EXPLORING THE RIBOSOMAL STRESS PATHWAY AS A TARGETABLE VULNERABILITY IN TP53 ABERRANT ACUTE LYMPHOBLASTIC LEUKEMIA

Writing Assignment

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Layman's Summary

Acute lymfoblastische leukemie (ALL) is een van de meest gediagnosticeerde kankers bij kinderen. Hoewel de overlevingskansen onder zowel kinderen als volwassenen erg zijn toegenomen, komen er ook nog steeds terugvallen voor. Een terugval is over het algemeen moeilijk te genezen. Vooral terugvallen die afwijkingen hebben in het gen *TP53* voorspellen een sombere uitkomst vanwege hun hoge resistentie tegen de meeste chemotherapeutica, maar ook tegen nieuwe immuuntherapieën die in de kliniek zijn gekomen. Daarom is er een dringende klinische behoefte aan betere therapieën voor leukemieën met afwijkingen in p53. In het afgelopen decennium is het proces dat voor nieuwe ribosomen zorgt, ook wel ribosoom biogenese (RiBi) genoemd, naar voren gekomen als een effectief doelwit bij kankertherapieën. Ribosomen zijn moleculaire motors die essentieel zijn voor de productie van alle nieuwe eiwitten in een cel. Het is bekend dat RiBi een rol speelt bij signaalroutes die belangrijk zijn bij de ontwikkeling van kanker. RiBi wordt daarom erg sterk gereguleerd om te voorkomen dat cellen transformeren naar een kankercel. Het monitoren van RiBi wordt gedaan door ribosomale stress-signaleringsroutes. Deze routes kunnen veel verschillende soorten afwijkingen in RiBi waarnemen en zorgen dat de cel actie onderneemt om dit te herstellen. Bij deze routes speelt het eiwit p53 vaak een grote rol, maar niet altijd. p53 is een eiwit die mega veel verschillende processen in de cel reguleert. Het is een erg belangrijk eiwit en is daarom ook het meest frequent gemuteerde eiwit in kanker. Ribosomale stressroutes blijken ook erg belangrijk te zijn voor de ontwikkeling van leukemie. Er zijn namelijk meerdere mutaties gevonden in ribosomale eiwitten die bij blijken te dragen aan de progressie van leukemie. In een onderzoek waar ze naar nieuwe doelwitten zoeken die kwetsbaar zijn in p53 afwijkende leukemiecellen, hebben ze het eiwit eEF2K gevonden als een nieuw kwetsbaar doelwit. Dit eiwit kan de algemene productie van eiwitten remmen. Momenteel wordt eEF2K ook onderzocht als mogelijk nieuw doelwit voor de behandeling van kanker. In deze review heb de ribosomale stressroute onderzocht als potentieel kwetsbaar target in p53 afwijkende leukemiecellen. Meer specifiek heb ik gekeken waardoor deze leukemie cellen met afwijkingen in p53 kwetsbaar zijn voor een verlies van eEF2K. Hiervoor heb ik eerst gekeken naar alle verschillende ribosomale stress routes, en naar hoe afwijkingen in RiBi en deze routes kunnen zorgen voor kanker ontwikkeling. Daarnaast heb ik gekeken hoe deze routes kunnen worden gebruikt als een doelwit voor de ontwikkeling van nieuwe en verbeterde anti-kanker therapieën. Ten slotte onderzocht ik de rol van eEF2K, zijn verband met ribosomale stress en zijn bijdrage aan de progressie van kanker.

Abstract

Acute Lymphoblastic Leukemia (ALL) is one of the most frequently diagnosed cancers in children. Although the survival rates have been increasing, relapses are still occurring, which are generally hard to cure. Especially relapses showing aberrations in *TP53* predict a dismal outcome due to their high resistance against most chemotherapeutic drugs, but also novel immune therapies that have entered the clinic. Therefore, there is an urgent clinical need for better therapies for TP53 deleted ALL. In the last decade the ribosome biogenesis (RiBi) pathway has emerged as an effective target in cancer therapy. RiBi is known to play a role in oncogenic signalling pathways and is therefore highly regulated to maintain cellular homeostasis. Monitoring of RiBi is managed by ribosomal stress signaling pathways that can sense both through p53 or be p53-independent. The role of the ribosomal stress pathway in the development of ALL is underlined by the presence of somatic mutations in several ribosomal proteins that all have been linked to contribute to T-ALL progression. In a study to find new vulnerabilities of *TP53* aberrant ALL, they identified eEF2K as a new vulnerable target. eEF2K is a protein which negatively regulates translation elongation and can be activated upon many different stresses, including ribosomal stress. eEF2K is also under investigation as a possible novel molecular target for cancer treatment. In this review I have explored the ribosomal stress pathway as a potential targetable vulnerability in p53 aberrant ALL in order to provide a mechanistic explanation for the observed synthetic lethality between loss of TP53 function and loss of eEF2K in ALL under conditions of cellular stress. For this I reviewed different p53-(in)dependent ribosomal stress signalling pathways, looked at how aberrations in RiBi and these pathways can be linked to cancer development, and how these pathways can be used as a target for the development of new and improved anticancer therapies. Finally, I examined the role of eEF2K, its connection to ribosomal stress and its contribution to cancer progression.

Introduction

Acute Lymphoblastic Leukaemia (ALL) is one of the most frequently diagnosed cancers in children [1]. ALL is a cancer of the lymphoid line of blood cells characterized by the development of large numbers of immature lymphocytes. Because of new advances, the survival rates of ALL patients have increased to 90% in children and 40% in adults [2]. However, relapses are still occurring, which are more difficult to cure. Especially relapses showing aberrations in the *TP53* gene, which occur in about 15% of ALL patients, predict a dismal outcome with survival rates below 30% [3]. These relapsed cancers are not only highly resistant to most of the chemotherapeutic drugs used to treat relapsed but also to novel immune therapies that have recently entered the clinic [3]. Therefore, there is an urgent clinical need for better therapies for TP53 deleted ALL.

In the last decade the ribosome biogenesis pathway has emerged as an effective target in cancer therapy [4–6]. Ribosomes are essential molecular machines made up of both RNA and protein, required for the production of all proteins the cell needs to function. Ribosome Biogenesis (RiBi), is an extraordinary complex process that takes place in the nucleolus and requires the coordination of numerous events and molecular players [6–8]. Cancer cells generally show an increase in RiBi, because of their high demand of proteins due to their unrestricted growth. RiBi plays therefore a central role in oncogenic signalling processes associated with several proto-oncogenes. Because RiBi is such an essential process, the cell has developed numerous processes that sense when this process is impaired to sustain cellular homeostasis. This monitoring of nucleolar function and coupling ribosome integrity to the cell cycle is managed by nucleolar or ribosomal stress signaling. The most important ribosomal stress pathways signal through activation of the tumor suppressor p53 causing a block in cell cycle and subsequent senescence or apoptosis [9,10]. However, several recent papers suggest the existence of a number of alternative pathways that control the relationship between ribosome biogenesis and cell proliferation, that are independent of p53 [11–16]. As many cancer cells are p53-deficient, these pathways could be of great importance for the development of novel drug targets for cancer treatments.

There are many cellular stresses that may affect RiBi causing an activation of the ribosomal stress pathway. These stresses include nutrient or growth factor deprivation, UV and gamma radiation, rRNA or ribosomal protein (RP) imbalances, oncogenes, replication stress, hypoxia and oxidative stress [10,11]. Because this pathway can sense a variety of different stresses, it ensures cells to adapt to new environments and therefore has an important anti-cancer role. Mutations affecting these pathways are therefore observed in different cancer types [6,17,18].

Next to cancer, RiBi also plays an important role in several congenital disorders known as Ribosomopaties [19,20]. These are developmental disorders that arise through impairments in RiBi and are characterized by haematological deficiencies and bone marrow failure. Classical ribosomopathies include Swachman-Diamond Syndrome (SDS), Diamond-Blackfan Anemia (DBA), Treacher-Collins syndrome (TCS) and the 5q-syndrome [20,21]. Most of these diseases arise through mutations in RP genes causing impaired RiBi and a deficiency in mature ribosomes, leading to a decrease in protein synthesis [20]. Impairments in RiBi also result in accumulation of free RPs, which can activate the ribosomal stress pathway causing excessive cell cycle arrest and apoptosis, leading to the observed developmental defects. Apparently, some cells are more vulnerable to these ribosomal defects, visible in the specific disease phenotype. Why specific cell types are more vulnerable to RP changes is still largely unknown. Contradictory, patients suffering from one of these diseases also show a higher predisposition to cancer development [8]. That specific mutations in RP genes are associated with cancer is demonstrated in the disease progression of T-ALL. Somatic mutations in RPL5, RPL11, RPL10 and RPL22 have all been linked to contribute to T-ALL progression [22,23]. This emphasizes the role of the ribosomal stress pathway in the development of this cancer type.

In a study to find new vulnerable targets for TP53-deleted ALL, a CRISPR-based screen was performed in the condition of cellular stress (unpublished data, van Leeuwen). This screen identified the eukaryotic elongation factor 2 kinase (eEF2K) as a new potential vulnerability. eEF2K is an unusual protein kinase that acts as a negative regulator of transcription elongation [24]. It is activated upon many different stresses, including nutrient stress, ER stress and ribosomal stress [25,26]. Because transcription elongation is a highly energy demanding process, the activity of eEF2K is tightly regulated by several signalling pathways including mTORC1, ERK and AMPK [24,27]. Impairments in the regulation of eEF2K is shown to contribute to cancer development. Overexpression of eEF2K is seen in different kinds of cancer and is often associated with poor survival in patients [28,29]. Although it seems paradoxical that high proliferation cells show high expression of a negative regulator of protein synthesis, there are multiple possible mechanisms described how eEF2K may promote cancer survival and tumor development [28,30,31].

eEF2K is under investigation as a possible novel molecular drug target for cancer treatment. Several studies have shown that knockdown of eEF2K slowed tumour growth [28,32]. Furthermore, inhibition of eEF2K is demonstrated to sensitize cells to conventional chemotherapeutics [32,33]. eEF2K inhibitors could therefore be used in combination therapies together with conventional chemotherapeutics to effectively kill cancer cells. However, effective inhibitors for eEF2K are still in development.

The aim of this review is to give an overview of the different ribosomal stress pathways and how impairments in RiBi can contribute to cancer development. Furthermore, I will give insight in why this pathway may represent a suitable target for the development of new and improved anticancer therapies. More specifically, I will try to give a mechanistic explanation for the observed synthetic lethality between loss of TP53 function and loss of eEF2K in ALL under conditions of cellular stress.

Chapter 1: Ribosome Biogenesis and the Ribosomal Stress pathway

Ribosome biogenesis is a highly complex process that involves many different events and molecular players. It is an essential process for the cell, as ribosomes synthesize all the proteins the cell needs to function. The entire process of RiBi is estimated to consume nearly 60% of cellular resources [7]. The cell has therefore developed numerous signalling processes that sense when this process is disrupted to maintain cellular homeostasis, together referred to as the ribosomal stress pathways [12,34]. The nucleolus, the place where RiBi mainly takes place, can in this way regulate important cellular processes such as cell cycle progression and cellular proliferation. In this first chapter I will describe the main steps involved in RiBi and explain the most important signalling processes involved in the ribosomal stress pathways.

The Process of Ribosome Biogenesis

Cytosolic ribosomes are composed of two different subunits, the small subunit (SSU) and the large subunit (LSU). Both subunits consist of several rRNA molecules and a lot of different ribosomal proteins (RPs) [7]. These factors together form the basis of the 80S ribosome. Most of the processes of RiBi take place in a specific location in the nucleus, the nucleolus. The size of the nucleolus reflects the rate of RiBi, thus cells that have a higher RiBi also show larger nucleoli [35]. The production of ribosomes involves all the three different RNA polymerases [7]. At first RNA polymerase I (Pol I) transcribes rDNA generating the 47S precursor RNA that is then further processed into the different rRNA molecules needed. For these further modifications small nucleolar RNAs (snoRNAs) play an important role. Secondly, RNA polymerase II (Pol II) transcribes all the ribosomal proteins, which are subsequently translated to proteins. This process takes place in the cytoplasm, so the proteins must be back transported into the nucleolus. Thirdly, RNA polymerase III (Pol III) transcribes the special 5S rRNA. All these factors are assembled in the nucleolus to form the pre-SSU and pre-LSU, which then are transported out of the nucleolus where they assemble to generate the final 80S ribosome.

As RiBi is an essential process for the cell, impairments are associated with different diseases and cancer development [20,21]. To maintain genomic and cellular homeostasis the cell has developed surveillance mechanisms that monitor aberrant ribosome production. The most important ribosomal stress pathways sense through activation of p53 leading to cell cycle arrest and play a pivotal role in maintaining the integrity of RiBi [9,10]. However, several recent papers describe other ribosomal stress pathways that signal independently of p53, also leading to cell cycle arrest or apoptosis [11–16]. Since more dan 50% of human cancers are p53 deficient, understanding these pathways could lead to novel molecular targets for p53-deficient cancer therapies. In the next section I will further elaborate on these different p53-dependent and p53-independent mechanisms.

p53-dependent Ribosomal Stress Pathways

The Impaired Ribosome Biogenesis Checkpoint (IRBC)

The most important ribosomal stress pathway is the Impaired Ribosome Biogenesis Checkpoint (IRBC) which is activated in response to numerous cellular stresses and is essential to maintain cellular homeostasis [9,10]. Stresses such as nutrient or growth factor deprivation, rRNA or RP imbalances, DNA damage, oncogenes, hypoxia, and oxidative stress all can interrupt ribosomal integrity and activate the IRBC. The most important players in this pathway are free ribosomal proteins (RPs), the E3-ubiquitin ligase mouse double minute-2 (MDM2) and the tumor suppressor p53 [10]. Under homeostatic conditions, MDM2 binds and ubiquitinates p53 targeting it for degradation (Fig. 1a). When ribosome integrity is lost there is an imbalance of ribosomal components, leading to unassembled RPs. These unassembled RP accumulate

and translocate from the nucleolus to the nucleoplasm, where they can bind to MDM2 and stabilise p53 (Fig. 1b). Recent studies have shown that as many as 16 RPs can interact with MDM2 and regulate p53 function [36]. Among them RPL11 and RPL5 are the most important players. Normally, excessive unassembled RPs in the nucleoplasm are targeted for proteasomal degradation, however, RPL11 and RPL5 escape this route by forming a complex with 5S rRNA, forming the pre-ribosomal complex 5S RNP [37] (Fig. 1b). This complex is also formed under normal conditions, but upon ribosomal stress there is a surplus of 5S RNP complex leading to MDM2 inhibition and p53 activation.

Unassembled RPs are the main regulators of the IRBC

Depletion of RPs from both the LSU as the SSU can lead to accumulation of RPL5 and RPL11, although these two are both part of the LSU. This is surprising since biogenesis of the LSU occurs independently of the SSU. One possible explanation is that depletion of SSU RPs influence the folding kinetics of the rRNA of the LSU, and thus slow down the assembly of the LSU resulting in unassembled RPs of the LSU [6]. Another explanation is that specific mRNAs of the LSU maintain translational activity upon ribosomal stress. Depletion of the SSU was namely shown to increase the production of RPL11. This is due to RPL11 mRNA having a specific 5'poly-pyrimidine tract (5'TOP), which maintains its translational activity upon depletion of the SSU [12]. Depletion of the SSU can therefore lead to excess of RPL11, which then blocks MDM2 activity and ultimately leads to a block in cell cycle progression and apoptosis. The imbalance of ribosomal components is thought to be one of the major reasons of ribosomal stress [10]. Equal amounts of RP components are essential for maintaining an adequate translational machinery. Ribosomal stresses that disrupt this balance, result in unassembled RPs which function as a regulatory unit that ensures crosstalk between RiBi, MDM2 and p53.

Other nuclear players involved in regulating the IRBC

Next to unassembled RPs, a few other nucleolar players also regulate p53 during ribosomal stress conditions. One of these players is the alternative reading frame protein p19Arf (mouse) p14ARF (human). Under normal conditions this protein is bound to nucleophosmin (NPM) which localises ARF to the nucleolus (Fig. 1a). Upon ribosomal stress ARF is translocated to the nucleoplasm where it binds to and blocks MDM2 activity, ensuring p53 accumulation leading to cell cycle arrest and apoptosis (Fig. 1b) [34]. Nucleostemin (NS) is another nuclear regulator of MDM2 function [17,38]. NS is a GTP-binding protein and highly expressed in various cancer cells and cancer stem cells [39]. GTP bound NS localises to the nucleoli, whereas GDP bound NS localises to the nucleoplasm. How NS regulates p53 activity is still under investigation, as it seems that both over-and-under-expression of NS leads to stabilisation and activation of p53 [12]. When overexpressed, NS can bind the central acidic region of MDM2, thereby preventing its folding and activity, allowing the accumulation of p53 (Fig. 1b). On the other hand, depletion of NS leads to disruption of ribosome production, causing accumulation of RPL5 and RPL11 and subsequent activation of the IRBC. Thus, the activity of MDM2 is blocked by NS itself when overexpressed, or by RPs when ribosome production is disrupted by NS depletion.

P53-independent Ribosomal Stress Pathways

Besides the important IRBC, several papers suggest the existence of alternative pathways controlling the relationship between ribosome biogenesis and cell proliferation, independent of p53 (Fig. 2). These pathways involve both RPs and other nuclear factors. As a large amount of cancer cells are p53-deficient, these pathways could be of great importance for the development of novel drug targets for cancer treatments as these cells are already deficient in p53 dependent ribosomal stress sensing.

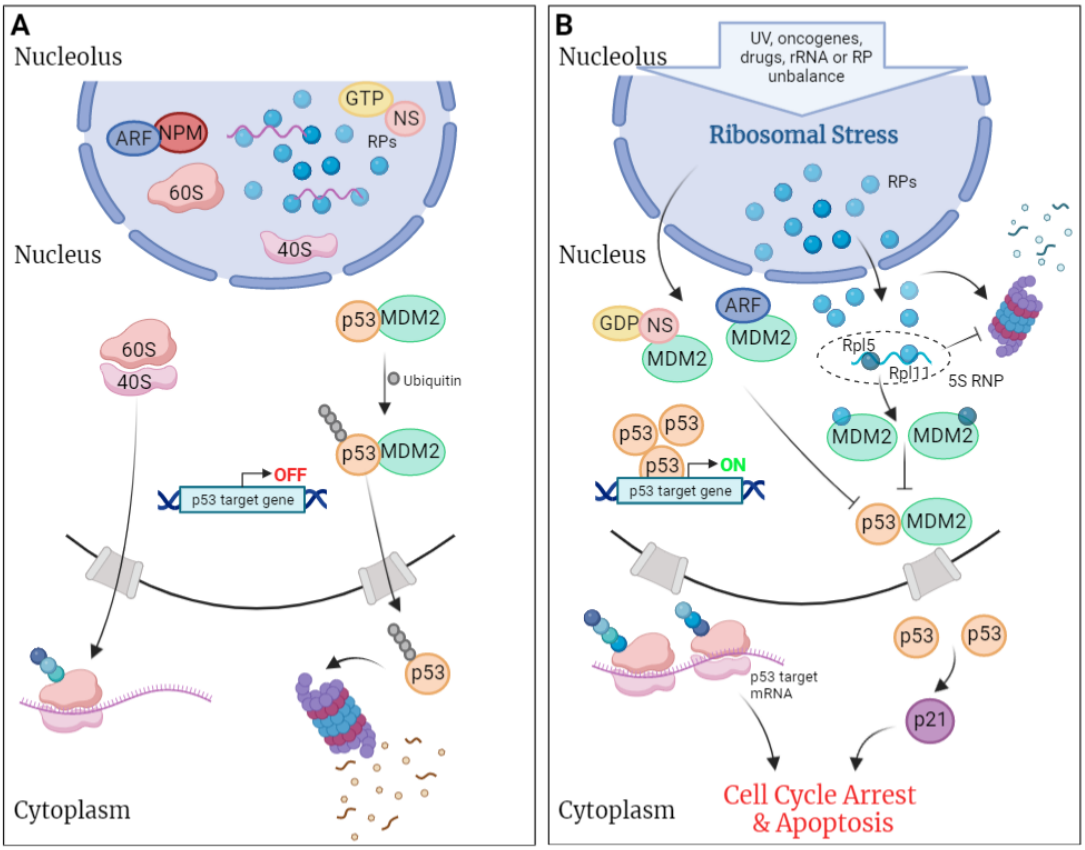

Figure 1: Schematic representation of the different p53-dependent ribosomal stress pathways in homeostatic and ribosomal stress conditions. A) Homeostatic conditions: Ribosomes are normally produced and MDM2 binds and ubiquitinates p53 targeting it for degradation. Nucleophosmin binds ARF and localises it to the nucleolus. GTP-bound NS also localizes to the nucleus. P53 target genes are off. B) Ribosomal stress: Ribosome integrity is lost and unassembled RPs translocate from the nucleolus to the nucleus, where most are targeted for degradation. However, Rpl5 and Rpl11 together with 5S rRNA form the 5SRNP complex which prevents them from degradation. Rpl5 and Rpl11 then bind to MDM2, preventing MDM2 p53 interaction. ARF and NS are translocated to the nucleoplasm where they can bind to and block MDM2 activity. This both ensures p53 accumulation leading to cell cycle arrest and apoptosis. Figure is created with BioRender.

E2F-1 and RPL11

The first mechanism I will describe involves the E2F transcription factor 1 (E2F-1), which is a transcription factor that controls expression of genes whose products are important for progression through the S phase. E2F-1 is known to be a mediator of both p53-dependent and p53-indepentend apoptosis [11,16]. The stability of E2F-1 is controlled by MDM2 that binds and protects E2F-1 from proteasome-dependent degradation (Fig. 2a). Donati et al., have shown that upon ribosomal stress RP11 is released from ribosomes and associates with MDM2 causing the release of E2F-1 and its subsequent degradation leading to cell cycle arrest [16] (Fig. 2a). Thus, RPL11 can regulate the activity of E2F-1, by interfering its interaction with MDM2, and thereby influencing the cell-cycle in a p53-independent manner.

P14ARF

In my review, I described ARF earlier as a regulator of p53-dependent nucleolar stress. Recent findings show that ARF is also involved in p53 independent cell cycle regulation [15]. These show that ARF can bind the oncogenes c-Myc and E2F-1, thereby inhibiting their transcriptional function, which leads to cell cycle arrest and apoptosis in absence of p53 (Fig. 2b). C-Myc and E2F-1 can on their turn regulate ARF expression, resulting in a p53-independent negative feedback mechanism. P14ARF is an alternative product from the *CDKN2A/2B* locus, which is mutated in more than 50% of T-ALL cases [40,41]. This suggests a possible driver role for p14ARF in T-ALL pathogenesis.

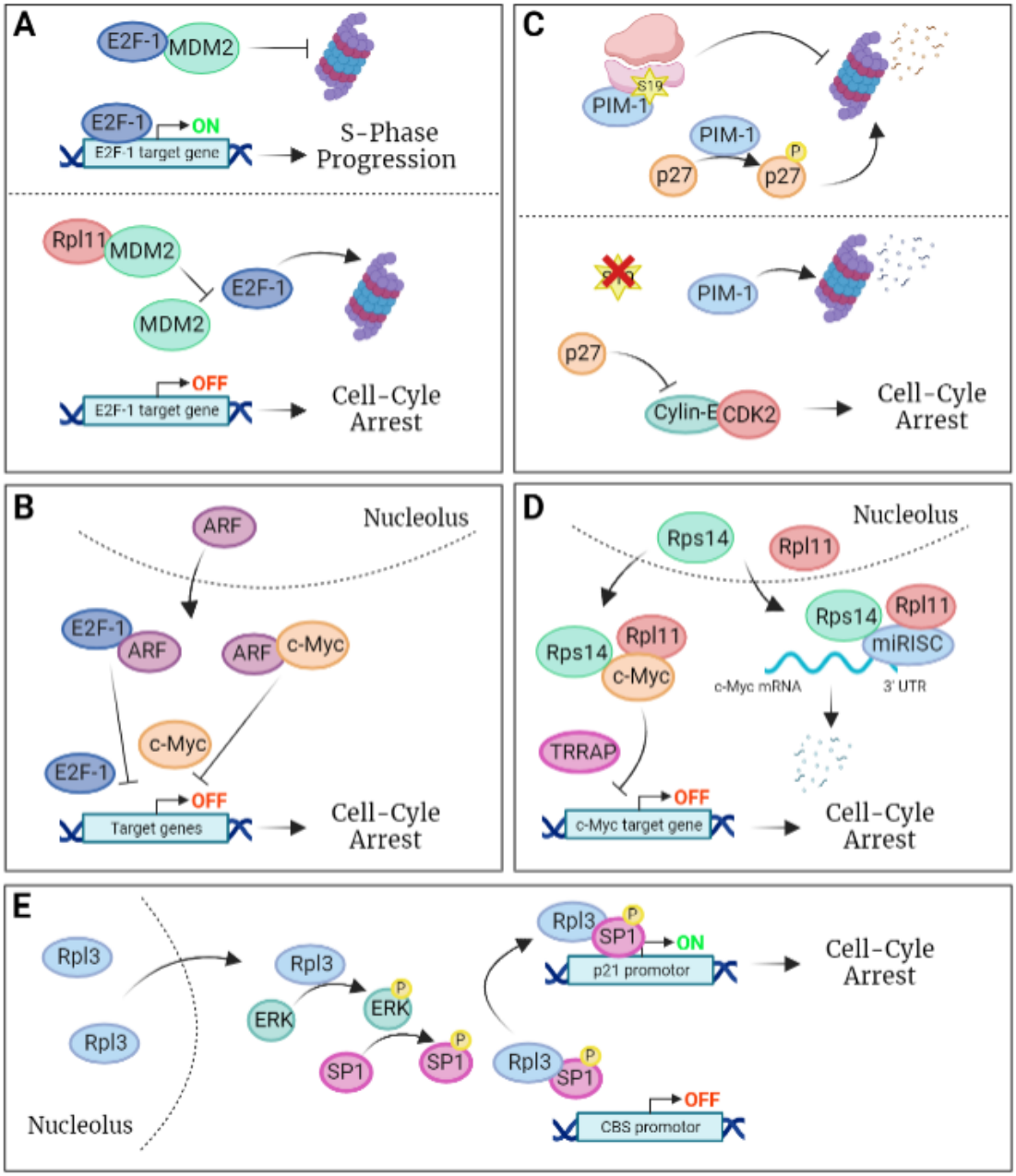

Figure 2 p53-independent ribosomal stress pathways. A) In homeostatic conditions (upper panel) MDM2 binds to E2F-1 thereby preventing its degradation. During ribosomal stress (lower panel) unassembled Rpl11 translocates to the nucleus, where it associates with MDM2 causing the release of E2F-1 and its subsequent degradation leading to cell cycle arrest. B) Upon ribosomal stress, ARF will translocate to the nucleus where it can bind E2F-1 and c-Myc, thereby inhibiting their transcriptional function, which leads to cell cycle arrest and apoptosis. C) In normal conditions (upper panel) PIM1 is stabilized by the Rps19 component of the ribosome. PIM1 can phosphorylate p27 targeting its degradation. Upon Rps19 knockout (lower panel), PIM1 is destabilized rendering it available for degradation. This causes stabilisation of p27 and consequently cell cycle arrest. D) Upon ribosomal stress, unassembled Rps14 and Rps11 translocate to the nucleus where they can bind to c-Myc, preventing the recruitment of the cofactor TRRAP to the promotor of c-Myc target genes. In addition, Rpl11 and Rps14 can through interaction with miRISC bind to the 3'UTR of the c-Myc mRNA, targeting it for degradation. This both leads to cell cycle arrest. E) Upon ribosomal stress, unassembled RPL3 can phosphorylate ERK, which further phosphorylates Sp1. Rpl3 can interact with phosphorylated Sp1 thereby displacing Sp1 from the CBS promotor to the p21 promotor, which results in cell cycle arrest. Figure is created with BioRender.

PIM1

Another protein that can play a part in p53-independent ribosomal stress is the kinase PIM1. This protein is a highly conserved serine/threonine kinase involved in cell cycle regulation and apoptosis. PIM1 can regulate cell cycle progression through phosphorylation of a myriad of known downstream targets, such as the cell cycle inhibitor p27, thereby marking it for degradation (Fig. 2c)[12]. Ribosomal stress and RP deficiencies can largely affect PIM1 protein stability. The interaction of PIM1 with the ribosomal protein RPS19 was demonstrated to play an important role in this. RPS19 deficient cells showed a decrease in PIM1 levels, due to PIM1 unable to interact with RPS19, rendering it available for proteasomal degradation [13]. This causes stabilisation of p27 and consequently cell cycle arrest (Fig. 2c). PIM1 can in this way induce cell cycle arrest in response to ribosomal stress, regardless of the p53 status of the cell. Interestingly PIM1 appears to play an important role in T-ALL disease progression. Several studies suggest that PIM1 may represent an attractive molecular target in human T-ALL [42,43]. Treatment of PIM inhibitors in combination with conventional chemotherapeutics improves leukemic survival of a PDX model of T-ALL. This underscores the contribution of PIM1 to cancer progression of T-ALL.

c-Myc and RPL11/RPS14

The oncogene c-Myc is a transcription factor that regulates the expression of numerous genes involved in cell growth and proliferation. These include many crucial factors involved in RiBi, for example the three RNA polymerases. Several papers have shown that some RPs can act as negative feedback regulators of c-Myc expression [44–47]. It was shown that RPL11 directly binds c-Myc, thereby preventing the recruitment of the co-factor TRRAP to the promotor of c-Myc target genes [46]. In addition, RPL11 can target the c-Myc mRNA for degradation, by recruiting the miRISC complex to the 3'-UTR of the c-Myc mRNA, through interaction with miR-24 and miR-130 [44] (Fig. 2d). Another study showed that RPS14 can negatively regulate c-Myc in the same manner as RPL11. RPS14 is also able to bind c-Myc, preventing recruitment of TRRAP to c-Myc target genes. Furthermore, RPS14 can induce c-Myc mRNA degradation through interaction with miR-145 of the miRNA pathway [47] (Fig. 2d). Thus, both RPL11 and RPS14 can negatively regulate c-Myc expression by inducing the degradation of the mRNA of c-Myc and by preventing the activation of c-Myc target genes.

RPL3 and p21

Next to RPL11 and RPS14, RPL3 is also described to play a role in p53-independent ribosomal stress. Besides being a component of the LSU, Russo et al,. describe that RPL3 has extra-ribosomal functions involved in cell cycle progression in response to drug-induced ribosomal stress. [11,14,48]. They have shown that RPL3 can induce G1 arrest through promoting the gene expression of the kinase inhibitor p21. RPL3 can phosphorylate the extracellular signal-regulated kinase (ERK), which is speculated to further phosphorylate Sp1 [14]. Sp1 can bind to RPL3, thereby promoting the binding to the p21 promoter and activating p21 gene transcription. Furthermore, RPL3 can displace Sp1 from the CBS promotor to the p21 promotor, in this way inhibiting CBS transcription (Fig. 2e). Taken together, RPL3 can regulate cell cycle progression by inducing G1 arrest through activation of p21 gene transcription.

Chapter 2: The link between excessive Ribosome Biogenesis and Cancer

Cancer cells require a lot of ribosomes to maintain high protein synthesis rates needed for their unrestricted growth. Excessive RiBi is generally believed to play a critical role in cancer initiation and progression. Over a century ago, cancer cells were already linked to aberrant increases in nucleolar size and number, most commonly reflecting upregulation of RiBi [8,35]. Both these characteristics are hallmarks of cancer cells and still used as markers of poor prognosis for many cancer types. In this chapter I will provide insight in how excessive RiBi is associated with cancer initiation and progression.

RiBi plays a central role in oncogenic signaling processes

Many oncogenic signaling processes are known to control ribosome synthesis and function. These pathways include RAS, PI3K/mTORC1 and c-Myc signaling. c-Myc is a prominent controller of RiBi, able to regulate the function of all the three RNA polymerases, important for RP and rRNA production [49]. One of the functions of c-Myc is directly controlling the expression of the upstream binding factor (UBF), an essential component of the Pol I transcription machinery, thereby influencing the transcription of rDNA [17,49]. Moreover, c-Myc can directly influence the expression of pre-rRNA genes and can increase 5S rRNA biosynthesis [50]. Overexpression of c-Myc can therefore lead to enhanced RiBi. The RAS/ERK pathway also plays an important role in the regulation of RiBi. ERK can regulate the phosphorylation and activation of UBF and transcription initiator factor IA (TIF-1A), thereby activating rRNA synthesis [51]. Similarly, PI3 K/AKT/mTORC1 signaling pathway also modulates the phosphorylation of UBF and TIF-IA [52]. These data illustrate that activating mutations in these signaling pathways could enhance ribosome biogenesis and trigger cancer cell proliferation.

In contrast, there are also numerous tumor-suppressors that can inhibit ribosome biogenesis, such as p53, PTEN, RB and ARF. p53 can inhibit the activity of Pol I by binding to the SL-1 complex, which inhibits the binding of the Pol I complex with the rDNA promotor [53]. RB can inhibit rDNA transcription by binding with the UBF of the Pol I, thereby preventing its recruitment to the rRNA promotor [54]. PTEN represses Pol I transcription by disrupting the SL1 complex [8]. These examples suggest that inactivation of tumor suppressors and/or overexpression of onco-genes can result in the hyper-activation of RiBi. However, these oncogenes and tumor suppressors also affect many other processes, which make the contribution of excessive RiBi to cancer initiation unclear.

Contribution of excessive RiBi to Cancer progression

Hyper-activation of RiBi can contribute to cancer progression by promoting cancer cell growth. Recent studies have shown that there are more mechanistic explanations how excessive RiBi can promote tumorigenesis, which includes regulating transcriptional programs supporting malignant transformation.

Altered pattern of translation

The first consequence of hyper-activated RiBi, is an increase in ribosomes abundance, resulting in increased global protein synthesis rates. Santagata et al., described a model in which this increased global protein synthesis rate can contribute to cancer tumorigenesis via the induction of the heat shock factor 1 (HSF1) translational program [55]. This program regulates the expression of heat-shock proteins (HSP), which are involved in diverse cellular mechanisms all important for cellular growth. The HSF1-regulated transcriptional program is associated with malignant transformation [56]. Santagata et al., have shown that inhibition of protein translation leads to an inhibition of the HSF1-regulated gene expression program, suggesting that protein translation and this HSF1-activation are coordinated. Excessive RiBi can in this way

alter the translational program of HSF-1. A second study observed that overexpression of RPL15 can increase the metastatic potential of several cancer types [57]. They showed that RPL15 overexpression resulted in enhanced translation of other ribosomal proteins, leading to increased amount of fully assembled ribosome and enhanced global translational activity. Furthermore, RPL15 overexpression is shown to promote the translation of E2F-regulated genes. Taken together, these studies show that an alteration in translation patterns caused by excessive RiBi can play an important role in the development and progression of cancer.

Proteotoxic stress

The increased protein production associated with excessive RiBi, may overwhelm the capacity of the chaperone network and the protein degradation pathways, leading to an increase in proteotoxic stress and activation of the unfolded protein response (UPR) [6,58]. Proteotoxic stress is also associated with an increase in reactive oxygen species (ROS), which may promote tumorigenesis due to the increase in oxidative DNA damage [59]. Alternatively, dysregulation of protein homeostasis under these conditions can lead to a global decline in cellular function and eventual cancer cell death.

These are two mechanistic explanations of how excessive RiBi can contribute to cancer progression. In the next chapter I will discuss how impairments in RiBi are associated with cancer progression.

Chapter 3: Ribosomopathies and their implications in Cancer Development

Aberrant Ribosome Biogenesis is associated with a broad spectrum of diseases together named ribosomopathies. The most common include Diamond Blackfan Anemia (DBA), Swachmand-Diamond Syndrome (SDS), Treacher-Collins syndrome (TCS), X-linked dyskeratosis congenita (DC), cartilage hair hypoplasia (CHH) and the 5q-syndrome [20,21,60]. These diseases are characterized by cellular hypo-proliferation phenotypes, such as haematological deficiencies, bone marrow failure and anaemia [61]. Most ribosomopathies arise through mutations in RP genes or other RiBi factors. This leads to a deficiency of mature ribosomes, which decreases protein synthesis and, as a result, causes hypo-proliferation. Impairments in RiBi also result in accumulation of free RP proteins and subsequent activation of the MDM2/p53 pathway leading to cell cycle arrest and apoptosis [6,10]. This overactivation of the IRBC pathway may further enhance the hypo-proliferative phenotypes associated with these diseases.

Contradictory, many ribosomopathies are also characterized by a predisposition to the development of cancer, a disease with a hyper-proliferative cellular nature [62]. The involvement of RP mutations in cancer is demonstrated by the occurrence of somatic of deletions and frameshift mutations in RP genes in various cancer types. It appears that 43% of human sporadic cancers contain mutations or deletions in chromosomal regions containing RP genes [63]. Cancers showing these RP mutations are also often p53-deficient. It is, however, unknown whether these mutations play an important role in neoplastic transformation. Although, a recent study suggested a driver role of RPL5 and RPL10 mutations in the disease progression of T-ALL [64]. This finding further emphasizes the importance of functional ribosomes in the disease progression of T-ALL.

Ribosomopathies and their oncogenic potential

It seems contradictory that mutations causing large activation of the tumor suppressor p53 are associated with cancer. However, multiple mechanisms have been described demonstrating the link between ribosomopathies and cancer development. In the next section I will describe these mechanisms more in detail.

Altered mRNA translation due to emergence of specialized “onco-ribosomes”

The first mechanisms I will describe includes the alteration of the composition of ribosomes upon RP haploinsufficiency. Ribosomes were generally believed to be homogenous molecules, that equally promote the translation of all mRNA molecules. Over the last decade, it has become clear that this is not the case and ribosomes are rather heterogenous molecules that play an important role in transcription regulation [65,66]. Ribosomes can thus differ in RP and rRNA composition, resulting in the generation of specialized ribosomes that can selectively translate specific mRNA molecules. This ribosome heterogeneity offers an extra step in the regulation of mRNA translation.

A number of studies show that, in addition to negatively impacting ribosome assembly, RP mutations can contribute to ribosome diversity, leading to ribosomes with altered functions [19,66–70]. These so called “onco-ribosomes” may translate differentially specific mRNAs beneficial for cancer progression, for instance increasing the expression of oncogenes and reducing that of tumor suppressors [67,71,72]. Furthermore, these “onco-ribosomes” display altered translational speed and fidelity. One way onco-ribosomes alter translational fidelity is by altered interactions of ribosomes with mRNA secondary regulatory structures, such as the Internal ribosomal entry sites (IRES). IRES elements are regulatory elements on the mRNA, which can recruit ribosomes independently of the canonical 5’cap-driven translation initiation. Recent studies show that mutations in RPs can influence the efficiency of IRES-mediated translation. One example is the

RPL10 R98S mutation found in T-ALL, which drives increased IRES-dependent translation of the anti-apoptotic factor BCL-2 [68]. This increased BCL-2 translation enables the RP mutated T-ALL cells to survive high levels of oxidative stress associated with this mutation, further described below. On the other hand, RP mutations may also lead to ribosomes with a reduced translation of IRES containing mRNAs. This is seen in DBA patients, where mutations in RPL11 and RPL19 can result in the downregulation of IRES-containing mRNAs of proliferation and differentiation factors *BAG1* and *CSDE1* [70]. This last example, however, cannot fully explain the cancer predisposition in DBA patients. The altered ribosomes in T-ALL can alter gene expression also using the programmed -1 ribosomal frameshift (-1 PRF) signals, which are another class of cis-acting mRNA control elements [19]. High rates of -1 PRF on a mRNA are associated with low rates of protein expression. The RPL10 R98S mutation in leukaemia displays lower rates of -1 PRF signalling in genes of the JAK-STAT signalling cascade, causing overexpression of JAK-STAT proteins, which can further influence cellular proliferation and survival [69]. Taken together, these examples show that mutations in RPs can lead to the formation of "onco-ribosomes" with altered mRNA expression profiles, enhancing the expression of growth-promoting and pro-oncogenic proteins that could further contribute to cancer progression.

An extra step in creating heterogenous ribosomes are differences in rRNA modification. 2'-O-methylation (2'-O-Me) is the most abundant rRNA modification and the pattern of these mutation is altered in cancer cells compared to their healthy counterpart. Interestingly, these modifications are associated with alterations in IRES-dependent translation initiation [5]. Differences in rRNA modifications can in this way also result in altered translation initiation.

Extra-ribosomal moonlighting functions of RPs

Besides their contribution in ribosomes, ribosomal proteins can have extra-ribosomal moonlighting functions that can be beneficial for cancer development. In chapter 1, I have already described some extra-ribosomal functions of RPs, mainly involved in the regulation of ribosomal stress pathways. RPL11 and RPL5 are, for example, the most important factors of MDM2-mediated p53 stabilisation and activation. Mutations in one of these two proteins, which is visible in DBA patients, are therefore associated with both ribosome biogenesis defects as cell cycle progression due to the inability to activate the tumor suppressor p53. This suggests that loss of the functions of RPL11 and RPL5 might predispose DBA patients to cancer development. Furthermore, RPL5 and RPL11 are also involved in the negative regulation of c-Myc expression. Mutations in these two RPs are suggested to contribute to oncogenic c-Myc overexpression. To support this notion, c-Myc upregulations are described in mouse lymphoma models harbouring RPL5 and RPL11 mutations [73]. Thus, mutations in RPL5 and RPL11 can result in dysregulated expression of TP53 and c-Myc, leading to elevated risks of cancer development. Many more extra ribosomal moonlighting functions of RPs that can be beneficial for cancer development are described in a recent review on this topic [65].

Metabolic alterations and Cellular stress

The role of defective ribosomes in the transition to cancer may also be more indirect. Emerging evidence underlines the role of ribosomes in the regulation of proteasomal degradation. Furthermore, ribosome integrity plays a role in cellular redox homeostasis. In this section, I will describe these metabolic alterations in response to ribosomal deficiencies and how these can contribute to cancer progression.

Deregulated protein degradation contributes to oncogenic potential

Recent studies show that there is a link a link between the functionality of ribosomes and proteasomes. A large study investigating interaction partners of ribosomes identified 15 proteasomal proteins as interaction partners of ribosomes [74]. This suggests that there are physical interactions between the ribosome and the proteasome. Ribosomopathies strengthen the idea that dysregulation of ribosomes has implications on the proteasome. The RPL10 R98S cell model showed both up-and downregulation of proteasomal units, including Psmb10 and Psmb9, which is associated with reduced chymotrypsin and caspase like proteasomal

activity [69]. This altered activity can enhance the stability of the oncogenic kinase Jak1, which contributes to the pathogenesis of T-ALL. Altered composition and activity of the proteasome is thus able to influence the stability of proteins, affecting the oncogenic potential of the cell.

Involvement oxidative stress in pathogenesis of ribosomopathies

A number of studies has shown a link between cellular redox homeostasis and ribosome integrity [6,19,69]. Ribosomopathies suffer from high cellular oxidative stress due to increased cellular ROS levels, which is demonstrated in models of DBA with RPL5 and RPL19 mutations [75]. Oxidative stress can result in mitochondrial dysfunction, which can contribute to the hypo-proliferative phenotype observed in these diseases. The involvement of oxidative stress in this phenotype is further demonstrated by antioxidant treatment, which reduce ROS levels and thereby completely rescues the growth defect [68]. This reinforces the significance of oxidative stress in the pathogenesis of ribosomopathies. However, the mechanism by which ribosome defects result in higher ROS levels and oxidative stress remain poorly understood. In the case of leukaemia, elevated ROS levels may be the cause of increased peroxisomal activation. Peroxisomes are oxidative organelles that produce high levels of hydrogen peroxide (H2O2). The RPL10 R98S mutation in T-ALL is shown to enhance the peroxisomal β-oxidation leading to oxidative stress through ROS accumulation [68]. This results in mitochondrial dysfunction and subsequent growth defects. Earlier, I described that this mutation is associated with an overexpression of the IRES-dependent mRNA BCL-2. This enhanced expression facilitates and enhances leukemic RPL10 R98S cell survival under high oxidative stress conditions. This paper furthermore shows that RPL10 R98S mutated cells are highly sensitive to BCL-2 inhibitors, highlighting BCL-2 inhibition as a novel therapeutic opportunity in RPL10 R98S defective T-ALL [68].

Transition from hypo- to hyper-proliferation

I have described several cellular changes induced by ribosome defects which can contribute to cancer development. Several of these findings can be integrated to form a model that can possibly explain the paradoxical evolution from hypo- to hyper-proliferative disease phenotypes in ribosomopathy patients (Fig. 3).

We have demonstrated that mutations in RP proteins can generate ribosomes with altered composition and function. These “onco-ribosomes” can shift the translational output resulting in increased expression of oncogenes and reduced expression of tumor suppressors. In addition, RP proteins can have extra-ribosomal moonlighting functions that can alter the expression of onco-genes and tumor suppressors such as p53 and c-Myc. Mutations, in these proteins can therefore lead to dysregulated expression of these genes leading to elevated risks of cancer development. Altered ribosomes can also influence metabolic alterations that can contribute to cancer progression. This includes the deregulation of protein degradation, which can result in enhanced stability of oncogenes. Furthermore, ribosomal defects are shown to induce elevated oxidative stress levels and the generation of high ROS levels which are highly toxic to cells and impairs their cellular growth. These high ROS levels, at first, mainly contribute to the hypo-proliferative nature of ribosomopathies. However, the oxidative damage caused by high ROS levels can also induce DNA damage, resulting in higher genomic instability. This will increase the chance of the induction of secondary mutations that can transition cells from a hypo- to a hyper-proliferation state. Thus, the high oxidative stress levels at first hinder cell growth, but can later enable increased mutagenesis, leading to the acquisition of rescuing mutations. To summarize, ribosome defects can result in altered gene expression, altered protein degradation and changes in cell metabolism that can promote genomic instability and secondary mutations, each of which contribute to the transition from a hypo- to a hyperproliferation state.

Besides this model to transition from a hypo- to hyper-proliferation state, we can ascribe a specific cancer-promoting activity of the ribosomal RPL10 R98S mutation to leukaemia formation. This mutation is accountable for around ~8% of paediatric T-ALL patients and promotes T-ALL pathogenesis in several ways

[23]. At first this mutation was associated with upregulation of the JAK-STAT signalling pathway, demonstrated in both mouse hematopoietic cell models as well as T-ALL patients [69]. This upregulation was the result of a decreased Jak1 proteasomal degradation and a reduced -1 PRF signalling and thus higher expression of JAK-STAT mRNAs. Secondly, this mutation is associated with high ROS levels, which are normally toxic to these cells. Due to increased translation of the IRES-dependent anti-apoptotic mRNA BCL-2, these cells are able to survive the high oxidative stress conditions, thereby inducing a growth advantage [68]. Both these pathways contribute to the cancer-promoting activity of the ribosomal RPL10 R98S mutation in T-ALL.

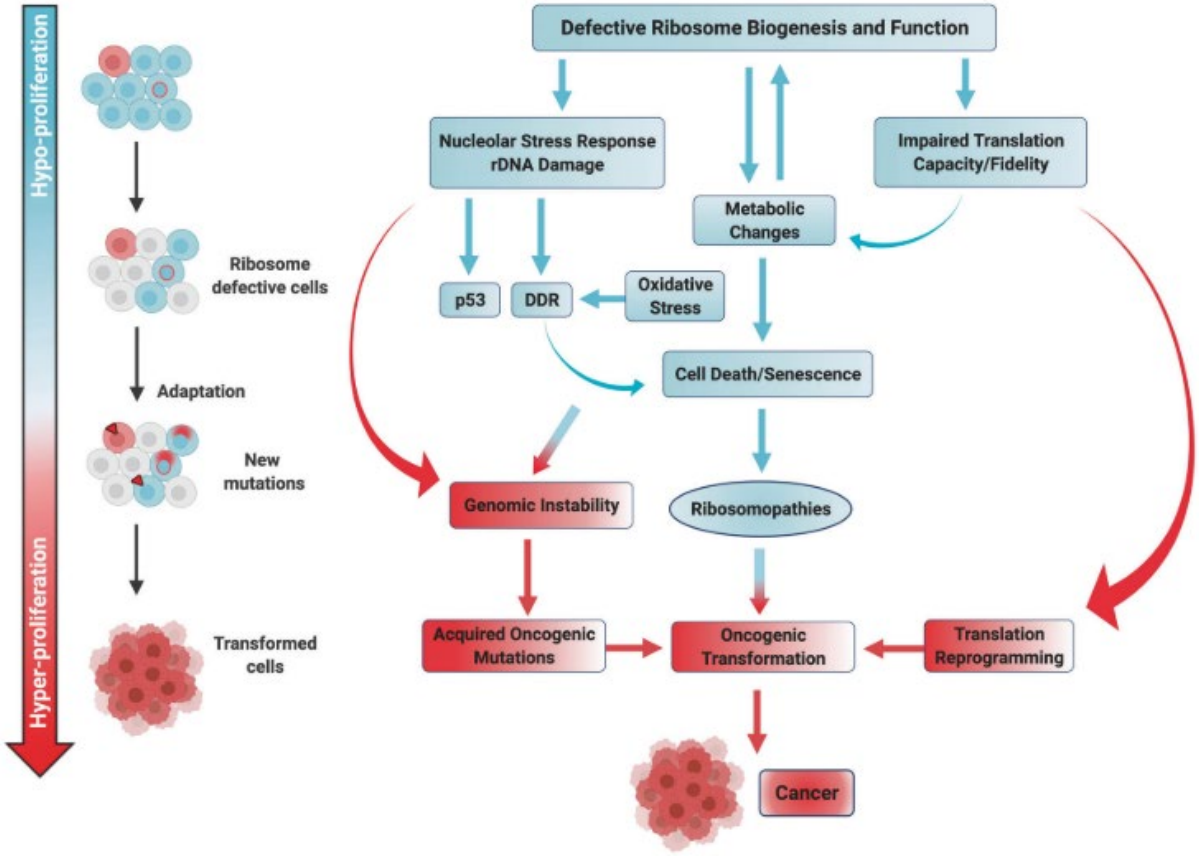

Figure 3 A model of the transition from a cellular hypo-proliferation to a hyper-proliferation state in ribosomopathies. Defective Ribosome biogenesis and function can activate the p53 dependent nucleolar stress response, but also the p53-independent DNA damage response (DDR). Defective ribosomes biogenesis and function can furthermore result in the emergence of specialized 'onco-ribosomes' which can lead to impaired translation capacity and fidelity. The regulation of cellular metabolism is also affected during ribosome defects. In turn, deregulation of metabolism leads to oxidative stress that further impairs ribosome biogenesis and ribosome function. All these stresses contribute to the hypo-proliferative cellular response including cell cycle arrest, senescence and apoptosis present in ribosomopathies. The high oxidative stress can induce DNA damage, resulting in higher genomic instability. This will increase the chance of the induction of secondary rescuing mutations. The impaired translational capacity can furthermore result in translational reprogramming to pro-survival mechanisms. These both underpin the transition from hypo-proliferation to hyper-proliferation phenotypes and cancer predisposition. Figure obtained from Kang et al. (2021) [38]

Chapter 4: Ribosome Biogenesis as an effective target for Cancer Therapies

Ribosome Biogenesis has recently emerged as a target for cancer therapy [5,6,63]. This is mainly because cancer cells produce more ribosomes, because of their great need for proteins and are thus more vulnerable to a reduction in RiBi than healthy cells. Several standard-of-care chemotherapeutics like actinomycin D, doxorubicin, camptothecin and 5-fluorouracil were shown to disrupt RiBi [76]. This shows that inhibition of this process may offer a general therapeutic strategy to block cancer cell proliferation. However, these drugs also induce a lot of other genotoxic effects, which make them not very selective. Therefore, many new drugs have been designed that selectively inhibit ribosomal biogenesis.

Ribosome biogenesis can be targeted in several ways, such as inhibiting RNA synthesis or inhibiting pre-rRNA processing. Furthermore, the function of ribosomes can also be targeted, especially the earlier described onco-ribosomes, which can have specific functions favourable for the cancer cell. In this chapter I will explain these different options and how RiBi or ribosome function can be targeted to inhibit cancer cell proliferation.

Targeting RiBi at the level of rRNA synthesis

rRNA is a critical component of the ribosome. Cancer cells show often an increase in the synthesis of rRNA. Several cancer therapeutics are reported to inhibit RNA synthesis, such as Actinomycin D. Here I will point out two promising drugs that target the function of the rRNA polymerase I, CX-5461 and BMH-21.

CX-5461 is a small molecule that selectively inhibits the transcription of rRNA by Pol I, but does not affect Pol II mRNA transcription or DNA synthesis. CX-5461 inhibits the initiation stage of rRNA synthesis by binding to G-quadruplex (G4) DNA motifs, abundant in rDNA. This prevents the binding of the SL1 transcription factor to the rDNA promotor, causing inhibition of the initiation of rRNA synthesis by Pol I [77]. This leads to senescence and autophagy, but not apoptosis in solid tumor cell lines. The cells don't go in apoptosis since a block in transcription initiation can be sensed as decreased nutrient availability, therefore activating autophagy instead of apoptosis. Autophagy is a process used by cancer cells to survive conditions of cellular stress, but an excess of autophagy can also lead to cell death [78]. This illustrates how CX-5461 can have potent and selective antitumor activity, by taking advantage of unique qualities of cancer cells and at the same time having a small effect on non-transformed cells. This small effect on non-transformed cells was also observed in in vitro studies, where CX-5461 was shown to inhibit a broad-spectrum of proliferative activity in cancer cells, but not affected the viability of non-transformed cells [77]. Furthermore, CX-5461 is demonstrated to produce antitumour responses against solid tumors in vivo. The selectivity of CX-5561 did not depend on the p53 mutational status of the cell, suggesting that CX-5461 induces autophagy in a p53-independent manner.

The effect of CX-5461 was also investigated in liquid cancers such as ALL. A study investigating the effect of CX-5461 on ALL cells, showed that CX-5461 specifically inhibits proliferation of ALL cells by inducing the caspase-dependent apoptosis pathway, in a p53 independent manner [79]. CX-5461 also activated the ATM/ATR pathway causing G2 phase cell cycle arrest. This arrest provides cells time to recover from the drug induced stress. Inhibition of ATR kinase functions by ATR inhibitors further enhanced the CX-5461 mediated apoptosis response and led to robust cell killing [79]. Taken together, CX-5461 represents a small molecule therapeutic agent that selectively targets Pol I transcription and induces autophagy and apoptosis in both solid and blood tumors. CX-5461 is currently in a phase I/II clinical trial for advanced breast cancer.

The second compound is BMH-21. BMH-21 is a potent anticancer small molecule that can inhibit Pol I transcription. It can bind to GC-rich sequences, which are present at a high frequency in rDNA genes [80]. The inhibition of rRNA synthesis by BMH-21 leads to segregation of nucleolar structures and altered localization of nucleolar proteins, such as UBF, NPM and nucleolin [80]. This can explain how BMH-21 can inhibit Pol I function, as these proteins are needed for transcription initiation. Furthermore, BMH-21 is shown to promote the degradation of RPA194, a protein of the LSU of Pol I, in an ubiquitin-proteasome-dependent manner [80]. This ability was independent of the *TP53* genetic status of the cancer cell line. However, degradation is not the primary event that leads to transcription inhibition, as RPA194 degradation was observed long after the rapid kinetics of Pol inhibition. The primary event leading to transcription inhibition likely includes the intercalation of BMH-21 with rDNA and the observed segregation of the nucleolus.

However, both BMH-21 and CX-5461 are not very selective for rDNA genes, as the DNA-motifs they bind are also present in other parts in the DNA. Furthermore, it appeared that both compounds have a selective toxicity towards cells with particular molecular profiles [5]. This makes it hard to predict their effects in an unknown molecular setting.

Targeting RiBi biogenesis at other levels than RNA synthesis

rRNA synthesis is only one of several steps of RiBi that represents a druggable target. Ribosome Biogenesis can also be inhibited by inhibition of pre-rRNA processing. Small nucleolar RNAs (snoRNAs) play a very important role in this process. Langhendries and colleagues have shown that knockdown of snoRNAs U3 and U8, which are important for the proper folding of pre-RNAs, result in a decrease in the tumorigenic potential of aggressive cancer cells [81]. They show that U3 or U8 depletion triggers the potent RP-MDM2-p53-dependent anti-tumor stress response, which leads to cell growth arrest and apoptosis.

In addition, RiBi can also be targeted at the level of ribosome assembly. The best inhibitor of this process is diazoborin, which inhibits the formation of the ribosomal LSU at a late stage in budding yeast [82]. Finally, anticancer drugs can be designed against chemical modifications of rRNAs, which also constitute a crucial step of RiBi and are shown to lead to the production of cancer ribosomes (see below).

Targeting the function of ribosomes

Besides inhibiting the formation of ribosomes, the function of ribosomes can also be targeted. As the primary role of ribosomes is to translate proteins, we could by targeting them modulate the translational capacity of cells, which is especially important in tumorigenesis. However, major challenges involving the specificity of this inhibition still exists, (i) How can we specifically target diseased cells and not healthy cells, (ii) Would this lead to a general decrease in translational activity or a specific subset of them? Despite these challenges, there are already several molecules in use to treat human diseases, including cancer [83].

Combination therapies are quite common in cancer treatments. Often this leads to less toxicity as the drugs can be used in lower concentrations. Combining inhibitors of ribosome biogenesis/function together with other anticancer treatments could prevent resistance and prolong the effects of individual drugs.

Targeting onco-ribosomes

As we have seen in chapter 3, ribosomes are heterogeneous particles that differ in their protein and RNA composition. We have seen that upon RP mutations, cancer cells can contain ribosomes with altered composition which can be beneficial for cancer progression, also referred to as onco-ribosomes. In addition, ribosome heterogeneity increases due to rRNA modifications, such as 2'-O-methylation (2'-O-Me) which is most abundant. It appears that onco-ribosomes display altered rRNA 2'-O-Me patterns, compared to their healthy counterparts. This makes it possible to develop specific inhibitors that target onco-ribosomes specifically, thereby killing only cancer cells without affecting healthy cells.

Chapter 5: eEF2K and its Connection to Cancer

In the previous chapter we have shown that the ribosome biogenesis pathway could be a potential druggable target for cancer therapies. Different steps in this pathway can be targeted, including inhibition of RNA synthesis, inhibition of pre-rRNA processing and inhibition of the function of ribosomes. The potential of targeting the ribosome biogenesis pathway in the context of TP53 deleted ALL has yet to be investigated.

To identify the Achilles Heel of TP53 deleted ALL cells, a very recent study has employed a CRISPR-based screen in TP53 knockout ALL cells, under conditions of cellular stress (unpublished data, van Leeuwen lab). This screen identified the eukaryotic elongation factor 2 kinase (eEF2K) as a potential target. eEF2K is an unusual protein kinase that acts as a negative regulator of transcription elongation [24]. It is activated upon many different stresses, including nutrient deprivation, ER stress, DNA damage and ribosomal stress [25,26]. It is particularly interesting that eEF2K can be activated upon ribosomal stress, which demonstrates a link between impaired ribosome biogenesis and transcription regulation. This suggests that the ribosomal stress pathway could represent a targetable vulnerability in TP53 deficient ALL cells.

In this chapter I will introduce eEF2K and describe its regulation. Furthermore, I will explain the contribution of eEF2K in cancer survival and progression, as overexpression of eEF2K is seen in different cancer types and is often associated with poor survival in patients. In addition, I will demonstrate eEF2K as a possible novel molecular target for cancer treatment. A schematic representation of the regulation of eEF2K and its contribution to cancer survival is visible in Figure 4.

Introduction eEF2K

eEF2K is a very unusual protein kinase, belonging to a small family of six known genes in the human genome, known as 'a-kinases' [24]. Their name is based on their preference to phosphorylate residues in a-helices [31]. a-kinases have very low sequence similarity with other kinases and are therefore structurally very different from main kinase super-families. eEF2K activity is mainly regulated by calmodulin (CaM) and its associated Ca2+ ions [24]. eEF2K can regulate protein elongation by phosphorylation of its only known substrate eEF2. eEF2 is a protein that helps ribosomes move along the mRNA during the elongation stage of protein synthesis, and its phosphorylation impairs its association with the ribosome, leading to elongation inhibition.

Translation elongation is a highly energy demanding process. To modulate the cellular energy demand and maintain cellular homeostasis, translation elongation and thus eEF2K activation, is negatively regulated by numerous cellular stresses, including nutrient deprivation, hypoxia, ER stress, DNA damage and ribosomal stress [30]. These stresses modulate several key signalling pathways such as mTORC1-, ERK- and AMPK-signaling, to affect eEF2K function [24,27]. This ensures the cell to regulate and modulate transcription elongation when the cell is under unfavourable conditions. For instance, under low energy levels, the AMP-activated kinase (AMPK), will inhibit transcription elongation by positively regulating eEF2K activity [24,31]. AMPK senses when cellular energy levels drop (low ATP levels) and phosphorylates a range of substrates to reduce energy demand and increase energy supply. It is therefore not surprising that AMPK will activate eEF2K, and thus inhibit transcription elongation. AMPK can regulate eEF2K function through directly phosphorylating it, but it likely also involves the inhibition of mTORC1 signaling, which is a negative regulator of eEF2K. mTORC1 is a key cellular sensor of the nutrient or energy status of the cell that can inhibit eEF2K function when the energy status of the cell is favourable. mTORC1 can be stimulated by hormones, growth factors and nutrients and inhibits eEF2K activity by phosphorylating eEF2K at a residue close to the CaM-binding motif [27]. This impairs CaM binding and results in inhibition of eEF2K. As

consequence, mTORC1 thus stimulates transcription elongation when conditions are favourable. The RAS/RAF/ERK pathway also negatively regulates eEF2K, by directly phosphorylating eEF2K at residue Ser359 or via p90RSK at Ser366 [24].

Another stress that can inhibit translation elongation is ribosomal stress. A recent study has shown that ribosomal stress can activate eEF2K, thereby inhibiting translation elongation [25]. This leads to a decrease in protein synthesis. This was observed in both p53-positive and p53-negative cells, suggesting that this phenomenon does not appear to directly correlate to the RP-MDM2-P53 pathway. This is a very important finding, as this demonstrates a link between ribosomal stress and eEF2K activity, even in TP53 deficient cells.

Furthermore, the same paper showed that, besides downregulating general protein synthesis, ribosomal stress can specifically increase the translation of specific mRNAs [25]. This includes mRNAs with a terminal oligopyrimidine (TOP) mRNA, which often encode ribosomal and translational factors. Upon ribosomal stress, TOP mRNAs were shown to be more associated with polysomes, both in p53-positive and p53-negative cells. Thus, ribosomal stress can in this way lead to a decrease in general protein synthesis, but increase in the translation of proteins needed to bring ribosome numbers to higher levels. This is a very clever mechanisms allowing cells to adequately respond to low ribosome levels. However, how ribosomal stress can activate eEF2K is still unknown. The writers of this paper suggest a possible role of the protein kinase PIM1, which we earlier described to interact with ribosomes and is destabilized upon ribosomal stress [13]. Whether this hypothesis is true remains to be investigated.

eEF2K promotes cancer survival and progression

Impairments in the regulation of eEF2K are known to contribute to cancer development. Overexpression of eEF2K is seen in different kinds of cancer and is often associated with poor survival in patients [28,29]. Although it seems paradoxical that highly proliferating cells show high expression of a negative regulator of protein synthesis, there are multiple possible mechanisms described how eEF2K may promote cancer survival and tumor development [28,30,31]. In this section some of these mechanisms will be discussed.

eEF2K protects cancer cells from cell death caused by nutrient deprivation

Several cancer types are known to be very sensitive to nutrient deprivation (ND) [84]. Anabolic processes typically driven by oncogenic pathways may have deleterious effects on the cell when nutrients are scarce. Therefore, nutrient deprivation is used as an cancer therapy in several cancer types including, breast cancer, glioma and T-ALL [85]. A paper by Leprivier et al., demonstrated that eEF2K, by inhibiting translation elongation, could protect cancer cells from cell death caused by low nutrient levels [28]. Inhibition of eEF2K was shown to induce cell death in triple-negative breast cancer, which is normally highly resistant to therapy [30,32]. eEF2K inhibition could thus render cells sensitive to nutrient starvation. In the context of ALL, we know that lymphocytes are particularly vulnerable to amino acid depletion therapies, such as asparaginase (ASNase) treatment [86,87]. Although this is not an essential amino-acid, lymphocytes are insufficiently able to synthesize this amino-acid themselves, making them particularly vulnerable against such therapies. This could explain why these cells are also vulnerable to loss of eEF2K.

eEF2K contributes to increased survival against chemotherapeutic drugs

In addition, several papers show increased cell sensitivity against chemotherapeutic drugs upon eEF2K inhibition. In triple-negative breast cancer xenografts in mice, silencing of eEF2K sensitized the cells to doxorubicin [32]. Similarly, inhibition of eEF2K by siRNA in pancreatic cancer cells, led to increased responsiveness to gemcitabine. In addition, inhibition of eEF2K in glioma cells enhanced the cytotoxicity of the AKT inhibitor MK-2206 [33]. This indicates that eEF2K somehow increases cell survival against these drugs.

The mechanisms for this phenomenon are, however, not known, but hypothesis include the contribution of eEF2k in controlling apoptotic signalling processes [88].

eEF2K promotes cell survival by alteration of synthesis of pro-survival proteins

Modulating protein elongation does not inhibit the synthesis of every protein in the same manner. As we have seen in chapter 3, ribosomes are heterogenous molecules and inhibition of transcription elongation can therefore result in a differential effect on specific proteins. Cancer cells can exploit this, leading to increased synthesis of specific cell survival-related proteins. In pancreatic cancer cells, eEF2K can modulate the expression of pro-survival protein transglutaminase (TG2). Inhibition of eEF2K leads to downregulation of TG2 resulting in caspase-dependent apoptosis through the induction of apoptosis inducting factor (AIF) [89]. In addition, RNA-mediated depletion of eEF2K in breast cancer cells in vivo, led to an increase in pro-oncogenic proteins, such as c-Myc and cyclin D. Furthermore, siRNA-mediated depletion of eEF2K in glioma cells has been shown to sensitize cells to the TNF-related apoptosis inducing ligand (TRAIL), accompanied by overexpression of pro-apoptotic Bcl-xL and downregulation of anti-apoptotic Bcl-2 [90]. To point out the importance of eEF2K, inhibition of eEF2K is associated with increased apoptotic cell death in different cancer types, including glioma, breast cancer and pancreatic cancer cells.

eEF2K can aid cell migration and invasion

Several reports suggest a role for eEF2K in aiding cell migration and invasion. Overexpression of eEF2K is associated with aggressive tumours and poor patient survival, possibly induced by increased cell proliferation, motility and invasion. In breast cancer cells, inhibition of eEF2K by miR603 and miR-877 decreased the expression of pro-invasion proteins Src/FAK, cyclin D1 and Akt [91]. The mechanisms how eEF2K can promote cancer cell migration remains unclear. Xie et al., suggested a role for integrins. They demonstrated that eEF2K can upregulate the expression of cell migration and invasion-related proteins, by upregulation of some integrins [92]. The importance of eEF2K in cell migration is emphasized by studies showing that inhibition of eEF2K drastically decreased the cell migration and invasion of lung, breast, pancreatic and glioma cancer cells [32,91].

eEF2K positively regulates autophagy

Finally, a number of studies show a role of eEF2K in the regulation and activation of autophagy [33,88]. Autophagy is an important process involved in the recycling of cellular components. It can respond to several stresses, such as ND and growth factor deprivation, which represents that this process is important for cancer cell survival. eEF2K and autophagy are both negatively regulated by mTORC1. Under stress conditions, mTORC1 is inhibited and loses its inhibitory effects on eEF2K, leading to increased autophagy. Furthermore, eEF2K is known to regulate the expression of major autophagy genes including BECN1 and autophagy related 7 (ATG7). However, in colon cancer cells, eEF2K was also shown to suppress autophagy [93]. Thus, the effects of eEF2K on autophagy are not always the same and are dependent on the cellular context.

Evidence that eEF2K can impede tumorigenesis

Although there is a lot of evidence that activation of eEF2K can promote cancer survival, there are also some papers that report that eEF2K can inhibit tumor progression. Faller et al., demonstrated that mTORC1-mediated inhibition of eEF2K is required for the proliferation of their APC-deficient cells, a colorectal cancer model. They also show that rapamycin treatment of APC-deficient adenomas can result in tumour cell growth arrest and differentiation [94].

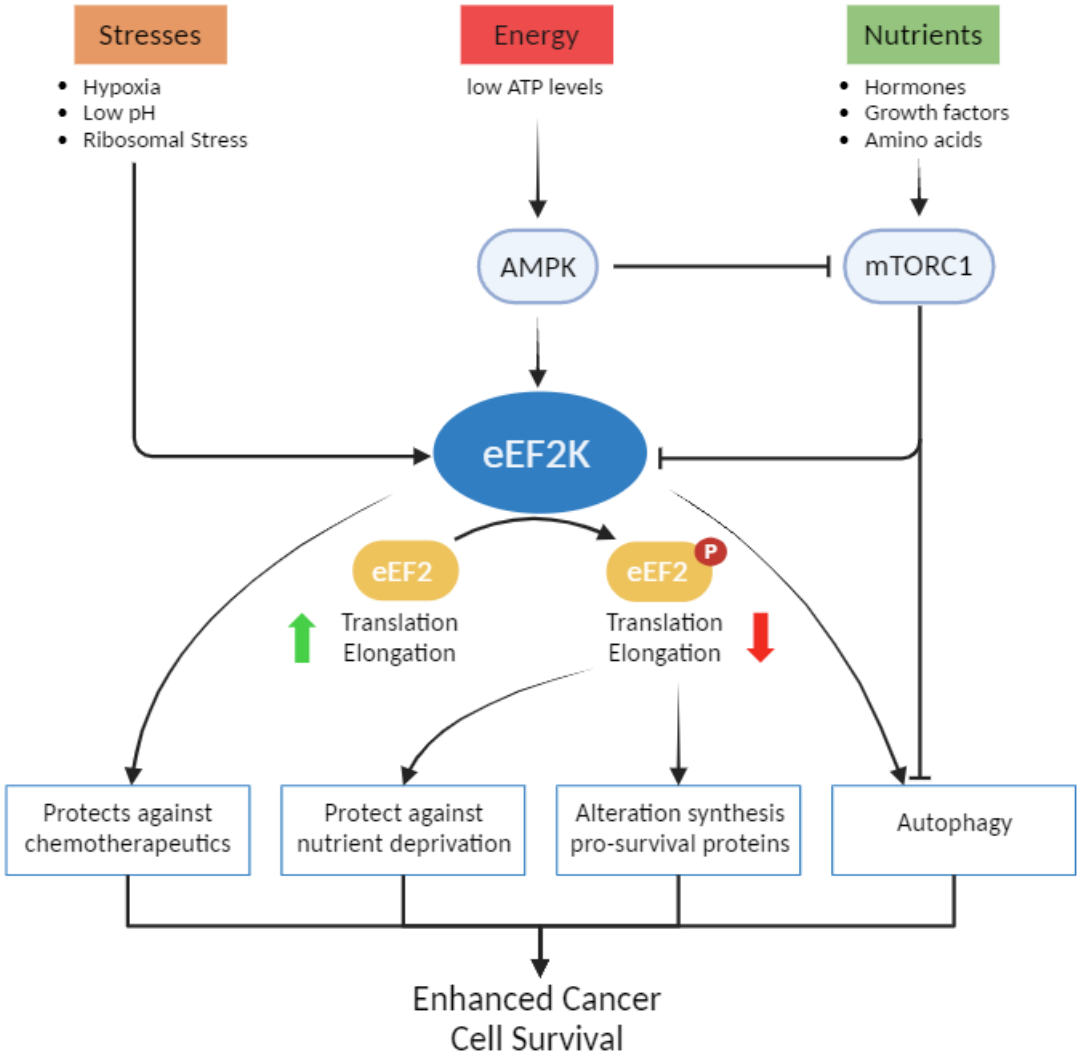

Figure 4 Regulatory networks of eEF2K and the consequences of eEF2K overexpression in cancer development. Highly simplified figure showing how different stresses, energy levels and nutrients can regulate eEF2K activity. Active eEF2K will phosphorylate its target eEF2K, which will result in inhibition of translation elongation. This inhibition can result in the protection of cells against nutrient deprivation and alteration in the synthesis of pro-survival proteins. Furthermore, overexpressed eEF2K can promote cancer cell survival by protecting against chemotherapeutics and by inducing autophagy. Image is created with BioRender.com.

eEF2K as a novel molecular drug target for cancer treatment

eEF2K is under investigation as a possible novel molecular drug target for cancer treatment. Several studies have shown that knockdown of eEF2K slowed tumour growth [28,32]. As earlier described, inhibition of eEF2K can sensitize cells to conventional chemotherapeutics. eEF2K inhibitors could therefore be used in combination therapies together with conventional chemotherapeutics to effectively kill cancer cells. However, effective inhibitors for eEF2K are still in development.

Inhibitors of eEF2K

Classical protein kinase inhibitors cannot inhibit eEF2K, because α-kinases show very low sequence homology with other protein kinase families [24]. On one hand, this makes it harder to develop newly specific compounds that can inhibit eEF2K. On the other hand, a specific inhibitor for eEF2K likely won't have large off-target effects. There is still lack of information regarding the three-dimensional structure of eEF2K, which complicates the development of novel effective inhibitors of eEF2K. Some inhibitors of eEF2K have

been synthesized, however, none of these show therapeutic potential. NH125, which was first reported as a histidine kinase inhibitor, was later also reported to inhibit eEF2K [95]. However, further work revealed that NH125 is not very specific and can also lead to the phosphorylation of eEF2 [96]. Currently, A-484954 is reported as the most specific and promising inhibitor of eEF2K. However, high micromolar concentrations of A-484954 are needed to inhibit eEF2K in cells [95]. These findings indicate that better inhibitors of eEF2K are needed for clinical applications.

Another possibility to inhibit eEF2K activation is by increasing its degradation. Several drugs are currently on the market that instead of inhibiting its target, increasing its association with the degradative pathway [97]. It remains to be shown whether this would be an effective option to target eEF2K.

Discussion

In this review I have explored the ribosomal stress pathway as a potential targetable vulnerability in p53 aberrant ALL in order to provide a mechanistic explanation for the observed synthetic lethality between loss of TP53 function and loss of eEF2K in ALL under conditions of cellular stress. For this I reviewed different p53-(in)dependent ribosomal stress signalling pathways, looked at how aberrations in RiBi and these pathways can be linked to cancer development, and how these pathways can be used as a target for the development of new and improved anticancer therapies. Finally, I examined the role of eEF2K, its connection to ribosomal stress and its contribution to cancer progression.

First, I explained that RiBi is an essential process that involves the coordination of numerous events and molecular players. To maintain genomic and cellular homeostasis the cell has developed multiple ribosomal stress pathways that monitor aberrant ribosome production. Most pathways sense trough p53, but pathways that signal independently of p53 are also observed. This suggests that even in a p53-deficient environment, ribosomal stress can lead to senescence and apoptosis.

Both overexpression and impaired RiBi can contribute to cancer development. Overexpressed RiBi is observed in many different cancer types [8]. Several oncogenic signaling processes and tumor suppressors can control ribosome synthesis and function. Inactivation of tumor suppressors and/or activation mutations in onco-genes leads to the hyper-activation of RiBi in order to facilitate cancer cell proliferation. Next to promoting cancer cell growth, excessive RiBi can also regulate transcriptional programs or increase the proteotoxic stress thereby supporting malignant transformation. However, these oncogenes and tumor suppressors also affect numerous other processes, which makes the contribution of excessive RiBi in cancer initiation difficult to assess.

Besides excessive RiBi, Impaired RiBi is also associated with cancer-predisposition. Mutations in RP genes or other RiBi factors can give rise to a broad spectrum of developmental disorders known as ribosomopathies. These are diseases characterized by hypo-proliferative phenotypes. In addition, ribosomopathies also predispose to cancer development, which is confirmed by the high percentage of somatic mutations affecting RP genes in human sporadic cancers. Interestingly, RPL5 and RPL10 mutations are suggested to play a driver role in the disease progression of T-ALL. The RPL10 R98S mutation is accountable for around ~8% of paediatric T-ALL patients, demonstrating its importance in disease initiation.

Different mechanisms have been proposed to explain how impairments in RiBi can contribute to tumorigenesis. In addition to negatively impacting ribosome assembly, RP mutations can also increase ribosome diversity, leading to ribosomes with altered functions. The RPL10 R98S mutation in T-ALL can alter mRNA translation by increasing the IRES-dependent translation of the anti-apoptotic factor BCL-2. It can furthermore reduce the -1 PRF signalling, resulting in the increase of JAK-STAT protein synthesis. Both these translational changes can influence cellular proliferation and survival. Moreover, RPs can have extra-ribosomal functions. For example, RPL5 and RPL11 regulate the expression of TP53 and c-Myc, thereby influencing cell cycle progression. Mutations in these RPs can therefore result in dysregulated expression of TP53 and c-Myc, leading to elevated risks of cancer development. At last, defective ribosomes can also have a more indirect effect to tumorigenesis. This includes deregulating the activity of the proteasome, affecting protein stability, which further contributes to the oncogenic potential of the cell.

Effective and selective targeting of RiBi is a promising cancer therapy strategy, particularly for overcoming chemoresistance to standard-of-care treatments. RiBi is mostly targeted at the level of rRNA synthesis. Recently, two specific RNA polymerase I inhibitors were developed to inhibit RiBi. One of these molecules, CX-5641 was shown to inhibit proliferation of ALL cells, by inducing the caspase-dependent apoptosis

pathway, in a p53-independent manner. This suggests that this drug will also inhibit RiBi in our situation. It would therefore be interesting to see whether CX-5461 also works as an effective cancer treatment in p53-deficient ALL. Another drug, BMH-21 was also identified as an effective inhibitor of RNA polymerase by inducing the segregation of nucleolar structures and alteration of the localization of nucleolar proteins [80]. However, both compounds bind to DNA motifs which are, besides abundant in rRNA genes also present in other parts of the DNA, which reduces their selectivity.

Ribosome biogenesis can also be targeted at other levels than RNA synthesis, such as pre-rRNA processing or ribosome assembly. In addition, the function of ribosomes can be targeted. However, this is challenging, as the drugs need to specifically target diseased cells but not healthy cells. Furthermore, it is not known whether targeting ribosome function would lead to a general decrease in transcriptional activity or only a specific subset. Different steps of RiBi can thus be targeted and could work as a cancer therapy. The potential of targeting the ribosomal biogenesis pathway in the context of TP53 deleted ALL has yet to be investigated.

Interestingly, p53-deficient ALL cells appear to be vulnerable to eEF2K loss. eEF2K is known to promote tumorigenesis by several mechanisms, and inhibition of eEF2K has shown to be an effective cancer treatment, especially to overcome resistance against conventional chemotherapeutics [32]. This suggests indeed that p53-deficient ALL cells, which are frequently resistant to conventional chemotherapeutics, may be responsive to eEF2K inhibition. However, efficient inhibitors of eEF2K have yet to be developed. The fact that eEF2K can be activated upon ribosomal stress even in a p53-deficient environment, confirms the link between RiBi and translational control, which further suggests that the ribosomal stress pathway could be a targetable vulnerability in TP53 deficient ALL cells.

For the future it would be interesting to look at the effectiveness of targeting the ribosomal biogenesis pathway in p53-deficient ALL cells. Initial experiments could use CX-5461 to see whether these cells are vulnerable to inhibition of rRNA synthesis. Also, to increase the potential of targeting eEF2K, better inhibitors of eEF2K have to be developed. As information of the three-dimensional structure of eEF2K is still missing, developing specific inhibitors is challenging. Therefore, looking into degraders, that could enhance eEF2K degradation may represent a viable alternative.

In conclusion, TP53-deficient ALL cells are vulnerable to loss of eEF2K, as this protein can protect the cells against nutrient deprivation, where some ALL therapies, such as ASNase treatment, are aimed at inducing nutrient stress and apoptosis induction. Furthermore, inhibition of eEF2K can sensitize cells to conventional therapeutics even in a p53-deficient environment. As ribosomal stress can activate eEF2K, targeting RiBi may represent a targetable vulnerability in p53 aberrant ALL. However, further research is needed to provide insights into the mechanisms by which this synthetic lethality arises.

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