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Ras oncogenes: split personalities

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Abstract

Extensive research on the Ras proteins and their functions in cell physiology over the past 30 years has led to numerous insights that have revealed the involvement of Ras not only in tumorigenesis but also in many developmental disorders. Despite great strides in our understanding of the molecular and cellular mechanisms of action of the Ras proteins, the expanding roster of their downstream effectors and the complexity of the signalling cascades that they regulate indicate that much remains to be learnt.

Discoveries made in the late 1970s and the early 1980s revealed that the transforming activities of the rat-derived Harvey and Kirsten murine sarcoma retroviruses contribute to cancer pathogenesis through a common set of genes, termed *ras* (for rat sarcoma virus). Soon thereafter, the use of recently developed techniques in gene transfer, DNA sequencing and DNA mapping led to the identification of *ras* genes as key players in experimental transformation as well as in human tumour pathogenesis. Unanswered by these initial discoveries, however, was the signalling context in which the Ras proteins operate — which proteins impart signals to Ras and how do the resulting functionally activated Ras proteins pass signals on to downstream targets within the cell? The answers to these questions were complicated by the discoveries of multiple Ras regulators and a large cohort of downstream effectors, each with a distinct pattern of tissue-specific expression and a distinct set of intracellular functions. Despite the lack of a complete understanding of the normal and pathological functions of Ras, significant progress has been made over the past three decades. Here, we chronicle some of these milestones, with particular emphasis on the role of Ras in normal and deregulated cellular physiology.

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DATABASES

ProteinDataBank: <http://www.pdb.org/pdb/home/home.do>

[1bkd](#) | [1wq1](#) | [4q21](#) | [5p21](#)

FirstGlance in Jmol (3D structures): <http://molvis.sdsc.edu/fgij/index.htm>

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[E1A](#) | [Ha-ras](#) | [Ki-ras](#) | [myc](#) | [N-ras](#) | [NF1](#) | [PTPN11](#) | [RIN1](#) | [SV40 large T antigen](#) | [Tiam1](#)

Interpro: <http://www.ebi.ac.uk/interpro>

[Cdc25](#) | [DH](#) | [PH](#) | [SH2](#) | [SH3](#)

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Cancer precursors

The cellular homologues of the viral Harvey and Kirsten transforming *ras* sequences were first identified in the rat genome in 1981 (REF. 1) and were subsequently found in the mouse² and human³ genomes. These discoveries revealed that the *ras* oncogenes behaved, at least superficially, like the *src* oncogene of Rous sarcoma virus (RSV), the origins of which were first reported in 1976 (REF. 4). Thus, protooncogenes residing in the genomes of normal cells can be activated into potent oncogenes by retroviruses, which acquire these sequences and convert them into active oncogenes. The Harvey sarcoma virus-associated oncogene was named *Ha-ras* (*H-ras* in mammals), whereas that of Kirsten sarcoma virus was termed *Ki-ras* (*K-ras* in mammals). Mutant alleles of these *ras* sequences were soon discovered in many human cancer cell lines, including those of the bladder, colon and lungs^{5–7} (TIMELINE).

Timeline. Key events in the field of Ras research

GAP, GTPase-activating proteins; GEF, guanine nucleotide-exchange factors; MAPK, mitogen-activated protein kinase; PI3K, phosphoinositide 3-kinase; PIK3A, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit- α ; PLC, phospholipase C; RalGDS, Ral guanine nucleotide-dissociation stimulator; SV40, simian virus-40; TERT, telomerase reverse transcriptase; TIAM1, T-cell lymphoma invasion and metastasis-1.

Detailed sequence analyses revealed that the oncogenic alleles invariably differed from their wild-type counterparts by point mutations that affect the reading frames of the various *ras* oncogenes. The resulting amino-acid replacements were usually found to affect residue 12, and also less commonly residues 13 and 61 (REFS 8–11). By 1983, the third member of the mammalian family of *ras*-related genes, *N-ras*, had been cloned from neuroblastoma and leukaemia cell lines^{12–15} (FIG. 1). This gene was also found to contain activating point mutations in certain human tumours.

The efficiency of the *ras* lesion in initiating cellular transformation in primary cells was soon questioned when it was discovered that a *ras* oncogene could not transform freshly isolated rodent embryo cells. Consequently, three reports that were published in 1983 described the ability of H-Ras-Val12 to transform primary cells that had previously been immortalized by either carcinogens¹⁶ or transfection with *myc*, SV40 large T antigen or adenovirus *E1A* oncogene^{17,18}. These findings extended the concept of multistep carcinogenesis and suggested that mutant Ras proteins can only transform (to a tumorigenic state) cells that have undergone predisposing changes. As verified in various cell types^{19–21}, these predisposing changes usually involved the acquisition by cells of an ability to proliferate indefinitely in culture — the phenotype of cell immortalization (BOX I).

Box 1

The Ras tumour-suppressor effect

Oncogenic Ras has been shown to cause senescence in primary cells through the activation of the p53–p21WAF and/or p16INK4A–Retinoblastoma (Rb) tumour-suppressor pathways^{212–214}. These observations suggested that the normal response of ‘uninitiated’ naive cells to hyperactive Ras signalling is to undergo cell-cycle arrest and/or senescence rather than unlimited proliferation and/or transformation. Indeed, the susceptibility of certain normal cells to Ras-mediated transformation would seem to rely mostly on prior inactivation of these tumour-suppressor pathways (for example, see REF. 215). In addition, the findings that Ras-induced senescence in primary cells explained the molecular basis of the oncogenic cooperation model that was proposed in 1983 (see REFS 17,18), as Ras-collaborating oncoproteins, such as E1A, SV40 and E6/E7 are all well-known inactivators of the Rb and the p53 pathways. Collectively, these observations suggested that Ras activation in normal cells causes a halt in cell proliferation rather than an initiation of tumorigenesis.

Other groups have reported that human embryonic fibroblasts are resistant to Ras-induced cellular senescence²¹⁶. Furthermore, *in vivo* expression of oncogenic K-Ras at levels comparable to those of its endogenous counterparts caused cellular transformation^{217,218}. These and similar experiments led to the finding that p16 was the key factor in determining whether cells become senescent or are transformed in response to Ras activation. High levels of *Ras* expression cause an acute elevation of p16, resulting in cell-cycle arrest. By contrast, moderate activation of Ras (such as that mimicked in the endogenous K-Ras mouse models) does not cause an acute p16 response, allowing Ras-induced transformation. Similarly, Ras activation in the background of suppressed p16 (for example, in cells maintained at low-stress-culture conditions or by using short hairpin RNA against p16) causes cellular proliferation.

Ras activation of p16 and p53 seem to depend, in part, on the activation of the p38 mitogen-activated protein kinase (MAPK) pathway²¹⁹ through unidentified mechanisms. Ras activation has been shown to cause elevated reactive oxygen species (ROS) levels²²⁰, and ROS promote p38 activation²²¹. However, whether ROS are the mediators

of Ras activation of the p38 MAPK remains to be fully delineated. The Ras-induced Raf-MAPK pathway might feed into the p53 pathway by activating the p38-regulated/activated protein kinase (PRAK), which in turn phosphorylates and activates p53 directly²²². MDM2, murine double minute-2; PI3K, phosphoinositide 3-kinase; p14/19ARF, p14ARF in humans and p19ARF in mice.

By 1984, mutant K-RAS alleles were found in lung carcinoma specimens, indicating that these mutations did not arise as a consequence of culturing cancer cells *in vitro*^{22,23}. Moreover, specific associations were found between the various RAS oncogenes and particular types of human cancer (TABLE 1). K-RAS mutations were frequent in pancreatic²⁴ and colonic carcinomas²⁵, H-RAS mutations were predominantly found in bladder carcinomas²⁶, and N-RAS mutations were linked primarily to lymphoid malignancies^{27–31} and melanomas³².

Experimentally induced tumours were also found to harbour mutant *ras* genes. For example, mouse mammary and skin tumours that were induced by the topical application of carcinogens contained H-Ras mutations^{33–35}, whereas carcinogen-induced thymomas carried activating N-Ras mutations³⁶. Other long-known carcinogens, such as ionizing radiation, were also found to cause K-Ras mutations in mice³⁷ as well as in tissue-culture cells³⁸. However, these discoveries provided no insight into the main puzzle surrounding the mutant, oncogenic *ras* alleles: why were the activating mutations invariably localized to a small number of sites in the reading frames of these genes?

Activated mutants and the GTPase cycle

Ras proteins were initially described to bind to guanine nucleotides in 1980 by Edward Scolnick's group, which was investigating the biochemical features of the viral H-Ras protein³⁹. This binding suggested that Ras might function analogously to the heterotrimeric G proteins, which had previously been found to possess an intrinsic GTP hydrolysis (or GTPase) activity that shuttles them from an active to an inactive signalling state. The reactivation of these G proteins occurred when their bound GDP nucleotide was ejected, making room for the binding of the more abundant cellular GTP (reviewed in REF. 40).

The actual biochemical proof that Ras proteins were indeed GTPases came several years later. In 1984, three groups reported that mutated Ras oncoproteins differ functionally from their normal counterparts^{41–43}. The oncogenic forms of Ras exhibited impaired GTPase activity, which suggested that the hydrolysis of GTP somehow terminates the activated state of the protein, which is consistent with the presumed analogy to the behaviour of G proteins. Furthermore, the link between the much-studied Gly-to-Val substitution of residue 12 of H-Ras and GTP hydrolysis was made the following year by Frank McCormick's group, which noted that antibodies that are specific to that region blocked GTP binding⁴⁴. Other oncogenic mutations (such as Gln61Leu in H-Ras) were also shown to impair GTP hydrolysis⁴⁵ and other oncogenic forms of Ras were later determined to be impaired in GTP hydrolysis (for example, REF. 46). However, the extent of such impairment did not always correlate with transformation, suggesting that compromised intrinsic GTP hydrolysis was necessary but not sufficient for aberrant Ras activation.

Post-translational modifications

Post-translational lipid processing of Ras was found to be another key determinant of its functioning. An initial study in this area, published in 1982, showed that the mature form of viral H-Ras localized to the cell membrane⁴⁷. Several months later it was demonstrated that viral H-Ras is palmitoylated at the C terminus; the resulting attached lipid moiety facilitated

its association with the membrane⁴⁸. The functional connection between this lipid modification and Ras function was made by Douglas Lowy's group in 1984, which showed that lipid binding and membrane association were actually required for the transforming activity of the viral H-Ras oncoprotein^{49,50}. Working with cellular H-Ras, Stuart Aaronson's group proceeded to demonstrate that this C-terminal processing and membrane recruitment of Ras is a prerequisite to its biochemical activation⁵¹.

The molecular mechanisms of Ras lipid processing were laid out over the subsequent 5 years through a series of observations using yeast genetics, protein biochemistry and *in vitro* cellular systems^{52–57} (FIGS 2,3). Indeed, the C-terminal CAAX motif, previously found to be important for Ras function, was found to be the target of a post-translational modification that involved the addition of a farnesyl isoprenoid lipid, catalysed by the enzyme farnesyl transferase (FTase). Subsequent studies determined that this prenylation reaction is followed by the proteolytic cleavage of the AAX sequence, catalysed by Ras-converting enzyme-1 (RCE1) and the carboxymethylation of the now terminal Cys residue by the isoprenylcysteine carboxymethyltransferase-1 (ICMT1) enzyme.

Although these CAAX-signal modifications appeared to be essential for the association of Ras with the plasma membrane, other studies identified the requirement for a second C-terminal signal that facilitates full membrane recruitment and hence full Ras function (for example, see REF. 57). For K-Ras-4B, this second signal is a string of positively-charged lys residues upstream of the C terminus that are sufficient to anchor the protein to the membrane. However, prenylated H-Ras, N-Ras and K-Ras-4A require a further palmitoylation step in which a palmitoyl moiety is attached to upstream C-terminal Cys residues before their anchoring in the membrane is stabilized. The enzymes that catalyse these various steps became attractive targets for drug development efforts.

Upstream regulation

The first indications that Ras activity is modulated by associated proteins came from the group of James Feramisco in 1984, who observed increased GTPase activity of cellular H-Ras in response to treatment with epidermal growth factor (EGF)⁵⁸. Indeed, Ras activation appeared to be vital for signalling by extracellular mitogens, as microinjected antibodies that were directed against Ras blocked the serum-stimulated cellular growth of NIH 3T3 cells⁵⁹ and the growth factor-induced differentiation of PC12 cells⁶⁰. At the time, multiple groups also described the activation or inhibition of Ras proteins by growth factors⁶¹ and cytokines^{62–64}. Similar observations made in non-mammalian systems, such as *Xenopus laevis* oocytes⁶⁵, suggested that Ras proteins, similar to G proteins, can function as transducers of signals arising from plasma-membrane-associated receptors⁶⁶.

Still, the nature of the factors that regulate the activation and inactivation of Ras proteins remained unclear. In fact, inactivators of Ras signalling were first identified with the discovery of Ras GTPase-activating proteins (GAPs). The first member of the RasGAP family was identified and purified by the groups of both McCormick and Scolnick and was found to possess GTPase-promoting functions that preferentially functioned on the normal, but not oncogenic, N-Ras or H-Ras proteins^{46,67–69}. The group of Stuart Aaronson reported 1 year later that platelet-derived growth factor (PDGF) receptor stimulation promoted Tyr phosphorylation of RasGAP, thereby enabling its membrane translocation⁷⁰ and providing the first molecular link between mitogenic signalling and Ras regulators. Soon thereafter, the second RasGAP, neurofibromin-1 (NF1), was identified and found to be disrupted in patients with neurofibromatosis^{71–74}. This finding provided the earliest indication of aberrant Ras signalling in a developmental disease (discussed below).

Although informative, these studies did not reveal the proteins that are responsible for the functional activation of Ras. Such proteins were named either guanine nucleotide-exchange factors (GEFs) or guanine nucleotide-releasing proteins (GRFs). The yeast cell-division-cycle-25 (*Cdc25*) protein (not to be confused with the cyclin-dependent kinase phosphatase of the same name) was the first RasGEF to be identified^{75,76} and was found to activate Ras by stimulating its guanine nucleotide-exchange activity.

The mammalian counterparts of *Cdc25* were characterized in cellular extracts several years later by a number of groups^{77–81}, in efforts that culminated with the cloning of the first mammalian RasGEF, termed son of sevenless (*SOS*) because of its contribution to the morphogenesis of the *Drosophila melanogaster* eye⁸². Indeed, SoS served as the prototype for a number of other proteins that exhibited accelerated guanine nucleotide-exchange activity towards Ras, collectively forming the *Cdc25* family of RasGEFs.

The connection between RasGEFs and upstream mitogenic signalling was described by a number of groups in 1993, when it was demonstrated that the adaptor protein growth factor receptor-bound protein-2 (*GRB2*) associates with the eGF receptor and concomitantly attaches to the mammalian SoS^{83–87}. Subsequently, several more Ras-specific GAPs and GEFs were characterized, each possessing, in addition to the GAP or *Cdc25* domains, a number of signalling motifs, such as the Src-homology-2 (*SH2*) or *SH3* modules, which enable their physical and functional interactions with a variety of regulatory partners^{88,89}. The molecular mechanisms of how these GAPs and GEFs regulate the activity of Ras GTPases became apparent only later.

Anatomy of Ras regulation

The sequence homologies between Ras proteins and the functionally analogous heterotrimeric G proteins (which were better characterized at the time) expedited much of the initial biochemical and structural investigations of Ras GTPases in the 1980s (for example, see REFS 90,91). Comparative analyses accompanied by site-directed mutagenesis studies identified residues in the Ras N and C termini as being important for GTP binding and cell transformation^{92–96}. Moreover, these studies produced a wealth of mutant Ras proteins that have proven to be of great use in deciphering downstream signal-transduction pathways.

Ras three-dimensional structure

Detailed insights into the three-dimensional fold of Ras proteins were provided in 1990 by crystallographic determinations of the structures of GDP- and GTP-bound Ras proteins^{97–100} and of their mutant variants^{101,102}. The overall Ras structure was shown to consist of a hydrophobic core of six stranded β -sheets and five α -helices that are interconnected by a series of ten loops (FIG. 4a). Five of these loops are situated on one facet of the protein and have crucial roles in determining the high affinity nucleotide interactions of Ras and in regulating GTP hydrolysis. In particular, the GTP γ -phosphate is stabilized by interactions that are established with the residues of loops 1, 2 and 4 (for example, Lys16, Tyr32, Thr35, Gly60 and Gln61; see FIG. 4b). A prominent role is attributed to Gln61, which stabilizes the transition state of GTP hydrolysis to GDP, in addition to participating in the orientation of the nucleophilic attack that is necessary for this reaction. As such, oncogenic mutations of Gln61 reduce the intrinsic GTP hydrolysis rate, thereby placing the Ras protein in a constitutively active state.

The structural differences between the RasGDP and the RasGTP conformations reside mainly in two highly dynamic regions, termed switch i (residues 30–40) and switch ii (residues 60–76). Both regions are required for the interactions of Ras with upstream as well

as downstream partners (see also FIG. 2a). The binding of GTP alters the conformation of switch i, primarily through the inward reorientation of the side chain of Thr35, thereby enabling its interactions with the GTP γ -phosphate as well as the Mg^{2+} ion. Similarly, the γ -phosphate induces significant changes in the orientation of the switch ii region through interactions it establishes with Gly60 (FIG. 4b).

GAP- and GEF-mediated regulation of Ras activity

The structural details of GAP-mediated and GEF-mediated regulation of Ras activity were ultimately laid out by the groups of both Alfred Wittinghofer and John Kuriyan in the late 1990s, when the structures of H-Ras in complex with the p120GAP GRD¹⁰³ (GAP-related domain) and that of H-Ras in complex with the catalytic domain of SoS¹⁰⁴ were reported (FIG. 4c,d,e). Importantly, the binding of the variable loop of GAP $\alpha 7$ to the switch I of Ras establishes the pairing specificity between GAP and Ras. This is followed by a high affinity interaction with the GAP Phe-Leu-Arg (FIR) motif, which stabilizes the two switch domains as a highly conserved GAP Arg-finger loop⁸⁸ inserts itself into an active site, provoking a ~1,000-fold acceleration in GTP hydrolysis.

An analogous mechanism has been described for the SOS-mediated ejection of guanine nucleotides from Ras. During the course of this interaction, an α -helical hairpin of the Cdc25 domain pries open the switch I and switch II domains of Ras, causing a series of side-chain rearrangements that involve Ala59, which reorients and inserts itself into the Mg^{2+} -binding cleft. These changes, together with structural perturbations of the phosphate-binding loop, cause a 10,000-fold enhancement in the GDP ejection rate and its preferential replacement in the nucleotide-binding site with GTP, which is approximately tenfold more abundant than GDP in the cytosol.

Downstream effector signalling

The mechanistic details of how Ras proteins function in cellular signalling were lacking for much of the 1980s. At the time, however, several Ras-induced changes in cellular biochemistry had been catalogued by a number of research groups. Among the earliest of such reports came from the Feramisco laboratory in 1986, which described the activation of phospholipase A₂, a calcium-dependent glycerophospholipid esterase, within minutes of the microinjection of Ras proteins into quiescent rat-embryo fibroblasts¹⁰⁵. Other groups reported a number of similar biochemical changes that are associated with Ras expression, such as the phosphorylation of mitochondrial proteins¹⁰⁶, the phosphorylation of plasma-membrane components¹⁰⁷, increased diacylglycerol levels^{108–110}, enhanced phospholipid metabolism and the activation of protein kinase C (PKC)^{111–113}. At the time, it was not clear whether such biochemical alterations were secondary, pleiotropic effects of the forced overexpression of Ras. In 1988, Fukami and colleagues showed that antibodies that were injected against phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) inhibited Ras-induced mitogenesis¹¹⁴, indicating that some of the downstream biochemical responses to Ras activation are actually crucial to its cell-biological effects. Experiments such as these fuelled the search for the direct downstream signalling effectors that interact directly with Ras and mediate its various functions (FIG. 5).

The connection with RAF1 and the MAPK cascade

In 1993, four groups reported the direct interaction of Ras with the RAF1 Ser/Thr kinase, which was the first bona fide mammalian Ras effector to be identified^{115–118}. RAF1, originally discovered through its association with avian and murine transforming retroviruses, was extensively characterized over subsequent years. This research revealed the ability of RAF1 to signal through a pathway that involves the mitogen-activated protein

kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase (MEK), ERK1/2, and the E26-transcription factor proteins (ETS). This signalling cascade became the prototype of a number of other plasma-membrane-to-nucleus signal-transduction pathways. Interestingly, the role of MAPK signalling in Ras biology, initially described in 1992 by leever, wood and colleagues^{119,120}, has been characterized by several groups and shown to be both sufficient and necessary for Ras-induced transformation of murine cell lines^{121–124}. Furthermore, the subsequent identification of B-Raf mutations in cancers, in non-overlapping frequencies with Ras mutations (for example, in melanoma and colon cancer¹²⁵) emphasized an important role for aberrant Raf–MEK–ERK signalling in oncogenesis.

Ras signals through PI3K and Ral proteins

A year later, two other Ras effectors were identified: the p110 catalytic subunit of the class I phosphoinositide 3-kinases (PI3Ks)¹²⁶ and the guanine nucleotide-exchange factors for the Ras-like (RalA and RalB) small GTPases (Ral guanine nucleotide-dissociation stimulator (RalGDS) and RalGDS-like protein (RGL))^{127–129}. As with Raf, PI3K activity was shown to be required for Ras transformation of NIH 3T3 cells¹³⁰. Indeed, the anti-apoptotic effects of Ras were ascribed to the pro-survival activities of activated PI3K signalling through a pathway that involves the Ser/Thr kinase AKT/protein kinase B and the transcription factor nuclear factor- κ B (NF- κ B), both of which have crucial roles in preventing anoikis^{131–133}.

Initial studies of the RalGDS–Ral pathway in rodent fibroblasts indicated a limited involvement of this effector pathway in Ras-mediated cell transformation^{134,135}. More recent studies in human cells, however, have suggested the opposite. Thus, activation of the RalGEF–Ral pathway alone, but not the PI3K or the Raf pathways, was sufficient to promote Ras transformation of human kidney epithelial cells¹³⁶. Furthermore, evidence from the laboratory of Michael White has recently suggested prominent but distinct roles for the highly related RalA and RalB GTPases in regulating cellular proliferation as well as countering apoptosis, respectively¹³⁷. Unresolved, however, are the mechanistic details of the downstream functions of Ral in normal and/or pathological settings.

Additional Ras effectors with diverse functions

Over the years, several other Ras effectors have been described, which include a number of proteins with diverse roles in cell physiology. These include phospholipase C- ϵ (PLC ϵ), T-cell lymphoma invasion and metastasis-1 (TIAM1), Ras interaction/interference protein-1 (RIN1), All (acute lymphoblastic leukaemia)-1 fused gene on chromosome 6 (AF-6, also known as afadin) protein, and the Ras association domain-containing family (RASSF) proteins. In 2001, PLC ϵ was described to be a direct effector of Ras^{138,139}. The activation of PLC ϵ enables it to cleave Ptdins(4,5)P₂ into inositol-1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG), promoting the release of Ca₂₊ and the activation of PKC, respectively. PLC ϵ contains a Cdc25 domain, which also places it upstream of Ras. Indeed, full length PLC ϵ or its isolated Cdc25 domain have been shown to activate the Ras–MAPK pathway¹⁴⁰.

TIAM1 was characterized by Channing Der's group as a Ras effector in 2002 (REF. 141). Independently, John Collard and colleagues showed that Tiam1^{−/−} mice exhibited resistance to Ras-induced skin carcinogenesis that was caused by 7,12-dimethylbenz(a)anthracene (DMBA) treatment, suggesting that TIAM1 activity is required for Ras transformation¹⁴². Of interest is the fact that the few tumours that developed in these knockout mice exhibited an elevated apoptotic index and were more invasive than those growing in wild-type mice. These findings suggest that there are potentially opposing roles for TIAM1 in the initiation, maintenance and progression of *ras*-driven tumours. It is worthwhile to note that mice that

are deficient for RalGDS, PLC ϵ or the p110 α subunit of PI3K all exhibit impaired Ras-induced skin cancers^{143–145}, which suggests a central role for all of these effectors in the processes of tumorigenesis.

Some of the less-characterized Ras effectors include AF-6, RIN1 and the RASSF proteins. AF-6 was identified in 1996 by Kozo Kaibuchi's group using glutathione *S*-transferase (GST)–RasGTP affinity chromatography¹⁴⁶. Subsequent sequence analysis indicated that AF-6 contains both microtubule- and actin-binding motifs, which suggested that AF-6 might participate in cytoskeletal activities downstream of Ras¹⁴⁷. Indeed, AF-6 was found to associate with proteins that are involved in regulating cell polarity (such as ponsin and profilin) and to localize to adherens junctions¹⁴⁸.

Ras-interacting proteins also include regulators with reported tumour-suppressor activities, such as RIN1 and the RASSF proteins. Originally identified in yeast, RIN1 was characterized as an effector for Ras in 1995 and was the first example of a downstream element that blocked Ras transformation¹⁴⁹. The high-affinity interactions of RIN1 with Ras suggest that one of the mechanisms through which it antagonizes Ras function could be through competition with Raf. Alternative models propose a mechanism in which RIN1, which is described as a GEF for RAB5-like proteins, can trigger endocytosis of Ras-stimulating growth factor receptors, such as the eGF receptor¹⁵⁰, thereby inhibiting Ras signalling. Recently, the *RIN1* gene was found to be silenced in breast-tumour cell lines¹⁵¹. Indeed, re-expression of RIN1 in cells from these lines blocked anchorage-independent growth *in vitro* and tumour formation *in vivo*¹⁵¹.

Three members of the RASSF proteins have been characterized to date. *RASSF5* (also known as NoRel) was isolated in 1998 (REF. 152) and was later shown to possess pro-apoptotic functions¹⁵³, as do *RASSF1* and *RASSF2* (REFS 154,155). Although the detailed mechanism of action for RASSF proteins has not been fully delineated, preliminary evidence points to the association of certain RASSF proteins with the mammalian sterile-20-like protein kinase-1 (*MST1*) and *MST2*; in *D. melanogaster* these kinases participate in a pathway that suppresses the activity of cyclin E and leads to cell-cycle arrest and apoptosis. Indeed, *Rassf1a*-knockout mice are more susceptible to spontaneous or induced tumorigenesis¹⁵⁶, which is consistent with the role of RASSF1A as a tumour suppressor.

A time and place for Ras activation

Mounting evidence indicates that the several Ras isoforms have differing functions in various tissues, despite a great deal of shared structural, biophysical and biochemical properties. The first evidence to support this hypothesis was derived from the association between activated *RAS* oncogenes and specific human cancers (TABLE 1). This association has been attributed, to a large extent, to the preferential expression of specific Ras isoforms in the affected organs¹⁵⁷. Indeed, experiments in mouse genetics have suggested that there are distinct functions of Ras isoforms in specific tissues throughout development. For example, K-Ras-4B, but not K-Ras-4A, is essential for embryogenesis, as K-Ras-4B-deficient embryos succumbed to anaemia, liver defects and cardiac abnormalities early in gestation^{158,159}. By contrast, H-Ras- and/or N-Ras-deficient animals develop normally and are viable^{160,161}, which suggests that their functions are mostly dispensable or at least redundant. More recently, mice in which the H-Ras coding sequence was knocked into the locus of K-Ras (resulting in the loss of detectable K-Ras protein) were also shown to be viable¹⁶², indicating that H-Ras can functionally replace K-Ras during embryogenesis when its expression is controlled by the K-Ras promoter. Most importantly, these findings suggest that the mortality of the K-Ras-knockout mice might not result from the intrinsic inability of other Ras isoforms to compensate for K-Ras functions, but might rather derive from the

inability of the other isoforms to be expressed in the same embryonic compartments as K-Ras.

The tissue-specific requirements of the various Ras isoforms might be explained by the contrasting abundance of these proteins in various tissues¹⁶³. Alternatively, these requirements might be explained by the disparity in the distributions and the activation outputs of these isoforms within the intracellular membrane compartments of various cell types. For example, plasma-membrane-tethered K-Ras can induce transformation, whereas mitochondrial K-Ras induces apoptosis¹⁶⁴. Moreover, although activated H-Ras is associated with both the Golgi apparatus and the endoplasmic reticulum (ER), only the ER-associated form can activate RAF1–ERK signalling¹⁶⁵. In contrast to the ER-tethered H-Ras¹⁶⁵, the Golgi-associated forms do not induce transformation¹⁶⁶, suggesting that the subcellular distribution of Ras effectors determines their activation by Ras and, in turn, regulate Ras functions.

Although extensive Ras research is conducted using activated mutants of Ras proteins that are overexpressed in cellular systems, it is clear that the subcellular distribution of these overexpressed hyperactive variants might differ significantly from that of their endogenous counterparts. Indeed, much of the growth factor-induced activation of Ras is reported to occur at the plasma membrane, not at the ER or the Golgi¹⁶⁷. Moreover, it seems that the functional activation of Ras depends on its release from lipid-raft microdomains in the plasma membrane and its redistribution to other nearby sites, enabling its physical engagement with various downstream effectors¹⁶⁸.

Beyond oncogenesis: Ras in development

In the 1990s, the discovery that some of the well-known Ras regulators are mutated in several congenital developmental disorders suggested that aberrant Ras regulation can lead to more than oncogenic transformation. Thus, the identification of mutations in Ras regulators (such as *NF1*; REFS 169–171) or downstream effectors (such as Raf¹⁷²) strongly indicated that deregulated Ras signalling can also cause aberrant development. Several developmental disorders, collectively known as cardio-facio-cutaneous (CFC) diseases, have been linked to Ras signalling (FIG. 5); these include neurofibromatosis type-1, Costello and Noonan syndromes.

Neurofibromatosis type-1 is a dominantly-transmitted familial cancer syndrome that is manifested by the accumulation of pigmented lesions in the skin and the eye, and by a predisposition to sporadic malignant outgrowths, such as neurofibromas, neurofibrosarcomas, pheochromocytomas, astrocytomas and juvenile myelomonocytic leukemia (*JMML*)¹⁷³. The syndrome is caused by a mutation in the *NF1* gene, which encodes neurofibromin-1, the second of the RasGAPs to be characterized^{71,73}. *Nf1*-mutant mice exhibit a multitude of abnormalities, including cardiovascular, haematopoietic and neural crest defects^{174,175}. Although expression of the RasGAP domain of NF1 in these mice rescued the cardiac and haematopoietic defects, it did not rescue the neural crest overgrowth, and mice died *in utero*¹⁷⁶. This suggests a causative role for the NF1–Ras axis in regulating developmental processes, at least in selected cell lineages of the developing embryo.

PTPN11 encodes Src-homology-2 domain-containing protein Tyr phosphatase-2 (*SHP2*), a non-receptor protein Tyr phosphatase that has a key role in conveying upstream growth factor signals to Ras¹⁷⁷. Mutations in *PTPN11* account for ~50% of Noonan syndrome cases¹⁷⁸ and are gain-of-function mutations that mostly deregulate the phosphatase activity and the substrate specificity¹⁷⁹ of SHP2. The syndrome is characterized by skeletal

abnormalities, learning disabilities, cardiac defects and symptoms that are consistent with JMML¹⁸⁰. Moreover, a direct causal link between *PTPN11* mutations and Ras signalling can be found in JMML. First, activating mutations in *PTPN11* cause deregulated ERK signalling and abnormal myeloid colony growth *in vitro*¹⁸¹. Second, activating *PTPN11* mutations are found in ~35% of cases of JMML, and the mouse model of Noonan syndrome (where the activated Asp61Gly-*Ptpn11* gene is expressed from the *Ptpn11* endogenous promoter) is also characterized by increased ERK activation¹⁸². Third, K-*Ras*-mutant mice develop JMML-like abnormalities and a significant number of human cases with JMML exhibit mutations in K-RAS¹⁸³. It is noteworthy that *PTPN11* mutations also cause LEOPARD syndrome, a disease that is characterized by cardiac and visual abnormalities, pulmonary stenosis, stunted growth and partial deafness¹⁸⁴. However, the involvement of Ras signalling in this syndrome has not yet been validated.

Costello syndrome is another CFC disease that is characterized by craniofacial abnormalities, impaired cardiac development and an increased predisposition to specific tumours, such as ganglioneuroblastomas, bladder carcinomas and rhabdomyosarcomas¹⁸⁵. The germline mutations that are associated with Costello syndrome mostly involve activating H-RAS mutations that affect codons 12, 13, 117 and 148. The most frequent lesion is the Gly12Ser mutation, which activates Ras, but to levels below those seen in the tumour-associated Gly12Val mutation¹⁸⁶.

Ras mutations have also been found in a subset of Noonan patients who do not harbour *PTPN11* mutations. These lesions are restricted to K-RAS and target residues Val14, Thr58, Val152, Asp153 and Phe156, all of which are thought to cause moderate upregulation of Ras activity¹⁸⁷. Other types of mutations that are found in patients with Noonan syndrome are *SOS1* mutations. These lesions are typically found within the Cdc25-regulatory DBI homology (DH) and/or pleckstrin homology (PH) domains and are thought to relieve the auto-inhibition that is imposed by the DH/PH domains on the RasGEF motif. Indeed, recent expression studies showed that Ras activation is elevated in cells that overexpress these *SOS1* mutants¹⁸⁸.

Antagonism of Ras: where are we?

The crucial role of prenylation in the cellular functions of Ras proteins has made the enzymes that catalyse the post-translational processing of Ras prime targets for rational drug design or compound-screening methodologies (FIG. 2). The first of such approaches adopted molecular mimicry, in which farnesyltransferase inhibitors (FTIs) that simulate the CAAX motif were used to compete with Ras for its post-translational processing enzymes, thus blocking the first step of Ras modification and thereby inhibiting its activity. Indeed, *in vivo* studies that were conducted with these inhibitors in 1995 were very promising, as FTIs caused regression of mouse mammary tumour virus-H-*ras* mammary carcinomas with no detectable systemic toxicity¹⁸⁹.

Although promising in principle, the FTI strategy has been hindered by two interrelated problems. Unlike H-Ras, both N-Ras and K-Ras were shown to become geranylgeranylated at their C termini following FTI treatment (and inhibition of FTase activity), which rendered them refractory to inactivation by FTIs¹⁹⁰. In addition, despite the success of FTIs in antagonizing N-Ras- and K-Ras-driven transformation^{191–193}, this function was later attributed to the ability of FTIs to inhibit the functions of other prenylated cellular proteins, such as Rheb (Ras homologue enriched in the brain), RhoB and the centromeric proteins CeNP-E or CeNP-F^{194–197}. These problems forced the development of alternative strategies to block Ras function.

Pharmacological inhibition of other steps of the Ras processing pathway have targeted the enzymes RCE1 and ICMT1. Although reports in the early 1990s suggested that blocking the proteolytic cleavage of Ras was not sufficient to inhibit its functions^{198,199}, more recent studies in mice suggested that Ras transformation is impaired in *Rce*-deficient animals. Indeed, conditional disruption of the *Rce1* allele caused ~50% reduction in the membrane-bound fraction of K-Ras and H-Ras, which is consistent with reduced xenograft tumour growth²⁰⁰. A similar approach was used to target ICMT1, causing a partial reduction in membrane association of K-Ras, which corresponded to the loss of its tumour-forming ability²⁰¹. Of note, drugs that inhibit ICMT1, such as methotrexate or cysmethynil, show effectiveness in tissue culture and animal models^{202,203}. However, in light of the extensive number of other CAAX-terminating proteins that serve as substrates for these two enzymes, in particular RhoGTPases, the selective and targeted nature of these approaches for Ras proteins remains unclear.

Over the years, more approaches have been developed to antagonize Ras functions *in vivo*. These include the inhibition of Ras expression by anti-sense RNA-based technology²⁰⁴, the use of anti-Ras ribozymes (hammerhead models; see REFS 205–207), or application of anti-Ras reoviral therapy^{208,209}. A significant effort has been made to develop low-molecular-weight chemical inhibitors of the downstream effectors of Ras, notably the Raf–MEK–ERK cascade (for example, Sorafenib²¹⁰ or CI-1040/PD184352, REF. 211). The long term future of these development efforts is hindered by difficulties in the mode of administration of such agents, their therapeutic index and their ability to durably suppress Ras activity *in vivo*.

Outlook

The advances achieved over the past 30 years have illuminated important details of the involvement of Ras in nearly every aspect of normal and aberrant cell physiology. Much of this knowledge has been gained using experimental approaches that relied primarily on forced overexpression of activated Ras proteins or total ablation of Ras activity in cellular systems. However, it is increasingly appreciated that ectopically expressed Ras proteins are often produced at aberrantly high levels and are often mis-localized to various subcellular sites. As such, it is imperative to revisit some of the fundamental questions regarding Ras function using experimental approaches that can more accurately mimic physiological levels of Ras expression. In addition, attention should be given to isoform-specific Ras signalling and to the question of how membrane micro-domains and cytoplasmic compartments determine the coupling of Ras to its effectors and downstream signalling pathways.

Finally, although much of the emphasis has been placed on H-, K- and N-Ras proteins, more attention must be focused on other, less-characterized family members, such as M-Ras and R-Ras, which are likely to reveal further novel insights into Ras biology. Similarly, additional effort is required to understand the full range of immediate downstream effectors of Ras beyond the canonical MAPK, PI3K and Ral proteins. With this expanding knowledge comes the hope that we might finally be able to exploit our knowledge of Ras biology for the development of truly effective therapeutics.

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Glossary

Rous sarcoma virus (RSV)	A retrovirus that was discovered in 1916 by Peyton rous by injecting a cell-free extract of chicken tumour into healthy chickens. The extract was found to induce oncogenesis in Plymouth rock chickens
G proteins	A family of proteins involved in second messenger cascades. They function as molecular switches, alternating between an inactive GDP-bound and active GTP-bound state
GTPase-activating protein	(GAP), A protein that stimulates the intrinsic ability of a GTPase to hydrolyse GTP to GDP. Therefore, GAPs negatively regulate GTPases by converting them from active (GTP bound) to inactive (GDP bound)
Guanine nucleotide-exchange factor	(GEF), A protein that facilitates the exchange of GDP for GTP in the nucleotide-binding pocket of a GTP-binding protein
Anoikis	The induction of programmed cell death by the detachment of cells from the extracellular matrix
Lipid raft	A membrane microdomain that is enriched in cholesterol, sphingolipids and lipid-modified proteins, such as glycosyl phosphatidylinositol (GPI)-linked proteins and palmitoylated proteins. These microdomains often function as platforms for signalling events
Cardio-facio-cutaneous diseases	Congenital developmental disorders caused by dysregulated ras signalling. These diseases are characterized by the accumulation of sporadic tumours as well as skeletal, cardiac and visual abnormalities
Neural crest	A group of embryonic cells that separate from the embryonic neural plate and migrate, giving rise to the spinal and autonomic ganglia, peripheral glia, chromaffin cells, melanocytes and some haematopoietic cells

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Figure 1. The ras family of small gTPases

a | The primary structure of Ras proteins. The Ras family of GTPases encompasses 36 genes, which encode 39 Ras proteins (20–29 kDa) in the human genome. Most of the Ras family proteins have been characterized and found to regulate important cellular processes, such as growth, cytoskeletal rearrangements, adhesion, motility and differentiation. Secondary structural elements are shown as arrows (for β -sheets, yellow) and cylinders (for α -helices, blue). Ras family members share substantial primary sequence homology in their N termini, particularly in the phosphate-binding loop (P-loop) and the nucleotide-sensitive switch I and II regions. The C terminus contains the membrane-targeting CAAX sequences²²³. H-Ras isoform-1 numbering indicated. **b** | Overall sequence comparison among Ras proteins. The Ras family radial tree was generated using matrices derived from a ClustalW multiple sequence alignment of the various Ras family members. a.a., amino acids.

Figure 2. C-terminal processing of ras proteins

a | C-terminal sequences. The C terminus is highly divergent among the Ras family members and contains the membrane-targeting sequences²²³. The C-terminal sequences of Kirsten- (K-), Harvey- (H-) and neuroblastoma- (N-) Ras proteins are shown. Cys residues in red or green are farnesylated or palmitoylated, respectively. **b** | C-terminal processing. A farnesyl pyrophosphate moiety (orange) is covalently attached to the newly-synthesized cytoplasmic Ras proteins by the enzyme farnesyltransferase (FTase)^{54–56}. This reaction is followed, in the endoplasmic reticulum, by the proteolytic cleavage of the last three amino-acid residues (AAX) by Ras-converting enzyme-1 (RCE1), and by the carboxymethylation of the last Cys residue by ICMT1 (for example, see REF. 224). The C-terminal Lys residues in K-Ras-4B are sufficient to anchor it in the membrane, whereas H-, N- and K-Ras-4A require a palmitoylation step (by a palmitoyltransferase (PTase)) in which a palmitoyl moiety (red) is attached to the C-terminal upstream Cys residues before their insertion in the membrane is stabilized⁵⁷. In the presence of FTase inhibitors, both K-Ras-4A and N-Ras can become prenylated by geranylgeranyl transferase²²⁵.

Figure 3. Recycling of ras proteins

Cytoplasmic Ras proteins traffic through the endoplasmic reticulum (ER) where they are post-translationally processed by farnesyltransferase. Farnesylated Kirsten (K)-Ras-4B is then transported directly to the plasma membrane through unknown mechanisms^{226,227}, whereas Harvey- (H-), neuroblastoma- (N-), and K-Ras-4A transit through the Golgi apparatus and undergo a palmitoylation step before homing to plasma-membrane invaginations enriched with the cholesterol-binding protein caveolin (see REF. 228). Efficient activation of these Ras proteins might require their localization to specific plasma-membrane microdomains (lipid raft or non-raft domains) that regulate the nature of the recruited Ras effectors (and hence the identities of the signalling pathways that are activated downstream of Ras) and the amplitude of the Ras signal activation¹⁶⁸. Endocytosed and de-palmitoylated H- and N-Ras are shuttled back from the plasma membrane to the Golgi²²⁹ where they are recycled. Alternatively, ubiquitylated plasma-membrane-tethered H- or N-Ras (representing ~2% of total cellular H- or N-Ras levels) are de-ubiquitylated and targeted to endocytic vesicles²³⁰. The nature and function of the signalling circuitry that Ras proteins execute in endosomes and other cytoplasmic organelles is an emerging area of intense investigation.

Figure 4. Anatomy of ras regulation

a | The structure of Ras. The Ras three-dimensional fold is shown to consist of six β -sheets and five α -helices interconnected by a series of ten loops. Crystallographic structures of inactive RasGDP⁹⁹ (2.0 Å resolution; Protein Data Bank code [4q21](#)) and active RasGppNHp⁹⁷ (1.35 Å resolution; PDB code [5p21](#)) are shown, with the nucleotide-sensitive switch I and II regions depicted in red and green, respectively. The GDP and GTP nucleotides are shown as balls. **b** | Nucleotide-dependent structural rearrangements. The differences between the inactive GDP-bound and the active GTP-bound Ras reside mainly in two regions, termed switch I (~residues 30–40) and switch II (~residues 60–76), both of which are required for the interactions of Ras with its regulators and effectors. The γ -phosphate induces significant changes in the orientation of the switch II region through the interactions that it establishes with Thr35 and Gly60. Notably, these two residues are among the most conserved residues in the GTPase family, suggesting that the mechanisms of GTP binding (and hydrolysis) are essentially the same among various members. **c** | Ras in complex with its regulators. The crystal structures of Ras in complex with the DBL homology (DH)/pleckstrin homology (PH) domain of son of sevenless¹⁰⁴ (SOS) (left; 2.8 Å resolution; PDB code [1bkd](#)) and with p120 Ras GTPase-activating protein¹⁰³ (p120GAP) are shown (right; 2.5 Å resolution; PDB code [1wq1](#)). **d** | The Ras guanine nucleotide-exchange factor (RasGEF) binding interface. The insertion of the α -helical hairpin of SOS (green; specifically Leu938 and Glu942) into the nucleotide pocket of Ras (yellow) reorients Ala59 and perturbs the phosphate-binding (P)-loop, facilitating guanine nucleotide ejection. **e** | The RasGAP binding interface. Similarly, the insertion of the catalytic Arg789 finger of GAP into the active site of Ras stabilizes Gln61 and contributes to GTP hydrolysis. All structures were visualized with DeepView-Swiss-PdbViewer, and images were generated with POV-Ray.

Figure 5. Ras signalling networks

Ras proteins function as nucleotide-driven switches that relay extracellular cues to cytoplasmic signalling cascades. The binding of GTP to Ras proteins locks them in their active states, which enables high affinity interactions with downstream targets that are called effectors. Subsequently, a slow intrinsic GTPase activity cleaves off the γ -phosphate, leading to Ras functional inactivation and thus the termination of signalling. This on-off cycle is tightly controlled by GTPase-activating proteins (GAPs) and guanine-nucleotide exchange factors (GEFs). GAPs, such as p120GAP or neurofibromin (NF1), enhance the intrinsic GTPase activity and hence negatively regulate Ras protein function. Conversely, GEFs (also known as GTP-releasing proteins/factors, termed GRPs or GRFs), such as RasGRF and son of sevenless (SOS), catalyse nucleotide ejection and therefore facilitate GTP binding and protein activation. The classical view of Ras signalling depicts Ras activation and recruitment to the plasma membrane following receptor Tyr kinase (RTK) stimulation by growth factors (GF). Activated Ras engages effector molecules — belonging to multiple effector families — that initiate several signal-transduction cascades. Outputs shown represent the main thrusts of the indicated pathways. Ras activation can also occur in endomembrane compartments, namely the endoplasmic reticulum and the Golgi. Activating mutations in the different components of the Ras–Raf–mitogen-activated protein kinase (MAPK) pathway are associated with the indicated developmental disorders, suggesting that MAPK-signal antagonism might be a rational approach to manage certain cardio-facio-cutaneous (CFC) syndromes. AF-6, acute lymphoblastic leukaemia-1 fused gene on chromosome 6; CD1, cadherin domain-1; CDC42, cell division cycle-42; ELK, ETS-like protein; ERK, extracellular signal-regulated kinase; ETS, E26-transcription factor proteins; Ins(1,4,5)P₃, inositol-1,4,5-trisphosphate; JNK, Jun N-terminal kinase; MEK, mitogen-activated protein kinase/ERK kinase; NF-κB, nuclear factor-κB; PI3K, phosphoinositide 3-kinase; PKB/C, protein kinase B/C; PLA/Cε/D, phospholipase A/Cε/D; RalBP1, Ral-binding protein-1; RASSF, Ras association domain-containing family; Rin1, Ras interaction/interference protein-1; SAPK, stress-activated protein kinase; SHP2, Src-homology-2 domain-containing protein Tyr phosphatase-2; TIAM1, T-cell lymphoma invasion and metastasis-1.

Table 1

Ras mutations in human cancers

Tissue	H-Ras	K-Ras	N-Ras
Adrenal gland	1%	0%	5%
Biliary tract	0%	32%	1%
Bone	2%	1%	0%
Breast	1%	5%	1%
Central nervous system	0%	1%	2%
Cervix	9%	8%	1%
Endometrium	1%	14%	0%
Eye	0%	4%	1%
Gastrointestinal tract (site indeterminate)	0%	19%	0%
Haematopoietic and lymphoid tissue	0%	5%	12%
Kidney	0%	1%	0%
Large intestine	0%	32%	3%
Liver	0%	7%	4%
Lung	1%	17%	1%
Meninges	0%	0%	0%
Oesophagus	1%	4%	0%
Ovary	0%	15%	4%
Pancreas	0%	60%	2%
Parathyroid	0%	0%	0%
Peritoneum	0%	6%	ND
Pituitary	2%	0%	0%
Placenta	0%	0%	0%
Pleura	0%	0%	0%
Prostate	6%	8%	1%
Salivary gland	16%	4%	0%
Skin	5%	2%	19%
Small intestine	0%	20%	25%
Stomach	4%	6%	2%
Testis	0%	5%	4%
Thymus	0%	15%	0%
Thyroid	4%	3%	7%
Upper aerodigestive tract	9%	4%	3%
Urinary tract	12%	4%	3%

Data derived from the Catalogue of Somatic Mutations in Cancer (COSMIC) of the Wellcome Trust Sanger Institute, Cambridge, UK. ND, not determined.