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Deregulation of Cell Growth and Malignant Transformation

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Abstract Cell growth and cell division are fundamental aspects of cell behavior in all organisms. Recent insights from many model organisms have shed light on the molecular mechanisms that control cell growth and cell division. A significant body of evidence has now been accumulated, showing a direct link between deregulation of components of cell cycle machinery and cancer. In addition, defects in one or more steps that control growth are important for malignant transformation, as many tumor suppressors and proto-oncogenes have been found to regulate cell growth. The importance of cell growth in tumor development is further supported by the discovery that rapamycin, an effective anticancer drug, inhibits a key regulator of protein synthetic machinery and cell growth, mammalian target of rapamycin (mTOR). In most cases, cell growth and cell division are coupled, thereby maintaining cell size within physiological limits. We believe that, in a long-term perspective, understanding how these two processes are coordinated *in vivo* and how their interplay is deregulated in a number of diseases, including cancer, may have a direct impact on the efficiency of modern therapeutics.

Cell growth, cell division, and cell death and differentiation are the most fundamental aspects of cell behavior (1). The size of an animal depends mainly on the size and number of the cells it contains (2). Cell growth and cell division are genetically regulated to ensure that animals grow to optimal size, and that their tissues are appropriately sized, precisely proportioned, and fully functional. In addition to genetic controls, cell growth, division, and final body size can also be influenced by external factors, such as nutrients and temperature in some organisms (3). Deregulation of the molecular mechanisms controlling cell growth results in cells of altered size and can lead to developmental errors and contribute to a variety of pathological conditions, including cancer, and inflammatory and metabolic diseases (4,5).

Cell Division

Over the last few decades, major advances have been made in understanding the machinery that controls cell progression through the cell cycle, in particular identifying cell cycle regulatory genes, including cyclins, cyclin dependent kinases (cdk) and their inhibitors, as well as genes that monitor the distinct steps of cell cycle progression, termed cell cycle checkpoint genes (6-8). A cell cycle checkpoint is defined as a regulatory pathway that controls the order and timing of cell cycle transitions, ensuring that critical events such as DNA replication, chromosome segregation, and probably many other metabolic events are completed with high fidelity (8-10). Mechanistically, a cell cycle checkpoint establishes the relationship between two unrelated biochemical

events, notably the incidence in which the lesion takes place and the second event that prevents the cell from progressing through the cell cycle until the first event has been repaired. The establishment of this relationship requires the presence of three components: 1) a defect; 2) a sensor, which detects the defect; and 3) a target, cell cycle regulator. The failure to activate these checkpoints allows the cell to divide when DNA is damaged or when chromosomes are incorrectly partitioned, leading to genomic instability. This is essential for the generation of cancer, as evidenced by high frequency of mutations of cell cycle checkpoint genes in human cancer (8).

Cell Growth

The term cell growth is used to collectively describe an increase in cell volume, cell mass, or biosynthetic rates. Yet it is poorly understood what cell growth really is and how it is regulated (2). Generally, cell size in many organisms correlates with ploidy of the cell, ie, the number of copies of the basic number of chromosomes (11). Apparently, ploidy increases are required to prevent genomic DNA from becoming limiting for cell growth (12). The large cell size that results from increased ploidy contributes to the formation of specialized cells and patterning of specific tissues (12). Furthermore, increased ploidy in cancer cells due to the accumulation of mutations, which increase chromosome missegregation, could be, at least partially, responsible for their increased cell growth potential (8). The control of cell growth involves balancing positive regulation of anabolic processes with negative regulation of catabolic processes by a number of signaling pathways that are regulated by growth factors, nutrients, and changes in cellular energy status (3,13-15). We know very little about the anabolic processes that these signaling pathways control in order to regulate cell growth. It has been proposed that one particular anabolic process, protein synthesis, may be a key determinant of cell growth (16). Certainly, increased rates of protein translation require synthesis of new ribosomes (17,18). Indeed, genes that control ribosome biogenesis and protein translation have been identified in yeast as critical regulators of cell growth and cell size (11,19,20). Growth is also coordinated with cell division in most dividing cells, probably to ensure that there are sufficient cellular components for survival and normal functioning of daughter cells (3,13,14,21). The molecular mechanisms of this coordination will be discussed in the next section. In addition to the temporal control of growth mentioned above, cell growth can be subjected to spatial constrains (2,13,22). For example, budding yeast and neurons can grow in polarized manner as a result of localized growth at one end of the cell. Understanding the molecular mechanism of the spatial control of cell growth is still in its infancy. Additionally, in multicellular organisms, growth of individual cells is controlled relative to overall body growth, such that the organs constituting the organism are properly proportioned (1).

Coordination of Cell Growth with Cell Cycle Progression

Numerous studies have emphasized that cell growth and cell division, even though separable processes, are coordinated in most dividing cells during animal development and adulthood (2,11,21,23,24). Thus, the terms cell growth (increase in cell mass) and cell division (increase in cell number) are very often incorrectly used synonymously. Although earlier studies in yeast pointed out to a tight coordination between cell growth and division, analyses of this phenomenon in many experimental systems in higher organisms commonly revealed a loose coordination (25). In extreme cases, growth and proliferation can occur in different phases of the life cycle. For example, the growing oocyte of *Xenopus laevis* increases in size 17,000-fold during oogenesis without cell division (25). Immediately after fertilization, the embryo goes through twelve rounds of cell division, without accompanying growth.

Coordination of cell growth and cell division could be accomplished by one or more of the following mechanisms: the dependency of cell cycle progression on growth, the dependency of cell growth on cell cycle progression, the coordinated control of cell growth and cell cycle progression, or the independent but coordinated control of cell growth and division (Fig. 1) (11). Of these mechanisms, the dependency of cell cycle progression on cell growth seems to be a predominant mechanism in yeast and, in some circumstances, in higher organisms such as *Drosophila* and mammals (Fig. 1A). Genetic evidence in yeast indicates that cell growth and cell cycle progression are two separable yet interconnected processes, and that

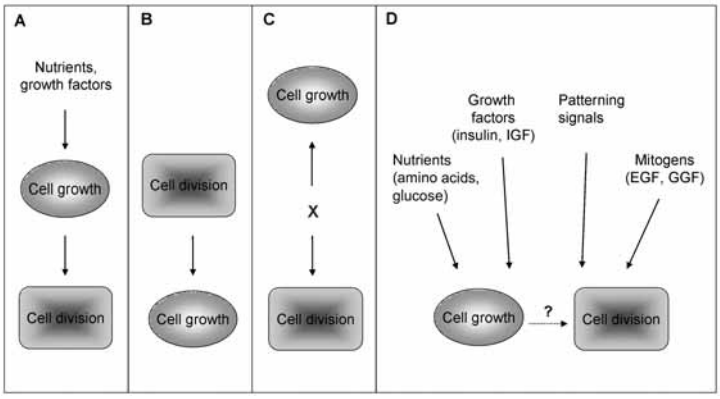

Figure 1. Different models explaining coordination between cell growth and cell division. **A.** The dependency of cell cycle progression on growth. **B.** The dependency of cell growth on cell cycle progression. **C.** The coordinate control of cell growth and cell cycle progression. X represents a putative coordinator of both cell growth and cell division. **D.** The independent but coordinated control of cell growth and division. Insulin growth factor (IGF), epidermal growth factor (EGF) and glial growth factor (GGF) are shown.

growth is dominant and rate limiting for cell division. This finding has been originally based on analyses of two types of proliferation mutants (23,24). One group of mutations found in cell cycle regulators blocked cell cycle progression but did not prevent cell growth. The other group of mutations prevented both cell growth and cell cycle progression. These mutations were in the genes that regulate biosynthetic pathways. Blocking cell growth in mammalian cells in culture by deprivation of nutrients, growth factors, or treatment with translational inhibitors leads to cell cycle block, usually in G1 phase (26-28). Conversely, abundant nutrients or activation of growth regulatory signaling pathways can increase the pace of the cell cycle progression (29,30).

It is yet not clear how the stimulation of cell growth drives cell cycle progression. Two possibilities have been proposed. One predicts that cell cycle machinery is somehow measuring cell size. Cell-size checkpoints were suggested to exist in yeast but their existence is questionable in mammalian cells (31,32). The second possibility is that cell cycle machinery responds to increased translation rates, which do not always correlate with cell size. In that situation, a critical translation rate of cell cycle regulators, such as cyclin Cln3 and Cdc25 phosphatase in yeast, might trigger cell cycle progression (33,34). This could explain, at least partially, how mammalian growth-promoting oncogenes stimulate cell division and possibly malignant transformation, and why some growth inhibitors are effective in treatment of human cancer (14). These issues will be discussed in more detail later in the text.

A model whereby cell division drives cell growth is not well experimentally supported (Fig. 1B). Acceleration of cell cycle in yeast, *Drosophila* and mammalian cells does not upregulate cell growth (mass accumulation), ultimately leading to generation of abnormally small daughter cells (21,24,35). This observation has had a huge impact on understanding the molecular mechanisms of tumorigenesis, implying that, in addition to accumulating mutations that deregulate cell cycle progression, tumor cell must acquire mutations that upregulate cell growth.

In the third model, cell growth and cell cycle progression are coordinated by a common signal(s) (Fig. 1C). Recently, several molecules that are thought to function specifically in cell cycle control, such as p53, retinoblastoma protein (Rb), and p14 alternative reading frame protein (p14ARF) have been implicated in the regulation of cell growth responses (4,36).

The problem is even more complicated in the context of an intact tissue, where multiple modes of regulation are possible, such as cell-cell communications, growth factors, patterning signals, hormones, and nutrients (Fig. 1D). Recent studies have helped in elucidating mode of coordination of cell growth and cell cycle progression in metazoans. Stimulation of growth of wing imaginal disc cells by overexpression of activators of

growth, such as dMyc and dRas1, stimulate G1/S cell cycle transition most likely by regulating the expression of cyclin E at the translational level (21,37). However, stimulation of growth in this experimental system is not sufficient to drive G2/M transition and cell division. An increased cell growth in the absence of cell cycle progression generates oversized cells. Even in the absence of cell division, size of wing compartment was normal, suggesting an enormous plasticity of these processes at the level of organ. The rate of cell division in this tissue is under the control of patterning signals that regulate G2/M transition via Cdc25/String phosphatase. Interestingly, dE2F can drive cell growth and cell division by regulating both cyclin E and Cdc25/String expression (21). One can assume that there are many different modes of coordination between cell growth and proliferation by diverse signaling pathways *in vivo*, and that they are used for distinct developmental purposes (25). Similar type of coordination has been shown in mammalian cells *in vitro*. Stimulation of Schwann cells with insulin-like growth factor 1 (IGF-1) increases growth, but does not drive their division. On the other hand, their stimulation with mitogenic glial growth factor 2 (GGF-2) increases overall rate of division, without stimulating cell growth. These findings show that increases of the rate of cell growth alone do not determine the cell cycle progression in these cells. Interestingly, when Schwann cells were simultaneously stimulated with both factors, the size of daughter cells immediately after division was variable and dependent on relative concentrations of IGF-1 and GGF-2 (35). These data are in favor of a model in which coordination of cell growth and division is flexible and dependent on the relative concentration of extracellular growth factors and mitogens.

Signaling Pathways in Regulation of Growth

Genetic analyses in *Drosophila melanogaster* showed that dRas, dMyc, and phosphatidylinositol 3-kinase (dPI3K) signaling cascades regulate cell growth (37-41).

Loss of dMyc in the developing *Drosophila* wing retarded cell growth and reduced cell size, whereas dMyc overproduction increased growth rates and cell size (37). dMyc causes dramatic increases in nucleolar mass, suggesting that it may mediate its growth effect by increasing protein synthetic capacity. This notion has been supported by messenger RNA (mRNA) expression profiling studies using microarrays in *Drosophila*, which indicate that dMyc primarily activates genes involved in ribosome biogenesis, mRNA processing, and translation (42,43). Functional and target identification studies of c-Myc in mammals concur with the work in *Drosophila* in supporting a role for c-Myc in regulating cellular growth via increased translation rates (44-47).

Similarly, loss of dRas reduced growth rates and cell size, and overproduction of dRas increased cell size and growth rates in the developing *Drosophila* wing (38). Same as dMyc, dRas promoted G1/S but not G2/M transition (38). Overproduction of dRas activated growth driver dMyc, suggesting that some effects of dRas on cell growth and G1/S transition are probably regulated by dMyc (38).

When PI3K is genetically activated or inhibited within developing *Drosophila* imaginal wing discs, organ size and cell size are increased or decreased, respectively (40,41). Although these changes in cell size are accompanied by a change in the cell number, cell size is also altered, demonstrating that the effect on growth is greater than on cell division.

It has been proposed that protein synthesis may be a key determinant of cell growth (16). Consistent with this, the Myc, Ras, and PI3K-signalling pathways increase the overall rate of protein synthesis by stimulating either the rates of translation initiation and elongation or ribosome biogenesis (14). Of these pathways, PI3K-signalling pathway recently received the most attention in the field of cell growth regulation. Therefore, we will further discuss its components, regulation, and relevance to the development of diseases.

PI3K-mTORC1 Signaling Pathway

The activation of PI3K signaling pathway is initiated by the binding of growth factors, such as insulin and insulin growth factor-1 (IGF-1) to their tyrosine kinase receptors, G-protein coupled receptors, but also by direct interaction with oncogenic Ras (Fig. 2) (48,49). The activation of PI3K leads to production of the lipid second messenger phosphatidylinositol 3,4,5-trisphosphates (PtdIns(3,4,5)P3) from phosphatidylinositol 4,5-diphosphates (PtdIns(4,5)P2) (49). The tumor sup-

pressor phosphatase and tensin homologue (PTEN) reverses the action of PI3K by dephosphorylating (PtdIns(3,4,5)P3) at D-3 position and is thus an essential suppressor of PI3K signaling, whose function is lost in various advanced-stage carcinomas (50,51). Phosphatidylinositol 3, 4,5-trisphosphate recruits proteins containing pleckstrin homology (PH) domain to the plasma membrane, thereby coupling PI3K signals to downstream effector molecules (48). Activation of one particular effector molecule, protein kinase B (PKB), following membrane translocation is essential in mediating the effects of PI3K, not only on cell growth, but also in regulating other metabolic and anti-apoptotic effects (Fig. 2) (52-55). PKB is activated by phosphorylation on two key residues: Thr308 by the phosphoinositide-dependent protein kinase (PDK1) and Ser 473 by mammalian target of rapamycin complex 2 (mTORC2) (Fig. 2) (56,57).

PKB activation is thought to regulate activation of downstream kinase mammalian target of rapamycin complex 1 (mTORC1) (58). The activation of mammalian target of rapamycin (mTOR) by PKB is indirect and involves inactivation of an inhibitor of cell growth, tumor suppressor tuberous sclerosis complex (TSC) composed of hamartin (TSC1) and tuberin (TSC2) heterodimer proteins (Fig. 2) (59). TSC mutations are associated with an autosomal dominant genetic disorder, tuberous sclerosis (TS), a disease that is associated with cancer susceptibility, including hamartomas in various organs (60). One feature of these tumors is that they contain large cells. In *Drosophila*, mutations in either *dTSC1* or *dTSC2* have been identified in screens for genes that suppress cell growth (61-65). It has been suggested that phosphorylation of TSC2 at PKB phosphorylation sites is important for inhibition of TSC complex and cell growth (66-68). However, a recent report in *Drosophila* presents results that are in contrast with this hypothesis (69). *TSC2* null flies are successfully rescued using wild-type *TSC2* or PKB-phosphorylation-site mutants of *TSC2*. These rescued mutant flies have similar sized cells compared with wild-type-rescued flies, suggesting that phosphorylation of TSC2 by PKB has no effect on function of TSC2 under conditions of normal PKB activation during *Drosophila* development. It is

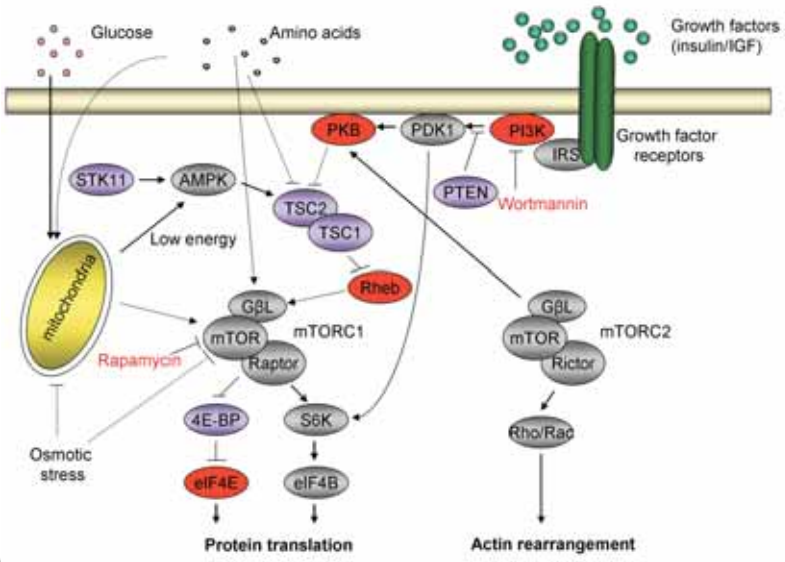

Figure 2. The PI3K-mTOR signaling network. In this model, mammalian target of rapamycin complex 1 (mTORC1) is shown at the center of the network that integrates the three inputs of nutrients (amino acids and glucose), growth factor stimulated phosphatidylinositol 3-kinase (PI3K) signals and cellular energy status in order to regulate translation and probably other aspects of cell growth. Two best characterized phosphorylation targets of mTORC1 that regulate translation, S6 kinase 1 (S6K1) and 4E binding protein 1 (4E-BP1), are shown. It is not known what activates mammalian target of rapamycin complex 2 (mTORC2). mTORC2 phosphorylates protein kinase B (PKB) and participates in its activation. This complex is also involved in the regulation of actin organization via Rho/Rac small guanosine triphosphatases (GTPases). Only mTORC1 is sensitive to rapamycin. Solid lines represent known interactions, whereas dashed lines indicate that uncertainty exists as to how these signals may feed into the pathway. Known proto-oncogenes are shown in red and tumor suppressors in purple.

still possible that under non-physiological conditions, PKB-mediated phosphorylation of TSC2 could be relevant for regulation of cell growth. Resolving this contradictory issue requires further investigation. Recently, a potential positive regulator of TSC1/2 has been identified, the adenosine monophosphate-activated protein kinase (AMPK) (Fig. 2) (70). AMPK is activated by adenosine triphosphate (ATP) depletion or energy stress and shunts protein and fatty acid synthesis and upregulates ATP-regenerative processes, such as glucose uptake and β-oxidation (71). It seems that the link between AMPK activation and inhibition is through phosphorylation-mediated enhancement of TSC1/2 activity (71). Consistent with the role of AMPK in the activation of TSC1/2, a positive regulator of AMPK, serine/threonine-protein kinase 11 (STK11) is mutated in another hamartoma-susceptibility disease, Peutz-Jeghers syndrome (72). The overexpression of TSC1/2 inhibits both nutrient- and growth factor-induced activation of mTORC1 downstream targets, S6 kinase 1 (S6K1) and 4E binding protein 1 (4E-BP1) (66,67,73). These results suggest that amino acids stimulate mammalian target of rapamycin (mTOR) signaling via inhibition of TSC1/2 function. However, the molecular mechanism of this inhibition is unknown. In conclusion, the TSC1/2 could be a link between growth factor signaling, nutrients, cellular energy status, and the mTOR signaling pathway (74).

Genetic and biochemical evidence has identified the small guanosine triphosphatase (GTPase) Rheb (Ras homologue enriched in brain) as a positive regulator of cell growth and downstream target of TSC1/2 (Fig. 2) (74). Mutations in *Drosophila Rheb* (*dRheb*) reduce cell growth and cell size, whereas dRheb overexpression increases growth and cell size (75-77). TSC2 has a domain that shares homology with GTPase, activating protein domains (GAP) and TSC2 functions as a Rheb-GAP in unstimulated cells (77-81). Under such conditions, Rheb is in guanosine diphosphate (GDP)-bound form and the mTORC1 signaling pathway is inhibited via unknown molecular mechanism. Following inactivation of TSC1/2 by PKB, a greater proportion of Rheb is in guanosine triphosphate (GTP)-bound form, and that correlates with stimulation of the mTORC1 signaling pathway (74). The functional importance of GTPase activating domain of TSC2 is further underscored by the observation that it is frequently mutated in tuberous sclerosis (TS) patients (60). TSC2 harboring a point mutation in the GTPase-activating domain, a mutation that is found in tuberous sclerosis, has no inhibitory effect on the activation of mTORC1 downstream targets when overexpressed in combination with TSC1 (73). Altogether, these results strongly suggest that TSC2-mediated regulation of GTPase activity of Rheb is essential for modulation of the mTORC1 signaling pathway. Biochemical analyses have shown that the overexpression of Rheb stimulates the phosphorylation of mTORC1 downstream targets, S6K1 and 4E-BP1, whereas the loss of Rheb function has the opposite effects (75-77,79,82). Furthermore, rapamycin, an inhibitor of mTORC1, blocks the effects of Rheb overexpression on S6K1 (79,81,82). These biochemical findings, in conjunction with detailed epistasis analyses in *Drosophila*, have supported the hierarchical relationship between TSC1/2, Rheb, and TOR in *Drosophila* and mammals (75-77). It is important to keep in mind that other possibilities are not ruled out by these experiments.

The conserved serine/threonine protein kinase TOR was originally identified genetically in budding yeast, where gain-of-function mutations in the *TOR1* and *TOR2* genes were shown to confer resistance to rapamycin, a fungal macrolide that inhibits yeast growth. Over the last decade, TOR has emerged as a critical regulator of the protein synthesis and cell growth in yeast, *Drosophila*, *Caenorhabditis elegans*, plants, and mammals. In addition to regulating protein synthesis, mTOR signaling may also regulate transcription, cell proliferation, cytoskeletal rearrangements, and autophagy (13,59). Although these TOR-mediated effects are physiologically relevant for the cell, they will not be discussed here due to space limitation. *TOR* mutant *Drosophila* shows a small cell size phenotype and reduced body size, which is consistent with a role of dTOR in regulating cell growth (83,84). It seems that dTOR, in addition to controlling cell autonomous growth, controls growth in a systemic fashion (85). mTOR exists in two distinct complexes within cells: one that contains raptor, GβL, mTOR (mTORC1), and another containing rictor, GβL, and mTOR (mTORC2) (Fig. 2) (59,86-89). The identification of the two structurally and functionally distinct multiprotein complexes has provided a molecular basis for understanding the complexity of mTOR signaling.

mTORC1 is sensitive to rapamycin and mediates the temporal control of cell growth, in part by regulating protein synthesis and mass accumulation through its substrates, S6K1 and 4EBPs (59,86,87, 90). mTORC2 is rapamycin insensitive and mediates the spatial control of cell growth by regulating a Rho GTPase signaling pathway, which impinges on the actin organization (88,89). Recently, mTORC2 was implicated in phosphorylation of PKB on Ser 473 (57). This observation suggests the existence of feedback mechanism in which TORC2 activation leads to positive regulation of PKB signaling pathway. Cell growth in higher organisms is tightly coupled to nutrient availability, growth factors and energy status of the cell. It seems that mTORC1 integrates all three inputs to control cell growth (59).

Yet, the primary stimulus for mTORC1 is still unclear. Regulation of TOR by nutrients is conserved from yeast to man (59). One possibility is that nutrients are the primary stimulus, whereas growth factor signaling may modulate the intensity of growth in certain tissues or during specific developmental stages. Alternatively, the primary stimulus in higher organisms might be growth factor signaling, whereas the nutrient signals subsequently modify the potency of the response. Activated mTORC1 regulates translation through hierarchical phosphorylation either by activation of S6K1/2 or by inactivation of eukaryotic translation initiation factor 4 (eIF4E) inhibitor, 4E-BP1 (Fig. 2) (90,91). It is poorly understood how cellular responses mechanistically regulate mTORC1 signaling. It seems that the stability of the mTOR-raptor interaction strengthens during nutrient deprivation, which correlates with a decrease in the *in vitro* kinase activity of mTORC1 (92). On the other hand, stimulation of leucine-starved cells with leucine reduces the amount of raptor associated with mTORC1 and correlates with increased mTORC1 in *in vitro* kinase activity (92). This regulation requires GβL, since, in its absence, the interaction between mTOR and raptor is insensitive to nutrients (87). It is not known whether amino acids activate mTORC1 directly or indirectly. The fact that insulin stimulation does not affect the stability of mTORC1 complex suggests that the main function of raptor is to couple nutrient signals to mTORC1, independently of growth factors (92). It is also not known how growth factor-activated Rheb mechanistically regulates the activity of the mTORC1 signaling pathway. Raptor also serves as a scaffold for the interaction between mTORC1 and its substrates, S6K1/2 and 4E-BP1 (93). In addition to nutrients and growth factors, mTORC1 seems to be a major integrator of the signals that convey the overall status of the cellular environment to the protein synthetic machinery and cell growth. As mentioned above, ATP depletion or energy stress could inhibit mTORC1 activity indirectly trough AMPK, a negative regulator of PI3K-mTORC1 signaling pathway (71). A direct activation of mTORC1 kinase activity by ATP had been reported (94). Because mitochondria are the energy factories of the cell, and protein synthesis is the major consumer of cellular energy, it makes sense that signaling networks have evolved to inform the protein synthetic machinery about the energetic status of the cell (92,94). Furthermore, mTORC1 has been suggested to respond to phosphatidic acid, second messengers in some mitogenic pathways and osmotic stress (92,95). How do mTORC1 substrates regulate protein synthesis? It had been originally proposed that mTOR, through S6K1/2, mediates phosphorylation of ribosomal protein S6, and facilitates translation of mRNAs that have an oligopyrimidine tract at their transcriptional start (5'-TOP mRNAs). They encode for components of the translational machinery, most notably ribosomal proteins and elongation factors 1A (EF1A) and 2A (EF2A) (96-99). Surprisingly, the analysis of compound *S6K1* (-/-) and *S6K2* (-/-) mice suggested that the 5'TOP mRNA regulation is S6K1/2 independent (Fig. 3) (100). The recruitment of 5'TOP mRNAs to polysomes was inhibited in serum stimulated *S6K1* (-/-) and *S6K2* (-/-) cells by mTORC1 inhibitor, rapamycin (100). Given that both *S6K* deficient *Drosophila* and *S6K1*-deficient mice had growth defects, it seems that S6K is essential for growth (101,102). Despite a growth defect, S6 phosphorylation was normal in *S6K1*-deficient mice, indicating that a mechanism, dependent on S6K1 and independent of S6 phosphorylation, is likely to drive growth. It still remains to be confirmed whether S6K1 potential substrates, eukaryotic translation initiation factor 4B (eIF4B) and S6K1 Aly/REF-like target (SKAR) protein, are mediators of S6K in regulating growth (Fig. 3) (103,104). The mTORC1 signaling mediates phosphorylation of 4E-BP proteins, which block translation initiation factor eIF4E (Fig. 3) (105,106). Upon phosphorylation, the 4E-BPs dis-

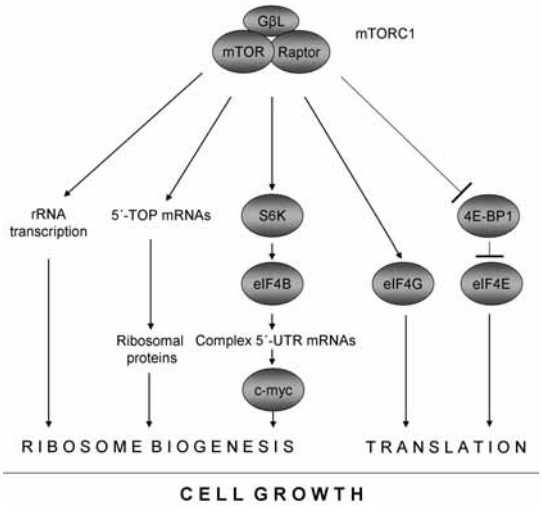

Figure 3. Pathways downstream of mTORC1 that regulate translation and cell growth. mTORC1 regulates translation by directly phosphorylating 4E-BP1, S6K, and eukaryotic translation initiation factor 4G (eIF4G). Phosphorylation of 4E-BP1 liberates eukaryotic initiation factor 4E (eIF4E), leading to upregulation of cap-dependent translation. Activated S6K phosphorylates eukaryotic initiation factor 4B (eIF4B), possibly leading to translation of messenger RNAs (mRNAs) that have a highly structured 5'-untranslated region (5'-UTRs), such as c-Myc. Increased expression of c-Myc could enhance ribosome biogenesis. Furthermore, mTORC1 also activates ribosome biogenesis by upregulating ribosomal RNA (rRNA) transcription and translation of mRNAs that have an oligopyrimidine tract at their transcriptional start (5'-TOP mRNAs).

lodge from eIF4E, allowing binding of eIF4E to the 5′ cap of mRNA (91,107). This activation step is necessary for the enhanced translation of mRNAs that exhibit extensive secondary structure in their 5′-untranslated regions (5′-UTR) and therefore have low affinity for translational machinery (90). More than thirty years ago, Harvey Lodish postulated that spectrum of translated mRNA varies with the overall rate of protein synthesis (108). Low affinity mRNAs are outcompeted with high affinity mRNAs when the overall rate of translation is reduced. In contrast, when the translational rates are upregulated, both mRNAs are translated. The fact that a very high proportion of low affinity mRNAs are those encoding oncoproteins, growth factors, survival factors, and cell cycle regulators, suggests one possible mechanism by which deregulation of components of PI3K-mTORC1 signaling pathway leads to malignant transformation (109). Furthermore, mTORC1 has been implicated in the regulation of translation initiation factors 4G (eIF4G) and 4B (eIF4B), and ribosomal RNA (rRNA) transcription (Fig. 3) (14,103,110,111). Taken together, there is ample evidence suggesting that the PI3K-mTORC1 signaling pathway increases the overall rate of protein synthesis by stimulating either the rates of translation initiation and elongation or ribosome biogenesis (14).

Role of PI3K-mTORC1 Signaling Pathway in Cancer

For a long time, it was speculated that deregulation of protein translation and cell growth participate in cancer pathogenesis. These speculations were supported by the observations that overexpression of eIF4E induces malignant transformation *in vitro* (112,113), as well as in transgenic mouse models (114). Furthermore, eIF4E expression is significantly increased in many cancers (115).

The amount of eIF4E is limiting in cell, and therefore, its overexpression is thought to enhance the translation of mRNA(s) that exhibit extensive secondary structure in their 5′-untranslated regions (5′-UTR), such as c-Myc, cyclin D1, fibroblast growth factor 2 (FGF2), and ornithine decarboxylase (ODC), and vascular endothelial growth factor (VEGF), which could enhance growth, proliferation, and angiogenesis (105,116-120). Deregulation of these molecules may, at least partially, explain malignant transformation by overexpressed eIF4E.

The evidence of causal relationship between deregulated protein synthesis and neoplastic transformation came from the discoveries that human cancer susceptibility syndromes have been attributed to mutations in tumor suppressor genes that control cell growth and the rate of protein synthesis, such as TSC1/2, PTEN, 4E-BP(s), and STK11 (60,72,121-123). This evidence is further supported by the discovery of a number of proto-oncogenes in PI3K-mTORC1 growth regulatory pathways, including PI3K, PKB, and Rheb (4,14,48,49,55,124). As mentioned above, deregulation of these molecules can lead to increases in eIF4E-dependent and -independent translation of mRNAs that encode proteins highly enriched in cancer-promoting functions (Fig. 3). It has to be kept in mind that mutations in components of the PI3K-mTORC1 growth regulatory pathway can also deregulate cell cycle progression, apoptosis, and many other cellular processes independently of translation, all of which

could contribute to malignant transformation and tumor progression (14,49,74).

The importance of the PI3K-mTORC1 signaling pathway in cancer is further underscored by the discovery that rapamycin, an effective anticancer drug is a specific mTORC1 inhibitor. Rapamycin is the most effective in the treatment of PI3K/PKB-overexpressing or PTEN- or TSC-deficient tumors, which rely extensively on mTORC1 pathway activation. The exact molecular mechanisms of its anti-tumor action are not known. Rapamycin probably, in addition to protein synthesis, inhibits many other mTORC1-mediated biological responses in tumor cells. Due to upregulation of mTORC1 signaling in neuroblastoma, glioblastoma, pancreatic carcinoma, and prostate cancer, these cells are particularly sensitive to rapamycin action (125,126). Furthermore, rapamycin is currently in clinical trials for the treatment of TS syndrome (14). Since the majority of advanced carcinomas loose both *PTEN* alleles, it is reasonable to speculate that rapamycin could show some beneficial effects in the treatment of these tumors.

Ribosome Biogenesis

To meet the increased demand for proteins during cellular adaptation to changing environments, the cell must increase translational capacity by upregulating ribosome biogenesis (17,18). It has been estimated that proliferating HeLa cell produces about 7,500 ribosomes per minute, which requires the synthesis of approximately 300,000 ribosomal proteins, transcription of about 150-200 ribosomal RNA genes, and numerous interactions with factors such as endo- and exoribonucleases, RNA helicases, assembly factors, and small nucleolar ribonucleoprotein particles (snoRNPs), putting an immense demand on cellular machinery (127). Indeed, ribosome biogenesis is the most energy consuming process in the exponentially proliferating cell (128). Correct assembly of ribosomes requires the coordination of synthesis and processing of rRNA, synthesis and transport of ribosomal proteins, and the concomitant assembly of ribosomal proteins into the pre-ribosomal subunits and transport of 40S and 60S subunits from the nucleolus to the cytoplasm (Fig. 4) (129).

The molecular mechanisms that regulate ribosome biogenesis in response to extracellular stimuli or changes in cellular metabolism are still poorly understood. As discussed above, growth regulation signaling networks upregulate ribosome biogenesis at the level of rRNA transcription, transcription of factors involved in ribosome biogenesis, and translation of 5′-TOP mRNAs (4,14, 110,111).

Ribosome biogenesis is precisely coordinated with different phases of the cell division cycle, ensuring that translation of mRNAs occurs at the appropriate levels and during a specific stage of the cell cycle (130). Recent evidence in yeast has suggested that intimate coordination between ribosome biogenesis and cell division could be achieved through interactions between ribosome synthesis factors and specific steps in cell division (131,132). Such interactions have not yet been demonstrated in mammalian cells.

Role of Ribosome Biogenesis in Diseases

No one would argue that upregulation in ribosome biogenesis is necessary to allow rapid cell growth and division in tumors. After all, cancer-associated morphological changes in the nucleolus were recognized a long time ago as a reliable marker of malignant transformation (133). Nucleoli readily stain with silver and their size and number directly reflect the rate of cell growth. In the clinical practice, these numbers are used as tools in determining the patients' prognosis in cancers such as those of the breast, liver, and lung (134).

However, a large body of recent evidence has suggested that quantitative and qualitative changes in ribosome biogenesis could lead to the development of a number of pathological processes, including cancer (Fig. 4) (4,14).

Several tumor suppressors and proto-oncogenes have been implicated in the regulation of ribosome biogenesis at the level of rRNA transcription (Fig. 4). Two tumor suppressors, Rb and p53 have been shown to downregulate the activity of Pol I and Pol III promoters, thus limiting the production of rRNA and transfer RNA (tRNA) molecules (4). Inactivating mutations of *Rb* and *p53* may, in addition to deregulation of cell division cycle, result in aberrant upregulation in rRNA synthesis (135). Mutations in *Drosophila* tumor suppressor *brain tumor* (*brat*) lead to the development of malignant brain tumors. The *brat*-mutant cells are larger than wild-type cells and they have enlarged nucleoli. The *brat* gene encodes a protein

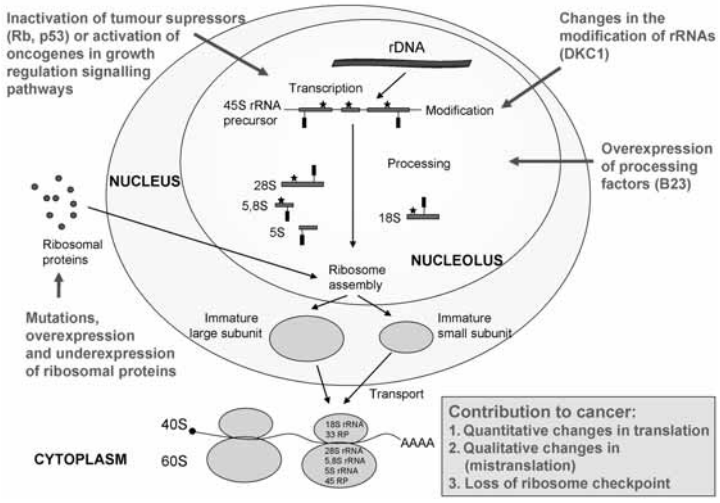

Figure 4. Deregulation of ribosome biogenesis and malignant transformation. Ribosome biogenesis involves coordination of synthesis, processing, and modification of rRNAs (pseudouridines and methyl groups are shown as stars and black rectangles, respectively), synthesis and import of ribosomal proteins, and the concomitant assembly of ribosomal proteins with rRNAs, and transport of 40S and 60S subunits from the nucleolus to the cytoplasm. In the cytoplasm, they become functional ribosome following initiation of translation. Shown are different steps in ribosome biogenesis that could contribute to malignant transformation, if deregulated.

that directly inhibits rRNA synthesis (136). Therefore, the loss of *p53*, *Rb*, or *brat* in tumors might result in malignant transformation through upregulation of ribosome biogenesis and protein synthesis (4).

Additionally, some positive regulators of rRNA transcription, such as extracellular regulated kinase (ERK) and PI3K, are either overexpressed or hyperactivated in many human tumors (49,137). These kinases also affect translational efficiency and many other biological processes in the cell. Therefore, it is difficult to causally relate their role in ribosome biogenesis to malignant transformation.

Overexpression of ribosomal proteins seems to be frequently associated with tumorigenesis (Fig. 4) (4,138,139). As discussed above, the activation of the PI3K-mTORC1 signaling pathway results in increased translation 5'TOP mRNAs, which encode many components of the translational apparatus, most notably ribosomal proteins (14). Hyperactivation of this signaling pathway might be responsible for the overexpression of ribosomal proteins in many human tumors. Furthermore, the proto-oncogene *c-Myc* that is deregulated through genomic aberrations in B cell lymphomas and some other malignancies upregulates transcription of genes that encode ribosomal proteins, factors involved in ribosome biogenesis and translation factors (140-142).

It is difficult to imagine how would an increase in the expression of individual components of a ribosome upregulate ribosome biogenesis and translation in tumor cells. One possible explanation is that these cells must acquire mutations in a number of tumor suppressors and oncogenes to upregulate the synthesis of all components of ribosomes, in order to increase ribosome biogenesis and translational capacity.

Surprisingly, overexpression of a single ribosomal protein, S3a, was able to induce transformation of NIH3T3 cells and induce formation of tumors in nude mice (143). The ability of S3a to induce transformation could be a consequence of its effects on protein translation, although such effects were not tested in this study. Since ribosomal protein S3a was shown to be endonuclease that cleaves DNA in response to ultraviolet irradiation, this extra-ribosomal function of S3a might be important for some aspects of tumor development (144). Similarly, many other ribosomal proteins have a second function apart from the ribosome. Understanding these functions could be relevant in understanding the role of ribosomal proteins in cancer (145). Although increases in the expression of ribosomal proteins have been correlated with a malignant transformation in many instances, some ribosomal protein genes are suggested to be haplo-

insufficient tumor suppressors in *Zebrafish* and *Drosophila* (146-148). How could decreases in the level of ribosomal proteins lead to tumorigenesis? The reduction in the number of ribosomes that is the result of ribosomal protein deficiency could lead to an impaired translation of specific mRNAs that require more direct contact with ribosomes. The identification of mRNAs that are deregulated in cells with lower number of ribosomes will improve our understanding of how deficiency in ribosomal proteins may contribute to the development of malignant tumors. The reduction in ribosome number could result in impaired translation of specific mRNAs that are coding tumor suppressors (14). However, as mentioned above, the possibility for an extraribosomal function for ribosomal proteins can not be easily excluded (145). Consistent with this notion, some cases of Diamond-Blackfan anemia in humans include also growth retardation and an increased susceptibility to hematopoietic tumors and are associated with mutations in the ribosomal protein S19 (149). It has yet to be determined if the S19 deficiency leads to malignant transformation through its effects on ribosomes and translation. Alternatively, S19 could function outside of ribosomes as a tumor suppressor. Interestingly, mice in which one allele of *S19* was inactivated by gene targeting did not develop any symptoms of Diamond-Blackfan anemia (150). The reason for this discrepancy is not understood.

In addition to changes in the expression of ribosomal components, a defect in modification of rRNA can be responsible for tumor predisposition (Fig. 4). Mutation in *dyskerin* (*DKC1*), a putative pseudouridine synthase, which mediates post-transcriptional modification of rRNA through site-specific conversion of uridine to pseudouridine, is the cause of a human disease, dyskeratosis congenita (DC), which is characterized by cancer predisposition (151). The role of *DKC1* mutation in the pathogenesis of malignant tumors was tested in a genetically defined mouse model. A complicating issue was that DKC1 has an additional role in the maintenance of telomeres, dysfunction of which can also lead to cancer (152). More than 50% of mice, having hypomorphic *DKC1* mutation, develop malignant tumors in the first generations, when telomeres are functional. These results suggest that the role of DKC1 in tumor suppression is related to its function in ribosome biogenesis (153). The reduction in modified uridines in the ribosome could affect the regions of the ribosome that are important for binding of tRNA and mRNA, leading to an impaired translation of tumor suppressors. Alternatively, a misfunctional ribosome could mistranslate mRNAs into aberrant oncogenic proteins.

Fidelity of Ribosome Biogenesis and Cell Cycle Progression

Since ribosome biogenesis is one of the most energy consuming processes in the exponentially proliferating cells, and errors in this process could affect the gene expression at the level of translation, the molecular mechanisms may have evolved to sense its fidelity (4,14,18). The responses to defects in ribosome biogenesis induced by mutations in genes that participate in this process or different types of stresses have been recently analyzed.

We generated a conditional deletion of both genes for ribosomal protein *S6* gene in the liver of adult mice using an interferon-inducible Mx-Cre system (154). Livers from fasted animals, deficient for S6 grew in response to nutrients, even though 40S ribosomal subunit biogenesis was abolished. However, hepatocytes failed to proliferate or induce cyclin E, following partial hepatectomy, despite formation of active cyclinD/cdk4 complexes. These results suggested that a defect in ribosome biogenesis might induce a checkpoint control that prevents cell cycle progression *in vivo*.

A recent observation linked defects in ribosome biogenesis and a p53-dependent cell cycle checkpoint control. A nucleolar block of proliferation 1 protein (BOP1), which is involved in rRNA processing, has been shown to cooperate with p53 in regulating G1/S transition (155). A dominant negative mutant form of BOP1 induced a cell cycle block in NIH 3T3-derived LAP3 cells, which was abrogated by inactivation of p53, although rRNA processing was still impaired. These results strongly support the existence of the molecular mechanisms that inhibit cell division in the presence of mutations in genes that are involved in ribosome biogenesis to prevent infidelity of translation and development of pathological processes, as discussed in the previous sections.

Ribosome biogenesis is a complex process that is extremely sensitive to changes in cellular metabolism, energy levels, nutrient availabil-

ity, and various chemical inhibitors (156). Recent experiments provided some insight into the molecular mechanisms that coordinate cell cycle progression with ribosome biogenesis under such conditions. For example, L5, L11, and L23 ribosomal proteins are released from the nucleolus, following the treatments that inhibit ribosome biogenesis, such as starvation for nutrients or incubation with a low concentration of actinomycin D *in vitro,* to interact with mouse double minute 2 homolog (MDM2) and inhibit its activity towards p53 (157-161). It has been proposed that they couple cell growth to the cell cycle. Cell growth would cause L5, L11, and L23 to be assembled into ribosomes, whereas inhibition of cell growth would release them from the nucleolus to inhibit MDM2 and activate p53, leading to the cell cycle block. Furthermore, p14ARF, which binds to MDM2 and negatively regulates its activity, is under normal condition sequestered in the nucleolus. Perturbation of the nucleolus during stress may release p14ARF from the nucleolus to the nucleoplasm, where it binds to MDM2 to inhibit its activity and p53 degradation, which may result in transcriptional activation of p21 and the cell cycle block (162,163). In addition, p14ARF was found to suppress the processing of pre-rRNA by interacting and inactivating the nucleolar protein B23, a nuclease that carries out early pre-RNA cleavage (36,164). These findings suggest that the MDM2-p14ARF-B23 interaction may function in coordinating ribosome biogenesis and cell growth with cell cycle progression under stress conditions. Therefore, it is not surprising that p14ARF is frequently mutated and B23 over-expressed in many tumors (165,166).

All together, alterations in ribosome biogenesis can lead to quantitative or qualitative defects in mRNA translation, which could have deleterious consequences on the cell (Fig. 4). A ribosome checkpoint is probably activated to either eliminate defective cells or prevent cell division under such conditions. We can speculate that failure to activate this checkpoint could lead to the development of number of diseases, including cancer.

Perspective

Although the regulation of the gene expression at the transcriptional level attracted most of the attention in the past, it is becoming increasingly evident that the control of mRNA translation is equally or even more important (167). Also, the molecular mechanisms that mediate and integrate many parameters of cell growth have been largely neglected, despite their relevance for biology and medicine (11). Hopefully, important progress will be made in understanding the molecular basis of a number of the diseases caused by deregulation of cell growth. It is safe to predict that in the near future much more will be learnt about the PI3K-mTOR signaling pathway and mechanisms of its activation. Genetic screens in model organisms such as *Drosophila* and *Caenorhabditis elegans*, genetic inactivation of individual molecules in the PI3K-mTOR signaling pathway in the mouse, and large scale analyses of protein-protein interactions in the pathway provide the most promising approaches in understanding the role of this pathway in an organismal context (168). In a broader perspective, it will be crucial to understand the contribution of growth deregulation to malignant transformation and tumor progression relative to other oncogenic events. Do mutations in growth regulation pathways cooperate with other oncogenic events during transformation process? In that sense, crosses between these mice and mice deficient in tumor suppressors such as p53 and Rb, will be particularly informative (169). Microarray based global analyses of mRNAs shifts into polysomes following deregulation of different components of the PI3K-mTOR signaling pathways will likely reveal novel translationally regulated target genes and help in elucidating the molecular mechanisms that contribute to malignant transformation and tumor progression (22,170). These studies will be of great importance for the identification of novel drug targets and development of effective anti-cancer drugs (14).

Although, a significant progress has been made recently in understanding the connections between ribosome biogenesis and cell cycle progression, this field is still in its early phase. Many effects of the increased rates of ribosome biogenesis on the cell division cycle are probably mediated through increases in the overall rate of protein synthesis as discussed above (14). Surprisingly, results in yeast have shown the existence of multiple interactions between diverse factors involved in ribosome biogenesis and specific steps in the cell division cycle (131,132). Discovery of the functional importance of these interactions

and their relevance for human diseases will be a huge challenge for the future. As discussed earlier in the text, a number of research groups pointed out to the existence of a cell cycle checkpoint that monitors fidelity of ribosome biogenesis (4,154-156). It will be necessary to discover the molecular steps involved in the activation of ribosome cell cycle checkpoint and understand molecular mechanisms of their activation. The most challenging questions in the field are related to the analysis of checkpoint responses induced by defects in ribosome biogenesis in genetically defined *in vivo* mammalian models. The gene targeting of ribosome biogenesis checkpoint genes in the mouse and phenotypic analysis of mutant mice will be the next line of investigations in this field. We hope that understanding the molecular steps involved in activation of ribosome cell cycle checkpoints will help in designing drugs for treatment of a number of diseases, including cancer.

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