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(57) Abstract: The present invention provides transgenic, large non-human animal models of diseases and conditions, as well as methods of using such animal models in the identification and characterization of therapies for the diseases and conditions.

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TRANSGENIC ANIMAL MODELS OF DISEASE

Field of the Invention

This invention relates to transgenic, non-human animal models of disease, cells that can be used to make such animals, and methods of using these animals and cells.

Background of the Invention

Many human diseases and conditions are caused by gene mutations. Substantial effort has been directed towards the creation of transgenic animal models of such diseases and conditions, to facilitate the testing of approaches to treatment, as well as to gain a better understanding of disease pathology. Early transgenic animal technology focused on the mouse, while more recent efforts, which have been bolstered by the development of somatic cell nuclear transfer, have included larger animals, including pigs, cows, and goats. This technology has resulted in the production of, for example, pigs in which the gene encoding α -1,3-galactosyltransferase has been knocked out, in efforts to generate organs that can be used in xenotransplantation (see, e.g., Lai et al., Science 295:1089-1092, 2002). Additional applications of this technology include the production of large quantities of human proteins (e.g., therapeutic antibodies; see, e.g., Grosse-Hovest et al., Proc. Natl. Acad. Sci. U.S.A. 101(18):6858-6863, 2004). Substantial benefits may be obtained by the use of somatic cell nuclear transfer technology in the production of large animal models of human disease.

An example of a disease caused by gene mutations is cystic fibrosis (CF), which is an inherited disease that affects many organs of the body, including the lungs, pancreas, sweat glands, liver, and organs of the reproductive tract. The disease is characterized by abnormalities in fluid secretion, which can lead to diverse physiological problems. For example, in the lungs of CF patients, secreted mucus is unusually heavy and sticky, and thus tends to clog small air passages, making it difficult for patients to breathe and leading to bacterial infection and inflammation. Repeated lung infections and blockages in CF patients can cause severe, permanent

lung damage. Other features of CF arise from the clogging of ducts leading from the pancreas to the small intestine, which blocks the transport of critical digestive enzymes such as amylase, protease, and lipase. This can lead to problems including incomplete digestion, diarrhea, bowel blockage, and weight loss. Digestive complications of CF can also be caused by blockage of liver bile ducts. Due to these and other features of the disease, CF causes progressive disability in patients and ultimately leads to early death.

CF is caused by the presence of a mutation in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein, which is a chloride channel found in the membranes of epithelial cells lining passageways of the lungs, liver, pancreas, intestines, and digestive tract, and in the skin. The disease is autosomal recessive, and thus CF patients have mutations in both CFTR alleles, while asymptomatic CF carriers have mutations in only one allele. There are more than 1,200 different known mutations of the CFTR gene that can lead to cystic fibrosis in humans, with some mutations causing milder symptoms than others. However, about 70% of people with CF have the disease due to a particular gene mutation, a deletion of three nucleotides, leading to the loss of a phenylalanine that is normally present at position 508 of the CFTR protein. This form of the disease, often referred to as $\Delta F508$ (CFTR- $\Delta F508$, also called F508del-CFTR), is both the most common and the most severe form of the disease. The loss of phenylalanine at position 508 results in improper CFTR protein folding, which causes retention of the mutant protein in the ER and targets it for degradation before it even reaches the cell membrane. Additionally, this deletion alters channel gating, reducing the rate of channel opening.

There is no cure for CF. Current approaches to treatment include the use of mucous thinning drugs, digestive enzyme supplementation, bronchodilators, respiratory therapy, antibiotics, and lung transplantation. Even given the availability of these approaches to treatment, as the disease progresses, patients typically suffer from an increasingly poor quality of life. New approaches to treating diseases such as CF, which may be identified, for example, by the use of large animal models, are therefore needed for this and other devastating diseases.

Summary of the Invention

The invention provides large, non-human animal models of human diseases or conditions, in which one or more genes associated with the diseases or conditions include one or more targeted mutations. The animals of the invention can be, for example, ungulates such as, e.g., pigs, cows, sheep, and goats. In one example, the disease or condition is cystic fibrosis and the gene including one or more mutations is a cystic fibrosis membrane transporter gene (CFTR).

The animal models of the invention can include the mutation(s) in one or both alleles of the gene in the genome of the transgenic animal, and the mutation(s) can result in full or partial inactivation of the gene(s). In one example, the mutation includes an insertion of an exogenous nucleic acid molecule and/or a transcription termination sequence. In another example, the mutation substantially eliminates expression of a functional gene product of the targeted gene in cells in which such expression normally takes place, absent the mutation. In the case of an animal with a mutation or mutations in both alleles of a gene, the mutation or mutations in each allele can be identical to one another or can be different.

The invention also provides isolated cells of transgenic, large non-human animal models of human diseases or conditions, in which one or more genes associated with the diseases or conditions include one or more targeted mutations. The animals can be, for example, ungulates, such as, e.g., pigs, cows, sheep, and goats. In one example, the disease or condition is cystic fibrosis and the gene including one or more mutations is a cystic fibrosis membrane transporter gene.

The invention further provides transgenic, large non-human animal models of human diseases and conditions (e.g., pigs), in which one or more endogenous genes associated with the diseases or conditions are knocked-out (i.e., genetically altered in such way as to inhibit the production or function of the product of the gene) and replaced with a homologous wild-type or mutated gene derived from a different animal (e.g., a human). In one example, a pig with its endogenous porcine CFTR knocked-out, expresses a human transgene encoding a CFTR gene, such as the CFTR- Δ 508 gene.

Examples of CFTR mutations that can be included in the animals (and cells) of the invention include (i) class I mutations, which result in little or no mRNA production, and thus little or no protein production (e.g., nonsense mutation (e.g., G542X), a frameshift mutation (e.g., 394delTT), a splice junction mutation (e.g., 1717-1GtoA)), (ii) class II mutations, which result in a protein trafficking defect where CFTR is made, but fails to traffic to the cell membrane (e.g., F508del), (iii) class III mutations, which result in CFTR trafficking to the cell membrane, but failing to be properly regulated or responding to cAMP stimulation (e.g., G551D, which fails to respond to cAMP stimulation), (iv) class IV mutations, which result in a CFTR channel function defect (e.g., R117H), and (v) class V mutations, which cause CFTR synthesis defects, resulting in reduced synthesis or defective processing of normal CFTR (e.g., missense mutation (e.g., A455E), or a mutation introduced by alternative splicing (e.g., 3849+10kbC→T). Additional mutations include 621+1→T, W1282X, R347P, S549I,N,R(A→C), R553X, and N1303K.

The cells of the invention can include the mutation(s) in one or both alleles of the genes in the genomes of the cells, and the mutation(s) can result in full or partial inactivation of the gene(s). In one example, the mutation includes an insertion of an exogenous nucleic acid molecule and/or a transcription termination sequence. In another example, the mutation substantially eliminates expression of a functional gene product of the targeted gene in cells in which such expression normally takes place, absent the mutation. In the case of a cell with a mutation or mutations in both alleles of a gene, the mutation or mutations in each allele can be identical to one another or can be different. In one example, the cells are fetal cells, such as fetal fibroblasts. Additional examples of cell types included in the invention are provided below.

The invention further provides methods of making transgenic, large non-human animal models of diseases or conditions, as described above and elsewhere herein. The methods can include the steps of: (i) introducing one or more mutations into an allele of one or more genes associated with a disease or condition in a cell (e.g., a fetal fibroblast) to generate a donor cell; (ii) introducing the nucleus of the donor cell into a recipient cell (e.g., an enucleated oocyte) to generate an embryo; and (iii) transferring the embryo into a surrogate female. The animals can be, for

example, ungulates, such as, e.g., pigs, cows, sheep, and goats. In one example, the disease or condition is cystic fibrosis and the gene including one or more mutations is a cystic fibrosis membrane transporter gene. In a variation of these methods, the donor cell includes one or more mutations in one allele of a gene, and the method is carried out to introduce one or more mutations into the other allele. In another example, the methods further involve breeding an animal that is born from the surrogate female to obtain a homozygous mutant.

The invention also includes methods of identifying therapeutic agents that can be used in the treatment of diseases or conditions (e.g., cystic fibrosis). These methods involve administering one or more candidate therapeutic agents to a transgenic animal, as described above, and monitoring the animal for one or more symptoms of the disease or condition. Detection of improvement in a symptom of the disease or condition indicates the identification of a compound that can be used in the treatment of the disease or condition.

The invention further provides methods of targeting the introduction of mutations into pig cells. These methods involve the steps of providing pig cells (e.g., fetal fibroblasts), using an adeno-associated viral vector to deliver a gene targeting construct to the isolated pig cells, in the absence of cell detachment and reattachment, and selecting gene-targeted clones. The cells are in culture for 30 days or less (e.g., 20 days or less; see below) during the targeting construct delivery and selection steps. These methods can be used, for example, for the introduction of a mutation into a cystic fibrosis transmembrane conductance regulator gene (e.g., the $\Delta F508$ mutation) in the pig cell. Information concerning other examples of mutations that can be used in the invention, as well as the use of the present methods to inactivate or replace genes (e.g., to replace pig genes with human genes), is provided below.

By “donor cell” is meant a cell from which a nucleus or chromatin material is derived, for use in nuclear transfer. As is discussed elsewhere herein, nuclear transfer can involve transfer of a nucleus or chromatin only, as isolated from a donor cell, or transfer of an entire donor cell including such a nucleus or chromatin material.

By “genetic modification,” “mutation,” or “disruption” of a gene (e.g., a CFTR gene) is meant one or more alterations in gene sequences (including coding

sequences and non-coding sequences, such as introns, promoter sequences, and 5' and 3'-untranslated sequences) that alter the expression or activity of this gene by, for example, insertion (of, e.g., heterologous sequences, such as selectable markers, and/or termination signals), deletion, frame shift mutation, silent mutation, nonsense mutation; missense mutation, point mutation, or combinations thereof. In one example, the amino acid sequence encoded by the nucleic acid sequence has at least one amino acid altered as compared to a naturally-occurring sequence. Examples of mutations include the insertion of a polynucleotide into a gene, the deletion of one or more nucleotides from a gene, and the introduction of one or more base substitutions into a gene. Preferred modifications of CFTR sequences are those that lead to one or more features of CF in transgenic animals including a mutation in, or disruption of, both CFTR alleles. As is discussed elsewhere herein, the modifications in the two CFTR alleles of such animals can be identical or different. Further, the modifications can result in a complete lack of functional CFTR production (as in the human $\Delta F508$ mutation), or can result in diminished functional CFTR production, as may be characteristic of less severe forms of the disease.

Examples of such mutations include but are not limited to: i) class I mutations, which result in little or no mRNA production, and thus little or no protein production (e.g., nonsense mutations, G542X; frameshift mutations, 394delTT; and splice junction mutations, 1717-1GtoA), ii) class II mutations, which result in a protein trafficking defect where CFTR is made, but fails to traffic to the cell membrane (e.g., F508del), iii) class III mutations, which are those in which CFTR traffics to the cell membrane, but fails to be properly regulated (e.g., G551D, which fails to respond to cAMP stimulation), iv) class IV mutations, which result in a CFTR channel function defect (e.g., R117H), and v) class V mutations, which cause CFTR synthesis defects, resulting in reduced synthesis or defective processing of normal CFTR (e.g., missense mutations, A455E; alternative splicing, 3849+10kbCtoT).

In one example, a mutation is introduced by the insertion of a polynucleotide (e.g., a positive selection marker, such as an antibiotic resistance gene (e.g., a neomycin resistance gene)) into an endogenous gene. Optionally, a mutation that is introduced into such an endogenous gene reduces the expression of the gene. If

desired, the polynucleotide may also contain recombinase sites flanking the positive selection marker, such as loxP sites, so that the positive selection marker may be removed by a recombinase (e.g., cre recombinase).

By “homologous” genes is meant a pair of genes from two animal species that encode proteins having similar functional and physical properties. The proteins encoded by homologous genes are often very similar in structure and function (although not always), and typically have a common evolutionary origin. The sequence identity is typically equal to or greater than 80% between two gene homologs. One example of a homologous gene pair is the porcine CFTR and human CFTR gene locus.

By “homozygous knock-out non-human mammal” is meant a mammal other than a human in which the two alleles of an endogenous gene (such as the CFTR gene) have been genetically targeted, resulting in a marked reduction or elimination of expression of a functional gene product, which is achieved by gene deletion or disruption. According to this invention, the genetic targeting event at both alleles may or may not be the same. Thus, a non-human mammal, in which the two alleles of an endogenous gene (such as a CFTR gene) have been genetically targeted by two different targeting vectors resulting in the null expression of the gene, would be considered as being a homozygous knock-out non-human mammal. An example of a “knock-in mutation” is one resulting in the insertion of a mutation into an endogenous gene, for example, introducing the $\Delta F508$ or another CF mutation into a CFTR gene.

By animal “knock-out” is meant an animal (e.g., a pig or mouse; also see other animals described herein) having a genome in which the function of a gene has been disrupted, or “knocked-out.” A common method of producing disabled genes using recombinant DNA technology involves inserting an antibiotic resistance gene into the normal DNA sequence of a clone of the gene of interest by homologous recombination. This disrupts the action of the gene, thereby preventing it from leading to the production of an active protein product. A cell (or cell nucleus) in which this transfer is successful can be injected into a recipient cell (e.g., an enucleated oocyte) to generate a transgenic animal by nuclear transfer. In another approach, the cell is injected into an animal embryo, producing a chimeric animal.

These animals are bred to yield a strain in which all of the cells contain the knocked-out gene.

By “recipient cell” is meant a cell into which a donor cell, a donor cell nucleus, or donor cell chromatin is introduced. Preferably, recipient cells are enucleated prior to nuclear transfer. Examples of recipient cells include oocytes, fertilized zygotes, and two-cell embryos.

By “transgenic, large non-human animal” is meant any non-human animal that includes a genetic modification, as defined herein. Examples of such animals include animals other than mice such as, for example, ungulates. Examples of ungulates that can be used in the invention include members of the orders *Perissodactyla* and *Artiodactyla*, such as any members of the family *Suidae*, and in particular any member of the genus *Sus*, such as *Sus scrofa*, which is also known as the domestic pig or a subspecies thereof (*Sus scrofa domestica*). In addition to porcine ungulates, additional ungulates that can be used in the invention include bovine, ovine, and caprine ungulates. Thus, for example, the invention can include the use of cows (e.g., *Bos taurus* or *Bos indicus*), sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, and elephants.

The invention provides several advantages, as it provides large, non-human animal models that can be used in the identification and characterization of therapies for genetic diseases. One example of such a disease is cystic fibrosis which, as discussed above, is a devastating disease, leading to increased levels of disability and, eventually, early death. Despite progress in understanding and treating CF, the pathogenesis of the disease is not well understood and therapies remain inadequate. A major impediment to answering questions is the lack of an animal model that shows disease similar to that in humans. Availability of a CF pig will allow investigators to address key problems that have persisted unresolved for years. As a result, it will be possible to develop new treatments, therapies, and preventions.

Further, given the close physiological relationship between humans and large animals, such as pigs, there is an increased likelihood that results obtained using the animal models of the invention can be applied to humans, relative to other animal models (e.g., mice, which do not develop the airway and pancreatic disease typical of

human CF). Specifically with respect to pigs, it is noted that pigs and humans have anatomical, histological, biochemical, and physiologic similarities. Further, pigs and humans possess similar abundance of submucosal glands and glycoprotein synthesis/secretion. In addition, pigs and humans have similar respiratory immune systems and pulmonary inflammatory responses, making the pig be a particularly good model for CF disease of humans. Further, the use of human sequences in large animals such as pigs, as in some examples of the invention, provides additional benefits of providing a system that is very similar to that of humans. The invention thus can be used to provide substantial benefits in the treatment of diseases and conditions caused by or associated with gene mutations, such as cystic fibrosis.

According to one particular aspect, the invention relates to an isolated cell of a pig model of cystic fibrosis, wherein a cystic fibrosis membrane transporter (CFTR) gene of the cell comprises a targeted mutation selected from deletion of phenylalanine 508 (Δ F508 mutation) and a null mutation.

According to another particular aspect, the invention relates to an isolated cell of a pig model of cystic fibrosis, in which an endogenous CFTR gene is knocked-out or inactivated, and a wild-type or Δ F508 CFTR gene from a different animal is expressed.

According to another particular aspect, the invention relates to a method of making a pig model of cystic fibrosis, the method comprising the steps of:

- i) introducing a Δ F508 or null mutation into an allele of a CFTR gene in a donor pig to generate a donor cell;
- ii) introducing the nucleus of the donor cell into a recipient porcine enucleated cell to generate an embryo; and
- iii) transferring the embryo into a surrogate female pig.

According to another particular aspect, the invention relates to a method of identifying a therapeutic agent that can be used in the treatment of cystic fibrosis, the method comprising administering a candidate therapeutic agent to a pig comprising cells comprising a targeted mutation in a CFTR gene and monitoring the pig for a symptom of cystic fibrosis, wherein the targeted mutation is a deletion of phenylalanine 508 (Δ F508 mutation) or a null mutation, and detection of improvement in a symptom of cystic fibrosis indicates the identification of a compound that can be used in the treatment of cystic fibrosis.

According to another particular aspect, the invention relates to a method of targeting the introduction of a mutation into a gene in a pig cell, the method comprising the steps of:

- providing pig cells,
- using an adeno-associated viral vector to deliver a gene targeting construct into the isolated pig cells, in the absence of cell detachment and reattachment, and
- selecting gene-targeted clones, wherein the targeting construct introduces a deletion of phenylalanine 508 ($\Delta F508$ mutation) or a null mutation, and the cells are in culture for 30 days or less during the targeting construct delivery and selection steps.

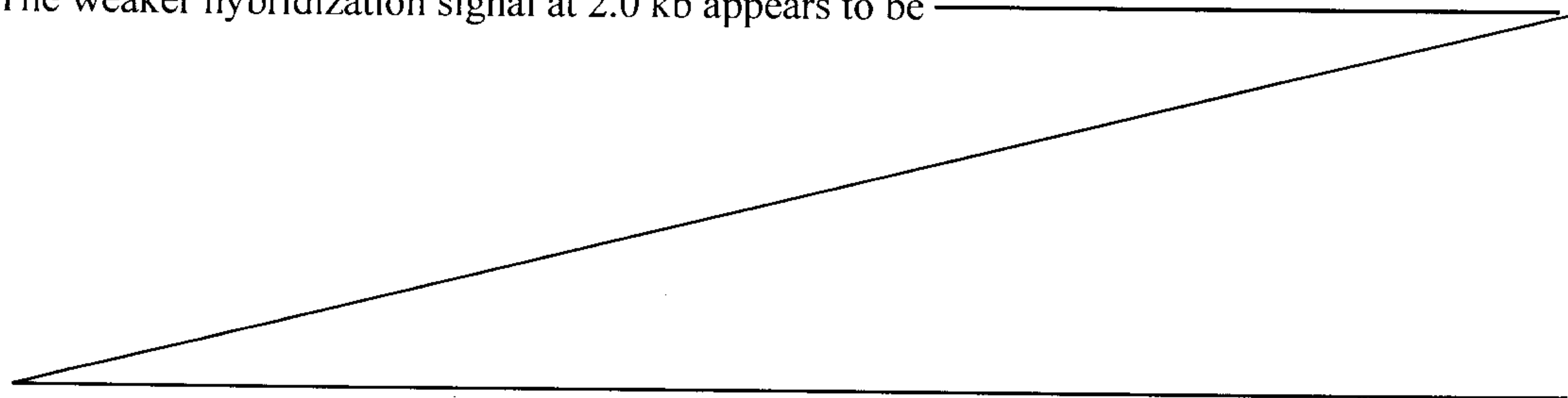
Other features and advantages of the invention will be apparent from the drawings, the detailed description, the experimental examples, and the claims.

Brief Description of the Drawings

Figure 1 is a graph showing *CFTR* expression in pig fetal fibroblasts. Data are quantitative RT-PCR of pig *CFTR* mRNA relative to *GAPDH* in primary pig fetal fibroblasts, nasal epithelia, and rectal epithelia. Similar results were obtained on two other occasions.

Figure 2 is a schematic representation of targeting constructs for homologous recombination for *CFTR-null* and *CFTR- $\Delta F508$* . Exons 8-11 of pig *CFTR* are depicted in black boxes. Neo^R contains a neomycin resistance cDNA driven by the PGK promoter and flanked by loxP sites. The engineered stop codon is indicated in the *CFTR-null* targeting vector. The positions of probes for Neo^R and *CFTR* Southern blots are indicated. PCR screen primers are depicted as arrowheads.

Figure 3 shows screening results from *CFTR-null* targeted pig fetal fibroblasts. A) Example of PCR results. Primers amplified a 2.0 kb product from the wild-type allele and 3.7 kb product from the *CFTR-null* allele. Lanes 5, 8, 9, 12, and 13 are examples of PCR-positive clones. B) Southern blot of the PCR gel using a Neo^R-specific biotin-labeled oligonucleotide. This assay confirms that the 3.7kb product contains the Neo^R sequence. The weaker hybridization signal at 2.0 kb appears to be



an artifact, with some of the targeted band co-migrating with the wild-type product. Note the differences in intensity of the two bands in panel A relative to panel B.

Figure 4 is a genomic Southern blot of DNA from *CFTR-null* targeted pig fetal fibroblasts. A) *Bgl*II-digested genomic DNA was hybridized with a probe that detects pig CFTR downstream of the targeting vector boundary. *CFTR-null*-targeted allele yields a ~9.7 kb band and wild-type is ~7.9 kb. These blots also allowed us to identify wells containing monoclonal colonies and those containing more than one type of G418^R colony. For example, wells 3 and 11 appeared to have more intense signals in the wild-type band than the targeted band, indicating that those wells likely contained one targeted clone and one or more random integration events. B) The same digested DNAs were hybridized with a Neo^R-specific probe. The *CFTR-null*-targeted band is at ~9.7 kb. Note that the band in lane 6 likely represents a random integration event, and lane 1 may have two random integration events. Wells 4, 5, 7, 8, 10, 12-14, 16, and 17 are examples of cells that may be ideal nuclear donors for generating a heterozygote animal

Figure 5 shows screening results from *CFTR-ΔF508* targeted pig fetal fibroblasts. A) Example of PCR results. Primers amplified a 2.0 kb product from the wild-type allele and a 3.7 kb product from the *CFTR-ΔF508* allele. B) Southern blot of the PCR gel using a *ΔF508* allele-specific biotin-labeled oligonucleotide. This assay confirms some of the 3.7 kb products contained the *ΔF508* mutation. Note that lanes 1, 2, and 4 contain clones that underwent homologous recombination but failed to carry the *ΔF508* mutation. On the right, wells contained either wild-type *CFTR* or *CFTR-ΔF508* plasmid DNA. This control is included to ensure that the assay Southern blot is specific to *ΔF508*.

Figure 6 is a Southern blot of amplified genomic DNA from *CFTR-ΔF508* targeted pig fetal fibroblasts. In contrast to our experience with the *CFTR-null* targeting, the *CFTR-ΔF508* targeted cells failed to proliferate after transfer to larger dishes. As a result, we were unable to obtain sufficient quantities of genomic DNA for a genomic Southern blot. Therefore, we used the relatively small amount of DNA for whole genome amplification. A) *Bgl*II-digested amplified genomic DNA was hybridized with a probe that detects pig *CFTR* downstream of the targeting vector

boundary. The *CFTR*- $\Delta F508$ -targeted allele yields a ~9.7 kb band and the wild-type is ~7.9 kb. B) Digested DNAs from similar clones were hybridized with a Neo^R-specific probe. The *CFTR*- $\Delta F508$ -targeted band is at ~9.7 kb. Note that all lanes in this Southern blot contain an intense band at ~5 kb. This band was also present in non-infected fibroblast control DNA wells. This probe is possibly hybridizing to the endogenous PGK promoter sequence, because the probe includes some PGK promoter sequence. Consistent with this, the Neo^R-probed blot in Fig. 4A also contains a faint band at 5 kb in all samples if markedly overexposed.

Figure 7 is a photograph of the first *CFTR*+/- piglet taken at one day of age.

Figure 8 is a Southern blot of genomic DNA from *CFTR*-targeted pigs. *Bgl*III-digested genomic DNA was hybridized with a probe that detects pig *CFTR* downstream of the targeting vector boundary, shown in Fig. 2. *CFTR*-null and *CFTR*- $\Delta F508$ -targeted alleles produced a ~9.7 kb band, and wild-type is ~7.9 kb. A) *CFTR*-null. Lanes 1-11 contain DNA from individual cloned pigs. Note that pig 10 was wild-type. WT well contains DNA from a wild-type control. B) *CFTR*- $\Delta F508$. Lanes 1-5 contain DNA from individual cloned pigs. Note that pig 4 was wild-type. WT well contains DNA from a wild-type control.

Figure 9 shows *CFTR* mRNA expression in *CFTR*+/- and *CFTR*+/ $\Delta F508$ pigs. A) Quantitative RT-PCR was used to measure wild-type *CFTR* mRNA levels in rectal epithelial samples from *CFTR*+/- and wild-type pigs. B) Quantitative RT-PCR was used to measure $\Delta F508$ -*CFTR* mRNA relative to wild-type mRNA levels in *CFTR*+/ $\Delta F508$ and wild-type pigs. Error bars represent S.D.

Figure 10 is a photograph of *CFTR* +/+, +/-, and -/- piglets at one day of age.

Figure 11 shows piglet genotyping by PCR. This gel shows the genotyping results of 15 piglets. Wild-type *CFTR* yielded a product of 2.0 kb and targeted *CFTR* yielded a product of 3.7 kb. NTC is no template control. *CFTR* +/+ control is in lane C1, *CFTR* +/- control is in lane C2, and *CFTR* -/- control is in lane C3.

Figure 12 shows nasal Vt in *CFTR* +/+ and *CFTR* -/- piglets. Amiloride, low chloride solution, isoproterenol, ATP, and CFTR inhibitor GlyH-101 were applied as indicated. Vertical (red) lines indicate positions where original traces were scanned and spliced together for the figure.

Figure 13 is a photograph of pancreata from CFTR $-/-$ and CFTR $+/+$ pigs.

Figure 14 is an amino acid sequence alignment of human, pig, and mouse CFTR. Transmembrane domains (TM), nucleotide-binding domains (NBD), and the R domain are boxed and labeled. Walker A and B motifs, signature motifs (SM), and F508 are shaded. The alignment was generated using ClustalW. The NBD boundaries are based on the NBD1 crystal with the NBD2 boundaries based on amino acids counting up from Walker A and down from Walker B.

Figure 15 shows that pig and mouse CFTR- Δ F508 produce some mature band C protein. The images show immunoprecipitated and *in vitro* phosphorylated wild-type and Δ F508 CFTR of human, pig, and mouse. A. Constructs were expressed for 24, 48, and 72 hours in COS7 cells. B and C. Constructs were expressed for 48 hours in NIH-3T3 (B) and LLC-PK1 (C) cell lines. H, human; P, pig; M, mouse. Bands B and C are indicated by arrows.

Figure 16 shows that fully glycosylated pig and mouse Δ F508 are not endoglycosidase-H sensitive. The images show immunoprecipitated and *in vitro* phosphorylated human, pig, and mouse wild-type and Δ F508 CFTR incubated in the presence (+) or absence (-) of 10 mU of endoglycosidase H. Human CFTR was from electroporated COS7 cells; we expressed pig and mouse CFTR using adenoviral vectors. The last 2 lanes are COS7 cells infected with Ad-GFP. Bands A, B, and C are indicated by arrows.

Figure 17 shows that human, pig, and mouse wild-type CFTR and pig and mouse CFTR- Δ F508 are expressed on the apical surface of differentiated airway epithelia. Immunostaining of differentiated human CF airway epithelia expressing human, pig, and mouse wild-type and Δ F508 CFTR. Data are X-Y (A,B,E,F,I,J) and X-Z (C,D,G,H,K,L) confocal images. CFTR immunostaining is in green and ZO-1 (tight junction) in red. Apical membrane is shown by arrow and filter (at the basal membrane) is indicated by dotted line. In panel B, faint staining of CFTR- Δ F508 is visible beneath the apical surface. Bar indicates 10 μ m.

Figure 18A shows single-channel currents from human, pig, and mouse wild-type and Δ F508 CFTR. Representative current traces from excised, inside-out patches of HeLa cells containing single channels of human, pig, and mouse wild-type

and $\Delta F508$ CFTR. Holding voltages were human at -80 mV, pig at -100 mV, mouse wild-type at -50 mV, and mouse $\Delta F508$ at -80 mV. Human tracings were from cells incubated at reduced temperature and then studied at 37°C and are taken from Teem et al. (Receptors Channels 4:63-72, 1996); pig and mouse channels were from cells incubated at 37°C and studied at ~25°C. Expanded tracings on bottom show sub-conductance in mouse wild-type and $\Delta F508$ CFTR. Figure 18B shows the properties of wild-type and $\Delta F508$ -CFTR. Data are mean $\pm$ SEM for single-channel conductance (g), open state probability (P_o), burst duration (BD), and interburst interval (IBI). $n=4-5$ membrane patches for each. Asterisks indicate $p<0.05$ compared to wild-type CFTR using Mann-Whitney Rank Sum test. Note that values for human CFTR and CFTR- $\Delta F508$ were taken from Teem et al. (Receptors Channels 4:63-72, 1996).

Figure 19 shows transepithelial currents in human CF airway epithelia expressing human, pig, and mouse CFTR and CFTR- $\Delta F508$. Examples of current traces of human, pig, and mouse wild-type CFTR and CFTR- $\Delta F508$ expressed in differentiated human CF airway. Agents were present during times indicated by bars.

Figure 20 shows the bumetanide-sensitive cAMP-stimulated current in differentiated CF airway epithelia. A. Currents in human and mouse airway epithelia expressing human, pig, and mouse wild-type CFTR and CFTR- $\Delta F508$ CFTR after subtraction of currents from GFP-expressing control epithelia. B. Bumetanide-inhibited current in CF epithelia expressing CFTR- $\Delta F508$ as a percentage of bumetanide-inhibited current in CF epithelia expressing wild-type CFTR of each species.

Detailed Description of the Invention

The invention provides animal models of human disease (e.g., cystic fibrosis (CF)), which can be used in the identification and characterization of approaches for treating the diseases and conditions. As is discussed further below, the animal models of the invention are large, non-human animals, such as pigs, which have been

genetically modified to include one or more mutations in a gene associated with a particular disease or condition (e.g., the cystic fibrosis transmembrane regulator (CFTR) gene in CF). The genetic modifications can result in the animals having one or more symptoms characteristic of the disease or condition. Animals exhibiting such symptoms are particularly advantageous in the development of therapeutic approaches, as candidate drugs and other approaches to treatment can be evaluated for effects on the symptoms in such animals. Thus, in addition to the animal models themselves, the invention also provides methods of using the animals for identifying and characterizing treatments. Further, the invention includes methods of making transgenic, large non-human animal models and cells that can be used in these methods. The animal models systems, methods, and cells of the invention are described further, below.

In addition to animals including knock-outs or mutations in endogenous genes, the invention also includes transgenic, large non-human animal models of human diseases and conditions (e.g., pigs), in which one or more endogenous genes associated with the diseases or conditions are knocked-out (i.e., genetically altered in such way as to inhibit the production or function of the products of these genes) and replaced with a comparable wild-type or mutated gene derived from a different animal (e.g., a human). In one example, a pig with its endogenous porcine CFTR knocked-out expresses a human transgene encoding a mutated CFTR protein, such as the CFTR- Δ 508 gene (i.e., a CFTR^{-/-}, hCFTR- Δ F508 pig). Alternatively, the human transgene may encode a normal, wild-type copy of a gene of interest (e.g., CFTR). These embodiments of the invention are especially useful for the generation of non-human animal models of human diseases and conditions that can be used to test existing and potential therapeutics that may only (or may preferentially) modulate or treat the disease when contacting, or being in the presence of, human copies of the disease gene or protein in question.

The invention is described herein in reference to animal models of CF, which are generated by mutation, deletion, or replacement of the CFTR gene. However, the methods of the invention are also applicable to the development of animal models of additional diseases and conditions, examples of which are provided below.

The transgenic animals of the invention can be made using the following general strategy. Briefly, the genome of a cell (e.g., a fetal fibroblast) from an animal of interest, such as a pig, is genetically modified by, for example, gene targeting by homologous recombination, to create a “donor cell.” According to the methods of the invention, the genetic modification results in at least partial inactivation of a gene associated with a particular disease or condition (e.g., a CFTR gene in CF), as will be described in further detail below. The nucleus of such a genetically modified donor cell (or the entire donor cell, including the nucleus) is then transferred into a so-called “recipient cell,” such as an enucleated oocyte. After activation and, typically, a brief period of *in vitro* culture, the resulting embryo is implanted into a surrogate female in which development of the embryo proceeds. Typically, the donor cell, oocyte, and surrogate female are of the same species, but the sources can be different species, as is known in the art.

Details of methods for making large genetically modified animals, such as pigs, according to the invention, are provided below. Additional information concerning methods for making genetically modified pigs and other large animals is known in the art and can also be used in the present invention (see, e.g., US 2005/0120400 A1; U.S. Patent No. 5,995,577; WO 95/16670; WO 96/07732; WO 97/00669; WO 97 00668; WO 2005/104835; Lai et al., *Reproductive Biology and Endocrinology* 1:82, 2003; Hao et al., *Transgenic Res.* 15:739-750, 2006; Li et al., *Biology of Reproduction* 75:226-230, 2006; Lai et al., *Nature Biotechnology* 24(4):435-436, 2006; Lai et al., *Methods in Molecular Biology* 254(2):149-163, 2004; Lai et al., *Cloning and Stem Cells* 5(4):233-241, 2003; Park et al., *Animal Biotechnology* 12(2):173-181, 2001; Lai et al., *Science* 295:1089-1092, 2002; Park et al., *Biology of Reproduction* 65:1681-1685, 2001).

The transgenic animals of the invention can be any non-human mammals, including, for example, ungulates. Examples of ungulates that can be used in the invention include members of the orders *Perissodactyla* and *Artiodactyla*, such as any members of the family *Suidae*, and in particular any member of the genus *Sus*, such as *Sus scrofa*, which is also known as the domestic pig or a subspecies thereof (*Sus*

scrofa domestica). In one specific example, the animal is a miniature swine that is a descendent from the miniature swine described by Sachs et al., Transplantation 22:559, 1976. In addition to porcine ungulates, additional ungulates that can be used in the invention include bovine, ovine, and caprine ungulates. Thus, for example, the invention can include the use of cows (e.g., *Bos taurus* or *Bos indicus*), sheep, goats, buffalos, antelopes, oxen, horses, donkeys, mule, deer, elk, caribou, water buffalo, camels, llama, alpaca, and elephants.

The invention includes animals in which only one allele of a targeted gene (e.g., CFTR) is disrupted, mutated, or replaced with the other allele remaining unaffected. These animals, which are referred to herein as “heterozygous” or “hemizygous” animals, can be used, for example, in breeding approaches to generate homozygous mutants, if desired, for example, in the case of diseases caused by homozygous recessive mutations. These animals can also be used as animal models themselves, in the case of diseases caused by autosomal dominant mutations.

Also included in the invention are homozygous mutant animals, in which both alleles of a target gene (e.g., CFTR) are disrupted or mutated, by the same or different mutations (or replaced with the same or different gene(s), optionally with the same or different mutations). In addition to being obtainable by breeding approaches involving hemizygous animals, homozygous mutant animals can also be obtained using an approach in which a cell (e.g., a fetal fibroblast) including a mutation in one allele, such as a cell obtained from an animal produced using the method summarized above, is subjected to gene targeting by homologous recombination to achieve modification of the remaining allele. The resulting donor cell can then be used as a source of a modified nucleus for nuclear transfer into a recipient cell, such as an enucleated oocyte, leading to the formation of a homozygous mutant embryo which, when implanted into a surrogate female, develops into a homozygous mutant animal.

A target gene (e.g., a CFTR gene) can be subject to genetic modification in any appropriate cell type of a species for which it is desired to create an animal model of a disease associated with mutation of the gene, according to the invention. As is understood in the art, it is necessary to be able to culture and carry out homologous recombination in a cell that is to be used as a donor cell. A particular example of such

a cell, which is described in more detail below in connection with pigs, in the experimental examples, is the fetal fibroblast. These cells can be obtained using, for example, the approach described in U.S. Patent Application Publication 2005/0120400 and other references cited herein.

The invention also includes the use of other cell types that may be present in the cell preparations obtained using the method described in U.S. Patent Application Publication 2005/0120400. Additional examples of cells that can be used as donor cells in making the transgenic animals of the invention include other fetal cells, placental cells, or adult cells. Specific examples of such cells for gene targeting include differentiated cells such as fibroblasts, epithelial cells, neural cells, epidermal cells, keratinocytes, hematopoietic cells, melanocytes, chondrocytes, B-lymphocytes, T-lymphocytes, erythrocytes, macrophages, monocytes, placental, and muscle cells.

If a cell to be genetically altered is derived from an embryo or a fetus, the cell (e.g., a fetal cell or placental cell) can be isolated at any time during the gestation period until the birth of the animal, which may or may not be itself genetically altered. In the case of a pig, such cells can be obtained, for example, between 20 to 90 days of gestation, between 25 to 60 days of gestation, between 30 to 45 days of gestation, or between 35 to 40 (e.g., at 35 days) of gestation. The time periods for obtaining cells from other animals is known in the art (see, e.g., WO 2005/104835).

Gene targeting carried out to make the cells and animals of the invention can result in gene inactivation by disruption, removal, modification, or replacement of target gene sequences. For example, inactivation can take place by the insertion of a heterologous sequence and/or a stop codon into a target gene. A specific example of this type of inactivation, in the context of a CFTR gene, is described in the experimental examples, below. As is known in the art, inserted sequences can replace previously existing sequences in a gene or can be added to such sequences, depending on the design of the targeting construct. Also as is known in the art, the design of targeting constructs can be altered, depending upon whether it is desired to completely knock out the function of a gene or to maintain some level of reduced function. In the case of CFTR, for example, complete knock out of function is consistent with the most common form of CF ($\Delta F508$; see above), but other, less

dramatic changes may be desirable for the generation of models of disease maintaining some CFTR function. Such changes may be achieved by, for example, replacement with sequences that are identical to wild-type sequences, except for the presence of specific mutations giving rise to features of the target disease. In other approaches, coding sequences are not altered or are minimally altered and, rather, sequences impacting expression of a target gene, such as promoter sequences, are targeted. In any case, selectable marker insertion is often desirable to facilitate identification of cells in which targeting has occurred. If desired, such markers or other inserted sequences can later be removed by, e.g., cre-lox or similar systems.

A CFTR^{-/-} (i.e., knock-out), hCFTR-ΔF508 pig can be made numerous ways, including, but not limited to: i) introducing a human CFTR-ΔF508 cDNA, partial human CFTR-ΔF508 gene, or entire human CFTR-ΔF508 gene into pig CFTR^{-/-} cells, selecting for human CFTR-ΔF508 expression, and using these cells as nuclear donors in somatic cell nuclear transfer, and ii) introducing a human CFTR-ΔF508 cDNA, partial human CFTR-ΔF508 gene, or entire human CFTR-ΔF508 gene into pig CFTR^{-/-} into matured oocytes, fertilizing, then transferring to a recipient female. The human CFTR sequence is described, for example, by Riordan et al., Science 245(4922):1066-1073, 1989 (erratum in Science 245(4925):1437, 1989)). Human, pig, and mouse CFTR sequences are also provided in SEQ ID NOs:1-6.

As is known in the art, targeted gene modification requires the use of nucleic acid molecule constructs having regions of homology with a targeted gene (or flanking regions), such that integration of the construct into the genome alters expression of the gene, either by changing the sequence of the gene and/or the levels of expression of the gene. Thus, to alter a gene, a targeting construct is generally designed to contain three main regions: (i) a first region that is homologous to the locus to be targeted (e.g., the CFTR gene or a flanking sequence), (ii) a second region that is a heterologous polynucleotide sequence (e.g., encoding a selectable marker, such as an antibiotic resistance protein) that is to specifically replace a portion of the targeted locus or is inserted into the targeted locus, and (iii) a third region that, like the first region, is homologous to the targeted locus, but typically is not contiguous with the first region of the genome. Homologous recombination between the

targeting construct and the targeted wild-type locus results in deletion of any locus sequences between the two regions of homology represented in the targeting vector and replacement of that sequence with, or insertion into that sequence of, a heterologous sequence that, for example, encodes a selectable marker. In the case of targeting transcriptionally inactive genes, such as, for example, the CFTR gene in fibroblasts, or a gene having only very low levels of transcription, the constructs of the invention can include a promoter, such as a PGK promoter, which drives expression of the selectable marker (e.g., Neo). Use of such promoters may not be required in cases in which transcriptionally active genes are targeted, if the design of the construct results in the marker being transcribed as directed by an endogenous promoter. Exemplary constructs and vectors for carrying out such targeted modification are described herein. However, other vectors that can be used in such approaches are known in the art and can readily be adapted for use in the invention.

In order to facilitate homologous recombination, the first and third regions of the targeting vectors (see above) include sequences that exhibit substantial identity to the genes to be targeted (or flanking regions). By “substantially identical” is meant having a sequence that is at least 80%, 90%, 95%, 98%, or 100% identical to that of another sequence. Sequence identity is typically measured using BLAST[®] (Basic Local Alignment Search Tool) or BLAST[®] 2 with the default parameters specified therein (see, Altschul et al., J. Mol. Biol. 215:403-410, 1990; Tatiana et al., FEMS Microbiol. Lett. 174:247-250, 1999). These software programs match similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications. Thus, sequences having at least 80%, 90%, 98%, 99%, or even 100% sequence identity with the targeted gene loci can be used in the invention to facilitate homologous recombination.

The total size of the two regions of homology (i.e., the first and third regions noted above) can be, for example, approximately 2-25 kilobases (e.g., 4-20, 5-15, or 6-10 kilobases), and the size of the second region that replaces a portion of the targeted locus can be, for example, approximately 0.5-5 kilobases (e.g., 1-4 or 3-4 kilobases). A specific example of such a construct is described below, in the experimental examples.

The targeting constructs can be included within any appropriate vectors, such as plasmid or viral vectors (e.g., adenovirus or adeno-associated virus vectors), which can be introduced into cells using standard methods including, for example, viral transduction, electroporation, or microinjection. One example employs an adeno-associated viral vector (AAV) (e.g., rAAV2, which can be made by standard methods using a pAV2 plasmid (ATCC 37216), rAAV1, and rAAV5).

The use of AAV to deliver the targeting construct offers many benefits. First, AAV1 (and other AAV serotypes) infects pig fetal fibroblasts with 95-100% efficiency. Second, AAV infection of pig fetal fibroblasts results in little or no cell toxicity. Third, AAV infection results in the delivery of a single-stranded gene targeting construct directly to the nucleus. Single-stranded gene targeting vectors are thought to yield more efficient gene targeting and result in a more favorable homologous recombination to non-homologous recombination ratio (Hendrie and Russell, *Molecular Therapy* 12(1):9-17, 2005).

The methods of the invention, employing AAV vectors, resulted in high levels of gene targeting efficiency in these somatic cells, as compared to prior methods. Central to the methods of the invention is the fact that the entire procedure was performed in a time-sensitive manner, because excessive cell culture time (more than 30 days) negatively impacts nuclear transfer efficiency (Lai et al., *Cloning and Stem Cells* 5(4):233-241, 2003). In one example, following fibroblast harvest from day 35 fetuses, the fetal fibroblast cells were frozen within 48 hours. The use of an AAV vector to deliver the gene targeting construct allowed targeting to begin 24 hours after thawing cells and required no cell detachment and re-attachment, which is required in other methods. Multiple cell detachment and re-attachment events (trypsinization) are thought to decrease the ability of a cell to serve as a nuclear donor in nuclear transfer. Further, G418 selection in 48 96-well plates prevents the need for the more conventional, time-consuming isolation of resistant clones with cloning rings. The screen for gene-targeted clones was designed such that all positive clones could be identified and frozen within a 3-5 day period. All clones were frozen by day 18, therefore the cells have been in culture approximately 20 days since being harvested from the fetus. This is an important aspect of the invention, because reduction of the

time in culture increases the likelihood that cells used as nuclear donors are viable, normal, and euploid.

Accordingly, the invention provides a method of gene-targeting cells, such as pig cells (e.g., pig fetal fibroblasts), in which the number of days in culture (during which targeting and selection takes place) is less than 30 days, e.g., 25-29, 20-24, 19, 18, 17, 16, 15, or fewer days. To facilitate this method, the selection can take place in multi-well plates, as described further below. Further, the cells may be frozen shortly after harvest (e.g., within 24, 48, or 96 hours). After cell thawing (or after harvest, if the cells are not previously frozen), gene targeting with an AAV vector can be carried out within, for example, 12, 24, 36, or 48 hours, without the use of multiple detachment/re-attachment events, and selection can proceed in an expedited manner, such as by use of multi-well plates (e.g., 96-well plates), prior to freezing.

Other types of vectors, or more specifically other types of targeting construct delivery methods, are available, and were used during initial attempts to disrupt the pig CFTR gene. Cell transfection methods, including calcium phosphate, lipofection, electroporation, and nuclear injection can be used to deliver the targeting construct, though the disadvantages of inefficient transfection efficiency, cell toxicity, requirement of a pure (clean) targeting construct DNA sample, and poor ratio of homologous recombination to non-homologous recombination far outweigh the benefit of ease. If the gene is transcriptionally active in the cell being used, then a promoterless selectable marker strategy can be employed, so that antibiotic resistance will only be found in cell that have had a recombination event within a transcribed unit.

Genetically targeted cells are typically identified using a selectable marker, such as neomycin. If a cell already contains a selectable marker, however, a new targeting construct containing a different selectable marker can be used. Alternatively, if the same selectable marker is employed, cells can be selected in the second targeting round by raising the drug concentration (for example, by doubling the drug concentration), as is known in the art. As is noted above, targeting constructs can include selectable markers flanked by sites facilitating excision of the marker sequences. In one example, constructs can include loxP sites to facilitate the efficient

deletion of the marker using the cre/lox system. Use of such systems is well known in the art, and a specific example of use of this system is provided below, in the experimental examples.

Upon obtaining cells in which a target gene (e.g., a CFTR gene) has been targeted (one or both alleles, as described above), nuclear transfer can be carried out. Optionally, the genetically modified nuclear donor cells can be frozen prior to nuclear transfer. Recipient cells that can be used in the invention are typically oocytes, fertilized zygotes, or two-cell embryos, all of which may or may not have been enucleated. Typically, the donor and the recipient cells are derived from the same species. However, it is possible to obtain development from embryos reconstructed using donor and recipient cells from different species.

Recipient oocytes can be obtained using methods that are known in the art or can be purchased from commercial sources (e.g., BoMed Inc., Madison, WI). As is known in the art, the donor nucleus or the donor cell itself can be injected into the recipient cell or injected into the perivitelline space, adjacent to the oocyte membrane. The nuclear transfer complex formed in this manner can be activated by standard methods, which may involve electrical fusion/activation or electrical fusion/chemical activation, as is described further below. Further processing of the nuclear transfer complex, including implantation of the complexes into surrogate mothers, is described further below.

The transgenic animals of the invention can be used in the identification and characterization of drug and other treatment methods for the disease or condition associated with mutation of the gene targeted according to the invention. In these methods, for example, a candidate therapeutic agent can be administered to an animal and the impact of the agent on a feature of the disease exhibited by the animal can be monitored. Optionally, the methods can also involve exposure of the animals to environmental or other conditions known to contribute to or exacerbate the disease or condition. For example, in the case of CF animal models having impaired respiratory function, the effect of the drug on such function can be assessed by measurement of standard respiratory parameters. In another example, in the case of animals exhibiting

impaired digestion, due to blockage of pancreatic and/or liver ducts, the effect of a treatment on digestion can be determined.

With the porcine model of the invention, it is possible to test hypotheses that lead to new treatments and to evaluate potential therapies for CF lung disease. The porcine model also makes it possible to assess electrolyte transport by porcine airway epithelia *in vitro* and *in vivo*, the volume of airway surface liquid *in vitro* and *in vivo*, the ion composition of airway surface liquid *in vitro* and *in vivo*, the airway surface liquid pH in the airway, and electrolyte transport in the small airways. It is also possible to measure respiratory mucociliary transport *in vitro* and *in vivo*. For assessing inflammation, several tests and assays can be carried out, including (but not limited to) assays of key markers of inflammation in amniotic fluid, fetal lung liquid, and bronchoalveolar lavage by using lung tissue histochemistry, large-scale gene expression profiling of pulmonary tissues, cytokine and cell assays, and proteomics. It is also possible to raise CF and non-CF piglets in isolators under completely germ free conditions and to test for the development of pulmonary inflammation, and then selectively expose the piglets to inflammatory stimuli including bacteria and viruses. In addition, investigators can test how loss of CFTR function in airway epithelia results in altered NF κ B signaling, the function of secreted epithelial antimicrobials/host defense proteins, and the consequences of loss of CFTR function in macrophages or neutrophils. The availability of the porcine CF model allows tests of the early manifestations of the CF, an important question that remains unanswered. The natural history of pulmonary infections in CF pigs can also be monitored, leading to a determination of whether the airway epithelia of CF pigs can be colonized by CF or porcine pathogens and/or non-pathogenic opportunistic organisms.

Although lung disease is the current main cause of mortality, patients suffer from CF disease in many other organs. Availability of a CF model allows new investigations and tests of therapeutics in the pancreas, intestine, sweat gland, liver, vas deferens, kidney, and other organs affected primarily or secondarily by CF. The screening methods of the invention can be carried out to test the efficacy of new compounds, combinations of new and old compounds, non-pharmaceutical treatments, and combinations of pharmaceutical and non-pharmaceutical treatments.

The invention has been described above in reference to mutation of the CFTR gene to generate non-human animal models of cystic fibrosis. As is stated above, the invention can also be used in the generation of transgenic, non-human animal models of other diseases and conditions associated with gene mutations. There are innumerable examples of such diseases and conditions known in the art, which can be included in this invention. Some specific examples are listed in the Table 1.

Table 1

Disease	Gene	Reference
Hypercholesterolemia Genomics Hum. and atherosclerosis	LDLR and APOE	Lusis et al., Annu. Rev. Genet. 5:189-218, 2004
Cancer 1997 and Ashworth, Oncogene.	p53, BRCA1 and 2	Levine-AJ, Cell, 88:323-331, Gudmundsdottir 25(43):5864-5874, 2006
Huntington's disease 28, 2007	huntingtin	Walker, Lancet 369(9557):218-
Duchenne muscular Neurol. (1):1-7, dystrophy	dystrophin	Deconinck and Dan. Pediatr 2007
Polycystic kidney disease	PKD1 and 2	Gattone V., Current Opinion in Pharmacology 5:535-542, 2005
Sickle-cell disease Pharmacol. Sci.	alpha/beta-globin	Steinberg MH, Trends 27(4):204-10, 2006
Hemophilia A 24;	Factor VIII	Bolton-Maggs and Pasi, Lancet 361(9371):1801-9, 2003
Ataxia-telangiectasia Mutat.	ATM	Concannon and Gatti, Hum. 10(2):100-7, 1997
Retinoblastoma 14(4):283-8, 1999	RB1	Lohmann, Hum. Mutat.

Possible mutations to these disease genes include knock-outs (by, e.g., insertion of a selection cassette), knock-ins (e.g., by point mutations that correspond to human disease mutations), and, in the case of Huntington's disease (and any other trinucleotide repeat expansion disorder family members), an expansion of the trinucleotide repeat to pathogenic sizes.

The following Examples are meant to illustrate the invention and are not meant to limit the scope of the invention in any way.

Experimental Examples

We wished to generate pigs with two different alterations in their *CFTR* gene, a null allele and the $\Delta F508$ mutation. A null allele would lack any CFTR function; it should therefore provide a valuable model for assessing the porcine CF phenotype, for comparing the consequences of other CF-associated mutations, for exploring pathogenesis, and for evaluating many therapeutic strategies. The $\Delta F508$ mutation deletes Phe508 and is the most common CF-associated mutation, accounting for ~70% of CF alleles (Zielenski et al., Annu. Rev. Genet. 29:777-807, 1995). In humans, this mutation disrupts processing of the protein, so that nearly all CFTR- $\Delta F508$ is retained in the endoplasmic reticulum (ER) and degraded, preventing maturation to the plasma membrane. In addition, this deletion reduces the activity of single CFTR channels and shortens their lifetime on the cell surface (Dalemans et al., Nature 354:526-528, 1991; Teem et al., Receptors Channels 4:63-72, 1996; Skach, Kidney Int. 57:825-831, 2000). Earlier work showed that reducing the incubation temperature and other interventions allowed some of the mutant protein to escape the ER and traffic to the cell surface, where it retained significant activity (Denning et al., Nature 358:761-764, 1992). These findings plus the prevalence of the $\Delta F508$ mutation have driven efforts to correct the CFTR- $\Delta F508$ defects (Lukacs et al., N. Engl. J. Med. 349:1401-1404, 2003; Verkman et al., Curr. Pharm. Des. 12:2235-2247, 2006). We have found that porcine CFTR- $\Delta F508$ showed at least partial processing *in vitro* (Ostedgaard et al., Proc. Natl. Acad. Sci. U.S.A. 104:15370-15375, 2007; also see below). A pig with the $\Delta F508$ mutation could be of value for understanding the mechanisms responsible for the CFTR- $\Delta F508$ biosynthetic defects *in vivo* and for developing pharmacological agents to correct the CFTR- $\Delta F508$ biosynthetic defects. To begin developing these porcine models of CF, we combined gene targeting and SCNT.

The following experimental examples describe the generation of cystic fibrosis pig models (*CFTR-null* and *CFTR- $\Delta F508$* alleles), an interspecies analysis of the $\Delta F508$ mutation, and approaches to making pigs expressing human CFTR sequences (e.g., human $\Delta F508$ CFTR).

I. Cystic Fibrosis Pig

Results

Fetal pig fibroblasts express little CFTR

We worked with fetal fibroblasts from domestic pigs (*Sus scrofa*) since they have been used successfully for transgenic SCNT (Park et al., Animal Biotechnology 12(2):173-181, 2001). Because a promoter-trap strategy was previously used in porcine fibroblasts (Lai et al., Science 295:1089-1092, 2002), we asked if *CFTR* is expressed in fetal fibroblasts. We used quantitative RT-PCR and compared the results to transcript levels in nasal and rectal epithelia, which are known to express *CFTR* at low levels (Trapnell et al., Proc. Natl. Acad. Sci. U.S.A. 88:6565-6569, 1991). Figure 1 shows that the primary fibroblasts produced very little *CFTR* mRNA. This result prevented the use of a promoter-trap strategy as was done for the only other gene targeted in pigs (Lai et al., Science 295:1089-1092, 2002; Dai et al., Nat. Biotechnol. 20:251-255, 2002).

Developing vectors to target the pig CFTR gene

We designed a “null” targeting construct to disrupt *CFTR* exon 10 with a neomycin resistance cassette (Neo^R) (Fig. 2). Because *CFTR* can exhibit some alternative splicing, we chose to disrupt exon 10, which encodes a portion of nucleotide binding domain 1; this exon is required for CFTR function. We also included an engineered stop codon at position 508. Therefore, F508X would be expected to trigger nonsense-mediated mRNA decay as well as prematurely interrupt any translation of *CFTR*. The $\Delta F508$ targeting vector was designed to delete residue Phe508 (Fig. 2). We also inserted a Neo^R in the intron downstream of exon 10 as a positive selection marker. In this vector, Neo^R was flanked by loxP sites so that it could be removed at a later time if it was found to markedly reduce the level of the *CFTR*- $\Delta F508$ mRNA, a situation encountered in some attempts to make a *CFTR*- $\Delta F508$ mouse (Colledge et al., Nat. Genet. 10:445-452, 1995; Zeiher et al., J. Clin. Invest. 96:2051-5064, 1995).

We initially used nuclear microinjection and then electroporation to deliver the null targeting vector to fetal fibroblasts. However, we recovered no clones with

homologous recombination. We then investigated AAV-mediated gene targeting, which has been used to deliver targeting vectors to cell lines and primary cells (Inoue et al., J. Virol. 73:7376-7380, 1999; Hirata et al., Nat. Biotechnol. 20:735-738, 2002; Porteus et al., Mol. Cell. Biol. 23:3558-3565, 2003; Russell et al., Nat. Genet. 18:325-330, 1998). Using an AAV vector has the advantage that it delivers single-stranded DNA to the nucleus, the amount of DNA per cell is small, and it can infect many cell types (Hendrie et al., Mol. Ther. 12:9-17, 2005). To first determine which AAV serotypes can infect pig fetal fibroblasts, we infected them with eGFP-expressing AAV1, 2, and 5 (each with AAV2 ITRs). Each AAV infected the cells with at least 50-80% efficiency, however, AAV1 appeared to infect nearly 100% of cells. Because of rAAV genome size constraints, the total length of the targeting vectors was limited to ~4.5 kb. Neo^R was centrally located in both vectors (Fig. 2).

AAV vectors introduced the CFTR-null and CFTR-ΔF508 alleles

We obtained fetal fibroblasts from males so that all of our clones would be male, which would allow us to more rapidly expand the number of animals. Primary cultures of pig fetal fibroblasts were infected with AAV1 carrying the null targeting vector. After 24 hours, cells were transferred to a series of 96-well plates. Approximately two weeks later, cells in each well of the 96-well plates were “replicated” by splitting among three plates: 96-well culture plates for cell expansion, 96-well culture plates for potential cryopreservation, and 96-well PCR plates for cell lysis.

We screened cell lysates by PCR to identify wells containing gene-targeted clones (Fig. 3A) and then hybridized with a Neo^R-specific probe to test for inclusion of this marker (Fig. 3B). We then froze positive clones; by that time, cells had been in culture 15-17 days. We also passaged positive clones from the “cell expansion” plates to generate DNA for genotype determination. Southern blots with *CFTR*- and Neo^R specific probes identified clones with a targeted *CFTR* allele that were free of random integration (Fig. 4). On average, 75% of PCR-positive clones were also positive by Southern blot and were clonal.

We used identical procedures to introduce the *CFTR*- $\Delta F508$ construct and screen for homologous recombinants. We identified numerous PCR-positive clones (Fig. 5A), that were confirmed by Southern blotting with a $\Delta F508$ allele-specific probe (Fig. 5B). Eighteen of 25 (72%) PCR-positive clones contained the F508 deletion. The other 28% failed to contain $\Delta F508$, suggesting that gene targeting had occurred, but crossing over was downstream of F508. Subsequent Southern blots revealed *CFTR*- $\Delta F508$ targeted clones (Figs. 6A and 6B).

Variability in homologous recombination depended on the donor

Over the course of these studies, we targeted the *CFTR* gene in fibroblasts derived from several fetuses. The fetuses were all siblings harvested from the same uterus at the same time. Yet, surprisingly, we saw a striking fetus-to-fetus variability in targeting frequency (Table 2, below). Even when fibroblasts from different fetuses were infected and screened at the same time, with the same reagents, and by the same people, pronounced differences occurred; an example is fetus 5 vs. fetus 7 in Table 2. These results suggest the difference was not due to experimental process.

Table 2

<u>Donor</u>	<u>G418-resistant (%)</u>	<u>Targeted/G418-resistant (%)</u>
9	0.13	0.03
5	0.09	10.93
7	0.09	0.07
2	0.17	7.29
3	0.18	7.22
4	0.15	0.027

CFTR targeting data from donor cells derived from multiple fetuses. "Donor" refers to the number of the donor for the fibroblasts. The percentage of G418-resistant cells was determined by dividing the number of G418-resistant clones by the number of AAV-infected cells *100. Targeted clones are those that were PCR-positive for homologous recombination.

SCNT produced gene-targeted piglets

To produce heterozygote pigs, we used the *CFTR-null* targeted fetal fibroblasts as nuclear donors for transfer to enucleated oocytes. Then to each of eight surrogate females, we transferred between 94 and 144 SCNT embryos. At 117-118 days of gestation (full term), we delivered piglets by Cesarean section. Five surrogates produced ten males; three surrogates did not produce offspring. Figure 7 shows the first *CFTR*^{+/-} piglet. Southern blots revealed that nine of the ten offspring were *CFTR-null* heterozygotes, and one was wild-type (Fig. 8). The *CFTR*^{+/-} males reached sexual maturity, and they sired numerous litters of heterozygote offspring, both males and females.

In addition, each of four surrogates received 103-185 *CFTR-ΔF508* SCNT embryos. Five males were recovered from three surrogates on days 116-117. Southern blots revealed that four were *CFTR-ΔF508* heterozygotes and one was a wild-type. The *CFTR-ΔF508* males have not yet reached sexual maturity. All of the *CFTR*^{+/-} and *CFTR*^{+/-}*ΔF508* were phenotypically normal.

The ΔF508 allele, but not the null allele, generated mRNA

We asked whether the targeted alleles were transcriptionally active in an epithelium where *CFTR* is normally expressed. We biopsied rectal epithelia and measured *CFTR* mRNA using quantitative RT-PCR. In *CFTR*^{+/-} animals, mRNA was present at ~50% of wild-type levels (Fig. 9A). We cannot be certain that the remaining mRNA arose from the non-targeted allele, however the result is consistent with disruption of one *CFTR* allele and nonsense-mediated mRNA decay.

To assess the influence of the Neo^R cassette that resides in the intron downstream of exon 10, we used probes specific for wild-type *CFTR* and *CFTR-ΔF508*. *CFTR-ΔF508* mRNA was present at 65-70% of wild-type levels (Fig. 9B). This expression level suggests that the retained Neo^R cassette has only minimal effects on transcription. Moreover, this amount of transcript is likely sufficient to produce relatively normal amounts of *CFTR-ΔF508* protein.

Mating of CFTR^{+/-} males and CFTR^{+/-} females

The CFTR^{+/-} males and CFTR^{+/-} females reached sexual maturity and were mated. To date, the sows have delivered two litters with a total of 16 piglets.

Figure 10 shows some of the piglets at one day of age. The piglets were active and could not be distinguished by appearance or behavior, a situation that is similar to humans with cystic fibrosis (CF), who appear identical at birth to those without CF.

Genotyping by PCR showed that the two litters had 8 CFTR^{+/+}, 3 CFTR^{+/-}, and 5 CFTR^{-/-} piglets (Fig. 11).

We assessed electrolyte transport in wild-type and CFTR^{-/-} piglet airway epithelia by measuring the transepithelial voltage (V_t) across nasal epithelia; we used methods identical to those used in humans (Fig. 12). As expected, amiloride inhibited V_t in both sets of piglets indicating the presence of ENaC Na⁺ channels. To test for the presence of CFTR Cl⁻ channels, we reduced the Cl⁻ concentration on the apical surface. The wild-type V_t hyperpolarized as observed in non-CF humans. However, in the CFTR^{-/-} piglets, V_t did not change, a finding identical to the result in CF humans. This result indicates that CFTR function is absent in the CF piglets. Adding isoproterenol, to increase intracellular levels of cAMP, failed to hyperpolarize V_t, and application of ATP, to increase the intracellular Ca²⁺ concentration, had only small effects on V_t in animals of both genotypes. These results indicate that the CFTR^{-/-} piglets do not have a prominent alternative Cl⁻ channel. In contrast, CFTR^{-/-} mice have a markedly increased alternative Cl⁻ channel that has been hypothesized to explain the fact that CF mice do not develop lung disease. Finally, addition of the CFTR inhibitor GlyH-101 inhibited V_t in non-CF, but not CF piglets. Together, these data indicate that CFTR is absent from the nasal epithelia in CFTR^{-/-} piglets. Importantly, the CF piglets show electrolyte transport similar to that in humans with CF and different from that in CF mice.

On gross inspection, the pancreas from the CFTR^{-/-} piglets was smaller, showed less distinct lobulation, and was redder than that from a CFTR^{+/+} piglet (Fig. 13). Of note, humans with severe CFTR mutations uniformly develop pancreatic insufficiency, whereas CFTR^{-/-} mice do not develop pancreatic disease.

The *CFTR*^{-/-} piglets developed meconium ileus, which occurs in 15% of humans with CF. In humans, meconium ileus is associated with severe mutations in the *CFTR* gene. Of note, meconium ileus does not occur in *CFTR*^{-/-} mice.

For unknown reasons, CF mice, but not CF humans, have white teeth. The color of the teeth in *CFTR*^{+/+} and *CFTR*^{-/-} piglets was identical, indicating that in this respect, the CF pigs are like humans rather than mice.

These results indicate that CF pigs have a phenotype like that in humans with CF, including electrolyte transport in airway epithelia, pancreatic disease, meconium ileus, and tooth color. Importantly, in each of these cases, they are different from CF mice. These data suggest that *CFTR*^{-/-} pigs will serve as an ideal model in which to study the pathogenesis of CF and to develop new treatments, as described herein.

Materials of Methods

Fetal fibroblasts

Fetal fibroblasts were isolated from day 35 fetuses as previously described (Lai et al., Cloning Stem Cells 5:233-241, 2003). Cells were grown at 39°C in F10 media (Invitrogen) containing 20% FCS and 30 µg/ml gentamicin. Fetus gender was determined by PCR amplification of the Y-chromosome-specific *Sry* gene (Pomp et al., J. Anim. Sci. 73:1408-1415, 1995).

Targeting vector construction

Genomic clone: Genomic DNA was isolated (Puregene, Gentra) from pig fetal fibroblasts. A 5683 bp PCR product including *CFTR* exon 10 and flanking intronic sequence was amplified from pig fetal fibroblast genomic DNA using primers GC1F and GC8R (for primer sequences see Table 3, below) and a high fidelity polymerase (PfuUltraTM, Stratagene). Primers were designed based on the domestic pig genomic sequence from the NIH Intramural Sequencing Center (NISC) Comparative Vertebrate Sequencing Project (Genbank: AC092478 and AC092497). This PCR product was subcloned into pCR-Blunt II-TOPOTM (Invitrogen), verified by sequencing (using primers GC1F-GC8R), and served as the template for PCR amplification of 5' and 3' targeting arms. This plasmid is referred to as pG16.

CFTR-KO construction: Using PCR, the 5' and 3' homologous recombination arms were amplified from pG16 and sequentially subcloned upstream and downstream of the Neo^R cassette in pPGK-Neo-I (a generous gift from Tim Ley, Washington University; Genbank Accession Number AF335419) such that the Neo^R cassette is in the opposite orientation to the *CFTR* sequence. Primers: 5' arm: G16-Neo5'F and G16-Neo5'R; 3' arm: G16-Neo3'F and G16-Neo3'R. The Neo^R cassette consists of a Neo^R cDNA driven by the PGK promoter and is flanked by loxP sites. In the resulting construct, the Neo^R cassette disrupts *CFTR* exon 10 immediately after an in-frame stop codon that was introduced to follow isoleucine 507. Thymidine 1531 is effectively deleted, becoming the first nucleotide of the stop codon. This targeting construct is referred to as pG16-Neo.

CFTR-ΔF508 construction: The *CFTR-ΔF508* targeting vector was constructed in a similar way using the following primers: 5' arm: dF-Neo 5'F-XhoI and dF-Neo 5'R-EcoRV; 3' arm: dF-Neo 3'F-BamHI and dF-Neo 3'R-HindIII. The nucleotides encoding F508 were subsequently deleted from exon 10 using PCR mutagenesis. This targeting construct is referred to as pdF-Neo.

Table 3

Primers

Name	Sequence (5'-3')
GC1F	TTTCTCTTCTGCCTATTTCCC (SEQ ID NO:7)
GC1R	AGAAAACACTGAAGGATGCCT (SEQ ID NO:8)
GC2F	GTTTCAAATAGTTACTCAGTTTGA (SEQ ID NO:9)
GC2R	CCTCCAACCTGACACTAATCTTCTCA (SEQ ID NO:10)
GC3F	GTAGAGCTGTCAGAGAAGTAA (SEQ ID NO:11)
GC3R	AAGCCACAGAAGCATATGCAT (SEQ ID NO:12)
GC4F	AATCACTCTCAGGATGCACAT (SEQ ID NO:13)
GC5F	ATACTCAGAACAGGAAGTGCT (SEQ ID NO:14)
GC5R	ATAGCATAAGCTTCACTGTGC (SEQ ID NO:15)
GC6F	TGTCAGTAGAGAATTAGAGATTA (SEQ ID NO:16)
GC6R	GCACTACTCACCTACATCCA (SEQ ID NO:17)
GC7F	ACCTGGAAGTTGGAACACTCA (SEQ ID NO:18)
GC7R	GAAGACCCTTTACCTTCTTCTA (SEQ ID NO:19)
GC8F	CATCCAGCTGCAAACAACATT (SEQ ID NO:20)
GC8R	AATTATGCCAAACTCCATCTTAT (SEQ ID NO:21)
Ex10a5F	AGAATTTCAATTCTGCTCTCAGT (SEQ ID NO:22)

Quantitative RT-PCR primers and probes

Pig *CFTR* and *GAPDH* expression in fetal fibroblasts, nasal and rectal tissue (Figure 1) and *CFTR* expression in *CFTR* +/- pigs (Figure 9A).

CFTR primers and probe

pCFTR-1819F (anneals within exon 18)

AGTGGGCTGTAAACTCCAGTATAGA

(SEQ ID NO:23)

pCFTR-1819R (anneals withinin exon 19)

CCTTCTGCCGGCATATCAATAAACT

(SEQ ID NO:24)

pCFTR-1819 probe (spans exon 18/19 junction) FAM-

ATCGCATCAAGCTATCC-NFQ

(SEQ ID NO:25)

GAPDH primers and probe

pGAPDH-TM-F AAGCTCATTTCTCGTACGACAAT (SEQ ID NO:26)

pGAPDH-TM-R GGAGGCCATGTGGACCAT (SEQ ID NO:27)

pGAPDH-TM probe FAM-TCCACCACCCTGTTGCT-NFQ (SEQ ID NO:28)

Pig *CFTR* and $\Delta F508$ -*CFTR* expression in *CFTR* +/- $\Delta F508$ pigs (Figure 9B). Primers are the same for both, probes are allele specific.

CFTR primers and probe

pCFTR-TM-F TCATGCCGGGGCACCATTAAA (SEQ ID NO:29)

pCFTR-TM-R CGCTTTGATGACACTCCTGTATCTA (SEQ ID NO:30)

pCFTR-TM probe FAM-ACACCAAAGATGATGTTTTTC-NFQ (SEQ ID NO:31)

 $\Delta F508$ primers and probe

delF-TM-Forward TCATGCCGGGGCACCATTAAA (SEQ ID NO:32)

delF-TM-Reverse CGCTTTGATGACACTCCTGTATCTA (SEQ ID NO:33)

delF-TM-Probe FAM-GAAACACCAATGATGTTTTTC-NFQ (SEQ ID NO:34)

PCR Primers and Probes. All DNA sequences are 5'-3'. FAM: 6-carboxyfluorescein; NFQ: Non Fluorescent Quencher

AAV production

The targeting vector sequences were amplified from pG16-Neo and pdF-Neo by PCR to include flanking SbfI sites and were subcloned into the rAAV2 proviral

plasmid, pAV2 (ATCC 37216). Because of AAV genome size constraints, the total length of the targeting vectors is ~4.5 kb with the NeoR cassettes centrally located (G16-Neo: 5' targeting arm = 1510 bp; 3' targeting arm = 1274 bp; NeoR cassette = 1706 bp. dF-Neo: 5' targeting arm = 1475 bp; 3' targeting arm = 1296 bp; NeoR cassette = 1706 bp). pAV2-G16-Neo was grown in SURE2TM cells (Stratagene) and purified via a CsCl₂ method (Sambrook, Fritz, and Maniatis, Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Plainview, NY, 1989). rAAV1 (with AAV2 ITRs) was prepared as previously described (Yan et al., J. Virol. 76:2043-2053, 2002). Helper-free virus stocks were treated with nuclease and purified by high-performance liquid chromatography. Physical titers of rAAV were determined by slot blot hybridization. These viruses are referred to as AAV-G16-Neo and AAV-dF-Neo.

Infection and selection

1.5x10⁶ fetal fibroblasts were thawed and plated on a 100 mm collagen-coated culture dish. 24 hours later, cells were infected with AAV-G16-Neo or AAV-dF-Neo (200 µl, 2.5x10¹² particles/ml). 24 hours later, cells were trypsinized and transferred to 48 96-well, collagen-coated plates (BD Biosciences). 48 hours later, G418 (100 µg/ml) was added to the cell media. 10 days later, each well was trypsinized (60 µl trypsin, 0.5% EDTA) and split among 3 different vessels. For cell freezing, 1/3 of the cells were transferred to 96-well collagen-coated culture dishes and returned to the incubator for growth. For cell propagation, 1/3 of the cells were transferred to 96-well collagen-coated culture dishes and returned to the incubator for growth. For PCR screening, 1/3 of the cells were transferred to 96-well PCR plates.

PCR screen and PCR Southern blot

Cells in the 96-well PCR plates were spun down and resuspended in lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-Cl, pH 8.5, 0.5% Nonidet P40,TM 0.5% TweenTM, 400 µg/ml Proteinase K) (McCreath et al., Nature 405:1066-1069, 2000). Most wells (~70%) contained only dead cell debris following selection, but all wells were processed to minimize human error. The cells were lysed for 30 min at

65°C, followed by 10 min at 95°C. 1 µl of lysate was used in a 50 µl PCR reaction. PCR conditions: 2 min at 95°C, 30 cycles of 95°C for 20 sec, 56°C for 20 sec, and 68°C for 4 min, then 68°C for 5 min. Primers Ex10a5F and GC7R are expected to amplify a 2.0 kb product from wild-type alleles and a 3.7 kb product from G16-Neo targeted alleles. PCR products were electrophoresed on 1.0% E-Gel 96 gels (Invitrogen). Positive PCR reactions were also electrophoresed on standard 1.0% agarose gels and transferred to a nylon membrane. The membranes were probed with biotin-labeled Neo-specific or $\Delta F508$ -allele-specific oligonucleotides and detected by chemiluminescence (North2South, Pierce).

Processing screen-positive cells

Following identification of PCR-positive clones, the corresponding cells from the “freezing” plate were grown to confluence (~10,000 cells). Cells were detached with 60 µl trypsin and 20 µl of detached cells were placed into each of 3 cryovials. Three hundred µl freezing media was added to each cryovial and the vials were transferred to an isopropanol cryofreezing container at -70°C. After 24 hours, the vials were transferred to liquid nitrogen. The corresponding cells from the propagation plate were transferred to 24-well plates, and subsequently to 6-well and 100 mm culture dishes. The sequential transfer to increasingly larger culture dishes was carried out to achieve consistent cell growth and viability.

Southern blotting

For CFTR-KO targeting, genomic DNA was isolated from 100 mm dishes (Gentra) and 10 µg was digested with *Bgl*III overnight. For *CFTR*- $\Delta F508$ targeting, genomic DNA was isolated from 24-well dishes. Ten ng was used for whole genome amplification (Repli-GTM, Qiagen) and 25 µg amplified DNA was digested with *Bgl*III overnight. Genomic digests were electrophoresed on a 0.7% agarose gel and transferred to a positively charged nylon membrane (Roche) by using an alkaline transfer procedure. Blots were pre-hybridized for 15 min at 65°C in Rapid-hyb buffer (Amersham). The blot was then hybridized in Rapid-hyb buffer with a ³²P-labeled probe specific for a region of *CFTR* that is outside of the targeting vector boundaries.

For Neo-specific probing, blots were either stripped, or, in most cases, the *Bgl*/II digest and Southern blot procedure was repeated using a ^{32}P -labeled Neo-specific probe.

Preparation of donor cells for SCNT

Frozen aliquots of *CFTR*-targeted cells were thawed at 37°C and pre-warmed in F-10 medium (Invitrogen) with 20% fetal calf serum (FCS). The cells were washed twice by centrifugation and cultured (F-10, Invitrogen; 20% FCS, Hyclone; gentamicin, 2.5 ng/ml FGF and G418, Invitrogen) for 1-2 days in 24-well collagen-coated plates (35-4408, Biocoat cellware). Confluent cells were dispersed with 0.05% trypsin/EDTA for 3-5 min at 38.5°C and 500 μL F-10 with 20 % FBS, followed by centrifugation twice at 3000 rpm for 5 min. The supernatant was removed, and the cells were resuspended in micromanipulation medium (25 mM HEPES, TCM199, Gibco; 0.3% BSA).

Oocyte maturation and SCNT

Oocytes were received from BoMed, Inc (Madison, WI) ~24 hours after placing them into maturation medium, and were then transferred to a 4-well dish and cultured for a total maturation of 42-44 hours at 38.5°C in a humidified atmosphere of 5% CO_2 in air. After 42-44 h of *in vitro* maturation, oocytes were stripped of their cumulus cells by gentle vortexing in 0.5 mg/mL hyaluronidase. After removal of the cumulus cells, oocytes with good morphology and a visible polar body (metaphase II) were selected and kept in micromanipulation medium at 38.5°C until SCNT.

SCNT was performed essentially as previously described (Lai et al., Science 295:1089-1092, 2002; Lai et al., Nat. Biotechnol. 24:435-436, 2006) in micromanipulation medium supplemented with 7.5 $\mu\text{g/mL}$ cytochalasin B. The metaphase II chromosomes and the polar body were aspirated by inserting a micropipette through the zona pellucida and aspirating the polar body and the adjacent cytoplasm into the pipette. Next a donor cell was aspirated into the same pipette, the pipette was inserted into the previously made hole in the zona pellucida, and the cell deposited under the zona pellucida. The nuclear transfer complex was fused in a medium with a low Ca^{2+} concentration (0.3 M mannitol, 0.1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.1

mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ and 0.5 mM HEPES), activated with 200 μM thimerosal for 10 min in the dark, and then rinsed and treated with 8 mM dithiothreitol (DTT) for 30 min. Finally the oocytes were rinsed to remove any traces of DTT (Lai et al., Nat. Biotechnol. 24:435-436, 2006). Following fusion/activation, oocytes were washed three times with PZM3 as previously described for 30 min (Im et al., Theriogenology 61:1125-1135, 2004). Those that had fused were cultured for 15-21 hours until surgical embryo transfer to a surrogate.

Surrogate preparation and embryo transfer

The embryonic cleavage rate was examined before transferring the reconstructed embryos into recipients. The recipients were synchronized by administering 18-20 mg Regumate and hCG as previously described (Lai et al., Cloning Stem Cells 5:233-241, 2003). Twelve surrogates on the first day of estrus (designated day 0) or the first day after standing estrus were used. Embryo transfer was performed surgically as previously described (Lai et al., Cloning Stem Cells 5:233-241, 2003) and 94 to 185 embryos were inserted into one oviduct through the ovarian fimbria. Surrogates were checked for pregnancy by abdominal ultrasound examination after day 21 and then checked weekly throughout gestation, and were allowed to go to term. A cesarean section was performed to recover the piglets on day 116-118. After delivery the piglets were provided medical care, fed colostrums, and initially raised on a commercial pig milk replacer until mature enough to be placed on standard pig diets.

Rectal biopsy

Pigs were lightly anesthetized with ketamine (20 mg/kg) and acepromazine (0.2 mg/kg). A 10 cm anoscope was partially inserted in the rectum and rectal tissue was collected using gastrointestinal biopsy forceps (2.2 mm). Tissue samples were immediately placed in RNAlaterTM (Ambion). Recovery from anesthesia was monitored continuously until the pigs returned to normal activity (2-4 hours).

Quantitative RT-PCR

Quantitative RT-PCR using TaqManTM chemistry and an ABI 7500 Fast Real-time PCR System was used to measure pig *CFTR* mRNA. Briefly, total RNA was isolated from fibroblasts or nasal and rectal biopsy tissue (RNeasyTM, Qiagen). First-strand cDNA was synthesized with random primers (SuperScript IIITM, Invitrogen). Sequence-specific primers and probes for pig *CFTR* and *GAPDH* were designed and ordered using Assays-by-designTM (Applied Biosystems). For measuring total *CFTR* mRNA primer/probe sets spanning exons 18 and 19 of *CFTR* and *GAPDH* were used in separate reactions. For measuring $\Delta F508$ mRNA levels, one primer set and two probes (*F508* and $\Delta F508$) were used in separate reactions. Primer and probe sequences are included in Table 2. TaqManTM Fast Universal PCR Master Mix was used for all reactions. The reaction volume was 20 μ l (10 μ l of 2x Master Mix without UNG, 1 μ l of 20x target primer and probe, 8 μ l of Nuclease-free water, and 1 μ l of cDNA sample). The reaction plates were covered with optical film and centrifuged briefly. The thermocycler conditions were as follows: 20 sec at 95°C, 40 cycles of 95°C for 3 sec and 60°C for 30 sec. All experiments were run in triplicate. Because the efficiencies of *CFTR* and *GAPDH* amplification were not equal, the relative quantification of transcript levels was performed using the standard curve method.

II. The $\Delta F508$ mutation – an interspecies analysis

The $\Delta F508$ mutation confers at least three defects on human *CFTR*: it reduces channel activity, it impairs processing, and it reduces the protein's stability at the cell surface. The $\Delta F508$ mutation inhibits gating of *CFTR* channels from three species (i.e., mouse, pig, and human) studied by the same mechanism, a reduced opening rate. In contrast, the characteristic processing defect observed in human *CFTR*- $\Delta F508$ is less severe in pig and mouse proteins. This conclusion is supported by our finding that the mouse and pig proteins showed complex glycosylation, were readily excised for patch-clamp experiments at 37°C, immunocytochemistry localized some of the protein to the apical membrane of airway epithelia, and corrected the Cl^- transport defect in CF airway epithelia more than did human *CFTR*- $\Delta F508$. This shows that

there is a gradient in the severity of the $\Delta F508$ processing defect, with human worse than pig, and pig somewhat worse than mouse. Additionally, this also shows that the processing defect and the functional defect in CFTR- $\Delta F508$ arise from different causes.

Vectors and expression

Human, pig, and mouse *CFTR* cDNA (SEQ ID NOs:1, 3, and 5) were amplified from *Homo sapiens*, domestic pig (*Sus scrofa*), and domestic mouse (*mus musculus*). We subcloned all three *CFTR* cDNAs into pcDNA3.1 (Invitrogen) and recombinant adenoviruses. For recombinant adenovirus of mouse CFTR, we had to remove intron 11 which had been inserted previously to stabilize the vector.

For protein processing studies, COS7 cells were electroporated; 3T3 and LLC-PK1 cells were transfected with plasmid and Lipofectamine 2000TM. For deglycosylation studies, COS7 cells were electroporated with human CFTR or infected with adenovirus encoding pig or mouse CFTR. For patch-clamp studies, HeLa cells were infected with adenovirus encoding mouse CFTR or transfected using a hybrid vaccinia virus system encoding pig CFTR. Expression in human and mouse airway epithelia was with recombinant adenoviruses. Murine tracheal epithelia were cultured from $\Delta F508/\Delta F508$ transgenic mice (CFTR^{tm1Kth}) or CFTR null mice expressing the intestinal FABP-CFTR (CFTR^{tm1Unc/FABP-CFTR}). In the absence of gene transfer, there were no cAMP-stimulated Cl⁻ currents in either mouse genotype.

Biochemical studies

COS7 cells were solubilized in lysis buffer with 1% TX-100TM and proteinase inhibitors. CFTR was immunoprecipitated with M3A7 antibody (Upstate Technology) and then *in vitro* phosphorylated as described previously. Note that the consensus phosphorylation sites and N-glycosylation sites are conserved in all three species (Fig. 14). Processing studies in NIH-3T3 and LLC-PK1 cells were carried out similarly to those in COS7 cells. For deglycosylation studies, membranes were isolated from COS7 cells and solubilized in LB plus 1% NP40TM (Pierce). Supernatants

were divided, immunoprecipitated, and resuspended with or without endoglycosidase H. Following incubation, precipitates were *in vitro* phosphorylated.

Immunocytochemistry

Three days following gene transfer, epithelia were fixed, permeabilized, and incubated with a mixture of anti-CFTR antibodies (M3A7, MM13-4 (Upstate Biotechnology) and 13-1 (R&D Systems) and anti-ZO-1 (Zymed) primary antibodies, followed by Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and examined by confocal laser scanning microscopy.

Electrophysiology

For Ussing chamber studies, transepithelial Cl⁻ current was measured 3 days following gene transfer using a Cl⁻ concentration gradient as previously described. For patch-clamp studies CFTR currents were studied in excised, inside-out membrane patches of HeLa cells as previously described. Channels were activated with the catalytic subunit of PKA and Mg-ATP; PKA was present in all cytosolic solutions that contained ATP. Holding voltage was -50 to -100 mV. Experiments were performed at 23-26°C. Data acquisition, processing, and analysis were performed as previously described. Data are mean ± SEM unless otherwise stated. P < 0.05 was considered statistically significant.

Sequence of pig CFTR

We cloned the pig CFTR cDNA and used it to predict the amino acid sequence (Fig. 14). The pig CFTR amino acid sequence is nearly 93% identical to that of human CFTR. For comparison, mouse CFTR shows 78% identity to human CFTR. The region immediately surrounding F508 is highly conserved.

Glycosylation of human, pig, and mouse CFTR- Δ F508

The pattern of human CFTR glycosylation changes as the protein migrates from the ER to the Golgi complex. The nascent protein lacking glycosylation is called “band A.” In the ER, CFTR undergoes core glycosylation and migrates more slowly during electrophoresis as “band B.” In the Golgi complex, more extensive glycosylation occurs, which further slows and broadens the electrophoretic migration of the “band C” form. Differences in glycosylation do not appear to affect function, but do provide a convenient way to assess the biosynthetic processing of CFTR. When we expressed wild-type human, pig, and mouse CFTR in the monkey kidney cell line COS7, we observed the typical appearance of bands B and C (Fig. 15A). Human CFTR- Δ F508 produced band B, but not band C, consistent with defective exit from the ER. This result agrees with many previous reports in several different cell lines. Surprisingly, in addition to band B, mouse CFTR- Δ F508 generated a significant proportion of band C protein. Pig CFTR- Δ F508 also produced a small amount of band C. These results suggested that some mouse and pig mutant protein may have trafficked to the Golgi complex.

To learn whether the differences between the three species of CFTR- Δ F508 depended on the primate COS7 cell line, we expressed the constructs in the mouse NIH-3T3 fibroblast line and the pig LLC-PK1 kidney cell line (Fig. 15B and C), as well as human HEK-293T cells (not shown). In each of these cell lines, human CFTR- Δ F508 generated only the band B form, whereas pig and mouse CFTR- Δ F508 produced both band B and some fully glycosylated protein, consistent with our studies in COS7 cells. We also noted that some of the wild-type and Δ F508 pig CFTR migrated slightly more rapidly than band B of either human or mouse.

To confirm that the high molecular mass C forms of pig and mouse CFTR- Δ F508 were due to complex glycosylation, we used endoglycosidase H digestion. Endoglycosidase H removes carbohydrate from proteins that contain only the sugar added in the ER, but it does not delete complex glycosylation added in the Golgi complex. Endoglycosidase H treatment shifted the migration of the band B form of all the proteins to the unglycosylated form (Fig. 16). However, like the band C form of the wild-type CFTRs, the fully glycosylated mouse and pig CFTR- Δ F508 were

resistant to endoglycosidase H, confirming that these proteins were glycosylated in the Golgi complex.

Expression of human, pig, and mouse CFTR- Δ F508 at the cell surface

To determine if the human, pig and mouse CFTR- Δ F508 were localized at the apical membrane of airway, we expressed the proteins in well-differentiated human CF airway epithelia and examined them with confocal immunocytochemistry. Consistent with earlier studies, wild-type human CFTR localized at the apical membrane and human CFTR- Δ F508 appeared to be expressed diffusely throughout the cell (Fig. 17). As expected from the biochemical studies, both pig and mouse wild-type were localized to the apical membrane. However, in contrast to human CFTR- Δ F508, both the pig and mouse mutants were also present in the apical portion of the airway cells.

Single channel gating of human, pig, and mouse CFTR- Δ F508

Most, although not all studies indicate that human CFTR- Δ F508 manifests a channel gating defect that reduces activity. To learn whether the Δ F508 mutation compromises the channel activity in pig and mouse CFTR, we studied excised, inside-out patches of membrane containing CFTR channels. We readily detected channels in patches taken from cells expressing pig and mouse CFTR- Δ F508 grown at 37°C, consistent with the conclusion that pig and mouse CFTR- Δ F508 are able to reach the cell membrane under physiological conditions. This contrasts with human CFTR- Δ F508, which must be incubated at lowered temperatures to produce significant amounts of cell surface protein. Phosphorylation by the catalytic domain of cAMP-dependent protein kinase (PKA) and cytosolic ATP were required for activity of all versions studied. The single channel conductances for the wild-type channels were human (8.3 pS) > pig (6.7 pS) > mouse (4.3 pS), and they were not significantly altered by the Δ F508 mutation (Fig. 18A and 18B). Lansdell et al. (J. Physiol. 508:379-392, 1998) reported that heavily filtering currents recorded from mouse CFTR revealed a subconductance state that was ~10% the amplitude of the full

conductance. With heavy filtering, we also observed the subconductance in both wild-type and $\Delta F508$ channels (Fig. 18A).

In the presence of PKA and 1 mM ATP, the open state probability (P_o) of wild-type CFTR varied in the order, pig (0.39) $\approx$ human (0.37) $>$ mouse (0.08) (Fig. 18B); the values for human were taken from our earlier study. In assessing mouse P_o , we did not take into account the subconductance state; as reported by Lansdell et al., it was very difficult to study due to its small single-channel conductance. The $\Delta F508$ mutation reduced the P_o of human CFTR to 27%, pig CFTR to 46%, and mouse CFTR to 50% of the corresponding wild-type channel (Fig. 18A and 18B). The cause of the reduced P_o was a decrease in the rate of channel opening without a significant alteration of burst duration (Fig. 18B). Thus, in all three species, the $\Delta F508$ mutation altered gating by a similar mechanism.

Transepithelial Cl⁻ current generated by human, mouse, and pig CFTR- $\Delta F508$

Because both pig and mouse CFTR- $\Delta F508$ were partially processed through the Golgi complex and likely targeted to the apical membrane, and because they both retained partial Cl⁻ channel activity, we predicted they would generate transepithelial Cl⁻ currents when expressed in well-differentiated CF airway epithelia. To assay transepithelial Cl⁻ transport, we mounted epithelia in modified Ussing chambers and measured transepithelial Cl⁻ current. We first inhibited Na⁺ current with amiloride, which hyperpolarizes the apical membrane voltage, increasing the driving force for Cl⁻ secretion through CFTR. Then, we increased CFTR activity by elevating cellular levels of cAMP with forskolin and IBMX. Finally, we reduced transepithelial Cl⁻ current by inhibiting the Na⁺-K⁺-Cl⁻ cotransporter with basolateral bumetanide; the resulting change in current provides a good measure of the Cl⁻ current. We chose bumetanide rather than CFTR inhibitors, because they can have different efficacy on CFTR from different species.

Expressing wild-type human CFTR produced significant transepithelial Cl⁻ current (Figs. 19 and 20), as previously described. The same was true for wild-type pig and mouse CFTR. As expected, human CFTR- $\Delta F508$ failed to generate much current. However, relative to the wild-type current of each species, both pig and

mouse CFTR- Δ F508 produced substantial transepithelial Cl^- currents (Fig. 20B). To determine if these results were limited to expression in human epithelia, we repeated the study using airway epithelia derived from CF mice; the results were qualitatively similar (Fig. 20A and 20B). Thus, pig and mouse CFTR- Δ F508 generated transepithelial Cl^- currents in CF airway epithelia from two different species. These results indicate that some pig and mouse CFTR- Δ F508 was present and active in the apical membrane of airway epithelia.

III. A transgenic, non-human animal model of cystic fibrosis using transgenic human CFTR- Δ F508

The above-mentioned defects between mutant mouse, pig, and human CFTR illustrate the need for the generation of a transgenic non-human animal model of CF that contains a CFTR- Δ F508 mutation that closely mimics the functional and processing characteristics of the human CFTR- Δ F508. The generation of a large animal, such as a pig, in which the endogenous CFTR gene is knocked-out and a human CFTR- Δ F508 transgene is introduced allows for the study of the causative factor of human CF without suffering from experimental artifacts introduced by the different physical and functional characteristics of the endogenous CFTR of the animal. Such models also facilitate the identification, characterization, and development of approaches (e.g., small molecule-based drugs) that can be used in CF therapy.

Generation of a CFTR-/- knock-out, human CFTR (hCFTR) transgenic pig

A yeast artificial chromosome (YAC) containing the entire ~230 kb human CFTR gene (wild-type or F508del) plus upstream and downstream sequences is introduced into pig CFTR-/- fetal fibroblasts. One specific example is YAC37AB12 (Anand et al., Genomics 9(1):124-130, 1991). This YAC has been used to complement null CF mice (Manson et al., EMBO 16(14):4238-4249, 1997) and to express human CFTR in Chinese hamster ovary cells (Mogayzel et al., Human Molecular Genetics 6(1):59-68, 1997). Any mutations, such as the CF F508del mutation, are introduced into the YAC by site-directed techniques that are well known

in the art. YAC delivery is accomplished, for example, via nuclear microinjection, lipid-mediated transfection, or fusion of fibroblasts with yeast spheroplasts. Since the human CFTR-expressing YAC contains an antibiotic selection marker (different from the marker used to make the CFTR^{-/-} cells), cells positive for YAC transgenesis are screened by antibiotic selection. Resistant cells are then screened by PCR, Southern blot, fluorescence *in situ* hybridization (FISH), and/or fiber-FISH to assess human CFTR integration, copy number, and integrity. These procedures are optimized to minimize the number of fibroblast doublings and time in culture. Cells deemed appropriate for nuclear transfer are then transferred to enucleated oocytes, fused, and electrically stimulated, and transferred to recipient females.

Alternatives to the YAC approach include the use of a minigene, which is a DNA sequence containing the human CFTR promoter and the entire cDNA (CDS and 5' and 3' UTRs) with the first one, two, or three introns still intact. This approach results in human CFTR that still has normal endogenous levels of expression without the large size of the entire gene (<40kb vs. >300kb). Additionally, human CFTR cDNA is introduced (i.e., no introns) with either a CFTR promoter, a promoter with CFTR-like tissue expression (e.g., cytokeratin 18 promoter), or a constitutive promoter such as the CMV promoter. In another alternative, the human CFTR and necessary regulation, selection, and tracking elements (e.g., promoter, antibiotic resistance gene, GFP, luciferase) are introduced into a pig fibroblast or fertilized embryo by means of a viral vector, such as a retrovirus or lentivirus. Each of these methods results in the random integration of the wild-type or mutated human CFTR gene in the pig genome, the exact location of which can be later identified.

Other Embodiments

Although the invention has been described above in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto.

Use in the claims and elsewhere herein of singular forms, such as “a” and “the,” does not exclude indication of the corresponding plural form, unless the context indicates to the contrary. Thus, for example, if a claim indicates the presence of “a” mutation in “a” gene, it can be interpreted as covering one or more mutations, in one or more genes, unless otherwise indicated.

Other embodiments are within the following claims.

CLAIMS:

1. An isolated cell of a pig model of cystic fibrosis, wherein a cystic fibrosis membrane transporter (CFTR) gene of the cell comprises a targeted mutation selected from deletion of phenylalanine 508 (Δ F508 mutation) and a null mutation.
2. The isolated cell of claim 1, wherein said targeted mutation in said CFTR gene is the Δ F508 mutation.
3. The isolated cell of claim 1, wherein said targeted mutation in said CFTR gene is a null mutation.
4. The isolated cell of any one of claims 1-3, wherein the mutation is present in one allele of the CFTR gene in the genome of the pig.
5. The isolated cell of any one of claims 1-3, wherein the mutation is present in both alleles of the CFTR gene in the genome of the pig.
6. The isolated cell of claim 3, wherein the mutation comprises an insertion of an exogenous nucleic acid molecule and/or a transcription termination sequence.
7. The isolated cell of claim 5, wherein the mutation substantially eliminates expression of a functional gene product of the targeted gene in cells in which such expression normally takes place, absent the mutation.
8. The isolated cell of claim 5, wherein the mutations in both alleles of the CFTR gene are identical to one another.
9. An isolated cell of a pig model of cystic fibrosis, in which an endogenous CFTR gene is knocked-out or inactivated, and a wild-type or Δ F508 CFTR gene from a different animal is expressed.
10. The isolated cell of claim 9, wherein the cell is CFTR^{-/-} (knock-out) and expresses a human wild-type or Δ F508 CFTR gene.
11. The cell of any one of claims 1-10, wherein the cell is a fetal cell.

12. The cell of any one of claims 1-11, wherein said cell is a fibroblast.
13. The cell of claim 1 or 2, wherein said cell comprises one or two $\Delta F508$ cystic fibrosis transmembrane conductance regulator mutations.
14. A method of making a pig model of cystic fibrosis, the method comprising the steps of:
- i) introducing a $\Delta F508$ or null mutation into an allele of a CFTR gene in a donor pig to generate a donor cell;
 - ii) introducing the nucleus of the donor cell into a recipient porcine enucleated cell to generate an embryo; and
 - iii) transferring the embryo into a surrogate female pig.
15. The method of claim 14, wherein the donor cell is a fetal fibroblast.
16. The method of claim 14 or 15, wherein the recipient cell is an enucleated oocyte.
17. The method of any one of claims 14-16, wherein the donor cell comprises a $\Delta F508$ or null mutation in one allele of the CFTR gene, and the method is carried out to introduce a $\Delta F508$ or null mutation into the other CFTR allele.
18. The method of any one of claims 14-17, further comprising breeding a male pig that is born from a surrogate female to a female pig that is born from a surrogate female to obtain a homozygous mutant.
19. The method of any one of claims 14-18, wherein the CFTR gene comprises the $\Delta F508$ mutation.
20. The method of any one of claims 14-18, wherein the CFTR gene comprises a null mutation.
21. The method of claim 20, wherein the CFTR gene is not expressed in the cell into which the mutation is introduced.
22. The method of any one of claims 14-21, wherein an adeno-associated virus vector is used to introduce the mutation into the allele of the CFTR gene.

23. A method of identifying a therapeutic agent that can be used in the treatment of cystic fibrosis, the method comprising administering a candidate therapeutic agent to a pig comprising cells comprising a targeted mutation in a CFTR gene and monitoring the pig for a symptom of cystic fibrosis, wherein the targeted mutation is a deletion of phenylalanine 508 (Δ F508 mutation) or a null mutation, and detection of improvement in a symptom of cystic fibrosis indicates the identification of a compound that can be used in the treatment of cystic fibrosis.

24. The method of claim 23, wherein said mutation in said CFTR gene is the Δ F508 mutation.

25. The method of claim 23, wherein said mutation in said CFTR gene is a null mutation.

26. The method of any one of claims 23-25, wherein the mutation is present in one allele of said CFTR gene in the genome of the pig.

27. The method of any one of claims 23-25, wherein the mutation is present in both alleles of the CFTR gene in the genome of the pig.

28. The method of any one of claims 23-27, wherein the mutation results in full or partial inactivation of the CFTR gene.

29. The method of claim 25, wherein the mutation comprises an insertion of an exogenous nucleic acid molecule and/or a transcription termination sequence.

30. The method of claim 27, wherein the mutation substantially eliminates expression of functional CFTR in cells in which such expression normally takes place, absent the mutation.

31. The method of claim 27, wherein the mutations in both alleles of the gene are identical to one another.

32. A method of targeting the introduction of a mutation into a gene in a pig cell, the method comprising the steps of:

providing pig cells,

using an adeno-associated viral vector to deliver a gene targeting construct into the isolated pig cells, in the absence of cell detachment and reattachment, and

selecting gene-targeted clones, wherein the targeting construct introduces a deletion of phenylalanine 508 (Δ F508 mutation) or a null mutation, and the cells are in culture for 30 days or less during the targeting construct delivery and selection steps.

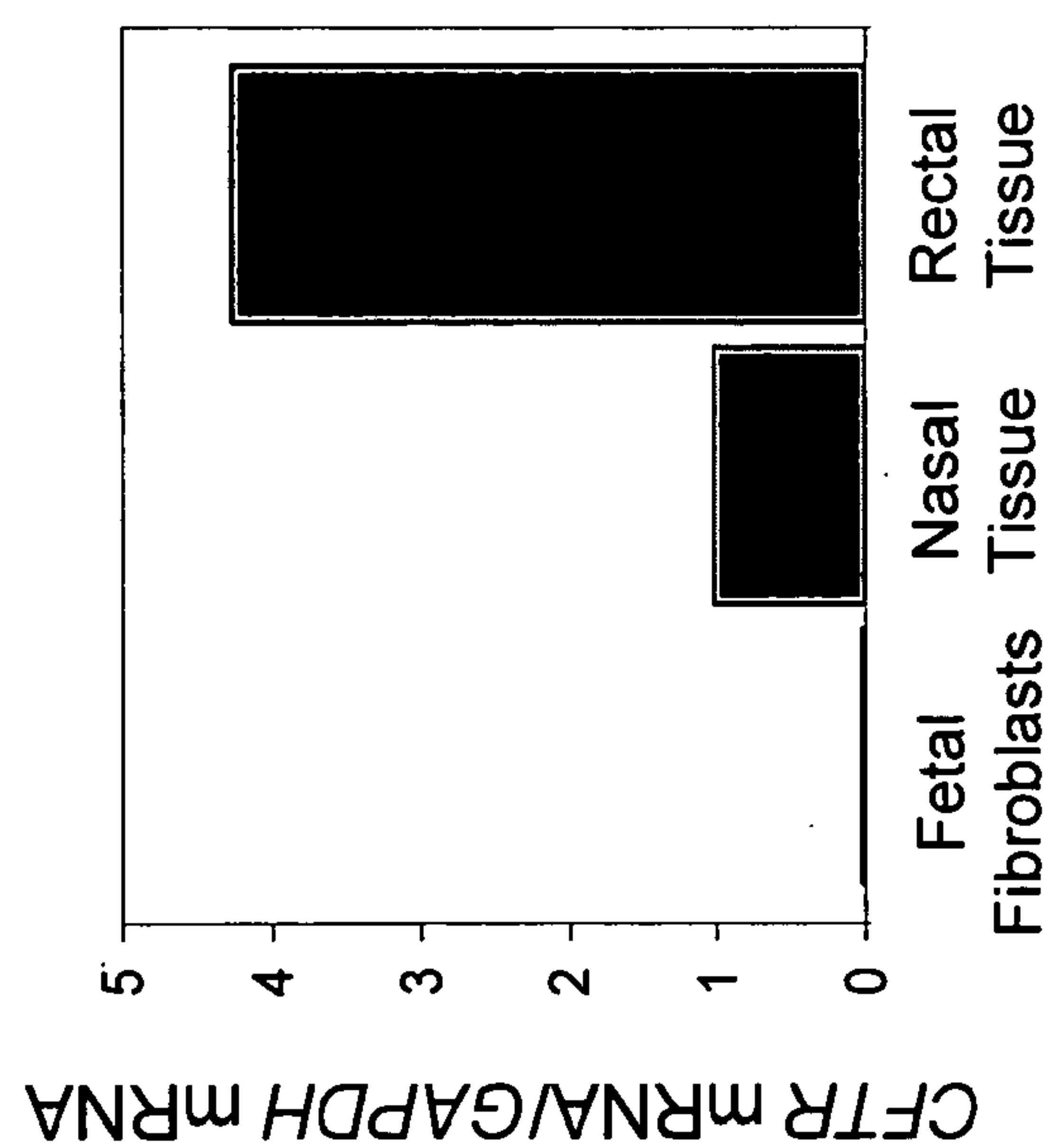
33. The method of claim 32, wherein the cells are fetal fibroblasts.

34. The method of claim 32 or 33, wherein the cells are in culture for 20 days or less during the targeting construct delivery and selection steps.

35. The method of claim 32, wherein the mutation is the Δ F508 mutation.

36. The method of claim 32, wherein the mutation is a null mutation.

Figure 1



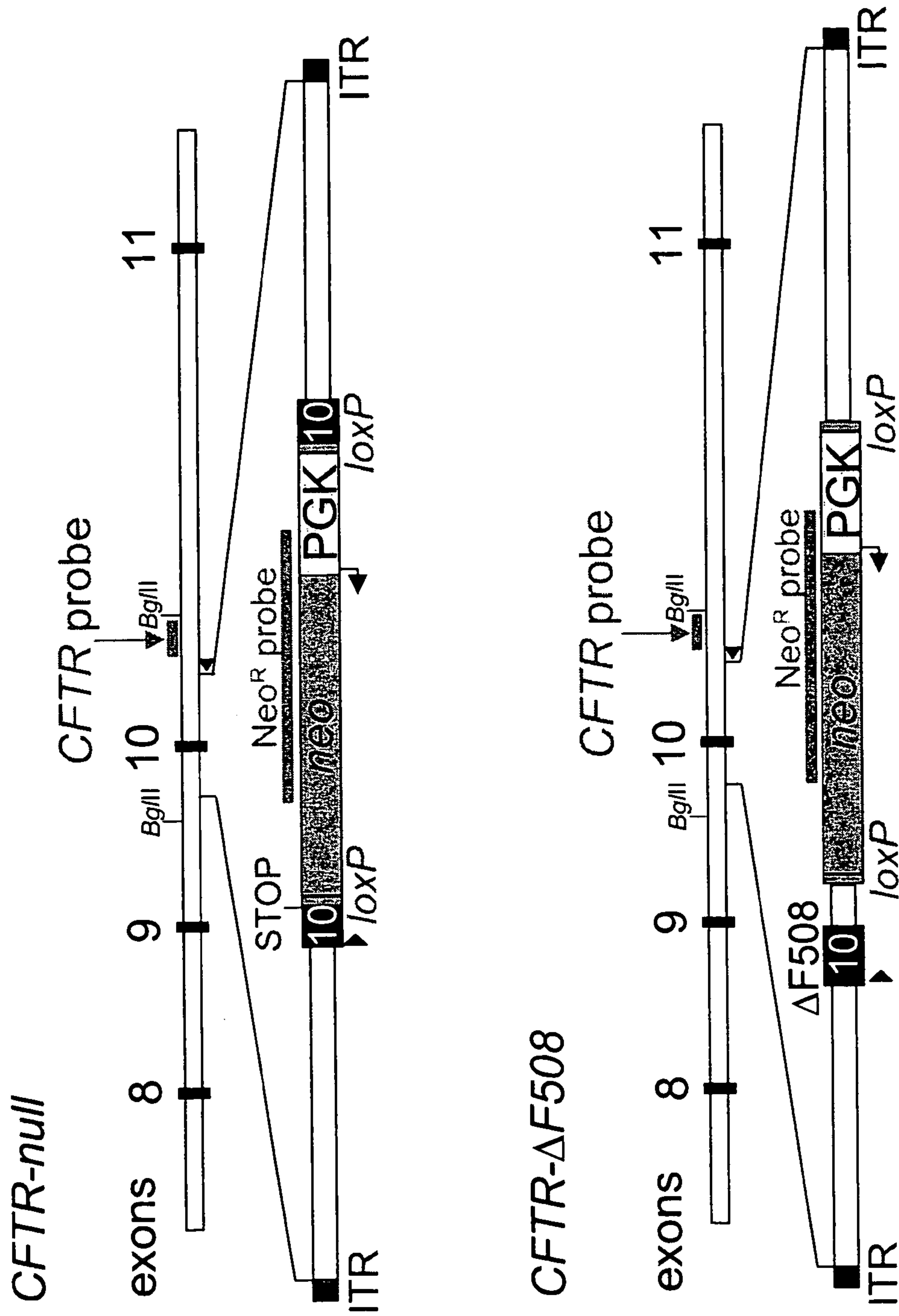


Figure 2

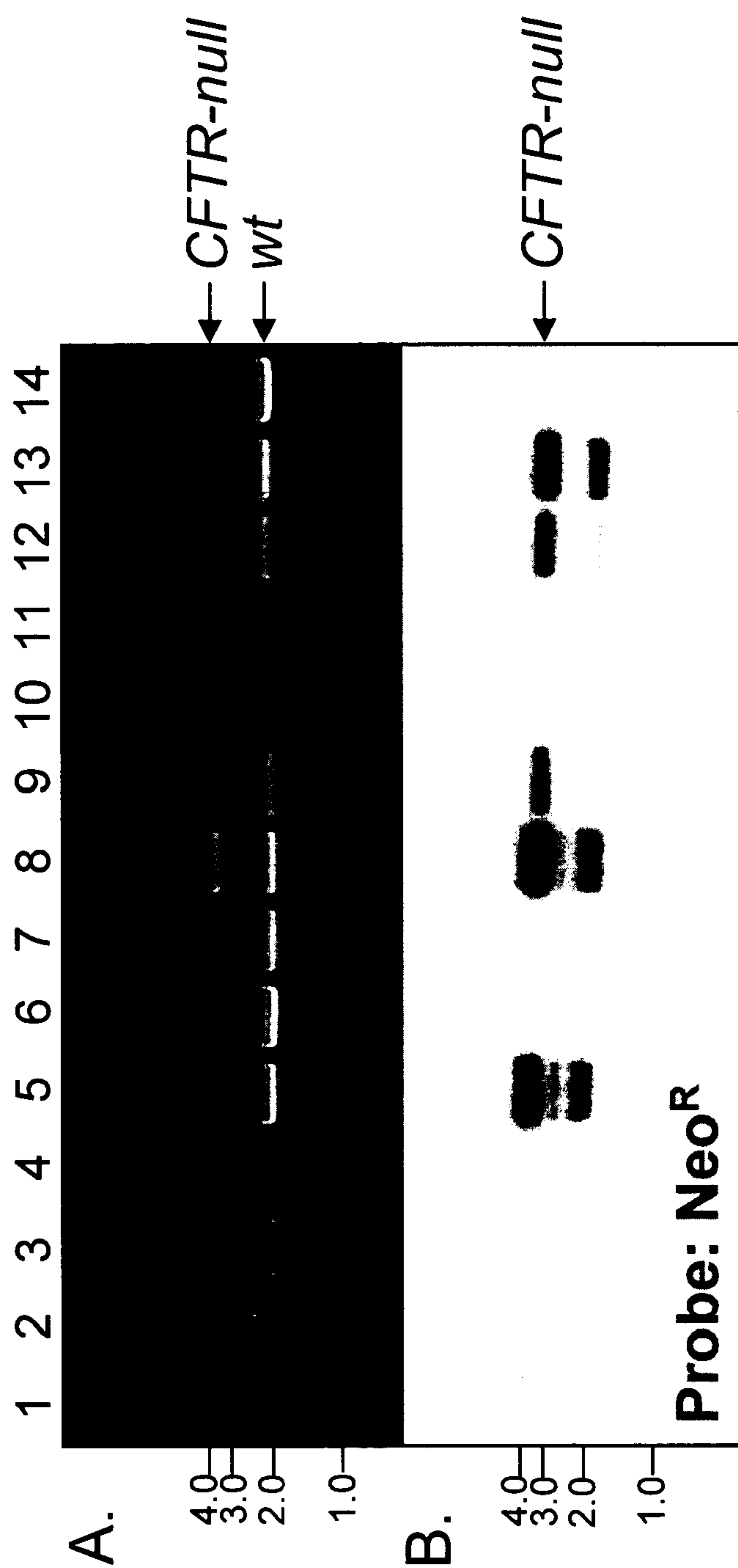


Figure 3

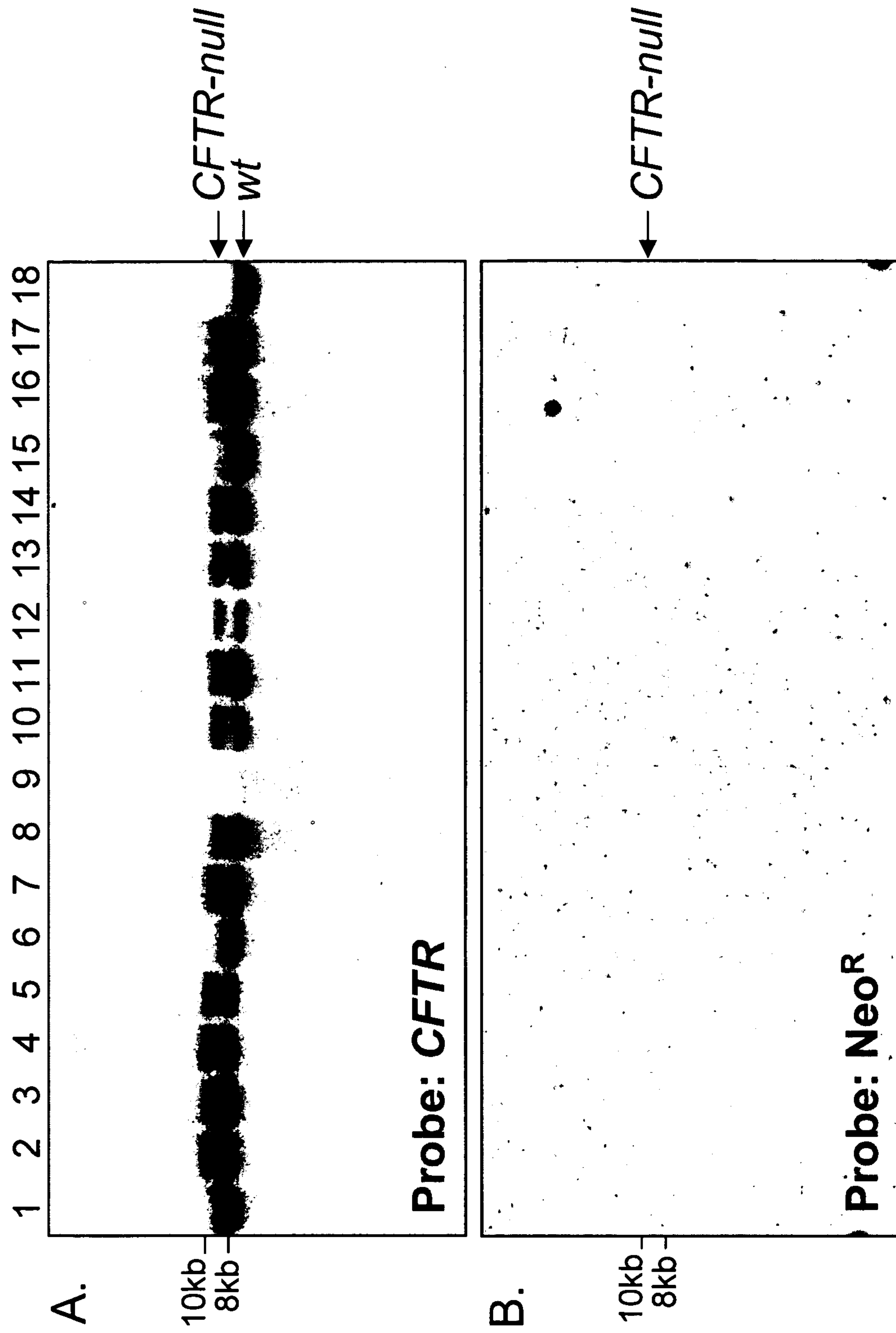


Figure 4

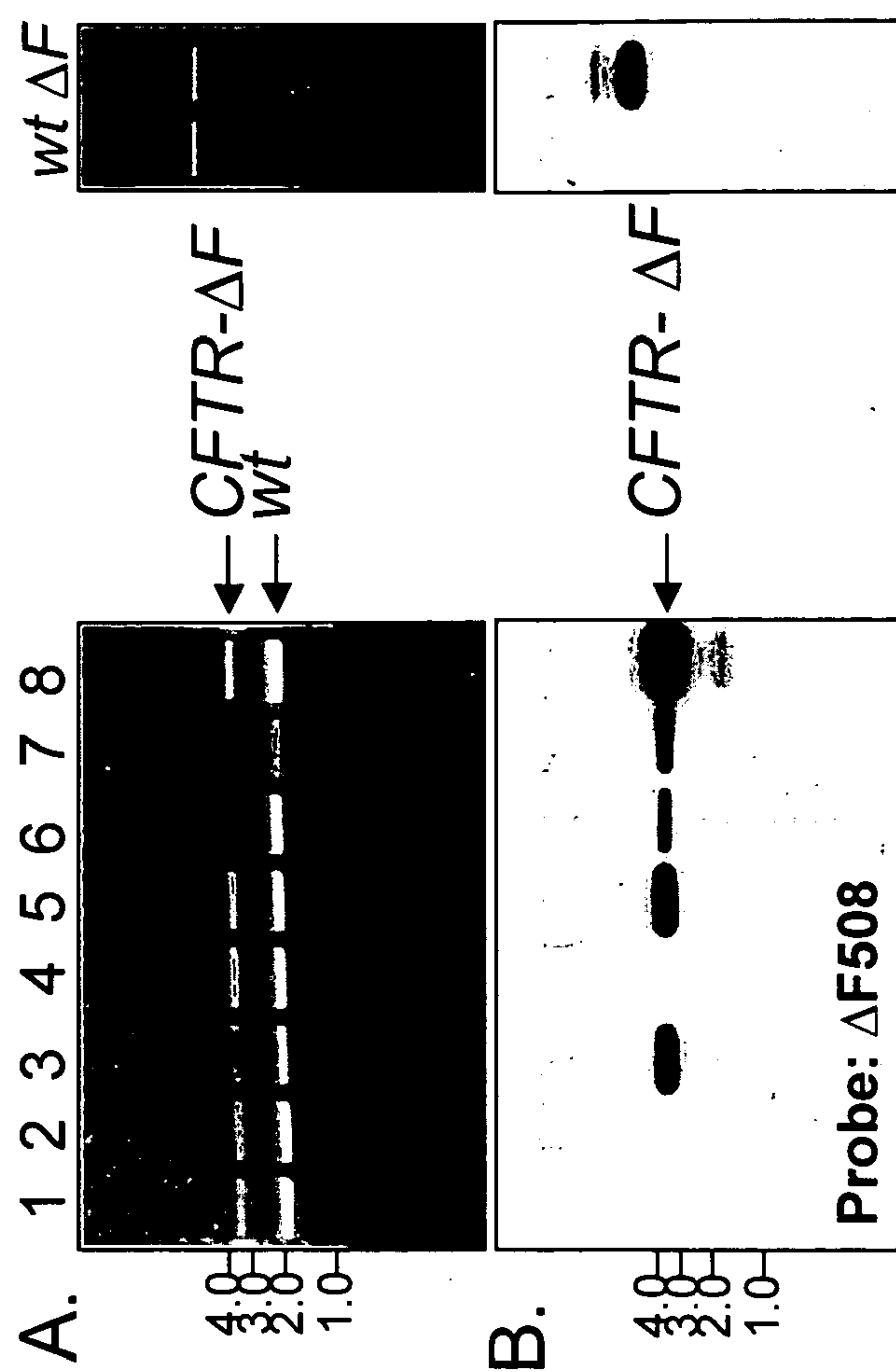


Figure 5

Figure 6

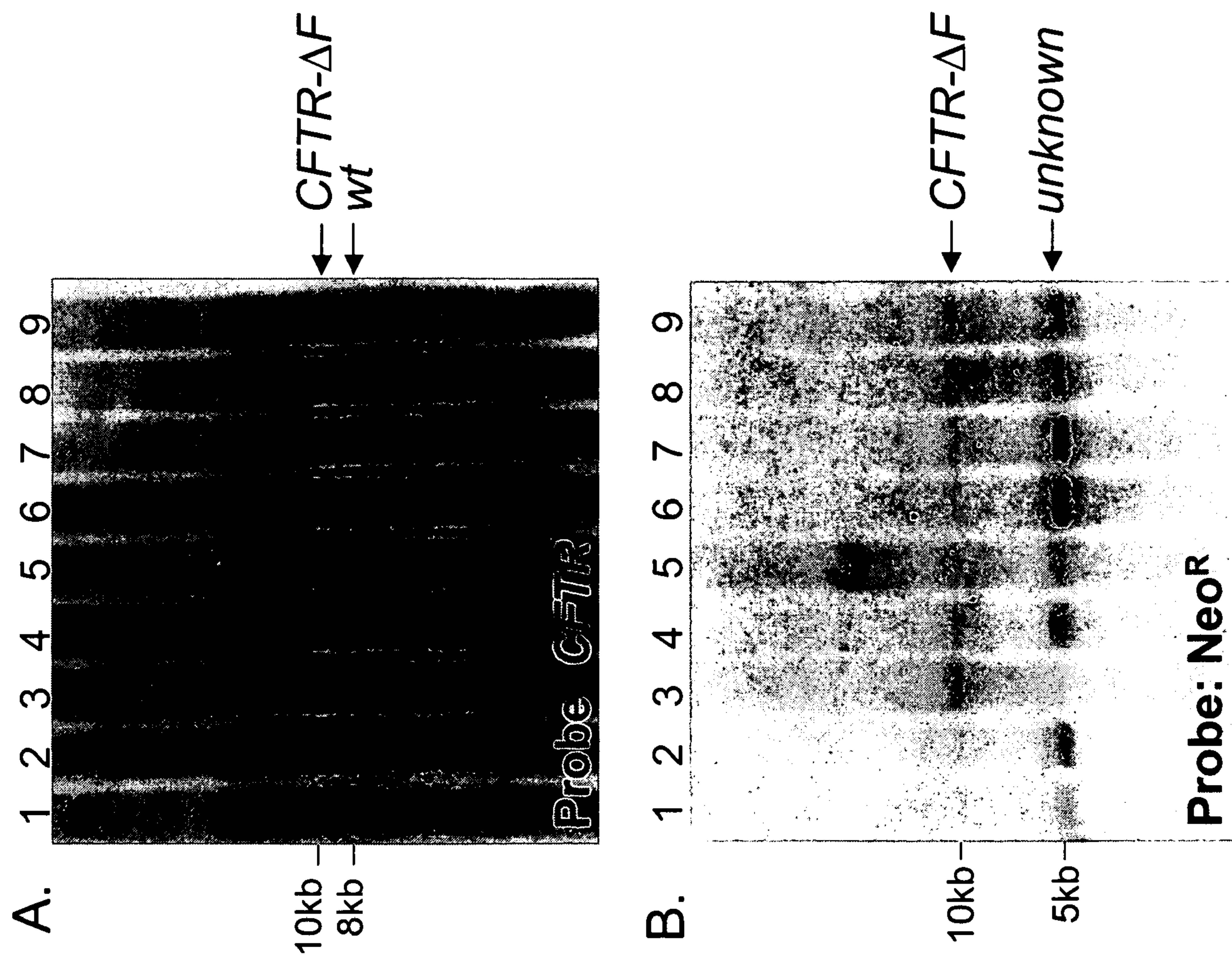




Figure 7

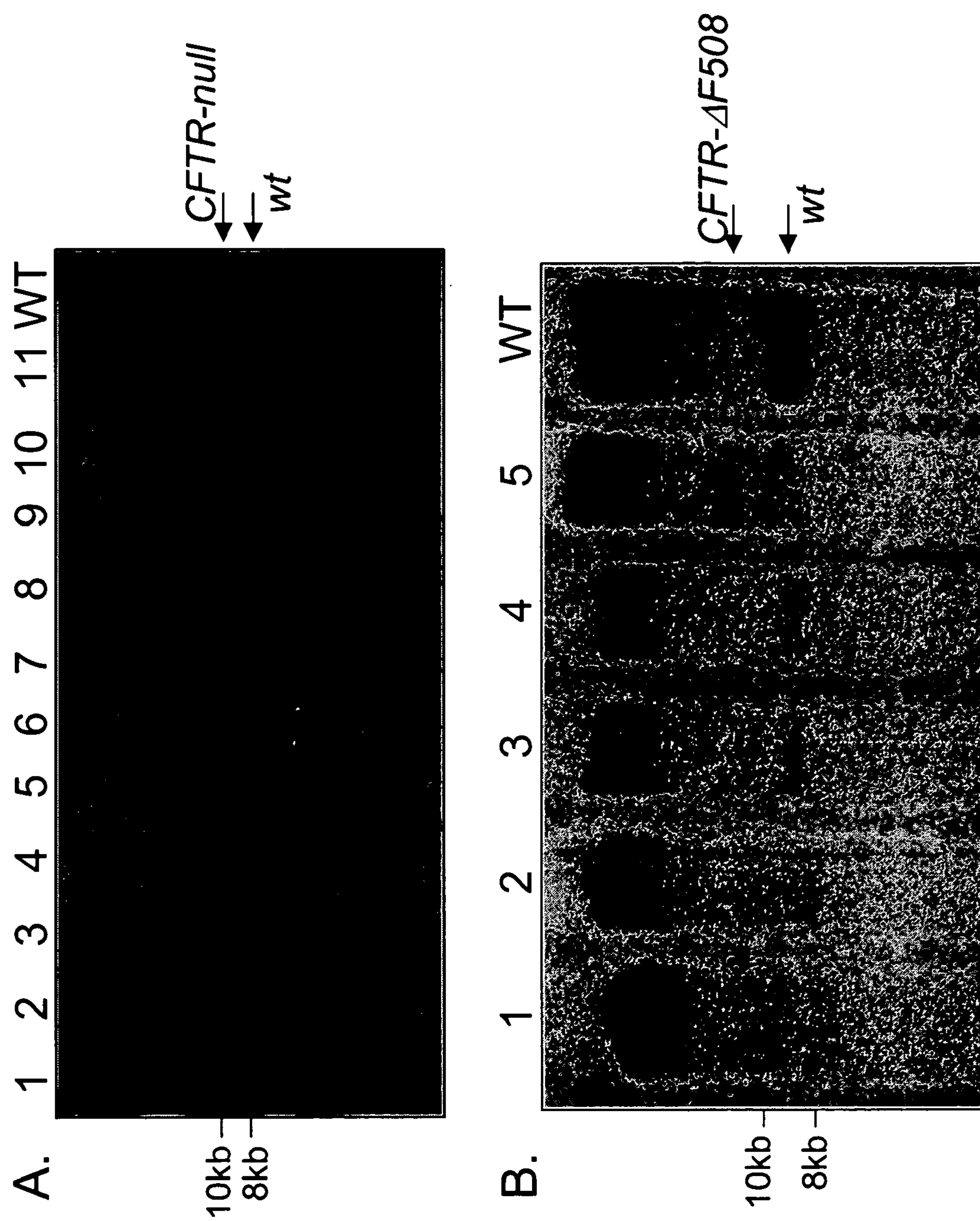


Figure 8

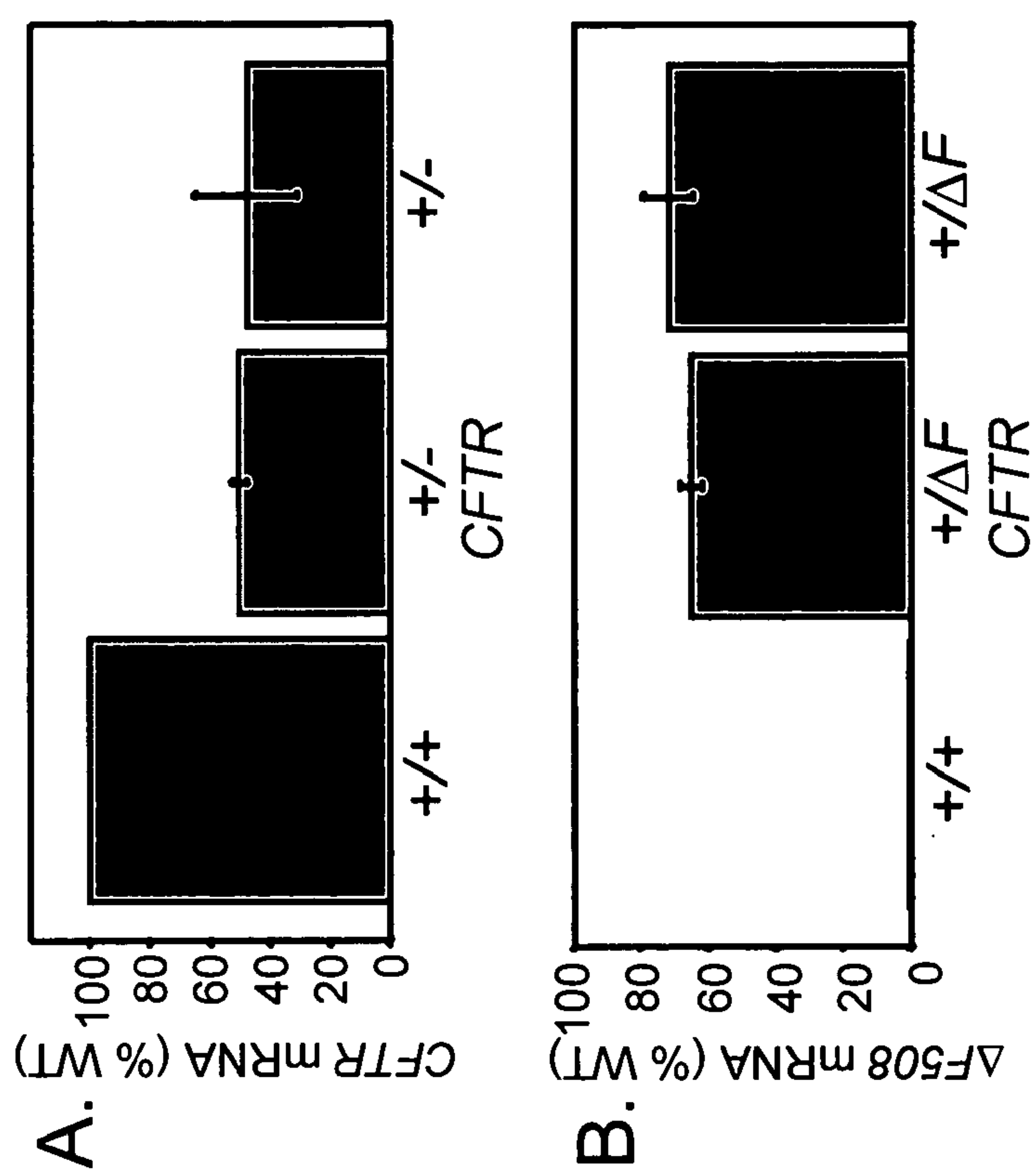


Figure 9



Fig. 10

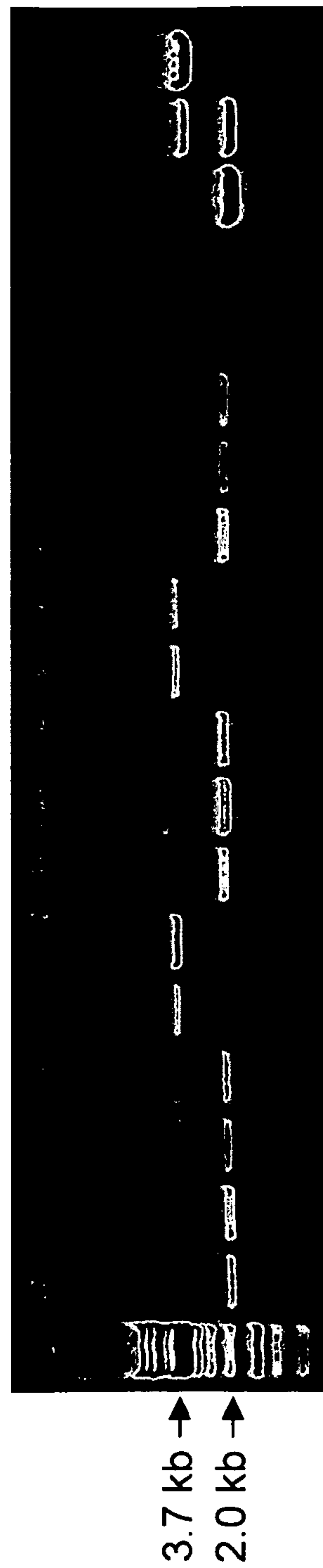


Fig. 11

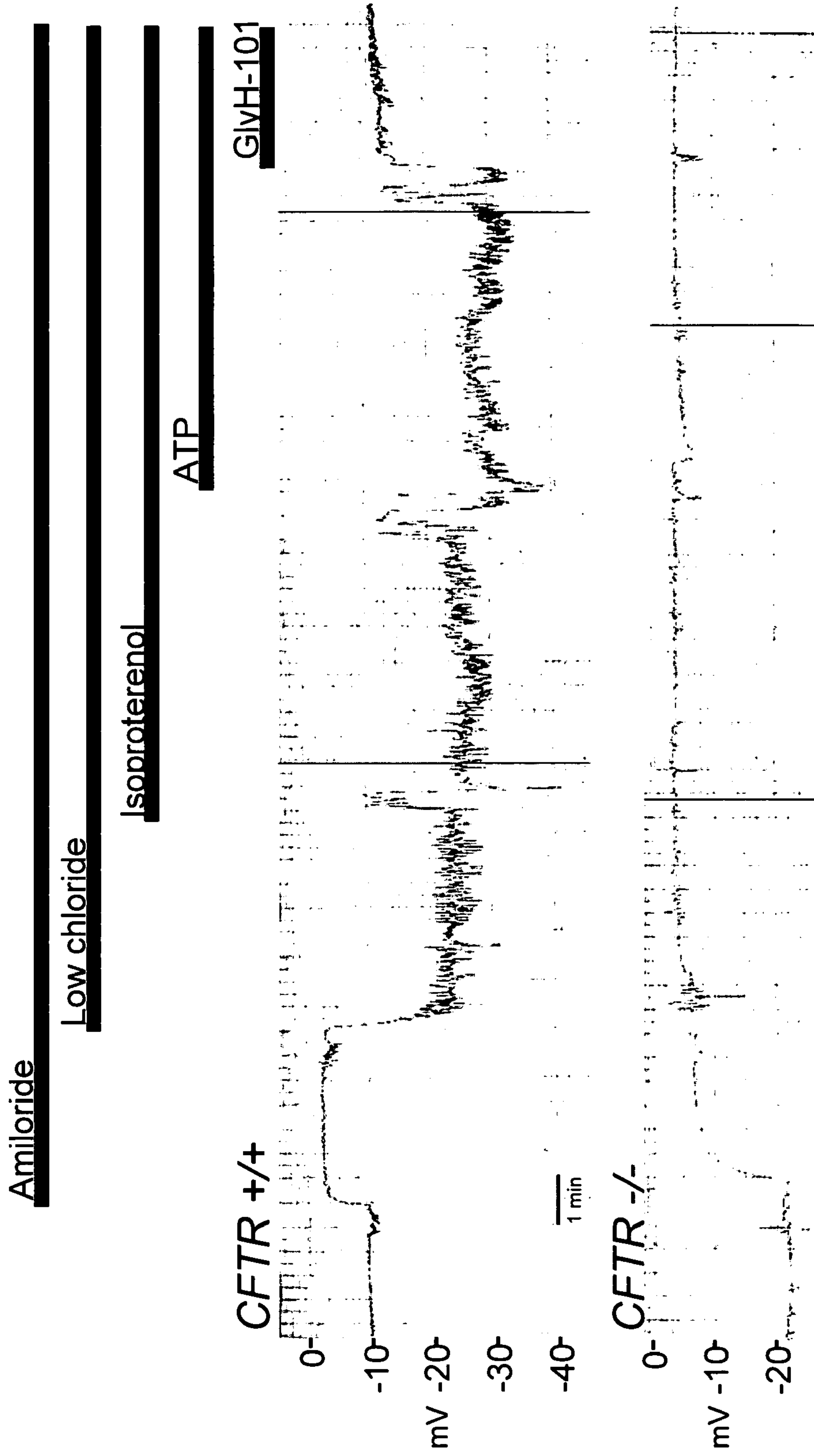


Fig. 12

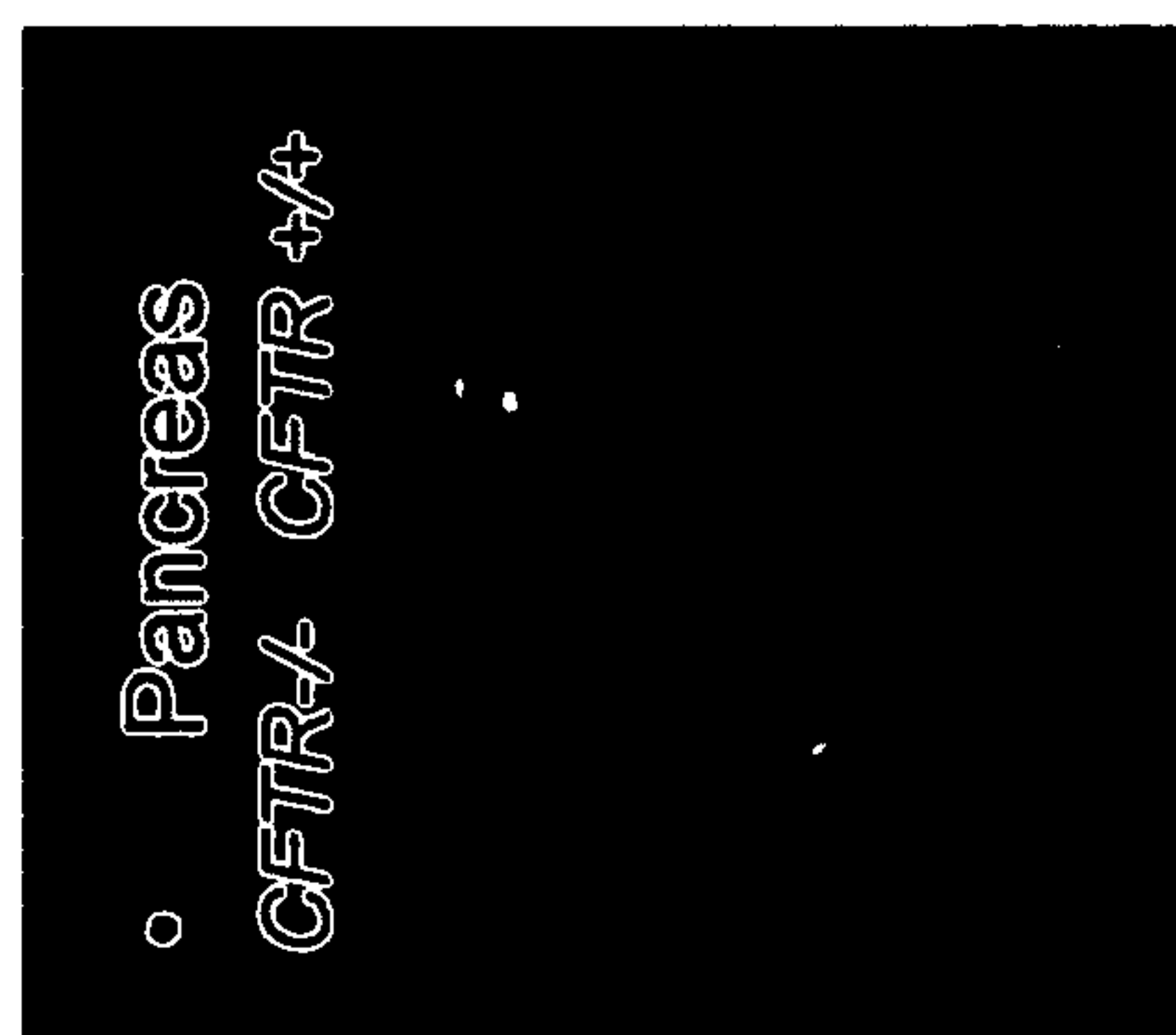


Fig. 13

Figure 14

human	1	MQRSPLEKASVVS	KLFFSWTRPILRKGYRQRL	ELSDIYQIPSVDSADNLSEKLEREW	DRELASKKNPKLINALRRCFFW	TM1	FMFYGIFLYI
pig	1	MQRSPLEKASIF	SKLFFSWTRPILRKGYRQRL	ELSDIYHSSSDSADNLSEKLEREW	DRELASKKNPKLINALRRCFFW		FMFYGIILYI
mouse	1	MQKSPLEKASF	ISKLFFSWTTPILRKGYRHH	LELSDIYQAPSADSADHLEKLEREW	DREQASKKNPQLIHALRRCFFW		FLFYGILLYI
human	91	GEVTKAVQPLLI	GRIIASYDPDNKEER	TM2	SIAYLGIGLCLLFIVRTLLI	HPAIFGLHHIGMQMRIA	MSLIYKTKLSSRVLDKISIGQL
pig	91	GEVTKAVQPLLI	GRIIASYDPDNKAER		SIAYLGVLGCLLFIVRTLLI	HPAIFGLHHIGMQMRIA	MSLIYKTKLSSRVLDKISIGQL
mouse	91	GEVTKAVQPVL	GRIIASYDPENKVER		SIAYLGIGLCLLFIVRTLLI	HPAIFGLHRIGMQMRTA	MSLIYKTKLSSRVLDKISIGQL
human	181	VSLLSNNLNKFDE	GLALAHFVWIAPLQVALLMGLI	TM3	WELLQ	TM4	ASAFCGLGFLIVLALFQAGLGRMMMKYRDQ
pig	181	VSLLSNNLNKFDE	GLALAHFVWIAPLQVTLMLGLI		WELLQ		ASAFCGLAFLVVLALFQAGLGRMMMKYRDQ
mouse	181	VSLLSNNLNKFDE	GLALAHFIWIAPLQVTLMLGLI		WDLIQ		ESAFCGLGLLIILVIFQAILGRMMVKYRDQ
human	271	SVKAYCWEEAME	KMIENLRQTELKLRKAAYVRYFNS	TM5	SAFFFSGFFVFLSVLPYALIK	TM6	GIILRKIFTTISFCIVLRMAVTRQFPWAVQT
pig	271	SVKAYCWEEAME	KMIENLRQTELKLRKAAYVRYFNS		SAFFFSGLFVFLSVLPYALIK		GIIMLRKIIFTTISFCIVLRMAVTRQFPWAVQT
mouse	271	SVKAYCWESAME	KMIENLRVLELKMTRKAAYMRFFTS		SAFFFSGFFVFLSVLPYTVIN		GIVLRKIIFTTISFCIVLRMSVTRQFPPTAVQI
human	361	WYDSLGAINKIQ	DFLQKQEQYKTL	NBD1	LEYNLTTFE	VVMENVTA	FWEEGFGLFEKAKQNNNNRKT
pig	361	WYDSLGAINKIQ	DFLQKQEQYKTL		LEYNLTTFE	VVMENVTA	FWEEGFGLFEKAKQNNNSRKISNGD
mouse	361	WYDSFGMIRKI	QDFLQKQEQYK		LVLEYNLMTTQ	IIMENVTA	FWEEGFGLLEKVKQSSNGDRKHSSDEN
human	451	GQLLAVAGSTG	AGKTSLLMMIMGELEP	Walker A	SEGKIKHSGRISFCSQFSWIMPGTI	F508	KENIIFGVSYDEYRYSVIKACQLEEDISKFAEKDNIV
pig	451	GQLLAVAGSTG	AGKTSLLMMIMGELEP		SEGKIKHSGRISFCSQFSWIMPGTI		KENIIFGVSYDEYRYSVIKACQLEEDISKFAEKDNIV
mouse	451	GEMLAITGSTG	SGKTSLLMLILGELE		ASEGIIKHSGRVSFCSQFSWIMPGTI		KENIIFGVSYDEYRYSVVKACQLOODITKFAEQDNTV
human	541	LGEGGITLGGQ	RARISLARAVYKDAD	SM	LYLLDSPFGYLDVLT	Walker B	TEKEIFESCVCCKLMANKTRI
pig	541	LGEGGITLGGQ	RARISLARAVYKDAD		LYLLDSPFGYLDVLT		TEKEIFESCVCCKLMANKTRI
mouse	541	LGEGGVTLGGQ	RARISLARAVYKDAD		LYLLDSPFGYLDVFT		EEQVFESCVCCKLMANKTRI
human	631	SELQNLQPDFS	SKLMGCDSDQFSA		ERRNSILTETLHRFS	F domain	LEGDAPVSWTETKQSFQKQGEFGEKRN
pig	631	SELQSRPDFS	SKLMGYDTDFQ		TAERRNSIITETLRRFS		LEGDASVSWNETKQSFQKQGEFGEKRN
mouse	631	SELQSLRPDFS	SKLMGYDTDFQ		TEERRSSILTETLRRFS		VDDSSAPWSKPK--QSFRTGEVGEKRN
human	721	MNGIEEDSDE	PLERRLSLVPDSE		QGEAILPRISVISTGPTLQARRRQSV		LNLMTHS-VNQQQNIHRKTTASTRKVSLAPQ
pig	721	MNGFEEDSGE	PLERRLSLVPDSE		HGEAILPRSNVINAGPTFQGRRRQSV		LNLMTRSSVNQQQSIHRKTATSTRKMSLVPQ
mouse	719	IDGESDDLQ--	--EKRLSLVPDSE		QGEAALPRSNMIATGPTFPGRRRQSV		LDLMTFT-PNSGSSNLQRTTSIRKISLVPQ
human	810	RRLSQETGLE	ISEEINEEDLKECF		DDMESIPAVTTWNTYLRYITVHKSI	TM7	IFVLIWCLVIFLAEVAASLVWLWLLGNTPLQDKGNSTHSR
pig	811	RRLSQDTGLE	ISEEINEEDLRECF		DDVESIPTVTTWNTYLRYVTVHKSI		IFVLIWCLVFLAEVAACLVWLCLLKKTSPQDKGNSTKGA
mouse	805	RRLSQDSTLN	ITEEINEEDLKECF		DDVIKIPVTTWNTYLRYFTLHKGI		LLVLIWCVLFLVEVAASLVWLWLLKNNPVNSGNGTKIS
human	900	NNSYAVIITST	SYVVFYIYVG	TM8	VADTLLAMGFFRGLPLVHTLITVSKILH		HKMLHSVLQAPMSTLNTLKAGGILNRFSKDIA
pig	901	NNSYAVIITST	SYVVFYIYVG		VADTLLALGLRGLPLVHTLITVSKILH		HRKMLHSVLQAPMSTLNTLKAGGILNRFSKDIA
mouse	895	NSSYVVIITST	SYVVFYIYVG		VADTLLALSLEFRLPLVHTLITASKILH		RKMLHSILHAPMSTISKLKAGGILNRFSKDIA
human	990	TI FDFIQLL	LIVIGAI	TM9	AVVAIQHYIFVATVPVIVAFIMLRAYFI	TM10	QTSQQLKQLESEGRSPIFTHLVTSLKGLWTLRAFRQPYFETLFH
pig	991	TI FDFIQLL	LIVIGAVVSVIKHYIFLATVPVIVAFILLRAYFI				HTSQQLKQLESEGRSPIFTHLITSLKGLWTLRAFRQPYFETLFH
mouse	985	TI FDFIQLV	FIVIGAIIVVSALQHYIFLATVPGLVVFILLRAYFI				HTAQQLKQLESEGRSPIFTHLVTSLKGLWTLRAFRQTYFETLFH
human	1080	KALNLHTANW	FLYLSTLRWFQMR	TM11	IEMIFVIFIAVTFISILTTGEGEGR	TM12	VGIIITLAMNIMSTLQWAVNSSIDVDSLMSRSRVFKFIDM
pig	1081	KALNLHTANW	FLYLSTLRWFQMR		IEMIFVIFIAVTFISILTTGEGEGT		VGIIITLAMNIMSTLQWAVNSSIDVDSLMSRSRVFKFIDM
mouse	1075	KALNLHTANW	FMYLATLRWFQMR		IDMIFVLFVIVTFISILTTGEGEGT		AGIIITLAMNIMSTLQWAVNSSIDTDSLMSRSRVFKFIDI
human	1170	PTEGK-PTK	STKPYKNGQLSKVMI	NBD2	IENSHVKDDIWPSGGQMTVKDLTAKYTEGGNAILEN	Walker A	SFISIPGQRVGLLGRTGSGKSTLLSAFI
pig	1171	PAEGDQPNF	SFKPSKDQLSKVMI		IENQHVKDDIWPSGGQMTVKDLTAKYVDGNAVLEN		SFISIPGQRVGLLGRTGSGKSTLLSAFI
mouse	1165	QTEESMYTQ	IIKELPREGSSDVLVIKNEHVKKSDIWPSGGEMVVKDLTVKYMDGNAVLEN		SFISIPGQRVGLLGRTGSGKSTLLSAFI		
human	1259	RLLNTEGEI	QIDGVSWDSITLQQWRKAFGVI		PQKVFI	SM	FSGTFRKNLDPYEQWSDQEIWKVADEVGLRSVIEQFPGKLDVFLVDGGCVLSH
pig	1261	RLLNTEGEI	QVDGVSWDSITLQQWRKAFGVI		PQKVFI		FSGTFRKNLDPYGQWSDQEIWKVAEEVGLRSVIEQFPGKLDVFLVDGGCVLSH
mouse	1255	RMLNIKGDI	EIDGVSWNSVTLOEWRKAFGVI		TQKVFI		FSGTFRQNLDPNGKWKDEEIWKVADEVGLKSVIEQFPGQLNFTLVDGGYVLSH
human	1349	GHKQLMCLARS	SVLSKAKI	Walker B	LLLDEPSAHLDPITYQIIRRTLKQAFADCTVILCEHRIEAMLE		CQFLVIEENKVRQYDSIQKLLNERSLFR
pig	1351	GHKQLMCLARS	SVLGAKI		LLLDEPSAHLDPITYQIIRRTLKQAFADCTVILCEHRIEAMLE		CQRFVIEENKVRQYDSIQRLLEKSLFR
mouse	1345	GHKQLMCLARS	SVLSKAKI		LLLDEPSAHLDPITYQVIRRVLQAFAGCTVILCEHRIEAMLCQ		RFLVIEESNVWQYDSLQALLSEKSI
human	1439	QAISPSDRV	KLFPHRNSSCKSKPQIAALKE		ETEEEVQDTRL		
pig	1441	QAISPLDRL	KLLPHRNSSKQSRSRKIAALKE		ETEEEVQETRL		
mouse	1435	QAISSEKMR	FFOGRHSSKHKPRTOITALKE		ETEEEVQETRL		

Figure 15

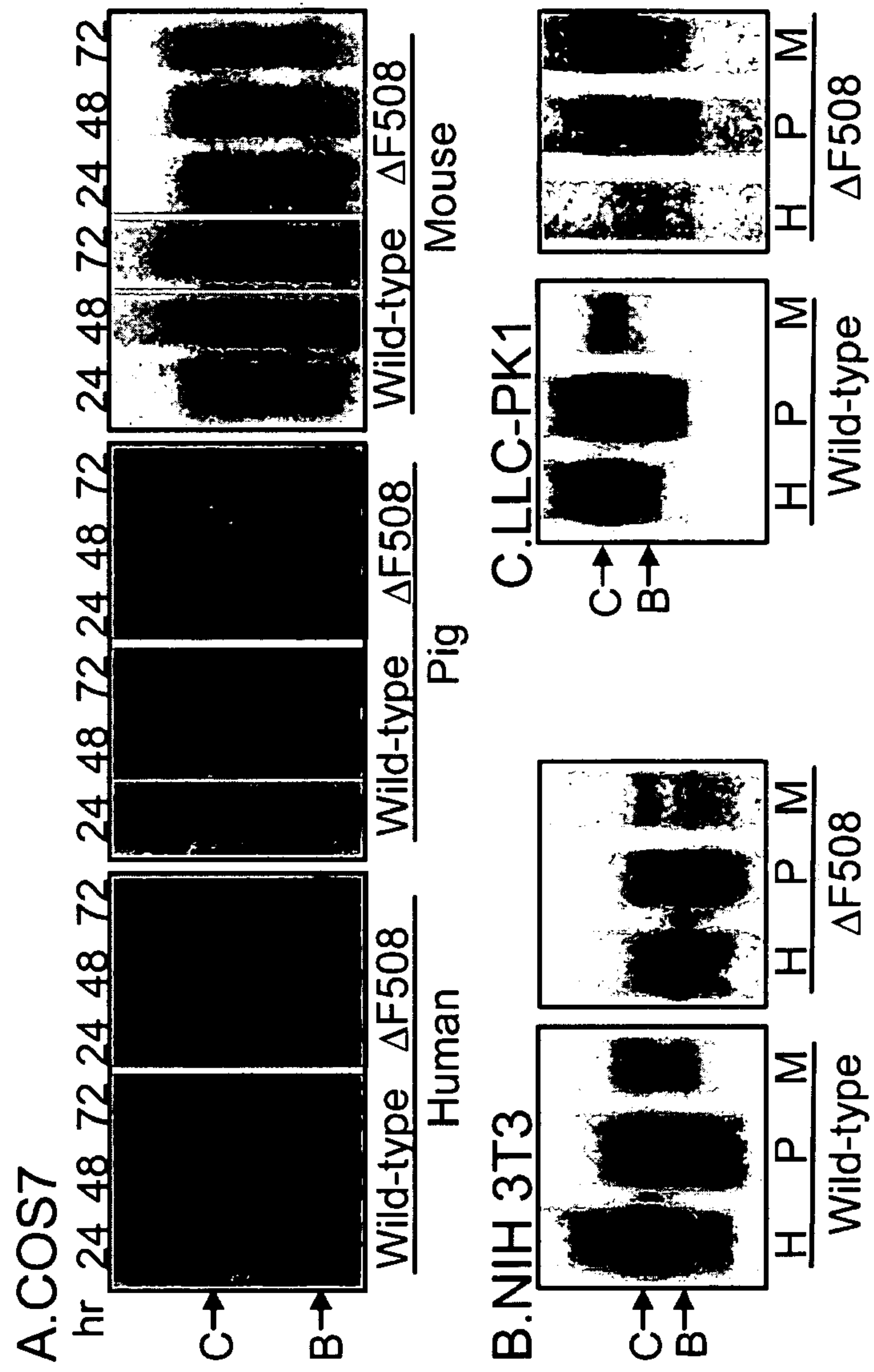
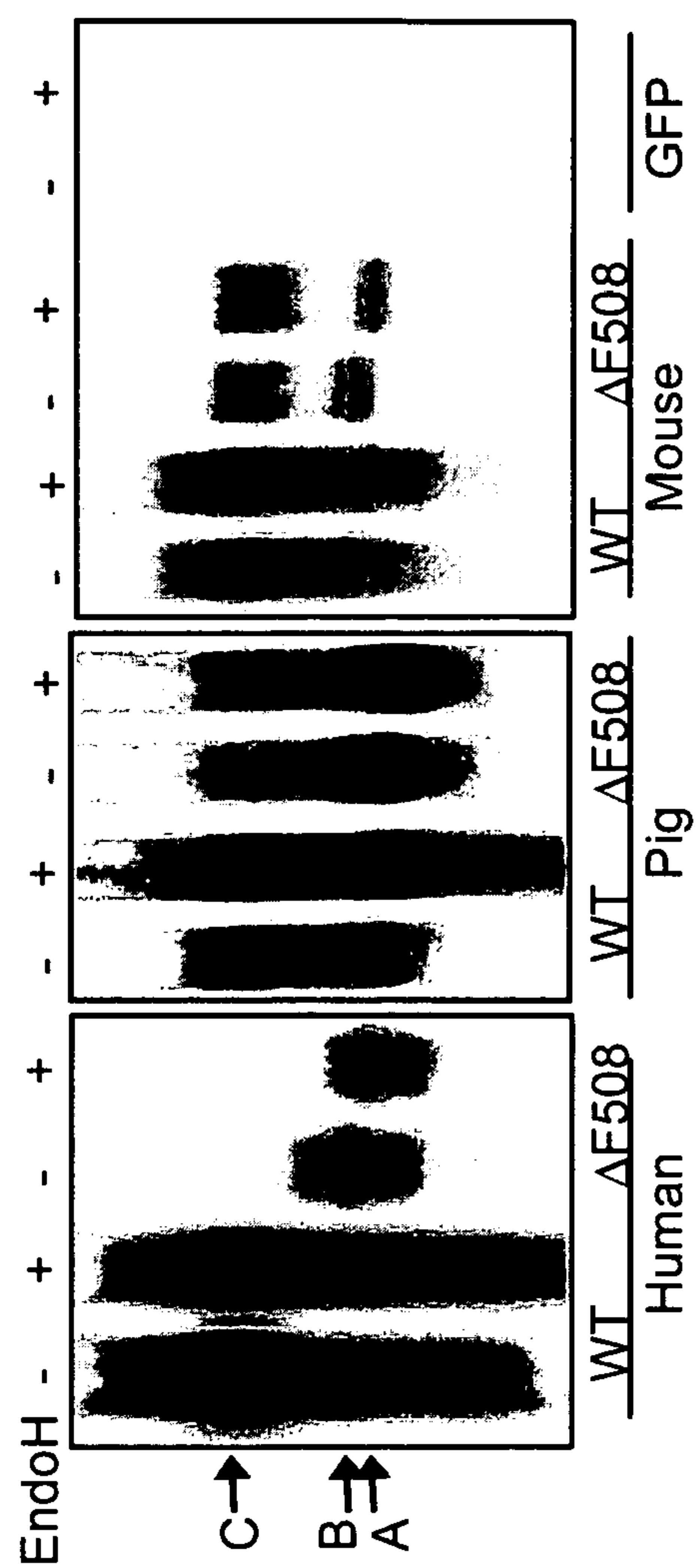


Figure 16



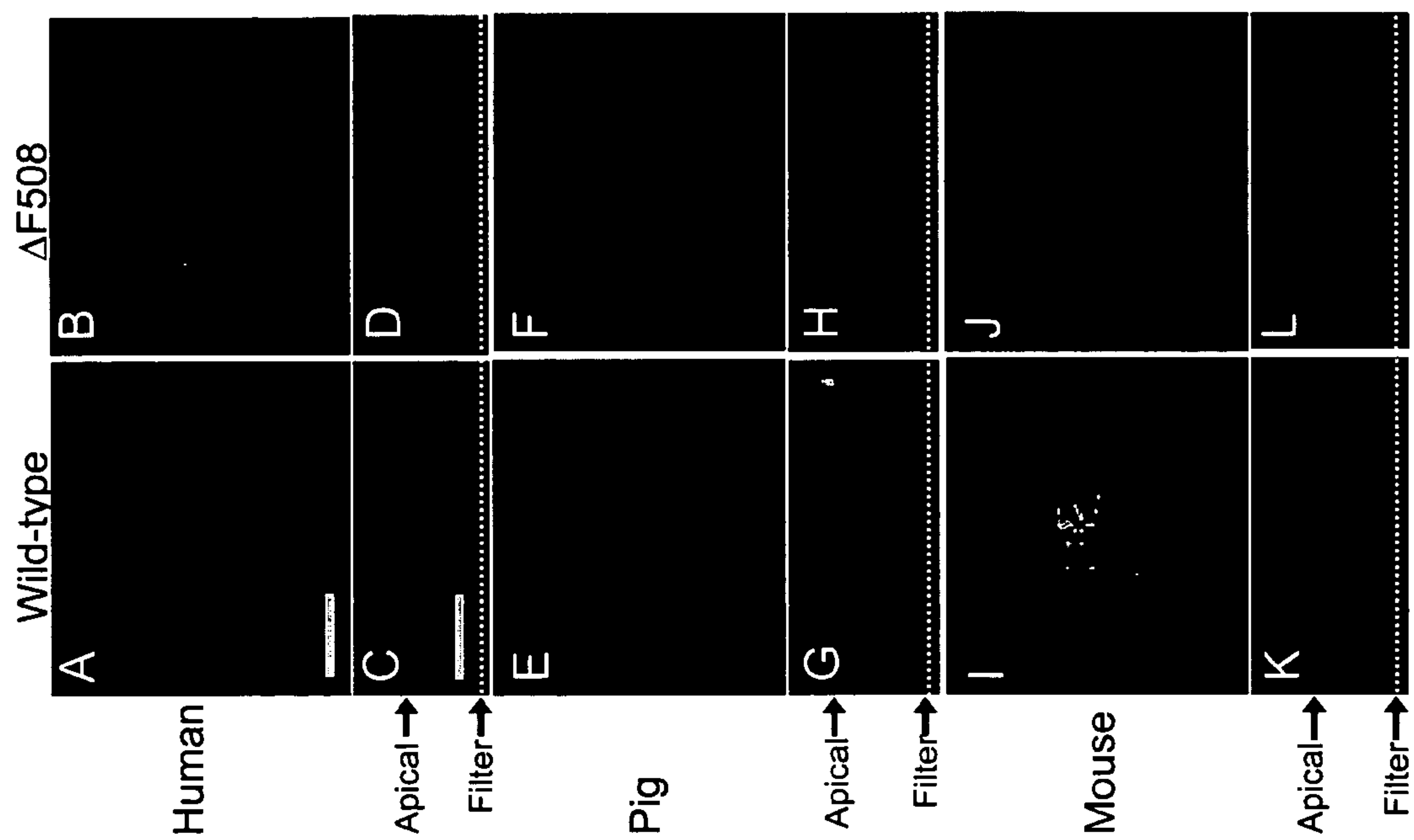


Figure 17

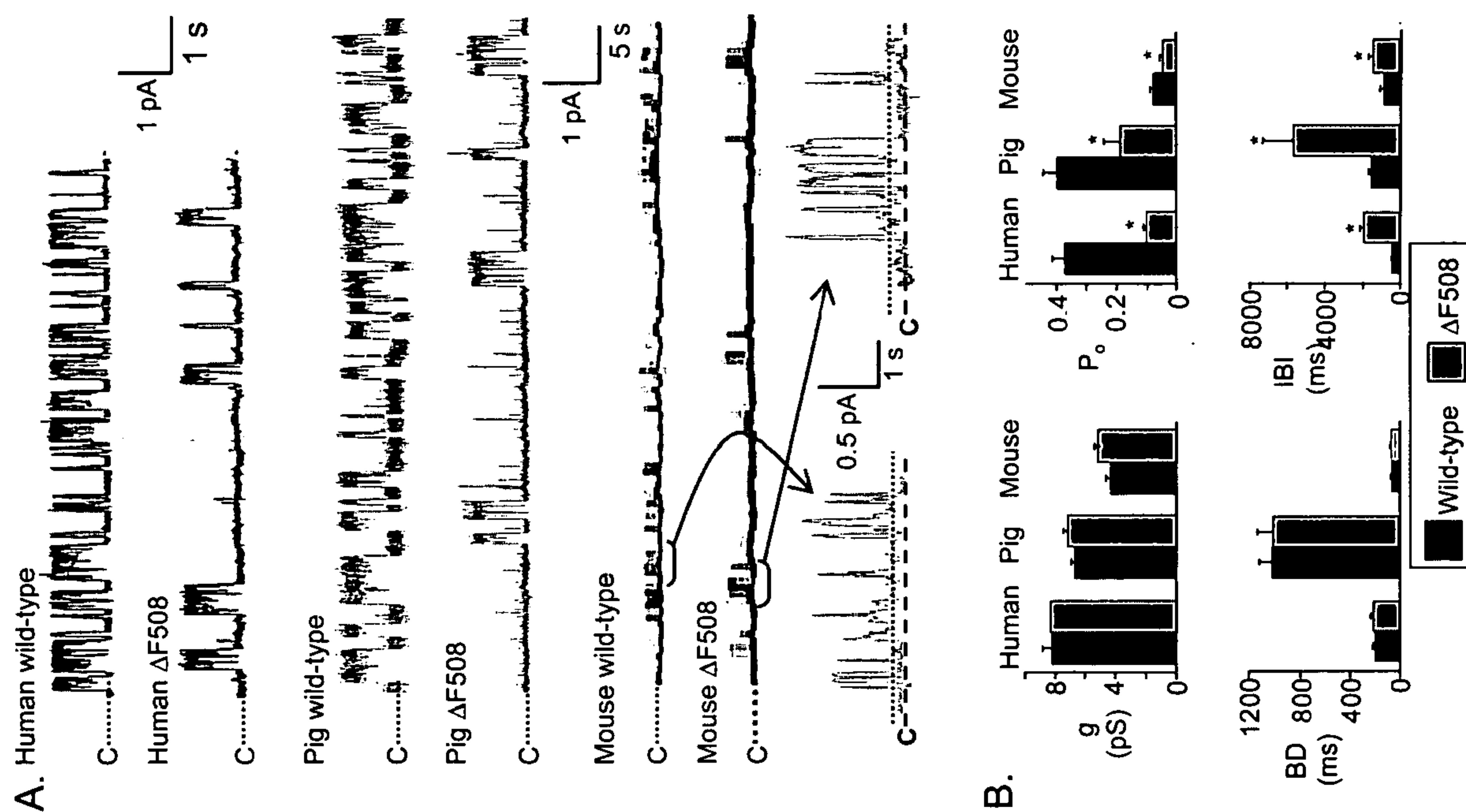


Figure 18

Figure 19

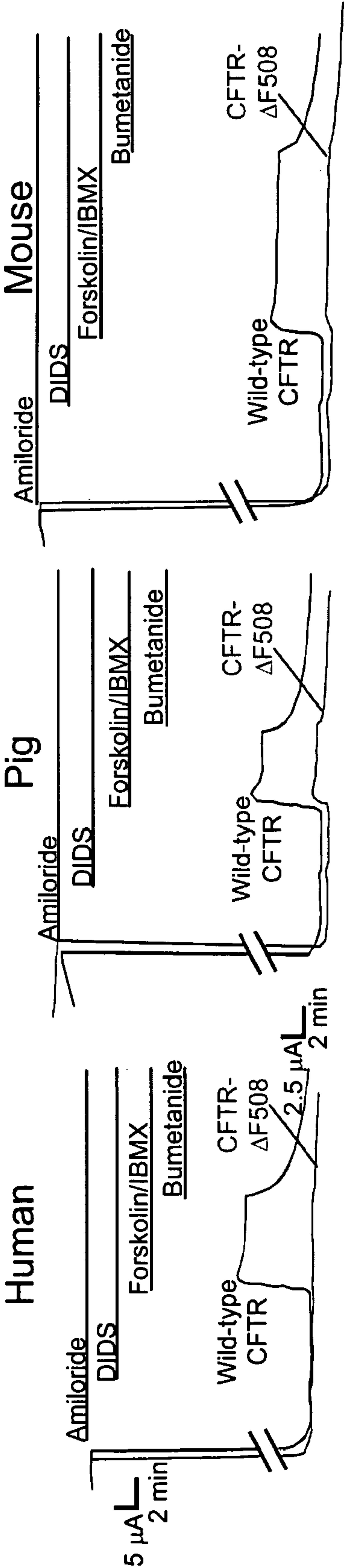


Figure 20

