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

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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 trabeculation and compaction. Further analysis demonstrated that Fkbp1a is a novel negative modulator of activated Notch1. Activated Notch1 (N1ICD) was significantly upregulated in Fkbp1a systemically deficient mice, implying its involvement in regulating cardiac trabeculation and compaction. Overexpression of Fkbp1a significantly reduced the stability of N1ICD and direct inhibition of Notch signaling significantly reduced hypertrabeculation in Fkbp1a-deficient mice. 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

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 hypertroabeculation (LVHT)] (Finagher, 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 1a (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...
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 Fkbplα-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 (Pushamforoush et al., 2004). The elevated level of Bmp10 observed in both Fkbplα 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 Fkbplα 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 at the distal end of the myocardium, where trabeculation initiates, and was significantly less abundant in endocardial cells near the proximal end of the trabecular endocardium (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 Fkbplα in regulating ventricular trabeculation and compaction, and its pathogenetic role in LVNC, we generated Fkbplα conditional knockouts using the Cre-loxP recombination system. Ablating Fkbplα in cardiac progenitor cells via the use of Nkx2.5Cre+ mice (Moses et al., 2001), we were able to generate Fkbplαlox/–;Nkx2.5Cre mice that recapitulate the ventricular hypertrabeculation and noncompaction with full phenotypic resemblance of systemic Fkbplα null mice. By contrast, ablation of Fkbplα using cardiomyocyte-specific Cre lines did not give rise to abnormal ventricular wall formation. Surprisingly, endothelial-restricted ablation of Fkbplα phenocopied the ventricular hypertrabeculation and noncompaction observed in Fkbplα systemically deficient mice, suggesting that endocardium plays an important role in regulating ventricular trabeculation and compaction. Biochemical and molecular analyses demonstrated that Fkbplα regulates Notch1-mediated signaling within developing endocardial cells. An excess of activated Notch1 is found in Fkbplα-ablated endothelial cells and is the likely cause of the observed Fkbplα mutant phenotypes. Treatment of Fkbplα-deficient mice with Notch inhibitors reduces the hypertrabeculation phenotype. Taken together, we have revealed a mechanism whereby Fkbplα modulates a critical level of Notch1 activity that is required for regulating ventricular chamber and wall formation.

MATERIALS AND METHODS

Generation of Fkbplα floxed and conditional knockout mice

The generation of Fkbplα floxed mice (Fkbplαlox/lox) has been described previously (Maruyama et al., 2011). To achieve cell lineage-restricted deletion of Fkbplα in the developing heart, Fkbplαlox 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 Fkbplα+/Cre mice followed by an additional intercross onto Fkbplαlox/lox mice. For the most part, we used Fkbplαlox/–;Cre and Fkbplαlox/–;Cre as the experimental group and Fkbplαlox/lox;Cre and Fkbplαlox/lox;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 at 4°C overnight. Fixed 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-Fkbplα (FKBP12) 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 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-Fkbplα 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 512x512 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 system (Vevo 770, VisualSonics) equipped with a 40 MHz mechanical scan (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 left ventricle. LVID and end-diastolic volume were calculated with Vevo Analysis software (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 512x512 pixel resolution with a Nikon 20× oil immersion objective (2 μm intervals).

Cell transfection and protein stability assay

Flag-tagged human FKBP1A 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, F804), 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 FKBP1A tagged with Flag epitope (FKBP1A-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
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 FKBPA-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 analysis.

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-EGFP. 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 Fkbp1aflox/flox 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 (Ptprc) and anti-Mac1 (Itgam) antibodies (all eBiosciences). The CD45− Mac1− CD31+ populations were sorted on a FACS Aria (Becton Dickinson). The resulting endothelial cells were cultured in EGM2 (endothelial growth medium-2, Lonza) with 10% FBS, and 7 days after initiating culture the adherent sorted endothelial cells were cultured in EGM2 (endothelial growth medium). N1ICD-expressing endothelial cells, X-tremeGENE HP DNA transfection reagents (Roche Diagnostics) were used according to the manufacturer’s instructions. To test Notch1 protein stability in endothelial cells, N1ICD-PCS2 was co-transfected into Fkbp1aflox/flox endothelial cells with empty pCDNA3 plasmid at a 10 to 1 ratio using FuGENE HD (Roche Applied Bioscience) according to the manufacturer’s instructions. Cells stably expressing N1ICD were selected by neomycin resistance with G418 (Invitrogen, 400 ng/ml) for 8 hours before being lysed in Ni-agarose lysis 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 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 Nkx2.5cre to ablate the Fkbp1a gene. Nkx2.5cre delivers Cre within cardiac progenitor cells in the cardiac crescent, which give rise to cardiomyocytes, endocardial 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). Fkbp1aΔN1ICD-Nkx2.5cre 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 Fkbp1aΔN1ICD 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, Fkbp1aΔN1ICD:H1-Ncx-cre mice demonstrated normal ventricular chamber formation and survived to adulthood. Histologically, no hypertrabeculation or noncompaction was found in Fkbp1aΔN1ICD: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 Fkbp1aΔN1ICD 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 Nkx2.5cre.
Despite this early Cre activity, Fkbp1a\(^{\text{lox/–}}\):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 hypertroebulation and noncompaction in Fkbp1a-deficient mice, and instead imply another cell lineage or lineages as the primary cause.

Epicardium has been implicated to play an important role in supporting ventricular wall growth and, potentially, compaction (Sucof et al., 2009). To test whether epicardial Fkbp1a contributes to hypertroebulation or noncompaction, we ablated Fkbp1a from epicardial cells using Tbx18\(^{\text{Cre–}}\) mice (Cai et al., 2008). Tbx18\(^{\text{Cre–}}\) 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\(^{\text{lox/–}}\):Tbx18\(^{\text{Cre–}}\) 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\(^{\text{lox/–}}\):Wnt1-cre mutants (Table 1; supplementary material Fig. S2). When considering the cell lineages marked by Nkx2.5\(^{\text{Cre–}}\), the endocardium remained untested. Therefore, we next sought to specifically ablate Fkbp1a from the endocardium.

### 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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<tbody>
<tr>
<td>Cardiac</td>
<td>Nkx2.5(^{\text{Cre–}})</td>
<td>100% embryonic lethality with ventricular hypertroebulation, 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 hypertroebulation, noncompaction and VSD</td>
<td>45, 22, 31 (E13.5 and E14.5)</td>
</tr>
<tr>
<td>Epicardial</td>
<td>Tbx18(^{\text{Cre–}})</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>
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*The number of mutant and control embryos analyzed from the indicated number of litters, respectively.

VSD, ventricular septal defect.

### 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 hypertroebulation 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\(^{\text{lox/–}}\):Tie2-cre mice phenocopied the ventricular hypertroebulation and noncompaction seen in Fkbp1a-deficient hearts (Fig. 3; Table 1). Approximately 35% (15/45) of E13.5-14.5 Fkbp1a\(^{\text{lox/–}}\):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\(^{\text{lox/–}}\):Tie2-cre embryos 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\(^{−/−}\) hearts, including various degrees of hypertroebulation, 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\(^{\text{lox/–}}\):Tie2-cre mice (compared with just ~5% of Fkbp1a\(^{−/−}\) mice) survived in utero, most of these survivors died within 8 weeks of birth, probably owing to compromised cardiac function (see below) and defects in T cell function (data not shown); the surviving Fkbp1a\(^{\text{lox/–}}\):Tie2-cre mice exhibited milder hypertroebulation and noncompaction phenotypes.
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 activities were significantly enhanced in both Dll4-transfected and Dll4/Notch1 co-transfected MEFs when compared with transfected wild-type MEFs (Fig. 6 A). 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 Fkbp1a−/−-deficient and wild-type control MEFs in 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 Fkbp1aflax/flax mouse endothelial cells (Fkbp1aflax/flax/Cre) (Fig. 6B). Hes1-luciferase reporter assays were performed in Fkbp1aflax/flax and Fkbp1aflax/flax/Cre cells (Fig. 6B). Similar to the finding in MEFs, Notch-dependent luciferase activities were significantly higher in Fkbp1aflax/flax/Cre than in Fkbp1aflax/flax endothelial cells (Fig. 6B).

We transfected activated Notch1 (N1ICD-PCS2) into Fkbp1aflax/flax endothelial cells in conjunction with pCDA3-FKBP1A (human) or control pCDA3-eGFP vector. The N1ICD protein level in of Fkbp1a-deficient, Fkbp1aflax/flax: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 Fkbp1aflax/flax: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 Fkbp1aflax/flax: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.

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. 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.

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 Fkbp1aflax/flax-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 Fkbp1aflax/flax-H1-Ncx-Cre heart. (C) Morphological and histological analysis of Fkbp1aflax/flax-H1-Ncx-Cre and control hearts at E14.5. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.
FKBP1A-overexpressing endothelial cells was significantly lower than in Fkbp1aflox/flox eGFP-expressing cells (Fig. 6C, top). As expected, overexpression of FKBP1A 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 FKBP1A-overexpressing endothelial cells (Fig. 6D). Notch1 mRNA levels were not altered in Fkbp1a-deficient or FKBP1A-overexpressing endothelial cells. However, the mRNA levels of the Notch1 downstream targets Efnb2, Hey1 and Hey2 were upregulated in Fkbp1a-deficient and downregulated in FKBP1A-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 FKBP1A (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 Fkbp1a-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 FKBP1A-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-Fkbp1a flox/flox/Cre cells. N1ICD-Fkbp1a flox/flox/Cre cells had a significantly slower degradation rate than that of N1ICD-Fkbp1a flox/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

**Fig. 3. Endothelial-restricted ablation of Fkbp1a using Tie2-cre transgenic mice.** (A) Dual immunofluorescence and PCR analyses to confirm the genetic ablation of Fkbp1a in developing endocardial/endothelial cells at E12.5. Representative confocal images of heart sections are co-stained with Alexa Fluor 647-conjugated anti-Fkbp1a antibody (red) and Alexa Fluor 488-conjugated anti-CD31 antibody (green). There is a significant reduction of Fkbp1a in the CD31-positive endothelial cells in Fkbp1aflox/–:Tie2-cre hearts. Beneath is shown a diagnostic genomic PCR analysis of DNA samples isolated from E13.5 heart alongside a schematic of the Fkbp1aflox/– allele showing the location of diagnostic primers p1, p2 and p3. E, exon. A specific p3-p2 band represents the allele after Cre-loxP-mediated recombination. (B) Morphological and histological analysis of Fkbp1aflox/–:Tie2-cre and control embryos and hearts at E14.5. Fkbp1aflox/–:Tie2-cre embryos are edematous (red arrow). The mutant hearts lack a normal ventricular groove (yellow arrows) and demonstrate hypertrabeculation and noncompaction (black arrows). RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; VSD, ventricular septal defect.
mutant embryos at E11.5, a stage prior to the appearance of the hypertrabeculation and noncompaction phenotypes (Chen et al., 2009). The pregnant \textit{Fkbp1a}+/− females that were bred with \textit{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 \textit{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. 8Bbc). Remarkably, ventricular septum formation was also greatly improved in all DBZ-treated \textit{Fkbp1a}-deficient hearts.

![Fig. 4. Histological and functional analyses of adult \textit{Fkbp1a}+/−:Tie2-cre mice.](image)

(A) (Left) Restricted growth of surviving \textit{Fkbp1a}+/−:Tie2-cre mice. (Right) Abnormal ventricular wall structure in \textit{Fkbp1a}+/−: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 \textit{Fkbp1a}+/−: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 \textit{Fkbp1a}+/− and \textit{Fkbp1a}+/−:Tie2-cre mouse hearts.](image)

(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 \textit{Fkbp1a}-deficient and \textit{Fkbp1a}+/−:Tie2-cre mutant hearts (arrows in b’ and c’). The boxed regions in a–c are magnified in a’–c’. (B) \textit{In situ} hybridization of downstream targets of Notch1 in \textit{Fkbp1a}+/−:Tie2-cre and control hearts at E12.5. Ephrin B2 (\textit{Efnb2}), neuregulin 1 (\textit{Nrg1}) and \textit{Hey1} are upregulated in endocardial cells in \textit{Fkbp1a}+/−:Tie2-cre hearts. \textit{Bmp10} is upregulated in both trabecular and compact myocardium. Interestingly, \textit{Hey2} expression is upregulated in trabecular myocardium compared with controls. Arrows indicate positive signals. (C) \textit{qRT-PCR} confirms the expression levels of Notch1, \textit{Efnb2}, \textit{Nrg1}, \textit{Hey1}, \textit{Hey2} and \textit{Bmp10} in \textit{Fkbp1a}+/−:Tie2-cre hearts at E13.5. Interestingly, despite the altered expression pattern, the overall expression level of \textit{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 pathogenetic 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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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References


Fig. S1. Analysis of X-Gal-stained E9-E18 embryos from H1-Ncx-cre; R26R indicator mice. (A-D) H1-Ncx-cre transgenic males were bred to R26R females. Whole-mount X-Gal-stained embryos were either photographed whole at E9 (A), E9.5 (B) or E12 (C) or partially dissected to expose the heart (D) at E18 to visualize Cre-expressing cells. Note that lacZ expression is restricted to the heart (A-C). Fetal hearts (D) continue to exhibit robust Cre activity within the ventricles and atria but now Cre activity is also observed within the smooth muscle cells lining the major lung vasculature.

Fig. S2. Normal ventricular wall formation upon myocardial, epicardial and neural crest cell-specific ablation of Fkbp1a. cTnt-cre mice were used to ablate Fkbp1a in early developing myocardium. Fkbp1a<sup>fl/+</sup>:cTnt-cre mice develop a normal ventricular wall. Several lines of evidence suggest that epicardial-derived signaling can exert effects on ventricular wall growth and formation. As Nkx2.5<sup>cre</sup> deletes Fkbp1a from the epicardial lineage, we examined whether Fkbp1a function within the epicardium regulates ventricular wall formation using Tbx18<sup>cre</sup> mice, which have previously been shown to drive Cre mainly to the developing epicardium and, potentially, a small population of cardiomyocytes. Similar to results obtained with cardiomyocyte-specific Cre drivers, Fkbp1a<sup>fl/+</sup>:Tbx18<sup>cre</sup> mice exhibit normal development of the ventricular wall and survive to adulthood. Additionally, neural crest cell-restricted ablation of Fkbp1a via Wnt1-cre mice also did not generate a left ventricular noncompaction phenotype. Combined, these data indicate that cardiomyocyte, epicardial and cardiac neural crest cell populations are not the primary cell types contributing to the ventricular hypertrabeculation and noncompaction phenotypes in Fkbp1a-deficient mice.
Fig. S3. Characterization of murine endothelial cells isolated from neonatal Fkbp1a<sup>fl</sup>/<sup>fl</sup> mice. (A) Initial isolation and characterization of Fkbp1a<sup>fl</sup>/<sup>fl</sup> endothelial cells using the endothelial surface marker CD31. (a) Flow cytometry analysis for CD31-positive cells. (b) Immunofluorescent staining using anti-CD31 antibody to confirm flow cytometry data. (B) Confirmation of endothelial cell morphology and molecular markers for Fkbp1a<sup>fl</sup>/<sup>fl</sup> endothelial cells by immunofluorescence staining. (a) Typical endothelial morphology of cells continuously cultured and maintained for 74 days (phase contrast image, ×100). (b,c) Representative immunofluorescence images of Fkbp1a<sup>fl</sup>/<sup>fl</sup> cells stained with antibodies against the endothelial surface markers Flk1 and Vcam1 (×200). (C) MatriGel tube-forming assay confirming the functionality of Fkbp1a<sup>fl</sup>/<sup>fl</sup> endothelial cells. Fkbp1a<sup>fl</sup>/<sup>fl</sup> endothelial cells start to form a tube after 5 hours of culture (a) and are able to form well-defined tubular structures within 20 hours (b).

Fig. S4. Analysis of N1ICD level in FKBPIA-overexpressing cells. (A) Western blot analysis shows that the N1ICD protein level is significantly lower in FKBPIA-HEK293 cells than in HEK293 control cells. (B) qRT-PCR analysis shows similar N1ICD mRNA level in FKBPIA-HEK293 and control cells.
Fig. S5. Assessment of the rate of increase of N1ICD in FKBP1A-HEK293 and HEK293 cells. There is no statistical difference in the rate of increase of N1ICD between FKBP1A-overexpressing FKBP1A-HEK293 and control HEK293 cells.

Fig. S6. Quantitative comparison of proliferative activities of DBZ-treated Fkbp1a mutant and control hearts. DBZ treatment significantly reduced cellular proliferative activity in both compact wall and trabecular myocardium of Fkbp1a-deficient hearts.