Fkbp1a controls ventricular myocardium trabeculation and compaction by regulating endocardial Notch1 activity

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INTRODUCTION

The formation of the ventricular walls is essential to cardiogenesis and is required for normal cardiac function (Moorman and Christoffels, 2003). Ventricular trabeculation and compaction are important morphogenetic events that are controlled by a precise balance between cardiomyocyte proliferation and differentiation during ventricular chamber formation and wall maturation. The trabeculation phase of cardiac development is initiated at the end of cardiac looping (E9.0 to E9.5 in mouse) and is defined as the growth of primitive cardiomyocytes to form the highly organized muscular ridges that are lined by layers of invaginated endocardial cells (Ben-Shachar et al., 1985). The newly formed trabeculae gradually contribute to the formation of the papillary muscles, the interventricular septum and cardiac conductive cells. Concomitant with ventricular septation, the trabeculae start to compact at their base adjacent to the outer myocardium, adding substantially to ventricular wall thickness and coincidently establishing coronary circulation (Wessels and Sedmera, 2003). A significant reduction in trabeculation is closely associated with myocardial growth arrest, whereas persistent trabeculation and/or a reduced level of compaction are associated with left ventricular noncompaction (LVNC) (Towbin, 2010) [also called left ventricular hypertrabeculation (LVHT)] (Finsterer, 2009). LVNC (MIM300183) is a distinct form of inherited cardiomyopathy (Pignatelli et al., 2003; Sandhu et al., 2008). Although some LVNC patients survive into adulthood (Weiford et al., 2004), severe cases accompanied with other cardiac defects (e.g. Ebstein’s anomaly) commonly die at a young age (Koh et al., 2009).

The underlying molecular and cellular mechanisms that orchestrate ventricular trabeculation and compaction, as well as the pathogenesis of LVNC, are poorly understood due to the heterogeneity of the patients and the lack of suitable genetic models (Srivastava and Olson, 2000). Genetic screening of isolated LVNC patients has identified a handful of gene mutations in sarcomere proteins (Klaassen et al., 2008; Xing et al., 2006). However, the genes that are linked to the more severe cases of pediatric LVNC have yet to be identified. Previously, we showed that mutant mice deficient in FK506 binding protein la (Fkbp1a, also known as FKBP12) develop multiple abnormal cardiac structures that phenocopy severe cases of LVNC, including a characteristic increase in the number and thickness of ventricular trabeculae (i.e. hypertrabeculation), deep intertrabecular recesses, lack of compaction that leads to a thin left ventricular wall (i.e. noncompaction) and a prominent ventricular septal defect (VSD) (Shou et al., 1998).

Fkbp1a is a ubiquitously expressed 12 kDa peptidyl-prolyl isomerase (Bierer et al., 1990; Schreiber and Crabtree, 1995). It binds to FK506 and rapamycin and inhibits calcineurin and mTOR activity. Fkbp1a is associated with multiple intracellular protein

SUMMARY

Trabeculation and compaction of the embryonic myocardium are morphogenetic events crucial for the formation and function of the ventricular walls. Fkbp1a (FKBP12) is a ubiquitously expressed cis-trans peptidyl-prolyl isomerase. Fkbp1a-deficient mice develop ventricular hypertrabeculation and noncompaction. To determine the physiological function of Fkbp1a in regulating the intercellular and intracellular signaling pathways involved in ventricular trabeculation and compaction, we generated a series of Fkbp1a conditional knockouts. Surprisingly, cardiomyocyte-restricted ablation of Fkbp1a did not give rise to the ventricular developmental defect, whereas endothelial cell-restricted ablation of Fkbp1a recapitulated the ventricular hypertrabeculation and noncompaction observed in Fkbp1a systemically deficient mice, suggesting an important contribution of Fkbp1a within the developing endocardia in regulating the morphogenesis of ventricular myocardium and trabeculation. Further analysis demonstrated that Fkbp1a is a novel negative modulator of activated Notch1. Activated Notch1 (N1ICD) was significantly upregulated in Fkbp1a systemically deficient mice, whereas endothelial cell-restricted ablation of Fkbp1a did not give rise to the ventricular hypertrabeculation and noncompaction. Our findings suggest that Fkbp1a-mediated regulation of Notch1 plays an important role in intercellular communication between endocardium and myocardium, which is crucial in controlling the formation of the ventricular walls.

KEY WORDS: FK506 binding protein 12, Ventricular hypertrabeculation/noncompaction, Notch1, Endocardial-myocardial signaling, Mouse
complexes (Ozawa, 2008; Wang and Donahoe, 2004), such as BMP/Activin/TGFβ type I receptors, Ca2+-release channels, and functionally regulates the voltage-gated Na+ channel (Maruyama et al., 2011). Previously, we revealed that Bmp10 is upregulated in Fkbp1a-deficient hearts. Bmp10-deficient mice die by E10.5 and exhibit hypoplastic and thin ventricular walls and impaired ventricular trabeculation (Chen et al., 2004). Transgenic overexpression of Bmp10 in embryonic heart led to ventricular hypertrabeculation (Pushnarmourous et al., 2004). The elevated level of Bmp10 observed in both Fkbp1a and Nkx2-5 knockout hearts correlates strongly with the ventricular hypertrabeculation and noncompaction phenotypes displayed in these mutants (Chen et al., 2009). However, the underlying mechanism by which Fkbp1a regulates Bmp10 expression and ventricular wall formation remains elusive.

Recently, it has been shown that endocardial Notch1 provides key spatial-temporal control of myocardial growth via regulation of Bmp10 and neuregulin 1 (Nrg1) (Grego-Bessa et al., 2007). Endocardium is primarily made up of endothelial cells. Activated Notch1 intracellular domain (N1ICD) was found to be more abundant in endocardial cells near the proximal end of the trabecular myocardium, where trabeculation initiates, and was significantly less abundant in the endocardial cells at the distal end of the trabeculae (Grego-Bessa et al., 2007). Ablation of Notch1 or its transcriptional co-factor Rhbpk within endothelial cells results in hypotrabeculation and, subsequently, early embryonic lethality (Del Monte et al., 2007; Grego-Bessa et al., 2007). Interestingly, both endocardially expressed Nrg1 and myocardially expressed Bmp10 were downregulated in endothelial-restricted Notch1 knockout hearts (Grego-Bessa et al., 2007). Collectively, these findings suggested a crucial role for endocardial Notch1 in regulating ventricular trabeculation.

To determine the cellular and molecular mechanism of Fkbp1a in regulating ventricular trabeculation and compaction, and its pathogenetic role in LVNC, we generated Fkbp1a conditional knockouts using the Cre-loxP recombination system. Ablating Fkbp1a in cardiac progenitor cells via the use of Nkx2.5Cre mice (Moses et al., 2001), we were able to generate Fkbp1aflox−/Nkx2.5cre mice that recapitulate the ventricular hypertrabeculation and noncompaction with full penetrance observed in systemic Fkbp1a null mice. By contrast, ablation of Fkbp1a using cardiomyocyte-specific Cre lines did not give rise to abnormal ventricular wall formation. Surprisingly, endothelial-restricted ablation of Fkbp1a phenocopied the ventricular hypertrabeculation and noncompaction observed in Fkbp1a systemically deficient mice, suggesting that endocardium plays an important role in regulating ventricular trabeculation and compaction. Biochemical and molecular analyses demonstrated that Fkbp1a regulates Notch1-mediated signaling within developing endocardial cells. An excess of activated Notch1 is found in Fkbp1a-ablated endothelial cells and is the likely cause of the observed Fkbp1a mutant phenotypes. Treatment of Fkbp1a-deficient mice with Notch inhibitors reduces the hypertrabeculation phenotype. Taken together, we have revealed a mechanism whereby Fkbp1a modulates a critical level of Notch1 activity that is required for regulating ventricular chamber and wall formation.

MATERIALS AND METHODS

Generation of Fkbp1a floxed and conditional knockout mice

The generation of Fkbp1a floxed mice (Fkbp1afllox) has been described previously (Maruyama et al., 2011). To achieve cell lineage-restricted deletion of Fkbp1a in the developing heart, Fkbp1aflox mice were crossed to various cell type-specific Cre mouse lines. To ensure efficient Cre-loxP recombination in these conditional genetic ablations, we first created Fkbp1afllox/Cre+ mice followed by an additional intercross onto Fkbp1afllox/Cre+ mice. For the most part, we used Fkbp1afllox/Cre+ as the experimental group and Fkbp1afllox+/Cre− and Fkbp1afllox+/Cre− as the control group. Animal protocols were approved by the Indiana University School of Medicine Institutional Animal Care and Research Advisory Committee.

Histological, morphological, whole-mount and section in situ hybridization, and immunohistochemical analyses

Embryos were harvested by cesarean section. Embryos and isolated embryonic hearts at specific stages were fixed with 4% paraformaldehyde overnight. Frozen sections of Embryos were paraffin embedded, sectioned (7 μm), and stained with Hematoxylin and Eosin. Whole-mount and section in situ hybridization were performed as previously described (Franco et al., 2001). In brief, complementary RNA probes for various cardiac markers were labeled with digoxigenin (DIG)-UTP using the Roche DIG RNA Labeling System according to the manufacturer’s guidelines. Immunohistochemical staining was performed using the staining system from Vector Laboratories according to the manufacturer’s instructions. The primary antibodies used in the immunohistochemical analyses were: anti-Fkbp1a (FKBPI2) antibody (Thermo Scientific, PA-026A), MF-20 anti-myosin heavy chain monoclonal antibody [Developmental Studies Hybridoma Bank (DSHB), University of Iowa], anti-Ki67 antibody (ab15580; Abcam), anti-CD31 (Pecam1) antibody (BD Biosciences, 553370) and anti-cleaved Notch1 (N1ICD) antibody (Cell Signaling, 2421S).

Whole-mount immunofluorescence staining and confocal microscopy imaging

Staining and microscopy procedures were as previously described (Chen et al., 2004). Embryos were harvested and washed three times in ice-cold PBS and fixed for 10 minutes in pre-chilled acetone before being treated with blocking solution containing 3% non-fat dried milk (Bio-Rad) and 0.025% Triton X-100 for 1 hour. Directly conjugated primary antibodies were then added to a final concentration of 1 μg/ml for 12-18 hours at 4°C. The anti-CD31 antibody (BD Biosciences) and the MF-20 monoclonal antibody (DSHB) were labeled with Alexa Fluor 488 and the anti-Fkbp1a antibody (Thermo Scientific) was labeled with Alexa Fluor 647 using a monoclonal antibody labeling kit (Molecular Probes). Samples were analyzed using a Bio-Rad MRC 1024 laser scanning confocal microscope equipped with a krypton-argon laser (488, 647 nm). Z-series were obtained by imaging serial confocal planes at 512×512 pixel resolution with a Nikon 20× oil-immersion objective (2 μm intervals).

Echocardiography

Mice were gently anesthetized with 1.5% isoflurane. Two-dimensional short-axis images were obtained with a high-resolution micro-ultrasound system (Vevo 770, VisualSonics) equipped with a 40 MHz mechanical scan probe. Fractional shortening (FS), ejection fraction, left ventricular internal diameter (LVID) during systole, LVID during diastole, end-systolic volume, and end-diastolic volume were calculated with Vevo Analysis software (version 2.2.3) as described previously (Zhu et al., 2009). LVID during systole and diastole were measured from M-mode recording at the level of the papillary muscles, whereas end-systolic and end-diastolic volumes were measured with B-mode recording in a plane containing the aortic and mitral valves.

Cell transfection and protein stability assay

Flag-tagged human FKBPIA cDNA was subcloned into the BamHI and EcoRI sites of the pCDNA3 vector (Invitrogen). N1ICD expression plasmid (N1ICD-PCS2) is a generous gift from Dr Raphael Kopan (Washington University). Western blots employed anti-Flag (Sigma, F8104), anti-N1ICD (Cell Signaling) and anti-tubulin (Sigma, T6199) antibodies. For the protein stability assay, the N1ICD-expressing plasmid was transfected into control HEK293 cells and those stably expressing human FKBPIA tagged with Flag epitope (FKBPIA-HEK293) using FuGENE HD (Roche) according to the manufacturer’s instructions. Cycloheximide (50 µg/ml, Sigma) was added to the medium for the indicated length of time. To inhibit proteasomes, transfected cells were treated with the proteasome inhibitor

DEVELOPMENT
MG-132 (10 μmol, EMD Chemicals) for various time periods. For detection of ubiquitylated N1ICD, histidine-tagged ubiquitin (His-ubiquitin) pCDNA3 plasmid was co-transfected with N1ICD expression plasmid into HEK293 or FKBP1A-HEK293 cells. After 30 hours, transfected cells were incubated with MG-132 (10 μmol) for 8 hours before being lysed in Ni-agarose lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 5 mM imidazole, 0.05% Tween 20, 100 μg/ml N-ethylmaleimide, and Complete Protease Inhibitor (Roche)). His-ubiquitin-conjugated proteins were enriched using Ni-NTA-agarose (Qiagen) from cells co-transfected with N1ICD and His-ubiquitin. The beads were washed ten times with Ni-agarose wash buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 0.05% Tween 20). In order to reduce nonspecific binding to beads as much as possible, nickel-binding proteins were resuspended in 2× sample buffer supplemented with 200 mM imidazole and heated for 10 minutes at 95°C before western blot with anti-N1ICD antibody.

Luciferase assay
Hes1-pGL3 luciferase plasmid and full-length Notch1 and DI4 expression plasmids have been described previously (McGill and McGlade, 2003). Fkbp1a knockout and control mouse embryonic fibroblasts (MEFs) and endothelial cells were isolated as described (Weaver et al., 1997). For the luciferase assay, Hes1-pGL3 reporter plasmid was transfected into Fkbp1a knockout or control MEFs and endothelial cells alone or with different combinations of Notch1, DI4 and Fkbp1a expression plasmids. Renilla luciferase plasmid was co-transfected as an internal control. Transfection efficiency was determined by co-transfection of pCDNA3-EGF. Luciferase activity was analyzed 36 hours after transfection with the Dual-Luciferase assay System (Promega) according to the manufacturer’s instructions.

Isolation and culture of endothelial cells
Murine endothelial cells were isolated from lung tissues of Fkbp1a<sup>flox/flox</sup> neonates (day 10). The harvested lung tissue was minced to small pieces and then digested with 0.25% collagenase A (Stem Cell Technologies) for 40 minutes followed by cell dissociation buffer (Life Technologies) in order to produce a single-cell suspension. The cell suspension was filtered through a 70-μm strainer and stained with anti-CD31, anti-CD45 (Ppcre) and anti-Mac1 (Igam) antibodies (all eBiosciences). The CD45<sup>−/−</sup> Mac1<sup>−/−</sup> CD31<sup>+</sup> endothelial cells were sorted on a FACS Aria (Becton Dickinson). The sorted endothelial cells were cultured in EGM2 (endothelial growth medium 2, Lonza) with 10% FBS, and 7 days after initiating culture the adherent cells were analyzed by FACS and confirmed by immunofluorescence staining for endothelial cell surface markers including CD31 (eBiosciences), Vcam1 (Santa Cruz) and Fk1 (Kdr) (eBiosciences) (see supplementary material Fig. S3A,B). To further validate the endothelial characteristics of the cells, we used an in vitro Matrigel tube-形成 assay (see supplementary material Fig. S3C). As previously described, we plated Fkbp1a<sup>flox/flox</sup> cells on a thin layer of Matrigel (Stem Cell Technologies) at 1×10<sup>4</sup> cells/well of a 96-well plate in ECM2 containing 10% FCS and allowed a tubular structure to form. Typical tube formation after 20 hours in culture (supplementary material Fig. S3) suggested that the isolated Fkbp1a<sup>flox/flox</sup> cells maintained endothelial cell characteristics. To generate Fkbp1a-deficient endothelial cells, Fkbp1a<sup>flox/flox</sup> endothelial cells were infected with recombinant adenosinie expressing Cre/eGFP (1×10<sup>4</sup> pfu). Cycloheximide (25 μg/ml) was added to the medium for the indicated length of time followed by western blot analysis.

RESULTS
Cardiac progenitor ablation of Fkbp1a
To determine whether the ventricular hypertrabeculation and noncompaction present in Fkbp1a-deficient mice is a primary cardiac defect, we used Nkx<sub>2.5</sub><sup>cre</sup> to ablate the Fkbp1a gene. Nkx<sub>2.5</sub><sup>cre</sup> delivers Cre within cardiac progenitor cells in the cardiac crescent, which give rise to cardiomyocytes, endothelial cells and to a subpopulation of cells from the septum transversum that gives rise to the epicardium, and within cardiac neural crest cells (Moses et al., 2001; Zhou et al., 2008). Fkbp1a<sup>flox/flox</sup>:Nkx2.5<sup>cre</sup> mice demonstrated fetal lethality, dying in utero between E14.5 and birth. The mutants phenocopied Fkbp1a-deficient mice, with profound hypertrabeculation, noncompaction and VSDs with 100% penetrance (Fig. 1; Table 1) (Shou et al., 1998), indicating that the ventricular hypertrabeculation and noncompaction within Fkbp1a systemic knockouts are primarily derived from defects in the cardiogenic program, and are not secondary to defects in another organ system.

Specific ablation of Fkbp1a within myocardial, epicardial and neural crest cell-restricted cell populations
To further determine the cell-autonomous contribution to the ventricular hypertrabeculation and noncompaction caused by Fkbp1a ablation, we crossedbred Fkbp1a<sup>flox</sup> mice onto the cardiomyocyte-specific sodium-calcium exchanger promoter H1-cre (H1-Ncx-cre) (Müller et al., 2002). The Cre activity in H1-Ncx-cre is observed in developing myocardium as early as E9.5 and in a cardiomyocyte-restricted manner throughout embryonic, fetal and adult life (supplementary material Fig. S1). To our surprise, Fkbp1a<sup>flox/flox</sup>:H1-Ncx-cre mice demonstrated normal ventricular chamber formation and survived to adulthood. Histologically, no hypertrabeculation or noncompaction was found in Fkbp1a<sup>flox/flox</sup>:H1-Ncx-cre hearts (Fig. 2; Table 1). Western blot, qRT-PCR and immunofluorescence analyses confirmed efficient ablation of Fkbp1a within the embryonic myocardium (Fig. 2). This finding suggests that cardiomyocytes are not the primary cell type contributing to the ventricular hypertrabeculation and noncompaction observed in mice with systemic Fkbp1a ablation.

To validate this observation, we crossedbred Fkbp<sup>flox</sup> mice to other cardiomyocyte-specific Cre transgenic lines, including cTnt-cre (Table 1) (Jiao et al., 2003; Wang et al., 2000). Consistently, all of these mutant mice exhibited normal ventricular chamber formation. cTnt-cre delivers Cre within the developing myocardium as early as E7.5 (Jiao et al., 2003), which is similar to that reported for Nkx2.5<sup>cre</sup>.
Despite this early Cre activity, Fkbp1a<sup>fl<sup>ox</sup>/−:cTnt-cre mice display normal ventricular trabeculation and compaction (supplementary material Fig. S2), further indicating that the abnormal ventricular development in Fkbp1a-deficient mice is not a direct function of Fkbp1a within cardiomyocytes. Similarly, cardiac development was reported to be normal in studies using MHC-cre and Mck-cre to delete Fkbp1a (Maruyama et al., 2011; Tang et al., 2004). Together, these data rule out cardiomyocytes as the primary contributing cell lineage to ventricular hypertrabeculation and noncompaction in Fkbp1a-deficient mice, and instead imply another cell lineage or lineages as the primary cause.

Epidermis has been implicated to play an important role in supporting ventricular wall growth and, potentially, compaction (Sucov et al., 2009). To test whether epicardial Fkbp1a contributes to hypertrabeculation or noncompaction, we ablated Fkbp1a from epicardial cells using Tbx18<sup>cre</sup> mice (Cai et al., 2008). Tbx18<sup>cre</sup> mice deliver Cre activity largely to epicardium, with a low activity in cardiomyocytes refined in ventricular septum (Cai et al., 2008; Christoffels et al., 2009). We found that Fkbp1a<sup>fl<sup>ox</sup>/−:Tbx18<sup>cre</sup></sup> mutants had normal ventricular wall formation (Table 1; supplementary material Fig. S2). Previously we showed that, despite low penetrance, Fkbp1a-deficient mice develop exencephaly (Shou et al., 1998), indicating potential involvement of cranial-facial neural crest cells. We used Wnt1-cre to ablate Fkbp1a from neural crest cell lineages (Jiang et al., 2000). Once again, we observed normal ventricular wall formation in Fkbp1a<sup>fl<sup>ox</sup>/−:Wnt1-cre mutants (Table 1; supplementary material Fig. S2). When considering the cell lineages marked by Nkx2.5<sup>cre</sup>, the endocardium remained untested. Therefore, we next sought to specifically ablate Fkbp1a from the endocardium.

**Endothelial-specific ablation of Fkbp1a**

The developing endocardium has an important role in regulating ventricular wall formation (Brutsaert, 2003). To test the contribution of endocardium to the hypertrabeculation and noncompaction phenotypes observed with systemic Fkbp1a ablation, we studied the endothelial-specific deletion of Fkbp1a using Tie2-cre (Tie2 is also known as Tek) transgenic mice (Constien et al., 2001; Gitler et al., 2004; Kisanuki et al., 2001). Tie2-cre mice deliver Cre activity to endothelial cells, including endocardial cells, as early as E9.0 (Kisanuki et al., 2001). Fkbp1a<sup>fl<sup>ox</sup>/−:Tie2-cre mice phenocopied the ventricular hypertrabeculation and noncompaction seen in Fkbp1a-deficient hearts (Fig. 3; Table 1). Approximately 35% (15/45) of E13.5-14.5 Fkbp1a<sup>fl<sup>ox</sup>/−:Tie2-cre embryos were edematous and appeared to have failing hearts as evidenced by the absence of effective perfusion. Hearts isolated from these E13.5-14.5 Fkbp1a<sup>fl<sup>ox</sup>/−:Tie2-cre mice were edematous and typically ‘pumpkin-shaped’, lacking the normal ventricular groove, a strong indication of abnormal ventricle chamber development (Fig. 3).

Histological analysis of these hearts confirmed multiple abnormal cardiac ventricular structures similar to those of Fkbp1a<sup>−/−</sup> hearts, including various degrees of hypertrabeculation, noncompaction and a thin compact wall. Prominent VSDs, including both membranous and muscular, were also observed in six of these 15 mutant hearts.

Although the majority of Fkbp1a<sup>fl<sup>ox</sup>/−:Tie2-cre mice (compared with just ~5% of Fkbp1a<sup>−/−</sup> mice) survived in utero, most of these survivors died within 8 weeks of birth, probably owing to compromised cardiac function (data not shown) and defects in T cell function (see below) and in thymus development (25, 25, 17). The number of mutant and control embryos analyzed from the indicated number of litters, respectively.

**Table 1. Phenotypes of cell lineage-restricted Fkbp1a mutants**

<table>
<thead>
<tr>
<th>Ablation</th>
<th>Cre line</th>
<th>Phenotype</th>
<th>n (mutant, control, litters)*</th>
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<tr>
<td>Cardiac</td>
<td>Nkx2.5&lt;sup&gt;cre&lt;/sup&gt;</td>
<td>100% embryonic lethality with ventricular hypertrabeculation, noncompaction and VSD</td>
<td>10, 12, 7</td>
</tr>
<tr>
<td>Cardiomyocyte</td>
<td>H1-Ncx-cre</td>
<td>100% viable and fertile, normal ventricular development</td>
<td>11, 35, 12</td>
</tr>
<tr>
<td>Endothelial</td>
<td>Tie2-cre</td>
<td>−35% with ventricular hypertrabeculation, noncompaction and VSD</td>
<td>45, 22, 31 (E13.5 and E14.5)</td>
</tr>
<tr>
<td>Epicardial</td>
<td>Tbx18&lt;sup&gt;cre&lt;/sup&gt;</td>
<td>100% viable and fertile, normal ventricular development</td>
<td>5, 5, 4</td>
</tr>
<tr>
<td>Neural crest cell</td>
<td>Wnt1-cre</td>
<td>100% survive to postnatal, normal ventricular wall development, but 100% die within 1 month of birth due to defect in thymus development</td>
<td>25, 25, 17</td>
</tr>
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</table>

*The number of mutant and control embryos analyzed from the indicated number of litters, respectively.

VSD, ventricular septal defect.
Fkbp1aflox/–:H1-Ncx-cre

The boxed regions of the merge are enlarged to the right. (endocardium is altered in Bessa et al., 2007). To define whether Notch1 activation within the expression, ventricular trabeculation and wall formation (Grego-Bessa et al., 2007). Recent studies have demonstrated that perturbation of Notch1 altered in Fkbp1aflox/flox mutants. of Fkbp1a-deficient, Fkbp1aflox/–:Tie2-cre and wild-type control hearts. Using immunohistochemistry, we were able to confirm that nuclear N1ICD was robustly distributed within the endocardial cells close to the proximal end of the trabecular myocardium of control hearts (Fig. 5A). By contrast, this characteristic pattern of N1ICD was clearly altered in Fkbp1a mutant hearts, as N1ICD was found in endocardial cells throughout and alongside the trabecular myocardium (Fig. 5A), suggesting that Notch1 is activated ectopically within Fkbp1a-deficient and Fkbp1aflox/–:Tie2-cre endocardium.

Furthermore, Hey1 and ephrin B2 (Efnb2), two well-known direct downstream targets of Notch1 that are expressed within endocardium (Grego-Bessa et al., 2007; Hainaud et al., 2006), were found significantly upregulated in the Fkbp1a-deficient endocardial cells, supporting the observation that there is increased Notch1 activity in Fkbp1a mutant endocardium (Fig. 5B,C). Interestingly, although the overall level of Hey2 expression did not appear to differ significantly in Fkbp1a mutant versus control heart (Fig. 5C), its expression pattern was significantly altered (Fig. 5B). In normal myocardium, Hey2 transcript is restricted to compact myocardium. We found that Hey2 expression is expanded into the trabecular myocardium in Fkbp1a mutant heart (Fig. 5B). Endocardial Nrg1 expression is downstream of Efnb2 and is downregulated in Notch1 mutant endocardium (Grego-Bessa et al., 2007). Both in situ hybridization and qRT-PCR analyses confirmed that Nrg1 was upregulated in Fkbp1aaflox/–:Tie2-cre heart, as was Bmp10 (Fig. 5B,C). Taken together, our data demonstrate that Fkbp1a is an important regulator for endocardial Notch1-mediated signaling during ventricular wall formation.

Fkbp1a regulates N1ICD stability and turnover

To further define the role of Fkbp1a in Notch1 activation, we isolated mouse embryonic fibroblasts (MEFs) from Fkbp1a-deficient and control embryos (E12.5) and analyzed Notch activity. Notch-dependent Hes1-luciferase reporter plasmid was transfected into Fkbp1a-deficient and wild-type control MEFs in combination with Notch1 and delta-like 4 (Dll4) expression plasmids. The level of luciferase activity reflects the level of activated Notch1 (McGill and McGlade, 2003). From four independent sets of experiments, we found that luciferase activities were significantly enhanced in both Dll4-transfected and Dll4/Notch1 co-transfected Fkbp1a-deficient MEFs when compared with transfected wild-type MEFs (Fig. 6A). Re-introduction of Fkbp1a into the Fkbp1a-deficient cells reduced luciferase activity to a level comparable to that in wild-type cells. Consistent with these findings, western blot analysis demonstrated that the endogenous N1ICD level is significantly higher in Fkbp1a-deficient than in wild-type MEFs (Fig. 6A). To validate this finding in endothelial cells, we prepared endothelial cells from Fkbp1aaflox/– neonatal mice (supplementary material Fig. S3). Fkbp1a-deficient endothelial cells were generated by transducing the cells with Ad-Cre/eGFP. Western blot confirmed the efficient ablation of Fkbp1a in Cre-transduced Fkbp1aaflox/– mouse endothelial cells (Fkbp1aaflox/–Cre) (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aaflox/– and Fkbp1aaflox/–Cre cells (Fig. 6B).

(Fig. 4). Echocardiographic analysis demonstrated severely compromised cardiac function in these mutants, consistent with the histological findings (Fig. 4). Collectively, our observations indicate that abnormal endocardial function is likely to be the primary cause of the hypertrabeculation and noncompaction phenotypes in Fkbp1a-deficient mutants.

Notch1 and downstream endocardial signaling are altered in Fkbp1a mutant hearts

Recent studies have demonstrated that perturbation of Notch1 signaling within the endocardium impairs cardiomyocyte Bmp10 expression, ventricular trabeculation and wall formation (Grego-Bessa et al., 2007). To define whether Notch1 activation within the endocardium is altered in Fkbp1a mutants, we compared the pattern of activated N1ICD in the developing ventricular endocardial cells

Fig. 2. Cardiomyocyte-restricted ablation of Fkbp1a using H1-Ncx-cre transgenic mice. (A) Dual immunofluorescence analyses confirm the genetic ablation of Fkbp1a in developing myocardium at E12.5. Representative confocal images of heart sections co-stained with Alexa Fluor 647-conjugated anti-Fkbp1a antibody (red) and Alexa Fluor 488-conjugated anti-myosin heavy chain antibody (MF-20; green). There is a significant reduction in Fkbp1a in Fkbp1aflox/–:H1-Ncx-cre myocardium. The boxed regions of the merge are enlarged to the right. (B) Western blot confirms the efficient removal of Fkbp1a in Fkbp1aflox/–:H1-Ncx-cre heart. (C) Morphological and histological analysis of Fkbp1aflox/–:H1-Ncx-cre and control hearts at E14.5. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.
Fkbp1aflox/flox FKBPA-overexpressing endothelial cells was significantly lower than in Fkbp1aflox/flox eGFP-expressing cells (Fig. 6C, top). As expected, overexpression of FKBPA reduced Notch-dependent Hes1-luciferase activity (Fig. 6C, bottom).

To determine whether Fkbp1a could affect endogenous Notch1 expression in endothelial cells, we compared Notch1 mRNA levels in Fkbp1a-deficient and human FKBPA-overexpressing endothelial cells (Fig. 6D). Notch1 mRNA levels were not altered in Fkbp1a-deficient or FKBPA-overexpressing endothelial cells. However, the mRNA levels of the Notch1 downstream targets Efnb2, Hey1 and Hey2 were upregulated in Fkbp1a-deficient and downregulated in FKBPA-overexpressing endothelial cells, consistent with the notion that Notch1 activities are impacted directly by the intracellular levels of Fkbp1a.

Previously, we established HEK293 cell lines with overexpression of Flag-tagged human FKBPA (FKBP1A-HEK293). When we attempted to overexpress N1ICD in HEK293 and FKBP1A-HEK293 cells, we found that the N1ICD protein level was significantly reduced in FKBP1A-HEK293 cells when compared with control HEK293 cells (supplementary material Fig. S4). Given that Notch activity is closely associated with ubiquitylation-mediated degradation, this finding, along with the observation that there is no change in Notch1 mRNA levels and that N1ICD protein is significantly higher in FKBPA-deficient cells, suggested that, mechanistically, Fkbp1a regulates Notch1 activity by modulating N1ICD stability. To test this hypothesis, we compared the N1ICD protein degradation rate in human FKBPA-overexpressing (i.e. FKBP1A-HEK293) and control HEK293 cells.

The rate of N1ICD degradation was significantly greater in FKBP1A-HEK293 cells (Fig. 7Aa,b). To validate this finding in endothelial cells, we generated stable N1ICD-overexpressing Fkbp1aflox/flox cells (N1ICD-Fkbp1a flox/flox) and compared their N1ICD degradation rate with that of N1ICD-Fkbp1aflox/flox/Cre cells. N1ICD-Fkbp1a flox/flox/Cre cells had a significantly slower degradation rate than that of N1ICD-Fkbp1aflox/flox cells (Fig. 7Ac,d).

We also evaluated the rate of N1ICD translation. There is no noticeable difference in N1ICD protein synthesis between FKBP1A-HEK293 cells and controls (Fig. 7B; supplementary material Fig. S5). We next blocked ubiquitylation-mediated protein degradation using the proteasome inhibitor MG-132, and assessed the amount of ubiquitylated N1ICD in FKBP1A-HEK293 and control cells. The amount of ubiquitylated N1ICD was significantly higher in FKBP1A-HEK293 cells than in HEK293 control cells (Fig. 7C). Taken together, these data indicate that Fkbp1a regulates the level of activated Notch1 by controlling its stability via ubiquitin-mediated protein turnover.

The γ-secretase inhibitor DBZ reduces hypertrabeculation in Fkbp1a-deficient hearts

Clearly, elevated Notch signaling is directly associated with abnormal trabeculation in Fkbp1a mutant embryos. We therefore reasoned that reducing Notch1 activation in vivo would be able to rescue the ventricular morphological defects in Fkbp1a mutant hearts, thereby validating the hypothesized mechanism. We applied the γ-secretase inhibitor DBZ (Milano et al., 2004) to Fkbp1a
mutant embryos at E11.5, a stage prior to the appearance of the hypertrabeculation and noncompaction phenotypes (Chen et al., 2009). The pregnant Fkbp1a+/− females that were bred with Fkbp1a+/− males were administrated with DBZ (10 μM/kg via intraperitoneal injection, n = 6 females) and vehicle (100 μl, n = 6 females). The embryos were harvested at E13.5 and processed for morphological and histological examination. Results show that administration of DBZ significantly diminished the hypertrabeculation phenotypes in Fkbp1a-deficient hearts when compared with vehicle-treated controls (Fig. 8A,Ba). The ratio of trabeculae thickness to compact wall thickness approached a normal morphological distribution (Fig. 8Bc). Remarkably, ventricular septum formation was also greatly improved in all DBZ-treated Fkbp1a-deficient hearts.

Fig. 4. Histological and functional analyses of adult Fkbp1aflox/+;Tie2-cre mice. (A) (Left) Restricted growth of surviving Fkbp1aflox/+;Tie2-cre mice. (Right) Abnormal ventricular wall structure in Fkbp1aflox/+;Tie2-cre hearts, with hypertrabeculation and noncompaction. Arrows indicate abnormal trabecular myocardia and ventricular septum. (B) M-mode echocardiographic analysis of 2-month-old adult males. One-second traces are shown. Both fractional shortening and ejection fraction are severely compromised in Fkbp1aflox/+;Tie2-cre mutants. Values indicate mean ± s.e.m.; n = 12; *P < 0.05. LVIDd, left ventricular internal diameter diastolic; LVIDs, left ventricular internal diameter systolic; FS%, fractional shortening; LV vol d, left ventricular volume diastolic; LV vol s, left ventricular volume systolic; EF%, ejection fraction; HR, heart rate.

Fig. 5. Assessment of Notch1-mediated signaling in the developing endocardium of Fkbp1a-deficient and Fkbp1aflox/+;Tie2-cre mouse hearts. (A) Immunohistological analysis using antibody specific to activated Notch1 (N1ICD). In control heart (WT), nuclear N1ICD is mainly located in endothelial cells at the proximal end of trabeculae (arrows in a’). By contrast, N1ICD was found throughout endothelial cells in both Fkbp1a-deficient and Fkbp1aflox/+;Tie2-cre mutant hearts (arrows in b’ and c’). The boxed regions in a–c are magnified in a’–c’. (B) In situ hybridization of downstream targets of Notch1 in Fkbp1aflox/+;Tie2-cre and control hearts at E12.5. Ephrin B2 (Efnb2), neuregulin 1 (Nrg1) and Hey1 are upregulated in endocardial cells in Fkbp1aflox/+;Tie2-cre hearts. Bmp10 is upregulated in both trabecular and compact myocardium. Interestingly, Hey2 expression is upregulated in trabecular myocardium compared with controls. Arrows indicate positive signals. (C) qRT-PCR confirms the expression levels of Notch1, Efnb2, Nrg1, Hey1, Hey2 and Bmp10 in Fkbp1aflox/+;Tie2-cre hearts at E13.5. Interestingly, despite the altered expression pattern, the overall expression level of Hey2 is not altered. Error bars indicate s.e.m. LV, left ventricle.
Consistent with the histological findings, in situ hybridization demonstrated that the levels of endocardial Efnb2 and Nrg1, as well as myocardial Bmp10, were significantly reduced in DBZ-treated as compared with vehicle-treated Fkbp1a−/− hearts (Fig. 8C). As further validation, Ki67 immunohistological staining showed that DBZ treatment resulted in strong suppression of cardiomyocyte proliferative activities in both trabecular and compact myocardium within Fkbp1a−/− hearts, showing a more wild-type growth profile (Fig. 8C; supplementary material Fig. S6). However, despite significant reductions in cardiomyocyte hyperproliferation and the myocardial hypertrabeculation phenotype of DBZ-treated mutant hearts, the thickness of the compact wall (ventricular compaction) did not seem to show significant improvement upon DBZ treatment, suggesting that a Notch-independent signaling pathway might contribute in part to the regulation of ventricular wall compaction. Taken together, these findings define an important role for Fkbp1a in regulating Notch1-mediated signaling during ventricular wall development.

**DISCUSSION**

Ventricular hypertrabeculation and noncompaction are congenital myocardial anomalies that result from disrupted development of the ventricular wall and have been increasingly recognized in the clinic (Chen et al., 2009). Genetic analysis of patients, mostly with isolated forms of ventricular noncompaction without CHDs (congenital heart defects), has demonstrated a broad genetic heterogeneity, suggesting a complex and/or multifactorial network contributing to the etiology and pathogenesis (Chen et al., 2009). Currently, surviving patients carrying FKBP1A mutations have not been reported. Given the pivotal role of Fkbp1a in ventricular wall formation and the embryonic lethal phenotype of Fkbp1a-deficient mice, patients with a germline FKBP1A loss-of-function mutation would seem to have little chance of survival in utero. However, mouse genetic manipulation provides an excellent opportunity to determine the cause and the pathogenic network that contributes to the ventricular hypertrabeculation and noncompaction, allowing for the identification of genetic pathways that regulate normal ventricular development.
As an isomerase, Fkbp1a presumably functions as a chaperone in maintaining the appropriate conformation of its substrate proteins/peptides, which could be relevant to the regulation of their functional state and/or stability (Lu et al., 2007). Our findings suggest that Fkbp1a has a role in regulating endocardial Notch1 activity, which is crucial to ventricular wall formation. Our findings are entirely consistent with recent reports by Mysliwiec and colleagues that upregulation of Notch1 in endocardial cells leads to ventricular hypertrabeculation and noncompaction (Mysliwiec et al., 2011; Mysliwiec et al., 2012). Most recently, Yang and colleagues demonstrated that upregulated Notch2 activity in Numb/Numb-like compound deficient mutants contributes to Bmp10 upregulation and ventricular hypertrabeculation and noncompaction phenotypes (Yang et al., 2012), further validating our conclusion. However, it should be noted that Tie2-Cre is not endocardial specific, but targets to all endothelial cells including coronary endothelial cells. Nfatc1-Cre expression is initially restricted to the endocardium, and subsequently marks cardiac vascular endothelial cells as the coronary vasculature system develops (Wu et al., 2012). Owing to the spatial-temporal pattern of Cre activity within these lines, we currently cannot distinguish the relative contribution of the different cardiac endothelial cell subtypes. Despite this limitation, it is nonetheless clear that Fkbp1a signaling in cardiac endothelial cells plays a major role in regulating ventricular trabeculation and compaction.

The biochemical mechanism by which Fkbp1a regulates Notch1 activity is likely to involve controlling the stability of N1ICD. Indeed, biochemical analyses in endothelial cells, MEFs and HEK293 cells indicate that Fkbp1a can modulate the stability of activated Notch1. It is well-known that Notch signaling is regulated by post-translational modification events. Activated N1ICD is cleared and thereby inactivated within the cell by ubiquitylation and subsequent proteolysis via the proteasome. This proteasome-dependent degradation of N1ICD is thought to be mediated by interactions of its PEST (proline, glutamic acid, serine and threonine) domain and a specific E3 ubiquitin ligase, Fbxw7 (Oberg et al., 2001). As a known cis-trans peptidyl-prolyl isomerase, one attractive hypothesis is that Fkbp1a regulates the conformation of N1ICD via the proline residues contained within its PEST domain. To test this hypothesis, we used co-immunoprecipitation to determine whether Fkbp1a is able to directly bind N1ICD. However, we have not been able to consistently detect a direct molecular interaction between N1ICD and Fkbp1a (data not shown). This negative result could be due to technical difficulties in capturing transient hit-and-run type interactions between Fkbp1a and N1ICD. Further study employing systematic biochemical analyses will be necessary to determine whether Fkbp1a directly interacts with N1ICD to facilitate its ubiquitylation, or operates via an indirect mechanism. In addition, as Fkbp1a interacts with several other protein complexes, our findings do not exclude the potential involvement of other signaling pathways in contributing to the ventricular wall defects in Fkbp1a mutant hearts. Indeed, although the Notch inhibitor DBZ profoundly reduced cellular hyperproliferation and hypertrabeculation, the noncompaction phenotype was barely affected by DBZ (Fig. 8). This could simply reflect the complex dynamics of ventricular compaction, which involves multiple Notch-mediated signaling pathways in multiple cardiac cell lineages, or there could be an additional unknown parallel signaling pathway(s) that synergistically controls ventricular compaction.

Endothelial cell-restricted ablation of Notch1 and Rbpjk downregulates the expression of both Efnb2 and Nrg1 within endothelial cells and Bmp10 within cardiomyocytes (Grego-Bessa et al., 2007). Both Bmp10 and Nrg1 have been suggested to have a major functional role in the spatial-temporal control of cardiomyocyte proliferation and ventricular trabeculation (Grego-
Bessa et al., 2007; Shi et al., 2003). Interestingly, Bmp10 expression can be specifically upregulated by Nrg1 (Kang and Sucov, 2005). In addition, Nrg1 hypomorphic mutants have a significantly reduced Bmp10 expression level (Lai et al., 2010). These findings, both in vitro and in vivo, suggest the interesting idea that Bmp10 is likely to be a downstream target of Nrg1. Endocardial-derived Nrg1 is of particular interest as it signals through its receptor complex, ErbB2/4, which is present only in adjacent cardiomyocytes (Lemmens et al., 2006), providing a unique mechanism for intercellular interactions between two different cardiac cell populations. In zebrafish, the heart is highly trabeculated. A recent study using zebrafish heart as a model system demonstrated that a Nrg1-ErbB2/4 signaling cascade has a dual function: in addition to promoting cardiomyocyte proliferation, it regulates cardiomyocyte delamination and migration (Liu et al., 2010). This dual activity ultimately controls trabeculation in zebrafish. Interestingly, analysis of endocardial-restricted Rbpjk knockouts has shown that exogenous Bmp10 rescues the observed myocardial proliferative defect, whereas Nrg1 does not (Grego-Bessa et al., 2007). Apparently, the Nrg1-ErbB cascade is likely to be part of the evolutionarily conserved mechanism for the regulation of cardiomyocyte delamination that allows for ventricular trabeculation (Liu et al., 2010) or for the regulation of cardiomyocyte differentiation via its pro-differentiation activity (Lai et al., 2010). It would be interesting to determine whether Bmp10 has a similar function in regulating cardiomyocyte proliferation and trabeculation in the highly trabeculated zebrafish heart.

In summary, our study demonstrates a novel genetic and biochemical role for Fkbp1a in regulating Notch1 activity within the endocardium that in part facilitates the intercellular interplay between endocardium and myocardium necessary to define the normal levels of ventricular trabeculation and compaction in the mammalian heart.

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