

THIS WEEK

EDITORIALS

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America's carbon compromise

As looming tax increases and budget cuts threaten to plunge the US economy back into recession, Congress should take a hard look at introducing a carbon tax as an important part of the solution.

This week, a reinvigorated Barack Obama returned to the White House knowing that he was poised on the edge of a fiscal cliff. Rather than relishing his victory last week, Obama must immediately set about crafting a compromise on deficit reduction with congressional leaders. The stakes could hardly be higher — for science, for US citizens and, indeed, for the world. In the event of failure, a budgetary time-bomb of tax increases and sweeping budget cuts will detonate on 2 January. As well as resulting in indiscriminate cuts to funds for scientific research and many other areas, it could knock the United States back into recession and deliver yet another blow to an already fragile global economy.

Faced with such dire consequences, one might expect that all the financial options would be on the table, especially the good ones. Unfortunately, this is not the case, at least not yet.

So far, lawmakers have rehashed long-standing disputes about the size of government and the social safety net, but have ignored ideas that could transform the fiscal challenge into an opportunity. One such proposal is the carbon tax, which could bring financial and political benefits for all and chart a new course forward for energy independence and global warming (see page 309). It is a solution that is every bit as improbable as it is logical, but one should remember Winston Churchill's assessment of the United States' tendency to do the right thing — once all the alternatives have been exhausted.

Just consider the possibilities. To put a levy on carbon would raise revenues that could be used to offset lower tax rates for individuals and businesses. This is what conservatives say they want to do. It would put more income — and thus choice — in the hands of consumers. Economists like the idea for more fundamental reasons. Generally, it is best to tax things that one wishes to discourage (such as smoking) rather than those that should be encouraged (such as working). Environmentalists like the idea of a carbon tax because it could generate some much-needed revenue for clean-energy research and development while reducing carbon emissions.

The numbers are not negligible. An analysis conducted in August by economists at the Massachusetts Institute of Technology (MIT) in Cambridge showed that a carbon tax of US\$20 per tonne of carbon from fossil fuels, if instituted in 2013 and increased by 4% per year, would raise \$1.5 trillion over the course of a decade. Averaged out, this amounts to \$150 billion annually — a sizeable chunk of the trillion-dollar deficits that the US government has been running in recent years. Scholars at the Brookings Institution, a centrist think tank in Washington DC, advocate ramping federal investments in energy research up from \$3.8 billion now to \$30 billion annually, to drive down the cost of low-carbon energy (including cleaner-burning coal). It is an ambitious proposal, and would leave a pile of cash that could be redistributed elsewhere for beneficial use.

Conservatives loathe taxes, and US politicians obsess over energy prices, but a revenue-neutral carbon tax would get around these

problems. The MIT analysis found that the economy benefited regardless of whether the money was reinvested in social programmes or redistributed in the form of lower taxes and cash payments to offset higher energy costs for the poor. For environmentalists, the problem with a carbon tax is that it does not technically limit emissions, but the MIT model suggests that it would perform quite well: carbon emissions fall to 14% below 2006 levels by 2020 as consumers and businesses find ways to reduce their energy use in response to higher prices.

"A carbon tax would depend on political courage and a break with party orthodoxy."

Opposition to the idea may not be what it was. For example, on 13 November, the American Enterprise Institute hosted a conference in Washington DC on the economics of a carbon tax. The institute is a conservative think tank, and its officials have previously raised doubts about climate science. The idea has also been bubbling up in other right-leaning think tanks as a conservative solution to reduce greenhouse gases.

The problem is that to enact a carbon tax would depend on political courage and a willingness to break with party orthodoxy, rare traits in Washington today. President Obama has made energy and climate part of his agenda for the second term, but his first — and perhaps biggest — opportunity to make good on that promise will come in the next few weeks. As US politicians contemplate diving into the fiscal abyss, they would be wise to consider a painless policy that benefits everyone. ■

A shaky restart

Japan still has lessons to learn from Fukushima if it is to convince the public about nuclear energy.

The nuclear disaster that followed the March 2011 tsunami in Japan uncovered serious flaws in the country's nuclear-safety regulations. Japan learned its lesson: it started putting a premium on safety, and is doing everything it can to assure a wary public that similar mistakes will not be made again. Well, that was the hope. Two recent revelations show that it could still do much more.

The country's Nuclear Regulation Authority (NRA) was set up to right the wrongs of the previous regulatory infrastructure. One of its first tasks was to draw up new safety standards for reactor operations. The NRA formed an investigation team of six experts, which held its first meeting on 25 October. The team is expected to submit its report in time for the NRA to put the standards up for public comment in the spring and to make them law in July 2013.

Last week, Japanese media reported that four of those experts have

received regular stipends or one-time grants from the nuclear industry. Nuclear engineer Akio Yamamoto of Nagoya University, for example, has received at least ¥50,000 (US\$630) over the past three years from each of three companies related to nuclear energy, including Nuclear Engineering, a firm in Osaka that is affiliated with Kansai Electric Power. Although there is no suggestion that Yamamoto has done anything wrong, he also received some ¥27 million in grants from eight nuclear-energy companies during that period, as well as an undisclosed amount from Mitsubishi Heavy Industries, which builds reactors.

An NRA spokesman defended the team's composition, arguing that the report will be used only as a reference for the five NRA commissioners who will ultimately decide on the policy. (Those commissioners do not have similar ties with industry.) If the NRA had tried to rule out everyone with any connection to the industry when choosing the experts, the spokesman said, it would have run out of people.

These are fair points, and the fact that the team members had to disclose their contributions at all was a laudable nod to transparency. But playing down the importance of the report by saying it will just be used as a reference is unconvincing.

Much of the uproar over the handling of the Fukushima disaster came from the public perception that conflicts of interests led regulators, who were too tightly tied to the nuclear industry, to favour cost-savings over safety. The NRA, created in large part as an answer to that criticism, has itself been lambasted for moving many staff from the old regulatory structure to the new organization, including its head, Shunichi Tanaka. It seems that Japanese policy-makers, despite the many public demonstrations, still haven't got to grips with the tendency for conflicts of

interest to lead to bad decisions and, even if they don't, to breed mistrust.

Similarly troubling is the rush with which the government reopened two of the country's shuttered nuclear reactors in July without fully evaluating the seismology of the ground beneath. Last week, at its second meeting, a subcommittee of the NRA could not confirm whether

"Japan was supposed to emerge with a new respect for reactor safety."

a fault line running under a seawater-intake channel — used to cool the reactors in an emergency — is active.

At stake is whether the fault is a landslide fault or a more dangerous, deeper tectonic one. The NRA has ordered Kansai Electric, the plant's operator, to dig trenches to investigate the geology more thoroughly. That should take less than two months, but existing facilities at the plant are in the way, making it much more complicated — and expensive.

Even if the risk from that fault is trivial, as many think, critics point out that the threat of shaking from nearby faults, the potential size of a tsunami and the possibility of structural defects like those found at Fukushima have not been adequately characterized.

Large sectors of the public opposed the reactor restarts with demonstrations of a fervour not seen in Japan in decades. The country had already proved that it could get by, at least in the short term, with no nuclear power. Some scientists had pointed out the uncertainty over the seismic fault, and suggested how to deal with it, before the reactors were restarted. Japan was supposed to emerge from the Fukushima crisis with a new respect for reactor safety and better awareness of the need to convince people of that safety. It hasn't made a very good start. ■

Save scientific sites

The push to conserve cultural-heritage sites must not leave out areas of interest to science.

It is possible for an outsider to visit and enjoy the ancient city of Samarkand in Uzbekistan, home to the fifteenth-century observatory of the astronomer Ulugh Beg. That is in part thanks to the United Nations Educational, Scientific and Cultural Organization (UNESCO) World Heritage Convention, which this week celebrates its 40th anniversary (see page 328). Samarkand was put on the World Heritage List in 2001; the listing gives it important protection from the ongoing political chaos that has followed the collapse of the Soviet Union.

The observatory must have been lovely during the two decades that it was active. Contemporary reports describe splendid architecture and exquisite tiling and mosaics. Frescos illustrating the orbits of the planets and the exact positions of stars adorned the observatory's inner walls. It was largely destroyed by God-fearing hordes in 1449, but the innovative work of its scientists survived to influence Western astronomy and algebra. Using a sextant 40 metres in radius, astronomers at the observatory recalculated the positions of nearly 1,000 stars and compiled their results in the widely translated 1437 star catalogue *Zij-i Sultani*. They stabilized the sextant by anchoring it in a 2-metre-wide trench dug into a hill in the plane of the meridian. The measurements were of unprecedented accuracy: the astronomers used them to recalculate trigonometric tables and to calculate the length of the sidereal year (the time taken for Earth to orbit the Sun once in relation to the fixed stars) to within one minute of the measurement now accepted. Archaeologists discovered the remains of the observatory in 1908.

A place on the World Heritage List means that a site must be maintained with international-standard conservation methods, and not spoiled with inappropriate building development. International inspection teams visit to ensure compliance. Inspections of Samarkand revealed in the mid-2000s that conservation was under par, and

that city planners, supported by local politicians, alarmingly failed to respect the integrity of the site. UNESCO called for increased monitoring of the site and threatened to place it on the List of World Heritage in Danger — although it has so far avoided this designation.

In the past few decades, scientists from many disciplines have developed techniques — ranging from lasers to nuclear technologies and microbiology — for conserving and restoring artworks and monuments. Scientists at the Foundation for Research and Technology — Hellas in Heraklion, Greece, for example, invented a laser with one beam in the ultraviolet range and another in the infrared to clean a frieze on the Parthenon, part of a World Heritage Site, without damaging its surface. The widely publicized work on the frieze was completed in 2005.

Understandably, scientists would like more funding to allow them to fine-tune such techniques. That is hard to justify generically — each archaeological site or monument has its own problems, with technical solutions that must be worked out on an individual basis. The story of Samarkand shows that politics — and thus the World Heritage List — is at least as important as science to the conservation of important monuments around the world. Funds for cultural-heritage technologies must be maintained as part of a broad approach to consider cultural heritage more widely. City and regional plans to cope with climate change, for example, should be required to consider the impact on cultural heritage. The European Commission is quietly voicing support for such a push, and it should be encouraged to speak louder.

Very few of the 962 entries on the World Heritage List involve scientific sites, perhaps because science is not automatically thought of as a part of culture. However, astronomers have begun to do something about this. In the 2009 International Year of Astronomy, astronomers worked with a UNESCO advisory group, the International Council on Monuments and Sites, to produce a list of astronomical sites that they think are, like the Samarkand Observatory, worth saving. These include the nineteenth-century Royal Observatory in Cape Town,

South Africa. If this site is designated as a World Heritage Site, interest surrounding it might encourage the much-desired development of science in the country. Other scientists should follow the astronomers' example. ■

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Be persuasive. Be brave. Be arrested (if necessary)

A resource crisis exacerbated by global warming is looming, argues financier Jeremy Grantham. More scientists must speak out.

I have yet to meet a climate scientist who does not believe that global warming is a worse problem than they thought a few years ago. The seriousness of this change is not appreciated by politicians and the public. The scientific world carefully measures the speed with which we approach the cliff and will, no doubt, carefully measure our rate of fall. But it is not doing enough to stop it. I am a specialist in investment bubbles, not climate science. But the effects of climate change can only exacerbate the ecological trouble I see reflected in the financial markets — soaring commodity prices and impending shortages.

My firm warned of vastly inflated Japanese equities in 1989 — the grandmother of all bubbles — US growth stocks in 2000 and everything risky in late 2007. The usual mix of investor wishful thinking and dangerous and cynical encouragement from industrial vested interests made these bubbles possible. Prices of global raw materials are now rising fast. This does not constitute a bubble, however, but is a genuine paradigm shift, perhaps the most important economic change since the Industrial Revolution. Simply, we are running out.

The price index of 33 important commodities declined by 70% over the 100 years up to 2002 — an enormous help to industrialized countries in getting rich. Only one commodity, oil, had been flat until 1972 and then, with the advent of the Organization of the Petroleum Exporting Countries, it began to rise. But since 2002, prices of almost all the other commodities, plus oil, tripled in six years; all without a world war and without much comment. Even if prices fell tomorrow by 20% they would still on average have doubled in 10 years, the equivalent of a 7% annual rise.

This price surge is a response to global population growth and the explosion of capital spending in China. Especially dangerous to social stability and human well-being are food prices and food costs. Growth in the productivity of grains has fallen to 1.2% a year, which is exactly equal to the global population growth rate. There is now no safety margin.

Then there is the impending shortage of two fertilizers: phosphorus (phosphate) and potassium (potash). These two elements cannot be made, cannot be substituted, are necessary to grow all life forms, and are mined and depleted. It's a scary set of statements. Former Soviet states and Canada have more than 70% of the potash. Morocco has 85% of all high-grade phosphates. It is the most important quasi-monopoly in economic history.

What happens when these fertilizers run out is a question I can't get satisfactorily answered and, believe me, I have tried. There seems to be only one conclusion: their use must be drastically reduced in the next 20–40 years or we will begin to starve.

The world's blind spot when it comes to the

fertilizer problem is seen also in the shocking lack of awareness on the part of governments and the public of the increasing damage to agriculture by climate change; for example, runs of extreme weather that have slashed grain harvests in the past few years. Recognition of the facts is delayed by the frankly brilliant propaganda and obfuscation delivered by energy interests that virtually own the US Congress. (It is not unlike the part played by the financial industry when investment bubbles start to form ... but that, at least, is only money.) We need oil producers to leave 80% of proven reserves untapped to achieve a stable climate. As a former oil analyst, I can easily calculate oil companies' enthusiasm to leave 80% of their value in the ground — absolutely nil.

The damaging effects of climate change are accelerating. James

Hansen of NASA has screamed warnings for 30 years. Although at first he was dismissed as a madman, almost all his early predictions, disturbingly, have proved conservative in relation to what has actually happened. In 2011, Hansen was arrested in Washington DC, alongside Gus Speth, the retired dean of Yale University's environmental school; Bill McKibben, one of the earliest and most passionate environmentalists to warn about global warming; and my daughter-in-law, all for protesting over a pipeline planned to carry Canadian bitumen to refineries in the United States, bitumen so thick it needs masses of water even to move it. From his seat in jail, Speth said that he had held some important positions in Washington, but none more important than this one.

President Barack Obama missed the chance of a lifetime to get a climate bill passed, and his great environmental and energy scientists John

Holdren and Steven Chu went missing in action. Scientists are understandably protective of the dignity of science and are horrified by publicity and overstatement. These fears, unfortunately, are not shared by their opponents, which makes for a rather painful one-sided battle. Overstatement may generally be dangerous in science (it certainly is for careers) but for climate change, uniquely, understatement is even riskier and therefore, arguably, unethical.

It is crucial that scientists take more career risks and sound a more realistic, more desperate, note on the global-warming problem. Younger scientists are obsessed by thoughts of tenure, so it is probably up to older, senior and retired scientists to do the heavy lifting. Be arrested if necessary. This is not only the crisis of your lives — it is also the crisis of our species' existence. I implore you to be brave. ■

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IT IS CRUCIAL THAT
SCIENTISTS
SOUND A MORE
REALISTIC,
MORE
DESPERATE,
NOTE ON GLOBAL
WARMING.

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RESEARCH HIGHLIGHTS

Selections from the
scientific literature

PHYSIOLOGY

How cold triggers fat formation

In frigid temperatures, mice ramp up the production of heat-generating brown fat by decreasing the levels of a small RNA molecule.

Because brown fat burns energy — unlike its unpopular cousin, white fat — its production is an attractive target for obesity and diabetes therapies. Markus Stoffel at the Swiss Federal Institute of Technology in Zurich and his colleagues found that exposure to cold reduced the expression of microRNA-133 in brown and subcutaneous white fat. Inhibiting miRNA-133 promoted brown-fat formation, whereas forcing miRNA-133 expression switched off brown-fat production.

The small RNA acted by directly inhibiting PRDM16, a protein that is central to the production of brown fat from white-fat-cell precursors. *Nature Cell Biol.* <http://dx.doi.org/10.1038/ncb2612> (2012)

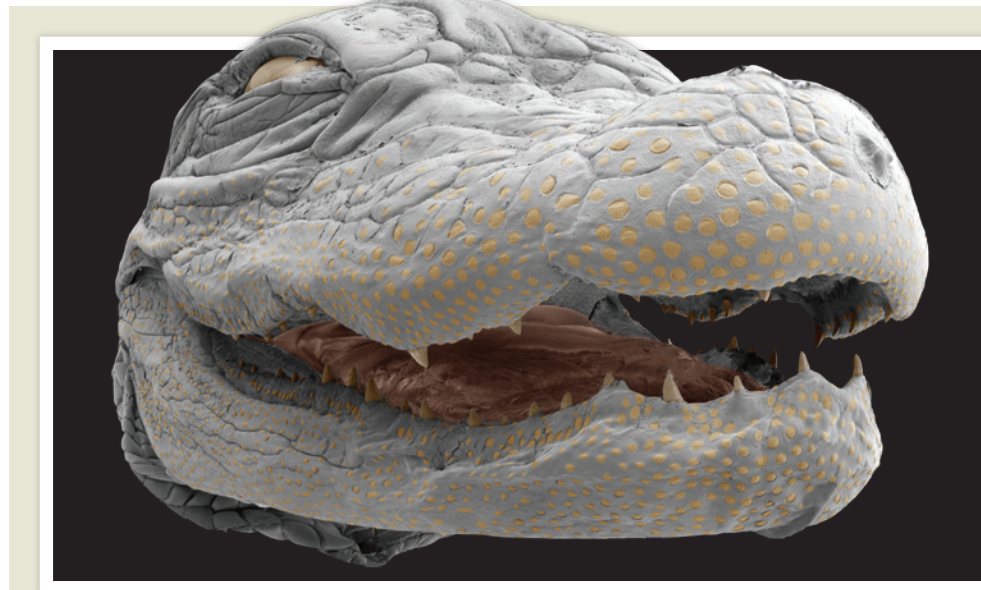
OCEANOGRAPHY

Thinning ice more fragile and mobile

The pronounced thinning of Arctic sea ice has made the ice pack more brittle and susceptible to wind drift.

The volume of Arctic sea ice decreased by one-third during 2007–11 compared with the 1979–2006 mean. In a model simulation, Jinlun Zhang at the University of Washington in Seattle and his colleagues demonstrate that the decline in volume substantially reduces the mechanical strength of the ice, thus boosting ice-drift speed and deformation rates.

Forecasts of ice-edge locations will become more challenging as the thinning



D. LEITCH/VANDERBILT BRAIN INST.

ZOOLOGY

Thick-skinned but sensitive

Crocodiles and alligators may sense their prey using tiny bumps on their mouths that are highly sensitive to touch.

Kenneth Catania and Duncan Leitch at Vanderbilt University in Nashville, Tennessee, investigated the raised bumps — called integumentary sensory organs — in 18 American alligators (*Alligator mississippiensis*; hatchling pictured) and 4 Nile crocodiles (*Crocodylus niloticus*). Confocal microscopy revealed that the bumps (pictured yellow), which are packed

most tightly around the teeth and mouth, share similar structures with tactile skin receptors in mammals. The bumps seemed to be insensitive to electrical current or water salinity, but showed nerve responses when stimulated with a range of levels of force — responding to low levels with a sensitivity exceeding that in primate fingertips.

The authors suggest that the animals use the sensitive bumps to locate prey, and to identify food and other items inside their mouths.

J. Exp. Biol. 215, 4217–4230 (2012)

and weakening of sea ice leads to a state of free drift, the authors note.

Geophys. Res. Lett. <http://dx.doi.org/10.1029/2012GL053545> (2012)

NEUROSCIENCE

Blind reading with sounds

Blind adults taught to ‘read’ using sounds that represent letters use the same area of the brain’s visual cortex that sighted humans use when reading.

Using a program that ‘describes’ images in sound,

Amir Amedi at the Hebrew University of Jerusalem in Israel and his team trained eight congenitally blind people to decipher the shapes of letters and objects such as faces and tools. The authors then imaged participants’ brains as they listened to sounds associated with letters or other objects. When the volunteers read using the sounds, they activated the same part of the visual cortex — the visual word form area (VWFA) — as sighted controls did when viewing letters.

The work shows that the VWFA is not dependent on visual information alone,

and suggests that the visual cortex may be trained to help recognize the environment, even in those who are blind from birth.

Neuron 76, 640–652 (2012)

MATERIALS

Counting loops in gels

Polymer networks, such as rubber and nylon, consist of linked chain-like or branched molecules that almost always contain loops — structural imperfections that weaken a network’s connectivity and

lower the material's elasticity.

Jeremiah Johnson and his colleagues at the Massachusetts Institute of Technology in Cambridge have developed a method to count the number of the most common loops in polymeric materials. The authors broke a hydrogel, a type of polymer network that soaks up water, into quantifiable fragments that reflected the connectivity of the original network, then used mass spectrometry to count the loops. They found that too many loops prevented the gel from forming.

The researchers say they are now using their method to correlate the effects of loops on the mechanical properties of a variety of polymer networks.

Proc. Natl Acad. Sci. USA
<http://dx.doi.org/10.1073/pnas.1213169109> (2012)

ANIMAL BEHAVIOUR

Wrens learn as embryos in the egg

A single song element is all that superb fairy-wren nestlings need to include in their begging calls to get fed by their mothers, and, in an unusual example of prenatal learning, the nestlings seem to learn this 'password' as embryos.

Adult superb fairy-wrens (*Malurus cyaneus*; pictured) use these begging calls to distinguish their offspring from those of two cuckoo species that often invade their nests. Sonia Kleindorfer at Flinders University in Adelaide, Australia, and her team analysed recordings of the fairy-wren calls and found that each nest had a common begging call different from those of all other

nest. That call contained a signature element also found in the call the mother made while incubating her eggs. When the team swapped eggs around across 22 nests, nestlings from those eggs begged using the calls of their foster, not their biological, mothers, suggesting that the calls were learned.

Curr. Biol. <http://dx.doi.org/10.1016/j.cub.2012.09.025> (2012)

NEUROTECHNOLOGY

Brain-machine does the two-step

Brain-machine interfaces (BMIs) detect and use brain activity to perform an intended task, and could be invaluable to people with paralysis. Typically, BMIs are able to process only single movements, but one developed by Ziv Williams at Harvard Medical School in Boston, Massachusetts, and his colleagues can control a series of motions — potentially expanding the complexity of tasks that BMIs can perform.

The team recorded brain activity in monkeys that were trained to move a computer cursor with their paws to each of two areas on a screen in a particular order. This revealed activity in two distinct groups of neurons in the brain's premotor cortex that was associated with each of the upcoming movements. The authors then programmed a computer to decode this signal from the brain and found that the mind-controlled computer moved the cursor at about the same speed that the monkeys achieved with their paws.

Nature Neurosci. <http://dx.doi.org/10.1038/nn.3250> (2012)

NEUROSCIENCE

When neurons mature too early

A genetic mutation linked to intellectual disability and autism causes the premature formation of functional connections between brain cells during a crucial window of development early in life.

COMMUNITY CHOICE

The most viewed papers in science

CHEMISTRY

Mega-MOF's super surface

HIGHLY READ
on pubs.acs.org
in September

Metal-organic frameworks, or MOFs, are of interest for applications such as catalysis and gas storage. Researchers now report a method that allowed them to synthesize these porous crystals with record-breaking surface areas.

Omar Farha at Northwestern University in Evanston, Illinois, and his colleagues created two copper-based MOFs, each with a surface area of approximately 7,000 square metres per gram. To help boost surface area, they used supercritical carbon dioxide to activate the MOFs, avoiding framework collapse, which can occur when the solvents used in MOF synthesis are removed.

Moreover, the authors calculated that by using acetylenes, rather than more bulky phenyls, as links in their framework, they could further increase the theoretical maximum surface area of MOFs to as high as 14,600 square metres per gram, roughly 40% higher than some previous estimates, the team suggests.

J. Am. Chem. Soc. 134, 15016–15021 (2012)

Mutations that inactivate one copy of the gene *SYNGAP1* often cause intellectual disability in humans. Gavin Rumbaugh of the Scripps Research Institute in Jupiter, Florida, and his team found that mice with a similar mutation produce neurons that mature too quickly after birth and become overactive in a brain region important for cognitive function. Mice with one copy of *SYNGAP1* have memory problems and are prone to seizures — a symptom in humans with the mutations.

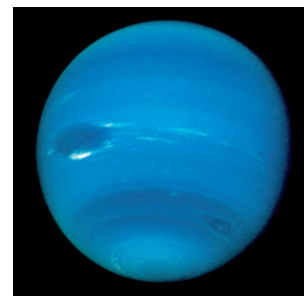
Correcting the mutation in mice after this developmental period had little effect on the symptoms, and introducing the mutation into adult mice did not affect neuronal function — suggesting that the activity of the *SYNGAP1* protein during this developmental window has long-lasting effects.

Cell 151, 1–15 (2012)

ASTRONOMY

More co-orbiters for Neptune

Some astronomers think that Neptune (pictured) can no longer capture objects whose



NASA/SPL

orbits around the Sun are similar to its own. But Carlos and Raúl de la Fuente Marcos at the Complutense University of Madrid in Spain, have discovered that four objects originally classified as minor planets are actually co-orbiters that joined Neptune's orbit as recently as 50,000 years ago.

The work brings to 14 the number of objects that, like Neptune, orbit the Sun every 165 years. The four latest objects are not in the plane of the Solar System and follow eccentric paths. One is likely to diverge from its current path just 2,000 years from now.

Astron. Astrophys. 547, L2 (2012)

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D. WATTS/NATUREPL.COM

SEVEN DAYS

The news in brief

RESEARCH

Stem-cell faker

A scientist who last month fabricated a story about using induced pluripotent stem cells to treat patients with heart failure (see *Nature* **491**, 7–8; 2012) has retracted two of his papers. Hisashi Moriguchi, of the University of Tokyo, retracted research in *Scientific Reports*. He also withdrew his claim to be affiliated with Massachusetts General Hospital and Harvard Medical School, and to have received approval for his work from an institutional review board.

Expensive errors

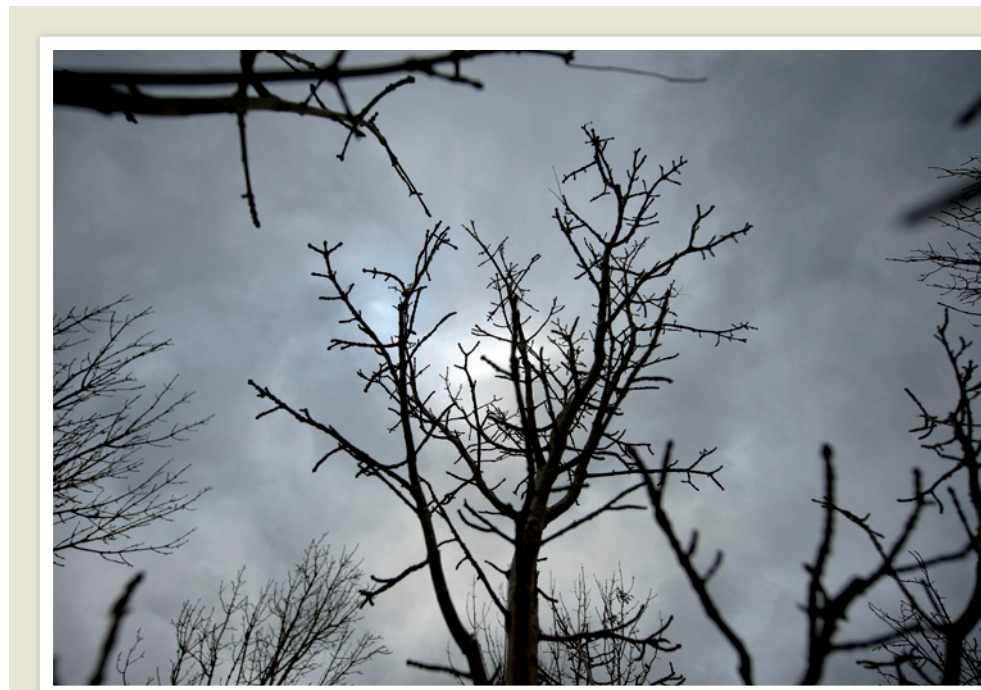
Mistakes in accounting for research projects funded by the European Union in 2011 may have amounted to 3% of the total spending in this area — around €360 million (US\$460 million), according to a report published on 6 November. The European Court of Auditors found that “over-declaration of costs by beneficiaries” was the most common mistake by claimants, but said that most errors were likely to be unintentional. See go.nature.com/nc69zw for more.

Snow survey starts

The Solid Precipitation Intercomparison Experiment, an international two-year project to measure the depth of snow and the amount of snowfall at 15 locations around the world, kicks off this week. See page 312 for more.

Ethics code

Members of the American Anthropological Association overwhelmingly approved new ethical guidelines for research, the professional body announced on 7 November. The rewrite was prompted by a controversy over anthropologists’ participation



B. CLARKE/GETTY

Killer fungus claims UK ash trees

‘Ash dieback’ is likely to kill nearly all of the United Kingdom’s 90 million ash trees, despite the announcement of a plan to control the fungal disease on 9 November. Diseased trees in nurseries will be destroyed, and an import ban

will stay in place. The fungus (*Hymenoscyphus pseudoalbidus*) was initially misidentified by mycologists, making it hard for the European Union to control its spread across the continent. See go.nature.com/anzy6u for more.

in US military efforts in Iraq and Afghanistan (see go.nature.com/so2doa). The new code is rooted in more flexible principles, such as avoiding harm to research subjects and obtaining their informed consent, and not the hard-and-fast rules that the previous ethics code had included.

federal budgets. Scientists hope that the new centre, which will ally the Charité university clinic with the Max Delbrück Center for Molecular Medicine, can rival research powerhouses in the United States and Britain. See page 317 for more.

Open-access funds

Britain’s research-funding agencies will spend more than £100 million (US\$159 million) over the next five years to pay for work that they funded to be free to read, the agencies announced on 8 November. The grants will come out of the United Kingdom’s science budget and will be awarded to universities and other research institutions. Research

Councils UK (an umbrella body for seven agencies that spend a total of £2.8 billion a year) has said that from April 2013, all work funded by the agencies must be published in an open-access format — but the grants for next year are sufficient to cover only 45% of papers. See go.nature.com/d28egt for more.

Fukushima clean-up

The company responsible for decontaminating the ruined Fukushima Daiichi nuclear power plant in Japan says that the cost of doing so will soar. The Fukushima plant suffered meltdowns in three reactors on 11 March 2011 after being stricken by an earthquake and tsunami. On 7 November,

POLICY

Berlin’s biomedicine

Berlin’s mayor Klaus Wowereit announced a deal on 6 November to form the Berlin Institute of Health, which will receive more than €300 million (US\$380 million) in extra funding over the next five years, 90% of it from

the Tokyo Electric Power Company, which owns the plant, announced that cleaning up the ruined reactors and surrounding countryside could cost ¥10 trillion (US\$126 billion) — double the size of the clean-up fund set aside by the government.

Space drive

The British contribution to the European Space Agency will rise by £60 million (US\$93 million) per year to £240 million, the UK chancellor George Osborne said in a speech at the Royal Society in London on 9 November. He also named eight technological areas in which the United Kingdom could be a world leader, including satellites and space, synthetic biology, advanced materials and regenerative medicine. See go.nature.com/mcyniw for more.

Fat tax abandoned

Denmark has abolished a tax on high-fat foods, one year into its controversial attempt to make its population healthier. The tax, which added 16 kroner (US\$2.70) for every kilogram of saturated fat in high-fat products, drove up food prices and put jobs at risk, the Danish Ministry of Taxation said. “We have to try improving the public health by other means,” said food minister Mette Gjeskov.

The country has also rejected a proposed tax on high-sugar foods. See go.nature.com/hkgys for more.

Advice at risk

A letter calling for the budget of the UK Parliamentary Office of Science and Technology (POST) to be protected from cuts was published on 7 November, signed by two former British science ministers, a Nobel laureate and many UK learned societies. POST, which provides politicians with analyses of scientific issues, could face cuts of up to £98,000 (US\$156,000), or 17% of its total budget. See go.nature.com/p55ezf for more.

PEOPLE

Palaeontologist dies

Farish Jenkins, a palaeontologist at Harvard University in Cambridge, Massachusetts, died on 11 November, aged 72. Jenkins was a noted science communicator and lecturer. His discovery with colleagues of the fossil remains of *Tiktaalik roseae* — a four-legged, fish-like creature that seemed to show the evolutionary transition of vertebrates from water to land — appeared on the 6 April 2006 cover of *Nature* and sparked a media frenzy. See go.nature.com/y5ehem for more.



Energetic farewell

Hu Jintao, China's outgoing president, said that protecting the environment must be a top priority for the country's next government. In his opening speech to the 18th National Congress in Beijing on 8 November, he told delegates that China must set a ceiling on its energy consumption. Hu (pictured) is likely to be succeeded this week as the head of China's Communist Party by Xi Jinping, who will then become the country's president in March 2013.

BUSINESS

Vaccine tribulations

A large phase III clinical trial of a malaria vaccine candidate, RTS,S/AS01, has reported disappointing results in infants who received their first injection between 6 and 12 weeks of age. The vaccine reduced the number of episodes of clinical malaria over 12 months by around a third for these babies, the key

COMING UP

20–21 NOVEMBER

The European Space Agency council meets in Naples, Italy, to put together a multi-year spending plan. www.esa.int

22–23 NOVEMBER

At a European Council meeting in Brussels, heads of state negotiate the European Union's budget for 2014–20, including the final amount apportioned to research. Around €80 billion (US\$100 billion) has been proposed for the research framework (see *Nature* **489**, 188–189; 2012). go.nature.com/d7rq5u

age group targeted by the trial. The protection is lower than that observed in a previous, smaller phase II trial of babies in the same age group and less than the 55% reported last year in a group of children who were vaccinated at between 5 and 17 months of age. See go.nature.com/gmw9ib for more.

Sequencing stir

Genetic-testing company 23andMe, based in Mountain View, California, will make anonymized customer data available to researchers, it said at the annual meeting of the American Society of Human Genetics in San Francisco, California, last week. At the same event, Pacific Biosciences of Menlo Park, California, announced technology improvements that allow genomic sections at least 5,000 base pairs long to be sequenced in a single read, compared with a few hundred base pairs for other technologies. See go.nature.com/jqfr3k for more.

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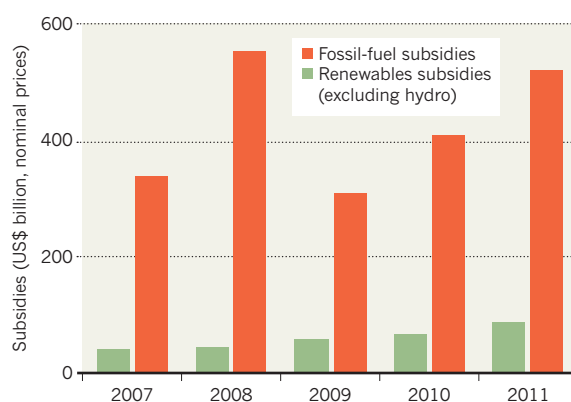
For daily news updates see: www.nature.com/news

TREND WATCH

Subsidies to lower the price of renewable energies, such as solar and wind, rose 24% on 2010 values to reach US\$88 billion in 2011, according to the International Energy Agency's *World Energy Outlook 2012*, released on 12 November. But that support was less than a sixth of the \$523 billion used to artificially reduce the cost of oil, natural gas and coal last year. Fossil-fuel subsidies largely track the price of crude oil. See go.nature.com/i5jt1v for more on this year's energy outlook.

FOSSIL-FUEL SUBSIDIES DWARF RENEWABLES AID

Higher energy prices and rising consumption of oil and gas led to a recent surge of subsidies for fossil fuels to US\$523 billion.



NEWS IN FOCUS

ALTERNATIVE MEDICINE Doubts greet positive outcome of heart therapy **p.313**

GENOMICS Sow's DNA yields silk purse for researchers **p.315**



GERMANY Creative funding for a biomedical powerhouse in Berlin **p.317**

BIOMEDICINE The man who challenged fertility dogma **p.318**



president has “taken major steps to reduce our carbon consumption, including setting higher fuel-efficiency standards for cars and trucks”. When he accepted that endorsement, Obama acknowledged that “climate change is a threat to our children’s future, and we owe it to them to do something about it”.

Yet this new opportunity to confront climate change and invest in science and technology comes with towering obstacles. The election did not end the polarization of Congress — Republicans retained control of the House of Representatives, and the Democrats only slightly strengthened their narrow majority in the Senate. And the ‘fiscal cliff’ looms large — automatic tax increases and spending cuts, the legacy of earlier budget battles, will hit on 2 January unless the outgoing Congress finds a way to avert them in the session that begins this week (see *Nature* **487**, 414–415; 2012).

The cuts, totalling some US\$136 billion, would apply to all discretionary spending next year, including defence, and would eat deeply into federal science budgets (see ‘At the precipice’). Congressional leaders expect Obama to play an active part in brokering a deal to avoid the fiscal cliff, which economists say could plunge the fragile US economy back into recession. The outcome will foreshadow Obama’s prospects for achieving other objectives — including those relevant to science — during his second term.

CLIMATE OPPORTUNITY

Obama may have to develop his climate plans without some high-profile lieutenants. Energy secretary Steven Chu and Environmental Protection Agency (EPA) administrator Lisa Jackson are both rumoured to be stepping down. During Obama’s first term, both became lightning rods for Republican attacks — Chu for his role in approving a \$535-million government loan guarantee to Solyndra, a solar-energy company that later went bankrupt, and Jackson for implementing greenhouse-gas regulations. But even without Chu or Jackson, the administration’s approach to renewable energy and global warming would change very little, says Michael Gerrard, director of the Center for Climate Change Law at Columbia University in New York. “I suspect we would have continuity in the broad policy approaches.”

Obama’s election victory, combined with growing alarm in the United States over ►

Re-elected US President Barack Obama has won four more years in which to cement his legacy.

POLICY

Obama reasserts research focus

But ‘fiscal cliff’ threatens science and climate goals.

BY ERIC HAND, IVAN SEMENIUK, JEFF TOLLEFSON AND MEREDITH WADMAN

Speaking last week in Washington DC, US President Barack Obama reminded voters of the plan they had effectively endorsed by re-electing him. One of his key objectives, Obama said, would be to ensure that the United States “is a global leader in research and technology and clean energy, which will attract new

companies and high-wage jobs to America”.

A different objective will be in the spotlight this week when Obama visits New York, a city still recovering from the damage caused by Hurricane Sandy on 29 October. Climate change could make storms like Sandy more common in the future. And New York’s mayor, Michael Bloomberg, cited climate concerns when he endorsed Obama for re-election (see *Nature* **491**, 167–168; 2012), saying that the

PEOPLE'S CHOICE

Science at stake in state proposals

During last week's national election, US voters also weighed in on state ballot measures that affect research.

Hope for higher education

California voters approved temporary tax increases expected to generate US\$6 billion annually for schools and community colleges through 2017, with smaller revenues through 2019. Passage of the measure also halted imminent state budget cuts that would have cost the University of California (UC) and the California State University system \$250 million each over one year. UC administrators expressed optimism that the measure heralds a more favourable fiscal and political climate for the institution, which has weathered years of state funding cutbacks.

"Education and research are multi-year endeavours," says Keith Yamamoto, vice-chancellor for research at UC San Francisco. "The impact of a lack of stable funding is substantial."

Steve Montiel, a UC spokesman, called the measure "a significant step towards the prospect of financial stability".

Experimentation with marijuana

Colorado and Washington became the first states to legalize marijuana for non-medical purposes, permitting purchase and use of the drug by adults aged 21 and older. Residents of Colorado can also possess up to six marijuana plants.

The new measures set the stage for a legal battle: federal law prohibits the substance, and the US Drug Enforcement Administration says that its policy "remains unchanged". Opponents fear that looser marijuana laws could lead to more drug abuse in the United States. In studies of

drug use in the Netherlands since the country's de facto legalization of marijuana in 1976, Robert MacCoun, a drug-policy expert at the University of California, Berkeley, has found only modest increases in marijuana use and no significant escalation to harder drugs (R. MacCoun and P. Reuter, *Science* **278**, 47–52; 1997).

"Of course, the Netherlands is a different country", but it provides some of the only available data worldwide, MacCoun says. The Washington measure would direct some marijuana tax revenue towards drug-abuse research.

No labelling for genetically modified food

California voters rejected a measure that would have made theirs the first state to require labelling of foods containing genetically modified organisms (GMOs).

Bob Goldberg, a plant geneticist at UC Los Angeles, who co-authored an argument against the proposal in the state's voting guide, calls the measure "ideological and not evidence-based".

Marion Nestle, a food-politics expert at New York University, argues that labelling might have promoted greater consumer trust in GMOs. "Not having a choice induces paranoia in people," she says.

A poll conducted by the California Business Roundtable, a business-advocacy group based in Sacramento, and the School of Public Policy at Pepperdine University in Malibu, California, found that public support for labelling was at around 65% in August, then plummeted in the month before the election. The opposing campaign — backed heavily by Monsanto, a producer of genetically modified seeds based in St Louis, Missouri — escalated its television advertising in the final weeks. **Helen Shen**

starting point, but we think it could become extremely attractive if the lawmakers begin to run out of options for generating revenue," says Mark Muro, a senior fellow at the Brookings Institution, a non-partisan think tank in Washington DC.

Although the idea of any tax — let alone one on carbon — is anathema to most conservatives on Capitol Hill, the idea offers something for everybody in the current budget crunch. A tax would reduce emissions and raise revenue for energy research and development — outcomes that environmentalists would welcome — and it would generate extra revenue that conservative lawmakers could use to offset lower tax rates on individuals and businesses.

In August, economists at the Massachusetts Institute of Technology (MIT) in Cambridge reported that the United States could raise \$1.5 trillion over ten years and reduce emissions to 14% below 2006 levels by 2020 by instituting a tax of \$20 per tonne of carbon in 2013 and increasing it by 4% a year. Less than 20% of that revenue would be enough to fund a massive boost in federal investments in clean-energy research and development — from \$3.8 billion in 2012 to \$30 billion annually, Muro says. The administration has yet to weigh in on the idea, and some on Capitol Hill think that is just as well. If pushed prematurely, the idea could wither in the political spotlight before lawmakers have a chance to fully consider its merits. "We're trying to generate interest," a senior House aide told *Nature*. "The more discussion there is, the better."

FUNDING FEARS

Basic research has historically fared well under Democratic and Republican administrations, but many university administrators were nonetheless relieved by the election's outcome. They wondered whether Republican candidate Mitt Romney would side with other Republicans — including Paul Ryan, his running mate and chairman of the House budget committee — who advocate severe spending cuts to government programmes as a way of reducing the deficit. "Thank God we don't have to find out," says Stewart Smith, dean for research at Princeton University in New Jersey.

Universities that receive grant money from federal science agencies are nonetheless bracing themselves for the fiscal cliff that awaits if Congress cannot reach a budget deal or find a way to extend the bargaining window

before 2 January. The administration's Office of Management and Budget estimates that most funding agencies would have their budgets slashed by 8.2% in the absence of an agreement. Claude Canizares, vice-president for research at MIT, says that the result

▶ severe weather events, such as Hurricane Sandy and the severe drought in the Midwest this summer, could bolster efforts to curb carbon emissions. Jackson laid the foundation for such reductions after the US Supreme Court ruled in 2007 that carbon dioxide is a pollutant, which allowed the EPA to regulate the gas under the Clean Air Act. Jackson went on to craft the first US greenhouse-gas regulations for vehicles, and in March proposed a rule that would effectively ban the construction of new coal-fired power plants unless they are equipped to capture and sequester roughly 50% of the carbon dioxide they emit. The agency next plans to propose rules for existing power plants, then oil refineries. The details of those rules are unclear. The EPA could, for

example, set energy-efficiency standards for different types of power plant or take a more flexible approach that would let states — which normally implement air-quality rules — decide how to proceed.

There is also an outside possibility that Congress's struggle to avoid the fiscal cliff could bring another approach to the fore: a carbon tax. To avoid the automatic spending cuts and tax rises, lawmakers must find new ways to raise revenue. So far, the House has focused on closing tax loopholes, but those efforts are expected to come up short. As an alternative, a carbon tax has been quietly gaining traction in policy meetings, even among some conservatives.

"A carbon tax is certainly not likely as a



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LEFT: T. WILLIAMS/CO ROLL CALL/GETTY
RIGHT: S. SHAVER/UP/EYEVINE

US Environmental Protection Agency administrator Lisa Jackson (left) and energy secretary Steven Chu (right) drew much Republican ire in 2008–12.

would be a loss of \$40 million in research revenue at the university, which drew \$619 million in research support in 2011. An 8% cut in funding to the US Department of Energy (DOE) would mean an 8% loss in staff at the DOE Princeton Plasma Physics Laboratory, of which Smith will become vice-president in January.

Advocates for biomedical research are equally concerned about the prospect of the cuts. “I don’t know how you spare anyone or anything” at the US National Institutes of Health (NIH), says Jennifer Zeitzer, director of legislative affairs at the Federation of American Societies for Experimental Biology in Bethesda, Maryland. The automatic cuts would slash the agency’s \$30.7-billion budget by \$2.5 billion, a portion of which would be exacted from every NIH institute and centre.

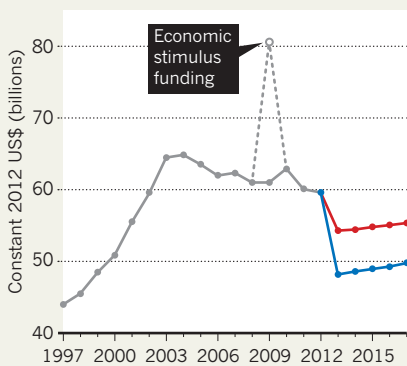
That prospect, in combination with other pressures on medical research budgets, is “chilling”, says Ann Bonham, chief scientific officer at the Association of American Medical Colleges in Washington DC. She notes that the Patient Protection and Affordable Care Act, the health-care-reform law that is the signature policy achievement of Obama’s first term and will ultimately extend health insurance to more than 30 million now-uninsured US citizens, also mandates a \$155-billion cut in government payments to hospitals. That could hurt the large teaching hospitals that support much US medical research (see *Nature* 487, 13–14; 2012). Bonham worries that the confluence of stresses will consign the biomedical research enterprise to “death by 1,000 cuts”.

Supporters of Obama’s health-care reform argue that it will eventually curb soaring US

government outlays for health care, thereby shrinking the deficit and producing more revenue for agencies, including the NIH. If the plan works, the reform is “likely to be a long-term gain for research”, says Ezekiel Emanuel, a medical ethicist and health-policy expert at the University of Pennsylvania in Philadelphia, who was the senior health-policy adviser to the White House budget office from 2009–11.

AT THE PRECIPICE

Annual US funding for non-military research and development will drop sharply if government-wide budget cuts take effect (red). If defence spending is spared, even deeper cuts will apply (blue).



Science could also benefit from another Obama goal: immigration reform. One result of such reform could be more ‘H-1B’ visas for foreign scientists and engineers, a need that both candidates emphasized during the campaign.

In the meantime, Obama’s science agenda

will depend on cooperation with a Congress that includes some new faces. Last week’s general election not only returned him to office and decided state ballot measures (see ‘People’s choice’); it also marked the end of some key lawmakers’ terms. The chairmanship of the Senate energy and water committee is being vacated by the retiring Jeff Bingaman, a Democrat from New Mexico. Some observers expect Ron Wyden, a Democrat from environmentally friendly Oregon, to fill the spot. Although generally liberal, Wyden is known for crossing the aisle to work with Republicans. “Wyden is a very thoughtful guy; he likes to think outside the box,” says Michael Lubell, director of public affairs for the American Physical Society in Washington DC.

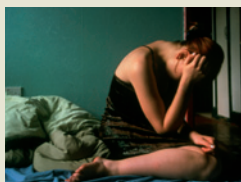
In the House, it’s unclear who will chair the spending subcommittee that funds the NIH. The current chairman, Denny Rehberg of Montana, relinquished his House seat in a failed bid for the Senate. The science, space and technology committee is losing its chairman, Ralph Hall of Texas, who is stepping down in accordance with Republican term limits. Jim Sensenbrenner of Wisconsin, Dana Rohrabacher of California and Lamar Smith of Texas all want the job. Lubell says that his money is on Smith, a co-sponsor of patent-reform legislation who has also tried to make it easier for foreign graduates with science degrees to remain in the United States.

For now, Obama’s victory has created an opening for compromise after two years of Congressional gridlock. With the fiscal clock ticking, the coming weeks may well set the tone for the next four years. ■ SEE EDITORIAL P. 301

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Kasprowy Wierch, a summit in Poland, will soon get new snow-measuring instruments.

CLIMATE SCIENCE

Snow survey hopes for avalanche of data

More accurate snowfall measurements could improve climate models and estimates of water resources.

BY JANE QIU

Mountains are barometers of climate change, but some of the simplest questions about them are the hardest to answer. How much snow coats their peaks and slopes, for example? And how do these frosty shrouds alter from year to year? This week, an international programme kicks off to answer those questions.

In a two-year project called the Solid Precipitation Intercomparison Experiment (SPICE), spearheaded by the World Meteorological Organization (WMO), climate scientists will deploy a suite of state-of-the-art snow gauges at 15 sites in geographically and climatically diverse countries around the world, up

to 4,318 metres above sea level (see 'White noise'). The goal is to make accurate measurements of snow depth and snowfall — the most fragile form of precipitation, which can elude or clog simple collecting devices — and come up with recommendations for the best ways to do snow surveys in different parts of the world. The results could improve climate models and help to predict permafrost stability, ecosystem changes and the availability of water resources in the coming decades.

"Snowfall is an important part of the global hydrological cycle," says Roger Atkinson, acting head of the WMO's Instruments and Methods of Observation Programme in Geneva, Switzerland. "If we can't accurately measure the amount of snowfall, then we won't

be able to know how much water we have and how it will change in the future."

Snowfall also "partly determines whether a glacier grows or retreats," says Zhang Yinsheng, a climate scientist at the Chinese Academy of Sciences' Institute of Tibetan Plateau Research in Beijing, who is not involved in the SPICE project. "People have been debating the fate of Himalayan glaciers for a long time, but we don't have a proper grasp of even the basics."

Although researchers can accurately assess some climate parameters such as temperature, pressure, wind speed and humidity, measuring snowfall remains challenging. Snowflakes are light and drift with the wind, and weather conditions can dramatically affect what proportion of snowfall is actually captured by researchers' cylindrical metal gauges, says climate scientist Rodica Nitu at Environment Canada in Gatineau, who leads the project. And when the temperature is around freezing, the damp snow tends to stick to the rim of the container, soon forming a cap and preventing further collection.

"Undercatch is a serious problem," says Roy Rasmussen, a climate scientist at the US National Center for Atmospheric Research in Boulder, Colorado. This is particularly the case with automatic gauges, which can capture as little as 20% of the actual snowfall, he says.

Unreliable snow readings introduce one of the greatest unknowns to climate models, hampering the ability to predict future changes in water resources and mountain hazards, says Rasmussen. And snowfall, like other forms of precipitation, is expected to increase as the globe warms. Better snow data could help modellers to predict the increase in snowfall, and whether it will be sufficient to offset the increased melting of glaciers.

The last major international effort to measure snow was more than 20 years ago, and "there has been a lot of progress since then," says Rasmussen. One of the main aims of the project is to test the range of recently developed sensors, gauges and windshields. For example, field observations show that shields to reduce the horizontal wind speed above the gauge increase collection enormously. "It's the most important factor for accurate snow measurements," says Rasmussen.

New ways of heating the measuring gauges should also prevent snow capping without causing evaporation or air turbulence that blows the snow away. The field is also switching from manual to automated instruments, enabling continuous measurements over large, hard-to-access areas. Relating the two data sets will make records of snow measurements continuous over time, says Nitu.

Zhang says that the project is timely and important, but that it misses crucial regions such as the Himalayas, where SPICE doesn't have a testing site. Early next year, Zhang and his colleagues will set up a network of

A. RADOSAVLJEVIC/ALAMY

stations across the Tibetan plateau and surrounding mountain ranges, at altitudes up to 6,000 metres, which will gather accurate snow measurements across the region that could augment SPICE's results.

In the longer term, however, "there will never be enough ground measurements to cover an entire mountain", says Michael Lehning, a climate scientist at the Swiss Federal Institute for Snow and Avalanche Research in Davos-Dorf, who is involved in the project. Results from SPICE will be used to calibrate airborne and satellite-based sensors, which use techniques such as microwave, radar and laser ranging to survey much larger areas.

"The idea is to push remote sensing to be accurate enough for use in mountains," says Lehning. "It's still a long way off, but SPICE is a good starting point." ■

WHITE NOISE

The SPICE project aims to test the most advanced techniques for measuring snowfall at sites around the world.



ALTERNATIVE MEDICINE

Chelation-therapy heart trial draws fire

Critics not persuaded that metal-snaring treatment works.

BY EWEN CALLAWAY

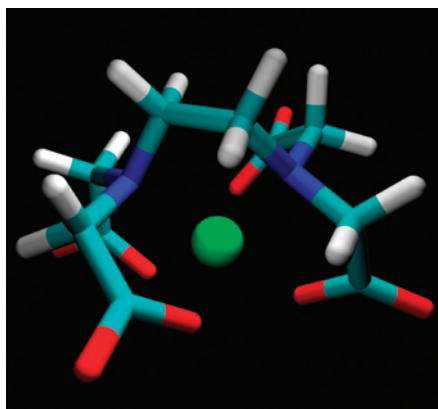
With millions of Americans regularly using complementary medicines, researchers usually applaud efforts to test and debunk folk treatments such as echinacea, a herbal supplement often deployed against the common cold. But what if a trial shows that an alternative therapy might work?

That is the case for a study funded by the US National Center for Complementary and Alternative Medicine (NCCAM), part of the National Institutes of Health (NIH) in Bethesda, Maryland. The trial hints that a fringe therapy intended to sop up metal ions in the blood might reduce participants' risk of heart attack. Critics are attacking both the rigour of the study and the records of some of its investigators, complicating the NCCAM's efforts to answer charges from some researchers that it funds quackery, and raising questions about whether the centre's US\$128-million annual budget is being spent wisely.

The Trial to Assess Chelation Therapy (TACT) was a 10-year, \$31.6-million study involving 1,708 participants at 134 centres. It aimed to test whether weekly infusions of a salt of ethylenediaminetetraacetic acid (EDTA) can lower the risk of repeat heart attacks. EDTA is a chelating agent: the molecule is peppered with electron-rich nitrogen and oxygen atoms, which can grab and hold onto positive metal ions (see picture). The US Food and Drug

Administration has approved one salt, calcium disodium EDTA, to treat lead poisoning.

Proponents of chelation therapy for heart disease initially speculated that EDTA could also cleanse the blood of calcium ions, a component of the atherosclerotic plaques that block blood vessels. But evidence against that hypothesis led them to suggest alternative mechanisms, for example that the molecule captures other metals, preventing heart-damaging inflammation. In spite of the uncertainty, the treatment is already big business: a 2007 US government survey estimated that, every year, 110,000 Americans undergo chelation therapy,



The electron-rich oxygen (red) and nitrogen (dark blue) atoms in ethylenediaminetetraacetic acid can grab and hold onto positive metal ions (green).

which can cost thousands of dollars per course.

According to TACT, which the NCCAM co-funded along with the National Heart, Lung, and Blood Institute (NHLBI), the therapy shows signs of working. On 4 November at the annual meeting of the American Heart Association in Los Angeles, California, trial leaders reported that 26% of patients who received infusions of disodium EDTA went on to suffer a heart attack, stroke or other heart problem, compared with 30% of patients on a placebo — a statistically significant difference.

Many medical researchers were quick to question the results. Perplexingly, the benefit was observed only among participants with diabetes, and 30% of participants dropped out of the trial, undermining comparison between the treatment and placebo. Critics also note that nearly two dozen trial co-investigators have been disciplined by state medical boards for infractions ranging from insurance fraud to providing ineffective treatments. "They offer aromatherapy, crystal therapy and every imaginable wacky form of medicine. You can't do high-quality research at sites like that," says Steven Nissen, a cardiologist at the Cleveland Clinic in Ohio. "We wasted \$30 million and 10 years on an unreliable study."

He worries that the research will be used to support unapproved chelation therapies, which have been linked to heart attacks and death. "Public harm is going to come out of this. People are going to get bilked out of a lot of money. People are going to die."

Kimball Atwood, an anaesthesiologist at Tufts University School of Medicine in Boston, Massachusetts, and one of TACT's most vociferous critics, argues that the trial has been troubled from the beginning. In a paper titled 'Why the NIH Trial to Assess Chelation Therapy (TACT) should be abandoned' (K. C. Atwood *et al. Medscape J. Med.* **10**, 115; 2008), he claimed that trial proponents had mischaracterized earlier studies of chelation therapy ►

► by suggesting that their results were equivocal and necessitated a larger follow-up. Atwood says that the earlier studies in fact found that the treatment was ineffective at preventing heart attacks.

In 2008, TACT was suspended after regulators learned that subjects were not being given calcium disodium EDTA, as implied on informed-consent forms — instead, they were being infused with the slightly different salt disodium EDTA, for which the FDA had revoked approval. The trial resumed after consent forms were reworded to include warnings, such as “death is a rare complication of EDTA infusions”.

Josephine Briggs, director of the NCCAM, declined to comment on TACT until the results are published in a journal. The principal investigator, cardiologist Gervasio Lamas of Mount Sinai Medical Center in Miami Beach, Florida, says that the study's findings were a surprise and deserve following up. He adds that the trial consent forms were approved by the NIH and multiple institutional review boards. Gary Gibbons, director of the NHLBI, says that his institute stands by the study's methodology.

But critics charge that TACT is simply the latest example of dubious research into unproven therapies supported by the NCCAM. Some argue that even high-quality studies would have little value, because negative results are unlikely to sway ardent practitioners. “Show me one alternative medication or procedure that was studied, found to not work, and was abandoned by practitioners. I'm not aware of any,” says Steven Novella, a neurologist at Yale University in New Haven, Connecticut. Briggs, who previously led the NIH's kidney-disease research, points out that echinacea sales fell after an NCCAM-funded study showed it was ineffective against colds (R. B. Turner *et al.* *N. Engl. J. Med.* 353, 341–348; 2005). With the centre's research showing that Americans spend about \$34 billion on alternative medicine each year, “we think it's really important to bring some science into this,” she says.

Briggs adds that the NCCAM's critics often misrepresent the centre's research, focusing on studies of herbal supplements such as lavender oil but ignoring multi-million-dollar grants for more-mainstream science. Among the largest studies funded by the centre this year are a computational analysis of the human microbiome and an effort to use brain imaging to understand and treat chronic back pain.

Novella and other NCCAM critics do praise Briggs for bringing increased accountability to the centre, and for boosting the rigour of the research it funds. But “even if you did pristine research under the NCCAM,” says Novella, “it's what you're studying that is the problem.” ■

BIOTECHNOLOGY

Pig geneticists go the whole hog

Genome will benefit farmers and medical researchers.

BY ALISON ABBOTT

T. J. Tabasco is something of a porcine goddess at the University of Illinois, Urbana-Champaign, where her ruddy, taxidermied head looks down from the office wall of geneticist Lawrence Schook. Now she has been immortalized in this week's *Nature*¹ — not by name, but by the letters of her DNA.

Scientists are salivating. For the past couple of decades they have been slowly teasing information from the pig genome, applying it to breed healthier and meatier pigs, and to try to create more faithful models of human disease. This week's draft sequence of T. J.'s genome (see page 393), with its detailed annotation — a ‘reference genome’ — will speed progress on both fronts, and perhaps even allow pigs to be engineered to provide organs for transplant into human patients. “Agriculture in particular will benefit fast,” says Alan Archibald of the Roslin Institute in Edinburgh, UK, one of the paper's lead authors. “The pig industry has an excellent track record for rapid adoption of new technologies and knowledge.”

T. J., a domestic Duroc pig (*Sus scrofa domestica*), was born in Illinois in 2001. The next year, Schook and his colleagues generated a fibroblast cell line from a small piece of skin from her ear and commissioned clones to be created from it, so that they could work on animals all with the same genome. One set of clones was created at the National Swine Resource and Research Center (NSRRC) in Columbia, Missouri, along with genetically engineered pigs with genes added or deleted to mimic human diseases. “Making such pigs has got increasingly easier as knowledge of the genome increases,” says physiologist Randall Prather, a co-director of the NSRRC, which is funded by the National Institutes of Health (NIH).

The NIH launched the NSRRC in 2003 to encourage research in pig disease models. Pigs are more expensive to keep than rodents, and they reproduce more slowly. But the similarities between pig and human anatomy and physiology can trump the drawbacks. For example, their eyes are a similar size, with photoreceptors similarly distributed in the retina. So the pig became the first model for retinitis pigmentosa, a cause of blindness. And four years ago, researchers created a pig model of cystic fibrosis² that, unlike mouse models, developed



T. J. Tabasco, star of the show.

symptoms resembling those in humans.

Geneticist and veterinarian Eckhard Wolf at the Ludwig-Maximilian University in Munich, Germany, has exploited the similarity between the human and pig gastrointestinal system and metabolism — like us, pigs will eat almost anything and then suffer for it — to develop models of diabetes. One pig model carries a mutant transgene that limits the effectiveness of incretin, a hormone required for normal insulin secretion³. Mice with the transgene developed unexpectedly severe diabetes, but the pigs have a more subtle pre-diabetic condition that better models the human disease. “This shows the importance of using an animal with a relevant physiology,” says Wolf.

Pig models are now being developed for other common conditions, including Alzheimer's disease, cancer and muscular dystrophy. This work will be enriched by the discovery, reported in the genome paper, of 112 gene variants that might be involved in human diseases. Knowledge of the genome is also allowing scientists to try to engineer pigs that could be the source of organs, including heart and liver, for human patients. Pig organs are roughly the right size, and researchers hope to create transgenic pigs carrying genes that deceive the immune system of recipients into not rejecting the transplants.

Back on the farm, early knowledge about the pig genome led to the discovery in 1991 of a gene involved in porcine stress syndrome, in which the stress of overheating, being moved or even having sex causes the animals to die suddenly⁴. It then became possible to test for the ►

► gene and select pig stocks free of it.

Having the full genome should also help investigators to breed out susceptibility to porcine reproductive and respiratory syndrome (PRRS), a viral disease costing the US pig industry US\$600 million per year. The PRRS Host Genetics Consortium, a network of US research groups, has identified a region on one chromosome that affects levels of virus in the blood during infection⁵. Archibald, who works on PRRS, says that the high-quality genome

sequence should help investigators zero in on the genes responsible.

But the pig genome is not just about applications. Lead co-author Martien Groenen, a genome researcher from Wageningen University in the Netherlands, has resequenced the genomes of scores of different strains of wild and domestic pigs, and used the information to show that the pig was domesticated independently in Asia and Europe. He has also started to work out which genes were involved in the

selection of desired traits — such as a longer spine to give more bacon — on different continents. “It’s curiosity-driven research, but it may also help animal breeders in the future,” he says. ■

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BUSINESS

Investment relief for biotech sector

Public markets provide cash injection for struggling field.

BY HEIDI LEDFORD

Robert Forrester gets a little giddy when he talks about the day his company went public. The otherwise understated chief operating officer of Verastem, a small biotechnology company developing drugs to target cancer stem cells, chuckles and bounces in his chair as he recounts key strategic decisions along the way to the company’s initial public offering (IPO) on 26 January, which raised US\$55 million.

Until recently, Verastem’s IPO would have stood little chance. Few biotech companies have braved an IPO in the years since the global recession hit, and those that did often took a beating in the public markets. Venture capitalists began to pull out of the sector. Colleagues scoffed when Forrester told them that Verastem, a young company in Cambridge, Massachusetts, with no clinical data was going public. “Many people said, ‘you’ve got to be kidding,’” he recalls.

But the IPO drought may be ending. This year has seen 12 biotech IPOs, and others are in the pipeline. So far, this has pumped some \$800 million into the sector, according to Renaissance Capital, an IPO-research company based in Greenwich, Connecticut. And biotech stocks are doing well — the NASDAQ Biotech Index has outperformed the NASDAQ Composite Index for the past 20 months (see ‘Bullish on biotech’). “If this trend holds, it could be great news for the sector,” says Josh Lerner, who studies venture capital at Harvard Business School in Boston, Massachusetts.

Restoring access to the public markets — particularly for young companies that have few fund-raising options left — can give companies

the capital they need to expand research programmes, hire more researchers or even just survive. It can also grant them access to ‘generalist’ investors who do not specialize in health care. “Public investors who may have been out of biotechnology for the past couple of years have started to move back in,” says James Healy, a general partner at venture-capital firm Sofinnova Ventures in Menlo Park, California.

Observers credit several factors for the rising investor confidence in biotech. Large pharmaceutical firms eager to restock drug pipelines are gobbling up smaller firms at high prices. Biotechnology companies have celebrated several high-profile successes in the past 18 months, with the US Food and Drug Administration approving groundbreaking drugs such as vemurafenib, a genetically tailored drug for advanced melanoma whose prowess in knocking out tumours is matched by its jaw-dropping price tag — more than

\$50,000 for six months of treatment.

Investors may also be drawn to the sector because of the poor performance of other industries, which are suffering more directly from the sluggish US economy, says Eric Schmidt, an analyst at investment bank Cowen and Company in New York. “Biotech earnings tend to grow independently of the economy, unlike electronics or consumer products,” says Schmidt. “Everybody needs medicine.”

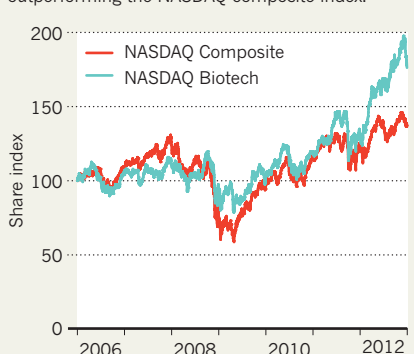
The wave of public investment could help to offset the dearth of venture capital. A survey released last year by the National Venture Capital Association, headquartered in Arlington, Virginia, showed that nearly 40% of venture capitalists had decreased their investment in biotech during the previous three years, put off by the long timelines and high risks of drug development. Several prominent health-care funds have closed altogether. “Biotech is a money-eating machine,” Lerner says. “The need for capital is so large, and given what’s happened to venture capital, having alternatives is important.”

Nowhere is that need greater than in young companies, the riskiest of all biotech investments, which have been among the hardest hit by the drop in venture funding. Verastem’s IPO money advanced the company’s business plan by two years; it should begin phase II trials of its leading compound by mid-2013, Forrester says.

But IPOs are not necessarily the answer for all struggling biotech ventures, cautions Brian Atwood, a managing director of Versant Ventures, a venture-capital firm in Menlo Park. He notes that many of the companies that pulled off IPO triumphs this year are unusual in some respect. Kythera Biopharmaceuticals of Calabasas, California, for example, is particularly appealing to investors because patients will have to pay out of their own pockets for its leading product — a fat-fighting injection designed to shrink double chins — rather than relying on health insurance and its accompanying cost controls. And Verastem’s Forrester can barely utter a sentence without referencing the company’s executives and scientific advisory board: a who’s who of Boston’s biomedical glitterati. Healy agrees: “It’s a higher-quality set of companies that have recently gone public compared with those that may have gone public five years ago.” ■

BULLISH ON BIOTECH

An index fund of biotech companies is outperforming the NASDAQ composite index.



SOURCE: NASDAQ

FUNDING

Berlin aims to create research powerhouse

German government finds a creative way to fund universities.

BY QUIRIN SCHIERMEIER

Berlin is poor but sexy," Klaus Wowereit once famously said of Germany's capital, where he has served as mayor since 2001. Now he is hoping that more money will buy it a little extra love, at least from biomedical researchers. Last week, he proudly announced an opulent deal to boost the city's scattered health-research base, sweetened with a hefty chunk of federal funding. Those in charge of the new Berlin Institute of Health (BIH) that was created by the deal believe that it could rival research powerhouses in the United States and Britain.

It also offers a model for circumventing the country's long-standing restrictions on federal funding of universities, a rule that baffles many outsiders. "That's the way forward, no matter what critics might say," says Wolfgang Herrmann, president of the Technical University of Munich, one of Germany's highest-ranked research universities.

The BIH will ally the Charité, Berlin's largest university clinic, with the Max Delbrück Center for Molecular Medicine (MDC), a national biomedical research centre. Over the next five years, the institute will receive more than €300 million (US\$380 million) in extra funding, 90% of which will come from federal budgets, with the remainder coming from the city and private sources. From 2018 onwards, the federal government will permanently support the BIH with an annual sum of €80 million. "That's a marvellous windfall," says Walter Rosenthal, the MDC's scientific director. "It could help us set up in Berlin something equivalent to the Howard Hughes Medical Institute in Maryland — it's a once-in-a-lifetime opportunity."

The MDC researches basic molecular medicine, but has little direct access to patients, so teaming up with the Charité should help to speed its discoveries to the clinic. Under the auspices of the BIH, molecular biologists and clinical researchers will join forces to tackle a spectrum of illnesses, from cancer to cardiovascular disorders, and neurodegenerative diseases such as Parkinson's and Alzheimer's. The BIH will complement Germany's six national health-research centres, which have narrower focuses. Work at the new institute, spread across Berlin's health campuses, could start next spring. Research coordinators at the Charité

and the MDC have put together a preliminary research plan, which an international evaluation team will review by March 2013.

A significant portion of the start-up cash, says Rosenthal, will be used to equip BIH groups with state-of-the-art sequencing, mass spectroscopy and bio-imaging technologies, and to expand the MDC's bioinformatics capacity. The newly equipped labs and secure long-term funding should lure biomedical researchers from around the world. Over the next 8 years, up to 60 collaborative groups could be set up, Rosenthal says.

"Recruitment is the bottleneck when it comes to turning Berlin into a health-science hub of truly international rank," says Claus Scheidereit, an oncologist who coordinates cancer research at the MDC. "If we can get over that, we can start to think really big."

The BIH is the German government's latest attempt to inject national funds into universities, circumventing a highly federalized system in which state governments jealously guard their responsibility for universities. The gov-

"It could help us set up in Berlin something equivalent to the Howard Hughes Medical Institute."

ernment's acclaimed 'excellence initiative', for example, invited German universities to compete for federal top-up grants and has so far generated thousands of new science jobs.

Collaborations that resemble the BIH, linking national research centres and universities, have also been forged in Karlsruhe, and between Jülich and Aachen.

A proposed amendment to Germany's constitutional law would allow the federal government to co-finance universities permanently, but it is unlikely to win the required two-thirds majority in parliament. Its opponents fear that the bill would allow Berlin to dictate national policy in areas, particularly secondary education, in which the states enjoy near-total control.

Scientists maintain that Germany's federalized university system is hampering the creation of national research hubs such as those in neighbouring Switzerland. "Why shouldn't the Technical University of Munich become Germany's federal institute of technology?" says Herrmann. "Because the rules don't allow it? If it helps our country, we should change them sooner rather than later." ■

Jonathan Tilly defied decades of dogma by suggesting that women can make new eggs throughout their lives. Now some of his critics are taking a second look.

Jonathan Tilly likes to gauge the significance of his work by the hair on the backs of his arms. “Look at it standing up,” he says, thrusting out his forearm on a mid-August afternoon. A reproductive biologist at Massachusetts General Hospital in Boston, Tilly was explaining a procedure to retrieve stem cells from the ovaries of a sterile woman. This experiment, he hopes, will help to quell criticism of his most controversial claim: that ovaries have the potential to make eggs indefinitely. This defies the long-held dogma that female mammals are born with all the oocytes (precursors to eggs) they will ever produce, a population that dwindles with age and is exhausted at menopause.

Tilly first challenged that doctrine in 2004, in a paper¹ suggesting that the oocytes in mouse ovaries are being replenished by stem cells. If properly understood, such cells could be harnessed to generate fresh eggs for women with fertility problems, or even achieve a goal Tilly has been pursuing for 25 years: delaying or halting menopause. “The hairs are still up,” Tilly says. It “happens every time I think about that experiment”.

He has since published a parade of headline-grabbing papers, culminating this year in a report² that he had isolated the elusive stem cells from human ovaries and coaxed them to develop into bona fide oocytes. But his work has been dogged by doubt. Some researchers question his methods and reasoning. Others have tried, and failed, to repeat his experiments. Tilly “always makes what I call ‘big satellites’, something tremendous in the sky,” says molecular biologist Kui Liu at the University of Gothenburg in Sweden. “He exaggerates,” Liu says, and produces a “big press release”. “A few years later, people realize, ‘Oh, not right.’”

Tilly says he has weathered a lot of attacks. “When I made the decision to pursue this, it was out of pure excitement that we found something that could revolutionize the field. It never even crossed my mind that it would be

so negative and so nasty. And it really is negative and nasty.”

But now the stand-off of mistrust, and sometimes open contempt,

has taken a strange twist. Two of Tilly’s most vociferous critics have become his collaborators: one serving on the board of advisers at his start-up company, OvaScience in Cambridge, Massachusetts; the other working directly with the stem cells that Tilly had isolated. “These cells are doing things *in vitro* that can really start to address scientific problems,” says Evelyn Telfer, a reproductive biologist at the University of Edinburgh, UK, who was doubtful of Tilly’s work in the past. “If we are really interested in the science ... then this is a great tool.”

A COUNTING PROBLEM

The ‘no new eggs’ doctrine has a long history. In 1951, the influential anatomist Solly Zuckerman, at the University of Birmingham, UK, performed an in-depth analysis of evidence available at the time. He concluded that none of it effectively countered a proposal from the 1870s stating that female mammals stop producing oocytes after birth³.

For the first 15 years of his career, Tilly focused mainly on programmed cell death, or apoptosis, and he was struck by the fact that no one had ever quantified the loss of eggs due to ovulation and natural oocyte death over time. So beginning around 1999, Tilly commandeered a microscope and mouse ovarian tissue in order to count the follicles, the cellular compartments in which oocytes develop, in mice at different ages. He found a mathematical imbalance: the number of degenerated follicles was three times higher than expected on the basis of the starting pool. If the mice were losing oocytes at this rate, their eggs should be depleted far sooner than they actually were. Something had to be replacing them, he concluded: stem cells were the likely culprit.

Few were willing to accept the idea. It took Tilly two years — and numerous rejections and revisions — to get the data published in *Nature*, in 2004. Controversy ensued over his methods as well as his conclusions. One critique said, for example, that it was “alarming” that Tilly used the rate of follicle disappearance in one mouse strain to calculate loss for another⁴.

Tilly dropped most of his apoptosis work and steered his entire lab towards proving the existence and functionality of these stem cells. “You are sort of standing on the precipice

wondering whether or not you should make the jump,” he says. “Getting the 2004 paper published was for me the jump, because there was no turning back at that point.”

A year later, Tilly reported that he had identified the source of these putative cells: bone marrow⁵. When he transplanted either marrow or blood from healthy mouse donors into sterile mice, the animals could produce cells that looked like oocytes. But he could not yet fertilize the resulting eggs and create embryos — the true test of an egg stem cell.

At least six groups challenged the bone-marrow finding. In one critique⁶, a group led by Telfer wrote that none of Tilly’s experiments had successfully been replicated, and that the results could be interpreted in other ways. Critics also asserted that Tilly was overreaching, particularly in media interviews.

In *The Boston Globe* in 2005, for example, Tilly is quoted as saying: “They’re your own cells; you don’t need anybody’s approval. They go right into your blood supply and go right to your ovaries, where they mature into eggs.” David Albertini, a reproductive biologist at the University of Kansas Medical Center in Kansas City, calls such claims outrageous: “A lot of us reproductive biologists feel that this is a frank travesty that has falsely raised the hopes of many women.”

Tilly defended his comments and challenged his peers to go back to their labs and reproduce his experiments. Several did. In 2006, stem-cell biologist Amy Wagers at Harvard University in Cambridge, Massachusetts, and her collaborators stitched together the circulatory systems of two mice⁷. One, the donor, expressed green fluorescent protein (GFP) in its cells. The other did not. The scientists found that although green, glowing, blood-borne cells could infiltrate the ovaries of the recipient mice, these cells acted like blood cells, not oocytes.

Tilly, in response, performed a similar experiment, showing that mice sterilized by chemotherapy could give birth after a bone-marrow transplant⁸. But the babies did not express GFP, indicating that the eggs from which they were derived came from the recipient, not the donor. Tilly argued that the bone marrow either protected existing oocytes or revived oocyte formation, but critics argued that the chemotherapy probably didn’t kill off all the recipient’s oocytes in the first place.

SHANGHAI SURPRISE

With little independent replication of his work, Tilly was standing alone through much of the fray. Then, in 2009, Ji Wu at Shanghai Jiao Tong University in China and her colleagues reported that they had isolated from mice what she called “female germline stem cells” — not from bone marrow, but from ovarian tissue⁹. When her team transplanted the cells into chemotherapy-treated female mice, they developed into mature oocytes, then fertilizable eggs and, the clincher, healthy pups.

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Although previously sceptical of the prospect, Telfer says that when she read Wu's paper in 2009 she paused, thinking "there must be something in this". She had met Tilly at the bar during a scientific meeting the year before and they talked about their differences. After Wu's paper, the two co-authored a commentary that articulated something like a truce¹⁰. "Although these findings do not establish that oogenesis occurs in adult females under physiological conditions," Tilly and Telfer wrote, "they strongly support the existence of [germline stem

cells] in adult mouse ovaries. If equivalent cells can be found in human ovaries, stem-cell-based rejuvenation of the oocyte reserve in ovaries on the verge of failure may one day be realized."

Many set out to replicate Wu's results, including Tilly. And in February, he reported the isolation of what he called "oogonial stem cells" from human ovaries². By injecting the cells into human ovarian tissue transplanted into mice, he was able to generate both follicles and what seemed to be mature oocytes (see *Nature* **483**, 16; 2012).

"So now we have two different labs, using conceptually a similar protocol, and both groups got confirmatory data," he says. "We felt at that point there should be no more debate."

But there was. Critics soon began pointing out a problem shared by both the teams' approaches. Each identify their respective stem cells using antibodies meant to bind a cell-surface protein, a common technique in cell biology. But the protein they target, called vasa, normally sits inside the cell, not on its surface. "There are a lot of people in the field struggling to understand how this can possibly work," says Patricia Hunt, a reproductive biologist at Washington State University in Pullman.

Tilly says that although mature oocytes do not express vasa on their surface, his cells — which are a cross between embryonic precursors and full-blown oocytes — do. Vasa, he says, becomes non-detectable on the cell surface as the cells mature into eggs. But, he adds, "We don't have any proof of that yet."

Liu in Sweden says that he initially believed Wu's paper when it came out. But his group could not repeat the technique. To bypass the cell-surface problem with vasa, Liu used an approach that tracks the protein inside the cells¹¹. He was able to extract ovarian, vasa-expressing cells, but none of them underwent division — a major criterion for stem cells.

Wu contends that her cell-isolation technique is not easy to perform and invites scientists to come to her lab to learn it. She

adds that Liu's group "didn't use our protocol of isolating the cells. So how to compare?" In fact, Tilly says that his lab had trouble repeating Wu's protocol, too. Eventually, his team retrieved cells but "found consistent oocyte contamination". He had to modify the protocol to retrieve the mouse and human oogonial stem cells, and they differed in size from those Wu had isolated. Wu says that her cells and Tilly's are probably "subtypes" of each other and that there is still "a lot of work to do" to figure out exactly how they are related.

Telfer, meanwhile, has begun to collaborate with Tilly. After going to Boston in 2011 to observe his human stem cells, she was impressed, and took a sample back to Scotland. Her team had worked out a culture system using mouse and cow tissues to grow egg precursor cells into fertilizable eggs entirely outside the body. With Tilly's cells she needed to adapt the technique for use in humans. "The first experiments blew me away, just blew me away," she says. Tilly's cells, she found, grew rapidly into oocyte-like structures. "I spent the whole night trying to find another explanation other than new follicles had formed," she says. "And I could not come up with one."

Telfer has applied for permission from the UK Human Fertilisation and Embryology Authority to attempt to fertilize the cells; such experiments are forbidden using US federal funding. If successful, the technique to make fertilizable human eggs outside the body could eventually be disseminated to fertility clinics throughout the world.

BOUNDARIES AND BACKLASH

The cells are also being used at OvaScience, which was founded in April 2011 and has secured US\$48 million in venture capital. The company is exploiting Tilly's cells in several ways. One aim is to rejuvenate egg cells from older women by adding fresh cytoplasm and mitochondria. The research builds on a controversial experimental fertility technique in which egg cells are injected with cytoplasm from another woman's eggs. The OvaScience approach would use mitochondria extracted from the mother's own oogonial stem cells, which Tilly says would be healthier than those from an ageing mother's eggs, and should skirt some of the ethical and safety questions raised by using donor mitochondria. OvaScience plans to begin clinical trials this year in collaboration with two Boston-based fertility clinics.

Tilly's oogonial stem cells will also serve as a screening tool for new drugs that might block

or boost egg production. Such drugs might help reverse infertility or even help delay or halt menopause. Albertini still worries that such claims inflate hopes but, like Telfer, he is trying to keep an open mind. The prospect of new models for screening fertility drugs convinced him to join OvaScience's scientific advisory board. "There are a lot of things that I know and I do that could be helpful to them," he says.

The company is swiftly moving forward, steered by the team that guided Sirtris, based in Cambridge, Massachusetts, a biotech firm focused on anti-ageing therapies. In fact, it was a collaboration between Tilly and Sirtris's founder, David Sinclair, at Harvard Medical School in Boston, that sparked the launch of OvaScience.

He and Tilly are "mutual admirers", Sinclair says, explaining that they joined forces in 2009 to explore the idea that egg quality declines with age because older eggs lack enough energy to support fertilization. Sinclair offers his anti-ageing expertise and his experience of controversy; some of the initial results on which Sirtris was founded could not be replicated and have been a source of contention in the field (see *Nature* **464**, 480–481; 2010).

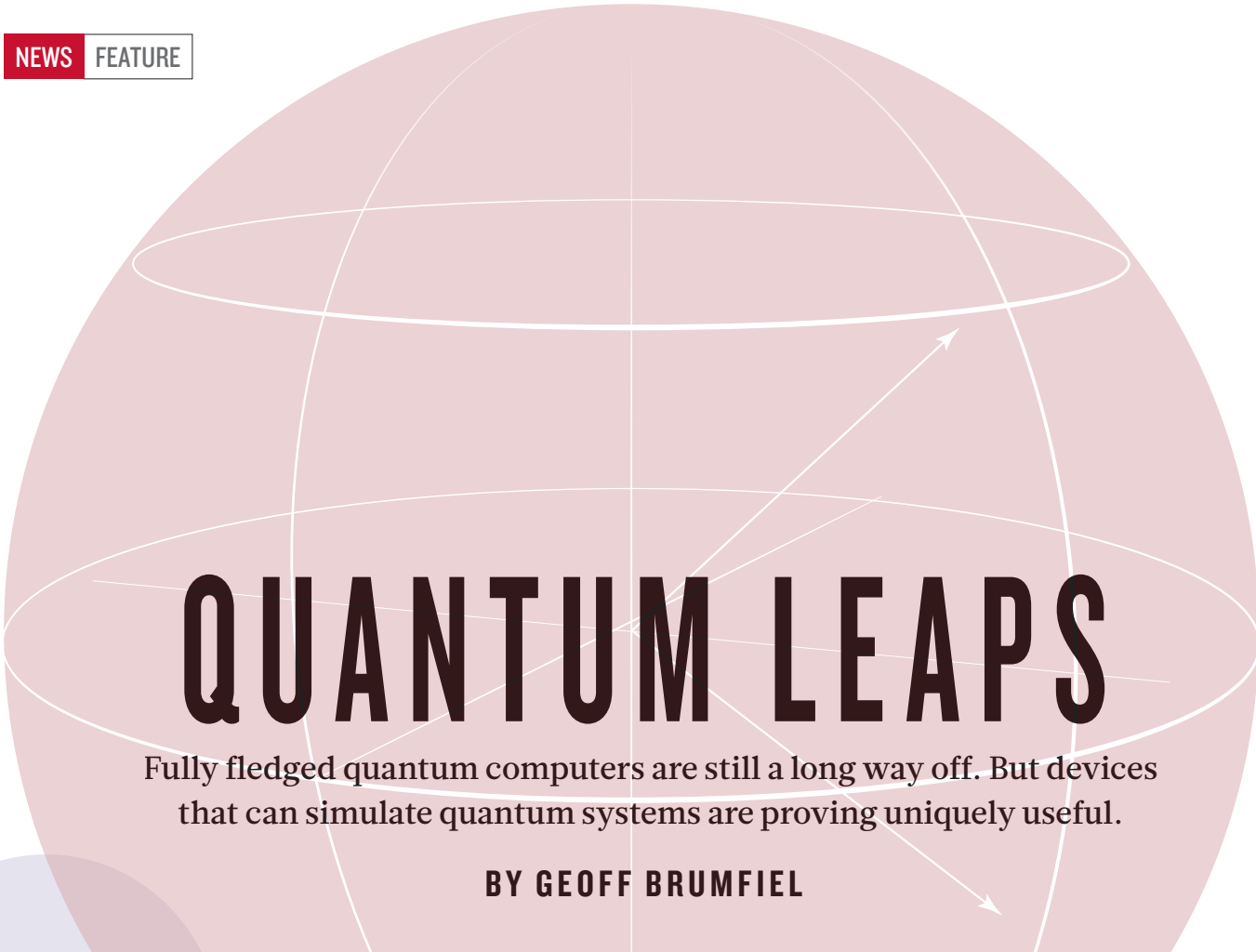
"It is an interesting team that Jon and I make," Sinclair says, "because the two of us push the boundaries of science. And both of us have encountered backlashes in doing so."

Still, despite his characteristic gumption and ebullience, Tilly seems to be burdened by the continual sparring. Although he's shifted much of his time to studying oogonial stem cells in the ovary, he still maintains that bone-marrow stem cells might also create new eggs. His critics disagree, and even if they accept the existence of oogonial stem cells, they still question whether such cells normally function to produce new eggs.

"The data provided so far don't support this concept," says Albertini. Tilly maintains that these stem cells must be doing something in the body. But in exasperation, he is willing to concede that it may not matter in the clinic. "If you could take these cells outside the body, and get them to make a functional egg that can make a normal healthy baby, what do you care about the physiology?" ■

Trisha Gura is a freelance writer in Boston, Massachusetts.

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QUANTUM LEAPS

Fully fledged quantum computers are still a long way off. But devices that can simulate quantum systems are proving uniquely useful.

BY GEOFF BRUMFIEL

When high-energy physicists announced in July that they had found the long-sought Higgs boson — their biggest find in decades — the thousands of individuals involved rightly held their heads high. But in some sense, they had already been beaten to the prize.

Months earlier, a team of nine physicists had taken a rarefied vapour of rubidium-87 atoms, cooled it down to very near absolute zero and used lasers to arrange the atoms into a tiny grid. The physicists then tweaked the temperature until the atoms neared a critical ‘phase transition’ — a point between two different behaviours, such as liquid water and solid ice. Monitoring their grid in this in-between region, the researchers saw an unusual wave of energy that appeared momentarily and then died away¹. Mathematically speaking, this behaviour was the same as the appearance and decay of a Higgs particle inside a particle collider.

“Obviously, it’s not at all the Higgs particle,” says Immanuel Bloch, the researcher who led the study at the Max Planck Institute for Quantum Optics in Garching, Germany. If nothing else, this particle moved in only two dimensions, whereas the Higgs moves in three. But the experiment is still helpful for particle physicists, says Bloch, because it gives them a new way to explore and test the complex quantum field theories that underlie the Higgs.

This experiment also put Bloch and his team at the vanguard of the rapidly growing field known as quantum simulation. The idea, broadly speaking, is to use orderly systems such as a grid of atoms to model much more complicated things — new particles, for example, or high-temperature superconductors. The behaviour of such systems cannot be derived by hand, and even the world’s fastest supercomputers can’t model them.

Quantum simulators are the lesser sibling of an idea in physics known as quantum computers, which have been touted for more than three decades as a way to do everything from complex modelling to code-breaking. What the simulators and computers share is an ability

to operate by the rules of quantum mechanics. Where they differ is in computational power: quantum computers are general-purpose machines able to carry out any possible algorithm, whereas quantum simulators have to be tailored specifically for the problem at hand. Current-generation simulators are also tough to control, and they may not be able to tackle every problem. Nevertheless, the simulators are much easier to build than quantum computers. And researchers say that the devices will soon be able to solve at least some quantum problems that can’t be tackled in any other way.

NUTS AND BOLTS

The world of quantum physics is full of theorems, but one goes unwritten: if you want to get noticed, show that your idea came from Richard Feynman.

Feynman, the mid-twentieth-century’s greatest theoretical physicist, came up with the idea of quantum simulation in 1981 when he was asked to deliver a keynote speech at the Massachusetts Institute of Technology (MIT) in Cambridge². He decided to talk about how physics might be simulated with computers and got straight to the core of the problem: computers run on certainty, but at a fundamental level, nature deals in probability. According to the laws of quantum mechanics, he knew, particles very rarely exist in one state or another, but instead live in a ‘superposition’ of two states at once. When observed, the paradox resolves itself according to the laws of statistics. For example, an electron’s ‘spin’ may orient itself in one direction half the time, and in the other direction for the other half.

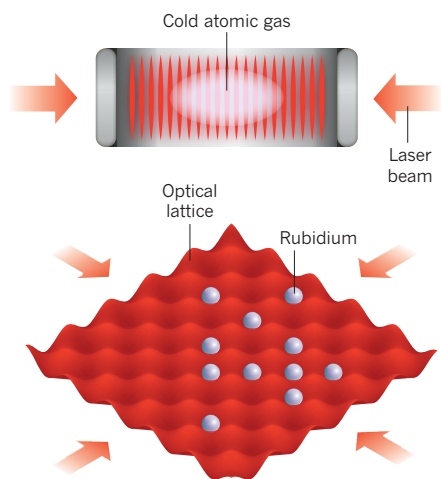
It is not hard to program a normal computer to model the probabilistic behaviour of that one electron, said Feynman. But particles do not live in isolation, and in quantum systems their probabilities are linked, or ‘correlated’. These correlations mean that every combination of particle states must be computed separately, and this creates an exponential rise in complexity. A system with three electrons has eight possible configurations, with eight probabilities to

QUANTUM BOARD GAMES

The set-ups of quantum simulators are different, but the concept is the same: first take atoms, ions or electrons, cool them to cryogenic temperatures and arrange them in an orderly grid. Then tune the interactions on the grid to mimic a more complex material.

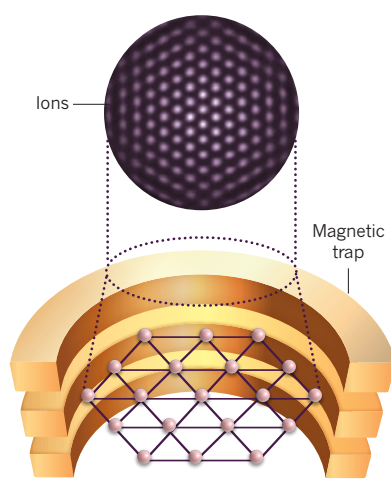
COLD ATOMS

Rubidium atoms are held in place by criss-crossed laser beams, which can also be used to tweak individual particles. A single pair of lasers holds the atoms in a one-dimensional column (top), whereas two pairs hold them in a grid (bottom). Some excitations in the grid system behave like the Higgs particle.



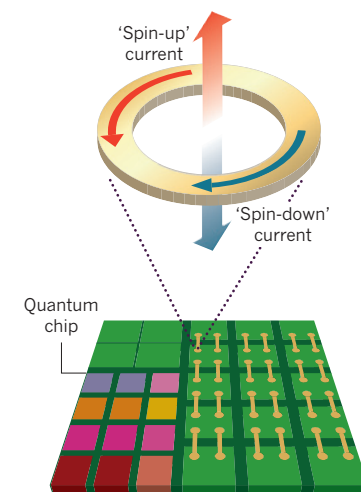
TRAPPED IONS

A combination of electric and magnetic fields trap charged, ionized atoms in an orderly grid. The ions wiggle and rotate in a way that mimics the interactions of quantum magnetism — a phenomenon that can't be simulated in classical systems.



SUPERCONDUCTING LOOPS

A quantized loop of current can flow clockwise, anticlockwise or in a superposition of both in a superconducting circuit (top). An array of such loops (bottom) can be manipulated to simulate various quantum systems — and perhaps even biological processes such as photosynthesis.



compute; 300 electrons create as many configurations as there are atoms in the known Universe.

Feynman spent most of his lecture trying to find a way out of this conundrum. It is not easy using ordinary computers, he concluded, but there is another possibility: build a computer that thinks in terms of probabilities. This quantum imitator, as he called it, would look a lot like whatever system you were trying to model. It wouldn't need to crunch every outcome, but instead would simply recreate the range of probabilities. Rather than delivering one solution, the imitator would deliver many, and the likelihood of each answer would create a probabilistic picture of how the complex system behaves. Feynman didn't do the maths, but he did conclude that almost any quantum system "can be simulated in every way, apparently, with little latticeworks of spins and other things".

At the time of Feynman's talk, the little lattices of which he spoke didn't exist. Quantum systems are extremely fragile, in the sense that almost any interaction with the outside world will destroy the delicate correlations. It has taken 30 years to develop the technology required to keep the particles isolated enough to finish the simulation unimpeded, yet interactive enough to let physicists extract the answer. But there are now several options. Bloch's group uses neutral atoms, other teams are combining electric and magnetic fields with lasers to trap ions of lighter atoms, such as beryllium. A third technique involves controlling eddies of current inside superconducting microcircuits, and a fourth uses quantum particles of light — photons — moving through microscopic waveguides (see 'Quantum board games').

All these techniques are rapidly increasing in their capabilities. In April, a group led by John Bollinger at the National Institute

of Standards and Technology in Boulder, Colorado, unveiled a two-dimensional system of hundreds of trapped ions that could simulate a form of quantum magnetism³. The simulator seems to work well for weak fields of the sort that can already be modelled on classical computers, says Bollinger. Now, with some modifications, he hopes to simulate strong magnetic fields, which are beyond the reach of even the most powerful supercomputers.

Bloch, meanwhile, is considering applications beyond the Higgs for a neutral-atom simulator. For example, the rubidium atoms in his lattice might be used to model a complex class of materials called high-temperature superconductors. These 'high- T_c ' materials can

conduct electrons with no resistance at temperatures much higher than conventional superconductors can — but for decades nobody has been able to understand why. Theorists have developed a number of competing models to explain the behaviour, but haven't been able to test them: the electrons in the superconductors are just too difficult to isolate and study. So Bloch

wants to use atoms as surrogates. By changing the intensity of the criss-crossing laser beams, atoms can be made to tunnel from one point in the lattice to another in a way that mimics the motion of electrons through the atomic lattice of a high- T_c material. At least some theories of high- T_c superconductivity should be checkable with Bloch's set-up.

Quantum simulators might even be able to model non-quantum problems, such as protein folding, that still require huge amounts of computing power to decipher. A group at the Canadian company D-Wave Systems in Burnaby and at Harvard University in Cambridge, Massachusetts, recently did just that by mathematically mapping

"IN A QUANTUM COMPUTER YOU HAVE TO MAKE SURE THAT NO PARTICLE MAKES A MISTAKE."

the folding problem onto a quantum system of 128 loops of current spinning on a superconducting chip⁴. Each loop could spin clockwise, anticlockwise or in a superposition of both directions simultaneously. The performance of the system wasn't great; in one of its protein-folding problems, it found the correct, experimentally determined, answer just 13 out of 10,000 times. Still, says Alán Aspuru-Guzik, a theoretical chemist from Harvard and co-author of the paper, "it's remarkable to me that it was possible to do it" at all.

GOAL CHANGE

Despite all the technical progress, however, the existing simulators are at best a limited approximation of Feynman's original vision — a fully fledged quantum computer that is 'universal', or able to execute any quantum algorithm and simulate any conceivable quantum system. Researchers have been exploring the potential applications of such a device ever since Feynman described it. Arguably the most important one came in 1994, when mathematician Peter Shor, now at MIT, laid out an algorithm that would allow a quantum computer to function as a powerful code-breaking machine⁵. Other quantum algorithms have followed, drawing many scientists (and several intelligence services) into the quest for quantum computing and sparking widespread efforts to create such a machine.

Yet building a powerful, universal quantum computer has proven to be a tough task. A true Feynman computer would be able to control thousands or millions of atoms at once, but most of the current systems face a trade-off between size and control. Bloch, for example, can hold as many as hundreds of thousands of atoms in his laser lattice, but he can't then set their quantum states individually. Other researchers have more control over individual atoms, but their systems, which use trapped ions of beryllium, can manage only a handful of atoms with exquisite precision. On top of this comes the omnipresent problem of disruptions from the outside world, which ruin delicate quantum states: even the tiniest bump will create a computational error.

With current systems so far from the ideal, quantum simulators have come to be seen as less of a stepping stone, and more of a goal in their own right. Simulators do not need to be as large as computers, and, crucially, because the answer is encoded as an average across all their atoms, they are believed to be tolerant of the outside disruptions. "In a quantum computer you have to make sure that no particle makes a mistake," says Ignacio Cirac, a theorist at the Max Planck Institute for Quantum Optics. "In a quantum simulation, if you have 100 particles and one of them is wrong, then 99 are still right."

Some see parallels to the middle of the last century, when scientists such as Vannevar Bush were experimenting with 'analog' computers made from resistors and capacitors. The machines were tailored to specific problems or to a class of problems, and could perform a simple set of operations on an input signal. Some of the devices could even perform mathematical calculations. In retrospect, they seem puny compared with digital computers, which use programmable combinations of transistors to perform practically any program. But they were fast, robust and valuable for applications that matched their architecture, says Seth Lloyd, a theoretical physicist and engineer at MIT. They were particularly good at controlling machinery, for example. "All the control circuits in the Saturn moon rocket were analog," Lloyd says.

Like analog computers, quantum simulators are closely tied to their constituent parts, and are less flexible than a true quantum computer. But Lloyd thinks that they might yet find their 'Moon shot' in problems of quantum complexity. For example, as microprocessors

shrink and new materials are engineered at a molecular level, quantum effects become more and more important. That, in turn, will lead to a dramatically growing need for quantum modelling that allows designers to understand and predict the materials' behaviour. At least some of those needs are going to be met by quantum simulators, Lloyd predicts. "What seems to be happening is that quantum simulators work on a variety of special cases," he says, "and the number of cases seems to be growing rather rapidly."

Aspuru-Guzik has one such process in mind: photosynthesis. When light strikes a leaf, it creates a pair of negative and positive charges that travel long distances to reaction centres, where they are used to make energy for the plant. The charge pairs may travel according to the rules of quantum mechanics: some researchers think that the collective wavefunction of the pairs spreads out across the light-absorbing chromophore molecules inside the leaf, allowing the pairs to move more efficiently than they would classically (see *Nature* **474**, 272–274; 2011).

Aspuru-Guzik and others think that a simulator could help them to pin down exactly how this happens. Photosynthesis is what Aspuru-Guzik calls a "dirty quantum system" — that is, it contains both quantum and classical elements. A little matrix of superconducting current loops might be perfect for modelling it, he argues, because the loops, too, are subject to noise from the outside world. It still wouldn't

be easy, however: Aspuru-Guzik estimates that something such as photosynthesis would require hundreds of quantum bits to simulate, and those systems, he predicts, are at least a decade away.

The ambitions of the scientists developing quantum simulators are considerably more modest. Most are starting their systems out on models that can be calculated with conventional supercomputers to prove that their simulators produce reliable results. Gradually, they plan to push their atoms, current loops or other little units to the point at which the supercomputers can no longer cope.

At that point, "the model that we're able to implement might not even correspond to a real material, but in a sense, who cares?", says Chris Monroe, a physicist at the University of Maryland in College Park. Even if they don't behave like a superconductor or a Higgs particle, the new systems may still be able to tell researchers a thing or two that their older machines can't. Eventually, Monroe and others believe that simulators will be tailored to model different things. Cold atoms, for example, might work best on superconductors, whereas ions could handle magnetism. Of course, there will still be quantum systems that are too tough for any set-up to tackle.

It may be a vision considerably less flashy than Feynman's universal quantum machine, yet within the physics community, quantum simulators are getting more attention than ever before. "Many physicists who sort of pooh-poohed the idea of quantum computing, especially ten years ago or so, they're now sort of embracing this," says Monroe. The systems may be less ambitious, but that may make them more achievable.

Lloyd puts it another way. "If life doles you quantum lemons, let's make quantum lemonade," he says. Simulators may not be as sweet as quantum computers, but "as long as the lemonade is tasty and refreshing, I think that's fine." ■

Geoff Brumfiel is a senior reporter at Nature.

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"THE MODEL THAT WE'RE ABLE TO IMPLEMENT MIGHT NOT EVEN CORRESPOND TO A REAL MATERIAL."

COMMENT

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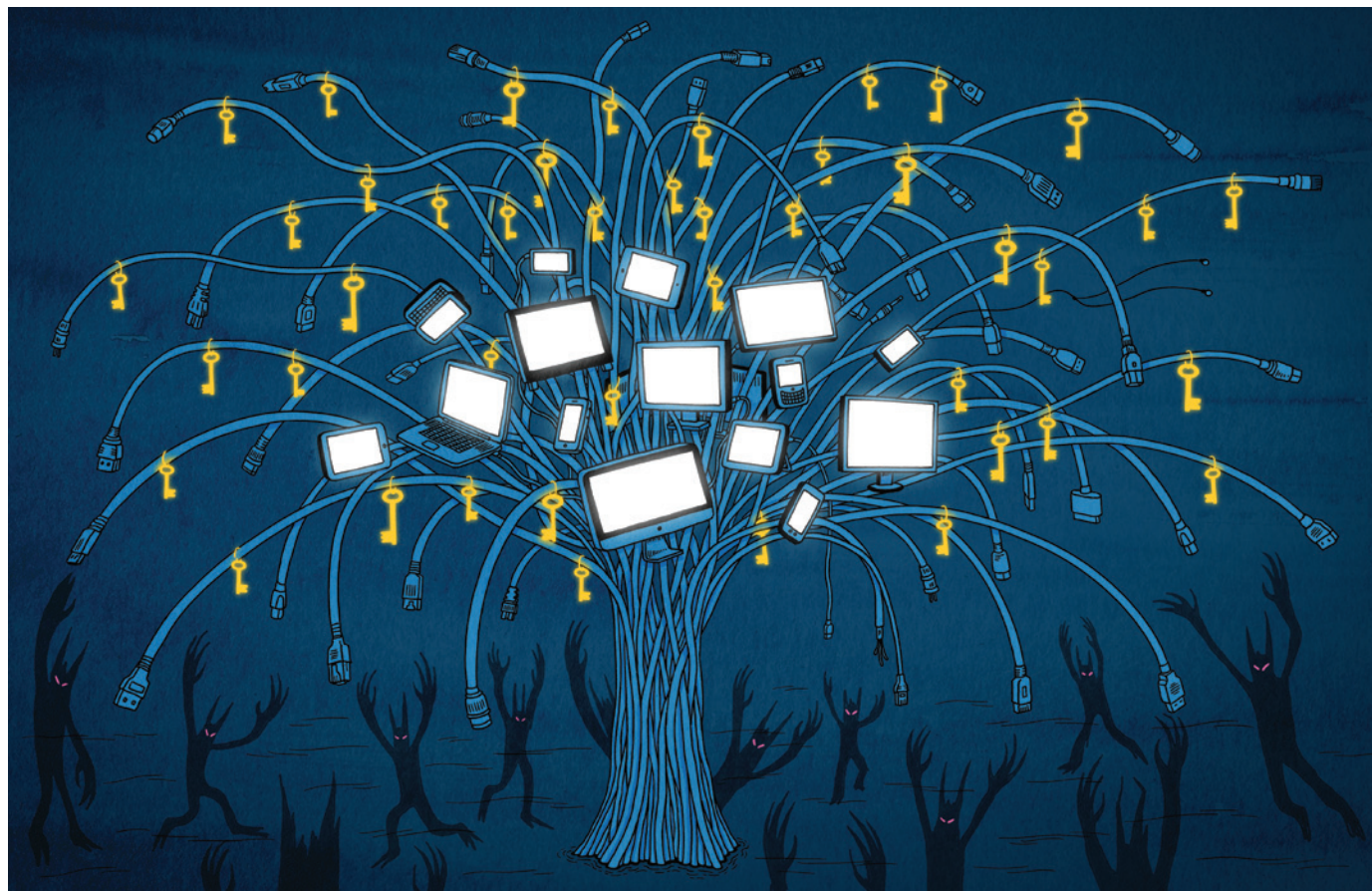
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ILLUSTRATION BY ANDREW RAE



Secure the Internet

Software engineers must close the loophole used to intercept online communications, say **Ben Laurie** and **Cory Doctorow**.

In 2011, a fake Adobe Flash updater was discovered on the Internet. To any user it looked authentic. The software's cryptographic certificates, which securely verify the authenticity and integrity of Internet connections, bore an authorized signature. Internet users who thought they were applying a legitimate patch unwittingly turned their computers into spies. An unknown master had access to all of their data.

The keys used to sign the certificates had been stolen from a 'certificate authority' (CA), a trusted body (in this case, the

Malaysian Agricultural Research and Development Institute) whose encrypted signature on a website or piece of software tells a browser program that the destination is bona fide. Until the breach was found and the certificate revoked, the keys could be used to impersonate virtually any site on the Internet.

Fake certificates are used by hackers and governments to harvest online communications. In 2011, for example, a hacker based in Iran stole the signing keys from DigiNotar, a Dutch CA that declared

bankruptcy soon afterwards. The keys were used to impersonate sites such as Facebook and Gmail in Iranian dissidents' browsers, allowing all of their messages to be read.

Certificates allow the web to work. They secure transactions and allow users to enter credit-card numbers, share data across networks or chat in private forums. Without certificates, hackers could easily stop, corrupt or eavesdrop on these exchanges. But certificates are in trouble. As more authorizing bodies are added to browsers' lists of trusted CAs, and as governments, ►

► hackers and unscrupulous insiders weaken the Internet's security system, it is becoming virtually impossible to know whether a connection is legitimate.

Many Internet technicians, ourselves included, agree that it is time to fix the problem. What remains is the substantial hurdle of reaching consensus about how.

The international Internet Engineering Task Force is adding registration information to the (already overburdened) domain name system (DNS). But websites can still be taken over. The Electronic Frontier Foundation (where C.D. is a fellow), based in San Francisco, California, has proposed a cryptographic protocol called Sovereign Keys (SK) that would make it impossible for a third party to impersonate any website.

"It is becoming virtually impossible to know whether a connection is legitimate."

A third effort is under way, led by a team (including B.L.) at Google, based in Mountain View, California. This protocol — called Certificate Transparency (CT) — is similar to SK, but it includes an independent cross-checking system. Release dates have not been set for either protocol, but CT has the potential to be rolled out sooner, through regular software updates for Google's web browser, Chrome. We see it as a stepping stone to a more ambitious system, such as SK.

We call on browser vendors to support a shift to a more secure system. There are economic barriers — no one is likely to make money from shoring up the Internet. But the risks of ignoring this security loophole are too great.

CROSS-TALK

Before your browser connects to a website, it asks your local network's DNS server for the numeric address corresponding to the website's domain name. (For example, one of the addresses for www.facebook.com is 66.220.149.11.) But DNS is not secure — its communications with browsers are unscrambled, and they are easy to intercept. Anyone sharing your network can steal your credit-card information or passwords.

To scramble messages and keep them private, you need encryption. If a browser has received a cryptographic certificate signed by a CA, its address bar shows a key or padlock icon. All browsers have pre-installed lists of trusted CAs against which to check certificates.

CAs do a lot of due diligence before issuing a certificate, but they are fallible. Cryptographic methods can be used to spot forgery and tampering, but not to distinguish real certificates issued by diligent CAs from those issued by mistake or by a

CA that has been conned or taken over.

The proliferation of CAs is putting the entire Internet at risk. Governments are not responding to the problem — indeed, some policy-makers have shown a remarkable willingness to undermine online security for law-enforcement reasons. India's government, for example, is seeking weaker security for Skype and BlackBerry mobile devices¹. In 2011, US lawmakers proposed the Stop Online Piracy Act, which would require DNS providers to return false results when users try to connect to sites accused of facilitating copyright infringement².

Luckily, software engineers are in a position to fix the certificate loophole.

CERTIFICATE TRANSPARENCY

CT and SK rely on a type of record that uses cryptographic methods to prove that none of its past entries has been erased — an 'untrusted, verifiable, append-only log'. This log is based on a mathematical principle called a Merkle tree — a hierarchy of linked items, or 'leaves'.

In CT, each leaf is a certificate. For a particular node in the tree, a value can be generated in a few steps on the basis of the values of other nodes. For a tree with, say, a million leaves, a verifier would have to track only 20 nodes to confirm any particular leaf. Even a tiny alteration throws off the calculations entirely. With Merkle trees it is possible to prove efficiently that a particular leaf is in the tree without revealing the contents of the other leaves. It is also impossible to fake such a proof for a leaf that is not in the tree.

Merkle-tree logs are stored on a small number of computers, or log servers. Every time a CA generates a new certificate, it sends a copy to all the log servers, which then return a cryptographically signed proof that the certificate has been added to the log. Browsers could be pre-configured with a list of verified log servers (in addition to the list of CAs now installed).

Periodically — perhaps hourly — a number of 'monitor' servers contact the log servers and ask for a list of all the new certificates for which they have issued proofs. These monitors — operated by companies, banks, individuals and service providers — would discover any unauthorized certificates, just as credit reports alert people to cards or loans issued falsely in their names.

This process works only if the log servers are honest; here, auditor servers come in. Every so often, a browser sends all the proofs it has received to a number of auditors — anyone may act as one, because the logs are public. If a proof has been signed by a log server but does not appear in its log, the auditor knows that something is wrong. Within an hour of committing their first transgressions, rogue CAs and

log servers could be detected and removed from browsers' lists.

Even with modest uptake, CT will begin cleaning up the Internet immediately on roll-out. Blocking will improve as more organizations, browser vendors and users participate. Initially, browsers that adopt CT will not be able to block connections for which no proofs are offered. After a year, Chrome will be updated to warn users before establishing a secure connection without a proof. Later, it will not connect to any site without one. We hope that other browsers will follow a similar path.

Also based on Merkle-tree logs, SK is a more theoretical approach. Instead of requiring CAs and domain registrars, SK issues a private key for each website to only one holder. No one else may use that key, so misuse by a third party is not a problem. Anything unverified is blocked, so SK would foil attempts by governments to use domain seizures to censor content that they find to be objectionable.

LONG-TERM GAIN

Through systems such as CT and SK, software engineers can and should solve the certificate problem. After all, the Internet is international and independent; governments cannot mandate a solution.

There are some economic barriers to improving security, but it is a worthwhile investment. CAs have no short-term incentive to support these measures, but the CA network offers the best avenue for rolling out steps such as CT. Similarly, someone must run the log servers, even though doing so will not lead to direct economic gain. But the long-term stability and security of the Internet is good for business. In line with this view, Google will run some log servers — but others should as well, so that we can avoid putting all of our eggs in one basket. Browser vendors should also commit to supporting CT.

History tells us that those who seek new avenues of attack will eventually find them. But this troubling breach must be closed down now. ■

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The global diversity of birds in space and time

W. Jetz^{1*}, G. H. Thomas^{2*}, J. B. Joy^{3*}, K. Hartmann⁴ & A. O. Mooers³

Current global patterns of biodiversity result from processes that operate over both space and time and thus require an integrated macroecological and macroevolutionary perspective^{1–4}. Molecular time trees have advanced our understanding of the tempo and mode of diversification^{5–7} and have identified remarkable adaptive radiations across the tree of life^{8–10}. However, incomplete joint phylogenetic and geographic sampling has limited broad-scale inference. Thus, the relative prevalence of rapid radiations and the importance of their geographic settings in shaping global biodiversity patterns remain unclear. Here we present, analyse and map the first complete dated phylogeny of all 9,993 extant species of birds, a widely studied group showing many unique adaptations. We find that birds have undergone a strong increase in diversification rate from about 50 million years ago to the near present. This acceleration is due to a number of significant rate increases, both within songbirds and within other young and mostly temperate radiations including the waterfowl, gulls and woodpeckers. Importantly, species characterized with very high past diversification rates are interspersed throughout the avian tree and across geographic space. Geographically, the major differences in diversification rates are hemispheric rather than latitudinal, with bird assemblages in Asia, North America and southern South America containing a disproportionate number of species from recent rapid radiations. The contribution of rapidly radiating lineages to both temporal diversification dynamics and spatial distributions of species diversity illustrates the benefits of an inclusive geographical and taxonomical perspective. Overall, whereas constituent clades may exhibit slowdowns^{10,11}, the adaptive zone into which modern birds have diversified since the Cretaceous may still offer opportunities for diversification.

Birds (class Aves) constitute a fascinating and widely-studied radiation. Analyses based on a very incomplete ‘tapestry’ phylogeny¹² suggested higher speciation and diversification rates in the tropics and in South relative to North America^{13–15}. In addition, numerous geographically disparate clades are considered exceptional radiations, including both New and Old World warblers in the Northern Hemisphere^{10,16}, island radiations such as Darwin’s finches¹⁷, and the explosively diversifying white-eyes that span much of the southern Old World¹⁸. The prevalence and implications of such rapidly radiating clades have not been put in broader context: how characteristic are shifts in diversification, and to what extent do clade-specific and tree-wide variation in diversification rate contribute to diversity dynamics across the extant tree of the entire class? Where do these radiating lineages occur, and how much do they contribute to current-day patterns of diversity in the highly diverse tropics compared to relatively depauperate higher latitudes?

We address these questions using the first set of complete phylogenies of extant bird species (9,993 species, see Methods), compiled in a Bayesian framework, and a new species-level measure of past diversification rate. The phylogeny builds on previously established deeper relationships and combines molecular data for 6,663 species with taxonomic constraints for data-deficient species to more fully account

for phylogenetic uncertainty. Lineages-through-time⁸ and novel diversification-rates-through-time plots (Fig. 1) indicate that net diversification leading to extant lineages generally increased from approximately

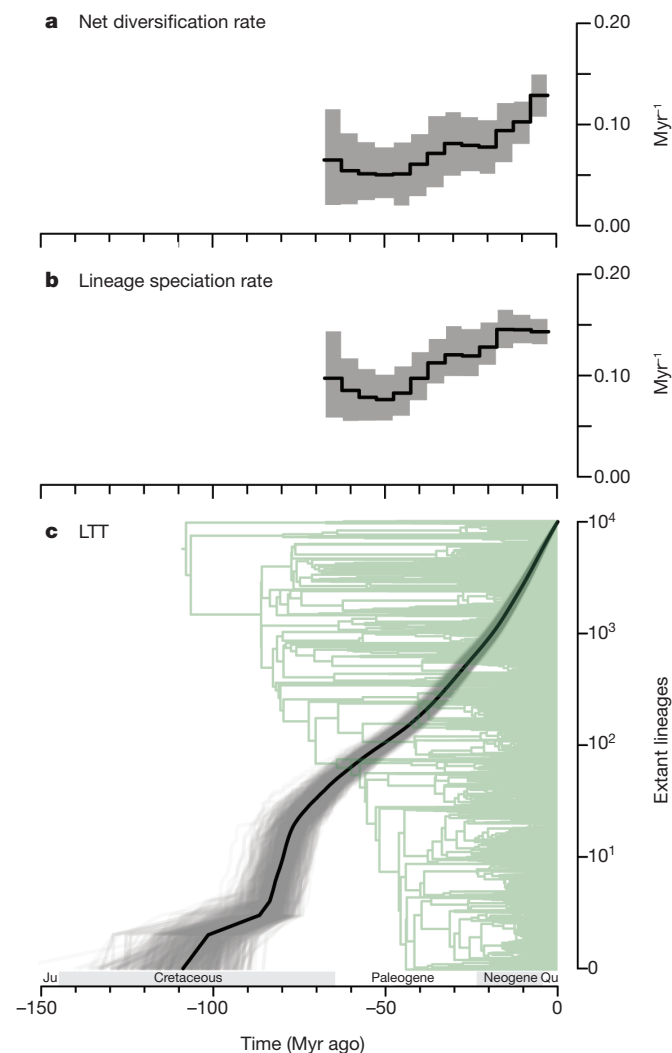


Figure 1 | Diversification of all birds through time. **a, b,** Estimates of the tree-wide lineage net diversification (speciation – extinction) rate (**a**) and speciation rate (**b**), calculated in 5 million year intervals (line segments). These are estimated to be very similar (see Supplementary Discussion). The shaded region represents the area between the 5th and 95th quantiles for 525 assessed trees with the mean rate traced in black. Intervals outside 67.5 and 2.5 Myr ago are not shown due to lack of data (≤ 30 lineages per interval) and the difficulty of accounting for ongoing speciation events, respectively. **c,** Lineage-through time plot for 1,000 trees (in grey), with mean waiting times to speciation in black. Green background is the tree depicted in Fig. 2. Geologic time periods are delineated at the bottom of the plot. Ju, Jurassic period; Qu, Quaternary period.

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50 million years (Myr) ago to at least 5 Myr ago. This contrasts markedly with patterns of decreasing net diversification reported for smaller clades for which a model of niche-filling coupled with long-term lineage competition¹⁹ has been invoked^{9,10,20–22}.

By far the best model for diversification (median Akaike Information Criterion improvement over all other models $\gg 1,700$; Supplementary Discussion and Table 1) is one where a subset of individual clades (identified in Fig. 2 and Table 1) are assigned their own constant and elevated diversification rates. This confirms a space- and time-dependent history of avian diversification, with most of the consistently identified rate increases occurring within the last ~ 50 Myr. Several of these shifts confirm previously identified highly diverse clades with key morphological and behavioural innovations or environmental opportunities, including hummingbirds, parrots and a number of songbird lineages. Some additional young and rapidly radiating groups with rates of per-lineage species accumulation ≥ 0.25 species per million years (Table 1) stand out. These include the previously unrecognized rapid radiations of both ducks and geese, and of select gulls, as well as recognized rapid radiations of ovenbirds plus woodcreepers²³, and of white-eyes¹⁸, all with extant diversity ranging from 44 to 300 species. Intriguingly, these recently rapidly radiating clades show no obvious restriction to parts of the phylogenetic tree (Fig. 2) or landmasses. Rapidly and slowly radiating clades are highly interspersed. Collectively, these tree-wide distributed bursts produce the overall average increase in tree-wide diversification rate (Fig. 1a). Importantly, these novel patterns assume rate constancy within clades, and so integrate (rather than conflict with) changing rates associated with, for example, diversity- or time-dependent slowdowns at smaller scales within individual clades^{9,10,20}.

To understand the consequences of rate variation across the tree for the present-day geographic variation in avian diversity in more detail, we calculate a new metric of species-level lineage diversification rate (DR) for every species. The measure captures the longer-term splitting rate leading to a given species, made possible by the full resolution of the phylogeny. It is directly related to clade level diversification rate measures (see Supplementary Methods), but offers species-level resolution. Mean species values range from 0.01 to 4.66 species Myr⁻¹ and roughly follow a lognormal distribution (Fig. 2, centre) with a geometric mean of 0.157 species Myr⁻¹ and only limited variation owing to

captured phylogenetic uncertainty (Supplementary Discussion Fig. 4). The species level detail illustrates the marked heterogeneity in diversification rates beyond the main shifts and identifies numerous 'hot' sections of recent rapid radiations dispersed widely across the avian tree of life.

This heterogeneity extends to the geographical prevalence of species with a signature of high past diversification. We find that the main geographic differences in diversification rate are east–west hemispheric, rather than latitudinal^{14,15} (see below). Average rates are distinctly lower in the Eastern (east of 28° W, DR = 0.145, $N = 5,810$) compared to the Western Hemisphere (DR = 0.177, $N = 4,183$, $P_{\text{avg}} < 0.05$, $f(P < 0.05) = 73/100$, Fig. 3). In contrast, there are no significant difference in rates ($P = 0.69$, $f(P < 0.05) = 0/100$) between Northern (DR = 0.161, $N = 5,084$) and Southern Hemisphere species (DR = 0.154, $N = 4,909$). Intriguingly, avian assemblages in Australia, Southeast Asia, Africa and Madagascar are characterized by particularly low average rates (below approximately 0.12 species Myr⁻¹ in most locations, compared to a global mean of 0.16). These regions also harbour substantially fewer than expected of the 25% of species with highest diversification rate ($< 15\%$ in most assemblages, Fig. 3d). One hypothesis for the patterns in Australia and Africa is regional density-dependent diversification, with early filling of ecological space by ancient radiations in their regions of origin²⁴. Generally, high diversification rates and large relative prevalence of top diversification rate species are found throughout higher-latitude North America, parts of north Asia and southwest South America—the main breeding areas of several of the rapidly radiating clades identified in Table 1 (including warblers, ducks, gulls and woodpeckers). These regions have all been characterized by strong climatic fluctuations from the Pliocene to the present (with, for example, notable emergence and size fluctuations of wetland and forest breeding habitats), which supports the suggestion that the geography of past climate dynamics has had a major role for today's diversity patterns¹⁴. Isolated locations such as islands have been proposed to strongly facilitate rapid radiations through the reduction in gene flow and novel ecological opportunities they often present after initial colonization^{17,25}. We confirm this globally, as non-pelagic birds with more than half of their range on islands ($N = 1,085$, DR = 0.203) have much larger diversification rates than predominantly mainland birds ($N = 8,629$, DR = 0.153; $P_{\text{avg}} < 0.001$,

Table 1 | Rate shifts found in at least 25% of sampled trees.

Node	English	Scientific	Age	r	Species
G	Select gulls	Select Laridae	4.6 (0.0)	0.74	44
U	White-eyes, select babblers	Zosteropidae, select Timaliidae	8.1 (0.1)	0.49	113
N	Ovenbirds, woodcreepers	Dendrocolaptidae, Furnariidae	17.8 (0.1)	0.28	285
[O]	- Ovenbirds	- Furnariidae	13.6 (0.1)	0.31	223
C	Select ducks, geese	Anseriformes	10.8 (0.1)	0.28	109
T	Babblers, white-eyes	Timaliidae, Zosteropidae	17.7 (0.1)	0.27	355
Y	Weavers, estrildid finches	Ploceidae, Estrildidae	21.9 (0.2)	0.24	278
H	Woodpeckers and allies	Ramphastidae, Indicatoridae, Picidae	36.4 (0.3)	0.17	361
[I]	- Woodpeckers	- Picidae	23.8 (0.2)	0.21	220
X	Passeroidea songbirds	Passeroidea	35.7 (0.1)	0.20	1,418
D	Select hummingbirds	Select Trochilidae	20.7 (0.1)	0.19	266
R	Select sylvioid songbirds	Select Sylvioidae	33.3 (0.1)	0.17	1,031
[S]	- Subset	- Subset	29.1 (0.1)	0.19	704
Q	Passerida songbirds	Passerida	44.7 (0.1)	0.16	3,574
M	New World suboscines	Tyrannides	52.4 (0.2)	0.16	1,227
P	Select Corvida songbirds	Select Corvida	32.1 (0.2)	0.16	442
F	Gulls, terns, auks, skuas, jaegers	Stercorariidae, Alcidae, Laridae	21.1 (0.1)	0.15	131
V	Muscicapoidea songbirds	Muscicapoidea	38.8 (0.1)	0.14	732
[W]	- subset	- Subset	29.4 (0.2)	0.15	603
J	Parrots, songbirds	Psittaciformes, Passeriformes	77.2 (0.2)	0.14	6,320
[L]	- Most songbirds	- Passeriformes	66.8 (0.2)	0.14	5,964
A	Fowl	Galloanserae	78.7 (0.2)	0.08	448
[B]	- Landfowl	- Galliformes	53.9 (0.3)	0.13	267
K	True parrots	Psittacidae	35.6 (0.3)	0.13	330
E	Pigeons, doves	Columbidae	33.4 (0.1)	0.12	289

Rate shifts were identified using MEDUSA⁵ (see Fig. 2 for position and prevalence). Pairs of nested shifts are combined when younger shifts (in square brackets) were depressed by the presence of older to $< 10\%$ occurrence. 'Node' refers to position on example tree (Fig. 2). 'Age' of node is the mean age in units million years (with s.e. calculated across trees) calculated across the posterior sample of trees and 'r' is the mean per lineage diversification rate (in units of Myr⁻¹, s.e. across trees < 0.01 for all) estimated for focal nodes of given species richness ('Species'). All clade-level rate estimates were inferred using Laser²⁸. The background rate as estimated with MEDUSA was $r = 0.056$ Myr⁻¹. Support for nodes was 1.00 for all except U (0.02), T (0.73), S (0.48), P (0.85) and E (0.93).

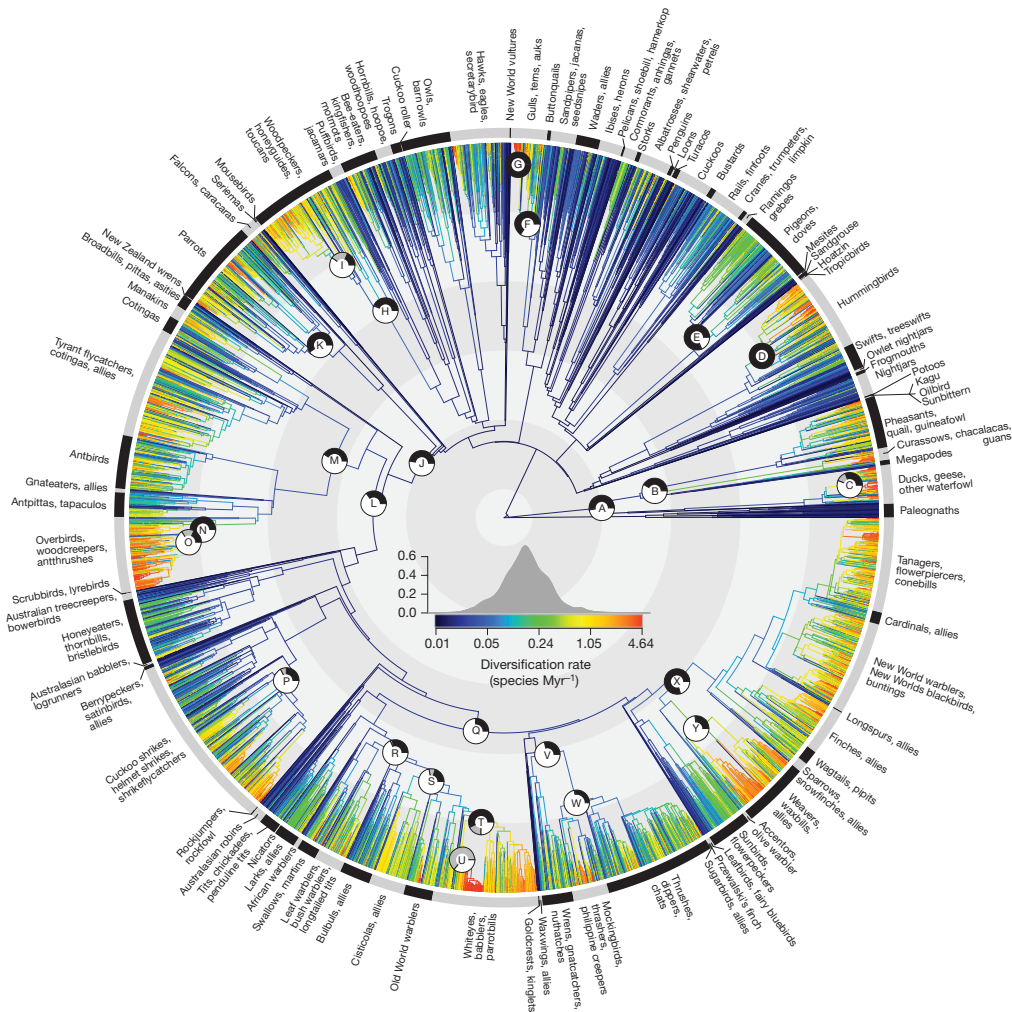


Figure 2 | Diversification across the avian tree. Diversification rate shifts identified by MEDUSA and the species level diversification rate metric (DR) are displayed on a representative avian tree. Nodes with shifts in diversification rate identified in at least 25% of the tested trees are indicated by pie-charts (labelled A–Y, see Table 1). Black and grey areas show the proportion of trees with a shift at the focal node and with shifts that are nested within (more recent than) the focal node, respectively. Shifts are counted only once (for example, shifts at node U do not contribute to the prevalence of nested shifts at node T). Prevalence of shifts may be lowered by a ‘trickle-down’ effect where combinations of nested shifts are rarely identified in the same tree (see also

Supplementary Discussion Fig. 6 for discussion). Branches are coloured according to the mean diversification rate of descendant branches. We colour branches for visualization purposes to highlight tree-wide variation in diversification rate and do not analyse values for internal branches. Diversification rate quantifies the splitting rate along branches leading to a species and offers species-level detail for clade-level diversification rate (see Supplementary Methods). The inset shows the scale and frequency distribution of diversification rate values across species. Concentric grey circles show time from the present in 20 million year intervals.

$f(P < 0.05) = 100/100$). Many islands, particularly those inhabited by passerines in the Pacific, North Atlantic and Caribbean, stand out for their high diversification rates, for example, as shown by the dramatic white-eye radiation (Table 1). As might be expected on the basis of species richness alone, passerines ($N = 5,966$, $DR = 0.179$) have on average much higher diversification rate values than non-passerines ($N = 4,027$, $DR = 0.131$), a difference captured by the diversification rate increase observed at the base of the Passeriformes (node ‘L’ in Table 1, Fig. 2). Accordingly, passerines have a strong influence on the geographic patterns of highest diversification rate (Fig. 3b, c). However, both major groups harbour high and low diversification rate species and both have major hemispheric differences as well as higher rates on islands. Intriguingly though, non-passerines, especially through groups such as water birds, and some gulls and woodpeckers, drive the higher rates in Asia and also show high rates in North America. Passerines show significant recent radiations in temperate Asia and North America. In particular due to the ovenbird and woodcreeper clades, passerines contribute strongly to high average diversification rates and present richness in South America, especially south

of the core rainforest belt. We propose that over the past 10–20 Myr the expansion of core temperate habitats⁴ as well as mountain uplifts and, more recently, the retreat of high-latitude glaciations (followed by range expansions and speciation), all contributed to these distinctive patterns.

These geographic patterns add an important dimension to perspectives that have emphasized latitude¹⁵ as a predictor of both diversification rate and standing diversity. In a highly simplifying global latitudinal view (Fig. 4), high diversification rate species in all tropical lowland regions seem generally outnumbered by species of lower diversification rate. Overall, the simple hypothesis that latitudinal diversity differences result from raw variation in net rates by latitude is rejected: mean diversification rate shows no significant trend with absolute latitude or between the tropics and higher latitudes (Fig. 4). This finding is consistent with the hypothesis that the greater combined age and expanse of tropical moist forests has facilitated greater species accumulation without necessarily facilitating greater rates of diversification⁴. The equatorial dip in average diversification rates is followed by a peak in the slightly drier and (over the past 10 Myr)

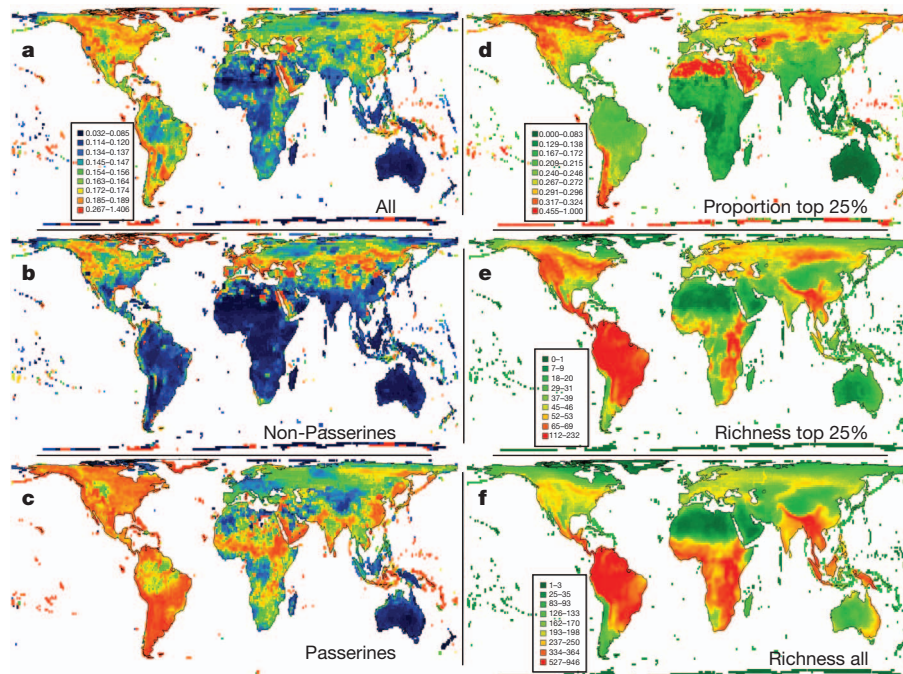


Figure 3 | Geographic variation in species-level lineage diversification rate and the richness of high-diversification rate species. **a–c**, Mean assemblage diversification rate (see Fig. 2), calculated as the geometric mean of all species in a grid cell assemblage, weighted by the inverse of their range size. **a**, All species; **b**, non-passerines; **c**, passerines. This visualization limits the overbearing

(pseudo-replicating) effect wide-ranging species have on perceived spatial patterns of assemblage summaries⁴. **d–f**, Relative (**d**) and absolute (**e**) richness of top 25% diversification rate species ($DR \geq 0.243$ species Myr^{-1}); **f** shows the richness of all 9,993 bird species for comparison. Grid cell size is 110×110 km for all panels (Behrman projection).

climatically more dynamic regions at around $10\text{--}15^\circ$ in both hemispheres and an overall decrease towards the high latitudes. However, as our spatial results indicate, the outcome of any such latitudinal comparisons, excepting perhaps a narrow equatorial band, will depend on hemisphere and region, and the inclusion, prevalence and current evolutionary dynamics of particular clades (see also ref. 3).

Our choice of a constant-rate birth model as a prior on diversification is conservative with respect to clade-level rate heterogeneity, increasing diversification towards the present and major hemispheric differences in lineage-diversification rate. Our results are also robust to differing phylogenetic hypotheses, including currently existing alternative backbone topologies and the inclusion of data-deficient species (see Supplementary Discussion). Indeed, our tree distribution was designed to both integrate the latest taxonomic data and to capture remaining phylogenetic uncertainty given current knowledge; this means it will not be suitable for some questions in avian systematics. In addition, the age of origination of crown group birds and the absolute diversification rates will be contingent on the specific fossil constraints used. Ongoing discovery of cryptic species may further affect rate estimates, especially in the tropics where taxonomists have been less active. Further, and importantly, our inference is necessarily limited to lineages leading to present-day species and cannot account for the non-random extinction of entire clades (for example, of the entire moa clade in New Zealand). Finally, the illustrated spatial patterns tie diversification rate to the occurrence of species today rather than the time of their origination when the distribution of bioclimatic zones and species may have been different. Nonetheless, the uncovered geographic heterogeneity in diversification rates seems pervasive and provides a first global integration of species-level diversification rate variation across both time and space. It may be that birds are distinctive in their pattern of an increasing diversification rate from ~ 50 Myr ago to the recent: the overall adaptive zone into which modern birds have diversified since at least the early Eocene may not be saturated, and opportunities for diversification may be expanding. Alternatively, if the evidence for saturation in other taxa is not due to sampling

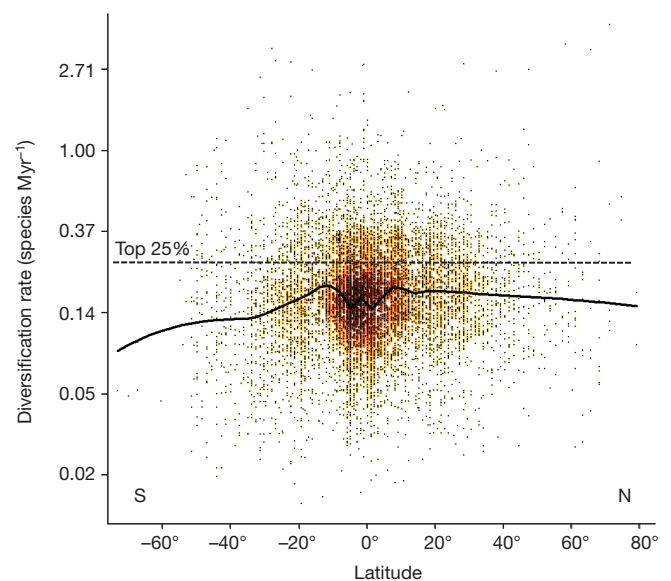


Figure 4 | Latitudinal gradient in species-level lineage diversification rate. Each black point represents a single species diversification rate (DR) at the centroid latitude of its global breeding range. Only the 75% species with small to moderate latitudinal extent ($<26.4^\circ$, $N = 7,493$) are included to ensure comparable centroid positions (see Supplementary Discussion Fig. 7 for very similar patterns and results including all species). There is no significant association between diversification rate and absolute centroid latitude ($P_{\text{avg}} = 0.51$, $f(P < 0.05) = 0/100$) or for intra- ($<23^\circ$ latitude) against extra-tropical centroid location ($P_{\text{avg}} = 0.16$, $f(P < 0.05) = 31/100$). The solid line is a loess smooth over all data (span = 0.2, degree = 2). The dashed line indicates the threshold identifying the quartile of species with highest diversification rate ($DR \geq 0.243$ Myr^{-1}). Darker brown shading highlights greater density of species points.

issues²⁶, then the difference may be due to the novel combined scale and resolution of our analyses, and smaller clades that are geographically or ecologically bounded may indeed saturate^{9–11}. We will need to compare global complete trees such as the one we present here with well-characterized component clades. We predict that constraint-induced slowdowns in net diversification will appear at smaller ecological and geographic scales, but that these do not combine to produce the same pattern at broader scales. This would be consistent with a classical view of multiple and ongoing, individually constrained adaptive radiations as a main driver of biodiversity¹⁹.

METHODS SUMMARY

Phylogenetic tree construction. Pseudo-posterior samples of complete avian trees were assembled as follows. (1) Every bird species was assigned to one of 158 clades identified using a backbone phylogeny²⁷. (2) Relaxed-clock trees were generated for each clade from sequence data. (3) Relaxed-clock trees for entire clades were generated combining species with and without genetic data: species without genetic information (3,330) were placed within their clade using constraint structures consistent with consensus trees from step (2) plus taxonomic information and branching times sampled from a pure birth model of diversification. (4) Final trees were assembled from the clade distributions plus samples of dated backbone trees from (one of two) distributions constructed using relaxed molecular clock techniques, 15 genes, ten fossil constraints and extensive topology constraints derived from published sources. For further information see Supplementary Methods, for trees, see Supplementary Results or <http://birdtree.org>.

Diversification analyses. We compared diversification models on 525 sampled trees: seven models fit smooth changes in rate²⁸, one searches for tree-wide rate shifts²⁹ and one identifies clades that have significantly elevated or depressed diversification rates relative to the entire tree⁵. To visualize diversification through time we estimated tree-wide speciation and extinction rates in five-million-year intervals using TreePar²⁹.

We measured the species-level lineage diversification rate (DR) for every species i as the inverse of its mean equal splits measure³⁰:

$$DR_i = \left(\sum_{j=1}^{N_i} l_j \frac{1}{2^{j-1}} \right)^{-1}$$

where N_i = number of edges on path from species i to the root and l_j = length of the edge j .

Comparative diversification rate analyses. For comparisons of diversification rate we used phylogenetic generalized least squares across 100 trees and list average P values and frequency (f) of trees with $P < 0.05$. For the island–mainland comparison we excluded 279 predominantly pelagic species.

Spatial analysis. We compiled non-invasive breeding distributions from the literature and select updates (see Supplementary Methods). We extracted these ranges over a 110 km equal area grid in Behrman projection excluding cells with <30% dry land or without off-shore islands, resulting in 2.43×10^6 occurrence records over 12,850 cells.

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Supplementary Information is available in the online version of the paper.

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Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to W.J. (walter.jetz@yale.edu) or A.O.M. (amooers@sfu.ca).

Little change in global drought over the past 60 years

Justin Sheffield¹, Eric F. Wood¹ & Michael L. Roderick²

Drought is expected to increase in frequency and severity in the future as a result of climate change, mainly as a consequence of decreases in regional precipitation but also because of increasing evaporation driven by global warming^{1–3}. Previous assessments of historic changes in drought over the late twentieth and early twenty-first centuries indicate that this may already be happening globally. In particular, calculations of the Palmer Drought Severity Index (PDSI) show a decrease in moisture globally since the 1970s with a commensurate increase in the area in drought that is attributed, in part, to global warming^{4,5}. The simplicity of the PDSI, which is calculated from a simple water-balance model forced by monthly precipitation and temperature data, makes it an attractive tool in large-scale drought assessments, but may give biased results in the context of climate change⁶. Here we show that the previously reported increase in global drought is overestimated because the PDSI uses a simplified model of potential evaporation⁷ that responds only to changes in temperature and thus responds incorrectly to global warming in recent decades. More realistic calculations, based on the underlying physical principles⁸ that take into account changes in available energy, humidity and wind speed, suggest that there has been little change in drought over the past 60 years. The results have implications for how we interpret the impact of global warming on the hydrological cycle and its extremes, and may help to explain why palaeoclimate drought reconstructions based on tree-ring data diverge from the PDSI-based drought record in recent years^{9,10}.

Drought is a major natural hazard that can have devastating impacts on regional agriculture, water resources and the environment, with far-reaching impacts in an increasingly globalized world¹¹. The perceived abundance of drought in the 2000s, such as long-term events in the western United States, southeast Australia and northeast China, and the recent short-term but severe events in Russia and the central United States, hint that climate change may be a forcing factor and this is only likely to get worse, given projected climate warming and precipitation changes for the twenty-first century^{1,2}. Many studies have attributed the severity and length of recent droughts to global warming^{5,12}. The Fourth Assessment Report (AR4) of the Intergovernmental Panel on Climate Change (IPCC) summarized the evidence in the following terms: “More intense and longer droughts have been observed over wider areas since the 1970s, particularly in the tropics and subtropics. Increased drying linked with higher temperatures and decreased precipitation has contributed to changes in drought”¹³. The AR4 drew heavily from historic analyses of the PDSI, which shows an increase in drought in the last few decades, regionally^{5,14} and globally⁴, that is commensurate with the increase in global temperatures. The AR4 summary has been substantially revised, however, in the recent IPCC Special Report on Extremes³ that notes the over-reliance on the PDSI and possible overestimation of the increase in regional and global drought.

The PDSI was developed originally as an agricultural monitoring tool in the United States in the 1960s¹⁵ that helped in allocating aid to stricken farmers. It is used pervasively for operational monitoring and increasingly in studies of climate change^{2,16}. Its popularity stems from

its ease of use and long history in agricultural applications. However, the PDSI has several shortcomings because of its simplicity^{3,17,18}, including the treatment of potential evaporation (PE, the evaporative demand of the atmosphere), which is calculated from temperature data by using the empirical Thornthwaite equation⁷. It has been well established that evaporation is a function of more than just temperature, and the correct physics includes radiative and aerodynamic controls on evaporative demand^{8,19–21}. Temperature-based PE methods apparently perform relatively well in climatological applications because air temperature is correlated with net radiation and humidity at weekly, monthly and subannual timescales²². However, estimating trends is problematic and there is extensive literature showing that temperature-based methods are flawed, inherently because the temperature state does not uniquely determine the evaporative flux (see Supplementary Information). In the context of climate change, the temperature-based approach responds to recent observed warming with increasing PE. When used in the PDSI model, which derives soil moisture from the balance between precipitation, evaporation and runoff, the increase in PE drives an increase in drought globally¹³ in addition to the impact of any changes in precipitation. However, numerous studies based on observations and detailed physical modelling have shown regional declines in evaporative demand over past decades as a result of various combinations of declining radiation, vapour-pressure deficit and/or wind speed⁶, despite generally increasing regional temperatures.

To resolve this discrepancy and provide an improved estimate of changes in global drought over the past 60 years based on better

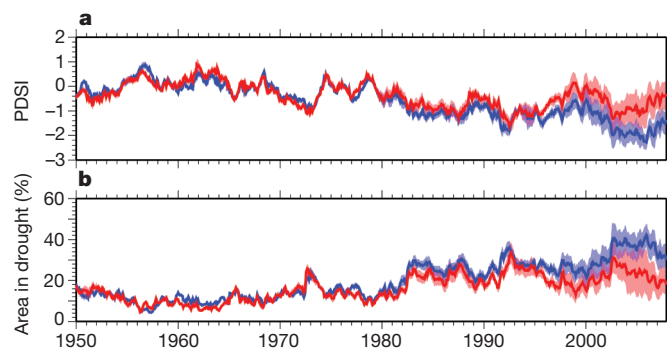


Figure 1 | Global average time series of the PDSI and area in drought. **a**, PDSI_Th (blue line) and PDSI_PM (red line). **b**, Area in drought (PDSI < -3.0) for the PDSI_Th (blue line) and PDSI_PM (red line). The shading represents the range derived from uncertainties in precipitation (PDSI_Th and PDSI_PM) and net radiation (PDSI_PM only). Uncertainty in precipitation is estimated by forcing the PDSI_Th and PDSI_PM by four alternative global precipitation data sets. Uncertainty from net radiation is estimated by forcing the PDSI_PM with a hybrid empirical–satellite data set³¹ and an empirical estimate. The other near-surface meteorological data are from a hybrid reanalysis–observational data set³¹. The thick lines are the mean values of the different PDSI data sets. The time series are averaged over global land areas excluding Greenland, Antarctica and desert regions with a mean annual precipitation of less than 0.5 mm d^{−1}.

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physics, we calculate global changes in the PDSI using two methods for PE. We use the Thornthwaite algorithm and a physically based estimate based on the currently accepted Penman–Monteith (PM) equation^{19,23} forced by our global meteorological data set and a set of alternative precipitation and net radiation data sets (see Methods). Because the PM is a more accurate, comprehensive and physically based model of PE (see Supplementary Information), it also has greater data requirements, which until recently has precluded its use at large scales. However, the increasing availability of detailed meteorological data from gridded ground observations, satellite remote sensing and atmospheric model reanalyses now makes it possible to calculate improved estimates that take into account radiative and aerodynamic controls. Recent studies have claimed that there is little difference between the PDSIs that use the Thornthwaite and PM algorithms (PDSI_Th and PDSI_PM, respectively)^{24,25} but this can be attributed to inconsistencies in the forcing data sets and simulation configuration (see Supplementary Information).

The global averaged time series of PDSI using the two PE methods (Fig. 1a) clearly shows the decreasing trend in the PDSI_Th since the 1970s but not for the PDSI_PM. Uncertainty due to precipitation and net radiation is estimated using four different global precipitation data sets and two different net radiation data sets, and is represented by the shading. The trend for 1950–2008 is $-0.037 \pm 0.004 \text{ yr}^{-1}$ ($P < 0.01$) and $-0.018 \pm 0.005 \text{ yr}^{-1}$ ($P < 0.01$) for the PDSI_Th and PDSI_PM, respectively. The more recent trend over 1980–2008 is similar for the PDSI_Th ($-0.032 \pm 0.008 \text{ yr}^{-1}$; $P < 0.01$) but is essentially zero for the PDSI_PM ($0.003 \pm 0.018 \text{ yr}^{-1}$; not significant). The global area in

drought (Fig. 1b) from 1980 to 2008 increases by $0.559 \pm 0.117\%$ per year ($P < 0.01$) for the PDSI_Th and increases by $0.078 \pm 0.205\%$ per year (not significant) for the PDSI_PM. Despite the smaller overall trend in PDSI_PM, there is still an increase in drought area, although this is seven times smaller than the PDSI_Th drought area trend. The spatial distribution of trends in the PDSI_Th (Fig. 2) shows drying across much of the global land, particularly over Africa and eastern Asia. In contrast, the PDSI_PM shows a mixture of drying and wetting that combines to give a smaller trend globally. The equivalent trends in the PE_Th are increasing everywhere (98% of land area), as expected given the global increase in temperature, but are a mixture of increases (58% of land area) and decreases (42%) for the PE_PM. The two methods disagree in the sign of the trends across much of northern South America, Central America, eastern North America, eastern sub-Saharan Africa, western Russia, southern and southeast Asia, and Australia. Because some of these regions are water-limited, the impact on actual evaporation, and therefore the PDSI, is small. However, in energy-limited regions such as northern Eurasia and the Amazon, the differences in PE translate into differences in the sign of the PDSI trends.

The results show that previous calculations of the increase in global drought are overestimated. However, there are several sources of uncertainty in our approach, not least from the errors in the meteorological data. We use contemporary data, which are the best that are currently available globally, but we recognize that they are not perfect. Nevertheless, the regions of decreasing PE trends estimated with the PM model are generally in agreement with the abundance of evidence

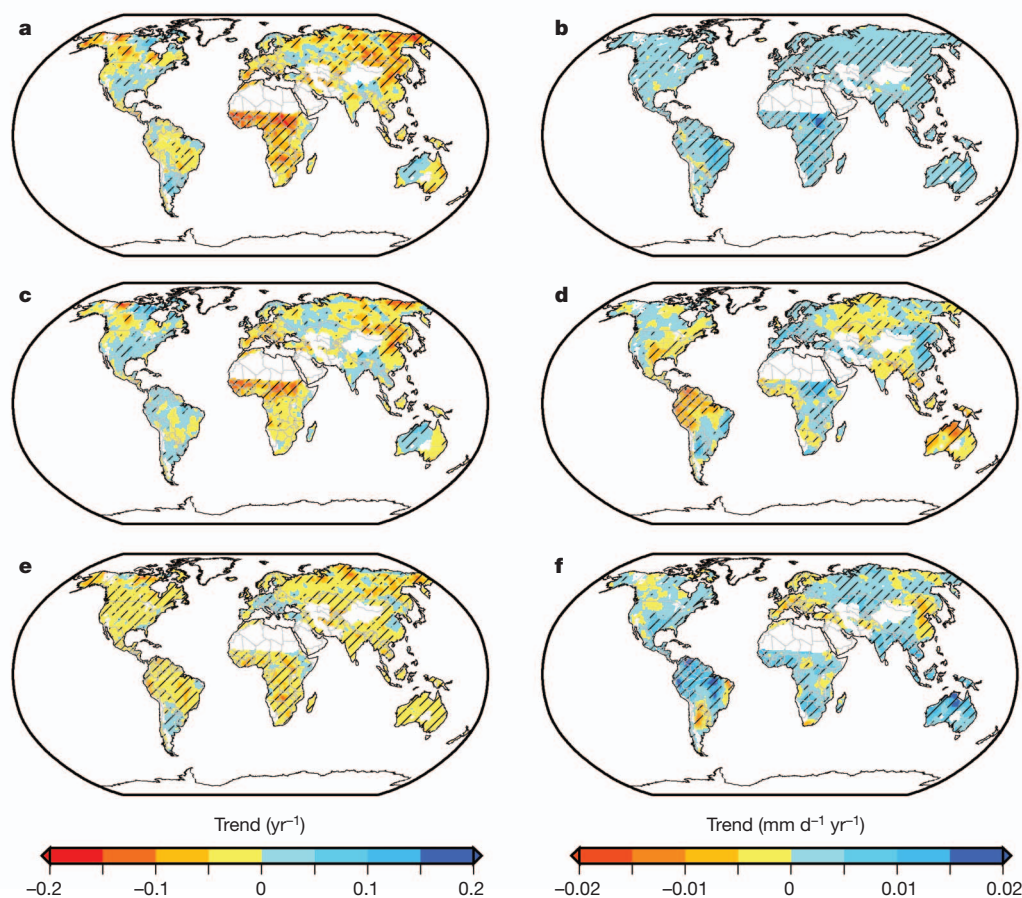


Figure 2 | Trends in the PDSI and PE. a, c, e, Non-parametric trends for 1950–2008 in annual average PDSI (averaged over the results using the four precipitation data sets and, for the PDSI_PM, also over the two net radiation data sets) from the PDSI_Th (a) and the PDSI_PM (c), and their difference (e). b, d, f, Non-parametric trends for 1950–2008 in annual average PE from the Thornthwaite equation

(b) and the PM equations (d), and their difference (f). Values are not shown for Greenland, Antarctica and desert regions with a mean annual precipitation of less than 0.5 mm d^{-1} . Statistically significant trends at the 95% level are indicated by hatching. The difference in trends in e and f and its statistical significance are calculated from the time series of differences between the two data sets.

of decreasing pan evaporation for many regions⁶, which has been attributed to global dimming, decreases in wind speed and in some places decreasing vapour-pressure deficit²⁰ (see Supplementary Information for further discussion). The temperature-based algorithm does not capture these trends. The trends in the radiation, wind-speed and humidity data used to calculate the PE_PM are generally consistent with regional observations, although increasing trends in downward longwave radiation and decreasing trends in wind speed from sparse monitoring networks are generally underestimated by the global data set. However, the possible underestimation of trends in downward longwave radiation cannot explain the difference between the PDSI_Th and PDSI_PM, and underestimation of wind-speed decline implies that the difference is probably a conservative estimate (see Supplementary Information). Furthermore, uncertainty due to the data on precipitation and net radiation as estimated from the standard deviation of PDSI trends for different precipitation and net radiation data sets is much smaller than the difference in PDSI trends due to the PE method. As these data sets improve and the uncertainties are reduced, the magnitude of the calculated trends in drought extent will change; however, the conclusion—that poor physical representation of potential evapotranspiration induces untenable estimates of long-term changes in drought—will remain.

Despite the long-standing consensus that the underlying science for temperature-based estimates of PE is flawed, compounded by the results of this and other studies^{6,21,22} that the flaws are manifested in errors in the estimations of the impact of warming on drought and hydrology in general, the reasons for the long and continued use of the PDSI_Th for climate studies in essentially its original form are a curiosity. The arguments justifying its use are generally based on the availability of data, but they are also probably related to its traditional use for mapping agricultural drought and allocating drought aid, in which comparisons—and thus division of aid—are based on spatial maps made over a short period (weeks to months). Those applications rely on spatial variations that are driven mainly by precipitation deficits, and so the PDSI_Th should do a reasonable job of distinguishing between regions of more or less drought severity at a given instant. However, in the assessment of long-term variations due to climate variability or change, a use for which the PDSI_Th was originally not designed, it seems that the over-sensitivity to changes in temperature, and other simplifications, compromises the comparisons in time. Palaeoclimate reconstructions of drought may be particularly susceptible because they are often developed by scaling tree-ring data to match the calculated PDSI_Th for their overlap period. For some regions, the tree-ring data, which reflect real variations in climatic and non-climatic factors (such as disturbances), diverge from the instrumental-based PDSI_Th in recent decades when warming has been most rapid (see, for example, refs 9, 10). Similarly, the ‘divergence problem’²⁶ as it relates to reconstructions of temperature from high-latitude and high-elevation tree-ring data may be associated with the assumption that temperature can be used as a surrogate for the controls on growth through variations in evapotranspiration, notwithstanding the competing impacts of other environmental factors (for example, higher concentrations of CO₂). This can lead to overestimation of past changes and conversely underestimation of recent trends in the context of the past.

The results of this study have implications for how we interpret the role of global warming in changes to the terrestrial hydrological cycle and its extremes, such as drought, and how we quantify the impacts of future climate change. Several regional studies^{5,12} have suggested that higher temperatures than normal were the cause for increased drought in recent years through increased evaporation. Yet there is evidence that the direct impact of temperature on drying may actually be a misinterpretation of feedbacks between the land and the atmosphere. It is more plausible that evaporation actually decreases during drought²⁷ because of less precipitation, and that drought drives increases in temperatures because there is less evaporative cooling and thus a higher sensible heat flux warming the air²⁸. Short-term temperature anomalies

are likely to be a response to drought, rather than a factor in forcing drought²⁹. Of concern is if the perceived influence of warming on drought as quantified by empirical approaches is extrapolated into the future and predictions of the impacts of climate change are likely to be overestimated^{21,22,30}. It is therefore essential to retain a perspective on the magnitude of impacts of global warming that is based on our physical understanding of the complex relationships between climate and hydrological variability. The use of physically realistic hydrological modelling merged with the wealth of *in situ* and satellite-based data sources has the potential to give better estimates of changes in global drought and its relationship with climate change.

METHODS SUMMARY

We quantify drought with the original and self-calibrating version of the PDSI model¹⁸, which uses the Thornthwaite algorithm (PDSI_Th), and a modified version (PDSI_PM) that uses the PM formulation for PE. The two models (PDSI_Th and PDSI_PM) are forced with precipitation and temperature data from our global meteorological data set³¹, which combines atmospheric reanalysis data with available remote sensing and ground observations and has been updated to 1948–2008. The meteorological data are adjusted to remove spurious trends due to observational system changes (see Supplementary Information). We also use a set of alternative global precipitation data sets to evaluate the impact of uncertainties in global precipitation trends on the drought trends, recognizing that precipitation is the main driver of drought variability but that there are uncertainties in precipitation trends at regional to global scales. The PDSI_PM model additionally requires radiation, humidity and other near-surface meteorological inputs, which are also taken from the updated meteorological data set (see Supplementary Information). Trends in annual mean values are calculated by using the non-parametric Mann–Kendall test and given as the median value across PDSI data sets derived from different precipitation data sets, with the standard deviation given after the $\pm$ sign and estimated by the scaled median absolute deviation. An α value of 0.05 is used to test for significance. The area in drought is calculated as the percentage of land area with a PDSI of less than -3 .

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions J.S. and E.F.W. conceived the study with inspiration from M.L.R. J.S. performed the analyses and mainly wrote the manuscript. E.F.W. and M.L.R. contributed to discussion and the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to J.S. (justin@princeton.edu).

METHODS

Palmer Drought Severity Index. Historically, the PDSI¹⁵ has been the tool of choice when monitoring and analysing drought occurrence, especially in the United States, where it is one component of the US National Drought Monitor³². It is generally calculated on weekly to monthly timescales and uses precipitation and temperature inputs to drive a simplified water-balance model with a generic two-layer soil model and meteorology that is normalized with a reference set of water balance terms. At continental to global scales its simplicity makes it an attractive choice for reconstructing drought records^{4,33}, for which it has also been shown to be a proxy for soil moisture⁴. It has been used to analyse continental-scale to global-scale, long-term variability in drought by several studies (see, for example, refs 4, 34, 35). The PDSI is generally well correlated with output from more comprehensive hydrological modelling³⁶ but diverges in cooler seasons and high latitudes, and substantially so in drier regions.

Despite this legacy, the PDSI has been shown to be unsuitable for widespread application and suffers from simplifications in its physical basis and soil hydrology^{17,37}. For example, its exclusion of cold-season processes makes it unsuitable for application in many parts of the world. The usual form of the PDSI algorithm uses several empirical constants to characterize the local climate. These were originally derived by Palmer using data from a number of climate divisions generally located in the midwestern United States and are therefore not representative of the whole globe. For example, the criteria for signalling the start and end of a drought are arbitrarily based on original data from the midwestern United States. Some of the shortcomings have been addressed by a self-calibrating version¹⁸ that removes the spatial inconsistencies.

We quantify drought with the original and self-calibrating version of the PDSI model¹⁸, which uses the Thornthwaite algorithm (PDSI_Th), and a modified version (PDSI_PM) that uses the PM formulation for PE.

Thornthwaite PE algorithm. PE is modelled in the PDSI by using the temperature-only-based Thornthwaite method. Thornthwaite⁷ correlated mean monthly temperature with PE, as determined from the water balance for valleys in the eastern USA, where sufficient moisture was available to maintain active transpiration. The Thornthwaite formula for monthly PE (mm) is

$$PE = 16d(10T/I)^a$$

where T is the mean temperature for the month (in °C) and d is a correction factor that depends on latitude and month. I is the annual thermal index,

$$I = \sum_{i=1}^{12} (T_i/5)^{1.514}$$

where the subscript i refers to the month of the year and a is an empirical factor,

$$a = 0.49 + 0.0179I - 0.0000771I^2 + 0.000000675I^3$$

Penman–Monteith PE algorithm. The PM approach²³ is generally accepted as the most comprehensive algorithm for modelling potential and actual evapotranspiration (given additional estimates of the plant and environmental resistance to atmospheric demand). It is derived from consideration of the equations of the surface energy balance by means of elimination of the surface temperature term. It forms the basis for the evaporation submodel of many distributed hydrological and land surface models, the latter of which often form the land component of coupled climate models, and has been used as the basis for regional and global retrievals of evapotranspiration based on satellite remotely sensed data (see, for example, refs 38–40). The PM equation given below models the diffusion of energy from plants or soil against stomatal and aerodynamic resistance, given inputs of net radiation, temperature, humidity and wind speed:

$$ET = \frac{\Delta R_{\text{net}} + (\rho c_p D / r_a)}{\Delta + \gamma(1 + r_s / r_a)}$$

where evapotranspiration (ET) is now in W m^{-2} , which can be converted into mm per month by dividing by the latent heat of vaporization of water, λ (J kg^{-1}). Δ (Pa K^{-1}) is the slope of the plot of saturated vapour pressure against air temperature, R_{net} is the net radiation (W m^{-2}), ρ is the density of air (kg m^{-3}), c_p is the specific heat of air at constant pressure ($\text{J kg}^{-1} \text{K}^{-1}$), D is the vapour-pressure deficit (Pa) and γ is the psychrometric constant (Pa K^{-1}). r_a and r_s are the aerodynamic and stomatal resistances (s m^{-1}), respectively. ET collapses to PE when the stomatal resistance is zero; the recommended form of the equation¹⁹, given as the sum of the radiative and aerodynamic components, is

$$PE = \frac{\Delta}{\Delta + \gamma} R_{\text{net}} + \frac{\gamma}{\Delta + \gamma} 6.43(1 + 0.536U)D$$

where PE is now in mm d^{-1} ; U is the wind speed (m s^{-1}) at 2 m height. In comparison with the Thornthwaite expression, which is based solely on temperature, the PM models evaporation as the combination of radiative and aerodynamic processes, thus giving a more realistic estimate and having the potential to be influenced by changes in humidity, radiation and wind speed, as well as temperature.

Global meteorological forcing dataset. The two PDSI models (PDSI_Th and PDSI_PM) are forced with precipitation and temperature data from our global meteorological data set. The PDSI_PM model additionally requires radiation, humidity and other near-surface meteorological inputs, which are also taken from this data set. The global meteorological forcing dataset³¹ combines reanalysis data and observations to form a global, long-term (1948–2008), 1.0°, 3-hourly data set of precipitation, surface radiation and near-surface meteorology. The data set is designed for forcing land-surface hydrological models and other physical models at large spatial (regional to global) and temporal (annual to decadal) domains. Thus the goal of the data set is to ensure robustness of long-term trends and variability. At the same time it adjusts the short-term (daily and diurnal) variations to match observational data where available and maintains interrelations between variables. The data set is based on the NCEP/NCAR reanalysis (NNR)⁴¹, which provides continuous records of atmospheric and land variables from 1948 to the present. The reanalysis data are corrected to remove biases at diurnal to annual timescales by merging with observational data for precipitation, air temperature, and shortwave and longwave radiation. Precipitation and temperature are scaled to match the Climatic Research Unit (CRU) TS3.0 data set⁴² on a monthly timescale. The diurnal cycle of precipitation is resampled from a statistical model derived from the Tropical Rainfall Measurement Mission (TRMM) Multi-Satellite Precipitation Analysis (TMPA) satellite-based data set⁴³. The diurnal range in air temperature is also adjusted to match the CRU TS3.0 data set. For 1984–2007, the shortwave and longwave radiation are scaled to match the NASA/GEWEX Surface Radiation Budget (SRB) satellite based data set⁴⁴ on a monthly timescale. The data set is available from <http://hydrology.princeton.edu/data.pgpf.php>.

Alternative precipitation data sets. A set of four global precipitation data sets is used to quantify the uncertainty in global precipitation. The data sets are CPC-Prec/L⁴⁵ (<ftp://ftp.cpc.ncep.noaa.gov/precip/50yr/>), Global Precipitation Climatology Centre V4 (ref. 46) (GPCC; <http://gpcc.dwd.de>), Climatic Research Unit TS3.0 (ref. 42) (<http://badc.nerc.ac.uk/browse/badc/cru>) and Willmott–Matsuura V2.01 (ref. 47) (http://climate.geog.udel.edu/~climate/html_pages/download.html).

Trend calculation. Trends in annual mean values are calculated by using the non-parametric Mann–Kendall test and given as the median value across PDSI data sets derived from different precipitation data sets, with the standard deviation given after the $\pm$ sign and estimated by the scaled median absolute deviation. An α value of 0.05 is used to test for significance. The area in drought is calculated as the percentage of land area with a PDSI of less than -3 .

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Spontaneous motion in hierarchically assembled active matter

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With remarkable precision and reproducibility, cells orchestrate the cooperative action of thousands of nanometre-sized molecular motors to carry out mechanical tasks at much larger length scales, such as cell motility, division and replication¹. Besides their biological importance, such inherently non-equilibrium processes suggest approaches for developing biomimetic active materials from microscopic components that consume energy to generate continuous motion^{2–4}. Being actively driven, these materials are not constrained by the laws of equilibrium statistical mechanics and can thus exhibit sought-after properties such as autonomous motility, internally generated flows and self-organized beating^{5–7}. Here, starting from extensile microtubule bundles, we hierarchically assemble far-from-equilibrium analogues of conventional polymer gels, liquid crystals and emulsions. At high enough concentration, the microtubules form a percolating active network characterized by internally driven chaotic flows, hydrodynamic instabilities, enhanced transport and fluid mixing. When confined to emulsion droplets, three-dimensional networks spontaneously adsorb onto the droplet surfaces to produce highly active two-dimensional nematic liquid crystals whose streaming flows are controlled by internally generated fractures and self-healing, as well as unbinding and annihilation of oppositely charged disclination defects. The resulting active emulsions exhibit unexpected properties, such as autonomous motility, which are not observed in their passive analogues. Taken together, these observations exemplify how assemblages of animate microscopic objects exhibit collective biomimetic

properties that are very different from those found in materials assembled from inanimate building blocks, challenging us to develop a theoretical framework that would allow for a systematic engineering of their far-from-equilibrium material properties.

We assembled active materials from microtubule filaments, which are stabilized with the non-hydrolysable nucleotide analogue GMPCPP, leading to an average length of 1.5 μm . Bundles were formed by adding a non-adsorbing polymer—poly(ethylene glycol) or PEG—which induces attractive interactions through the well-studied depletion mechanism. To drive the system far from equilibrium, we added biotin-labelled fragments of kinesin-1, a molecular motor that converts chemical energy from ATP hydrolysis into mechanical movement along a microtubule⁸. Kinesins were assembled into multi-motor clusters by tetrameric streptavidin, which can simultaneously bind and move along multiple microtubules, inducing inter-filament sliding (Fig. 1a). In this respect, our experiments build upon important earlier work that demonstrated the formation of asters and vortices in networks of unbundled microtubules and kinesin^{9,10}. However, compared to these dispersed networks, the proximity and alignment of depletion-bundled microtubules greatly increases the probability of kinesin clusters simultaneously binding and walking along neighbouring filaments, thus enhancing the overall activity.

Motor-induced sliding of aligned microtubules depends on their relative polarity. Kinesin clusters generate sliding forces between microtubules of opposite polarity, whereas no sliding force is induced between microtubules of the same polarity^{11–13}. To study the dynamics

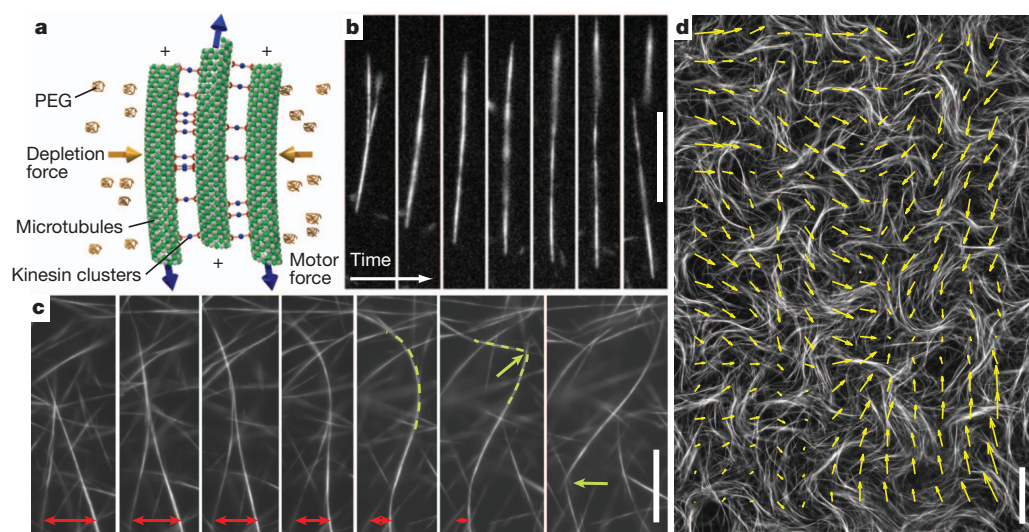


Figure 1 | Active microtubule networks exhibit internally generated flows. **a**, Schematic illustration of an extensile microtubule-kinesin bundle, the basic building block used for the assembly of active matter. Kinesin clusters exert inter-filament sliding forces, whereas depleting PEG polymers induce microtubule bundling. **b**, Two microtubule bundles merge and the resultant bundle immediately extends, eventually falling apart. Time interval, 5 s; scale bar, 15 μm . **c**, In a percolating microtubule network, bundles constantly merge (red arrows), extend, buckle (green dashed lines), fracture, and self-heal to produce a robust and highly dynamic steady state. Time interval, 11.5 s; scale bar, 15 μm . **d**, An active microtubule network viewed on a large scale. Arrows indicate local bundle velocity direction. Scale bar, 80 μm .

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of active bundles, we confined a dilute suspension of microtubule bundles to quasi-two-dimensional (2D) chambers. No sliding is observed for isolated bundles, indicating that they are locally polarity sorted. However, owing to passive diffusion, quasi-static bundles occasionally encounter each other and join as a result of attractive depletion interactions. Merging bundle domains are equally likely to have the same or opposite polarity, the latter case resulting in their relative sliding and even bundle disintegration (Fig. 1b). In one case, two polarity-sorted bundles met with an initial anti-parallel orientation and promptly slid off each other. Subsequently, they rotated by 180° and rebound with relative orientation opposite to their original, and this time no sliding occurred (Supplementary Video 1). Overall, the active microtubule bundles almost exclusively exhibited extensile behaviour, in contrast to actin/myosin bundles, which preferentially contract¹⁴. Consequently, as we show next, gels constituted from extensile microtubule bundles are strikingly different from actin/myosin gels, which exhibit bulk contraction^{15,16}.

Increasing the concentration of extensile microtubule bundles led to the formation of percolating bundled active networks (BANs) with unique properties (Methods). For example, in contrast to classical polymer gels, which respond only passively to externally imposed stresses, BANs exhibited internally generated fluid flows (Fig. 1d, Supplementary Video 2). These highly robust spatiotemporally chaotic flows occurred throughout millimetre-sized samples and can persist for up to 24 h, limited only by the available chemical fuel (ATP) produced through the regeneration system. To gain insight into the microscopic dynamics that drive the formation of such patterns, we visualized constituent microtubule bundles at high magnification. Similar to the dilute case, we observed extension of bundles due to internal polarity sorting. However, in contrast to the dilute case, the extending microtubule bundles were now connected to a viscoelastic network. This caused them to buckle at a critical length scale¹⁷ and subsequently fracture into smaller fragments which quickly recombined with surrounding bundles of random relative polarity, generating further extension.

Thus, BAN dynamics are determined by cycles of microtubule bundles undergoing polarity sorting, extension, buckling, fracturing and subsequent recombination (Fig. 1c, Supplementary Video 3). The emergent patterns, involving thousands of microtubule bundles, are qualitatively similar to those observed in other non-equilibrium systems such as swarming bacteria or swimming spermatozoa^{18,19}. Theoretical coarse-grained models of extensile rod suspensions predict the occurrence of hydrodynamic instabilities that may underpin such large-scale collective effects^{20,21}. Being constructed from the bottom up, the properties of BANs are easily controlled and optimized. For

example, kinesin walking velocity is tuned by ATP concentration, allowing us to examine how active flows depend on the underlying microscopic dynamics (Supplementary Video 4)⁸.

To quantify fluid flow, we embedded micrometre-sized beads into the BANs (Fig. 2a, Supplementary Video 5). The beads were coated with a polymer brush to suppress their depletion-induced binding to microtubule bundles. In the absence of ATP, the tracer particles probed a passive viscoelastic network, and their mean square displacement (MSD) was sub-diffusive (Fig. 2b). At intermediate ATP concentrations, MSDs became super-diffusive at longer timescales, while remaining sub-diffusive at shorter timescales; indicating that the beads were being advected by internally generated flows. Similar effects were observed for beads suspended in active bacterial baths²². At saturating ATP concentrations, MSDs were essentially ballistic; that is, beads moved along straight paths. At longer timescales, all MSDs should become diffusive; however, this requires measuring long trajectories that are not accessible to our imaging system.

We also examined the spatial structure of internally generated fluid flow by calculating the equal-time velocity–velocity correlation function between bead pairs, $\langle V(R) \cdot V(0) \rangle$, where R is their lateral separation (Fig. 2c). The correlation functions decayed exponentially and become anti-correlated at approximately $200 \mu\text{m}$. The decay of $\langle V(R) \cdot V(0) \rangle$ taken at different ATP concentrations collapsed onto a universal curve when rescaled by the peak velocity $\langle V(0)^2 \rangle$, revealing that the spatial structure of the internally generated fluid flow is independent of kinesin velocity (Fig. 2c). For saturating ATP concentrations, the average bead velocity ($\sim 2.2 \mu\text{m s}^{-1}$) was approximately three times the maximum kinesin velocity⁸. Previous work on unbundled microtubule–motor mixtures described temporally transient dynamics before settling into a quasi-static state consisting of either aster or vortex defects¹⁰.

BANs are fundamentally different at both macroscopic and microscopic scales. At macroscopic scales, BANs exhibit steady-state permanent flows that persist for days and are only limited by ATP availability and stability of the constituent proteins. At microscopic scales, BAN dynamics is driven by cascades of microtubule bundles extending, buckling, self-fracturing and self-healing. Such unique dynamics inherently demands the presence of a depletion agent, absent in previous studies. Furthermore, BANs are very robust, exhibiting the same behaviour over a wide range of microscopic control parameters (0.4% to 10% PEG, $0.5\text{--}5 \text{ mg ml}^{-1}$ microtubules, $10 \mu\text{M}$ to 3 mM ATP, $1.5\text{--}120 \mu\text{g ml}^{-1}$ kinesin). Finally, as we show next, BANs under confinement exhibit qualitatively distinct behaviour from those of unbundled microtubule–kinesin networks²³.

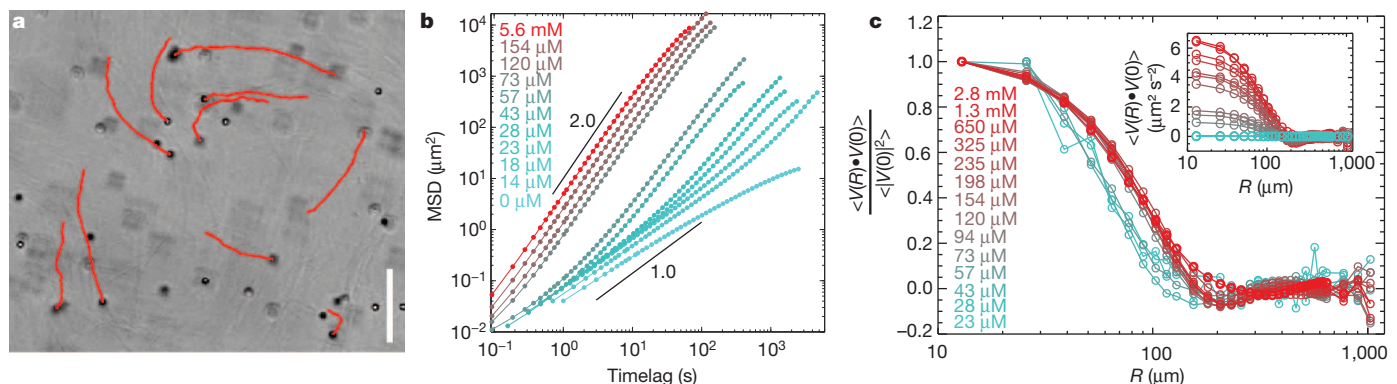


Figure 2 | ATP concentration controls dynamics of active microtubule networks. **a**, Tracer particles embedded in BANs indicate local fluid flow. Trajectories of the particles (red paths) reveal highly non-Brownian motion. Scale bar, $80 \mu\text{m}$. **b**, Mean square displacements of tracer beads plotted as a function of time for different ATP concentrations (colour-coded on the figure). Lines indicate a diffusive exponent of 1.0 and a ballistic exponent of 2.0. **c**, Normalized spatial velocity–velocity correlation functions as a function of

lateral separation for varying concentrations of ATP (colour-coded on the figure). The velocities were determined using equal time intervals of 5 s. When normalized by the peak velocity $\langle V(0)^2 \rangle$, the correlation functions rescale onto a universal curve, revealing a characteristic length scale that is independent of ATP concentration. Inset, bare spatial correlation functions reveal that average velocity depends on ATP concentration.

It is well known that with increasing concentration, rod-like molecules undergo a transition to a nematic liquid-crystalline phase. To explore this regime we created a flat 2D oil–water interface stabilized with a surfactant, with BANs being dispersed in aqueous phase. Over time extensile microtubule bundles adsorbed onto the PEG brush formed by the surfactant molecules, eventually covering the entire surface with a dense liquid-crystalline monolayer of locally aligned bundles. The adsorbed 2D layer constituted an active microtubule liquid-crystalline phase, characterized by fast streaming flows and defect unbinding (Supplementary Video 6). Further information about the nature of active microtubule liquid crystals can be garnered by examining the structure and dynamics of their defects. In general, active liquid crystals can have either nematic symmetry^{24,25} (as found in monolayers of amoeboid cells or vertically shaken monolayers of granular rods^{26,27}) or polar symmetry (which is found in motility assays at high concentrations^{5,28}). Polar liquid crystals form vortex and aster defects^{5,29}.

In contrast, we found that active microtubule liquid crystals form disclination defects of charge $1/2$ or $-1/2$, implying the presence of nematic symmetry (Fig. 3a–c). This is expected because the basic building blocks of these materials are symmetrically extensile microtubule bundles. In equilibrium liquid crystals, defects are largely static structures whose presence is determined by either internal frustrations or external boundary conditions. In contrast, defects in active microtubule nematics exhibited unique spatiotemporal dynamics. They were created as uniformly aligned nematic domains extend, buckle and internally self-fracture (Fig. 3d), similar to the dynamical cascades

of microtubule bundles in 3D active networks. Once created, a fracture line terminated with a pair of oppositely charged disclination defects. Eventually, even as the fracture self-healed, the defects remained unbound and streamed around until eventually annihilating with oppositely charged defects (Supplementary Video 6). The rates of defect creation and annihilation were balanced, creating steady-state streaming dynamics that persisted for many hours. These observations exemplify how active nematics are fundamentally different from equilibrium ones, in which fractures, internal streaming flows and spontaneous unbinding of defect pairs are never observed.

In a biological context, active fluids are frequently confined to the cytoplasm and it has been proposed that such confinement leads to emergence of coherent flows that can enhance cellular transport, a phenomenon known as cytoplasmic streaming^{30,31}. For this reason we encapsulated BANs in aqueous droplets emulsified in fluorinated oil. When squeezed between two surfaces, such water-in-oil active droplets exhibited an unforeseen emergent property: persistent autonomous motility. This motility was highly robust, limited only by the sample lifetime, which was typically a few days (Fig. 4a, Supplementary Video 7). Instead of moving along straight lines, the active emulsion droplets preferentially moved in periodic patterns, with their average velocities reaching up to $\sim 1 \mu\text{m s}^{-1}$. In comparison, droplets without any chemical fuel did not exhibit any motion (Fig. 4b).

To elucidate the microscopic mechanism that drives droplet motility, we imaged the internal microtubule dynamics. For small droplets (less than $30 \mu\text{m}$), microtubule bundles extended and pushed against

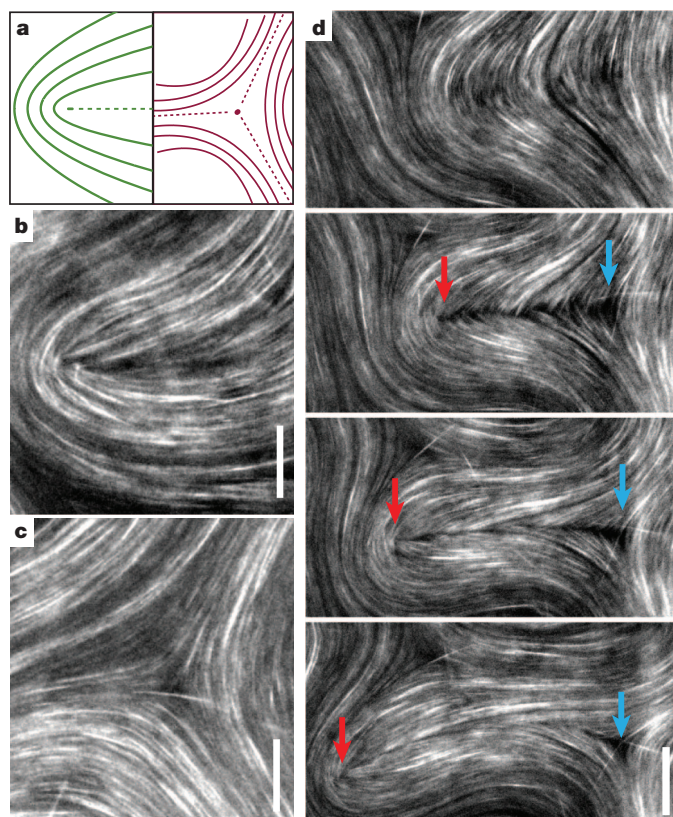


Figure 3 | Dynamics of 2D streaming nematics confined to fluid interfaces. **a**, Schematic illustrations of the nematic director configuration around disclination defects of charge $1/2$ (left) and $-1/2$ (right). **b**, **c**, Active liquid crystals exhibit disclinations of both $1/2$ (top) and $-1/2$ (bottom) charge, indicating the presence of nematic order. Scale bars, $15 \mu\text{m}$. **d**, A sequence of images demonstrates buckling, folding and internal fracture of a nematic domain. The fracture line terminates with a pair of oppositely charged disclination defects (red arrow tracks $1/2$ disclination; blue arrow tracks $-1/2$ disclination). After the fracture line self-heals, the disclination pair remains unbound. Time lapse, 15 s ; scale bar, $20 \mu\text{m}$.

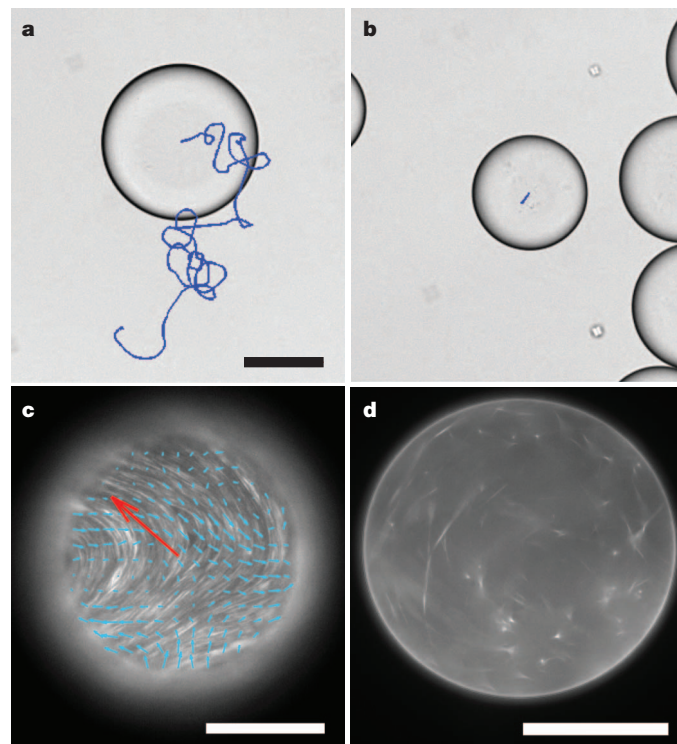


Figure 4 | Motile water-in-oil emulsion droplets. **a**, Droplets containing extensile microtubule bundles exhibit spontaneous autonomous motility when partially compressed between chamber surfaces. A droplet trajectory taken over a time interval of 33 min is overlaid onto a bright-field droplet image. Scale bar for **a** and **b** is $80 \mu\text{m}$. **b**, In the absence of ATP, passive droplets exert no internal forces, and the only contribution to their movement is minor drift. **c**, Fluorescence image of active microtubule bundles that spontaneously adsorb onto the oil–water interface. The resulting active liquid crystalline phase exhibits streaming flows, indicated with blue arrows. The red arrow indicates the direction of instantaneous droplet velocity. The image is focused on the droplet surface that is in contact with the coverslip. Scale bar, $100 \mu\text{m}$. **d**, Image of the droplet taken at a midplane indicates that the droplet interior is largely devoid of microtubule bundles. Scale bar, $100 \mu\text{m}$.

the water–oil interface, reaching a quasi-static state. Unable to buckle, they remained dispersed throughout the droplet and no motility was observed. In contrast, when confined within larger droplets, active microtubule bundles exhibited dramatically different behaviour. Similar to the flat surface, they adsorbed onto the oil–water interface, exhibiting coherent spontaneous flows (Supplementary Videos 8, 9). When in frictional contact with a hard surface, these internal flows drove the motility of the entire droplet (Fig. 4c). Scanning through the bulk of these motile droplets revealed that their interiors are largely devoid of microtubule bundles relative to their surface (Fig. 4d). The critical droplet size required for the formation of active liquid crystals was roughly similar to the collective buckling length scale that characterizes flows of 3D BANS. Spontaneous flows that drive droplet motility bear resemblance to cytoplasmic streaming observed in *Drosophila* oocytes³⁰.

Equilibrium nematics confined to spherical surfaces exhibit interesting defect configurations that have been proposed as building blocks for assembly of higher-order equilibrium structures³². Our experiments provide a new opportunity to explore how dynamic defects of active nematics on spheres can be harnessed to control droplet motility. At the next level of hierarchy, they also enable future studies in which emulsions are used for studying synchronization, crystallization and jamming of self-propelled spheres at high concentrations³³.

Conventional polymer gels, liquid crystals and emulsions are quintessential systems of soft condensed-matter physics. Studies of such materials have resulted in numerous fundamental advances and technological applications. However, the properties of these materials are limited by the laws of equilibrium statistical mechanics. Here, starting with simple extensible bundles and culminating in the assembly of motile droplets, we have demonstrated the hierarchical assembly of active gels, liquid crystals and emulsions. Liberated from equilibrium constraints, the tunable emergent properties of such far-from-equilibrium materials are strikingly different from their passive analogues, enabling further development of the statistical mechanics of active matter.

METHODS SUMMARY

Microtubules were polymerized to a concentration of 6 mg ml^{−1} with GMPCPP. The non-hydrolysing analogue of GTP reduces the microtubule nucleation barrier, resulting in very short filaments. Left at room temperature, the filaments anneal end-to-end, slowly increasing in average length. The behaviour of active microtubule networks is very sensitive to the length distribution of constituent filaments. After polymerization, microtubules used for active gel experiments were left at room temperature for two days, while microtubules used to assemble active 2D nematics were left for one. They had average lengths of 1.5 μm and 1.5 μm, respectively. Biotin-labelled kinesin fragments (401 amino acids of the amino-terminal motor domain of *Drosophila melanogaster* kinesin) were purified from *Escherichia coli*. Motor clusters were assembled by mixing biotin-labelled K401 with tetrameric streptavidin at a molar ratio of 1.7:1. Active mixtures consisted of microtubules, kinesin clusters, depleting polymer (PEG, 20 kDa), anti-bleaching agents, and an ATP regenerating system. The regenerating system used phosphoenol pyruvate (PEP) and pyruvate kinase/lactate dehydrogenase (PK/LDH) to maintain the ATP concentration at a constant level, allowing us to tune the kinesin velocity by controlling the initial ATP concentration. At the highest PEP concentrations, the regenerating system kept samples active for tens of hours. Samples were observed in a conventional flow cell and surfaces were coated with a repulsive polyacrylamide brush to suppress adsorption of proteins onto walls due to depletion or other non-specific interactions. The behaviour of active samples was monitored by visualizing microtubules with fluorescence microscopy, and by tracking 3-μm beads with bright-field microscopy. Beads were coated with poly-L-lysine-poly(ethylene glycol) (PLL-PEG) block copolymers, which suppressed their sticking to the microtubule bundles. Emulsions were made with a PFPE-PEG-PFPE surfactant (E2K0660) in HFE7500 oil.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions T.S., D.T.N.C., S.J.D. and Z.D. conceived the experiments and interpreted the results. T.S., D.T.N.C. and S.J.D. performed the experiments. T.S. and D.T.N.C. conducted data analysis. T.S., D.T.N.C. and Z.D. wrote the manuscript. M.H. synthesized surfactant and contributed to the assembly of active emulsions.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to Z.D. (zdoggic@brandeis.edu).

METHODS

Tubulin purification and microtubule polymerization. Tubulin was purified from bovine brain through two cycles of polymerization–depolymerization in high-molarity 1,4-piperazinediethanesulphonic (PIPES) buffer³⁴. The purified protein had a concentration of 7.4 mg ml⁻¹ in M2B buffer (80 mM PIPES, pH 6.8, 1 mM EGTA, 2 mM MgCl₂) and was stored at -80 °C. Tubulin was subsequently recycled and flash-frozen at a concentration of 13.2 mg ml⁻¹ in liquid nitrogen using thin-walled polymerase chain reaction (PCR) tubes. For fluorescence microscopy, tubulin was labelled with Alexa Fluor 647 (Invitrogen, A-20006) by a succinimidyl ester linker; absorbance spectroscopy indicated that 29% of monomers were labelled³⁵. Recycled tubulin was copolymerized with fluorescently labelled tubulin, producing microtubules with 3% of labelled monomers. The polymerization mixture consisted of 4.5 µl (13.2 mg ml⁻¹) unlabelled tubulin, 0.9 µl (7.4 mg ml⁻¹) Alexa-647-labelled tubulin, 1.4 µl glycerol (to 15% of final volume), 0.56 µl GMPCPP (10 mM)³⁵ (Jena Biosciences, NU-4056), 0.47 µl dithiothreitol (DTT, 20 mM), and M2B buffer to a final volume of 9.4 µl (for a final tubulin concentration of 6.8 mg ml⁻¹). The suspension was incubated for 30 min at 37 °C, and subsequently diluted to 6 mg ml⁻¹ with M2B. Microtubules were annealed at room temperature for 2 days before using them for mixing experiments. This resulted in an average microtubule length of 1.5 µm.

Kinesin–streptavidin complexes. K401, which consists of 401 amino acids of the N-terminal motor domain of *D. melanogaster* kinesin, was purified as previously published³⁶. The protein was frozen at 0.7 mg ml⁻¹ in 50 mM imidazole (pH 6.7), 4 mM MgCl₂, 2 mM DTT, 50 µM ATP and 36% sucrose buffer. Kinesin–streptavidin complexes were assembled by mixing 7 µl of freshly thawed K401 solution containing 3 mM DTT with 8 µl of 0.35 mg ml⁻¹ streptavidin (Invitrogen, S-888). The mixture was incubated on ice for at least 10 min before diluting with M2B to a final volume of 28 µl.

Polymer-coated sterically repulsive microspheres. Coating microspheres with a polymer brush leads to a steric repulsion that prevents their adsorption onto surfaces³⁷. Negatively charged polystyrene beads were coated with a block-copolymer (PLL-PEG), consisting of poly-L-lysine backbone and poly(ethylene glycol) side chains. The positively charged lysine groups electrostatically attach to the bead surface, leaving the PEG chains extending off the surface. PLL-PEG is synthesized by conjugating *N*-hydroxysuccinimidyl ester of methoxypoly(ethylene glycol) propionic acid (Laysan Bio, MPEG-SCM-20K-1g, molecular mass 20 kDa) with poly-L-lysine (Sigma, P7890-100MG, molecular mass 20 kDa) in 50 mM sodium borate buffer, pH 8.5. The concentration of lysine amino acids is 10 mM and the concentration of 20-kDa PEG molecules is 2.86 mM. This mixture is incubated at room temperature with gentle mixing for an hour and is subsequently transferred into a dialysis membrane (pore size 5 kDa). It is dialysed two times against Millipore-purified H₂O each time for more than 8 h.

PLL-PEG block-copolymer was added to 3-µm polystyrene beads decorated with sulphate groups in a low salt buffer. The final mixture containing 10 µl PLL-PEG, 100 µl HEPES (100 mM, pH 7.5), 12 µl bead stock (8% w/v) and 878 µl H₂O was incubated for 1 h, stirred gently and sonicated every 20 min. Subsequently, the beads were centrifuged for 5 min (5,000g) and resuspended in M2B buffer at ~1.3% w/v. Beads were imaged using bright-field microscopy and their positions were tracked using well-established protocols³⁸.

Observation chambers for optical microscopy. Microscope glass slides and coverslips were coated with a polyacrylamide brush to prevent non-specific adsorption of protein³⁹. Slides were first rinsed and sonicated with hot water containing 0.5% detergent, then with ethanol, and finally with 0.1 M KOH. Subsequently, the slides were soaked in a mixture of 98.5% ethanol, 1% acetic acid, and 0.5% of the silane-bonding agent 3-(trimethoxysilyl) propylmethacrylate (Acros Organics, 216551000) for 15 min. Slides were rinsed a final time and immersed in a 2% w/v aqueous solution of acrylamide. 35 µl per 100 ml of tetramethylethylenediamine (TEMED) and 70 mg per 100 ml of ammonium persulphate were added to the acrylamide solution to promote polymerization of the polyacrylamide brush that is covalently attached to the glass surfaces. Slides and coverslips were stored in a suspension of polymerized acrylamide and used for up to two weeks. Each slide was rinsed and air-dried immediately before use.

Assembly of active microtubule bundles. To study the dynamics of dilute microtubule bundles we confined a low concentration of microtubules to a quasi-2D observation chamber. In the absence of molecular motors, depletion interaction causes formation of microtubule bundles with mixed polarity. Introducing kinesin clusters induces relative sliding and eventual disintegration of bundles in which microtubules have mixed polarity while leaving intact bundles in which microtubules have the same polarity. This leads to a steady state that consists only of bundles with the same polarity. In the dilute limit (0.25 mg ml⁻¹) the average bundle length is 9 µm and the average cross-section contains approximately 10–20 microtubule bundles. Similar bundle thicknesses are measured in 3D bundled active networks.

Active microtubule networks. Several initial mixtures were prepared separately and then combined into the final solution. Individual components were dissolved and frozen separately in M2B buffer, and then thawed freshly for use. We included anti-oxidant components (listed below) and trolox (Sigma, 238813) to avoid photo-bleaching during fluorescence imaging. Anti-oxidant solution 1 (AO1) contained 15 mg ml⁻¹ glucose and 2.5 M DTT. Anti-oxidant solution 2 (AO2) contained 10 mg ml⁻¹ glucose oxidase (Sigma G2133) and 1.75 mg ml⁻¹ catalase (Sigma, C40). Second, a high-salt M2B solution (69 mM MgCl₂) was included to raise the MgCl₂. Lastly, ATP-regenerating components, including enzyme mixture pyruvate kinase/lactate dehydrogenase (PK/LDH, Sigma, P-0294) and phosphoenol pyruvate (PEP) were included in the final mixture. Because ATP is hydrolysed by kinesin activity, the PK/LDH uses PEP as a fuel source to convert ADP back into ATP at a rate that is much faster than the ATP hydrolysis. Thus constant ATP concentration is maintained throughout the experiments until all the PEP is exhausted⁴⁰.

The components described above were mixed without microtubules and in large volumes to reduce pipetting inaccuracies, while not wasting the valuable tubulin. The 'active pre-mixture' contained the following: 1.3 µl AO1, 1.3 µl AO2, 1.7 µl ATP (from stocks with different ATP concentrations), 1.7 µl PK/LDH, 2.9 µl high-MgCl₂-M2B, 3 µl of sterically repulsive beads (3 µm beads, ~1.3% w/v), 6 µl trolox (20 mM), 8 µl PEP (200 mM), 8 µl PEG (6% w/w in M2B), 4 µl kinesin–streptavidin mix, and 12.1 µl M2B buffer. To prepare the final active mixture, 5 µl of the active pre-mixture was mixed with 1 µl of the 6 mg ml⁻¹ microtubule solution, resulting in a final microtubule concentration of 1 mg ml⁻¹.

2D active microtubule nematics at oil–water interface. A 90-µm polyacrylamide flow cell was constructed as described above. HFE7500 oil with 2% (v/v) PFPE-PEG-PFPE surfactant was flowed into the cell and immediately displaced by flowing in the active microtubule mixture. The oil preferentially wets the interface, leaving a perfectly stable flat 2D oil–water interface decorated with the surfactant. The BANs were allowed to adsorb onto the oil–water interface and then imaged with fluorescence microscopy. Experiments indicate that the PEG is necessary for the adsorption of the microtubule bundles onto the surfactant monolayer and that active nematics assemble on flat as well as curved surfaces. For these experiments, 1-day-old microtubules are used, resulting in tighter nematic defects. Furthermore, the presence of surfactant is essential for the preservation of the streaming state and adsorption is only observed in active samples. In the absence of ATP, bundles do not adsorb onto the interface, instead forming a bulk rigid gel.

Light microscopy. Active microtubule networks were viewed with epi-fluorescence microscopy (Nikon Eclipse Ti microscope). AlexaFluor 647-labelled microtubules were illuminated with a 120-W metal halide light source (X-cite 120) and a fluorescent filter cube (Semrock, Cy5-4040B-NTE). Images were acquired with a cooled charge-coupled device (CCD) camera (Andor Clara). To view a large sample area, we used the motorized stage and the Ti microscope's Perfect Focus system to acquire adjacent fields of view, which were subsequently stitched together using a MatLab routine. 3-µm tracer particles were imaged with standard bright-field microscopy, and particle tracking was performed with Matlab software³⁸. Particle image velocimetry of the bundle flows was performed using the freely available Matlab-based package PIVlab (version 1.11).

Active mixtures in aqueous emulsion droplets. The active microtubule network in aqueous suspension was mixed with HFE7500 oil at a ratio of 1:5 and vortexed briefly five times to create water droplets within the oil medium. The aqueous droplets were stabilized with a 2% v/v solution of PFPE-PEG-PFPE surfactant (E2K0660)⁴¹. Glass slides and cover slips were cleaned using the protocol previously described for preparation of polyacrylamide-coated slides. The emulsion was co-flowed into the microscope chamber with extra HFE7500 oil to disperse the droplets. Within the droplets, the dynamics of microtubule bundles were viewed with fluorescence microscopy, while the motion of the droplets themselves was examined with bright-field microscopy.

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An ultraviolet-radiation-independent pathway to melanoma carcinogenesis in the red hair/fair skin background

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People with pale skin, red hair, freckles and an inability to tan—the ‘red hair/fair skin’ phenotype—are at highest risk of developing melanoma, compared to all other pigmentation types¹. Genetically, this phenotype is frequently the product of inactivating polymorphisms in the melanocortin 1 receptor (*MC1R*) gene. *MC1R* encodes a cyclic AMP-stimulating G-protein-coupled receptor that controls pigment production. Minimal receptor activity, as in red hair/fair skin polymorphisms, produces the red/yellow pheomelanin pigment, whereas increasing *MC1R* activity stimulates the production of black/brown eumelanin². Pheomelanin has weak shielding capacity against ultraviolet radiation relative to eumelanin, and has been shown to amplify ultraviolet-A-induced reactive oxygen species^{3–5}. Several observations, however, complicate the assumption that melanoma risk is completely ultraviolet-radiation-dependent. For example, unlike non-melanoma skin cancers, melanoma is not restricted to sun-exposed skin and ultraviolet radiation signature mutations are infrequently oncogenic drivers⁶. Although linkage of melanoma risk to ultraviolet radiation exposure is beyond doubt, ultraviolet-radiation-independent events are likely to have a significant role^{1,7}. Here we introduce a conditional, melanocyte-targeted allele of the most common melanoma oncoprotein, BRAF^{V600E}, into mice carrying an inactivating mutation in the *Mclr* gene (these mice have a phenotype analogous to red hair/fair skin humans). We observed a high incidence of invasive melanomas without providing additional gene aberrations or ultraviolet radiation exposure. To investigate the mechanism of ultraviolet-radiation-independent carcinogenesis, we introduced an albino allele, which ablates all pigment production on the *Mclr*^{e/e} background. Selective absence of pheomelanin synthesis was protective against melanoma development. In addition, normal *Mclr*^{e/e} mouse skin was found to have significantly greater oxidative DNA and lipid damage than albino-*Mclr*^{e/e} mouse skin. These data suggest that the pheomelanin pigment pathway produces ultraviolet-radiation-independent carcinogenic contributions to melanomagenesis by a mechanism of oxidative damage. Although protection from ultraviolet radiation remains important, additional strategies may be required for optimal melanoma prevention.

To study the role of pigmentation in BRAF^{V600E} melanoma development, we used a series of genetically matched mice on the C57BL/6 background with various pigmentation phenotypes (Fig. 1a). To mimic dark-skinned individuals with a high eumelanin-to-pheomelanin ratio, we used mice with the wild-type C57BL/6 pigmentation phenotype (‘black’). To mimic individuals with the red hair/fair skin phenotype who carry a high pheomelanin-to-eumelanin ratio, we used mice

with premature termination of the *Mclr* transcript (*Mclr*^{e/e}, ‘red’)⁸. To mimic individuals with albinism who have no melanin, we used mice with an inactivating mutation at the tyrosinase locus (*Tyr*^{c/c}, ‘albino’)⁹. Because tyrosinase is the initial and rate-limiting enzyme in melanin synthesis, albino melanocytes do not produce any pigment, but are normal in number and viability¹⁰.

We generated two variants of each pigmentation phenotype. One variant contains melanocytes in the dermis. A second matched variant contains transgenic stem cell factor expressed under the keratin 14 promoter (*K14-SCF*), which mimics SCF expression in human epidermal keratinocytes and results in epidermal melanocyte localization¹¹.

To create a genetic context primed for the induction of melanoma we also introduced into each of our six variants a system for inducible, melanocyte-specific expression of oncogenic BRAF^{V600E} (ref. 12). In humans, mice and zebrafish, expression of BRAF^{V600E} in melanocytes primarily causes benign nevi, rather than melanoma^{12–15}. In this context, malignant melanoma progression is thought to be constrained by

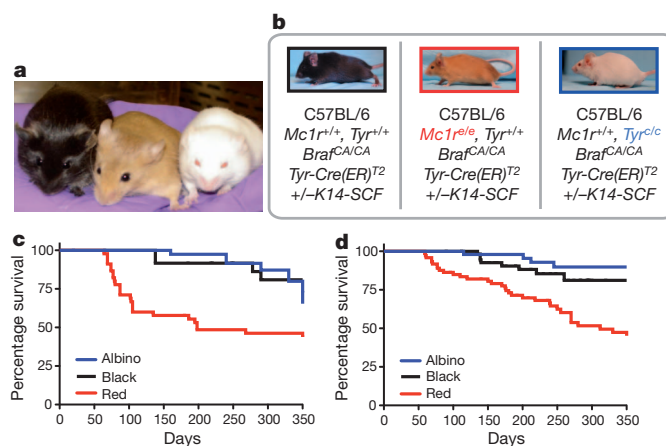


Figure 1 | Without ultraviolet radiation, *Braf*^{CA} red mice have an increased rate of melanoma development relative to black and albino *Braf*^{CA} animals. **a**, C57BL/6 pigmentation variants with epidermal melanocytes (*K14-SCF*). From left to right: black (wild type), red (*Mclr*^{e/e}) and albino (*Tyr*^{c/c}). **b**, Genotype of animals used for experimental studies. **c**, Percentage survival of pigmentation variants not carrying the *K14-SCF* transgene, that is, no epidermal melanocytes ($n_{\text{black}} = 28$, $n_{\text{red}} = 40$, $n_{\text{albino}} = 48$). $P_{\text{black-albino}} = 0.250$, $P_{\text{black-red}} = 0.003$, $P_{\text{albino-red}} = 0.003$. **d**, Percentage survival of pigmentation variants carrying the *K14-SCF* transgene, that is, epidermal melanocytes ($n_{\text{black}} = 49$, $n_{\text{red}} = 77$, $n_{\text{albino}} = 41$). $P_{\text{black-albino}} = 0.103$, $P_{\text{black-red}} = 0.009$, $P_{\text{albino-red}} < 0.0001$.

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oncogene-induced senescence¹⁵. Consistent with this, expression of BRAF^{V600E} in conjunction with silencing of *PTEN*, *TP53* or *CDKN2A* leads to development of malignant melanoma^{12–14}. However, spontaneous progression of BRAF^{V600E}-expressing melanocytes to malignant melanoma has been reported following a long latency period in C57BL/6 mice, although this phenomenon was not seen on an outbred model^{12,16,17}.

We initially produced six groups of BRAF^{V600E} inducible ('*Braf*^{CA}') mice representing three pigmentation variants ('black', 'red' and 'albino') with or without epidermal melanocytes (+/- *K14-SCF*, Fig. 1b). Melanocyte-selective expression of BRAF^{V600E} was achieved by tamoxifen-mediated activation of Cre recombinase (*Tyr-Cre(ER)*^{T2}) in adult mice carrying the *Braf*^{CA} allele. The animals were then followed without environmental genotoxic stressors (such as ultraviolet radiation). Black and albino *Braf*^{CA} mice developed similarly low rates of melanoma after a long latency (regardless of *K14-SCF* status). In contrast, red *Braf*^{CA} mice developed melanomas at an accelerated rate with >50% having tumours after 1 year, regardless of *K14-SCF* status (Fig. 1c, d).

The tumours on the black, red and albino backgrounds were grossly amelanotic and largely on the dorsal trunk (within the tamoxifen-treated areas). Occasionally, a tumour would develop on the ventral trunk, tail or paw, which may reflect a predictable spread of tamoxifen secondary to grooming. (Fig. 2a–c). The tumours, which were primarily dermal, were generally amelanotic on the red and albino backgrounds, whereas the melanomas in black mice often had superficial pigmentation adjacent to the epidermis (Fig. 2d–f). Regardless of pigmentation background, the tumours were histologically similar with spindle cell features which were not easily distinguishable from tumours on C57BL/6 *Braf*^{CA}-*Pten*^{flox/flox} animals generated in parallel (compare Fig. 2 with Supplementary Fig. 1). On closer examination, occasional red-BRAF^{V600E} tumour cells were found to contain melanin (Fig. 2g, h). It was further possible to increase pigmentation in the most superficial melanoma cells with topical application of forskolin, an adenylate cyclase agonist known to stimulate skin pigmentation¹⁸ (Fig. 2i). The limited induction of pigmentation is likely related to the poor tissue penetration of forskolin, but nonetheless demonstrates the ability of the melanoma cells to become hyper-pigmented *in vivo* upon activation of cAMP signalling.

Tumours on all three pigmentation backgrounds stained positively for S100, a standard immunohistochemical melanoma marker (Fig. 2j). In addition, reverse transcriptase polymerase chain reaction (RT-PCR) showed that the tumours consistently express the melanocytic pigment genes *M-Mitf*, *Dct*, *Tyrp1* and *Tyr* (Fig. 3a, d and data not shown). In addition, occasional HMB45⁺ cells could be found by immunofluorescence (Supplementary Fig. 2). The tumours on all three pigmentation backgrounds were locally invasive to fat and skeletal muscle with active mitoses. Although no gross visceral organ metastases were observed, small clusters of cells expressing gp100, the pre-melanosome-associated glycoprotein (Pmel/gp100/HMB45), could be found in skin draining lymph nodes (Fig. 2k).

Using a primary cell line derived from one of the red mouse melanomas, we observed that forskolin upregulated the expression of the melanocyte-specific isoform of *Mitf* (*M-Mitf*), and produced a marked increase in expression of the *Dct* and *Tyrp1* pigment genes, consistent with the ability of the cells to respond to melanocytic differentiation signals (Fig. 3a).

To determine whether the melanoma cells were dependent on the presumed oncogenic driver BRAF^{V600E}, we tested their response to small molecule inhibitors of BRAF or MEK (also known as MAP2K). Treatment with the oncogenic BRAF inhibitor, PLX4720, or the MEK inhibitor, U0126, prevented melanoma cell proliferation *in vitro*, and PLX4720 blocked tumour cell growth *in vivo*, consistent with a dependency of these tumours on the BRAF^{V600E} oncoprotein (Fig. 3b, c). BRAF inhibition also elevated the expression of melanocytic genes as previously reported in human melanomas (Fig. 3d)¹⁹.

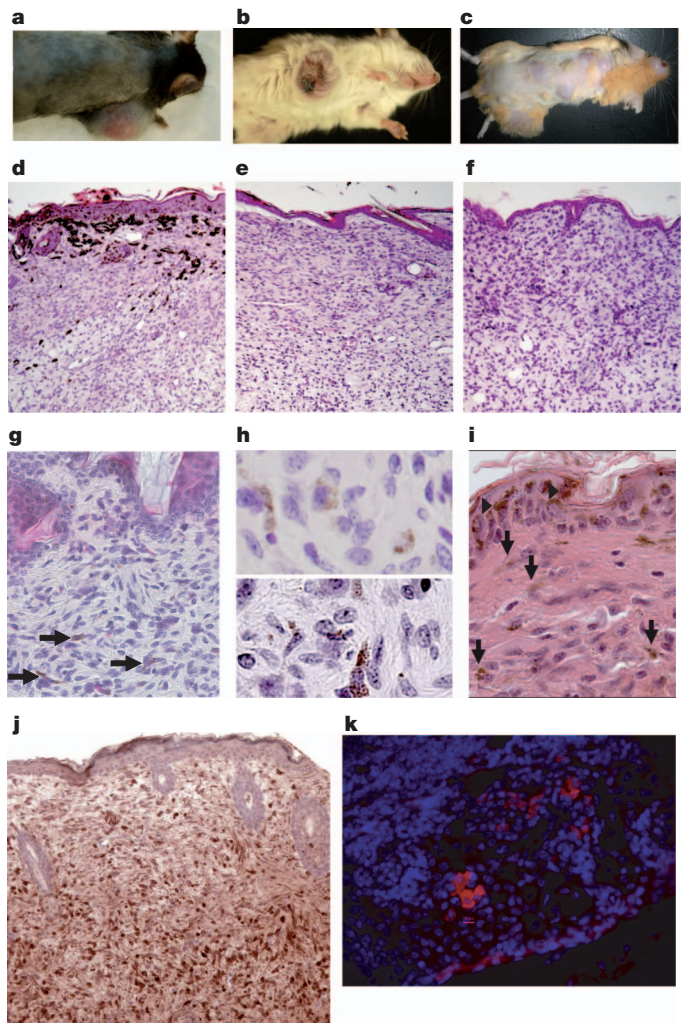


Figure 2 | Melanomas on all three pigmentation variants are morphologically similar and exhibit common histologic features. a–c, Melanomas on black (a), albino (b) and red (c) mice are grossly amelanotic. d–f, Histologically, black (d), albino (e) and red (f) melanomas are also mostly amelanotic, although superficial tumour cells in black-BRAF^{V600E} tumours carry melanin (original magnification, ×10). g, Red-BRAF^{V600E} melanomas can also carry pigment (arrows) (original magnification, ×20). h, Further magnification of two red melanomas also illustrates pigmented tumour cells (original magnification, ×100). i, Forskolin induces epidermal pigmentation (arrowheads) and mild tumour cell pigmentation (arrows) (original magnification, ×20). j, Tumour cells stain positive for S100 (original magnification, ×10). k, Skin-draining lymph nodes carry gp100⁺ cells (red) (scale bar, 10 μm).

Because inactivating mutations in *Mc1r* alter cAMP levels in the cell, red mice undoubtedly have numerous intracellular pathway differences relative to wild-type *Mc1r*^{E/E} (black) animals, including altered DNA repair²⁰. We therefore wished to study whether the pheomelanin pigment pathway itself has an intrinsic mechanistic role, or whether it is merely a marker of melanoma risk. To investigate this question we introduced the albino tyrosinase (*Tyr*^{c/c}) allele into the red *Mc1r*^{E/E} background to test melanoma incidence in albino-*Mc1r*^{E/E} animals, which retain low MC1R activity and also lack all pigment production (Fig. 4a). A melanocyte-targeted *LacZ* transgene was used to confirm that the albino allele does not alter melanocyte number in these mice (Supplementary Fig. 3a, b)¹⁰. As shown in Fig. 4b, the albino allele profoundly protected red mice from melanoma. The rare albino-*Mc1r*^{E/E} melanomas occurred after long latency and had the same amelanotic, S100⁺, histologic features as the other pigmentation variant *Braf*^{CA} animals (Supplementary Fig. 4a–c). This observation suggests

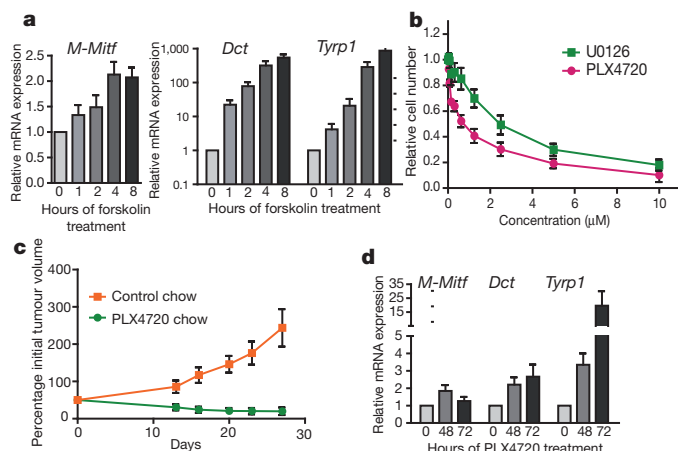


Figure 3 | Tumour cells from a red-*Braf*^{CA} animal behave like classic *BRAF*^{V600E} melanomas after cAMP upregulation or BRAF inhibition. **a**, Forskolin (20 μ M) upregulates expression of melanocytic markers ($n = 4$). **b**, MAPK inhibition by PLX4720 or U0126 decreases melanoma cell proliferation ($n = 3$). **c**, PLX4720 blocks melanoma growth *in vivo* ($n = 3$). **d**, PLX4720 (2 μ M) upregulates expression of melanocytic markers. Relative mRNA expression normalized to 18S ribosomal RNA and 0 h time point. Error bars denote s.e.m.

that the pheomelanin synthesis pathway is necessary for the high rate of ultraviolet-radiation-independent melanoma in the red mice.

Previous studies have demonstrated that ultraviolet radiation amplifies reactive oxygen species (ROS) production and subsequent

oxidative DNA damage in the skin of pigmented mice²¹. Cells with high pheomelanin levels that receive ultraviolet radiation have been found to carry particularly high levels of oxidative damage^{4,5}. Because darkly pigmented individuals carry both pheomelanin and eumelanin, it has been proposed that their lower melanoma risk may result from eumelanin intermediates and polymers absorbing ROS and functioning as *in vivo* antioxidants^{22,23}.

To determine whether ROS-mediated oxidative DNA damage is affected by the pheomelanin synthesis pathway, levels of 8,5'-cyclo-2'-deoxyadenosine (cdA) and 8,5'-cyclo-2'-deoxyguanosine (cdG) were measured in DNA isolated from skin of red-*Mc1r*^{e/e} and albino-*Mc1r*^{e/e} mice, using a previously reported liquid chromatography-tandem mass spectrometric method²⁴ (Fig. 4c). These two ROS-mediated cyclopurines are unlikely to be artificially induced during sample preparation and are quite stable^{25,26}. Significantly, replication studies in *Escherichia coli* have shown that S-cdA and S-cdG can lead to A-to-T and G-to-A mutations at frequencies of 11% and 20%, respectively²⁷. Comparing cyclopurine levels in the skin of various pigmentation-variant mice, it was found that the levels of cdA and cdG are significantly higher in skin from red-*Mc1r*^{e/e} mice compared to skin from albino-*Mc1r*^{e/e} animals (Fig. 4d, e). This observation indicates that activation of the pheomelanin synthesis pathway results in increased oxidative DNA damage. Correlative evidence for increased cellular oxidative stress was also found in the observation that red-*Mc1r*^{e/e} mouse skin carries higher levels of lipid peroxides, a product of ROS-mediated lipid damage (Fig. 4f).

The findings reported here indicate that in the context of oncogenic BRAF activation, individuals carrying red hair/fair skin *MC1R* polymorphisms have an increased risk of melanoma, owing to both poor

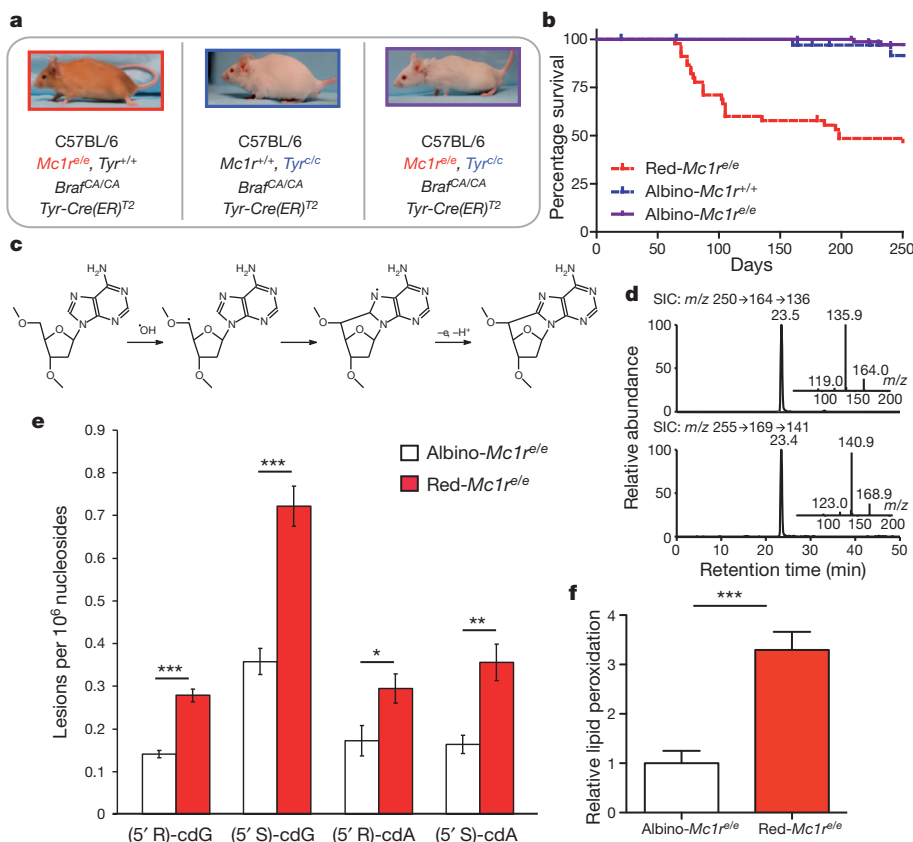


Figure 4 | The ultraviolet-radiation-independent propensity of red *Braf*^{CA} mice to develop melanoma is dependent on pigment production. **a**, Genotypes of mice studied. **b**, The albino allele protects *Mc1r*^{e/e} mice from melanoma development ($n_{\text{red}} = 40$, $n_{\text{albino}} = 48$, $n_{\text{albino-red}} = 90$). $P_{\text{albino-albino-red}} = 0.308$, $P_{\text{albino-red}} < 0.0001$, $P_{\text{red-albino-red}} < 0.0001$. **c**, ROS react with purine nucleosides to produce 8,5'-cyclopurine lesions (cdA shown).

d, Selected-ion chromatograms for DNA from albino-*Mc1r*^{e/e} mouse skin. The insets show the positive-ion MS/MS spectra for unlabelled and labelled S-cdA. **e**, Both diastereomers of cdA and cdG are significantly higher in red-*Mc1r*^{e/e} skin ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. **f**, Lipid peroxide levels are significantly higher in red-*Mc1r*^{e/e} skin ($n = 3$), *** $P < 0.0001$.

protection from environmental carcinogens like ultraviolet radiation, and also via intrinsic carcinogenic features of pheomelanin synthesis; potentially via pheomelanin itself, an intermediate of pigment synthesis or a by-product of the pathway.

In humans, there are multiple *MC1R* polymorphisms with varied perturbation of receptor function that produce a red hair/fair skin phenotype; however, a unifying feature of these various polymorphisms is a high pheomelanin-to-eumelanin ratio, which is also produced by the *Mc1r*^{e/e} allele in mice. Recent data intriguingly demonstrated that black animals overexpressing hepatocyte growth factor (HGF) who receive ultraviolet radiation are at a higher risk of melanoma than their albino counterparts²¹. Although the present study reveals a small difference between black and albino BRAF^{V600E}-driven melanomas in the presence of K14-SCF (Fig. 1d), the effect did not reach statistical significance ($P = 0.103$), perhaps signifying oncogene-specific differences. It seems that the effects of pigmentation and ultraviolet radiation are likely to work together in determining melanoma risk.

The photometer used for our laboratory's routine calibration (International Light 1400) was unable to detect any measurable ultraviolet radiation in our mouse cages during ambient light exposure. However, strong epidemiological work links ultraviolet radiation to melanoma, and the current data do not diminish the importance of sun exposure as a key contributing factor¹. In humans, it is likely that the ultraviolet-independent effects act in concert with ultraviolet-mediated cellular toxicity. In agreement with published studies, ultraviolet radiation at a UV-A/UV-B ratio similar to that found in sunlight (10 J cm⁻² UV-A and 0.65 J cm⁻² UV-B) was found to exacerbate oxidative damage selectively in red mouse skin as measured by levels of lipid peroxidation^{4,5} (Supplementary Fig. 5a). Studies are underway to investigate whether ultraviolet radiation is able to alter the red-BRAF^{V600E} tumour phenotype. Preliminary studies examining the effect of visible light (180 J cm⁻²) did not reveal significantly altered lipid peroxidation in any pigmentation context ($P = 0.4506$). Perhaps, however, there is a trend towards an increased level of lipid peroxidation in red mouse skin (Supplementary Fig. 5b).

Further evidence suggesting an ultraviolet-radiation-independent red hair/fair skin melanoma risk is the observation that although darker-skinned individuals have a significantly lower risk of melanoma than lighter-skinned individuals, the sun protective factor (SPF, a measurement of sunburn protection) of darker skin has been estimated at only in the range of SPF 2.0–4.0 (ref. 28). In addition, sunscreen (typically SPF 20–40) has shown weak efficacy in protecting against melanoma, unlike its protection against cutaneous squamous cell carcinoma^{29,30}. There are numerous potential explanations for the sunscreen-melanoma data including the possibility that ultraviolet radiation shielding may protect against only one of several carcinogenic mechanisms—with the intrinsic pheomelanin pathway representing an additional contributor to melanomagenesis via ultraviolet-radiation-independent means. These data are not evidence against a role for ultraviolet radiation in melanomagenesis. Indeed, the effect of ultraviolet radiation is likely to exacerbate this mechanism, such that ultraviolet radiation shielding and sunscreen remain extremely important for skin cancer prevention. However, further preventative strategies may be essential to optimally diminish melanoma risk in the most susceptible individuals.

METHODS SUMMARY

Mice. At 6–10 weeks of age, mice were treated topically with 20 mg ml⁻¹ tamoxifen for 5 days. For topical darkening, 20% coleus extract was applied as previously described¹⁷. For *in vivo* PLX4720 studies, animals were given *ad libitum* mouse chow containing 2% PLX4720 by weight. All studies and procedures involving animal subjects were approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital and Dana-Farber Harvard Cancer Center, and were conducted strictly in accordance with the approved animal handling protocol.

Morphological examination. Histology and immunostaining were performed according to established protocols using anti-S100 (Dako), anti-DCT (Santa

Cruz), anti-C5 MITF (tissue culture supernatant), anti-HMB45 (Santa Cruz) and anti-gp100 (Abcam) antibodies.

Primary cell culture. Primary tumour was digested overnight and grown in DMEM. Proliferation was measured by the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Quantitative RT-PCR. Messenger RNA expression of melanocytic markers was determined using intron-spanning mouse-specific primers with SYBR FAST qPCR master mix (Kapa Biosystems).

Measurement of 8,5'-cyclopurine-2'-deoxynucleosides. cda and cdG levels were measured as previously published²⁴. Briefly, nuclear DNA was isolated from mouse skin, digested to nucleosides, separated by high-performance liquid chromatography (HPLC) and analysed by liquid chromatography–multi-stage mass spectrometry (LC–MS/MS/MS).

Measurement of lipid peroxidation. Mouse skin was irradiated with ultraviolet (10 J cm⁻² UV-A and 0.65 J cm⁻² UV-B), or visible light (180 J cm⁻²). After homogenization, lipid peroxidation was measured with the OxiSelect TBARS Assay Kit (Cell Biolabs).

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions D.M. and D.E.F. conceived and planned the project. M.M., M.W.B. and K.M.H. provided the mice carrying the *PTEN*, *BRAF^{V600E}* and *Tyr-Cre(ER)^{Y2}* alleles. D.M. performed the mouse work with help from A.M., J.L., S.P.D. and K.C.R. Histology was performed by D.M., X.L., J.C.V. and J.A.D. with support from D.A.H. Pathological analysis was provided by M.P.H., J.K.L. and M.C.M. *In vitro* studies were performed by D.M. with help from A.M. and J.L. J.A.W. generated the primary mouse cell line. J.W., C.R.G. and Y.W. collected DNA from mouse skin and performed LC-MS/MS/MS. The manuscript was written by D.M. and D.E.F. All authors discussed the results and commented on the manuscript.

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METHODS

Mice. All animals used for breeding were backcrossed a minimum of six generations onto the C57BL/6 genetic background (this corresponds to a >98.4% C57BL/6 congenic animal, <http://jaxmice.jax.org/support/nomenclature/tutorial.html>). The black (wild type), red (*Mc1r^{+/e}*) and albino (*Tyr^{e/c}*) animals were purchased from Jackson Laboratories. *K14-SCF* animals were acquired from T. Kunisada. Genotyping of each litter, including the *Tyr-Cre(ER)^{T2}*, *Braf^{CA}* and *PTEN^{flox/flox}* alleles was performed as previously published^{10,11}. At 6–10 weeks of age the dorsal fur was trimmed using animal shears with a 0.25 mm head and the mice were treated topically with 20 mg ml⁻¹ tamoxifen for 5 consecutive days. For tumour darkening, a 20% solution of *Coleus forskohlii* root extract (80 µM forskolin) was topically applied daily as previously described¹⁷. For *in vivo* PLX4720 studies, animals were given *ad libitum* mouse chow containing 2% PLX4720 by weight or control chow acquired from Plexikon. All studies and procedures involving animal subjects were approved by the Institutional Animal Care and Use Committees of Massachusetts General Hospital and Dana-Farber Harvard Cancer Center and were conducted strictly in accordance with the approved animal handling protocol.

Dissection and histology. Tissues of interest were photographed, excised, weighed, rinsed in PBS, fixed in 10% neutral-buffered formalin, rinsed in PBS, and stored in 70% ethanol. Formalin-fixed tissues were paraffin embedded (FFPE) and sectioned (3–5 µm) using standard procedures. Morphological analysis was performed using multiple independent samples per site/organ (5 to 9 samples per genotype) as well as >6 animals. Two pathologists (J.K.L., M.P.H.) independently examined the histopathology of the tumour samples. Digitization and image capture was performed using an Olympus DP70 digital camera (Olympus) connected to an Olympus BX51 light microscope or a Scanscope whole-slide scanning system (Aperio).

Immunohistochemistry. For immunohistochemistry, sections were deparaffinized with xylene and hydrated with a graded series of alcohol. Sections were boiled in 50 mM Tris-buffer (pH 9) or citrate for antigen retrieval and rinsed in PBS. Sections were blocked in 1% BSA, 0.1% Triton X-100 PBS, incubated with 1:200 dilutions of rabbit anti-S100 (Dako), 1:100 dilutions of goat anti-DCT (Santa Cruz), 1:200 dilutions of mouse anti-HMB45 (Santa Cruz) and 1:200 dilutions of mouse anti-gp100 (Abcam) antibodies, followed by visualization with appropriate secondary antibodies conjugated to Alexa594 or Alexa488 (1:500). Appropriate controls for specificity of staining were included and images were captured using an upright fluorescence microscope (Eclipse 90i, Nikon). To identify epidermal melanocytes, skin from reporter mice carrying the various pigmentation alleles and the *K14-SCF* transgene as well as a *DCT-LacZ* reporter allele was cryosectioned and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and nuclear fast red counterstaining.

Primary cell culture. Tumour cells were digested overnight in 10 mg ml⁻¹ collagenase and 1 mg ml⁻¹ hyaluronidase. Initially tumour cells were grown in RPMI media with HEPES and 20% serum. Subsequently tumour cells were grown in DMEM media with 10% serum. Proliferation after 72 h of PLX4720 (Chemietek) and U0126 (Cell Signaling) was determined by the CellTiter-Glo Luminescent Cell Viability Assay (Promega).

Quantitative RT-PCR. RNA was collected from primary cultured tumour cells treated for varying times with forskolin or PLX4720 using the RNeasy Plus mini kit (Qiagen). mRNA expression of melanocytic markers was determined using intron-spanning mouse-specific primers with Kapa SYBR FAST qPCR master mix (Kapa Biosystems). Expression was normalized to 18S rRNA and 0 h time points. Primer sequences used: *Mitf* forward GCCTGAAACCTTGCTATGCTGGAA, *Mitf* reverse AAGGTACTGCTTTACCTGGTGCCT, *Dct* forward AGGTACCATCTGTTGTGGCTGGAA, *Dct* reverse AGTTCCGACTAATCA GCGTTGGGT, *Typr1* forward TGGGGATGTGGATTTCTCTC, *Typr1* reverse AGGGAGAAAGAAGGCTCCTG, *18S* forward AGGTTCTGGCCAACGG TCTAG, *18S* reverse CCCTCTATGGGCAATTTT.

Extraction of nuclear DNA from mouse skin tissues. Nuclear DNA was isolated from mouse skin using a high-salt method. Tissues were ground under liquid nitrogen into fine powder using a mortar and pestle. A nuclei lysis buffer containing 20 mM Tris (pH 8.3), 20 mM EDTA, 400 mM NaCl, 1% SDS (w/v) and 0.05% proteinase K (w/v) was added to the tissue and incubated in a water bath at 55 °C overnight. Half volume of saturated NaCl solution was added to the digestion mixture, incubated at 55 °C for 15 min then centrifuged at ~12,000g

for 30 min. The supernatant was collected and centrifuged again. The nucleic acids in the supernatant were precipitated with cold ethanol, dissolved in water and incubated in the presence of 0.03% RNase A (w/v) and 0.25 U µl⁻¹ of RNase T1 at 37 °C overnight, and subsequently extracted with an equal volume of chloroform/isoamyl alcohol (24:1, v/v) twice. The DNA was then precipitated from the aqueous layer by cold ethanol, centrifuged at 10,000g at 4 °C for 15 min, washed twice with 70% cold ethanol and dried under vacuum. The DNA pellet was dissolved in deionized water and quantified by using ultraviolet absorption spectrophotometry.

Enzymatic digestion of nuclear DNA. Nuclease P1 (16 U), phosphodiesterase 2 (0.025 U), 20 nmol of erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) and a 30 µl solution containing 300 mM sodium acetate (pH 5.6) and 10 mM zinc chloride were added to 200 µg of DNA. In this context, EHNA served as an inhibitor for deamination of 2'-deoxyadenosine to 2'-deoxyinosine (dI) induced by adenine deaminase. The above digestion was continued at 37 °C for 48 h. To the digestion mixture were then added alkaline phosphatase (10 U), phosphodiesterase 1 (0.0125 U) and 60 µl of 0.5 M Tris-HCl buffer (pH 8.9). The digestion was continued at 37 °C for 2 h and subsequently neutralized by addition of formic acid. To the mixture were then added uniformly ¹⁵N-labelled standard lesions, which included 400 fmol of R-cdG, 150 fmol of S-cdG, 80 fmol of R-cdA and 40 fmol of S-cdA. The enzymes in the digestion mixture were subsequently removed by chloroform extraction twice. The resulting aqueous layer was subjected to off-line high-performance liquid chromatography (HPLC) separation for the enrichment of the lesions under study, following our previously described procedures²⁶.

Liquid chromatography–multi-stage mass spectrometry (LC–MS/MS/MS) analysis. The LC–MS/MS/MS experiments were conducted using an LTQ linear ion trap mass spectrometer using our recently described conditions²⁶. Briefly, the amounts of cdA and cdG lesions in each nucleoside sample were calculated based on the ratios of peak areas found in the selected-ion chromatograms for the analyte (for example, the 23.5 min peak in the top panel of Fig. 4d for S-cdA) and the corresponding stable isotope-labelled standard (for example, the 23.4 min peak in the bottom panel of Fig. 4d for the ¹⁵N-labelled S-cdA), the known amount of uniformly ¹⁵N-labelled standard was added to the nucleoside mixture (for example, 40 fmol for S-cdA), and calibration curves. The calibration curves were constructed from the same LC–MS/MS/MS analyses of a series of mixtures with known compositions of the unlabelled cdA, cdG and constant amounts of the corresponding uniformly ¹⁵N-labelled standards, as described previously²⁶. The lesion formation frequencies as shown in Fig. 4e were then calculated by dividing the amounts of cdA and cdG in the sample with the total amount of nucleosides present.

Skin irradiation and lipid peroxide measurement. Six-week-old mice were euthanized and fur was removed using animal shears with a 0.25 mm head. Twelve sections of skin, each with an area of 1 cm², were removed from each mouse and placed in 35-mm dishes on ice after adherence to Whatman filter paper suspended in PBS. For each ultraviolet and visible light study, six sections of skin from each mouse were placed in the dark on ice as controls. For ultraviolet studies, six sections of skin from each mouse were irradiated on ice with 10 J cm⁻² UV-A and 0.65 J cm⁻² UV-B at an irradiance of 6.67 mW cm⁻² using a Sylvania 350 Blacklight (Osram Sylvania). This ultraviolet distribution is comparable to natural sunlight (96.65% UV-A and 3.35% UV-B). Two mice of each pigmentation type were used for a total of *n* = 12 skin samples for each condition. For visible light studies, six sections of skin from each mouse were irradiated on ice with 180 J cm⁻² visible light from a Dolan-Jenner A3200 Fiber-Lite Illuminator at an irradiance of 200 mW cm⁻². The illuminator bulb was fit with a Thorlabs FEL0400 Edgepass ultraviolet filter with a transmission of <0.001% for wavelengths <400 nm, such that no irradiation output was detectable in the ultraviolet range below 400 nm. One mouse of each pigmentation type was used for a total of six skin samples for each condition. Following treatment, skin sections were flash-frozen and homogenized in PBS containing the antioxidant butylated hydroxytoluene (BHT) to prevent further lipid peroxidation, using a Qiagen TissueLyser II. Homogenized samples were centrifuged and supernatants were collected. Protein content of each sample was determined by Coomassie Plus Protein Assay, and samples were diluted with PBS plus BHT for normalization of sample concentration (Thermo Scientific). Lipid peroxidation of each irradiated set of sample was determined using an OxiSelect TBARS Assay kit and normalized to its unirradiated control (Cell Biolabs).

Analyses of pig genomes provide insight into porcine demography and evolution

A list of authors and their affiliations appears at the end of the paper

For 10,000 years pigs and humans have shared a close and complex relationship. From domestication to modern breeding practices, humans have shaped the genomes of domestic pigs. Here we present the assembly and analysis of the genome sequence of a female domestic Duroc pig (*Sus scrofa*) and a comparison with the genomes of wild and domestic pigs from Europe and Asia. Wild pigs emerged in South East Asia and subsequently spread across Eurasia. Our results reveal a deep phylogenetic split between European and Asian wild boars ~1 million years ago, and a selective sweep analysis indicates selection on genes involved in RNA processing and regulation. Genes associated with immune response and olfaction exhibit fast evolution. Pigs have the largest repertoire of functional olfactory receptor genes, reflecting the importance of smell in this scavenging animal. The pig genome sequence provides an important resource for further improvements of this important livestock species, and our identification of many putative disease-causing variants extends the potential of the pig as a biomedical model.

The domestic pig (*Sus scrofa*) is a eutherian mammal and a member of the Cetartiodactyla order, a clade distinct from rodent and primates, that last shared a common ancestor with humans between 79 and 97 million years (Myr) ago^{1,2} (<http://www.timetree.net>). Molecular genetic evidence indicates that *Sus scrofa* emerged in South East Asia during the climatic fluctuations of the early Pliocene 5.3–3.5 Myr ago. Then, beginning ~10,000 years ago, pigs were domesticated in multiple locations across Eurasia³ (Frantz, L. A. F. *et al.*, manuscript submitted).

Here we provide a high-quality draft pig genome sequence developed under the auspices of the Swine Genome Sequencing Consortium^{4,5}, established using bacterial artificial chromosome (BAC)⁶ and whole-genome shotgun (WGS) sequences (see Methods and Supplementary Information). The assembly (Sscrofa10.2) comprises 2.60 gigabases (Gb) assigned to chromosomes with a further 212 megabases (Mb) in unplaced scaffolds (Table 1 and Supplementary Tables 1–3).

Genome annotation

A *de novo* repeat discovery and annotation strategy (Supplementary Fig. 8) revealed a total of 95 novel repeat families, including: 5 long interspersed elements (LINEs), 6 short interspersed elements (SINEs), 8 satellites and 76 long terminal repeats (LTRs). The relative content of repetitive elements (~40%, Supplementary Figs 9 and 10) is lower than reported for other mammalian genomes. The main repetitive element groups are the LINE1 and glutamic acid transfer RNA (tRNA^{Glu})-derived SINEs or PRE (porcine repetitive element). The expansion of PRE is specific to the porcine lineage. Phylogenetic analysis of LINE1 and PRE (Supplementary Figs 13 and 14) indicates that only a single lineage of each is currently active and that the main expansion of both LINE1 and PRE occurred in the first half of the Tertiary period. Smaller expansions, particularly in LINE1, have occurred since, but recent activity is very low (Supplementary Information).

Annotation of genes, transcripts and predictions of orthologues and paralogues was performed using the Ensembl analysis pipeline⁷ (Table 1 and Supplementary Figs 3–7). Further annotation for non-protein-coding RNAs (ncRNAs) was undertaken with another analysis pipeline (Supplementary Information and Supplementary Table 4).

Evolution of the porcine genome

Evolution of genes and gene families

To examine the mutation rate and type of protein-coding genes that show accelerated evolution in pigs, we identified ~9,000 as 1:1 orthologues within a group of six mammals (human, mouse, dog, horse, cow and pig). This orthologous gene set was used to identify proteins that show accelerated evolution in each of these six mammalian lineages (Supplementary Information). The observed number of synonymous substitutions per synonymous site (dS) for the pig lineage (0.160) is similar to that of the other mammals (0.138–0.201) except for the mouse (0.458), indicating similar evolutionary rates in pigs and other mammals. The observed dN/dS ratio (ratio of the rate of non-synonymous substitutions to the rate of synonymous substitutions) of 0.144 is between those of humans (0.163) and mice (0.116), indicating an intermediate level of purifying selection pressure in the pig. Genes showing increased dN/dS ratios in each lineage were analysed using DAVID⁸ to examine whether these rapidly evolving genes were enriched for specific biological processes. Most lineages show different fast-evolving pathways, but some pathways are shared (Fig. 1).

Immune genes are known to be actively evolving in mammals^{9,10}. Because many immune genes were not included in the analysis of 1:1 orthologues, we examined a randomly selected subset of 158 immunity-related pig proteins for evidence of accelerated evolution (Supplementary information). Twenty-seven of these genes (17%)

Table 1 | Assembly and annotation statistics

Assembly	Placed	Unplaced	Annotation*
Total length	2,596,639,456	211,869,922	21,640 protein-coding genes
Ungapped length	2,323,671,356	195,490,322	380 pseudogenes
Scaffolds	5,343	4,562	2,965 ncRNAs†
Contigs	73,524	168,358	197,675 gene exons
Scaffold N50	637,332	98,022	26,487 gene transcripts
Contig N50	80,720	2,423	

* Numbers refer to the annotation performed by Ensembl (release 67). Results of an independent annotation by the NCBI can be obtained from <http://www.ncbi.nlm.nih.gov/mapview/stats/BuildStats.cgi?taxid=9823&build=4&ver=1>.

† An improved ncRNA annotation with 3,601 ncRNAs and structured elements is available as a separate track in Ensembl version 70 and for download from <http://rth.dk/resources/rnannotator/susscr102>. N50, 50% of the genome is in fragments of this length or longer.

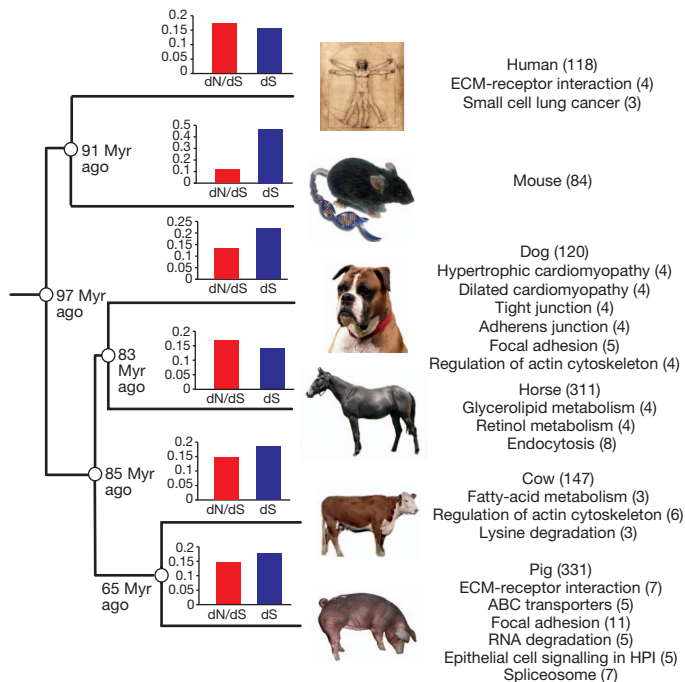


Figure 1 | Phylogeny of the six mammals used in the dN/dS analysis. KEGG pathways with genes that show accelerated evolution for each of the six mammals used in the dN/dS analysis. The bar charts show the individual dN/dS and dS values for each of the six mammals. The dN/dS and dS values refer to the time period of each of the six individual lineages. The number of proteins that show significantly accelerated dN/dS ratios in each lineage varies from 84 in the mouse to 311 in the pig lineage. Pathways significantly ($P < 0.05$) enriched within this group of genes are also shown with the number of genes shown in brackets. HPI, *Helicobacter pylori* infection.

demonstrated accelerated evolution (Supplementary Table 8). A parallel analysis of 143 human and 145 bovine orthologues revealed very similar rates of evolution (18% in human and 12% in cattle, respectively). Using a branch-site analysis, we detected accelerated evolution of amino acids in PRSS12, CD1D and TRAF3 specific to pig (positive selection on pig branch), as well as amino acids in TREM1, IL1B and SCARA5 specific to pig and cow (positive selection on the cetartiodactyl branch).

Further analysis of porcine immune genes (Supplementary Table 5) revealed evidence for specific gene duplications and gene-family expansions (Supplementary Tables 6 and 7). The analysis of this second cetartiodactyl genome indicates that some expansions are cetartiodactyl-specific (cathelicidin) whereas others are ruminant/bovine-specific (β -defensins, C-type lysozymes) or potentially porcine-specific (type I interferon, δ subfamily).

Pigs have at least 39 type I interferon (IFN) genes, which is twice the number identified in humans and significantly more than in mice. We also detected 16 pseudogenes in this family. Cattle have 51 type I IFNs (13 pseudogenes), indicating that both bovine and porcine type I IFN families have undergone expansion. This is particularly important for interferon subtypes δ (IFND), ω (IFNW) and τ (IFNT); pigs and cattle are evolving species-specific subtypes of IFND and IFNT, respectively. Both species are expanding the IFNW family and share many more IFNW isoforms than other species. Thus, expansion of interferon genes is not ruminant-specific as proposed earlier¹⁰, although duplication within some specific sub-families seems to be either bovine- or porcine-specific.

Within the immunity-related genes annotated, we found evidence for duplication of six immune-related genes: *IL1B*, *CD36*, *CD68*, *CD163*, *CRP* and *IFIT1*, and one non-immune gene, *RDH16*. The *CD36* gene is also duplicated in the bovine genome, whereas the *IL1B* gene duplication, where evidence for a partial duplication was

reported previously¹¹, is unique in mammals. Other key immune genes in the major histocompatibility complex, immunoglobulin, T-cell-receptor and natural killer cell receptor loci have been characterized in detail^{12–19} (Supplementary Information).

Another significant porcine genome expansion is the olfactory receptor gene family. We identified 1,301 porcine olfactory receptor genes and 343 partial olfactory receptor genes²⁰. The fraction of pseudogenes within these olfactory receptor sequences (14%) is the lowest observed in any species so far. This large number of functional olfactory receptor genes most probably reflects the strong reliance of pigs on their sense of smell while scavenging for food.

Conservation of synteny and evolutionary breakpoints

Alignment of the porcine genome against seven other mammalian genomes (Supplementary Information) identified homologous synteny blocks (HSBs). Using porcine HSBs and stringent filtering criteria, 192 pig-specific evolutionary breakpoint regions (EBRs) were located. The number of porcine EBRs (146, Supplementary Table 11 and Supplementary Fig. 16) is comparable to the number of bovine-lineage-specific EBRs (100) reported earlier using a slightly lower resolution (500 kilobases (kb)), indicating that both lineages evolved with an average rate of ~ 2.1 large-scale rearrangements per million years after the divergence from a common cetartiodactyl ancestor ~ 60 Myr ago². This rate compares to ~ 1.9 rearrangements per million years within the primate lineage (Supplementary Table 11). A total of 20 and 18 cetartiodactyl EBRs (shared by pigs and cattle) were detected using the pig and human genomes as a reference, respectively.

Pig-specific EBRs were enriched for LTR endogenous retrovirus 1 (LTR-ERV1) transposons and satellite repeats (Supplementary Table 12), indicating that these two families of repetitive sequences have contributed to chromosomal evolution in the pig lineage. Different families of transposable elements seem to have been active in the cetartiodactyl ancestor. The cetartiodactyl EBRs are enriched for LINE1 elements and tRNA^{Glu}-derived SINEs. tRNA^{Glu}-derived SINEs, previously found over-represented in cetartiodactyl EBRs defined in the bovine genome¹⁰, originated in the common ancestor of cetartiodactyls²¹. Our observation that these elements are also enriched in porcine EBRs strongly supports the hypothesis that active transposable elements promote lineage-specific genomic rearrangements.

A stringent set of porcine to human one-to-one orthologues using the MetaCore database revealed that porcine EBRs and adjacent intervals are enriched for genes involved in sensory perception of taste ($P < 8.9 \times 10^{-6}$; FDR < 0.05), indicating that taste phenotypes may have been affected by events associated with genomic rearrangements. Pigs have a limited ability to taste NaCl²². *SCNN1B*, a gene encoding a sodium channel involved in the perception of salty tastes, is located in a porcine-specific EBR. Another gene, *ITPR3*, encoding a receptor for inositol triphosphate and a calcium channel involved in the perception of umami and sweet tastes, has been affected by the insertion of several porcine-specific SINE mobile elements into its 3' untranslated region (3' UTR), consistent with our observation of a higher density of transposable elements in EBRs. In addition to 8 bitter taste receptor genes annotated by Ensembl and which were used in the gene enrichment analysis, we identified 9 intact genes, to give a total number of 17 TAS2R receptors in the pig (Supplementary Table 13). This compares to 18 intact bitter taste receptors in cattle, 19 in horse, 15 in dog and 25 in humans^{23,24}. Of the 14 bitter taste receptor genes that were mapped to a specific pig chromosome (SSC), 10 were found near 2 EBRs on SSC5 and SSC18 (Supplementary Tables 13 and 15). We also found that at least four taste receptors (*TAS1R2*, *TAS2R1*, *TAS2R40* and *TAS2R39*) have been under relaxed selection (Supplementary Information). Pigs are not sensitive to bitter tastes and tolerate higher concentrations of bitter compounds than humans^{22,25}. Thus, pigs can eat food that is unpalatable to humans. A review of the porcine taste transduction network (Supplementary Fig. 17) revealed additional genes affected by rearrangements that affect apical and taste

receptor cell' processes. Together with the observed over-representation of genes related to 'adrenergic receptor activity' and 'angiotensin and other binding' categories in the pig EBRs (Supplementary Fig. 18), our data indicate that chromosomal rearrangements significantly contributed to adaptation in the suid lineage.

Population divergence and domestication

Divergence between Asian and European wild boar

We investigated the evolution within *Sus scrofa* in Eurasia by sequencing ten individual unrelated wild boars from different geographical areas. In total, 17,210,760 single nucleotide polymorphisms (SNPs) were identified among these ten wild boars. The number of SNPs segregating in the four Asian wild boars (11,472,192) was much higher than that observed in the six European wild boars (6,407,224) with only 2,212,288 shared SNPs. This higher nucleotide diversity was visible in the distribution of heterozygous sites of the Asian compared to the European wild boar genomes (Fig. 2). Phylogenomic analyses of complete genome sequences from these wild boars and six domestic pigs revealed distinct Asian and European lineages (Supplementary Fig. 23) that split during the mid-Pleistocene 1.6–0.8 Myr ago (Calabrian stage, Frantz, L. A. F. *et al.*, manuscript submitted). Colder climates during the Calabrian glacial intervals probably triggered isolation of populations across Eurasia. Admixture analyses (Supplementary Information) within Eurasian *Sus scrofa* disclosed gene flow between the northern Chinese and European populations consistent with pig migration across Eurasia, between Europe and northern China, throughout the Pleistocene. Our demographic analysis on the whole-genome sequences of European and Asian wild boars (Fig. 3) revealed an increase in the European population after pigs arrived from China. During the Last Glacial Maximum (LGM; ~20,000 years ago)²⁶, however, Asian and European populations both suffered population bottlenecks. The drop in population size was more pronounced in Europe than Asia (Fig. 3), suggesting a greater impact of the LGM in northern European regions and probably resulting in the observed lower genetic diversity in modern European wild boar.

The deep phylogenetic split between European and Asian wild boars is further supported by our observation of 1,272,737 fixed differences between the six European and four Asian wild boars, 1,706 of which are non-synonymous mutations in 1,191 different genes. Genes involved in sensory perception, immunity and host defence were among the most rapidly evolving genes (Supplementary Table 28), further strengthening the conclusions from our analysis of immunity-related pig proteins. This conclusion is further supported by our observation that these genes are also enriched in porcine segmental duplications (Supplementary Information).

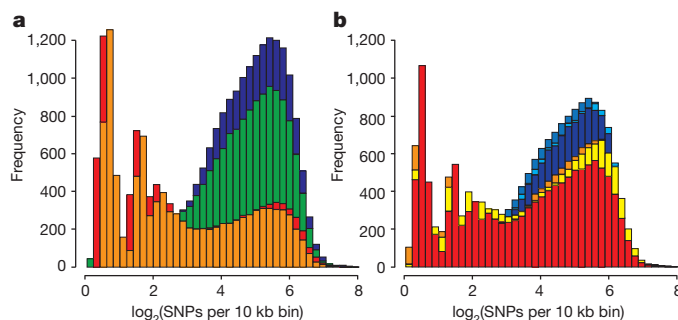


Figure 2 | Distribution of heterozygosity for individual pig genomes. Shown is the distribution of the heterozygosity as the $\log_2$ (SNPs) per 10k bin. **a**, Wild *Sus scrofa*: blue, south China; green, north China; orange, Italian; red, Dutch. **b**, Breeds: blue, Chinese breeds (Jiangquhai, Meishan, Xiang); red-yellow, European breeds (Hampshire, large white, landrace). Note that the Hampshire breed is a North American breed of European origin.

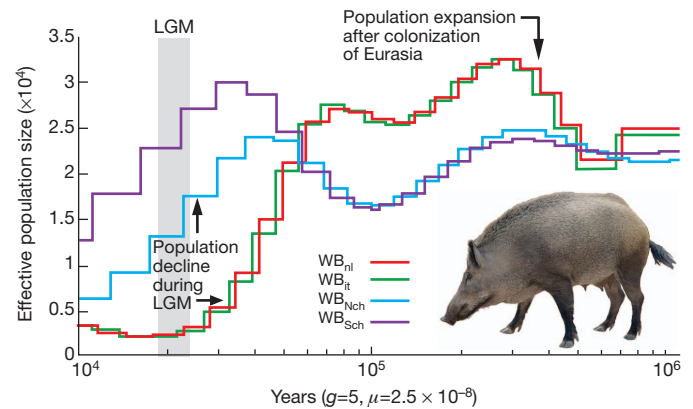


Figure 3 | Demographic history of wild boars. Demographic history was inferred using a hidden Markov model (HMM) approach as implemented in pairwise sequentially Markovian coalescence (PSMC)⁴⁵. In the absence of known mutation rates for pig, we used the default mutation rate for human (μ) of 2.5×10^{-8} . For the generation time (g) we used an estimate of 5 years. The Last Glacial Maximum (LGM) is highlighted in grey. WB_{Nl}, wild boar Netherlands; WB_{It}, wild boar Italy; WB_{Nch}, wild boar north China; WB_{Sch}, wild boar south China.

To investigate further whether specific regions in the genome of European and Asian wild boar have been under positive selection, a selective sweep analysis was performed on the ten wild boar genome sequences using an approach similar to that recently described in the comparison of Neanderthal and *Homo sapiens* genomes²⁷. Regions in the genome under strong positive selection after the divergence of these two populations are expected to share fewer derived alleles. Using stringent criteria (Supplementary Information), we identified a total of 251 putative selective sweep regions, with an average size of 111,269 base pairs (bp), together comprising around 1% of the genome and harbouring 365 annotated protein-coding genes (Supplementary Table 26). Many of these regions (112) do not contain any currently annotated protein-coding genes. In contrast, the 10 putative selective sweep regions located between positions 39–43 Mb on SSC3 together harbour 93 genes. This SSC3 region (Supplementary Fig. 25) is directly adjacent to the centromere and exhibits low recombination rates²⁸. Low recombining regions have been shown to be more prone to selective sweeps and meiotic drive^{29,30}. Although similar large putative selective sweep regions close to the centromere were only observed on SSC6 between positions 56.2–57.5 Mb, on most chromosomes selective sweep regions tended to cluster in the central part of chromosomes, thus exhibiting a clear correlation with regions of low recombination (Supplementary Fig. 27). As expected, regions with the highest nucleotide differentiation between European and Asian wild boars were observed in high recombination regions towards the end of the chromosomes on both metacentric and acrocentric chromosomes²⁸.

The putative selective sweep regions displayed significant over-representation of genes involved in RNA splicing and RNA processing, indicating possible changes in the regulation of genes at the level of RNA processing (Supplementary Table 27). Several of these genes (*CELF1*, *CELF6*, *WDR83*, *RBM39*, *RBM6*, *HNRNPA1*, *HNRNPM*) are involved in alternative splicing, and, small differences in expression might affect alternatively spliced transcripts of specific genes. Evolution of regulatory splicing factors such as heterogeneous ribonucleoprotein particle (hnRNP) proteins has been proposed as an evolutionary model for alternative splicing³¹, and genetic variation in such factors can affect alternative splicing and result in different phenotypes or disease³². Our observation that specific genes involved in splicing show accelerated evolution in the pig lineage (Fig. 1) supports this hypothesis. Of particular interest is the selective sweep region observed at position 26 Mb on SSC3 around the *ERI2* gene (Fig. 4), which encodes ERI1 exoribonuclease family member 2. Different gene variants have

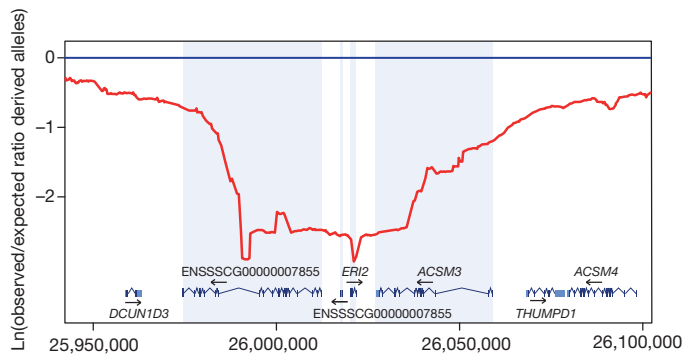


Figure 4 | Putative selective sweep region around the *ERI2* gene on SSC3. The y axis shows the log-transformed value of the ratio for the observed/expected derived allele frequency using a sliding window at a bin size of 50,000 bp. The x axis shows the position on SSC3 in base pairs.

been fixed in European and Asian wild boar coding for proteins that differ at two amino acid positions: Cys52Arg and His358Leu encoded by exons 3 and 9 of the *ERI2* gene, respectively. The precise function of *ERI2* is unknown but the *ERI1* exoribonuclease family members have been shown to be involved in mRNA decay³³ and in *Caenorhabditis elegans* *ERI-1* has been shown to be involved in the degradation of microRNAs (miRNAs)³⁴.

Independent domestication and admixture events in domestic breeds

A phylogenetic tree constructed using four European wild boar and domestic pigs and six East Asian wild boar and domestic pigs revealed a clear distinction between European and Asian breeds, thus substantiating the hypothesis that pigs were independently domesticated in western Eurasia and East Asia³. An admixture analysis revealed European influence in Asian breeds, and a ~35% Asian fraction in European breeds (Supplementary Table 24). These results are consistent with the known exchange of genetic material between European and Asian pig breeds³⁵. We also observed that European breeds form a paraphyletic clade, which cannot be solely explained by varying degrees of Asian admixture (Supplementary Information). Within each continent, our analysis revealed different degrees of relatedness between breeds and their respective wild relatives (Supplementary Table 20).

During domestication, pigs were often allowed to roam in a semi-managed state and recurrent admixture between wild and domesticated individuals was not uncommon, especially in Europe³⁵. Thus, the most likely explanation for the paraphyletic pattern seen in domestic individuals is a long history of genetic exchange between wild and domestic pigs.

The pig as a biomedical model

The pig is an important biomedical model and the ability to generate transgenics and knockouts in combination with somatic nuclear cloning procedures has resulted in a number of models for specific human diseases³⁶. Naturally occurring mutations also offer opportunities to use pigs as biomedical models^{37,38}. To explore the potential for natural models further, predicted porcine protein sequences were compared with their human orthologues. We observed 112 positions where the porcine protein has the same amino acid that is implicated in a human disease (Supplementary Table 29). Most of these changes in humans have been shown to increase risk in multifactorial traits such as obesity (*ADRB3*, *SDC3*) and diabetes (*PPP1R3A*, *SLC30A8*, *ZNF615*) or shown to result in relatively mild phenotypes (for example, dyslexia: *KIAA0319*) or late-onset diseases such as Parkinson's disease (*LRRK2*, *SNCA*) and Alzheimer's disease (*TUBD1*, *BLMH*, *CEP192*, *PLAU*). These porcine variants are of interest, as they will allow detailed

characterization in an experimental model organism whose physiology is very similar to that of human.

Among 32,548 non-synonymous mutations identified by sequencing 48 individual pigs, representing 8 different European and Asian breeds and wild boars³⁹, 6 protein variants implicated in human disease were identified (Supplementary Table 30). In addition, another 157 nonsense mutations in 142 genes were identified, 11 of which have also been implicated in human disease (Supplementary Table 31). Most of these 11 variants were only observed in a heterozygous state and those for which homozygous individuals were observed probably result in either a mild phenotype (*ASS1*, mild form of citrullinaemia in humans) or in phenotypes unlikely to affect the fitness of wild boars (*RBBP8*, pancreatic carcinomas). Our estimate for the average number of nonsense mutations per individual (~30) is smaller than that observed in humans⁴⁰ despite the observed threefold higher nucleotide diversity in pigs³⁹. This is in agreement with the higher effective population size in the pig compared to that for the human population, which exhibited a strong bottleneck followed by an exponential increase in size during recent history⁴¹.

When considering pig-to-human xenotransplantation, porcine endogenous retroviruses (PERVs) pose a risk of zoonotic infection. The pig genome contains fewer endogenous retroviruses than many vertebrates, including humans and mice, and most PERVs were characterized as defective. However, the potential risk posed by reactivation of rare replication-competent PERVs and defective PERVs by recombination remains, as shown for murine ERVs (XMRV)⁴². Most PERVs consist of γ and γ -like groups (68%), with β -retroviral ERVs comprising the second most abundant group (Supplementary Fig. 15). Our phylogenetic study shows a particularly close relationship between the most intact $\gamma 1$ group of PERVs ($\gamma 1$) and murine γ -ERVs, suggesting a potential recent instance of murine-to-porcine transmission of $\gamma 1$ ERVs (Supplementary Fig. 15). We identified 20 almost intact PERV $\gamma 1$ loci (Supplementary Table 10), none of which contained a complete set of *gag*, *pol* or *env* open reading frames, indicating that these proviruses are not replicable. We also identified four β -retroviral PERVs, each containing defects, primarily in *env*. These were distantly related to intracisternal type A particle (IAP) proviruses of mice and the mouse mammary tumour virus (MMTV)-like (HML) proviruses of humans. None of the above loci was shared in more than 120 pigs tested, indicating considerable PERV polymorphisms.

Conclusion

The draft pig genome sequence reported here has illuminated the evolution of *Sus scrofa* and confirmed its speciation in South East Asia and subsequent domestication at multiple regions across Eurasia. The high-quality annotated reference genome sequence has already proven to be a critical framework for comparing individual genomes^{39,43,44} and its value is further illustrated in associated papers published elsewhere (<http://www.biomedcentral.com/series/swine>). The genome sequence also provides a valuable resource enabling effective uses of pigs both in agricultural production and in biomedical research.

METHODS SUMMARY

Assembly. We constructed a hybrid *de novo* assembly based primarily on sequences from BAC clones sequenced clone-by-clone and supplemented with Illumina whole-genome shotgun (WGS) reads. BAC clones were selected from the high-resolution physical (BAC contig) map⁶ with CHORI-242 library clones prepared from DNA from a single Duroc sow (Duroc 2-14) chosen preferentially. The WGS sequence data were generated using DNA isolated from the same animal. The BAC-derived sequence data were assembled into sequence contigs using Phrap on a clone-by-clone basis and subsequently independently assembled WGS contigs (Supplementary Information) were used to extend BAC clone-derived sequence contigs and to close gaps between clone-derived contigs. Further details and other methods are described in Supplementary Information.

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Author Information The final assembly (Sscrofa10.2) has been deposited in the public sequence databases (GenBank/EMBL/DBJ) under accession number AEMK01000000. The primary source of the Sscrofa10.2 assembly is the NCBI ftp site (http://ftp.ncbi.nih.gov/genbank/genomes/Eukaryotes/vertebrates_mammals/Sus_scrofa/Sscrofa10.2/). The chromosomes are CM000812–CM00830 and CM001155. They are built from 5,343 placed scaffolds, with GenBank accession numbers GL878569–GL882503 and JH114391–JH118402. The 4,562 unplaced scaffolds of Sscrofa10.2 have accessions in the ranges GL892100–GL896682 and JH118403–JH118999. Illumina sequences for the sequenced wild boars and individuals of the other breeds, aligned against build10.2, have been deposited in the European Nucleotide Archive (ENA) under project number ERP001813. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.A.M.G. (martien.groenen@wur.nl) or A.L.A. (alan.archibald@roslin.ed.ac.uk). This work is licensed under a Creative Commons Attribution-NonCommercial-ShareAlike 3.0 Unported licence. To view a copy of this licence, visit <http://creativecommons.org/licenses/by-nc-sa/3.0/>

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Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes

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Pancreatic cancer is a highly lethal malignancy with few effective therapies. We performed exome sequencing and copy number analysis to define genomic aberrations in a prospectively accrued clinical cohort ($n = 142$) of early (stage I and II) sporadic pancreatic ductal adenocarcinoma. Detailed analysis of 99 informative tumours identified substantial heterogeneity with 2,016 non-silent mutations and 1,628 copy-number variations. We define 16 significantly mutated genes, reaffirming known mutations (*KRAS*, *TP53*, *CDKN2A*, *SMAD4*, *MLL3*, *TGFBR2*, *ARID1A* and *SF3B1*), and uncover novel mutated genes including additional genes involved in chromatin modification (*EPC1* and *ARID2*), DNA damage repair (*ATM*) and other mechanisms (*ZIM2*, *MAP2K4*, *NALCN*, *SLC16A4* and *MAGEA6*). Integrative analysis with *in vitro* functional data and animal models provided supportive evidence for potential roles for these genetic aberrations in carcinogenesis. Pathway-based analysis of recurrently mutated genes recapitulated clustering in core signalling pathways in pancreatic ductal adenocarcinoma, and identified new mutated genes in each pathway. We also identified frequent and diverse somatic aberrations in genes described traditionally as embryonic regulators of axon guidance, particularly *SLIT/ROBO* signalling, which was also evident in murine Sleeping Beauty transposon-mediated somatic mutagenesis models of pancreatic cancer, providing further supportive evidence for the potential involvement of axon guidance genes in pancreatic carcinogenesis.

Pancreatic cancer is the fourth leading cause of cancer death, with an overall 5-year survival rate of $<5\%$, statistics that have not changed in almost 50 years¹. Advances in neoadjuvant and adjuvant chemotherapeutic regimens have resulted in some improvement in outcome, but pancreatectomy remains the single most effective treatment modality for pancreatic cancer, and offers the only potential for cure. Only 20% of patients present with localized, non-metastatic disease which is suitable for resection². Those who undergo resection and receive adjuvant therapy have a median survival of 12–22 months and a 5-year survival of 20–25%³. Existing systemic therapies are only modestly effective and the median survival for patients with metastatic disease remains 6 months. Genomic characterization of pancreatic ductal adenocarcinoma (PDAC), which accounts for over 90% of pancreatic cancer, has so far focused on targeted polymerase chain reaction (PCR)-based exome sequencing of primary and metastatic lesions propagated as xenografts or cell lines⁴. A deeper understanding of the underlying molecular pathophysiology of the clinical disease is needed to advance the development of effective therapeutic and early detection strategies.

Clinical cohort

A cohort of 142 consecutive patients with primary operable, untreated PDAC who underwent pancreatectomy with curative intent (pre-operative clinical stages I and II) were recruited, and consent was obtained for genomic sequencing through the Australian Pancreatic Cancer Genome Initiative (APGI), the Baylor College of Medicine Pancreatic Cancer Genome Project and the Ontario Institute for Cancer Research Pancreatic Cancer Genome Study (ABO collaboration) between June 2005 and June 2011 as part of the International Cancer Genome Consortium (ICGC)⁵. Detailed clinico-pathological characteristics of the cohort demonstrated features typical of resected PDAC with regard to tumour size, grade, lymph node metastasis and survival when compared to multiple retrospectively acquired cohorts^{6–8}, defining the accrued population as representative of the clinical disease in the community (Supplementary Table 1 and Supplementary Fig. 1).

Cellularity and mutation detection

A major challenge in genomic sequencing is the low malignant epithelial cell content of many cancers, which can adversely impact on the sensitivity of mutation detection. Most sequencing studies so far have used samples with $>70\%$ tumour cellularity, or cell lines/xenografts^{4,9}. To implement genomic sequencing approaches in clinical practice, it is imperative to efficiently and accurately detect actionable mutations in diagnostic clinical samples. We devised methodologies to overcome the challenges associated with extensive desmoplastic stroma that is characteristic of the majority of PDAC, and these strategies facilitated the discovery of novel molecular mechanisms in the pathophysiology of this disease. The cellularity of each primary sample was estimated through pathological review, deep amplicon-based sequencing of exons 2 and 3 of *KRAS* (average depth of $1,000\times$), and single nucleotide polymorphism (SNP) array-based cellularity estimates using a novel algorithm (qpure)¹⁰. *KRAS* mutations were identified in 93% of 142 cases and tumour cellularity ranged from 5% to 85% with a mean of 38% (Supplementary Table 2, Supplementary Figs 2 and 3, and Supplementary Methods).

To inform cellularity thresholds for subsequent analyses, we defined the impact of stromal DNA content on mutation detection by exome capturing and sequencing different mixtures of cancer cell line and matched germline DNA (100%, 80%, 60%, 40%, 20% and 10% cell line DNA) when sequenced to a depth of $70\times$ coverage. Using these data as a standard, the median sensitivity to detect true positives across all samples in the cohort with greater than 20% epithelial cellularity was estimated at 45% (Supplementary Table 3). An informative cohort of 99 patients who had greater than 20% cellularity and/or ≥ 10 validated somatic mutations was taken forward for further analysis.

Mutation detection and CNV analysis

We performed hybrid-selection-based capture and sequencing of the entire exomes of tumour and matched normal DNA derived from all 142 patients using a combination of capture systems and next-generation sequencing platforms (see Supplementary Methods). The

Table 1 | Mutations in pancreatic ductal adenocarcinoma (*n* = 99)

Mutation class	Total
Missense	1,684
Nonsense	99
Splice site	89
Insertion/deletion	144
Non-silent	2,016
Silent	611

sequence depths at each site (APGI 65×, BCM 104× and OICR 205×) were adopted to ensure suitable sensitivity across their respective cohorts (Supplementary Table 3). In the informative 99 samples, we detected 2,627 high-confidence mutations, 2,016 of which were non-silent (Table 1). A total of 1,502 of these events (1,350 non-silent) were independently validated via an orthogonal sequencing method (see Supplementary Methods). The average number of mutations detected per patient was 26 (range 1–116), consistent with the expected sensitivity based on cellularity estimates and previous studies^{4,11} (Supplementary Table 2). We confirmed the high prevalence of genetic aberrations known to be important in PDAC and observed mutations in 38 of the 79 genes (48% overlap) that occurred more than once previously reported by ref. 4, and 186 of all 998 mutated genes (19% overlap) in that study. We also defined a large number of novel mutations (1,456 genes), most of which occurred at low frequency (see Supplementary Tables 4–6 and Supplementary Fig. 4 for detailed comparisons). The observed transversion/transition rates in the cohort correlated closely with those previously reported in PDAC cell lines and xenografts (Supplementary Table 7).

Significant mutated gene analysis¹² of genes with non-silent mutations that occurred in 2 or more individual cancers identified 16 genes in the top 20 mutated genes in 2 of 3 stringent analytical approaches (Table 2, Supplementary Table 8 and Supplementary Methods) and reaffirmed the importance of mutations known to occur in PDAC: *KRAS*, *TP53*, *CDKN2A*, *SMAD4*, *MLL3*, *TGFBR2*, *ARID1A* and *SF3B1*. Novel significantly mutated genes included additional genes involved in chromatin modification (*EPC1* and *ARID2*) and *ATM*, recently implicated as a PDAC susceptibility gene through bi-allelic inactivation in a case of familial PDAC (germline mutation and loss of heterozygosity (LOH) in the tumour)¹³. Aberrations of *ATM* occurred in 8% of our cohort (mutated in 5%, LOH or loss in 5%, with two patients exhibiting both mutation and LOH or loss) and mutations detected in other genes not previously reported: *ZIM2*, *MAP2K4*, *NALCN*, *SLC16A4* and *MAGEA6* (Table 2). GISTIC2.0¹⁴ identified 30 genes affected by copy-number alterations (Q value <0.0001) and included losses of *CDKN2A* and *SMAD4* (Supplementary Table 4).

Table 2 | Significantly mutated genes in pancreatic ductal adenocarcinoma

Gene symbol	Gene name and protein function	SB mutagenesis*	shRNA†
<i>KRAS</i>	Oncogene; GTPase; activation of MAPK activity	Yes	Yes
<i>TP53</i>	Tumour suppressor p53; DNA damage response	–	Yes
<i>CDKN2A</i>	Cyclin-dependent kinase inhibitor 2A; G1/S transition of mitotic cell cycle; tumour suppressor	Yes	–
<i>SMAD4</i>	Mothers against decapentaplegic homologue 4; BMP signalling pathway	Yes	Yes
<i>MLL3</i>	Myeloid/lymphoid or mixed-lineage leukaemia protein 3; DNA binding; regulation of transcription	Yes	Yes
<i>TGFBR2</i>	Transforming growth factor-β receptor type II; regulation of growth	Yes	–
<i>ARID1A</i>	AT-rich interactive domain-containing protein 1A; SWI/SNF complex; chromatin modification	Yes	Yes
<i>ARID2</i>	AT-rich interactive domain-containing protein 2; chromatin modification	Yes	–
<i>EPC1</i>	Enhancer of polycomb homologue 1; histone acetylation	Yes	–
<i>ATM</i>	Ataxia telangiectasia mutated; DNA damage response	–	Yes
<i>SF3B1</i>	Splicing factor 3B subunit 1; nuclear mRNA splicing	–	Yes
<i>ZIM2</i>	Zinc finger imprinted 2; regulation of transcription	–	Yes
<i>MAP2K4</i>	Dual specificity mitogen-activated protein kinase kinase 4; Toll-like receptor signalling pathway	Yes	Yes
<i>NALCN</i>	Sodium leak channel non-selective protein; sodium channel activity	–	Yes
<i>SLC16A4</i>	Solute carrier family 16 member 4; monocarboxylate transporter	–	Yes
<i>MAGEA6</i>	Melanoma-associated antigen 6; protein binding	–	ND

ND, not determined.

* Significant insertion sites in two independent Sleeping Beauty mutagenesis screens^{17,18}.

† *In vitro* shRNA screens in 102 cancer cell lines with effect on cell survival¹⁹.

Pathways in pancreatic cancer

To better understand potential underlying mechanisms of importance in PDAC, we performed a series of pathway analyses using genes that were recurrently mutated in two or more individuals using GeneGO¹⁵, and identified mechanisms known to be important in cancer: G1/S checkpoint machinery ($P = 1.49 \times 10^{-3}$), apoptosis ($P = 1.32 \times 10^{-4}$), regulation of angiogenesis ($P = 7.72 \times 10^{-4}$) and TGF-β signalling ($P = 9.50 \times 10^{-4}$). Interestingly, novel gene signatures were enriched in our cohort, including axon guidance ($P = 5.30 \times 10^{-5}$) (Supplementary Table 9). The inclusion of mutation data for 24 cases from ref. 4 strengthened the association of axon guidance ($P = 3.3 \times 10^{-7}$), and was more evident still when all mutated genes in our data set were used as input ($P = 4.67 \times 10^{-8}$).

Functional relevance of genomic events

Differentiating somatic driving events of carcinogenesis from passenger mutations is a major challenge in cancer genomics¹⁶. Despite significant advances in computational algorithms, experimental evidence of functional relevance is paramount. We used data from three published experimental biological screens to infer functional consequences for the individual genomic events and the pathways we identified. These included data from two independent Sleeping Beauty transposon (SB) mutagenesis screens in *Kras* transgenic mouse models of PDAC^{17,18} and an *in vitro* short hairpin RNA (shRNA) screen which examined the consequences of downregulating 11,194 putative cancer genes on survival in a panel of 102 cell lines (13 pancreatic)¹⁹ (Supplementary Methods and Supplementary Figs 5 and 6). Data from these screens confirmed the functional importance of *KRAS*, *TP53*, *CDKN2A* and *SMAD4* mutations and attributed potential functional relevance to most significantly mutated genes—*MLL3*, *TGFBR2*, *SF3B1*, *EPC1*, *ARID1A*, *ARID2*, *MAP2K4*, *ATM*, *NALCN*, *ZIM2*, *SLC16A4* (Table 2)—and many genes mutated at low frequency (Supplementary Table 4).

Pathway analysis of high confidence insertions in SB transposon mutagenesis screens demonstrated enrichment for axon guidance genes ($P = 1.6 \times 10^{-3}$), providing independent supportive evidence for a potential role in the pathogenesis of PDAC. In these screens, 14 genes involved in axon guidance pathways were detected (5 genes common to both). In addition, a further 32 genes were mutated in at least one SB pancreatic tumour (out of 21) but did not meet the significance threshold with the stringent analyses that were applied¹⁷ (Supplementary Tables 10 and 11).

Axon guidance pathway genes

The class of genes traditionally described for their roles in axon guidance (semaphorins, slits, netrins and ephrins) are important regulators of

normal neuronal migration and positioning during embryonic development. More recently, they have been implicated in cancer cell growth, survival, invasion and angiogenesis²⁰; however, the incidence of aberrations in these genes in cancer is largely unknown. We identified recurrent mutations and copy-number variations (CNVs) of axon guidance pathway genes in this cohort (Fig. 1 and Supplementary Table 4): *SLIT2* and *ROBO2* mutations were present in 5% of patients, with focal copy-number losses of *ROBO1*, and *SLIT2* detected by GISTIC2.0 analysis and confirmed by manual review, potentially having an impact on a further 15% of the cohort, suggesting that aberrant SLIT/ROBO signalling is potentially a common feature of PDAC (Figs 1 and 2). In addition, we used targeted PCR-based sequencing of an additional 30 cases of PDAC for axon guidance genes and identified mutations in *ROBO1* in two patients and additional mutations in *SLIT2* and *ROBO2* (one patient each). Low mRNA expression of the *ROBO2* receptor was associated with poor patient survival ($P = 0.04$). Furthermore, high mRNA expression of *ROBO3*, a known inhibitor of *ROBO2* signalling²¹, demonstrated an appropriate reciprocal inverse association with poor survival ($P < 0.006$) (Fig. 2).

Class 3 semaphorins (*SEMA3A* and *SEMA3E*) exhibited significant amplification in 18% of patients and an additional 3% harboured mutations (Fig. 1). Semaphorins signal through neuropilin and plexin receptors to elicit their effects²². *SEMA3A* amplification correlated with high mRNA expression on microarray ($P = 0.03$), and high mRNA expression of *SEMA3A* and *PLXNA1*, another molecule central to semaphorin signalling, were both associated with poor patient survival on univariate analysis (Fig. 3a), and were independently prognostic on multivariate analyses with clinico-pathological variables (Supplementary Table 12).

To elucidate further the significance of the observed CNV events, we reviewed copy number, CNV segment size and changes in heterozygosity of axon guidance genes in a recent independent CNV analysis of 39 fine-needle aspiration biopsies²³ and the 16 PDAC cell lines in the

CONAN database (<http://www.sanger.ac.uk/cosmic>)²⁴. Overall, the predominant changes recapitulated our studies, showing frequent focal losses within genes involved in SLIT/ROBO signalling, and gains in genes involved in canonical semaphorin signalling (Supplementary Tables 4, 13 and 14).

To assess whether dysregulation of axon guidance genes is associated with early neoplastic transformation, as are many developmental signalling pathways, we examined mRNA expression in murine models of early pancreatic carcinogenesis (*in vitro* acinar-to-ductal metaplasia and *in vivo* pancreatic injury). Expression levels of components of SLIT/ROBO and semaphorin signalling changed progressively from normal pancreas, through acinar-to-ductal metaplasia and pancreatic injury to genetically engineered murine PDAC, indicating a role for the dysregulation of these axon guidance genes in tumour initiation and progression (Fig. 3b and Supplementary Table 15).

Discussion

We devised methodologies to optimize mutation detection for clinical samples in a large cohort of patients and reaffirm known mutations in PDAC, better define their prevalence in a large cohort of early PDAC, and identify potential novel drivers in this disease. Somatic mutations in *ATM* were identified in a significant proportion of patients (8%), highlighting the importance of BRCA-mediated DNA damage repair mechanisms in sporadic PDAC as well as familial disease¹³. Previously, mutations in individual genes involved in chromatin remodelling such as *ARID1A*²⁵ have been described and additional genes identified here (*EPC1* and *ARID2*) infer that chromatin remodelling may have an important role in PDAC, along with other cancer types²⁶.

Novel mutations in genes traditionally described for their roles in axon guidance were also observed by a combination of genomic data and supportive experimental evidence from independent murine SB mutagenesis screens. Axon guidance is integral to organogenesis,

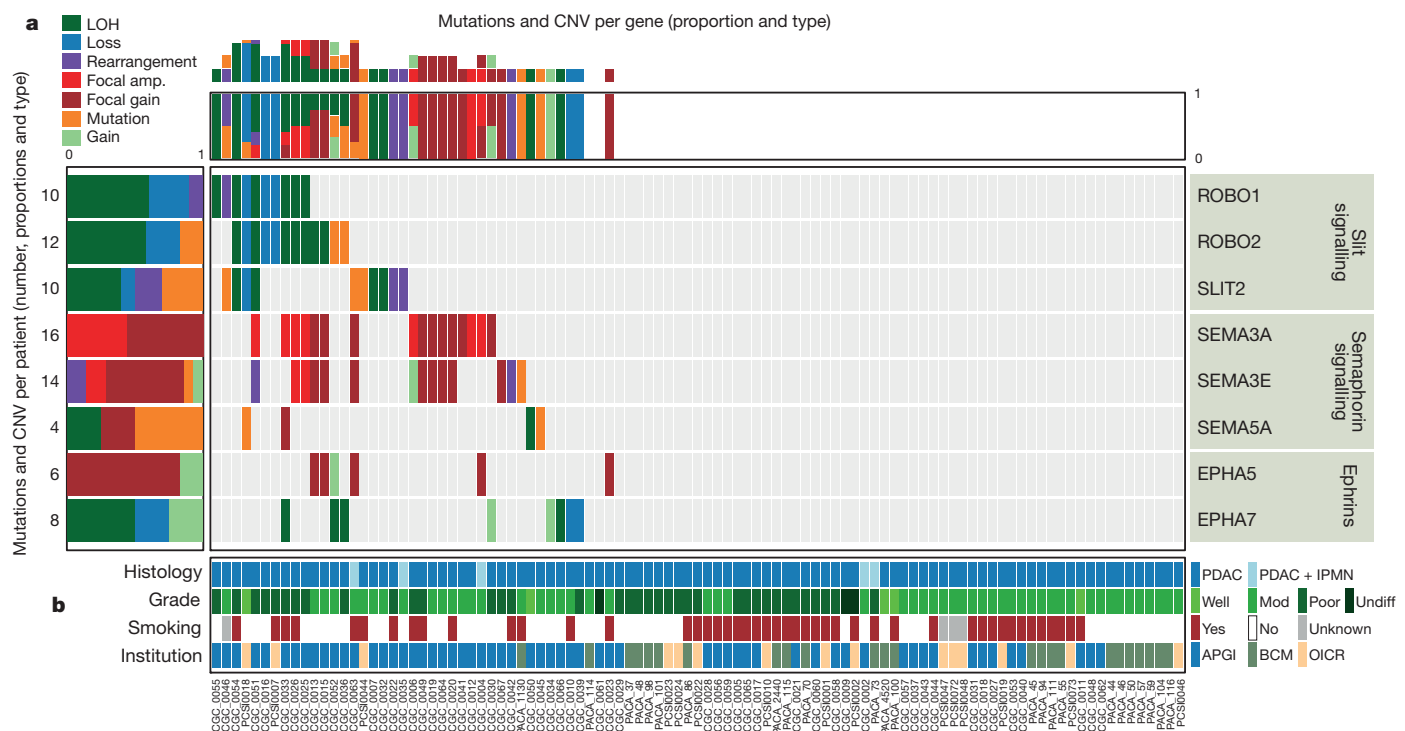


Figure 1 | Mutations and copy number variation in axon guidance genes. Axon guidance pathway genes with recurrent mutations and/or copy-number changes defined by GISTIC2.0 analysis ($Q < 0.2$), and manually reviewed for focal alterations. **a**, SNV and CNV frequency per patient with gene-centric summary (left) and patient-centric summary (top); numbers of patients with mutations and proportion of each event are presented. Please see

Supplementary Table 4 for further details. **b**, Clinico-pathological variables for individual patients. APCI, Australian Pancreatic Cancer Genome Initiative; BCM, Baylor College of Medicine; IPMN, intraductal papillary mucinous neoplasm; Mod, moderately differentiated; OICR, Ontario Institute for Cancer Research; PDAC, pancreatic ductal adenocarcinoma; Undiff, undifferentiated.

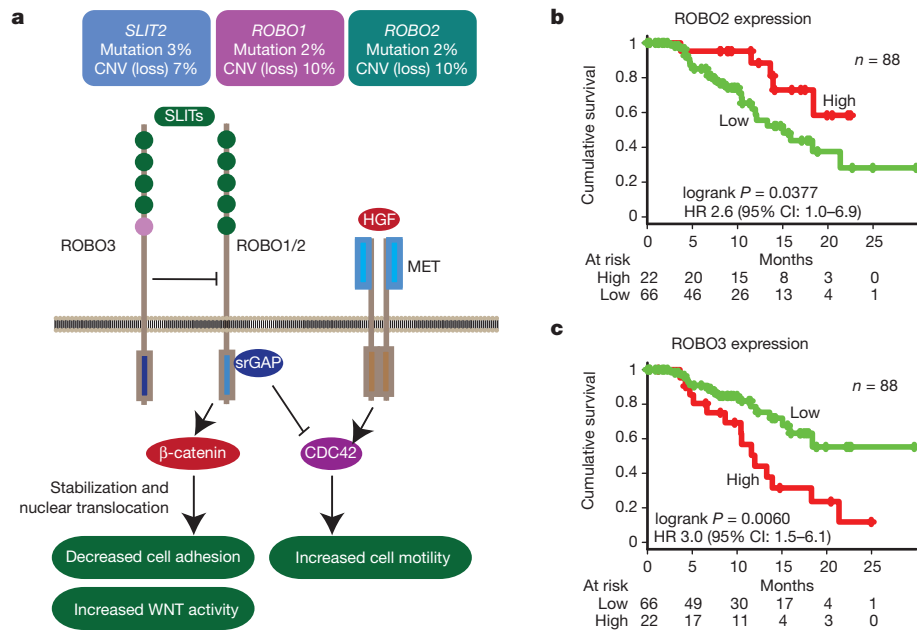


Figure 2 | SLIT/ROBO signalling in pancreatic ductal adenocarcinoma. **a**, SLIT/ROBO signalling normally enhances β -catenin complex formation with E-cadherin and suppresses WNT signalling activity. Loss of ROBO1/2 signalling promotes stabilization of β -catenin, which decreases E-cadherin complex formation and cell adhesion and augments WNT signalling activity through increased nuclear translocation of β -catenin. In addition, SLIT/ROBO signalling can downregulate MET signalling activity; loss of ROBO signalling activity promotes MET signalling downstream and may have an impact on

therapeutic strategies aimed at inhibiting MET activity at the receptor level. (Adapted from ref. 20.) Aberrations in *SLIT2* and/or *ROBO1/2* affected 23% of patients (6% mutated with 1 patient showing mutations in both *SLIT2* and *ROBO2*), with 18% demonstrating CNV corresponding to loss of the gene. **b**, **c**, High expression of SLIT receptor ROBO2 was associated with a better prognosis (**b**), and high expression of ROBO3, an inhibitor of ROBO2, showed an inverse relationship, with high levels associated with poor survival (**c**). HR, hazard ratio.

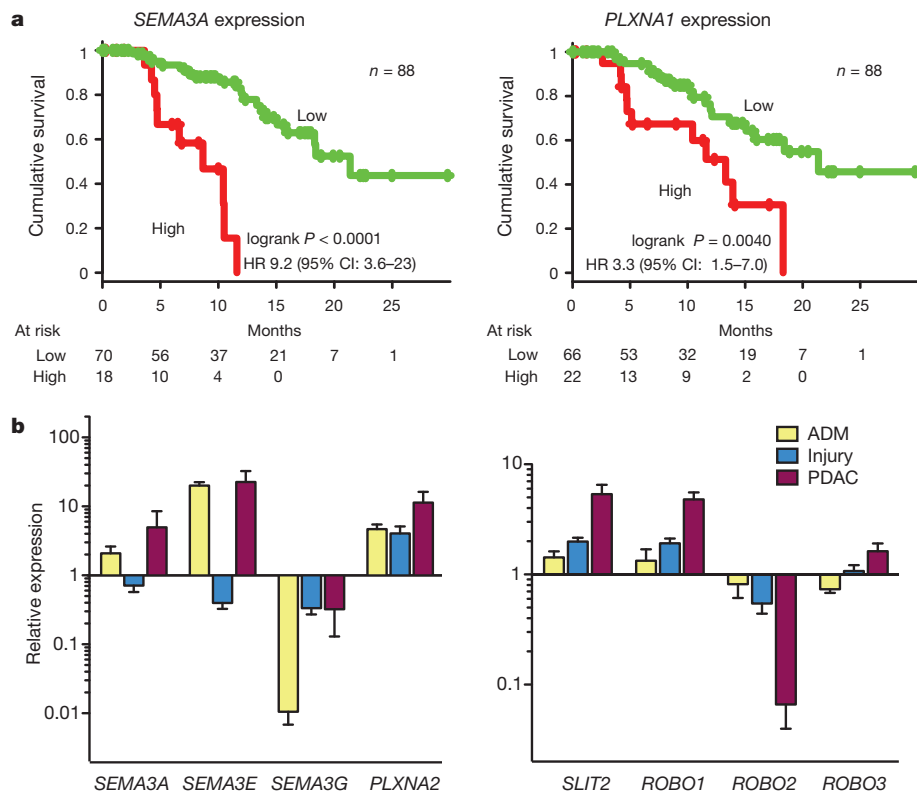


Figure 3 | Axon guidance genes in human and murine pancreatic ductal adenocarcinoma. **a**, Kaplan–Meier survival curves showing co-segregation of aberrant expression of components of semaphorin signalling with outcome. Amplification at *SEMA3A* and *PLXNA1* loci was associated with high mRNA expression and both are independent poor prognostic factors. **b**, Quantitative RT–PCR for components of semaphorin and SLIT/ROBO signalling in murine

models of early (acinar-to-ductal metaplasia (ADM) and pancreatic injury) and established PDAC in genetically engineered mice with a *Pdx1*-promoter-driven activating mutation of *Kras* and mutant *Tp53* allele (*Pdx1-Cre; LSL-Kras^{G12D}; LSL-Trp53^{R172H}*). Error bars represent standard error of the mean (see Supplementary Table 15 for details).

regeneration, wound healing and other basic cellular processes^{22,27}. The widespread genomic aberrations observed here in axon guidance genes suggests that they may have a role in PDAC, joining mounting evidence in other cancers^{20,28}, including a recent report demonstrating *ROBO2* mutations in liver-fluke-associated cholangiocarcinoma²⁹. In addition, evidence from cancers of the lung, breast, kidney and cervix implicate aberrant SLIT/ROBO signalling in carcinogenesis²⁰; *Robo1* knockout mice develop bronchial hyperplasia and focal dysplasia, and inactivation of *Slit2* and *Slit3* leads to the development of hyperplastic disorganized lesions in the breast²⁰. Upregulation of MET and WNT signalling have important roles in PDAC, and recent data indicate that SLIT/ROBO signalling modulates MET and WNT signalling activity through CDC42 and β -catenin, respectively²⁰. Loss of SLIT/ROBO signalling can potentially be an alternative mechanism for deregulating these pathways downstream of their receptors, and in addition could influence the activity of inhibitors that target these upstream components, for example, MET inhibitors (Fig. 2).

Class 3 semaphorins are the only secreted semaphorins in vertebrates. They regulate cell growth, invasiveness and angiogenesis, and are highly expressed in metastatic cells in many cancer types^{30,31}. Although aberrant semaphorin signalling in cancer seems to be organ specific³², our finding that high expression of *SEMA3A* and its receptor *PLXNA1* co-segregates with poor patient survival is supported by a previous study that reported this association and also demonstrated promotion of invasiveness of PDAC cell lines by *SEMA3A*³¹. Therapeutics targeting molecules involved in axon guidance have been developed as potential strategies to facilitate neuronal regeneration after injury³³, but are yet to be assessed for their role in cancer treatment.

As illustrated here, global genomic analysis of large, well-annotated and clinically homogeneous cohorts of patients can identify mechanisms that are common among genomically diverse cancers, and will be pivotal in the development of novel therapeutic strategies that are guided by the determination of the molecular phenotype of individual patients³⁴. Future work will be required to determine which key components, when damaged, drive the disease, and these mechanisms will need to be assessed in molecularly well-characterized preclinical models³⁵. The potential therapeutic strategies identified will then require testing in appropriate clinical trials that are specifically designed to target subsets of patients stratified according to well-defined molecular markers^{36,37}.

METHODS SUMMARY

Sample acquisition and processing. Samples used were prospectively acquired and restricted to primary operable, non-pretreated pancreatic ductal adenocarcinoma. Representative sections were reviewed independently by at least one additional pathologist with specific expertise in pancreatic diseases. Samples either had full face frozen sectioning performed in optimal cutting temperature (OCT) medium, or the ends excised and processed in formalin to verify the presence of carcinoma in the sample to be sequenced and to estimate the percentage of malignant epithelial nuclei in the sample relative to stromal nuclei. Macrodissection was performed if required to excise areas that did not contain malignant epithelium. **Sequencing.** Cellularity of each tumour sample was estimated with pathology review, deep sequencing of *KRAS* and a method developed using genome-wide SNP array data (qpure¹⁰). Exon capture was performed using the SureSelect II or Nimblegen capture methods and paired-end sequenced on the SOLiD (v4) or GAI/HiSeq platforms. Somatic mutations were called and then verified on the Ion Torrent Personal Genome Machine (Life Technologies Corporation) and 454 (Hoffman-La Roche Limited).

Analysis. Significantly mutated genes were identified using the Genome MuSiC package¹². DNA copy number analyses were performed using the Illumina HumanOmni1 Quad genotyping arrays and GenoCN software. Recurrent and significant copy number changes were identified using GISTIC2.0¹⁴. Functional enrichment of gene categories was assessed using the Metacore package (Thomson-Reuters Corporation) and the MSigDB v3.0 database³⁸. All sample information and data for mutation, copy number and expression analyses were submitted to the ICGC DCC at <http://dcc.icgc.org/>. A complete description of the materials and methods including approvals for human research and animal experimentation is provided in Supplementary Information.

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Broad and potent neutralization of HIV-1 by a gp41-specific human antibody

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Characterization of human monoclonal antibodies is providing considerable insight into mechanisms of broad HIV-1 neutralization. Here we report an HIV-1 gp41 membrane-proximal external region (MPER)-specific antibody, named 10E8, which neutralizes ~98% of tested viruses. An analysis of sera from 78 healthy HIV-1-infected donors demonstrated that 27% contained MPER-specific antibodies and 8% contained 10E8-like specificities. In contrast to other neutralizing MPER antibodies, 10E8 did not bind phospholipids, was not autoreactive, and bound cell-surface envelope. The structure of 10E8 in complex with the complete MPER revealed a site of vulnerability comprising a narrow stretch of highly conserved gp41-hydrophobic residues and a critical arginine or lysine just before the transmembrane region. Analysis of resistant HIV-1 variants confirmed the importance of these residues for neutralization. The highly conserved MPER is a target of potent, non-self-reactive neutralizing antibodies, suggesting that HIV-1 vaccines should aim to induce antibodies to this region of HIV-1 envelope glycoprotein.

Induction of an antibody response capable of neutralizing diverse HIV-1 isolates is a critical goal for vaccines that protect against HIV-1 infection. Potentially the greatest obstacle to achieving this goal is the extraordinary diversity that develops in the target of neutralizing antibodies, the envelope glycoprotein (Env). Although vaccines have thus far failed to induce broadly neutralizing antibody responses, there are examples of chronically infected patients with sera that neutralize highly diverse HIV-1 isolates^{1–8}. These individuals provide evidence that it is possible for the human antibody response to neutralize highly diverse strains of HIV-1, although the mechanisms by which such responses are induced or mediated remain incompletely understood^{9,10}.

Recently, isolation and characterization of human monoclonal antibodies from cells of chronically infected patients have provided considerable advances in understanding the specificities and mechanisms underlying broadly neutralizing antibody responses to HIV-1. Env exists on the virion and infected-cell surface as a trimer of heterodimers made up of gp120 and gp41 subunits. For some time, only a small number of broadly neutralizing monoclonal antibodies had been isolated, consisting of one antibody that binds the CD4-binding site on gp120 (b12), one that binds a glycan configuration on the outer domain of gp120 (2G12), and three that bind the membrane-proximal external region (MPER) on gp41 (2F5, Z13e1 and 4E10)^{11–13}. More recently, considerably more broad and potent antibodies have been discovered that target the CD4-binding site of the envelope protein^{14–17} (for which VRC01 is a prototype) and glycan-containing regions of the variable 1 (V1)/V2 and V3 regions of gp120^{4,18–20} (for which PG9 and PGT128 are prototypes). The specificities of these new antibodies are providing important information regarding antigen targets on Env to which the humoral immune response might be directed to mediate broad and potent neutralization. However, evidence for these specificities in many chronically infected patients within our HIV-1-infected cohort¹ is lacking, suggesting that broad and potent neutralization may be mediated by other specificities.

Here we report isolation of a broad and potent gp41 MPER-specific human monoclonal antibody, 10E8, from an HIV-1-infected individual with high neutralization titres. 10E8 is among the most broad and potent antibodies thus far described, and lacks many of the characteristics previously thought to limit the usefulness of MPER-specific antibodies in vaccines or passive therapies, including lipid binding and auto-reactivity. In addition, the crystal structure of 10E8, along with biochemical binding studies, demonstrate that the breadth of 10E8 is mediated by its unique mode of recognition of a structurally conserved site of vulnerability within the gp41 MPER.

10E8 isolation and neutralizing properties

To understand the specificities and binding characteristics that underlie a broadly neutralizing antibody response we developed techniques that permitted isolation of human monoclonal antibodies without previous knowledge of specificity²⁰. Serum from one donor, N152, exhibited neutralizing breadth and potency in the top 1% of our cohort against a 20 cross-clade pseudovirus panel (Supplementary Table 1)¹. Peripheral blood CD19⁺IgM[−]IgD[−]IgA[−] memory B cells from this patient were sorted and expanded for 13 days with interleukin (IL)-2, IL-21 and CD40-ligand expressing feeder cells. The supernatants of ~16,500 B-cell cultures were screened and IgG genes from wells with neutralization activity were cloned and re-expressed²¹ and two novel antibodies (10E8 and 7H6) were isolated.

Nucleotide sequence analysis of DNA encoding 10E8 and 7H6 revealed that both were IgG3 antibodies and were somatic variants of the same IgG clone. These antibodies were derived from *IGHV3-15*05* and *IGLV3-19*01* germline genes, and were highly somatically mutated in variable genes of both heavy chain (21%) and λ light chain (14%) compared to germ line. These antibodies also possessed a long heavy-chain complementarity-determining 3 region (CDR H3) loop composed of 22 amino acids (Fig. 1a). The heavy chains of 10E8 and

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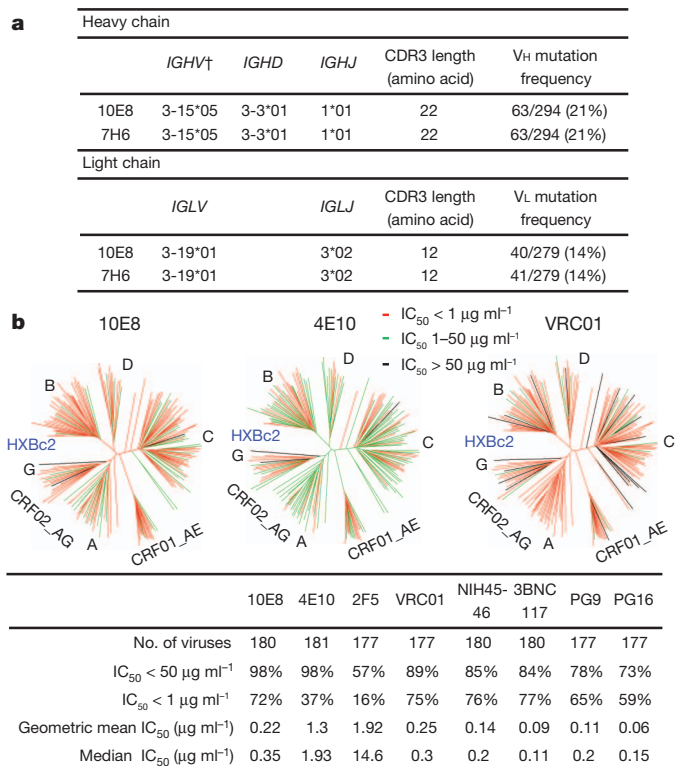


Figure 1 | Analyses of 10E8 sequence and neutralization. **a**, Inferred germline genes encoding the variable regions of 10E8 and 7H6. Dagger footnote symbol indicates that germline alleles were determined using the IMGT database (<http://imgt.org>). **b**, Neutralizing activity of antibodies against a 181-isolate Env-pseudovirus panel. Dendrograms indicate the gp160 protein distance of HIV-1 primary isolate Env glycoproteins. Letters indicate clade. Data below the dendrogram show the number of tested viruses, the percentage of viruses neutralized and the geometric mean IC₅₀ for viruses neutralized with an IC₅₀ < 50 µg ml⁻¹. Median titres are based on all tested viruses, including those with IC₅₀ > 50 µg ml⁻¹, which were assigned a value of 100.

7H6 were identical and there were only two residue differences in the light chain (Supplementary Fig. 1)²².

To assess neutralization activity of the clonal variants, they were initially tested against 5 Env pseudoviruses (Supplementary Table 1a), and monoclonal antibody 10E8 was selected for further study. To determine whether the neutralization activity of 10E8 was representative of the overall neutralization specificity present in patient N152 donor serum, the neutralization panel was expanded to 20 Env pseudoviruses, and 10E8 was tested in parallel with N152 donor serum. Although there were some similarities in the pattern of neutralization of highly resistant variants, a correlation of the neutralization half-maximum inhibitory concentration (IC₅₀) of monoclonal antibody 10E8 and inhibitory dilution (ID₅₀) of N152 serum did not achieve statistical significance ($P = 0.11$; Supplementary Fig. 2 and Supplementary Table 1b). This finding indicates that although 10E8 may have a major role, the full breadth of neutralization of HIV-1 by N152 serum is probably mediated by an amalgam of 10E8-like or other antibodies.

To compare the neutralization potency and breadth of 10E8 with other broadly neutralizing anti-HIV-1 antibodies, 10E8 was then tested in a 181-isolate Env-pseudovirus panel in parallel with 4E10, 2F5, VRC01, NIH45-46, 3BNC117, PG9 and PG16 (Fig. 1b and Supplementary Table 1c). At an IC₅₀ below 50 µg ml⁻¹, 10E8 neutralized 98% of the tested pseudoviruses compared to 98% for 4E10 and 89% for VRC01. However, at an IC₅₀ below 1 µg ml⁻¹, 10E8 neutralized 72% of the tested viruses compared to 37% for 4E10. The median and geometric mean IC₅₀ values for 10E8 were below 1 µg ml⁻¹. Thus, 10E8 mediates broad and potent neutralization against a large range

of viruses and the potency is comparable to some of the best available monoclonal antibodies.

10E8 epitope specificity and binding

To map the epitope of the 10E8 antibody, we tested binding to different subregions of Env by enzyme-linked immunosorbent assay (ELISA). 10E8 bound strongly to gp140, gp41 and the 4E10-specific MPER peptide, but not to gp120 (Fig. 2a). To map further the 10E8 epitope within the MPER, we examined binding of 10E8 to overlapping peptides corresponding to the 2F5 (656–671), Z13e1 (666–677) and 4E10 (671–683) specificities. 10E8 bound to the full MPER and the 4E10-specific peptides, but not 2F5- or Z13e1-specific peptides. Within the 4E10 epitope, when a peptide with a truncated carboxy terminus was tested, 4E10.19 (671–680), 10E8 binding was weakened considerably, indicating that the three terminal amino acids of the MPER—Tyr681, Ile682 and Arg683—were crucial for 10E8 binding (Supplementary Fig. 3a). Consistent with these results, only the full MPER and 4E10-specific peptides blocked 10E8-mediated neutralization of the chimeric C1 virus, which contains the HIV-2 Env with the HIV-1 MPER (Supplementary Fig. 3b). Taken together, these data indicate that the minimal 10E8 epitope is located within residues 671–683 of the MPER, although additional contacts towards the amino terminus of the MPER could not be excluded.

To map more precisely the epitope of 10E8, a panel of alanine mutant peptides scanning MPER residues 671–683 was used to block 10E8 neutralization of the chimeric C1 virus (Fig. 2b)²³. MPER peptides with alanine substitutions at Trp 672, Phe 673 or Thr 676 failed to block 4E10 or 10E8 neutralization, indicating that these residues are critical for both 4E10 and 10E8 binding. Residues Asn 671 and Arg 683, both of which are not required for 4E10 binding, were found to be critical for 10E8 binding and neutralization (Supplementary Table 2 and Fig. 2b). We also tested the ability of 10E8 to neutralize HIV-1 JR2 pseudoviruses with alanine substitutions in MPER residues 660–683 (Supplementary Table 3). Consistent with the effects of alanine substitutions on peptide binding, residues Asn 671 and Arg 683 were critical for 10E8, but not 4E10, neutralization. Individual alanine substitutions at residues 671–673, 680 and 683 resulted in reduced neutralization sensitivity to 10E8 most apparent at the IC₉₀ level rather

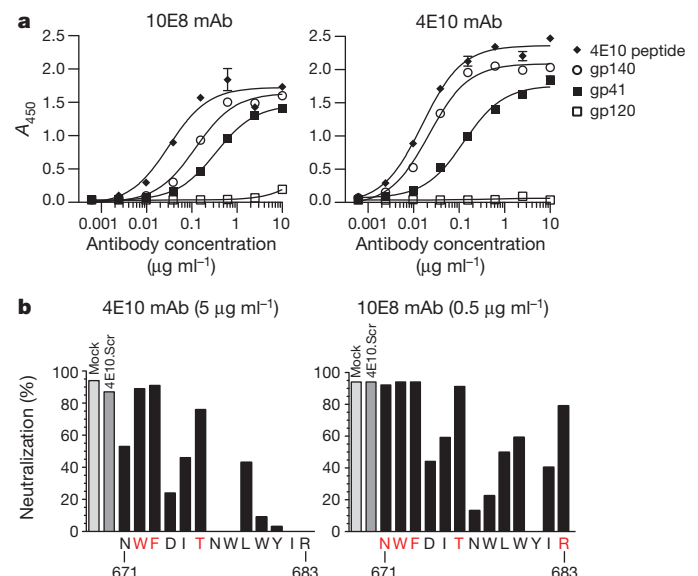


Figure 2 | Binding specificity of 10E8. **a**, ELISA binding of monoclonal antibody (mAb) 10E8 or 4E10 to gp140, gp120, gp41 or 4E10 peptide. Error bars denote one standard error of the mean (s.e.m.). **b**, Inhibition of monoclonal antibody 10E8 or 4E10 neutralization of C1 HIV-2/HIV-1 MPER virus by 4E10 alanine scanning peptides. Residues shown in red indicate positions for which the alanine mutant peptide did not block neutralization.

than the IC₅₀ level. Although the mechanism for this phenomenon is unclear, a similar effect has been observed previously when MPER mutations cause partial resistance to 4E10²⁴. Taken together, these results indicated that 10E8 recognized a novel epitope that overlaps the known 4E10 and Z13e1 epitopes but differs in a critical dependence on binding to Asn 671 and Arg 683, the last residue of the MPER.

We next investigated whether the greater neutralization potency of 10E8 compared to other MPER antibodies was a result of higher binding affinity to the MPER. Capture of a biotinylated peptide encompassing the full MPER (656–683) to a surface-plasmon resonance (SPR) chip allowed the binding kinetics of antigen-binding fragments (Fabs) 10E8, 2F5 and 4E10 to be examined. In contrast to its higher neutralization potency, the dissociation constant (K_d) of 10E8 to this MPER peptide was weaker than that of 2F5 and 4E10: 17 nM for 10E8 versus 3.8 nM for 2F5 and 0.74 nM for 4E10 (Supplementary Fig. 4). Therefore, the affinity of 10E8 for the MPER in a soluble peptide format did not explain its greater neutralization potency compared to other MPER-specific antibodies.

Prevalence of 10E8-like antibodies

We next investigated the prevalence of MPER-specific and 10E8-like neutralizing antibodies in our cohort of HIV-1-infected donors. We selected 78 sera from our cohort with a neutralization ID₅₀ > 100 against at least one pseudovirus in a five-virus mini-panel¹. The median time since diagnosis of these donors was 13.5 years, median CD4 count was 557 cells μl^{-1} , median plasma HIV RNA 5,573 copies ml^{-1} , and they were not receiving antiretrovirals. We tested neutralization against the HIV-2/HIV-1 chimaera C1 (Supplementary Table 4)²⁵. Of 78 sera, 21 exhibited neutralization activity against the HIV-2/HIV-1 C1 virus (Supplementary Table 5). To map the region that was targeted by these sera, neutralization was measured using seven HIV-2/HIV-1 chimaeras containing subdomains of the MPER (Supplementary Table 4)²⁵. Of the 21 sera with neutralization activity against the entire MPER, 8 exhibited a neutralization pattern similar to that observed for 10E8, which entailed neutralization of only those HIV-2/HIV-1 chimaeric viruses that contained the terminal residue of the MPER Arg 683 (C4, C4GW and C8; Supplementary Table 5). For further confirmation of these results, we used peptides corresponding to different portions of the MPER to block sera neutralization of the HIV-2/HIV-1 chimaera C1 (Supplementary Table 6). Of the eight sera found to have a 10E8-like pattern based on neutralization of the chimaeras, three were blocked by peptides consistent with 10E8-like activity. An additional three of the eight 10E8-like sera were blocked by peptides in a pattern consistent with a combination of 10E8- and Z13e1-like antibodies. The six patients whose sera had 10E8-like activity did not differ from the remaining 72 patients with regard to clinical course or HIV neutralization (Supplementary Fig. 5, legend). Overall, 27% of the tested patient sera exhibited anti-MPER neutralizing activity. This prevalence is considerably higher than observed in previous work, possibly related to selection of donors with known neutralizing activity^{8,26–28}. Furthermore, 8% of the tested sera had 10E8-like antibodies (Supplementary Fig. 5), indicating that 10E8-like antibodies are not rare.

Analysis of 10E8 autoreactivity

A property common to the previously characterized MPER monoclonal antibodies 2F5 and 4E10 is that they crossreact with self-antigens²⁹. In addition, binding to both the cell membrane and the Env trimer is thought to be important for optimal neutralization by these antibodies and this autoreactivity may be an obstacle to the elicitation of similar antibodies by a vaccine^{29,30}. Surface plasmon resonance analysis showed that 10E8 did not bind to anionic phospholipids, such as phosphatidyl choline-cardiolipin (PC-CLP) and phosphatidyl choline-phosphatidyl serine (PC-PS) liposomes (Fig. 3a). 10E8 also did not bind HEp-2 epithelial cells, in contrast to 2F5 and 4E10 that bound in a cytoplasmic and nuclear pattern (Fig. 3b). Additionally, 10E8 did not bind auto-antigens, such as Sjogren's syndrome antigens A and B, Smith antigen,

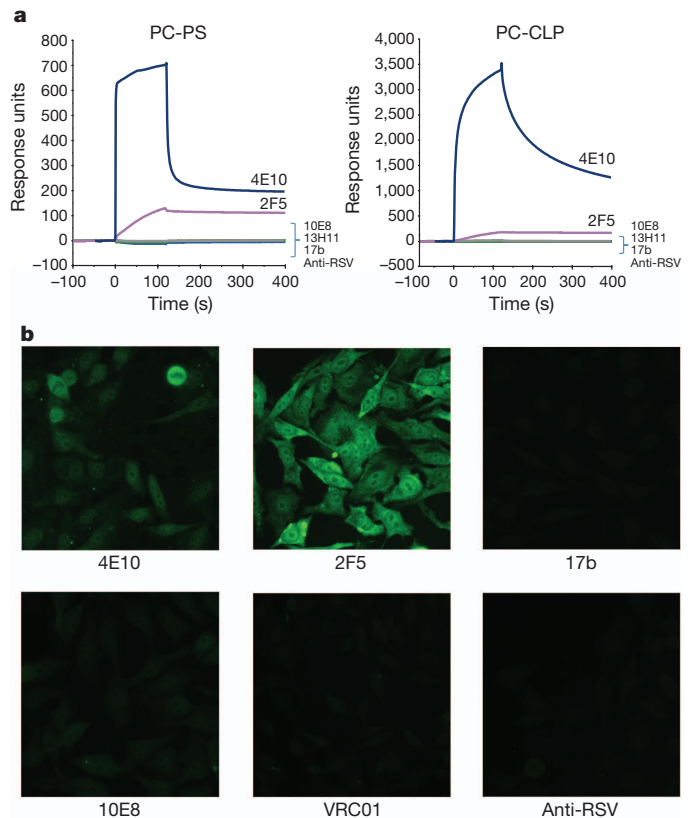


Figure 3 | Analysis of 10E8 autoreactivity. **a**, SPR analysis of 10E8 binding to anionic phospholipids. 10E8 was injected over PC-CLP liposomes or PC-PS liposome immobilized on the Biacore L1 sensor chip. 4E10 and 2F5 were used as positive controls and 13H11, 17b and anti-RSV F protein as negative controls. **b**, Reactivity of 10E8 with HEp-2 epithelial cells. Controls are as above with VRC01 added as an additional negative control. Antibody concentration was 25 $\mu\text{g ml}^{-1}$. All pictures are shown at $\times 400$ magnification.

ribonucleoprotein, scleroderma 70 antigen, Jo1 antigen, centromere B and histone (Supplementary Table 7). Taken together, these results suggest that 10E8, in contrast to other MPER antibodies, is not autoreactive.

Virion accessibility of 10E8

The 2F5 and 4E10 antibodies have been shown to bind relatively poorly to the HIV-1 envelope spike on the surface of infected cells or to cell-free virions, and react more efficiently after Env engagement of the CD4 receptor³¹. We measured binding to cleaved, full-length envelope spikes on HIV_{JRFL} transfected cells (Supplementary Fig. 6a). Although 10E8 bound less efficiently than other antibodies such as VRC01 or F105, where accessibility is not an issue, it bound more efficiently than either 2F5 or 4E10. In contrast to results of alanine substitution, a mutation in the 4E10 (F673S) region in full-length HIV_{JRFL} envelope spikes enhanced 10E8 binding although the mechanism remains unclear. A mutation in the 2F5 (K665E) region had no influence on 10E8 binding. These data indicate that 10E8 has modestly greater access to the MPER epitope on the cell surface than either 2F5 or 4E10.

To assess binding to cell-free virus, we incubated virions with antibody, washed out unbound antibody, and tested neutralization^{31–33}. During washing, antibodies that cannot access their Env target on free virions will be largely removed and therefore neutralization will be diminished. As a control, neutralization of the HXBc2 isolate was not diminished by washing, because the MPER region is accessible on this laboratory-adapted isolate³¹. Washing also had little impact on neutralization of JRFL by VRC01. Consistent with previous work, 2F5 and 4E10 neutralization of most virus isolates tested was substantially diminished after washing (Supplementary Fig. 6b)^{31,34}. In contrast to 2F5 and

4E10, washing had a smaller effect on 10E8 neutralization of most viruses tested, as measured by the area under the curve or analysis of the fold-change in neutralization at a fixed inhibitory concentration (Supplementary Fig. 6c). Although 10E8 is not fully able to access its epitope on the native viral spike similarly to VRC01, under most experimental conditions tested it was better able to access its epitope than either 2F5 or 4E10.

Structure of the 10E8–gp41 complex

To provide an atomic-level understanding of the interaction of 10E8 with HIV-1, we crystallized the Fab of 10E8 in complex with a peptide encompassing the entire 28-residue gp41 MPER (residues 656–683). Monoclinic crystals diffracted to 2.1 Å resolution, and structure solution and refinement to $R_{\text{cryst}} = 18.01\%$ ($R_{\text{free}} = 21.76\%$) revealed two complexes in the asymmetric unit (heretofore referred to as complexes 1 and 2) (Supplementary Table 8). Overall, 10E8 bound to one face of the MPER peptide, which formed two helices, each 15–20 Å in length and oriented 100° relative to each other (Fig. 4a). Electron density was observed for the entire MPER, ranging from Asn 656 to Arg 683 (Leu 660 to Arg 683 for complex 2), with the highest degree of ordered density observed from residue Trp 666 within the N-terminal helix through to Arg 683 of the C-terminal helix (Supplementary Fig. 7). Analysis of main-chain dihedral angles (Supplementary Table 9) indicated that the N-terminal α -helix extends from residue Asn 657 to Ala 667, tightens into a 3_{10} -helix

between residues Ser 668 and Leu 669, before turning at residues Trp 670 and Asn 671. The C-terminal α -helix, capped by Asn 671, starts at residue Trp 672 and extends to residue Arg 683, the final residue of the MPER (Fig. 4a, b).

The 10E8 antibody contacts the gp41 MPER primarily through its heavy chain, although crucial contacts are also mediated by the light chain CDR L3 (Fig. 4a–c and Supplementary Tables 10–12). Three predominant loci of interaction are observed between the antibody and gp41 (Supplementary Tables 13 and 14): one between residues of the tip of the CDR H3 loop and the tip of the C-terminal helix of the peptide, a second between residues of the CDR H2 loop and residues of the hinge region of the peptide, and a third at the juncture of the three heavy-chain CDR loops and the light chain CDR L3, which form a hydrophobic cleft that holds residues of the beginning of the MPER C-terminal helix (Fig. 4b).

10E8–gp41 interface

To complement the results observed for the mutagenesis of the highly conserved 10E8 epitope (Fig. 4d, f, h and Supplementary Tables 2 and 3), each residue of the 10E8 paratope, as determined from the crystal structure, was individually mutated to alanine and the resulting 25 10E8 variants assessed for affinity to a soluble MPER peptide. Overall, the most pronounced effects of the alanine mutations on the binding affinity of 10E8 to a soluble MPER peptide occurred within residues of the CDR H3 loop, although mutations

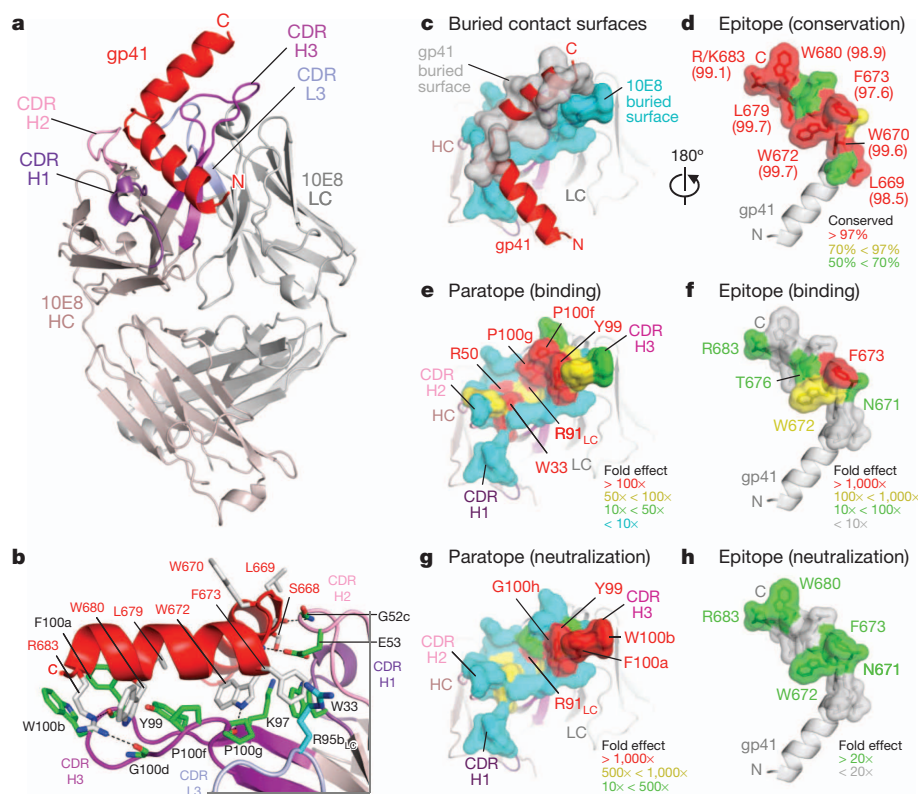


Figure 4 | Crystal structure of 10E8 Fab in complex with its gp41 MPER epitope. **a**, 10E8 recognizes a highly conserved gp41 helix to neutralize HIV-1. Fab 10E8 is shown in ribbon representation (shades of violet for heavy chain (HC) and of grey for light chain (LC)) in complex with a gp41 peptide (red) that encompasses the MPER (Asn 656–Arg 683). **b**, Interface between 10E8 and gp41 with select 10E8 side chains (green, heavy chain; cyan, light chain) and gp41 side chains (grey) in stick representation. In analogy to a hand, the hinge can be viewed as being gripped by a thumb (represented by the CDR H2), the C-terminal helix as being suspended along a corresponding extended forefinger (represented by the CDR H3), and residues that commence the C-terminal helix as being caught in the cleft between the thumb and forefinger (represented

by the juncture of the CDR loops). (See ref. 22 for numbering.) **c**, **d**, Buried contact surfaces and epitope conservation. An examination of the buried contact surface on gp41 (grey; **c**) reveals that highly conserved epitope residues (labelled, **d**) are buried by 10E8 (Supplementary Tables 11 and 12; conservation percentages provided in parentheses). **e**–**h**, Alanine mutagenesis of paratope and epitope. Residues at the tip of the 10E8 CDR H3 loop and within the hydrophobic cleft are crucial for recognition of gp41 and for virus neutralization (Supplementary Tables 15 and 16), as mapped onto the buried 10E8 contact surface (**c**, **e**, **g**). These results mirror the effects of alanine scan mutations of the 10E8 epitope (Supplementary Tables 2 and 3), as mapped onto the buried gp41 contact surface (**f**, **h**).

found in the 4E10 epitope, the additional 10E8-bound residue Lys/Arg683 is critical to neutralization. In addition to other differences in binding based on structural analyses noted above, it is possible that the additional potency of 10E8 compared to 4E10 against naturally occurring viral variants may be mediated through binding of highly conserved residues Trp 680 and Lys/Arg 683 that directly interact with the 10E8 CDRH3.

Discussion

10E8 is a broad and potent neutralizing antibody with important implications for efforts to induce such antibodies with vaccines. Previous MPER antibodies were limited in potency, and had a more limited ability to access MPER on Env of primary isolates. In addition, lipid binding and autoreactivity were thought to be characteristics of MPER antibodies and important obstacles to their elicitation by vaccines^{9,29,30}. However, 10E8 lacks each of these characteristics. In addition, antibodies with a similar specificity were not rare in our chronically infected cohort. This suggests that 10E8-like antibodies were not deleted from the repertoire because of autoreactivity. These results further suggest that 10E8-like antibodies might be raised in a larger fraction of HIV-uninfected persons receiving a vaccine designed to elicit these antibodies without the B-cell defects of chronic HIV infection. Design of such a vaccine will probably require not only presentation of an intact 10E8 epitope but also use of a platform sufficiently immunogenic to drive the evolution of 10E8-like antibodies.

The extraordinary breadth and potency of 10E8 seems to be mediated by its ability to bind highly conserved residues within MPER. Although the epitope of 10E8 overlaps those of known monoclonal antibodies such as 4E10, it differs in recognition surface, angle of approach, lipid binding and self-reactivity. Alanine scanning, structural analysis and paratope analysis each indicate that 10E8 makes crucial contacts with highly conserved residues Trp 672, Phe 673, Trp 676 and Lys/Arg 683. The extraordinary breadth of some potent monoclonal antibodies, for example that bind the CD4 binding site, is thought to be conferred by blocking a functionally important site that is critical for viral entry. Whether 10E8 impairs Env function or simply acts by binding highly conserved residues remains to be determined. Nonetheless, the breadth and potency of 10E8 demonstrates a conserved site of gp41 vulnerability (Fig. 5b) that is an important target antigen for HIV neutralization and that will probably reinvigorate interest in MPER-based HIV vaccine design.

METHODS SUMMARY

Peripheral blood CD19⁺IgM⁺IgD⁺IgA⁺ B cells were sorted by flow cytometry, plated at 4 cells per well, and expanded with cytokines and feeder cells. B-cell culture supernatants were screened by microneutralization against HIV_{MN.03} and HIV_{Bal.26} pseudoviruses. IgG genes from wells with neutralization activity were cloned and re-expressed in 293T cells. Breadth of neutralizing activity was confirmed against a 181-isolate Env pseudovirus panel. Specificity was determined by alanine scanning peptides and mutant Env pseudoviruses. Lipid binding and autoreactivity of 10E8 were measured by SPR plasmon resonance, indirect immunofluorescence on HEP-2 cells and bead arrays. Binding of HIV envelopes on transfected 293 cells was detected by flow cytometry. After pre-incubation with antibody, the impact of washing virions before infecting TZM-bl cells was used to measure access to viral MPER. The frequency of HIV-1⁺ sera with a given specificity was measured by the ability to neutralize HIV-2/HIV-1 chimaeras containing portions of the MPER. 10E8 was co-crystallized with a peptide encompassing the entire 28-residue gp41 MPER (residues 656–683). Structure determination revealed two complexes in the crystal asymmetric unit. Analysis of differences between the two complexes enabled essential interactions to be discerned. The paratope, as defined by residues in the antibody that showed reduced solvent accessibility when in complex with gp41, was subjected to comprehensive alanine scan, with each of the 25 10E8 alanine mutants assessed by SPR for recognition of gp41 and for neutralization of a panel of 5 pseudotyped viruses. The sequence of the patient plasma viral RNA was derived using limiting dilution RT-PCR.

Full Methods and any associated references are available in the online version of the paper.

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Author Contributions M.C., J.H., L.L., G.O., J.R.M. and P.D.K. designed the study, analysed the data, and prepared this manuscript. J.H. and L.L. performed B-cell sorting, antibody cloning, epitope mapping assay, MPER-specific neutralizing sera screening and assessed the impact of sequence variation on 10E8 neutralization. M.K.L. and J.R.M. tested the breadth and potency of 10E8. B.C., S.K.S. and R.W. performed the infected cell surface staining and antibody-virion washout assays. S.M.A. and B.F.H. performed the autoreactivity assays. G.O., Y.Y. and P.D.K. performed 10E8 structural analysis, with T.W. and B.Z. assisting with paratope alanine scanning. R.T.B. screened the B-cell culture supernatants for neutralization activity. H.I. sequenced the patient N152 virus. S.A.M. led the clinical care of the patients. M.C., L.L., N.A.D.-R. and N.S.L. optimized B-cell culture protocol.

Author Information The nucleotide sequence of 10E8 heavy and light chains have been submitted to GenBank under accession numbers JX645769 and JX645770. Coordinates and structure factors for 10E8 Fab in complex with the gp41 MPER have been deposited with the Protein Data Bank under accession code 4G6F. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.C. (mconnors@nih.gov).

METHODS

Study patients. We selected the plasma and peripheral blood mononuclear cells (PBMCs) from the HIV-1-infected patients enrolled in the National Institute of Health under a clinical protocol approved by the Investigational Review Board in the National Institute of Allergy and Infectious Diseases (NIAID-IRB). All participants signed informed consent approved by the NIAID-IRB. The criteria for enrolment were as follows: having a detectable viral load, a stable CD4 T-cell count above $400 \text{ cells } \mu\text{l}^{-1}$, being diagnosed with HIV infection for at least 4 years, and off antiretroviral treatment for at least 5 years. On the basis of the locations of current and former residences, all patients were presumed to be infected with clade B virus. Donor N152 was selected for B-cell sorting and antibody generation because his serum neutralizing activity is among the most potent and broad in our cohort. He is a slow progressor based on criteria described previously⁴¹. At the time of leukapheresis, he had been infected with HIV-1 for 20 years, with CD4 T-cell counts of $325 \text{ cells } \mu\text{l}^{-1}$, plasma HIV-1 RNA values of $3,811 \text{ copies ml}^{-1}$ and was not on antiretroviral treatment.

Viruses and plasmids. HIV-1 JR2 MPER alanine mutant pseudovirus plasmids were obtained from M. Zwick (The Scripps Research Institute). HIV-2/HIV-1 chimaeras were provided by G. Shaw and L. Morris.

Memory B-cell staining, sorting and antibody cloning. Staining and single-cell sorting of memory B cells were performed as follows. PBMCs from HIV-1-infected donor N152 were stained with antibody cocktail consisting of anti-CD19-PE-Cy7 (BD Bioscience), IgA-APC (Jackson ImmunoResearch Laboratories Inc.), IgD-FITC (BD Pharmingen) and IgM-PE (Jackson ImmunoResearch Laboratories Inc.) at 4°C in dark for 30 min. The cells were then washed with 10 ml PBS-BSA buffer and re-suspended in $500 \mu\text{l}$ PBS-BSA. A total of 66,000 $\text{CD19}^+\text{IgA}^-\text{IgD}^-\text{IgM}^+$ memory B cells were sorted using a FACSaria III cell sorter (Becton Dickinson) and re-suspended in IMDM medium with 10% FBS containing 100 U ml^{-1} IL-2, 50 ng ml^{-1} IL-21 and $1 \times 10^5 \text{ ml}^{-1}$ irradiated 3T3-MSCD40L feeder cells⁴². B cells were seeded into 384-well microtitre plates at a density of 4 cells per well in a final volume of $50 \mu\text{l}$. After 13 days of incubation, $40 \mu\text{l}$ of culture supernatants from each well were collected and screened for neutralization activity using a high-throughput micro-neutralization assay against HIV-1_{MN.3} and HIV-1_{Bal.26}. B cells in each well were lysed with $20 \mu\text{l}$ lysis buffer containing $0.25 \mu\text{l}$ of RNase inhibitor (New England Biolabs Inc.), $0.3 \mu\text{l}$ of 1 M Tris pH 8 (Quality Biological Inc.) and $19.45 \mu\text{l}$ DEPC-treated H_2O . The plates with B cells were stored at -80°C .

The variable region of the heavy chain and the light chain of the immunoglobulin gene were amplified by RT-PCR from the wells that scored positive in both the HIV-1_{MN.3} and HIV-1_{Bal.26} neutralization assay. The cDNA product was used as template in the PCR reaction. To amplify the highly somatically mutated immunoglobulin gene, two sets of primers as described previously²¹ were used in two independent PCRs. One set of primers consisted of the forward primers and the reverse primers specific for the leader region and constant region of IgH, Igk or Igl, respectively. The other set of primers consisted of the forward primer mixes specific for FWRI and respective reverse primers specific for the IgH, Igk and Igl J genes. All PCRs were performed in 96-well PCR plates in a total volume of $50 \mu\text{l}$ containing 20 nM each primer or primer mix, 10 nM each dNTP (Invitrogen), $10 \mu\text{l}$ $5\times$ Q-solution (Qiagen) and 1.2 U HotStar Taq DNA polymerase (Qiagen). From the positive PCR reactions, pools of the V_H or V_L-region DNA were ligated to a pCR2.1-Topo-TA vector (Invitrogen) for sequencing before cloning into the corresponding Ig γ 1, Igk and Igl expression vector. $10 \mu\text{g}$ of heavy and light chain plasmids, cloned from the same well and combined in all possible heavy and light chain pairs, were mixed with $40 \mu\text{l}$ FuGENE 6 (Roche) in $1,500 \mu\text{l}$ DMEM (Gibco) and co-transfected into 293T cells. The full-length IgG was purified using a recombinant protein-A column (GE Healthcare).

Neutralization assays. Neutralization of the monoclonal antibodies was measured using single-round HIV-1 Env-pseudovirus infection of TZM-bl cells⁴³. HIV-1 Env pseudoviruses were generated by co-transfection of 293T cells with pSG3 delta Env backbone and a second plasmid that expressed HIV-1 Env. At 72 h after transfection, supernatants containing pseudovirus were harvested and frozen at -80°C until further use. In the neutralization assay, $10 \mu\text{l}$ of fivefold serially diluted patient serum or monoclonal antibody was incubated with $40 \mu\text{l}$ of pseudovirus in a 96-well plate at 37°C for 30 min before addition of TZM-bl cells. After 2 days of incubation, cells were lysed and the viral infectivity was quantified by measuring luciferase activity with a Victor Light luminometer (Perkin Elmer). The 50% inhibitory concentration (IC_{50}) was calculated as the antibody concentration that reduced infection by 50%. Antibody epitopes were mapped using HIV-1 JR2 MPER alanine mutant pseudoviruses in a TZM-bl assay.

HIV-2/HIV-1 chimaera neutralization. HIV-2/HIV-1 C1 chimaera (HIV-2 virus 7312A with HIV-1 gp41 MPER)²⁵ was used in the competition assay. A fixed concentration of MPER peptide was incubated with serially diluted 2F5,

4E10, Z13e1 or 10E8 antibody at 37°C for 30 min before incubation with HIV-2/HIV-1 C1 chimaera. Wild-type HIV-2 virus 7312A was used as a control. Antibody epitope mapping was completed by adding $10 \mu\text{l}$ 10E8 monoclonal antibody to $5 \mu\text{l}$ serial dilutions of 4E10 peptide or its alanine mutants at 37°C for 30 min before the addition of HIV-2/HIV-1 C1 chimaera. The degree to which peptides blocked antibody-mediated neutralization was calculated as the fold change in the IC_{50} value of the antibody in the presence of 4E10 alanine mutants compared to the wild-type peptide. The precise binding region within the MPER targeted by patient serum or antibodies was determined using the HIV-2/HIV-1 chimaeras containing different portions of HIV-1 MPER, such as C1 (HIV-2 Env with HIV-1 MPER), C1C (HIV-2 Env with clade C MPER), C3 (HIV-2 Env with 2F5 epitope), C4 (HIV-2 Env with 4E10 epitope), C6 (HIV-2 Env with short 4E10 epitope NWFDIT), C7 (HIV-2 Env with short 2F5 epitope ALDKWA) and C8 (HIV-2 Env with both Z13 and 4E10 epitope). Fivefold diluted patient serum or monoclonal antibody was incubated with chimaera in a 96-well plate at 37°C for 30 min before addition of TZM-bl cells. The specificities within patient sera were confirmed by blocking neutralization of the C1 chimaera with $25 \mu\text{g ml}^{-1}$ of 2F5, 4E10, MPER, Bal.V3, control peptide, or $50 \mu\text{g ml}^{-1}$ of Z13 peptide.

ELISA assays. Each antigen at $2 \mu\text{g ml}^{-1}$ was coated on 96-well plates overnight at 4°C . Plates were blocked with BLOTTO buffer (PBS, 1% FBS, 5% non-fat milk) for 1 h at room temperature, followed by incubation with antibody serially diluted in disruption buffer (PBS, 5% FBS, 2% BSA, 1% Tween-20) for 1 h at room temperature. $1:10,000$ dilution of horseradish peroxidase (HRP)-conjugated goat anti-human IgG antibody was added for 1 h at room temperature. Plates were washed between each step with 0.2% Tween 20 in PBS. Plates were developed using 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) and read at 450 nm.

Autoreactivity assays. Binding of 10E8 to phospholipid was measured by SPR conducted on a BIAcore 3000 instrument and data analyses were performed using the BIAevaluation 4.1 software (BIAcore) as described previously³⁰. Phospholipid-containing liposomes were captured on a BIAcore L1 sensor chip, which uses an alkyl linker for anchoring lipids. Before capturing lipids, the surface of the L1 chip was cleaned with a 60-s injection of 40 mM octyl- β -D-glucopyranoside, at $100 \mu\text{l min}^{-1}$, and the chip and fluidics were washed with excess buffer to remove any traces of detergent. Monoclonal antibodies were then injected at $100 \mu\text{g ml}^{-1}$ at a flow rate of $30 \mu\text{l min}^{-1}$. After each antibody injection, the surface was again cleaned with octyl- β -D-glucopyranoside, and 5-s injections of each 5 mM HCl, then 5 mM NaOH, to clean any adherent protein from the chip.

Reactivity to HIV-1 negative human epithelial (HEp-2) cells was determined by indirect immunofluorescence on slides using Evans Blue as a counterstain and FITC-conjugated goat anti-human IgG (Zeus Scientific)²⁹. Slides were photographed on a Nikon Optiphot fluorescence microscope. Regarding Fig. 3b, koda-chrome slides were taken of each monoclonal antibody binding to HEp-2 cells at a 10-s exposure, and the slides scanned into digital format. The Luminex AteNA Multi-Lyte ANA test (Wampole Laboratories) was used to test for monoclonal antibody reactivity to SSA/Ro, SS-B/La, Sm, ribonucleoprotein (RNP), Jo-1, double-stranded DNA, centromere B, and histone and was performed as per the manufacturer's specifications and as previously described²⁹. Monoclonal antibody concentrations assayed were 50, 25, 12.5 and $6.25 \mu\text{g ml}^{-1}$. $10 \mu\text{l}$ of each concentration were incubated with the luminex fluorescent beads and the test performed per the manufacturer's specifications.

Fluorescence-activated cell sorting (FACS) staining of cell-surface HIV-1 Env. FACS staining was performed as previously described^{31,44}. Forty-eight hours after transfection, cells were collected and washed in FACS buffer (PBS, 5% HIFBS, 0.02% azide) and stained with monoclonal antibodies. The transfected cells were suspended in FACS buffer and were incubated with the antibodies for 1 h at room temperature. The monoclonal antibody-cell mixture was washed extensively in FACS buffer and phycoerythrin (PE)-conjugated goat anti-human secondary antibody (Sigma) was added for 1 h at a 1:200 dilution, followed by extensive washing to remove unbound secondary antibody. The antibody-PE-stained cells were acquired on a BD LSRII instrument and analysed by FlowJo.

Antibody-virus washout experiments. From a starting concentration of 2 mg ml^{-1} , $12.5 \mu\text{l}$ of fivefold serially diluted antibodies in PBS were added to $487.5 \mu\text{l}$ of DMEM containing 10% heat-inactivated FBS and $15 \mu\text{l}$ of pseudovirus such that the final concentrations of antibodies were $50 \mu\text{g ml}^{-1}$ to $0.08 \mu\text{g ml}^{-1}$ in a total volume of $500 \mu\text{l}$. In the 'no inhibitor' control, the same volume of PBS was added instead of antibody. The reaction mixture was incubated for 30 min at 37°C . The $250 \mu\text{l}$ reaction mixture was diluted to 10 ml with complete DMEM, centrifuged at 25,000 r.p.m. in a SW41 rotor, for 2 h at 4°C . The virus pellet was then washed two additional times with 10 ml of PBS. During the washing steps, the virus-antibody complex was centrifuged at 40,000 r.p.m. for 20 min at 4°C . After the final wash, $250 \mu\text{l}$ of DMEM was added to the washed virus pellet and it was re-suspended by gentle shaking at 4°C for 30 min. A total of $100 \mu\text{l}$ of the suspended virus was used to infect $100 \mu\text{l}$ of TZM-bl cells ($0.2 \text{ million per ml}$), in

duplicate. From the remaining 250 µl of reaction mixture, an equal volume of the antibody virus mixture was used as a 'no washout' control. Plates were incubated at 37 °C in a CO₂ incubator for 2 days. After 2 days, the luciferase assay was done as described previously⁴⁵. The data were then plotted to determine the neutralization mediated by the antibodies in 'wash' or 'no wash' conditions.

Structure determination and analysis. The antigen binding fragment of 10E8 (Fab) was prepared using LysC digestion, as previously described⁴⁶. The IgG was first reduced with 100 mM dithiothreitol (DTT) for 1 h at 37 °C, followed by 1 h of dialysis in HEPES, pH 7.6, to reduce the DTT concentration to 1 mM. Antibodies were then dialysed against 2 mM iodoacetamide for 48 h at 4 °C, and subjected to a final dialysis against HEPES, pH 7.6, for 2 h. After reduction and alkylation, antibodies were cleaved with Lys-C (Roche). Fab was purified by ion exchange (Mono S) and size-exclusion chromatography (S200). Purified 10E8 Fab was incubated with tenfold excess peptide RRR-NEQELLELDKWASLWNWFDITNWLWYIR-RRR (American Peptide) and the complex then set up for 20 °C vapour diffusion sitting-drop crystallizations on a Honeybee 963 robot. A total of 576 initial conditions adapted from the commercially available Hampton (Hampton Research), Precipitant Synergy (Emerald Biosystems) and Wizard (Emerald Biosystems) crystallization screens were set up and imaged using the Rockimager (Formulatrix), followed by hand optimization of crystal hits. Crystals grown in 40% PEG 400, 0.1 M NaCitrate, 0.1 M Tris pH 7.5 diffracted to 2.1 Å resolution in a cryoprotectant composed of mother liquor supplemented with 15% 2R-3R-butanediol and excess peptide. After mounting the crystals on a loop, they were flash cooled and data collected at 1.00 Å wavelength at SER CAT ID-22 or BM-22 beamlines (APS) and processed using HKL-2000⁴⁷. Structures were solved through molecular replacement with Phaser^{48,49}, using a previously obtained free structure of 10E8 as a search model. Refinement of the structure was undertaken with Phenix⁵⁰, with iterative model building using Coot⁵¹. The structure was validated with MolProbity⁵², yielding 97% and 99.8% of residues falling within most favoured and allowed Ramachandran regions, respectively. The structure was analysed with APBS⁵³ for electrostatics, Ligplot⁵⁴ for direct contacts, PISA⁵⁵ for buried surface areas, and lsqkab (ccp4 Package⁵⁶) for r.m.s.d. alignments. Helical wheels were generated using the program Pepwheel (<http://150.185.138.86/cgi-bin/emboss/pepwheel>). All graphics were prepared with Pymol (PyMOL Molecular Graphics System).

Assessment of binding affinities of 10E8 and 10E8 variants to the gp41 MPER. Surface-Plasmon Resonance (SPR) (Biacore T200, GE Healthcare) was used to assess binding affinity of wild-type 10E8 to a gp41 MPER peptide. A biotinylated peptide composed of residues 656–683 of the gp41 MPER (RRR-NEQELLELDKWASLWNWFDITNWLWYIR-RRK-biotin; American Peptide) was coupled to a biacore SA chip to a surface density of 20–50 response units (RU). The 10E8 fragment of antigen binding (Fab) was then flowed over as analyte at concentrations ranging from 0.25 nM to 125 nM, at twofold serial dilutions, with association and dissociation phases of up to 5 min, at a flow rate of 30 µl min⁻¹. The binding of the 2F5 and 4E10 Fab controls to the same peptide was examined under identical conditions.

Binding affinities of the 10E8 paratope alanine mutants to the MPER were also assessed with SPR, but using an antibody capture method. A Biacore CM5 chip was amine-coupled with anti-human Fc antibody to high surface densities of ~10,000 RU. The 10E8 paratope variant IgGs were then captured to between 1,500–2,500 RU and a peptide composed of residues 656–683 of the gp41 MPER (RRR-NEQELLELDKWASLWNWFDITNWLWYIR-RRR) flowed over as analyte at twofold serial dilutions starting at 500 nM (with the exception of HCD30A, W100bA, S100cA, P100fA, which started at 250 nM). Association and dissociation phases spanned 3 min and 5 min, respectively, at a flow rate of 30 µl min⁻¹. Binding sensograms were fit with 1:1 Langmuir models using Biacore BioEvaluation Software (GE Healthcare). In all cases, Biacore HBSEP+ buffer was used (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.1% P-20).

PCR amplification and sequencing. Extraction of viral RNA from plasma and cDNA synthesis were performed as previously described⁵⁷. Single molecules of a 588-bp fragment, encompassing the MPER region of the HIV-1 envelope gene, obtained through limiting dilution, were PCR-amplified with the Expand High Fidelity PCR System (Roche Applied Science) using the following primer sets: +7789 (sense) 5'-TCTTAGGAGCAGCAGGAAGCACTATGGG-3' and -8524 (antisense) 5'-GTAAGTCTCTCAAGCGGTGGTAGC-3' in a first round reaction; +7850 (sense) 5'-ACAATTATTGTCTGGTATAGTGCAACAGCA-3' and -8413 (antisense) 5'-CCACCTTCTCTTCGATTCCTTCGG-3' in a second round reaction. Each round of PCR consisted of 25 cycles, with the initial denaturation at 94 °C for 2 min, followed 25 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min, with the final extension at 72 °C for 7 min. The PCR products were purified with the QIA quick PCR purification kit (Qiagen), and then cloned into pCR2.1-TOPO vector (TOPO TA Cloning kit, Invitrogen) for sequence analysis of individual molecular clones. The DNAs from 18 independent clones were sequenced with the ABI BigDye Terminator v3.1 Ready Reaction Cycle Sequencing kit (Applied Biosystems) and analysed with the ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

Statistical analysis. The relationship between the potency of N152 patient serum and 10E8, and the relationship between 10E8 variant binding and neutralization were evaluated by the Spearman rank method.

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The Mu transpososome structure sheds light on DDE recombinase evolution

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Studies of bacteriophage Mu transposition paved the way for understanding retroviral integration and V(D)J recombination as well as many other DNA transposition reactions. Here we report the structure of the Mu transpososome—Mu transposase (MuA) in complex with bacteriophage DNA ends and target DNA—determined from data that extend anisotropically to 5.2 Å, 5.2 Å and 3.7 Å resolution, in conjunction with previously determined structures of individual domains. The highly intertwined structure illustrates why chemical activity depends on formation of the synaptic complex, and reveals that individual domains have different roles when bound to different sites. The structure also provides explanations for the increased stability of the final product complex and for its preferential recognition by the ATP-dependent unfoldase ClpX. Although MuA and many other recombinases share a structurally conserved ‘DDE’ catalytic domain, comparisons among the limited set of available complex structures indicate that some conserved features, such as catalysis *in trans* and target DNA bending, arose through convergent evolution because they are important for function.

Mobile DNA elements are important in many aspects of biology, such as disease, evolution and the spread of antibiotic resistance, and the recombinases they encode, including MuA, are useful genetic tools^{1,2}. The DNA transposition system of bacteriophage Mu was the first to be developed *in vitro*³. MuA, many other DNA transposases, and retroviral integrases share a conserved RNaseH-like or DDE catalytic domain, named for the three Mg²⁺-binding carboxylates in their active sites⁴. Structural studies have lagged behind biochemical ones: only three family members have been co-crystallized in active, DNA-bound complexes, and only one with target DNA^{5–7}. Despite mechanistic similarities, only the catalytic domain is conserved among all of these, and their overall architectures are completely different⁴. More examples are needed to understand the diverse ways in which these enzymes harness a common catalytic domain to accomplish transposition. The richness of the known biochemistry for bacteriophage Mu, from assembly of the initial complex to targeted disassembly of the product complex, makes it a particularly informative example for structural studies.

The first steps of Mu transposition (Fig. 1) are common to many other DNA transposition systems as well as retroviral integration: (1) pairing of the mobile element's ends by the recombinase to form a ‘transpososome’ or ‘intasome’; (2) hydrolytic nicking at the bacteriophage–host junction; and (3) attack of the newly freed 3' hydroxyls on a target DNA (strand transfer), creating a new connectivity. Bacteriophage Mu uses this mechanism to form a lysogen, and to replicate when it becomes lytic. During the lytic phase, host enzymes are recruited to convert the branched product into replication forks, resulting in duplication of the entire bacteriophage genome. However, during initial lysogen formation, the ‘flanking host’ DNA (grey in Fig. 1) consists only of extra sequences appended during packaging of the bacteriophage Mu DNA into bacteriophage capsids. In this case, a poorly understood signal causes the transposase to cleave both strands at each genome end, leading to a simple insertion without replication^{8,9}.

MuA is chemically active only when incorporated into transpososomes that pair the two ends of the phage genome, and thus assembly of this complex is a regulatory step. Mu transpososomes become

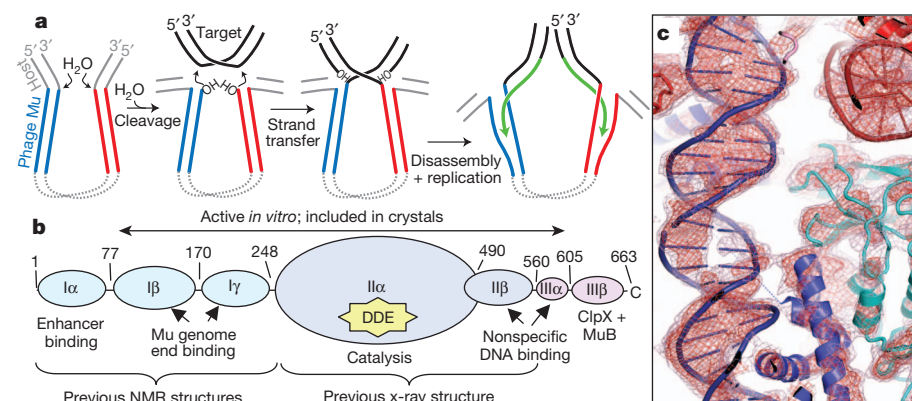


Figure 1 | Transposition pathway and structure determination. **a**, Cartoon of transposition. The transposase (MuA) pairs the bacteriophage genome ends (blue and red). At each end, the same active site catalyses the attack of H₂O at the phage–host junction and then the direct attack of the phage 3'-OH on target DNA ('strand transfer'). Target binding is nonspecific, and there is a 5-bp stagger between the sites of attack. Host and target DNAs may be entire circular replicons. After the ATP-dependent unfoldase ClpX disassembles the final strand transfer complex, the 3' hydroxyls are used as replication primers, resulting in duplication of the bacteriophage genome. Our crystals contain the strand transfer product (third panel). **b**, Domain structure of MuA. **c**, Experimental electron density map after phase improvement with Parrot superimposed on the model (contours are 1.2 and 2σ).

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increasingly stable as the reaction progresses^{10,11}. After strand transfer the complex is so stable that the enzyme MuA does not actually turn over. The strand transfer reaction can only be reversed if the complex is disrupted, for instance by heating to 75 °C (refs 12, 13). This may be a thermodynamic necessity for a reaction in which the first step (hydrolysis) is committed, yet the second step (strand transfer) is chemically isoenergetic, with no net change in the number of phosphodiester bonds. *In vivo*, Mu transpososomes must be disassembled by the ATP-dependent unfoldase ClpX before DNA replication can proceed^{14–17}. From the transpososome structure it is possible to derive explanations for the increased stability of the final complex and its preferential recognition by ClpX.

Crystallization of the strand transfer complex was facilitated by two observations. First, despite a lack of target sequence specificity, MuA attacks mismatch-containing target DNA with single-nucleotide precision (Supplementary Fig. 1)¹. Second, the natural transpososome assembly pathway (described below) can be simplified under permissive conditions *in vitro*. The resulting active complexes contain four copies of the MuA protein and two copies of a ~50-base-pair (bp) DNA derived from the bacteriophage genome's right end, each carrying two MuA binding sites (termed R1 and R2)^{18,19}. Modelling based on the structure of these complexes suggests that transpososomes formed on full left and right ends are quite similar.

Overall architecture

Viewed in isolation, the five domains of each subunit resemble beads on a string (Supplementary Fig. 2). However, when all four subunits are assembled on the DNA, they intertwine to form a network of protein–protein and protein–DNA interactions (Fig. 2 and Supplementary Video 2). The overall transpososome resembles a pair of scissors, with the bacteriophage end DNAs forming the handles and the sharply bent target DNA the blades. A 34 Å resolution electron microscopy (EM) reconstruction of Mu transpososomes in the absence of target DNA found a similar V-shape, although the arms were shorter and the accompanying electron spectroscopic imaging predicted a more contorted path for the DNA²⁰. Within the transpososome, most of the individual protein domains perform different roles in the R1- versus R2-bound subunits. Where a system encoded by a larger genome might, during the course of evolution, use two separate polypeptides, bacteriophage Mu re-uses the same sequence to perform different functions within the complex.

Catalysis and Mu DNA end-binding *in trans*

The catalytic sites lie within domain II α of MuA. In agreement with biochemical studies, only the R1-bound subunits' active sites engage with DNA, and they do so *in trans*: for example, the dark-blue subunit

binds the blue Mu DNA via domains I β and γ , whereas its active site domain docks at the red Mu DNA–target junction (the interdomain linkers are too short for any other connectivity) (Fig. 3). First characterized for Mu transposition^{21,22}, such *trans* catalysis is a recurring theme in DNA transposition, and helps to render chemical activity dependent on full complex assembly⁴. Domains II α and II β of the R2 subunits bridge the two Mu end DNAs and have a primarily structural role. II α of each R2 subunit interacts with the DNA-binding domain (DBD) I β of the subunit bound to the R1 site of the same DNA segment, whereas II β of each R2 subunit binds to the opposite DNA segment. Biochemical studies had predicted domain II β to interact with the target DNA, which does occur in the R1 subunits²³.

Domains I β and I γ of MuA recognize the specific binding sites on the bacteriophage DNA ends and their positions agree with footprinting and mutagenesis data^{24,25}. The closest structural match to MuA's tandem DNA binding domains is the centromere binding protein CENP-B, which probably evolved from an ancestral transposase²⁶. The eukaryotic mariner family Mos1 and Tc3 transposase structures also include tandem DBDs^{7,27}. In both, contacts between the DBDs equivalent to domain I β of MuA mediate synapsis of the two transposon ends. In the Mu transpososome, only the R1-bound DBDs mediate protein–protein contacts: I β as described above, and I γ to both II α of the R1 subunit at the other end of the Mu bacteriophage and III α of the R2 subunit at the same end of the Mu bacteriophage. The importance of these interactions is underscored by the high sequence conservation within the interaction surfaces (Supplementary Fig. 3).

Although the resolution precludes detailed analysis of DNA bending, the path of the backbone is clear. The R2 site is bent by ~28°, largely through compression of the major groove around domain I β , which agrees with DNase I hypersensitive sites and the CENP-B–DNA structure^{10,25,28}. The R1 site is less bent (~17°). Stronger bending there would cause a steric clash between domain I γ of the R1-bound subunit and the β -barrel (II β) of the adjacent R2 subunit. We propose that the R1 site straightens somewhat upon transpososome formation. The formation of favourable protein–DNA and protein–protein contacts within the transpososome could offset the cost of weakening contacts between DNA and domain I β .

Larger DNA conformational changes may occur on transpososome formation, as indicated by solution experiments that found ~90° bends in monomeric MuA–DNA complexes²⁹. In monomeric MuA, domain II α could interact *in cis* with its own I β domain and additional DNA bending might be induced by electrostatic interactions with II β and III α . A different but stable monomer conformation would raise the energy barrier to spontaneous tetramer formation, making it more amenable to regulation. It could also prevent premature encounters between the active site and the DNA.

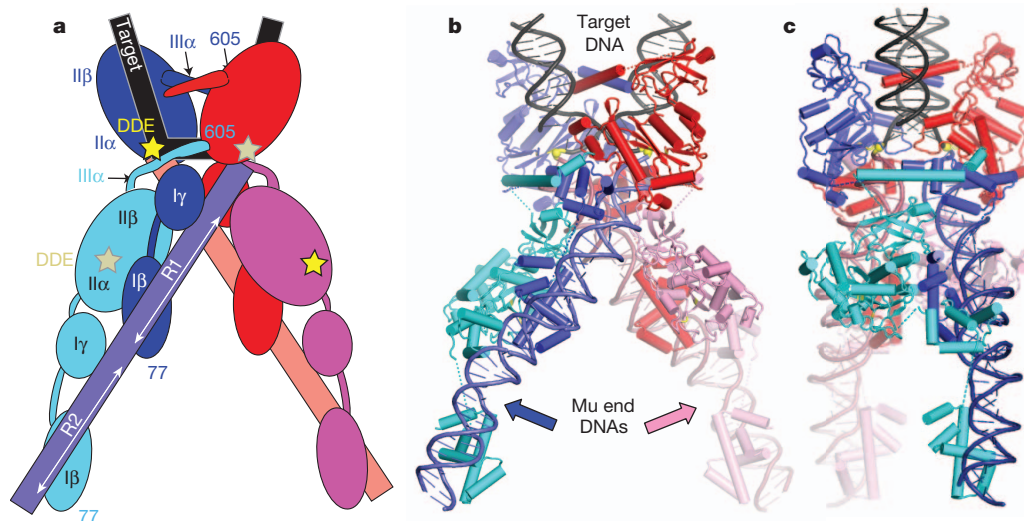


Figure 2 | Transpososome structure. The complex sits on a crystallographic two-fold symmetry axis (vertical) that relates the blue and red halves. The pale- and dark-coloured subunits adopt different conformations within the homotetramer. DNA colours match Fig. 1. **a**, Cartoon. Catalytic sites are marked as yellow and tan stars (facing the viewer or the background, respectively) and domains of the blue subunits are labelled. **b**, Ribbon drawing, with the scissile phosphate groups shown as yellow spheres. **c**, Same drawing as in **b**, rotated ~90° about a vertical axis.

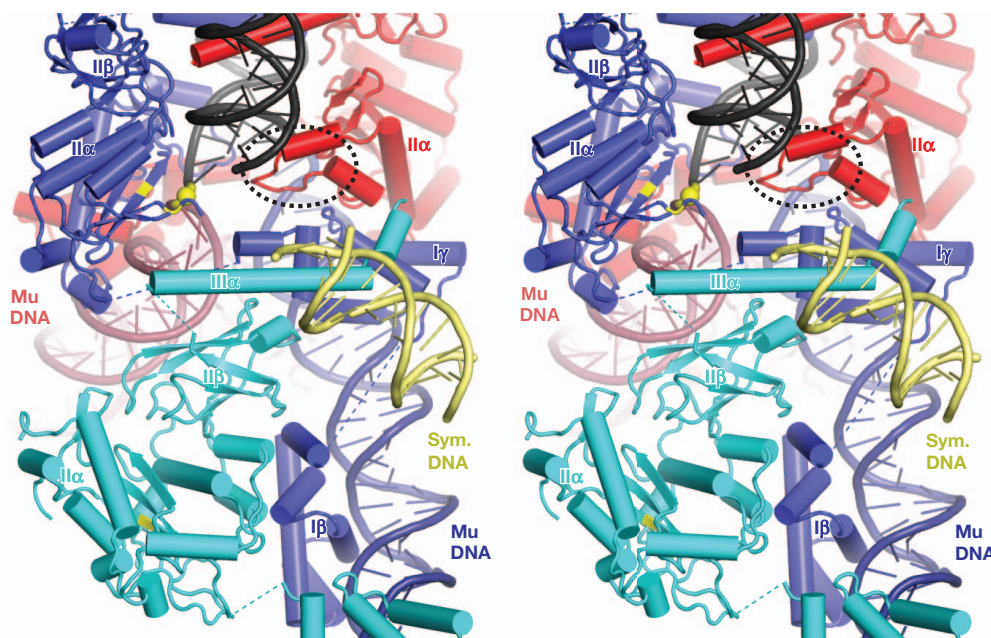


Figure 3 | Stereo close-up view of interactions near the Mu DNA–target junction. Colours are the same as in Fig. 2. A segment of DNA from a symmetry-related complex (yellow) binds the positively charged domain III α of the R2-bound subunit (cyan). If the red Mu end DNA were extended to include flanking host DNA, it could lie where the yellow DNA does. The yellow sphere marks the phosphate group at the Mu–target DNA junction, and the main chains of the two active site D residues are also yellow (a third active site residue lies on a helix that could not be modelled). The loop that extends from domain II α (~amino acids 410–430) to interact with the black target DNA is circled on the red subunit.

Target DNA

The target DNA is bent through a total of $\sim 140^\circ$. Protein–DNA contacts are mediated by the R1 subunits' domains II α , II β and III α and extend to all but the outermost base pairs of the target DNA, as predicted by footprinting³⁰. A long loop (residues 410–430) extends from each catalytic domain (II α) underneath the target. This loop could be fit onto experimental electron density by a rigid-body rotation from its position in the 2.8 Å domain II α β structure (Protein Data Bank accession 1bcm)³¹. The positively charged β-barrel, domain II β , interacts loosely with the outer end of the target. Although poorly ordered, its position is defined by the SeMet signal from two adjacent Met residues. The relative orientation between domains II α and II β of the R1 subunit is shifted slightly from that in the unbound protein and in the R2 subunit. This alters the connecting loop, which also lies near the target, but could not be modelled. Finally, the domain III α s of the R1 subunits pair to form a coiled coil on the concave side of the bent target DNA.

Two roles for domain III α

Domain III α , the final ~ 45 residues in our structure, also has different roles in the R1 compared with the R2 subunits. It is highly positively charged and binds DNA as an isolated peptide³². The domain III α s of the R1 subunits appear to stabilize the bent target DNA in two ways: (1) alleviating electrostatic repulsion between the sides of the U-shaped target; and (2) physically trapping the target DNA within the complex. In the absence of target DNA, they must be either mobile or in a different location. As the carboxy terminus of MuA contains a ClpX binding tag, rearrangement of the R1 subunits' domain III α s upon target binding could allow ClpX to preferentially recognize the final strand transfer complex for ATP-dependent disassembly. Furthermore, it is these end-most subunits that ClpX preferentially unfolds^{33,34}.

Initial transpososome assembly requires domain III α on the R2-bound, but not the R1-bound, subunits^{22,35,36}. The structure suggests that the R2 subunits' domain III α s stabilize the complex by wrapping around the other subunits near the active site. The domain III α s may also anchor the flanking host DNA (Figs 1 and 3). The construct crystallized included minimal flanking host DNA, but if extended, it could bind the R2 subunit's III α domain, occupying a spot where symmetry-related DNA interacts in the crystal. This model agrees with footprinting data that predicted a large distortion, and would prevent steric clashes between the flanking host and target DNAs^{10,30}. Domain III α was reported to have cryptic nuclease activity that might cleave the flanking DNA flap after the initial insertion reaction^{8,32}. Alternatively,

movement of domain III α (triggered by an unknown signal) might deliver the uncleaved strand to the DDE site for hydrolysis.

Transpososomes with full left and right ends

Although the complex that we crystallized is highly active *in vitro*, its assembly requires high protein and DNA concentrations or 'permissive' solvent conditions¹⁸. The natural system is more complicated and provides an interesting example of templated complex assembly. The two ends of bacteriophage Mu genome carry different arrays of three MuA binding sites each, and the left end also binds the DNA-bending protein HU (Fig. 4). Conversion of an initial pairing of right and left ends to an active complex is stimulated by transient binding of the amino-terminal domains of several MuA subunits to an internal enhancer element^{37,38}. However, if complexes assembled this way are

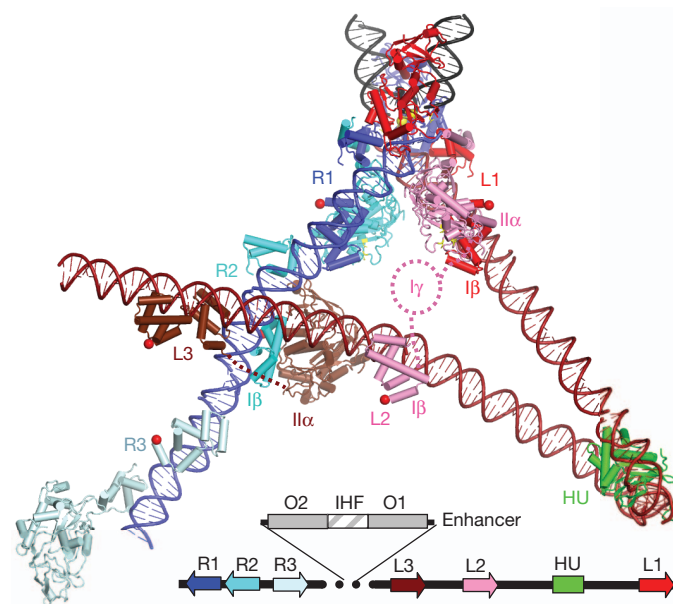


Figure 4 | Model for a transpososome assembled on full left (reddish) and right (blue) bacteriophage ends. The N terminus of each domain I β is marked with a red sphere to show the approximate position of domain I α , which transiently binds the enhancer. Domains discussed in the text are labelled. Inset: cartoon of the bacteriophage Mu genome ends and internal enhancer element.

treated with a high salt wash, an active complex remains that contains only four subunits of MuA, contacting only the R1, R2 and L1 DNA sites^{10,19}. Thus, the other binding sites are important for assembly but not for the final activity.

Modelling showed that the functional part of the tetrameric assembly, where Mu ends join to target DNA, can be identical in the crystallized (R1R2)₂ complex and in the full left plus right complex (Fig. 4). Subunits R1, R2, L1 and domains II and III of the L2 subunit were modelled directly from the crystal structure. The HU-induced bend allows a single protomer of MuA to bind the L2 site via domain I β while its domains II and III form part of the core complex. Domain I γ acts as a linker, which explains why L2 is the only site where I γ doesn't bind DNA²⁵.

Additional interactions involving the L3- and R3-bound subunits may temporarily hold the components together while the intertwining of protein and DNA needed to form a transpososome at the Mu–host junction occurs. We modelled interactions between the L3 subunit's domain II α and R2's I β based on those seen in the crystal between the R2- and R1-bound subunits. The R3 subunit's role is unclear, but if the HU-induced bend were relaxed, similar cross-end interactions might occur between the L3 and R3 subunits.

Topological studies predicted two right-handed superhelical crossings within the transpososome^{39,40}. One such crossing occurs in the crystal structure, near the junction with target DNA (R1 over L1 in Fig. 4). A second crossing (L3 over R2) results from the severe bend induced by HU in the model, in conjunction with a smaller bend in the L2 site. Restraint of two supercoils within such short segments of DNA is consistent with observations that supercoiling stimulates transpososome assembly⁴¹.

During assembly on intact bacteriophage DNA, domain I α transiently binds an internal enhancer, which contains two clusters of MuA binding sites termed O1 and O2. There are too many degrees of freedom to add the enhancer to our model. However, our model does agree with data showing that O1-bound proteins interact with both L3 and R1 and that proteins bound to the somewhat longer O2 may contact both R3 and L1^{42,43}. The multiple protein–protein interaction surfaces of MuA may help to stabilize an initial pairing of the bacteriophage ends, but interactions between the wrong partners could slow down the transition to a final, cleavage-ready complex. The enhancer may stimulate this transition by preventing unproductive interactions among L- and R-bound subunits as well as by aligning them for productive ones.

Such an elaborate assembly process is not limited to Mu: many other mobile elements also require seemingly 'extra' recombinase subunits⁴⁴. Although the details vary among these systems, they may all be using the same fundamental strategy of using additional subunits to temporarily stabilize pairing of the element end DNAs while a complicated, intertwined structure forms at the 3' ends. The additional complexity may also provide additional opportunities for regulation. Finally, for mobile elements that are present in high copy, it may help to ensure that the two ends paired in a single transpososome belong to the same copy of the mobile element.

Convergent and divergent evolution

Many DNA transposases and retroviral integrases share a structurally conserved DDE or RNaseH-like catalytic domain, indicative of divergence from a common ancestor. However, this is the only domain conserved among these diverse recombinases. Comparison of the four reported structures of DDE recombinases in complex with substrate DNAs shows that other recurring features may reflect convergent evolution for functional reasons (Fig. 5). All four complexes are held together by intertwined networks of protein–protein and protein–DNA contacts, although different domains mediate those contacts⁴. Mos1 and MuA do have structurally related bipartite DNA-binding domains, but even those domains form different protein–protein contacts in their respective transpososomes⁷.

Despite the diversity of these complexes, catalysis is always in *trans*: the subunit that catalyses DNA cleavage and joining on one mobile element

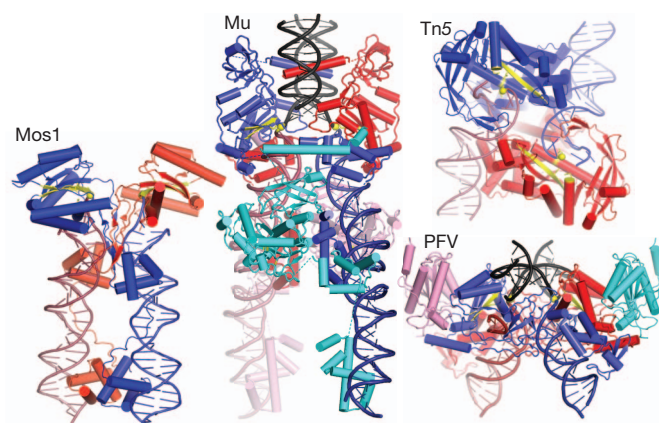


Figure 5 | Comparison of DDE recombinase–DNA complexes. The mobile element ends are red and blue, and target DNA (where included) is black. Subunits that carry out the chemical reactions are red and blue; additional subunits are pink and cyan. Active site residues, scissile phosphate groups, and the two β -strands of the conserved catalytic domain that carry the catalytic D residues are in yellow. Mos1 is a Tc1/mariner family eukaryotic DNA transposon; Tn5 is a bacterial DNA transposon; and PFV is a mammalian retrovirus^{5–7}. Mos1 and Tn5 require only a dimer for activity, whereas Mu transposase and PFV integrase require tetramers. In the PFV structure, only the catalytic domains of the additional subunits were visible (pink and cyan).

end binds to specific sequences on the other end. This feature ensures that the chemical reactions at the two element ends are coordinated, because the complex requires proper pairing of the ends for assembly.

Another recurring feature is strong bending of the target DNA. Target DNA bound by the prototype foamy virus (PFV) intasome is also bent, although not quite as severely as that in the Mu transpososome⁶. The Mos1 transpososome was crystallized without target DNA, but additional end DNAs found in the crystal bind where target is expected to, and in a way that requires target bending⁷. Modelling of target DNA onto the Tn5 transpososome structure also requires bending, which agrees with biochemical data for the related Tn10 system^{5,45}. Outside of the catalytic domain, contacts to the target DNA vary widely among these structures. Why then have they all evolved to strongly bend the target DNA? As noted for the PFV structure, target bending may help render strand transfer irreversible by straining the DNA conformation such that the ends snap away from the active site after strand transfer. This may be a source of the product binding energy that drives forward the otherwise-isoenergetic strand transfer reaction. The overall conformation of the target DNA in the Mu transpososome resembles that bound by integration host factor (IHF). In that case, a nick at the kink does enhance affinity by allowing the ends to spring apart⁴⁶.

The DDE catalytic domain is thus a conserved module that has been co-opted by numerous mobile elements to perform similar chemical reactions. However, other similarities in the way that it has been harnessed to mobilize these elements seem to reflect convergent evolution to satisfy functional requirements.

METHODS SUMMARY

We determined the structure of the final strand transfer complex, which contains a tetramer of MuA, two copies of the bacteriophage end DNA, and one target DNA (Supplementary Fig. 1 and Supplementary Video 1). Crystallizations used a slightly truncated protein, MuA(77–605), which is active *in vitro* and lacks only the N-terminal enhancer-binding domain and the C-terminal domain that interacts with ClpX and MuB, a second bacteriophage-encoded protein that helps deliver an appropriate target DNA under non-permissive conditions⁴⁷. The bacteriophage end DNA mimics pre-cleaved right ends, and the 35-bp target DNA contains a central G•G mismatch. Although MuA displays little sequence specificity for target DNA, it attacks mismatch-containing DNA with single-nucleotide precision¹. This feature facilitated production of a homogeneous sample for crystallization.

Phases were determined by MIRAS using three derivatives. The crystals diffracted anisotropically to 5.2 Å, 5.2 Å and 3.7 Å along the three principal axes.

Model building was possible despite the low resolution because >90% of the protein structure had been previously determined as isolated domains^{31,48,49}. Placement of the protein domains was verified by SeMet data, and the DNA sequence register by an additional data set collected from crystals where every T on one strand had been substituted with 5-bromodeoxyuridine (BrdU) (Supplementary Fig. 4). No previous structure was available for domain III α , which comprises ~45 amino acids that are strongly predicted to form one long helix followed by a short one. Although density for these helices was visible, the sequence register is uncertain. The complex lies on a crystallographic two-fold axis such that the asymmetric unit contains half a transpososome. After highly restrained refinement, *R* and *R*_{free} values were 39.3% and 43.7%, well within the range expected for a low-resolution structure.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions S.P.M. carried out most of the crystallographic work, Y.Z.P. grew the first diffracting transpososome crystals and assisted with all other aspects of the project, and P.A.R. designed the project and assisted in computational work and interpretation of the results.

Author Information Coordinates and structure factors were deposited at the Protein Data Bank under accession 4fcy. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to P.A.R. (PRice@uchicago.edu).

METHODS

Overview. We determined the structure of the final strand transfer complex, which contains a tetramer of MuA, two copies of the bacteriophage end DNA, and one target DNA (Fig. 2 and Supplementary Video 1). Crystallizations used a slightly truncated protein, MuA(77–605), which is active *in vitro* and lacks only the N-terminal enhancer-binding domain and the C-terminal domain that interacts with ClpX and MuB, a second bacteriophage-encoded protein that helps deliver an appropriate target DNA under non-permissive conditions⁴⁷. The bacteriophage end DNA mimics pre-cleaved right ends, and the 35-bp target DNA contains a central G•G mismatch. Although MuA displays little sequence specificity for target DNA, it attacks mismatch-containing DNA with single-nucleotide precision¹. This feature facilitated production of a homogenous sample for crystallization.

Phases were determined by MIRAS using three derivatives. The crystals diffracted anisotropically to 5.2 Å, 5.2 Å and 3.7 Å along the three principal axes. Model building was possible despite the low resolution because >90% of the protein structure had been previously determined as isolated domains^{31,48,49}. Placement of the protein domains was verified by SeMet data and the DNA sequence register by an additional data set collected from crystals where every T on one strand had been substituted with BrdU (Supplementary Fig. 4). No previous structure was available for domain III α , which comprises ~45 amino acids that are strongly predicted to form one long helix followed by a short one. Although density for these helices was visible, the sequence register is uncertain. The complex lies on a crystallographic two-fold axis such that the asymmetric unit contains half a transpososome. After highly restrained refinement, *R* and *R*_{free} values were 39.3% and 43.7%, well within the range expected for a low-resolution structure.

Expression and purification of the MuA transposase. The pMK599 plasmid, a pET3c derivative that contains the *MuA* open reading frame coding for residues 77–605, was a gift from the Mizuuchi laboratory⁵⁰. This plasmid was transformed into *Escherichia coli* Rosetta pLysS strain (EMD Biosciences) for protein overexpression. After plating transformants, a starter culture was prepared by inoculating multiple colonies into LB media (with 100 μ g ml⁻¹ ampicillin) and growing at 37 °C until the absorbance at 600 nm (*A*₆₀₀) was ~0.7. Typically, 100 ml of starter culture was prepared per litre of final culture. After the addition of starter culture to fresh ampicillin-containing LB media, cells were grown to *A*₆₀₀ of ~0.8, then protein expression was induced with IPTG (added to a final concentration of 0.5 mM). Cells were collected 2 h after induction by centrifugation at ~8,000g for 10 min, and cell pellets were stored at -80 °C for later use.

Cell pellets were re-suspended in a lysis buffer (25 mM HEPES (pH 7.50), 1 mM EDTA, 1 M NaCl, 10% sucrose, 10% glycerol, 5 mM DTT, 200 μ g ml⁻¹ lysozyme, protease inhibitor cocktail from Roche Diagnostics), sonicated, and centrifuged at 40,000g for 1 h (18,000 r.p.m. in a SS-34 rotor). Ammonium sulphate was added to the supernatant to 30% saturation to precipitate the protein. The pellet was collected by centrifugation, and redissolved in buffer A (20 mM MES (pH 5.5), 0.5 mM EDTA, 5% glycerol, 0.2 M NaCl and 1 mM DTT). The protein sample was filtered before loading onto a heparin affinity column (GE Healthcare). Proteins were eluted with salt gradient from 0.2 M to 2.0 M NaCl. To improve the purity, MuA-containing fractions were rechromatographed on heparin after dialysis into buffer A. The protein was then dialysed into buffer A again and loaded onto a Mono-S column (GE Healthcare). A gradient similar to that from the heparin affinity purification was applied. Fractions containing MuA were pooled and dialysed at 4 °C into 20 mM HEPES (pH 7.5), 0.5 mM EDTA, 0.2 M ammonium sulphate, 20% glycerol, and 1 mM DTT. The protein was concentrated to approximately 10 mg ml⁻¹, and stored at -80 °C. Minimal nuclease contamination was detected when samples (0.5 mg ml⁻¹ final concentration) were incubated for 2 h at 37 °C in 10 mM HEPES pH 7.5 with supercoiled plasmid DNA, 50 mM NaCl and 10 mM MgCl₂.

SeMet-labelled MuA(77–605) was prepared similarly except that cells were grown differently⁵¹. Instead of using LB, the cells were inoculated in M9 media plus 0.4% glucose, 10 mM NaCl, 0.1 mM CaCl₂, 2 mM MgSO₄ and 100 mg ml⁻¹ ampicillin until *A*₆₀₀ reached ~0.5. An amino acid cocktail containing L-isoleucine, L-leucine, L-lysine, L-phenylalanine, L-threonine and L-valine was then added to a final concentration of 100 mg of each amino acid per litre. Seleno-DL-methionine (Sigma) was also added to a final concentration of 60 mg l⁻¹. The culture was grown for 15 more minutes before 0.5 mM IPTG was added. Cells were collected after 3 h of induction.

Preparation of Mu end and target DNA. Mu end DNA duplexes were designed to contain the R1 and R2 binding sites for MuA. Each duplex was prepared by mixing four single strands in equal molar amounts. The oligonucleotides used for the structure determination are listed below: TL, 5'-GCTTGAAGCGGCGCA CGAAAAACGCG-3'; TR, 5'-AAAGCGTTTACGATAAATGCGAAAAC-3'; BL, 5'-AACGCTTTCGCGTTTTTCGTGCGCCGCTTCA-3'; BR, 5'-CGGTT TTCGCATTTATCGTGA-3'. These strands were heated at 80 °C for 20 min, and annealed by slow cooling to room temperature. The final concentration of

the duplex DNA is 0.2 mM in TEN buffer (10 mM Tris-HCl and 0.5 mM EDTA, 100 mM NaCl, pH 8.0). The resulting DNA mimics the product of initial DNA cleavage by MuA, and has a three-nucleotide 5'-overhang on the uncleaved strand, and a two-nucleotide 5'-overhang on the other end. Each strand of the resulting duplex is nicked at a position that does not interfere with transpososome assembly.

The target DNA contains a central mismatch and was designed to be asymmetric to avoid hairpin formation during annealing. The target DNA was prepared in a manner similar to that of the Mu end DNA using the following oligonucleotides: (1) 5'-TATCGCAACAACACATCGGATAACCATAAGTAA TA-3'; (2) 5'-TATTACTTATGGTTATCGGATGTGTTGTTGCGATA-3'.

All unmodified oligonucleotides were obtained from IDT Technologies. Brominated oligonucleotides (discussed below) that were used in this study to validate the sequence of the donor DNA and the location of the target DNA were obtained from Yale University's Keck Facility. Because brominated oligonucleotides are photolabile, they were handled in the dark as much as possible.

Crystallization and data collection. Strand-transfer complexes were assembled by mixing the target DNA, Mu end DNA, and MuA protein in 1:1.4:3.7 molar ratios in a solution containing 10 mM MgCl₂, 25 mM HEPES (pH 7.5), 10 mM DTT, 0.02% Zwittergent, 14% glycerol, and 0.2 M (NH₄)₂SO₄. This was incubated for at least 1 h at room temperature to ensure completeness of the strand transfer reaction. Although DMSO is usually added to stimulate assembly of transpososomes with two right end DNAs, we found that it was not necessary at the high protein and DNA concentrations used for crystallographic work¹⁸. Crystallization trials were then performed using the hanging-drop vapour diffusion method: drops contained a 1:1 mixture of complex stock solution (~2 mg ml⁻¹) and well, and were incubated at 19 °C. Crystals appeared in 22–28% (v/v) PEG400, 0.1 M HEPES (pH 7.50), and 0.2 M MgCl₂, and grew to their full size in 2–4 weeks. Tantalum bromide derivatives were obtained by soaking the crystals of the strand-transfer complex for 1–8 days with 0.4 mM [Ta₆Br₁₂]²⁺ cluster (Jena Bioscience) in a solution that mimicked the condition of the drop. For derivatization with mercury, crystals were soaked in 32% PEG400, 0.1 M HEPES (pH 7.5), 0.2 M MgCl₂ and 0.1 mM mersalyl acid (Sigma) for 1–2 days. For derivatization with selenium, crystallization setups were done with SeMet protein. And for that with bromine, the brominated donor DNA where every thymine on the T-rich strand (oligos BL & BR) was replaced with BrdU was used. All crystals were frozen in liquid N₂ directly from the drop.

Numerous data sets were collected from several different beamlines at the Advanced Photon Source in Argonne. Many crystals were screened at BIO-CARS 14-BM. All data sets used for the final phasing and refinement were collected at SBC-CAT 19-ID beamline at 100K temperature. For the SeMet data, data sets collected from two different crystals were merged to improve completeness of data, especially at the low-resolution shells. X-ray data collected from the native and Ta-derivative crystals were integrated and scaled with HKL3000 suite and the others with HKL2000⁵². A summary of the data collection statistics is shown in Supplementary Table 1.

Structure determination and refinement. The toehold in solving the structure of the Mu transpososome was a single Tantalum bromide cluster (Supplementary Table 1). This cluster was initially found using direct methods in SHELXD⁵³ from a 5-day-soaked Ta-data set, and was consistent with the anomalous difference Patterson maps⁵⁴ generated from other Ta-data sets where crystals were soaked for 1, 3, 5, 7 and 8 days. SIRAS phases from this one cluster were generated using MLPHARE and were used in anomalous difference Fourier methods to determine the substructure for the mercury derivative⁵⁵. We used SIRAS phases calculated from the Hg derivative to confirm independently the Ta site. After several rounds of difference Fourier calculation, we were able to locate the rest of the heavy atoms. Final MIRAS phases were generated from 4 [Ta₆Br₁₂]²⁺, 3 Hg and 17 Se sites. Reasonable figures of merit were obtained before density modification: 0.41 for centric and 0.25 for acentric reflections. With 77% solvent in the crystal, further phase improvement was achieved by density modification using Parrot⁵⁶. Electron density maps generated showed clear density for the DNA as well as tubular densities that represent protein helices. The protein structure was initially modelled by docking previously determined structural domains (I β , I γ and catalytic domains) of the MuA transposase into the density using Se peaks as markers. The bromine sites, despite not being included in calculating phases due to the low resolution of that data set, were particularly useful in guiding the model building for the donor DNA. We also have a low-resolution data set from a crystal that contains a symmetric brominated target DNA: 5'-TATCGCAACAACACA TCGGATGTGTTGTTGCGATA-3'. Bromine peaks obtained from this particular crystal was useful in confirming the location of our target DNA (Supplementary Fig. 4).

The transpososome lies on a crystallographic two-fold axis such that the asymmetric unit comprises half of a transpososome: two MuA protomers, one Mu end DNA, and

half target DNA. The initial model revealed possible loose contacts between two crystallographically related copies of domain II β (a β -barrel). To improve the diffraction of our crystals, we engineered three sets of mutations (Quickchange, Stratagene) in that region of contact: a single M521W mutation and two double mutations, M521W/N525L and M521L/N525L. The latter led to an improvement of the resolution along the best diffracting axis of our 'native data set' from 4.2 to 3.7 Å.

The structure was modelled in COOT⁵⁷, and refined using PHENIX⁵⁸. Density was visible for several sections that unfortunately could not be modelled due to the resolution: for example, the linker between domains I β and I γ lies in the minor groove as seen for Mos1 and CENP-B, and the region around the third active site residue of the R1-bound subunits, which clearly changes conformation from the inactive form seen in isolated domain II structures. Multiple restraints were used during refinement due to the low resolution of the data. These include H-bond restraints on the DNA base pairs, secondary structure restraints, model restraints where models of the individual domains of the MuA transposase were obtained from the PDB, NCS restraints, and Ramachandran restraints. Nine TLS groups were used: (1) R1 β and the DNA with which it is interacting; (2) R2 β and DNA; (3) R1 γ and DNA; (4) R2 γ and DNA plus R2-domain III α ; (5) R2-domain II including the β -barrel; (6) R1-domain II without the β -barrel; (7) R1- β -barrel; (8) R1-domain III α ; and (9) target DNA including the sequences after the CA step in the donor DNA (that do not interact directly with the DNA binding domains). As categorized by PROCHECK, the percentage of residues in the following regions of the Ramachandran plot were: favoured/allowed/generous/disallowed = 93.8/5.4/0.2/0.5. Several variations on this protocol were tried. Simply removing the Ramachandran restraints made very little difference, probably because most of the model was already restrained to previously determined domain structures. The unrestrained Ramachandran plot statistics were: favoured/allowed/generous/disallowed = 90.1/9.0/0.4/0.5, and $R_{\text{work}}/R_{\text{free}}$ were 40.1/43.6% (as opposed to 39.3/43.7% with restraints). Superimposing the two structures (refined $\pm$ Rama restraints) revealed some slight differences in residues 347–356. This is the region where Ramachandran outliers were observed. However, upon inspection of the experimental map and the difference maps, it was difficult to discern which was more correct. Hence, we are choosing to report a structure that has better geometry. We also tried refinement with DEN⁵⁹, but it only improved the R_{free} by 0.3% and greatly degraded the Ramachandran plot. Our structure may be an unusual test case for DEN because of the low resolution of our data and the high quality of our individual domain models.

During the initial rounds of refinement, progress stalled when R_{free} was $\sim$ 49%. However, after ellipsoidal truncation and anisotropic scaling were performed on the native data set using the Diffraction Anisotropy server⁶⁰, the R_{free} considerably improved to $\sim$ 44%. The server truncated the data set to 3.7 Å along c^* and 5.2 Å along a^* and b^* . The final refined structure has an R_{work} and R_{free} of 39.30% and 43.70%, respectively.

Modelling the full transpososome. To model the full complex, additional model B-form DNA coordinates was created using the W3DNA server⁶¹. DNA and protein coordinates were manipulated in both pymol and coot. Subunits R1,

R2, L1 and domains II and III of the L2 subunit in the model could be taken directly from the crystal structure. To model the other subunits, we docked domain I β of the R2 subunit and the DNA segment it binds onto the appropriate site in the modelled DNA. The L1 and L2 binding sites are separated by an $\sim$ 80-bp segment where the DNA bending protein HU binds. Modelling of the HU-induced bend was based on the structure of a closely related IHF–DNA complex and on footprinting data for HU synergistically bound within this loop^{62,63}. Modelled DNA for the L end was broken and appropriate sections abutted to the ends of the DNA in the IHF–DNA structure. We justified some additional bending of the DNA on the L2 end of the HU site based on the symmetry-related DNA in the IHF structure, and the fact that IHF- and HU-induced bends are known to be flexible. Bending of the model DNA in the L2 binding site was based on bending seen crystallographically in the R2 site. In modelling the R3-bound subunit, the other domains simply followed I β as a rigid body, which gives only a rough placement of domain II. For the L3-bound subunit, we modelled an interaction between its domain II and the R2 subunit's domain I β based on the II–I β interactions seen in the crystal.

Figures were prepared using Pymol (The PyMOL Molecular Graphics System, Version 1.3).

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Quantum-dot spin-photon entanglement via frequency downconversion to telecom wavelength

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Long-distance quantum teleportation and quantum repeater technologies require entanglement between a single matter quantum bit (qubit) and a telecommunications (telecom)-wavelength photonic qubit^{1–5}. Electron spins in III–V semiconductor quantum dots are among the matter qubits that allow for the fastest spin manipulation^{6,7} and photon emission^{8,9}, but entanglement between a single quantum-dot spin qubit and a flying (propagating) photonic qubit has yet to be demonstrated. Moreover, many quantum dots emit single photons at visible to near-infrared wavelengths, where silica fibre losses are so high that long-distance quantum communication protocols become difficult to implement¹⁰. Here we demonstrate entanglement between an InAs quantum-dot electron spin qubit and a photonic qubit, by frequency downconversion of a spontaneously emitted photon from a singly charged quantum dot to a wavelength of 1,560 nanometres. The use of sub-10-picosecond pulses at a wavelength of 2.2 micrometres in the frequency downconversion process provides the necessary quantum erasure to eliminate which-path information in the photon energy. Together with previously demonstrated indistinguishable single-photon emission at high repetition rates^{11,12}, the present technique advances the III–V semiconductor quantum-dot spin system as a promising platform for long-distance quantum communication.

A quantum communication network¹ will consist of stationary matter qubits and flying photonic qubits. The two key technologies for quantum communication networks are quantum teleportation from a photonic qubit to a matter qubit or vice versa, and a quantum repeater to create and store the entangled states of remote matter qubits. These two core technologies rely on the ability to create entanglement between a matter qubit and a photonic qubit over long distances, followed by a Bell state measurement that transforms the matter qubit–photonic qubit entanglement into the teleportation of a given unknown quantum state, or into matter–matter entanglement^{13–18}. Matter–photon and matter–matter entanglement generation have been shown in several ionic^{4,16,17} and atomic^{2,5,18} systems, while matter–photon entanglement was recently observed in nitrogen-vacancy diamond colour centres³. However, these systems suffer from low photon collection efficiencies and relatively long optical recombination times, which can only partially be overcome by cavity–quantum electrodynamic (cavity–QED) solutions¹. In addition, none of them use telecom-wavelength (1.5 μm) photons that would allow long-distance entanglement distribution. Combining a solid-state monolithic cavity–QED system with a fast quantum emitter^{19–21} could solve the low photon yields. In the InAs quantum-dot system, subnanosecond optical recombination times have previously led to the demonstration of high single-photon rates when the quantum dots are embedded in high-quality optical cavities^{8,9,20,21}. Moreover, spin control of charged InAs quantum dots was shown to be feasible on picosecond timescales

while preserving spin coherence^{6,7,22}, making it a promising candidate as a quantum network technology. However, spin–photon entanglement has yet to be established in this system, and its emission wavelength does not match the low-loss wavelength range of silica fibres. In this work, we address both these challenges with an ultrafast frequency downconversion technique based on a periodically poled lithium niobate (PPLN) waveguide device²³. Its timing resolution enables demonstration of entanglement between a single InAs quantum-dot spin qubit and a photonic qubit at 910 nm, while the target wavelength (1,560 nm) would permit long-distance quantum communication.

The physical system used in our experiment is presented in Fig. 1a–d. It consists of a single electron-doped InAs quantum dot, embedded in a low- Q microcavity (see also Methods). With an external magnetic field oriented in the Voigt geometry (perpendicular to the growth direction/optical axis), two Λ -systems are formed, where an optically active, excited state is coupled to each of two ground states. The excited states are so-called trion states, as they consist of three particles: two electrons, paired into a singlet state, and an unpaired hole ($|\uparrow\downarrow\uparrow\rangle$ and $|\uparrow\downarrow\downarrow\rangle$). Both trion states are connected to each of the electron spin states, $|\downarrow\rangle$ and $|\uparrow\rangle$, in a Λ -configuration. These Λ -systems have been extensively studied²⁴, and were previously used for initialization²⁵ and coherent manipulation of the electron spin states^{6,7,22,26}. The optical selection rules are indicated in Fig. 1a, and were verified by polarization-selective photoluminescence (Fig. 1b). In addition, initialization, rotation and measurement of the spin state of the quantum dot are realized, using all-optical techniques as reported previously^{6,7} (see Fig. 1c, d and Methods). By selectively exciting one of the trion states, for example, the $|\uparrow\downarrow\uparrow\rangle$ -state, spontaneous emission decay in a Λ -system leads to entanglement between the emitted photon and the electron spin^{3,4}:

$$|\psi\rangle = \frac{1}{\sqrt{2}}(|\uparrow\rangle \otimes |iH; \omega + \delta\omega\rangle + |\downarrow\rangle \otimes |V; \omega\rangle) \quad (1)$$

Here $\delta\omega$ refers to the difference in energy (colour) of the emitted photons, determined by the electron Zeeman energy, and H and V to their polarization (see Fig. 1c); ω refers to the frequency (wavelength) of the V-polarized photon (910.10 nm). Previous coherent spin manipulation experiments^{6,7} relied on a relatively large Zeeman splitting $\delta\omega$ between the respective electron spin states, both for high-fidelity spin initialization and readout, and for ultrafast coherent control of the spin through a combination of stimulated-Raman transitions and Larmor precession. In the present experiment, this energy separation is set at about $2\pi \times 17.6$ GHz (external magnetic field $B = 3$ T, Fig. 1d), leaving room for which-path information to leak out into the environment via the photon energy, which makes verification of entanglement between the polarization of the photon and the electron spin challenging. Upon detection of an emitted photon in

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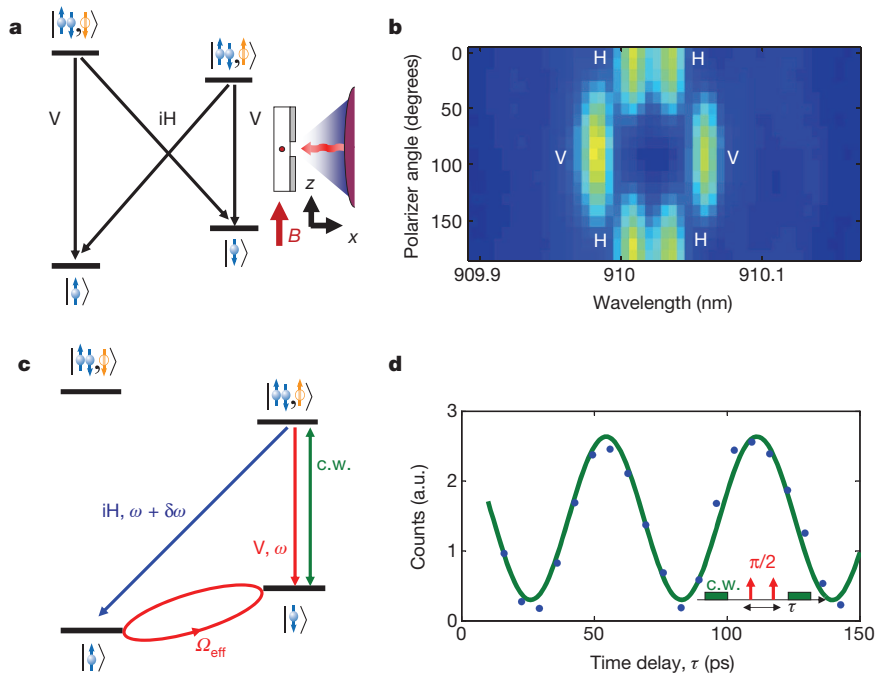


Figure 1 | Level structure of quantum dot and spin manipulation. **a**, Level structure of an electron-doped quantum dot, with the magnetic field in-plane (Voigt geometry, boxed). V and H refer to linear polarizations, either perpendicular (V) or parallel (H) to the magnetic field. Blue and orange arrows refer to the electron and (trion-) hole states, respectively. **b**, Verification (by polarization-selective magnetophotoluminescence) of the polarization selection rules of the studied electron-doped quantum dot. **c**, Level structure and optical spin manipulation scheme used. $\delta\omega$, Larmor precession frequency ($2\pi \times 17.6$ GHz for $B = 3$ T); ω , frequency of a V-polarized photon (wavelength 910.10 nm for $B = 3$ T); c.w., narrowband, continuous wave laser used for initialization and readout; Ω_{eff} , effective spin Rabi frequency resulting from manipulation by detuned ($\Delta = 300$ GHz), circularly polarized optical pulses^{6,7}. **d**, Ramsey interference experiment, demonstrating coherent control of the electron spin qubit. Blue, raw data, for $\pi/2$ - $\pi/2$ interference; green, least squares fit. For a 3 T magnetic field, the Larmor precession frequency $\delta\omega = 2\pi \times 17.6$ GHz. Inset, pulse scheme used.

the rotated polarization basis (say, $|\sigma^+\rangle$ -photon) at time t_1 , the resulting spin state evolves as follows:

$$|\psi_{\text{spin},\sigma^+}(t)\rangle = \frac{1}{\sqrt{2}} \left(e^{i\delta\omega(t-t_1)} |\uparrow\rangle - |\downarrow\rangle \right) \quad (2)$$

Owing to the large Zeeman splitting, a small uncertainty Δt_1 in the photon's arrival time can result in a large uncertainty in the phase of the spin state ($\delta\omega \times \Delta t_1$) and therefore a reduction in the entanglement visibility. Lowering the magnetic field further reduces the fidelity of the single-photon-based spin readout⁶ and is therefore not a viable option in our experiment. Previous experiments in ionic⁴ and NV-diamond systems³ relied on the speed (timing jitter) of commercial single-photon detectors to fix the phase of the coherent spin precession, or, equivalently, to act as a quantum eraser²⁷ of the relatively small energy difference between different branches of the Λ -system (Supplementary Information). Such high bandwidth/fast detection techniques have also been employed to increase visibility in photon-photon interference experiments¹². However, the much larger energy difference in our system requires a more advanced quantum eraser technique.

In order to tackle the large energy difference, we use a frequency downconversion technique that converts a single 910-nm photon into a single 1,560-nm photon with sub-8-ps timing resolution; this extreme timing resolution is sufficient to erase the which-path frequency information. The technique consists of mixing the single 910-nm photon with a few-picosecond pump light pulse at 2.2 μm in a PPLN waveguide device, producing a 1,560-nm photon via the process of difference-frequency generation (Methods). After narrowband filtering at 1,560 nm, the frequency-downconverted photon can be detected on a superconducting nanowire single-photon detector²⁸ (SNSPD) conditional on the exact overlap of the single 910-nm photon and the 2.2- μm pump pulse. The detection of the 1,560-nm photon therefore heralds a time-accurate measurement of the 910-nm photon: only if the 910-nm photon entered the PPLN waveguide exactly at the same time as the 2.2- μm pulse can a 1,560-nm photon be generated.

The system diagram of the conversion set-up is indicated in Fig. 2a. Using cross-correlation with a bright, 3-ps 910-nm pulse (Fig. 2b), we can infer a timing resolution of 8 ps or better for the PPLN waveguide device (Methods). As 8 ps correspond to an effective bandwidth of more

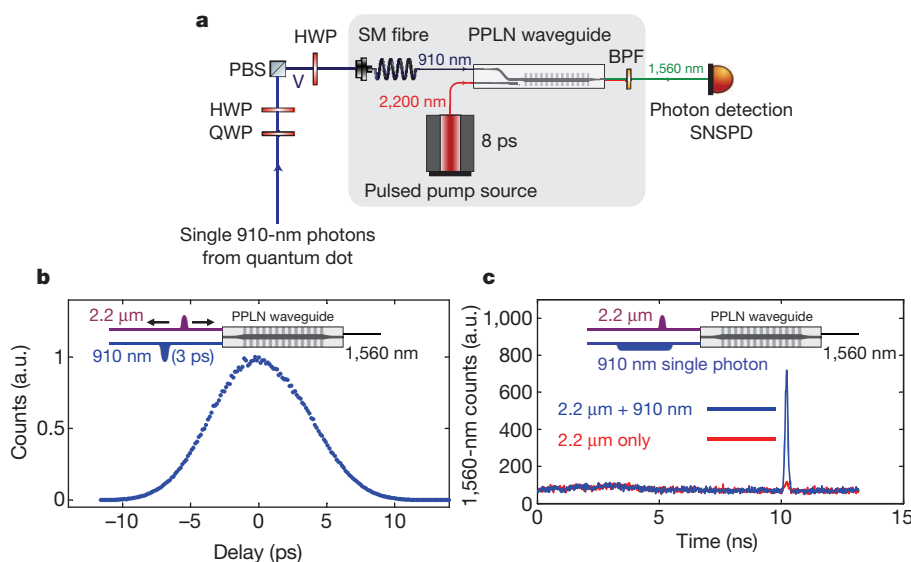


Figure 2 | Ultrafast conversion to 1,560 nm. **a**, Schematic overview of the conversion technique used (Methods). The 910-nm photon polarization is measured, with high timing accuracy, by a combination of a polarization analysing stage, a PPLN downconverter and an SNSPD. PBS, polarizing beamsplitter; HWP, half-wave plate; QWP, quarter-wave plate; SM, single-mode; BPF, 1,560 nm bandpass filter. **b**, Timing resolution of the 1,560-nm conversion technique, measured by cross-correlating the 2.2- μm conversion pulse with a classical, 3-ps pulse at 910 nm (inset). From these data, we can infer a sub-8-ps resolution for the arrival time of a single photon from the quantum dot. **c**, Performance of the conversion technique at single-photon levels, measured using an SNSPD. For single, 910-nm photons at the input (blue trace), the residual noise (red trace) can be seen to be well below the single-photon level (Methods). Inset, cross-correlation set-up.

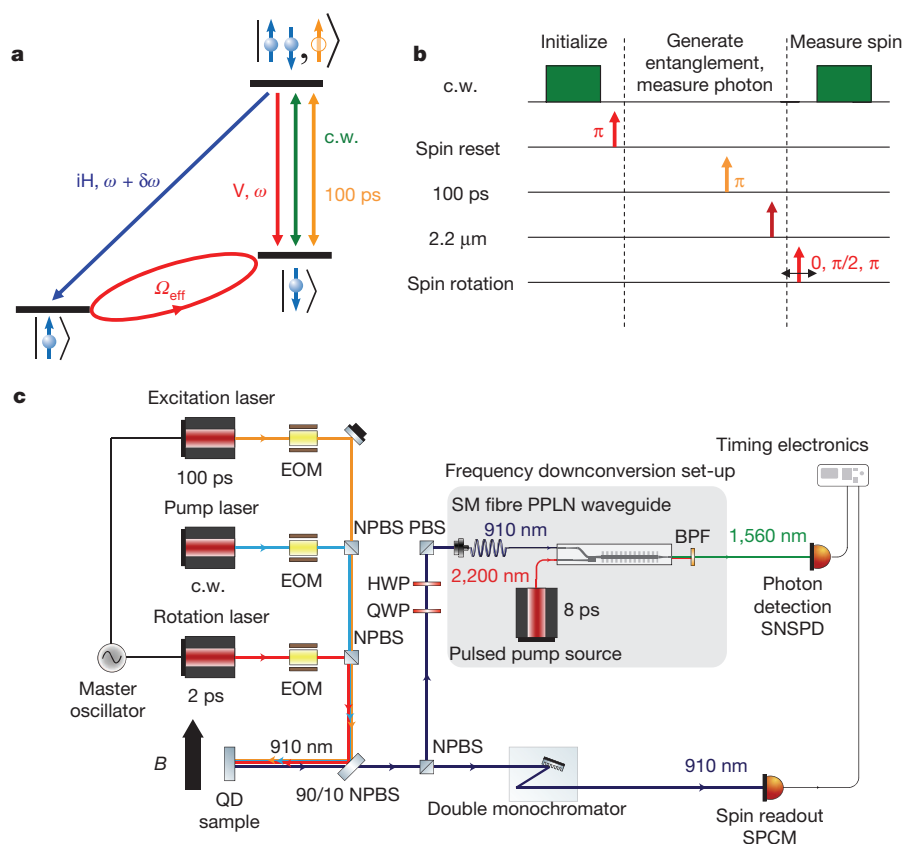


Figure 3 | Quantum-dot manipulation scheme for spin-photon entanglement verification. **a**, Schematic overview of the quantum-dot manipulation techniques used in the experiment. ‘100 ps’ indicates the resonant, 100-ps laser pulse used to excite the system into the $|\uparrow\downarrow\rangle$ -state; ‘c.w.’ indicates the continuous wave (c.w.) laser used for initialization into the $|\uparrow\rangle$ -state, and for readout of the $|\downarrow\rangle$ -state. $\delta\omega$, Spin Larmor precession frequency; Ω_{eff} , effective spin Rabi frequency, resulting from manipulation by 3-ps, detuned optical pulses^{6,7}. For the classical correlation measurements, manipulation of the spin using the H-branch was used as well, whereas the entanglement result was obtained using only V-branch pumping. **b**, Timing

diagram of the pulse sequence used in the experiment. Note the three cycles used in the experiment: initialization through optical pumping and spin rotation, generation of entanglement using 100-ps laser pulses and spontaneous emission decay, followed by ultrafast photon conversion to a telecom wavelength, and spin measurement through a combination of spin rotation (measurement basis change), optical pumping and single-photon detection. The cycle time of a single shot of the experiment was chosen to be either 39 or 52 ns. **c**, Schematic diagram of the set-up used in the experiment. EOM, electro-optic modulator; NPBS, non-polarizing beamsplitter. See text for other components.

than 100 GHz, this is more than sufficient to erase the information inherent in the Zeeman energy of 17.6 GHz at 3 T (Supplementary Information). In addition, the conversion and filtering technique results in almost noise-free signal transduction to the telecom band, as shown in Fig. 2c. We measure the polarization state of 910-nm photons using a polarization analysing stage consisting of a quarter-wave plate and a half-wave plate and a polarizer, followed by a single downconversion set-up that provides the necessary timing accuracy (filtering) in order to verify entanglement at 910 nm. The polarization selective operation of PPLN waveguides does not permit a full photonic polarization qubit at 910 nm to be directly downconverted to a polarization qubit at 1,560 nm. However, and as a straightforward extension of the work presented in this Letter, mapping the polarization qubit into a dual-rail qubit²⁹, using two separate time-resolved downconverters, would realize a spin-entangled 1,560-nm qubit, suitable for long-distance quantum communication¹⁰ (Supplementary Information).

We analyse spin-photon entanglement using a combination of the time-resolved conversion technique and previously established spin initialization, manipulation and readout techniques^{6,7}. Using 3-ps-long, 1-nm red-detuned optical pulses in a Ramsey interferometry set-up, we can completely control the state of any arbitrary electron spin superposition, with fidelities around 95% for the particular quantum dot used in this work (Supplementary Information). The spin state is measured using an optical pumping scheme^{6,7}, which is also used to initialize the system into the $|\uparrow\rangle$ -state. Both processes emit a single

910-nm H-polarized photon that can be detected after frequency and polarization filtering from the optical pumping laser^{6,7} (Fig. 3a and Methods). For a 13-ns spin interrogation time, readout and initialization fidelities of $\geq 96\%$ can be inferred from the time-resolved emission decay, mainly limited by residual leakage of the continuous-wave laser used for optical pumping (Supplementary Information). The full control sequence of the optical pulses is indicated in Fig. 3b, and consists of three stages. First, the system is initialized into either the $|\downarrow\rangle$ -state or the $|\uparrow\rangle$ -state by a combination of optical pumping and spin rotation with a π -pulse. Then, a 100-ps optical π -pulse resonant with the $|\downarrow\rangle$ - $|\uparrow\downarrow\rangle$ -transition (or $|\uparrow\rangle$ - $|\uparrow\downarrow\rangle$ -transition) excites to the trion state, after which spontaneous emission occurs into the electron spin states. The difference between the spontaneous emission decay time (600 ps) and the excitation pulse duration (100 ps) allows for temporal filtering of the spontaneously emitted single photons. In addition, for the classical correlation measurements (see below), cross-polarization filtering can be used to further suppress noise from the reflection of the 100-ps excitation laser. The spontaneously emitted single photons are then sent to a polarization analysing stage, after which they are converted to 1,560 nm using the PPLN waveguide, and detected by an SNSPD. The next stage of the experiment consists of the spin analysis, which is performed through a combination of spin rotations (these implement an effective measurement basis change) and the optical pumping/readout cycle. We measure the correlations between the polarization of the 910-nm photon and the spin state through a timing histogram analysis

of the photons detected in the conversion and measurement cycles (Fig. 3c and Methods).

Using our time-resolved downconversion technique, we measure the classical (computational basis) correlations between the spin along the magnetic field axis (z), and the H–V polarization of the photon

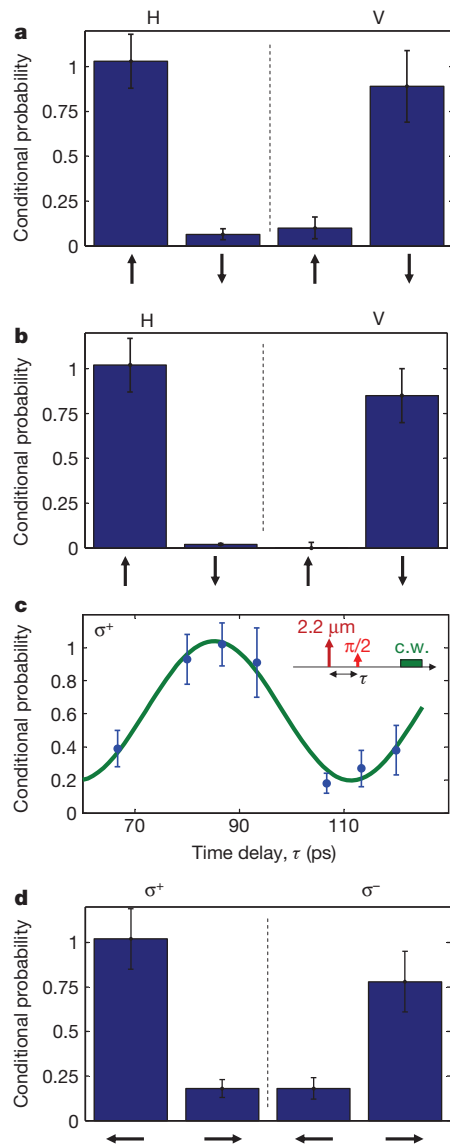


Figure 4 | Spin-photon entanglement verification. **a**, Classical (computational basis) spin-photon correlations, measured through downconversion to 1,560 nm. The black arrows (see also **b**, **d**) refer to the spin orientation in the computational (vertical arrows) or rotated (horizontal arrows) basis. **b**, Classical (computational basis) spin-photon correlations, measured at 910 nm, without downconversion. **c**, Spin-photon entanglement: on time-resolved detection of a $\sigma^{+,-}$ -downconverted photon, the electron spin starts precessing due to the electron Zeeman energy (Larmor precession). By changing the arrival time of the subsequent $\pi/2$ -pulse, this coherent spin precession can be mapped into a Ramsey fringe, where the population in the $|\downarrow\rangle$ -state oscillates as a function of time, and depends on the helicity of the downconverted photon (the Ramsey fringes for opposite helicities are in antiphase). This oscillatory spin signal then results in an oscillatory, periodic coincidence count rate between the downconverted single photon and the spin-measurement photon. Blue, raw data from histogram analysis; green, least-squares, sinusoidal fit to the data. Inset, relative timings used. 2.2 μm indicates the 2.2- μm conversion pump pulse; $\pi/2$ shows the $\pi/2$ spin rotation pulse; c.w. indicates the continuous wave (c.w.), spin measurement laser at 910 nm. **d**, Spin-photon correlations in the rotated bases, demonstrating entanglement. Error bars, ± 1 s.d. (**a–d**), due to Poissonian statistics ($n_{\text{events}} \approx 50\text{--}100$).

(Fig. 4a). For an H-polarized photon, we measure an excellent correlation with the $|\uparrow\rangle$ -spin-state in the subsequent spin measurement cycle. Likewise, for a V-polarized photon, we see a strong correlation with the $|\downarrow\rangle$ -spin-state. We can directly compare these correlations with the ones obtained without ultrafast downconversion, using a commercial single-photon detector (Fig. 4b). The results are in good agreement, and differ only due to small amounts of residual noise in the down-conversion process (Supplementary Information).

Verification of entanglement requires observation of correlations in a rotated basis of the photon polarization and the spin as well. When we measure the photon in the circular polarization basis ($|\sigma^+\rangle$, $|\sigma^-\rangle$), we measure the spin in the basis of $|\rightarrow\rangle = \frac{1}{\sqrt{2}}(|\uparrow\rangle + |\downarrow\rangle)$ and $|\leftarrow\rangle = \frac{1}{\sqrt{2}}(|\uparrow\rangle - |\downarrow\rangle)$. After detection of a $|\sigma^+\rangle$ ($|\sigma^-\rangle$)-downconverted photon at time t_1 , the spin is projected into the $|\leftarrow\rangle$ -state ($|\rightarrow\rangle$), which subsequently evolves in time due to Larmor precession:

$$|\psi_{\text{spin}}(t)\rangle = \frac{1}{\sqrt{2}} \left(e^{i(\delta\omega)(t-t_1)} |\rightarrow\rangle \mp |\leftarrow\rangle \right) \quad (3)$$

Here $\delta\omega$ corresponds to the Zeeman frequency of $2\pi \times 17.6$ GHz. By scanning the arrival time of a $\pi/2$ spin rotation pulse in a Ramsey interferometer^{6,7} (Fig. 4c and Methods) we can trace out this coherent oscillation, and verify entanglement. From the minima and maxima of these coherent oscillations, we can derive, as in Fig. 4d, the photon-spin correlations for $|\sigma^+\rangle$ ($|\sigma^-\rangle$) downconversion. For a particular photon polarization ($|\sigma^+\rangle$, $|\sigma^-\rangle$) and arrival time of the $\pi/2$ spin rotation pulse, a correlation can be measured, as expected. Subsequently changing the arrival time of the $\pi/2$ -pulse by half a Larmor period results in an anticorrelation (see also Fig. 4c). Together, these results demonstrate spin-photon entanglement. We estimate the entanglement fidelity^{3,4} to be 0.8 ± 0.085 (Methods and Supplementary Information), which exceeds the classical limit of 0.5 by more than three standard deviations^{3,4}.

Whereas our entanglement verification technique relies on time-resolved frequency conversion, resulting in filtering out only those 910-nm photons that exactly overlap with the arrival time of the 2.2- μm pump pulse, a more generic downconversion technique with a continuous wave pump laser at 2.2 μm and coincidence detection of two photons from separate quantum-dot sources could be used in future experiments in order to obtain spin-spin entanglement¹⁷ (Supplementary Information).

We have demonstrated high-fidelity spin-photon entanglement in a single InAs quantum dot using an ultrafast downconversion technique to the lowest-loss telecom wavelength (1,560 nm). When combined with ultrafast control of InAs quantum-dot electron spins^{6,7} and fast radiative decay of indistinguishable single photons from such quantum dots embedded in optical cavities^{11,12,20,21}, our results should enable quantum state transfer from a flying qubit to a stationary qubit or vice versa, and entanglement distribution between two remote matter qubits in long-distance quantum networks.

We note that, contemporaneously with this work, another group verified spin-energy entanglement between an InAs quantum-dot electron spin and a spontaneously emitted photon at 966 nm (ref. 30).

METHODS SUMMARY

Quantum dot and optical control. All results are obtained from a single quantum dot, emitting at 910 nm, embedded in a planar microcavity⁷. An external magnetic field in Voigt geometry splits the electron spin and trion states (Fig. 1a). A 0.68 NA aspheric lens focuses the pump and rotation lasers onto the sample. The coherent manipulation techniques are identical to those reported previously⁷, with fidelities (initialization, readout, coherent rotation) around 95% or higher (Supplementary Information). After initialization, the $|\uparrow\downarrow\rangle$ -state is excited by a 100-ps mode-locked-laser pulse, synchronized with the spin-rotation laser. Photoluminescence is collected in a confocal set-up, and split into two branches. One branch is cross-polarized and sent through a double-monochromator onto a single-photon counter (SPCM) for spin-state analysis. The other branch is sent to a polarization analysing stage, after which it is sent to the downconversion set-up.

Downconversion, data acquisition and processing. The 2.2- μm light pulses needed for conversion are generated in a PPLN chip by mixing 3-ps, 911-nm pulses from the spin-rotation laser with narrowband, continuous wave 1,560-nm light. A PPLN waveguide converts 910-nm photons into 1,560-nm photons, conditional on overlap with the 2.2- μm pulses. An SNSPD subsequently detects the 1,560-nm photons. The SPCM and SNSPD signals are combined on a timing analyser, which allows for signal gating in post-processing. The noise in the conversion process is well below the single-photon level (between 4:1 and 10:1 signal-to-noise ratio). The correlation data are obtained from the coincidences between the downconverted single photons and the single photons used for spin detection, through post-processing of the data stream from the timing analyser, and normalized to uncorrelated events (Supplementary Information). The entanglement fidelity analysis follows the same procedure used in ion-trap⁴ and NV-diamond³ spin-photon entanglement experiments, and is estimated to be around 0.8 ± 0.085 , well above the classical limit of 0.5.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions S.M., C.S., M.K. and S.H. grew and fabricated the samples. K.D.G. and Y.Y. designed the experiment. K.D.G., J.S.P., L.Y., P.L.M., C.M.N. and N.Y.K. performed the optical experiments. J.S.P. designed and fabricated the PPLN waveguides. J.S.P. and L.Y. developed the 2.2- μm set-up and the 1,560-nm filtering design. C.M.N. and R.H.H. packaged, characterized and implemented the SNSPD detectors. Y.Y., M.M.F., E.A. and A.F. guided the work. K.D.G. wrote the manuscript with input from all authors.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to K.D.G. (kdegreve@stanford.edu).

METHODS

Device design. The quantum-dot sample used is similar to the ones used previously to investigate ultrafast optical control and single spin echoes⁷. Compared to those experiments, the dot density was reduced, and the wavelength used was blue-shifted (910 nm). An asymmetric, low- Q cavity (10 nm FWHM, centred at 910 nm) redirects the spontaneous emission preferentially in one direction, increasing collection efficiency and reducing required peak powers for coherent control⁷. An external magnetic field (Voigt geometry, perpendicular to the optical axis) splits both the electron spin states and the excited, trion states. The resulting Λ -systems are indicated in Fig. 1a, and the polarization selection rules are verified by means of polarization-selective photoluminescence (half-wave plate and polarizing beamsplitter, Fig. 1b).

Spin control and single-photon collection. The quantum-dot device is cooled to 1.6 K inside a superconducting magnetic cryostat (Oxford Spectromag; the magnetic field used varies between $B = 3$ and 6 T). A 0.68 NA aspheric lens inside the cryostat focuses the pump and rotation lasers onto the sample, which is scanned relative to the lens by means of slip-stick piezo-electric positioners (Attocube Systems). The coherent manipulation techniques used are identical to those reported previously⁷. A narrowband c.w. laser (New Focus Velocity) is used for spin initialization and readout, resonant to the $|\downarrow\rangle-|\uparrow\downarrow\uparrow\rangle$ -transition (910.10 nm for a 3 T magnetic field), and switched on and off by a fibre-based EOM (EOSpace). The interrogation time is 13 ns, with an initialization and readout fidelity of about 96% or higher (Supplementary Information). Coherent spin rotations are performed in a Ramsey-interferometry set-up, using pulses from a mode-locked laser (3-ps pulse duration, centre wavelength 911 nm, Spectra-Physics Tsunami), which are delayed relative to each other through a retroreflector on a motorized stage. Individual pulses are selected through free-space EOMs (Conoptics), which are double-passed in order to increase the extinction ratio. The selective excitation of the $|\uparrow\downarrow\uparrow\rangle$ -state before spontaneous emission is realized through a combination of optical pumping into the $|\uparrow\rangle$ -state, rotation by an optical π -pulse into the $|\downarrow\rangle$ -state, followed by the application of a 100-ps pulse (optical π -pulse) from another mode-locked laser (Spectra-Physics Tsunami), resonant with the $|\downarrow\rangle-|\uparrow\downarrow\uparrow\rangle$ -transition. This laser is synchronized with the rotation laser (Spectra-Physics Lok-to-Clock system). Accurate polarization control limits the probability of excitation into the $|\uparrow\downarrow\uparrow\rangle$ -state to less than 1%. Another fibre-optic modulator allows for pulse-picking of the 100-ps excitation pulses. The single-photon photoluminescence is collected in a confocal set-up, and split into two branches by a non-polarizing beamsplitter. One branch is cross-polarized with respect to the initialization and optical pumping lasers, and sent through a double-monochromator onto a single-photon counter for spin-state analysis (Perkin-Elmer SPCM; 20% quantum efficiency, 170 Hz ungated dark count rate). The other branch is sent to a polarization analysing stage (quarter- and half-wave plate and polarizer), after which it is coupled into single-mode fibres and sent to the downconversion set-up. The polarization analysing stage is carefully calibrated, in order to account for residual birefringence in the setup. All EOMs are controlled by mutually synchronized pulse-pattern generators (76 MHz Tektronix and 10 GHz Anritsu PPG), that are themselves synchronized to the repetition rate of the mode-locked lasers. In contrast to previous experiments, no software-based lock-in technique was used. Instead, spatial, polarization, wavelength and time-filtering are used to separate reflected light from the single photons.

Downconversion set-up. The 2.2- μ m light pulses needed for conversion are generated by a difference-frequency generation (DFG) process that mixes the 3-ps, 911-nm pulses from the mode-locked laser with narrowband, c.w. 1,560-nm light in an MgO-doped, PPLN chip. The 1,560-nm light is modulated by a fibre-optic

modulator, and amplified by erbium-doped fibre amplifiers. After wave mixing, the residual 1,560-nm and 911-nm light is filtered out through a combination of dichroic and absorptive filters. The resulting pulse width depends on the exact power and wavelength used for the DFG process, but is measured to be between 3 and 8 ps.

A PPLN waveguide efficiently converts 910-nm, spontaneously emitted photons to 1,560-nm photons, conditional on overlap with the 2.2- μ m pulses. Residual scattered light from the 910-nm and 2.2- μ m branches is eliminated through a fibre-Bragg grating and a long-pass filter. The 1,560-nm photons are subsequently detected on an SNSPD, maintained at 2 K, with 14% system detection efficiency, 40-Hz ungated dark count rate and 100-ps FWHM timing jitter. Timing analysis is performed on a timing analyser (PicoQuant HydraHarp), used in time-tagged time-resolved (TTTR) mode, which allows for accurate gating of the signals of both the SPCM and the SNSPD in post-processing, thereby drastically reducing the effects of dark counts. The overall signal-to-noise ratio is limited by residual leakage of the 2.2- μ m light, dark counts within the timing response of the SNSPD, and residual reflected light from the 100-ps excitation pulses, but is in general well below the single-photon level (signal-to-noise ratio ranging between 4:1 and 10:1 for the experiments described in this work). The timing of the 2.2- μ m light is chosen such that the subsequent $\pi/2$ -pulse arrives well within the T_2^* -dephasing time of the quantum dot; similar results can be obtained for times up to microseconds by implementing a spin echo technique to overcome shot-to-shot dephasing⁷.

Data analysis and entanglement fidelity. The correlation data obtained in this work are the result of a histogram analysis, performed on the coincidence count rate between the downconverted single photons, and the single photons used for spin detection (Supplementary Information). The coincidence count rate is obtained through post-processing of the TTTR data stream, and comparing the coincidences within the same experimental run to those in subsequent, uncorrelated ones. The repetition rate is set at 39 or 52 ns, and the 0.1% single photon efficiency and time-gated frequency downconversion result in a 1,560-nm single-photon detection and entanglement generation rate of some 2–5 Hz. In combination with another 0.1% single-photon detection efficiency in order to detect the spin state, this results in an average coincidence rate of some 2–5 mHz. We emphasize that these losses are predominantly due to the inefficiency of extracting a single photon from the quantum dot, which can be significantly improved by accurate cavity design⁸. The conversion process in itself, while lossy due to the aggressive time-filtering in order to obtain good timing resolution, is rather effective, with internal quantum efficiencies estimated above 80%, and filtering losses of several dB maximum.

The entanglement fidelity analysis follows the same procedure used in the ion-trap⁴ and NV-diamond³ spin-photon entanglement experiments:

$$F \geq F_1 + F_2,$$

$$F_1 = \frac{1}{2} \left(\rho_{H\uparrow, H\uparrow} + \rho_{V\downarrow, V\downarrow} - 2\sqrt{\rho_{H\downarrow, H\downarrow}\rho_{V\uparrow, V\uparrow}} \right),$$

$$F_2 = \frac{1}{2} \left(\rho_{\sigma+\leftarrow, \sigma+\leftarrow} - \rho_{\sigma+\rightarrow, \sigma+\rightarrow} + \rho_{\sigma-\rightarrow, \sigma-\rightarrow} - \rho_{\sigma-\leftarrow, \sigma-\leftarrow} \right).$$

Here $\rho_{H\uparrow, H\uparrow}$ etc. refer to elements of the spin photon density matrix, which can be associated with the observed correlations in our experiment. For the values obtained in our work, $F_1 = 0.44 \pm 0.06$, $F_2 = 0.36 \pm 0.06$, from which we obtain a bound on the quoted fidelity: $F \geq 0.8 \pm 0.085$.

Structural insight into the type-II mitochondrial NADH dehydrogenases

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The single-component type-II NADH dehydrogenases (NDH-2s) serve as alternatives to the multisubunit respiratory complex I (type-I NADH dehydrogenase (NDH-1), also called NADH:ubiquinone oxidoreductase; EC 1.6.5.3) in catalysing electron transfer from NADH to ubiquinone in the mitochondrial respiratory chain¹. The yeast NDH-2 (Ndi1) oxidizes NADH on the matrix side and reduces ubiquinone to maintain mitochondrial NADH/NAD⁺ homeostasis. Ndi1 is a potential therapeutic agent for human diseases caused by complex I defects^{2–9}, particularly Parkinson's disease, because its expression restores the mitochondrial activity in animals with complex I deficiency. NDH-2s in pathogenic microorganisms are viable targets for new antibiotics^{10,11}. Here we solve the crystal structures of Ndi1 in its substrate-free, NADH-, ubiquinone- and NADH-ubiquinone-bound states, to help understand the catalytic mechanism of NDH-2s. We find that Ndi1 homodimerization through its carboxy-terminal domain is critical for its catalytic activity and membrane targeting. The structures reveal two ubiquinone-binding sites (UQ_I and UQ_{II}) in Ndi1. NADH and UQ_I can bind to Ndi1 simultaneously to form a substrate–protein complex. We propose that UQ_I interacts with FAD to act as an intermediate for electron transfer, and that NADH transfers electrons through this FAD–UQ_I complex to UQ_{II}. Together our data reveal the regulatory and catalytic mechanisms of Ndi1 and may facilitate the development or targeting of NDH-2s for potential therapeutic applications.

Electron transport chains, which comprise oxidative phosphorylation complexes I to V in the inner membrane of mitochondria, serve as the core components during respiration¹². Complex I catalyses the transfer of two electrons from NADH to reduce ubiquinone to ubiquinol, and is the entry point for a large fraction of the electrons that traverse the respiratory chain^{13,14}. This catalysis is the rate-limiting step in respiration and is central to energy metabolism¹⁵. Three different types of NDH are known: proton-pumping NDH (NDH-1); non-proton-pumping NDH (NDH-2); and the rare sodium-pumping NDH, called NQR^{1,15,16}.

First identified in domestic yeast^{17,18}, NDH-2 is a ~50-kDa, monotopic membrane protein with no energy-transducing capacity. Subsequently, multiple NDH-2s were found in various fungi, plants and primitive eukaryotic cells^{1,19}. Genome-wide analysis has shown that a majority of organisms, such as *Escherichia coli*, contain both NDH-1 and NDH-2. However, some species have only NDH-2 (Supplementary Fig. 1, Supplementary Data 1 and Supplementary Table 1). Ndi1, one of the NDH-2s in *Saccharomyces cerevisiae*, faces the inside surface of the mitochondrial inner membrane, similar to NDH-1²⁰. Here we report the crystal structures of Ndi1 in its apo and substrate-bound states.

Ndi1 forms a globular α/β structure that can be divided into three domains (Fig. 1a and Supplementary Fig. 2). Similar to other NADH- and FAD-associated enzymes, Ndi1 contains two canonical Rossmann

domains with an FAD molecule buried deep in the first one (residues 49–412, referred to as the active domain hereafter) (Fig. 1 and Supplementary Fig. 3). A search of the Dali database²¹ revealed that the NADH- and FAD-associated enzymes are the closest structural homologues of the Ndi1 active domain, with most of them having a root mean squared deviation (r.m.s.d.) of less than 2 Å when compared with the Ndi1 active domain. The structures of Ndi1 and other NADH- and FAD-associated enzymes differ in their C-terminal domains (CTDs; Fig. 1b). Ndi1 CTD is highly conserved among NDH-2s from different species (Supplementary Fig. 2 and Supplementary Data 1 and 2) and contains three β -strands (β 19– β 21) and two α -helices (α 15 and α 16) (Fig. 1a and Supplementary Fig. 2). The two α -helices form a helix–turn–helix motif that extensively interacts with the active domain, whereas the three β -strands make fewer contacts with the active domain (Supplementary Fig. 4).

In all the structures, each asymmetric unit contains an Ndi1 homodimer (Fig. 2a). Interactions between the two Ndi1 monomers are mainly mediated through helix α 16 of the CTD (Fig. 2b and Supplementary Fig. 5a, b), burying a surface area of 5,982 Å². Consistent with this, we eluted wild-type Ndi1 from a gel filtration column with a native molecular mass of ~150 kD, corresponding to the calculated size of an Ndi1 dimer (Supplementary Fig. 6a). The linear relationship between Ndi1 concentration and its activity indicated that a single enzyme catalysed the electron transfer reaction (Supplementary Fig. 6b). To investigate whether Ndi1 was oligomeric *in vivo*, we co-expressed Ndi1–FLAG and Ndi1–Myc proteins in yeast cells and performed immunoprecipitation assays. As shown in Fig. 2c, the two Ndi1 proteins interacted with each other, indicating that Ndi1 formed an oligomer in yeast cells. Deletion of α 16 compromised the interaction between Ndi1–FLAG and Ndi1–Myc, and deletion of both α 15 and α 16 abolished the interaction (Fig. 2c). This result indicated that the CTD is important for Ndi1 oligomerization. We further confirmed the functional importance of the CTD through a rescue assay. The plasmid carrying wild-type Ndi1 but not the mutants with α 16 or α 15 and α 16 deleted, rescued the yeast *ndi1Δ* phenotype (Supplementary Fig. 5c). Thus, Ndi1 functions as a homodimer both *in vitro* and *in vivo*.

The membrane anchoring of *E. coli* NDH-2 is required for its activities²². One side of the Ndi1 homodimer contains a large hydrophobic surface, which is formed by the solvent-exposed residues from the CTD and surrounded by numerous positively charged patches (Fig. 2a, d). In solution, the hydrophobic surface was shielded by the detergent molecules used for protein purification, because several detergent molecules bound to this region in the structures of Ndi1 (Supplementary Fig. 7). This suggested that the hydrophobic surface could have a role in the mitochondrial membrane localization of Ndi1. Indeed, fractionation indicated that wild-type Ndi1 protein was exclusively found in the membrane fraction. In contrast, a large pool of the

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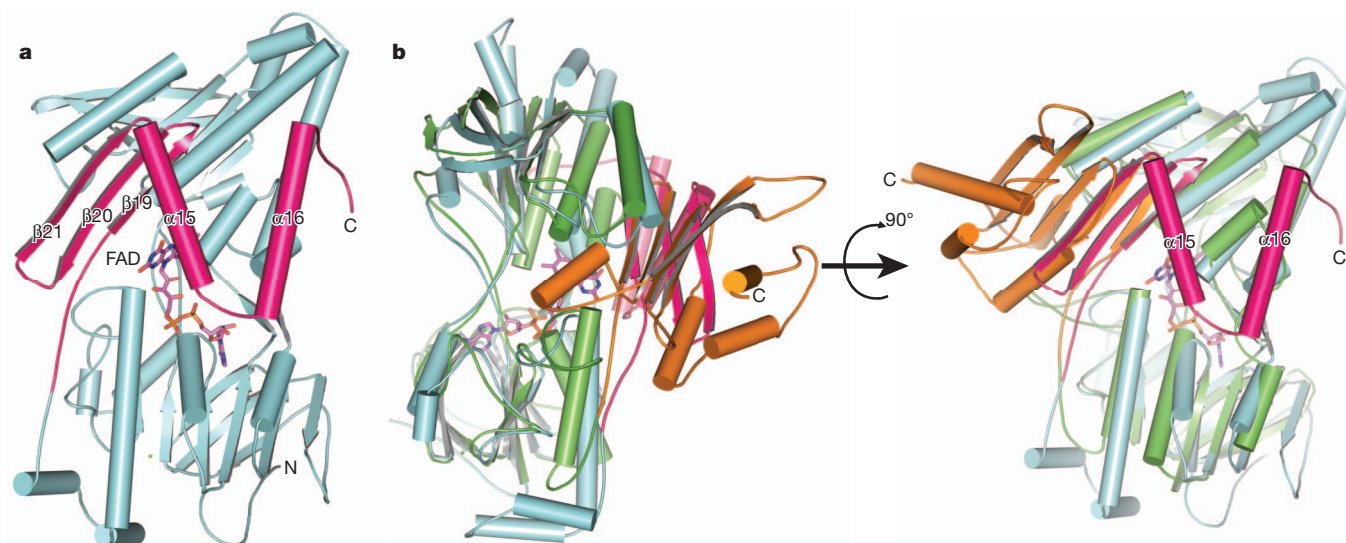


Figure 1 | Ndi1 has a unique CTD. **a**, Overall structure of Ndi1. The CTD (residues 439–513) of Ndi1 is coloured in pink and the active domain in cyan. FAD is shown in stick representation. **b**, Two views of the structural alignment

between Ndi1 and the Pdr–Pdx complex from *Pseudomonas putida* (Protein Data Bank ID, 3LB8). The CTD of Pdr is coloured in orange and the remaining part in green. Colour codes for Ndi1 are the same as those in **a**.

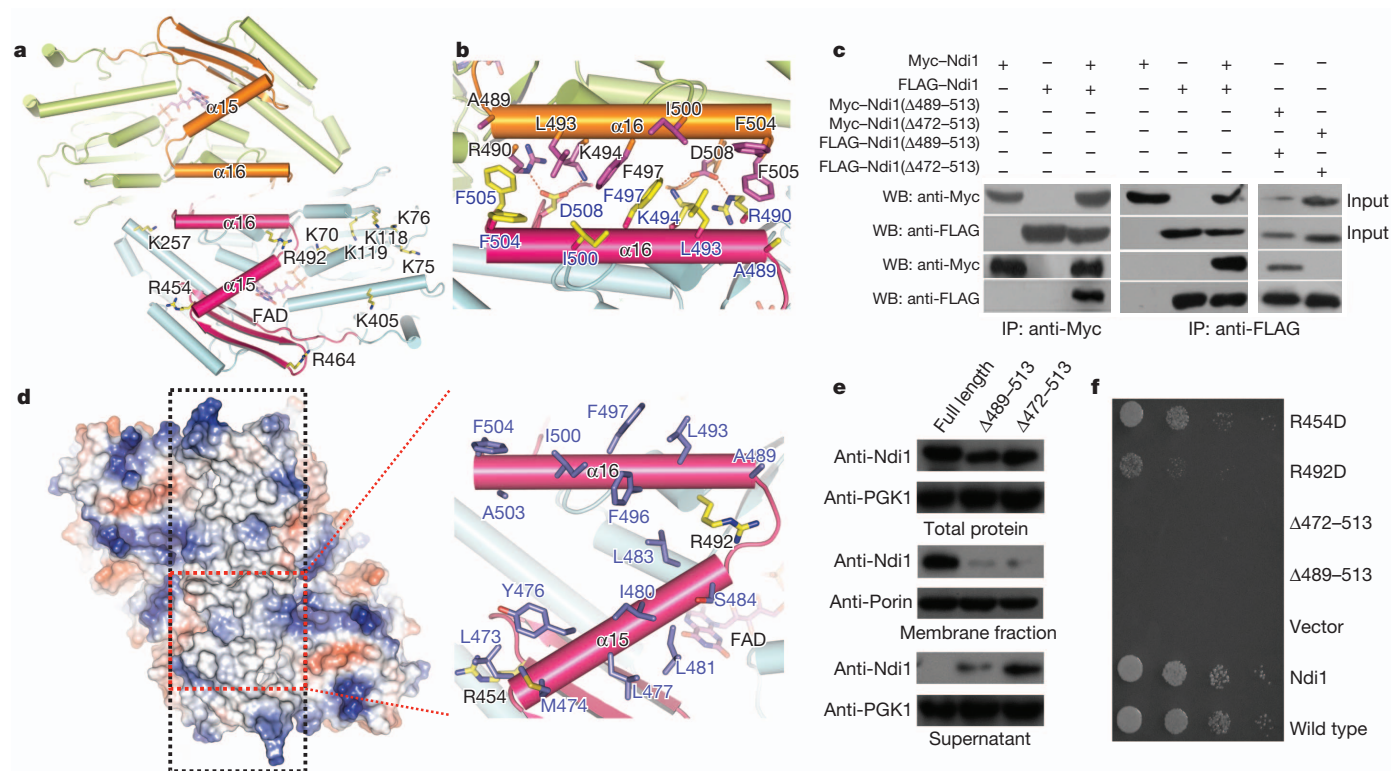


Figure 2 | The CTD mediates Ndi1 homodimerization and membrane attachment. **a**, Ndi1 forms a homodimer in crystals. One protomer is coloured as in Fig. 1a and the other one is coloured in orange (CTD) and lime (remainder). The conserved, positively charged residues on the membrane attachment side of one protomer are shown as yellow sticks. **b**, Detailed interactions between $\alpha 16$ of the two Ndi1 protomers. **c**, Ndi1 forms a dimer *in vivo*. Top: western blots of lysates (input) of *ndi1Δ trp1Δ* cells expressing the wild-type and the mutant forms of Ndi1–Myc, Ndi1–FLAG or both. Proteins were detected by western blot with anti-Myc and anti-FLAG antibodies. Bottom: Cell lysates in the top panels were subjected to anti-FLAG or anti-Myc immunoprecipitation (IP) followed by anti-Myc and anti-FLAG immunoblotting (WB). **d**, A large hydrophobic surface formed by the CTD in

the Ndi1 homodimer. Left: electrostatic surface of the Ndi1 homodimer. The hydrophobic surface is highlighted with a dashed box. Right: the side chains of the surface residues from $\alpha 15$ and $\alpha 16$ of the CTD. For clarity, only one Ndi1 monomer is shown. **e**, The CTD is essential for Ndi1 membrane attachment. The lysates of *ndi1Δ* cells transformed with vectors encoding the indicated forms were fractionated into three samples: total protein, membrane fraction and supernatant. The samples were subjected to western blots. The loading amounts of the samples were examined using anti-Porin for the membrane fractions and anti-PGK1 antibody for the total protein and supernatant fractions. **f**, Mutations compromising Ndi1 membrane attachment cause growth defects in yeast cells.

mutant Ndi1 protein with $\alpha 15$ and $\alpha 16$ deleted was detected in the supernatant fraction (Fig. 2e), supporting a role for the two α -helices in Ndi1 membrane anchoring. The two α -helices were required for yeast viability, because their deletion resulted in a lethal phenotype (Fig. 2f).

We then solved the structure of the NADH-bound Ndi1. As observed in other NADH and FAD reductases, NADH binds to the second Rossmann domain and is fully accessible to FAD from the matrix side (Supplementary Fig. 8). Stabilized by a number of hydrogen-bonding interactions, NADH binding causes a slight conformational change in this domain (Supplementary Fig. 9a, b). Supporting their functional importance, single mutations of the NADH-interacting residues caused a lethal phenotype in yeast (Supplementary Fig. 9c). The nicotinamide ring of NADH is orientated in parallel with the *re*-face of the FAD isoalloxazine ring, with the distance between C4 of NADH and N5 of FAD being 3 Å, indicating that a direct electron transfer between them can occur.

The structure of the ubiquinone-bound form was determined by co-crystallizing Ndi1 with ubiquinone in the absence of NADH. One ubiquinone molecule (UQ_I) with a well-defined density is located close to FAD (Fig. 3a) and lies in the groove formed by $\beta 19$ – $\beta 21$ and $\alpha 15$ from the CTD (Supplementary Figs 10a, b). The distance between the centre of the FAD isoalloxazine ring and UQ_I is about 7.8 Å. This site was occupied by a Triton X-100 (TRT) molecule in the apo structure (Supplementary Fig. 7), explaining why the protein extracted with *n*-dodecyl- β -D-maltopyranoside (DDM) but not with TRT contained bound ubiquinone^{23,24}. Mutations of the residues that interact with UQ_I markedly attenuated the enzymatic activity of Ndi1 and

compromised yeast survival (Supplementary Figs 10c, d). Although comparatively less well defined, a second ubiquinone molecule (UQ_{II}) was assigned to the stretch of density adjacent to UQ_I (Fig. 3a). This assignment was consistent with the recent studies indicating that Ndi1 contains both a bound ubiquinone and a catalytic ubiquinone at similar sites^{24,25}. Both ubiquinone molecules are located on the *si*-face of the isoalloxazine ring of FAD and extend their aliphatic side chains towards the large hydrophobic surface (Supplementary Fig. 10a). The less well-defined UQ_{II} forms fewer contacts with Ndi1 than does UQ_I (Supplementary Fig. 10b).

Putidaredoxin reductase (Pdr) is an NADH-dependent enzyme and transfers electrons to P450 through forming a complex with the [2Fe–2S]-containing protein putidaredoxin²⁶ (Pdx). As anticipated, the structure of Pdr resembles that of the Ndi1 active domain. UQ_{II} in Ndi1 is similarly positioned relative to the [2Fe–2S] cluster in Pdx. The distance between the centre of the isoalloxazine ring and the UQ_{II} centre is about 11.2 Å, comparable to the 12 Å distance between FAD and the [2Fe–2S] cluster in the Pdr–Pdx complex (Supplementary Fig. 11). These structural observations suggest that it is spatially possible for the electron to be transferred from reduced FAD ($FADH_2$) to UQ_{II} .

Previous studies suggested that Ndi1 could form a ternary complex with NADH and ubiquinone²³. We therefore soaked the ubiquinone-bound Ndi1 crystals with NADH and solved the structure of the Ndi1–NADH–ubiquinone complex (Fig. 3b). UQ_{II} was not observed in the complex. Because the crystals of the complex were obtained in the presence of oxygen, it might represent an enzyme–substrate complex

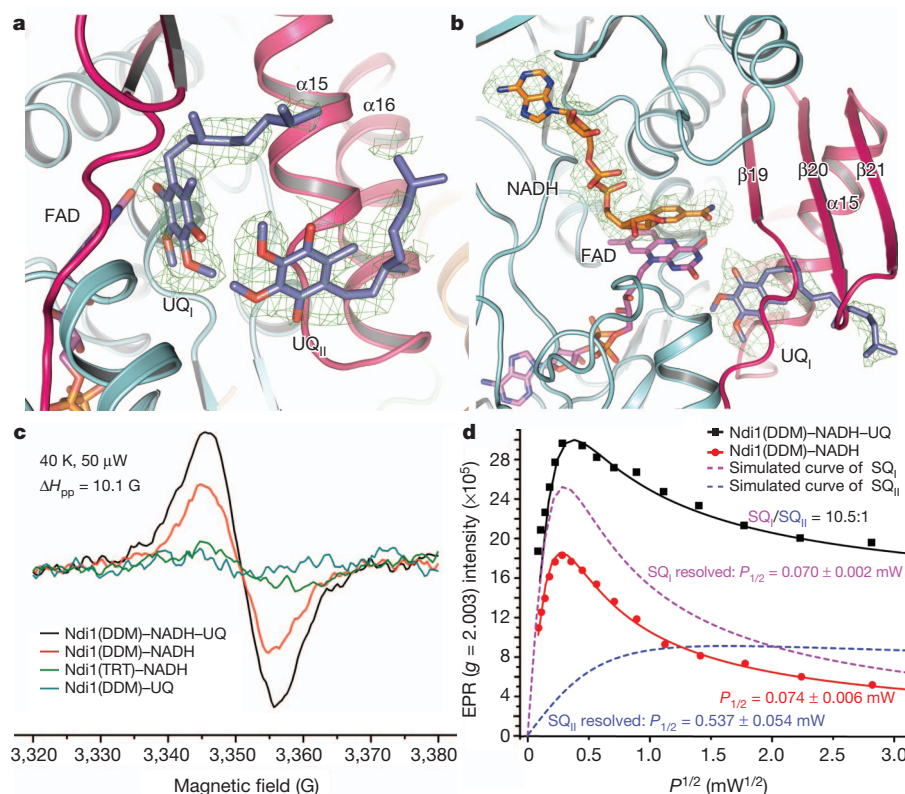


Figure 3 | Electron transfer of Ndi1 involves two ubiquinone molecules. **a**, Two ubiquinone molecules bind to Ndi1. The ubiquinone-binding sites in the Ndi1–ubiquinone complex are shown in ribbon representation. The ubiquinone and FAD molecules are shown as sticks and labelled. The $F_o - F_c$ omit density (green) is contoured at 3.5 σ . **b**, Ribbon representation of the reaction centre in the Ndi1–NADH–ubiquinone complex. The $F_o - F_c$ omit density (green) is contoured at 3.5 σ . **c**, Generation of Ndi1-catalysed radicals requires both NADH and ubiquinone. The EPR spectra of the $g = 2.003$ signals (g , electron g -factor) arise from ubisemiquinones of Ndi1 proteins as indicated.

ΔH_{pp} , peak-to-peak width. **d**, Ndi1-catalysed generation of two populations of radicals. The power saturation behaviours of the $g = 2.003$ EPR signals of Ndi1 were monitored at 40 K. All experimental parameters except the microwave power were the same as in **c**. The ubisemiquinone signal of Ndi1(DDM)–NADH (red) was fitted by the equation described in Methods, and that of the sample with excessive ubiquinone (black) was resolved as the sum of two components. The theoretical curves for these two components are shown by the purple and blue dotted lines. Two sets of half-saturation parameters ($P_{1/2}$) and the relative concentration ratio are indicated.

rather than a metastable enzyme–intermediate complex. Comparison with the apo-Ndi1 structure shows that Ndi1 undergoes almost no conformational changes when bound by ubiquinones (r.m.s.d, 0.22 Å) or NADH–ubiquinone (r.m.s.d, 0.26 Å). The UQ_I -binding site is almost identical in the two structures, suggesting that UQ_I may act as a cofactor together with FAD to mediate electron transfer (Supplementary Fig. 12).

Our structures reveal that Ndi1 possesses a unique CTD (Fig. 1 and Supplementary Fig. 2) that mediates the Ndi1 dimerization required for its activity (Figs 2a–c and Supplementary Fig. 5). The CTD-mediated Ndi1 dimerization creates a large hydrophobic surface at one side (Fig. 2d) and a hydrophilic groove at the other (Supplementary Fig. 13). The biochemical assay (Figs 2e, f) supports there being a role for the hydrophobic surface in targeting Ndi1 to the mitochondrial membrane. The positively charged patches around the hydrophobic surface could also contribute to the membrane localization of Ndi1, presumably through interactions with the phosphate groups of membrane-localized phospholipids. Further investigations are needed to understand the specific mitochondrial membrane targeting of Ndi1. Additionally, the CTD is directly involved in the recognition of ubiquinones by Ndi1 (Figs 3a, b). Taken together, we conclude that the unique CTD of the single-component enzyme Ndi1 enables it to perform the electron transfer function of the multi-component NDH-1s.

Like many ubiquinone-reactive enzymes^{27–30}, Ndi1 has two ubiquinone-binding sites (Fig. 3a). UQ_I may not readily exchange with the ubiquinone pool, because it forms extensive interactions with the CTD of Ndi1. In contrast, UQ_{II} may better serve this purpose because it has much fewer contacts with Ndi1 (Supplementary Figs 10a, b). Our structures suggest that there is an electron transfer pathway from NADH to UQ_{II} , with UQ_I acting as an intermediate together with FAD. FAD first accepts two electrons from NADH to form $FADH_2$. Several possible mechanisms could be responsible for subsequent electron transfer. $FADH_2$ could transfer one electron to UQ_I and one to UQ_{II} , reducing both to ubisemiquinones. Then a disproportionation reaction between them could occur, restoring UQ_I to its previously oxidized state and converting UQ_{II} into ubiquinol. Alternatively, $FADH_2$ could transfer the electrons one by one to UQ_{II} through the bound UQ_I , reducing UQ_{II} to ubiquinol. It is also possible that $FADH_2$ transfers both electrons successively to UQ_I and that the two electrons are then further transferred to UQ_{II} consecutively. The net results of these three different pathways are the same: two electrons from NADH are transferred through the FAD– UQ_I complex to UQ_{II} . All three mechanisms involve ubisemiquinone radicals. Indeed, we detected the NADH–ubiquinone-dependent ubisemiquinone radicals in the Ndi1-catalysed electron transfer reaction using electron paramagnetic resonance (EPR) (Fig. 3c). Moreover, the power saturation experiments suggested the existence of two different populations of ubisemiquinone radicals (Fig. 3d). Future studies, such as EPR experiments at different pHs and redox titrations, are needed to establish the detailed electron transfer pathway of Ndi1.

Our study demonstrates that the electron transfer activity of the single-component Ndi1 is enabled by its unique CTD. Our data also support a model in which Ndi1 catalyses the electron transfer from NADH to UQ_{II} through a mechanism mediated by the FAD– UQ_I intermediate. This information could be valuable in guiding the engineering of Ndi1 for therapy and the design of antiparasitic inhibitors of NDH-2s.

METHODS SUMMARY

The wild-type and mutant forms of Ndi1 were expressed and purified from *E. coli* strain C43. Crystals were grown at 18 °C by the hanging-drop vapour diffusion method. Diffraction data sets were collected at beamline BL17U of the Shanghai Synchrotron Radiation Facility. The structures were solved by single-wavelength anomalous diffraction and molecular replacement methods. Data collection and structure refinement statistics are summarized in Supplementary Table 2. The

EPR data were collected at the High Magnetic Field Laboratory of Chinese Academy of Sciences.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.

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Author Contributions M.Y. designed and directed the project. Y.F., W.L., J. W., J.G., D.X. and J.-W.W. purified the proteins, grew the crystals, collected data, solved the crystal structures and performed the *in vitro* activity analyses. Y.L. and Q.Z. performed the genome analysis of the NDHs. B.Z. and J.L. performed the *in vivo* biological analyses. Y.F., K.W. and C.T. performed the EPR analyses. M.Y. analysed the data and wrote the paper with the help of all the authors.

Author Information The atomic coordinates and structure factors of the apo-, NADH-, ubiquinone- and NADH-ubiquinone-bound forms of Ndi1 have been deposited in the Protein Data Bank under accession codes 4G6G, 4G6H, 4G74 and 4G73, respectively. Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to M.Y. (maojunyang@tsinghua.edu.cn).

METHODS

Genome-wide analysis of phylogenetic relationships. NDH sequences from representative species of Prokaryota, Protista, Plantae, Fungi and Metazoa were used to investigate the evolutionary history of this gene family. Because some of the selected species were available only with draft genomes, two methods were used to obtain the sequences. The genes from completely sequenced genomes were obtained from KEGG (the Kyoto Encyclopedia of Genes and Genomes) ORTHOLOGY Database (KEGG ORTHOLOGY Entry K03885). (The locus tags of NDHs are listed in Supplementary Table 1.) The genes from draft genomes (*Saccharomyces mikatae* and *Saccharomyces bayanus*; see Supplementary Data 2) were identified by TBLASTN searches using three full-length NDH protein sequences of *S. cerevisiae*. The collected sequences were aligned using the MUSCLE (multiple sequence comparison by log-expectation) program³¹. The alignment was further adjusted manually using BIOEDIT³². Phylogenetic relationships among the NDHs were reconstructed using a maximum-likelihood procedure in PHYML³³ with the Jones–Taylor–Thornton amino-acid substitution model and 100 bootstrap replicates. (The tree and alignment are available as Supplementary Fig. 1 and Supplementary Data 1 and 2.) Furthermore, we obtained the subunit numbers of NADH:ubiquinone oxidoreductase (complex I) of each species from the KEGG PATHWAY Database (pathway entry, ko00190).

Protein expression and purification. The complementary DNA of Ndi1 from *S. cerevisiae* was cloned into pQE-80L vector. The Ndi1 mutants were generated by two-step PCR and were subcloned, overexpressed and purified in the same way as wild-type protein. Selenomethionine (Se-Met)-labelled protein was purified similarly. Overexpression of Ndi1 was induced in *E. coli* strain C43 using 0.5 mM isopropyl- β -D-thiogalactopyranoside when the cell density reached $D_{600\text{ nm}} = 1.0$. After growth for 10–12 h at 35 °C and 80 r.p.m., the cells were collected, homogenized in a buffer containing 50 mM Tris (pH 8.0) and 1 mM EDTA, and disrupted by sonication. Cell debris was removed by low-speed centrifugation for 15 min. The supernatant was collected and applied to ultracentrifugation at 150,000g for 1 h. The membrane fraction was collected and suspended in 50 mM Tris (pH 7.6), 200 mM NaCl, 0.1 mM EDTA and 10% glycerol (buffer A). After the addition of Triton X-100 at a final concentration of 0.3% (w/v) and 1 mM PMSF, the sample was incubated for 2 h with slow stirring at 4 °C. Following another ultracentrifugation step at 150,000g for 30 min, the supernatant was collected and loaded onto Ni²⁺-nitrilotriacetate affinity resin (Ni-NTA, Qiagen) pre-equilibrated with buffer A containing 0.1% Triton X-100. Subsequently, the resin was washed three times, each time with 5 ml of buffer A containing 0.1% Triton X-100 and 8 mM histidine. Then the protein was eluted with buffer A containing 0.02% Triton X-100 and 200 mM histidine. The purified Ndi1 protein was concentrated and subjected to gel filtration chromatography (Superdex-200 10/30, GE Healthcare) in a buffer containing 25 mM Tris (pH 7.6), 300 mM NaCl, 5 mM DTT, 0.1 mM EDTA and 0.02% Triton X-100. The peak fractions were collected for biochemical analyses or crystallization.

Crystallization, data collection and structure determination. Crystals were grown at 18 °C by the hanging-drop method by mixing 1 μ l of protein (10 mg ml⁻¹) with 1 μ l of reservoir solution (3.8 M potassium formate, 2% w/v polyethylene glycol monomethyl ether 2,000 (pH 4.2 or 10.2)). The crystals appeared overnight and grew to full size in about four to five days. To obtain the NADH-bound Ndi1 crystals, we soaked the crystals in cryo-protectant with 10 mM NADH for about 1–3 min. To obtain the ubiquinone-bound Ndi1 crystals, we made fresh 50 mM ubiquinone-4 (UQ₄) stock solution in DMSO. Ndi1 and ubiquinone were co-crystallized with a final concentration of 0.5 mM UQ₄. To get the NADH-ubiquinone-bound Ndi1 crystals, we soaked the ubiquinone-bound Ndi1 crystals in cryo-protectant with 10 mM NADH for about 1–3 min. The crystals were then transferred to liquid nitrogen.

All the data were collected at Shanghai Synchrotron Radiation Facility beamline BL17U and were integrated and scaled using the HKL2000 package³⁴. Further processing was carried out using programs from the CCP4 suite³⁵. Data collection statistics are summarized in Supplementary Table 2. The self-rotation function with POLARREN revealed a prominent peak at the section of $k = 180^\circ$, indicating the presence of a non-crystallographic two-fold symmetry axis. SHELXD³⁶ was used to locate the positions of selenium sites, and HA_NCS³⁷ independently identified a two-fold non-crystallographic symmetry axis from the anomalous sites. The non-crystallographic symmetry from selenium sites has the same orientation as that from the self-rotation function, which verified the validity of the anomalous scatter positions. The identified anomalous scattering sites were input to PHASER³⁸ for single-wavelength anomalous diffraction phasing. The real-space constraints were applied to the electron density map in DM³⁹. The final electron density map was of sufficient quality for BUCCANEER⁴⁰ to be able to build almost the complete model. The final model rebuilding was performed using COOT⁴¹ and the protein structure was refined with PHENIX⁴² against the high-resolution native data using non-crystallographic symmetry and stereochemistry information as

restraints. Other structures were solved using molecular replacement. Data collection and structure refinement statistics are summarized in Supplementary Table 2.

Yeast strains and plasmids. The strains used in this study were derived from haploid strain BY4742 (*MAT α his3 Δ 1 leu2 Δ 0 lys2 Δ 0 ura3 Δ 0*). *ndi1 Δ* was generated by disruption of the *NDI1* gene through homologous recombination with KanMX4 as the replacement marker. Screen of positive colonies was achieved by spreading cells on YPD (yeast extract/peptone/dextrose) medium plus 0.4 mg ml⁻¹ G418. Growth defects on YPG (yeast extract/peptone/glycerol) medium were used to confirm the disruption. *ndi1 Δ trp1 Δ* was generated by replacing the *TRP1* gene of *ndi1 Δ* with *LEU2*. After selection on the SD-Leu medium, the colonies were further streaked on SD-Trp plates to confirm the deletion.

The vector pADH1–YES2 was modified from the pYES2 vector from Invitrogen, in which the *GAL1* promoter was replaced with an *ADH1* promoter for the purpose of constitutive expression of the target gene. pYES2–Ndi1 and pADH1–YES2–Ndi1 were generated by insertion of the complementary DNA of Ndi1 between the restriction sites EcoRI and XhoI of the vectors pYES2 and pADH1–YES2, respectively. The Ndi1 mutants were generated by two-step PCR and were subcloned into the two vectors using the same approach. To obtain pYES2–Myc–Ndi1 and pYES3–FLAG–Ndi1 constructs, DNA sequences corresponding to Myc (EQKLISEEDL) and FLAG (DYKDDDDK) tags were inserted into the complementary DNA sequence of Ndi1 of pYES2–Ndi1 and pYES3–Ndi1 constructs, respectively, just after its mitochondrial targeting sequence.

Yeast growth assay. For growth on agar plates, yeasts expressing wild-type and mutated *NDI1* genes in pYES2 or pADH1–YES2 vector were inoculated into liquid SD–Ura medium for overnight culture. After normalization of $D_{660\text{ nm}}$ to 0.4, the cells were spotted in tenfold serial dilutions on SG–Ura and SD–Ura plates. The plates were incubated at 30 °C for three days before the pictures were taken. SD–Ura medium contains 2% (w/v) glucose, 0.5% (w/v) ammonium sulphate, 0.17% yeast nitrogen base with essential amino acids and other required nutrients except uracil. SG–Ura medium contains all the same components as SD–Ura except that the 2% (w/v) glucose is replaced with 2% (v/v) glycerol. For growth on agar plates, 2% agar was added.

Enzyme assays for Ndi1. The enzymatic activity of wild-type and mutant Ndi1 proteins was measured spectrophotometrically using NADH and UQ₁ as substrates. The standard assay was carried out at 30 °C in a 1.8-ml reaction mixture containing 50 mM MOPS (pH 7.0), 1 mM EDTA and varying amounts of NADH, UQ₁ and enzymes. Reactions were initiated by the addition of the enzyme to the reaction mixture. The progress of the reaction was monitored continuously by following the formation of NAD⁺ at 340 nm, using a Lambda 45 spectrophotometer (PerkinElmer Life Sciences) equipped with a magnetic stirrer in the cuvette holder. The activities were determined using an extinction coefficient for NADH of 6,220 cm⁻¹ M⁻¹ at 340 nm.

Co-immunoprecipitation experiments. Co-immunoprecipitation was performed as follows. For wild-type Ndi1, *ndi1 Δ trp1 Δ* cells were transformed with vectors pYES2–Myc–Ndi1 and pYES3–FLAG–Ndi1. After selection, the cells were grown in 50 ml of SGE–Ura medium to exponential phase before the cells were lysed by French press with extraction buffer (PBS, 0.1% Triton X-100 and protease inhibitor cocktail (Sigma)). The lysates were centrifuged at 15,000g for 30 min at 4 °C, and the supernatants were then incubated overnight with anti-FLAG or anti-Myc antibodies (Sigma) conjugated to protein A/G agarose. The agarose beads were washed three times with extraction buffer, boiled in SDS–polyacrylamide gel electrophoresis (PAGE) loading buffer, subjected to the SDS–PAGE and analysed by western blotting. Co-immunoprecipitation experiments for the truncated forms of Ndi1 were performed similarly.

Membrane fractionation. For membrane fractionation assay, *ndi1 Δ* cells transformed with pYES2–Ndi1 vectors were cultured overnight in SD–Ura medium and then transferred to SGE–Ura. After growing to the exponential phase, cells were collected by centrifugation. The cells were broken in lysis buffer (PBS buffer with protease inhibitors), and after centrifugation at 4,300g for 15 min the supernatants were obtained as the sample of total proteins. The solution of total proteins was then further ultracentrifuged at 250,000g for 1 h, and the resulting supernatant and pellet taken as samples of supernatant and membrane fractions, respectively. The total protein, supernatant and membrane fractions were subjected to western blotting.

EPR characterization of ubisemiquinone radical. Four different samples prepared from Ndi1 proteins extracted using Triton X-100 (Ndi1–TRT) or DDM (Ndi1–DDM) were tested: NADH–UQ₁–Ndi1–DDM, NADH–Ndi1–DDM, NADH–Ndi1–TRT and UQ₁–Ndi1–DDM. All EPR samples were prepared in anaerobic conditions after purification of the Ndi1 protein. The Ndi1 protein was added to a final concentration of 50 μ M and incubated with or without 5 mM UQ₁. After the addition of 2 mM NADH and incubation for 1 min, the sample was transferred to an EPR tube before a quick freezing to liquid-helium

temperature (around 2 K). EPR measurements were conducted on a Bruker X-band (9.4 GHz) EMSplus10/12 spectrometer. A cylindrical resonator (ER 4119hs TE011) was used for data collection. During EPR data acquisition, the sample temperature was maintained by an Oxford Instrument ESR 910 liquid-helium continuous-flow cryostat. A high-sensitivity EPR spectrum was obtained by temperature optimization from 2 to 200 K, and most of the EPR spectra were collected at an optimized temperature, 40 K. The EPR data acquisition parameters were as follows: modulation amplitude, 5.0 G; microwave power, 50 μ W; sweep time, 40 s; time constant, 81.92 ms; sample temperature, 40 K.

A power saturation experiment was implemented with the microwave power ranging from 44 to 14 dB (or 8 μ W to 8 mW) with a step size of 2 dB. The power saturation data was fitted following the equation $D = \sum_{i=1}^n C_i \sqrt{P(1 + P/P_{1/2,i})^{-0.5b_i}}$, in which C_i is the relative concentration of the i th free radical, $P_{1/2,i}$ is the half-saturation parameter and b_i is the homogeneity parameter. All the EPR data were collected at the High Magnetic Field Laboratory of Chinese Academy of Sciences.

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