p53 (refs 4, 8). This link between E2F-1 function and $p14^{ARF}$ expression provides an explanation for the ability of oncogenic stimuli that could deregulate E2F-1 activity, such as defects in the RB pathway or activation of oncogenes such as Ras, E1A or Myc, to activate p53 (refs 9–12). Thus, abnormal proliferation, resulting in deregulated E2F-1 activity, would induce $p14^{ARF}$ and stabilize p53 (Fig. 1d). This would lead to cell-cycle arrest or apoptosis unless a second lesion occurred, such as a mutation in $p14^{ARF}$ or p53 itself.

This model is supported by the observation that tumours containing $p14^{ARF}$ mutations can tolerate the retention of wild-type p53 (refs 4, 8), strongly suggesting that loss of this pathway linking deregulated E2F-1 to the activation of p53 is an essential step in malignant progression.

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$p19^{ARF}$ links the tumour suppressor p53 to Ras

Normal healthy cells possess safeguard mechanisms that sense oncogenic signals and trigger anti-tumorigenic responses that limit the proliferative potential of cells harbouring active oncogenes[1]. In particular, expression of the Ras oncogene in normal primary cells causes a cell-cycle arrest that involves the activation of the tumour-suppressor protein p53 (ref. 2). It has recently been reported that the tumour-suppressor $p19^{ARF}$ can activate p53 (refs 3–5). Here we show that $p19^{ARF}$ in the mouse (the human homologue is called $p14^{ARF}$) is essential for the activation of p53 in response to oncogenic Ras. These results, together with the finding that $p19^{ARF}$ does not mediate the activation of p53 by DNA damage[6], dissociate the activation of p53 into two pathways: one pathway is induced by DNA damage and is independent of $p19^{ARF}$, whereas the other pathway is induced by oncogenic Ras and is dependent on $p19^{ARF}$.

Under normal cellular conditions, p53 is maintained at relatively low levels because it has a short half-life. However, in the presence of DNA damage or oncogenic stresses, p53 accumulates rapidly through post-transcriptional mechanisms and gains full potential as a transcriptional activator[7]. The protein $p19^{ARF}$, one of the two tumour-suppressors encoded by the *INK4a-ARF* locus[8], activates p53 both by neutralizing MDM2, which destabilizes p53, and by interacting directly with p53 (refs 3–5). DNA damage does not increase the level of $p19^{ARF}$, and the activation of p53 in response to DNA damage does not require $p19^{ARF}$ (ref. 6). These observations led us to explore the possibility that $p19^{ARF}$ is involved in the activation of p53 in response to oncogenic stimuli.

We have analysed the effect of an oncogenic form of the Ras protein (H-RasG12V, hereafter abbreviated as RasV12) on the steady-state levels of $p19^{ARF}$ mRNA (Fig. 1a). The levels of $p19^{ARF}$ mRNA were increased significantly (between five- and tenfold) by RasV12 (Fig. 1a). To test the role of $p19^{ARF}$ on the accumulation of p53 triggered by RasV12, we measured endogenous p53 levels in primary mouse embryonic fibroblasts (MEFs) derived from wild-type and *ARF*-null embryos[6] (provided by C. Sherr). As previously described[2], we found that the levels of p53 were increased significantly by RasV12 in wild-type MEFs (Fig. 1b, compare lanes 1 and 2). In contrast, p53 levels in $ARF^{-/-}$ MEFs remained unchanged in the presence of RasV12 (Fig 1b, compare lanes 3 and 4). These results show that $p19^{ARF}$ is required for the accumulation of p53 in response to oncogenic Ras.

We have also determined the effect of RasV12 on the proliferation of wild-type and $ARF^{-/-}$ MEFs (Fig. 1c). Wild-type cells infected with a retrovirus expressing RasV12 had significantly reduced proliferative activity, and most adopted a flat and extended morphology with cytoplasmic vacuolization (not shown), all features of senescence-like arrest[2]. In contrast, whole cell populations of $ARF^{-/-}$ MEFs expressing RasV12 proliferated at rates higher than their vector-infected counterparts. In this respect, $ARF^{-/-}$ cells expressing oncogenic RasV12 behave similarly to $p53^{-/-}$ cells[2]. These results extend the previously reported observation[6] that monolayers of $ARF^{-/-}$ MEFs transfected with oncogenic Ras develop foci of tumorigenic cells.

The induction of the tumour-suppressor protein $p16^{INK4a}$ has also been implicated in the cell-cycle arrest elicited by oncogenic Ras[2]. However, the complex genomic organization of the *INK4a-ARF* locus[8] complicates the interpretation of some of the observations made with cells that lack p16 because of the elimination of exons 2 and 3, henceforth referred to as $(INK4a\text{-}ARF)^{\Delta 2,3}$ cells (ref. 9). These cells retain exon 1β, which encodes for the active domain of $p19^{ARF}$ (refs 4, 5, 10), and express an aberrant mRNA containing exon 1β (our unpublished observations). Therefore, it is possible that $(INK4a\text{-}ARF)^{\Delta 2,3}$ cells produce

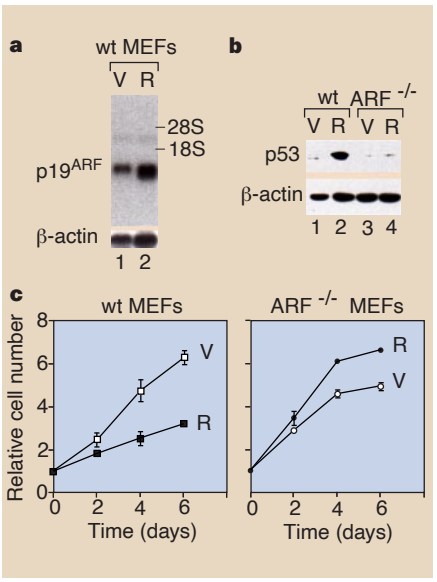

Figure 1 $p19^{ARF}$ is essential for the p53-dependent arrest provoked by Ras. **a,** $p19^{ARF}$ mRNA levels are increased in response to oncogenic Ras. Primary wild-type (wt) MEFs minimally passaged in culture were infected with replication-deficient retroviruses containing H-RasV12 (R) or the corresponding empty vector (V) (ref. 2). After selection of the infected cells, total RNA was extracted and probed with a labelled polymerase chain reaction fragment corresponding to exon 1β of the *INK4a-ARF* locus (top). To control for equal loading and transfer, blots were re-probed to detect actin (bottom). **b,** p53 accumulation requires $p19^{ARF}$. Primary MEFs of the indicated genotype were infected with empty vector (V) or H-RasV12 (R), as in **a**. Endogenous p53 levels were measured by immunoblotting with an anti-p53 antibody (top). To control for equal loading and transfer, blots were then re-probed to detect actin (bottom). **c,** $ARF^{-/-}$ cells do not undergo proliferation arrest in response to oncogenic Ras. Growth curves of primary MEFs of the indicated genotype infected with empty vector (V) or H-RasV12 (R), as in **a**. The complete experiment was repeated three times, with similar results.

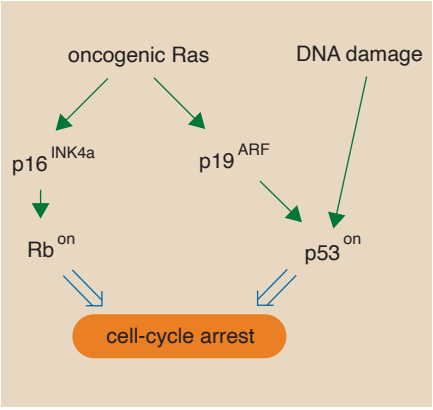

Figure 2 Anti-tumorigenic responses mediated by the products of the *INK4a-ARF* locus. Rb, retinoblastoma.

a peptide containing a functional $p19^{ARF}$ domain. It has previously been reported that $(INK4a\text{-}ARF)^{\Delta 2,3}$ cells do not arrest when oncogenic Ras is introduced but are still able to induce p53 (ref. 2). In the light of our present results, this suggests that $(INK4a\text{-}ARF)^{\Delta 2,3}$ cells retain a functional, or partly functional, *ARF* gene. Taken together, these data indicate that oncogenic Ras elicits an anti-tumorigenic response mediated by the upregulation of both $p19^{ARF}$ and $p16^{INK4a}$, which in turn activate the tumour suppressors p53 and retinoblastoma, respectively (Fig. 2).

Other oncogenes that are mechanistically unrelated to Ras have recently been reported to activate p53 in a $p19^{ARF}$-dependent manner, resulting in a pro-apoptotic state[11,12]. We speculate that $p19^{ARF}$ senses unscheduled entry into the S phase of the cell cycle. Homozygous loss of the *INK4a-ARF* locus is common in human tumours. The results reported here, together with those of previous reports[2,6,11,12], indicate that the loss of $p19^{ARF}$ and $p16^{INK4a}$ renders cells unprotected against the action of oncogenes.

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Impacts on Earth in the Late Triassic

Spray *et al.*[1] postulate that five widely dispersed terrestrial impact structures with very similar geological age estimates (about 214 million years ago, in the Late Triassic epoch) are evidence of a multiple impact event. Most notably, the three largest impact structures, Saint Martin in western Canada (~40 km diameter), Manicouagan in eastern Canada (~100 km diameter), and Rochechouart in France (~25 km diameter), plot at virtually the same palaeolatitude in a continental reconstruction. Spray *et al.* suggest that this apparent crater chain was produced within hours as a series of coaxial projectiles collided in rapid succession with the rotating planet Earth, and drew analogies to the recent collision sequence of fragmented comet Shoemaker–Levy 9 with Jupiter.

However, published palaeomagnetic data for the Manicouagan[2,3] and Rochechouart[4] impact structures argue strongly against such a closely timed origin for these ancient events. This is because the characteristic remanent magnetizations of the melt rocks, including the most rapidly cooled glassy phases, indicate formation in a Late Triassic palaeomagnetic dipolar field of normal polarity at Manicouagan but of reverse polarity at Rochechouart.

These impact events must therefore have been separated temporally by at least the few thousand years[5] it takes for a geomagnetic polarity reversal to take place, a process which in any case occurred relatively infrequently (at an average rate of about twice per million years[6,7]) in the Late Triassic. Thus, although there is an interesting concentration of impact events in the Late Triassic, the opposite geomagnetic polarities recorded by the Manicouagan and Rochechouart melt rocks appear to preclude a synchronous multiple impact origin.

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Spray replies — Kent raises an interesting point regarding the proposed late Triassic multiple-impact event[1]. It might appear contradictory that the Manicouagan (~100 km diameter) and Rochechouart (~25 km diameter) impact structures possess normal and reversed geomagnetic reversals, respectively, if they were formed within hours of each other, as we suggested[1]. However, palaeomagnetic fields are acquired when magnetic mineral phases pass through their Curie points (the temperatures at which iron minerals assume magnetic order and remain with their magnetic moments parallel to the Earth's magnetic field at that time). Critically, this does not necessarily coincide with the time of formation of the host rocks.

Rochechouart possesses a thin, sporadically developed impact melt layer (4 m thick at most). The palaeomagnetic data of Pohl and Soffel[2] were obtained from glass-bearing impact breccias and lithic breccias. The high-clast/low-melt content would have resulted in these rocks cooling below their Curie points (for example, approximately 580 °C for pure magnetite) within a short geological period, probably less than 100 years. This would be due to the cooling effect of the entrained cold clasts and the relatively rapid conductive and convective cooling of such a thin melt layer.

In contrast, Manicouagan, as a much larger impact structure, possesses an extensive and significantly thicker melt sheet (> 230 m and probably 500 m in total original thickness). Glass-rich breccias were not sampled as part of palaeomagnetic studies at Manicouagan[4,5], but crystalline melt sheet was sampled and many of the samples were medium-grained. It would have taken thousands, perhaps tens of thousands, of years for such a body of superheated melt to cool below the relevant Curie points. For example, Onorato *et al.*[3] calculate that it would have required about 1,600 years for the centre of the melt sheet to reach its solidus (915 °C) and up to 10,000 years to cool to about 600 °C. This is for a body estimated at 200 m thick. We know now that 500 m is a more realistic thickness, so the estimates[3] are on the low side.

Consequently, although both Manicouagan and Rochechouart could have been formed within hours of each other, the resulting impact-generated rocks would have reached their Curie points at times sufficiently different to allow for a natural geomagnetic reversal to have taken place, accounting for the different magnetic polarities of these impact structures.

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