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Abstract

The synthesis of transfer RNA (tRNA) is controlled by TFIIIB and TFIIIC factors which recruit RNA polymerase III (Pol III) to DNA and the MAF1 protein directly interacting with polymerase. Remarkable evolutionary conservation of the eukaryotic Pol III machinery suggests a common mechanism of regulation through cellular signaling. Here, we summarize the results of studies describing Pol III regulation through TFIIIC, TFIIIB, and MAF1 in yeast and in humans. TFIIIC and TFIIIB are multi-protein complexes regulated at the level of expression of individual subunits, as well as through phosphorylation and interaction with partner proteins. The interaction between TFIIIC and TFIIIB, as well their association with tRNA genes, are dynamic. The mechanism underlying Maf1 regulation of tRNA synthesis, including the differential effect on subsets of Pol III genes, is conserved in both yeast and humans.

Keywords	RNA polymerase III; tRNA; TFIIIB; TFIIIC; Maf1
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To Whom It May Concern:

We are submitting an invited review “Regulation of tRNA synthesis by the general transcription factors of RNA polymerase III, TFIIIB and TFIIIC, and by the MAF1 protein” by Graczyk, Cieśla and myself for the BBA special issue titled as “Regulation of tRNA synthesis and modification”. I hope you will find it interesting.

Sincerely yours,

Magdalena Boguta

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Regulation of tRNA synthesis by the general transcription factors of RNA polymerase III, TFIIIB and TFIIIC, and by the MAF1 protein

A brief, informative title: Regulation of tRNA synthesis by TFIIIB, TFIIIC and Maf1

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Key words: RNA polymerase III; tRNA; TFIIIB; TFIIIC; Maf1

ABSTRACT

The synthesis of transfer RNA (tRNA) is controlled by TFIIIB and TFIIIC factors which recruit RNA polymerase III (Pol III) to DNA and the MAF1 protein directly interacting with polymerase. Remarkable evolutionary conservation of the eukaryotic Pol III machinery suggests a common mechanism of regulation through cellular signaling. Here, we summarize the results of studies describing Pol III regulation through TFIIIC, TFIIIB, and MAF1 in yeast and in humans.

TFIIIC and TFIIIB are multi-protein complexes regulated at the level of expression of individual subunits, as well as through phosphorylation and interaction with partner proteins. The interaction between TFIIIC and TFIIIB, as well their association with tRNA genes, are dynamic. The mechanism underlying Maf1 regulation of tRNA synthesis, including the differential effect on subsets of Pol III genes, is conserved in both yeast and humans.

INTRODUCTION

Transfer RNAs (tRNA) are molecules that serve as adaptors for translation, linking messenger RNA (mRNA) with amino acid sequences of proteins. tRNAs are transcribed by RNA polymerase (Pol) III, a highly evolutionarily conserved complex that consists of 17

subunits [1]. Apart from tRNA, Pol III synthesizes several other essential components of the protein biosynthetic machinery, including 5S rRNA and 7SL RNA and a subset of small noncoding RNAs required for the maturation of other RNA molecules and for control of translation. The rate of Pol III transcription is tightly regulated in response to different conditions.

For unicellular eukaryotes, such as yeast (*Saccharomyces cerevisiae*), coordination of tRNA levels and ribosome function is important for optimal utilization of nutrients and for survival. Consequently, tRNA transcription by Pol III is down-regulated in the stationary phase by nutrient starvation or a non-fermentable carbon source, secretion defects, DNA damage, and chemical treatments.

Pol III in mammals is subject to much broader regulatory influences. Pol III activity is decreased in response to differentiation and stress signals, such as starvation, DNA damage, and hypoxia [2-4] and increased in response to growth factors, hormones, nutrients, and inflammatory signals [5-7]. As there is a spectrum of cues that modulate Pol III activity both in yeast and mammals, appropriate mechanisms governing the control over this crucial enzyme function in both species.

In eukaryotes, Pol III recruitment to tRNA genes is mediated by two general transcription factors, TFIIIB and TFIIIC; and the contribution of gene-specific regulatory proteins is not yet known. Pol III transcription can be also globally down-regulated. In yeast cells, repressive signals for Pol III converge on a central negative regulator, the MAF1 protein [8]. The function of MAF1 as a Pol III repressor is conserved among eukaryotes, including mammals; however, mammalian cells contain an expanded repertoire of regulatory circuits and elements that modulate Pol III activity. Several other important regulators, including p53, Myc, and Rb, also directly control Pol III.

Here, we briefly describe the mechanisms underlying the assembly of the Pol III initiation factors, TFIIB and TFIIIC. We also review the modes of tRNA transcription regulation both in yeast and mammals, focusing on MAF1 and the two general Pol III transcription factors, TFIIIB and TFIIIC.

TRANSCRIPTION FACTORS, TFIIIB AND TFIIIC, AND tRNA GENES

The Pol III system is highly conserved in eukaryotes. This system includes TFIIIB, which is required for Pol III recruitment and transcription initiation, and TFIIIC, which locates Pol III-transcribed genes and recruits TFIIIB. tRNA genes contain two internal promoters, box A

and box B, which are specifically recognized and bound by TFIIIC, a large multi-subunit protein complex. Both yeast and human TFIIIC consist of six polypeptides organized into two globular domains, τA and τB (**Table 1**). τB binds to the B box with high affinity and favors A box binding by τA (reviewed in [9, 10]). In yeast, all tRNA genes are occupied by TFIIIC [11]; however, in mammalian cells, not all tRNA genes are transcriptionally active [12-14]. The correlation between TFCIII binding and transcription activity and the mechanisms responsible for directing TFIIIC to individual genes remain unknown.

Both in yeast and in mammals, TFIIIB consists of three subunits as follows: TFIIB-related Brf1, TATA-box binding protein (TBP), common also for the other two RNA polymerases, and Pol III-specific subunit Bdp1. TFIIIB plays an important role in recruiting Pol III to its target genes; however the recruitment of TFIIIB itself may be one of the most important steps in the regulation of transcription by Pol III. TFIIIB is also required and sufficient to sustain multiple rounds of transcription [15], which makes it a good target for regulation. Accordingly, in mammalian cells, several proteins that regulate tRNA transcription extensively exploit modulation of TFIIIB (see below). Strikingly, this does not occur in yeast cells. On the other hand, recent work has suggested a possible molecular mechanism of TFIIIB recruitment to its target genes [16].

a. The role of TFIIIC in TFIIIB recruitment in yeast

Yeast TFIIIB assembles upstream of the start site of transcription through interaction between Brf1 and Bdp1 with Tfc4, a subunit of the TFIIIC τA module. Based on yeast two-hybrid system and genetic analyses, recruitment of both the Brf1 and Bdp1 is directed by the tetratricopeptide repeat domains (TPR) of Tfc4 [17-19] (for a more detailed description, see a review by A. Vannini in this issue).

The first model of TFIIIC architecture was recently proposed based on structural information on individual subunits and cross-linking data [16]. This model allows for mapping and analysis of TFIIIC mutations that have been previously described and to design polypeptides for biochemical pull-down experiments aimed at studying Tfc4 interaction with Tfc3 subunit of τB and subunits of TFIIIB, Brf1, and Bdp1. The model assumes an initial step of Brf1 recruitment through binding to the N-terminal domain of Tfc4, followed by TBP recruitment via Brf1 and Tfc8 subunit of τB, and, finally, binding of Bdp1 to Tfc4. Competition between Bdp1 and Tfc3 τB subunits to bind the same domain of Tfc4 suggests that recruitment of Bdp1 induces a conformational change, leading to displacement of τB

module and, consequently, to dissociation of TFIIIC from the gene. This hypothesis stipulates that TFIIIB recruitment occurs only under optimal conditions for transcription [16].

Earlier work argues against this proposed model, as co-immunoprecipitation studies performed in extracts from living cells have revealed that interactions between TFIIIC and TFIIIB occur at higher rates in repressive rather than optimal conditions [20]. TFIIIC-TFIIIB interactions indicate that the PIC complex may be quickly reconstructed through transfer of the TFIIIB to the tRNA gene and that transcription will be restored under favorable growth condition. Furthermore, dissociation of TFIIIC (the whole complex or the τB module only) from tRNA is questionable. Studies applying *in vitro* transcription system have shown that TFIIIC is required for TFIIIB and Pol III assembly but is displaced from DNA in post-initiation step [21, 22]; in addition, studies have shown that *in vitro* transcription of human tRNA genes with TATA boxes does not require TFIIIC [23]. *In vivo* data show, however, that TFIIIC is present at all transcriptionally-active Pol III genes, although its absolute binding efficiency is relatively low compared to TFIIIB and Pol III [24, 25].

Interestingly, the association between TFIIIC with and III-transcribed genes greatly increases during acute repression [20, 24], suggesting that it may counteract Pol III progression during elongation. This proposal is further supported by a recent genome-wide analysis of nascent transcripts attached to Pol III, in which a strikingly uneven polymerase distribution along the transcription units was observed, suggesting regional slow-down of elongation or transient pausing of the polymerase [26]. Inspection of individual tRNA genes showed a predominant pattern with a high density of nascent transcripts over the 5′ end and a weaker peak before the 3′ end of the gene. In addition, the 5′ and 3′ peaks of transcribing Pol III coincided with the beginning of the A box and the B box of the internal promoter, respectively, suggesting that TFIIIC bound to A and B boxes could slow the Pol III elongation rate leading to transient pausing. The role of TFIIIC during Pol III transcription is not clear and requires further investigation.

In contrast to the increased occupancy of TFIIIC under stress conditions, the binding of TFIIIB to tRNA gene is reduced (**Figure 1**). Analysis of the occupancy of tRNA genes by Pol III machinery revealed a decrease in the recruitment of TFIIIB along with Pol III during the stationary growth phase, as well as during nutrient deprivation, rapamycin, and hydroxyurea treatment [11, 24, 27, 28]. As described below, occupancy of Pol III factors on tRNA genes is controlled by phosphorylation of the subunits, regulation of their expression levels, and interaction with regulatory proteins.

b. Regulation of TFIIIB recruitment to mammalian tRNA genes

Pol III transcription in mammalian cells is directly inhibited by proteins targeting TFIIIB by modulating its association with TFIIIC and DNA. With the exception of the down-regulation of transcription 1 (Dr1), most regulatory proteins, such as retinoblastoma (RB), p107, p130, and p53, are tumor suppressors. Since there are several excellent and detailed reviews available [29-31], here, we are only briefly presenting the subject.

The tumor-suppressor pocket protein RB and its close homologs, p107 and p130, play key roles in regulating progression of the cell cycle from the G1 to S phase [32]. In the early G1 phase, Pol III activity is very low and RB activity is very high, indicating that this protein is responsible for down-regulation of Pol III [33]. The p107 and p130 proteins act in the same manner as RB, which plays a dominant role in regulating Pol III transcription [34]. The tumor- suppressor p53 protein also negatively regulates Pol III activity. In undamaged cells, p53 is present at very low levels and is persistently targeted for degradation by Mdm2 ubiquitin ligase [35]. Under stressful conditions, such as DNA damage, p53 is stabilized and induces cell cycle arrest or apoptosis [35]. From a mechanistic point of view, the p53 mode of action in Pol III regulation relies on its ability to associate with TBP, preventing TFIIIB from binding to TFIIIC and preventing Pol III recruitment [2].

Regardless of the mechanism driving p53-dependent Pol III downregulation, the question arises why Pol III transcription, from evolutionarily point of view, is directly regulated by p53 since p53 activation eventually leads to activation of RB [36], which also represses Pol III? This trait may provide a backup mechanism to assure Pol III inhibition even if one of these important regulators fails. On the other hand, DNA repair occurs under genotoxic stress, such as methane methylsulfonate or UV treatment, when p53 is rapidly activated and Pol III activity is down-regulated [2]. Interestingly, transcription-coupled DNA repair does not occur in actively transcribed tRNA genes, as in Pol II-transcribed genes [37]. It has been suggested that the occupancy of active tRNA genes by transcription factors and polymerase may restrain DNA repair machinery from binding to these genes [37]. Damman and colleagues (1997) concluded that, since several genes encode one tRNA, there may not be a selective pressure to repair them and, therefore, dedicated mechanisms would not be needed. However, a lack of repair mechanisms would lead to accumulation of mutations and rapid divergences of tRNA sequences, which contradicts the fact that tRNA sequences are relatively well conserved in evolution [38]. As this study was performed on human fibroblasts, which may have partially or completely lost p53 activity during the course of immortalization, a lack of enhanced DNA repair might have been a result of non-repressed transcription of tRNA rather than the lack of

evolutionary need to repair multicopy genes such as these encoding tRNAs. In this regard, p53 would serve to rapidly quench Pol III activity and allow DNA repair to proceed. To validate this hypothesis, one should compare mutation accumulation within transcriptionally active and inactive tRNA genes in p53-competent cells and in cells with inactivated p53.

Dr1 (also known as NC2β) is another negative regulator of Pol III that affects the formation of a functional TFIIIB complex in mammals. Dr1 is an evolutionarily-conserved regulator that associates with TBP and represses basal and activated Pol II transcription [39, 40]. Interestingly, a yeast homolog of Dr1 was identified that also inhibits tRNA transcription when overexpressed in yeast cells [41]. In mammalian cells, Dr1, together with its dimerization partner, DRAP1, associates with Pol III-transcribed genes, including tRNA genes [42]. Dr1 has been proposed to specifically disrupt binding of Brf1 to TBP, thereby inactivating TFIIIB [43]. While Dr1 is clearly implicated in Pol III down-regulation, the physiological role of this phenomenon is unknown.

c. Modulating TFIIIB/C subunit levels

Several *in vitro* studies in yeast, mouse, and human cells suggest that TFIIIB or TFIIIC are very often limiting factors in Pol III-dependent transcription, possibly as a result of sequestering (as described above) and/or post-translational modifications (see below). Regulation of transcription factor subunit expression may constitute an additional mechanism that modulates Pol III activity. In yeast, Brf1 levels are regulated in response to growth conditions since in cells grown to the early stationary phase, Brf1 protein abundance decreases, which is correlated with the reduction of Pol III-dependent transcription [44] (**Figure 2A**). Both *in vivo* and *in vitro* data suggest that Brf1 is a limiting factor for Pol III transcription [45]. However, Pol III repression from chlorpromazine treatment results from defects in TFIIIB-DNA complex assembly but is not correlated with altered levels of TFIIIB or TFIIIC subunits [8].

Mammalian cells have similar mechanisms. All three subunits of TFIIIB are regulated by several regulatory pathways, which may affect their levels and/or impose posttranslational modifications (**Figure 2B**). Brf1, Bdp1, and TBP levels are modulated by various pro-growth, proliferative, or stressful stimuli. Brf1 and TBP subunits appear to be the most extensively regulated, which is likely the result of their role in the initiation of transcription by Pol III (as the other two RNA polymerases, in the case of TBP). The multiplicity of signals that mammalian cells are exposed to within organisms, as compared to yeast, increase the complexity of networks that govern transcription (**Figure 2B**).

Evolutionarily-conserved mitogen-activated protein (MAP) kinases are crucial mediators of transcriptional responses to extracellular signals, including growth factors, hormones, cytokines, and environmental stresses [46, 47]. In mammals, there are three major groups of MAP kinases as follows: c-Jun N-terminal kinases (JNKs), extracellular signal-regulated kinases (ERKs), and p38 MAP kinases [46]. MAP kinases target several transcription factors, transcriptional regulators, and chromatin proteins, and have been implicated in the regulation of transcription by all three RNA polymerases [46, 47].

Brf1, Bdp1, and TBP expression is differentially regulated by JNK [48]. In mouse embryonic fibroblasts and human hepatocellular carcinoma cells, JNK1 stimulates expression of Brf1 and TBP by phosphorylating Elk-1, an ETS domain-containing transcriptional activator, and by inducing its association with the promoters of Brf1- and TBP-encoding genes [48]. Although Bdp1 levels are dependent on JNK1 and Elk-1, Bdp1 is not directly regulated by either of these proteins; instead, Bdp1 regulation is reliant on Elk-1 up-regulation of TBP expression, which in turn stimulates Bdp1 expression [48]. JNK2 kinase down-regulates Brf1, Bdp1, and TBP expression. While the precise mechanism of this phenomenon remains to be elucidated, this mechanism may involve negative regulation of JNK2 through phosphorylation of Elk-1 or other transcription factors [48].

The most canonical substrate of JNK is c-Jun, a subunit of the AP-1 transcription factor [46]. AP-1 refers to a dimeric transcription factor that consists of two proteins from the JUN, FOS, and ATF families [49]. Interestingly, hepatocellular carcinoma cells treated with alcohol have increased c-Jun levels and exhibit enhanced binding of c-Jun to TBP and Brf1 promoters, which in consequence elevates their expression and drives tRNA transcription [50]. In breast epithelial cells treated with ethanol, TBP and Brf1 levels are also up-regulated [51]. In these cells, however, JNK1 stimulates expression of ERα (estrogen receptor α), which directly binds to the Brf1 promoter and increases its expression. This increase in TBP levels may be directly mediated by c-Jun, as has been shown in hepatocellular carcinoma cells [50].

Another example substantiating the role of MAP kinases in regulating TFIIIB subunits is extracellular signal-regulated kinase (ERK)-dependent upregulation of Brf1 during the hypertrophic growth of cardiomyocytes [52]. Since Elk-1 is also an ERK target, it is possible that, in cardiomyocytes, ERK drives Brf1 expression by modulating Elk-1 binding to the Brf1 promoter. This possibility, however, has not been experimentally tested and, therefore, the role of JNK1 cannot be excluded. Furthermore, stimulation of mouse epidermal cells with

epidermal growth factor (EGF) induces Pol III activity, and TBP expression, which is dependent on ERK, JNK, and p38 [7].

MAP kinases play extensive roles in regulating levels of TFIIIB subunits in mammals; however, how these signalling branches are selected and what determines their selection is currently unknown. Furthermore, the role of MAP kinases does not seem to be limited to the regulation of TFIIIB component levels, as they are also implicated in TFIIIB subunit phosphorylation (see further below). TBP down-regulation by MAF1 potentially competes with Elk-1 in binding to the TBP promoter, although the mechanism underlying MAF1 binding requires further investigation [9].

There is a scarcity in the literature regarding how TFIIIC subunits are regulated. A unique mode of autoregulation has been reported for the yeast subunit Tfc6 [53]. The promoter of the gene encoding Tfc6 contains a functional regulatory region called the extra TFIIIC site 6 (ETC6), which comprises the B box element that binds the TFIIIC complex; through this binding, TFIIIC down-regulates expression of its own subunit, Tfc6. This tight control of Tfc6p levels could be important for regulating global tRNA synthesis.

Altered expression of some TFIIIC subunits in mammalian cells has been correlated with infection and disease. Increased expression of two subunits of TFIIIC – TFIIIC220 and TFIIIC110 – has been shown in cells transformed with simian virus SV40 [54]. Similarly, five of six TFIIIC subunits (TFIIIC63, TFIIIC220, TFIIIC102, TFIIIC110, and TFIIIC90) are expressed at abnormally high levels in ovarian tumors [55]. The proto-oncogene c-Myc, which is frequently activated in many cancers, may drive this up-regulation, as several studies have shown that expression of TFIIIB and TFIIIC subunits may be regulated by c-Myc (reviewed in [56]). However, the functional relevance of this regulation has not yet been investigated directly. Given that the expression of several Pol III and Pol I core subunits may be regulated by c-Myc, the coordinated regulation of protein synthesis required for cell growth and division may also be carried out by c-Myc [56].

d. TFIIIB and TFIIIC factors as subjects to phosphoregulation

Various environmental conditions work through multiple signaling pathways to alter the phosphorylation state of components of Pol III machinery in both yeast and mammals, thereby regulating tRNA transcription. Several global proteomic-based studies have identified that all three yeast TFIIIB subunits and five subunits of TFIIIC (Tfc1, Tfc3, Tfc4, Tfc6, and Tfc7) are phosphoproteins [57-59]. Tfc1, Tfc3, and Tfc4 are phosphorylated *in vivo* [60], while Tfc6 and Tfc7 are phosphorylated *in vitro* [61]. The significance of these findings have

not yet been established but we do know that dephosphorylation of yeast TFIIIC reduces its binding to tRNA genes and reduces Pol III transcriptional activity [62]. Five of six subunits of human TFIIIC have also been shown to be phosphorylated, yet, the sixth subunit of human TFIIIC, TFIIIC35, had not been identified at that time; thus, it cannot be excluded that it is also a subject for this modification [63]. However, both the functional relevance of these phosphorylations and their regulations remain to be elucidated.

Contrary to TFIIIC, phosphorylation of yeast and mammalian TFIIIB subunits has been much better studied. Yeast TBP is phosphorylated by casein kinase 2 (CK2) [64], which is responsible for the active *in vitro* and *in vivo* transcription of tRNA genes [65]. In unstressed cells, the regulatory subunit of CK2 binds to TBP and is required for high CK2 activity and Pol III transcription. Under stressful conditions like DNA damage, catalytic CK2 subunits dissociate from TBP-CK2 complexes, resulting in reduced Pol III activity [66].

Bdp1, another subunit of yeast TFIIIB, is also regulated by phosphorylation in a stress-dependent manner. During exponential growth, Bdp1 is phosphorylated on four sites; however, under conditions that inhibit RNA transcription, such as rapamycin or chlorpromazine treatment, Bdp1 is dephosphorylated. Three kinases are implicated in Bdp1 phosphorylation as follows: cAMP-dependent kinase PKA, TOR-regulated kinase Sch9, and protein kinase CK2 [67]. Yeast Brf1 was identified as a phosphoprotein, but neither the kinase responsible nor the role of Brf1 phosphorylation has been determined [57, 58].

tRNA transcription in mammalian cells fluctuates during the cell cycle; it is lowest in mitotic cells and in the early G1 phase [68]. Although the retinoblastoma protein pRB regulates this process [69], several lines of evidence have shown that, during mitosis, all subunits of mammalian TFIIIB are hyperphosphorylated, which may constitute an additional mechanism of Pol III regulation [68, 70, 71]. The kinase responsible for TBP phosphorylation during mitosis, as well the residues modified, are not known. TBP hyperphosphorylation alone cannot account for Pol III repression, since addition of excess TBP to mitotic cellular extracts does not restore Pol III activity *in vitro* [70]. Indeed, phosphorylation of Bdp1 and Brf1 by CK2 and the Polo-like kinase 1 (Plk1) play a role in Pol III repression during mitosis [72, 73]. CK2 mediates Bdp1 phosphorylation at multiple sites, preventing its association with chromatin; this may also hold true for tRNA, given that Bdp1 is required for transcription of all classes of Pol III genes, although it has been shown only for U6 gene (a type III promoter-containing Pol III gene) [73].

Plk1 kinase belongs to a family of serine/threonine kinases that plays multiple roles in cell cycle progression [74]. Plk1 levels begin to increase during the G2 phase and reach their

peak during the M phase, triggered by the phosphorylation of threonine 210 by the Aurora A kinase [75]. Activated Plk1 phosphorylates Brf1 at threonine 270, which is associated with Pol III inhibition [72]. Importantly, the expression of a mutant form of Brf1 (T270D substitution), which mimics constitutive phosphorylation by Plk1, represses Pol III activity; this process can be alleviated by co-expression of mutant Brf1 that cannot be phosphorylated (T270A substitution) [72].

Contrary to mitotic cells, in asynchronously growing cells or during interphase, CK2 and Plk1 stimulate Pol III regulation [72, 76]. While CK2-dependent Brf1 phosphorylation (at a currently unknown site or sites) is required for increased Pol III activity in asynchronously growing cells, Brf1 is phosphorylated at serine 450 throughout entire cell cycle as mediated by Plk1; substituting this serine with alanine generates Brf1 that is no longer able to support high levels of tRNA 5S RNA transcription [72]. TBP is also phosphorylated in asynchronously growing cells but the modified residue or residues and the role of this modification are unknown [77]. As previously mentioned, yeast TBP is phosphorylated by CK2; therefore, is possible that mammalian TBP is also a substrate of this kinase [20].

Overall, the data described above indicate that CK2 and Plk1 serve a dual role in regulating Pol III activity in coordination with the cell cycle by mediating phosphorylation of two components of TFIIIB, Bdp1, and Brf1. During interphase or in asynchronously growing cells, CK2 and Plk1 promote TFIIIB binding to TFIIIC, thus stimulating transcription. During mitosis, phosphorylation of Brf1 and Bdp1 result in dissociation of the latter from the chromatin, while Brf1 and TBP, intriguingly, remain associated; nevertheless, Pol III activity is down-regulated [72, 73, 76].

Pol III transcription in mammals is also controlled by phosphorylation of TFIIIB subunits by the MAP kinase ERK2. Brf1 is directly phosphorylated by ERK2 at serine 145 in serum-stimulated mouse embryonic fibroblasts, and mutations of the ERK2 docking or phosphoacceptor site reduce Pol III activity [78]. ERK inhibition prevents TFIIIB from binding to TFIIIC without affecting Brf1-TBP interaction and restrains Pol III recruitment; Brf1 phosphorylation at S145 therefore stimulates TFIIIB and Pol III to its target genes [78]. ERK2 plays a role in phosphorylating Brf1, as it has been shown that upon serum-stimulation of mouse fibroblasts and upon phorbol myristate acetate (PMA) treatment of human histiocytic lymphoma U937 cells, TBP is phosphorylated in an ERK-dependent manner [79]. However, it is not clear whether ERK2 directly phosphorylates TBP, at which sites, or how this affects tRNA transcription.

Mammalian Pol III is also dependent on the activity of a lipid and protein phosphatase, PTEN, which is one of the most frequently mutated tumor-suppressors in human cancers. PTEN is a negative regulator of the PI3K/Akt/mTOR signalling pathway which influences multiple cellular functions, including cell growth and proliferation [80]. Upregulation of PTEN, which is associated with inhibition of the PI3K/Akt/mTOR signalling pathway, results in down-regulation of Pol III activity and tRNA synthesis [81]. Depletion of PTEN, which is associated PI3K/Akt/mTOR signalling pathway activation, results in up-regulation of Pol III activity. Interestingly, PTEN differentially modulates the phosphorylation state of Brf1 and Bdp1. In cells with high PTEN activity, Brf1 is hypo-phosphorylated and Bdp1 hyper-phosphorylated; in cells with depleted PTEN, Brf1 is hyper-phosphorylated and Bdp1 hypo-phosphorylated. Furthermore, induction of PTEN results in reduced association of Brf1, Bdp1, and TBP with tRNA genes; thus, PTEN negatively regulates TFIIIB recruitment to Pol III promoters. Although it is clear that PTEN affects Pol III transcription, it remains to be determined which kinases and phosphatases are directly involved in this process and which residues of Brf1 and Bdp1 are affected. As inactivation of Bdp1 by phosphorylation is consistent with that observed in mitotic cells (see above), it is likely that hyper-phosphorylation of this protein serves as a mechanism to prevent it entering into the TFIIIB complex.

In summary, multiple pathways modulate tRNA transcription both in yeast and in mammals. Protein synthesis and, therefore, tRNA transcription adjusts to changing conditions and, in mammalian cells, its regulation depends on developmental stages or phases of the cell cycle [82]. Strikingly, most of the abovementioned proteins that regulate Pol III in mammals have been implicated in tumorigenesis. The most common tumor suppressors inhibit Pol III activity, whereas oncogenes stimulate Pol III activity. These activities have tremendous consequences, as misregulation of these proteins in cancer cells inevitably affects Pol III transcription. Indeed, Pol III activity has been shown to be upregulated in many types of cancer [30].

REGULATION OF TRNA SYNTHESIS BY MAF1

a. The family of eukaryotic MAF1 proteins

MAF1 is a global transcriptional repressor of Pol III originally discovered in *S. cerevisiae* [83]. The sequence of the MAF1 protein is conserved among eukaryotes and contains three signature domains, A, B, and C boxes, not found in any other polypeptide.

MAF1 orthologues function as Pol III repressors in fungi, flies, worms, mammals, plants, and parasites [84-89]. Studies of MAF1 in various organisms (eg. parasites, plants) have been reported even ahead the respective Pol III transcription systems were characterized. Generally, a single-copy gene encodes MAF1; however, *Trypanosoma brucei* has two nearly identical MAF1 genes in its genome [88]. The structures of MAF1 proteins seem to be conserved since MAF1 from the citrus plant adopts the same structural fold as human MAF1 [90, 91]. The A, B, and C regions in the protein sequence do not correspond to structural modules or defined surface patches, but, in yeast, interaction between these regions is required for MAF1 function in Pol III repression [92]. According to molecular studies, the A and B regions of the human MAF1 protein form a complex with RNA pol III large subunits and Brf1, respectively, whereas the C region regulates MAF1 stability [93, 94].

Several MAF1 orthologues are targets of phosphorylation. Multiple phosphorylation sites are found in the long linker between the A and B domains of yeast MAF1 but are more uniformly distributed in the worm and human MAF1, which contain shorter linkers between domains. Different types of MAF1 observed on polyacrylamide gels have been interpreted as phosphorylated and dephosphorylated forms; in addition, two separate species of human MAF1 differing by molecular mass have also been reported [93].

Yeast MAF1, contrary to human MAF1, contains two conserved nuclear localization signals (NLS) [83]. The activity of yeast MAF1 is regulated through phosphorylation state-dependent distribution between the nucleus and the cytoplasm (see [95] for detailed mechanism). Whereas nuclear MAF1 function as Pol III repressor, a role of MAF1 in the cytoplasm is so far unexplored. In parasites, mammals and plants, but also in some yeast strains, MAF1 is predominantly nuclear [88, 90, 96, 97].

b. Molecular mechanism of the repression of tRNA transcription by MAF1

Molecular mechanisms underlying the repression of tRNA transcription by MAF1 has mostly been studied in yeast and in humans. In yeast, MAF1 does not bind directly to tRNA genes; instead it physically interacts with Pol III, especially under repressive conditions [20, 28]. Analysis of the Pol III structure in complex with MAF1 showed that MAF1 binds to the Pol III clamp at the rim of the cleft and re-arranges the C82-C34-C31 sub-complex, which is required for transcription initiation [91]. This localization is consistent with biochemical and genetic interactions of MAF1 with the N-terminal region of C160, which forms most of the clamp [28, 94, 98]. By relocating a specific WH domain of the C34 subunit of Pol III, MAF1 weakens the interaction between C34 and the Brf1 subunit of the TFIIIB initiation factor, thus

preventing formation of a closed Pol III complex, but exactly how MAF1 is recruited to Pol III during ongoing transcription is unknown [91, 99]. MAF1 does not bind to a preassembled Pol III-Brf1-TBP-DNA initiation complex, and the interactions of Pol III with MAF1 and Brf1-TBT-DNA are mutually exclusive [91, 100]. MAF1 does not impair Pol III elongation to the end of the template or affect the Pol III distribution along the transcription units [26, 91].

c. MAF1 repression of Pol II genes

Several independent studies have indicated that MAF1-mediated repression of transcription is not limited to Pol III. Human MAF1 has been proposed to repress all three RNA polymerases by inhibiting expression of TBP [85]. MAF1-directed repression of target human Pol II genes (PTEN and FASN) affects physiological processes, such as reproduction, cancerogenesis, and lipid homeostasis [101]. Moreover, inactivation of yeast genes encoding MAF1 prevents the induction of gluconeogenesesis when cells are transferred from medium with glucose to a non-fermentable carbon source [102]. The effects of MAF1 proteins on cell physiology, possibly uncoupled from Pol III repression, are beyond the scope of this article and are referred to in another review [101].

Human Pol II genes, such as CDKN1A and GDF15, are repressed by MAF1 in a Pol III-dependent fashion [103]. Promoters of the CDKN1A and GDF15 genes contain MIR elements, which are short interspersed nuclear elements (SINE) repeats that may be potentially transcribed by Pol III. Experimental data has convincingly shown that repression is mediated through binding of MAF1 to promoter-associated SINEs; this is the first time that MAF1 has been shown to bind specific DNA sequences [103]. Contrary, MAF1 knockdown permits Pol III binding to SINEs, possibly through enhanced binding of TBP, allowing for TFIIIB recruitment. According to this novel mechanism, MAF1-dependent recruitment of Pol III to Pol II promoter-associated SINEs stimulates R-loop formation and activates Pol II-directed transcription [103]. CDKN1A is a cyclin-dependent kinase that inhibits cell-cycle progression through interaction with cyclins and cyclin-dependent kinases; GDF15 is another proliferation gene. As a result, inhibition of CDKN1A or GDF15 by MAF1 may regulate cellular processes, such as proliferation, differentiation and apoptosis, increasing the repertoire of potential effects of MAF1 on cell physiology. MAF1 is also associated with the Pol I promoter region of the procyclin gene and Pol II transcribed SL RNA gene in procyclic forms of *Trypanosoma brucei* and, perhaps, plays a role in their regulation [88].

d. MAF1 as a signalling pathway mediator

Signalling pathways target MAF1 to modulate phosphorylation status, thereby mediating various stress signals to determine levels of tRNA synthesis by Pol III. In yeast, MAF1 is the only Pol III negative regulator that acts as an effector of several signalling pathways [28]. In addition to the down-regulation that normally occurs in the stationary phase, MAF1 is required for Pol III repression following rapamycin treatment, starvation, secretion defects, and oxidative and replication stress [8, 20, 27, 28, 104, 105]. The main MAF1 phosphatase is protein phosphatase 4 (PP4), which directly interacts with MAF1 [106]; however, PP2A phosphatase is also involved [28]. The molecular mechanisms that trigger MAF1 activity in response to different signalling pathways are only partially understood.

Under favorable growth conditions, MAF1 is phosphorylated and cannot interact with Pol III. Phosphorylation of yeast MAF1, some of which occurs in the vicinity of an NLS, is mediated by Sch9 [105], c-AMP-dependent protein kinase A (PKA), TORC1, and casein kinase II (CK2) [96, 107, 108]. Sch9 may be a main kinase for yeast MAF1, since inactivation of all potential Sch9 phosphorylation sites, which are the same as PKA sites, promotes nuclear localization of MAF1 and increases MAF1-Pol III association [105]. Remarkably, yeast Sch9 is phosphorylated and activated by TORC1; thus, TORC1 could potentially control yeast MAF1 indirectly. MAF1 is also involved in Sch9-mediated regulation of lifespan, as the extended lifespan of *sch9Δ* cells is reversed in the absence of MAF1 and the overproduction of MAF1 has an opposite effect lifespan [109]. Moreover, the effect of MAF1 on lifespan regulation by Sch9 is independent of tRNA levels, which are unchanged when MAF1 is overproduced. Thus, MAF1 independently influences cell signalling.

The mammalian TOR kinase (mTOR) localizes to tRNA genes through its interaction with TFIIIC [97]. mTOR-mediated phosphorylation of MAF1 functionally contributes to regulation of the repressive activity of MAF1 at the chromatin [6, 110]. Current data suggest that MAF1 from the citrus plant may similarly be regulated by an as-yet uncharacterized homolog of mTOR [90].

Yeast MAF1 is also a target of CK2 kinase [108], which is enriched on promoters of Pol III genes. Through association with Pol III, MAF1 is located in close proximity to CK2. CK2 phosphorylation of MAF1 is correlated with important events required for Pol III activation, including release of MAF1 from chromatin and dissociation of MAF1 from the Pol III complex [108]. CK2 is a promiscuous kinase and MAF1 is not its only substrate associated with Pol III activity, as the components of Pol III machinery in yeast and humans (TFIIIB subunits and the SNAP190 factor) are phosphorylated or controlled by CK2 [66, 73, 76, 111].

e. Variability of MAF1-dependent regulation among tRNAs

Both yeast and human MAF1 proteins regulate levels of tRNAs to various extents [12, 26, 112]. The relative transcription intensity of Pol III across yeast nuclear tRNA genes was compared under near optimal growth conditions and under stressful conditions. Reduced transcription was observed for nearly all tRNAs under stressful conditions; however, the degree of repression was highly variable among the tRNA genes, with a subset of tRNA genes markedly less repressed (**Figure 3**) [26, 112]. Similarly, Pol III has different enrichment effects on isogenes, indicating different transcriptional activity on gene copies within families. The heterogeneity of tRNA repression in the wild type is substantially reduced in a mutant lacking MAF1, providing genome-wide evidence that a subset of "housekeeping" tRNA genes has low responsiveness to both environmental and cellular signals. Notably, this group contains at least one tRNA per amino acid [26].

In contrast to yeast, not all Pol III genes are actively transcribed in mammals – silent Pol III genes are not occupied by Pol III or MAF1 [12]. Importantly, like in yeast, there is a class of "housekeeping" Pol III human genes that are not regulated by external signals and MAF1; at least some of these genes bind MAF1 [12].

f. Indirect effect of MAF1 on post-transcriptional steps of tRNA biosynthesis

tRNA biosynthesis in the eukaryotic cell is a multi-step pathway that involves transcription, 5' and 3' end maturation, exportation from the nucleus, intron removal, and numerous nucleotide modifications. MAF1-mediated control of tRNA transcription in response to environmental conditions must be coupled with regulation of the subsequent steps in tRNA maturation. Uncoupling of these processes in yeast through MAF1 inactivation results in an accumulation of high levels of tRNA precursors, including both primary transcripts and end-processed intron-containing tRNA precursors [113]. Additionally, the anti-suppressor phenotype of the yeast MAF1 deletion mutant (*maf1Δ*) suggests that tRNAs overproduced in the absence of MAF1 are not fully functional [114].

Viability of the yeast *maf1Δ* strain allows for identification of the steps in the tRNA maturation pathway saturated by the increased amounts of primary transcripts (**Figure 3**). One such process is the nuclear export of tRNA by Los1 exportin [113], which is regulated in coordination with tRNA transcription. MAF1 is phosphorylated under favorable growth conditions in glucose medium, thus preventing Pol III repression, and Los1 is localized in the nuclear membrane, providing active tRNA export. Following a shift to repressive conditions, tRNA transcription is inhibited due to MAF1 dephosphorylation and tRNA export is lowered

by localization of Los1 to the cytoplasm. Growth conditions have a substantial effect on the relative levels of pre-tRNA and mature tRNA species at several different levels of transcription and export regulation. In contrast, transcription and export are uncoupled in strains lacking MAF1, which results in abnormally high levels of pre-tRNA.

Arimbasseri and colleagues (2017) solved a long-term conundrum by explaining why tRNAs overproduced in the absence of MAF1 are not fully functional [115]. Their elegant work makes a convincing case that saturation of dimethyltransferase Trm1 plays a crucial role in how MAF1 affects tRNA suppression. Their results demonstrated that increases or decreases in global Pol III activity leads to inverse changes in the efficiency of m^2_2G26 modification of specific tRNAs. Thus, the previously unknown link connecting Pol III activity and m^2_2G26 efficiency is a limiting amount of Trm1. This link has been conserved through evolution, as the authors showed that the increase of m^2_2G26 content in specific tRNAs in response to starvation was also detected in human embryonic kidney cells.

Finally, sequencing of tRNA precursors on the global scale revealed that efficiency of 3' end modification of tRNA precursors by CCA is lower in the *maf1Δ* mutant, especially under repressive conditions [116]. This result implicates decreased Cca1 activity in *maf1Δ* mutants upon the shift to unfavorable growth conditions, which may contribute to the accumulation of pre-tRNA in the nuclei of *maf1Δ* cells, as reported previously [113]. These changes in pre-tRNA cellular dynamics can have effects on programmed shifts in translation.

CONCLUSIONS AND PERSPECTIVES

TFIIIC and TFIIIB auxiliary factors and the negative regulator MAF1 are the unique elements of Pol III transcription machinery that distinguish it from other polymerases. Activity of these regulatory factors is controlled in response to external signals and affects the efficiency of tRNA transcription. The current review describes the known and hypothetical mechanisms by which the activity of Pol III regulators is controlled in lower and higher eukaryotes.

Although atomic models of Pol III preinitiation complex and Bdp1 have been reported [117, 118], the mode by which Pol III interacts with the negative regulator MAF1 is not yet known and the mechanism of repression requires further study. The differential specificity of MAF1 toward various genes likely relies on additional factors interacting with Pol III chromatin, which also requires further elucidation.

The function of TFIIIC and TFIIIB in recognition of tRNA genes and in the location of polymerases is conserved among eukaryotes; this conservation suggests analogous

mechanisms for regulation of their activities. While the activities of human Pol III auxiliary factors have been examined *in vitro* in the respective fractions of cellular extracts, yeast TFIIIC and TFIIIB have been evaluated using genetic approaches and two-hybrid systems. These data, together with recent results generated by crystallography and crosslinking, have allowed for the creation a model of yeast TFIIIC architecture and formulation of a hypothesis of how TFIIIB is recruited under optimal transcription conditions. This hypothetical mechanism has yet to be confirmed by *in vivo* evidence.

The activities of TFIIIC and TFIIIB are regulated at the level of expression of individual subunits, their phosphorylation, and interaction with partner proteins. Subunits of TFIIIB have been studied extensively in humans but much less is known about the control of TFIIIC activity. Although the majority of TFIIIC subunits are phosphorylated, regulatory function of their phosphorylation invites further study. TFIIIB expression and activity are regulated by several pathways, which may modulate their levels and/or impose posttranslational modifications. TFIIIB subunits are modulated in response to various pro-growth, proliferative, or stress stimuli; moreover, proteins targeting TFIIIB and modulating its association with TFIIIC and DNA have been shown to affect tRNA transcription in mammalian cells, and among these regulatory proteins are tumor suppressors such as p53 and retinoblastoma protein. While regulatory proteins that interact with TFIIIB have not been reported in yeast, these may still exist.

In summary, a plethora of factors influence tRNA transcription, especially in mammalian cells, but it is unclear why mammalian cells would need all of these different positive and negative regulators. This abundance, at first glance, seems to be superfluous; however, when one considers the higher complexity of mammalian cells in comparison to unicellular eukaryotes, these regulators may act within completely different stages of the cell cycle, under different physiological conditions, or developmental stage of an organism. The fine-tuning of tRNA transcription plays an important role in cell physiology, and deregulation of Pol III transcription has been implicated in a variety of human diseases, including cardiovascular disorders and cancer [30, 119].

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FIGURE LEGENDS

Figure 1. TFIIIB and TFIIIC are inversely correlated with tRNA genes. In favorable growth conditions, when TFIIIB shows strong binding to tRNA genes, TFIIIC is only weakly associated with these genes, and Pol III has high transcriptional activity [20]. Conversely, in unfavorable growth conditions, TFIIIB dissociates from tRNA genes and TFIIIC strongly binds to these genes, and Pol III has low transcriptional activity [28].

Figure 2. Control of TFIIIB expression and phosphorylation in yeast and mammals. (A) The mechanisms that regulate TFIIIB subunits are mostly unknown. Phosphorylation of Bdp1 by PKA, Sch9, and CK2 has a stimulatory effect on tRNA transcription [67]. Similarly, phosphorylation of TBP by CK2 up-regulates Pol III activity [64, 66]. Phosphorylation of Brf1 by an unknown kinase is associated with increased Pol III activity, and, finally, an unknown mechanism reduces Brf1 levels in the stationary phase of growth, which correlates with downregulation of Pol III activity [44]. In mammalian cells **(B)**, TFIIIB subunits are mostly under the control of MAP kinase pathways, which regulate their phosphorylation and levels by modulating the activity of other transcription activators [7, 48, 50, 52, 78]. TFIIIB subunits are also phosphorylated by CK2 (Brf1, Bdp1) [73, 76] and Plk1 (Brf1) [72] in a cell cycle-dependent manner. TBP is also extensively phosphorylated under various conditions [70, 77, 79]; however, the role of these modifications is not clear. Tumor-suppressor PTEN indirectly and differentially modulates the phosphorylation state of Brf1 and Bdp1 [81]; the expression of Brf1 and TBP is also stimulated by c-Myc (reviewed in [56]). Given the number of regulatory influences, Brf1 and TBP may play a central target in TFIIIB regulation.

Figure 3. Model of Maf1-dependent regulation of tRNA transcription and posttranscriptional steps of tRNA biosynthesis in yeast. Maf1 is a global negative regulator of Pol III transcription; however, for reasons unknown, a subset of housekeeping tRNAs (encircled with dashed circle) exhibit low responsiveness to Maf1. Primary Pol III-synthesized transcripts are end-processed in the nucleus following CCA addition at the 3' end. Intron-containing pre-tRNAs are exported to the cytoplasm by Los1 exportin. Following pre-tRNA splicing on mitochondrial outer membranes, tRNAs are charged with amino acids and delivered to ribosomes. Turnover of mature tRNAs is controlled by the rapid decay (RTD) pathway and tRNAs are efficiently modified both in the nucleus and cytoplasm. Some processes in tRNA biogenesis that are regulated by growth conditions and/or indirectly depend on Maf1 are marked with red frames. Cca1 nucleotidyltransferase, Los1 exportin and

Trm1 modification enzyme are saturated in the absence of Maf1 [113, 120, 121], and tRNA rapid decay and control of translation fidelity are Maf1-dependent [114, 122].

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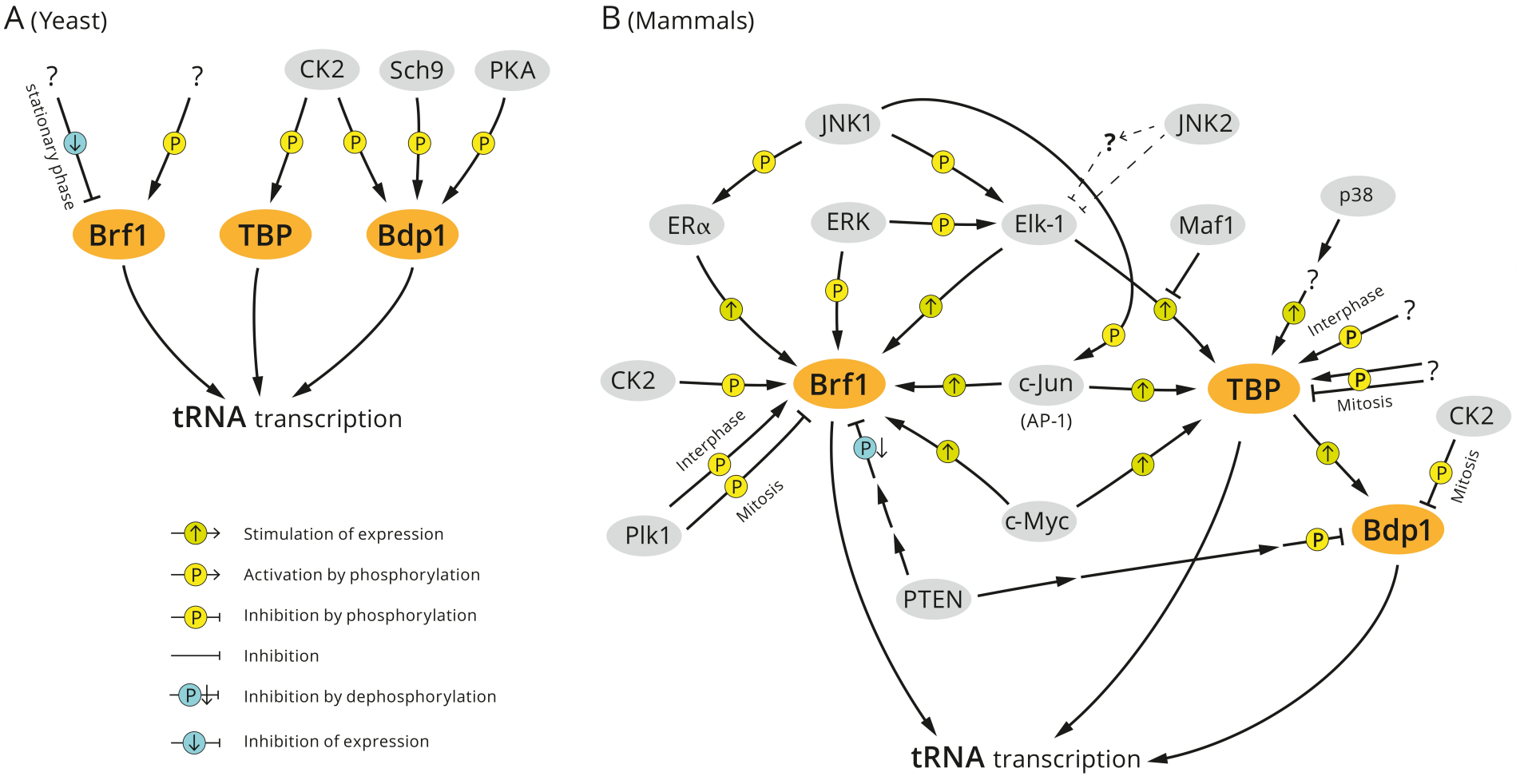
A (Yeast)
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CK2
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PKA
stationary phase
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Stimulation of expression
Activation by phosphorylation
Inhibition by phosphorylation
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Inhibition of expression
B (Mammals)
JNK1
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ERα
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Elk-1
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p38
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Brf1
c-Jun
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TBP
Interphase
Mitosis
Plk1
c-Myc
PTEN
Bdp1
tRNA transcription

favourable growth conditions

occupancy on tRNA genes

TFIIIB↑

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transcription **ON**

unfavourable growth conditions

occupancy on tRNA genes

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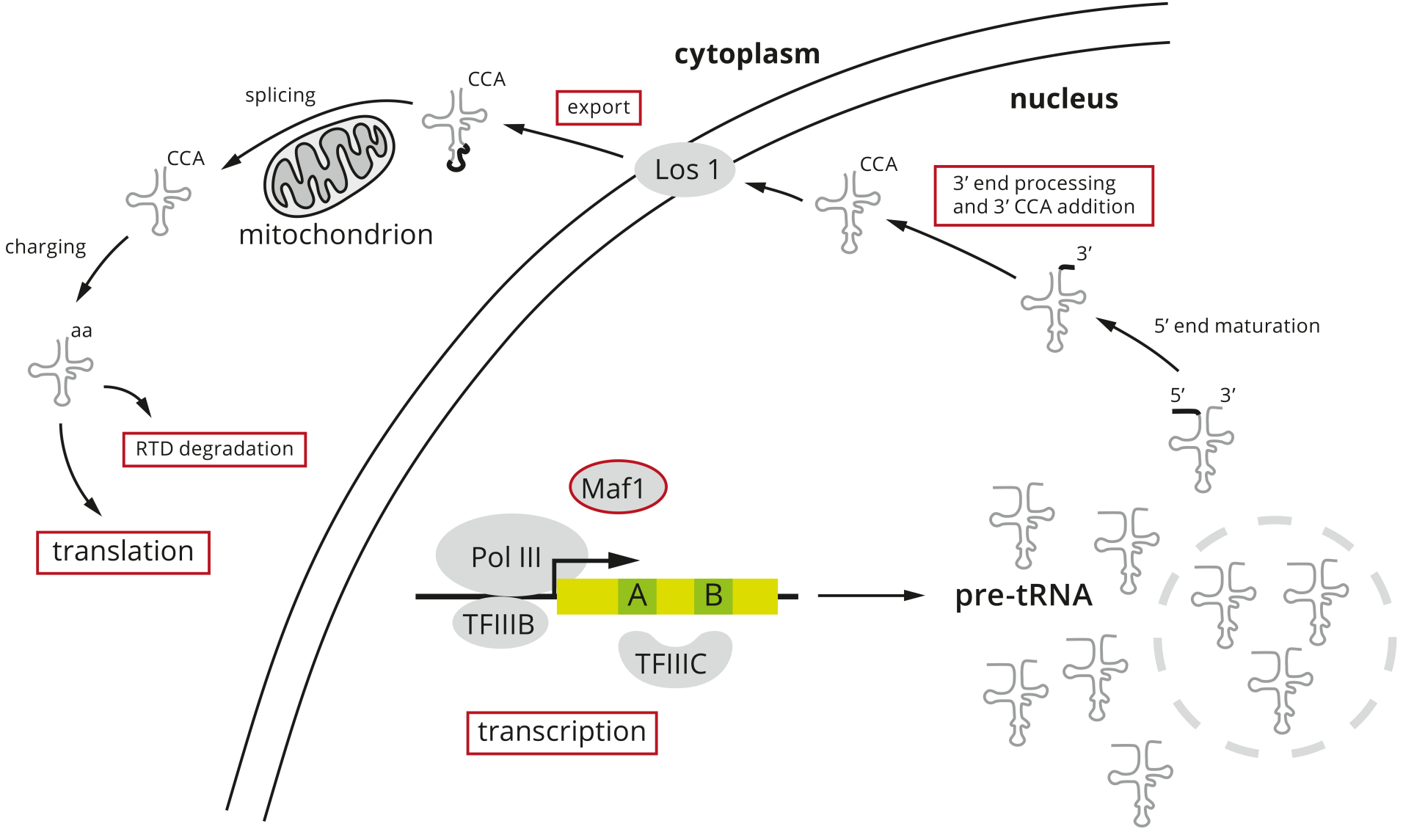
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