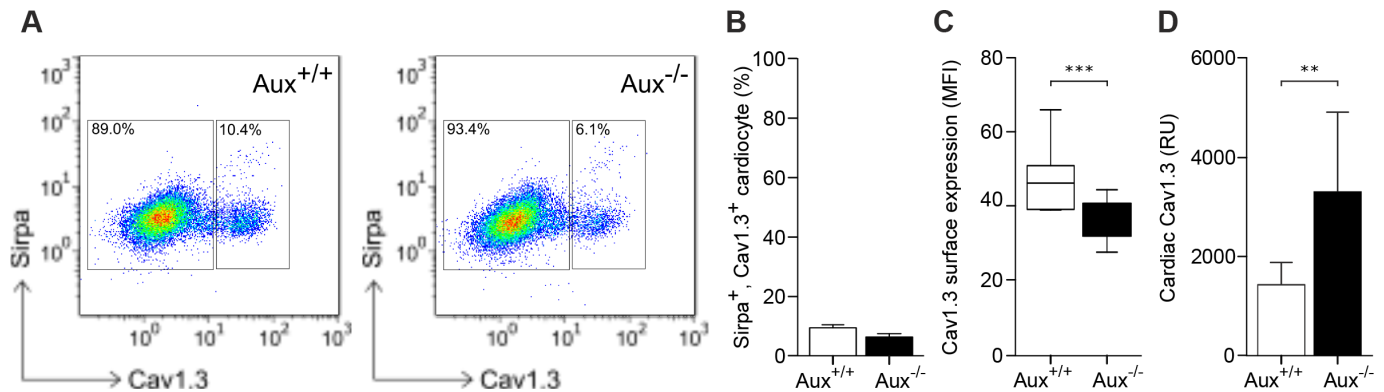
(Fluo-4-AM) and time lapse imaging. Auxilin-deficient cardiomyocytes presented a pronounced irregular oscillation pattern (figure 5A,B and online supplemental movies 1 and 2), with reduced mean frequency and increased variability of ( $\text{Ca}^{2+}$ )<sub>i</sub> oscillations compared with wild-type cells (figure 5C,D), though the total number or number of Ca-oscillating cells per area did not differ between wild-type and auxilin-deficient cultures (figure 5E). Strikingly, auxilin-deficient cells also appeared unable to organise into well-connected cellular networks in vitro, in contrast to wild-type cells (figure 5F). Cross-correlation analysis of cardiomyocyte ( $\text{Ca}^{2+}$ )<sub>i</sub> activity demonstrated lower connectivity among auxilin-deficient cells (figure 5G), as well as a decreased mean path length reflecting an impaired capacity to communicate with cells at greater distances (figure 5H). Auxilin-deficient cells were, however, capable of interacting in so-called small-world networks (figure 5I).

#### Auxilin deficiency leads to decreased cell surface expression of the calcium channel $\text{Ca}_v1.3$ on cardiocytes

Given the described role of auxilin in the clathrin-mediated endocytic process and our finding that auxilin-deficient cardiomyocytes display a disturbed calcium homeostasis, we hypothesised that absence of auxilin may impair the recycling of calcium channels to the plasma membrane of cardiomyocytes. Flow cytometry analysis of mouse neonatal *Sirpa*<sup>+</sup> cardiocytes revealed that the proportion of cells expressing the calcium channel  $\text{Ca}_v1.3$  was comparable in auxilin-deficient and wild-type mice (figure 6A and B), but that  $\text{Ca}_v1.3$  cell surface expression was significantly lower in *Sirpa*<sup>+</sup> $\text{Ca}_v1.3$ <sup>+</sup> auxilin-deficient cells compared with wild-type cells (p<0.01, figure 6C). Conversely, cardiac expression levels of  $\text{Ca}_v1.3$  RNA transcripts were significantly higher in auxilin-deficient neonatal mice compared with wild-type mice (figure 6D), indicating that decreased  $\text{Ca}_v1.3$  expression



**Figure 5** Auxilin deficiency causes impaired calcium homeostasis and decreased intercellular connectivity in neonatal primary cardiomyocytes. (A) Phase-contrast images of primary neonatal cardiomyocytes of wild type and auxilin knockout mice in cultured monolayers. (B) Time lapse images of  $(Ca^{2+})_i$  transients in spontaneously oscillating cardiomyocytes isolated from neonatal mice and loaded with Fluo4-AM. Examples of  $(Ca^{2+})_i$  recordings from individual cardiomyocytes are shown. Videos of these cultures presented in online supplemental movie 2. (C, D) Frequency of  $(Ca^{2+})_i$  oscillation transients and coefficient of variation in wild-type and knockout neonatal cardiomyocytes. Data are based on measurements from  $n=7$  (wild-type) and  $n=6$  (auxilin-knockout independent experiments with a mean of 165 cells analysed per experiment, each conducted with cells pooled from littermates ( $\geq 5$  pups)). (E)  $(Ca^{2+})_i$  transients measurements in neonatal cardiomyocyte cultures showing the number of cells with  $(Ca^{2+})_i$  transients per area and the total number of cells per area from wild-type vs auxilin knockout mouse pups. (F) Functional cell connection maps illustrating significantly correlated, thus connected, pairs of representative cultured cardiomyocytes. Multicoloured bar indicates correlation coefficient, with higher values representing a stronger correlation between the activities of the cells connected by the line. (G) Connectivity index in neonatal cardiomyocytes. (H) Lambda index representing the shortest mean path length in neonatal cardiomyocytes. (G–I) Representation of cardiomyocyte organisation into small-world networks in wild-type vs knockout cultures. Data are based on measurements from  $n=7$  (wild-type) and  $n=6$  (auxilin-knockout) independent experiments with a mean of 165 cell analysed per experiment, each conducted with cells pooled from littermates ( $\geq 5$  pups). Au: arbitrary units. scale bars 100  $\mu$ m. Results are shown as mean  $\pm$  SE; two-tailed Student's t-test, \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 6** Auxilin deficiency leads to decreased cell surface expression of the Ca<sub>v</sub>1.3 calcium channel in primary neonatal cardiomyocytes. (A, B) Flow cytometry analysis of wild-type and auxilin-deficient SIRPa and Ca<sub>v</sub>1.3 double-stained primary mouse neonatal cardiomyocytes. (C) Mean fluorescence intensity (MFI) of Ca<sub>v</sub>1.3 cell surface expression in SIRpa<sup>+</sup>Ca<sub>v</sub>1.3<sup>+</sup> primary neonatal cardiomyocytes from wild-type vs auxilin-deficient mice. Calculations are based on n=9 independent experiments per genotype with pooled cells from littermates (≥5 pups per experiment). (D) Cardiac RNA expression of Ca<sub>v</sub>1.3 in primary neonatal cardiomyocytes from wild-type (n=5) and auxilin-deficient mice (n=5). Expression levels are relative to cardiac TAF8 expression. Results are shown as mean±SEM or minimal and maximal values; Mann-Whitney test, \*\*p<0.01, \*\*\*p<0.001.

on the plasma membrane of auxilin-deficient cells was not due to a general decrease in expression, and further suggesting that auxilin-deficient cardiomyocytes upregulate the transcription of Ca<sub>v</sub>1.3 to compensate for decreased protein levels on the cell surface.

#### Auxilin-deficient mice display Chb abnormalities during fetal development

To address whether the lack of auxilin affects fetal heart function in vivo, we monitored developing mice in utero by Doppler echocardiography. Notably, we observed several different CHB-related cardiac pathologies in auxilin-deficient mice at the fetal stage (figure 7A–F, online supplemental movies 3 and 4). Both the AV-time and isovolumetric contraction time were prolonged in auxilin knockout fetuses compared with wild-type fetuses (figure 7G,H). Furthermore, auxilin-deficient fetuses displayed abnormal heart rates and arrhythmias, including frequent ectopic beats generated in the atria and/or ventricles (figure 7I,J and online supplemental table 4). Interestingly, the effect was gene-dosage dependent as heterozygous animals showed an intermediate phenotype (figure 7G–J). Of note, the number of ectopic beat observations among auxilin knockout animals peaked at gestational day 13 (figure 7K), which corresponds to the window of disease onset for CHB in humans. Importantly, the cardiac abnormalities we observed in auxilin-deficient mice in utero are similar to those observed in human fetuses with CHB, as exemplified by one of our recorded human fetal case presenting with ectopic tachycardia at gestational age 21 weeks (figure 7L,M), and progressing to CHB at gestational age 24 weeks (figure 7N,O, online supplemental movies 5 and 6).

#### DISCUSSION

Given the low recurrence rate of CHB despite the persistence of autoantibodies in the mothers, fetal genetic factors have been suggested to contribute to disease development. Here, we identify auxilin/*DNAJC6* as a novel fetal susceptibility gene for CHB and report that decreased cardiac expression associates with the disease genotype. We further demonstrate that auxilin under normal circumstances is highly expressed in the fetal heart, and that auxilin deficiency impairs cardiomyocyte performance in vitro and leads to cardiac CHB abnormalities in vivo, thereby directly linking a novel susceptibility gene with disease mechanism and providing a functional basis for how decreased

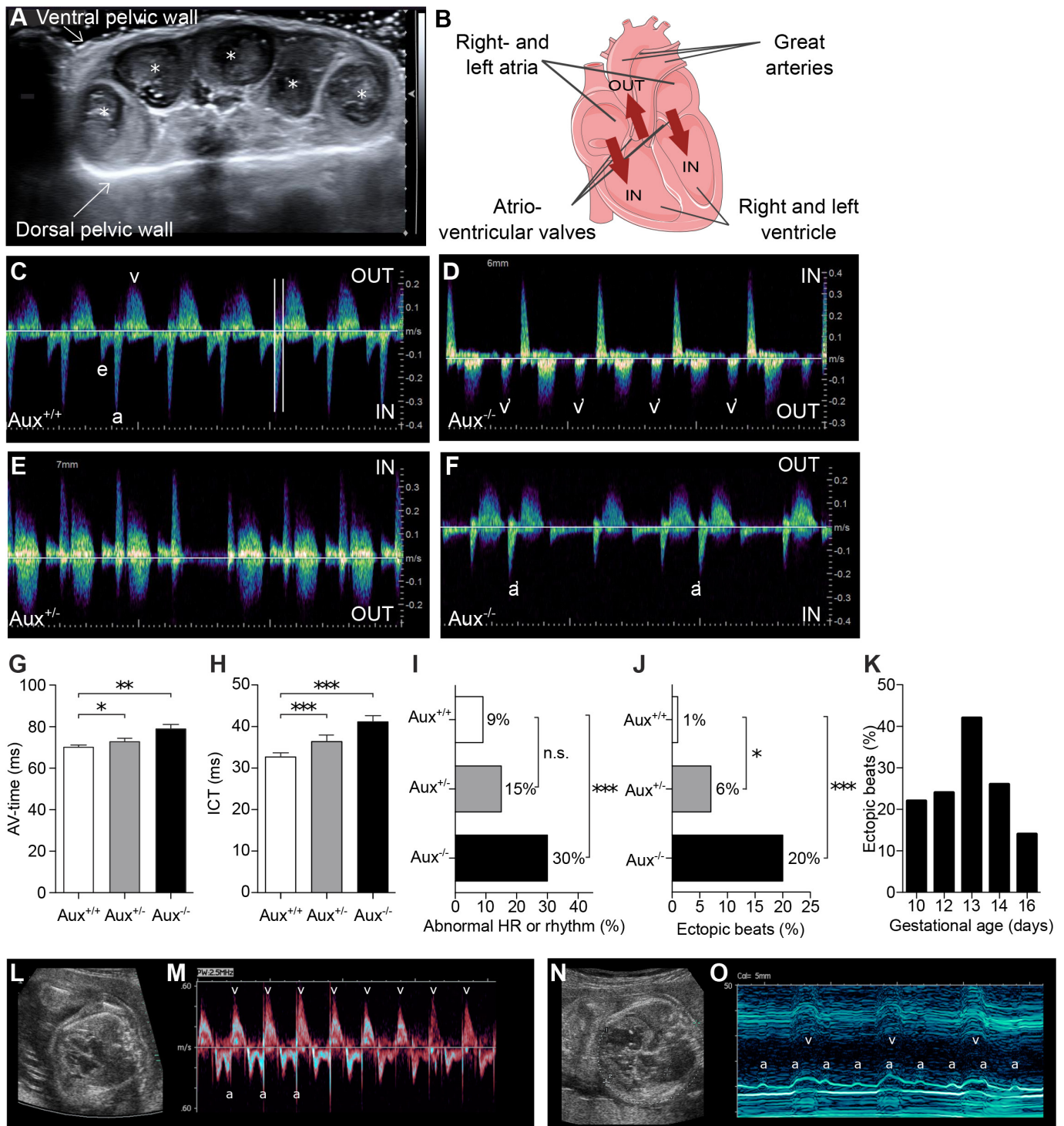
expression of auxilin may contribute to the development of CHB.

The majority of mothers of children with CHB carry autoantibodies to the Ro/SSA autoantigens and will thus have genetic traits reflecting their autoimmune status.<sup>25 26</sup> In order to segregate these potentially confounding maternal disease traits and identify genetic traits specific to CHB, we chose to perform a genome-wide association study using a family-based setup including individuals with CHB and their unaffected first-degree relatives. Interestingly, we found that the SNPs most significantly associated with CHB were located outside the human leucocyte antigen (HLA) region, in contrast to a previously published genome-wide association study in which the SNPs most significantly associated with CHB were found in the HLA region.<sup>27</sup> This discrepancy may be explained by the fact that the latter study<sup>27</sup> was based on a C-C set-up, and that its findings may therefore reflect inherited maternal traits linked to the autoimmune status of the mothers rather than CHB-specific disease traits.

Our family-based analysis strategy uncovered several CHB-specific polymorphisms across the whole genome, and additional validation of association combined with cardiac eQTL analysis identified auxilin/*DNAJC6* as the primary candidate for a susceptibility gene for CHB. This prompted us to further investigate the auxilin expression pattern. Indeed, auxilin expression and function had mainly been described in neuronal tissue,<sup>28 29</sup> and its potential involvement in heart function was unknown. Surprisingly, we found that auxilin was not only expressed in the heart, but that its levels were also markedly higher in fetal compared with adult cardiac tissue. In addition, we observed that the homologous protein GAK, which has been suggested to act as a functional substitute for auxilin,<sup>30</sup> was expressed only at low levels in the fetal heart, but at higher levels than auxilin in the adult heart. These findings, therefore, provide a rationale as to why a decreased expression of auxilin would be associated with a fetal cardiac phenotype.

Auxilin operates in the clathrin-mediated endocytic process,<sup>31</sup> and absence of auxilin may therefore impair the recycling of ion channels or other molecules important for cardiac function to the plasma membrane of cardiomyocytes. This in turn could explain the lower cellular connectivity and communication as well as the decreased and less well-coordinated Ca<sup>2+</sup> oscillations we observed in auxilin-deficient cardiomyocytes.





**Figure 7** Auxilin deficiency causes cardiac abnormalities in vivo during fetal development. (A) Transsectional abdominal ultrasound view showing individual mouse fetuses (\*) in utero. (B) Illustration of spatial and directional relationships between ventricular inflows and outflows registered in (C–F). (C–F) Echocardiographic Doppler flow velocity recordings from wild-type and auxilin-deficient fetuses, with cardiac inflow (IN) through atrioventricular valves and outflow (OUT) in the great arteries. (C) Normal recording showing two-peaked inflow with early passive e-wave (e), higher a-wave (a), and ventricular outflow (v). Vertical lines denote one AV-time interval. (D) Ventricular ectopic beats (VES) (v') in bigeminy. (E) Mobitz type II, second-degree AV-block. (F) Conducted premature ectopic supraventricular beats (SVES) (a'). (G, H) mechanical AV-time interval and isovolumetric contraction time (ICT), Kruskal-Wallis and Dunn's post hoc tests. Results are shown as mean $\pm$ SE. (I, J) Proportion of fetuses with abnormal heart rate (HR) or rhythm (I), or with ectopic beats (SVES, VES) (J),  $\chi^2$  test. Auxilin<sup>+/+</sup> (n=147), auxilin<sup>+/-</sup> (n=89), and auxilin<sup>-/-</sup> (n=131) fetuses (G–J). (K) percentages of auxilin<sup>-/-</sup> fetuses with ectopic beats according to gestational age. Percentages are calculated based on the total number of auxilin<sup>-/-</sup> fetuses at each gestational age. I–o a human fetal case of junctional ectopic tachycardia progressing to CHB. (L, M) normal appearing heart with ectopic tachycardia at gestational age (GA) 21 weeks. (N, O) Complete CHB at GA 24 weeks with bradycardia and dilated echogenic heart. \*P<0.05, \*\*p<0.01, \*\*\*p<0.001.

In support of this hypothesis, we found that auxilin-deficient cardiac cells displayed lower levels of the calcium channel  $\text{Ca}_v1.3$  on their plasma membrane compared with wild-type cells. Interestingly,  $\text{Ca}_v1.3$ -deficient mice have been reported to exhibit cardiac abnormalities such as sinus bradycardia and AV block at birth,<sup>32,33</sup> suggesting that decreased expression of  $\text{Ca}_v1.3$  on the surface of auxilin-deficient cells may contribute in part to the cardiac abnormalities we observed in auxilin-deficient mice in utero. Indeed, we show here that auxilin-deficient mice develop cardiac abnormalities in utero similar to early CHB manifestations, such as ectopic beats, arrhythmias and prolongation of the AV time. AVBII/III was observed, but the occurrence did not reach statistical significance.

Calcium channels, including  $\text{Ca}_v1.3$ , have been described as potential targets of autoantibodies from mothers of children with CHB,<sup>34–36</sup> and maternal antibodies were reported to inhibit  $\text{Ca}_v1.3$  calcium currents in exogenous expression systems.<sup>34</sup> A genetically determined lower cardiac auxilin expression, resulting in decreased calcium channel presence on the cell surface, may thus synergize with the inhibitory effect of maternal autoantibodies to further diminish  $\text{I}_{\text{Ca,L}}$  current density and overall cardiomyocyte performance. Interestingly, fetal cardiomyocytes do not yet possess a fully developed sarcoplasmic reticulum, and the excitation-contraction coupling is thus largely dependent on cell surface calcium channels.<sup>37,38</sup> By contrast, adult cardiac cells rely mainly on sarcoplasmic calcium stores. Decreased expression of auxilin resulting in lower surface expression of calcium channels would therefore have a larger impact on cardiomyocyte function in the fetal heart than in the adult heart, rendering fetal cardiac cells particularly susceptible to the pathogenic effects of maternal antibodies while maternal cardiac cells are left relatively unaffected. This in turn might begin to explain why, despite the presence of circulating autoantibodies, cardiac manifestations similar to CHB are not detected in mothers of children with CHB.

Considering the role of auxilin in the clathrin-mediated endocytosis process, it is probable that auxilin deficiency may affect the presence of many different molecules to the plasma membrane of cardiac cells. Here, we limited our investigation to the  $\text{Ca}_v1.3$  calcium channel as a proof of concept; however, it is likely that auxilin deficiency alters the surface expression also of other molecules, such as other ion channels or connexins involved in cardiac function, which in turn may contribute to CHB development. In addition, although we here focus on auxilin/*DNAJC6* as a susceptibility gene for CHB, it is likely that other genetic variants may contribute directly or indirectly to cardiomyocyte performance and hence also affect susceptibility to CHB. However, CHB is a rare disease in the general population, occurring in about 1 in 20 000 births,<sup>21</sup> and establishing large cohorts of patients that would enable the detection of many different risk variant combinations remains a challenge. The main limitations of this study are linked to this rarity of the studied condition, and includes the employed threshold at  $p \leq 1 \times 10^{-4}$  for the family-based association for disease traits and the lack of a replication cohort of patients with CHB.

In all, we identify auxilin/*DNAJC6* as a novel susceptibility gene for CHB and demonstrate a previously unreported role for auxilin in fetal cardiac function, revealing in particular that auxilin is necessary for cardiac cells to maintain normal calcium homeostasis and establish functional networks. The disease-associated genetic variant, leading to decreased auxilin expression, may thus affect fetal myocardial function, both mechanically and electrically, as part of CHB. The involvement of maternal autoantibodies in CHB has long been recognised,

especially regarding the establishment of inflammation and subsequent scarring of the AV node.<sup>39,40</sup> However, the mechanisms underlying the early phases and cardiac manifestations of NLE other than complete AV block remain unclear.<sup>41</sup> Importantly, we show here that auxilin-deficient mice develop cardiac abnormalities in utero similar to CHB manifestations such as prolonged AV time interval and isovolumetric contraction time, ectopic beats and arrhythmias, and provide a mechanistic basis as to how lack of auxilin may underlie such features at the molecular and cellular level. Identification of auxilin/*DNAJC6* as a susceptibility gene for CHB that directly impacts cardiac function therefore begins to elucidate the tissue-dependent pathogenic mechanisms involved in CHB. This, in turn, shifts the focus from solely trying to prevent the pathogenic effects of maternal antibodies and instead taking into account intrinsic cardiac defects affecting fetal heart function, thus opening the road to conceiving new screening and therapeutic strategies for this often lethal condition.

#### Author affiliations

<sup>1</sup>Department of Medicine, Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden

<sup>2</sup>Technical University of Denmark, Lyngby, Denmark

<sup>3</sup>Department of Women's and Children's Health, Karolinska Institutet, Stockholm, Sweden

<sup>4</sup>Department of Physiology and Experimental Medicine, Hospital for Sick Children, Washington, DC, USA

<sup>5</sup>Department of Clinical Neuroscience, Karolinska Institutet, Stockholm, Sweden

<sup>6</sup>Pediatric Cardiology, Hospital for Sick Children, Toronto, Ontario, Canada

<sup>7</sup>Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

<sup>8</sup>National Institutes of Health, Bethesda, Maryland, USA

<sup>9</sup>Evergrande Center for Immunologic Diseases, Harvard Medical School, Boston, Massachusetts, USA

<sup>10</sup>Broegelmann Research Laboratory, Department of Clinical Science, University of Bergen, Bergen, Norway

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**Collaborators** The Swedish congenital heart block study group: Aurelie Ambrosi, Amanda Skog Andreasson, Gunnar Bergman, Håkan Eliasson, Eva Fernlund, Fredrik Gadler, Anders Jonzon, Ingrid Kockum, Linda Lagnefeldt, Malin Hedlund, Mats Mellander, Sabrina Meisgen, Johannes Mofors, Vijole Ottosson, Annika Rydberg, Stina Salomonsson, Thomas Skogh, Sven-Erik Sonesson, Elke Theander, Joanna Tingström, Guđný Ella Thorlacius, Marie Wahren-Herlenius, Annika Öhman.

**Contributors** SM and MW-H designed the study with input from MH, AA, VKK, EH, AE, IK and S-ES. Members of TSCHBSG, AF-C, AH, TO, PE, JM and KG-D assisted in identification and characterisation of patients and controls. LG and RMH provided study material. SM, MH, VO, LB, LS, SR, LM, WN and S-ES performed the experiments. SM, MH, LF, GET, VO, DR, DF, LB, JM, EH, IK and S-ES analysed the data. SM, AA, GET and MW-H wrote the manuscript, and all authors participated in manuscript preparation until its final form. MW-H is the study guarantor.

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#### ORCID iD

Marie Wahren-Herlenius <http://orcid.org/0000-0002-0915-7245>

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