Resistence of Acta2<sup>R149C/+</sup> mice to aortic disease is associated with defective release of mutant smooth muscle α-actin from the chaperonin-containing TCP1 folding complex

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Pathogenic variants of the gene for smooth muscle α-actin (ACTA2), which encodes smooth muscle (SM) α-actin, predispose to heritable thoracic aortic disease. The ACTA2 variant p.Arg149Cys (R149C) is the most common alteration; however, only 60% of carriers have a dissection or undergo repair of an aneurysm by 70 years of age. A mouse model of ACTA2 p.Arg149Cys was generated using CRISPR/Cas9 technology to determine the etiology of reduced penetrance. Acta2<sup>R149C/+</sup> mice had significantly decreased aortic contraction compared with WT mice but did not form aortic aneurysms or dissections that followed to 24 months, even when hypertension was induced. In vitro motility assays found decreased interaction of mutant SM α-actin filaments with SM myosin. Polymerization studies using total internal reflection fluorescence microscopy showed enhanced nucleation of mutant SM α-actin by formin, which correlated with disorganized and reduced SM α-actin filaments in Acta2<sup>R149C/+</sup> smooth muscle cells (SMCs). However, the most prominent molecular defect was the increased retention of mutant SM α-actin in the chaperonin-containing t-complex polypeptide folding complex, which was associated with reduced levels of mutant compared with WT SM α-actin in Acta2<sup>R149C/+</sup> SMCs. These data indicate that Acta2<sup>R149C/+</sup> mice do not develop thoracic aortic disease despite decreased contraction of aortic segments and disrupted SM α-actin filament formation and function in Acta2<sup>R149C/+</sup> SMCs. Enhanced binding of mutant SM α-actin to chaperonin-containing t-complex polypeptide decreases the mutant actin versus WT monomer levels in Acta2<sup>R149C/+</sup> SMCs, thus minimizing the effect of the mutation on SMC function and potentially preventing aortic disease in the Acta2<sup>R149C/+</sup> mice.

The natural history of root or ascending thoracic aortic aneurysms is to asymptptomatically enlarge over time, which

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agonists but have normal vascular development and life expectancy (10). Despite the hypotension, Acta2−/− mice develop aortic root and ascending aneurysms by 6 months of age, and increasing blood pressure significantly accelerated enlargement of the aortic root and ascending aorta (11). Both aortic tissue and explanted SMCs from Acta2−/− aortas show increased levels of reactive oxygen species, which activates nuclear factor-κB signaling and increases angiotensin II receptor type Ia expression, thus potentiating angiotensin II signaling in vascular SMCs without an increase in local angiotensin II levels. Losartan, an angiotensin II receptor blocking agent, attenuated aneurysm formation, supporting that signaling through the angiotensin II receptor type Ia was in part responsible for thoracic aneurysm formation in the Acta2−/− mice (11).

To confirm pathogenic pathways responsible for thoracic aortic disease in individuals with ACTA2 mutations, the heterozygous R149C missense mutation was introduced into mice. Despite decreased force generation in the aorta and in vitro evidence that the mutant SM α-actin alters interaction with binding proteins, the Acta2R149C/+ mice do not form thoracic aortic aneurysms or dissect, even when biomechanical forces on the ascending aorta are increased. Surprisingly, our studies show that the mutant SM α-actin remains associated with chaperonin-containing TCP-1 (CCT) complex responsible for folding monomeric actins, which leads to reduced amounts of the mutant SM α-actin available for cellular functions compared with WT SM α-actin in SMCs. These results suggest that lower levels of available mutant SM α-actin compared with WT SM α-actin may underlie the decreased penetrance of thoracic aortic disease in ACTA2 R149C patients.

Results

Decreased contraction of the aorta of Acta2R149C/+ mice

A mouse model of ACTA2 p.Arg149Cys was generated by introducing the mutation into the endogenous Acta2 allele using CRISPR/Cas9 technology in C57BL/6NJ mice and was designated as Acta2R149C/+ (Fig. S1A). No homozygous mice were born with heterozygous mating, suggesting the Acta2R149C/R149C mice are not viable. Sequencing of the Acta2 complementary DNA isolated from aortic tissue confirmed expression of the mutant and WT alleles (data not shown). To confirm production of the mutant protein, actin isoform content was analyzed by two-dimensional gel electrophoresis, followed by immunoblot analyses using a pan-actin antibody (Fig. 1A). Trace amounts of nonspecific bands most likely represent post-translational modification of the actin. Expression of both WT and mutant SM α-actin was confirmed in the SM-dependent organs of Acta2R149C/+ mice, including the aorta and bladder (Figs. 1A and S1B). Levels of contractile and SMC differentiation markers were assessed in aortic tissue lysates and showed increased levels of SM myosin heavy chain but decreased levels of calponin in mutant aorta compared with WT aorta, whereas there were no changes in SM α-actin and SM22α (Fig. 1B and Table S1).

The blood pressures of WT and Acta2R149C/+ mice at 2 months of age did not differ (mutant versus WT systolic blood pressure: 121 ± 3.6 mm Hg versus 121 ± 3.5 mm Hg, p = 0.93, and diastolic blood pressure: 90 ± 2.9 mm Hg versus 93 ± 3.9 mm Hg, p = 0.48; n = 5 per genotype). Force generation in aortic rings from both ascending and descending thoracic aorta from the Acta2R149C/+ mice was decreased compared with the WT mice (Fig. 1C). The KCl-induced force development, resulting from membrane depolarization, was decreased by 55 to 60% in the mutant ascending aorta and 60 to 65% in the mutant descending thoracic aorta. Similarly, the phenylephrine (PE)-induced force development acting via G-protein–coupled α-adrenergic receptors was decreased by 65 to 70% in both ascending and descending mutant thoracic aorta. Thus, the aortic rings from both ascending and descending thoracic aorta showed decreased force generation in Acta2R149C/+ mice compared with WT mice. Phosphorylation of the SM myosin regulatory light chain (RLC) initiates SMC contraction. Mice were treated with PE and assessed for differences in the extent of RLC phosphorylation in the aortic segments (Fig. 1D). PE treatment increased RLC phosphorylation in ascending aorta to 30% and in descending thoracic aorta to 40% in both the Acta2R149C/+ and WT mice. There was an increase in the ratio of tissue weight to length in both ascending and descending thoracic aorta in the Acta2R149C/+ mice without differences in total RLC protein expression (Fig. 1E). In contrast to decreased contraction observed in aortic tissues of mutant mice, where SM α-actin is the predominant actin isoform, there were no significant differences in contractile responses to KCl or carbachol or in levels of RLC phosphorylation in urinary bladder strips between mutant and WT mice (Fig. S1C). Together, our data indicate altered force generation in the aortas of Acta2R149C/+ mice with no differences in RLC phosphorylation.

Acta2R149C/+ mice have increased aortic medial area but no thoracic aortic disease

Acta2R149C/+ and WT mice have similar body weight and survival over 24 months (p = 0.08; Gehan–Breslow–Wilcoxon test). Echocardiography assessment of the aorta over 24 months (104 weeks) indicates that the aortic diameter of Acta2R149C/+ mice is initially smaller than the WT mice in young mice, but after 30 weeks of age, the mutant and WT aortas are of the same diameter and remain similar until 104 weeks of age when the mutant aorta is again smaller (Fig. 2A). Appraisal of the aortic pathology at 5 months of age indicated that the Acta2R149C/+ aortic walls are significantly thicker (p = 0.045), with widening of the space between the elastin lamellae, and there is a corresponding decrease in cell density (p = 0.0012) (Fig. 2B and Table 1). There is no increase in elastin breaks (p = 0.503) or proteoglycan deposition (p = 0.108).

Increased blood pressure is a major risk factor for thoracic aortic aneurysm growth and progression to acute aortic dissections in humans and mouse models, including the Acta2−/− mouse model (11, 12). Therefore, we increased biomechanical forces on the ascending aorta using both surgical transverse
aortic constriction (TAC) in the arch and treatment with L-Nω-nitroarginine methyl ester (L-NAME; Cayman Chemical) and a high salt diet. The increase in blood pressure using either method resulted in mild impairment of left ventricular systolic function in mutant mice when compared with WT mice (Figs. S2 and S3) (13). Despite the increased pressures on the ascending aorta with TAC, the aortas in the Acta2R149C+/+ mice did not enlarge to a greater extent than the WT aortas, and no mice experienced aortic rupture (Fig. 2C). Similarly, treatment with L-NAME and a high salt diet increased blood pressures to the same extent in both the WT and mutant mice (Table S2). There was no difference in aortic diameter and no aortic ruptures in either WT or mutant mice treated with L-NAME and a high salt diet (Fig. 2D).

Biochemical characterization of R149C SM α-actin predicts decreased force generation

We biochemically characterized the SM α-actin with the R149C mutation to better understand its physiological impact. When HIS-tagged SM α-actin R149C was expressed in the baculovirus/Sf9 cell expression system, good yields of soluble expressed protein were obtained, but no mutant actin protein bound to the HIS affinity purification column. We hypothesized that the HIS tag may be inaccessible because of retention of mutant actin inside its folding complex, the CCT complex. Supporting this conclusion is the fact that in vitro–translated mammalian skeletal muscle α-actin binds to but is not released from the heterologous yeast CCT complex. Altering a single amino acid residue in skeletal muscle α-actin, changing asparagine 299 to a threonine, (N299T) results in the release of skeletal muscle α-actin from the yeast CCT complex and successful in vitro purification (14). Based on these data, N299T was introduced into the R149C and WT SM α-actin constructs. This strategy successfully allowed the R149C/N299T SM α-actin expressed in Sf9 cells to bind to the HIS column and be purified for subsequent biophysical studies, supporting the hypothesis that retention in the Sf9 CCT-bound mutant actin resists aortic disease

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CCT prevented purification of the R149C single mutant SMα-actin.

To determine if SMα-actin R149C is retained in the mammalian CCT complex, ACTA2 WT and mutant constructs were translated using a rabbit reticulolysate system, which includes the CCT complex. The translated proteins were analyzed on nondenaturing gels, and a higher and lower molecular weight band was present, corresponding to CCT-bound and CCT-unbound SMα-actin (Fig. 3A). A skeletal muscle α-actin (ACTA1) variant, E259V, that was previously shown to have increased association with the CCT complex was used as a positive control (15). Significantly more ACTA2 R149C monomers associated with the CCT complex than the WT or N299T actins. Introduction of the N299T mutation into the R149C construct led to the mutant actin being released from the CCT complex similar to WT. Quantitation of reaction products from six in vitro translation assays indicates significantly increased ratio of CCT bound to released actin for R149C actin compared with either WT actin (1.14 ± 0.47 versus 0.42 ± 0.1, p = 0.01) or R149C/N299T actin (0.64 ± 0.22, p = 0.048).

Using SMα-actin isolated from the baculovirus/Sf9 cell expression system, we assessed the ability of the altered SMα-actins to form filaments and interact with a subset of actin-binding proteins in vitro (7, 8). Because R149C is not released from the CCT complex and therefore cannot be

### Table 1

| Mouse genotype | Wall thickness (mm) | Cells (×10^5)/mm² | Lamellae layers | Elastin breaks |
|----------------|---------------------|-------------------|-----------------|---------------|
| WT             | 0.13 ± 0.02         | 823.81 ± 64.02    | 7.8 ± 0.58      | 0.7 ± 0.37    |
| Acta2^{R149C/+}| 0.179 ± 0.02        | 491.75 ± 22.32    | 8.4 ± 0.32      | 1.1 ± 0.43    |

*Data were obtained from five mice per group. Error bars indicate SD.

*p < 0.05.
puriﬁed, R149C/N299T SM α-actin was used for these studies. Polymerization of individual ﬁlaments in real time was followed by total internal reﬂection ﬂuorescence (TIRF) microscopy, and the rate of growth of actin ﬁlaments quantiﬁed as a function of actin concentration (Fig. 3B). The slope of this plot is the assembly rate, the γ-intercept is the disassembly rate, and the x-intercept is the critical concentration. The R149C/N299T and N299T ﬁlaments had a similar critical concentration as WT that indicates similar ﬁlament stability, although the assembly rate of R149C/N299T was reduced to ~30% (Table 2). Filaments formed from WT, N299T, and R149C/N299T were incubated with various concentrations of coﬁlin
CCT-bound mutant actin resists aortic disease

Table 2
Polymerization rates of WT and mutant actins

| Actin          | Assembly rate (subunits-μM⁻¹-s⁻¹) | Disassembly rate (subunits-s⁻¹) | Critical concentration (mM) |
|----------------|-----------------------------------|--------------------------------|-----------------------------|
| WT             | 16.5 ± 0.4                        | 0.5 ± 0.2                      | 47.8 ± 4.6                  |
| N299T          | 13.2 ± 3.6                        | 0.1 ± 0.02                     | 8.7 ± 0.6                   |
| R149C/N299T    | 10.8 ± 1.5                        | 0.4 ± 0.6                      | 37.0 ± 5.4                  |

Errors indicate SD.

A Data from published work (7).

B Data were obtained from two experiments using two independent protein preparations.

C Data were obtained from four experiments using two independent protein preparations.

The Altered SMC phenotype and decreased mutant SM α-actin levels in Acta2R149C/+ SMCS

To further assess how the R149C SM α-actin disrupts cellular function, SMCs were explanted from the ascending aorta of the Acta2R149C/+ and WT mice. Acta2R149C/+ SMCs are differentiated to the same extent as WT SMCs based on similar SMC contractile proteins (Fig. 4A and Table S1). Furthermore, SM α-actin is in polymerized (F-actin) state with little to no monomeric actin (G-actin) present in either mutant or WT SMCs (Fig. 4B). Transforming growth factor beta treatment appropriately increases F-actin levels. Immunofluorescence also shows that Acta2R149C/+ SMCs assemble SM α-actin filaments, but the filaments appear longer and less robust than filaments in WT SMCs (Figs. 4C and S4). Super-resolution microscopy confirmed an altered actin filament structure in the Acta2R149C/+ SMCs (Fig. 4D). Actin filaments in the Acta2R149C/+ cell appeared more dispersed and less organized with fewer stress fiber/bundle-like structures. The mutant SMCs had filaments that aligned in all directions and covered the cell more uniformly, leading to a less oriented network than in the WT SMCs. Fourier transform of the images shows many high-frequency bands for the WT SMC that are not present in the Acta2R149C/+ SMCs, indicating more ordered actin structures in the WT SMCs (Fig. 4, left). Approximately 9 million actin molecules were identified in both WT and Acta2R149C/+ SMCs, consistent with similar levels of actin expression. The distribution of the minimum distance between two identified actin protomers for both the WT and Acta2R149C/+ SMCs was fit to the sum of two Gaussian peaks. The main peak at ~4 nm corresponds to the distance between neighboring protomers within an actin filament, and the second peak at 6 to 8 nm corresponds to either the next protomer within the same filament or an actin protomer in a neighboring filament (Fig. 4E, right). On average, the distance for the second peak fits was slightly less for WT than for the Acta2R149C/+ SMCs, confirming that the WT has more bundle-like structures, whereas the mutant cell actin is less organized.

Since the R149C mutant SM α-actin is retained in the CCT complex in both yeast cells and rabbit reticulolysates, we sought to determine whether there is increased retention of the R149C mutant SM α-actin in the CCT complex in Acta2R149C/+ SMCs. After immunoprecipitation of the CCT complex using an antibody directed against the TCP1α subunit, followed by immunoblot analyses for SM α-actin, we consistently found increased SM α-actin associated with the CCT complex in the Acta2R149C/+ SMCs when compared with WT SMCs (Fig. 4F). Based on two-dimensional gel electrophoresis, we observed that the cytoplasmic levels of mutant were lower than the WT SM α-actin in the Acta2R149C/+ SMCs (Fig. 4G).

Discussion

Clinical data from families with the ACTA2 R149C variant found that thoracic aortic disease was associated with significantly decreased penetrance; only 60% of carriers have aortic disease by 70 years of age (6). In comparison, FBN1 pathogenic variants are essentially fully penetrant in individuals with Marfan syndrome (17). Here, we show that Acta2R149C/+ mice do not have thoracic aortic disease, thus recapitulating the

Altered SMC phenotype and decreased mutant SM α-actin levels in Acta2R149C/+ SMCs

To further assess how the R149C SM α-actin disrupts cellular function, SMCs were explanted from the ascending

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|----------------|-----------------------------------|--------------------------------|-----------------------------|
| WT             | 16.5 ± 0.4                        | 0.5 ± 0.2                      | 47.8 ± 4.6                  |
| N299T          | 13.2 ± 3.6                        | 0.1 ± 0.02                     | 8.7 ± 0.6                   |
| R149C/N299T    | 10.8 ± 1.5                        | 0.4 ± 0.6                      | 37.0 ± 5.4                  |

Errors indicate SD.

A Data from published work (7).

B Data were obtained from two experiments using two independent protein preparations.

C Data were obtained from four experiments using two independent protein preparations.

to assess their susceptibility to severing. Surprisingly, N299T filaments showed extreme resistance to coflin cleavage even as high as 600 nM coflin (Table 3). R149C/N299T filaments showed a higher frequency of cleavage than N299T filaments, but the absolute frequency was much lower than for WT filaments. Thus, we were not able to conclusively determine how the presence of the R149C alteration affects coflin severing of filaments.

We assessed the interaction of R149C/N299T with profilin, an actin-binding protein that affects the free G-actin pool available for polymerization and found that R149C/N299T binds profilin with an affinity of 2.7 μM, comparable to the value of 3.0 μM previously obtained for WT (Fig. 3C) (7). In contrast, we identified that formin caused more than a 3-fold increase in filament number for R149C/N299T when compared with the N299T control (Table 4). We also assessed the interaction of the SM α-actins with myocardin-related transcription factor-A (MRTF-A), which serves as a coactivator for SMC-specific gene expression (16). In the cytoplasm, each MRTF-A molecule can bind up to five actin monomers in a cooperative fashion. R149C/N299T binds approximately 2-fold less to MRTF-A than WT (Kd of 3.3 μM versus 1.8 μM for WT, but the binding is very cooperative with a Hill coefficient of 4.7, compared with the value of three for WT α-actin. (Fig. 3D) (7, 8).

Finally, the interaction of the WT and mutant actin filaments with phosphorylated SM myosin to generate motion was assessed by an in vitro motility assay. With phalloidin-stabilized actin filaments, the R149C/N299T filaments showed slower speeds than either WT or N299T filaments, with the largest difference occurring in the presence of tropomyosin (Fig. 3E and Table S3). Larger reductions in speed were observed with R149C/N299T filaments that were not stabilized with phalloidin, both in the absence or the presence of tropomyosin (Fig. 3F and Table S3). The slower speeds with which SM myosin moves R149C/N299T actin filaments suggests that this important actin-binding partner interacts less favorably with mutant actin filaments, which could result in decreased force generation by the mutant SMCs as we observed in vivo previously.
decreased penetrance observed in patients with the ACTA2 R149C mutation. The most dramatic biochemical change associated with the R149C SM α-actin was the retention of the mutant actin with the CCT complex, leading to reduced cytosolic levels of the mutant α-actin compared with WT α-actin in SMCs. Importantly, haploinsufficiency of ACTA2 is not established to predispose to highly penetrant HTAD in humans or mice. Thus, the amount of mutant SM α-actin released from the CCT complex could potentially determine whether an individual with the ACTA2 R149C variant has aortic disease or not.

Actins cannot fold spontaneously and require the CCT complex to achieve their native conformation (18). Previous studies have identified both skeletal and cardiac α-actin mutations that result in α-actin folding defects, such as impaired release from the CCT complex or the cochaperone, prefoldin (15, 19). For example, ACTA1 L94P and E259V are recessive missense mutations in skeletal muscle α-actin that predispose to nemaline myopathy. L94P and E259V lack release of the α-actin from the CCT complex and prefoldin, respectively, with such affinity that none of the mutant α-actin is released from these complexes (15). Patients homozygous for these ACTA1 mutations have severe nemaline myopathy, but heterozygous parents are unaffected. These data indicate that individuals with heterozygous ACTA1 alterations, leading to no mutant α-actin released from the CCT complex, have half normal levels of WT skeletal α-actin and no myopathy. Other ACTA1 missense mutations are not sequestered in the CCT complex, and a mutant protein is produced, leading to myopathy in the heterozygous state. These data indicate that individuals with heterozygous ACTA1 L94P and E259V lack release of the mutant α-actin from the CCT complex and prefoldin, and half normal levels of WT skeletal α-actin do not cause myopathy. Based on these data, we hypothesize that the amount of ACTA2 R149C variant released from the CCT complex could determine the penetrance of aortic disease in both mice and humans with this variant.

We previously found that a rare MYH11 missense variant, R247C, alters myosin function and decreases contraction of aortic segments but does not lead to thoracic aortic disease in mice (20). When this MYH11 variant was crossed with Acta2−/− mice, aortic dilation in the double mutant mouse model was augmented when compared with the Acta2−/− mice alone (21). The degree in the decrease of aortic ring contraction did not correlate with rate of aortic enlargement in these studies. Thus, variants in genes triggering HTAD can disrupt protein function and aortic contraction but not lead to thoracic aortic disease. As mentioned previously, we hypothesize that there is a threshold for the levels of mutant α-actin that triggers thoracic aortic disease, but our data indicate that the threshold may not directly correlate with further decreases in aortic contraction. In the case of patients with the ACTA2 R149C alteration, our data suggest that a factor contributing to the penetrance of thoracic aortic disease may be the degree of disruption of actin filaments by varying amounts of mutant SM α-actin released from the CCT complex. Additional genetic studies of patients with ACTA2 R149C pathogenic variants may identify novel genetic variants that modulate the penetrance of aortic disease in these patients and provide insight into factors that alter the release of the mutant α-actin from the CCT complex.

A second mutation N299T in the R149C actin was introduced to allow the release from the CCT folding complex, and the R149C/N299T actin was thus used to assess the defects in biochemical properties caused by the R149C mutation in comparison with WT and N299T actin. In contrast to other mutant actins we have studied, R149C/N299T formed stable filaments, bound normally to profilin, and co-operatively bound to MRTF-A (7, 8). A previously studied ACTA2 mutation, R179H, had a severe polymerization defect with a critical concentration ~40-fold higher than WT, whereas another mutation, R258C, formed only slightly less stable filaments. Both these mutant SM α-actins were released from the CCT complex at levels similar to WT SM α-actin. A feature common to both R258C and R179H actin was their enhanced affinity for profilin, which was not shared by R149C/N299T. The poorly polymerizing R179H showed little co-operative binding to MRTF-A. Interaction of R149C/N299T with the actin-nucleator formin increased filament number to a larger extent than the N299T control. Binding of formin to R179H actin, in contrast, suppressed nucleation and slowed polymerization rates. The actin filaments in the Acta2+/−R149C/− cell

### Table 3
**Severing frequency of WT and mutant actin filaments by coflin**

| Frequency (min⁻¹ μm actin⁻¹ ×10⁵) | 50 nM coflin | 100 nM coflin | 300 nM coflin | 600 nM coflin |
|-----------------------------------|-------------|--------------|--------------|--------------|
| WT                                | 237 ± 90    | 259 ± 123.2  | 293 ± 160.6  | 149.7 ± 49.5 |
| N299Tb                            | 3.6 ± 0.6   | 0.8 ± 2.0    | 2.4 ± 3.8    | 2.0 ± 3.3    |
| R149C/N299Tb                       | 11.6 ± 18   | 28.7 ± 18.6  | 40.0 ± 21.8  | 45.4 ± 31.2  |

There were statistically significant differences between groups as determined by a one-way ANOVA for each coflin concentration (50 nM coflin, p = 3.68 × 10⁻⁹; 100 nM coflin, p = 6.5 × 10⁻⁵; 300 nM coflin, p = 1.6 × 10⁻⁹; and 600 nM coflin, p = 5.9 × 10⁻⁷). Error bars indicate SD.

### Table 4
**Increase of WT and mutant actin filaments in the presence of formin**

| Actin         | 0 nM formin | 50 nM formin |
|---------------|-------------|--------------|
| WT            | 1           | 2.4 ± 0.6    |
| N299T         | 1           | 1.5 ± 0.5    |
| R149C/N299T   | 1           | 5.4 ± 2.3    |

One-way ANOVA followed by a post hoc Tukey’s honest significant difference test showed no significant differences neither between WT and N299T (p = 0.699) nor between WT and R149C/N299T (p = 0.083). The difference between N299T and R149C/N299T was significant (p = 0.03). Data were obtained from three experiments using three different protein preparations.
appear more dispersed with less stress fiber/bundle-like structures and a less oriented appearance compared with filaments in WT cells. The hyperactive nucleation of R149C monomers by formin may enable filament formation at more dispersed locations compared with WT, which in turn would decrease bundling and possibly decrease the ability to generate force. A common feature of all three mutant actins is an impaired interaction with SM myosin, which predicts reduced contraction rate and force generation. Consistent with the in vitro data, ex vivo analyses of contractility in aortic rings confirm decreased force generation by the Acta2R149C/− mice. D, STORM images of cells expressing WT (left) or R149C (right) actin. E, left, the Fourier transform of the images showed many high-frequency bands for the WT cell. Right, histogram of the distance of each actin molecule’s closest neighbor in WT and R149C cells. The solid lines are the sum of two Gaussian peaks for WT (black) and R149C (blue). The dashed line shows individual Gaussian peaks for WT (3.5 ± 2.2 and 6.4 ± 4.6 nm) and R149C (4.1 ± 3.0 and 7.7 ± 5.5 nm). F, the CCT complex was immunoprecipitated using antibodies directed against CCT1 followed by immunoblot analyses for SM α-actin, revealing increased SM α-actin associated with the CCT complex in mutant SMCs and no SM α-actin associated with the CCT complex in WT SMCs. G, 2D gel electrophoresis showed decreased R149C α-actin content compared with WT α-actin in the Acta2R149C/− SMCs. CCT, chaperonin-containing TCP-1; MHC, myosin heavy chain; SMC, smooth muscle cell.
with WT mice. We noted a transient decrease in ejection fraction in hypertensive WT mice treated with L-NAME at 14 weeks and a slower sustained decrease in ejection fraction of WT TAC mice compared with mutant TAC mice with transient increases in stroke volume and cardiac output. These varying responses to biomechanical stress may reflect a difference in fibrotic remodeling of the heart in mutant versus WT mice, which will be assessed in future studies.

Our data support that the presence of the R149C SM α-actin in the Acta2<sup>R149C/+</sup> mice decreases aortic contraction but does not cause thoracic aortic disease, which reflects the decreased penetrance of thoracic aortic disease in patients with this ACTA2 variant. The impaired release of mutant SM α-actin from the CCT complex decreases the level of mutant to WT SM α-actin in the cytoplasm, and clinical studies indicate that ACTA2 variants resulting in a mutant protein are more likely to be disease-causing variants than variants causing haplodeficiency, thus affirming that the amount of mutant actin produced is a major factor in determining whether an individual has disease or not. These data therefore provoke a hypothesis that the amount of R149 SM α-actin released from the CCT may determine whether the mutant SM α-actin causes thoracic aortic disease or not in a given individual. Since ACTA2 R149C mutation carriers have either thoracic aortic disease or early onset coronary artery disease, the unanswered question is whether the increased retention of the mutant SM α-actin in the CCT complex conversely increases the risk for coronary artery disease.

**Experimental procedures**

Additional information on methodologies is presented in the supporting information.

**Engineering and maintenance of the Acta2<sup>R149C/+</sup> mice**

All mouse experiments were approved and performed in accordance with institutional guidelines set forth by the Institutional Animal Care and Use Committee for the University of Texas Health Science Center at Houston (UTHealth; AWC-18-0173) and the National Institutes of Health Guidelines for the care and use of laboratory animals. CRISPR/Cas9 genome editing was used to introduce the ACTA2-R149C mutation into C57BL/6NJ mice (The Jackson Laboratory; catalog no. 005304).

**Preparation of SM strips from mice and force measurements**

Mice were euthanized by exposure to an anesthetic, 2.5% avertin (450 mg/kg intraperitoneal), one time only for a terminal procedure (the dissection and harvesting of ascending aorta). The aorta was isolated with endothelial cells removed by gentle swabbing and excessive adventitia removed by dissection. Ascending (2–3 mm) and descending thoracic (5 mm) aortic segmental rings were mounted by triangular wires to isometric force apparatus as previously described (20). Aortic rings were passively stretched to 1.8 to 2.0 g and remained quiescent for 60 min before precontraction with 65 mM KCl in Krebs–Ringer solution. Aortic rings were also treated with 10 μM PE, an α-adrenergic agonist. Force measurements were normalized as grams of developed force per tissue wet weight.

The urothelium and adventitia were removed from isolated urinary bladder, and the SM layer dissected into longitudinal strips (0.5 × 0.5 × 8.0 mm) was mounted on an isometric force apparatus in physiological salt solution as previously described (20). Strips were equilibrated and stretched 1.2 times slack length. After 30 min of equilibration, the strips were precontracted with 65 mM KCl three times and then stimulated with 10 μM carbachol, a muscarinic agonist (Sigma). Force measurements were recorded isometrically by a Grass FT03 force transducer connected to Powerlab 8/SP data acquisition unit (AD Instruments). Stresses (Newtons per square meter) were calculated to normalize contraction responses to bladder tissue cross-sectional areas.

**Measurement of myosin RLC phosphorylation**

Isolated tissues were snap frozen by clamps prechilled in liquid nitrogen for protein phosphorylation measurements. Frozen muscles were processed as described previously (20). Muscle proteins in 8 M urea sample buffer were subjected to urea/glycerol-PAGE at 400 V for 80 min to separate non-phosphorylated and monophosphorylated RLC. Following electrophoresis, protein was transferred to polyvinylidene difluoride membranes and probed with antibodies against SM-α-actin from the CCT-bound mutant actin resists aortic disease

**Isoelectric focusing analysis**

Aorta and bladder tissue samples (~5 mg) were collected from WT and Acta2<sup>R149C/+</sup> mice and homogenized directly in ReadyPrep 2-D Starter Kit Rehydration/Sample Buffer (Bio-Rad), then subjected to fractionation in a 24 cm, pH 4 to 7, immobilized pH gradient gel strip (GE Healthcare). Samples were focused for a total of 100 kVh in a PROTEAN IEF Cell (Bio-Rad). After isoelectric focusing, the portion of gel strip corresponding to pH 5.1 to 5.4 was subjected to 10% SDS-PAGE and immunoblotted using routine procedures.

**Mouse echocardiography**

Echocardiography was performed with an ultrasound system (Vevo 3100 imaging system; MX550D, 40 MHz transducer; VisualSonics) at different time points indicated. Anesthesia was induced with 4% isoflurane and maintained at 1.5 to 2%; mice were then restrained supine on a heated platform to keep body temperature at 38 °C. Images were acquired and stored as a digital cine loop for offline calculations. Measurements of the aortic diameter were done in at least three separate heartbeats, and the mean of the measurements was calculated. Data analysis was performed blinded.

**Hypertensive treatment and blood pressure measurement**

High salt diet (8% NaCl diet from Harlan Laboratories) and L-NAME (3.0 g/l in drinking water) were used to induce...
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hypertension in age-matched and sex-matched Acta2R149C/+ and WT mice. L-NAME water was changed every day. Measurement of blood pressures in conscious mice was performed with a tail cuff blood pressure analyzer designed with volume-pressure recording technology (Model Coda; Kent Scientific Technology). Body temperature of the mice was maintained at 37 °C during measurement. Data from measurements for 3 consecutive days were used, and the mean value of the readings for each day per mouse was averaged and taken as the single blood pressure measurement for that animal.

TAC

After initial echocardiography, mice at the age of 3 months were anesthetized with isoflurane (4% for induction and 2% for maintenance) and transferred to a heated platform. Anesthetized mice were intubated, followed by midline cervical incision, to expose the aorta. Aortic constriction was achieved by placing a 7.0 nylon suture ligature against a 27-gauge needle on transverse aorta. The needle was removed promptly to create an aortic constriction of 0.4 mm in diameter, and the chest was sealed. The mice were maintained on a heating pad for recovery. Ketoprofen (5 mg/kg subcutaneously) was administered every 24 h for 3 days after surgery. Echocardiography was performed on all surviving mice each week for 4 weeks after surgery.

Aortic histopathology

Paraffin-embedded aorta tissue cross sections (5 μm) were stained with H&E, Verhoeff–Van Gieson, and Movat according to the standard protocols. Specimens were imaged using a Leica microscope. Quantification of aortic wall thickness was performed using ImageJ (the National Institutes of Health). Cell density and elastin breaks were quantified using the cell counter function. All quantitative analyses were performed by two or more researchers.

SMC isolation and culture

SMCs were explanted from the ascending aortas of age-matched and gender-matched Acta2R149C/+ and WT littermates as previously described (https://bio-protocol.org/e2045). SMCs were maintained in SM basal medium (Promo Cell) supplemented with 20% fetal bovine serum (Gibco), insulin, epidermal growth factor, fibroblast growth factor (Promo Cell), Hepes (Millipore Sigma), sodium pyruvate (Millipore Sigma), l-glutamine (Millipore Sigma), and antibiotic/antimycotic (Millipore Sigma). SMCs were serum starved overnight, lysed, and fractionated using the CellLytic NuCLEAR Extraction Kit (Sigma; NXTRACT-1KT).

Immunofluorescence

Cells were seeded on 22 mm cover slips and attached overnight. Cells were fixed in 4% paraformaldehyde in 0.1 M PBS. The SMCs were permeabilized with 0.3% Triton X-100 for 15 min, blocked in 1% bovine serum albumin in PBS for 1 h, and then incubated with α-actin primary antibody at 4 °C overnight. Cells were incubated with Alexa Fluor 488 Goat-antimouse at room temperature for 1 h and Texas Red-X phalloidin for 40 min. Immunofluorescent images were obtained using the Leica DMi8 SPE confocal microscopy at the indicated magnification.

Immunoprecipitation

Cells were lysed in 20 mM Tris, pH 8, 137 mM NaCl, 1% NP-40, and 2 mM EDTA and incubated in 10 mM Tris (pH 8), 150 mM NaCl, 0.1 mM EGTA, 20% glycerol, and 0.2% NP-40 and TCP1α antibody overnight at 4 °C with normal rabbit immunoglobulin G used as the control. Protein A/G magnetic beads were washed in 10 mM Tris, pH 8, 150 mM NaCl, 1% NP-40 three times, and incubated with samples at 4 °C for 3 h. Protein was eluted in 2× Laemmli buffer (Bio-Rad) by heating the beads at 70 °C for 10 min and collecting the eluate using magnetic separation.

Super-resolution microscopy

Super-resolution images were obtained on a commercial Nikon N-STORM super-resolution microscope. Raw images were processed with the ImageJ plugin ThunderStorm to obtain the final super-resolution images (25). See supporting information for additional details.

Actin cloning and expression

The base SM α-actin–thymosin construct described previously was recloned into pFastBac (Thermo Fisher Scientific) and mutated to contain either N299T or R149C/N299T (7–9). Recombinant baculovirus was produced using the Bac-to-Bac Baculovirus expression system (Thermo Fisher Scientific). See supporting information for additional details.

Reticulocyte lysate in vitro translation assays

ACTA2 and ACTA1 were each cloned into the vector CMVTnT (Promega; catalog no. L5620) behind the T7 promoter. An additional 30 polyA tail was added after each gene for mRNA stability. All DNA preps in the reticulocyte study were eluted in RNase-free water. The SM α-actin variants introduced by site-directed mutagenesis were R149C, N299T, and F-to-G actin assay

The fraction of F-to-G actin was assayed using the G-actin/F-actin In Vivo Assay Kit (Cytoskeleton).

Subcellular fractionation

SMCs were serum starved overnight, lysed, and fractionated using the CellLytic NuCLEAR Extraction Kit (Sigma; NXTRACT-1KT).

Western blotting

Lysates from whole cells or tissues were fractionated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane according to the standard protocols. Immunoblots were quantitated with ImageJ.

F-to-G actin assay

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and R149C/N299T. All constructs were sequenced to verify mutagenesis and confirm the absence of PCR-induced errors. The individual SM α-actin variants were expressed in vitro in reticulocyte lysates using the Promega Transcription Translation Reaction Protocol (400 ng DNA) and labeled with 35S methionine. Reaction products were analyzed on 4.5% non-denaturing Safer gels with ATP and autoradiography to determine the protein levels of CCT-bound actin (26).

**Biochemical assays of actin**

In vitro motility and TIRF microscopy assays were previously described (7, 8). TIRF microscopy used for the polymerization assays in the absence or the presence of actin-binding proteins was carried out on a Nikon ECLIPSE Ti microscope with through-objective type TIRF. The speed at which rhodamine phalloidin–stabilized actin filaments were moved by phosphorylated SM myosin was determined using a semiautomated tracking program that allowed analysis of large numbers of filaments, which were then fitted to a Gaussian distribution as previously described (7). For speed of movement using filaments that were not stabilized with phalloidin, WT filaments were formed from 25% rhodamine-labeled WT actin and 75% unlabeled WT. N299T and N299T/R149C filaments were formed from 25% rhodamine-labeled N299T actin and 75% of unlabeled N299T or N299T/R149C actin. Filament speed was tracked manually with ImageJ because the automated program could not detect the dimmer filaments. See supporting information for additional details.

**Statistical analysis**

All data are representative of at least three separate experiments unless noted otherwise. Difference in survival was determined by a Gehan–Breslow–Wilcoxon test. A two-tailed unpaired t test or one-way ANOVA followed by a Tukey’s honest significant difference post hoc test was used to determine statistical significance between groups. A p value less than 0.05 indicated statistical significance.

**Data availability**

The data underlying this article are available from the corresponding author upon reasonable request.

**Supporting information**—This article contains supporting information (7).

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: ACTA1, gene for skeletal muscle α-actin; ACTA2, gene for smooth muscle α-actin; CCT, chaperonin-containing TCP-1; HTAD, heritable thoracic aortic disease; L-NAMe, l-N-nitroarginine methyl ester; MRTF-A, myocardin-related transcription factor-A; PE, phallopinephrine; RLC, regulatory light chain; SM, smooth muscle; SMG, smooth muscle cell; TAC, transverse aortic constriction; TIRF, total internal reflection fluorescence.

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