exons to ensure that the PCR product represents the specific messenger RNA species and not genomic DNA. PCR was performed using Taq DNA polymerase (Eppendorf). The DNA sequence of the primers is summarized in Supplementary Information.

DNA ploidy and polymorphism

DNA contents per cell were determined by staining cells with propidium iodide and subsequent FACS analysis.

Genomic DNA were extracted from embryonic stem cells, bone marrow cells, and BMESL cells. DNA was amplified using microsatellite primers (D9MIT48, Research Genetics) detecting polymorphisms between the bone marrow genome (mostly C57BL/6) and the embryonic stem cell genome (129/Sv), separated on 5% agarose gel and visualized by ethidium bromide staining.

Received 17 January; accepted 27 February 2002.
Published online 13 March 2002, DOI 10.1038/nature730.

1. Weissman, I. L. Translating stem and progenitor cell biology to the clinic: barriers and opportunities. *Science* **287,** 1442–1446 (2000).
2. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. & Campbell, K. H. Viable offspring derived from fetal and adult mammalian cells. *Nature* **385,** 810–813 (1997).
3. Clarke, D. L. *et al.* Generalized potential of adult neural stem cells. *Science* **288,** 1660–1663 (2000).
4. Pittenger, M. F. *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284,** 143–147 (1998).
5. Ferrari, G. *et al.* Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279,** 1528–1530 (1998).
6. Petersen, B. E. *et al.* Bone marrow as a potential source of hepatic oval cells. *Science* **284,** 1168–1170 (1999).
7. Theise, N. D. *et al.* Liver from bone marrow in human. *Hepatology* **32,** 11–16 (2000).
8. Lagasse, E. *et al.* Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. *Nature Med.* **6,** 1229–1234 (2000).
9. Brazelton, T. R., Rossi, F. M. V., Keshet, G. I. & Blau, H. M. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290,** 1775–1779 (2000).
10. Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. & McKercher, S. R. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science* **290,** 1779–1782 (2000).
11. Krause, D. S. *et al.* Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105,** 369–377 (2001).
12. Hadjantonakis, A. K., Gertsenstein, M., Ikawa, M., Okabe, M. & Nagy, A. Generating green fluorescent mice by germline transmission of green fluorescent ES cells. *Mech. Dev.* **76,** 79–90 (1998).
13. Kawasome, H. *et al.* Targeted disruption of $p70^{s6k}$ defines its role in protein synthesis and rapamycin sensitivity. *Proc. Natl Acad. Sci. USA* **95,** 5033–5038 (1998).
14. Minamino, T. *et al.* MEKK1 suppresses oxidative stress-induced apoptosis of embryonic stem cell-derived cardiac myocytes. *Proc. Natl Acad. Sci. USA* **96,** 15127–15132 (1999).
15. Hamazaki, T. *et al.* Hepatic maturation in differentiating embryonic stem cells in vitro. *FEBS Lett.* **497,** 15–19 (2001).
16. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transciption factor Oct4. *Cell* **95,** 379–391 (1998).
17. Nishimoto, M., Fukushima, A., Okuda, A. & Muramatsu, M. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. *Mol. Cell. Biol.* **19,** 5453–5465 (1999).
18. Kawasaki, H. *et al.* Induction of midbrain dopaminergic neurons from ES cells by stromal cell-derived inducing activity. *Neuron* **28,** 31–40 (2000).
19. Everett, C. A. & West, J. D. The influence of ploidy on the distribution of cells in chimaeric mouse blastocysts. *Zygote* **4,** 59–66 (1996).
20. Tada, M., Takahama, Y., Abe, K., Nakatsuji, N. & Tada, T. Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. *Curr. Biol.* **11,** 1553–1558 (2001).
21. Tada, M., Tada, T., Lefebvre, L., Barton, S. C. & Syrani, M. A. Embryonic germ cells induce epigenetic reprogramming of somatic nucleus in hybrid cells. *EMBO J.* **16,** 6510–6520 (1997).
22. Parwaresch, M. R., Kreipe, H. & Radzun, H. J. Human macrophage hybrid forming spontaneous giant cells. *Virchows Arch. B* **51,** 89–96 (1986).
23. Chiozzi, P. *et al.* Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z/P2X7 receptor. *J. Cell Biol.* **138,** 697–706 (1997).
24. Falzoni, S. *et al.* The purinergic P2Z receptor of human macrophage cells. Characterization and possible physiological role. *J. Clin. Invest.* **95,** 1207–1216 (1995).
25. Enelow, R. I., Sullivan, G. W., Carper, H. T. & Mandell, G. L. Induction of multinucleated giant cell formation from in vitro culture of human monocytes with interleukin-3 and interferon-β: comparison with other stimulating factors. *Am. J. Resp. Cell Mol. Biol.* **6,** 57–62 (1992).

Supplementary Information accompanies the paper on *Nature*'s website (http://www.nature.com).

Acknowledgements

The authors are indebted to A. Nagy for providing embryonic stem cell lines; Y. Yoneda for discussion; J. Crawford, D. Steindler, S. May and S. Sugrue for critical reading of the manuscript; and G. Brown, M. Jorgenson, A. Meacham, N. Devine, and Diagnostic Cytogenetics for technical assistance. This work was supported by grants from the National Institutes of Health (to N.T. and E.W.S.).

Competing interests statement

The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to N. T. (e-mail: terada@pathology.ufl.edu).

Changing potency by spontaneous fusion

Qi-Long Ying*, Jennifer Nichols*, Edward P. Evans† & Austin G. Smith*

** Centre for Genome Research, University of Edinburgh, The King's Buildings, West Mains Road, Edinburgh EH9 3JQ, UK*
† Department of Zoology, University of Oxford, South Parks Road, Oxford OX1 3PS, UK

Recent reports have suggested that mammalian stem cells residing in one tissue may have the capacity to produce differentiated cell types for other tissues and organs[1–9]. Here we define a mechanism by which progenitor cells of the central nervous system can give rise to non-neural derivatives. Cells taken from mouse brain were co-cultured with pluripotent embryonic stem cells. Following selection for a transgenic marker carried only by the brain cells, undifferentiated stem cells are recovered in which the brain cell genome has undergone epigenetic reprogramming. However, these cells also carry a transgenic marker and chromosomes derived from the embryonic stem cells. Therefore the altered phenotype does not arise by direct conversion of brain to embryonic stem cell but rather through spontaneous generation of hybrid cells. The tetraploid hybrids exhibit full pluripotent character, including multilineage contribution to chimaeras. We propose that transdetermination consequent to cell fusion[10] could underlie many observations otherwise attributed to an intrinsic plasticity of tissue stem cells[9].

The capacity to generate differentiated cell types representing all three definitive germ layers has traditionally been considered a property reserved to cells of the inner cell mass and epiblast in the early embryo and derivative embryonal carcinoma and embryonic stem (ES) cells *in vitro*[11,12]. Recently, however, remarkable potencies have been ascribed to stem cells isolated from various fetal and adult tissues including the central nervous system (CNS). CNS stem cells have been reported to contribute to haematopoietic lineages when injected into irradiated mice[6], to produce muscle when co-cultured with skeletal myoblasts[13], and to colonize multiple fetal lineages when introduced into pre-implantation embryos[5]. A further circumstance in which nervous system stem cells seem to change determination is during co-culture with differentiating ES cells, when they form multinucleated myotubes[5]. We investigated the basis of these phenomena and in particular whether during co-culture CNS cells may first convert to pluripotent ES cells as a route to generating other cell types.

We used distinct transgenic markers to isolate and identify descendants of both the ES cells and the CNS cells (Fig. 1). ZIN40 mice constitutively co-express resistance to the selection agent G418 and nuclear β-galactosidase activity[14]. Oct4-GiP mice express puromycin resistance and green fluorescent protein (GFP) exclusively in pluripotent and germline cells under direction of regulatory sequences of the mouse *Oct4* gene[15]. Neurosphere cultures[5] were initiated from dissociated forebrains of transgenic fetuses at embryonic day 14.5 carrying either of these markers. Neurospheres were expanded for 3 days and then combined with HT2 ES cells. HT2 cells carry the hygromycin phosphotransferase–herpes simplex virus (HSV) thymidine kinase fusion gene (*Hytk*)[16] inserted into the *Oct4* locus by homologous recombination[17,18]. Selection was subsequently applied with G418 or puromycin as appropriate to eliminate the HT2 cells. After 2–4 weeks, proliferating cells with undifferentiated ES cell morphology re-emerged (Fig. 2b). Colonies were recovered from each of 23 independent co-cultures. These included one experiment in which fetal telencephalic cells were combined directly with ES cells without prior preparation of

neurospheres. Cells surviving selection expressed nuclear β-galactosidase (ZIN40 cells) or cytoplasmic GFP (Oct4-GiP cells) as appropriate. Expression of the Oct4-GiP transgene (Fig. 1b) is striking because, like Oct4 itself, this transgene is expressed only in germline and pluripotent cells and is not active in CNS cells *in vivo* or in primary culture (ref. 15 and our unpublished observations). GFP activity is therefore indicative of at least partial epigenetic reprogramming of the somatic cell genome to a state of pluripotency. Thus it appeared that ES cells could be generated from fetal brain cells.

We then tested these cells for expression of the hygromycin resistance marker carried only by HT2 ES cells (Fig. 1a). The cultures were completely resistant to hygromycin. Furthermore, they were sensitive to gancyclovir, which kills only cells harbouring the HSV *Tk* transgene. To exclude the possibility that inefficient selection could have given rise to mixed cultures, we generated several clonal derivatives by limiting dilution. The clonal isolates all exhibited fetal and ES cell resistance/sensitivity phenotypes. Therefore transgenic selection markers of different origin are co-expressed in individual cells.

We inferred that the selected cells were likely to be hybrids. Consistent with this, they had enlarged nuclei with multiple nucleoli (Fig. 2a, b). To test this interpretation directly, we prepared metaphase chromosome spreads (Fig. 2c). Analysis of 18 independent isolates revealed a tetraploid or near-tetraploid complement in all cases. The ES cells are male, and where a female fetus had been used as the source of brain cells, the sex chromosome complement was XXXY. The strain origin of chromosomes 8 and 14 can readily be discriminated owing to polymorphisms in centromeric heterochromatin[19]. Both strain 129 chromosomes of ES cell origin and non-strain 129 chromosomes of transgenic mouse origin were present in three separate isolates examined in this way (Fig. 2d).

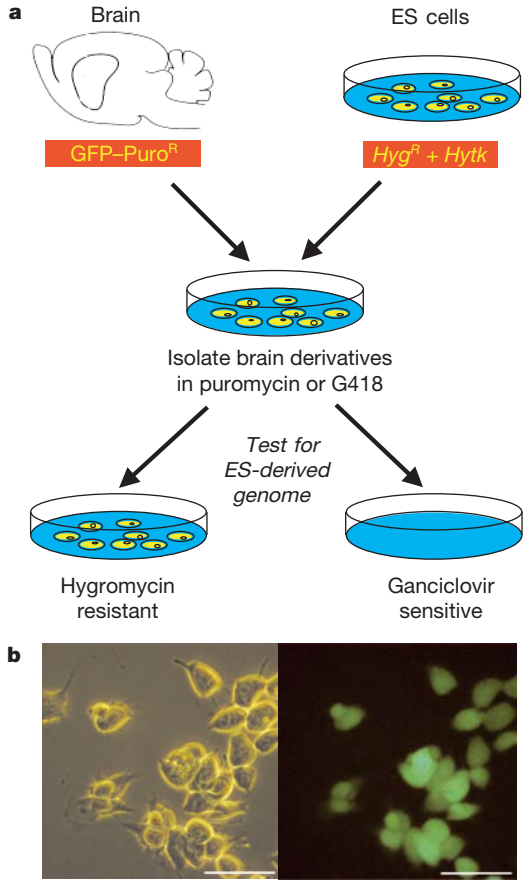

Figure 1 Isolation and identification of descendants of ES and CNS cells. **a**, Diagram of experimental strategy. **b**, Expression of GFP in undifferentiated cells isolated after co-culture of Oct4-GiP fetal brain cells with HT2 ES cells and selection in puromycin.

These observations can be explained only by the formation of cell hybrids between CNS and ES cells.

The hybrid cells expressed the ES cell markers[12] alkaline phosphatase and stage-specific embryonic antigen (SSEA)-1 (data not shown). They expressed the essential pluripotent cell transcription factor Oct-4[18,20] as well as the Oct4-GiP transgene. Like normal ES cells, they were dependent on the self-renewal factor leukaemia inhibitory factor (LIF) to suppress differentiation[12]. On aggregation they formed embryoid bodies[21] containing both extra-embryonic endoderm and spontaneously contracting cardiomyocytes (Fig. 3). Embryoid bodies treated with retinoic acid[22] gave rise to neurons. Thus the hybrids have *in vitro* self-renewal and differentiation properties similar to those of regular ES cells[12]. Hybrids produced by electrofusion of ES cells with thymocytes have similarly been found to exhibit ES cell characteristics[23].

We examined the capacity for incorporation into embryonic development by blastocyst injection. Contributions to fetal tissues were detected in 8 of 23 transferred embryos by β-galactosidase staining for the ZIN40 marker (Fig. 4a). The contributions were modest by comparison with standard ES cells and uneven between tissues, but this is expected owing to competitive overgrowth of tetraploid hybrid cells by diploid host cells[24]. Interestingly, 1 of 14 live-born mice showed overt coat-colour chimaerism (Fig. 4b). This animal was euthanized and we analysed the internal organs for expression of nuclear β-galactosidase. Staining was detected in intestine, kidney, heart and most prominently in liver (Fig. 4c). A proportion of tetraploid hepatocytes is normally present in the liver, which may account for the persistent contribution of hybrid cells in this organ. These observations establish that the presence of a genome derived from a brain cell is not prohibitive for multilineage contribution of ES cell hybrids.

Finally we investigated whether similar fusion events could occur with cells isolated from adult brain. A modified schedule for co-culture and selection was adopted to allow for the comparatively slower proliferation rate of adult-derived neurospheres. Neurospheres were established from transgenic mice that express ubiqui-

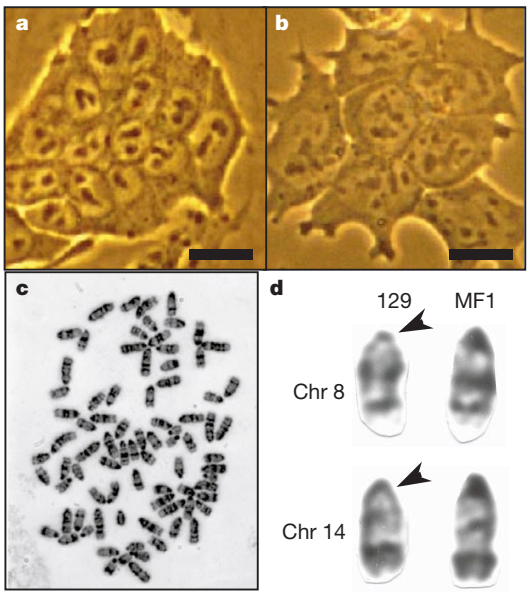

Figure 2 Morphology and chromosomal constitution. **a**, **b**. Photomicrographs of parental ES cells (**a**) and cells isolated after co-culture (**b**) showing enlarged nuclei with multiple nucleoli in cells with otherwise typical ES cell morphology. Scale bar, 25 μm. **c**, Metaphase spread showing tetraploid chromosome complement after co-culture of adult brain neurospheres and ES cells. **d**, Strain 129 and non-strain 129 chromosomes in cells derived from co-culture. Arrowheads indicate the polymorphic centromeric C bands that are diagnostically reduced in strain 129 chromosomes and large in MF1 or C57BL/6 chromosomes of the transgenic mice.

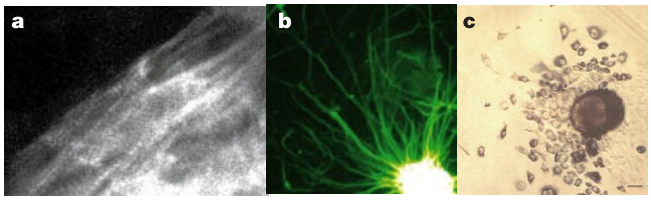

Figure 3 Pluripotency of hybrid cells. Embryoid bodies were prepared from hybrid cells and outgrowths analysed by immunostaining for markers of cardiomyocytes (β-actinin, **a**), or, if treated with retinoic acid, neurons (type III tubulin, **b**), or by *in situ* hybridization (**c**), which revealed *Sparc* messenger RNA in migratory cells of typical parietal endoderm morphology.

tously the tauGFP fusion protein linked with puromycin resistance[25]. The lateral ventricles of an 8-week-old female mouse were dissociated and neurospheres were propagated for 2 weeks as described[5]. These neurosphere cells were combined in a 10:1 ratio with ES cells in adherent culture. The co-culture was maintained in neural stem cell medium supplemented with LIF for 1 week. The culture was then transferred to ES cell medium and expanded for a further week before applying puromycin selection. After 2 weeks of selection, proliferating colonies of tauGFP-expressing ES cells appeared. These cells had large nuclei and multiple nucleoli as above. Chromosome preparations confirmed that they were tetraploid with an XXXY complement (Fig. 2c) and contained both strain 129 and non-strain 129 chromosomes 8 and 14.

The frequency of hybrid isolation in these experiments is between 10^{-4} and 10^{-5} per brain cell plated, although note that we have made no efforts at optimization. There is no fusogen present under our culture conditions, implying that the hybrids are generated by spontaneous fusion between ES cells and CNS cells. The phenomenon is not dependent on the use of HT2 cells because an independently derived ES cell line, 46C, was used in the fusion with adult neurosphere cells. We have also obtained hybrids from co-cultures between ES cells. Spontaneous fusion between mammalian cells was originally documented in 1961[26,27]. This provided the foundation for development of somatic cell genetics and the appreciation that cell specification can be reprogrammed in hybrids[10]. This work has been overlooked in recent reports of transdetermination and transdifferentiation[1–8,13], resulting in a major challenge to the concept of progressive lineage restriction during development[9]. However, as we have shown here, the fact that mammalian cells can spontaneously form hybrids presents an alternative explanation for the apparent ability of cells from one tissue to generate progeny of another type. Future claims of cell plasticity should therefore examine the presence not just of donor cell markers but also of host cell markers in any putative transdetermination product. □

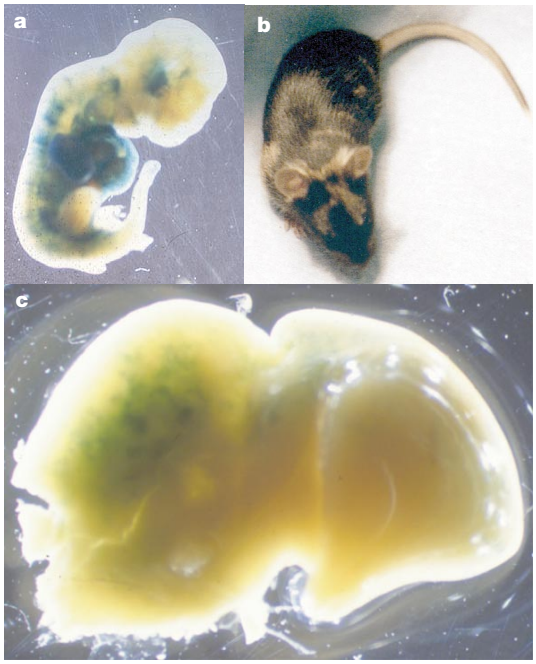

Figure 4 Contribution of ZIN40/HT2 hybrid cells to chimaeras. **a**, β-galactosidase staining of fetal chimaera at embryonic day 11.5, slightly retarded. **b**, Adult mouse chimaera. **c**, β-galactosidase staining of liver from an adult mouse chimaera.

Methods

Embryonic stem cells and hybrid cells were maintained in LIF-supplemented medium without feeders[28]. HT2 and 46C ES cells are of pure inbred 1290la origin. Transgenic mice are on mixed 129 × MF1 (ZIN40 and Oct4-GiP) and 129 × C57BL/6 (tauGFP) backgrounds. Neurosphere cultures were initiated and expanded in DMEM/F12 medium with N2 and B27 supplements plus fibroblast growth factor-2 (10 ng ml^{-1})[5]. Dissociated fetal neurosphere cells and ES cells were mixed in a 2:1 ratio and plated on dishes coated with poly-D-lysine and laminin at 1–1.5 × 10^4 cells cm^{-2}. Cultures were maintained in neural stem cell medium for 48 h, then changed to ES cell medium containing fetal calf serum and LIF. G418 (200–400 μg ml^{-1}) was added after a further 2 days of co-culture, but puromycin (1 μg ml^{-1}) only after 6 days because the Oct4-GiP transgene is not expressed in brain cells. In the case of adult neurosphere cells, the co-culture was initiated at a 10:1 ratio of brain cells to ES cells and maintained for 2 weeks before application of puromycin selection. ES cell differentiation and chimaera production followed standard protocols[29].

Received 28 November 2001; accepted 27 February 2002.
Published online 13 March 2002, DOI 10.1038/nature729.

1. Brazelton, T. R., Rossi, F. M., Keshet, G. I. & Blau, H. M. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290,** 1775–1779 (2000).
2. Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. & McKercher, S. R. Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* **290,** 1779–1782 (2000).
3. Krause, D. S. *et al.* Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105,** 369–377 (2001).
4. Lagasse, E. *et al.* Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. *Nature Med.* **6,** 1229–1234 (2000).
5. Clarke, D. L. *et al.* Generalized potential of adult neural stem cells. *Science* **288,** 1660–1663 (2000).
6. Bjornson, C. R., Rietze, R. L., Reynolds, B. A., Magli, M. C. & Vescovi, A. L. Turning brain into blood: a hematopoietic fate adopted by adult neural stem cells *in vivo*. *Science* **283,** 534–537 (1999).
7. Ferrari, G. *et al.* Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279,** 1528–1530 (1998).
8. Orlic, D. *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* **410,** 701–705 (2001).
9. Blau, H. M., Brazelton, T. R. & Weimann, J. M. The evolving concept of a stem cell: entity or function? *Cell* **105,** 829–841 (2001).
10. Ephrussi, B. *Hybridization of Somatic Cells* (Princeton Univ. Press, Princeton, 1972).
11. Gardner, R. L. & Beddington, R. S. Multi-lineage 'stem' cells in the mammalian embryo. *J. Cell Sci.* **10 (Suppl.),** 11–27 (1988).
12. Smith, A. in *Stem Cell Biology* (ed. Marshak, D. R.Gardner, R. L.Gottlieb, D.) 205–230 (Cold Spring Harbor Laboratory Press, New York, 2001).
13. Galli, R. *et al.* Skeletal myogenic potential of human and mouse neural stem cells. *Nature Neurosci.* **3,** 986–991 (2000).
14. Mountford, P. & Smith, A. G. Internal ribosome entry sites and dicistronic RNAs in mammalian transgenesis. *Trends Genet.* **11,** 179–184 (1995).
15. Yeom, Y. I. *et al.* Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. *Development* **122,** 881–894 (1996).
16. Lupton, S. D., Brunton, L. L., Kalberg, V. A. & Overell, R. W. Dominant positive and negative selection using a hygromycin phosphotransferase–thymidine kinase fusion gene. *Mol. Cell. Biol.* **11,** 3374–3378 (1991).
17. Mountford, P. *et al.* Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. *Proc. Natl Acad. Sci. USA* **91,** 4303–4307 (1994).
18. Niwa, H., Miyazaki, J. & Smith, A. G. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. *Nature Genet.* **24,** 372–376 (2000).
19. Akeson, E. C. & Davisson, M. T. in *Genetic Variants and Strains of the Laboratory Mouse* (eds Lyon, M., Rastan, S. & Brown, S.) 1506–1509 (Oxford Univ. Press, Oxford, 1996).
20. Nichols, J. *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct-4. *Cell* **95,** 379–391 (1998).
21. Doetschman, T. C., Eistetter, H., Katz, M., Schmidt, W. & Kemler, R. The *in vitro* development of blastocyst-derived embryonic stem cell lines: formation of visceral yolk sac, blood islands and myocardium. *J. Embryol. Exp. Morphol.* **87,** 27–45 (1985).
22. Bain, G., Kitchens, D., Yao, M., Huettner, J. E. & Gottlieb, D. I. Embryonic stem cells express neuronal properties *in vitro*. *Dev. Biol.* **168,** 342–357 (1995).
23. Tada, M., Takahama, Y., Abe, K., Nakatsuji, N. & Tada, T. Nuclear reprogramming of somatic cells by *in vitro* hybridization with ES cells. *Curr. Biol.* **11,** 1553–1558 (2001).
24. Nagy, A. *et al.* Embryonic stem cells alone are able to support fetal development in the mouse. *Development* **110,** 815–821 (1991).
25. Pratt, T., Sharp, L., Nichols, J., Price, D. J. & Mason, J. O. Embryonic stem cells and transgenic mice ubiquitously expressing a tau-tagged green fluorescent protein. *Dev. Biol.* **228,** 19–28 (2000).
26. Barski, G., Sorieul, S. & Cornefert, F. "Hybrid" type cells in combined cultures of two different mammalian cell strains. *J. Natl Cancer Inst.* **26,** 1269–1291 (1961).
27. Sorieul, S. & Ephrussi, B. Karyological demonstration of hybridization of mammalian cells *in vitro*. *Nature* **190,** 653–654 (1961).

28. Smith, A. G. Culture and differentiation of embryonic stem cells. *J. Tissue Culture Methods* **13,** 89–94 (1991).
29. Robertson, E. J. *Teratocarcinoma and Embryo-derived Stem Cells: A Practical Approach* (IRL, Oxford, 1987).

Acknowledgements

H. Niwa and I. Chambers generated the HT2 ES cells and Marios Stavridis the 46C cells. We thank C. Graham for directing us to the original studies of Barski and Ephrussi and are grateful to C. Blackburn and A. Medvinksy for comments on the manuscript. This research was supported by the International Human Frontiers Science Program and by the Medical Research Council and Biotechnology and Biological Sciences Research Council of the UK.

Competing interests statement

The authors declare competing financial interests: see the website (http://www.nature.com) for details.

Correspondence and requests for materials should be addressed to A.G.S. (e-mail: austin.smith@ed.ac.uk).

Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus

Jianhang Jia*†, Kazuhito Amanai*†, Gelin Wang*†, Jiong Tang‡, Bing Wang* & Jin Jiang*

* *Center for Developmental Biology and Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9133, USA*
‡ *Division of Cell and Molecular Biology, University of Texas Southwestern Medical Center, Dallas, Texas 75390-9133, USA*
† *These authors contributed equally to this work*

The *Drosophila* protein Shaggy (Sgg, also known as Zeste-white3, Zw3) and its vertebrate orthologue glycogen synthase kinase 3 (GSK3) are inhibitory components of the Wingless (Wg) and Wnt pathways[1]. Here we show that Sgg is also a negative regulator in the Hedgehog (Hh) pathway. In *Drosophila*, Hh acts both by blocking the proteolytic processing of full-length Cubitus interruptus, Ci (Ci155), to generate a truncated repressor form (Ci75), and by stimulating the activity of accumulated Ci155 (refs 2–6). Loss of *sgg* gene function results in a cell-autonomous accumulation of high levels of Ci155 and the ectopic expression of Hh-responsive genes including *decapentaplegic* (*dpp*) and *wg*. Simultaneous removal of *sgg* and *Suppressor of fused*, *Su(fu)*[7], results in wing duplications similar to those caused by ectopic Hh signalling. Ci is phosphorylated by GSK3 after a primed phosphorylation by protein kinase A (PKA), and mutating GSK3-phosphorylation sites in Ci blocks its processing and prevents the production of the repressor form. We propose that Sgg/GSK3 acts in conjunction with PKA to cause hyperphosphorylation of Ci, which targets it for proteolytic processing, and that Hh opposes Ci proteolysis by promoting its dephosphorylation.

The Hh family of secreted proteins controls cell growth and patterning in many principal developmental processes in both vertebrates and invertebrates[8]. Moreover, mutations in components of the Hh signalling pathway have been implicated in many human disorders, including cancer[9]. During *Drosophila* limb development, posterior (P)-compartment cells express and secrete Hh that induces adjacent anterior (A)-compartment cells to express target genes including *dpp*, *wg* (leg only) and *patched* (*ptc*) by regulating the transcription factor Ci[4,10,11]. In A-compartment cells distant from the AP compartment boundary, Ci is processed to generate a truncated repressor form (Ci75) that represses a subset of Hh-responsive genes including *dpp*[2,4]. In A-compartment cells adjacent to the AP compartment border, Hh signalling blocks Ci processing to generate Ci75, and causes the accumulation of full-length Ci (Ci155)[2]. In addition, high levels of Hh stimulate a distinct transcriptional activation activity of Ci155, which is required for the expression of Hh-responsive genes such as *ptc*[3,4,12].

In both wing and leg discs, loss of *sgg* function in the A compartment either by using *sgg* mutations or by overexpressing a dominant negative form of GSK3 (DN-GSK3)[13] causes the accumulation of high levels of Ci155 in a cell-autonomous fashion without affecting *ci-lacZ* expression (Fig. 1a, b, e, h, j–l, and see Supplementary Information). In wing discs, anterior sgg^- cells or DN-GSK3-expressing cells located outside the wing pouch region ectopically express *dpp*, which is repressed by Ci75 (Fig. 1c, d). However, anterior sgg^- cells do not ectopically activate *ptc*, which is activated by Ci155 (Fig. 1f). In leg discs, anterodorsal sgg^- cells distant from the AP boundary ectopically express *wg* and low levels of *dpp* (Fig. 1g–i), a phenotype similar to that associated with *sgg PKA* double-mutant cells in which both Wg and Hh signalling pathways are ectopically activated[14]. As in the case of wing discs,

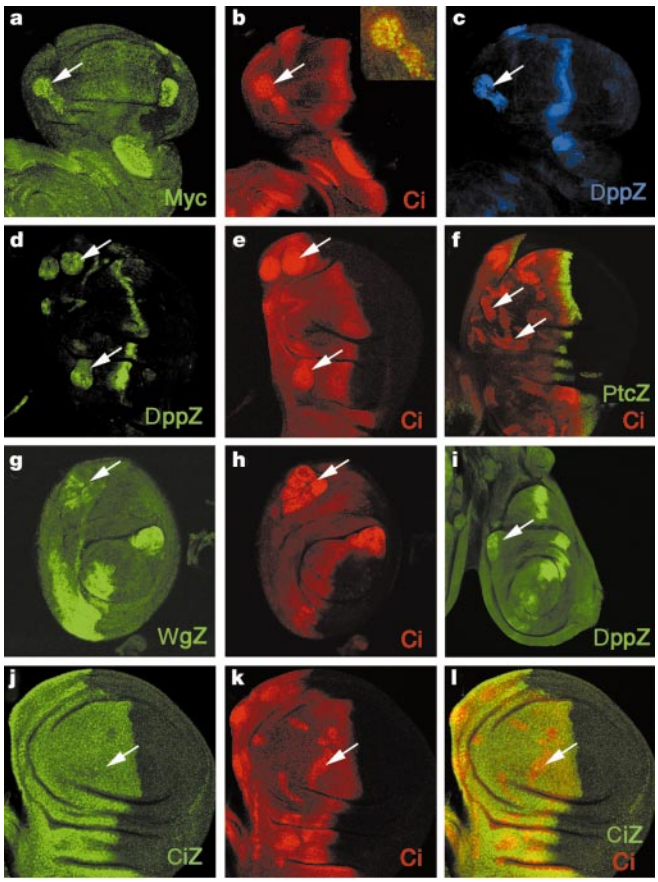

Figure 1 Ectopic Hh signalling activity in sgg^- cells. **a**–**c**, A wing disc carrying sgg^- clones and showing the expression of a marker gene (**a**), Ci155 (**b**) and *dpp-lacZ* (**c**). sgg^- cells are recognized by strong Myc staining. Anterior sgg^- cells accumulate high levels of Ci155 and ectopically express *dpp-lacZ*. The inset in **b** shows an enlarged view of the anterior sgg^- clone (arrow in **a**). **d**, **e**, A wing disc containing clones of cells that express *UAS-DN-GSK3* using *actin5c > CD2 > Gal4*. Anterior DN-GSK3-expressing cells accumulate high levels of Ci155 (arrows in **e**) and ectopically express *dpp-lacZ* (arrows in **d**). **f**, A wing disc carrying sgg^- mutant clones and exhibiting the expression of Ci155 and *ptc-lacZ*. Anterior sgg^- mutant clones do not ectopically activate *ptc-lacZ* (arrows). **g**–**i**, Leg discs carrying sgg^- clones and showing the expression of *wg-lacZ* (**g**), Ci155 (**h**) and *dpp-lacZ* (**i**). Anterodorsal sgg^- cells accumulate high levels of Ci155 (**h**) and ectopically express low levels of *wg-lacZ* (**g**) and *dpp-lacZ* (**i**). **j**–**l**, A wing disc carrying sgg^- clones and showing the expression of *ci-lacZ* and Ci155. sgg^- cells (arrow) do not express *ci-lacZ* at higher levels.