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RNA polymerase III repression by the Retinoblastoma tumor suppressor protein

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Abstract

The Retinoblastoma (RB) tumor suppressor protein regulates multiple pathways that influence cell growth, and as a key regulatory node, its function is inactivated in most cancer cells. In addition to its canonical roles in cell cycle control, RB functions as a global repressor of RNA polymerase (Pol) III transcription. Indeed, Pol III transcripts accumulate in cancer cells and their heightened levels are implicated in accelerated growth associated with RB dysfunction. Herein we review the mechanisms of RB repression for the different types of Pol III genes. For type 1 and type 2 genes, RB represses transcription through direct contacts with the core transcription machinery, notably Brf1-TFIIB, and inhibits preinitiation complex formation and Pol III recruitment. A contrasting model for type 3 gene repression indicates that RB regulation involves stable and simultaneous promoter association by RB, the general transcription machinery including SNAPc, and Pol III, suggesting that RB may impede Pol III promoter escape or elongation. Interestingly, analysis of published genomic association data for RB and Pol III revealed added regulatory complexity for Pol III genes both during active growth and during arrested growth associated with quiescence and senescence.

Keywords

RNA polymerase III; Retinoblastoma; transcription; repression; chromatin; cell cycle

1. Introduction - The Retinoblastoma Tumor Suppressor Protein

Mutations in the RB1 gene, encoding the Retinoblastoma (RB) tumor suppressor protein were originally identified as culpable in a rare childhood eye tumor known as Retinoblastoma [1]. In early studies of retinoblastoma cell lines, tumor-associated mutations were correlated with either undetectable RB1 mRNA or mRNA of a decreased molecular size [2, 3], consistent with the loss of RB expression in these pediatric tumors. Subsequently, RB1 mutations were observed in other classes of cancers, especially small-cell lung carcinomas [4], wherein genetic lesions at the RB1 locus are as penetrant as in retinoblastoma (reviewed in [5]). These early studies suggested an important role for RB in prevention of adult-onset malignancies, and indeed, dysregulation of the RB pathway via

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direct mutation of RB or through indirect modulation of other regulatory components is so frequently observed as to be recognized as a hallmark of cancer [6, 7].

The function of RB in tumor suppression has been an actively pursued topic with early studies linking RB function to cell cycle control. RB is a 110 kDa protein which becomes phosphorylated when cells are rapidly cycling [8–11] and dephosphorylated in growth arrested cells [12]. Microinjection of recombinant RB into nocodazole-synchronized cells early in the G1 phase resulted in a pronounced G1 cell cycle block that was not observed when RB was injected in asynchronously growing cells [13]. These studies linked the changes in RB phosphorylation status to cell cycle control, a process centrally important to regulated proliferation. RB post-translational modification is facilitated by the cyclin dependent kinase (cdk) 4/ cyclin D and cdk2/cyclin E complexes, whose activities are important for G1 progression [14–16]. RB was found to associate with the E2F family of transcription factors and impede their function in RNA polymerase (Pol) II activation of cell cycle target genes [17–20]. A prevalent model emerged that RB prevents transcription of key genes required for DNA synthesis in early G1 and cyclin/cdk phosphorylation relieves RB repression (Figure 1). In addition to cell cycle control, RB is dephosphorylated as cells differentiate, implicating RB function in developmental processes [9]. RB also participates in regulation of apoptotic responses [21–23], and it is clear that RB governs multiple aspects of cell fate choices that are deregulated in tumorigenesis.

2. RNA polymerase III activity in cancer – The biosynthetic capacity hypothesis

The prevalent model for RB function in attenuating cancer progression attributes RB mutation with the loss of E2F repression and cell cycle control. However, this cell cycle-centric model does not account for a simple requirement that accelerated proliferation by cancer cells must also involve increased synthesis of cellular components to match their increased rate of division. In part, the requisite increased biosynthetic capacity for rapid proliferation is achieved by increased output by Pol III, which is responsible for the production of the 5S ribosomal (r) RNAs, all transfer (t) RNAs, and a multitude of other non-protein coding RNAs [24], many of which are involved in protein output (reviewed in [25, 26]). Many of these well established genes and some newly identified loci have been recently characterized as bona fide Pol III targets in mammalian cells in genomic ChIP-seq experiments [27–29] that further revealed a surprising complexity to Pol III regulation and function.

Consistent with their essential role in active growth, Pol III transcripts are frequently elevated in tumor cells [30–33], and many of these same cells exhibit defects in the RB pathway. Indeed, the RB tumor suppressor was shown to repress global Pol III transcription both in vitro and in vivo [34]. From these early studies, a biosynthetic capacity hypothesis was proposed that Pol III inhibition by tumor suppressors, including RB, limits cellular proliferation [34, 35]. Supporting the concept that Pol III activity is limiting to robust cancer proliferation, the forced reduction of Pol III activity in mouse xenograft assays could inhibit tumor formation [36]. An integral connection between enhanced Pol III activity and cellular dysfunction in human cancer is also suggested from the observation that scleroderma patients who exhibit autoantibodies against Pol III also are more likely to be diagnosed with some forms of cancer than similar patients who don't express elevated Pol III antibodies [37]. These observations hint that Pol III levels or activity may be a prelude to clinically evident disease in a subset of cancer patients. Pol III-associated structures and activity may also serve as useful biomarkers for cancer diagnosis and disease staging as some Pol III transcripts are localized to nuclear structures termed perinucleolar compartments (PNC) [38], and increased PNC prevalence has been positively correlated with tumor progression

and poor prognosis for patient survival [39–43]. While the function of the PNC remains enigmatic, these observations provide support of the hypothesis that increased steady state levels of Pol III transcripts are an important contributing factor in cancer progression.

3. RNA Polymerase III transcription during cell cycle progression

The products of Pol III target genes play essential roles in cell growth, and consequently they are often maintained at very high levels during periods of active proliferation. Many of these RNAs exhibit long half lives, and the combination of high steady state levels and long half lives has often lead to the assumption that these “housekeeping genes” are constitutively active. However, it has become clear that Pol III transcription is dynamically regulated both during development and throughout the cell cycle (Figure 2). The highest levels of Pol III activity are typically observed in late G1, S, and G2 phases and lowest levels prevalent during early G1 and M phases [44–48], as determined using extracts prepared from staged cells. While global Pol III activity fluctuates during the cell cycle, Pol III target genes exhibit distinct factor requirements, and its activity at specific loci is governed by stage-specific and gene-specific mechanisms, dependent upon underlying promoter structures, as described in more detail below.

Pol III transcribed genes are grouped into three general types (Figure 3). Both type 1 (5S rRNA) and type 2 (tRNA) Pol III responsive genes contain intragenic promoter elements that recruit TFIIA plus TFIIC, or just TFIIC, respectively (reviewed in [25, 26]). In both types, promoter recognition is followed by recruitment of the Brf1-TFIIB complex, composed of the TATA box binding protein (TBP) [49–52], Brf1 [53–57], and Bdp1 [56, 58–60]. In contrast, Type 3 genes contain extragenic promoter elements, and promoter recognition is accomplished by a multiprotein complex called the small nuclear RNA activating protein complex (SNAPc) [61–66], also known as the PSE transcription factor (PTF) [67–70]. SNAPc cooperates for promoter recognition with a related but different TFIIB complex that contains TBP and Bdp1, but Brf2 instead of Brf1 [56, 57, 71, 72]. Pol III is directly recruited by TFIIB complexes [50, 73–75], and these complexes have emerged as the preeminent signaling nexus governing Pol III activity (Figure 4). Interestingly, both the protein kinase CK2 [48, 76–78] and the Polo-like kinase 1 (Plk1) [79] phosphorylate TFIIB with both positive and negative roles in Pol III regulation, stimulating activity during interphase but inhibiting activity during mitosis. In both signaling pathways, TFIIB phosphorylation disrupts Bdp1 chromatin association during mitosis with consequent diminished transcription [46, 80, 81]. One prediction is that CK2 can phosphorylate Bdp1 to affect global Pol III activity, while Plk1 phosphorylates Brf1 and affects only type 1 and 2 genes, providing regulatory flexibility within the Pol III network. CK2 can also phosphorylate SNAPc [82] and affect cooperative DNA binding by SNAPc and TBP [83, 84], but any contributions to type 3 gene regulation during cell cycle progression have not been established. In addition to regulated output during the S phase and M phase of the cell cycle, Pol III activity is generally low during early G1 phase. Interestingly, a robust increase in steady state B2 RNA levels was observed after serum stimulation [85], and this rapid elevation in endogenous B2 transcripts was sensitive to ERK kinase inhibition, linking increased Pol III output to the Ras pathway. Like the other kinase pathways described above, ERK can interact with and phosphorylate the Brf1 component of TFIIB. However, the ERK docking site is absent in Brf2, suggesting that type 3 genes might respond differently to signaling through the Ras pathway

4. Role of RB in RNA polymerase III transcription

Early observations suggested that Pol III activity is diminished in early G1 phase of the cell cycle, a period that correlates with increased RB activity [47, 86], and thus efforts were

initiated to investigate the contribution of RB to Pol III regulation. From these pioneering studies, a clear role emerged for RB [34] and its related family members p107 and p130 [87]. The first report connecting RB regulation to Pol III transcription demonstrated that RB-deficient human Saos2 osteosarcoma cells support elevated Pol III transcription as compared to RB-proficient U2OS osteosarcoma cells. Primary mouse fibroblasts deficient for RB also supported elevated Pol III transcription as compared to matched wild type MEFs in nuclear run-on assays, thus providing strong evidence that RB functions as a global repressor of Pol III activity in vivo [34]. Pol III transcription of an adenovirus VAI construct, a type 2 gene, could also be repressed by recombinant RB minimally containing the AB pocket and C-terminal domains. This observation was significant because previous studies on RB repression of E2F-mediated Pol II transcription demonstrated that these same regions were important for cell cycle control and tumor suppression [88–91]. In addition, the RB-related family members p107 and p130 were found to repress Pol III activity [87], and as these RB family members exhibit different activity profiles during cell cycle progression [92–94], these members might contribute to regulatory capability at distinct cell cycle stages [95] and in different tissue types.

A potential repression mechanism for type 1 and type 2 gene regulation, shown in Figure 5, was first established when it was observed that RB could co-purify with Brf1-TFIIB, both in co-immunoprecipitation assays and in biochemical fractionation of Brf1-TFIIB from transcriptionally active cell extracts [96, 97]. This connection had direct functional implications given the important role for the Brf1-TFIIB complex in Pol III recruitment. Consistent with the notion that the RB-Brf1-TFIIB connection is relevant, only the hypophosphorylated and active form of RB commonly observed in early G1 phase was found to associate with Brf1-TFIIB [95]. In vitro, Brf1-TFIIB activity became limiting upon RB addition to Pol III repression assays [96, 97], suggesting that this complex is the functional target for RB during repression. In addition to Brf1-TFIIB, RB was observed to associate with both Brf1-TFIIB and TFIIC2 in one study [97], and a competitive equilibrium model of RB repression was proposed wherein RB disrupts communication between TFIIC2 and Brf1-TFIIB [97]. Both of these studies led to the model that RB represses Pol III transcription by disrupting key interactions among the basal transcription machinery [96, 97].

During this initial period of RB experimentation for Pol III repression, concomitant studies of Pol II regulation had demonstrated that RB could inhibit cell cycle gene expression by stably associating with E2F at target gene promoters [98]. Some of these studies further indicated that RB directly disrupts preinitiation complex assembly involving TFIID and TFIIA on naked DNA templates [99]. An alternative mechanism involving chromatin modification was suggested from studies demonstrating that E2F/RB interacts with and recruits histone deacetylases (HDAC) [100–102] and ATP-dependent chromatin remodeling complexes [103, 104] to Pol II-transcribed target genes activated by E2F. In these studies, HDAC inactivation with trichostatin A also inhibited the RB-mediated repression. RB was further demonstrated to co-purify with E2F-1, HDAC1 and DNA methyltransferase (DNMT) 1 with RB and DNMT1 cooperating to repress transcription of E2F-activated genes [105]. In this E2F model, RB repression involves chromatin regulation at both the DNA and histone levels. Interestingly, RB repression of Ad VAI gene expression by Pol III was not sensitive to HDAC inhibition with trichostatin A, suggesting that a similar chromatin-based repression mechanism might not be involved for type 2 genes. Results from template commitment assays instead suggested that recombinant RB could disrupt a pre-formed preinitiation complex and in coimmunoprecipitation studies RB impeded Brf1 association with TFIIC and with Pol III, but not with another member of the TFIIB complex, TBP [106]. These observations reinforced the concept that RB disrupts communication between members of the Pol III general transcription machinery to enact

repression. In contrast with the prevalent RB-E2F model for repression of cell cycle genes, stable association by RB at a type 2 locus was not observed [107], suggesting that RB prevents pre-initiation complex assembly by blocking Brf1-TFIIB loading, or in cases wherein the Brf1-TFIIB/TFIIIC complex is formed, RB may utilize a transient hit and run mechanism to disassemble Brf1-TFIIB from the preinitiation complex.

Although RB represses Pol III globally [34], distinct factors are involved in Pol III transcription of the divergent type 3 target genes, and consequently a different repression mechanism is employed to regulate expression of genes within this category [108]. Most notably, RB was observed to stably associate with the endogenous RNU6-1 locus in RB proficient cells [107]. Thus, a model involving a simple hit and run mechanism was not supported, but rather, RB repression more closely resembled some aspects of the E2F model that require substantial RB residency at target promoters. Robust protein-protein interactions between RB and two components of SNAPc, SNAP43 and SNAP50, and with TBP were observed in vitro, suggesting that promoter targeting involves multiple contacts within the preinitiation complex. Both promoter targeting and general transcription factor interactions with RB were reliant upon the AB pocket domain and C terminal region. In these studies, RB did not interact with Brf2 in vitro. Thus, RB-TFIIB interactions may be more critical for type 1 and type 2 gene repression than for type 3 genes. Nonetheless, TBP activity was limiting during RB repression assays in vitro [108] and it remains possible that Brf2-TFIIB plays an important role in RB repression. Interestingly, RB association with the RNU6-1 locus was detected simultaneously with components of SNAPc and Pol III [107], a phenomenon that was also observed using a U6-1 reporter gene in vitro, suggesting that RB does not necessarily disrupt preinitiation complex assembly. A model was proposed that RB inhibits Pol III promoter escape or elongation during repression of type 3 genes (Figure 6).

5. Genome-wide analyses of RB and RNA polymerase III

To better understand the relationship between global Pol III regulation and RB function in tumor suppression, we compared the outcomes of ChIP-Seq experiments that examined genomic association by Pol III [27] and RB during active proliferation and growth arrest in human IMR90 fibroblasts [109]. As shown in Figure 7, RB was detected at both type 2 and 3 Pol III genes in actively growing cells. RB was found to associate with more type 3 genes during arrested growth, both during quiescence induced by serum starvation and senescence induced by stable expression of a transgene expressing dominant active Ras. Interestingly, increased RB association at all three types of genes was noted during senescence. As induction of senescence represents a major mechanism for tumor suppression by RB, these observations are consistent with Pol III repression contributing to key cancer prevention networks.

Surprisingly, the type 2 gene category contained numerically the greatest number of loci that were associated with RB, an unexpected outcome based on a repression model wherein RB primarily blocks preinitiation complex assembly, as noted above. A closer examination of the relative peak positions for RB and Pol III shows that RB was bound remarkably close to the locations occupied by Pol III at some of these sites, consistent with an intimate relationship between RB and the Pol III machinery (Figure 8). In some cases, a single RB peak was observed at a locus that harbors a cluster of tRNA genes, raising the possibility that RB may extend its reach to multiple genes. It should be noted that the functional significance of RB binding at these loci has not been experimentally tested, nor has the binding of RB and Pol III at these loci been determined contemporaneously, such as through sequential ChIP assays. One likely possibility is that RB association at some type 2 genes is fortuitous, and is due to its binding at other nearby target genes, as has been observed for other Pol II factors [28, 110]. Indeed, the position of the RB peaks relative to Pol III was variable among type 2

genes with RB located upstream from the transcription start site in some examples, but downstream from the termination site in others (Supplemental Table S1). The variable positioning of these peaks indicates that an association between RB and other factors besides Brf1-TFIIB likely underlies RB recruitment to these sites. At this time, whether the tethering of RB to neighboring Pol II-transcribed genes can influence expression of a proximally located Pol III gene is uncertain. In an alternative interpretation of this data, stable RB association at type 2 loci is directly relevant to Pol III regulation and could be an important component of the repression mechanism. Indeed, not all tRNA genes are equivalently bound by Pol III, even within an amino acid isotype family, a property that is evolutionarily conserved [111], and thus, RB might be recruited only to tRNA genes that are poised for Pol III transcription. In this case, RB might fine tune Pol III transcript levels via regulation of only select targets within each category depending upon the growth conditions and gene utilization by the Pol III machinery.

As noted for type 2 genes, RB also resided at some, but not all, type 3 genes in actively growing cells with RB residency observed at an increased number of loci during growth arrest. This observation is additionally consistent with a role for RB in regulation of some type 3 genes during regular cell cycle progression. Surprisingly, RB did not associate with all members within a gene family, best exemplified by the U6 gene family, which contains five actively transcribed members [112], but with only two of these exhibiting RB association. The identity of type 3 genes considered in this analysis and their RB status is shown in Table 1. Some type 3 genes did not exhibit RB association under any conditions, even though these putative RB targets harbored SNAPc and TBP (data not shown), which were previously suggested as critical for RB recruitment [107]. In vitro repression assays suggest that at least one of these candidates that lacked RB association, the Y1 gene, was sensitive to RB expression during transient transfection but repression was not as robust as other type 3 genes that were positive for RB in vivo [107]. The significance of these different binding patterns for RB repression remains to be determined, but nonetheless raises the possibility that RB may utilize different mechanisms for repression of genes within a particular family. Whether these RB negative type 3 genes are simply not targeted for repression at all or instead are repressed by a mechanism whereby RB inhibits stable PIC formation but does not reside at the loci, reminiscent of that proposed for type 1 and type 2 gene regulation, is unknown.

As with the type 2 genes, the peak locations for type 3 RB positive genes and the Pol III apparatus suggest an intimate relationship between the repressor and the basal apparatus, and is consistent with previous ChIP assays that examined RB binding at the RNU6-1 locus in human RB proficient cells [107]. Nonetheless, in those studies, RB was observed to co-occupy the RNU6-1 core promoter with SNAPc and TBP, and thus it is surprising that the peak of RB binding at type 3 loci in IMR90 fibroblasts was not within the core promoter but rather was variably located within the enhancer regions (Figure 9). Thus, one question remains as to how RB can establish contacts with the preinitiation complex from this distal location. It was previously demonstrated that the U6-1 promoter harbors a nucleosome positioned between the PSE and the DSE that is important for activated Pol III transcription [113]. DNA wrapping around the nucleosome juxtaposes the activator, Oct1, and its target, the core transcription factor, SNAPc [113–115]. In this scenario, RB positioned distally to the nucleosome would similarly be brought into position to its targets within the core machinery by DNA wrapping. Consistent with the involvement of chromatin modifying factors in RB regulation of Pol II transcription, RB expression by transient transfection facilitated DNMT1 recruitment to the RNU6-1 locus [116], suggesting that this and other chromatin modifying factors contribute to Pol III regulation during tumor suppression, a topic for future efforts. Together, these studies have uncovered an unanticipated diversity of RB tumor suppressor function in the control of Pol III.

Supplementary Material

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Acknowledgments

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Figure 1. RB repression of cell cycle target genes is relieved by cyclin/cdk phosphorylation
 During early G1, RB can form stable complex at cell cycle target gene promoters through interactions with the E2F/DP transcriptional activator, interfering with transcriptional activation. Repression can be mediated via disruption of the general transcription factors (GTFs) or via recruitment of chromatin modifying factors. Later in G1, RB is phosphorylated by the cyclin D/cdk4 and cyclin E/cdk2 kinases to disrupt E2F interaction and relieve repression.

Figure 2. Pol III activity is dynamically regulated during cell cycle progression

During G0 and early G1, Pol III transcription is repressed by RB and its related family members, p107 and p130. Upon RB phosphorylation by cyclin/cdk, Pol III activity increases until peak activity is achieved during late G1 and S phases. In some contexts, Pol III activity increases in early G1 after serum stimulation via Ras activation and ERK stimulation of Brf1-TFIIB in a process that precedes cyclin/cdk activation. The protein kinase CK2 and Polo-like kinase both exhibit positive and negative effects on Pol III transcription, stimulating activity in S phase but inhibiting activity during mitosis. The mechanism for Pol III regulation by each of these factors is different for the distinct types of Pol III genes, as dictated by underlying differences in promoter architecture and factors involved.

Figure 3. Pol III transcribes target genes with divergent promoter structures

Both type 1 (5S rRNA) and type 2 (tRNA) genes exhibit intragenic promoter elements while type 3 genes contain extragenic promoter elements. Transcription from the U6-1 gene is stimulated by a nucleosome that is positioned between the enhancer and core promoter regions. In all cases, Pol III transcription is terminated at a string of thymidine residues (t).

Figure 4. Type 2 and type 3 genes utilize different but overlapping sets of factors for Pol III transcription

(A) Schematic representation of the factors used for Pol III transcription from type 2 promoters. The A and B boxes within the intragenic promoter are recognized by the multiprotein TFIIC complex. Promoter targeting of the Brf1-TFIIB complex, composed of Brf1, TBP, and Bdp1, is facilitated by communication between TFIIC and Brf1-TFIIB. The Bdp1 component of the Brf1-TFIIB complex plays an important role in direct Pol III recruitment. (B) Schematic representation of the factors used for Pol III transcription from type 3 promoters. Core promoter recognition is accomplished by cooperative DNA binding by the multiprotein SNAP complex and a distinct Brf2-TFIIB complex, composed of Brf2,

TBP, and Bdp1. The juxtaposition of the PSE and TATA box dictates that these genes are transcribed by Pol III. DNA binding by SNAPc is also stimulated by the Oct-1 and STAF activator proteins with direct protein-protein contacts potentially enabled by DNA wrapping around a nucleosome positioned between the DSE and PSE.

Figure 5. RB disrupts preinitiation complex assembly at type 2 genes to enact repression
 (A) Assembly of the TFIIC/Brf1-TFIIB preinitiation complex enables active Pol III transcription at type 2 genes. (B) RB interacts with the Brf1 component of the TFIIB complex to prevent preinitiation complex assembly and Pol III recruitment. In vitro template commitment assays indicate that RB can also disrupt a preformed preinitiation complex in a possible hit and run mechanism.

Figure 6. RB forms a stable association with SNAPc, TBP, and Pol III at type 3 genes during repression

(A) Protein contacts from the Oct-1 and STAF activators stimulate preinitiation complex assembly by SNAPc and Brf2-TFIIB during activated Pol III transcription. (B) RB can interact with multiple subunits within the preinitiation complex, including SNAP43, SNAP50, and TBP to form a stable association at the promoter along with Pol III. In this model, stable promoter binding by RB inhibits Pol III promoter escape or elongation, either directly or through effects on chromatin structure at both the histone and DNA levels.

Figure 7. Genome-wide analysis of RB association at Pol III transcribed genes

Chip-Seq data describing the association by RB at genomic targets during active and arrested growth (quiescence, senescence) in human IMR90 fibroblasts [109] was compared to that observed for Pol III [27] with the percentage of RB positive genes for each gene type indicated. Type 3 genes exhibited the greatest percentage of members that were associated with RB, whereas the greatest number of RB positive loci was observed for type 2 genes. The fold enrichment observed for RB positive loci is shown in the supplemental table S2.

Figure 8. RB can associate with local but variable locations at type 2 genes

Schematic representation of the relative peak positions for Brf1 (dashed red), Bdp1 (dashed blue), Pol III (green), and RB (dashed orange). In this example of a tRNA^{Ala} gene, the RB peak was located downstream of the termination site. In other cases, the peak of RB sequence reads was located upstream and adjacently to the peak locations for Brf1 and Bdp1 (not shown). In some examples of clustered tRNA genes, RB associated with only a single member (not shown).

Figure 9. RB associates at variable locations within the enhancer regions of type 3 genes
 Schematic representation of relative peak positions for RB (orange) and Pol III (green) at the RNU6 (ATAC) and RNU6-1 loci. The dashed lines indicate the relative peak positions for SNAP45 (yellow) and Bdp1 (blue) that was observed at the RNU6 (ATAC) loci. In this experiment, association by SNAPc and TFIIB at the RNU6-1 locus were not observed (106) and these peaks are included solely for reference.

Table 1

Comparison of RB and Pol III occupancy at type 3 genes.

Type 3 Pol III ⁺ Genes	Condition			
	<i>Growth</i>	<i>Quiescence</i>	<i>Senescence</i>	
7SK	+	+	+	RB Positive
RNaseP	+	+	+	
U6ATAC	+	+	+	
MRP	-	+	+	
U6-1	-	+	+	
U6-2	-	-	+	RB Negative
U6-7	-	-	-	
U6-8	-	-	-	
U6-9	-	-	-	
Y1	-	-	-	
Y3	-	-	-	
Y4	-	-	-	
Y5	-	-	-	