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(54) Title: DIAGNOSIS OF CILIOPATHIES

(57) Abstract: The invention provides, *inter alia*, reagents and methods for diagnosing ciliopathies in a human or animal. Furthermore, the present invention relates to a knockout mouse, more specifically to a mouse in which the Cby1 gene is disrupted and which exhibits ciliary disease.

DIAGNOSIS OF CILIOPATHIES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of the priority date of U.S. provisional Application No. 61/162,983, filed March 24, 2009. For the purpose of any U.S. patent that may issue from the instant application, this prior provisional application is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

This invention relates to proteins, such as antibodies, that specifically bind a Chibby protein (Cby), and more particularly to the use of such antibodies in, for example, the diagnosis of ciliary disease.

BACKGROUND

Primary ciliary dyskinesia (PCD) is a rare genetic disorder that affects organ systems that rely on ciliary activity for proper function. PCD patients commonly suffer from chronic inflammation and infection of the upper respiratory structures (primarily the sinuses, ears, nose, and pharynx) and lower respiratory structures (including the bronchi, bronchioles, and alveoli within the lungs). As ciliated cells are also found in the ependymal lining of the brain, people with PCD may experience chronic headaches and hydrocephalus. In addition, cilia line the fallopian tubes, and the spermatozoal flagella have a core structure that is identical to cilia. As a result, female PCD patients may have difficulty conceiving or experience miscarriages and ectopic pregnancies. Male patients may be infertile.

As the movement of the cilia contributes to organ placement in the developing embryo, approximately 50% of the PCD population has a condition called *situs inversus totalis* where the abdominal organs, including the heart, liver, spleen, and intestines, are on the opposite side of the abdominal cavity relative to their usual position. This subset of patients is said to have Kartagener Syndrome. The organs may function normally in their mirror-image position, but serious defects have also been reported.

SUMMARY

Early diagnosis and prophylactic treatment can be important in preventing or limiting the damage associated with PCD. Currently, the only definitive way to diagnose PCD is through a biopsy to assess ciliary morphology and orientation. Nasal nitric oxide is low in PCD, and this measurement shows some promise as a screening test. However, there is still a great need for methods and devices that can be used to conveniently and sensitively detect and characterize impaired ciliary function.

The present invention features methods of using agents that detect the expression of a Chibby-encoding gene (*e.g.*, *Cby1*) or the presence of a Chibby protein (*e.g.*, a Chibby-binding molecule). The methods can be used to identify animals (*e.g.*, humans), with malfunctioning cilia. In another aspect, the invention features a *Cby1* knockout mouse and cells or tissues derived therefrom, which can be used to identify the physiological role(s) of *Cby1*, and which can serve as a model system in which to test possible therapeutics for cilia-related diseases, in particular PCD. In another aspect, the invention features methods of identifying agents that may, as an unwanted side effect, produce secondary dyskinesia. For example, therapeutic agents in development or chemical or biological compounds under consideration for use (*e.g.*, in an environmental setting or in a personal care product such as a cosmetic, lotion, cream, shampoo, diaper, wound dressing, or the like) can be tested to determine whether the agents or compounds inhibit the expression or activity of Chibby and, therefore, may promote secondary dyskinesia. These methods can be carried out by providing a tissue in which Chibby expression and/or activity is within normal limits, exposing the tissue to a test substance, and determining whether the test substances reduces the expression or activity of Chibby. The tissue can comprise isolated cells or tissues (*e.g.*, in tissue culture) or tissue *in vivo* (*e.g.*, in an animal model).

More specifically, the invention includes methods of diagnosing a subject for a condition associated with impaired ciliary function (*e.g.*, PCD). The methods include providing a sample (*e.g.*, a tissue sample) from the subject and exposing the sample to an agent or agents (*e.g.*, a nucleic acid probe or pair of PCR primers)) that detects Chibby-encoding mRNA or a molecule that specifically binds a Chibby protein (*e.g.*, an anti-Chibby antibody). The exposure can occur for a time and under conditions in which the

agent(s) specifically bind the Chibby-encoding mRNA or the molecule specifically binds Chibby protein. For example, binding can occur within a sample kept at room temperature or maintained at physiological temperatures. Samples in which mRNA is detected can be tested at even higher temperatures to minimize any non-specific binding. Typically, binding between molecules (*e.g.*, between an antigen and its cognate antibody) can occur within minutes or hours, and the diagnostic and screening methods described here can be carried out on that time scale. A decrease in binding of the molecule to the Chibby protein, relative to a reference standard, indicates that the subject has a condition associated with impaired ciliary function. Similarly, where a nucleic acid probe indicates decreased *Cby1* expression, relative to a reference standard, the patient from whom the sample was obtained may have or may develop a condition associated with impaired ciliary function.

From a clinical perspective, the subject is most likely to be a human patient. However, other mammals can be examined for diagnostic purposes, and the knockout mice described herein (or cells or tissues obtained therefrom) can be examined in the context of a screen for PCD therapeutics. Thus, the methods can include detecting *Cby1* expression or Chibby protein expression, including expression within samples of human or knockout mouse tissue in the presence of one or more possible therapeutic agents (*e.g.*, a small molecule or protein). The results of such screening assays can be interpreted in view of controls or reference standards.

A Chibby protein within the tissue can form a complex with a Chibby-binding molecule. For example, a Chibby-binding antibody (*e.g.*, the monoclonal antibody 8-2) can form an immune complex with Chibby by reacting a sample (*e.g.*, a test sample containing an unknown amount of Chibby) with the antibody. As a reference, one can include either a positive or a negative control sample (or both) in the analysis, and the amount of the immune complex formed in the sample (*i.e.*, the test sample) can be compared to the amount in one or both of the control samples. The immune complex can be detected by any of a number of methods. For example, where the assay is an immunoassay, one can detect the immune complex by Western blotting, ELISA, immunoprecipitation, or immunofluorescence staining.

While the molecule that specifically binds Chibby may be, as discussed, an antibody, an antigen-binding fragment thereof, or any of the other immunoglobulins or immunoglobulin-type molecules known or described herein (*e.g.*, an scFv), the invention is not so limited. The methods of diagnosing PCD, of screening for therapeutic agents that can be used in the treatment of PCD, and of identifying agents or compounds that may cause unwanted secondary ciliary dyskinesia can be carried out using any detectable Chibby-binding molecule. In addition to antibodies, these molecules include non-immunoglobulin scaffolds such as adnectins, affibodies, anticalins, DARPins (designed ankyrin repeat proteins), engineered Kunitz-type inhibitors, protein A, the lipocalins, and modified fibronectin domains. These and other binding molecules can be equipped with prescribed binding functions, and they are known in the art. See, *e.g.*, Gebauer and Skerra, *Curr. Opin. Chem. Biol.* 13:245-255, 2009; Skerra, *Curr. Opin. Biotechnol.* 18:295-304, 2007; and Stumpp *et al.*, *Drug. Disc. Today* 13:695-701, 2008.

Ciliary disease can be diagnosed when Chibby is present at reduced levels, mislocalized, or otherwise deregulated. For example, mislocalization of the Chibby protein, as revealed by histological staining, indicates that the subject has a condition associated with impaired ciliary function.

As noted, the Chibby-binding molecule can be an antibody. The diagnostic methods and screens for agents, including therapeutic agents, described herein can be carried out with antibodies (*e.g.*, the classic tetrameric antibody of the IgG class) or with an antigen-binding fragment thereof, including a single chain antibody (scFv). Polyclonal antibodies can also be used. In other embodiments, the antibodies can be human or humanized antibodies, single-domain antibodies, or deimmunized antibodies.

Samples provided for the present methods can be obtained from a patient suspected of having a ciliary disease or considered at risk for developing a ciliary disease. These include diseases characterized by upper and/or lower respiratory tract infection, chronic headaches, hydrocephalus, infertility, polycystic kidney disease, and heart, liver, spleen, pancreatic, and intestinal defects. Samples can be obtained from, for example, the respiratory tract, a sinus cavity, the ear, nose, or throat, the kidney, the liver, or the fallopian tube. The sample can also be semen.

The Chibby protein can be encoded by CBY1 (human) mapping to 22q13.1 (GenBank Ref Seq Accession Number NM_015373; GeneID 25776) or can have an amino acid sequence that is at least 70% identical to SEQ ID NO:1 (GenBank Ref Seq Accession Number NP_056188).

The invention also provides kits for performing the methods of the invention. The kits of the invention can include a probe that specifically binds or amplifies a Chibby-encoding nucleic acid and/or a Chibby-binding molecule. The kits may also include other reagents for preparing the tissue, applying the probe or binding molecule, or detecting any complex formed between the probe and *Cby1* mRNA or between a Chibby-binding molecule and Chibby. Instructional materials, in print, audio, or video form, can also be included. For example, the invention features kits that include a molecule that specifically binds Chibby (*e.g.*, an antibody) and instructions directing the application of the molecule to a tissue sample obtained from a patient suspected of having a condition associated with impaired ciliary function. The reagents can be those useful in an immunoassay, and can facilitate or optimize detection of the reaction product. Other reagents within the kit can be used to quantitate Chibby expression or quantify the extent of any mislocalization.

As indicated above, the invention also features mice homozygous (or heterozygous) for the defect of the *Cby1* gene and tissues or cells (*e.g.*, germ cells) isolated from such an animal model.

The details of one or more embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description, examples, and from the claims.

DETAILED DESCRIPTION

The present invention is based, in part, on the discovery that Cby is an important mediator of ciliary function and, in particular, in ciliary conditions associated with PCD. More specifically, the present invention is related to the discovery that mislocalization or reduced levels of Cby are important factors in the pathogenesis of diseases and disorders of the cilia. The present invention provides methods for the diagnosis of a condition associated with impaired ciliary function by examining a cell or tissue sample from a

subject. The methods can be repeated over time, allowing one to monitor the presence of Cby, and decisions about treatment can be made based on the outcome.

Samples may be derived from a variety of sources, including cells or tissues affected by PCD. The samples can be obtained in a number of ways, including by nasal scrapings and nasal biopsy. Once obtained, a sample can be kept in solution or can be, for example, fixed or bound to a substrate. It may also be frozen and used in whole or in part at a later time. A sample can refer to any material suitable for testing for the presence of Cby or suitable for screening for molecules that bind to Cby or fragments thereof. Methods for obtaining such samples are within the level of skill in the art.

The sample can be exposed to a molecule that binds Chibby, such as an antibody that specifically binds a Cby polypeptide or a mutant or fragment thereof. Cby antibodies may be generated using methods well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, single chain antibodies, as well as Fab fragments, including $F(ab')_2$ and F_v fragments. Fragments can be produced, for example, by a Fab expression library. As noted above, non-immunoglobulin scaffold proteins can also be used to detect Chibby proteins.

Antibodies to Cby can be used to identify Cby or fragments thereof in tissue, *e.g.*, from a biopsy (*e.g.*, a kidney biopsy). The amount of Cby present could be determined, for example, by quantitative image analysis. The antibody administered in the method can be the intact antibody or antigen binding fragments thereof, such as Fab, $F(ab')_2$, and F_v fragments, which are capable of binding the epitopic determinant. The antibodies used in the method can be polyclonal or, more preferably, monoclonal antibodies. Monoclonal antibodies with different epitopic specificities are made from antigen containing fragments of the protein by methods well known in the art.

A target polypeptide, such as Cby, or an agent that modulates the activity and/or expression of Cby, can be evaluated to determine regions of high immunogenicity. Methods of analysis and epitope selection are well known in the art. See, *e.g.*, Ausubel *et al.*, eds., *Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York N.Y.* 1988. Analysis and selection can also be accomplished, for example, by various software packages, such as LASERGENE NAVIGATOR software (DNASTAR; Madison Wis.). The peptides or fragments used to induce antibodies should be antigenic,

but are not necessarily biologically active. Preferably, an antigenic fragment or peptide is at least 5 amino acids in length, more preferably, at least 10 amino acids in length, and most preferably, at least 15 amino acids in length. It is preferable that the antibody-inducing fragment or peptide is identical to at least 70% (*e.g.*, 75%, 80%, 85%, 90%, 95%, 98%, or 99%) of SEQ ID NO:1.

MPFFGNTFSPKKTPPRKSASLSNLHSLDRSTREVELGLEYGSPTMNLAGQSLKFE NGQWIAETGVSGGVDRREVQRLRRRNQQLEEENNLLRLKVDILLDMLSESTAES HLMEKELDELRISRKRK (SEQ ID NO:1).

Methods for the production of antibodies are well known in the art. For example, various hosts, including goats, rabbits, rats, mice, humans, and others, may be immunized by injection with the target polypeptide or any immunogenic fragment or peptide thereof. Depending on the host species, various adjuvants may be used to increase immunological response. Such adjuvants include, but are not limited to, Freund's adjuvant, mineral gels such as aluminum hydroxide, and surface-active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, KLH, and dinitrophenol. Among adjuvants used in humans, BCG (bacilli Calmette-Guerin) and *Corynebacterium parvum* are especially preferable.

Monoclonal and polyclonal antibodies may be prepared using any technique which provides for the production of antibody molecules by continuous cell lines in culture. Techniques for *in vivo* and *in vitro* production are well known in the art. See, *e.g.*, Pound, *Immunochemical Protocols, Humana Press, Totowa N.J.,* 1998; Harlow and Lane, *Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York N.Y.*, 1988. The production of chimeric antibodies is also well known, as is the production of single-chain antibodies. See, *e.g.*, Morrison *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6851-6855, 1984; Neuberger *et al.*, *Nature* 312:604-608, 1984; Takeda *et al.*, *Nature* 314:452-454, 1985. Antibodies with related specificity, but of distinct idiotypic composition, may be generated, for example, by chain shuffling from random combinatorial immunoglobin libraries. See, *e.g.*, Burton, *Proc. Natl. Acad. Sci. USA* 88:11120-11123, 1991.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening immunoglobulin libraries or panels of highly

specific binding reagents. See, *e.g.*, Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA* 86:3833-3837, 1989; Winter and Milstein, *Nature* 349:293-299, 1991. Antibody fragments which contain specific binding sites for the target polypeptide may also be generated. Such antibody fragments include, but are not limited to, $F(ab')_2$ fragments, which can be produced by pepsin digestion of the antibody molecule, and Fab fragments, which can be generated by reducing the disulfide bridges of the $F(ab')_2$ fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity. See, *e.g.*, Huse *et al.*, *Science* 254:1275-1281, 1989.

Antibodies can be tested for anti-target polypeptide activity using a variety of methods well known in the art. Various techniques may be used for screening to identify antibodies having the desired specificity, including various immunoassays, such as enzyme-linked immunosorbent assays (ELISAs), including direct and ligand-capture ELISAs, radioimmunoassays (RIAs), immunoblotting, and fluorescent activated cell sorting (FACS). Numerous protocols for competitive binding or immunoradiometric assays, using either polyclonal or monoclonal antibodies with established specificities, are well known in the art. See, *e.g.*, Harlow and Lane. Such immunoassays typically involve the measurement of complex formation between the target polypeptide and a specific antibody. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on the target polypeptide is preferred, but other assays, such as a competitive binding assay, may also be employed. See, *e.g.*, Maddox *et al.*, *J. Exp. Med.* 158:1211, 1983.

In the present invention, diagnostic applications include those using "human" or "humanized" antibodies directed to Cby or fragments thereof. Humanized antibodies are antibodies, or antibody fragments, that have the same binding specificity as a parent antibody, (*i.e.*, typically of mouse origin) and increased human characteristics. Humanized antibodies may be obtained, for example, by chain shuffling or by using phage display technology. For example, a polypeptide comprising a heavy or light chain variable domain of a non-human antibody specific for a Cby is combined with a repertoire of human complementary (light or heavy) chain variable domains. Hybrid pairings specific for the antigen of interest are selected. Human chains from the selected

pairings may then be combined with a repertoire of human complementary variable domains (heavy or light) and humanized antibody polypeptide dimers can be selected for binding specificity for an antigen. Techniques described for generation of humanized antibodies that can be used in the method of the present invention are disclosed in, for example, U.S. Pat. Nos. 5,565,332; 5,585,089; 5,694,761; and 5,693,762. Furthermore, techniques described for the production of human antibodies in transgenic mice are described in, for example, U.S. Pat. Nos. 5,545,806 and 5,569,825.

Assays will typically provide for detectable signals associated with the binding of the compound to a protein or cellular target. Binding can be detected by, for example, fluorophores, enzyme conjugates, and other detectable labels well known in the art. See, *e.g.*, Enna *et al.*, *supra*. The results may be qualitative or quantitative.

For screening the compounds for specific binding, various immunoassays may be employed for detecting, for example, human or primate antibodies bound to the cells. Thus, one may use labeled anti-hIg, *e.g.*, anti-hIgM, hIgG or combinations thereof to detect specifically bound human antibody of the galactosyl epitope. Various labels can be used such as radioisotopes, enzymes, fluorescers, chemiluminescers, particles, etc. There are numerous commercially available kits providing labeled anti-hIg, which may be employed in accordance with the manufacturer's protocol.

In another embodiment, the present diagnostic and/or prognostic methods can be carried out using nucleic acid probes to detect, characterize, and quantify *Cby1* in a sample obtained from a patient. If desired, *Cby1* can be isolated and/or quantitated. For example, *Cby1*-specific hybridization probes can be used to detect, characterize by length, and quantify mRNA by, for example, Northern blot of total or poly-A^+-selected RNA samples. The probes can also be used as hybridization probes to detect, characterize by location, and quantify mRNA by *in situ* hybridization to tissue sections. See, *e.g.*, Schwarchzacher *et al.*, *In Situ* Hybridization, Springer-Verlag New York (2000).

Preferably, in the diagnostic methods of the present invention, normal or standard values for Chibby gene or protein expression are established in order to provide a basis for the diagnosis of the existence of a ciliary disease or disorder or a predisposition to a ciliary disease or disorder. In one of the methods of the present invention, this is

accomplished by combining body fluids or cell extracts taken from normal subjects with antibody to Cby under conditions suitable for complex formation. Such conditions are well known in the art. The amount of standard complex formation may be quantified by comparing levels of antibody-target complex in the normal sample with a dilution series of positive controls, in which a known amount of antibody is combined with known concentrations of purified Cby. Standard values obtained from normal samples may be compared, for example, in a specific embodiment, with values obtained from samples from subjects suspected of having ciliary disease or disorder, or having a predisposition to ciliary disease or disorder, associated with PCD. Deviation between standard and subject values establishes the presence of, or predisposition to, the disease state.

Monoclonal antibodies can be detected by methods discussed, for example, *supra*. Monoclonal antibodies against Cby can be conjugated to an appropriate enzyme such as horseradish peroxidase, protein ferritin, enzyme alkaline phosphatase, β-D-galactosidase *etc*. These enzyme-linked antibody preparations can be mixed with, for example, tissue samples that contain unknown amounts of Cby in an indirect ELISA. Direct or sandwich ELISAs could also be performed using the same antibodies.

RIA techniques may also be used to measure levels of Cby in, for example, tissue. For example, Cby may be radioactively labeled and mixed with monoclonal antibodies specific for Cby and a tissue sample containing an unknown amount of unlabeled Cby. Binding competition between the labeled and unlabeled Cby with the monoclonal antibody occurs. By measuring the amount of radioactivity of the reaction mixture, the amount of Cby present in the sample can be quantitatively determined. See, *e.g.*, U.S. Patent Nos. 4,438,209 and 4,591,573. Non-competitive RIAs can also be performed.

The present invention provides kits for detecting Cby in samples, in particular, in solid samples. In a preferred embodiment, the diagnostic kits of the present invention contain reagents for measuring levels of Cby in tissue samples. In a particular embodiment, this kit comprises a monoclonal antibody specific for Cby bound to a support and a second monoclonal antibody specific for the first antibody and labeled. The kit further comprises reagents for detecting the enzyme-labeled monoclonal antibody. The reagent kit employs immunological methods in measuring Cby in the tissue sample, thus allowing for the detection and monitoring of ciliary disorders and

diseases. In another embodiment, the kit comprises a radio-labeled or fluorescein-labeled antibody in place of the enzyme-labeled antibody.

In one embodiment, the diagnostic kit of the present invention comprises elements useful in the detection of Cby in tissue samples, using immunohistochemical techniques. The kit could be used in conjunction with, for example, a software program which allows for quantitative measurement of the levels of Cby in the tissue sample by image analysis or other comparative techniques. See, *e.g.*, Riser *et al.*, 1996, *supra*.

In a preferred embodiment, the diagnostic kit of the present invention is packaged and labeled, for example, in box or container which includes the necessary elements of the kit, and includes directions and instructions on the use of the diagnostic kit.

The present invention provides mice that are homozygous for the defect of the Cby1 gene, in which no functional Cby1 is produced. The Cby1 knockout mouse (*Cby1-/-*) serves as a tool for directly identifying the physiological role of Cby1, and to provide a model animal useful in the study of the cause of, and in the development of means for the treatment of, ciliary-related diseases, in particular PCD, based on findings obtained with such mice.

The present invention further provides mice that are heterozygous for the defect of the Cby1 gene. The mice may be used not only as means for reproduction of mice that are homozygous for the defect of the Cby1 gene, through their cross-fertilization and examination of the presence/absence of the Cby1 gene product, but also for the same purpose as the homozygous mice as they themselves have at least a potential defect concerning ciliary function.

The present invention also provides tissues of mice that are mono- or heterozygous for the defect of the Cby1 gene. Such tissues may be used as a test system for the development of therapeutic drugs for ciliary disease. The test system can be configured as a high-throughput screen and may be automated.

The present invention also provides cells of mice that are homo- or heterozygous for the defect of the Cby1 gene. Such cells may be used as an *in vitro* test system for the development of therapeutic drugs for ciliary disease. Further, germ cells or fertilized eggs of mice that are homozygous for the defect of the Cby1 gene can be used for the production of mice that are homozygous for the defect of the Cby1 gene.

Cby knockout (*Cby1-/-*) mice develop upper respiratory infections and are unable to clear bacteria due to the absence of mucociliary transport (MCT). The MCT defects result, at least in part, from a marked paucity of motile cilia in the nasal epithelium possibly due to the abnormal transport or docking of the basal bodies to the apical membrane. This respiratory phenotype is similar to the clinical features observed in PCD patients. Cby is normally localized at the base of the cilia in cultured MDCK2 cells and in nasal epithelial tissue. The perturbation of cilia and the corresponding effects on MCT render *Cby1-/-* mice particularly suitable for studies of PCD and other chronic upper respiratory diseases.

The majority of Cby knockout (*Cby1-/-*) mice display premature death before or around weaning after 2-3 days of weight loss. All *Cby1-/-* mice develop rhinitis and sinusitis. When challenged with *Pseudomonas* isolates, *Cby1-/-* mice are unable to clear the bacteria from the nasal cavity. Notably, *Cby1-/-* mice exhibit a complete absence of mucociliary transport due to a marked paucity of motile cilia in the nasal epithelium. Moreover, ultrastructural studies reveal impaired basal body docking to the apical surface of multi-ciliated cells. In support of these phenotypes, Cby protein is localized at the base of cilia. As the phenotypes of *Cby1-/-* mice bear striking similarities to PCD, *Cby1-/-* mice may prove to be a useful model for this condition.

In the present invention, "defect of the *Cby1* gene" means that no functional Cby protein is produced due to the disruption of the *Cby1* gene. The present invention described above and defined by the appended claims can be utilized as a model animal and a screening system for the investigation of the mechanisms causing ciliary diseases, and for the development of therapeutic drugs for such diseases.

EXAMPLES

We first characterized the expression of mouse *Cby* in embryonic day (E) 9.5-11.5 embryos by whole mount *in situ* hybridization analysis using a probe specific for the *Cby* coding region. Our analysis showed that *Cby* is ubiquitously expressed at E9.5-E11.5. Northern blot analysis revealed that *Cby* is expressed in multiple adult tissues and throughout embryogenesis. These data establish a broad expression profile for *Cby* in the

developing mouse embryo, as well as the presence of *Cby* transcripts in multiple adult tissues.

In order to investigate Cby function, we performed germline inactivation by removing the entire coding region of the mouse *Cby1* gene. Gross morphological evaluation of newborns did not reveal apparent defects. However, by two weeks of age, *Cby1-/-* pups were runted and were anemic, as evidenced by decreased hematocrit on complete blood count analysis and reduced subcutaneous fat.

To better understand the association of *Cby* gene ablation with runting, we performed survival analysis of pups produced by Cby heterozygous (*Cby1+/-*) parents. The survival analysis showed that *Cby1-/-* mice stratified into two groups: the majority of them died by postnatal day (P) 25 (KO-D) while a small percentage started to gain weight and survived for more than 18 months (KO-S). However, all surviving *Cby1-/-* mice (7/32) continued to have low body mass compared to wild-type (*Cby1+/+*) mice. At all stages analyzed, *Cby1+/-* mice were indistinguishable from *Cby1+/+* counterparts. These data demonstrate that the gross physiological effect of loss of *Cby* on mouse development results in lowered body mass followed by early postnatal lethality. The exact causes of their postnatal death, however, remain unknown.

Histopathological phenotyping demonstrated that all *Cby1-/-* mice, both those that die postnatally and those that survive, develop sinusitis. This presents as early as P7 and persists throughout the lifetime of the surviving adults. Mice with severe sinusitis also had otitis media.

To further probe sinus function we tested the susceptibility of *Cby1-/-* mice to bacterial infection. We intranasally infected five *Cby1-/-,* five *Cby1+/-*, and six *Cby1+/+* mice with *Pseudomonas aeruginosa*, a pathogenic bacterium frequently associated with chronic airway infection in patients with cystic fibrosis (CF) and PCD (Gibson *et al.*, *Am. J. Respir. Crit. Care Med.* 168:918-51, 2003). We found that at 72 h post-infection, infected littermate controls efficiently cleared bacteria and had no sinus inflammation as revealed by histological analysis of the upper airways. In contrast, all *Cby1-/-* mice had accumulated abundant mucus and bacterial debris leading to severe inflammation and distorted morphology of sinuses. Concurrent colony forming units (CFU) analysis revealed that both *Cby1-/-* and control mice successfully cleared the bacteria from the

lungs. Bacterial challenge, therefore, revealed further phenotypic abnormalities, as *Cby1-/-* mice develop sinus and middle ear inflammation and fail to clear bacteria from the sinuses.

Defects in bacterial clearance can have multiple etiologies as illustrated by PCD and CF. Although respiratory phenotypes observed in PCD and CF patients are quite similar, the molecular and cellular mechanisms underlying these phenotypes differ. As previously mentioned, abnormal airway cilia structure or motility underlie PCD phenotypes (Livraghi and Randell, *Toxicol. Pathol.* 35:116-29, 2007). By contrast, human patients with CF caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) exhibit abnormal ion and water transport secondary to defects in Cl^- conductance. This leads to depletion of the peri-ciliary aqueous layer in the respiratory system with accumulation of abnormally viscous mucus that adheres to the epithelial surfaces and cannot be transported by cilia (Livraghi and Randell, *Toxicol. Pathol.* 35:116-29, 2007).

To address potential ion channel deficiencies in *Cby1-/-* mice, we determined Na^+ absorption in response to amiloride and Cl^- secretion in response to UTP and forskolin in tracheas from *Cby1+/+* and *Cby1-/-* mice. The tracheal bioelectrics were identical between the two genotypes, indicating that ion channel function was not affected. These results argue against the involvement of ion channel defects in the airway pathology of *Cby1-/-* mice.

Next, we examined ciliary function by measuring MCT in nasopharyngeal cavities. As expected, control mice exhibited normal MCT of endogenous particles in the nasal cavity. Strikingly, the *Cby1-/-* mice exhibited an MCT rate of zero. The cilia of the control mice generated steady flow of mucus, whereas in three of the four *Cby1-/-* mice studied, mucus movements were completely absent. In the fourth *Cby1-/-* mouse we observed small islands of local mucus movement but this was not sufficient to generate detectable MCT. Thus, *Cby1-/-* mice develop sinusitis and otitis media due to a failure of the mucociliary defense system in the nasal passages and possibly in the Eustachian tube.

Defects in MCT may be caused either by abnormal cilia structure (*e.g.* absence of outer, or inner dynein arms or radial spokes) or by reduced numbers of cilia. To examine cilia structure we analyzed nasopharyngeal cilia by transmission electron microscopy

(TEM). Cilia from *Cby1-/-* mice exhibit apparently normal ciliary ultrastructure with a 9+2 microtubular arrangement and the presence of outer dynein arms. Basal bodies in *Cby-/-* mice also show normal nine-triplet structure. Similarly, we did not detect any apparent defects in structure of bronchial cilia from *Cby1-/-* mice. In the course of our TEM studies we noticed that *Cby1-/-* mice have an abnormally low abundance of cilia in their nasopharynx. A marked paucity of cilia was also observed upon analysis of *Cby1-/-* nasopharyngeal tissue at P0 ruling out the possibility that loss of cilia is secondary to chronic inflammation. To verify our observation we performed scanning electron microscopy (SEM) analysis of proximal lung airways from adult *Cby1-/-* and *Cby1+/+* mice. Consistent with the TEM results, we found that the number of cilia is dramatically decreased in the *Cby1-/-* tissue when compared to that from *Cby1+/+* controls. Taken together, we conclude that loss of Cby results in a paucity of cilia in respiratory tracts, which most likely accounts for the lack of MCT activity in *Cby1-/-* mice.

Based on the data presented so far, we hypothesized that the ciliary defects in *Cby1-/-* mice might be caused by altered docking or transport of basal bodies to the apical membrane. Through detailed EM analysis, we found that a significant number of basal bodies failed to position apically and dock at the plasma membrane in *Cby1-/-* nasal ciliated cells. This is not attributable to a general loss of apical-basal polarity in these cells since apical marker ZO1 and actin filaments as well as basolateral marker E-cadherin remained unaffected in *Cby1-/-* mice. As cilia extend from basal bodies, compromised basal body docking may explain, at least in part, the defective ciliogenesis in *Cby1-/-* mice.

Given the involvement of *Cby* in cilia pathology, we then asked if Cby protein is localized in a manner consistent with this pathology. Double immunostaining with an antibody against acetylated-α-tubulin (cilia marker) revealed that Cby is localized at the ciliary base in multi-ciliated cells of the adult *Cby1+/+* nasal epithelium while no Cby staining was detected in the *Cby1-/-* tissue. In good agreement with these data, Cby protein is expressed in ciliated cells in developing lung airways and esophagus at E18.5. Additionally, endogenous Cby was detected at the base of primary cilia in cultured MDCK2 cells. We also noted that Cby co-localizes with the centrosomal marker γ-tubulin. Several ciliopathy-associated proteins have been shown to localize to both

centrosomes and basal bodies, including CEP290 that is involved in Joubert syndrome (Valente *et al.*, *Nat. Genet.* 38:623-5, 2006) and ALMS1 that is mutated in Alstrom syndrome (Li *et al.*, *PLoS Genet.* 3:e8, 2007). Cby localization at the base of the cilia further supports our notion that Cby is directly involved in ciliogenesis.

Cby acts as an antagonist of Wnt/β-catenin signaling in mammalian cultured cells and *Drosophila melanogaster* embryos. Hence we evaluated the status of β-catenin-dependent transcription in nasopharyngeal epithelia from *Cby1-/-* mice and age-matched controls using quantitative PCR for the known direct β-catenin targets, *Axin2* and *CyclinD1*. We observed a mild but consistent increase in the expression of these genes in the *Cby1-/-* tissue. Similar results were obtained for primary mouse embryonic fibroblasts (PMEFs) derived from *Cby1-/-* and *Cby1+/+* embryos. These data are consistent with Cby being a β-catenin antagonist (Takemaru *et al.*, *Nature* 422:905-9, 2003), and suggest that mild chronic activation of Wnt/β-catenin signaling may contribute to the ciliary phenotypes of *Cby1-/-* mice. Multiple components of the Wnt/β-catenin pathway such as APC, β-catenin and Dishevelled (Dvl) have been shown to localize to the ciliary base (Corbit *et al.*, *Nat. Cell Biol.* 10:70-6, 2008; Gerdes *et al.*, *Nat. Genet.* 39:1350-60, 2007; Park *et al.*, *Nat. Genet.* 2008). We therefore analyzed the localization of β-catenin and Dvl1 in nasal ciliated cells from *Cby-/-* mice and littermate controls. Lateral membranous β-catenin and apical Dvl1 localization remained unchanged in the absence of Cby. Worthy of note, contrary to β-catenin localization at primary cilia in cultured PMEFs (Corbit *et al.*, 2007), we did not detect β-catenin at motile cilia, implicating that its localization at the ciliary base may be unique to primary cilia.

The airway phenotypes of *Cby1-/-* mice bear strong similarities to those of human PCD patients. PCD is frequently associated with mutations in genes encoding dyneins, including *DNAH1* and *DNAH5* (Olbrich *et al.*, *Nat. Genet.* 30:143-4, 2002; Pennarun *et al.*, *Am. J. Hum. Genet.* 65:1508-19, 1999). Our qPCR analysis demonstrated that inactivation of *Cby* did not significantly affect levels of *DNAH1* and *DNAH5* transcripts in nasal epithelial tissue.

Presentation of sinusitis and otitis media, and an inability to clear bacteria from the sinuses upon intranasal infection, underlie similarities between the phenotypic defects found in *Cby-/-* mice and in human patients with PCD. This is especially evident when *Cby-/-* mice are exposed to bacterial challenge, exacerbating the already inflamed state of their upper airway. About half of PCD patients have laterality defects and many are subfertile (Meeks and Bush, *Pediatr. Pulmonol.* 29:307-16, 2000; Noone *et al.*, *Am. J. Respir. Crit. Care Med.* 169:459-67, 2004). To date, we have not observed left-right patterning defects such as *situs inversus* in *Cby1-/-* mice, suggesting that Cby is not essential for proper structure and function of motile 9+0 cilia in the embryonic node. We noticed partially penetrant infertility in *Cby1-/-* males. All these observations signify similarities between phenotypes of *Cby-/-* mice and human patients with PCD.

Current animal models of PCD with respiratory pathology have abnormal ciliary structure. The axonemal dynein heavy chain 5 (*DNAH5* gene) mutant mice lack outer dynein arms, and *Poll-Dpcd*-deficient mice (*poll* and *Dpcd* encode DNA polymerase λ and a protein of unknown function, respectively) lack inner dynein arms (Ibanez-Tallon *et al.*, *Hum. Mol. Genet.* 11:715-21, 2002; Kobayashi *et al.*, *Mol. Cell Biol.* 22:2769-76, 2002). While the majority of PCD patients also show defects in airway cilia structure, about 10% of "atypical" PCD cases show cilia with normal axoneme structure (Livraghi and Randell, *Toxicol. Pathol.* 35:116-29, 2007; Zariwala *et al.*, *Annu. Rev. Physiol.* 69:423-50, 2007). This allows us to speculate that a subset of PCD may be caused by mutations in the *Cby* gene. Thus, *Cby1-/-* mice might serve as a model for "atypical" cases of PCD. Given that the existing mouse models of PCD exhibit very high incidence of hydrocephalus and perinatal lethality, surviving *Cby1-/-* mice may also be useful as an animal model for long-term studies of chronic upper airway infections (Livraghi and Randell, *Toxicol. Pathol.* 35:116-29, 2007).

In the following paragraphs, we describe some of the techniques used to generate the data discussed above.

Northern blots were purchased from OriGene and hybridized with a [^{32}P]-labeled full-length mouse *Cby* cDNA probe. The probe was synthesized by random priming using the DECAprime II Kit (Ambion).

To generate Cby knockout mice, a BAC (bacterial artificial chromosome) clone containing the *Cby* gene was isolated from the mouse 129 BAC library (Research Genetics). The BAC clone was characterized by restriction mapping and sequencing. An upstream 4.1-kb *BamH*I-*Stu*I fragment and a downstream 4.1-kb XbaI-SmaI fragment were subcloned into PGKneoF2L2DTA targeting vector (a gift from Dr. Philip Soriano, Fred Hutchinson Cancer Research Center) on either side of the neomycin resistance cassette in order to replace the whole *Cby* coding sequence. The linearized construct was electroporated into R1 embryonic stem cells, and transfectants resistant to G418 were screened for homologous recombination by Southern blot analysis. Of 17 neomycin-resistant clones analyzed, 5 (29%) contained correctly targeted *Cby* allele. Three independent targeted cell lines were separately injected into blastocysts through the transgenic mouse facility at the University of Washington. Chimeric males were crossed with female C57BL/6 mice, and germline transmission was detected by the presence of agouti-colored offspring and further confirmed by Southern blot analysis. All three ES clones successfully underwent germline transmission, and all three mouse lines showed identical phenotypes. The mice were backcrossed to C57BL/6 mice at least five times (≥N5 generation). Genotyping was performed by PCR; primer sequences for genotyping were as follows:

P1, 5'-TGCCATTACACGAGACTAGACAG-3' (SEQ ID NO:2)

P2, 5'-AGACACCAGTGTCAAGAGGTGAG-3' (SEQ ID NO:3)

P3, 5'-CTTTCTAGAGAATAGGAACTTCGG-3' (SEQ ID NO:4)

Mice were fed irradiated Picolab Rodent Diet 20 #5053 (PMI Nutrition International) and reverse osmosis water. All supplies entering animal rooms were autoclaved and rooms were maintained at 70-74°F, 45-55% humidity, with 28 air changes/h 12/12-h light/dark cycle. All animal procedures were approved by the University of Washington (UW) Animal Care and Use Committee.

Whole mount *in situ* hybridizations were carried out as previously described (Cygan *et al.*, *Development* 124:5021-32, 1997).

For histological analysis, mice were euthanized by CO_2 asphyxiation in accordance with UW Animal Care and Use Committee procedures. Complete necropsies were performed and tissues were dissected and immersion-fixed in 10% neutral buffered

formalin. Skulls were decalcified in Cal-rite (Richard-Allan Scientific). Samples were then processed routinely by paraffin embedding. All tissues were initially stained with hematoxylin and eosin (H&E), examined histologically and selected sections were also stained with giemsa or periodic acid Schiff.

For cross-section TEM of the mainstem bronchus, tissue was removed, fixed in 2% glutaraldehyde/2% formaldehyde in 0.1 M Sorenson's buffer and post-fixed in 1% osmium tetroxide in Sorenson's buffer. Samples were then embedded in epon resin (Poly/Bed812, Polysciences) and 90 nm sections were cut, stained with 7% uranyl acetate/0.3% lead citrate, and viewed using a Zeiss EM900 electron microscope. For cross-section TEM of the nasopharynx, tissue was removed, fixed in 2.5% glutaraldehyde/2% formaldehyde in 0.1 M Cacodylate buffer and post-fixed in 1% osmium tetroxide in Cacodylate buffer. Samples were then embedded in epon resin (Poly/Bed812, Polysciences) and 70 – 90 nm sections were cut, stained with 6% aqueous uranyl acetate and Reynold's lead citrate, and viewed using a JEOL 1230 electron microscope with a Gatan 2K x 2K CCD camera.

For scanning electron microscopy, adult lungs were inflation-fixed with 4% PFA and proximal lung tissue removed. The tissue was then dehydrated using a graded ethanol series to 100% and transferred to 100% hexamethyldisilazane (HMDS; Electron Microscopy Sciences) through a graded series of ethanol-HMDS mixtures. The specimens were air-dried, mounted on SEM stubs and sputter-coated with gold before examination with a LEO1550 scanning electron microscope at 10 kV using a Robinson back scatter detector.

For bacterial challenge, an overnight culture of PAO-SC11, a mutant of PAO1 *Pseudomonas aeruginosa* (Chugani and Greenberg, *Microb. Pathog.* 42:29-35, 2007), was diluted 1:10 in Luria broth (LB) and grown for another hour to an OD of ~ 1.0 in the morning of infection. The bacteria were harvested by centrifugation, resuspended in PBS with 10 mM $MgCl_2$, and diluted to the desired concentration. Actual counts were determined by serial dilution and plating on LB agar plates. Mice were anesthetized with 130 mg/kg ketamine / 9 mg/kg xylazine and infected intranasally with 30 µl of PAO-SC11. A group of control mice was euthanized immediately after infection to determine actual deposition in the lungs. Lungs were homogenized in PBS containing 10 mM

$MgCl_2$ and 0.05% Triton X-100, and colony forming unit (CFU) analysis was performed by serial dilution and plating on LB and *Pseudomonas* isolation agar plates (Becton Dickinson). At 72 h post-infection *Cby-/-* and control mice were euthanized, lungs were collected for CFU analysis and sinuses were fixed and subjected to histological analysis as described above. GraphPad Prism software was utilized to analyze CFU results using one-way ANOVA test.

For tracheal electrophysiology, mice were euthanized by CO_2 asphyxiation, the trachea was isolated, split longitudinally, removed from mouse, and positioned on the $0.025cm^2$ aperture of an Ussing chamber as previously described (Grubb *et al.*, *Am. J. Physiol.* 267:C293-300, 1994). The tissue was bathed bilaterally in Krebs Ringer bicarbonate buffer and studied under short circuit current (Isc) conditions (Grubb *et al.*, *Am. J. Physiol.* 267:C293-300, 1994). To assess the magnitude of electrogenic Na^+ absorption, amiloride (10^{-4} M) was added apically after a 30-minute equilibration period. Five minutes later, UTP (10^{-4} M) (induces Cl^- secretion via the Ca^{2+} activated Cl^- conductance) was added, followed by apical forskolin (10^{-5} M) (induces an increase in Cl^- secretion by increasing cAMP levels). All drugs were purchased from Sigma-Aldrich.

For mucociliary transport measurements, mice were euthanized by CO_2 inhalation and the lower jaw removed, exposing the hard and soft palate. An incision was made through the skin and fascia over the most caudal palatine fold. The skin and fascia were then carefully removed from the soft palate by peeling the tissue caudally using fine forceps. Once the skin and fascia were stripped from the soft palate, the very thin ventral wall of the nasopharyngeal meatus became evident and the beating cilia on the apical side of the membrane were seen clearing debris caudally. Next, water-equilibrated mineral oil was applied to the membrane to prevent desiccation. As the cilia are on the opposite side of the membrane (and the nasopharyngeal meatus is not actually opened), the mineral oil did not contact the cilia. For image acquisition, the preparation was placed under a dissecting scope. A video camera (MTI) was mounted on the scope and interfaced with a VHS recorder and monitor. Before MCT was recorded, a slide micrometer was placed on the stage of the dissecting scope and the image recorded. When the data were reduced, the video monitor was calibrated for distance using the recorded image of the slide micrometer. Once the video was recorded, MCT was determined by playing the video

back and determining the time it took endogenously secreted mucus and other inhaled debris present in the nasal cavity prior to euthanasia to traverse the calibrated distance (usually an *in vivo* distance of 0.5-1 mm) on the monitor screen. Time was recorded with a stopwatch (accurate to 0.01 sec) and MCT was calculated as mm/min.

For RNA extraction and real-time RT-PCR, total RNA was purified from mouse primary embryonic fibroblasts and from nasopharyngeal epithelia using the RNeasy Mini Kit (Qiagen), with DNase digestion by the RNase-free DNase Set (Qiagen). First-strand cDNA synthesis and PCR amplification were performed with 100 ng of RNA template using the iScript One-Step RT-PCR Kit with SYBR Green (BioRad) and the MiniOpticon real-time PCR detection system (BioRad) according to the manufacturer's instructions. The primer pairs used were: *CyclinD1* (5′-TGTTCGTGGCCTCTAAGATGAAG-3′ (SEQ ID NO:5) and

5′-AGGTTCCACTTGAGCTTGTTCAC-3′) (SEQ ID NO:6);

Axin2 (5′-CTCCCCACCTTGAATGAAGA-3′ (SEQ ID NO:7) and

5′-ACATAGCCGGAACCTACGTG-3′) (SEQ ID NO:8);

and *ARBP* (5′-TGTTTGACAACGGCAGCATTT-3′ (SEQ ID NO:9) and

5′-CCGAGGCAACAGTTTGGGTA-3′) (SEQ ID NO:10). The level of transcripts for *ARBP* was used as an internal standard. Samples were analyzed in triplicate.

For immunofluorescence staining, MDCK2 cells were grown to confluency and then serum starved for 48 h to induce ciliation. Cells were fixed either in 4% PFA or in ice-cold methanol, washed in PBS, permeabilized with 0.1% Triton X-100 in PBS, and incubated with primary and secondary antibodies. The nasal epithelium was removed, processed as for routine histological analysis, and sections were incubated with primary and secondary antibodies. Stained sections were mounted with ProLong Gold antifade reagent with DAPI (Invitrogen). Images were acquired at room temperature using a Leica SP1/MP confocal microscope (40x and 100x oil lenses) and Leica confocal software. Images were analyzed and 3D visualized using Imaris software and assembled in figure format in Adobe Photoshop. Primary antibodies were as follows: anti-acetylated α-tubulin (Sigma; 1:10,000), anti γ-tubulin (Sigma; 1:400), Dvl1 (Santa Cruz Biotechnology; 1:500), ZO1 (Zymed Laboratories; 1:500), E-cadherin (BD Transduction

Laboratories; 1:500), 15B8 β-catenin (Sigma; 1:500). We also used Alexa Fluor 488 phalloidin (Invitrogen; 1:100) to visualize actin cytoskeleton. Rabbit anti-Cby polyclonal antibody was raised against the N-terminal portion of mouse Cby and purified by antigen affinity chromatography at Covance, and used at a 1:500 dilution. Alexa secondary antibodies were purchased from Molecular Probes (Invitrogen Molecular Probes).

WHAT IS CLAIMED IS:

1. A kit comprising:

a molecule that specifically binds Chibby; and

instructions directing the application of the molecule to a tissue sample.

2. The kit of claim 1, wherein the molecule that specifically binds Chibby is an immunoglobulin.

3. The kit of claim 1, wherein the molecule that specifically binds Chibby is an adnectin, an affibody, an anticalin, a designed ankyrin repeat protein, an engineered Kunitz-type inhibitor, a protein A, a lipocalin, or a modified fibronectin domain.

4. The kit of any of claims 1-3, wherein the tissue sample has been obtained from a patient suspected of having a condition associated with impaired ciliary function.

5. The kit of any of claims 1-3, wherein the tissue sample comprises isolated cells or tissues maintained in tissue culture.

6. A method of diagnosing a subject for a condition associated with impaired ciliary function, the method comprising providing a tissue sample from the subject and exposing the sample to a molecule that specifically binds a Chibby protein, wherein the exposure occurs for a time and under conditions in which binding between the molecule and Chibby can occur.

7. The method of claim 6, wherein a decrease in binding of the molecule to the Chibby protein, relative to a reference standard, indicates that the subject has a condition associated with impaired ciliary function.

8. The method of claim 6 or claim 7, wherein mislocalization of the Chibby protein, as revealed by binding of the molecule to the Chibby protein, indicates that the subject has a condition associated with impaired ciliary function.

9. The method of any of claims 6-8, wherein the tissue sample is a sample from the respiratory tract, a sinus cavity, the ear, the kidney, the liver, or the fallopian tube.

10. The method of any of claims 6-9, wherein the condition is primary ciliary dyskinesia.

11. The method of any of claims 6-10, wherein the subject is a human.

12. The method of any of claims 6-11, wherein the molecule is an immunoglobulin.

13. the method of claim 12, wherein the immunoglobulin is an antibody or an antigen binding fragment thereof.

14. The method of claim 13, wherein the antibody is a polyclonal antibody, a monoclonal antibody, a humanized antibody, a single chain antibody, a single-domain antibody or a deimmunized antibody.

15. The method of any of claims 6-14, wherein the Chibby protein is encoded by CBY1 (human) mapping to 22q13.1 (GenBank Ref Seq Accession Number NM_015373; GeneID 25776) or has an amino acid sequence that is at least 70% identical to SEQ ID NO:1 (GenBank Ref Seq Accession Number NP_056188).

16. A method of determining whether an agent may cause secondary dyskinesia, the method comprising

(a) providing a sample comprising a ciliated cell expressing Chibby;

(b) exposing the sample to the agent; and

(c) assessing Chibby expression in the sample, wherein a decrease in the expression of Chibby or a change in the expected cellular location indicates that the agent may cause secondary dyskinesia.

17. A mouse homozygous for the defect of the Cby1 gene.

18. A mouse heterozygous for the defect of the Cby1 gene.

19. A tissue isolated from the mouse of claim 17 or claim 18.

20. A cell isolated from the mouse of claim 17 or claim 18.

21. The cell of claim 20, wherein the cell is a germ cell.