Conditional depletion of the RNA polymerase I subunit PAF53 reveals that it is essential for mitosis and enables identification of functional domains

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Running title: PAF53 is essential for cell growth

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Abstract

Our knowledge of the mechanism of rDNA transcription has benefitted from the combined application of genetic and biochemical techniques in yeast. Nomura’s laboratory developed a system in yeast to identify genes essential for ribosome biogenesis. Such systems have allowed investigators to determine if a gene was essential and to determine its function in rDNA transcription. However, there are significant differences in both the structures and components of the transcription apparatus and the patterns of regulation between mammals and yeast. Thus, there are significant deficits in our understanding of mammalian rDNA transcription. We have developed a system combining CRISPR/Cas9 and an auxin-inducible degron that enables combining a “genetics-like” approach with biochemistry to study mammalian rDNA transcription. We now show that the mammalian orthologue of yeast RPA49, PAF53, is required for rDNA transcription and mitotic growth. We have studied the domains of the protein required for activity. We have found that the C-terminal, DNA-binding domain (tWH), the heterodimerization and the linker domain were essential. Analysis of the linker identified a putative helix-turn-helix (HTH) DNA-binding domain. This helix-turn-helix (HTH) constitutes a second DNA-binding domain within PAF53. The HTH of the yeast and mammalian orthologues is essential for function. In summary, we show that an auxin-dependent degron system can be used to rapidly deplete nucleolar proteins in mammalian cells, that PAF53 is necessary for rDNA transcription and cell growth, and that all three PAF53 domains are necessary for its function.

Ribosome biogenesis is essential for homeostasis and cell growth, mitotic or hypertrophic. This complex process is
energetically expensive, consuming up to 60% of a yeast cell’s RNA synthetic capacity (1,2). As such, it is subject to multiple levels of regulation. The rate-limiting step in this complex process is transcription of the pre-riboosomal RNA (rRNA) genes (rDNA) by RNA polymerase I (Pol I)(3-5). Abnormalities in the rates of rRNA synthesis and ribosome biogenesis cause a broad range of human diseases, the ribosomopathies (6-10). Furthermore, the pleomorphic nucleoli of tumor cells, the sites of rDNA transcription, are characteristic of neoplasia and reflect dysregulation of rRNA synthesis in cancer (11-15). rDNA transcription requires a unique set of transcription factors not used by the other nuclear polymerases, and we are just beginning to understand the biochemistry and functional interactions of these mammalian factors. Many of the genes whose protein products are involved in ribosome biogenesis were included in CRISPR screenings of essential genes (16-19) and in high-throughput screening of mammalian cells for proteins that affect nucleolar number (20,21). While these procedures reveal proteins involved in ribosome biogenesis, they do not provide for biochemical analysis of function. Nomura’s laboratory demonstrated a screen for mutants deficient for transcription by yeast RNA polymerase I (22,23). These “rrn” mutants identified genes encoding unique subunits of Pol I (22,24), as well as genes encoding other components of the committed template. The development of an in vitro transcription system (25-29) led to the combination of genetic and biochemical approaches that enhanced our understanding of the mechanism of rDNA transcription in yeast.

A fully functional molecule of yeast RNA polymerase I consists of 15 subunits. This total includes the core Pol I, a heterodimer of RPA49-RPA34.5 and RRN3. Five of the core subunits are shared with the other two polymerases and two are shared with Pol III (30,31).

Two of the Pol I subunits, yeast RPA34.5 and RPA49 form a heterodimer with still poorly defined roles in rDNA transcription. The heterodimer of RPA49/RPA34.5 is easily dissociable from the polymerase, and the association of the mammalian orthologues PAF53/PAF49 with Pol I is subject to growth-related regulation (32-35).

While *S. cerevisiae* RPA49 is not essential for viability (36), deletion of yeast RPA49 results in colonies that grow at 6% of the wild type rate at 25C (36). Similarly, when the *S. pombe* orthologue, RPA51, was deleted (37), specific rDNA transcription was reduced 70% (no effect on nonspecific polymerase activity). Deletion of the other partner in the heterodimer, RPA34.5, has a minor effect on growth or rRNA synthesis, but results in a polymerase that loses the RPA49 subunit upon purification (38). Biochemical purification of Pol I results in two fractions, one of which does not contain either RPA49 or RPA34.5 (39). This is complemented by the observation that a large fraction of the polymerase particles prepared for cryo-EM are free Pol I enzymes that “either lacked the RPA49/RPA34.5 subcomplex or displayed a flexible clamp-stalk region” (40). Interestingly, most of the interactions between the heterodimer and Pol I in yeast appear to be mediated by the RPA49 subunit (41). The N-termini of RPA34.5 and RPA49 are required for heterodimerization. The heterodimer stimulates polymerase
nuclease activity and has a triple β-barrel domain similar to the core of TFIIF and the Pol III heterodimer of C37/C53 (42-44). The C-terminus of yeast RPA49 contains a domain with dual winged helices (tandem winged helix, t-WH) (42) that is capable of DNA-binding and resembles a similar element in TFIIE (42) and the Pol III subunit RPC34 (45,46). Mutations within the tWH of RPA49 result in increased sensitivity to 6-azauracil and mycophenolic acid and defects in transcription elongation (47) and lower levels of recruitment of Pol I and Rrn3 at the promoter (36,47).

The mammalian orthologues of yeast RPA49 and RPA34.5, referred to as PAF53, and PAF49, respectively, are essential for promoter-specific transcription (32,33). Yamamoto et al. reported that PAF53 and PAF49 were associated with a fraction of the core Pol I molecules that are active in transcription (32). Hannan et al. confirmed this observation and estimated that 60% of the polymerase molecules in a rat hepatoma cell line contained PAF53 (48). Yamamoto et al. also reported on the growth-dependent nucleolar localization of PAF49, a result subsequently confirmed by our laboratory (34,35,49). Most recently, several CRISPR/Cas9 based screenings of the mammalian genome identified PAF53 and PAF49 as being "essential" genes (16,17), which was confirmed when we found that we could not isolate cell lines that failed to express PAF53 (50) following CRISPR/CAS9 mutagenesis.

The assembly of Pol I-specific "polymerase associated factors" (Rrn3 and the heterodimer of PAF49 and PAF53) with Pol I is a necessary step in rDNA transcription (25,28,32,33,37,47,49,51-74). While genetic studies in yeast and KO studies in mammalian cells demonstrate that the PAF53/PAF49 complex is essential for cellular physiology (17,42,50,75), their roles in rDNA transcription are unknown. Moreover, because there are significant differences in both the structures and components of the transcription apparatus and the patterns of regulation between mammals and yeast, studies on yeast rDNA transcription leave significant holes in our understanding of mammalian rDNA transcription. As transcription by Pol I is essential for cell growth or viability (76-79), it has been impossible to generate cell lines in which these essential genes are knocked out (17,50). While shRNA and siRNA will knockdown the levels of the proteins, this can take days, depending upon the half-life of the protein, and allows compensatory mechanisms to occur. To facilitate the study of rDNA transcription in mammalian cells, we sought to develop a system that would allow us to rapidly knockdown the protein levels and replace them with mutants.

Auxins (AUX), such as indole-3-acetic acid (IAA), promote the interaction between SCFTIR1 (TIR1), an auxin-dependent E3 ubiquitin ligase (80-84) and the AUX or IAA family of transcription factors (85,86). In the presence of auxin, TIR1 associates with the AUX/IAA transcription factor. This induces the rapid ubiquitination and subsequent proteasome-dependent degradation of transcription repressors that contain an auxin inducible degron (AID). The AID from the IAA17 protein of Arabidopsis thaliana has been used to program proteins for rapid degradation in yeast and animal cells (80,81,87).

We investigated the use of that system to study transcription by Pol I in mammalian cells focusing on PAF53. Using CRISPR/Cas9, we tagged the C-
terminus of the endogenous PAF53 genes with a 43 amino acid AI) from IAA17 (aa 68-111) using homology directed repair and a donor fragment with short homology arms (~125 bp). When cells that expressed TIR1 from Oryza sativa (81) (tagged with a nuclear localization signal (NLS-TIR1)) and PAF53-AID were treated with IAA, the PAF53-AID was eliminated within one hour. This resulted in the inhibition of rDNA transcription and caused cell cycle arrest. Using cell proliferation as the phenotype, we investigated the domains of PAF53. \textit{In silico} analysis of mammalian PAF53 revealed structures strikingly similar to that of yeast RPA49. These included the N-terminal dimerization domain, a linker and the C-terminal tandem winged helix. We have previously confirmed the functional homology of the N-terminal dimerization domain (88) to yeast RPA49, and we now demonstrate that the C-terminal tWH has DNA-binding activity similar to that of the yeast domain. We found that deletion of the N-terminal dimerization domain significantly inhibited the ability of ectopic PAF53 to rescue cell division. Interestingly, deletion of the C-terminal tWH had an intermediate effect on rescue, while deletion of the linker inactivated PAF53. We identified a DNA-binding motif in the linker and found that DNA-binding by the linker was required for both PAF53 and RPA49 activity. Finally, our data imply that the binding of PAF53 to Pol I requires dimerization with PAF49 a result that would be different from that found in yeast.

\textbf{Results}

Our first experiments were designed to determine whether a nucleolar protein could be targeted for degradation by TIR1. As shown in Figure 1, we found that when amino acids 1-229 of \textit{A. thaliana} IAA17 were linked to H2B tagged with YFP and an AID (Figure 1B), the protein was degraded when HEK293 cells were cotransfected with a vector expressing TIR1 and treated with IAA. Subsequently, we found that the same AID could target PAF53 expressed transiently (Figure 1C). We also found that the 43 amino acid degron (aa 68-111) of IAA17 would drive auxin-dependent degradation in HEK293 cells (Figure 1C). We then examined the possibility of using an Aux/IAA 13–amino acid domain II consensus sequence, aa 82-94, that has been shown to work in plants (82). That domain did not work in our cells (Lanes 5 and 6, Figure 1C). These experiments also serve to demonstrate that activation of TIR1 by IAA did not target the endogenous PAF53 (lower band in Panels C and D). Subsequently, we have used the 43 amino acid degron as the AID in our constructs. Nishimura \textit{et al.} (80) reported that the AID tag could be on either the N- or C-termini of proteins and still support auxin induced degradation. As shown in Figure 1D, we confirmed that in mammalian cells expressing TIR1, the AID can be on either terminus of the target protein, and as discussed below, it can be internal.

Based on these observations, we established cell lines that constitutively expressed TIR1. However, we found that these cell lines did not degrade AID-tagged PAF53 as rapidly as predicted (data not shown) from the transient transfection experiments. We considered two models. Either we did not have the same levels of TIR1 expression in the stable cell lines as in the transiently transfected cell lines, or the TIR1 was not localizing to the nucleus/nucleolus. We
examined the sequence of *Oryza sativa* TIR1 and found it did not contain a nuclear localization signal (NLS). A monopartite NLS (PAAKRVKLD) was added to the N-terminus of TIR1 and cells that constitutively expressed NLS-TIR1 were selected with hygromycin. One clone, 569, that demonstrated the rapid degradation of transiently transfected PAF53-AID was chosen for further cloning experiments. Clone 569 was then subject to CRISPR driven homology directed repair to introduce the AID onto the C-terminus of the endogenous PAF53 genes using the strategy summarized in Figure 2A. After selection and cloning by limiting dilution, the clones were screened by western blots for expression of the chimeric PAF53 to select for cells homozygous for AID-PAF53 expression (Figure 2B). The two forms of PAF53 seen in lane 1 are the result of tagging only one allele and lane 2 represents the results of tagging both alleles. Further, recombination was confirmed by PCR for the recombinant insert (Figure 2C). The PCR products were cloned and four clones were sequenced. The sequences were identical, and did not demonstrate errors in recombination (data not shown).

Clones that only expressed the AID-tagged PAF53 were selected, expanded and stored. We found that the anti-PAF53 antibody recognized two bands in some of the clones (Figure 2D). The major band, migrating at ~58 kDa, was at the molecular mass predicted for the AID-tagged PAF53 (P-A). The upper band migrated with a mass predicted if it were the complete PAF53-AID-F2A-NPT chimeric protein (P-A-N), ~87 Kda, due to incomplete F2A-dependent cleavage. When IAA was added to these cells, we observed a rapid degradation, within one hour, of both bands that reacted with anti-PAF53 antibodies (Figure 2D, lanes 2 and 4). This confirmed that they were the bands predicted and demonstrated that the presence of an AID in the middle of a protein could target the protein for degradation.

As introduced, the role(s) of yeast RPA49 in rDNA transcription are not clear. When mammalian cells were screened for genes “required for proliferation and survival”, Wang *et al.* reported that the PolR1E (PAF53) gene was essential. This result was confirmed by Bertomeu *et al.* (16) and others (50). Thus, we sought to determine the specific effect of knocking down PAF53 using the AID as both physiological and biochemical results should be apparent very rapidly.

As shown in Figure 3A, treatment of clone 763 that expressed both TIR1 and AID-tagged PAF53 for three hours with IAA resulted in the inhibition of rDNA transcription as demonstrated by metabolic labeling of total cell RNA. This experiment has been reproduced at least three times and we observed at least 99% inhibition of synthesis of the 47S pre-rRNA in all experiments. The inhibition of rDNA transcription can induce a phenomenon referred to as “nucleolar stress” or “ribosome stress”. The cellular responses to this stress can include cell cycle arrest or cell death (77-79,89-94). When clone 763 cells were exposed to IAA, they appeared to complete one round of division and then arrest (Figure 3B). The cell cycle arrest was stable for up to nine days with no apparent increase in cell death. To confirm that the cell-cycle arrest was due to the knockdown of PAF53, we sought to determine if the ectopic expression of the protein would rescue the arrest. Clone 763 cells were transfected with pCDNA3.1 driving the expression of mouse PAF53 and a vector driving the
expression of eGFP two days before the cells were treated with IAA. Control cells were transfected with an empty vector and the vector driving eGFP. Subsequently, the number of viable cells (trypan blue exclusion) was measured in each of the three groups. As shown in Figure 3, expression of ectopic PAF53 rescued rDNA transcription (Figure 3A, lanes 4) and prevented cell cycle arrest (Figure 3B). Western blot analysis (Figure 3C) confirmed the expression of the mouse homolog and the lack of expression of the endogenous AID-PAF53 throughout the time course of the experiment.

Clone 763 cells depleted of PAF53 did not demonstrate a net proliferation. This could be the product of cell cycle arrest or a steady-state relationship between cell division and cell death. In order to examine this question, we examined the cells for trypan blue dye exclusion. We did not observe a significant increase in trypan blue staining following treatment with IAA, indicating that we were not causing cell death (data not shown). The inhibition of rDNA transcription has been shown to cause cell cycle arrest at the M/G1 boundary. However, the effect of rapidly targeting rDNA transcription has not been previously studied. Previous studies have used Cre-dependent recombination to knockdown RRN3 and UBF (95,96), two components of the rDNA transcription apparatus (97). This process takes much longer than an hour and can cause apoptosis (96). As shown in Figure 4, twenty-four hours after the depletion of PAF53, there was no alteration in the distribution of cells through the cell cycle. However, after two days, we observed a decrease in the percentage of the cells in G1 and an increase in the percent of cells in S (Figure 4B). The increase in the S/G1 ratio was significant after 48 hours (Figure 4C). Interestingly, we did not observe a significant accumulation of cells in either the G1 or G2 checkpoints.

As shown in Figure 5, yeast RPA49 can be considered to consist of three domains an N-terminal dimerization domain that mediates the interaction with RPA34.5, a C-terminal tandem winged-helix (t-WH) that has DNA-binding capacity and a linker domain between the two [(88) and SWISSMODEL, Q9GZS1, see also PDB entry 3NF1]. Both SWISS MODEL and I-TASSER predict structures for mouse and human PAF53 similar to that of yeast RPA49 despite <25% sequence identity (98-102). We have previously demonstrated that the N-terminal domains of mouse PAF53 and PAF49 are required for dimerization, and are predicted to contain the triple barrel β-structure found in the RPA49/RPA34.5 heterodimer and in TFIIF (42,44,88,103). Interestingly, I-Tasser and SWISSMODEL (Q9GZS1) predict similar structures for the C-terminal domains of the yeast (NP_014151.1) and mammalian orthologues (human NP_071935.1 or AAH14331.1) despite their being only 22% identical. While it has been demonstrated that the t-WH of yeast RPA49 has DNA-binding activity, the same has not been reported for the predicted t-WH of the mammalian orthologues. As shown in Figure 6A, we determined that human PAF53 was capable of binding to DNA. Moreover, we found that the N-terminal dimerization domain was not required for DNA-binding (Figure 6B. aa109-435; lanes 2 and 3). The t-WH domain, amino acids 216-435, also bound DNA (lanes 4 and 5). However, when the N-terminal WH was deleted, the remainder lost DNA-binding activity (lanes 6 and 7). Thus, both the yeast and mammalian orthologues

These results suggest that the N-terminal dimerization domain is not essential for DNA binding in mammalian cells, whereas the C-terminal t-WH domain is crucial for DNA binding in yeast. Moreover, the yeast RPA49 t-WH domain shows a higher degree of conservation in mammalian orthologues, indicating a conserved role in DNA binding.
contain N-terminal dimerization domains and C-terminal DNA-binding domains.

We then sought to determine which, if any, of the domains of the protein were essential for cell cycle progression. Two days prior to treatment with IAA, cells were transfected with vectors expressing various deletion mutants of mouse PAF53 (Figure 7A). We found that a construct lacking the N-terminal dimerization domain (PAF53 aa109-435) failed to restore cell division (Figure 7D). The N-terminal dimerization domain (PAF53 aa1-160) itself did not rescue cell division either (Figure 7C). Surprisingly, we observed partial rescue with a construct that did not contain the C-terminal tWH, but did contain most of the dimerization domain and the linker (PAF53aa1-222) (Figure 7B). These three constructs would appear to be properly folded, as deletion of the dimerization domain did not inhibit DNA binding by the t-WH (Figure 6B lanes 4 and 5) and deletion of the t-WH did not inhibit dimerization (88). Further, all three constructs were expressed for the duration of the experiment (Figures 7E and F). Thus, the linker region between the dimerization and tWH domains appears to be a functional domain.

When we examined the structure of the linker, we noted that both mammalian PAF53 and yeast RPA49 contain a helix-turn-helix motif (HTH). It has been reported that the linker spans the cleft in Pol I (31,104). We hypothesized that the HTH might come in contact with the template and that the linker might participate in transcription due to DNA-binding activity. When we expressed His-PAF53 aa1-222 and tested it in a DNA-binding assay, we found that the purified, recombinant protein had DNA-binding activity (Figure 8). To examine the role of the putative HTH in DNA binding, we mutated each of the helices individually (Figure 9, panels A and B) and tested equal amounts of the recombinant proteins for DNA binding activity. As shown in figure 9, panel C, mutation of either of the helices significantly inhibited DNA binding. Thus, mammalian PAF53 has two domains with DNA-binding activity, the tWH and the helix-turn-helix of the “linker”. Subsequently, we carried out rescue experiments using PAF53 constructs that contained similar mutations of the HTH. As shown in Figure 10, cells expressing PAF53 with the HTH mutated failed to divide following IAA treatment.

To understand if the PAF53 HTH is functionally conserved across species, we used yeast growth assays to examine the effect of an HTH deletion in the yeast PAF53 orthologue RPA49, and compared its growth to several well-characterized RPA49 mutant variants. Consistent with previous reports, transforming the RPA49 deletion strain with the wild-type RPA49 expression vector or a mutant variant lacking the non-essential N-terminal dimerization domain significantly restored cell growth at 30°C similarly to wild-type RPA49 (38,42,47) (Figure 11). Likewise, expression of an RPA49 variant lacking the C-terminal tWH failed to rescue growth and remained slow growing as previously observed (36,42,47) (Figure 11). Finally, we tested the RPA49 variant lacking the HTH, which retained a slow growth phenotype (Figure 11), indicating that the HTH is important for RPA49 function in yeast. The slow growth of the HTH deletion was further exacerbated at 18°C (Figure 11). This is a common characteristic of yeast strains with defects in ribosome biogenesis (105). Interestingly, the mutants with deletions of the HTH domain grew less well at 30C
than cells transfected with empty vector (pRS424). Together, these results are consistent with our PAF53 cell viability assays, further emphasizing the functional importance of the HTH in Pol I transcription across species.

**Discussion**

We have established that the TIR1, auxin-dependent degron system can be used to rapidly deplete nucleolar proteins. Our data demonstrate the rapid, (<1hr.) auxin-dependent degradation of a nucleolar protein, PAF53 and the feasibility of using this approach to study the function of those proteins in mammalian cells following depletion or replacement with mutant forms of the protein. In our initial investigations of the feasibility of this system, we found that we had to optimize the system in order to apply it to nucleolar proteins.

We found that the degradation of a nuclear protein was facilitated when we added a nuclear localization signal to osTIR1. We also found that the minimal conserved core of domain II of the IAA17 degron was insufficient to target degradation, and that the 44 AA degron we used, could be placed on the N- or C-terminal of the target or internal.

The yeast RPA34.5 and RPA49, and their mammalian homologs, PAF49 and PAF53, are structurally similar. Figure 5 compares the structures of yeast RPA49 and the structure predicted for human PAF53 by I-TASSER (101,102,106). The three domains, dimerization, linker and tandem winged helix (tWH), of RPA49 are indicated (88).

As discussed, yeast RPA49 is not essential for cell division (see Figure 11, pRS424 empty vector). When we depleted PAF53, our HEK293 cells failed to divide. A priori, we expected to see arrest at either the G1/M or G2/S interfaces. However, we did not. There was a slight accumulation of cells in S, but no distinct arrest in either G1 or G2. This is in contrast to the patterns observed when RRN3 and UBF were knocked down (95,96). In those instances, the investigators observed accumulation of the cells in either G1 (RRN3) or G2 (UBF) concomitant with a minor inhibition of the distribution of cells in S or its abrogation. Our observation requires further examination. One possibility for this result would be if the cells were proceeding through the cell cycle at a greatly reduced rate.

The interaction of PAF53 with PAF49 requires the first 160 amino acids of PAF53 (88) and the amino acids 41-86 of PAF49 (88). We have previously reported that when PAF49, the mammalian homolog of RPA34.5, is expressed ectopically it binds to Pol I in the absence of ectopic PAF53 (88). Our observation that a form of PAF53 which lacks the heterodimerization domain (PAF53 aa109-435) does not rescue cell growth is strongly supportive of a model in which a significant portion of the interaction of PAF53 with Pol I is dependent upon its N-terminus and perhaps the dimerization domain. That is, the interaction of mammalian PAF53 with PAF49 is necessary for PAF53 function.

The tWH domain of RPA49 has been demonstrated to be a DNA-binding domain, and models of rDNA transcription suggest that the tWH of RPA49 may have multiple roles in rDNA transcription including the stabilization of the open complex by binding upstream DNA (42,107). We have found that the putative tWH of mammalian PAF53 has DNA binding activity and that the C-
terminus of PAF53 is required for full activity. This suggests an essential role for the tWH in mammalian cells. Engel et al. suggest that the tWH domain may move with upstream DNA facilitating the transition from initially transcribing complex to elongating complex and stabilizing that structure(108). This would be consistent with the finding that the tWH domain enhances processivity in vitro (42).

As shown in Figure 5C, we found that PAF53_1-222 was nearly eighty percent as effective as intact PAF53 in its ability to support cell division. In contrast, a mutant that lacks a significant fraction of the linker (aa1-160) was essentially inactive in this assay. When combined with the finding that the dimerization domain itself was necessary to rescue cell growth fully, these results would argue for an essential role for the linker.

Experiments on yeast RPA49 had not predicted a role for the “linker”. The observation that in silico analysis of the linker of mammalian PAF53 predicts a structure similar to that observed for the yeast argues for a conserved function. The predicted structure for mammalian PAF53 contains a helix-turn-helix domain (HTH), a potential DNA-binding domain, that lies very close to the DNA just downstream of where the bubble would form in the closed complex (PDB 5W66). Further, the construct PAF53_1-160 clips the c-terminal helix within this HTH. We then found that this region has DNA-binding activity, and that mutagenesis of the helices alone or in combination (data not shown), inhibited DNA-binding by PAF53_1-222. Likewise, deletion of the HTH in yeast resulted in a molecule that fails to rescue cell growth, consistent with a conserved and essential role for RPA49 in Pol I transcription. Interestingly, the structure of the linker has not always been clearly discerded in structures of Pol I (104), possibly due to dynamics in its interactions with core Pol I or the template. It has been proposed that the linker spans the active site cleft and may in fact play a role in the process of proceeding from initiation to escape (104,109). We are determining the role of the HTH in transcription, e.g. the transition from initiation to elongation.

The placement of the linker vis-a-vis the template in PDB5W66 led us to model a potential interaction with the DNA template. As shown in Figure 12, the linker can be modeled to interact with the DNA template in the closed complex and with one strand of DNA in the open complex. As RPA49 is a single-stranded nucleic acid binding protein it is possible that the interaction of the linker with the template participates in melting, while the interaction of the tWH facilitates elongation.

Our results demonstrate that all three domains of PAF53 are necessary for mitotic cell growth. (1,110-112). The role(s) each of these domains play during rDNA transcription will be the subject of future studies. Most particularly, we found a requirement for both the linker and the heterodimerization domains of PAF53 (88). This latter finding suggests that PAF53 function may be dependent upon the interaction with PAF49. This is in contrast to what is observed in S. cerevisiae and bears further scrutiny. Yeast expressing a mutant RPA49 in which the dimerization domain has been deleted grew nearly as well as wild type at 30C and 18C (Figure 11).

Previously, our lab has demonstrated that inhibiting rRNA synthesis in cancer cells causes cell death while normal cells arrest (113), and ribosome biogenesis has become a target for cancer chemotherapy (14,114-116).
Understanding the role PAF53 plays in Pol I transcription could provide novel drug targets that could be utilized in cancer therapy.

**Experimental Procedures**

**Cell Culture, Transfection, Selection and Analysis**

HEK293 cells (ATCC) were cultured as recommended in DMEM containing 10% FBS and Invitrogen Antibiotic-Antimycotic. Cells were routinely passed at 1:4 dilutions every third day and were not used after the 18th passage. Transfection of 60% confluent HEK293 cells was carried out as described (49,88) using PEI (117). After eight hours, the medium was changed. When selecting for stable transfections, the selection antibiotic was added 48 hr. post transfection. The sequences encoding NLS-TIR1 were cloned in pcDNA3.1/Hygro(+), and selection for expression of NLS-TIR1 required hygromycin (100 μg/ml). When the goal was to recombine into the PAF53 gene, the cells were given puromycin 24 hours following transfection. Forty-eight hours later, the medium was changed to medium free of puromycin, but containing G418 (500 μg/ml) to select for recombinants. In order to determine if specific constructs rescued the ability of cells to divide, the cells were cotransfected with a vector expressing the PAF53 construct to be tested and a vector expressing eGFP to mark the transfected cells. Two days later, 500μM indole acetic acid (IAA) was added to the culture medium. IAA (Abcam) was made fresh by dissolving in water immediately before use. After the indicated time, the number of green fluorescent cells in the population was determined by FACS analysis. At least 10,000 cells were counted for each analysis, which was carried out in triplicate for each time point of an experiment. Cell counts, trypan blue exclusion and cell cycle analysis was carried out as described (113,118).

Metabolic labeling with [3H]-uridine was done as described previously (113). Briefly, clone 763 cells were transfected with empty vector or vector expressing mouse PAF53. 48 hours later, the cells were treated with IAA or vehicle. Three hours later, [3H]-uridine was added to the medium. After 30 minutes, the cells were harvested and RNA was isolated. The isolated RNA was fractionated by agarose gel electrophoresis, stained with ethidium bromide (Ethidium Br), photographed, transferred to PVDF. The filters were dried and impregnated with Enhance (Perkin Elmer) and subject to fluorography as described previously (113).

**Yeast growth assays**

The yeast RPA49 deletion strain used in this study was BY27858/NOY2305 (MATa ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11,15 can1-1 rpa49Δ::His3MX6) and was obtained from the Nomura collection available from the Genetic Resource (NBRP, Japan). This strain was transformed with various high copy number TRP1 marker RPA49 expression plasmids to test their effect on yeast growth. The entire RPA49 gene locus starting 360 base pairs upstream of the ATG start codon and 143 base pairs downstream of the TAG stop codon was cloned into the 2-micron pRS424-TRP1 marker plasmid and the indicated deletion mutations were generated by Quickchange PCR mutagenesis. Yeast cell viability assays were performed on glucose complete (GluC) plates lacking Tryptophan (Trp). Cells were cultured overnight in GluC-Trp media at 30°C until saturated. Equivalent amounts of saturated overnight culture was pelleted
and washed two times in sterile, deionized H2O. Cells were resuspended in 1mL of fresh sterile, deionized H2O and prepared using 5-fold serial dilutions. Cells were spotted onto GluC-Trp plates and grown at 30°C and 18°C to examine yeast cell growth.

**Constructs**

The wild-type and deletion clones of mouse PAF53 used in these experiments were described previously (49,88) and is NCBI Reference Sequence: NP_073722.1. In these clones, PCR was used to insert a FLAG-tag onto the N-terminus of wild-type mouse PAF53 cloned in pCDNA3.1 downstream of the CMV promoter. The deletion clones, 1-160 and 1-220 were cloned in pEBG-GST (Addgene). The vector expressing eGFP was described previously (49). All constructs were confirmed by sequencing. The vector expressing AID-H2B-YFP was obtained from Addgene.

**gRNA Design**

We used CHOPCHOP (http://chopchop.cbu.uib.no/) as described (119) to design gRNA. BsmB1 linkers were added to two gRNA targeting exon 12 of human PAF53, TAATCTTCTCCGCTTTGCCAGG and TAGACGCATGCTTTCCAGACAGG (the PAM is underlined), and the oligos were then annealed following a standard protocol and ligated into the vector (120,121), plentiCRISPR v2 (Addgene, (122)). MilliporeSigma supplied the oligonucleotides. The constructs were confirmed by sequencing. The use of plentiCRISPR v2, which is constructed around a 3rd generation lentiviral backbone, allows for the simultaneous infection/transfection of the vector for the expression of Cas9 and gRNA and for selection for puromycin resistance.

**Homologous Recombination**

An oligonucleotide was designed that contained 125 nucleotides of PAF53 coding sequence in frame with DNA that codes for amino acids 68-111 of A. thaliana IAA17. This was followed by GSG and the sequence coding for a F2A self-cleaving peptide (123-125) (VKQTLNFDLLKLADVESNPGP) which was upstream of the sequence coding for neomycin phosphotransferase, a stop codon and the next 125 nucleotides of 3’ noncoding PAF53 exon (Figure 2). The oligonucleotide was synthesized by Genewiz and cloned into pUC57Kan. Cells were transfected with plentiCRISPRv2 coding for the gRNAs that target exon 12 along with the plasmid containing the oligonucleotide for recombination. Twenty-four hours following transfection, puromycin was added to the culture medium (6 μg/ml). After another 48 hr., the medium was changed to medium containing G418 (500 μg/ml) to select for recombination. After 72 hr., the surviving cells were subject to cloning by limiting dilution. After cloning, the success of recombination was confirmed by PCR, as described previously, using the forward and reverse primers, 5’-CAGTCATGTGAGGGTGCTCTCCAGTTC TTCTG-3’ and 5’-GCCCTTGAAGAACTCGTCAAGAAGGC -3’ primers indicated in Figure 2 (50) and western blots for PAF53. The PCR products were cloned in PCR blunt II TOPPO (Invitrogen). Both strands of four separate clones were sequenced. Analysis demonstrated that the sequence of all four clones were identical.
Western Blotting, Protein Purification and EMSA

Cell lysis and western blotting was carried out as described (126) using antibodies to PAF53 (48). The anti-PAF53 antibodies were either raised to recombinant PAF53 in our laboratory (48) or obtained from Proteintech Group and recognizes both human and mouse PAF53. The antibody from our laboratory has been validated previously (35,48), and the depletion of endogenous PAF53 results in a loss of immunoreactivity for both proteins. Recombinant proteins were expressed in either BL21 or Rosetta (Novagen) cells, purified as described previously using either His-, FLAG- or GST- tags (49,55,88) and analyzed by SDS-PAGE and Coomassie Brilliant Blue R staining. To quantitate the amount of protein used in the electrophoretic mobility shift analysis (EMSA), the purified proteins were electrophoresed in parallel with BSA standards. The gels were then stained with Coomassie Brilliant Blue R, scanned with a Chemi-Doc MP Imaging system (Bio-Rad) and the amount of recombinant protein determined by plotting a standard curve for the BSA (127). EMSA was carried out as described previously (55). Binding was carried out in 10% glycerol, 50 mM KCl, 5 mM MgCl₂ and 1 mM DTT using fluorescent tagged DNA, nucleotides -30 to + 10 of the rat rDNA promoter, and no competitor. Gel electrophoresis was carried using Tris (25mM)/glycine (0.19M) buffer at pH 8.3. The gels were pre-electrophoresed for 30 min at 60V before loading and running in the dark. Western blots and shifts were visualized with a ChemDoc MP (Bio-Rad).

Statistical Analysis

All experiments were reproduced at least three times with three technical replicates each time. Quantitative results that required comparisons between groups were subject to statistical analysis using two-tailed Student’s t test for two groups or one way ANOVA followed by Dunnett’s multiple comparison test to determine significant differences among more than two groups. Data met assumptions of the tests (i.e., normal distribution, similar variance). Normality of our data was determined via a D’Agostino-Pearson omnibus normality test and a Shapiro-Wilk normality test. To determine whether the variance differed between groups, an F test was performed when comparing two groups and a Bartlett’s and Brown-Forsythe test was used to compare the variance of more than two groups.
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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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Figure 1. The AID of Auxin/IAA17 of *A. thaliana* imparts auxin-dependent degradation to nucleolar proteins. A. Schematic of IAA17 and the regions used to transfer auxin-dependent degradation. The consensus sequence from Domain II is shown. B. H2B-GFP-IAA17 is targeted for auxin-dependent degradation in HEK293 cells that express TIR1. HEK293 cells constitutively expressing TIR1 were transfected with a plasmid (pcDNA3.1) coding for H2B-GFP-IAA17 for 24 hr., and then treated with 500 μM indole acetic acid (IAA) in water. Amino acids 1-229 of *A. thaliana* IAA17 were linked to H2B tagged with YFP and an AID. Bar = 20 μM. C. Amino acids 1-229 and 68-111 can program PAF53 for auxin-dependent degradation, but the degron core, amino acids 82-
94, does not. In lanes 1-4, ectopic PAF53 was visualized with the anti-PAF53 antibody. In lanes 5 and 6, the ectopic AID-PAF53 was FLAG tagged and visualized with anti-FLAG antibody as the ectopic protein migrated too close to the endogenous protein to be clearly visualized with anti-PAF53 antibody. D. Amino acids 1-229 program PAF53 for auxin-independent degradation if placed on either the N-or C-termini of the protein. The TIR1 used in these experiments did not contain a nuclear localization signal. The analyses presented were carried out after three hours of auxin treatment (500 μM of IAA (+ auxin) or water (− auxin). Western blots for PAF53 were carried out as described (48).
Figure 2. Targeting PAF53 with an AID. A) Design of the oligonucleotide used to recombine the AID sequence into the endogenous PAF53 gene. The oligonucleotide contained 125 bp of exon 12 of the human PAF53 gene in frame with aa 68-111 of IAA17. This was followed, in frame, by GSG, an F2A sequence, DNA coding for neomycin phosphotransferase (NPTII), a stop codon and 125 bp of 3' noncoding sequence from the PAF53 gene. The oligonucleotide was cloned into pUC57Kan, and linearized plasmid was co-transfected along with pLentiCRISPRv2 as described in Materials and Methods. Stable recombinants were selected with G418 and subject to dilution cloning. (B) Examples of monoallelic recombination (lane 1) and biallelic recombination (lane 2) as determined by western analysis with anti-PAF53 antibody. C) PCR analysis of
homozygous recombinants using the PCR primers indicated in Panel A. D) Treatment with 500 μM IAA results in the degradation of PAF53-AID in one hour. The cells of Clone 763 demonstrated two proteins that reacted with anti-PAF53 antibody. The major band, migrating at ~ 58 kDa, was at the molecular mass predicted for the AID-tagged PAF53 (P-A). The upper band migrated with a mass predicted if it were the complete PAF53-AID-P2A-NPT chimeric protein (P-A-N), ~ 87 Kda.
Figure 3. Depletion of the endogenous PAF53-AID results in (A) the inhibition of rDNA transcription and (B) the arrest of cell proliferation, which can be rescued by the ectopic expression of PAF53. A) Metabolic labeling demonstrates the inhibition of pre-47S rRNA synthesis upon depletion of PAF53. Forty-eight hours after clone HEK763 cells, that expressed both TIR1 and AID-tagged PAF53, were transfected with empty vector or vector coding for ectopic PAF53, they were treated with 500 μM IAA for three hours. Following treatment, [3H]-uridine was added to the media for 30 minutes and total RNA was isolated and analyzed as described in Materials and Methods (113). B) Cells
cultured in 30 mm wells were treated with 500 μM IAA at “0” time. All cells were transfected at -48 hr. with either empty vector (control) or pcDNA3.1 expressing wild-type mouse PAF53 (+PAF53). Cells were then harvested at the times indicated and the number of cells determined as described (113). The growth curves were fitted with a polynomial. N=3, +/- Std. Dev. C) The expression of ectopic mouse PAF53 is maintained over the seven day period of the experiments described in Panel A. Cells were treated with IAA as indicated and harvested immediately after the onset of treatment or at the times indicated. The cells were lysed in HEPES lysis buffer and the expression of endogenous or ectopic PAF53 determined by western analysis with anti-PAF53 antibody.
Figure 4. Depletion of PAF53 causes an increase in the S/G1 ratio of cell cycle distribution. (A) FACS analysis of cells at the indicated times post exposure to IAA. (B) Quantitation of the distribution of cells in G_1, S or G_2 following IAA treatment. Three independent repeats of the analyses presented in Panel A were each carried out in triplicate. The data were analyzed by one-way ANOVA. (C) The ratio of cells in S and G1 was calculated for the data presented in Panel B. Significance was determined by a one-way ANOVA. *= 0.05-0.01; **= 0.01 -0.001 ; ***= 0.001 -0.0001;****= <0.0001.
Figure 5. Domains of Yeast RPA49 and Human PAF53. The structure of yeast RPA49 was adapted from pdb5w66. The structures of human PAF53 were generated by SWISS MODEL (98,99) and I-TASSER (101,102,106) as indicated. The dimerization and tWH domains for yeast RPA49 are indicated. Similar structures are apparent in the predicted structures of human PAF53. The domains of yeast RPA49 are adapted from Geiger et al. (42).
Figure 6

A. **PAF53**
   - **Wild-type**
   - tWH
   - linker
   - dimerization

B. **EMSA**
   - **SDS-PAGE**
   - PAF53
   - PAF53-DNA
   - DNA

C. **EMSA**
   - **SDS-PAGE**
   - DNA only
   - 109-420
   - 216-420
   - 326-420
Figure 6. The putative tWH structure of mouse PAF53 has DNA-binding activity. (A) SWISS-MODEL predictions for the structures of the PAF53 constructs used in these experiments. The predicted structure generated by SWISS-MODEL terminates at amino acid 420. (B) Purified, full-length mouse PAF53 has DNA-binding activity. DNA-binding assays (2 pmol DNA) with increasing amounts of PAF53 (0.1-0.5 µg) are presented in lanes 2-4 of the right hand figure in panel B. A coomassie stained gel of the protein used is presented in the left hand figure in panel A. (C) Deletion mutagenesis of PAF53 demonstrates that the PAF53 lacking the N-terminal dimerization domain has DNA-binding activity and that mutagenesis of the tWH inhibits DNA-binding. The indicated mutants were used in the band-shift assays in two different concentrations (+, 0.25 µg and ++, 0.5 µg) in the assays. No shift was seen when two µg of 326-435 was used (data not shown). A coomassie stained gel of the GST-tagged proteins used is presented in the right hand figure in panel C. Intervening lanes containing different amounts of the purified proteins were removed for the composite image. The amounts of the proteins used in the EMSA were normalized to the coomassie stained bands. The conditions for the EMSA were as described in Materials and Methods.
Figure 7. PAF53 function requires all three domains of the protein. Forty-eight hours prior to treatment with IAA, cells were cotransfected with vectors expressing eGFP and either wild-type PAF53 or the indicated deletion mutants of mouse PAF53. At day 0, cells were then treated with IAA (500 μM) to deplete endogenous PAF53. At day 0 or 7 days after IAA, cells were harvested and counted. The growth of the cells transfected with
mutant forms of PAF53 is presented relative to the growth of cells transfected with wild-type PAF53 in matched experiments. (A) PAF53 constructs used in the rescue assays. The predicted structure generated by SWISS-Model terminates at amino acid 420. (B) In comparison to wild-type PAF53, PAF531-222 was approximately 80% as active in rescuing cell division. (C) Further deletion of the linker domain inhibits rescue. PAF531-160 did not rescue cell division. (D) The N-terminal dimerization domain is required to rescue cell division. Deletion of the N-terminal dimerization domain, as in PAF53109-435, inhibits the ability of PAF53 to rescue cell proliferation. The dotted blue lines indicate the cell number that was observed when the cells were treated with IAA and transfected with an empty vector. (E and F) Western blots for PAF53 demonstrate that all three of the deletion mutants tested were expressed for the duration of the experiment. In panels B, C and D, each point represents a separate biological replicate. The error bars indicate the mean +/- S.D. ** Significance was determined by a two-tailed t-test.
Figure 8. **Amino acids 1-222 of PAF53 have DNA-binding activity.** His-PAF53\textsubscript{1-222} (1-2 \mu g), lacking the C-terminal tWH domain, was expressed in BL21, purified by IMAC, analyzed by SDS-PAGE (insert) and assayed for DNA binding activity using the same fragment as in Figure 6.
Figure 9. The helix-turn-helix domain of the linker of PAF53 is responsible for DNA-binding activity. A. Sequences of wild-type and mutant forms of PAF53 1-222. I-tasser prediction of the structure of PAF53 amino acids 140-180 demonstrating (in yellow) the glycine substitution in the two predicted helices. C. Mutation of amino acids 149-151 (H1) or 164-166 (H2) (glycine substitution) reduces DNA-binding activity demonstrated by amino acids 1-222 of PAF53. One-two micrograms of each mutant were used in the
EMSA. The various mutants of His-PAF531-222, lacking the C-terminal tWH domain, were expressed in Rosetta DE3 (Novagen), purified by IMAC, analyzed by SDS-PAGE (insert) and assayed for DNA binding activity using the same fragment as in Figure 6. The coomassie stained SDS-PAGE is a composite picture from which intervening lanes containing different amounts of the purified proteins were removed.
Figure 10. PAF53 function requires the linker HTH to function. Forty-eight hours prior to treatment with IAA, cells were cotransfected with vectors expressing eGFP and either wild-type PAF53 or the indicated HTH mutants of mouse PAF53 or mouse PAF531-222. Cells were then treated with IAA (500 µM) to deplete PAF53. Five days after IAA, cells were harvested and GFP+ cells counted. The growth of the cells transfected with the indicated forms of PAF53 is presented relative to the growth of cells transfected with empty vector in matched experiments. Each point represents a separate biological replicate. ** Significance was determined by a one way ANOVA. The error bars indicate the mean +/- S.D.
Figure 11. Spot assays of yeast transformed with various high copy pRS424 RPA49 expression vectors. The rpa49Δ deletion strain was transformed with the indicated RPA49 expression vectors or with empty pRS424. These strains were tested for growth by plating 5 ul of serial diluted cells onto GluC-Trp plates incubated at either 30°C or 18°C.
Figure 12. Position of RPA49 in the Pol I PIC with respect to either closed or open complex DNA. The RPA49 HTH (red) is in close proximity to closed DNA and spans where DNA is melted and opened in the Pol I PIC. Closed DNA within the active site and cleft was modeled using B-form duplex DNA (104). Pymol was used to visualize the models and colored accordingly.
Conditional depletion of the RNA polymerase I subunit PAF53 reveals that it is essential for mitosis and enables identification of functional domains
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