DATA SUPPLEMENT

Supplemental Methods

Antibodies, Constructs, and Reagents: The SB 431542 compound, cyclohexamide, and anti-SMα-actin, and Flag antibodies were purchased from Sigma. The C-term specific anti-FAK and anti-ERK1/2 antibodies were purchased from Upstate. The anti-phospho Y397FAK antibody was purchased from BioSource and the Texas-Red phalloidin was purchased from Molecular Probes. SM-22 antibody was a generous gift from Mario Gimona and SM-MHC antibody was obtained from U. Groeschel-Stewart. TGF-β was purchased from Calbiochem. The GFP and GFP-FRNK adenoviruses were generated and expanded as described previously.[1] The promoter reporter constructs: SM α-actin (from -2560 to +2784), SM22 (from -450 to +88) and SM-MHC (from -4200 to +11600) luciferase constructs used have been previously described.[2] 6xSBE-luciferase was a gift from Li Li (Wayne State University).[3] Flag-FAK was a generous gift from Dr. Tom Parsons (University of Virginia) and was previously described.[4]

Cell Culture, Transfections, and Reporter Assays

Aortic vascular smooth muscle cells (SMC) were cultured from C57bl6 mice or Wistar rats using enzymatic digestion as previously described.[5] Cells were maintained in Delbucco's modified eagle medium with F12 supplemented with 10% fetal bovine serum and 0.5% penicillin/streptomycin and used from passage 8-16. 10T1/2 cells were obtained through ATCC and were maintained in Dulbucco's modified eagle medium supplemented as above. Chick proepicardial cells were a generous gift from Mark Majesky. Cells were induced to differentiate into coronary SMC (cSMC) by serum-starvation for 7 days as previously described.[6] For reporter assays, cells were transfected

with appropriate constructs using either Superfect (Qiagen) or Trans-IT (Mirus) transfection reagents according to manufacturer's protocol. Following transfections, cells were lysed in Glo Lysis Buffer and assayed using Steady Glo Luciferase Assay system (Promega) as directed by the manufacturer. In some experiments, cells were transduced with GFP or GFP-FRNK adenoviurs (10 m.o.i) prior to experimentation.

Immunocytochemistry and detection of BrdU incorporation in cultured cells

Cells were processed for immunocytochemistry using previously published methods.[7] In brief, cells were fixed with 4% paraformaldehyde, permeabilized with 4% Triton X-100 in PBS, incubated with SM α-actin (1:100) for two hours. After washing with PBS, slides were incubated for 1 hour with FITC-conjugated donkey anti-mouse antibodies (2 μg/ml) or Texas Red-conjugated phalloidin to detect filamentous actin.

To measure proliferating cells, BrdU (Sigma, 30 μg/mL) was administered to SMC grown on chamber slides. After 4 hrs, cells were fixed in 4% paraformaldehyde and stained using a BrdU detection kit (Invitrogen).

Western Analysis

To examine protein levels, lysates from cells or tissues were prepared by lysing in RIPA buffer with protease and phosphatase inhibitors as previously described.[8] Protein concentration was determined by using a colorimetric BCA assay (Pierce). Lysates were electrophoresed on an 11% polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with antibodies for c-terminal FAK (Upstate), SM α-actin (Sigma), FAK pY397 (BioSource), SM-myosin heavy chain (generated by U. Groeschel-Stewart), and SM-22 (generous gift from Mario Gimona).

Real-Time Quantitative RT-PCR

Tissue or cells were lysed in TRIzol (Invitrogen) and total RNA was isolated accoroding to the manufacturer's protocol. Expression was measured using the ABI Prism 7700 TaqMan system. Primers and fluorescent probes were designed for the following genes: FRNK (Forward: GCTGCATTCTGAGGCGTTTA, Reverse: CAGGATTGTGCACCACCAG, Probe: AGCCAGGACTGAGACGCCGCC), FAK (Forward: GAAAGCAGTAGTGAGCCAACC, Reverse: GAGACTGTCCACTATCTTCTG, Probe: CTCCATGCCTGATAATACTGGCCCAG), SM-22 (Forward: TGCAGTGTGGCCCTGATGT, Reverse: TGCTCAGAATCACGCCATTCT, Probe: AGATCGTGGGCGCCTGGGCT), and SM α-actin (Forward: CGCTGTCAGGAACCCTGAGA, Reverse: CGAAGCCGGCCTTACAGAG, Probe: CAGCACAGCCCTGGTGTGCGAC). 18S primers and probes were a generous gift from Hyung-Suk Kim. Primers and probes for smoothelin were used as described previously.[9]

Transwell assay

SMC were trypsinized and resuspended in DMEM:F12 containing 1% penicillin/streptomycin and 0.1% bovine serum albumin. Approximately 20,000 cells were plated in serum-free media on transwell filters (8 μm pore size) precoated with FN, using 10% serum-containing medium as the chemoattractant. After 7 hours, the cells were fixed in 4% paraformaldyhyde and the remaining cells in the upper chamber were removed with a cotton swab. Migrated cells were counted by indirect fluorescence.

Animal Procedures

Mice with germline deletion of FRNK were generated by homologous recombination as previously reported and were backcrossed to the C57/Bl6 strain at least

6 times before experimentation.[10] Genotypes were obtained from tail snip DNA using PCR analysis for primers specific to FRNK.[10]

Conscious blood pressure was measured in mice aged 13 weeks using a tail cuff detection system (Hatteras). Over a period of 20 minutes, 20 measurements were taken every day for 6 consecutive days. Data is presented as an average of measurements for all days.

To measure unconscious blood pressure, mice were anaesthetized with isofluorane, and the left carotid artery was exposed. A pressure transducer (Millar) was inserted through the carotid into the aorta to measure aortic pressure. Phenylephrine was administered through a catheter inserted into the jugular vein. Between doses of phenylephrine, each animal was allowed to recover its blood pressure to resting levels.

For injury studies, mice aged 9-10 weeks were anesthetized using isofluorane and a suture was tied around the left common carotid artery just below the bifurcation as previously described.[11] Animals were allowed to recover and were sacrificed at various times following injury. For RNA studies, a 3mm portion of the carotid was dissected out from 1 mm below the site of ligation (left), or 1mm below the bifurcation (right). For histology, the carotids were removed *en bloc* and sectioned serially from 2-3 mm away from the site of injury.

In situ hybridization

Tissues from 1 week post-natal C57black6 mice were harvested, fixed and embedded in paraffin. Serial sections (12 μm) were hybridized in the absence or presence of a FRNK-specific digoxingenin-labeled antisense RNA probe O/N at 42^{o} C. Sections were washed and incubated with an alkaline phosphatase-conjugated anti-

digoxigenin Ab (Roche; 1:1,000 O/N at 4^{o}C). Slides were developed using NBT/BCIP as substrates (purple staining) and counterstained with methylene-green (1%).

BrdU incorporation, Immunohistochemistry and Trichrome/Elastin Stain

Pregnant mothers were injected with BrdU (30 mg/kg) 1 day prior to giving birth. Wild type (+/+) or FRNK$^{-/-}$ (-/-) pups were sacrificed at postnatal day 4 and aortas were removed, paraffin embedded, and sectioned. An antibody to BrDU was used to detect proliferating cells within the media. Positively stained area was quantified with ImageJ software.

For general tissue immunohistochemistry, tissue from mouse pups or post-surgical animals was harvested, formalin-fixed, and paraffin-embedded. Sections (8-9 μm) were dehydrated, permeabilized, blocked, and incubated with an antibody for SM α-actin (Sigma). Antibody binding was detected with HRP-linked secondary antibody. Sections were developed with diamino benzidine and subsequently counterstained with methyl green. For morphometric measurements, a modified Masson's trichrome/Verhoeff stain was used to localize elastin. We used ImageJ (NIH) to measure circumference of the lumen, internal elastic lamina (IEL), and external elastic lamina (EEL), along with area of the media and lumen.

Supplemental Discussion

While we did not observe significant hemodynamic differences in adult FRNK$^{-/-}$ mice in comparison to littermate controls, we cannot currently rule out the possibility that significant changes might be apparent at earlier time points. Indeed, we found that SM marker gene expression was significantly reduced in FRNK-/- vessels from 2-3 weeks following birth but was normalized by 8 weeks (a time point prior to our hemodynamic assessment). However, our data obtained from adult mice does rule out a function for FRNK as a signaling molecule in the regulation of α-adrenergic-dependent vasoconstriction. In addition, these data provide evidence that baseline hemodynamics are not altered in FRNK-/- mice prior to our surgical manipulation, lending credence to a specific function for FRNK in the re-conversion of SMC from a synthetic to contractile phenotype. Nonetheless, it will be of future interest to evaluate vascular reactivity and blood pressure in younger FRNK-/- mice.

FRNK belongs to a sub-class of smooth muscle-specific genes that are regulated in a CArG-independent fashion including aortic carboxypeptidase-like protein (ACLP), cysteine-rich protein 2 (CRP2), and histidine-rich calcium-binding protein (HRCBP) ACLP exhibits an expression profile similar to that of FRNK, with protein levels increasing in VSMC after carotid artery ligation, while canonical smooth muscle genes decrease.[12] ACLP is also SRF-independent; rather, its promoter is activated by transcription factors Sp1 and Sp3.[12] Interestingly, much like FRNK, CRP2 seems to be most robust in arterial smooth muscle, and its expression is also regulated by TGF-β.[13, 14] CRP2 expression is dependent upon an 800 bp region of its promoter that is independent of Sp1 and Sp3 but dependent on TGF-β mediated activation of ATF2.[14, 15] Although

HRCBP is expressed in all three muscle types (skeletal, cardiac, and smooth) it constitutes another example of a smooth muscle-selective protein expressed in a CArG-independent fashion.[16] Expression of HRCBP is dependent on a highly conserved myocyte ehancer factor 2 (MEF2) site within the promoter.[16] Studies are currently underway in our laboratory to define to what extent FRNK is regulated by some of these known CArG-independent mechanisms that drive SM-selective transcription.

We recently found that like FRNK expression, deletion of FAK from SMC (by homologous recombination) resulted in enhanced TGF-β dependent SM marker gene expression (un-published observations; RLS, LSS, CPM, and JMT). Thus, we postulate that direct modulation of FAK activity (or the intrinsic shifting of FAK/FRNK expression) likely mediates a balance between SMC migratory and contractile capacities necessary for proper vascular development and injury repair. There are several putative mechanisms whereby FAK activity might limit TGF-β dependent responses. FAK is a multifunctional protein that associates with a number of adapter molecules through well-defined protein interaction sites, and regulates downstream activation of MapKinases and small GTPases. FAK activation is required for full ERK activity in some models and it has been previously shown that ERK-dependent activation of the SRF co-factor, Elk-1, negatively regulates SMC marker gene expression by competitively interfering with the myocardin binding to SRF [17-20]. RhoA activation has also been shown to be an important determinant of SMC differentiation marker gene expression and previous studies have shown that FAK-null fibroblasts exhibit enhanced basal RhoA activity, indicating the possibility that this pathway might also be involved in promoting SMC maturation in FRNK expressing or FAK-null SMC [21]. Interestingly, we recently reported that the FAK-

interacting protein leupaxin shuttles from focal adhesions to the nucleus, where it acts as an SRF co-factor to enhance SM marker gene expression [22]. Furthermore, we showed that expression of a constitutively active FAK variant leads to sequestration of leupaxin within focal adhesions and reduces leupaxin-dependent gene transcription [22]. Studies to determine the relative contributions of (or relationship between) leupaxin, Map Kinases, or Rho A in regulating SMC phenotypes in FRNK expressing and/or FAK-null SMC are currently ongoing in our laboratory.

Supplemental Figure Legends

Online Figure I. FRNK is expressed in coronary SMC. Western blot of FRNK and FAK levels in proepicardial cells (PEC),bovine endothelial cells (BEC), human coronary SMC (HuCSMC), and human aortic SMC (HuAoSMC). Blots were probed with an antibody that detects FAK and FRNK.

Online Figure II. FRNK expression is tightly regulated but myocardin family members do not affect FRNK promoter activity. A. Western blot of Rat SMC treated with cyclohexamide (CHX, 100μg/mL) for indicated times. Densitometry is shown below. **B.** 10T1/2 or VSMC were transfected with 6kb FRNK-Luc or TK-Luc and assayed for luciferase activity 48 hours later. **C.** Rat aortic SMC were transfected with FRNK-Luc or SMα–actin-Luc in the presence of empty vector, myocardin, or MRTF-A and were processed for luciferase activity at 48 hr.

Online Figure III. Ectopic expression of FRNK regulates SM marker gene expression but does not alter myocardin levels. A, C. SM-MHC and SMα-actin luciferase assay in 10T1/2 cells expressing GFP, GFP-FRNK, or SuperFAK (SFAK) performed in 10% serum-containing media (Mean +/- SEM; n>/=3) **B.** Rat aortic SMC were infected with GFP or GFP-FRNK and qRT-PCR was used to measure myocardin expression. Data is normalized to the presence of 18S and is presented as the mean +/- SEM (n =3). **D.** Rat aortic SMC were co-transfected with SM22-luciferase reporter construct and with either empty vector (EV), SFAK, FAK, Y397FFAK. Western blotting was performed 48 hrs following transfection.

Online Figure IV. FRNK$^{-/-}$ mice do not display abnormal homeostasis. **A.** Wt (+/+) and FRNK$^{-/-}$ (-/-) mice aged 13 weeks were subjected to tail cuff measurement of systolic blood pressure. Data is presented as an average of the calculated mean for each mouse over 6 days, +/- SEM. **B.** Blood pressure was measured in anaesthetized mice aged 14 weeks by aortic catheterization during administration of phenylephrine (PE) into the jugular vein. Data are expressed as the mean +/- SEM. **C.** Aortic medial thickness was measured using ImageJ software in Wt (+/+) and FRNK$^{-/-}$ (-/-) mice aged 13 wks from formalin-fixed, paraffin-embedded sections stained with SM α-actin. Data is expressed as the mean +/- SEM. Sample histology with staining for SM α-actin from mice aged 13 weeks is shown at right.

Online Figure V. FRNK$^{-/-}$ mice show no differences in vessel growth in response to carotid artery ligation. Wild type (+/+) and FRNK$^{-/-}$ (-/-) mice were subjected to ligation of the left carotid artery for 14 days. Measurements were taken from trichrome/elastin stained sections using ImageJ (NIH). **A.** The distance between the IEL and EEL in the right and left carotid was measured at 9 points along the 3mm region of remodeled vessel for each individual animal. Data is presented as the average of the mean for each animal, +/- SEM. **B.** The circumference of the lumen, IEL, and EEL was measured three times from the left carotid of each animal. **C.** Ratio of intimal area:medial area in the left, injured carotid. Data for each panel is presented as the average of the mean for each animal, +/- SEM.

Online Figure VI. FRNK-/- cells are morphologically similar to wild type SMC, but have lower levels of SM α-actin staining. FRNK$^{-/-}$ (-/-) or WT (+/+) cells were maintained in serum, fixed in 4% paraformaldyhyde, and stained with an antibody for SM α-actin and phalloidin.

Supplemental References

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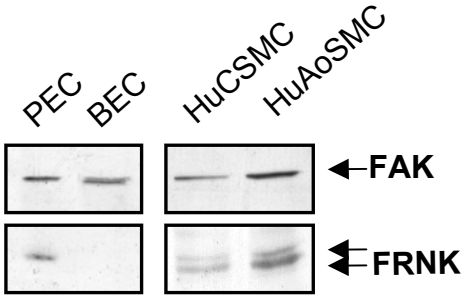

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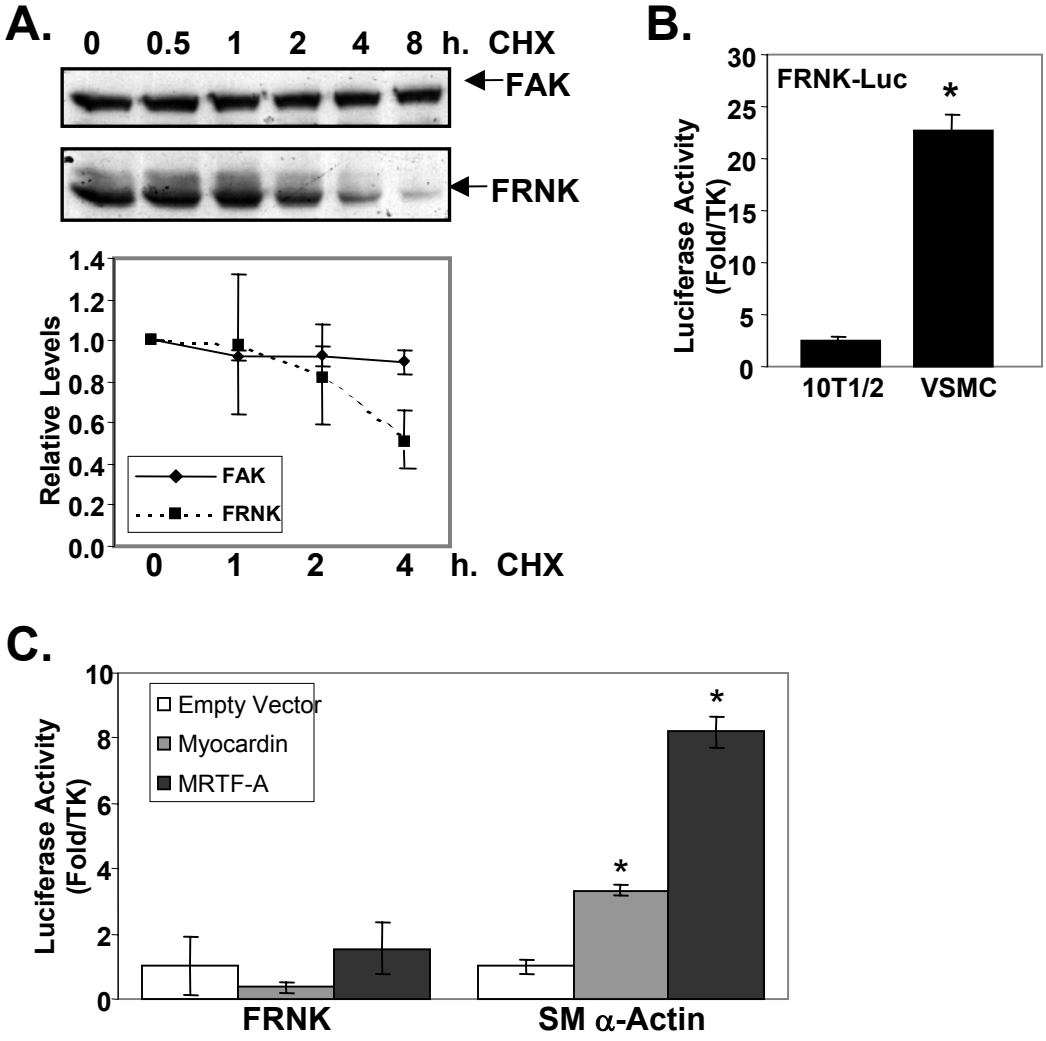

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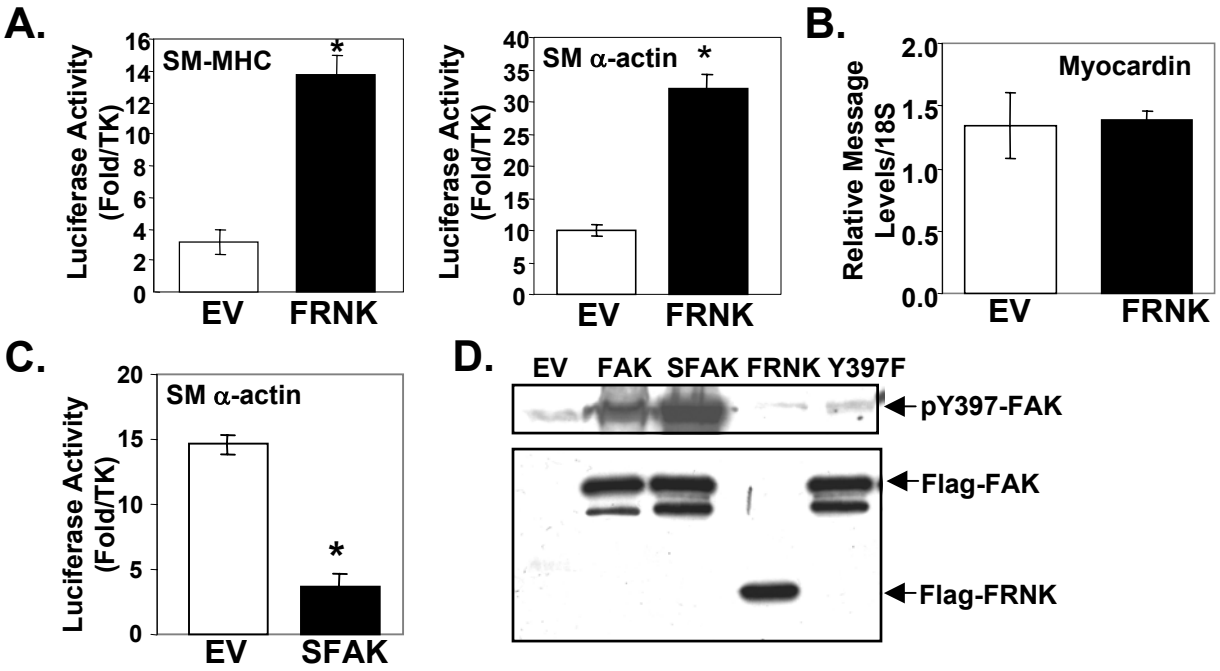

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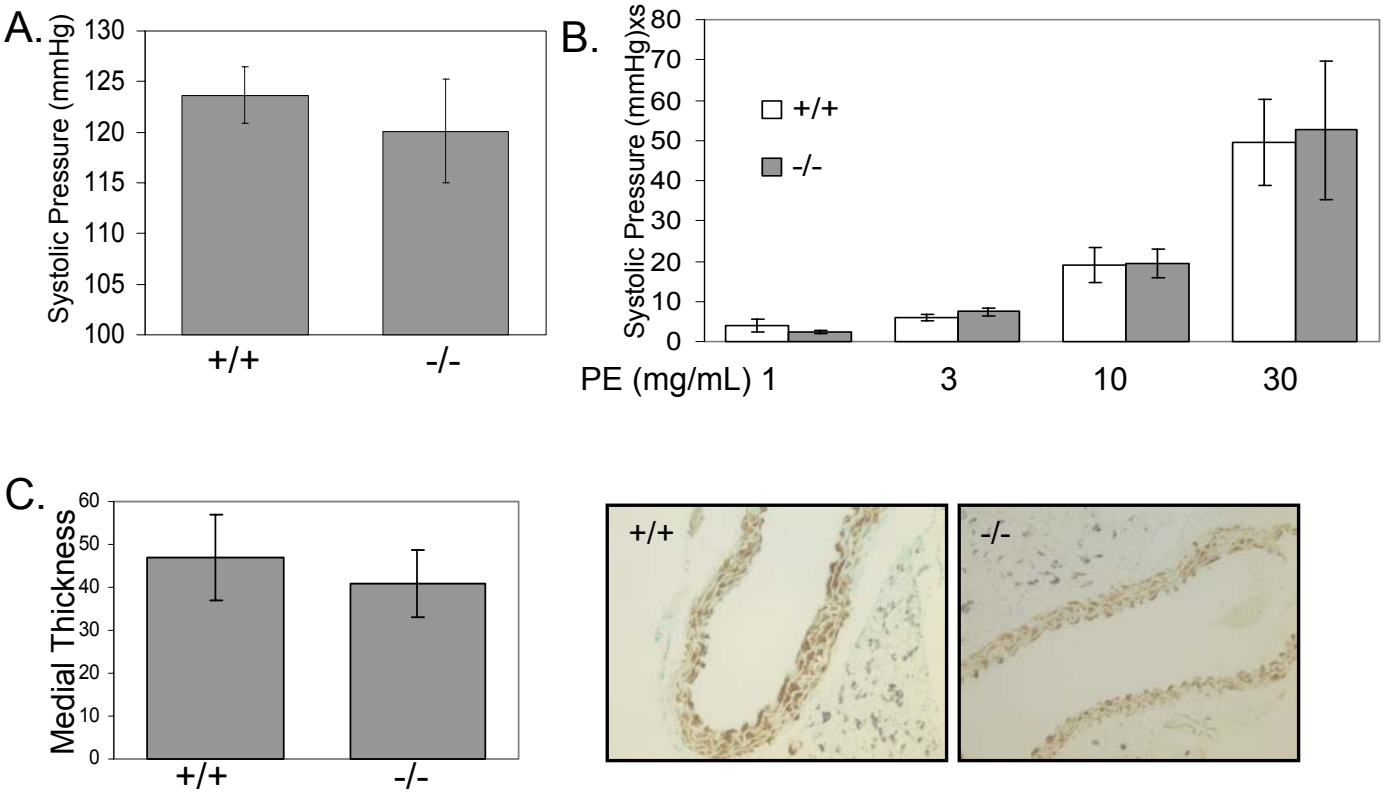

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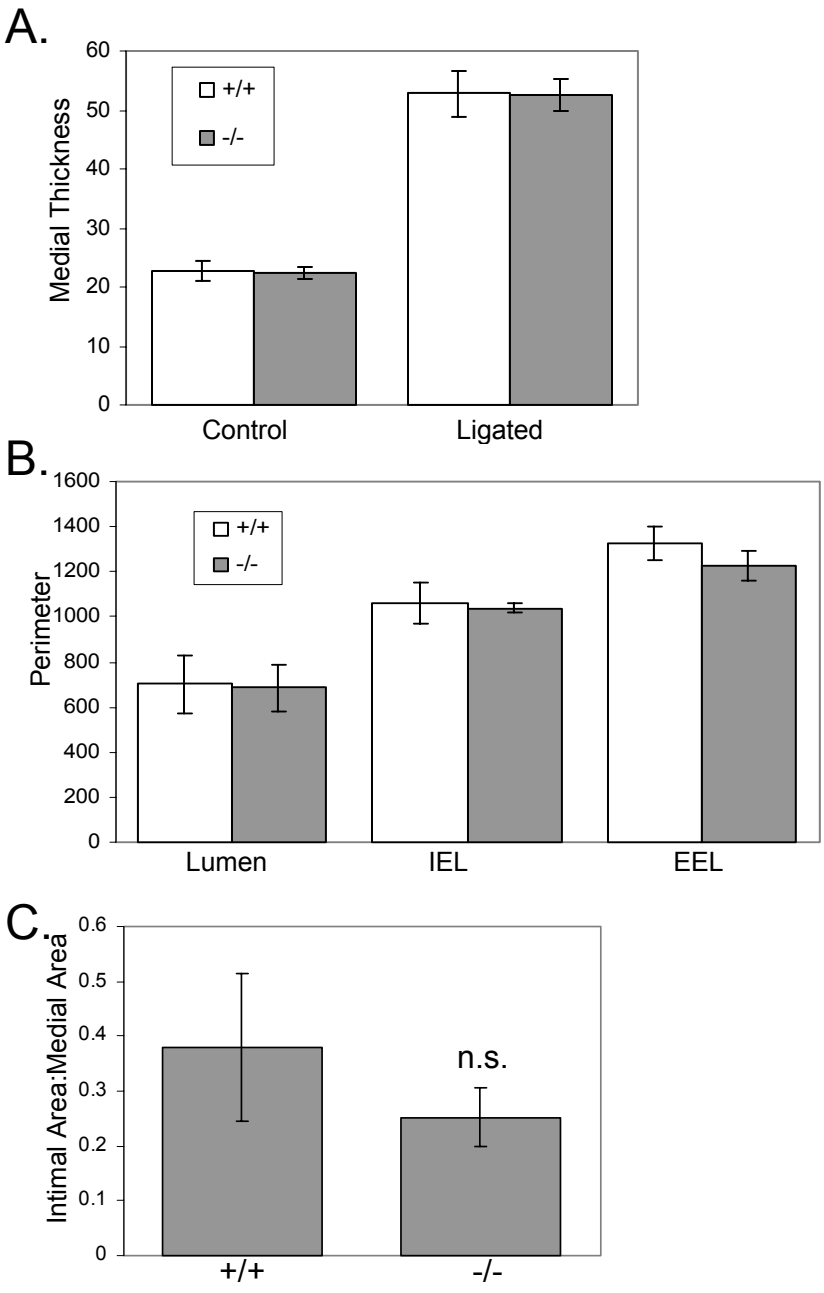

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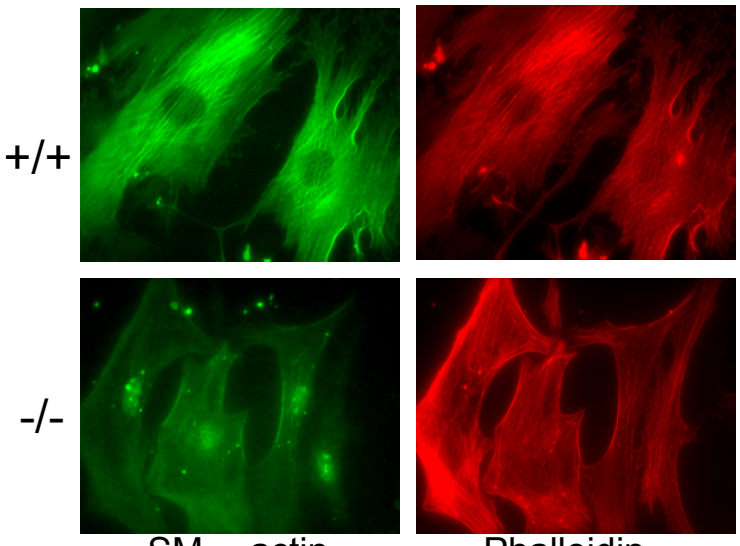
Online Figure V



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Online Figure VI