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Jagged1 intracellular domain-mediated inhibition of Notch1 signalling regulates cardiac homeostasis in the postnatal heart

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Aims Notch1 signalling in the heart is mainly activated via expression of Jagged1 on the surface of cardiomyocytes. Notch controls cardiomyocyte proliferation and differentiation in the developing heart and regulates cardiac remodelling in the stressed adult heart. Besides canonical Notch receptor activation in signal-receiving cells, Notch ligands can also activate Notch receptor-independent responses in signal-sending cells via release of their intracellular domain. We evaluated therefore the importance of Jagged1 (J1) intracellular domain (ICD)-mediated pathways in the postnatal heart.

Methods and results In cardiomyocytes, Jagged1 releases J1ICD, which then translocates into the nucleus and down-regulates Notch transcriptional activity. To study the importance of J1ICD in cardiac homeostasis, we generated transgenic mice expressing a tamoxifen-inducible form of J1ICD, specifically in cardiomyocytes. Using this model, we demonstrate that J1ICD-mediated Notch inhibition diminishes proliferation in the neonatal cardiomyocyte population and promotes maturation. In the neonatal heart, a response via Wnt and Akt pathway activation is elicited as an attempt to compensate for the deficit in cardiomyocyte number resulting from J1ICD activation. In the stressed adult heart, J1ICD activation results in a dramatic reduction of the number of Notch signalling cardiomyocytes, blunts the hypertrophic response, and reduces the number of apoptotic cardiomyocytes. Consistently, this occurs concomitantly with a significant down-regulation of the phosphorylation of the Akt effectors ribosomal S6 protein (S6) and eukaryotic initiation factor 4E binding protein1 (4EBP1) controlling protein synthesis.

Conclusions Altogether, these data demonstrate the importance of J1ICD in the modulation of physiological and pathological hypertrophy, and reveal the existence of a novel pathway regulating cardiac homeostasis.

Keywords Cardiac hypertrophy • Cardiomyocyte differentiation • Notch signalling • Jagged1-intracelullar domain

1. Introduction

The Notch pathway is an evolutionarily conserved communication system between adjacent cells expressing Notch receptors (Notch1–4) and membrane-bound ligands of the Jagged (Jagged 1,2) or Delta-like (Dll1,3,4) family.[1] Notch signalling is crucial for cardiac morphogenesis during development. In particular, Notch regulates myocyte proliferation in the trabeculate myocardium and valve formation.[2] Furthermore, Notch controls proliferation and differentiation both in committed cardiac progenitor cells and in immature cardiomyocytes.[3–6] Importantly, the Notch pathway is also essential for the maintenance of structural and functional integrity of the postnatal heart.[7–11] In the adult heart, Jagged1-mediated Notch signalling is activated upon damage and limits the extent of the hypertrophic response in stressed cardiomyocytes.[7,11] On the contrary, pharmacological and genetic inhibition of Notch signalling exacerbates cardiac hypertrophy following increased workload.[7] Accordingly, transgenic (TG) mice overexpressing Jagged1 on the surface of cardiomyocytes demonstrated a limited hypertrophic and fibrotic response to pressure overload, and cardiac precursor expansion.[11] Along the same line, forced activation

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of the Notch pathway in the infarcted heart following induced cardiomyocyte-specific Notch1 expression or intramyocardial delivery of a Notch1 pseudoligand increased survival, improved cardiac function, and minimized fibrosis.[8,9]

Canonical Notch signalling is initiated in the signal-receiving cell by interaction of Notch receptors with their ligands. This event triggers the sequential cleavage of the Notch receptor by A Disintegrin and Metalloproteinase (ADAM) and a γ-secretase complex, and then the release of the Notch intracellular domain (NICD), which enters the nucleus, interacts with RBP-Jκ and other co-activators to activate target gene expression.[1] Prototypical target genes of Notch are transcriptional repressors of the Hes and Hey family. Importantly, endocytosis of the ligand in the signal-sending cell is required for induction of Notch receptor proteolysis.[12] Indeed, ubiquitylation-dependent internalization of the ligand is thought to exert a pulling force on the receptor to expose its cleavage sites and thereby promote enzymatic cleavage and downstream activation. For this reason, truncated forms of Jagged and Delta, lacking ubiquitylation-sensitive intracellular domains, are unable to activate Notch signalling.[13,14]

Interestingly, Notch ligands can also be cleaved in signal-sending cells by the ADAM metalloprotease and the γ-secretase complex following ligand–receptor interaction.[15–18] This process induces the release of an intracellular fragment of the ligand, which then translocates into the nucleus. This raises therefore the interesting possibility that ligand activation could produce cellular responses within signal-sending cells. In this context, the Jagged1 intracellular domain (J1ICD) has been shown to regulate cell transformation and proliferation.[15,19] Similarly, the nuclear translocation of X-Serrate-1ICD in *Xenopus* was shown to inhibit primary neurogenesis.[20] Finally, Delta-like 1 ICD production was demonstrated to block proliferation, cell motility, and neuronal migration,[21,22] independently of its activity as a Notch receptor ligand.[23,24]

Given the crucial role of Notch signalling in cardiac homeostasis, we evaluated therefore the importance of Jagged1-mediated pathways in postnatal cardiomyocyte differentiation and growth. We demonstrated that Jagged1 is indeed cleaved in cardiomyocytes and produces a J1ICD fragment, which can be detected in the nucleus of responding cells. To investigate Jagged1-mediated effects in the heart, we therefore generated TG mice carrying a cardiomyocyte-specific and inducible form of J1ICD. In the neonatal heart, J1ICD accelerates the maturation of cardiomyocytes, in part via inhibition of the Notch signalling pathway. In the adult heart, J1ICD production modulates hypertrophic remodelling during the response to pressure overload. Altogether, these data suggest an important role for Notch ligand-mediated signalling in the developing and the adult heart.

2. Methods

For detailed methods, see Supplementary material online.

2.1 Transgenic (TG) mER-J1ICD-mER mice

The rat J1ICD was isolated from the pTOPO-J1ICD plasmid (kindly provided by Matthew J. LaVoie, Harvard Medical School, Boston, USA), and fused at its C- and N-terminal ends to amino acids 281–599 of the mutated (G525R) mouse oestrogen receptor (mER; a kind gift from Michael Reth, Max-Plank Institute, Freiburg, Germany). The resultant fragment was subcloned downstream of the α-myosin heavy chain (α-MHC) promoter. The α-MHC-mER-J1ICD-mER construct was linearized and injected into the male pronucleus of fertilized B6D2F1 embryos. The resulting TG mice were backcrossed into the C57/BL6 background strain. Nuclear translocation of the transgene was induced by intraperitoneal (i.p.) injection of 20 mg/kg of tamoxifen (Sigma, Saint Louis, MO). In some experiments, mice were injected with MG132 i.p. at 7 mg/kg.

2.2 Animal experiments

Mice were housed under standard conditions. Ten-week-old mice were anaesthetized by i.p. injection of ketamine/xylazine/acepromazine (65/15/2 mg/kg body weight). Mice were placed on a warm pad for maintenance of body temperature. Transaortic constriction (TAC) was created using a 7.0-silk suture tied twice around the aorta between the right innominate artery and the left common carotid and a predetermined-gauge needle size (25 gauge). The needle was then gently retracted leaving a restriction of the same calibre among different mice. For animals undergoing a sham operation, the ligature was placed in an identical location but was not tied. Animals were injected daily with 20 mg/kg of tamoxifen i.p. (Sigma) or vehicle (95% ethanol and sunflower oil only). Adult mice were sacrificed by CO_2 inhalation and subsequent cervical dislocation. Neonatal mice were sacrificed by rapid decapitation. Animal experiments were approved by the Government Veterinary Office (Lausanne, Switzerland) and performed according to the guidelines from Directive 2010/63/EU of the European Parliament.

2.3 Echocardiography

Transthoracic echocardiography was performed using a 30 MHz probe and the Vevo 770 Ultrasound machine (Visualsonics, Toronto, ON, Canada) under mild anaesthesia with 1–1.5% isoflurane. Diastolic and systolic internal ventricular septum (IVSd and IVSs), diastolic and systolic left ventricular free posterior wall thickness (LVPWd and LVPWs), and left ventricular internal end-diastolic and end-systolic chamber (LVIDd and LVIDs) dimensions were measured three times on M-mode images. LV fractional shortening (%FS) and ejection fraction (%EF) were also calculated. The right ventricle internal diameter and wall thickness were also measured on parasternal short-axis view, using papillary muscles as reference points.

2.4 Statistical analysis

In this study, data are expressed as mean ± SEM. One-way ANOVA with Benferroni's multiple comparison test was used to assess significance of differences between experimental groups. Two-sided Student's *t*-test was used when only two groups are compared. *P* values of <0.05 were considered as significant. Analysis was performed using the GraphPad Prism version 5.04 (GraphPad Software, Inc.).

3. Results

3.1 J1ICD inhibits canonical Notch activation in cardiomyocytes

Evidence suggested that membrane-bound Notch ligands released intracellular fragments within signal-sending cells to activate Notch receptor-independent cellular responses. Since Jagged1 was demonstrated to be the predominant form expressed in the heart, we examined whether J1ICD could be produced in primary cardiomyocytes. Jagged1 and J1ICD were immunoprecipitated from neonatal cardiomyocyte lysates using antibodies directed against the C-terminal end of the protein. We indeed identified a 20 kDa fragment corresponding to the expected size for J1ICD (*Figure 1A* and *B*). This fragment accumulated following proteasome inhibition using MG132. Furthermore, J1ICD release was blocked by the γ-secretase inhibitor, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), demonstrating the involvement of γ-secretase-mediated Jagged1 cleavage in J1ICD production. J1ICD was also produced in the postnatal and adult heart as well as in isolated adult cardiomyocytes (*Figure 1C*). We next determined in which cellular compartments, Jagged1 and J1ICD,

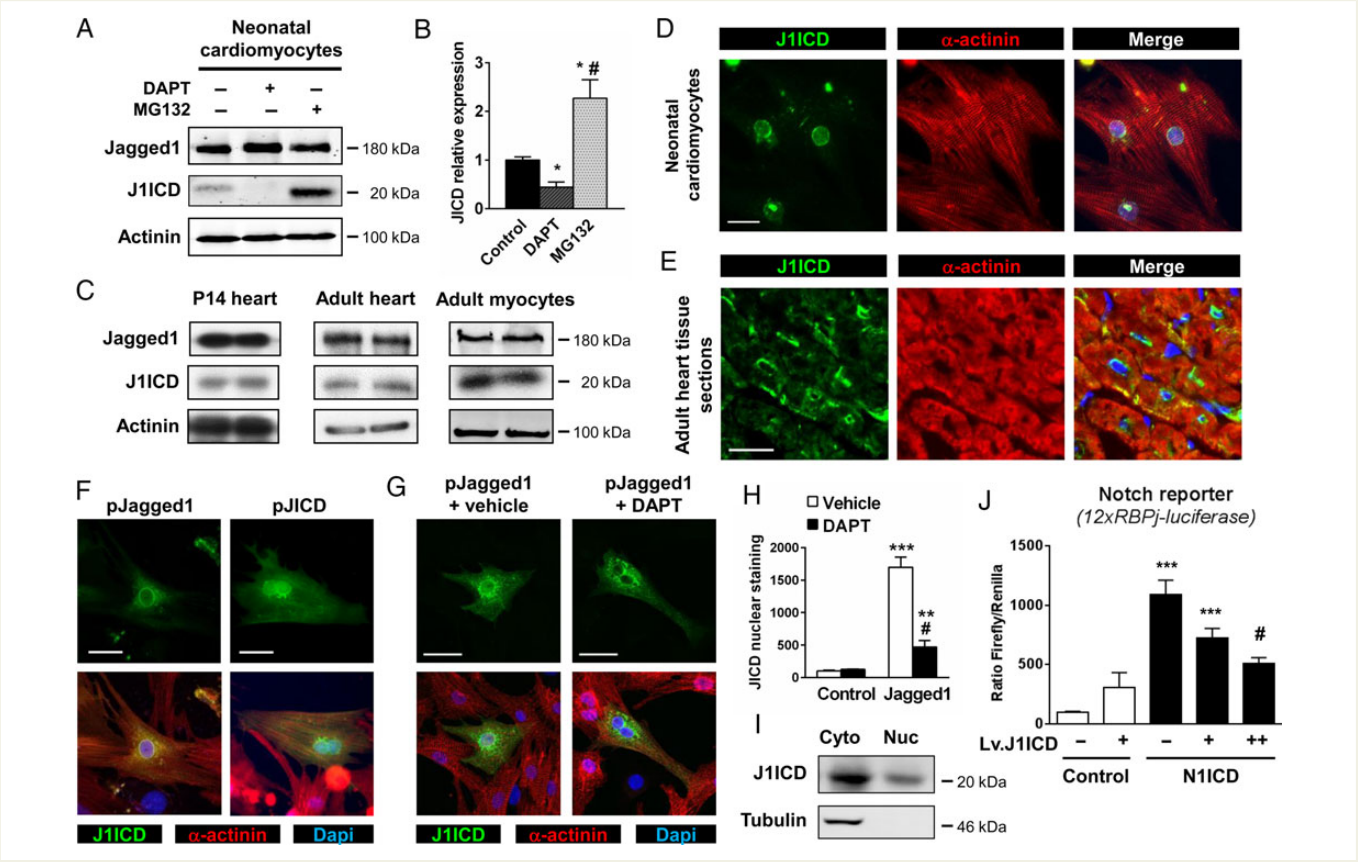

Figure 1 Jagged1 cleavage in cardiomyocytes produces J1ICD. (*A*) Neonatal cardiomyocytes were treated with either DAPT (10 μM) or MG132 (10 μM) for 12 h. Immunoprecipitated full-length Jagged1 and J1ICD proteins were detected by western blotting. Anti-α-actinin immunoblot was performed as a loading control. (*B*) J1ICD levels were quantified by densitometry. $^{*}P \leq 0.05$ vs. vehicle, $^{\#}P \leq 0.05$ vs. DAPT + MG132. (*C*) Immunoprecipitated Jagged1 and J1ICD in postnatal hearts of P14 and adult mice, and isolated adult mice cardiomyocytes. (*D*) Neonatal cardiomyocytes or (*E*) adult heart sections from Jagged1-overexpressing mice were stained with anti-Jagged1 (green) and anti-α-sarcomeric-actinin antibodies (red). Nuclei were stained with DAPI (blue). Scale bar, 20 μm. (*F*) Cardiomyocytes were transfected with either pcDNA-Jagged1 or pcDNA-J1ICD vector and stained as in *D*. Images in *D*–*F* are representative of four-independent experiments. (*G*) Confocal images of Jagged1-expressing cells treated with either vehicle or DAPT (10 μM) for 12 h. (*H*) Nuclear J1ICD staining obtained in *G* was quantified and normalized to the nucleus area. Bar graphs are mean ± SEM from three experiments. $^{**}P \leq 0.01$; $^{***}P \leq 0.001$ vs. control; $^{\#}P \leq 0.001$ vs. Jagged1 + DAPT. (*I*) J1ICD immunoprecipitation in cytoplasmic and nuclear fractions of neonatal myocytes. (*J*) Neonatal cardiomyocytes were transfected with either pcDNA (control) or pcDNA-NICD together with a Notch reporter (12xRBPJk-luciferase) and an internal control (pCMV-Renilla). Cells were then infected for 24 h with different amounts of a J1ICD-expressing lentiviral vector (Lv.J1ICD) or a control vector as indicated. Notch transcriptional activity was determined by quantification of luciferase (Firefly) and Renilla luminescence. Data are mean ± SEM from three experiments. $^{***}P \leq 0.001$ vs. control; $^{\#}P \leq 0.01$ vs. N1ICD.

were expressed. In untransfected cardiomyocytes, antibodies directed against J1ICD, which recognize both full-length Jagged1 and J1ICD, marked the cytoplasm as well as the perinuclear and nuclear regions (*Figure 1D*). To further analyse subcellular localization, we took advantage of TG mice overexpressing Jagged1 specifically in cardiomyocytes, which were recently established in our laboratory (*Figure 1E*).[11] Analysis of heart sections stained with antibodies against J1ICD confirmed the presence of the fragment at the membrane, in the cytoplasm, and in the perinuclear region. Then, we transfected cardiomyocytes *in vitro* with either a Jagged1 or a J1ICD encoding plasmid (*Figure 1F*). Following Jagged1 or J1ICD overexpression, J1ICD was readily detected in all three cellular compartments. Furthermore, nuclear localization was more apparent in cells transfected with J1ICD, supporting translocation of this fragment into the nucleus. Importantly, nuclear staining is markedly diminished in Jagged1-overexpressing cardiomyocytes treated with DAPT, indicating that J1ICD production and translocation were dependent on γ-secretase-mediated cleavage of full-length Jagged1 (*Figure 1G* and *H*). Using purified cytoplasmic/membrane and nuclear fractions from neonatal cardiomyocytes and immunoblotting against J1ICD, we further demonstrated the presence of J1ICD in both compartments (*Figure 1I*). We next evaluated the effects of J1ICD on Notch signalling in isolated cardiomyocytes. Notch transcriptional activity was measured using a RBP-Jκ-responsive luciferase (12xRBP-Jκ-Luc) reporter gene. Therefore, neonatal cardiomyocytes were transfected with the 12xRBP-Jκ-Luc reporter, which was induced by forced Notch1 intracellular domain (N1ICD) expression. Cardiomyocytes were then infected with increasing amounts of a lentiviral vector encoding J1ICD (Lv.J1ICD). Lv.J1ICD had no impact on basal luciferase activity. In contrast, Lv.J1ICD significantly decreased N1ICD-induced transcriptional activity in a dose-dependent manner (*Figure 1J*), indicating that J1ICD produced an important inhibitory effect on Notch signalling.

3.2 TG mice with inducible cardiomyocyte-specific J1ICD expression

To investigate the effects of Jagged1 signalling on postnatal cardiac growth and function, we generated an inducible form of J1ICD, in which J1ICD was fused to two mutant oestrogen receptor hormone-binding domains to generate the mER-J1ICD-mER fragment. The mER sequences in the construct confer tamoxifen-mediated inducibility in cardiomyocytes and prevent potential detrimental effects of transgene expression during development. The suitability of the system was

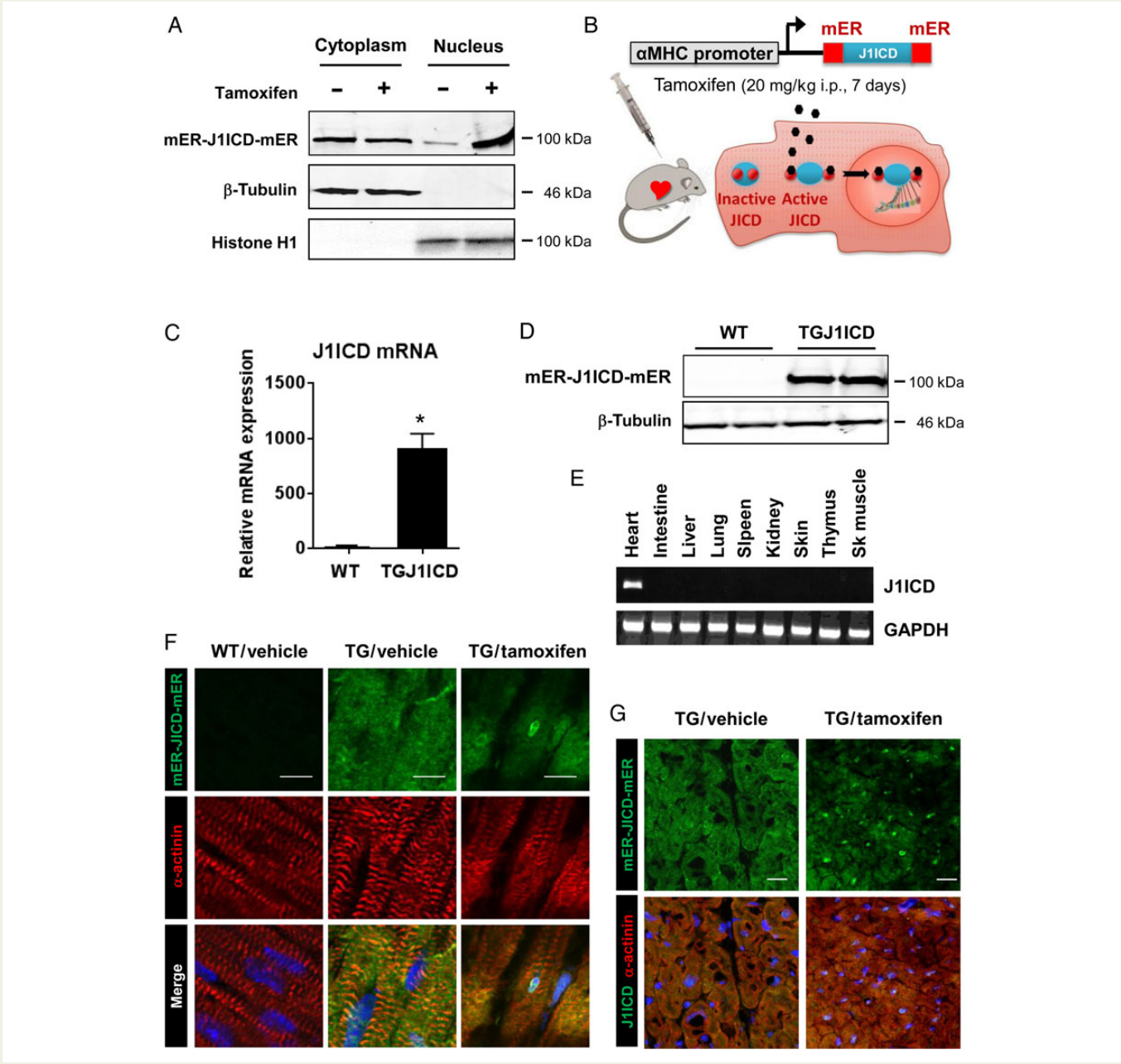

Figure 2 TG mice with cardiac-specific expression and inducible activation of J1ICD. (*A*) HEK 293 cells transfected with pEF1α-mER-J1ICD-mER were treated with 4-OH-tamoxifen (1 μM) or DMSO. Cytoplasmic and nuclear fractions were separated and assessed for J1ICD expression by western blot. Anti-α-tubulin and anti-histone H1 immunoblots were performed as loading controls. (*B*) Schematic representation of the α-MHC:mER-J1ICD-mER cDNA used to generate TGJ1ICD mice (top). In the absence of tamoxifen, the mER-J1ICD-mER fusion protein forms a complex with HSP proteins and is sequestered in the cytosol. Tamoxifen injections release mER-J1ICD-mER and induce its nuclear translocation (bottom). (*C*) Quantitative RT-PCR analysis of the transgene expression. Results were normalized to endogenous Gapdh and Jagged1 expression and were expressed as mean ± SEM ($n = 10–15$ mice per group; $^*P \leq 0.01$). (*D*) Anti-Jagged1 immunoblot performed on whole heart extracts from WT or TGJ1ICD mice. (*E*) RT-PCR specific for the transgene and Gapdh gene were performed on different organs of TGJ1ICD mice to show the cardiac-specific expression of the transgene. (*F* and *G*) Heart sections from WT or TGJ1ICD mice, treated with vehicle or tamoxifen for 48 h, were immunostained with anti-Esr1 antibody, which recognizes the mER motif of the transgene (green) and anti-α-sarcomeric-actinin antibody (red). Nuclei were stained with DAPI (blue). Scale bar, 10 μm (*F*) and 20 μm (*G*). Images in *F* and *G* are representative of at least five mice per group.

then tested in transfected HEK 293 cells. As expected, the mER-J1ICD-mER fusion protein was found primarily in the cytoplasm in vehicle-treated cells, whereas it was predominantly detected in the nuclear fraction in cells treated with tamoxifen (*Figure 2A*). We next generated TG mice expressing mER-J1ICD-mER under control of the cardiomyocyte-specific α-MHC promoter (herein TGJ1ICD; *Figure 2B*). Three different TG lines were initially produced. Characterization demonstrated no obvious phenotypic difference between these lines. We present therefore data obtained using one particular line. Significant cardiac expression of the transgene was readily detected by RT-PCR and western blot in TG mice (*Figure 2C* and *D*), and its expression was restricted to the heart (*Figure 2E*). Similarly, immunostaining of heart sections from TGJ1ICD mice showed that J1ICD was sequestered in the cytoplasm of cardiomyocytes in the absence of tamoxifen, whereas tamoxifen administration allowed the TG protein to enter the nucleus (*Figure 2F*). Of note, all cardiomyocytes demonstrated nuclear staining after tamoxifen treatment (*Figure 2G*).

3.3 J1ICD accelerates cardiomyocyte maturation *in vitro* via inhibition of Notch signalling

We first evaluated the importance of J1ICD on neonatal cardiomyocyte proliferation and maturation. Ventricular cardiomyocytes from neonatal wild-type (WT) and TGJ1ICD mice were therefore isolated and cultured in the presence of tamoxifen. We first analysed the effects of tamoxifen-induced J1ICD activation on Notch signalling. Immunostaining against the activated form of Notch1 (N1ICD) was used to detect α-actinin-positive cardiomyocytes actively signalling via the Notch1 receptor (see Supplementary material online, *Figure S1A*). Quantitative analysis showed a smaller percentage of N1ICD-positive cardiomyocytes in the population derived from TGJ1ICD TG mice following tamoxifen treatment (see Supplementary material online, *Figure S1B*), confirming that nuclear translocation of J1ICD inhibited Notch signalling. Inhibition of Notch signalling was also demonstrated by the significant down-regulation of Notch target gene expression, Hes1 and Hey2, in TGJ1ICD cardiomyocytes compared with WT cultures (see Supplementary material online, *Figure S1C*). Down-regulation of the Notch pathway has been associated with differentiation and maturation in cardiomyocytes. Interestingly, there was a greater proportion of cardiomyocytes with striated α-actinin in TGJ1ICD vs. WT cultures (see Supplementary material online, *Figure S1A* and *B*). This observation is consistent with J1ICD activation promoting cardiomyocyte differentiation via Notch signalling inhibition. Finally, we determined the number of proliferating neonatal cardiomyocytes after incorporation of the nucleoside analogue, 5-bromo-2-deoxyuridine (BrdU). Fewer BrdU-positive cardiomyocytes were present in TGJ1ICD cultures (see Supplementary material online, *Figure S1D* and *E*). Overall, these data indicated that J1ICD has inhibitory effects on Notch signalling, leading to decreased myocyte proliferation and accelerated cardiomyocyte maturation.

3.4 Accelerated cardiomyocyte maturation in neonatal TGJ1ICD mice following J1ICD activation

To assess whether J1ICD had an impact on cardiomyocyte maturation *in vivo*, neonatal WT and TGJ1ICD mice received daily injections of tamoxifen from birth to postnatal day 14 (P14), i.e. during the time period in which cardiomyocytes gradually exit from the cell cycle.[25,26] As observed in isolated cells, Notch signalling was down-regulated in cardiomyocytes in the neonatal TG myocardium (*Figure 3A* and *B*). Indeed, the number of N1ICD-positive cardiomyocytes was significantly lower in the heart of TGJ1ICD vs. WT mice at P7 and P14, respectively. Importantly, the expression of the Notch target genes, such as Hey1, Hey2, and Hes1, was significantly decreased in TGJ1ICD hearts when compared with WT. Jagged1 expression was also down-regulated in TG hearts, whereas expression of the Notch1 receptor was similar to what observed in WT (*Figure 3C*). Interestingly, the inhibitory effects of J1ICD activation on Notch signalling were particularly evident at P7. Previous studies suggested that NICD could be regulated via targeted ligand intracellular domain-mediated degradation.[27,28] To investigate whether the negative regulation exerted by J1ICD on Notch signalling occurred via a similar mechanism, anti-J1ICD immunoprecipitation was performed from WT and TGJ1ICD heart lysates, and the presence of J1ICD and N1ICD in immunoprecipitates was revealed by immunoblotting (*Figure 3D*). N1ICD co-immunoprecipitated with J1ICD in WT hearts, indicating that J1ICD physically interacts with N1ICD. The amounts of N1ICD were significantly reduced in extract from TGJ1ICD mouse hearts, consistent with J1ICD-mediated N1ICD degradation. Indeed, N1ICD was protected from degradation in TG hearts in the presence of the ubiquitin proteasome inhibitor, MG132.

We then followed cardiac growth in WT and TGJ1ICD mice following tamoxifen administration, and found that the heart in TGJ1ICD mice was characterized by a significantly different growth curve than that observed in WT animals (*Figure 4*). Specifically, the TG hearts demonstrated a slight increase in ventricular mass at P7, but a decreased weight and dimensions at P14 (*Figure 4A* and *B*). Importantly, cardiomyocyte apoptosis was not increased in 2-week-old TGJ1ICD mice, as detected using a TUNEL assay, excluding that cell death participates in the reduction of cardiac mass in TG hearts (data not shown). Cardiomyocytes from TGJ1ICD mice were found significantly larger at P4 and P7 but smaller at P14 (*Figure 4C*). Since cardiomyocyte differentiation and maturation are usually inversely correlated with proliferation, we evaluated cardiomyocyte mitosis in neonatal WT and TGJ1ICD hearts. At P4, the number of PH3-positive cardiomyocytes was reduced in TG hearts when compared with WT (*Figure 4D* and *E*). These findings were confirmed using BrdU incorporation to detect proliferating cardiomyocytes (*Figure 4F*). When a single dose of BrdU was administrated, the number of BrdU-positive cardiomyocytes was found lower at P4 in TG vs. WT hearts. These data are consistent with J1ICD-activated cardiomyocytes having an increased propensity to exit the cell cycle and to mature during the first days after birth. In agreement with this, the decreased ratio between expression of the fetal (β-MHC) and adult (α-MHC) isoforms of the MHC in TG hearts at P4 demonstrated early maturation (see Supplementary material online, *Figure S2B*).

3.5 Compensatory induction of the Wnt and AKT signalling pathways in the neonatal heart upon J1ICD activation

Although J1ICD-mediated pathways reduced cardiomyocyte proliferation during the first week of age, TGJ1ICD mice eventually demonstrated a compensatory increase in cardiomyocyte proliferation. Indeed, cardiomyocyte mitosis decreased rapidly after birth in WT hearts as assessed by the number of PH3-positive cells and by BrdU incorporation (*Figure 4E* and *F*). In contrast, the J1ICD-activated cardiomyocyte population was characterized by a rebound in

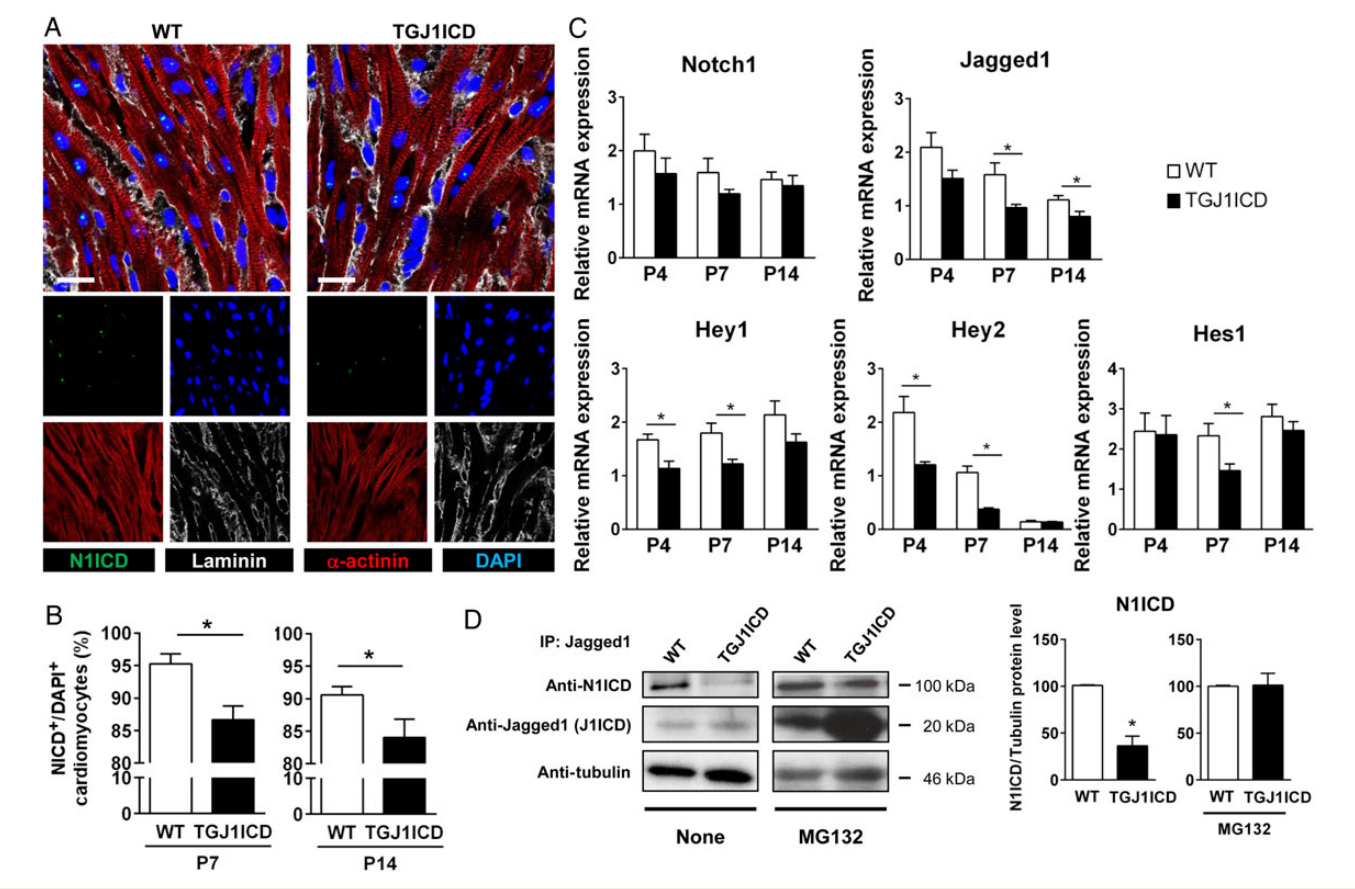

Figure 3 Notch activation is decreased in neonatal TGJ1ICD mice. WT and TGJ1ICD neonatal mice treated daily with tamoxifen were sacrificed at postnatal days P0, P4, P7, and P14. (*A*) Confocal images of heart sections immunostained against N1ICD (green), laminin (red), and α-sarcomeric-actinin (white). Nuclei were stained with DAPI (blue). Scale bar, 20 μm. (*B*) The number of NICD-positive cardiomyocytes at P7 and P14 was quantified on images depicted in *A*, and expressed as % of total DAPI-positive cardiomyocytes ($n = 4–6$ mice per group). (*C*) RNA expression of Notch target genes was analysed by quantitative RT-PCR. Results, normalized to Gapdh, represent fold change relative to the WT group at P0 and are expressed as mean ± SEM ($n = 4$ mice per group and per time point; $^*P \leq 0.05$). (*D*) Tamoxifen-treated mice were injected or not with MG132 from P9 to P14. J1ICD immunoprecipitation was performed using anti-Jagged1 antibody on whole heart extracts. Immunoprecipitates were immunoblotted with anti-N1ICD, anti-Jagged1 to detect J1ICD, and anti-tubulin antibodies. A quantification of N1ICD band intensity relative to tubulin is shown on the right panel ($n = 5–6$ mice per group). Bar graphs are mean ± SEM. $^*P \leq 0.05$ vs. WT.

proliferative activity at P7. Sustained cardiomyocyte proliferation in the TG group was also confirmed by a significant up-regulation of cyclin B1 and cyclin D1 mRNA expression during the recovery period (see Supplementary material online, *Figure S2A*). Finally, the β- to α-MHC ratio was increased at P14, attesting the more immature phenotype of proliferating cardiomyocytes (see Supplementary material online, *Figure S2B*). These findings suggested that cardiomyocytes with reduced Notch activity secondary to transgene activation could activate compensatory signalling pathways to stimulate myocyte division.

The Wnt pathway has been implicated in cardiogenesis[29] and is known to interact with the Notch pathway to regulate cell fate decision during cardiac differentiation.[30–32] In particular, canonical Wnts, including Wnt3A, Wnt6, Wnt8, and β-catenin signalling, negatively influence cardiac specification and terminal cardiac differentiation.[33–38] On the other hand, the non-canonical Wnt pathway is known to promote cardiac differentiation.[39–41] We thus measured the expression of different Wnt ligands and Wnt regulators, specific of both canonical and non-canonical Wnt signalling, in neonatal WT and TGJ1ICD mice. Expression levels were determined at P7 during the peak of the compensatory proliferative response (*Figure 5A*). Wnt3A and Wnt8 levels were undetectable, and Wnt6 was not significantly changed. However, the Wnt inhibitors, such as Dkk1, Axin2, and Wif1, were significantly down-regulated in TG at P7, consistent with an activation of canonical Wnt signalling in TG hearts during the proliferative phase. To confirm activation of the Wnt pathway at P7, we determined the amounts of activated (dephosphorylated) and total β-catenin in WT vs. TGJ1ICD heart extracts by immunoblotting (*Figure 5B* and *C*). The ratio of active to total β-catenin was increased in TGJ1ICD hearts, supporting a compensatory response via the Wnt pathway in neonatal TG mice. Finally, the expression level of the Wnt inhibitors Sfrp1 and Sfrp2 was unchanged. Moreover, Wnt11, a component of the non-canonical Wnt pathway, was down-regulated (*Figure 5A*). This is coherent with the immature state of TG cardiomyocytes around P14. Overall, these data suggest that cardiac canonical and non-canonical Wnt pathways were regulated in a way to counterbalance J1ICD-mediated Notch signalling inhibition. Interestingly, the expression of Wnt ligands and Wnt inhibitors as well as the active/total

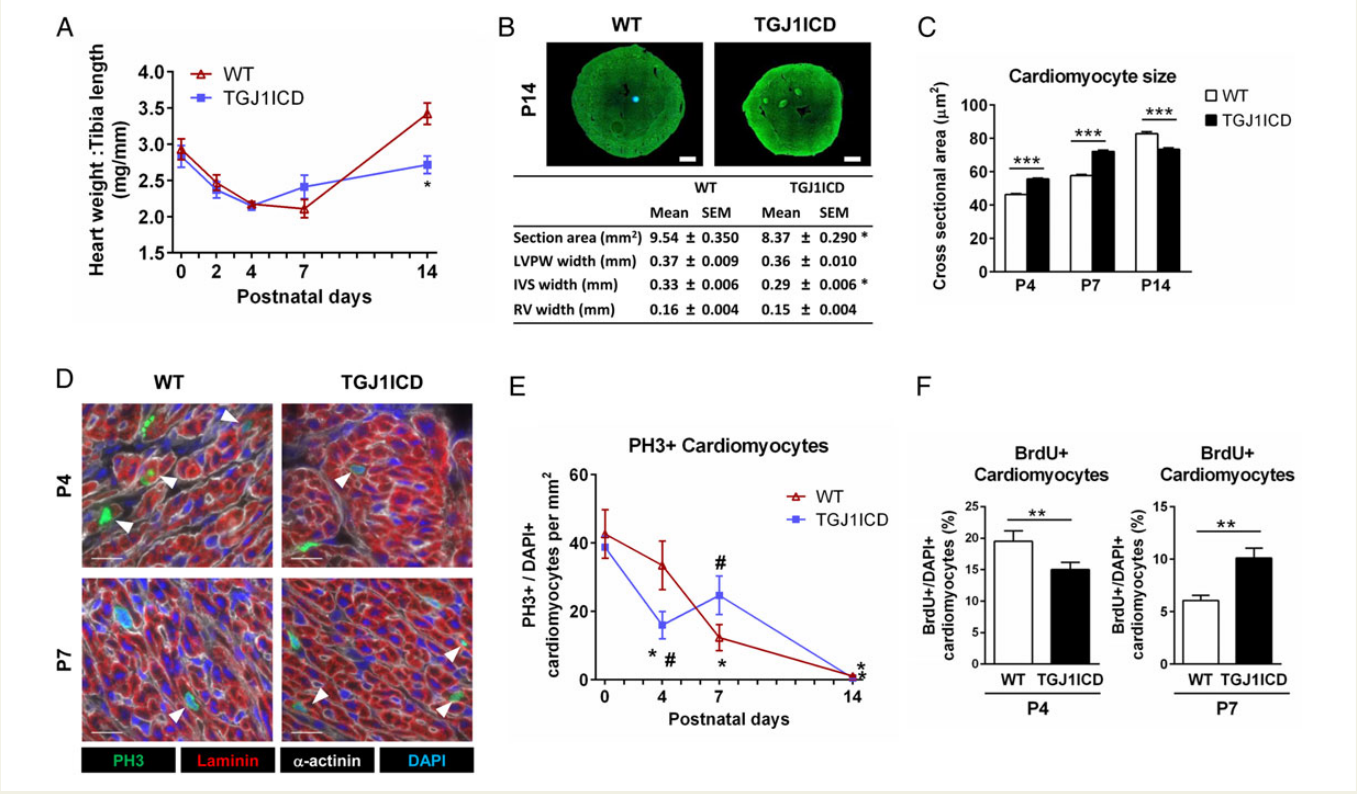

Figure 4 Altered cardiomyocyte maturation in neonatal TGJ1ICD mice following J1ICD activation. TGJ1ICD neonatal mice treated daily with tamoxifen were sacrificed at postnatal days P0, P2, P4, P7, and P14. (*A*) The ventricular weight to tibia length ratio of WT and TGJ1ICD mice. (*B*) Heart sections of 2-week-old mice (top) were used to measure cardiac dimensions (bottom). LVPW: left ventricular posterior wall; IVS: interventricular septum; RV: right ventricle. Scale bar, 0.5 mm. (*C*) Cross-sectional areas of ≥1000 myocytes per group were measured on laminin-stained heart sections. *B* and *C*; $n = 6$ mice per group. $^{*}P \leq 0.05$, $^{***}P \leq 0.01$ vs. WT. (*D*) Heart sections were immunostained against PH3 (green), α-sarcomeric-actinin (red), and laminin (white). Nuclei were stained with DAPI (blue). Arrows indicate PH3-positive cardiomyocytes. Scale bar, 20 µm. (*E*) Quantification of PH3-positive cardiomyocytes ($n = 4–8$ mice per group; $^{*}P \leq 0.05$ vs. P0, $^{\#}P \leq 0.05$ vs. WT). (*F*) Mice received BrdU at P4 and P7, and were analysed for BrdU incorporation at P14. Bar graphs are mean ± SEM. $n = 4–5$ mice per group. $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ vs. WT.

β-catenin ratio were not altered in TGJ1ICD mice at P4 (see Supplementary material online, *Figure S3*), suggesting that positive regulation of the Wnt pathway observed at P7 occurred secondary to J1ICD-mediated Notch signalling inhibition.

The mTOR/Akt signalling pathway also plays an important role in physiological proliferation and growth in the postnatal heart.[42] We measured therefore the activity of this pathway 1 week after birth and observed a significant increase of Akt phosphorylation at Thr308 and Ser473 in TG hearts, which demonstrated full activation of Akt at this particular time point. Consistently, phosphorylation of Akt downstream targets, namely ribosomal S6 protein (S6) and eukaryotic initiation factor 4E binding protein1 (4EBP1), was increased in TGJ1ICD mice (*Figure 5D* and *E*).

3.6 Attenuated cardiac hypertrophic response to pressure overload in adult TGJ1ICD mice following J1ICD activation

To evaluate the impact of J1ICD activation in the adult heart, WT and TGJ1ICD mice received daily injections of tamoxifen during 1 week. However, echocardiographic analysis of cardiac dimensions and function did not show any differences between WT and TGJ1ICD mice (see Supplementary material online, *Table S1*). Although Notch signalling can be readily detected in isolated adult cardiomyocytes and in the adult WT heart, Notch signalling was unaffected by J1ICD activation under normal conditions (see Supplementary material online, *Figure S4*). These findings are not surprising, given the fact that the Notch pathway is minimally activated in the unstressed adult heart. We therefore subjected the adult heart to pressure overload using the TAC model (*Figure 6*). In this case, the number of N1ICD-positive cells increased in the WT heart under stress, indicating that the Notch pathway was induced. In contrast, stimulated Notch activity was blunted in TGJ1ICD mice, as assessed by the lower number of N1ICD-positive cells in the stressed TG heart following tamoxifen-induced J1ICD activation (*Figure 6A* and *B*). We then evaluated the hypertrophic response in the stressed WT and TGJ1ICD hearts. Compared with WT mice, the development of cardiac hypertrophy was significantly attenuated in TGJ1ICD mice in response to pressure overload as assessed by a decreased LV mass to tibia length ratio (*Figure 6C*; see Supplementary material online, *Table S1*). Echocardiographic analysis also showed reduced septal and ventricular wall thicknesses, and suggested improved function in TGJ1ICD mice after TAC (*Table 1*). Importantly, limited hypertrophy in TGJ1ICD hearts after TAC was associated with a significant reduction in cardiomyocyte size (*Figure 6A* and *D*). Finally, the number of apoptotic cells was also reduced (*Figure 6E*). Cardiac markers of stress were, however, not different in the two genotypes

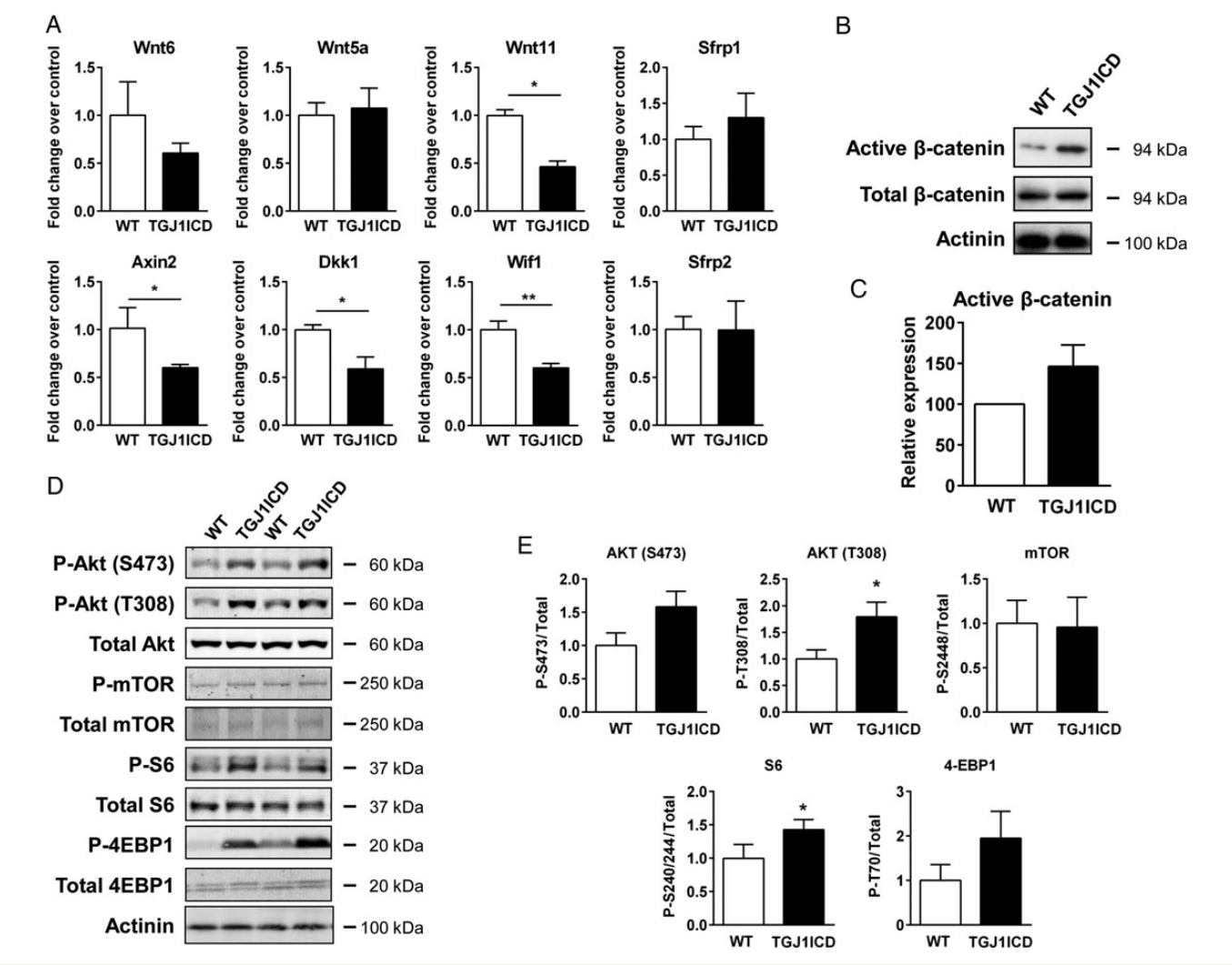

Figure 5 J1ICD-mediated Notch inhibition affects AKT and Wnt signalling pathways. WT and TGJ1ICD neonatal mice were treated daily with tamoxifen until postnatal day P7. (*A*) Quantitative RT-PCR analysis of Wnt ligands and Wnt inhibitors. Results, normalized to Gapdh, represent fold change relative to WT. (*B*) The active (dephosphorylated) and the total form of β-catenin were analysed by western blot. An immunoblot anti-α-actinin is shown as a loading control. (*C*) The active-β-catenin immunoblot was quantified and normalized to total β-catenin. (*D*) Western blot analysis of AKT and its downstream targets. (*E*) Bands intensities were quantified by densitometry and the ratios of the phosphorylated to total signals were normalized to the intensity of the WT group. (*A*, *C*, and *E*) Bar graphs are mean ± SEM ($n = 5–7$ mice per group; $^*P \leq 0.05$ vs. WT).

after TAC (see Supplementary material online, *Figure S5*). Quantification of collagen content by Masson trichrome staining, and measurement of fibrosis-associated gene expression, showed that TGJ1ICD mice developed a normal fibrotic response after TAC (*Figure 6F* and *G*). Altogether, these results indicated that J1ICD activation limited cardiomyocyte hypertrophy and apoptosis induced by pressure overload in adult TGJ1ICD mice without affecting fibrosis.

The Akt pathway promotes cardiomyocyte proliferation and survival in the postnatal heart.[43] Furthermore, Akt also regulates the development of cardiac hypertrophy in adulthood through activation of mTOR and its effector proteins, S6 and 4EBP1.[44–47] Therefore, the Akt pathway has been implicated in the stimulation of cardiomyocyte growth and survival during the response of the heart to stress. Indeed, pressure overload in WT mice induced phosphorylation of Akt, mTOR, S6, and 4EBP1, all components of the mTOR complex 1 (mTORC1) pathway controlling protein synthesis (*Figure 7*). Consistent with J1ICD activation having a negative action on cardiac hypertrophy, phosphorylation of S6 and 4EBP1 was abolished in TGJ1ICD mice after TAC. Interestingly, this occurred concomitantly with a phosphorylation of Akt at T308. To identify at which level the pathway was regulated, we measured the expression of a number of regulators, among them Phosphatase and Tensin homolog (PTEN), phosphoinositide-dependent protein kinase-1 (PDK1), Proviral Integration Site 1 (Pim-1), Tuberous Sclerosis Complex (TSC)1, and TSC2. However, none of them demonstrated different expression between the two genotypes (data not shown), indicating that the inhibitory effects on S6 and 4EBP-1 phosphorylation occurred downstream mTORC1. Finally, the Wnt pathway is also required for adaptive cardiac remodelling.[30,48,49] Accordingly, we observed a significant up-regulation of the Wnt ligand expression accompanied with a down-regulation of the expression of the Wnt inhibitors Sfrp1 and Wif1, and Axin2, a negative regulator of canonical Wnt, in both genotypes after TAC (see Supplementary material online, *Figure S6A*). This was

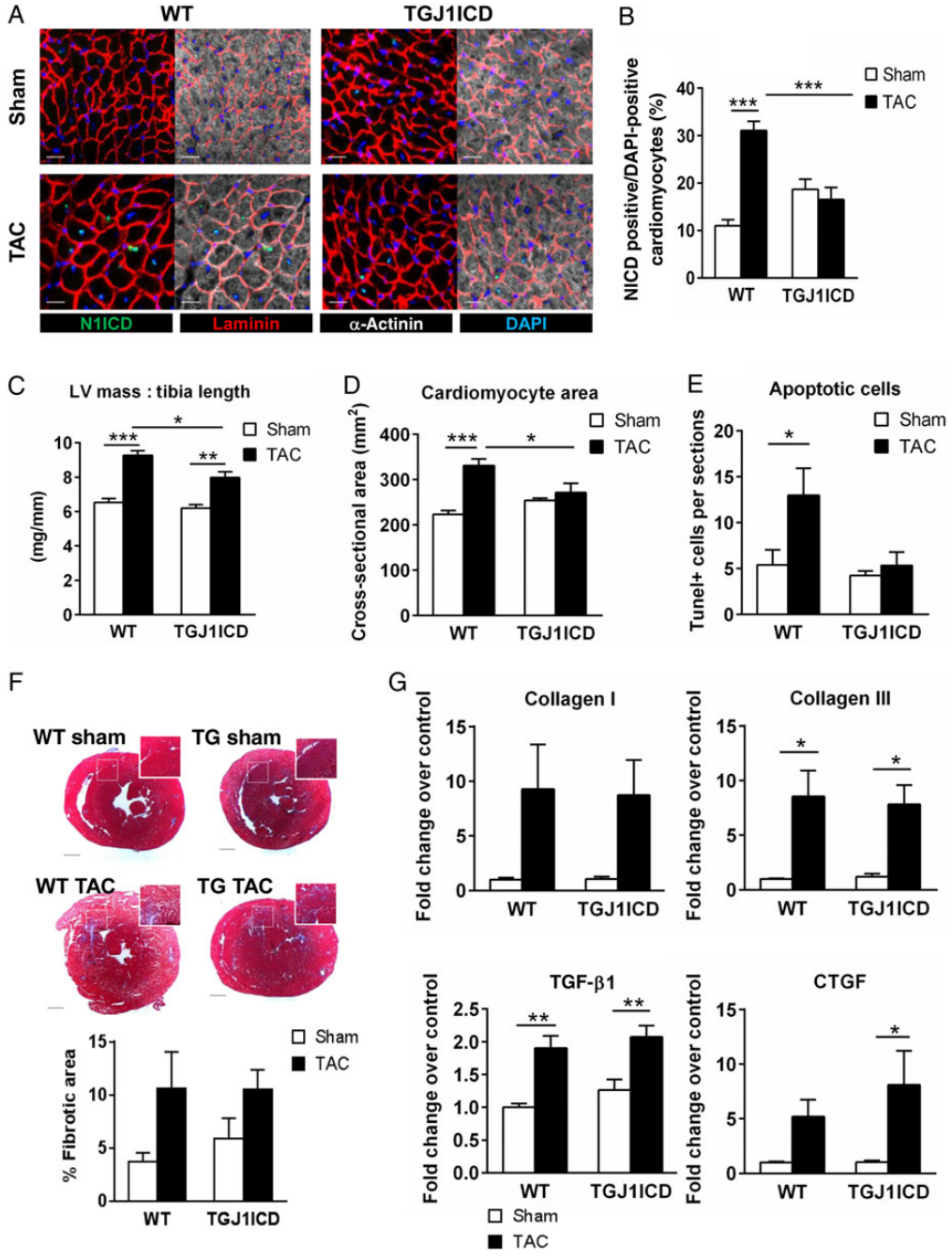

Figure 6 Attenuated hypertrophic response to pressure overload in adult TGJ1ICD hearts. Adult WT and TGJ1ICD littermates were subjected to TAC or sham operation and were treated with tamoxifen for 1 week. (*A*) Heart sections were immunostained against NICD (green), laminin (red), and α-sarcomeric-actinin (white). Nuclei were stained with DAPI (blue). Scale bar, 20 μm. (*B*) The number of NICD-positive cardiomyocytes was quantified. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ vs. control or vs. indicated values. (*C*) LV mass normalized to tibia length in TAC or sham operated WT and TGJ1ICD mice. (*D*) Cross-sectional areas of ≥3000 myocytes per group were measured on laminin-stained heart sections. (*E*) Number of TUNEL-positive cells per heart sections. (*F*) Top, Masson Trichrome staining. Scale bars, 2 mm. Bottom, the fibrotic area was quantified and expressed as the percentage of the total section area. (*G*) Quantitative RT-PCR analysis of fibrosis markers. Results, normalized to Gapdh, represent fold change relative to WT sham. (*B*–*G*) Bar graphs are mean ± SEM ($n = 6$–7 mice per group; $^{*}P \leq 0.05$; $^{***}P \leq 0.01$ vs. indicated values).

correlated by an increase in the active form of β-catenin (see Supplementary material online, *Figure S6B* and *C*). Altogether, these data indicated a similar activation of the Wnt pathway in the stressed heart in both genotypes. However, the Wnt pathway was not differentially activated in WT vs. TGJ1ICD mice.

4. Discussion

Notch signalling has been appreciated primarily as a one-way communication system between two adjacent cells. One cell expresses a Notch ligand such as Jagged1, which then sends a signal to a receiving

Table 1 Echocardiographic parameters of cardiac dimension and function in adult TGJ1ICD TG mice following TAC

	WT		TGJ1ID	
	Sham	TAC	Sham	TAC
IVSd (mm)	0.71 ± 0.01	0.99 ± 0.02[a]	0.71 ± 0.02	0.94 ± 0.03[a]
IVSs (mm)	0.91 ± 0.02	1.33 ± 0.04[a]	0.95 ± 0.03	1.28 ± 0.04[a]
LVIDd (mm)	4.24 ± 0.11	3.93 ± 0.06[a]	4.09 ± 0.05	3.77 ± 0.05[a]
LVIDs (mm)	3.18 ± 0.11	2.88 ± 0.09	2.95 ± 0.09	2.61 ± 0.14
LVPWd (mm)	0.69 ± 0.01	1.00 ± 0.02[a]	0.69 ± 0.01	0.95 ± 0.03[a]
LVPWs (mm)	0.89 ± 0.01	1.22 ± 0.03[a]	0.94 ± 0.03	1.25 ± 0.04[a]
LV mass (mg)	109.3 ± 4.82	154.1 ± 5.65[a]	102.5 ± 3.84	135.3 ± 5.77[a,b]
%FS	25.2 ± 0.98	26.7 ± 1.61	27.8 ± 1.66	30.8 ± 3.37
%EF	50.0 ± 1.62	52.6 ± 2.51	54.2 ± 2.60	58.2 ± 5.02

[a]TAC vs. sham.
[b]TG vs. WT.

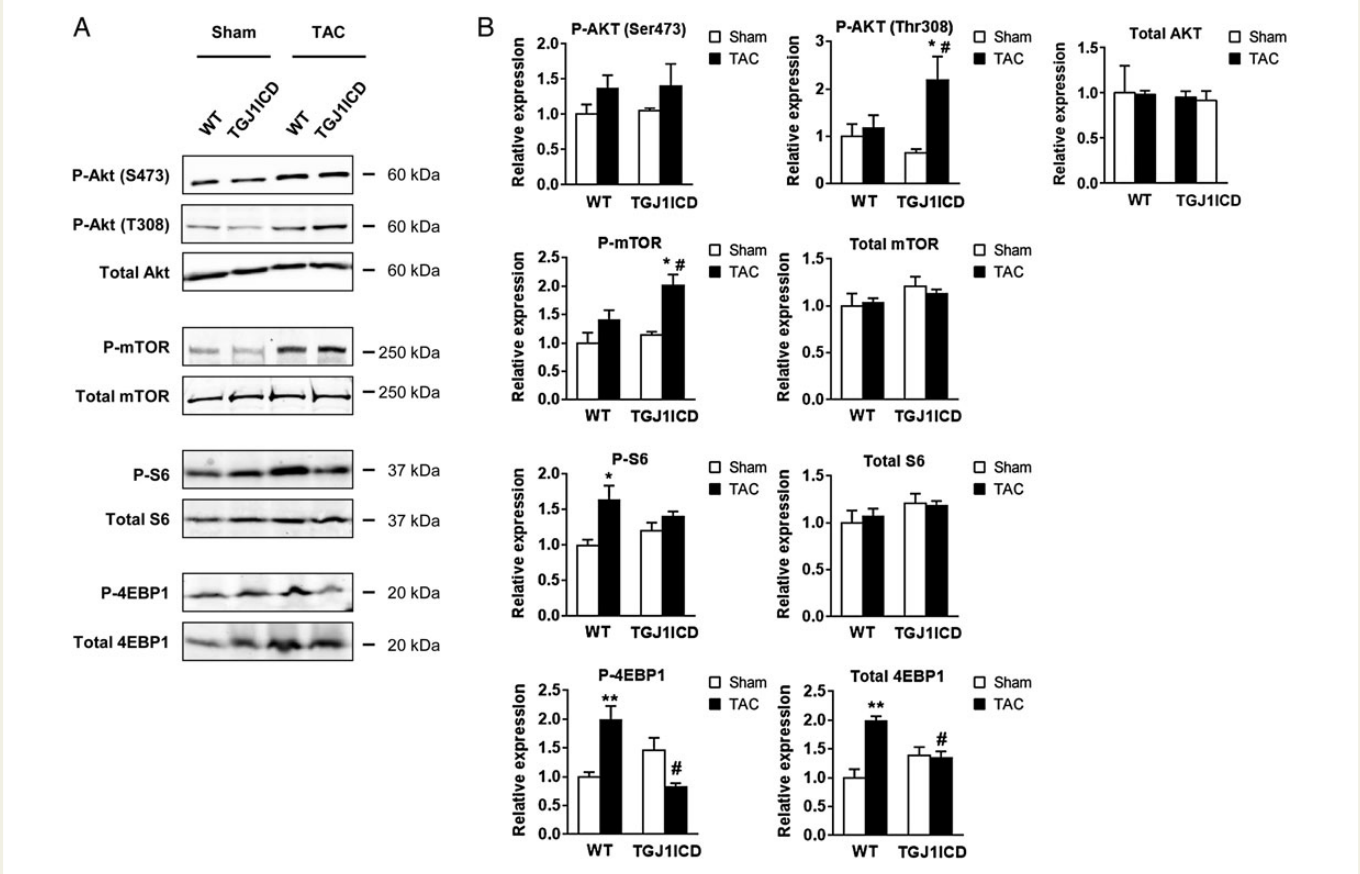

Figure 7 Crosstalk between J1ICD signalling and the AKT pathway. (*A*) Phosphorylated and total AKT, mTOR, S6, and 4EBP1 were analysed by western blot in adult WT and TGJ1ICD mice subjected to TAC or sham operation and treated with tamoxifen for 1 week. (*B*) Immunoblots were quantified. Bar graphs represent the ratio of the phosphorylated to total signals normalized to the WT sham group ($n = 3–6$ mice per group; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$ vs. WT sham; $^{\#}P \leq 0.05$ vs. WT TAC).

cell expressing a Notch receptor.[1] In some instances, however, Notch ligand-mediated events have been described.[15–18] In accordance with these previous findings, we present the first evidence supporting a functional role for the intracellular domain of the Notch ligand Jagged1 in cardiomyocytes during physiological and pathological growth. We demonstrated that cardiomyocytes endogenously release J1ICD upon receptor ligand activation following γ-secretase-dependent cleavage of full-length Jagged1. The fragment then translocates into the nucleus, where it exerts negative regulatory effects on Notch transcriptional activity. Nuclear translocation of J1ICD has been described

in other cell types, where it controls gene expression and modifies cell phenotype via Notch receptor-independent pathways.[15,19,20,28,50] Indeed, the intracellular portion of the ligand appears to modulate cell fate in cancer cells or even in cells committed to the neurogenic lineage during development.[19,21–24] Nevertheless, the mechanism by which Notch ligands influence gene transcription remains poorly understood. Notch ligand intracellular domains do not contain DNA-binding motives, suggesting that they indirectly modulate gene expression by interacting with transcription factors or other molecular co-activators.[19,21–24,51] Competition for the γ-secretase complex between Notch receptors and ligands during activation might also account for modulation of Notch receptor-associated pathways.[15] Interestingly, recent data showed that J1ICD as well as Dll1-ICD directly interact with NICD, and inhibit Notch signalling by promoting degradation of the NICD–RBP-Jκ complex via the ubiquitin proteasome.[27,28] Our own data are consistent with this model. Indeed, N1ICD co-precipitated with J1ICD in WT hearts, but the levels of NICD were largely diminished in TGJ1ICD hearts, suggesting that the increased concentrations of J1ICD accelerated NICD degradation. In support of this, the proteasome inhibitor, MG132, restored normal levels of co-precipitated NICD. Moreover, the numbers of N1ICD-positive cardiomyocytes decreased significantly upon induction of J1ICD translocation into the nucleus. Therefore, it is likely that high J1ICD concentrations in TG mice reduce nuclear levels of N1ICD in cardiomyocytes, and thereby down-regulates the expression of Notch target genes such as Hes1, Hey1, and Hey2. Moreover, lower amounts of N1ICD-positive cardiomyocytes are also measured in adult TGJ1ICD mice after stress, suggesting that similar inhibition of the Notch1 signalling pathway occurs during the adaptive response of the heart to damage. Altogether, these data support a role for J1ICD as a compensatory factor that limits excessive Notch signalling. In this context, whether or not Notch signalling directly triggers Jagged1 cleavage remains to be established.

Inhibition of the Notch1 pathway following J1ICD activation reduces mitosis and stimulates terminal differentiation in cultured neonatal cardiomyocytes as shown by increased sarcomeric organization. Similarly, decreased cardiomyocyte division and increased cardiac maturation characterize the TGJ1ICD heart shortly after birth. In TGJ1ICD neonates, increased cardiomyocyte size compensates initially for decreased myocyte proliferation. Indeed, cardiomyocyte cross-sectional area is augmented in 4- and 7-day-old TG mice, leading to an increased heart weight to tibia length ratio at 1 week of age. These results are consistent with previous observations, showing that pharmaceutical or genetic blockade of the Notch pathway results in a significant reduction of cardiac precursor cell and immature cardiomyocyte proliferation while favouring differentiation into mature cardiomyocytes.[4–9] In particular, newborn mice treated with the γ-secretase inhibitor, DAPT, are characterized by low myocyte formation. In turn, defective cardiomyocyte production triggers a compensatory response in the viable cardiomyocyte population that eventually leads to overt cardiac hypertrophy.[4] In contrast to general Notch inhibition in DAPT-treated cardiac cells, however, the action of the J1ICD transgene is restricted to α-MHC-expressing cardiomyocytes, excluding thereby direct effects on undifferentiated precursor cell differentiation.

In response to J1ICD-mediated inhibition of Notch signalling in the neonatal heart, we observed a new wave of cardiomyocyte proliferation at Day 7 after birth, most probably as an attempt to compensate for the deficit in myocyte number. This indicates that pathways are activated to overcome Notch inhibition and force cardiomyocytes to re-enter the cell cycle. Such compensatory response appears to be, in part, mediated through activation of Wnt signalling in the neonatal heart. Indeed, several lines of evidence suggested that the canonical Wnt/β-catenin pathway negatively regulates cardiac differentiation and promotes proliferation. For instance Wnt3A, Wnt6, and Wnt8 have been shown to interfere with cardiomyocyte formation at the late stage of development.[33,34,36–38] Conversely, Wnt inhibitors, such as Dkk1 and Wif1, favour terminal cardiac differentiation and morphogenesis.[33,36] Moreover, gain- and loss-of-function studies have shown that non-canonical Wnt11 is necessary for cardiogenesis.[33,39–41] We measured therefore the expression of canonical and non-canonical Wnt components in WT and TGJ1ICD mice at 1 week of age. We found that inhibitors of canonical Wnt were indeed down-regulated. Accordingly, β-catenin signalling was stimulated as shown by the greater active/total β-catenin ratio in TG hearts. Moreover, cardiogenic Wnt11 expression was reduced in TG mice. Altogether, this indicates that canonical Wnt signalling is activated, likely diminishing thereby cardiogenic differentiation and facilitating proliferation. Expression of Wnt signalling targets, such as cyclin B1 and D1, was stimulated, further supporting the existence of compensatory feedback mechanisms between the Notch and Wnt pathways to preserve adequate numbers of cardiomyocytes in the neonatal heart.

The Akt pathway is also crucial for physiological proliferation and survival of cardiomyocytes in the postnatal heart.[42,46,47] Concomitant to canonical and non-canonical Wnt modulation in TG newborns, we observed indeed a significant up-regulation of the Akt signalling pathway 1 week after birth. Indeed, phosphorylation of downstream targets of Akt involved in myocyte growth, such as S6 and 4EBP1, was significantly increased upon J1ICD activation. Accordingly, cyclin D1, which is also an Akt target controlling myocyte proliferation,[52] was up-regulated in TGJ1ICD hearts. Interestingly, Notch signalling has been implicated in immature cardiomyocyte proliferation, in part via up-regulation of cyclin D1,[5,6] and has been shown to induce Akt phosphorylation in the heart,[8] confirming crosstalk between these two important pathways to maintain an active cardiomyocyte cell cycle. Overall, these data suggested that Wnt and AKT signalling pathways block maturation and stimulate proliferation in the cardiomyocyte population to compensate for early cardiac maturation following J1ICD-induced Notch inhibition. However, this mitotic activity is only transient and does not fully counteract the action of J1ICD. At the end of the second week, the heart of TG mice is smaller than that of the WT littermates.

Activation of overexpressed J1ICD in cardiomyocytes in the unstressed adult heart has no impact on Notch signalling, and on cardiac morphology and function. This is in accordance with previous findings demonstrating that, in the adult heart, Notch is only activated following damage.[7,9,11] In contrast, J1ICD activation in the adult heart after TAC-induced pressure overload blunts significantly the hypertrophic response and reduces the number of apoptotic cardiomyocytes. This occurs concomitantly with a dramatic reduction of the number of Notch1 signalling cardiomyocytes. Blockade of the Notch pathway in the heart is expected to exacerbate hypertrophy.[7] Therefore, the fact that cardiac growth is reduced in TG mice indicates that J1ICD-mediated Notch inhibition stimulates a strong compensatory response via down-regulation of growth promoting pathways in stressed cardiomyocytes. Interestingly, the fibrotic response is not affected, consistent with a cell autonomous effect of transgene expression in cardiomyocytes. These data should be, however, put into perspective with results we obtained recently using a different TG model, in which full-length

Jagged1 was overexpressed specifically in cardiomyocytes.[11] High Jagged1 expression on the surface of cardiomyocytes in this model sends signals to surrounding cells, including cardiomyocytes, fibroblasts, and cardiac precursor cells. This creates a situation, in which both hypertrophy and fibrosis are reduced, favouring thereby the emergence of cardiac precursor cells. What the present study therefore suggests is that J1ICD production in cardiomyocytes could significantly contribute to reduce hypertrophy in Jagged1-overexpressing cardiomyocytes.

Wnt signalling was not differentially modulated between WT and TGJ1ICD mice, suggesting that this pathway was not involved in determining the cardiac phenotype in adult TGJ1ICD. However, we found significant modulation of the Akt pathway following J1ICD activation. Phosphorylation of S6 and 4EBP1, two effectors of the Akt/mTORC1 pathway controlling protein synthesis, is increased in the stressed heart of WT mice, but abolished in the heart of TGJ1ICD mice. This is consistent with the reduced cardiac hypertrophy observed in TG mice subjected to pressure overload. Akt activity depends on upstream activation of the phosphoinositide 3-kinase (PI3K), leading to phosphorylation of Akt at Thr-308 by the PDK1 and at Ser-473 by the mTORC2 pathway.[42] Phosphorylation at Ser-473 was shown to be important for the development of hypertrophy. In the present study, phosphorylation at Ser-473 is not different in the two genotypes, indicating that regulation occurs at a different level. Thr-308 Akt phosphorylation is largely increased in TG hearts. This might reflect positive feedback operating on Akt in face of S6 and 4EBP1 dephosphorylation. Therefore, since all regulators of the Akt pathway upstream of mTORC1 appear activated, it suggests that the blockade of the pathway occurs downstream of this complex. Finally, Akt has also been shown to produce beneficial effects via reduced apoptosis during pathological remodelling. Reduced compensatory hypertrophy in TGJ1ICD TG might also reflect increased cardiomyocytes survival via activation of the Akt pathway. Accordingly, TGJ1ICD mice showed reduced myocyte apoptosis in the heart under pressure overload.

Altogether, we have described a novel mechanism that modulates Notch signalling in the heart during physiological and pathological hypertrophy. Molecular mechanisms implicate production and translocation into the nucleus of the intracellular domain of Jagged1 to blunt Notch1 receptor signalling. This J1ICD-mediated pathway appears therefore to play a significant role to counterbalance exacerbated Notch activation in the damaged heart. In this context, crosstalk between the Notch, Wnt, and Akt pathways fine-tunes the hypertrophic response in cardiomyocytes to enhance adaptation to stress.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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