RAS in cellular transformation and senescence

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Introduction to RAS

In 1964, a transforming retrovirus was described that produced tumours in mice [1]. The Harvey rat sarcoma virus, aptly named H-RAS after the discovering scientist, encoded an oncogene that had been hijacked from its host. A similar virus was isolated in 1970 and was named the Kirsten rat sarcoma virus or K-RAS [2]. In 1982, the human genes homologous to the viral genes were elucidated and were designated c-H-RAS and c-K-RAS [3], and subsequently the third and final RAS family member was isolated from human neuroblastoma samples and termed c-N-RAS. An examination of other human cancers also revealed RAS genes capable of transforming mammalian cells, and interestingly, these alleles were transforming due to point mutations that inactivated the proteins' intrinsic guanine triphosphatase (GTPase) activity [4,5].

RAS functions downstream of mitogenic growth factor receptors. Following the ligation with growth factors, these receptor tyrosine kinases undergo autophosphorylation and thereby recruit adaptor proteins that bind guanine nucleotide exchange factors (GEFs) (for review, see [6]). GEFs catalyse the exchange of guanine diphosphate (GDP) for guanine triphosphate (GTP) on small GTP-binding proteins, including RAS. GTP-bound RAS subsequently interacts with a number of effectors to regulate cellular proliferation and survival, notably including the RAF family of proteins and type I phosphoinositol-3-kinase (PI3K). RAS proteins mutated at codons 12, 13, or 61 are rendered constitutively GTP bound and consequently have increased affinity to RAF proteins and PI3K, leading to activation of downstream pathways.

The RAF family of serine/threonine kinases consists of three members: ARAF, BRAF, and CRAF [6]. Following activation by RAS, RAF kinases phosphorylate Mitogen-Activated Protein Kinase (MEK) kinases (MEK1 and MEK2), resulting in their activation. MEK proteins subsequently phosphorylate Extracellular Regulated MAP Kinase (ERK) (ERK1 and ERK2) in the cytoplasm, resulting in their activation and nuclear translocation. ERK kinases have a variety of cytoplasmic and nuclear targets, importantly including ETS transcription factors to regulate the expression of many pro-proliferative genes.

RAS-GTP also interacts with and stimulates the activity of PI3K [6]. Upon activation, PI3K catalyses the conversion of phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P2) to produce phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3). Increased local concentrations of PtdIns(3,4,5)P3 result in recruitment of the kinase PDK1 via its pleckstrin homology domain, which phosphorylates and activates the kinase AKT. AKT in turn promotes cellular survival and activates mammalian target of rapamycin (mTOR) to increase ribosomal biogenesis and messenger RNA (mRNA) translation. Additionally, active PI3K also stimulates the GTP-binding protein RAC, a regulator of the actin cytoskeleton. Therefore, rather than relying upon a single pathway for cellular transformation, it is hypothesised that numerous alterations in multiple biochemical pathways collectively promote cellular transformation by oncogenic RAS.

Oncogenic RAS mutations are the most common oncogenic events identified in human tumours, being found in 30% of human cancers. KRAS is the most commonly mutated member of the RAS family, present in over 90% of ductal pancreatic cancers, 40–50% colorectal cancers, and 30% of non-small cell lung cancers (6). A detailed characterisation of the cellular and molecular pathways altered by oncogenic RAS has been pursued in order to identify potential therapeutic targets for such cancers, but despite its expected role in promoting tumourigenesis, ectopic expression of oncogenic RAS in primary cell cultures induces a paradoxical irreversible growth arrest known as oncogene-induced senescence (OIS) [7]. OIS is characterised by a flattened cellular morphology, a large nucleus with a prominent nucleolus, chromatin reorganisation, and activation of the p53 and $p16^{INK4a}$ pathways [7]. Although ectopic expression of oncogenic RAS in primary cells induces OIS, the exact molecular mechanisms remain unclear. Studies have

indicated that the ability of RAS to induce senescence depends upon the RAF/MEK/ERK and p38MAPK pathways [8–12] and is accompanied by upregulation of $p16^{INK4A}$, p53, $p14/p19^{ARF}$, and/or $p21^{WAF1}$ [7,13] and silencing of E2F target genes [14]. This review will explore the pathways that mediate RAS-induced senescence, as well as how the dosage of RAS may determine the cellular fate between OIS and transformation.

p53 as a sensor of oncogenic stress

The p53 tumour suppressor functions as a cellular gatekeeper to prevent aberrant cell cycle progression. p53 levels are determined by the relative levels of the E3 ubiquitin ligase MDM2 (HDM2 in humans) and the MDM2 inhibitor $p14/p19^{ARF}$ (reviewed in [15]). In the setting of limiting $p14/p19^{ARF}$ levels, MDM2 targets p53 for proteasomal degradation. $p14/p19^{ARF}$ interferes with the E3 ubiquitin ligase function of MDM2 to stabilise p53 and increase its activity. p53 is also stabilised by phosphorylation via a variety of kinases, preventing its interaction with MDM2. Following stabilisation, p53 has both transcription-dependent and -independent targets to induce senescence, apoptosis, cell cycle arrest, autophagy, metabolic changes, and other cellular processes. The p53 protein is required for the onset of OIS following ectopic oncogenic RAS expression [7]. Expression of oncogenic RAS leads to the transcriptional upregulation of $p14/p19^{ARF}$ via RAF/MEK/ERK signalling and p38MAPK activation via MKK3/6. Activation of p38MAPK leads to phosphorylation and activation of p53 [16]. Similarly, the p38MAPK target PRAK has been shown to phosphorylate p53 on serine 37, which is required for the induction of RAS-induced senescence [17]. Cells deficient for PRAK fail to undergo OIS upon ectopic mutant RAS expression and instead are more tumourigenic. Similarly, $PRAK^{-/-}$ mice are more susceptible to DMBA/TPA-induced skin tumourigenesis. Thus, p53 is a critical regulator of RAS-induced senescence and is activated by multiple mechanisms.

The requirement for $p16^{INK4A}$ for the onset of senescence

$p16^{INK4a}$ is an inhibitor of the cyclin D-dependent kinases CDK4 and CDK6 (for review, see [18]). These cyclin-dependent kinases are responsible for the phosphorylation of retinoblastoma-associated protein (RB) and its related family members (p130 and p107), resulting in the activation of the E2F transcription factor family and transcription of S phase-specific cell cycle genes. In the presence of $p16^{INK4a}$, the cell cycle is inhibited due to repression of E2F target genes, which are required for entry into the S phase of the cell cycle. Expression of ectopic oncogenic RAS leads to increases in the levels of $p16^{INK4a}$ protein. $p16^{INK4a}$ was implicated functionally in OIS by the observation that expression of ectopic oncogenic RAS in $p16^{INK4a-/-}$ MEFs results in transformation rather than senescence [7]; however, this effect was likely due to the concomitant deletion of the linked $p14/p19^{ARF}$ gene. The specific mechanism underlying the role of $p16^{INK4a}$ in senescence induction in human cells is not entirely clear; however, $p16^{INK4a}$ plays a role in the RB-dependent chromatin reorganisation characteristic of senescent cells [19]. Senescent cells develop foci of heterochromatin (SAHF) that are distinct from that of proliferating or quiescent cells [14], which coincide with RB-dependent repression of heterochromatic genes. Many of these genes are targets of the E2F transcription factor. In addition to its effects on the RB tumour suppressor pathway, $p16^{INK4a}$ also facilitates the formation of heterochromatic foci [19]. $p16^{INK4a}$ is required for the establishment of SAHF, but not their maintenance [19]. Therefore, $p16^{INK4a}$ may play a dual role in senescence induction through the regulation of RB and the establishment of SAHF.

RAS-induced senescence: The DNA damage response

Cells respond to DNA damage by arresting the cell cycle and initiating DNA repair mechanisms. Following the detection of DNA damage (single-stranded or double-stranded break), a multi-protein complex assembles at the site of damage followed by activation of the PI3K-family members ataxia telangiectasia gene product (ATM) and ATR. These proteins directly and indirectly activate p53 by phosphorylation. Following ectopic expression of oncogenic RAS, a brief period of increased proliferation ensues prior to the induction of senescence. This period of proliferation puts stress on the cell, leading to activation of a stress checkpoint. Di Micco and colleagues found robust activation of a DNA damage response (DDR) following expression of $H\text{-}RAS^{V12}$ [20]. Interestingly, inactivation of components of the DDR could block RAS-induced senescence and allow for transformation. Additionally, $H\text{-}RAS^{V12}$ expression increased the number of active replicons during DNA replication, but led to replication fork instability or stalling. Therefore, activation

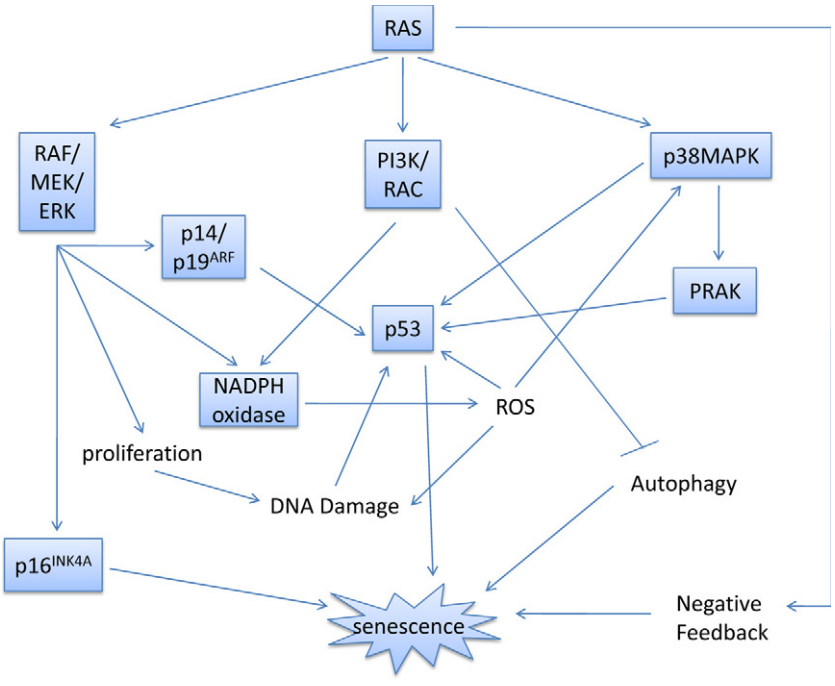

Fig. 1. Induction of OIS by oncogenic RAS. Oncogenic RAS promotes generation of ROS, activation of the DNA damage response, transcriptional upregulation of $p14/19^{ARF}$ and $p16^{INK4A}$, and activation of p53. These OIS pathways converge on p53 and $p16^{INK4A}$ to induce senescence.

of a DNA damage response induces senescence in response to overexpression of oncogenic RAS.

Reactive oxygen species production

The mitogenic response to RAS was demonstrated to involve the production of reactive oxygen species (ROS), including superoxide and hydrogen peroxide [21]. Oncogenic RAS promotes the assembly of a superoxide generating enzyme complex containing Rac-GTP, NADPH oxidase (Nox), NADPH and additional proteins [22]. Activation of this complex occurs by increased transcription of the Nox mRNA due to RAF/MEK/ERK signalling, and increased Rac-GTP binding to the Nox complex due to activation of PI3K or Tiam1 [23,24]. As ROS are highly reactive and can damage DNA, protein, RNA, and lipids, production of ROS can result in cell cycle arrest, senescence, or even cell death. Indeed, Lee and colleagues found that induction of senescence by RAS was due to the production of ROS, and culturing mutant RAS-overexpressing cells in antioxidants or in low oxygen conditions prevented the onset of senescence [25]. The exact mechanism of ROS-induced senescence is unknown but likely involves a combination of factors. As ROS damages DNA [26], subsequent activation of the DNA damage response pathway results in p53 activation and cell cycle arrest. p53 is also a direct sensor of ROS, because cells exposed to H2O2 demonstrate increased levels and activity of p53 [27]. ROS can promote the stabilisation of both p53 mRNA and protein, leading to increased activation of downstream targets [27,28]. ROS can also activate signalling pathways upstream of known senescence effectors. For example, the p38MAPK pathway is activated by ROS and p38MAPK can directly phosphorylate p53 on serine 33, resulting in the activation of p53 transcriptional activity [16,29]. Therefore, elevated production of ROS by ectopic RAS expressing cells activates several pathways that converge on p53 to induce senescence (Fig. 1).

Negative feedback and senescence

Neurofibromatosis type 1 causes hyperactivation of RAS due to inactivation of the RAS-GTPase activating protein neurofibromatosis-related protein-1 (NF1). Cichowski and colleagues found that acute NF1 deficiency in primary human fibroblasts rapidly induced senescence, and this correlated with attenuation of the PI3K/AKT and RAF/MEK/ERK pathways due to expression of RAS pathway negative regulators, including Spred, Sprouty and Map kinase phosphatases [30]. Ectopic introduction of an

activated RAF allele similarly resulted in decreased levels of RAS-GTP, attenuation of the PI3K pathway and induction of senescence, suggesting decreased PI3K signalling is universally important for continued proliferation. Attenuation of the PI3K pathway by ectopic oncogenic RAS has additionally been shown to be correlated with the onset of autophagy, and follows the initial proliferative burst [31]. Autophagy is the organised process of recycling cellular proteins and organelles, and may be important for maintaining the high metabolic rate of senescent cells, despite their non-proliferative state. Blocking the induction of autophagy with short hairpin RNA (shRNA) vectors targeting the autophagy effector ATG5 delays the onset of senescence and increases cellular proliferation. Therefore, following the initial hyperactivation of downstream pathways by ectopically expressing RAS or deleting NF1, subsequent activation of negative feedback loops results in the attenuation of these pathways, which contributes to the senescence response.

RAS-induced transformation: a question of dosage?

Following the observation that RAS proteins induce senescence in primary cells, it was postulated that tumours could only form when RAS mutations occurred in cells that had also acquired mutations in genes involved in the senescence response, such as p53 or $p16^{INK4a}$. In support of this hypothesis, frequent alterations in RAS mutant tumours include the tumour suppressors p53, $p16^{INK4a}$, and $p14/p19^{ARF}$. On the other hand, recent data suggests that the RAS-induced senescence response may be an effect of gene dosage.

While ectopic expression of RAS in fibroblasts results in hyperactivation of RAS effector pathways, stabilisation of p53 and senescence, physiological expression of $K\text{-}RAS^{G12D}$ from its endogenous promoter fails to hyperactivate downstream pathways [32]. Levels of activated ERK and AKT are slightly attenuated by endogenous $K\text{-}RAS^{G12D}$, and $p19^{ARF}$ and p53 levels increase but to a lesser extent than in ectopic RAS expressing cells [32]. In contrast to ectopic RAS-induced senescence, endogenous expression of $K\text{-}RAS^{G12D}$ results in immortalisation and partial transformation of murine fibroblasts, and tumourigenesis *in vivo* with no obvious signs of senescence [32].

In support of the idea that these effects are due to gene dosage, a comparison of high and low expression levels of $H\text{-}RAS^{V12}$ in the mouse mammary gland was performed [33]. Chodosh and colleagues found that high levels of $H\text{-}RAS^{V12}$ induced a transient period of mammary epithelial cell proliferation followed by induction of senescence, with increased levels of the senescence markers p53, $p19^{ARF}$, $p16^{INK4A}$ and PML. Deficiency in the senescence effectors $p16^{INK4A}/p19^{ARF}$ negated the senescence response. In contrast, low levels of $H\text{-}RAS^{V12}$ expression that approximated endogenous RAS promoted continuous proliferation and hyperplasia. These mammary cells lacked detectable senescence markers and eventually progressed to tumours. Therefore, unlike high levels of oncogenic RAS expression, a low level of oncogenic RAS expression does not induce a senescence response *in vivo*, suggesting that OIS may only occur in certain situations in tumour evolution.

Concluding remarks

Although the many mechanisms of RAS-induced senescence are complex, they are not isolated pathways. They converge on common effectors, such as p53 and $p16^{INK4a}$, to execute the senescence response, and have common second messengers, such as ROS, that can activate several pathways including the DNA damage response, p53, and p38MAPK (Fig. 1). It is likely that the combination of these pathways pushes the ectopic RAS expressing cell over the threshold resulting in senescence. Gene dosage and thresholds need to be taken into account when studying proteins such as RAS. Indeed, although high levels of oncogenic RAS can induce senescence in cell culture it is unclear how and when senescence occurs in human tumour progression. As RAS overexpression and gene amplification is a common feature of malignant tumours [34], the acquired mutations of p53, $p16^{INK4a}$ and $p14/p19^{ARF}$ may allow for the increased signalling needed for progression to the malignant state and the avoidance of the senescence checkpoint caused by RAS pathway hyperstimulation. Therefore, a deeper understanding of the ability of RAS mutant cells to withstand oncogenic stress will likely inform the development of successful therapeutics against tumours harbouring this oncogene.

Conflict of interest statement

None declared.

Abbreviations

- Ataxia Telangiectasia gene product (ATM): mutated in familial syndrome with role in DNA damage response.

- Extracellular Regulated MAP kinase (ERK): synonym for MAP kinase.
- GTP, GDP: nucleotide phosphate forms of Guanine that are important in the synthesis of RNA and also bind to "G-proteins" in the cell.
- GTPase: enzymatic hydrolysis of GTP to yield GDP and inorganic phosphate. Can be intrinsic to the G-protein itself, or allosterically stimulated by GAPs.
- Mammalian target of Rapamycin (mTOR): raptor, a binding protein of mTORC. Important role in the control of protein synthesis and response to nutrient deprivation.
- Messenger RNA: form of RNA that is translated to protein.
- Mitogen Activated Protein Kinases: MAP kinases, synonym of ERK.
- NADPH: reduced form of NADP and a co-factor for many cellular processes in the control of oxidative stress and other important processes.
- Neurofibromatosis related protein-1 (NF-1): autosomal dominant disease resulting in high risk of schwannomas and peripheral nerve sheath tumours. NF-1 is a GAP for Ras-GTP.
- Oncogene-induced: a cellular state caused by the oncogenic expression.
- Retinoblastoma: autosomal dominant cancer syndrome including retinal tumours, osteosarcomas in children. Rb is a major control tumour suppressor gene of the cell cycle.
- Short hair-pin RNA (ShRNA): term to describe a plasmid DNA form of RNA interference.

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