frame to generate the plasmids p(*rec8–GFP*) and p(*rad21–GFP*).

Monitoring segregation of centromeres tagged with GFP during meiosis. *rec8*Δ cells tagged with GFP at the *lys1* locus were crossed with non-tagged *rec8*$^+$ cells or *rec8*Δ cells. For this purpose, both types of cell were cultured to log phase, collected by centrifugation, mixed and then spotted on a sporulation plate SPA. This allows subsequent synchronous conjugation and meiosis. When cells undergoing meiosis I and II became abundant in the population, cells were fixed with cold methanol and zygotes monitored for GFP and DAPI.

Spore analysis. *h*$^+$/*h*$^-$ *cdc2-L7 rec8*Δ diploid cells carrying heterozygous centromere-linked markers (*lys1*, *pat1* and *ade6*) and isogenic *rec8*$^+$ cells were sporulated on a YPD plate. At 33 °C they preferentially formed two spores that had undergone only meiosis I; at 25 °C they further underwent meiosis II and formed four spores. Dyads or tetrads were dissected and incubated on YE plates at 25 °C for 7 days. Fully viable dyad pairs were further tested for auxotrophy by replica plating on minimal plates at 25 °C, and for the *pat1* phenotype by incubating the replicated plate at 33 °C.

Synchronous meiosis. To induce synchronous meiosis, we used a temperature sensitive *pat1-114* allele[12]. For FISH experiments, a diploid strain *h*$^+$/*h*$^+$ *cdc2-L7/cdc2-L7 pat1-114/pat1-114 rec8-GFP/rec8-GFP lys1-131/lys1*$^+$ *ade6-M210/ade6-M216* was cultured in EMM-N at 25 °C for 16 h, and G1 cells were collected by elutriation. We placed these cells were at 34 °C and added 0.5 g l^{-1} of NH_4Cl; after 2 h cultures were shifted to 30 °C.

Detection of Rec8 phosphorylation. Synchronous meiosis was induced in diploid *pat1 ced2 rec8–HA* cells. Cell extracts were prepared every 1 h and Rec8–HA was detected by western blotting using anti-HA antibodies (12CA5). Extracts from the 4-h sample were incubated with and without calf intestine alkaline phosphatase (Sigma), or with phosphatase plus a phosphatase inhibitor mix (60 mM β-glycerophosphate, 15 mM nitrophosphate, 1 mM NaV_3), and were analysed by western blotting.

GFP fluorescence and FISH. For GFP fluorescence, cells were fixed by cold methanol, stored at −20 °C, washed and suspended in PEMS buffer (100 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$, 1.2 M sorbitol) with DAPI. The method for FISH in fission yeast was performed as previously described[25]. To digest the meiotic cell wall, we used ten times the concentration of the enzymes (zymolyase at 10 mg ml^{-1} and novozyme at 2 mg ml^{-1}) and incubated at 36 °C for 30 min. We used a digoxigenin-labelled centromere repeat probe[13] and purified anti-GFP antibodies (a gift from K. Sawin).

Chromosome spreads. Spread nuclei were prepared as described[26]. For immunostaining, previous methods were followed[27], using anti-GFP antibodies diluted 1:50 in PBS for the first antibody, and Cy3-conjugated goat anti-rabbit IgG diluted 1:200 for the second antibody.

Assay of reductional segregation in mitotic cells expressing Rec8–GFP. Diploid *h*$^+$/*h*$^-$ *rad21::ura4*$^+$/*rad21::ura4*$^+$ *ura4-D6/ura4-D6 leu/leu1 ade6-M210/ade6-M216* cells carrying p(*rad21–GFP*) or p(*rec8–GFP*) cultured without adenine were moved to medium with adenine. After culturing for six generations, they were spread on adenine-limiting plates and examined for adenine auxotrophy. Cells that had undergone reductional segregation of the *ade6* allele could be detected because they formed pink colonies. By microscopic inspection of each pink colony, we excluded haploid or aneuploid colonies caused by chromosome loss or missegregation.

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Creation of human tumour cells with defined genetic elements

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During malignant transformation, cancer cells acquire genetic mutations that override the normal mechanisms controlling cellular proliferation. Primary rodent cells are efficiently converted into tumorigenic cells by the coexpression of cooperating oncogenes[1,2]. However, similar experiments with human cells have consistently failed to yield tumorigenic transformants[3–5], indicating a fundamental difference in the biology of human and rodent cells. The few reported successes in the creation of human tumour cells have depended on the use of chemical or physical agents to achieve immortalization[6], the selection of rare, spontaneously arising immortalized cells[7–10], or the use of an entire viral genome[11]. We show here that the ectopic expression of the

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telomerase catalytic subunit (*hTERT*)[12] in combination with two oncogenes (the simian virus 40 large-T oncoprotein and an oncogenic allele of H-*ras*) results in direct tumorigenic conversion of normal human epithelial and fibroblast cells. These results demonstrate that disruption of the intracellular pathways regulated by large-T, oncogenic *ras* and telomerase suffices to create a human tumor cell.

One ostensibly important difference between rodent and human cells derives from their telomere biology. Murine somatic cells express telomerase activity and have much longer telomeres[13] than their normal human counterparts, which lack telomerase activity[14,15]. Because normal human cells progressively lose telomeric DNA with passage in culture, telomeric erosion is thought to limit cellular lifespan[16]. Ectopic expression of the *hTERT* gene, which encodes the catalytic subunit of the telomerase holoenzyme, enables some[17] but not all (ref. 18, W. C. Hahn and R. A. Weinberg, unpublished observations) pre-senescent primary human cells to multiply indefinitely. Moreover, ectopic *hTERT* enzyme expression enables telomerase-negative, transformed cells to bypass crisis, a proliferative barrier after senescence characterized by widespread cell death[19]. Together, these studies indicate that telomerase can confer replicative immortality on certain human cell types. Because almost all human tumours have detectable telomerase activity[15], we reasoned that telomerase might contribute to the tumorigenic conversion of human cells.

To determine whether human cells immortalized by the ectopic expression of *hTERT* were tumorigenic, we serially introduced combinations of *hTERT* and an oncogenic *ras* (H-*ras*V12) allele[20] by using amphotropic retroviruses into human embryonic kidney (HEK) cells that express large-T to bypass senescence (HA1 cells)[21] (Fig. 1). We also introduced large-T and combinations of *hTERT* and oncogenic *ras* into early-passage (passage 5) normal human BJ fibroblasts (Fig. 1). For each infection, parallel cultures were infected with control retroviruses specifying only a drug resistance gene as controls. Polyclonal, mass-infected populations as well as clonal isolates were obtained for each combination of serially introduced genes. We estimate that approximately 60 population doublings were expended during the process of serially introducing large-T, *hTERT* and *ras*. We could not propagate cells expressing only *hTERT* and *ras*, as these cells entered a senescent state immediately after the introduction of *ras*[20], confirming that *hTERT* does not abrogate a *ras*-induced growth arrest (data not shown)[22]. Amounts of large-T expressed were similar in all cell populations (Fig. 1a) and cell clones (data not shown), and increased expression of the Ras protein was observed in the expected cell populations (Fig. 1b) and clones (data not shown) after infection with a vector transducing the *ras* oncogene. We also confirmed that the ectopic expression of *hTERT* (Fig. 1c) resulted in telomerase activity in both HEK and BJ cells (Fig. 1d). This telomerase activity resulted in both telomere elongation (Fig. 2a) and stabilization (Fig. 2b) in these cells, as assessed by Southern blotting of genomic DNA. Expression of the *ras* oncogene did not affect the ability of telomerase to maintain telomeres in these cells (Fig. 2a, b).

Expression of *hTERT* led to the immortalization of BJ fibroblasts expressing large-T, recapitulating the results seen previously with HA1 and pre-senescent BJ cells (Fig. 3)[17,21]. In contrast, telomerase-negative polyclonal (Fig. 3a, b) and monoclonal (data not shown) cell populations expressing only large-T and *ras* entered crisis within

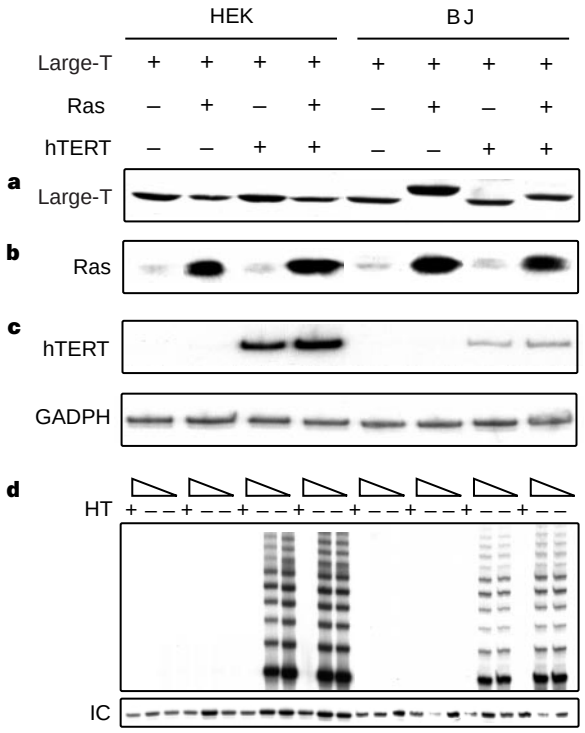

Figure 1 Expression of large-T, *ras* and telomerase. Immunoblotting for the large-T of relative molecular mass 80,000 (M_r 80K) (**a**) and M_r 21K Ras (**b**) proteins confirmed equal expression of large-T and overexpression of Ras in the indicated cells. The anti-Ras antibody (259) used in this study recognizes a determinant common to both wild-type and mutated Ras. Large-T was not detected in uninfected HEK and BJ cells (data not shown). The slower migration of large-T in the presence of *ras* expression was seen only in BJ cells and was observed with two different anti-large-T monoclonal antibodies. **c**, Expression of *hTERT* transcripts was confirmed by RT-PCR. The *hTERT*-specific primers amplified a 175 bp product. Primers specific for glyceraldehyde-3-phosphate dehydrogenase (GADPH) (220 bp product) confirmed that equal amounts of RNA were present in each sample. **d**, Cellular extracts (200 and 20 ng) were tested for telomerase activity by using the PCR-based TRAP assay; heat-treated samples (HT) were included as controls. IC refers to an internal PCR standard to demonstrate the absence of PCR inhibitors in the cellular extracts.

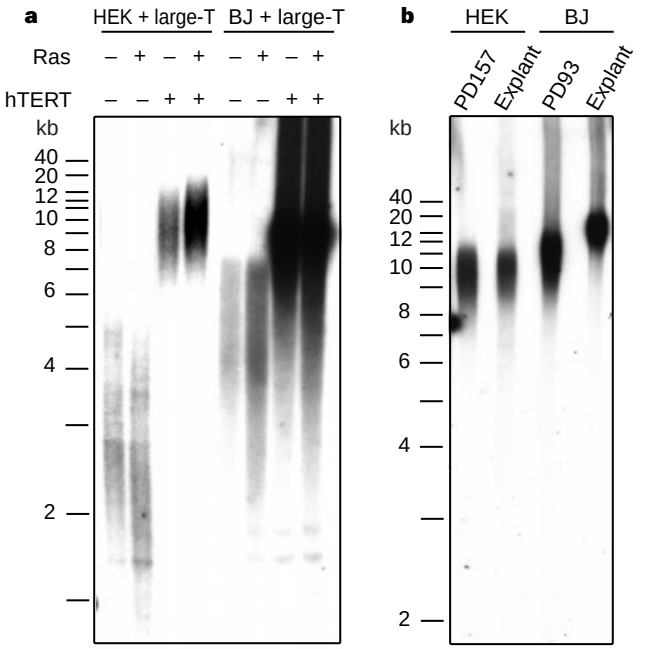

Figure 2 Expression of *hTERT* stabilizes telomere length. **a**, Telomere length for HEK and BJ cells was analysed by hybridization of genomic DNA with a telomere-specific oligonucleotide probe after 6 population doublings. The positions of size standards (kb) are indicated at the left. **b**, Telomere lengths for HEK and BJ cells expressing large-T, *hTERT* and *ras* at late passage (HEK, population doubling 157; BJ population doubling 93) and after recovery (explant) from a tumour formed in a nude mouse.

Table 1 Anchorage-independent growth of large-T-expressing HEK and BJ cells

Experiment 1*

	Number of colonies after 21 d								
	HEK + LT				BJ + LT				Calu-1
No. of cells seeded	hTERT⁻ Ras⁻	hTERT⁻ Ras⁺	hTERT⁺ Ras⁻	hTERT⁺ Ras⁺	hTERT⁻ Ras⁻	hTERT⁻ Ras⁺	hTERT⁺ Ras⁻	hTERT⁺ Ras⁺	(control)
10^4	10	40	4	160	0	3	0	145	200
10^3	0	12	0	80	0	0	0	40	75

Experiment 2†

	Number of colonies after 21 d				
	HEK; LT + hTERT + Ras		BJ; LT + hTERT + Ras		293
No. of cells seeded	Parental	Explanted	Parental	Explanted	(control)
10^5	3,460	2,850	4,046	4,858	2,220
10^4	785	434	524	492	286
10^3	106	34	50	64	29

The lung cancer cell line Calu-1 and the adenovirus-transformed cell line 293 were used as positive controls.
* Anchorage-independent growth of HEK and BJ cells with combinations of large-T (LT), *hTERT*, and *ras*. Six clonal isolates from HEK or BJ cells expressing only LT and *hTERT* never formed colonies, whereas clonal isolates from HEK or BJ cells expressing LT, *hTERT* and *ras* produced variable numbers of colonies (HEK 0–40 and BJ 7–247; 10^4 cells seeded). The number of colonies formed by such cells correlated with the expression of *ras* in each clonal isolate (data not shown).
† Anchorage-independent growth of HEK and BJ cell populations before (parental) and after (explanted) passage in a nude mouse.

10 population doublings, and no immortal clones arose spontaneously from these cultures after two months of culture, confirming earlier observations[4]. Oncogenic *ras* led to clear morphological transformation (data not shown) but had only a minor effect on the growth rate in monolayers of BJ fibroblasts expressing both large-T and *hTERT* (Fig. 3b).

We then ascertained the ability of these various cell types to grow in an anchorage-independent fashion, one of the hallmarks of the tumorigenic state. We observed efficient colony formation in soft agar only with cells expressing the combination of large-T, *ras* and *hTERT* (Table 1). Although occasional colonies were seen with cells expressing only large-T and *ras*, these were both significantly less numerous as well as markedly smaller in size. Furthermore, when cells expressing only large-T, large-T and *ras*, or large-T and *hTERT* were introduced into immunodeficient nude mice, no tumours were observed even after three months of observation, although the telomerase-expressing human breast cancer cell lines BT549 or SW613, used as controls, readily formed tumours in this assay (Table 2).

In marked contrast, when cells expressing large-T, *ras* and *hTERT* were introduced into nude mice, rapidly growing tumours were repeatedly observed with high efficiency (Table 2, Fig. 4c). Histologically, these converted HEK cells formed malignant tumour nodules composed of cells with cytoplasmic vacuoles, whereas the BJ fibroblasts were transformed into large, malignant, undifferentiated, spindle to epithelioid-shaped cells (data not shown). Thus, the ectopic expression of these three genetic elements seemed to be sufficient to confer tumorigenic potential on both HEK cells and human fibroblasts.

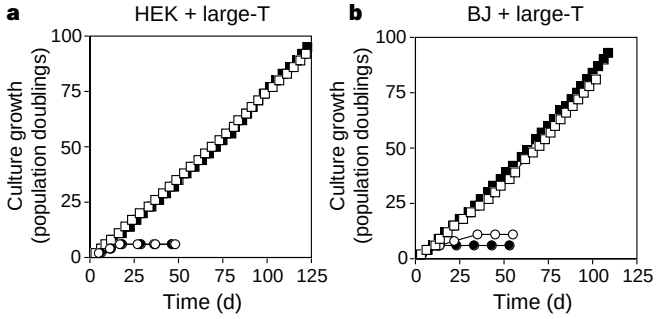

Figure 3 Expression of *hTERT* immortalizes large-T-expressing HEK and BJ cells. Growth of HEK (**a**) and BJ (**b**) cell populations is shown. Cells lacking *hTERT* (circles) entered crisis and could no longer be passaged. Filled symbols represent cells that also expressed oncogenic *ras*. Squares indicate cells expressing *hTERT*.

But it remained possible that additional genetic alterations were required beyond these three changes for these cells to become tumorigenic. Several observations made such a scenario unlikely. We observed no lag in the outgrowth of tumours after injection of these cells into mice (Fig. 4c). Analysis of retroviral integration sites by Southern blotting revealed that the cell populations expressing large-T, *hTERT* and *ras* were polyclonal both before injection into nude mice and after recovery from them (Fig. 4b). Explanted tumour cells were morphologically indistinguishable (data not shown), had similar telomere lengths (Fig. 2b), grew *in vitro* at the same rate (Fig. 4a) and formed similar numbers of anchorage-independent colonies to the parental cells (Table 1, Experiment 2). Furthermore, when these explanted tumour cells were reinoculated into nude mice, they formed tumours with similar kinetics to those seen after injection of the parental cells (Fig. 4c). Taken together, these findings suggest that the tumorigenic growth exhibited by these cells is not the consequence of additional, rare stochastic events occurring *in vivo* after inoculation of these cells.

We conclude that the ectopic expression of a defined set of genes, specifically large-T, *ras* and *hTERT*, suffices to convert normal human cells into tumorigenic cells. These results begin to define the biochemical pathways that must be disrupted to create tumorigenic human cells from normal mesenchymal or epithelial precursors. This number is likely to involve changes in at least four distinct signalling pathways, minimally one more than is needed to transform rodent cells. In addition to changes required in the mitogen-response pathway activated by *ras* and in the telomere maintenance pathway represented by *hTERT*, large-T perturbs at least two distinct cellular control pathways through its ability to bind and

Table 2 Formation of tumours in immunodeficient nude mice

Cells	Genotype	Number of tumors/number of injections
HEK + LT	hTERT⁻, Ras⁻	0/6
	hTERT⁻, Ras⁺	0/6
	hTERT⁺, Ras⁻	0/8
	hTERT⁺, Ras⁺	8/8
BJ + LT	hTERT⁻, Ras⁻	0/4
	hTERT⁻, Ras⁺	0/4
	hTERT⁺, Ras⁻	0/4
	hTERT⁺, Ras⁺	4/4
BT549		8/8
SW613		8/8

Results are shown for polyclonal populations. Analysis of two individual clones derived from each population gave identical results; 2×10^6 cells were injected in each experiment. LT, large-T.

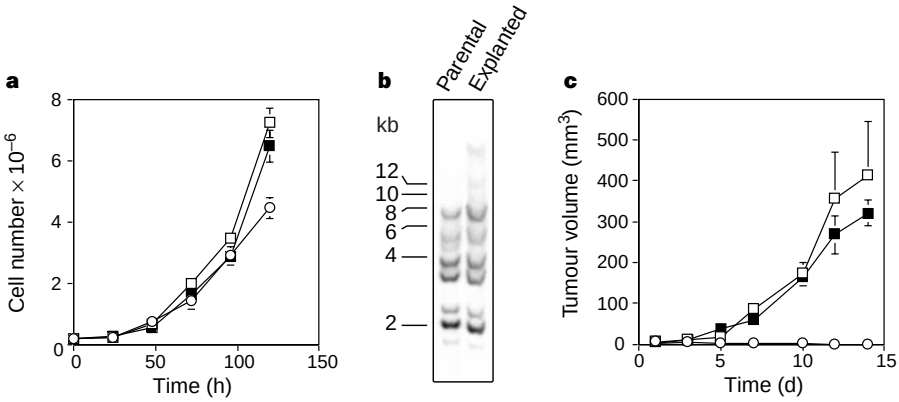

Figure 4 Growth properties and clonality of tumorigenic cells. **a**, Explanted or parental HEK cells expressing large-T, *ras* and *hTERT* grew at equivalent rates in culture. HEK cells expressing large-T and *hTERT* but lacking (open circles) or expressing (open squares) *ras* were compared with explanted tumour cells (filled squares). **b**, Comparison of retroviral integration sites in parental or explanted HEK cells expressing large-T, *ras* and *hTERT* demonstrates that such cells are polyclonal. An *Eco*RV–*Sal*I fragment of the pBABE-hygro-hTERT retrovirus[21] was used as a probe. The positions of size standards (kb) are indicated at the left. **c**, Explanted tumour cells grew at the same rate as parental HEK cells expressing large-T, *ras* and *hTERT* on reinoculation into nude mice. Symbols are the same as in **a**. Results are means ± s.d. for six experiments.

functionally inactivate the cellular pRB and p53 tumour-suppressor proteins. Others have recently reported that the introduction of *hTERT*, *ras* and the human papillomavirus E6 and E7 proteins (which, like large-T, inactivate p53 and pRB) together did not succeed in allowing normal human fibroblasts to grow in an anchorage-independent fashion[22]. Because large-T is also known to perturb other cellular targets[23,24], the possibility remains that one or more of these other pathways must also be disrupted for tumorigenic conversion to occur. Nevertheless, our findings indicate that only in the context of *hTERT* expression do alterations in these other cellular pathways lead to tumorigenesis in human cells.

It is now highly likely that telomere maintenance, achieved either by *hTERT* expression or by an alternative mechanism[25], contributes directly to oncogenesis by allowing pre-cancerous cells to proliferate beyond the number of replicative doublings allotted to their normal precursors. Although the expression of telomerase does not by itself lead to a tumorigenic phenotype (Table 2)[22,26], telomere maintenance facilitated by *hTERT* expression *in vivo* might cooperate with additional oncogenic mutations to create a malignantly transformed clone. We suspect that the small, abortive soft agar colonies seen here in the absence of ectopic *hTERT* expression are one manifestation of replicative mortality that occurs in the absence of telomere maintenance. The present observations support the notion that telomere maintenance is essential for the formation of human tumour cells. We suggest that identical rules will be found to apply to autochthonously arising human tumour cells. □

Methods

Generation of cell lines. To create amphotropic retroviruses, Phoenix cells were transfected with pBABE-hygro-hTERT[21] and pBABE-puro-*ras*-V12 (a gift from S. Lowe) by calcium-phosphate precipitation. These ecotropic retroviral supernatants and supernatants collected from the large-T retroviral producer line ψ_2: simian virus 40 (ref. 27) were used to infect the amphotropic packaging cell line PT67 (Clontech Laboratories). The resulting supernatants were used to infect HEK (HA1)[21] cells or foreskin fibroblasts (BJ). Retroviral constructs were introduced serially; drug selection was used to purify cell populations between infections. Cells were selected in hygromycin (100 μg ml^{-1}, 7 d), puromycin (0.5 μg ml^{-1}, 2–3 days) or neomycin (400 μg ml^{-1}, 7 days), respectively. Retroviral vectors carrying only drug resistance genes were used as controls. In all cases, the point at which a culture reached confluence in a 10-cm culture dish after the last viral infection was designated population doubling 0; this point represents at least 70 population doublings from the original primary culture. Cells were considered to have entered crisis when they could no longer be passaged and exhibited widespread cell death.

Telomerase assays, polymerase chain reaction with reverse transcription (RT-PCR), telomere analysis and immunoblotting. Cellular extracts were assayed for telomerase activity with a PCR-based telomeric repeat amplification protocol (TRAP) assay[28]. For RT-PCR, total cellular RNA was prepared from cells by using RNazol (Tel Test B). In each reaction, 100 ng of total RNA was used with primers specific for retrovirally encoded *hTERT* (5′-GACACACATTCCACAGGTCG-3′ and 5′-GACTCGACACCGTGTCACCTAC-3′) or primers specific for human GADPH (5′-GAGAGACCCTCACTGCTG-3′ and 5′-GATGGTACATGACAAGGTGC-3′) with the RTth kit (Perkin Elmer). The RT reaction was performed for 10 min at 70 °C followed by 30 cycles of PCR (94 °C, 45 s; 60 °C, 45 s; 72 °C, 90 s). Telomere length was measured by hybridizing a ^{32}P-labelled telomeric $(CCCTAA)_3$ probe to *Hin*fI- and *Ras*I-digested genomic DNA. Immunoblotting of total cellular extracts (75 μg) was performed with the rat anti-Ras antibody 259 and the mouse anti-LT antibody Pab 101 (Santa Cruz Biotechnology).

Analysis of retroviral integration sites. Genomic DNA (15 μg) from parental and explanted tumour cells was digested with *Eco*RI and *Bam*HI and hybridized to a ^{32}P-labelled *Eco*RV–*Sal*I fragment of the *hTERT* cDNA under conditions that did not permit the detection of genomic fragments of *hTERT*.

Soft agar assays. Soft agar assays were performed as described[29]. Cultures were coded and scored in a blinded fashion by a second observer.

Tumorigenicity assays. Immunodeficient mice (Balb/c–ByJ-Hfh11^{nu}; Jackson Laboratory) were maintained in pathogen-free conditions. Mice were γ-irradiated with 400 rad before injection to suppress natural killer cells[30]; tumours also formed in mice that had not been irradiated, but with a 1–2 week latency period. Cells (2×10^6) were injected subcutaneously into mice anaesthetized with Metofane; tumours were measured every 2–3 d. Mice were when tumours exceeded 1 cm. In experiments where cells were reisolated, tumours were removed surgically, minced, incubated in a dilute (0.15%) solution of collagenase for 2 h, washed, and placed in culture. Tumour volume was calculated with the formula $4/3\pi r^3$, where r is the radius of the tumour.

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Essential role for oncogenic Ras in tumour maintenance

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Advanced malignancy in tumours represents the phenotypic endpoint of successive genetic lesions that affect the function and regulation of oncogenes and tumour-suppressor genes[1]. The established tumour is maintained through complex and poorly understood host–tumour interactions that guide processes such as angiogenesis and immune sequestration. The many different genetic alterations that accompany tumour genesis raise questions as to whether experimental cancer-promoting mutations remain relevant during tumour maintenance. Here we show that melanoma genesis and maintenance are strictly dependent upon expression of H-Ras^{V12G} in a doxycycline-inducible H-Ras^{V12G} mouse melanoma model null for the tumour suppressor INK4a. Withdrawal of doxycycline and H-Ras^{V12G} down-regulation resulted in clinical and histological regression of primary and explanted tumours. The initial stages of regression involved marked apoptosis in the tumour cells and host-derived endothelial cells. Although the regulation of vascular endothelial growth factor (VEGF) was found to be Ras-dependent *in vitro*, the failure of persistent endogenous and enforced VEGF expression to sustain tumour viability indicates that the tumour-maintaining actions of activated Ras extend beyond the regulation of VEGF expression *in vivo*. Our results provide genetic evidence that H-Ras^{V12G} is important in both the genesis and maintenance of solid tumours.

a

Number of mice	Genotype Tyr–rtTA	Tet–Ras	INK4a	Doxycycline induction	Number of mice with melanoma	Latency (days ± s.d.)
40	+	+	−/−	Yes	10	60 ± 26
23	−	+	−/−	Yes	0	—
12	+	+	−/−	No	0	—

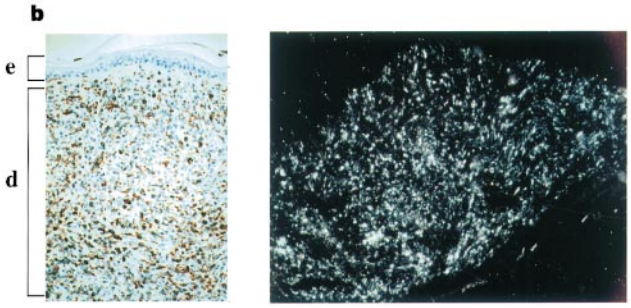

Figure 1 Inducible Tyr/Tet–Ras transgenic mice on INK4a-deficient background developed cutaneous melanomas. **a**, Summary of tumour incidence in the Tyr/Tet–Ras–INK4a-null colony and impact of doxycycline treatment. **b**, Left: anti-TRP1 staining of a primary cutaneous melanoma. Note strong immunoreactivity in dermal nodule with overlying intact epidermis; e, epidermis; d, dermis. Right: *in situ* hybridization for H-Ras^{V12G} transcript in a primary melanoma nodule.

To develop a cancer model in which dominantly acting oncoproteins are somatically regulated *in vivo*, transgenic mouse lines harbouring the reverse tetracycline transactivator[2] under the control of the tyrosinase gene promoter–enhancer elements (designated Tyr–rtTA) and another containing the H-Ras^{V12G} open reading frame driven by a minimal promoter containing multimerized tet-operons[2,3] (designated Tet–Ras) were inter-crossed with INK4a$^{+/-}$ mice to generate cohorts of single and double transgenic mice (designated Tyr/Tet–Ras) that were homozygous null for INK4a. Upon weaning, a subset of single and double transgenic INK4a$^{-/-}$ mice were given doxycycline in their drinking water[4]. In the doxycycline-treated group, 10 out of 40 Tyr/Tet–Ras INK4a$^{-/-}$ mice developed melanomas with an average latency of 60 days (Fig. 1a). In contrast, the untreated Tyr/Tet–Ras INK4a$^{-/-}$ mice ($n = 12$) or treated Tet–Ras INK4a$^{-/-}$ ($n = 23$) did not develop melanomas. The Tyr/Tet–Ras INK4a$^{-/-}$ melanomas shared all of the macroscopic features of the constitutive Tyr–Ras INK4a$^{-/-}$