Transcription Factor p63 Directly Regulates Signaling Pathways Involved in Epidermal Fate Specification

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Contents

Abstract

Transcription factor ΔNp63 is involved in stem cell maintenance and cell fate specification after mutations in the DNA binding region of p63 were found to be associated with multiple developmental abnormalities in humans. Deletions of the DNA binding region in mice produce severe phenotypic effects such as limb truncation and loss of the epidermis. Although the effects of p63 mutations are well documented, a mechanism by which p63 mutations produce these effects remained elusive. In this thesis I show that transcription factor ΔNp63 regulates components of the Wnt signaling pathway required for the specification and subsequent differentiation of skin lineages. p63 directly regulates Wnt10b, Wnt4, BMPR1B, and β-Catenin through enhancer recognition and transcriptional activation. ΔNp63 stimulates the Wnt signaling cascade by up-regulating several Wnt ligands as well as β-Catenin. Deletions of ΔNp63 enhancers for Wnt10b in vivo result in its reduced expression in the skin and associated appendages. Taken together, these findings reveal a role for p63 in regulating components of the Wnt signaling cascade important for epidermal fate specification.

Background Knowledge

Introduction

Transcription Factor p63

Transcription factor p63 is a homologue of the tumor suppressor p53, and a member of the p53 family of transcription factors, consisting of: p53, p63 and p73 (Mills, 2005). p63 exhibits pioneer activity, characterized by the ability to bind condensed chromatin, much like p53 (Sammons et al., 2015). There exists at least two isoforms of the p63 gene, each directing the expression of two fundamentally different classes of protein. These two forms can be distinguished by the existence of an N-terminal transactivation (TA) domain. The isoform lacking this domain is referred to as the N-terminally truncated (ΔN) isoform (Yang et al., 1998). Current research supports that ΔNp63 isoforms act as both repressors and activators, however the specific targets of p63, either activated or repressed, are largely unknown (Westfall and Pietenpol, 2004).

Although the specific targets are unknown, heterozygous germline mutations in p63 result in a range of human syndromes involving defective development of the limbs, skin and its associated structures (van Bokhoven and McKeon, 2002). p63 KO mice die at birth and show severe developmental abnormalities, such as limb

truncations, defects in the epidermis, as well as its appendages (Mills et al., 1999; Yang et al., 1999). The surface epithelium of these mice is thin, lacks stratification, and does not express markers of epithelial differentiation. The epithelial phenotype is either a result of a lack of commitment to epidermal lineages (Mills et al., 1999), or a lack of proliferative potential in epidermal stem cells (Yang et al., 1999).

p63-associated syndromes in humans have three primary characteristics: ectodermal dysplasia, split hand/foot malformation, and orofacial clefting. Ectodermal dysplasia is characterized by the abnormal development of ectoderm-derived tissues, such as skin, hair, teeth, nails and several exocrine glands. Split hand foot malformation manifests as the malformation of the hands and feet, often with a median cleft. Digits 2, 3, and 4 are typically absent, known as ectrodactyly. Syndactyly, the fusion of digits, can also be observed. Orofacial clefting typically presents as cleft lip/palate. Orofacial clefting is primarily observed in a complex syndrome, but has been observed alone (Leoyklang et al., 2006). Split Hand/Foot Malformation also occurs outside of other syndromes (Ianakiev et al., 2000). Currently, p63 mutations have been identified in Rapp Hodgkin Syndrome (RHS) (Kantaputraet al., 2003), Ankyloblepharon-Ectodermal Defects-cleft lip/palate Syndrome (AEC) (Payne et al., 2005), Limb Mammary Syndrome (LMS) (van Bokhoven et al., 2001), and Ectrodactyly, Ectodermal Dysplasia and Cleft lip/palate syndrome (EEC) (Celli et al., 1999).

Role of Enhancers

Enhancers are a class of DNA regulatory sequences that can affect gene expression. Enhancers are typically a few hundred base pairs in length and contain a short transcription factor recognition sequence, which functions to recruit transcription factors to that site (Spitz and Furlong, 2012). Enhancers activate transcription independent of their location, distance or orientation to the promoters of genes (Banerji et al., 1981). Enhancers can also activate transcription of genes within another chromosome (Geyer et al., 1990). Current understanding of enhancers describes them as clusters of DNA sequences capable of binding transcription factors that interact with components of the mediator complex or transcription factor II D (TFIID). Through this binding enhancers are able to help recruit RNA polymerase II (RNAPII) and promote transcription (Maston et al., 2006; Malik and Roeder, 2010). Enhancers play an important role in tissue specific gene expression through cell-type specific occupancy (Ong and Corces, 2011).

Enhancers have a role in development through transcriptional regulation (Spitz and Furlong, 2012). A number of studies have been performed showing that mutations in enhancer have resulted in limb malformations, or in some cases truncation (VanderMeer and Ahituv, 2011). One such example is the enhancer MFCS1, or ZRS, an enhancer for Shh that regulates Shh from nearly one million base pairs away (Lettice et al., 2003). During limb development MFCS1 is active in the

posterior limb bud mesenchyme, and its activity is critically required for normal limb development in mice (Sagai et al., 2005). This serves as evidence to the cell-type specific occupation of enhancers required for development, and can be seen in Figure 1. Point mutations within MFCS1 cause limb malformations, such as preaxial polydactyly (Lettice et al., 2003). This finding substantiates cis-regulatory mutations in enhancers leading to limb malformations and developmental defects.

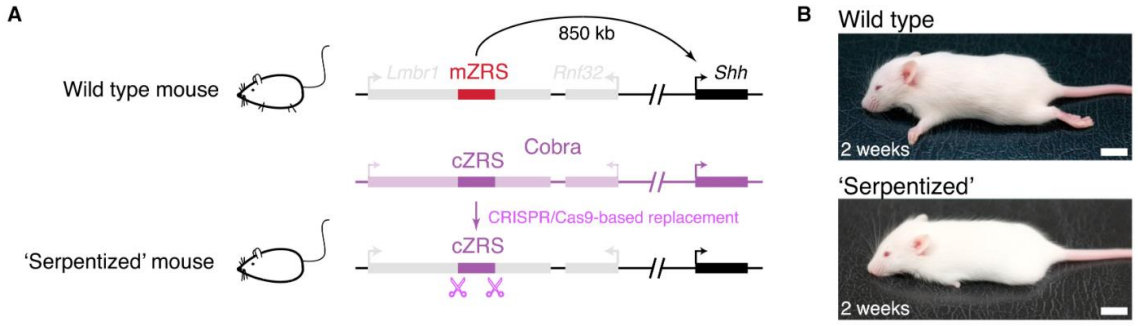


Figure 1. A, CRISPR replacement of the ZRS enhancer for Shh with an orthologous cobra enhancer. B, Phenotypic analysis of CRISPR deletion shows mice without limbs, referred to as serpentized. (Kvon et al., 2016)

Wnt Signaling

p63 has roughly 5800 targets in the human genome, with more than 3000 targets occurring within 5kb upstream or 1kb downstream of well characterized genes, including multiple components of the Wnt signaling cascade (Yang et al., 2006). Wnt ligands interact with multiple receptors, each activating a unique signaling pathway. Wnt activates the canonical Wnt/β-catenin cascade, the non-

canonical planar cell polarity (PCP) pathway, and the Wnt/Ca2+ pathway, although the most well studied pathway is the canonical pathway (Clevers, 2006). Signaling in the canonical pathway is initiated when a Wnt protein released from, or present on the cell surface, binds to a Frizzled (Fz) receptor. Fz receptors are low-density lipoprotein receptor-related proteins located at the cell surface transducing a signal to a wide variety of intracellular proteins. These proteins include Dishevelled (Dsh), glycogen synthase kinase-3β (GSK-3β), Axin, Adenomatous Polyposis Coli (APC), and the transcriptional regulator, β-catenin. β-catenin is a potent signal inside the cell, as β-catenin levels are typically kept at a low level due to continuous proteasome degradation controlled by GSK-3β, APC, and Axin. Canonical Wnt signaling increases intracellular levels of β-catenin by inhibiting degradation, allowing β-catenin to activate transcription factors lymphoid enhancer-binding factor 1 (LEF1) and T cell-specific transcription factor (TCF). A large number of Wnt targets have been identified, including Wnt proteins, which affect self-regulation (Logan, 2004).

Wnt signaling is involved in both development and stem cell self-renewal (Molofsky et al., 2004, Cadigan et al., 1997). Mutant analysis has revealed a wide variety of Wnt functions in development, and loss of a single Wnt gene can produce dramatic phenotypes. Loss of Wnt3a results in Paraxial mesoderm defects, deficiency in neural crest derivatives, reduction in dorsolateral neural precursors in the neural tube, and tailbud defects (Aulehla et al. 2003; Galceran et al. 1999, 2000; Ikeya et al. 1997; Lee et al. 2000; Yoshikawa et al. 1997). While loss of Wnt4 results in defects in female development, the absence of the Mullerian duct, as well as

defects in adrenal gland development (Heikkila et al. 2002, Mulroy et al. 2002, Vainio et al. 1999). Finally mutations in the Wnt10b gene have been associated with Split Hand/Foot Malformation, specifically in conjunction with p63 mutations (Ugur et al., 2008). Wnt regulates stem cell self-renewal by sustaining expression of the pluripotent state-specific transcription factors Oct-3/4, Rex-1 and Nanog. Oct-3/4 and Rex-1 have been studied as representative transcription factors involved in controlling the pluripotent state of stem cells, and Wnt activation positively regulates these transcription factors (Sato et al., 2004). Wnt ligands promote self-renewal of mouse hematopoietic stem cells through the upregulation of HoxB4 and Notch-1, which are involved in hematopoietic stem cell proliferation (Reya et al., 2003).

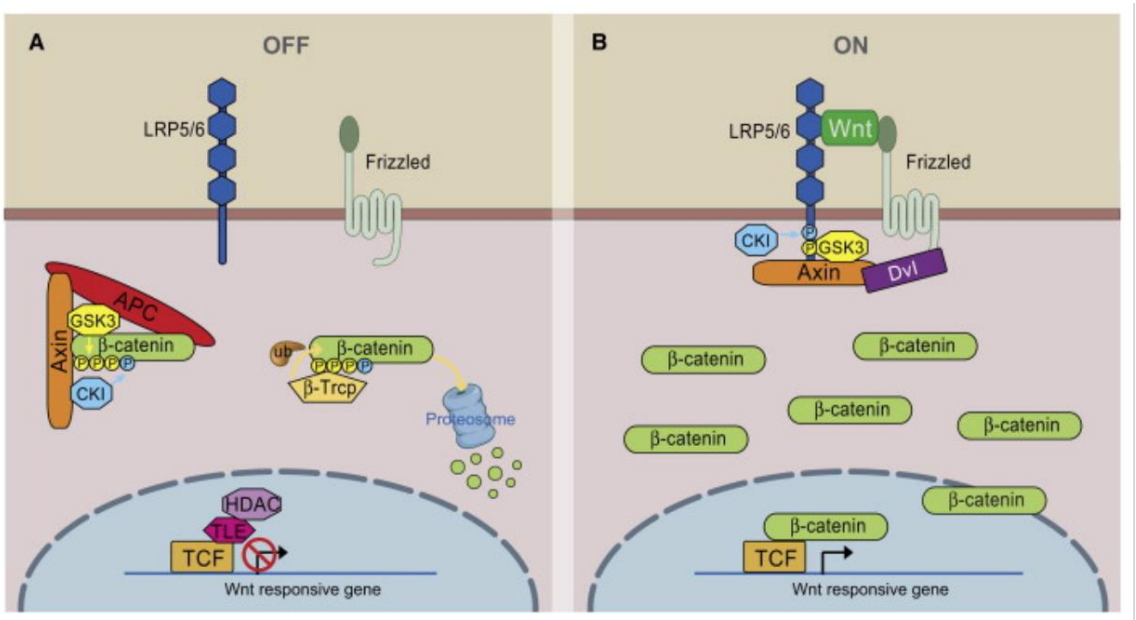


Figure 2. Illustration of the Wnt signaling cascade. Presence of the Wnt ligand results in GSK-3β complex failing to form. Without the GSK-3β degradation complex, β-catenin is able to promote transcription of downstream target genes (MacDonald et al., 2009).

BMP Signaling

Bone Morphogenic Protein (BMP) signaling is intertwined with Wnt signaling in many biological processes, including stem cell maintenance, cell fate specifications, organogenesis, and carcinogenesis (Logan and Nusse, 2004; Moon et al., 2004; Varga and Wrana, 2005; Hardwick et al., 2008). Although BMP and Wnt signaling act independently of each other, they are often expressed in complementary or overlapping fashion (Itasaki and Hoppler, 2010). BMP can act either antagonistically or synergistically in differing cell types (Azpiazu et al., 1996; Carmena et al., 1998). BMP signal transduction begins with a BMP ligand binding two distinct type II and type I serine/threonine kinase receptors (Shi and Massagué, 2003). The serine/threonine kinase domains of type II receptors are constitutively active, and phosphorylate Gly-Ser (GS) domains in the type I receptors upon ligand binding, leading to the activation of type I receptor kinases (Miyazono et al., 2005). Following receptor activation, signals are transmitted through Smad-dependent and Smad-independent pathways, including ERK, JNK, and p38 MAP kinase pathways (Derynck et al., 2001). However, Smads are the major signal transducers for the serine/threonine kinase receptors. Upon activation by type II receptors, type I receptors phosphorylate receptor-regulated Smads (R-Smads), which form complexes with common-partner Smads (Co-Smads) (Heldin et al., 2007). The R-Smad/Co-Smad complexes then enter the nucleus and regulate transcription by interacting with transcription factors and recruiting co-activators or co-repressors (Miyazano et al., 2000).

As previously stated, BMP forms a sort of "cross-talk" with Wnt signaling depending on cell type, an example of such can be seen in stem cell proliferation and maintenance. Either BMP or Wnt signaling can sustain the maintenance of the pluripotent state. In the case of Wnt signaling, a pharmacological GSK3β inhibitor is sufficient to maintain the undifferentiated state of ES cells (Sato et al., 2004). BMP can maintain pluripotency through upregulation of *Id* genes, downstream target of BMP signaling (Ying et al., 2003). However, in the case of proliferation BMP acts as antagonist to the promotion of stem cell self renewal by Wnt signaling (He et al., 2004). In skin development, the interplay of Wnt and BMP signaling are critical for specification of embryonic skin, a diagram of this interplay can be seen in Figure 2. In early ectodermal progenitor cells, Wnt signaling blocks the ability to respond to FGFs, allowing them to respond to BMP signaling and adopt an epidermal fate. As development progresses, the single-layered embryonic epidermis continue to express Wnt. Cells that fail to respond to Wnt, are specified to become epidermal cells through BMP, FGF and Notch signaling. Cells that do respond to Wnt signaling also respond to FGF and BMP inhibitory signals from the mesenchyme. As a result, the epidermis and hair placode is primarily patterned by the inhibition of BMP inhibitory signals and Wnt activating signals (Hardy, 1992; Davidson, 1983; Petiot et al., 2003; Jung et al., 1998; Noramly and Morgan, 1998; Botchkarev et al., 1999).

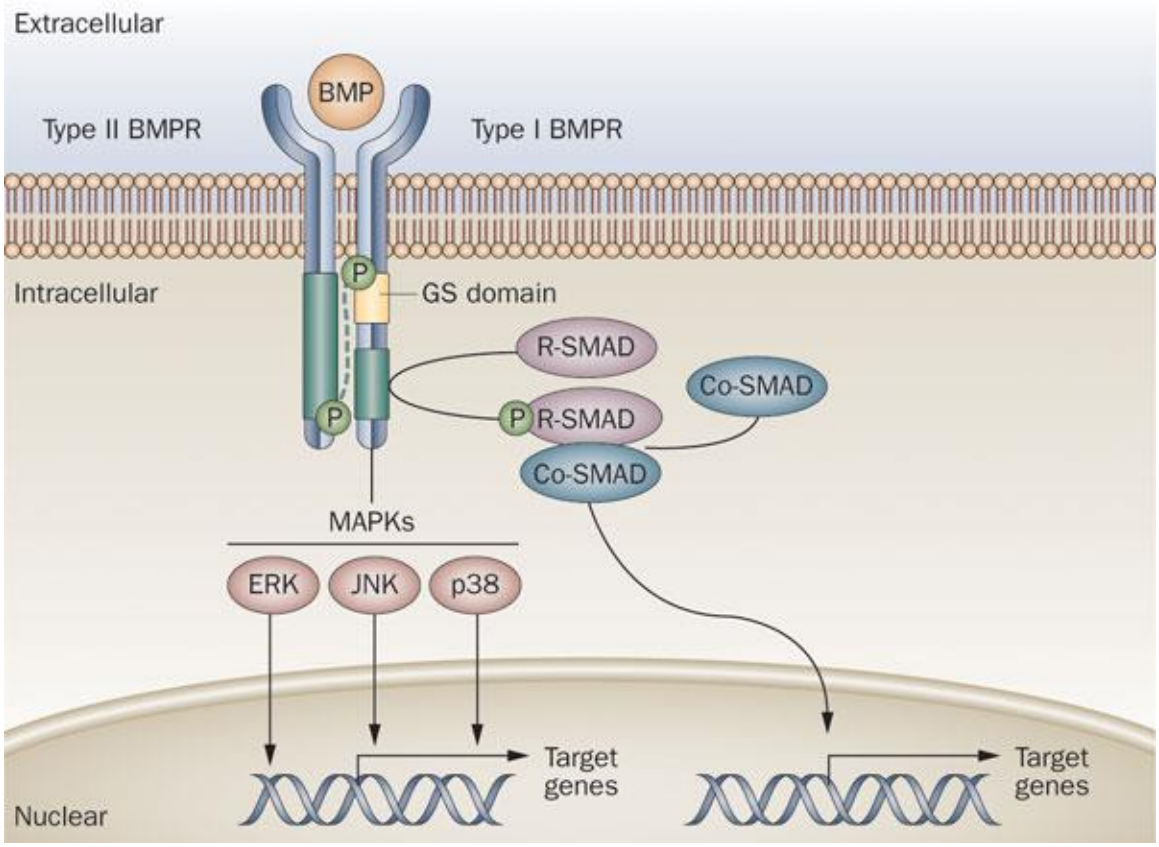


Figure 3. Illustration of BMP Signaling. BMP signaling is capable of affecting target genes through both MAPK and SMAD pathways. Presence of BMP ligand activates a kinase cascade, where terminal signals enter the nucleus and promote transcription (Shore and Kaplan, 2010).

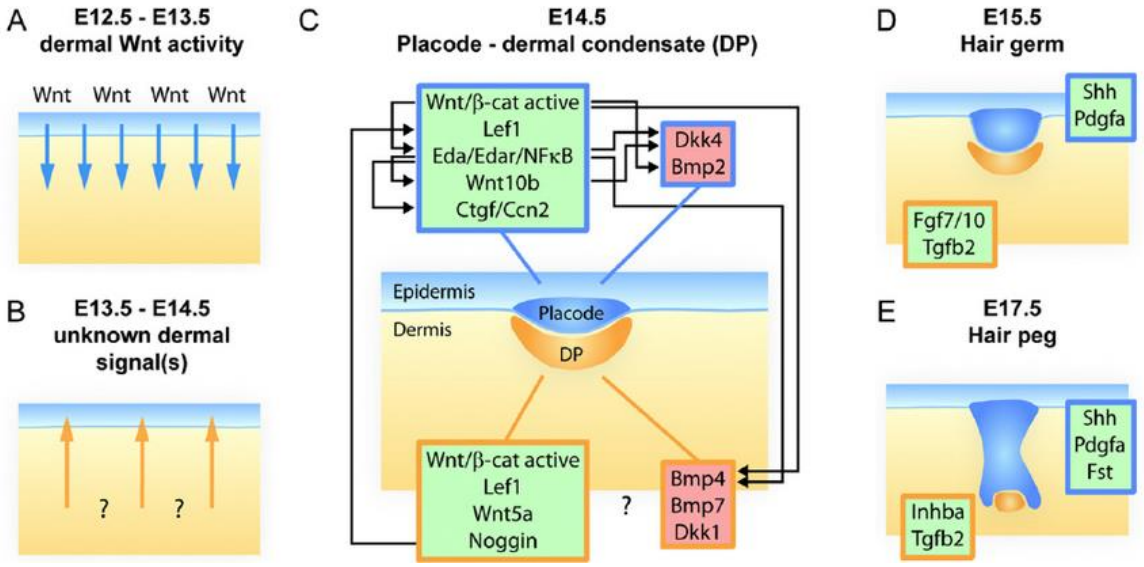


Figure 4. A, Wnt signaling from the epidermis at the E12.5 stage. B, The dermal produced signals are largely unknown, but some feedback loop has been hypothesized. C, Interplay of multiple signaling events in the placode at E14.5, both Wnt and BMP signaling are involved in patterning. D, Shh and FGFs are involved in Hair germ formation at E15.5. E, Formation of the hair peg at E17.5 involves Shh and Tgfb2 signaling. (Sennet et al., 2012)

Experimental Design

Key Molecular Techniques

Luciferase assays are a common technique for studying many aspects of the cell (Fan and Wood, 2007), but are especially useful when studying the promoter activity of cloned fragments in vitro. The luciferase protein was first cloned in 1985 (De Wet et al., 1985) and has remained common in biological research due to its high sensitivity and high correlation between expression and luminescence. The luciferase protein is also useful due to its lack of post-translational processing (De Wet et al., 1987). As a whole, the luciferase protein is well suited for use in gene expression analysis in vitro.

Promoter assays allow for the study of promoter effect on transcription by cloning different promoters or transcriptional regulation elements upstream of luciferase cDNA and then quantifying differential transcription through luminescence. When studying the effects of enhancers, expression vectors are constructed with the enhancer of interest cloned upstream of a minimal promoter which drives luciferase expression. This vector is then transfected into a given cell line where the vector can be expressed. Following incubation, expression levels are determined by measuring luminescence. This system operates on the assumption

that the varying effects of enhancers will differentially enrich promoters, which will result in varied driving of gene expression. Thus, weaker enhancers will result in lower luminescence, while more potent enhancers will result in greater luminescence. However, luciferase assays often have a high degree of variance in their results due to the high sensitivity of Firefly luciferase, and often require multiple repeats. Differences in transfection efficiency were corrected by using a dual reporter assay, which normalizes luciferase activity to a co-transfected second reporter.

Although promoter assays provide a powerful tool for quantifying gene expression in vitro, they are susceptible to the same limitations of other in vitro experiments. The development of CRISPR allows for the introduction of targeted genomic alterations into living cells and organisms, making CRISPR a powerful tool for biological research in vivo (Doudna, 2014). Before the introduction of CRISPR, inducing precise, targeted genome alterations were limited to certain organisms such as homologous recombination in yeast or recombineering in mice. These techniques often required drug-selectable markers or resulted in residual sequences associated with the modification method, such as *loxP* sites from Cre recombinase-mediated excision (Nagy, 1999). Targeted genome editing using CRISPR provides a general method for inducing targeted deletions, insertions and precise sequence changes in a broad range of organisms and cell types. The high efficiency of genome editing eliminates the need for additional sequences, such as drug-resistance marker genes, and additional modifications to remove them.

The Cas9 protein, essential to the CRISPR/Cas9 genome editing, functions as a dual-RNA–guided DNA endonuclease that utilizes a tracrRNA:crRNA duplex (Deltcheva et al., 2011). The tracrRNA:crRNA duplex was later modified to contain only a single sgRNA, containing a 5' 20-nucleotide guide sequence to determine the target, as well as a 3' double stranded structure that binds to the Cas9 protein (Jinek et al., 2012). The Cas9 protein is a large multifunctional protein with two nuclease domains, HNH and RuvC-like (Makarova et al., 2006). The Cas9 protein will remain inactive until bound by the sgRNA (Jinek et al., 2012). Once activated, the HNH domain cleaves the DNA strand complementary to the sgRNA, while the RuvC-like domain cleaves the opposite strand (Gasiunas et al., 2012). By modifying the sequence of the 20-nucleotide guide it is possible to target specific regions of DNA, as long as those regions are located adjacent to an NGG PAM sequence (Jinek et al., 2012). Upon sgRNA binding, the Cas9 protein undergoes a conformational change, resulting in the active form (Jinek et al., 2012). Cas9 will then bind the DNA region complementary to the sgRNA sequence and the HNH and RuvC-like domains will cleave the DNA 3 bases upstream of the PAM sequence (Anders et al., 2014).

Using the CRISPR/Cas9 system allows for the targeted excision of p63 enhancer sites through the genome, and thus the creation of an in vivo model for p63 regulation. p63-bound DNA regions located proximally to genes of interest were characterized enhancer sites, and although this method of enhancer characterization produces high probability sites, it should be noted that enhancers could act on genes from long-range distances as well. However, by excising p63 enhancer regions it is possible to study the effects of p63-regualte gene expression,

and whether those effects corroborate previous insights into p63's role as a transcriptional regulator.

Methods

CRISPR Cloning

Using the DNA 2.0 program, sgRNA were designed both upstream and downstream of enhancer regions. BbsI recognitions sites were added at the 5'-end to facilitate cloning. Primers were produced by Eurofins Genomics. Following sgRNA production, primers were annealed in a thermal cycler using a standard annealing protocol. During this time, the px459 vector obtained from Dr. Dongmei Wang was digested using the restriction enzyme BbsI. The vector was then purified using the OMEGA EZNA DNA Purification kit. Following purification, the annealed sgRNA were ligated into the vector using a 3:1 ratio. Ligation was accomplished using the Quick Ligase protocol from New England BioLabs, Inc. The ligation product was then transformed into 50 µl of Stable3 competent cells, and plated on Ampicillin plates. Plates were cultured at 37 °C for 16 hours. Colony PCR was performed on 8 colonies from each plate, and successful ligation was verified by gel electrophoresis. Colonies containing the desired insert were transferred to 6 mL of ampicillin containing LB

and cultured for 16 hours. The plasmid was then extracted using OMEGA EZNA Plasmid Mini Kit.

sgRNA Production

To produce the sgRNA for mouse injections, Eurofins Genomics produced primers containing the T7 promoter upstream of the sgRNA sequence. These primers were then used to produce PCR fragments, with the sgRNA containing pX459 plasmid serving as template. These fragments were purified first using the EZNA DNA Purification kit, and then further purified using the Qiagen DNA Purification kit, which also served to further concentrate the fragments. These fragments then served as template for in-vitro transcription to produce sgRNA. In-vitro transcription was performed using the ThermoFisher MEGAshortscript™ T7 Transcription Kit. 200 ng of template was used in the reaction, and transcription was performed for three hours. The transcripts were then purified using the ThermoFisher MEGAclear™ Transcription Clean-Up kit and quantified by NanoDrop.

Mouse Injections

sgRNA were be combined with the Cas9 construct and used in pronuclear injections into zygotes. Zygotes were transferred at the two cell stage to pseudo-pregnant mice. Embryos were then allowed to develop normally, and collected at E13.

Enhancer Amplification and Vector Preparation

Wild type enhancer sites were created through PCR amplification. Phusion polymerase was used due to its increased accuracy compared to Taq. Fragments of approximately 250 bp were amplified from wild type genomic mouse DNA. These fragments were then used as templates for mutant fragments. Targeted mutations were introduced in high consensus bases in the HOMER motif. Three sites were chosen in each binding site and mutated from either G or C to A. Utilizing primers that spanned the enhancer site; mismatches were introduced in the primers, allowing for mutant fragment formation. Overlapping fragments were then annealed and further amplified to produce mutant fragments. Both wild type and mutant fragments utilized KpnI and HindIII cloning sites, and were coned into the pGL4.23-GW vector (Addgene).

Luciferase Assay

Mouse Keratinocyte, mk, cells were cultured in E Low Ca^{2+} media, and then seeded at 20k cells per well into a 24-well plate. Cells were cultured for six hours to allow the cells to adhere, and then transfected. Transfections were accomplished using the Mirus TransIT-LT1 Transfection reagent. 5 replicates were performed for both mutant and wild type enhancers. Each replicates consisted of the following complex: 50 µl of P-Media, 50 ng of enhancer containing pGL4 vector, 2 ng Renilla, 348 ng of MIGR, and 1.2 µl of TransIT-LT1. Complexes were thoroughly mixed, and

allowed to rest for 30 minutes. 50 µl of transfection complex was then added drop-wise to each well. Cells were culture for 48 hours following transfection. Transfection efficiency was visualized using MIGR. E Low Ca^{2+} media was aspirated off, and cells were washed with PBS. Cells were then lysed in Passive Lysis Buffer for 15 minutes. Following lysis, the assay was performed. The assay was performed using the Promega Dual-Luciferase Assay Reporter System. 20 µl of lysis product was combined with 50 µl of LARII and initial luminescence was measured. 50 µl of Stop & Glo was then added and the second luminescence was measured.

Implications

Results

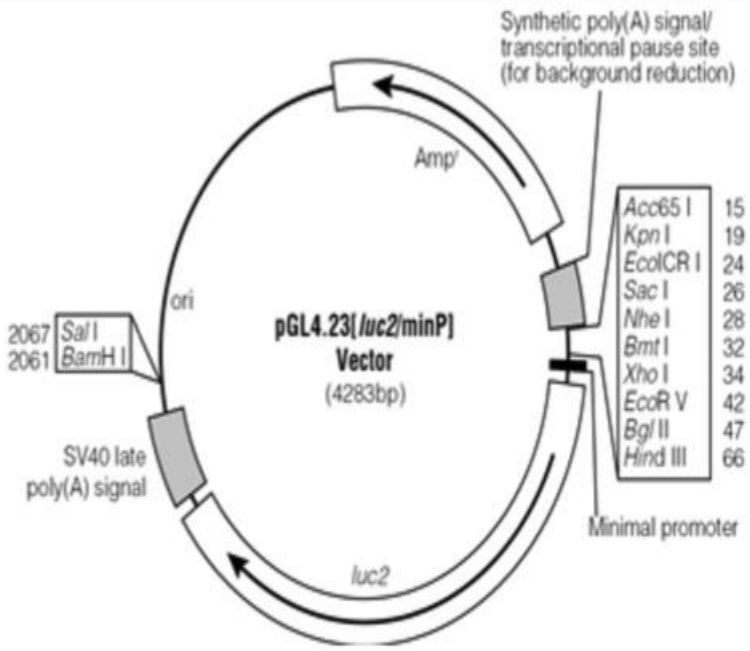
Using the ATAC-Seq and CHIP-Seq data obtained from Dr. Fan, expected p63 enhancer sites were identified and several enhancers were selected as candidates. The following enhancers were selected: Wnt10b, Wnt4, β-Catenin, and BMPR1B. To analyze the binding effect of p63, both wild type and mutant forms of the enhancers were created through PCR. For mutant enhancers, three point mutations were introduced in the binding motif. These point mutations correlate with high consensus sites from the HOMER motif, and were changed to adenine residues to

disrupt p63 binding (Fig. 6A). These PCR fragments were then ligated into the pGL4.23 vector (Fig. 5A) via the KpnI and HindIII restriction sites. Successful ligations were first verified by restriction digest (Fig. 5B), and were then sequenced to validate successful mutation or amplification of wild type enhancers (Fig. 5C). Vectors were then transfected into mouse keratinocyte cells, and allowed to incubate for 48 hours. Enhancer activity was quantified using a dual reporter luciferase assay (Fig. 6C). β-Catenin and BMPR1B showed the largest change in luciferase expression with 16- and 18-fold reduction in expression respectively. Wnt10b and Wnt4 followed with 9- and 4-fold reductions. Enhancer strength was then compared to the pGL3 control and pGL3 basic vectors (Fig. 6D). Both Wnt4 and Wnt10b enhancers resulted in stronger luciferase expression than the pGL3 control vector, with β-Catenin and BMPR1B vectors exhibiting slightly less transcriptional activity.

Following the success of the promoter assay and subsequent demonstration of p63 regulation in vitro, I sought to demonstrate p63 regulation in vivo. Again using CHIP-Seq and ATAC-Seq data, the p63 enhancer site for Wnt10b was selected for deletion via the CRISPR/Cas9 genome editing system. To interrupt p63 binding of enhancer sites, sgRNA were designed flanking either side of the enhancer, located in chromosome region chr15:98775830-98776513 (Fig. 7A), to fully excise the enhancer (Fig. 8A). sgRNA were cloned into the pX459 vector using BbsI restriction sites (Fig. 7B). Successful cloning was verified by sequencing (Fig. 7C). sgRNA were then produced via T7 in vitro transcription (Fig. 7D) from the pX459 vector template and combined with Cas9 for pronuclear injections. Following blastocyst

implantation, embryos were collected at E13. Successful deletion was first verified by PCR and gel electrophoresis (Fig. 8B), and samples from animals exhibiting the knockout band were sequenced to validate complete excision (Fig 8C). The complete embryonic epidermis was harvested and used to test Wnt10b expression levels verified by qRT-PCR (Fig. 8D). It was found that excising the p63 enhancer upstream of Wnt10b resulted in a 60% reduction of the transcribed mRNA in vivo, thus indicating that p63 regulates Wnt10b by binding enhancers and promoting transcription.

A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Vector
(4283bp)
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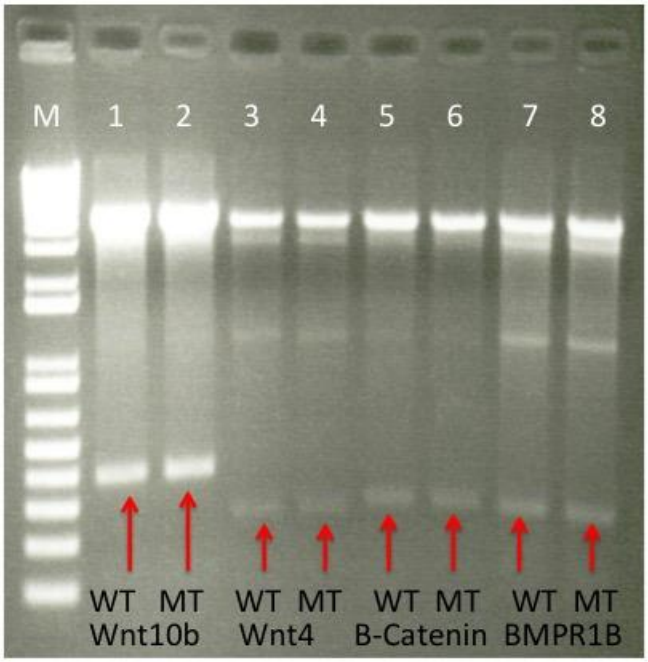
2061 BamH I
SV40 late
poly(A) signal
luc2
Acc65 I 15
Kpn I 19
EcoICR I 24
Sac I 26
Nhe I 28
Bmt I 32
Xho I 34
EcoR V 42
Bgl II 47
Hind III 66
Minimal promoter

B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Wnt10b Wnt4 B-Catenin BMPR1B

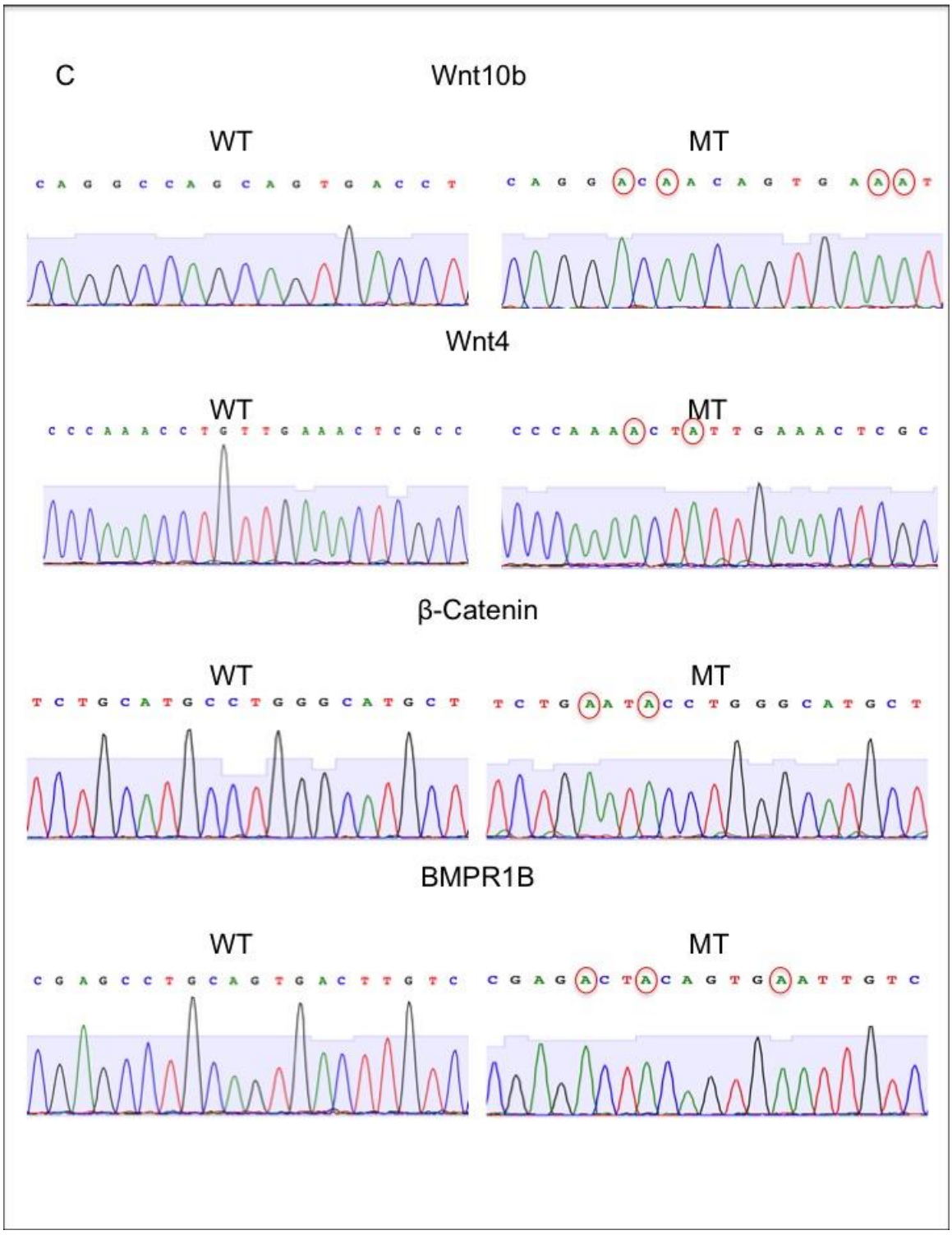
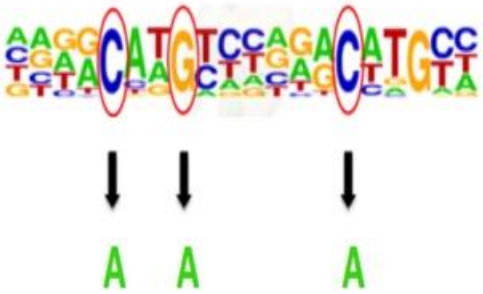

C
Wnt10b
WT
MT
Wnt4
WT
MT
β-Catenin
WT
MT
BMPR1B
WT
MT

Figure 5. A, vector map for the pGL4.23 expression vector, shows the KpnI and HindIII restrictions sites used for cloning. B, Restriction digest verification for all 8 vector constructs. Lane 2: Wnt10b Wild Type, Lane 3: Wnt10b Mutant, Lane 4: Wnt4 Wild Type, Lane 5: Wnt 4 Mutant, Lane 6: β-Catenin Wild Type, Lane 7: β-Catenin Mutant, Lane 8: BMPR1B Wild Type, Lane 9: BMPR1B Mutant. Red arrows indicate released enhancer fragments. Marker used was 1Kb+. C, Trace files showing successful cloning of enhancer sites, mutations are circled in red.

A


A
A
A

B


Wnt10b
Wild
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Mutant
GGACAACAGTGAAATG
Wnt4
Wild
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Mutant
AAACTATTGAAACTCG
B-Catenin
Wild
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BMPR1B
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Mutant
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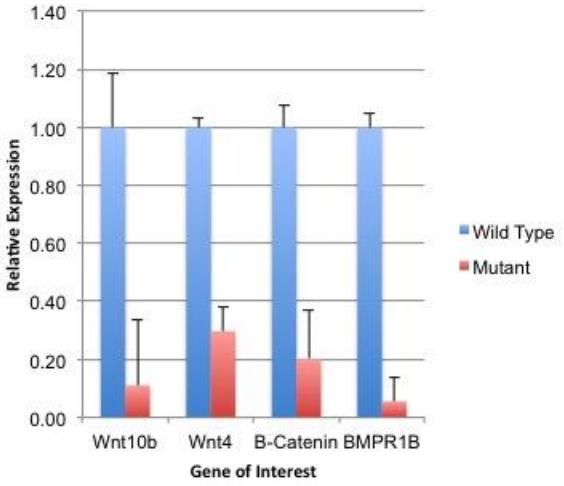
C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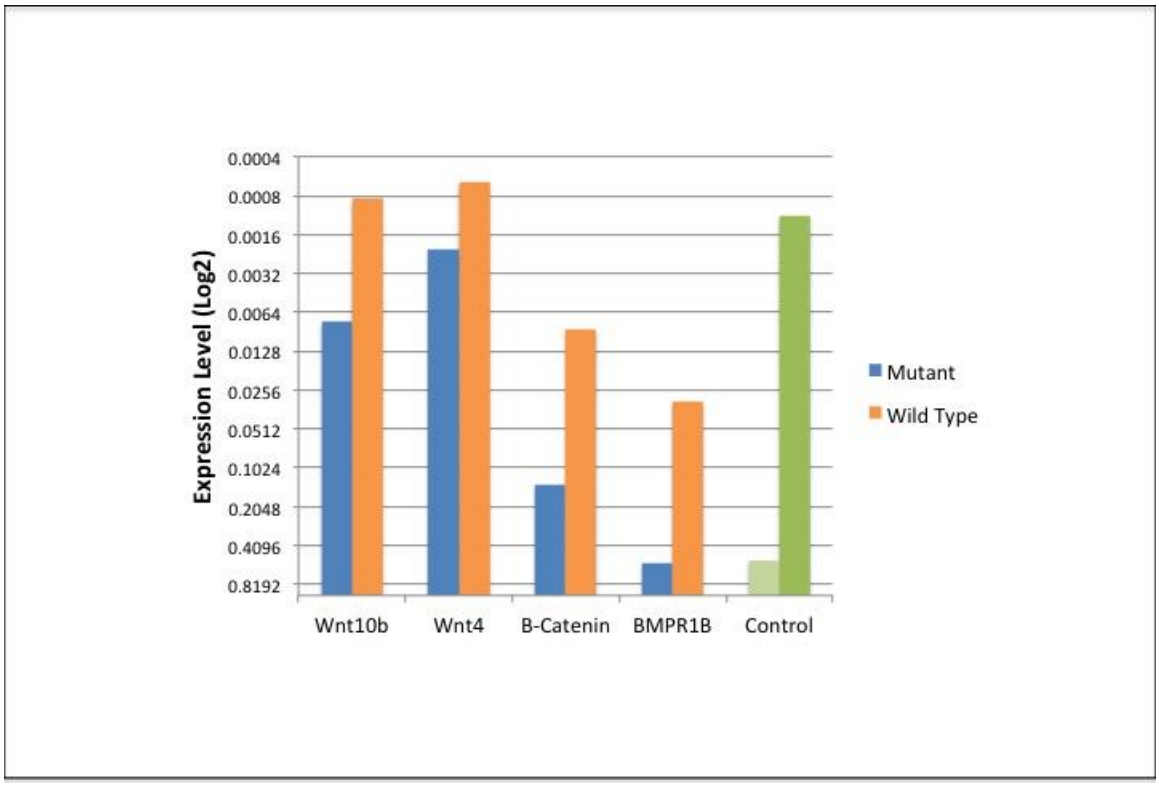
Wnt4
B-Catenin
BMPR1B
Gene of Interest
Wild Type
Mutant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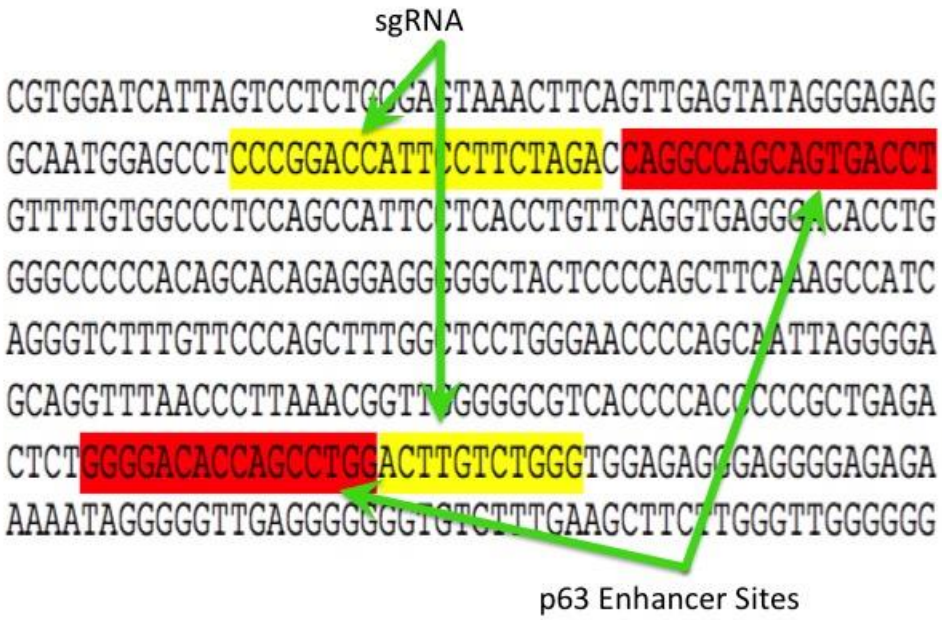
0.4096
0.8192
Expression Level (Log2)
Wnt10b
Wnt4
B-Catenin
BMPR1B
Control
Mutant
Wild Type

Figure 6. A, HOMER motif of p63, where red circles indicates nucleotides that were high consensus and targeted for point mutation. As illustrated, these nucleotides were mutated from either guanine or cytosine into adenine. B, Sequencing results from the eight vector constructs was annealed to indicate points of mutation in the mutant enhancers. C, dual reporter assay shows the relative expression of each wild type-mutant pair. Error bars indicate standard deviation. n=5. T-Test indicates $p < 0.005$. D, dual reporter assay shows enhancer strength compared to the pGL3 control and pGL3 basic vectors. n=5. T-Test indicates $p < 0.005$.

A


sgRNA
CGTGGATCATTAGTCCTCTCCGAGTAAACTTCAGTTGAGTATAGGGAGAG
GCAATGGAGCCTCCCGGACCATTCCTTCTAGACCAGGCCAGCAGTGACCT
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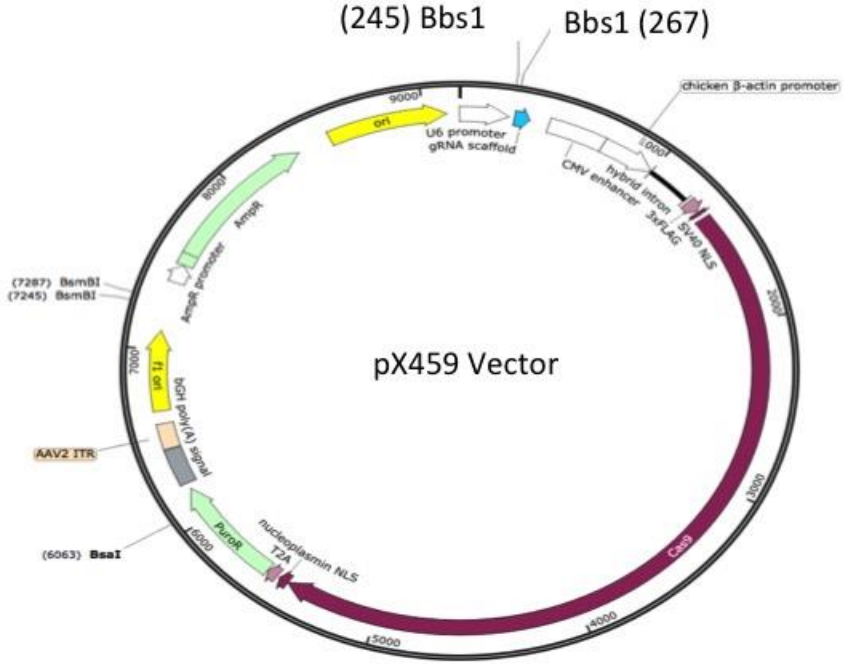
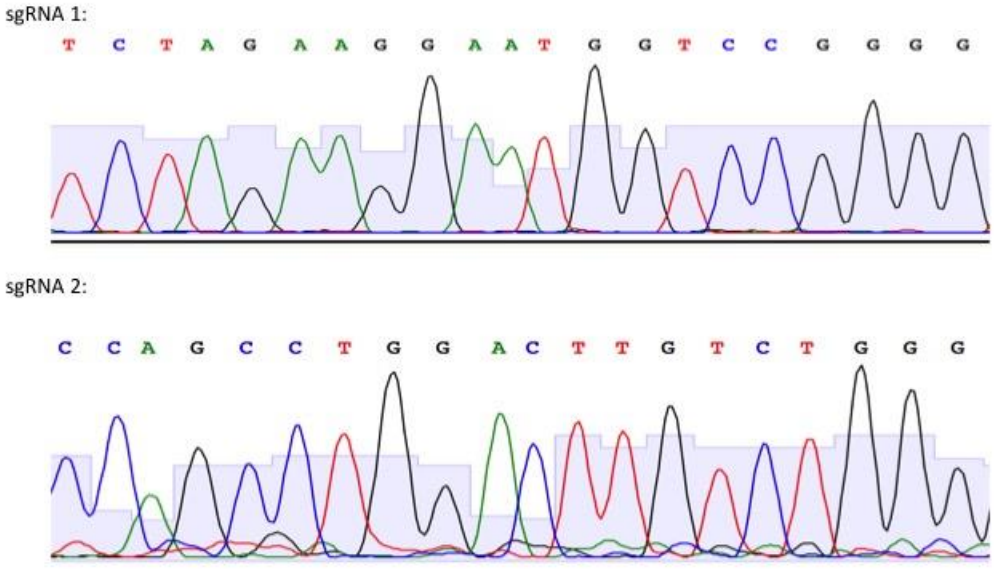
GGGCCCCCACAGCACAGAGGAGGGGCTACTCCCCAGCTTCAAAGCCATC
AGGGTCTTTGTTCCCAGCTTTGGCTCCTGGGAACCCCAGCAATTAGGGGA
GCAGGTTTAACCCTTAAACGGTTGGGGGCGTCACCCCACCCCCGCTGAGA
CTCTGGGGACACCAGCCTGGACTTGTCTGGGTGGAGAGGAGGGGAGAGA
AAAATAGGGGGTTGAGGGGGGGTGTCTTTGAAGCTTCTTGGGTTGGGGGG
p63 Enhancer Sites

B


(245) Bbs1
Bbs1 (267)
chicken β-actin promoter
ori
U6 promoter
gRNA scaffold
CMV enhancer
hybrid intron
3xFLAG
SV40 NLS
Cas9
nucleoplasmin NLS
T2A
PuroR
AmpR promoter
AmpR
f1 ori
bGH poly(A) signal
AAV2 ITR
(7287) BsmBI
(7245) BsmBI
(6063) BsaI
pX459 Vector

C


sgRNA 1:
T C T A G A A G G A A T G G T C C G G G G
sgRNA 2:
C C A G C C T G G A C T T G T C T G G G

D

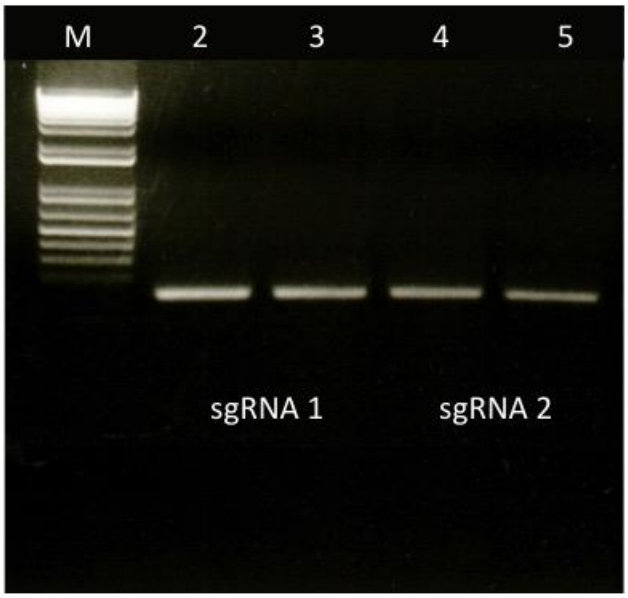

M
2
3
4
5
sgRNA 1
sgRNA 2

Figure 7. A, Chromosome region chr15:98775830-98776513, the p63 enhancer site for Wnt10b. Yellow highlighting indicates sgRNA sequence, while red highlighting indicates p63 binding sites. B, pX459 vector map, BbsI restriction sites are indicated. C, Sequencing results from vector constructs containing sgRNA. D, Gel electrophoresis indicating successful amplification of sgRNA template used for in vitro transcription. Lanes 2 and 3: sgRNA 1, Lanes 4 and 5: sgRNA 2. Marker used was 1kb+.

A

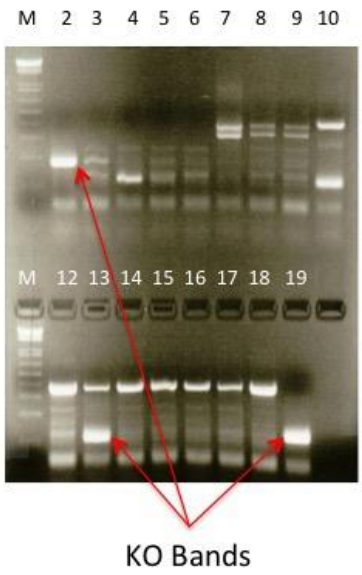
sgRNA 1:

CCCGGACCATTCCTTCTAGA

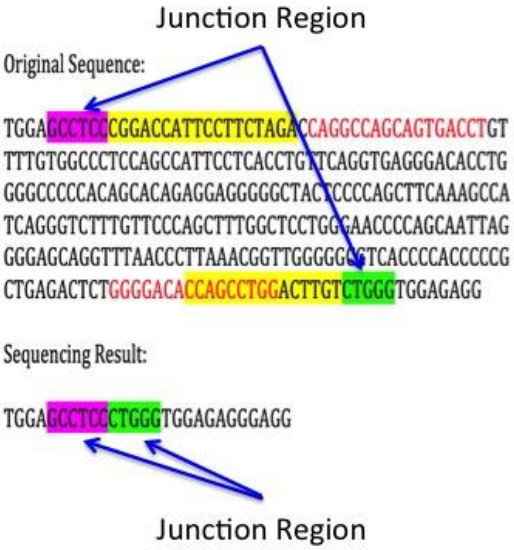
sgRNA 2:

CCAGCCTGGACTTGTCTGGG

B



C



D

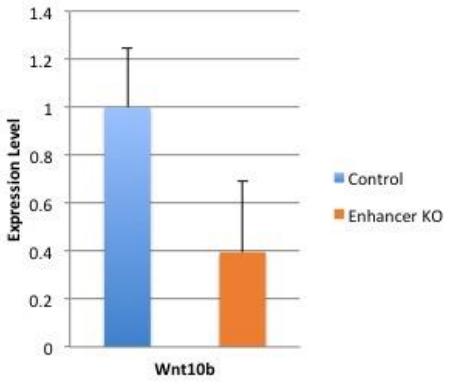


Figure 8. A, sgRNA design for the Wnt10b p63 enhancer site. B, gel electrophoresis following genotyping of the injected mice. Lanes 2, 13, and 19 show the KO band, and are indicated by red arrows. 1kb+ was used as the Ladder. C, sequencing from the animal genotyped in lane 19. Red text is indicative of p63 binding sites; yellow highlighted regions are the sgRNA sequence. Purple, green, and blue highlighting are used as reference points to illustrate the junction region occurring between the purple and green high lighting. D, qRT-PCR data from Dr. Xiying Fan for the three KO mice. GAPDH and HPRT were used as reference genes. Error bars indicate standard deviation. n=3. T-Test indicates $p < 0.05$.

Discussion

I have provided the missing link in p63's regulatory role in cell fate specification by signaling cascade. This is the first demonstration that p63 directly binds enhancers and regulates several genes involved in signaling pathways. Interrupting p63 binding is sufficient to decrease expression levels of target genes, and has the potential to impede signaling pathways.

In vivo analysis not only substantiated this finding, but also shines light on gene expression regulation through noncoding DNA elements. Enhancers are unique when compared to other transcription regulation elements in that they can function independently of proximity to other regulatory elements. Enhancers act on genes whether the enhancer is located upstream, downstream, or within the gene itself. This is primarily accomplished through DNA looping and compaction, which results in regions of DNA that are linearly distant being physically close. The ability of enhancers to work both in the forward and reverse direction allows them to take advantage of DNA looping to act on distant promoters.

Enhancers have been thought to provide a mechanism for cell specific fate specification by regulating signaling pathways. I have shown that p63 does in fact regulate signaling pathways, and it could therefore be speculated that p63 is actively involved in cell fate specification. As seen in the promoter assay data, (Fig. 6D) enhancers are not created equally and even if the same transcription factor binds it can result in differential gene expression. Based on sequencing results these

enhancers vary in sequence, which provides an avenue for altered binding affinity of p63. As evidenced by the introduction of point mutations, interrupting binding affinity is sufficient to reduce luciferase expression, but some minimal expression still occurs. Thus, it is possible to conclude that varying sequences in enhancer sites are responsible for regulating the binding affinity of p63, and ultimately gene expression. I have verified that p63 regulates the Wnt/β-Catenin signaling pathway involved in limb bud and skin development.

Together with previous proposed mechanisms, I demonstrated that p63 plays a mechanistic role in the development of p63-associated syndromes such as EEC. Although the in vivo research still needs to be completed to verify this finding, the in vitro models shown here indicate how a loss of p63 binding could result in the disruption of signaling pathways responsible for maintaining normal development. These insights ultimately lead to a greater understanding of p63-associated syndromes, and will provide guidance when researching possible genetic alterations to correct both syndromic and non-syndromic malformations. Finally, this research has resulted in a greater understanding of how transcription factors might be involved in cell fate specification through regulation of signaling pathways via enhancers. This finding could provide the basis for new technologies such as targeted stem cell differentiation by up-regulating target genes or signaling pathways involved in cell fate specification.

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Enhancers can activate transcription independent of their location, distance or orientation with respect to the promoters of genes

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