Supplemental Material

Online Methods:

Mice: All C57/Bl6 mice (Taconic) used for pluronic applications and carotid ligation injury models were 10-18 week old males and ApoE$^{-/-}$ were 15-18 week old males. All mice were kept on a standard 12 hour light/dark cycle and fed a normal chow diet. Food and water was available ad-libatum. Mice containing a heterozygous deletion of Cx43 (Cx43$^{+/-}$, as described)[1] were bred to produce litters containing wild type (Cx43$^{+/+}$), heterozygous (Cx43$^{+/-}$) and knockout (Cx43$^{-/-}$) newborns mice for isolation of aortic tissue. Tail snips from each newborn mouse were used for genotyping and loss of Cx43 further confirmed by Western blotting. All animals were used according to the University of Virginia Animal Care and Use Committee guidelines as previously described.[2]

The Cx43 MAPK knock-in mice (Cx43-MK4A) were made using a v6.5 ES cell line derived from C57Bl/6J and 129S6SvEV F1 hybrid mice. The knock-in vector contained a 5' homology arm, the non-coding Exon 1, a neo/PGK cassette flanked by loxP sites followed by Exon2 that contains the whole Cx43 coding sequence including the mutations (S255A, S262A, S279A and S282A) and a 3' homology arm. After electroporation, ES cells were screened with long range PCR using primers amplifying from the homology arms to the floxed neomycin cassette, and homologous recombination was confirmed by Southern blotting to ensure intact homologous recombination into the Cx43 locus (**Online Figure I**). The mice were interbred with B6.C-Tg (CMV-cre) 1Cgn/J mice (Jackson) to remove the neomycin selection cassette and then backcrossed to C57Bl/6J mice 6 times to omit the Cre gene and approach strain homogenicity. All mice used for experimentation were 10-18 week old males.

Vascular smooth muscle cell (VSMC) isolation: Newborn mice were euthanized by decapitation and descending aortas removed, placed into a wash solution (HBSS,1% BSA, 1% Glutamine, 1% sodium pyruvate, L-ascorbic acid w/v 10%w/v, pyruvate 20% w/v, Fungizone 0.1%), and dissected horizontally and longitudinally. Explants, were placed in 75 mm flasks containing 2 mL of Amniomax C-100 media plus supplement (Gibco), and incubated at 37ºC, 5% CO_2 for 2 weeks with media changed every 3 days. After VSMC outgrowth, explants were removed and media replaced with fresh supplemented Amniomax media for 1 week (**Online Figure II**). Cells were trypsinized, re-plated in the same 75mm flask (Passage 1, P1) and grown to near confluence. Media was then exchanged for DMEM F12 containing 20% FBS (Defined, Hyclone) with 1% pen/strep, 1% glutamine for 48 hour, VSMC trypsinized and re-plated in 225 mm flasks (P2) and grown to near confluence. Finally media was exchanged for growth media (10% FBS in DMEM F12 with 1% pen/strep, 1% glutamine) for 48 hours before cells being trypsinized and re-plated into 225 mm flasks (P3). Cells were used between P4 and P8 for all experiments.

Carotid surgeries: Pluronic application surgeries were performed as described.[2] Briefly, mice were anesthetized by intraperitoneal injection of ketamine-xylazine, the left common carotid artery exposed, and PDGF (200 ng) mixed in pluronic gel (F-127) was applied to the outside of the carotid wall. Forty-eight hours after the surgery, mice were euthanized with an intraperitoneal injection of 60-90 mg/kg pentobarbital, mice were perfused with 4% PFA and both treated (left common) and control (right common) carotids removed and stored in 4% PFA overnight for whole mount analysis or immuno-sectioning. For ultrastructure transmission electron microscopy (TEM) and immune-TEM (i-TEM) vessels were fixed in 4% PFA containing gluteraldehyde (2.5% or 0.5% respectively). For in vivo cell proliferation studies, mice were pre-

injected with 5-ethynyl-2'-deoxyuridine (EDU) prior to surgeries and carotids removed post-treatment for whole mount analysis of EDU incorporation into VSMC (n=4, as described).[2]

Carotid ligation (injury) surgeries were performed as previously described.[3] Briefly, mice were anesthetized by intraperitoneal injection of ketamine-xylazine, the left common carotid artery exposed and carefully freed from surrounding tissue. A 6/0 silk suture was run under the carotid artery distal to the bifurcation site and two consecutive, flat square knots were tied tight enough to restrict blood flow. Control mice were surgical controls, where carotid artery was exposed and freed of surrounding tissue as described above, but no suture was applied. At 2 weeks after surgery, mice were euthanized with an intraperitoneal injection of 60-90 mg/kg pentobarbital, mice were then perfused with a krebs buffered solution containing heparin followed by 4% PFA in krebs solution. Carotids were then removed and stored in 4% PFA overnight for whole mount analysis or immuno-sectioning. We characterized neointima formation in C57Bl/6 mice to occur at between 1-3mm distal of the ligation site as has been previously described.[3] For analysis of neointima formation in vessels, areas corresponding to the adventitia (immediately surrounding the media), the media (containing VSMC), neointima (containing VSMC) and lumen were measured on H&E stained cross sections of carotid vessels were measured using MetaMorph image analysis software (n=7, **Online Figure III**).

In-vitro cell proliferation: VSMC were plated at 5x10^4 cells in 6 well plates for 24 hours in growth media and cell cycle stall induced by switching to a low serum media (0.5% FBS) for 48 hours followed by transfection with plasmids (5 µg DNA each, $Cx43^{-/-}$ VSMC only,) using Lipofectamine 2000 for 18 hours. Cells were then incubated with EDU (5 µg/mL) with or without the addition of PDGF (10 ng/mL) for 24 hours in low serum media. In studies of reduced gap junction communication, carbenoxolone (25 □M) was applied to cells for 30 mins prior to the addition of PDGF and EDU for 24 hours. Cells were trypsinized, pelleted, re-suspended in 0.5 mL PBS then fixed using 4% PFA and stored at 4°C. Cell proliferation was measured by flow cytometry of Alexa 488 labeled EDU as previously described.[2]

Plasmids: A DNA construct for full length rat Cx43 (Cx43, a.a.1-382,[4] **Online Figure IV**) in vector pcDNA3.1 was grown in DH5α cells (Invitrogen). In experiments, control vector was empty pcDNA3.1. The Cx43 C-terminus ($Cx43^{CT}$, rat, a.a. 236-382, **Online Figure IV**) DNA construct sub-cloned in frame with the glutathione S-transferase (GST) tag in pGEX-6P-2 plasmids was kindly provided by Dr P. Sorgen (as described [5]) and was grown in BL21-codon plus cells (Stratagene/ Agilent technologies< Santa Clara, CA). Full length human cyclin E (cyclin E1, transcript variant 2, NM 057182.1) inserted in the pGEX-2T plasmid, kindly provided by Dr B. Lüscher,[6] was transformed in BL21-codon plus cells. All DNA sequencing was performed by the UVA sequencing core facility.

Site directed mutagenesis: Primers for site directed mutagenesis of Cx43/ $Cx43^{CT}$ were designed using QuikChange primer design program and mutagenesis performed using the QuikChange kit (Agilent, Stratagene, Santa Clara, CA). Primers were designed against MAPK serines (Cx43: S255, S262, S279, S282 or corresponding $Cx43^{CT}$: S20, S27, S44, S47), to alanine (non-phosphorylated, $Cx43^{MK4A}$/$Cx43^{CTMK4A}$) or to aspartate (phospho-mimetic, $Cx43^{MK4D}$/ $Cx43^{CTMK4D}$) See **Online Figure IV.** To produce Cx43CT constructs used for transfection of $Cx43^{-/-}$ cells a stop codon was inserted immediately 5' of a.a. 382 of the $Cx43^{CT}$, $Cx43^{CTMK4A}$ and $Cx43^{CTMK4D}$ constructs by site directed mutagenesis. Constructs were then excised using BamH1 and Xho1 from the pGEX-6P-2 vector and ligated to the pcDNA3.1 vector. Following mutagenesis, plasmids were sequenced using primers designed against the T7 promoter in the pcDNA3.1 vector and against Cx43 (for full length Cx43) or against the pGEX expression vector or the T7 promoter for $Cx43^{CT}$ plasmids.

Bacterial protein expression and purification: Connexin 43^{CT} proteins were purified as described,[5] with the following alterations to the protocol. Plasmids were transformed into BL21 competent bacterial cells. Bacterial clones grown in 2 L of LB broth were induced at 0.5 OD_{600} with 1 mM isopropyl-1-thio-B-D-galactopyranoside (IPTG) for 3 hours. Bacteria were pelleted and re-suspended (1 g/ 5 mL) in protein buffer: PBS (pH 7.4); NaCl (190 mM); dithiothreitol (DTT, 1mM); Pefabloc (0.1 mM) and complete protease inhibitor cocktail (1:100, Sigma) then stored at -80 °C. Cells were thawed and membranes disrupted by microfluidics then lysates cleared by centrifugation (100,000 x *g*, 1 hour, 4°C). Supernatants were incubated with 4 mL of gluthatione-sepharose beads (Glutathione-sepharose 4 fast flow, GE Lifesciences) for 3 hours, flow rate 0.5 mL/min at 4°C. Protein bound beads were washed with 40 column volumes of protein buffer, followed by 20 column volumes of protein buffer without Pefabloc or protease inhibitors and GST cleavage performed by incubation with PreScission (80U) at 4°C overnight with rocking. Eluted $Cx43^{CT}$ proteins were further purified by size exclusion (320 mL Superdex 75 size exclusion column, GE Healthcare Bio-Sciences Corp., Piscataway, NJ) to greater than 90% purity. Eluted $Cx43^{CT}$ samples were collected and concentrated using Amicon centriplus 10 filters.

Cyclin E was prepared as described[7] with the following modifications. Cyclin E transformed BL21 bacterial cells were grown overnight then media inoculated to 0.1 OD. Bacteria were grown in 4 L to 1.0 OD at 37 °C, chilled to 16 °C on ice, grown at 16 °C for 1 hour then protein expression induced with ITPG (0.1 mM) for 24 hours at 16 °C. Bacteria were then pelleted and re-suspended in protein buffer and protein purification was performed as described for $Cx43^{CT}$ proteins except cyclin E-GST was eluted from the beads (without cleavage) in a solution of PBS plus L-glutathione (1 mM) prior to size exclusion purification (as above).

Analytical size exclusion chromatography (ANSEC): Prior to analysis, sample buffers were altered to pH 6.5, KCl (40 mM) with no DTT using PD10 de-salting columns (GE Lifesciences) followed by concentration of proteins to approximately 15-20 mg/mL. Proteins analyzed by ANSEC were at a final concentration of 350 μM for $Cx43^{CT}$ and 75 μM for cyclin E-GST with a molar ratio of 4.7:1 (43^{CT}: cyclin E-GST) for combined samples. Samples were run on a Bio-Silect SEC 250-5 column with a bed volume of 14 mL (300x7.8 mm, Biorad; Hercules, CA) either as single proteins or as $Cx43^{CT}$ + cyclin E-GST co-incubated for 5 minutes prior to loading on ANSEC column. Eluted fractions from the column were analyzed by Western Blot for the presence of Cx43 and cyclin E. Molecular weight standards of blue dextran (~2,000 kDa; void volume), alcohol dehydrogenase (150 kDa) and albumin (66 kDa) were run using the above conditions and eluted in fractions 11, 16 and 17, respectively.

Cell culture, total protein isolations: Following treatments, all cells were harvested in cold lysis buffer 1: PBS (pH 7.4) containing: NaCl (125 mM); EDTA (5 mM); sodium deoxycholate (1%); triton X-100 (0.5%); sodium orthavanadate (500 μM); AEBSF (10 μM); protease inhibitor cocktail (1:100, Sigma) and phosphatase inhibitor cocktail 2 + 3 (1:100, Sigma). All isolations were performed at 4 °C, samples were dounced 30 times on ice, incubated with rotation for 30 mins at 4 °C and centrifuged at 14,000g for 5 minutes. Cleared lysates were used for Western blot and co-immunoprecipitation analysis. For experiments investigating Retinoblastoma (Rb) and its phosphorylation by Western blot, total protein lysates were sonicated immediately following harvest in lysis buffer 1 and used without centrifugation. Proteins samples were quantified by bradfords assay prior to loading and equal loading confirmed using GAPDH or □-tubulin.

Cell culture, membrane preparations: Membrane fractions were harvested as previously described.[2] Briefly, following treatments all cells were harvested in lysis buffer 2: PBS (pH 7.4)

containing: NaCl (125 mM); sodium orthavanadate (500 μM); AEBSF (10 μM); protease inhibitor cocktail (1:100, Sigma) and phosphatase inhibitor cocktail 2 + 3 (1:100, Sigma). All isolations were performed at 4 °C, samples were dounced 30 times on ice, incubated with rotation for 30 mins at 4 °C and centrifuged at 14,000g for 5 minutes. Cleared lysates were then centrifuged at 100,000g at 4 °C for 1 hour and pellets (membrane fractions) resuspended in lysis buffer 1. Harvested membrane fractions were dounced 30 times on ice, incubated with rotation for 30 mins at 4 °C and centrifuged at 14,000g for 5 minutes. Cleared lysates were used for Western blot and co-immunoprecipitation analysis. Proteins were quantified by bradfords assay prior to loading and equal loading confirmed on Western Blot using GAPDH as previously described for membrane proteins.[8] Membrane sample purity was determined by through lack of detection of □-tubulin by Western blot.

Co-immunoprecipitation: Vascular smooth muscle cells ($Cx43^{+/+}$) were switched to low serum media for 72 hours prior to treatment with PDGF (10 ng/mL) for 24 hours. The VSMC isolated from $Cx43^{-/-}$ in growth medium were transfected by Nucleofector (Lonza) using 10 μg of each plasmids for 18 hours followed by incubation with PDGF for 24 hours. Following treatments all cells were harvested as described above for total protein lysates or membrane proteins. Dynabeads coated with antibodies for either Cx43, cyclin E or CDK 2 were incubated with lysates for 3 hours at 4 °C followed by 4 washes in lysis buffer 1 prior to elution of bound proteins from the beads using 5x lamelli buffer for Western Blot analysis (as described).[9]

Scrape load dye transfer (SLDT): Cells stalled were transfected (as above, using Lipofectamine) then treated with PDGF (10 ng/mL), carbenoxolone (25 □M) plus PDGF, or no treatment (control). Twenty four hours after treatment, lucifer yellow (0.5 mg/mL in PBS) was applied to cells then a scrape was created using a 23G needle, with 3 scrapes per well. After 10 minute incubation at 37°C, cells were washed in PBS and fixed for 5 mins in 4% PFA (as described)[10]. Images of dye transfer were captured on an Olympus FVX with a 20x 0.6 NA water immersion lens. Distance of dye transfer was quantified from the wound edge using MetaMorph imaging software.

Cell Treatments: For inhibition of Cx43:cyclin interactions, cell cycle stalled $Cx43^{+/+}$ VSMC were pre-treated with Erk inhibitors, U0126 (10 μM, VWR,[11] kindly provided Dr Norbert Leitinger) or Roscovitine (15 μM, Selleck)[12] or with DMSO (1 μl/mL, control) prior to treatment with PDGF (10 μg/mL).

Antibodies: Immunocytochemistry, i-TEM and Western blot analysis of samples were performed using antibodies against: Cx43 (polyclonal, Sigma);[2] MAPK phosphorylated-Cx43 (Cx43-P, polyclonal);[2] cyclin E (monoclonal and polyclonal Abacam);[13, 14] cyclin D1 (monoclonal Abcam);[15] p21 (monoclonal, Santa Cruz);[16] p27 (monoclonal, Santa Cruz);[17] CDK2 (monoclonal, BD biosciences);[18] eNOS (polyclonal, BD biosciences);[19] prolyl-4-hydroxylase (polyclonal, Abcam);[20] SM α-actin (polyclonal, sigma);[21] SM-22α (polyclonal, Abcam);[22] total Retinoblastoma (Rb clone 4H1, polclonal Cell Signaling);[23] phosphorylated Rb^{780} (monoclonal Cell Signalling);[24] phosphorylated $Rb^{807/811}$ (monoclonal, Cell Signalling).[25] Antibodies were visualized with donkey anti-rabbit or anti-mouse Alexa 594/ Alexa 488 for immunocytochemistry or using 680/800 nm conjugated secondary antibodies (LI-COR) for Western blotting.

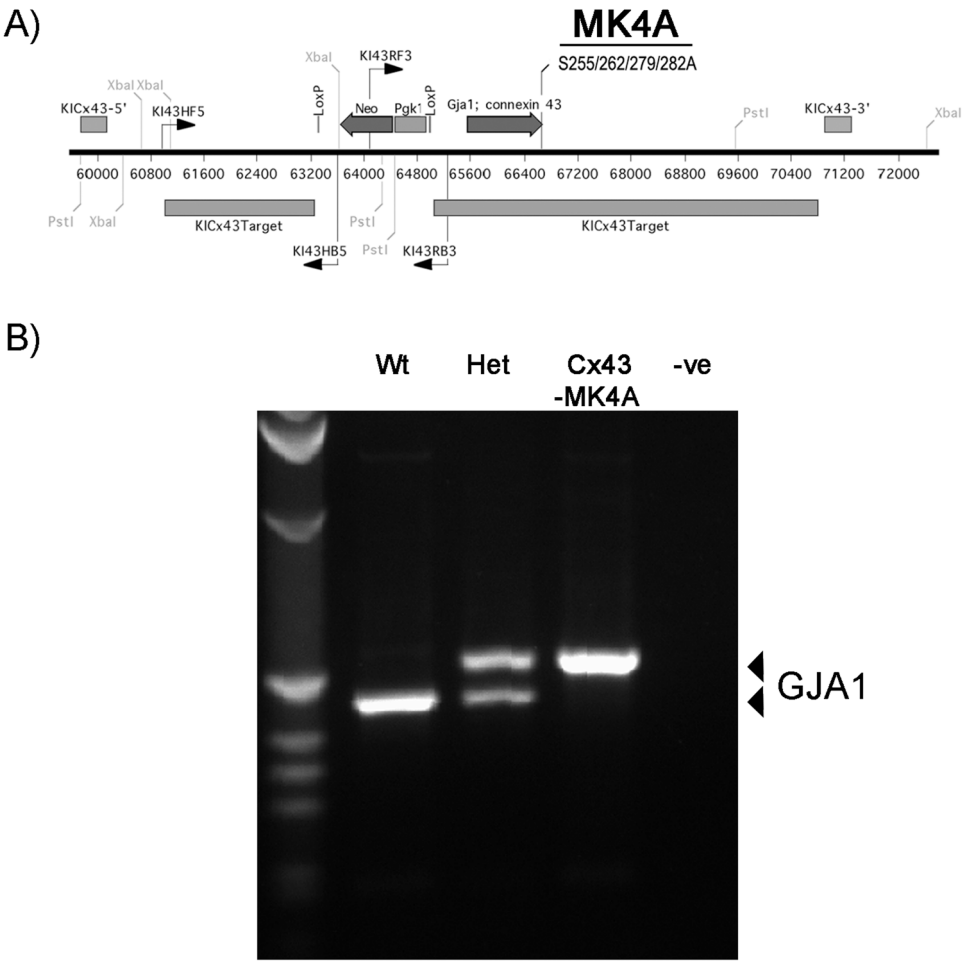
Immunocytochemistry: All immunofluorescence was performed as described.[26] In all images Cx43 and cyclin E were detected using secondary antibodies coupled to Alexa 594 and Alexa 488 respectively with nuclei identified through 4',6-diamidino-2-phenylindole (DAPI) staining. For quantification of pixel intensities in carotids, regions corresponding to VSMC layers were placed between layers of elastic lamina using MetaMorph imaging software (as described).[2, 3] In

each image, at least three areas of VSMC were recorded; at least three images were used per mouse per treatment (n = 4).

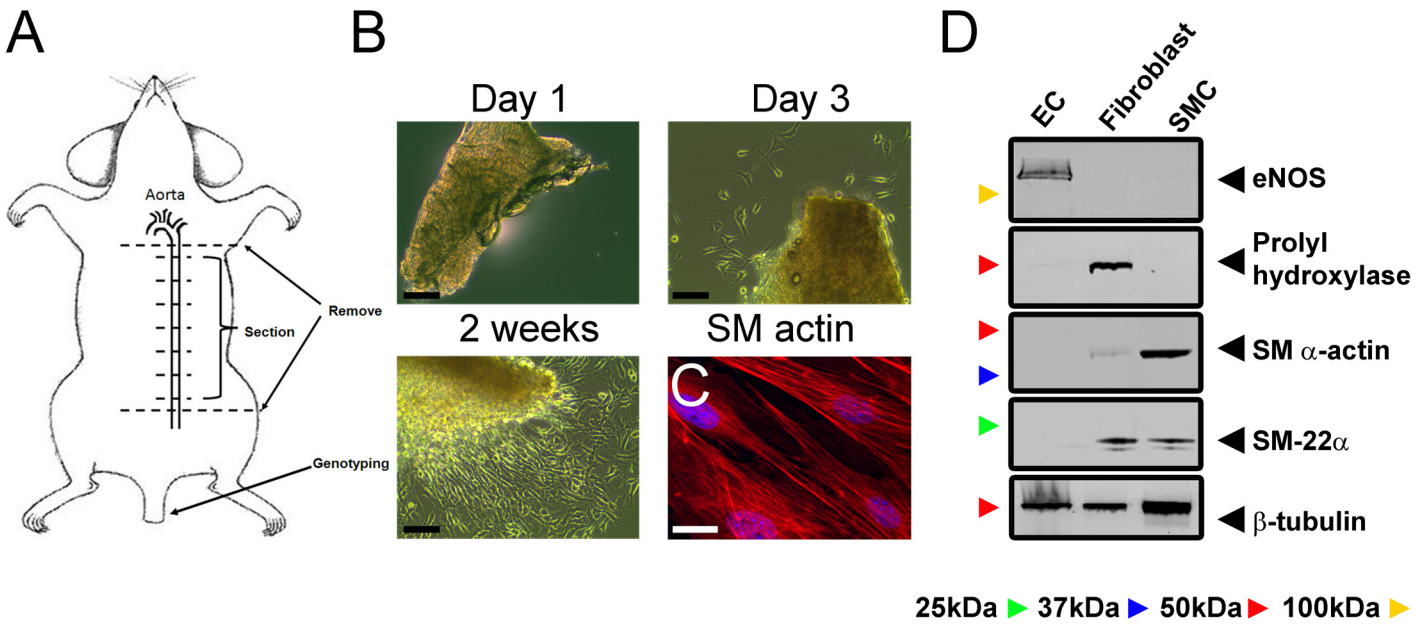
Transmission electron microscopy: Carotid samples (isolated as above) were analyzed by TEM or i-TEM as previously described.[2, 26] Analysis of protein interactions on i-TEM was performed using differing sized immuno-gold beads to detect: Cx43/ Cx43-P (25 nm), CDK2 (15 nm) and cyclin E (10 nm). To determine the distance relationship between the proteins, the distance between the edges of each bead type and its closest surrounding bead was measured using Metamorph imaging software. An n=20 interactions was measured for each treatment with representative images shown.

Statistical analysis: 1-way or 2-way ANOVA followed by Bonferroni's post-test were used for comparisons between 3 treatment groups and student t-test used for comparisons of 2 treatment groups. A P value of <0.05 was significant, in all images * is $P < 0.05$, ** is $P<0.01$ and *** is $P< 0.001$.

Online Figure I



Online Figure II



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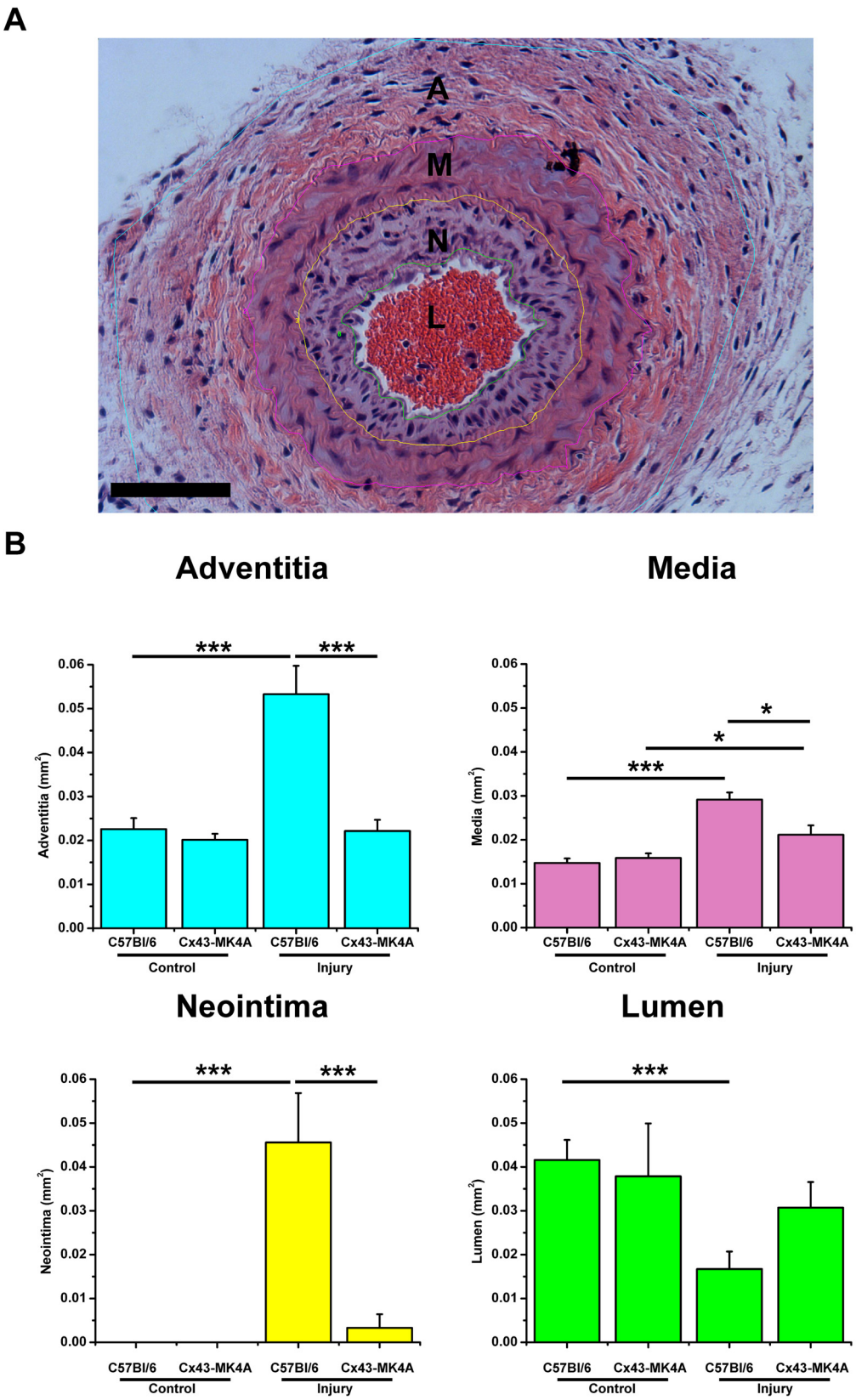

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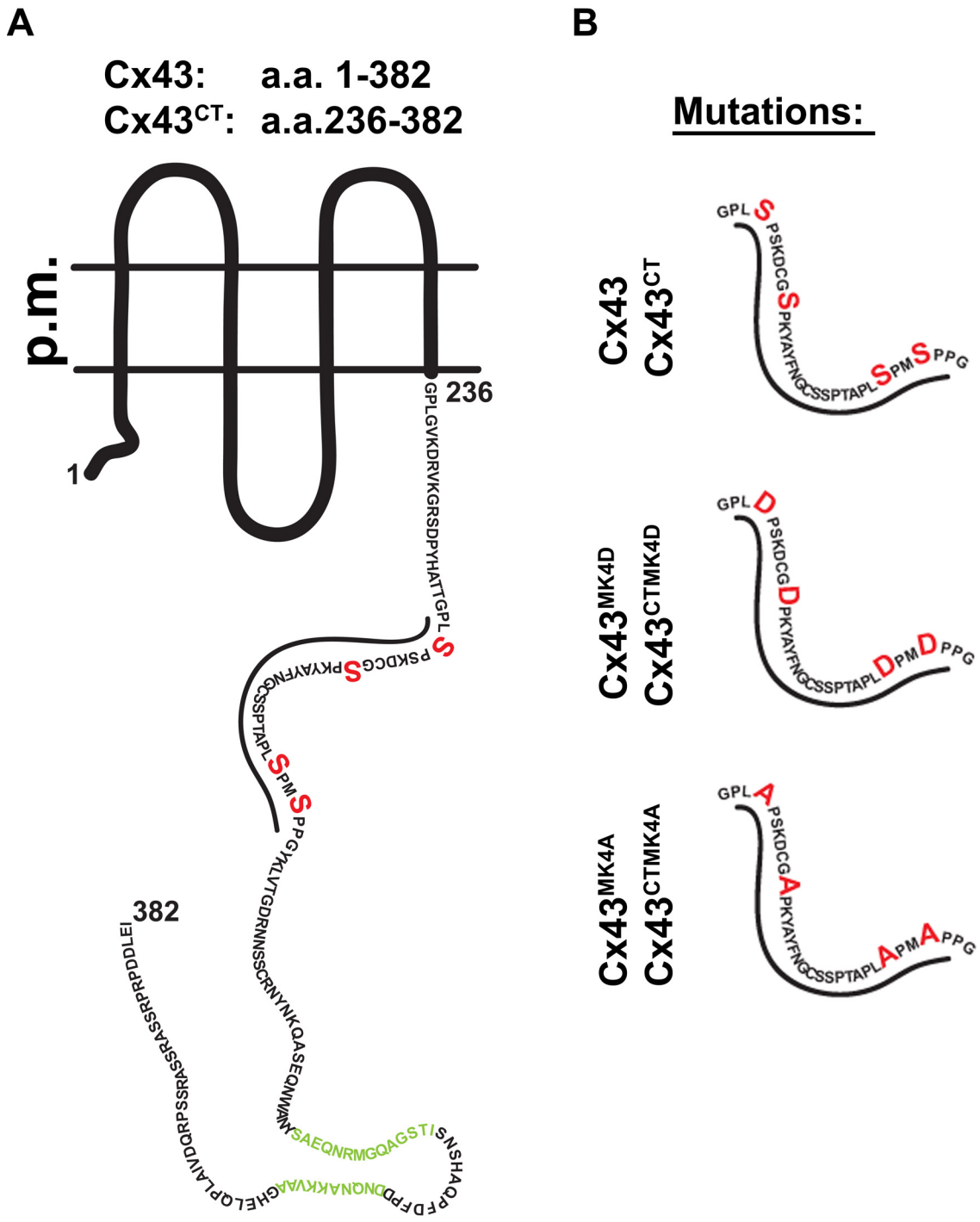

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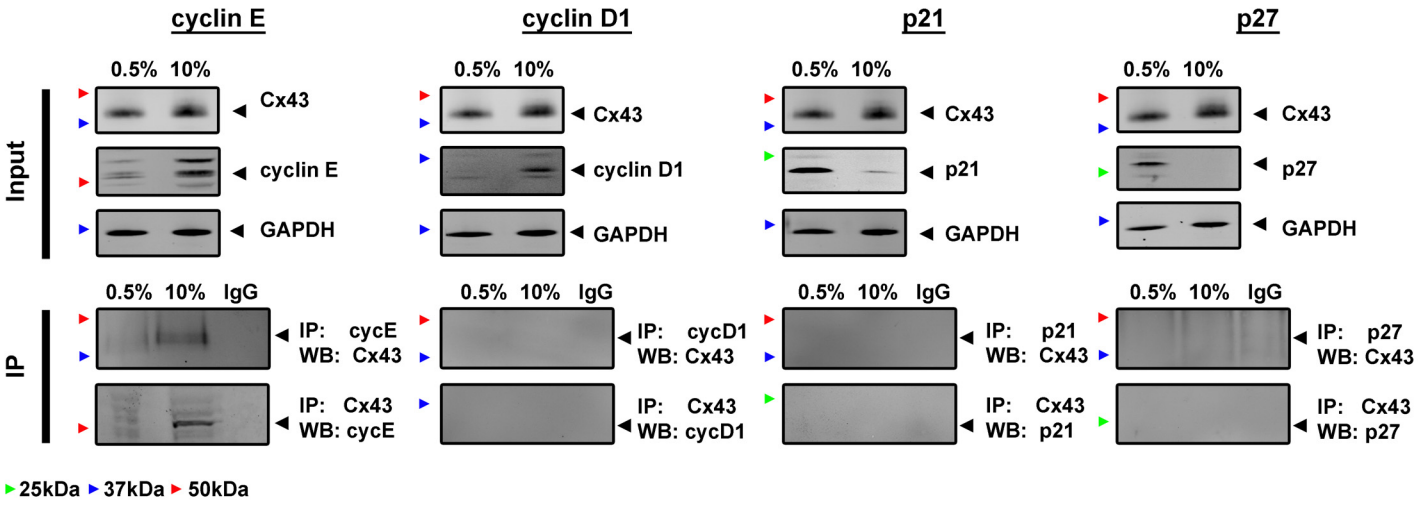
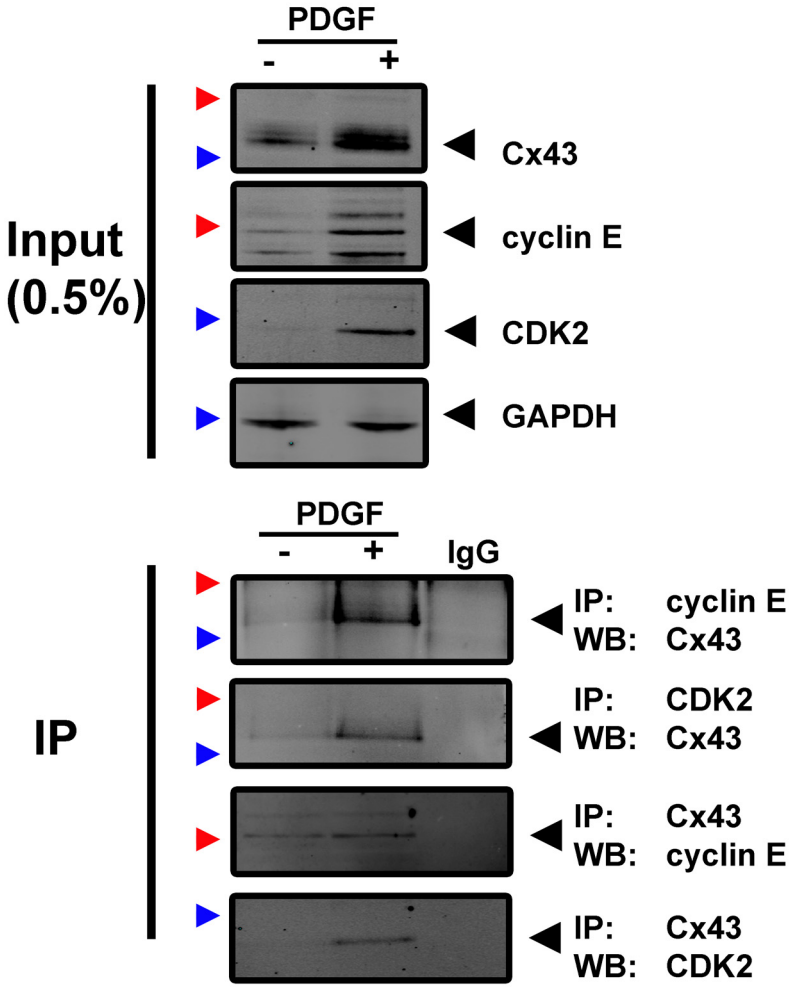
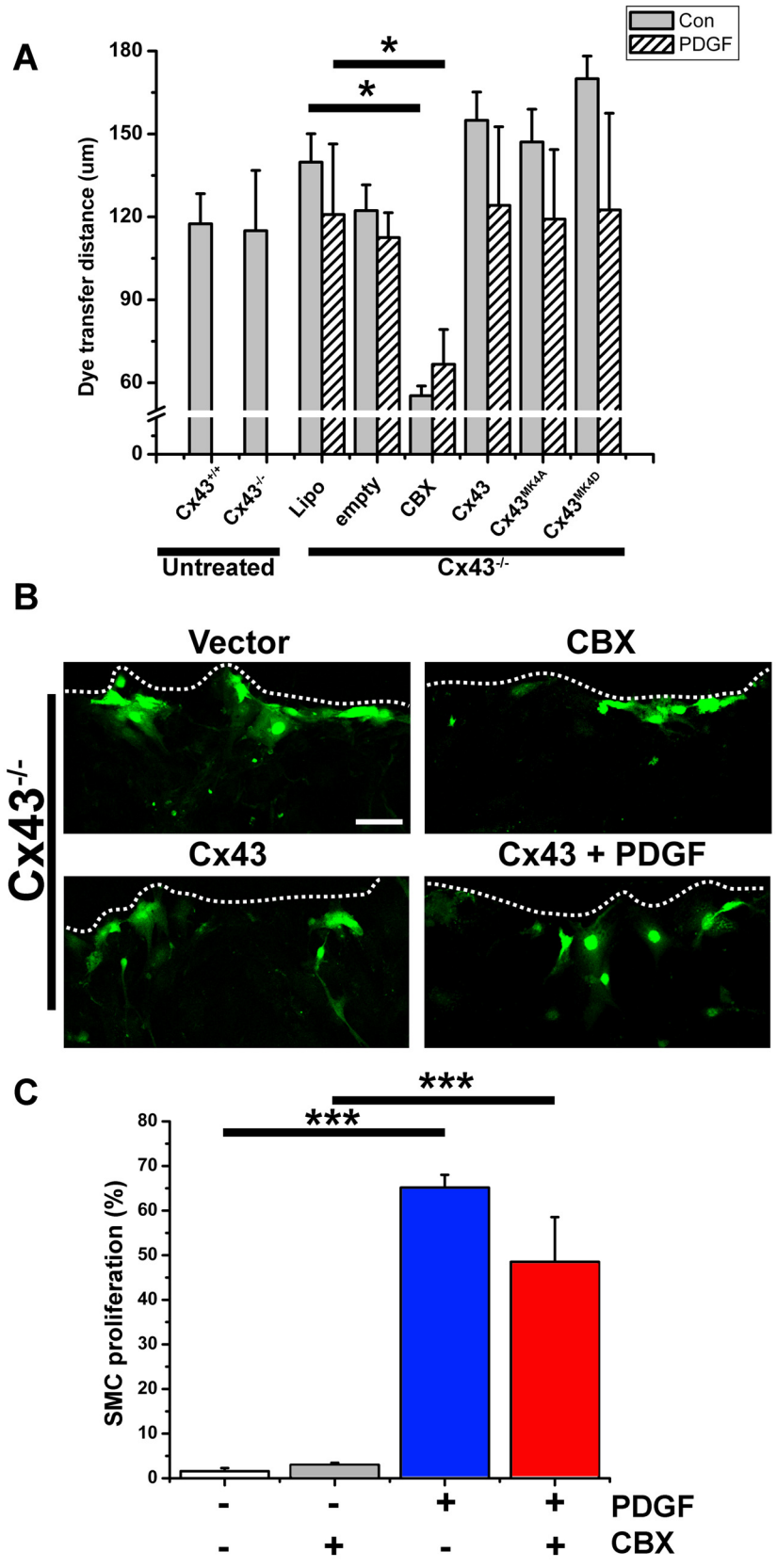


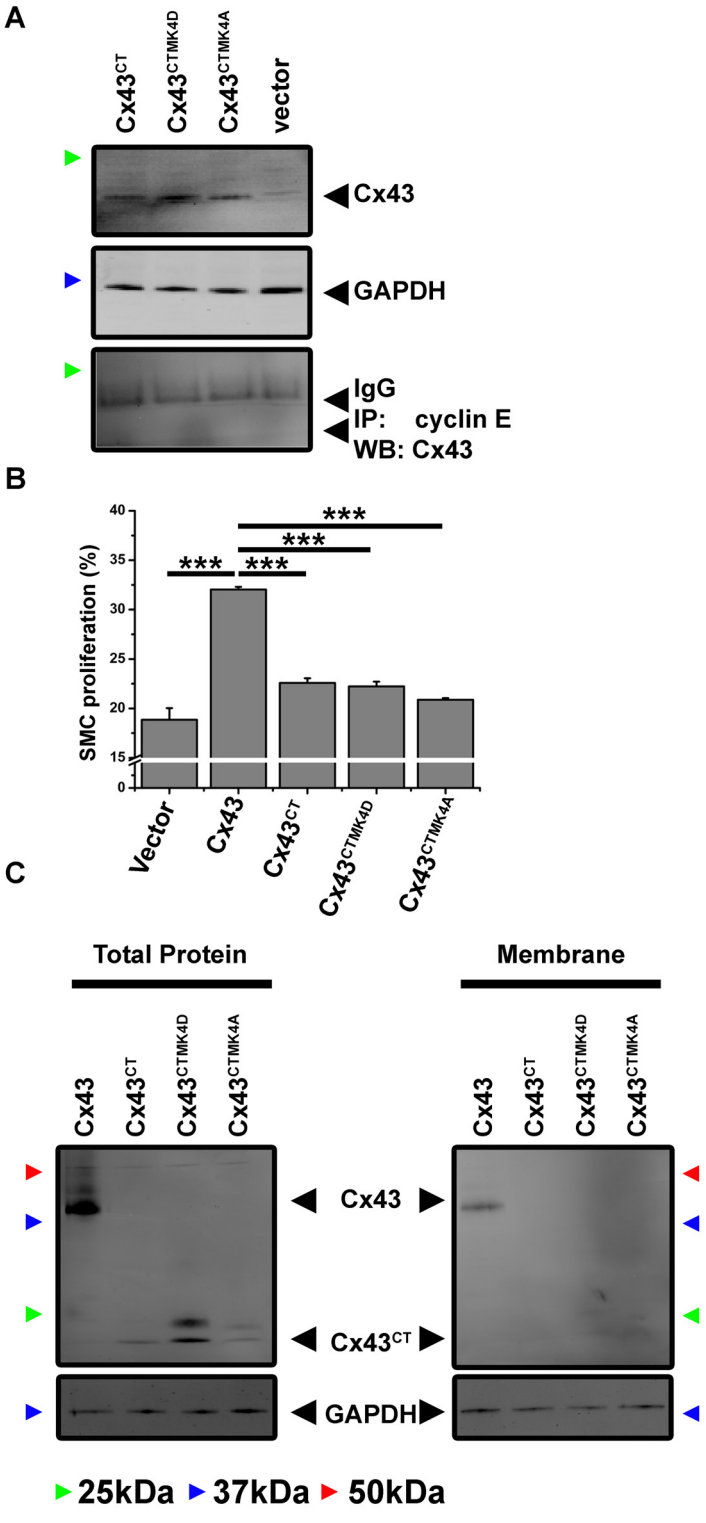
Online Figure VI



Online Figure VII



Online Figure VIII



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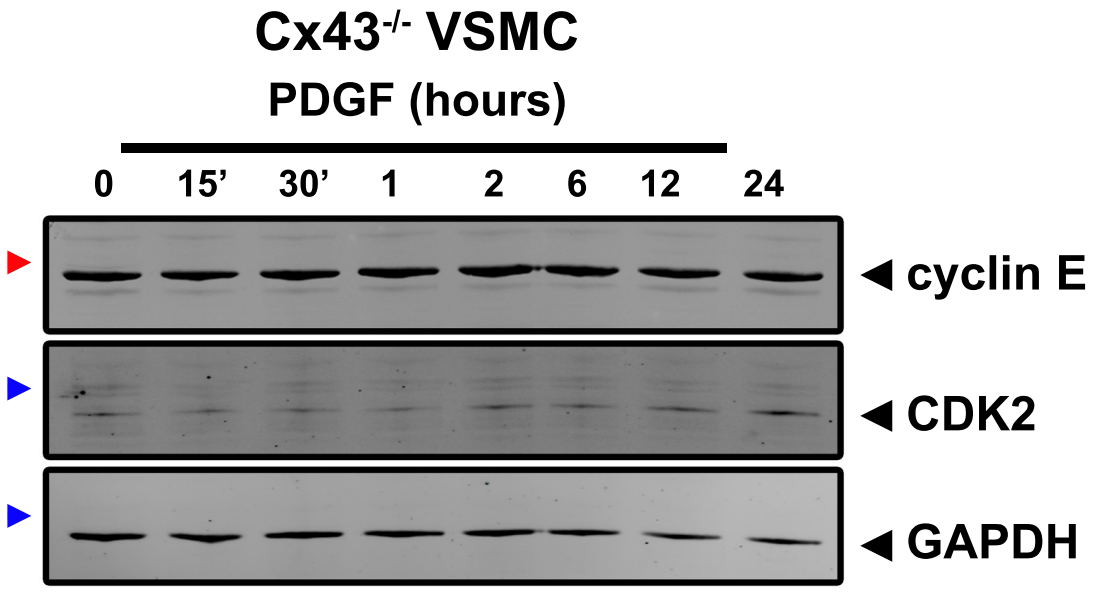
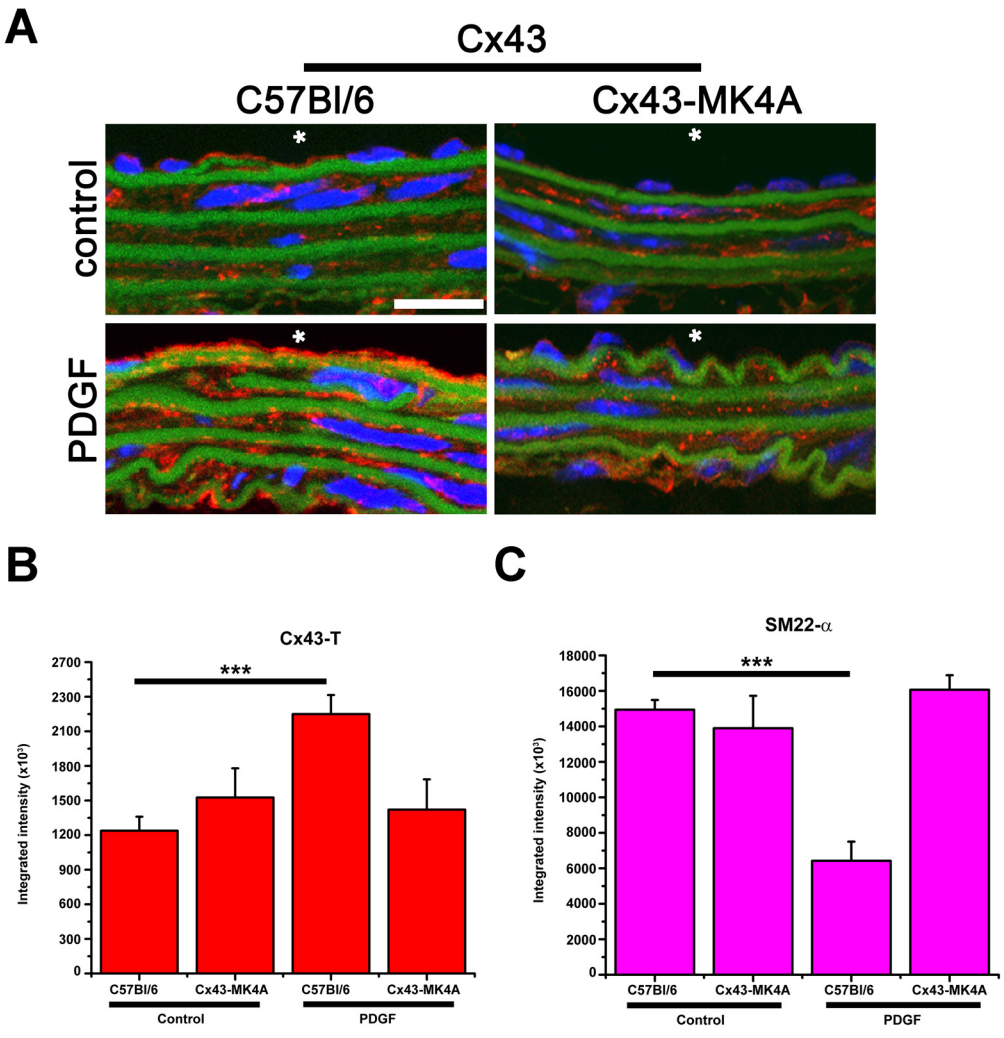


Online Figure X



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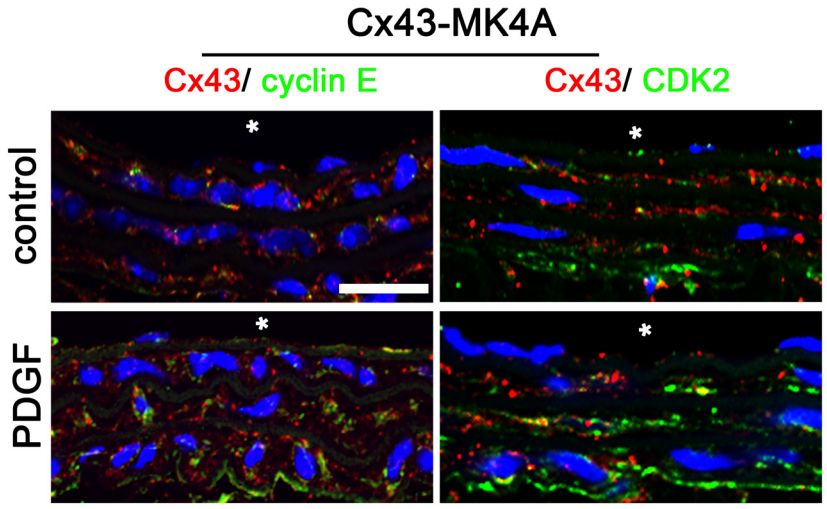

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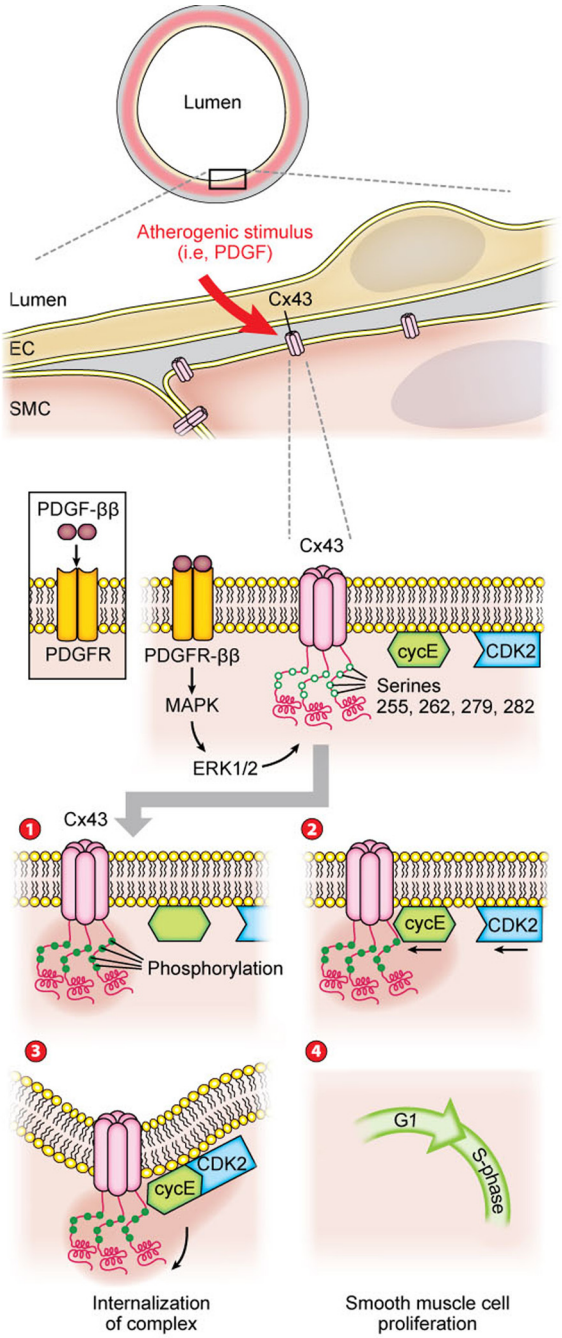

Online Table I

Cx43 Input		
n=3	**mean**	**SE**
Empty	0	0
Cx43	5.07524	0.46426
Cx43^{MK4D}	4.37382	0.50823
Cx43^{MK4A}	4.75226	0.8343

Cx43 IP		
n=3	**mean**	**SE**
Empty	0	0
Cx43	4.77667	2.33989
Cx43^{MK4D}	4.22333	2.35404
Cx43^{MK4A}	0	0

Ratio (IP:Input)		
n=3	**mean**	**SE**
Empty	0	0
Cx43	1.0063	0.50397
Cx43^{MK4D}	1.0758	0.67533
Cx43^{MK4A}	0	0

Online Figure Legends

Supplementary Figure I: Generation of Cx43-MK4A mice: In **A**, the scheme for the knock-in vector highlights site of insertion of Cx43 knock in (KI) coding sequence including the mutations (S255A, S262A, S279A and S282A). PCR analysis of DNA samples taken from wild type (wt), heterozygous and Cx43-MK4A demonstrates the slower migrating band that is seen for Cx43-MK4A construct (**B**).

Supplementary Figure II: Isolation of aortic VSMC. Newborn mice (around 2 hours old) taken from breeding pairs of $Cx43^{+/-}$ mice were euthanized by decapitation and the descending aortas removed (**A**). Explants were plated in supplemented Amniomax media and VSMC sprouting observed over a 2 week period before removal of explants and re-plating of VSMC (Passage 1, **B**). Cells were confirmed to be VSMC by immunofluorescence based on the presence of SM α-actin (Red, **C**) with nuclei labeled using DAPI (Blue, **C**). In **B** the scale bars are: top left is 200 μm, top right and lower left are 50 μm and in **C** the scale bar is 10 □m. Cells isolated from newborn mouse aortas of C57Bl/6 mice were confirmed to be VSMC through the absence of the endothelial cell marker eNOS as shown in endothelial cells (EC, HUVEC cells), the absence of fibroblast marker prolyl-4-hydroxylase as shown in primary human fibroblasts (Fibroblast) and by the presence of smooth muscle markers SM α-actin and SM-22α (**D**). These studies identified that cells isolated from the aortas of newborn mice were primarily VSMC. In **D**, black arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25 kDa, blue is 37 kDa, red is 50 kDa, Yellow is 100 kDa.

Supplemental Figure III: Vessel wall measurements in carotid ligation mice. Areas corresponding to the adventitia (A), media (M), neointima (N) and lumen (L) were measured on H&E sections from control and injury mice for C57Bl/6 and Cx43-MK4A mice with MetaMorph imaging software (**A**). In **A** the scale bar is 100 μm. Areas were converted from pixels to mm^2 and statistical analysis performed comparing controls versus injury in C57Bl/6 and Cx43-MK4A mice, n=7 for each condition (**B**). In C57Bl/6 mice following injury, we observed increases in adventitial and media thicknesses as well as the development of neointima and consequently reductions in lumen diameter. In Cx43-MK4A mice, we did not observe thickening of the adventitial layer or significant formation of neointima (**B**). However, alterations within the media layer were evident with an increased media thickness, which was significantly less than in C57Bl/6 mice (**B**). In graphs, "*" indicates $P<0.05$ and "***" indicates $P<0.001$ (**B**, n=7)

Supplemental Figure IV: Plasmid generation and mutagenesis. Plasmids for full length Cx43 (Cx43, a.a. 1-382, pCDNA 3.1) or Cx43 C-terminus ($Cx43^{CT}$, a.a. 236-382, pCDNA3.1 or p-GEX6P2) (**A**) were sequenced then mutagenesis performed to produce the phospho-mimetic form (Aspartate, D) or null phosphorylation forms (Alanine, A) at the MAPK serines representative of S255, S262, S279 and S282 in the C-terminus (**B**). Amino acid regions highlighted in green show the known α-helical regions within the Cx43 C-terminus.

Supplementary Figure V: Cx43 interacts with cyclin E in aortic smooth muscle cells. Mouse aortic VSMC ($Cx43^{+/+}$) were stalled in 0.5% serum media for 72 hours followed by 24 hours in either 0.5% or 10% serum as indicated. Cells were lysed in lysis buffer 1 and cleared lysates incubated with Dynabeads coated with Cx43, cyclin E, cyclin D1, $p21^{waf1/cip1}$ or $p27^{kip1}$ antibodies as labeled. Proteins eluted from beads were analyzed by Western blot using antibodies directed against Cx43, cyclin D1, cyclin E, $p21^{waf1/cip1}$, $p27^{kip1}$. Representative Western Blots (input) demonstrate that in cells treated with 10% serum, expression levels of Cx43, cyclin E and CDK2 are increased, whereas expression of the cell cycle inhibitors $p21^{waf1/cip1}$, $p27^{kip1}$ are decreased. Co-Immunoprecipitation (IP) studies demonstrate that of the proteins tested only Cx43 and cyclin E form in complex in proliferating cells (10% serum). Black

arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25kDa, blue is 37 kDa, red is 50kDa.

Supplemental Figure VI: PDGF promotes formation of complexes between Cx43, cyclin E and CDK2 in vitro. Mouse aortic VSMC ($Cx43^{+/+}$) were stalled in 0.5% serum media for 72 hours followed by 24 hours in either 0.5% or 10 ng/mL PDGF in 0.5% serum as indicated. Cells were lysed in lysis buffer 1 and cleared lysates were incubated with dynabeads coated with Cx43, cyclin E or CDK2 antibodies as labeled. Proteins eluted from beads were analyzed by Western blot using antibodies directed against Cx43, cyclin E or CDK2. Representative Western Blots (input) demonstrate that in cells treated with PDGF, expression levels of Cx43, cyclin E and CDK2 are increased. Co-Immunoprecipitation (IP) studies demonstrate that Cx43, cyclin E and CDK2 form in complex in PDGF treated cells. Black arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25kDa, blue is 37 kDa, red is 50kDa.

Supplemental Figure VII: Effects of transfection on gap junction communication. Confluent monolayers of $Cx43^{+/+}$ or $Cx43^{-/-}$ cells in low serum were transfected with Cx43 plasmids as described in supplemental materials and lucifer yellow dye transfer was assessed by SLDT in control (untreated) or PDGF treated cells (**A**). In both untreated $Cx43^{+/+}$ and $Cx43^{-/-}$ cells, dye transfer from the edge of the wound to surrounding cells was identified. Transfection of $Cx43^{-/-}$ cells with plasmids containing full length Cx43 did not produce significant alterations in dye transfer in untreated or PDGF treated conditions (**A-B**). Reductions in dye transfer were observed following treatments of $Cx43^{-/-}$ cells with the gap junctional inhibitor CBX (25 □M) in both untreated and PDGF treated cells (**A**). In **B** representative images show dye transfer in vector transfected, CBX treated $Cx43^{-/-}$ cells and in untreated and PDGF treated Cx43 transfected $Cx43^{-/-}$ cells. In B the dashed line represents the edge of the scrape, scale bar is 40 □m. $Cx43^{+/+}$ cells pre-treated CBX (25 □M) did not demonstrate significantly reduce VSMC proliferation following treatment with PDGF as compared to PDGF treated (**C**). In graphs, "*" indicates P<0.05 and "***" indicates P<0.001 (**A**, n=3 and **C**, n=6)

Supplemental Figure VIII: Expression of free Cx43CT proteins in $Cx43^{-/-}$ VSMC. Mouse aortic VSMC ($Cx43^{-/-}$) in low serum were transfected with $Cx43^{CT}$ plasmids as described in supplemental materials. Following PDGF treatments cells were lysed in lysis buffer 1 and cleared lysates were incubated with dynabeads coated with cyclin E antibodies. Proteins eluted from beads were analyzed by Western blot using antibodies directed against Cx43. Representative Western Blots (input) demonstrate that free $Cx43^{CT}$, $Cx43^{CTMK4A}$, $Cx43^{CTMK4D}$ proteins can be expressed in the $Cx43^{-/-}$ cells. Co-Immunoprecipitation (IP) studies demonstrate that free $Cx43^{CT}$ proteins do not co-precipitate with cyclin E in PDGF treated $Cx43^{-/-}$ cells (**A**). VSMC proliferation was increased in $Cx43^{-/-}$ cells transfected to express full length Cx43 but not free $Cx43^{CT}$, $Cx43^{CTMK4A}$, $Cx43^{CTMK4D}$ proteins in response to PDGF treatments (**B**). Comparisons of proteins isolated form transfected $Cx43^{-/-}$ cells demonstrate the both full length and free $Cx43^{CT}$ proteins can be identified in total protein lysates but only full length proteins can be identified in the cell membranes (**C**). In **A** and **C**, black arrowheads identify the expected molecular weight for each protein, colored arrowheads correspond to molecular weights: green is 25kDa, blue is 37 kDa, red is 50kDa. In graphs "***" indicates P<0.001 (**B**, n=4)

Supplemental Figure IX: Effects of PDGF treatments on cyclin E and CDK2 in $Cx43^{-/-}$ cells. Mouse aortic VSMC ($Cx43^{-/-}$) grown in low serum for 72 hours were treated with PDGF and harvested at specific timepoints over the course of 24 hours as described in supplemental materials and methods. Cells were lysed in lysis buffer 1 and cleared protein lysates were analyzed by Western Blot for cyclin E an CDK2 expression. PDGF treatments did not produce notable increases in cyclin E expression but CDK2 proteins were found to be increased by 24 hours.

Supplemental Figure X: Cx43 expression in Cx43-MK4A carotid VSMC. Carotid sections were analyzed by immunofluorescence for protein expression using antibodies directed against Cx43. In each image red represents Cx43, green represents internal elastic lamina, blue indicates nuclei (DAPI) and "*" represents the luminal side of the vessels. Scale bar is 20 □m. Immunofluorescence quantification of Cx43 (**B**) and SM22-α (**C**) from carotid VSMC layers. Treatment of carotids with PDGF increases Cx43 expression in VSMC from C57Bl/6 but not Cx43-MK4A mice (n=4).

Supplemental Figure XI: Cx43 interactions in Cx43-MK4A carotid VSMC. Carotid sections from Cx43-MK4A mice were analyzed by immunofluorescence for protein expression and co-localization using antibodies directed against Cx43, cyclin E or CDK2. In each image red represents Cx43, green represents either cyclin E or CDK2 (as labeled), blue indicates nuclei (DAPI) and "*" represents the luminal side of the vessels, scale bar is 20 □m. No co-localization (orange) was detected in either control or treated vessels from Cx43-MK4A mice.

Supplemental Figure XII: Schematic of Cx43 phosphorylation and control of VSMC proliferation. VSMC proliferation in atherogenesis is associated to increases in PDGF and alterations in the expression of Cx43 (**A**). PDGF which acts via its receptor activates an ERK signaling pathway that induces phosphorylation of Cx43 at the MAPK serines (**B**). Following phosphorylation of Cx43 MAPK serines (**C1**), both cyclin E and CDK2 interact with the C-terminus of Cx43 (**C2**). Following interactions, the protein complex becomes internalized (**C3**). The interaction of Cx43 and cyclin E is critical in the regulation of VSMC proliferation (**C4**).

Online Table I: Quantification of Cx43 expression in transfected Cx43$^{-/-}$ cells. Western blot band intensities for Cx43 protein in transfected (**top**) and transfected co-immunprecipitation (**middle**) samples were used to calculate a ratio (IP: Input, **bottom**) for each sample. Samples were quantified using Licor Odyssey imaging software and analysis performed by Origin Pro software (n=3).

Supplemental Method References

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