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## Restoring ciliary function to differentiated Primary Ciliary Dyskinesia cells with a lentiviral vector

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### Abstract

Primary ciliary dyskinesia is a genetically heterogeneous autosomal recessive disease in which mutations disrupt ciliary function, leading to impaired mucociliary clearance and life-long lung disease. Mouse tracheal cells with a targeted deletion in the axonemal dynein intermediated chain gene *Dnaic1* differentiate normally in culture but lack ciliary activity. Gene transfer to undifferentiated cultures of mouse *Dnaic1*<sup>-/-</sup> cells with a lentiviral vector pseudotyped with avian influenza hemagglutinin restored *Dnaic1* expression and ciliary activity. Importantly, apical treatment of well-differentiated cultures of mouse *Dnaic1*<sup>-/-</sup> with lentiviral vector also restored ciliary activity, demonstrating successful gene transfer from the apical surface. Treatment of *Dnaic1*<sup>flox/flox</sup> mice expressing an estrogen responsive Cre recombinase with different doses of tamoxifen indicated that restoration of ~20% of ciliary activity may be sufficient to prevent the development of rhinosinusitis. However, while administration of a  $\beta$ -galactosidase expressing vector to control mice demonstrated efficient gene transfer to the nasal epithelium, treatment of *Dnaic1*<sup>-/-</sup> mice resulted in a low level of gene transfer, demonstrating that the severe rhinitis present in these animals impedes gene transfer. The results demonstrate that gene replacement therapy may be a viable treatment option for primary ciliary dyskinesia, but further improvements in the efficiency of gene transfer are necessary.

### Keywords

cilia; airway; lentiviral; bronchiectasis; rhinosinusitis; DNAI1

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#### Conflict of interest

Dr. J.C. Olsen is a named inventor on patents involving EIAV-based gene transfer technology and has received royalties for the use of EIAV-based vectors. All other authors declare no potential conflict of interest.

#### Supplementary information

Supplementary information is available at Gene Therapy's website.

## Introduction

Primary ciliary dyskinesia (PCD) is a rare genetic disease in which mutations in critical genes impair the function of motile cilia resulting in reduced or absent mucociliary clearance (MCC) <sup>1,2</sup>. Patients with PCD, as a result of the defect in MCC, suffer from neonatal respiratory distress, chronic rhinosinusitis, otitis media, and repeated airway infections, resulting in bronchiectasis <sup>1,2</sup>. Additionally, abnormalities of situs occur with increased frequency in this population <sup>3,4</sup>, and males with PCD are typically infertile <sup>1,2</sup>. Current treatments are designed to reduce the incidence and severity of respiratory tract infections, as there are no corrective treatments available <sup>5,6</sup>.

Recent genetic studies have identified some of the genes mutated in PCD, including components of the dynein motors (*DNAI1*, *DNAI2*, *DNAH5*, *DNAH11*), radial spokes (*RSPH4A*, *RSPH9*) and other genes involved in ciliary assembly (*KTU*, *LRRC50*) <sup>7</sup>. Because of the genetically heterogeneous nature of the disease, which makes it unlikely that a single drug can be identified to treat/correct each causative mutation, gene transfer may be a viable therapeutic option for PCD. For example, a drug that corrects a specific defect in one axonemal protein would be unlikely to correct mutations in a different axonemal protein. In contrast, if a suitable gene transfer vector were available, by simply replacing the cDNA the vector could be used to correct most, if not all, PCD causing mutations. Heterozygotes have apparently normal ciliary function, indicating that dominant negative mutations are not common, and the conducting airways, which are the site of the most serious pathology, are relatively accessible to administration of a viral vector <sup>1,2</sup>.

Much prior effort has been devoted to developing gene transfer as a therapy for other airway diseases, most notably, cystic fibrosis. These studies have identified several challenges to successful gene therapy, including the resistance of well-differentiated (polarized) airway epithelium to apical transduction by viral vectors, the difficulties of obtaining long-term expression, and the development of an immune response to many vector components <sup>8-10</sup>. Although gene therapy of airway diseases has not yet been successful, researchers are continuously making advances in vector design and production. For example, several groups have identified viral envelope proteins useful for targeting lentiviral vectors to the apical membrane of differentiated airway epithelial cells <sup>11-14</sup>. Our group has recently demonstrated that pseudotyping lentiviral vectors with an avian influenza hemagglutinin (HA) protein allows efficient apical transduction of differentiated airway epithelial cells <sup>15,16</sup>. The incorporation of endogenous cellular promoters and the use of integrating lentiviral vectors to transduce airway epithelium in animal models have resulted in stable, long-term expression <sup>11-14,17</sup>.

To test the hypothesis that gene transfer could restore normal ciliary activity to PCD cells, we utilized an inducible mouse model of PCD we have previously characterized <sup>18</sup>. In this model, activation of an estrogen responsive Cre recombinase (CreER) by tamoxifen treatment causes the deletion of two exons flanked by loxP sites in the axonemal dynein intermediate chain 1 (*Dnaic1*) gene. The mouse *Dnaic1* protein is 82% identical to the human *DNAI1* protein, and mutations in *DNAI1* cause about 10% of PCD cases <sup>19-22</sup>. The deletion disrupts the structure of critical WD40 domains in the *Dnaic1* protein and prevents

the assembly of the outer dynein arm<sup>23</sup>, resulting in immotile cilia and a PCD phenotype. Unlike traditional knock-out models, the use of an inducible system avoids the complications of neonatal hydrocephalus<sup>24, 25</sup>, heart defects<sup>26, 27</sup>, and other situs abnormalities that frequently occur in PCD mice, allowing us to study adult PCD animals<sup>18</sup>.

In this report, we have tested the ability of an HA-pseudotyped lentiviral vector to restore ciliary activity to both undifferentiated and differentiated PCD cells in vitro. We also utilized our inducible mouse model to estimate the level of gene transfer required to prevent upper airway disease and to investigate the turnover of a ciliary protein, two important aspects that will have to be considered when designing clinical trials for PCD. Finally, we examined the effect of preexisting rhinosinusitis on the ability of the HA-pseudotyped lentiviral vector to transduce the nasal epithelium of PCD animals.

## Results

### Construction of lentiviral vectors

A full-length mouse cDNA for *Dnaic1* was cloned into a lentivirus gene transfer vector (SIN6.1CB-W) based on the equine infectious anemia virus (EIAV,<sup>15</sup>) and the construct was verified by direct sequencing (Fig. 1a). The *Dnaic1* cDNA was under control of hybrid promoter consisting of the human CMV enhancer followed by the chicken  $\beta$ -actin promoter (CB) that is ubiquitously expressed<sup>16</sup>. Additional vectors expressing the reporter genes EGFP, firefly luciferase, or  $\beta$ -galactosidase ( $\beta$ -gal) from the same construct were utilized as controls in these studies<sup>16</sup>. Vectors were also constructed that contained an internal ribosomal entry site (IRES) after the *Dnaic1* cDNA followed by a cDNA encoding EGFP, however, these vectors were found to be inefficient. Viral particles pseudotyped with influenza hemagglutinin (HA) from fowl plague virus were produced by transfection of 293T cells as previously described<sup>16</sup>. Transduction of 293 cells with *Dnaic1*-encoding lentivirus resulted in expression of a protein of the correct size that reacted with a purified monoclonal antibody against human DNAI1 on Western blotting (Fig. 1b,<sup>18</sup>) confirming the vector was expressing full-length *Dnaic1*.

### Gene transfer to undifferentiated PCD cells restores ciliary activity

To test the hypothesis that exogenous expression of *Dnaic1* could restore ciliary activity to *Dnaic1*<sup>-/-</sup> (PCD) cells, mouse tracheal epithelial (MTE) cells from *Dnaic1*<sup>flox/flox</sup> mice that also express CreER were cultured at an air/liquid interface (ALI) on porous inserts<sup>18</sup>. Cells were treated with tamoxifen to activate CreER and induce the deletion of *Dnaic1* and generate PCD cells as previously described<sup>18</sup>. After the cells reached confluence, but before ciliated cell differentiation was visible (day 5 of culture), cultures were treated apically with *Dnaic1*-encoding HA-EIAV lentivirus and then monitored for the appearance of ciliary activity. As a negative control, cultures received either no vector or were transduced with a lentiviral vector expressing EGFP from the CB promoter. Each experiment also included cultures that were not treated with tamoxifen or cultures from heterozygous mice (*Dnaic1*<sup>flox/wt</sup>, CreER<sup>+</sup>) treated with tamoxifen as a positive control for ciliogenesis. As expected, the percentage of the culture surface area demonstrating active ciliary beating in the PCD cells and PCD cells transduced with the EGFP expressing control vector was extremely low,

averaging <1% in 4 separate experiments (Supplemental Video S1). Transduction of PCD cells with a *Dnaic1* expressing lentivirus resulted in a clearly visible increase in ciliary activity, seemingly in parallel with the development of ciliary activity in the positive control cultures. In four separate experiments, treatment of undifferentiated cultures of PCD cells with lentivirus expressing *Dnaic1* increased the level of ciliary activity significantly over untreated PCD cultures. At the time when ciliary activity was maximal in the vector-treated cultures, the level of ciliary activity averaged ~10% of the positive control cultures, compared to ~0.4% for the PCD cultures ( $p=0.04$  by student's t-test,  $n=4$ ; Figure 2a). Measurement of ciliary beat frequency (CBF) using the SAVA system demonstrated that there was no significant difference in CBF between the positive control cultures and the virally transduced cultures (heterozygote=18.4 Hz; experimental=18.2 Hz;  $p=0.42$  by student's t-test,  $n=9$ ; Supplemental Video S2, S3). Figure 2b illustrates the typical time course of the development of ciliary activity in an experiment in which treatment with a lentiviral vector expressing *Dnaic1* from the CB promoter increased ciliary activity from 0.1% to 8.5%. Ciliary surface activity in the positive control cultures typically reached 40-50%, depending on the culture and the time of measurement. Ciliary activity was maintained in the virally transduced cultures for the remainder of the experiment, usually 2-3 weeks, at which time ciliary activity in the positive control cultures was also diminishing due to the limited lifespan of primary cultures.

At the end of these experiments, individual cultures were harvested for various additional analyses. Qualitative analysis of cellular DNA was performed by PCR amplification using primers specific for deleted exons of *Dnaic1* followed by gel electrophoresis. Because the primers span the intron between exons 17 and 18, it is possible to distinguish products derived from the endogenous genomic *Dnaic1* (333 bp) and products derived from the vector encoded cDNA (223 bp). These experiments confirmed the essentially complete deletion of the endogenous *Dnaic1* in cultures treated with tamoxifen (as shown previously; Fig. 3a in ref. 18) and the presence of the integrated viral genome in cultures treated with vector (2/2 cultures; data not shown). Treatment of PCD cultures with *Dnaic1* expressing lentivirus also resulted in easily detectable levels of *Dnaic1* RNA. Quantitative RT-PCR using exon 17-18 specific primers demonstrated that virally transduced cultures expressed *Dnaic1* RNA at levels between 7 and ~140-fold higher than the PCD cultures when analyzed 4 weeks after transduction ( $n=3$ ; data not shown).

### Apical gene transfer to differentiated PCD cells restores ciliary activity

Gene transfer to differentiated airway epithelial cells from the apical surface has been shown to be a major obstacle to successful gene therapy of lung diseases, including cystic fibrosis. To determine if apical delivery of an HA-EIAV lentivirus encoding wild-type *Dnaic1* to fully-differentiated PCD cells could restore ciliary activity, tamoxifen-treated MTE cells from *Dnaic1*<sup>fllox/fllox</sup>/CreER<sup>+</sup> animals were cultured at an ALI for 19 days. At this time, all positive control (non-PCD) cultures were well-differentiated and displayed easily visible ciliary activity covering 35-65% of the culture surface, while the PCD cultures had few or no actively beating ciliated cells. Apical treatment of the PCD cultures with *Dnaic1*-expressing lentiviral vectors again resulted in an increase in ciliary activity. The percentage of the surface area of the vector-treated cultures increased to levels as high as 8.8%, while

the PCD cultures again averaged less than 1.0%. The average ciliary activity in treated cultures was 10-fold higher than that in the PCD cultures (6.1% vs 0.6%;  $p=0.02$  by student's t-test,  $n=4$ ). Compared to the positive control cultures that had an average of 56% of their surface covered by active ciliated cells, treatment with the lentiviral vector restored ~10% of the normal level of ciliary activity (Fig. 3a). Active ciliated cells appeared in the cultures 5-6 days after vector application (Fig. 3b), and increased in number over the next several days. RT-PCR analysis demonstrated full-length *Dnaic1* RNA in differentiated PCD cultures treated with vector, and qRT-PCR with primers specific for exons 17-18 demonstrated levels several thousand-fold that observed in the PCD cultures ( $n=3$ ; data not shown). The amount of intact *Dnaic1* RNA in the cultures transduced after differentiation appeared greater than that found in cultures transduced early in culture; this most likely reflects the increased cell turnover in the cultures transduced early due to the longer time interval between the viral treatment and the RNA isolation in the two groups. Similarly, Western blot analysis showed the levels of intact *Dnaic1* protein to be higher in the virally transduced differentiated cultures than in the virally transduced undifferentiated cultures. *Dnaic1* protein was expressed in the late cultures at almost 60% of the level in positive control cultures, while no full-length *Dnaic1* protein was detectable in the cultures that were transduced at earlier time points (data not shown).

In contrast to the cultures treated with vector before differentiation, in which the appearance of ciliary activity roughly paralleled the development of ciliary activity in the positive controls, we noticed a delay of 5-6 days before ciliary activity was observed in the already differentiated PCD cultures following vector treatment (compare Figure 2b and 3b which are from the same experiment). One hypothesis for the delay in restoration of ciliary activity to the differentiated PCD cells is that the incorporation of the vector-expressed wild-type *Dnaic1* protein into the already fully assembled ciliary axonemes may be rate-limiting. Very little is known about the time required for assembly or turnover of axonemal proteins in mammalian systems, and this may be an important consideration when designing or testing treatments for PCD. Therefore we took advantage of the inducibility of our system to study the turnover of the intact *Dnaic1* protein in cultured tracheal epithelial cells. In these studies, MTE cells from *Dnaic1*<sup>flox/flox</sup>/CreER<sup>+</sup> mice were cultured at an ALI in the absence of tamoxifen until ciliated cell differentiation was well-established (14-21 days). The percentage of the culture surface area covered with actively beating cilia was measured and the cultures were then treated with tamoxifen (1  $\mu$ M tamoxifen in media on day 0, 2, and 4) to induce the deletion of exons 17-18 from *Dnaic1*. Cultures were harvested at various times during treatment and the amount of intact *Dnaic1* gene and the level of *Dnaic1* protein were measured, along with the amount of ciliary activity remaining. As expected, treatment with tamoxifen rapidly induced the deletion of the floxed *Dnaic1*, decreasing the percentage of intact *Dnaic1* to ~60% 48 hrs after tamoxifen addition and to < 25% after 5 days of tamoxifen treatment (Fig. 4). Similarly, the amount of intact *Dnaic1* protein in the cellular (soluble) fraction of total cell lysates decreased rapidly over the first 5 days of treatment, while the level of deleted *Dnaic1* protein increased (Fig. 4 and supplemental Fig. S1). In contrast, the level of intact *Dnaic1* protein in the axonemal fraction (pellet) decreased at a slower rate, with over 50% of the untreated control level remaining 5 days after initiation of treatment and almost 30% remaining 14 days after initiation of treatment (Fig. 4 and

supplemental Fig. S1). The high levels of intact Dnaic1 protein remaining in the axonemal fraction were reflected in even higher levels of ciliary activity, with over 90% of the activity of untreated control cultures maintained at 5 days, and >70% maintained at 14 days (Fig. 4). These results suggest that Dnaic1, and likely other ciliary proteins, are very stable once they are incorporated into the axonemal structure. Further, these results suggest that the delay in the restoration of ciliary activity in well-differentiated PCD cultures may be due to the relatively slow incorporation of the newly synthesized Dnaic1 protein into pre-existing axonemes.

The above studies clearly demonstrate that apical treatment with a lentiviral vector can restore ciliary activity to PCD cells in vitro, however the percentage of cells corrected was only 10-15% of the positive control cultures. To determine if this level of transfer might be sufficient to improve the outcome of PCD patients, we treated *Dnaic1*<sup>flox/flox</sup>/CreER<sup>+</sup> mice with different doses of tamoxifen to induce different levels of *Dnaic1* gene deletion. We also included several *Dnaic1*<sup>WT/flox</sup>/CreER<sup>+</sup> animals that were not treated with tamoxifen, but because of low levels of leakiness (i.e. CreER activation and deletion of the target gene in the absence of tamoxifen treatment<sup>18</sup>), also had varying levels of genomic *Dnaic1* deletion. Mucociliary clearance was measured in the nasopharynx as previously described<sup>18</sup>.

Following the measurement of MCC, the nasal cavity was fixed for histology and the level of intact genomic *Dnaic1* remaining was determined in excised tracheal tissue by qPCR. As shown in Fig. 5a, the rate of mucociliary clearance was directly correlated with the level of intact genomic *Dnaic1* remaining ( $R^2=0.7$ ). As noted in our previous study<sup>18</sup>, most animals with < 20% of intact genomic *Dnaic1* remaining had essentially no mucociliary clearance, although 2 animals with 16% and 19% *Dnaic1* had MCC rates of ~2 mm/min and 1 animal with 22% of the wild-type level of intact genomic *Dnaic1* had a MCC rate of 3.8 mm/min. Examination of the nasal cavity of these animals revealed essentially normal histology in 2 while the third showed severe mucus accumulation and sinusitis (Fig. 5b). In contrast, all animals with lower levels of intact genomic *Dnaic1* (<15%) had extensive mucus accumulation in the nasal cavity, in agreement with previous studies of these PCD mice. Together, these studies indicate that successful gene transfer of as little as 20% of the normal level of a ciliary gene may be able to restore a low level of MCC and significantly lessen the severity of disease.

To determine if the HA-pseudotyped lentiviral vector could efficiently transduce PCD airway epithelium in vivo, we administered vector to groups of PCD and control animals by nasal inhalation. To measure both the level of gene transduction and the stability of expression, we first utilized a vector expressing both firefly luciferase and  $\beta$ -gal from a single CB promoter construct<sup>16</sup>. Four days after vector administration, animals were anesthetized and luciferase activity was measured in the nasal cavity as described in methods. Clear evidence of gene transfer to the control animals was observed, with an average luciferase activity ~8-fold higher than the untreated (no virus) negative control animal ( $1.6 \times 10^6 \pm 2.9 \times 10^5$  photons/sec/cm<sup>2</sup> (avg.  $\pm$  S.E.M., n=6) vs.  $2.1 \times 10^5 \pm 8.0 \times 10^4$  photons/sec/cm<sup>2</sup> (avg.  $\pm$  S.E.M. of 3 repeat measures); Fig. 6). In contrast, only one of the PCD animals exhibited a substantial level of luciferase activity ( $1.0 \times 10^6$  photons/sec/cm<sup>2</sup>), with the PCD animals together averaging  $4.6 \times 10^5 \pm 1.5 \times 10^5$  photons/sec/cm<sup>2</sup> (avg  $\pm$



S.E.M., n=5; significantly different from controls with  $p < 0.005$  by student's t-test) The level of luciferase activity was increased in the control animals at 7 and 12 days after treatment, while the level of activity in the PCD animals, although increased, remained significantly lower than the controls. At day 7, the control animals averaged  $1.3 \times 10^7 \pm 2 \times 10^6$  compared to  $1.7 \times 10^6 \pm 8.4 \times 10^5$  for the PCD animals; at day 12 the control animals averaged  $2.4 \times 10^7 \pm 5.9 \times 10^6$  while the PCD animals averaged  $2.2 \times 10^6 \pm 1.2 \times 10^6$  (significantly different at both days with  $p < 0.007$ ). After one month, the level of luciferase activity declined in both groups, and was no longer significantly different between them, although the average activity remained higher in the controls (Fig. 6). To examine the cellular distribution of viral mediated gene transfer, animals were euthanized and tissues were processed for detection of  $\beta$ -gal. Sections were prepared and  $\beta$ -gal positive cells were enumerated in the nasal cavity and trachea. In agreement with the luciferase data, there were significantly fewer  $\beta$ -gal positive cells in the nasal cavity of the PCD mice compared to the control animals ( $2.2 \pm 2$  vs  $116 \pm 54$ , mean  $\pm$  S.E.M. in 3 sections/ animal; n=5;  $p=0.03$  by student's t-test). In contrast, there was no significant difference in the numbers of  $\beta$ -gal positive mice in sections of the tracheas from PCD mice compared to the control animals ( $174 \pm 107$  vs  $30 \pm 24$ ; n=5;  $p > 0.11$  by student's t-test), indicating that the inhibition of gene transfer was limited to the site of disease pathology, i.e., the nasal cavity. To further examine the distribution of virally transduced cells in the PCD animals compared to controls, additional groups of PCD and control animals were treated with an HA-pseudotyped lentivirus expressing only  $\beta$ -gal. Animals were again administered vector by nasal inhalation, but were euthanized and examined for positive gene transfer about the time of maximum luciferase expression (Fig. 6; 12 days after treatment). In confirmation of our earlier study<sup>16</sup>, control animals displayed a high level of gene transfer to the ciliated epithelium of the nasal cavity.  $\beta$ -gal positive cells were easily identified along the septum, nasal turbinates, and throughout the nasopharynx, while olfactory tissue was routinely negative (Fig. 7a, c). PCD animals displayed little or no gene transfer to the nasal epithelium (Fig. 7b, d). While control animals averaged over 500  $\beta$ -gal positive cells, PCD animals had significantly fewer positive cells ( $532 \pm 42$ ; n=7 vs.  $62 \pm 31$ ; n=9;  $p=1.3 \times 10^{-7}$  by student's t-test). In contrast, transfer to the trachea was again similar between the two groups (control= $285 \pm 31$ , PCD= $298 \pm 39$ ;  $p > 0.4$  by student's t-test, Fig 7e, f, g, h). These data clearly demonstrate that the accumulated mucus and/or inflammation in the nasal cavity of the PCD animals prevent efficient gene transfer.

## Discussion

Primary ciliary dyskinesia is a genetically heterogeneous disease that occurs with an incidence of approximately 1:16,000, and is therefore classified as a “rare” or “orphan” disease. Currently there are no specific treatments available for PCD, and individuals with PCD suffer from repeated respiratory tract infections eventually leading to bronchiectasis<sup>2,5</sup>. Our laboratories have been investigating the development of gene replacement therapy as a treatment for PCD and other respiratory diseases, including cystic fibrosis. To investigate the pathogenesis and treatment of PCD, we have previously developed an inducible mouse model of PCD that does not develop hydrocephalus or

developmental (heart) defects<sup>18</sup>. In this work, we have utilized this model to investigate several aspects of gene therapy as a treatment for PCD.

Our initial studies demonstrated that expression of the wild-type *Dnaic1* from a lentiviral vector successfully restored ciliary activity to cultures of undifferentiated PCD cells. Measurements of ciliary beat frequency were not significantly different between the virally transduced cultures and the controls, suggesting that expression of the wild-type *Dnaic1* was sufficient to restore normal ciliary activity to the PCD cells. This is consistent with an earlier study in which a VSV-g pseudotyped lentiviral vector was used to express DNAI1 in human PCD cells<sup>28</sup>. While it may be expected that expression of the wild-type protein will also lead to the correct orientation and regulation of ciliary activity required for efficient mucociliary clearance, further studies are required to test this hypothesis.

Importantly, for delivery of a gene transfer vector to the airways by inhalation, the vector must be able to transduce the airway epithelium via the apical surface. We have recently reported that influenza HA-pseudotyped vectors can mediate efficient gene transfer to murine airways<sup>16</sup>. In this study, we found that apical administration of influenza HA-pseudotyped vector expressing *Dnaic1* to fully differentiated cultures of PCD cells restored ciliary activity to a similar level as did treatment of undifferentiated cells. In cultures that were examined at the end of the experiment, we observed higher levels of *Dnaic1* RNA and protein in cultures that were transduced late in culture compared to those transduced early. Although we think it is likely this difference is due to the longer time period between transduction and analysis allowing for greater turnover (loss) of the transduced cells in the cultures treated early, other possibilities need to be considered. For example, it is possible that in the late stage cultures the PCR analysis was detecting residual vector RNA. In both conditions, the maximum level of correction achieved was approximately 10%; it is unclear at this time what the rate-limiting step for correction is. Interestingly, the appearance of ciliary activity in apically transduced differentiated cultures required several days, whereas expression of a reporter protein (e.g., EGFP) is typically observed in <72 hrs (e.g.,<sup>15</sup>). This observation prompted us to examine the stability the *Dnaic1* protein by taking advantage of the inducible nature of our model. Unlike *Chlamydomonas*, an organism that rapidly sheds and regrows its flagella<sup>29</sup>, there is little known about the turnover of respiratory ciliary proteins. Our studies indicated that although the Cre-mediated deletion of *Dnaic1* and the decline in the level of cytoplasmic *Dnaic1* protein occurred rapidly after tamoxifen treatment, the level of axonemal *Dnaic1* protein and ciliary activity declined at a much slower rate. These studies demonstrate that once ciliary proteins are assembled into axonemal structures they exhibit an increased stability. These results also suggest that the process of *Dnaic1* assembly into outer dynein arms (ODA) and the incorporation of sufficient ODA into the existing ciliary axonemes to restore motility requires several days. Alternatively, it is possible that the vector is transducing undifferentiated or preciliated cells that are then undergoing ciliated cell differentiation. In our previous study, 80% of the cells transduced by the HA-pseudotyped vector were identified as ciliated cells, making this seem less likely<sup>15</sup>. Additional studies using a vector expressing both a reporter and the *Dnaic1* protein are necessary to distinguish between these two possibilities.



Although the studies reported here demonstrate that ciliary activity can be restored to individual PCD cells by gene transfer, it is not known what level of gene transfer will be necessary to prevent or reduce the disease symptoms caused by PCD. Efficient mucociliary clearance in the airways requires the continuous transport of mucus, from the bronchioles to the larynx, and so the distribution as well as the number of cells transduced will be important determinants of success. To investigate the level of gene transfer required, we again took advantage of the inducible nature of our model system to generate individual animals with varying levels of *Dnaic1* deletion. Measurement of MCC rates in the nasopharynx and examination of nasal histology showed that animals with ~ 20% of the wild-type level of intact genomic *Dnaic1* remaining had some MCC remaining and most were free of obvious disease. Although this number (20%) is clearly an approximation, we have successfully transduced up to 40% of the surface epithelial cells in the trachea and large airways of mice using HA-pseudotyped lentiviral vector <sup>16</sup>, suggesting that improvements in vector design may make gene therapy for PCD possible.

An additional barrier to gene transfer to the airways of adult individuals with PCD, cystic fibrosis, or other diseases is the existence of disease-associated pathology, including the presence of excess mucus and/or the presence of inflammatory cells and mediators that may reduce or prevent gene transfer. Mice with a deletion of *Dnaic1* invariably develop rhinosinusitis with an accumulation of mucus and inflammatory cells in the nasal cavity and sinuses. Administration of an HA-pseudotyped vector to groups of control and PCD mice by nasal inhalation revealed a profound inhibition of gene transfer to the nasal cavity due to the presence of rhinosinusitis. In contrast, gene transfer to the trachea and lower airways of PCD mice was not significantly affected, demonstrating that the vector was able to successfully transduce regions of the airways lacking the excess mucus and inflammation present in the nasal cavity. However, additional strategies will be required for successful gene transfer to the airways of individuals with pre-existing disease. These may include pre-treatment to reduce the level of mucus accumulation and/or inflammation prior to administration of the vector. Alternatively, administration of vector to affected individuals early in the course of the disease, perhaps prenatally <sup>30</sup>, may allow successful gene transfer before the development of severe symptoms.

In summary, the results presented here demonstrate that restoration of ciliary activity to PCD cells by gene transfer to the apical surface of airway epithelial cells is possible using an HA-pseudotyped lentiviral vector, and that successful transfer of ~20% of the normal endogenous level of the mutated gene may be sufficient to prevent or mitigate the consequences of disease. Using an inducible mouse model of PCD that allows studies of adult animals, these studies have also demonstrated a significant inhibition of gene transfer to the nasal cavity as a result of pre-existing rhinosinusitis.

## Methods

### Generation of animals

The generation and breeding of the *Dnaic1*<sup>flx/flx</sup> mice expressing CreER from the ROSA promoter has been described <sup>18</sup>. These mice are maintained on a mixed background (C57Bl6/129) and when treated with tamoxifen provide a long-lived animal model of PCD.

All procedures using animals were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill, and were conducted in accordance with policies for the ethical treatment of animals established by the National Institutes of Health.

### Cloning of Dnaic1

The cloning of mouse Dnaic1 into the lentiviral vector was performed using standard molecular biology procedures and reagents. RNA was isolated from cultured mouse tracheal epithelial cells and a full-length cDNA for mouse Dnaic1 was amplified using PfuUltra polymerase (Stratagene, Santa Clara, CA) and primers ACCaagcttGCAATGCCCTCGAAACAG (sense) and TTTtctagaCTGGGCCATACTCTCAGGTTTT (anti-sense). The product was cloned into the TOPO vector (TOPO-TA cloning kit; Invitrogen) and sequenced. A clone with the correct sequence was used to amplify the Dnaic1 cDNA using Fidelity Fast Start polymerase (Roche Diagnostics, Indianapolis, IN) with primers incorporating Sall and NotI restriction sites (AATTgtcgaccgGCAATGCCCTCGAAACAGat; TTTgcgcccgCTGGGCCATACTCTCAGGTTTT). The product was digested, ligated into the lentiviral vector SIN6.1CB-W, sequenced, and used for production of vector.

### Production of Lentiviral vectors

Recombinant EIAV-based lentiviral vector stocks were generated by transient transfection of 293T-based TAB22 cells as previously described<sup>16,31</sup>. Briefly, lentiviral plasmids were transfected into 293T cells using the standard calcium phosphate method. After a 48 h incubation cell supernatant containing lentiviral vectors was harvested, clarified by low speed centrifugation (100 g, 10 min), and filtered through 0.2 µm PES membranes. Vector was concentrated by high speed centrifugation at 5000 g (Beckman Avanti J-E centrifuge, JS-5.3 rotor) for 21 h at 8°C. Vector pellets were suspended in vector formulation buffer (5 mM Hepes (pH 7.4), 37 mM NaCl and 40 mM lactose) and stored at -80°C. Recombinant lentiviral vector stocks were titered on 293T cells by serial dilution as previously described<sup>16</sup>. Titers for these experiments ranged from 6-10 × 10<sup>7</sup> infectious units per ml (IFU).

### Western blotting of Dnaic1

Detection of Dnaic1 protein by Western blotting was performed using a mouse monoclonal antibody generated against a synthetic peptide from the human DNAI1 protein<sup>18</sup> using standard procedures. For analysis of mouse tracheal epithelial cells, total cell lysates were prepared in Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Ciliary axonemes were isolated from cultured mouse tracheal epithelial cells as previously described<sup>32</sup> and prepared in gel-loading buffer. For studies of protein turnover, both the soluble (cytoplasmic) and pellet (axonemal) fractions from individual cultures were analyzed. Protein samples were fractionated on 4 to 12% Bis-Tris gradient gels (Invitrogen, Carlsbad, CA), transferred to nitrocellulose membranes, and probed using Amersham ECL Plus reagents (GE Healthcare, Buckinghamshire, UK), all according to manufacturer's instructions. For normalization of loading, Western blots of cytoplasmic extracts were reprobed with an antibody to GAPDH (Sigma, St. Louis, MO), and axonemal pellets were

reprobed with mouse anti-acetylated  $\alpha$ -tubulin (Invitrogen). Quantification of signals was performed on an Odyssey Imaging System (Licor, Lincoln, NB).

### **Culture, tamoxifen treatment, and transduction of MTE cells**

Procedures for the isolation and culture of mouse tracheal epithelial (MTE) cells have been described in detail previously<sup>18,33</sup>. To generate PCD cells for gene transfer studies, MTE cells from *Dnaic1<sup>fllox/fllox</sup>; CreER<sup>+/-</sup>* mice were seeded on collagen coated Millicell Cell Culture Inserts (12 mm; 0.4  $\mu$ m, Millipore) and treated with 1  $\mu$ M tamoxifen for the first 5-7 days of culture. As we have observed no difference between heterozygous and wild-type cultures, mice from both genotypes were used as controls. Lentiviral vectors with a titer of  $6-10 \times 10^7$  infectious units (IFU) per ml (33  $\mu$ L) were applied to the apical surface of washed cultures (day 5 or 19) and allowed to incubate for 3-4 hours before the supernatant was removed. Cultures were washed and refed 48 hrs after infection.

For *Dnaic1* turnover studies, differentiated cultures of MTE cells from *Dnaic1<sup>fllox/fllox</sup>; CreER<sup>±</sup>* mice were cultured in the absence of tamoxifen until ciliated cell differentiation was apparent (day 14-21). Experimental cultures were then treated with 1  $\mu$ M tamoxifen in media on day 0, 2, and 4, while positive control cultures received only media. Ciliary activity was measured and parallel cultures were harvested for DNA, RNA, and protein analysis. Four separate experiments were performed with similar results, although not all data were collected from each experiment.

### **Measurement of ciliary activity**

The extent of ciliary activity and measurements of ciliary beat frequency (CBF) were performed essentially as previously described<sup>18</sup> using a Nikon Eclipse TE2000 microscope and the SAVA software package<sup>34</sup>. Briefly, for measurements of the percentage of active ciliated surface area, nine different low-magnification fields (one central, eight peripheral) from each insert were measured. Typically, 2-3 inserts were measured and averaged at each time point. Because the absolute level of ciliogenesis varied between cultures, data is also expressed as the percentage of positive control cultures. For comparison between vector-treated and untreated cultures, the data shown in Figures 2a and 3a is taken from the time-point when vector-treated cultures showed maximal ciliary activity as a percentage of positive control. For determining CBF, positive control (heterozygous) cultures and PCD cultures treated with vector on day 5 were washed free of mucus and debris, equilibrated at 37° C, and 9 random fields were analyzed on day 25 (20 days after vector treatment).

### **Measurement of *Dnaic1* RNA and DNA**

RNA and DNA were isolated from MTE cell cultures or tail snips using RNeasy or DNeasy reagents and kits (Qiagen Inc., Valencia, CA) according to the manufactures protocols. DNA and RNA concentrations were determined by spectrophotometry. Qualitative PCR analysis of samples was performed by using primers (TGCTCCAAATCCTACTCCAG and AACACAGTGGGAAGAGTACGG) that are internal to the deleted region of *Dnaic1* to amplify cellular DNA followed by gel electrophoresis using standard procedures. The primers produce a 323 bp product with intact genomic DNA, no product from the deleted genomic DNA (tamoxifen-treated), and a 223 bp product from the vector encoded cDNA.

Quantitative PCR to determine the amount of intact *Dnaic1* in various samples was performed using LightCycler SYBR Green reagents (Roche Applied Science, Mannheim, Germany) and a Roche LightCycler using the same primers as previously published<sup>18</sup>. Each sample was analyzed in duplicate. Relative changes in the level of *Dnaic1* were calculated from the efficiency of the PCR reaction and the cross-point deviation between samples. For experiments in which the level of *Dnaic1* was being quantified, signals were normalized to the single-copy reference gene  $\beta$ -ENaC (*Scnn1b*)<sup>18</sup>; for the estimation of levels of *Dnaic1* mRNA, signals were normalized to the level of the ciliated cell-specific gene dynein axonemal heavy chain 5 (*Dnahc5*) using primers GTCTGGATGGGCGGCATGACTA and ATCCTGTAGCCCCTCGAGCTCA.

### Tamoxifen treatment of mice

To generate PCD mice, *Dnaic1<sup>flox/flox</sup>; CreER<sup>+/-</sup>* mice, 6-9 weeks old, were treated with tamoxifen (five intraperitoneal injections of 75  $\mu$ g/g body weight<sup>18</sup>). For the experiment shown in Fig. 5, animals were treated with 2 or 3 injections of tamoxifen to generate mice with varying levels of intact genomic *Dnaic1* remaining. Additional *Dnaic1<sup>flox/wt</sup>; CreER<sup>+/-</sup>* mice with varying levels of intact genomic *Dnaic1* (due to the spontaneous activation of CreER) and *Dnaic1<sup>wt/wt</sup>* mice were included in the study. After allowing sufficient time for ciliated cell turnover (>6 mos; <sup>18</sup>), mucociliary clearance was measured in the nasopharynx, the nasal cavity was fixed in formalin for histology, and the percentage of intact genomic *Dnaic1* remaining was determined in tracheal tissue.

### Measurement of MCC

Measurement of mucociliary clearance in the nasopharynx was performed as previously described in detail<sup>18</sup>. In this procedure, the transport of endogenous particles is recorded with a video camera after exposing the nasopharynx by dissection immediately after euthanizing the animal. The time required for the particles to move a calibrated distance (0.5-1 mm) is measured and the MCC rate reported for each animal is the average speed of 5-10 particles.

### Treatment of Mice with lentiviral vectors

Lentiviral vectors were administered to *Dnaic1<sup>-/-</sup>; CreER<sup>+/-</sup>* (PCD) and control mice by nasal inhalation<sup>16</sup>. Control animals included both *Dnaic1<sup>+/-</sup>* and *Dnaic1<sup>+/+</sup>* littermates; both were treated with tamoxifen. Mice were anesthetized with isoflurane and the concentrated vector solution ( $7 \times 10^8$  IFU) was administered dropwise to the tip of the nose in a small volume (20-25  $\mu$ l), where it was spontaneously inhaled into the nasal cavity.

### Quantification of luciferase

For live animal imaging of nasal luciferase expression<sup>16</sup>, mice were anesthetized with isoflurane and D-luciferin (Gold Biotechnology, St Louis, MO, USA) substrate (20  $\mu$ l of 25mg/ml D-luciferin in PBS) was delivered by intranasal administration<sup>35</sup>. Mice were imaged using the IVIS Lumina optical imaging system (Caliper Life Sciences, Hopkinton, MA, USA) and images of luciferase activity were collected using a charge-

coupled device camera interfaced to a computer. Data is presented as the average  $\pm$  S.E.M. in photons/sec/cm<sup>2</sup>.

### Quantification of $\beta$ -galactosidase

Tissue from the lungs, trachea and nasal cavity of treated animals were prepared and stained with X-Gal Solution as previously described<sup>16</sup>. For quantification, sections were scanned at high resolution (2400 dpi) and stained (blue) cells were counted. To quantify transduction in the nasal cavity, multiple sections (3 or 4) at different levels of the nasal cavity were counted for each animal and the total number of stained cells were compared. In the first experiment, sections were prepared from levels 9, 11, and 23; in the second experiment, sections were prepared from levels 5, 9, 11, and 23, based on the system of Mery et al.<sup>36</sup>. For quantification in the trachea, a single section the length of the trachea was evaluated; both sides of the longitudinal section through the trachea were counted. Data is reported as mean  $\pm$  S.E.M.

### Statistical Analyses

Results between PCD and vector-treated groups were compared using a student's t-test with a p-value of 0.05 accepted as significant. In experiments where more than two groups were being compared, an ANOVA was first performed to determine significance. For the data shown in Figure 5a, the coefficient of determination ( $R^2$ ) was determined by linear regression.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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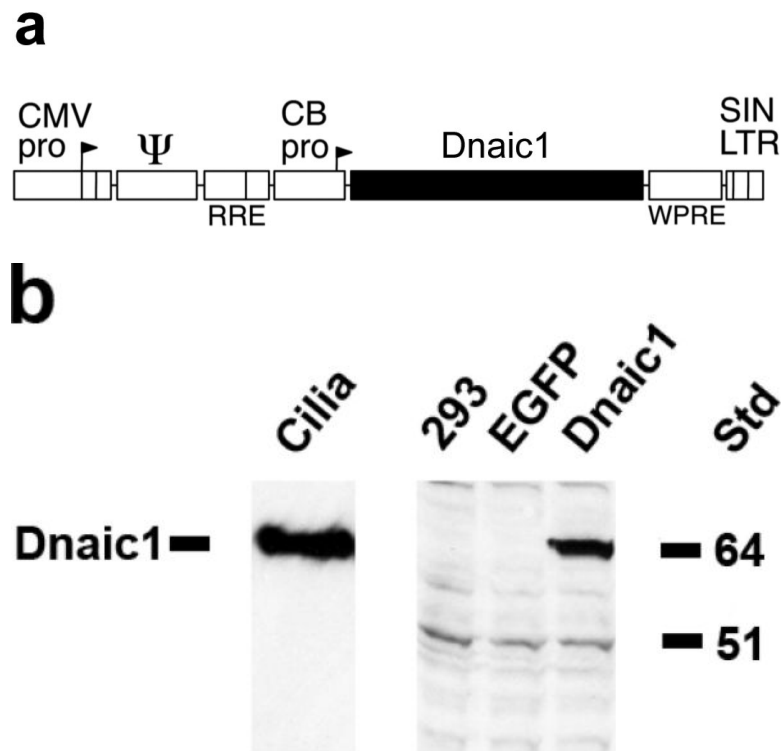
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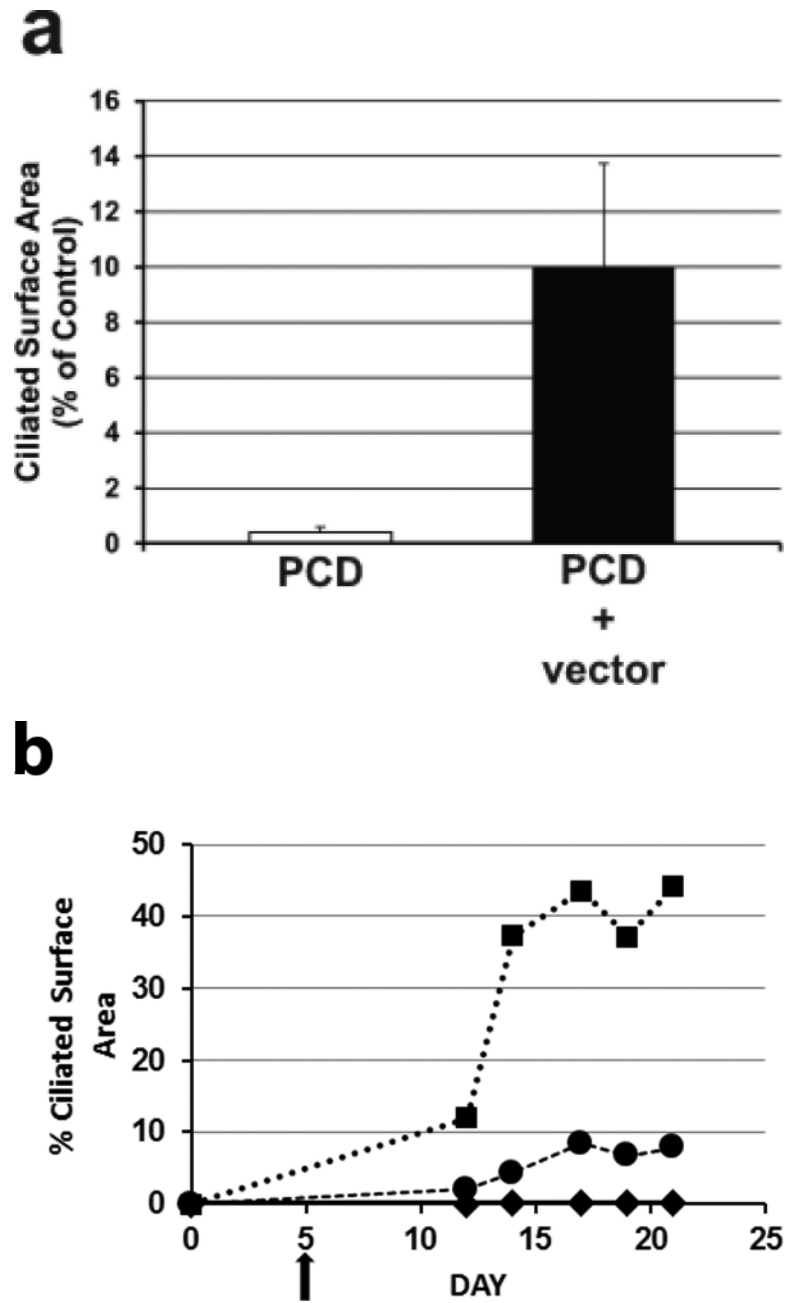


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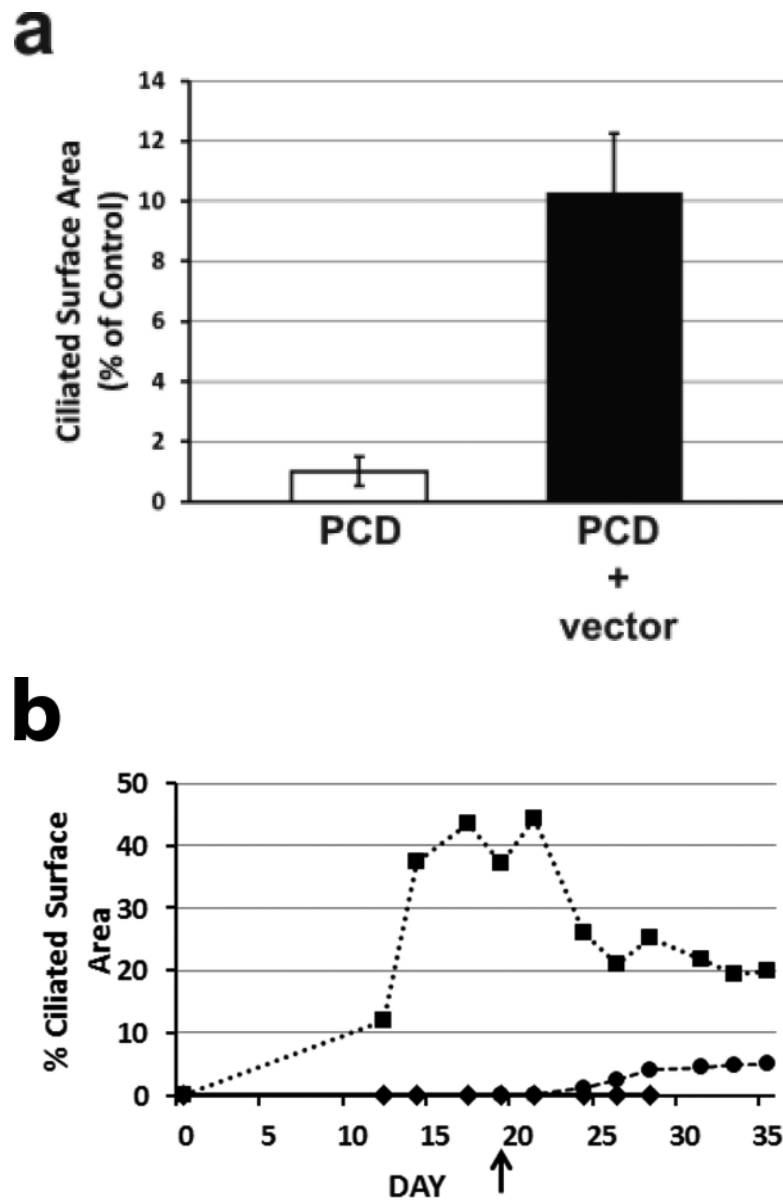
**Figure 1.**

a) Diagram of the lentiviral gene transfer construct used in these studies. The mouse *Dnaic1* cDNA was expressed from a hybrid CMV enhancer/chicken  $\beta$ -actin promoter (CB). The vector also contains an upstream CMV enhancer/promoter fused to the R and U5 domains of the EIAV long terminal repeat (LTR) sequence, the viral Rev Responsive Element (RRE), RNA encapsidation sequence ( $\Psi$ ), the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and a 3' self-inactivating (SIN) LTR sequence. b) Western blot demonstrating expression of full-length *Dnaic1* protein. Cultures of 293 cells were untreated (negative control) or transduced with lentiviral vectors expressing EGFP (EGFP) or *Dnaic1* (*Dnaic1*) from the CB promoter. Purified mouse cilia (cilia) isolated from tracheal cell cultures<sup>32,33</sup> served as a positive control. Molecular size markers are shown (Std.).



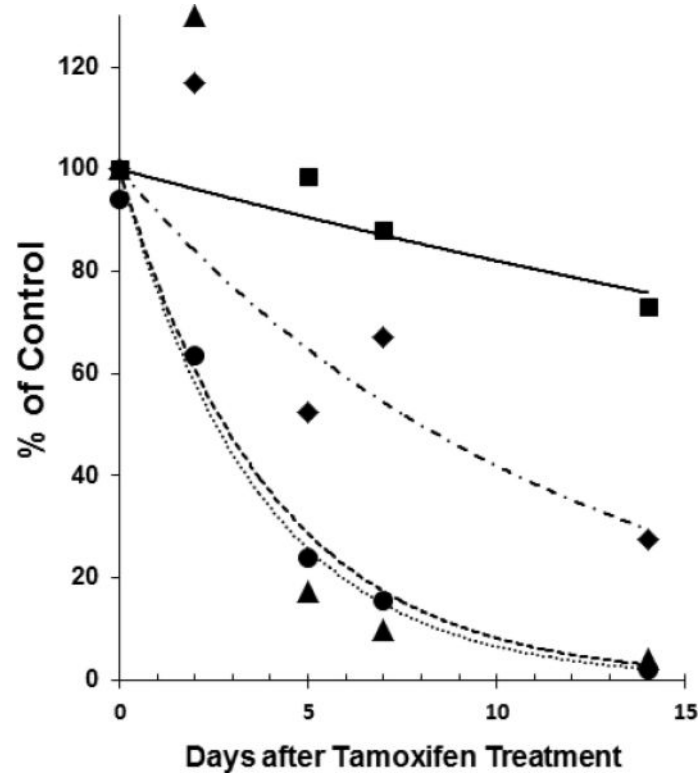
**Figure 2.**

a) Ciliary activity as a percentage of surface area in PCD cultures and undifferentiated PCD cultures transduced with a lentiviral vector expressing *Dnaic1*. Data are expressed as the percentage of parallel positive control cultures and represents the average  $\pm$  S.E.M. of 4 separate experiments. b) Representative experiment showing the increase in ciliary activity vs. time in culture of positive control cells (squares), PCD cells (diamonds), or in undifferentiated PCD cells transduced apically with the vector (circles). Vector was administered on day 5 (arrow).



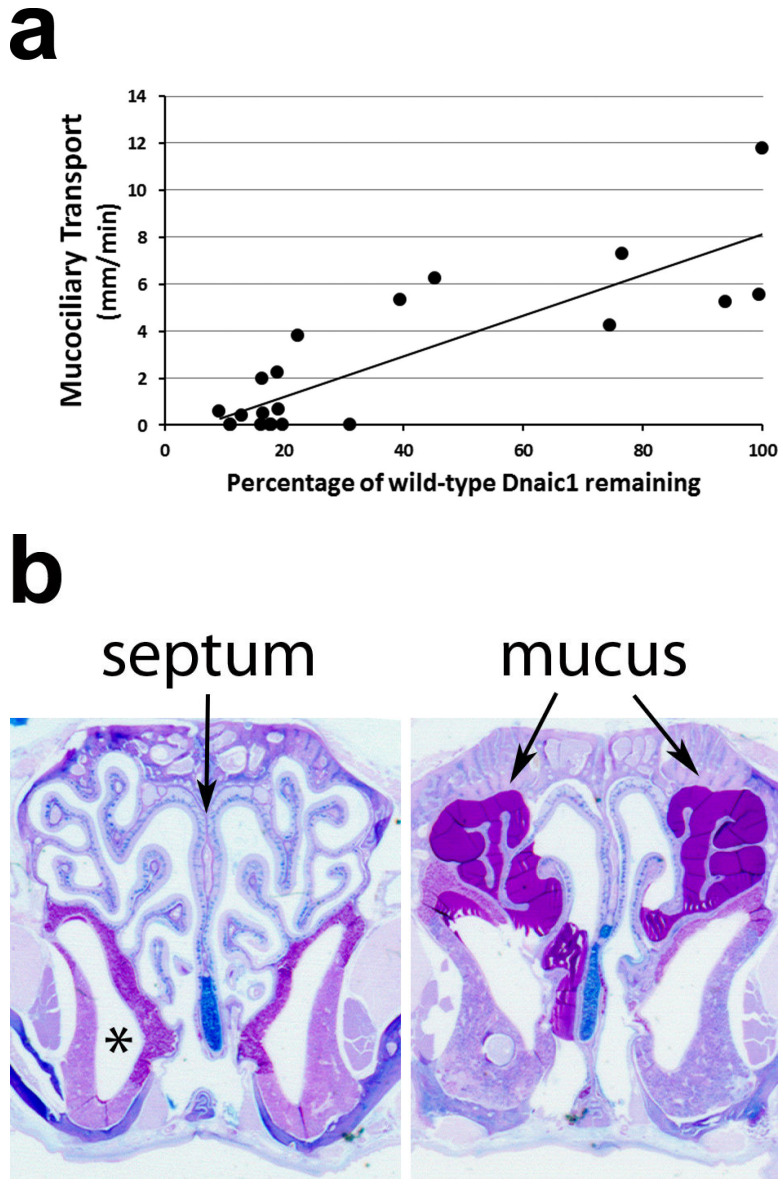
**Figure 3.**

a) Ciliary activity as a percentage of surface area in PCD cultures and differentiated PCD cultures transduced apically with a lentiviral vector expressing *Dnaic1*. Data are expressed as the percentage of parallel positive control cultures and represents the average  $\pm$  S.E.M. of 4 separate experiments. b) Same experiment as in 2b showing the increase in ciliary activity vs. time in culture of positive control cells (squares), PCD cells (diamonds) or in differentiated PCD cells transduced apically with the vector (circles). Vector was administered on day 19 (arrow).



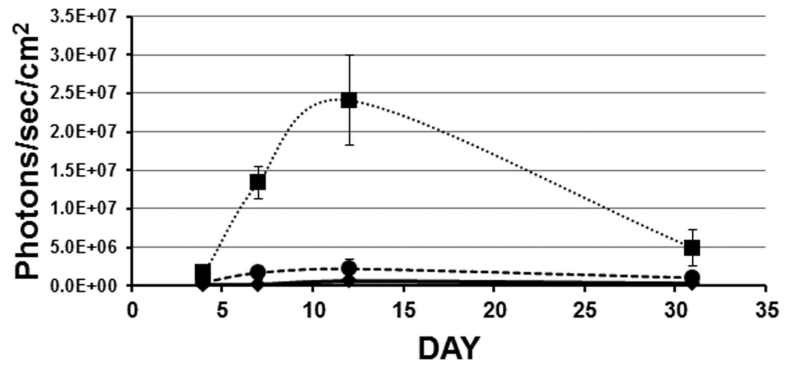
**Figure 4.**

Turnover of *Dnaic1* protein in ciliated cells. Differentiated cultures of *Dnaic1*<sup>flx/flx</sup> CreER<sup>+</sup> cells were treated with tamoxifen (day 0, 2, and 4) and the percentage of intact *Dnaic1* DNA (circles), cytoplasmic protein (triangles), and axonemal protein (diamonds) were measured over time, along with the level of ciliary activity (squares). Measurements were taken before treatment (day 0) and at various times after treatment with tamoxifen was begun. Data shown are from a single experiment. See text for details.

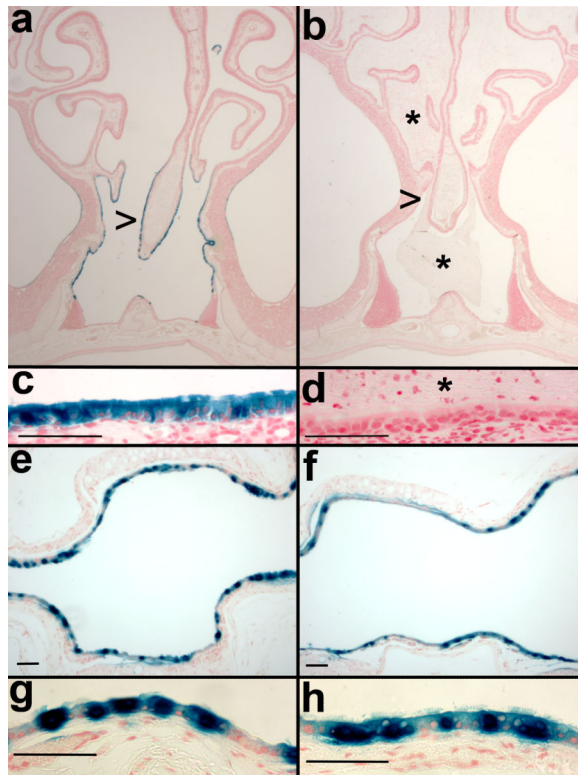


**Figure 5.** a) Relationship between level of intact genomic *Dnaic1* and MCC. Most animals with >20% *Dnaic1* remaining had MCC rates > 2 mm/min and no evidence of disease. b) Histology of the nasal cavity of animals with 16% (left) and 19% (right) of the wild-type level of *Dnaic1* showing the absence (left) or presence (right; arrow) of rhinosinusitis. The maxillary sinus (\*) and nasal septum (arrow) are indicated (left). Sections were stained with alcian blue-periodic acid Schiff.





**Figure 6.** Time course of luciferase activity in the nasal cavity of control (squares; n=6) and PCD (circles; n=5) mice transduced with a lentiviral vector. Data = average  $\pm$  S.E.M. Untreated (no virus) negative control (diamonds) exhibited  $<1 \times 10^6$  photons/sec/cm<sup>2</sup> at all time points.



**Figure 7.**

Gene transfer in the nasal cavity (a, b, c, d) and trachea (e, f, g, h) of control (a, c, e, g) and PCD (b, d, f, h) mice. HA-pseudotyped lentivirus expressing  $\beta$ -gal was administered to mice by nasal inhalation and 12 days later tissues were stained for the expression of  $\beta$ -gal and paraffin sections were evaluated. Both the nasal cavity and the trachea exhibited positive  $\beta$ -gal staining in the control mice. In PCD animals the level of gene transfer in the nasal cavity was greatly reduced (note the mucus (\*) in b, d; compare with Figure 5), while gene transfer to the trachea was not impeded. Nasal sections (a, b, c, d) are from  $\sim$ level 23<sup>36</sup>. Arrowheads pointing at the nasal septum in a, b, indicate the location of the higher magnification images in c, d. Sections were counter-stained with neutral fast red. Scale bars =50  $\mu$ m in c, d, e, f, g, h.