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Supporting Online Material for

Losartan, an AT1 Antagonist, Prevents Aortic Aneurysm in a Mouse Model of Marfan Syndrome

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Materials and Methods

Mice

All experiments were performed using a previously described mouse line harboring *Fbn1* mutation C1039G[1]. All mice were back-crossed (>9 generations) into the C57BL/6J background, allowing valid comparisons between litters. Mice were sacrificed with an inhalation overdose of halothane (Sigma-Aldrich, St. Louis). Immediately following sacrifice, the abdominal aorta was transected and both left and right ventricles were flushed with phosphate-buffered saline (PBS; pH 7.4). Latex was injected into the left ventricular apex until visible from the transected aorta. Mice were fixed in 10% buffered formalin for 48 hours, after which the heart and aorta were removed en bloc. The ascending aorta was transected just above the level of the aortic valve and 2-3 mm transverse sections were mounted in 4% agar prior to paraffin embedding. Sections (5 μM) were stained with hematoxylin and eosin or Verhoeff's-van Gieson (VVG) stains. TUNEL-staining was performed using the TdT-FragEL DNA Fragmentation Detection Kit (Oncogene Research Products, Boston). Immunohistochemistry was performed using a polyclonal antibody recognizing pSmad2 (Cell Signaling Technology, Danvers). Slides were examined at 40x magnification using an Eclipse E400 microscope (Nikon Inc., Tokyo).

TGFb Neutralizing Antibody

Wild-type and *Fbn1*$^{C1039G/+}$ mice were injected intraperitoneally with TGFβ NAb, a panspecific antibody against TGFβ 1, 2, and 3 (R&D Systems, Minneapolis) once every two weeks beginning at 7 weeks of age and continuing for 2 months. The TGFβ NAb was reconstituted to 10 mg/ml using PBS (pH 7.4) for the high dose treatment group receiving 10 mg/kg (n=9) or to 1 mg/ml for the low dose treatment group receiving 1 mg/kg (n=11). Rabbit IgG (10mg/kg; Zymed Laboratories, Inc, San Francisco) was administered as a negative control (n=10).

Prenatal drug treatment

Female *Fbn1*$^{C1039G/+}$ mice underwent timed matings with wild-type male mice. At 14.5d post-coitum, pregnant female *Fbn1*$^{C1039G/+}$ mice were treated with oral losartan (0.6 g/L in drinking water; n=10), propranolol (0.5 g/L; n=6) or placebo (n=12). Therapy was continued throughout lactation and after weaning until 10 months of age. Mice were sacrificed and examined using the techniques described above. Propranolol was used for comparison with losartan because ß-adrenergic receptor blockade is the current albeit controversial standard of care to modulate abnormal growth of the aortic root in MFS.

Postnatal drug treatment

Beginning at 7 weeks of age, wild-type and *Fbn1*$^{C1039G/+}$ mice were treated with oral losartan (0.6 g/L in drinking water; n=5), propranolol (0.5 g/L; n=7) or placebo (n=10). Mice were continued on oral therapy for 6 months and then sacrificed.

Echocardiography

All echocardiograms were performed on awake, unsedated mice using the VisualSonics Vevo 660 V1.3.6 imaging system and a 40 or 60-MHz transducer (model RMV603, VisualSonics, Inc., Toronto). For the mice in the TGFβ NAb experiment, baseline and monthly echocardiograms were performed. For the postnatal losartan therapy group, echocardiograms were performed at baseline and every other month until the time of sacrifice. The aorta was imaged in the parasternal long axis view, and 3 measurements were obtained at the level of the sinuses of Valsalva at each time point by an observer blinded to genotype and treatment arm. All echocardiographic studies were performed by 2 individuals with extensive experience with mouse echocardiography, and all studies were interpreted by a single echocardiographer who was blinded to both mouse genotype and treatment arm.

Analysis of wall thickness and architecture

Wall thickness and architecture were analyzed using 5 μM cross-sections of the aortic root stained with VVG. Wall thickness at 16 different representative locations was measured and averaged by an observer blinded to genotype and treatment arm for each mouse. Wall architecture at 4 sites was assessed and averaged by 3 separate blinded observers using a scale of 1 (indicating no breaks in the elastic fiber) through 4 (indicating diffuse disruption) for each mouse.

Lung analysis

Histologic and morphometric analyses of the lung were performed in 8 month old female mice after 6 months of treatment with losartan or placebo. Morphometry was performed after standardized inflation, with analysis of at least 10 fields per lung. Both lungs were examined and there were at least 4 mice per group. These methods were previously described[2].

Statistical analysis

One way ANOVA was used to evaluate significance between groups with $p<0.05$ considered statistically significant.

References:

1. Judge DP, Biery NJ, Keene DR, Geubtner J, Myers L, Huso DL, Sakai LY, Dietz HC (2004) Evidence for a critical contribution of haploinsufficiency in the complex pathogenesis of Marfan syndrome. J Clin Invest 114:172-81.
2. Neptune ER, Frischmeyer PA, Arking DE, Myers L, Bunton TE, Gayraud B, Ramirez F, Sakai LY, Dietz HC (2003) Dysregulation of TGF-beta activation contributes to pathogenesis in Marfan syndrome. Nat Genet 33:407-11.

Legends for supplemental figures:

Supplemental Fig. 1. Characterization of the ascending aortic wall in wild-type **(A, C, E)** and *Fbn1*$^{C1039G/+}$ **(B, D, F)** mice. **(A, B)** VVG stain for elastin demonstrating diffuse disruption of elastic lamellae in *Fbn1*$^{C1039G/+}$ mice, **(C,D)** trichrome (blue) stain demonstrating increased collagen deposition in *Fbn1*$^{C1039G/+}$ mice. **(E, F)** Nuclear pSmad2, a marker of TGFβ signaling demonstrating marked increased pSmad2 in the *Fbn1*$^{C1039G/+}$ mice (arrows represent positive nuclei). Scale bars **(A-F)**, 40μm.

Supplemental Fig. 2 Gross inspection of ascending aortic dimension (arrowheads) after latex injection in representative wild-type **(A)** and *Fbn1*$^{C1039G/+}$ mice treated prenatally with placebo **(B)**, propranolol **(C)** or losartan **(D-F)**. Scale bars **(A-F)**, 4 mm.

Supplemental Fig. 3. Echocardiographic characterization of the aortic root during postnatal treatment with losartan. **(A)** Representative measurement of the aortic root (arrowheads) in the parasternal long axis view from wild-type and *Fbn1*$^{C1039G/+}$ mice. **(B)** Absolute aortic root measurements before treatment at 7 weeks of age (green bars) and after 6 months of treatment (red bars). A significant difference in absolute aortic root size is observed in wild-type, and *Fbn1*$^{C1039G/+}$ mice treated with placebo and propranolol but not those treated with losartan. $*p<0.01$, $**p<0.0001$, $***p<0.002$, †p=0.33

Supplemental Fig. 4. TUNEL staining for apoptosis in wild-type **(A)**, *Fbn1*$^{C1039G/+}$ mice treated with placebo **(B)**, propranolol **(C)** and losartan **(D)**. Dashed line demarcates junction between aortic media (right) and adventitia (left). Arrowhead, representative positive nuclei in the aortic media. No apparent differences are seen between genotypes and treatment arms. Scale bars **(A-D)**, 20μm.

Figure S1

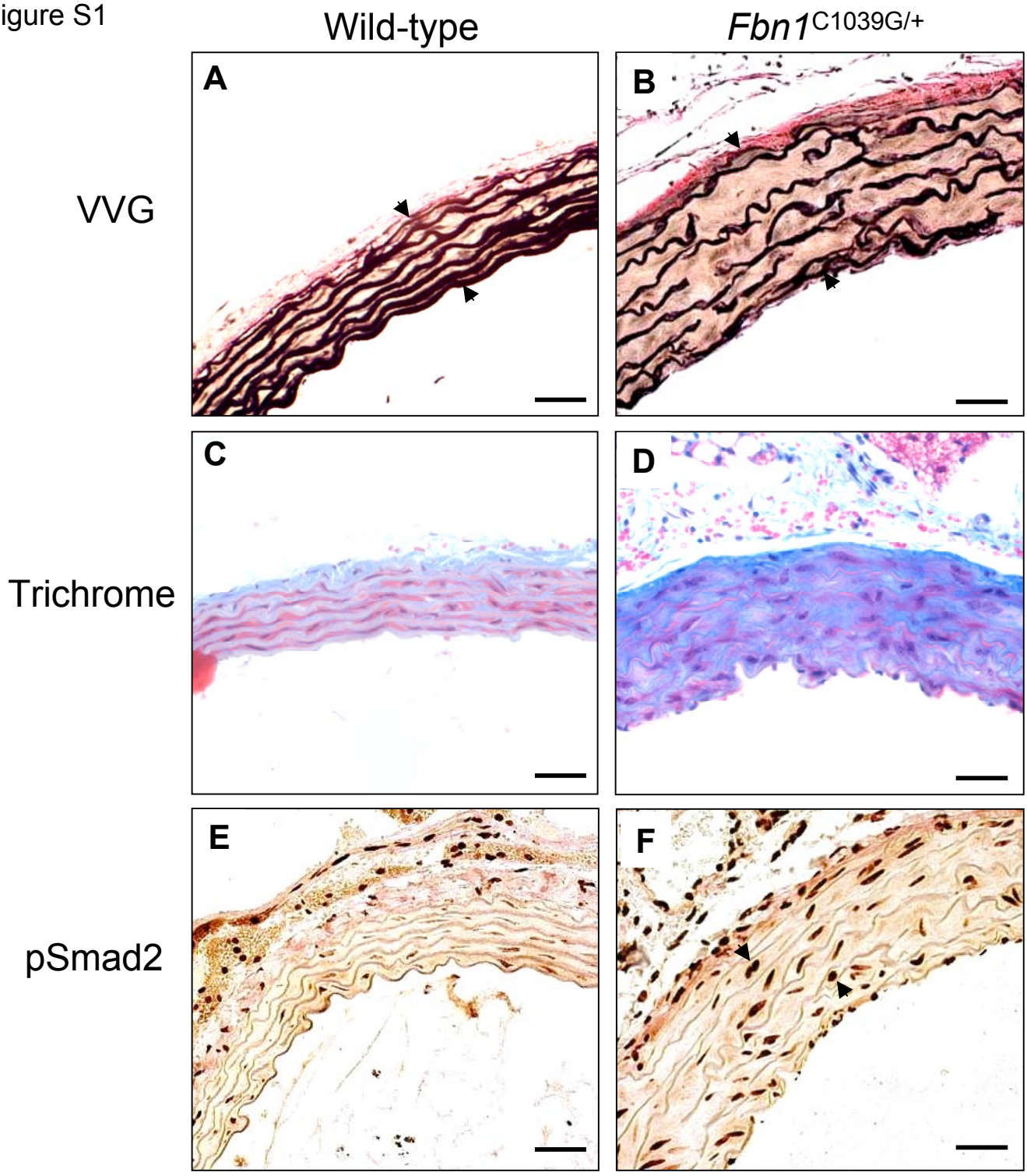


Figure S2

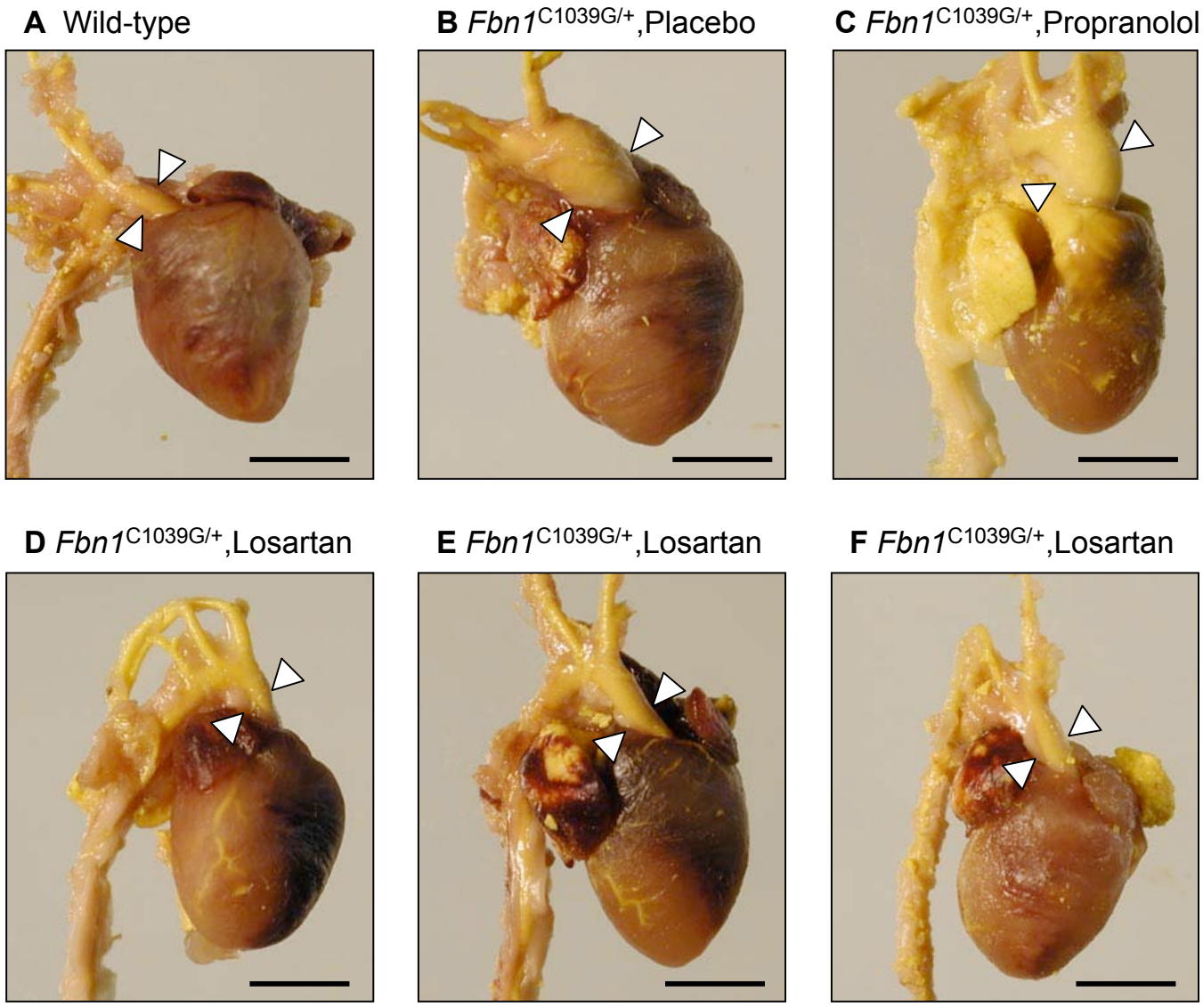

A Wild-type
B Fbn1C1039G/+,Placebo
C Fbn1C1039G/+,Propranolol
D Fbn1C1039G/+,Losartan
E Fbn1C1039G/+,Losartan
F Fbn1C1039G/+,Losartan

Figure S3

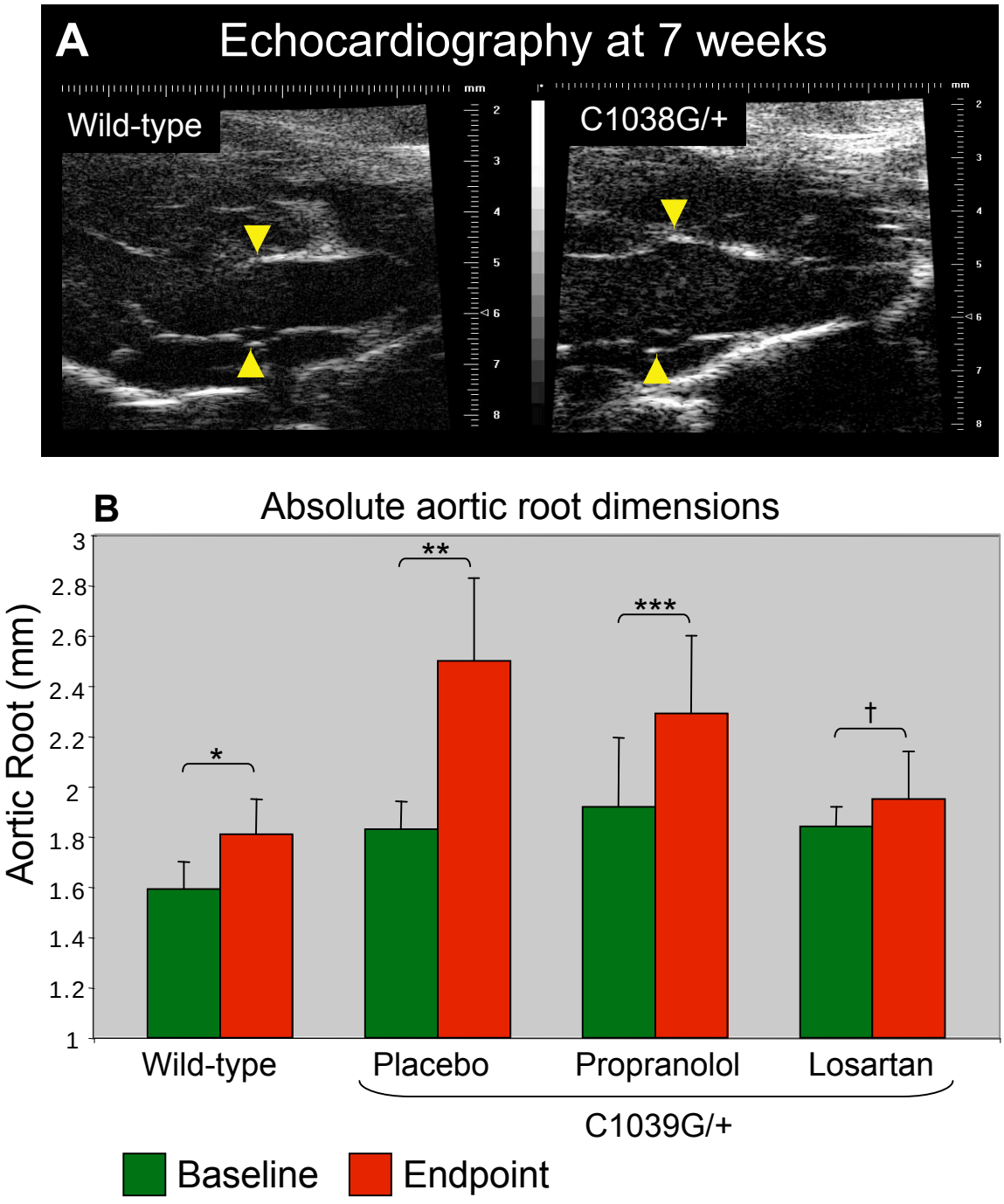
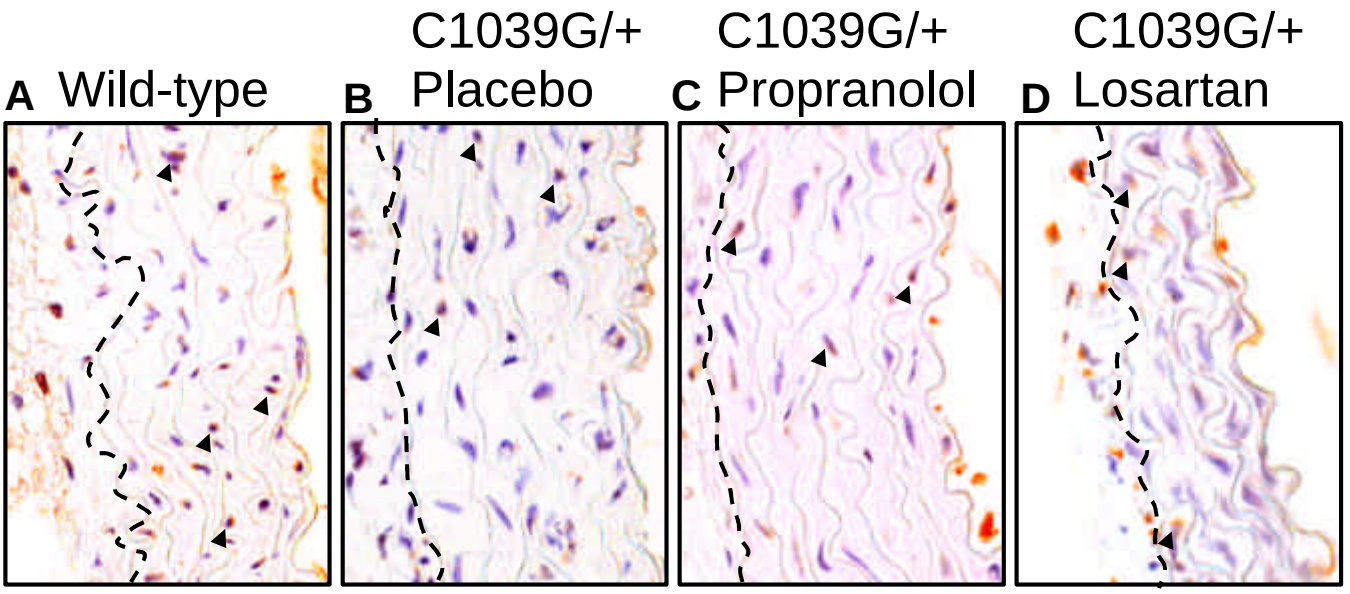

A
Echocardiography at 7 weeks
Wild-type
C1038G/+
B
Absolute aortic root dimensions
Aortic Root (mm)
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
*
**
***
†
Wild-type
Placebo
Propranolol
Losartan
C1039G/+
Baseline
Endpoint

Figure S4


A Wild-type
B C1039G/+ Placebo
C C1039G/+ Propranolol
D C1039G/+ Losartan