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Fibulin-1 is required during cardiac ventricular morphogenesis for versican cleavage, suppression of ErbB2 and Erk1/2 activation and to attenuate trabecular cardiomyocyte proliferation

Marion A. Cooley[1], **Victor M. Fresco**[1], **Margaret E. Dorlon**[1], **Waleed O. Twal**[1], **Nathan V. Lee**[2], **Jeremy L. Barth**[1], **Christine B. Kern**[1], **M. Luisa Iruela-Arispe**[2], and **W. Scott Argraves**[1,3]

[1]Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, Charleston, SC 29425 USA

[2]Department of Molecular, Cell and Developmental Biology and Molecular Biology Institute, University of California, Los Angeles, CA 90095 USA

Abstract

Background—Trabeculation is an integral component of cardiac ventricular morphogenesis and is dependent on the matrix metalloproteinase, ADAMTS1. A substrate of ADAMTS1 is the proteoglycan versican which is expressed in the developing ventricle and which has been implicated in trabeculation. Fibulin-1 is a versican and ADAMTS1-binding extracellular matrix protein required for ventricular morphogenesis. Here we investigated the involvement of fibulin-1 in ADAMTS1-mediated cleavage of versican in vitro, and the involvement of fibulin-1 in versican cleavage in ventricular morphogenesis.

Results—We show that fibulin-1 is a cofactor for ADAMTS1-dependent in vitro cleavage of versican V1, yielding a 70-kDa amino-terminal fragment. Furthermore, fibulin-1-deficiency in mice was found to cause a significant reduction (>90%) in ventricular levels of the 70-kDa versican V1 cleavage product and a 2-fold increase in trabecular cardiomyocyte proliferation. Decreased versican V1 cleavage and augmented trabecular cardiomyocyte proliferation in fibulin-1 null hearts is accompanied by increased ventricular activation of ErbB2 and Erk1/2. By contrast, versican deficiency was found to lead to decreased cardiomyocyte proliferation and reduced ventricular trabeculation.

Conclusion—We conclude that fibulin-1 regulates versican-dependent events in ventricular morphogenesis by promoting ADAMTS1 cleavage of versican leading to suppression of trabecular cardiomyocyte proliferation mediated by the ErbB2-Map kinase pathway.

Keywords

trabeculation; ventricular noncompaction; versican; fibulin-1; ADAMTS-1; ErbB2; Brg1; Erk1/2; DPEAAE; cardiomyocyte; knockout

[3]Address correspondence to: W. Scott Argraves, Ph.D., Department of Regenerative Medicine and Cell Biology, Medical University of South Carolina, 173 Ashley Avenue, Charleston, SC 29425-2204 Tel.: (843) 792-5482, Fax: (843) 792-0664; argraves@musc.edu.

Competing interests statement
The authors declare no competing financial interests.

Introduction

Cardiac trabeculae are ridges of endothelium lined myocardium that protrude into the lumen of the ventricular chamber (Sedmera et al., 1997; Sedmera et al., 2000). These structures are formed during development through a process that involves myocyte proliferation and migration toward the endocardium (Ong et al., 1998). With progressive development, trabeculae coalesce into the compact layer of the myocardium, the interventricular septum and papillary muscles (Rychterova, 1971; Sedmera et al., 2000).

Studies of mouse mutants have provided insights into the molecular mechanisms of trabeculation (Chen et al., 2009). Mutants have been described that display abnormalities in trabeculation that range from a lack of trabeculae to an increase in trabecular cardiac myocyte densities. For example, a lack of trabeculation is associated with loss of function mutations in genes related to Notch and ErbB signaling (i.e., *Notch1, Nrg1, Bmp10, ErbB2, ErbB4, Pofut1, RBPJk*) (Gassmann et al., 1995; Lee et al., 1995; Grego-Bessa et al., 2007; Okamura and Saga, 2008) as well as extracellular matrix (ECM) related genes *Has2* (Camenisch et al., 2000) and other genes including *Brg1* (Stankunas et al., 2008). By contrast, genes including *Fkbp1a, Peg1*, *Jarid2,* and *ADAMTS1*, when deleted lead to augmented trabeculation (Shou et al., 1998; Takeuchi et al., 1999; King et al., 2002; Stankunas et al., 2008).

Evidence for direct and indirect interactions between many of the gene products associated with trabeculation is the basis for an integrated trabeculation regulatory network. For example, Brg1 serves as a transcriptional repressor of a secreted matrix metalloproteinase (MMP), ADAMTS1, expressed by the ventricular endocardium during early stages of trabeculation (Stankunas et al., 2008). ADAMTS1 cleaves the proteoglycan versican (Sandy et al., 2001), which is expressed by the trabeculated ventricular myocardium (Henderson and Copp, 1998; Stankunas et al., 2008). Versican interacts with the glycosaminoglycan, hyaluronan, which is synthesized by both the ventricular myocardium and endocardium (Stankunas et al., 2008) via the hyaluronan synthase, Has2. Under conditions of Has2-deficiency, the lack of hyaluronan leads to defective activation of several ErbB receptor tyrosine kinases, ErbB2 and ErbB3 (Camenisch et al., 2002). The molecular basis of hyaluronan-mediated activation of these receptors is not clear, but may involve hyaluronan being in complex with versican (Koyama et al., 2007). Indeed, versican has been shown to stimulate phosphorylation of ErbB1 (Wu et al., 2004; Xiang et al., 2006) through a process that appears to involve direct interaction between ErbB1 and the G3 domain of versican, which contains two EGF-like motifs (Zhang et al., 1998). This interaction leads to signaling events that include Erk1/2 phosphorylation, increased ErbB1 expression (Wu et al., 2004) and ErbB1-dependent cell proliferation (Zhang et al., 1998).

Fibulin-1 is an ECM protein that when deleted in mice leads to cardiac abnormalities including thin ventricular myocardium (Cooley et al., 2008). Furthermore, fibulin-1 interacts with two key components of the trabeculation regulatory network, versican and ADAMTS1 (Aspberg et al., 1999; Lee et al., 2005). Based on findings that fibulin-1 serves as a cofactor for ADAMTS1-mediated cleavage of the versican related proteoglycan, aggrecan (Lee et al., 2005), as well as a cofactor for ADAMTS5-mediated cleavage of versican (McCulloch et al., 2009), fibulin-1 may also promote ADAMTS1-mediated cleavage of versican. The significance of putative fibulin-1/ADAMTS1-mediated cleavage of versican in the context of ventricular trabeculation is not known, but the versicanase activity of ADAMTS family members in several tissues appears to be important to regulation of the growth promoting effects of versican (McCulloch et al., 2009; Kern et al., 2010). Here we investigated the involvement on fibulin-1 in versican cleavage in association with cardiac ventricular morphogenesis using a fibulin-1-deficient mouse model (Cooley et al., 2008).

Results

Fibulin-1 acts as a cofactor for ADAMTS1-mediated cleavage of versican

ADAMTS1 cleaves the proteoglycans aggrecan and versican (Kuno et al., 2000; Sandy et al., 2001). Fibulin-1 is a cofactor for both ADAMTS1-mediated cleavage of aggrecan (Lee et al., 2005) and ADAMTS5-mediated cleavage of versican (McCulloch et al., 2009). Using purified components, we evaluated the ability of fibulin-1 to influence *in vitro* cleavage of versican by ADAMTS1. As shown in Figure 1, incubation of versican with ADAMTS1 resulted in the production of an ~70 kDa fragment capable of binding to biotinylated hyaluronan. The fragment size and ability to bind hyaluronan was consistent with it being the 70 kDa fragment known to be liberated from the amino terminus of versican V1 by ADAMTS1 cleavage (Sandy et al., 2001) and which contains a hyaluronan binding site (Matsumoto et al., 2003). This was supported by the finding that the fragment reacted with antibody to the neo epitope (DPEAAE) created as a result of the ADAMTS1 cleavage of versican (Fig. 1). Addition of fibulin-1 to the reaction mixture increased the amount of 70 kDa fragment produced by ~2-fold. These findings suggest that fibulin-1 promotes ADAMTS1 mediated cleavage of versican V1.

Coincident expression of fibulin-1 and versican V0/V1 in the trabecular layer of the embryonic mouse heart

Fibulin-1 deficiency in mice had been shown to lead to abnormalities of the ventricular myocardium (Cooley et al., 2008), but fibulin-1 expression in the developing cardiac ventricles had not been established. Immunohistological analysis showed fibulin-1 to be in close proximity to the ventricular endocardium lining the trabeculae in embryonic hearts from E10.5 to E15.5 (Figs. 2A-C, arrowheads, and Fig. 3A and *D*). Little to no fibulin-1 immunolabel was detectable in the compact myocardial layer at these stages (Fig. 2). Analysis of LacZ expression in the hearts of mice bearing β-galactosidase under the control of the fibulin-1 promoter showed pronounced fibulin-1 expression in the trabecular myocardium (Fig. 2D and *E*). Little or no fibulin-1-LacZ expression was detected in endocardial cells that overlay the trabeculae (Fig. 2E). Fibulin-1-LacZ expression was also observed in epicardial cells as well as cells sparsely spread throughout the compact myocardium (Fig. 2D and *F*). Analysis of RNA from embryonic ventricles shows that fibulin-1 transcript levels (both C and D variants) are highest at E9.5 and gradually decline through E16.5 (Fig. 4A and *B*).

We next sought to compare the pattern of ventricular expression of fibulin-1 with that of versican, specifically the versican variants that serve as ADAMTS1 substrates, V0 and V1. Using antibodies that detect the GAG beta domain common to both V0 and V1 versican variants, immunohistological analysis showed that V0/V1 variants are deposited in the ECM space underlying the trabecular endocardium (Fig. 3B and *E*), similar to fibulin-1. By analysis of ventricle RNA preparations using qPCR, we found that versican V0/V1 transcript levels were high at E9.5 and subsequently decline through E16.5 (Fig. 4C), similar to the profile of expression of fibulin-1.

Versican V0/V1 cleavage in the developing heart

Previous studies have shown that ADAMTS1 is expressed by the endocardium that overlies the developing trabeculae (Stankunas et al., 2008). ADAMTS1 expressed in this region has been implicated in versican cleavage to yield the anti-DPEAAE reactive amino terminal versican V1 fragment (Stankunas et al., 2008). By immunohistological analysis we observed little anti-DPEAAE immunostaining in the ECM space underlying the trabecular endocardium at E11.5 (Fig. 3C), whereas later in development (i.e., E13.5), anti-DPEAAE

immunostaining was apparent in this region (Fig. 3F). Relatively strong anti-DPEAAE immunoreactivity is also apparent in the epicardium at E13.5.

Immunoblot analysis of extracts of embryonic mouse heart ventricles was used as an additional approach to evaluate the kinetics of expression of versican V0/V1, the 70-kDa amino terminal versican V1 cleavage fragment and fibulin-1 during ventricular development. The specificity of the DPEAAE antiserum was verified using the DPEAAE peptide to block antibody interaction with the 70 kDa fragment (*data not shown*). In agreement with findings from qPCR analysis (Fig. 4), levels of versican V0/V1 (detected with a GAG beta domain antiserum) and fibulin-1 in the ventricle extracts were found to be high at E9.5 and decline between E11.5 and E12.5 (Fig. 5). By contrast, levels of the anti-DPEAAE-reactive 70 kDa fragment of versican V1 sharply increase after E9.5 and appear to peak around E11.5 and diminish thereafter (Fig. 5). Together, the findings indicate that during ventricular morphogenesis, diminishing levels of versican V0/V1 mRNA and protein are accompanied by an increase in cleavage of versican V1 to yield the 70 kDa amino terminal fragment. The profile of 70 kDa fragment expression during ventricular morphogenesis (Fig. 5C) peaked at E11.5 with levels of both ADAMTS1 and ADAMTS5 mRNA peaking at E12.5 (Fig. 4D and *E*). By contrast, the expression of ADAMTS9 remained relatively constant through the E9.5-16.5 period of ventricular morphogenesis (Fig. 4F).

Ventricle extracts from fibulin-1 nulls have reduced levels of versican V1 cleavage fragment

We next evaluated the influence of fibulin-1 deficiency on the levels of the 70 kDa versican cleavage fragment in embryonic cardiac ventricle extracts. By immunoblot analysis it was found that there was a 92% decrease in the level of the anti-DPEAAE reactive 70 kDa fragment of versican V1 in extracts from E13.5 fibulin-1 null ventricles as compared wildtype (Fig. 6) ($p=0.01$, $n=4$ for each genotype). A similar reduction in the level of 70 kDa fragment was observed in ventricle extracts from E15.5 fibulin-1 nulls versus wildtype embryos (*data not shown*). By contrast, immuno-slot blot analysis using antiserum to an epitope present in the versican GAG beta domain of both intact versican V0/V1 and the carboxy terminal versican V0/V1 cleavage fragment, showed no significant difference in immunoreactive material between extracts from ventricles of null and wildtype embryos ($p=0.45$, $n=4$ for each genotype) (Fig. 6). Similarly, levels of the link protein, Hapln1, were not significantly different between wildtype and fibulin-1 null extracts (Fig. 6). These findings suggest that fibulin-1 is crucial for generation of the 70 kDa fragment of versican V1 during cardiac development.

Fibulin-1 deficiency does not influence expression of ADAMTS1 or versican in embryonic ventricles

qPCR was performed on E13.5 ventricular RNA preparations to evaluate the effect of fibulin-1 deficiency on expression of ADAMTS family members known to cleave versican including ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS9 and ADAMTS20 (Sandy et al., 2001; Somerville et al., 2003; McCulloch et al., 2009). The results showed that fibulin-1 deficiency did not significantly alter the expression of ADAMTS1, ADAMTS4, ADAMTS5, ADAMTS9 and ADAMTS20 (Fig. 7A). Furthermore, versican V0/V1 transcript levels in ventricular RNA from fibulin-1 nulls were not significantly different from those of wildtype hearts (Fig. 7B). These findings strengthen the possibility that the basis for reduced levels of 70 kDa cleavage product in fibulin-1 nulls relates to the absence of a critical ADAMTS cofactor (i.e., fibulin-1) rather than a reduction in levels of versican V0/V1 or the major ADAMTS versicanases expressed in the heart.

Versican V0/V1 promotes ventricular cell proliferation and trabeculation

During the E9.5 to E16.5 period of ventricular morphogenesis, the decline in both V0/V1 versican RNA and protein levels (Figs. 4C, 5A and 5B) coincides with a decrease in proliferation as evidenced by a decrease in expression of the cell proliferation marker, Ki67 (Fig. 8A) and an increase in the expression of cyclin kinase inhibitor, p21Cip1 (Fig. 8B). V1 versican has been shown to augment proliferation *in vitro* (Zhang et al., 1998) and findings showing reduced trabeculae in mice having increased levels of anti-DPEAAE reactive versican cleavage product (Stankunas et al., 2008) support the concept that versican V0/V1 influences proliferation associated with ventricular trabeculation. Using antibody to the GAG beta domain contained in the core proteins of both V0 and V1 variants we performed immunohistological analysis of E9.5 hearts from wildtype embryos and embryos homozygous for a transgene insertion into the versican gene (i.e, versican$^{hdf/hdf}$ mice). Unlike wildtype E9.5 hearts, in which versican V0/V1 was prominent in the ECM underlying the ventricular endocardium (Fig. 9A), versican V0/V1 immunostaining was absent from the hearts of versican$^{hdf/hdf}$ mice (Fig. 9B). We also observed that ventricular trabeculation in E9.5 versican$^{hdf/hdf}$ mice was greatly reduced as compared to wildtype (Fig. 9C and *D*). We also assessed proliferation in the hearts of versican$^{hdf/hdf}$ E9.5 embryos and observed a significant reduction in the level of cardiomyocyte (i.e., αSM actin positive) proliferation in versican$^{hdf/hdf}$ hearts as compared to wildtype (Fig. 9E). These findings indicate that versican V0/V1 is critical to cardiomyocyte proliferation in the process of ventricular trabeculation.

Fibulin-1 suppresses ventricular cell proliferation

We next determined whether the observed reduction in the level of the versican V1 70 kDa cleavage product in fibulin-1 nulls was associated with augmentation in ventricular cell proliferation. To do this, heart sections from fibulin-1 null and wildtype embryos were probed with antibodies to detect cells expressing Ki67 and phosphohistoneH3 (PHH3), markers of proliferation (Fig. 10). The results show an ~2-fold increase ($p<0.05$) in the numbers of both Ki67 positive and PHH3 positive cardiomyocytes (i.e., cells expressing α-sarcomeric actin) in the trabeculae of fibulin-1 null hearts (Fig. 10E and *F*). No significant changes in numbers of Ki67 positive or PHH3 positive cardiomyocytes was observed in either the compact layer or interventricular septum of fibulin-1 nulls. The total number of trabecular cardiomyocytes was also measured by assessing the number of Draq5 stained nuclei within α-sarcomeric actin positive cells. The results showed an approximate ~2 fold increase in the number of cardiomyocytes in the trabeculae of fibulin-1 null hearts as compared to wildtype (Fig. 10G). Together these findings indicate that reduced versican cleavage in fibulin-1 nulls is associated with increased trabecular cell proliferation.

Fibulin-1 deficiency augments activation of ErbB2 and intermediates of the Map kinase pathway

We next sought to establish the consequences of fibulin-1 deficiency on versican related signaling events important in the regulation of trabecular cell proliferation. Versican has been shown to stimulate phosphorylation of ErbB1 (Wu et al., 2004; Xiang et al., 2006) and Erk and to promote ErbB1-dependent cell proliferation (Zhang et al., 1998). Defective versican signaling may also contribute to the lack of trabecular myocardial cell proliferation seen in ErbB2 and ErbB4 nulls given that reduced ErbB2 and ErbB3 activation has been associated with hyaluronan deficiency (Camenisch et al., 2002). Using qPCR we found that all four ErbB family members were expressed in the developing cardiac ventricle (Fig. 11) and that their expression declined over the E9.5-16.5 time frame, resembling the patterns of fibulin-1, versican V0/V1 and MKi67. Employing a multiplex bead assay, levels of phosphorylated ErbB2 and Erk1/2 were measured in ventricle extracts from embryonic

hearts. As a result it was found that pErbB2 and pErk1/2 levels were increased in extracts of ventricles from fibulin-1 null embryos at E13.5 as compared to wildtype embryos (Fig. 12).

Discussion

Our studies indicate that fibulin-1 is required for ADAMTS1-mediated cleavage of versican in the developing cardiac ventricles. We also demonstrate that fibulin-1 deficiency leads to increased trabecular cardiomyocyte proliferation, an outcome similar to what occurs in ADAMTS1 deficiency (Stankunas et al., 2008). These findings, together with our evidence that versican V0/V1 is crucial for proliferation of cardiomyocytes leads to the conclusion that fibulin-1-mediated cleavage of versican by ADAMTS1 is required to suppress proliferation of ventricular cardiomyocytes during heart development. Furthermore, our findings indicate that a component of the process by which fibulin-1 attenuates ventricular cell proliferation involves its ability to suppress ErbB2 signaling normally required for trabecular cardiomyocyte proliferation (Lee et al., 1995; Liu et al., 2010).

Our findings show that ADAMTS1 expression peaks during ventricular morphogenesis coincident with peak levels of versican V1 cleavage product. These findings are consistent with those from Brg1 ablation studies indicating that ADAMTS1 is the principal ADAMTS family member mediating versican V1 cleavage during ventricular morphogenesis (Stankunas et al., 2008). Brg1 ablation was found to derepress ventricular expression of ADAMTS1, leading to an increase in versican V1 cleavage in the ventricles as evident by an increase in anti-DPEAAE immunostaining (Stankunas et al., 2008). Importantly, Brg1 ablation did not alter the expression of ADAMTS5, ADAMTS9 or ADAMTS20 (Stankunas et al., 2008). This, together with our findings that fibulin-1 acts as a cofactor for ADAMTS1 cleavage of versican V1 *in vitro* and that versican V1 cleavage is reduced by >90% in the ventricles of fibulin-1 nulls, indicates that fibulin-1 functions to promote ADAMTS1-mediated cleavage of versican during cardiac ventricular development. Our findings do not preclude the possibility that fibulin-1 deficiency could also reduce the activity of the other ADAMTS family members expressed in the ventricles and thereby amplify the negative effect on versican cleavage (Kern et al., 2010). Similar to the ventricular phenotype resulting from ADAMTS1 deficiency, ADAMTS9 deficiency has been reported to lead to a hypertrabeculation-like phenotype, although it remains to be established whether this deficiency produces a reduction in ventricular versican proteolysis. We also cannot exclude the possibility that fibulin-1 deficiency reduces ADAMTS cleavage of substrates other than versican (Porter et al., 2005). Of the known ADAMTS1 substrates, only nidogen-1 has been implicated in the formation of cardiac myocardium, such that combined deficiency of nidogen-1 and nidogen-2 results in a thin myocardium and hypoplastic trabeculae (Bader et al., 2005).

The observed decline in ventricular versican V0/V1 expression over the E9.5-E16.5 time frame (Fig. 5) correlates precisely with the decreasing mitotic index profile within the trabecular layer (Toyoda et al., 2003) as well as the decreasing ventricular expression of MKi67 (Fig. 8A), cyclin D1 and CDK4 (Nakajima et al., 2011) and the increasing expression of the cell-cycle inhibitory factor, p21Cip1 (Fig. 8B). In addition to diminishing versican transcript levels through this time frame, another factor contributing to the decreased levels of versican expression is its proteolysis by ADAMTS1, a process that increases in the ventricle after E9.5. The onset of ADAMTS1 proteolysis of versican is crucial to normal cardiac trabeculation as revealed by Brg1 ablation studies showing that premature derepression of ADAMTS1 expression results in earlier than normal cleavage of versican and reduced trabeculation (Stankunas et al., 2008). By contrast, under conditions of reduced versican cleavage resulting from deficiency of the ADAMTS1 cofactor, fibulin-1, proliferation persists in the ventricle beyond the period during which it normally subsides

(Fig. 10). Together, the findings suggest that attenuation of versican signaling by reduction of transcription and proteolytic cleavage of versican (specifically the V1 variant) may be required to quell ventricular cell proliferation during the E9.5-16.5 time frame.

Several studies have demonstrated that ErbB2 is required for trabecular cardiomyocyte proliferation (Lee et al., 1995; Liu et al., 2010). Furthermore, suppressed activation of ErbB2 occurs in mice deficient in the versican-binding glycosaminoglycan, hyaluronan (Camenisch et al., 2002), which also display a lack of trabeculation (Camenisch et al., 2000). These findings, together with evidence that versican V0/V1 is required for cardiomyocyte proliferation and ventricular trabeculation (Fig. 9) suggest that hyaluronan-bound versican may drive trabecular cardiomyocyte proliferation via an ErbB2-dependent signaling mechanism, which includes downstream activation of components of the MAPK/ERK pathway. As we show here, under conditions in which versican proteolysis is reduced as a result of an absence of fibulin-1, we not only observed increased ventricular cell proliferation, but also significantly increased activation of ventricular ErbB2 accompanied by increased activation of downstream Erk1/2 (Fig. 12). The possibility that this increased ErbB2-Erk1/2 activation and increased cardiomyocyte proliferation underlies observed abnormalities of the ventricular myocardium in fibulin-1-deficient mouse hearts i.e., thin myocardium and expanded trabecular layer (Cooley et al., 2008), is consistent with findings of Nakamura et al. (2007) showing that increased Erk1/2 activation in ventricular myocardium leads to increased proliferation of cardiomyocytes and ventricular noncompaction.

Experimental Procedures

Gene-targeted mice

The fibulin-1 deficient and versican$^{hdf/hdf}$ mouse strains used in this study were described previously (Mjaatvedt et al., 1998; Cooley et al., 2008). The fibulin-1 targeted strain was maintained on a mixed 129P2/OlaHsd:C57BL/6 background whereas the versican hdf strain was maintained on a C57BL/6 background.

Antibodies

Guinea pig anti-ADAMTS1 polyclonal antibody has been described previously (Lee et al., 2005). Rabbit anti-ADAMTS1 polyclonal antibody was obtained from Abcam (28284, Cambridge, MA). Goat anti-human cartilage link protein/HAPLN1 (C14) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The rabbit anti-fibulin-1 polyclonal (rb1323) antiserum has been described previously (Argraves et al., 1989; Argraves et al., 1990). Rabbit anti-versican GAG beta domain serum was raised against a fusion protein containing mouse versican V1 residues 1360-1439 (Acc. no.: Q62059;GI:10893583) (Kern et al., 2007) and was provided by Dr. Stan Hoffman (Medical University of South Carolina, Charleston, SC). These residues are contained in the GAG beta domain of both the V0 and V1 variants of versican. Rabbit polyclonal antibody against the C-terminal sequence (DPEAAE) of the fragment of versican V0/V1 generated as a result of proteolytic cleavage (Sandy et al., 2001) was purchased from Affinity BioReagents (Golden, CO). Rabbit anti-actin (A2668), mouse monoclonal anti-α-smooth muscle actin (A2547) and mouse monoclonal anti-α sarcomeric actin were purchased from Sigma Chemical Co. (St. Louis, MO). Rat anti-Ki67 was purchased from Dako (Carpinteria, CA). Rabbit anti-phosphohistone H3 was purchased from Cell Signaling (Carlsbad, CA).

Proteins

Versican, purified from the conditioned medium of cultured human smooth muscle cells as described (Sandy et al., 2001), was obtained from Dr. John Sandy (Rush University Medical

Center, Chicago, IL). ADAMTS1 was prepared as described previously (Rodriguez-Manzaneque et al., 2000). Fibulin-1 was purified from human placenta as described (Godyna et al., 1994).

In vitro versican cleavage assay

Versican (3.5 μg) either alone or in the presence of ADAMTS1 (0.5–1 μg) was incubated in 60 μl of 50 mM Tris, pH 7.4, 10 mM $CaCl_2$, 80 mM NaCl for 2 h at 37°C containing fibulin-1 or BSA (1 μg each). The digests were subjected to chondroitinase ABC digestion (Lee et al., 2005) and separated on 10% SDS-polyacrylamide gels, transferred to Optitran nitrocellulose membrane (Schleicher and Schuell, Keene, NH). Versican was detected with biotinylated hyaluronan (Sigma) and avidin-HRP (Abcam). ADAMTS1 was detected in immunoblotting with guinea pig ADAMTS1 antibody and fibulin-1 was detected with monoclonal antibody 3A11. The 70 kDa versican V1 cleavage fragment was detected with anti-DPEAAE.

Immunoblot analysis of ventricle extracts

Embryonic hearts were removed from embryos of varying stage (E9.5-E16.5), atria, aortae and pulmonary arteries removed, the ventricular portions immediately frozen and the remainder of the embryo used for genotyping. Frozen ventricles of the same stage and genotype were combined and extracted in 6 M urea, 50 mM Tris pH 7.4 buffer containing proteinase inhibitors (Complete Mini with EDTA, Roche Diagnostics Corp., Indianapolis, IN) using disposable pestles (Sigma). The extracts were clarified by centrifugation at 14,000 × *g* at 4°C. Protein levels in the supernatants were quantified using the BCA method (Pierce, Rockford, IL). Equal amounts of proteins were subjected to Western or immunoslot blotting using PVDF membranes (Thermo Scientific, Hudson, NH). As a loading control, blots were probed with actin antibody.

Immunohistochemistry

Embryos were harvested from timed pregnant females, a segment isolated for genotyping and the remaining portions of embryos fixed in methacarn. Hearts were removed from the fixed embryos and embedded in paraffin and sectioned at 5 μm thickness. Immunohistochemical staining was performed on deparaffinized and rehydrated sections subjected to antigen unmasking using a protocol with high temperature citric acid (H-3300, Vector Laboratories Burlingame, CA). Sections were incubated with rabbit anti-fibulin-1 (15 μg/ml), anti-α-smooth muscle actin (5 μg/ml, Sigma), anti-versican GAG beta, anti-DPEAAE (20 μg/ml), anti-Ki67 (15 μg/ml) or anti-phosphohistone H3. Bound antibodies were detected with fluorescently conjugated secondary antibodies (Invitrogen). Nuclei were labeled with 0.25 mM Draq5 (Cell Signaling Technology, Danvers, MA) in PBS for 5 min prior to a final series of washes in PBS. Sections were analyzed using a Leica TCS SP2 AOBS Confocal Microscope System (Leica Microsystems Inc., Exton, PA).

β-galactosidase analysis

Embryonic hearts were bisected and fixed in 4% paraformaldehyde/PBS for 1 h followed by a 12 h incubation in PBS containing 0.02% sodium deoxycholate and 0.01% NP-40 at 4°C. To detect β-gal activity, hearts were incubated in PBS containing 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$ and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Pierce), for 1–4 h at 37°C. The hearts were transferred to buffered formalin for 1 h and then subjected to cryosectioning.

RNA purification and RT-QPCR

Ventricles of wildtype and fibulin-1 null mice were collected at various stages, stored in RNAlater RNA Stabilization Reagent (Qiagen, Valencia, CA), and total RNA was isolated using a Qiagen RNeasy Mini Kit. cDNA was prepared from total RNA (500ng) using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's specifications. cDNA preparations were diluted to 100 μl, and 2 μl were used in 10-μl reactions with the SsoFast EvaGreen Supermix reagent (Bio-Rad). The oligonucleotide primers (Integrated DNA Technologies, Coralville, IA) used in qPCR reactions are described in Table 1. Thermal cycling was performed using a Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad); all reactions were performed in triplicate. The resulting data were analyzed with the PCR Miner web tool (Zhao and Fernald, 2005) to calculate reaction efficiencies and cycle thresholds; the method of Liu et al. (2002) was employed to calculate starting fluorescence values (mRNA levels) for genes of interest and reference genes (Hprt, Sdha, Ywhaz and Tbp). Genes of interest were standardized in relation to the geometric mean of the reference genes.

Bio-Plex Phosphoprotein Detection Assay

Embryos were harvested and genotyped as described above. Hearts were isolated and aortic and pulmonary blood vessels removed along with the atria. The ventricular tissues were extracted with Bio-Plex cell lysis buffer (Bio-Rad, Hercules, CA) and protein concentration determined by the Bio-Rad DC protein assay (Bio-Rad). Levels of phospho-ErbB2 and phospho-Erk1/2 in the extracts were determined using multiplex bead assay kits (Bio-Rad) and a Bioplex-200 instrument (Bio-Rad).

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Key Findings

- Fibulin-1 promotes ADAMTS1-mediated cleavage of versican.
- Fibulin-1 deficient embryonic cardiac ventricles have reduced levels of the 70-kDa amino-terminal fragment of versican V1 containing a carboxy terminal DPEAAE sequence.
- Fibulin-1 deficient embryonic cardiac ventricles have increased trabecular cardiomyocyte proliferation.
- Fibulin-1 deficient embryonic cardiac ventricles have increased levels of ErbB2 and Erk1/2 activation.
- Hearts from versicanhdf/hdf embryos lack versican V0/V1 and show a lack of trabeculae.

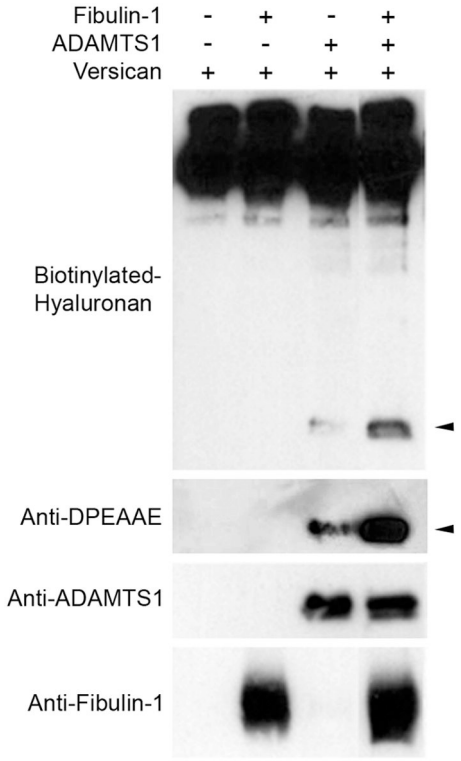

Figure 1. Fibulin-1 promotes the cleavage of versican by ADAMTS1

Versican was incubated plus or minus ADAMTS1 and fibulin-1. The mixtures were subjected to chondroitinase ABC digestion, separated by SDS-PAGE and immunoblotted with antibodies to fibulin-1, the neo-epitope DPEAAE or ADAMTS1. Blots were also probed with biotinylated hyaluronan and avidin-HRP to detect forms of versican containing the hyaluronan binding domain. *Arrowheads* point to the 70-kDa polypeptide band.

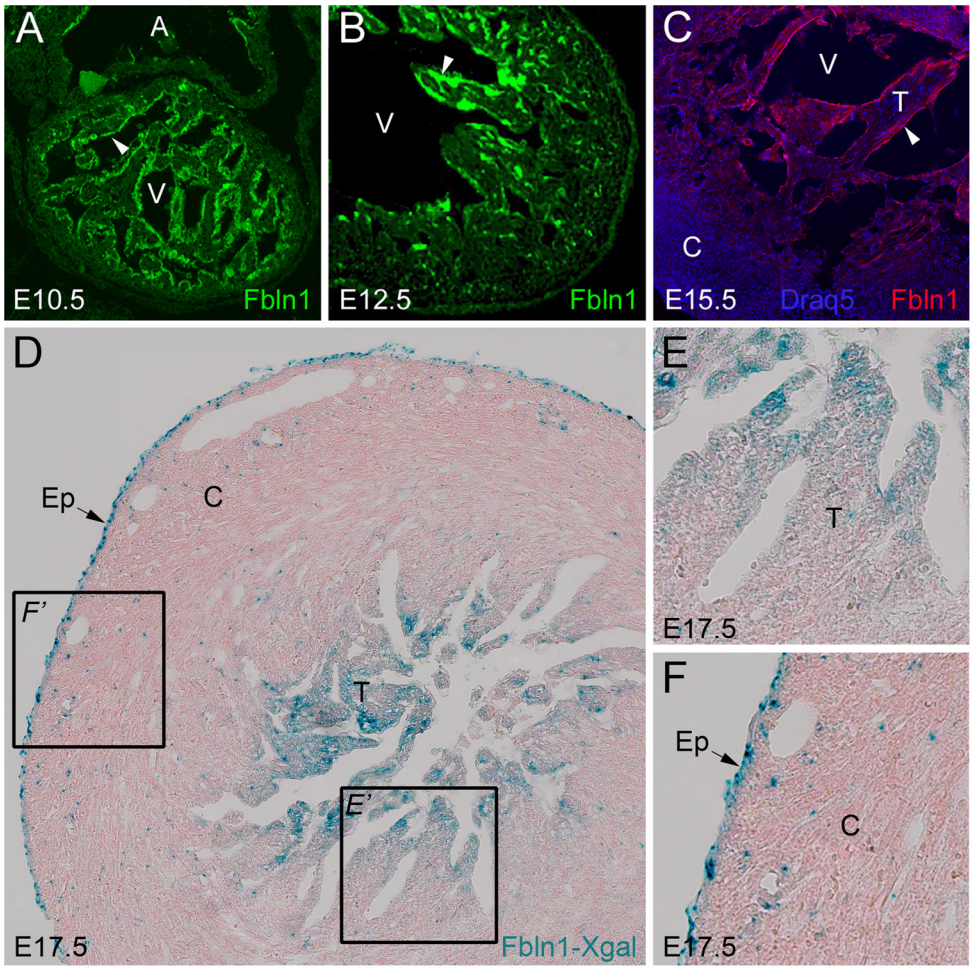

Figure 2. Fibulin-1 expression in cardiac ventricles of the embryonic mouse

A and ***B*** are sections of paraffin embedded embryonic hearts immunolabeled with antibodies to fibulin-1 (Fbln1; *green*). ***C*** shows a single optical section of a whole mount E15.5 heart cut to reveal ventricular chambers and labeled using antibodies to fibulin-1 (Fbln1; *red*). Nuclei were stained using Draq5 (*blue*). *Arrowheads* indicate areas with apparent subendocardial accumulation of fibulin-1 immunostaining. ***D–F***, show Xgal staining in the ventricular region of an E17.5 embryo heterozygous for the Fbln1 allele bearing β-galactosidase under the control of the fibulin-1 promoter. A, atrium; V, ventricle; T, trabeculae; C, compact myocardium; Ep, epicardium.

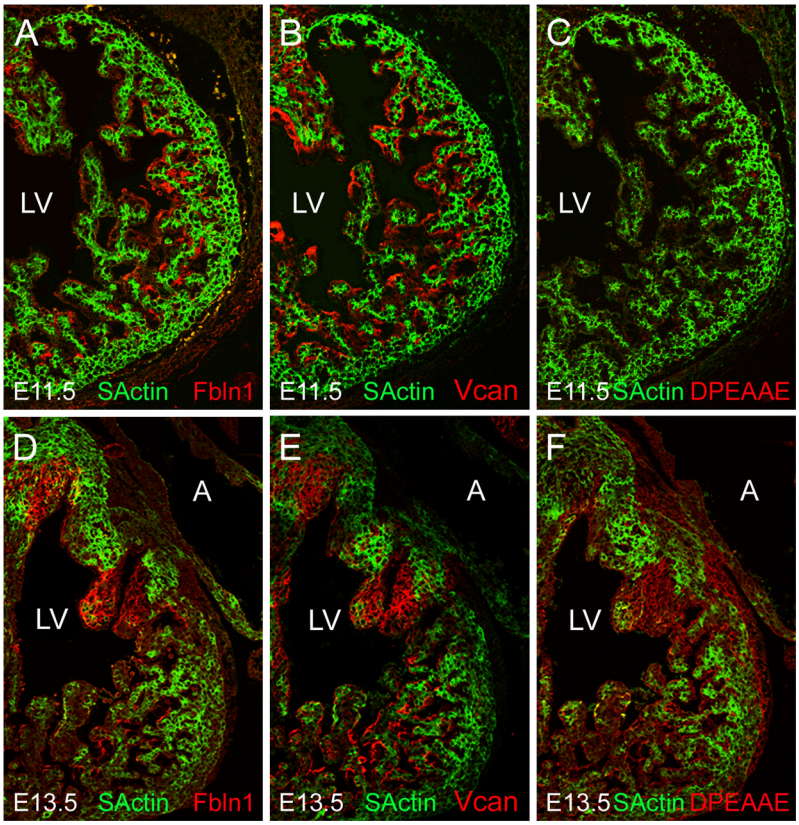

Figure 3. Fibulin-1 and V0/V1 versican are present in the trabecular ECM prior to the appearance of the anti-DPEAAE reactive V1 cleavage product

Shown are sections of E11.5 and E13.5 wildtype hearts immunolabeled with antibodies to fibulin-1 (Fbln1; *red*), versican V0/V1 (Vcan; *red*), 70 kDa V1 fragment of versican (DPEAAE; *red*) and smooth muscle actin (SActin; *green*). LV, left ventricle; A, atrium.

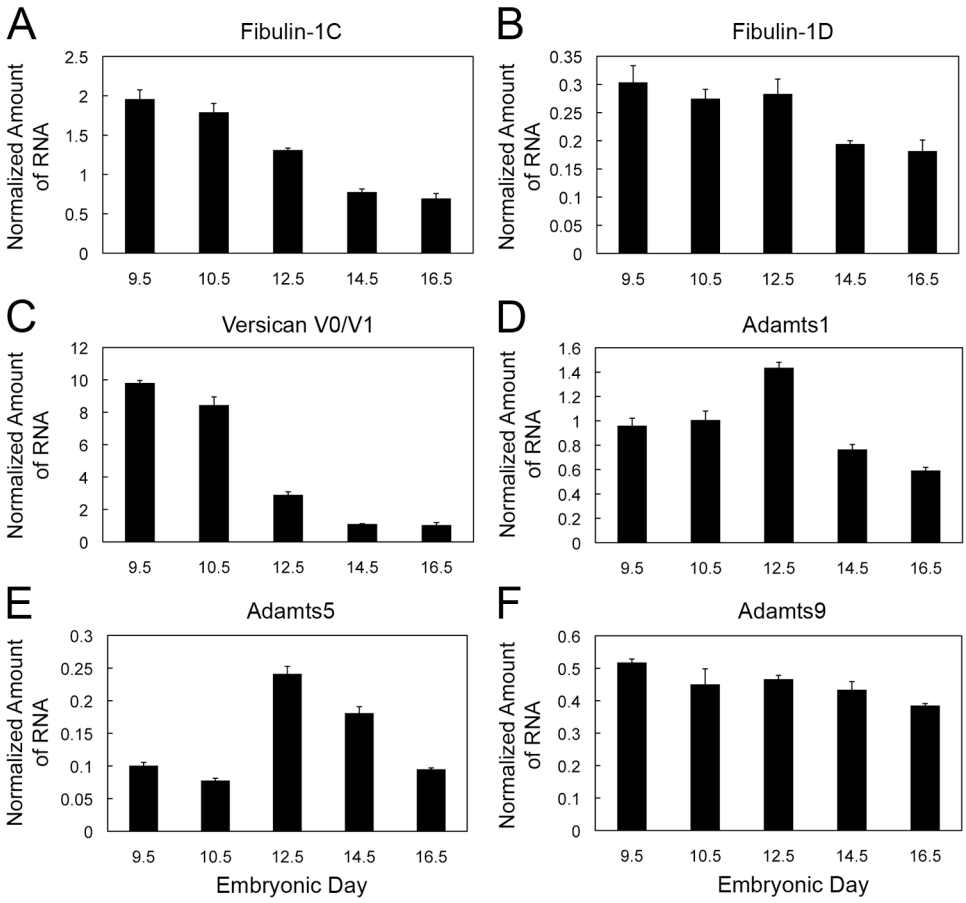

Figure 4. qPCR analysis of fibulin-1, versican and ADAMTS1/5/9 expression during ventricular morphogenesis

qPCR was performed on cardiac ventricle RNA preparations from E9.5-16.5 wildtype embryos. The plotted values show relative amounts of mRNA based on triplicate reactions of RNA pools for each stage (i.e., 15 ventricles for E9.5; 10 for E10.5; 2 for E12.5; 2 for E14.5 and 2 for E16.5). Bars correspond to STDV of the mean. Values are standardized in relation to four reference genes.

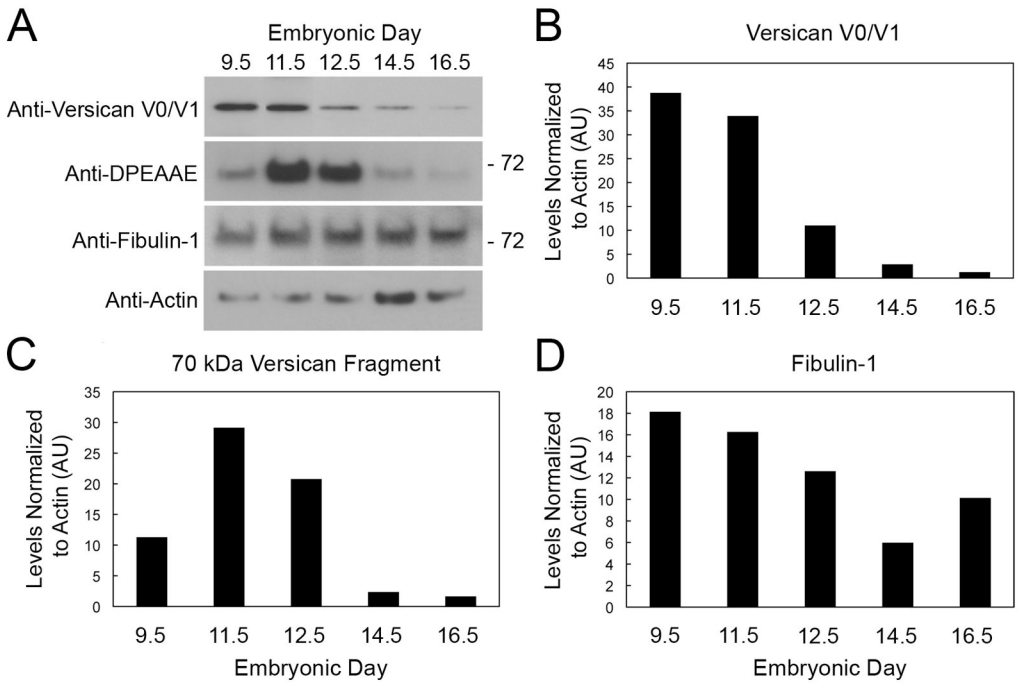

Figure 5. Changes in levels of V0/V1 versican, the 70 kDa vesican cleavage product and fibulin-1 during ventricular development

A,Immuno-slot blot detection of versican V0/V1 (Anti-versican V0/V1) and Western blot detection of the 70 kDa V1 fragment of versican (Anti-DPEAAE) and fibulin-1 (Anti-Fibulin-1) in ventricle extracts (i.e., 15 ventricles for E9.5; 2 for E11.5; 2 for E12.5; 2 for E14.5 and 2 for E16.5. ***B–D***, densitometric analysis of levels of versican V0/V1, 70 kDa versican cleavage fragment and fibulin-1 shown in *A*, normalized to levels of actin for each stage. The results are representative of two experiments.

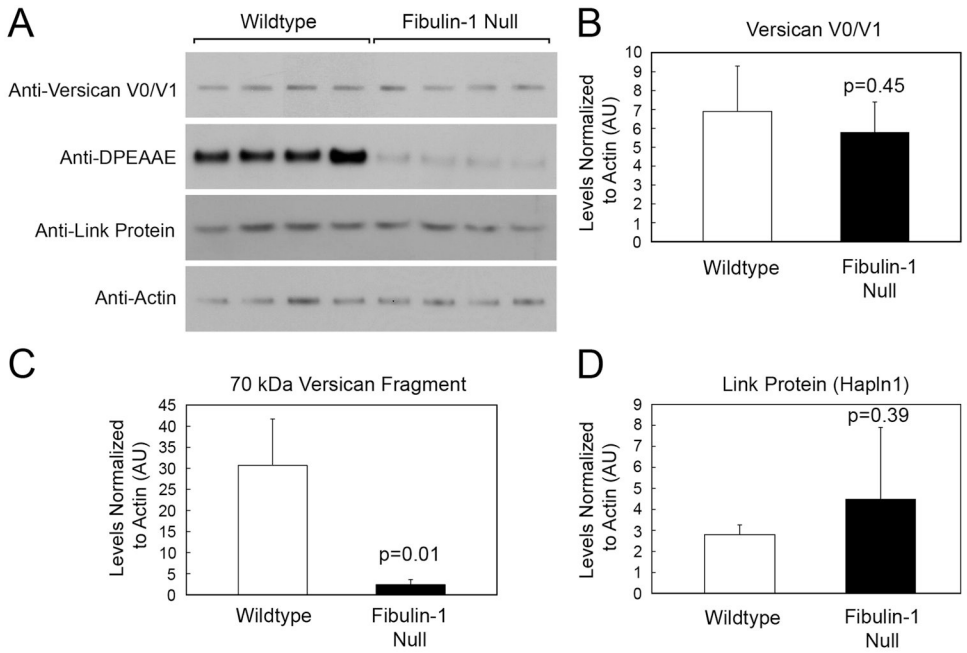

Figure 6. Cardiac ventricles from fibulin-1 nulls have reduced levels of versican V1 cleavage fragment

A, heart ventricle extracts from four individual wildtype and four fibulin-1 null E13.5 embryos were subjected to immunoslot blot analysis with versican GAG beta domain antiserum (anti-versican V0/V1) or subjected to Western blot analysis using anti-DPEAAE, anti-link protein (HAPLN1) and anti-actin IgGs. ***B–D***, densitometric analysis of levels of versican V0/V1, 70 kDa versican cleavage fragment and link protein shown in *A*, normalized to levels of actin. Plotted data are means ± STDV. Indicated p-values were calculated using unpaired Student's *t*-test.

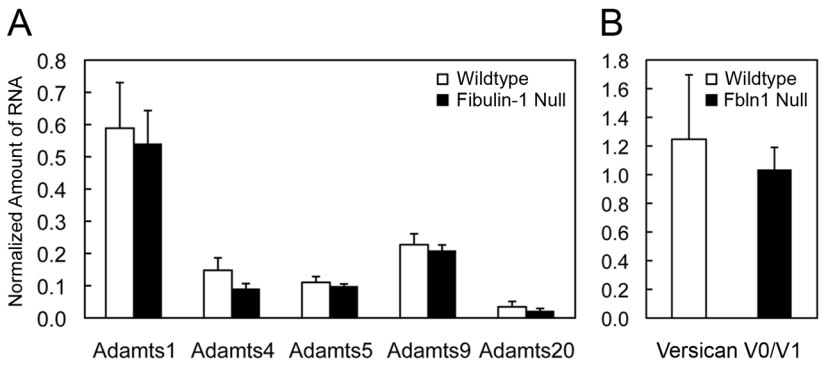

Figure 7. Fibulin-1 deficiency does not alter ventricular expression of versican or versicanases of the ADAMTS family

A shows qPCR analysis of members of the ADAMTS family known to cleave versican in E13.5 wildtype and Fbln1 null ventricles. ***B***, shows qPCR analysis of V0/V1 mRNAs in E13.5 wildtype and Fbln1 null ventricles. qPCR was performed on cardiac ventricle RNA preparations from E13.5 wildtype and fibulin-1 null embryos. The plotted values show mean relative amounts of mRNA ± STDV (n = 4 for each genotype). Values are standardized in relation to four reference genes. Unpaired Student's *t*-tests for all pairs did not demonstrate significance at 0.05.

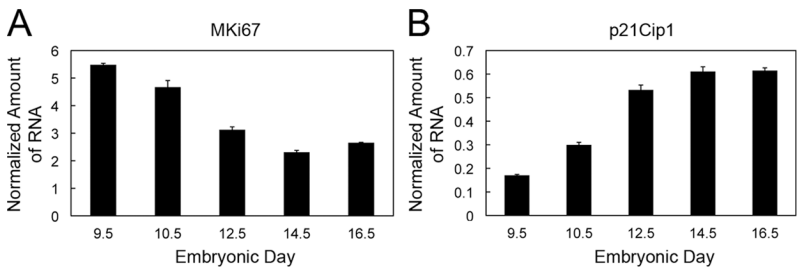

Figure 8. MKi67 and p21Cip1 expression during ventricular morphogenesis

qPCR analysis of MKi67 (***A***) and p21Cip1 (***B***) was performed on cardiac ventricle RNA preparations from E9.5-16.5 wildtype embryos. The plotted values show relative amounts of mRNA based on triplicate reactions of RNA pools for each stage (i.e., 15 ventricles for E9.5; 10 for E10.5; 2 for E12.5; 2 for E14.5 and 2 for E16.5). Bars correspond to STDV of the mean. Values are standardized in relation to four reference genes.

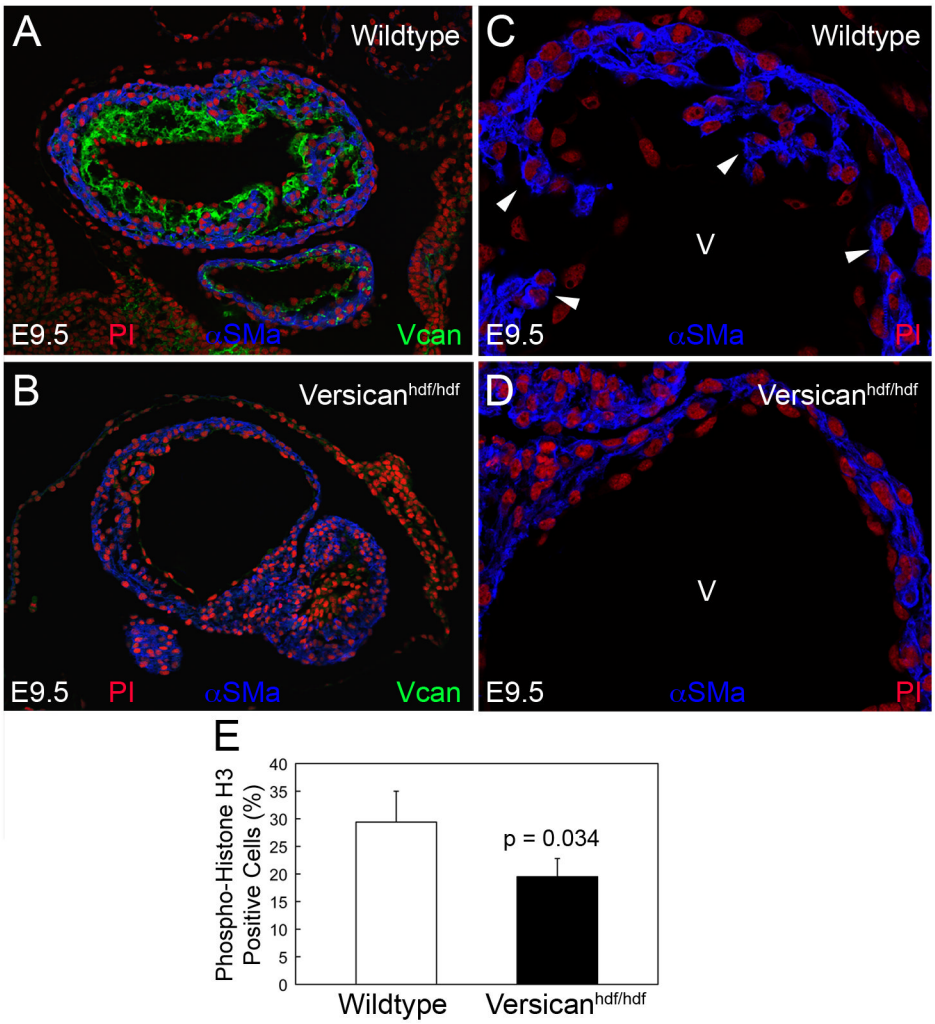

Figure 9. Hearts of versican$^{hdf/hdf}$ mice lack expression of versican V0/V1, have reduced trabeculation and reduced cardiomyocyte proliferation

A and ***B*** are sections of E9.5 cardiac ventricles from WT (*A*) and versican$^{hdf/hdf}$ mice (*B*) immunolabeled with antibodies to versican GAG beta domain containing V0/V1 (*green*), α smooth muscle actin (αSMa; *blue*). Nuclei were stained using propidium iodide (PI; *red*). ***C*** and ***D*** are sections of E9.5 cardiac ventricles from WT (*C*) and versican$^{hdf/hdf}$ mice (*D*) immunolabeled with antibodies to α smooth muscle actin (αSMa; *blue*). Nuclei were stained using propidium iodide (PI; *red*). V, ventricle; *arrowheads*, trabeculae. **E**, shows a graph depicting the percentage of phosphohistone H3 (PHH3)-positive cardiomyocytes (i.e., PHH3 stained nuclei in αSM actin-positive cells) in E9.5 hearts of control and versican$^{hdf/hdf}$ embryos as a function of the total number of cardiomyocytes (i.e., total number of PI stained nuclei in αSM actin-positive cells). The data was derived from the analysis of 4 E9.5 versican$^{hdf/hdf}$ hearts and 3 control hearts (1 WT and 2 versican$^{+/hdf}$ heterozygotes). Plotted values are from counts of 2682 cells from versican$^{hdf/hdf}$ hearts and 1185 cells from control hearts. The plotted mean percentage values are ± STDV.

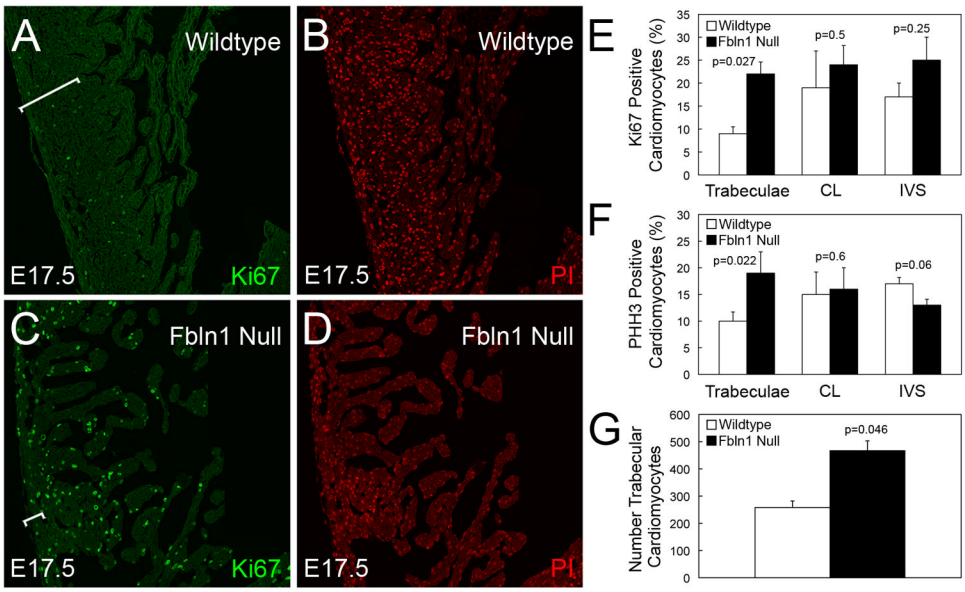

Figure 10. Fibulin-1-deficiency leads to increased proliferation in the trabeculae layer

A–D show right ventricular regions of hearts from E17.5 wild-type (+/+) and fibulin-1 null (−/−) embryos immunolabeled using anti-Ki67 (*A* and *C*) or stained using propidium iodide (PI) (*B* and *D*). Brackets in *A* and *C* indicate compact layer of myocardium. ***E***, shows a graph depicting the percentage of Ki67-positive cardiomyocytes (α-sarcomeric actin positive cells) as a function of the total number of Draq5 stained nuclei per microscopic fields from trabecular layer (TL), compact layer (CL) or interventricular septum (IVS) regions. The data was derived from the analysis of sections from 2 hearts of each genotype, measuring Ki67 positive-α-sarcomeric actin positive cells from the TL (1758 WT;4092 KO), CL (1096 WT;1577 KO) and IVS (1680 WT;1488 KO). ***F***, shows a graph depicting the percentage of phosphohistoneH3 (PHH3)-positive cardiomyocytes (α-sarcomeric actin positive cells) as a function of the total number of Draq5 stained nuclei per microscopic fields from TL, CL or IVS regions. The data was derived from the analysis of sections from 2 hearts of each genotype, measuring PHH3 positive-α-sarcomeric actin positive cells from the TL (2098 WT;2426 KO), CL (1593 WT;888 KO) and IVS (1629 WT;1062 KO). ***G***, shows total number of Draq5 stained nuclei within α-sarcomeric actin positive cells in 11 microscopic fields of uniform area within the trabecular regions of 2 hearts of each genotype. The plotted values in *E–G* are ± STDV and indicated p-values were calculated using unpaired Student's *t*-test.

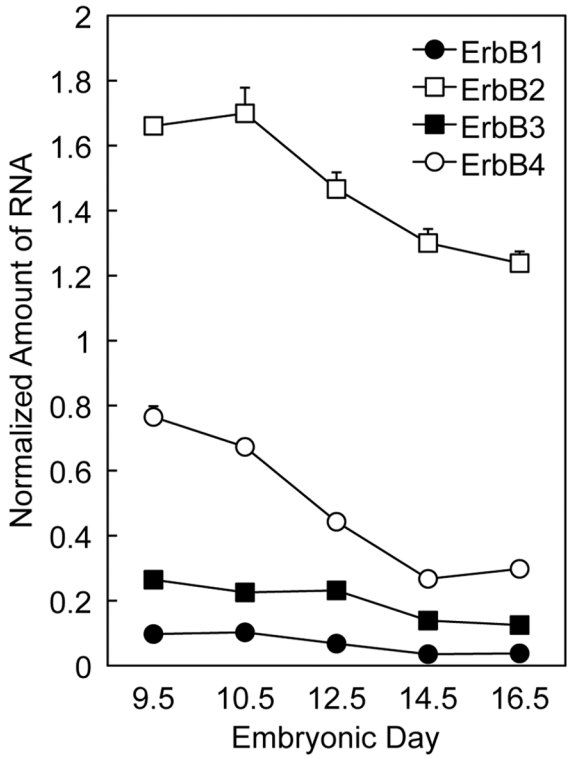

Figure 11. qPCR analysis of the expression of ErbB receptor family members during ventricular morphogenesis

qPCR analysis of ErbB1, ErbB2, ErbB3 and ErbB4 was performed on cardiac ventricle RNA preparations from E9.5-16.5 wildtype embryos. The plotted values show relative amounts of mRNA based on triplicate reactions of RNA pools for each stage (i.e., 15 ventricles for E9.5; 10 for E10.5; 2 for E12.5; 2 for E14.5 and 2 for E16.5). The plotted values are means ± STDV and are standardized in relation to four reference genes.

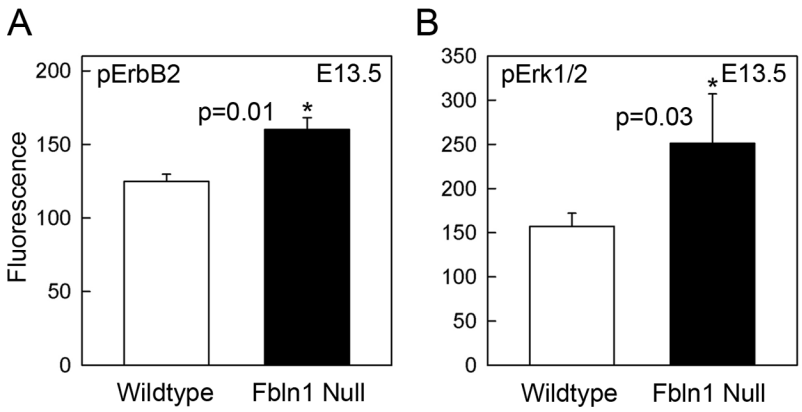

Figure 12. Multiplex bead array analysis of phospho-ErbB2 and phospho-Erk1/2 levels in cardiac ventricle extracts from E13.5 wildtype and fibulin-1 null embryos

The assay was performed on extracts of ventricles isolated from E13.5 fibulin-1 null (−/−) (n=3) and wild-type (+/+) embryos (n=3) (***A*** and ***B***). pErbB2 levels were normalized to total ErbB2 and pErk1/2 levels were normalized to total Erk levels. Plotted data are means ± STDV. Indicated p-values were calculated using unpaired Student's t-test.

Table 1

qPCR primer sequences

Gene	Target sequence ID No.	Forward Primer 5′-3′	Reverse Primer 5′-3′
Adamts1	NM_024400	ACACTGGCGGTTGGCATCGT	GCCAGCCCTGGTCACCTTGC
Adamts4	NM_172845	GCCCATGGACTGGGTTCCGC	TGCCACCCGGGGCTCCAATA
Adamts5	NM_011782	GGGCGCCCTTGTTGCTGCTA	GTGTTTCCTCCCCCTGCGGC
Adamts9	NM_175314	TGGCAATTGTGGGGACGTTTCAT	ACCAAGGCAGCACAATTCTTCCCA
Adamts20	NM_001164785	TCGGGGACCATGGAAGTCGGT	ACACCAGGACAGTTGATGGTCAGC
p21Cip1	NM_007669	GGAGTCAGGCGCAGATCCACAG	AGCGCATCGCAATCACGGCG
Erbb1	NM_207655	TCGAAAGCGTACACTACGCCGC	TGCCAAATGCTCCCGAACCC
Erbb2	NM_001003817	GCCGCGGGTACCCAAGTGTG	GAGAGGCTGGCATTGGCGGG
Erbb3	NM_010153	GTCGCTGCTTCGGGCCCAAT	CAAGGGGCGCTGGACACCTG
Erbb4	NM_010154	ATCTCCTCCGCGTGCTCGCA	GAGAAGCGCTGGGCTGGACG
Fbln1C	NM_010180	GCTTCCGCTGTCTGTCCTTTGAATG	GGCACTCCTGGTTCTCATGGC
Fbln1D	NM_010180	GCTTCCGCTGTCTGTCCTTTGAATG	GCAGGCCTCATCGTTGGGAC
Hprt1	NM_013556.1	TCCTCCTCAGACCGCTTTT	CCTGGTTCATCATCGCTAATC
Mki67	NM_001081117	GAGCGGCGGCCAGAGCTAAC	ATCGCCGCTCCGCTTGATGG
Sdha	NM_023281.1	TGTTCAGTTCCACCCCACA	CACGACACCCTTCTGTGATG
Tbp	NM_013684.1	GGGGAGCTGTGATGTGAAGT	CCAGGAAATAATTCTGGCTCA
Versican V0/V1[1]	NM_001081249	GGAAGCTGCAGAAGCTAGGCGTGGCCAG	CATCAGGCTCACCACTTGAAACATGGTC
Ywhaz	NM_011740.2	TTTCCCAGCCTTAAAAGGTCTAA	CGATGACGTCAAACGCTTC

[1]Primers are contained within the GAG beta coding region of exon 8.