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PAF53 is essential in mammalian cells: CRISPR/Cas9 fails to eliminate PAF53 expression

Lawrence I. Rothblum[1,*], **Katrina Rothblum**[1], and **Eugenie Chang**[1]

[1]Depoartment of Cell Biology, The University of Oklahoma College of Medicine, Oklahoma City, OK 73104

Abstract

When mammalian cells are nutrient and/or growth factor deprived, exposed to inhibitors of protein synthesis, stressed by heat shock or grown to confluence, rDNA transcription is essentially shut off. Various mechanisms are available to accomplish this downshift in ribosome biogenesis. Muramatsu's laboratory (1) first demonstrated that mammalian PAF53 was essential for specific rDNA transcription and that PAF53 levels were regulated in response to growth factors

While S. cerevisae A49, the homologue of vertebrate PAF53, is not essential for viability (2), deletion of yA49 results in colonies that grow at 6% of the wild type rate at 25C. Experiments described by Wang et al. (3) identified PAF53 as a gene "essential for optimal proliferation". However, they did not discriminate genes essential for viability. Hence, in order to resolve this question, we designed a series of experiments to determine if PAF53 was essential for cell survival. We set out to delete the gene product from mammalian cells using CRISPR/CAS9 technology.

Human 293 cells were transfected with lentiCRISPR v2 carrying genes for various sgRNA that targeted PAF53. In some experiments, the cells were cotransfected in parallel with plasmids encoding FLAG-tagged mouse PAF53. After treating the transfected cells with puromycin (to select for the lentiCRISPR backbone), cells were cloned and analyzed by western blots for PAF53 expression. Genomic DNA was amplified across the "CRISPRd" exon, cloned and sequenced to identify mutated PAF53 genes.

We obtained cell lines in which the endogenous PAF53 gene was "knocked out" only when we rescued with FLAG-PAF53. DNA sequencing demonstrated that in the absence of ectopic PAF53 expression, cells demonstrated unique means of surviving; including recombination or the utilization of alternative reading frames.

We never observed a clone in which one PAF53 gene is expressed, unless there was also ectopic expression In the absence of ectopic gene expression, the gene products of both endogenous genes were expressed, irrespective of whether they were partially mutant proteins or not.

[*]Corresponding author: Lawrence-rothblum@ouhsc.edu.

Keywords

ribosomal RNA; nucleolus; RNA polymerase I; rDNA; PAF53; A49; CRISPR/Cas9; transcription

Introduction

When mammalian cells are nutrient and/or growth factor deprived, exposed to inhibitors of protein synthesis or grown to confluence, rDNA transcription can be repressed by as much as 80–90%. Various mechanisms can be applied to accomplish this downshift in ribosome biogenesis. In some instances, RNA polymerase I (Pol I) is modified such that it is incapable of specific initiation (4–13). One of the gene products responsible for this is *Rrn3* (7), a protein whose association with Pol I is reversible (only ~10% of the Pol I molecules in the cell contain Rrn3). Rrn3 plays a key role in initiation by serving as a bridge between RNA polymerase I and core factor in yeast or SL-1 in mammals (13–16) and by binding to the DNA in both mammals and yeast (17). Phosphorylation modulates the assembly of Rrn3 with Pol I (16, 18) and may determine that Rrn3 functions stoichiometrically (19).

Serum deprivation also causes the dissociation of the PAF53 (PolR1E) and PAF49 (CD3EAP) heterodimer from the polymerase (1, 20–22). PAF53 and PAF49 are the mammalian homologues of yeast rpa49 (yA49) and yeast rpa34.5 (yA34), respectively. The yeast yA34/yA49 heterodimer may play more than one role in yeast rDNA transcription (described below). Muramatsu's laboratory (1) first demonstrated that mammalian PAF53 was essential for specific rDNA transcription and that PAF53 levels were regulated. In contrast, Seither *et al.* reported "constitutive association of PAF53 with Pol I." (23) We have reproduced and extended Muramatsu's lab's observations (21, 22). Interestingly, the yeast heterodimer is easily dissociated from the rest of Pol I, producing a polymerase with impaired transcriptional activity as compared to the complete polymerase (24). In mammals, the heterodimer appears to mediate the interaction between Pol I and UBF (25).

While *S. cerevisae* A49, the homologue of PAF53, is not *essential* for viability (2), deletion of yA49 results in colonies that grow at 6% of the wild type rate at 25C (2). Similarly, when the *S. pombe* homologue was deleted (26), specific rDNA transcription was reduced 70% (no effect on nonspecific polymerase activity), cells failed to grow at 25C and grew at half the wild-type rate at 30C. Deletion of yA34 results in a polymerase that loses the yA49 subunit upon purification (27). A CRISPR screening of the mammalian genome identified PAF53 and PAF49 as being "essential" genes (3). However, it is not known if the deletion of the genes results in reduced viability or merely impaired cell division rates as seen in yeast.

The role(s) of these two proteins in rDNA transcription are still not resolved. While rDNA transcription by Pol I and the ensuing ribosome biogenesis is essential for cell homeostasis and/or growth, it is still not clear if PAF53 and PAF49 are essential for cell division. Recently, (3, 28) the CRISPR/Cas system was used to carry out genome wide queries to identify essential genes in the human genome. Several of the genes identified as being essential were components of the ribosomal DNA (rDNA) transcription apparatus. This included the genes encoding Rrn3, PAF49 and PAF53. In fact, the experiments described by Wang *et al.* (3) identified genes "essential for optimal proliferation", not necessarily genes

essential for viability. Hence, in order to resolve this question, we designed a series of experiments to determine if PAF53 was essential for cell survival.

In one experiment we designed a guide RNA to target exon 2 in the human PAF53 gene. Using that gRNA, we attempted to knock-out the gene and cloned any cells that survived the "knock-out". When we carried out western analysis to determine the expression of PAF53 in those cells, we found that all of the clones expressed a protein that was immunoreactive. In addition, we amplified the "mutated" exon 2 and sequenced the PCR products to determine the nature of putative insertions or deletions within the exon. These results confirmed the western analyses, the cells used alternative reading frames within exon 2 resulting in the expression of a full length PAF53. In a parallel experiment, we carried out the same CRISPR/Cas treatment, but also transfected the cells with a vector that would ectopically express mouse FLAG-PAF53 to see if we could "rescue" the cells with an ectopic form of the PAF whose cDNA was resistant to the gRNA directed to the human coding sequence. We failed to obtain cloned cell lines that did not express PAF53, whether human or mouse. Moreover, the only cell lines that did not express the human form of PAF53 expressed the mouse protein. Thus, our results are consistent with the model that PAF53 is essential for cell survival.

Materials and Methods

gRNA design

We used CHOP**CHOP** (http://chopchop.cbu.uib.no/) as described (29) to design gRNA. Bsmb1 linkers were added to gRNA targeting exon 2, ACAAAGATTCCACCAACCCCAGG and GAACAAAGATTCCACCAACCCCAGG (the PAM is underlined), and the oligos were then annealed following a standard protocol and ligated into the vector (30, 31), plentiCRISPR v2 (Addgene, (32)) and 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.

Expression of ectopic PAF53

The mouse PAF53 clone used in these experiments was described previously (22). In this clone, PCR was used to insert a FLAG-tag onto the N-terminus of mouse PAF53 cloned in pCDNA3 downstream of the CMV promoter.

Transfection and Western blotting

Transfection of 60% confluent 293 cells was carried out as described (22, 33) using PEI (34). In the rescue experiment, the cells were first transfected with a vector driving mouse PAF53 (FLAG-tagged or FLAG-tagged and AID tagged). Twenty-four hours later, they were transfected with a vector coding for puromycin acetyl-transferase, Cas9 and the sgRNA (pLentiCRISPR v2). Twenty-four hours following transfection, puromycin was added to the culture to select for transfected cells. Forty-eight hours later, the puromycin was removed and the cells allowed to grow for four days. At that time, the surviving cells were subjected to cloning by limiting dilution and the clones were expanded to 60 mm dishes. Cell lysis and

western blotting was carried out as described (35) using antibodies to either FLAG or PAF53 ((36)).

Mutation Analysis

Primers were designed that spanned exon 2 of the human PAF53 gene. Genomic DNA was isolated using the Guide-it Mutation Detection kit (Clontech) and 5 μl of diluted lysate was used in a PCR reaction. The PCR products from each PCR reaction were cloned in TOPO-TA Cloning pCR4-TOPO (Invitrogen) and miniprep DNA was sequenced. Multiple clones of the PCR products from each mammalian cell clone were sequenced.

Materials

HEK293 cells were cultured in DMEM-10% FBS and routinely passed every third day. Cells, at 60% confluence were transfected as described above. Twenty-four hours later, puromycin was added and the cultures maintained in the presence of puromycin. Subsequently, the culture medium was replaced with medium without puromycin and the cells cultured for 6 days to allow for selection. In these experiments we did not apply any other selection pressure.

Results

Rescue Experiments

The first series of experiments was designed to yield clones of 293 cells that expressed mouse FLAG-PAF53 and not the endogenous human PAF53. We termed these “rescue” experiments. The possible results included four classes of clones: 1) Clones that would express “wild-type” human PAF53, and ectopic mouse PAF53; 2) Clones that would express ectopic mouse PAF53; 3) Clones that would express “wild-type” human PAF53; and 4) clones that expressed neither. Figure 1, panel A provides western blots demonstrating that we isolated the first three classes of clones. Western blots demonstrated that Clone 4 is a “class 1 clone” and expresses both ectopic and endogenous PAF53 genes, clone 8, a “class 2 clone”, expressed only the mouse gene and clone 14, a “class 3 clone”, expresses the human gene product. We screened more than thirty clones, and did not find any clones that did not express PAF53.

In order to determine the nature of the mutations, we amplified exon 2 from isolated genomic DNA, cloned the PCR products and sequenced the cloned DNA. Sequencing of exon 2 from Clone 4, Figure 1, panel B., demonstrated that one endogenous allele had an in-frame deletion of six base pairs, causing the deletion of two amino acids. On the other hand, the second endogenous allele was not expressed due to recombination with a fragment from an intron (stop codon) from the microcephalin gene of chromosome 8. Thus, clone 4 expressed PAF53 from one endogenous allele as well as the ectopic protein. Analysis of the sequences of exon 2 in Clone 8, Figure 1, panel C., demonstrated that both of the endogenous alleles were mutated. There is a single base pair insertion in one of the genes that results in a stop codon. The second gene contains a two base pair deletion that alters the reading frame and results in a stop codon after splicing. Hence, there is no expression of the endogenous PAF53. Interestingly, sequencing of the cloned exon sequences in Clone 14,

Figure 1, panel D., demonstrated a deletion of 12 nucleotides that results in the synthesis of a protein that lacks four amino acids, but is otherwise intact.

These experiments demonstrated the feasibility of replacing the endogenous human transcription factor with a mouse protein. However, they also suggested that the protein was required for cell survival, we did not identify any clones that did not express either human or mouse PAF53. To examine this question in more detail, the experiment was repeated with the exception that the cells were not cotransfected with the vector expressing mouse PAF53. Following transfection and puromycin selection, the surviving cells were cloned by limiting dilution. Once colonies were in 30 mm dishes, whole cell extracts were prepared and analyzed by western blotting and DNA was isolated from the colony, exon 2 of PAF53 was amplified, cloned and sequenced.

As shown in Figure 2A, all of the colonies obtained expressed proteins that cross-reacted with anti-PAF53 antibody. However, some of the colonies expressed proteins that clearly migrated differently on SDS-PAGE. In order to determine the nature of the putative mutant proteins, we sequenced exon 2 in each of the colonies.

As shown in Figure 2, we found that colonies that expressed PAF53 from two alleles were the only colonies that survived the selection process. The cells that survived were those that either did not demonstrate evidence of Cas9 activity or that had recombination such that the exons contained an open reading frame. In most instances the alternative open reading frame was the result of the deletion of one or two amino acids and was encoded by the exon itself. In some instances, clone PAF53A (Fig. 2B) for example, we found the insertion of as many as 19 amino acids from another source of DNA. Thus, the wild type sequence, STNPRKRNQRIL, was interrupted with 19 amino acids, resulting in STNQPGTAQLGHARADLGEQRPPRKRNQRIL. Interestingly, this was found in both alleles in this clone. We found no modification of exon 2 in the chromosomes from Clone B, *i.e.* no evidence for Cas9 activity. On the other hand, in other clones, such as PAF53C (Fig. 2D), the sequence of one of the PAF53 genes demonstrated a deletion of 3 nucleotides, one amino acid, while the second gene had an insertion of 54 nucleotides that coded for 18 amino acids, LGNVRQKIPSTDLESDLQ, in frame with the coding sequence of PAF53. Hence, the two bands for PAF53 in the western blot. Clone D (Fig. 2E) had two different mutations in the two PAF53 genes. In one allele, we found a deletion of one amino acid that once again resulted in an alternative reading frame mutation or eight amino acids, GRGINGSW at the for the exon. The mutation in the second gene was slightly more complex. We found a single nucleotide substitution mutation, that did not change a codon, and an in-frame nine nucleotide deletion that deleted the amino acids STN from the sequence N-NKDSTNPRKRNQRIL-C. Clone E (Fig. 2F) also had two classes of mutations in the PAF53 genes. One class was similar to that found in clone D, a deletion of nine nucleotides that resulted in the deletion of the amino acids STN from the sequence N-NKDSTNPRKRNQRIL-C. The second was a single nucleotide insertion that resulted in an alternative reading frame N-NKDSTNPGRINGSW-C in place of N-NKDSTNPRKRNQRIL-C.

Discussion

We have described attempts to utilize CRISPR/Cas9 to delete a factor, PAF53, that is part of the Pol I transcription apparatus. While first attempts to eliminate expression of the protein failed, they provide the basis for interesting speculation. While scoring of our pools for in/dels indicated a high efficiency of mutagenesis, none of the isolated clones demonstrated that we had eliminated expression of PAF53. We have characterized over 50 puromycin-resistant clones, and have not isolated any that did not express PAF53. This suggested that the protein was essential for cell survival. This was examined in two sets of experiments wherein we used the same gRNA that targeted exon 2 of the gene and provided an alternative form of mouse PAF53, All of the clones obtained expressed PAF53, endogenous or ectopic, depending upon the experiment. In an unpublished experiment, we targeted two different exons and supplemented with a vector that would express an ectopic form of PAF53 that was resistant to the gRNAs used. In this experiment, we were able to eliminate expression of the endogenous gene, but all of the clones obtained expressed the ectopic protein (data not shown). Thus, while CRISPR has greatly facilitated our ability to modify gene expression, it would seem additional technology will be required to eliminate, at least in a short time frame, the expression of essential gene products. We are presently examining the use of CRISPR to introduce a auxin-inducible degron into the PAF53 gene (37–41). That should allow us to reversibly eliminate expression of the protein.

While we have failed to isolate clones that did not express PAF53. It is theoretically possible that this was due to our experimental design. The experimental process requires that the cells proliferate at a “reasonable” rate, both during the initial selection with puromycin and once subject to cloning by limiting dilution. Thus, cells that would replicate at a significantly reduced rate, might be overgrown in the first phase of the experiment, resulting in their underrepresentation in that population. Moreover, their “lack” of proliferation would preclude their isolation in the second phase of the experiment. Thus, it is not clear if the use of CRISPR/Cas9 alone will allow us to determine if PAF53 is essential for cell viability. Our future goal is to generate a homogenous cell population in which we can program the degradation of PAF53 through the use of a degron. Then we should be able to determine if the lack of PAF53 “prevents” or “inhibits” proliferation. Further, it is possible that the successful deletion of PAF53 will result in cell death due to the inhibition of rDNA transcription (42–49).

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Abbreviations

rDNA	ribosomal DNA
sgRNA	small guide RNA
CRISPR	Clustered regularly interspaced short palindromic repeats

Cas9	CRISPR-associated protein-9 nuclease
Pol I	RNA polymerase I
PAF53	Polymerase Associated Factor 53Kda

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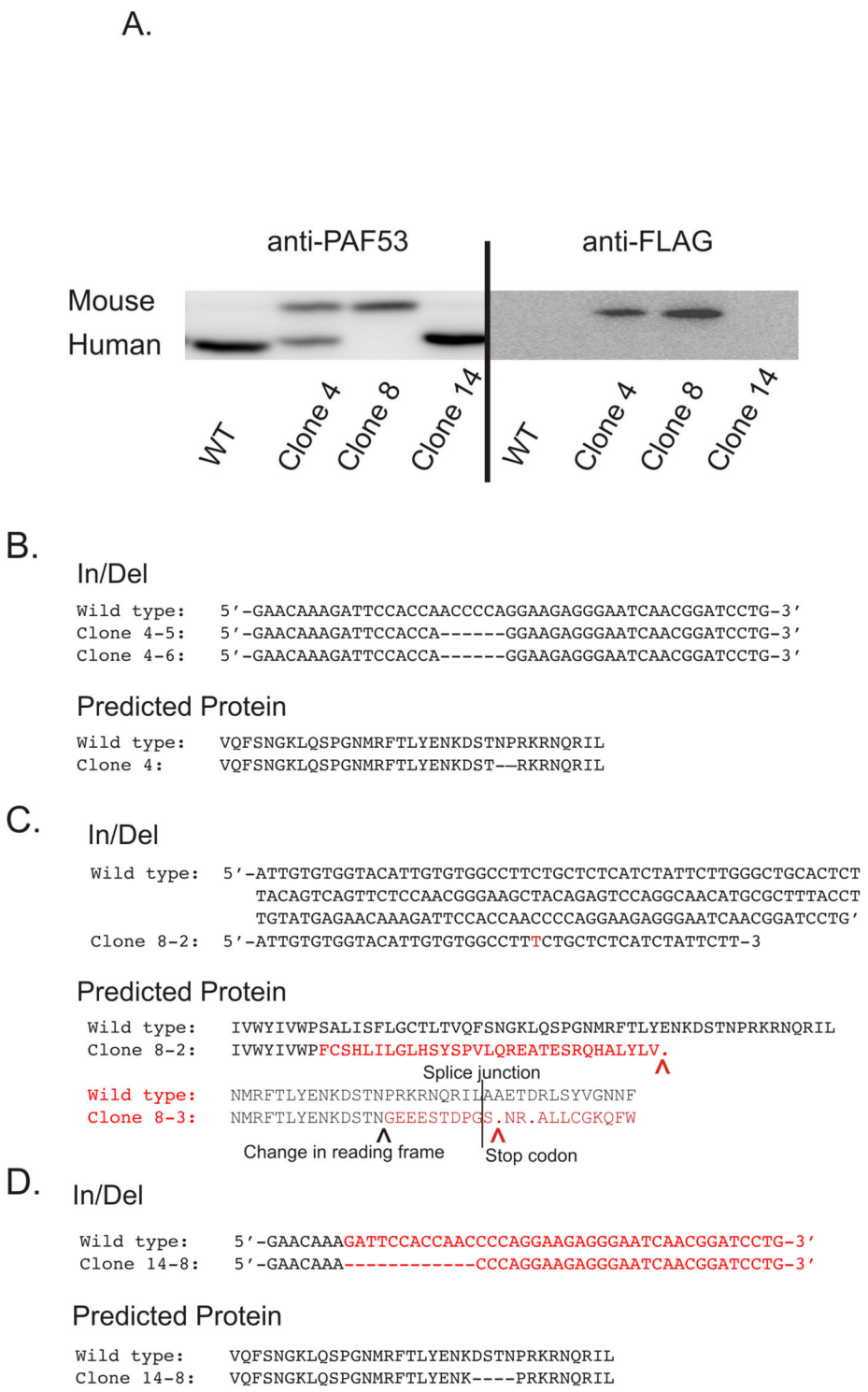

Figure 1.

Analysis of clones resulting from CRISPR of exon 2 of PAF53 and rescue with ectopic FLAG-PAF53. A. Western blots demonstrating the knockout of expression of endogenous PAF53 and rescue with FLAG-PAF53. B. Sequence analysis of exon 2 of the resulting

clones. clone 4 contained one wild type gene. Both of the endogenous genes wee mutated in clone 8. Three PCR clones from clone 14 were sequenced. Two wee mutant and one was wild type. The second numbers in the sequences refer to the cloned PCR products from the genomic DNA from the clonal cell lines.

Figure 2.

Western blot analysis demonstrating the mobility of PAF53 in clones after CRISPR/Cas9 mutagenesis of exon 2 and the sequences of the exons of the clonal cell lines. These cells were not provided with an alternative, gRNA-resistant form of PAF53. B.–F. Sequence analysis of exon 2 of the resulting clonal cell lines. Three or more PCR clones from each cell line were sequenced. The second numbers in the sequences refer to the cloned PCR products from the genomic DNA from the clonal cell lines.