CMLS, Cell. Mol. Life Sci. 57 (2000) 1613–1636
1420-682X/00/111613-24 $ 1.50 + 0.20/0

CMLS Cellular and Molecular Life Sciences

Review

Ras proteins in the control of the cell cycle and cell differentiation

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Received 8 March 2000; received after revision 24 May 2000; accepted 24 May 2000

Abstract. The Ras family of small GTPases includes three closely related proteins: H-, K-, and N-Ras. Ras proteins are involved in the transduction of signals elicited by activated surface receptors, acting as key components by relaying signals downstream through diverse pathways. Mutant, constitutively activated forms of Ras proteins are frequently found in cancer. While constitutive Ras activation induces oncogenic-like transformation in immortalized fibroblasts, it causes growth arrest in primary vertebrate cells. Induction of p53 and cyclin-dependent kinase inhibitors such as $p15^{INK4b}$, $p16^{INK4a}$, $p19^{ARF}$, and $p21^{WAF1}$ accounts for this response. Interestingly, while *ras* has usually been regarded as a transforming oncogene, the analysis of Ras function in most of the cellular systems studied so far indicates that the promotion of differentiation is the most prominent effect of Ras. While in some cell types, particularly muscle, Ras inhibits differentiation, in others such as neuronal, adipocytic, or myeloid cells, Ras induces differentiation, in some cases accompanied by growth arrest. Several possible mechanisms for the pleiotropic effects of Ras in animal cells are discussed.

Key words. Ras; cell cycle; differentiation.

Ras families

Ras proteins comprise a group of 20- to 25-kDa proteins that serve as molecular switches in pivotal processes governing cellular growth and differentiation. In mammals, the Ras superfamily of GTPases contains over 65 members. Based on their functions and structures, this superfamily has been subdivided into six families represented by Ras, Rho, Rab, Ran, Rad, and Arf [1, 2]. The Ras family itself is subdivided, based on homology within the effector domain region, into the subfamilies: Rap (Rap-1A, Rap-1B, Rap-2A, and Rap-2B); R-Ras (R-Ras, TC21/R-Ras2, and R-Ras3); Ral (RalA and RalB), Rheb, M-Ras [3–6], and the Ras subfamily that includes H-Ras, K-RasA, K-RasB, and N-Ras, on which this review will focus.

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ras genes

ras genes have been remarkably well conserved through eukaryotic evolution from fission yeast to humans, such that mammalian *ras* can restore a wild-type phenotype to *ras*-deficient yeast mutants [1]. Mammals encode three *ras* genes, H-*ras*, K-*ras*, and N-*ras*, which are expressed in every tissue and cellular type although the expression pattern varies depending on the organ and the stage of development [7]. These three genes exhibit a similar genetic structure with four exons and a 5′ non-

coding exon (exon ∅). K-*ras* possesses two alternative fourth exons (4A and 4B) that give rise to K-RasA and K-RasB proteins, respectively, which differ solely in their 25 carboxy-terminal amino acids [1, 8]. *ras* genes, however, differ extensively in their intronic organization, both in sequence and size, resulting in genes with remarkable size variation: K-*ras* spans more than 35 kbp; N-*ras*, 7 kbp, and H-*ras*, 3 kbp in humans. With respect to their regulatory elements, all *ras* genes are regulated by a TATA-less, GC-rich promoter, typical of housekeeping genes [1].

Ras proteins

Structure and function. The product encoded by the *ras* genes is a protein of 188 amino acids (189 in K-RasB) of approximately 21 kDa. The p21 proteins encoded by the three human genes are homologous in their first 164 amino acids, the first 86 N-terminal residues being identical, and the identity is up to 79% within the following 78 residues. However, they differ completely in the 25 carboxy-terminal residues, with the sole exception of cysteine 186. For this reason, the segment comprising residues 165–185 has been termed the heterogeneous region [8] (fig. 1). Ras biological activity resides in what has been termed the effector domain (amino acids 32–40) by means of which it binds to the different effector molecules [9]. However, five noncontiguous segments: residues 5–63, 77–92, 109–123, 139–165 and the carboxyl terminal CAAX box [8, 10] are also essential for Ras function (fig. 1).

The hallmark of Ras function is its regulated transit between an inactive state, in which it is bound to guanine dinucleotides (GDPs), and an active state bound to guanine trinucleotides (GTPs). This cycle is tightly regulated by at least two types of regulatory proteins: guanine nucleotide exchange factors (GEFs) that catalyze the GDP/GTP exchange, and GTPase-activating proteins (GAPs) that enhance the intrinsic capacity of Ras to hydrolyze GTP into GDP, thereby returning Ras to the inactive state [1, 8]. Structural differences between the active and inactive states are apparently restricted to the switch I (residues 30–38) and switch II (residues 60–76) regions [11]. The switch I region comprises the main binding site for effector molecules and GAPs, while the switch II region is involved in part in the interaction with GEF regions [9, 12, 13]. The position of these regions within the Ras molecule is indicated in figure 1.

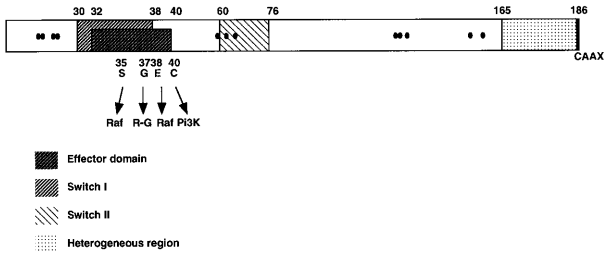

Figure 1. Structure of Ras proteins. The different regions with special relevance to Ras function are boxed. Dots indicate the position of transforming mutations found in human tumors. The effector domain, switch I and switch II regions are described in the text. Differences among the three Ras proteins accumulate in the C-terminal heterogeneous region. Mutations at the indicated residues within the effector domain yield proteins that signal only to Raf, PI3K and Ral-GDS ('R-G').

Posttranslational modifications. To be functional, Ras proteins must associate with the inner side of the plasma membrane. Oncogenic Ras proteins harboring mutations that render Ras cytosolic lose their transforming activity [14]. This association with the membrane is brought about by posttranslational modifications within the carboxyl-terminal CAAX box. Cys186 in the CAAX motif is first farnesylated by the addition of a C_{15} polyisoprenyl moiety. This is then followed by the proteolytic removal of the three AAX amino acids C-terminal to Cys186 and subsequently by the carboxymethylation of the now C-terminal, farnesylated Cys186 [8]. These modifications result in a more hydrophobic protein with greater affinity for the membrane. However, we now know that these modifications are not enough to provide stable binding to the membrane. For this purpose, a second attachment is required. In H-Ras, N-Ras, and K-RasA this is achieved by the addition of a palmitoyl group to a cysteine in the hypervariable region. K-RasB possesses a lysine-rich polybasic C-terminal segment, believed to interact electrostatically with phospholipids [15]. It is now becoming apparent that the different Ras proteins traffic by different routes to their site of action in the membrane and that all the targeting information is contained within the hypervariable regions of the respective Ras [16].

Ras activation

The presence of oncogenically mutated *ras* genes has been detected in up to 35% of human tumors [17, 18], although the frequency of each *ras* oncogene varies depending on the tissue. H-*ras* mutations are preferentially found in skin and squamous head and neck tumors. K-*ras* mutations are mainly detected in adenocarcinomas, cholangiocarcinomas, colorectal tumors, and are almost omnipresent in pancreatic carcinomas. Finally, N-*ras* mutations are common in hematopoietic malignancies, mainly acute myeloblastic leukemias and myelodysplasic syndromes [18, 19]. Activating mutations are mainly found in codons 12, 13, 59,

61, and 63, and their biochemical consequence is an impaired intrinsic GTPase activity rendering the protein resistant to the GAP activity exerted by regulatory GAP proteins such as p120 and NF-1. On the other hand, mutations in codons 16, 19, 116, 117, 119, 144, and 146 result in facilitated GDP/GTP exchange [8]. In addition, there are examples of tumors where Ras is overexpressed [1, 17].

Ras effector pathways

Extracellular stimuli of diverse nature, whether mediated by cell surface receptors of the tyrosine kinase, nonreceptor tyrosine kinase, or the G protein-coupled types, all converge in Ras, which is transiently activated upon the receipt of these stimuli [20]. Activated Ras then relays its signals downstream through association with a growing number of signalling intermediaries that interact with Ras-GTP [21]. This interaction is exerted mainly through the effector domain, some flanking residues, and the switch II region [12, 22] and is brought about by the conformational change that occurs upon the exchange of GDP for GTP. The specific binding to the different effector molecules is dependent on specific amino acids within the effector domain, as shown in figure 1 [23–25]. To date, the criteria followed to establish a bona fide Ras effector are: (i) preferential binding to the GTP-bound form of Ras, (ii) binding to a region within the effector domain, this binding eliciting the activation of the effector molecule with a subsequent biochemical and/or biological effect, and (iii) dysfunction of the putative effector molecule abolishes at least part of Ras-mediated effects [10]. These guidelines have yielded three proteins that are now well established as Ras effectors in mammalian cells: Raf, PI3K, and Ral-GDS. A growing number of Ras-interacting proteins such as MEKK1, Rin 1, AF-6 and Nore-1 [10, 21, 26, 27] await further confirmation as Ras effectors.

Raf and the ERK pathway

The first Ras effector identified was Raf-1. Raf binds Ras in a GTP-dependent fashion both in vitro and in vivo [28–31]. This association brings about the recruitment of Raf to the membrane, where it is activated by still poorly understood mechanisms [32, 33]. Once activated, Raf then stimulates MAP-kinase extracellular signal-regulated kinase (MEK), by serine phosphorylation [34]. Activated MEK functions as a dual-specificity kinase and phosphorylates threonine and tyrosine residues in, hitherto its sole substrates, mitogen-activated protein kinases p44 extracellular signal-regulated kinase (ERK1) and p42 ERK2 [35–38]. ERKs have a wide spectrum of substrates in different cellular locations including cytoplasmic proteins such as SOS, MEK, Rsk, PHAS-1, or PLA_2, and nuclear proteins that are phosphorylated upon ERK translocation to the nucleus, mainly transcription factors such as Elk-1, Ets-2, C/EBP, and SMADs [39–41]. The importance of the Raf/ERK pathway is underscored by the profound effects that blockade of this pathway by pharmacological agents, dominant inhibitory mutants of Raf, MEK, and ERKs, or microinjected antibodies against these molecules has on Ras biological roles in most cell types [21, 42]. Moreover, activation of the ERK pathway is sufficient to induce cellular transformation or differentiation in diverse cell types [43, 44]. However, in other cases, transformation by the constitutive activation of the ERK pathway by MEK is blocked by abrogating Ras function [45], indicating that in some cellular settings, activation of the ERK pathway might not be sufficient for inducing a transformed phenotype and may require additional Ras signals.

Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI3K) is a lipid kinase that catalyzes the phosphorylation of phosphoinositides in the 3′ position of the inositol ring in response to diverse stimuli. PI3K is composed of a regulatory p85 subunit, of which five isoforms have been identified, and a catalytic p110 subunit, with five isoforms described so far, which exhibit different substrate specificity and regulation [46, 47]. Two of these, p110α and β, have been shown to bind to the GTP-bound form of Ras through the effector domain. There is now ample evidence that Ras controls p110 activity and that PI3K is responsible for some of the biological effects of Ras [47–49]. The effect of Ras on PI3K activity is, in most cases, modest. However, Ras synergizes with p85 to induce PI3K activation, implying that several signals may be required for full PI3K activation. Activation of PI3K is sufficient to induce transcription of the *fos* promoter, but PI3K stimulation of DNA synthesis seems to require concomitant ERK stimulation [50–52]. To date, the main substrates of PI3K are Akt/PKB, and p70 ribosomal protein S6 kinase $p70^{S6K}$ [53, 54], which participates in transcriptional control. Activation of Akt generates a potent anti-apoptotic signal and regulates metabolic routes through the phosphorylation of glycogen synthase kinase 3 (GSK3) among other substrates [55, 56].

Ral-GDS and the Ral GTPase pathway

Ral-GDP dissociation stimulator (Ral-GDS) and its related proteins Rgl, Rgl2, and Rlf, all of them exchange factors for the GTPase Ral, have been shown to bind the GTP form of Ras [57–60]. Ral-GDS binds to

Ras in response to growth stimulatory signals and promotes GDP/GTP exchange on Ral [61]. Ral dominant inhibitory mutants can diminish Ras-induced transformation of NIH3T3 by more than 60% [62, 63]. In contrast, activated Ral blocks PC12 differentiation, opposing the effect of other Ras effectors [64], suggesting that Ral plays an important role in mediating the biological effects of Ras. In this aspect, Ral synergizes with Raf in the activation of the *fos* promoter [65]. With respect to its substrates, Ral has been shown to activate phospholipase D (PLD), with the participation of the GTPase Arf [66]. On the other hand, Ral interacts in its GTP form with a GAP protein for the Cdc42/Rac-1 GTPases named Ral-interacting protein 1 (RIP1; also known as RLIP76 or RalBP) [67, 68]. This interaction provides a direct connection of Ras with the Rho family GTPases that, although they cannot be considered direct Ras effectors, greatly influence Ras function. In addition to the activation of the effectors mentioned above, phospholipid-derived second messengers are also components of the Ras response. As such, Ras activates phosphatidylcholine-specific PLD (PC-PLD), and PLD activation by protein kinase C is greatly enhanced in Ras-transformed cells [69, 70].

Rho family GTPases

The role of Rho family GTPases in the control of cell proliferation and regulation of the actin cytoskeleton has been extensively reviewed [71–73]. It is now well-established that Rac-1, Rho A, and Cdc42 are involved in Ras transformation. All three cooperate with the focus-forming activity of Ras, while dominant inhibitory mutants abrogate the transforming potential of Ras in fibroblasts [74–77]; however, how these interactions occur is still not fully understood. The mechanisms coupling Ras and Rho GTPases are also not clarified. Potential mediators include p190 Rho-GAP that can interact with p120 Ras-GAP, the Ral-binding protein RIP, and activation of PI3K [72]. The fact that Ras exchange factors, like SOS and Ras-GRF, possess Dbl homology (DH) domains, shown to catalyze GDP/GTP exchange over Rho GTPases [78, 79], is also a potential link between Ras and Rho proteins that warrants further exploration. With regard to their effectors, Rho GTPases regulate at their origin the signalling cascades that lead to the activation of the MAP kinases Jun N-terminal kinase (JNK) and p38 [39, 40, 80], which ultimately impinge in the regulation of diverse cytoplasmic proteins and exchange factors [39]. On the other hand, they are also essential to control the dynamics of the actin cytoskeleton [73]. These processes are carried out by association with an ever-growing number of effector proteins that directly bind Rho GTPases. To date, there are 25-odd proteins that bind Rac-1 and/or Cdc42, these proteins having in common the presence of a so-called CRIB (Cdc42/Rac-interactive binding) domain. Rho interacts directly with more than 15 proteins of diverse activities [72, 73].

Ras and cell cycle control

There is nowadays no doubt as to the pivotal role that Ras proteins display as signal transducers, upon the stimulation of most types of cells with almost every known stimulus and, in the same fashion, in the instigation of malignant transformation. Both aspects of Ras biology have been extensively reviewed [8, 17, 81]. However, how Ras-mediated events impinge in the control of the cell cycle regulatory machinery, which ultimately decides the fate of a cell, has been an obscure field until very recently.

Cell cycle control is a complex process involving many different proteins. Cell cycle progression is essentially controlled by the activity of a family of protein kinases, the cyclin-dependent kinases (CDKs), their regulatory activating subunits, the cyclins, and a group of inhibitory proteins, the CDK inhibitors (CKIs), in addition to several other phosphatases and kinases that exert regulatory roles over these proteins. The molecular basis and regulation of cell cycle control has been thoroughly reviewed [82–85]. The mechanisms by which Ras regulates the activities of these main players in the control of cell cycle progression is presently a subject of intense investigation. Today, information is accumulating rapidly to explain the influences of Ras on the cell cycle, and is finally casting some light to reveal why Ras induces so many, sometimes apparently opposed, responses in different cells. Ras involvement in cell cycle control has been recently reviewed [10, 86–88].

Ras activity in the G_0/G_1 transition

Seminal experiments demonstrated that microinjection of activated Ras proteins induced the entry into S phase and DNA synthesis in quiescent cells. In the same fashion, abrogation of Ras activity by microinjection of anti-Ras antibodies resulted in the arrest of cells growing in serum and of cells transformed with oncogenes encoding tyrosine kinases. These experiments were the first indication that Ras function was required for G_1 transition. Interestingly, Ras activity was found to be dispensable once the cells had entered S phase until the next cycle [89–91]. This was quite in agreement with the well-established model in which G_1 entry would be triggered by 'competence factors' such as mitogens like epidermal growth factor (EGF), while 'progression factors' would drive the passage through G_1 until the

'restriction point' at late G_1 is reached. Indeed, one study demonstrated elegantly that Ras has two peaks of activation during G_1 transition: the first is achieved rapidly after mitogen stimulation, presumably at the G_0/G_1 entry, and a second peak, much more potent, occurs after mid-G_1 [92] that would, theoretically, stimulate the drive through the restriction point, thus explaining the necessity for Ras activity throughout G_1. Surprisingly, in fibroblasts, only the first burst of Ras activity is accompanied by ERK activation. This may not be a general characteristic of cell cycle progression, as regenerating hepatocytes exhibit two maximums of ERK activation that coincide with the aforementioned Ras peaks [93]. However, activation of the ERK pathway by Ras-independent means during the second peak cannot be discounted. On the other hand, constitutive activation of PI3K is sufficient to promote entry into S phase, but not progression throughout the entire cell cycle [50–52, 94], suggesting the necessity for additional signals to PI3K for the completeness of the cycle. Overall, the effects and patterns of activation of the different Ras effector pathways could imply that they may be playing distinct roles at different points in the cell cycle and that their individual activities may be required at different stages.

Ras and cyclin-CDK regulation

A decisive event in the progression from G_1 into the S phase is the phosphorylation of the retinoblastoma gene product (Rb). Under resting conditions, the Rb protein is hypophosphorylated, sequesters unproductively transcription factors of the E2F family, and recruits histone deacetylase (HDAC), thus repressing the transcription of genes essential for cell cycle progression [95, 96]. The onset of the G_1 phase triggers the expression of cyclin D1 which binds to and activates CDK4/6. The complex cyclin D1-CDK4/6 phosphorylates Rb at multiple sites. The hyperphosphorylated Rb loses its affinity for E2F, which leads to the disassembly of the E2F-HDAC complex, thus allowing gene transcription and cell cycle progression. Ras is directly involved in the control of this process by inducing the expression of cyclin D1 in various cellular systems [97–102]. This regulation takes place, at least partially, by the AP-1-like sequences present in the cyclin D1 promoter [103] and is mainly mediated by the ERK pathway [104–107]. However, recent reports indicate that other Ras effectors such as PI3K can also regulate the expression [108] and stability [109, 110] of cyclin D1 and, subsequently, transit into the S phase and DNA synthesis [111]. A similar situation occurs in T cells, in which E2F-mediated transcription is dependent on PI3K/Akt [112, 113]. Transcription from the cyclin D1 promoter can also be triggered by signals from Ral and Rac GTPases [114, 115].

Although induction of cyclin D1 is a necessary requirement for Ras-induced mitogenesis and tumorigenesis [116], this may not be the only mechanism through which Ras controls the Rb phosphorylation state and, consequently, G_1 progression. Cyclin D1 cooperates with Ras in the transformation of primary murine cells [117, 118], indicating that additional Ras signals may be promoting cell cycle progression. In this context, while the shortening of the G_1 phase detected in Ras-transformed cells can be associated with increased expression of cyclin D1, constitutive overexpression of cyclin D1 that accelerates G_1 progression is not sufficient to transform cells [97, 101]. Likewise, ectopic cyclin D1 expression alone is insufficient to override G_1 arrest induced by the expression of a dominant inhibitory Ras [119]. Moreover, while Ras and cyclin D1 are required at similar times during serum-induced cell cycle entry of quiescent NIH3T3 cells, this is not the case in continuously cycling cells in which Ras activity is only required at the G_2 phase to promote passage through the next cycle, while cyclin D1 is required throughout the G_1 phase [120, 121]. Overall, these data indicate clearly that Ras is unlikely to exert control on cell cycle progression solely through cyclin D1.

Ras also regulates the expression of other cyclins such as cyclin D3, A, and E, and of members of the E2F family of transcription factors [102]. Activated Ras also induces cyclin E expression in *Drosophila* cells [122]. There are indications that Raf can also induce the expression of cyclin E and A, thus regulating CDK2 activity [99, 123]. Recent reports indicate that the ability of oncogenic Ras to drive the expression of cyclin A is a critical step for the induction of anchorage-independent growth, a hallmark of transformation [124, 125], and all three aforementioned Ras effector pathways are required for the regulation of the cell cycle machinery in this process [126]. Although regulation of the CDK2 complex can take place by controlling the expression of its regulatory cyclins, it is mainly achieved through the degradation of the inhibitory $p27^{KIP1}$ protein. Several studies indicate that the downregulation of $p27^{KIP1}$ at the late G_1 phase requires Ras activity [101, 102, 127, 128]. The lack of $p27^{KIP1}$ degradation can be sufficient to stop Ras-induced proliferation [99].

$p27^{KIP1}$ degradation induced by Ras is exerted mainly through the ERK pathway [101, 127–129]. Indeed, $p27^{KIP1}$ is a substrate of ERK and phosphorylated $p27^{KIP1}$ cannot bind and inhibit CDK2 [127]. In addition, activation of ERK by MEK1 leads to early degradation of $p27^{KIP1}$ [111]. However, the requirement for ERK signaling in $p27^{KIP1}$ degradation may be indirect and could be restricted to the titration of $p27^{KIP1}$ by the cyclin D-CDK4/6 complex and does not exclude the involvement of other pathways. PI3K may also be required for $p27^{KIP1}$ degradation either through Akt or

p70 [112, 113]. Rho GTPase has also been implicated in the degradation of $p27^{KIP1}$, as inhibition of Rho function blocks growth factor-mediated $p27^{KIP1}$ degradation [130], while constitutively active Rho promotes the activation of the cyclin E-CDK2 complex in the absence of growth stimulation [131]. Recent reports also indicate that Ras can regulate $p27^{KIP1}$ at the transcriptional level through AFX-like transcription factors. Overexpression of AFX blocks the G_1 transition by inducing $p27^{KIP1}$ expression, but AFX-mediated transcription is negatively regulated by Ras through a mechanism that involves Ral and PI3K/Akt signals [132].

As the Rb 'pocket' protein is a key sensor in G_1 progression, much attention has been focused on the role played by Rb in Ras-mediated cell cycle regulation. Inhibition of Ras activity by neutralizing antibodies or the expression of dominant interfering mutants results in the blockade of cell cycle progression in asynchronous wild-type, but not in Rb-null mutant cells. Loss of Rb, however, does not completely overcome the requirement for Ras signals. While Rb-deficient, continuously cycling cells fail to arrest when Ras function is inhibited, the mitogen-induced G_0/G_1 transition is effectively blocked [133, 134]. This requirement for Ras may be explained by the need for cyclin E/CDK2 activity that can also be triggered by Ras and is still required in the absence of Rb [129]. Inhibition of Ras also results in hypophosphorylation of Rb, which can be rescued by forced expression of cyclin D1 [129, 133]. This result indicates that signals originating in Ras are necessary for cyclin D/CDK-mediated Rb inactivation. Interestingly, while Ras regulates Rb activity, this effect seems to be bidirectional as Rb can also regulate Ras function by yet unveiled processes [135].

Ras as cell growth inhibitor: CKI regulation and Ras

Unlike NIH3T3, Rat-2, and other established cell lines, Ras oncogenes are incapable of transforming primary cell lines. To do so, they require the participation of a cooperating nuclear oncogene such as SV40 large T antigen or c-*myc*. [136–138]. Strikingly, introduced alone to these primary cells, oncogenic Ras causes growth arrest. In recent years, an explanation for these, apparently contradictory, effects of activated Ras has started to emerge. In primary cells, Ras induces the expression of CDK inhibitors, resulting in cell cycle arrest through at least three pathways: the $p16^{INK4a}$-Rb pathway, the $p19^{ARF}$-p53 pathway, and a p53-independent $p21^{WAF1}$ pathway. Cooperating oncogenes, such as the SV40 large T antigen or adenoviral E1A protein, block p53 or Rb function, indicating the necessity for downregulating these cell cycle regulators to achieve a fully transformed phenotype. Several mechanisms have been proposed to explain the cooperation between Ras and Myc to promote transformation, including induction of CDC2 [139], induction of cyclin E [129, 140], Myc stabilization by Ras [141], and induction of c-Myc expression by Ras/Raf [142]. In addition, both Ras (see above) and c-Myc [143, 144] inactivate $p27^{KIP1}$.

Ras-expressing rodent and human primary fibroblasts exhibit a morphology and molecular markers characteristic of cellular senescence [145]. In a fashion reminiscent of its upstream activator, activated Raf also induces G_1 arrest, accumulation of hypophosphorylated Rb, and cell senescence by a mechanism that involves the upregulation of the inhibitors $p16^{INK4a}$ and $p21^{WAF1}$, depending on the cell type [146, 147], and these same effects are observed following the ectopic expression of upstream components of the ERK cascade such as MEK [146, 148]. Interestingly, the intensity of the signal decides cell fate. Only high levels of Raf activity are able to induce senescence, whereas less intense Raf activation induces cyclin D1 expression and drives the cell through the completion of the cell cycle [123, 149, 150]. This phenomenon may reflect a security mechanism by means of which, under normal conditions, cells in which an abnormally high Ras pathway signal would lead to deregulated cell cycle progression, cell division and, ultimately, oncogenic growth are driven to a proliferative 'dead end.'

Despite these data, there are so far no reports with a side-by-side comparison of the effects of the three Ras proteins on growth inhibition of primary fibroblasts. H-ras has recently been shown to be a somewhat more potent growth inhibitor than K- or N-*ras* in the K562 myeloid cell line [151]. Extension of these studies to primary fibroblasts and other cell types is expected.

Ras and the $p16^{INK4a}$/Rb and $p19^{ARF}$/p53 pathways. One mechanism by which Ras-mediated growth inhibition takes place is through induction of $p16^{INK4a}$ and $p15^{INK4b}$ expression [145, 152]. These two proteins are members of the INK4 protein family and are potent inhibitors of CDK4 and CDK6. Following CDK4 and CDK6 inhibition, Rb is not hyperphosphorylated and G_1 progression is arrested [153]. Thus, by this pathway, oncogenic Ras arrests cell cycle progression through the participation of Rb. Accordingly, the expression of oncogenic Ras in mouse fibroblasts deficient for $p16^{INK4a}$ does not result in growth inhibition but in cell transformation [154]. Moreover, NIH3T3 fibroblasts, the prototypical cell line used for Ras transformation assays, are deficient in the $p16^{INK4a}$ gene [153] and are easily transformed by oncogenic Ras. Thus, depending on the genetic background, Ras may have opposite effects.

A second mechanism for Ras-mediated growth arrest is the induction of $p19^{ARF}$, p53, and (through p53) $p21^{WAF1}$ [87, 102, 123, 137, 145–148, 150]. The INK4a-ARF locus encodes two tumor-suppressor proteins:

$p16^{INK4a}$ and $p19^{ARF}$. As described recently, Ras induces the expression of $p19^{ARF}$ [155]. $p19^{ARF}$ in turn inhibits Mdm2 activity [156–162]. Mdm2 is required for ubiquitin-dependent p53 degradation, and therefore the inactivation of Mdm2 by $p19^{ARF}$ results in the accumulation of active p53 [163–167]. By this pathway, oncogenic Ras arrests the cell cycle through p53. Thus, primary mouse fibroblasts deficient for $p19^{ARF}$ can be transformed by Ras alone [168]. In addition, $p19^{ARF}$ potently suppresses oncogenic transformation of primary cells by a combination of Ras and nuclear oncogenes such as c-*myc*, but this function is abrogated by the neutralization of p53 [157]. In this respect, it has been shown that, in mice, $p19^{ARF}$ is essential for the activation of p53 in response to oncogenic Ras [155]. On the other hand, recent results also suggest that $p19^{ARF}$ induces growth arrest and senescence in mouse embryo fibroblasts by a p53-independent pathway [169]. Regarding the individual share that each of the p16INK4a/Rb and p19ARF/p53 pathways contribute to the biological outcome resulting from Ras hyperactivity, it is noteworthy that there are important differences between mouse and human fibroblasts. In murine fibroblasts devoid of either p53 or $p16^{INK4a}$, *ras* oncogenes provoke cell transformation instead of growth arrest or senescence. On the other hand, in human primary fibroblasts, inactivation of both the $p16^{INK4a}$/Rb and $p19^{ARF}$/p53 pathways is required for Ras-induced transformation [145].

Ras and $p21^{WAF1}$. The above-mentioned findings imply that, in primary cells, oncogenic Ras elicits an anti-proliferative response brought about by the combination of at least two pathways: upregulation of $p16^{INK4a}$ that impinges on the activation of the tumor suppressor protein Rb, and upregulation of $p19^{ARF}$ which in turn activates the 'guardian of the genome' p53. $p21^{WAF1}$ is a major transcriptional target of p53 [170] that encodes an inhibitory protein for CDK2 and Cdc2 activities, resulting in cell cycle arrest [171]. In primary cells, $p21^{WAF1}$ induction is a consequence of Ras-mediated upregulation of p53 [145, 148]. Thus, Ras activity, through $p19^{ARF}$ induction and Mdm2 inactivation, results in p53 accumulation and subsequent $p21^{WAF1}$ upregulation.

The induction of $p21^{WAF1}$ plays an important role in the anti-proliferative response generated by Ras in rat primary Schwann cells [172]. In mouse fibroblasts, cell cycle arrest elicited by Ras also depends on p53 [145, 173], while $p21^{WAF1}$-deficient (but not p53-deficient) fibroblasts retain an anti-proliferative and senescent response to oncogenic H-*ras* [173]. High Raf activity also results in growth arrest [123, 147, 150], but in contrast to Ras-mediated growth inhibition, cell cycle arrest in response to acute Raf activation is p53 independent but p21 dependent [123, 150].

In this respect, although $p21^{WAF1}$ induction is mainly a consequence of p53 upregulation in primary cells, a p53-independent pathway for Ras-mediated $p21^{WAF1}$ upregulation has also been described [123, 150, 151]. It must also be noted that other p53 target genes can induce growth arrest (e.g., 14-3-3σ or Gadd45). So Ras may also be exerting cell cycle arrest through the participation of these or similar proteins. The upregulation of $p21^{WAF1}$ by Ras occurs mostly at the transcriptional level, and Sp1 and E2F-1 sites in the $p21^{WAF1}$ promoter have been identified as the Ras-responsive elements [151, 174, 175]. Unfortunately, the mechanisms responsible for $p15^{INK4b}$, $p16^{INK4a}$, and $p19^{ARF}$ upregulation by Ras are so far unknown.

Interestingly, in Swiss 3T3 cells, induction of $p21^{WAF1}$ by Ras has been related to the activation of Rho, such that in the presence of elevated Rho activity, Ras is not capable of inducing $p21^{WAF1}$ [176], which suggests that a certain level of Rho activation may contribute to prevent a Ras-induced anti-proliferative effect in tumor cells. This is consistent with the previously described requirement for Rho in Ras-induced transformation [75]. It should be noted that all the previous reports showing $p21^{WAF1}$ induction by Ras were carried out in cells expressing either p53, $p16^{INK4a}$, or $p19^{ARF}$. Interestingly, Ras-mediated growth arrest and $p21^{WAF1}$ elevation have also been found in human leukemia cells devoid of p53, $p15^{INK4b}$, $p16^{INK4a}$, and $p19^{ARF}$ [151]. Thus, there must be a connection between Ras and $p21^{WAF1}$ upregulation that functions independently of p53 and the INK4 family inhibitors.

So, as depicted in figure 2, Ras promotes the induction of at least five proteins which may induce cell cycle arrest: $p15^{INKb}$, $p16^{INK4a}$, $p19^{ARF}$, $p21^{WAF1}$, and p53. These proteins are components of three anti-proliferative pathways ending in Rb phosphorylation, p53 induction, and p53-independent $p21^{WAF1}$ upregulation, respectively. Finally, it must be stressed that the pathways responsible for Ras-stimulated growth arrest seem to be strictly dependent on the cell type involved. While all three pathways (Rb, p53, $p21^{WAF1}$) seem to be operational in primary fibroblasts, this may not be the case for other cells, and cell-specific differences are likely to operate.

Ras and differentiation

Despite the enormous amount of relevant information on Ras activities in signal transduction, its biological function in development and differentiation of vertebrate tissues remains largely unexplained. Targeted deletion of the three *ras* genes in mice results in distinct phenotypes, suggesting different roles for the three Ras proteins. K-*ras*-deficient mouse embryos die after 12.5

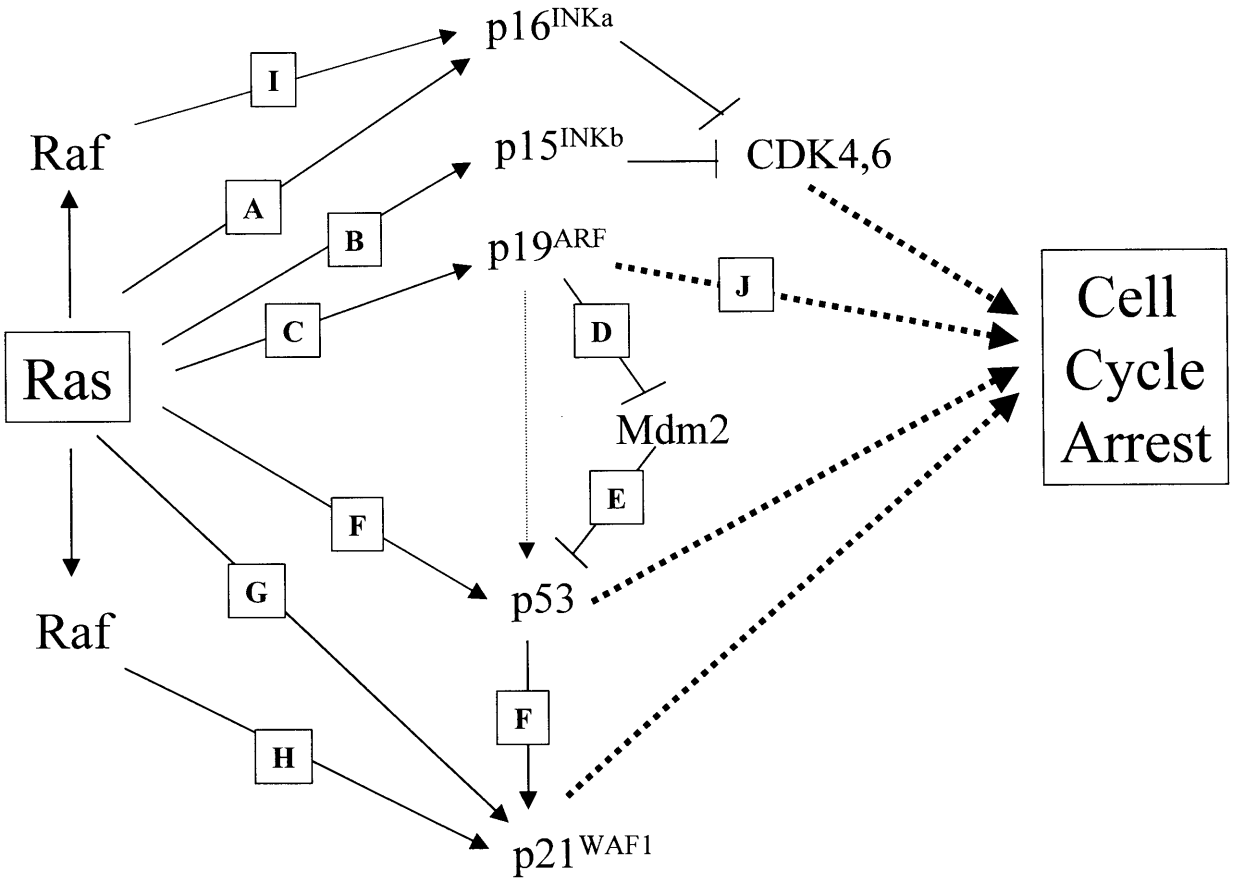

Figure 2. Oncogenic Ras may induce growth arrest through different pathways. Most of these pathways have been unveiled in primary cells (human and mouse fibroblasts, rat Schwann cells), although some have also been studied in transformed cells [102, 326]. Thick dotted lines indicate multistep mechanisms. A, Ras induces $p16^{INK4a}$ [145, 154], which provokes cell cycle arrest through the inhibition of CDK4,6 kinases and accumulation of hypophosphorylated Rb; B, Ras induces $p15^{INK4b}$ [152], another inhibitor of CDK4 and 6; C, Ras induces $p19^{ARF}$ [155]; D, $p19^{ARF}$ inactivates Mdm2 [156–162]; E, Mdm2 promotes degradation of p53 [165–167, 329], and thus, the overall effect of $p19^{ARF}$ induction is the upregulation of p53 (thin dotted line) which causes cell cycle arrest by inducing the expression of $p21^{WAF1}$ and other target genes; F, a number of reports demonstrate that Ras induces p53, although the involvement of ARF/Mdm2 has not been tested [102, 148, 150], and in these cases Ras also induces $p21^{WAF1}$ as a result of p53 accumulation; G, several reports indicate that Ras induces $p21^{WAF1}$ [174–176], in some cases in a p53-independent way [150, 151], and in at least one report, $p21^{WAF1}$ induction was also $p15^{INKb}$, $p16^{INK4a}$ and ARF independent [151]; H, Raf, a Ras effector kinase, also induces $p21^{WAF1}$ [123, 150, 172], and this induction can be p53-independent [123, 150]; I, both Raf [147, 172] and the major Raf effector MEK [146, 148] also induce $p16^{INK4a}$; J, $p19^{ARF}$ can also negatively regulate the cell cycle by a p53-independent mechanism [169].

days of development, indicating that this isoform is essential for embryonic development [177, 178]. In contrast, N-*ras*-null homozygous mutant mice are born and grow normally [179]. This is also the case in H-*ras*-deficient mice (E. Santos, personal communication).

ras was originally identified as the prototypical oncogene because Ras mutations were frequently found in human and experimental tumors [17] and because *ras* can transform murine immortalized fibroblasts and, in cooperation with other oncogenes, primary fibroblasts [1]. However, as described below, the prevailing effect of ectopic expression of Ras oncoproteins, in the majority of cells in culture, is differentiation rather than transformation. An indirect clue suggesting a role for Ras proteins in differentiation is that, while the three *ras* genes are expressed in all murine and human tissues, there is a marked differential expression of the *ras* genes during development, suggestive of distinct roles depending on the tissue [7, 180]. We will briefly review the information gathered on Ras function in some important differentiation models.

Neuronal cells

The first indication that Ras could promote cell differentiation was obtained in a neuronal cell line, PC12,

which derives from a rat pheochromocytoma. Activated, oncogenic versions of H-*ras* [181, 182], K-*ras* [182], and N-*ras* [183] induce neuronal-like differentiation accompanied by growth arrest and neurite formation. The overexpression of wild-type Ras also induces PC12 differentiation [184]. These results are consistent with early reports pointing to the brain as one of the tissues with higher Ras expression, as assessed by Northern analysis [7], immunohistochemical staining [180], or immunoblotting [185]. PC12 can be readily differentiated by nerve growth factor (NGF). The differentiation induced by NGF has been associated with prolonged Ras activity [186, 187] and the dominant-negative mutant H-RasN17 blocks NGF-induced neuronal differentiation [188, 189]. Thus, the differentiation mediated by ectopic expression of oncogenic Ras seems to mimic the events triggered by NGF. The signaling pathway mediating Ras-induced PC12 differentiation was later found to include Raf [190] and MAPK [45]. More recent reports indicate that the effectors of Ras-mediated differentiation in PC12 are Raf and PI3K but not Ral-GDS [64].

Ras is also involved in the differentiation of other neuronal cell lines. For example, ectopic expression of RasN17 in rat hippocampal H19-7 cells blocks bFGF-mediated differentiation. The same result is observed with a dominant-negative Raf [191]. Likewise, NGF-induced differentiation in two other neuroblastoma cell lines (IMR-32 and SK-N-SH) downregulates the levels of the Ras GTPase-activating proteins neurofibromin and GAP, with a concomitant increase in GTP-bound Ras [192]. Ras is also involved in the transduction of the NGF signal in primary neurons [193], and Ras activity is both necessary and sufficient for the survival of this type of neuron [194].

The differentiation-enhancing effect of Ras in neuronal cells is not restricted to NGF-induced differentiation. The SK-N-BE neuroblastoma cell line can be differentiated with retinoic acid. Although Ras activation is not observed under these circumstances [195], transfection with the exchange factor Ras-GRF results in enhanced retinoic acid-induced differentiation [196].

On the other hand, in vivo data indicate that the involvement of Ras in neuronal differentiation is not applicable to all types of neuronal cells. Sympathetic neurons, enteric neurons, and adrenal chromaffin cells all derive from the neural crest. During development these cells migrate to their respective body locations where they proliferate and differentiate. The effect of activated H-Ras has been tested in transgenic mice where the transgene was expressed under the control of the dopamine-β-hydroxylase promoter, which restricts expression to sympathetic cells. The mice present ganglioneuromas in the adrenal gland and preaortic sympathetic ganglia, but neurons of cervical and enteric ganglia differentiate normally despite H-Ras oncoprotein expression [197].

Finally, data from Ras-deficient mice indicate that Ras may also be required for neuron survival. Thus, K-*ras*$^{-/-}$ mice exhibit increased apoptosis in spinal chord motoneurons [177]. This may seem inconsistent with the observation in Ras-GAP knockout mice, in which Ras activity would be expected to be upregulated, but which also show extensive neuronal cell death in the anterior neural tube and cranial neural crest [198], suggesting that a strictly regulated and balanced Ras function may be necessary in this biological process.

Myeloid cells

Along with neuronal cells, myeloid cells, constitute the cellular model where Ras effects on differentiation have been more extensively studied. The role of *ras* genes in hematopoietic cell differentiation is paradoxical. On one hand, activating mutations in N-*ras* are among the most frequent genetic alterations detected in human acute myeloid leukemias (AMLs) and myelodysplasic syndromes (MDS). The reported frequencies of *ras* mutations range from 25 to 44% in AML [19], although some studies have demonstrated no correlation between *ras* mutation and poor prognosis [199, 200]. The highest frequencies of *ras* mutations are detected in MDS. In chronic myelomonocytic leukemia, a subtype of MDS, *ras* mutations appear in 32 to 65% of the cases [19]. Juvenile chronic myeloid leukemia (JCML) represents a noteworthy example of Ras involvement in human leukemogenesis. JCML is a myeloproliferative syndrome which clinically resembles chronic myeloid leukemia, but occurs in children and lacks the translocation generating the Bcr-Abl fusion protein, present in all classical chronic myeloid leukemias. JCML patients usually carry either activating *ras* mutations or loss of the neurofibromin type 1 (NF-1) gene [201]. Consistently, JCML patients lacking NF-1 have elevated levels of Ras-GTP [202] and mice whose hematopoietic system is reconstituted with NF-1-deficient stem cells develop a myeloproliferative syndrome similar to human JCML [203].

Ectopic expression of activated *ras* genes in myeloid-derived cell lines has yielded divergent results with respect to differentiation. Some reports show that *ras* oncogenes abrogate cytokine requirements in growth factor-dependent cell lines. This is the case for the murine cell lines PB-3c [204], NSF/N1.H7 [205], FDC-P1 [206], FDC-P2 [207], and for human TF-1 cells [208]. Mutant H- and N-Ras also inhibit chemically induced erythroid differentiation of the rat erythroleukemia D5A1 cell line [209]. However, in other models, *ras* oncogenes promote or enhance differentiation. For example, retroviral transduction of activated H-*ras* in

human myeloid P39 cells results in an enhancement of retinoic acid-induced differentiation to the monocytic-granulocytic lineage [210]. And in human myeloid leukemia U937 cells, an H-RasD12 mutant induces monocytic differentiation accompanied by growth inhibition [208]. 32D is a nontumorigenic interleukin (IL)-3-dependent murine myeloid cell line which undergoes granulocyte differentiation when stimulated with granulocyte-colony-stimulating factor (G-CSF) [211]. Infection with Harvey or Kirsten viruses does not abrogate IL-3 dependence. Moreover, the infected cells show characteristics of monocytic differentiation, which is more pronounced in K-*ras*-expressing cells [212]. Nevertheless, in these cells, expression of H-RasN17 blocks proliferation [213], suggesting that Ras activity, despite increasing the expression of differentiation markers, is required to maintain a proliferative state.

In contrast to the above-mentioned results, other reports describe Ras-induced erythroid differentiation, as is the case in TF-1 cells [214] and monocytic differentiation in mouse FDC-P1 cells transduced with v-H-*ras* [215]. In this case, Ras also induces a tumorigenic phenotype that resembles that found in primary hematopoietic precursors (see below). The remarkable heterogeneity of the cell lines utilized in these experiments may partially account for these contradictory results.

In contrast, in other myeloid cells, expression of Ras oncogenic forms induces growth arrest without significant differentiation. In the case of K562, a cell line derived from a CML, a systematic comparison of H-, K-, and N-*ras* oncogenes has demonstrated that the three Ras proteins induce a pronounced growth arrest, with H-*ras* displaying the most potent antiproliferative effect [151]. This result would help explain the absence of Ras mutations in CML, even in the terminal blastic phase of the disease [216]. Consistent with this, a recent report shows that the activity of MAPK, a major Ras effector, is not required for growth of several human myeloid leukemia-derived cell lines [217].

In contrast with the divergent results encountered in cultured myeloid cell lines, in vivo data indicate that the primary effect of Ras overexpression in early myeloid cells is the promotion of differentiation, while still stimulating proliferation. Ras promotes differentiation in bone marrow hematopoietic precursors. Early work demonstrated that infecting with Kirsten and Harvey viruses erythroid precursors from mice spleen treated with phenylhydrazine resulted in the formation of colonies of differentiated erythroid cells [218]. Harvey virus-infected murine bone marrow cells give rise to monocyte-macrophage colonies where the vast majority of the cells undergo terminal macrophage differentiation [219]. As in murine cells, retroviral transduction of *ras* oncogenes into human bone marrow cells does not result in the appearance of blastic cells or growth factor independence, reminiscent of transformed cells [220]. When erythroid differentiation was analyzed in human $CD34^+$ cells infected with a mutant N-Ras, virus erythroid progenitors expressing N-*ras* exhibited an increased cell doubling time, down-regulation of the differentiation rate, and increased cell death during the differentiation process [221]. Consistently, transfecting human $CD34^+$ with N-*ras* antisense oligodeoxynucleotides results in a drop in the number of granulocyte-macrophage colony-forming units (CFU-GM), macrophage colony-forming units (CFU-M) and megakaryocyte colony-forming units (CFU-Meg), [222] suggesting that Ras is required for differentiation of hematopoietic cells.

Finally, the effect of oncogenic Ras on differentiation versus transformation of hematopoietic cells in vivo has been addressed by repopulating mice bone marrow with Ras-expressing cells [223–225]. Infection of bone marrow cells with a retrovirus carrying activated H-*ras* produces T-cell lymphomas in 70% of the animals, despite the presence of the proviral genome in both myeloid and lymphoid cells [223]. In another study, using a different retroviral H-ras vector, infected mice developed T-cell or B-cell lymphomas, but again no neoplasias of myeloid origin [224]. Furthermore, while v-H-*ras* transforms oncogenically only lymphoid precursors, it enhances monocytic-macrophagic differentiation. Moreover, H-*ras* promotes the formation of monocyte-macrophage colonies, which undergo terminal differentiation [224]. Last, mice reconstituted with bone marrow cells infected with activated N-*ras* develop myeloid disorders in about 60% of the animals. These neoplasias are characterized by the excessive proliferation of myeloid cells that eventually undergo differentiation. These conditions resemble human CML or AML but accompanied by differentiation [225]. Induction of apoptosis in lymphocytes may account for the absence of lymphoid leukemias in mice with N-*ras*-reconstituted bone marrow [225]. It is interesting that while H-*ras* induces lymphoid malignancies (thymic lymphomas and B-cell leukemias), N-*ras* promotes myeloproliferative disorders. The data gathered so far are consistent with a model in which antiproliferative defects are a primary consequence of N-*ras* mutations in hematopoietic cells, and secondary transforming events would be required for the development of myeloid leukemia [225]. The phenotype of *ras* knockout mice also supports a role for K-*ras* and N-*ras* in hematopoiesis. As mentioned above, two groups have reported that K-$ras^{-/-}$ embryos die between the 12th and 14th day of gestation and, in one of the reports, this lethality is attributed to a failure in hepatic hematopoiesis [177, 178].

Lymphoid cells

Experiments with transgenic mice carrying activated or dominant negative *ras* genes under the control of lymphocyte-specific promoters point to a major role for *ras* in the commitment of lymphoid lineages. In transgenic mice expressing a dominant negative Ras mutant (H-RasN17) in B-cell progenitors, the progression to pro- and pre-B-cell stages can be almost completely inhibited. The development of these cells, where Ras function has been suppressed, is arrested at a very early stage, prior to the formation of the pre-B receptor [226]. The differentiation-promoting effect of Ras at different stages of lymphocytic development has also been demonstrated in Ras transgenic mice expressing Ras. Activated Ras induces the progression of RAG1-deficient pro-B cells to cells that share many characteristics with precursor pre-B cells [227]. These findings suggest that Ras signals are required for B-cell development. In pro-B cells, activated Ras induces developmental progression by activating both differentiation and survival programs [228]. Likewise, activated Ras expression in mice deficient for the assembly of the heavy-chain variable-region genes drives the progression of B lineage cells beyond the developmental checkpoint otherwise controlled by the μ heavy chain [228].

Similarly, work with transgenic mice has demonstrated that Ras enhances T-cell differentiation. Expression of activated Ras in $CD4^-CD8^-$ (double-negative) prothymocytes in vivo promotes their differentiation into small $CD4^+CD8^+$ (double-positive) cortical thymocytes. However, Ras expression in double-positive cells does not cause proliferation or maturation to single-positive thymocytes [229]. Accordingly, in mice expressing RasN17 in T cells, the proliferation in response to T-cell receptor (TCR) stimulation is blocked and positive selection of thymocytes is impaired. In contrast, negative selection occurs normally in the presence of H-*ras*N17 or dominant-negative MEK-1 transgenes [230, 231]. Therefore, signalling through Ras is sufficient to promote differentiation of double-negative to double positive-cells, but further differentiation requires the activity of additional signalling pathways. These results have been partially reproduced in $CD4^+CD8^+$ thymocytes of the DPK cell line, in which downregulation of CD8 occurs upon transfection of an activated *ras* gene. However, the cells were not fully differentiated, indicating that Ras signalling provides only a partial signal for double-positive thymocyte differentiation [232].

Epithelial cells

Murine primary epithelial keratinocytes in culture undergo terminal differentiation (formation of cornified cells) when exposed to media with high Ca^{2+} concentrations [233]. In these cells, calcium-induced differentiation is blocked upon infection by Kirsten or Harvey viruses, although different reports disagree as to whether the cells are growth arrested or continue to proliferate [234–236]. Inhibition of calcium-induced differentiation is consistent with the high incidence of H-*ras* mutations detected in chemically induced papillomas and squamous cell carcinomas in mice [237–240]. Conversely, the incidence of *ras* mutations in human squamous cell carcinomas is very low. Interestingly, mutations in K- and N-*ras* genes are infrequent in experimental and human tumors [241–244]. Introduction of the v-H-*ras* gene induces the expression of keratins 8 and 18 both in cultured mouse keratinocytes [245] and in the MCA3D cell line [246]. These keratins are characteristic of embryonic and adult simple epithelia cells, but not of stratified squamous epithelia such as skin, thus suggesting that the H-*ras* oncogene would alter the normal differentiation program of epidermal cells. In contrast to the former results, normal human keratinocytes transduced with the v-H-*ras* gene form a noninvasive and normally differentiated epidermis after grafting to nude mice [247]. Consistent with this, the immortalized human keratinocytic cell line HaCaT transfected with H-*ras*V12 showed no correlation between Ras levels and malignancy in transplantation experiments [248, 249]. Thus, some variations notwithstanding, the differentiation potential is not significantly reduced in these clones, whatever the levels of *ras* oncogene expression or the malignant properties of the cells.

Data from experimental and human skin carcinogenesis are also consistent with a differentiation-promoting effect of activated Ras. Keratoacanthomas are keratinocyte-derived tumors that eventually regress by a process of redifferentiation. Keratoacanthomas induced in rabbit skin by dimethylbenzanthracene show a high frequency of H-*ras*-activated genes carrying a mutation in codon 61. The same result occurs in human keratoacanthomas, although mutations affect both the 12th and 61st codons [250, 251]. Moreover, mutated H-*ras* transcripts are up to threefold more abundant than the nonmutated H-*ras* mRNA [252]. In line with these findings, transgenic rabbits in which the expression of H-RasV12 has been targeted to keratinocytes has recently been shown to develop multiple keratoacanthomas that regress spontaneously [253]. Together, these findings indicate that oncogenic Ras expression in skin cells is compatible with keratinocyte differentiation. However, Ras may play a role in tumor progression in the presence of other concomitant oncogenic alterations, which would explain the high incidence of Ras mutation in skin cancers.

Muscle cells

Muscle cells are one of the exceptions regarding the pro-differentiation effects of Ras oncoproteins. Oncogenic forms of N-*ras* and H-*ras* prevent skeletal myoblast differentiation as shown in murine myoblastic cell lines such as C2C12 [254, 255], BC3H1 [256], and C3H10T1/2 [257], and rat 23A2 [258]. In these cells, Ras-mediated inhibition of muscular differentiation correlates with a shutdown in the transcription of muscle-specific genes [256, 259, 260]. Moreover, H-*ras*-induced inhibition of differentiation is associated with a downregulation in the expression of MyoD, a helix-loop-helix transcription factor that acts as a myogenic determinant [257, 261]. Interestingly, an activated R-Ras mutant (R-RasQ87L) enhances differentiation of C2C12 cells. Thus, the differentiation inhibition effect is restricted to the Ras subfamily [262]. This effect can help explain the differentiation-inhibiting effect of Ras on muscle precursor cells. In this context, in the rat myoblast line 23A2 transfected with oncogenic N-*ras* or H-*ras*, the secretion of an unidentified inhibitor of skeletal myoblast differentiation is triggered [263]. Furthermore, these findings are the first to implicate an autocrine/paracrine mechanism in the inhibition of myogenic differentiation by oncogenic Ras. In rat 23A2 cells, the differentiation-defective phenotype established by Ras does not depend on MAPK activation [258]. A recent report suggests that the inhibition of myoblast differentiation is mediated by the Ral-GDS signalling pathway [264]. An inhibitory effect of Ras on muscle differentiation seems to be in contrast with the high Ras expression levels detected in adult differentiated muscle cells. It must be noted, however, that the above examples all refer to oncogenic Ras, which exerts a more potent signal than wild-type Ras even when the latter is expressed at high levels, as in adult skeletal muscle. Finally, it is noteworthy that K-*ras*$^{-/-}$ mice exhibit abnormally thin ventricular walls [177]. Thus, cardiac muscle appears to be one of the tissues affected by K-*ras* deficiency. Taken together, the reported data indicate that muscle is the tissue where Ras exerts the most clear inhibitory effect on differentiation. However, recent data challenge this view, as the microinjection of activated *ras* genes in regenerating muscle induces the expression of muscle-specific genes, suggesting that Ras can also promote myocytic differentiation under some circumstances [265].

Adipocytes

Mouse 3T3-L1 cells differentiate into adipocytes upon exposure to insulin or insulin-like growth factor-I (IGF-I). Expression of an N-*ras* oncogene (N-*ras*K61) leads to differentiation of these cells into adipocytes, in the absence of insulin or IGF-I [266]. Ras also mimics insulin action in other respects, such as hexose transport [267]. Ras is strongly activated in response to insulin stimulation in parental 3T3-L1 cells [268]. On the other hand, recent results suggest that oncogenic *ras* induces proliferation instead of adipogenic differentiation of 3T3-L1 cells when Notch-1 expression is downregulated, implicating Notch-1 in the interpretation of Ras activation as a proliferation- or differentiation-inducing signal in these cells [269]. Concerning the effector pathways involved in these processes, Ras-induced MAPK activation is not required for the differentiation of 3T3-L1 cells and, in fact, MAPK has been shown to antagonize differentiation [270].

Another model where Ras induces adipocytic differentiation is in brown adipocytes. Transfection of rat fetal brown adipocytes with SV40 large T antigen results in an immortalized cell line, while transfection of the T antigen gene together with H-*ras*K12 yields a transformed phenotype. However, in the latter case, cells exhibit a phenotype with differentiation traits, such as a higher lipid content and upregulated fatty acid synthase and uncoupling protein expression [271]. Furthermore, brown adipocytes expressing activated Ras develop insulin insensitivity, showing an impairment in the insulin receptor autophosphorylation and augmented glucose uptake in an insulin-independent manner [272, 273]. Using conditional Ras expression vectors, Ras has been shown to induce the expression of IGF-I and IGF-I receptor. As IGF-I mediates adipocytic differentiation, an IGF-I autocrine/paracrine loop might be implicated in the brown adipose tissue differentiation process induced by oncogenic Ras [274]. However, the Ras-induced differentiation effect requires survival factors, since Ras induces apoptosis in a MAPK-independent manner in serum-deprived brown adipocytes [275].

Other cellular differentiation models in vertebrates

The data accumulated so far suggest that Ras acts as an inhibitor of melanocytic differentiation while inducing transformation. Ras mutations are a frequent finding in human melanomas, with a reported incidence ranging from 4 to 36% of the cases. Interestingly, most of these mutations occur in N-*ras* codon 61 [276–279]. Transgenic mice in which H-RasV12 expression is restricted to melanocytes show melanocytic hyperplasia. In these animals, treatment with topical carcinogens results in an increased incidence of melanomas [280]. Melanocyte-specific expression of H-RasV12 concomitant with the loss of the p16^{INK4a} gene enhances the development of spontaneous melanomas [281]. In this system, the supression of Ras expression results in the regression of the melanomas, demonstrating that Ras signals are required for the maintenance of the tumor [282]. In agreement, a dominant-negative RasN17 or the MAPK

inhibitor PD98059 induce differentiation in murine B16 melanoma cells [283]. Although the effect of activated Ras has not been directly tested in this cell line, the inactivation of Ras or MAPK is associated with melanocytic differentiation, a finding at odds with the previously discussed requirement of MAPK for the neuronal differentiation of PC12 and with the roles of Ras and MAPK in adipogenic differentiation of 3T3-L1 cells.

The formation of parietal endoderm is one of the first differentiation processes during mouse development, and F9 embryonal carcinoma cells have proved to be a valuable model to study such phenomena. Treatment of F9 cells with retinoic acid induces differentiation into primitive endoderm cells, while commitment toward parietal endoderm is promoted by subsequent addition of parathyroid hormone. Activated H-*ras* induces differentiation to primitive endoderm while inhibiting the differentiation toward parietal endoderm [284, 285]. Consistent with this effect, retinoic acid-induced differentiation towards primitive endoderm is accompanied with increased Ras activity [285].

Rat endometrial cells can be transformed by SV40 T antigen, giving immortalized cell lines. Ectopic expression of activated H-*ras* enhances the transformed characteristics of these cells, but the expression of glandular and decidual endometrial cell markers recovers [286]. A parallel case occurs in rat granulosa cells. These cells when transformed by SV40 are less differentiated, with loss of progesterone synthesis. However, transfection of H-*ras*V12 restores progesterone production [287]. These two cases where Ras enhances the expression of differentiation markers in cells previously immortalized by SV40 T antigen is reminiscent of the fetal brown adipocyte model described above.

The CaCo2 cell line derives from a human colon carcinoma that does not carry mutations in K- or H-*ras* genes. Sublines obtained by retroviral transduction of H-*ras*V12 grow more slowly in low serum and re-express several markers of intestinal brush border [288]. Likewise, while transfection of oncogenic K-*ras* into the HD6-4 colon epithelial cell line abrogates some differentiated characteristics, such as $\beta 1$ integrin maturation, adhesion to collagen, and cell apicobasal polarity, transfection with wild-type K-*ras* or oncogenic H-*ras* do not exert these effects [289]. These differences are consistent with the frequent activation of K-*ras* but not H-*ras* found in colon cancer.

Oncogenic Ras inhibits differentiation of the FRTL-5 and WRT cell lines, immortalized cell lines derived from rat thyroid epithelium. Thyroid epithelial cells in culture respond to thyroid-stimulating hormone (TSH) both by proliferating and turning on the expression of thyroid-specific genes. Thus, in these cells, proliferation and differentiation are not mutually exclusive. Proliferation of FRTL-5 and WRT depends on Ras activity, as it is abrogated by a dominant negative Ras mutant [290, 291]. Ectopic expression of oncogenic H- and K-Ras results in hormone-independent proliferation and supression of thyroid differentiation, accompanied by inhibition of thyroid transcription factor-1 and -2 (TTF-1 and TTF-2) [290, 292, 293]. Conversely, other results do not seem consistent with the previous data. Thus, a constitutively activated MAPK does not alter the thyroid differentiation program [294], and an H-Ras mutant that selectively signals through PI3K (H-RasV12C40) promotes proliferation of WRT cells without inhibiting thyroid-specific gene expression [295]. Moreover, a recent report indicates that, in contrast to the findings in rat immortalized cell lines, activated Ras (H-RasV12) induced proliferation accompanied with differentiation in human primary follicular epithelial thyroid cells, suggesting that the inhibition of differentiation depends on other abnormalities present in immortalized cells [296].

Drosophila and *Artemia*

Genetic analysis in *Drosophila melanogaster* has permitted the dissection of the role of Ras in the development of a discrete body structure, the insect eye. The *Drosophila* eye is composed of approximately 800 identical units called ommatidia, each of which contains eight photoreceptors cells (termed R1–R8) plus 12 non-neuronal cells (four cone cells and eight accessory cells) disposed in an orderly pattern. This pattern originates from an undifferentiated mass of pluripotent cells in the larva, the eye imaginal disc. Neuronal differentiation of R7 cells is the best understood event in ommatidial assembly. This is initiated by the binding of the ligand Boss (Bride-of-Sevenless), expressed exclusively in the R8 cells, to Sev (Sevenless), a receptor-tyrosine kinase transmembrane receptor expressed in the R7 cell. Sev initiates a signalling cascade mediated by DRas1, the *Drosophila* orthologue to mammalian Ras [297]. Remarkably, this cascade was found to be assembled by components with a high homology to and the same function as those found in mammalian cells: Drk (an SH3-SH2-SH3 adapter), Sos (a guanine nucleotide exchange factor), DRas1, DRaf, Dsor (a MAPKK), and Rolled (MAPK) [298]. Expression of an activated form of Ras1 (V12) activates the Ras cascade in nonneuronal cone cells, transforming them into R7 cells. This disrupts the normal ommatidial array and produces an externally rough eye. The phenotype was originally described as a 'scar' in the eye [299]. This R7 specification is independent of *sev*. Thus Ras1 activation is sufficient to promote neuronal differentiation in these eye precursor cells.

Finally, Ras activity during *Drosophila* differentiation is not restricted to the eye. For example, Ras plays roles in *Drosophila* oogenesis and imaginal disc differentiation. Ectopic expression of Ras inhibits follicle cell differentiation [300]. Another interesting hint revealed by *Drosophila* analysis is that Ras is not essential for proliferation: animals lacking *ras* complete several rounds of cell division before arresting development as abnormal embryos [301]. Activated Ras1 (Ras1V12) induces hyperplasic growth and increases cell death in imaginal tissues [302]. Moreover, in cells of wing imaginal discs, Ras is not an essential requirement for cell proliferation, but it promotes growth and increases cell size. However, cells expressing activated Ras are able to differentiate at the appropriate time in development [122]. Interestingly, as in mammal primary cells (see above), activation of Ras in *Drosophila* cells is not sufficient to accelerate cell division.

Another example of Ras involvement in arthropod early development is that observed in the brine shrimp *Artemia* (*A. franciscana* or *A. salina*). Encysted *Artemia* gastrulae can persist in a latent state for long periods of time. When cysts are exposed to appropriate conditions of humidity and temperature, development reassumes, leading to the hatching of a swimming larva. Although intense morphogenesis takes place, little cell proliferation occurs prior to hatching. The *Artemia* genome has at least one *ras* homologue, with biochemical properties similar to those of mammalian Ras [303, 304]. *ras* mRNA is already present in the cysts, but Ras protein and GTP-binding activity are very low. However, Ras protein levels rise after hatching. Thus, in this model, Ras expression is linked to cell proliferation rather than to tissue differentiation.

Caenorhabditis and *Dictyostelium*

The *ras* homologous gene in *Caenorhabditis elegans* (*let-60RAS*) was originally isolated as a gene that controls the switch between vulval and hypodermal cell fates during vulval induction [305, 306]. The 184-amino acid Let-60 Ras protein is similar in overall sequence to the three mammalian Ras proteins but, interestingly, it is more similar to K- and N-*ras* than to H-*ras* [307]. *let-60* is the only *ras* gene detected so far in *C. elegans*. Constitutive activation of Let-60 causes a multivulva phenotype [306], while heterozygous worms carrying a dominant negative *let-60RAS* show a vulvaless phenotype [307]. A mutation that suppresses the Let-60Ras-mediated multivulva phenotype has been described. The mutated gene, *sur-1/mpk-1*, encodes a MAPK homologue. *C. elegans* homologues for other genes of the Ras-MAPK pathway, such as *mek-2* and *ksr-1*, were subsequently cloned and all of them suppressed the multivulval phenotype [308–311]. These findings provide evidence for the extreme conservation of the Ras-Raf-MAPK-mediated signalling pathway along the phylogenetic scale [312, 313].

In *C. elegans*, Ras and many of its upstream regulators and downstream targets have been found to play a decisive role in multiple developmental events. Animals homozygous for a *let-60* null allele die in the first of the four larval stages. However, mosaic analysis of Ras function reveals no clear role in cellular proliferation in late development. Instead, Ras is required for the establishment of a few temporally and spatially distinct cell fates [307, 314]: (i) Male copulatory spicule formation. Spicules are structures used by male worms to locate the vulva and anchor the male cloaca to the vulva during sperm transfer. (ii) Correct migration of sex-myoblast cells during the third larval stage. (iii) The three genes of the Ras cascade, *let-60ras*, *mpk-1/sur-1*, and *mek-2*, are required for meiotic cycle progression. As a result, mutations in these genes give rise to a sterile phenotype. (iv) Specification of the P12 neuronal lineage [307]. (v) Recently, Ras has been found to play a functional role in transduction of olfactory stimuli, via MAPK activation [315], this being the first instance where Ras function has been related to the nervous system.

Dictyostelium discoideum is probably the simplest living model known where the term 'cell differentiation' can be correctly used. This organism can live as isolated cells (amoebae) which, in response to low nutrient signals, aggregate to form a 'slug' which later differentiates into a fruiting body that contains at least two cell types: stalk and spores. cAMP acts as a potent chemotactic molecule in the process of amoebal aggregation. In this organism, Ras activity impairs differentiation. *D. discoideum* expresses five *ras* genes at different stages of development. *ras*D and *ras*G products are closely related to mammalian Ras proteins, whereas *rasB*, *rasC*, and *rasS* are more divergent. *rasD* is induced by cAMP and expressed after aggregation, while *ras*G is expressed prior to germination and during vegetative growth [316–319]. Thus, *Dictyostelium* is the only organism expressing different *ras* genes at different stages of development. *Dictyostelium* expressing a *ras*D(G12T) mutant exhibits reduced chemotactic sensitivity and formation of aberrant multitipped aggregates that are blocked from further differentiation [320]. Cells expressing the *ras*G(G12T) activated gene show impaired aggregation [321], and null mutants with abolished expression of RasG show defects in cell motility and cytokinesis, but are able to grow [322]. Thus, activated Ras abrogates normal differentiation of *D. discoideum*.

Ras and differentiation: concluding remarks

When looking at the differentiation models described above, the main conclusion that can be drawn is that an important, and probably the most prevalent effect of Ras is to promote cell differentiation. This is particularly clear in neuronal and adipocytic differentiation models. It also seems to be the predominant effect in the in vivo myeloid, lymphoid, and epidermal cell differentiation, at least during their earlier stages. However, in those hematopoietic cell lineages, Ras-promoted differentiation does not bring about growth arrest. Furthermore, data indicating that Ras mediates inhibition of muscle and thyroid differentiation has recently been challenged [265, 295, 296]. The effects of activated *ras* on the differentiation of the aforementioned cell systems are summarized in table 1. These effects are, in most cases, in sharp contrast to those elicited in immortalized rodent fibroblasts, such as NIH3T3, where Ras promotes cell proliferation and transformation into an 'oncogenic-like' phenotype. Historically, fibroblast transformation was the first biological read-out of Ras activity and has therefore imposed the view of *ras* as the 'typical transforming oncogene.' However, having first assayed Ras activity in, for example, PC12 rather than NIH3T3 cells, *ras* should be re-evaluated today as a 'typical' differentiating gene. Several plausible hypotheses for this 'revisionist role' are listed below.

Table 1. Examples of Ras effect on differentiation of some cells in culture. See text for details and references.

Cells or cell line (cell type, species)	Differentiation	Effect
PC12 (rat pheochromocytoma cell line)	Neuronal	Induction
32D (mouse myeloid precursor cell line)	Monocytic	Induction
P39 (human myelodysplasic syndrome cell line)	Myeloid	Induction
Bone marrow cells (mouse)	Macrophagic	Induction
B cell precursors (transgenic mice)	Lymphoid B	Induction
T cell precursors (transgenic mice)	Lymphoid T	Induction
DPK (mouse pre-T cell line)	Lymphoid T	Induction
F9 (mouse embryonal carcinoma)	Primitive endoderm	Induction
3T3-L1 (mouse pro-adipocytes)	Adipocytic	Induction
Fetal brown adipocytes (rat)	Brown adipocytic	Induction
CaCo2 (human colon carcinoma cell line)	Intestinal brush border	Induction
Regenerating muscle (rat)	Muscular	Induction
FRTL-5, WRT (rat thyroid follicular cell lines)	Thyroid epithelium	Inhibition
FD5A1, SKT6 (rat, mouse erythroleukemia cell lines)	Erythroid	Inhibition
BALB/MK (mouse keratinocytes cell line)	Epidermal	Inhibition
C3H10T1/2, C2C12, BC3H1 (mouse myoblasts cell lines)	Muscular	Inhibition
23A2 (rat myoblast cell line)	Muscular	Inhibition

The view of Ras provoking either transformation or differentiation depending on the cellular model utilized raises two important and essentially unanswered questions.

1) How can Ras exert such opposing effects, ranging from oncogenic transformation to differentiation, depending on the cell type? And how does Ras drive differentiation into such distinct lineages within the same animal, as happens, for example, in *C. elegans*?

2) How can the differentiation-promoting (and therefore antiproliferative) effects of *ras* genes be reconciled with the common finding of *ras* mutations in many human and experimental tumors? Differentiation and proliferation being, usually, incompatible, one would expect that cells harboring a 'differentiation gene' should not be selected during tumor development. Moreover, in all the systems tested, differentiation is induced much more potently by *ras* genes carrying 'oncogenic' mutations (i.e., in codons 12 or 61) than by their wild-type versions. The scenario is particularly puzzling in the case of myeloid cells, where Ras mediates cell growth arrest and differentiation in ex vivo models but *ras* mutations are frequent in tumoral conditions such as AML.

Several hypotheses have been proposed to explain these conflicting pleiotropic effects, and all rely on the presence of tissue- or cell type-specific regulatory mechanisms or molecules, of the Ras signaling pathways at their multiple levels. For some models, there is experimental evidence to support these views [reviewed in ref. 323].

1) Differential signalling kinetics and intensity. For example, stimuli that provoke a transient activation of the Ras-MAPK pathway would trigger proliferation, whereas sustained activation of this pathway would trigger differentiation. In rat pheochromocytoma PC12 cells, transient Ras activity induces cell proliferation, while a maintained activation results in neuronal differentiation [186]. Furthermore, different effects may depend on the intensity of the signal. This has been demonstrated in rodent primary fibroblasts. In this system, a low-intensity Ras (and Raf) signal induces proliferation, while a high-intensity signal induces growth inhibition [123, 145, 150]. A similar effect may conceivably be operating in differentiation.

2) The presence of tissue-specific downstream effectors. This hypothesis can explain why different genes are activated in different tissues by similar upstream signals, to induce distinct cellular phenotypes. For example, depending on the cell in question, the predominant

pathway departing from Ras could be Raf-MAPK, PI3K, or the Ral-GDS pathway. An example has again been found in PC12 cells. In this system, Raf and PI3K mediate Ras-induced cell cycle arrest and differentiation. However, Ral-GDS activity suppresses growth arrest and neurite outgrowth induced by NGF treatment. Thus, Ras could promote both anti-differentiation and pro-differentiation effects through the activation of distinct effector proteins [64]. Likewise, not all effectors may act as such in all cell types, which may affect the Ras response. For example, in brown adipocytes, PI3K does not seem to act as a Ras effector [273].

3) Integration of signals at the level of gene expression would result in distinct responses. For example, different signalling pathways might converge upon common elements in the promoters of target genes. Thus, Ras effects on a gene, or set of genes, could be counteracted or amplified by other signalling pathways, depending on the tissue. An example is the mentioned effect of Notch-1 abrogating the Ras-mediated differentiation of pro-adipocytes [269]. In the case of muscle differentiation suppression [263] or induction of brown adipocyte differentiation [274], autocrine/paracrine mechanisms have been proposed to operate. In these models, Ras would be inducing the secretion of factors that inhibit or promote differentiation, respectively.

4) In some cell types, growth arrest triggers a differentiation program. Actually, in many cellular models, differentiation appears to be a consequence of drug or cytokine-induced growth arrest. Thus, it is likely that the primary effect of Ras is to inhibit or retard growth, and differentiation would be a consequence of Ras-mediated cell cycle arrest rather than a direct effect of Ras. However, Ras would not drive differentiation in cells where differentiation is not linked to growth arrest.

Finally, another unexplored issue is a possible differential role of the three Ras proteins in differentiation. While Ras proteins are ubiquitously expressed and the three genes are induced by growth factors [1, 324, 325], there are tissue- and developmental stage-specific differences in the expression of the three Ras isoforms [7, 180, 326], supporting the view that each Ras protein may serve a specialized function. Although not yet fully unveiled, there are some differences in the biochemical effects of Ras proteins [reviewed in refs 10, 327]. A recent report on a genome-wide survey of Ras target genes reveals that, although they have many common targets, there are also isoform-specific downstream targets [328]. Divergent effects have been described after the repopulation of mouse bone marrow with cells expressing H-*ras* or N-*ras* [223–225]. Another puzzling finding is that H- or N-Ras inhibit muscular differentiation of myoblastic cell lines (see above) while the muscular system develops normally in H- and N-*ras*-null mice. K-*ras*-deficient mice embryos die after 12.5 days of development and exhibit early hematopoietic defects. These phenotypes are exacerbated in K-*ras*$^{-/-}$/N-*ras*$^{+/-}$ mice, where embryos die with severe anemia [177–179]. These results suggest that the three Ras isoforms are not equivalent in their roles in differentiation. Unfortunately, to date no systematic comparisons of the effects of the three *ras* genes in a particular differentiation model have been reported.

Acknowledgements. We are grateful to M. Dolores Delgado, Ricardo Escalante, Margarita Lorenzo, Miguel Quintanilla, Leandro Sastre, and Manuel Serrano for critical reading of parts of the manuscript. We also thank Angel Pellicer, Angela Tyner, and Eugenio Santos for disclosing information prior to publication. Work in the laboratories of the authors is supported by grants PM98-109 and FD97-0241 (to J.L.) and PM98-0131 and a grant from Fundacion Marcelino Botín (to P.C.).

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