ROS as a tumour suppressor?

Matthew R. Ramsey and Norman E. Sharpless

Senescence is an important mechanism for suppressing mammalian tumours and it may also contribute to aging. A new study suggests that changes in the metabolism of oxygen radicals are important for establishing senescence and blocking cytokinesis to ensure senescent cells never divide again.

Reactive oxygen species (ROS) serve many cellular functions; for example, second messenger, anti-bacterial agent, mutagen, aging-accelerant and growth stimulant. With regard to neoplasia, the view has generally been that ROS cause cancer through a number of mechanisms, including the induction of DNA damage and alteration of intracellular signaling[1]. A provocative new report from Takahashi *et al.* on page 1291 of this issue[2], however, suggests that ROS have an unexpected role in inducing and maintaining senescence-induced tumour suppression.

Senescence is a specialized form of terminal differentiation that is usually irreversible and induced by a number of stimuli associated with neoplastic growth, such as oncogene activation and telomere dysfunction. In particular, ROS is known to induce senescence in a number of systems, although the *in vivo* significance is unclear and the mechanism is not well understood. Although well-described in cultured cells for over four decades, the role of senescence in intact organisms had, until recently, been controversial. Several reports (reviewed in ref. 3), have demonstrated that senescence is not merely an artifact of cell culture but occurs *in vivo* to limit tumour growth, even in very young mice and humans. Perhaps the most compelling demonstration of this occurs in dysplastic naevi — a pigmented skin lesion thought, in some cases, to be a precursor of malignant melanoma. Unexpectedly, a high frequency of naevi contain signature oncogenic mutations associated with melanoma, yet such naevi do not proliferate and instead exhibit classic features of senescence[4,5]. Therefore, although most of us have these common cutaneous lesions, even as children, only an unlucky few will ever develop melanoma because these would-be cancers are usually checked by senescence.

A vexing problem for the field, however, has been determining how senescence, which occurs in many distinct cell types in response to a wide variety of cues, is different in biochemical terms from reversible forms of growth arrest. For example, although senescence is closely associated with activation of the $p16^{INK4a}$ tumour suppressor gene, it has never been clear why other cell-cycle inhibitors, such as $p18^{INK4c}$, are not. One model proposes that senescence results from a prolonged and potent anti-proliferative signal (for example, as conveyed by $p16^{INK4a}$ or *p53* activation) coupled with other ill-defined senescence-promoting stimuli. The nature of these additional stimuli has been unclear, but has been suggested to be forms of 'cellular stress',

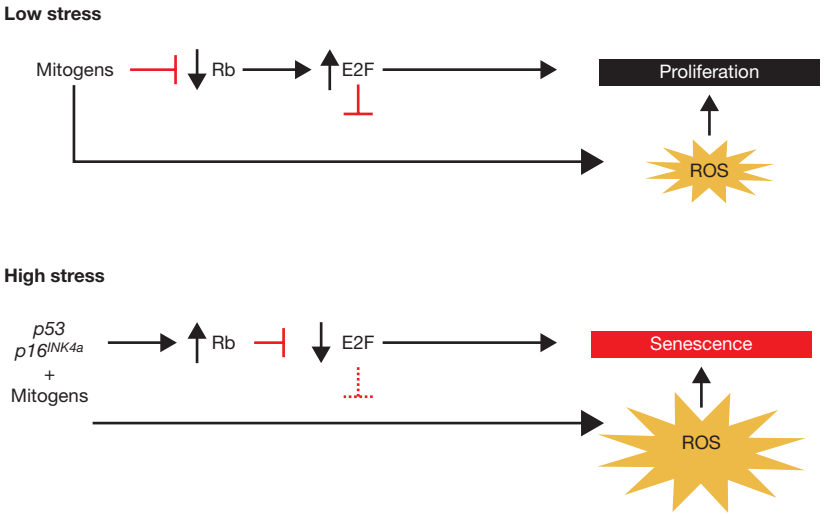

Figure 1 Senescence requires a mitogenic signal under conditions of cellular stress. During low-stress conditions, mitogens inactivate Rb and therefore activate E2F to induce proliferation. The authors suggest that as part of its S-phase promoting activity, E2F activation decreases ROS levels by regulating genes involved in ROS production and metabolism. Therefore, under these settings, mitogenic signals are matched by low levels of ROS and proliferation ensues. In conditions of high cellular stress, however, tumour suppressor genes such as *p53* and $p16^{INK4a}$ are activated, leading to inhibition of E2F, through Rb. In this setting, mitogenic stimulation is not accompanied by E2F activation and ROS levels accumulate to senescence-promoting levels. The authors suggest that high levels of ROS also induce a PKC-mediated block of cytokinesis as part of the senescence programme.

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including ROS. It is this question that the study by Takahashi *et al.* addresses.

To understand the mechanics of senescence, the authors used an immortalized human fibroblast line containing a temperature-sensitive mutant of the SV40 large T-antigen that functions by binding and inactivating both the Rb and p53 tumour suppressor proteins at permissive temperature. Normally, Rb binds to E2F transcription factors to prevent the induction of genes required for the completion of the cell cycle, whereas the p53 transcription factor induces a number of genes that block proliferation. The activation of p53 and/or Rb is required for senescence in this system, and therefore growth at the permissive temperature blocks the induction of senescence. This system also allows for the reactivation of these anti-proliferative pathways when the cells are switched to the restrictive temperature, which inactivates the large T-antigen, activates Rb–p53 and results in senescence. The authors showed that when these cells were grown under standard conditions, including media with high-serum supplementation, they senesced rapidly after being switched to the restrictive temperature. Moreover, once senescence was established, these cells were not able to re-enter the cell cycle when switched to the permissive temperature. Therefore, in agreement with previously published data[6,7], established senescence of these human cells does not require persistent p53 or Rb activity, and is extremely difficult, if not impossible, to reverse.

The authors next examined what features of cell culture were able to cooperate with Rb–p53 activation to induce senescence. When the large T-antigen was inactivated in cells cultured in media with reduced serum supplementation, cells ceased proliferation but did not become senescent. Instead, these cells re-entered the cell cycle normally when they were switched back to the permissive temperature. This result suggests that senescence requires both a potent anti-proliferative signal (for example, $p16^{INK4a}$ and *Rb* activation), and the presumably strong mitogenic signals associated with culture in high serum media (Fig. 1). This finding is consistent with a previous study that showed that activation of the ERK–MAPK pathway, a strong proliferative signal in many cell types, is necessary for the establishment of senescence in a related cell-culture model[8]. Establishing senescence also requires that this period of conflict lasts at least a few days[6], during which time widespread and characteristic changes in chromatin architecture occur, associated with the production of senescence-associated heterochromatic foci[9].

Because of prior work linking ROS and senescence in human fibroblasts[10], the authors went on to examine the level of ROS in proliferating and senescent cells. As expected, they found that ROS levels were more elevated in cells grown in high serum than in low serum media. Surprisingly, growth arrest resulting from changing to the restrictive temperature markedly increased the levels of ROS. Moreover, fully senescent cells demonstrated even higher levels of ROS, and these increased levels persisted after returning the cells to the permissive temperature. Importantly, the authors could block the establishment of senescence by treating the cells with an oxygen radical scavenger, suggesting that ROS promotes senescence. The authors make a particularly tantalizing speculation as to how this process may work: Rb, through E2F transcription factors, regulates enzymes (for example, MnSOD, GPX and catalase) involved in ROS production and metabolism. Therefore, mitogenic stimulation induces a growth-related production of ROS, while concomitant Rb activation induces an enzymatic production of ROS and impedes ROS degradation, suggesting these cellular signals converge to produce sharply elevated ROS levels (Fig. 1). In agreement with this hypothesis, the authors showed that $p16^{INK4a}$ expression in normal fibroblasts, which presumably does little more than activate Rb, induced ROS levels to almost the same levels as the strong proliferative signals associated with the ectopic expression of oncogenic Ras. Because of convincing data linking ROS and aging, many experts in the field have wondered whether ROS promotes senescence by inducing $p16^{INK4a}$ expression and *Rb* activation. Although this may be, the authors show that, in fact, $p16^{INK4a}$–Rb activation can induce ROS.

To understand how ROS may help to enforce the senescent state, the authors focused on protein kinase C. In agreement with previous work[11], the authors showed that increased levels of ROS were associated with the formation of the activated, catalytic fragment of PKCδ, which, in turn, seems to lead to the further production of ROS, thus establishing a self-sustaining activation loop. As PKCδ activation has been linked to G2–M cell-cycle arrest[12], the authors astutely realized that this finding may explain a long-known feature of senescent cells: on mitogen stimulation, senescent cells will synthesize a modest amount of new DNA, but will not traverse the cell cycle[6,7]. Through a variety of genetic and pharmacologic approaches, the authors show that PKCδ activation induces a sharp reduction in WARTS protein expression (a kinase required for cytokinesis) through an unknown mechanism. Taken together, these data suggest that the PKCδ-mediated depletion of WARTS is an important component of the senescence program. Although these experiments are convincing, one can imagine other mechanisms whereby high levels of ROS may contribute to senescence, and it could be argued that other likely effects of elevated levels of ROS in senescent cells are not considered by this model.

The findings of Takahashi *et al.* seem to define another function for ROS — enforcer of senescence. Given the evidence that ROS promote mammalian aging, this conclusion will be satisfying to researchers in gerontology who believe senescence contributes to aging, as this work better explains the known links between ROS and senescence. The widespread belief that anti-oxidants may retard mammalian aging is not challenged by these data. However, this is not the case for the belief that scavengers of ROS (for example, anti-oxidants) should prevent cancer. In contrast to the well-described tumour-promoting activities of ROS, this work suggests a means whereby ROS significantly participate in an anti-cancer mechanism of undisputed importance. Human clinical-trial data suggests the authors may be on to something: two large, blind, randomized trials of supplementation with beta-carotene, a moderate anti-oxidant, have been conducted in patients at increased risk for cancer (current and former smokers), and both yielded the same result — beta-carotene supplementation increased lung cancer risk[13,14]. Although there are other tenable explanations for these findings[15], the notion that ROS contributes to tumour suppression as suggested by Takahashi *et al.* may be a potential reason as to why extended anti-oxidant supplementation does not seem effective in cancer chemoprevention. □

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Ahead of the curve: mitochondrial fusion and phospholipase D

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A new study has identified a novel phospholipase D (PLD) that is located in the mitochondrial outer membrane and is required for organelle fusion. As PLD-catalysed production of fusogenic lipids is critical for many examples of membrane fusion (such as those mediated by SNAREs), this startling finding raises the possibility that mitochondria use a mechanism common to other cellular fusion events.

The relative rates of mitochondrial fusion and division control the striking array of mitochondrial shapes and numbers observed in different eukaryotic cell types[1]. Mitochondrial morphology can range from the hundreds of small, ovoid organelles seen in hepatocytes to the long, spiral tubule found at the base of a sperm flagellum. In addition to shape and number, mitochondrial dynamics are critical for normal organelle function. For example, mice defective in mitochondrial fusion die early in development[2], whereas yeast mitochondrial-fusion mutants rapidly lose their mitochondrial DNA and are defective in oxidative phosphorylation[1]. Moreover, organelle fusion and division also seem critical for the dramatic membrane remodelling events facilitating cytochrome *c* release during apoptosis[3,4].

Before the study by Choi *et al.*[5] on page 1255 of this issue, three proteins were known to mediate mammalian mitochondrial fusion. Two related proteins, Mitofusin 1 (Mfn1) and Mitofusin 2 (Mfn2), are anchored in the outer membrane with a GTPase domain facing the cytosol[6]. Mfn1 and Mfn2 are at least partially redundant, as cells lacking Mfn1 show a reduction in mitochondrial fusion, whereas fusion is absent in cells lacking both proteins. Mitofusins are homologous to fusion proteins first identified in *Drosophila* (fuzzy onions)[7] and yeast (Fzo1p)[8,9]. In yeast *fzo1* mutants, cells contain many small mitochondrial fragments instead of the 5–10 tubular mitochondria observed in wild-type cells, and a defect in mitochondrial fusion in *fzo1* mutants can be directly demonstrated using a cell-mating assay[1]. The fragmentation of mitochondria in *fzo1* mutants depends on mitochondrial division[10]. In particular, yeast mitochondrial fission requires the dynamin-related GTPase, Dnm1p, and cells lacking this protein contain a single mitochondrion consisting of a network of interconnected tubules. Strikingly, *dnm1 fzo1* double mutants contain relatively normal, tubular-shaped mitochondria, suggesting that mitochondrial shape and number in cells is controlled, at least in part, by a balance between division and fusion[10]. The third mammalian protein required for mitochondrial fusion is OPA1, a dynamin-like GTPase[11]. Alternative splicing and proteolytic processing produces multiple forms of OPA1 located in the inner membrane and intermembrane space. Highlighting the pivotal role of fusion in mitochondrial function, defects in both Mfn2 and OPA1 have been implicated in neurodegenerative disorders[6,11].

Mfn1, Mfn2 and OPA1 do not resemble other fusion proteins (such as the SNARES of the exocytic–endocytic pathway), raising the possibility that mitochondria fusion occurs by a very distinct mechanism.

A potential new and exciting mitochondrial fusion component was found in a search of the human genome for proteins distantly related to the well-characterized PLD1 and PLD2 enzymes. These classical PLDs cleave phosphatidylcholine to produce phosphatidic acid[12]. Choi *et al.* identified a PLD-like protein called MitoPLD, which they showed resides in the mitochondrial outer membrane and is expressed in a wide array of tissues. Providing a clue to its function, alignments showed that mitoPLD actually resembles bacterial cardiolipin synthase more than PLD1 or PLD2. In eukaryotes, cardiolipin — a 'double' phospholipid with four fatty acid tails — is found in mitochondrial membranes and is synthesized by an inner membrane-localized cardiolipin synthase that is very different from PLD1, PLD2 or MitoPLD. Using a bacterially produced MitoPLD, Choi *et al.* found that it does not make cardiolipin, but instead seems to catalyse the reverse reaction, hydrolysing cardiolipin to generate phosphatidic acid. Although MitoPLD resembles PLD1 and PLD2 in its production of phosphatidic acid, MitoPLD contains only half of the site necessary for catalysis. The authors show that the mitochondrial protein dimerizes, which presumably generates the active form of enzyme.

A big surprise came from the observation that MitoPLD is required for mitochondrial fusion, which was monitored through fusion of HeLa cells that individually expressed mitochondrial-targeted GFP or dsRed. When MitoPLD activity was inhibited using RNA interference or by expressing a dominant-negative version of MitoPLD, most of the mitochondrial fragments within the fused cells contained only GFP or dsRed. In contrast, cells with

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