GEMC1 is a critical regulator of multiciliated cell differentiation

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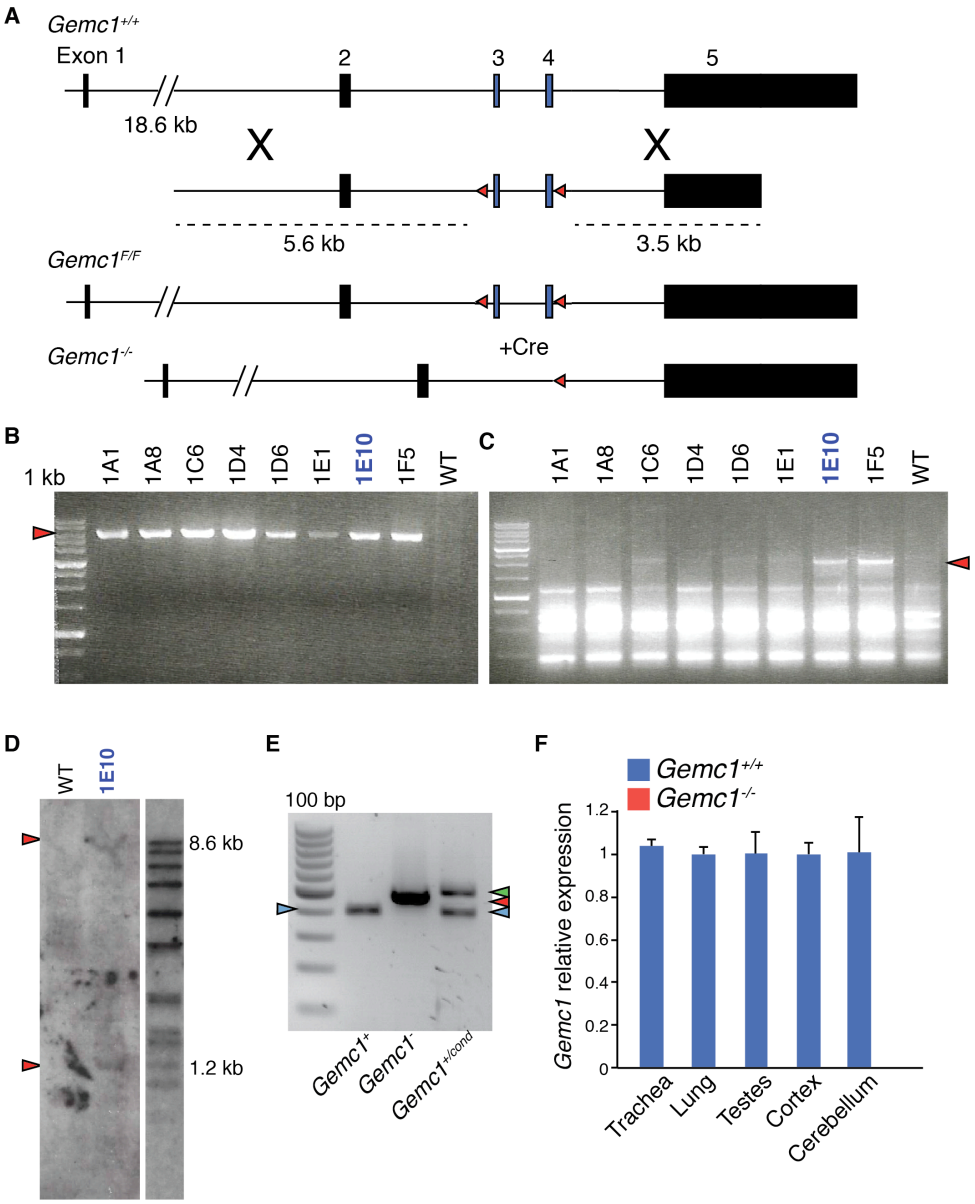
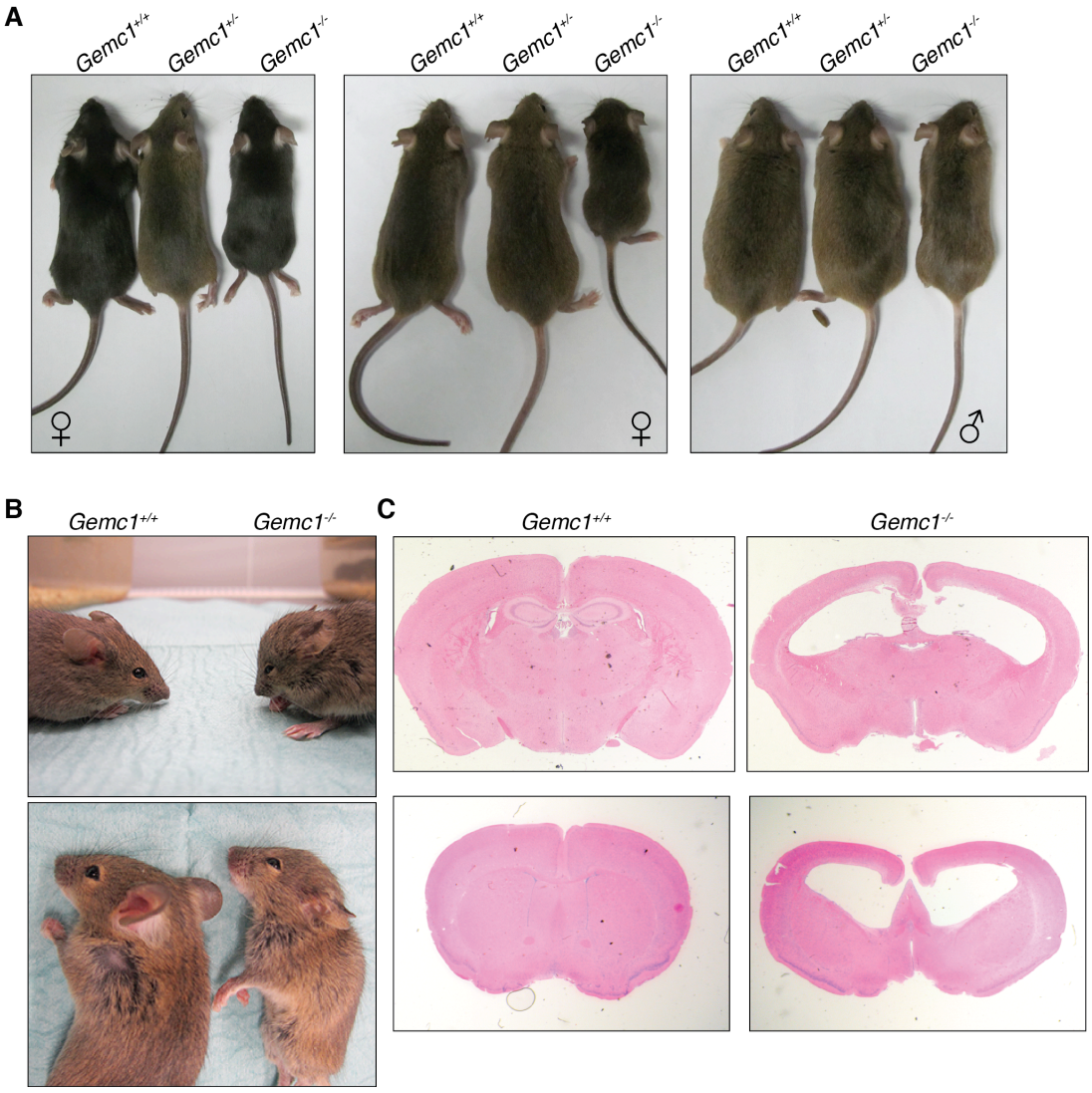
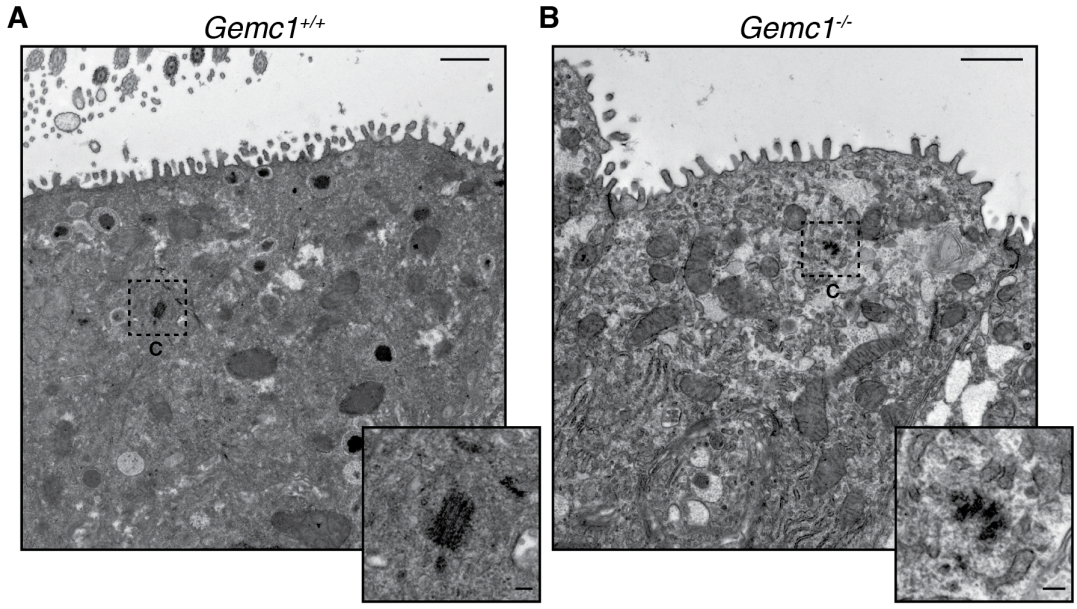


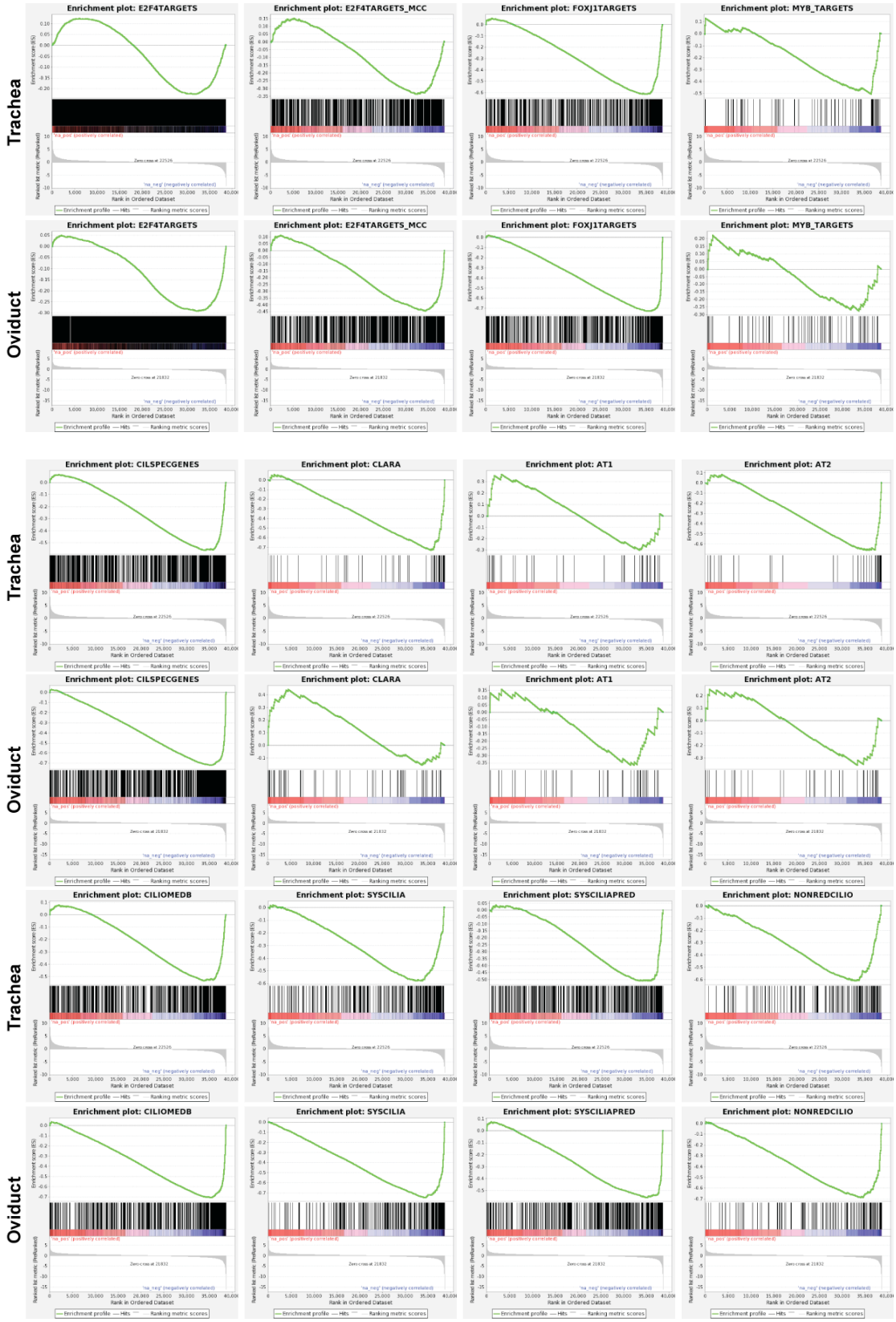
Appendix Figure S1. Generation of mice lacking GEMC1. A. Design of the targeting construct used to delete exons 3 and 4 of mouse *Gemc1*. B. and C. Targeting was checked by multiple long range PCR strategies. The band expected in correctly targeted embryonic stem cell clones (indicated by letter/number codes) is indicated with a red arrow and one of the clones used to generate mice is indicated in blue (1E10). The reaction in B is specific for detecting the 5'homology arm and in C the 3'loxP site. D. Clones were screened for single insertions by Southern blotting after PvuII digest of genomic DNA. Expected bands (9.5 kb) for the correct insertion are indicated by red arrowheads (bottom band results from partial restriction digest). Closest size markers from the same gel are indicated on right. E. Examples of PCR genotyping for the wild type (blue arrowhead), deleted (red arrowhead) and floxed (green arrowhead) allele. *Gemc1* was deleted by crossing with Sox2-Cre transgenic mice(Hayashi et al, 2002). All subsequent analyses were performed in Cre negative animals. F. *Gemc1* expression is not detected in multiple tissues of *Gemc1*$^{-/-}$ mice by quantitative real time PCR (Taqman). Values for *Gemc1*$^{-/-}$ are relative to the corresponding wild type tissue (each set to 1) See Appendix materials and methods for additional information.

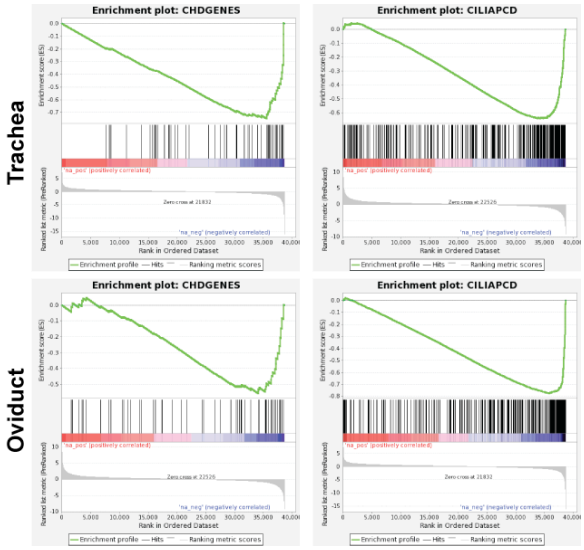


Appendix Figure S2. Examples of littermate animal size and hydrocephaly. A. Examples of 1 month old littermate animals of the specified sex and genotype (top row). B. Example of cranial swelling in a *Gemc1*$^{-/-}$ mouse with hydrocephaly compared to a littermate control. C. Additional examples of symmetrical hydrocephaly in coronal sections from 2 pairs of littermate animals.

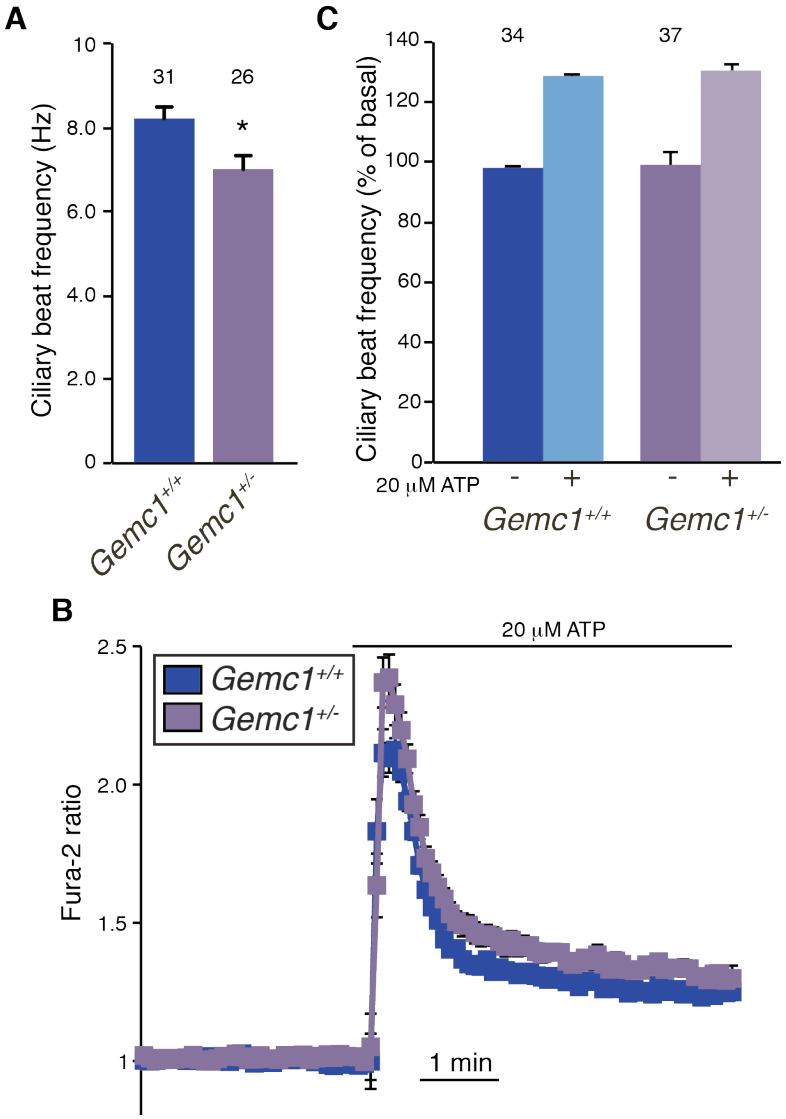


Appendix Figure S3. Detection of centrioles by TEM. Examples of undocked centrioles identified in tracheal sections from the indicated genotypes. Deuterosome mediated centriole expansion is not readily detected in the SC like cells of the *Gemc1*$^{-/-}$ trachea compared to MCCs from wild type mice (Fig 4C). Scale bars = 1 μm or 100 nm in the higher magnification insets.


Trachea
Oviduct
Enrichment plot: E2F4TARGETS
Enrichment plot: E2F4TARGETS_MCC
Enrichment plot: FOXJ1TARGETS
Enrichment plot: MYB_TARGETS
Enrichment plot: CILSPECGENES
Enrichment plot: CLARA
Enrichment plot: AT1
Enrichment plot: AT2
Enrichment plot: CILIOMEDB
Enrichment plot: SYSCILIA
Enrichment plot: SYSCILIAPRED
Enrichment plot: NONREDCILIO
Rank in Ordered Dataset
Enrichment score (ES)
Ranked list metric (PreRanked)
Enrichment profile
Hits
Ranking metric scores



Appendix Figure S4. Geneset enrichment analysis (GSEA) plots for each tissue generated from microarray data (Datasets EV1 and EV2). Genesets used are provided with their abbreviated name and associated references in Appendix Table S1.



Appendix Figure S5. Ca2+ and ciliary beat frequency (CBF) responses to ATP in ciliated tracheal cells. A. CBF was measured in *Gemc1*$^{+/+}$ and *Gemc1*$^{+/-}$ tracheal cultures (this was impossible in *Gemc1*$^{-/-}$ animals due to the lack of MCCs). Spontaneous CBF of ciliated cells obtained from *Gemc1*$^{+/-}$ mice was slightly lower than *Gemc1*$^{+/+}$ cells (measured at RT). The number of cilia recorded for each condition is shown above the graph. Mean CBF and SEM are plotted. * P<0.05 Student t-test. B. Time course of mean Ca2+ responses to 20 μM ATP in *Gemc1*$^{+/+}$ (n=70) and *Gemc1*$^{+/-}$ (n=27) ciliated tracheal cells. C. Mean normalized CBF response (% basal CBF) measured before and after 3 minutes in the presence of 20 μM ATP in *Gemc1*$^{+/+}$ and *Gemc1*$^{+/-}$ cells. No significant differences were observed between genotypes. Error bars indicate SEM.

Human *GMNC* 3'UTR

TGACCCCTCTTTTATCACAAAGCACTGCCACGAACTCTGTTTACAAAGACCTCTCTTGCACTTGAATTTGACTATGTGGA
ACACAGAAGCTGTTGCTTAGACTGTCTCCTGCCACTTTAAATGTCACTCTCAATTACCCACTTAAATCTGCAGGGAATGG
CTTCCAAGAATCCATAATGAAACGTGTCCGCGCTTCTTTTACCTAAATTTGACTTGTTTACACACCAATACCTGCTATTG
ACATCCTCTCTTTGCCCTATACTTGATAAGGTATTTTCCACTCACTTCTATATTCCTTTAAGACCTAGATACATTTTTCC
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TTCTGAAAACTGTCTTTAGAATGAGCCCTATTTTTGCTTCAAGTACTTGCTACTGCATGTTCAAATAGCTGAGGGTGGGG
TTGAAACAAGTGTCTTTGCCCATCACCACTACTCCTGAAGACATAATGTGGATGTGTGTAGCATTATGTTCATGGCAGTT
CAAAAAGTTCAGTGCCCCATAGTGGCATTGACAAAGCCTCTTAAAACAACTGTAACTCCACTAATAATCAATATCACAGA
TAAACTTTAAAATTACAAAGAATTTAAATTATTTTTAAATGCCTCATGATGTATCAGCTTCTATATGTACCCAGCATCAT
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CCAGAATAATTTGCACATCCTGCATGTGCACCTATGTTTTTTGGACAAACTTCAATTAATGATGAAATCTTTCATGTTGA
AACCTGTTCTCATTTTTCTCTTTTCCACATCTCAATAAGTCATCTTGCACAATCAATATCTCGTAGGTCAACTGTGTTGG
TTAGGATTCTTTGTAACAGAGACATGCCTTTCATTGTTGTTTCTGTTTGGTTGTTGCTTTATTTTGGTGATGTAAGCAAA
GGGTCTGTTTTATAAAGTTTTGTTGCCACCCACTTTTAAAAATAATTTATGAAGAAAATCCAATATAGTAATTAATATTA
CTCTCTGGAGTACTTTAAGAAGTAGAACACATTCCATTTTCTAAAAAATAAAATAAAATAAATAACTGTGCCTTTTACAT
TCTGGTATCTGGGCCCCTGGTCGGTGAAATTTGTCGCTTTTTAGAATAAGAGATTTAAGGATTCGAATTATTGTACTTTA
TGACACTAGGAATTGAGAAAGTAATATAACTAATTTTGAAAGTCTAATAATCAAGCTGTAAAAGGAAATGTATGAGTTAA
CAAATATTTATTTTGCATTCTTTGTGTGCCTTATCATGTGTTAAGTACCATGAGGGTACACCCAGGCTTAGCACAATGCA
TTTGGTATACAAAACCATGTAAAATGTCATAGCTTTTGATAGCTTTTAAATGAAACTTAATTTTGGCCCAGTTTAAAGAT
ATTACCGAACATTTGGGATAAAATGTGATGGTTTCCTGGCTTCTATCATGGACGATGTTATGGAAGACAATTACCACGTT
TTTGAAATCATGTCTATAAATATTTAATATCCATTTAAATTATGTATAGAAAATTATTTTGTTCATGTTTTAGAAATTAA
ACATTTCTTTCACCTGA

CPEB consensus motif (CPE)

Mir449/34 target site

Musashi binding element

Polyadenylation signal

Appendix Figure S6. Predicted 3'UTR regulatory sites in *GEMC1*: The 3'UTR sequence of *GEMC1 (GMNC)* was analyzed using UTRscan(Grillo et al, 2010), DIANA Tools(Paraskevopoulou et al, 2013) and the CPE translational group server(Pique et al, 2008) to identify putative regulatory regions.

Abbreviated name	***References***	***Notes***
E2F4 targets	(Lee et al, 2011)	ChIPseq of E2F4 targets in B-lymphoblastoid cell lines.
E2F4 targets _MCC	(Ma et al, 2014)	Expression of a dominant negative E2F4 in Xenopus.
FoxJ1 targets	(Choksi et al, 2014)	FoxJ1 overexpression in transgenic zebrafish.
Myb targets	(Zhao et al, 2011)	ChIPseq in myeloid progenitors
CHD genes	(Li et al, 2015)	Chemical mutation and exome sequencing in mice to identify congenital heart defects reveals a role for cilia.
Cilia genes PCD	(Geremek et al, 2011)	Bronchial biopsies of PCD patients identifies cilia related genes.
Cilspecgenes, Clara, AT1, AT2	(Treutlein et al, 2014)	Single cell sequencing to ID markers of MCCs and other cell types of the lung.
CILIOME-DB	(Berman et al, 2003; Blacque et al, 2005; Blacque et al, 2004; Bloodgood & Salomonsky, 1994; Chiang et al, 2006; Cole, 2003; Cuvillier et al, 2000; Dawe et al, 2005; Evans et al, 2006; Fan et al, 2004; Ferrante et al, 2006; Hildebrandt & Otto, 2005; Huang et al, 2004; Kamiya, 2002; Kovar et al, 2001; Kulaga et al, 2004; Kurvari et al, 1996; Low et al, 2006; Mahjoub et al, 2002; May et al, 2005; Mollet et al, 2005; Murayama et al, 2005; Nakamura et al, 1996; Nishimura et al, 2004; Nishimura et al, 2005; Norrander et al, 2000; Ou et al, 2005; Patel-King et al, 2004; Pedersen et al, 2003; Pfannenschmid et al, 2003; Ponting, 2006; Quarmby, 2000; Rupp & Porter, 2003; Smith et al, 2006; Stephens & Lemieux, 1999; Stoetzel et al, 2006; Streets et al, 2006; Swoboda et al, 2000; Williams & Nelsen, 1997; Yanagisawa & Kamiya, 2004; Yen et al, 2006)	Ciliome database compiled from multiple organisms.

SYSCILIA (Cilia gold standard) and SYSCILIAPRED (predicted cilia genes)	(Arnaiz et al, 2010; Arnaiz et al, 2009; Blacque et al, 2005; Boesger et al, 2009; Broadhead et al, 2006; Cao et al, 2006; Chen et al, 2006; Efimenko et al, 2005; Hamosh et al, 2005; Keller et al, 2005; Kilburn et al, 2007; Kim et al, 2010; Kubo et al, 2008; Laurencon et al, 2007; Liu et al, 2007; Mayer et al, 2009; Mayer et al, 2008; McClintock et al, 2008; Muller et al, 2010; Nogales-Cadenas et al, 2009; Ostrowski et al, 2002; Pazour et al, 2005; Reinders et al, 2006; Ross et al, 2007; Smith et al, 2005; Stolc et al, 2005; Stubbs et al, 2008; van Dam et al, 2013; Wigge et al, 1998)	SYSCILIA consortium validated list of cilia related genes and predicted cilia genes.
CILIOPATHIES	(Bachmann-Gagescu et al, 2015; Boon et al, 2014; Hamosh et al, 2005; Roosing et al, 2015; Shaheen et al, 2015; Wallmeier et al, 2014; Wheway et al, 2015)	Genes implicated in or screened for in human ciliopathies.
Nonredcilio	All sets and(Hoh et al, 2012)	

Appendix Table S1. Genesets used for GSEA analysis. Abbreviations complete references and hyperlink to source (where applicable). See Dataset EV2 for more details.

Appendix Materials and Methods

Phylogenetic analysis

To check the evolutionary relationships among different proteins (MCIDAS, GEMC1, and GEMININ) we screened for homologues in the representative proteomes database rp55(Chen et al, 2011) to account for diversity while reducing the noise. Full sequences were used for queries in iterative hidden Markov-based searches using HMMER3(Eddy, 2011) until convergence was reached. The best scoring hits were proteins annotated as GEMC1, MCIDAS, and GEMININ, and the common region was the coiled-coil region common to the three of them. Coiled-coil regions are a challenge for accurate detection of homology as they may provide highly significant e-values while not being homologues(Mistry et al, 2013). To alleviate this problem, in addition to standard searches, we also used a specific scoring matrix derived from coiled-coil regions(Surkont & Pereira-Leal, 2015). To identify potential problematic sequences we excluded those exhibiting biases of the same order of magnitude as the scores in further analyses (for more details, (Eddy, 2011)). The filtered sequences were aligned to the profile of the Geminin domain (PFAM PF07412), and were also aligned using MAFFT(Katoh & Standley, 2013). The resulting sequence alignments were very similar. To infer phylogenies we used probabilistic inference with PhyML(Guindon et al, 2009) where two models of evolution were compared, one being derived from coiled-coil regions(Surkont & Pereira-Leal, 2015), while the other was estimated from the sequence alignment using ProTtest(Abascal et al, 2005). Phylogenetic analyses were run in 16 nodes in a cluster under the mpi-PhyML version of the program. All the parameters were estimated from the model, and we ran 1000 bootstrap replicates in unrooted trees.

Southern blotting

Digoxigenin labelled probes for neomycin to screen for multiple integrants were prepared by PCR in which the standard dNTP mix is replaced by: dATP 1mM, dCTP 1mM, dGTP 1mM, dTTP 0.6 mM, DIG-11-dUTP 0.4 mM. (Roche). Probe was purified by ethanol precipitation and resuspended in TE. Probe was diluted to 10-20ng/ml in 10ml of hybridization solution (DIG Easy Hyb, Roche). Genomic DNA (5 μg) from selected ES cell clones was digested in PvuII (NEB) overnight and run on a 0.7% agarose gel. DNA was transferred to Zeta Probe GT membrane (Bio-Rad) by capillary alkaline transfer in 0.4N NaOH and the membrane was hybridized with DIG labelled probe overnight at 42°C following manufacturers recommendations. Following stringent washes, the probe was detected with CDP-star (Roche). (Primer seqs for NEO probe; Neo int fwd 1; CCATGGGATCGGCCATTGAAC, Neo int rev 1; CCATGGGTCACGACGAGATCATCG).

Long-range PCR

Long-range PCR reactions were performed using SequalPrep (Life Technologies) following the manufacturer recommendations. The following primers were used for the detection of the 5'homology arm: GF3 (CAATGAGTCCGGCACATTCTCAAC) and 5'ULAR3 (CACAACGGGTTCTTCTGTTAG), 3'homology arm: DloxPF (GAGATGGCGCAACGCAATTAA) and GR4 (CAATGACTTTCCACTAGGCTACACTAACAC), and the 3'loxP site: DloxPR (TGAACTGATGGCGAGCTCAGACC) and 3'diagfwd2 (TCATGCTGGAGTTCTTCGCCC).

Mouse genotyping

Genomic DNA was extracted from samples of mouse tail and lysed and digested with Proteinase-K (0.4 mg/ml in 10 mM Tris-HCl, 20 mM NaCl, 0.2% SDS, 0.5 mM EDTA) overnight at 56ºC. DNA was recovered by isopropanol precipitation, washed in 70% ethanol, dried and resuspended in 10 mM TE for use in PCR reactions. The following primers were used to detect the wild type and floxed (conditional) alleles: UF (GAGTTGGGAACTTGGCTGAGCC) and UR (TGAGAGAGCTGAGCAGCTCC), and the deleted allele: UF and DR (CTCTGAACTGTAAGGCAGCTCG). PCR reactions were

performed with Taq polymerase (Biotools) and run for 35 cycles with an annealing temperature of 58ºC.

Generation of inducible cell lines.

GEMC1 inducible expression systems were obtained transfecting U2OS-TRex™ cell line or T98G-derivative cell lines that stably express the Tet-repressor protein (Mailand and Diffley, 2005) with pcDNA4/TO vectors containing GFP-FLAG, GFP-FLAG-GEMC1 full length or GFP-FLAG-GEMC1 deletion mutants. Clones selection was obtained with Zeocin 100 µg/ml. U2OS-TRex™ were grown in cell media containing Tetracycline-free serum. Tetracycline at a final concentration of 1 µg/ml was added in order to induce protein expression.

Ciliary beat frequency

In order to test the effect of GEMC1 on ciliary activity we measured spontaneous CBF and the effects of ATP in *Gemc1*$^{+/+}$ and *Gemc1*$^{+/-}$ primary tracheal cultures using high-speed digital video microscopy as previously described(Lorenzo et al, 2008).

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