Supplemental Materials and Methods:

Gene-Targeted Mice: Filamin-A floxed mice were bred with the Cre lines to obtain conditional KO animals. As Filamin-A is X-linked in both mouse and humans, we primarily focused our analyses on male mice, although some female mice have been characterized for phenotypic differences. No difference in phenotype is observed between male and female animals. Conditional deletion of Filamin-A does not cause lethality as anticipated (Table S1).

Western Analyses: HH40 chick mitral valve fibroblasts were grown in culture following isolation and harvested at passage numbers 1-3. Cells were lysed in 1X RIPA and equal amounts of protein were subjected to Western Analyses using antibodies described above (1:1000 dilution for each). Equal amounts of growth medium were loaded in each of their respective lanes (15 μL each).

Volumetric Quantification by Reconstruction: Three-dimensional reconstructions were performed using Amira 5.3.3 software (Visage Imaging, Andover, MA). 80-100 5 mm thick sections were used to generate each E17.5 or P1 reconstruction. Hematoxylin and Eosin stained slides were used in the analyses. Volumetric measurements were generated from $\geq$3 hearts from each genotype. Final data are presented as average volumes obtained from Amira 3D reconstructions of the cKO valves compared to WT.

TG2/Serotonylation Assays: Mitral valve fibroblasts were obtained from fetal chick (HH40) and solubilized using standard 1X RIPA buffer as described previously. Samples were incubated in the presence or absence of TG2 antagonist, Cystamine (10mM) in a calcium rich buffer (250 mM Tris-Cl, pH 7.4; 33mM NaCl, 250mM $CaCl_2$, 1X protease inhibitor-Sigma), for 30 minutes at 37^{o}C. Co-IP reactions were performed using a

serotonin antibody (Pierce, 1:100). Immunocomplexes were recovered using protein-A/G agarose beads (Roche), spun, and washed 3 times in 1X TBST. Proteins were solubilized in 2X SDS-PAGE buffer and subjected to Western analyses and probed for Filamin-A. Confirmatory Co-IP's were performed using lysate from cultured chick fetal mitral valve fibroblasts. Cultures were obtained by trypsinizing 120 fetal chick MV's and plated in 1X M199, 1% chick serum, 1% ITS, pen/strep. Cells were passaged a maximum of 3 times after which they were used for TG2/Serotonylation assays in which we incorporate biotinylated serotonin (100 mM) into the reaction cocktail. For synthesis of the biotinylated serotonin, serotonin hydrochloride (Tocris, Inc.) and EZ-Link Sulfo-NHS-LC-LC-Biotin (Thermo Scientific) were used. Equimolar amounts of serotonin and Sulfo-NHS-LC-LC-Biotin were reacted at room temperature using pyridine as the solvent system. The reaction mixture was frozen, lyophilized, and purified by reverse-phase HPLC; product was validated via mass spectroscopy (Figure S8) and stored at -20°C before use. Filamin-A antibody (Epitomics, 1:100) was used to IP immunocomplexes followed by Western blotting and detection using a streptavidin-HRP antibody. All co-IP experiments were performed a minimum of 3 times, each providing consistent results.

Matrix Compaction Assays: Collagen Compaction Assays: Assays were performed with cultured fetal chick mitral valve fibroblasts ($5X10^5$/ml) of passage 3 or less. Rat tail collagen (2mg/ml-BD biosciences) was neutralized with 10N NaOH and cells were added. Collagen/Cell mixture was quickly added to 4-well nunc cultured dishes and placed in incubator for polymerization. Polymerized gels were released from the wells and allowed to be free-floating in low serum containing chick media (1X M199, 1%ITS,

0.1% Chick Serum) for 5 days. Each day area measurements were scored and % compaction was calculated based on (Ao/A) as previously described [17]. The following day chemicals were added (serotonin, clomipramine, cystamine, fluoxetine—Tocris, Inc.) at appropriate doses as described in results. Data represent a minimum of 4 individual experiments for each pharmacological intervention and were repeated a minimum of 3 times. Fibrin Compaction Assays: Primary E17.5 anterior mitral leaflet cultures were established by microdissecting out the anterior leaflets on Filamin-A cKO and WT valves and placing the entire valve in culture (48-well for each leaflet). Cells were grown in culture for ~2 weeks to generate enough cells to perform compaction assays. Media used for growth: 1X MEM, 10% FBS, 1X Pen/Strep, 1X L-glutamine, 1X Na-pyruvate, 1X Vitamin-mix (Invitrogen), 1X non-essential amino acids, 5mM HEPES, 10 mM Glucose, 1mM ascorbate. 50,000 cells were resuspended in fibrinogen (5mg/ml—Sigma), 1 unit/mL thrombin and added to each well of a BSA treated 96-well dish. Gels contracted over 24 hour period and scored for area changes as described above. Data represent a minimum of 5 experiments from 4 cKO and 5 WT littermate cells and were repeated in quadruplicate.

Statistical Consideration: Statistical significance was determined using the Student t-test (2-tailed, type 2), with significance ($P<.05$). Statistical data are presented as the mean $\pm$ one standard deviation from the mean.

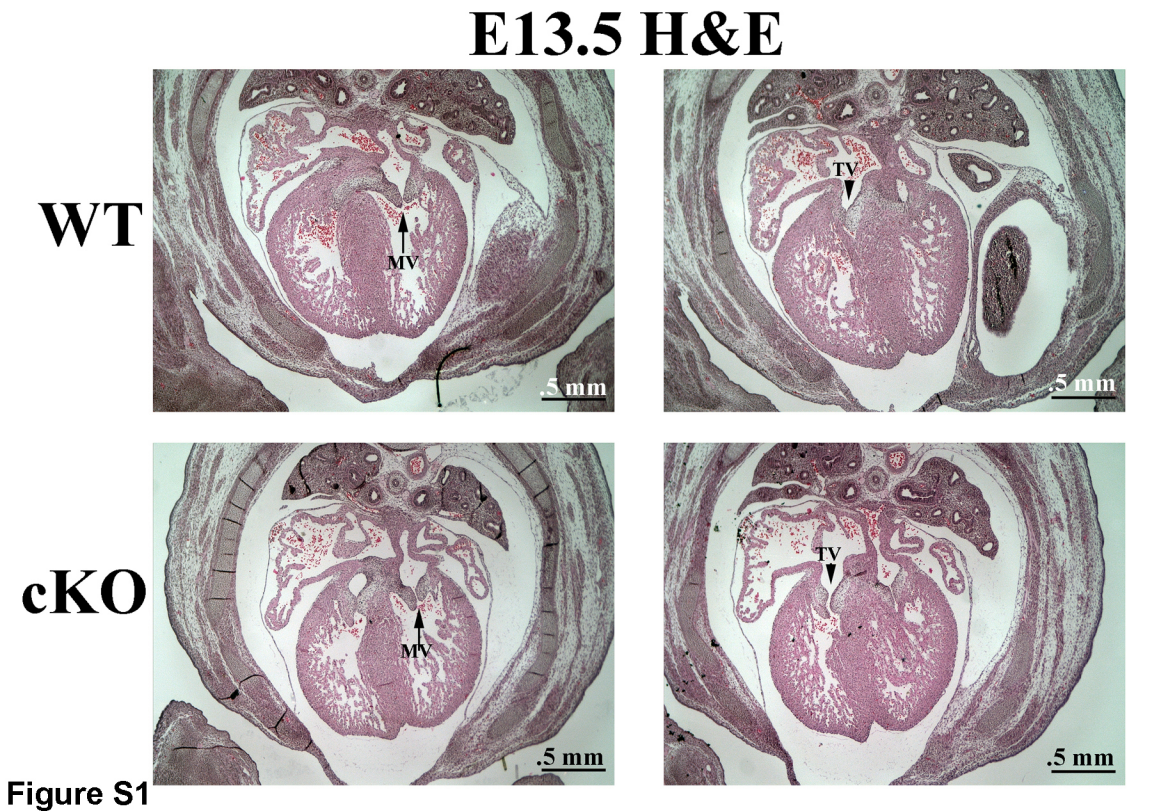


Figure S1

Endocardial Removal of Filamin-A the Valves Result in Enlareged Leaflets

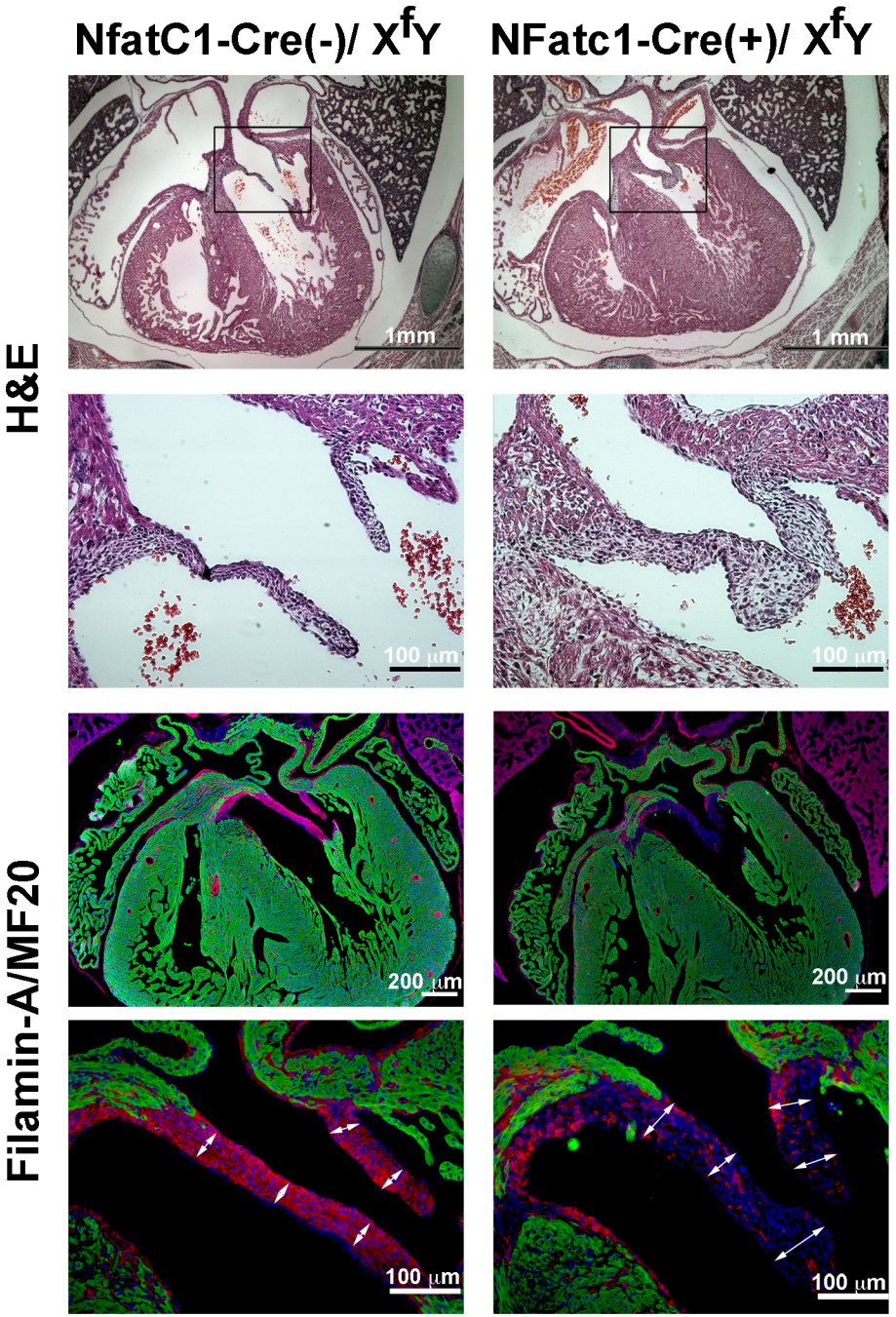
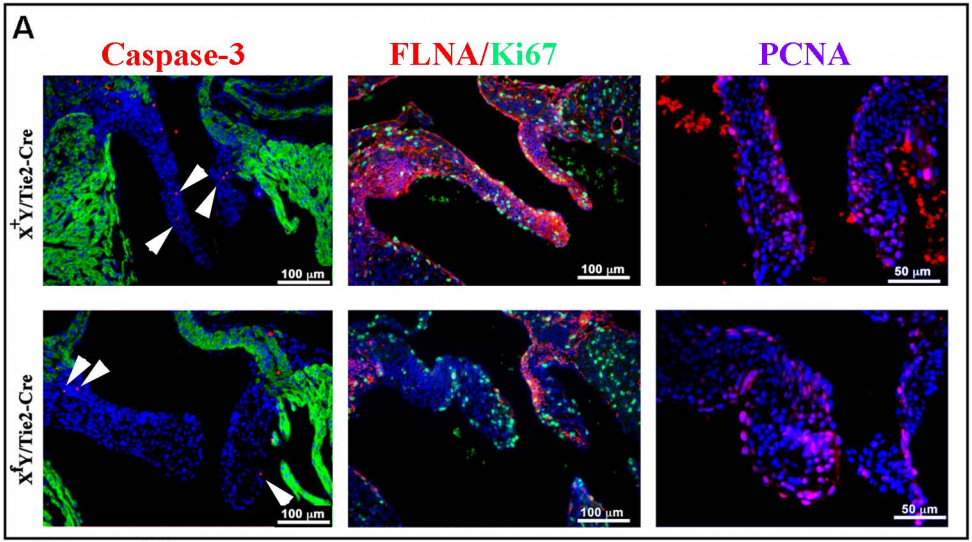


Figure S2


A
Caspase-3
FLNA/Ki67
PCNA
X+Y/Tie2-Cre
XfY/Tie2-Cre
100 μm
100 μm
50 μm
100 μm
100 μm
50 μm

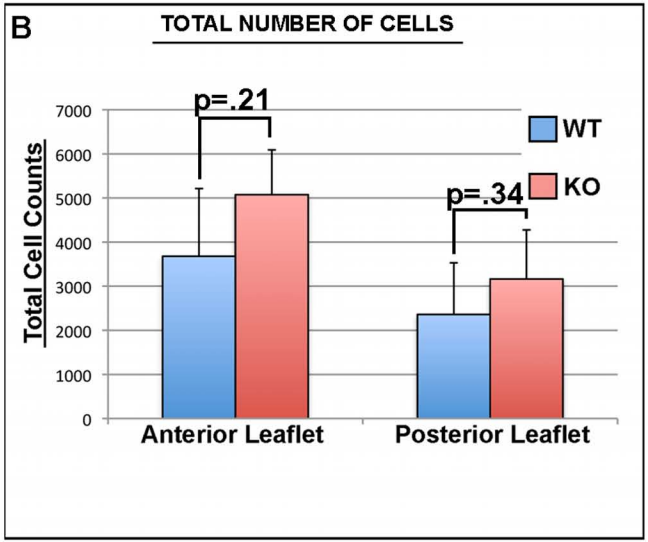

B
TOTAL NUMBER OF CELLS
Total Cell Counts
7000
6000
5000
4000
3000
2000
1000
0
p=.21
p=.34
WT
KO
Anterior Leaflet
Posterior Leaflet

Figure S3

Matrix Changes in Filamin-A cKO

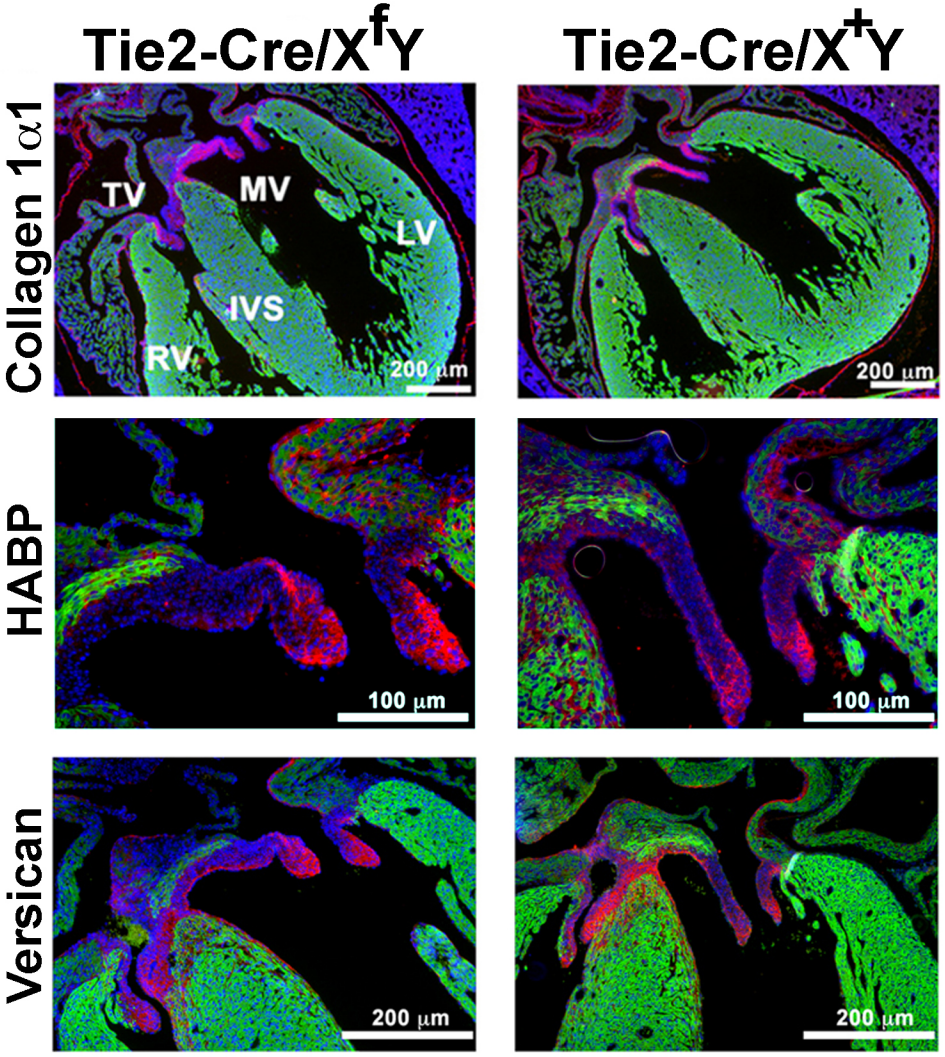


Figure S4

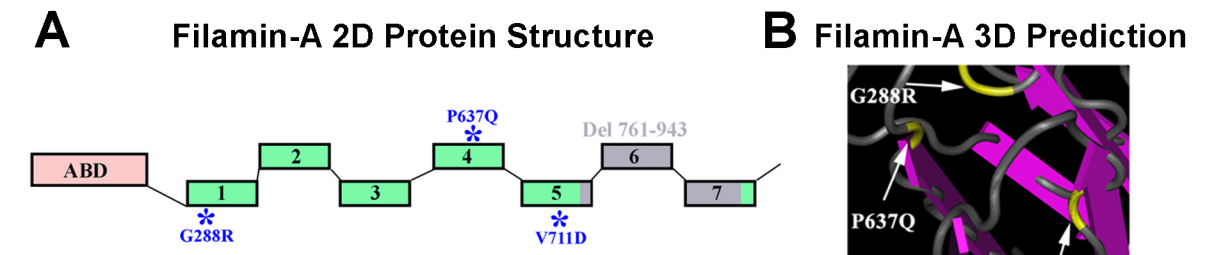

A
Filamin-A 2D Protein Structure
ABD
1
2
3
4
5
6
7
P637Q
Del 761-943
G288R
V711D
B
Filamin-A 3D Prediction
G288R
P637Q
V711D

Figure S5

E15.5

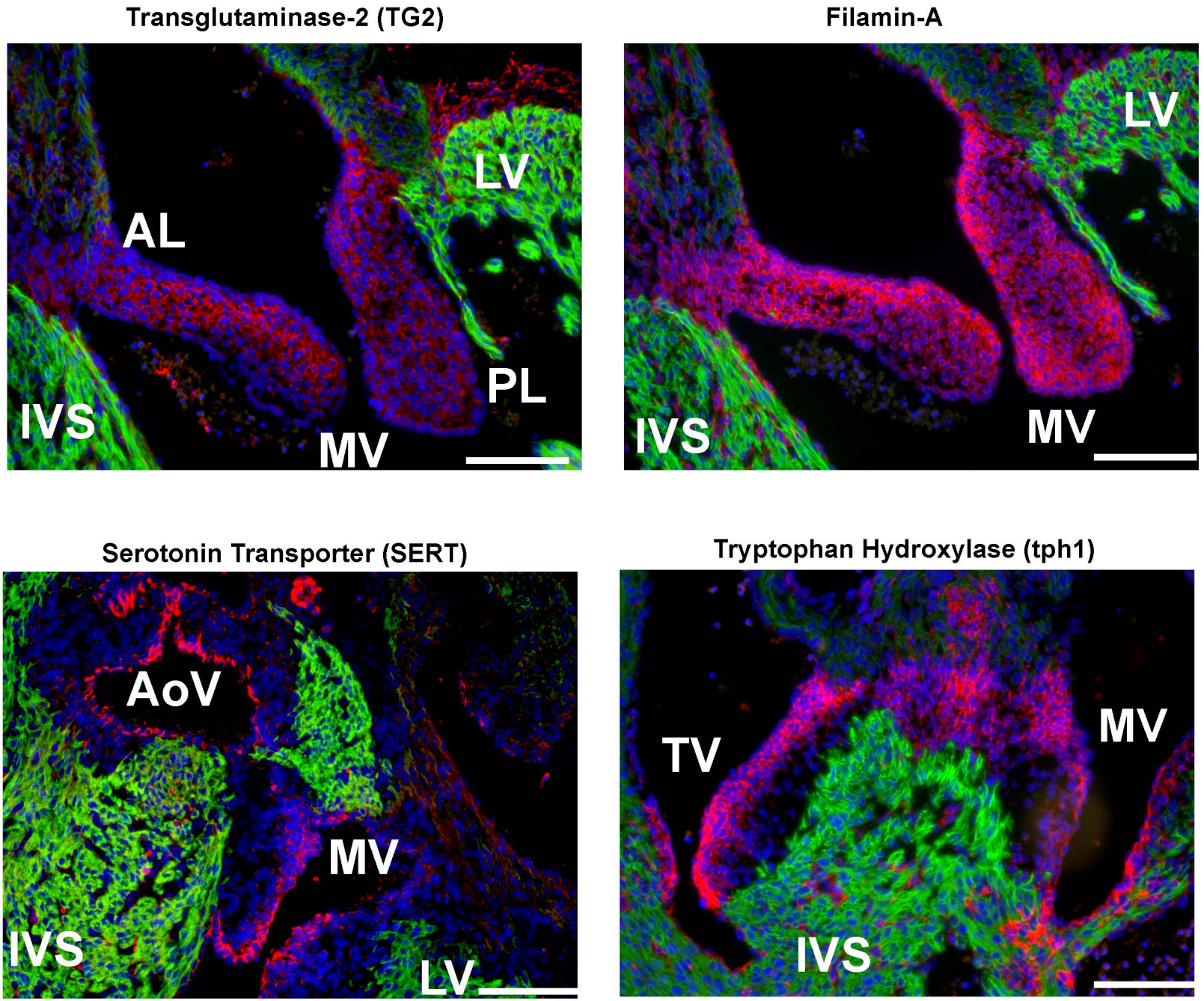


Figure S6

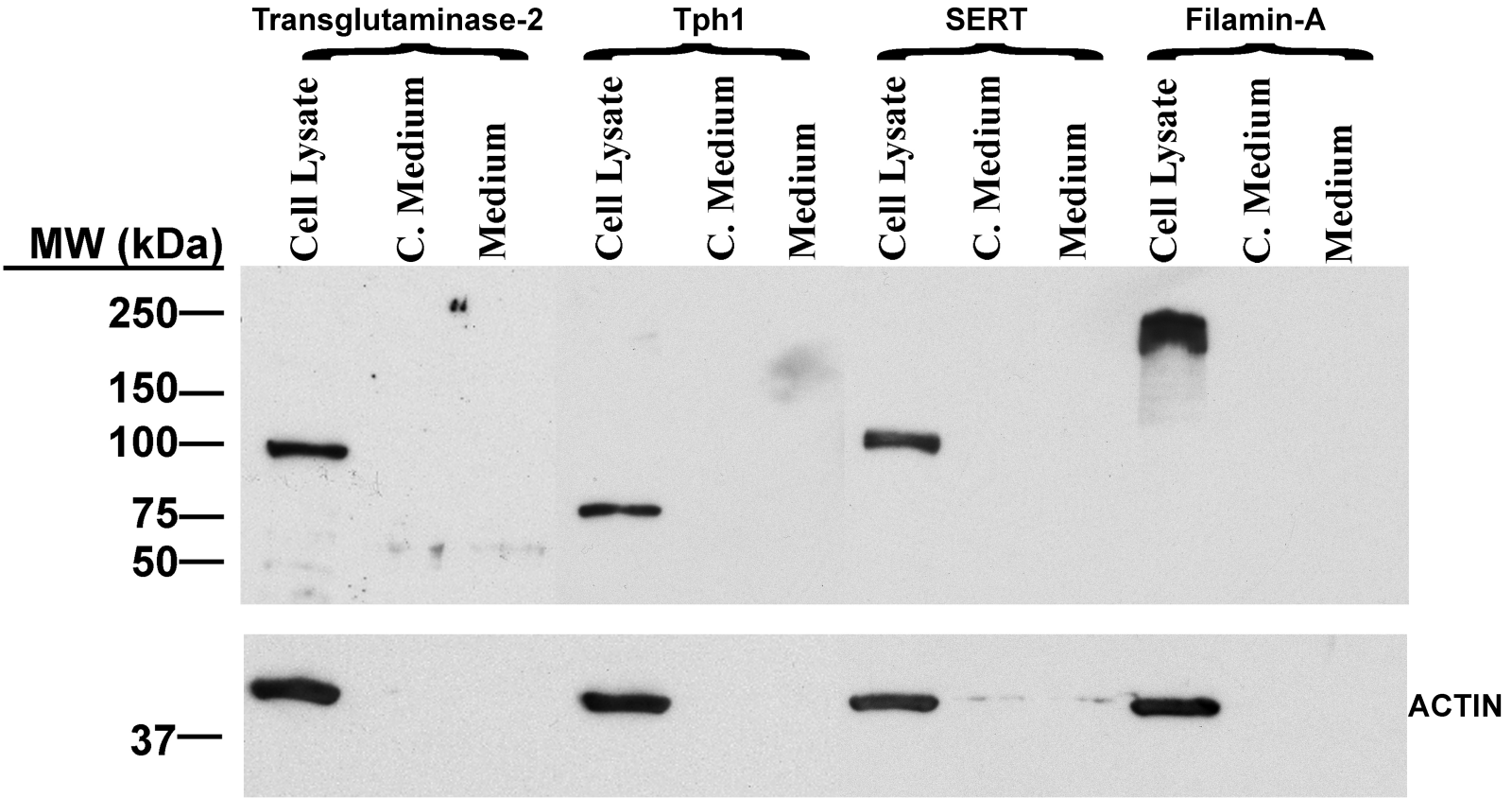

Transglutaminase-2
Tph1
SERT
Filamin-A
Cell Lysate
C. Medium
Medium
MW (kDa)
250
150
100
75
50
37
ACTIN

Figure S7

Mass Spec of Biotinylated-Serotonin

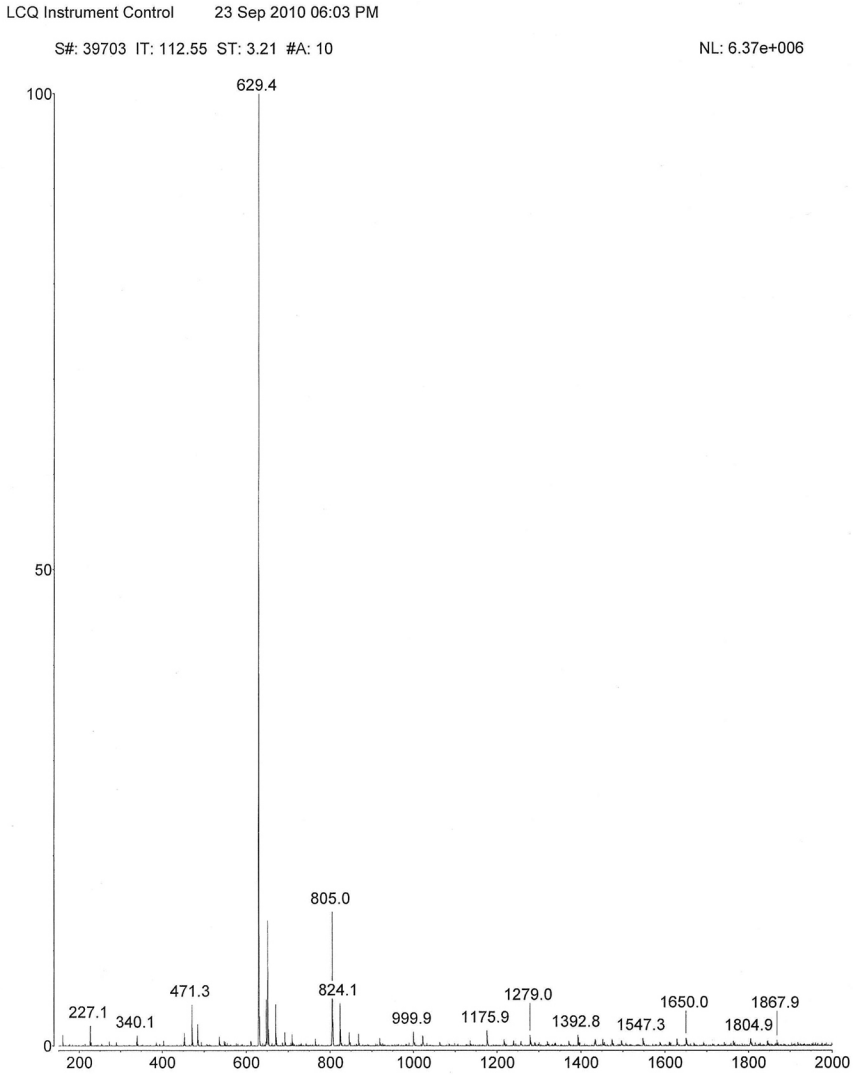


Figure S8

Table S1 Lethality Indices of Filamin-A cKO mice

		males				females					
		xfy		x+y		xfxf		xfx+		x+x+	
		cre+	cre−	cre+	cre−	cre+	cre−	cre+	cre−	cre+	cre−
NfatC1-Cre/Filamin-A	sire x+y/ N(+/-) dam x^fx^f	47/ 49.5%	48/ 50.5%	N/A	N/A	N/A	N/A	46/ 49%	48/ 51%	N/A	N/A
Tie2-Cre/Filamin-A	sire x^+y/ T(+/−) dam x^fx^+	11/ 32%	6/ 18%	6/ 18%	11/ 32%	N/A	N/A	7/ 30%	9/39%	2/9%	5/22%
	sire x^fy/ T(+/−) dam x^fx^+	18/30%	23/38%	13/21%	7/11%	11/ 20%	19/ 29%	21/ 32%	15/23%	N/A	N/A
	sire x^fy/ T(+/−) dam x^fx^f	16/ 70%	7/30%	N/A	N/A	6/35%	11/65%	N/A	N/A	N/A	N/A

Figure S1: Filamin-A cKO Mice do not Exhibit Morphological Defects at E13.5. Comparable sections of WT and cKO mice were evaluated at E13.5 by histology. No appreciable defects in the structure of either the developing mitral and/or tricuspid valve are observed.

Figure S2: Conditional KO of Filamin-A using Cardiac Specific NfatC1-Cre Results in Comparable Defects Observed using Tie2-Cre. Filamin-A floxed mice were bred with the NfatC1-Cre mice and valve phenotypes were investigated by histological methods. This Cre-model resulted in a hypomorph of Filamin-A in the AV leaflets and exhibited a similar phenotype observed with the Tie2-Cre. Whereas the Tie2-Cre resulted in nearly 100% removal of Filamin-A expressing cells from the anterior leaflet, the NfatC1-Cre was ~75% efficient as shown in the panel in the bottom right. Leaflet enlargement is observed in the NfatC1-Cre model consistent with findings in the Tie2-Cre/FLNA mice. Arrows demonstrate valve width throughout the leaflets

Figure S3: Neither Cell Proliferation nor Apoptosis are Regulated by Filamin-A in the Developing Valves. (**A**) Ki67, PCNA, and activated caspase-3 stainings were performed and indicate that Filamin-A deficient cells do not exhibit significant increase in proliferation or apoptosis (arrows) compared to Filamin-A containing cells. (**B**) Total number of cells was counted in both the anterior and posterior mitral leaflets. Although there was a trend towards increased numbers of cells, this did not reach statistical significance. N>3 for each genotype

Figure S4: Matrix Accumulation is Not a Major Contributor to the Valve Phenotype. IHC for Collagen I, Hyaluronan Binding Protein (HABP), and Versican shows no significant changes in matrix production and/or distribution. Additionally, these matrix molecules do not show localization throughout the Filamin-A deficient anterior leaflet, suggesting loss of Filamin-A does not lead to primary defects in matrix production at E17.5. MF20-green, nuclei-blue. All images are taken at the same microscope settings.

Figure S5: Analyses of XMVD Causing Point Mutations. (**A**) Schematic of the amino end of Filamin-A protein showing where the point mutations (blue) and deletion mutation (gray) are present. ABD-Actin binding domain. (**B**) Insilico 3D modeling using Cn3D software. The mutation residues are depicted by arrows and are labeled in yellow. Each pathogenetic mutation faces an inward pocket, which suggests its importance in either conformation stability or binding interfaces.

Figure S6: IHC for Filamin-A, TG2, SERT, and Tph1 at E15.5. Wild-type mice at E15.5 were sectioned and stained for each marker (presented in red). Whereas TG2 expression overlaps with filamin-A and, to a lesser degree, Tph1, little overlap in expression is observed with the serotonin transporter (SERT). Scale bars=50μm. IVS=interventricular septum, LV-Left Ventricle, AL & PL-Anterior and Posterior Mitral leaflets, MV-Mitral Valve.

Figure S7: Western Analyses for TG2, Tph1, SERT and Filamin-A. Western analyses were conducted on primary fetal mitral valve fibroblasts to determine expression of proteins. Each of the proteins examined were appreciably expressed by primary valve fibroblasts and were not secreted into the media. Equal amounts of medium were added to the gels.

Figure S8: Mass Spec Analyses of Biotinylated-Serotonin. Molecular mass of the reacted product as shown by tall peak is the same as theoretical molecular weight of biotinylated-serotonin. See Materials and Methods for more details.

Table S1: Conditional Filamin-A Deficiency Does Not Cause Lethality. Table showing total number of mice weened. Two different Cre-models were used in the study and the genotype and sex of all mice are represented in the table. To date, we have not observed any issue with lethality in either of our Filamin-A cKO strains.