Barriers to Ras transformation

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Tumorigenesis invariably requires multiple hits. New data shows that carcinogenesis originating from *Ras* mutations also requires increased Ras levels and a block of cellular senescence. Intriguingly, low non-transforming levels of *Ras* do not trigger senescence, suggesting that this programme is only activated in response to *bona fide* cancer threats.

It has been increasingly clear that cells on the verge of forming tumours undergo altruistic responses such as apoptosis or cellular senescence. The senescence-induced permanent proliferative arrest may confine tumours to a benign stage[1] and recruit cells of the innate immune system for their elimination[2]. Although senescence-activated β-galactosidase (β-Gal) staining at pH 6 (ref. 3) and, more recently, other markers[4], have in principle permitted detection of senescent cells *in vivo*, it remains difficult to detect senescent cells in animal models of carcinogenesis and patient biopsies. Nevertheless, it is clear that not all oncogene-expressing cells end as senescent cells[5,6]. What then is the relationship between the expression of an activated oncogene and the senescence programme? This is precisely the question that Sarkisian *et al.*[7] set out to answer on page 493 of this issue.

The authors used an elegant approach to modulate the levels of expression of oncogenic *Ras* in the mammary gland using a doxocyclin-inducible promoter[1]. They found that near physiological levels of oncogenic *Ras* expression increased cell proliferation leading to hyperplasic lesions. No signs of cellular senescence were associated with these lesions, indicating that low levels of Ras are not sufficient to induce cellular senescence *in vivo*. On the other hand, high levels of expression of oncogenic *Ras* during puberty (the time at which most of the mammary gland develops in mice) lead to induction of a senescence programme characterized by persistent growth arrest, senescence markers such as senescence-associate β-Gal and plasminogen activator inhibitor, as well as expression of senescence regulators such as PML, p53, p21, $p19^{Arf}$ and $p16^{Ink4a}$. In addition, the oestrogen receptor was relocalized from the nucleus to the cytoplasm in cells expressing high levels of Ras, indicating that senescent cells may not respond to oestrogenic stimulation during puberty. As a consequence, Ras activation inhibited the elongation of mammary ducts stimulated by oestrogens. Importantly, high levels of expression of oncogenic *Ras* did not induce senescence in the mammary glands of mice harbouring deletion of the *Ink4a–Arf* locus, as previously reported in cell culture models of Ras-induced senescence[8]. Clearly, only high levels of Ras can trigger a cellular signal that induces the senescence programme. It will be very interesting to determine whether only high Ras levels can induce DNA-damage signals, given that the DNA damage response (DDR) is critical for oncogene-induced senescence[9–11].

As low levels of Ras cannot induce senescence, the key question for the senescence model is whether low levels of Ras are actually transforming? Sarkisian *et al.*[7] show that low levels of Ras are not directly transforming *in vivo*. Hence, their work is consistent with the model of senescence as a tumour-suppression mechanism activated in response to an aberrant signal that can lead to malignant transformation if unchecked[8]. One might expect that the senescence programme evolved to respond only to direct oncogenic threats, avoiding the unnecessary accumulation of senescent cells. In the oncogenic Ras transgenic mice of Sarkisian *et al.*[7], cells expressing low levels of Ras only progressed into malignant lesions when Ras levels increased, and where the senescence checkpoint was evaded. The authors proposed a three-stage model for Ras-induced tumorigenesis (Fig. 1). The priming lesion is a mutation in the *Ras* gene that produces a constitutive active allele of *Ras*. This mutation increases cell proliferation and leads to hyperplastic lesions, but is not sufficient for transformation. A second hit is necessary to increase Ras levels and consequently provide the oncogenic signal. Nevertheless, cells bearing high levels of Ras cannot form a tumour because of the senescence barrier. Tumours only arise if a third hit disables the senescence programme. The nature and timing of this third hit during the carcinogenesis process remain unclear.

The etiology of the first hit has been clear for some time. It is well established that many carcinogens can lead to Ras mutations[12]. In the classic two-stage carcinogenesis model, carcinogens that induce *Ras* mutations induce benign papillomas, but not cancers. Application of the carcinogen dimethyl benzanthracene (DMBA) to the skin of mice induced cellular senescence[13], suggesting that tumour progression of cells with endogenous oncogenic *Ras* was prevented. As Sarkisian *et al.*[7] suggest that Ras levels must be high to induce senescence, we should expect that carcinogens must be sufficient to induce both *Ras* mutations and high Ras activity in the skin of mice. However, it is unclear which mechanism may increase Ras levels in cells expressing mutant *Ras*. In the mammary gland, Sarkisian *et al.*[7] found that upregulation of ligands of the EGF-R pathway correlated with the increase in transgenic *Ras* levels. Although there is a potential caveat with this observation, in that an artificial promoter was employed to drive Ras expression, such ligands may also increase endogenous Ras levels and/or signalling in cells with mutations in

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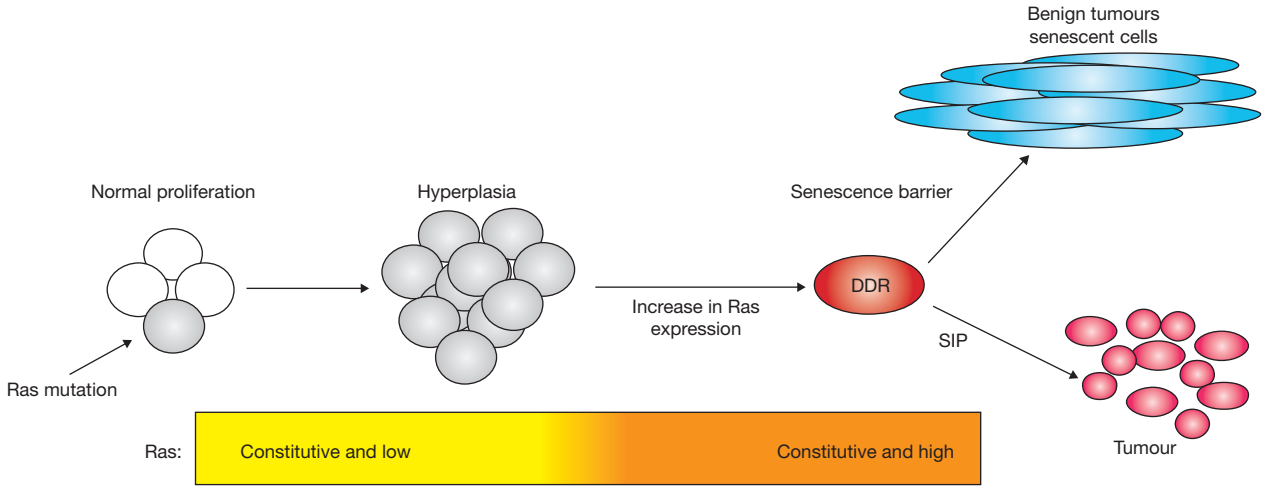

Figure 1 The three-stage carcinogenesis model for Ras-induced tumours according to Sarkisian *et al.*[7]. Ras mutations are a necessary condition to promote cell proliferation. However, low levels of Ras are not transforming. Therefore, a second hit must increase Ras levels and/or signalling. High Ras activity does not transform cells because counteractive senescence responses triggered by the DNA-damage response (DDR) prevent their proliferation. Cells may evade senescence because of changes (genetic or epigenetic) that inactivate the tumour suppressors that control cellular senescence (senescence inhibitory processes, SIP). These changes may arise at any moment along the carcinogenesis process before the cells actually commit to an irreversible senescent arrest.

endogenous *Ras*[12]. It is interesting to consider the likelihood that tumour-suppression mechanisms evolved to prevent cancer at the level of Ras-expression. The fact that Ras levels are controlled by the *let-7* family of micro-RNAs, and the levels of these micro RNAs are often downregulated in lung tumours is consistent with this notion[14]. A practical consequence of the new stage in carcinogenesis proposed by Sarkisian *et al.*[7] is the possibility of developing drugs to prevent the shift from low Ras to high Ras signalling.

How then do tumours arise in spite of the mechanisms controlling Ras levels and the senescence programme, and what is the cellular target of the third hit? In the two-stage carcinogenesis model, tumours arise after application of substances known as tumour promoters. A logical hypothesis is that tumour promoters may block senescence. However, none of the well-known tumour promoters (including TPA and okadaic acid) block Ras-induced senescence in culture (our unpublished observations). The studies of Sarkisian *et al.*[7] offer a different explanation. They studied the reversibility of senescence in epithelia expressing high levels of Ras to analyse this issue. Although the relevant model for real cancers is a lesion that starts with low Ras levels and then progress to high Ras levels, we can still learn about the reversibility of the senescence process *in vivo* from their experiments. They observed that epithelial senescent cells disappeared after Ras signalling was terminated. This was associated with markers of apoptotic cell death and, notably, the substitution of senescent cells with a normal mammary epithelial tree. These data indicate either that some senescent cells can revert to proliferation, or that some tissue stem cells with the potential to regenerate the gland were not irreversibly arrested by high *Ras* expression.

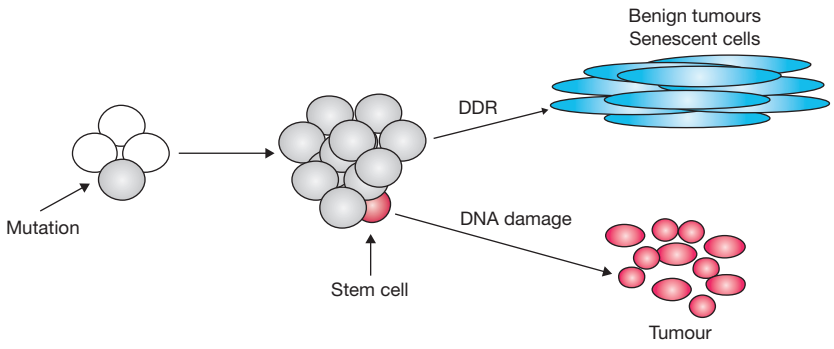

Figure 2 Senescence-resistant stem cells may be the most common targets of Ras-induced tumours *in vivo*. According to this model, most cells expressing oncogenic Ras succumb to the senescence process. However, tissue stem cells (in red) are resistant to the process and, under the influence of oncogene-induced DNA damage, accumulate critical mutations that allow malignant transformation. Resistance to senescence may be an intrinsic property of stem cells, or the stem-cell niche may control it.

To distinguish between reversion and replacement, the authors used a technique that labels senescent cells with BrdU. A pulse of BrdU was used to selectively label the cells that grow after induction of oncogenic *Ras*. Only cells that arrest shortly thereafter, because of senescence, retain the label, whereas dividing cells lose it by dilution. To mark the cells that resume proliferation after stopping Ras expression, Ki67, another proliferation marker, was used. The authors found no overlap between the two staining methods, suggesting that the cells resuming proliferation after withdrawal of Ras expression were not the cells that retained BrdU labelling (senescent cells). The data is consistent with the hypothesis that senescent cells are irreversibly arrested. The authors propose that non-senescence cells, perhaps stem cells, that remained silent among the epithelial masses of senescent cells, were responsible for regenerating the gland after senescent cells died because of lack of Ras signalling. By extension, these stem or progenitor cells, resistant to Ras-induced senescence, could be the real targets of the carcinogenesis process (Fig. 2). These questions highlight the current technical problems in evaluating senescence *in vivo*: a single

cell that escapes the programme may be sufficient to go on to form a tumour. Growth arrest is not necessarily specific for senescent cells, as most adult cells are quiescent (including stem cells). The senescence-associated β-Gal marker correlates with the senescent phenotype, but does not label all arrested cells and does not reflect the mechanisms responsible for the irreversibility of the senescent arrest. Molecular markers of the DNA-damage signalling response[9–11], the senescence associated heterochromatin foci (SAHFs)[15] and the PML bodies[16], which are functionally linked to the senescence programme, could be more reliable predictors of the stability of the senescence phenotype. Whether or not oncogenic Ras can induce these senescence traits in all cells *in vivo* remains to be investigated. As oncogene-induced senescence involves DNA damage[9–11], cells evading senescence could potentially accumulate critical mutations to develop a full malignant phenotype (Fig. 2). Resistance to senescence may be an intrinsic property of certain stem or progenitor cells and it may be controlled by host factors (for example, cytokines in the stem cell niche). Mouse models of oncogene-induced senescence, such as the one presented here by Sarkisian *et al.*[7], are ideal to find answers to these questions.

COMPETING FINANCIAL INTERESTS
The authors declare that they have no competing financial interests.

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Anchoring RCC1 by the tail

Paul R. Clarke

Generation of GTP-bound Ran on chromatin by its guanine nucleotide-exchange factor RCC1 provides a spatial signal that controls nuclear-envelope formation, nucleo-cytoplasmic transport and mitotic spindle assembly. A study now identifies an unusual post-translational modification, α-N-methylation of the amino-terminal tail of RCC1, which anchors the protein on chromosomes.

The intracellular organization of eukaryotic cells requires communication between organelles and subcellular structures to ensure their proper arrangement and to coordinate their reorganization — for example, during the cell-division cycle. Localization of enzymes provides a way to signal the presence of specific structures and to attract molecular partners. On page 596 of this issue, Chen *et al.*[1] identify a highly unusual post-translational modification that chromosomally anchors RCC1, the guanine nucleotide-exchange factor for the Ran GTPase[2]. This localization is essential for normal mitosis, when chromosomes influence the assembly and function of the mitotic spindle[3].

Ran is a member of the Ras superfamily of small GTPases. Similarly to other GTPases, Ran switches between between GDP- and GTP-bound conformations that determine its molecular interactions. Unusually, Ran is an abundant and mainly diffusible protein that is spatially controlled by the distinct localization of regulators of its GDP–GTP cycle. Ran–GAP1, the GTPase activating factor that stimulates GTP hydrolysis by Ran, is excluded from the nucleus. In contrast, RCC1 is localized to chromosomes throughout the cell cycle[4,5] where it generates Ran–GTP, which directs nuclear-envelope formation, nucleo-cytoplasmic transport and mitotic spindle assembly[6]. Disrupting the interaction of RCC1 with chromatin perturbs the normal gradient of Ran–GTP concentration and the ability of chromosomes to direct spindle assembly[4,7]. Chen *et al.*[1] now show that RCC1 is modified by α-amino methylation of the N-terminal residue throughout the cell cycle, and demonstrate that this modification is critical to stabilize the interaction between RCC1 and chromatin during mitosis. This provides a new post-translational mechanism by which intracellular protein localization can be directed.

Ran–GTP controls the formation and dissociation of complexes between proteins carrying targeting motifs and transport factors of the importin-β family. During nuclear import, Ran–GTP binds importin-β and disrupts its interaction (commonly via importin-α) with the nuclear-localization signal in an imported cargo protein, releasing the cargo in the nucleoplasm. Conversely, the interaction of the export factor Crm1 with a nuclear-export signal in a protein is stabilized when Ran–GTP binds Crm1, causing export of the complex from the nucleus. Even when the nuclear envelope breaks down during mitosis in animal and plant cells, these reactions continue to provide a mechanism to release factors from inhibitory complexes or to promote their localization to specific structures, thereby directing the organization of the mitotic spindle during prometaphase and reassembly of the nuclear envelope at telophase.

As the localization of RCC1 is critical for the spatial organization of Ran–GTP, the molecular details of the interaction of RCC1 with chromatin and how it is regulated are important questions. RCC1 has a core seven-bladed propeller structure[8]. Its interaction with chromatin involves contacts with histones H2A and H2B[9] and is modulated by Ran[4,10], which interacts with the opposite side of the propeller[8]. However, the localization of RCC1 to chromatin is strongly dependent on a flexible N-terminal tail that extends beyond the core propeller structure[4,8]. Deletion of the tail

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