Limited restoration of cystic fibrosis lung epithelium *in vivo* with adult marrow derived cells

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Online Data Supplement

METHODS

Animals

Adult male (6-12 weeks) C57Bl/6 mice and transgenic C57Bl/6 mice constitutively expressing green fluorescent protein (GFP) under the control of the ubiquitin promoter (courtesy of Phillippa Marrack PhD, National Jewish Medical and Research Center, Denver CO) were utilized as donors (25). Adult female (8-12 weeks) “gut-corrected” *Cftr* KO mice (*Cftr*$^{tm1Unc-}$TgN(FABP)CFTR), courtesy of Anna van Heeckeren DVM, Case Western Reserve University) were used as recipients (26). Mice were housed in pathogen-free barrier facilities (ventilator racks) in the Small Animal Facility at the University of Vermont (UVM). All studies were subject to IACUC review at UVM and conformed to institutional and AAALAC standards for humane treatment of laboratory animals.

Bone Marrow Harvest: Total Marrow Cells

Adult male GFP mice (6-12 weeks) were euthanized by intraperitoneal overdose of sodium pentobarbital. The legs and pelvis were removed and overlying skin, muscle, and soft tissue removed by blunt dissection. The femur and tibia from each leg and the pelvis were isolated and residual tissue removed by rubbing with a sterile-gauze cover sponge. The bones were rinsed in 70% ethanol and stored in culture medium (RPMI 1640, Cellgro) with 2% FBS, 1% L-Glut, 1% P/S. Under sterile conditions, the ends of each bone were cut off with a scalpel blade and the marrow flushed out by inserting a 25 gauge syringe and rinsing with medium until clear. Recovered total marrow cells were centrifuged (500g x 5

min), washed once with fresh medium, and then re-suspended in fresh medium (Dulbecco's modification of Eagle's medium, Cellgro) with 15% FBS, 1% L-Glut, 1% P/S (2,3). The cells were passed through a 100 μm filter to remove bone fragments and debris and total number of cells counted with a hemocytometer. On average, approximately 150 x 10^6 total marrow cells were recovered from a single mouse.

Isolation, Culture, and Transplantation of Stromal Marrow Cells

Total marrow cells isolated from C57Bl/6 mice and obtained as described above were seeded into 10 cm plastic petri culture dishes (Corning) at a density of 10^5-10^6 cells/cm^2 and placed in a tissue culture incubator (37ºC, 5% CO_2) overnight. Non-adherent cells were subsequently removed and discarded. Adherent cells, reflecting primarily mesenchymal stem cells (27), were cultured for 7-10 days in DMEM culture medium with 15% serum. Cells were then harvested by gentle scraping, centrifuged (250g x 5 min), washed once with fresh medium, re-suspended in 1 ml fresh medium, and total cell number counted using a hemocytometer. In preliminary studies, we found that marrow from 2 donor mice was necessary to yield approximately 1-2 x 10^6 adherent cells.

On the day of marrow cell administration, non-irradiated female *Cftr* KO mice received tail vein injection of 10^6 cells/mouse (13).

Myeloablation and Transplantation of Total Marrow Cells

As the *Cftr* KO mice are on a mixed strain background (C57BL/6, 129, FVB/NJ) it was necessary to deplete T cells from the donor marrow cells to minimize the risk of early graft versus host disease (GVHD) (28). In brief, filtered total marrow cells were incubated in 0.83% NH_4Cl in water for 5 min at 37ºC to lyse red blood cells. Following washing with culture medium to remove lysed cell fragments, cells were incubated on ice for 30 min with 0.1 µg/ml rat α-mouse CD3 mAb (Pharmingen, Torrey Pines, CA). Cells were then incubated in a T25 plastic flask (Corning) for 45 min at 4°C on a rocking platform with a goat anti-rat IgG magnetic bead conjugate (1:1, v:v) (Polysciences Warrington, PA). The flask was placed onto a BioMag separating magnet for 1 minute and then the medium removed, washed once and then re-suspended in sterile PBS and total remaining cells counted on a hemocytometer. In preliminary studies, we found that, on average, marrow from one donor mouse yielded approximately 40-50 x 10^6 total cells following T-cell depletion.

On the day of donor cell administration, adult female *Cftr* KO (recipient) mice underwent total body irradiation (800 rads) using a cesium-137 cell irradiator (Nordion International ISO 1000, Model B, Vancouver, British Columbia). Approximately four hours after irradiation, mice received re-suspended total bone marrow cells (20 X 10^6 cells/mouse) by tail vein injection. Mice were subsequently housed in barrier conditions and fed sterilized food and antibiotic treated water (200 mg sulfamethoxazole and 40 mg trimethoprim per 5 ml sterilized water). Engraftment was assessed 30 days after transplantation by

determining the percent of peripheral leukocytes expressing GFP using flow cytometry.

Naphthalene-Induced Lung Injury

Naphthalene (275 mg/kg body weight of a stock solution of 10 ml/kg in sterile corn oil (Mazola)) was administered by intraperitoneal injection to a) naive adult female *Cftr* KO mice three days prior to administration of cultured stromal cells; b) chimeric adult female *Cftr* KO mice one month after transplantation with total marrow cells (29).

Assessment of Donor-Derived Cells in Recipient Lungs

Recipient female *Cftr* KO mice were euthanized by lethal overdose of pentobarbital at the indicated times after transplantation and/or induction of lung injury. The chest was opened *in situ* and the vasculature perfused by infusing 10-20 ml of normal saline into the right ventricle. The trachea was cannulated with a small gauge butterfly syringe and the lungs removed en bloc. 4% paraformaldehyde was infused into the trachea until the lungs were inflated and the lungs subsequently gravity fixed at 20 cm pressure for 2 hours at 4ºC. Lungs were also immersed in the paraformaldehyde during this period. Following fixation, lungs were paraffin embedded and 5 μm cut sections mounted on glass slides. Hematoxylin and eosin-stained sections were assessed for inflammation and injury.

Detection of donor-derived cells was determined by the presence of Y chromosome positive cells in lung sections detected using the method we have

developed (30). We have found this method to be sensitive and specific for detection of donor marrow cells *in situ* in recipient lungs; direct fluorescence or immunostaining for GFP was not further required to detect donor marrow cells *in situ* in these studies. In brief, murine Y paint probe was digoxin labeled (DIG-Nick Translation Mix, Roche, Penzberg, Germany), denatured at 75ºC for 7 min and re-annealed in the presence of mouse Cot-1 DNA (Invitrogen, CA) at 37ºC for at least 1 hour to reduce non-specific hybridization. De-paraffinized 5 µm mounted lung sections were immersed in 10 mM sodium citrate for 15 min at 96°C. After denaturation in 60% formamide/2XSSC at 70°C, slides were incubated overnight with digoxigenin-labeled Y paint probe. Unbound probe was removed by washing in 50% formamide/2X SSC at 42º C for 2 minutes and slides were then incubated for 1 hour at room temperature with Cy3-conjugated mouse anti-digoxigenin (Jackson Immuno Research, West Grove PA, 1:100 dilution). The presence of probe hybridization signal was assessed by fluorescence microscopy prior to the immunohistochemical detection step.

Detection of cell type specific phenotypic markers was subsequently done on the same histologic sections using antibodies directed against epithelium and leukocytes. Rabbit anti-mouse CFTR, (gift of Diane Krause MD PhD, Yale University) was used at 1:100 dilution. The antibody binds to the epitope LGRIIASYDPENKVERC, located at the N-terminal part of mouse *Cftr*. Specificity of the anti-CFTR antibody was verified by competition experiments using blocking CFTR peptide (Figure E1 A,B) in sections of a positive control (C57Bl/6) mouse lung and by lack of signal in a negative control (naïve *Cftr* KO) mouse

lung (Figure E1 C). Peptide blocking was done by incubating antibody solution at room temperature for 3 hours in 10 mM PBS , 0.5% BSA, 0.1% Triton X-100, with or without 1 μg/ml of blocking CFTR peptide, gift of Diane Krause MD PhD, Yale University. Tubes were centrifuged 10 min. at max speed in benchtop centrifuge at 4ºC. Supernatant was collected and applied to slides.

Rabbit anti-mouse CFTR (Alomone Labs) was used at 1:8000 dilution. The antibody reacts with an epitope ((C)KEETEEEVQDTRL) located at the C-terminal part of the protein. Positive and negative (naïve *Cftr* KO) mouse lungs controls (Fig. E2) were processed in parallel.

Other antibodies that were used include: rabbit anti-mouse Clara cell secretory protein, (CCSP, gift of Barry Stripp PhD., University of Pittsburgh, 1:15000 dilution); rabbit anti-human pro surfactant protein C (proSP-C, Chemicon, CA, 1:1000 dilution); rat anti-mouse CD45, (Caltag Laboratories, Burlingame, CA, 1:500 dilution), mouse anti-human pan-cytokeratin (Sigma, St. Louis, MO, 1:200 dilution). Zenon Mouse Labeling kit (Molecular Probes) was used to directly label mouse antibody. Secondary detection was done using AlexaFluor 488 donkey anti-goat, AlexaFluor 488 goat anti-rabbit and AlexaFluor 647 goat anti-rat antibodies (Molecular Probes) at a 1:500 dilution. All antibodies were diluted in 10mM PBS containing 0.5% BSA and 0.1% Triton-X. For nuclear staining, sections were incubated with Hoechst 33342 dye (Molecular probes, Eugene, OR) at 1:1000 dilution for ten minutes at room temperature.

Sections were systematically visualized with a Zeiss LSM 510 META confocal microscope and an Olympus BX50 confocal microscope equipped with a krypton-argon mixed gas multiline laser.

Images were acquired by LSM software and Bio-Rad Lasersharp 2000 software, (PSI Inc., League City, TX).

For cell count of Y chromosome positive, CD 45 negative, CCSP positive cells, approximately 20,000 CCSP-expressing cells were examined on 8-12 non-adjacent 5μm sections per lung. Similarly, for counting of *Cftr* positive cells, multiple sections equivalent to approximately 10,000 CCSP positive cells, were evaluated. For cell count of Y chromosome positive, CD 45 negative, proSP-C positive cells, 20 random 60X fields were evaluated per section. 5 sections were examined for each lung.

Isolation of mature leukocytes

Mature murine bone marrow neutrophils were isolated from donor (adult transgenic GFP) mice as previously described (31). In brief, marrow from femurs and tibias was flushed with Hank's balanced salt solution (HBSS) and layered on a three step Percoll gradient. After centrifugation at 1,060g for 30 min., neutrophils were recovered and checked for purity (>95%). Alveolar macrophages were isolated from donor (adult transgenic GFP) mice by bronchoalveolar lavage (BAL) (32). In brief, the trachea was exposed by dissection and cannulated with a 23 gauge butterfly syringe. The lungs were then lavaged *in situ* three times with 1.0 ml of 0.1M EDTA in sterile PBS (32).

This yielded a population of cells >99% alveolar macrophages as determined by cytospin preparations. Peripheral lymphocytes were isolated from donor (adult transgenic GFP) mice spleens as previously described (33). In brief, spleen was harvested by dissection and lymphocytes isolated by centrifugation in LSM Lymphocyte Separation Medium (ICN/Cappel, Aurora OH) according to manufacturer's instructions. Cells were washed with saline solution prior to RNA extraction.

Analysis of *Cftr* expression by RT-PCR

Total RNA was isolated from cells and homogenates of left lung lobe tissue with TRIzol reagent (Invitrogen, Carlsbad CA), and treated with RQ1 RNase-free DNase (Promega, Madison WI) to eliminate any eventual contamination with genomic DNA. 1 µg of total RNA was subjected to reverse transcription (SuperScript II, Invitrogen) and cDNA was then amplified by PCR with Taq polymerase (Promega). *Cftr* was amplified using forward exon 9 primer (mCF11, 5'-CTT GTG GGA AAT CCT GTG CTG AA), and reverse exon 11 primer (mCF12, 5'-CCT TCT CCA AGA ACT GTG TTG TC) (34). Expected size of the PCR product was 329 bp. For GAPDH amplification the following primers were used: 5'-ACG ACC CCT TCA TTG ACC TC, forward, and 5'-TTC ACA CCC ATC ACA AAC AT, reverse. β-actin was amplified using forward primer 5'-TCC TTC GTT GCC GGT CCA CA and reverse primer 5'-CGT CTC CGG AGT CCA TCA CA, that amplified a 508 bp fragment. 40 cycles of amplification were performed using a DNA Engine thermocycler (MJ Research, Watertown, MA). Control

reactions without reverse transcription were included. The expected sizes of of the PCR products were checked by electrophoresis after migration on a 1.5% agarose gel.

Flow Cytometry

Peripheral leukocytes obtained from chimeric mice one month after transplant were assessed via flow cytometry with a COULTER EPICS XL Analyzer (Coulter Corporation, Miami FL) and 10,000 events were assessed for the percent of GFP positive cells with WinMDI software. Lung homogenates, prepared from recipient mouse lungs as previously described (35) were also assessed for % GFP and CD45 positive and negative cells following incubation with rat anti-mouse CD45 (Caltag Laboratories, Burlingame, CA, 1:500 dilution).

Statistical Analyses

Data are expressed as the mean ± standard deviation. Poisson analysis for rare events was applied to evaluate the distribution of rare donor-derived cells among the different experimental conditions (36).

FIGURE LEGENDS

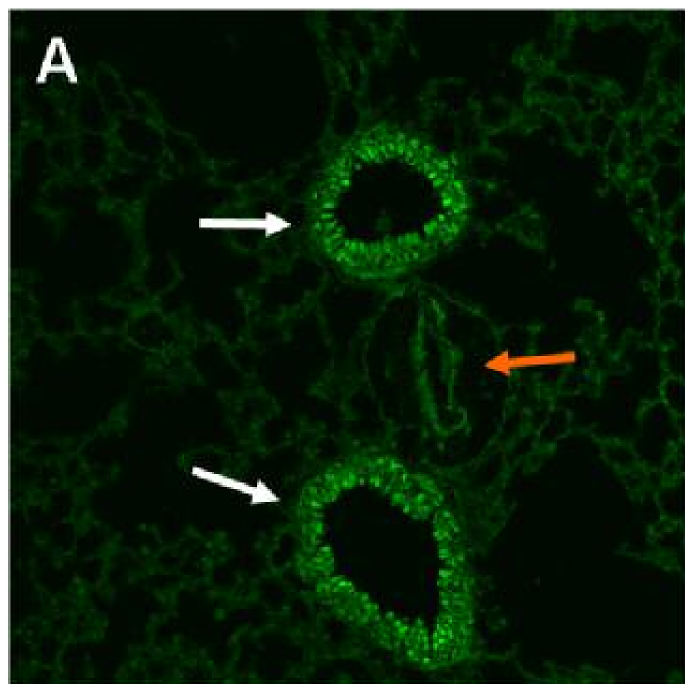
Figure E1 – Assessment of the specificity of the anti-CFTR antibody that binds to epitope LGRIIASYDPENKVERC, located at the N-terminal part of mouse *Cftr*. Serial sections of a positive control lung (C57Bl/6 mouse) were stained with anti-CFTR antibody in either the absence (A) or presence (B) of 1 µg/ml blocking CFTR peptide. White arrows indicate airway epithelium; orange arrow points to a blood vessel. A negative control (naïve *Cftr* KO mouse) stained lung section is depicted in (C). Original Magnification 200X.

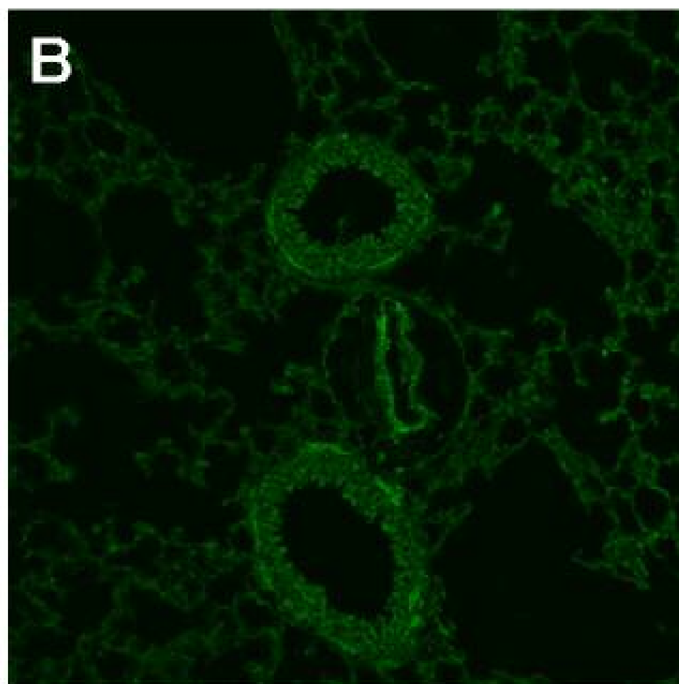
Figure E2 – Control of specificity for the anti-CFTR antibody that interacts with the epitope (C)KEETEEEVQDTRL, located at the C-terminal part of the protein. Serial sections of a positive control lung (C57Bl/6 mouse) (A, B) and of a negative control (*Cftr* KO mouse) (C, D) are shown. Section depicted in A and C were stained with anti-CFTR antibody. B and D show sections incubated with secondary antibody only. White arrows indicate airway epithelium. Original Magnification 100X.

Figure E3 – No RT control for the analysis of *Cftr* expression by RT-PCR. Primers for *Cftr* amplification were located in different exons as follows: forward exon 9 primer (mCF11, 5'-CTT GTG GGA AAT CCT GTG CTG AA); reverse exon 11 primer (mCF12, 5'-CCT TCT CCA AGA ACT GTG TTG TC) (34).

Figure E4 - Localization of donor-derived cells in lungs of chimeric female Cftr KO mice following transplantation with male GFP mouse stromal marrow cells. Each staining is shown independently on his channel. A) Hoechst 33342 nuclear staining; B) Y probe - Cy3; C) CFTR – AlexaFluor 488; D) Cytokeratin - AlexaFluor 647; E) Overlay. Original magnification 1000X.

Fig. E1





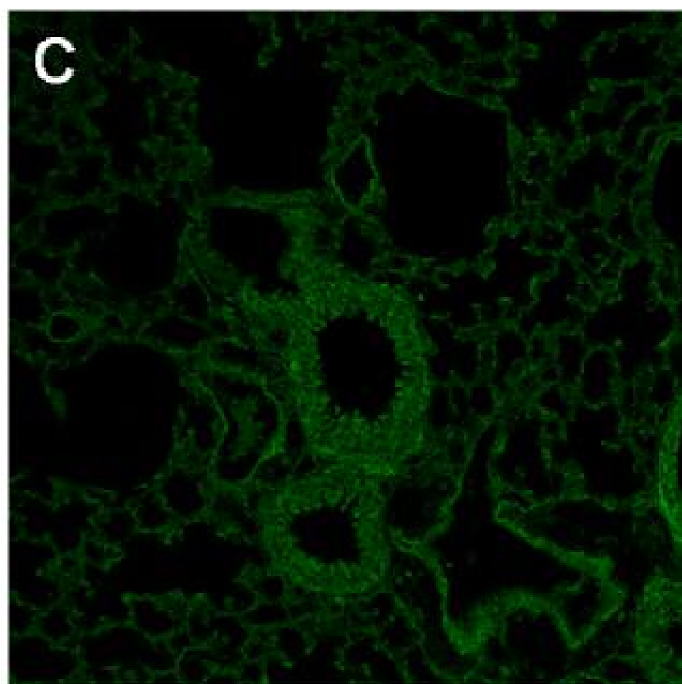
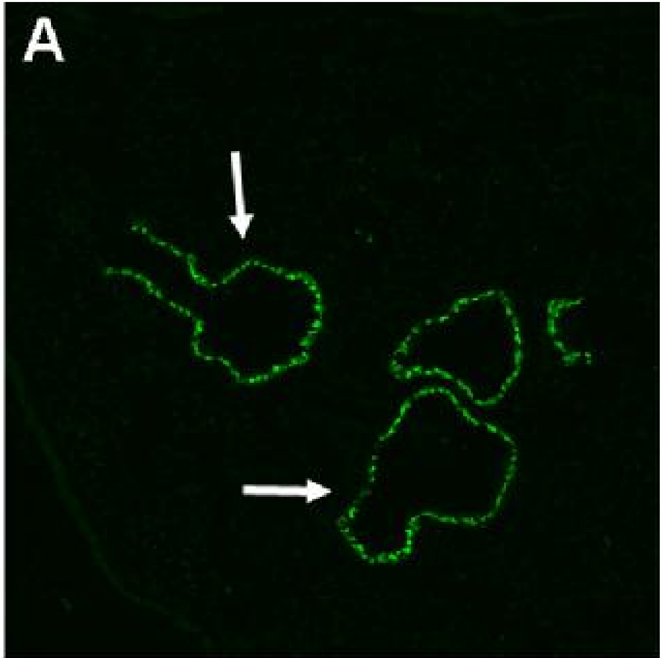
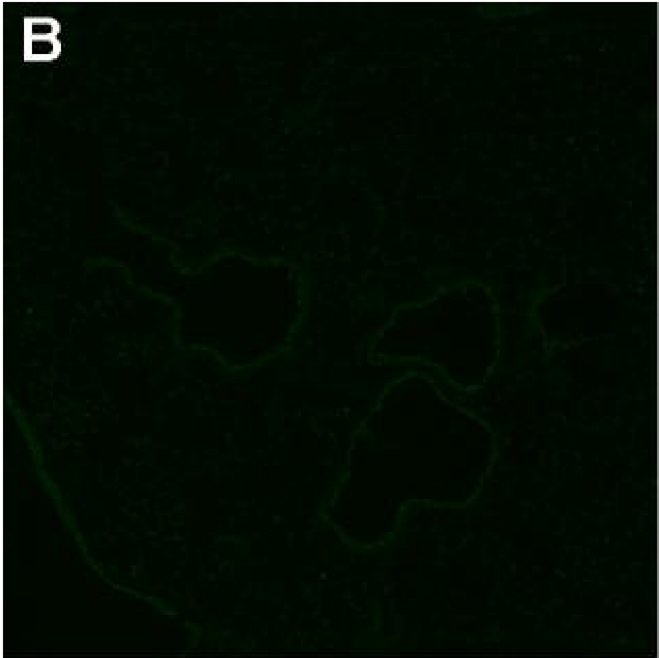
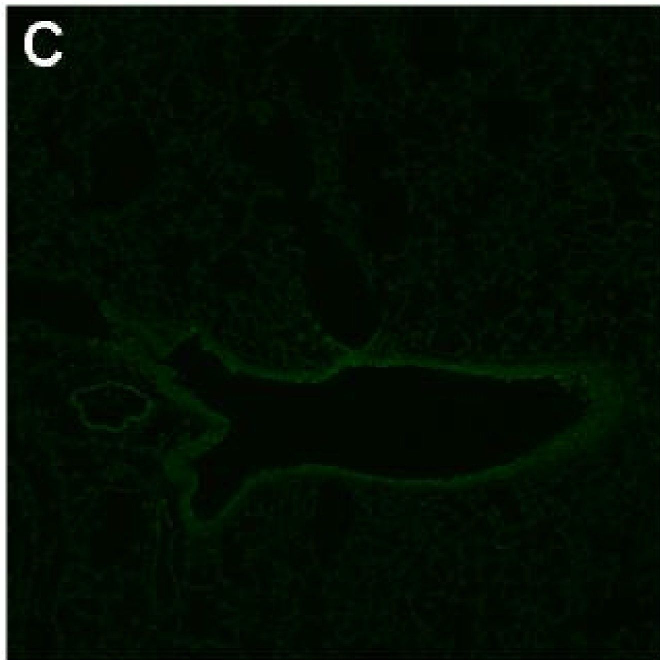


Fig. E2







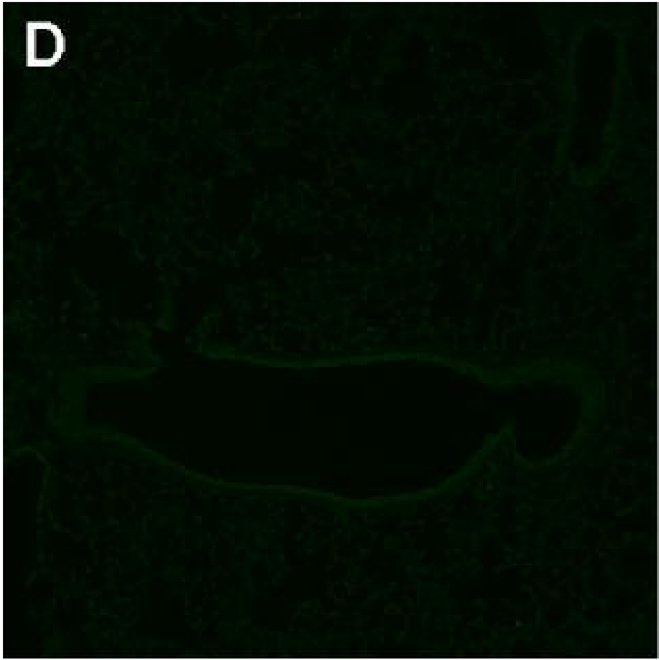


Fig. E3

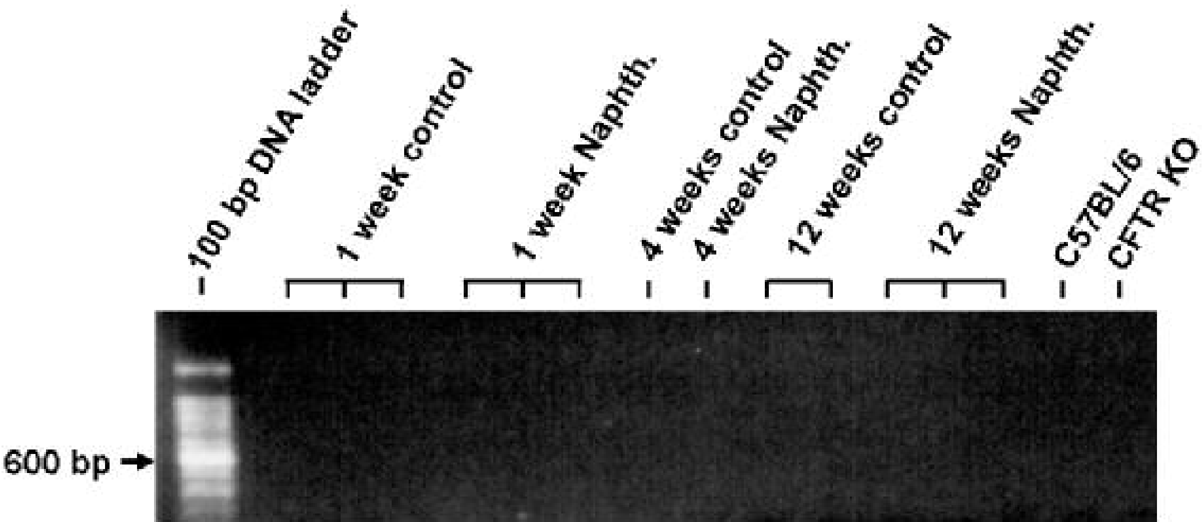


Fig. E4