Origin and function of stomata in the moss *Physcomitrella patens*

Caspar C. Chater1, Robert S. Caine2, Marta Tomek3, Simon Wallace4, Yasuko Kamisugi5, Andrew C. Cuming5, Daniel Lang3, Cora A. MacAlister6, Stuart Casson7, Dominique C. Bergmann8, Eva L. Decker3, Wolfgang Frank3, Julie E. Gray7, Andrew Fleming2, Ralf Reski3,10* and David J. Beerling2*

Stomata are microscopic valves on plant surfaces that originated over 400 million years (Myr) ago and facilitated the greening of Earth’s continents by permitting efficient shoot-atmosphere gas exchange and plant hydration1. However, the core genetic machinery regulating stomatal development in non-vascular land plants is poorly understood2-4 and their function has remained a matter of debate for a century5. Here, we show that genes encoding the two basic helix-loop-helix proteins PpSMF1 (SPEECH, MUTE and FAMA-like) and PpSCREAM1 (SCRM1) in the moss *Physcomitrella patens* are orthologous to transcriptional regulators of stomatal development in the flowering plant *Arabidopsis thaliana* and essential for stomata formation in moss. Targeted *P. patens* knockout mutants lacking either PpSMF1 or PpSCRM1 develop gametophytes indistinguishable from wild-type plants but mutant sporophytes lack stomata. Protein-protein interaction assays reveal heterodimerization between PpSMF1 and PpSCRM1, which, together with moss-angiosperm gene complementations, suggests deep functional conservation of the heterodimeric SMF1 and SCRM1 unit is required to activate transcription for moss stomatal development, as in *A. thaliana*6. Moreover, stomata-less sporophytes of ΔpPpSMF1 and ΔpPpSCRM1 mutants exhibited delayed dehiscence, implying stomata might have promoted dehiscence in the first complex land-plant sporophytes.

Colonization of terrestrial environments by green plants approximately 500 Myr ago established the basis for the emergence of complex land-based ecosystems that fundamentally transformed the biogeochemical cycling of carbon, water and energy7,8. Fossils suggest stomata originated on the small leaves of the early phylogenetic inference robustly suggests that PpSMF1 and PpSCRM2 are co-orthologous to *AfFAMA* which, in *Arabidopsis*, is essential for guard cell fate. Both analyses robustly reject a (co-)orthologous relationship of the SMF genes in *Physcomitrella* and *Selaginella* with the MUTE/SPCH clade, as suggested by our earlier phylogenetic analysis6. Reasoning that genes encoding stomatal regulators would be preferentially expressed in the stomata-bearing sporophyte, we interrogated microarray datasets20 and *P. patens* transcriptome atlas results21 that identified PpSMF1, PpSMF2 and PpSCRM1 as strong candidates because of their upregulation in the sporophyte relative to protonemal tissue, as supported by quantitative polymerase chain reaction with reverse transcription.

1Departamento de Biología Molecular de Plantas, Instituto de Biotecnología, Universidad Nacional Autónoma de Mexico, Cuernavaca 62210, Mexico.
2Department of Animal and Plant Sciences, University of Sheffield, Sheffield S10 2TN, UK.
3Plant Biotechnology, Faculty of Biology, University of Freiburg, Schaeznestraße 1, 79104 Freiburg, Germany.
4Royal College of Veterinary Surgeons, Belgravia House, 62–64 Horseferry Road, London SW1P 2AF, UK.
5Centre for Plant Sciences, University of Leeds, Leeds LS2 9JT, UK.
6Department of Molecular Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan 48109-1048, USA.
7Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, UK.
8HHMI and Department of Biology, Stanford University, Stanford, California 94305-5020, USA.
9Plant Molecular Cell Biology, Faculty of Biology, Ludwig-Maximilians-Universität München, LMU Biocenter, Großhaderner Straße 2, 82152 Planegg-Martinsried, Germany.
10BIOS – Centre for Biological Signalling Studies, 79104 Freiburg, Germany. These authors contributed equally to this work. e-mail: ralf.reski@biologie.uni-freiburg.de; d.j.beerling@sheffield.ac.uk

© 2016 Macmillan Publishers Limited, part of Springer Nature. All rights reserved.
Figure 1 | The genome of the moss *P. patens* encodes orthologues of the bHLH transcription factors regulating stomatal development in flowering plants.

**a**, Image of a developing *P. patens* sporophyte; the arrow indicates the region of stomatal placement. **b**, Stacked image of an excised sporophyte with stomata (orange/brown pores) forming a ring around the capsule base. Scale bar, 100 µm. **c**, Close-up of the sporophyte epidermis with single-celled guard cells and central pores. Scale bar, 25 µm. **d**, Bootstrapped maximum-likelihood phylogenies of the SMF gene family comprising the FAMA, SPCH and MUTE subfamilies and the SCRM/ICE gene family in sequenced land plants. Internal node names in red indicate inferred subfamily ancestry. Internal nodes are coloured to indicate either duplication (red), speciation (green) or haplotype (blue) origin of the descendant nodes. Edge values represent bootstrap values. External node names comprise species abbreviations, original accession codes of the protein sequences (available at [https://phytozome.jgi.doe.gov/pz/portal.html](https://phytozome.jgi.doe.gov/pz/portal.html)) and accepted gene names of experimentally studied representatives in red. Species abbreviations in five-letter code: *Arabidopsis thaliana*, *Populus trichocarpa*, *Oryza sativa*, *Sorghum bicolor*, *Selaginella moellendorfii* and *Physcomitrella patens*. **f–h**, Relative expression of *PpSMF1*, *PpSMF2* and *PpSCRM1* in the developing sporophyte (grey bars) and protonema tissue (black bars) analysed by qRT–PCR. Error bars indicate standard error of the mean (s.e.m.). Three replicates per tissue type were used.
transcription (qRT–PCR) (Fig. 1f–i; Supplementary Figs 1–2). Additionally, PpSCRM1 is the most highly expressed of the four PpSCRM paralogues across P. patens tissues including developing sporophytes23 (Supplementary Fig. 2). Based on these analyses, we investigated the role of PpSMF1, PpSMF2 and PpSCRM1 in regulating stomatal formation in P. patens by generating targeted gene deletion mutants using homologous recombination. Altogether, we generated two independent knockout lines for each of PpSMF1, PpSMF2 and PpSCRM1. Flow cytometry analyses verified gametophytes of all the mutants were haploid, as in the wild-type, and not polyploid transformants (Supplementary Fig. 3).

Stomata of P. patens form exclusively during the sporophyte stage of the life cycle (Fig. 1a) and are restricted to a small area around the capsule base (Fig. 1b). P. patens lacks the early meristematic lineage for stomata seen in A. thaliana. Instead, the formation of a cell equivalent to a guard mother cell (GMC) is specified3 which, in common with the closely related Funaria hygrometrica23, appears to undergo an incomplete symmetric division leading to the formation of a single guard cell and a central pore (Fig. 1c). Strikingly, in both ΔPpSMF1 and ΔPpSCRM1 mutant lines, the stomatal developmental programme is halted, resulting in no mature guard cells. Instead, only pavement-like cells develop and in ΔPpSCRM1 very occasionally cells form that enter the stomal lineage but fail to mature into stomata (Fig. 2a,b). In contrast, ΔPpSMF2 mutants develop normal wild-type stomata (Fig. 2a,b). We confirmed integration of the transgenes at the targeted loci and verified absence of gene expression in all mutant lines using genomic PCR and RT–PCR. (Fig. 2c; Supplementary Figs 4–6). Closer anatomical inspection revealed a correlation between the presence of stomata and of sub-stomatal cavities, pointing to functional stomata: sectioning of sporophytes revealed loss of stomata in ΔPpSMF1 and ΔPpSCRM1 was accompanied by the loss of sub-stomatal cavities, whereas in the wild type and in ΔPpSMF2 stomata and sub-stomatal cavities were present (Supplementary Fig. 7). We found no differences in sporophyte sizes between the different mutants and wild-type lines (Supplementary Fig. 8). These results establish PpSMF1 and PpSCRM1, but not PpSMF2, as essential for the formation of stomata in P. patens. Our targeted knockout results are independently supported by cross-species gene complementation studies in which PpSMF1, but not PpSMF2, partially complemented A. thaliana mule and fama mutants6. Taken together, these data strengthen our hypothesis that a single ancestral PpSMF1-like gene and a SCRm partner were responsible for stomatal development in early land plants.

Because group Ia bHLH proteins are obligate heterodimers with group III bHLHs in A. thaliana, we next used bimolecular fluorescence complementation (BiFC) assays7 and yeast two-hybrid (Y2H) experiments14 to determine direct protein–protein interactions between PpSMF1 and PpSCRM1 in vivo. Transient co-expression of PpSMF1::YPFPn and PpSCRM1::YFPc, as well as PpSMF1::YFPc and PpSCRM1::YPFPn, resulted in strong yellow fluorescent protein (YFP) fluorescence in the nuclei of Allium cepa cells, whereas no YFP fluorescence was detected in controls (Fig. 3a; Supplementary Fig. 9). Specific interaction of PpSMF1 and PpSCRM1 was also demonstrated by Y2H experiments. PpSMF1 and PpSCRM1 fused with Gal4-DB alone showed no transcriptional activation, but strong activation was observed by using PpSMF1 as bait and PpSCRM1 as prey (Fig. 3b–d). These results support PpSMF1 and PpSCRM1 as physically interacting heterodimeric partners. Furthermore, their nuclear localization is consistent with a role as DNA-binding transcription factors, reinforcing functional orthology to the A. thaliana group Ia and IIb bHLHs, respectively.

The BiFC and Y2H results suggest that PpSMF1–PpSCRM1 heterodimerization could occur in P. patens cells because of highly conserved protein–protein interactions. In silico analysis of the putative key domains involved in DNA binding during heterodimerization suggests that an E-box binding domain (EBD) in PpSMF1 and PpSMF2, a corresponding DNA binding domain in PpSCRM1 and coiled-coil domains in both peptides are conserved between P. patens and A. thaliana (Supplementary Fig. 10). However, PpSMF2 expression is very low compared with PpSMF1 and it is therefore unsurprising there is no aberrant phenotype in ΔPpSMF2 mutants despite key regulatory motifs being present (Supplementary Fig. 9).

Conservation of functional motifs of PpSMF1 and PpSCRM1, which are both strongly expressed in the sporophyte23, taken together with our experimental data (Figs 1–3), suggests that a heterodimeric bHLH partnership first existed in the ancestor of mosses and flowering plants which could both initiate and complete stomatal development.

Having produced mosses with stomata-less sporophytes, we next addressed the longstanding mystery relating to stomatal function in an early diverging non-vascular land plant lineage26,27. Current opinion suggests moss stomata facilitate nutrient and water transport and gas exchange in the developing sporophyte26. Current opinion suggests moss stomata facilitate nutrient and water transport and gas exchange in the developing sporophyte26. Current opinion suggests moss stomata facilitate nutrient and water transport and gas exchange in the developing sporophyte26,27 and also assist dehiscence and release of spores during sporophyte maturation24, when pores become less able to close. We tested the function of stomata in P. patens in this context by tracking the development and subsequent dehiscence of the sporophytes in the wild type and mutants (Fig. 4). The absence of stomata had no effect on spore development, morphology or viability in lines of ΔPpSMF1 and ΔPpSCRM1 as determined using scanning electron microscopy and brightfield microscopy and spore germination assays, respectively (Supplementary Figs 11 and 12). In contrast, observations of sporophyte development over time indicated that stomata-less ΔPpSMF1 and ΔPpSCRM1 mutants showed significantly (P < 0.01) delayed capsule dehiscence relative to the wild type during the late stages of development, as measured by the percentage of open capsules and timing of dehiscence (Fig. 4; Supplementary Figs 13–14). Although the reduced sporophyte of Physcomitrella is different from that of larger complex mosses, such as Funaria, our data suggest stomata during late-stage sporophyte development may function in a similar manner, aiding capsule dehiscence26. Intriguingly, delayed sporophyte dehiscence in P. patens seems to be decoupled from the browning of the sporophyte capsule, which is commonly assumed to be an indicator of capsule and spore maturation. As indicated by our quantitative analysis of the transition of capsule colouring (Supplementary Fig. 15), ΔPpSMF1 capsules did not reveal any significant deviation from the wild type. However, in young green sporophytes of P. patens, and F. hygrometrica, stomata open and close in response to cues, such as light and abscisic acid, through molecular pathways co-opted from the gametophyte27,30, suggesting gas exchange functionality. A complex picture of stomatal function in early land plant lineage sporophytes is therefore emerging relating to age, and possibly environmental conditions, but with stomatal action ultimately linked to reproductive success.

We propose that an ancestral land plant possessed a multifunctional ancestral dimer, comprising ancient variants of PpSMF1 and PpSCRM1, which was sufficient to initiate and drive stomatal development in the early sporophyte. Specifically, results from our experiments with knockout mutants in the moss P. patens, belonging to an extant lineage of non-vascular land plants with stomata, and our protein–protein interaction evidence support the notion that a FAMA-like and SCRm-like bHLH partnership was responsible for the origin of stomata in the earliest vascular land plants over 400 Myr ago. Remarkably, the origin of this genetic system that gave rise to stomata, together with those for roots10,11 and leafy shoots12, ultimately helped facilitate the evolutionary radiation of plants on land leading to increases in terrestrial ecosystem complexity and primary production13,31 that supported a burgeoning diversity of life on the continents.
Figure 2 | PpSMF1 and PpSCRM1 are required for stomatal development in the moss *P. patens*. a, Stacked ultraviolet fluorescence images (upper panel), scanning electron microscope images (middle panel) and bright-field images (bottom panel) showing the spore capsule base and epidermal close-ups from *P. patens* wild-type (WT), ΔPpSMF1, ΔPpSMF2 and ΔPpSCRM1 knockout mutants, respectively. The top panel wild-type representative is from the Villersexel K3 ecotype of *P. patens*, the middle panel wild-type representative is from the Gransden D12 ecotype and the bottom panel wild-type representative relates to the Gransden 2004 ecotype. There were no discernible differences between the sporophytes of the different background lines. For both of the ΔPpSCRM1 lines generated, we observed one such instance of aborted stomata (see bottom right panel) in the seven capsules of each line surveyed. Scale bars, 50 µm (top and middle panels); 15 µm (bottom panel). b, Number of stomata formed per sporophyte in two independent lines of each genotype versus wild-type controls. Error bars indicate 1 s.e.m. for ΔPpSMF1 and ΔPpSCRM1 and the corresponding wild types, seven capsules of each line were analysed. For ΔPpSMF2 and wild-type background, five capsules were surveyed. A one-way ANOVA was performed to test for differences between the wild-type and ΔPpSMF2 lines and no significant differences (denoted ns) were found. c, RT-PCR to confirm loss of the respective transcript in each of the *P. patens* knockout lines (top panel). A Rubisco (RBCS) control was run to verify the integrity of the produced cDNA (bottom panel). For labelling purposes, the wild types Villersexel K3, Gransden D12 and Gransden 2004 are denoted Vx, GrD12 and Gr04. For PpSMF2, two bands were amplified in the control for which the smaller 239 bp product represents the size expected for PpSMF2.
Figure 3 | BiFC and yeast two-hybrid assays demonstrating PpSMF1 and PpSCRM1 protein-protein interactions. a, Representative bright-field, fluorescence and overlay/merged images of BiFC analysis showing pairwise combinations of bHLH constructs, each fused with a complementary N- or C-terminal part of the YFP molecule (YFPn and YFPc fusions, respectively). In the intact A. cepa epidermis, using BiFC, PpSMF1 and PpSCRM1 showed strong heterodimerization in the nuclei. Controls are described in Supplementary Fig. 9. Scale bars, 100 μm. b,c Yeast two-hybrid analysis showing growth on minimal medium (b) and growth on stringent-selection medium (c). Blue indicates reporter activation. d, Key to patch plate assays shown in b,c.
protocol. The resulting cDNA was used for PCR amplification (35–40 cycles) (Supplementary Table 1 for primers). At the end of the PCR program, samples were loaded into wells for agarose gel (1% w/v) electrophoresis and visualized by a UVitec digital camera. Primer sequences were designed and selected using Primer3 (http://frodo.wi.mit.edu/primer3/).

Molecular analysis. Three replicates of 7 day old protonemata grown on BCDAT, and three replicates of peat-pellet-derived sporophytes were harvested from two peat-pellets per replicate to generate sufficient RNA (approx. 300 capsules per replicate). RNA was extracted and processed using the above-described methods. Before DNase treatment and cDNA synthesis, the quality of this RNA was assessed using the Nanodrop (ThermoScientific) to ensure the same amount of RNA in all replicates prior to downstream applications. Relative qRT-PCR was performed using the Rotor-Gene SYBR Green PCR Kit (400 ng) on a Corbett Rotor Gene 6000 (Qiagen) following the manufacturer’s protocols. Relative quantification was performed by normalizing the take-off value and amplification efficiency of the genes analysed relative to three housekeeping genes

Microscopic analysis. For epidermal phenotyping, five to seven mature sporophytes of each line, and the corresponding wild type, were removed from individual peat-pellet-grown gametophores beneath a Leica MZFLIII stereomicroscope. Capsules were stored in a modified Carnoy’s solution (2:1 ethanol:acetic acid) for a period of 2 weeks before dissection. Dissected sporophytes were viewed with an Olympus BX51 microscope and photomicrographs taken using an Olympus D717 camera. Images were analysed using ImageJ software.

Sporophyte maturation and dehiscence. Gametophores were cultivated from sporophytes on agar plates with Knop medium including microelements26. Individual 3 week old colonies were identified and transferred to Knop plates. Between eight and ten plants were isolated per plate and generating at least five plates per line. Plates were sealed with 7/8 of Parafoil and 1/8 of Micropore film and grown under long day conditions at 25 °C. After 5 weeks, plates were transferred into climate cabinets with short day conditions at 15 °C, sealed with Parafoil and grown for 4 weeks until formation of gametangia. Fertilization was initialized by soaking plates with sterilized water (re-closed with Parafoil), re-opening the plates after 5 days to remove the water, rescaling with Micropore film and then cultured for 3–6 weeks at 15 °C short-day conditions. Developing sporophytes were recorded and traced by marking and numbering them on the plate lids as they appeared.

Data availability. All moss mutants described here were deposited in the International Moss Stock Center IMSC (Supplementary Table 2).
14. Horst, N. A. et al. A single homeobox gene triggers phase transition, embryogenesis and asexual reproduction. Nat. Plants 2, 15209 (2016).
15. Raven, J. A. Selection pressures on stomatal evolution. New Phytol. 153, 371–386 (2002).
16. MacAlister, C. A., Ohashi-Ito, K. & Bergmann, D. C. Transcription factor control of asymmetric cell divisions that establish the stomatal lineage. Nature 445, 537–540 (2007).
17. Chinnusamy, V. et al. ICE1: a regulator of cold-induced transcription and freezing tolerance in Arabidopsis. Genes Dev. 17, 1043–1054 (2003).
18. Olsen, J. L. et al. The genome of the seagrass Zostera marina reveals angiosperm adaptation to the sea. Nature 530, 331–335 (2016).
19. Ran, J.-H., Shen, T.-T., Liu, W.-J. & Wang, X.-Q. Evolution of the bHLH genes involved in stomatal development: implications for the expansion of developmental complexity of stomata in land plants. PLoS ONE 8, e78997 (2013).
20. O'Donoghue, M. T. et al. Genome-wide transcriptomic analysis of the sporophyte of the moss Physcomitrella patens. J. Exp. Bot. 64, 3567–3581 (2013).
21. Ortiz-Ramirez, C. et al. A transcriptome atlas of Physcomitrella patens provides insights into the evolution and development of land plants. Mol. Plant 9, 205–220 (2016).
22. Sack, F. D. & Paolillo, D. J. Incomplete cytokinesis in Funaria. Am. J. Bot. 72, 1325–1333 (1985).
23. Weinithal, D. & Tafira, T. Imaging protein-protein interactions in plant cells by bimolecular fluorescence complementation assay. Trends Plant Sci. 14, 59–63 (2009).
24. Ito, T. et al. A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc. Natl Acad. Sci. USA 98, 4569–4574 (2001).
25. Haig, D. Filial mistletoes: the functional morphology of moss sporophytes. Ann. Bot. 111, 337–345 (2013).
26. Merced, A. & Renzaglia, K. S. Moss stomata in highly elaborated Oedipodium (Oedipodiaceae) and highly reduced Ephemerum (Pottiaceae) sporophytes are remarkably similar. Am. J. Bot. 100, 2318–2327 (2013).
27. Chater, C. et al. Regulatory mechanism controlling stomatal behavior conserved across 400 million years of land plant evolution. Curr. Biol. 21, 1025–1029 (2011).
28. Garner, D. L. B. & Paolillo, D. J. J. On the functioning of stomates in Funaria. Bryologist 76, 423–427 (1973).
29. Merced, A. & Renzaglia, K. S. Patterning of stomata in the moss Funaria: a simple way to space guard cells. Ann. Bot. 117, 985–994 (2016).
30. Lind, C. et al. Stomatal guard cells co-opted an ancient ABA-dependent desiccation survival system to regulate stomatal closure. Curr. Biol. 25, 928–935 (2015).
31. Franks, P. J. & Beerling, D. J. Maximum leaf conductance driven by CO2 effects on stomatal size and density over geologic time. Proc. Natl Acad. Sci. USA 106, 10343–10347 (2009).
32. Rensing, S. A. et al. The Physcomitrella genome reveals evolutionary insights into the conquest of land by plants. Science 319, 64–69 (2008).
33. Cove, D. The moss, Physcomitrella patens. J. Plant Growth Regul. 19, 275–283 (2000).
34. Wallace, S. et al. Conservation of Male Sterility 2 function during spore and pollen wall development supports an evolutionarily early recruitment of a core component in the sporopollenin biosynthetic pathway. New Phytol. 205, 390–401 (2015).
35. Reki, R. & Abel, W. O. Induction of budding on chloronemata and caulonemata of the moss, Physcomitrella patens, using isopentenyladenine. Planta 165, 354–358 (1985).
36. Egener, T. et al. High frequency of phenotypic deviations in Physcomitrella patens plants transformed with a gene-disruption library. BMC Plant Biol. 2, 6 (2002).
37. Frank, W., Ratnadewi, D. & Reki, R. Physcomitrella patens is highly tolerant against drought, salt and osmotic stress. Planta 220, 384–394 (2005).
38. Hohe, A., Rensing, S. A., Mildner, M., Lang, D. & Reki, R. Day length and temperature strongly influence sexual reproduction and expression of a novel MADS-box gene in the moss Physcomitrella patens. Plant Biol. 4, 595–602 (2002).
39. Hohe, A. et al. An improved and highly standardised transformation procedure allows efficient production of single and multiple targeted gene-knockouts in a moss, Physcomitrella patens. Curr. Genet. 44, 339–347 (2004).
40. Kamisugi, Y., Canning, A. C. & Cove, D. J. Parameters determining the efficiency of gene targeting in the moss Physcomitrella patens. Nucleic Acids Res. 33, 10 (2005).
41. Luna, E. et al. Plant perception of β-aminobutyric acid is mediated by an aspartyl-tRNA synthetase. Nat. Chem. Biol. 10, 450–456 (2014).

Acknowledgements
We thank R. Haas and T. Fulton for excellent technical assistance. R.S.C. was supported by a NERC studentship. D.C.B is a GBMF investigator of the Howard Hughes Medical Institute. D.J.B. acknowledges funding through an ERC Advanced Grant (CDREG, 322998). R.R. acknowledges funding through the Excellence Initiative of the German Federal and States Governments (EXC294). A.C.C. and Y.K. acknowledge support from BBSRC (Grant numbers BB/F001797/1 and BB/R006710/1).

Author contributions
C.C.C., R.S.C., W.F., J.E.G., A.F., D.J.B. and R.R. designed the study, C.C.C., R.S.C., D.L. and W.F. contributed materials and advice. A.C.C. constructed vectors C.C.C., R.S.C., D.L. and R.J.E.G. addressed to R.R. and D.J.B. Hybrids were produced by R.S.C. and R.R. were carried out by K.Y. and J.E.G. DNA restriction fragment length polymorphism (RFLP) and Southern blot hybridization of the knockout mutant lines. D.J.B., R.R. and S.C. carried out the paper version of the manuscript. D.J.B. acknowledges funding through an ERC Advanced Grant (CDREG, 322998). R.R. acknowledges funding through the Excellence Initiative of the German Federal and States Governments (EXC294). A.C.C. and Y.K. acknowledge support from BBSRC (Grant numbers BB/F001797/1 and BB/R006710/1).

Additional information
Supplementary information is available for this paper. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to R.R. and D.J.B.

Competing interests
The authors declare no competing financial interests.