Subcellular localization of MC4R with ADCY3 at neuronal primary cilia underlies a common pathway for genetic predisposition to obesity

Jacqueline E. Siljee, Yi Wang, Adelaide A. Bernard, Baran A. Ersoy, Sumei Zhang, Aaron Marley, Mark Von Zastrow, Jeremy F. Reiter and Christian Vaisse

Most monogenic cases of obesity in humans have been linked to mutations in genes encoding members of the leptin-melanocortin pathway. Specifically, mutations in MC4R, the melanocortin-4 receptor gene, account for 3–5% of all severe obesity cases in humans. Recently, ADCY3 (adenylyl cyclase 3) gene mutations have been implicated in obesity. ADCY3 localizes to the primary cilia of neurons, organselles that function as hubs for select signaling pathways. Mutations that disrupt the functions of primary cilia cause ciliopathies, rare recessive pleiotropic diseases in which obesity is a cardinal manifestation. We demonstrate that MC4R colocalizes with ADCY3 at the primary cilia of a subset of hypothalamic neurons, that obesity-associated MC4R mutations impair ciliary localization and that inhibition of adenylyl cyclase signaling at the primary cilia of these neurons increases body weight. These data suggest that impaired signaling from the primary cilia of MC4R neurons is a common pathway underlying genetic causes of obesity in humans.

Most mammalian cells, including neurons, each possess a single, immotile primary cilium, an organelle that transduces select signals. Defects in the genesis or function of primary cilium cause a range of overlapping human diseases, collectively termed ciliopathies. Several ciliopathies, such as Bardet–Biedl syndrome and Alström syndrome, cause obesity, and mutations in genes encoding ciliary proteins, such as CEP19 and ANKRD26, cause nonsyndromic obesity in mice and humans. Although the mechanisms underlying a number of ciliopathy-associated phenotypes, such as polycystic kidney disease or retinal degeneration, have been at least partly elucidated, how ciliary dysfunction leads to obesity remains poorly understood. Ubiquitous ablation of the primary cilia of neurons in adult mice causes an increase in food intake and obesity, thus suggesting that ciliopathy-associated obesity involves the postdevelopmental disruption of anorexigenic neuronal signals. Recently, genetic and epigenetic studies have suggested a role of ADCY3 variations in human obesity, and loss-of-function mutations in Adcy3 in mice have been found to lead to a severe obesity phenotype. ADCY3, a member of the adenylyl cyclase family that mediates Gs signaling from G-protein-coupled receptors (GPCRs), is specifically expressed at the primary cilia of neurons.

The melanocortin 4 receptor (MC4R) is a Gs-coupled GPCR that transduces anorexigenic signals in the long-term regulation of energy homeostasis. Heterozygous mutations in MC4R are the most common monogenic cause of severe obesity in humans, and individuals with homozygous null mutations display severe early-onset obesity. Similarly, in mice, deletion of Mc4r causes severe obesity. MC4R is a central component of the melanocortin system, a hypothalamic network of neurons that integrates information about peripheral energy stores and that regulates food intake and energy expenditure. Despite being a major target for the pharmacotherapy of obesity, little is known about the subcellular localization of MC4R.

When expressed in unciliated heterologous cells, MC4R traffics to the cell membrane. However, in ciliated cells such as mouse embryonic fibroblasts (MEFs), retinal pigment epithelium (RPE) or inner-medullary collecting duct (IMCD3) cells, we found that a previously well-characterized, functional, C-terminally GFP-tagged MC4R (MC4R-GFP) localized to primary cilia (Fig. 1a). In a quantitative assay developed in IMCD3 cells, we found that the ciliary enrichment of MC4R was comparable to that of Smoothened (SMO), a known cilium-enriched protein, and was the strongest among members of the melanocortin-receptor family (Fig. 1b).

We set out to determine whether, and to what extent, MC4R localizes to primary cilia in mice. Most of the anorexigenic activity of MC4R is due to its function in a subset of single minded 1 (SIM1)-expressing neurons of the paraventricular nucleus of the hypothalamus (PVN), and all MC4R-expressing neurons in the PVN express SIM1 (ref. 2). Using a transgenic mouse line in which GFP was expressed in all SIM1-expressing neurons, we first investigated whether SIM1-expressing PVN neurons were ciliated. We found that adenylyl cyclase 3 (ADCY3)-positive primary cilia were present in most SIM1-expressing neurons of the PVN (Supplementary Fig. 1).

Previous attempts to determine the subcellular localization of MC4R in vivo in mice have been unsuccessful, owing to the small number of neurons in which it is expressed, its low abundance and the lack of tractable antibodies. To circumvent these limitations, we used Cas9-mediated recombination in mouse zygotes to insert a GFP tag in frame at the C terminus of the endogenous Mc4r locus (Fig. 2a). The MC4R-GFP/+ knock-in mice did not have an obvious energy-metabolism phenotype and were fertile, thus suggesting that the C-terminal GFP did not substantially impair the trafficking or function of MC4R in these mice. Confocal imaging of the PVN of these mice demonstrated that MC4R and ADCY3 colocalized to the primary cilia of a subset of PVN neurons (Fig. 2b–i).
If MC4R localization to primary cilia is essential for its function, we reasoned that human obesity-causing mutations in MC4R might impair its function by compromising its ciliary localization. Heterozygous MC4R mutations are the most common genetic cause of severe childhood obesity, and more than 50 obesity-associated mutations in MC4R have been described (Supplementary Fig. 2). Functional assessment of the effects of these mutations in nonciliated cells indicated that many of these mutations disrupt trafficking of the receptor to the membrane or impair ligand activation (Supplementary Fig. 2). In nonciliated HEK293 cells, eight obesity-associated MC4R mutant proteins (p.Arg7His, p.Gly231Ser, p.Arg236Cys, p.Leu250Gln, p.Ile130Thr) traffic normally to the cell membrane and respond normally to α-melanocyte-stimulating hormone (α-MSH) activation. To determine whether any of these mutants caused altered ciliary localization of MC4R, we quantified their ciliary enrichment in IMCD3 cells (Fig. 3a). We found that p.Pro230Leu and p.Arg236Cys significantly decreased MC4R ciliary localization. Notably, these two alterations are located...
in the third intracellular domain of MC4R (Supplementary Fig. 2), a domain previously implicated in the ciliary localization of other GPCRs.

To further determine whether the MC4R p.Pro230Leu alteration alters ciliary localization in vivo, we injected adeno-associated viruses (AAVs) expressing MC4R p.Pro230Leu–GFP and MC4R-GFP in a Cre-dependent fashion into Sim1-Cre transgenic mice (Fig. 3b–d). The human wild-type MC4R-GFP localized to primary cilia of Sim1-expressing PVN neurons (Fig. 3e–h), thus confirming that the human receptor also traffics to the cilia in vivo. In contrast, MC4R p.Pro230Leu–GFP did not colocalize with ADCY3 in primary cilia (Fig. 3i–l). Together, these results suggested that MC4R mutations...
may cause human obesity by altering the ciliary localization of the receptor.

If MC4R and ADCY3 function at the primary cilia to regulate body weight, we predicted that specific inhibition of adenylyl cyclase at the primary cilia of MC4R-expressing neurons should be sufficient to cause obesity. Specific inhibition of adenylyl cyclase at the primary cilia of neurons can be achieved by expression of a constitutively active version of the cilia-specific G\textsubscript{i}-protein-coupled receptor GPR88 (GPR88 p.Gly283His, denoted GPR88\textsuperscript{a}). GPR88\textsuperscript{a} was delivered to Sim1-expressing neurons of the PVN through the same approach used for the hMC4R-GFP double-floxed inverted orientation (DIO)-AAV (Fig. 3), but a high level of virus was delivered at the midline to ensure high coverage of PVN neurons (Supplementary Fig. 3). Because visualization of cilia expression requires confocal imaging, a DIO-AAV for mCherry expression was co-injected with the DIO-AAV for expression of a Flag-tagged version of GPR88\textsuperscript{a} to allow the accuracy of the targeting and the coverage of the PVN at the end of the experiment to be determined.
in each mouse (Supplementary Fig. 3). Weight-paired littermate mice injected with only DIO-AAV–mCherry were used as controls. After AAV injections, mice in which Flag-GPR88 was expressed at the primary cilia of Sim1-expressing PVN neurons increased their food intake and gained significantly more weight than did controls (Fig. 4), thus demonstrating that adenylyl cyclase signaling at the primary cilia of these neurons is essential for the regulation of body weight.

Together, our data suggest that impaired signaling from the primary cilia of MC4R-expressing neurons is a common pathway for syndromic and nonsyndromic causes of monogenic obesity in humans. Our data do not indicate, however, that primary cilia are necessary for Gs coupling and ADCY activation by MC4R, because these processes occur in nonciliated cells. Instead, our data suggest that this signaling must occur at the primary cilia, because impairing localization of MC4R at the primary cilia or inhibiting ADCY at the primary cilia impaired regulation of body weight. This functional link between MC4R and cilopathy-associated obesity parallels findings underlying other human cilopathy-associated phenotypes. For example, syndromic and nonsyndromic polycystic kidney disease is linked to impaired function of polycystin 1 and 2, proteins expressed at the primary cilia of renal tubular cells, whereas impaired function of rhodopsin in the anterior segment of retinal cells, a specialized primary ciliary, is a common pathway for both common and cilipathy-associated retinal phenotypes. Our findings also provide important new insights into the subcellular basis underlying the relationship between short-term regulation of food intake and long-term regulation of energy homeostasis. PVN MC4R-expressing neurons are part of a neuronal circuitry implicated in short-term control of feeding, because they receive synaptic gabanergic and glutamatergic inputs, particularly from synaptic inputs from the arcuate nucleus of the hypothalamus. MC4R itself, however, controls long-term energy homeostasis, as evidenced by the phenotype of MC4R-deficient mice or humans, and both MC4R ligands have a slower effect on food-intake regulation. In strong support of this model, a recent report has elegantly established that the PVN MC4R-expressing neurons receive fast-acting food-intake-regulating synaptic inputs from the arcuate nucleus that are postsynaptically modulated by MC4R through its slower-acting neuropeptide ligands α-MSH and AGRP.

Our finding that MC4R localizes to the primary cilia of MC4R PVN neurons provides for a subcellular compartmentalization of the slower signaling by the endogenous MC4R ligands, thus allowing for independent control of long-term energy homeostasis in neurons also implicated in the short-term regulation of food intake.

Methods

Methods, including statements of data availability and any associated accession codes and references, are available at https://doi.org/10.1038/s41588-017-0020-9.

Received: 13 June 2017; Accepted: 15 November 2017; Published online: 8 January 2018

References

1. Lubrano-Berthelier, C. et al. Melanocortin 4 receptor mutations in a large cohort of severely obese adults: prevalence, functional classification, genotype-phenotype relationship, and lack of association with binge eating. J. Clin. Endocrinol. Metab. 91, 1811–1818 (2006).
2. Vasse, C. et al. Melanocortin-4 receptor mutations are a frequent and heterogeneous cause of morbid obesity. J. Clin. Invest. 106, 253–262 (2000).
3. Vasse, C., Clement, K., Guy-Grand, B. & Froguel, P. A frameshift mutation in human MC4R is associated with a dominant form of obesity. Nat. Genet. 20, 113–114 (1998).
4. Stergioulas, E. et al. Genome-wide association study of height-adjusted BMI in childhood identifies functional variant in ADCYS5. Obesity (Silver Spring) 22, 2252–2259 (2014).
5. Wang, Z. et al. Adult type 3 adenylyl cyclase-deficient mice are obese. PLoS One 4, e6979 (2009).
6. Bishop, G. A., Berbari, N. F., Lewis, J. & Mykytyn, K. Type III adenylyl cyclase localizes to primary cilia throughout the adult mouse brain. J. Comp. Neurol. 505, 562–571 (2007).
7. Reiter, J. F. & Leroux, M. R. Genes and molecular pathways underpinning ciliopathies. Nat. Rev. Mol. Cell Biol. 18, 533–547 (2017).
8. Goetz, S. C. & Anderson, K. V. The primary cilium: a signalling centre during vertebrate development. Nat. Rev. Genet. 11, 331–344 (2010).
9. Green, J. A. & Mykytyn, K. Neuronal ciliary signaling in homeostasis and disease. Cell. Mol. Life Sci. 67, 3287–3297 (2010).
10. Hildebrandt, F., Benzing, T. & Katsanis, N. Ciliopathies. N. Engl. J. Med. 364, 1533–1543 (2011).
11. Vaisse, C., Reiter, J. F. & Berbari, N. F. Cilia and obesity. Cold Spring Harb. Perspect. Biol. 9, a028217 (2017).
12. Shalata, A. et al. Morbid obesity resulting from inactivation of the ciliary protein CEP19 in humans and mice. Am. J. Hum. Genet. 93, 1061–1071 (2013).
13. Acz, P. et al. A novel form of ciliopathy underlies hyperphagia and obesity in Ank2Δ6 knockout mice. Brain Struct. Funct. 220, 1512–1518 (2015).
14. Davenport, J. R. et al. Disruption of intraflagellar transport in adult mice leads to obesity and slow-onset cystic kidney disease. Curr. Biol. 17, 1586–1594 (2007).
15. Lis, L., Shen, C., Seed Ahmed, M., Ostenson, C. G. & Gu, H. F. Adenylyl cyclase 3: a new target for anti-obesity drug development. Obes. Rev. 17, 907–914 (2016).
16. Krashes, M. J., Lowell, B. B. & Garfield, A. S. Melanocortin-4 receptor-regulated energy homeostasis. Nat. Neurosci. 19, 206–219 (2016).
17. Huizar, D. et al. Targeted disruption of the melanocortin-4 receptor results in obesity in mice. Cell 88, 131–141 (1997).
18. Morton, G. J., Meek, T. H. & Schwartz, M. W. Neurobiology of food intake and disease. Nat. Rev. Neurosci. 15, 367–378 (2014).
19. Lubrano-Berthelier, C. et al. Intracellular retention is a common characteristic of childhood obesity-associated MC4R mutations. Hum. Mol. Genet. 12, 145–153 (2003).
20. Aanstad, P. et al. The extracellular domain of Smoothened regulates ciliary localization and is required for high-level Hh signaling. Curr. Biol. 19, 1034–1039 (2009).
21. Corbit, K. C. et al. Vertebrate Smoothened functions at the primary cilium. Nature 437, 1018–1021 (2005).
22. Balthasar, N. et al. Divergence of melanocortin pathways in the control of food intake and energy expenditure. Cell 123, 493–505 (2005).
23. Garfield, A. S. et al. A neural basis for melanocortin-4 receptor-regulated appetite. Nat. Neurosci. 18, 863–871 (2015).
24. Bromberg, Y., Overton, J., Vasse, C., Leibl, R. L. & Rost, B. In silico mutagenesis: a case study of the melanocortin 4 receptor. FASEB J. 23, 3059–3069 (2009).
25. Colton, M. et al. Association of functionally significant Melanocortin-4 but not Melanocortin-3 receptor mutations with severe adult obesity in a large North-American case control study. Hum. Mol. Genet. 18, 1140–1147 (2009).
26. Erosy, B. A. et al. Mechanism of N-terminal modulation of activity at the melanocortin-4 receptor GPCR. Nat. Chem. Biol. 8, 725–730 (2012).
27. Hinney, A. et al. Melanocortin-4 receptor gene: case-control study and transmission disequilibrium test confirm that functionally relevant mutations are compatible with a major gene effect for extreme obesity. J. Clin. Endocrinol. Metab. 88, 4258–4267 (2003).
28. Berbari, N. F., Johnson, A. D., Lewis, J. S., Askwith, C. C. & Mykytyn, K. Identification of ciliary localization sequences within the third intracellular loop of G protein-coupled receptors. Mol. Biol. Cell 15, 1540–1547 (2008).
29. Marley, A., Choy, R. W. & von Zastrow, M. GPR88 reveals a discrete function of primary cilia as selective insulators of GPCR cross-talk. PLoS One 8, e70857 (2013).
30. Fenselau, H. et al. A rapidly acting glutamatergic ARC–PVH satiety circuit postsynaptically regulated by α-MSH. Nat. Neurosci. 20, 42–51 (2017).
analyzed the data and wrote the paper. S.Z. performed experiments. A.A.B. performed experiments and analyzed data relevant to Fig. 4. B.A.E. conceived and performed experiments and analyzed data relevant to Fig. 2. A.M. and M.V.Z. contributed regents and expertise relevant to Fig. 4.

**Competing interests**
The authors declare no competing financial interests.

**Additional information**
Supplementary information is available for this paper at https://doi.org/10.1038/s41588-017-0020-9.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to C.V.

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.
For immunofluorescence staining, cells were fixed with 4% PFA for 20 min at 4 °C, according to the manufacturer's protocol. After reaching confluency, cells were (CCF) with 10% FBS (MEF). Effectene (Qiagen) was used for transfections (CCF, UCSF), 10% FBS, and 2 mM GlutaMAX (Gibco) (IMCD3) or DMEM 3% goat serum. Primary antibodies were added in binding buffer and incubated at 37 °C and 5% CO2 and cultured in 50% DMEM/50% Ham's F-12 nutrient mix MEFs (NIH/3T3 ATCC CRL-1658) and RPE (ATCC CRL-4000)) were maintained Studies in cell lines. Expression plasmids.

Methods

Quantification of ciliary localization in cultured cells. A three-plane z stack of transiently transfected IMCD3 cells was acquired on an Olympus IX-70 microscope, and the best focus of average was recorded in Metamorph software (Molecular Devices). Relative ciliary enhanchment was calculated in Matlab software as the ratio between the green-channel pixel intensity of GFP-chimera expression at the primary cilium versus the pixel intensity of the cell body, wherein the cilium was defined by acetylated-tubulin staining recorded in the red channel.

In vivo studies in mice. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco. Zygote injection and implantation were performed at the transgenic core of the Gladstone Institute.

Generation of Mc4r-GFP knock-in mice. Superovalated female FVB/N mice (4 weeks old) were mated to FVB/N stud males. Fertilized zygotes were collected for 1 h with secondary antibodies and then 5 min with Hoechst 33342 at room temperature, and were mounted in Opti-MEM (Life Technologies) for 24 h to induce ciliation. Plasmid-transfected cells were fixed and stained before imaging by confocal microscopy. For immunofluorescence staining, cells were fixed with 4% PFA for 20 min at 4 °C, permeabilized in binding buffer (10% BSA, 2% goat serum and 0.02% sodium azide in PBS) with 0.3% Triton X-100 (Sigma) and blocked in binding buffer with 3% goat serum. Primary antibodies were added in binding buffer and incubated overnight at 4 °C. Subsequently, cells were washed in PBS, incubated for 1 h with secondary antibodies and then for 5 min with Hoechst 33342 at room temperature, and were mounted in Prolong Gold (Life Technologies). GFP was detected with chicken polyclonal anti-GFP (Abcam, ab19790). Primary cilia were detected with mouse monoclonal anti-acetylated tubulin (Sigma, T7451). The secondary antibodies were goat anti-chicken Alexa Fluor 488 (Invitrogen, A11039) and goat anti-mouse Alexa Fluor 555 (Invitrogen), A21244. Nuclei were labeled with To-Pro3 (Invitrogen) or Hoechst 33342 (Invitrogen).

Microscopes. Imaging of transfected immortalized cells was performed on a Zeiss Upright Axioskope 2 Plus fluorescence microscope and/or on a Leica SL, a Leica SP5 or a Zeiss LSM 780 confocal microscope.

Immunofluorescence studies of mouse hypothalamic. Mice were perfused transcardially with PBS followed by 4% paraformaldehyde fixation solution. Brains were dissected and postfixed in fixation solution at 4 °C overnight, soaked in 30% sucrose solution overnight, embedded in O.C.T. (Tissue-tek, Sakura Finetek USA), frozen and cut into 35-μm coronal sections. After being washed, sections were incubated for 1 h (3% normal goat serum in PBS, 0.2% Triton X-100 and 0.2% sodium azide) then incubated with primary antibodies (chicken anti-GFP (Abcam, ab19790), rabbit anti-ADCY3 (Santa Cruz Biotechnology, sc-588) or mouse anti-Flag M1 (Sigma, F3100) overnight at 4 °C. Sections were extensively washed in PBS, and then incubated with secondary antibodies (goat anti-chicken Alexa Fluor 488 (Invitrogen, A11039), goat anti-mouse Alexa Fluor 488 (Invitrogen, A11001), or goat anti-rabbit Alexa Fluor 633 (Invitrogen, A21071)).

Image capture and processing. Whole-section images were generated with an ApoTome microscope. Confocal images were generated with a Zeiss LSM 780 confocal microscope. In confocal images, MC4R-GFP was labeled with Alexa 488, and the neuronal cilia marker ADCY3 was labeled with Alexa 647. Mice were tested over 96 continuous hours, and the data from the middle 48 h were analyzed.

In vivo studies in mice. Mice expressing Cre under the control of the Sim1-cre promoter, Tg(Sim1-EGFP)AX55Gsat, were obtained from the Mutant Mouse Repository. Tg(Sim1-EGFP)AX55Gsat, were obtained from the Gladstone Institute.

Generation and injection of AAVs. DIO-AAV–MC4R-GFP, DIO-AAV–MC4R p.Pro230Leu–GFP and DIO-AAV–GPR88* plasmids were generated by replacing hChR2(I343R)-EYFP in pAAV-Ef1a-DIO- hChR2(I343R)-EYFP-WPRE- pA (obtained from K. Deisseroth) with hMC4RGFP, P230LMc4RGFP or GPR88(G283H), respectively. AAV DJ were prepared and titrated by the Stanford Neuroscience Viral Core, which also provided the stock mCherry–DIO-AAV (GVVC-AAV-14).

DIO-AAV particles were injected in the PVN in Tg(Sim1-cre)Lowel mice to anatomically and genetically restrict expression to Sim1-expressing PVN neurons. For experiments presented in Fig. 3, 0.2 µl of DIO-AAV–MC4R-GFP or DIO-AAV–MC4R p.Pro230Leu–GFP was stereotactically injected unilaterally in the PVN (coordinates: AP = −0.8, ML = 0.2, DV = −5.2). Mice were sacrificed 7 d after the injections. For experiments presented in Fig. 4 (and Supplementary Fig. 3), DIO-AAV–mCherry with or without DIO-AAV–Flag-GPR88* was stereotactically injected in 1 µl at the midline just above the third ventricle (coordinates: AP = −0.8, ML = 0.0, DV = −5.2). Weight was measured for 2 months, after which the mice were sacrificed to confirm the site of injection. Mice with missed injections were excluded before data analysis. mCherry expression was assessed in all mice through wide-field microscopy to verify the accuracy and extent of the AAV injection and GPR88* expression, because in mice injected with both DIO-AAV–mCherry and DIO-AAV–Flag-GPR88*, neurons were infected with both viruses (Supplementary Fig. 3).

Mouse metabolism studies. For experiments presented in Fig. 4 (and Supplementary Fig. 3), mice were single housed after AAV injections. Weight was measured for 2 months. Food intake was measured with a CLAMS system (Columbus Instruments) at baseline and 6 weeks after AAV injections. Mice were tested over 96 continuous hours, and the data from the middle 48 h were analyzed.
Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

### Experimental design

1. **Sample size**
   - Describe how sample size was determined.
   - Sample sizes were chosen based upon the estimated effect size drawn from previous publications and from the performed experiments.

2. **Data exclusions**
   - Describe any data exclusions.
   - No data points were excluded in analysis.

3. **Replication**
   - Describe whether the experimental findings were reliably reproduced.
   - All attempts at replication were successful (details are indicated on figures/results).

4. **Randomization**
   - Describe how samples/organisms/participants were allocated into experimental groups.
   - All mice were paired at baseline by body weights and litters.

5. **Blinding**
   - Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   - NA

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. **Statistical parameters**
   - For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a | Confirmed
   --- | ---
   X | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   X | A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   X | A statement indicating how many times each experiment was replicated
   X | The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   X | A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   X | The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   X | A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   X | Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study. Metamorph/Excel/Prism 7

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company. No restrictions

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species). All antibodies are used and validated in published papers.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used. Cell line were initially purchased from ATCC

b. Describe the method of cell line authentication used. No additional authentication used

c. Report whether the cell lines were tested for mycoplasma contamination. Cells were not tested for mycoplasma contamination but such contamination would not have allowed for proper ciliation

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use. No commonly misidentified cell lines were used

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study. Mice were used. All information regarding sex, strains, origin of the animals and genotypes are indicated in the result, figures or method section of the manuscript

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants. Provide all relevant information on human research participants, such as age, gender, genotypic information, past and current diagnosis and treatment categories, etc. OR state that the study did not involve human research participants.