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Supplemental Material

Supplemental Methods

Generation of the *Cx40$^{fl/fl}$* mice

A genomic clone derived from 129SV mice (9 kb HindIII-HindIII fragment, a kind gift of Dr. David Paul, Harvard Medical School, Boston, MA) and spanning the Cx40 exon 2 region was subcloned in pBluescript-KS. The gene-targeting construct was generated by replacing the HindIII-ScaI fragment, by the LoxP – *neo*^{r} – LoxP – Cx40 ORF - LoxP cassette including Cx40 exon 2 upstream (Hind III-PmlI) and downstream (SnaI-SnaI) regions available for homologous recombination (Figure S1A). Transfection, selection (Figure S1B), and screening were performed as described by Verbeek et al. (1995)[1] except that the selection medium contained 300 µg/ml neomycin and, in a subsequent selection round, 300 µg/ml G418. Clones bearing a targeted Cx40 allele were identified by Southern blot analysis of KpnI-digested genomic DNA using an exon 2-spanning probe. Hybridizing bands of 4 kb or 5.3 kb indicate the presence of Cx40 wild-type or Cx40 mutant alleles, respectively. From 900 *neo*^{r} clones isolated, 17 (2%) had acquired the ne*o*^{r} insertion by homologous recombination. Of these clones, 4 did not show random integration; i.e. these 4 clones were killed by the G418 selection and were propagated from duplicate plates. Two clones were selected for microinjection of day 3.5 C57BL/6 embryos. Of these two clones, chromosomal counts were performed (Figure S1C) and found normal. Male chimeric offspring were bred with C57BL/6 females and the tail DNA of agouti offspring was analyzed for the presence of the mutated allele. Tail DNA from the offspring was analyzed by PCR for the presence of the mutated allele. The following primers were used: mCx40-46: 5′-GTGACATGACCTGGATCTCTGGAG-3′ and mCx40-53: 5’-GCCATCCTCTGCTACATATGCAG-3’; as well as the nested set mCx40-46a: 5’-

GTTAGAATCAATCCGACTCAC-3’ and mCx40-53a: 5′- GCAGCTAGAGCCATGAGTC , giving rise to a 555 bp (wild-type allele) and a 697 bp (mutated allele; including loxP) band. Heterozygous offspring carrying the mutation were backcrossed for two generations with Balb/c animals (Charles River, Iffa Credo, Lyon) and subsequently intercrossed. Homozygous *Cx40*$^{fl/fl}$ and wild-type littermates were selected to establish two independent *Cx40*$^{fl/fl}$ mouse strains with indistinguishable phenotype and the proper (Balb/c/Ola129) wild-type control strain. Then, *Cx40*$^{fl/fl}$ mice were backcrossed for 12 generations to the C57BL/6 background. Efficacy of *in vivo* recombination was first tested by crossing *Cx40*$^{fl/fl}$ mice with Cre-deleter mice.[2] PCR was performed on genomic DNA isolated from tail-tips to evaluate excision of exon2 of Cx40 and presence of Cre. Offspring of this crossing was heterozygous for the floxed Cx40 allele and half of the population expressed Cre. Figure S1D shows that mice that lack expression of Cre (lanes 3-5, 8 and 9) show the typical two banded pattern of wild-type (555 bp) and floxed Cx40 (697 bp). However, in mice expressing Cre (lanes 1,2 and 6,7) only the wild-typeCx40 band was detected while the higher band of 697 bp was absent, indicative of effective recombination.

Atherosclerosis-susceptible mice with endothelial-specific deletion of Cx40 and EGFP mice

For deletion of the *Cx40* gene specifically in endothelium *in vivo*, we used a mouse strain expressing the Cre under control of *Tie2* transcriptional elements.[3] Endothelial specificity of recombination using this strain has been described previously.[4] Thus, we interbred *Cx40*$^{fl/fl}$ mice with atherosclerosis-susceptible *ApoE*$^{-/-}$ mice to generate *Cx40*$^{fl/fl}$ *ApoE*$^{-/-}$ mice (control group 2; C2). Of note, wild-type mice do not develop advanced atherosclerosis. Interbreeding of *ApoE*$^{-/-}$ mice with mice harboring the Cre recombinase coding sequence under the control of the endothelial-specific Tie2 promoter resulted in *Tie2Cre*$^{+}$ *ApoE*$^{-/-}$ mice (control group 1;

C1). Finally, groups C1 and C2 were interbred to generate the *Tie2Cre*$^{+}$ *Cx40*$^{fl/fl}$ *ApoE*$^{-/-}$ mice (Cx40del group). Genotypes of mice were checked by PCR using above described protocols.

Transgenic mice expressing ubiquitously the enhanced green fluorescent protein (EGFP) by a CMV-β-actin promoter or mice in which EGFP expression is under the control of the *Cx40* gene (Cx40$^{EGFP/+}$ mice) were also used.[5,6] "Okabe" mice were kindly provided by Drs Hererra and Swetloff, Geneva University Medical School, Switzerland. All animal procedures were performed according to National Veterinary Guidelines and approved by the Ethics Committees of the individual Universities.

Experimental interventions on mice

Atherosclerosis. We induced atherosclerosis in control and Cx40del mice by feeding them from the age of 10 weeks with a high-cholesterol diet for 5 or 10 weeks. There were no significant differences in plasma lipids, leukocyte counts, and animal weights, between the three groups, both before and after the high-cholesterol diet (data not shown). Protocols for quantification of atherosclerosis have been previously described in detail.[10]

Heart rates and arterial pressure measurements. All animals were anesthetized with isoflurane and implanted with microminiaturized radiotelemeters (PA-C20, Data Sciences Int., St Paul, MN) with catheter placed into the left carotid artery as described.[8] Arterial pressure was recorded in conscious animals once daily for 5 d. at the same time starting from the day of operation. Heart rate was determined offline from the pressure curve.

Vasomotor responses in isolated aortas. Mice were anesthetized with pentobarbital sodium (40 mg/kg body weight, i.p.) and the descending thoracic aorta with intact endothelial layer was isolated and dissected free from surrounding tissue and cut into rings (3 mm in length). The rings were suspended in a Multi-Myograph System (Model 610M, Danish Myo Technology A/S, Denmark) as described.[9] Aortic rings were allowed to equilibrate for 45 min and progressively stretched to a passive tension of 2.5 mN which results in an optimal length–

tension relationship. The contractility was examined with the responses to KCl (100 mM) or NE (10^{-9} to 10^{-5} M); the endothelium-dependent and –independent relaxations were examined with the concentration response curves to ACh (10^{-9} to 10^{-5} M) or SNP (10^{-10} to 10^{-5} M). Relaxations were expressed as percentage of decrease in tension of the contraction to NE ($3x10^{-7}$ M). Contractions were presented as percentage of tension compared to KCl.

Lung inflammation studies. Alveolar recruitment of neutrophils was measured in response to *Pseudomonas aeruginosa* LPS intra-tracheally (IT) instilled in anesthetized mice. Neutrophils were collected by BAL 3, 6, 12 or 24 h after LPS treatment. The collected cells were counted and stained with May-Grunwald-Giemsa for microscopic identification. In the absence of LPS, 90-95% of the cells were alveolar macrophages, the remaining being lymphocytes. In the presence of LPS, the proportion of neutrophils increased with time of stimulation to reach 80% of the alveolar cell population within 12 h.

Macrophage isolation. Mice were intraperitoneally injected with 1 ml thioglycollate 4% (Sigma). After 3–4 d, mice were killed and leukocytes were obtained by peritoneal washing using 5 mM EDTA in PBS. Peritoneal macrophages were then purified by magnetic cell sorting with CD11b microbeads and LS columns (Miltenyi Biotec) or by using a differential attachment procedure.

Western blotting and immunohistochemistry. Western blotting and immunostainings were performed as previously described[7,10] using antibodies against Cx43 (BD Transduction), Cx40 (Chemicon or ADI), Cx37 (ADI), von Willebrand factor (Dako) or CD73 (Santa Cruz or Pharmingen). An anti β-actin (Pharmingen) was used as control for protein loading. Fluorescent cells were viewed on an inverted TMD-300 microscope (Nikon) equipped with a 40X phase 3 DM objective with a numerical aperture of 0.7 (Zeiss). Images were captured with a Visicam digital camera (Visitron Systems) connected to a personal computer running Metafluor 4.01 software (Universal Imaging).

Adhesion assays

Adhesion assays were carried out using monolayers of bEnd.3 cells and H36.12j cells, a mononuclear mouse cell line as previously described,[11] or neutrophils collected by BALs from mice subjected to lung inflammation with 100 ng LPS for 24h, as described above. Leukocytes were labeled with 5-(and-6)-carboxyfluoresceine diacetate (Molecular probes), according to the manufacturer's protocols. Labeled leukocytes were added onto a confluent monolayer of bEnd.3 cells grown on gelatin-coated plastic dishes for 30 or 90 min at 37°C. At the end of the adhesion period, dishes were carefully washed not to detach adherent cells and fixed with 4% paraformaldehyde. Adherent leukocytes were counted under a microscope equipped for fluorescence detection using a 20X objective. One hundred fields were considered and adherent leukocytes were expressed as number per field.

RNA silencing, RNA anti-sense and RT-PCR

bEnd.3 cell monolayers were transfected for 12h with small interfering RNA for Cx40, Cx26 or CD73 (all from Santa Cruz) using lipofectamine (Invitrogen) and allowed to recover in complete culture medium for an additional 12h. Transfection conditions were optimised using the Block-it™ Fluorescent Oligo (Invitrogen). In some experiments, bEnd.3 cells were pre-incubated for 24 h with 10µM of Cx40 sense (5'-GTA TCA TGC CAT CCC AG-3') or anti-sense (5'-GAA GCT CCA ATC GCC CAT-3') phosphorothioated oligonucleotides. RNA was extracted with TRIzol (Invitrogen) and further purified using Quick Prep Micro mRNA Purification Kit (Amersham). Reverse transcription and PCR were performed for Cx40 and GAPDH using the following primer pairs: Cx40 sense (5′-ATG GGT GAC TGG AGC TTC C-3′), Cx40 anti-sense (5′-CAC AAA GAT GAT CTG CAG TAC CC-3); GAPDH sense (5'-TGG TAT CGT GGA AGG ACT CAT GAC-3'), GAPDH anti-sense (5'-ATG CCA GTG ACG TTC CCG TTC AGC-3').

CD73 activity

To determine CD73 activity, bEnd.3 cells were washed in a phosphate free incubation medium and in the absence of nucleotide. The reaction was started by the addition of 1 mM AMP in a solution containing (in mM): 2 $MgCl_2$, 120 NaCl, 5 KCl, 10 glucose, 20 Hepes (pH 7.4). After 40 min, the solution was collected and the reaction stopped by adding 5% (w/v) TCA and placed on ice. The release of inorganic orthophosphate (Pi) was detected by the malachite green method.[12] The Pi released from cells incubated without nucleotide was subtracted from the total Pi released during the incubation. CD73 activity (nmol/40 min) is calculated from a standard curve generated using increasing concentration of KH_2PO_4 as a source of Pi. For each experiment, the CD73 inhibitor AMP-CP was included as an additional control condition to ensure that Pi was released from the AMP-dependent CD73 activity.

To evaluate nucleotides hydrolysis by native CD73 in lung microvessels, cryosections were fixed in 4% paraformaldehyde and preincubated for 45 min at room temperature in 0.25 mM sucrose, 2 mM $CaCl_2$ and 50 mM Tris-maleate, pH 7.4. Enzymatic assay was carried out at 37°C the same buffer complemented with 2 mM $Pb(NO_3)_2$, 5 mM $MnCl_2$, 3% dextran T-250 and 100 µM AMP as substrate. In control experiments, the substrate was omitted. The Pi released from nucleotide hydrolysis is captured by lead and visualized by precipitation with 1% $(NH_4)_2S$. Sections were then mounted with Eukitt (Kindler GmbH & CO) and photographed. Intensity of lead precipitation per lung alveolar septa surface was quantified using an algorithm created in Metamorph (Universal imaging).

Hela cell co-culture

Parental and Cx40-expressing Hela cells were kindly provided by Dr. Klaus Willecke (Bonn University, Germany).[13] Either the wild-type (WT) or the Cx40-expressing (Cx40) cells were plated on the bottom of 5 µm-pores Transwell inserts (Costar) before Cx40-expressing cells

were seeded on the top of the insert. Cells were seeded at high density (200’000 cells) and cultured for 48h to allow the formation of cell-cell contacts within the pores, as previously reported.[14] Cells in the bottom chamber were then exposed to NECA for 3 hours prior to addition of leukocytes (THP-1, a human monocytic cell line) onto Cx40-expressing cells in the top chamber. THP-1 cells were first labeled -(and-6)-carboxyfluoresceine diacetate and then allowed to adhere for 30 min. The number of adherent leukocytes was expressed as described above.

Supplemental Figures and Figure Legends

Figure S1: Generation of mice with a conditional mutation in the *Cx40* gene

A: Schematic representation of the gene-targeting construct. **B:** Procedures used to select clones bearing the Cx40 allele at the appropriate integration site. **C:** Karyotyping on the selected clones revealed normal chromosome numbers. **D:** PCR analysis on mice heterozygous for the floxed Cx40 allele revealed two bands, representing wild-type Cx40 and floxed Cx40 (bottom panel), in mice not expressing the Cre recombinase (top panel). As expected, only the wild-type Cx40 gene product was detected in mice expressing Cre recombinase.

Figure S2: Endothelial-specific Cx40 deletion in *ApoE*$^{-/-}$ mice does not alter aortic function

A: Maximal contraction obtained with 100 mM KCl in aortic rings from control (C1 and C2, black and open bars) and Cx40del (hatched bar) mice. N=6 per group. **B:** Contraction of aortic rings from control (C1, C2, black and open squares) and Cx40del (triangles) mice in response to norepinephrine (NE). Values are expressed as percentage of KCl-induced maximal contraction. N=6 per group. **C,D:** Endothelium-dependent relaxations in response to acetylcholine (ACh) and endothelium-independent relaxations in response to the NO donor sodium nitroprusside (SNP) in aortic rings of control (C1 and C2, black and open squares) and Cx40del mice (triangles). N=6 per group. Responses for different concentrations were compared using analysis of variance for multiple comparisons (ANOVA with Bonferroni multiple comparison test). Contraction in response to KCl or NE was not significantly different (P=0.51) in aortic rings from controls and Cx40del mice. Likewise, endothelium-dependent (ACh) and -independent (SNP) relaxation were comparable between the three groups (P=0.82 and 0.89, respectively).

Figure S3: Cx40del mice exhibit accelerated atherosclerosis

Representative photographs (N=3-5) of aortic sinuses (**A**) and arches (**B**) from C1 (left), C2 (middle) and Cx40del (right) mice that had received 5 weeks high-cholesterol diet. Samples were stained with Sudan-IV (red) for lipids and counterstained with Mayer Hemalum (purple).

Figure S4: Cx40 is not expressed by monocytes and neutrophils

Fluorescent (**A,B,D,E**) and phase contrast (**C,F**) images of neutrophils (**A-C**) and monocytes (**D-F**) collected respectively from the alveolar space or peritoneal cavity of Okabe mice with systemic expression of EGFP or in mice in which EGFP expression is under the control of the *Cx40* gene ($Cx40^{EGFP/+}$ mice). EGFP was not detected in both neutrophils (**B,C**) and monocytes (**E,F**) collected from $Cx40^{EGFP/+}$ mice. Bar represents 200 µm in panels A-C and 50 µM in panels D-F. **G,H:** Western blots for EGFP (top panels) and β-actin (bottom panels) in total proteins isolated from 1: parental Hela cells, 2; Hela cells transfected with a plasmid encoding for EGFP, 3: leukocytes from wild-type mice, 4: leukocytes from $Cx40^{EGFP/+}$ mice, 5: leukocytes from "Okabe" mice. Leukocytes are monocytes isolated from the peritoneal cavity in **G** and neutrophils collected from the alveolar space in **H**.

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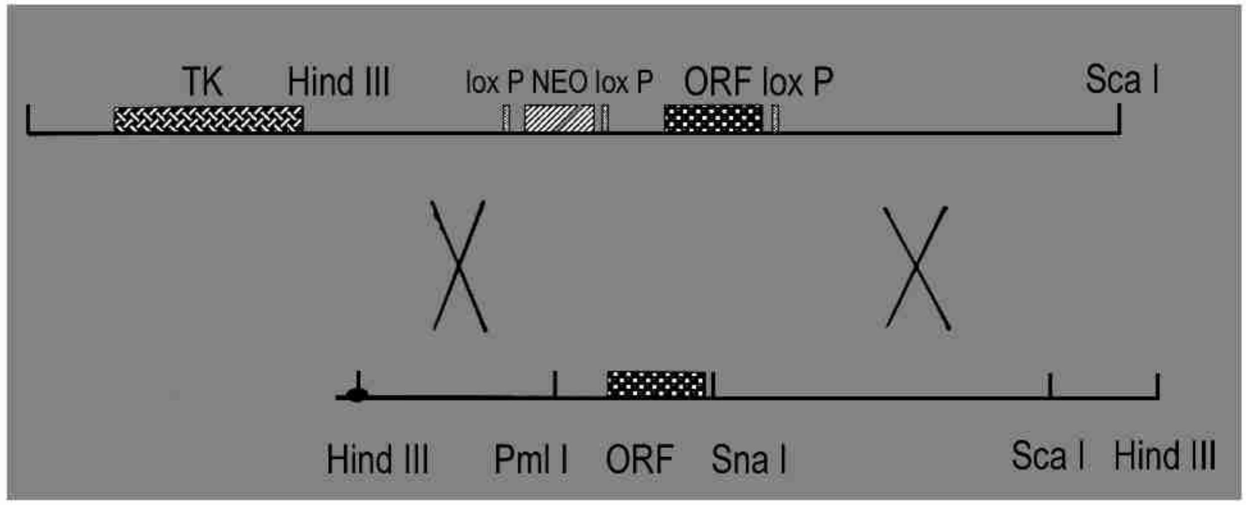
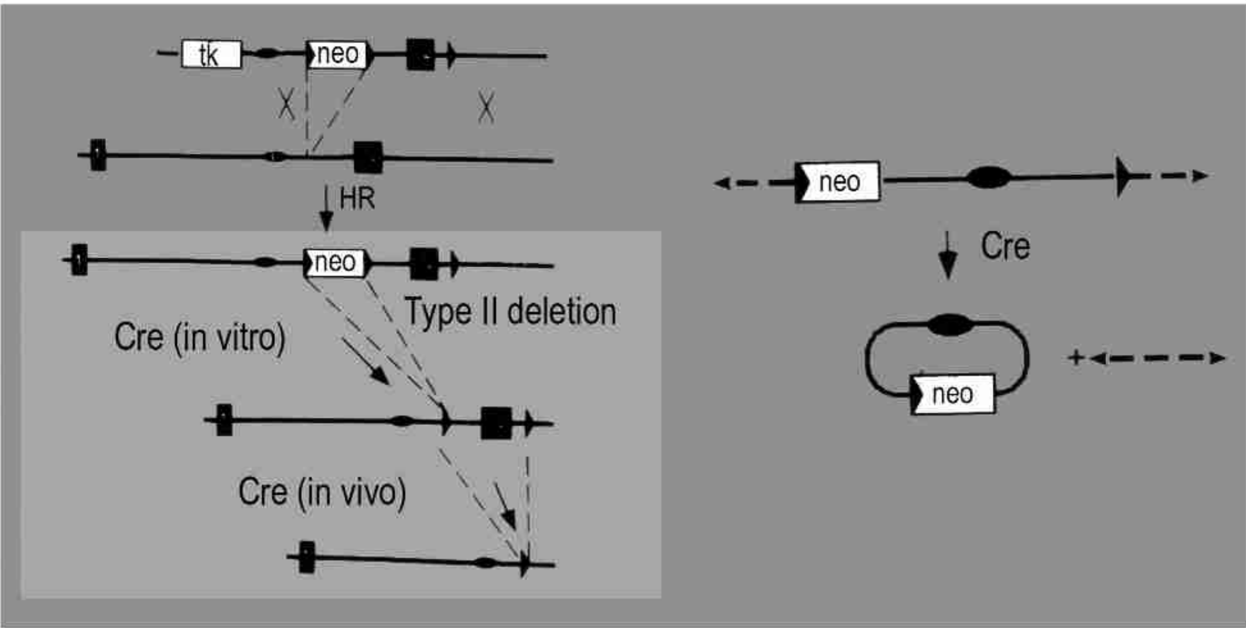
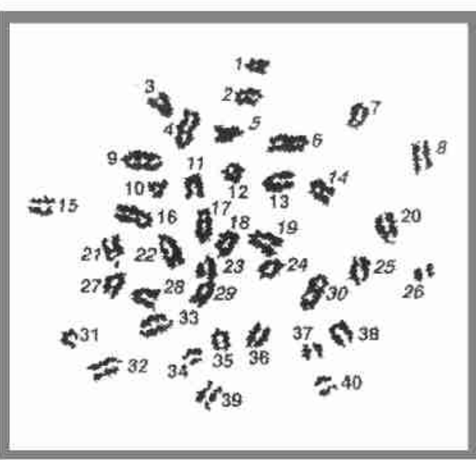






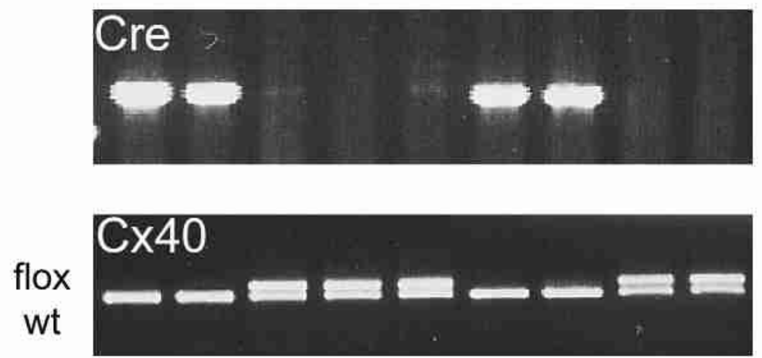
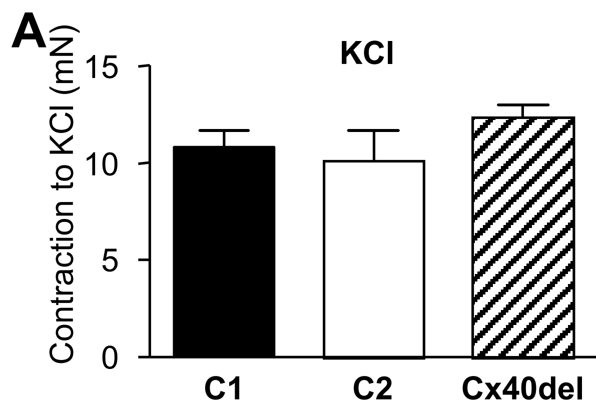
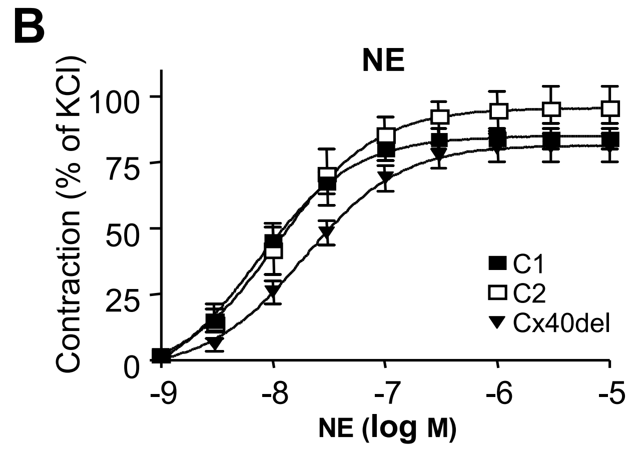
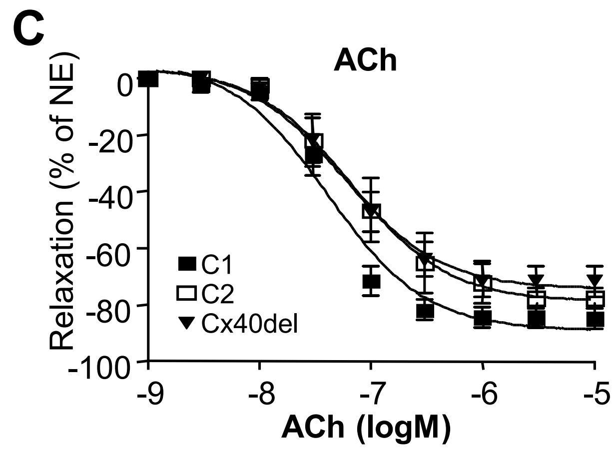


Figure S1







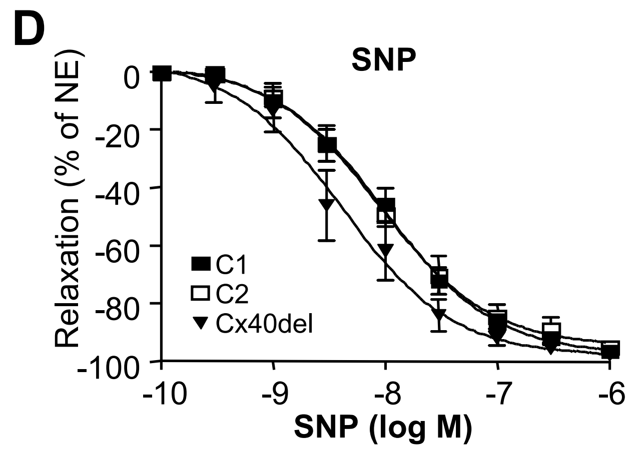


Figure S2

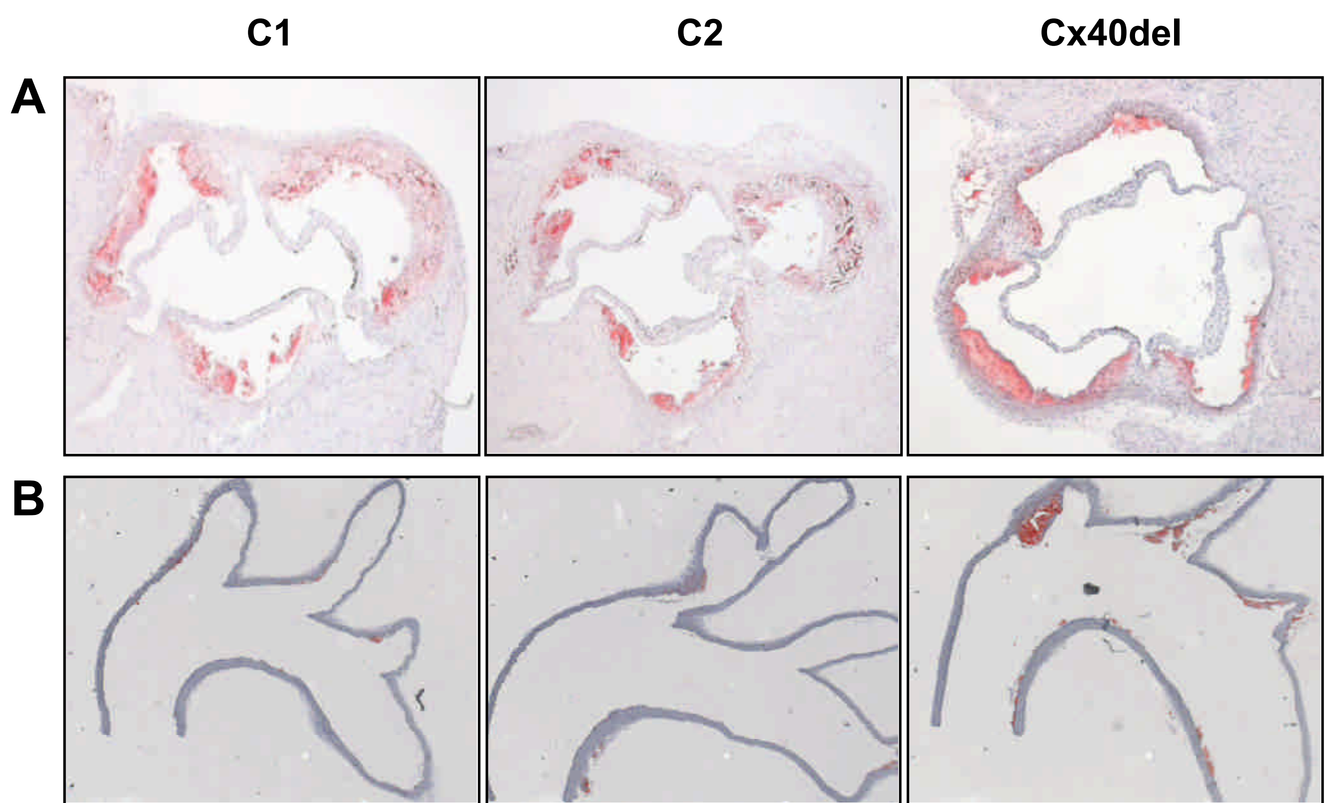
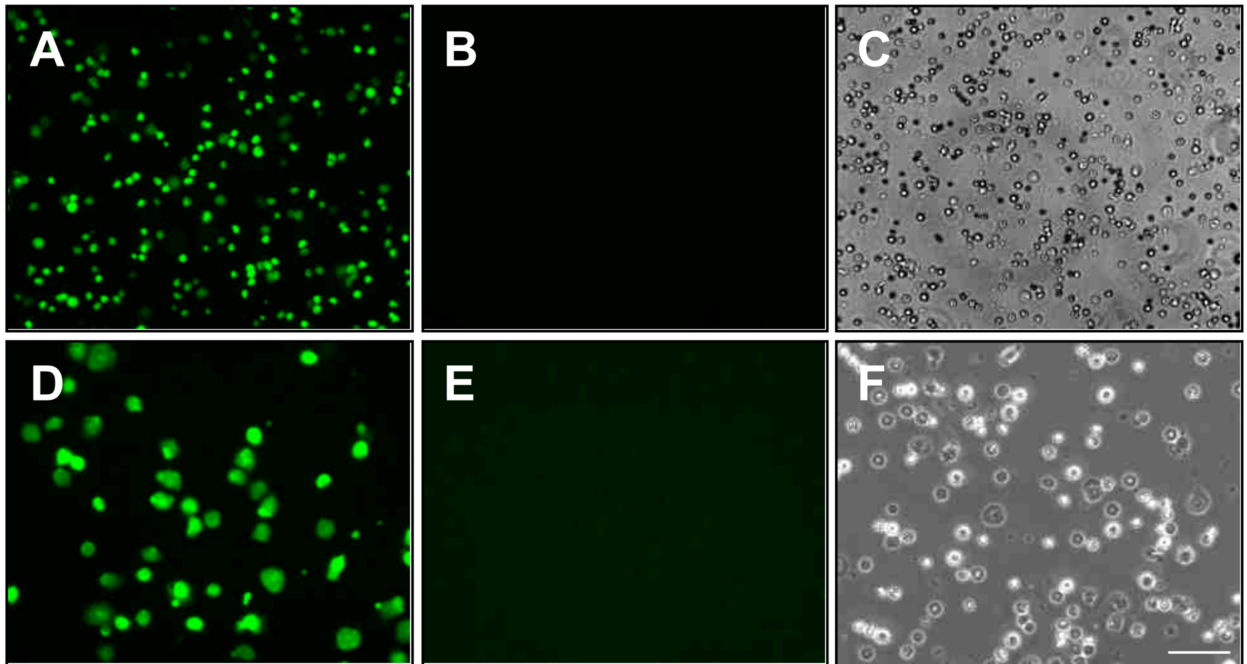


Figure S3



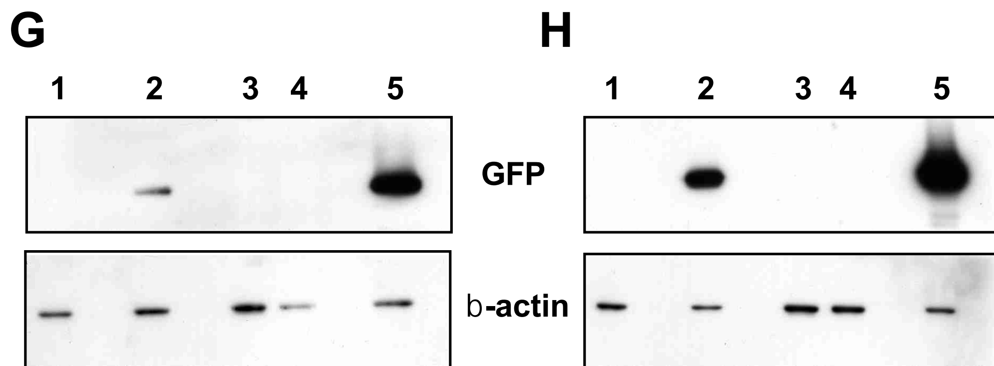


Figure S4