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PABPN1, a target of p63, modulates keratinocyte differentiation through regulation of p63α mRNA translation

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Abstract

p63 is expressed from two promoters and produces two N-terminal isoforms, TAp63 and ΔNp63. Alternative splicing creates three C-terminal isoforms ρ63α/β/δ whereas alternative polyadenylation in coding sequence (CDS-APA) creates two more C-terminal isoforms p63γ/ε. While several transcription factors have been identified to differentially regulate the N-terminal p63 isoforms, it is unclear how the C-terminal p63 isoforms are regulated. Thus, we determined whether PABPN1, a key regulator of APA, may differentially regulate the C-terminal p63 isoforms. We found that PABPN1 deficiency increases p63γ mRNA through CDS-APA. We also found that PABPN1 is necessary for p63α translation by modulating the binding of translation initiation factors (eIF4E and eIF4G) to p63α mRNA. Moreover, we found that the p53 family, especially p63α, regulates PABPN1 transcription, suggesting that the mutual regulation between p63 and PABPN1 forms a feedback loop. Furthermore, we demonstrated that PABPN1 deficiency inhibits cell growth, which can be rescued by ectopic ΔNp63α. Finally, we showed that PABPN1 controls the terminal differentiation of HaCaT keratinocytes by modulating ΔNp63α expression. Taken together, our findings suggest that PABPN1 is a key regulator of the C-terminal p63 isoforms through CDS-APA and mRNA translation and that the p63-PABPN1 loop modulates p63 activity and the APA landscape.

Keywords

p63; PABPN1; alternative polyadenylation; mRNA translation; keratinocyte differentiation

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CRediT Statement

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Data Availability Statement

No datasets were generated or analyzed during the current study.

Conflict Of Interest

The authors declare no competing interests.

Introduction

p63, a member of the p53 family of transcription factors, regulates a diverse array of biological processes, including stem cell maintenance, epithelial cell differentiation, senescence and tumorigenesis (Crum and McKeon 2010; Vanbokhoven et al. 2011). The p53 family consists of p53, p63 and p73, all of which share high sequence similarity (Harms et al. 2004; Yang et al. 2002). Like p53 and p73, p63 is expressed from two different promoters forming two classes of N-terminally distinct isoforms: TA and ΔN (Yang et al. 1998) (Figure 1A). The TA isoform contains an N-terminal activation domain similar to the one present in p53, and thus can activate similar target genes for tumor suppression (Dohn et al. 2001; Harms et al. 2004). The ΔN isoform, on the other hand, harbors a different activation domain, allowing the ΔN isoform to regulate growth-promoting targets (Helton et al. 2006). The TA and ΔN isoforms are also expressed as at least five additional isoforms (α, β, γ, δ and ε) that differ in their C-termini (Mangiulli et al. 2009) (Figure 1A). The α/β/δ isoforms are formed by alternative splicing of exons 12-13 and thus have similar 3'UTR sequences from exon 14 (Mangiulli et al. 2009). However, γ and ε isoforms are produced by alternative polyadenylation (APA) of PAS sites present in exon 10' and intron 10, respectively.

Several p63 mouse models recapitulate the crucial role of p63 in development and cancer. Mice deficient in all p63 isoforms are born with severe developmental defects, including lack of skin, limbs, hair, and mammary gland, and die soon after birth (Mills et al. 1999; Yang et al. 1999). Most of these phenotypes are also present in mice lacking ΔNp63, suggesting an indispensable role of ΔNp63 isoforms in the ectodermal lineage development (Romano et al. 2012). On the other hand, TAp63-deficient mice are born normal but show aging and female germ cell defects (Su et al. 2009; Suh et al. 2006). Moreover, the TAp63 knockout mice are tumor-prone, reinforcing its role as a p53-like tumor suppressor (Su et al. 2010). Conversely, ΔNp63 isoforms behave as oncogenes and are often amplified/overexpressed in cancers (Chakrabarti et al. 2014; Graziano and De Laurenzi 2011). Considering the important and differential roles of p63 isoforms in normal physiology and disease, there is an increasing need to identify factors that control and regulate various N- and C-terminal isoforms. Although several studies have focused on regulation and function of ΔN and TA isoforms (Romano et al. 2012; Su et al. 2010; Su et al. 2009), much less is known about how C-terminal isoforms are regulated.

Cleavage and polyadenylation (CPA) is an essential process in 3' end maturation of mRNAs, that involves endonucleotic cleavage and subsequent polyadenylation of RNAs (Proudfoot 2016). CPA sites, also called polyadenylation sites or poly(A) sites (PAS), consist of several cis elements, including the core AAUAAA hexamer, upstream UGUA and U-motifs and downstream U/GU-rich motif (Shi and Manley 2015). A majority of eukaryotic genes possess multiple PASs mostly in the 3'UTRs and some in the intronic regions. Using alternate PASs, APA generates different mRNA species that contain varying lengths of 3'-UTRs (3'-UTR-APA) or varying coding sequence (CDS-APA) (Tian and Manley 2016). Recently, APA has emerged as a vital player in development, stress response and disease pathogenesis including cancer (Elkon et al. 2013; Xiang et al. 2018). Various studies have identified factors that are involved in APA (Li et al. 2015; Tian and Manley 2016). One such factor is PABPN1, an RNA-binding protein that was discovered through its ability to bind

mRNApoly(A) tails (Wahle 1991). PABPN1 regulates APA by binding and suppressing the use of weak PASs (Jenal et al. 2012; De Klerk et al. 2012).

Here, we analyzed the p63 gene, which contains multiple APA sites. Alternative usage of these sites leads to formation of isoforms that differ in coding sequences. We further identified PABPN1 as one of the factors that regulates CDS-APA of the p63 gene. In this regard, we showed that knock-down of PABPN1 leads to increased expression of p63γ isoform by APA. Notably, we also demonstrate that PABPN1 can regulate mRNA translation of the p63α isoforms. Moreover, we showed that p63α can repress PABPN1 transcription and thus, the mutual regulation between p63 and PABPN1 represents a feedback loop. Functionally, we showed that PABPN1 regulates keratinocyte proliferation and differentiation via ΔNp63α.

Results

Human and mouse p63 gene contains multiple polyadenylation sites

The canonical hexamer, AAUAAA, and its common variant, AUUAAA (Proudfoot 2011; Tian and Manley 2016), are found in 70-75% of mouse and human genes (Tian et al. 2007). These hexamers play a key role in transcriptional termination and their mutation leads to defective 3' end processing (Sheets et al. 1990). Since p63γ/ε are formed by CDS-APA, we manually scanned for these PASs in the human p63ε/γ-specific exons 10a/10' and in α/β/δ 3'UTR in exon 14 (Figure 1A). As illustrated in Figure 1A, we found one canonical PAS in p63γ-specific exon 10' and one in p63ε-specific exon 10a, suggesting that these PASs may be utilized for CDS-APA. We also found two canonical hexamers and two variants in human p63 exon 14, suggesting that p63α/β/δ may undergo 3'UTR-APA (Figure 1A).

Next, to examine if any of the manually annotated sites is utilized, we searched the latest polyA_DB3 database that catalogs CPA sites in multiple genomes (Wang et al. 2018). Unlike its earlier versions that were based on ESTs, PolyA_DB3 contains PASs mapped by the 3' region extraction and deep sequencing (3'READS), making it more accurate and quantitative. Based on the database (Figure 1B-C and Supplementary Tables S1-S2), we found two conserved canonical PASs between mouse and human: one in exon 10' and the other in exon 14. We also found two intronic PASs closer to the promoters in both mouse and human, which are likely to be cryptic. In addition, we found one variant hexamer each in mouse exon 10' and human exon 14, indicating that mouse p63γ and human p63 α/β/δ have a transcript with a shorter 3'UTR. However, we did not find any PAS in p63ε-specific exon 10a, suggesting that p63ε isoform may not be frequently expressed. Since the canonical PAS in exon 10' is used to generate p63γ and the one in exon 14 to generate the predominant p63α, we decided to focus on these two conserved PASs and p63α/γ for further studies.

PABPN1 differentially regulates p63α and p63γ isoforms via APA

Recently, several 3' end formation factors have been shown to regulate APA (Li et al. 2015), including PABPN1 (De Klerk et al. 2012). As PABPN1 regulates both CDS-APA and 3'UTR-APA by suppressing the usage of weaker PASs (Jenal et al. 2012; De Klerk et al. 2012; Li et al. 2015), we examined whether PABPN1 plays a role in p63 APA. To test this,

PABPN1 was knocked down in HaCaT keratinocytes that express high levels of ΔNp63. We observed that p63γ mRNA and protein were increased dramatically upon PABPN1 KD (Figure 2A-B), suggesting that PABPN1 KD leads to increased usage of the weaker p63γ specific PAS. Notably, although PABPN1 KD did not seem to alter p63α mRNA levels, there appeared to be a robust decrease in p63α protein levels, thus showing a differential effect on the regulation of p63α and p63γ isoforms (Figure 2A-B). To confirm this, we attempted to knock out PABPN1 using CRISPR-Cas9. We were able to generate multiple *PABPN1*$^{+/-}$ but not *PABPN1*$^{-/-}$ cell lines, suggesting that PABPN1 is necessary for cell survival, which is consistent with a previous PABPN1 knock-out mouse study (Vest et al. 2017). Nevertheless, we found that in *PABPN1*$^{+/-}$ cells, the levels of p63γ mRNA and protein were increased whereas the level of p63α protein but not mRNA was decreased (Figure 2C-D). As PABPN1 can also regulate 3'UTR-APA (Jenal et al. 2012; De Klerk et al. 2012) and that p63α 3'UTR contains at least 2 PASs, we examined if PABPN1 regulates the length of p63α 3'UTR. For this, RT-PCR was performed to measure the levels of p63α mRNAs with varying lengths of 3'UTRs in PABPN1-KD HaCaT cells. As seen in Supplementary Figure S1, the abundance of p63α mRNA with short or long 3'UTR was not altered, suggesting that PABPN1 does not regulate p63α 3'UTR-APA.

As p63 plays a vital role in tumor suppression and promotion, we examined if PABPN1 regulates p63 isoforms in various cancer cell lines. We found that PABPN1 KD had similar effects on p63 isoforms in breast cancer cell line MCF-7 and pancreatic cancer cell line Mia-PaCa2 as seen in HaCaT cells (Figure 2E-H). As ΔNp63α is amplified in SCC and serves as a prognostic marker, we examined ΔNp63α/γ and found that ΔNp63γ CDS-APA was also regulated by PABPN1 deficiency in Fadu, SCC9 and ME180 (Figure 2I-N). Since we did not have p63γ-specific antibody, we determined whether the band observed in immunoblots is indeed p63γ isoform. We found that the band was seen to be increased in cells with PABPN1 KD alone but decreased in cells concurrently transfected with PABPN1 and p63γ siRNAs (Supplementary Figure S2). As a control, p63γ protein was absent in p63γ KO ME180 cells (Supplementary Figure S2). Taken together, we conclude that PABPN1 differentially regulates p63α and p63γ expression via CDS-APA.

PABPN1 regulates p63α expression through mRNA translation

Based on the observation that PABPN1 KD had no effect on the p63α mRNA levels but decreased the p63α protein, we reasoned that PABPN1 may regulate p63α mRNA translation. Although PABPN1 is mainly involved in nuclear pre-mRNA polyadenylation and poly(A) tail length control, the fission yeast orthologue of PABPN1 is known to associate with polysomes and translating mRNPs (Lemieux and Bachand 2009). In addition, PABPN1 appears to regulate anti-apoptotic protein XIAP expression via translation (Davies et al. 2008). Thus, ^{35}S metabolic labeling was performed to determine the rate of nascent protein synthesis in HaCaT cells in which one allele of the PABPN1 gene was knocked out. Notably, we found that the level of newly synthesized p63α protein was significantly decreased by PABPN1 deficiency in *PABPN1*$^{+/-}$ HaCaT cells (Figure 3A).

To confirm the role of PABPN1 in p63α translation, we examined the binding of the translation initiation factors eIF4E and eIF4G to p63α mRNA by RNA-immunoprecipitation

(RNA-IP) assay. As seen in Figure 3B, the binding of both eIF4E and eIF4G to the p63α mRNA was decreased by PABPN1 deficiency in *PABPN1*$^{+/-}$ cells as compared to isogenic control cells, supporting the idea that PABPN1 is necessary for p63α translation.

PABPC1, a functionally related cytoplasmic PABP protein, is known to directly bind to eIF4G and facilitate mRNA circularization (Imataka et al. 1998; Tarun and Sachs 1996; Wells et al. 1998), which is necessary for the assembly of the translation initiation complex (Jackson et al. 2010). Thus, we examined if PABPN1 associates with eIF4G. To test this, immunoprecipitation followed by western blot was performed. We found that a minute fraction of eIF4G was detected in anti-PABPN1 complexes in HaCaT cells (Figure 3C). Likewise, a fraction of PABPN1 was detected in anti-eIF4G complexes (Figure 3D), suggesting that PABPN1 regulates p63α translation by modulating the binding of eIF4G to p63α mRNA.

PABPN1 transcription is regulated by the p53 family

Several p53 family modulators, such as Mdm2 and Rbm38, are in turn transcriptionally regulated by p53 family proteins (Barak et al. 1993; Shu et al. 2006). Thus, we examined if PABPN1 is regulated by p63. For this, RT-PCR was performed to measure whether PABPN1 mRNA levels were altered in p53-null RKO cells upon ectopic expression of TAp63α. As a positive control, p21, a well-known target of the p53 family (el-Deiry et al. 1993), was measured and found to be induced by ectopic TAp63α (Figure 4A). In contrast, we found that the level of PABPN1 mRNA was decreased by ectopic TAp63α (Figure 4A). We and others have showed that various p63 isoforms have both common and unique transcriptional activities (Dohn et al. 2001; Harms et al. 2004; Helton et al. 2006). To test whether PABPN1 is differentially regulated by various p63 isoforms, the level of PABPN1 was measured in p53-null H1229 cells upon transient expression of myc-tagged TAp63α, ΔNp63α, TAp63γ and ΔNp63γ. As expected, p21 mRNA was induced by the potent transactivator TAp63α or TAp63γ but little if any by ΔNp63α or ΔNp63γ (Figure 4B). Notably, both TAp63α and ΔNp63α seemed to repress PABPN1 mRNA levels whereas TAp63γ or ΔNp63γ overexpression had no effect (Figure 4B). Consistently, the level of PABPN1 protein was reduced by overexpression of TAp63α or ΔNp63α but not TAp63γ or ΔNp63γ (Figure 4C). To confirm these observations, the levels of PABPN1 mRNA and protein were examined in a set of H1299 cell lines that can inducibly express various isoforms of p63. Indeed, the levels of PABPN1 mRNA and protein were decreased in two separate clones upon induction of TAp63α or ΔNp63α (Figure 4D-G) but not TAp63γ (Supplementary Figure S3A-B).

If PABPN1 is directly repressed by p63α, we reasoned that reduction in p63α protein would lead to increased PABPN1 expression. To test this, PABPN1 was measured in p63α-knockout Mia-PaCa2 and HaCaT cell lines, which predominantly express TAp63α and ΔNp63α isoforms, respectively. We found that both PABPN1 mRNA and protein were increased in p63α-knockout Mia-PaCa2 and HaCaT cells as compared to their respective isogenic controls (Figure 4H-K). In contrast, the levels of PABPN1 mRNA/protein were not significantly altered by knockout of p63γ (Supplementary Figures S3C-F).

Since p53 and p63 regulate a set of common and unique target genes, we tested if p53 also regulates PABPN1 expression. We found that the PABPN1 mRNA and protein were

decreased by inducible expression of p53 in p53-null H1299 cells (Supplementary Figure S4A-B) as well as by transient overexpression of p53 in $p53^{-/-}$ RKO or H1299 cells (Supplementary Figure S4C-D). Notably, we found that PABPN1 protein level was decreased by DNA damage in p53-competent but not p53-KO RKO cells (Supplementary Figure S4E). These data suggest that PABPN1 expression can be repressed by DNA damage in a p53-dependent manner and that PABPN1 is a p53 family target.

While a majority of the well-defined target genes are activated by the p53 family, there have been several genes whose expression is repressed by the p53 family (Abraham et al. 2018; Leonard et al. 2011; Ramsey et al. 2011; Truong et al. 2006; Yugawa et al. 2010). The p53 family proteins are known to activate and repress their target genes by binding to a p53 response element (p53-RE) within the promoter or intronic region of the target gene. Upon searching for a potential p53-RE in the PABPN1 gene, we found a putative p53-RE consisting of three half-sites from −969 to −936 upstream of the PABPN1 transcription start site (Figure 4L). To determine if the p53-RE in the PABPN1 promoter is recognized by TAp63α, ΔNp63α or p53, chromatin immunoprecipitation (ChIP) assay was perform ed with H1299 cells uninduced or induced to express TAp63α or ΔNp63α, or with RKO cells mock treated or treated with campothecin to induce p53 protein expression. The binding of TAp63α, ΔNp63α and p53 to the p53-RE2 in the p21 promoter was measured and used as a positive control (Figure 4M-P). We found TAp63α, ΔNp63α and p53 recognized the p53-RE in the PABPN1 promoter region (Figure 4N-P).

To assess if the PABPN1 promoter p53-RE is responsive to p53 family, we generated two luciferase reporter constructs containing a PABPN1 promoter with or without the p53-RE (Figure 4Q). Since ECT2 is a repressive target of p53 (Scoumanne and Chen 2006), a luciferase reporter carrying the ECT2 promoter was used as a positive control. Indeed, the luciferase activity under the control of the ECT2 promoter was dramatically repressed by p53 and to a lesser extent by both TAp63α and ΔNp63α (Figure 4R). Similarly, we found that the luciferase activity for the reporter harboring the p53-RE from the PABPN1 promoter was significantly reduced to 50% by TAp63α, ΔNp63α and p53 as compared to a control reporter (Figure 4R). In contrast, TAp63α, ΔNp63α and p53 had no effect on the luciferase activity for the reporter under the control of the PABPN1 promoter without the p53-RE (Figure 4R). These observations suggest that TAp63α, ΔNp63α and p53 can robustly repress the PABPN1 transcription by binding to the p53-RE within the PABPN1 promoter.

PABPN1 regulates keratinocyte proliferation and differentiation via ΔNp63α

PABPN1 has been shown to regulate cell proliferation and differentiation (Apponi et al. 2010). Similarly, ΔNp63α plays a key role in keratinocyte proliferation and differentiation (Barbieri et al. 2006; King et al. 2003). Thus, we examined if PABPN1 regulates keratinocyte cell proliferation via ΔNp63α by colony formation assays. We showed that the proliferation rate of HaCaT keratinocytes was substantially reduced by depletion of PABPN1 in *PABPN1*$^{+/-}$ HaCaT cells as compared to isogeneic control cells (Figures 5A-C). To determine if the reduced proliferation is in part caused by reduction of ΔNp63α expression, ΔNp63α was overexpressed in isogenic control and *PABPN1*$^{+/-}$ clones. Indeed, we found that ΔNp63α overexpression was able to rescue the proliferation defect seen in

PABPN1$^{+/-}$ clones (Figures 5A-C). In a parallel approach, short-term cell proliferation assay (growth curve) was performed with isogenic control and *PABPN1*$^{+/-}$ HaCaT cells upon transient overexpression of ΔNp63α. Similar results were seen (Figure 5D-E) as with colony formation assay. Interestingly, when plotted as % rescue, ectopic expression of ΔNp63α was able to restore the proliferation efficiency of *PABPN1*$^{+/-}$ clones to that of isogenic control cells (Figure 5F). In addition, we examined the role of increased p63γ protein by PABPN1 knockdown in keratinocyte proliferation. For this, a p63γ specific siRNA was used to knock down p63γ in *PABPN1*$^{+/-}$ and isogenic control HaCaT cells. Colony formation assay was performed and showed that knockdown of p63γ led to further decreased cell proliferation in both *PABPN1*$^{+/-}$ and isogenic control cells (Supplementary Figures S5A-C), consistent with our previous study (Yan et al. 2017). Our findings thus provide evidence that ΔNp63α, but not ΔNp63γ, is a mediator of PABPN1 in regulating keratinocyte cell proliferation.

ΔNp63α controls several genes involved in the terminal differentiation of keratinocytes (Barbieri et al. 2006). In addition, the level of ΔNp63α protein is found to be decreased upon keratinocyte differentiation (Zhang et al. 2010). Consistent with this, either overexpression or knockdown of ΔNp63α is shown to inhibit terminal differentiation as measured by cornified envelope formation (Barbieri et al. 2006; King et al. 2003). As PABPN1 is required for p63α expression, we examined if down-regulation of PABPN1 has an effect on cornified envelope formation in HaCaT keratinocytes via decreased p63α expression. For this, two independent *PABPN1*$^{+/-}$ HaCaT clones, which have a reduced level of ΔNp63α protein (Figure 5G), were induced to differentiate by serum deprivation along with increased Ca^{2+} concentration. Immunoblotting showed that involucrin (IVL), a marker for differentiation, was increased in isogenic control cells but little if any in *PABPN1*$^{+/-}$ HaCaT clones (Figure 5G). We also found that PABPN1 levels were slightly increased upon differentiation (Figure 5G), which is likely due to de-repression of PABPN1 transcription by decreased expression of ΔNp63α. Since PABPN1 is primarily localized in nucleus, we examined if keratinocyte differentiation modulates PABPN1 localization. To test this, immunofluorescence assay was performed and showed that PABPN1 nuclear localization was not altered during keratinocyte differentiation (Supplementary Figures S6A-B). Moreover, as PABPN1-KD increases p63γ expression, we determined whether p63γ plays a role in keratinocyte differentiation. We found that upon differentiation, the level of IVL expression was similarly induced in *p63γ*$^{-/-}$ cells as compared to isogenic control cells (Supplementary Figure S7), suggesting that p63γ is not a major isoform for keratinocyte differentiation. Furthermore, cornified envelope formation assay was performed and showed that the number of cornified envelopes were markedly decreased in *PABPN1*$^{+/-}$ as compared to isogenic control HaCaT cells (Figure 5H).

Discussion

p63 is a vital gene involved in development and disease. Many studies have defined how the p63 gene is regulated, most of which focus on the regulation of TA and ΔN isoforms (Romano et al. 2012; Su et al. 2010; Su et al. 2009). Here we define a novel pathway that regulates p63 isoforms by APA. We showed that human and mouse p63 genes possess multiple PASs in the 3'UTRs and in the internal alternate exons. The usage of the APA site in the exon 10' leads to expression of the p63γ isoform. Recently, PABPN1 was shown to

bind weaker PAS sites and suppress their usage (Jenal et al. 2012; De Klerk et al. 2012). In this study, we found that down-regulation of PABPN1 increases the usage of the p63γ-specific PAS, suggesting that the PAS site in exon 10' is a weak PAS site. Based on our results and published studies (Jenal et al. 2012; De Klerk et al. 2012), we propose a model for how p63γ is regulated by PABPN1 via CDS-APA (Figure 5I).

Yeast Pab2, a PABPN1 orthologue, has been shown to associate with several ribosomal proteins, general translation factors and polyribosomes, implying a role in translational regulation (Lemieux and Bachand 2009). Likewise, recent studies showed that PABPN1 interactome contains several ribosomal proteins and general translation factors (Banerjee et al. 2019). PABPN1 is also found to regulate XIAP mRNA translation (Davies et al. 2008) and plays a role in the initial pioneer round of translation in the cytoplasm (Ishigaki et al. 2001; Sato and Maquat 2009). Here, we found that PABPN1 KD decreases p63α expression without affecting p63α mRNA, which is confirmed by decreased synthesis of nascent p63α protein by PABPN1 deficiency. We also showed that PABPN1 associates with the translation initiation factor eIF4G and that PABPN1 deficiency leads to decreased binding of the translation initiation factors eIF4E and eIF4G to p63α mRNA. Thus, it is possible that the poly(A) tail-bound PABPN1 is involved in the initial round of p63α mRNA translation by modulating the binding of eIF4F to p63α mRNA. Considering that PABPN1 and PABPC1 are found to interact with each other on the poly(A) tail of mRNAs (Rufener and Mühlemann 2013), it is also possible that the poly(A) tail-bound PABPN1 is replaced by the abundant cytoplasmic PABPC1 and then facilitates mRNA loop formation for efficient translation through interaction with eIF4G. Together, we propose a model for how PABPN1 regulates p63α mRNA translation (Figure 5J). Since the model is speculative, further studies are warranted to explore the mechanism by which PABPN1 regulates p63α mRNA translation.

Like several p53 family modulators, such as Mdm2 and Rbm38 that are also regulated by p53 family (Barak et al. 1993; Shu et al. 2006), we found that PABPN1 is transcriptionally regulated by p53 and p63α but not p63γ. This is not surprising since p63α but not p63γ protein contains the C-terminal SAM and TID domains, both of which have been shown to be necessary for p63α repression activities (Ghioni et al. 2002; Serber et al. 2002). Although p63 is mainly shown to activate its target genes, there have been several genes whose expression is repressed by p63 by recruiting transcriptional inhibitors, such as the HDAC complex (Abraham et al. 2018; Leonard et al. 2011; Ramsey et al. 2011; Truong et al. 2006; Yugawa et al. 2010). Similarly, while there have been numerous reports showing direct repression by p53 (Ho and Benchimol 2003; Menendez et al. 2009; Wang et al. 2010; Wang et al. 2009), there is an extensive debate about whether p53 can directly repress a target gene (Fischer et al. 2014). While our experiments indicate that the p53 family represses PABPN1 transcription via a p53-RE in the PABPN1 promoter, we cannot rule out the possibility of an indirect mechanism. Of note, as opposed to ΔNp63α, TAp63α exists as an inactive dimer in oocytes and forms an active tetramer upon phosphorylation (Deutsch et al. 2011; Tuppi et al. 2018). Although our experiments used TAp63α overexpression, it was still able to induce p21 and repress PABPN1 expression. Thus, we could see further repression of PABPN1 if TAp63α is activated by DNA damage (Tuppi et al. 2018). Nonetheless, the mutual regulation between PABPN1 and p53/p63α implicates a role of APA in the DNA damage

response as well as a role of the p53 family in regulating the APA landscape (Dubbury et al. 2018; Zheng et al. 2018).

PABPN1 is classified as a general factor for CPA since it binds to poly(A) tails of mRNAs. Thus, PABPN1 is hypothesized to regulate a large number of its mRNA targets. Surprisingly, PABPN1 deficiency only regulates a limited set of protein coding genes in human cells and fission yeast (Beaulieu et al. 2012; Lemieux and Bachand 2009). Here, we have identified p63 as a unique target of PABPN1. Since precise levels of ΔNp63α protein are crucial for keratinocyte proliferation (Barbieri et al. 2006; King et al. 2003), we found that PABPN1 deficiency inhibits cell proliferation by reducing ΔNp63α expression. Interestingly, during keratinocyte differentiation, ΔNp63α expression was decreased whereas PABPN1 was induced. Considering the mutual regulation between PABPN1 and ΔNp63α, it is likely that decreased ΔNp63α leads to de-repression of PABPN1 transcription and consequently increased expression of PABPN1. It should be noted that both overexpression and knockdown of ΔNp63α are shown to inhibit terminal differentiation as measured by cornified envelope formation (Barbieri et al. 2006; King et al. 2003). Consistently with this notion, we observed that PABPN1-deficient HaCaT cells in which ΔNp63α expression is suppressed are resistant to keratinocyte differentiation. Thus, the breakage of the PABPN1-ΔNp63α loop impedes keratinocyte differentiation.

Based on our results, p63γ regulation by PABPN1 induced APA does not seem to alter keratinocyte proliferation or differentiation. This could be because the amount of ΔNp63γ in keratinocytes is lower compared to the major isoform ΔNp63a. As p63γ is a major isoform involved in muscle differentiation (Cefalù et al. 2015), it could serve as a good system to study the biological effect of PABPN1-p63γ axis. Considering that mutations in the PABPN1 gene lead to muscle-related disease oculopharyngeal muscular dystrophy (OPMD) (Brais et al. 1998) and the fact that p63γ isoform plays a critical role in muscle differentiation (Cefalù et al. 2015), it is likely that the PABPN1- p63γ axis plays a role in the pathogenesis of OPMD, which merits further investigation.

Taken together, we identified two unique mechanisms by which PABPN1 differentially regulates p63 isoforms: p63γ via CDS-APA and p63α (and likely p63β/δ) via mRNA translation. Finally, we identified a p63-PABPN1 feedback loop and provided evidence that PABN1 plays a critical role in keratinocyte proliferation and differentiation by regulating ΔNp63α translation.

Materials and Methods

See the Supplementary Materials online for detailed experimental methods.

RNA isolation and RT-PCR

Total RNA was harvested and isolated using TRIzol reagent (Invitrogen). 1μg total RNA was used to synthesize cDNA using RevertAid First Strand cDNA Synthesis kit according to the manufacturer's protocol (ThermoFisher Scientific™). The primers used to amplify various mRNAs and the PCR conditions used are described in the Supplementary materials.

RNA-IP assay

RNA immunoprecipitation was carried out as described previously (Peritz et al. 2006). Briefly, isogenic control and *PABPN1*$^{+/-}$ HaCaT cells were grown to 80-90% confluence in a 100 mm dish (~1 x 10^7 cells) and cell extracts were prepared in immunoprecipitation buffer (10mM HEPES, pH 7.0, 100mM KCl, 5mM MgC12, 0.5% Nomdet P-40, and 1mM DTT), pre-cleared with protein A/G beads, and then incubated with 2 μg of anti-eIF4E, anti-eIF4G or an isotype control IgG overnight at 4 °C. The RNA-protein immunocomplexes were brought down using magnetic protein A/G beads (MedChemExpress). Following washings and RNA-purification, RT-PCR analysis was carried out to determine the levels of p63α and actin transcripts.

Chromatin immunoprecipitation (ChIP) assays

ChIP assay was performed as described previously (Qian et al. 2012). H1299 cells were induced to express TAp63α and ΔNp63α for 18 hours, whereas RKO cells were treated with 200 nM campothecin to induce p53 expression for 16 hours, followed by fixing and harvesting cells for ChIP assays. The binding of various p53 family proteins to the PABPN1 promoter was detected by PCR with forward primer, 5'- GGC TTT GAA TTC CCT GCA CAT T -3', and reverse primer, 5'- CAG GAG TTT CAG ACC AGC CT -3'. The primers used to amplify the p53-RE2 within the *p21* promoter were forward primer, 5'- GGT CTG CTA CTG TGT CCT CC -3', and reverse primer, 5'- CAT CTG AAC AGA AAT CCC AC -3'.

Statistical Analysis

Data are presented as means ± standard error of the mean (SEM) or means ± standard deviation (SD). The *p* values were calculated using the unpaired, two-tailed Student's *t*-tests, and $p < 0.05$ was considered statistically significant. For each experimental data point, $n = 3$. Excel (Microsoft, Redmond, WA) was used for statistical analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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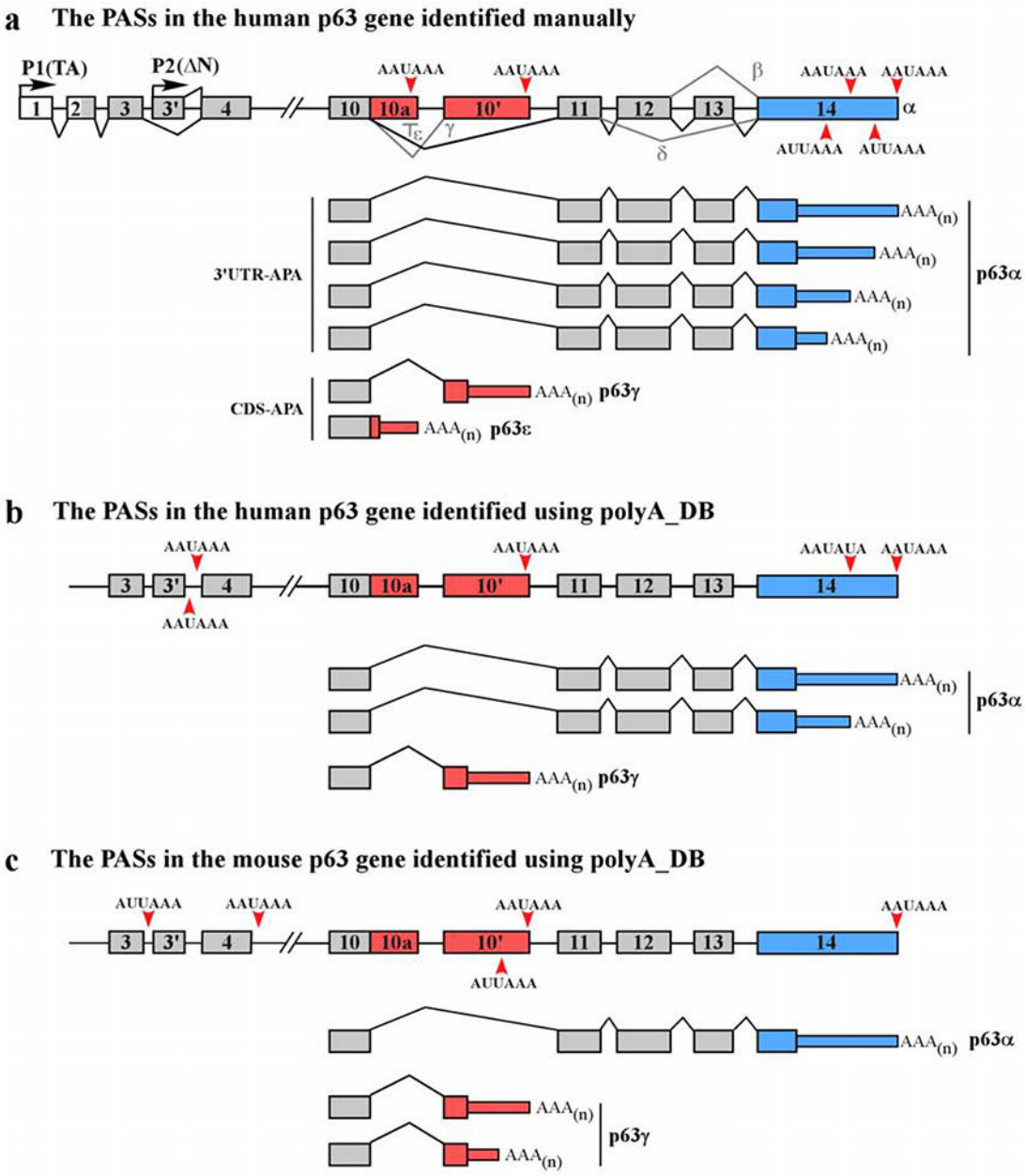


Figure 1- Human and mouse p63 gene contains multiple polyadenylation sites.

(A) Top: Schematic of the mammalian p63 gene along with the two separate promoters and multiple alternative splicing events. It also depicts the manually identified PASs in the human p63 gene. Red boxes-internal exons having PASs; blue box-3' most exon having PASs. Bottom: The putative mRNA species that would be formed if the manually identified PASs are used. For simplicity, only the p63α mRNA species with varying 3'UTR lengths are shown as the β and δ isoforms could also form mRNA with similar 3'UTR lengths. (B) The PASs in the human p63 gene identified using polyA_DB (V3.2) database. The exons and other regions are labeled same as in (A). The putative mRNA formed by usage of cryptic PASs in the N-terminal are not shown. (C) The PASs in the mouse p63 gene identified using polyA_DB (V3.2) database are depicted as in B.

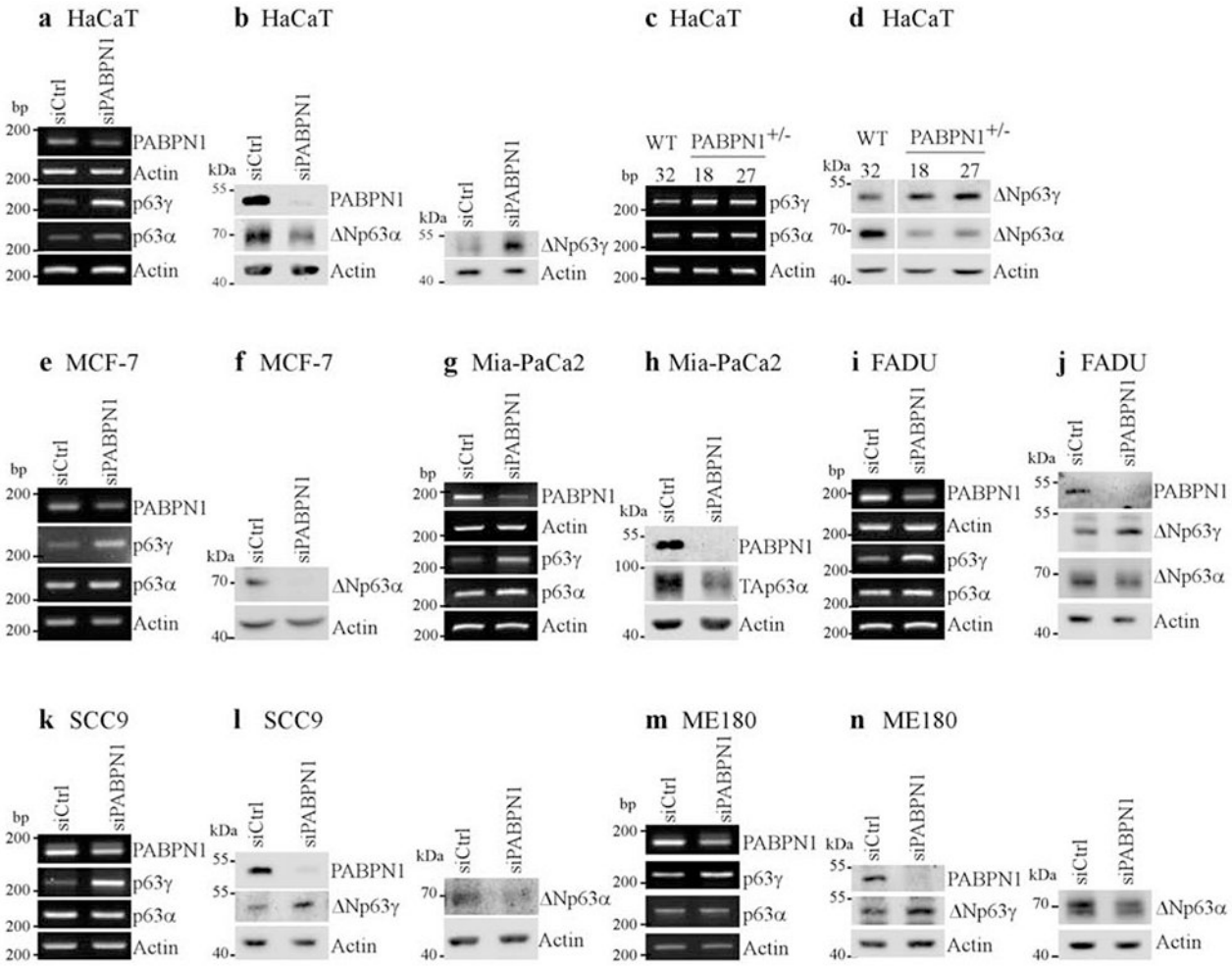


Figure 2 - PABPN1 differentially regulates p63α and p63γ isoforms via APA.

(A, E, G, I, K and M) Indicated cells were transfected with 25 nM control or PABPN1 siRNA for 72 hours followed by RNA isolation and RT-PCR for p63 isoforms. (B, F, H, J, L and N) Indicated cells were treated as in A, E, G, I, K and M followed by immunoblot of various proteins. (C and D) *PABPN1*$^{+/-}$ HaCaT cell lines were generated using CRISPR-Cas9 system and examined for the mRNA and protein levels of the indicated genes by RT-PCR and Western blotting, respectively. The immunoblots shown in (D) were spliced from the same gel and had the same exposure time. The knockdown experiments were performed at least two times in all the indicated cell lines.

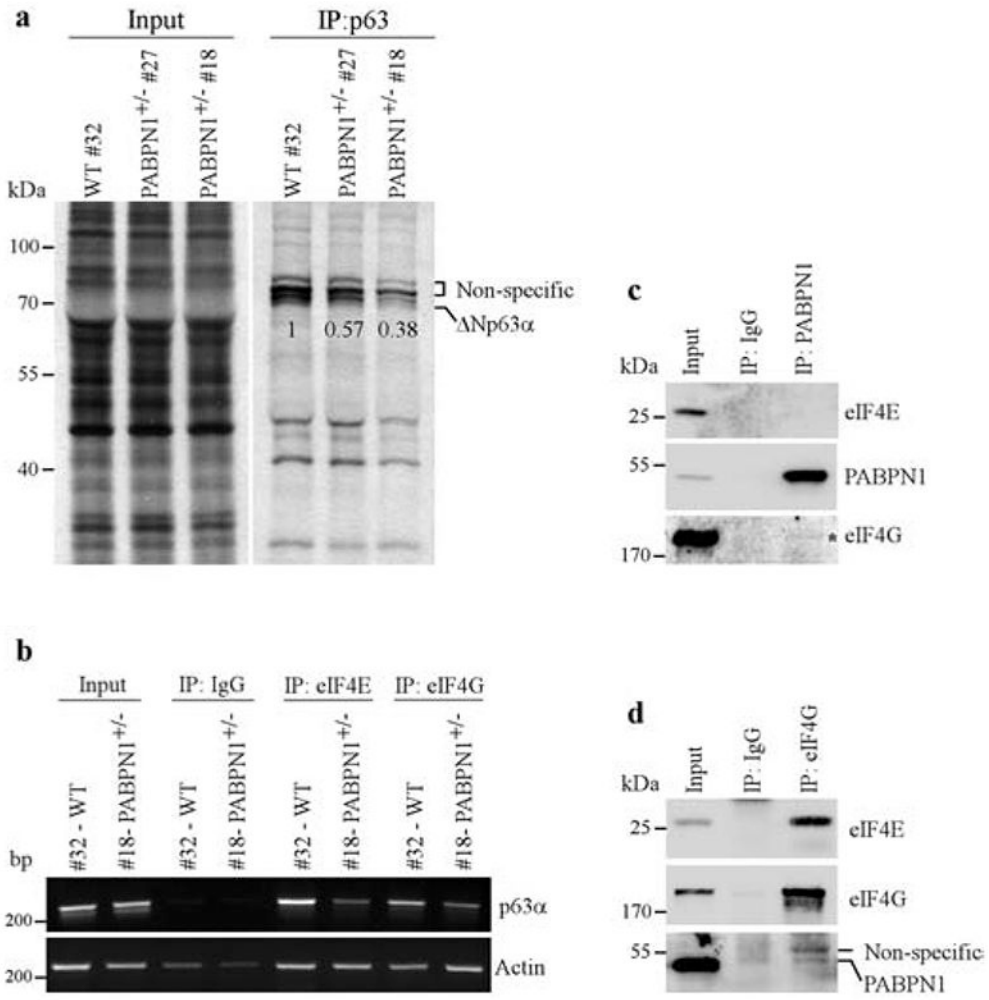


Figure 3 - PABPN1 regulates p63α expression through mRNA translation.

(A) Isogenic control and *PABPN1*$^{+/-}$ HaCaT clones were labeled with ^{35}S-methionine for 30 min and then used for immunoprecipitation (IP) with 2 μg of anti-p63. The immunoprecipitates were separated on 8% SDS-PAGE and p63 was visualized by autoradiography. (B) RNA-IP using anti-eIF4E or anti-eIF4G antibody was performed with lysates from isogenic control or *PABPN1*$^{+/-}$ HaCaT clones to determine the binding of eIF4E or eIF4G to p63α mRNA. Pull-down of actin mRNA was used as a positive and loading control. (C and D) HaCaT cell lysates were immunoprecipitated with anti-PABPN1 (C) or anti-eIF4G (D) followed by immunoblotting with the indicated antibodies. Each dataset is representative of two independent experiments.

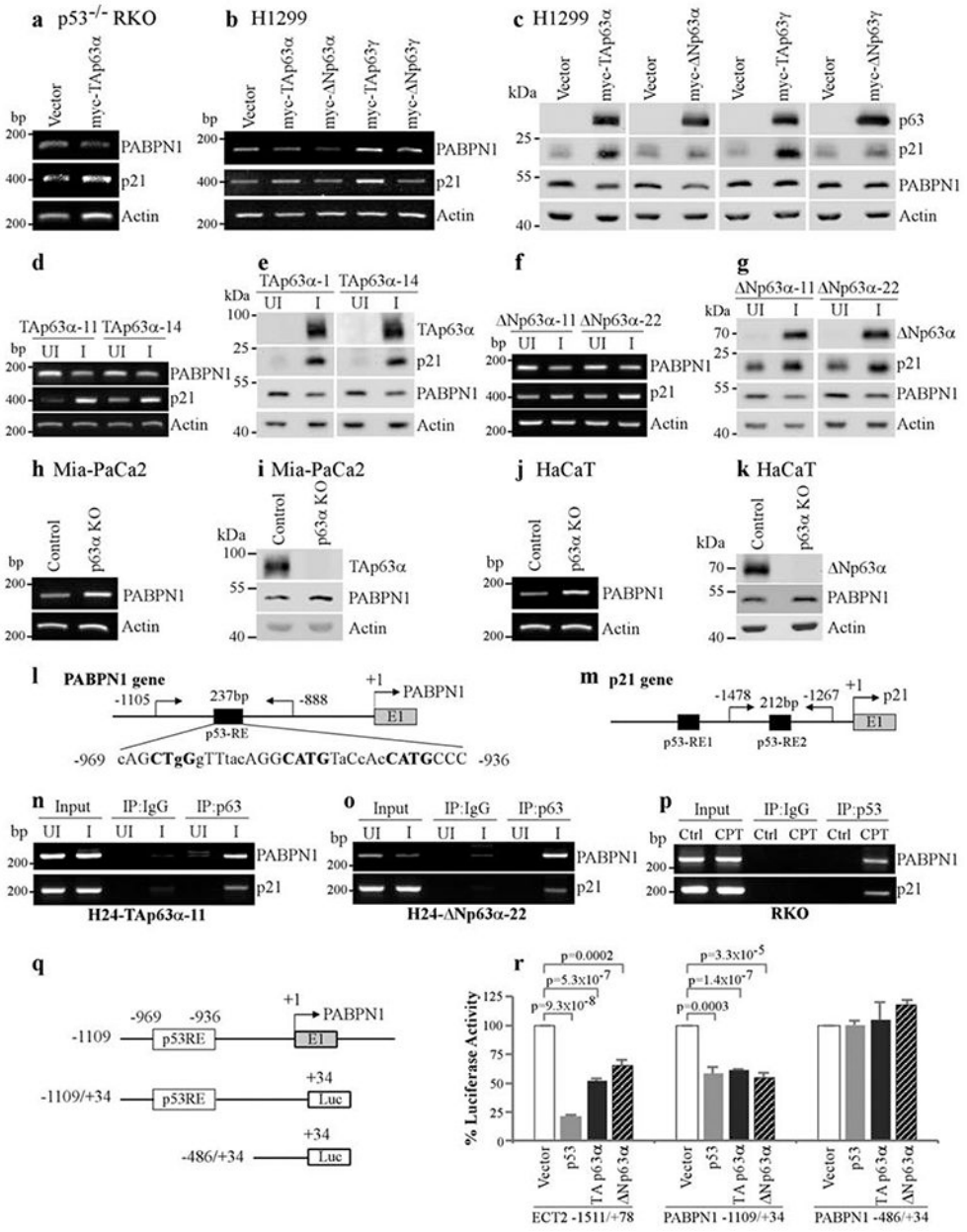
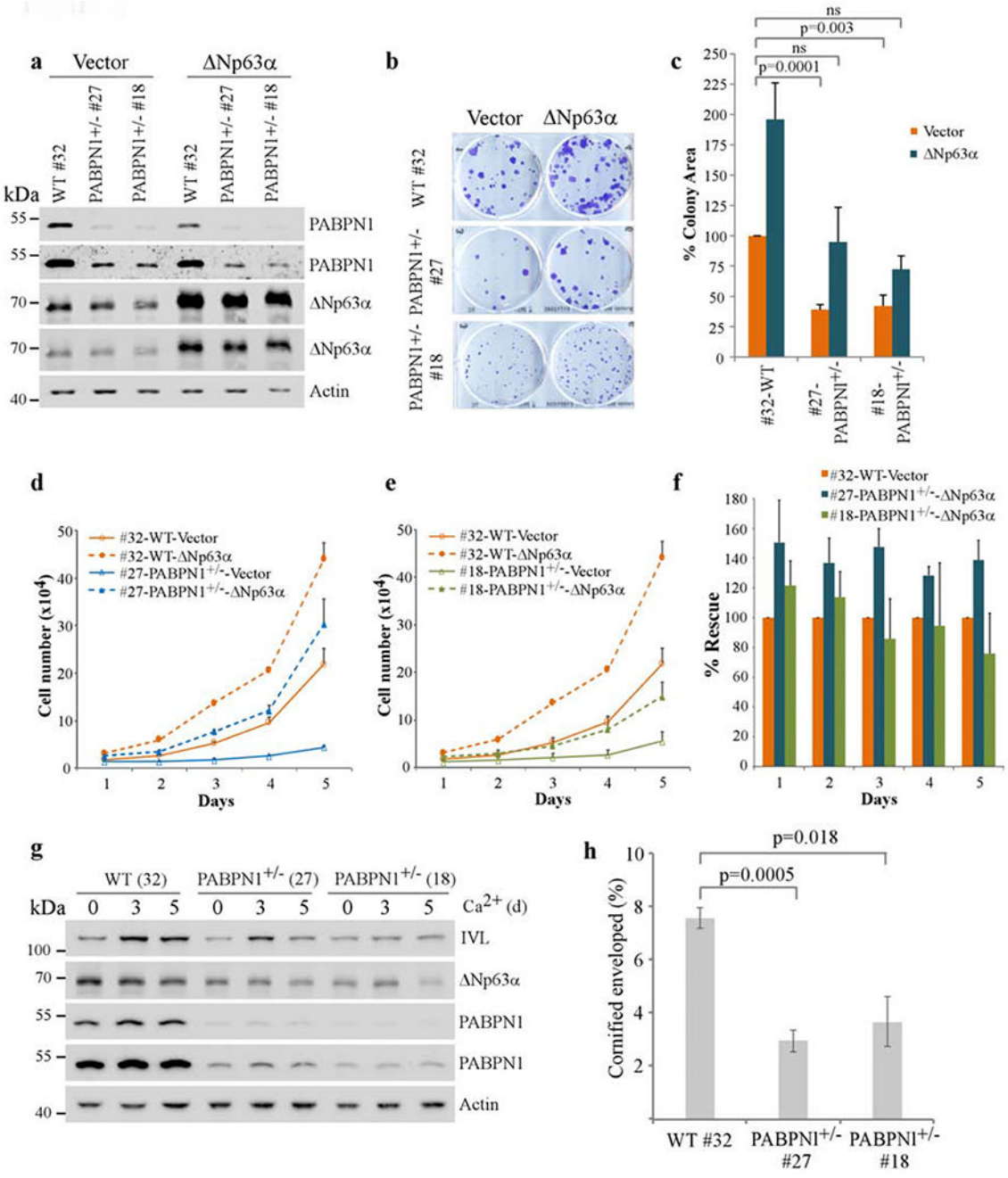


Figure 4 - PABPN1 transcription is regulated by p63α.

(A) p53$^{-/-}$ RKO cells were transiently transfected with either empty vector or myc-TAp63α for 24 hours followed by RNA isolation and RT-PCR for the indicated mRNAs using specific primers. p21 was used as a positive control and actin was used as a loading control. (B and C) H1299 cells were transiently transfected with the indicated constructs for 24 hours followed by RT-PCR (B) or immunoblotting (C) with the indicated antibodies. (D and E) Two H1299 cell lines were induced to express TAp63α for 24 hours followed by RT-PCR (D) and immunoblotting (E) to measure PABPN1 mRNA and protein, respectively. (F and G) Two different H1299 clones were un-induced or induced to express ΔNp63α for 24 hours followed by RT-PCR (F) or immunoblotting (G) with the indicated antibodies. (H-K) p63α-KO MiaPACA2 (H and I) and p63α-KO HaCaT (J and K) along with their respective

isogenic control cells were examined by RT-PCR (H and J) and immunoblotting (I and K) to measure PABPN1 mRNA and protein, respectively. (L-M) Putative p53-RE site in the PABPN1 promoter. Also shown are the locations of primers on PABPN1 and p21 promoters for chromatin immunoprecipitation (ChIP) assays. (N and O) ChIP was performed with cell lysates from TAp63α-expressing (H24-p63α-11) (N) or ΔNp63α-expressing (H24-ΔNp63α-22) H1299 cells (O) using p63 antibody. PCR was performed to determine the binding of p63 to the p53 RE in the PABPN1 promoter. The binding of p63 to the p53-RE2 in the p21 promoter was used as a positive control. (P) RKO cells were treated with 200 nM campothecin for 16 hours followed by ChIP using p53 antibody. The experiment was performed as in (C and D). (Q) Schematic showing the position of the p53-RE in the PABPN1 promoter and constructs generated for luciferase reporter assays. (R) Luciferase reporter activity for the PABPN1 promoter constructs showed in (Q) was measured in H1299 cells, which were transfected with an empty vector or pcDNA3 expressing wild-type p53, myc-TAp63α or myc-ΔNp63α for 24 hours. The luciferase construct carrying the ECT2 promoter was used as a positive control. Data are represented as mean ± SD from three replicates.



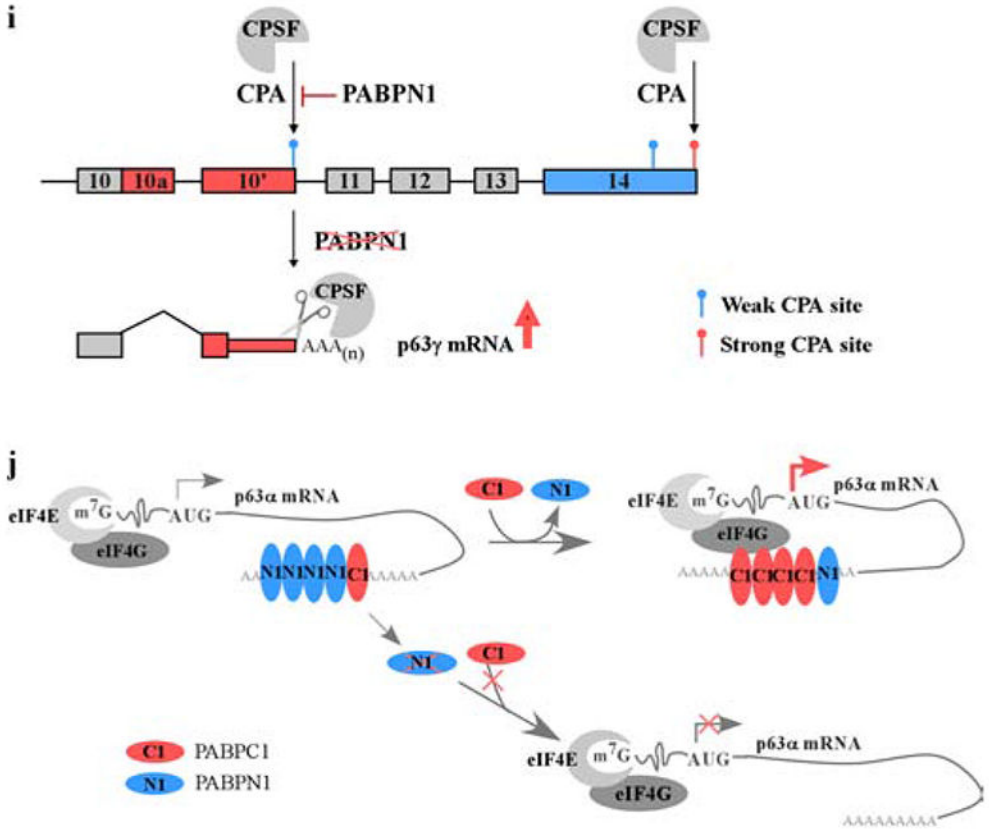


Figure 5 - PABPN1 regulates keratinocyte proliferation and differentiation via ΔNp63α.

(A) Isogenic control and two *PABPN1*$^{+/-}$ HaCaT clones were transfected with an empty vector or pcDNA3 expressing myc-ΔNp63α for 48 hours, and then analyzed by western blotting with the indicated antibodies. (B) Isogenic control and *PABPN1*$^{+/-}$ HaCaT clones were plated for colony formation assays 24 h after transfection as in (A) and grown for two weeks followed by fixing and staining with crystal violet. Three independent colony formation assays were performed. (C) The colonies obtained from the experiment in (B) were quantified using Colony Area in ImageJ software and plotted as percentage of colony area. The density of colonies from isogenic control cells transfected with an empty vector was set at 100%. Data are represented as mean ± SEM, n=3. (D-E) Isogenic control, *PABPN1*$^{+/-}$ HaCaT clone #27 (D) and #18 (E) were transfected with an empty vector or pcDNA3 expressing myc-ΔNp63α, and then counted over a 5 day period. The graphs are presented as mean ± SEM from three independent experiments. (F) Percentage rescue of cell proliferation by ectopic expression of myc-ΔNp63α in *PABPN1*$^{+/-}$ HaCaT cells from experiments in (D-E). The rate of cell proliferation by isogenic control cells transfected with an empty vector was set at 100%. Bars represent mean ± SEM from three independent experiments. (G) Isogenic control and *PABPN1*$^{+/-}$ HaCaT clone #27 and #18 were grown to confluence and then incubated in 0.1% FBS plus 1.5 mM calcium for 0-5 days. Cell lysates were collected and then analyzed by western blotting for the levels of IVL, ΔNp63α, PABPN1 and actin. Data is representative of two independent experiments. (H) The experiment was performed as in (G) except the culture time was extended to 11 d for cornified cell envelope formation. The number of cornified cell envelopes was counted and

expressed as percentage of total cells (mean ± SEM, n=3). (I) A model for how p63γ is regulated by PABPN1 via CDS-APA. PABPN1 inhibits CPSF mediated CPA within p63γ specific exon. However, upon depletion or mutation of PABPN1, this inhibition is lost resulting in increased CPA within the p63γ specific exon and increased p63γ transcription. (J) A model for how p63α is regulated by PABPN1 via mRNA translation. Poly(A) tail-bound PABPN1 is replaced by abundant cytoplasmic PABPC1, which facilitates mRNA loop formation via interaction with eIF4G. PABPN1 deficiency attenuates the recruitment of PABPC1 to poly(A) tail, decreasing mRNA loop formation and subsequently p63α mRNA translation.