Review

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Synthesis of the ribosomal RNA precursor in human cells: mechanisms, factors and regulation

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Abstract: The ribosomal RNA precursor (pre-rRNA) comprises three of the four ribosomal RNAs and is synthesized by RNA polymerase (Pol) I. Here, we describe the mechanisms of Pol I transcription in human cells with a focus on recent insights gained from structure-function analyses. The comparison of Pol I-specific structural and functional features with those of other Pols and with the excessively studied yeast system distinguishes organism-specific from general traits. We explain the organization of the genomic rDNA loci in human cells, describe the Pol I transcription cycle regarding structural changes in the enzyme and the roles of human Pol I subunits, and depict human rDNA transcription factors and their function on a mechanistic level. We disentangle information gained by direct investigation from what had apparently been deduced from studies of the yeast enzymes. Finally, we provide information about how Pol I mutations may contribute to developmental diseases, and why Pol I is a target for new cancer treatment strategies, since increased rRNA synthesis was correlated with rapidly expanding cell populations.

Keywords: human transcription; ribosomal RNA precursor; RNA polymerase I; structural biochemistry

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1 Introduction

The central dogma of molecular biology includes the flow of genetic information from DNA over RNA to complete proteins (Crick 1970). Essential to this process, transcription from DNA to RNA is enabled by DNA-dependent RNA polymerases (Pols) and translation from RNA to proteins is performed by ribosomes with the help of aminoacylated tRNAs. In eukaryotes, at least three specific nuclear RNA polymerases have evolved which are highly conserved throughout evolution (Roeder and Rutter 1969; Werner and Grohmann 2011).

Among the three Pols, overall structure and function is conserved as well as the steps of the transcription cycle, but transcription associated factors vary, thus contributing to enzyme specificity and specialization (Engel et al. 2018; Girbig et al. 2022; Vannini and Cramer 2012). Pol II decodes protein-coding genes to mRNA and produces many small regulatory RNAs. tRNAs, 5S rRNA and other long non-coding RNAs such as U6 RNA or 7SK RNA are produced by Pol III, the largest of the three Pols (Dieci et al. 2007). Unlike the other polymerases, Pol I transcribes only one single gene, the rRNA precursor gene (Goodfellow and Zomerdijk 2013; Moss et al. 2007; Pitts and Laiho 2022). In mammals, the resulting 47S (or referred to as 45S) pre-rRNA is processed in the three rRNAs 28S, 18S, and 5.8S, which, together with the 5S rRNA and ribosomal proteins (r-proteins), form the ribosome, the cellular machinery responsible for translation of proteins (Klinge and Woolford 2019). The amount of ribosomes is a critical determinant of cell growth (Chaillou et al. 2014) and rRNA transcription by Pol I is a key regulatory step during ribosome biogenesis (Grummt 1999; Reeder 1999). Thus, Pol I transcription must be tightly regulated and responds to the growth status of the cell as well as to nutrient availability (Goodfellow and Zomerdijk 2013). In humans, Pol I transcripts accounts for about 80 % of total cellular RNA (Palazzo and Lee 2015), which gives rise to up to 10 million ribosomes within one cell (Wolf and Schlessinger 1977). Furthermore, considering the close link between Pol I transcription and cellular growth, it's not surprising that many protooncogenes and tumor suppressors are involved in the regulation of this process (Moss et al. 2007; Pitts and Laiho 2022)

and deregulation is observed in many different types of cancer and a range of severe developmental and neurological disorders. This review aims to bring together mechanistic insights of human Pol I on a structural and functional level and tries to highlight some similarities and differences between human and yeast Pol I.

2 The loci of Pol I transcription – ribosomal DNA organization

Pol I transcription, pre-rRNA processing and the first steps of ribosome biogenesis take all place within a distinct subcompartment of the nucleus, the nucleolus. The mammalian nucleus can harbor more than one nucleolus which originate from different nucleolar organizer regions (NORs) (McStay 2016; Schöfer and Weipoltshammer 2018). In humans, five NORs exist in the haploid genome, located at the acrocentric chromosomes 13, 14, 15, 21, and 22, all together containing 200–300 ribosomal genes (Henderson et al. 1972) (see Figure 1A). Each NOR consists of many ribosomal (r)DNA gene repeats mainly in head-to-tail orientation and has the potential to form a nucleolus. While it was observed that all NORs in non-transformed human cell lines are active, there is evidence that individual NORs might be transcriptionally silent in cancer cell lines (Roussel et al. 1996). Ultrastructural analysis of active nucleoli revealed nucleolar sub-compartments. Fibrillar centres and associated dense fibrillar centres are the location of pre-rRNA synthesis and co-transcriptional rRNA processing, whereas further steps of ribosomal subunit maturation occur in granular centres (Scheer et al. 1993; Schöfer and Weipoltshammer 2018). The 5S rRNA gene array transcribed by Pol III is located on chromosome 1 and not physically connected to the NORs in human cells but may associate with

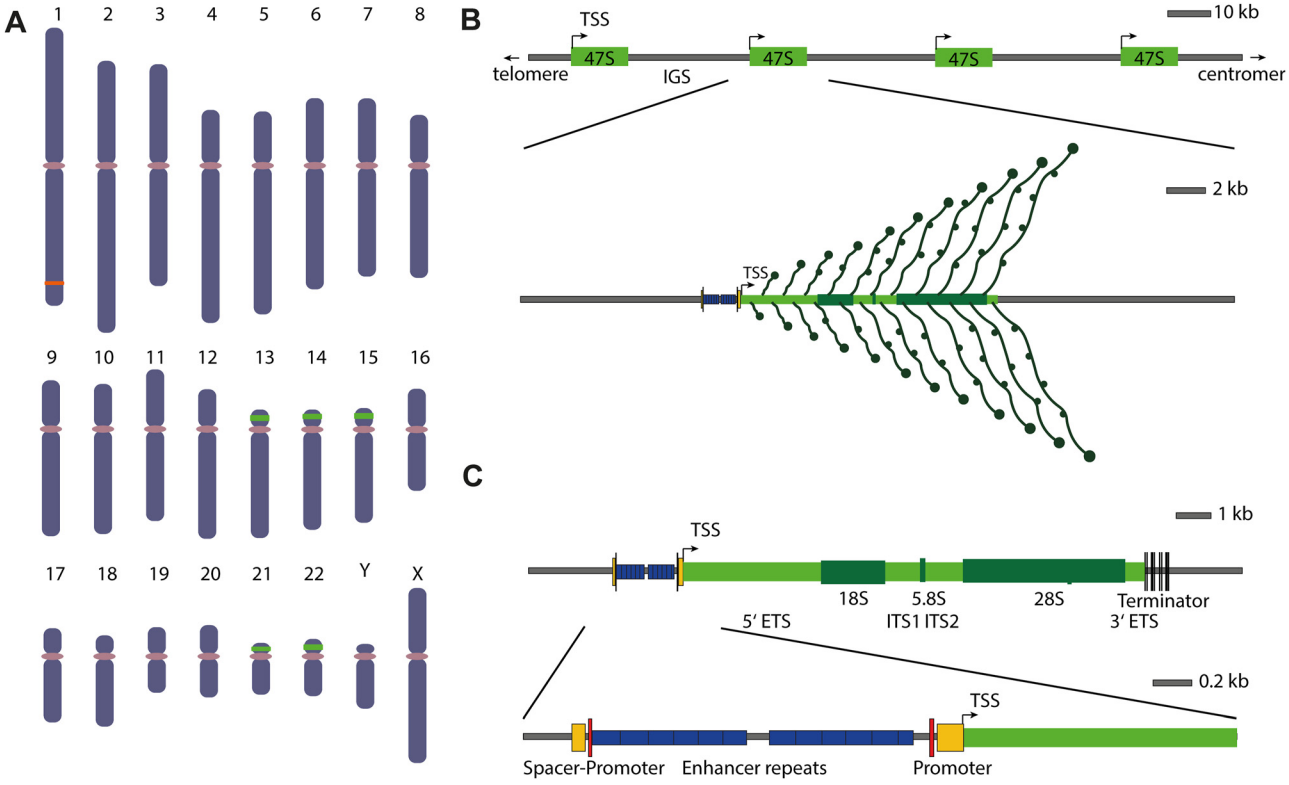

Figure 1: The genomic rDNA locus in human cells. (A) Schematic representation of human chromosomes with centromeres (labeled in pink). The genomic loci of 47S rDNA repeats (labeled in green) and of 5S rDNA repeats (labeled in orange) are shown. (B) Zoom-in into a genomic rDNA locus: on top visualization of the 47S pre-rRNA gene with the transcription start site (TSS; labeled with an arrow) and the intergenic spacer region (IGS) in relation to telomere and centromere. Bottom: Schematic representation of a 'Miller chromatin spread' depicting an actively transcribed gene. The rDNA gene with IGS (grey), enhancer repeats (blue), spacer and 47S rDNA promoters (yellow), and the transcribed region (green) are outlined. Elongating Pol I molecules transcribing the 47S rDNA sequence synthesize the pre-rRNA (fibrils extending from the transcribed region). Ball-like structures represent co-transcriptional pre-ribosomal assembly intermediates. (C) The transcribed region (green) is composed of the 5′ external transcribed spacer (ETS), 18S rRNA (dark green), internal transcribed spacer (ITS) 1, 5.8S rRNA (dark green), ITS2, 28S rRNA (dark green) and 3′ ETS. Binding sites for the transcription termination factor 1 (TTF-I) downstream of the spacer promoter or upstream of the 47S rDNA promoter, and at the 3′ end of the 47S rRNA gene are shown in red. Enhancer repeats (blue) are located in between the two promoter regions (yellow). A scale bar for DNA length is shown on the right top corner of each schematic representation.

nucleoli (Matera et al. 1995; Németh et al. 2010). 47S pre-rRNA processing includes co- and posttranscriptional endo- and exonucleolytic cleavage and assembly with ribosome biogenesis factors and r-proteins. A majority of the multiple steps leading to mature ribosomes take place in the nucleolus, in the nucleus, and lastly in the cytoplasm (Klinge and Woolford 2019). Mature ribosomes consist of the 60S large subunit comprising 28S, 5.8S and 5S rRNAs as well as around 50 r-proteins and the 40S small subunit including 18S rRNA and about 33 r-proteins, which form the 4 MDa cellular protein-producing machinery.

Pol I transcription from active rDNA genes is highly processive, leading to a dense packing of Pol I enzymes in the transcribed rRNA gene region. This has been observed for many organisms by electron microscopy of so-called 'Miller chromatin spreads' (Miller and Beatty 1969; Miller and Bakken 1972). In these structures, also referred to as 'Christmas trees', the rDNA bound by many elongating Pol I enzymes forms the stem of the tree, while the synthesized pre-rRNA extends from the stem forming the branches with terminal ball-like structures of pre-rRNA assembly intermediates (Neyer et al. 2016; Osheim et al. 2004) (Figure 1B). The mammalian rDNA gene consists of a spacer promoter with a binding site for a transcription termination factor (TTF-I) followed by enhancer repeats, another TTF-I binding site, the actual promoter with the transcription start site (TSS), the transcribed 47S pre-rRNA coding sequence followed by several TTF-I binding sites, and an intergenic spacer (IGS) spanning the region between the genes (Potapova and Gerton 2019). The transcribed region is composed of the 5′ external transcribed spacer (ETS), 18S rRNA, internal transcribed spacer (ITS) 1, 5.8S rRNA, ITS2, 28S rRNA and 3′ ETS (Figure 1C).

Pol I recognizes only one specific promoter which is composed of a core promoter located around the transcription start site (TSS) (around −45 to +20) and the upstream control element (UCE) or upstream promoter element (UPE) (around −234 to −107) (Jones et al. 1988). The DNA sequence of the promoter region is poorly conserved among species which may explain the species specificity of transcription initiation factors of the Pol I transcription system (Moss et al. 2007). Instead, DNA-structure rather than sequence has been suggested to be a common feature of Pol I promoters including a conserved DNA bendability and meltability which might assist transcription initiation (Engel et al. 2017).

3 Pol I structure and subunit function

Pol I itself is a large protein complex of about 600 kDa, composed of 14 subunits in yeast and 13 in metazoans and most other species (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). In human cells, only ~10 % of Pol I extracted from the nucleus is in an initiation-competent state (Pol Iβ), whereas remaining Pol I (Pol Iα) represents elongating Pol I which is not active in promoter-dependent *in vitro* transcription (Miller et al. 2001). One obvious difference between the two states is the stable association of the conserved initiation factor RRN3 to Pol Iβ which is required to stabilize the initiation competent form of the enzyme. The two different mammalian Pol I states resemble the two Pol I containing fractions derived from cell extracts of the yeast *Saccharomyces cerevisiae* (from here on called yeast) (Bier et al. 2004; Milkereit and Tschochner 1998).

With a range of purification strategies available (Engel 2016; Moreno-Morcillo et al. 2014; Pilsl et al. 2016) and a growing toolkit of cryo-EM sample preparation and screening techniques (Amann et al. 2023; Pilsl et al. 2022) in development, cryo-EM structures of yeast Pol I in different functional states were determined (Pilsl and Engel 2022), soon followed by cryo-EM reconstructions of hPol I. Recently, structures of the human enzyme in different functional states were obtained which help to distinguish conserved from organism-specific features (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). While we reported the structure of monomeric hPol I (Daiß et al. 2022), Misiaszek et al. determined an elongation complex (EC) reconstruction in a post-translocated state, an open complex (hPol I bound to a transcription bubble without RNA) and a co-structure with initiation factor RRN3 (Misiaszek et al. 2021). Zhao et al. report reconstructions of elongating polymerase in pre-translocated, post-translocated and backtracked states (Zhao et al. 2021).

3.1 Overall architecture

The overall structure of human and yeast Pol I is conserved and resembles the bi-modular architecture of Pol II with a DNA-binding, central cleft, 'core' and 'shelf' modules (Cramer et al. 2000; Cramer et al. 2001) and two additional protruding subcomplexes, the RPA49/34 heterodimer on the core module and the stalk at the shelf module (Daiß et al. 2022; Engel et al. 2013; Fernández-Tornero et al. 2013; Misiaszek et al. 2021; Zhao et al. 2021). The core polymerase is composed of the five common subunits shared between all three polymerases, RPABC1, RPABC2, RPABC3, RPABC4, and RPABC5, of two subunits shared with Pol III, RPAC1, and RPAC2, as well as the Pol I-specific subunits RPA1, RPA2, and RPA12. RPA12 binds to the RPA2 lobe-structure and interacts and anchors the two other Pol I-specific lobe-binding subunits RPA49 and RPA34. The stalk is constructed by the Pol I-specific subunit RPA43 and in yeast additionally by A14

Table 1: Nomenclature and function of Pol I subunits and associated factors in yeast and human.

S. cerevisiae Pol I	*H. sapiens* Pol I	*H. sapiens* Pol II	*H. sapiens* Pol III	Function
A190	RPA1	RPB1	RPC1	Active center
A135	RPA2	RPB2	RPC2	Active center
AC40	RPAC1	RPB3	RPAC1	
AC19	RPAC2	RPB11	RPAC2	
Rpb5	RPABC1	RPABC1	RPABC1	
Rpb6	RPABC2	RPABC2	RPABC2	
Rpb8	RPABC3	RPABC3	RPABC3	
Rpb12	RPABC4	RPABC4	RPABC4	
Rpb10	RPABC5	RPABC5	RPABC5	
A12.2	RPA12	RPB9 & TFIIS	RPC10	RNA cleavage, proofreading, termination
A49	RPA49	TFIIFα TFIIEβ	RPC5	Initiation, elongation
A34.5	RPA34	TFIIFβ	RPC4	Initiation, elongation
A43	RPA43	RPB7	RPC8	Open complex formation, elongation, termination
A14		RPB4	RPC9	Open complex formation, elongation, termination
			RPC3	Open complex stabilization
			RPC6	Open complex stabilization
			RPC7	Open complex stabilization

(Engel et al. 2013; Fernández-Tornero et al. 2013; Heiss et al. 2021) (see Table 1).

The Pol I cleft (Figure 2, green), into which DNA is loaded, is localized in between the clamp (Figure 2, pink) and protrusion domains on the upstream edge (Figure 2, dark blue), and between lobe (Figure 2, blue) and jaw domains (Figure 2, light blue) on the downstream edge. Next to the clamp, the stalk (Figure 2, slate) is bound to the core polymerase. In yeast Pol I, clamp, stalk and shelf module build a rigid mobile element, in which the shelf module consists of the cleft and foot domain of A190 as well as Rpb5 and Rpb6 which are homologs to RPA1, RPABC1 and RPABC2, respectively (Fernández-Tornero 2018). Protrusion, lobe, and jaw on the other side of the cleft are ordered from the upstream to the downstream edge of the enzyme. Subunit RPA12 (Figure 2, dark orange) and the heterodimer RPA49/34 (Figure 2, light purple/light pink) on the opposite side of the polymerase compared to the stalk make their contacts to the core Pol I via the lobe domain. The funnel (Figure 2, yellow-green) and pore (Figure 2, yellow) are located underneath the lobe/jaw region and the cleft and enable NTPs and the C-terminal domain of RPA12 to reach the active center (Figure 2, orange). The active center is located behind the cleft and terminated by the wall (Figure 2, dark green) which is located at the upstream side of the polymerase and helps binding the promoter DNA together with the protrusion in yeast (Neyer et al. 2016; Tafur et al. 2016).

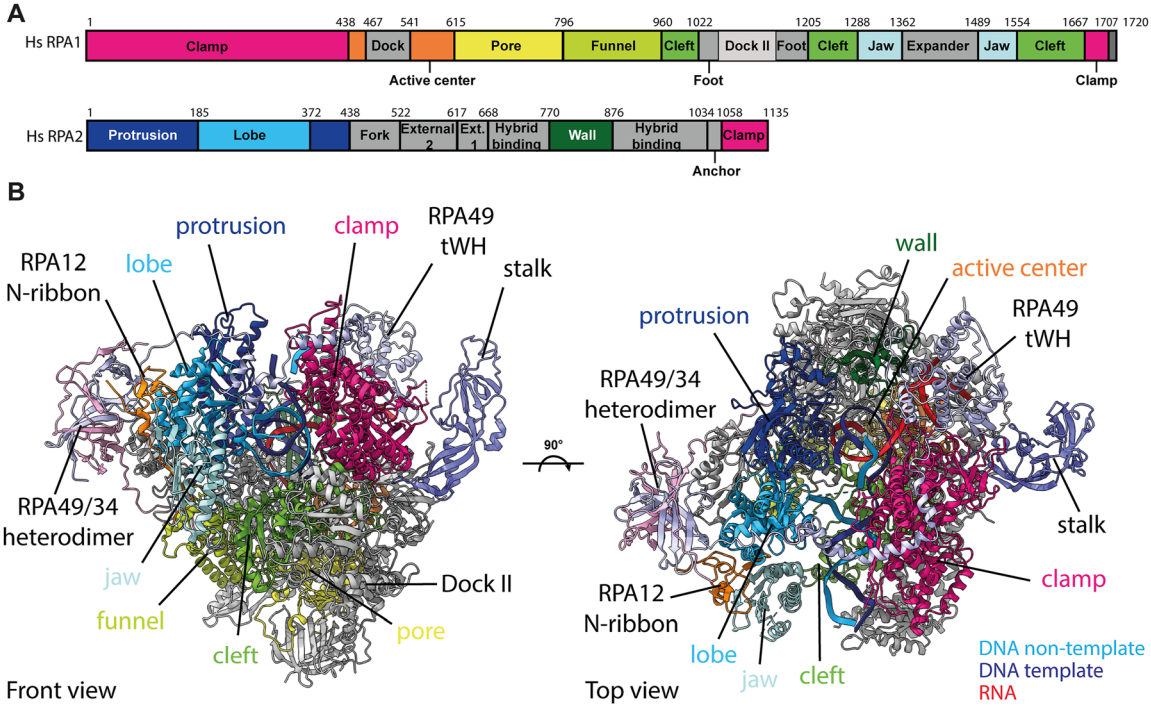

Figure 2: Functional sub-domains of human Pol I. (A) Schematic representation of human subunits RPA1 and RPA2 (to scale) with domain boundaries. (B) Cartoon representation of elongating hPol I (PDB 7OB9) outlining functional sub-domains in the 3D model (colored as in A). Specific subunits are depicted: RPA49 (light purple), RPA34 (light pink), RPA12 (dark orange) and RPA43 (stalk; slate).

3.2 Clamp/stalk region

The stalk subcomplex can carry out multiple functions including promotion of open complex formation, the increase of processivity and augmentation of transcription termination (Werner and Grohmann 2011). No homologue for the yeast subunit A14 was identified in humans using sequence-based searches on DNA and protein levels (Russell and Zomerdijk 2006). Indeed, compositional analysis by mass spectrometry in combination with structural analysis verified that the purified human enzyme comprises only a single-subunit stalk lacking A14 (Daiß et al. 2022; Misiaszek et al. 2021), and uncovering that a 13-subunit Pol I composition is the predominant configuration of Pol I throughout eukaryotic evolution whereas the 14-subunit configuration of *Saccharomycotina* is essentially an outlier of evolution (Daiß et al. 2022).

The cryo-EM reconstructions of hPol I further demonstrated that the clamp/stalk region of the human enzyme shows increased flexibility compared to the yeast enzyme. This causes technical challenges which were overcome with different strategies: Misiazsek et al. used focused refinement and classification to improve the density of the clamp/stalk region, allowing modelling of these parts using a subset of ~5 % of high-quality particles (Misiaszek et al. 2021). Zhao et al. used chemical crosslinking to improve density quality in this region (Zhao et al. 2021). For monomeric, endogenous hPol I, neither strategy resulted in improved cryo-EM densities of flexible regions (Daiß et al. 2022), indicating that the hPol I clamp/stalk region inherently shows a reduced rigidity and does apparently not adopt a defined state in the absence of substrate. Nevertheless, the C-terminal region of the stalk-subunit RPA43 remained flexible in all reconstructions (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). Overall, the stalk subcomplex is flexibly linked to hPol I, likely owing to a reduced interaction surface with the polymerase core. Apparently, an N-terminal region of human Pol I subunit RPA43 (aa 28–44) partially occupies the position of yeast Pol I subunit A14 (Misiaszek et al. 2021). In yeast, this N-terminal region of RPA43 interacts with a small clamp region of subunit RPA2 (aa 1135–1168) and an N-terminal helix in the common Pol subunit Rpb6 (residues 52–68) (Zhao et al. 2021), which both may have co-evolved with subunit A14 (Misiaszek et al. 2021). Hence, clamp and stalk are especially separated in the human enzyme, leading to or co-inciting with a more closed clamp in the human enzyme (Zhao et al. 2021). In line with this, the hPol I EC shows increased contacts between clamp domain and engaged DNA, contributing to a tightly closed clamp and apparently resulting elongation complexes with an increased stability (Zhao et al. 2021). This is well in line with the facts that an enhanced hPol I EC stability of the human enzyme compared to its yeast counterpart was described and that elongation rates increased ~50 % from 60 nt/s (yeast) to 91 nt/s in human cells (Albert et al. 2011; Dundr et al. 2002; French et al. 2003; Zhao et al. 2021). During initiation upon RRN3 binding, a 'flipping' of the stalk was observed by a ~5 Å movement of the distal part of RPA43, whereas the polymerase-proximal part of RPA43 remains stably anchored to the Pol I core (Misiaszek et al. 2021). An enhanced flexibility within the clamp/stalk region was previously described in *S. pombe* Pol I reconstructions compared to *S. cerevisiae* ones, but is even more pronounced in the human enzyme (Heiss et al. 2021).

3.3 Lobe-binding subunits RPA12 and RPA49/34

Subunit RPA12 is important for proper termination and promotes RNA cleavage and proofreading activity of Pol I in yeast (Schwank et al. 2022). The C-terminal ribbon (C-ribbon) of RPA12 resembles the C-ribbon of the Pol II elongation factor TFIIS which can enter the funnel to reach the newly synthesized RNA in a backtracked state (Kettenberger et al. 2003) and hence enables the intrinsic RNA cleavage activity of Pol I (Engel et al. 2013; Fernández-Tornero et al. 2013; Jennebach et al. 2012). This C-ribbon has an alternative binding site on the external domains of subunit RPA2 in yeast (Tafur et al. 2019) and is connected to an N-ribbon domain that is stably associated with the Pol I lobe. The linker between both RPA12 domains is flexible, allowing detachment of the C-ribbon and reaching the active center between the funnel helices and the pore domain to promote RNA cleavage (Jennebach et al. 2012; Scull et al. 2021). The N-terminal domain of RPA12 resembles the N-terminal domain of Pol II subunit RPB9 and binds the lobe domain of Pol I subunit RPA2 (Ruan et al. 2011).

The different structures of hPol I show a similar mechanism of the C-terminal ribbon of RPA12, which can invade the active center between the funnel helices and the pore domain in order to catalyze the cleavage of backtracked RNA (Zhao et al. 2021). This RPA12 C-ribbon domain was shown to be flexible in pre- and post-translocated EC states in hPol I, but inserted into the funnel in the backtracked state, in an open complex reconstruction and the co-structure of human Pol I with RRN3 (Misiaszek et al. 2021; Zhao et al. 2021). In the backtracked state of the human enzyme showing an engaged RPA12 C-ribbon, the funnel is slightly wider and the gating tyrosine (RPA2 Tyrosine 687) is opened to allow the accommodation of backtracked RNA (Zhao et al. 2021).

Interestingly, the flipping of this gating tyrosine is induced by the insertion of RPA12 and does not require the presence of a backtracked RNA in the inactive open complex state (Misiaszek et al. 2021).

The subunits of heterodimer RPA49/34 interact via their N-terminal dimerization domains and contact the Pol I core via the RPA2 lobe and the N-ribbon domain of RPA12. In yeast, the heterodimer was shown to functionally support initiation as well as elongation stages (Beckouet et al. 2008; Geiger et al. 2010; van Mullem et al. 2002). The RPA49/34 dimerization domain structurally and functionally resembles Pol II transcription factor TFIIF (Geiger et al. 2010). RPA34 possesses a linker ARM running along the core polymerase and a large flexibly linked, non-conserved C-terminal domain (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). RPA49 contains a linker including a helix-turn-helix (HTH) motif and a tandem winged helix (tWH) domain, comparable to Pol II transcription factor TFIIE (Geiger et al. 2010). Both HTH and tWH of RPA49 possess DNA binding activity with the tWH showing a sequence preference for promoter DNA in the yeast and the human system (Geiger et al. 2010; McNamar et al. 2021). Upon Pol I cleft contraction, the linker locates above the cleft and contacts the non-transcribed DNA strand during transcription initiation (Han et al. 2017; Sadian et al. 2019) in yeast and thus likely also in human cells.

The divergent C-terminal tail of RPA34 is flexible in all single particle cryo-EM reconstructions of hPol I, similar to its non-conserved yeast counterpart in subunit A34.5 (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). The comparable part of subunit RPC5 in human Pol III is also flexible in cryo-EM reconstructions, but was shown to comprise two consecutive, flexibly linked but ultimately well-folded tandem winged helix domains that play a role in Pol III complex stability (Ramsay et al. 2020). The ARM region of subunit RPA34 connects the N-terminal dimerization domain with a flexible C-terminal tail and diverges from yeast to human, but binds the core polymerase in the same region and possesses the same function of helping to anchor RPA34 to the core (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). In yeast, contacts between the Pol I core and subunit A34.5 are mostly formed by charged residues and hydrogen bonds, whereas the human counterpart is formed by two contacting hydrophobic patches and charged residues of the RPA34 ARM domain are oriented in a distal direction and might function as an additional interaction platform (Misiaszek et al. 2021). The N-terminal dimerization domains of RPA34 and RPA49 are visible in all reconstructions, despite showing rather weak density in most reconstructions that may indicate a flexible association (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). The linker, helix-turn-helix motif and tWH domains of RPA49 are flexible in the monomeric state in both, human and yeast enzymes (Daiß et al. 2022; Neyer et al. 2016; Torreira et al. 2017). In the inactive open complex and RRN3-engaged states, these regions were also shown to be flexible in the human enzyme (Misiaszek et al. 2021; Zhao et al. 2021). However, intense focused classification and -refinement of cryo-EM data allowed modelling of the region in a subset of particles (Misiaszek et al. 2021). Despite low conservation from yeast to human, RPA49 is structurally conserved with the linker spanning over the cleft and the tWH binding the clamp near the RNA exit channel (Misiaszek et al. 2021). Superimposing the different structures of the human enzyme reveals that the tWH domain would clash with bound RRN3, indicating a role in RRN3--release during promoter escape (Misiaszek et al. 2021).

In line with the interaction surface being poorly conserved, the strength of RPA49/34 association with the Pol I core varies among species (McNamar et al. 2021; Penrod et al. 2012). Even though the yeast subcomplex associates with Pol I over the entire transcribed region of the rRNA gene (Beckouet et al. 2008; Rossi et al. 2021), and the heterodimer remains stably bound to Pol I upon ion-exchange chromatography (Engel 2016), it is not stable in high salt conditions, which is also the case for the subcomplex of mouse Pol I (Albert et al. 2011; Hanada et al. 1996). In fission yeast, structural analysis of elongating and monomeric Pol I indicates a flexible or even dissociated RPA49/34 heterodimer, while both subunits stably co-purify and show defined cryo-EM density in inactive Pol I dimers (Heiss et al. 2021). For rat Pol I, it was shown that less than 60 % of affinity purified Pol I molecules carry subunit RPA49 concluding that different Pol I populations that vary in heterodimer occupancy may be present in cells (Hannan et al. 1998; McNamar et al. 2021).

RPA49/34 may be a center of hPol I regulation as the sub-complex can be dissociated under certain circumstances and can get lost during purifications (Daiß et al. 2022; Penrod et al. 2012; Russell and Zomerdijk 2006). Accordingly, specific regulatory modifications of the subunits, such as acetylation, have been observed in mammals (Chen et al. 2013). Furthermore, detailed functional analysis of the yeast RPA49/34 uncovered diverse functions: During initiation the tWH domain of RPA49 might help to recruit transcription initiation factor RRN3 and facilitates its release upon promoter escape (Beckouet et al. 2008; Han et al. 2017). During elongation the heterodimer enhances

processivity, stimulates RNA cleavage activity and facilitates the passage through nucleosomes (Merkl et al. 2020).

3.4 Metazoan-specific 'dock II' domain

One striking observation in the human Pol I structures was the discovery of a previously undescribed structural domain within its largest subunit RPA1. This domain was termed 'dock II' and resembles an HMG-box fold situated within the foot domain of the polymerase (Figure 2) (Daiß et al. 2022). Three-dimensional fold analysis *in situ* revealed that the domain is closely related to the structure of HMG-box 5 within the hPol I transcription factor UBF (Daiß et al. 2022). Unlike most HMG-boxes, the dock II domain is incapable of DNA-binding, but may serve as a protein-protein interaction surface. Interestingly, dock II is located within the foot domain, which is known to be a protein-protein interaction hotspot in Pol II (Bernecky et al. 2016; García-López et al. 2011; Plaschka et al. 2015). Additionally, it was shown that the foot domain of Pol II is rearranged in mammals compared to yeast, creating an enhanced interaction platform (Bernecky et al. 2016). It can therefore be speculated, that the expanded size of the foot domain in human Pols may allow an increased number of transcription factors interactions in metazoan cells. In line with this, phylogenetic analysis confirms a high conservation of the domain in metazoans, but an absence in other organisms (Daiß et al. 2022).

The search for possible interaction partners of the human dock II domain yielded Topoisomerase 2a (Top2a) as a likely candidate (Daiß et al. 2022). Topoisomerase type II, controlling DNA topology in an ATP-dependent manner via double-strand breaks, is expressed as two isoforms in vertebrates, with Top2a being expressed in proliferating cells and Top2b throughout the cell cycle (Lee and Berger 2019; Morotomi-Yano and Yano 2021). Top2a was shown to be involved in hPol I transcription initiation (Ray et al. 2013) and can be stimulated by an interaction with the HMG-box of the protein HMGB1 (Stros et al. 2007). Interestingly, this specific HMG-box also does not directly interact with DNA, too (Stros et al. 2007). Supporting a role in rDNA transcription, an enrichment of Top2a at the rDNA locus was uncovered by ChIP-Seq analysis in mouse cells. Unlike transcription initiation factors, however, Top2a apparently binds the entire gene including promoter and transcribed regions (Daiß et al. 2022), resembling the binding pattern observed for the six HMG-boxes containing factor UBF (Herdman et al. 2017). Additionally, a physical interaction between Top2a and Pol I, as well as Top2a and UBF were described (Daiß et al. 2022; Ray et al. 2013). To date, the exact functions of the six HMG-boxes within UBF remain unclear. UBF is conserved throughout metazoans, hinting at a possible co-evolution with the 'dock II' domain (Daiß et al. 2022).

The exact role of such interactions is currently unknown. It might be speculated that Top2a – once recruited with the help of the dock II domain - may assist Pol I initiation by releasing supercoils occurring upon DNA melting. Alternatively, Top2a may behave similar to a transcription elongation factor by releasing DNA-supercoils downstream of the polymerase assisting Pol I progression through the gene, especially during the first round of transcription. More likely, however, Top2a may bind to UBF, increasing Top2a concentration all over the rRNA gene which might transiently interact with hPol I whenever necessary (Daiß et al. 2022).

4 Pol I activation mechanism and transcription cycle

4.1 Activating Pol I for transcription

The two largest subunits, RPA1 and RPA2, form the active center and are responsible for the enzymatic activity. The DNA-mimicking 'expander' loop of subunit RPA1 becomes structured in inactive yeast Pol I conformations and resembles the DNA backbone location in the cleft thus preventing unspecific DNA binding (Engel et al. 2013; Fernández-Tornero et al. 2013; Heiss et al. 2021). This element is flexible in RPA1 in hPol I reconstructions, but appears to be conserved among organisms (Daiß et al. 2022; Misiaszek et al. 2021). The bridge helix within RPA1 (P961-L996 in human RPA1) is involved in the nucleotide addition cycle and resembles a "nanomechanical switchboard" required for physical translocation after successful phosphodiester bond formation in yeast Pol II (Brueckner and Cramer 2008; Brueckner et al. 2009; Weinzierl 2011). In inactive monomeric hPol I the RPA1 bridge helix is partially unwound but fully folds in the elongation complex (Misiaszek et al. 2021), as also observed for yeast Pol I. Upon activation, the "expander" element retracts to allow for DNA binding and contraction of the active center cleft concomitant with folding of the bridge helix in order to enable RNA synthesis in the yeast and human enzymes (Engel et al. 2013; Fernández-Tornero et al. 2013; Misiaszek et al. 2021). Such contraction of the active center cleft is accomplished by a movement of the clamp and stalk region and is apparently specific to Pol I (Engel et al. 2018; Fernández-Tornero 2018).

The movement of stalk and clamp regions is essential for proper Pol I activity in yeast, especially during Pol I activation by contraction. hPol I structures now indicate, that cleft contraction upon activation is a conserved feature

throughout evolution. Similar to yeast, maximal cleft contraction is observed during elongation, and a slight widening takes place during backtracking (Zhao et al. 2021), probably partially loosening DNA-contacts. In line with this, monomeric hPol I shows such flexibility that density for the clamp region is not observed in cryo-EM reconstructions indicating that it may not adapt a defined state at all (Daiß et al. 2022). Therefore, it can be concluded that activation by contraction is a conserved feature of the rDNA transcription system important for regulation and efficiency (Hori et al. 2023).

4.2 hPol I throughout the transcription cycle

The transcription cycle of hPol I is essentially conserved from yeast to human, comprising the three stages: initiation, elongation and termination being extensively studied in yeast (Girbig et al. 2022; Engel et al. 2018). At actively transcribed, open genes, the promoter is bound by Pol I specific transcription initiation factors which help to recruit RRN3-bound Pol I molecules. During transcription initiation, structural rearrangements of Pol I take place leading to the contraction of the Pol I active center cleft alongside DNA duplex melting and stabilization of the opened transcription bubble. Rrn3 association is phosphorylation-dependent (Milkereit and Tschochner 1998) and is lost during promoter escape in yeast and mammals (Beckouet et al. 2008; Herdman et al. 2017) (Figure 3; Supplementary Figure 1). After promoter clearance and dissociation of Rrn3, Pol I enters a productive elongation phase. During elongation the active center cleft is fully contracted in yeast (Heiss et al. 2021; Neyer et al. 2016; Tafur et al. 2016; Tafur et al. 2019) and human enzymes (Misiaszek et al. 2021; Zhao et al. 2021). In case of pausing, stalling or incorrect nucleotide incorporation, Pol I employs its built-in cleavage and backtracking activity to resume elongation (Lisica et al. 2016). Pol I transcription is terminated by the transcription termination factor TTF-I which binds to multiple binding sites at the end of the rRNA gene (Németh et al. 2013). After transcription termination, RRN3 can re-associate with Pol I and transcription can be re-initiated at the promoter. A schematic overview of the hPol I transcription cycle and the positioning of Pol I transcription factors on a human rRNA gene are shown in Figure 3.

4.3 Dimerization

In addition to the 'activation by contraction' mechanism for Pol I, 'hibernation by dimerization' of Pol I was first suggested by structural and biophysical studies with *S. cerevisiae* Pol I and later with the *S. pombe* enzyme (Engel et al. 2013; Engel et al. 2016; Heiss et al. 2021; Pilsl et al. 2016) and also observed *in vitro* and in yeast cells (Torreira et al. 2017). For *S. cerevisiae* and *S. pombe*, Pol I homodimerization is achieved by divergent structural arrangements. This raises the question whether the dimerization-mechanism might be conserved throughout evolution. Nevertheless, *in vitro* analyses of human Pol I did not provide evidence that dimerization occurs in metazoans. While this does not exclude that dimerization is possible in a salt- and concentration-dependent manner similar to yeast, but with the equilibrium shifted to the monomeric population, current evidences suggest that Pol I dimerization is exclusive to yeast species.

4.4 Activity and proofreading of human Pol I

Comparison of highly purified yeast and human Pol I *in vitro* revealed a reduced cleavage and proof-reading activity of the human enzyme (Daiß et al. 2022). Specifically, hPol I backtracking was not as deep as backtracking of its yeast counterparts and a large number of hPol I molecules reads through specific pausing sites incorporating wrong nucleotides *in vitro* while yeast enzymes are precisely stalled in case of nucleotide misincorporation. As discussed above, this may be related to an increased flexibility of the clamp/stalk domain within the human enzyme compared to yeast. This might be the cause for the reduced proof-reading and cleavage activity. Interestingly, specific mutations of yeast Pol I were also able to create a yeast Pol I version that is faster but more error prone without impacting cell viability (Darrière et al. 2019). Hence, quantity of pre-rRNA synthesis may be relatively more important than quality throughout evolution (Hori et al. 2023). This raises the question whether the tolerated error rate within rRNA synthesis may be higher in metazoans compared to single cell eukaryotes and whether compensation mechanisms in quality control coevolved at a similar rate. Nevertheless, it cannot be excluded that the *in vivo* situation differs and additional factors coordinate with human Pol I to improve proof-reading during rRNA synthesis in cells.

5 Factors involved in Pol I transcription

To ensure proper Pol I transcription, many basal and regulatory factors assist the polymerase in cells. In mammals, transcription factors RRN3, UBF and 'selectivity factor 1' (SL1) enable proper transcription initiation. RRN3 is

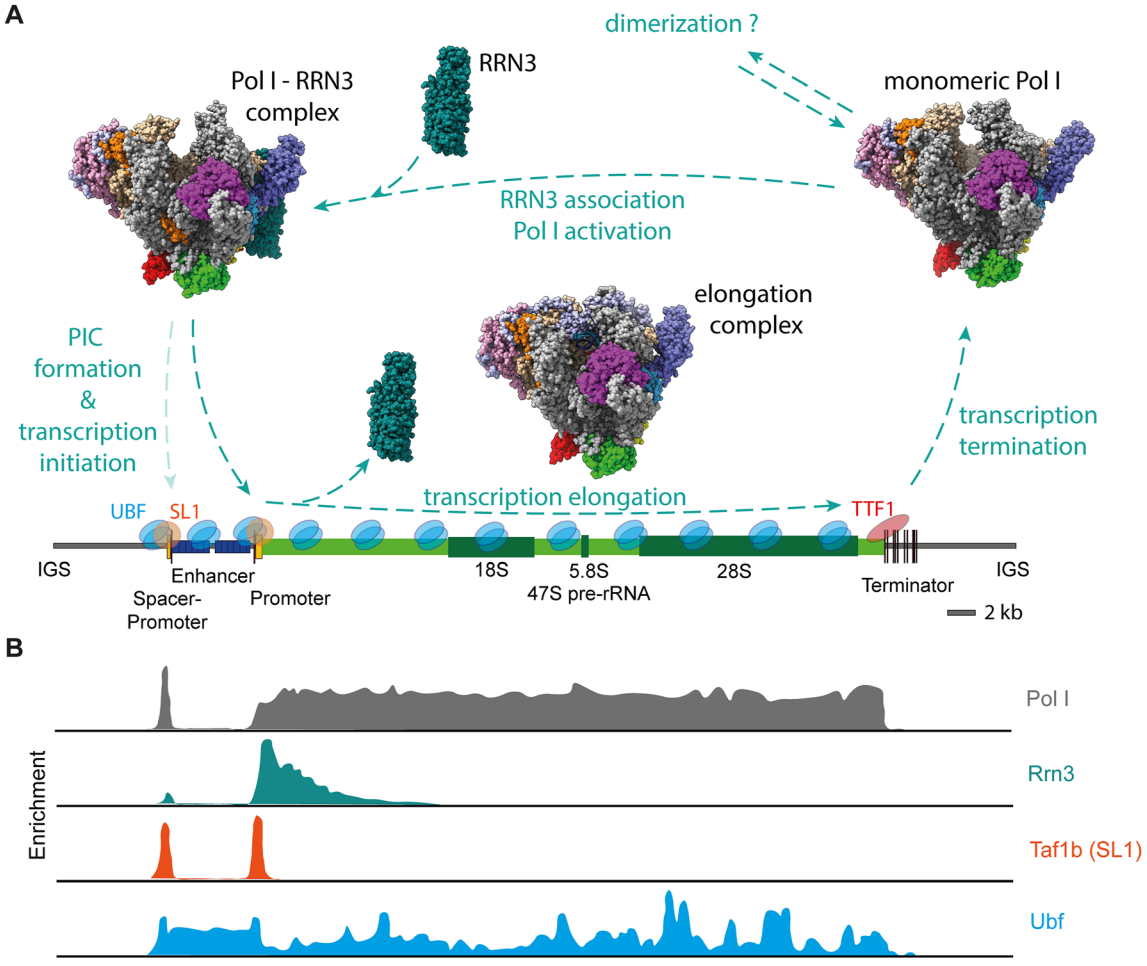

Figure 3: hPol I transcription cycle. (A) Monomeric Pol I (colored by subunits) can be activated by RRN3 (teal) association and primed for initiation. Promoter-bound UBF (blue) and SL1 (orange) recruit the Pol I – RRN3 complex and transcription initiation takes place. After promoter escape and RRN3 dissociation, Pol I enters productive transcription elongation. Terminator-bound factor TTF-I (red) is essential for transcription termination. After termination, monomeric Pol I can re-enter into the cycle. Monomeric yeast Pol I can also be stored in the form of hibernating dimers. Bottom: Schematic representation of the rDNA genomic locus as in Figure 1. Pol I subunit colors are: RPA1 grey, RPA2 wheat, RPAC1 red, RPAC2 yellow, RPABC1 magenta, RPABC2 hafnium, RPABC3 green, RPABC4 lemon, RPABC5 density, RPA43 slate, RPA12 orange, RPA49 light blue, RPA34 light pink. (B) Localization of mammalian Pol I, RRN3, TAF1B as a subunit of SL1 and UBF is shown schematically in relation to panel A (ChIP data from mouse cells as described in (Daiß et al. 2022)). Pol I is enriched at the spacer-promoter and binds over the whole transcribed region. RRN3 dissociates during promoter escape before the polymerase enters a productive elongation phase and is only slightly enriched at the stalled polymerases at the spacer-promoter. TAF1B as a component of SL1 clearly localizes to the spacer and gene promoters. UBF binds at the promoter and the whole gene body.

essentially conserved from yeast to human (Moss et al. 2007), whereas core promoter binding factor SL1 includes the homologues of yeast core factor (CF) subunits, but also includes the additional subunits TAF1D and TBP (Comai et al. 1992; Heix et al. 1997; Gorski et al. 2007) (see Figure 4 for domain organization and conservation of initiation factors). Yeast CF consists of subunits Rrn6, Rrn7 and Rrn11 in a 1:1:1 stoichiometry, binds the core promoter DNA and directly recruits the Pol I-Rrn3 complex (Engel et al. 2017; Han et al. 2017; Knutson and Hahn 2013; Knutson et al. 2014). The mammalian homo-dimeric UBF is distantly related to the Hmo1 protein and the UBF CTD shows similarities to the C-terminal domain of Net1 in the yeast system (Albert et al. 2013; Hall et al. 2006; Hannig et al. 2019; Merz et al. 2008; Schächner et al. 2022). As UBF, the non-homologous yeast multi-subunit 'upstream activating factor' (UAF) containing subunits Rrn5, Rrn9, Rrn10, Uaf30 and histones H3 and H4 (Keener et al. 1998) binds the UPE, but has so far no mammalian counterpart. UAF together with TBP increases transcription from basal levels 10–50 fold by bridging between CF and UAF via direct protein-protein interactions and likely independent from its known DNA-binding activity (Keener et al. 1998; Kramm et al. 2019; Steffan et al. 1996; Steffan et al. 1998). Furthermore, UAF prevents rDNA transcription by Pol II

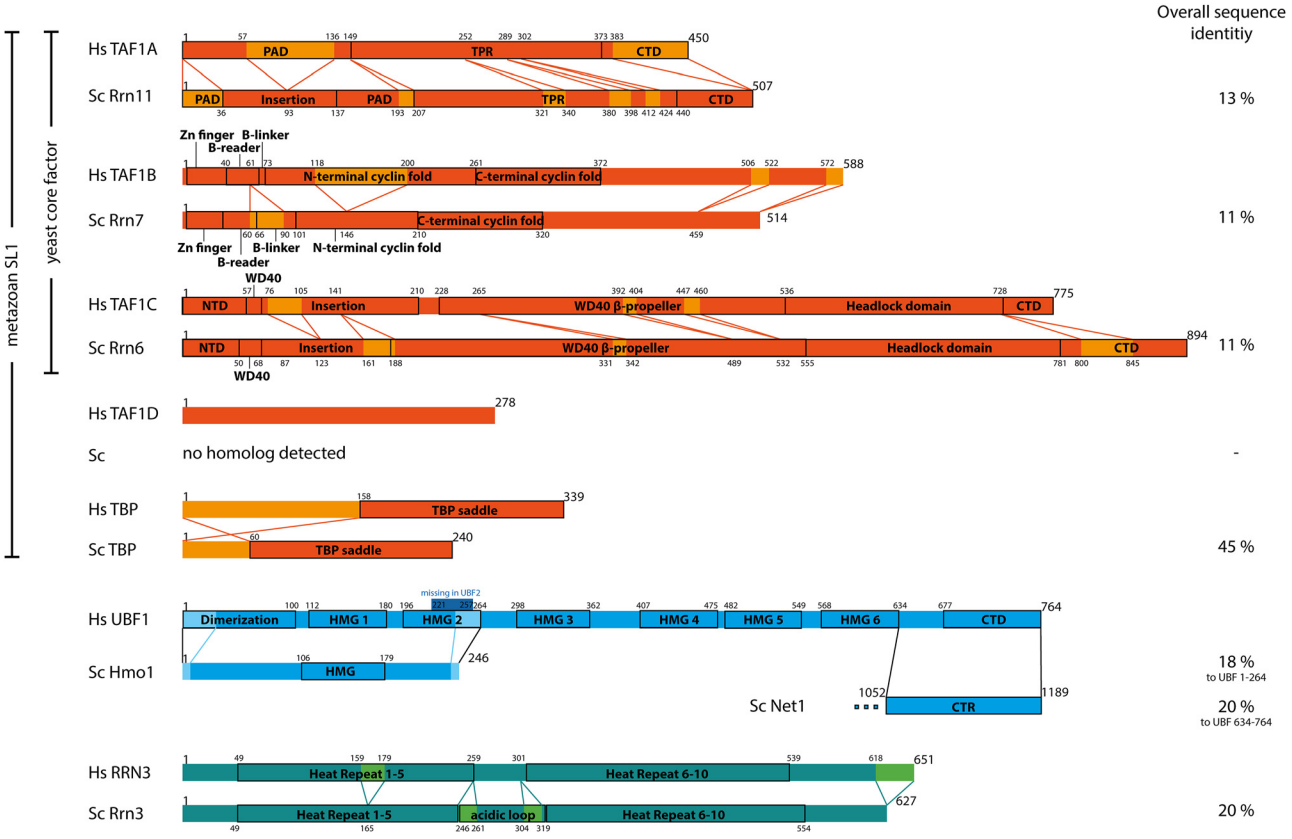

Figure 4: Pol I transcription initiation factor. Schematic domain architecture of the Pol I transcription initiation factors with largest differences to their yeast homologs: TAF1A (Rrn11), TAF1B (Rrn7), TAF1C (isoform 2; Rrn6), TAF1D, TBP (TBP), UBF (Hmo1/Net1) and RRN3 (Rrn3). SL1 subunits are shown in orange, UBF in blue and RRN3 in teal. Subdomains and insertions/deletions of 10 or more residues are indicated.

(Baudin et al. 2022; Oakes et al. 1999; Siddiqi et al. 2001; Smith et al. 2018).

5.1 Initiation factor RRN3

RRN3 association to Pol I leads to structural rearrangements in yeast and human enzymes (Blattner et al. 2011; Engel et al. 2016; Misiaszek et al. 2021; Pilsl et al. 2016; Torreira et al. 2017) and is regulated by post-transcriptional modifications, including changes during the transcription cycle and only a minor portion of Pol I is bound to RRN3 at the same time (Milkereit and Tschochner 1998; Bodem et al. 2000; Cavanaugh et al. 2002; Bier et al. 2004; Blattner et al. 2011). With its direct interaction with SL1 and Pol I including binding to RPA43 (Bodem et al. 2000; Cavanaugh et al. 2002; Milkereit and Tschochner 1998; Peyroche et al. 2000), RRN3 plays a central role in Pol I recruitment to the promoter and bridging Pol I to promoter bound initiation factors (Peyroche et al. 2000; Russell and Zomerdijk 2006). RRN3 stabilizes the monomeric, initiation competent Pol I and thus formation of the pre-initiation complex and the formation of the first phosphodiester bonds (Bodem et al. 2000; Engel et al. 2016; Pilsl et al. 2016), making the factor essential during initiation (Blattner et al. 2011; Schnapp and Grummt 1991; Schnapp et al. 1993). Additionally, human RRN3 may directly interact with DNA *in vitro* (Stepanchick et al. 2013). RRN3 is a target of regulation by post-translational modification as described below.

5.2 Selectivity factor 1

Initiation factor SL1 is composed of the three yeast core factor homologues TAF1A (Rrn11), TAF1B (Rrn7), TAF1C (Rrn6), and includes TBP and TAF1D (Comai et al. 1992; Gorski et al. 2007; Heix et al. 1997; Russell and Zomerdijk 2006). TAF12 was proposed to constitute an additional subunit of the SL1 complex *in vivo* (Denissov et al. 2007). SL1 weakly interacts with Pol I subunit RPA34 (Panov et al. 2006b), whereas TAF1B and TAF1C can interact directly with RRN3 (Bodem et al. 2000; Miller et al. 2001; Moorefield et al. 2000; Russell and Zomerdijk 2006). SL1 is essential for mammalian Pol I transcription (Comai et al. 1992; Russell and

Zomerdijk 2006; Tremblay et al. 2022; Zomerdijk et al. 1994) and directs species-specificity, hence its name (Comai et al. 1992; Heix et al. 1997; Murano et al. 2014; Russell and Zomerdijk 2006). For instance, Pol I and UBF are interchangeable between mice and human, but SL1 is not and only promotes rDNA transcription of the rDNA gene from its original source organism (Bell et al. 1990; Comai et al. 1992). Overall, SL1 functions in promoter recognition and binding of the core promoter element and then recruits RRN3-bound Pol I to the promoter (Friedrich et al. 2005; Miller et al. 2001; Tremblay et al. 2022). However, stable association of SL1 to the promoter requires UBF, otherwise SL1 cannot associate efficiently (Bell et al. 1990; Rong et al. 2007). Without SL1, UBF frequently de- and associates from/with promoter DNA, but after binding of SL1, both factors stay stably bound over many rounds of transcription initiation (Russell and Zomerdijk 2006). Further supporting functional cooperativity of SL1 and UBF binding to the promoter, DNase I footprinting seems to be in line with mutual stabilization of the factors (Jantzen et al. 1992). In yeast, the SL1-homologue CF induces a characteristic 30° bent in the proximal promoter DNA duplex (Engel et al. 2017). Since the principal components are conserved throughout organisms, also in metazoans, it was speculated that a defined DNA bendability upstream of the transcription start site may biophysically define rDNA promoters, rather than a conserved DNA sequence (Jackobel et al. 2019). This hypothesis stands at the time of writing, as structural analyses and *in vitro* structure-function studies of the human initiation components are awaited.

5.3 Upstream binding factor

UBF assembles as a homodimer (Jantzen et al. 1992; McStay et al. 1991) and specifically binds to the UPE and the core promoter and functions as an architectural protein at the rDNA promoter (Jantzen et al. 1992). UBF additionally binds throughout the transcribed rDNA region, defining the structure of actively transcribed genes (Hamdane et al. 2014; Herdman et al. 2017; O'Sullivan et al. 2002). Thereby, UBF replaces nucleosomes on active rDNA genes generating a nucleosome depleted region (NDR), and plays a role in regulating Pol I elongation (Herdman et al. 2017; Stefanovsky et al. 2006; Tremblay et al. 2022).

UBF is composed of an N-terminal dimerization domain responsible for homodimerization, followed by six consecutive HMG-box domains and a highly charged, acidic C-terminal domain (CTD) (Jantzen et al. 1990; Jantzen et al. 1992; McStay et al. 1991). HMG-boxes 1-3 function in DNA binding which induces bending of a ~140 bp long DNA to a 360° loop (McStay et al. 1991; Neil et al. 1996; Putnam et al. 1994; Stefanovsky et al. 2001). HMG box 1 appears to be necessary and sufficient for sequence specific promoter binding (Jantzen et al. 1992). Binding and recruiting SL1 occurs via the CTD of UBF in a phosphorylation-dependent manner and might involve subunits TBP and TAF1A of SL1 (Kwon and Green 1994; Tuan et al. 1999; Voit et al. 1992). The interaction between UBF and TBP relies on the conserved C-terminal region of TBP (Kwon and Green 1994). Promoter-bound UBF functions during Pol I promoter escape at the transition between initiation and elongation (Panov et al. 2006a) and can also directly interact with RPA49 and RPA34 (Panov et al. 2006c). Interestingly, UBF is expressed in two splice variants, the full-length UBF1 and UBF2 lacking a part of the DNA-binding HMG box 2 (Jantzen et al. 1992; O'Mahony and Rothblum 1991; Rong et al. 2007). Both, UBF1 and UBF2, bind throughout the rDNA gene in the NDR, but only UBF1 appears to be bound at the spacer and gene promoters (Tremblay et al. 2022). This observation is astonishing as it was shown that HMG box 1 is necessary and sufficient for promoter sequence specificity (Jantzen et al. 1992) and although box 1 is present in both splice variants, the two variants show different localizations. While the reason of this remains unclear, cooperativity with SL1 may play a role in the process.

Although, UBF is not conserved from yeast to human, some similarities to different yeast proteins can be found. UPE binding is performed by the yeast multi-subunit UAF (Keener et al. 1997), whereby it does not have any reported homology with UBF and uses a hexameric histone-like scaffold for DNA recognition (Baudin et al. 2022). In yeast, HMG-box containing protein Hmo1 shows homology with UBF HMG boxes 1 and 2, and has functional similarities by binding throughout the rDNA gene and stabilizing the NDR (Albert et al. 2013; Hall et al. 2006; Merz et al. 2008; Schächner et al. 2022; Wittner et al. 2011). Moreover, the C-terminal domain (CTD) of UBF might share similarities with the C-terminal region (CTR) of yeast Net1 (aa 1052–1189), which stimulates Pol I transcription *in vitro* and *in vivo* (Hannig et al. 2019).

5.4 Additional factors

Besides the above factors, numerous other proteins participate in Pol I transcription and its regulation (see also chapter 6). One of these factors, Transcription Termination Factor I (TTF-I) is a myb-domain containing factor related to the yeast proteins Reb1/Nsi1 the termination site of human rDNA genes (Németh et al. 2013). As mentioned before, TTF-I has also binding sites within the promoter region (Figure 1C),

and this constellation is as well conserved in yeast. Besides its role in termination, TTF-I may additionally act as a replication fork barrier (Akamatsu and Kobayashi 2015; Gerber et al. 1997), or activate and silence transcription (Längst et al. 1997; Längst et al. 1998; Santoro et al. 2002; Strohner et al. 2004). Another factor necessary for termination is PTRF (Pol I transcript release factor), helping to release Pol I from the DNA and afterwards allowing Pol I to be primed for re-initiation by re-association of Rrn3 or being stored (Jansa et al. 1998).

6 Regulation of Pol I transcription

Considering the importance of Pol I transcription with its direct link to cell growth and cell division, the transcription system must be tightly regulated (Russell and Zomerdijk 2006). One regulatory strategy is the activation ('opening') and inactivation ('closing') of single rDNA genes. However, this mechanism was shown to play a minor role in regulation of the transcriptional output of Pol I in mammals and yeast (Fahy et al. 2005; French et al. 2003; Stefanovsky and Moss 2006). Even in fast growing cells only about 50 % of genes are transcriptionally active and in an open chromatin state. Whereas Pol I transcription drops by about 90 % in stationary phase cells, opened genes are only reduced about half (Fahy et al. 2005). Additionally, under growth stimulation only Pol I transcription increases several-fold, whereas the number of active genes stays constant (Stefanovsky and Moss 2006). Furthermore, phenotypic analysis of yeast strains carrying a reduced number of rDNA repeats are viable with no apparent growth defect under normal conditions while all repeats remain open and constitutively activated (French et al. 2003). Whereas epigenetic control of repeat activation may have more importance in mammalian cells (Bersaglieri and Santoro 2019), in yeast the presence of multiple rDNA gene copies appears to be required for genome stability and control repeat amplification (Ide et al. 2010), rather than transcription boosting (Hori et al. 2023). Collectively, these experiments suggest that Pol I transcription is mainly regulated at the level of initiation controlling the polymerase load of rDNA genes.

Post-transcriptional modifications of proteins involved in Pol I transcription initiation in response to signal transduction pathways might be involved in the regulation of rRNA synthesis. Initiation factors RRN3 and UBF as well as Pol I subunits RPA1, RPA2 and RPA49/34 are phosphoproteins (Dephoure et al. 2008; Gadal et al. 1997; Hannan et al. 1998; Mayya et al. 2009; Olsen et al. 2010; Panova et al. 2006a) and therefore may serve as regulation hubs. For example, the PKC ('Protein kinase C') pathway, which monitors cell surface integrity, is able to repress Pol I transcription in case of secretory defects in yeast (Kamada et al. 1995; Warner 1999). Additionally, phosphorylation of UBF at its CTD by CK2 ('casein kinase 2') leads to enhanced UBF-SL1 interactions and thereby contributes to Pol I transcription activity adjustment to cellular growth (Jantzen et al. 1992; Tuan et al. 1999; Voit et al. 1995). Overall, UBF activity and PIC (pre-initiation complex) formation is regulated by the growth-dependent MAPK ('mitogen-activated protein kinase') and ERK ('extracellular signal-regulated kinase') pathways as well as the growth-dependent and nutrient-sensing mTOR ('mammalian target of rapamycin') and stress dependent JNK2 ('c-Jun N-terminal kinase 2') pathways (Russell and Zomerdijk 2006). Furthermore, regulation of Pol I association of RRN3 in a phosphorylation-dependent manner is an important regulatory strategy allowing the cell to rapidly react to changing nutrient availability and other stress condition via a combination of RSK ('ribosomal s6 kinase') and ERK and mTOR pathways as well as the JNK pathway with activation of repression of rRNA synthesis (Cavanaugh et al. 2002; Mayer et al. 2004, 2005; Moss et al. 2007; Russell and Zomerdijk 2006; Warner 1999; Zhao et al. 2003). During nutrient or energy shortage, the phosphorylation status of RRN3 is changed in a way that avoids interaction of RRN3 with promoter-bound SL1 by various kinases and phosphatases including AMPK ('AMP-activated protein kinase') (Chen et al. 2013; Hoppe et al. 2009; Mayer et al. 2004). Notably, these post-translational modification (PTM)-dependent regulation strategies all rely on a modulation of initiation activity. Since the mechanism of Pol I initiation is distinct from Pol II and III (Engel et al. 2018) and apparently allows the highest possible initiation rates, any interference with initiation factor affinity consequently leads to strong, direct effects and drastically reduces transcriptional output. In line with this, a single phospho-mimicking mutation in Rrn3 leads to severe growth defects in yeast cells and prevents Pol I association *in vitro* (Blattner et al. 2011). Additionally, however, acetylation of subunit RPA49 was described as an additional point of regulation (Chen et al. 2013). This is achieved by the interplay of CBP ('CREB binding protein') and SIRT7 ('NAD-dependent protein deacetylase sirtuin-7') (Chen et al. 2013). SIRT7 is able to catalyze subunit deacetylation leading to increased Pol I transcription and is regulated at the step of nucleolus-localization of the factor in response to different stress conditions (Chen et al. 2013). In fact, upregulation of SIRT7 expression was associated with several cancers, emphasizing its important role in cell growth regulation in mammals (Chen et al. 2013; Kim et al. 2013). As described above, another level of regulation might be the association of the heterodimer RPA49/34 to Pol I which at least seems to be species-specific (Penrod et al. 2012).

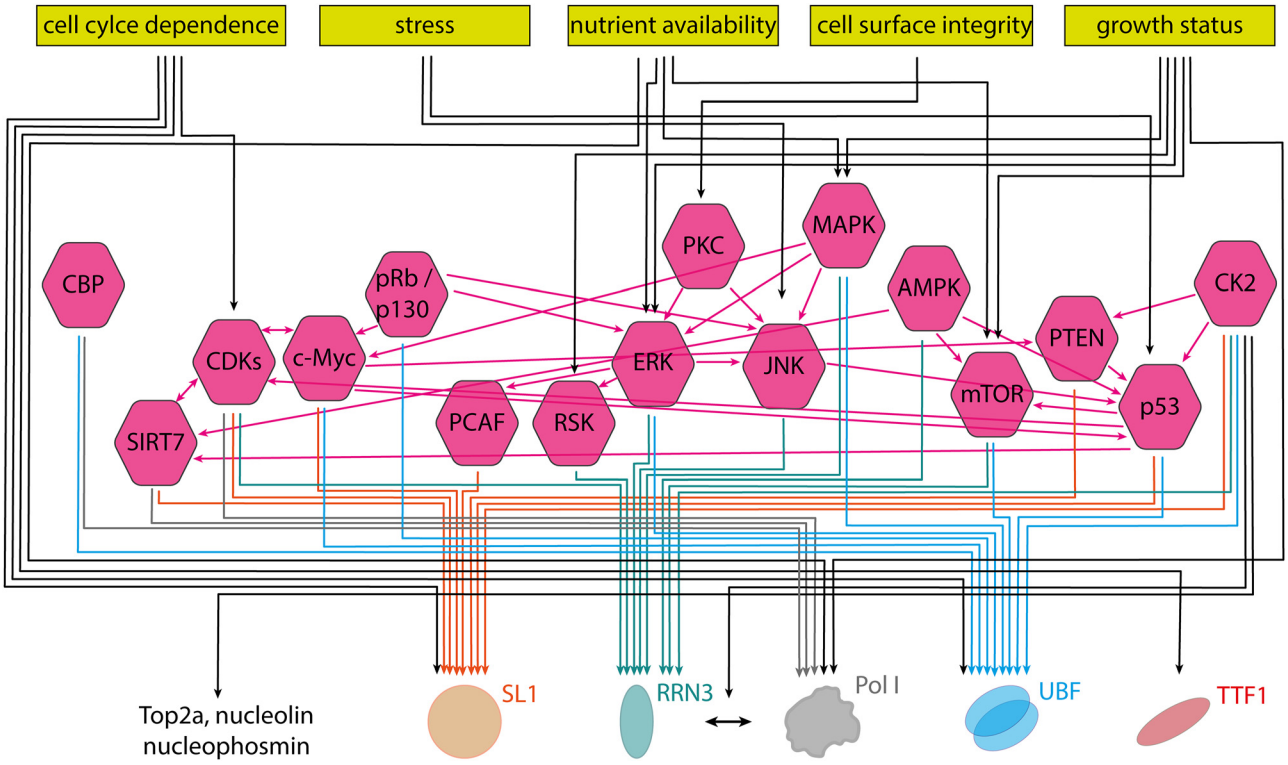

Figure 5: Schematic overview of the Pol I regulation network. Schematic overview of the regulation network of Pol I transcription: different cellular conditions (yellow) activate divers signaling pathways and proteins (pink) that regulate one or more factors involved in Pol I transcription (arrow in the color of the regulated protein). Cross-talk between the different signaling pathways is indicated with arrows (pink). Regulation network was summarized using various references (Claypool et al. 2004; Drygin et al. 2010; Ferreira et al. 2020; Grummt 2003; Mayer et al. 2004; Panova et al. 2006c; Russell and Zomerdijk 2006; Zhao et al. 2003).

Although many of the known influences of regulation include PTM of initiation factors regulation of transcription elongation also seems to play a role (Moss et al. 2007; Stefanovsky et al. 2006) and some factors may prevent complete pre-rRNA synthesis by blocking elongation in a road-block-like fashion (Antony et al. 2022). Figure 5 visualizes the regulatory network of human Pol I activity in a composite manner.

In many cancer cells, Pol I transcription is upregulated to cater to the increased requirement for ribosomes in rapidly growing cells. However, the underlying molecular mechanisms are diverse and remain elusive. For example, also the well-known oncogene Myc was shown to control Pol I transcription, which could represent one early step in oncogenesis in some cases (Arabi et al. 2005; Grandori et al. 2005; Russell and Zomerdijk 2006). Additionally, it was shown that protein kinases have the potential to upregulate rDNA transcription in cancer cells, including CK2 and ERK, which lead to UBF and RRN3 phosphorylation (Bierhoff et al. 2008; Lin et al. 2006; Murano et al. 2014; Panova et al. 2006b; Stefanovsky et al. 2001). Furthermore, mutations in UBF may underly some forms of acute myeloid leukemia (AML) and related pathologies (Duployez et al. 2023; Umeda et al. 2022). Taking this into account, drugs downregulating Pol I transcription are potential promising anticancer drugs, which started to be evaluated and will be further investigated (Ferreira et al. 2020). In fact, targeting of initiation complex formation (Andrews et al. 2021) or prevention of promoter escape (Mars et al. 2020) appear to be promising strategies underlying the action if drug candidate compounds relaying effects via the nucleolar stress surveillance pathway (Hannan et al. 2022).

7 Mutations within hPol I associated with inherited diseases

Several developmental and neurodegenerative diseases are caused or accompanied by mutations in Pol I subunits, some of them being negative-dominant. Knowing the 3D structure of the human Pol I enzyme allows positioning of these mutations and enables us to draw conclusions about the

Table 2: Known mutations in Pol I related to human disease.

Mutation	Potential impact on Pol I (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021)
Acrofacial dysostosis*, *Cincinnati type	
RPA1 V1299F (Weaver et al. 2015)	Might destabilize RPA1 fold intrinsically and RPA12 association
RPA1 E593Q (Weaver et al. 2015)	Might affect nucleotide addition cycle
Treacher Collins syndrome	
RPA2 S682R (Sanchez et al. 2020)	Might destabilize the bridge helix
RPA2 R1003 C/S (Sanchez et al. 2020)	Might affect nucleotide association
RPAC1 R279Q/W (Dauwerse et al. 2011; Thiffault et al. 2015)	Might destabilize RPA34 interaction
RPAC2 E47K (Dauwerse et al. 2011)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
RPAC2 T50I (Dauwerse et al. 2011)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
RPAC2 L51R (Dauwerse et al. 2011)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
RPAC2 G52E (Dauwerse et al. 2011)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
RPAC2 L55V (Schaefer et al. 2014)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
RPAC2 R56C (Dauwerse et al. 2011)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
RPAC2 L82S (Dauwerse et al. 2011)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
RPAC2 G99S (Dauwerse et al. 2011)	Might destabilize RPAC2 fold intrinsically and RPAC1-RPAC2 interaction
Treacle protein mutations	Mutations in a Pol I regulating protein
Hypomyelinating leukodystrophy	
RPC1 mutations	affects Pol III transcription system
RPC2 mutations	affects Pol III transcription system
RPAC1 T26I (Gauquelin et al. 2019; Thiffault et al. 2015)	Might predominantly affect the Pol III system
RPAC1 T27A (Gauquelin et al. 2019)	Might predominantly affect the Pol III system
RPAC1 P30S (Gauquelin et al. 2019)	Might predominantly affect the Pol III system
RPAC1 N32I (Gauquelin et al. 2019; Thiffault et al. 2015)	Might predominantly affect the Pol III system
RPAC1 M65V (Gauquelin et al. 2019; Thiffault et al. 2015)	Might destabilize RPAC1 fold intrinsically
RPAC1 N74S (Gauquelin et al. 2019; Thiffault et al. 2015)	Might predominantly affect the Pol III system
RPAC1 V94A (Gauquelin et al. 2019; Thiffault et al. 2015)	Might destabilize RPAC1 fold intrinsically
RPAC1 I105F (Gauquelin et al. 2019)	Might predominantly affect the Pol III system
RPAC1 H108Y (Gauquelin et al. 2019)	Might predominantly affect the Pol III system
RPAC1 R109H (Gauquelin et al. 2019; Thiffault et al. 2015)	Might predominantly affect the Pol III system
RPAC1 A117P (Gauquelin et al. 2019)	Might destabilize RPAC1 fold intrinsically
RPAC1 G132D (Gauquelin et al. 2019; Thiffault et al. 2015)	Might destabilize RPAC1 fold intrinsically
RPAC1 C146R (Gauquelin et al. 2019; Thiffault et al. 2015)	Might destabilize RPAC1 fold intrinsically
RPAC1 R191Q (Gauquelin et al. 2019; Thiffault et al. 2015)	Might destabilize RPAC1 fold intrinsically
RPAC1 I262T (Gauquelin et al. 2019; Thiffault et al. 2015)	Might destabilize RPAC1 fold intrinsically
RPAC1 T313 M (Gauquelin et al. 2019)	Might destabilize RPAC1 fold intrinsically
RPAC1 E324K (Gauquelin et al. 2019; Thiffault et al. 2015)	Might destabilize RPAC1 fold intrinsically
juvenile neurodegenerative phenotype akin to the HL-phenotype	
RPA1 S934L (Kara et al. 2017)	Might destabilize contacts to RPA2

effects such mutations may have on a structure-function level (Table 2) (Daiß et al. 2022; Misiaszek et al. 2021; Zhao et al. 2021). Mutation E593Q in the largest Pol I subunit RPA1 underlies Acrofacial dysostosis, Cincinnati type and is located near the active center of the enzyme. The mutation might therefore have an effect on the nucleotide addition cycle as this amino acid is located close to the catalytic triad (D588, D590, and D592 coordinate the active site magnesium ion). Another mutation within subunit RPA1 (V1299F) leads to the same phenotype and could destabilize the RPA1 fold itself, and/or affects association of subunit RPA12 to the core polymerase. A disease clearly associated with the Pol I

transcription is a juvenile neurodegenerative phenotype akin to the HL-phenotype caused by a mutation within RPA1 which seems to destabilize RPA1-RPA2 contacts. The hypomyelinating leukodystrophy (HL) phenotype is caused by mutations in the Pol I and III shared subunit RPAC1 as well as Pol III subunits RPC1 and RPC2 and is therefore not easily classified as Pol I or Pol III disease. Many of the mutations within the shared subunit (RPAC1: T26I, T27A, P30S, N32I, N74S, I105F, H108Y, R109H) most likely only affect Pol III when evaluating the structures of both enzymes (Daiß et al. 2022; Girbig et al. 2021; Li et al. 2021; Misiaszek et al. 2021; Ramsay et al. 2020; Wang et al. 2021; Zhao et al. 2021), but others (M65V, V94A, A117P, G132D, C146R, R191Q, I262T, T313 M, E324K) might affect the fold of subunit RPAC1 itself and therefore have the potential to affect Pol I and Pol III transcription system. Altogether, HL seems to be often caused by Pol III defects only, but in other cases it cannot be excluded that Pol I defects also play a role in the disease. The Treacher Collins syndrome is another disease which cannot be classified as Pol I or Pol III-associated disease per se as it is caused by mutations in the shared subunits RPAC1 and RPAC2 as well as within RPA2 and the TCOF1 gene. Mutations within RPAC2 (E47K, T50I, L51R, G52E, L55V, R56C, L82S, G99S) seem to destabilize the RPAC2 fold itself as well as its interaction with subunit RPAC1 and might affect both, Pol I and Pol III. However, mutation R279Q/W of subunit RPAC1 seems to affect predominantly the Pol I system as the mutation might destabilize the interaction of RPA34 with the Pol I core but has no obvious defects in Pol III. Pol I specific mutations within RPA2 might destabilize the bridge helix (S682R) or affect the nucleotide addition cycle (R1003 C/S) and mutations in the Treacle protein could affect Pol I transcription via its regulatory role. Hence, Treacher Collins Syndrome in contrast to the HL phenotype seems to be caused predominantly by Pol I defects, but with some mutations an additional Pol III defect seems to be very likely. Taken together, it seems that point mutations situated on the interfaces of Pol I (and/or III) subunits resulting in mild effects on secondary structure folds or complex integrity underly the developmental/neurodegenerative diseases.

8 Outlook

With the structure of human Pol I determined by single particle cryo-EM, all structures of yeast and mammalian DNA-dependent RNA polymerases are now available. The structures allowed a detailed analysis of structural and functional conservation among species and across transcription systems, as well as explaining how disease-causing mutations might affect the activity of the enzyme. This information now supports the basic understanding of disease mechanisms, may facilitate drug development and lays the groundwork for further research. While the key concepts of the Pol I activity cycle and its regulatory mechanisms appear to be conserved, the roles and structural basis of regulation by organism-specific factors in response to external or cell cycle-dependent queues remains poorly understood. How human Pol I initiation, elongation, backtracking, proof-reading, termination, and chromatin interaction are achieved on a mechanistic level, what the exact functions of mammalian-specific domains are and how mutations in the Pol I system exactly contribute to human diseases will be key questions over the following years. Some of these questions can now be addressed on the basis of recent analyses, such as the nature of and reason for the apparently decreased proof-reading efficiency and the apparently increased nucleotide-incorporation error-rates of human compared to yeast Pol I. Especially the precise role of Top2a in interplay with the human Pol I transcription system, function(s) of the metazoan dock II domain and consequences of the absence of stalk-subunit A14 in humans will remain challenging to study. However, answers to these questions hold the promise to give insights in why the Pol I transcription system, highly specialized in the synthesis of one specific RNA is of such importance to all eukaryotes, even though yeast cells in which rRNA is synthesized by Pol II are viable and do not require Pol I (Nogi et al. 1991).

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