

RNA POLYMERASES I AND III, GROWTH CONTROL AND CANCER

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Abstract | Transcription of rRNA and tRNA genes by RNA polymerases I and III is essential for sustained protein synthesis and is therefore a fundamental determinant of the capacity of a cell to grow. When cell growth is not required, this transcription is repressed by retinoblastoma protein, p53 and ARF. However, inactivation of these tumour suppressors in cancers deregulates RNA polymerases I and III, and oncoproteins such as Myc can stimulate these systems further. Such events might have a significant impact on the growth potential of tumours.

rRNA
(ribosomal RNA). An RNA that carries out essential structural and catalytic roles within the ribosome. The Pol I products (the 28S, 18S and 5.8S rRNAs) are sometimes referred to as the large rRNAs to distinguish them from the Pol III product 5S rRNA.

tRNA
(transfer RNA). A short RNA that functions as an essential adaptor to translate the genetic information carried by mRNA into the sequence of amino acids in a polypeptide. This is possible because each tRNA only recognizes a particular amino acid and matches it to specific codons in the mRNA.

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Under a light microscope, one of the most striking visual features of cancer cells is their highly enlarged nucleoli, which are commonly used by pathologists to identify tumour formation^{1–3}. Although the association of nucleolar hypertrophy with cancer has been appreciated since 1896 (REF. 4), the significance of this obvious connection is usually overlooked by cancer biologists. Only recently has molecular biology begun to explain how the nucleolus erupts into hyperactivity during tumorigenesis. Like an angry volcano, it has a dangerous and profound impact on its surroundings.

Nucleoli are dynamic ribosome factories that form within the nucleus around the tandem arrays of rRNA genes. These templates are transcribed by RNA polymerase (Pol) I to produce a long precursor that is rapidly processed into the 18S, 5.8S and 28S rRNA molecules that carry out essential structural and catalytic functions within the ribosomal core. As transcription proceeds, ribosomal proteins assemble around the nascent rRNA, and this assembly process requires the incorporation of 5S rRNA, which, like tRNA, is synthesized by Pol III (BOX 1). In some species, Pol III transcription occurs in the nucleolus, whereas in others it is nucleoplasmic and its products then move to nucleoli for processing and maturation^{5–9}. Accumulation of Pol III transcripts around the nucleolus is especially evident in transformed cells¹⁰. It is the dense concentration of macromolecules at the sites of rRNA synthesis that accounts for the visibility

of nucleoli, which have no membranes and disappear if transcription is curtailed. Conversely, nucleoli expand when Pol I transcription is elevated. The inflated nucleoli of cancer cells are therefore a clear indication that rRNA synthesis is abnormally high. The generality of this phenomenon in cancers argues urgently that it is fundamental to the process of tumorigenesis. This review summarizes the substantial recent advances that have been made in understanding how transcription by Pol I and Pol III changes during tumour development. It will also consider how these changes might influence the growth of cancer cells.

Consequences of deregulated Pol I and Pol III. Although often used rather loosely, the term growth is defined as an increase in cell mass and should not be confused with proliferation, which is an increase in cell number. Cell-cycle progression and proliferation cannot occur in the absence of adequate cell growth^{11–14}, and because 80–90% of the dry mass of a cell is protein, it is inevitable that the rate of protein accumulation dictates the rate of growth (mass increase)^{15,16}. Even a 50% decrease in translation can cause fibroblasts to exit the cell cycle and quiesce, which is clear evidence of the dependence of proliferation on growth¹⁷. In most cell types, the number of mRNA molecules exceeds the number of ribosomes and so the overall rate of protein synthesis is limited by ribosome availability^{15,18}. The production of rRNA

Box 1 | Eukaryotic RNA polymerases and their products

In eukaryotes, the transcription of nuclear genes is shared by three RNA polymerases (Pols), each of which is essential for viability. Pol I has 14 subunits, whereas Pol II and Pol III have 12 and 17 subunits, respectively. Pol I is dedicated exclusively to transcribing the rRNA genes, of which there are ~400 copies in humans. Pol II transcribes the protein-encoding genes, as well as many genes that encode small nuclear (sn)RNA molecules. Pol III synthesizes various short untranslated RNA molecules, including 5S rRNA, tRNA, 7SL RNA (an essential component of the SIGNAL-RECOGNITION PARTICLE), 7SK RNA (which regulates Pol II transcription), MRP, U6 and H1 RNA molecules (which are required for post-transcriptional processing of rRNA, mRNA and tRNA, respectively). Pol III also transcribes the SHORT INTERSPERSED NUCLEAR ELEMENTS (SINES), including *Alu* genes, of which there are over a million in humans. Despite having the smallest number of templates, it is thought that Pol I can contribute up to ~70% of all nuclear transcription in actively growing cells, with Pol II and Pol III providing ~20% and ~10%, respectively.

is a limiting step in ribosome accumulation, as there is little or no wastage — almost all rRNA gets incorporated into the ribosomes¹⁹. Indeed, rates of protein synthesis correlate strongly with the RNA content of a cell, which comprises 95% rRNA and tRNA¹⁵. Therefore, Pol I and Pol III need to maintain high rates of transcription to sustain the synthesis of ribosomes, and thereby protein, that underlies cell growth.

Clear evidence of this requirement for elevated Pol I and Pol III transcription has come from studies that

were designed to specifically restrict the synthesis of rRNA or tRNA. For example, halving the concentration of a tRNA can cause a threefold increase in cell-mass doubling time, as was shown by deleting surplus copies of the INITIATOR tRNA gene in *Saccharomyces cerevisiae*²⁰. In addition, the hypertrophic growth of cardiomyocytes that occurs in response to adrenergic stimuli can be blocked by using an antisense approach to curtail the induction of rRNA synthesis²¹.

Transcription intermediary factor (TIF)IA is a Pol-I-specific transcription factor that is required for promoter recruitment of Pol I (FIG. 1). A dominant-negative mutant of TIFIA can suppress cell-cycle progression in proliferating HEK293T tumour cells²², presumably by restricting ribosome production and thereby halting growth. An even more striking result was obtained using an activated version of TIFIA to increase Pol I transcription specifically: despite the high rate at which the HEK293T cell line proliferates, a substantial acceleration in proliferation was observed²². This shows that rRNA synthesis is limiting for the proliferation of these highly transformed cells. Proliferation can also be stimulated in tumour cells by overexpressing **nucleophosmin**, a protein that is involved in pre-rRNA processing²³. The inescapable conclusion is that a developing cancer might achieve a proliferative advantage by raising its production of rRNA.

Co-regulation of Pol I and Pol III by ERK

The above-mentioned study also showed one way in which a tumour can increase its rate of rRNA synthesis. The activated TIFIA mutant that had such profound effects on cell behaviour carried a single substitution in its phosphoacceptor site for the mitogen-activated protein kinase (MAPK) extracellular signal-regulated kinase (ERK); the natural serine was changed to aspartate in this mutant to mimic constitutive phosphorylation²². This is thought to facilitate the function of TIFIA, which acts as a bridge between the promoter-selectivity factor **SL1** and Pol I (FIG. 1), thereby stimulating recruitment of the latter to promoters. Conversely, in the dominant-negative TIFIA mutant that caused restriction of Pol I transcription and therefore restriction of proliferation, the serine residue was substituted by alanine, thereby precluding phosphorylation and inhibiting Pol I recruitment to promoters²².

As well as binding to TIFIA, ERK also binds and phosphorylates upstream binding factor (**UBF**), which interacts directly with Pol I promoters, and the Pol-III-specific general transcription factor **TFIIIB**, which is responsible for recruiting Pol III to its promoter templates^{24,25}. Phosphorylation by ERK can regulate the DNA-binding properties of UBF²⁴, whereas it enhances the ability of TFIIIB to bind to Pol III and to another Pol-III-specific general transcription factor, **TFIIIC**²⁵. By activating TIFIA, UBF and TFIIIB, ERK is able to co-regulate Pol I and Pol III directly, thereby ensuring that the synthesis of 5.8S, 18S and 28S rRNA is coordinated with the production of 5S rRNA and tRNA. This might be of great importance to the cellular economy,

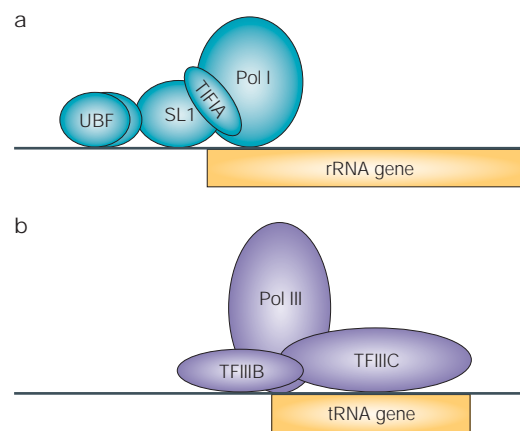


Figure 1 | Basal human Pol I and Pol III transcription complexes. **a** | Assembly of the RNA polymerase (Pol I) pre-initiation complex involves the synergistic action of the homodimeric DNA-binding factor upstream binding factor (UBF) and the promoter-selectivity factor SL1, which consists of the TATA-binding protein (TBP) and three TBP-associated factors (TAFs). An initiation-competent subpopulation of Pol I molecules is bound by the transcription intermediary factor (TIF)IA polypeptide, which interacts with SL1 and is essential for productive Pol I recruitment. Pol I also associates with at least two other essential factors, TIFIC and general transcription factor TFIIH, which are not shown (see REF. 148 for a detailed review). **b** | Most Pol-III-transcribed genes have internal promoters within the transcribed region, which are recognized by the large, five-subunit factor TFIIIC. In turn, TFIIIC recruits TFIIIB, which is composed of TBP and the TAFs BRF1 and BDP1. TFIIIB recruits Pol III and helps it initiate transcription (see REF. 149 for a detailed review).

7SL RNA
An essential RNA component of the signal-recognition particle.

SIGNAL-RECOGNITION PARTICLE
A complex that is responsible for inserting nascent polypeptides into or through cell membranes; it identifies an N-terminal signal sequence that is carried by proteins destined for secretion or membrane localization.

SHORT INTERSPERSED NUCLEAR ELEMENT (SINE)
A pseudogene derived from tRNA or 7SL RNA that is propagated by retrotransposition. SINES are typically 200–300 bp long and contain functional Pol III promoters. They are highly abundant in mammalian genomes, especially in humans, where the *Alu* SINE family constitutes ~10% of the genomic DNA.

INITIATOR tRNA
The tRNA that is responsible for bringing the first amino acid to the start of the message.

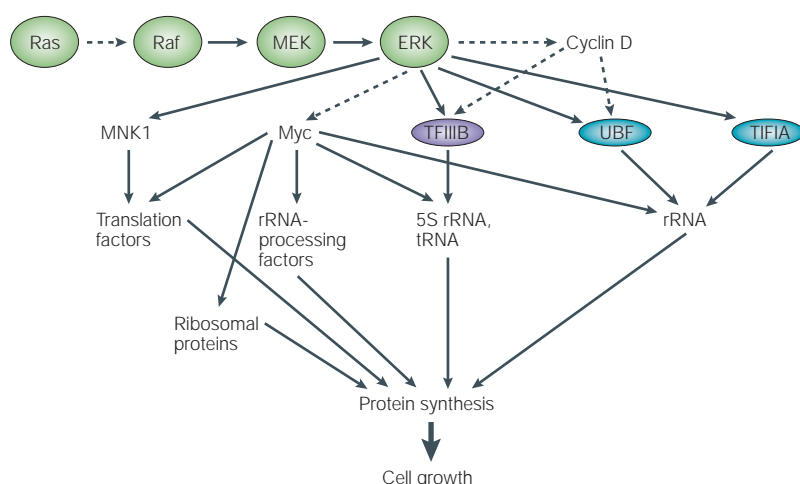


Figure 2 | **ERK targets that stimulate protein synthesis and therefore cell growth.**

Extracellular signal-regulated kinases (ERKs) are activated in response to mitogens through a signal-transduction cascade that involves Ras, Raf and the ERK kinase MEK. ERK has been shown to act directly on upstream binding factor (UBF) and transcription intermediary factor (TIF)IA, so as to stimulate rRNA synthesis by RNA polymerase (Pol) I. It also directly activates general transcription factor (TF)IIIB to increase transcription by Pol III of genes that encode tRNA and 5S rRNA. Another direct target of ERK is the kinase MAPK-interacting kinase-1 (MNK1), which phosphorylates and activates eukaryotic translation-initiation factor (eIF)4E. In addition, Myc is stabilized when the ERK pathway is stimulated. Many Myc targets are involved in ribosome biogenesis and other aspects of protein synthesis, including genes that encode tRNA, rRNA, enzymes involved in rRNA processing, translation factors and many ribosomal proteins. ERK activation also stimulates cyclin-D1 production, which leads to phosphorylation of retinoblastoma protein (RB; not shown) and therefore the derepression of UBF and TFIIIB. There are many other targets of the ERK pathway, which are not shown. Some of these might also impact on growth and/or promote cell-cycle progression, including the transcription factors activator protein-1 (AP-1) and TATA-binding protein (TBP). The interrupted lines indicate the presence of intermediate steps.

as these four rRNA species are required in equimolar quantities, and a significant imbalance in their concentrations would be extremely wasteful, considering that ~80% of the energy of a cell can be spent in generating the translation apparatus²⁶.

ERK responds to mitogenic signals that activate receptors on the surface of the cell; this triggers a signalling cascade that involves the GTPase Ras, the kinase Raf and the ERK kinase MEK²⁷. Activation of this pathway is sufficient to stimulate transcription by Pol I and Pol III, whereas inhibitors of the cascade have the opposite effect^{22,24,25}. It has been estimated that ERK is abnormally active in ~30% of human tumours, most commonly due to the oncogenic mutation of Ras²⁷. This is likely to lead to a coordinate increase in transcription by Pol I and Pol III. The eukaryotic translation-initiation factor (eIF)4E also responds to ERK activation, which allows an efficient induction of protein synthesis that spurs cells into growth. In addition, ERK stimulates the expression of some Pol-II-transcribed genes, both by activating transcription factors such as ELK and by increasing chromatin accessibility at specific promoters; some of the genes that are induced in this way encode members of the activator protein-1 (AP-1) protein family that stimulates proliferation²⁸. Another target of ERK is the gene that encodes TATA-BINDING PROTEIN (TBP), the activation

of which can raise transcription by all three nuclear RNA polymerases^{29–34}. At the same time, ERK induces the production of cyclin D1 and its assembly into a functional kinase complex, which phosphorylates and inactivates the retinoblastoma protein (RB) — a potent inhibitor of growth and proliferation. A direct target for ERK activation is carbamoyl phosphate synthetase II (CPS II), which catalyses the rate-limiting step in the production of pyrimidine nucleotides, which form the building blocks for RNA and DNA synthesis. Induction of transcription by Pol I and Pol III is therefore part of an extensive programme of growth-promoting changes that are invoked by the ERK pathway, several of which impact on ribosome production (FIG. 2).

Myc, a master regulator of protein synthesis Another target of ERK is the oncoprotein Myc, which is stabilized following phosphorylation, leading to a significant increase in its concentration³⁵. Like ERK, Myc has been shown to target several components of the protein-synthesis pathway^{26,36}. So, as a result of ERK activation, Myc causes a rapid increase in translation and growth, which is a prelude to cell-cycle progression^{26,37}. Indeed, in some cell types Myc can induce growth in the absence of a proliferative response^{38–42}. Conversely, loss of Myc function compromises growth and protein synthesis^{39,43}.

Myc is a transcription factor that binds and regulates a large number of genes, including many that encode ribosomal proteins, rRNA-processing factors and translation factors^{37,44–52}. Furthermore, Myc is a potent direct activator of tRNA and 5S rRNA synthesis⁵³. Unlike most Myc target genes, these Pol-III-transcribed genes do not contain a DNA sequence that is recognized directly by Myc. Nevertheless, CHROMATIN IMMUNOPRECIPITATION has confirmed that endogenous Myc is associated with tRNA and 5S rRNA genes *in vivo*^{53,54}. This can be explained by its interaction with the essential Pol-III-specific factor TFIIIB⁵³. Unlike the Pol III templates, the rRNA repeats contain several copies of the E-box DNA motif that is bound by Myc. In keeping with this, when Myc expression is induced, rRNA concentrations rise and nucleoli inflate^{42,46,55}. Conversely, the expanded nucleoli of liver cancer cells deflate when a *c-Myc* transgene is inactivated in mice⁵⁶. Part of the ability of Myc to stimulate rRNA synthesis is due to its induction of UBF expression⁵⁵. However, there is also a direct effect on Pol I transcription, with Myc binding to SL1 and promoting its recruitment to promoters (C. Grandori, N. Gomez-Roman, Z. Felton-Edkins, R. Eisenman and R. White, unpublished observations). In addition, Myc can stimulate rRNA processing and maturation⁵². It is therefore not surprising that the expression levels of rRNA and tRNA fall when endogenous Myc is removed by genetic knockout or RNA interference^{43,53,54}. Conversely, enlarged nucleoli and elevated rRNA synthesis are seen in knockouts of the Myc antagonist mitotic-arrest-deficient-like protein-1 (MAD1; REF. 55).

TATA-BINDING PROTEIN (TBP). A small, highly conserved protein that binds the TATA motif in gene promoters, but is also used by genes that lack TATA boxes. TBP is essential for the expression of rRNA and all Pol-III-transcribed genes, as well as many Pol II templates.

CHROMATIN IMMUNOPRECIPITATION A technique for determining whether a protein binds to a particular region of the genome *in vivo*. It involves treating live cells with formaldehyde to form nonspecific crosslinks between the DNA and any associated proteins. The cells are then lysed, the genomic DNA is sheared into small fragments and the protein of interest is immunoprecipitated. Any protein-associated DNA is then removed and analysed by PCR.

Deregulation of Myc is a common feature of some tumour types, including **Burkitt lymphomas**, neuroblastomas and colon carcinomas^{36,57}. Indeed, it has been estimated that one in seven cancer deaths in the United States involve deregulated Myc³⁶. Although not yet confirmed, it seems a safe bet that such deregulation will impact strongly on the output of Pol I and Pol III.

The TOR pathway

Like Myc, the target of rapamycin (**TOR**) pathway is a key regulator of cell growth and proliferation^{58–60}. It functions primarily through changes to the protein-synthesis apparatus, stimulating translation initiation and inducing ribosome production^{58–60}. In both mammals and budding yeast, this involves the activation of Pol I transcription^{61–67}. In rodent fibroblasts, the TOR pathway stimulates phosphorylation of UBF in its C-terminal domain, thereby promoting its interaction with SL1 (REF 65). Consistent with this, SL1 occupancy of rRNA promoters increases rapidly following activation of the TOR pathway with insulin-like growth factor⁶⁶. TIFIA has also been implicated as a target of TOR signalling in yeast⁶⁴ and mammals⁶⁷, although the latter is controversial⁶⁵. As one would expect, Pol III transcription is also highly sensitive to the TOR pathway, and again this is apparent in both budding yeast⁶² and mammals (E. L. Graham, R. J. White and P. H. Scott, unpublished observations). However, the mechanism (or mechanisms) that are responsible for Pol III activation by TOR have yet to be established.

Overexpression or mutation of TOR has not been observed in human cancers, but it is nevertheless frequently hyperactive, due to changes in components of the upstream pathways that regulate its function, including Ras, PTEN, AKT2 and phosphatidylinositol 3-kinase^{68–71}. Such events are likely to contribute to the elevated output of Pol I and Pol III.

Overexpression of UBF and TFIIC in tumours

The activation of rRNA and tRNA synthesis that is induced by Myc, TOR or ERK occurs as part of a coordinated programme that involves several aspects of cellular behaviour that respond to growth-promoting stimuli. However, some types of tumour show specific changes to the Pol I and Pol III machinery that seem to be independent of these pathways. For example, overexpression of the Pol I transcription factor UBF was detected in 12 out of 17 hepatocellular carcinomas, when compared with matched healthy liver tissue from the same patients⁷². Similarly, a study of nine ovarian carcinomas found that each overexpressed the Pol-III-specific factor TFIIC⁷³. Elevated TFIIC concentrations are also found in several fibroblast cell lines that have been transformed by the DNA tumour viruses SV40 and POLYOMAVIRUS, when compared with untransformed parental cells⁷⁴. TFIIC is a complex of five subunits and the mRNAs that encode each of these subunits were consistently elevated in each of the ovarian carcinomas and transformed fibroblast cell lines^{73,74}. This was not a simple response to proliferative changes, since the concentrations of these mRNAs

are unaffected by serum withdrawal in culture⁷³. Instead, the data argue that TFIIC overexpression is a more specific feature of transformation in these cell types.

These observations have important implications. The activation of Pol I and Pol III is one of many changes in cell behaviour that occur in response to ERK, TOR or Myc. As such, it is difficult to tell which of these changes are most important during tumorigenesis; some might be crucial, whereas others could be peripheral effects. However, as TFIIC is a dedicated, Pol-III-specific transcription factor with no other known functions, its consistent elevation in ovarian carcinomas argues strongly that there is specific selective pressure to increase Pol III output as these tumours develop.

Evading the guardian

The tumour suppressor **p53** regulates the transcription of many genes, including those for rRNA and tRNA, which it can strongly repress both *in vitro* and *in vivo*^{75–80}. When bound by p53, TFIIB cannot be recruited to promoters, as shown by chromatin immunoprecipitation of tRNA genes in living cells⁷⁹. p53 has been dubbed 'the guardian of the genome' because of its key protective role in cells⁸¹. In response to various environmental stresses, including hypoxia, ribonucleotide depletion and exposure to genotoxins, p53 is induced to initiate complex response pathways that culminate either in apoptosis or in the arrest of both growth and cell-cycle progression^{82,83}. Under such circumstances, curtailing biosynthesis might be important for maintaining cell viability. Reducing the production of rRNA and tRNA might be especially advantageous when ribonucleotide pools are depleted and, indeed, we find that such conditions do trigger a p53-dependent repression of Pol III transcription (J. Morton and R. White, unpublished observations).

In fibroblasts from p53-knockout mice, tRNA and rRNA synthesis is elevated markedly^{76,77}. Such deregulation might also occur in cancers where p53 function is compromised. Around half of all sporadic human tumours carry a mutation in one *p53* allele combined with deletion of the other^{84,85}. In most cases, the mutation lies in the central domain of p53 (residues 100–300), which it needs to repress transcription by Pol I or Pol III (REFS 78,86,87). Tumour-derived substitutions in this region can disrupt its ability to inhibit reporters of Pol I or Pol III activity, although this is not always the case^{78,87}. Inherited mutations in p53 cause **Li–Fraumeni syndrome**, which is characterized by a familial predisposition to cancer⁸⁸. Pol III transcriptional activity was found to be abnormally elevated in untransformed primary cells from eight of the ten patients with Li–Fraumeni syndrome that were tested⁸⁷. Some cancers (5–10%) overexpress the oncogenic human homologue of mouse double minute-2 (**HDM2**), which targets p53 for degradation^{89,90}. As expected, expression of a Pol III reporter can be released from the repressive effects of p53 by HDM2

POLYOMAVIRUS
A small DNA tumour virus that causes tumours in mice.

(REF. 87). The human papillomavirus E6 oncoprotein triggers p53 degradation in cervical carcinomas and also derepresses Pol III transcription⁸⁷. Overall, it is believed that p53 function is compromised in most tumours, either directly by mutation or through the aberrant activity of its regulators^{90,91}. In a significant proportion of these cases, it is likely that Pol I and/or Pol III have become derepressed.

A key upstream controller of p53 is the tumour suppressor **ARF**, which provides a first line of defense against hyperproliferative signals that are provoked by oncogenic stimuli^{90,91}. Such stresses induce ARF activity, which raises p53 concentrations by binding to HDM2 and inhibiting its ability to catalyse p53 degradation^{83,90,91}. Not only does ARF trigger a p53 response that represses Pol I transcription, it also blocks the production of mature rRNA by inhibiting processing of the primary transcript⁸⁰. The latter activity is p53 independent and maps to a region of ARF that is especially well conserved through evolution and is required for it to suppress proliferation⁸⁰. ARF interacts with several proteins that are involved in ribosome biogenesis²³. One of these is nucleophosmin (B23), a nucleolar endoribonuclease that is required for rRNA processing²³. Binding of ARF promotes the ubiquitylation and degradation of nucleophosmin, thereby blocking a specific step in the maturation of rRNA²³. Oncogenic Ras can induce nucleophosmin, but only in cells that lack ARF²³. Sherr and colleagues have suggested that the primordial role of ARF might have been to slow down ribosome biogenesis in response to hyperproliferative stresses that arise through the activation of oncogenes. Its subsequent linkage to p53 might have evolved to provide a more efficient checkpoint for coupling ribosome production with p53-dependent inhibitors of cell-cycle progression⁸⁰.

It is also noteworthy that specific blocks in rRNA processing have been shown to trigger p53-mediated reversible cell-cycle arrest^{92,93}. Indeed, the arrest that is induced in this manner is even more efficient than that produced by p21, a model inhibitor of cyclin-dependent kinases⁹². A p53 response is also induced by elevated concentrations of ribosomal protein **L11**, which can bind and inactivate HDM2 (REFS 94,95). Perhaps L11 accumulates if the availability of other ribosomal components is restricted, so its accumulation indicates a problem with ribosome production. Such observations support the belief that p53 senses 'nucleolar stress' and thereby helps to ensure that cell-cycle progression is coupled to ribosome biogenesis (FIG. 3). Indeed, Rubbi and Milner have presented striking evidence that nucleolar disruption might actually be the trigger for all p53 responses⁹⁶. In this model, anything that threatens healthy growth by disrupting the synthesis of ribosomes will stabilize p53 and thereby trigger cell-cycle arrest or apoptosis^{96,97}.

Throwing off the chains of RB

Control of growth and proliferation by RB. The growth and proliferation of untransformed mammalian cells is constrained by RB — an important tumour

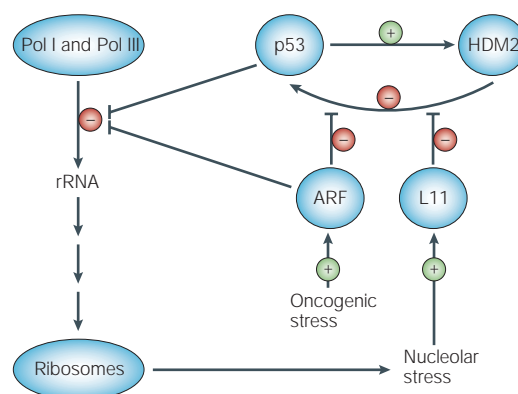


Figure 3 | Cross-talk between p53, ARF and ribosome biosynthesis. Transcription of the human homologue of the mouse double minute-2 (*HDM2*) gene is activated by p53. HDM2 binds and ubiquitylates p53, causing its export from the nucleus and/or degradation. The ARF tumour-suppressor protein and ribosomal protein L11 can protect p53 from HDM2. ARF and p53 both inhibit rRNA synthesis, which is required for ribosome biogenesis. ARF is activated by hyperproliferative stresses, which is caused by the aberrant activation of oncogenes. Perturbations of ribosome synthesis might be sensed as nucleolar stress by L11.

suppressor that prevents cell-cycle entry in the absence of appropriate mitogenic signals. It is widely believed that cancers cannot develop unless RB function is compromised in some way^{98,99}. A large number of protein partners have been reported to bind RB, and this might be one reason why its influence is both powerful and pleiotropic¹⁰⁰. A prevalent view has been that the activity of RB is explained by its ability to repress E2F, but there are flaws in this model¹³. E2F can induce cell-cycle progression, which is consistent with its ability to activate genes that encode proteins such as cyclin E, which help drive the cell cycle^{101,102}. However, E2F does not promote the increase in mass that constitutes cell growth^{41,103}. Cell-cycle arrest, in itself, need not prevent cells from growing, yet growth is curtailed in response to RB (otherwise the cells would get bigger and bigger). The interaction with E2F is therefore insufficient to explain the growth-control function of RB. However, RB is two orders of magnitude more abundant than E2F and has been shown to bind and regulate a diverse range of proteins, including many transcription factors^{100,104–106} (FIG. 4). It is highly unlikely that its pleiotropic effects are achieved solely through interactions with just one of these targets. Indeed, the RB mutant R661W remains capable of suppressing cell-cycle progression and tumour formation, despite being unable to bind or regulate E2F^{107–109}. A challenging but very important task will be to determine how its many partners contribute to the key functions of RB.

RB interacts with UBF and TFIIB. A clear link to growth control is provided by UBF and TFIIB, which are targeted by RB to achieve repression of Pol I and Pol III, respectively^{110–116} (BOX 2). When bound by RB, TFIIB is unable to interact with TFIIC or Pol III and so is sequestered in an inactive complex that is not

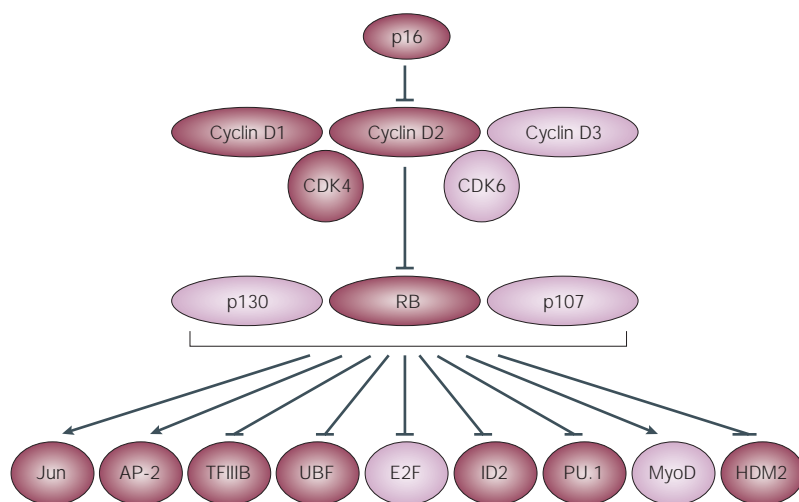


Figure 4 | The RB pathway has several effects on transcription. The pocket proteins retinoblastoma protein (RB), p107 and p130 regulate a wide range of transcription factors, including activator protein-2 (AP-2), Jun (which forms part of the AP-1 transcription factor), E2F, inhibitor of DNA binding-2 (ID2), human homologue of mouse double minute-2 (HDM2), MyoD, PU.1, TFIIIB and upstream binding factor (UBF)¹⁰⁰. Other targets have also been identified, which are not shown. Although the pocket proteins are inhibitory towards most of these transcription factors, they can stimulate the action of AP-2, Jun and MyoD. When bound to cyclins D1, D2 or D3, cyclin-D-dependent kinases CDK4 and CDK6 can inactivate the pocket proteins by phosphorylating them. These kinases are themselves inhibited by p16. Many components of this pathway are mutated or overexpressed in cancers (shown in dark pink). The components in light pink are rarely, if ever, mutated or aberrantly expressed.

recruited to the promoter^{116,117}. An exception is provided by U6 SMALL NUCLEAR (SN)RNA genes, which use a different combination of factors from most Pol III templates; chromatin immunoprecipitation revealed the presence of RB at U6 genes in several cell types¹¹⁶. For the Pol I system, RB inhibits transcription by binding UBF and preventing it from recruiting SL1 and the stimulatory cofactor CREB-binding protein (CBP)^{115,118}. RB accumulates in the nucleolus when the demand for rRNA decreases, under conditions such as cell confluence or during the differentiation of monocytes^{110,119}. These activities help ensure that the output of Pol I and Pol III is restricted under conditions that are inappropriate for growth. Binding of RB to UBF and TFIIIB is maximal in growth-arrested cells, but RB dissociates following mitogenic stimulation^{119,120}. In this way, RB contributes to the mitogen sensitivity of these systems, such that rRNA and tRNA synthesis rises when cells are actively growing and cycling. (FIG. 5) RB has two relatives, known as p107 and p130, with which it shares 30–35% identity¹⁰⁶. p130 carries out a similar role to RB in repressing UBF and TFIIIB when growth is inappropriate^{118,120–122}. By contrast, p107 does not regulate UBF, although it can bind and repress TFIIIB^{121,122}. These effects must have an enormous influence on nuclear activity, given that Pol I and Pol III together can account for up to 80% of all transcription¹²³.

Pol I and Pol III are deregulated when RB is inactivated in tumours. As mentioned above, the loss of RB function might be an obligatory step in tumour development. Theoretically, this could provide a universal

route towards unfettered transcription by Pol I and Pol III, although the reality is undoubtedly more complex. Many human malignancies carry mutations in RB, including the retinoblastomas where the gene was first discovered^{124–126}. Often in such cases, RB expression is ablated completely, a situation that is modelled by mice carrying a targeted disruption of the *Rb1* gene, where synthesis of tRNA and 5S rRNA is substantially elevated¹¹¹. Other tumours express mutant forms of RB and in these cases the mutation almost always disrupts a domain that is known as the large pocket (residues 393–869), which is necessary and sufficient for the suppression of growth and proliferation^{125,127,128}. A homologous region is found in p107 and p130, so these three factors are referred to as the pocket proteins. The large pocket domain is required for RB to bind UBF and TFIIIB, and to regulate Pol I and Pol III (REFS 110–113,118). Several cases have been described where pocket function is compromised by point mutations that arose in cancers¹²⁵. For example, the function of RB as a tumour suppressor was lost in a small-cell lung carcinoma through substitution of residue 706 (REF. 129). This and similar tumour-derived mutations are sufficient to prevent it from regulating Pol I and Pol III (REFS 54,110,111,118,130). One can infer that Pol III transcription will be derepressed in cancers that have sustained such mutations. However, this might not be enough to derepress Pol I transcription, because of the functional redundancy of RB with p130 (REFS 118,122). Unlike Pol III, Pol I activity seems normal in RB-knockout mouse embryonic fibroblasts and only increases if p130 is ablated as well¹²². It is important to establish if this also applies to other cell types, especially those of epithelial origin, from which most tumours arise.

Mutations in p107 or p130 are extremely rare, but their functions are often compromised in parallel with RB inactivation through other changes that occur commonly in cancers^{91,106,131}. Most prevalent among these is the hyperactivity of cyclin-dependent kinases that phosphorylate and inactivate the pocket proteins⁹¹. For example, cyclin D1 is overexpressed in >50% of breast carcinomas and cyclin-dependent kinase (CDK)4 is overexpressed in ~40% of glioblastomas^{91,132}. Furthermore, a large proportion of cancers fail to express p16, a specific inhibitor of cyclin-D-dependent kinases; examples of this include 30% of breast carcinomas and 80% of pancreatic carcinomas^{91,133}. Such aberrations cause hyperphosphorylation of the pocket proteins, which prevents their interactions with UBF and TFIIIB^{119,120}. In addition, the cyclin-D- and cyclin-E-dependent kinases have been shown to activate Pol I transcription directly by phosphorylating UBF^{134,135}. Deregulated CDKs are therefore likely to stimulate the synthesis of rRNA and tRNA in many tumour types.

The E7 oncoprotein of the human papillomaviruses is known to bind and neutralize the pocket proteins in cervical carcinomas^{136–138}. An E7 peptide can dissociate RB from UBF *in vitro*¹¹⁰. Furthermore, Pol III reporter activity is stimulated by E7 in transfected cells, but not

U6 SMALL NUCLEAR RNA (U6 snRNA). snRNA molecules are small, untranslated RNA molecules that function in the nucleus by guiding the assembly of macromolecular complexes on the target RNA to allow site-specific modifications or processing reactions to occur. U6 snRNA is an essential small nuclear (sn)RNA that is required for pre-mRNA splicing.

Box 2 | The case for UBF and TFIIB as bona fide RB targets

More than 70 transcription factors have been reported to bind retinoblastoma protein (RB)¹⁰⁰. Although this sounds unlikely, RB is hundreds of times more abundant than most transcription factors and therefore has the potential to interact with many partners. Furthermore, many of the interactions might be restricted to particular times or cell types. In principle, RB could indeed be involved in regulating this diversity of targets, thereby functioning as a master controller that integrates several activities. However, not all the reported RB-binding partners have strong credentials as bona fide RB targets; for example, some interactions have only been observed *in vitro* or under conditions of overexpression¹⁰⁰. By contrast, a wealth of biochemical and genetic data establish that transcription by RNA polymerase (Pol) I and Pol III is regulated by RB through its interactions with upstream binding factor (UBF) and the general Pol-III-specific transcription factor TFIIB, respectively. In both cases, regulation has been confirmed by four independent laboratories^{110–112,114,115,122,145}. The evidence can be summarized as follows:

- Pol III transcription is elevated in primary fibroblasts from RB-knockout mice^{53,111,120}. The effect can be reproduced *in vitro* using extracts of these cells¹¹³. Pol I transcription is normal in such cells, but is elevated in RB^{-/-} p130^{-/-} double-knockout fibroblasts¹²².
- Nucleolar localization of RB is observed in many cell types^{110,119,146,147}.
- Chromatin immunoprecipitation revealed the presence of endogenous RB at chromosomal U6 snRNA genes in several types of living cell¹¹⁶.
- Overexpression of RB represses transcription by Pol I and Pol III in transfected cells^{54,110–112,115,117,130,119}. Repression is abolished if the transfected RB carries point mutations in its pocket domain^{54,111,112,130}.
- Recombinant RB inhibits transcription by Pol I and Pol III *in vitro*^{110–114,116,117,122,139,145}. This repression can be prevented by adenoviral E1A¹¹¹, simian virus 40 (SV40) large T antigen¹³⁹ or by point mutations in the RB pocket^{110–112}.
- RB-mediated repression of Pol I or Pol III transcription can be reversed by overexpression of UBF or TFIIB, respectively^{110,112–114,145}. This implicates UBF and TFIIB as targets of RB.
- UBF and TFIIB bind in GST PULL-DOWN ASSAYS to wild-type, but not mutant, forms of recombinant RB^{110,112–116,118,121,122}.
- Endogenous RB co-immunoprecipitates with endogenous UBF and TFIIB^{74,113,118,120–122,139}. This interaction is compromised by viral E7 (REF 110) or large T antigens of SV40 and polyomavirus^{74,139} and when RB is inactivated by phosphorylation^{118,120}.
- Primary fibroblasts from RB-knockout mice have a specific elevation of TFIIB activity¹¹³.

by E7 mutants that are defective in RB binding^{121,139}. The pocket proteins can also be bound and neutralized by the E1A oncoprotein of adenovirus and the LARGE T ANTIGENS of polyomavirus and SV40 (REFS 140–144). These have all been shown to derepress Pol III transcription, both *in vitro* and *in vivo*^{74,111,139}. Furthermore, the interaction between RB and TFIIB was found to be substantially diminished in fibroblasts that were transformed by polyomavirus or SV40 (REFS 74,139). Release of TFIIB from the inhibitory influence of RB is therefore a feature of cells that are transformed by several DNA tumour viruses.

The combined data indicate that Pol III transcription can be derepressed if RB function is compromised, be it through oncogenic mutation, hyperphosphorylation by cyclin-dependent kinases or the binding of transforming proteins, such as E7. Since one or another of these aberrations is believed to occur in most, if not all, human tumours, they have the potential to provide a universal route to deregulate Pol III transcription in cancers. Concomitant neutralization of RB and p130 is also likely to derepress Pol I transcription in many cancer types, although mutation of RB alone might be insufficient.

Implications

Although concrete evidence is still lacking, it is likely that tumour suppressors can use the repression of Pol I and/or Pol III to achieve growth restraint. It can hardly be a coincidence that these systems are targeted

directly by RB, p53 and ARF. It is unquestionable that growth can be prevented when the synthesis of rRNA or tRNA becomes limiting. The important issue is the extent to which this happens under physiological circumstances. Genetic analysis found a 2.4-fold reduction of tRNA concentrations by RB in serum-starved mouse embryonic fibroblasts¹²⁰. It has yet to be established whether this is sufficient to limit mammalian cell growth, but such a reduction is enough to increase substantially the cell-mass doubling time of yeast²⁰. Growth of rat cardiomyocytes can be prevented by using an antisense UBF construct to specifically block the 37% increase in rRNA synthesis that normally accompanies serum stimulation in this system²¹. The use of knockout mice revealed that RB and the related protein p130 together suppress rRNA production by ~40% (REF 122). Such quantitative comparisons make it seem likely that growth rates might indeed become limited through the action of pocket proteins on UBF and TFIIB. This might only be the case under particular conditions, whereas other pathways could mediate growth restraint in different cell types or circumstances. Nevertheless, the control points that are provided by Pol I and Pol III could carry out crucial regulatory functions in some situations. The loss of such controls during tumour development might constitute an important step towards neoplastic growth.

It is clear that cancer involves significant changes to the transcription factors that are employed by Pol I and Pol III. The challenge is now to determine whether

GST PULL-DOWN ASSAY

A technique that allows the detection of proteins that bind *in vitro* to an immobilized recombinant protein fused to glutathione S-transferase (GST).

LARGE T ANTIGEN

The transforming oncoprotein of the DNA tumour virus simian virus 40 (SV40).

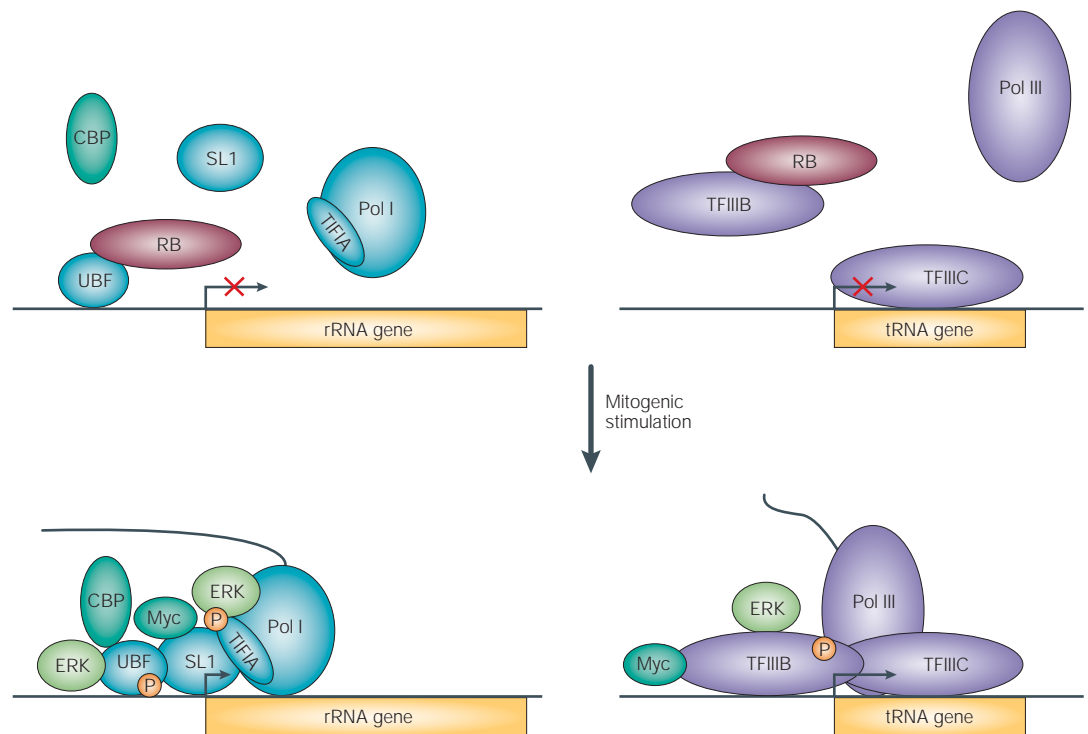


Figure 5 | **Induction of rRNA and tRNA synthesis by mitogens.** In serum-starved cells, the output of RNA polymerase (Pol I and Pol III) is relatively low. This is partly due to the effects of retinoblastoma protein (RB), which binds upstream binding factor (UBF) and Pol-III-specific general transcription factor TFIIB, and thereby represses Pol I and Pol III transcription, respectively. RB prevents UBF from interacting with promoter-selectivity factor SL1 and cofactor CBP (CREB-binding protein) and prevents TFIIB from binding Pol III and TFIIC. Mitogens activate cyclin-dependent kinases, which phosphorylate RB, causing it to dissociate from UBF and TFIIB. The related pocket protein p130 behaves in a similar manner to RB. Mitogenic stimulation induces Myc, which interacts with SL1 and TFIIB and stimulates production of rRNA and tRNA, respectively. Extracellular signal-regulated kinase (ERK) is also activated in response to mitogens and increases transcription by binding and phosphorylating transcription intermediary factor (TIF)1A, UBF and TFIIB. P, phosphate.

perturbations to these systems are necessary or sufficient to allow passage along the multistep pathway to carcinogenesis. A strong indication that this is the case is provided by the findings that a growing number of unrelated tumour suppressors and oncogene products target these systems directly and wrestle to control their output. The selective overexpression of Pol-I- or Pol-III-specific

factors in certain types of malignancy is also suggestive^{72,73}. The fact that elevated rRNA synthesis has recently been shown to accelerate proliferation of transformed cells²² provides a further reason for believing that these systems have a profound impact on cancer biology. If so, they could offer unexplored opportunities for therapeutic intervention.

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Competing interests statement

The author declares no competing financial interests.

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