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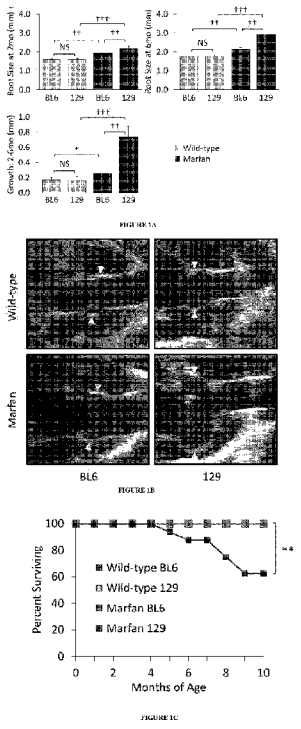
(54) Title: MAP KINASE PATHWAY TARGETS FOR THE TREATMENT OF MARFAN SYNDROME

(57) Abstract: The instant disclosure provides methods and compositions for the diagnosis, treatment and prevention of Marfan syndrome and related diseases, disorders and conditions. The disclosure further provides pharmaceutical compositions and kits for the diagnosis, treatment and prevention of Marfan syndrome and related diseases, disorders and conditions.



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MAP KINASE PATHWAY TARGETS FOR THE TREATMENT OF MARFAN SYNDROME

CROSS REFERENCE TO RELATED APPLICATION(S)

[0001] The present application claims the benefit of U.S. Provisional Patent Application No. 62/466,197 filed March 2, 2017, the entire contents of which are incorporated by reference herein.

GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant No.: R01 122586 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] Marfan syndrome (MFS) is a systemic connective tissue disorder with autosomal dominant inheritance, caused by mutations in the FBN1 gene. It has cardinal features involving the ocular, skeletal and cardiovascular systems, with the major cause of mortality resulting from aortic root dilatation, dissection and rupture.

[0004] Many of the features of Marfan syndrome are common in the general population and represent a tremendous public health burden. These include aortic aneurysm (1-2% of the population at large), mitral valve prolapse (~7%), emphysema (11%), scoliosis (0.5%), cataract (30%), arthritis (very common), and myopathy (many common genetic and acquired forms).

SUMMARY

[0005] As described below, the present disclosure features compositions and methods for the treatment of Marfan syndrome diseases and disorders.

[0006] In one aspect, the instant disclosure provides a method for treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition involving administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling, thereby treating the subject.

[0007] In one embodiment, the agent that modulates the activity of MAP kinase pathway signaling is a MAP kinase pathway inhibitor. In certain embodiments, the agent that

modulates the activity of MAP kinase pathway signaling is an inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof. Optionally, the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is an antisense agent or a double-stranded nucleic acid, optionally a siRNA or shRNA specific for MMP17, MAP2K6 or MAP3K4.

[0008] In certain embodiments, the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is specific for a nucleic acid molecule having at least a 50% sequence identity to SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8.

[0009] In certain embodiments, the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is specific for a nucleic acid molecule set forth as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8.

[0010] In another embodiment, the agent that modulates the activity of MAP kinase pathway signaling is a small molecule. Optionally, the agent that modulates the activity of MAP kinase pathway signaling comprise Batimastat, GI 254023X, GM 6001, TMI 1, WAY 170523, WX-554 or combinations thereof.

[0011] In certain embodiments, the agent that modulates the activity of MAP kinase pathway signaling is an antibody.

[0012] In some embodiments, the Marfan syndrome-associated disease or disorder is a clinical condition associated with Marfan syndrome. Optionally, the disease or disorder is an aneurysm, an aortic aneurysm, or emphysema. In certain embodiments, the disease or disorder is an aneurysm.

[0013] Another aspect of the disclosure provides a method for treating a subject having Marfan syndrome or a Marfan-associated condition involving identifying a variant allele of MMP17, MAP2K6 or MAP3K4 that produces elevated expression and/or activity of a gene product of the MMP17, MAP2K6 or MAP3K4 gene; and replacing the variant allele of MMP17, MAP2K6 or MAP3K4 with an allele of MMP17, MAP2K6 or MAP3K4 that does not produce elevated expression and/or activity of a gene product of the MMP17, MAP2K6 or MAP3K4 gene, thereby treating the subject.

[0014] In one embodiment, the step of replacing the variant allele is performed via a CRISPR-Cas9 gene replacement process. Optionally, the step of replacing is performed ex vivo. In a related embodiment, re-introduction to the subject of a cell manipulated ex vivo to perform gene replacement treats the subject.

[0015] In certain embodiments, the variant allele of MMP17, MAP2K6 or MAP3K4 that produces elevated expression and/or activity of a gene product of the MMP17, MAP2K6 or MAP3K4 gene comprises a nucleic acid sequence having at least a 50% sequence identity to SEQ ID NOS: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23.

[0016] In certain embodiments, the variant allele of MMP17, MAP2K6 or MAP3K4 that produces elevated expression and/or activity of a gene product of the MMP17, MAP2K6 or MAP3K4 gene comprise nucleic acid sequences set forth as SEQ ID NOS: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23.

[0017] An additional aspect of the disclosure provides a pharmaceutical composition for the treatment of a disease or disorder characterized by aberrant MAP kinase pathway expression or activity, where the pharmaceutical composition includes an agent that modulates the activity of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof.

[0018] A further aspect of the disclosure provides a kit for identifying a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition that includes primers specific for amplification of MMP17, MAP2K6 and/or MAP3K4; a probe oligonucleotide, optionally fluorescently labeled, that specifically detects the presence of a MMP17, MAP2K6 and/or MAP3K4 amplicon, wherein detection of elevated expression of MMP17, MAP2K6 and/or MAP3K4 identifies the subject as having or at risk of developing Marfan Syndrome or a Marfan-associated condition; and instructions for use.

[0019] In certain embodiments, the kit further includes a pharmaceutical composition of the instant disclosure.

[0020] Another aspect of the disclosure provides a method for identifying and treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition involving: isolating a sample from the subject; assessing the expression level of MMP17, MAP2K6 and/or MAP3K4 in the sample, assessing the level of activity of a gene product of MMP17, MAP2K6 and/or MAP3K4 in the sample, and/or genotyping MMP17, MAP2K6 and/or MAP3K4 in the sample; identifying elevated expression of MMP17, MAP2K6 and/or MAP3K4 in the sample, identifying elevated activity of a gene product of MMP17, MAP2K6 and/or MAP3K4 in the sample, and/or identifying a genotype of MMP17, MAP2K6 and/or MAP3K4 in the sample that causes elevated expression and/or activity of MMP17, MAP2K6 and/or MAP3K4, or of a gene product thereof; and administering a therapy for Marfan Syndrome or a Marfan-associated condition to the subject, thereby treating the subject.

[0021] In certain embodiments, a method for treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprises administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling and/or expression, function or activity of epidermal growth factor receptor (EGFR); thereby treating the subject.

[0022] In certain embodiments, the therapy for Marfan Syndrome or a Marfan-associated condition comprises administration of a TGFβ NAb, losartan and/or an inhibitor of MMP17, MAP2K6 and/or MAP3K4, or of a gene product thereof.

[0023] *Definitions*

[0024] By "agent" is meant any small molecule chemical compound, antibody, nucleic acid molecule, or polypeptide, or fragments thereof.

[0025] By "ameliorate" is meant decrease, suppress, attenuate, diminish, arrest, or stabilize the development or progression of a disease.

[0026] By "alteration" is meant a change (increase or decrease) in the expression levels or activity of a gene or polypeptide as detected by standard art known methods such as those described herein. As used herein, an alteration includes a 10% change in expression levels, preferably a 25% change, more preferably a 40% change, and most preferably a 50% or greater change in expression levels.

[0027] By the terms "conjugated," "linked," "attached," "fused" and "tethered," when used with respect to two or more moieties, means that the moieties or domains are physically associated or connected with one another, either directly or via one or more additional moieties that serve as a linking agent, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, e.g., physiological conditions. The linkage can be based on genetic fusion according to the methods known in the art or can be performed by, e.g., chemical cross-linking. The compounds and targeting agents may be linked by a flexible linker, such as a polypeptide linker or a synthetic linker. The polypeptide linker can comprise plural, hydrophilic or peptide-bonded amino acids of varying lengths. The term "associated" will be used for the sake of brevity and is meant to include all possible methods of physically associating each compound with one or more desired molecules or agents.

[0028] By "detectable label" is meant a composition that when linked to a molecule of interest renders the latter detectable, via spectroscopic, photochemical, biochemical,

immunochemical, or chemical means. For example, useful labels include radioactive isotopes, magnetic beads, metallic beads, colloidal particles, fluorescent dyes, electron-dense reagents, enzymes (for example, as commonly used in an ELISA), biotin, digoxigenin, or haptens. The detectable label is associated with a composition of interest by any possible methods of physically associating each compound to a detectable label.

[0029] By "disease" is meant any condition or disorder that damages or interferes with the normal function of a cell, tissue, or organ. Examples of diseases include Marfan Syndrome.

[0030] As used herein, the term "EGFR inhibitor" is intended to include therapeutic agents that inhibit, downmodulate, suppress or downregulate EGFR signaling activity. The term is intended to include chemical compounds, such as small molecule inhibitors (e.g., small molecule tyrosine kinase inhibitors) and biologic agents, such as antibodies, interfering RNA (shRNA, siRNA), soluble receptors and the like.

[0031] By "effective amount" is meant the amount of a required to ameliorate the symptoms of a disease relative to an untreated patient. The effective amount of active compound(s) used to practice the present disclosure for therapeutic treatment of a disease varies depending upon the manner of administration, the age, body weight, and general health of the subject. Ultimately, the attending physician or veterinarian will decide the appropriate amount and dosage regimen. Such amount is referred to as an "effective" amount.

[0032] The disclosure provides a number of targets that are useful for the development of highly specific drugs to treat or a disorder characterized by the methods delineated herein. In addition, the methods of the disclosure provide a facile means to identify therapies that are safe for use in subjects. In addition, the methods of the disclosure provide a route for analyzing virtually any number of compounds for effects on a disease described herein with high-volume throughput, high sensitivity, and low complexity.

[0033] By "fragment" is meant a portion of a polypeptide or nucleic acid molecule. This portion contains, preferably, at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% of the entire length of the reference nucleic acid molecule or polypeptide. A fragment may contain 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100, 200, 300, 400, 500, 600, 700, 800, 900, or 1000 nucleotides or amino acids.

[0034] "Hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleobases.

For example, adenine and thymine are complementary nucleobases that pair through the formation of hydrogen bonds.

[0035] By "inhibitory nucleic acid" is meant a double-stranded RNA, siRNA, shRNA, or antisense RNA, or a portion thereof, or a mimetic thereof, that when administered to a mammalian cell results in a decrease (e.g., by 10%, 25%, 50%, 75%, or even 90-100%) in the expression of a target gene. Typically, a nucleic acid inhibitor comprises at least a portion of a target nucleic acid molecule, or an ortholog thereof, or comprises at least a portion of the complementary strand of a target nucleic acid molecule. For example, an inhibitory nucleic acid molecule comprises at least a portion of any or all of the nucleic acids delineated herein.

[0036] By "marker" is meant any protein or polynucleotide having an alteration in expression level or activity that is associated with a disease or disorder.

[0037] As used herein, "obtaining" as in "obtaining an agent" includes synthesizing, purchasing, or otherwise acquiring the agent.

[0038] "Primer set" means a set of oligonucleotides that may be used, for example, for PCR. A primer set would consist of at least 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 30, 40, 50, 60, 80, 100, 200, 250, 300, 400, 500, 600, or more primers.

[0039] By "reduces" is meant a negative alteration of at least 10%, 25%, 50%, 75%, or 100%.

[0040] By "reference" is meant a standard or control condition.

[0041] A "reference sequence" is a defined sequence used as a basis for sequence comparison. A reference sequence may be a subset of or the entirety of a specified sequence; for example, a segment of a full-length cDNA or gene sequence, or the complete cDNA or gene sequence. For polypeptides, the length of the reference polypeptide sequence will generally be at least about 16 amino acids, preferably at least about 20 amino acids, more preferably at least about 25 amino acids, and even more preferably about 35 amino acids, about 50 amino acids, or about 100 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least about 50 nucleotides, preferably at least about 60 nucleotides, more preferably at least about 75 nucleotides, and even more preferably about 100 nucleotides or about 300 nucleotides or any integer thereabout or therebetween.

[0042] By "siRNA" is meant a double stranded RNA. Optimally, an siRNA is 18, 19, 20, 21, 22, 23 or 24 nucleotides in length and has a 2 base overhang at its 3' end. These dsRNAs can be introduced to an individual cell or to a whole animal; for example, they may be introduced systemically via the bloodstream. Such siRNAs are used to downregulate mRNA levels or promoter activity.

[0043] By "specifically binds" is meant a compound or antibody that recognizes and binds a polypeptide of the disclosure, but which does not substantially recognize and bind other molecules in a sample, for example, a biological sample, which naturally includes a polypeptide of the disclosure.

[0044] Nucleic acid molecules useful in the methods of the disclosure include any nucleic acid molecule that encodes a polypeptide of the disclosure or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. Nucleic acid molecules useful in the methods of the disclosure include any nucleic acid molecule that encodes a polypeptide of the disclosure or a fragment thereof. Such nucleic acid molecules need not be 100% identical with an endogenous nucleic acid sequence, but will typically exhibit substantial identity. Polynucleotides having "substantial identity" to an endogenous sequence are typically capable of hybridizing with at least one strand of a double-stranded nucleic acid molecule. By "hybridize" is meant pair to form a double-stranded molecule between complementary polynucleotide sequences (e.g., a gene described herein), or portions thereof, under various conditions of stringency. (See, e.g., Wahl, G. M. and S. L. Berger (1987) *Methods Enzymol.* 152:399; Kimmel, A. R. (1987) *Methods Enzymol.* 152:507).

[0045] For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium

dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred: embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

[0046] For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42 C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

[0047] By "substantially identical" is meant a polypeptide or nucleic acid molecule exhibiting at least 50% identity to a reference amino acid sequence (for example, any one of the amino acid sequences described herein) or nucleic acid sequence (for example, any one of the nucleic acid sequences described herein). Preferably, such a sequence is at least 60%,

more preferably 80% or 85%, and more preferably 90%, 95% or even 99% identical at the amino acid level or nucleic acid to the sequence used for comparison.

[0048] Sequence identity is typically measured using sequence analysis software (for example, Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, BLAST, BESTFIT, GAP, or PILEUP/PRETTYBOX programs). Such software matches identical or similar sequences by assigning degrees of homology to various substitutions, deletions, and/or other modifications. Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. In an exemplary approach to determining the degree of identity, a BLAST program may be used, with a probability score between e-3 and e-100 indicating a closely related sequence.

[0049] By "subject" is meant a mammal, including, but not limited to, a human or non-human mammal, such as a bovine, equine, canine, ovine, or feline.

[0050] Ranges provided herein are understood to be shorthand for all of the values within the range. For example, a range of 1 to 50 is understood to include any number, combination of numbers, or sub-range from the group consisting 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50.

[0051] As used herein, the terms "treat," treating," "treatment," and the like refer to reducing or ameliorating a disorder and/or symptoms associated therewith. It will be appreciated that, although not precluded, treating a disorder or condition does not require that the disorder, condition or symptoms associated therewith be completely eliminated.

[0052] Unless specifically stated or obvious from context, as used herein, the term "or" is understood to be inclusive. Unless specifically stated or obvious from context, as used herein, the terms "a", "an", and "the" are understood to be singular or plural.

[0053] Unless specifically stated or obvious from context, as used herein, the term "about" is understood as within a range of normal tolerance in the art, for example within 2 standard deviations of the mean. About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from context, all numerical values provided herein are modified by the term about.

[0054] The recitation of a listing of chemical groups in any definition of a variable herein includes definitions of that variable as any single group or combination of listed groups. The recitation of an embodiment for a variable or aspect herein includes that embodiment as any single embodiment or in combination with any other embodiments or portions thereof.

[0055] Any compositions or methods provided herein can be combined with one or more of any of the other compositions and methods provided herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[0056] Figures 1A-1C show the aortic phenotype of pure C57BL/6J (BL6) and 129S6/SvEvTac (129) mice. Figure 1A: Aortic root size (mm) in BL6 (n=16) and 129 (n=13) wild-type (WT) mice, and BL6 (n=15) and 129 (n=15) *Fbn1*$^{C1039G/+}$ (MFS) mice at 2 and 6 months of age, and postnatal aortic root growth (mm) from 2 to 6 months; Figure 1B: Representative parasternal long axis echocardiograms at 6 months of the aortic root (yellow arrow) and ascending aorta (red arrow) in WT and MFS mice on BL6 and 129 strains; Figure 1C: Survival curve to 10 months for WT and MFS mice on pure BL6 and 129 strains. *<0.05, **<0.01, †<0.001, ††<0.0001, †††<0.00001, NS non-significant.

[0057] Figures 2A-2E show the molecular mechanisms driving aortic disease in MFS mice. Figure 2A: Western blot analysis of the aortic root of 4 WT and 4 MFS mice on BL6 and 129 strains at 10 months of age; Figure 2B: Aortic root growth (mm) from 2 to 6 months in placebo- (n=12) and losartan-treated (n=9) 129 WT mice, and placebo- (n=11) and losartan-treated (n=8) 129 MFS mice; Aortic root growth from 2 to 4 months in placebo- (n=13) and RDEA119-treated (n=5) 129 WT mice, and placebo- (n=13) and RDEA119-treated (n=10) 129 MFS mice; Figure 2C: Survival curve up to 8 months of treatment in placebo-and losartan-treated 129 WT and MFS mice; Figure 2D: Western blot analysis of the aortic root of 3 placebo- and 3 losartan-treated 129 WT and MFS mice at 10 months of age; Figure 2E: Western blot analysis of the aortic root of 3 placebo- and 3 RDEA119-treated 129 WT and MFS mice at 4 months of age. Plac: placebo; Los: losartan; RDEA: RDEA119. *<0.05, **<0.01, †<0.001, ††<0.0001, †††<0.00001, NS non-significant.

[0058] Figures 3A-3G show the mapping modifier loci of aortic aneurysm in MFS mice. Figure 3A: Single QTL genome scan of intercrossed MFS mice that possessed either a 'BL6-like' aortic root size (<2.20mm; n=35) or a '129-like' aortic root size (>2.70mm; n=40) at 6 months of age; genome wide significance LOD score of 3.82 is indicated by the red line;

2 loci exceeded this threshold, with individual LOD scores and adjusted p-values indicated; Figure 3B: Aortic root size (mm) at 6 months of age in F2 generation intercrossed BL6/129 MFS mice, stratified by number of 129 alleles in the 2 regions of interest (average n per group = 13); Figure 3C: Aortic root size (mm) at 6 months of age in MFS mice lacking either one or two functional *Mmp17* and *Map2k6* alleles (n≥10 per group); Figure 3D: Western blot analysis of the aortic root at 10 months of age in 3 WT mice, 3 pure 129 MFS mice, 3 pure BL6 MFS mice, and 3 MFS mice either retaining (+/+) or lacking (-/-) functional *Mmp17* and *Map2k6* alleles; Figure 3E: Western blot analysis of Egfr activation in the aortic root at 10 months of age in 4 pure BL6 and pure 129 WT and MFS mice, as well as 3 MFS mice either retaining (+/+) or lacking (-/-) functional *Mmp17* and *Map2k6* alleles; Figure 3F: Aortic root growth (mm) from 2 to 4 months of age in placebo-(n=12) and erlotinib-treated (n=6) 129 WT mice, and placebo- (n=11) and erlotinib-treated (n=11) 129 MFS mice; Figure 3G: Western blot analysis at 4 months of age in 3 placebo- and 3 erlotinib-treated 129 WT and MFS mice. Chr: Chromosome; M17: Mmp17; M2: Map2k6; Plac: placebo; Erlo: erlotinib. *<0.05, **<0.01, †<0.001, ††<0.0001, NS non-significant.

[0059] Figures 4A-4E show the identification of a modifier locus for aortic aneurysm in MFS patients. Figure 4A: Pedigrees of 5 MFS families recruited for genome wide linkage analysis; yellow indicates MFS patients with mild aortic disease, black indicates MFS patients with severe aortic disease, grey indicates patients with indeterminate data, and white indicates family members who do not have MFS; Figure 4B: Parametric linkage analysis revealed 1 locus that surpassed a genome wide significance LOD score of 3.3, which is indicated by the red line; Figure 4C: Quantitative PCR analysis of *MAP3K4* mRNA expression from cultured dermal fibroblasts of 2 controls, 2 MFS patients from family B with mild aortic disease and 2 MFS patients from family B with severe aortic disease; all biological samples were run in experimental triplicate and normalized to β-actin; Figure 4D: Aortic root growth from 2 to 6 months of age in WT mice either retaining (+/+; n=12) or haploinsufficient (+/- n=7) for Map3k4, and MFS mice either retaining (n=8) or haploinsufficient (n=7) for Map3k4; Figure 4E: Western blot analysis of the aortic root at 10 months of age in 3 WT and 3 MFS mice either retaining or haploinsufficient for Map3k4. M3k4: Map3k4; *<0.05, **<0.01, †<0.001, NS non-significant

[0060] Figure 5 shows the ascending aortic size (mm) in BL6 (n=16) and 129 (n=13) WT mice, and BL6 (n=15) and 129 (n=15) MFS mice at 2 and 6 months of age, and postnatal

growth (mm) from 2 to 6 months. AA: Ascending aorta; *<0.05, **<0.01, †<0.001, ††<0.0001, NS non-significant.

[0061] Figure 6 shows the systolic blood pressure (BP) at 6 months of age in WT and MFS mice on pure BL6 and 129 strains (n=5 per group). NS: non-significant.

[0062] Figure 7 shows the weight (in grams) at 2 months of age in female and male WT and MFS mice on pure BL6 and 129 strains (n>10 per group). NS non-significant.

[0063] Figure 8 shows representative sections of lung alveoli, and mean linear intercept (MLI; μm) in 10-month old WT and MFS mice on pure BL6 and 129 strains (n=5 per group). Line: 50μm. **<0.01, †<0.001, ††<0.0001, NS non-significant.

[0064] Figure 9 shows representative spine x-rays and mean modified kyphosis index (length of yellow arrow; mm) in 10-month old WT and MFS mice on pure BL6 and 129 strains (n=10 per group). Line: 10mm. *<0.05, ††<0.0001, †††<0.00001, NS non-significant.

[0065] Figure 10 shows the aortic root size (mm) at 6 months of age in WT and MFS mice on pure (F0 generation) BL6 and 129 strains (n as per Figure 1A), and F1 generation intercrossed WT (n=20) and MFS (n=21) mice. †<0.001, ††<0.0001, NS non-significant.

[0066] Figure 11 shows the Mmp17 C-terminal DNA and amino acid sequences, illustrating the 9 amino acid truncation in the BL6 mouse strain (SEQ ID NO: 24) relative to the 129 strain (SEQ ID NO: 25). The human MMP17 sequences (SEQ ID NO: 26) are displayed for comparison. The DNA sequence highlighted in red identifies the 129 codon that corresponds to the BL6 stop codon. The amino acid highlighted in red represents the predicted start site of the GPI anchor in each sequence.

DETAILED DESCRIPTION

[0067] A need exists for methods and compositions for the diagnosis and treatment of Marfan syndrome and associated diseases, disorders and conditions.

[0068] The instant disclosure is based, at least in part, upon the discovery that upregulation of expression and/or activity of certain MAP kinase pathway components drives Marfan Syndrome (MFS) development in a mouse model of Marfan Syndrome. Genetic backcrosses were performed between mice of an MFS model and a non-MFS-afflicted strain of mice, thereby identifying genetic loci associated with MFS. In particular, loci that caused elevated expression and/or activity of MMP17, MAP2K6 or MAP3K4, or of gene products thereof, were identified as contributing to MFS and related conditions in such mice.

Modulation of MAP kinase pathway signaling, and particularly inhibition and/or gene replacement of MMP17, MAP2K6 and/or MAP3K4, or of gene products thereof, is thereby discovered to be a therapeutic approach for MFS. MFS diagnosis is also aided by this identification of MAP kinase pathway signaling components/loci indicative of development of MFS in a subject.

[0069] *Marfan Syndrome (MFS)*

[0070] Marfan syndrome (MFS) is an autosomal dominant connective tissue disorder that includes a predisposition for aortic root aneurysm and aortic rupture. MFS is caused by a deficiency of the microfibrillar constituent protein fibrillin 1 that is imposed by heterozygous mutations in FBN1.

[0071] Marfan syndrome (MFS) is a systemic disorder with autosomal dominant inheritance and a prevalence of approximately 1 per 5,000 population (Pyeritz, R.E. & McKusick, V.A. (1979) *N Engl J Med.* 300, 772-777). The syndrome shows no racial preference and both sexes are affected equally. It has been estimated that 25% of cases occur due to spontaneous mutations. While this condition shows high penetrance, marked interfamilial clinical variability is the rule (Pyeritz, R.E. et al. (1979) *Birth Defects Orig Artic Ser.* 15, 155-178). Historic lack of a specific biochemical or genetic marker of disease, coupled with the variability in clinical presentation, has frustrated diagnosis of equivocal cases and has likely contributed to a significant underestimation of the prevalence of disease.

[0072] The cardinal features of this disorder involve the ocular, skeletal, and cardiovascular systems. Cardiovascular pathology, including aortic root dilatation, dissection, and rupture, pulmonary artery dilatation, myxomatous valve changes with insufficiency of the mitral and aortic valves, and progressive myocardial dysfunction, is the leading cause of mortality in the MFS. The majority of fatal events associated with untreated MFS occur in early adult life. In a prospective study of 72 patients in 1972, the average age of death was 32 years (Murdoch, J.L. *et al.* (1972) *N Engl J Med.* 286, 804-808).

[0073] Skeletal involvement is evident in nearly all people with MFS. Progressive anterior chest deformity or scoliosis can cause cardiopulmonary dysfunction and commonly require surgical correction. Joint instability can cause physical disability and predispose to premature arthritis. Lung disease most commonly manifests with spontaneous pneumothorax and has been identified in 4-11% of MFS patients (Wood, J.R., *et al.* (1984) *Thorax.* 39, 780-784; Hall, J.R., *et al.* (1984) *Ann Thorac Surg.* 37, 500-504).

[0074] Many of the features of Marfan syndrome are common in the general population and represent a tremendous public health burden. These include aortic aneurysm (1-2% of the population at large), mitral valve prolapse (~7%), emphysema (11%), scoliosis (0.5%), cataract (30%), arthritis (very common), and myopathy (many common genetic and acquired forms).

[0075] Previous work has implicated enhanced TGFβ signaling in MFS disease pathogenesis. In *Fbn1*$^{C1039G/+}$ mice (hereafter termed MFS mice), a well-validated model of MFS, multiple phenotypic manifestations, including aortic aneurysm, developmental emphysema, mitral valve disease and skeletal muscle myopathy, can be attenuated by administration of TGFβ neutralizing antibody (TGFβ NAb) or the angiotensin-II (Ang-II) type 1 receptor blocker (ARB) losartan, in association with blunted Smad2/3 activation (Habashi *et al.*, *Science* 312, 117; Neptune *et al.*, *Nature Genetics* 33, 407; Ng *et al.*, *Journal Clinical Investigation* 114, 1586; Cohn *et al.*, Nature Medicine 13, 204).

[0076] It is well-recognized that the severity of aortic disease and other systemic manifestations in patients with MFS is highly variable. While the causes for this are not well understood, there is clearly a strong genetic component, since members of the same family often share similar manifestations and outcomes. Despite this, there is very limited correlation between the type or location of a patient's FBN1 mutation and their disease. This implies that there may be other major modifier genes which determine the penetrance and/or severity of a patient's disease.

[0077] A need exists for methods and compositions for the treatment of Marfan syndrome and associated diseases, disorders and conditions, e.g., diseases, disorders and conditions associated with aberrant MAP kinase pathway component expression and/or activity.

[0078] *MAP Kinase Pathway Signaling*

[0079] A mitogen-activated protein kinase (MAPK or MAP kinase) is a type of protein kinase that is specific to the amino acids serine, threonine, and tyrosine (i.e., a serine/threonine-specific protein kinase). MAPKs are involved in directing cellular responses to a diverse array of stimuli, such as mitogens, osmotic stress, heat shock and proinflammatory cytokines. They regulate cell functions including proliferation, gene expression, differentiation, mitosis, cell survival, and apoptosis (Pearson G, *et al.* (Apr 2001). *Endocrine Reviews*. 22 (2): 153–83).

[0080] MAP kinases are found in eukaryotes only, but they are fairly diverse and encountered in all animals, fungi and plants, and even in an array of unicellular eukaryotes.

[0081] MAPKs belong to the CMGC (CDK/MAPK/GSK3/CLK) kinase group. The closest relatives of MAPKs are the cyclin-dependent kinases (CDKs; Manning G, *et al.* (Dec 2002). *Science*. 298 (5600): 1912–34)).

[0082] The first mitogen-activated protein kinase to be discovered was ERK1 (MAPK3) in mammals. Since ERK1 and its close relative ERK2 (MAPK1) are both involved in growth factor signaling, the family was termed "mitogen-activated". With the discovery of other members, even from distant organisms (e.g. plants), it has become increasingly clear that the name is a misnomer, since most MAPKs are actually involved in the response to potentially harmful, abiotic stress stimuli (hyperosmosis, oxidative stress, DNA damage, low osmolarity, infection, etc.). Because plants cannot "flee" from stress, terrestrial plants have the highest number of MAPK genes per organism ever found. Thus the role of mammalian ERK1/2 kinases as regulators of cell proliferation is not a generic, but a highly specialized function.

[0083] Most MAPKs have a number of shared characteristics, such as the activation dependent on two phosphorylation events, a three-tiered pathway architecture and similar substrate recognition sites. These are the "classical" MAP kinases. But there are also some ancient outliers from the group as sketched above, that do not have dual phosphorylation sites, only form two-tiered pathways, and lack the features required by other MAPKs for substrate binding. These are usually referred to as "atypical" MAPKs (Coulombe P, Meloche S (Aug 2007). *Biochimica et Biophysica Acta*. 1773 (8): 1376–87). It is yet unclear if the atypical MAPKs form a single group as opposed to the classical ones.

[0084] Mitogen-activated protein kinases are catalytically inactive in their base form. In order to become active, they require (potentially multiple) phosphorylation events in their activation loops. This is conducted by specialized enzymes of the STE protein kinase group.

[0085] In the case of classical MAP kinases, the activation loop contains a characteristic TxY (threonine-x-tyrosine) motif (TEY in mammalian ERK1 and ERK2, TDY in ERK5, TPY in JNKs, TGY in p38 kinases) that needs to be phosphorylated on both the threonine and the tyrosine residues in order to lock the kinase domain in a catalytically competent conformation. *In vivo* and *in vitro*, phosphorylation of tyrosine precedes

phosphorylation of threonine, although phosphorylation of either residue can occur in the absence of the other.

[0086] This tandem activation loop phosphorylation (that was proposed to be either distributive or processive, dependent on cellular environment) is performed by members of the Ste7 protein kinase family, also known as MAP2 kinases. MAP2 kinases in turn, are also activated by phosphorylation, by a number of different upstream serine-threonine kinases (MAP3 kinases). Because MAP2 kinases display very little activity on substrates other than their cognate MAPK, classical MAPK pathways form multi-tiered, but relatively linear pathways. These pathways can effectively convey stimuli from the cell membrane (where many MAP3Ks are activated) to the nucleus (where only MAPKs may enter) or to many other subcellular targets.

[0087] In comparison to the three-tiered classical MAPK pathways, some atypical MAP kinases appear to have a more ancient, two-tiered system. ERK3 (MAPK6) and ERK4 (MAPK4) were recently shown to be directly phosphorylated and thus activated by PAK kinases (related to other MAP3 kinases; Déléris P, *et al.* (Feb 2011). *The Journal of Biological Chemistry*. 286 (8): 6470–8). In contrast to the classical MAP kinases, these atypical MAPKs require only a single residue in their activation loops to be phosphorylated. The details of NLK and ERK7 (MAPK15) activation remain unknown.

[0088] Inactivation of MAPKs is performed by a number of phosphatases. A very conserved family of dedicated phosphatases is the so-called MAP kinase phosphatases (MKPs), a subgroup of dual-specificity phosphatases (DUSPs; Theodosiou A, Ashworth A (Jun 2002). *Genome Biology*. 3 (7): reviews3009.1–reviews3009.10). As their name implies, these enzymes are capable of hydrolyzing the phosphate from both phosphotyrosine and the phosphothreonine residues. Since removal of either phosphate groups will greatly reduce MAPK activity, essentially abolishing signaling, some tyrosine phosphatases are also involved in inactivating MAP kinases (e.g. the phosphatases HePTP, STEP and PTPRR in mammals).

[0089] As mentioned above, MAPKs typically form multi-tiered pathways, receiving input several levels above the actual MAP kinase. In contrast to the relatively simple, phosphorylation-dependent activation mechanism of MAPKs and MAP2Ks, MAP3Ks have stunningly complex regulation. Many of the better-known MAP3Ks, such as c-Raf, MEKK4 or MLK3 require multiple steps for their activation. These are typically allosterically-

controlled enzymes, tightly locked into an inactive state by multiple mechanisms. The first step en route to their activation consists of relieving their auto-inhibition by a smaller ligand (such as Ras for c-Raf, GADD45 for MEKK4 (Miyake Z, *et al.* (Apr 2007). *Molecular and Cellular Biology*. 27 (7): 2765–76) or Cdc42 for MLK3 (Du Y, *et al.* (Dec 2005). *The Journal of Biological Chemistry*. 280 (52): 42984–93)). This commonly (but not always) happens at the cell membrane, where most of their activators are bound (note that small G-proteins are constitutively membrane-associated due to prenylation). That step is followed by side-to-side homo- and hetero-dimerization of their now accessible kinase domains. Recently determined complex structures reveal that the dimers are formed in an orientation that leaves both their substrate-binding regions free (Rajakulendran T, *et al.* (Sep 2009). *Nature*. 461 (7263): 542–5). Importantly, this dimerization event also forces the MAP3 kinase domains to adopt a partially active conformation. Full activity is only achieved once these dimers transphosphorylate each other on their activation loops. The latter step can also be achieved or aided by auxiliary protein kinases (MAP4 kinases, members of the Ste20 family). Once a MAP3 kinase is fully active, it may phosphorylate its substrate MAP2 kinases, which in turn will phosphorylate their MAP kinase substrates.

[0090] The ERK1/2 pathway of mammals is probably the best-characterized MAPK system. The most important upstream activators of this pathway are the Raf proteins (A-Raf, B-Raf or c-Raf), the key mediators of response to growth factors (EGF, FGF, PDGF, etc.); but other MAP3Ks such as c-Mos and Tpl2/Cot can also play the same role. All these enzymes phosphorylate and thus activate the MKK1 and/or MKK2 kinases, that are highly specific activators for ERK1 and ERK2. The latter phosphorylate a number of substrates important for cell proliferation, cell cycle progression, cell division and differentiation (RSK kinases, Elk-1 transcription factor, etc.).

[0091] In contrast to the relatively well-insulated ERK1/2 pathway, mammalian p38 and JNK kinases have most of their activators shared at the MAP3K level (MEKK1, MEKK4, ASK1, TAK1, MLK3, TAOK1, etc.). In addition, some MAP2K enzymes may activate both p38 and JNK (MKK4), while others are more specific for either JNK (MKK7) or p38 (MKK3 and MKK6). Due to these interlocks, there are very few if any stimuli that can elicit JNK activation without simultaneously activating p38 or reversed (Cargnello M, Roux PP (Mar 2011). *Microbiology and Molecular Biology Reviews*. 75 (1): 50–83). Both JNK and p38 signaling pathways are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in adaptation to stress, apoptosis

or cell differentiation. JNKs have a number of dedicated substrates that only they can phosphorylate (c-Jun, NFAT4, etc.), while p38s also have some unique targets (e.g. the MAPKAP kinases MK2 and MK3), ensuring the need for both in order to respond to stressful stimuli.

[0092] ERK5 is part of a fairly well-separated pathway in mammals. Its sole specific upstream activator MKK5 is turned on in response to the MAP3 kinases MEKK2 and MEKK3. The specificity of these interactions are provided by the unique architecture of MKK5 and MEKK2/3, both containing N-terminal PB1 domains, enabling direct hetero-dimerization with each other (Nakamura K, Johnson GL (Sep 2003). *The Journal of Biological Chemistry*. 278 (39): 36989–92). The PB1 domain of MKK5 also contributes to the ERK5-MKK5 interaction: it provides a special interface (in addition to the D-motif found in MKK5) through which MKK5 can specifically recognize its substrate ERK5 (Glatz G, *et al.* (Mar 2013). *The Journal of Biological Chemistry*. 288 (12): 8596–609). Although the molecular-level details are poorly known, MEKK2 and MEKK3 respond to certain developmental cues to direct endothelial formation and cardiac morphogenesis. While also implicated in brain development, the embryonic lethality of ERK5 inactivation due to cardiac abnormalities underlines its central role in mammalian vasculogenesis (Regan CP, *et al.* (Jul 2002). *Proc. Nat'l Acad. Sci.* USA. 99 (14): 9248–53). It is notable, that conditional knockout of ERK5 in adult animals is also lethal, due to the widespread disruption of endothelial barriers (Hayashi M, Lee JD (Dec 2004). J*ournal of Molecular Medicine*. 82 (12): 800–8). Mutations in the upstream components of the ERK5 pathway (the CCM complex) are thought to underlie cerebral cavernous malformations in humans.

[0093] As typical for the CMGC kinase group, the catalytic site of MAP kinases has a very loose consensus sequence for substrates. Like all their relatives, they only require the target serine / threonine amino acids to be followed by a small amino acid, preferably proline ("proline-directed kinases"). But as SP/TP sites are extremely common in all proteins, additional substrate-recognition mechanisms have evolved to ensure signaling fidelity (Garai Á, *et al.* (Oct 2012). *Science Signaling*. 5 (245): ra74). Unlike their closest relatives, the cyclin-dependent kinases (CDKs), where substrates are recognized by the cyclin subunit, MAPKs associate with their substrates via auxiliary binding regions on their kinase domains. The most important such region consists of the hydrophobic docking groove and the negatively charged CD-region. Together they recognize the so-called MAPK docking or D-motifs (also called kinase interaction motif / KIM). D-motifs essentially consist of one or two

positively charged amino acids, followed by alternating hydrophobic residues (mostly leucines), typically upstream of the phosphorylation site by 10-50 amino acids (Reményi A, *et al.* (Dec 2005). *Mol. Cell.* 20 (6): 951–62). Many of the known MAPK substrates contain such D-motifs that can not only bind to, but also provide specific recognition by certain MAPKs. Interestingly, D-motifs are not restricted to substrates: MAP2 kinases also contain such motifs on their N-termini that are absolutely required for MAP2K-MAPK interaction and MAPK activation (Bardwell AJ, *et al.* (May 2009). *The Journal of Biological Chemistry.* 284 (19): 13165–73). Similarly, both dual-specificity MAP kinase phosphatases and MAP-specific tyrosine phosphatases bind to MAP kinases through the same docking site (Goldsmith EJ (Dec 2011). *Science Signaling.* 4 (204): pe47; Huang Z, Zhou B, Zhang ZY (Dec 2004). *The Journal of Biological Chemistry.* 279 (50): 52150–9). D-motifs can even be found in certain MAPK pathway regulators and scaffolds (e.g. in the mammalian JIP proteins).

[0094] Other, less well characterized substrate-binding sites also exist. One such site (the DEF site) is formed by the activation loop (when in the active conformation) and the MAP kinase-specific insert below it. This site can accommodate peptides with an FxFP consensus sequence, typically downstream of the phosphorylation site (Sheridan DL, *et al.* (Jul 2008). *The Journal of Biological Chemistry.* 283 (28): 19511–20). Note that the latter site can only be found in proteins that need to selectively recognize the active MAP kinases, thus they are almost exclusively found in substrates. Different motifs may cooperate with each other, as in the Elk family of transcription factors, that possess both a D-motif and an FxFP motif. The presence of an FxFP motif in the KSR1 scaffold protein also serves to make it an ERK1/2 substrate, providing a negative feedback mechanism to set the correct strength of ERK1/2 activation.

[0095] Since the ERK signaling pathway is involved in both physiological and pathological cell proliferation, it is natural that ERK1/2 inhibitors would represent a desirable class of antineoplastic agents. Indeed, many of the proto-oncogenic "driver" mutations are tied to ERK1/2 signaling, such as constitutively active (mutant) receptor tyrosine kinases, Ras or Raf proteins. Although no MKK1/2 or ERK1/2 inhibitors were developed for clinical use, kinase inhibitors that also inhibit Raf kinases (e.g. Sorafenib) are successful antineoplastic agents against various types of cancer (Kim DH, Sim T (Mar 2012). *Arch. Pharmacol Res.* 35 (4): 605–15; Matsuda Y, Fukumoto M (Dec 2011). *Medical Molecular Morphology.* 44 (4): 183–9).

[0096] *MMP17*

[0097] The *MMP17* gene encodes a member of the peptidase M10 family and membrane-type subfamily of matrix metalloproteinases (MMPs). Proteins in this family are involved in the breakdown of extracellular matrix in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling, as well as in disease processes, such as arthritis and metastasis. Members of this subfamily contain a transmembrane domain suggesting that these proteins are expressed at the cell surface rather than secreted. The encoded preproprotein is proteolytically processed to generate the mature protease. This protein is unique among the membrane-type matrix metalloproteinases in that it is anchored to the cell membrane via a glycosylphosphatidylinositol (GPI) anchor. Elevated expression of the encoded protein has been observed in osteoarthritis and multiple human cancers.

[0098] An illustrative amino acid sequence (SEQ ID NO: 1) of human Mmp17 is NP_057239.4:

MRRRAARGPGPPPPGPGLSRLPLPLLLLLALGTRGGCAAPAPAPRAEDLSLGVEW
LSRFGYLPPADPTTGQLQTQEELSKAITAMQQFGGLEATGILDEATLALMKTPRCSLP
DLPVLTQARRRRQAPAPTKWNKRNLSWRVRTFPRDSPLGHDTVRALMYYALKVWS
DIAPLNFHEVAGSAADIQIDFSKADHNDGYPFDGPGGTVAHAFFPGHHHTAGDTHFD
DDEAWTFRSSDAHGMDLFAVAVHEFGHAIGLSHVAAAHSIMRPYYQGPVGDPLRY
GLPYEDKVRVWQLYGVRESVSPTAQPEEPPLLPEPPDNRSSAPPRKDVPHRCSTHFD
AVAQIRGEAFFFKGKYFWRLTRDRHLVSLQPAQMHRFWRGLPLHLDSVDAVYERTS
DHKIVFFKGDRYWVFKDNNVEEGYPRPVSDFSLPPGGIDAAFSWAHNDRTYFFKDQ
LYWRYDDHTRHMDPGYPAQSPLWRGVPSTLDDAMRWSDGASYFFRGQEYWKVLD
GELEVAPGYPQSTARDWLVCGDSQADGSVAAGVDAAEGPRAPPGQHDQSRSEDGY
EVCSCTSGASSPPGAPGPLVAATMLLLLPPLSPGALWTAAQALTL

[0099] The corresponding nucleic acid sequence (SEQ ID NO: 2) encoding human Mmp17 is NM_016155:

agtccggcgggggcgccgcggagagcggagggcgccgggctgcggaacgcgaagcggagggcgcgggaccctgcacg
ccgcccgcgggcccatgtgagcgccatgcggcgccgcgcagcccggggacccggcccgccgcccccagggcccggactctcg
cggctgccgctgccgctgctgctgctgctggcgctggggacccgcgggggctgcgccgcgcccgcacccgcgccgcgcgccga
ggacctcagcctgggagtggagtggctaagcaggttcggttacctgcccccggctgaccccacaacagggcagctgcagacgcaa
gaggagctgtctaaggccatcacagccatgcagcagtttggtggcctggaggccaccggcatcctggacgaggccaccctggccct
gatgaaaaccccacgctgctccctgccagacctccctgtcctgacccaggctcgcaggagacgccaggctccagcccccaccaagt

ggaacaagaggaacctgtcgtggagggtccggacgttcccacgggactcaccactggggcacgacacggtgcgtgcactcatgta
ctacgccctcaaggtctggagcgacattgcgcccctgaacttccacgaggtggcgggcagcgccgccgacatccagatcgacttctc
caaggccgaccataacgacggctacccttcgacggccccggcggcaccgtggcccacgccttcttccccggccaccaccacacc
gccggggacacccactttgacgatgacgaggcctggaccttccgctcctcggatgcccacgggatggacctgtttgcagtggctgtc
cacgagtttggccacgccattgggttaagccatgtggccgctgcacactccatcatgcggccgtactaccagggcccggtgggtgac
ccgctgcgctacgggctcccctacgaggacaaggtgcgcgtctggcagctgtacggtgtgcgggagtctgtgtctcccacggcgca
gcccgaggagcctcccctgctgccggagcccccagacaaccggtccagcgcccgcccaggaaggacgtgccccacagatgca
gcactcactttgacgcggtggcccagatccggggtgaagctttcttcttcaaaggcaagtacttctggcggctgacgcgggaccggca
cctggtgtccctgcagccggcacagatgcaccgcttctggcggggcctgccgctgcacctggacagcgtggacgccgtgtacgagc
gcaccagcgaccacaagatcgtcttctttaaaggagacaggtactgggtgttcaaggacaataacgtagaggaaggatacccgcgcc
ccgtctccgacttcagcctcccgcctggcggcatcgacgctgccttctcctgggcccacaatgacaggacttatttctttaaggaccagc
tgtactggcgctacgatgaccacacgaggcacatggaccccggctaccccgcccagagccccctgtggaggggtgtccccagcac
gctggacgacgccatgcgctggtccgacggtgcctcctacttcttccgtggccaggagtactggaaagtgctggatggcgagctgga
ggtggcacccgggtacccacagtccacggcccgggactggctggtgtgtggagactcacaggccgatggatctgtggctgcgggc
gtggacgcggcagaggggccccgcgcccctccaggacaacatgaccagagccgctcggaggacggttacgaggtctgctcatgc
acctctggggcatcctctccccgggggccccaggcccactggtggctgccaccatgctgctgctgctgccgccactgtcaccaggc
gccctgtggacagcggcccaggccctgacgctatgacacacagcgcgagcccatgagaggacagaggcggtgggacagcctgg
ccacagagggcaaggactgtgccggagtccctgggggaggtgctggcgcgggatgaggacgggccaccctggcaccggaaggc
cagcagagggcactgcccgccagggctgggcaggctcaggtggcaaggacggagctgtcccctagtgagggactgtgttgactga
cgagccgaggggtggccgctccagaagggtgcccagtcaggccgcaccgccgccagcctcctccggccctggagggagcatctc
gggctgggggcccaccctctctgtgccggcgccaccaaccccacccacactgctgcctggtgctcccgccggcccacagggcct
ccgtccccaggtccccagtggggcagccctccccacagacgagccccccacatggtgccgcggcacgtcccccctgtgacgcgtt
ccagaccaacatgacctctccctgctttgtaaaaaaaaaaaaaaaaaaa

[0100] Known MMP17 inhibitors include those recited in Table 1.

[0101] Table 1 – MMP17 Inhibitors

Compound	Action	Cas Number
Batimastat	Potent, broad spectrum MMP inhibitor	130370-60-4
GI 254023X	Selective ADAM10 metalloprotease inhibitor	260264-93-5
GM 6001	Broad spectrum MMP inhibitor	142880-36-2

TMI 1	Adam 17 (TACE) and MMP inhibitor; orally bioavailable	287403-39-8
WAY 170523	Potent and selective inhibitor of MMP-13	307002-73-9

[0102] Table 2 – dbSNP MMP17 Variants

SNP ID	Chr 12 pos	Sequence Context	Type
rs7302578	131,831,385(+)	GAGGT(C/T)GTGGG (SEQ ID NO: 9)	intron-variant
rs7312725	131,849,044(+)	ggctc(C/T)gccgt (SEQ ID NO: 10)	intron-variant
rs7314389	131,829,208(+)	TGGCC(C/T)TCAGC (SEQ ID NO: 11)	intron-variant, upstream-variant-2KB
rs7484461	131,848,633(+)	ccatg(C/G)ctctg (SEQ ID NO: 12)	intron-variant
rs7484577	131,840,427(+)	TCGGA(C/T)GACTC (SEQ ID NO: 13)	intron-variant

[0103] *MAP2K6*

[0104] Dual specificity mitogen-activated protein kinase kinase 6 also known as MAP kinase kinase 6 (MAPKK 6) or MAPK/ERK kinase 6 is an enzyme that in humans is encoded by the *MAP2K6* gene, on chromosome 17 (Han J, *et al.* (Feb 1996). *The Journal of Biological Chemistry.* 271 (6): 2886–91).

[0105] MAPKK 6 is a member of the dual specificity protein kinase family, which functions as a mitogen-activated protein (MAP) kinase kinase. MAP kinases, also known as extracellular signal-regulated kinases (ERKs), act as an integration point for multiple biochemical signals. This protein phosphorylates and activates p38 MAP kinase in response to inflammatory cytokines or environmental stress. As an essential component of p38 MAP

kinase mediated signal transduction pathway, this gene is involved in many cellular processes such as stress-induced cell cycle arrest, transcription activation and apoptosis (Entrez Gene: MAP2K6 mitogen-activated protein kinase kinase 6".).

[0106] An illustrative amino acid sequence (SEQ ID NO: 3) of human Map2k6 is NP_002749.2:

MSQSKGKKRNPGLKIPKEAFEQPQTSSKACISIGNQNFEVKADDLEPIMELGRGA
YGVVEKMRHVPSGQIMAVKRIRATVNSQEQKRLLMDLDISMRTVDCPFTVTFYGAL
FREGDVWICMELMDTSLDKFYKQVIDKGQTIPEDILGKIAVSIVKALEHLHSKLSVIH
RDVKPSNVLINALGQVKMCDFGISGYLVDSVAKTIDAGCKPYMAPERINPELNQKG
YSVKSDIWSLGITMIELAILRFPYDSWGTPFQQLKQVVEEPSPQLPADKFSAEFVDFTS
QCLKKNSKERPTYPELMQHPFFTLHESKGTDVASFVKLILGD

[0107] The corresponding nucleic acid sequence (SEQ ID NO: 4) encoding human Map2k6 is NM_002758.3:

agttccaagtttggagcttttagctgccagccctggcccatcatgtagctgcagcacagccttccctaacgttgcaactgggggaa
aaatcactttccagtctgttttgcaaggtgtgcatttccatcttgattccctgaaagtccatctgctgcatcggtcaagagaaactccacttg
catgaagattgcacgcctgcagcttgcatctttgttgcaaaactagctacagaagagaagcaaggcaaagtcttttgtgctcccctcccc
catcaaaggaaaggggaaaatgtctcagtcgaaaggcaagaagcgaaaccctggccttaaaattccaaaagaagcatttgaacaacc
tcagaccagttccacaccacctcgagatttagactccaaggcttgcatttctattggaaatcagaactttgaggtgaaggcagatgacct
ggagcctataatggaactgggacgaggtgcgtacggggtggtggagaagatgcggcacgtgcccagcgggcagatcatggcagt
gaagcggatccgagccacagtaaatagccaggaacagaaacggctactgatggatttggatatttccatgaggacggtggactgtcc
attcactgtcacctttatggcgcactgtttcgggagggtgatgtgtggatctgcatggagctcatggatacatcactagataaattctaca
aacaagttattgataaaggccagacaattccagaggacatcttagggaaaatagcagtttctattgtaaaagcattagaacatttacatag
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[0108] Another illustrative amino acid sequence (SEQ ID NO: 5) of human Map2k6 is NP_001317379.1:

MELGRGAYGVVEKMRHVPSGQIMAVKRIRATVNSQEQKRLLMDLDISMRTVDC
PFTVTFYGALFREGDVWICMELMDTSLDKFYKQVIDKGQTIPEDILGKIAVSIVKALE
HLHSKLSVIHRDVKPSNVLINALGQVKMCDFGISGYLVDSVAKTIDAGCKPYMAPER
INPELNQKGYSVKSDIWSLGITMIELAILRFPYDSWGTPFQQLKQVVEEPSPQLPADKF
SAEFVDFTSQCLKKNSKERPTYPELMQHPFFTLHESKGTDVASFVKLILGD

[0109] The corresponding nucleic acid sequence (SEQ ID NO: 6) encoding human Map2k6 is NM_001330450:

ttgctgcaatccgaacttgaggaggggtggagtctgttcagttctgtttctccttgccgaagtgtggtctttggagctaagtgaaga
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[0110] Known MAP2K6 inhibitors include those recited in Table 3.

Table 3 – MAP2K6 Inhibitors

Compound	Action	Mechanism of Action
WX-554	inhibitor	MEK inhibitor

Table 4 - dbSNP MAP2K6 Variants

SNP ID	Chr 17 pos	Sequence Context	Type
rs707247	69,430,471(-)	TTCTT(A/T)CAGGC (SEQ ID NO: 14)	intron-variant
rs707248	69,416,490(-)	CTAAA(G/T)GCCAT (SEQ ID NO: 15)	intron-variant
rs731606	69,430,375(-)	TTCCA(A/T)TTGGC (SEQ ID NO: 16)	intron-variant
rs732322	69,422,847(-)	tggcc(C/T)aggag (SEQ ID NO: 17)	intron-variant
rs739559	69,494,089(-)	CCCTT(A/G)TTATT (SEQ ID NO: 18)	intron-variant

[0111] *MAP3K4*

[0112] Mitogen-activated protein kinase kinase kinase 4 is an enzyme that in humans is encoded by the MAP3K4 gene (Takekawa M, *et al.* (Oct 1997). *EMBO J.* 16 (16): 4973–82; Entrez Gene: MAP3K4 mitogen-activated protein kinase kinase kinase 4").

[0113] The central core of each mitogen-activated protein kinase (MAPK) pathway is a conserved cascade of 3 protein kinases: an activated MAPK kinase kinase (MAPKKK) phosphorylates and activates a specific MAPK kinase (MAPKK), which then activates a specific MAPK. While the ERK MAPKs are activated by mitogenic stimulation, the CSBP2 (p38α) and JNK MAPKs are activated by environmental stresses such as osmotic shock, UV irradiation, wound stress, and inflammatory factors. This gene encodes a MAPKKK, the MEKK4 protein, also called MTK1. This protein contains a protein kinase catalytic domain at the C terminus. The N-terminal nonkinase domain may contain a regulatory domain. Expression of MEKK4 in mammalian cells activated the CSBP2 (p38α) and JNK MAPK pathways, but not the ERK pathway. In vitro kinase studies indicated that recombinant MEKK4 can specifically phosphorylate and activate PRKMK6 (MKK6) and SERK1 (MKK4), MAPKKs that activate CSBP2 (p38α) and JNK, respectively but cannot phosphorylate PRKMK1 (MKK1), an MAPKK that activates ERKs. MEKK4 is a major

mediator of environmental stresses that activate the p38 MAPK pathway, and a minor mediator of the JNK pathway. Two alternatively spliced transcripts encoding distinct isoforms have been described (Entrez Gene: MAP3K4 mitogen-activated protein kinase kinase kinase 4").

[0114] An illustrative amino acid sequence (SEQ ID NO: 7) of human Map3k4 is NP_005913.2:

MREAAAALVPPPAFAVTPAAAMEEPPPPPPPPPPPPEPETESEPECCLAARQEGTL
GDSACKSPESDLEDFSDETNTENLYGTSPPSTPRQMKRMSTKHQRNNVGRPASRSNL
KEKMNAPNQPPHKDTGKTVENVEEYSYKQEKKIRAALRTTERDHKKNVQCSFMLD
SVGGSLPKKSIPDVDLNKPYLSLGCSNAKLPVSVPMPIARPARQTSRTDCPADRLKFF
ETLRLLLKLTSVSKKKDREQRGQENTSGFWLNRSNELIWLELQAWHAGRTINDQDF
FLYTARQAIPDIINEILTFKVDYGSFAFVRDRAGFNGTSVEGQCKATPGTKIVGYSTH
HEHLQRQRVSFEQVKRIMELLEYIEALYPSLQALQKDYEKYAAKDFQDRVQALCLW
LNITKDLNQKLRIMGTVLGIKNLSDIGWPVFEIPSPRPSKGNEPEYEGDDTEGELKELE
SSTDESEEEQISDPRVPEIRQPIDNSFDIQSRDCISKKLERLESEDDSLGWGAPDWSTEA
GFSRHCLTSIYRPFVDKALKQMGLRKLILRLHKLMDGSLQRARIALVKNDRPVEFSE
FPDPMWGSDYVQLSRTPPSSEEKCSAVSWEELKAMDLPSFEPAFLVLCRVLLNVIHE
CLKLRLEQRPAGEPSLLSIKQLVRECKEVLKGGLLMKQYYQFMLQEVLEDLEKPDC
NIDAFEEDLHKMLMVYFDYMRSWIQMLQQLPQASHSLKNLLEEEWNFTKEITHYIR
GGEAQAGKLFCDIAGMLLKSTGSFLEFGLQESCAEFWTSADDSSASDEIRRSVIEISRA
LKELFHEARERASKALGFAKMLRKDLEIAAEFRLSAPVRDLLDVLKSKQYVKVQIPG
LENLQMFVPDTLAEEKSIILQLLNAAAGKDCSKDSDDVLIDAYLLLTKHGDRARDSE
DSWGTWEAQPVKVVPQVETVDTLRSMQVDNLLLVVMQSAHLTIQRKAFQQSIEGL
MTLCQEQTSSQPVIAKALQQLKNDALELCNRISNAIDRVDHMFTSEFDAEVDESESV
TLQQYYREAMIQGYNFGFEYHKEVVRLMSGEFRQKIGDKYISFARKWMNYVLTKC
ESGRGTRPRWATQGFDFLQAIEPAFISALPEDDFLSLQALMNECIGHVIGKPHSPVTG
LYLAIHRNSPRPMKVPRCHSDPPNPHLIIPTPEGFSTRSMPSDARSHGSPAAAAAAAA
AAVAASRPSPSGGDSVLPKSISSAHDTRGSSVPENDRLASIAAELQFRSLSRHSSPTEE
RDEPAYPRGDSSGSTRRSWELRTLISQSKDTASKLGPIEAIQKSVRLFEEKRYREMRR
KNIIGQVCDTPKSYDNVMHVGLRKVTFKWQRGNKIGEGQYGKVYTCISVDTGELM
AMKEIRFQPNDHKTIKETADELKIFEGIKHPNLVRYFGVELHREEMYIFMEYCDEGTL
EEVSRLGLQEHVIRLYSKQITIAINVLHEHGIVHRDIKGANIFLTSSGLIKLGDFGCSVK
LKNNAQTMPGEVNSTLGTAAYMAPEVITRAKGEGHGRAADIWSLGCVVIEMVTGK

RPWHEYEHNFQIMYKVGMGHKPPIPERLSPEGKDFLSHCLESDPKMRWTASQLLDH SFVKVCTDEE

[0115] The corresponding nucleic acid sequence (SEQ ID NO: 8) NM_005922 encoding human Map3k4 is:

gatctgggaggcttgtccctcgccgcccaccgtagccccggcgctcggccggtcgccgtttccaagatggccgcggcgcgca cggctcctgcggcggggtagaggcggaggcggagtcgagtcactcccgcacttcggggctccggtgccccgcgccaggctgcag cttactgcccgccgcggccatgcggggctccgtgcacggatgagagaagccgctgccgcgctggtccctcctcccgcctttgccgtc acgcctgccgccgccatggaggagccgccgccaccgccgccgccgccaccaccgccaccggaacccgagaccgagtcagaac ccgagtgctgcttggcggcgaggcaagagggcacattgggagattcagcttgcaagagtcctgaatctgatctagaagacttctccga tgaaacaaatacagagaatctttatggtacctctccccccagcacacctcgacagatgaaacgcatgtcaaccaaacatcagaggaat aatgtggggaggccagccagtcggtctaatttgaaagaaaaaatgaatgcaccaaatcagcctccacataaagacactggaaaaaca gtggagaatgtggaagaatacagctataagcaggagaaaaagatccgagcagctcttagaacaacagagcgtgatcataaaaaaaat gtacagtgctcattcatgttagactcagtgggtggatctttgccaaaaaaatcaattccagatgtggatctcaataagccttacctcagcct tggctgtagcaatgctaagcttccagtatctgtgcccatgcctatagccagacctgcacgccagacttctaggactgactgtccagcag atcgtttaaagtttttgaaactttacgacttttgctaaagcttacctcagtctcaaagaaaaaagacagggagcaaagaggacaagaaaa tacgtctggtttctggcttaaccgatctaacgaactgatctggttagagctacaagcctggcatgcaggacggacaattaacgaccagg acttctttttatatacagcccgtcaagccatcccagatattattaatgaaatccttactttcaaagtcgactatgggagcttcgcctttgttaga gatagagctggttttaatggtacttcagtagaagggcagtgcaaagccactcctggaacaaagattgtaggttactcaacacatcatgag catctccaacgccagagggtctcatttgagcaggtaaaacggataatggagctgctagagtacatagaagcactttatccatcattgca ggctcttcagaaggactatgaaaaatatgctgcaaaagacttccaggacagggtgcaggcactctgtttgtggttaaacatcacaaaag acttaaatcagaaattaaggattatgggcactgttttgggcatcaagaatttatcagacattggctggccagtgtttgaaatcccttcccctc gaccatccaaaggtaatgagccggagtatgagggtgatgacacagaaggagaattaaaggagttggaaagtagtacggatgagagt gaagaagaacaaatctctgatcctagggtaccggaaatcagacagcccatagataacagcttcgacatccagtcgcgggactgcatat ccaagaagcttgagaggctcgaatctgaggatgattctcttggctggggagcaccagactggagcacagaagcaggctttagtagac attgtctgacttctatttatagaccatttgtagacaaagcactgaagcagatggggttaagaaagttaattttaagacttcacaagctaatgg atggttccttgcaaagggcacgtatagcattggtaaagaacgatcgtccagtggagttttctgaatttccagatcccatgtggggttcaga ttatgtgcagttgtcaaggacaccaccttcatctgaggagaaatgcagtgctgtgtcgtgggaggagctgaaggccatggatttaccttc attcgaacctgccttcctagttctctgccgagtccttctgaatgtcatacatgagtgtctgaagttaagattggagcagagacctgctgga gaaccatctctcttgagtattaagcagctggtgagagagtgtaaggaggtcctgaagggcggcctgctgatgaagcagtactaccagtt catgctgcaggaggttctggaggacttggagaagcccgactgcaacattgacgcttttgaagaggatctacataaaatgcttatggtgta ttttgattacatgagaagctggatccaaatgctacagcaattacctcaagcatcgcatagtttaaaaaatctgttagaagaagaatggaatt tcaccaaagaaataactcattacatacggggaggagaagcacaggccgggaagcttttctgtgacattgcaggaatgctgctgaaatc tacaggaagttttttagaatttggcttacaggagagctgtgctgaattttggactagtgcggatgacagcagtgcttccgacgaaatcagg

aggtctgttatagagatcagtcgagccctgaaggagctcttccatgaagccagagaaagggcttccaaagcacttggatttgctaaaat
gttgagaaaggacctggaaatagcagcagaattcaggctttcagccccagttagagacctcctggatgttctgaaatcaaaacagtatg
tcaaggtgcaaattcctgggttagaaaacttgcaaatgtttgttccagacactcttgctgaggagaagagtattattttgcagttactcaatg
cagctgcaggaaaggactgttcaaaagattcagatgacgtactcatcgatgcctatctgcttctgaccaagcacggtgatcgagcccgt
gattcagaggacagctggggcacctgggaggcacagcctgtcaaagtcgtgcctcaggtggagactgttgacaccctgagaagcat
gcaggtggataatcttttactagttgtcatgcagtctgcgcatctcacaattcagagaaaagctttccagcagtccattgagggacttatga
ctctgtgccaggagcagacatccagtcagccggtcatcgccaaagctttgcagcagctgaagaatgatgcattggagctatgcaacag
gataagcaatgccattgaccgcgtggaccacatgttcacatcagaatttgatgctgaggttgatgaatctgaatctgtcaccttgcaacag
tactaccgagaagcaatgattcaggggtacaattttggatttgagtatcataaagaagttgttcgtttgatgtctggggagtttagacagaa
gataggagacaaatatataagctttgcccggaagtggatgaattatgtcctgactaaatgtgagagtggtagaggtacaagacccaggt
gggcgactcaaggatttgattttctacaagcaattgaacctgcctttatttcagctttaccagaagatgacttcttgagtttacaagccttgat
gaatgaatgcattggccatgtcataggaaaaccacacagtcctgttacaggtttgtaccttgccattcatcggaacagcccccgtcctat
gaaggtacctcgatgccatagtgaccctcctaacccacacctcattatccccactccagagggattcagcactcggagcatgccttccg
acgcgcggagccatggcagccctgctgctgctgctgctgctgctgctgctgttgctgccagtcggcccagcccctctggtggtga
ctctgtgctgcccaaatccatcagcagtgcccatgataccaggggttccagcgttcctgaaaatgatcgattggcttccatagctgctga
attgcagtttaggtccctgagtcgtcactcaagccccacggaggagcgagatgaaccagcatatccaagaggagattcaagtgggtc
cacaagaagaagttgggaacttcggacactaatcagccagagtaaagatactgcttctaaactaggacccatagaagctatccagaag
tcagtccgattgtttgaagaaaagaggtaccgagaaatgaggagaaagaatatcattggtcaagtttgtgatacgcctaagtcctatgat
aatgttatgcacgttggcttgaggaaggtgaccttcaaatggcaaagaggaaacaaaattggagaaggccagtatgggaaggtgtac
acctgcatcagcgtcgacaccggggagctgatggccatgaaagagattcgatttcaacctaatgaccataagactatcaaggaaactg
cagacgaattgaaaatattcgaaggcatcaaacaccccaatctggttcggtattttggtgtggagctccatagagaagaaatgtacatctt
catggagtactgcgatgaggggactttagaagaggtgtcaaggctgggacttcaggaacatgtgattaggctgtattcaaagcagatc
accattgcgatcaacgtcctccatgagcatggcatagtccaccgtgacattaaaggtgccaatatcttccttacctcatctggattaatcaa
actgggagattttggatgttcagtaaagctcaaaaacaatgcccagaccatgcctggtgaagtgaacagcaccctggggacagcagc
atacatggcacctgaagtcatcactcgtgccaaaggagagggccatgggcgtgcggccgacatctggagtctggggtgtgttgtcata
gagatggtgactggcaagaggccttggcatgagtatgagcacaactttcaaattatgtataaagtggggatgggacataagccaccaa
tccctgaaagattaagccctgaaggaaaggacttcctttctcactgccttgagagtgacccaaagatgagatggaccgccagccagct
cctcgaccattcgtttgtcaaggtttgcacagatgaagaatgaagcctagtagaatatggacttggaaaattctcttaatcactactgtatgt
aatatttacataaagactgtgctgagaagcagtataagccttttaaccttccaagactgaagactgcacaggtgacaagcgtcacttctc
ctgctgctcctgtttgtctgatgtggcaaaaggccctctggagggctggtggccacgaggttaaagaagctgcatgttaagtgccattac
tactgtacacggaccatcgcctctgtctcctccgtgtctcgcgcgactgagaaccgtgacatcagcgtagtgttttgacctttctaggttca
aaagaagttgtagtgttatcaggcgtcccataccttgtttttaatctcctgtttgttgagtgcactgactgtgaaaccttaccttttttgttgttg
ttggcaagctgcaggtttgtaatgcaaaaggctgattactgaaatttaagaaaaaggttctttttttcaataaatggtttatttttaggaaagctc
aaaaaaaaaaaaaaaaaa

Table 5 - dbSNP MAP3K4 Variants

SNP ID	Chr 06 pos	Sequence Context	Type
rs1000277	161,105,793(-)	ACTAA(C/T)CAAAT (SEQ ID NO: 19)	intron-variant
rs1001756	161,105,475(+)	aaatg(A/G)cgagg (SEQ ID NO: 20)	intron-variant
rs1001808	161,057,414(+)	GAGCT(A/G)TAGAT (SEQ ID NO: 21)	intron-variant
rs1001809	161,057,478(+)	gtttc(C/G)aaacc (SEQ ID NO: 22)	intron-variant
rs10080366	161,019,422(+)	tcttt(C/T)tattt (SEQ ID NO: 23)	intron-variant

[0116] *Agents of the Disclosure*

[0117] The disclosure provides agents to modulate the expression or activity of a MAP kinase pathway component, e.g., MMP17, MAP2K6 and/or MAP3K4, or a gene product thereof. In one embodiment, the agent is an inhibitor or antagonist of a MAP kinase pathway component, e.g., MMP17, MAP2K6 and/or MAP3K4, or a gene product thereof, that selectively blocks MAP kinase pathway signaling. In a particular embodiment, the agent is an inhibitor of MMP17, MAP2K6 and/or MAP3K4, or a gene product thereof. Non-limiting illustrative examples include small molecules (e.g., of Tables 1 and 3), antibodies, antisense and/or siRNA molecules directed to inhibit MMP17, MAP2K6 and/or MAP3K4, or a gene product thereof.

[0118] Agents useful in the methods of the disclosure can be nucleic acid molecules, e.g., antisense, ribozyme, or RNA interference technology, e.g., siRNA molecules corresponding to a portion of the nucleotide sequence encoding a component member of the MAP kinase pathway (e.g., a nucleic acid encoding Mmp17, Map2k6 and/or Map3k4).

[0119] Antisense polynucleotides may act by directly blocking translation by hybridizing to mRNA transcripts or degrading such transcripts of a gene. The antisense molecule may be recombinantly made using at least one functional portion of a gene in the antisense orientation as a region downstream of a promoter in an expression vector.

Chemically modified bases or linkages may be used to stabilize the antisense polynucleotide by reducing degradation or increasing half-life in the body (e.g., methyl phosphonates, phosphorothioate, peptide nucleic acids). The sequence of the antisense molecule may be complementary to the translation initiation site (e.g., between -10 and +10 of the target's nucleotide sequence).

[0120] Ribozymes catalyze specific cleavage of an RNA transcript or genome. The mechanism of action involves sequence-specific hybridization to complementary cellular or viral RNA, followed by endonucleolytic cleavage. Inhibition may or may not be dependent on ribonuclease H activity. The ribozyme includes one or more sequences complementary to the target RNA as well as catalytic sequences responsible for RNA cleavage (e.g., hammerhead, hairpin, axehead motifs). For example, potential ribozyme cleavage sites within a subject RNA are initially identified by scanning the subject RNA for ribozyme cleavage sites which include the following trinucleotide sequences: GUA, GUU and GUC. Once identified, an oligonucleotide of between about 15 and about 20 ribonucleotides corresponding to the region of the subject RNA containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render candidate oligonucleotide sequences unsuitable. The suitability of candidate sequences can then be evaluated by their ability to hybridize and cleave target RNA. The ribozyme may be recombinantly produced or chemically synthesized.

[0121] siRNA refers to double-stranded RNA of at least 20-25 basepairs which mediates RNA interference (RNAi). Duplex siRNA corresponding to a target RNA may be formed by separate transcription of the strands, coupled transcription from a pair of promoters with opposing polarities, or annealing of a single RNA strand having an at least partially self-complementary sequence. Alternatively, duplexed oligoribonucleotides of at least about 21 to about 23 basepairs may be chemically synthesized (e.g., a duplex of 21 ribonucleotides with 3' overhangs of two ribonucleotides) with some substitutions by modified bases being tolerated. Mismatches in the center of the siRNA sequence, however, abolishes interference. The region targeted by RNA interference should be transcribed, preferably as a coding region of the gene. Interference appears to be dependent on cellular factors (e.g., ribonuclease III) that cleave target RNA at sites 21 to 23 bases apart; the position of the cleavage site appears to be defined by the 5' end of the guide siRNA rather than its 3' end. Priming by a small amount of siRNA may trigger interference after amplification by an RNA-dependent RNA polymerase.

[0122] *CRISPR-Cas*

[0123] In certain aspects inhibition of MAP kinase components can be achieved by administration of inhibitory nucleic acids (e.g., dsRNAs, siRNAs, antisense oligonucleotides, etc.). It is also contemplated that CRISPR-Cas (e.g., CRISPR-Cas9) methods can be used to excise and replace MAP kinase pathway component alleles identified to carry a deleterious mutation and/or variant that predisposes/contributes to MFS. Such variant alleles include the variant alleles identified in mice, for at least MMP17 and MAP2K6 herein, and further including similar human alleles of these genes, as well as of MAP3K4. Such methods can be performed upon the cells of a subject *in vivo* or *ex vivo*. Use of any combination of the above and/or other known MAP kinase component modulators is also contemplated.

[0124] The CRISPR-Cas system is known in the art. Non- limiting aspects of this system are described in U.S. Patent No. 8,697,359, issued April 15, 2014, the entire content of which is incorporated herein by reference.

[0125] Non-limiting examples of Cas proteins include Casl, CaslB, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8, Cas9 (also known as Csnl and Csxl2), Cas 10, Csyl, Csy2, Csy3, Csel, Cse2, Cscl, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmrl, Cmr3, Cmr4, Cmr5, Cmr6, Csbl, Csb2, Csb3, Csxl7, Csxl4, Csx10, Csxl6, CsaX, Csx3, Csxl, Csxl5, Csfl, Csf2, Csf3, Csf4, homologs thereof, or modified versions thereof. These enzymes are known; for example, the amino acid sequence of *S. pyogenes* Cas9 protein may be found in the SwissProt database under accession number Q99ZW2. In some embodiments, the unmodified CRISPR enzyme has DNA cleavage activity, such as Cas9. In some embodiments, the CRISPR enzyme is Cas9, and may be Cas9 from *S. pyogenes* or *S. pneumoniae*. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands at the location of a target sequence, such as within the target sequence and/or within the complement of the target sequence. In some embodiments, the CRISPR enzyme directs cleavage of one or both strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, a vector encodes a CRISPR enzyme that is mutated with respect to a corresponding wild-type enzyme such that the mutated CRISPR enzyme lacks the ability to cleave one or both strands of a target polynucleotide containing a target sequence. For example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from *S. pyogenes* converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include,

without limitation, H840A, N854A, and N863A. In aspects of the invention, nickases may be used for genome editing via homologous recombination.

[0126] In general, a guide sequence is any polynucleotide sequence having sufficient complementarity with a target polynucleotide sequence to hybridize with the target sequence and direct sequence-specific binding of a CRISPR complex to the target sequence. In some embodiments, the degree of complementarity between a guide sequence and its corresponding target sequence, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g., the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies, ELAND (Illumina, San Diego, Calif.), SOAP (available at soap.genomics.org.cn; Ruiqiang Li, Yingrui Li, Karsten Kristiansen, Jun Wang; SOAP: short oligonucleotide alignment program, *Bioinformatics*, Volume 24, Issue 5, 1 March 2008, Pages 713–714,), and Maq (available at maq.sourceforge.net; Heng Li, Jue Ruan, and Richard Durbin. *Genome Res*. 2008. 18: 1851-1858). In some embodiments, a guide sequence is about or more than about 5, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 35, 40, 45, 50, 75, or more nucleotides in length. In some embodiments, a guide sequence is less than about 75, 50, 45, 40, 35, 30, 25, 20, 15, 12, or fewer nucleotides in length. The ability of a guide sequence to direct sequence-specific binding of a CRISPR complex to a target sequence may be assessed by any suitable assay. For example, the components of a CRISPR system sufficient to form a CRISPR complex, including the guide sequence to be tested, may be provided to a host cell having the corresponding target sequence, such as by transfection with vectors encoding the components of the CRISPR sequence, followed by an assessment of preferential cleavage within the target sequence, such as by Surveyor assay as described herein. Similarly, cleavage of a target polynucleotide sequence may be evaluated in a test tube by providing the target sequence, components of a CRISPR complex, including the guide sequence to be tested and a control guide sequence different from the test guide sequence, and comparing binding or rate of cleavage at the target sequence between the test and control guide sequence reactions. Other assays are possible, and will occur to those skilled in the art.

[0127] EGFR Inhibitors

[0128] In certain embodiments, a method for treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprises administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling and/or expression, function or activity of epidermal growth factor receptor (EGFR); thereby treating the subject. In certain embodiments, the agent that modulates the activity of MAP kinase pathway signaling is an inhibitor of the MAP kinase pathway. In certain embodiments, the agent that modulates the activity of MAP kinase pathway signaling is an inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof. In certain embodiments, the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is an antisense agent or a double-stranded nucleic acid, optionally a siRNA or shRNA specific for MMP17, MAP2K6 or MAP3K4. In certain embodiments, the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is specific for a nucleic acid molecule set forth as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8. In other embodiments, the agent that modulates the activity of MAP kinase pathway signaling comprise Batimastat, GI 254023X, GM 6001, TMI 1, WAY 170523, WX-554 or combinations thereof. Any combination of inhibitors can be used, for example, an siRNA and a compound, e.g. GI 254023X, and/or an antibody.

[0129] In certain embodiments, the agent that modulates expression, function or activity of epidermal growth factor receptor (EGFR) is an EGFR inhibitor comprising: an antibody, interfering RNAs microRNAs, shRNA, small molecule or combinations thereof.

[0130] In certain embodiments, the EGFR inhibitor comprises: gefitinib, erlotinib, lapatinib, Brigatinib (AP26113), Afatinib (BIBW2992), Neratinib (HKI-272), AZD3759, AZ5104, CL-387785 (EKI-785), Canertinib (CI-1033), Poziotinib (HM781-36B), Osimertinib (AZD-9291), PD168393, CNX-2006, Rociletinib (CO-1686, AVL-301), WZ4002, Pelitinib (EKB-569), AC480 (BMS-599626), TAK-285, CUDC-101, AEE788 (NVP-AEE788), CP-724714, Dacomitinib (PF299804, PF299), AG-490 (Tyrphostin B42), AST-1306, OSI-420, WHI-P154, WZ3146, WZ8040, AZD8931 (Sapitinib), PKI-166, PD158780, AG 1478, PD153035 HCl, Icotinib, Varlitinib, AZD-9291, AEE788 (NVP-AEE 788), AG-1478 (NSC 693255), AG-490, Anlotinib, ARRY-380, BIBX 1382, BMS-690514, cetuximab, panitumumab, zalutumumab, nimotuzumab, matuzumab or mAb 806. An anti-EGFR antibody comprises cetuximab, matuzumab, panitumumab, nimotuzumab or mAb 806 and a small molecule EGFR inhibitor comprises gefitinib, lapatinib, canertinib, erlotinib HCL, pelitinib, PKI-166, PD158780, or AG 1478. In certain embodiments, combinations of

agents, e.g. antibody or small molecules, can be administered as part of the therapy. In other embodiments, an inhibitor of transforming growth factor β (TGFβ) is also administered.

[0131] In certain embodiments, a pharmaceutical composition comprises a therapeutically effective amount of at least one agent that modulates expression, function or activity of MMP17, MAP2K6, MAP3K4, epidermal growth factor receptor (EGFR), or combinations thereof.

[0132] An exemplary anti-EGFR antibody is cetuximab (ERBITUX™). Cetuximab is commercially available from ImClone Systems Incorporated. Other examples of anti-EGFR antibodies include matuzumab (EMD72000), panitumumab (VECTIBIX™; Amgen); nimotuzumab (THERACIM™) and mAb 806. An exemplary small molecule inhibitor of the EGFR signaling pathway is gefitinib (IRESSA™), which is commercially available from AstraZeneca and Teva. Other examples of small molecule inhibitors of the EGFR signaling pathway include erlotinib HCL (OSI-774; TARCEVA™, OSI Pharma); lapatinib (TYKERB™, GlaxoSmithKline); canertinib (canertinib dihydrochloride, Pfizer); pelitinib (Pfizer); PKI-166 (Novartis); PD158780; and AG 1478 (4-(3-Chloroanillino)-6,7-dimethoxyquinazoline).

[0133] The EGFR inhibitor may be an EGFR tyrosine kinase inhibitor, or may alternatively target the extracellular domain of the EGFR target. In certain embodiments, the EGFR inhibitor is a tyrosine kinase inhibitor such as Erlotinib, Gefitinib, or Lapatinib, or a molecule that targets the EGFR extracellular domain such as Cetuximab or Panitumumab.

[0134] In certain embodiments, the EGFR inhibitor is an anti-EGFR antibody, preferably a monoclonal antibody, such as Cetuximab or Panitumumab.

[0135] Cetuximab and Panitumumab are currently the clinically mostly used anti-EGFR monoclonal antibodies. However, further anti-EGFR monoclonal antibodies are in development, such as Nimotuzumab (TheraCIM-h-R3), Matuzumab (EMD 72000), Zalutumumab (HuMax-EGFr), Nimotuzumab and Sym 004. Similarly, Erlotinib, Gefitinib, Lapatinib and Regorafenib are currently the clinically mostly used tyrosine kinase EGFR inhibitors. However, further tyrosine kinase EGFR inhibitors are in development, such as Canertinib (CI-1033),Neratinib (HKI-272), Afatinib (BIBW2992), Dacomitinib (PF299804,PF-00299804), TAK-285, AST-1306, ARRY334543, AG-1478 (Tyrphostin AG-1478), AV-412, OSI-420 (DesmethylErlotinib), AZD8931, AEE788 (NVP-AEE788), Pelitinib (EKB-569), CUDC-101, AG 490, PD153035 HCL, XL647, Ruxolitinib, and BMS-

599626 (AC480).The method according to the invention may also be used to predict response to these tyrosine kinase EGFR inhibitors or any other tyrosine kinase EGFR inhibitors that might be further developed, in particular if the patient is suffering from of lung cancer (in particular non-small cell lung cancer, NSCLC), pancreatic cancer, or head and neck cancer (in particular squamous cell carcinoma of the head and neck (SCCHN)).

[0136] TGFβ Inhibitors

[0137] Transforming growth factor beta (TGFβ) has emerged as a candidate mediator of disease pathology and a key therapeutic target in MFS (Massagu, J., *et al. Cell* 103, 295-309 (2000)). The TGFβ family of cytokines influences a diverse repertoire of cellular processes including cell proliferation, survival and synthetic activity. The TGFβ ligand isoforms bind to a cell surface complex of type 1 and 2 TGFβ receptor subunits (TβRI and TβRII respectively). These in turn phosphorylate the canonical cytoplasmic receptor-activated SMAD (R-SMADs) proteins, SMAD2 or SMAD3. TGFβ receptors can also initiate activation of noncanonical cascades including the mitogen activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK1/2), Jun N-terminal kinase (JNK1/2) and p38. There is a clear signature for increased TGFβ signaling in patients and mice with MFS, as evidenced by the accumulation of activated SMAD2/3, ERK1/2, and p38 (pSMAD2/3, pERK1/2, and pp38, respectively) in diseased tissues (Neptune, E.R. *et al. Nature Genetics* 33, 407-11 (2003); Ng, C.M. *et al.* J. *Clin. Invest.* 114, 1586-92 (2004); Cohn, R.D. *et al. Nature Medicine* 13, 204-10 (2007); Carta, L. *et al. J. Biol. Chem.* 27, 5630-6 (2009)). Furthermore, therapies that associate with increased TGFβ activation in MFS mice, such as calcium channel blockers, exacerbate aortic disease (Doyle, J.J. *et al. eLife* 4:e08648 (2015)), while those that attenuate TGFβ signaling and related pathways, such as TGFβ neutralizing antibody (NAb), the angiotensin-II (Ang-II) type 1 receptor blocker (ARB) losartan, or the inhibitor of ERK1/2 activation RDEA119, can suppress aortic disease in MFS mice (Habashi, J.P. *et al. Science* 312, 117-21 (2006); Holm, T.M. *et al. Science* 15, 358-61 (2011); Habashi, J.P. *et al. Science* 15, 361-5 (2011)).

[0138] The major classes of TGFβ inhibitors include ligand traps, antisense oligonucleotides (ASO), small molecule receptor kinase inhibitors and peptide aptamers. Ligand traps include anti-ligand neutralizing antibodies and soluble decoy receptor proteins that incorporate the ectodomains from either TGFβ receptor II (TβRII) or TGFβ receptor III (TβRIII)/betaglycan protein. Neutralizing antibodies have been raised against individual ligands or may be designed to block all three human isomers TGFβ 1, -2 and -3. ASO can also be used to reduce the bioavailability of active TGFβ ligands by blocking de novo

synthesis of TGFβ. ASOs are single-stranded polynucleotide molecules, usually 13-25 nucleotides in length, that hybridize to complementary RNA, inhibiting mRNA function, and preventing protein synthesis through accelerated mRNA degradation by RNase H or steric blockade. Another therapeutic strategy is to block TGFβ receptor I (TβRI) activity through the use of small molecule receptor kinase inhibitors that act via ATP-competitive inhibition of the catalytic domain of the serine-threonine kinase of the TβRI (ALK5-Receptor). Lastly, targeting intracellular TGFβ signaling molecules, such as Smads, is possible with the use of peptide aptamers. Aptamers are small peptide, DNA or RNA molecules containing a target-binding and a scaffolding domain that stabilize and interfere with the function of the target.

[0139] Accordingly, in some embodiments, an inhibitor of TGFβ or TGFβ signaling is administered as part of a therapeutic regimen. TGFβ signaling controls proliferation, cellular differentiation, and other functions in a variety of cell types, and can play a role in cell cycle control, regulation of the immune system, and development in certain cell types. Inhibition of TGFβ signaling may include inhibition of any TGFβ signaling pathway and/or member of the TGFβ superfamily including ligands such as TGFβ1, TGFβ2, TGFβ3, inhibins, activin, anti-mullerian hormone, bone morphogenetic protein, decapentaplegic and Vg-1; receptors such as TGFβ type I receptor, TGFβ type II receptor, ALK1, ALK2, ALK3, ALK4, ALK5, ALK6, ALK7 and ALK8; and downstream effectors such as R-SMAD and other SMAD proteins (e.g., SMAD1, SMAD2, SMAD3, SMAD4, SMAD5).

[0140] In some embodiments, the activity of one or more TGFβ receptors is inhibited. In some embodiments, one or more TGFβ receptor-ligand interactions are inhibited. In some embodiments, a TGFβ type I receptor is inhibited. A TGFβ type I receptor may include one or more of ALK1, ALK2, ALK3, ALK4, ALK5, ALK6, ALK7 and ALK8. In some embodiments, the TGFβ receptor is ALK5.

[0141] A TGFβ inhibitor (e.g., a TGFβ signaling inhibitor) may be an ALK5 inhibitor, in some embodiments. An ALK5 inhibitor may bind to ALK5 or one or more ALK5 ligands or both. An ALK5 inhibitor may bind to ALK5 or one or more downstream SMAD proteins or both. An ALK5 inhibitor may disrupt one or more ALK5-ligand interactions or may disrupt one or more ALK5-SMAD interactions. In some embodiments, an ALK5 inhibitor blocks phosphorylation of SMAD2. ALK5 inhibitors may include one or more small molecule ALK5 inhibitors. In some embodiments, an ALK5 inhibitor is an ATP analog.

[0142] A TGFβ inhibitor of the present invention interacts with a TGFβ receptor, inhibits the signaling of the receptor, interacts with a TGFβ protein, and/or inhibits the transcription and/or translation of the gene encoding TGFβ-1, -2, and/or -3. The TGFβ inhibitor of the present invention inhibits TGFβ1, TGFβ2, and/or TGFβ3; in case an inhibitor inhibits all three TGFβ isotypes, the inhibitor is a pan-specific inhibitor. Examples of TGFβ inhibitors include: A) a small molecule inhibitor of TGFβ receptor type I kinase (ALK5; an inhibitor is an ALK5 inhibitor), B) a neutralizing anti-TGFβ-1, -2, -3 antibody or TGFβ binding fragments thereof, C) a neutralizing anti-TGFβ receptor type I, type II or type III antibody or TGFβ receptors binding fragments thereof, D) an antisense oligonucleotide specific for mRNA encoding TGFβ1, -2, and -3 isotypes or other components of TGFβ signaling assembly optionally comprising a modified nucleoside such as 2'-O, 4'-C-methylene linked bicyclic ribonucleotides, known as locked nucleic acids LNA (e.g., oxy-LNA, amino-LNA, thio-LNA), phosphorodiamidate morpholino oligomers (PMO), phosphorothioate (PS), 2'-O-methyl (2'-Ome), 2'-fluoro (2'-fluoro (2'-F), or 2'-methoxyethyl (2'-MOE) derivatives, E) an antisense RNA molecule specific for TGFβ2-mRNA like belagenpumatucel-L and/or TGFβ1-mRNA or TGFβ3-mRNA or other components of mRNA encoding TGFβ signaling assembly, F) a silencing RNA molecule (siRNA) specific for mRNA encoding TGFβ1, -2, and/or -3 isotypes or other components of TGFβ signaling assembly, G) a short hairpin RNA (shRNA) specific for mRNA encoding TGFβ1, -2, and/or -3 isotypes or other components of TGFβ signaling assembly, H) a miRNA molecule specific for mRNA encoding TGFβ1, -2, and/or -3 isotypes or other components of TGFβ signaling assembly, I) an aptamer and/or spiegelmer molecule specific for TGFβ1, -2, and/or -3 isotypes or other components of the TGFβ signaling assembly, and/or J) a ribozyme molecule specific for mRNA encoding TGFβ1, -2, and/or -3 isotypes or other components of the TGFβ signaling assembly.

[0143] ASOs are single-stranded polynucleotide molecules comprising 13-25 nucleotides, preferably 15-20 nucleotides, more preferred 15, 16, 17, 18, 19, 20, 21, 22 or 23 nucleotides, that hybridize to complementary RNA, inhibiting mRNA function, and preventing protein synthesis for example through accelerated mRNA degradation by RNase H or steric blockade.

[0144] In certain embodiments, a TGFβ inhibitor binding to TGFβ receptor type I, such as a non-peptide, small molecule inhibitor of ALK-5 kinase from dihydropyrolopyrazole derivatives (e.g., LY2157299, LY21109761, LY580276, LY550410, GW788388), pyrazole derivatives (e.g., LY364947, HTS-466284, SM305, SB525334, A-83-01), quinazoline

derivatives (e.g., SD-208), or imidazole derivatives (e.g., SM16, SB431542, or SB505124). SD-208 for example is a small molecule inhibitor of type I kinase of TGFβ receptor with IC_{50}=49 nM, which inhibits TGFβ signal transduction in a dose-dependent fashion, as for example measured by p-SMAD2 western blot analysis.

[0145] A human pan-anti-TGFβ antibody binding to and neutralizing all three isotypes of TGFβ (e.g., TGFβ1, 2, 3) like GC-1008, a neutralizing high-affinity antibody or antigen-binding immunoglobulin single variable domain or polypeptide thereof, that neutralize mature human TGFβ1 (like CAT 192), or hTGFβ2 (like CAT 152), and neutralizing anti-TGFβ receptor type II antibody (like D10) or an antibody fragment thereof which binds and neutralizes human TGFβ receptor type II, a fusion protein comprising a TGFβ type II receptor fusion protein, a small molecule or peptide consisting of, for example, 11-50 amino acid residues blocking assembly of the TGFβ signaling complex extra- and intracellularly.

[0146] *Pharmaceutical Compositions of the Invention*

[0147] The agents described herein can be formulated into pharmaceutical compositions for the treatment of the diseases, disorders and conditions disclosed herein. The language "pharmaceutical composition" includes preparations suitable for administration to mammals, e.g., humans. When the compounds used in the methods of the present disclosure are administered as pharmaceuticals to mammals, e.g., humans, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99.5% (more preferably, 0.5 to 90%) of active ingredient in combination with a pharmaceutically acceptable carrier.

[0148] The phrase "pharmaceutically acceptable carrier" is art recognized and includes a pharmaceutically acceptable material, composition or vehicle, suitable for administering compounds of the present disclosure to mammals. The carriers include liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject agent from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: sugars, such as lactose, glucose and sucrose; starches, such as corn starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate;

powdered tragacanth; malt; gelatin; talc; excipients, such as cocoa butter and suppository waxes; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; esters, such as ethyl oleate and ethyl laurate; agar; buffering agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and other non-toxic compatible substances employed in pharmaceutical formulations.

[0149] Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

[0150] Examples of pharmaceutically acceptable antioxidants include: water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (13HT), lecithin, propyl gallate, α-tocopherol, and the like; and metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

[0151] Formulations of the present disclosure include those suitable for oral, nasal, topical, transdermal, buccal, sublingual, rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient that can be combined with a carrier material to produce a single dosage form will generally be that amount of the compound that produces a therapeutic effect. Generally, out of one hundred percent, this amount will range from about 1 percent to about ninety-nine percent of active ingredient, preferably from about 5 percent to about 70 percent, most preferably from about 10 percent to about 30 percent.

[0152] Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present disclosure with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association a compound of the present disclosure with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

[0153] Formulations of the disclosure suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of a compound of the present disclosure as an active ingredient. A compound of the present disclosure may also be administered as a bolus, electuary or paste.

[0154] In solid dosage forms of the disclosure for oral administration (capsules, tablets, pills, dragees, powders, granules and the like), the active ingredient is mixed with one or more pharmaceutically acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; humectants, such as glycerol; disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; solution retarding agents, such as paraffin; absorption accelerators, such as quaternary ammonium compounds; wetting agents, such as, for example, cetyl alcohol and glycerol monostearate; absorbents, such as kaolin and bentonite clay; lubricants, such a talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, and mixtures thereof; and coloring agents. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid compositions of a similar type may also be employed as fillers in soft and hard-filled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

[0155] A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (for example, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

[0156] The tablets, and other solid dosage forms of the pharmaceutical compositions of the present disclosure, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well

known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid compositions that can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions that can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

[0157] Liquid dosage forms for oral administration of the compounds of the disclosure include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluent commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

[0158] Besides inert dilutents, the oral compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

[0159] Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

[0160] Formulations of the pharmaceutical compositions of the disclosure for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the disclosure with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a

suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

[0161] Formulations of the present disclosure which are suitable for vaginal administration also include pessaries, tampons, creams, gels, pastes, foams or spray formulations containing such carriers as are known in the art to be appropriate.

[0162] Dosage forms for the topical or transdermal administration of a compound of this disclosure include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active compound may be mixed under sterile conditions with a pharmaceutically acceptable carrier, and with any preservatives, buffers, or propellants that may be required.

[0163] The ointments, pastes, creams and gels may contain, in addition to an active compound of this disclosure, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

[0164] Powders and sprays can contain, in addition to a compound of this disclosure, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

[0165] Transdermal patches have the added advantage of providing controlled delivery of a compound of the present disclosure to the body. Such dosage forms can be made by dissolving or dispersing the compound in the proper medium. Absorption enhancers can also be used to increase the flux of the compound across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the active compound in a polymer matrix or gel.

[0166] Ophthalmic formulations, eye ointments, powders, solutions and the like, are also contemplated as being within the scope of this disclosure.

[0167] Pharmaceutical compositions of this disclosure suitable for parenteral administration comprise one or more compounds of the disclosure in combination with one or more pharmaceutically acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into

sterile injectable solutions or dispersions just prior to use, which may contain antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents.

[0168] Examples of suitable aqueous and nonaqueous carriers that may be employed in the pharmaceutical compositions of the disclosure include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

[0169] These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents that delay absorption such as aluminum monostearate and gelatin.

[0170] In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

[0171] Injectable depot forms are made by forming microencapsule matrices of the subject compounds in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of drug to polymer, and the nature of the particular polymer employed, the rate of drug release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations are also prepared by entrapping the drug in liposomes or microemulsions that are compatible with body tissue,

[0172] The preparations of the present disclosure may be given orally, parenterally, topically, or rectally. They are of course given by forms suitable for each administration

route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, etc. administration by injection, infusion or inhalation; topical by lotion or ointment; and rectal by suppositories. Oral administration is preferred.

[0173] The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

[0174] The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

[0175] These compounds may be administered to humans and other animals for therapy by any suitable route of administration, including orally, nasally, as by, for example, a spray, rectally, intravaginally, parenterally, intracistemally and topically, as by powders, ointments or drops, including buccally and sublingually.

[0176] Regardless of the route of administration selected, the compounds of the present disclosure, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present disclosure, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

[0177] Actual dosage levels of the active ingredients in the pharmaceutical compositions of this disclosure may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient.

[0178] The selected dosage level will depend upon a variety of factors including the activity of the particular compound of the present disclosure employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compound employed,

the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

[0179] A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the disclosure employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved.

[0180] In general, a suitable daily dose of a compound of the disclosure will be that amount of the compound that is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. Generally, intravenous and subcutaneous doses of the compounds of this disclosure for a patient, when used for the indicated analgesic effects, will range from about 0.0001 to about 100 mg per kilogram of body weight per day, more preferably from about 0.01 to about 50 mg per kg per day, and still more preferably from about 1.0 to about 100 mg per kg per day. An effective amount is that amount treats a disease, disorder or condition set forth herein.

[0181] If desired, the effective daily dose of the active compound may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms.

[0182] While it is possible for a compound of the present disclosure to be administered alone, it is preferable to administer the compound as a pharmaceutical composition.

[0183] Certain aspects of the disclosure involve administration of nucleic acid agents. Nucleic acid agents can be effectively delivered to a subject as stabilized agents, with such stability often provided via lipid nanoparticle (LNP) encasement of active nucleic acid agents and/or modification of therapeutic nucleic acid agents with one or more stabilizing modifications, including, e.g., 2'-O-alkyl modifications (including 2'-O-methyl), 2'-F modifications, backbone modifications, locked nucleic acid (LNA) configurations, GalNAc modifications, cholesterol conjugates, etc. Such modifications are known in the art and can be readily employed by the skilled artisan for delivery of the nucleic acid agents of the instant disclosure.

[0184] *Methods of Treatment*

[0185] As used herein, the term "Marfan syndrome or associated diseases, disorders and conditions" is intended to mean Marfan syndrome or any one of the multitude of diseases disorders or conditions that is caused or associated with the biochemical events that cause Marfan syndrome, e.g., the aberrant expression or activity of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof. Exemplary conditions include aneurysm, an aortic aneurysm, valve disease, emphysema, myopathy, scoliosis, or eye disease. Exemplary eye diseases include cataracts, myopia, glaucoma, and retinal detachment. Moreover, Marfan syndrome or associated diseases, disorders and conditions include diseases and disorders that related to muscle growth, maintenance, or regeneration, e.g., muscular dystrophies such as Duchenne muscular dystrophy. Further, the disease or disorder can be a lung disease or disorder, e.g., emphysema, pneumothorax, and COPD.

[0186] The term "treated," "treating" or "treatment" includes the diminishment or alleviation of at least one symptom associated or caused by Marfan syndrome, or an associated disease, disorder or condition. For example, treatment can be diminishment of one or several symptoms of a disease or disorder or complete eradication of the disease or disorder, e.g., Marfan syndrome.

[0187] The term "subject" is intended to include organisms, e.g., prokaryotes and eukaryotes, which are capable of suffering from or afflicted with Marfan syndrome, or a disease, disorder or condition related thereto. Examples of subjects include mammals, e.g., humans, dogs, cows, horses, pigs, sheep, goats, cats, mice, rabbits, rats, and transgenic non-human animals. In certain embodiments, the subject is a human, e.g., a human suffering from, at risk of suffering from, or potentially capable of suffering from a Marfan syndrome, or a disease, disorder or condition related thereto.

[0188] The agents and pharmaceutical compositions of the disclosure can be administered to a subject to treat or prevent diseases, disorders and conditions associated with aberrant expression or activity of a MAP kinase pathway component, e.g., *MMP17*, *MAP2K6* and/or *MAP3K4*, or a gene product thereof. In one embodiment, the agents and pharmaceutical compositions are used to treat or prevent Marfan syndrome or diseases or disorders associated with Marfan syndrome.

[0189] In one embodiment, the agents or pharmaceutical compositions are administered in an effective amount using a dosing schedule determined by a medical

provider to treat or prevent a disease or disorder set forth herein. The agents or pharmaceutical compositions can be administered in a variety of methods, as described herein and known to one of skill in the art.

[0190] In one aspect, the disclosure provides a method for preventing in a subject, a disease or condition associated with an aberrant expression or activity of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof, by administering to the subject an agent which modulates expression or activity of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof. Subjects at risk for a disease which is caused or contributed to by aberrant expression or activity of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof, can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the aberrancy of expression or activity of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof, such that a disease or disorder is prevented or, alternatively, delayed in its progression.

[0191] Another aspect of the disclosure pertains to methods of modulating expression or activity of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof, for therapeutic purposes. Accordingly, in an exemplary embodiment, the modulatory method of the disclosure involves contacting a cell with an agent that modulates one or more of the activities of a component of a MAP kinase signaling pathway, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof. An agent that modulates expression or activity of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof can be an agent as described herein, such as a nucleic acid, a polypeptide, or a small molecule. In one embodiment, the agent inhibits one or more activities of a MAP kinase pathway component, e.g., *MMP17, MAP2K6* and/or *MAP3K4*, or a gene product thereof, and/or inhibits one or more components of the MAP kinase pathway. Examples of such inhibitory agents include antisense *MMP17, MAP2K6* and/or *MAP3K4* nucleic acid molecules, anti-Mmp17, -Map2k6 and/or -Map3k4 antibodies, and small molecule inhibitors of Mmp17, Map2k6 and/or Map3k4. These modulatory methods can be performed in vitro (e.g., by culturing the cell with the agent) or, alternatively, in vivo (e.g., by administering the agent to a subject). As such, the present disclosure provides methods of treating an individual afflicted with a disease or disorder characterized by aberrant MAP

kinase pathway signaling, e.g., Marfan syndrome or an associated disease or disorder. In one embodiment, the method involves administering an agent, or combination of agents that modulates MAP kinase pathway signaling.

[0192] The disclosure further provides kits comprising agents or pharmaceutical compositions of the disclosure and instructions for use. In one embodiment, the kits of the disclosure are for the treatment of diseases and disorders characterized by aberrant MAP kinase pathway signaling. In a related embodiment, the MAP kinase pathway signaling associated disease or disorder is Marfan syndrome or a disease or disorder related to Marfan syndrome.

EXAMPLES

[0193] It should be appreciated that the invention should not be construed to be limited to the examples that are now described; rather, the invention should be construed to include any and all applications provided herein and all equivalent variations within the skill of the ordinary artisan.

[0194] *Example 1: Involvement of MAP Kinase Pathway Components Map3k4, Map2k6 and Mmp17 in Marfan Syndrome*

[0195] It has been shown that the C57BL/6 and Sv129 mouse backgrounds exhibit strain-specific modulatory effects on TGFβ deficiency phenotypes, and this may relate to their influence on downstream TGFβ signaling (Bourdeau, *et al. American Journal Pathology* 158, 2011; Warshamhabashiana *et al. Am J Resp Cell Mol Biol* 27, 705). With this in mind, it was examined whether the Sv129 background could exacerbate a MFS mouse model phenotype. MFS model mice from a pure C57BL/6J (hereafter termed BL6) background were backcrossed for greater than 10 generations onto a 129S6/SvEvTac (hereafter termed 129) background.

[0196] 129 MFS mice exhibited a highly significant increase in aortic root size, post-natal aortic root growth and premature lethality from dissection, compared with BL6 MFS counterparts, in association with increased activation of both Smad2/3 and Erk1/2 cascades. All aortic parameters in 129 MFS mice were fully rescued by both losartan and RDEA119. 129/MFS mice also showed worse lung emphysema and spine kyphosis, with significant correlation between aortic root size and these phenotypes, indicating deleterious systemic genetic modification in 129 MFS mice. Wild type (WT) mice on the two background strains showed no difference in any parameter, indicating MFS disease-specific modification.

[0197] To map potential modifier loci, a large seven generational pedigree of intercrossed BL6 MFS and 129 MFS mice was generated. The resulting offspring were then classified as either BL6-like, 129-like or indeterminate, based on aortic size and shape at 6 months of age. Genome mapping of forty BL6-like and forty 129-like MFS mice revealed 2 QTLs on chromosomes 5 and 11 that strongly linked with severe aortic phenotype (LOD=4.76 and 4.78, respectively) and reached genome wide significance (p=0.008 for both), with evidence of epistasis between the loci (MfLOD=12.8; p=0.0006).

[0198] Causative genetic variation was searched for in these QTL regions using the mouse genome project, and one likely causal variant was identified in each locus, that was predicted to be both functionally deleterious and mechanistically linked to Mapk signaling. On chromosome 5, a missense point mutation in Mmp17 (rs29636438; p.X579W) was predicted to alter the C-terminal region, modify attachment of the GPI anchor, and affect its cell surface expression, causing greater activation of Mapk signaling in 129 MFS mice. On chromosome 11, a missense point mutation in Map2k6 (rs51129320; p.G76E) was predicted to cause greater activation of Mapk signaling in 129 MFS mice. Haploinsufficiency for Map2k6 and Mmp17 rescued aortic root growth in 129 MFS mice by approximately 50%, while knockout of these two genes reduced aortic root growth in 129 MFS mice to a rate indistinguishable from that of their BL6 MFS counterparts.

[0199] Genetic modifiers of disease progression in MFS patients were then investigated. Five exceptional families were identified to possess defined and typical FBN1 mutations, that showed discrete intrafamilial variation in the penetrance of vascular disease. Genome-wide linkage in these five families identified a major modifier locus for vascular disease in MFS, encompassing a 5.5 Mb linkage interval on chromosome 6 (LOD=4.0). While the protective haplotype varied between families, all patients with mild disease (20/20) shared a 3.9 Mb familial haplotype that was only observed in 2/18 severely affected family members ($p<0.0001$). Interestingly, this region includes *MAP3K4*, which lies directly upstream of *MAP2K6* in the Mapk signaling cascade. Furthermore, haploinsufficiency for Map3k4 led to a full rescue of aortic root growth in MFS mice, to a rate indistinguishable from that of WT littermates.

[0200] Thus, a common pathway of genetic modification in MFS has herein been identified, which provides a rationale for exploration of therapies that target genes of this pathway/these pathways, particularly including *MMP17, MAP2K6* and *MAP3K4*. Given that a number of MFS-related conditions also appear to be driven by increased TGFβ signaling

(Gallo *et al. J. Clin. Investigation* 124, 448; Doyle *et al. Nature Genetics* 44, 1249; Lindsay *et al., Nature Genetics* 44, 922), these pathways are likely also to hold broader clinical relevance. While losartan has been previously described as providing some degree of rescue of aortic root growth in patients with MFS (Lacro, *et al., New England Journal of Medicine* 371, 2061), there is still significant room for additional pharmacological interventions that can ameliorate the potentially devastating cardiovascular, skeletal and ocular consequences of the disease. Whilst surgical intervention exists for many complications, these often involve major procedures that have significant morbidity and/or mortality associated with them. Furthermore, they often require ongoing additional treatment (e.g., lifelong use of anticoagulation after aortic root replacement) which too possess risk for significant complications. The instant disclosure therefore seeks to provide a MFS therapy possessing the ability to prevent these deleterious outcomes from ever occurring, thereby negating the need for major surgical intervention.

[0201] *Example 2: Materials and Methods*

[0202] *Mice:* All mice were cared for under strict compliance with the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. The *Fbn1*$^{C1039G/+}$ line was initially maintained in a pure C57BL/6 background (backcrossed for greater than 9 generations), allowing for valid comparisons, prior to backcrosses with Sv29 mice, performed as described above. In order to further accommodate the potential for temporal- or background-specified variation, all comparisons were made between contemporary littermates when possible. Mice were sacrificed with an inhalation overdose of halothane (Sigma-Aldrich, St. Louis). Mice underwent immediate laparotomy, descending abdominal aortic transection, and PBS (pH 7.4) was infused through the right and left ventricles to flush out the blood. Mice that were analyzed for aortic histology had latex injected under low pressure into the left ventricular apex until it was visible in the descending abdominal aorta. Mice were fixed for 24 hours in 10% buffered formalin, after which the heart, aorta and lungs were removed and stored in 70% ethanol.

[0203] *Echocardiography:* Nair hair removal cream was used on all mice the day prior to echocardiograms. All echocardiograms were performed on awake, unsedated mice using the Visualsonics Vevo 660 V1.3.6 imaging system and a 30 MHz transducer. Mice were imaged at baseline, and at six months, when also sacrificed. The aorta was imaged using a parasternal long axis view. Three separate measurements of the maximal internal dimension at the sinus of Valsalva and proximal ascending aorta were made from distinct

captured images and averaged. All imaging and measurements were performed by a cardiologist who was blinded to genotype.

[0204] *Histological and Morphometric Analysis:* Latex-infused ascending aortas were transected just above the level of the aortic valve, and 2-3mm transverse sections were mounted in 4% agar prior to paraffin fixation. Five micrometer aortic sections underwent Verhoeff-van Giesen (VVG) staining and were imaged at 40x magnification, using a Nikon Eclipse E400 microscope. Wall thickness of the aortic media was measured by a single blinded observer at 16 different locations around the most proximal ascending aortic ring and averaged. Wall architecture of 4 representative sections for each mouse was assessed by the same 3 blinded observers and graded on an arbitrary scale of 1 (indicating no breaks in the elastic fiber) to 5 (indicating diffuse fragmentation), and the results were averaged. Elastic fiber content was quantified in four separate representative images of each section of the most proximal ascending aorta by a single blinded observer, using NIS Elements Advanced Research (Nikon, Japan). The aortic media and the elastic fibers were individually outlined and their areas calculated. The respective areas were averaged from all the images of a given aortic section and the ratio of elastic fiber content to total aortic media was determined. Aortic root size was measured by echocardiography at six months (baseline before treatment).

[0205] *Example 3: Identification of Major Genetic Modifiers of Aortic Aneurysm in Marfan Syndrome.*

[0206] Marfan syndrome (MFS) is an inherited connective tissue disorder caused by heterozygous mutations in the *FBN1* gene. The major cause of mortality is aortic aneurysm, dissection and/or rupture.

[0207] *Materials and Methods*

[0208] *Mouse lines:* Mice were cared for under compliance with the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. *Fbn1*$^{C1039G/+}$ mice on a pure C57BL/6J background were already onsite. Mice on a pure 129S6/SvEvTac background were obtained from Taconic Biosciences (Rensselaer County, NY, USA). The *Fbn1*$^{C1039G/+}$ mice on a pure C57BL/6J background were bred into a pure 129S6/SvEvTac background and backcrossed for >10 generations. Mice haploinsufficient or knockout for Mmp17 (B6.129P2-*Mmp17*^{tm1Dgen}/J; #005824), Map2k6 (B6.129S1-*Map2k6*^{tm1Flv}/J; #008382) and Map3k4 (B6.129S6-*Map3k4*^{tm1Flv}/J; #008375) were obtained from the Jackson Laboratory (Bar Harbor,

ME, USA), and bred to *Fbn1*C1039G/+ mice. Animals were checked daily for death and all mice found dead were immediately autopsied to assess for evidence of aortic dissection. Mice that were sacrificed were done so using a lethal dose of ketamine and acepromazine (1ml of 10mg/ml acepromazine in 10mls of 100mg/ml ketamine; MWI Vet, ID, USA). At the time of sacrifice, mice underwent immediate laparotomy and descending abdominal aortic transection. Phosphate buffered saline (PBS) was infused through the right and left ventricles to flush out the blood.

[0209] *Mouse drug treatments:* Losartan was dissolved in drinking water and filtered to reach a concentration of 0.6g/L, giving an estimated daily dose of 60 mg/kg/day, as described previously (Habashi, J.P. *et al. Science* 312, 117-21 (2006)). Mice were started on losartan at 2 months of age and continued for 8 months. Placebo-treated animals received regular drinking water. RDEA119 was reconstituted in 10% 2-hydroxypropyl-beta-cyclodextrin (Sigma-Aldrich, MO, USA) dissolved in PBS, and administered twice daily by oral gavage at a dose of 25 mg/kg, as described previously (Holm, T.M. *et al. Science* 15, 358-61 (2011)). Treatment was initiated at 2 months of age and continued for 2 months. 10% 2-hydroxypropyl-beta-cyclodextrin dissolved in PBS was administered as a control. Erlotinib was reconstituted in 1% methylcellulose (Sigma-Aldrich) dissolved in PBS, and administered daily by oral gavage at a dose of 50mg/kg. Treatment was initiated at 2 months of age and continued for 2 months. 1% methylcellulose dissolved in PBS was administered as a control.

[0210] *Mouse echocardiography:* Nair hair removal cream was used on all mice the day prior to echocardiography. All echocardiograms were performed on awake, unsedated mice using the VisualSonics Vevo 2100 imaging system and a 30 MHz transducer (Fujifilm VisualSonics, ON, Canada). The aorta was imaged using a parasternal, long-axis view. Three separate measurements of the maximal internal dimension at the sinus of Valsalva and proximal ascending aorta were made from distinct captured images and averaged. All imaging and measurements were performed blinded to genotype and treatment arm.

[0211] *Mouse blood pressure analysis:* Blood pressures were analyzed by taking 20 tail cuff blood pressures per day over 5 days in each mouse to habituate the mice to the tail cuff blood pressure system (BP-2000, Visitech Systems, USA), and the blood pressures obtained on the last day were averaged.

[0212] *Mouse aorta Western Blot analysis:* Mice that were sacrificed for Western Blot analysis had their proximal ascending aortas immediately dissected, flash frozen in liquid

nitrogen and stored at -80°C until further processing. Protein was extracted using the reagents and protocol from a Total Protein Extraction Kit containing protease inhibitor and Protein Phosphatase Inhibitor Cocktail (Millipore, MA, USA). Aortas were homogenized using a pellet pestle motor (Kimble-Kontes, NJ, USA) as per the extraction kit protocol. Samples were then stored once more at -80°C until Western blot analysis was performed. Aortic tissue homogenates were dissolved in sample buffer, run on a NuPAGE Novex 4-12% Bis-Tris Gel (Invitrogen, CA, USA), and transferred to nitrocellulose membranes using the iBlot transfer system (Invitrogen). Membranes were washed in PBS and blocked for 1 hour at room temperature with 5% instant non-fat dry milk dissolved in PBS containing 1% Tween-20 (Sigma-Aldrich; PBS-T). Equal protein loading of samples was determined by a protein assay (Bio-Rad, CA, USA) and confirmed by probing with an antibody against β-Actin (Sigma-Aldrich #A5316). Membranes were probed overnight at 4° centigrade with primary antibodies against pSmad2 (Cell Signaling, MA, USA #3108), pSmad3 (Cell Signaling #9250), pErk1/2 (Cell Signaling #4370), pMek1 (Cell Signaling #9154), pp38 (Cell Signaling #4511), pJnk1/2 (Santa Cruz #6254), pPkcβ (Cell Signaling #9371), and pEgfr (Cell Signaling #3777), dissolved in PBS-T containing 5% milk. Blots were then washed in PBS-T and probed with HRP-conjugated anti-rabbit or anti-mouse secondary antibodies (GE Healthcare, UK #NA934) dissolved in PBS-T containing 5% milk for 1 hour at room temperature. Blots were then washed in PBS-T, developed using SuperSignal West Femto HRP substrate (Pierce Scientific, IL, USA), exposed to BioMax Scientific Imaging Film (Sigma), and quantified using ImageJ analysis software (NIH, MD, USA).

[0213] *Mouse lung tissue collection and analysis:* Mice that were sacrificed for lung histology had their trachea intubated with a 20-gauge blunt needle; 0.5% agar was infused under low and constant pressure using direct manometry to inflate the lungs. The trachea was then tied-off using an 8-0 Vicryl suture and the needle was removed. Samples were fixed for 24 hours in 10% buffered formalin and then stored in 70% ethanol. Individual lobes of the lungs were mounted in 4% agar and fixed in paraffin. Five micrometer lung sections underwent hematoxylin and eosin staining and were imaged at 10x magnification, using a Nikon Eclipse E400 microscope. Five fields were analyzed for each lobe of each lung by a single blinded observer, and a mean linear intercept was calculated as described previously (Neptune, E.R. *et al. Nature Genetics* 33, 407-11 (2003)).

[0214] *Mouse skeletal X-ray analysis:* Mice undergoing spine X-ray were anesthetized using a combination of ketamine and xylazine (1ml of 100mg/ml ketamine and 100µl of

100mg/ml xylazine in 10mls of PBS; MWI Vet). They were placed in the left lateral decubitus position on a radiolucent platform with adjacent scale bar and imaged at 1x magnification using a Faxitron MX20 (Faxitron, AZ, USA). The output electronic file was processed using ImageJ analysis software (NIH). Spine kyphosis was quantified using a modified kyphosis index (Laws, N., Hoey, A. *J. Appl. Physiol.* 97, 1970-7 (1985)). In brief, a line was drawn from the anterior superior iliac spine to the point of maximal lordosis of the cervical spine. A line was then drawn perpendicular from this line to the anterior aspect of the vertebral body at the point of maximal thoracic kyphosis.

[0215] *Mouse whole genome analysis*: SNPs were selected from the Illumina mouse medium density linkage panel array that were divergent between the C57BL/6J and 129S6/SvEvTac background strains. Genotyping was performed using an Illumina mouse medium density linkage panel, and was run using standard GoldenGate chemistry on an iScan microarray scanner (Illumina, CA, USA). Genotypes were called using Illumina GenomeStudio software (GenomeStudio 2011.1, Genotyping Module 1.9.4 and Gentrain version 1.0). The average physical coverage was 2.9 Mb, the maximum gap was 22.4 Mb and there were 10 gaps >12 Mb. The genotype quality score cut-off for a "no call" genotype was 0.25. Only SNPs of sufficient quality were used for downstream analysis. R/qtl software (rqtl.org; Broman K.W., Sen Ś. (2009) Introduction. In: A Guide to QTL Mapping with R/qtl. Statistics for Biology and Health. Springer, New York, NY) was utilized to calculate LOD scores for the whole-genome SNP analysis. All of the animals for which there was both genotype and phenotype data were included in the analysis; mice were only included if they were either BL6 or 129 throughout the whole ROI on a given chromosome. First, the "scanone" function was used to perform a single-QTL genome scan utilizing a nonparametric model and 1000 permutations. The results were expressed as a LOD score and p-value. The "scanone" function calculated the genome-wide LOD significance threshold for this dataset as 3.82. Next, the "scantwo" function was used to perform a two-dimensional genome scan with a two-QTL model utilizing a binary model. The recommended significance threshold for a mouse intercross using this function was a MfLOD >9.1. Since the "scantwo" function does not compute a p-value, we performed ANOVA plus a post-hoc linear trend test of the raw continuous data to generate a p-value for the 129 allele dose-response graph.

[0216] *Families with Marfan syndrome:* The 5 families were recruited from the Connective Tissue Clinic at Johns Hopkins Hospital and Ghent University Hospital. The diagnosis of MFS was made in accordance with the revised Ghent nosology (Loeys, B.L., *et al.*

Revised Ghent criteria for the diagnosis of Marfan syndrome (MFS) and related conditions. *J. Med. Genet.* 47, 476-485 (2010)). Echocardiograms were performed and interpreted as previously described (Brooke B.S., *et al. N. Engl. J. Med.* 358, 2787-95 (2008)). Aortic root aneurysm was defined by a maximal aortic root Z score >2.0. All blood samples and skin biopsies were collected in compliance with the Institutional Review Board after informed consent was obtained. DNA was extracted from 5 mL whole blood using a DNeasy blood and tissue kit (Qiagen, CA, USA). For the purposes of genome wide linkage analysis, individuals harboring an *FBN1* mutation were classified as affected; they were assigned a severe status if they had an aortic root Z score >3.0, a history of aortic root dissection or a history of aortic root surgery; they were assigned a mild status if they had an aortic root Z-score <2.0 or had reached age 60 years without prior aortic root dissection or aortic root surgery; those with a Z score 2.0-3.0 were classed as indeterminate. Individuals without an *FBN1* mutation were classified as unaffected.

[0217] *Human whole genome analysis:* The DNA was digested, amplified, and hybridized to either a Genome-Wide Human SNP Nsp/Sty Array 5.0 or 6.0 according to the manufacturer's recommended protocol (Affymetrix, CA, USA). The arrays were then scanned using a GeneChip Scanner 3000 7G (Affymetrix). The genomic location of each marker was determined from the Affymetrix Genetic Map (Affymetrix). SNPs were selected that were present on both the Affymetrix 5.0 and 6.0 Array using PLINK. Allele frequencies for this SNP set were calculated using PLINK. Tests for Hardy-Weinberg equilibrium (HWE) and Mendelian errors were calculated for autosomal SNPs using Pedstats. Tests for HWE were calculated for sex chromosome SNPs using PLINK, and Mendelian errors using Pedstats. Subsequently, the following quality control filters were applied using PLINK; <1% missing data, minor allele frequency (MAF) >0.2, no Mendelian errors and HWE p>0.05. Statistically unlikely genotypes were then filtered using the error-checking algorithm in MERLIN/MinX. As a last step, SNPs that were in complete linkage disequilibrium with a proximate SNP (r^2=1) were excluded using PLINK to produce a list of SNPs of outstanding quality. Prior to genome wide linkage analysis, the dataset was simplified whilst maintaining uniform coverage across the genome by only including every 10th consecutive SNP. Parametric linkage analysis was performed following the maximized maximum LOD score (MMLS) procedure with the restricted set of SNPs using MERLIN/MinX. The analysis was performed twice, once under a dominant model and once under a recessive model, considering a low sporadic rate (0.0002), an arbitrary penetrance (0.50 and 0.80 for one and two alleles respectively), and arbitrary gene

frequencies (0.01 for the dominant model and 0.1 for the recessive model). The limits of the regions of interest were defined by the closest neighboring upstream and downstream SNP markers with LOD scores >1 levels below the peak identified by parametric linkage analysis. Haplotypes were established for all genotyped individuals across the regions of interest. Since the mode of inheritance and the penetrance of the modifier region were uncertain, the segregation of haplotypes between individuals classified as affected with mild disease was examined first. Next individuals classified as affected with severe disease were incorporated into this analysis. The NCBI mapviewer (cbi.nlm.nih.gov/projects/mapview; Wolfsberg, T. G. 2010. Using the NCBI Map Viewer to Browse Genomic Sequence Data. *Current Protocols in Bioinformatics*. 29:1.5:1.5.1–1.5.25.) was used to identify candidate genes contained within haplotypes of interest.

[0218] *Human DNA sequencing analysis:* Candidate genes were cycle sequenced across all exons, exon/intron boundaries and potential non-coding functional variants from the ENCODE dataset (defined as SNPs in regions associated with DNase hypersensitivity, transcription factor occupancy, histone modification and a MAF <0.15) in 2 individuals with mild disease and 2 individuals with severe disease from each family. Firstly, targeted segments of DNA were amplified by PCR using a DNA Engine Dyad thermal cycler (Bio-Rad). Next, the samples were purified using QIAquick PCR purification kit (Qiagen). Finally, cycle sequencing was performed using BigDye Terminator v3.1 kit and an ABI 3730xl DNA Analyzer/sequencing machine in accordance with the manufacturer's instructions (Life Technologies, CA, USA).

[0219] *Human dermal fibroblast quantitative RT-PCR expression analysis:* Primary human dermal fibroblasts were derived from forearm skin biopsies from 2 control individuals, 2 patients in family B with severe aortic disease and 2 patients from family B with mild aortic disease. Cells were incubated in T-75 flasks at 37°C with 95% air and 5% CO2 whilst submerged in Dulbecco's modified eagle medium (Gibco, Life Technologies) containing 10% fetal bovine serum (Sigma-Aldrich), antibiotics and antimycotic (Gibco). Media was replaced every 3 days. Cells were passaged at 95-100% confluence. Cells were collected using TRIzol (Life Technologies) and immediately stored at -80°C. RNA was extracted using a miRNeasy mini kit according to the manufacturer's instructions (Qiagen). Quantitative RT-PCR was performed using a RNA-to-CT 1-step Kit and an ABI 7900HT Fast Real-Time PCR System in accordance with the manufacturer's instructions (Life Technologies). The following pre-

validated probes were used for analysis: Hs01060665 (*β-ACTIN*) and Hs00245958_m1 (*MAP3K4*) (Life Technologies).

[0220] *Statistical analysis:* All quantitative data are shown as bar graphs produced using Excel (Microsoft, WA, USA). Mean ± 2 standard errors of the mean (SEM) are displayed. Statistical analyses were performed using two-tailed *t* tests. A p-value <0.05 was considered statistically significant for all tests.

[0221] *Results*

[0222] Mice were characterized as heterozygous for a disease-causing *Fbn1* allele (*Fbn1*$^{C1039G/+}$) on a pure C57BL/6J mouse background (hereafter termed BL6 MFS mice). These mice have been shown to recapitulate multiple manifestations of the disease, including aortic root aneurysm, developmental lung emphysema, and skeletal deformity (Neptune, E.R. *et al. Nature Genetics* 33, 407-11 (2003); Habashi, J.P. *et al. Science* 312, 117-21 (2006); Judge, D.P. *et al. J. Clin. Invest.* 114, 172-81 (2004)), although the severity of the manifestations is on the milder end of the human phenotypic spectrum. In the present study, these *Fbn1*$^{C1039G/+}$ mice were backcrossed greater than 10 generations onto a 129S6/SvEvTac background (hereafter termed 129 MFS mice), to assess the impact of mouse strain on MFS disease phenotype. Aortic root size at 2 and 6 months of age, and post-natal aortic root growth from 2 to 6 months, were greater in 129 MFS mice, compared to their BL6 MFS counterparts (Figures 1A, 1B yellow arrows). This strain-dependent modification of aortic size was absent in wild-type (WT) littermates, showing it to be a MFS disease-specific effect. Furthermore, the 129 strain did not exacerbate growth of the more distal ascending aorta in MFS mice (Figures 5, 1B red arrows), indicating that this strain-specific exacerbation only occurs at sites of underlying disease predisposition, as opposed to a more generalized loss of arterial homeostasis.

[0223] Compared to BL6 MFS mice, 129 MFS animals died prematurely secondary to aortic dissection and/or rupture (Figure 1C). This was independent of hemodynamic status, weight or gender (Figures 6, 7). Compared to BL6 MFS animals, 129 MFS mice also had worse lung disease as measured by mean linear intercept (MLI; Figure 8; Neptune, E.R. *et al. Nature Genetics* 33, 407-11 (2003)), and worse spine kyphosis as measured by a modified kyphosis index (KI; Figure 9; Laws, N., Hoey, A. *J. Appl. Physiol.* 97, 1970-7 (1985)). Of note, in 129 MFS mice there was an individual correlation between aortic root size and both lung MLI (r^2=0.75, p=0.012) and spine KI (r^2=0.30, p=0.013).

[0224] To assess the mechanism driving disease exacerbation, Western blot analysis was performed on the aortic root of 10-month old animals. Compared to BL6 MFS mice, 129 MFS animals showed greatly enhanced activation of pathways previously shown to be operative in MFS mice, including Smad2/3, Erk1/2 and its upstream activator Mek1, p38, and PKCI3 (Figure 2A). Jnk1/2 activation remained unaffected, suggesting selective, rather than global modulation of MAPK signaling. There were no differences in activation of any of these pathways in WT animals on the two backgrounds, indicating MFS disease-specific modification. Treatment of 129 MFS mice with either the ARB losartan or the inhibitor of Erk1/2 activation RDEA119, reduced aortic root growth to a rate equal to that of WT animals (Figure 2B). Furthermore, losartan ameliorated premature lethality in 129 MFS mice (Figure 2C). This correlated with a reduction in both canonical (Smad2) and noncanonical (Erk1/2, p38) TGFI3 signaling cascades in losartan-treated 129 MFS mice (Figure 2D), and a selective reduction in noncanonical (Erk1/2, p38) cascades in RDEA119-treated 129 MFS animals (Figure 2E). Taken together, these data illustrate that the 129 strain exacerbates signaling cascades known to be upregulated in BL6 MFS mice, and therapies targeting these pathways are highly efficacious even against the more severe disease seen in 129 MFS animals.

[0225] To identify modifier loci for the MFS aortic phenotype, MFS mice were interbred on the 2 pure strains to generate MFS animals on a mixed genetic background. The F1 generation MFS mice displayed an aortic root size that was intermediate between the 2 parental strains (Figure 10). Mice from the F1 and later generations were interbred to produce a 7-generation pedigree that contained circa 300 MFS mice possessing a range of aortic aneurysm severity. Genome-wide linkage analysis was performed on 35 MFS mice with mild aortic aneurysm (root size <2.20mm at 6 months), and 40 MFS mice with severe aortic aneurysm (root size >2.70mm at 6 months). Two loci were identified that strongly linked with aortic severity on chromosomes 5 and 11, both of which exceeded the LOD score threshold of 3.82 to achieve genome wide significance (Figure 3A), with evidence of epistasis between the loci (MfLOD=12.8). The regions of interest (ROI) were defined as those flanked by individual markers that surpassed genome wide significance; these included 14.7Mb on chromosome 5 (128,433,315-143,149,098 bp) and 8.9Mb on chromosome 11 (106,799,515-115,722,120 bp). When 129 allele dose at these ROI was plotted against aortic root size at 6 months of age in the F2 generation interbred mice, there was a clear dose-response, with more 129 alleles (0 to 4) correlating with increased aortic size (p=0.0006; Figure 3B).

[0226] Table 6 shows candidate functional variants that differed between the BL6 and 129 strains within the regions of interest on chromosomes 5 and 11 identified using the mouse genome project online repository that were disfavored by PROVEAN.

[0227] Table 6

Chr	Gene	Residue	Score	Prediction
5	*Mmp17*	p.X579W	9aa deletion	Stop-Loss
5	*Tfr2*	p.G42V	-3	Disfavored
5	*Zkscan*	p.R246W	-3	Disfavored
5	*Fzd10*	p.S389R	-1	Disfavored
5	*Rimbp2*	p.D1062H	-1	Disfavored
11	*Map2k6*	p.G76E	-2	Disfavored
11	*Bptf*	p.R2360C	-3	Disfavored
11	*Helz*	p.L1498P	-3	Disfavored
11	*Sdk2*	p.L1501P	-3	Disfavored
11	*Ccdc46*	p.E507G	-2	Disfavored
11	*Grin2c*	p.P1098H	-2	Disfavored
11	*Slc9a3r1*	p.P182S	-1	Disfavored
11	*Grin2c*	p.P970L	-1	Disfavored

[0228] Candidate functional variants were sort within both loci that differed between the 2 strains using the mouse genome project online repository (Table 6). Only 1 variant at each locus was predicted to be both functional and located in a gene likely to influence TGF3/MAPK signaling. On chromosome 11, a missense point mutation was identified in *Map2k6*, a known member of the MAPK signaling cascade, which was predicted to significantly alter protein function (rs51129320; n.11:110490856-110490856G>A; c.G227A; p.G76E; PROVEAN score -3.66). On chromosome 5, the BL6 strain encodes a premature termination codon in *Mmp17* relative to the 129 strain (rs29636438; n.5:129606538-129606538G>A; c.A1737G; p.X579W),

which creates a truncated protein that is missing the terminal 9 amino acids (Figure 11). This hydrophobic sequence at the C-terminus is critical to the protein's ability to anchor to the extracellular side of the cell membrane via a GPI anchor. Loss of the terminal 9 amino acids in BL6 mice was predicted to significantly alter the hydrophobicity profile of the C-terminal segment, reducing the efficiency of GPI attachment and decreasing the amount of functional protein at the cell surface compared to the 129 strain (Galian C., *et al. J. Biol. Chem.* 287, 16399-409 (2012); Yan W., *et al. J. Mol. Biol.* 275, 25-33 (1998)). Given that both *Map2k6* and *Mmp17* are expressed in the vasculature and are reported to be positive effectors of TGF3/MAPK signaling (Paye, A., *et al. Cancer Res.* 74, 6758-70 (2014); Han, J., *et al. J. Biol. Chem.* 271, 2886-91 (1996)), it was hypothesized that both variants would likely induce a loss of function on the BL6 background.

[0229] *Mmp17* and *Map2k6* knockout mice (*Mmp17*$^{-/-}$ and *Map2k6*$^{-/-}$) were obtained from Jackson laboratories on a mixed BL6/129 background. These were crossed to 129 MFS mice to generate MFS animals with predominant but variable 129 strain content that were haploinsufficient or fully deficient for one or both genes. Mice deficient in both genes were born at expected Mendelian ratios and survived to adulthood without apparent deleterious consequence. MFS mice possessing the 129 sequence at the two genes of interest fully recapitulated both the aortic root size and biochemical signaling seen in pure 129 MFS mice (Figures 3C, 3D). Furthermore, aortic root size at 6 months was dependent on the number of functional *Mmp17* and *Map2k6* alleles, with MFS mice lacking both genes having an aortic size that was indistinguishable from that of pure BL6 MFS animals (Figure 3C). Western blot analysis of the aortic root in 10-month old MFS mice showed that knockout of both *Mmp17* and *Map2k6* led to attenuation of canonical and noncanonical signaling cascades to values at or below those of BL6 MFS mice (Figure 3D). Selective knockout of *Mmp17* led to an intermediate suppression of these signaling cascades, indicating that the two genes act in an additive manner.

[0230] Consistent with prior work showing that Mmp17 can potentiate MAPK signaling by enhancing EGFR activation independent of catalytic activity (Galian C., *et al. J. Biol. Chem.* 287, 16399-409 (2012)), it was found that increased Egfr phosphorylation in the aortic root of 129 MFS mice relative to BL6 MFS animals, which was fully normalized by *Mmp17* knockout, was independent of *Map2k6* status (Figure 3E). Furthermore, treatment with the EGFR inhibitor erlotinib abrogated aortic root growth in 129 MFS mice (Figure 3F), in association with reduced activation of Egfr, Erk1/2, and p38 (Figure 3G). Hence EGFR

inhibition may represent a novel, clinically-available, therapeutic strategy that can help ameliorate aortic growth in MFS.

[0231] In parallel, 5 MFS families were recruited that showed discrete intrafamilial variation in the severity of vascular disease among age- and gender-matched *FBN1* mutation carriers (Figure 4A). Parametric linkage analysis was performed to identify variation conferring a mild aortic disease phenotype. This revealed a single locus harboring protective genetic variation on chromosome 6 that exceeded the genome-wide significance LOD score of 4.0 (Figure 4B). While there was no common haplotype across families, all 5 families showed a positive LOD score in this region, with all 20 individuals classified as having mild disease sharing a 3.9Mb familial haplotype between markers rs676017 and rs6455736. The association between the presence of the protective haplotype and disease status (mild aortic disease 20/20; severe aortic disease 1/18) was statistically significant ($p<0.0001$). Of the 32 genes in this region, *MAP3K4* represented the outstanding candidate, based upon the fact that it lies directly upstream and is a direct activator of MAP2K6, one of the two modifier genes identified in MFS mice.

[0232] While direct sequencing of all exons, exon/intron boundaries and potential non-coding functional variants from the ENCODE dataset in the 5 MFS pedigrees did not yield a candidate variant in *MAP3K4*, we did observe a significant reduction in *MAP3K4* expression in dermal fibroblasts of patients with mild disease compared to those with severe disease (Figure 4D). Next, MFS mice to mice haploinsufficient for Map3k4 (*Map3k4*$^{+/-}$) were bred to test for genetic interaction. Aortic root growth from 2 to 6 months was reduced in MFS mice haploinsufficient for *Map3k4*, compared to MFS littermates that retained both alleles (Figure 4D). Western blot analysis of the aortas of 10-month old mice confirmed that Map3k4 levels were reduced in these animals, as was activation of both Erk1/2 and p38 (Figure 4E).

[0233] Taken together, this confluence of discovery-based and hypothesis-driven methodologies has informed disease pathogenesis in MFS and should provide confidence regarding the potential of therapeutic strategies directed against these novel target proteins. It demonstrates the power of harnessing nature's ability to generate phenotypic variability in pathological disease states through modifying genetic variation. It also highlights the utility of using mouse models to inform human disease modifier studies, since the latter can be limited by a paucity of families with discrete intrafamilial phenotypic variation, small pedigrees, allelic heterogeneity at the primary disease locus, and late onset declaration of phenotypic variation. Such issues can be mitigated by concomitant utilization of robust, validated animal models.

[0234] A role for EGFR activation in MFS has not been recognized previously; whether this relates to a failed regulatory role of fibrillin-1, or represents a more indirect consequence of events in MFS pathogenesis remains to be determined. Such mechanisms could include EGFR transactivation via known modulators such as TGFβ (Uchiyama-Tanaka Y., *et al. Kidney Int.* 62, 799-808 (2002); Vinals F., Pouyssegur J. *Mol. Cell Biol.* 21, 7218-30 (2001)), and/or Ang-II (Mehta P.K., Griendling K.K. *Am. J. Physiol. Cell Physiol.* 292, C82-97 (2007); Higuchi S., *et al. Clin. Sci.* 112, 417-28 (2007)). Interestingly, Mmp2 and 9 are both activated in MFS mice in an Erk1/2-dependent manner (Xiong W., *et al. Circ. Res.* 110, e92-e101 (2012)), and can also mediate Ang-II-dependent Egfr transactivation and consequent Erk1/2 activation in vascular cells. Furthermore the Efgr inhibitor erlotinib can abrogate Ang-II infusion-mediated TGFI3 induction in vascular tissues, all of which suggests that deleterious feedforward loops involving TGFβ, Ang-II and Egfr could influence MFS disease pathogenesis.

[0235] This work supports the observations that both Smad2/3 and Erk1/2 signaling cascades are activated in MFS mice, with a direct correlation between the extent of activation of these proteins and phenotypic severity. It also illustrates prominent activation of p38 in this MFS mouse model, recapitulating prior observations in fibrillin-1 null mice (Carta, L. *et al. J. Biol. Chem.* 27, 5630-6 (2009)); this may relate to a hypomorphic *Map2k6* allele on the BL6 background previously preventing its robust activation. Interestingly, Ang-II mediated Egfr transactivation has been shown to drive dual Erk1/2 and p38 activation in VSMCs, in the absence of Jnk1/2 upregulation, a direct recapitulation of our findings. In contrast, prior work has suggested that Map3k4 modulates p38 and Jnk1/2 activation without affecting Erk1/2 (Yan W., *et al. J. Mol. Biol.* 275, 25-33 (1998)).

[0236] Finally, this work highlights a role for Mmp17 in the modulation of aortic disease in MFS mice. It may achieve this effect through modulation of Egfr, as the data herein evidences.

[0237] *Incorporation by Reference*

[0238] The contents of all references, patents, pending patent applications and published patents, cited throughout this application are hereby expressly incorporated by reference.

[0239] *Equivalents*

[0240] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed is:

1. A method for treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising:

administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling; thereby treating the subject.

2. The method of claim 1, wherein the agent that modulates the activity of MAP kinase pathway signaling is an inhibitor of the MAP kinase pathway.

3. The method of claim 1, wherein the agent that modulates the activity of MAP kinase pathway signaling is an inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof.

4. The method of claim 3, wherein the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is an antisense agent or a double-stranded nucleic acid, optionally a siRNA or shRNA specific for MMP17, MAP2K6 or MAP3K4.

5. The method of claim 4, wherein the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is specific for a nucleic acid molecule set forth as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8.

6. The method of claim 1, wherein the agent that modulates the activity of MAP kinase pathway signaling is a small molecule.

7. The method of claim 1, wherein the agent that modulates the activity of MAP kinase pathway signaling comprise Batimastat, GI 254023X, GM 6001, TMI 1, WAY 170523, WX-554 or combinations thereof.

8. The method of claim 1, wherein the agent that modulates the activity of MAP kinase pathway signaling is an antibody.

9. The method of claim 1, wherein the Marfan syndrome-associated disease or disorder is a clinical condition associated with Marfan syndrome.

10. The method of claim 9, wherein the disease or disorder is an aneurysm, an aortic aneurysm, or emphysema.

11. The method of claim 9, wherein the disease or disorder is an aneurysm.

12. A method for treating a subject having Marfan syndrome or a Marfan-associated condition comprising:

identifying a variant allele of MMP17, MAP2K6 or MAP3K4 that produces elevated expression and/or activity of a gene product of the MMP17, MAP2K6 or MAP3K4 gene; and

replacing the variant allele of MMP17, MAP2K6 or MAP3K4 with an allele of MMP17, MAP2K6 or MAP3K4 that does not produce elevated expression and/or activity of a gene product of the MMP17, MAP2K6 or MAP3K4 gene; thereby treating the subject.

13. The method of claim 12, wherein the step of replacing is performed via a CRISPR-Cas9 gene replacement process.

14. The method of claim 12, wherein the step of replacing is performed *ex vivo*.

15. The method of claim 14, wherein re-introduction to the subject of a cell manipulated *ex vivo* to perform gene replacement treats the subject.

16. The method of claim 12, wherein the variant allele of MMP17, MAP2K6 or MAP3K4 that produces elevated expression and/or activity of a gene product of the MMP17, MAP2K6 or MAP3K4 gene comprise nucleic acid sequences set forth as SEQ ID NOS: 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 or 23.

17. A pharmaceutical composition for the treatment of a disease or disorder characterized by aberrant MAP kinase pathway expression or activity, wherein the pharmaceutical composition comprises an agent that modulates the activity of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof.

18. The pharmaceutical composition of claim 17, wherein the disease or disorder is Marfan syndrome or a clinical condition associated with Marfan syndrome.

19. The pharmaceutical composition of claim 17, wherein the disease or disorder is an aneurysm, an aortic aneurysm, or emphysema.

20. The pharmaceutical composition of claim 17, wherein the disease or disorder is aneurysm.

21. The pharmaceutical composition of claim 17, wherein the agent is an inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, or is a compound comprising Batimastat, GI 254023X, GM 6001, TMI 1, WAY 170523, WX-554 or combinations thereof.

22. A kit for identifying a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising:

primers specific for amplification of MMP17, MAP2K6 and/or MAP3K4;

a probe oligonucleotide, optionally fluorescently labeled, that specifically detects the presence of a MMP17, MAP2K6 and/or MAP3K4 amplicon, wherein detection of elevated expression of MMP17, MAP2K6 and/or MAP3K4 identifies the subject as having or at risk of developing Marfan Syndrome or a Marfan-associated condition; and
instructions for use.

23. The kit of claim 22, wherein the disease or disorder is Marfan syndrome or a clinical condition associated with Marfan syndrome.

24. The kit of claim 23, wherein the disease or disorder is an aneurism, an aortic aneurism, or emphysema.

25. The kit of claim 24, wherein the disease or disorder is an aneurism.

26. The kit of claim 22, further comprising a pharmaceutical composition of claim 17.

27. A method for identifying and treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising:

isolating a sample from the subject;

assessing the expression level of MMP17, MAP2K6 and/or MAP3K4 in the sample,

assessing the level of activity of a gene product of MMP17, MAP2K6 and/or MAP3K4 in the sample, and/or genotyping MMP17, MAP2K6 and/or MAP3K4 in the sample;

identifying elevated expression of MMP17, MAP2K6 and/or MAP3K4 in the sample,

identifying elevated activity of a gene product of MMP17, MAP2K6 and/or MAP3K4 in the sample, and/or identifying a genotype of MMP17, MAP2K6 and/or MAP3K4 in the sample that causes elevated expression and/or activity of MMP17, MAP2K6 and/or MAP3K4, or of a gene product thereof; and

administering a therapy for Marfan Syndrome or a Marfan-associated condition to the subject, thereby treating the subject.

28. The method of claim 27, wherein the therapy for Marfan Syndrome or a Marfan-associated condition comprises administration of a TGFβ neutralizing antibody (Nab), losartan and/or an inhibitor of MMP17, MAP2K6 and/or MAP3K4, or of a gene product thereof.

29. A method for treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising:

administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling and/or expression, function or activity of epidermal growth factor receptor (EGFR); thereby treating the subject.

30. The method of claim 29, wherein the agent that modulates the activity of MAP kinase pathway signaling is an inhibitor of the MAP kinase pathway.

31. The method of claim 29, wherein the agent that modulates the activity of MAP kinase pathway signaling is an inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof.

32. The method of claim 31, wherein the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is an antisense agent or a double-stranded nucleic acid, optionally a siRNA or shRNA specific for MMP17, MAP2K6 or MAP3K4.

33. The method of claim 31, wherein the inhibitor of MMP17, MAP2K6 or MAP3K4, or of a gene product thereof, is specific for a nucleic acid molecule set forth as SEQ ID NO: 2, SEQ ID NO: 4, SEQ ID NO: 6 or SEQ ID NO: 8.

34. The method of claim 29, wherein the agent that modulates the activity of MAP kinase pathway signaling comprise Batimastat, GI 254023X, GM 6001, TMI 1, WAY 170523, WX-554 or combinations thereof.

35. The method of claim 29, wherein the agent that modulates the activity of MAP kinase pathway signaling is an antibody.

36. The method of claim 35, wherein the agent that modulates expression, function or activity of epidermal growth factor receptor (EGFR) is an EGFR inhibitor comprising: an antibody, interfering RNAs microRNAs, shRNA, small molecule or combination thereof.

37. The method of claim 36 wherein EGFR inhibitor comprises: gefitinib, erlotinib, lapatinib, Brigatinib (AP26113), Afatinib (BIBW2992), Neratinib (HKI-272), AZD3759, AZ5104, CL-387785 (EKI-785), Canertinib (CI-1033), Poziotinib (HM781-36B), Osimertinib (AZD-9291), PD168393, CNX-2006, Rociletinib (CO-1686, AVL-301), WZ4002, Pelitinib (EKB-569), AC480 (BMS-599626), TAK-285, CUDC-101, AEE788 (NVP-AEE788), CP-724714, Dacomitinib (PF299804, PF299), AG-490 (Tyrphostin B42), AST-1306, OSI-420, WHI-P154, WZ3146, WZ8040, AZD8931 (Sapitinib), PKI-166, PD158780, AG 1478, PD153035 HCl, Icotinib, Varlitinib, AZD-9291, AEE788 (NVP-AEE

788), AG-1478 (NSC 693255), AG-490, Anlotinib, ARRY-380, BIBX 1382, BMS-690514, cetuximab, panitumumab, zalutumumab, nimotuzumab, matuzumab or mAb 806.

38. The method of claim 37, wherein an anti-EGFR antibody comprises cetuximab, matuzumab, panitumumab, nimotuzumab or mAb 806.

39. The method of claim 37, wherein a small molecule EGFR inhibitor comprises gefitinib, lapatinib, canertinib, erlotinib HCL, pelitinib, PKI-166, PD158780, or AG 1478.

40. The method of claim 29, further comprising an inhibitor of transforming growth factor β (TGFβ).

41. A pharmaceutical composition comprising a therapeutically effective amount of at least one agent that modulates expression, function or activity of MMP17, MAP2K6, MAP3K4, epidermal growth factor receptor (EGFR), or combinations thereof.

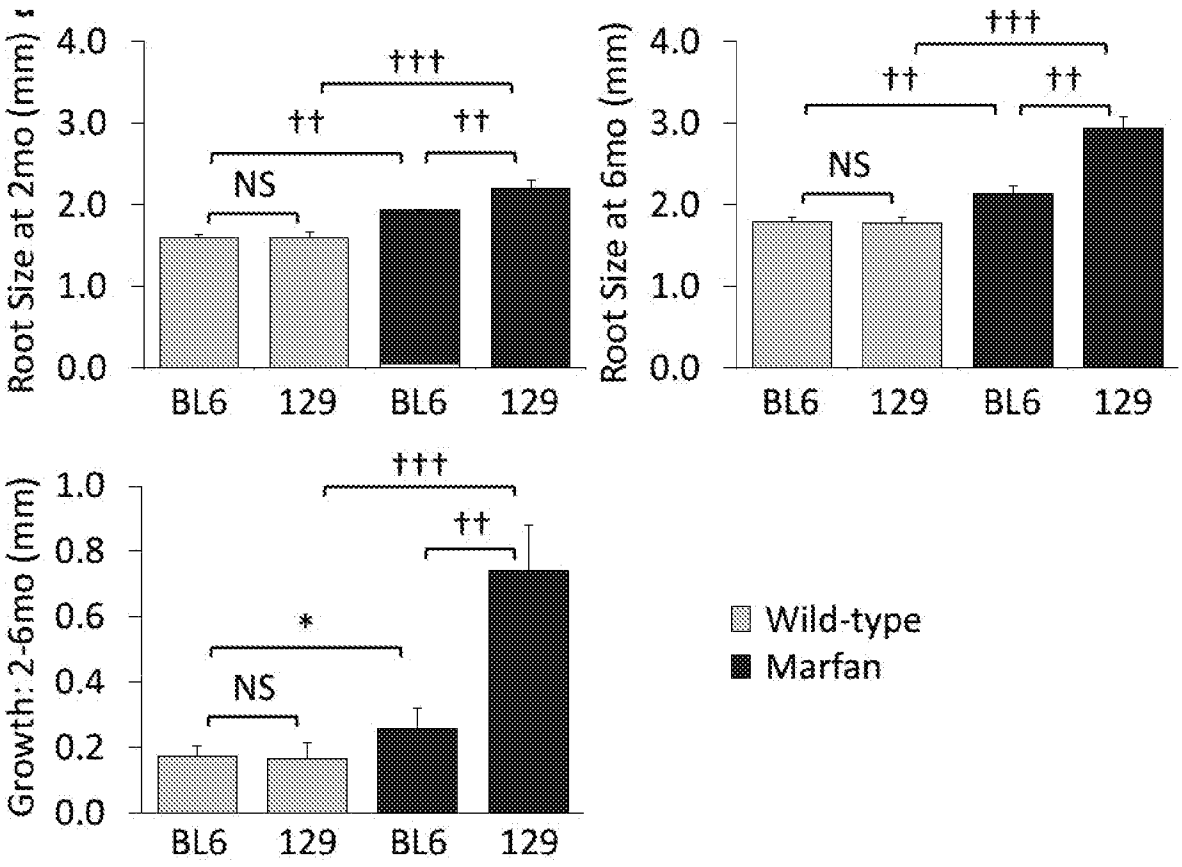
Root Size at 2mo (mm)
4.0
3.0
2.0
1.0
0.0
NS
††
†††
††
BL6
129
BL6
129
Root Size at 6mo (mm)
4.0
3.0
2.0
1.0
0.0
NS
††
†††
††
BL6
129
BL6
129
Growth: 2-6mo (mm)
1.0
0.8
0.6
0.4
0.2
0.0
NS
*
††
†††
BL6
129
BL6
129
Wild-type
Marfan

FIGURE 1A

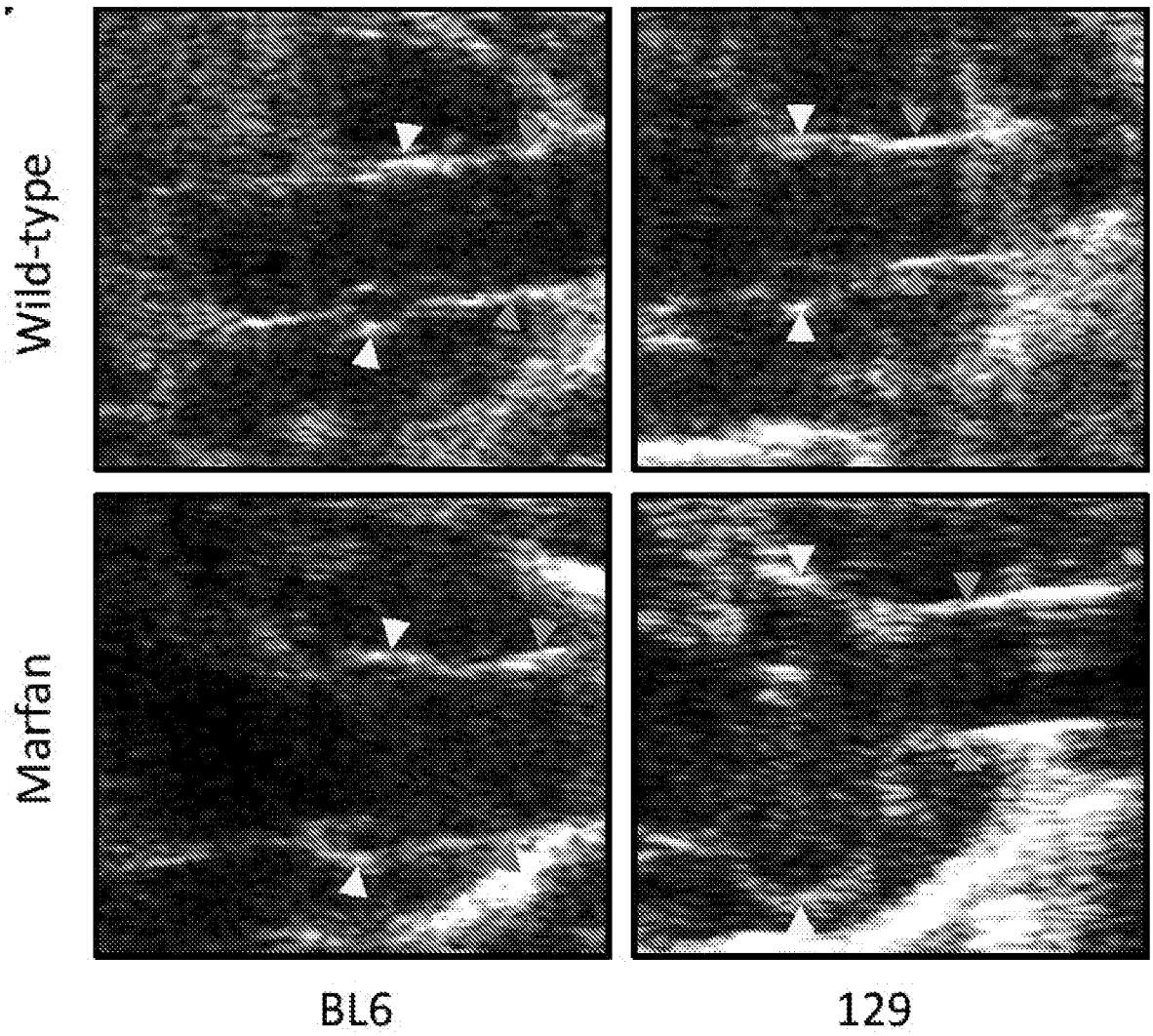

FIGURE 1B

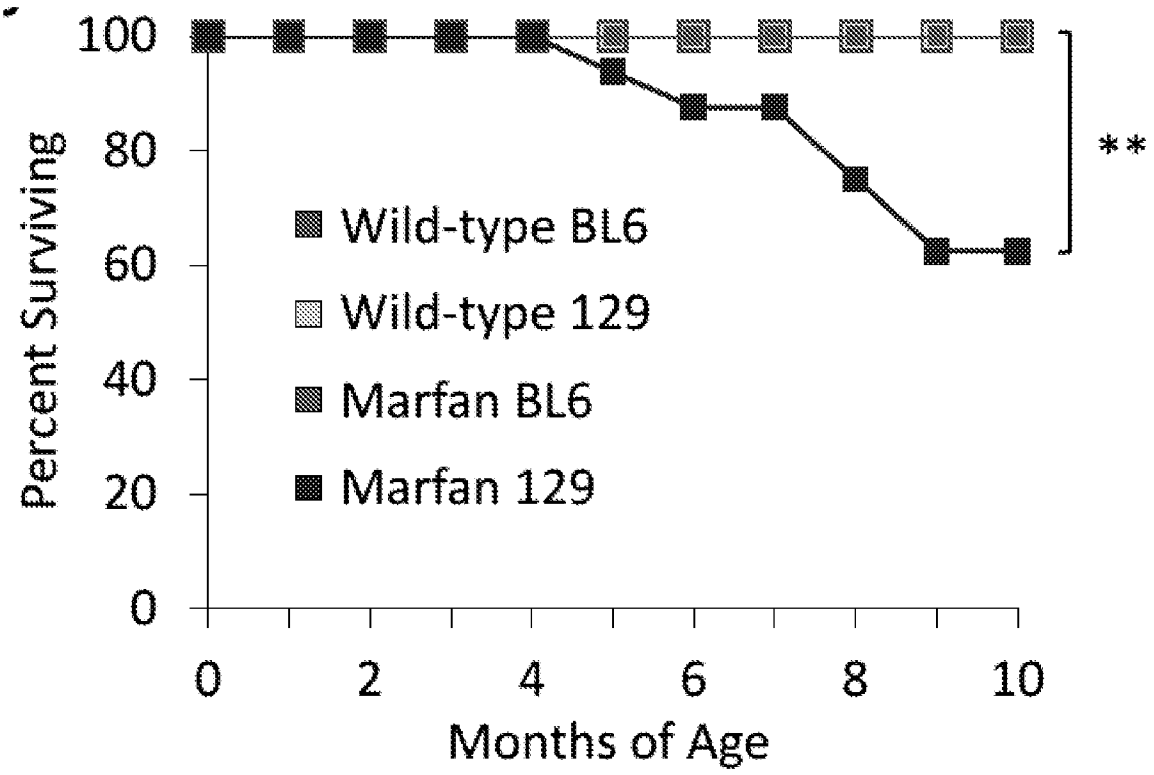
100
80
60
40
20
0
Percent Surviving
Wild-type BL6
Wild-type 129
Marfan BL6
Marfan 129
**
0
2
4
6
8
10
Months of Age

FIGURE 1C

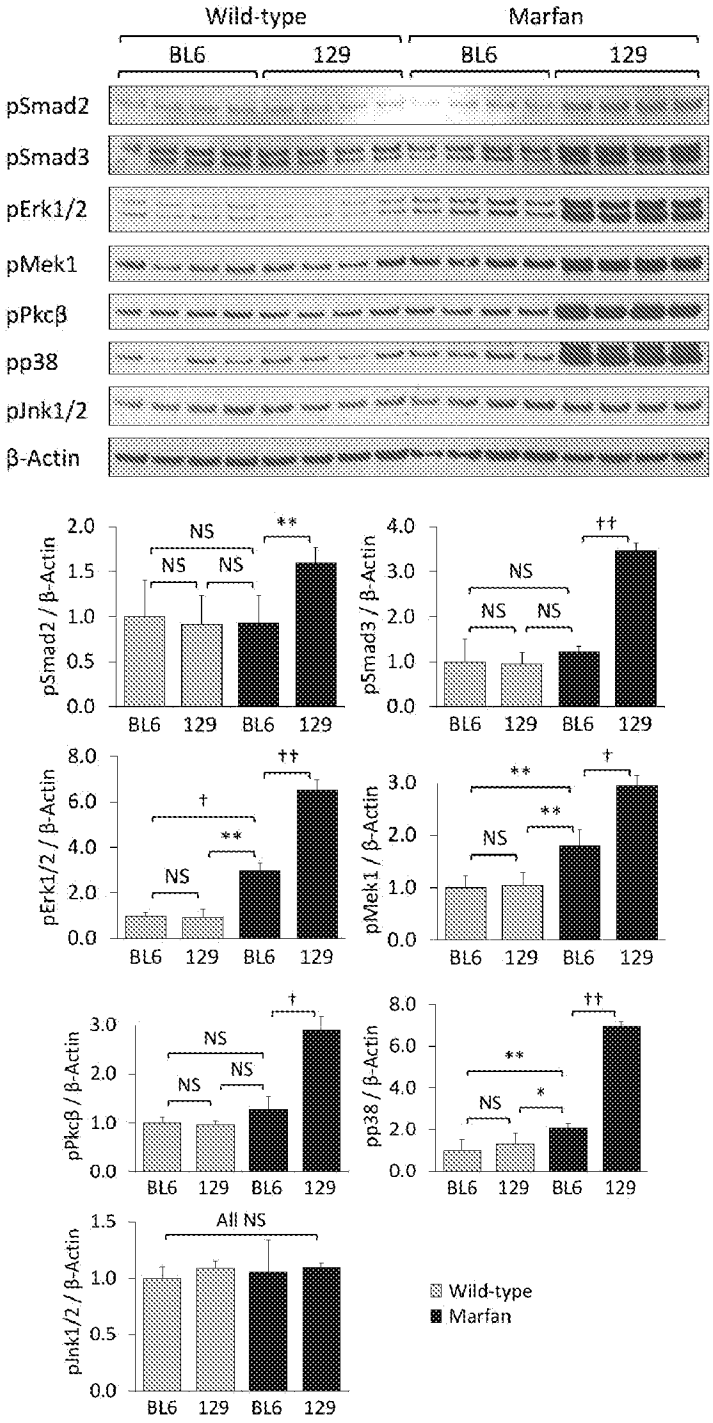
Wild-type
Marfan
BL6
129
BL6
129
pSmad2
pSmad3
pErk1/2
pMek1
pPkcβ
pp38
pJnk1/2
β-Actin
pSmad2 / β-Actin
pSmad3 / β-Actin
pErk1/2 / β-Actin
pMek1 / β-Actin
pPkcβ / β-Actin
pp38 / β-Actin
pJnk1/2 / β-Actin
NS
**
††
†
*
All NS
Wild-type
Marfan

FIGURE 2A

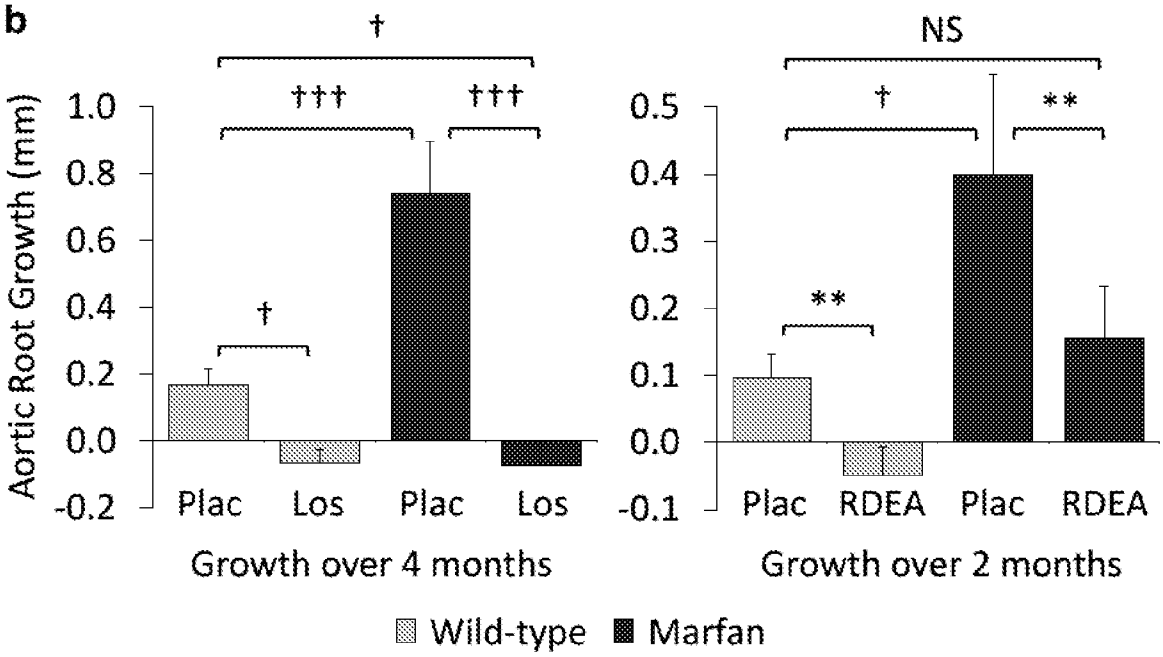

FIGURE 2B

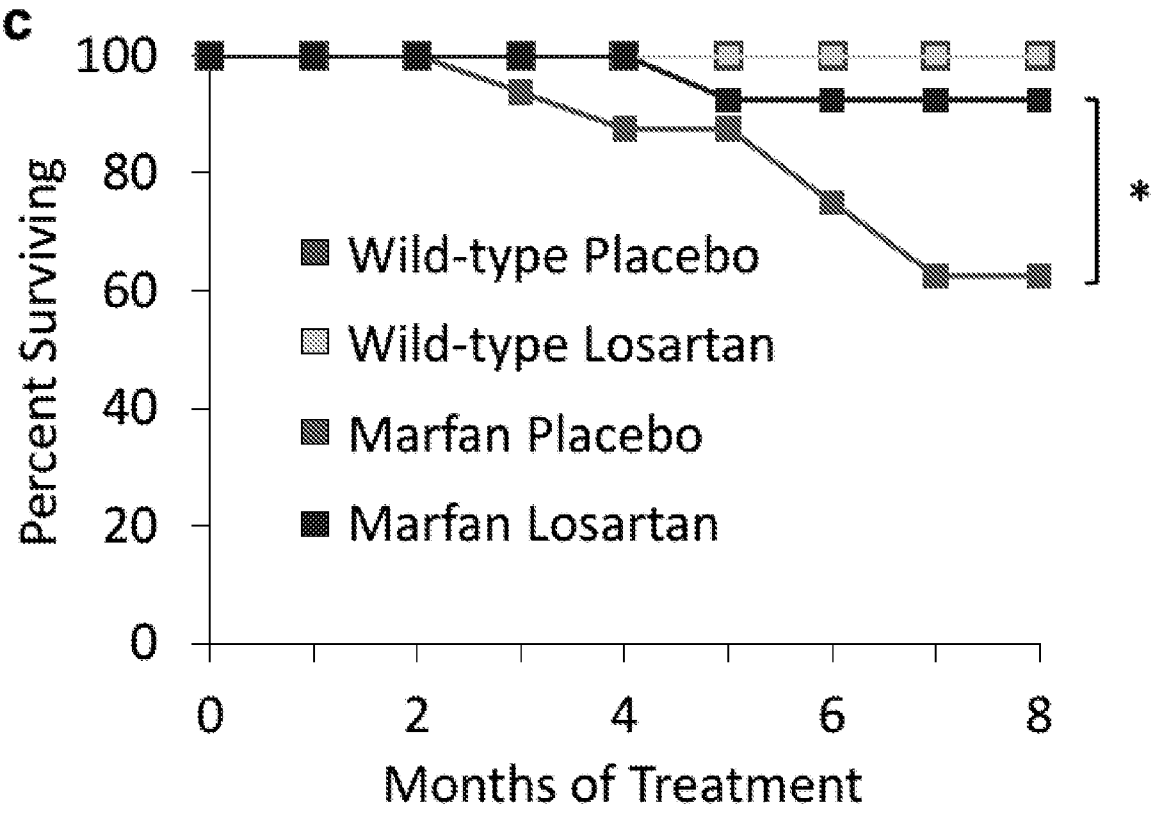

FIGURE 2C

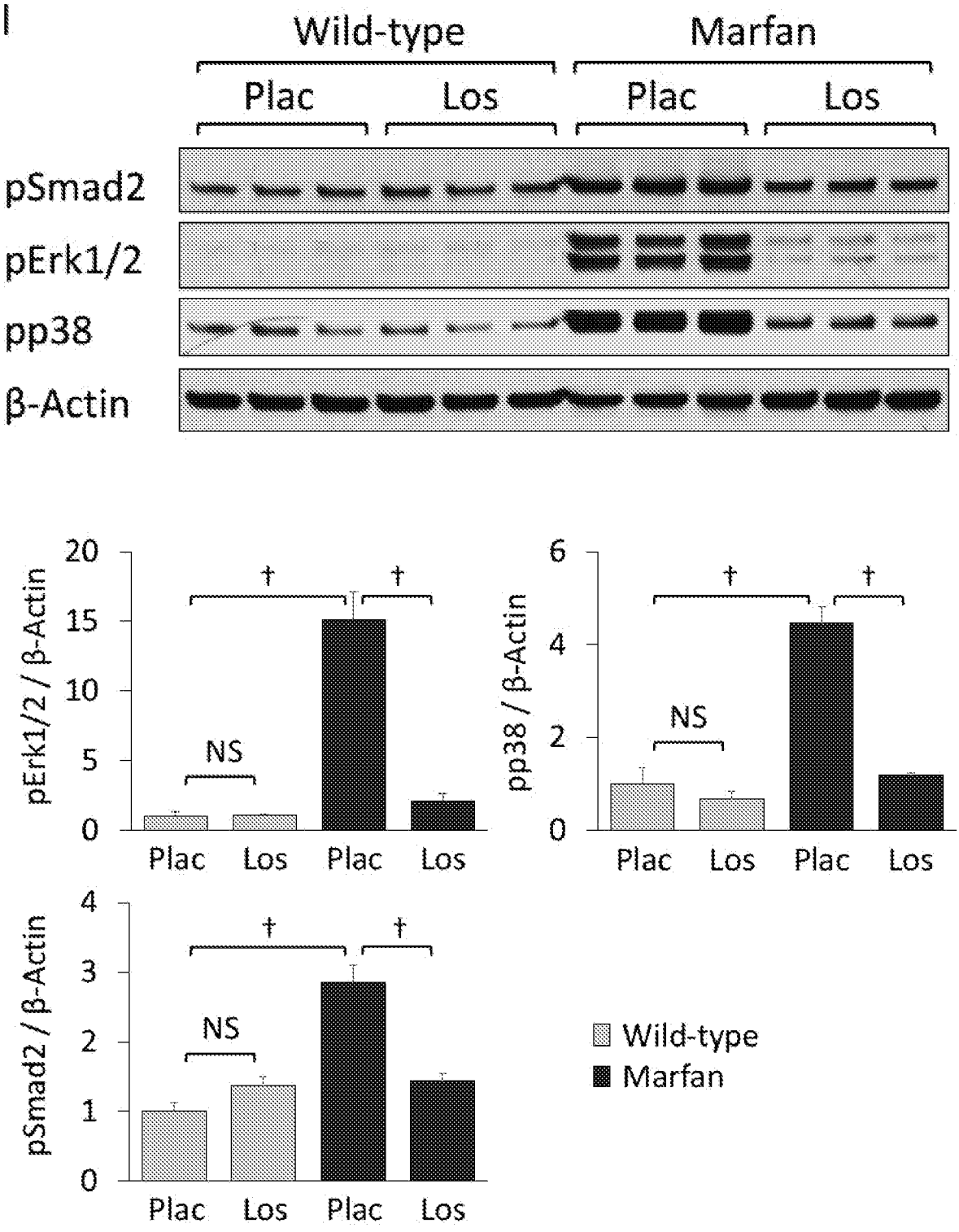
I
Wild-type
Marfan
Plac
Los
Plac
Los
pSmad2
pErk1/2
pp38
β-Actin
pErk1/2 / β-Actin
20
15
10
5
0
†
†
NS
Plac
Los
Plac
Los
pp38 / β-Actin
6
4
2
0
†
†
NS
Plac
Los
Plac
Los
pSmad2 / β-Actin
4
3
2
1
0
†
†
NS
Plac
Los
Plac
Los
Wild-type
Marfan

FIGURE 2D

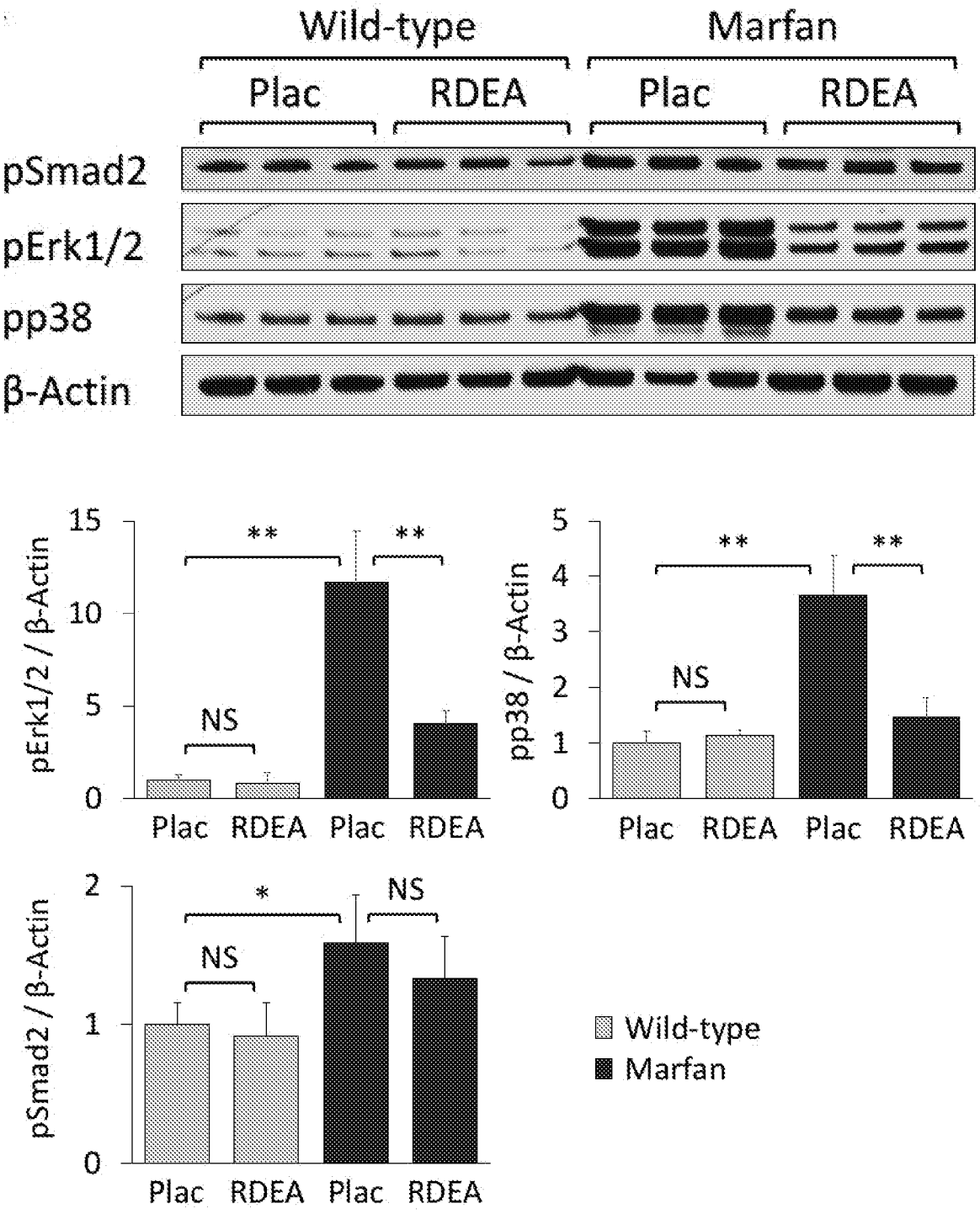
Wild-type
Marfan
Plac
RDEA
Plac
RDEA
pSmad2
pErk1/2
pp38
β-Actin
pErk1/2 / β-Actin
15
10
5
0
**
**
NS
Plac
RDEA
Plac
RDEA
pp38 / β-Actin
5
4
3
2
1
0
**
**
NS
Plac
RDEA
Plac
RDEA
pSmad2 / β-Actin
2
1
0
*
NS
NS
Plac
RDEA
Plac
RDEA
Wild-type
Marfan

FIGURE 2E

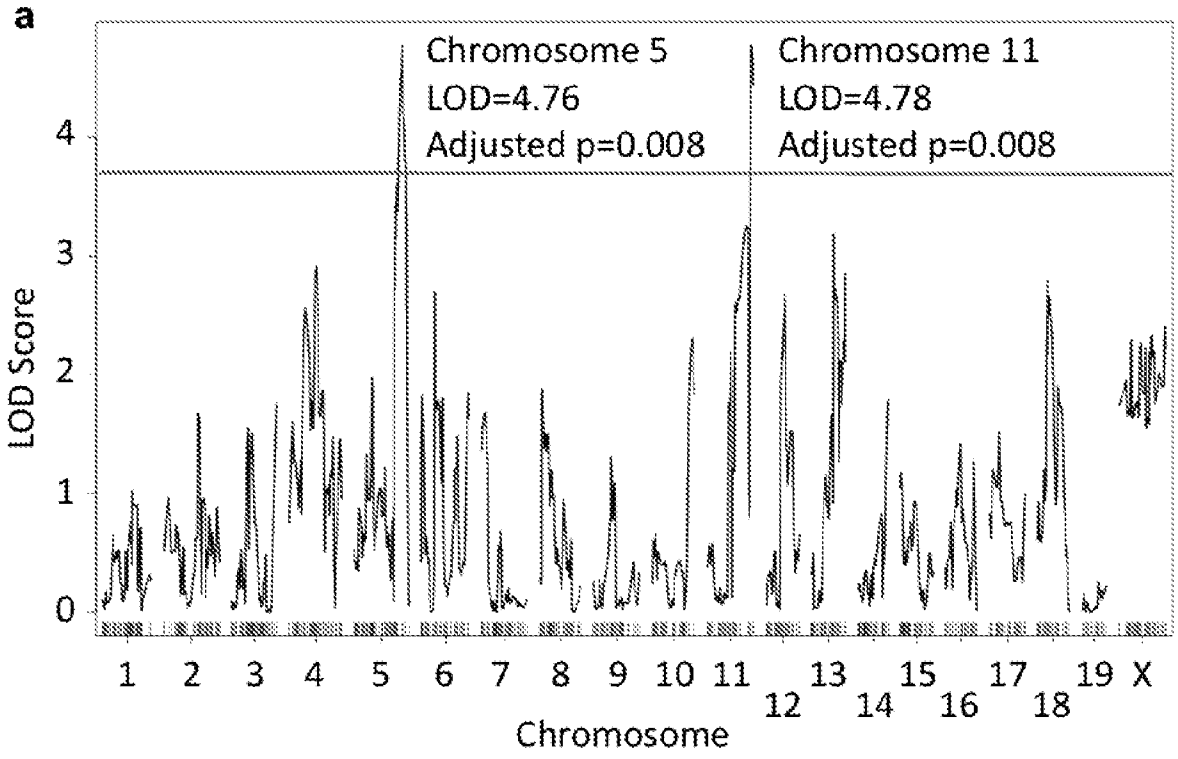

FIGURE 3A

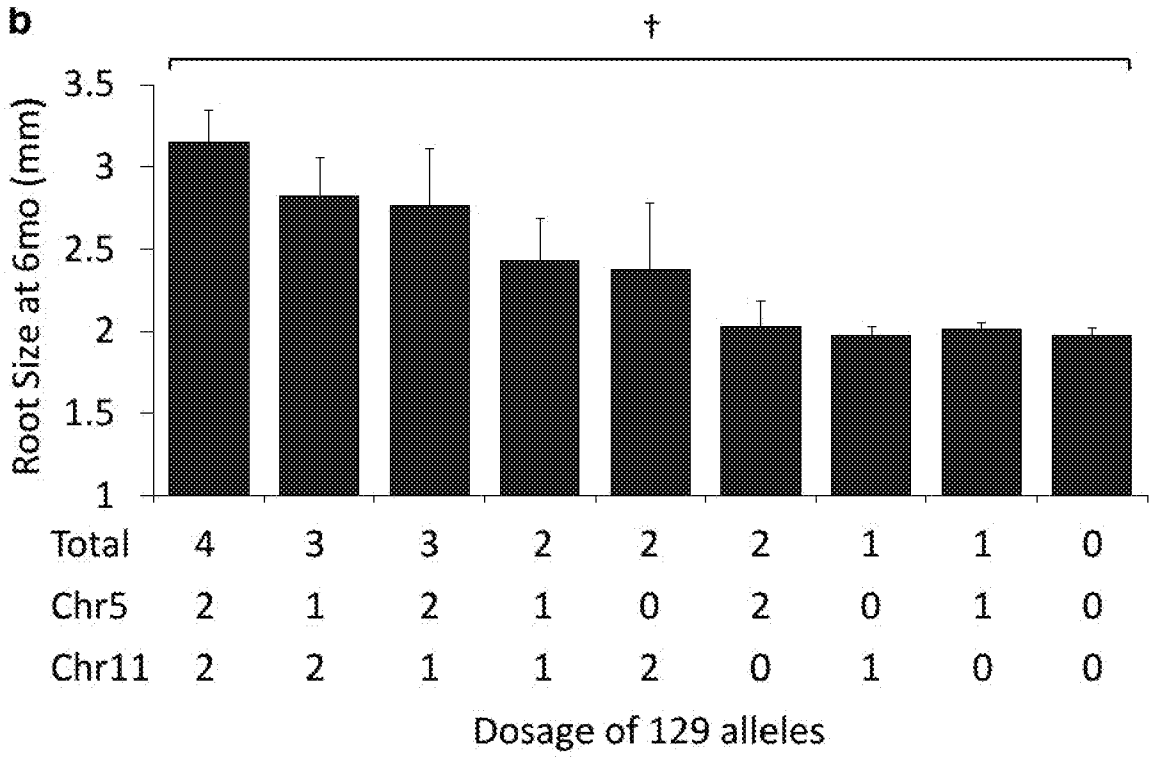

FIGURE 3B

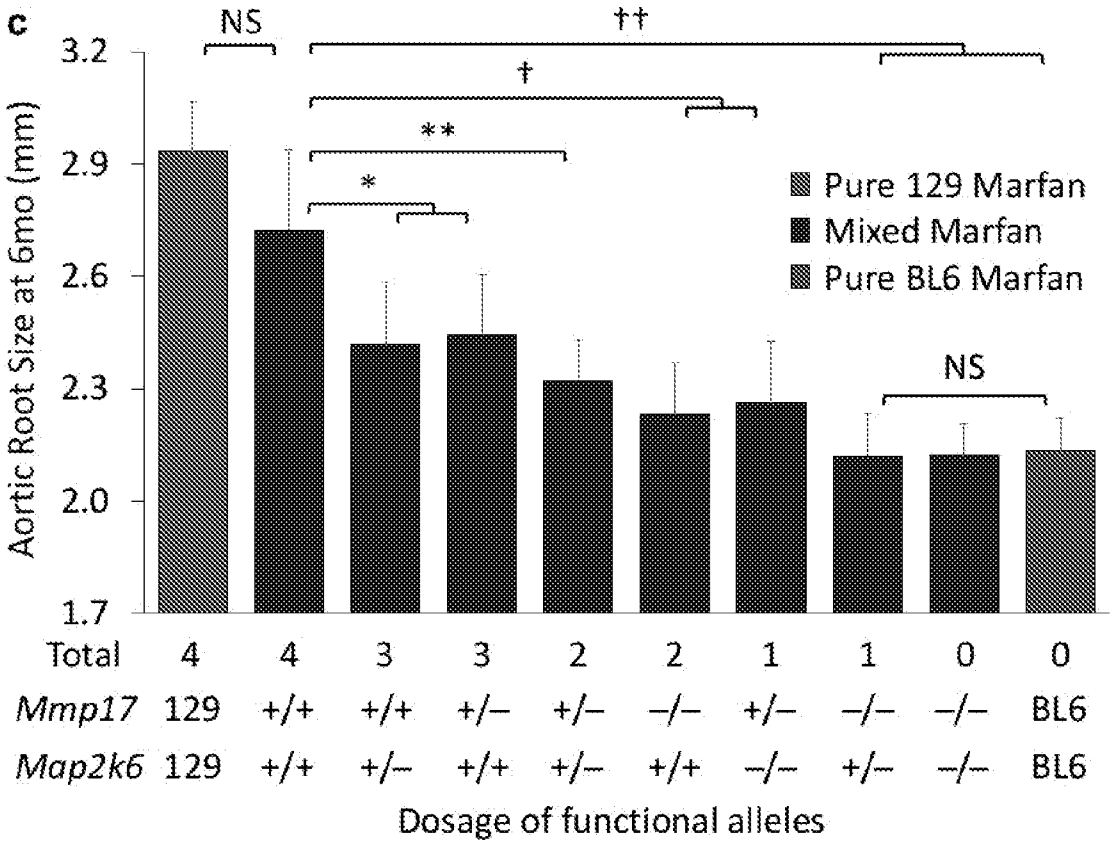
c
NS
††
†
**
*
3.2
2.9
2.6
2.3
2.0
1.7
Aortic Root Size at 6mo (mm)
Pure 129 Marfan
Mixed Marfan
Pure BL6 Marfan
NS
Total 4 4 3 3 2 2 1 1 0 0
Mmp17 129 +/+ +/+ +/– +/– –/– +/– –/– –/– BL6
Map2k6 129 +/+ +/– +/+ +/– +/+ –/– +/– –/– BL6
Dosage of functional alleles

FIGURE 3C

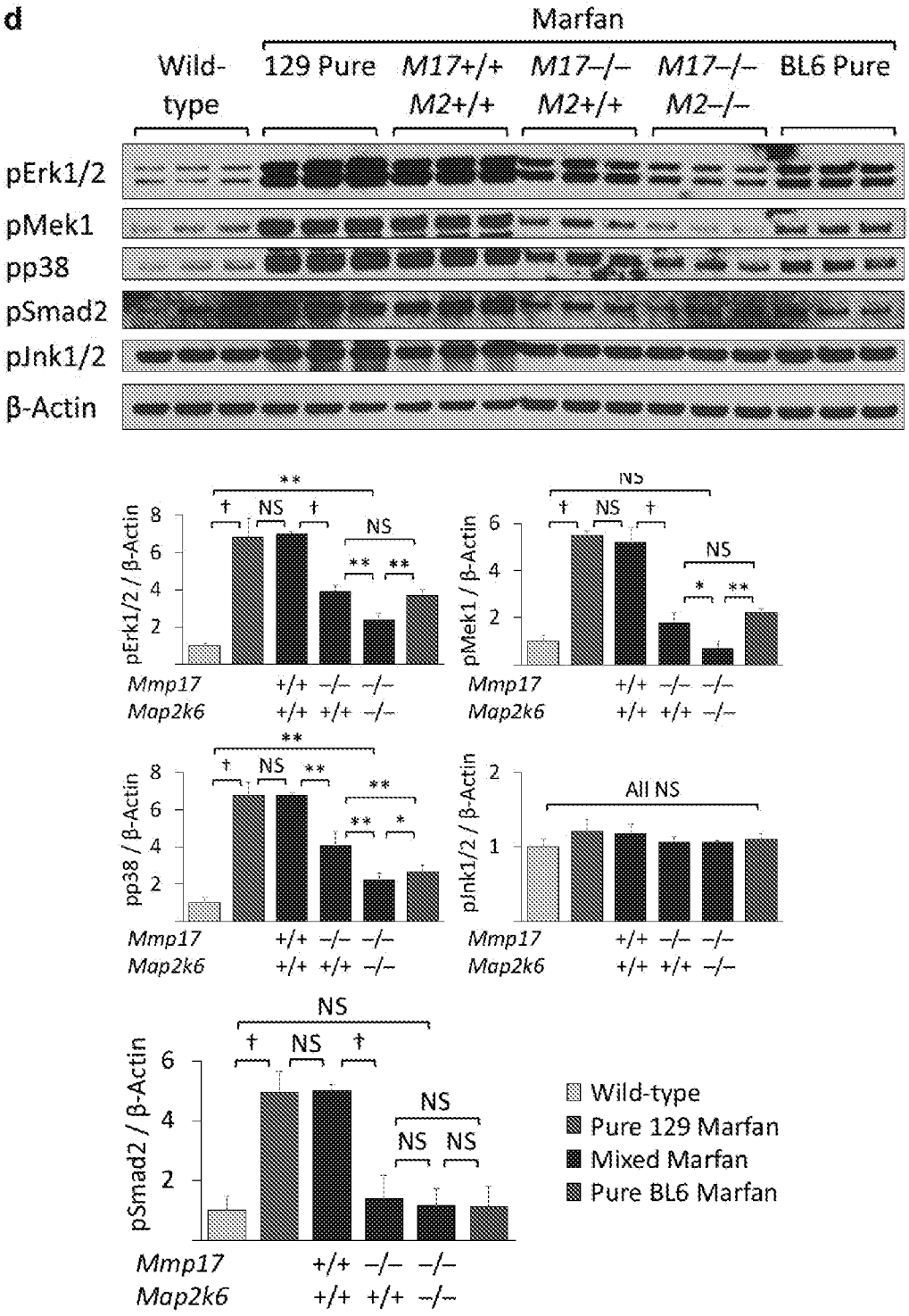
d
Marfan
Wild-type
129 Pure
M17+/+ M2+/+
M17–/– M2+/+
M17–/– M2–/–
BL6 Pure
pErk1/2
pMek1
pp38
pSmad2
pJnk1/2
β-Actin
pErk1/2 / β-Actin
pMek1 / β-Actin
pp38 / β-Actin
pJnk1/2 / β-Actin
pSmad2 / β-Actin
Mmp17
Map2k6
+/+ –/– –/–
+/+ +/+ –/–
NS
All NS
Wild-type
Pure 129 Marfan
Mixed Marfan
Pure BL6 Marfan

FIGURE 3D

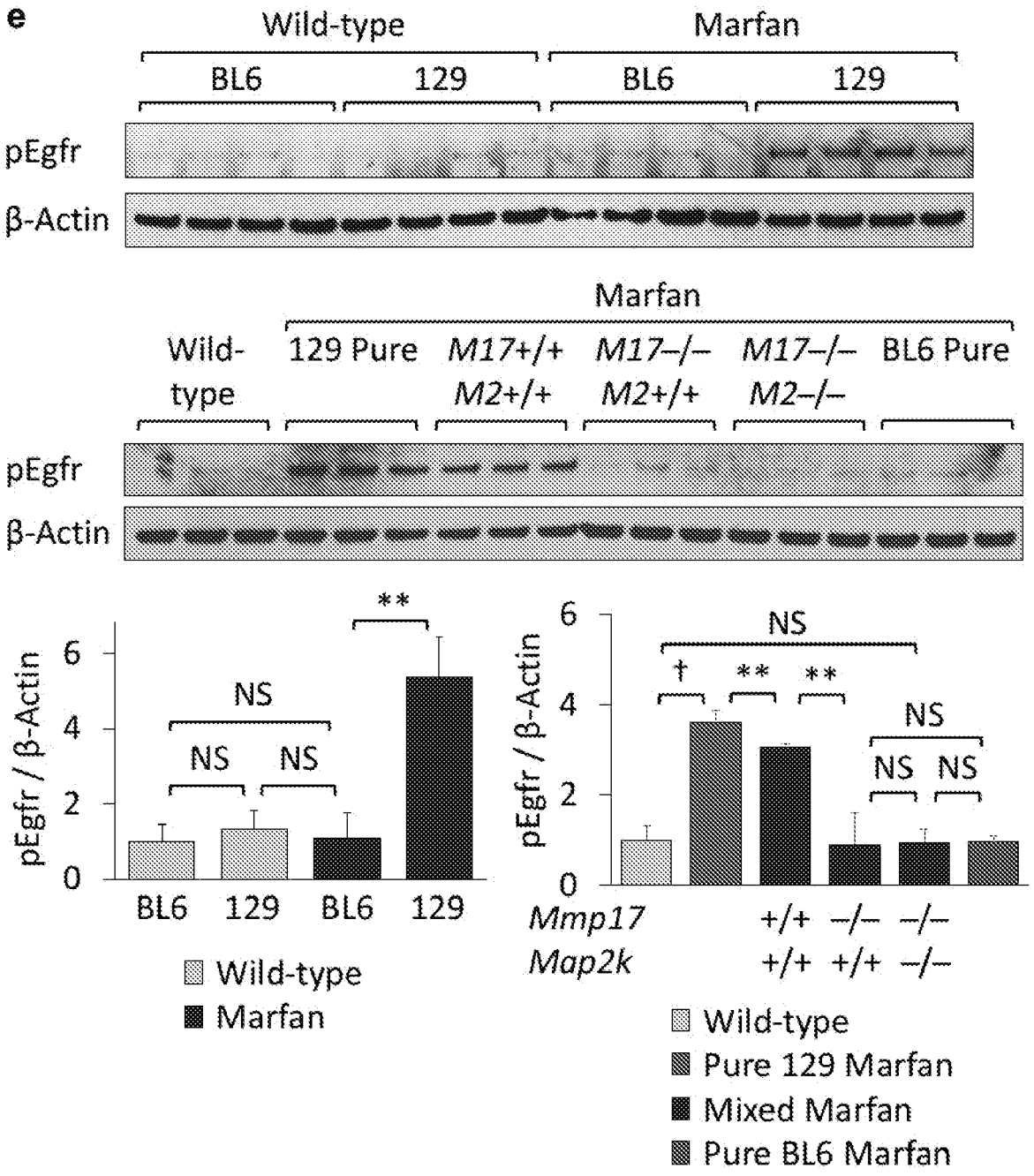
e
Wild-type
Marfan
BL6
129
BL6
129
pEgfr
β-Actin
Marfan
Wild-type
129 Pure
M17+/+ M2+/+
M17–/– M2+/+
M17–/– M2–/–
BL6 Pure
pEgfr
β-Actin
pEgfr / β-Actin
**
NS
NS
NS
BL6
129
BL6
129
Wild-type
Marfan
pEgfr / β-Actin
NS
†
**
**
NS
NS
NS
Mmp17
Map2k
+/+ –/– –/–
+/+ +/+ –/–
Wild-type
Pure 129 Marfan
Mixed Marfan
Pure BL6 Marfan

FIGURE 3E

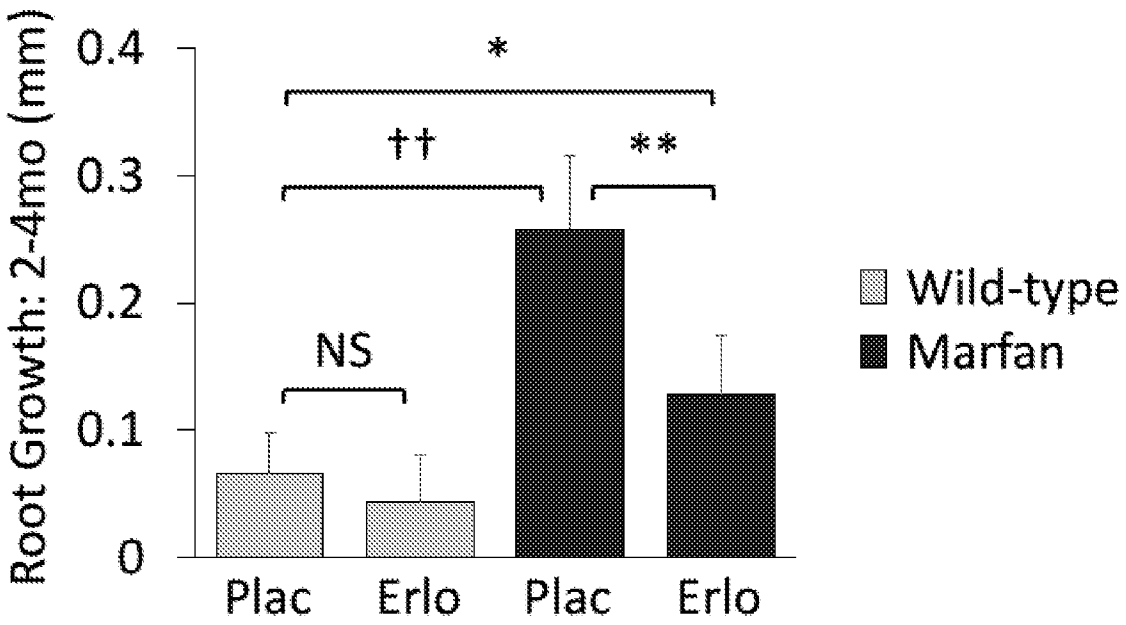
Root Growth: 2-4mo (mm)
0.4
0.3
0.2
0.1
0
*
††
**
NS
Plac
Erlo
Plac
Erlo
Wild-type
Marfan

FIGURE 3F

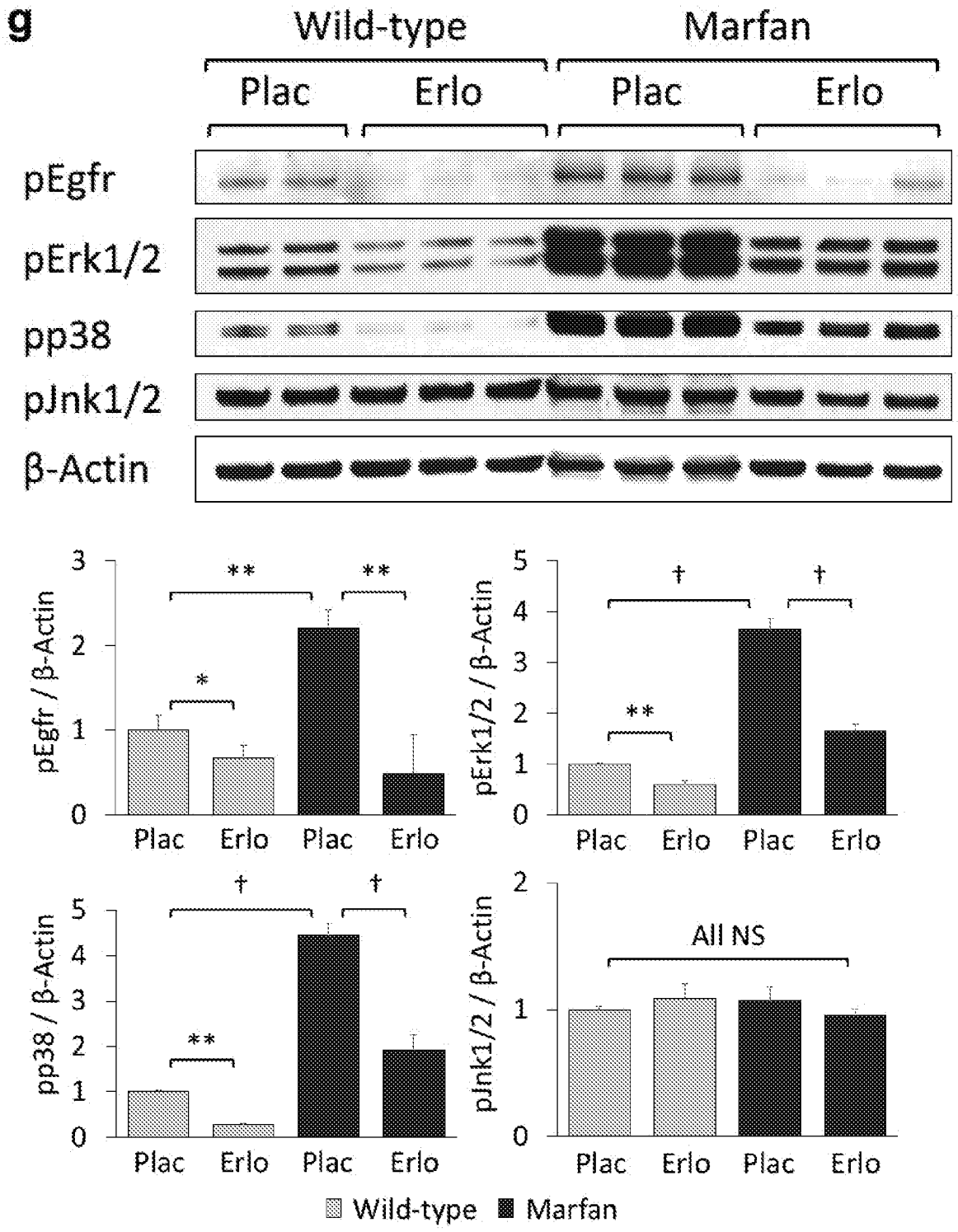
g
Wild-type
Marfan
Plac
Erlo
Plac
Erlo
pEgfr
pErk1/2
pp38
pJnk1/2
β-Actin
pEgfr / β-Actin
pErk1/2 / β-Actin
pp38 / β-Actin
pJnk1/2 / β-Actin
All NS
Wild-type
Marfan

FIGURE 3G

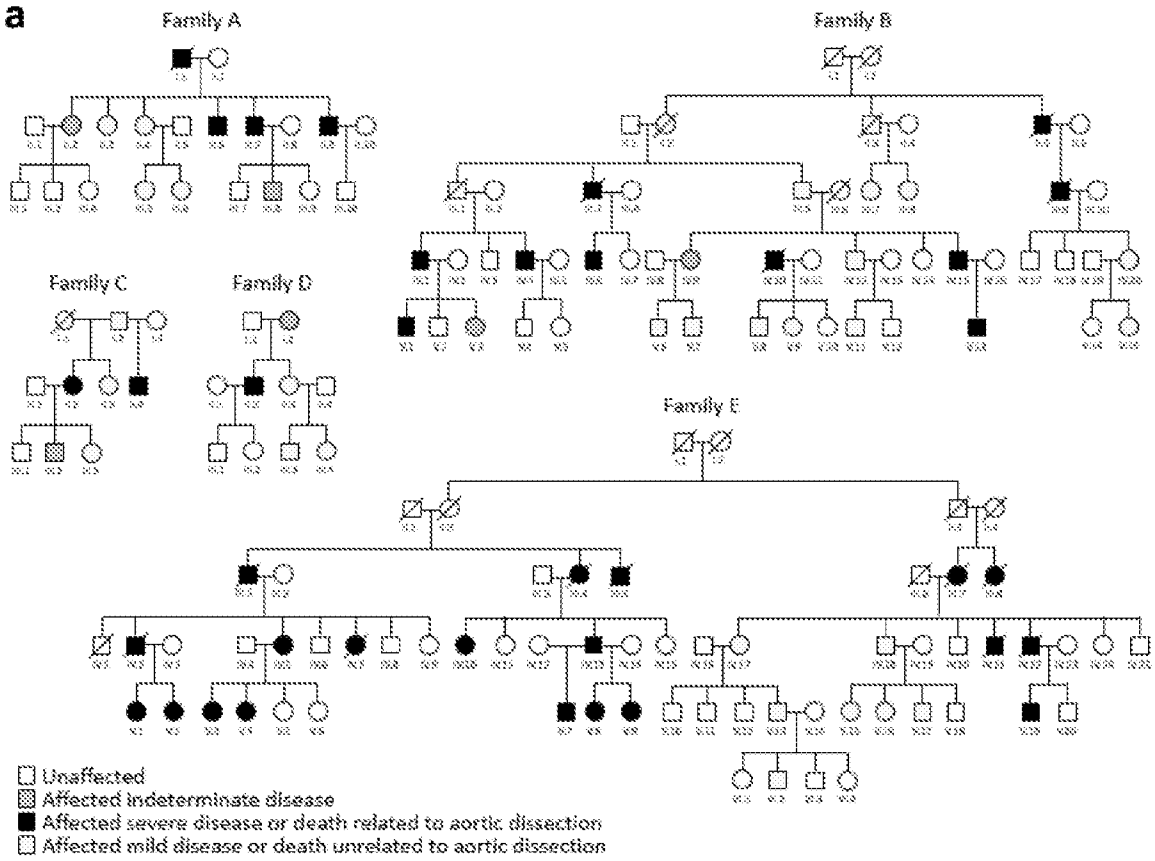
a
Family A
Family B
Family C
Family D
Family E
Unaffected
Affected indeterminate disease
Affected severe disease or death related to aortic dissection
Affected mild disease or death unrelated to aortic dissection

FIGURE 4A

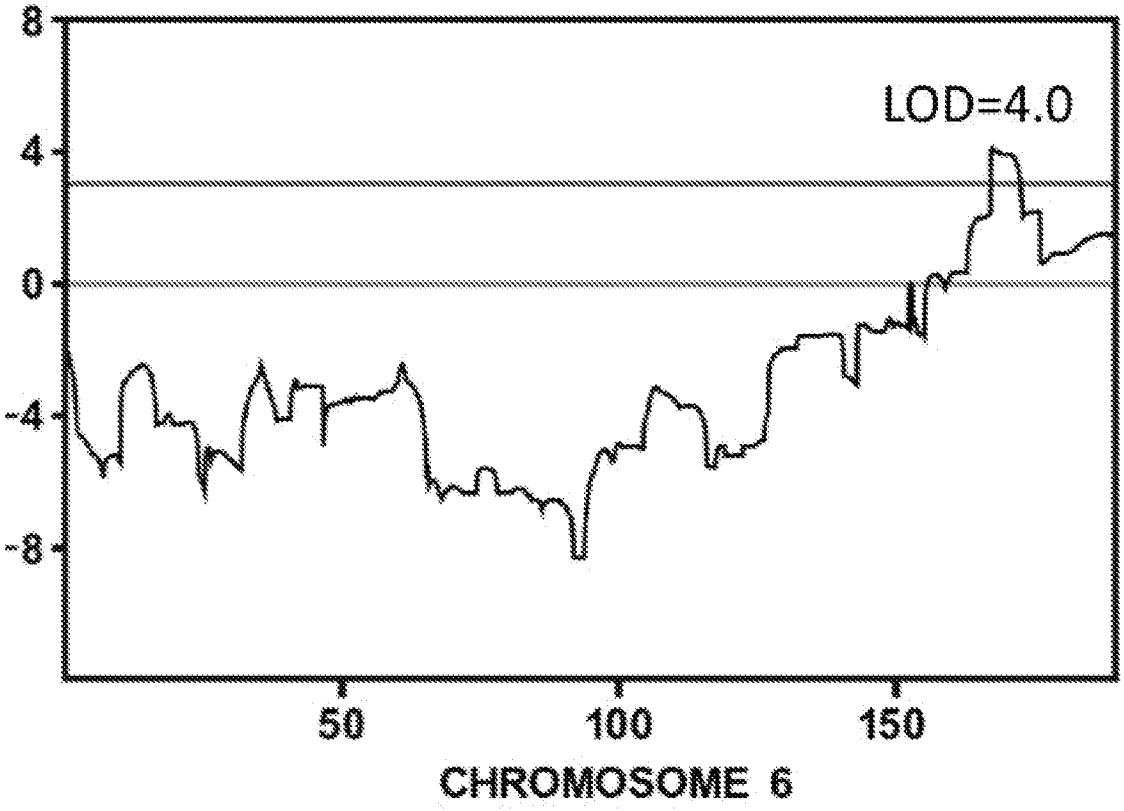

FIGURE 4B

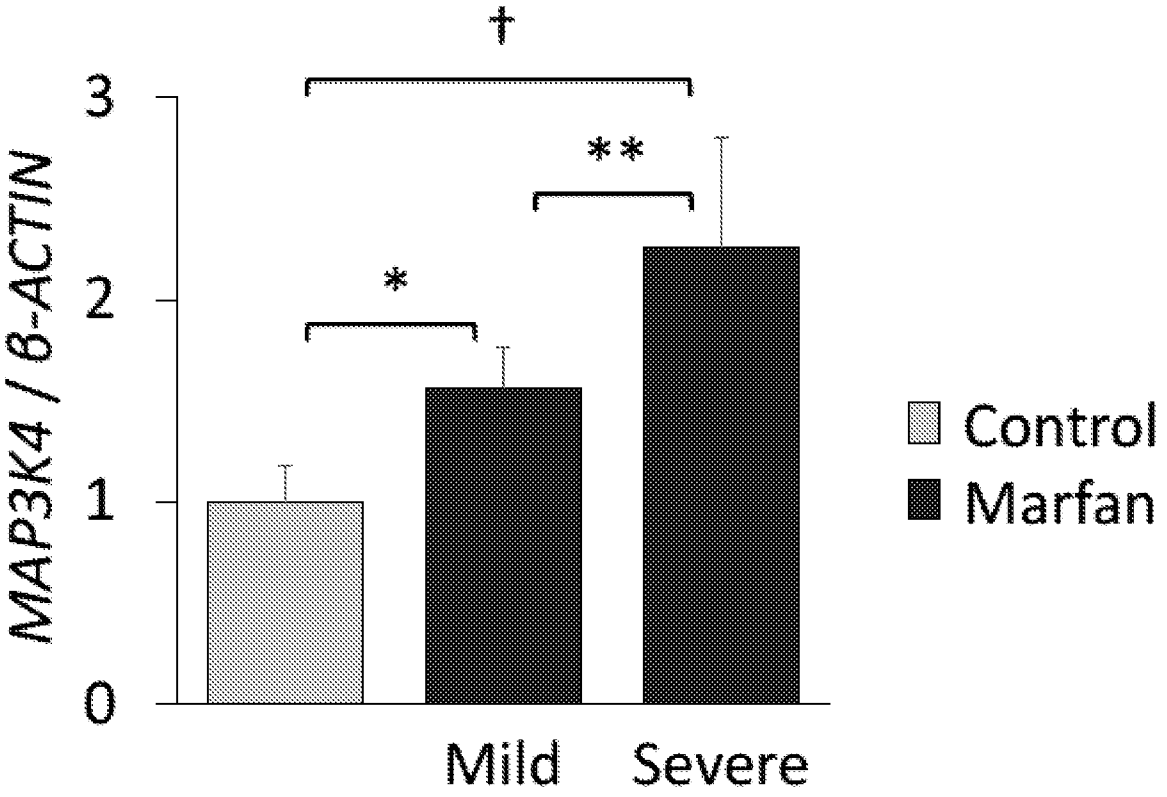

FIGURE 4C

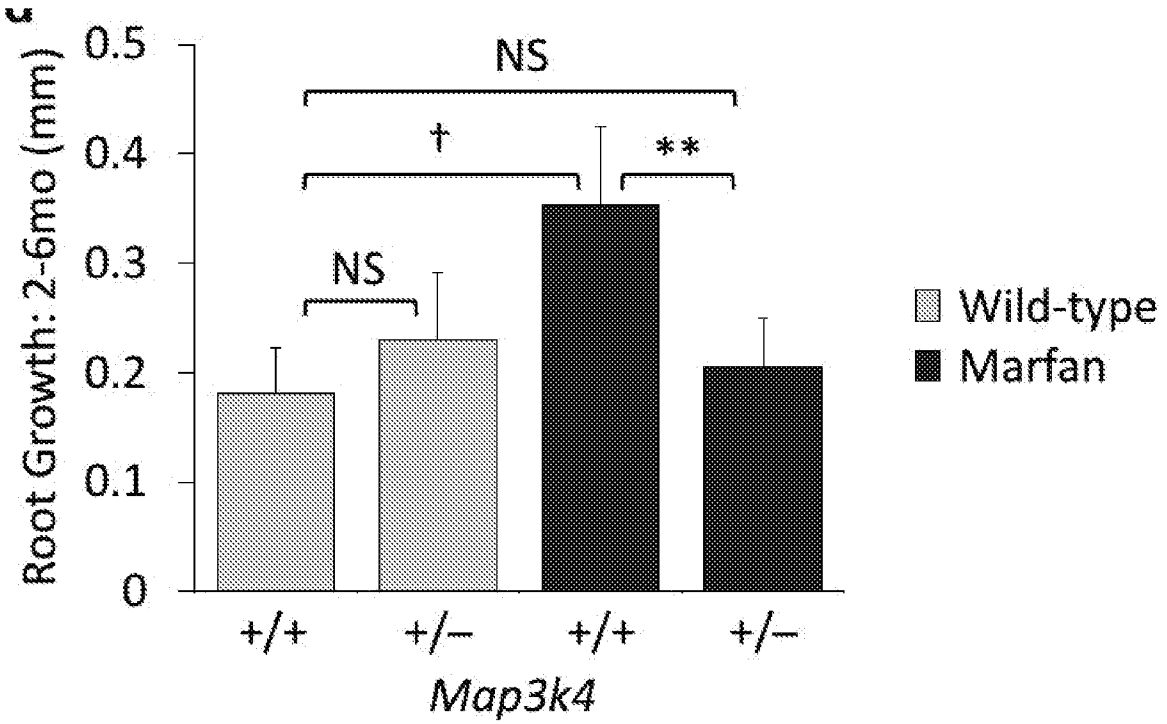

FIGURE 4D

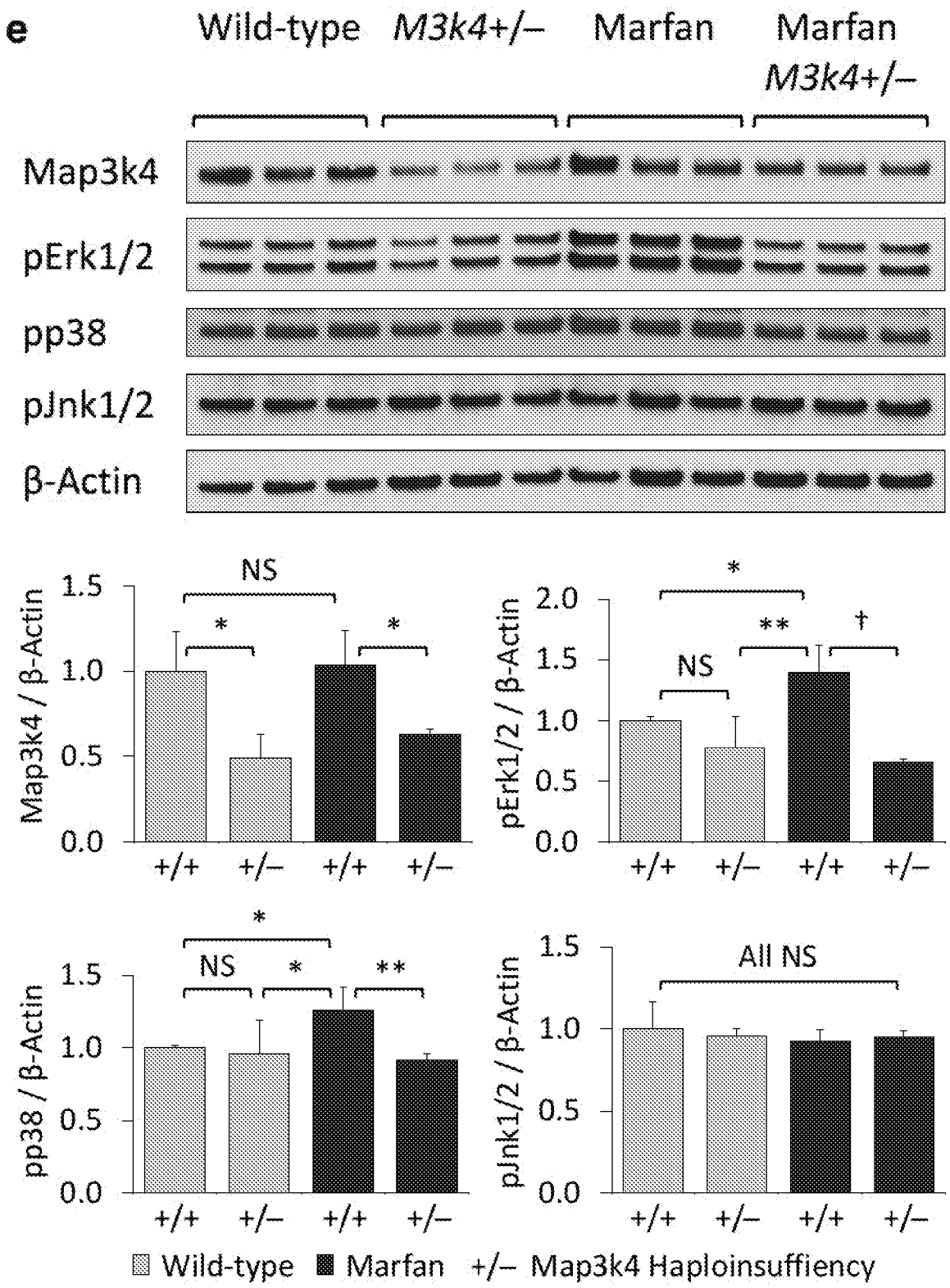
e
Wild-type
M3k4+/–
Marfan
Marfan M3k4+/–
Map3k4
pErk1/2
pp38
pJnk1/2
β-Actin
Map3k4 / β-Actin
pErk1/2 / β-Actin
pp38 / β-Actin
pJnk1/2 / β-Actin
NS
All NS
+/+
+/–
Wild-type
Marfan
+/– Map3k4 Haploinsuffiency

FIGURE 4E

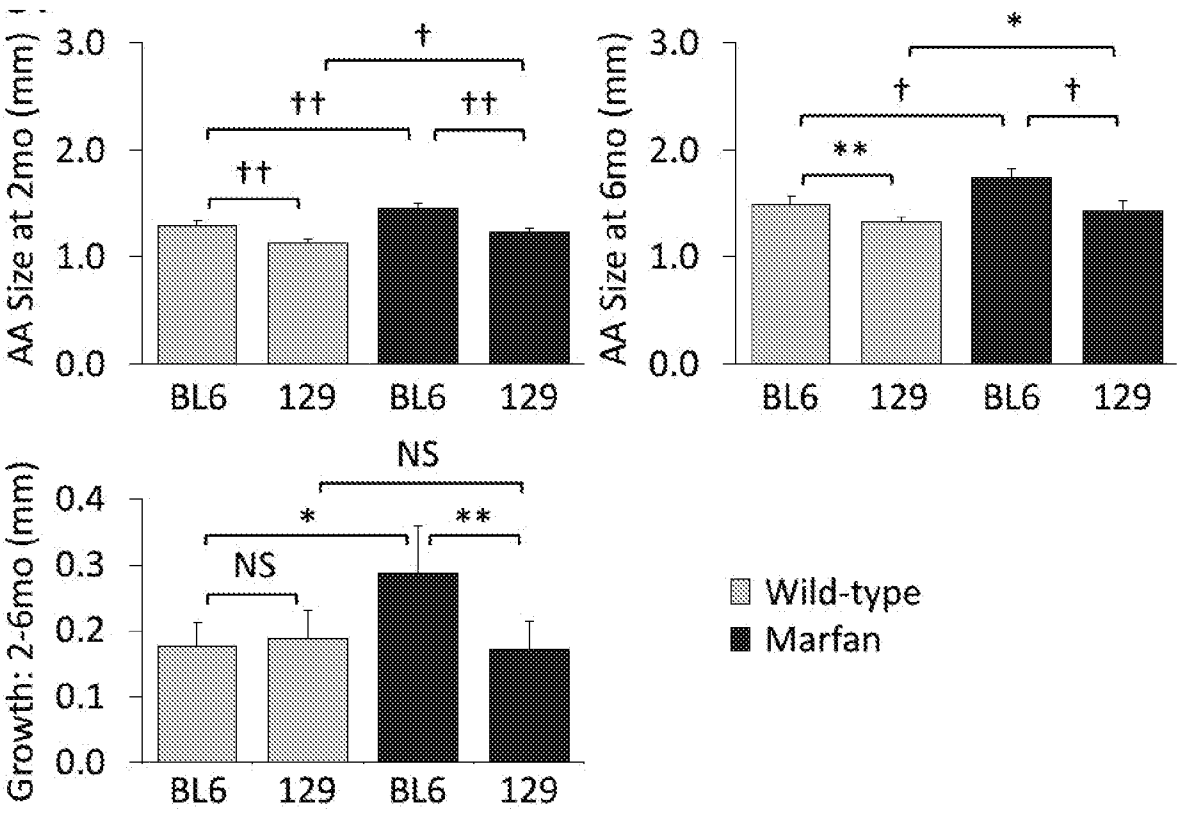

FIGURE 5

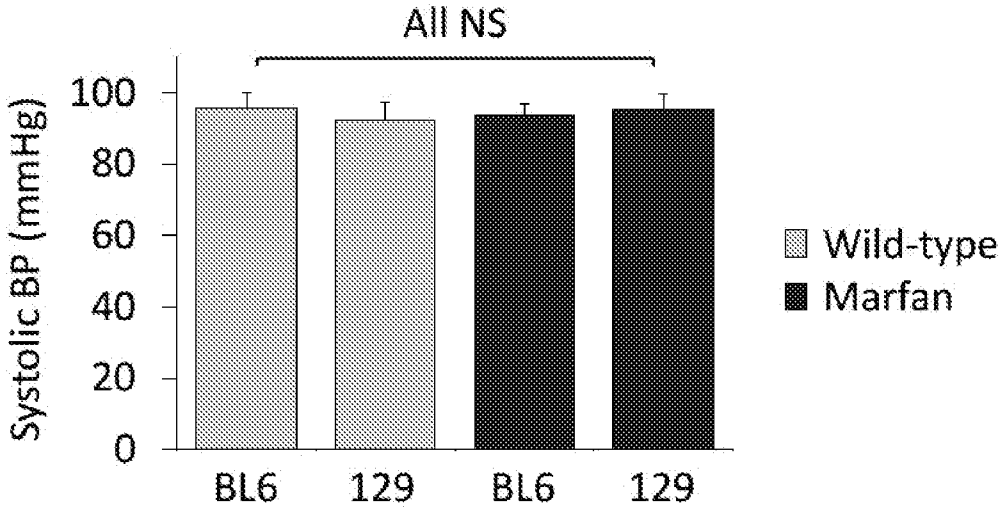

FIGURE 6

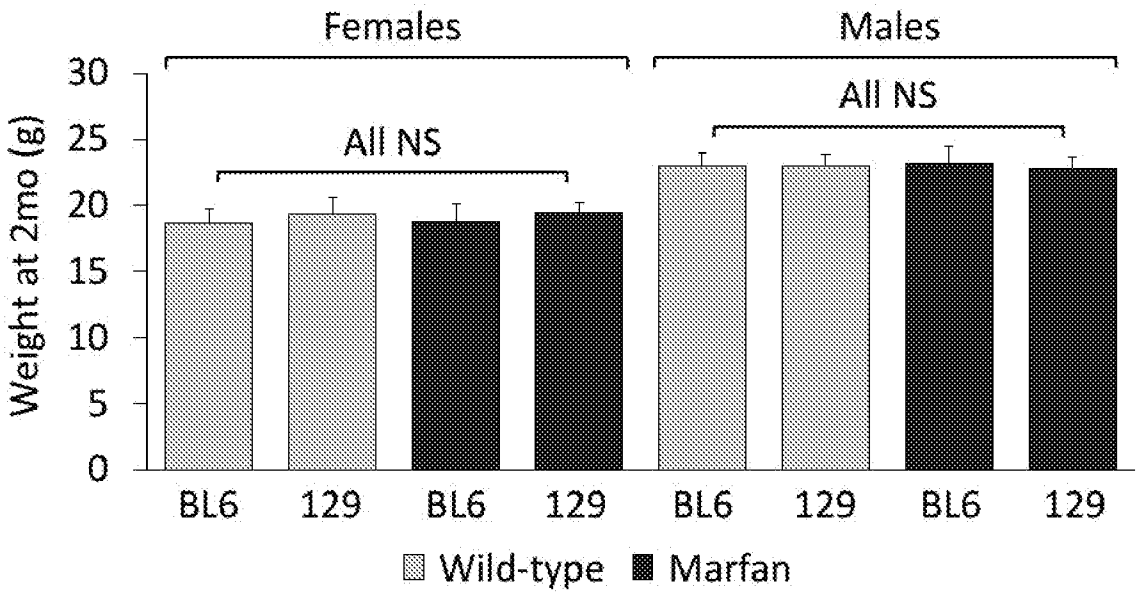

FIGURE 7

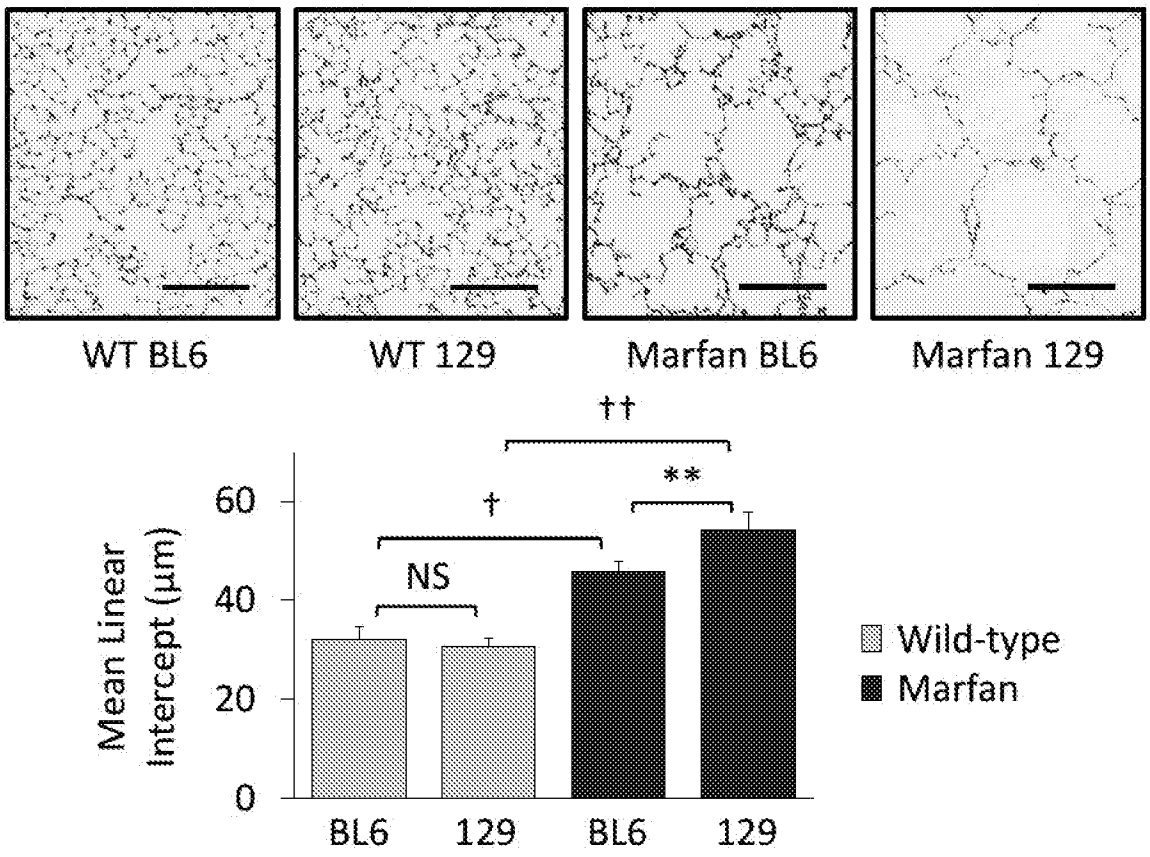

FIGURE 8

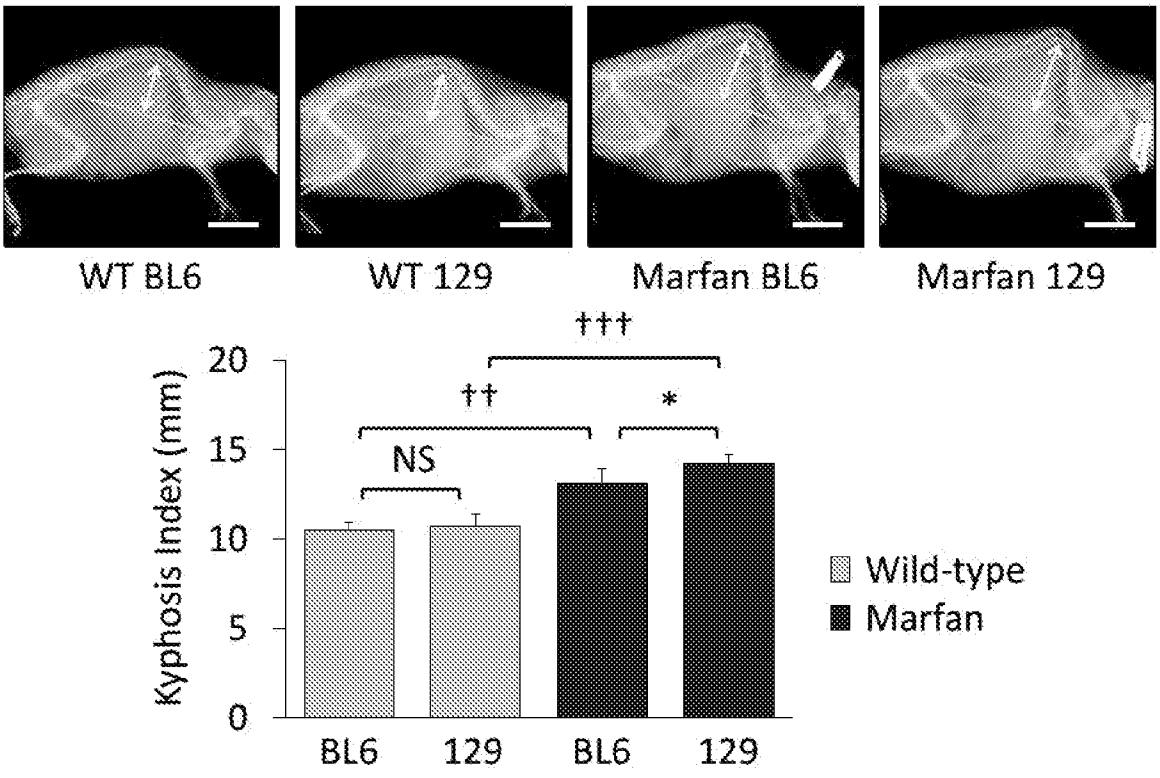

FIGURE 9

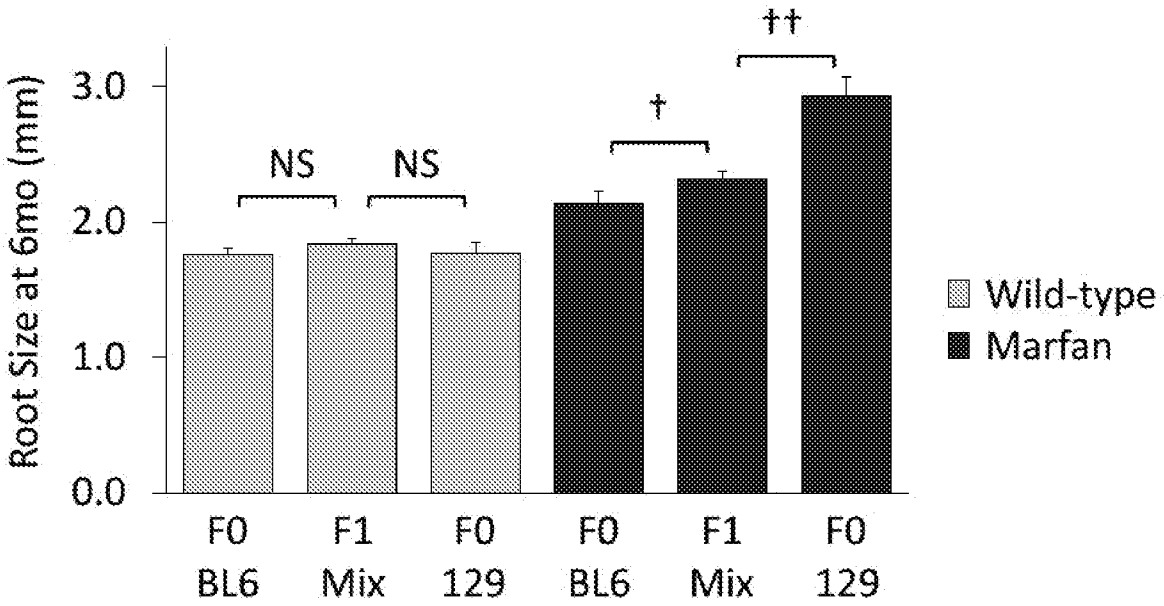

FIGURE 10

Mus musculus: C57BL6/J Strain (SEQ ID NO: 24)

DNA ACTTCAGACGCACACAGGTTGGCACTGCCATCTCTGCTGCTTCTGACTCCA

Protein -T--S--D--A--H--R--L--A--L--P--S--L--L--L--L--T--P-

DNA CTGCTGTGGGGCCTGTGA

Protein -L--L--W--G--L--*-

Mus musculus: 129S6/SvEvTac Strain (SEQ ID NO: 25)

DNA ACTTCAGACGCACACAGGTTGGCACTGCCATCTCTGCTGCTTCTGACTCCA

Protein -T--S--D--A--H--R--L--A--L--P--S--L--L--L--L--T--P-

DNA CTGCTGTGGGGCCTGTGGACCTCAGTCTCTGCCAAGGCATCCTGA

Protein -L--L--W--G--L--W--T--S--V--S--A--K--A--S--*-

Homo Sapiens (SEQ ID NO: 26)

DNA CCGGGGGCCCCAGGCCCACTGGTGGCTGCCACCATGCTGCTGCTGCTGCCG

Protein -P--G--A--P--G--P--L--V--A--A--T--M--L--L--L--L--P-

DNA CCACTGTCACCAGGCGCCCTGTGGACAGCGGCCCAGGCCCTGACGCTATGA

Protein -P--L--S--P--G--A--L--W--T--A--A--Q--A--L--T--L--*-

FIGURE 11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20692

A. CLASSIFICATION OF SUBJECT MATTER
IPC - C12N 9/64; C12Q 1/68; C12Y 304/24 (2018.01)
CPC - C12N 9/6491; C12Q 1/6827, 1/6883, 1/6837; C12Y 304/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	WO 2014/037316 A1 (UNIVERSITE DE LIEGE) 13 March 2014; page 9, paragraph 3; page 12, paragraph 4	41 --- 3-4, 6, 17-21, 27-28, 31-32
X -- Y	US 2010/0034806 A1 (DIETZ, HC et al.) 11 February 2010; abstract; paragraphs [0012], [0016]-[0017], [0169]	1-2, 9-11, 29-30 --- 3-4, 6-8, 17-21, 27-28, 31-32, 34-35
Y	(HOLM, TM et al.) Noncanonical TGFbeta Signaling Contributes to Aortic Aneurysm Progression in Marfan Syndrome Mice. Science. 15 April, 2011; Vol. 332, No. 6027; pages 358-361; abstract; page 258, column 2, paragraph 1; figure 1A; DOI: 10.1126/science.1192149	1-4, 6-11, 17-21, 27-32, 34-35
Y	(CUSSAC, D et al.) Alpha2beta-Adrenergic Receptor Activates MAPK via a Pathway Involving Arachidonic Acid Metabolism, Matrix Metalloproteinases, and Epidermal Growth Factor Receptor Transactivation. Journal of Biological Chemistry. 12 March, 2002; Vol. 277, No. 22; pages 19882-19888; abstract; page 19886, column 2, paragraph 2; DOI: 10.1074/jbc.M110142200	7, 34
Y	(BURKHARD, K et al.) Use of Inhibitors in the Study of MAP Kinases. Methods in Molecular Biology. 2010; Vol. 661; pages 1-15; page 2, paragraph 1; page 4, paragraph 3; DOI: 10.1007/978-1-60761-795-2_6	8, 35

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 July 2018 (12.07.2018)	30 JUL 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20692

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	(PAYE, A et al.) EGFR Activation and Signaling in Cancer Cells Are Enhanced by the Membrane-Bound Metalloprotease MT4-MMP. Cancer Research. 15 October, 2014; Vol. 74, No. 23; pages 6758-6770; page 6760, column 2, paragraph 2; DOI: 10.1158/0008-5472.CAN-13-2994	22-25
A	(ALONSO, MM et al.) Deficiency of MMP17/MT4-MMP Proteolytic Activity Predisposes to Aortic Aneurysm in Mice. Circulation Research. 11 May, 2015; Vol. 117, No. 2; pages 1-73; page 15, paragraphs 1-2; page 18, paragraph 2; DOI: 10.1161/CIRCRESAHA.117.305108	22-25
A	US 2010/0209923 A1 (NIIKAWA, N et al.) 19 August, 2010; abstract; paragraphs [0012], [0039], [0046], [0057]	22-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20692

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

An Invitation to Furnish Nucleotide and/or Amino Acid Sequence Listing and to Pay, Where Applicable, Late Furnishing Fee ("ISA/225") was mailed on 14 March 2018 (14.03.2018). The electronic sequence listing submitted on 11 April 2018 (11.04.2018) in response to the ISA/225 is acknowledged, however it does not comply with the standard provided for in Annex C of the Administrative Instructions. Therefore, the international search has been carried out only to the extent possible.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20692

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 5, 16, 33
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 5, 16, and 33 are not searchable for the following reason: The ISA/225 was mailed on 14 March 2018. The applicant did not, within the prescribed time limit, pay the required late furnishing fee for the furnishing of a sequence listing in response to an invitation under Rule 13ter.1(a) or (b). Accordingly, ISA/US cannot consider the sequence listing submitted on 11 April 2018.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 26
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

-***- Please See Within the Next Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Group 1+ Claims 1-4, 6-11, 17-25, 27-32, 34-35 and 41

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

International application No.
PCT/US18/20692

-***-Continued from Box III Observations where unity of invention is lacking -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-4, 6-15, 17-25, 27-32, 34-41, and an inhibitor of MMP17 (therapy) are directed toward methods for identifying and treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition; and pharmaceutical compositions and kits therefor.

The methods, compositions and kits will be searched to the extent they encompass a therapy encompassing an inhibitor of MMP17 (therapy). Applicant is invited to elect additional therapy(ies), with a specified type of therapy (e.g. allele replacement therapy) for a specified target (e.g. MAP2K6) for each elected therapy, to be searched. Additional therapy(ies) will be searched upon the payment of additional fees. It is believed that claims 1, 2, 3 (in-part), 4 (in-part), 6 (in-part), 7 (in-part), 8 (in-part), 9 (in-part), 10 (in-part), 11 (in-part), 17 (in-part), 18 (in-part), 19 (in-part), 20 (in-part), 21 (in-part), 22 (in-part), 23 (in-part), 24 (in-part), 25 (in-part), 27 (in-part), 28 (in-part), 29, 30, 31 (in-part), 32 (in-part), 34 (in-part), 35 (in-part), and 41 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass an inhibitor of MMP17 (therapy). Applicants must specify the claims that encompass any additionally elected therapy(ies). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be a therapy encompassing an inhibitor of MAP2K6 (therapy).

No technical features are shared between the therapies of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a method for treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising: administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling; thereby treating the subject; a pharmaceutical composition for the treatment of a disease or disorder characterized by aberrant MAP kinase pathway expression or activity, comprising a therapeutically effective amount of at least one agent that modulates expression, function or activity; a method for treating a subject having Marfan syndrome or a Marfan-associated condition comprising: identifying a variant allele that produces elevated expression and/or activity of a gene product; and replacing the variant allele with an allele of that does not produce elevated expression and/or activity of a gene product; thereby treating the subject; a kit for identifying a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising: primers specific for amplification; a probe oligonucleotide, optionally fluorescently labeled, that specifically detects the presence of an amplicon, wherein detection of elevated expression of a target identifies the subject as having or at risk of developing Marfan Syndrome or a Marfan-associated condition; and instructions for use; a method for identifying and treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising: isolating a sample from the subject; assessing the expression level of a marker in the sample, assessing the level of activity of a gene product of the marker in the sample, and/or genotyping a marker gene in the sample; identifying elevated expression of the marker in the sample, identifying elevated activity of a marker gene product of in the sample, and/or identifying a genotype of a marker gene in the sample that causes elevated expression and/or activity of the marker gene, or of a gene product thereof; and administering a therapy for Marfan Syndrome or a Marfan-associated condition to the subject, thereby treating the subject; a method for treating a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising: administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling and/or expression, function or activity of epidermal growth factor receptor (EGFR); thereby treating the subject; these shared technical features are previously disclosed by US 2010/0034806 A1 to Dietz et al. (hereinafter 'Dietz') in light of evidence provided by the article 'TGF Beta signalling and its role in tumour pathogenesis' by Kaminska et al. (hereinafter 'Kaminska') and in view of US 2010/0209923 A1 to Niikawa et al. (hereinafter 'Niikawa') and WO 2016/186772 A2 to Genzyme Corporation (hereinafter 'Genzyme').

Dietz discloses a method for treating a subject having or at risk of developing Marfan Syndrome (a method for treating a subject having or at risk of developing Marfan Syndrome; abstract, paragraph [0012]) comprising: administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling (comprising: administering to the subject an effective amount of an agent that modulates the activity of TGFBeta (MAP kinase pathway signaling); paragraph [0012], see Kaminska, Figure 2, legend, wherein TGFB activates several MAPKs); thereby treating the subject (thereby treating the subject; paragraph [0012]); a pharmaceutical composition for the treatment of a disease or disorder characterized by aberrant MAP kinase pathway expression or activity (a pharmaceutical composition for the treatment of a disease or disorder characterized by aberrant TGFBeta expressoin (MAP kinase pathway expression or activity); paragraph [0027]), comprising a therapeutically effective amount of at least one agent that modulates expression, function or activity (comprising a therapeutically effective amount of at least one agent that modulates expression, function or activity; paragraphs [0012], [0027]); a method for identifying and treating a subject having or at risk of developing Marfan Syndrome (a method for diagnosing (identifying) and treating a subject having or at risk of developing Marfan Syndrome; paragraphs [0012], [0080]): isolating a sample from the subject (isolating tissue (a sample) from the subject; paragraphs [0012], [0080]); assessing the expression level of a marker in the sample (assessing the expression level of TGFBeta (a marker) in the sample; paragraphs [0012], [0080]); identifying elevated expression of the marker in the sample (identifying elevated expression of the marker in the sample; paragraphs [0012], [0080]); and administering a therapy for Marfan Syndrome to the subject, thereby treating the subject (administering a therapy for Marfan Syndrome to the subject, thereby treating the subject; paragraph [0012]); a method for treating a subject having or at risk of developing Marfan Syndrome (a method for treating a subject having or at risk of developing Marfan Syndrome; paragraph [0012]) comprising: administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling and/or expression; thereby treating the subject (administering to the subject an effective amount of an agent that modulates the activity of MAP kinase pathway signaling and/or expression; thereby treating the subject; paragraph [0012]). Dietz further discloses wherein Marfan syndrome is associated with an Fbn1 allele encoding a cysteine substitution in an EGF-like domain of fibrillin-1 (wherein Marfan syndrome is associated with an Fbn1 allele encoding a cysteine substitution in an EGF-like domain of fibrillin-1; paragraph [0169]); and a kit with instructions for use (a kit with instructions for use; paragraph [0034]).

-***-Continued Within the Next Supplemental Box-***-

-***-Continued from Previous Supplemental Page:

Dietz does not disclose a method for treating a subject having Marfan syndrome comprising: identifying a variant allele that produces elevated expression and/or activity of a gene product and replacing the variant allele with an allele of that does not produce elevated expression and/or activity of a gene product; a kit for identifying a subject having or at risk of developing Marfan Syndrome or a Marfan-associated condition comprising: primers specific for amplification; a probe oligonucleotide, optionally fluorescently labeled, that specifically detects the presence of an amplicon, wherein detection of elevated expression of a target identifies the subject as having or at risk of developing Marfan Syndrome or a Marfan-associated condition; and instructions for use.

Niikawa discloses diagnosis of Marfan syndrome (diagnosis of Marfan syndrome; abstract) using primers specific for amplification (using primers specific for amplification; paragraphs [0046], [0057]) and a probe for said diagnosis (a probe for said diagnosis; paragraph [0012]), wherein the probe is fluorescently labeled (wherein the probe is fluorescently labeled; paragraph [0039]).

Genzyme discloses methods for treating a disease or disorder associated with a mutation (methods for treating a disease or disorder associated with a mutation; abstract), comprising repairing a gene with a mutation (comprising repairing a gene with a mutation; paragraph [0178]), including the Fbn1 gene mutation associated with Marfan syndrome (including the Fbn1 gene mutation associated with Marfan syndrome; table 1).

It would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Dietz to have used primers for amplification, as disclosed by Niikawa, of a gene allele associated with Marfan syndrome, as disclosed by Dietz, and detecting the amplification product, or specific allele using a fluorescently labeled probe in order to enable diagnosis of Marfan syndrome in order to identify patients in need of the treatment disclosed by Dietz, and to provide a kit therefor, including instructions, based on the disclosure of Dietz, in order to more readily enable said diagnosis. It further would have been obvious to a person of ordinary skill in the art at the time of the invention was made to have modified the disclosure of Dietz to have provided a method of treating Marfan syndrome, including replacing or repairing a variant allele associated with Marfan syndrome, as disclosed by Genzyme, and with the overexpression of TGFBeta, as disclosed by Dietz, as an alternative or supplementary means of modifying the level and/or activity of TGFBeta in a subject with Marfan syndrome.

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by a combination of the Dietz, Miikawa and Genzyme references, unity of invention is lacking.