

A non-canonical Hippo signaling pathway regulates DeltaNp63 in cancer cells

Ana Maria Low Calle

Submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy
under the Executive Committee
of the Graduate School of Arts and Sciences

COLUMBIA UNIVERSITY

2023

© 2022

Ana Maria Low Calle

All Rights Reserved

Abstract

A non-canonical Hippo signaling pathway regulates DeltaNp63 in cancer cells

Ana Maria Low Calle

The p63 transcription factor, a member of the p53 family, plays an oncogenic role in squamous cancers, while its expression is often repressed in breast cancers. In the canonical conserved Hippo pathway, known to play a complex role in regulating the growth of cancer cells, the protein kinases Mammalian Ste20 like kinases 1/2 (MST) and Large tumor suppressor kinases 1/2 (LATS) act sequentially to phosphorylate and inhibit the Yes-associated Protein/Transcriptional coactivator PDZ binding transcription factors (YAP/TAZ). We found that in the MCF10A mammary epithelial cell line and in squamous and breast cancer cell lines, expression of deltaNp63 RNA and protein is strongly repressed by inhibition of specific components of the Hippo pathway in a manner that is independent of p53. While the Hippo pathway protein kinases MST1/2 and LATS1 are required for p63 expression, the next step of the pathway namely phosphorylation and degradation of the YAP/TAZ transcriptional activators, is not required for repression of p63. This suggests that regulation of p63 expression occurs by a non-canonical version of the Hippo pathway. Interestingly, we observed that experimentally lowering p63 expression leads to increased Yes Associated Protein protein levels, thereby constituting a feedback loop. In addition, p63 loss reduces the growth of MCF10A and squamous cancer cell lines. These results, which reveal the intersection of the Hippo and p63 pathways, may prove useful for the control of their activities in cancer cells.

Table of Contents

List of Figures.....	iii
List of Tables.....	iv
Acknowledgments	v
Preface.....	vii
Chapter 1: Introduction.....	1
1.1 The p53 family of transcription factors.....	1
1.2 The p63 transcription factor and its role in development of epithelial tissues .	3
1.3 Role of the p63 transcription factor in cancer	7
1.3.1 p63 in Squamous Cancers.....	8
1.3.2 p63 in Breast Cancer	11
1.4 Signaling pathway regulation of Δ Np63 expression.....	14
1.5 The Canonical Hippo Pathway and Its Role in Cancer	20
1.5.1 Mammalian Ste20-like kinases 1 and 2: MST1 and MST2	21
1.5.2 The adaptor protein Salvador: SAV1	23
1.5.3 The Mps One Binder 1A/1B adaptor proteins: MOB1A and MOB1B	24
1.5.4 The Large Tumor Suppressor Kinases: LATS1 and LATS2	27
1.5.5 The Yes-Associated Protein and The Transcriptional Co-Activator with PDZ-binding motif: YAP and TAZ.....	29
1.6 Upstream Regulators of the Hippo Pathway.....	34
1.7 Hippo Pathway Interactions with p63.....	37
1.8 Chapter 1 Figures and Tables	39

Chapter 2: A non-canonical Hippo pathway represses the expression of Δ Np63	56
2.1 Abstract	56
2.2 Introduction.....	57
2.3 Materials and Methods	59
2.4 Results.....	62
2.5 Discussion	68
2.6 Acknowledgements	70
Chapter 3: p63 regulates the acinar architecture in mammary epithelial cell 3D culture model	87
3.1 Introduction.....	87
3.2 Materials and Methods	89
3.3 Results.....	93
3.4 Discussion	98
3.5 Chapter 3 Figures	103
Chapter 4: Future Directions	110
References.....	118

List of Figures

Figure 1-1. The p63 Transcription Factor Isoforms	39
Figure 1-2. Expression of TP63 mRNA Across Human Cancers	40
Figure 1-3. Genomic Alterations in the TP63 Gene Across Cancer Types.....	41
Figure 1-4. TP63 mRNA in Normal Tissue Samples Compared to Breast Cancer Subtype Samples and in Breast Cancer Metastasis	42
Figure 1-5 TP63 mRNA in Lung, Cervical, Head and Necks and Esophageal Squamous Cancer samples at different disease stages.....	43
Figure 1-6. The Canonical Hippo Pathway.....	44
Figure 2-1. p53-Independent Regulation of p63 by the Hippo Pathway Kinases MST1 and MST2.....	72
Figure 2-2. LATS1 and MOB1A Regulate p63 Expression.....	74
Figure 2-3. MST Regulate p63 Expression in Breast and Squamous Cancer Cell Lines.	76
Figure 2-4. Repression of p63 after MST1/2 Inhibition is Independent of Yap and Taz.	77
Figure 2-5. p63 Inhibits Yap and Taz Activity.	79
Figure 2-6. p63 Facilitates Growth.	81
Figure 3-1. Lumen Clearing in MCF10A Represses GATA3 and increase BIM.	103
Figure 3-2: Δ Np63 Maintains the Structure of MCF10A in 3D culture.	105
Figure 3-3: Δ Np63 α Represses GATA3 and the Luminal Phenotype in 3D.	107
Figure 3-4: p63 Overexpression and ZEB1 and 2 Ablation Partially Rescue EMT.....	108

List of Tables

Table 1. List of p63 Target Genes Associated with worse survival in Squamous Cancers	
Patients	45
Table 2. Regulators of Δ Np63 Expression	51
Table 3. YAP/TAZ Gene Signature	53
Supplemental Table 1. Primer Sequences	86

Acknowledgments

I would like to thank Carol for giving me the opportunity to join her lab, for her constant support, her bright ideas, and helpful discussions. Her passion for science is inspiring. I would like to thank Ron for his guidance, for teaching me side by side, and for his invaluable suggestions and ideas. I thank my advisors for their mentorship, and for encouraging me to read and dig deeper despite the failures. Both their expertise and vision helped me move this project forward. Thank you.

To my committee members, Iva Greenwald and Richard Baer, thank you for your time and your valuable suggestions through the years. An exceptional thanks to Yan Zhu for generously agreeing to be on my thesis committee at my defense and for seeing me through this last stage of my Ph.D. I want to thank our collaborators Anil Rustgi, Alison Taylor, and James Hudspeth for giving us cell lines and reagents to complete this project. I would like to thank Hana Ghoneima for all her help with some of the experiments of this project. To the Prives lab members, I want to thank Ella Freulich for being a fantastic lab manager, for her knowledge, technical expertise, and support; and for bringing so much joy to the lab every day. I am grateful for my lab friends: Chen, the best bench mate I could ever wish for; thank you for your advice and invaluable help through this Ph.D. Alyssa and Divya, thank you for your support, advice, and for our lunches and yogurt breaks in and out of the lab. You guys made this journey fun despite the long hours and multiple failures. Josh, thank you for your words of encouragement and your excellent suggestions. I also want to thank the current Prives lab members Bitu, Asja, Rafa, Kausik, Kazu, and Tatsu for their valuable suggestions in lab meetings and for all our shared lunches.

I am incredibly grateful to John Hunt for his invaluable help and support at the beginning of this Ph.D. Special thanks to my dear friends Albert Tsai, Elena Carazo, and Marlene Buckmaster. I would have never survived this journey without you; thank you for being there when I needed you the most and for all the good memories. You will always have a place in my heart. I would like to thank Jaime E. Castellanos for being my mentor before grad school, and giving me many tools I use every day the lab. Thank you for helping me get here.

I would also like to thank my cousin Ana Cecilia Calle for being such a great writing partner. To the rest of my wonderful family, thanks for your constant love and support during this journey. Last but not least, I want to thank my parents, Martha Lucia, Eduardo, and my brother Juan Francisco, for their invaluable support, empathy, and loving encouragement that kept me going and kept sane despite all the failures. I love you guys; I would not be here without you.

Preface

Cancer is an imbalance between cell growth, proliferation, and cell death. Millions of people are diagnosed with this disease worldwide every year. This dissertation discusses the transcription factor p63 in two types of cancers, squamous and breast, and its regulation by the Hippo pathway. The role of p63 in cancer is context-dependent; while most breast cancers lose p63 expression, squamous cancers retain and amplify its expression.

This project aimed to understand the role of p63 in breast and squamous cancers. For this purpose, the introduction in chapter 1 will describe the characteristics and functions of the p63 transcription factor in normal tissues and development, followed by its specific role in squamous and breast cancers. I will then discuss how multiple signaling pathways regulate the expression of p63. Finally, the Hippo signaling pathway will be described, along with its activation in normal tissues and its dysregulation in cancer.

Chapter 2 includes the findings of my main project describing the role of a non-canonical Hippo pathway in regulating p63's expression in breast and in squamous cancers that require p63 for growth.

Chapter 3 summarizes the typical architecture of the mammary gland and the preliminary results of studying the role of p63 in a 3D culture model of mammary epithelial cells. P63's relationship to epithelial-mesenchymal transition and the mammary gland architecture will be discussed along with the future directions of this project.

Finally, chapter 4 will discuss the future directions of my main project described in chapter 2, with questions and potential future experiments needed to address those questions.

Chapter 1: Introduction

Tissues require a balance between controlled cell proliferation, differentiation, and cell death. This balance allows the repair and healthy function of tissues. Cancer develops when this balance is altered by the uncontrolled proliferation of cells, leading to abnormal growth within organs and the formation of tumors. This often leads to metastasis, invasion of neighboring tissues, and death. The role of the p63 transcription factor in cancer is context-dependent; while most breast cancers lose p63 expression, squamous cancers retain and amplify its expression in the disease. The following sections will summarize the role of the p63 transcription factor in normal tissues, breast and squamous cancers. The second part, will describe the Hippo signaling pathway, its components and activation in normal tissues, and its dysregulation in cancer.

1.1 The p53 family of transcription factors

The p53 family of transcription factors includes p53, the most famous tumor suppressor protein, and the proteins p63 and p73. However, the family members have a shared structure and can transcribe some gene targets in common. They differ in their biological functions, target genes, and roles in tumorigenesis. P53 is a tumor suppressor, more than 50% of human cancers have p53 mutations, and p53 null mice develop embryonically normal but later develop soft tissue sarcomas (Donehower et al. 1992). In contrast, p63 null mice die early after birth due to dehydration caused by developmental defects in epithelial tissues (Yang et al. 1998), and p73 null mice have developmental defects in the nervous system (Yang et al. 2000).

The domain architecture of all the p53-family members consists of an N-terminal Transactivation domain (TA) followed by a central DNA binding domain (DBD) and an oligomerization domain (OD) at the C-terminus (Yang et al. 1998).

P53 plays a critical role as a tumor suppressor, evidenced in the high frequency of mutations in the p53 gene present in human cancers (Freed-Pastor and Prives 2012) and families suffering from Li-

Fraumeni syndrome. This syndrome is a rare hereditary condition caused by germline mutations in p53 that give carriers a very high risk of developing tumors in multiple organs at a very young age (Beckerman and Prives 2010; McBride et al. 2014). In addition, p53 null mice at six months of age spontaneously develop soft tissue sarcomas, such as osteosarcomas and lymphomas (Donehower et al. 1992). P53 function as a tumor suppressor dependent on its capacity to transactivate multiple gene targets in response to different types of stress, such as DNA damage, activation of oncogenes, and hypoxia, among others (Beckerman and Prives 2010). Under cellular stress, p53 is stabilized and translocated to the nucleus, where it binds to its DNA response element to transcribe cell-cycle arrest (p21), apoptosis (Bax, Noxa), and senescence genes (Beckerman and Prives 2010). Due to its capacity to induce arrest and cell death, p53 protein levels are tightly regulated in unstressed cells by its negative regulator Mouse Double Minute 2 (MDM2). This E3 ligase targets p53 for ubiquitin-mediated proteasomal degradation (Beckerman and Prives 2010). P53's transcriptional activation of arrest, apoptosis, and senescence programs protects tissues by allowing repair or elimination of damaged cells, preventing the propagation of mutations that could lead to the development of cancer.

The other family member p73, unlike p53, is rarely lost or mutated in cancers. On the contrary, several isoforms of p73 can be overexpressed in certain cancers, like non-small cell cancers, breast, stomach and esophagus adenocarcinomas (Rozenberg et al. 2021). In mouse models, the loss of one or both copies of p73 leads to spontaneous formation of lung adenocarcinomas and lymphomas in mice (Flores et al. 2005; Tomasini et al. 2008). In these cancers, p73 contributes to oncogenesis by promoting growth, inflammation, and angiogenesis, among others (Rozenberg et al. 2021).

The last family member, p63 unlike p53, is rarely mutated in cancers and plays a role in the embryonic development of epithelial tissues (Melino et al. 2015). P63 null mice have developmental defects of epithelial tissue, lack limbs, craniofacial structures, and glandular epithelial tissues, including the mammary gland, and die postnatally of dehydration caused by lack of skin (Mills et al. 1999; Yang et al. 1999). The TP63 gene is located at chromosome arm 3q27-29 and has a higher sequence homology to

p73 than p53 (Yang et al. 1998). The p63 transcription factor will be explained in more detail In the following section.

1.2 The p63 transcription factor and its role in development of epithelial tissues

The TP63 gene has ten different isoforms (Figure 1-1) that originate from alternative promoter usage and alternative splicing. The isoforms are divided into two main groups, the TA full length and the Δ N isoform truncated at the 5' end. The TA isoform is transcribed from the P1 promoter upstream of exon 1. The Δ N isoform is transcribed from the P2 promoter located in the middle of exon 3, so this isoform starts from a truncated exon named exon 3' (Figure 1-1) (Yang et al. 1998; Melino 2011). Each TA and Δ N isoform can generate five additional isoforms formed by alternative splicing and truncations at the 3' end: the full-length α isoform and the β , γ , δ and ϵ isoforms (Yang et al. 1998; Mangiulli et al. 2009).

The p63 full length protein structure consists of an N-terminal Transactivation domain (TA) that shares 22% identity with p53, followed by a central DNA binding domain (DBD) that is 60% similar to p53 and an oligomerization domain (OD) at the C-terminus which is 38% identical to p53 (Yang et al. 1998). These three domains form the basic architecture of all the p53-family members (Figure 1-1). In addition, p63 has a second transactivation domain (TA2) after the OD, present only in the α and β isoforms (Ghioni et al. 2002; Duijf et al. 2002), followed by a SAM (Sterile α -motif) domain present only in the α isoforms, in other transcription factors and signaling molecules (Thanos and Bowie 1999). Germline mutations in the SAM domain, associated with a developmental syndrome with severe skin erosions, causes protein aggregates inhibiting the transcriptional activity of p63 proteins in humans (Russo et al. 2018). At the end of the C-terminus an Inhibitory Domain (ID) gives transcriptional inhibitory properties to the α isoforms by promoting the binding and inhibition of the Transactivation domain (TA) inducing a closed inactive conformation of the TAp63 α isoform (Serber et al. 2002; Straub et al. 2010). In addition, the C-terminal TI

domain of the $\Delta Np63\alpha$ isoform interacts with histone deacetylases to induce transcriptional repression of apoptotic genes (Puma) in squamous cancer cell lines (Ramsey et al. 2011).

Expression of different p63 isoforms vary depending on the tissue. A recent study by Marshall and collaborators, using RNAseq data from human tissues, analyzed the exon-junction reads to evaluate the abundance of each p63 isoform across 36 human tissues (Marshall et al. 2021). The analysis found that the majority of tissues expressed very low levels of p63 (less than two transcripts per million), and only a subset of tissues, primarily epithelial, showed high levels of p63 mRNA (more than two transcripts per million). These include the skin, esophagus mucosa, salivary glands, vagina, cervix, mammary gland, prostate, testis, bladder and skeletal muscle (Marshall et al. 2021). The analysis of the N-terminal isoforms revealed that the $\Delta Np63$ (exon junctions 3' to 4) was the predominant isoform making between 80–100% of the total p63 transcripts present in the tissues with high expression in the skin, breast, esophagus, vagina, and prostate (Marshall et al. 2021). Interestingly, 100% of the transcripts in the bladder correspond to the ΔN isoform, while in the testis, 96% correspond to the TA isoform (Marshall et al. 2021). Similarly, the C-terminal isoform expression revealed that the α isoform is the most abundant in tissues with high p63 expression, such as the skin, esophagus, and mammary gland. In the testis where the TA isoform is the most abundant, TAp63 α is expressed exclusively. Skeletal muscles were the exception, expressing only the γ isoform, TAp63 γ (Marshall et al. 2021). This study illustrates p63's restricted tissue expression and confirms the pattern of p63 expression previously observed by histochemistry in mouse and human tissues (Yang et al. 1998; Como et al. 2002).

The importance of p63 expression in epithelial tissues is evidenced in TP63 null mouse models that showed the critical role of p63 in the development of skin and glandular tissues, and the morphogenesis of the limb and craniofacial structures (Mills et al. 1999; Yang et al. 1999). In humans, seven different syndromes have been associated with heterozygous mutations in TP63 that cause developmental disorders characterized by craniofacial malformations and orofacial clefting, absence or malformation of fingers, nails, teeth, and mammary glands (Harazono et al. 2022).

The $\Delta Np63\alpha$ isoform, besides being the most highly expressed isoform in the skin and glandular epithelium, is the isoform responsible for skin development in mice. Ablation of the $\Delta Np63$ isoform exclusively by replacing the 3' exon with GFP without affecting the TA isoforms, found that $\Delta Np63$ null mice had only small patches of skin with upper layer differentiation, but with low expression of the proliferation marker Ki67. Indicating that the loss of the ΔN isoform accelerated differentiation, by decreasing the proliferation capacity of the keratinocytes (Romano et al. 2012). In contrast, TA null mice in the skin had blistering, premature aging and wound healing defects, phenotypes that are consequence of impaired proliferation and increased senescence of keratinocytes (Su et al. 2009). These results demonstrate different but complementary roles of the ΔN and TA isoforms, where both isoforms are required for the proper development of the skin layers. ΔN isoforms are necessary for proliferation, development, and differentiation of the skin, while TA isoforms are required to restrict the excessive proliferation of the skin stem cells to prevent premature senescence.

TAp63 isoforms respond to DNA damage or other types of stress in epithelial tissues, neurons, and germline cells (Su et al. 2013). While TAp63 γ had the highest activation of a p53 reporter gene, similar to WT p53 (Yang et al. 1998). TAp63 α isoforms is mostly expressed in the testis, and ovaries and oocytes where it is the most abundant (Marshall et al. 2021; Suh et al. 2006). TAp63 α expression is high in oocytes during meiotic arrest until its expression decreases before entry into ovulation (Suh et al. 2006). TAp63 α is essential for DNA damage induced apoptosis in oocytes (Suh et al. 2006). Since the period between arrest and ovulation can last several years in humans, TAp63 α is present in cells in an inactive state, then is activated by phosphorylation upon DNA damage (Deutsch et al. 2011). In contrast, the $\Delta Np63$ isoform acts as an apoptosis inhibitor after UV light-induced DNA damage when constitutively expressed in the upper layers of the skin (Liefer et al. 2000). In the adult mouse mammary gland, $\Delta Np63\alpha$ acts as a survival factor that prevents excessive apoptosis during the post-lactation involution period (Yallowitz et al. 2014), since p63 +/- mice have increased apoptosis and increased activation of Stat3 and p53 pathways, compared to p63+/+ (Yallowitz et al. 2014). $\Delta Np63$ isoform regulates proliferation, inhibits apoptosis and regulates differentiation in keratinocytes and mammary epithelial cells, while the TAp63 isoforms regulate their terminal differentiation, restrict proliferation and regulate apoptosis of epithelial cells upon DNA damage.

The Δ Np63 isoform has an apoptosis inhibitory role, when the Δ Np63 α isoform, was coexpressed with WT p53 and other p63 isoforms, it had a dominant-negative inhibitory activity towards the TAp63 γ isoforms, inhibiting their transactivation of a p53 response element reporter (Yang et al. 1998). The dominant-negative activity is due to the Inhibitory Domain (ID) domain present in the α isoform (Straub et al. 2010), The N-terminally truncated domain exclusive to the Δ N isoform, made of 14 amino acids present in the 3' Exon, have a reduced capacity to transactivate p53 response elements compared to the TAp63 isoforms (Yang et al. 1998). Later, it was recognized that the Δ Np63 α isoform also has transcriptional activation capacity of its own (Ghioni et al. 2002). Initial analysis of p63 targets showed that p63 binding is enriched in genes involved in cell cycle and cell proliferation (Yang et al. 2006). Other genes are associated with Notch, Wnt, and Transforming Growth factor β signaling (Yang et al. 2006). p63 binds to promoters of cell adhesion molecules like tight junctions, adherens junction, and focal adhesion genes (Yang et al. 2006). Inducing proliferation and differentiation of the skin's stem cells, Δ Np63 directly regulates the expression of keratinocyte differentiation genes, like basal Keratins 5 and 14 (Romano et al. 2009), as well as Fras1 (Fraser Extracellular Matrix Complex Subunit 1, a gene important for attachment of the basal layer to the extracellular matrix) and IK β Kinase (the negative regulator of NF κ B transcription factor) required for terminal differentiation of keratinocytes (Koster et al. 2007). In the mammary gland Δ Np63 α is present in myoepithelial cells (Barbareschi et al. 2001) and induces the expression of EGFR receptor and cell adhesion molecules like integrins α 3, α 5, α 6, β 1, and β 4, necessary for attachment of epithelial cells to the extracellular matrix and (Carroll et al. 2006).

Several studies have attempted to identify a *bona fide* list of genes regulated by p63, with inconsistent results. As an attempt to identify consistent targets across multiple datasets, a recent analysis combined 11 RNAseq and ChIPseq data sets from 9 different cell types (keratinocytes, immortalized keratinocytes HaCat, the foreskin fibroblast BJ, mammary epithelial cells MCF10A, squamous cell carcinomas H226 and KYSE70, head and neck squamous cell line FaDu, and pancreatic adenocarcinoma BxPC3 and SUIT2). This analysis defined a p63 gene regulatory network of 180 genes, including direct targets that have p63 binding sites proximal to the transcription start site, or indirect targets with p63 binding sites associated with distal enhancers (Riege et al. 2020). A gene set enrichment analysis found among

the p63 regulated genes cell cycle regulator genes such as E2F, MYC, and the DREAM complex (dimerization partner retinoblastoma-like, E2F and MuvB complex), and genes involved in mammary stem cell activity, epithelial development, and SCCs growth (Riege et al. 2020). Further analysis of the TF binding enrichment among p63 target genes, revealed that p63 upregulated targets also have binding sites for Kruppel Like Factor 4 (KLF4), MYC, and SMAD4 transcription factors. This suggests cooperation between p63 and other transcription factors to regulate transcription, rather than a direct regulation of those targets by p63.

The cooperation of p63 with other factors to induce transcription, has been shown in primary fibroblasts differentiated to keratinocytes by the combined overexpression of KLF4 and p63 (Lin-Shiao et al. 2019). This cooperation with KLF4 allows p63 to establish the enhancers needed to express epithelial and craniofacial development genes (Lin-Shiao et al. 2019). In fact, the majority of p63 regulated genes have p63 bound to enhancer regions at intergenic and intronic regions, rather than binding to promoters or transcription start sites (Lin-Shiao et al. 2019; Riege et al. 2020; Saladi et al. 2017). In summary, p63 helps establish enhancers needed for the expression of epithelial and development genes and induces their expression indirectly through binding to enhancer regions rather than direct transcriptional activation at the transcription start site.

1.3 Role of the p63 transcription factor in cancer

The limited expression of TP63 to specific tissues in humans is also seen across human cancers. TP63 is highly expressed compared to normal tissue, in head and neck, lung, cervical and esophageal squamous carcinomas, as well as bladder carcinoma, thymomas, and the lymphoid neoplasm Diffuse Large B-cell lymphomas (Figure 1-2). TP63 mRNA levels are also present in prostate adenocarcinomas and breast invasive carcinomas but at lower levels compared to normal tissues (Figure 1-2). Similarly, the TP63 gene is most frequently amplified at a genomic level in squamous cancers of the lung, head and neck, cervical and esophageal, but it is rarely mutated, truncated or deleted (Figure 1-3). In addition, based on the analysis of more than one thousand squamous carcinoma patient samples, the Δ Np63 isoform was preferentially expressed over the TA isoform due to methylation at the P1 promoter (Campbell et al. 2018).

This suggests that p63 could play an oncogenic role in squamous cancers. In contrast, the TP63 mRNA levels in breast cancers are much lower than in normal tissues, and the frequency of amplifications is also very low. Suggesting that p63 plays a less clear role in breast cancers (Figure 1-3). The following section will summarize some of the functions of p63 in squamous and breast cancers reported in the literature.

1.3.1 p63 in Squamous Cancers

Squamous cell carcinomas (SCC) are cancers originating from the epithelium of the air, digestive or genitourinary tract, which can give origin to different types of cancers: cervical, lung, esophageal, bladder, head and neck (Campbell et al. 2018). These types of SCC have been associated with exposure to risk factors such as smoking, alcohol, ultraviolet light from the sun, and human papillomavirus infection (Dotto & Rustgi, 2016). The basal layer of epithelial tissues separates the mesenchyme from the epithelium and is made of highly proliferative cells with a big nucleus. These proliferative cells give origin to more differentiated cells, generating successive layers of smaller and flattened cells until they reach the last layer of terminally differentiated, no dividing, flat cells. The name of this terminal layer of flat cells or “squames” gives this type of cancers their name (Dotto & Rustgi, 2016).

SCCs have a common signature of genomic alterations present in different cancers. A computational study of almost 10000 pan-cancer samples was analyzed by copy number alterations, DNA methylation, mRNA, miRNA, and protein expression. The study found that five types of SCC clustered together and shared common signatures independent of their tissue of origin (Campbell et al. 2018). The highest mutation frequency was in TP53, followed by PIK3CA (p110 α protein of the phosphatidylinositol 3-kinase) and NOTCH1 (Campbell et al. 2018). In addition, components necessary for cell metabolism growth and survival were increased across SCC, genes related to the PI3 kinase-Akt-mTOR pathway, and hotspot missense activating mutations in HRAS, Map kinase MAPK1, and RAC1 (Campbell et al. 2018). Although SCCs originate from different tissues, they share common genes and gene alterations associated with a squamous signature. These alterations are overexpression of SOX2 and TP63, specifically the Δ Np63 isoform, since the P1 promoter (the TA isoform promoter) is methylated (Campbell et al. 2018). The elevated mRNA levels and high frequency of genomic amplifications at the TP63 locus found in SCCs,

suggest that p63 has an oncogenic role in these cancers. The next section will summarize some examples of the functions attributed to p63 in SCCs in the literature.

Δ Np63 α expression is essential for oncogenic transformation of keratinocytes, a necessary step for SCCs development. Furthermore, high expression of Δ Np63 α protects keratinocytes from undergoing senescence induced by oncogenic Ras, increasing survival and tumor formation in xenograft models (Keyes et al. 2011). Δ Np63 α inhibits senescence through the transcriptional upregulation of lymphoid-specific helicase (Ish a chromatin remodeler) (Keyes et al. 2011). Similarly, *in vivo* after skin exposure to the mutagenic agent DMBA that induces RAS mutations, a mice model with skin induced Δ Np63 α overexpression, had higher incidence of SCCs, compared to WT mice (Devos et al. 2017). Keratinocytes derived from Δ Np63 overexpressing mice, proliferated faster and were less senescent. Indicating that Δ Np63 facilitates oncogenic transformation of skin keratinocytes by increasing proliferation and delaying senescence (Devos et al. 2017). Another potential oncogenic role of Δ Np63 is inhibition of apoptosis, by its dominant-negative activity towards other p53 family members transactivation activity (Yang et al. 1998). In addition, Δ Np63 can bind to other p53 family members, such as TAp73 and mutant p53, but not with WT p53 (Gaiddon et al. 2001). Δ Np63 inhibits the transcriptional activity of TAp73 β , preventing expression of apoptotic genes, such as puma and noxa, in head and neck SCCs (Rocco et al. 2006). Δ Np63 α regulates proliferation and survival, while inhibits apoptosis and senescence in keratinocytes and SCCs, supporting its role as oncogene in SCCs development.

P63 regulates growth and proliferation *in vivo* and *in vitro* through multiple mechanisms. Δ Np63 regulates a signature of 28 genes targets associated with low probability of survival in SCCs patients (see Table 1); some of them are cell cycle related genes such as E2F4, E2F6, SIN3A, E2F1, FOXM1, NFYA, and NFYB, and the MYC/MAX transcription factors are among p63-upregulated genes (Riege et al. 2020). One of those mechanisms is transcriptional regulation of signaling components. *In vivo* in a SCC mouse model, gene expression analysis found that Δ Np63 induced genes related to Fibroblast Growth Factor Receptor signaling (FGFR2 and FGFR3). FGRF genes are important for SCCs growth because ablation of p63 decreased tumor volume, proliferation, increased apoptosis and caused tumor regression in mouse

SCCs (Ramsey et al. 2013). In addition, ablation of p63 also downregulated integrin signaling genes expressed in epithelial cells such as Integrin- α 6 and extracellular matrix molecules, Collagens 16a1 and 17a1 and lamin- β 3 (Ramsey et al. 2013). Δ Np63 also regulates epithelial cell differentiation specific genes, such as Keratin 6A and 14 present in the basal layers of the skin (Boldrup et al. 2007). This indicates the importance of Δ Np63 transcriptional program to maintain the epithelial differentiation state in SCCs.

Other signaling pathway regulated by Δ Np63 is hyaluronic acid signaling. Δ Np63 transcriptionally activates CD44 receptor expression in head and neck SCC (Boldrup et al. 2007). CD44 is the hyaluronic acid receptor (an extracellular matrix component) associated with a stem cell phenotype, since only cells positive for CD44 can generate tumors *in vivo* (Prince et al. 2007). Δ Np63 regulates CD44 expression by direct binding to its promoter. In addition, Δ Np63 induces the expression of the hyaluronic acid synthase (HAS3) while repressing the hyaluronidase genes (HYAL-1 and HYAL-3) in head and neck squamous cancers, increasing the total levels of hyaluronic acid *in vitro* and in xenograft models *in vivo*. The upregulation of HAS3 increases the activation by phosphorylation of EGFR; while loss of p63 or ablation of HAS3, impairs the activation by phosphorylation of EGFR (Compagnone et al. 2017). This suggests that Δ Np63 increases Hyaluronic acid synthesis and receptor expression, which activates EGFR signaling, required for proliferation of SCCs.

In addition, Δ Np63 increases anchorage independent growth through the transcriptional repression of the Hippo signaling negative regulator WWC1 (WW and C2 Domain Containing 1, also known as Kibra). In consequence Δ Np63 repression of WWC1, leads to activation of YAP1 (the transcriptional activator of the Hippo pathway which will be explained in detail in section 1.5.5) impairing its phosphorylation and cytoplasmic retention (Saladi et al. 2017), interestingly, in SCCs amplifications in chromosome 11q are associated with increased expression of YAP1. In addition, genomic amplifications on Δ Np63 are also frequent in SCCs, but are mutually exclusive with YAP1 amplifications (Campbell et al. 2018). This mutual exclusivity suggests the existence of 2 different oncogenic paths one driven by genomic amplifications of Δ Np63 and the other by amplifications of YAP1, suggesting redundancy in their functions, negative regulation between them, or one factor could activate the other as shown by Saladi et al. (2017).

Δ Np63 can regulate tumor growth through the transcriptional activation of genes involved in metabolism regulates enhancer mediated expression of the glucose transporter GLUT1 (SLC2A1), which increases glucose uptake, increasing proliferation and anchorage-independent growth in SCCs, as well as increased tumor growth in xenograft models (Hsieh et al. 2019).

In summary, Δ Np63 induces proliferation *in vitro* and *in vivo* and anchorage independent growth, through multiple mechanisms such as regulation of cell cycle genes, regulation of different signaling pathways such as FGFR2, CD44 and EGFR and increasing glucose uptake through GLUT1 expression.

1.3.2 p63 in Breast Cancer

Breast cancer is the most commonly diagnosed cancer, with an estimated 2.3 million new cases in 2020, representing 11.3% of total new cancer cases and 6.9% of total cancer deaths. Breast cancer is the 5th cause of cancer mortality worldwide (Sung et al. 2021). Breast cancer is a heterogeneous disease classified in the clinic in four molecular subtypes Luminal A, Luminal B, Her2 Enriched, and Triple Negative. *Luminal A* is positive for the expression of Estrogen Receptor (ER), Progesterone Receptor (PR), and negative for the expression of Her2 (ERBB2 receptor). It has low cell proliferation rates, lower-grade (less invasive), and good prognosis. The *luminal B* subtype also has ER and PR expression but is lower than the luminal A subtype; it can be Her2 positive or negative and has a higher grade (more invasive) than the luminal A, higher cell proliferation, and an intermediate prognosis. *Her2 enriched* has amplifications and increased expression of the Her2 gene; it does not express ER or PR, it has higher grade and higher proliferation rate, so it is aggressive. Still, it responds to Her2 targeted therapies and has an intermediate prognosis. *Triple Negative*, also called *basal-like*, has no expression of ER, PR, or Her2. It has a high grade (invasive), high cell proliferation rate, and poor prognosis (reviewed in Harbeck et al., 2019). All breast cancers arise in the lobules, which are the functional units of the terminal ducts (Harbeck et al., 2019). The lobular units consist of two main types of cells: epithelial cells that are polarized and surround a central lumen, and basal cells or myoepithelial cells that are located adjacent to the luminal cells, in contact with the basement membrane and the stroma (Inman et al. 2015). Myoepithelial cells in the breast tissue express Δ Np63 α , the most abundant isoform in breast tissue (Barbareschi et al. 2001; Marshall et al. 2021).

TP63 mRNA expression in breast cancers is much lower compared to normal tissues (Figure 1-2) and genomic amplifications at the TP63 locus are rare in breast cancers (Figure 1-3), compared to squamous cancers. When breast cancer samples are stratified by molecular subtype, the p63 mRNA levels are also much lower compared to normal tissues (Figure 1-4 A). In addition, when breast cancer samples are classified based on the presence or absence of metastasis, the levels of p63 mRNA decrease in cases with metastasis (Figure 1-4 B). Interestingly, in a Her2 breast cancer mouse model, a similar reduction in p63 expression was observed during cancer progression from hyperplasia to invasive carcinoma (Memmi et al. 2015). Analysis of TCGA breast cancer samples shows that the p63 mRNA levels in breast cancers decrease in advanced invasive stages of (Figure 1-4 B, C). This evidence suggests that the tumorigenesis process in breast tissue downregulates p63 mRNA, and the metastatic process downregulates it further. This evidence makes p63's role in breast cancer context dependent. The following sections will discuss evidence in the literature supporting p63's role as an oncogene and as a tumor suppressor.

The importance of p63 for the development of the mammary gland is evident since the p63 null mice lack mammary glands (Mills et al. 1999; Yang et al. 1999). Indeed, a subpopulation of p63 positive myoepithelial cells has stem cells properties, with the capacity to self-renew and regenerate the entire tissue in mice (Prater et al. 2014; Shackleton et al. 2006; Stingl et al. 2006). A mammary reconstitution assay of the fat pad in mice showed that $\Delta Np63$ overexpression increases the capacity of mammary epithelial cells to reconstitute the mammary gland ductal tree (Chakrabarti et al. 2014). In this same model p63 has oncogenic functions, it regulates the expression of Wnt signaling genes like Fzd7 receptor and the ligand Wnt5A in a triple negative basal-like breast cancer mouse model, and p63 increased tumor formation capacity of basal-like breast cancer cell lines MDA-231 and Sum-1315 and breast cancer samples (Chakrabarti et al. 2014). This study shows that $\Delta Np63$ regulates the proliferation capacity of mammary epithelial cells and basal-like breast cancer cells.

A different oncogenic role of p63, is regulation of Hedgehog signaling by transcriptional activation of the genes Sonic Hedgehog (the ligand), Patched1 (Ptch1, the receptor), and Gli2 (GLI family zinc finger 2, the downstream target) in a Her2 mouse tumor model. $\Delta Np63$ increased cell proliferation, self-renewal,

and *in vivo* tumor proliferation of mouse mammary tumor cells (Memmi et al. 2015). In addition, Δ Np63 increases EGFR signaling through transcriptional upregulation of the Hyaluronic acid receptor CD44 and increases synthesis of the CD44 ligand, hyaluronic acid, by mRNA upregulation of the hyaluronic acid synthase 3 in basal breast cancer cells, increasing their proliferation and self-renewal (Gatti et al. 2018). Interestingly, elevated expression of CD44 is associated with self-renewal capacity and proliferation of breast cancer cells (Gatti et al. 2018). The data described above illustrates that Δ Np63 transcriptional activation of different signaling pathway components regulates growth in breast cancer cells.

P63's most controversial role is its effect on epithelial cell migration, which appears to be in some contexts positively regulated while in others is inhibited by p63. Regarding its role in migration, Δ Np63 can induce migration by transcriptional upregulation of the gene Missing In Metastasis Protein (MTSS1) a protein involved in the regulation of the actin cytoskeleton, in basal breast cancer cell line HCC1954 (Giacobbe et al. 2016). In addition, p63+ and Keratin 14+ cells have been shown to lead the collective invasion of primary tumor organoids in 3D, where strands of cells detach from the tumor as a group and penetrate the extracellular matrix (Cheung et al. 2013). The basal cells positive for p63 and Keratin 14 localize at the invasive edge of the strands, in contact with the matrix. Interestingly, even luminal B type of tumors that express very low levels of p63, have cells that acquire basal markers (p63 and keratin 14) when grown in collagen matrixes in 3D (Cheung et al. 2013). Keratin 14 positive cells at the leading edge, were observed in multiple human breast cancer samples from Luminal A, B, and triple negative subtypes (Cheung et al. 2013). *In vivo*, Keratin 14 was present at the edge of cells that had metastasized to the lung (Cheung et al. 2013). P63 is required for the collective invasion of these types of tumors since invasive strands coming out of the tumor disappear almost entirely with the loss of p63, and can induce cell migration in luminal A, B, and triple negative subtypes of breast cancer (Cheung et al. 2013). Even though the exact mechanism behind collective invasion is still unknown, it is likely Δ Np63 induced expression of extracellular matrix attachment genes, such as Integrin α 6 and β 4 is required for this 3D migration/invasion, evidenced by the presence of p63 positive cells only at the edges of the invasive front.

The evidence of $\Delta Np63$ as a migration/metastasis inhibitor comes from several studies that showed that p63 loss increases migration in basal-like breast cancer cell lines (Orzol et al. 2016). Similarly, in non-transformed mammary epithelial MCF10A, mutant Ras, mutant PI3K, and Her2 overexpression repressed $\Delta Np63\alpha$ expression and induced an epithelial mesenchymal transition, increased cell migration in 2D, increased invasion in 3D, and tumor metastasis (Yoh et al. 2016; Hu et al. 2017). The evidence supporting $\Delta Np63$ inhibition of EMT and migration in MCF10A and other basal-like cancer cells, supports its role as a potential tumor suppressor, usually evaluated in 2D wound healing and transwell assays. However, evidence from studies in 3D tumor organoids suggests that cancer cells require p63 for collective invasion required for metastasis *in vivo* and invasion *in vitro* in 3D tumor models. Based on this discrepancy, the contribution of $\Delta Np63$ seems to be context dependent, as it inhibits migration in 2D and is required for collective invasion in 3D. Therefore, in order to better understand the context dependent role of p63 in migration, further studies are necessary using more physiological 3D tumor models, different types of breast cancers, and different extracellular matrices, to clarify the role of p63 in migration and metastasis.

P63's role as a suppressor of migration and metastasis, could explain why its expression decreases in advanced stages of breast cancers (Figure 1-4 C), compared to squamous cancers that maintain high levels of p63 expression even in advanced stages (Figure 1-5), which suggests that p63 could be an oncogenic driver in SCCs that depend on p63 for growth.

1.4 Signaling pathway regulation of $\Delta Np63$ expression

The mechanisms that control $\Delta Np63\alpha$ expression could have therapeutic potential for the treatment of squamous cancers and breast cancers with high expression of p63. The factors and signaling pathways that regulate the expression of $\Delta Np63$ will be described in the following section and are summarized in Table 2.

Epithelial Growth Factor Receptor and other cell surface receptors

Corneal epithelial stem cells in the limbus (the transition zone between the cornea and conjunctiva) in the eye, depend on $\Delta Np63\alpha$ expression for proliferation (Cheng et al. 2009). Keratinocyte growth factor

(KGF) through the KGF Receptor (an alternative splice variant of Fibroblast Growth Factor Receptor 2 expressed in Keratinocytes) regulates $\Delta Np63\alpha$ expression and proliferation in a dose-dependent manner in limbal epithelial cells. KGF induces the expression of $\Delta Np63\alpha$ through p38 MAP kinase-dependent signaling as treatment with a p38 inhibitor suppressed $\Delta Np63\alpha$ expression in corneal epithelial stem cells (Cheng et al. 2009).

Epithelial Growth Factor Receptor (EGFR) is a gene target of $\Delta N63\alpha$ in mammary epithelial cell line MCF10A (Carroll et al. 2006). At the same time, upon treatment with EGF, EGFR increases $\Delta N63\alpha$ expression through Phosphoinositide 3-Kinase pathway in keratinocytes (Barbieri et al. 2003). Similarly, activation of EGFR with EGF increased the expression of $\Delta Np63\alpha$ through activation of STAT3, while the inhibition of EGFR (with a tyrosine kinase inhibitor) decreased $\Delta Np63\alpha$'s expression in SCCs (Ripamonti et al. 2013). Interestingly, the EGFR-STAT3- $\Delta Np63\alpha$ signaling axis is required for growth of SCCs after radiation treatment (Ripamonti et al. 2013). In contrast, in mammary epithelial cells, constitutive active PI3K signaling, induced by overexpression of mutant RAS, mutant PI3K and HER2, repressed $\Delta Np63\alpha$ expression (Yoh et al. 2016; Hu et al. 2017). In this model, FOXO3a is responsible for the constitutive expression of $\Delta Np63\alpha$. AKT activation downstream of PI3K, phosphorylates FOXO3a leading to its nuclear exclusion and $\Delta Np63\alpha$ repression (Yoh et al. 2016; Hu et al. 2017). This evidence suggests that keratinocytes and SCCs regulate $\Delta Np63\alpha$ expression through different downstream targets genes, leading to opposite effects in mammary epithelial cells.

Epithelial Mesenchymal Transition related factors

The epithelial–mesenchymal transition (EMT) is a transcriptional program that occurs during embryonic development, wound healing, and tissue homeostasis (Stemmler et al. 2019). EMT activates a mesenchymal phenotype that induces cell motility, loss of epithelial cell polarity, and loss of cell-cell contacts. EMT is activated by transcription factors such as Snail (also known as SNAI1), Slug (also known as SNAI2), Twist-related protein 1 (TWIST1), zinc-finger E-box-binding homeobox 1 (ZEB1), and ZEB2 (Stemmler et al. 2019). Similarly, overexpression of constitutively active forms of NF- κ B, Ras and PI3K induced EMT in mammary epithelial cells (Chua et al. 2007; Yoh et al. 2016; Hu et al. 2017). In addition,

overexpression of EMT transcription (ZEB2, Snail and Slug) suppresses $\Delta p63$ expression in SCC and mammary epithelial cells as well (Higashikawa et al. 2007; Chua et al. 2007; Herfs et al. 2010). At the same time, ablation of $\Delta Np63$ induced EMT in mammary epithelial cells, keratinocytes and SSCs (Yoh et al. 2016; Mehrazarin et al. 2015). Since $\Delta Np63$ regulates expression of cell adhesion molecules and epithelial genes, is not surprising that EMT would induce changes in $\Delta Np63$ expression. In fact, loss of $\Delta Np63$ seems to be a requirement for this process to take place. Whether rescue of $\Delta Np63$ is enough to reverse the EMT phenotype is still unclear. However, $\Delta Np63$ overexpression inhibits EMT induced migration (Hu et al. 2017) which indicates that $\Delta Np63$ exerts inhibitory effects on EMT.

NF κ B signaling pathway

Nuclear factor kappa B (NF- κ B) is a heterodimeric transcription factor that is inactivated in the cytoplasm by binding to inhibitor-of-kappaB (I κ B) (Ghosh and Karin, 2002; Karin and Lin, 2002). In mouse keratinocytes, activation of NF- κ B transcription factor induced repression of $\Delta Np63$, mediated by ankyrin repeats SH3 domain and protein rich region containing protein 2 (ASPP2). ASPP2 binds to I κ B, releasing NF- κ B from inhibition (Tordella et al. 2013). ASPP2 is induced upon keratinocyte differentiation and represses $\Delta Np63$ (Tordella et al. 2013). Similarly, in mammary epithelial cells, overexpression of a constitutively active form of NF- κ B (with reduced affinity for I κ B α) induced EMT and increased Zeb1, and Zeb2 overexpression and repressed p63 expression (Chua et al. 2007). In summary, NF- κ B regulates $\Delta Np63$ expression in mammary epithelial cells and keratinocytes, regulates differentiation and EMT.

Notch signaling pathway

The Notch signaling pathway consists of four Notch receptors, Notch 1-4, and 5 different ligands Delta-like 1, 3, and 4 (DLL1, DLL3, and DLL4), and Jagged 1 and 2 (JAG1 and JAG2) (Bray 2016). When the pathway gets activated, the ligand expressed in a neighboring cell, binds to the Notch receptor expressed in a target cell, inducing the proteolytic cleavage of the receptor by the protease ADAM on the extracellular side, and the γ -secretase protease on the intracellular side. This releases the Notch Intracellular Domain (NICD), which interacts with DNA-binding protein CBF1–Suppressor of Hairless–LAG1 (also known as RBPJ, to stimulate gene expression (Bray 2016).

Several studies have shown that Δ Np63 and Notch signaling antagonize each other in human mammary epithelial cells and keratinocytes. Constitutive Notch activation through NICD overexpression, induces luminal cell differentiation, and decreased the number of p63 positive cells and colonies, in mouse mammary basal/stem cells (Bouras et al. 2008). Similarly, in keratinocytes, constitutive active Notch1 signaling, repressed Δ Np63 expression (Nguyen et al., 2006). In addition, ablation of Δ Np63 in basal mammary cells, induced luminal cell characteristics, which phenocopies Notch activation (Yalcin-Ozuysal et al. 2010). Together these observations suggest that active notch represses p63 and induces luminal cell characteristics. In contrast, inhibition of Notch with γ -secretase inhibitor or knockdown of the CBF1 coactivator, induced expansion of p63 positive basal cells, and impaired luminal cell differentiation in mammary epithelial cells (Bouras et al. 2008; Yalcin-Ozuysal et al. 2010). In keratinocytes, inhibition of Notch signaling by ablation of Notch1 receptor, increased Δ Np63 expression (Nguyen et al., 2006). In addition, overexpression of Δ Np63 phenocopied Notch inhibition, impairing luminal cell differentiation, and increasing basal cells keratin expression (Yalcin-Ozuysal et al. 2010). These results indicate that Notch signaling and p63 counteract each other. Notch represses p63 and induces luminal differentiation, while p63 inhibits Notch signaling, impairing luminal differentiation and maintaining the basal cell phenotype.

In keratinocytes, deletion of Notch1 receptor, increased Δ Np63 and interferon regulatory factor 3 (IRF3) expression, while Notch activation repressed Δ Np63 and downregulated IRF3 and IRF7 transcription factors mRNA (Nguyen et al., 2006). In addition, overexpression of IRF7 rescued Δ Np63 mRNA expression upon Notch activation (Nguyen et al., 2006), suggesting that IRF7 is responsible for Δ Np63 expression in keratinocytes. Yet, Δ Np63's mRNA is only partially downregulated by combined ablation of IRF7 and IRF3 together, but not separately (Nguyen et al., 2006). In addition, the effects of IRFs on Δ Np63 protein levels were not evaluated in this study. Even though the results are inconclusive, the observations suggest that activation of Notch signaling represses Δ Np63 mRNA partially through downregulation of IRF7, and at the same time suggests that other factors besides IRF7 are required for Δ Np63's constitutive expression.

Hedgehog signaling pathway

The Hedgehog signaling pathway is involved in development and differentiation of cells. When the pathway is inactive the Patched1 (PTCH1) receptor keeps the transmembrane protein Smoothed (SMO) inhibited, allowing the Gli transcription factor to be proteolytically processed to generate the GliR form that lacks the C-terminal domain and acts as repressor (Zhou and Jiang 2022). When the ligands Sonic Hedgehog (SHH), Indian Hedgehog or Desert Hedgehog bind the receptor PTCH1, the pathway is activated and releases SMO from inhibition. This prevents the proteolysis of Gli, which is turned into a full length activator GliA that enters the nucleus to induce transcription. There are three Gli members: Gli1, Gli2, and Gli3, both Gli2 and Gli3 are proteolytically processed to generate GliR when the signaling is off, and Gli1 acts only as a transcriptional activator, induced as a positive feedback loop to amplify the signal (Zhou and Jiang 2022).

Activation of Hedgehog signaling by overexpression of the ligand Ihh, repressed Δ Np63 α and promoted the transcription of the TAp63 isoform through activation of Gli3, increasing the proliferation of mammary epithelial cells (Li et al. 2008a). Interestingly, Gli3 knockdown also repressed Δ Np63 α and promoted TAp63 transcription, however, to a lesser degree compared to Ihh (Li et al. 2008a). This apparent contradiction suggests that the lack of Gli3R (the truncated form present when signaling is off) is responsible for Δ Np63 α repression and not the activation of Gli3 full length. In addition, the complexity of this pathway increases with the fact that Δ Np63 transcriptionally regulates multiple components of this pathway, such as the negative regulator suppressor of fused (SUFU), the ligand Sonic Hedgehog (Shh), the transcriptional activator Gli2, and the receptor Ptch1 (Chari et al. 2013; Memmi et al. 2015). At the same time Δ Np63 represses Indian Hedgehog ligand in mammary stem cells (Li et al. 2008a). These results indicate a reciprocal relationship between Hedgehog signaling which represses Δ Np63, and Δ Np63 which transcriptionally regulates Hedgehog signaling components in a feedback loop.

Wnt β -Catenin pathway

Wnt signaling is activated by binding Wnt ligands, which are secreted Cys-rich proteins, to Frizzled receptors. There are more than 15 WNT receptors and co-receptors. The combination of a receptor with a specific WNT ligand determines the downstream pathway (Niehrs 2012). The downstream pathway can be

canonical (β -catenin-dependent) or non-canonical (β -catenin-independent). When active, the canonical, catenin dependent pathway recruits the kinase GSK3 β to the membrane, where it is inhibited. This induces stabilization and nuclear translocation of β -catenin, which acts as co-activator of T-cell factor (TCF)/lymphoid enhancer-binding factor (LEF) transcription factors (Niehrs 2012). In mice Wnt9 ligand is induced by Pbx1/2 transcription factors during craniofacial development. This activates the WNT β -Catenin pathway and its downstream transcriptional regulator Lef1/TCF. The Δ Np63 promoter is activated by Wnt signaling through Lef1/TCF downstream factor during embryonic development in mice (Ferretti et al. 2011). In addition, the WNT β -Catenin pathway positively regulates the expression of Δ Np63 through the limb-bud and heart (LBH) factor during puberty in the mouse mammary gland (Lindley et al., 2015). LBH is a downstream transcriptional target of Lef1/TCF upon stimulation of Wnt signaling with Wnt3a, which activates the LBH promoter (Rieger et al. 2010). LBH is expressed in basal cells of the mammary epithelia and is required for the growth of the mammary gland in puberty (Lindley et al., 2015). LBH increases transcription of Δ Np63 α in mammary epithelia and downregulates TAp63 α (Lindley et al., 2015). These results indicate that WNT β -Catenin pathway positively regulates Δ Np63 α expression through Lef1/TCF and LBH factors, during development and puberty in mice.

The Hippo signaling pathway

The hippo pathway can positively or negatively regulate Δ Np63, this regulation will be explained in detail at the end of this chapter (Section 1.7). The findings described in Chapter 2 will also compliment what is known about p63 regulation by the Hippo pathway.

In summary, multiple factors from different signaling pathways regulate through repression or activation, the expression of Δ Np63 in mammary epithelial cells, keratinocytes and SCCs. Since multiple interactions and crosstalk exist between pathways, it is unlikely that a unique factor would control Δ Np63 α ; most likely various factors in combination respond to multiple signaling inputs that at the end determine the level of expression of p63.

1.5 The Canonical Hippo Pathway and Its Role in Cancer

The hippo pathway is a tumor suppressor pathway. It was discovered in *Drosophila* in genetic screens for mutations that induce excess proliferation of cells in tissues (Justice et al. 1995; Xu et al. 1995). The Hippo pathway is one of the eight signaling pathways most commonly altered in human cancers (Sanchez-Vega et al. 2018) based on findings by The Cancer Genome Atlas (a program that genetically characterized twenty thousand primary cancer samples matched to standard tissue samples across 33 different types of cancers (*The Cancer Genome Atlas Program - National Cancer Institute, 2018*)).

The first member of the hippo pathway found in initial screens in *Drosophila* was the warts (*wts*) kinase orthologue to the human Large tumor suppressor (LATS) kinase (Justice et al. 1995; Xu et al. 1995). Another component Salvador (*sav*) was found as a binding partner of the warts kinase (Tapon et al. 2002), and it is required for apoptosis and cell cycle exit (Kango-Singh et al. 2002; Tapon et al. 2002). Years later mutations in the Hippo gene, a Ser/Thr Kinase (*hpo*), homolog of the Mammalian Ste20 kinases 1 and 2 (MST1 and 2), caused the same phenotype of excess growth in tissues as *sav* and *wts* (Kango-Singh et al. 2002; Tapon et al. 2002). Hippo gives the pathway its name due to the large head phenotype it causes in flies when it is mutated (Udan et al. 2003). Another gene in the pathway is Mob as a tumor suppressor (*mats*), whose loss of function also increased cell proliferation (Lai et al. 2005). Finally, it was found that the functional effector downstream of the pathway's kinases was Yorkie (*yki*) (ortholog of Yes associated protein- Yap- in humans), whose *overexpression* phenocopied excess growth and impaired apoptosis of the loss of function of *hpo*, *wts*, *mats*, and *sav*. Yki loss of function caused organs of small size similar to the overexpression of *hpo* and *wts* (Huang et al. 2005). The Wts kinase binds and phosphorylates *yki* resulting in the inhibition of its transcriptional activity (Huang et al. 2005).

In humans, the core Hippo pathway (Figure 1-6) consists of MST1 and 2 kinases (Mammalian sterile like-20) that bind and phosphorylate the adaptor protein Salvador (SAV1, also known as WW45), increasing their kinase activity (Callus et al. 2006; Bae et al. 2017). MST activates the human Large Tumor Suppressor Kinase (LATS) (Chan et al. 2005). The scaffold proteins MOB1A and B induce the activation of LATS1/2 along with MST1 and 2 (Hergovich et al. 2006; Praskova et al. 2008). Active LATS1/2 negatively

regulate the transcriptional activators Yes Associated Protein (YAP) and Transcriptional co-Activator with PDZ-binding motif (TAZ, also known as WW domain-containing transcription regulator protein 1 or WWTR1) (Kanai et al. 2000; Zhao et al. 2007; Lei et al. 2008). Once phosphorylated, YAP and TAZ are sequestered in the cytoplasm and targeted for degradation (Hao et al. 2008; Lei et al. 2008; Liu et al. 2010; Zhao et al. 2007, 2010). When kinases LATS1/2 are inactive, YAP and TAZ get translocated to the nucleus where they interact with TEAD transcription factors to induce transcription of growth-inducing genes like Connective Tissue Growth Factor (CTGF) (Zhang et al. 2009; Zhao et al. 2008) (Figure 1-6). Each component of the core Hippo pathway will be explained in more detail below.

1.5.1 Mammalian Ste20-like kinases 1 and 2: MST1 and MST2

The Mammalian Ste20-like kinases 1 and 2 (MST1/2) are Ser/Thr kinases, that induce apoptosis when transiently overexpressed (Graves 1998). MST1 and MST2 are activated by autophosphorylation in the kinase activation loop at T183 in MST1 and T180 in MST2 (Praskova et al. 2004). Homodimerization of MST2 through its C-terminal domain (named SARAH after **SAV-RASSF-HIPPO**, from the *Drosophila* homologs) is required for MST2 autophosphorylation at T180, which activates the downstream phosphorylation of MOB1, LATS1/2 and YAP (Chan et al. 2005; Ni et al. 2013; Tran et al. 2020). Autophosphorylation is critical for MST's kinase activity since phosphomutant T180A is inactive (Ni et al. 2013). MST localizes to the cell membrane binding the scaffold protein Salvador (SAV1) in a SARAH domain dependent manner (Yin et al. 2013). This step increases MST kinase activity by bringing their kinase domains close together (Tran et al. 2020). On the other hand, MST binding to a different adaptor the RASSF protein, prevents MST2 autophosphorylation by forming MST heterodimers (Ni et al. 2013). The K473 residue in MST2 is vital to allow the heterodimer formation with RASSF; the K473A mutation blocks the binding to RASSF and heterodimer formation, increasing MST2 autophosphorylation (Ni et al. 2013). MST1/2, in their heterodimer form, are found *in vivo* and have lower kinase activity towards MOB1 proteins and are less effective at inhibiting YAP/TAZ than homodimers (Rawat et al. 2016).

MST kinases also interact with phosphatases, since a time course treatment with Okadaic acid (a PP2 phosphatase inhibitor and activator of MST1/2) revealed that at the latest time points MST binds

phosphatase PP6 and SLAMP (a member of the STRIPAK PP2A phosphatase complex) as a feedback mechanism to turn off the pathway (Couzens et al. 2013). MST autophosphorylates its linker domain at seven phosphorylation sites, and only four of them, T349, T356, T364, T378 in MST2, are required for MOB1A/B binding and phosphorylation as well as downstream pathway activation (Ni et al. 2015). When phosphorylated, the other three phosphorylation sites in the linker domain at T325, T336, and T378 allow the binding of the STRIPAK phosphatase complex to MST to decrease its autophosphorylation (Ni et al. 2015; Zheng et al. 2017). This suggests that MST autophosphorylations can have opposite effects on downstream signaling. Some MST autophosphorylations allow the activation of MOB1A and the pathway, while other autophosphorylations recruit phosphatases to decrease MST activity and turn off the pathway.

MST1/2 are also required for cell cycle progression; their activity increases in mitosis and deletion of MST delays mitotic exit, MST1 and MST2 allow chromosomes to bind to microtubules (Oh et al. 2010).

Since the Hippo pathway is a potent regulator of organ size, it is expected that it would play a role in cancer development when it is dysregulated. The RasV12 mutation, common in some cancers, induced the formation of MST1/2 heterodimers with lower kinase activity towards MOB1, compared to homodimers. In consequence, heterodimers inhibit YAP less efficiently, than homodimers (Rawat et al. 2016). Ras induced MST1/2 heterodimer formation, which caused the transformation of MEFs, inducing anchorage independent growth (Rawat et al. 2016). If MST1/2 heterodimer formation is impaired by expression of either MST1 or MST2 alone in MEFs, then anchorage independent growth is inhibited (Rawat et al. 2016). This suggests that Ras induced transformation of MEFs is dependent on MST1/2 heterodimer formation.

Mouse models have been established to understand the role of MST kinases in mammalian cancer. The loss of MST1/2 impaired embryonic development. Mice hemizygous for either MST1 or MST2 died at several months of age of hepatocellular carcinoma, while others had enlarged livers (Zhou et al. 2009). Postnatal loss of MST1/2 in mice in the liver increased cell proliferation and had hepatocellular carcinomas with decreased phosphorylation of MOB and YAP (Lu et al. 2010; Zhou et al. 2009). Restoring MST1 expression in an hepatocellular carcinoma cell line decreased proliferation, increased apoptosis, and

increased YAP and MOB phosphorylation (Zhou et al. 2009). Another mouse model that studied MST1/2 in different tissues found that postnatal loss of MST1/2 caused higher susceptibility to liver cancer and enlarged stomach, liver, heart, and spleen organs (Song et al. 2010). In addition, the inhibition of MST1/2 with the small molecule inhibitor XMU-MP1 activates YAP and increases proliferation and liver repair after injury (Fan et al. 2016). All these studies suggest that the requirement of MST1 and 2 as tumor suppressors varies depending on the tissue and that the liver particularly relies on MST1/2 activity to regulate proliferation and prevent tumorigenesis.

1.5.2 The adaptor protein Salvador: SAV1

Salvador also known as WW45 in humans, is an adaptor protein of the MST1/2 kinases and it is their phosphorylation target (Callus et al. 2006). Its role was defined in human cells using the ACHN human cancer cell line that is SAV1 null and has low levels of YAP S127 phosphorylation (Dong et al. 2007). In this cell line, only the expression of LATS1/2, but not of MST1/2, was able to rescue the phosphorylation of YAP. To fully rescue the ability of MST1/2 to promote YAP phosphorylation, coexpression of MST along with SAV was required. This result placed SAV1 in the Hippo pathway downstream of MST1/2 and upstream of LATS1/2 (Dong et al. 2007).

SAV1 recruits MST1/2 to the plasma membrane, induces formation of MST homodimers, which induces their auto-phosphorylation and activation (Callus et al. 2006; Yin et al. 2013). In addition, SAV1 maintains MST1/2 activity by protecting them from interacting with the phosphatase complex STRIPAK (Bae et al. 2017). Despite the role of SAV in MST activation, others have found that SAV1 is not essential for inhibiting YAP/TAZ. On the one hand, livers from SAV1 null mice still retain partial phosphorylation of LATS and YAP (Yin et al. 2013). In addition, CRISPR knockout of SAV1 or MST1/2 did not block YAP/TAZ phosphorylation after serum starvation of HEK293T cells (Plouffe et al. 2016). This result indicates that LATS can be activated independently of MST.

One of the molecules that can activate LATS independent from MST and SAV is Neurofibromin 2 (NF2, also known as Merlin). NF2 is a membrane cytoskeleton adaptor protein, that causes tumors of the

nervous system known as Neurofibromatosis II and other cancers such as mesotheliomas when mutated (Yin et al. 2013). Livers from SAV1 or NF2 null mice have impaired phosphorylation of LATS and YAP (Yin et al. 2013). Interestingly, SAV1/NF2 double knockouts have even lower levels of YAP phosphorylation compared to either SAV1 or NF2 single knockouts. This indicates that both proteins SAV1 and NF2 are required for the complete activation of LATS1/2 and phosphorylation of YAP. These results show that SAV activation of MST is not the only existing mechanism to activate LATS. In addition, kinases such as TAO kinase 1-3 or MAPK4 can also activate upstream components of the Hippo pathway. TAO kinase 1-3 phosphorylate and directly activate MST1/2 (Boggiano et al. 2011; Poon et al. 2011). MAPK4 can activate LATS independent of MST (Meng et al. 2015; Zheng et al. 2015; Plouffe et al. 2016). Therefore, the requirement of SAV1 for the inhibition of YAP/TAZ may be cell-type and stimuli dependent, so further studies are required to clarify the circumstances that regulate their roles in the Hippo pathway.

Despite that SAV1 is not required to activate Hippo signaling *in vitro*, *in vivo* SAV1 loss can induce cancer and developmental defects in mouse models. Mouse null for SAV have defects in the skin and intestine, have decreased apoptosis, increased proliferation, and defects in differentiation of the skin layers and the intestinal villi (Lee et al. 2008). SAV loss also induced liver tumors, similar to MST1/2 loss, however the type of liver tumors was different. SAV loss, increased proliferation of liver progenitor oval cells, and induced cholangiocarcinomas, while in MST1/2 ablation induced hepatocellular carcinoma in mice (Lu et al. 2010; Lee et al. 2010). The results described above suggest that SAV is required as a tumor suppressor in mouse models in a tissue-specific manner. Tumors formed in SAV null mice are different from tumors formed in MST1/2 null mice, suggesting that the downstream signaling effects induced by SAV1 could be different from those induced by MST1/2.

1.5.3 The Mps One Binder 1A/1B adaptor proteins: MOB1A and MOB1B

Mps One Binder 1A/1B (MOB1A/B) are 96% identical adaptor proteins, that are part of the core Hippo pathway (Stavridi et al. 2003). MOB1A/B act as kinase activating proteins; their binding to the membrane induces the rapid recruitment and activation of LATS (Hergovich et al. 2006). Both MOB1A and B bind to active MST1/2 kinases and get phosphorylated at T12 and T35 (Praskova et al., 2008). MOB1A/B

once phosphorylated bind to LATS increasing its activation, by phosphorylation at T1079 by MST and by phosphorylation on its autoactivation loop at S909 in LATS1 (Praskova et al., 2008). Both phosphorylations are necessary to completely activate LATS. MOB1A/B are essential for LATS1/2 activation and therefore for the inhibition of YAP/TAZ, since CRISPR Knock out of both MOB1A and B in Hek293T cells, severely impaired YAP/TAZ phosphorylation (Plouffe et al. 2016).

Okadaic acid is phosphatase PP2A inhibitor and since PP2A inhibits MST kinases by decreasing their phosphorylation levels, then okadaic acid activates the Hippo pathway increasing MST1/2 activity by maintaining their phosphorylation levels high and therefore their activity. The increase in MST1/2 kinase activity induced by Okadaic treatment, increases MOB1A/B phosphorylation and binding to MST1/2, as well as binding to their upstream regulator proteins SAV1 and RASSF (Couzens et al. 2013). In addition, a time course treatment with Okadaic acid combined with proteomics, revealed that the core Hippo pathway follows a sequence of activation steps, first MOB1 is first bound to LATS kinases, then MOB1 binds to MST, followed by binding to SAV and RASSF adaptor proteins. Finally, longer activation of the pathway with Okadaic acid induced binding of the complex to the phosphatase PP6 (a member of the PP2A family of phosphatases) (Couzens et al. 2013). This suggests that extended activation of the pathway, induces the recruitment of phosphatases to the kinase complex, as a negative feedback mechanism to turn off the pathway. Further structural studies of MOB1A/B binding to MST and LATS, support the above mentioned activation steps of the core kinase cascade: first, active MST autophosphorylates multiple residues in its linker domain (between the kinase and SARAH domains) (Ni et al. 2015). Second, these phosphosites on MST work as docking sites for MOB1A/B binding. Third, MOB1A/B binds to LATS. Fourth, MST phosphorylates MOB1A/B at T12 and T35 and LATS1 at T1079 (Ni et al. 2015). This phosphorylation triggers a conformational change that releases MOB1A/B bound to LATS, from MST (Ni et al. 2015; Xiong et al. 2017). In the final step, MOB1A/B bound to LATS, stimulates its autophosphorylation and full activation (Ni et al. 2015).

Mass spectrometry studies revealed that MOB1A and B could have different binding partners despite having almost identical protein sequences. MOB1A interacts more abundantly with LATS1 and the

binding does not increase further with Okadaic acid treatment. MOB1B binds less to LATS1 and upon Okadaic acid treatment MOB1B binding to LATS1 increases (Couzens et al. 2013; Xiong et al. 2017). In addition, MOB1A binds to LATS2 less than it binds to LATS1 (Couzens et al. 2013; Xiong et al. 2017). MOB1A binding to LATS2 increased with longer treatments of pathway activation, since okadaic treatment increased their binding (Couzens et al. 2013; Xiong et al. 2017), which suggests that the binding is dependent on the proteins phosphorylation. In addition, MOB1B binds to the phosphatase PP6 while MOB1A does not. These data suggest that MOB1A interacts more abundantly with LATS1 than MOB1B. In addition, MOB1A also binds to LATS1 more than it does to LATS2. This evidence further demonstrates that the isoforms of MOB1 despite their sequence similarity, are possibly functionally different since they have different binding partners and respond differently to the activation of the pathway by phosphatase inhibition.

Both MOB1A/B have also been shown to have Hippo independent roles; they regulate cytokinesis in mitosis by regulating the stability of the microtubules. MOB1 loss prevents the separation of the two daughter cells due to increased acetylation of the microtubules in the middle bridge between the cells (Florindo et al. 2012).

Regarding their role in cancer, loss of MOB1A and MOB1B induced osteosarcomas, fibrosarcomas, tumors in the skin, liver, breast, lung, and salivary glands in mice (Nishio et al. 2012). MOB1A/B also have roles in development that are tissue dependent. MOB1A/B loss in keratinocytes, decreased LATS1/2 phosphorylation and increased nuclear YAP localization, inducing skin hyperplasia with increased cell divisions with less apoptosis in the suprabasal layer of the skin, as well as terminal differentiation defects of skin keratinocytes (Nishio et al. 2012). These results indicate that the loss of MOB1A/B causes defects in cell cycle exit and differentiation, that increase proliferation of keratinocytes, illustrating an important role of MOB1A/B in skin development. In addition, primary keratinocytes had multiple centrosomes and micronuclei, which indicates that MOB1A/B regulates chromosomal segregation (Nishio et al. 2012).

MOB1A/B loss can have developmental effects that do originate from excess proliferation, but increased apoptosis instead. Mice with chondrocyte specific deletion of MOB1A/B have chondrodysplasia

(decreased thickness of the cartilage zone and shorter bones), defect caused by impaired cell differentiation. Chondrocytes have impaired ossification due to excess YAP/TAZ activity, which repressed SOX9 gene, required for extracellular matrix gene expression and cartilage differentiation decreasing the overall size of the cartilages and bones (Goto et al. 2018). This indicates that MOB1A/B have tumor suppressor roles in multiple tissues, as well as an important role in development regulating cell proliferation, apoptosis, chromosomal segregation, as well as terminal differentiation of skin keratinocytes and chondrocytes.

1.5.4 The Large Tumor Suppressor Kinases: LATS1 and LATS2

LATS1/2 negatively regulate the transcriptional activators Yes Associated Protein (YAP) and TAZ (also known as WWTR1) by phosphorylation (Zhao et al. 2007; Lei et al. 2008). Once phosphorylated, YAP and TAZ are sequestered in the cytoplasm and targeted for degradation (Hao et al. 2008; Lei et al. 2008; Liu et al. 2010; Zhao et al. 2007, 2010). LATS1/2 are activated by MST1/2 by phosphorylation at T1079 in LATS1 and T1041 in LATS2 (Chan et al. 2005; Praskova et al. 2008; Hoa et al. 2016). MOB1A/B are essential for this phosphorylation step by bringing LATS1/2 close to MST and they are also vital to activate LATS autophosphorylation at S909 in LATS1 and S872 in LATS2 to make LATS fully active (Hergovich et al. 2006; Praskova et al. 2008; Ni et al. 2015; Hoa et al. 2016).

LATS1/2 can be activated independently of MST through the protein Merlin (also known as Neurofibromin 2 –NF2), which activates LATS1/2 upon disruption of actin cytoskeleton polymerization or inhibition of RHOA (Yin et al. 2013). NF2 also recruits LATS1/2 directly to the plasma membrane independently of MST1/2 (Yin et al. 2013). By increasing LATS1/2 at the plasma membrane it allows their interaction with SAV1 and MST activating them (Yin et al. 2013).

LATS2 also has cell cycle related roles. Spindle damage impairs proper chromosomal segregation during mitosis and the cells could have an additional cell cycle, becoming tetraploid. A tetraploidy checkpoint normally blocks additional rounds of DNA duplication in cells with already duplicated DNA (Aylon et al. 2006). LATS2 stabilizes p53 and increases its total levels by preventing its ubiquitination by MDM2,

after mitotic spindle damage. Active p53 then induces LATS2 mRNA expression in G2/M, establishing a feedback loop (Aylon et al. 2006). LATS2 and p53 are required to induce cell cycle arrest upon tetraploidy. Without either one, LATS2 or p53, cells continue duplicating their DNA in each successive cycle becoming tetraploid (Aylon et al. 2006). Whether MST1/2 are required for LATS2 stabilization of p53 and induction of cell cycle arrest is unclear, since their role was not directly evaluated.

LATS1 can have functions independent from LATS2. Higher levels of LATS1 expression are associated with lack of response to Sorafenib therapy (a chemotherapeutic agent a kinase inhibitor) in hepatocellular carcinoma patients (Tang et al. 2019a). LATS1 induced resistance to Sorafenib treatment through the inhibition of Sorafenib induced autophagy in hepatocellular carcinomas. Ablation of LATS1 but not LATS2 increased cell death and autophagy upon Sorafenib treatment (Tang et al. 2019a). The inhibition of autophagy was independent of the LATS1 kinase activity, instead LATS1 as an scaffolding protein binds and stabilizes the autophagy protein Beclin1 (Tang et al. 2019a). Indicating that LATS1 has kinase independent, as well as LATS2 independent functions that regulate the response to therapeutic agents.

LATS1 can also regulate cell differentiation and protein stability in a LATS2 independent manner. LATS1/2 are required to maintain the basal/myoepithelial phenotype of primary breast cells (Britschgi et al. 2017). Since LATS1/2 ablation induces YAP/TAZ dependent luminal differentiation and upregulation of Estrogen Receptor α levels (Britschgi et al. 2017). However, only LATS1 is required to induce protein degradation of Estrogen Receptor α , possibly to maintain low basal levels of Estrogen receptor to maintain the basal cell phenotype of mammary epithelial cells (Britschgi et al. 2017).

LATS kinases, are tumor suppressors that negatively regulate YAP/TAZ. LATS1 null mice are viable but have higher incidence of ovarian cancer and soft tissue sarcomas and treatment with carcinogens, which decreases their survival (St John et al. 1999). Human sarcomas have reduced or non-detectable expression of LATS1 due to promoter methylation (Hisaoaka et al. 2002). In human breast cancers have promoter methylation at the LATS1 and LATS2 promoters (Takahashi et al. 2005). Malignant mesothelioma tumor samples have LATS2 deletions, truncations and mutations in the coding region that

decrease total LATS2 expression (Murakami et al. 2011). LATS1/2 deletions induced renal carcinoma, by increasing YAP/TAZ activity in renal epithelium in adult mice (Carter et al. 2021). LATS1/2 kinases are important tumor suppressors necessary to inhibit YAP/TAZ induced proliferation. Their activity is necessary to prevent sarcomas, mesotheliomas, renal and ovarian carcinomas *in vivo*, suggesting that their activity is required in a tissue and cell type dependent manner.

1.5.5 The Yes-Associated Protein and The Transcriptional Co-Activator with PDZ-binding motif:

YAP and TAZ

The Yes-Associated protein (YAP) is a potent regulator of organ size by increasing cell proliferation and resistance to apoptosis (Dong et al. 2007). It is a transcriptional activator that binds to the SH3 domain of the Yes tyrosine kinase (Sudol 1994). Its role as a transcriptional activator was found in a yeast two hybrid screen for protein interactors with the PY motif (PPPY) of the transcription factor PEBP2 (This motif is conserved and is required for PEBP2's transcriptional activity) (Yagi et al. 1999). YAP contains a proline rich domain at its N-terminus, two central WW domains, an SH3 domain, a coiled-coiled domain followed by a transcriptional activation domain at its C-terminus (Sudol 1994; Yagi et al. 1999; Kanai et al. 2000; Zhao et al. 2007, 2010).

YAP is negatively regulated by the LATS1/2 kinases of the Hippo pathway. YAP is phosphorylated by LATS2, causing a shift in its electrophoretic motility when LATS2 is overexpressed (Zhao et al. 2007). LATS2 in combination with MST2, MOB1 and SAV caused the highest motility shift in YAP, induced the cytoplasmic localization of YAP and decreased the transcriptional activity of TEAD (Zhao et al. 2007) Showing that the highest phosphorylation occurs when both types of kinases and adaptor proteins are overexpressed (Zhao et al. 2007)..

Active unphosphorylated YAP increases the transcriptional activity of TEAD transcription factors (Zhao et al. 2007) YAP does not have DNA binding activity of its own, instead TEAD transcription factors are the major binding partners of YAP and are required to induce gene expression. This was evidenced by the lack of transcriptional activity observed in the S94A YAP mutant that cannot interact with TEAD

transcription factors (Zhao et al. 2008). The YAP-TEAD transcriptional activity is essential for the proliferative effects induced by the YAP 5SA constitutively active mutant in MCF10A cells, since ablation of their target gene Connective Tissue Growth Factor (CTGF), decreased proliferation and anchorage independent growth in 2D and 3D (Zhao et al. 2008). This suggests that YAPs transcriptional activity mediated by TEAD binding, is important for cell growth.

YAP is phosphorylated by LATS2 on five amino acid residues; S61, S109, S127, S163, and S381 (Zhao et al. 2007) and by LATS1 on S61, S109, S127, and S164, (Hao et al. 2008). LATS1 phosphorylates and inactivates YAP1 nuclear localization and transcriptional activity (Hao et al. 2008). The phosphorylation site at S127 is the main site that controls YAP nuclear localization; when this site is phosphorylated, YAP interacts with 14-3-3 protein and is retained in the cytoplasm (Zhao et al. 2007). The S127A mutant as well as the mutations in all 5 sites (5SA YAP), makes YAP resistant to inhibition by MST and LATS, and it is constitutively localized in the nucleus (Zhao et al. 2007; Hao et al. 2008). While individual mutations of the other phosphosites had no effect on YAP (Zhao et al. 2007). LATS1/2 phosphorylation of YAP at S381 does not change its cellular localization but decreases YAP protein stability, by allowing the interaction with the β -transducin repeat containing protein E3 ligase (β -TCRP also known as FBXW7) necessary for YAP1's ubiquitination and degradation (Zhao et al. 2010). LATS phosphorylation of YAP at S381 is required for subsequent phosphorylation by Casein Kinase δ/ϵ (CK1 δ/ϵ) at a phosphodegron at S384 and S387, which is required for the ubiquitination and degradation of YAP (Zhao et al. 2010).

YAP's cellular localization and protein stability are controlled by cell density. When cells are confluent YAP phosphorylation at S127 increases and YAP is localized in the cytoplasm, where it is ubiquitinated and degraded (Zhao et al. 2010). In sparse cells, YAP is less phosphorylated, stabilized and translocated to the nucleus where it activates transcription (Zhao et al. 2010, 2007). This indicates that LATS1/2 kinase activity (phosphorylation of YAP) increases with cell density. Since NF2 (Neurofibromin 2 or Merlin) activates LATS1/2 by recruiting them to the plasma membrane, in an MST1/2 independent manner (Yin et al. 2013).

TAZ is a YAP homolog (also known as WW domain-containing transcription regulator protein 1 or WWTR1) that was initially described as a 14-3-3 binding protein (Kanai et al. 2000). 14-3-3 is a cytoplasmic protein that binds to phosphoproteins (Pennington et al. 2018). TAZ N-terminus has the 14-3-3 binding domain followed by one WW domain required for binding to PPXY motifs (present in the transcriptional activation domain of transcription factors) (Kanai et al. 2000). After the WW domain is the transcriptional activation domain essential for TAZ target gene transcription, and at the coil-coiled domain and the conserved PDZ-binding domain at the C-terminus (Kanai et al. 2000). TAZ is inhibited by the Hippo pathway kinase LATS1/2 by phosphorylation at four residues S66, S89, S112 and S311 (Lei et al. 2008). TAZ phosphorylation at S89 at its N-terminus regulates its binding to 14-3-3 and its cytoplasmic localization, inhibiting its nuclear import and transcriptional activity (Kanai et al. 2000; Lei et al. 2008). Of all its phosphosites, S89 has the strongest effect, since S89A mutants are resistant to LATS inhibition (Lei et al. 2008).

Overexpression of TAZ, followed by affinity purification and mass spectrometry, found that all TEAD transcription factors 1-4 are binding partners of TAZ (Zhang et al. 2009). TAZ activation of TEAD-dependent transcription was confirmed through a luciferase screen of a human transcription factors library fused with the Gal4 DNA binding domain dependent luciferase reporter. TAZ overexpression activated TEAD-dependent luciferase activity (Zhang et al. 2009). When overexpressed TAZ induces growth, migration, and epithelial mesenchymal transition (EMT) in MCF10A cells (Lei et al. 2008). The CTGF gene was identified as a direct transcriptional target of TAZ and TEAD and is responsible for the growth and migration effects of TAZ in MCF10A cells (Zhang et al. 2009). A mutant of TAZ, S51A, that is TEAD binding defective, acts as a dominant negative, that inhibits TEAD dependent activation of CTGF, growth, migration, anchorage independent growth and EMT in MCF10A cells (Zhang et al. 2009). In parallel, it was found that TEAD binding is required for the nuclear retention of TAZ (Chan et al. 2009). WT TAZ is cytoplasmic, constitutively active S89A TAZ is nuclear, while TEAD binding defective mutant S89A TAZ F52/53A is retained in the cytoplasm (Chan et al. 2009). S89A TAZ induces anchorage independent growth while TEAD binding defective does not (Chan et al. 2009). TEAD fusion with TEAD binding defective mutant S89A TAZ (F52/53A S89A TAZ) restores its nuclear localization and anchorage independent growth (Chan

et al. 2009). It rather demonstrates that TAZ require binding to TEAD transcription factors for their nuclear localization, for their gene expression, growth, migration and anchorage independence effects.

The Hippo pathway negatively regulates TAZ activity by phosphorylation and cytoplasmic localization of TAZ, but LATS activity also regulates TAZ protein stability. TAZ phosphorylation by LATS on S311 allows TAZ interaction with the E3 ligase β -TRCP (Zhao et al. 2010). LATS phosphorylation at S311 is also required for the phosphorylation by Casein Kinase 1 δ/ϵ at S314. This creates a phosphodegron that is recognized by the β -TRCP E3 ligase, which ubiquitinates and targets TAZ for degradation (Liu et al., 2010). This follows the same mechanism described for YAP (Zhao et al. 2010). Similarly, TAZ is negatively regulated by cell density, in low density cells TAZ is active and its protein levels are high protein, while in high density cell culture conditions TAZ is ubiquitinated and degraded (Liu et al., 2010). Together these observations indicate that YAP/TAZ exist at very low levels in higher cell density, with LATS kinases increasing their activity at high cell density.

TAZ protein stability is additionally regulated by a second phosphodegron present at the N-terminus of TAZ (Huang et al. 2012). TAZ is phosphorylated by GSK3 (Glycogen synthase kinase-3) at S58 and S62, this allows TAZ degradation by β -TCRP E3 ligase (Huang et al. 2012). Active Phosphatidyl Inositol 3 Kinase pathway counteracts TAZ degradation, by AKT phosphorylation and inhibition of GSK3 and TAZ protein stabilization (Huang et al. 2012). In fact, the phosphodegron mutant S58/62A TAZ not only has higher protein stability than WT TAZ, but also induces growth and migration in MCF10A cells (Huang et al. 2012). Indeed, breast cancer cell lines with constitutively active Phosphatidyl Inositol 3 Kinase pathway have higher TAZ levels than their WT counterparts (Huang et al. 2012). This suggests that TAZ can induce growth in cancers with mutated or altered PI3K pathway and suggests that the therapeutic targeting of TAZ or GSK3 could be valuable to decrease growth in those tumors.

Once stabilized and activated YAP and TAZ regulate each other in a negative feedback loop. YAP knockdown induces the upregulation of TAZ protein, while overexpression of constitutively active YAP 5SA downregulates endogenous TAZ protein (Moroishi et al. 2015). Likewise, overexpression of active TAZ 4SA

downregulates YAP protein levels (Moroishi et al. 2015). The mechanism behind this negative feedback is the upregulation of LATS2 mRNA in a TEAD-dependent manner by active YAP/TAZ. In addition, active YAP/TAZ can also induce expression of Neurofibromin 2 (NF2) mRNA (Moroishi et al. 2015), which activates LATS kinases by recruitment to the plasma membrane (Yin et al. 2013). This demonstrates that YAP and TAZ through TEAD dependent transcription inhibit their own activation, in a negative feedback loop.

YAP is a potent regulator of organ size, since inducible YAP expression in the liver, significantly increased the organ's size even a few weeks after doxycycline treatment (Dong et al. 2007). Increasing cell proliferation and inhibiting apoptosis in the liver (Dong et al. 2007). YAP overexpression induced growth stimulatory genes such as SOX4, Myc, and the apoptosis inhibitor gene BIRC5/Survivin in the liver (Dong et al. 2007). Mice with overexpression of YAP develop hepatocellular carcinoma as early as 3 weeks of age, and have a mean survival of 7 weeks upon YAP overexpression (Dong et al. 2007). The overexpression of the phosphomutant S127A YAP under an ubiquitously expressed locus, had a similar effect increasing the liver's size 4-fold and causing dysplasia in the small intestine (Camargo et al. 2007). In the normal intestine, cell proliferation occurs exclusive in the progenitor/stem cell compartment located in the crypt between villi, where endogenous YAP is highly expressed also found (Camargo et al. 2007). YAP S127A overexpression in the intestine increased cell proliferation with loss of cell differentiation markers (Camargo et al. 2007). This evidence indicates that YAP controls organ size, increasing proliferation and expansion of undifferentiated progenitor cells in different tissues, such as liver and intestine, in mice.

Evidence in the literature also supports YAP and TAZ oncogenic roles in human breast and squamous cancers (SCCs). YAP is frequently amplified in mouse mammary tumors (Overholtzer et al. 2006). YAP overexpression induces EGF independent growth in 3D, invasive morphology, EMT and migration and anchorage independent growth, in the mammary cell line MCF10A (Overholtzer et al. 2006). YAP overexpression in MCF10A increased tumor size in xenograft models (Zanconato et al. 2015). The constitutively active form of YAP S127A increases tumor growth and metastasis in a TEAD-dependent

manner, in breast and melanoma cancer cell lines (Lamar et al. 2012). YAP required for tumor formation in a Polyoma middle T antigen mammary gland tumor mouse model (Chen et al. 2014). In addition, YAP and TAZ are frequently amplified in SCCs (Campbell et al. 2018; Wang et al. 2019a, 2018), they are required for squamous carcinoma formation (Debaugnies et al. 2018; Vincent-Mistiaen et al. 2018) and growth (Hiemer et al. 2015, 2015; Wang et al. 2019a; Li et al. 2019; Saladi et al. 2017). In addition, in breast cancers YAP/TAZ cooperate with AP-1 transcription factor to regulate cell cycle and growth inducing genes such as Cyclin A2, Cell Division Cycle 25A, Cell Division Cycle 6, among others (Zanconato et al. 2015). In squamous cell carcinomas YAP/TAZ regulate the transcription of genes important for cell cycle progression and survival, such as Polo-like Kinase, Aurora Kinase A, Cyclin Dependent Kinase 2, CyclinE2, Cell Division Cycle 6, proliferating cell nuclear antigen (PCNA) and the inhibitor of apoptosis BIRC5/Survivin (Hiemer et al. 2015). A YAP/TAZ gene signature is associated with low probability of survival in squamous cancers (Table 1).

In breast cancers, TAZ levels are higher in invasive metastatic breast cancers and TAZ increases the migration and self-renewal of breast cancer stem cells (Bartucci et al. 2015; Cordenonsi et al. 2011). Consistent with YAP/TAZ's increase growth in 3D, and anchorage independence, migration, EMT and invasion in MCF10A and breast cancer cells (Lei et al. 2008; Chan et al. 2009; Zhang et al. 2009). In addition, a phosphodegron mutant S58/62A TAZ induced growth and migration in MCF10A cells, and breast cancers with active PI3K mutations have higher levels of TAZ (Huang et al. 2012). This suggest that YAP/TAZ play oncogenic roles in human cancers increasing proliferation, anchorage independent growth and migration in breast and squamous cancer cells. Suggesting that therapeutic approaches designed to target YAP/TAZ for degradation or inhibition of their interaction with TEAD, could be beneficial for treatment some human cancers.

1.6 Upstream Regulators of the Hippo Pathway

The Hippo pathway responds to different types of stimuli. The upstream regulators of the hippo pathway are growth factors, small G Protein Coupled Receptors, cellular and energy stress, cell-cell contacts, cell polarity, mechanical signals from the extracellular matrix and the cell cytoskeleton (Zheng

and Pan 2019). Some of the upstream regulators of the Hippo pathway and their effects will be summarized next.

Several protein kinases have been described for the Hippo pathway upstream of MST. Tao Kinases 1/2/3 can phosphorylate MST1/2 (Boggiano et al. 2011; Poon et al. 2011). Alternatively, TAO kinases can activate LATS1/2 in an MST independent manner (Plouffe et al. 2016). Similarly, MAP4K 4/6/7 family kinases are also upstream of MST1/2 or can act in parallel to MST1/2, to activate LATS1/2 in the Hippo pathway (Meng et al. 2015; Plouffe et al. 2016; Zheng et al. 2015). This was demonstrated by the simultaneous ablation of all Tao Kinases 1/2/3 and MAP4K 4/6/7 simultaneously, phenocopied YAP/TAZ activation observed after LATS1/2 knock out, in Hek293T. However, MST1/2 knockout did not phenocopied YAP/TAZ activation induced by LATS1/2 ablation. This indicates that LATS can be activated in parallel to MST1/2 by other kinases (TAO and MAPK4). However, the context that induces the activation of those alternative kinases and differences in their downstream signals are still unknown.

Growth factors and membrane receptors can activate the Hippo pathway. The Epidermal Growth Factor (EGF) inhibits the Hippo pathway via phosphoinositide 3-kinase (PI3K) recruitment of phosphoinositide-dependent kinase-1 (PDK1) to the plasma membrane. PDK1 activation inhibits the formation of a complex between MST, SAV, and LATS, which inactivates the Hippo pathway, activating YAP nuclear translocation and gene expression (Fan et al. 2013). In addition, activation of the EGF receptor (EGFR) activates YAP/TAZ by direct phosphorylation of MOB1A/B at Y95, Y114, and Y117 that results in LATS1/2 inactivation (decreased phosphorylation at T1079), independent of MST1/2 activity in SCCs (Ando et al. 2021). These results suggest that EGFR tyrosine phosphorylations on MOB1, are inhibitory and decrease MOB1 activation of LATS1, without affecting their binding. Since MOB1 phosphorylations at the MST site were not affected (Ando et al. 2021), it suggests that EGFR phosphorylations in MOB1 decrease MST1/2 binding to LATS1/2, decreasing the pT1079 phosphorylation the MST site on LATS. Another mechanism downstream of EGF, is mutant Ras activates YAP by promotion MST1-MST2 heterodimer formation, that have lower kinase activity against YAP/TAZ (Rawat et al. 2016).

Different components of serum inactivate the Hippo pathway kinases. In serum, lysophosphatidic acid (a lipid) and Sphingosine-1 phosphate activate the G12/13 coupled Receptors (GPCR) which inhibits LATS1/2 and activates YAP/TAZ (Yu et al. 2012). In contrast, activation of G coupled protein Receptors with glucagon or epinephrine had the opposite effect, activated LATS and inhibited YAP/TAZ activity. Epinephrine increased cyclic AMP levels, which activated Protein Kinase A. Direct activation of Protein Kinase A with forskolin and with a cyclic AMP analog, increased LATS activation and inhibited YAP/TAZ (Yu et al. 2012). These studies evidence the complexity this pathway, where different soluble and growth factors can regulate the Hippo kinases through different mechanisms that either activate or inhibit YAP/TAZ. This demonstrates that the downstream response of the pathway is context and stimuli dependent.

Cellular stressors can activate the pathway as well. Oxidative stress induced with hydrogen peroxide treatment and osmotic stress induced with excess glucose, activate MST1/2 autophosphorylation and increase YAP inhibition (Praskova et al. 2004). Low energy levels induced by glucose starvation activate AMP-activated Kinase (sensor of ATP/AMP energy levels in the cell) and the LATS kinases, which results in phosphorylation of YAP at S127 and its inhibition (DeRan et al. 2014). Similarly, glucose but not 2-Deoxy-Glucose treatment (Glucose molecule that can be metabolized), decreases S127 YAP phosphorylation and activates YAP transcription (DeRan et al. 2014). These results indicate that the balance between cellular stressors, growth stimulatory signals and soluble factors, regulate cell proliferation, through the downstream activation of the Hippo pathway and inhibition YAP/TAZ.

RHOA kinase and actin polymerization are suppressors of the Hippo pathway through different Hippo signaling components. One of them is the Angiomiotin family of proteins (AMOT, AMOTL1 AND AMOTL2) which stimulate autophosphorylation and activation of LATS. AMOTs in response to Actin depolymerization, act as scaffold proteins that couple SAV MST MOB1 and LATS activity with the cytoskeleton dynamics to inhibit YAP (Mana-Capelli and McCollum 2018). Another component is the membrane protein Merlin/NF2 mentioned in previous sections, that activates LATS1/2 independently of MST1/2, by recruiting them directly to the plasma membrane, upon disruption actin cytoskeleton polymerization or RHOA inhibition (Yin et al. 2013). These proteins AMOT and NF2 are relevant for

activation of the Hippo pathway upon cell detachment from the extracellular matrix since RHOA activation and actin polymerization events are downstream of integrin signaling upon binding to the extracellular matrix.

In summary, the Hippo pathway inhibits YAP/TAZ using multiple signaling components outside the core kinase cascade. This allows the cells to respond to different types of stimuli from cellular stressors, growth factors and soluble factors, to cell density and integrin-extracellular matrix signaling. In this way the Hippo pathway regulates organ size and cancer development based on multiple cellular and environmental signals.

1.7 Hippo Pathway Interactions with p63

Several types of interactions have been described in the literature between the Hippo pathway and Δ Np63 transcription factor in cancer cells. Most of those interactions occur between YAP transcriptional activator and p63, the following section will describe some of those interactions.

The first type of interaction is YAP and TAZ inhibit Δ Np63 expression. Overexpression of TAZ increased migration and repressed Δ Np63 expression in mammary epithelial cells MCF10A, through TAZ-TEAD direct binding to the Δ Np63 promoter (Valencia-Sama et al. 2015). In addition, overexpression of constitutively active S127A YAP repressed Δ Np63 while YAP knockdown upregulated Δ Np63 in a mouse lung cancer model (Gao et al. 2014). The overexpression of YAP, which repressed p63, induced differentiation of lung cancers to adenocarcinomas (originates from alveolar epithelial cells), while overexpression Δ Np63 induced differentiation to the squamous type (originates from basal epithelial cells) (Gao et al. 2014). This differentiation between adeno and squamous cancers induced by p63 loss or gain affects the cancer's therapeutic response (Gao et al. 2014).

In the context of SCC YAP stabilizes Δ Np63 protein upon hippo inhibition. YAP activation stabilized Δ Np63 α protein in SCC under low attachment conditions (Fisher et al. 2016; Grun et al. 2018). Either ablation of endogenous YAP or Δ Np63 α , decreased growth under low attachment conditions, indicating

that YAP stabilization of Δ Np63 protein is important to induce growth, under low attachment conditions. Similarly, upon integrin signaling Δ Np63 α protein was stabilized in a active YAP dependent manner (Fisher et al. 2016; Grun et al. 2018).

The third type of interaction is Δ Np63 α induced activation of YAP through inhibition of Hippo signaling. Δ Np63 upregulates YAP mRNA (Li et al. 2017; Saladi et al. 2017) and activates YAP protein by repressing its negative regulators WWC1 (Kibra, an activator of LATS kinases) and MST2 kinases (Saladi et al. 2017; Li et al. 2017). In this way Δ Np63 can upregulate YAP mRNA and protein levels by direct repression of Hippo signaling components.

The fourth type of interaction is Hippo pathway activation of Δ Np63 α expression. The Hippo pathway kinases MST1/2 induce expression of Δ Np63 α upon oxidative stress (Wang et al. 2019b). Under oxidative stress MST1/2 phosphorylation of FOXO3a induces upregulation of Δ Np63 expression, independent of YAP in breast cancer cells (Wang et al. 2019b).

In conclusion, the interactions between the Hippo pathway and Δ Np63 are diverse and contradictory depending on the context since YAP can either stabilize or repress Δ Np63. At the same time Δ Np63 can activate YAP by inhibition of Hippo. In addition, Hippo can activate Δ Np63 expression independent of YAP activation. Since Hippo is activated or inhibited in a context dependent manner (as described in Section 1.6) more studies are needed to clarify the regulators and upstream signals responsible for the repression of Δ Np63, so these signals can be used for SCC targeted therapies. The findings described in chapter 2 will add to the current knowledge of p63's interactions with the Hippo pathway.

1.8 Chapter 1 Figures and Tables

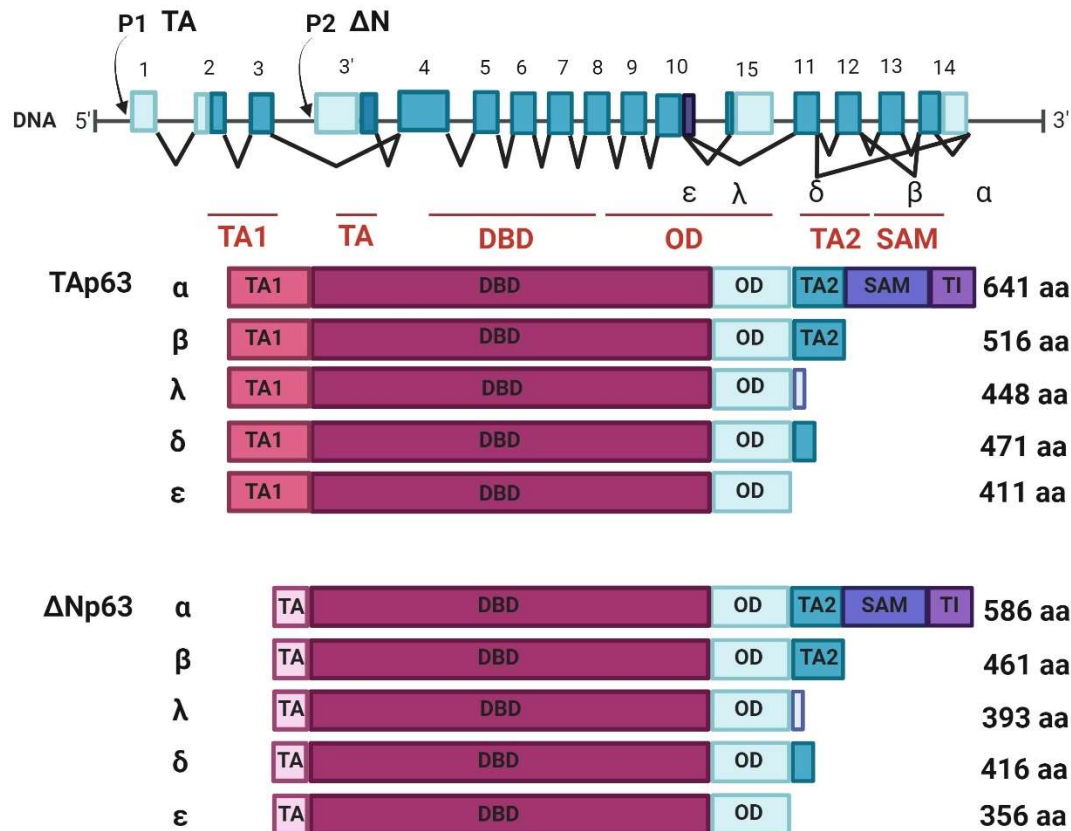


Figure 1-1. The p63 Transcription Factor Isoforms.

Exon and promoter structure of the p63 isoforms and their sequence similarity with the p53 protein structure. Two different alternative promoters P1 in exon 1 and P2 in exon 3 generate the TA and ΔN isoforms respectively. The isoforms α, β and γ are produced by alternative splicing, while the δ and ε are produced by exon skipping or early transcription termination. Diagram of the different p63 isoforms TA, ΔNp63, and α – ε isoforms. The different domains are: TA1 Transactivating Domain, TA Truncated Transactivating domain present only in the ΔN isoforms, DBD DNA binding domain, OD oligomerization domain, TA2 Second Transactivation Domain present only in the α and β isoforms, SAM Sterile α-motif domain, TI Transcription inhibitory domain. Modified from (Melino 2011; Yi et al. 2020). Created with Biorender.com

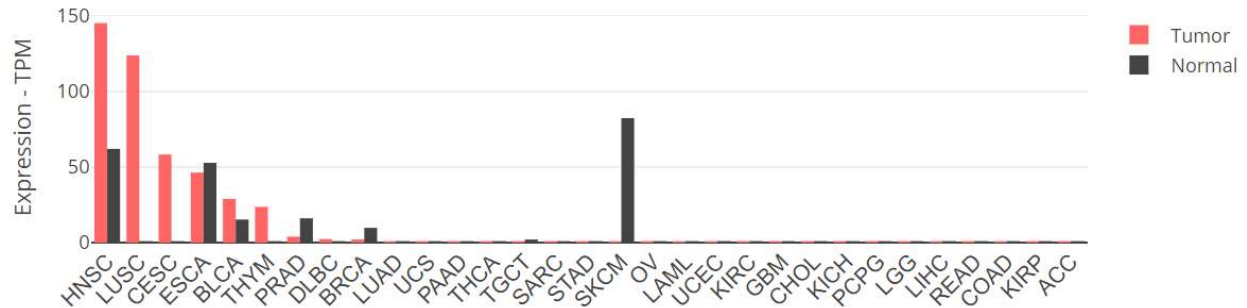


Figure 1-2. Expression of TP63 mRNA Across Human Cancers.

Relative mRNA levels (Transcripts per Million) of TCGA cancer samples compared to normal tissues. Cancer type abbreviations: (HNSC) Head and Neck squamous cell carcinoma, (LUSC) Lung squamous cell carcinoma, (CESC) Cervical squamous cell carcinoma and endocervical adenocarcinoma, (ESCA) Esophageal carcinoma, (BLCA) Bladder Urothelial Carcinoma, (THYM) Thymoma, (PRAD) Prostate adenocarcinoma, (DLBC) Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, (BRCA) Breast invasive carcinoma, (LUAD) Lung adenocarcinoma, (UCS) Uterine Carcinosarcoma, (PAAD) Pancreatic adenocarcinoma, (THCA) Thyroid carcinoma, (TGCT) Testicular Germ Cell Tumors, (SARC) Sarcoma, (STAD) Stomach adenocarcinoma, (SKCM) Skin Cutaneous Melanoma, (OV) Ovarian serous cystadenocarcinoma, (LAML) Acute Myeloid Leukemia, (UCEC) Uterine Corpus Endometrial Carcinoma, (KICH) Kidney Chromophobe, (GBM) Glioblastoma multiforme, (CHOL) Cholangio carcinoma, (KIRC) Kidney renal clear cell carcinoma, (PCPG) Pheochromocytoma and Paraganglioma, (LGG) Brain Lower Grade Glioma, (LIHC) Liver hepatocellular carcinoma, (READ) Rectum adenocarcinoma, (COAD) Colon adenocarcinoma, (KIRP) Kidney renal papillary cell carcinoma, (ACC) Adrenocortical carcinoma.

Created with Gene Expression Profiling Interactive Analysis (RRID:SCR_018294) (<http://gepia.cancer-pku.cn> accessed in March 2020) (Tang et al. 2019b).

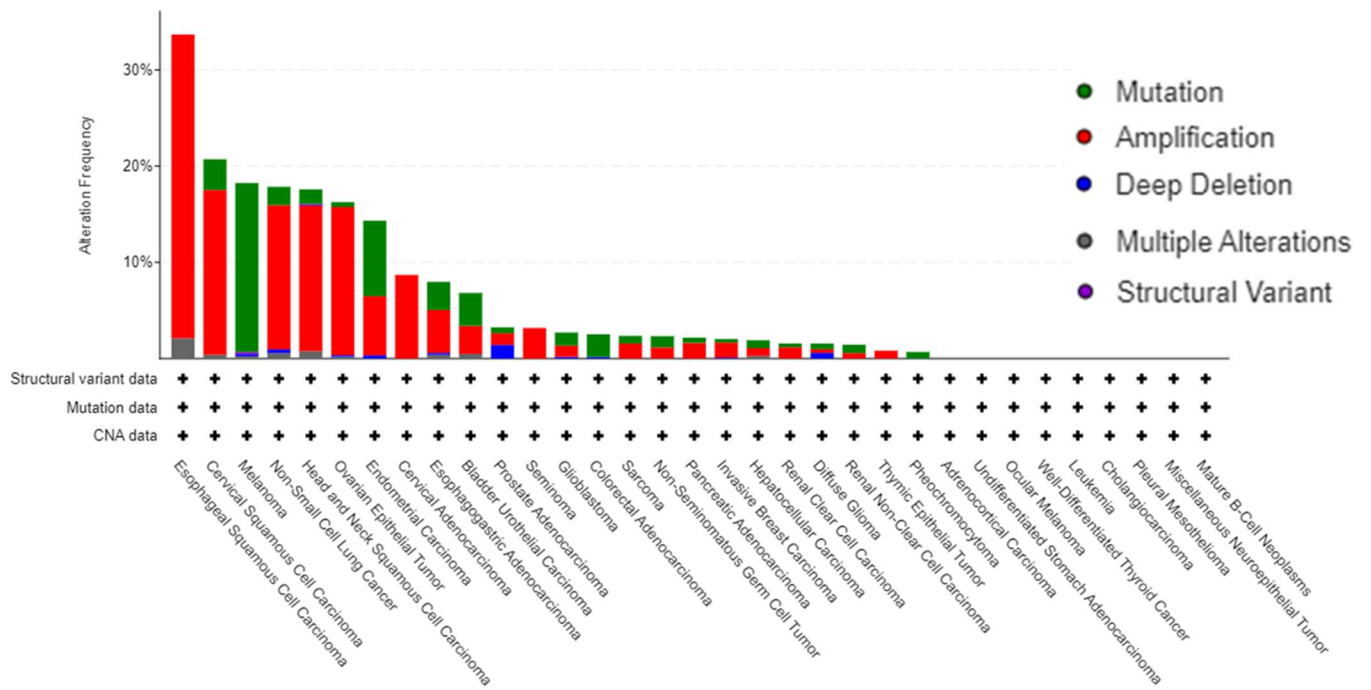


Figure 1-3. Genomic Alterations in the TP63 Gene Across Cancer Types.

Bar graph of the frequency and type of genomic alterations found in the TP63 gene in human cancers (Pan-cancer analysis of whole genomes ICGC/TCGA, Nature 2020) created with CBioportal website (Accessed March 2020) (Cerami et al. 2012).

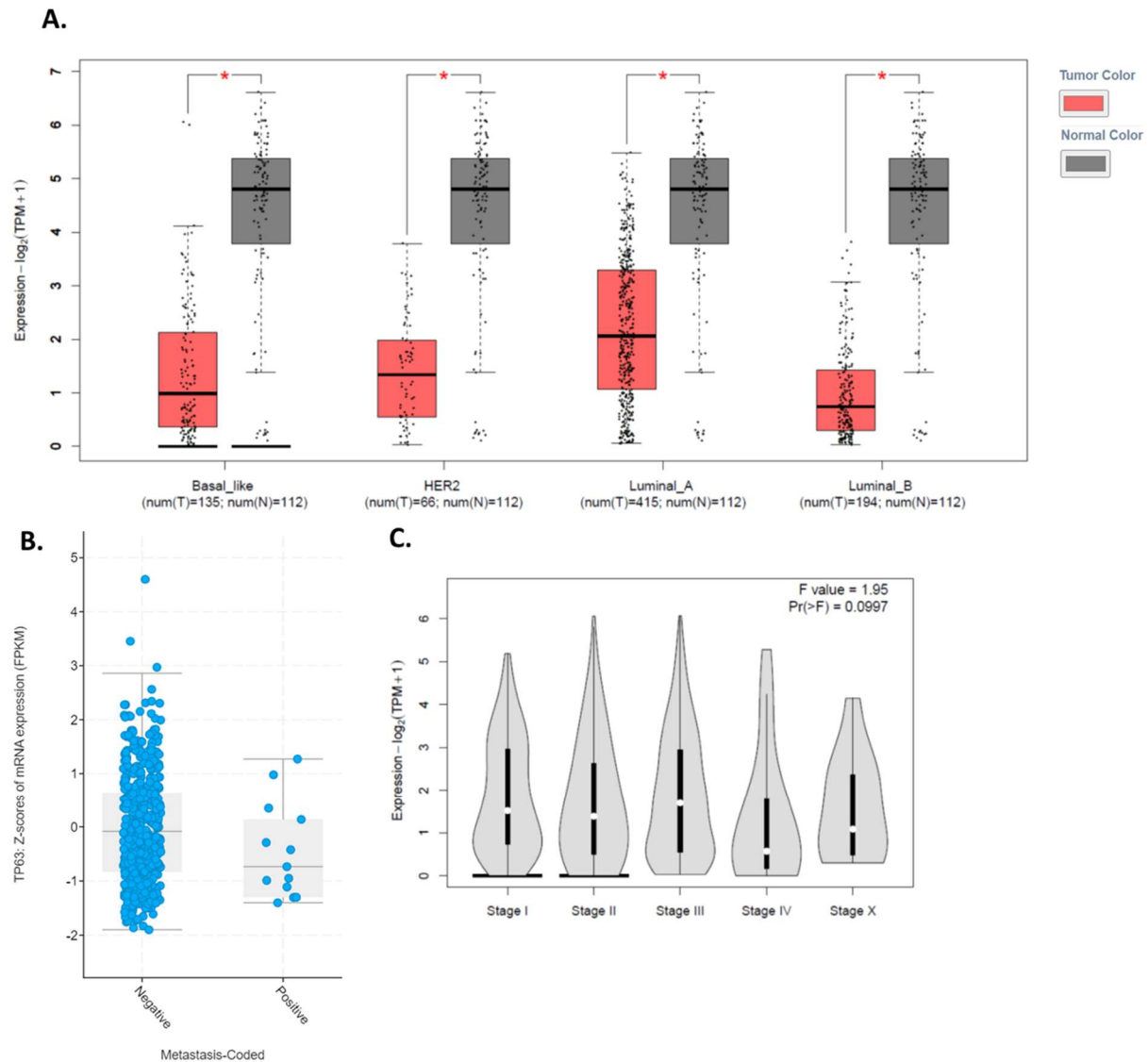


Figure 1-4. TP63 mRNA in Normal Tissue Samples Compared to Breast Cancer Subtype Samples and in Breast Cancer Metastasis.

A. Relative mRNA levels (Transcript per Million) comparing Breast cancer subtypes vs normal tissue samples from the TCGA database. * $p < 0.01$. Created with Gene Expression Profiling Interactive Analysis (RRID:SCR_018294) (<http://gepia.cancer-pku.cn> accessed in March 2020) (Tang et al. 2019b). **B.** Box plots of the relative mRNA levels (Z score of Fragments Per Kilobase of transcript per Million mapped reads) of Breast cancer tumor samples negative or positive for metastasis (N=519 samples). Created with CBioportal website (accessed in March 2020) (Cerami et al. 2012). **C.** Violin Plots of relative mRNA levels (Transcript per Million) in breast cancer samples from less invasive (Stage I) to more invasive (Stage IV).

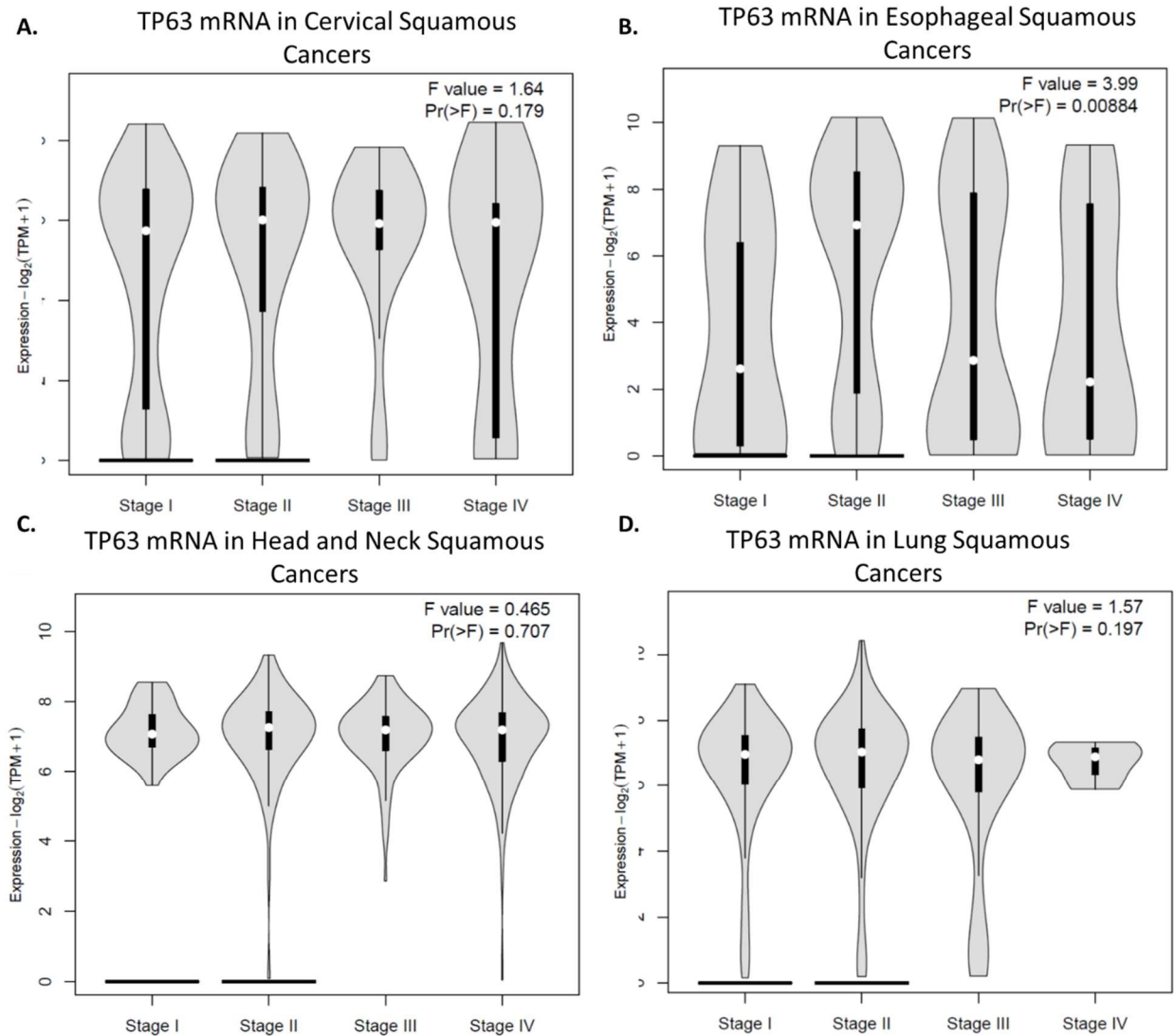


Figure 1-5. TP63 mRNA in Lung, Cervical, Head and Necks and Esophageal Squamous Cancer samples at different disease stages.

A. Violin Plots of TP63 relative mRNA levels (Transcript per Million) in cervical squamous cancer samples classified by stage from less invasive (Stage I) to more invasive (Stage IV). **B.** Same as A. for Esophageal

squamous cancer samples classified by stage **C**. Same as A. for head and neck squamous cancer samples classified by stage. **D**. Same as A. for lung squamous cancer samples classified by stage. Created with Gene Expression Profiling Interactive Analysis (RRID:SCR_018294) (<http://gepia.cancer-pku.cn> accessed in March 2020) (Tang et al. 2019b).

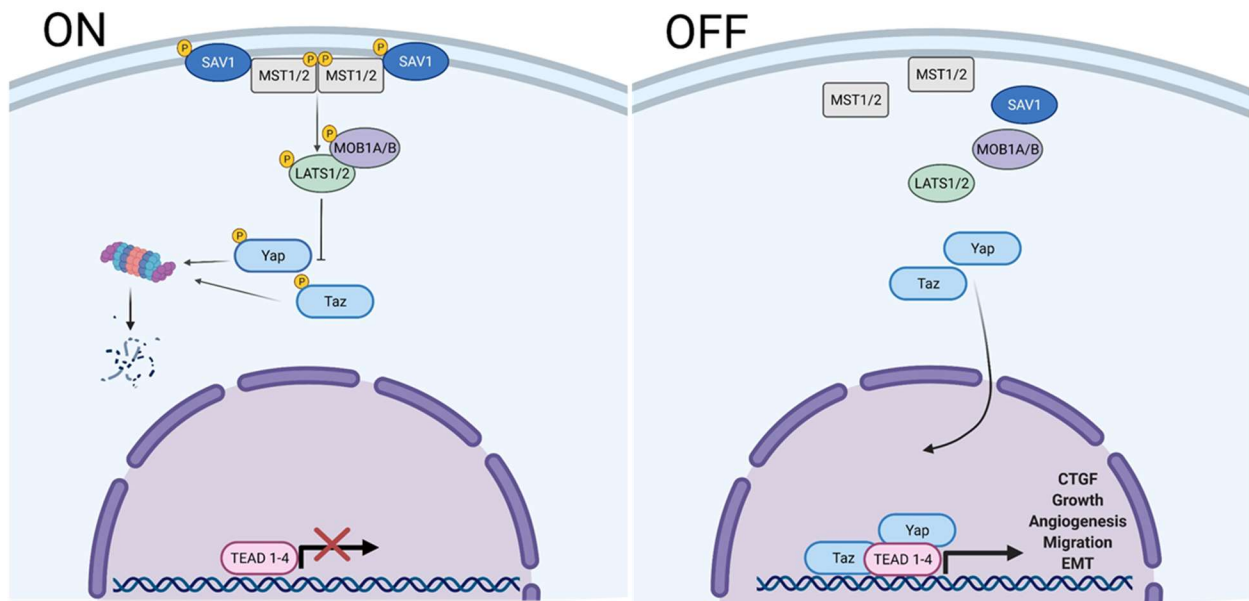


Figure 1-6. The Canonical Hippo Pathway.

Left Hippo ON shows the canonical Hippo pathway core components, when activated. The adaptor protein SAV allows the homodimer formation of MST1/2 by recruiting them to the membrane allowing their autophosphorylation and activation. This creates phosphorylation sites in their linker domain that allow the binding of MOB1A/B to MST1/2. This allows the binding of LATS1/2 to this complex. This way MST1/2 can phosphorylate MOB1A/B and activate LATS1/2. The phosphorylation of MOB1A/B by MST induces a conformational change that releases MOB bound to LATS from MST kinases, and increases the autophosphorylation of LATS to fully activate them. Active LATS kinases phosphorylate YAP/TAZ transcriptional activators to inhibit their nuclear translocation and induce their degradation by ubiquitination.

Right Hippo OFF represents the core components when the pathway is inhibited. When the core kinase

cascade is inhibited, YAP/TAZ are not degraded and can translocate to the nucleus and bind to TEAD transcription factors to activate transcription of genes involved in growth, migration, EMT among others.

Created with Biorender.com

Table 1. List of p63 Target Genes Associated with worse survival in Squamous Cancers Patients

Modified from (Riege et al. 2020)

Gene Symbol	Gene Name	Gene Pathway
LAD1	Ladinin 1	actin cytoskeleton basement membrane cadherin binding extracellular exosome structural molecule activity
TMEM40	Transmembrane Protein 40	Predicted to be integral component of membrane
FGFBP1	Fibroblast Growth Factor Binding Protein 1	cell-cell signaling fibroblast growth factor binding negative regulation of cell population proliferation plasma membrane endothelial cell proliferation involved in sprouting angiogenesis positive regulation of cell migration involved in sprouting angiogenesis positive regulation of fibroblast growth factor receptor signaling pathway signal transduction
IL1B	Interleukin 1 Beta	positive regulation of gene expression inflammatory response positive regulation of NF-kappaB transcription factor activity cytokine-mediated signaling pathway positive regulation of angiogenesis positive regulation of heterotypic cell-cell adhesion positive regulation of interferon-gamma production positive regulation of interleukin-6 production positive regulation of protein phosphorylation positive regulation of vascular endothelial growth factor production
FAT2	FAT Atypical Cadherin 2 Protocadherin Fat 2	adherens junction epithelial cell migration calcium ion binding cell-cell adhesion cell-substrate adhesion extracellular exosome

		homophilic cell adhesion via plasma membrane adhesion molecules
FOSL1	FOS Like 1, AP-1 Transcription Factor Subunit	regulation of transcription by RNA polymerase II DNA-binding transcription activator activity, RNA polymerase II-specific cellular defense response cellular response to extracellular stimulus chemotaxis female pregnancy in utero embryonic development integrated stress response signaling negative regulation of cell population proliferation
LPAR3	Lysophosphatidic Acid Receptor 3	G protein-coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger adenylate cyclase-activating G protein-coupled receptor signaling pathway bleb assembly gene expression lipid binding lysophosphatidic acid receptor activity positive regulation of MAPK cascade positive regulation of cytosolic calcium ion concentration regulation of metabolic process
MMP14	Matrix Metalloproteinase 14	metalloendopeptidase activity proteolysis Golgi lumen collagen catabolic process extracellular matrix disassembly zymogen activation angiogenesis branching morphogenesis of an epithelial tube cell motility craniofacial suture morphogenesis cytoplasmic vesicle embryonic cranial skeleton morphogenesis endodermal cell differentiation endothelial cell proliferation
RASSF6	Ras Association Domain Family Member 6	protein binding apoptotic process regulation of apoptotic process signal transduction Members of this family form the core of a highly conserved tumor suppressor network, the Salvador-Warts-Hippo (SWH) pathway. The protein encoded by this gene is a Ras effector protein that induces apoptosis
S100A2	S100 Calcium Binding Protein A2	protein binding identical protein binding endothelial cell migration calcium-dependent protein binding cellular component
SFN	Stratifin or 14-3-3sigma	cadherin binding cellular protein localization

		<p>cytoplasm establishment of skin barrier extracellular space intrinsic apoptotic signaling pathway in response to DNA damage keratinization keratinocyte development negative regulation of cysteine-type endopeptidase activity involved in apoptotic process negative regulation of keratinocyte proliferation negative regulation of protein kinase activity phosphoprotein binding positive regulation of cell growth positive regulation of protein export from nucleus protein kinase C inhibitor activity regulation of cyclin-dependent protein serine/threonine kinase activity regulation of epidermal cell division</p>
VSNL1	Visinin Like 1	<p>protein binding calcium ion binding cytosol membrane negative regulation of insulin secretion positive regulation of exocytosis positive regulation of insulin secretion involved in cellular response to glucose stimulus</p>
FERMT1	FERM Domain Containing Kindlin 1	<p>cell adhesion focal adhesion positive regulation of integrin activation actin filament binding basement membrane organization cell junction cell-matrix adhesion establishment of epithelial cell polarity integrin-mediated signaling pathway keratinocyte migration and proliferation negative regulation of canonical Wnt signaling pathway negative regulation of stem cell proliferation</p>
IRF6	Interferon Regulatory Factor 6	<p>DNA-binding transcription factor activity, RNA polymerase II-specific cell development cell junction cranial skeletal system development immune system process keratinocyte differentiation limb development mammary gland epithelial cell differentiation</p>
PTH1LH	Parathyroid Hormone Like Hormone	<p>adenylate cyclase-activating G protein-coupled receptor signaling pathway peptide hormone receptor binding cAMP metabolic process cell-cell signaling cytoplasm</p>

		epidermis development proliferation negative regulation of chondrocyte regulation of chondrocyte differentiation regulation of gene expression skeletal system development
XDH	Xanthine Dehydrogenase	Purine degradation Oxidation of hypoxanthine to xanthine
HMGA2	High Mobility Group AT-Hook 2	positive regulation of transcription, DNA SMAD binding mesenchymal cell differentiation 5'-deoxyribose-5-phosphate lyase activity C2H2 zinc finger domain binding DNA (apurinic or apyrimidinic site) endonuclease activity base-excision repair cAMP response element binding cell division
CSTA	Cystatin A	cytosol cysteine-type endopeptidase inhibitor activity cornified envelope negative regulation of endopeptidase activity negative regulation of peptidase activity cell-cell adhesion keratinocyte differentiation negative regulation of proteolysis peptidase inhibitor complex
CYP27B1	1-alpha-hydroxylase	calcidiol 1-monooxygenase activity mitochondrial outer membrane vitamin D metabolic process G1 to G0 transition calcium ion homeostasis calcium ion transport iron ion binding negative regulation of cell growth positive regulation of keratinocyte differentiation positive regulation of vitamin D receptor signaling pathway response to estrogen response to interferon-gamma
FEZ1	Fasciculation And Elongation Protein Zeta	cell adhesion cellular response to growth factor stimulus establishment of cell polarity gamma-tubulin binding mitochondrion morphogenesis negative regulation of autophagosome assembly
GPX2	Glutathione Peroxidase 2	cellular oxidant detoxification electron transfer activity glutathione peroxidase activity peroxidase activity response to oxidative stress
NIPAL4	Magnesium transporter NIPA4	magnesium ion transmembrane transport magnesium ion transmembrane transporter activity

		magnesium ion transport
PP4R4	Protein Phosphatase 4 Regulatory Subunit 4	negative regulation of phosphoprotein phosphatase activity protein serine/threonine phosphatase complex regulation of protein serine/threonine phosphatase activity
RNASE7	Ribonuclease A Family Member 7	defense response to Gram-positive and negative bacterium innate immune response ribonuclease activity antibacterial humoral response antimicrobial humoral immune response mediated by antimicrobial peptide defense response to fungus endonuclease activity
SERPINB13	Serpin Family B Member 13	protease binding cysteine-type endopeptidase inhibitor activity serine-type endopeptidase inhibitor activity negative regulation of endopeptidase activity negative regulation of keratinocyte apoptotic process regulation of proteolysis response to UV
COL17A1	Collagen Type XVII Alpha 1 Chain	extracellular matrix structural constituent conferring tensile strength basement membrane cell-cell junction cell-matrix adhesion collagen trimer epidermis development extracellular matrix organization extracellular region hemidesmosome assembly
TRIM7	Tripartite Motif Containing 7	E3 ubiquitin-protein ligase
PBX1	PBX Homeobox 1	DNA-binding transcription factor activity, RNA polymerase II-specific animal organ morphogenesis anterior/posterior pattern specification branching involved in ureteric bud morphogenesis embryonic limb morphogenesis embryonic organ development embryonic skeletal system development
SPOCK1	SPARC or Osteonectin Testican-1	cell adhesion cysteine-type endopeptidase inhibitor activity metalloendopeptidase inhibitor activity negative regulation of cell-substrate adhesion regulation of cell growth serine-type endopeptidase inhibitor activity plasma proteoglycan containing chondroitin- and heparan-sulfate chains.
COBL	Cordon-Bleu WH2 Repeat Protein	actin filament network formation actin filament polymerization cell cortex plasma membrane

		<ul style="list-style-type: none"> ruffle embryonic axis specification floor plate development positive regulation of ruffle assembly
FABP5	Fatty Acid Binding Protein 5	<ul style="list-style-type: none"> epidermis development fatty acid transport glucose metabolic process lipid metabolic process negative regulation of glucose transmembrane transport positive regulation of peroxisome proliferator Intracellular carrier for long-chain fatty acids selectively delivers fatty acids from the cytosol to the nucleus
CYP1B1	Cytochrome P450 Family 1 Subfamily B Member 1	<ul style="list-style-type: none"> monooxygenase activity oxidoreductase activity lipid metabolic process endoplasmic reticulum membrane estrogen metabolic process retinol metabolic process aromatase activity

Table 2. Regulators of Δ Np63 Expression

Pathway/Factor	Transcription Factor	ΔN mRNA Repression/Activation	Model	Reference
NFkB NFkB overexpression induced EMT	NFkB	Repression	MCF10A	(Chua et al., 2007)
EMT Zeb1 overexpression induced EMT and repressed p63	Zeb1	Repression	MCF10A	(Chua et al., 2007)
NFkB Repression of Δ Np63 is mediated by ASPP2 inhibition of I κ B which activates RelA/p65	NF- κ B RelA/p65	Repression	Mouse keratinocytes	(Tordella et al. 2013)
EMT EMT transcription factors Snail Slug	Snail Slug	Repression	SCC	(Higashikawa et al. 2007)
EMT GRHL2 when overexpressed increased Δ Np63 expression Δ Np63 downregulation represses GRHL2	GRHL2 (reciprocal regulation with Δ Np63)	Activation	normal human epidermal keratinocytes and SCC	(Mehrazarin et al. 2015)
EMT Zeb2 overexpression binds to E-boxes at the p63 promoter	Zeb2	Repression	Lkb1 deficient Lung adenocarcinoma mouse model	(Gao et al., 2014)
EMT Snail and Slug when overexpressed suppressed Δ Np63 α expression	Snail and Slug	Repression	SCC cell lines	(Herfs et al. 2010)
Ras-PI3K FOXO3 Inhibition by AKT phosphorylation. AKT is activated by mutant Ras, mutant PI3K and Her2 overexpression	FOXO3a	Activation	MCF10A	(Hu et al., 2017)
EGFR Activation of EGFR, activates STAT3 which activates p63 expression	STAT3	Activation	SCC cell lines	(Ripamonti et al. 2013)
KGFR Activation of KGFR through p38 MAP Kinase signaling regulates Δ Np63 α and proliferation corneal epithelial stem cells. Increased the levels of SP1 transcription factor	KGFR through p38 MAP Kinase	Activation	Corneal epithelial stem cells	(Cheng et al. 2009)
Wnt β-Catenin LBH increases transcription of Δ Np63 α in mammary epithelial cells	LBH limb-bud and heart transcriptional activator	Activation	basal mammary stem cells	(Lindley et al., 2015)

<p>Wnt β-Catenin ΔNp63 promoter is activated by Wnt signaling through activation of downstream factor Lef1/TCF</p>	Lef1/TCF	Activation	embryonic development in mice	(Ferretti et al. 2011)
<p>Notch Activation of Notch signaling repressed p63 by downregulation of IRF7. Overexpression of IRF7 counteracts Notch repression of p63</p>	IRF7	Activation	Keratinocytes	(Nguyen et al., 2006).
<p>Notch Notch activation induced luminal differentiation and overexpression of p63 and inhibition of Notch inhibited the luminal phenotype in mammary stem cells</p>	Unknown	Repression	mammary stem cells	(Yalcin-Ozuysal et al. 2010)
<p>Hedgehog The activation of Gli3 with Ihh and Knockdown of Gli3/Gli3R repress ΔNp63 and induce TAp63.</p>	lack of Gli3R Gli3	Repression	mammary epithelial cells	(Li et al. 2008a)
<p>Hippo Active S127A YAP induced adenocarcinoma differentiation, which induces p63 repression</p>	YAP	Repression	Lung carcinoma mouse model	(Gao et al., 2014)
<p>Hippo pS89A mutant TAZ and WT TAZ overexpression binds the ΔNp63 promoter</p>	TAZ	Repression	MCF10A	(Valencia-Sama et al., 2015)

Table 3. YAP/TAZ Gene Signature

Modified from (Wang et al. 2018)

Gene Symbol	Gene Name	Gene Pathways
CYR61 (CCN1)	Cysteine Rich Angiogenic Inducer 61	cell adhesion chemotaxis extracellular matrix binding growth factor binding heparin and integrin binding angiogenesis labyrinthine layer blood vessel development negative regulation of apoptotic process negative regulation of cell death
CTGF (CCN2)	Connective Tissue Growth Factor	angiogenesis cell adhesion cell differentiation cell migration cell-matrix adhesion collagen-containing extracellular matrix epidermis development extracellular matrix constituent secretion fibroblast growth factor receptor signaling pathway fibronectin binding
LATS2	Large Tumor Suppressor 2	protein serine/threonine kinase activity hippo signaling G1/S transition of mitotic cell cycle protein serine/threonine/tyrosine kinase activity spindle pole cell division negative regulation of canonical Wnt signaling pathway negative regulation of cyclin-dependent protein serine/threonine kinase activity negative regulation of protein localization to nucleus positive regulation of apoptotic process protein phosphorylation protein serine kinase activity regulation of organ growth regulation of transforming growth factor beta receptor signaling pathway
AMOTL2	Angiomotin Like 2	protein binding Wnt signaling pathway actin cytoskeleton organization angiogenesis bicellular tight junction establishment of cell polarity involved in ameboidal cell migration hippo signaling regulation of cell migration
NUAK2	NUAK Family Kinase 2	protein serine/threonine kinase activity cellular response to glucose starvation Serine/tyrosine kinase activity ATP binding

		actin cytoskeleton organization apoptotic process intracellular signal transduction negative regulation of apoptotic process
AXL	AXL Receptor Tyrosine Kinase	peptidyl-tyrosine phosphorylation phagocytosis ATP binding actin cytoskeleton blood vessel remodeling cell migration cellular response to extracellular stimulus cellular response to hydrogen peroxide cellular response to interferon-alpha inflammatory response
ANKRD1	Ankyrin Repeat Domain 1	RNA polymerase II-specific DNA-binding transcription factor binding cellular response to mechanical stimulus cellular response to TGF beta stimulus cellular response to tumor necrosis factor histone deacetylase binding negative regulation of transcription p53 binding positive regulation of DNA damage response, signal transduction by p53 class mediator positive regulation of apoptotic process positive regulation of protein secretion
TGFB2	Transforming Growth Factor Beta 2	epithelial to mesenchymal transition SMAD protein signal transduction BMP signaling activation of protein kinase activity negative regulation of angiogenesis negative regulation of epithelial cell proliferation positive regulation of cell growth pathway-restricted SMAD protein phosphorylation positive regulation of cell adhesion mediated by integrin positive regulation of epithelial to mesenchymal transition, response to wounding
PTPN14	Protein Tyrosine Phosphatase Non-Receptor Type 14	protein tyrosine phosphatase activity negative regulation of cell population proliferation peptidyl-tyrosine dephosphorylation protein dephosphorylation receptor tyrosine kinase binding regulation of protein export from nucleus regulation of transcription, DNA-templated transcription coregulator activity
NT5E	5'-Nucleotidase Ecto	plasma membrane, cytosol 5'-nucleotidase activity ATP metabolic process DNA metabolic process adenosine biosynthetic process calcium ion homeostasis response to ATP, dephosphorylation
FOXF2	Forkhead Box F2	DNA-binding transcription activator activity, RNA polymerase II-specific epithelial to mesenchymal transition establishment of planar polarity of embryonic

		extracellular matrix organization regulation of protein polyubiquitination
DOCK5	Dedicator Of Cytokinesis 5	guanyl-nucleotide exchange factor activity GTPase activator activity positive regulation of epithelial cell migration positive regulation of substrate adhesion-dependent cell spreading small GTPase mediated signal transduction
ASAP1	ArfGAP With SH3 Domain	GTPase activator activity cadherin binding cell projection membrane positive regulation of GTPase activity positive regulation of membrane tubulation positive regulation of podosome assembly
RBMS3	RNA Binding Motif Single Stranded Interacting Protein 3	poly(A) binding, RNA binding mRNA 3'-UTR binding negative regulation of canonical Wnt signaling negative regulation of gene expression positive regulation of gene expression ribonucleoprotein complex
MYOF	Myoferlin	extracellular exosome phospholipid binding plasma membrane repair cytoplasmic vesicle intracellular membrane-bounded organelle membrane fusion nuclear envelope plasma membrane organization
ARHGEF17	Rho Guanine Nucleotide Exchange Factor 17	guanyl-nucleotide exchange factor activity actin cytoskeleton organization small GTPase mediated signal transduction
CCDC80	Coiled-Coil Domain Containing 80	basement membrane extracellular matrix organization fibronectin binding heparin binding regulation of cell-substrate adhesion

Chapter 2: A non-canonical Hippo pathway represses the expression of Δ Np63

Ana Maria Low-Calle, Hana Ghoneima, Chen Katz, David Tong, Adriana M. Cuiibus, Carol Prives and Ron Prywes

Department of Biological Sciences

Columbia University

New York NY 10027

Email mrp6@columbia.edu and clp3@columbia.edu

Author Contributions A.M.L.C., C.P. and R.P. designed the experiments; A.M.L.C., H.G., R.P., A.M.C., D.T. and C.K. performed the experiments, A.M.L.C., H.G., R.P., A.M.C., C.K., C.P. and R.P. analyzed the data, and A.M.L.C., C.P., and R.P. wrote the manuscript.

Keywords Δ Np63, Hippo, Lats, Mst, Transcriptional Repression, Breast cancer, Squamous Cancer

2.1 Abstract

The p63 transcription factor, a member of the p53 family, plays an oncogenic role in squamous cancers, while in breast cancers its expression is often repressed. In the canonical conserved Hippo pathway, known to play a complex role in regulating growth of cancer cells, the protein kinases MST1/2 and LATS1/2 act sequentially to phosphorylate and inhibit the YAP/TAZ transcription factors. We found that in the MCF10A mammary epithelial cell line as well as in squamous and breast cancer cell lines, expression of Δ Np63 RNA and protein is strongly repressed by inhibition of certain components of the Hippo pathway in a manner that is independent of p53. While the Hippo pathway protein kinases MST1/2 and LATS1 are required for p63 expression, the next step of the pathway, namely phosphorylation and degradation of the YAP/TAZ transcriptional activators is not required for repression of p63. This suggests that regulation of p63 expression occurs by a non-canonical version of the Hippo pathway. Interestingly, we observed that

experimentally lowering p63 expression leads to increased YAP protein levels, thereby constituting a feedback loop. In addition, p63 loss reduces growth of MCF10A and squamous cancer cell lines. These results, which reveal the intersection of the Hippo and p63 pathways, may prove useful for the control of their activities in cancer cells.

2.2 Introduction

The p53 family member p63 is a transcription factor that was initially described to play a role in the embryonic development of the skin and glandular epithelial tissues such as the breast (Yang et al. 1999; Mills et al. 1999). p63 has two major alternative isoforms at the 5' end of the gene encoding TAp63 and the truncated isoform Δ Np63; and each of these have three additional splice variants at the 3' end; α , β , and γ (Yang et al. 1998) thereby creating six p63 variants with distinct N-and C-termini. The Δ Np63 α isoform is the predominant isoform expressed in normal stratified and glandular epithelial tissues (Como et al. 2002). p63 regulates the expression of epithelial-specific genes and regulates survival, cell adhesion, and keratin gene expression in breast epithelial cells and squamous cell carcinomas (SCC) (Carroll et al. 2006; Boldrup et al. 2007). The p63 locus is frequently amplified in primary lung and head and neck squamous cell carcinomas (Hibi et al. 2000) and in a more recent study the expression of the Δ Np63 isoform was enriched across multiple types of SCC (Campbell et al. 2018). Copy number gain at the p63 locus at Chromosome 3q28 was found in SCCs and showed a correlation with elevated expression of Δ Np63 (Campbell et al. 2018).

In breast cancers, the role of p63 is more complex, suggesting it can possess either tumor suppressor or growth promoting activities. On the one hand p63 expression is often lower in breast tumors than in normal tissue and it has been suggested that p63 can play a role as a suppressor of epithelial to

mesenchymal transition (EMT) and metastasis (Yoh et al. 2016; Hu et al. 2017). Yet p63 can also promote growth of basal-like breast cancer cells (Chakrabarti et al. 2014; Orzol et al. 2016).

Other genes that have been found to be frequently amplified in SCC are the Hippo pathway transcriptional activators YAP and TAZ (Campbell et al. 2018; Wang et al. 2019a). Each of these have been found to induce growth in both breast cancer cells and SCC (Chan et al. 2008; Cordenonsi et al. 2011; Zanconato et al. 2015; Li et al. 2019).

The Hippo pathway is a tumor suppressor signaling pathway that negatively regulates YAP and TAZ (Zhao et al. 2007; Lei et al. 2008). The upstream MST1 and 2 (Mammalian Sterile-like-20) protein kinases serve to phosphorylate and activate the LATS1 and 2 protein kinases (Large Tumor Suppressor Kinase) (Chan et al. 2005). Activated LATS1/2 phosphorylate the transcriptional activators YAP and TAZ which are then sequestered in the cytoplasm and targeted for degradation (Zhao et al. 2007; Hao et al. 2008; Lei et al. 2008; Zhao et al. 2010). When LATS1/2 are inactivated, YAP and TAZ are translocated to the nucleus where they interact with TEAD transcription factors to induce expression of growth-inducing genes such as Connective Tissue Growth Factor (CTGF) and others (Zhao et al. 2008; Zhang et al. 2009). Additional main components of this pathway are MOB1A and MOB1B which bind to LATS1/2 (Hergovich et al. 2006; Praskova et al. 2008) and SAV1 which binds to MST1/2 (Callus et al. 2006; Bae et al. 2017; Lin et al. 2020).

We previously found that oncogenic transformation of MCF10A cells with H-RAS or PIK3CA leads to the epithelial-mesenchymal transition (EMT) and repression of p63 expression (Yoh et al. 2016). Suppression of p63 expression also induces EMT. We were therefore interested in cellular signaling pathways that might control p63 expression. In addition, suppression of p63 expression may be useful therapeutically in SCCs where its expression is elevated. Since both p63 and YAP/TAZ have been shown to play roles in breast and squamous cancers, we considered the possibility of a connection between Hippo pathway components, YAP/TAZ, and p63. Our research has revealed unexpected links between p63 and this pathway which are described in this study.

2.3 Materials and Methods

Cell culture

The MCF10A immortalized non-transformed mammary epithelial cell line (a gift obtained from David Weber, University of Maryland School of Medicine) were grown in DMEM/F12 (Thermo Fisher Scientific, 11320033) with 5% horse serum (Thermo Fisher Scientific, 16050122), penicillin-streptomycin solution (Thermo Fisher Scientific, 15140122), 10 µg/mL insulin (Sigma-Aldrich, 91077C), 0.5 µg/mL hydrocortisone (Sigma-Aldrich, H0888) and 20 ng/mL Epidermal Growth Factor (PeproTech, AF-100-15). MCF10A CRISPR p53 Knock out (KO) clones were generated using CRISPR/Cas9 genome editing as previously described (Venkatesh et al. 2020). Esophageal squamous cell carcinoma cell lines TE5 and TE14 were a gift from Anil Rustgi, Herbert Irving Comprehensive Cancer Center, Columbia University Irving Medical Center and were grown in RPMI 1640 (Thermo Fisher Scientific, 11875119) supplemented with 10% fetal bovine serum (Gemini Bio-Products, 900108H) and penicillin-streptomycin solution. The Cal27 tongue squamous cell carcinoma cell line was a gift from Alison Taylor, (Columbia University Irving Medical Center) and was maintained in DMEM (Thermo Fisher Scientific, 12100061) supplemented with 10% fetal bovine serum and penicillin streptomycin solution. Breast cancer cell lines HCC1937 (Triple negative, primary ductal carcinoma) and HCC1954 (HER2 positive, ductal carcinoma) were obtained from the High-Throughput Screening facility of the Columbia University Genome Center. These cell lines were grown in RPMI 1640 with 10% fetal bovine serum and penicillin-streptomycin solution. Cells were detached with trypsin-EDTA (0.05%) (Thermo Fisher Scientific, 15400054) and counted before seeding for experiments using trypan blue in a Countess 3 Automated Cell Counter (Thermo Fisher Scientific). All cell lines were grown at 37°C with 5% CO₂.

siRNA transfection

For siRNA transfections, cells (1.5×10^5) were plated in 6 well plates and 24 h later each siRNA (17 nM) was transfected with Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, 13778150) following manufacturer's instructions. Cells were harvested 72 h post transfection. The following siRNAs were used siC (Silencer™ Select Negative Control No. 1, Thermo Fisher Scientific, 4390843), sip63#1 (Thermo Fisher Scientific, 4392420, ID s16411), sip63#2 (Thermo Fisher Scientific, 4392420, ID s531582), FoxO3#1

(Thermo Fisher Scientific, 4427037, ID s5260), and FoxO3#2 (Thermo Fisher Scientific, 4427037, ID s5261), siYap#1 (Thermo Fisher Scientific, 4427037, ID s20366), siYap#2 (Thermo Fisher Scientific, ID s20367), siTaz#1 (Thermo Fisher Scientific, 4392420, ID s24787), and siTaz#2 (Thermo Fisher Scientific, 4392420, ID s24788). The following Smartpool siRNAs were used: siRNA negative control (siC) (Dharmacon, D-001810-10-05), siMst1 (Dharmacon, L-004157-00-0005), siMst2 (Dharmacon, L-004874-00-0005), siLats1 (Dharmacon, L-004632-00-0005), siLats2 (Dharmacon, L-003865-00-0005), siMob1a (Dharmacon, L-021097-00-0005), siMob1b (Dharmacon, L-018359-01-0005) and siSav1 (Dharmacon, L-013070-01-0005). To decrease levels of YAP and TAZ proteins, siRNAs (11 nM) were transfected into wells and 24 h post transfection fresh media with drug was added and cells were harvested 24 h later.

Cell growth assay

Cells (4×10^4) were seeded in 24 well plates; 24 h later siRNAs (25 nM) were transfected into wells along with Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, 13778150) following manufacturer's instructions. At 96 h post transfection cell numbers were measured by adding 300 μ L/well of a 1:1 mix of complete medium and luminescent reagent Cell Titer-Glo 2.0 Assay (Promega, G9241) then plates were shaken in the dark at room temperature for 10 min. The cell suspension (100 μ L) from each well was added to 96 well white plates with clear bottoms (Thermo Fisher Scientific, 07-200565). Luminescence was read in the Biotek Synergy H1 Multi-Mode plate reader (Biotek Instruments), using complete medium Cell Titer-Glo mix as a blank. The luminescence units from each well were normalized to its corresponding DMSO or siRNA control.

Inhibitors

XMU-MP1 (Selleck Chemicals, S8334) is an inhibitor of MST1/2 (Fan et al. 2016). Truli (Gift from Dr. J. Hudspeth and CSNPharm, CSN26140) is an inhibitor of LATS1/2 (Kastan et al. 2021). Verteporfin (Selleck Chemicals, S1786) is an inhibitor of YAP binding to the TEAD transcription factors (Liu-Chittenden et al. 2012).

Immunoblot

Total protein lysates were obtained by washing the cells with ice cold phosphate buffered saline (PBS), and cells were lysed with RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate, and 1% NP40) supplemented with fresh protease inhibitors (3 µg/mL Leupeptin, 1 µg/mL macroglobulin, 1 µM benzamidine, 0.5 µM PMSF) and phosphatase Inhibitors (EMD Millipore, 524625). Cell lysates were incubated 10 minutes on ice and centrifuged at 13000 rpm for 10 minutes. The protein concentrations sample supernatants were measured with the Bio-Rad protein assay dye reagent (Bio-Rad, 5000006). Equivalent amounts of protein samples were mixed with Laemmli buffer (100 mM Tris-HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 5% glycerol) and incubated at 95°C for 5 min. Lysates (35 µg protein) were run on 10% SDS-polyacrylamide gels-and transferred to a nitrocellulose membrane (Bio-Rad, 1620115). After blocking for 10 min with 5% nonfat dried milk in PBS with 0.05% Tween-20 (Sigma-Aldrich, P1379) (PBST), the membrane was incubated with primary antibodies diluted in 0.1% milk in PBST at 4°C overnight. The following primary antibodies from Cell Signaling Technologies were used: GAPDH (1:10000, 5174), MST1 (1:500, 3682), MST2 (1:1000, 3952), LATS2 (1:500, 5888), pS127-YAP (1:8000, 13008), YAP (1:4000, 14074), pS89-TAZ (1:500, 59971), TAZ (1:500, 71192), CTGF (1:300, 86641), MOB1A/B (1:500, 13730), SAV1 (1:1000, 13301), and FOXO3 (1: 1000, 2497). In addition, primary antibodies to LATS1 (1:1000, Millipore, MABS1823) and p63 (1:500, Biocare Medical, CM163A) were used. The secondary antibodies, peroxidase-conjugated Anti-Rabbit IgG (Sigma-Aldrich, A6154) and anti-mouse IgG (Sigma-Aldrich, A4416), were incubated for 1 h at room temperature at 1:5000 dilution in 0.5% milk PBST for 1 h. Membranes were imaged with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, 322106) or Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500).

Quantitative RT-PCR

Total RNA (1000 ng) isolated with the RNAeasy mini kit (Qiagen, 74106), was converted to cDNA with the QuantiTect Reverse Transcription Kit (Qiagen, 205313) following manufacturer's instructions. Real-time PCR was performed in the StepOne Plus thermal cycler (Applied Biosystems) with Applied Biosystems Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, 4368708) and primers (0.2 µM) following the program: 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, 60°C for

one minute. Melting curves were performed between 60°C -95°C with 1°C/sec. The relative expression of each gene was calculated according to the $\Delta\Delta C_t$ method expression using the C_t values of the housekeeping gene RPL32 and normalized to the siRNA control or DMSO treated condition. For the list of primers used see Supplemental Table 1.

Wound healing assay

MCF10A cells (1.5×10^5) were plated in 6 well plates, and 24 h later were transfected with siRNA (26 nM). After 48 h they were detached with Accutase (MP biomedical, 100449) and 1×10^6 cells were plated for each condition. After 24 h the scratch wound was made with a P200 tip drawn across each well and medium was replaced with serum- and growth factor- free DMEM/F12 media with Mitomycin C (2 $\mu\text{g/ml}$, Sigma-Aldrich, M-4287), to prevent the wound closing due to cell growth. Pictures were taken with an inverted microscope (Nikon TS2) using a 4X lens at 0 h and 12 h after the wound was made. The wound area was measured with ImageJ software (U. S. National Institutes of Health, Bethesda, Maryland, USA), and the average wound closing taken from three independent experiments each with three technical replicates each was calculated. The wound area was calculated based on the area at 0 h minus the area at 12 h, normalized to the initial area of the wound at time 0 h.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Software version 9.3.1 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). For simple comparisons between two treatments Student's t-test was used. For analysis of three or more treatments One-way ANOVA with Dunnett's multiple comparisons test was performed. Statistical significance is represented in the graphs as: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$.

2.4 Results

The Hippo pathway kinases MST1 and MST2 regulate the levels of p63

Given the increased expression of p63 in squamous cell carcinomas and its reduced expression in breast carcinomas, we were interested in how p63 might be regulated. The Hippo signaling pathway interacts in multiple ways with members of the p53 tumor suppressor family (Furth et al. 2018), which provided the impetus to evaluate whether pharmacological inhibition of the Hippo pathway had any effect on expression of p63. For this purpose we used the immortalized untransformed mammary epithelial cell line MCF10A (Soule et al. 1990), that we previously showed expresses mainly the Δ Np63 α isoform of p63 (Yoh et al. 2016). To pharmacologically inhibit the pathway we used XMU-MP1, a small molecule inhibitor of the uppermost protein kinases in the pathway, namely MST1 and MST2 (Fan et al. 2016). After treatment of the MCF10A cells with XMU-MP1 for 24 hours we observed a significant downregulation of p63 expression at the mRNA and protein levels (Figure 2-1 A, B). As expected (Zhao et al. 2010; Fan et al. 2016), inhibition of MST1/2 also led to a decrease in phospho-YAP levels and an increase in total YAP levels (Figure 2-1 A). TAZ and phospho-TAZ levels were similarly regulated. The upregulation of YAP and TAZ proteins along with decreased phosphorylation at the LATS target sites on YAP (Ser127) and TAZ (Ser89) indicated that the kinase activity of LATS was suppressed due to the MST1/2 inhibitor (Figure 2-1 A). In addition, expression of the well-known YAP/TAZ target gene CTGF was strongly induced (Figure 2-1 A, B) (Zhao et al. 2008; Zhang et al. 2009). To confirm that the effect of the inhibitor was through MST1/2, we used siRNA to suppress the levels of the kinases MST1 and MST2 individually and together and observed an increase in the protein levels of YAP and TAZ and upregulation of CTGF in all three conditions compared to the control siRNA (Figure 2-1 C, D). CTGF induction was the highest with Mst2 knockdown while p63 suppression was slightly stronger with Mst1 siRNA (Figure 2-1 C, D). These results further demonstrate that both MST1/2 are required for p63 expression.

Since MCF10A cells harbor wild-type p53 and p53 is known to be a phosphorylation target of LATS (Aylon et al. 2006; Furth et al. 2015) we asked whether p53 was involved in the repression of p63 after the inhibition of MST1/2. When parental MCF10A cells and two p53 CRISPR KO clones were treated with XMU-MP1, p63 was downregulated to the same extent in all three cell lines (Figure 2-1 E, F) indicating that the effect of MST1/2 inhibition on p63 expression is independent of p53.

LATS1 and MOB1A regulate p63 expression

Since both MST1 and 2 are required for p63 expression in MCF10A cells, we evaluated whether their downstream protein kinases targets, LATS1 and 2, were also required. We first treated MCF10A with the LATS1/2 inhibitor Truli (Kastan et al. 2021) for 24 h which led to a 91% downregulation of p63 mRNA and a 7-fold induction of CTGF mRNA (Figure 2-2 B). p63 reduction and CTGF induction were also seen at the protein level along with decreased phosphorylation of YAP, and upregulation the total levels of YAP and TAZ proteins (Figure 2-2 A). There was also a mobility shift of phospho-TAZ protein, suggesting a change in its modifications due to LATS inhibition.

To confirm the inhibitor results, we ablated Lats1, Last2 and both Lats1/2 with siRNA to reduce their levels. Knockdown of LATS1, but not LATS2, downregulated p63 at the protein and mRNA levels (Figure 2-2 C, D) even though siRNA knockdown of both Lats1 and Lats2 were required for full activation of CTGF expression and decreased phospho-YAP and phospho-TAZ levels (Figure 2-2 C, D). These results showing that LATS1 is preferentially required for p63 repression, suggested that the repression of p63 might be through an independent process that is distinct from the activation of YAP and TAZ.

To further evaluate the requirement of the Hippo pathway for p63 expression, we tested cofactors of the MST and LATS kinases, SAV1 and MOB1A/B, respectively. When siRNAs directed against MOB1A, MOB1B or SAV1 were used, only MOB1A was required for p63 expression (Figure 2-2 E, F). We did not observe an effect of MOB1B siRNA on p63 or CTGF expression, but could not confirm this at the protein level due to lack of an antibody that can distinguish between A and B isoforms, although the siRNA for MOB1A abolished the lower band while the MOB1B siRNA abolishes the upper band (Figure 2-2 E). It is possible that MOB1B may simply be less abundant than MOB1A in MCF10A cells such that it is not required for Lats1/2 activity. The effects of SAV1 siRNA were relatively mild, despite general reports that SAV1 is required for MST activity (Callus et al. 2006; Bae et al. 2017). While SAV1 siRNA increased CTGF protein and mRNA levels, there was no effect on p63 mRNA and a modest reduction of p63 protein (Figure 2-2 E, F). Further studies will be required to understand the role of SAV1 in this system. Nevertheless, the results with MOB1A further show the requirement of the Hippo pathway for p63 expression.

MST and LATS regulate p63 expression in breast and squamous cancer cell lines

Since MCF10A is a non-transformed immortalized mammary epithelial cell line, it was of interest to evaluate whether p63 is regulated by the Hippo pathway in cancer cells as well. We selected previously described Δ Np63 positive breast cancer cell lines HCC1937 (triple negative) and HCC1954 (HER2 positive) (Gatti et al. 2018) and the squamous carcinoma cell lines TE5, TE14 and Cal27 with high expression of Δ Np63 (Supplemental Figure S-1 for relative expression of p63 mRNA). Treatment of HCC1937 and HCC1954 cells with the XMU-MP1 inhibitor of MST1/2 led to strong downregulation of Δ Np63 protein and mRNA (Figure 2-3 A, B). This correlated with markedly increased expression of the YAP/TAZ target gene CTGF (Figure 2-3 A, B) even though YAP and TAZ protein levels were only modestly increased in both cell lines (Figure 2-3 A). In the squamous cancer cell lines, we also observed suppression of Δ Np63 mRNA and protein upon treatment with XMU-MP1, though the extent varied (Figure 2-3 C, D). CTGF mRNA and protein induction also varied, with the strongest activation in Cal27 cells (Figure 2-3 C, D). The reduction in phospho-YAP levels by XMU-MP1 treatment was also particularly strong in Cal27 cells (Figure 2-3 C). These results demonstrate that MST kinase activity is required to maintain p63 expression in multiple cancer cell lines.

We next asked whether the LATS kinases were also involved in regulating p63 in cancer cells. We inhibited LATS1/2 activity with the inhibitor Truli and found that p63 was significantly downregulated in HCC1937 and HCC1954 cells (Supplemental Figure S-2 A, B). A similar repression of p63 mRNA was found in the squamous cancer cells TE5, TE14 and Cal27 (Supplemental Figure S-2 C, D) while p63 protein levels were clearly downregulated in the Cal27 cell line, with smaller changes in the other lines (Supplemental Figure S-2 C, D), the strongest repression of p63 protein was found in the Cal27 cell line despite being one of the cell lines with the highest relative expression of p63 mRNA (Supplemental Figure S-1). CTGF was only upregulated two fold in the breast cancer cell lines, with increased CTGF protein levels only seen in the HCC1954 cell line (Supplemental Figure S-2 B). In response to LATS inhibition, we observed a decrease in phospho-YAP and a mild increase of total levels of YAP in both cell lines; TAZ levels increased mostly in HCC1937 cells (Supplemental Figure S-2 A). While phospho-YAP inhibition was

seen in each of the squamous cell lines, the increase in total YAP levels was seen the clearest in Cal27 cells (Supplemental Figure S-2 C). We can conclude from these results that in breast and squamous carcinoma cells MST and LATS activity are required for the expression of p63. However, the effects of the inhibition of these kinases on the levels of YAP, TAZ, and CTGF varied depending on the cell line and suggested to us that the p63 repression and CTGF induction pathways are different.

Repression of p63 after MST1/2 inhibition is YAP/TAZ independent

YAP and TAZ are inhibited by LATS phosphorylation (Zhao et al. 2007; Lei et al. 2008; Zhao et al. 2010) which led us to ask whether they are also involved in the repression of p63 caused by MST inhibition. To address this, we used two approaches. We inhibited YAP transcriptional activity with the drug Verteporfin that impairs YAP interaction with TEAD transcription factors (Liu-Chittenden et al. 2012) and we suppressed YAP and TAZ levels with two different siRNAs. In both cases we observed that neither the inhibitor (Figure 2-4 A, B) nor the siRNA (Figure 2-4 C, D) had any effect on the repression of p63 induced by XMU-MP1 inhibition of MST; p63 was still suppressed at the mRNA level despite the lack of YAP/TAZ protein and activity. We confirmed that YAP/TAZ inhibition was effective by observing a significant drop in the induction of CTGF mRNA and protein (Figure 2-4 A-D). These results indicate that YAP/TAZ activity is not required for the repression of p63 upon MST inhibition and implicate the involvement of other factors downstream of LATS1 that are not part of the canonical pathway.

p63 inhibits YAP and TAZ activity

Since regulating Hippo pathway had such a strong effect on p63 expression, we hypothesized that there might be reciprocity such that p63 might impose feedback inhibition on the Hippo pathway. When we tested this by treating MCF10A cells with two different siRNAs to p63, and found that indeed p63 loss increased the mRNA and protein levels of the YAP/TAZ target gene CTGF (Figure 2-5 A, B). In line with this p63 siRNAs increased the total levels of YAP and TAZ proteins, although their respective mRNA levels were not changed (Figure 2-5 A, B). As there was only a slight increase in YAP and TAZ phosphorylation (Figure 2-5 A), this means that the proportion of phosphorylated proteins actually goes down compared to the increase in the total levels of these two proteins. We also tested whether p63 loss could also increase

YAP/TAZ activity after XMU-MP1 treatment by measuring induction of the CTGF gene and found increased induction of CTGF when p63 levels were suppressed with siRNAs (Figure 2-5 C). These results show that p63 serves to restrict YAP/TAZ levels and hence dampen their transcriptional activity in MCF10A cells. Note that we also found that p63 siRNAs increased YAP protein levels in the three SCC cell lines used (Figure 2-5 D), while YAP mRNA was unaffected (Figure 2-5 E), suggesting that, as with MCF10A cells, the regulation of YAP protein is post-transcriptional. The effects on TAZ protein levels were more varied in the SCC cell lines. While p63 siRNAs increased TAZ levels in TE5 cells, they remained low in TE14 cells and high in Cal27 cells (Figure 2-5 D). As expected p63 siRNAs induced CTGF expression in TE5 cells where both YAP and TAZ protein levels increased (Figure 2-5 E). In TE14 cells there was little induction of CTGF, perhaps due to the low relative levels of YAP and TAZ in these cells (Figure 2-5 D,E). In Cal27 cells p63 siRNAs induced CTGF expression, likely due to the increase in YAP levels, although TAZ levels did not change (Figure 2-5 D, E). Despite the variability of the results in these cell lines, our data show clearly that p63 inhibits YAP levels in both MCF10A and SCC cell lines and supports a pathway where repression of p63 expression by MST inhibition leads to greater YAP levels and therefore greater induction of CTGF.

p63 facilitates growth

To evaluate whether the targeting of p63 has an impact on growth of cancer cells, we knocked down p63 with siRNA in MCF10A, TE5 and Cal27 cells. We found that the reduction of p63 levels decreased the growth by approximately 50% (Figure 2-6 A, B). As YAP/TAZ have been shown to be involved in cell motility (Mason et al. 2019; Chan et al. 2008; Valencia-Sama et al. 2015), we wanted to evaluate whether the transient loss of p63, could also increase motility. We found that the loss of p63 increased the motility of MCF10A cells in a wound assay (Supplemental Figure S-3). While the two p63 siRNAs increased motility to different extents, both were significant. These results show that p63 loss can lead to increased motility, consistent with our previous results of an increase in EMT and mesenchymal properties (Yoh et al. 2016). It is possible that the increase in YAP/TAZ levels caused by p63 depletion may be contributing to the increased motility.

2.5 Discussion

Here we describe that p63 expression is regulated by the Hippo pathway in different types of cells including non-transformed mammary MCF10A cells, breast cancer, and tongue and esophageal squamous carcinoma cells. Further our data indicate that the Hippo pathway regulates the expression of Δ Np63 in a non-canonical manner. While the MST1/2, LATS1 and MOB1A factors are required, regulation of p63 is independent of YAP and TAZ (Figure 2-6 C). In addition, we found that p63 feeds back into the pathway, causing the inhibition of YAP/TAZ, since p63 loss increased YAP/TAZ levels and the induction of the YAP target gene CTGF.

Surprisingly, in our experimental setting, we found that LATS1 but not LATS2 was preferentially required for the expression of p63, even though CTGF induction required the inhibition of both LATS1 and LATS2. LATS1-specific effects have been seen before: LATS1 inhibited autophagy in a kinase-independent manner while LATS2 had no effect (Tang et al. 2019a). Moreover, in an affinity proteomics study done by immunoprecipitation with overexpressed MOB1A/B proteins, MOB1A bound LATS1 proteins more abundantly compared to LATS2, which suggests a stronger interaction with LATS1 (Xiong et al. 2017). This could explain the difference between LATS1 and LATS2 in their regulation of p63 expression. The preferential inhibition of LATS1 may provide a means to repress p63 expression without the potential side effects or toxicity of inhibiting both LATS1 and LATS2.

MOB1A and MOB1B are scaffold proteins that activate LATS kinases. Phosphorylation of MOB1 by MST1/2 allows MOB1 binding to LATS1/2 (Praskova et al. 2008); in turn LATS1/2 can then be phosphorylated and activated by MST1/2 (Hergovich et al. 2006; Praskova et al. 2008; Ni et al. 2015). We found that MOB1A proteins were required for maintenance of p63 expression while there was little effect of MOB1B depletion, despite the fact that their protein products are 95% identical (Stavridi et al. 2003). It is possible, however, that this is due to the higher expression of MOB1A compared to MOB1B (Figure 2-2 E and our previous RNAseq data (Yoh et al. 2016)). Alternatively, MOB1A binds LATS1 more compared to MOB1B (Xiong et al. 2017). Since p63 repression and CTGF induction were similarly sensitive to MOB1A depletion, this suggests that a common pathway involving MOB1A and LATS1 is involved.

The protein SAV1 binds to and is required for MST1/2 recruitment to the plasma membrane (Callus et al. 2006; Yin et al. 2013) and promotes the auto-phosphorylation and activation of MST1/2 by preventing its interaction with the phosphatase STRIPAK (Bae et al. 2017). In contrast to expectations, SAV1 depletion did not inhibit MST activity enough to cause the downregulation of p63 mRNA, while it only increased CTGF expression moderately (Figure 2-2 F). Although the extent of depletion of SAV1 protein and mRNA by siRNAs appears strong (Figure 2-2 E, F), it is possible that residual SAV1 is sufficient to maintain p63 levels. Alternatively, MST1/2 may be able to function independently of SAV1 in a pathway to maintain p63 expression. Surprisingly, SAV1 loss decreased p63 protein levels, but not p63 mRNA (Figure 2-2 E, F) suggesting post-transcriptional regulation. p63 protein stability has been found to be regulated by the E3 ubiquitin ligases Pirh2, WWP1, and ITCH among others (Jung et al. 2013; Li et al. 2008b; Rossi et al. 2006). Interestingly, ITCH is also an E3 ligase for the Hippo kinase LATS1 (Ho et al. 2011). Further studies will be required to determine whether LATS1 and SAV1 regulate p63 protein stability through any of these E3 ligases.

The most striking deviation from the canonical Hippo pathway that we discovered was that YAP and TAZ are not required to repress p63 levels in multiple cell lines. While many transcription factors have been reported to regulate Δ Np63 expression (Fisher et al. 2020) it's unclear which, if any such factors, may be acting here. We tested FOXO3 as a potential regulator (Hu et al. 2017; Wang et al. 2019b), but found no role (Supplemental Figure S-4). Further studies will be required to identify the steps in the pathway between LATS1 and Δ Np63. In contrast to our results, overexpression of TAZ was shown to repress Δ Np63 expression (Valencia-Sama et al. 2015). This difference may be due to differential effects exerted by the overexpression of TAZ alone compared to the inhibition of endogenous MST1/2 that we reported here. Other non-canonical (YAP/TAZ-independent) pathway functions for LATS kinases have been found. LATS1 can target Estrogen Receptor α for ubiquitin-mediated degradation (Britschgi et al. 2017) and LATS2 binds to and inhibits MDM2 to prevent p53 degradation (Aylon et al. 2006). In addition, LATS2 but not LATS1 inhibits the maturation and subsequent nuclear translocation of the mevalonate pathway transcription factor SREBP2 (Aylon et al. 2016). Together with our results, this suggests that there are additional targets of the LATS kinases besides YAP and TAZ that can mediate effects of the Hippo pathway.

Endogenous p63 has been shown to increase squamous cancer growth (Ye et al. 2014; Li et al. 2015; Compagnone et al. 2017) and survival (Rocco et al. 2006) and we have shown a similar effect in MCF10A and squamous cancer cell lines. It's unclear which targets of p63 promote growth of these cells, however it may be related to p63's role in supporting the epithelial differentiation state (Riege et al. 2020; Soares and Zhou 2018). It's unlikely that YAP and TAZ targets are involved, as we found that p63 inhibits YAP and TAZ levels. In addition, we found that the transient loss of p63 increased the motility of MCF10A cells in a wound assay (Supplemental Figure S-3), similar to the increased migration seen in a transwell assay in p63 shRNA stable cell lines (Hu et al. 2017). This is consistent with p63's role as a suppressor of the epithelial-mesenchymal transition and may contribute to increased invasiveness of breast cancer cells where p63 expression is suppressed (Yoh et al. 2016). Since YAP/TAZ can increase cell motility (Mason et al. 2019; Chan et al. 2008; Valencia-Sama et al. 2015), the increase of YAP and TAZ due to p63 depletion may contribute to the increase in motility in MCF10A cells.

In conclusion, we found a pharmacological and molecular way to downregulate the levels of p63 in breast and squamous carcinoma cells as well as immortalized mammary cells. This occurs through inhibition of MST1/2 and LATS1 kinases in the core Hippo pathway. This defines an alternative Hippo pathway, independent of YAP/TAZ, that may also regulate other factors besides p63. We propose that these kinases and associated factors involved in p63 regulation could be useful targets for further development of therapies for cancers that show a strong dependency on p63 for growth. It will be important to take into account that their targeting has the potential to increase YAP/TAZ levels and their induction of cellular growth. This complication could be accommodated by inhibition of YAP/TAZ with an existing drug like Verteporfin. Further understanding of this pathway to identify factors that are specific to Hippo regulation of p63 levels will also be useful for finding specific inhibitors of this wing of the Hippo pathway.

2.6 Acknowledgements

We thank Ella Freulich for her help in tissue culture and technical assistance and Joshua Choe for his helpful advice and suggestions. We thank the current and former members of the Prives Lab, for their

advice and useful discussions. We thank Anil Rustgi, Alison Taylor and David Weber for providing the cell lines we used in this study and James Hudspeth for providing us with the LATS small molecule inhibitor Truli. This work was supported by NCI grant CA87497 C.P.

2.7 Figures and Tables

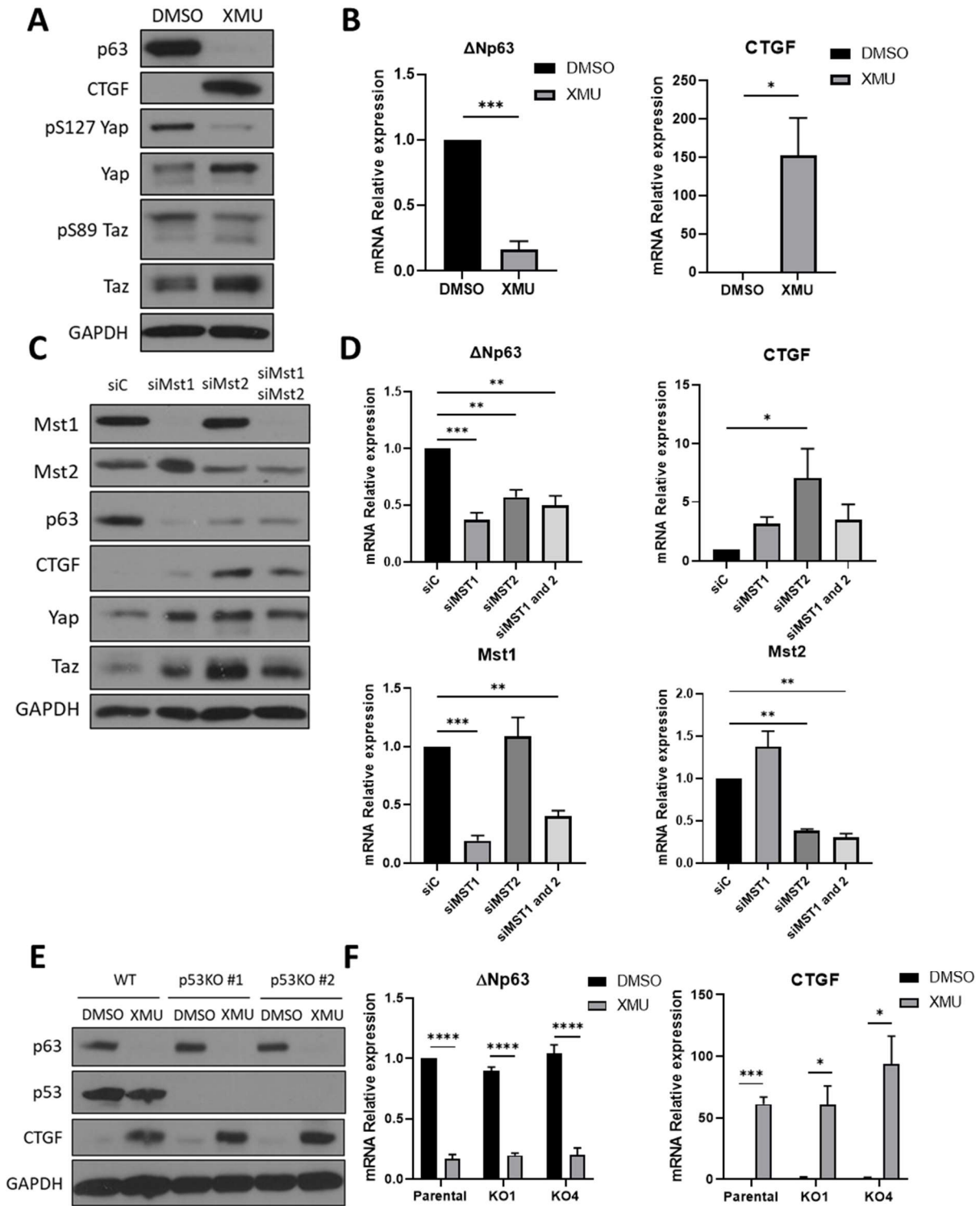


Figure 2-1. p53-Independent Regulation of p63 by the Hippo Pathway Kinases MST1 and MST2.

A. Representative immunoblot analysis of the indicated proteins after treatment of MCF10A cells with the MST1/2 inhibitor XMU-MP1 (5 μ M) for 24 h. Primary antibodies were used to detect the proteins or their phosphorylated forms as indicated. **B.** RT-qPCR analysis of CTGF and Δ Np63 expression in MCF10A cells treated as in A. Student's t-test was performed. **C.** MCF10A cells were transfected with Smartpool siRNA against Mst1, Mst2 or both for 72 h. Followed by immunoblotting analysis as in A. **D.** RT-qPCR of MCF10A cells transfected for 72 h with Smartpool siRNA against Mst1, Mst2 or both. ANOVA with pairwise comparisons against the siControl were performed. **E.** Immunoblot of wild-type MCF10A (WT) and two p53 CRISPR-generated p53 knock-out (KO) clones treated with XMU-MP1 (5 μ M) or DMSO for 24 h. **F.** RT-qPCR analysis of the indicated genes in the cell lines in E. Student's t-tests were performed. Bars represent the mean \pm SEM of three independent experiments with three technical replicates. Immunoblots are a representative experiment of three independent experiments. Statistical significance represented with * for p-value <0.05, ** for p-value<0.01, *** for p-value<0.005, **** for p-value<0.001

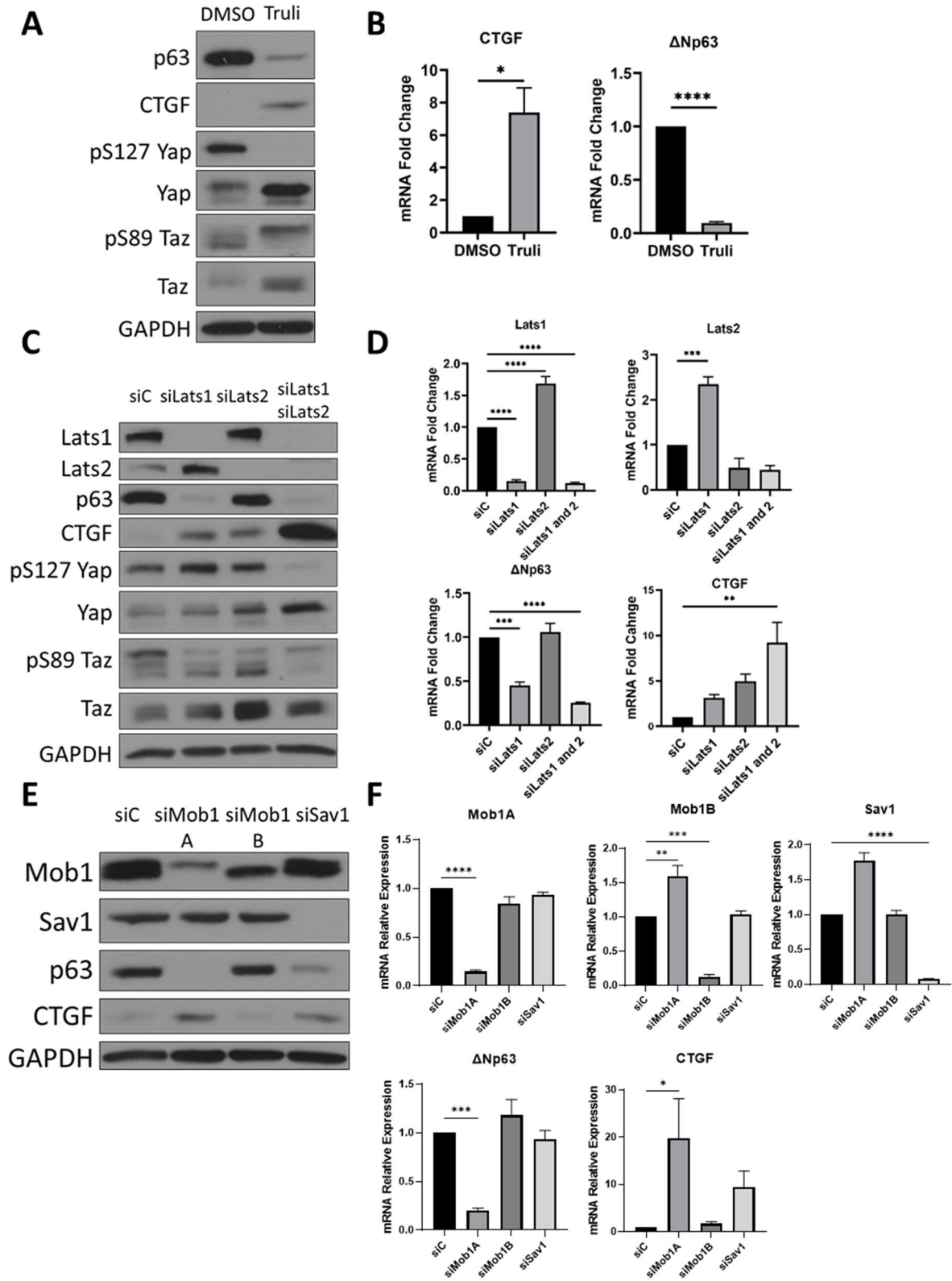


Figure 2-2. LATS1 and MOB1A Regulate p63 Expression.

A. Immunoblot analysis of the indicated proteins in MCF10A cells treated with DMSO or the LATS1/2 inhibitor Truli (5 μ M) for 24 h. **B.** qRT-PCR analysis of MCF10A cells treated as in A. Student's t-tests were performed **C.** Immunoblot analysis of the indicated proteins in MCF10A cells that were transfected with Smartpool siRNA against Lats1, Lats2 or both, for 72 h. **D.** qRT-PCR of MCF10A cells transfected as in C. **E.** Immunoblot analysis of the indicated proteins in MCF10A cells that were transfected with Smartpool siRNA against Mob1A, Mob1 or Sav1 for 72 h. **F.** qRT-PCR of MCF10A cells transfected as in E. For the immunoblots, one representative blots of three independent experiments is shown. For qRT-PCR, bars represent the mean \pm SEM of three independent experiments with three technical replicates normalized to the siRNA control (siC) or DMSO conditions. Statistical analysis was Student t-test in B and one-way ANOVA with Dunnett's multiple comparisons test was performed in D. and F.

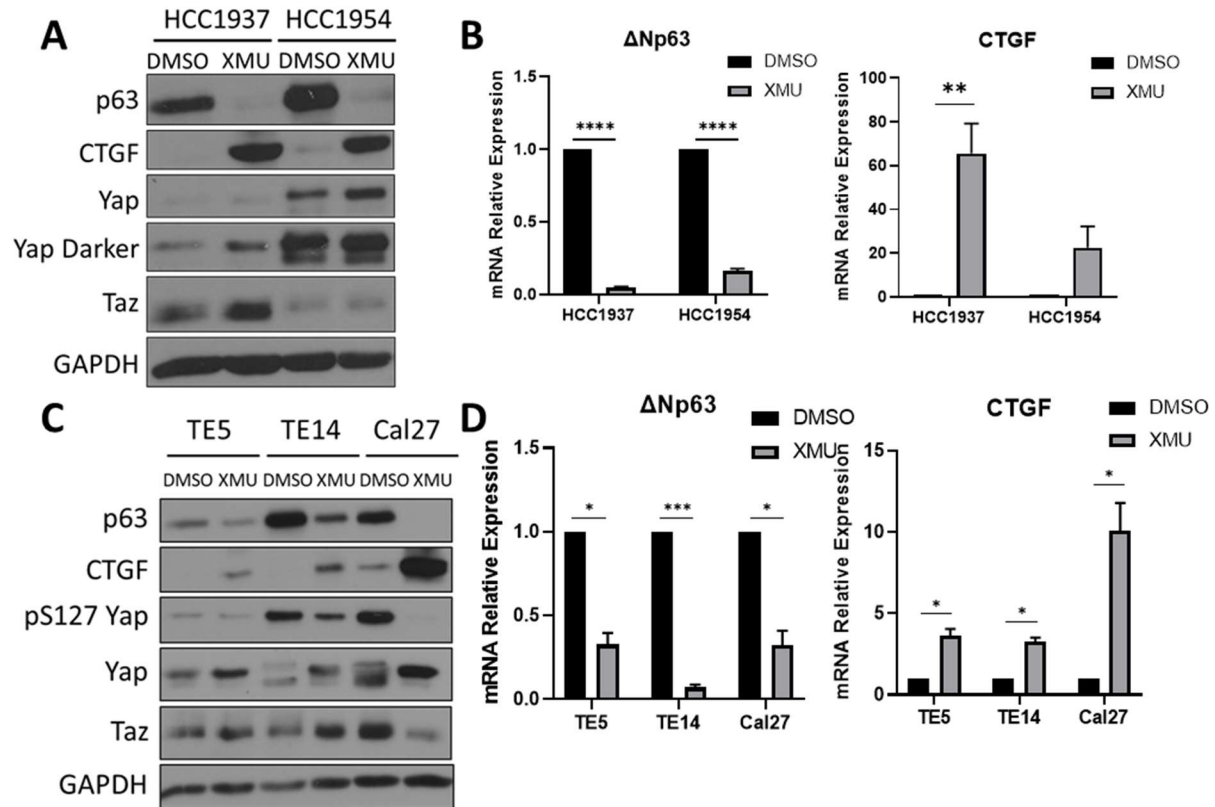


Figure 2-3. MST Regulate p63 Expression in Breast and Squamous Cancer Cell Lines.

A. Immunoblot analysis of HCC1937 and HCC1954 breast cancer cell lines after treatment with DMSO or XMU-MP1 (5 μ M) for 24 h. **B.** RT-qPCR analysis of HCC1937 and HCC1954 cells that had been treated as in A. **C.** Immunoblot of proteins in the TE5, TE14, and Cal27 squamous cancer cell lines treated with XMU-MP1 (5 μ M) for 24 h. **D.** RT-qPCR of squamous cancer cells treated with XMU-MP1 (5 μ M) for 6 h. For the immunoblots, a representative blot of three independent experiments is shown. For RT-qPCR, bars represent the mean \pm SEM of three independent experiments with three technical replicates normalized to the DMSO condition. Statistical analysis was Student t-test.

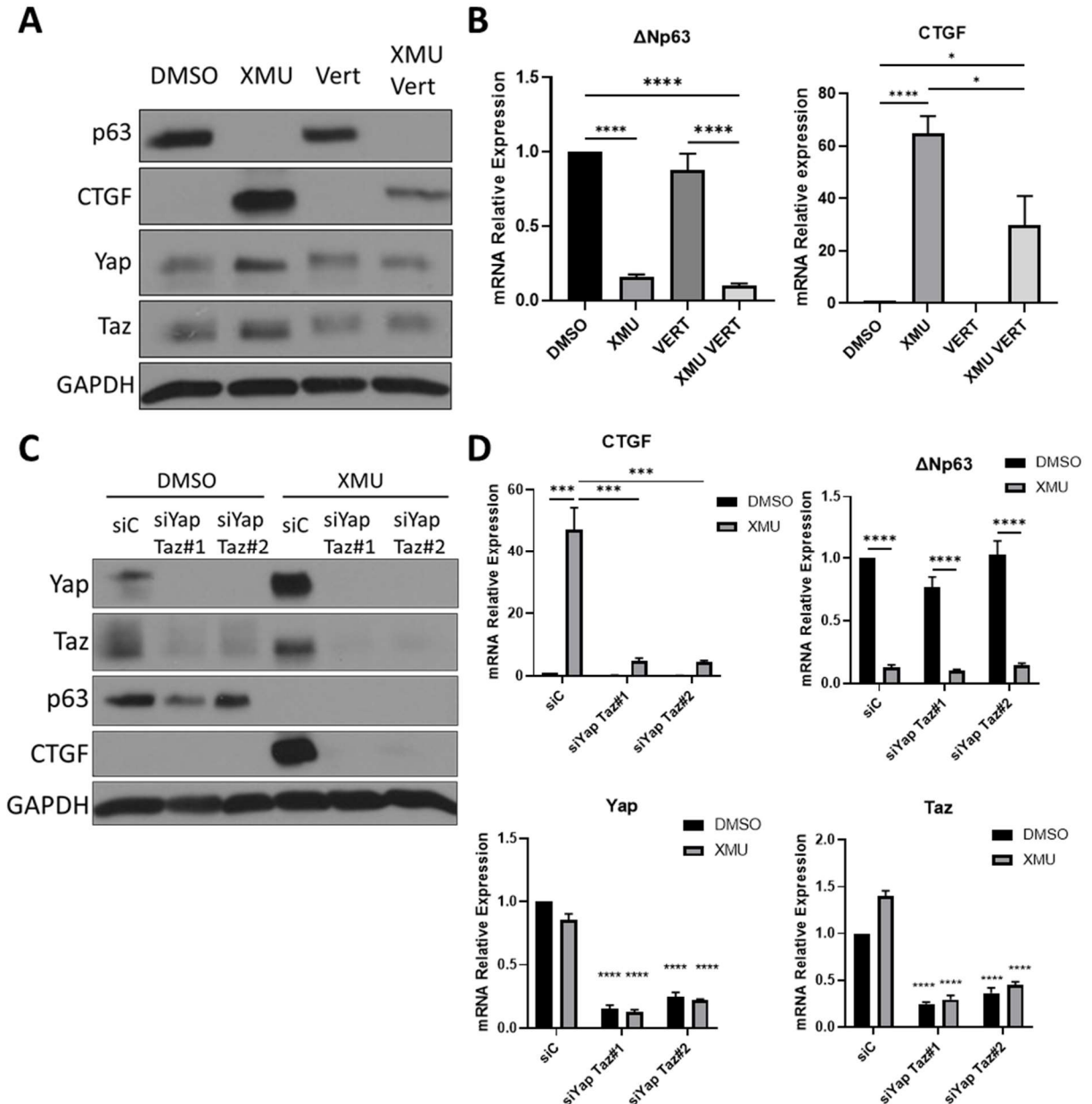


Figure 2-4. Repression of p63 after MST1/2 Inhibition is Independent of Yap and Taz.

A. Immunoblot analysis of MCF10A cells that were treated with DMSO or XMU-MP1 (5 μ M), the Yap/Taz inhibitor Verteporfin (4 μ M), or a combination of both inhibitors for 24 h. **B.** RT-qPCR of MCF10A cells treated as shown in A. Bars represent the mean \pm SEM of four independent experiments with three technical replicates, normalized to DMSO. **C.** Immunoblot of MCF10A cells transfected with the combination two different siRNAs against Yap and Taz for 24 h, then treated with 5 μ M of XMU-MP1 for 24 h. **D.** RT-qPCR

of MCF10A cells transfected with siRNAs against Yap and Taz for 24 h, then treated with 5 μ M of XMU-MP1 for 24 h. The immunoblots in A and C represent four (A) or three (C) independent experiments, respectively. Bars represent the mean \pm SEM of three independent experiments with three technical replicates, normalized to DMSO treated siControl. Statistical analysis was ANOVA with Dunnett's multiple comparisons test.

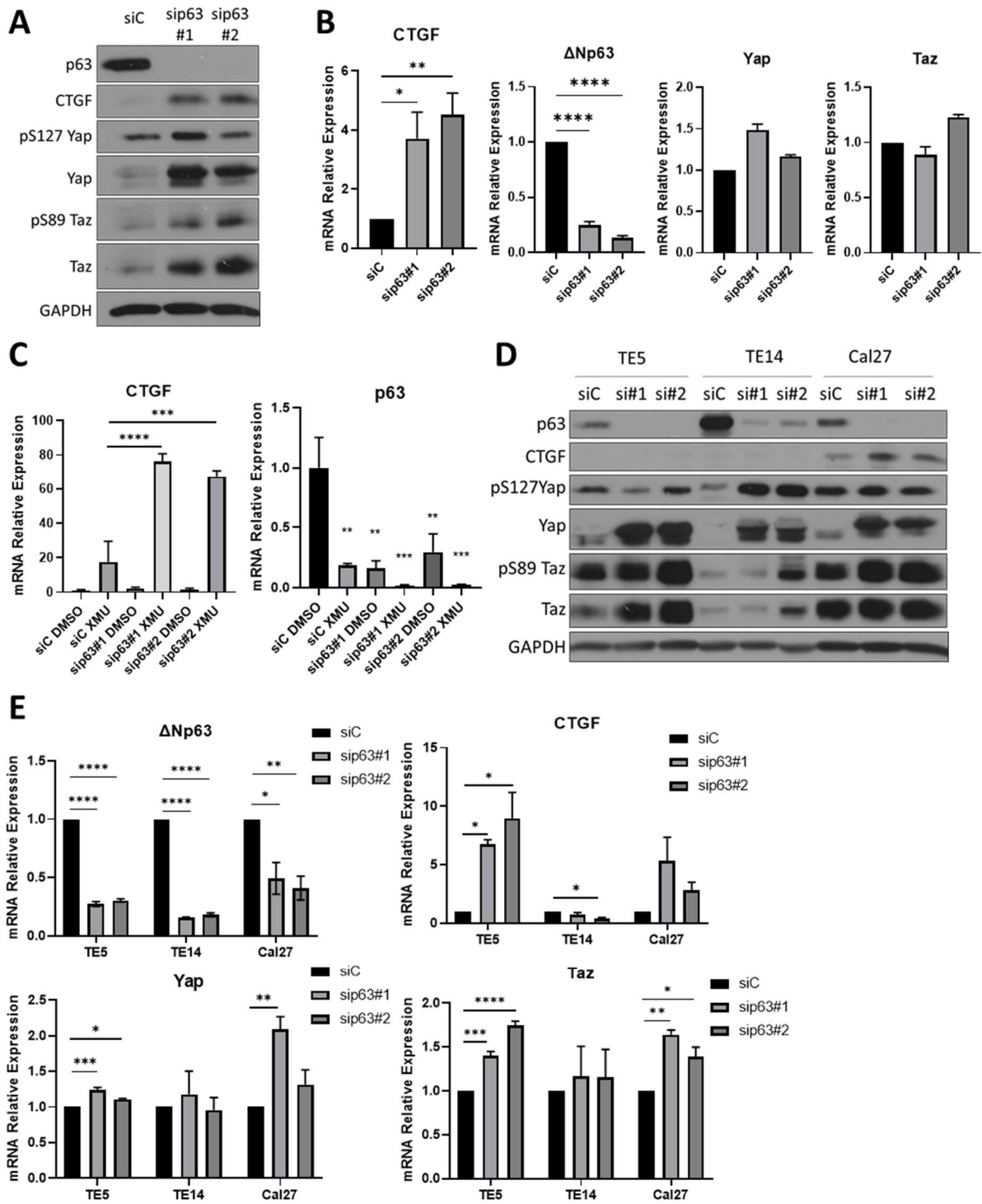


Figure 2-5. p63 Inhibits Yap and Taz Activity.

A. Immunoblot analysis of MCF10A cells that were transfected with control siRNA (siC) or two siRNAs against p63 for 72 h. **B.** RT-qPCR of Δ Np63, CTGF, YAP and TAZ expression in same cells treated as in A. **C.** RT-qPCR analysis of MCF10A cells that were transfected with control siRNA (siC) or two siRNAs against p63 (sip63#1 and sip63#2) for 72 h then treated for 6 h with DMSO or XMU (5 μ M). **D.** Immunoblot analysis of TE5, TE14 and Cal27 SCC cells transfected with siC or two siRNAs against p63 for 72 h as in A. **E.** qRT-PCR of TE5, TE14 and Cal27 cells transfected with siC or two siRNAs against p63 for 72 h as in B. For the immunoblots, a representative of three independent experiments is shown. For RT-qPCR, bars represent the mean \pm SEM of three independent experiments with three technical replicates, normalized to siC. For CTGF in A, four independent experiments were performed. Statistical analysis was One-Way ANOVA with Dunnett's multiple comparisons test.

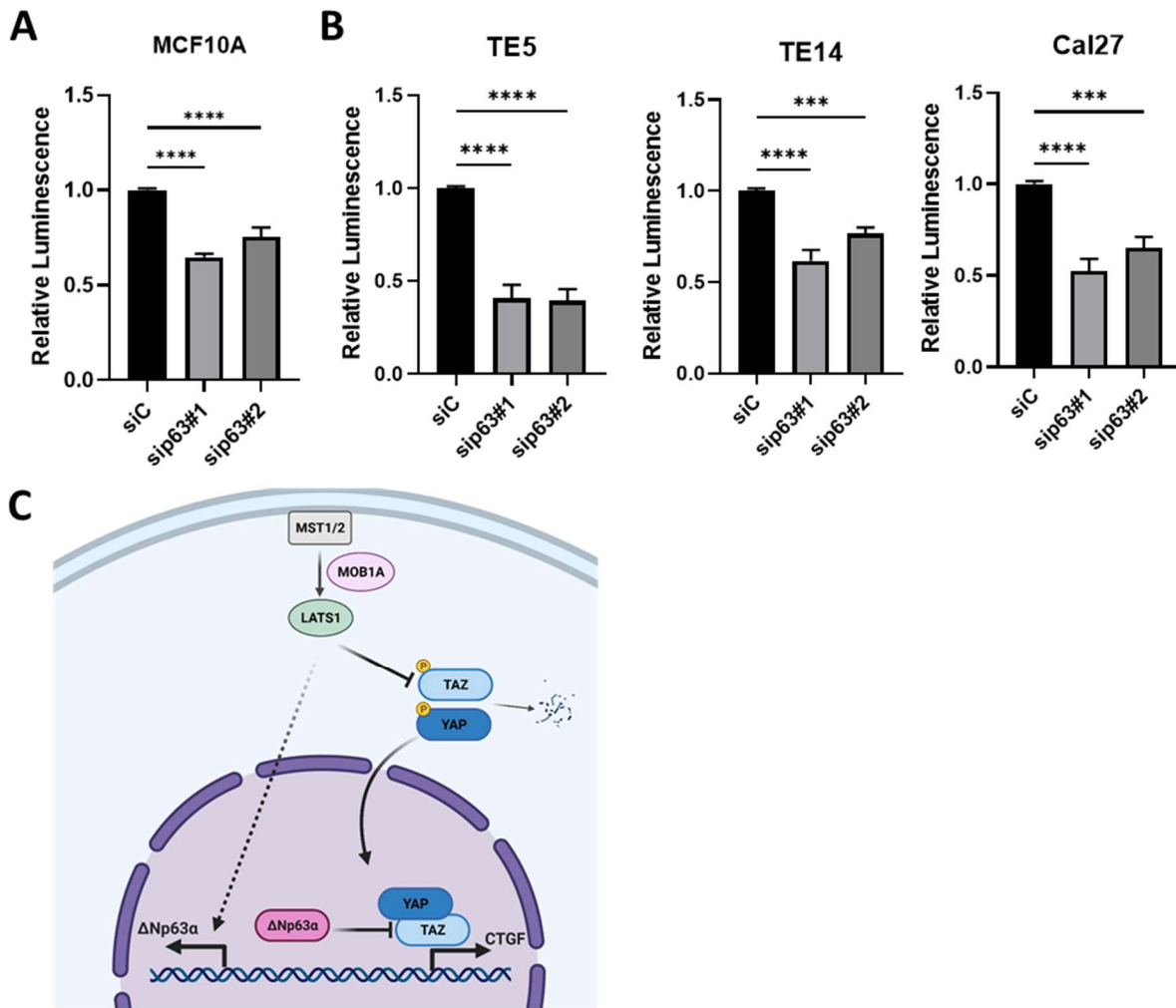
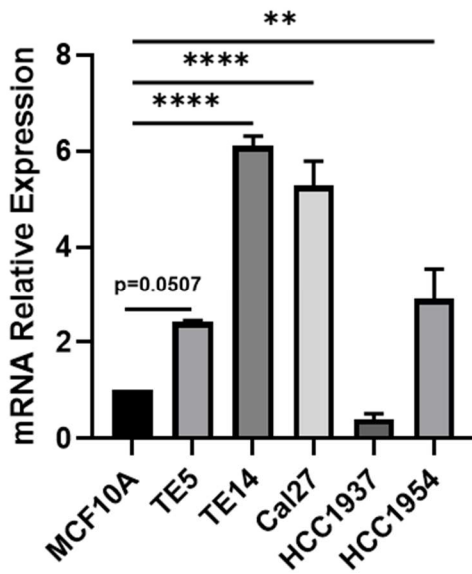


Figure 2-6. p63 Facilitates Growth.

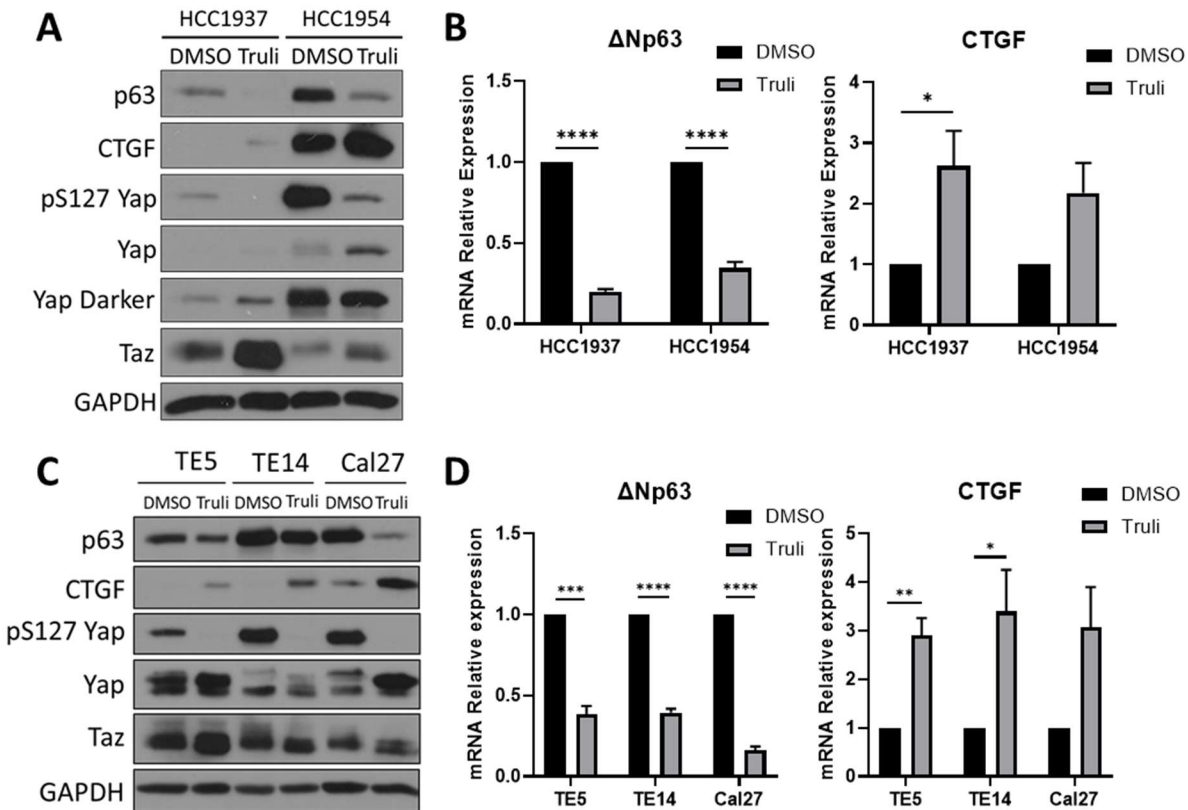
A. Growth of MCF10A cells transfected with siControl (siC) or p63 siRNAs for 72 h. Levels of viable cells were measured with Cell Titer-Glo, as relative luminescence units normalized to the siC. Bars represent the mean \pm SEM of four independent experiments. **B.** Levels of viable TE5, TE14 and Cal27 SCC cells transfected with siC or p63 siRNAs for 96 h measured as in A. Three independent experiments were performed. Bars represent the mean \pm SEM of three independent experiments. Statistical analysis was ANOVA with Dunnett's multiple comparisons test **C.** Model of non-canonical Hippo pathway regulation of p63 and feedback inhibition of YAP and TAZ by p63. MST1 and MST2 via MOB1A and LATS1 maintain

Δ Np63 transcription independent of YAP and TAZ. Δ Np63 is required to maintain low levels of YAP and TAZ and thereby prevent activation of Hippo targets such as CTGF. Created with BioRender.com



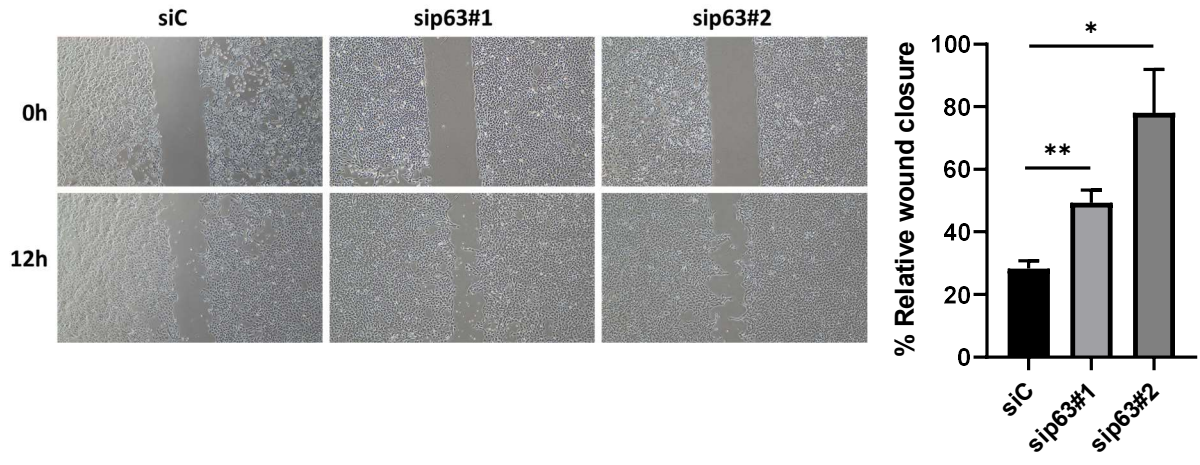
Supplemental Figure S-1. Comparison of Δ Np63 mRNA Expression Across Cell Lines.

The expression of Δ Np63 was determined by qRT-PCR and normalized to MCF10A levels. Bars represent the mean \pm SEM of three independent experiments. Statistical analysis was ANOVA with Dunnett's multiple comparisons test against the levels in the MCF10A cell line.



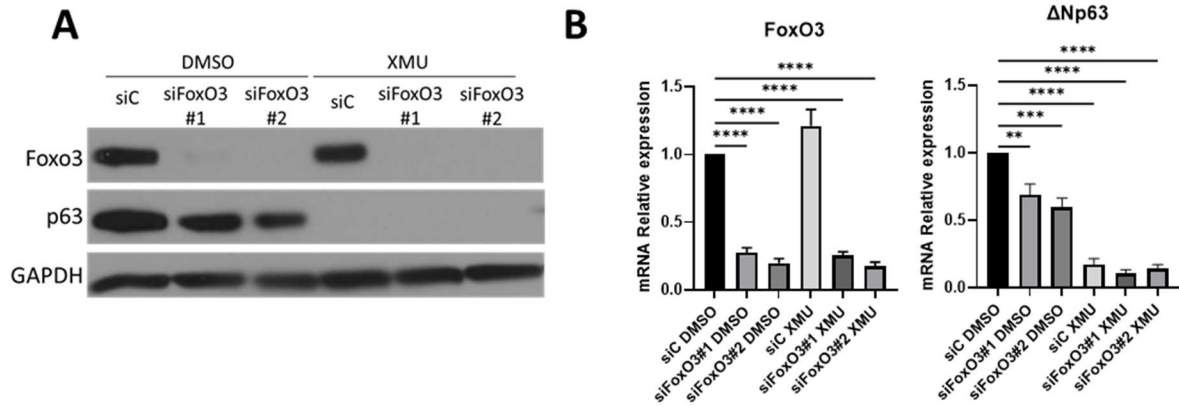
Supplemental Figure S-2. LATS Regulate p63 Expression in Breast and Squamous Cancer Cell Lines.

A. Immunoblot analysis of breast cancer cells in A that were treated with Truli (20 μM) for 24 h. **B.** RT-qPCR of breast cancer cells treated as shown in A. **C.** Immunoblot of squamous cancer cells treated with Truli (20 μM) for 24 h. **D.** RT-qPCR of squamous cancer cells treated as shown in C. For the immunoblots, a representative blot of three independent experiments is shown. For RT-qPCR, bars represent the mean ± SEM of three independent experiments with three technical replicates normalized to the DMSO condition. Statistical analysis was Student t-test.



Supplemental Figure S-3. p63 Inhibits Cell Migration.

Wound scratch assay of MCF10A cells that were transfected with siC or sip63 siRNAs for 72 h. The percentage of wound closure was calculated as the average of 4 independent experiments. Statistical analysis was by Student's t-test.



Supplemental Figure S-4. FoxO3 is Not Required for ΔNp63 Expression in MCF10A Cells.

A. Immunoblot of MCF10A cells that were transfected with control or two different FoxO3 specific siRNAs for 48 h and then were treated for 24 h with XMU-MP1 (5 μM). **B.** qRT-PCR analysis for ΔNp63 and FoxO3 expression treated as in A. Bars represent the mean ± SEM of three independent experiments with three technical replicates.

Supplemental Table 1. Primer Sequences

Gene	Forward sequence	Reverse sequence
RPL32	TTCCTGGTCCACAACGTCAAG	TGTGAGCGATCTCGGCAC
CTGF	GGAGTGGGTGTGTGACGAG	CTTCCAGTCGGTAAGCCGC
Δ Np63	AAACAATGCCAGACTCAATTT	GTGGTCTGTGTTATAGGGACTG
MST1	CAATGCAGAGGATGAGGAAGAG	TCTTGCCAAAGCTGTTGATCT
MST2	GCATGAACCCTTCCCTATGTC	AACCGCATCTGTAGTTCTTCTAAA
LATS1	AAATCTCATCAGCAGCGTCTAC	CTCATTTGATCCTGGGCATCTT
LATS2	TGGTCACATTAAACTCACAGATTTT	GTTAGACACATCATCCCAGAGG
SAV1	TCTGGTACCTGCAAATCCATATC	GGAAGAGTTCCCACTTCAGAAT
MOB1A	CCCGTGAGCTAAGGACGGT	AGGAAGCTCATCTTCGGTCC
MOB1B	GTCTCCTGTTCCATTTCGCCT	AAGAAGCTCATGTTGGCCGC
YAP	TTTTGGCTGCCACCAAGCTA	AATTCAGTCTGCCTGAGGGC
WWTR1 (Taz)	GGAGCTCATGAGGCAGGAAG	CCTGAACTGGGGCAAGAGTC
FOXO3	GGGCAAAGCAGACCCTCAA	GTCCACTTGCTGAGAGCAGA

Chapter 3: p63 regulates the acinar architecture in mammary epithelial cell 3D culture model

3.1 Introduction

The mammary gland epithelium consists of two cellular types, luminal and basal cells. The luminal cells are polarized and surround a central lumen, the basal cells or myoepithelial cells are located adjacent to the luminal cells in contact with the basement membrane and the stroma (Inman et al. 2015; Visvader and Stingl 2014). The basal and luminal cells are organized in branching ducts that end in secretory alveoli (Visvader and Stingl 2014). In normal breast tissue, the $\Delta Np63$ transcription factor is present in myoepithelial cells, forming a single and continuous layer in direct contact with the stroma, that at the same time supports the luminal cells (Ribeiro-Silva et al. 2003). $\Delta Np63$ positive cells co-stain with α -Smooth Muscle Actin a marker of myoepithelial cells, and with basal keratins (KRT) 5 and 14. Also myoepithelial cells are negative for KRT 7 and 8 which are proteins present in luminal cells (Ribeiro-Silva et al. 2003; Wang et al. 2002). Among this $\Delta Np63$ positive population of cells, a bipotent progenitor exists that can reconstitute the full mammary epithelium (Prater et al. 2014; Rios et al. 2014; Shackleton et al. 2006; Stingl et al. 2006). This bipotent progenitor has a basal cell phenotype and has the cell surface markers $CD49^{hi}$ (Integrin $\alpha 6$, a well-known p63 target gene, Carroll et al. 2006), $CD29^{hi}$ (Integrin $\beta 1$), $CD24^{+/mod}$ (sialoglycoprotein) and $Sca1^{low}$ (Stem cells antigen-1) (Shackleton et al. 2006; Shehata et al. 2012; Stingl et al. 2006).

The $\Delta Np63$ gene belongs to the p53 family of transcription factors, and is required for the proliferation of epithelial cells, the expression of basal cell genes, and for the expression of genes from multiple signalling pathways involved in mammary epithelial cell proliferation and cell differentiation (Romano et al. 2009; Nguyen et al. 2006; Memmi et al. 2015; Chakrabarti et al. 2014; Lindley et al. 2015). P63's important role in proliferation and epithelial cell differentiation is strongly evidenced in p63 null mice that have limb and craniofacial malformations, lack mammary glands and die perinatally of dehydration due to lack of skin (Mills et al. 1999; Yang et al. 1999). The p63 transcription factor regulates an adhesion

molecules transcriptional program in the human MCF10A cell line, this program includes genes such as Integrins $\alpha 6$ and $\beta 1$ as well as extracellular matrix proteins like fibronectin and collagen (Carroll et al. 2006). As previously shown by our lab and others, ablation of $\Delta Np63$ in the mammary epithelial cell line MCF10A induced an Epithelial to Mesenchymal Transition (EMT) (Yoh et al. 2016; Hu et al. 2017). EMT is the morphological and transcriptional alteration that causes changes in cytoskeletal organization, loss of cell-cell adhesion molecules, changes in cell morphology to be like mesenchymal spindles, and their apicobasal polarity is replaced by front-rear polarity (Shibue and Weinberg 2017). These changes increase their motility and their ability to degrade and reorganize the extracellular matrix, allowing for the invasion of neighboring tissues (Shibue and Weinberg 2017).

Luminal cells are positive for the transcription factor GATA3 (GATA Binding Protein 3) and the luminal Keratins 7, 8 and 18 (Cimpean et al. 2008; Usary et al. 2004). GATA3 is present in the luminal cells of the mammary gland and is absent from myoepithelial cells (Kouros-Mehr et al. 2006). Mice with GATA3 deletions have defects in the development of the ductal tree. The ducts cannot invade the stroma which means they lack the typical branching morphology, and the ductal tree lacks luminal epithelial cells (Kouros-Mehr et al. 2006). These ductal trees have over proliferation of undifferentiated luminal cells that lack the epithelial differentiation markers like E-Cadherin, Estrogen receptor- α , β -Casein, and KRT18 (Asselin-Labat et al. 2007; Kouros-Mehr et al. 2006). In mice lacking GATA3, the luminal cells are lost due to cell detachment and Caspase-3 induced apoptosis (Kouros-Mehr et al. 2006).

The laminin enriched extracellular matrix (Matrigel) 3D culture system has been used to evaluate the *in vitro* architecture of mammary ducts to model the behavior of tumorigenic and non-tumorigenic mammary epithelial cell lines (Kenny et al. 2007; Lee et al. 2007). This architecture reflects the gene expression of the cell line since different invasiveness morphologies cluster together based on their gene expression profiles (Kenny et al. 2007). In addition, signal transduction pathways due to the extracellular matrix in 3D are integrated differently in ways that cannot be observed in monolayer cultures (Kenny et al. 2007). The MCF10A a cell line has been used extensively to model the mammary architecture in 3D, is derived from a mastectomy of a 36-year-old woman with non-malignant fibrocystic disease (Soule et al.

1990). The immortalized MCF10A cells lack amplification, rearrangement, or mutational activation of HER-2 or Ras, and their karyotype is diploid (Soule et al. 1990). In 3D culture on top of matrigel, MCF10A cells form growth-arrested, single-layered, rounded acinar structures that develop a hollow lumen after 10 days of culture on top of Matrigel (Debnath et al. 2002). These spheres mimic the formation of mammary ducts. Hence, this system can be used to identify molecular mechanisms involved in mammary duct formation and the disruption of the mammary epithelium which occurs during early tumor progression (Shore and Rosen 2014).

We aim with this project to study the role of Δ Np63 in the mammary epithelial cells under more physiological conditions using the 3D culture model in which epithelial cells interact in 3 dimensions with extracellular matrix components. This system can also be used to study how these cellular interactions affect the expression of p63.

3.2 Materials and Methods

Cell culture

MCF10A cells (immortalized non-transformed mammary epithelial) were a gift from David Weber, University of Maryland School of Medicine, Baltimore, were grown in DMEM/F12 (Thermo Fisher Scientific, 11320033) with 5% horse serum (Thermo Fisher Scientific, 16050122), penicillin-streptomycin solution (Thermo Fisher Scientific, 15140122), 10 μ g/mL insulin (Sigma-Aldrich, 91077C), 0.5 μ g/mL hydrocortisone (Sigma-Aldrich, H0888) and 20 ng/mL Epidermal Growth Factor (PeproTech, AF-100-15). MCF10A CRISPR p53 Knock out (KO) clones were generated using CRISPR/Cas9 genome editing as previously described (Venkatesh et al. 2020). MCF10A stable cell Vector-shControl and sh Δ Np63 were described previously (Yoh et al. 2016). HEK293T cells were grown in DMEM supplemented with 10% fetal bovine serum. Cells were detached with trypsin-EDTA (0.05%) (Thermo Fisher Scientific, 15400054) and counted before seeding for experiments using trypan blue in a Countess 3 Automated Cell Counter (Thermo Fisher Scientific). All cell lines were grown at 37°C with 5% CO₂. Cholera Toxin from *Vibrio Cholerae* (C8052,

Sigma Aldrich) and Doxycycline (D9891, Sigma Aldrich) was added to the growth media as indicated in the results and figure legends.

Retroviral constructs and stable cell lines

The pCW-3xflag- Δ Np63 α plasmid was cloned as follows: pCW-MECOM2 was digested with NheI and BamHI and ligated to a PCR fragment of 3XFlag- Δ Np63 from pBabePuro-3XFlag- Δ Np63. The PCR primers had NheI and BamHI ends and the PCR fragment was digested with NheI and BamHI and ligated to the pCW fragment to make pCW-3XFlag- Δ Np63 α . pCW-MECOM2 vector is a derivative of pCW-57.1. The 3XFlag- Δ Np63 α is an all-in-one lentiviral vector expressing Δ Np63 α under a Doxycycline inducible promoter with a puromycin resistance gene and the tet activator rtTA under control of a PGK (3-phosphoglycerate kinase) promoter. HEK293T cells were seeded in 10 cm dishes 3×10^6 per plate, 24 h later cells were transfected with Lipofectamine™ 2000 (Thermo Fisher Scientific, 11668019) with 5 μ g of empty vector plasmid pCW57.1 (a gift from David Root, Addgene, plasmid 41393) or pCW-3xflag- Δ Np63 α and the lentivirus and 5 μ g of each packaging plasmid pSPAX2 (Addgene plasmid 12259) and pMD2.G (Addgene, plasmid 12260) (gifts from Didier Trono). Media was replaced after 24 h and the virus was harvested between 48-72 h post-transfection. MCF10A cells were transduced with 8 μ g/ml of polybrene (EMD Millipore, TR-1003-G) and freshly harvested virus filtered with 0.45 μ m filter. MCF10A cells were selected 48 h post-infection with 2 μ g/ml of puromycin and maintained with 1 μ g/ml (Invivogen, ant-pr-1). Doxycycline (D9891, Sigma Aldrich) was added to the growth media to induce gene expression as indicated in the results and figure legends.

siRNA transfection

For siRNA transfections 1.5×10^5 cells were plated in 6 well plates, 24 h later 17 nM of each siRNA was transfected with Lipofectamine™ RNAiMAX (Thermo Fisher Scientific, 13778150) following the manufacturer's instructions. The following siRNAs were used siC (Silencer™ Select Negative Control No. 1, Thermo Fisher Scientific, 4390843), siGata3#1 (Thermo Fisher Scientific, 4427037, ID s5600), siGata3#2 (Thermo Fisher Scientific, 44427037, ID s5601), siZeb1#1 (Thermo Fisher Scientific, 4392420, ID s229971), siZeb1#2 (Thermo Fisher Scientific, 4392420, ID s19032), siZeb2#1 (Thermo Fisher

Scientific, 4392420, ID s5261), siZeb2#2 (Thermo Fisher Scientific, 4427037, s19033). Cells were harvested 72 h post-transfection and lysed in RIPA buffer following the protocol described below.

3D cell cultures

Following the manufacturer's instructions and based on the published protocol (Debnath et al. 2002), 8 well chamber slides and 12 well plates were covered with 20 μ L and 100 μ L of Matrigel® Growth Factor Reduced Phenol Red Free (Corning, 356231) respectively, and placed in a 37°C incubator for 15 min. MCF10A cells were detached with Trypsin and counted with trypan blue with the Countess automatic cell counter (Thermo Scientific). 4×10^3 cells/well were seeded in 8 well chamber slides, 1.5×10^5 cells were seeded in 12 well plates in triplicate on top of Matrigel®. MCF10A cells were plated in assay medium as described previously (Debnath et al. 2002) supplemented with 2% Matrigel®. Media was replaced with fresh medium with Matrigel® every day for 4 days. On day 12, the cells on 8 well chambers were fixed for immunofluorescence and the cells seeded on the 12 well plates were harvested following the immunoblot protocol below.

Immunoblot

Cells seeded in 2D in 6 wells were washed in ice-cold PBS 1X, then lysed on ice in 80 μ L of RIPA buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS), and 1% NP40) supplemented with fresh protease inhibitors (3 μ g/mL Leupeptin, 1 μ g/mL macroglobulin, 1 μ M benzamidine, 0.5 μ M of PMSF) and phosphatase Inhibitors (EMD Millipore, 524625). Cells grown in 3D in 12 well plates, were harvested with 500 μ L/well of Corning Cell Recovery Solution (Thermo Fisher Scientific, CB-40253), incubated for 20 min on ice, followed by centrifugation at 4000 rpm for 5 min at 4°C. Pellets from 3D cultures were washed twice with Cell Recovery Solution and lysed with RIPA buffer with inhibitors. Cell lysates were incubated for 10 minutes on ice and centrifuged at 13000 rpm for 10 minutes. The protein concentration of each sample supernatant was measured with Bradford protein assay dye reagent (Bio-Rad, 5000006). Equivalent amounts of protein across samples were mixed with Laemlli buffer (100mM Tris-HCl pH 6.8, 2% SDS, 5% 2-Mercaptoethanol, 5% glycerol) and incubated at 95°C for 5min. Lysates (35 μ g protein) were loaded on each lane in a 10% SDS-polyacrylamide gel;

samples were separated at 120 V and transferred to a nitrocellulose membrane (Bio-Rad, 1620115). After blocking for 20 min with 5% nonfat dried milk in PBS with 0.05% Tween-20 (Sigma-Aldrich, P1379) (PBST), the membrane was incubated with primary antibodies diluted in 0.1% milk in PBST at 4°C overnight. The following primary antibodies from Cell Signaling Technologies were used: p63 α (1:1000, 4892S), GAPDH (1:10000, 5174), GATA3 (1: 1000, 5852S), Bim (1: 1000, 2933S), N-Cadherin (1: 1000, 13116S), Zeb1 (1: 500, 70512T). In addition, the primary antibodies to the following proteins were used: Zeb2 (1: 500, Abcam, ab138222), E-cadherin (1:500 Santa Cruz sc-8426), p63-4A4 (1:500, Biocare Medical, CM163A), KRT8 (1: 1000, Invitrogen, PA5-29607), KRT14 (1: 1000, Abcam ab7800), KRT5 (1:1000, Abcam ab52635), Fibronectin (1: 1000, Sigma-Aldrich, F3648), Vimentin (1: 1000, Abcam ab8978), E-Cadherin (1:1000, Santa cruz sc-8426) and Flag-M2 (1:1000 Sigma-Aldrich A2220). The secondary antibodies peroxidase-conjugated anti-Rabbit IgG (Sigma-Aldrich, A6154) and anti-mouse IgG (Sigma-Aldrich, A4416), were incubated for 1 h at room temperature at a 1:5000 in 0.5% milk PBST. Membranes were imaged with Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific, 322106) or Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500).

Immunofluorescence

At day 12 cells plated in 8 well chamber slides were washed with PBS and fixed in 4% Paraformaldehyde, followed by 3 washes with PBS. Next, cells were incubated in 3% Triton X-100 with 10% goat serum (EMD Millipore-Sigma S26) and 1% Bovine Serum Albumin in PBS with 0.1% tween (PBST) shaking for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C KRT8 (1:50 Thermo Fisher Scientific PA528985), KRT14 (1:100 Abcam Ab780), p63 4A4 (1:50 Biocare CM163A) GATA3 (1:20 cell signaling 5852S), Flag-M2 (1:50 Sigma-Aldrich A2220) in 5% goat serum in PBST, followed by 3 washes with PBST. Secondary antibodies Mouse Alexa fluor 594 (Thermo Scientific, A-11005) and Anti-Rabbit Alexa Fluor 488 (Thermo Scientific, A-11034) were incubated for 2 h shaking with 10% goat serum and 1% Bovine Serum Albumin in PBST for 2 h at room temperature followed by 3 washes with PBST and 1 quick wash with PBS followed by mounting in Vectashield with DAPI (Vector Laboratories, H-1200). Samples were stored at 4°C until imaging them in a confocal microscope Zeiss LSM 700. Images were taken in Z-stack mode with crosssections every 4 μ m from top to bottom of each acinar structure.

3.3 Results

MCF10A with filled lumens have high GATA3 and low BIM expression

In this project we aimed to evaluate the role of Δ Np63 in human mammary tissue architecture using the immortalized non-tumorigenic human mammary epithelial cell line MCF10A. This cell line forms spherical, single-layered hollow acinar structures when grown on Matrigel in 3D culture conditions (Debnath et al. 2002). However, we found that the MCF10A cell line grown in the lab had partially filled lumens (Figure 3-1 A; MCF10A cell line B). To test whether there was a problem with the 3D culture technique, seeded at the same time MCF10A cells from Dr. Joan Brugge, the cell line originally used to describe the 3D hollow lumen model (called from now on MCF10A cell line A) (Debnath et al. 2002). In contrast to our cell line, this cell line A formed acini with only one layer of cells and hollow lumen after 10 days in 3D culture (Figure 3-1 A). Both cell lines were grown under the same conditions and plated at the same time (Figure 3-1 A). Due to this striking difference, we sent the cell lines for verification and genotyping by ATCC. The results showed that both cell lines were genotypically identical and matched the MCF10A reference cell line from ATCC and did not have contamination with other cell lines.

MCF10A has properties of myoepithelial cells and expresses basal keratins KRT 5 and 14 and at the same time express KRTs 7, 8, and 18 common to luminal breast cells (Paine et al. 1992). This dual keratin phenotype suggests that MCF10A cells have a progenitor or stem-cell phenotype (Paine et al. 1992). In addition, studies in mice showed that myoepithelial cells have stem cell characteristics with the potential to reconstitute the entire ductal tree from one type of cell (Prater et al. 2014). We therefore evaluated whether the acinar structures we observed in MCF10A cell line B were due to the myoepithelial cells undergoing differentiation induced by the 3D culture conditions. To test this, we performed immunostaining for luminal and basal Keratin (KRT) proteins by confocal microscopy of 12-day 3D cultures. We found that the MCF10A B cells formed rounded acinar structures that are partially filled with cells (Figure 3-1 B). More surprising, they have a similar structure to the human breast ductal tissue, with an external layer of myoepithelial cells positive for the myoepithelial KRT14 protein and an internal layer of epithelial cells positive for luminal KRT8 protein (Figure 3-1 B).

To evaluate when the luminal cells appear, we did a time-course experiment, every 4 days until 12 days 3D cultures were fixed and stained for KRT8 (luminal) and 14 (basal). We found that cell line A was hollow at day 12 and expressed weaker levels of KRT14 and only KRT8 in some cells in the lumen at day 8 and 12, while Δ Np63 was expressed throughout (Figure 3-1 C and D). In contrast, at day 4 cell line B (never hollow) had basal-like cells that expressed KRT14 and Δ Np63. At later times two layers of cells were clearly apparent, the external layer was positive of KRT14 and Δ Np63 demonstrating a basal-like phenotype, while the internal layer was positive for KRT8 consistent with a luminal phenotype (Figure 3-1 C). At day 12 the cells in the basal layer were positive for Δ Np63 with an internal (or second layer of cells) that is positive for the luminal transcription factor GATA3 (seen as green staining) further suggesting that this layer of cells is luminal-like (Figure 3-1 D). The basal cells of the first outer layer are also positive for GATA3 (Figure 3-1 D). The transcription factor GATA3 is required for luminal differentiation of cells in the mammary gland, for the formation of the ductal tree, and expression of luminal cell genes Estrogen Receptor and KRT18 (Asselin-Labat et al. 2007). Together these results show that the MCF10A line B forms acini similar to the normal ductal structure containing an external layer of basal myoepithelial cells (KRT14 and Δ Np63 positive) and an internal layer of luminal cells (KRT8 and GATA3 positive).

The cells in the center of the acini have been reported to die by apoptosis (Debnath et al. 2002) and indeed we found that the MCF10A line A cells were hollow suggesting that cells at the center had undergone apoptosis. With MCF10A line B we found that cells remained at the center of the acini and stained positive for GATA3 (Figure 3-1 D). This suggests that with this cell line and conditions, the apoptosis in the lumen is less efficient and instead the differentiation of the basal and luminal layers occurs.

Studies in MCF10A 3D cultures demonstrated that the lumen clearing is induced by apoptosis mediated by the BIM protein (Reginato et al. 2003). To test if differences in BIM induction account for the defect in lumen clearing between cell line A and B, we collected cells at different time points growing in 3D and evaluated by immunoblot the expression of Δ Np63 GATA3 and BIM. No differences in the levels of Δ Np63 were found overtime in any of the cell lines (Figure 3-1 E). However, we observed big differences in the expression of GATA3 and the proapoptotic protein BIM, the hollow cell line A expressed high levels

of BIM as early as day 4 and decreased at day 12 and 16, consistent with the clearing of the lumen at day 10 (Figure 3-1 E). In contrast, the non-hollow cell line B had very low levels of BIM that peaked at day 8. It also had higher levels of GATA3 protein that decreased slightly at day 12 and 16, compared to cell line A (that gave hollow lumen) and that had extremely low levels of GATA3 (Figure 3-1 E). Thus, these results suggest that cell line B has impaired lumen clearing shown as lack of BIM expression. The difference in GATA3 may also contribute to the lack of lumen clearance as it allows the survival and prevents the detachment of mammary cells in the lumen as has been shown *in vivo* (Kouros-Mehr et al. 2006).

Additional studies on the induction of hollow lumen demonstrated that increased levels of cyclic Adenosine Monophosphate (cAMP) are responsible for apoptosis in the lumen of the acini in MCF10A (Debnath et al. 2002; Nedvetsky et al. 2012). Treatments with Forskolin, Cholera Toxin, and cAMP, activators of protein kinase A (PKA), induce apoptosis and hollow lumen formation in MCF10A cells (Nedvetsky et al. 2012). To test if the lack of cyclic AMP signaling was the cause of lumen filling, the MCF10A cell line B was treated with 100 ng/ml of cholera toxin for 10 days and immunofluorescence was done for basal and luminal KRT markers. We found that upon treatment with cholera toxin the lumen was partially hollow as seen by DAPI staining with some KRT8 positive cells (Figure 3-1 F). This effect was not observed in all the acinar structures. The partial effect on lumen clearing suggests that PKA and cyclic AMP may control the formation of hollow lumen. Further studies are required with other activators of PKA, such as Forskolin, to confirm this as well as knockdown experiments.

p63 is required to maintain the acinar structure of MCF10A cells in 3D culture

Interestingly, despite that the MCF10A cell line B do not cleared the lumen, it has a similar structure to the mammary gland tissue with two cells types expressing basal and luminal proteins. To test the role of Δ Np63 in the differentiation of these cell types, we grew a stable cell line expressing shRNA against Δ Np63 in 3D. It was previously described by our lab that the loss of Δ Np63 induces EMT in 2D (Yoh et al. 2016). As expected by the EMT result, the cells could not form acinar rounded structures compared to the shRNA control (Vector) in 3D (Figure 3-2 A). Instead, the sh Δ Np63 formed disorganized tubular structures, possibly formed by the loss of cell adhesion induced by EMT (Figure 3-2 A). EMT was confirmed by immunoblots

for the expression of mesenchymal proteins Zeb1, Vimentin, N-Cadherin, and lack of E-cadherin (Figure 3-2B). The disorganized tubular structures observed in the sh Δ Np63 cell line also expressed very low levels of basal KRT14 and KRT5 and luminal KRT8 (Figure 3-2 D, E). Interestingly, the expression of the luminal transcription factor GATA3 was increased after the ablation of Δ Np63 when grown in 3D, but not 2D (Figure 3-2 B, D). However, there was not a specific area of cells positive for GATA3 (Figure 3-2 C). The change in the MCF10A shp63 cells grown in 3D is not the same as the differentiation to luminal cells since the KRT8 marker is expressed at very low levels in the shp63 cells (Figure 3-2 D, E). However, these results suggest that p63 is required for basal KRT expression as well as differentiation of the cells into luminal-like cells in 3D. Since the shp63 cells have undergone EMT (Yoh et al. 2016), these results also suggest that mesenchymal cells cannot form acini.

Δ Np63 α represses GATA3 and the luminal phenotype in 3D

Since the acinar structures formed with the MCF10A B cell line contain both basal and luminal layers, this gives us the opportunity to test whether Δ Np63, which is normally expressed in the basal layer alters the formation of the luminal layer. For this purpose, we used a Doxycycline inducible retroviral system to overexpress 3xflag- Δ Np63 α in a stable cell line.

Initially, we confirmed the overexpression of p63 by immunoblot and immunofluorescence with Flag and p63 α antibodies after treatment with doxycycline for 24 h (Figure 3-3 A, B). In 3D, the overexpression of p63 for 12 days did not change the morphology nor the size of the acini in 3D cultures (Figure 3-3 C). However, the immunofluorescence for GATA3 (as a luminal marker) and Flag (for p63) revealed that the overexpression of Δ Np63 α caused the suppression of GATA3 in the luminal cells compared to the control and no effect on hollow lumen was observed (Figure 3-3 D). Immunoblotting of the cells grown in 2D and 3D revealed a downregulation of GATA3 protein upon Δ Np63 α overexpression in 3D while GATA3 expression in 2D was undetectable (Figure 3-3 E). This suggests that Δ Np63 α suppresses the luminal differentiation that occurs in 3D. As Δ Np63 α is normally expressed in the basal myoepithelial cells, but not the luminal cells of the mammary ducts, these results suggest that the repression of Δ Np63 α expression is critical for the proper luminal differentiation in 3D.

Δ Np63 partially rescues EMT

Since Mesenchymal to Epithelial Transition (MET) has been reported to be an important step after EMT in the progression of metastases (Ocaña et al. 2012; Stankic et al. 2013; Tsai et al. 2012), we tested whether EMT induced by ablation of Δ Np63 α is reversible by restoring the Δ Np63 α expression to rescue EMT induced by Δ Np63 loss. We used an shp63 stable cell that overexpressed Δ Np63 α under a Doxycycline inducible promoter. Cells with the 3xflag Δ Np63 α construct or its control vector were grown in 3D for 12 days with or without 0.1 μ g/ml of Doxycycline. First, the overexpression Δ Np63 α in the sh Δ Np63 stable cell line was confirmed by immunoblot of cells grown in 3D (Figure 3-4 A). The overexpression Δ Np63 α for 12 days induced the repression of the mesenchymal proteins Fibronectin, N-Cadherin, and Zeb1, but induced no change in E-Cadherin or vimentin proteins. As a positive control, the expression of the Δ Np63 α target gene, Integrin- α 6 was confirmed (Carroll et al. 2006) (Figure 3-4 A). In addition, the overexpression of Δ Np63 α downregulated the luminal transcription factor GATA3. Despite the changes observed in the expression of mesenchymal proteins, the overexpression of Δ Np63 α was not enough to rescue the disorganized structures induced by EMT upon ablation of p63 (Figure 3-4 B). This suggest that the induction of the EMT program is not reversible by p63 expression.

Decreased mesenchymal protein expression suggests that Δ Np63 α can be a partial suppressor of EMT as well as a repressor of GATA3. The increase in GATA3 observed upon p63 loss (Figure 3-2 D) suggests GATA3 as a possible regulator of EMT. To test this hypothesis GATA3 expression was ablated with siRNA for 72h in 2D. Despite the successful knockdown no effect was observed on the expression of the mesenchymal proteins Zeb1, N-Cadherin, E-Cadherin, or vimentin (Figure 3-4 C). This indicates that high levels GATA3 are not sufficient to cause EMT nor enough to reverse the EMT caused by p63 knockdown (Figure 3-4 C). Thus, our data indicate that Δ Np63 α can suppress luminal differentiation and partially suppress EMT and Gata3 expression, suggesting that other factors involved in EMT once activated, cannot be directly suppressed by Δ Np63 α or that other factors independent of Δ Np63 α and GATA3 are involved.

To test whether other factors were required for EMT besides the loss of p63, the ZEB1 transcription factor was knocked down for 72h in 2D in the shp63 cell line with and without overexpression of Δ Np63 α with Doxycycline. ZEB1 induces EMT and represses E-cadherin and has also been shown to repress Δ Np63 expression (Chua et al. 2007). The knockdown of ZEB1 with two different siRNAs was not enough to rescue the expression of E-Cadherin and did not impair the expression of the EMT markers, fibronectin, and N-Cadherin (Figure 3-4 D). This indicates that neither the loss of ZEB1 nor GATA3, nor restoring Δ Np63 expression alone are enough to reverse the EMT phenotype.

Previously our lab shows that the loss of Δ Np63 in MCF10A not only increased ZEB1 expression but also ZEB2, both EMT-inducing transcription factors (Yoh et al. 2016). We therefore decided to co-knockdown ZEB1 and ZEB2 with two different siRNAs for 72 h. Interestingly, the double knockdown rescued the expression of E-Cadherin in a Δ Np63 α -independent manner (Figure 3-4 E). This indicates ZEB1 and ZEB2 are indeed repressors of E-cadherin in these cells and that Δ Np63 is not required for the expression of E-Cadherin when ZEB1 and 2 are suppressed (Figure 3-4 E).

In summary, the results above mentioned suggest that Δ Np63 α not only controls the architecture of the acinar structures in 3D but also represses the luminal cell phenotype and at the same time, Δ Np63 α partially rescues impairs the EMT phenotype. The combination of Δ Np63 α overexpression with ablation of ZEB1 and ZEB2 reversed the EMT phenotype strongly. In particular, the loss of ZEB1 and ZEB2 was enough to rescue the expression of E-Cadherin. Interestingly, there was stronger repression of N-cadherin expression in cells with Δ Np63 α expression in combination with ZEB1/2 knockdown (Figure 3-4 E). This suggests that parts of the EMT phenotype may be regulated separately by Δ Np63 α and ZEB1/2 pathways.

3.4 Discussion

The aim of this project was to study in a physiologically relevant system such as matrigel 3D culture, the role of p63 in the regulation of the mammary epithelial architecture, as a tool to model of early tumorigenesis since p63 expression decreases in breast cancers (Figure 1-4). However, our cell line did

not form hollow lumens despite being characterized by ATCC as the same MCF10A reference cell line and discarding any contaminations with other cell lines. Despite those limitations, the model was useful to us to evaluate the effects that p63 has on EMT and luminal differentiation under more physiological conditions, because instead of the hollow lumen we observed luminal differentiation of the remaining cells present in the lumen.

Our results suggest that lack of lumen clearance observed in MCF10A A cell line could be due to low levels of BIM proapoptotic protein and high levels of GATA3 luminal protein. A similar phenotype was observed *in vivo* in mice null for BIM, which have mammary glands with filled lumens (Mailleux et al. 2007). However, further experiments are required to confirm if the lack of lumen clearance is caused by low BIM levels and instead the luminal differentiation with GATA3 occurs. To confirm this knockdown of BIM in the MCF10A cell line A should be evaluated if it could induce a similar phenotype of basal and luminal cell differentiation as the cell line B.

Previous studies demonstrate that hollow lumen is induced by increased cAMP levels, by treatment with forskolin or cholera toxin, known activators of PKA (Debnath et al. 2002; Nedvetsky et al. 2012), suggesting that apoptosis in the lumen requires cAMP/PKA dependent signaling. Multiple attempts to reproduce the cholera toxin-induced hollow lumen result were made (Figure 3-1 F), unfortunately, most were unsuccessful. This suggests that other PKA agonists like forskolin or cAMP analogs need to be tested to confirm the hollow lumen formation capacity in the cell line B.

Apoptosis in the lumen has been associated with the loss of cell attachment (Debnath et al. 2002; Reginato et al. 2003). In addition, anchorage-independent growth in MCF10A is induced by overexpression of phosphomutant YAP 5SA and TAZ 4SA (Zhao et al. 2008; Zhang et al. 2009), suggesting that the Hippo pathway could control anoikis and anchorage-independent growth via inhibition of YAP/TAZ. We hypothesize that active Hippo signaling (MST and LATS) could induce anoikis and lumen clearance in 3D, blocking the GATA3 luminal differentiation. Since loss of GATA3 induced detachment and apoptosis of the luminal cells in the mammary gland *in vivo* (Kouros-Mehr et al. 2006). To test this, first immunoblots of 3D

comparing the activation levels of the core Hippo pathway (LATS1/2, MOB1A/B, MST1/2, YAP, and TAZ) could indicate if MCF10A cell line A (hollow) has more active Hippo pathway compared to cell line B (not hollow). If this is the case, YAP or TAZ knockdown with shRNA, as well as knockdown of GATA3 could induce hollow lumen formation, or impair luminal differentiation. In addition, overexpression of a dominant-negative form of TAZ (without the transactivation domain or a TEAD binding mutant S51A) could inhibit the luminal differentiation (Zhang et al. 2009). If this is the case, the differentiation of the GATA3 positive cells that fill the lumen should be dependent on the transcriptional activity of TAZ and TEAD. If that is the case, knockdown of LATS could inhibit the hollow lumen formation in the MCF10A cell line A, and induce GATA3 luminal differentiation instead.

Activation of PKA with forskolin or increased levels of cAMP with Cholera toxin, induce hollow lumen formation in MCF10A (Nedvetsky et al. 2012). Activation of PKA with forskolin activates LATS (Yu et al. 2012). Actin polymerization, RHOA signaling, and integrin signaling can inhibit LATS (Plouffe et al. 2016). These results suggest that activation of PKA with forskolin and Cholera toxin could induce hollow lumen. To test this hypothesis, treatment of MCF10A B cells with PKA agonist such as Forskolin or cAMP should induce hollow lumen, while LATS knockdown should fill the lumens and induce luminal differentiation. Combined treatment of Forskolin with LATS knockdown should impair hollow lumen formation, if PKA activation of LATS is responsible for the hollow lumen. In addition, LATS can be activated by Merlin (also known as Neurofibromin 2 –NF2) upon disruption of actin cytoskeleton polymerization (Yin et al. 2013). If LATS is required for lumen formation, then overexpression NF2, could induce hollow lumen and impair luminal differentiation by activation of LATS.

Additional evidence that supports the Hippo pathway as a potential regulator of luminal differentiation is that LATS1 is required to maintain the basal phenotype (KRT14 expression) of primary breast epithelial cells (Britschgi et al. 2017) and maintains the expression of p63 in MCF10A (our results described in chapter 2). The loss of LATS1/2 and LATS1 alone induced luminal differentiation of basal primary breast epithelial cells in a YAP/TAZ and Estrogen Receptor α dependent manner (Britschgi et al. 2017). This suggests that LATS1 or LATS1/2 cknockdown could inhibit the hollow lumen formation in the

MCF10A, by induction of GATA3 luminal differentiation. In contrast, LATS activation through PKA activation with cAMP or forskolin, could inhibit luminal differentiation.

Other signaling pathways that have the potential to regulate the hollow lumen and differentiation in 3D culture are those that can induce basal to luminal differentiation and are known regulators of p63 expression, such as the Wnt β -catenin signaling, hedgehog, and notch signaling (mentioned in Signaling pathway regulation of Δ Np63 section 1.3).

Hedgehog signaling in absence of ligand, induce the Gli3 Repressor (Gli3R) by proteolytic cleavage of full length Gli3. Active PKA phosphorylation upon forskolin treatment is required for this cleavage (Wang et al. 2000). Upon activation with the Indian hedgehog ligand, full length Gli3 induced the repression of Δ Np63 and increased expression of TAp63 (Li et al. 2008a). In addition, loss of Ptch1, the negative regulator of Hedgehog signaling, increased expression of GATA3 in mammary epithelial cells *in vivo* (Li et al. 2008a). To test the role of hedgehog signaling in the MCF10A 3D culture model, the inhibition of hedgehog signaling by activation of PKA, with Forskolin, or overexpression of Gli3R or Patch1 in MCF10A, could maintain Δ Np63 expression, repress GATA3 and inhibit luminal differentiation.

Notch signaling could modulate apoptosis and cell differentiation in MCF10A 3D cultures. Active notch signaling, by overexpression of the Notch Intracellular Domain, induces the luminal phenotype and represses Δ Np63 expression in mammary epithelial cells (Yalcin-Ozuysal et al. 2010). In contrast, inhibition of Notch signaling with a γ -secretase inhibitor or knockdown of the Notch transcriptional activator CBF inhibits luminal cell differentiation (Bouras et al. 2008). Similarly, overexpression of Δ Np63 induces the basal cell phenotype and partially antagonizes Notch signaling impairing differentiation of luminal cells (Yalcin-Ozuysal et al. 2010). To test the role of Notch signaling in the MCF10A 3D culture model the treatment of MCF10A B 3D cultures with γ -secretase inhibitor or knockdown of CBF, could inhibit the luminal differentiation and maintain p63 expression, which could phenocopy our observations that the overexpression of p63 repressed GATA3 expression in the lumen (Figure 3-3 D).

Finally, the WNT β -Catenin pathway positively regulates the expression of Δ Np63 through its downstream transcriptional activator limb-bud and heart (LBH) factor, required for activation of Δ Np63 expression in the mammary gland in puberty (Lindley et al., 2015). In addition, the Pbx1/2 transcription factors induce expression of Wnt9 ligand which activates the downstream transcriptional regulator Lef1/TCF, which binds the Δ Np63 promoter, and activates a Δ Np63 luciferase promoter (Ferretti et al. 2011). To test the role of Wnt signaling in the MCF10A 3D culture model, knockdown of LBH transcription factor, inhibition of β -Catenin with small molecule inhibitor, or siRNA against the Wnt9 ligand, could induce the formation of luminal cells in 3D and repress Δ Np63 expression. This would indicate that the WNT β -Catenin pathway suppresses the luminal phenotype while inducing Δ Np63 expression.

This section suggests a potential role of Wnt, Notch, hedgehog and Hippo signaling pathways in the regulation of Δ Np63 expression and luminal cell differentiation in the mammary gland, which indicate that these pathways are good candidates to regulate the hollow lumen formation and luminal cell differentiation in MCF10A 3D cultures. This suggests that a very complex interplay between multiple signaling pathways regulate the luminal cell differentiation of breast epithelial cells. Δ Np63 expression and PKA activation are common downstream targets of these pathways, indicating that potential crosstalks exist between them that possibly regulate the hollow lumen formation, the expression of Δ Np63 and the differentiation of basal and luminal cells in the mammary gland.

3.5 Chapter 3 Figures

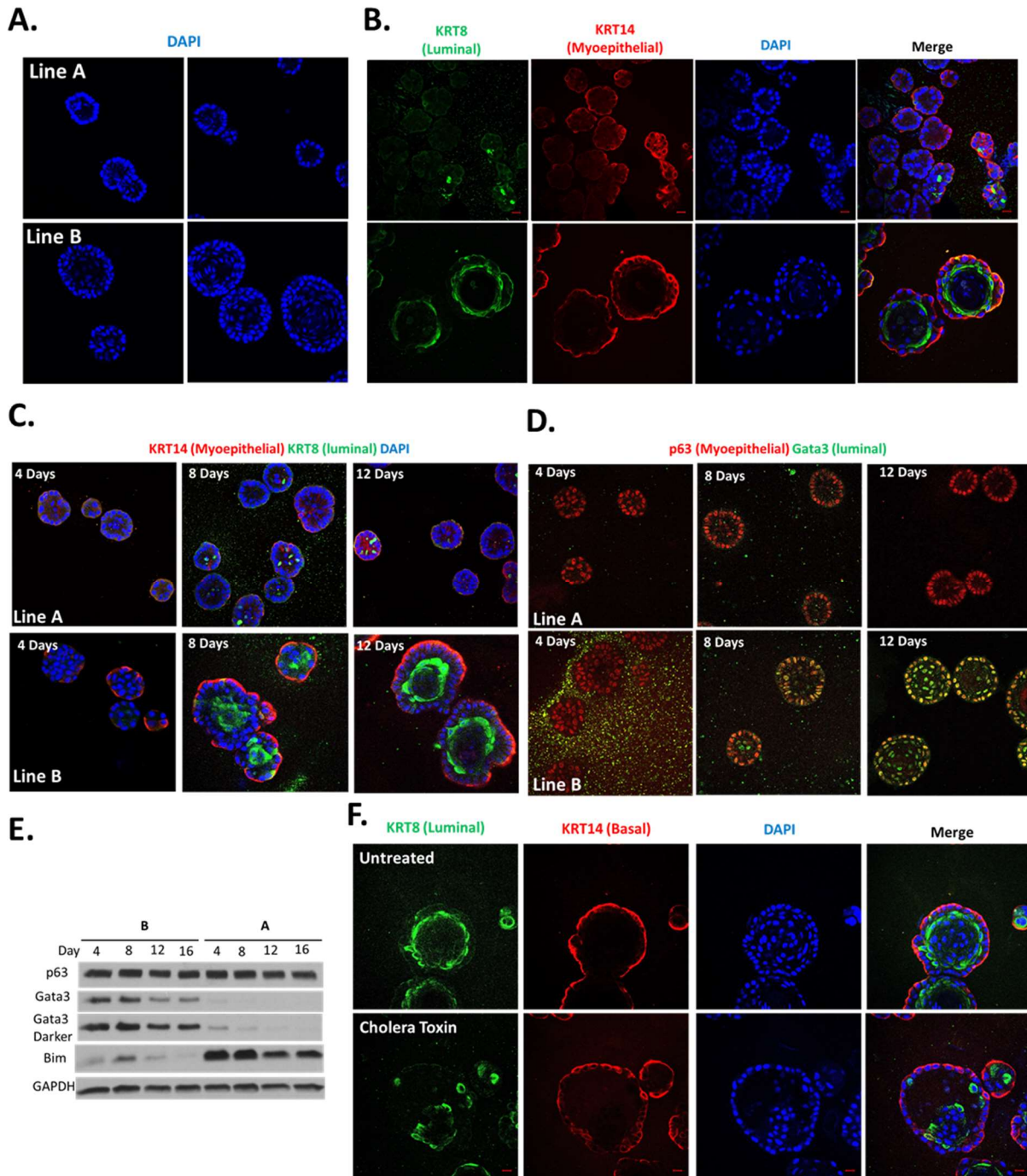


Figure 3-1. Lumen Clearing in MCF10A Represses GATA3 and increase BIM.

A. Immunofluorescence Images of 2 different fields of MFC10A 3D cultures stained with DAPI nuclear stain of 2 different cell lines one that forms hollow lumens Line A, and one cell line grown in our lab called Line B that is not hollow. **B.** Immunofluorescence of 3D cultures of MCF10A grown for 12 days on top of matrigel.

KRT8 stains luminal cells and KRT14 stains myoepithelial or basal cells and DAPI. **C.** Time-course experiments of 3D cultures grown in 3D for 4, 8, and 12 days co-stained for KRT8, KRT14, and DAPI. **D.** Same cells as in C, but co-stained for p63 (Basal cell marker) and Gata3 (luminal marker). **E.** Immunoblot of 4, 8, 12, and 16 days 3D cultures of MCF10A lines A and Line B **F.** Immunofluorescence of 3D cultures cell line B treated with 100 ng/ml of cholera toxin for 10 days.

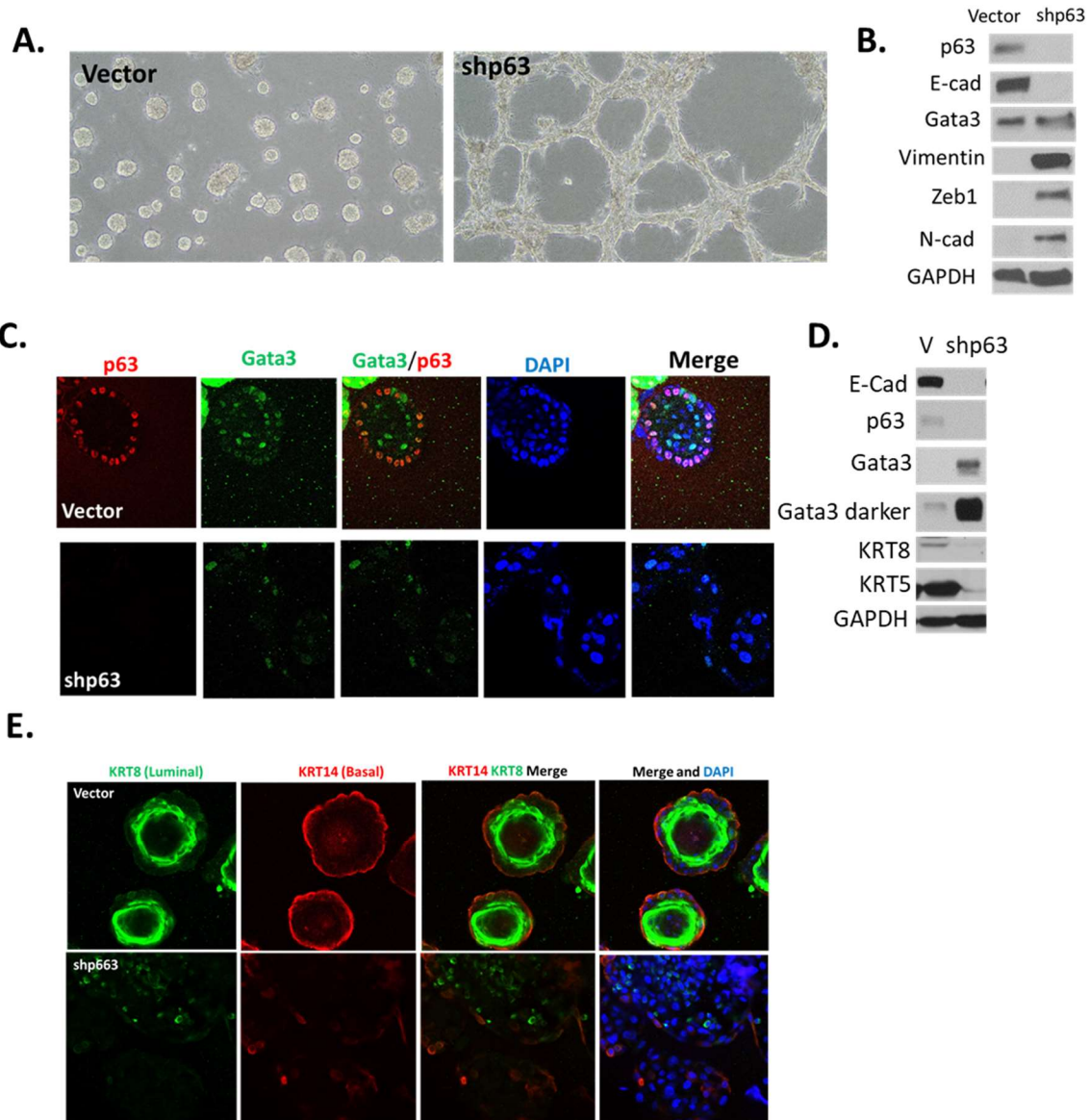


Figure 3-2. Δ Np63 Maintains the Structure of MCF10A in 3D culture.

A. Phase-contrast images of MCF10A stable cell lines expressing the empty vector or shRNA against Δ Np63 grown in 3D culture for 12 days. **B.** Immunoblot of vector and shp63 MCF10A cells grown in 2D **C.** Immunostaining of shp63 and vector cells grown in 3D for 12 days, stained with anti p63, Gata3 and Dapi. **D.** Immunoblot of the same cells grown in 3D for 12 days as shown in C. **E.** Immunostaining of shp63

and vector cells grown for 12 days, stained with KRT8, KRT14, and Dapi.

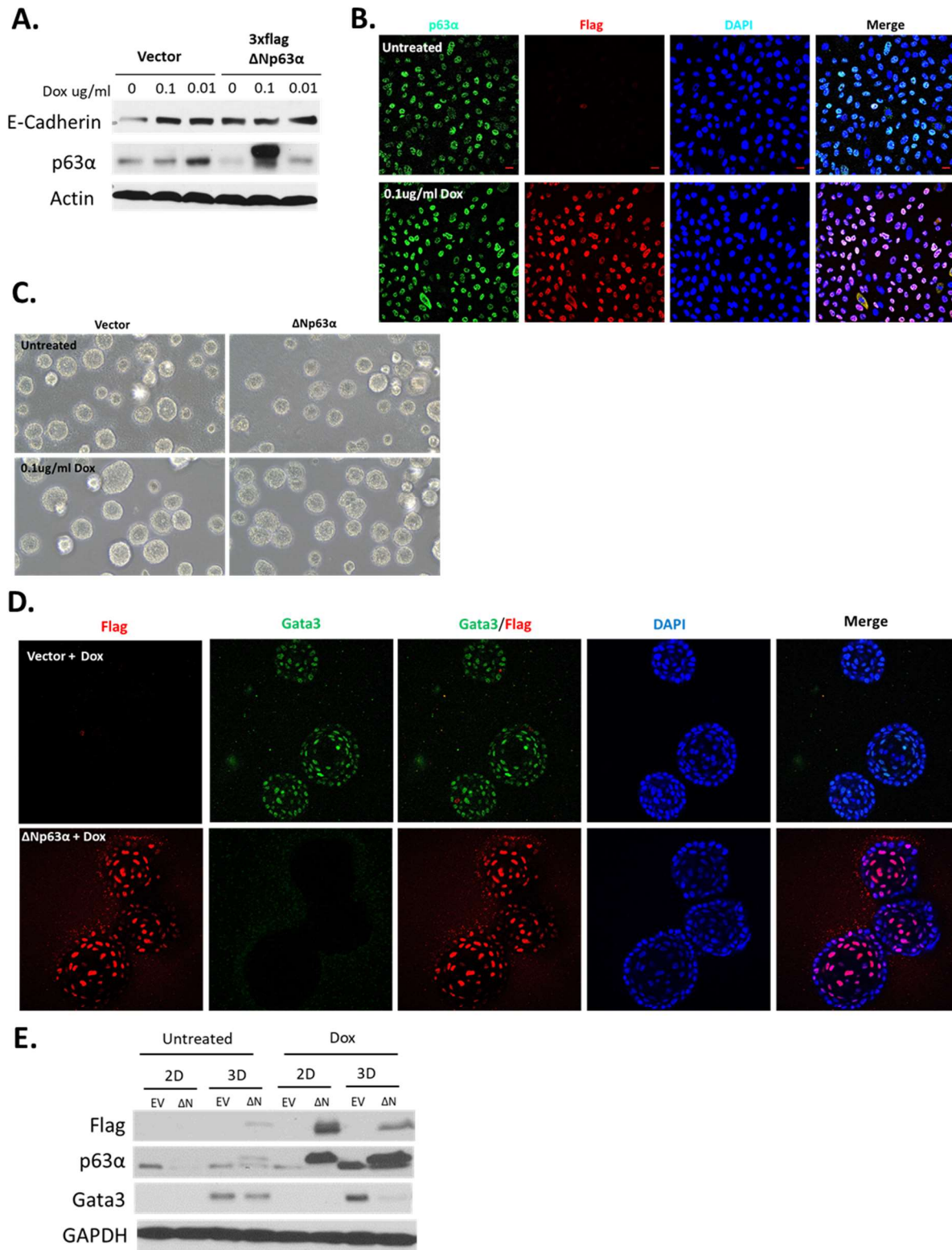


Figure 3-3. Δ Np63 α Represses GATA3 and the Luminal Phenotype in 3D.

A. Immunoblot of MCF10A tetOn inducible stable cell that overexpresses 3xflag- Δ Np63 α , induced with 0, 0.1, and 0.01 μ g/ml of Doxycycline. **B.** Immunostaining to show the nuclear localization of overexpressed 3xflag- Δ Np63 α , with anti-flag antibody after treatment for 24 h with 0.1 μ g/ml of Doxycycline. **C.** Phase-contrast images of MCF10A stable cell lines empty vector or 3xflag- Δ Np63 α treated with Doxycycline for 12 days. **D.** Immunostaining for Flag and Gata3 of the same cells as shown in C. **E.** Immunoblot of same cells in grown in 2D treated for 48 h with Doxycycline and in 3D for 12 days treated with and without Doxycycline.

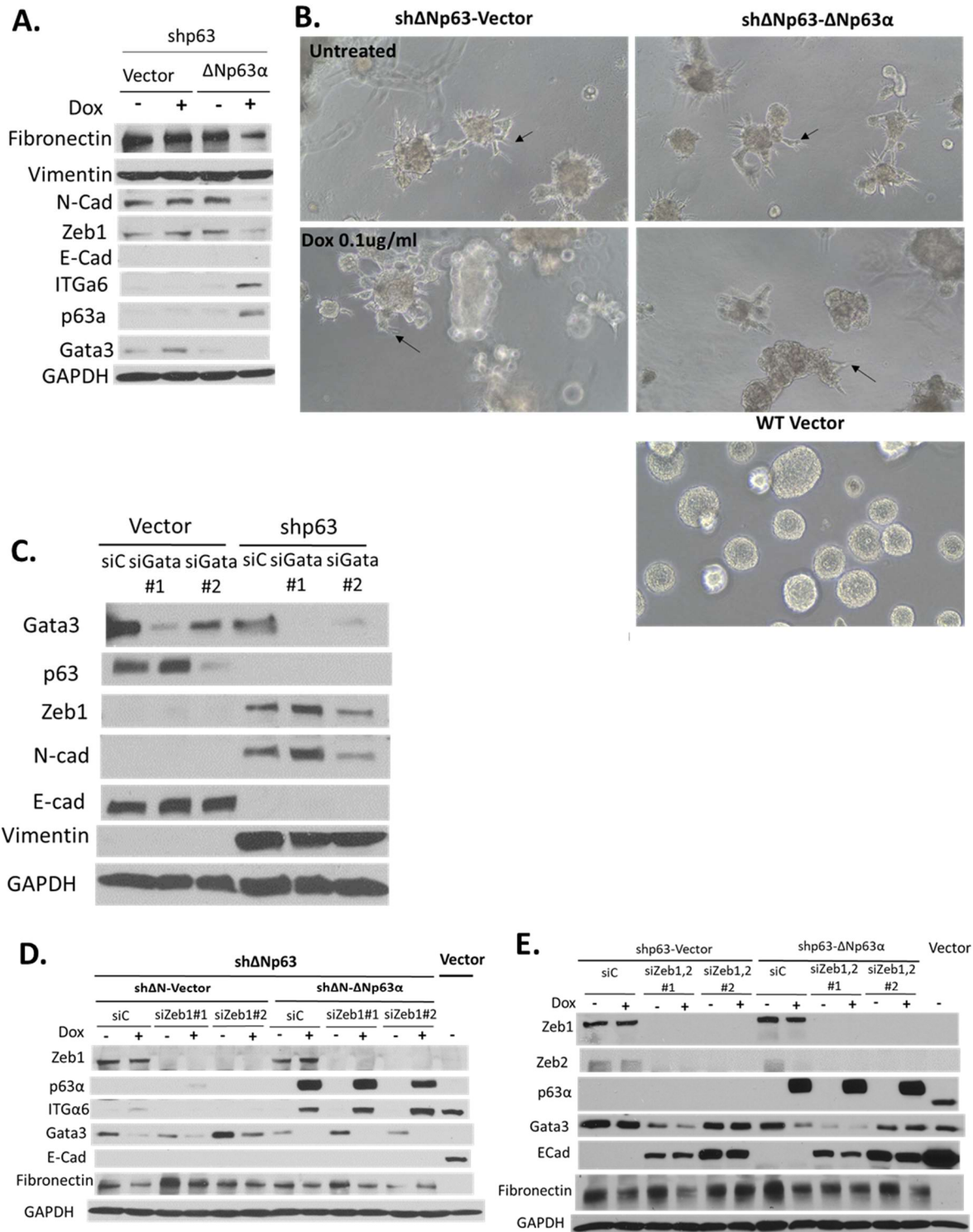


Figure 3-4. p63 Overexpression and ZEB1 and 2 Ablation Partially Rescue EMT.

A. MCF10A shp63 stable cell lines overexpressing tetOn inducible empty vector or 3xflag- Δ Np63 α were treated with 0.3 μ g/ml of Doxycycline for 12 days. **B.** Phase-contrast images of 3D cultures of MCF10A shp63 stable cell lines overexpressing tetOn inducible empty vector or 3xflag- Δ Np63 α were treated with 0.1 μ g/ml of Doxycycline for 12 days **C.** Knockdown of Gata3 for 72 h with 2 different siRNAs in MCF10A. **D.** MCF10A shp63 stable cell lines overexpressing tetOn inducible empty vector or 3xflag- Δ Np63 α were treated with 30 ng/ml of Doxycycline and transfected with control or 2 siRNAs against EMT transcription factor Zeb1 for 72 h. **E.** MCF10A shp63 stable cell lines overexpressing tetOn inducible empty vector or 3xflag- Δ Np63 α were treated with 50ng/ml of Doxycycline and cotransfected with control or 2 siRNAs against EMT transcription factor Zeb1 and Zeb2 for 48 h.

Chapter 4: Future Directions

Δ Np63 α depending on the context, can be an oncogene that induces growth and proliferation transactivating cell cycle genes, epithelial development and differentiation genes, mammary stem cell, and cell adhesion molecules (Riege et al. 2020). On the other hand, Δ Np63 α can be a tumor suppressor that inhibits migration and EMT in 2D models and at the same time it can be an oncogene that allows collective invasion in metastasis *in vivo* and in 3D tumor models (Cheung et al. 2013). In addition, Δ Np63 expression is different depending on the cancer type, in squamous cancers Δ Np63 α is amplified at a high frequency and its expression is higher compared to normal tissues (Figure 1-2). On the other hand, the expression of p63 decreases in breast cancers compared to normal tissues (Figure 1-2), and genomic amplifications are rare in prostate and breast cancers where its role as a driver oncogene is unlikely.

Δ Np63 α 's confusing role in cancer could be clarified by better understanding how its expression is controlled and how p63 itself might signal back into those same pathways. Multiple signaling pathways have been demonstrated to control the expression of p63 in mammary epithelial cells and skin keratinocytes including the EGFR, Ras, PI3K, Wnt β -catenin, Hedgehog and Notch pathways (Table 2). The results presented in chapter 2, reveal the Hippo pathway as a new regulator of Δ Np63 expression.

The regulation of p63 by a non-canonical Hippo pathway in normal and cancer cells reveals a new mechanism of regulation of this factor (Chapter 2 and Figure 2-6). At the same time, it opens up new avenues and raises questions that can be addressed next with the potential experiments suggested by the following strategies.

We found that MST1/2 and LATS1 are required for expression of p63, but not LATS2. It has been found that LATS can be activated independently of MST (Yin et al. 2013). It is interesting to evaluate whether the mechanism used by MST and LATS is the same to repress p63, or whether they regulate p63 independently from each other. One strategy is to evaluate whether p63 repression induced by MST1/2 knockdown can be rescued by LATS1 overexpression or by activation of LATS1/2 with a PKA agonist (e.g.

Forskolin) which can activate LATS (Yu et al. 2012). If LATS1 overexpression is enough to rescue p63 expression upon MST knockdown, that would suggest that MST1 activation of LATS1 is indeed required for p63 expression. However, if LATS1 knockdown is not enough to rescue p63 expression after ablation of MST, that would suggest that MST1/2 and LATS1 use independent mechanisms to induce p63 expression.

We also showed that both MST1 and 2 knockdown downregulated p63 independent of SAV1 (Chapter 2, Figure 2-2 E, F). SAV1 is required for MST homodimer formation and activation, while RASSF proteins have been shown to induce the formation of MST1/2 heterodimers (Rawat et al. 2016). It is possible that MST1/2 heterodimers are required for p63 expression rather than homodimers. To test whether heterodimers are involved in regulation of p63 expression, then knockdown of RASSF proteins and its effect on p63 expression could be evaluated. Additionally, rescue of p63 expression after MST1/2 co-knockdown, by combined expression of both MST1/2 but not overexpression of either MST1 or 2 alone. Would indicate that indeed, heterodimers are required in regulation of p63 expression and would explain why SAV1 is not required in these cells.

Our data show that MOB1A protein is more abundant than MOB1B, and we hypothesize that abundance is the reason why MOB1A is required for p63 expression rather than MOB1B. To confirm if this is the only reason, p63 repression induced by MOB1A knockdown, could be rescued with MOB1A or MOB1B overexpression. If p63 expression is rescued only with MOB1A, it suggests that a mechanism exclusively associated with MOB1A is responsible for the induction of p63 expression. In that case, a different hypothesis is that MOB1A interacts strongly with LATS1 compared MOB1B. Indeed that seems to be the case, since coimmunoprecipitation coupled with mass spectrometry in HEK293, revealed that overexpressed MOB1A binds more to LATS1 than LATS2 (Xiong et al. 2017). To test if this is the case in MCF10A, overexpression of flag-MOB1A and LATS1/2 followed by coimmunoprecipitation of MOB1A, and immunoblotting for LATS1 or LATS2. Since since MOB1A interaction with LATS1 is phosphorylation dependent (Praskova et al., 2008) and Okadaic acid treatment (PP2A phosphatase inhibitor) increases the binding of proteins which interaction is dependent on phosphorylation (Xiong et al. 2017). The

coimmunoprecipitation experiments of MOB1 proteins should include Okadaic acid or DMSO control treatment. If the hypothesis is correct, LATS1 should coimmunoprecipitate more with MOB1A compared to LATS2. Another experiment to test the relevance of MOB1A over MOB1B, is to evaluate whether the overexpression of a phosphomutant form (T12A the MST site) of MOB1A but not MOB1B, can repress p63 expression, acting as a dominant negative preventing MST induced LATS activation through MOB1 phosphorylation.

Another interesting finding is that SAV1 seems to control p63 protein stability without decreasing its mRNA. To test whether SAV1 knock down decreases the half-life of p63; cyclohexamide (inhibition of translation) treatment to compare p63 protein stability effects upon SAV1 knockdown. In addition, ubiquitination levels of p63 could be tested by knocking down SAV1 and treating the cells with the proteasome inhibitor MG132, followed by immunoprecipitation of p63 and immunoblot with ubiquitin. If SAV1 knockdown increases p63's ubiquitination, then immunoblotting of p63 should reveal the typical ladder characteristic of ubiquitinated proteins. If that is the case, then SAV1 knockdown in combination with several well known p63 E3 ligases such as Pirh2, ITCH, WWP1 could be tested (Jung et al. 2013; Li et al. 2008b; Rossi et al. 2006). Interestingly, ITCH is also the E3 ligase of LATS1 (Ho et al. 2011). SAV1 loss triggers p63's degradation knockdown of any of the E3 ligases upon SAV1 loss, should rescue the levels of p63. If none of those E3 ligases are involved, doing immunoprecipitation coupled with mass spectrometry for SAV1 could reveal potential protein interactors of SAV1.

Our results showed that inhibition of MST1/2 and LATS1/2 kinases repress p63 expression in both breast and squamous cancers, however it is still unclear whether LATS1 is the only kinase required for p63 expression in squamous and breast cancer cells. To answer this question knockdown of LATS1, LATS2 independently, as well as MOB1A and MOB1B, in one breast and squamous cell lines, could confirm that a non-canonical MOB1A-LATS1 dependent Hippo pathway regulates p63 expression in these cancer cells.

The results presented in chapter 2 revealed that repression of p63 after MST1/2 inhibition is YAP/TAZ independent (Figure 2-4). It is important to confirm this in a breast and a squamous cell line. In

addition, the same experiment should be evaluated upon inhibition of LATS1/2 with Truli, to confirm the YAP/TAZ independent repression of p63 occurs upon LATS inhibition.

In order to identify the potential targets downstream of LATS1 responsible for p63's repression or constitutive expression, a high throughput assay is required. One possibility is to find interactors of LATS1, other than YAP/TAZ, by co-immunoprecipitation of overexpressed LATS1 followed by mass spectrometry. MCF10A cells overexpressing LATS1 treated with DMSO, Okadaic acid (to maintain the phosphorylation status of the proteins in case the interaction is phosphorylation dependent) or Truli. LATS1 interactors should bind strongly to LATS1 upon Okadaic treatment (if the interaction is phosphorylation dependent) compared to DMSO, and the binding should decrease upon LATS kinase inhibition with Truli. This experimental setting will help identify new protein interactors of LATS1.

Multiple factors have been shown to be involved in p63 repression (Fisher et al. 2020; Yoh and Prywes 2015) (Sumarized in Table 2). Several of those candidates could be individually knocked down to evaluate their effect on p63 expression. Alternatively an unbiased approach could be to use a transcription factor library fused with to GAL4 transcription factor DNA binding domain, for their capacity to activate a luciferase reporter expression driven by several Gal4 binding response elements (Zhang et al. 2009). Transfection of this library could help identify transcription factors that are regulated by MST or LATS inhibition. MCF10A transfected with this library would be treated with XMU-MP1 (MST1/2 inhibitor), Truli (LATS inhibitor). As an alternative to these inhibitors an MCF10A shLATS1 stable cell line could be used. The result should show higher luciferase for the transcription factors that are activated upon MST or LATS inhibition, or lower luciferase signal for the transcription factors that are inhibited after the treatment. An alternative approach is to CRISPR knock-in of a luciferase gene under the P2 Δ Np63 promoter in MCF10A, and use it in a screen with a CRISPR library of Transcription factors, in combination with Truli, XMU or siLATS1 treatment. The factors when knocked out maintain high luciferase expression despite the MST or LATS inhibition, would be those responsible for the repression. While the factors that reduce the P2 Δ Np63 promoter luciferase activity, after knock out, would be those responsible for maintaining p63 endogenous expression.

Our data showed that p63 inhibits YAP and TAZ activity. To evaluate whether YAP/TAZ localize to the nucleus after ablation of p63, could be evaluated by immunofluorescence and nuclear cytoplasmic fractionation, as well as immunoprecipitation of YAP/TAZ with TEAD1 transcription factor after p63 knockdown. Evaluation of individual p63 target genes that have been also shown to be part of the Hippo pathway (such as SFN, RASSF6 and FAT2 (Riege et al. 2020)) could be knockdown to evaluate if they can activate YAP/TAZ upon knockdown. Alternatively, a two step unbiased approach could be used. First, ChIP seq with an antibody against p63 in MCF10A cells could help identify potential p63 targets. Second, to identify novel genes that are negative regulators of YAP/TAZ, a whole genome CRISPR screen lentiviral library could be used (with a puromycin selection for an efficient knock out), in a MCF10A stable cell line with luciferase gene reporter under the control of TEAD1 response element. The knocked out genes that induce high TEAD dependent luciferase reporter activation, would identify genes that negatively regulate YAP/TAZ. To crossreference the genes identified in both approaches, the p63 ChIP seq genes and the screen of YAP/TAZ negative regulators, would identify the genes that are also targets of p63.

Our results suggest that p63 loss decreases cell growth. This result will be confirmed with a growth curve upon p63 knockdown, or immunofluorescence to detect BrdU incorporation, to measure proliferation. In addition, to evaluate cell death after p63 loss, live/dead staining (calcein for living cells and Ethidium Bromide for dead) quantified by FACS analysis. This would demonstrate whether p63 loss induce any cell death. In addition, our results showed that p63 loss decreased growth, despite the increase in YAP/TAZ transcriptional activity. This suggests that the increase in YAP/TAZ is not enough to induce proliferation upon p63 loss and that p63 regulates growth through YAP/TAZ unrelated mechanisms.

In addition, measuring viability and cell death after knockdown of MST1, LATS1 and p63 coupled with simultaneous ablation of YAP/TAZ with siRNA, would reveal if a synergistic reduction of proliferation occurs with ablation of both p63 and YAP/TAZ simultaneously. In addition, rescuing cell proliferation and viability after p63 loss, with overexpression of p63, YAP, constitutively active YAP (5SA mutant), or YAP dominant negative forms defective of TEAD binding (YAP S94A mutation) (Zhao et al. 2008). Would demonstrate that WT YAP overexpression keeps the cells proliferating after ablation of p63. A rescue in

viability is expected for the phosphomutant (5SA) and WT forms of YAP, but not with the dominant negative YAP.

Δ Np63 α 's complex role in cancer could be clarified by better understanding how its expression is controlled and how p63 itself signals back into some of these pathways. Multiple signaling pathways have been demonstrated to control the expression of p63 in mammary epithelial cells and skin keratinocytes, and those pathways are listed in Table 2 and described in more detail in Chapter 1.

The fact that multiple signaling pathways have similar effects on p63 expression can be context dependent, but also suggests the existence of crosstalk connections between these pathways, revealing a very complex regulatory system that supports p63's expression. In fact, Hippo has been shown to crosstalk with several of these signaling pathways that will be described below.

EGFR activation increases Δ Np63 through STAT3 in squamous cell carcinomas (Barbieri et al. 2003; Ripamonti et al. 2013), while constitutively active Ras, PI3K and HER2 overexpression (ERBB2 receptor from the EGFR family) suppress Δ Np63 in mammary epithelial cells (Yoh et al. 2016; Hu et al. 2017). Hippo is activated by EGFR signaling and activates YAP via impaired complex formation between PDK1-MST-SAV in mammary epithelial cells (Fan et al. 2013). Additionally, EGFR activates YAP/TAZ upon EGF treatment, independent of PI3K pathway (Ando et al. 2021). EGFR directly phosphorylates tyrosines in MOB1, leading to LATS1/2 inactivation (decreased T1079 phosphorylation). EGFR activation does not impair MOB1 binding to LATS and MST1/2 kinase phosphorylation of MOB1 was not affected (Ando et al. 2021). Suggesting that EGFR activation decreases LATS activation, independent of MST and MOB1 binding to LATS. EGFR inhibition with erlotinib, inhibits YAP/TAZ in squamous cancers of the lung and head and neck (Ando et al. 2021).

The Wnt pathway induces the expression of Δ Np63 through the limb-bud and heart (LBH) transcription factor in the mammary gland in mice (Lindley et al., 2015). In addition, several connections between the Hippo pathway and Wnt- β -catenin have been suggested. YAP/TAZ are sequestered in the

same degradation complex as axin and β -Catenin (Azzolin et al. 2014). Activation of the Hippo pathway inhibits Wnt- β -catenin signaling (Imajo et al. 2012) and a non-canonical Wnt signaling pathway also activates YAP/TAZ (Park et al. 2015). These interactions illustrate a complex regulation between Wnt signaling and Hippo.

The hedgehog signaling pathway transcriptional activator Gli3 represses Δ Np63 expression (Li et al. 2008a), and Hippo and Hedgehog are also connected, since overexpression of active YAP blocks hedgehog target gene expression and GLI1 transcriptional activity (Tariki et al. 2014). Conversely, knockdown of YAP upregulates GLI1 and hedgehog target gene expression, while activation of hedgehog signaling increases YAP levels and promotes YAP/TAZ transcriptional activity (Tariki et al. 2014). Active YAP counteracts GLI activity, while active hedgehog promotes YAP activity. In addition, cell density regulates hedgehog and hippo signaling in opposite ways, high density increases hedgehog targets, while YAP/TAZ are phosphorylated and degraded and their targets are downregulated in high density conditions (Tariki et al. 2014).

Future experiments that aim to find the factors responsible for suppression of p63 should consider potential connections and crosstalks between Hippo and other pathways. To assess whether other pathways are activated in response to the inhibition of Hippo, first the expression of Wnt, Notch, and Hedgehog gene targets upon Hippo inhibition, should be evaluated. Second, the activation of components of these pathways should be evaluated by phosphorylation status, protein levels or luciferase reporter assays. This would help understand whether factors known to regulate p63's expression from other pathways (Gli3, LBH, STAT3 and others) cooperate with the Hippo pathway to activate p63.

An additional layer of complexity of this system is that Δ Np63 itself regulates the expression of genes involved in regulating several signaling pathways, such as WNT signaling receptor FZD7 among others are targets of Δ Np63 in primary mammary epithelial cells (Chakrabarti et al. 2014). The Notch signaling ligands JAGGED1 and JAGGED2 are targets of Δ Np63, as well as the NOTCH target gene HES1 is a repression target of p63 (Candi et al. 2006; Nguyen et al. 2006). In addition, Δ Np63 induces Sonic

Hedgehog and its downstream target GLI2 and PATCH1 (Memmi et al. 2015) and EGFR are also a targets of $\Delta Np63$ (Carroll et al. 2006). In addition, the Hippo pathway is an additional regulator of p63 expression in breast and squamous cancers, and $\Delta Np63$ can also regulate genes involved in Hippo signaling, such as 14-3-3 sigma (SFN), RASSF proteins and YAP1 (Saladi et al. 2017; Riege et al. 2020).

In summary, regulation of p63 expression in cells is the result of a complex network of multiple pathways such as Wnt, Notch, EGFR and Hedgehog and now based on our results, the Hippo pathway. In addition, p63 can transcriptionally induce components of these pathways, adding to the complexity of its own regulation. Hence, targeting p63 using pathway specific inhibitors could lead to synergistic effects mediated by p63 downregulation other signaling components, affecting the activation of other pathways. Hence, targeting p63 with inhibitors would likely have off target effects due to crosstalk between pathways. In order to target p63 to suppress cancer growth, further studies are required to understand how interactions between multiple pathways regulate p63 in order to efficiently inhibit growth and use potential off target effects that would synergize with other pathways to decrease growth.

References

- Ando T, Arang N, Wang Z, Costea DE, Feng X, Goto Y, Izumi H, Gilardi M, Ando K, Gutkind JS. 2021. EGFR Regulates the Hippo pathway by promoting the tyrosine phosphorylation of MOB1. *Commun Biol* **4**: 1237.
- Asselin-Labat M-L, Sutherland KD, Barker H, Thomas R, Shackleton M, Forrest NC, Hartley L, Robb L, Grosveld FG, van der Wees J, et al. 2007. Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. *Nat Cell Biol* **9**: 201–209.
- Aylon Y, Gershoni A, Rotkopf R, Biton IE, Porat Z, Koh AP, Sun X, Lee Y, Fiel M-I, Hoshida Y, et al. 2016. The LATS2 tumor suppressor inhibits SREBP and suppresses hepatic cholesterol accumulation. *Genes Dev* **30**: 786–797.
- Aylon Y, Michael D, Shmueli A, Yabuta N, Nojima H, Oren M. 2006. A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes Dev* **20**: 2687–2700.
- Azzolin L, Panciera T, Soligo S, Enzo E, Bicciato S, Dupont S, Bresolin S, Frasson C, Basso G, Guzzardo V, et al. 2014. YAP/TAZ Incorporation in the β -Catenin Destruction Complex Orchestrates the Wnt Response. *Cell* **158**: 157–170.
- Bae SJ, Ni L, Osinski A, Tomchick DR, Brautigam CA, Luo X. 2017. SAV1 promotes Hippo kinase activation through antagonizing the PP2A phosphatase STRIPAK. *Elife* **6**.
- Barbareschi M, Pecciarini L, Cangi MG, Macrì E, Rizzo A, Viale G, Doglioni C. 2001. p63, a p53 homologue, is a selective nuclear marker of myoepithelial cells of the human breast. *The American journal of surgical pathology* **25**: 1054–1060.
- Barbieri CE, Barton CE, Pietenpol JA. 2003. Δ Np63 α Expression Is Regulated by the Phosphoinositide 3-Kinase Pathway. *Journal of Biological Chemistry* **278**: 51408–51414.
- Bartucci M, Dattilo R, Moriconi C, Pagliuca A, Mottolese M, Federici G, Benedetto AD, Todaro M, Stassi G, Sperati F, et al. 2015. TAZ is required for metastatic activity and chemoresistance of breast cancer stem cells. *Oncogene* **34**: 681–690.
- Beckerman R, Prives C. 2010. Transcriptional Regulation by P53. *Cold Spring Harb Perspect Biol* **2**: a000935.

- Boggiano JC, Vanderzalm PJ, Fehon RG. 2011. Tao-1 Phosphorylates Hippo/MST Kinases to Regulate the Hippo-Salvador-Warts Tumor Suppressor Pathway. *Developmental Cell* **21**: 888–895.
- Boldrup L, Coates P, Gu X, Nylander K. 2007. Δ Np63 isoforms regulate CD44 and keratins 4, 6, 14 and 19 in squamous cell carcinoma of head and neck. *The Journal of Pathology* **213**: 384–391.
- Bouras T, Pal B, Vaillant F, Harburg G, Asselin-Labat M-L, Oakes SR, Lindeman GJ, Visvader JE. 2008. Notch Signaling Regulates Mammary Stem Cell Function and Luminal Cell-Fate Commitment. *Cell Stem Cell* **3**: 429–441.
- Bray SJ. 2016. Notch signalling in context. *Nat Rev Mol Cell Biol* **17**: 722–735.
- Britschgi A, Duss S, Kim S, Couto JP, Brinkhaus H, Koren S, De Silva D, Mertz KD, Kaup D, Varga Z, et al. 2017. The Hippo kinases LATS1 and 2 control human breast cell fate via crosstalk with ER α . *Nature* **541**: 541–545.
- Callus BA, Verhagen AM, Vaux DL. 2006. Association of mammalian sterile twenty kinases, Mst1 and Mst2, with hSalvador via C-terminal coiled-coil domains, leads to its stabilization and phosphorylation. *The FEBS Journal* **273**: 4264–4276.
- Camargo FD, Gokhale S, Johnnidis JB, Fu D, Bell GW, Jaenisch R, Brummelkamp TR. 2007. YAP1 Increases Organ Size and Expands Undifferentiated Progenitor Cells. *Current Biology* **17**: 2054–2060.
- Campbell JD, Yau C, Bowlby R, Liu Y, Brennan K, Fan H, Taylor AM, Wang C, Walter V, Akbani R, et al. 2018. Genomic, Pathway Network, and Immunologic Features Distinguishing Squamous Carcinomas. *Cell Reports* **23**: 194-212.e6.
- Candi E, Terrinoni A, Rufini A, Chikh A, Lena AM, Suzuki Y, Sayan BS, Knight RA, Melino G. 2006. p63 is upstream of IKK α in epidermal development. *J Cell Sci* **119**: 4617–4622.
- Carroll DK, Carroll JS, Leong C-O, Cheng F, Brown M, Mills AleaA, Brugge JS, Ellisen LW. 2006. p63 regulates an adhesion programme and cell survival in epithelial cells. *Nature Cell Biology* **8**: 551–561.
- Carter P, Schnell U, Chaney C, Tong B, Pan X, Ye J, Mernaugh G, Cotton JL, Margulis V, Mao J, et al. 2021. Deletion of Lats1/2 in adult kidney epithelia leads to renal cell carcinoma. *J Clin Invest* **131**. <https://www.jci.org/articles/view/144108> (Accessed February 20, 2022).

- Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E, et al. 2012. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* **2**: 401–404.
- Chakrabarti R, Wei Y, Hwang J, Hang X, Andres Blanco M, Choudhury A, Tiede B, Romano R-A, DeCoste C, Mercatali L, et al. 2014. Δ Np63 promotes stem cell activity in mammary gland development and basal-like breast cancer by enhancing Fzd7 expression and Wnt signalling. *Nature Cell Biology* **16**: 1004–1015.
- Chan EHY, Nousiainen M, Chalamalasetty RB, Schäfer A, Nigg EA, Silljé HHW. 2005. The Ste20-like kinase Mst2 activates the human large tumor suppressor kinase Lats1. *Oncogene* **24**: 2076–2086.
- Chan SW, Lim CJ, Guo K, Ng CP, Lee I, Hunziker W, Zeng Q, Hong W. 2008. A Role for TAZ in Migration, Invasion, and Tumorigenesis of Breast Cancer Cells. *Cancer Res* **68**: 2592–2598.
- Chan SW, Lim CJ, Loo LS, Chong YF, Huang C, Hong W. 2009. TEADs Mediate Nuclear Retention of TAZ to Promote Oncogenic Transformation. *J Biol Chem* **284**: 14347–14358.
- Chari NS, Romano RA, Koster MI, Jaks V, Roop D, Flores ER, Teglund S, Sinha S, Gruber W, Aberger F, et al. 2013. Interaction between the TP63 and SHH pathways is an important determinant of epidermal homeostasis. *Cell Death Differ* **20**: 1080–1088.
- Chen Q, Zhang N, Gray RS, Li H, Ewald AJ, Zahnow CA, Pan D. 2014. A temporal requirement for Hippo signaling in mammary gland differentiation, growth, and tumorigenesis. *Genes Dev* **28**: 432–437.
- Cheng C-C, Wang D-Y, Kao M-H, Chen J-K. 2009. The growth-promoting effect of KGF on limbal epithelial cells is mediated by upregulation of Δ Np63 α through the p38 pathway. *Journal of Cell Science* **122**: 4473–4480.
- Cheung KJ, Gabrielson E, Werb Z, Ewald AJ. 2013. Collective Invasion in Breast Cancer Requires a Conserved Basal Epithelial Program. *Cell* **155**: Cell.
- Chua HL, Bhat-Nakshatri P, Clare SE, Morimiya A, Badve S, Nakshatri H. 2007. NF- κ B represses E-cadherin expression and enhances epithelial to mesenchymal transition of mammary epithelial cells: potential involvement of ZEB-1 and ZEB-2. *Oncogene* **26**: 711–724.

- Cimpean AM, Suciuc C, Ceausu R, Tatucu D, Muresan AM, Raica M. 2008. Relevance of the immunohistochemical expression of cytokeratin 8/18 for the diagnosis and classification of breast cancer. *Rom J Morphol Embryol* **49**: 479–83.
- Como CJD, Urist MJ, Babayan I, Drobnjak M, Hedvat CV, Teruya-Feldstein J, Pohar K, Hoos A, Cordon-Cardo C. 2002. p63 Expression Profiles in Human Normal and Tumor Tissues. *Clin Cancer Res* **8**: 494–501.
- Compagnone M, Gatti V, Presutti D, Ruberti G, Fierro C, Markert EK, Vousden KH, Zhou H, Mauriello A, Anemone L, et al. 2017. Δ Np63-mediated regulation of hyaluronic acid metabolism and signaling supports HNSCC tumorigenesis. *Proc Natl Acad Sci U S A* **114**: 13254–13259.
- Cordenonsi M, Zanconato F, Azzolin L, Forcato M, Rosato A, Frasson C, Inui M, Montagner M, Parenti AR, Poletti A, et al. 2011. The Hippo Transducer TAZ Confers Cancer Stem Cell-Related Traits on Breast Cancer Cells. *Cell* **147**: 759–772.
- Couzens AL, Knight JDR, Kean MJ, Teo G, Weiss A, Dunham WH, Lin Z-Y, Bagshaw RD, Sicheri F, Pawson T, et al. 2013. Protein Interaction Network of the Mammalian Hippo Pathway Reveals Mechanisms of Kinase-Phosphatase Interactions. *Science Signaling* **6**: rs15–rs15.
- Debaugnies M, Sánchez-Danés A, Rorive S, Raphaël M, Liagre M, Parent M, Brisebarre A, Salmon I, Blanpain C. 2018. YAP and TAZ are essential for basal and squamous cell carcinoma initiation. *EMBO Rep* **19**: e45809.
- Debnath J, Mills KR, Collins NL, Reginato MJ, Muthuswamy SK, Brugge JS. 2002. The Role of Apoptosis in Creating and Maintaining Luminal Space within Normal and Oncogene-Expressing Mammary Acini. *Cell* **111**: 29–40.
- DeRan M, Yang J, Shen C-H, Peters EC, Fitamant J, Chan P, Hsieh M, Zhu S, Asara JM, Zheng B, et al. 2014. Energy Stress Regulates Hippo-YAP Signaling Involving AMPK-Mediated Regulation of Angiotensin-like 1 Protein. *Cell Reports* **9**: 495–503.
- Deutsch GB, Zielonka EM, Coutandin D, Weber TA, Schäfer B, Hannewald J, Luh LM, Durst FG, Ibrahim M, Hoffmann J, et al. 2011. DNA Damage in Oocytes Induces a Switch of the Quality Control Factor TAp63 α from Dimer to Tetramer. *Cell* **144**: 566–576.

- Devos M, Gilbert B, Denecker G, Leurs K, Mc Guire C, Lemeire K, Hochepped T, Vuylsteke M, Lambert J, Van Den Broecke C, et al. 2017. Elevated Δ Np63 α Levels Facilitate Epidermal and Biliary Oncogenic Transformation. *Journal of Investigative Dermatology* **137**: 494–505.
- Donehower LA, Harvey M, Slagle BL, McArthur MJ, Montgomery CA, Butel JS, Bradley A. 1992. Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* **356**: 215–221.
- Dong J, Feldmann G, Huang J, Wu S, Zhang N, Comerford SA, Gayyed MF, Anders RA, Maitra A, Pan D. 2007. Elucidation of a Universal Size-Control Mechanism in Drosophila and Mammals. *Cell* **130**: 1120–1133.
- Dotto GP, Rustgi AK. 2016. Squamous Cell Cancers: A Unified Perspective on Biology and Genetics. *Cancer Cell* **29**: 622–637.
- Duijf PHG, Vanmolkot KRJ, Propping P, Friedl W, Krieger E, McKeon F, Dötsch V, Brunner HG, van Bokhoven H. 2002. Gain-of-function mutation in ADULT syndrome reveals the presence of a second transactivation domain in p63. *Human Molecular Genetics* **11**: 799–804.
- Fan F, He Z, Kong L-L, Chen Q, Yuan Q, Zhang S, Ye J, Liu H, Sun X, Geng J, et al. 2016. Pharmacological targeting of kinases MST1 and MST2 augments tissue repair and regeneration. *Science Translational Medicine* **8**: 352ra108-352ra108.
- Fan R, Kim N-G, Gumbiner BM. 2013. Regulation of Hippo pathway by mitogenic growth factors via phosphoinositide 3-kinase and phosphoinositide-dependent kinase-1. *PNAS* **110**: 2569–2574.
- Ferretti E, Li B, Zewdu R, Wells V, Hebert JM, Karner C, Anderson MJ, Williams T, Dixon J, Dixon MJ, et al. 2011. A Conserved Pbx-Wnt-p63-Irf6 Regulatory Module Controls Face Morphogenesis by Promoting Epithelial Apoptosis. *Developmental Cell* **21**: 627–641.
- Fisher ML, Balinth S, Mills AA. 2020. p63-related signaling at a glance. *Journal of Cell Science* **133**: jcs228015.
- Fisher ML, Kerr C, Adhikary G, Grun D, Xu W, Keillor JW, Eckert RL. 2016. Transglutaminase Interaction with α 6/ β 4-Integrin Stimulates YAP1-Dependent Δ Np63 α Stabilization and Leads to Enhanced Cancer Stem Cell Survival and Tumor Formation TG2 in Skin Cancer Stem Cells. *Cancer Res* **76**: 7265–7276.

- Flores ER, Sengupta S, Miller JB, Newman JJ, Bronson R, Crowley D, Yang A, McKeon F, Jacks T. 2005. Tumor predisposition in mice mutant for p63 and p73: Evidence for broader tumor suppressor functions for the p53 family. *Cancer Cell* **7**: 363–373.
- Florindo C, Perdigão J, Fesquet D, Schiebel E, Pines J, Tavares AA. 2012. Human Mob1 proteins are required for cytokinesis by controlling microtubule stability. *J Cell Sci* **125**: 3085–3090.
- Freed-Pastor WA, Prives C. 2012. Mutant p53: one name, many proteins. *Genes Dev* **26**: 1268–1286.
- Furth N, Aylon Y, Oren M. 2018. p53 shades of Hippo. *Cell Death & Differentiation* **25**: 81–92.
- Furth N, Bossel Ben-Moshe N, Pozniak Y, Porat Z, Geiger T, Domany E, Aylon Y, Oren M. 2015. Down-regulation of LATS kinases alters p53 to promote cell migration. *Genes Dev* **29**: 2325–2330.
- Gaiddon C, Lokshin M, Ahn J, Zhang T, Prives C. 2001. A Subset of Tumor-Derived Mutant Forms of p53 Down-Regulate p63 and p73 through a Direct Interaction with the p53 Core Domain. *Mol Cell Biol* **21**: 1874–1887.
- Gao Y, Zhang W, Han X, Li F, Wang X, Wang R, Fang Z, Tong X, Yao S, Li F, et al. 2014. YAP inhibits squamous transdifferentiation of Lkb1-deficient lung adenocarcinoma through ZEB2-dependent DNp63 repression. *Nat Commun* **5**: 4629.
- Gatti V, Fierro C, Compagnone M, Giangrazi F, Markert EK, Bongiorno-Borbone L, Melino G, Peschiaroli A. 2018. Δ Np63 regulates the expression of hyaluronic acid-related genes in breast cancer cells. *Oncogenesis* **7**: 65.
- Ghioni P, Bolognese F, Duijf PHG, van Bokhoven H, Mantovani R, Guerrini L. 2002. Complex Transcriptional Effects of p63 Isoforms: Identification of Novel Activation and Repression Domains. *Mol Cell Biol* **22**: 8659–8668.
- Giacobbe A, Compagnone M, Bongiorno-Borbone L, Antonov A, Markert EK, Zhou JH, Annicchiarico-Petruzzelli M, Melino G, Peschiaroli A. 2016. p63 controls cell migration and invasion by transcriptional regulation of MTSS1. *Oncogene* **35**: 1602–1608.
- Goto H, Nishio M, To Y, Oishi T, Miyachi Y, Maehama T, Nishina H, Akiyama H, Mak TW, Makii Y, et al. 2018. Loss of Mob1a/b in mice results in chondrodysplasia due to YAP1/TAZ-TEAD-dependent repression of SOX9. *Development* **145**: dev159244.

- Graves JD. 1998. Caspase-mediated activation and induction of apoptosis by the mammalian Ste20-like kinase Mst1. *The EMBO Journal* **17**: 2224–2234.
- Grun D, Adhikary G, Eckert RL. 2018. NRP-1 interacts with GIPC1 and $\alpha 6/\beta 4$ -integrins to increase YAP1/ Δ Np63 α -dependent epidermal cancer stem cell survival. *Oncogene* **37**: 4711–4722.
- Hao Y, Chun A, Cheung K, Rashidi B, Yang X. 2008. Tumor Suppressor LATS1 Is a Negative Regulator of Oncogene YAP. *Journal of Biological Chemistry* **283**: 5496–5509.
- Harazono Y, Morita K, Tonouchi E, Anzai E, Takahara N, Kohmoto T, Imoto I, Yoda T. 2022. TP63 mutation mapping information in TP63 mutation-associated syndromes. *Advances in Oral and Maxillofacial Surgery* **5**: 100253.
- Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, Ruddy K, Tsang J, Cardoso F. 2019. Breast cancer (Primer). *Nature Reviews: Disease Primers*.
<http://www.proquest.com/docview/2296106130/abstract/4E5CC5AEE9B4490CPQ/2> (Accessed January 17, 2022).
- Herfs M, Hubert P, Suarez-Carmona M, Reschner A, Saussez S, Berx G, Savagner P, Boniver J, Delvenne P. 2010. Regulation of p63 Isoforms by Snail and Slug Transcription Factors in Human Squamous Cell Carcinoma. *Am J Pathol* **176**: 1941–1949.
- Hergovich A, Schmitz D, Hemmings BA. 2006. The human tumour suppressor LATS1 is activated by human MOB1 at the membrane. *Biochemical and Biophysical Research Communications* **345**: 50–58.
- Hibi K, Trink B, Patturajan M, Westra WH, Caballero OL, Hill DE, Ratovitski EA, Jen J, Sidransky D. 2000. AIS is an oncogene amplified in squamous cell carcinoma. *Proceedings of the National Academy of Sciences* **97**: 5462–5467.
- Hiemer SE, Zhang L, Kartha VK, Packer TS, Almershed M, Noonan V, Kukuruzinska M, Bais MV, Monti S, Varelas X. 2015. A YAP/TAZ-Regulated Molecular Signature Is Associated with Oral Squamous Cell Carcinoma. *Molecular Cancer Research* **13**: 957–968.
- Higashikawa K, Yoneda S, Tobiume K, Taki M, Shigeishi H, Kamata N. 2007. Snail-induced down-regulation of Δ Np63 α acquires invasive phenotype of human squamous cell carcinoma. *Cancer Res* **67**: 9207–9213.

- Hisaoka M, Tanaka A, Hashimoto H. 2002. Molecular Alterations of h-warts/LATS1 Tumor Suppressor in Human Soft Tissue Sarcoma. *Lab Invest* **82**: 1427–1435.
- Ho KC, Zhou Z, She Y-M, Chun A, Cyr TD, Yang X. 2011. Itch E3 ubiquitin ligase regulates large tumor suppressor 1 stability [corrected]. *Proc Natl Acad Sci U S A* **108**: 4870–4875.
- Hoa L, Kulaberoglu Y, Gundogdu R, Cook D, Mavis M, Gomez M, Gomez V, Hergovich A. 2016. The characterisation of LATS2 kinase regulation in Hippo-YAP signalling. *Cellular Signalling* **28**: 488–497.
- Hsieh M-H, Choe JH, Gadhvi J, Kim YJ, Arguez MA, Palmer M, Gerold H, Nowak C, Do H, Mazambani S, et al. 2019. p63 and SOX2 Dictate Glucose Reliance and Metabolic Vulnerabilities in Squamous Cell Carcinomas. *Cell Reports* **28**: 1860-1878.e9.
- Hu L, Liang S, Chen H, Lv T, Wu J, Chen D, Wu M, Sun S, Zhang H, You H, et al. 2017. Δ Np63 α is a common inhibitory target in oncogenic PI3K/Ras/Her2-induced cell motility and tumor metastasis. *Proc Natl Acad Sci USA* **114**: E3964–E3973.
- Huang J, Wu S, Barrera J, Matthews K, Pan D. 2005. The Hippo Signaling Pathway Coordinately Regulates Cell Proliferation and Apoptosis by Inactivating Yorkie, the Drosophila Homolog of YAP. *Cell* **122**: 421–434.
- Huang W, Lv X, Liu C, Zha Z, Zhang H, Jiang Y, Xiong Y, Lei Q-Y, Guan K-L. 2012. The N-terminal Phosphodegron Targets TAZ/WWTR1 Protein for SCF β -TrCP-dependent Degradation in Response to Phosphatidylinositol 3-Kinase Inhibition*. *Journal of Biological Chemistry* **287**: 26245–26253.
- Imajo M, Miyatake K, Iimura A, Miyamoto A, Nishida E. 2012. A molecular mechanism that links Hippo signalling to the inhibition of Wnt/ β -catenin signalling. *EMBO J* **31**: 1109–1122.
- Inman JL, Robertson C, Mott JD, Bissell MJ. 2015. Mammary gland development: cell fate specification, stem cells and the microenvironment. *Development* **142**: 1028–1042.
- Jung Y-S, Qian Y, Yan W, Chen X. 2013. Pirh2 E3 ubiquitin ligase modulates keratinocyte differentiation through p63. *J Invest Dermatol* **133**: 1178–1187.

- Justice RW, Zilian O, Woods DF, Noll M, Bryant PJ. 1995. The Drosophila tumor suppressor gene *warts* encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. *Genes Dev* **9**: 534–546.
- Kanai F, Marignani PA, Sarbassova D, Yagi R, Hall RA, Donowitz M, Hisaminato A, Fujiwara T, Ito Y, Cantley LC, et al. 2000. TAZ: a novel transcriptional co-activator regulated by interactions with 14-3-3 and PDZ domain proteins. *EMBO J* **19**: 6778–6791.
- Kango-Singh M, Nolo R, Tao C, Verstreken P, Hiesinger PR, Bellen HJ, Halder G. 2002. Shar-pei mediates cell proliferation arrest during imaginal disc growth in Drosophila. *Development* **129**: 5719–5730.
- Kastan N, Gnedeva K, Alisch T, Petelski AA, Huggins DJ, Chiaravalli J, Aharanov A, Shakked A, Tzahor E, Nagiel A, et al. 2021. Small-molecule inhibition of Lats kinases may promote Yap-dependent proliferation in postmitotic mammalian tissues. *Nat Commun* **12**: 3100.
- Kenny PA, Lee GY, Myers CA, Neve RM, Semeiks JR, Spellman PT, Lorenz K, Lee EH, Barcellos-Hoff MH, Petersen OW, et al. 2007. The morphologies of breast cancer cell lines in three-dimensional assays correlate with their profiles of gene expression. *Molecular Oncology* **1**: 84–96.
- Keyes WM, Pecoraro M, Aranda V, Vernersson-Lindahl E, Li W, Vogel H, Guo X, Garcia EL, Michurina TV, Enikolopov G, et al. 2011. Δ Np63 α Is an Oncogene that Targets Chromatin Remodeler Lsh to Drive Skin Stem Cell Proliferation and Tumorigenesis. *Cell Stem Cell* **8**: 164–176.
- Koster MI, Dai D, Marinari B, Sano Y, Costanzo A, Karin M, Roop DR. 2007. p63 induces key target genes required for epidermal morphogenesis. *PNAS* **104**: 3255–3260.
- Kouros-Mehr H, Slorach EM, Sternlicht MD, Werb Z. 2006. GATA-3 Maintains the Differentiation of the Luminal Cell Fate in the Mammary Gland. *Cell* **127**: 1041–1055.
- Lai Z-C, Wei X, Shimizu T, Ramos E, Rohrbaugh M, Nikolaidis N, Ho L-L, Li Y. 2005. Control of Cell Proliferation and Apoptosis by Mob as Tumor Suppressor, Mats. *Cell* **120**: 675–685.
- Lamar JM, Stern P, Liu H, Schindler JW, Jiang Z-G, Hynes RO. 2012. The Hippo pathway target, YAP, promotes metastasis through its TEAD-interaction domain. *PNAS*.
<https://www.pnas.org/content/early/2012/08/09/1212021109> (Accessed June 18, 2020).

- Lee GY, Kenny PA, Lee EH, Bissell MJ. 2007. Three-dimensional culture models of normal and malignant breast epithelial cells. *Nature Methods* **4**: 359–365.
- Lee J-H, Kim T-S, Yang T-H, Koo B-K, Oh S-P, Lee K-P, Oh H-J, Lee S-H, Kong Y-Y, Kim J-M, et al. 2008. A crucial role of WW45 in developing epithelial tissues in the mouse. *EMBO J* **27**: 1231–1242.
- Lee K-P, Lee J-H, Kim T-S, Kim T-H, Park H-D, Byun J-S, Kim M-C, Jeong W-I, Calvisi DF, Kim J-M, et al. 2010. The Hippo-Salvador pathway restrains hepatic oval cell proliferation, liver size, and liver tumorigenesis. *Proc Natl Acad Sci U S A* **107**: 8248–8253.
- Lei Q-Y, Zhang H, Zhao B, Zha Z-Y, Bai F, Pei X-H, Zhao S, Xiong Y, Guan K-L. 2008. TAZ promotes cell proliferation and epithelial-mesenchymal transition and is inhibited by the hippo pathway. *Mol Cell Biol* **28**: 2426–2436.
- Li J, Li Z, Wu Y, Wang Y, Wang D, Zhang W, Yuan H, Ye J, Song X, Yang J, et al. 2019. The Hippo effector TAZ promotes cancer stemness by transcriptional activation of SOX2 in head neck squamous cell carcinoma. *Cell Death Dis* **10**: 1–15.
- Li N, Singh S, Cherukuri P, Li H, Yuan Z, Ellisen LW, Wang B, Robbins D, DiRenzo J. 2008a. Reciprocal Intraepithelial Interactions Between TP63 and Hedgehog Signaling Regulate Quiescence and Activation of Progenitor Elaboration by Mammary Stem Cells. *Stem Cells* **26**: 1253–1264.
- Li X, Ottosson S, Wang S, Jernberg E, Boldrup L, Gu X, Nylander K, Li A. 2015. Wilms' tumor gene 1 regulates p63 and promotes cell proliferation in squamous cell carcinoma of the head and neck. *BMC Cancer* **15**: 342.
- Li Y, Kong F, Shao Q, Wang R, Hu E, Liu J, Jin C, He D, Xiao X. 2017. YAP Expression and Activity Are Suppressed by S100A7 via p65/NFκB-mediated Repression of ΔNp63. *Molecular Cancer Research* **15**: 1752–1763.
- Li Y, Zhou Z, Chen C. 2008b. WW domain-containing E3 ubiquitin protein ligase 1 targets p63 transcription factor for ubiquitin-mediated proteasomal degradation and regulates apoptosis. *Cell Death & Differentiation* **15**: 1941–1951.
- Liefer KM, Koster MI, Wang XJ, Yang A, McKeon F, Roop DR. 2000. Down-regulation of p63 is required for epidermal UV-B-induced apoptosis. *Cancer Res* **60**: 4016–4020.

- Lin Z, Xie R, Guan K, Zhang M. 2020. A WW Tandem-Mediated Dimerization Mode of SAV1 Essential for Hippo Signaling. *Cell Reports* **32**: 108118.
- Lindley LE, Curtis KM, Sanchez-Mejias A, Rieger ME, Robbins DJ, Briegel KJ. 2015. The WNT-controlled transcriptional regulator LBH is required for mammary stem cell expansion and maintenance of the basal lineage. *Development* dev.110403.
- Lin-Shiao E, Lan Y, Welzenbach J, Alexander KA, Zhang Z, Knapp M, Mangold E, Sammons M, Ludwig KU, Berger SL. 2019. p63 establishes epithelial enhancers at critical craniofacial development genes. *Sci Adv* **5**: eaaw0946.
- Liu C-Y, Zha Z-Y, Zhou X, Zhang H, Huang W, Zhao D, Li T, Chan SW, Lim CJ, Hong W, et al. 2010. The hippo tumor pathway promotes TAZ degradation by phosphorylating a phosphodegron and recruiting the SCF{beta}-TrCP E3 ligase. *J Biol Chem* **285**: 37159–37169.
- Liu-Chittenden Y, Huang B, Shim JS, Chen Q, Lee S-J, Anders RA, Liu JO, Pan D. 2012. Genetic and pharmacological disruption of the TEAD–YAP complex suppresses the oncogenic activity of YAP. *Genes Dev* **26**: 1300–1305.
- Lu L, Li Y, Kim SM, Bossuyt W, Liu P, Qiu Q, Wang Y, Halder G, Finegold MJ, Lee J-S, et al. 2010. Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. *Proceedings of the National Academy of Sciences* **107**: 1437–1442.
- Mailleux AA, Overholtzer M, Schmelzle T, Bouillet P, Strasser A, Brugge JS. 2007. BIM Regulates Apoptosis during Mammary Ductal Morphogenesis, and Its Absence Reveals Alternative Cell Death Mechanisms. *Developmental Cell* **12**: 221–234.
- Mana-Capelli S, McCollum D. 2018. Angiomotins stimulate LATS kinase autophosphorylation and act as scaffolds that promote Hippo signaling. *Journal of Biological Chemistry* **293**: 18230–18241.
- Mangiulli M, Valletti A, Caratozzolo MF, Tullo A, Sbisà E, Pesole G, D'Erchia AM. 2009. Identification and functional characterization of two new transcriptional variants of the human p63 gene. *Nucleic Acids Res* **37**: 6092–6104.
- Marshall CB, Beeler JS, Lehmann BD, Gonzalez-Ericsson P, Sanchez V, Sanders ME, Boyd KL, Pietsenpol JA. 2021. Tissue-specific expression of p73 and p63 isoforms in human tissues. *Cell Death Dis* **12**: 745.

- Mason DE, Collins JM, Dawahare JH, Nguyen TD, Lin Y, Voytik-Harbin SL, Zorlutuna P, Yoder MC, Boerckel JD. 2019. YAP and TAZ limit cytoskeletal and focal adhesion maturation to enable persistent cell motility. *J Cell Biol* **218**: 1369–1389.
- McBride KA, Ballinger ML, Killick E, Kirk J, Tattersall MHN, Eeles RA, Thomas DM, Mitchell G. 2014. Li-Fraumeni syndrome: cancer risk assessment and clinical management. *Nat Rev Clin Oncol* **11**: 260–271.
- Mehrazarin S, Chen W, Oh J-E, Liu ZX, Kang KL, Yi JK, Kim RH, Shin K-H, Park N-H, Kang MK. 2015. The p63 Gene Is Regulated by Grainyhead-like 2 (GRHL2) through Reciprocal Feedback and Determines the Epithelial Phenotype in Human Keratinocytes. *J Biol Chem* **290**: 19999–20008.
- Melino G. 2011. p63 is a suppressor of tumorigenesis and metastasis interacting with mutant p53. *Cell Death Differ* **18**: 1487–1499.
- Melino G, Memmi EM, Pelicci PG, Bernassola F. 2015. Maintaining epithelial stemness with p63. *Science Signaling* **8**: re9–re9.
- Memmi EM, Sanarico AG, Giacobbe A, Peschiaroli A, Frezza V, Cicalese A, Pisati F, Tosoni D, Zhou H, Tonon G, et al. 2015. p63 Sustains self-renewal of mammary cancer stem cells through regulation of Sonic Hedgehog signaling. *Proc Natl Acad Sci U S A* **112**: 3499–3504.
- Meng Z, Moroishi T, Mottier-Pavie V, Plouffe SW, Hansen CG, Hong AW, Park HW, Mo J-S, Lu W, Lu S, et al. 2015. MAP4K family kinases act in parallel to MST1/2 to activate LATS1/2 in the Hippo pathway. *Nat Commun* **6**: 8357.
- Mills AA, Zheng B, Wang X-J, Vogel H, Roop DR, Bradley A. 1999. p63 is a p53 homologue required for limb and epidermal morphogenesis. *Nature* **398**: 708–713.
- Moroishi T, Park HW, Qin B, Chen Q, Meng Z, Plouffe SW, Taniguchi K, Yu F-X, Karin M, Pan D, et al. 2015. A YAP/TAZ-induced feedback mechanism regulates Hippo pathway homeostasis. *Genes Dev* **29**: 1271–1284.
- Murakami H, Mizuno T, Taniguchi T, Fujii M, Ishiguro F, Fukui T, Akatsuka S, Horio Y, Hida T, Kondo Y, et al. 2011. LATS2 Is a Tumor Suppressor Gene of Malignant Mesothelioma. *Cancer Research* **71**: 873–883.

- Nedvetsky PI, Kwon S-H, Debnath J, Mostov KE. 2012. Cyclic AMP regulates formation of mammary epithelial acini in vitro ed. A. Yap. *MBoC* **23**: 2973–2981.
- Nguyen B-C, Lefort K, Mandinova A, Antonini D, Devgan V, Della Gatta G, Koster MI, Zhang Z, Wang J, di Vignano AT, et al. 2006. Cross-regulation between Notch and p63 in keratinocyte commitment to differentiation. *Genes Dev* **20**: 1028–1042.
- Ni L, Li S, Yu J, Min J, Brautigam CA, Tomchick DR, Pan D, Luo X. 2013. Structural basis for autoactivation of human Mst2 kinase and its regulation by RASSF5. *Structure* **21**: 1757–1768.
- Ni L, Zheng Y, Hara M, Pan D, Luo X. 2015. Structural basis for Mob1-dependent activation of the core Mst-Lats kinase cascade in Hippo signaling. *Genes Dev* **29**: 1416–1431.
- Niehrs C. 2012. The complex world of WNT receptor signalling. *Nat Rev Mol Cell Biol* **13**: 767–779.
- Nishio M, Hamada K, Kawahara K, Sasaki M, Noguchi F, Chiba S, Mizuno K, Suzuki SO, Dong Y, Tokuda M, et al. 2012. Cancer susceptibility and embryonic lethality in Mob1a/1b double-mutant mice. *J Clin Invest* **122**: 4505–4518.
- Ocaña OH, Córcoles R, Fabra Á, Moreno-Bueno G, Acloque H, Vega S, Barrallo-Gimeno A, Cano A, Nieto MA. 2012. Metastatic Colonization Requires the Repression of the Epithelial-Mesenchymal Transition Inducer Prrx1. *Cancer Cell* **22**: 709–724.
- Oh HJ, Kim MJ, Song SJ, Kim T, Lee D, Kwon S-H, Choi E-J, Lim D-S. 2010. MST1 Limits the Kinase Activity of Aurora B to Promote Stable Kinetochore-Microtubule Attachment. *Current Biology* **20**: 416–422.
- Orzol P, Nekulova M, Holcakova J, Muller P, Votesek B, Coates PJ. 2016. Δ Np63 regulates cell proliferation, differentiation, adhesion, and migration in the BL2 subtype of basal-like breast cancer. *Tumor Biol* **37**: 10133–10140.
- Overholtzer M, Zhang J, Smolen GA, Muir B, Li W, Sgroi DC, Deng C-X, Brugge JS, Haber DA. 2006. Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. *PNAS* **103**: 12405–12410.
- Paine TM, Soule HD, Pauley RJ, Dawson PJ. 1992. Characterization of epithelial phenotypes in mortal and immortal human breast cells. *International journal of cancer* **50**: 463–473.

- Park HW, Kim YC, Yu B, Moroishi T, Mo J-S, Plouffe SW, Meng Z, Lin KC, Yu F-X, Alexander CM, et al. 2015. Alternative Wnt Signaling Activates YAP/TAZ. *Cell* **162**: 780–794.
- Pennington KL, Chan TY, Torres MP, Andersen JL. 2018. The dynamic and stress-adaptive signaling hub of 14-3-3: emerging mechanisms of regulation and context-dependent protein–protein interactions. *Oncogene* **37**: 5587–5604.
- Plouffe SW, Meng Z, Lin KC, Lin B, Hong AW, Chun JV, Guan K-L. 2016. Characterization of Hippo Pathway Components by Gene Inactivation. *Molecular Cell* **64**: 993–1008.
- Poon CLC, Lin JI, Zhang X, Harvey KF. 2011. The Sterile 20-like Kinase Tao-1 Controls Tissue Growth by Regulating the Salvador-Warts-Hippo Pathway. *Developmental Cell* **21**: 896–906.
- Praskova M, Khoklatchev A, Ortiz-Vega S, Avruch J. 2004. Regulation of the MST1 kinase by autophosphorylation, by the growth inhibitory proteins, RASSF1 and NORE1, and by Ras. *Biochem J* **381**: 453–462.
- Praskova M, Xia F, Avruch J. 2008. MOBKL1A/MOBKL1B phosphorylation by MST1 and MST2 inhibits cell proliferation. *Curr Biol* **18**: 311–321.
- Prater MD, Petit V, Alasdair Russell I, Girardi RR, Shehata M, Menon S, Schulte R, Kalajzic I, Rath N, Olson MF, et al. 2014. Mammary stem cells have myoepithelial cell properties. *Nature Cell Biology* **16**: 942–950.
- Prince ME, Sivanandan R, Kaczorowski A, Wolf GT, Kaplan MJ, Dalerba P, Weissman IL, Clarke MF, Ailles LE. 2007. Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. *Proc Natl Acad Sci U S A* **104**: 973–978.
- Ramsey MR, He L, Forster N, Ory B, Ellisen LW. 2011. Physical association of HDAC1 and HDAC2 with p63 mediates transcriptional repression and tumor maintenance in squamous cell carcinoma. *Cancer Res* **71**: 4373–4379.
- Ramsey MR, Wilson C, Ory B, Rothenberg SM, Faquin W, Mills AA, Ellisen LW. 2013. FGFR2 signaling underlies p63 oncogenic function in squamous cell carcinoma. *J Clin Invest* **123**: 3525–3538.
- Rawat SJ, Araiza-Olivera D, Arias-Romero LE, Villamar-Cruz O, Prudnikova TY, Roder H, Chernoff J. 2016. H-ras Inhibits the Hippo Pathway by Promoting Mst1/Mst2 Heterodimerization. *Curr Biol* **26**: 1556–1563.

- Reginato MJ, Mills KR, Paulus JK, Lynch DK, Sgroi DC, Debnath J, Muthuswamy SK, Brugge JS. 2003. Integrins and EGFR coordinately regulate the pro-apoptotic protein Bim to prevent anoikis. *Nat Cell Biol* **5**: 733–740.
- Ribeiro-Silva A, Ramalho LN, Garcia SB, Zucoloto S. 2003. The relationship between p63 and p53 expression in normal and neoplastic breast tissue. *Archives of pathology & laboratory medicine* **127**: 336–340.
- Riege K, Kretzmer H, Sahm A, McDade SS, Hoffmann S, Fischer M. 2020. Dissecting the DNA binding landscape and gene regulatory network of p63 and p53 eds. A. Lal, M.E. Murphy, and J.-C. Bourdon. *eLife* **9**: e63266.
- Rieger ME, Sims AH, Coats ER, Clarke RB, Briegel KJ. 2010. The embryonic transcription cofactor LBH is a direct target of the Wnt signaling pathway in epithelial development and in aggressive basal subtype breast cancers. *Mol Cell Biol* **30**: 4267–4279.
- Rios AC, Fu NY, Lindeman GJ, Visvader JE. 2014. In situ identification of bipotent stem cells in the mammary gland. *Nature* **506**: 322–327.
- Ripamonti F, Albano L, Rossini A, Borrelli S, Fabris S, Mantovani R, Neri A, Balsari A, Magnifico A, Tagliabue E. 2013. EGFR through STAT3 modulates $\Delta N63\alpha$ expression to sustain tumor-initiating cell proliferation in squamous cell carcinomas. *Journal of Cellular Physiology* **228**: 871–878.
- Rocco JW, Leong C-O, Kuperwasser N, DeYoung MP, Ellisen LW. 2006. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. *Cancer Cell* **9**: 45–56.
- Romano R-A, Ortt K, Birkaya B, Smalley K, Sinha S. 2009. An Active Role of the ΔN Isoform of p63 in Regulating Basal Keratin Genes K5 and K14 and Directing Epidermal Cell Fate ed. P. Callaerts. *PLoS ONE* **4**: e5623.
- Romano R-A, Smalley K, Magraw C, Serna VA, Kurita T, Raghavan S, Sinha S. 2012. $\Delta Np63$ knockout mice reveal its indispensable role as a master regulator of epithelial development and differentiation. *Development* **139**: 772–782.

- Rossi M, Aqeilan RI, Neale M, Candi E, Salomoni P, Knight RA, Croce CM, Melino G. 2006. The E3 ubiquitin ligase Itch controls the protein stability of p63. *Proc Natl Acad Sci U S A* **103**: 12753–12758.
- Rozenberg JM, Zvereva S, Dalina A, Blatov I, Zubarev I, Luppov D, Bessmertnyi A, Romanishin A, Alsoulaiman L, Kumeiko V, et al. 2021. Dual Role of p73 in Cancer Microenvironment and DNA Damage Response. *Cells* **10**: 3516.
- Russo C, Osterburg C, Sirico A, Antonini D, Ambrosio R, Würz JM, Rinnenthal J, Ferniani M, Kehrlöesser S, Schäfer B, et al. 2018. Protein aggregation of the p63 transcription factor underlies severe skin fragility in AEC syndrome. *Proceedings of the National Academy of Sciences* **115**: E906–E915.
- Saladi SV, Ross K, Karaayvaz M, Tata PR, Mou H, Rajagopal J, Ramaswamy S, Ellisen LW. 2017. ACTL6A Is Co-Amplified with p63 in Squamous Cell Carcinoma to Drive YAP Activation, Regenerative Proliferation, and Poor Prognosis. *Cancer Cell* **31**: 35–49.
- Sanchez-Vega F, Mina M, Armenia J, Chatila WK, Luna A, La KC, Dimitriadoy S, Liu DL, Kantheti HS, Saghaflinia S, et al. 2018. Oncogenic Signaling Pathways in The Cancer Genome Atlas. *Cell* **173**: 321-337.e10.
- Serber Z, Lai HC, Yang A, Ou HD, Sigal MS, Kelly AE, Darimont BD, Duijf PHG, van Bokhoven H, McKeon F, et al. 2002. A C-Terminal Inhibitory Domain Controls the Activity of p63 by an Intramolecular Mechanism. *Mol Cell Biol* **22**: 8601–8611.
- Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat M-L, Wu L, Lindeman GJ, Visvader JE. 2006. Generation of a functional mammary gland from a single stem cell. *Nature* **439**: 84–88.
- Shehata M, Teschendorff A, Sharp G, Novcic N, Russell IA, Avril S, Prater M, Eirew P, Caldas C, Watson CJ, et al. 2012. Phenotypic and functional characterisation of the luminal cell hierarchy of the mammary gland. *Breast Cancer Research* **14**. <http://breast-cancer-research.biomedcentral.com/articles/10.1186/bcr3334> (Accessed April 27, 2017).
- Shibue T, Weinberg RA. 2017. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. *Nature Reviews Clinical Oncology*. <http://www.nature.com/doi/10.1038/nrclinonc.2017.44> (Accessed April 21, 2017).

- Shore AN, Rosen JM. 2014. Regulation of mammary epithelial cell homeostasis by lncRNAs. *The International Journal of Biochemistry & Cell Biology* **54**: 318–330.
- Soares E, Zhou H. 2018. Master regulatory role of p63 in epidermal development and disease. *Cell Mol Life Sci* **75**: 1179–1190.
- Song H, Mak KK, Topol L, Yun K, Hu J, Garrett L, Chen Y, Park O, Chang J, Simpson RM, et al. 2010. Mammalian Mst1 and Mst2 kinases play essential roles in organ size control and tumor suppression. *PNAS* **107**: 1431–1436.
- Soule HD, Maloney TM, Wolman SR, Peterson WD, Brenz R, McGrath CM, Russo J, Pauley RJ, Jones RF, Brooks SC. 1990. Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer research* **50**: 6075–6086.
- St John MA, Tao W, Fei X, Fukumoto R, Carcangiu ML, Brownstein DG, Parlow AF, McGrath J, Xu T. 1999. Mice deficient of Lats1 develop soft-tissue sarcomas, ovarian tumours and pituitary dysfunction. *Nat Genet* **21**: 182–186.
- Stankic M, Pavlovic S, Chin Y, Brogi E, Padua D, Norton L, Massagué J, Benezra R. 2013. TGF- β -Id1 Signaling Opposes Twist1 and Promotes Metastatic Colonization via a Mesenchymal-to-Epithelial Transition. *Cell Reports* **5**: 1228–1242.
- Stavridi ES, Harris KG, Huyen Y, Bothos J, Verwoerd P-M, Stayrook SE, Pavletich NP, Jeffrey PD, Luca FC. 2003. Crystal Structure of a Human Mob1 Protein: Toward Understanding Mob-Regulated Cell Cycle Pathways. *Structure* **11**: 1163–1170.
- Stemmler MP, Eccles RL, Brabletz S, Brabletz T. 2019. Non-redundant functions of EMT transcription factors. *Nature Cell Biology* **21**: 102–112.
- Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ. 2006. Purification and unique properties of mammary epithelial stem cells. *Nature*.
<http://www.nature.com/doi/10.1038/nature04496> (Accessed April 27, 2017).
- Straub WE, Weber TA, Schäfer B, Candi E, Durst F, Ou HD, Rajalingam K, Melino G, Dötsch V. 2010. The C-terminus of p63 contains multiple regulatory elements with different functions. *Cell Death Dis* **1**: e5–e5.

- Su X, Chakravarti D, Flores ER. 2013. p63 steps into the limelight: crucial roles in the suppression of tumorigenesis and metastasis. *Nature Reviews Cancer* **13**: 136–43.
- Su X, Paris M, Gi YJ, Tsai KY, Cho MS, Lin Y-L, Biernaskie JA, Sinha S, Prives C, Pevny LH, et al. 2009. TAp63 Prevents Premature Aging by Promoting Adult Stem Cell Maintenance. *Cell Stem Cell* **5**: 64–75.
- Sudol M. 1994. Yes-associated protein (YAP65) is a proline-rich phosphoprotein that binds to the SH3 domain of the Yes proto-oncogene product. *Oncogene* **9**: 2145–2152.
- Suh E-K, Yang A, Kettenbach A, Bamberger C, Michaelis AH, Zhu Z, Elvin JA, Bronson RT, Crum CP, McKeon F. 2006. p63 protects the female germ line during meiotic arrest. *Nature* **444**: 624–628.
- Sung H, Ferlay J, Siegel RL, Laversanne M, Soerjomataram I, Jemal A, Bray F. 2021. Global Cancer Statistics 2020: GLOBOCAN Estimates of Incidence and Mortality Worldwide for 36 Cancers in 185 Countries. *CA: A Cancer Journal for Clinicians* **71**: 209–249.
- Takahashi Y, Miyoshi Y, Takahata C, Irahara N, Taguchi T, Tamaki Y, Noguchi S. 2005. Down-Regulation of LATS1 and LATS2 mRNA Expression by Promoter Hypermethylation and Its Association with Biologically Aggressive Phenotype in Human Breast Cancers. *Clinical Cancer Research* **11**: 1380–1385.
- Tang F, Gao R, Jeevan-Raj B, Wyss CB, Kalathur RKR, Piscuoglio S, Ng CKY, Hindupur SK, Nuciforo S, Dazert E, et al. 2019a. LATS1 but not LATS2 represses autophagy by a kinase-independent scaffold function. *Nat Commun* **10**: 5755.
- Tang Z, Kang B, Li C, Chen T, Zhang Z. 2019b. GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis. *Nucleic Acids Res* **47**: W556–W560.
- Tapon N, Harvey KF, Bell DW, Wahrer DCR, Schiripo TA, Haber DA, Hariharan IK. 2002. salvador Promotes Both Cell Cycle Exit and Apoptosis in Drosophila and Is Mutated in Human Cancer Cell Lines. *Cell* **110**: 467–478.
- Tariki M, Dhanyamraju PK, Fendrich V, Borggreffe T, Feldmann G, Lauth M. 2014. The Yes-associated protein controls the cell density regulation of Hedgehog signaling. *Oncogenesis* **3**: e112.
- Thanos CD, Bowie JU. 1999. p53 Family members p63 and p73 are SAM domain-containing proteins. *Protein Sci* **8**: 1708–1710.

- Tomasini R, Tsuchihara K, Wilhelm M, Fujitani M, Rufini A, Cheung CC, Khan F, Itie-Youten A, Wakeham A, Tsao M-S, et al. 2008. TAp73 knockout shows genomic instability with infertility and tumor suppressor functions. *Genes Dev* **22**: 2677–2691.
- Tordella L, Koch S, Salter V, Pagotto A, Doondeea JB, Feller SM, Ratnayaka I, Zhong S, Goldin RD, Lozano G, et al. 2013. ASPP2 suppresses squamous cell carcinoma via RelA/p65-mediated repression of p63. *Proceedings of the National Academy of Sciences* **110**: 17969–17974.
- Tran T, Mitra J, Ha T, Kavran JM. 2020. Increasing kinase domain proximity promotes MST2 autophosphorylation during Hippo signaling. *J Biol Chem* **295**: 16166–16179.
- Tsai JH, Donaher JL, Murphy DA, Chau S, Yang J. 2012. Spatiotemporal Regulation of Epithelial-Mesenchymal Transition Is Essential for Squamous Cell Carcinoma Metastasis. *Cancer Cell* **22**: 725–736.
- Udan RS, Kango-Singh M, Nolo R, Tao C, Halder G. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nature Cell Biology* **5**: 914–20.
- Usary J, Llaca V, Karaca G, Presswala S, Karaca M, He X, Langerød A, Kåresen R, Oh DS, Dressler LG, et al. 2004. Mutation of *GATA3* in human breast tumors. *Oncogene* **23**: 7669–7678.
- Valencia-Sama I, Zhao Y, Lai D, Janse van Rensburg HJ, Hao Y, Yang X. 2015. Hippo Component TAZ Functions as a Co-repressor and Negatively Regulates Δ Np63 Transcription through TEA Domain (TEAD) Transcription Factor. *J Biol Chem* **290**: 16906–16917.
- Venkatesh D, O'Brien NA, Zandkarimi F, Tong DR, Stokes ME, Dunn DE, Kengmana ES, Aron AT, Klein AM, Csuka JM, et al. 2020. MDM2 and MDMX promote ferroptosis by PPAR α -mediated lipid remodeling. *Genes Dev* **34**: 526–543.
- Vincent-Mistiaen Z, Elbediwy A, Vanyai H, Cotton J, Stamp G, Nye E, Spencer-Dene B, Thomas GJ, Mao J, Thompson B. 2018. YAP drives cutaneous squamous cell carcinoma formation and progression ed. M.C. Frame. *eLife* **7**: e33304.
- Visvader JE, Stingl J. 2014. Mammary stem cells and the differentiation hierarchy: current status and perspectives. *Genes & Development* **28**: 1143–1158.
- Wang B, Fallon JF, Beachy PA. 2000. Hedgehog-Regulated Processing of Gli3 Produces an Anterior/Posterior Repressor Gradient in the Developing Vertebrate Limb. *Cell* **100**: 423–434.

- Wang L, Zhang Z, Yu X, Huang X, Liu Z, Chai Y, Yang L, Wang Q, Li M, Zhao J, et al. 2019a. Unbalanced YAP-SOX9 circuit drives stemness and malignant progression in esophageal squamous cell carcinoma. *Oncogene* **38**: 2042–2055.
- Wang X, Mori I, Tang W, Nakamura M, Nakamura Y, Sato M, Sakurai T, Kakudo K. 2002. p63 expression in normal, hyperplastic and malignant breast tissues. *Breast Cancer* **9**: 216–219.
- Wang Y, Li J, Gao Y, Luo Y, Luo H, Wang L, Yi Y, Yuan Z, Jim Xiao Z-X. 2019b. Hippo kinases regulate cell junctions to inhibit tumor metastasis in response to oxidative stress. *Redox Biol* **26**. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6582208/> (Accessed March 23, 2020).
- Wang Y, Xu X, Maglic D, Dill MT, Mojumdar K, Ng PK-S, Jeong KJ, Tsang YH, Moreno D, Bhavana VH, et al. 2018. Comprehensive Molecular Characterization of the Hippo Signaling Pathway in Cancer. *Cell Reports* **25**: 1304-1317.e5.
- Xiong S, Couzens AL, Kean MJ, Mao DY, Guettler S, Kurinov I, Gingras A-C, Sicheri F. 2017. Regulation of Protein Interactions by Mps One Binder (MOB1) Phosphorylation. *Mol Cell Proteomics* **16**: 1111–1125.
- Xu T, Wang W, Zhang S, Stewart RA, Yu W. 1995. Identifying tumor suppressors in genetic mosaics: the *Drosophila* *lats* gene encodes a putative protein kinase. *Development* **121**: 1053–1063.
- Yagi R, Chen L-F, Shigesada K, Murakami Y, Ito Y. 1999. A WW domain-containing Yes-associated protein (YAP) is a novel transcriptional co-activator. *EMBO J* **18**: 2551–2562.
- Yalcin-Ozuyisal Ö, Fiche M, Guitierrez M, Wagner K-U, Raffoul W, Brisken C. 2010. Antagonistic roles of Notch and p63 in controlling mammary epithelial cell fates. *Cell Death and Differentiation* **17**: 1600–1612.
- Yallowitz AR, Alexandrova EM, Talos F, Xu S, Marchenko ND, Moll UM. 2014. p63 is a prosurvival factor in the adult mammary gland during post-lactational involution, affecting PI-MECs and ErbB2 tumorigenesis. *Cell Death Differ* **21**: 645–654.
- Yang A, Kaghad M, Wang Y, Gillett E, Fleming MD, Dötsch V, Andrews NC, Caput D, McKeon F. 1998. p63, a p53 Homolog at 3q27–29, Encodes Multiple Products with Transactivating, Death-Inducing, and Dominant-Negative Activities. *Molecular Cell* **2**: 305–316.

- Yang A, Schweitzer R, Sun D, Kaghad M, Walker N, Bronson RT, Tabin C, Sharpe A, Caput D, Crum C, et al. 1999. p63 is essential for regenerative proliferation in limb, craniofacial and epithelial development. *Nature* **398**: 714–718.
- Yang A, Walker N, Bronson R, Kaghad M, Oosterwegel M, Bonnini J, Vagner C, Bonnet H, Dikkes P, Sharpe A, et al. 2000. p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. *Nature* **404**: 99–103.
- Yang A, Zhu Z, Kapranov P, McKeon F, Church GM, Gingeras TR, Struhl K. 2006. Relationships between p63 Binding, DNA Sequence, Transcription Activity, and Biological Function in Human Cells. *Molecular Cell* **24**: 593–602.
- Ye S, Lee KB, Park MH, Lee J-S, Kim SM. 2014. p63 regulates growth of esophageal squamous carcinoma cells via the Akt signaling pathway. *Int J Oncol* **44**: 2153–2159.
- Yi M, Tan Y, Wang L, Cai J, Li X, Zeng Z, Xiong W, Li G, Li X, Tan P, et al. 2020. TP63 links chromatin remodeling and enhancer reprogramming to epidermal differentiation and squamous cell carcinoma development. *Cell Mol Life Sci* **77**: 4325–4346.
- Yin F, Yu J, Zheng Y, Chen Q, Zhang N, Pan D. 2013. Spatial organization of Hippo signaling at the plasma membrane mediated by the tumor suppressor Merlin/NF2. *Cell* **154**: 1342–1355.
- Yoh K, Prywes R. 2015. Pathway Regulation of p63, a Director of Epithelial Cell Fate. *Frontiers in Endocrinology* **6**. <http://journal.frontiersin.org/article/10.3389/fendo.2015.00051/abstract> (Accessed June 8, 2016).
- Yoh KE, Regunath K, Guzman A, Lee S-M, Pfister NT, Akanni O, Kaufman LJ, Prives C, Prywes R. 2016. Repression of p63 and induction of EMT by mutant Ras in mammary epithelial cells. *Proc Natl Acad Sci U S A* **113**: E6107–E6116.
- Yu F-X, Zhao B, Panupinthu N, Jewell JL, Lian I, Wang LH, Zhao J, Yuan H, Tumaneng K, Li H, et al. 2012. Regulation of the Hippo-YAP Pathway by G-Protein-Coupled Receptor Signaling. *Cell* **150**: 780–791.
- Zanconato F, Forcato M, Battilana G, Azzolin L, Quaranta E, Bodega B, Rosato A, Bicciato S, Cordenonsi M, Piccolo S. 2015. Genome-wide association between YAP/TAZ/TEAD and AP-1 at enhancers drives oncogenic growth. *Nat Cell Biol* **17**: 1218–1227.

- Zhang H, Liu C-Y, Zha Z-Y, Zhao B, Yao J, Zhao S, Xiong Y, Lei Q-Y, Guan K-L. 2009. TEAD transcription factors mediate the function of TAZ in cell growth and epithelial-mesenchymal transition. *J Biol Chem* **284**: 13355–13362.
- Zhao B, Li L, Tumaneng K, Wang C-Y, Guan K-L. 2010. A coordinated phosphorylation by Lats and CK1 regulates YAP stability through SCF -TRCP. *Genes & Development* **24**: 72–85.
- Zhao B, Wei X, Li W, Udan RS, Yang Q, Kim J, Xie J, Ikenoue T, Yu J, Li L, et al. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes & Development* **21**: 2747–2761.
- Zhao B, Ye X, Yu J, Li L, Li W, Li S, Yu J, Lin JD, Wang C-Y, Chinnaiyan AM, et al. 2008. TEAD mediates YAP-dependent gene induction and growth control. *Genes Dev* **22**: 1962–1971.
- Zheng Y, Liu B, Wang L, Lei H, Pulgar Prieto KD, Pan D. 2017. Homeostatic Control of Hpo/MST Kinase Activity through Autophosphorylation-Dependent Recruitment of the STRIPAK PP2A Phosphatase Complex. *Cell Rep* **21**: 3612–3623.
- Zheng Y, Pan D. 2019. The Hippo Signaling Pathway in Development and Disease. *Developmental Cell* **50**: 264–282.
- Zheng Y, Wang W, Liu B, Deng H, Uster E, Pan D. 2015. Identification of Happyhour/MAP4K as Alternative Hpo/Mst-like Kinases in the Hippo Kinase Cascade. *Dev Cell* **34**: 642–655.
- Zhou D, Conrad C, Xia F, Park J-S, Payer B, Yin Y, Lauwers GY, Thasler W, Lee JT, Avruch J, et al. 2009. Mst1 and Mst2 maintain hepatocyte quiescence and suppress hepatocellular carcinoma development through inactivation of the Yap1 oncogene. *Cancer Cell* **16**: 425–438.
- Zhou M, Jiang J. 2022. Gli Phosphorylation Code in Hedgehog Signal Transduction. *Front Cell Dev Biol* **10**: 846927.
2018. The Cancer Genome Atlas Program - National Cancer Institute. <https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga> (Accessed February 27, 2022).