As mentioned earlier, it is possible that the empty class II molecules originate from low-affinity MHC–peptide complexes that easily dissociate. We view this possibility as unlikely, because such empty molecules would then be observed on other antigen-presenting cells. Thus, it appears that dendritic cells do indeed transport these empty class II molecules to the cell surface to capture extracellular peptides. In this case, does the invariant chain, expressed on the surface of several antigen-presenting cells, chaperone these MHC molecules to the surface before class II heterodimers form? In dendritic cells, but not in B cells, newly synthesized invariant-chain–MHC-class-II complexes travel via the cell surface en route to endosomal compartments[13]. Surface and extracellular proteases could degrade invariant-chain molecules engaged in these complexes, leaving empty class II molecules on the cells. Alternatively, dendritic cells may have developed an invariant-chain-independent pathway to recycle empty class II molecules to the cell surface.

Now that we know that dendritic cells have an external pathway for antigen presentation, it will be important to determine the origin of such exogenous peptides. Here, too, Santambrogio *et al.* offer relevant insight: they show that immature dendritic cells, but not B cells, are able to proteolyse extracellular antigens through the secretion of serine proteases[2]. These proteases seem to be able to degrade numerous substrates, including casein, ovalbumin, hen egg lysozyme, myelin basic protein and bovine serum albumin. Experiments using inhibitors of non-serine proteases (cysteine protease, metalloproteinase, and acid protease) indicated that these are not involved in degradation of extracellular proteins. Santambrogio *et al.* studied only a few substrates, however, and many other antigens would need to be tested before a conclusion can be reached in this matter. Genomics studies also indicate that dendritic cells may be host to a considerable number of proteases. However, the proteolytic potential of dendritic cells does not exclude the possibility that the peptides that bind to the dendritic-cell empty MHC class II molecules may derive from proteolysis in a different cell (such as a macrophage that has degraded a microbe).

We can only speculate about the immunological consequences of these empty MHC class II molecules on immature dendritic cells. Obviously, this extracellular pathway for antigen processing and presentation may be required to generate immunity to pathogens that block intracellular antigen processing. Alternatively, these empty molecules may be low-avidity ligands for antigen receptors of CD4-positive T cells, and may represent the mechanism by which dendritic cells maintain such T cells in the periphery[14]. Last but not least, because the direct binding of peptides on the surface of immature dendritic cells may not trigger the expression of co-stimulatory molecules that act with MHC–peptide complexes to stimulate T cells, the extracellular formation of MHC–peptide complexes may represent a mechanism to induce tolerance rather than immunity. To address these issues, we will need *in vivo* model systems in which antigen presentation occurs solely through empty MHC class II molecules on dendritic cells. Establishing the ultimate significance of Santambrogio *et al.*'s findings[1,2] will pose a considerable challenge. □

Jean Davoust and Jacques Banchereau are at the Baylor Institute for Immunology Research, 3434 Live Oak, Dallas, Texas 75204, USA.
e-mail: j.banchereau@baylordallas.edu

1. Santambrogio, L., Sato, A. K., Fischer, F. R., Dorf, M. E. & Stern, L. J. *Proc. Natl Acad. Sci. USA* **96,** 15050–15055 (1999).
2. Santambrogio, L. *et al. Proc. Natl Acad. Sci. USA* **96,** 15056–15061 (1999).
3. Cresswell, P. *Annu. Rev. Immunol.* **12,** 259–293 (1994).
4. Banchereau, J. & Steinman, R. M. *Nature* **392,** 245–252 (1998).
5. Banchereau, J. *et al. Annu. Rev. Immunol.* **18,** 767–811 (2000).
6. LaPan, K. E., Klapper, D. G. & Frelinger, J. A. *Hybridoma* **11,** 217–223 (1992).
7. Stern, L. J. & Wiley, D. C. *Cell* **68,** 465–477 (1992).
8. Garban, F. *et al. Blood* **87,** 3970–3976 (1996).
9. Andersson, T., Patwardhan, A., Emilson, A., Carlsson, K. & Scheynius, A. *Arch. Dermatol. Res.* **290,** 674–680 (1998).
10. Sherman, M. A., Weber, D. A., Spotts, E. A., Moore, J. C. & Jensen, P. E. *Cell. Immunol.* **182,** 1–11 (1997).
11. Busch, R., Cloutier, I., Sekaly, R. P. & Hammerling, G. J. *EMBO J.* **15,** 418–428 (1996).
12. Drenou, B. *et al. J. Immunol.* **163,** 4115–4124 (1999).
13. Saudrais, C. *et al. J. Immunol.* **160,** 2597–2607 (1998).
14. Brocker, T. *J. Exp. Med.* **186,** 1223–1232 (1997).

p53: only ARF the story

Alison C. Lloyd

Two tumour-suppressor proteins — p16^{INK4A} and p19^{ARF} — encoded by the same genetic locus both have a role in arresting the cell-division cycle. New results have revealed further complexities in the pathways involved.

Primary mammalian cells, when explanted into culture and propagated, divide a limited number of times before they irreversibly cease to proliferate — a state known as replicative senescence. This limitation to proliferative lifespan has been proposed to act as an intrinsic defence against cancer. Perhaps less fortuitously, the limited proliferative capacity of our cells may also be responsible for certain aspects of ageing. Two tumour-suppressor proteins, p16^{INK4A} and p19^{ARF} — encoded by a single genetic locus — have been implicated in the onset of senescence. However, the relative contributions of each protein have been controversial. On page 148 of this issue, Carnero and colleagues[1] dissect the role of each of these proteins in the senescence process and, by characterizing downstream pathways, they identify a novel mechanism by which p19^{ARF} can cause an arrest in the cell-proliferation cycle.

The *INK4A* locus consists of two overlapping genes that encode the two unrelated proteins p16^{INK4A} and p19^{ARF}. Overexpression of either protein results in cell-cycle arrest, although they act through distinct mechanisms: p16^{INK4A} downregulates proteins that inhibit the retinoblastoma protein (Rb), and p19^{ARF} acts by increasing levels of p53 (refs 2, 3, Fig. 1). Cellular levels of both p16^{INK4A} and p19^{ARF} increase progressively as cells approach the end of their proliferative lifespan. The requirement for p19^{ARF} in the senescence process has been established by the observation that primary fibroblasts isolated from p19^{ARF}-deficient animals appear to proliferate indefinitely[4]. The role of p16^{INK4A} is less clear. Both proteins are also induced in primary fibroblasts in response to expression of oncogenic (tumour-promoting) Ras protein, leading to premature entry into senescence[5,6]. p19^{ARF} is also induced in cells that overexpress the oncogenic proteins Myc or E1A, and is partly responsible for the cell death induced by the expression of these proteins[7,8]. Both p16^{INK4A} and p19^{ARF} are frequently found to be inactivated in human tumours, and p19^{ARF}-deficient mice are prone to develop tumours[4]. Whether these proteins act as tumour suppressors mainly by limiting proliferative lifespan or by protecting against oncogenic activation remains unresolved.

Carnero *et al.* set out to disentangle the roles of p16^{INK4A} and p19^{ARF} in replicative senescence[1]. To do this, they made use of viral vectors constructed to express 'antisense' RNA that was complementary to the 'sense' messenger RNA encoding p16^{INK4A} or p19^{ARF}. They could thus specif-

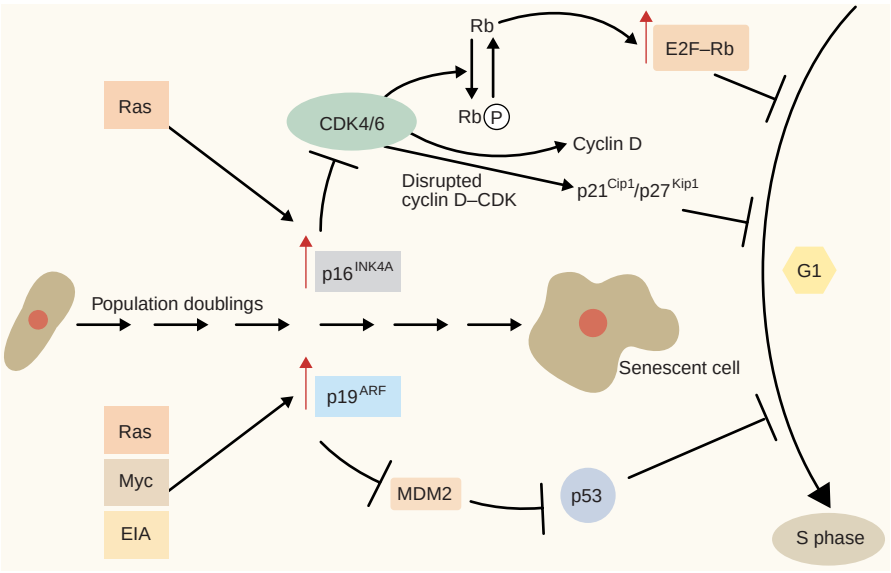

Figure 1 **Increased levels of $p16^{INK4A}$ or $p19^{ARF}$ result in cell-cycle arrest. $p16^{INK4A}$ inhibits cyclin-dependent kinases (CDKs) 4 and 6. As these CDKs can no longer phosphorylate the retinoblastoma protein (Rb), hypophosphorylated Rb accumulates. Hypophosphorylated Rb both inhibits transcriptional activation by E2F and enhances the repression of E2F. Inhibition of the cell cycle ensues. In addition, by disrupting cyclin D–CDK complexes, $p16^{INK4A}$ can release the CDK inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, which inhibit CDK2 activity. Increased levels of $p19^{ARF}$ inhibit the ability of MDM2 to mediate p53 degradation. p53 levels rise and inhibit cell-cycle progression, in part because of the induction of $p21^{Cip1}$ (not shown). Expression of the oncogenic proteins Ras, Myc and E1A also leads to raised levels of $p16^{INK4A}$ and/or $p19^{ARF}$. Circled 'P', phosphorylation. Red arrows indicate increased protein levels.**

ically target each mRNA, resulting in reduced levels of either $p16^{INK4A}$ or $p19^{ARF}$. Working with mouse embryo fibroblasts, Carnero *et al.* assessed whether or not these antisense vectors resulted in the cells bypassing replicative senescence. They did this in one of two ways, either by analysing the ability of pools of cells to be passaged, without a measurable slowdown in proliferation, beyond the time point at which the parental cells would normally enter senescence, or by assessing the ability of presenescent cells to form clonal colonies when plated at low density. Cells that manage to bypass replicative senescence are termed 'immortal'.

Carnero *et al.*'s antisense vectors worked efficiently and specifically, enabling the authors to show conclusively that reductions in the level of expression of either $p16^{INK4A}$ or $p19^{ARF}$ result in increased numbers of cells bypassing the senescent checkpoint. These immortalized cells maintain high amounts of the alternative protein product (that is, $p19^{ARF}$ when a vector directed towards $p16^{INK4A}$ is used, and vice versa), indicating that the level of each inhibitor reached at senescence is insufficient alone to trigger a proliferation arrest. This result argues for a role of both proteins in regulating proliferative lifespan. The $p19^{ARF}$ antisense vector appears to bypass proliferative senescence more efficiently than the $p16^{INK4A}$ antisense vector, although this finding is difficult to interpret, as the former vector appears to be more effective at lowering protein levels. It is also hard to determine whether the abrogation of either $p16^{INK4A}$ or $p19^{ARF}$ activity is sufficient to bypass senescence, or whether the loss of expression of either protein only increases the frequency of immortalization. The inefficiency with which the antisense-vector-expressing cells form colonies (less than one in 1,000 cells plated form colonies) may indicate that bypassing senescence is still a relatively rare event and that loss of $p16^{INK4A}$ or $p19^{ARF}$ is simply a contributory factor in this process. But with this type of assay it is not possible to distinguish between low survival rates and low rates of immortalization — and so this question remains unanswered.

Carnero and colleagues[1] also made use of 'reversible' antisense vectors. In these vectors, the authors included sites at which a protein called CRE recombinase acts. Carnero *et al.* first infected fibroblasts with these new vectors, and saw a decrease in the amounts of $p19^{ARF}$ or $p16^{INK4A}$. Then, they infected the cells with virus encoding CRE recombinase. This protein, acting at its target sites in the antisense vector, excised the antisense RNA corresponding to $p16^{INK4A}$ or $p19^{ARF}$. Removal of the antisense constructs in the immortalized cells led to the re-expression of the proteins and a block in the ability of the cells to form colonies. These experiments reinforce the view that both proteins contribute to the senescence phenotype and show that the unknown signals that upregulate $p16^{INK4A}$ and $p19^{ARF}$ levels toward the end of a cell's proliferative lifespan remain active in these immortalized cells.

Interestingly, Carnero *et al.* found that whereas cells expressing the constitutive $p19^{ARF}$ antisense vector and in which $p16^{INK4A}$ was re-expressed continued to proliferate, the re-expression of $p19^{ARF}$ in cells expressing the $p16^{INK4A}$ antisense vector resulted in the cells becoming senescent. So, it seems that cells that have lost $p19^{ARF}$ expression can proliferate with high $p16^{INK4A}$ levels, but cells without $p16^{INK4A}$ cannot proliferate with high levels of $p19^{ARF}$. These results lend further weight to the idea that the loss of $p19^{ARF}$ bypasses senescence more efficiently than the loss of $p16^{INK4A}$. In support of this suggestion, it is noteworthy that $p19^{ARF}$ levels are lower in cells immortalized by the $p16^{INK4A}$ antisense vector than in presenescent cells, suggesting that a selection against high $p19^{ARF}$ expression takes place during the immortalization process.

How do the proteins encoded by the *INK4A* locus inhibit the cell cycle during senescence? $p16^{INK4A}$ acts through the Rb pathway to inhibit the transcription factor E2F (Fig. 1). As expected, Carnero *et al.* found that a viral protein (E7) that overcomes the inhibition of this pathway was able to alleviate the growth arrest induced by re-expression of $p16^{INK4A}$. Also as expected (because $p16^{INK4A}$ does not act through p53), abrogation of p53 signalling by a dominant-negative mutant of p53 had no effect. Surprisingly, however (because $p19^{ARF}$ does work through p53), and in contrast to previous reports[4,9], dominant-negative p53 was unable to overcome the growth arrest induced by re-expression of $p19^{ARF}$. Moreover, $p19^{ARF}$ re-expression induced arrest in p53-deficient fibroblasts. Thus, $p19^{ARF}$ can negatively regulate the cell cycle by a p53-independent mechanism, as well as by a p53-dependent one.

Loss of Rb activity alone was also unable to overcome the $p19^{ARF}$-induced arrest; however, expression of $p16^{INK4A}$ antisense vector or E2F-1 in p53-deficient cells blocked the inhibitory effects of $p19^{ARF}$. So $p19^{ARF}$ appears to activate both the p53-dependent cell-cycle checkpoint and a second checkpoint, the latter of which can be overcome by inactivating the Rb pathway.

From these results, one might assume that

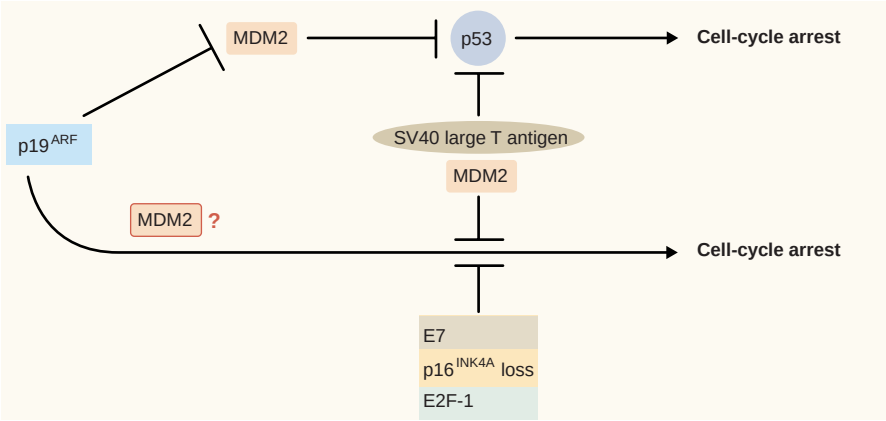

Figure 2 **p19^{ARF} induces cell-cycle arrest by p53-dependent and p53-independent mechanisms[1]. Top, p19^{ARF} inhibits the MDM2-dependent degradation of p53, probably by sequestering MDM2 in the nucleolus. Bottom, p19^{ARF} can also cause a cell-cycle arrest in cells lacking normal p53 function. The arrest in these cells appears to require MDM2 and can be overcome by inhibiting retinoblastoma protein (Rb) function (by means of loss of p16^{INK4A} or expression of E2F-1 or E7). Cells expressing proteins that can abrogate both the Rb and the p53 pathways, such as simian virus 40 (SV40) large T antigen and MDM2, are insensitive to the growth-inhibitory effects of p19^{ARF}. Thus either loss or overexpression of MDM2 appears to overcome the p53-independent, p19^{ARF}-induced cell-cycle arrest.**

the loss of p53 activity should not be sufficient for the immortalization of primary mouse embryo fibroblasts. If this were the case, and contrary to previous reports[4], it would be predicted that the loss of p16^{INK4A} or p19^{ARF} in addition to the loss of p53 should be selected for in immortalized clones. Indeed, this appears to be observed. Carnero and colleagues' analysis of clones isolated from p53-deficient fibroblasts revealed that several had lost p16^{INK4A} or p19^{ARF} expression or both. This situation seems to be analogous to that found in human cells, where both the Rb and the p53 pathways may need to be abrogated to bypass the senescence checkpoint and achieve an extended proliferative lifespan. The reason that immortalization occurs more readily in rodent cells than human cells may reflect an overall difference in genetic stability, rather than a difference in the number of checkpoints that must be bypassed.

p19^{ARF} regulates p53 levels by inhibiting a protein called MDM2. MDM2 reduces p53 levels by tagging it with multiple copies of the ubiquitin molecule and transporting it out of the nucleus to the cytoplasm, where its ubiquitin tag marks it out for degradation. p19^{ARF} is localized to the nucleolus. p19^{ARF} relocates MDM2 to the nucleolus, inhibits MDM2's ubiquitinating activity, and prevents MDM2 from escaping from the nucleus; as a consequence, p53 levels in the nucleoplasm rise[10,11]. MDM2 appears to be responsible for constitutively maintaining low levels of p53, as microinjection, into normal cells, of antibodies that block the interaction between the two proteins leads to an increase in p53 levels[12]. Genetic evidence also points to a critical interplay between p53 and MDM2: an MDM2 deletion is lethal at a very early stage in mouse development, whereas mice lacking both MDM2 and p53 develop to term.

So, is abrogation of MDM2 activity involved in the p19^{ARF}-induced, p53-independent cell-cycle arrest? Genetic evidence suggests that this is unlikely, as MDM2 and p53 double-null animals develop normally. Instead, Carnero *et al.*[1] show that p19^{ARF} is unable to induce senescence in fibroblasts lacking both MDM2 and p53. This indicates that MDM2 may have a role in mediating the p19^{ARF}-induced arrest (Fig. 2), but by a distinct mechanism, in that abrogation of MDM2 activity mediates the p53-dependent growth arrest, but MDM2 activity is required to signal the p19^{ARF}-induced, p53-independent arrest. This result predicts a tumour-suppressor function for MDM2 in the absence of p53. But, at least in mice, this does not appear to be the case: animals lacking both proteins develop tumours at the same rate as do animals lacking just p53 (ref. 13). Carnero *et al.* also show that overexpression of MDM2 can overcome a p19^{ARF}-induced arrest. MDM2 has been reported to have p53-independent functions in the oncogenic transformation of cells in culture and in inducing tumours *in vivo*[14]. Indeed, MDM2 binds to Rb and has been reported to activate E2F activity directly[15]; so, one might have expected that MDM2 overexpression would overcome the p19^{ARF}-induced arrest. However, we need to develop a model in which either loss or overexpression of MDM2 is able to overcome a p19^{ARF}-induced arrest. This is reminiscent of the situation with E2F-1 — either loss of E2F-1's repressor activity or induction of its transcriptional activity can contribute positively to cell-cycle progression[16] — and perhaps a similar mechanism may be involved for MDM2.

p19^{ARF} appears to be both a key regulator of proliferative lifespan and an important tumour-suppressor protein, so its mechanism of action is of great interest. Carnero *et al.*'s identification[1] of a p19^{ARF}-dependent arrest pathway that is independent of p53 regulation will undoubtedly fuel intensive efforts to uncover the mechanisms and the molecules involved.□

Alison C. Lloyd is a Cancer Research Campaign Senior Cancer Research Fellow at the MRC Laboratory for Molecular Cell Biology, University College London, Gower Street, London WC1E 6BT, UK.
e-mail: alison.lloyd@ucl.ac.uk

1. Carnero, A., Hudson, J. D., Price, C. M. & Beach, D. H. *Nature Cell Biol.* **2**, 148–155 (2000).
2. Chin, L., Pomerantz, J. & DePinho, R. A. *Trends Biochem. Sci.* **23**, 291–296 (1998).
3. Sherr, C. J. *Genes Dev.* **12**, 2984–2991 (1998).
4. Kamijo, T. *et al. Cell* **91**, 649–659 (1997).
5. Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D. & Lowe, S. W. *Cell* **88**, 593–602 (1997).
6. Palmero, I., Pantoja, C. & Serrano, M. *Nature* **395**, 125–126 (1998).
7. de Stanchina, E. *et al. Genes Dev.* **12**, 2434–2442 (1998).
8. Zindy, F. *et al. Genes Dev.* **12**, 2424–2433 (1998).
9. Pomerantz, J. *et al. Cell* **92**, 713–723 (1998).
10. Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J. & Bar-Sagi, D. *Nature Cell Biol.* **1**, 20–26 (1999).
11. Tao, W. & Levine, A. J. *Proc. Natl Acad. Sci. USA* **96**, 6937–6941 (1999).
12. Blaydes, J. P. & Wynford-Thomas, D. *Oncogene* **16**, 3317–3322 (1998).
13. Jones, S. N. *et al. Proc. Natl Acad. Sci. USA* **93**, 14106–14111 (1996).
14. Jones, S. N., Hancock, A. R., Vogel, H., Donehower, L. A. & Bradley, A. *Proc. Natl Acad. Sci. USA* **95**, 15608–15612 (1998).
15. Yap, D. B., Hsieh, J. K., Chan, F. S. & Lu, X. *Oncogene* **18**, 7681–7689 (1999).
16. Weinberg, R. A. *Cell* **85**, 457–459 (1996).

erratum

A typographical error appeared in Anne-Odile Hueber's News and Views article "CD95: more than just a death factor?" (*Nature Cell Biol.* **2**, E23–E25; 2000). Caspases act at aspartate residues within their targets, rather than at cysteine residues as stated.

correction

The legend to Figure 1 of the meeting report published in January (L.M. Machesky and M. Schliwa, *Nature Cell Biol.* **2**, E17–E18; 2000) should have concluded with the following sentence: 'Reprinted from A.B. Verkhovsky, T.M. Svitkina and G. G. Borisy, "Self-polarization and directional motility of cytoplasm", *Current Biology* **9**, 11–20, copyright (1999), with permission from Elsevier Science.'