RAS AND RHO GTPases IN G1-PHASE CELL-CYCLE REGULATION

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As *RAS* mutations are among the most frequent alterations in human cancers, RAS proteins and their signalling pathways have been studied intensively. Here, we outline the contributions of H-RAS, N-RAS and K-RAS to cell-cycle progression and cell growth. We also summarize recent results that indicate how other members of the RAS-GTPase subfamily — including E-RAS, RHEB, R-RAS, TC21 and RAL, as well as RHO GTPases — promote proliferation by regulating the transcription, translation and degradation of key cell-cycle components.

The prototypical RAS GTPase proteins — H-RAS, N-RAS and K-RAS — were first identified as the products of active oncogenes in human tumours. In addition to their ability to de-regulate replication in cancer cells, these GTPases also contribute to cell-cycle regulation in normal, non-transformed cells. H-RAS, N-RAS and K-RAS are highly related, sharing approximately 84% identity — their divergence being almost exclusively confined to the carboxyl terminus. Although there might be subtle differences in the signalling pathways that they activate and in their subcellular distribution, these three proteins similarly promote proliferation and regulate cell-cycle progression. Accumulating evidence indicates that, in addition to these well-characterized RAS GTPases, other RAS- and RHO-family proteins (FIG. 1; BOX 1) also have roles in regulating proliferation. In fact, the possibility exists that cell-cycle regulation, by direct or indirect means, is a general function of these GTPases.

The influence of the H-RAS, K-RAS and N-RAS proteins on specific components of the cell-cycle machinery has been comprehensively investigated and we summarize the salient findings below. The role of other RAS-subfamily members has been highlighted by recent reports revealing that RHEB (RAS homologue enriched in brain) has a central role in protein translational control and cell growth, and that embryonic-stem-cell-expressed E-RAS has a significant role in embryonic-stem-cell proliferation (BOX 2). The RAS-subfamily members RALA, TC21 (also known as R-RAS2) and R-RAS have also been shown to regulate signalling pathways that ultimately control cell-cycle progression.

The most well-studied members of the RHO-GTPase family are RHOA, RAC1 and CDC42. The contributions of each to cell-cycle regulation have been the focus of intense research for several years. Similar to the RAS GTPases, RHO proteins affect key components of the cell-cycle machinery through multiple mechanisms.

The G1 and S phases are not the only cell-cycle phases that are influenced by RAS and RHO proteins: accumulating evidence indicates that these GTPases are essential at all points in the cell cycle. Moreover, RAS might also negatively regulate cell-cycle progression in specific cell contexts. However, these topics would be appropriate for reviews in their own right. For the purposes of this review, we concentrate on the contribution of RAS- and RHO-family proteins to progression from G1 to S phase.

G1-phase cell-cycle progression

The eukaryotic cell cycle (FIG. 2) is made up of four distinct phases: the first gap phase (G1 phase); the DNA synthesis phase (S phase); the second gap phase (G2 phase); and finally, mitosis (M phase). Cell-cycle progression in metazoans requires stimulation by growth factors up to a stage late in G1 phase that is referred to as the restriction point. Once past this point, cells are refractory to both mitogenic and anti-mitogenic signals until the next G1 phase.

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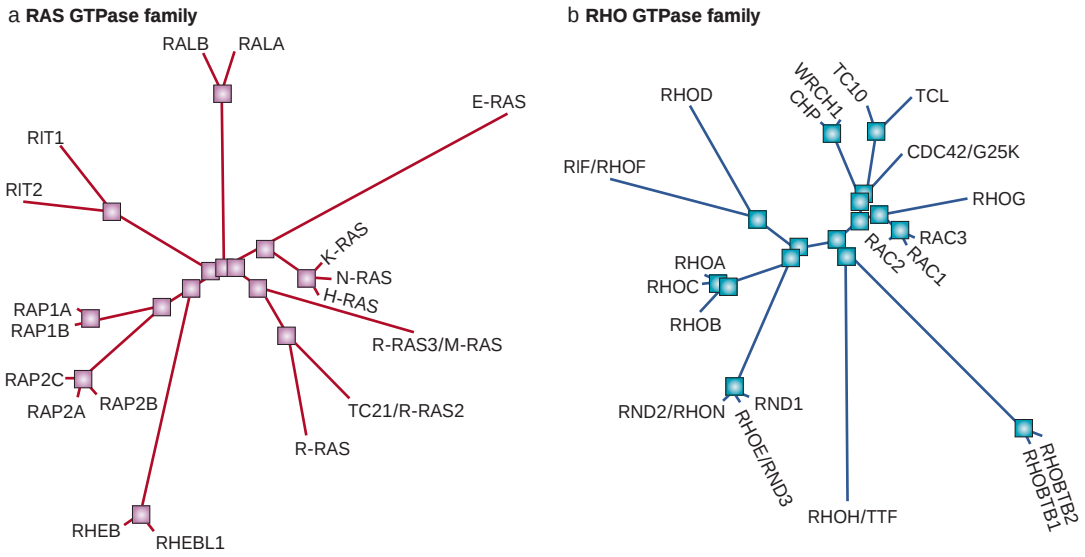


Figure 1 | **RAS- and RHO-GTPase families.** The figure shows the relationships between human RAS- and RHO-family members, the protein sequences of which were aligned into unrooted dendrograms using the MultAlin multiple-sequence-alignment program (see Online links). Filled squares represent points of divergence. Line lengths are proportional to the degree of sequence diversity. Of the RAS and RHO GTPases that undergo alternative splicing, only the K-RAS4B, RAC1A and CDC42 isoforms have been included. For illustrative purposes, the distances separating H-, K- and N-RAS from each other, RAP1A from RAP1B, RAP2A–C from each other, RHEB from RHEBL1, RHOA from RHOC, RAC1–3 from each other, RND1–3 from each other, RHOBTB1 from RHOBTB2, and CHP from WRCH1 have been slightly exaggerated. For RHOBTB1 and RHOBTB2, only the RHO GTPase domain was used in the alignment. RHOBTB3 was not included in the alignment because of its large divergence from the RHO family.

Progression through the cell cycle is driven by the activities of the cyclin-dependent kinases (CDKs), in association with their regulatory subunits, cyclins (reviewed in REF. 1). D-type cyclins (D1, D2 and D3) form active complexes with CDK4 or CDK6, whereas cyclins E1 and E2 work in combination with CDK2. Each cyclin–CDK complex phosphorylates a set of substrates that includes members of the retinoblastoma (RB) family of 'pocket proteins'. Hyperphosphorylation of RB inhibits its ability to sequester members of the E2F transcription-factor family. Liberated E2F complexes drive the transcription of genes that encode the proteins required for S-phase DNA synthesis, such as cyclin A, thymidine kinase and dihydrofolate reductase.

Although active cyclin-D–CDK complexes are the first to be detected after mitogenic stimulation of QUIESCENT cells, both cyclin-D- and cyclin-E-associated CDKs are believed to be required for G1-phase progression. The activities of cyclin–CDK complexes are modulated by the binding of two classes of CDK inhibitor (CDKI) that have differing mechanisms of action. The INK4 CDKI proteins ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$) inhibit CDK activity through direct 1:1 interactions with, and sequestration of, CDKs. However, the 'CIP/KIP'-family CDKIs ($p21^{WAF1/CIP1}$ (p21), $p27^{KIP1}$ (p27) and $p57^{KIP2}$) bind to cyclin–CDK complexes. At high levels, p21 and p27 function as cyclin-E–CDK2 inhibitors, resulting in cell-cycle arrest. At lower levels, however, p21 and p27 actually promote the assembly, stability and nuclear retention of cyclin-D–CDK4 and cyclin-D–CDK6 complexes — the catalytic activities of which are inefficiently inhibited *in vivo* by associated CIP/KIP proteins. Cyclin-D–CDK4 and cyclin-D–CDK6 complexes also promote cell-cycle progression by functioning as a sink for p21 and p27, thereby relieving cyclin-E–CDK2 complexes from p21/p27-mediated inhibition (FIG. 3b). Therefore, the relative levels of cyclin, CDK and CDKI proteins determine whether a cell will progress through the G1 phase and on through the remaining phases of the cell cycle.

Regulation of the cell cycle by RAS proteins

Early studies established the central position of the prototypical RAS GTPases in cell-cycle regulation. Microinjection of RAS-neutralizing antibodies, or introduction of a DOMINANT-NEGATIVE form of RAS, blocked growth-factor-induced S-phase entry[2,3]. Conversely, quiescent non-proliferating cells could be pushed back into the cell cycle by the microinjection of oncogenic H-RAS protein, independently of growth-factor stimulation[2,4].

Working downstream of RAS GTPases to mediate cell-cycle regulation is a three-component mitogen-activated protein kinase (MAPK) cascade, consisting of the RAF, MEK (MAPK and extracellular signal-regulated kinase (ERK) kinase) and ERK/MAPK proteins. In addition, the phosphatidylinositol 3-kinases (PI3Ks) and the RAL-activating RALGDS proteins also signal downstream of RAS (FIG. 4).

The principal function of RAS in G1–S-phase progression is to inactivate RB and relieve cells from its growth-inhibitory actions — cells without RB no longer require RAS activity[5,6]. Both the ERK/MAPK and the

QUIESCENT
The state of a cell that has exited the cell cycle and is in the G0 ('resting') phase.

DOMINANT-NEGATIVE
A protein containing a mutation that adversely affects the function of the corresponding, normal wild-type protein within the same cell. For small GTPases, dominant-negatives are inactive proteins with a reduced affinity for GTP that inhibit the wild-type proteins by binding and sequestering guanine-nucleotide-exchange factors.

Box 1 | RAS GTPase proteins

Small GTPase proteins (so named because of their low molecular weight (~20–35 kDa) relative to heterotrimeric GTPases) are evolutionarily conserved and are found across organismal kingdoms. The first small GTPase to be isolated was H-RAS; as a result, small GTPases have since been assembled into the RAS superfamily. On the basis of primary sequences, the mammalian small GTPases can be subdivided into the RAS, RHO, ARF, RAB, RAN and RAD/GEM subfamilies.

The RAS subfamily includes: H-RAS, K-RAS (4A and 4B), N-RAS, E-RAS, R-RAS, TC21/R-RAS2, M-RAS/R-RAS3, RALA, RALB, RAP1A, RAP1B, RAP2A, RAP2B, RAP2C, RIT1, RIT2, RHEB and RHEBL1. The best-characterized RAS-family members are H-RAS, K-RAS and N-RAS, which regulate a variety of biological processes that contribute to cell-cycle progression and, in pathophysiological conditions, to cancer.

The RAS-related RHO GTPases include: RHOA, RHOB, RHOC, RHOD, RHOE/RND3, RHOG, RHOH/TTF, RND1, RND2/RHON, RAC1 (A and B), RAC2, RAC3, CDC42/G25K, WRCH1, CHP, TC10, TCL, RIF/RHOF, RHOBTB1, RHOBTB2 and RHOBTB3. The best-studied members of the RHO subfamily are RHOA, RAC1 and CDC42, which share approximately 30% amino-acid identity with their RAS homologues. The classical function of RHOA, RAC1 and CDC42 is regulation of the actin cytoskeleton. However, RHO proteins have also been implicated in transcriptional regulation, protein translation, proliferation, motility, apoptosis and membrane trafficking.

PI3K pathways make key contributions to RB inactivation during G1 phase, as inhibition of either pathway blocks the proliferation of wild-type cells, but not *RB*-null cells[7]. Interestingly, however, even in cells that lack RB, cell-cycle re-entry from the G0 quiescent state requires RAS function but not ERK/MAPK activity, indicating that an alternative RAS effector pathway is responsible for this transition. As detailed in the following sections, each of the ERK/MAPK and PI3K pathways impinges, either individually or together, on the regulation of specific cell-cycle components that contribute to proliferation.

Cyclin D1. Mitogen-stimulated induction of cyclin D1 (FIG. 3a) is one of the key events that is required for RB phosphorylation and consequent G1-phase progression. Although the expression of active RAS is sufficient to induce cyclin-D1 expression in growth-factor-deprived quiescent cells[8], the results that were obtained when actively cycling cells were microinjected with a neutralizing anti-RAS antibody indicated that cyclin-D1 expression requires RAS function in the preceding G2 phase[9].

The expression of cyclin D1 and its assembly into a complex with CDK4 or CDK6 both require RAS activation of the RAF–MEK–ERK/MAPK pathway[10–13]. RAS-induced *cyclin-D1* transcription results from sustained activation of this pathway, which leads to elevated expression of transcription factors such as FRA1, FRA2, c-JUN and JUNB[14,15]. Heterodimerization of these transcription factors and binding to the AP-1 SITE in the *cyclin-D1* promoter results in increased transcription[11]. *Cyclin-D1* transcription is also facilitated by ERK1- and ERK2-mediated phosphorylation and consequent inhibition of the transcriptional co-repressor TOB[16]. RAS activation of PI3K also contributes to increased *cyclin-D1* transcription; cooperativity between the ERK/MAPK and PI3K pathways indicates that each pathway might use separate mechanisms to activate *cyclin-D1* transcription[17].

The regulation of cyclin-D1 protein stability is also important in generating active G1-phase CDK complexes. Phosphorylation of cyclin D1 by glycogen-synthase kinase-3β (GSK3β) results in the ubiquitylation and PROTEASOME-mediated degradation of cyclin D1 (REF. 18). However, PI3K signalling to AKT/protein kinase B (PKB) results in inhibition of GSK3β, thereby enhancing cyclin-D1 protein stability. RAS also works through PI3K to promote translation of *cyclin-D1* messenger RNA[19]. Therefore growth-factor-induced regulation of *cyclin-D1* transcription, stabilization of cyclin-D1 protein and cyclin-D1 assembly with CDK4 or CDK6 are regulated primarily through RAS-dependent pathways. The crucial importance of cyclin D1 for RAS-induced cell proliferation is shown by the resistance of mice that lack cyclin D1 to the induction of breast cancer by oncogenic H-Ras in mammary epithelial cells[20].

p27 and p21 CDK-inhibitor proteins

Mitogen-induced downregulation of p27 expression occurs through transcriptional and post-transcriptional mechanisms and can be prevented by dominant-negative RAS[10,21,22]. RAS-mediated activation of the RAF–MEK–ERK/MAPK pathway promotes cell-cycle progression by reducing p27 CDKI levels (FIG. 3c) through enhanced proteolysis[14,23] and decreased protein synthesis, resulting in increased CDK activity[24]. Furthermore, phosphorylation of p27 by cyclin-E–CDK2 and/or cyclin-A–CDK2 on Thr187 (REFS 25,26) results in the ubiquitylation and proteasome-mediated degradation of p27. However, sustained ERK/MAPK activation can also lead to p27 degradation independently of CDK2 activity[14,23], which is consistent with results that were obtained from a mouse knock-in model that showed a Cdk2-independent mechanism of p27 degradation in G1 phase[27].

The PI3K effector pathway has also been reported to have a role in the regulation of p27 expression[21,28,29]. RAS represses p27 transcription by inactivating members of the FORKHEAD TRANSCRIPTION-FACTOR FAMILY through PI3K and RAL-induced phosphorylation[29–31]. The PI3K pathway also regulates the proteasome-mediated degradation of p27 (REFS 21,28), possibly through the transcriptional induction of $p45^{SKP2}$ (REF. 28), the F-BOX component of the G1-phase E3 UBIQUITIN LIGASE, SCF (SKP1/CUL1/F-box protein).

Counter-intuitively, mitogens elevate p21 levels through RAS (FIG. 3a) and RAF–MEK–ERK/MAPK signalling[32,33]. This RAS- and ERK/MAPK-induced p21 elevation is the result of increased *p21* transcription[32]. The seemingly paradoxical pattern of p21 regulation by RAS might be explained by the role of p21 as a promoter of cyclin-D1–CDK assembly, nuclear retention and stability[34–36] — it functions as a positive factor for cell-cycle progression at lower expression levels. Therefore, the moderate levels of RAS activation that are achieved by mitogenic signalling result in a balance between increased p21 and decreased p27 levels that do not merely permit, but actually promote, cell proliferation.

AP-1 SITE
The palindromic DNA sequence TGACTCA, which serves as a binding site for transcription-factor complexes formed from heterodimers of FOS- and JUN-family proteins.

PROTEASOME
A large multisubunit protease complex that selectively degrades multi-ubiquitylated proteins. It contains a 20S particle that incorporates the catalytic activity, and two regulatory 19S particles.

FORKHEAD TRANSCRIPTION-FACTOR FAMILY
A family consisting of more than 40 members, which belong to the winged-helix class of DNA-binding proteins and are involved in diverse cellular functions, including glucose metabolism, apoptosis and cell-cycle regulation.

F-BOX
A domain found in the F-box family of proteins that binds and recruits protein substrates to SKP1/CUL1/F-box protein (SCF) E3 ubiquitin ligases. F-box proteins mediate the interaction between the substrate and the ubiquitin ligase, which results in substrate ubiquitylation and degradation by the proteasome.

Box 2 | E-RAS — embryonic-stem-cell-expressed RAS GTPase

The E-RAS GTPase is closely related to, although on a separate evolutionary branch from, the H-RAS, N-RAS and K-RAS proteins (~45% identity; FIG. 1). Previously, E-RAS was erroneously thought to be encoded by a non-productive pseudogene called *HRASP*[134]. Both human and mouse orthologues of E-RAS have specific amino-acid differences from the prototypical RAS proteins; these differences make E-RAS GTPase-deficient and, therefore, constitutively active[135].

Expression of E-Ras has been reported to be restricted to undifferentiated embryonic stem cells, with no detectable expression in differentiated embryonic stem cells or somatic tissues from adult mice[135]. Immunoprecipitation experiments showed that E-Ras interacts with phosphatidylinositol 3-kinase (Pi3k), but not with Raf1 or B-Raf. Consistent with these results, undifferentiated embryonic stem cells were shown to be sensitive to Pi3k inhibition, but not to Mek inhibition. Pi3k inhibition resulted in reduced cyclin-D1 levels and G1-phase arrest[136]. Pi3k regulation of cyclin-D1 levels in undifferentiated embryonic stem cells was mediated primarily through increased mRNA translation and decreased glycogen synthase kinase-3β (Gsk3β)-induced protein degradation. In addition, Pi3k activity was found to be independent of serum stimulation in undifferentiated embryonic stem cells[136], consistent with persistent activation by constitutively GTP-bound E-Ras. E-Ras, therefore, seems to have evolved to specifically regulate cell growth in undifferentiated embryonic stem cells.

RAS GTPases and mRNA translation

Mitogenic stimulation of cells leads to enhanced rates of mRNA translation and synthesis of proteins that are required for cell growth and G1-phase progression. RAS-regulated signalling pathways are intimately involved in transducing mitogenic signals to the translational apparatus.

Recruitment of mRNAs by polysomes. Recent findings indicate that the main contribution of RAS to protein expression might occur at the level of mRNA translation and not gene transcription. Studies of the consequences of blocking RAS and AKT/PKB signalling on the profiles of total mRNA and POLYSOME-associated mRNA revealed only moderate effects on global gene transcription, but profound effects on polysome-associated mRNA levels[37]. Specific mRNAs that are involved in cell-cycle regulation, including *p27* and *cyclin D2*, were found to be differentially recruited to ribosomes and then translated in response to RAS and AKT/PKB signalling. Therefore, a key mechanism of RAS-induced cell proliferation might involve enhanced translation of a pool of pre-existing mRNAs.

Regulation of TOR by RAS GTPases

One of the master regulators of translation control is the mammalian target of rapamycin (TOR) protein, which controls the translational apparatus through protein phosphorylation (FIG. 5). The requirement for TOR in cell-cycle progression was shown by studies with the inhibitor rapamycin, which blocks mitogen- and oncogene-induced proliferation (reviewed in REF. 38). The growth-inhibitory effects of rapamycin have been associated with *p27* induction and decreased *cyclin-D1* expression[39–42].

Key regulators of translation that are controlled by TOR include the p70 ribosomal S6 kinases (S6K1 and S6K2) and 4E-BP1. Both 4E-BP1 and S6K pathways contribute to the regulation of cell-cycle progression by TOR. For example, elevated expression of 4E-BP1 phenocopies the effects of rapamycin (decreased cyclin D1 and elevated p27 levels) in MCF7 (human breast carcinoma) cells[43]. Sequestration of the translation-initiation factor eIF4E by 4E-BP1 might block cyclin-D1 translation by preventing eIF4E-induced transport of *cyclin-D1* mRNA from the nucleus to the cytoplasm[44]. Similarly, microinjection of neutralizing anti-S6K antibodies blocks mitogen-induced G1–S-phase progression[45].

S6K1 and S6K2 phosphorylate the S6 protein of the 40S ribosomal complex, which stimulates the translation of mRNAs with 5′ terminal oligopyrimidine (TOP) TRACTS that code for the components of the protein synthesis apparatus (reviewed in REF. 46). Phosphorylation of 4E-BP1 by TOR dissociates 4E-BP1 from eIF4E, a translation initiation factor that binds the 5′-cap structure of mRNA transcripts (FIG. 5). Free eIF4E is then also able to bind eIF4G, a scaffolding protein that is involved in the assembly of the translation-initiation complex eIF4F (reviewed in REF. 47).

The TOR–S6K pathway is regulated by signals that are transmitted by PI3K in response to mitogen stimulation and nutrient supply. PI3K functions through AKT/PKB-mediated phosphorylation and inhibition of a suppressor complex (FIG. 6) that is composed of tuberous sclerosis-1 (TSC1) (also known as hamartin) and TSC2 (also known as tuberin), which function as negative regulators of the TOR pathway in *Drosophila melanogaster*[48–50] and mammals[51–56]. *TSC* gene mutations result in a predisposition to at least two cancer-related diseases; tuberous sclerosis and lymphangioleiomyomatosis. How TSC1 and TSC2 constrain the

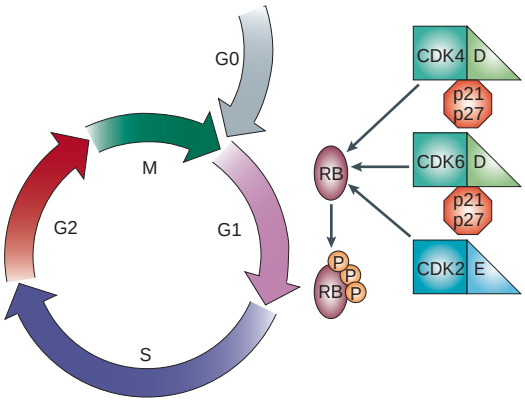


Figure 2 | **The eukaryotic cell cycle.** The cell cycle is composed of four distinct phases: the first gap phase (G1); the DNA synthesis phase (S); the second gap phase (G2); and finally mitosis (M). Quiescent cells that have 'dropped out' of the cell cycle are in G0 phase. Movement through the cell cycle is driven by the activities of complexes of cyclins and cyclin-dependent kinases (CDKs), which phosphorylate retinoblastoma (RB)-family 'pocket proteins', thereby blocking their growth-inhibitory functions and permitting cell-cycle progression. Advancement through G1 phase is facilitated by the D-type cyclins (D1, D2, D3, light-green triangles), which form active complexes with CDK4 or CDK6, and E-type cyclins (E1, E2, light-blue triangles) in combination with CDK2. Cyclin-D–CDK4 and cyclin-D–CDK6 complexes are active when associated with p21 or p27 CDK inhibitors (CDKIs), but only cyclin-E–CDK2 complexes that are free of CDKIs are active *in vivo*.

E3 UBIQUITIN LIGASE
The final enzyme complex in the ubiquitin-conjugation pathway. E3 enzymes transfer ubiquitin from previous components of the pathway to the substrate protein to form a covalently linked ubiquitin–substrate conjugate, which is then degraded by the proteasome.

POLYSOME
Or polyribosome; two or more ribosomes attached to different points on the same strand of mRNA.

TOP TRACTS
Terminal oligopyrimidine (TOP) tract. An uninterrupted sequence of 4–20 pyrimidines that is typically found in the 5′-untranslated region of messenger RNAs that encode components of the mammalian translational apparatus.

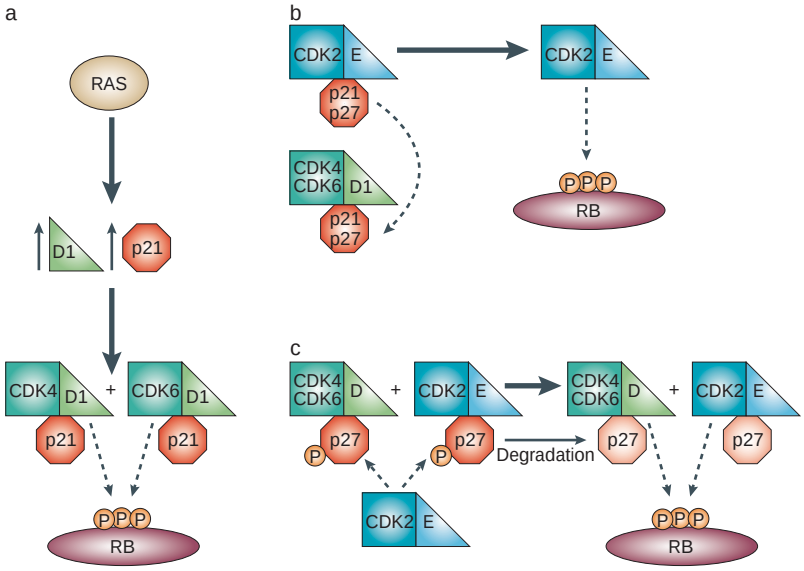


Figure 3 | **RAS effects on cell-cycle components. a** | RAS signalling elevates cyclin-D1 levels by influencing the transcription, translation and protein stability of cyclin D1. An increase in p21 levels results from direct effects on transcription and indirect effects (through cyclin D1) on protein stability. Cyclin D1 and p21 then drive the formation of active cyclin-D1–CDK4 (cyclin-dependent kinase-4) and cyclin-D1–CDK6 complexes, which promote cell-cycle progression through phosphorylation of retinoblastoma (RB) proteins. **b** | Once free of associated p21 or p27 (one mechanism for this being proteasome-mediated degradation of p21 and p27), the elevated levels of cyclin-D1–CDK4 and cyclin-D1–CDK6 function as a sink for p21 and p27 that would otherwise be associated with cyclin-E–CDK2 complexes (dashed arrow). This results in activation of the cyclin-E–CDK2 complexes, increased RB phosphorylation and progress through G1 phase. **c** | Active cyclin-E–CDK2 also phosphorylates p27 that is associated with cyclin-D–CDK4, cyclin-D–CDK6 or cyclin-E–CDK2, on Thr187, leading to p27 ubiquitylation and proteasome-mediated degradation. This leads directly to increased levels of active cyclin-E–CDK2 complexes, and indirectly activates cyclin-E–CDK2 by increasing the availability of cyclin-D–CDK4 and cyclin-D–CDK6 for p27 sequestration, as described in **b**. Further CDK2-independent mechanisms that lead to p27 degradation in response to RAS and RAF–MEK–ERK/MAPK signalling have been implied. ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase.

TOR–S6K pathway and therefore suppress tumorigenesis is unclear, but might involve their influence over the cell cycle[57–59], as changes in protein synthesis through translational control are essential to balance cell growth with division.

Regulation of S6K and 4E-BP1 by RAS GTPases

In addition to the regulation of TOR activity, PI3K signalling directly inputs into S6K though the activation of phosphoinositide-dependent kinase-1 (PDK1), which phosphorylates a site in S6K that is essential for its activation (FIG. 6). The RAF–MEK–ERK/MAPK pathway has also been implicated in the phosphorylation and regulation of 4E-BP1 and S6K1/2 (FIG. 5).

The RAF–MEK–ERK/MAPK pathway has been reported to be both necessary and sufficient for 4E-BP1 phosphorylation in some cell types[60], but not in others[61,62]. Whether ERK/MAPK-mediated 4E-BP1 phosphorylation promotes eIF4E dissociation from 4E-BP1 or prevents new complex formation is not clear[47,60]. In addition to regulating 4E-BP1 by direct phosphorylation, ERK/MAPK decreases 4E-BP1 expression in haematopoietic cells by transcriptional repression[63].

RAS and ERK/MAPK might also have mitogen-specific roles in S6K1 regulation. Neither has been reported to contribute to platelet-derived growth factor (PDGF)-induced S6K1 activation[64], whereas S6K1 activation by granulocyte-macrophage-colony-stimulating factor (GM-CSF) is MEK-dependent[65]. MEK-dependent phosphorylation at three ERK/MAPK consensus sites in the carboxyl terminus of S6K2 is required for full responsiveness to activating stimuli, whereas mutation of these sites reduces, but does not eliminate, S6K1 responsiveness[66]. Recent data indicate that ERK/MAPK might also regulate S6K/4E-BP1 upstream of TOR through MEK-dependent phosphorylation of TSC2 (REF. 67; FIG. 6).

Control of growth and proliferation by TSC1/2 and RHEB. GTPases are typically regulated through the opposing actions of guanine nucleotide-exchange factors (GEFs) and GTPase-ACTIVATING PROTEINS (GAPs). GEFs activate GTPases by promoting the exchange of GDP for GTP; GAPs inactivate GTPases by stimulating GTP hydrolysis (BOX 3).

Mutations in GEFs and GAPs are associated with several human diseases (for example, the NF1 RAS GAP is mutated in neurofibromatosis[68]; the TIAM1 RAC GEF in renal cell carcinomas[69]). In addition, the most severe forms of tuberous sclerosis are associated with mutations in a region of TSC2 that has homology to RAP1 GAP[70]. Although TSC2 shows GAP activity towards RAP1 and RAB5 *in vitro*[71,72], its physiologically relevant *in vivo* substrate has only recently been identified as the RAS-family member RHEB[73–75], which is an essential component of the TOR–S6K pathway[73,74,76,77]. RHEB and its closest relative, RHEBL1 (RHEB-like-1 52% identity), form a divergent branch of the RAS family (FIG. 1). A replication-promoting role for RHEB was originally postulated following the observations that Rheb synergizes with Raf1 to transform NIH-3T3 fibroblasts[78]; that Rheb is upregulated in Ras-transformed cells[78] and in tumour cell lines[79]; and that deletion of the *Rheb* gene in fission yeast results in growth arrest[80].

Genetic screens in *D. melanogaster* have recently identified Rheb as a growth promoter[76,77,81]. Partial loss of Rheb function results in reduced fly size, which is associated with decreased cell size and number. Conversely, overexpression of Rheb *in vivo* is sufficient to increase cell size and DNA content and to promote growth during nutrient starvation[76,77,81]. EPISTASIS analysis of RHEB, TSC1/2 and components of the PI3K and TOR pathways place RHEB downstream of PI3K and the TSC complex, but upstream of TOR (FIG. 6).

The results from these studies with *D. melanogaster* have been confirmed and extended in tissue-culture models. Ectopic expression of RHEB is sufficient to achieve TOR phosphorylation[53], phosphorylation and activation of S6K1 (REFS 73,74,77,82,83), and phosphorylation[74,82,83] and release of 4E-BP1 from eIF4E (REF. 82). Consistent with RHEB functioning downstream of the TSC complex, loss of TSC1/2 — and, therefore, loss of GAP activity towards RHEB — also leads to constitutive S6K1 and 4E-BP1 phosphorylation[53,54,74]. Indeed,

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).

EPISTASIS
A genetic interaction between two alleles. Epistatic analysis studies the genetic interaction between gene products in a signalling pathway. By determining the phenotypes of single and double mutants, the functional order of the components can be inferred.

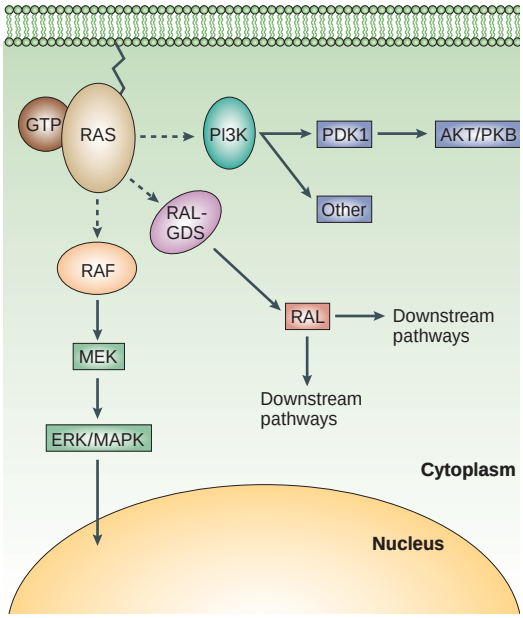


Figure 4 | **RAS effector pathways.** Active GTP-bound RAS stimulates a variety of effector signalling pathways. The three best-characterized are the RAF–MEK–ERK/MAPK, RAL and phosphatidylinositol 3-kinase (PI3K) pathways. Other RAS effectors have been identified, but their roles in cell-cycle regulation are less clearly defined. In the first of these pathways, GTP-bound RAS activates the serine/threonine kinase RAF, which, in turn, activates the dual-specificity tyrosine/threonine kinase MEK. Active MEK then phosphorylates ERK/MAPK, which translocates to the nucleus and activates a variety of transcription factors. RAS also activates the RAL GTPase through the activation of RAL guanine-nucleotide-exchange factors such as RALGDS. Furthermore, RAS binds and activates the catalytic subunit of PI3K, a lipid kinase that generates phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) second messengers, which alter the conformation and localization of the phosphoinositide-dependent kinase-1 (PDK1) and AKT/protein kinase B (PKB) kinases — ultimately leading to AKT/PKB activation. PtdIns(3,4,5)P_3 produced by PI3K also activates numerous other signalling pathways, which are not discussed here. ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase.

overexpression of TSC1/2 is sufficient to inhibit RHEB-mediated S6K1 activation and 4E-BP1 phosphorylation[74,82,83]. Unlike wild-type TSC2, TSC2 proteins with disease-associated point mutations in the GAP domain are unable to inhibit RHEB function or activate RHEB–GTP hydrolysis[73–75,82], which indicates that a primary function of TSC2 is to regulate RHEB–GTP levels through its GAP activity. It is likely that RHEB is the direct *in vivo* target of the TSC2 GAP, as TSC2 and RHEB form a complex[73] that is sufficient to promote GTP hydrolysis *in vitro*[75,82].

Overexpression of Rheb in *D. melanogaster* S2 cells promotes G1–S-phase progression, whereas RNA-INTERFERENCE-mediated knockdown of endogenous Rheb causes G1-phase arrest[81]. Analysis of cell-cycle components that are influenced by TSC1/2 or TOR indicates that potential RHEB targets might include p27 and cyclin E. TSC1/2 promotes accumulation of p27 protein[57], whereas AKT/PKB-dependent inactivation of TSC1/2 is required for p27 downregulation and cell-cycle progression[84]. In addition, deletion of *D. melanogaster* TOR inhibits cyclin-E expression and cell proliferation[85]. As discussed above, cyclin-D1 expression lies downstream of TOR and therefore might also be positively regulated by RHEB.

Further investigation of TSC1/2 and RHEB is needed to shed more light on their regulation and their crucial cell-cycle targets. In particular, clarification is needed of how the GAP activity of the TSC1/2 complex is regulated and how RHEB–GTP influences TOR activity. Furthermore, although most small GTPases are regulated through the combined actions of GEFs and GAPs (BOX 3), is not yet clear whether there is a RHEB-GEF or whether RHEB is regulated solely through GAP-mediated inactivation.

Comparing the phenotypes of RHEB and TOR loss-of-function mutants indicates that there might be other RHEB targets in addition to TOR, S6K1 and 4E-BP1. For example, *D. melanogaster* Tor mutants are viable[85], whereas loss of Rheb is lethal[77]. In this respect, it is interesting that TSC1/2 exists in signalling complexes that are distinct from those that regulate the TOR–S6K pathway[86].

Additional RAS-family GTPases

RAL GTPases (FIG. 1) — which have been implicated as important components of oncogenic transformation that are induced by RAS[87,88] — and TC21/R-RAS2 (REF. 89) also promote cell-cycle progression in their own right.

RAL proteins. RAL increases *cyclin-D1* transcription, probably through the activation of the transcription factor nuclear factor κB (NF-κB) and the subsequent association of NF-κB with NF-κB-responsive elements in the *cyclin-D1* promoter[17,90]. In addition, RAL induces phosphorylation and consequent inhibition of the forkhead transcription factor FOXO4/AFX, leading to decreased p27 transcription[31]. Interestingly, despite sharing 78% identity, the human RALA and RALB proteins seem to control distinct biological functions, with RALA contributing to cell proliferation and RALB promoting cell survival[91]. These functional differences might result from sequence divergence in the carboxy-terminal regions of RALA and RALB, which might lead to distinct patterns of subcellular localization.

R-RAS proteins. Numerous lines of evidence indicate that the R-RAS proteins (R-RAS, TC21/R-RAS2 and M-RAS/R-RAS3; FIG. 1) contribute to cell-cycle progression. Such evidence includes the finding that microinjection of recombinant R-RAS protein into quiescent cells stimulates G1–S phase progression[92]. Although these proteins have been grouped together largely on the basis of sequence similarity, there are distinct functional differences — for example, TC21 is a potent oncogene in NIH-3T3 cells[93], whereas R-RAS is poorly transforming[94]. There seem to be significant differences in the activation of the RAF–MEK–ERK/MAPK pathway by R-RAS

RNA INTERFERENCE
The use of double-stranded RNAs, with sequences that precisely match a given gene, to 'knock down' the expression of that gene by directing RNA-degrading enzymes to destroy the encoded mRNA transcript.

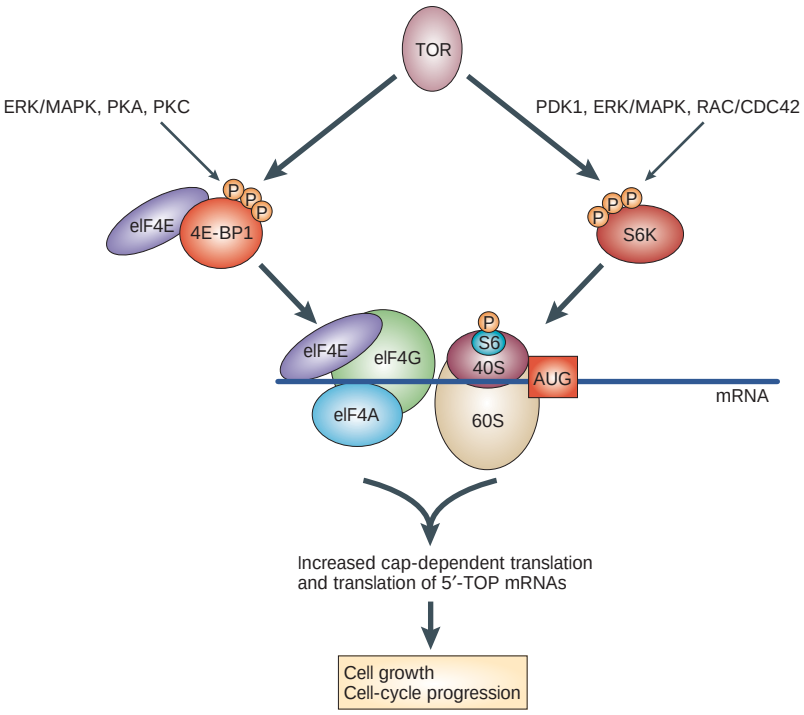


Figure 5 | **Regulation of translation by the target of rapamycin.** In response to nutrients or mitogenic signalling, target of rapamycin (TOR) phosphorylates 4E-BP1 and S6 kinase (S6K). Phosphorylation by TOR primes each molecule for further phosphorylation events that are required for inhibition or activation of 4E-BP1 and S6K, respectively. So, protein kinase A (PKA) and protein kinase C (PKC), in addition to the RAF–MEK–ERK/MAPK pathway, might regulate 4E-BP1 phosphorylation, resulting in liberated eIF4E being recruited into the translation-initiation-factor complex (eIF4F) that includes eIF4A and eIF4G. Assembly of this complex promotes translation that is dependent on the 5′-cap structure of mRNA. Meanwhile, the phosphatidylinositol 3-kinase (PI3K) effector phosphoinositide-dependent kinase-1 (PDK1) contributes to S6K activation by phosphorylating the T-LOOP in the catalytic domain. Other positive regulators of S6K phosphorylation and activation include the RAF–MEK–ERK/MAPK pathway and RAC/CDC42 and their effectors — mixed-lineage kinase-3 (MLK3) and PKCζ. S6K phosphorylates the S6 subunit of the 40S ribosome, which enhances translation of mRNAs with a 5′- terminal oligopyrimidine (TOP) tract (representing as many as 200 genes, including components of the translational apparatus). Enhanced protein synthesis allows cell growth and, in turn, cell-cycle progression. AUG, translation-initiation codon; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase.

proteins. R-RAS seems to be unable to activate this pathway, whereas TC21 and M-RAS do so weakly[92–96]. Despite the lower magnitude of RAF–MEK–ERK/MAPK activation by R-RAS proteins relative to classical RAS proteins, TC21-transformed cells are still dependent on ERK/MAPK activity for progression into S phase[93]; transformation by R-RAS and M-RAS might share a similar requirement. R-RAS proteins, like E-RAS (BOX 2), are significant activators of PI3K[89,94,95], and TC21 has also been shown to activate the RAL[89] and NF-κB signalling pathways[96]. These signal transduction pathways probably converge to collectively mediate R-RAS-induced effects on the cell cycle.

RHO GTPases and cell-cycle regulation

Early indications that RHO GTPases contribute to cell-cycle progression were the observations that inactivation of Rho by the *Clostridium botulinum* C3 ADP-ribosyl transferase, or microinjection with dominant-negative forms of Rac1 or Cdc42, blocked mitogen-stimulated G1–S phase progression in Swiss 3T3 fibroblasts[97,98]. Conversely, microinjection of active RhoA, Rac1 or Cdc42 into quiescent cells was sufficient to induce G1–S-phase progression[98]. Since these initial reports, RHO GTPases have been linked to the regulation of specific cell-cycle components.

CDK inhibitors. The ability of RHOA to repress p21 was revealed during investigations into oncogenic-RAS-induced G1–S-phase progression in Swiss 3T3 fibroblasts[99]. High-intensity signalling by RAS, RAF or MEK leads to p21-mediated growth inhibition in a variety of cell lines[99–104], but this can be overcome by RHO-mediated suppression of p21 transcription[99,104]. One reported mechanism of RHOA-induced p21 repression in vascular smooth muscle and erythromyeloblast D2 cells is phosphorylation, and consequent cytoplasmic retention, of active ERK/MAPK by the RHO effector RHO kinase (ROCK)[105,106]. However, RHOA-mediated suppression of p21 does not require ROCK function in normal and RAS-transformed fibroblasts, or in colon carcinoma cell lines[107–109], indicating that RHOA-induced p21 transcriptional regulation is mediated by cell-type-dependent signalling pathways.

RHO activation allows mitogen-stimulated cells to progress through the cell cycle. However, in the absence of adhesion or under conditions of cell confluence, RHO is inactive[108,110] and p21 expression remains high[33], thereby functioning as a monitor of the cellular environment and as an adhesion-dependent cell-cycle checkpoint. Activation of RAC1 or CDC42 by growth factors, or by cell attachment that is mediated through integrin proteins, might also influence cell-cycle progression through modulation of p21 levels by activating its ubiquitin-independent proteasome-mediated degradation[111].

Modulation of CDKI levels seems to be a recurrent theme in cell-cycle regulation by RHO GTPases. Inhibition of RHOA was reported to elevate p27 protein levels[22,24,112,113], whereas expression of active RHOA decreased these levels[24,113,114], possibly by inducing cyclin-E–CDK2 activity[115]. However, it remains to be determined whether the effects of RHOA on cyclin-E–CDK2 activity and consequent p27 degradation are the result of p21 repression[99,104,116], increased cyclin-E expression[117], or another mechanism. Inhibition of RHOA was also reported to increase *p27* mRNA translation through a 'RHO-responsive' element in the 3′-untranslated region of *p27* (REF. 118).

Cyclin-D1 *transcription.* RAS-mediated induction of *cyclin-D1* transcription results from prolonged activation of the RAF–MEK–ERK/MAPK pathway[12,15]. Accumulating evidence now indicates that sustained RAF–MEK–ERK/MAPK activation requires growth-factor-receptor signalling functioning through RAS, together with integrin-derived adhesion signals that operate through RHO GTPases[109,119–121]. For instance, RHOA was found to be necessary for sustained ERK/MAPK activation in response to simultaneous

T-LOOP
A structural loop that is highly conserved in the catalytic domains of protein kinases. Phosphorylation of this transactivation loop is often required for full catalytic activity.

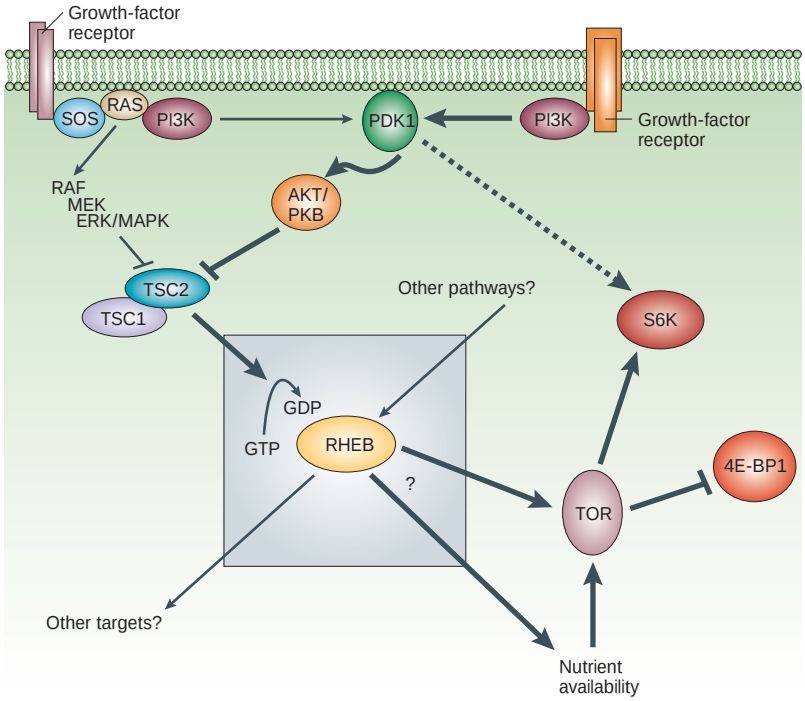


Figure 6 | **Regulation of TOR/S6K/4E-BP1 by the TSC–RHEB pathway.** Insulin and other growth factors activate phosphatidylinositol 3-kinase (PI3K), either through recruitment of the PI3K regulatory subunit to the appropriate receptor tyrosine kinase, or through the activation of RAS by receptor-associated guanine-nucleotide-exchange factors (GEFs) such as son-of-sevenless (SOS). PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3), which recruits phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane. PDK1 phosphorylates the activation loop of both AKT/protein kinase B (PKB) and S6 kinase. AKT/PKB-mediated phosphorylation of tuberous sclerosis (TSC)2 inhibits the TSC complex. Signalling through the RAF–MEK–ERK/MAPK pathway also leads to phosphorylation and inhibition of the TSC complex. The GTPase-activating protein (GAP) domain of TSC2 would otherwise promote GTP hydrolysis on the RAS homologue RHEB, reducing levels of the active, GTP-bound form. Therefore, inhibition of the TSC complex promotes RHEB activation, which signals to target of rapamycin (TOR), which, in turn, phosphorylates S6K and 4E-BP1. Other pathways of RHEB regulation might exist — there might be a RHEB GEF, or post-translational modifications such as FARNESYLATION. How RHEB activates TOR is unclear (denoted by ? in the figure), but it might be either direct, or indirect, through the regulation of nutrient availability. Results from genetic analysis in *Drosophila melanogaster* indicate that RHEB might also have targets other than TOR. ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK and ERK kinase.

FARNESYLATION
A post-translational modification in which a farnesyl group (a hydrophobic group of three isoprene units) is conjugated to proteins, such as RAS GTPases, that contain a carboxy-terminal CAAX motif. Farnesylation promotes attachment of the modified proteins to membranes.

STRESS FIBRES
A component of the actin cytoskeleton that consists of contractile bundles of actin and myosin II, which terminate in adhesion plaques that link the actin cytoskeleton to the cell surface. Stress fibres are involved in cell adhesion and the generation of tensile force.

stimulation of the fibroblast-growth-factor receptor and $\alpha_5\beta_1$ integrins (the main receptor for the matrix component fibronectin)[120]. This RHOA-derived signal that permits sustained ERK/MAPK activation requires the activity of LIM kinase, which is phosphorylated and activated by ROCK, and intact actin STRESS FIBRES to allow clustering of $\alpha_5\beta_1$ integrins[109,121]. Prolonged ERK/MAPK activity for several hours leads to G1-phase expression of cyclin D1 and subsequently to progression through G1 to S phase[15,22,119].

Surprisingly, however, although inhibition of Rho, Rock or Lim kinase blocked sustained Erk/Mapk activity, it actually led to rapid Rac- or Cdc42-dependent cyclin-D1 induction in response to mitogenic stimulation of murine fibroblasts[109,120,121]. So, although RHO, ROCK and LIM kinase are required for sustained RAS activation, they seem to work downstream of RAC or CDC42 to repress early cyclin-D1 induction in response to mitogenic stimuli[109,120,121] and thereby might normally function to prevent premature cell-cycle entry. The ability of LIM kinase to inhibit RAC- or CDC42-induced cyclin-D1 expression is independent of its effects on the actin cytoskeleton, but it does require LIM kinase to be localized to the nucleus[121].

Therefore, in fibroblasts, RHO functions as the master adhesion-dependent regulator of cyclin-D1 expression, and so the duration of the G1 phase is responsive to the formation of actin stress fibres and the induction of intracellular tension. When RHO, ROCK and LIM kinase are active, early G1-phase induction of cyclin D1 by RAC or CDC42 is blocked, but sustained ERK/MAPK activity results in cyclin-D1 induction in mid-G1 phase. When RHO, ROCK and LIM-kinase signalling are low, mitogen-stimulated ERK/MAPK activity is transient but RAC–CDC42 signalling leads to early cyclin-D1 expression and an accelerated G1 phase. It is interesting to note that ROCK signalling is down-regulated in RAS-transformed cells[108], which might allow RAC–CDC42 signalling to work in parallel with the RAS–RAF–MEK–ERK/MAPK pathway to elevate cyclin-D1 expression.

The studies outlined above indicate that there are at least two mechanisms for mitogen-induced cell-cycle entry: one is dependent on RAS and ROCK signalling to promote sustained ERK/MAPK activity and the consequent accumulation of cyclin D1; another functions through RAC to promote ERK/MAPK-independent cyclin-D1 expression. These findings have important implications for our understanding of cell-cycle control, as it has generally been assumed from previous studies that ERK/MAPK activation is universally required for cell-cycle entry. A challenge for the future will be to determine what mechanisms are used in different cell types.

Overexpression of mutant forms of RAC1 or CDC42 that lack GTPase activity has been found to be sufficient to induce cyclin-D1 expression and/or promoter activity[17,122–125]. The stimulatory effect of RAC1/CDC42 on *cyclin-D1* transcription is probably mediated through NF-κB[122] and not through AP-1-mediated transcription[123]. The link between RAC1 and CDC42 signalling to NF-κB activation has not been precisely defined and might involve many separate inhibitor-of-NF-κB (IκB) kinase (IKK)-dependent and IKK-independent pathways[126].

Activation of *cyclin-D1* transcription is not unique to RAC1 and CDC42 within the RHO GTPase family. The TC10 GTPase — which interacts with the EXO70 component of the exocyst complex to regulate insulin-stimulated translocation of the GLUT4 glucose transporter — promotes transcription from the *cyclin D1* promoter[127], possibly through the activation of NF-κB[128].

Regulation of mRNA translation by RAC or CDC42

Translational control through the TOR–S6K pathway is another regulatory mechanism through which RAC and CDC42 might influence cell-cycle progression. Wild-type RAC and CDC42 are each necessary for S6K1

activation in response to growth-factor stimulation, whereas constitutively active RAC or CDC42 is sufficient to activate S6K1 in the absence of mitogens[129–131]. S6K1 activation has been reported to involve the formation of signalling complexes that include S6K1 and active RAC or CDC42, and is possibly mediated by the RAC/CDC42 effector proteins protein kinase Cζ (PKCζ)[129] and mixed-lineage kinase-3 (MLK3; REF. 131). S6K activity was found to be required for cyclin-E1 induction by CDC42; however, rather than increasing *cyclin-E1* mRNA translation, S6K causes increased transcription by an unknown mechanism[132].

Unlike fibroblasts, in which *cyclin-D1* transcription is a key determinant of protein levels, *cyclin-D1* mRNA translation might be a crucial step in endothelial and epithelial cells[19,133]. Activation of $\alpha_5\beta_1$ integrin was required for mitogen-dependent RAC activation and *cyclin-D1* mRNA translation in human umbilical endothelial cells[133]. Cyclin-D1 expression and DNA synthesis were prevented by dominant-negative RAC, whereas active RAC was sufficient to rescue cyclin-D1 synthesis and cell-cycle progression in cells plated on a matrix that did not support $\alpha_5\beta_1$ activation[133].

Conclusions and perspectives

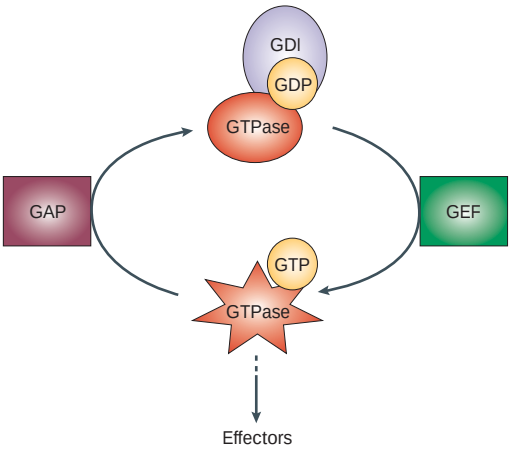
The prototypical RAS GTPases H-RAS, N-RAS and K-RAS signal through many pathways that influence the cell-cycle machinery at numerous levels, making these RAS proteins master regulators of cell proliferation. However, replication-promoting abilities are not unique to these members of the RAS superfamily; additional RAS- and RHO-family GTPases also influence cell-cycle progression. In some cases this is achieved using the same signalling pathways as the prototypical RAS proteins, as seen in TC21 activation of RAL signalling, or the regulation of PI3K by E-RAS and R-RAS proteins. In other cases the influence on the cell cycle might be accomplished in a less direct manner, such as the RHEB-induced effects on protein translation and cell growth, or the contribution of RHO signalling to sustained ERK/MAPK signalling through the induction of stress-fibre formation and consequent integrin clustering. The recurring theme linking the RAS and RHO families is that, by one means or another, these GTPases contribute to cell proliferation.

Indeed, the recent findings reviewed above indicate that a general function of RAS and RHO GTPases is to promote cell-cycle progression and proliferation. One caveat, of course, is that negative findings are less likely to be published, and therefore studies that show a lack of effect of these proteins do not make their way into the general awareness.

Irrespective of whether all, or only some, RAS and RHO proteins regulate proliferation, the large number of GTPases that have been shown to influence the cell cycle evokes the rather confusing image of these multiple inputs converging simultaneously. Instead, cell-type-specific, as well as spatial and temporal, factors probably dictate which pathways have dominant roles and which have accessory or permissive functions. Given that many experiments have made use of overexpressed constitutively active or dominant-negative GTPases to assess their contributions to cell-cycle regulation, it is not entirely surprising that the results are occasionally confusing, if not actually conflicting. Dominant-negative GTPases have been a valuable research tool. However, given the large number of RAS and RHO GTPases, and the even larger number of GEFs, it is difficult to unquestioningly accept as true that dominant-negative GTPases are as selective in their actions as was originally believed.

More-precise analysis in genetically tractable organisms should allow the contributions of the less well studied RAS and RHO proteins to be determined and the relative importance of each GTPase to be resolved. Similarly, RNA interference will enable researchers to examine how individual GTPases contribute to the regulation of proliferation in mitogen- and cell-specific contexts. We have attempted to reconcile conflicting results wherever possible, but some apparent inconsistencies might reflect bona fide cell-specific — and possibly even species-specific — differences. Ultimately, however, the goal of a complete and accurate picture of how RAS- and RHO-family proteins collectively and coordinately regulate the cell cycle will be achieved.

Box 3 | The GTPase cycle



RAS-family proteins are low-molecular-weight guanine-nucleotide-binding proteins. They are inactive when bound to GDP and active when bound to GTP. Regulation of this molecular switch mechanism occurs through a GDP–GTP cycle that is controlled by the opposing activities of guanine nucleotide-exchange factors (GEFs), which catalyse the exchange of GDP for GTP, and GTPase-activating proteins (GAPs), which increase the rate of GTP hydrolysis to GDP (see diagram). In the case of RHO proteins, another layer of regulation is provided by RHO–GDP-dissociation inhibitors (RHOGDIs), which sequester RHO away from the GDP–GTP cycle. GTPases interact with various effector proteins, which influence the activity and/or localization of these effectors; this ultimately influences cell-cycle progression.

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