THE TUMOR SUPPRESSING ROLES OF TISSUE STRUCTURE IN CERVICAL CANCER DEVELOPMENT

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DEDICATION

I dedicate this dissertation to my parents Van Duc Nguyen and Sinh Thi Pham and my boyfriend Justin. Without their love and support I would never have reached this point.

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ABSTRACT

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THE TUMOR SUPPRESSING ROLES OF TISSUE STRUCTURE IN CERVICAL CANCER DEVELOPMENT

Cervical cancer is caused by the persistent infection of human papilloma virus (HPV) in the cervix epithelium. Although effective preventative care is available, the widespread nature of infection and the variety of HPV strains unprotected by HPV vaccines necessitate a better understanding of the disease for development of new therapies. A major tumor suppressing mechanism is the inhibition of cell division by tissue structure; however, the underlining molecular circuitry for this regulation remains unclear. Recently, the Yap transcriptional co-activator has emerged as a key growth promoter that mediates contact growth arrest and limits organ size. Thus, we aimed to uncover upstream signals that connect tissue organization to Yap regulation in the inhibition of cervical cancer. Two events that disrupt tissue structure were examined including the loss of the tumor suppressor LKB1 and the expression of the viral oncogene HPV16-E6. We identified that Yap mediates cell growth regulation downstream of both LKB1 and E6. Restoration of LKB1 expression in HeLa cervical cancer cells, which lack this tumor suppressor, or shRNA knockdown of LKB1 in NTERT immortalized normal human dermal keratinocytes, demonstrated that LKB1 promotes Yap phosphorylation, nuclear exclusion, and proteasomal degradation. The ability of phosphorylation-defective Yap mutants to rescue LKB1 phenotypes, such as reduced cell proliferation and cell size, suggest that Yap inhibition contributes to LKB1 tumor suppressor function(s). Interestingly, LKB1's suppression of Yap activity required neither the canonical Yap kinases, Lats1/2, nor metabolic downstream targets of LKB1, AMPK and mTORC1. Instead, the scaffolding protein NF2 was required for LKB1 to induce a specific actin cytoskeleton structure that associates with Yap suppression. Meanwhile,

HPV16-E6 promoted Yap activation in all stages of keratinocyte differentiation. E6 activated the Rap1 small GTPase, which in turn promoted Yap activity. Since Rap1 does not mediate differentiation inhibition caused by E6, E6 may play a role in promoting cell growth through Rap1-Yap activation rather than preventing growth arrest through the disruption of differentiation. Altogether, the LKB1-NF2-Yap and E6-Rap1-Yap pathways represent two examples of a novel phenomenon, whereby the structure of a cell directly influences its gene expression and proliferation.

Lawrence A. Quilliam, PhD, Chair

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
AMPK	AMP-activated protein kinase
BMP	Bone morphogenetic proteins
BSA	Bovine serum albumin
Cdc42	Cell division cycle 42
CIN	Cervical intraepithelial neoplasia
CK	Casein kinase
CRTC2	CREB regulated transcription coactivator 2
Dco	Discs overgrown
Dlg	Discs large
DMSO	Dimethyl sulfoxide
E6AP	E6-associated protein
E6TP1	E6-targeted protein 1
E-cadheren	Epithelial cadherin
ECM	Extracellular matrix
EMT	Epithelial-mesenchymal transition
Epac2	Exchange protein directly activated by cAMP 2
ErbB	Epidermal growth factor receptor
ERK	Extracellularly-regulated kinase
ERM	Ezrin radixin moesin
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FOXO3	Forkhead box O 3
G-actin	Globular actin
GAP	Guanosine triphosphatase activating protein
GDP	Guanosine diphosphate
GEF	Guanosine nucleotide exchange factor
GFP	Green fluorescent protein
GLUT1	Glucose transporter 1
Grb2	Growth factor receptor-bound protein 2
GST	Glutathione S-transferase
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphatase
HDAC5	Histone deacetylase 5
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hpo	Hippo
HPV	Human papilloma virus
hTERT	human telomerase reverse transcriptase
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
Lats	Large tumor suppressor
Lft	Lowfat
LKB1	Liver kinase B1
MAP	Microtubule-associated protein
MAP4K4	Mitogen-activated protein kinase kinase kinase kinase 4
MAPK	Mitogen-activated protein kinase
MAPT	Microtubule-associated protein Tau
MARK	Microtubule affinity-regulating kinases

Mats	Mob as tumor suppressor
MEK	MAPK/ERK kinase
Merlin	Moesin-ezrin-radixin-like protein
Mst	Mammalian STE20-like protein kinase
mTOR	Mammalian target of Rapamycin
mTORC1	mTOR complex 1
NF2	Neurofibromin 2
PAK1	p21-activated kinase 1
PALS1	Protein associated with Lin Seven 1
PAR1	Partitioning defective 1
PATJ	PALS1-associated tight junction protein
PBS	Phosphate buffered saline
PDZ	Post-synaptic density, Discs large, Zonula occludens
PI3K	Phosphatidylinositol 3-kinases
PKA	Protein kinase A
pRb	Retinoblastoma protein
Rac1	Ras-related C3 Botulinum toxin substrate 1
Rap	Ras-associate protein
Ras	Rat sarcoma
RASSF1A	Ras association (RalGDS/AF-6) domain family member 1A
Rho	Ras homologous
RTK	Receptor tyrosine kinases
RUNX	Runt-related transcription factor
S6K	S6 ribosomal protein kinase
Sav	Salvador
SCCA	Squamous cell carcinoma
Scrib	Scribbled homolog
SDS-PAGE	Sodium-dodecyl sulfate polyacrylamide gel electrophoresis
Sipa1L1	Signal-induced proliferation-associated 1-like protein 1
SOS	Son of sevenless
STAT	Signal transducers and activators of transcription
STK	Serine/threonine kinase
STRAD	STE20-related adaptor protein
Taz	Tafazzin
TEAD	Tea-domain
TGF	Transforming growth factor
TSC2	Tuberous sclerosis protein 2
Wnt	Wingless/integrated
Wts	Warts
WWC1	WW and C2 domain containing 1
WWTR1	WW domain containing transcription regulator 1
Yap	Yes tyrosine kinase-associated protein
Yki	Yorkie
ZO	Zona occluden
β-TRCP	Beta-transducin-repeat-containing protein

CHAPTER 1. INTRODUCTION

1.1. CERVICAL CANCER EPIDEMIOLOGY AND CAUSAL FACTORS

Cervical cancer is responsible for 275,000 deaths every year making it the third most common cause of cancer-related death in women worldwide (*1-3*). Persistent infection by certain strains of the Human Papilloma Virus (HPV) has been shown to be the major cause of these cervical cancer cases. This discovery has led to very effective preventative treatments such as routine screening and HPV vaccines (*3*). Unfortunately, not all strains of HPV are blocked by these vaccinations and there is a lack of routine screening in many regions of the world. Therefore, cervical cancer remains a major health concern worldwide.

Nearly 100 different types of papillomavirus have been identified, of which more than 35 types of HPV have been isolated in neoplastic lesions of the anogenital tract (*1*). These viral infections can be sexually transmitted or occur from mother to child. Both men and women can be infected by HPV. However, women are more at risk for HPV-related cancers because estrogen may promote cancer development and the internal locations of cervical tumors hinder detection until late in the diseases development (*1, 4*). HPV infects undifferentiated basal cells of the epithelia, where its genome is maintained at a low copy number. As the cells become increasingly differentiated in the upper dermal layers, the viral genome is amplified and encapsulated virons are sloughed off in the dead squames (*2*). Some strains of HPV are associated with benign lesions or warts. These strains, including HPV 6 and HPV 11, are considered “low risk.” High risk strains include HPV 16, 18, 31, and 45. These strains have transforming potential and can replicate in more differentiated epithelial layers. Thus, these high risk strains are more effective at preventing the host cell from terminally differentiating or dying (*5, 6*). Consequently, the high risk strains 16 and 18 account for over 70% of all cervical carcinomas (*1*).

After infection by high risk HPV, cervical cancer begins with an asymptomatic stage where abnormal epithelial (skin) cells are confined to a lesion known as cervical intraepithelial neoplasia (CIN) in the epithelium of the cervix. This stage is followed by a transition stage

characterized by moderate to severe dysplasia, in which small clusters of neoplastic cells proliferate and may micro-invade the basement membrane. However, these dysplastic cells are still confined to the CIN lesion. If left untreated, the tumor progresses to the invasive stage where the tumor cells extend deep into the underlying stroma of the cervix (Figure 1-1). Ultimately, cancerous cells will invade into blood vessels and lymphatic channels allowing metastatic spread of these cells to other organs (*1*).

Although high-risk HPV strains are detected in almost 100% of cervical cancer samples, cooperative mutations contribute significantly to the development of this disease (*1, 2*). Indeed, a great number of HPV infections are resolved spontaneously without developing into cervical cancer (*4*). Persistent viral infection causes host cells to have genomic instability that might lead to mutation in tumor suppressing genes. These cooperative mutations play important roles in the degree of malignancy and patient survival (Figure 1-1). An example of cooperative mutation is the loss of LKB1 tumor suppressor. Patients with cervical cancer that is deficient for LKB1 have a median survival of 13 months while survival associated with LKB1-wild type tumors is greater than 100 months (*7*). A different example, glucose transporter 1 is highly expressed while the differentiation promoter, Notch 1 is strongly suppressed in high-risk HPV lesions, cervical cancer, and metastatic cancer compared to preneoplastic lesions (*8*). Therefore, new therapeutic treatments will require a better understanding of the viral oncoproteins HPV-E6 and E7 as well as tumor suppressing mechanisms that were lost during this cancer progression.

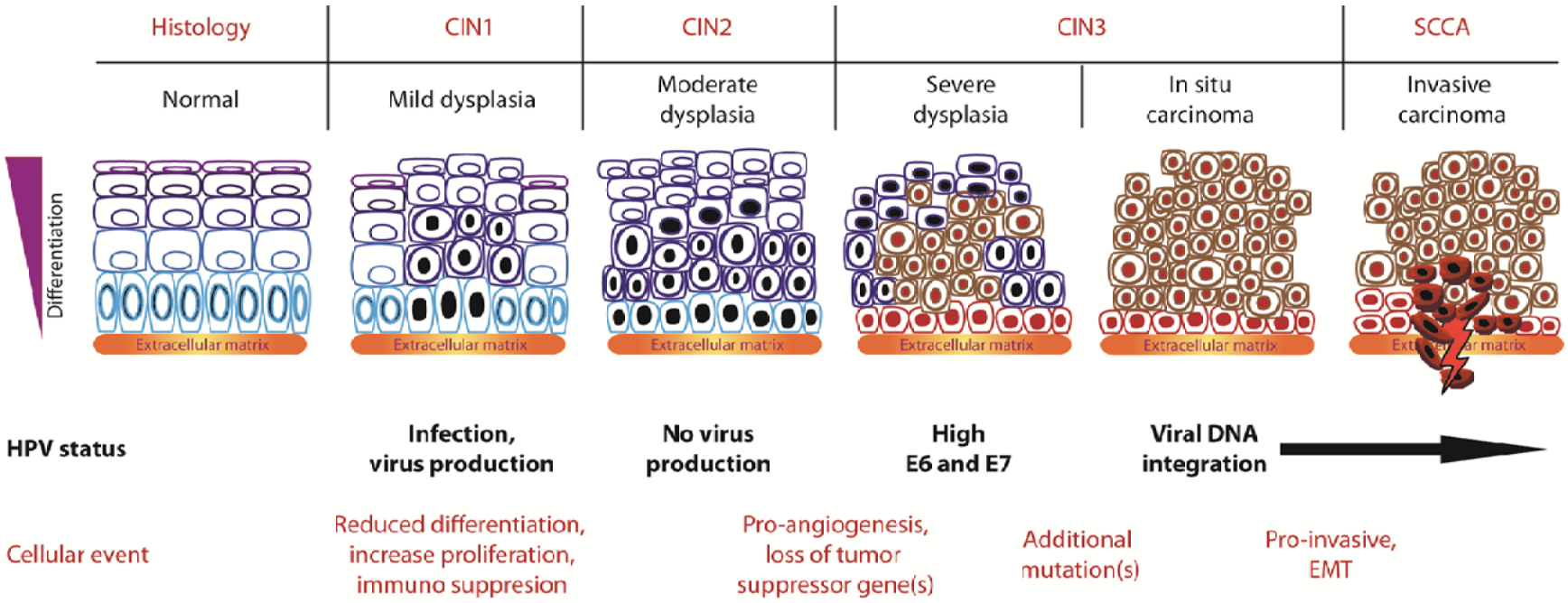

Figure 1-1. Cervical cancer progression illustration.

Upon Human Papillomavirus (HPV) infection, keratinocytes progress through various stages of dysplasia to become cancerous. Although the viral oncogenes HPV-E6 and HPV-E7 promote large number of cellular events leading to cancer, additional loss of tumor suppressor gene(s) are also essential in the cell's progression to invasive carcinoma. Two major morphological changes observed in this process are the loss of apical-basal polarity and the increase in cellular proliferation. The integration between polarity establishment and the control of proliferation remains unclear and it the focus of this dissertation.

1.2. NORMAL DIFFERENTIATION PROCESS AND CELL-CELL JUNCTIONS IN THE CREATION OF EPITHELIAL (SKIN) TISSUE

The formation of normal epithelial tissue structure not only protects the body from environmental insults it also serves to prevent the development of neoplastic growths. Breakdown in this tissue structure is associated with tumor growth, including cervical cancer. The epithelial structure consists of multiple cellular layers. The innermost layer of the epidermis is basal layer, followed by spinuous layer, granual layer, transitional layer, and cornified layer at the skin surface (Figure 1-2). These layers are made of several cell types including melanocytes, Langerhans (immune) cells, and the most abundant type, keratinocytes. Keratinocytes provide major structural components to the barrier layers of the skin (*9*). Keratinocytes first arise in the basal layer. Following cell division, most cells undergo a programmed process called differentiation as they migrate upward and away from the basal layer that retains the stem cells. As keratinocytes differentiate, they change both biochemically and morphologically. Matured keratinocytes contain two major cyto-structures called the intermediate filaments and the cornified envelope. The intermediate filaments are assembled from keratin monomers and the cornified envelope is assembled from involucrin and several membrane proteins (*10*). Keratinocytes with these cyto-structures attach to neighboring cells through adhesion proteins and lipids released from lamellar granules (Figure 1-2).

The differentiation process of keratinocytes is regulated both spatially and temporally by cell-cell adhesions and external stimuli such as vitamin A and calcium. Vitamin A drives differentiation upon being metabolized through various intermediates to retinoic acid, which binds to nuclear retinoic acid receptors. These receptors play an important role in modulating the transcriptions of genes that influence the differentiation process (*11*). Extracellular calcium level increases in the upper epithelial layers and this promotes the activation of proteins that mediate cell cohesion in the adherens junctions and tight junctions. Meanwhile, intracellular calcium

regulates the formation of desmosomal junctions and various calcium pump mutations lead to skin diseases such as Hailey-Hailey disease and Darier disease (*12-14*). Additionally, calcium activates transglutaminase, a protein at the interface between the living epidermal layers and the dead surface layer. This results in transglutaminase covalently attaching involucrin to membrane proteins for the formation of the cornified envelope surrounding the cells (*10*).

Depending on their differentiation stages, different types of cell junctions facilitate adhesion of keratinocytes to each other. Adherens and desmosomal junctions exist in the majority of skin layers, with the exception of the cornified layer. Tight junctions are formed at the granual layer just below the surface and specialized corneodesmosomal junctions are at the uppermost layer (Figure 1-2). Adherens junctions couple intercellular adhesion to the cytoskeleton therefore they relate a number of signaling cues important for cell shape, division, growth, and apoptosis. Adherens junctions are composed of strands of either the classical cadherin/catenin protein complexes or the nectin/afadin protein complexes. Classical cadherins are calcium-dependent cell adhesion molecules that interact with α and β-catenins via their cytoplasmic tail. Nectins are IgG-like cell adhesion molecules that bind to afadin. Both of these receptor complexes bind to and regulate the actin cytoskeleton. In the skin, desmosomal junctions play the major role in cell cohesion. Desmosomal junctions are composed of the desmosomal cadherins, which are members of the cadherin superfamily. The extracellular ends of desmosomal cadherins interact with each other, while the intracellular ends bind to adaptor proteins to form desmosomal plaques that anchor to keratin filaments. Many different types of desmosomal cadherins are continuously recycled or replaced throughout the life cycle of the keratinocytes. This dynamic process allows for changes in the composition of proteins in the desmosomes, their sizes, and stability according to the cell's stage of differentiation. Tight junctions form the barrier of the skin that regulates transcellular water, ions, and larger molecules. Tight junctions occur in both simple (single layer) epithelium and stratified (multi layers) epithelium where they separate the apical membrane domain from the basolateral part of the cell. The apical domain of the cell faces the lumen,

exterior environment while the basolateral part of the cell interacts with other cells or the cell-extracellular matrix (ECM). As cells enter the granual layer of the skin, tight junction proteins including Claudins, Occluden, and Zona Occluden (ZO) are assembled to form the tight junction structure. It remains unclear what signals in the granual layer promote this assembly since many tight junction components are expressed throughout the epidermal layers. Ultimately, keratinocytes are terminally differentiated to become corneocytes and commit to an epidermal programmed cell death similar to apoptosis (*15*). This dead surface layer of the skin (cornified layer) is structurally relevant to the skin's protective function. It consists of cells in cornified envelopes that are attached to each other by corneodesmosomal junction, which are static (unable to be recycled) desmosomal junctions. The corneodesmosomes are eventually degraded by adequate hydrolyses and corneocytes are sloughed off in dead squames (*16*).

The differentiation process allows keratinocytes to form various types of junctions. Conversely, the formation of cell junctions influences the differentiation process and the epithelial structure. These events are interconnected for the creation of a cohesive and flexible epithelium.

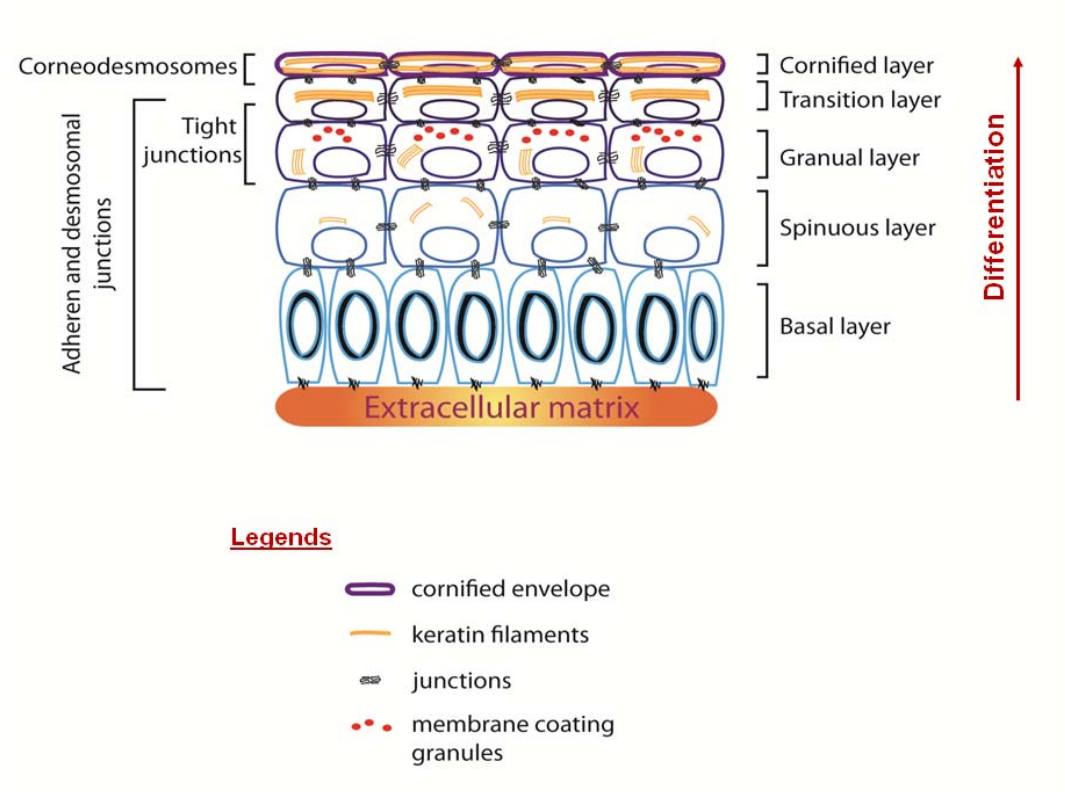

Figure 1-2. The differentiation process is required for the formation of functional layers of the skin.

Keratinocytes arise from the basal layer. As these cells proliferate and migrate upward, they undergo a programmed process where they differentiate into mature keratinocytes or corneocytes. Corneocytes have two major cyto-structures called the intermediate filaments and the cornified envelope. The intermediate filaments are assembled from keratin monomers and the cornified envelope is assembled from involucrin and several membrane proteins. Keratinocytes with these cyto-structures attach to neighboring cells through adhesion proteins and lipids released from the lamellar granules, forming a cohesive and flexible epithelium.

1.3. EPITHELIAL TISSUE STRUCTURE IS A MAJOR TUMOR SUPPRESSING MECHANISM

The epidermis turns over at an incredibly fast rate of 14 days to maintain an adult's skin. This equates to the creation of roughly 2 billion new epithelial cells each year (*9*). These cells often come in contact with cancer promoting agents such as UV light, free radicals, and chemical carcinogens. However, epithelial cancer is prevented in part by the tumor suppressing properties of the epithelial tissue structure.

Epithelial cells stop growing as they differentiate and are compacted in the epidermal layers. This is a common property of all differentiated tissues in a multicellular animal. The control of proliferation in conjunction with the regulation of cell death governs the shape and size of an organ during embryonic development, maintains tissue homeostasis, and allow for rapid growth in wound healing. As a tissue's structure increases in complexity, the regulation of growth and death become more intricate. The current perspective is that cancer cells arise upon defects in such regulation. For instance, the loss of E-cadheren and integrins, and hyper-activation of growth factor receptors such as ErbB2, TGF, and FGF receptors lead to disruption of tissue organization and the development of cancer (*17, 18*). Additionally, signaling pathways with important roles during embryonic development are often activated in cancers. This includes the JAK/STAT, Notch, MAPK/ERK, PI3K/AKT, NF-kB, Wnt and TGF-β pathways (*19*). Conversely, maintaining tissue homeostasis strongly inhibits cancer cell growth (*20*). Specifically, the skin structure suppresses the initiation of cancer by chemical carcinogens. This effect was discovered in the 1940s and is referred to as the "mutation/tumor suppressor" property of the skin (*21, 22*). A more recent study also found that epithelial tissue architecture provides resistance to c-myc induced cell growth, while disrupted structure cannot (*23*). Consistently, the loss of cell structure and differentiation is directly associated with cervical cancer. Indeed, physicians categorize tumor malignancy (also known as the grades of cervical intraepithelial

neoplasia (CIN)) by the degree of tissue structure disruption (Figure 1-1). The lowest risk of CIN progression to Squamous Cell Carcinoma (SCCA) is with CIN1, where undifferentiated cells and abnormal stratification (layer organization) are limited to the upper two-third of the epithelium. The highest risk is with CIN3, where abnormal cells occupy the full thickness of the epithelium and a complete loss of polarized tissue structure is observed (*24*).

Inhibition of proliferation by cell-cell contact is also observed in cultured immortalized mammalian cells. Non-cancerous cells grow to confluence and stop proliferating as they come in contact with each other. This phenomenon is called cell-cell contact inhibition of cell division (also known as postconfluence inhibition). Mirroring *in situ* carcinoma, the tumorigenicity of a number of cell lines are inversely correlated with their sensitivity to growth inhibition by contact with other cells (*25*). Thus, contact inhibition is a common assay for the study of proliferative regulation using cultured cells. This has been further refined by the development of three dimensional (3D) cultures. In addition to cell-cell interaction available in 2D monolayer, 3D culture provides cell-extracellular matrix (ECM) interactions due to cell adhesion to a laminin-rich matrix (Matrigel). Non-cancerous breast epithelial cells grow into acini in 3D culture. These acini represent a simple form of tissue structure. This culturing method confirmed that growth promoting pathways cause disturbance to the polarized structure, and the suppression of these signals restore acini architecture (*17*).

1.4. THE TRANSCRIPTIONAL CO-ACTIVATOR YAP REGULATES TISSUE HOMEOSTASIS

The Hippo pathway has recently emerged as a key inhibitor of epithelial cell proliferation to regulate tissue homeostasis (*26*). First identified in *Drosophila*, the Hippo pathway is a kinase cascade involving the kinase Hippo that phosphorylates and activates Warts. Active Warts then phosphorylates and inhibits the transcriptional co-activator Yorkie through cytoplasmic retention. Thus, Yorkie cannot localize to the nucleus and promote the transcription of proliferative genes once the Hippo kinases are activated. This pathway is conserved in mammalian cells with many redundancies (Table 1-1). Mst1 and 2 (mammalian ortholog of Hippo) activate Lats1 and 2 (Warts), which in turn suppresses Yap and Taz (Yorkie). In mammalian cell, Yap and Taz cooperate with transcription factors that are downstream of major developmental and cancer-promoting pathways such as SMADs (TGFβ and BMP pathways), β-catenin (WNT pathway), and TEAD (*27*). Thus, the Hippo kinases Mst and Lats function to inhibit cancer growth (*28-30*). During the formation of apical-basal polarity in epithelial cell, mammalian Yap, or its fly ortholog Yorkie, is down-regulated through interaction with cell junction proteins, such as α-catenin and the atypical cadherin, Fat, respectively (*31, 32*). Conversely, constitutive Yap expression induces epithelial-mesenchymal transition (EMT) in mammary cells grown in three-dimensional culture (*33*), indicating its oncogenic potential. Consistently, many studies have implicated Hippo signaling with cancer development. For instance, Yap overexpression (*34*) or loss of upstream suppressors Mst, Salvador, or Merlin/NF2 (*35*) lead to hepatomegaly and liver carcinomas. Furthermore, multiple human cancers have elevated Yap protein and nuclear localization (*36*).

Table 1-1. *Drosophila* members of the Hippo pathway and their mammalian orthologs.

Core components of the Hippo pathway (Hpo-Wts-Yki) were first identified in *Drosophila*, their mammalian orthologs were later found to have similar functions. These components' names are listed below.

Type	Human		Fly	
	Name	Abbreviation	Name	Abbreviation
Kinase	Mammalian STE20-like protein kinase	MST1/2 or STK3/4	Hippo	Hpo
Kinase	Large tumor suppressor	LATS1/2	Warts	Wts
Scaffold	Salvador homolog 1	Sav1 or WW45	Salvador	Sav
Scaffold	Mps One Binder kinase activator-like 1B	Mob1	Mob-as-tumor-suppressor	Mats
transcriptional co-activator	Yes tyrosine kinase-associated protein	Yap1/2	Yorkie	Yki
transcriptional co-activator	WW domain containing transcription regulator 1	Taz or WWTR1		

Since mammalian tissue structure is more complex, regulation of growth and death is also more intricate and fine-tuned in mammalian cells (Figure 1-3). The suppression of Yap is not an exception. Mass spectrometry analysis of Yap isolated from non-cancerous breast epithelial cells reveals 10 different phosphorylated serines. Guan's lab reported that five of these sites can be phosphorylated by Lats1 and Lats2 (Warts orthologs), including the key phosphorylation of serine 127 (*37*). In disagreement with this report, Sudol reported that Akt is the kinase that phosphorylates Yap on this site (*38*). Nonetheless, both labs agree that this phosphorylation site allows for 14-3-3 binding and consequently nuclear exclusion of Yap. Additionally, Yap contains a Post-synaptic density, Discs large, Zonula occludens (PDZ)-binding domain at its C-terminus. This binding domain allows Yap binding to Zona Occluden 2 (ZO-2) and is required for the transportation of Yap into the nucleus (*39*). Aside from 14-3-3 and ZO-2 binding, the localization of Yap is also regulated by mechanical forces such as stress fibers and adhesion surfaces (*40, 41*). Meanwhile, the phosphorylation of Serine 381 by Lats 1 and 2 was shown to prime Yap for further phosphorylation by Casein Kinases δ and ε. This event creates a phosphodegron sequence for an E3 ligase, β-TRCP, to bind to Yap for ubiquitylation and proteasomal degradation (*42*). Double mutation of S127 and S381 stabilizes Yap and promotes oncogenic phenotypes, but neither mutation alone can induce cellular transformation (*42*). Thus, Yap is regulated both spatially (nuclear exclusion) and temporally (degradation).

The signaling of Hippo kinases upstream of Yki is well worked out in *Drosophila* in contrast to events regulating Yap in mammals. The *Drosophila* membrane complex Fat-Lft-Dco translates extracellular stimuli to Expanded-Merlin-Kibra and nonconventional myosin Dachs. These proteins and complexes in turn promote the activation of Hippo kinases and Yorkie cytosolic retention. In contrast, the compositions of mammalian protein complexes that suppress Yap remain unclear. Controversy regarding Yap regulators in mammalian cells likely stems from redundant kinases, scaffolding proteins, and differential signals. In response to E-cadherin engagement, Lats is activated and Yap is suppressed; however, Mst 1 and 2 are not required for

Lats activation (*43*). Alternatively, in keratinocytes and in respond to mechanical forces, Yap is phosphorylated independent of Mst and Lats (*32, 41*). Whether these results suggest redundancy of Hippo kinases in certain mammalian systems will require further investigation. More importantly, no complex similar to the fly's Fat-Lft-Dco or Dachs has been identified. The hint that Dco is closely related to Casein Kinase 1 δ/ε, which have been found to phosphorylate and cause Yap degradation, suggest the conservation of such an inhibitory complex (*42*). Therefore, discoveries of upstream signals that connect tissue organization to Yap regulation in mammalian cells are of tremendous interests.

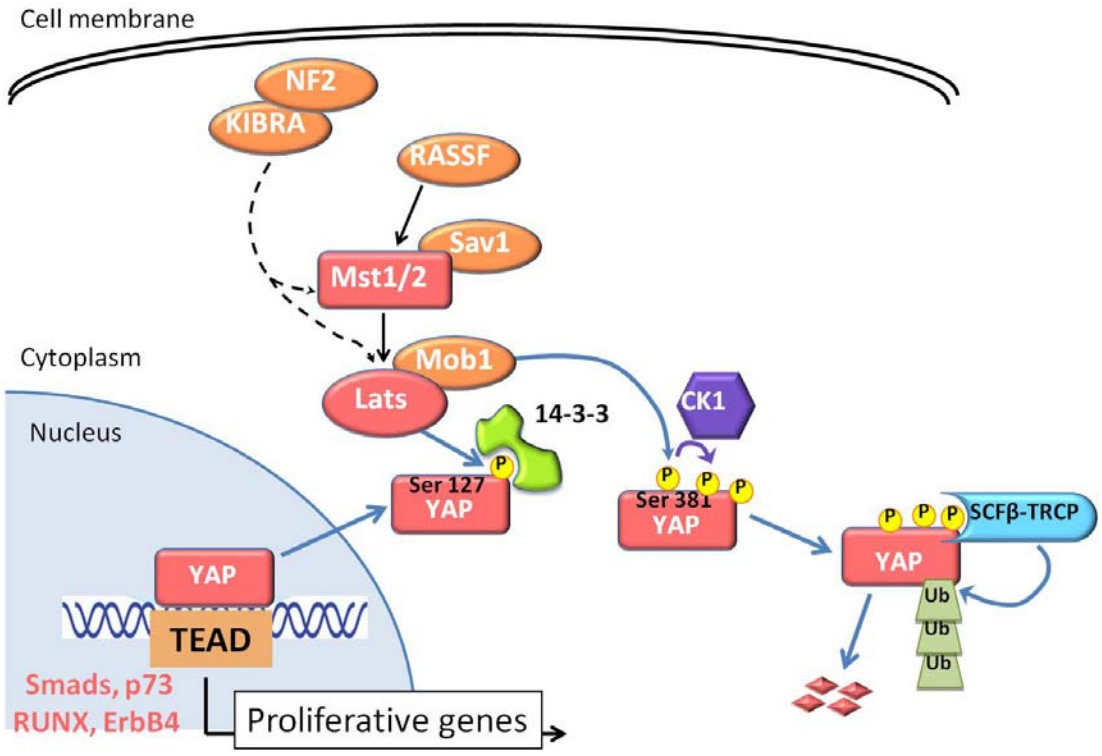

Figure 1-3. Yes-associated protein (Yap) is the endpoint of the Hippo kinase cascade that modulates tissue homeostasis.

Yap and its homolog Taz (or WWTR1) are subjected to regulation by hyper-phosphorylation by LATS1/2 and Casein kinase (CK1δ/ε), leading to nuclear exclusion and proteasomal degradation. These events prevent Yap/Taz from binding to and enhance transcriptional function of factors such as Tea-domain (TEAD) family, SMAD family, p53-related protein (p73), Runt-related transcription factor (RUNX), and Epidermal growth factor receptor 4 (ErbB4).

Unlike in Drosophila system, only a few upstream regulators, which relay signals from the cell membrane to the Hippo kinases, are established. These includes the neurofibromatosis type 2 (NF2 or Merlin) tumor suppressor, WW and C2 domain containing 1 (WWC1 or KIBRA), and Ras association (RalGDS/AF-6) domain family member 1 (RASSF1A).

1.5. THE LIVER KINASE B1 (LKB1/ STK11) CONTROLS BOTH METABOLISM AND TISSUE STRUCTURE

Another major regulator of tissue structure and organ size is the serine–threonine protein kinase LKB1 (liver kinase B1). Despite its name, LKB1 is ubiquitously expressed and it controls a wide range of cellular functions that include metabolism, polarity, and proliferation (*44*). LKB1 heterozygous patients suffer from Peutz-Jeghers syndrome that is characterized by gastrointestinal hamartomas and increased cancer predisposition. LKB1 inactivation is also frequently observed in sporadic non-small cell lung and cervical carcinomas (*44*, *45*).

LKB1 functions in complex with the pseudokinase STRAD and the scaffolding protein MO25. This LKB1 complex directly phosphorylates and activates AMP-activated protein kinase (AMPK) and 12 related kinases (*46*) (Figure 1-4). In response to the high levels of AMP accumulated during glucose starvation, AMPK suppresses mTOR complex 1 (mTORC1), a central regulator of cell metabolism. The suppression of mTORC1 by AMPK is mediated by the activation of a GTPase-activating protein (GAP) called tuberous sclerosis protein 2 (TSC2). TSC2 in turn inactivates a small GTPase Rheb, a potent activator of mTORC1. mTORC1 is reported to phosphorylate a number substrates including S6 ribosomal protein kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1), both involved in promotion of protein synthesis and cell size. mTORC1 also regulates many other cellular processes including protein synthesis, autophagy, lipid synthesis, mitochondrial metabolism/biogenesis, and cell cycle (*47*). LKB1 promotes efficient AMP-induced activation of AMPK and inhibition of mTORC1, thus inducing growth arrest in response to metabolic stress (*48*). Furthermore, AMPK also regulates transcription factors; activating FOXO3 and inhibiting CRTC2, HDAC5, and p300 (*49*). These transcription factors mediate transcriptional control of metabolism. Thus, AMPK/mTORC1 is regarded as the canonical pathway that mediates LKB1 tumor suppressor effects.

The role of LKB1 in suppressing gastrointestinal hamartomas, non-small cell lung, and cervical carcinomas implies LKB1 tumor suppressor function in the epidermis. Indeed, knockdown of LKB1 results in a loss of epithelial organization and increases Myc-dependent cell proliferation (*23*). Remarkably, LKB1 is the only protein whose activation is sufficient to polarize intestinal epithelial cells even in the absence of cell–cell contacts (*50*). The promotion of polarity by LKB1 is mediated by many signaling pathways (*49*). In general, LKB1 and its complex phosphorylate, translocate, and activate key kinases. These kinases include the microtubule affinity-regulating kinases (MARKs, also known as partitioning defective 1, par-1, kinases) family, the kinase suppressor of Ras 1 (KSR1), mammalian STE20-like protein kinase 4 (Mst4), AMPK, and brain-specific kinases (BRSK 1/2 or snRNP assembly-defective SAD1) (*49*). These kinases participate in a number of conserved polarity complexes to phosphorylate many substrates (illustrated in Figure 1-4). These substrates in turn facilitate the dynamics of microtubules and actin cytoskeleton for the formation of cell polarity. For instance, LKB1 phosphorylates and activates MARKs (par-1). MARKs in turn phosphorylate microtubule-associated proteins 2 and 4 (MAP2 and 4) and Tau (microtubule-associated protein tau, MAPT) for the regulation of microtubules (*51-53*). Alternatively, MARKs antagonize Dishevelled through phosphorylation and differentially regulate β-catenin and c-Jun N-terminal kinase (JNK) pathways downstream of Wnt activation (*54*) or E-cadherin engagement (*55*). Aside from metabolic control, LKB1-induced AMPK activation also promotes ZO-1 redistribution and enhancement of tight junctions (*49*). Additionally, upon E-cadheren engagement, the translocation of LKB1 complex with STRAD and MO25 recruits kinases like Mst4, which in turn activate Ezrin for the formation of brush border polarity (*56, 57*). In short, cellular polarity is a balancing act of cytoskeleton adjustment and protein distribution with LKB1 playing an essential part in regulation of key kinase activities.

While it is clear that LKB1 promotes cell polarization, it is unclear how tissue structure arising from this polarization leads to growth arrest. This thesis is the first study to examine the

possibility of cross-talk between LKB1 and the Hippo pathway in the suppression of cancer growth.

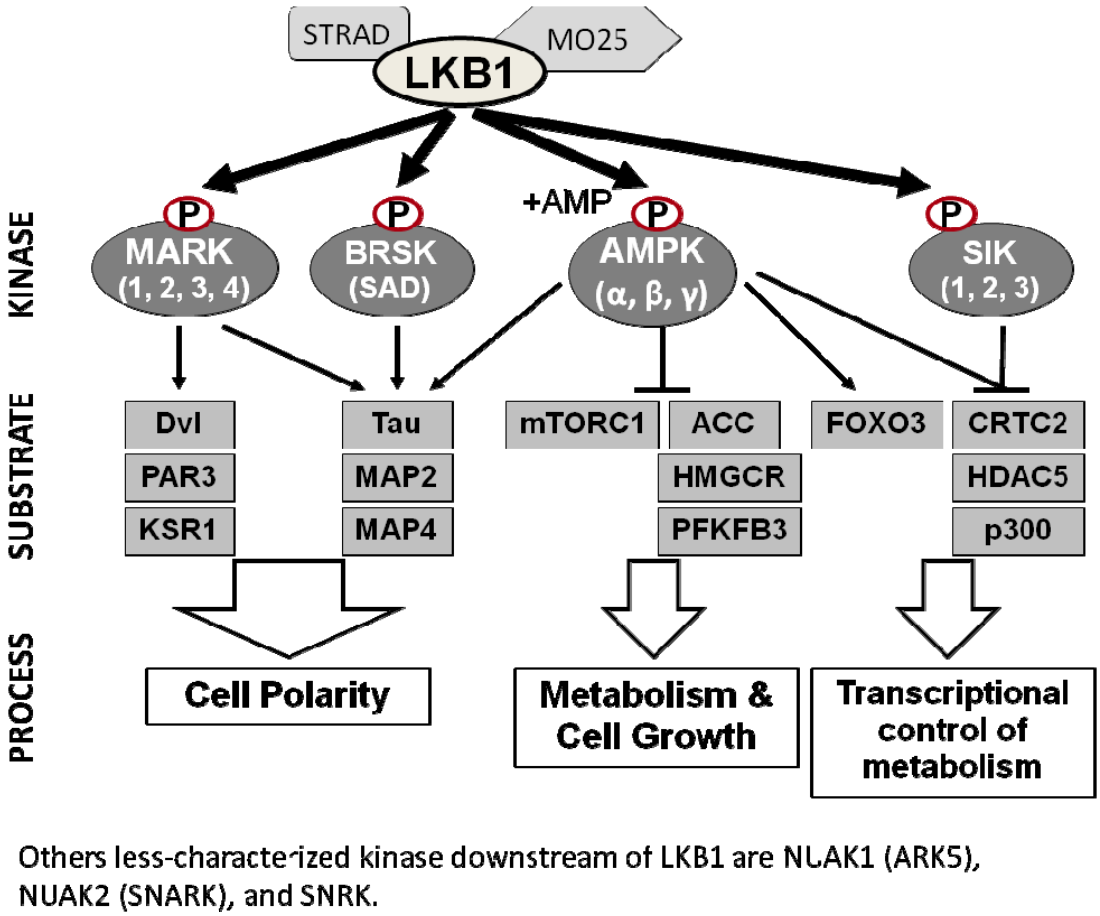

Figure 1-4. LKB1 is a master kinase that controls a wide range of substrates and cellular functions.

LKB1, in complex with the pseudokinase STRAD and the scaffolding protein MO25, directly phosphorylates and activates AMP-activated-protein-kinase (AMPK α, β, and γ) and 12 related kinases.

These families of kinases are: Microtubule affinity-regulating kinases (MARK 1, 2/PAR1, 3, and 4), Brain-specific kinase (BRSK 1, 2/snRNP assembly-defective SAD1), Salt-inducible kinase (1, 2/QIK, and 3/QSK), SNF-related kinase (SNRK), SNF1-like kinase1/AMPK-related kinase 5 (NUAK1/ARK5), and SNF1/AMP kinase-related kinase (NUAK2/SNARK).

The above kinases, in turn, phosphorylate and regulate a variety of substrates includes: Mammalian-target-of-rapamycin Complex 1 (mTORC1), acetyl-CoA carboxylase (ACC), HMG-CoA reductase (HMGCR), H6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 (PFKFB3) that regulate metabolism and cell growth. Forkhead box O3 (FOXO3), CREB-regulated transcription coactivator 2 (CRTC2), histone deacetylase 5 (HDAC5), and p300 histone acetyltransferase regulate transcription of metabolic genes. Dishevelled (Dvl), partitioning defective 3 (PAR3), kinase suppressor of Ras1 (KSR1), microtubule-associated protein (MAP), and microtubule-associated protein tau (Tau) that plays different roles in establishment of cell polarity.

1.6. THE SCAFFOLDING PROTEIN NF2 SUPPRESSES PROLIFERATION, IN PART, THROUGH THE INHIBITION OF YAP

A key suppressor of cell growth in response to cell-cell contact is Neurofibromatosis type 2 (NF2), also known as Moesin-ezrin-radixin-like protein (Merlin). The NF2 gene was first discovered due to its loss/mutation causing a dominant inherited tumor predisposition syndrome. Despite confusion due to the disease name, NF2 is not related to neurofibromatosis type 1 (cause by the mutation of the NF1 gene). NF2 is still classified as neurofibromatosis due to historical diagnosis even though NF2 patients infrequently have neurofibromas. In contrast, neurofibromatosis type 2 syndrome is characterized by the development of multiple schwannomas and meningiomas. These tumors cause a variety of symptoms such as hearing loss, imbalance, nausea or true vertigo, and a predisposition to other types of tumors (*58*). About 70% of NF2 patients have skin tumors ranging from intracutaneous lesions to more deep-seated subcutaneous tumors. These patient phenotypes strongly suggest the role of NF2 as a tumor suppressor. Similarly, NF2 heterozygous mice develop a variety of malignant tumors (*59, 60*). The NF2 deficient tumors derived from these mice have a greatly increase metastatic potential (*59*). Other studies also confirmed that NF2 plays a role in suppressing cancer cell proliferation, migration, and survival in melanoma (*61*), glioma (*62*), mesothelioma, and meningioma cells (*63, 64*).

NF2 (or Merlin) suppresses cell growth though its membrane-cytoskeleton scaffolding functions. The loss of NF2 leads to the activation of integrins, growth factor receptors, and receptor tyrosine kinases (RTKs) (*65-68*). The upregulation of integrins, such as integrin α6-β1 and -β4, enhances cell adhesion to the ECM and proliferation, while decreasing apoptosis (*66, 69*). NF2-deficient schwannoma cells release insulin-like growth factor-binding protein-1 (IGFBP-1) that functions through β1 integrin to activate focal-adhesion-kinase (FAK) and Src to promote growth (*69*). Additionally, NF2 forms a complex with E/N-cadherins and β-catenin;

thus, schwannoma cells have disrupted adherens junctions and upregulation of the Wnt/β-catenin pathway (*70*). Alternatively, NF2 promotes the endocytosis and recycling of at least four types of RTKs, including ErbB, vascular endothelial growth factor receptor (VEGFR), insulin-like growth factor 1 receptor (IGF1R), and platelet derived growth factor receptor (PDGFR) (*71*). The increased surface availability of these receptors leads to a strong activation of Ras/Raf/MEK/ERK and PI3K/AKT pathways in NF2 deficient cancer cells (*72*). Yet another mechanism of NF2 action is activation and membrane recruitment of the small GTPases Rac1, RhoA, Cdc42, and Ras (*68, 73-75*). Activation of these small GTPases might be a result of the above membrane proteins upregulation or a direct suppressing effect by NF2. For instance, NF2 expression might disrupt a complex composed of the ezrin/radixin/moesin (ERM) proteins, Grb2, SOS, Ras, and filamentous actin; therefore, NF2 inhibits growth factor signals mediated by Grb2-SOS (*68*). Alternatively, NF2 suppresses the Rac and Cdc42 downstream effector p21 activated kinases (PAKs) and subsequently JNK (*74, 76*). Thus, the loss of NF2 causes cell growth through the activation of a small GTPase at the membrane.

Interestingly, NF2 is structurally similar to the ERM proteins (*72*). Like ERM proteins, NF2 binds to and regulates the actin cytoskeleton. Unlike ERM proteins, the disruption of intramolecular interaction ("open" conformation) causes NF2 translocation away from the membrane (*77, 78*). A negative feedback exists whereby PAK phosphorylates NF2 on Serine 518 and dissociates NF2 from the cell membrane (*77-79*). Alternatively, cyclic AMP-dependent protein kinase A (PKA) also phosphorylates NF2 at this same residue (*80*). This phosphorylation promotes heterodimerization of NF2 and Ezrin (an ERM protein) which blocks NF2's suppression of cell growth (*77*). Additionally, homozygous knockout of NF2 is embryonic lethal to both mice and flies (*81, 82*), suggesting a non-redundant role of NF2 in development.

In addition to the above functions, NF2 (Merlin) also regulates the Hippo/Yap pathway. Similar to the Expanded-Merlin-Kibra complex in *Drosophila*, mammalian NF2 functions with Kibra and Expanded to regulate contact growth inhibition through the inhibition of Yki/Yap (*61,*

63, 64, 83). The knockdown of NF2 increases Yap expression and nuclear localization, and Yap activation is required for the increased proliferation and loss of contact inhibition in NF2-deficient mesothelioma and meningioma cells (*63, 64*). In melanoma and glioma cells, NF2 suppression of Yap is mediated by the activation of the Hippo kinase Mst and Lats (*61-63*). Surprisingly, in breast epithelial MCF10A cells, NF2 activates Lats independent of both Mst1 and 2 (*43*). A different study in epithelial cells, which confirmed NF2 negative regulation of Yap, found Yap phosphorylation independent of both Mst and Lats (*32*). These results suggest a possible redundancy of kinases in the epithelial cell while the scaffolding function of NF2 might be essential for bringing together growth inhibitory complexes.

1.7. HPV-E6 AND ITS DOWNSTREAM TARGETS CONTRIBUTE TO CERVICAL CANCER DEVELOPMENT

1.7.1. HPV-E6 – a viral protein that inhibits differentiation, apoptosis, and cellular polarity

As mentioned in section 1-1, persistent infection of high-risk HPV strains is the most common initiating event in the development of cervical cancer. Among viral proteins expressed early in the life cycle of HPV, two viral proteins E6 and E7 are responsible for cellular transformation. E7 binds to the retinoblastoma protein (pRb) and disrupts its complex formation with E2F transcription factors. Thus E7 can remove pRb's inhibition of the cell cycle and promote proliferation (*2, 84, 85*). E6 interacts with various cellular proteins and concomitantly with an E3 ubiquitin ligase called E6AP (*86, 87*). These interactions promote E6AP dependent-ubiquitination and proteasomal degradation of proteins that bind to the E6-E6AP complex. The major effects of E6, including the prevention of apoptosis and differentiation, are dependent on its degradation of the p53 tumor suppressor. p53 directly regulates a number of pro-apoptotic genes

as well as the Notch transcription factor, which plays an important role in promoting differentiation (*88, 89*).

However, p53 inhibition does not account for all E6 effects as some E6 mutants defective in p53 binding/degradation are still capable of preventing keratinocyte differentiation and apoptosis. Blocking these two processes leads to genomic instability (*84, 90, 91*). Furthermore, E6 from high risk HPV strains also enhances cancer malignancy by increasing cell spreading and adhesion. Therefore, other cellular targets of this multifunctional E6 protein are also of research interest for therapeutic purpose. Interestingly, high risk HPV-E6 is reported to promote the degradation of a number of PDZ domain-containing proteins such as hScrib, hDlg, MAGI, MUPP1, PATJ, and PTPN3 (*92*). These proteins are reported as parts of various polarity complexes and have crucial roles in cell junctions and tissue structure (*93, 94*). Consistent with this observation, the PDZ domain-binding motif was required for high-risk 31E6 to promote keratinocytes growth on organotypic raft cultures (*95*). These results suggest a possible role of E6 in disruption of contact inhibition.

1.7.2. Rap1 – a small GTPase with roles in junction formation and cell adhesion

A screen for cellular proteins targeted for degradation by the high risk HPV 16E6 identified E6-targeted protein 1 (E6TP1, Sipa1L1) (*96, 97*). Like p53, E6TP1 was also shown to be ubiquitinated by E6AP and targeted for proteasomal degradation (*98*). E6TP1 was later found to be a GTPase-activating protein (GAP), which deactivates a family of small molecular switch proteins, Ras-proximate protein (Rap) (*96*). There are several closely related members of the Rap family include Rap1A, Rap1B, Rap2A, Rap2B and Rap2C. Rap proteins are members of the Ras family. Like Ras, Rap shuttles between active GTP-bound and inactive GDP-bound states, upon activation by guanine nucleotide exchange factors (GEFs) and deactivation by GAPs (*99*). Rap1 and Rap2 exhibit 60% sequence identity and share a number of GEFs and GAPs. Redundant and distinct functions of Rap1 and Rap2 are still under investigation. Rap1 and Rap2 were first

considered as Ras antagonists because they compete for the Ras-binding domain of Raf-1 (also known as c-Raf) but do not activate it. This competition for Raf binding by Rap interferes with Ras transformation. Later, Rap1 was found to promote proliferation in cells that contain B-Raf through activation of the ERK mitogen-activated protein kinases pathway (*100*). Additionally, Rap2 may activate JNK through MAP4K4 to promote growth (*101*). Despite Rap1's possible roles in cell growth and transformation, its most notable roles are in cell junction formation, cell migration, and cancer invasiveness (*102-109*). In immune cells, Rap1 activates various integrins to regulate cell migration in response to chemokine signals. Increased Rap1 activity is associated with various types of leukemia and their malignancies (*99, 110, 111*). Rap1 also plays an essential role in the hematopoietic system where stem cells' attachment to their niche is important for their maintenance and functions (*99, 112*). On the other hand, Rap1 activation of integrins leads to increase cell-cell adhesion of endothelial and epithelial cells. Rap1 is also required for the maturation of adherens junctions through its regulation of vascular endothelial (VE)-cadherin (*113*) and epithelial (E)-cadherin (*105*). Since Rap1 regulates both cell-cell and cell-extracellular matrix (ECM) adhesion, it may have positive or negative influences on cancer development, depending on the specific cell type and its micro-environment (*99*). Intriguingly, Mina Bissell's lab reported that Rap1 is a crucial regulator of breast epithelial acinar structure in three-dimensional assays (*114*). Active Rap1 promotes cell growth and suppression of Rap1 activity restores tissue polarity and induces lumen formation (*114*). Thus at least in breast epithelial tissue, Rap1 up-regulation is a promoter of cancer malignancy.

1.8. HELA CELL AND NORMAL KERATINOCYTE (NTERT) ARE USED IN THIS STUDY

In order to understand the connection between tissue structure and growth control, this study utilized two cell lines: HeLa and normal keratinocytes (NTERT). These cell lines represent both ends of the spectrum in cervical cancer development. HeLa is a cervical cancer cell line that has not been manipulated to grow in cell culture. This cell line was isolated from a patient named Henrietta Lacks in 1951. HeLa has the high-risk HPV-18E6 and 18E7 genes integrated into its genome. HeLa also has 84 chromosomes (four copies of chromosome 12, three of 6, 8, and 17) and c-myc amplification. Although HeLa has been passaged for many decades in the tissue culture, its genome has remained astonishingly stable. This cell culture model interested us because HeLa cells have completely lost contact inhibition of growth and do not have LKB1 expression (*115*). Extraordinarily, LKB1 restoration to HeLa cells alone can recue cell polarity and contact inhibition, as further discussed in the results section of this thesis. This rescue also did not require the overexpression of LKB1 scaffold proteins such as STRAD and MO25. Thus, the ability to control the polarity of a cancer cell with a single protein expression is a great research tool.

On the other end of the spectrum, NTERT keratinocytes originated from normal human keratinocytes immortalized with hTERT. These cells have spontaneously lost $p16^{INK4A}$ expression bypassing the p16-imposed control of the pRb pathway. The molecular event for p16 loss has not been determined; although, reports suggest the mutation of the *trans*-acting regulator of p16. NTERT cells have normal p53 expression, EGF-dependent proliferation, and are sensitive to growth inhibition by phorbol esters (*116*). They also retain LKB1 expression and contact inhibition of cell division. Furthermore, they retain normal expression of differentiation markers such as keratin and involucrin, and form normal differentiating epidermal layers in organotypic culture (*116*). Additionally, NTERT cells have a normal genome without HPV integration. Thus,

NTERT cells are a normal and differentiable cell model which we can use to understand LKB1 and E6 downstream effects on tissue structure and Yap.

1.9. RATIONALE AND CENTRAL HYPOTHESIS

The *overarching goal* of this dissertation is to identify the molecular mechanisms that underline the ability of tissue structure to inhibit Yap and ultimately to suppress oncogenesis of cervical cancer. This central goal was formulated based on the published literature of Yap dominant roles in cell proliferation and Yap suppression in response to tissue structure formation and contact inhibition of cell division. Two events that lead to cervical cancer were examined in the context of Yap regulation: the loss of LKB1 tumor suppressor and the expression of the HPV-E6 viral oncogene. The *rationale* for the studies of these proteins is that both LKB1 and HPV-E6 affect cellular polarity and tissue structure. Furthermore, clinical studies showed that the loss of LKB1 associates with malignancy and greatly worsens the prognosis of HPV-positive cervical cancer (*7, 115, 117*). Thus, my *central hypothesis* is that Yap mediates cell growth regulation downstream of LKB1 loss and HPV-E6 expression in the promotion of cervical cancer. To test this hypothesis, three Aims were pursued: 1) Determine LKB1 regulation of Yap and cellular proliferation. 2) Examine the requirement of NF2 (Merlin), actin reorganization, and cellular polarity for LKB1 suppression of Yap. 3) Investigate the role of HPV-E6 and its downstream target E6TP1–Rap pathway in the inhibition of cell differentiation and the promotion of Yap activity.

CHAPTER 2. MATERIALS AND METHODS

Some text in this Chapter is copied from a publication by Nature Publishing Group:

Nguyen HB, Babcock JT, Wells CD, and Quilliam LA. LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap. Oncogene (2012) doi:10.1038/onc.2012.431

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2.1. GENERAL CELL CULTURE METHODS

NTERT keratinocytes cells (*116*) were grown in GIBCO keratinocyte media at 60 μM $CaCl_2$ supplemented with bovine pituitary extract (BPE), epidermal growth factor (EGF), and penicillin-streptomycin antibiotic mixture. For passaging, cells were washed once with Phosphate Buffered Saline (PBS), treated for 5 mins with Trypsin EDTA, and resuspended in Dulbecco's Modified Eagle Medium (DMEM)/ 10% fetal bovine serum (FBS) (Atlanta Biologicals). This step was added due to the lack of trypsin EDTA inactivating agents in normal keratinocyte media. The mixture was then spun down at 800 rpm for 5 mins. The high calcium media was removed and replaced with growth media. NTERT cells were induced to differentiate with 2.8 mM $CaCl_2$ added to growth media. Cells were frozen slowly in 10% DMSO + complete keratinocyte media. HeLa, 293gp, and 293T cells were grown in DMEM/antibiotics /10% FBS. The passaging method was the same as for NTERT cells except the spin down step was not necessary. Cells were frozen slowly in 10% DMSO + 20% FBS + DMEM.

2.2. GENERATION OF STABLE CELL LINES USING LENTI-VIRUS

Expression vector carrying gene of interest or pLKO vector carrying shRNA in combination with packaging plasmids (pRSV-REV, pMDLg/RRE, and VSV-G lenti) were transfected into 293T cells via calcium phosphate precipitation. Half the medium volume (example 5 ml for 10 cm plate) was changed next day. The following day, lenti-viruses were harvested and passed through 0.45 μm filters. Target cells were plated into filtered lenti-viral media containing a final concentration of 5 μg/ml polybrene. 48 hrs later, cells were selected in 1μg/ml puromycin until mock-transduced cells (no plasmid) were dead (usually 2 days exposure). Certain stable cell lines with growth disadvantage such as HeLa cells expressing LKB1 cells will lose expression after roughly 3 passages.

2.3. GENERATION OF STABLE CELL LINES USING RETRO-VIRALLY EXPRESSED HPV-16E6

pLXSN retroviral vector encoding HPV-16E6 was obtained from Dr. Roman's lab. Retrovirus was made by Lipofectamine-Plus transfection of this construct into Phoenix-ampho SD3443 cells (already stably express retroviral coating proteins). Alternatively, pLXSN-E6 in combination with VSV-G retro plasmid was transfected into 293gp cells via calcium phosphate precipitation or XtremeGeneHP. Half the medium was replaced next day. The following day, retro-viruses were harvested and passed through 0.45 μm filters. Viral supernatants with 1μg/ml polybrene were used to infect NTERT cells. After 3-6 hrs, cells were washed with PBS and returned to normal keratinocyte medium. Plates were split after 2 days and selected with 200 μg/ml G418 to establish stably expressing cell lines.

2.4. PLASMIDS, ANTIBODIES, AND CELL LYSIS BUFFERS

LKB1 was subcloned into pCDH (Systems Biosciences) following PCR amplification using EcoRI/NotI. Two plasmids available in the pCDH system: pCDH-Hygro (for untagged LKB1) and pCDH-Puro (LKB1 separate from GFP by a cleavable peptide). Both plasmids are for the generation of stable cell lines using lenti-virus delivering system. Alternatively, LKB1 was subclone into pQCXI-puro (Clonetech) via NotI/EcoRI sites for flag tagged protein. Rap1 WT and mutants were subcloned from pFlag-CMV using BglII/EcoRI sites and compatible overhang BamHI/EcoRI sites to pEGFP. Subsequently, the GFP-Rap1 fusion fragments were subcloned into pCSCG lenti viral vector using AgeI/SalI sites with partial SalI digestion. Note that pCSCG does not have mammalian selection. The GAP proteins such as E6TP1, KIAA1389, and SIPA were subcloned into donor vector 309-linker of the Creator System using a two-step-procedure. This vector 309-linker was modified from v309 to have polylinker with additional cloning sites

(see Appendix for more details). The two-step-cloning procedure involved subcloning the N-terminal portion (from 5'-AscI site up to an internal restriction site) following PCR amplification. Then the C-terminal portion was inserted to the same plasmid using internal and 3'end restriction sites. The N-termini of E6TP1, KIAA1389, and SIPA were flanked by AscI/HindIII, AscI/XhoI, and BamHI/StuI, respectively. Similarly, the C-termini were moved with HindIII/XbaI, XhoI/SpeI, and StuI/XbaI, respectively. The latter sites (XbaI and SpeI) were destroyed during cloning, thus flanking these GAPs remained AscI/PacI sites. RapGAPs were subcloned on the second reading frame into v309-L2 with modified polylinker using BglII/XbaI sites. Once again these sites were destroyed during cloning. cDNAs in donor vector 309 were recombined into acceptor vector with various tags such as myc, flag, GFP, CFP, and RFP using a homemade recombinase enzyme. Other plasmids included 5xGal4-luc (*118*). Gal4-Tead4, 2xFlagYap WT, S127A, S5A were purchased from AddGene (#24640, 19045, 27370, and 27371). pCDH-FlagYap WT and mutants were subcloned from above plasmids into pCDH-linker (a derivative of pCDH-Hygro containing a polylinker of multiple cloning sites) using NotI/XbaI. Vector YFP-occluden, cerulean-H2B, and *botulinum* C3 expression constructs were gifts from Clark Wells lab. Non-targeting controls were a scrambled luciferase sequence in pLKO.1 (Addgene #1864) for shRNA and ON-TARGET Plus (Dharmacon D001810-10-05) for siRNA.

Antibodies: Phospho-YapS127, LKB1, S6, phospho-S6, Phospho-acetyl coA carboxylase (ACC) and total ACC (Cell signaling), total Yap (Abnova), b-actin and U1snRNP (Santa Cruz), GAPDH (Meridian Life Science), NF2 and Taz from Wells lab (Cell signaling).

RIPA lysis buffers for whole cell lysate (WCL): buffer consisted of 50 mM Tris7.4, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% SDS. Additives were phosphatase inhibitors 5 mM NaF and 10 mM beta-glycerol phosphate, and proteasome inhibitors PMSF and aprotinin concentrations

2.5. TRANSIENT TRANSFECTION METHODS

2.5.1. Calcium Phosphate precipitation cellular transfection method

This method was used to transfect 293T cells for protein expression or lenti virus generation. In short, the appropriate amount of plasmid DNA, $CaCl_2$, and water were mixed. An equal volume of 2X HEPES buffered saline (HBS – 6.5g HEPES, 8g NaCl, 0.2g Na2HPO4-7H2O in 500 mL water, pH 7.05) was added to the DNA mixture drop-wise with continuous vortex for form a fine precipitation. After 20 mins at room temp, the final mixture was added to plates of 293T cells. For virus generation, 2 to 3 million cells per 10 cm plate were required.

2.5.2. XtremeGene (Roche) transfection method

This method caused minimal cytotoxicity and could be used for a number of different cell lines including HeLa cells. Roche's protocol was followed. In short, serum- and antibiotic-free DMEM was used. Plasmid DNA and transfecting reagent were incubated in this media for 15 to 30 mins. The mixture was then added to the cells (in regular growth medium), followed by next day media change.

2.5.3. Lipofectamine-2000 (Invitrogen) transfection method

This method was more cytotoxic but was required for the efficient transfection of siRNA. Briefly, cells were washed once with PBS and placed in serum- and antibiotic-free DMEM. Plasmid DNA and transfecting reagent were also incubated in this media for 15 to 30 mins. The mixture was then added to the cells for 3 to 4 hrs. Then cells were washed and placed back in normal media.

Note: a number of transfecting reagents and methods were tried on NTERT keratinocytes; however, transfection efficiency was low 10 to 20% at best. Thus, NTERT had to be infected for gene expression or knockdown studies.

2.6. THE EXPRESSION AND PURIFICATION OF GST-FUSION PROTEINS IN BACTERIA

pGEX RalGDS RBD or other proteins with GST-fusion were retransformed into E.coli strain Rosetta for protein production. Small culture was grown at 37C overnight with Ampicillin antibiotic before transfer to 500 mL culture. Large culture was grown to ~OD 0.6 to 0.8 at 600 nm (usually 3 hrs). Bacteria cells were induced with IPTG at 18C overnight. Upon harvesting, cells were lysed in GST Bacterial Lysis Buffer (20 mM Tris 8.0, 500 mM NaCl, 1 mM EDTA, 1% TritonX-100). Three different methods can be used to homogenize the bacterial mixture: French Press, freeze-thaw and passage through a 20G syringe, or sonication. A combination of syringe passage and sonication were acceptable. Homogenous mixture were spun down at 10,000 rpm for 20 minutes and the supernatant were collected contain GST-fusion proteins. These can be load onto PBS washed GSH beads for precipitation assay of snap freeze in aliquots.

2.7. RAP1 ACTIVITY ASSAY

NTERT keratinocyte were differentiated for the indicated days. Cells were lysed in cold condition to prevent GTP hydrolysis such as cold PBS wash, cold tumble, and cold RAL Lysis Buffer (50 mM Tris pH 7.4, 200 mM NaCl, 2.5 mM MgCl2, 10% Glycerol, and 1% IGEPAL) supplement with proteasome inhibitors. Rap1-GTP was measured by precipitation of Rap1-GTP from described keratinocyte lysates using a GST fusion protein containing the ras binding domain (RBD) of RalGDS immobilized on agarose beads as described (*119*). Bound proteins were eluted off the beads using sample buffer, size-separated by SDS-PAGE, transferred to Immobilon membrane, and blotted with Ras 142-24E05 antibody (that recognizes Rap1). 2 to 5% input lysate were also immunoblotted with Ras142 antibody to compare total Rap1 levels between samples.

2.8. THE RELATIVE QUANTIFICATION OF RNA LEVELS

RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's protocol. Briefly, cells were homogenized in TRIzol reagent prior to the addition of chloroform. Following phase separation, RNA was precipitated from the aqueous fraction using 50% isopropanol, washed with 70% ethanol, and dissolved in RNase-free water. First strand cDNA synthesis using M-MuLV reverse transcriptase was performed according to New England Biolabs protocol. This protocol involves 2 μg RNA, oligo dT primer, dNTP, RNAse inhibitor (Promega), buffer, and reverse transcriptase. Since oligo dT was used as primer, only mRNA containing polyA should be made into cDNA. Next, qRT-PCR detection of transcripts was performed using LightCycler-RNA Master Mix (Roche) and the Roche Universal Probes Library according to the manufacturer's protocol. Primer sequences and probe numbers are listed in the appendix. CTGF mRNA levels were normalized to GAPDH or RPLP0 (ribosomal protein, large, P0) expression. It is worth noting that the mRNA level of actin changes dramatically and GAPDH level also exhibited a small change upon LKB1 knockdown in NTERT cells; thus, RPLP0 is the only reliable standard in this case.

2.9. THREE DIMENSION MATRIGEL ASSAY (*17*)

Each stable cell line was trypsinized and 2 x 10^5 cells were plated between two layers of growth factor reduced Matrigel matrix (BD). Briefly, plates were pre-coated with a thin layer of Matrigel for 20 mins at 37C. After addition of cells, 10% Matrigel solution was used as top overlay. Full serum media was changed every 2 days. After 8-10 days, cell clumps were visualized using a Stereo Microscope (Nikon SMZ1500).

2.10. TEAD REPORTER ASSAY

0.1 μg Gal4-Tead4 and 0.8 μg 5xGal4-luc were transfected using either Lipofectamine2000 (Invitrogen) or XtremeGene HP (Roche). Two days later, firefly luciferase signals were measured in cell lysates using Promega kit (E153A and E1483) and a BioSystem Luminometer.

2.11. CELL ACCUMULATION ASSAY

Stable cell lines were seeded sparsely at 5 x 10^4 cells/plate into six or nine identical 35 mm plates. At the indicated times, three of these plates were trypsinized and resuspended in 500 μl media. Immediately, 50 μl of the cell suspension were mixed with 50 μl trypan blue and cells counted on a hemocytometer.

2.12. NUCLEAR FRACTIONATION METHOD

After PBS wash, attached cells were incubated in Fractionation Buffer (10 mM HEPES 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2% IGPAL) for 15 mins at 4C. If phospho-proteins were measured, the aforementioned phosphatase inhibitors were added. The IGPAL detergent amount was increased up to 1% for differentiated NTERT cells for cleaner fractionation. Cells were spun for 10 mins at 3,000 rpm. The cytoplasmic fraction was removed and remaining cell nuclei pellets were washed twice and resuspended in RIPA buffer for 10 mins. Nucleic fraction was collected by spinning for 10 mins at 14,000 rpm.

2.13. FLUORESCENCE MICROSCOPY

For immunofluorescence staining, cells were plated on treated cover glass (Deckglaser) placed inside culture plates. Two days later, cover glasses were carefully removed, washed, and fixed with 100% methanol at -20C for 8 minutes. Endogenous Yap was stained with Yap antibody and Texas red secondary antibody (Invitrogen T862) according to Cell Signaling's immunofluorescence protocol. Fluorescence images were taken with AxioVisionCam using a Zeiss Observer confocal microscope.

For live cell imaging of fluorescent proteins, cells were seeded sparsely on MatTek glass bottom plates (P35G-0-14-C). Two days after transfection of fluorescent protein-encoding plasmids, cells were viewed with the above Zeiss microscope.

2.14. CELL SIZE ANALYSIS

Cells were plated on 60 mm plates two days ahead of analysis. Cells were trypsinized and resuspended in DMEM/10% FBS media. The mixture was then spun down, media was removed and replaced with 200 to 500 μl of PBS + 0.2% BSA. Cell sizes were analyzed with a FACSCalibur machine. First measurement by red/green channels separated different populations with high or low LKB1-GFP expression. The mean forward scatter (FSC) of each population above was calculated and normalized to vector control to obtain relative cell size. Three samples of each population were analyzed for average relative cell sizes ± std dev.

2.15. DRUGS, EGTA TREATMENT, AND NUTRIENT STRESS

The following drug concentrations and manufacturers catalog numbers were used: 100 μg/ml cycloheximide (Sigma #66-81-9), 10 μM MG132 (CalBiochem #474790), 250 μM Torin1 (N.S.Gray DFCI).

EGTA treatment of HeLa cells with LKB1 restoration was incubation with DMEM 10% FBS, 25 mM HEPES, and 4 mM EGTA. Since DMEM has 2.8 mM Ca^{2+}, this treatment was closed to 1 mM EGTA treatment.

Nutrient stress: glucose starvation used glucose deficient DMEM (GIBCO 11966) with 10% FBS, Glutamine starvation use DMEM no glutamine.

2.16. WESTERN BLOT, DENSITOMETRY, AND STATISTICS

WB (immunoblot): WCL or immunoprecipitates were resolved on 10-12% SDS-PAGE and transfer to PVDF-FL membranes for immunoblotting. Immunoreactive bands were visualized with ECL, ECL Prime (GE Healthcare, Piscataway, NJ), or Supersignal Femto (Pierce) reagents. Films were developed using ALL-PRO 100 Plus processor. Alternatively, P-Yap and total Yap proteins (antibodies from different species) were viewed simultaneously using the Odyssey infrared imaging system (Biosciences).

Densitometric images from three independent experiments were quantified using ImageJ software (NIH). Normalized data is presented as mean + standard deviation (error bars).

P-values from Student T-test. ANOVA one-way were done with Origin 8.5.1 software.

CHAPTER 3. LKB1 REGULATES AMPK/MTOR-INDEPENDENT CELL GROWTH AND PROLIFERATION VIA THE INHIBITION OF YAP

Some text and figures in this Chapter are copied from a publication by Nature Publishing Group:

Nguyen HB, Babcock JT, Wells CD, and Quilliam LA. LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap. Oncogene (2012) doi:10.1038/onc.2012.431

3.1. INTRODUCTION

Growth and development are regulated by a balance between proliferation and apoptosis that is linked to tissue structure through poorly understood mechanisms. Deregulation of this balance results in the hyperproliferation of cancer cells as well as gross changes in cell morphology and tissue organization (*120*). For instance, the malignancy of cervical cancer, which arises from the epithelial layer of the cervix, is categorized by the degree of tissue disorganization. Thus, tissue structure is believed to be a tumor suppressing mechanism that controls cell proliferation. Recently, two crucial pathways have emerged that govern this mechanism: the Hippo pathway and signals triggered by LKB1. The Hippo pathway inhibits Yap transcription co-activator from promoting transcription of proliferative genes. Coordinately, Hippo suppression of Yap mediates contact inhibition of cell division, regulates tissue maintenance, and is a major regulator of organ size (*29, 49*). On the other hand, the tumor suppressor LKB1 is a master regulator of metabolism and apical-basal polarity. LKB1 reduces cell size and cell cycle through the central regulators of metabolism AMPK and mTORC1 (*49*). Additionally, LKB1 provides resistance to hyperplasia through establishment of epithelial structure (*23*); however, it is unclear how LKB1-promoted polarity suppresses cell growth. Despite the strong logical link between these two pathways, no evidence of their association has previously been reported.

Using HeLa cells that lack LKB1 expression, or alternatively suppressing its expression in non-transformed human keratinocytes, we now show that the regulation of cell size and proliferation by LKB1 are at least in part mediated by Yap inhibition. In HeLa, LKB1 restoration increased Yap phosphorylation, leading to nuclear exclusion and decreased stability. LKB1-induced growth arrest and cell size shrinkage could be partially reversed by expression of phosphorylation-defective Yap mutants. Similarly, LKB1 knock-down by shRNAs in keratinocytes increased cell sizes and endogenous transcription of CTGF, a Yap downstream

effect. Interestingly, these phenomena occurred independently of mTOR, AMPK or Lats1/2 activation, suggesting a non-canonical pathway links LKB1 activity to Yap phosphorylation. This work identifies a novel link between two major tumor suppressor pathways and a potential mechanism for cell polarity to restrict cancer cell growth.

3.2. RESULTS

3.2.1. LKB1 functions in promoting apical-basal polarity

Epithelial tissue structure is established through the formation of cell-cell junctions and apical-basal polarity whereby certain organelles and proteins are asymmetrically distributed. Interestingly, LKB1 is the only reported protein whose activation is sufficient to polarize intestinal epithelial cells in the absence of cell–cell contacts (*50*). Reciprocally, knockdown of LKB1 results in a loss of epithelial organization and increases Myc-dependent cell proliferation (*23*). Since cervical cancer cells such as HeLa have lost apical-basal polarity and lack LKB1 expression, HeLa was chosen as our cancer model cell line. Using lentiviral transduction, LKB1 was stably expressed in HeLa cells (HeLa LKB1), in contrast no LKB1 was detected in HeLa cells stably expressing GFP alone (HeLa Vec) (Figure 3-1, left panel). Alternatively, lenti-virus carrying shRNAs targeting LKB1 were used to suppress its endogenous expression in 293T kidney cells or NTERT non-transformed human keratinocytes (Figure 3-1, left panel). Consistent with previous report of LKB1 function, phase contrast images of cells with LKB1 restoration and knockdown showed that LKB1 promotes an epithelial-like morphology even in cancer cell lines (Figure 3-1, right panel). This polarized morphology promoted by LKB1 was even more pronounced when HeLa LKB1 cells were grown in three-dimensional matrix (in later Figure 3-6 A).

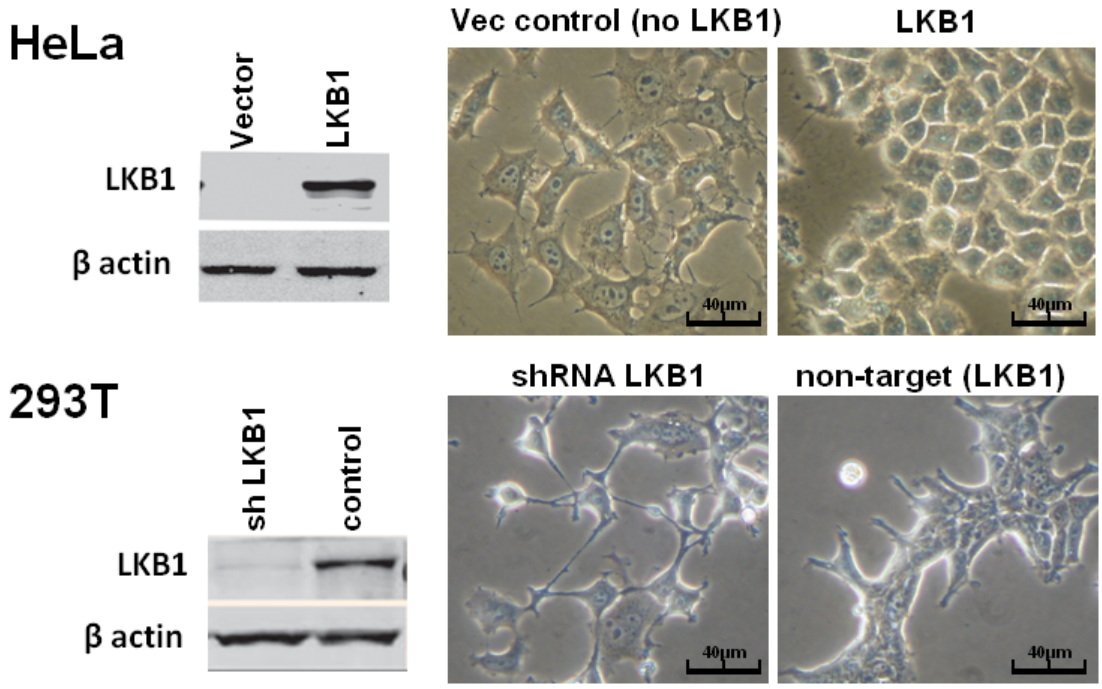


Figure 3-1. LKB1 expression promotes an epithelial-like cell morphology.

HeLa cells, that lack LKB1 expression, were transduced with lentivirus expressing LKB1 or GFP vector control. Alternatively, endogenous expression of LKB1 in 293T cells was suppressed using target specific shRNA. LKB1 expression was confirmed with immublot (left panel) and phase-contrast images of these cells were taken (right panel).

3.2.2. LKB1 tumor suppressor is a novel inhibitor of Yap oncoprotein

The Hippo pathway functions as part of a crowd control mechanism to limit organ size by reducing cell proliferation (*29*). In cultured cells, Hippo signaling is activated by high cell density, leading to phosphorylation and inhibition of the transcriptional co-activator Yap (*29*). Meanwhile, the tumor suppressor LKB1 promotes polarity as seen in above figure. To investigate the potential for crosstalk between LKB1 and the Hippo pathway, the effect of LKB1 expression on the inhibitory phosphorylation site Yap^{S127} (*37*), was measured. Correlated with epithelial-like morphology even at low cell density, LKB1 expression raised the phospho-YapS127/total Yap ratio to a level comparable to that seen in the NTERT non-cancerous immortalized human keratinocytes (Figure 3-2 A). Alternatively, the knockdown of LKB1 using two different shRNAs in NTERT cells promotes an increase in Yap protein level (Figure 3-2 B). These data suggested an inhibitory relationship between LKB1 and Yap.

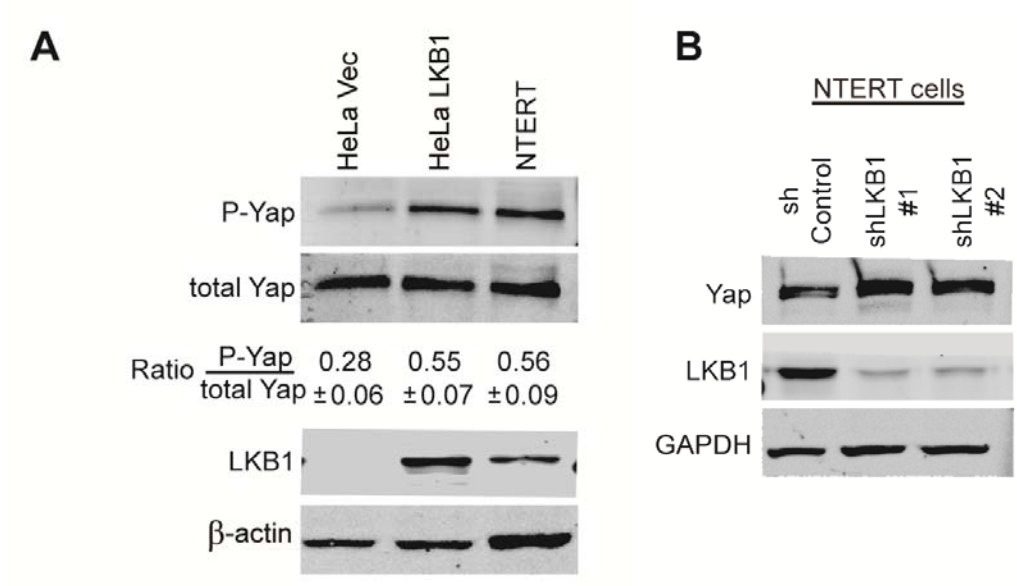


Figure 3-2. LKB1 increases Yap phosphorylation and knockdown of LKB1 increases total Yap protein.

A. The relative levels of Yap, phospho-YapSer127, LKB1 and -actin were detected by immunoblot analysis of HeLa cell lysates prepared following infection with lentivirus expressing LKB1 (HeLa LKB1) or GFP control (HeLa Vec) vectors and compared to uninfected NTERT immortalized human keratinocytes. The mean ratio of phospho-YapSer127/YapTotal from three experiments is indicated below a representative immunoblot. Error was computed as standard deviation of the mean.

B. NTERT cells were infected with two independent lentiviruses encoding LKB1 shRNAs (#1 and #2) or a non-specific control sequence. After stable selection on puromycin, the relative levels of endogenous LKB1 and GAPDH from these lysates were measured by immunoblot.

Blots are representatives of at least three independent experiments.

3.2.3. LKB1 expression reduced proliferative gene transcription downstream of Yap in HeLa and NTERT

Yap binds and stimulates the transcriptional activity of Tea-domain (TEAD) transcription factors to promote cell growth (*121*). Thus, Yap activity was inferred by TEAD-induced gene expression from a luciferase reporter system. HeLa cells were co-transfected with TEAD reporter and LKB1, a kinase dead LKB1, or vector control. The expression of LKB1 reduces the luciferase signals by approximately 5 fold. This inhibition required LKB1 kinase activity since the kinase-dead mutant failed to suppress luciferase activity (Figure 3-3 A). To test the effect of LKB1 on exogenously expressed Yap, we transfected cells with various ratios of LKB1- and Yap-encoding plasmids. Remarkably, this reporter system is sensitive to as little as 20 ng of exogenous Yap. While no changes were observed upon transfection of control vector, increasing the amount of transfected LKB1-encoding plasmid resulted in dose-dependent inhibition of Yap activity (Figure 3-3 B). Another measure of Yap activity is the transcription of connective tissue growth factor (CTGF) (*121*). Consistent with TEAD-luciferase data, LKB1 restoration drastically reduced CTGF mRNA level (Figure 3-3 C). Conversely, we employed shRNAs to examine the effect of endogenous LKB1 knockdown on Yap activity in NTERT keratinocytes. Suppression of LKB1 expression by two separate shRNAs greatly enhanced CTGF transcription as determined by qRT-PCR in NTERT (Figure 3-3 D) and TEAD activity in 293T cells (Figure 3-3 E). These data clearly indicated that LKB1 expression inhibits Yap transcriptional function.

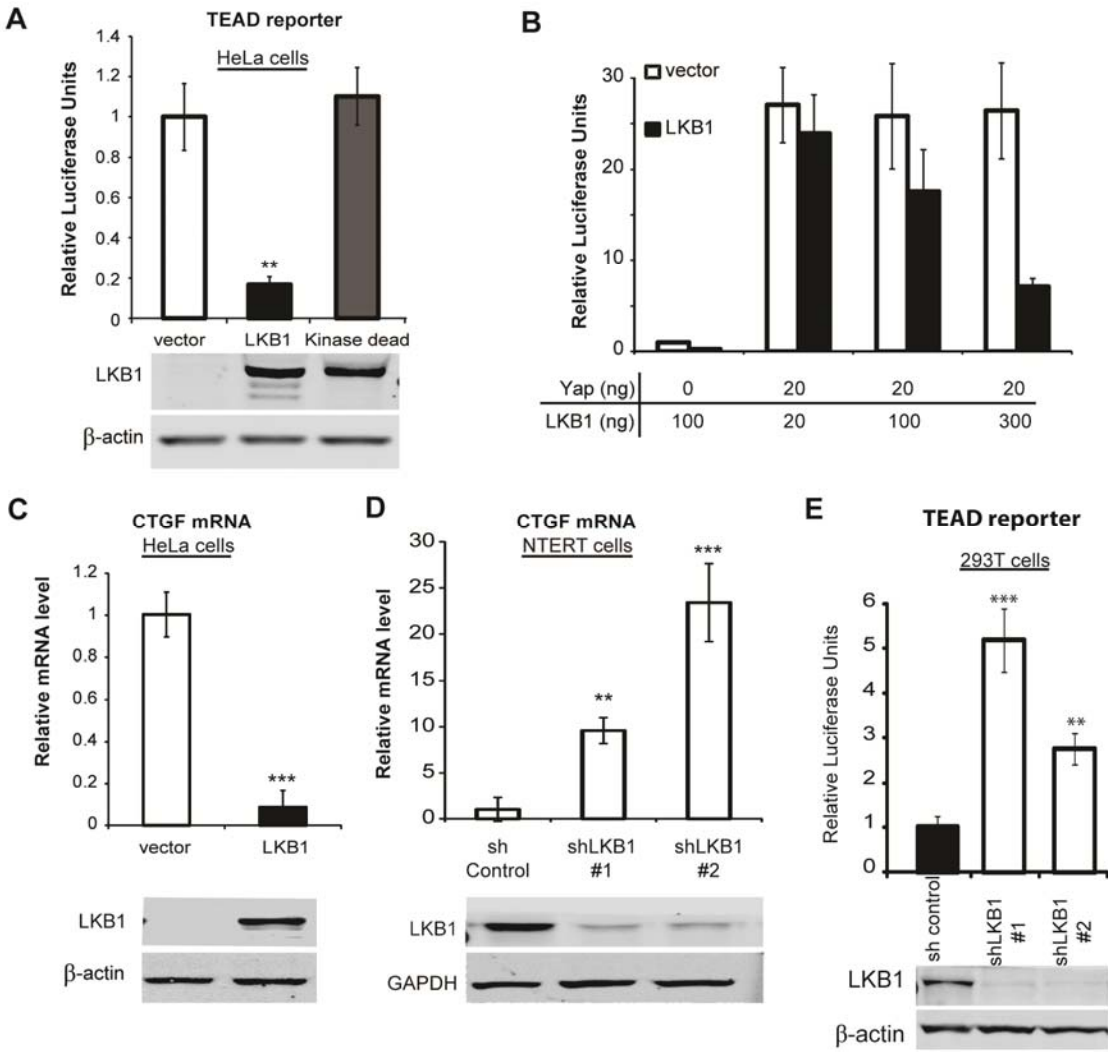


Figure 3-3. LKB1 inhibits Yap dependent transcription in HeLa, 293T, and NTERT cells.

A. HeLa cells were co-transfected with a luciferase reporter system containing a TEAD response element along with WT LKB1, kinase-dead LKB1, or empty vector. Yap-dependent transcription was inferred from luciferase signals after 48 hr and LKB1 expression confirmed by immunoblot.

B. TEAD-driven transcription was measured as in part A using HeLa cells transfected with the indicated amount of LKB1 and Yap expressing plasmids (fixed Yap level).

C. Endogenous HeLa cell CTGF transcript was measured by RT-PCR following infection with control or LKB1-encoding plasmids. Stable LKB1 expression confirmed by immunoblot.

D. CTGF mRNA was measured in NTERT cells as in part B following stable expression of LKB1 shRNAs #1 or #2 or a non-specific control sequence. Knockdown confirmed by immunoblot.

E. TEAD assay as in part A and knockdown as in part D using 293T cells.

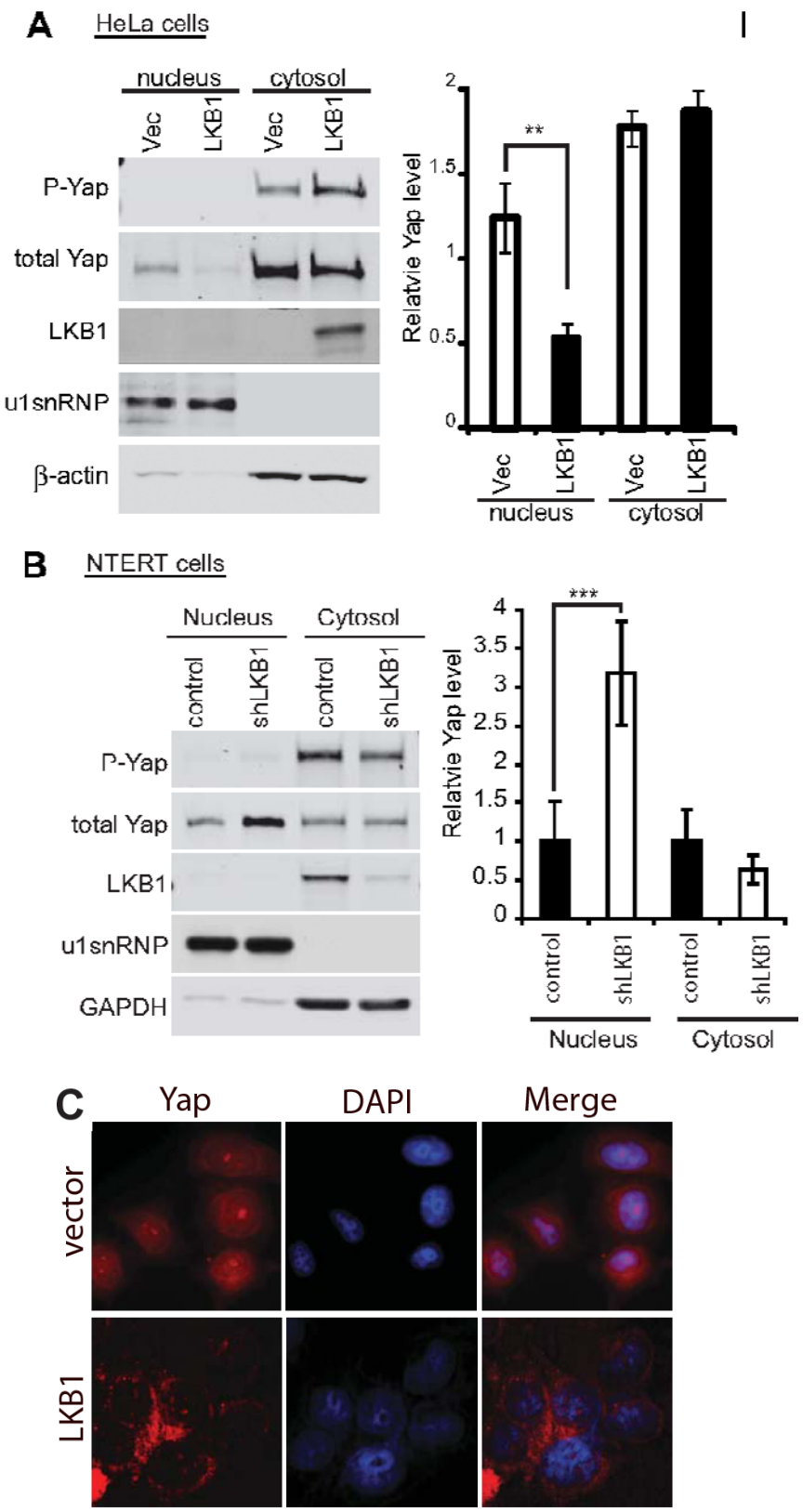
*Luciferase data are representatives of at least three independent experiments performed in triplicate. qRT-PCR is representative of two experiments performed in quadruplicate. Error was computed as standard deviation of the mean. Significance was computed by student T-test where p-values are indicated by * < 0.05; ** < 0.01; *** < 0.001.*

3.2.4. LKB1 regulates Yap via cytosolic retention and proteasomal degradation of Yap protein

Yap dependent transcription is inhibited via the hyperphosphorylation of Yap on multiple sites. Currently, there are 5 known Lats1/2 phosphorylation sites on Yap (*42*). Among these, phospho-serine 127 allows for 14-3-3 binding and Yap cytoplasmic retention (*38*). In order to evaluate whether this spatial regulation occurs in LKB1 cells, we employed both nuclear fractionation and fluorescence microscopy. Firstly, hypotonic lysis was used to separate nucleic and cytoplasmic fractions. Endogenous Yap from both fractions was then detected by western blotting. We independently fixed cells and visualized the subcellular localization of endogenous Yap protein by immunofluorescence microscopy. Consistent with the increased phospho-YapS127 level in LKB1 expressing cells, both localization methods showed a significant decrease in nuclear Yap protein in HeLa LKB1 cells comparing to HeLa Vec cells of the same plating density (Figures 3-4 A and C). Consistently, knockdown of LKB1 in NTERT cells caused a significant increase in nuclear Yap (Figures 3-4 B). We concluded that LKB1 restoration increases S127 phosphorylation, promoting Yap nuclear exclusion.

Since proteasomal degradation is another important Yap regulating mechanism (*29*), we examined whether LKB1 add-back decreases Yap protein stability. To monitor the stability of Yap, HeLa LKB1 and HeLa Vec cells were treated with cycloheximide (CHX) to inhibit new protein synthesis. Plated at low density, HeLa Vec exhibited insignificant decrease in Yap protein level over 6 hours of CHX treatment. Meanwhile, Yap level rapidly decreased in LKB1 cells (Figure 3-4 D). To confirm that the decrease in Yap level was due to proteasomal degradation, we used the proteasome inhibitor, MG132. As previously observed, we saw a significant decrease in Yap level in LKB1 cells treated with CHX alone compared to DMSO control. However, addition of MG132 along with CHX returned Yap protein to control level (Figure 3-4 E). These results are consistent with previously shown LKB1 knockdown in NTERT cells caused an increase in total

cellular Yap protein (Figure 3-2 B). Altogether, these results indicated that LKB1 decreases Yap stability by promoting its proteasomal degradation.


A
HeLa cells
nucleus
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Vec
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LKB1
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LKB1
u1snRNP
β-actin
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2
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1
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Vec
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control
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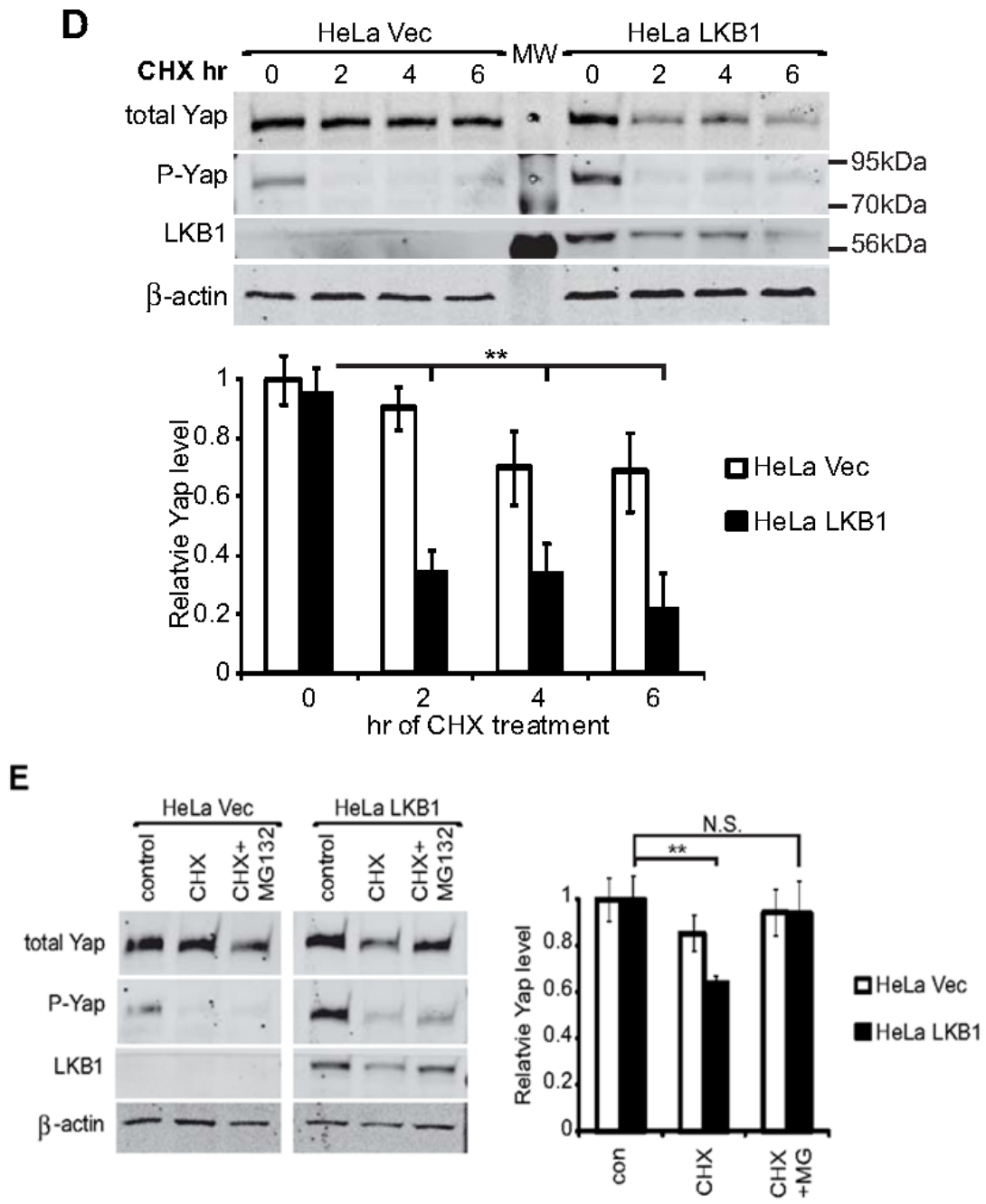


Figure 3-4. LKB1 inhibits Yap nuclear localization and promotes the degradation of Yap protein by the proteasome.

A. The relative levels of Yap, phospho-YapSer127, LKB1, U1snRNP (nuclear marker), and β-actin (cytosolic marker) were measured by immunoblot from nucleus- and cytosol-enriched fractions prepared from HeLa cells stably expressing LKB1 (HeLa LKB1) or GFP control vector (HeLa Vec). The average levels of Yap protein from three experiments are indicated in right panel next to a representative blot in left panel.

B. NTERT cells were treated as in part A to measure Yap levels in nuclear and cytosol enriched fractions.

C. HeLa LKB1 and HeLa Vec cells were fixed and immunostained for Yap. Nuclei were counterstained with DAPI.

D. HeLa LKB1 and HeLa Vec cells grown at low density were treated with 100 μg/ml cycloheximide (CHX) for the indicated times. The levels of Yap, phospho-YapSer127, LKB1 and β-actin were measured by immunoblot analysis from cell lysates (upper panel). The average Yap levels from three independent experiments were also computed (lower panel).

E. HeLa LKB1 and HeLa Vec cells were treated with CHX and 10 μM MG132 for 2 hr. Protein levels were then measured by immunoblot analysis (left panel). The average levels of Yap from three independent experiments are also shown (right panel).

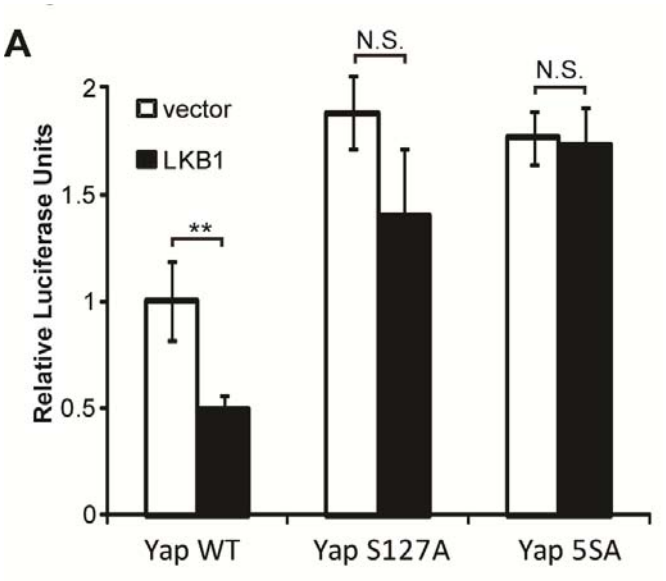
Errors and significances are represented as in Figure 3-3.

3.2.5. Mutation of phosphorylation sites on Yap desensitize Yap from LKB1 effects and rescue LKB1 suppression of proliferation

Figure 3-1 demonstrated that LKB1 expression induces phosphorylation of Yap^{Ser127}. This correlated with decreased Yap nuclear localization and transcriptional co-activation. We hypothesized that if Yap phosphorylation was the key event triggered by LKB1 expression to regulate gene expression and cell growth, then blocking such phosphorylation event(s) would rescue LKB1 tumor suppressor phenotypes. Two phosphorylation-defective Yap mutants were used in addition to a wild type (Yap^{WT}) construct to test this hypothesis: Yap^{S127A} has Ser 127 mutated to Ala while the Yap^{5SA} mutant lacks all five reported Lats phosphorylation sites (S61, S109, S127, S164, and S381) (*42*). The Yap^{5SA} mutant additionally has Ser 128, 131, and 163 mutated to Ala; however, these mutations do not affect cell growth and are not Lats phosphorylation sites (*37*). The TEAD-luciferase reporter assay was used as above to monitor Yap activity following transient transfection. While Yap^{WT} activity was inhibited by LKB1, the S5A mutant was insensitive to LKB1 action (Figure 3-5 A). A statistically insignificant but reproducible inhibition of Yap^{S127A} activity by LKB1 suggested that additional Yap phosphorylation sites might also play a role in suppressing its activity (Figure 3-5 A). These results suggested that Yap phosphorylation is required for LKB1 to suppress gene expression.

A major effect of LKB1 tumor suppressor expression was a strong reduction in proliferation of HeLa cells. This effect was measured by directly counting trypan-blue excluded cells on the indicated days. Correlated with the increase in Yap phosphorylation, LKB1 expression causes over 2-fold reduction of proliferation in HeLa LKB1 cells that was similar to the growth rate of NTERT cells (Figure 3-5 B). Since the transcriptional activity of Yap phospho-mutants was not inhibited by LKB1, we next addressed whether these mutants could rescue the suppression of proliferation seen following LKB1 expression. Lenti-viruses encoding LKB1 and various Yap transcripts were used to co-infect HeLa cells. After puromycin selection, cell accumulation was monitored as part B. Despite LKB1 suppressing endogenous Yap activity, the

expression of the Yap^{5SA} mutant significantly promoted cell proliferation (Figure 3-5 D). Consistent with the transcriptional activity data (Figure 3-5 A), the Yap^{S127A} mutant had a weaker effect than Yap^{5SA} on rescuing cell proliferation. Meanwhile, Yap^{WT} expression did not significantly change cell growth (Figure 3-5 D). In the absence of LKB1, the overexpression of Yap WT and mutants also did not significantly change cell accumulation (Figure 3-5 C). We concluded that the hyper-phosphorylation of Yap contributes to LKB1-mediated suppression of cell proliferation.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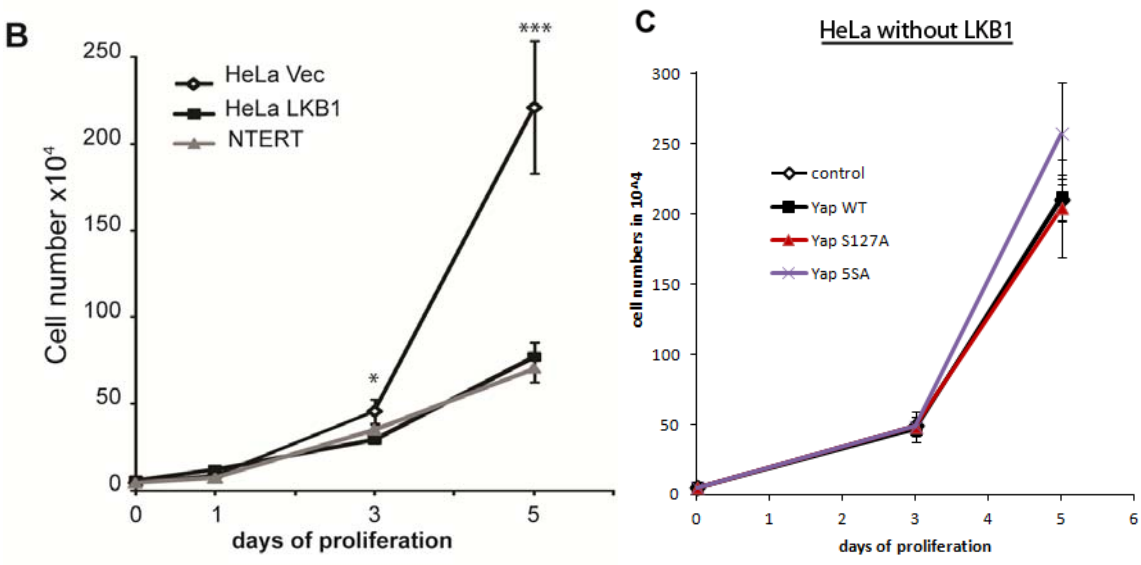
Yap S127A
Yap 5SA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HeLa Vec
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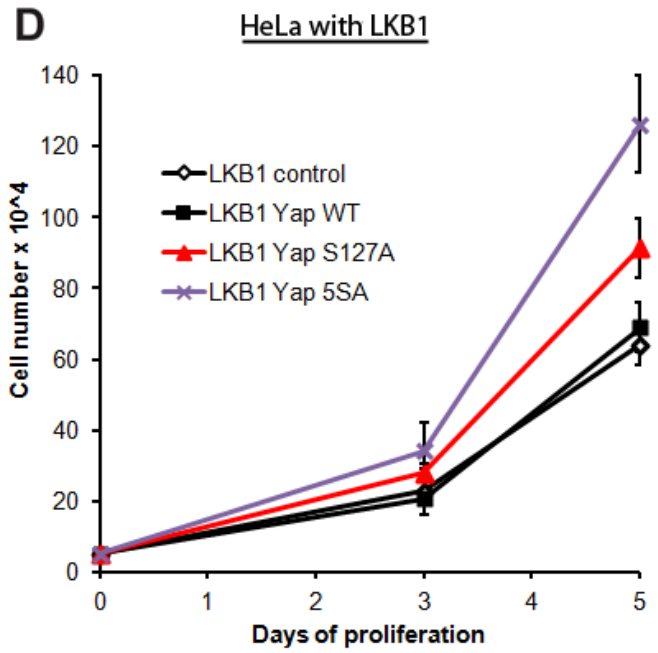


Figure 3-5. The ability of LKB1 to suppress Yap dependent transcription and cell growth is partially reversed by phosphorylation-deficient Yap mutants.

A. HeLa cells were transfected with Yap- wild type (WT), S127A, or 5SA in combination with vector control or LKB1. TEAD dependent transcription was measured as described in Fig 6a.

B. After seeding $5x10^4$ cells/plate, trypan blue-excluded cells were counted in triplicate on the indicated days by hemocytometer. The mean number of HeLa Vec (◊) or HeLa LKB1 (■), and NTERT immortalized human keratinocytes (▲) were plotted.

C. HeLa cells were infected with lenti-viruses carrying vector control (◊), Yap^{WT} (■), Yap^{S127A} (▲), and Yap^{5SA} (x) mutants. Following hygromycin selection, cell accumulation was measured as in part B.

D. HeLa cells were co-infected with LKB1 virus and others viruses as in part C. Following puromycin selection for LKB2 expression, cell accumulation was measured as in part B.

Error and significance are represented as described in Figure 3-3.

3.2.6. Yap^{5SA} mutant rescued cellular proliferation but not cell-cell compaction promoted by LKB1

Recently, the technique of three dimensional (3D) cultures has been developed for better understanding of tissue polarity. In addition to cell-cell interaction available in 2D monolayer, 3D culture also provides cell-ECM interaction due to Matrigel matrix enrichment of the laminin substrate. Many cancer promoting pathways were confirmed to cause disturbance to the polarized structure of epithelial acini, and the suppression of these signals restore acini structure in 3D culture (*17*). Therefore, we examined the effect of LKB1 and Yap^{5SA} expression on HeLa cell growth in a 3-dimensional Matrigel matrix. The above stable cell lines were plated at low density, submerged in two layers of matrix for 8 to 10 days. Consistent with our previous data from 2-dimensional culture, HeLa cells have completely loss cell polarity; they grow and spread into flat invasive colonies. The restoration of LKB1 significantly reduced cell growth in Matrigel as well as promoting polarized cell clumps (Figure 3-6 A, bottom left panel). Interestingly, Yap^{5SA} expression greatly increased cell proliferation both in the presence and absence of LKB1 but did not disrupt LKB1-induced cell-cell compaction. Somewhat vary from 2D result, the expression of either Yap^{WT} or Yap^{S127A} did not altered cell growth pattern nor cell cohesion (Figure 3-6 B). This result further supported the role of Yap hyper-phosphorylation in mediating LKB1 suppression of proliferation but not LKB1 promotion of polarity.

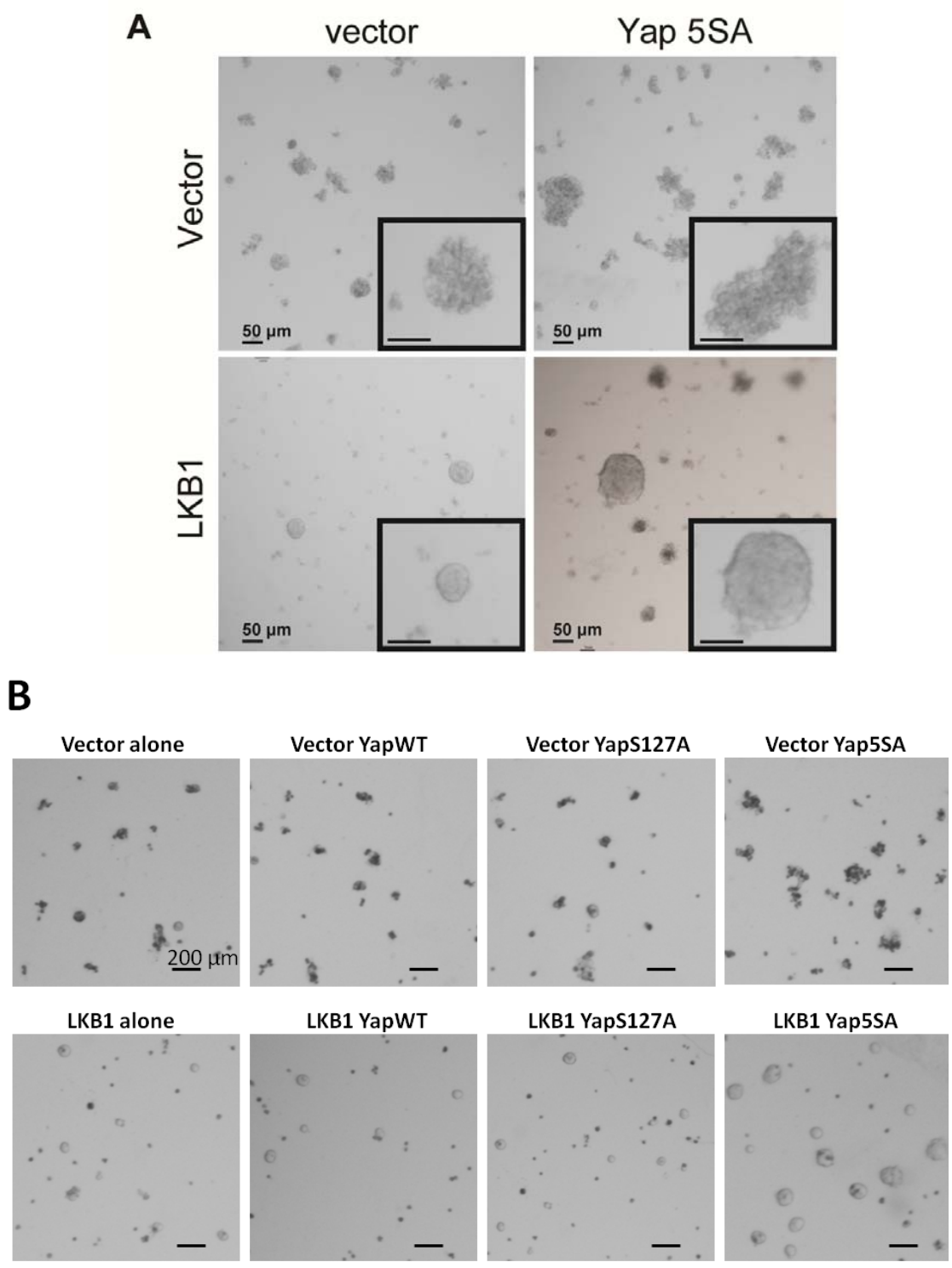


Figure 3-6. The expression of Yap^{5SA} mutant rescues LKB1 growth suppression effect but not LKB1 polarity effect in 3D Matrigel culture.

HeLa cells stably expressing the indicated cDNAs were imaged after 10 days culture in Matrigel. Representative images are shown. Insets are 2-fold increase magnification of colonies.

3.2.7. Phosphorylation-defective Yap mutants partially reversed cell size reduction by LKB1

Cell size is a complex phenotype that is often influences by proliferative state and could be the outcome of various distinct pathways (*122, 123*). Various studies have reported the regulation of cell and organ size by both LKB1 and the Hippo pathway. For example, conditional knockout of murine LKB1 resulted in increased islet beta cell volume (*124*). Meanwhile, Yap conditional overexpression in other tissues was reported to cause liver enlargement and thickening of epidermal layers (*34, 125*). Therefore, we examine whether LKB1 regulates HeLa cell size by inhibiting Yap. Utilizing the HeLa LKB1 and HeLa Vec cells described above, we first confirmed the ability of LKB1 to reduce cell size. The lentiviral pCDH plasmid encodes for both GFP and LKB1, thus LKB1 levels could be correlated with GFP fluorescence. Two distinct populations were observed in GFP/LKB1 add-back cells; those that expressed high levels of recombinant proteins and those that had much lower levels (Figure 3-7 A). While the relative volume of low GFP/LKB1-expressing cells was similar to HeLa Vec cells, consistent with previous reports (*45, 124*), the cells highly expressing LKB1 had significantly smaller average size (Figure 3-7 B). Meanwhile, knockdown of LKB1 in NTERT cells increased cell volume (Figure 3-7 C) confirming LKB1 function in cell size reduction.

To address whether the inhibition of Yap contributed to this effect, lenti-viruses carrying Yap^{WT} or the phosphorylation-defective S127A or 5SA mutants were co-transduced with LKB1-encoding virus into HeLa cells. While Yap^{WT} had no effect, Yap^{S127A} and Yap^{5SA} mutants significantly rescued LKB1-induced cell size reduction (Figure 3-7 D). The Yap^{5SA} mutant was most effective, again suggesting contribution of phospho-sites other than S127 in mediating LKB1's effects. However, even Yap^{5SA} expression did not completely return HeLa cells to control size (Figure 3-7 D). Thus, inhibition of Yap may be just one factor contributing to suppression of cell volume by LKB1.

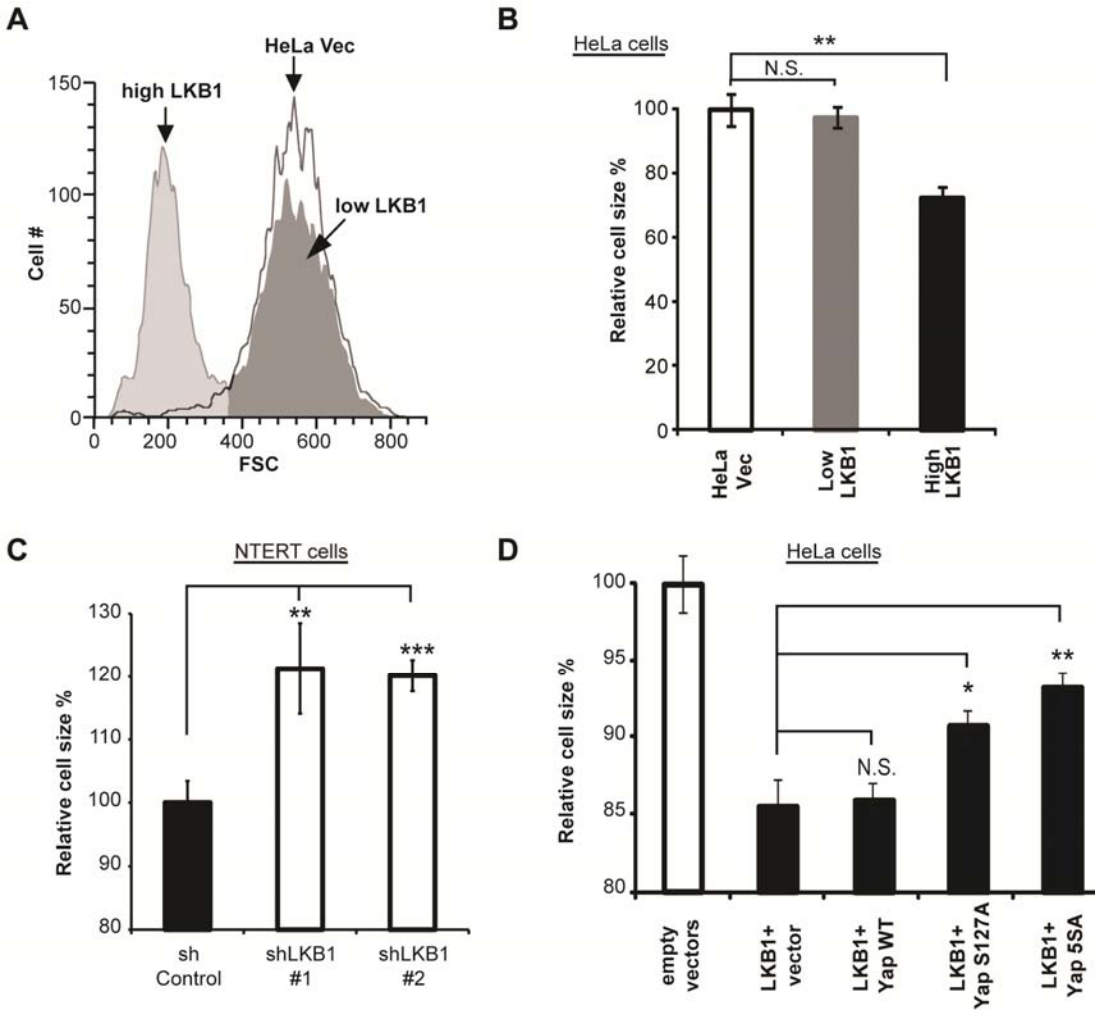


Figure 3-7. LKB1-induced cell shrinkage is partially reversed by the expression of Yap^{S127A} or Yap^{5SA}.

A. HeLa cells infected with a lentivirus carrying LKB1 and GFP (separated by a T2A self-cleaving peptide) or GFP alone were analyzed by flow cytometry forward scatter (FSC). Two sub-populations were observed in GFP/LKB1 add-back cells: one with high and one with low GFP signal.

B. The mean FSC of each population described in part A was calculated as an arbitrary unit of cell size, which was then normalized to vector control to obtain relative cell size. Three samples of each population were analyzed and the average relative sizes presented.

C. NTERT cells with LKB1 knockdown or non-target control were treated as in part A and B.

D. Stable cell lines generated from co-infection with LKB1 and Yap transcripts as described in Figure 3b were sorted by GFP signals/LKB1 expression. Cell sizes were then analyzed. The mean FSCs were normalized to vector control, averaged from three samples, and presented as mean ± S.D.

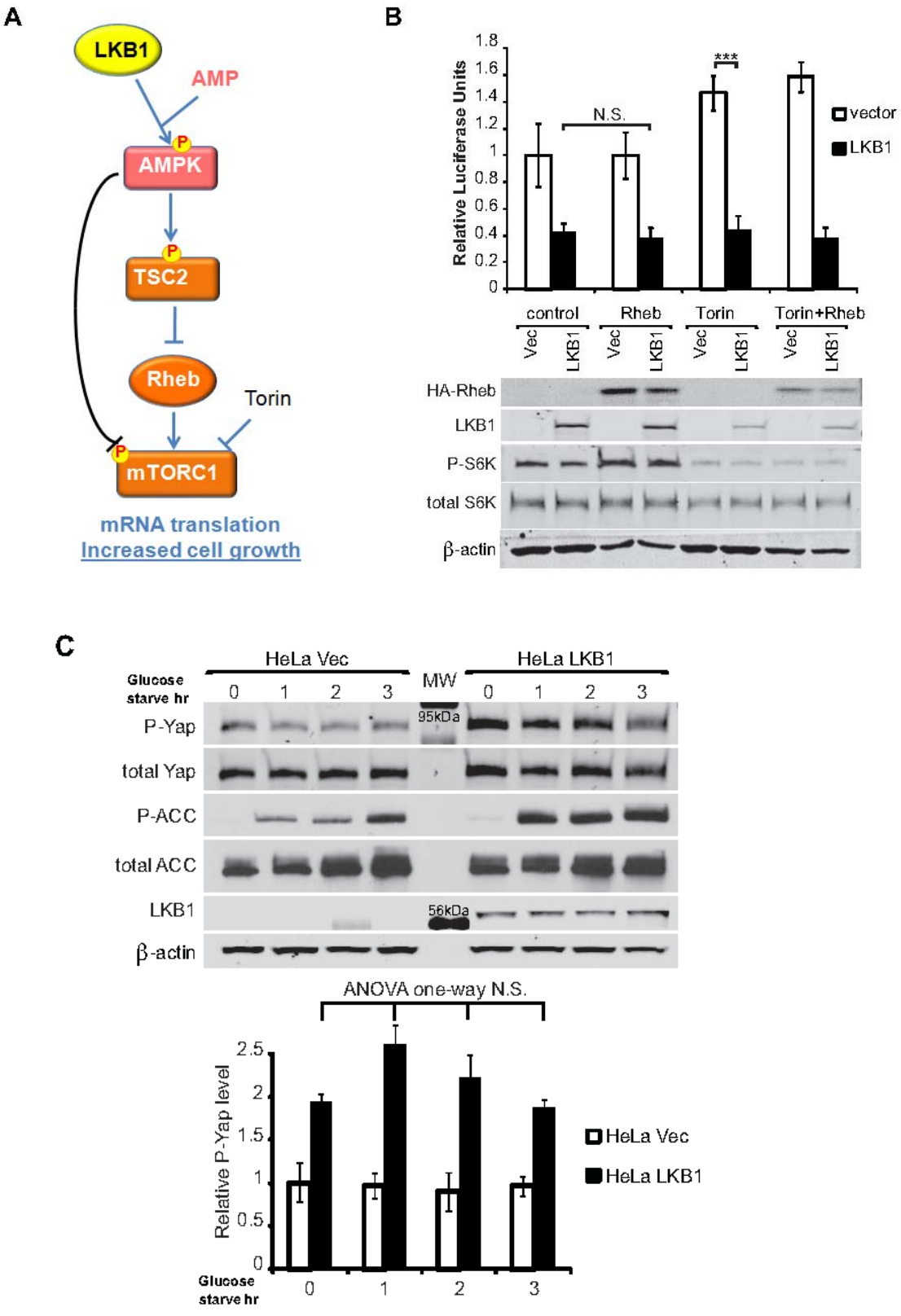
*All results are representative of at least two independent experiments performed in triplicate. * $p < 0.05$; ** $p < 0.01$; N.S. not significant*

3.2.8. Neither AMPK nor mTOR activity mediated LKB1's regulation of Yap

Numerous studies have reported that LKB1 regulates cell size through activation of AMPK and subsequent inhibition of mTORC1-induced protein synthesis via phosphorylation of TSC2 and/or raptor (*49*). Since Yap phosphorylation-defective mutants were found above to reverse LKB1-induced cell size reduction, we next addressed whether LKB1 regulates Yap via mTOR activation. The ATP analogue Torin1 (*126*) was used to effectively block both mTORC1 and C2 activities. To activate mTORC1, we overexpressed its upstream regulator, Rheb (*119*). mTORC1 activates S6 kinase (S6K), promoting ribosomal S6 protein phosphorylation to control protein synthesis and cell size (*127*). Thus we used phospho-S6K as a read-out for mTORC1 activity (Figure 3-8 A). As expected, Torin1 reduced and Rheb increased phospho-S6K level. However, changes in mTORC1 activity did not influence the ability of LKB1 to inhibit Yap activity (Figure 3-8 B). This suggested that LKB1 inhibits Yap independently of mTOR. Since Yap mutants only partially rescued LKB1 effects on cell size and proliferation (Figures 3-5 D, 3-6, and 3-7 D), we speculate that Yap and mTOR mediate independent aspects of signaling downstream of LKB1. Surprisingly, the inhibition of mTORC1 by the drugs Torin1 (Figure 3-8 B) and rapamycin, or by glutamine starvation (Figure 3-8 D) caused a small but significant increase in Yap activity (ANOVA one-way $p<0.01$). Since mTORC1 is a major metabolic sensor, this enhancement implies a possible negative feedback regulation between these two pathways downstream of LKB1.

LKB1 directly phosphorylates AMPK, enabling efficient activation upon AMP elevation, thus allowing cells to quickly response to metabolic stress (*49*). In order to examine possible mTOR-independent effects of AMPK on Yap, we induced metabolic stress via glucose starvation. AMPK phosphorylates acetyl-CoA carboxylase (ACC) to reduce glucose metabolism (*128*). Therefore we measured phospho-ACC as a positive control for AMPK activation. In this experiment, Yap^{S127} phosphorylation was measured instead of Yap transcriptional activity due to the short duration of the experiment precluding significant changes in luciferase expression.

Although AMPK was strongly activated by 1-3 hr of glucose starvation (large increase in ACC phosphorylation), Yap phosphorylation remained unchanged (Figure 3-8 C). Thus we concluded that LKB1 inhibits Yap independently of AMPK activation.


A
LKB1
AMP
AMPK
TSC2
Rheb
Torin
mTORC1
mRNA translation
Increased cell growth
B
Relative Luciferase Units
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
***
vector
LKB1
control
Rheb
Torin
Torin+Rheb
Vec
LKB1
HA-Rheb
LKB1
P-S6K
total S6K
β-actin
C
HeLa Vec
HeLa LKB1
Glucose starve hr
0 1 2 3
MW
95kDa
56kDa
P-Yap
total Yap
P-ACC
total ACC
LKB1
β-actin
ANOVA one-way N.S.
Relative P-Yap level
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HeLa Vec
HeLa LKB1
Glucose starve hr
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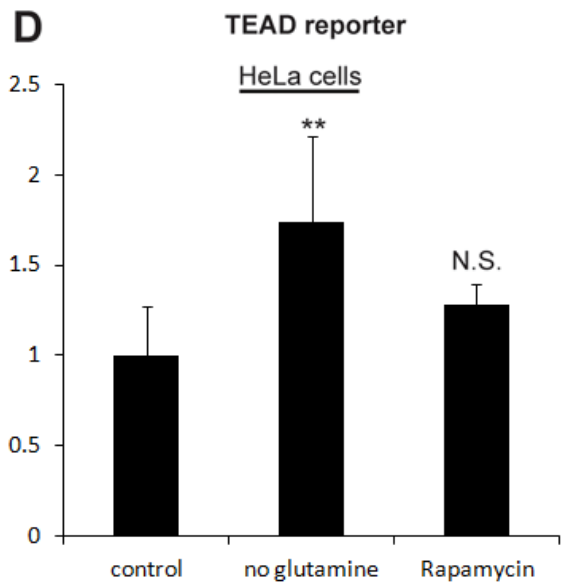


Figure 3-8. LKB1 regulates Yap independently of mTOR or AMPK activation.

A. LKB1 directly phosphrylates and activates AMPK, which in turn inhibits mTORC1 directly and through inactivation of Rheb activator.

B. HeLa cells were co-transfected with LKB1, Rheb and/or control vector as indicated. After 24 hr, cells were treated with the mTOR kinase inhibitor Torin1 (250 nM) for 16 hr. TEAD-luciferase activity was then measured as in Figure 3-3. HA-Rheb, LKB1, phospho-S6K, total S6K and β-actin were measured as controls for protein expression and mTOR activity.

C. HeLa Vec and HeLa LKB1 cells were glucose starved for up to 3 hr as indicated. Lysates were then immunoblotted to measure phospho-Yap^{Ser127}, Yap, phospho- and total acetyl CoA carboxylase (ACC), LKB1 and β-actin. AMPK activation was confirmed by increased ACC phosphorylation. The average levels of phospho-Yap^{Ser127} from three experiments are indicated in lower panel.

D. HeLa cells were transfected with TEAD reporters and treated with Rapamycin for 24hrs or glutamine starve overnight

Figures B and C are representative of three experiments, Figure D is representative of two experiments. Errors and significance are as described in Figure 3-3. ANOVA one way was performed to compare groups of four data points. N.S. not significant

3.2.9. LKB1 suppression of Yap function might not require Lats1/2 kinases

Neither one of the five phosphorylation sites included in the Yap^{5SA} matches LKB1 consensus substrate motif thus it is likely that LKB1 suppression of Yap is indirect. The consensus motif is DFGØsnXØXXgX--XLXTØCGSPXYAaPE with Ø represents a large hydrophobic residue; X, any amino acid; s, n, g and a preferences for Ser, Asn, Gly and Ala, respectively (*46*). The ability of Yap^{5SA} to revert the effects of LKB1 expression additionally suggested Lats1/2 kinases may couple LKB1 to Yap suppression due to Guan's report of Lats1/2 kinases phosphorylating these sites. To examine this, we transiently knocked down Lats1 and/or 2 using siRNA and measured Lats protein levels and TEAD-driven luciferase activity. Surprisingly, suppression of Lats1/2 expression did not impact the ability of LKB1 to suppress Yap activity (Figure 3-9 A). Although Lats1/2 knockdown partially rescued TEAD activity, LKB1 caused the same fold of activity suppression with and without Lats knockdown. Additionally, we immunoblotted HeLa LKB1 and control cell lysates for the Mst phosphorylate and activating site on Lats1 site T1079. LKB1 expression causes a decrease in both P-Lats and total Lats protein (Figure 3-9 B with total Lats quantified in right panel). This evidence argued against Lats mediating LKB1 effect. Together, these result suggested that either LKB1 regulated Yap activity via a non-Hippo pathway or there were redundancy in kinases that phosphorylate Yap in the mammalian system.

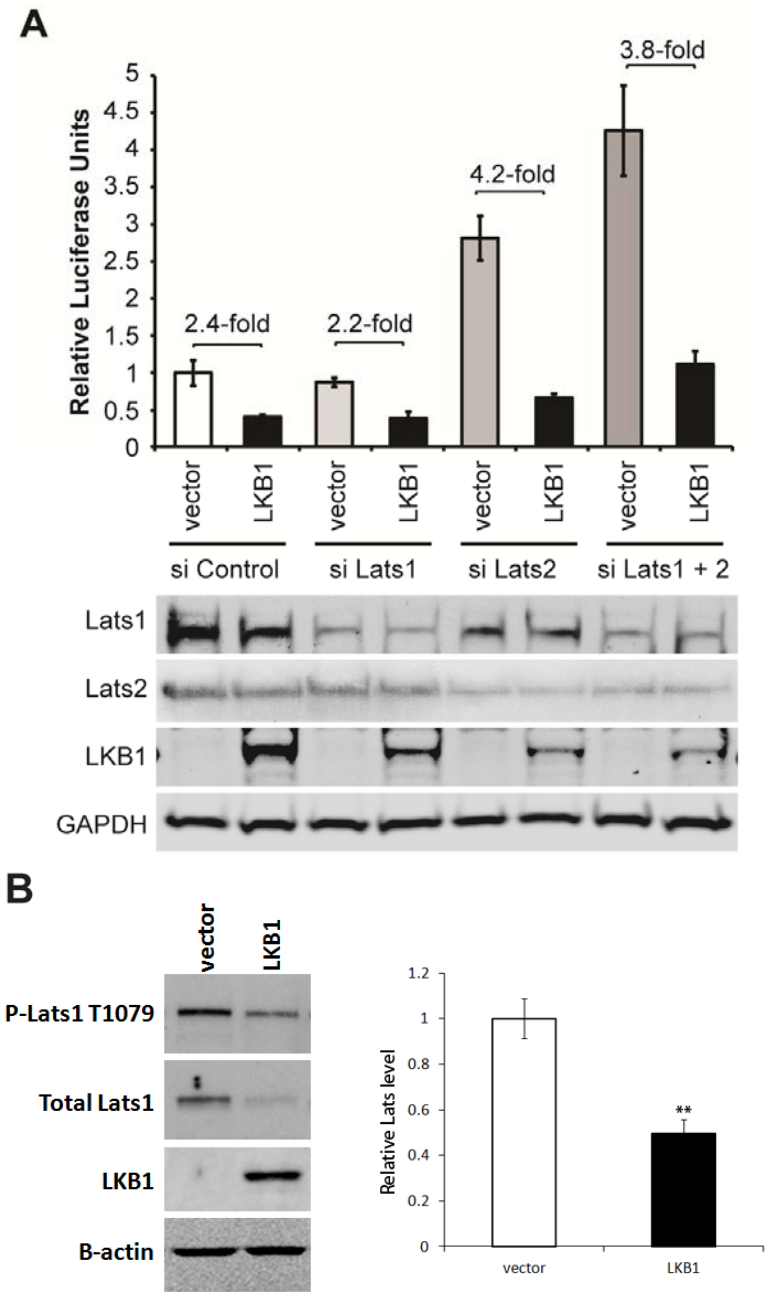


Figure 3-9. Yap suppression by LKB1 does not require Lats kinases.

A. HeLa cells were transiently transfected with indicated siRNAs, LKB1 or vector control, and TEAD luciferase plasmids. TEAD-luciferase activity as well as Lats 1, Lats2, LKB1 and GAPDH were then measured.

B. WCL from HeLa cells stably expressing LKB1 were immunoblotted for Lats1 kinase, LKB1, and β-actin (left panel). The average levels of Lats1 protein from three blots were quantified in right panel.

All results are representatives of at least three independent experiments. Error and significance are represented as described in Figure 3-3.

3.3. DISCUSSION

3.3.1. A novel polarity-growth connection in which LKB1 suppresses Yap leading to decreased cell proliferation and cell size

The architecture of a cell contributes to its survival, proliferation and function. A key component of this architecture is the establishment of apical-basal polarity whereby certain organelles and proteins are asymmetrically distributed. Polarity is established though a variety of signaling complexes that are integrated into the control of proliferation by poorly defined mechanisms. Recently, the Hippo (Mst-Lats-Yap) signaling pathway has emerged as a major growth regulator with its endpoint, phosphorylation and inactivation of the Yap transcriptional co-activator, being regulated by cell polarity/structure (*32, 40*). Interestingly, the LKB1 tumor suppressor is currently the only protein reported to cause cellular polarization in a cell autonomous fashion (*50*). The data reported here reveal a novel polarity-growth connection in which LKB1 activity regulates the subcellular distribution and proteasomal degradation of Yap leading to decreased cell proliferation and cell size (illustrated in Figure 3-10).

The master kinase LKB1 is required for epithelial structure-related resistance to hyperplasia (*23*). LKB1 promotes epithelial structure at least in part by regulating the Par-3/Par-6/atypical protein kinase C tight junction complex (*129*); however, how this structure prevent cell division is largely unknown. Consistent with a previous report in intestinal epithelial cells (*50*), expression of LKB1 autonomously promotes the polarization of HeLa cells in 2- and 3-D cultures. LKB1 also suppresses cell proliferation and its re-expression in HeLa cells reduced their growth rate to that of non-transformed human keratinocytes. Since the Yap transcriptional co-activator is a key regulator of cell growth in response to cell polarity, we examined the effect of LKB1 by its re-expression in HeLa cells and suppression of endogenous LKB1 gene expression in NTERT or 293T cells. LKB1 expression inhibited the ability of both endogenous and exogenously expressed Yap to induce the activation of a well-characterized luciferase-coupled

TEAD response element as well as endogenous CTGF transcription. These data demonstrated for the first time that Yap activity is closely associated with LKB1 status of tumor cells. Furthermore, LKB1 is known to suppress growth through the activation of AMPK and subsequently the inhibition of mTOR activity and protein synthesis. Indeed, no other signaling pathway downstream of LKB1 has been found to be activated in human tumors following LKB1 loss (*49, 130*). However, neither the activation of AMPK by glucose starvation nor pharmacological inhibition of mTOR activity impaired LKB1-mediated Yap phosphorylation. Therefore, the Yap transcriptional co-activator mediates LKB1's tumor suppressor functions independent of the canonical AMPK/mTOR pathway. Since Yap directly promotes the expression of proliferative genes, these findings indicate an alternative mechanism for LKB1-mediated suppression of cancer cell growth.

In many developmental systems and malignancies, the localization, integrity, and function of Yap are tightly regulated by its phosphorylation status. Consistently, we found that each of these parameters was closely linked to LKB1 expression in HeLa and NTERT cells. Specifically, LKB1 promoted phosphorylation of Ser127 that correlated with nucleus to cytoplasmic localization and enhanced Yap degradation. The decrease in nuclear Yap levels in LKB1 expressing cells also correlated with reduced proliferation of 2 and 3 dimensional cell cultures as well as cell size. Importantly, the expression of phosphorylation-resistant Yap mutants but not the overexpression of WT Yap reversed all impacts of LKB1 expression on gene expression, cell growth and cell size. These data support the notion that LKB1 impacts cell growth via Yap phosphorylation and inhibition.

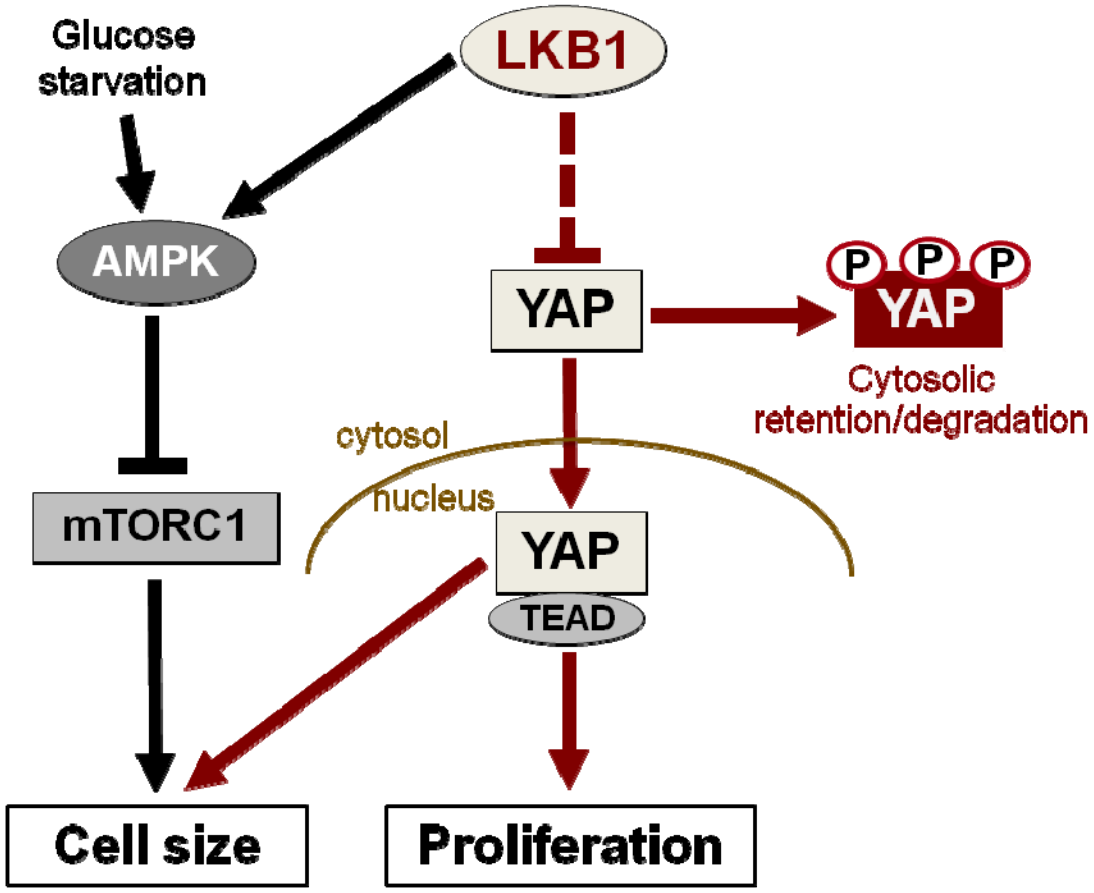


Figure 3-10. LKB1 suppression of Yap is a novel polarity-growth connection.

Aside from the regulation of metabolic sensors AMPK/mTORC1, LKB1-induced cell polarization impacts cell growth through the suppression of Yap transcription co-activator. This suppression is caused by the cytosolic retention and proteasomal degradation of Yap proteins as a result of Yap hyper-phosphorylation.

Red connecting lines in above diagram represent novel findings while black lines represent previously reported results. Arrows are promoting events while T-ended lines stand for inhibition. Dashed line is indirect effect.

3.3.2. The mechanism of Yap regulation is an integration of multiple pathways and requires the hyper-phosphorylation of Yap

Mass spectrometry analysis of Yap protein isolated from MCF10A cells done by Guan's lab reveals 10 unique phosphorylation sites (*42*). Five of these sites match the consensus sequence of Lats1/2 includes S61, S109, S127, S164, and S381. Interestingly, unlike the other phosphorylation sites, the mutation of S381 to alanine did not increase YAP electrophoretic mobility when isolated protein was run on gel that specifically retards phosphorylated proteins (Figure 1B of this report). Nonetheless, using a S381/S384 phospho-specific antibody, Lats1/2 was found to phosphorylate Yap on this S381 site and prime Yap for further phosphorylation by casein kinase δ/ε on nearby S384 and S387 sites. The phosphorylation of these sites in combination with nearby sequence (called phosphodegron sequence) is a recognition signal for the binding of the E3 ubiquitin ligase β-TRCP for Yap degradation. Lats1/2 were also required for the phosphorylation of Ser127 for Yap cytosolic retention. Altogether, the hyperphosphorylation inhibits Yap activity and the ability of Yap phospho-mutants to partially reversed the effects of LKB1 expression were consistent such a role. The Yap^{5SA} mutant has all five phosphorylation sites mutated, including the S381 priming site for degradation of Yap, while the Yap^{127A} mutant only has mutation at the cytosolic retention signal. Both the Yap^{5SA} and Yap^{127A} mutants were resistant to LKB1 suppression using the TEAD reporter assay; however, we observed a small but reliable suppression of the Yap^{127A} mutant by LKB1. Yap S127 and S381 double mutant (Yap^{2SA}) likely has the same effect as Yap^{5SA} (*42*). Consistent with previous reports indicating greater activity of the Yap^{5SA} mutant (*42*), it was also more effective than Yap^{127A} at rescuing LKB1 growth suppression in 2D cultures. Strikingly, the expression of the degradation-resistant Yap^{5SA} mutant reverted LKB1's growth inhibitory activity without disrupting LKB1-induced cell cohesion in 3D culture (most evident in Figure 3-6 A). Meanwhile, neither Yap^{WT} nor Yap^{127A} mutant has any effect with or without LKB1 in this condition (Figure

3-6 B). Thus, these results suggest that degradation rather than mere exclusion from the nucleus plays a role in LKB1-induced events.

None of the phosphorylation sites targeted in the Yap^{5SA} mutant match an LKB1 consensus motif, suggesting indirect kinase or phosphatase regulation. Since the AMPK-related kinase, NUAK1/ARK5 is a substrate of LKB1 that phosphorylates Lats (*131*), we first considered it as a mediator of Lats/Yap regulation. However, phosphorylation by ARK5 has been documented to destabilize Lats1 (*131, 132*) which would likely reduce phospho-Yap levels. Consistent with this report, we also observed a decrease in Lats1 protein level upon LKB1 expression, which could be related to ARK5 activity (Figure 3-9 B). Since LKB1 inhibits Yap, this decrease in Lats protein level is inconsistent with Lats or ARK5 mediating the inhibitory LKB1 effect. While we did observe a faster turn-over of Yap protein upon LKB1 expression, similar to that reported seen upon Lats activation (*42*), we did not see a role of Lats in Yap regulation by LKB1. The knockdown of Lats1/2 expression in HeLa cells did not prevent LKB1 from inhibiting Yap (Figure 3-9 A). Although Lats1/2 expression was not completely suppressed, this data strongly suggested that LKB1-induced inhibition of Yap does not require the Lats kinases. Conditional knock out of Mst1/2 in mouse or siRNA knock down of Mst1/2 and Lats1/2 similarly did not change Yap phosphorylation pattern or TEAD activity in keratinocytes (*32*). Additional reports similarly described increased Yap phosphorylation and nuclear exclusion in the absence of the Mst/Lats kinases (*41, 133*). These results together suggest that at least in mammalian epithelial cells, a non-Mst/Lats kinase might also be involved in the suppression of Yap activity.

CHAPTER 4. THE SCAFFOLD NF2 IS REQUIRED FOR LKB1 TO PROMOTE ACTIN REORGANIZATION AND YAP INHIBITION

One figure in this Chapter is copied from a publication by Nature Publishing Group:

Nguyen HB, Babcock JT, Wells CD, and Quilliam LA. LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap. Oncogene (2012) doi:10.1038/onc.2012.431

4.1. INTRODUCTION

Previously in chapter 3, we identified Yap as a novel inhibitory target that mediates the growth suppressing effect of LKB1. This chapter 4 is devoted to the molecular mechanism by which LKB1 activity causes Yap nuclear exclusion and degradation. Since the metabolism regulators AMPK/mTORC1 are not involved in Yap suppression, LKB1 promotion of cellular polarity is the focus of the current chapter.

LKB1 promotes cellular polarity through the activation of MARK family, Mst4, AMPK, and BRSK kinases (*49*). These downstream kinases are part of various polarity complexes and in turn facilitate the assembly of microtubules and actin cytoskeleton. Additionally, many scaffolding proteins that conduct signals from plasma membrane to the cytoskeleton also contribute to cell polarity. For instance, LKB1 activates MARK2, which directly phosphorylates Tau. This event promotes the destabilization of microtubules (*51-53*). Alternatively, the kinase Mst4 is recruited to the cell membrane through its direct binding with the scaffolding protein MO25α (a part of LKB1 complex). This recruitment leads to the phosphorylation and activation of Ezrin (an ERM protein) and consequently the formation of brush borders (*56, 57*). Thus the organization of cytoskeleton and the membrane localization of scaffolding proteins are important consequences of LKB1 activation.

On the other hand, the scaffolding protein NF2 mediates contact-dependent inhibition. NF2 inhibits cell growth both through membrane-cytoskeleton signaling and the Hippo/Yap pathway. The loss of NF2 results in up-regulation of membrane associated proteins such as integrins, growth factor receptors, and receptor tyrosine kinases (RTKs) (*65-68*). NF2 also inhibits Rac1-mediated activation of PAK. These regulations of RTKs and Rac1-PAK by NF2 reduce MAPK signaling as well as cell proliferation (*61, 72, 134*). Additionally, NF2 is a negative regulator of Yap (*35, 64, 83*). NF2 expression activates the Hippo kinases, Mst and Lats

(*61-63*). In epithelial cells, NF2 could also suppress Yap without the activation of Mst1/2 (*43*) and Lats (*32*). Nonetheless, the inhibition of Yap contributes to NF2 tumor suppressing effect.

NF2 shares structural homology with ERM proteins. Similar to ERM, NF2 binds to actin and influence cytoskeletal assembly. Unlike ERM proteins, the disruption of intramolecular interaction ("open" conformation) causes NF2 translocation away from the membrane (*77, 78*). This "open" conformation also promotes heterodimerization of NF2 and Ezrin (an ERM protein) which blocks NF2's suppression of cell growth (*77*). Interestingly, Ezrin is activated as a consequence of LKB1 expression (*135*). Given NF2 functions both as Yap suppressor and actin regulator, this chapter will examine the potential role of NF2 in mediating LKB1-Yap regulation.

4.2. RESULTS

4.2.1. LKB1 promotes an epithelial-like morphology that is associated with increased Yap phosphorylation

Low activity of Yap is associated with differentiated morphology. For epithelial cells, this morphology involves an increase in cell polarity and the formation of cell junctions (*32*). As previously discussed, the suppression of Yap proteins is facilitated by Yap phosphorylation. This event leads to the redistribution of Yap from the nucleus to the cytosol and cell junctions. Yap junctional sequestration is mediated by the interaction with membrane proteins such as a 14-3-3/α-catenin complex, Fat atypical cadherin, and the Amot family (*31, 32, 136*). Additionally, LKB1 restoration promoted an epithelial-like morphology with cell-cell cohesion in 2D and 3D culture (Figure 3-1, 3-6, and 4-1 A). In order to dissect the mechanism of Yap suppression by LKB1, we first confirmed whether cellular structure induced by LKB1 mediated Yap phosphorylation.

LKB1 promotion of epithelial architecture was examined by imaging live cells expressing fluorescently-tagged proteins. Two plasmids expressing pYFP-occluden (a component

of tight junctions) and pCFP-histone 2B (a nuclear marker) were co-transfected with vector or untagged LKB1 into HeLa cells. Fluorescence images taken from these live cells showed a junctional distribution of occluden in LKB1-expressing cells (Figure 4-1 B).

Since adherens junctions rely on Ca^{2+}-dependent homotypic interaction of E-cadherin molecules (*109*), we disrupted cell contacts by chelating extracellular calcium and examined the effect on Yap phosphorylation. Upon chelation of Ca^{2+} with EGTA, the peripheral localization of occluden in LKB1-expressing cells was lost within 6 minutes (Figure 4-1 B). Ca^{2+}-chelation also drastically decreased Yap^{Ser127} phosphorylation in a time dependent manner in LKB1 cells. In contrast, no significant change was observed in the already low P-Yap level of vector control cells (Figure 4-1 C). Surprisingly, the total Yap level in LKB1 cells was also slightly decreased (Figure 4-1 C). This suggested that cell polarity, induced by LKB1, impacts Yap phosphorylation and disruption of such structure lead to Yap degradation.

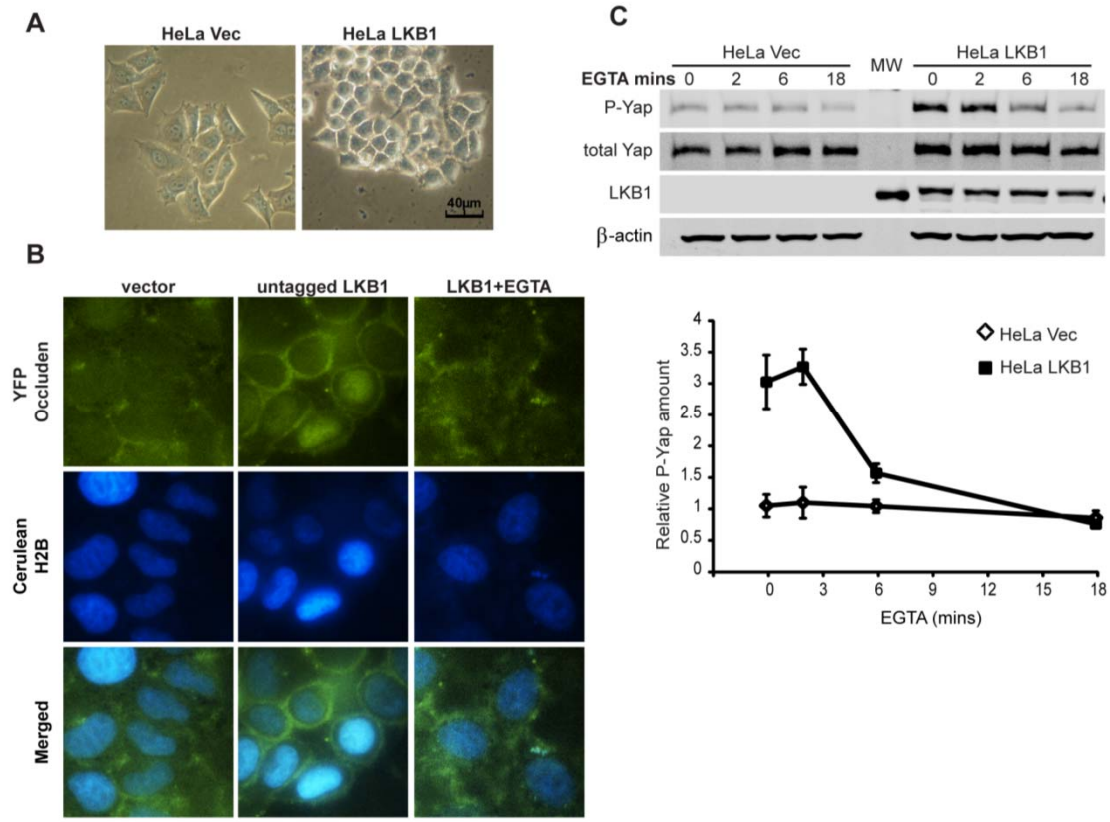


Figure 4-1. Cellular structure promoted by LKB1 plays a role in creating and maintaining elevated phospho-Yap levels.

A. Phase-contrast images suggested HeLa LKB1 cells have increase cell-cell attachment/cohesion.

B. Cells were plated on glass-bottom MatTek dishes. YFP-tagged occluden (a component of tight junctions) and cerulean histone 2B (a nuclear marker) were co-transfected with vector or untagged LKB1 into HeLa cells. Fluorescent live cell images were taken using a confocal microscope from vector control, LKB1 cells, and LKB1 cells after 6 min EGTA treatment.

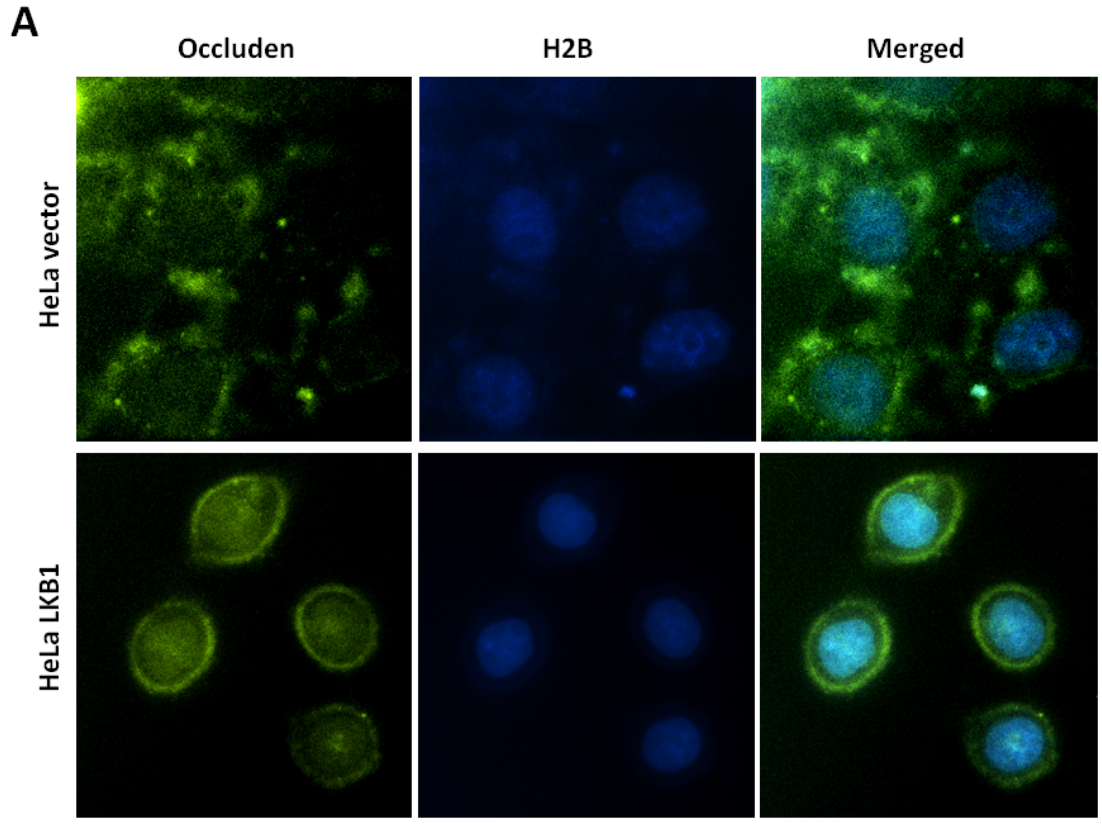
C. HeLa Vec and HeLa LKB1 cells were treated with EGTA to disrupt adherens junctions for indicated times. Lysates from these cells were immunoblotted for phospho-Yap^{Ser127}, total Yap, LKB1 and β-actin (upper panel). The average levels of phospho-Yap^{Ser127} of Vec (◊) and LKB1 (■) cells from three experiments were also computed (lower panel).

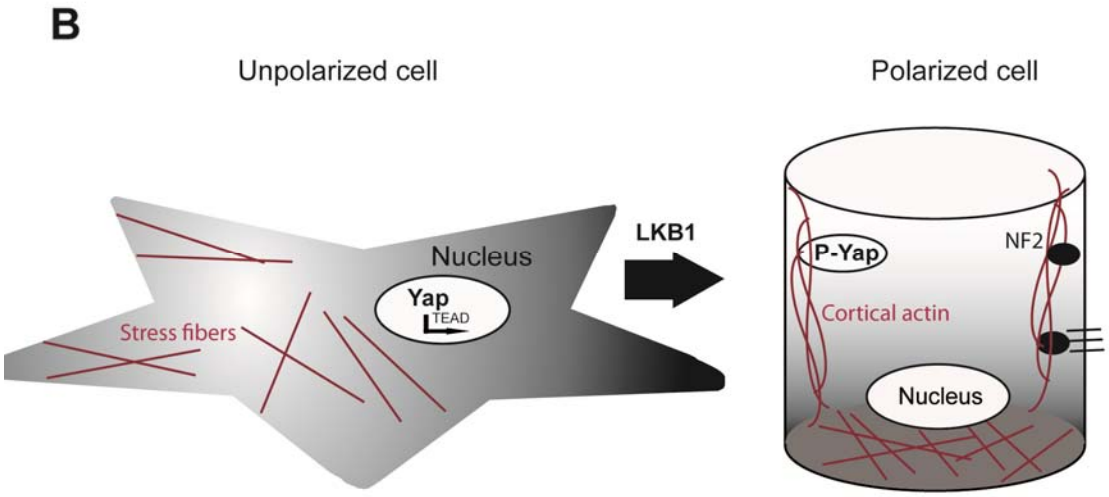
Blot and fluorescence images are representative. Error and significance are as described in Figure 3-3.

4.2.2. LKB1 promotes Occluden redistribution and the remodeling of actin structure

When HeLa cells with LKB1 expression were plated at low density so that cell-cell contact did not exist, individual cell autonomously established a distinct cylindrical cell shape. This was consistent with Clevers' report that LKB1 activation can polarize dissociated intestinal epithelial cells (*50*). Remarkably, even without cell cohesion, LKB1 promotes the redistribution of Occluden from the cytosol to cell membrane (Figure 4-2 A). This observation was done using the same live cell imaging technique previously described in Figure 4-1. This result suggests that while an extrinsic factor like cell-cell adhesions may enhance the organization of cellular components, such effect is an intrinsic property of an individual cell.

Cell morphologies are established by the organization of microtubules and actin structures. Since actin and not microtubules have been shown to regulate Yap localization (*40, 41*), we focused on whether LKB1 affects actin remodeling. Fully polarized epithelial cell with apical-basal protein distribution have two types of filament actin structure: cortical actin and stress fiber (*137*) (illustrated in Figure 4-2 B). Cortical actin structures are meshwork of filaments just beneath the plasma membrane. Meanwhile, stress fibers are contractile actin-myosin filament bundles that may provide driving force for the cell movement (*137, 138*). In order to examine actin filaments, we expressed LifeAct-GFP, a fluorescently-tagged peptide that specifically binds the polymerized F-actin and not monomeric G-actin (*139*). Fluorescence live cell images were taken from HeLa LKB1 and control cells previously transfected with LifeAct. Remarkably, LKB1 promoted an intricate actin structure even in the absence of cell-cell junctions (Figure 4-2 C). The increase in actin structure was associated with the cylindrical morphology. It is possible that this actin organization allowed the peripheral distribution of occluden previously observed in Figure 4-2 A.


A
Occluden
H2B
Merged
HeLa vector
HeLa LKB1


B
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Polarized cell
Nucleus
Yap
TEAD
Stress fibers
LKB1
P-Yap
NF2
Cortical actin
Nucleus

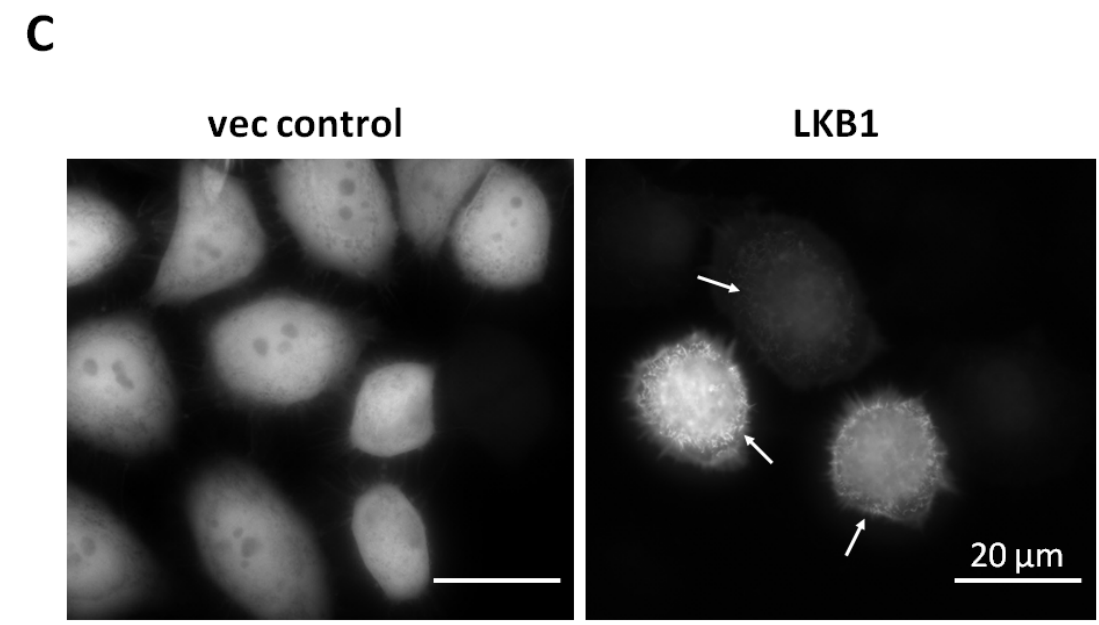


Figure 4-2. LKB1 promotes the organization of actin structure even in the absence of cell-cell contact.

A. Occluden and histone 2B in HeLa control or LKB1 cells at low density were visualized as described in Figure 4-1. Regardless of cell-cell junction formation, occluden is localized in a structure that polarized cells autonomously.

B. cartoon illustrating the two types of actin cytoskeleton that promotes cell shapes.

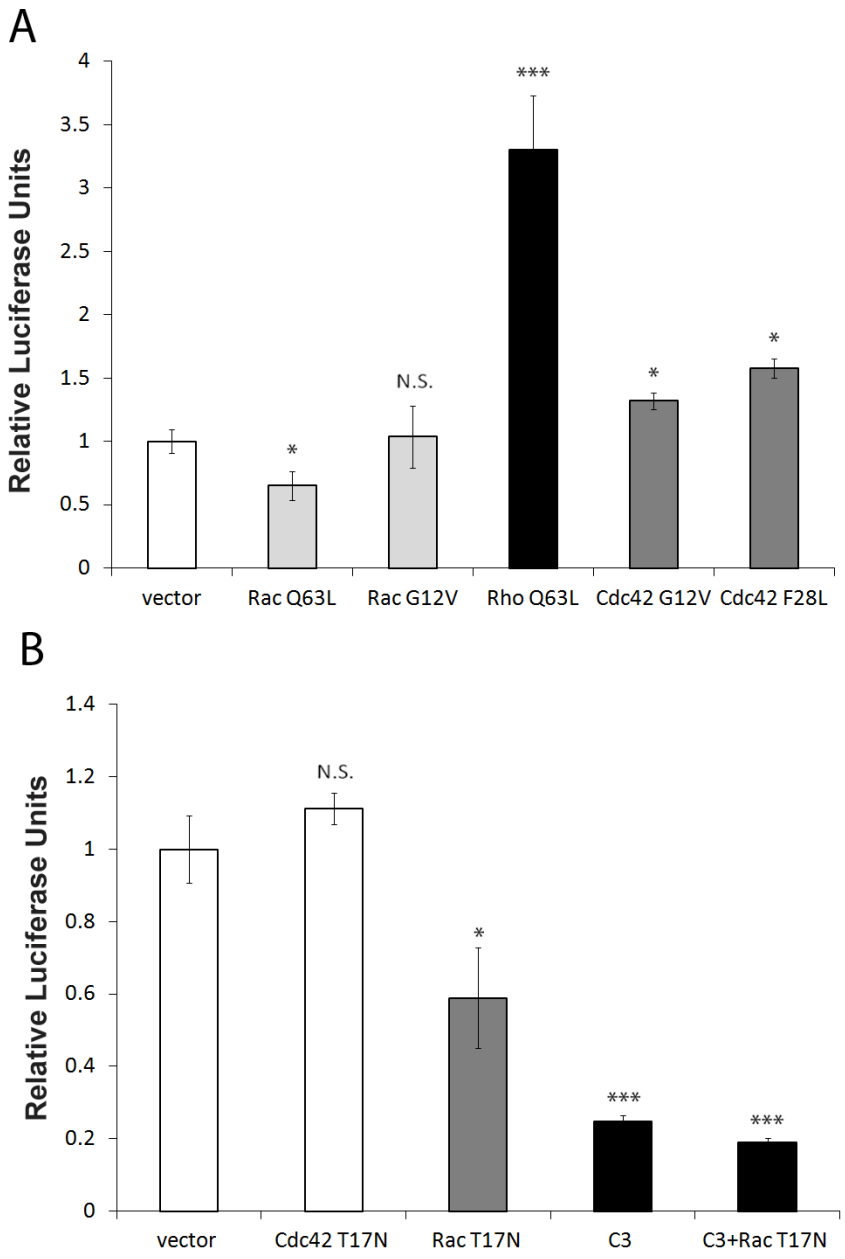
C. HeLa cells stably expressing untagged-LKB1 or control cells were transfected with plasmid expressing LifeAct, a GFP fusion peptide that specifically binds to F-actin but not G-actin. Actin structures in these live cells were visualized on MatTek plates using a fluorescence microscope as previously described. Pictures taken from the mid-section of the cells, white arrows denote actin structures.

Fluorescence images are representative.

4.2.3. An unclear role of Rho in mediating LKB1 effect

There are three major small GTPases responsible for cytoskeleton regulation: Rho, Rac, and Cdc42. LKB1 promotes actin remodeling that associated with Yap suppression. Thus we examined the effects of these GTPases on Yap using the TEAD reporter assay. Several constitutively active $RhoA^{Q63L}$, Rac^{G12V}, Rac^{Q63L}, $Cdc42^{G12V}$, or the fast cycling $Cdc42^{F28L}$ mutants were transiently expressed in HeLa cells in combination with TEAD reporter. $RhoA^{Q63L}$ strongly increased TEAD luciferase activity; in contrast, the expression of activated Rac and Cdc42 mutants did not change TEAD activity (Figure 4-3 A). Similarly, specific inhibition of Rho activity (*140*) by expressing the *Clostridium botulinum* exoenzyme C3 drastically reduces luciferase signals (Figure 4-3 B). Interestingly, the expression of dominant negative Rac^{T17N} also results in a suppression of Yap downstream transcription. However, this suppression by Rac^{T17N} is less than C3 effect and does not enhance C3 effect (Figure 4-3 B). The Rac^{T17N} mutant works by competing with wild-typed protein in binding to Rac activators- GEFs but cannot be activated. Thus, it is likely that Rac^{T17N} inhibition of Yap is an off-target effect, whereby Rac^{T17N} competes for GEF(s) that activate both Rho and Rac. Thus out of the three cytoskeleton regulators, only Rho is confirmed as a potent activator of Yap.

Furthermore, two recent studies reported that stress fibers control Yap nuclear retention and thus its transcriptional activity (*40, 41*). These reports are consistent with our result since only the Rho GTPase promotes stress fiber formation. Next we examined if the disruption of stress fibers couples LKB1 to the suppression of Yap activity. Interestingly, although $RhoA^{Q63L}$ mutant strongly activates Yap, LKB1 expression promoted a similar fold of Yap suppression in both vector control and $RhoA^{Q63L}$ cells (Figure 4-3 C). Additionally, a previous report showed that stress fiber disruption may preferentially affect the phosphorylation and stability of the Yap-related Taz protein (*41*). Therefore, it remains unclear whether LKB1 disrupts stress fibers or Rho activation is a parallel pathway that converges on Yap/Taz regulation.


A
Relative Luciferase Units
4
3.5
3
2.5
2
1.5
1
0.5
0
***
*
N.S.
*
*
vector
Rac Q63L
Rac G12V
Rho Q63L
Cdc42 G12V
Cdc42 F28L
B
Relative Luciferase Units
1.4
1.2
1
0.8
0.6
0.4
0.2
0
N.S.
*
***
***
vector
Cdc42 T17N
Rac T17N
C3
C3+Rac T17N

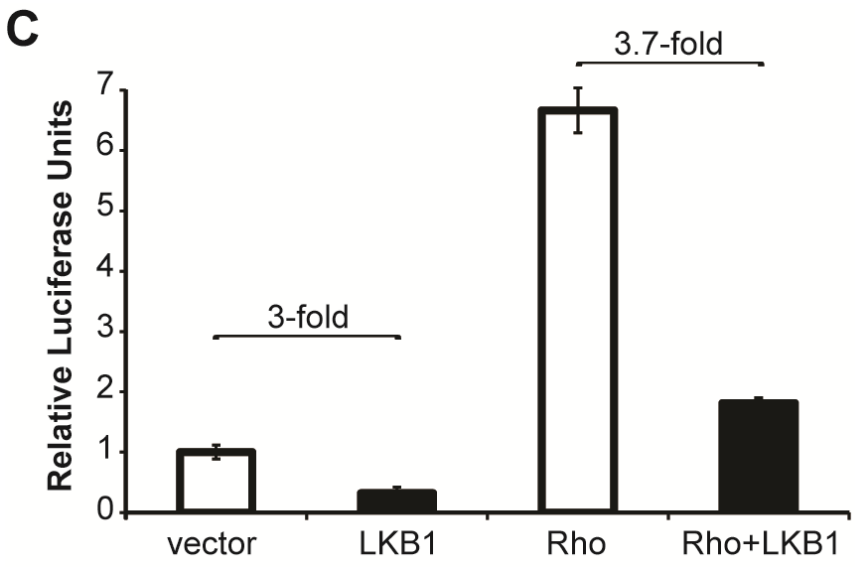


Figure 4-3. Rho is a potent activator of Yap but it is unclear whether the inhibition of Rho mediates LKB1 effect.

A. HeLa cells were co-transfected with plasmids expressing various Rac, Rho, Cdc42 constitutive active mutants, or empty vector control and TEAD reporter. Yap activity was inferred from luciferase signals as previously described in Figure 3-3.

B. Similar to experiment in part A, the effects of Cdc42, Rac dominant negative mutants, and a C3 *botulinum* toxin that specifically inhibit Rho were compared to vector control. Additionally, Rac^{T17N} mutant and C3 were combined to examine additive effect.

C. Once again, TEAD dependent transcription was measured in HeLa with constitutively active $RhoA^{Q63L}$ mutant in combination with vector control or LKB1 expressing plasmid.

Data representation of at least three independent experiments performed in triplicate. Error and significance are represented as described in Figure 3-3.

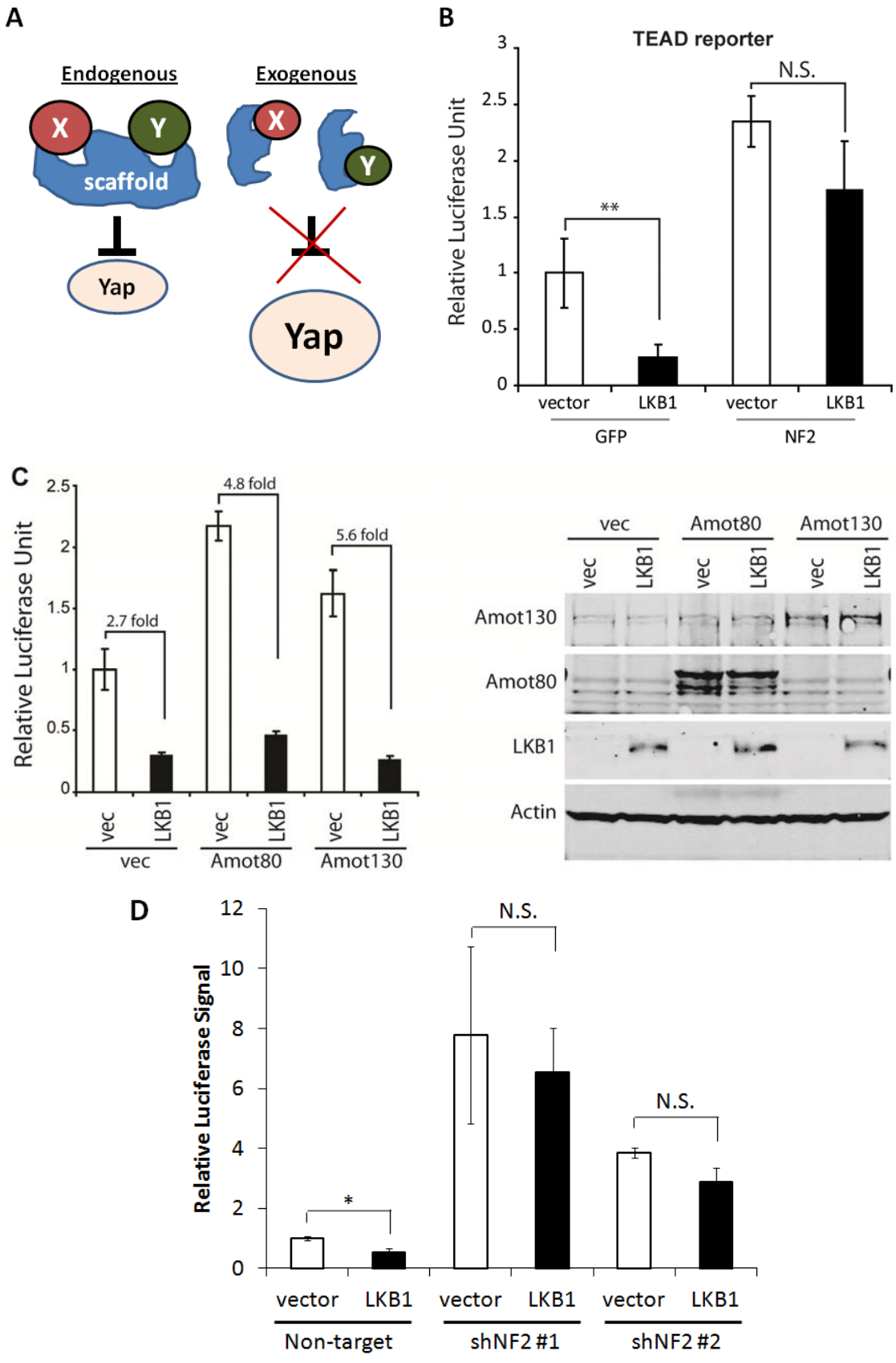
4.2.4. NF2 is required for LKB1 to suppress Yap

A number of scaffolding proteins regulate contact growth inhibition through Yap suppression. These include 14-3-3, the Amot family, and NF2 (Merlin). 14-3-3 couples Yap binding to α-catenin at the adherens junctions. Meanwhile, the Amot family binds to and sequesters Yap at the tight junctions. The Amot family consists of several scaffolding proteins named Amot80, Amot130, AmotL1, and AmotL2. Additionally, NF2 (Merlin) relates signals from the membrane to the activation of the Hippo kinases and Yap suppression.

In order to identify whether one or more of these scaffold proteins mediate LKB1 effect, two spliced variants of Amot, Amot130 and Amot80, or the NF2 protein were co-transfected in combination with LKB1. Yap downstream activity in these cells was then measured by TEAD reporting assay as previously described. Unexpectedly, both Amot and NF2 overexpression increased Yap activity despite their previously reported roles as Yap suppressors (Figure 4-4 B and C). However, this result is not unusual for inhibitory complexes, whereby the expression of the scaffold component may disrupt the complex stoichiometry (illustrated in Figure 4-4 A). Thus it is possible that Amot130 or NF2 overexpression break apart the necessary complexes for Yap suppression. The overexpression of both Amot spliced variants did not affect the ability of LKB1 to suppress Yap activity (Figure 4-4 C). Although further investigation is required to confirm whether LKB1 function independent of other Amot family members, at least Amot80 and 130 did not mediate LKB1's effect. In contrast, exogenous NF2 completely disrupts LKB1 function (Figure 4-4 B).

NF2 relates external stimuli to cytoskeleton regulation. This function is achieved through NF2 binding to both actin cytoskeleton and the cytoplasmic ends of cell surface glycoproteins (*78*). Given our previous results implicating actin reorganization in Yap inhibition, the result of NF2 overexpression was confirmed with the knock down of endogenous NF2 in HeLa cells. Cell lines with NF2 knockdown were generated with two different shRNAs, delivered by lenti-viral system. Both knockdown cell lines were transfected with LKB1 or control vector and TEAD

reporter for the measurement of Yap downstream transcription. As expected, both NF2 knockdown cell lines had significant increase of Yap activity, similar to NF2 expression. Furthermore, NF2 knockdown completely abolished LKB1's ability to inhibit Yap (Figure 4-4 D). Additionally, stable NF2 knockdown and LKB1 expression were achieved with dual lenti-viral infections. Whole lysates from these cells were immunoblotted for P-Yap, total Yap, LKB1, NF2, and GAPDH. Consistent with activity data, Yap phosphorylation promoted by LKB1 was completely eliminated by the knockdown of NF2 (Figure 4-4 E). Thus far, NF2 is the only protein that is absolutely required for LKB1 suppression of Yap.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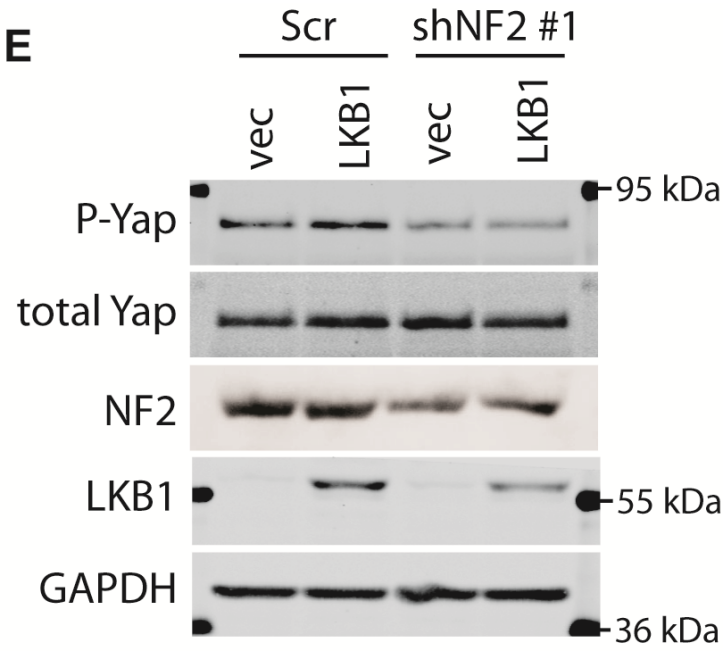

Figure 4-4. NF2 (Merlin) is required for LKB1 to inhibit Yap.

A. Illustration of how the overexpression of one component in an inhibitory complex, in particular a scaffold protein, may have the reverse effect on Yap activity.

B. HeLa cells were transiently transfected with indicated NF2, LKB1 or vector control, and TEAD luciferase plasmids. TEAD-luciferase activity was then measured as previously described.

C. HeLa cells were transiently transfected as in part B except with indicated Amot cDNA. TEAD-luciferase activity. Overexpression of Amot proteins was confirmed by western blot.

D. HeLa cells were infected with lentivirus expressing shRNA targeting NF2 or non-target control. Subsequently, cells were transfected with TEAD reporter and LKB1 or vector control. Yap activity was inferred from luciferase signals.

E. NF2 knockdown HeLa as in part D were also co-infected with lentivirus expressing LKB1 or vector control. Lysates from these cells were immunoblotted for P-Yap, total Yap, LKB1, NF2, and GAPDH.

All results are representatives of at least three independent experiments, luciferase experiments were performed in triplicate. Error and significance are represented as described in Figure 3-3.

4.2.5. NF2 is required for LKB1 to promote a specific actin structure that associates with lower Yap activity

Previous result in Figure 4-4 identifies that NF2 is required for LKB1 to suppress Yap. We also established that an epithelial-like cell shape promoted by LKB1 is associated with Yap phosphorylation (Figure 4-1). Additionally, NF2 has an actin binding mechanism that is unique from conventional ERM proteins. NF2 binds to actin directly through its N-terminal domain or indirectly through βII-spectrin or fodrin (*78*). Therefore, we asked whether NF2 is required for LKB1 to promote the epithelial-like morphology.

HeLa cells with stable NF2 knockdown and LKB1 restoration were generated by co-infection with lenti-viruses. In a similar process to Figure 4-2 C, LifeAct was used to visualize polymerized actin in these cells. Intriguingly, the knockdown of NF2 disrupts the epithelial-like shape that was induced by LKB1 expression (Figure 4-5 bottom right panel). Consistent with NF2's role in suppressing Rac and Rho GTPase, cells with NF2 knockdown alone has more ruffles than non-targeted control shRNA infected cells (Figure 4-5 bottom left). Both in the presence and absence of LKB1, cells with NF2 knockdown have an increase in cell spreading. This spreading is a mesenchymal-like morphology that associated with higher Yap activity. However, the knockdown of NF2 did not completely prevent actin polymerization in LKB1 cells. Thus, further investigation is needed to confirm the requirement of this actin structure in the suppression of Yap.

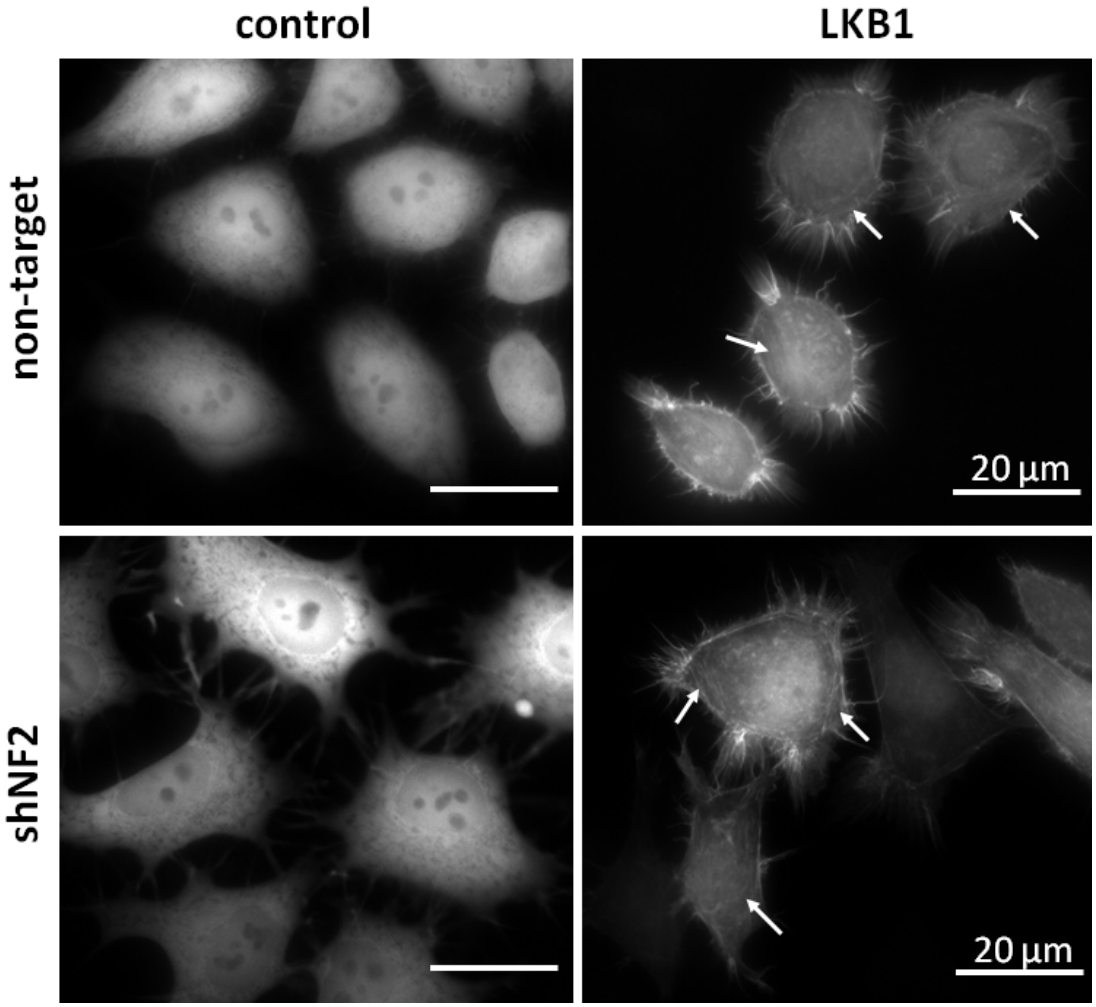

Figure 4-5. NF2 is required for LKB1 to promote an epithelial-like cell shape that is associated with lower Yap activity.

HeLa cells were co-infected with lenti-virus carrying untagged LKB1or pCDH-Hygro vector control and NF2 targeted shRNA or non-target control similar to Figure 4-3 E. Upon selection for LKB1 using 200 µg/ml hygromycin B, cells were transfected with LifeAct plasmid and actin structures were visualized as in Figure 4-2 C. Shown images were taken near the base of the cells, white arrows denote actin structures.

Fluorescence images are representative.

4.4. DISCUSSION

4.3.1. Actin structure reorganization might be an inside-out signaling event that links LKB1 expression to Yap suppression

The inhibition of proliferation by cell-cell contacts is often thought of as an extrinsic event. Here, we presented evidence of this growth regulation as both outside-in and inside-out signaling. In the former case, outside stimuli are sensed by membrane associated proteins, conducted by scaffold proteins, and resulting in the regulation of transcriptional factors. In this study, the destruction of LKB1-induced structure by disrupting adherens junctions completely suppressed Yap phosphorylation, which associates with Yap inhibition. Since LKB1 promotes a rapid turn-over of Yap protein (Figure 3-4 D), the small decrease in total Yap level in LKB1 cells following EGTA treatment (observed in Figure 4-1 C) suggests that P-Yap is degraded in this process. Thus, it is likely that most of the Yap protein in LKB1-expressing cells is phosphorylated and sequestered at the junctions prior to its degradation. This result represents an outside-in mechanism where junction formation causes the rapid turn-over of Yap protein and growth arrest.

On the other hand, LKB1 can promote an epithelial-like, cylindrical cell shape even in the complete absence of neighboring cells. This epithelial-like shape is associated with the formation of an actin cytoskeleton network and the relocalization of the tight junction protein occluden. This observation is very similar to Clevers' work in intestinal cells, except the actin and occluden redistribution appear somewhat different (*50*). While LKB1-activated intestinal cells have actin caps that promote a brush border with ZO-1 surrounding these caps, HeLa cells have actin structures that promote an epithelial-like shape and occluden surrounding the cells. This morphological difference might be due to the original functions of these cells as intestinal or cervical lining, respectively. While more evidence is needed, at least in epithelial cell, it is unmistakable that LKB1 can promote a global effect on actin reorganization in a cell autonomous

fashion. This new actin structure in turn may direct Yap proteins to degradation sites and inhibit cell growth. Additionally, as junction-associated proteins like occluden are moved to the cell periphery, LKB1 expression may allow epithelial cells to efficiently form adhesions once cells come in contact with one another. The cell autonomous cytoskeletal structure and its susceptibility to form junctions represent a form of inside-out signaling that might further enhance the above outside-in regulation.

4.3.2. The scaffold NF2 is required for LKB1 to reorganize actin structure and Yap suppression

In mammalian cells, a number of scaffolding proteins, such as 14-3-3, Amot family, Kibra, and NF2, have been reported to suppress Yap activity. These scaffolds are found close to the membrane and often interact with junctional proteins. For instance, 14-3-3 binds to both phosphorylated Yap and the junctional protein α-catenin in order to promote Yap sequestration to the adherens junction. Amot family members suppress Yap in similar fashion except the sequestration is to tight junctions and the actin cytoskeleton (*136*). Others proteins such as Kibra and NF2 have been shown to promote the activation of Mst-Lats in response to contact stimuli like E-caheren engagement. Other cell junction-associated proteins (PALS1, PATJ, MUPP1, and ZO2) that in turn associate with various Ser/Thr protein kinases also have been reported to interact with Yap or TAZ (reviewed in (*27*)). While our previous results suggest kinase(s) other than Lats also phosphorylate Yap, scaffolding proteins might be required to bring together inhibitory complexes for Yap suppression.

Here, we established that the scaffold protein NF2 is required for LKB1 to cause the formation of a particular actin network associated with Yap inhibition (illustrated in Figure 4-6). Correspondingly, the knockdown of NF2 by two different shRNAs completely abolished LKB1's effects on Yap downstream transcription and Yap phosphorylation. This result is particularly interesting since NF2's ability to suppress cell growth and to regulate actin cytoskeleton depends

on its phosphorylation status (*78*). Under disadvantaged growth conditions such as low serum or high density, NF2 exists at the membrane in a "closed" conformation with its N- and C-terminus binding to each other. Phosphorylation of NF2 on Serine 518 or mutations found in NF2 patients disrupt this intramolecular interaction and "open" NF2 dissociates from the membrane. Unlike ERM proteins, NF2 "closed" conformation is regarded as the "active" conformation due to NF2 functioning to suppress growth in this form. Meanwhile, "open" NF2 is redistributed from membrane to the cytosol (*78, 80*). A recent report suggested that "open" NF2 heterodimerizes with Ezrin although its function remains unclear (*80*). However, Rho (and not Rac or Cdc42) has been shown to translocate ERM proteins to the apical membrane and the actin protrusions (*141*). Since we observed that Rho specifically promotes Yap activity, it is possible that ERM proteins may also play a role in this actin organization. Furthermore, LKB1 restoration activates a number of kinases. It is possible that NF2 phosphorylation and de-phosphorylation allow dynamic movement of NF2 for cytoskeleton remodeling. This hypothesis is supported by the result that NF2 is required for LKB1 to form a distinct cylindrical cell shape as the result of F-actin formation (Figure 4-4). Interestingly, LKB1 can still promote some actin polymerization in the absence of NF2 but the overall structure is not the same as with NF2. Alternatively, Rho activation, which promotes actin stress fiber instead of cortical actin, increases Yap activity and does not completely reverse LKB1 suppression of Yap. Therefore, a specific actin structure promoted by LKB1 is associated low Yap activity. More investigation is needed to confirm whether a juxta-membrane actin structure is required for Yap phosphorylation and degradation to occur.

An alternative hypothesis is that NF2 is required for intracellular trafficking of Yap and Yap inhibitory complexes to an appropriate location in growth permissive or growth suppressive conditions. In *Drosophila*, Yorkie (Yap ortholog) can be suppressed independent of phosphorylation via direct binding with Expanded, Warts (Lats), and Hippo (Mst) (*142, 143*). This direct association suppresses cell growth through Yorkie translocation to the cell membrane.

Interestingly, Expanded is a FERM-domain protein that complexes with NF2 (Merlin) and Kibra in both *Drosophila* and mammalian cells (*35, 83, 143, 144*). Thus, NF2 might play a part in trafficking complexes that travel along the actin cytoskeleton. Various reports highlighting NF2 roles in vesicles trafficking (*145, 146*) and membrane lipid rafts association (*147*) support this possibility. While the exact mechanism of NF2 function requires more investigations, our results clearly define the role of NF2 scaffolding protein and the actin cytoskeleton in LKB1 regulation of Yap activity.

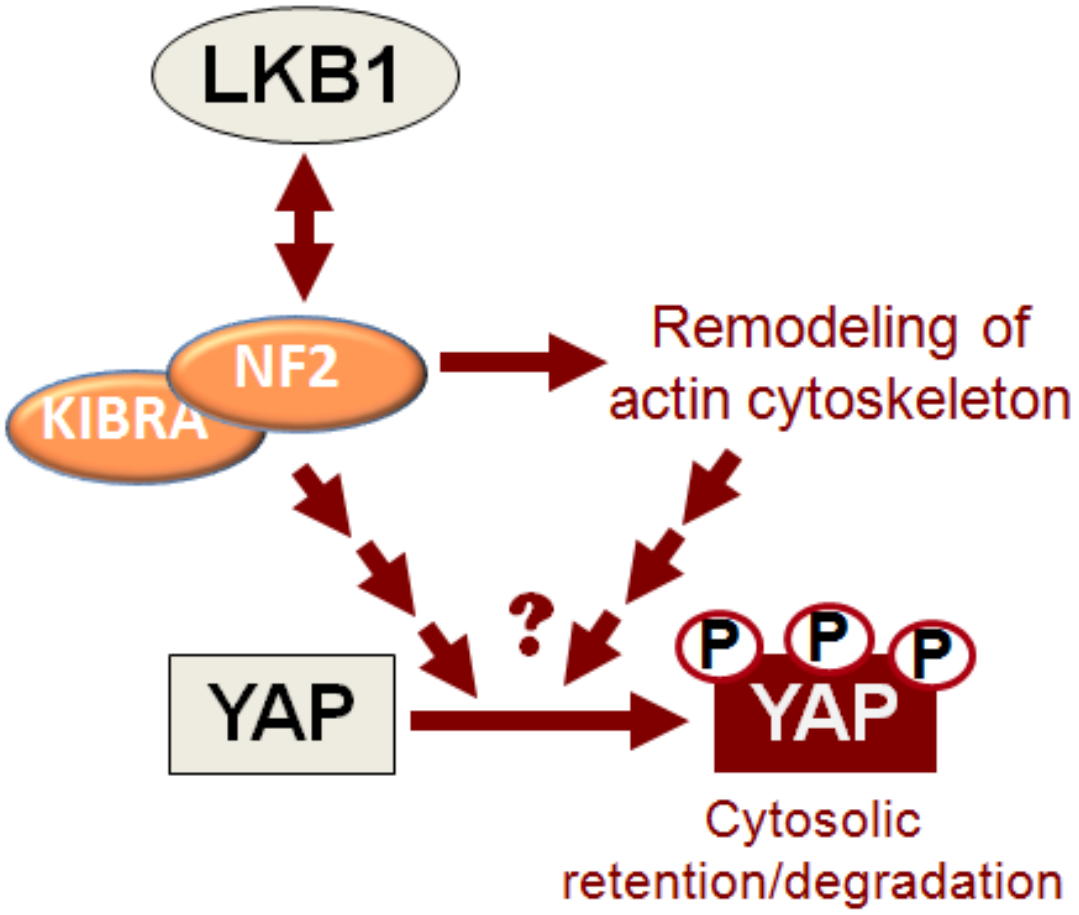

Figure 4-6. The scaffolding protein NF2 and the actin cytoskeleton play an essential role in LKB1 suppression of Yap activity.

NF2 is required for LKB1 to promote an epithelial-like cell shape that is associated with Yap inhibition. This distinct morphology might be caused by the remodeling of actin cytoskeleton. While NF2 plays essential role in Yap suppression, it remains unclear how Yap phosphorylation and degradation occur.

CHAPTER 5. THE VIRAL ONCOGENE HPV-E6 PROMOTES YAP ACTIVITY LIKELY THROUGH RAP1B ACTIVATION

5.1. INTRODUCTION

The HPV-E6 gene from high-risk papilloma viruses is one of two viral oncogenes that causes the development of cervical cancer (*2*). E6 interacts with various cellular proteins and with an E3 ubiquitin ligase called E6AP. This event promotes ubiquitination of E6 target proteins and ultimately their degradation (*86, 87*) (illustrated in Figure 5-1 A). The major target of E6 is the tumor suppressor p53. Upon E6 expression, the degradation of p53 contributes to the inhibition of apoptosis and the reduction of downstream transcriptional targets of Notch required for differentiation (*88, 89*). Additionally, E6 from high risk HPV also enhances cancer malignancy by increasing cell spreading, adhesion, and promotes the thickening of the epidermis. However, the molecular signaling underlining these p53-independent functions of E6 remains elusive. Interestingly, E6 contains a PDZ domain-binding motif that is required for high-risk strain 31E6 to promote keratinocytes growth on organotypic raft cultures (*95*). This motif allows E6 to bind and promote the degradation of a number of proteins containing PDZ domain(s) such as hScrib, hDlg, MAGI, MUPP1, PATJ, and PTPN3 (*92*). These PDZ-containing proteins are present in various polarity complexes and have crucial roles in cell junction formation and tissue structure. These observations encouraged us to examine a possible role of E6 in disruption of contact inhibition and Yap suppression. This chapter provides evidence that proliferation promoted by E6 might be the result of increase Yap activity.

An unbiased screen for cellular targets of the high risk 16E6 resulted in the identification of E6-targeted protein 1 (E6TP1 also known as Sipa1L1). E6TP1 is a GAP that deactivates a small GTPase family of Ras-related proteins (Rap) (*96, 97*) (illustrated in Figure 5-1 B). The Rap family consists of several closely related members called Rap1A, Rap1B, Rap2A, Rap2B, and Rap2C with notable roles in junction formation, cell migration, and cancer invasiveness (*102-109*). Importantly, Rap1 was shown as a crucial regulator of breast epithelial acinar structure in three dimensional culture. Activated Rap1-GTP promotes cell growth and suppression of Rap1

activity restores tissue polarity and induces lumen formation (*114*). Given E6's ability to disrupt tissue structure and Yap suppression's dependency on cell polarity, Rap1 was a prominent candidate for mediating E6 crosstalk with Yap. Here, we confirmed that Rap1 is activated by E6 in keratinocytes. Intriguingly, Rap1B specifically promoted Yap activation downstream of E6; however, Rap1 functions independent of the cell differentiation status.

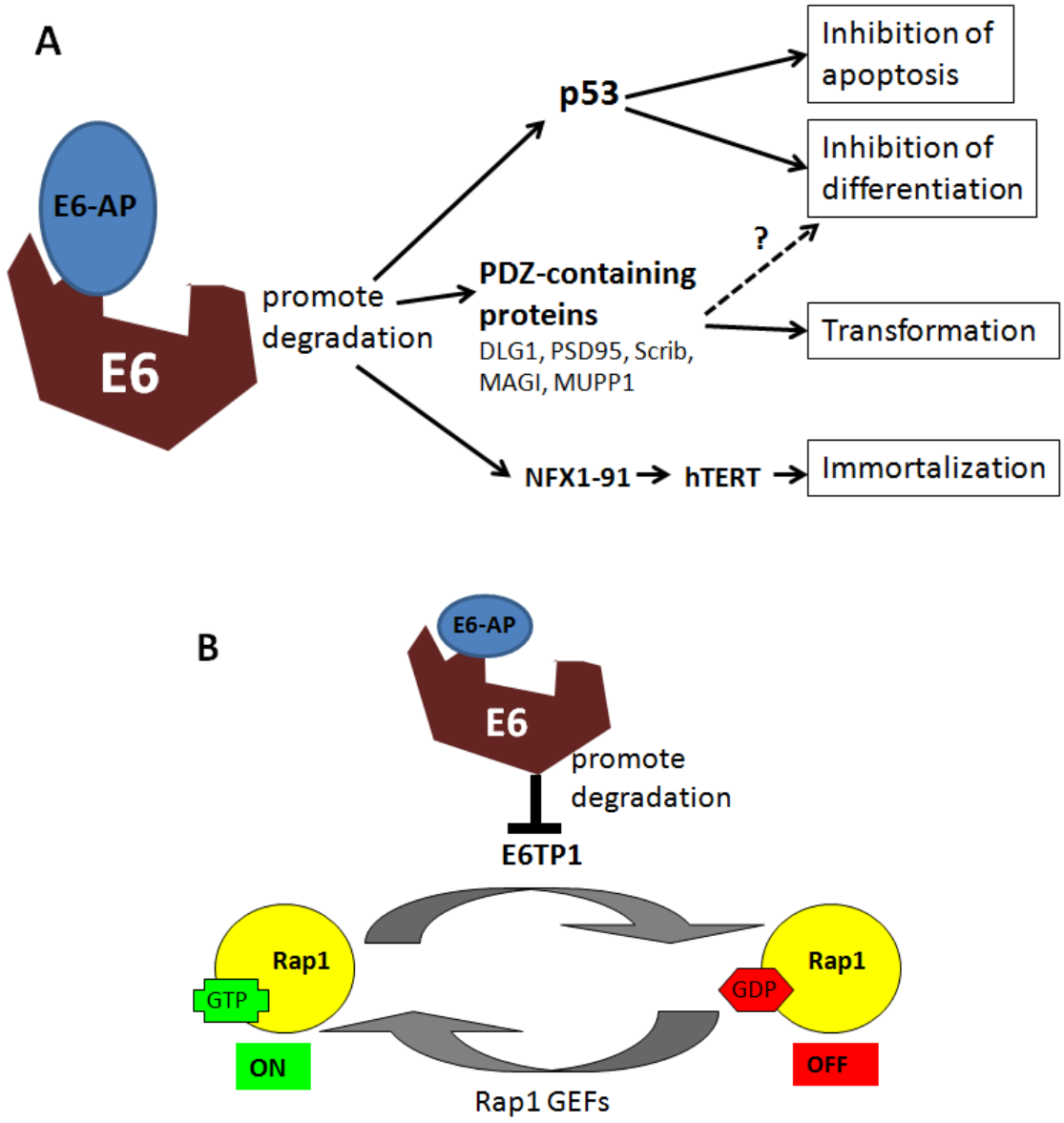

Figure 5-1. HPV-E6 disrupts normal cellular processes and contributes to cancer development.

A. E6 binds to E6-AP ubiquitin ligase and promotes the degradation of many tumor suppressors such as p53, PDZ-containing proteins, and hTERT inhibitor NFX1-91. These events contribute to a number of cancer promoting phenotypes including inhibition of differentiation.

B. E6 promotes the degradation of E6TP1, a Rap1 GAP, which functions to deactivate a small molecular switch Rap1. This event is predicted to increase GTP bounded active Rap1.

5.2. RESULTS

5.2.1. Yap phosporylation level increases with keratinocyte differentiation

A major effect of high-risk E6 expression is the disruption of the keratinocytes differentiation process. This normal process is essential for the establishment of epithelial tissue structure through the increase of junctions and the reduction of cell proliferation (*10*). The inhibition of Yap is reported to play a key role in reducing the cell's proliferative capacity (*26, 32*). Therefore, we first confirmed Yap suppression by phosphorylation during normal differentiation. In tissue culture of primary keratinocytes, this differentiation process can be induced by bringing the calcium level in the media to 2.8 mM. Although NTERT are immortalized cells, they retain the capacity to differentiate (Figure 5-2 A). Cell lysates taken from NTERT keratinocytes at the denoted dates of differentiation were immunoblotted for P-Yap, total Yap, and GAPDH levels (Figure 5-2 B). Consistent with previous reports on epithelial tissue immuno-staining (*26*), Yap phosphorylation greatly increased with the differentiation of NTERT cells. Surprisingly, total Yap levels also mildly increase with differentiation, possibly through the stabilization of P-Yap at cell junctions.

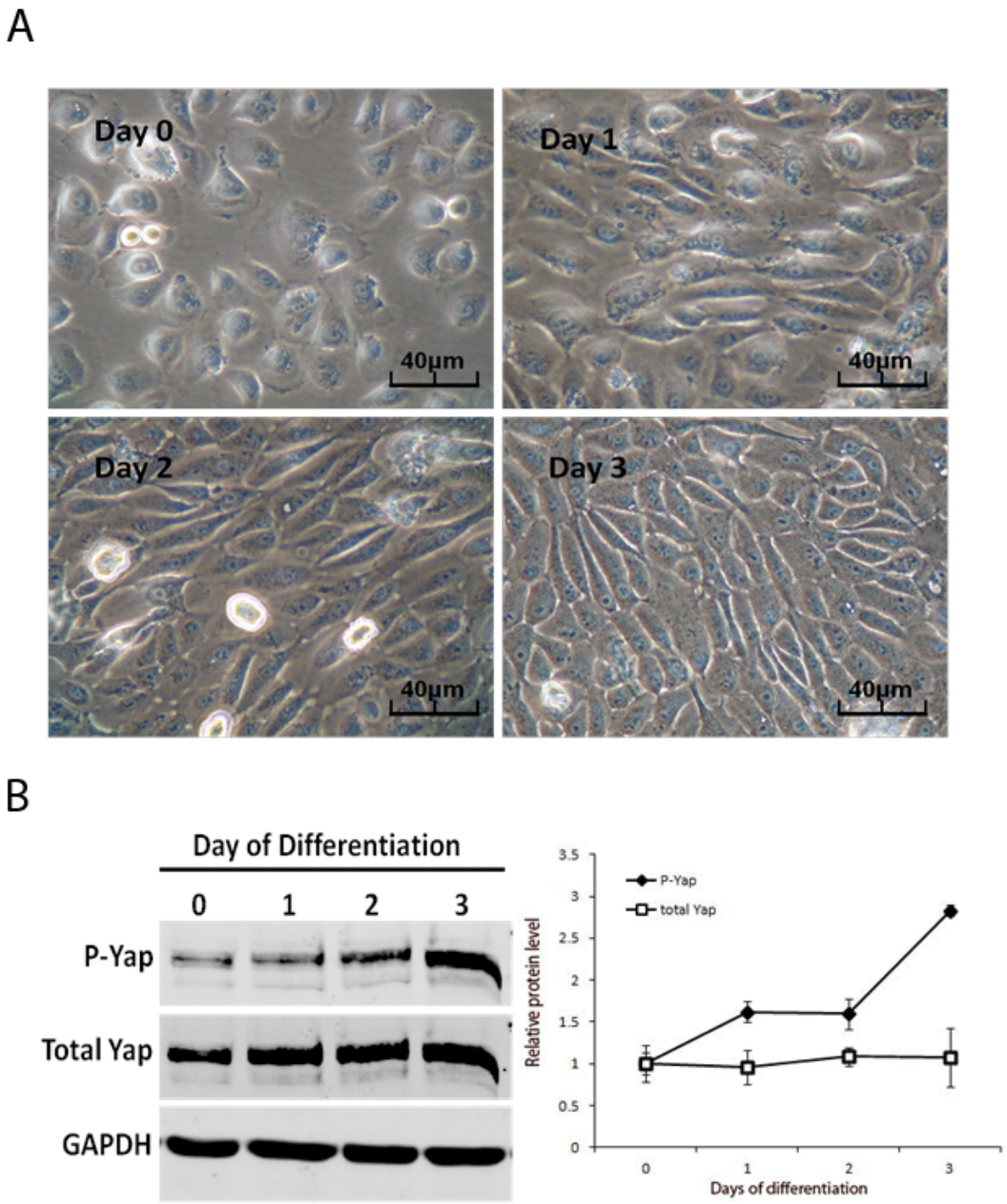

Figure 5-2. Yap phosporylation level increases with cellular differentiation.

A. Non-cancerous NTERT keratinocyte are grown to high density and differentiated with 2.8 mM Calcium.

B. Whole cell lysate (WCL) of NTERT at various day of differentiation is immunoblotted for Yap^{S127} phosphorylation and total Yap protein. The mean protein level from three experiments is indicated next to a representative blot.

Error was computed as standard deviation of the mean.

5.2.2. HPV-E6 reduces Yap phosphorylation and promotes Yap nuclear localization during keratinocyte differentiation

The normal differentiation process promotes the inactivation of Yap (Figure 5-2 B). Additionally, a major cancer promoting effect of E6 is the inhibition of differentiation. Therefore, we investigated whether E6 promotes Yap activity during differentiation. NTERT keratinocytes were used to study the effect of E6 in isolation from other HPV proteins. A retro-viral system was used to stably express either the high-risk 16E6 (NTERT E6) or vector control (NTERT control). These stable cell lines were differentiated using calcium for the indicated numbers of day(s). Whole lysates from NTERT E6 or NTERT control cells were immunoblotted for the level of P-Yap, total Yap protein, and β actin. Meanwhile, E6 activity was also confirmed via immunoblot of p53 and the differentiation marker involucrin. Consistent with the previous result, P-Yap levels greatly increase with differentiation. Interestingly, there was a significant increase of inactive P-Yap in E6 expressing cells at every stage of differentiation (Figure 5-3 A).

Yap phosphorylation is a reliable marker for the inhibition of Yap. However, a reduction of P-Yap level could be due to the disruption of cell junctions rather than a decrease in the rate of phosphorylation. Since E6 promotes the degradation of many junctional proteins (*95*), it was possible that E6 reduces P-Yap level through a reduction of Yap accumulation at the cell junctions. NTERT cells stably expressing E6 or vector control were induced to differentiate for 2 days. Cytosol and nuclear enriched fractions from these cells were then immunoblotted for P-Yap, total Yap, and p53 to measure the effect of E6. Additionally, GAPDH and Laminin A/C were used as markers for successful cytosolic and nucleic protein fractionation, respectively. Consistent with the above whole cell lysate blot (Figure 5-3 B), E6 expression reduces P-Yap level in the cytosol-enriched fraction. Significantly, E6 increases Yap nuclear localization, suggesting that E6 indeed promotes Yap activity during differentiation.

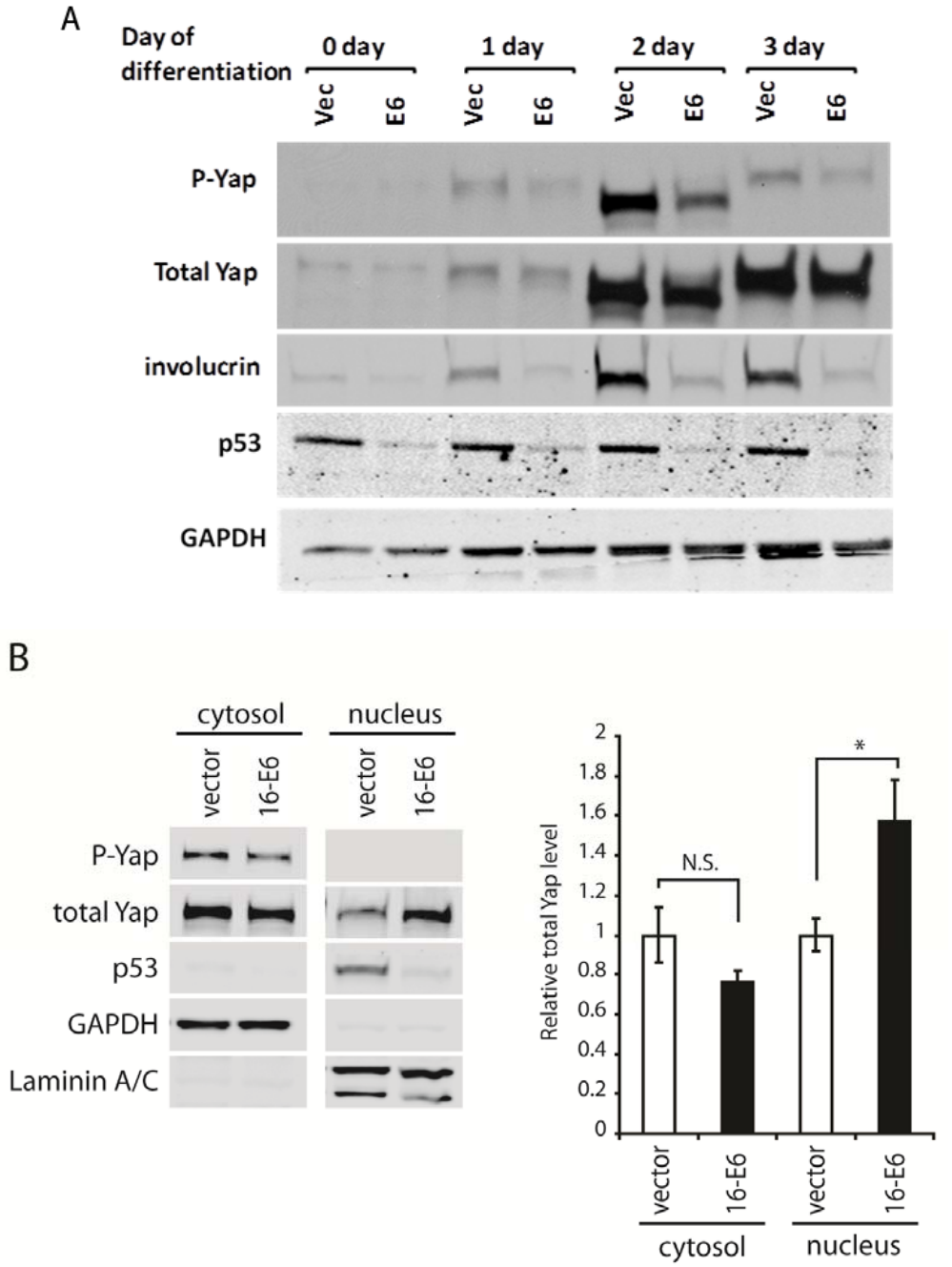

Figure 5-3. HPV-E6 inhibits cell differentiation and promotes Yap activation.

A. NTERT keratinocytes stably expressing E6 (NTERT E6) and vector control cells were differentiated for the indicated day(s). Lysate from these cells were immunoblotted for P-Yap, total Yap, involucrin - a marker of differentiation, p53, and GAPDH.

B. NTERT with E6 or control were differentiated for 2 days then subjected to nuclear fractionation and immunoblot as described in Figure 3-4 A/B. Representative blots are shown in left panel next to the quantification of average Yap protein from three independent experiments in the right panel.

Errors and significance are as described in Figure 3-3.

5.2.3. Rap1 GTPase is activated by HPV-E6; however, Rap1 does not mediate E6 differentiation inhibition

Next, we focused on identifying a molecular pathway that mediates Yap activation by E6. A prominent candidate was Rap1, a member of the Ras family of small G proteins. Rap1 plays an important role in cell adhesion through its integrin and E-cadherin regulation (*102-109*). Additionally, Rap1 activation disrupts tissue structure and Rap1 suppression restores epithelial acinar architecture (*114*). Band's lab reported that E6 induces Rap1 activation via the degradation of a Rap1 GAP named E6TP1. To confirm this result, previously described NTERT E6 or control cells were differentiated for the indicated days. The levels of Rap activation in these cells were measured by specific precipitation of the GTP-bound active Rap protein (Rap-GTP) from the cell lysates. Rap1-GTP was then measured by immunoblot using a Rap1 specific antibody. 2% input from whole cell lysate was also blotted as control for equal total Rap1 proteins. Consistent with E6 targeting Rap GAPs, E6 expression causes an increase in Rap1 activation regardless of differentiation stage. Surprisingly, Rap1-GTP amount increases with cell differentiation in both cell lines (Figure 5-4 A). Given that E6 inhibits differentiation, this increase in Rap1-GTP during normal differentiation suggests that Rap1 activation by E6 might serve a different function.

A major function of E6 is to inhibit the differentiation of keratinocytes from mesenchymal into epithelial cells. Meanwhile, the suppression of Yap and cell growth is associated with an epithelial phenotype. Although the above result suggested that Rap1 does not mediate E6-induced differentiation, we needed to rule out the deregulation of Rap1 in contributing to differentiation inhibition and consequently Yap suppression. Two different approaches were utilized: the overexpression of activated Rap1 mutants to mimic E6, and Rap1 knockdown to reverse E6's effect on cell differentiation. In the first approach, lenti-virus carrying two different constitutively active Rap1A mutants (63E and 28L) were utilized to infect NTERT keratinocytes alongside vector control lenti-virus. The 63E mutant is GTPase defective while the 28L is a fast-cycling mutant. Both mutants have enhanced activation due to spending increased

time in the GTP-bound state. Although no drug selection was needed, the Rap1 protein was GFP tagged and infection efficiencies were confirmed by both fluorescence microscopy and immunoblot for GFP. Inhibition of calcium-induced differentiation was measured by immunoblotting cell lysates for the epithelial marker, involucrin. Surprisingly, compared to the large reduction of involucrin induced by 16-E6 (Figure 5-3 A), both activated Rap1 mutants had no effect on this differentiation marker (Figure 5-4 B). The second approach examined whether shRNA-targeted knockdown of both Rap1A and Rap1B could partially reverse E6's suppression of differentiation. Two different shRNAs for Rap1B and one for Rap1A were used in various combinations to knock down both Rap1 proteins, which reduced the amount of Rap1-GTP, in NTERT E6 or control cell. Upon selection for stable knockdown, cells were differentiated and their lysates were immunoblotted for involucrin. Endogenous Rap1 was also monitored to confirm successful knockdown. Consistent with the Rap1 overexpression data, Rap1 knock down also did not affect the differentiation of either E6 or control cells (Figure 5-4 C). Altogether, these observations indicate that Rap1 activation by E6 does not affect keratinocyte differentiation.

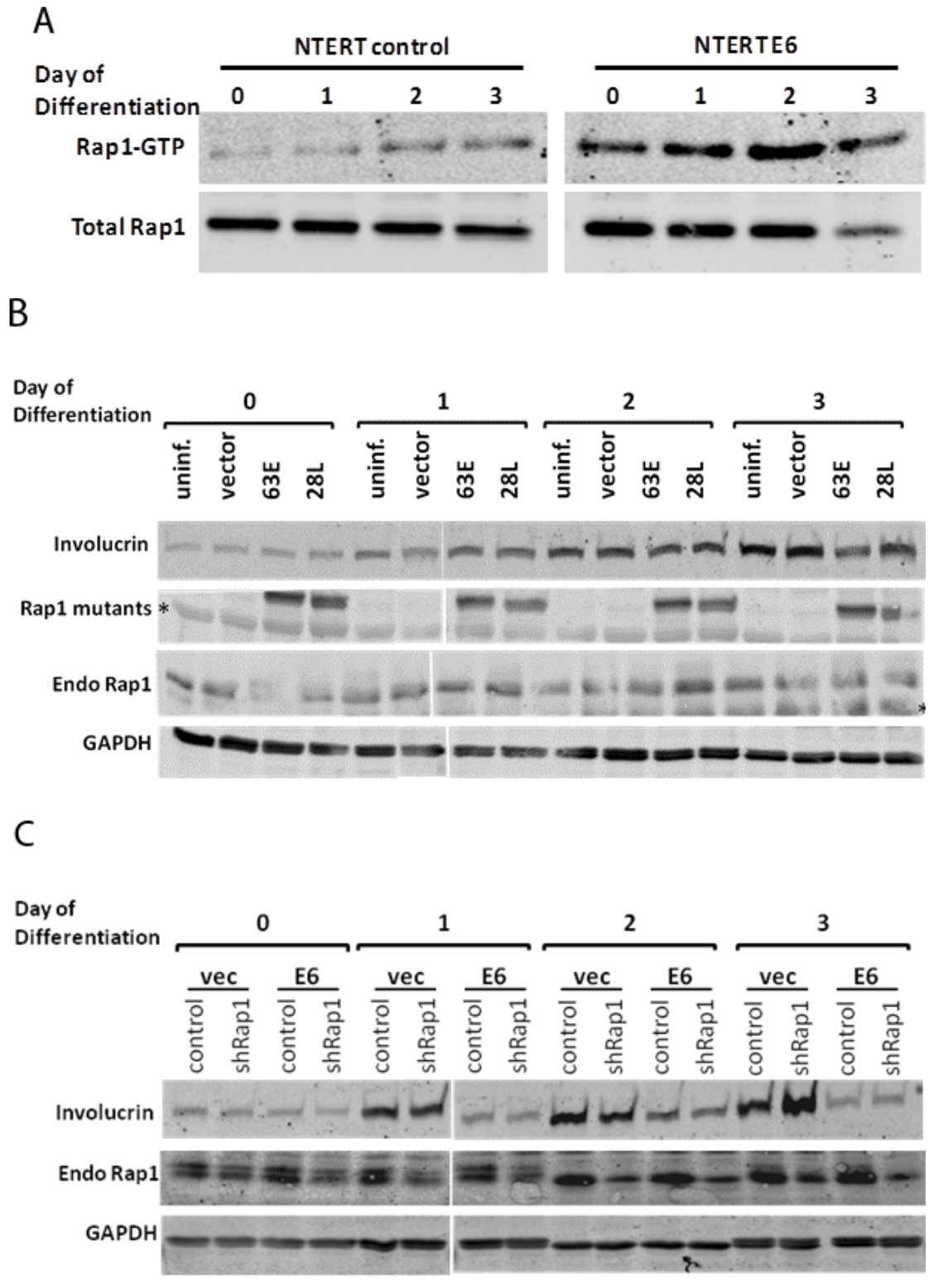

Figure 5-4. Rap1 is activated by E6; however, Rap1 does not effect keratinocyte differentiation.

A. Rap1 activation was measured by precipitation of the active GTP-bound form of Rap1 from NTERT E6 or control cells (described in Figure 5-3 A), using RalGDS RBD beads. Bounded Rap-GTP proteins were immunoblotted, while 2% whole cell lysate input was blotted for total Rap1 protein.

B. Using a lenti-virus system, two activated mutants of Rap1, 63E and 28L, were overexpressed in NTERT cells. Uninfected control, vector control, and cells stably expressing Rap1 mutants were then differentiated for the indicated days. Neither Rap1 mutant could mimic the effect of E6 to inhibit cell differentiation as measured by immunoblot of the epithelial marker, involucrin. Asterisk indicates non-specific band.

C. Control or E6-expressing NTERT cells were infected with lenti-virus expressing shRNA against Rap1. Lysates from these cells following the indicated days of differentiation were immunoblotted for involucrin marker, endogenous Rap1, and GAPDH.

Figure A, B, and C are the representatives of two experiments.

5.2.4. Rap1B likely mediates Yap activation by HPV-E6 independent of cell differentiation

Although changes in Rap1 activity did not disrupt keratinocyte differentiation, Rap1 over-activation has been shown to cause the destruction of tissue architecture and leads to tumor progression (*114*). Since E6 expression promotes both Yap nuclear localization (Figure 5-3) and Rap1 activation (Figure 5-4 A), we were interested in whether Rap1 activation by E6 increases Yap activity independent of cell differentiation. In order to study Rap1's effect in the presence of E6, we utilized HeLa cells that already express high-risk 18E6 and 18E7 proteins. Using the TEAD reporter assay described in Figure 3-3, Yap downstream activity was measured following the overexpression of closely related Rap family members Rap1A, Rap1B, Rap2A, and Rap2B. Interestingly, while Rap2 and Rap1A expressions mildly affected Yap activity in comparison to vector control, Rap1B specifically promoted Yap activation (Figure 5-5 A).

In order to confirm the ability of Rap1B to promote Yap activity, two different shRNA targeting Rap1B and one targeting Rap1A were transfected to HeLa cells together with TEAD activity reporters. Consistent with overexpress data, the knockdown of Rap1A had only a small effect while the knockdown of Rap1B vastly reduced TEAD transcriptional activity (Figure 5-5 B). Alternatively, we overexpressed two Rap GAPs (Rap1gap and E6TP1) that indiscriminately deactivate Rap proteins and two Rap GEFs (EPAC and C3G) that activate Rap. Similar to the knockdown of Rap1 proteins, the expression of GAP reduced Yap activity. Conversely, the expression of GEF promoted Yap downstream activity (Figure 5-5 C). Thus, although Rap1 does not mediate E6's inhibition of differentiation, these data suggest it is possible for Rap1B to promote Yap activity in HPV-positive cells.

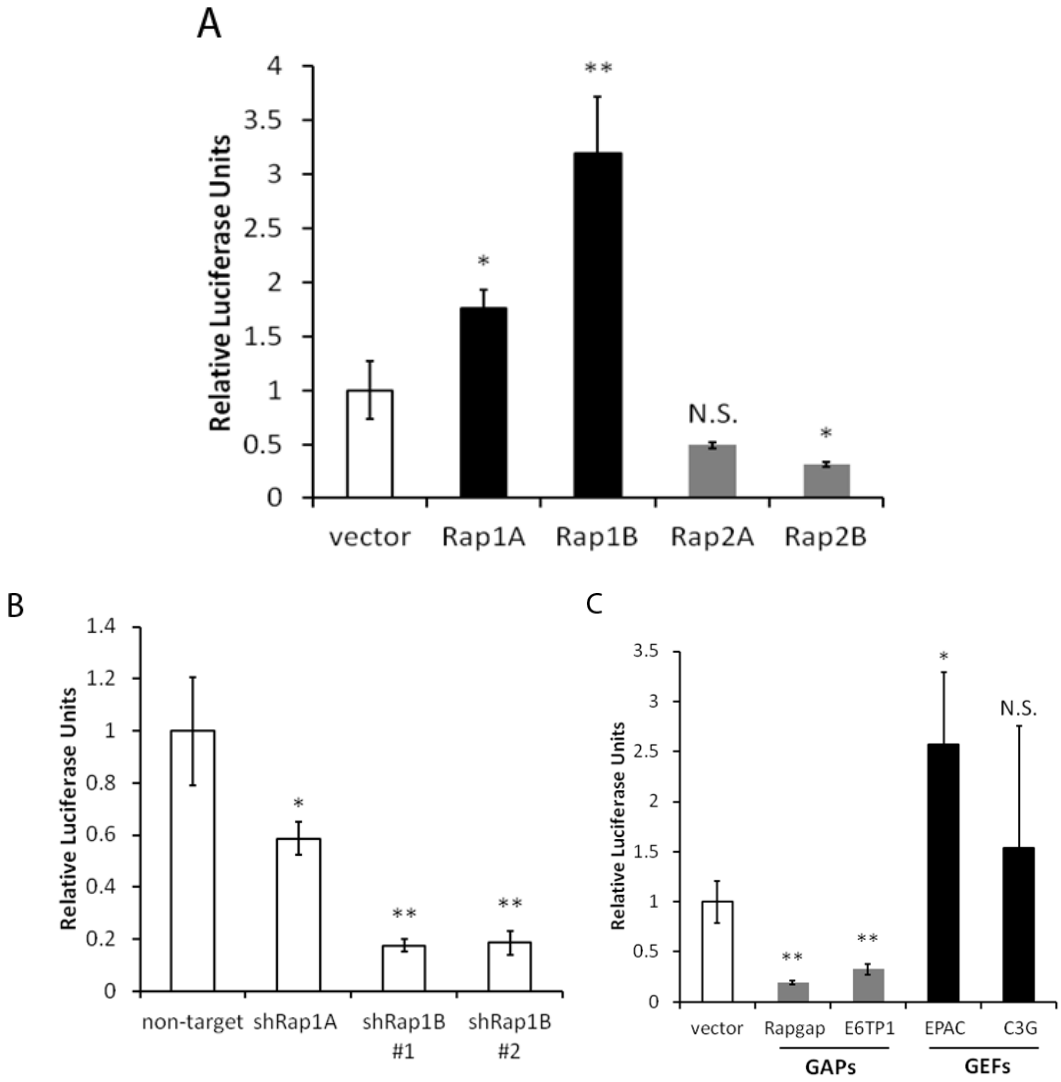

Figure 5-5. Rap1B specifically promotes Yap activity and might mediate Yap activation by HPV-E6.

A. HeLa cells (that are HPV18-positive), were transfected with TEAD reporter plasmids and plasmids expressing the indicated Rap family members or vector control. Yap activity was inferred from luciferase signal as described in Figure 3-3.

B. Similarly HeLa cells were transfected with plasmids containing shRNA targeting the indicated Rap1 member and Yap downstream transcriptional activity was examined.

C. Consistently, overexpression of Rap1 GAPs increased while and Rap GEFs decreased TEAD signals.

Figure A and B are representative of two independent experiments each performed in triplicate. Errors and significance are as described in Figure 3-3.

5.3. DISCUSSION

5.3.1. HPV-E6 promotes growth during differentiation likely through Rap1B-Yap activation

The viral oncoproteins E6 and E7 are the causal factors of cervical cancer development. The current perspective is that E7 promotes cell growth while E6 prevents cell death and differentiation (*1, 2, 148*). However, E6 has been shown to promote cell growth in some cases (*90, 95, 149*) prompting us to examine the effects of E6 on the progrowth transcription factor Yap. The expression of E6 in NTERT keratinocytes reduces Yap phosphorylation and increases nuclear Yap levels throughout the differentiation process. Since calcium-induced differentiation of keratinocyte suppresses Yap, the question remains whether E6 promotes growth or E6 prevents growth arrest through its disruption of differentiation. Here, we found evidence that E6 promotion of Yap activity is associated with the activation of the Rap1 small GTPase. The expression of high risk HPV-16E6 in non-cancerous keratinocytes strongly increases Rap1-GTP levels at all stages of differentiation. Additionally, in HeLa cells with E6 expression, the expression of Rap1 (but not Rap2) increased Yap downstream activity. Similarly, the knockdown of Rap1 decreased TEAD activity. Rap1B knockdown was more effective at reducing TEAD activity than Rap1A knockdown. Therefore our data suggest the possibility that HPV-E6 activates Rap1B which in turn may promotes Yap activity. The observation that Rap1 promotes Yap activity without effecting cell differentiation suggests that E6 regulation of Yap is growth promoting and not a result of growth arrest. However, the confirmation of this result will require experiments examine keratinocyte growth in 3D raft culture.

It is worth noting that E6 harbors a PDZ-binding motif, which might play a crucial role in E6's ability to cause the thickening of the epidermis (*95*). This PDZ-binding motif enables E6 to target a number of PDZ domain-containing polarity-associated proteins for degradation. Interestingly, at least two E6 targets, MUPP1 and PATJ have been shown to interact with Yap.

On the other hand, both Yap and Taz contain a PDZ-binding motif at their C terminus. At least for Yap2, this PDZ-binding motif is required for Yap binding to zonula occluden 2 (ZO-2) and Yap shuttling to the nucleus (*39, 150*). Thus, one may speculate that E6 releases Yap from membrane proteins through the degradation and disruption of polarity-associated complexes. Furthermore, E6TP1 and several other Rap1 GAPs all have a highly conserved (80 to 90% identity) PDZ domain. Interestingly, a non-synonymous amino acid polymorphism, A739T, in the PDZ domain of a Rap1 GAP called Sipa1 (Signal-induced proliferation-associated protein 1, also known as Spa-1) causes a drastic increase in the metastatic potential of breast cancer (*151*). Sipa1 is also shown to regulate the trafficking to the membrane of a protein channel Aquaporin 2 (*152*). Consistently, a number of studies support the role of Rap1 in protein-trafficking regulation. For instance, Rap1 regulates the endocytic recycling of E-cadherin to promote stem cell renewal (*153*). Alternatively, Rap1 cooperates with p38 MAPK and MAPK kinase 3/6 to couple receptor endocytosis with long term depression of neuronal synapses (*154*). Therefore, it is possible that Rap1 activation by E6 promotes the shuttling of Yap to the nucleus for the transcription of proliferative genes.

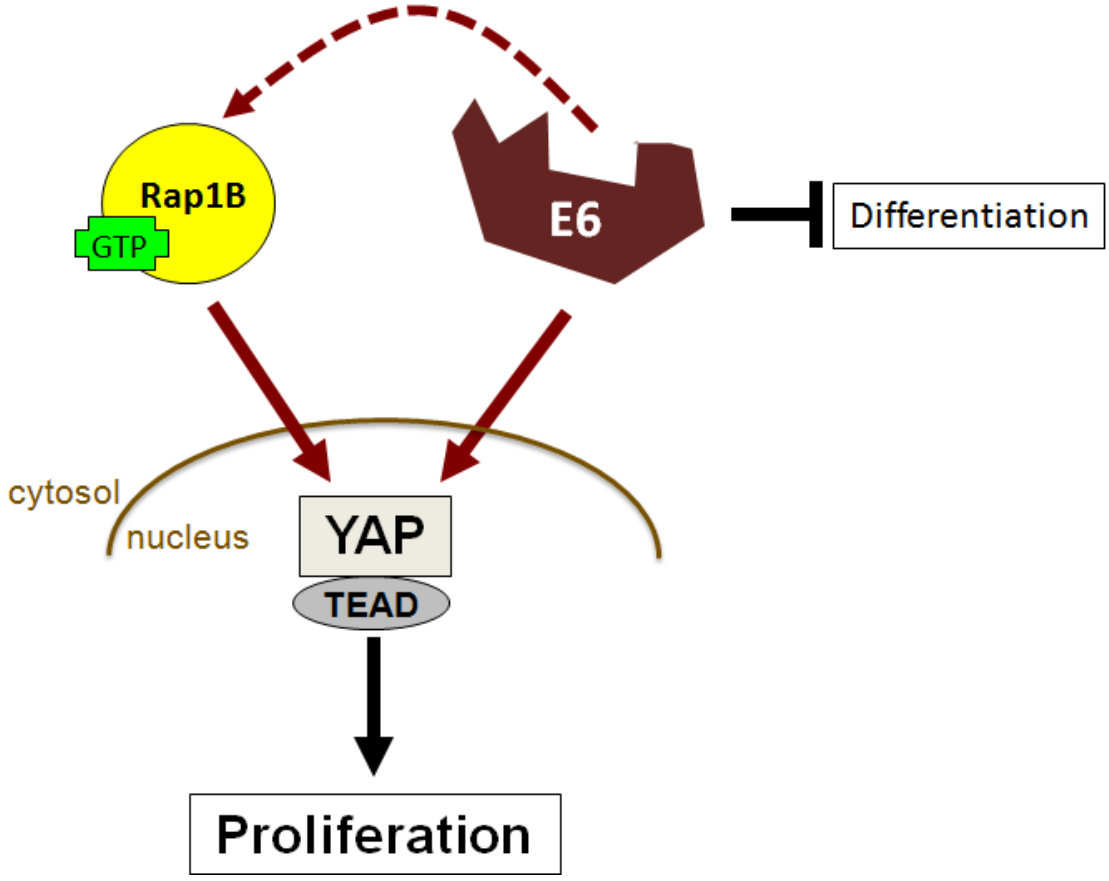

Figure 5-6. HPV-E6 likely promotes keratinocyte growth through Rap1-Yap activation.

The viral oncogene E6 promotes Yap activity, which otherwise is suppressed by cell differentiation. E6 also causes Rap1 activation. Since Rap1B specifically and potently increase Yap downstream transcription and Rap1 does not mediate the effect of E6 on differentiation, it is likely that E6 increases Yap function through Rap1.

5.3.2. Possible distinct functions of various Rap family members

Rap1A, Rap1B, Rap2A, Rap2B, and Rap2C are members of the Ras superfamily. These Rap proteins are encoded by five different genes which most studies have shown to be largely redundant. Interestingly, our data indicates that these Rap family members may have distinct effects on Yap activity. The general perspective is that Rap family members are differentially expressed in various cell types during different stages of development. Rap1 and Rap2 share a number of redundant functions. Rap1A was first discovered as a Ras antagonist which competes with the Ras-binding domain of Raf (c-Raf) preventing Ras from activating it. Thus Rap was found to suppress cancer growth through interference with Ras-signaling. Later, Rap1 was found to promote proliferation in cells that contain B-Raf through the ERK mitogen-activated protein kinases pathway (*100*). Additionally, Rap2 may activate JNK through MAP4K4 to promote cell growth (*101*). Therefore, the availability of Rap downstream effectors dictates their downstream effects. This fact underscores the tissue specificity of Rap family members and the different roles these family members may play in different contexts. Like Ras, Rap shuttles between active GTP-bound and inactive GDP-bound states, upon activation by GEFs and deactivation by GAPs (*99*). Rap1 and Rap2 exhibit 60% sequence identity and these Rap family members share a number of GEFs and GAPs. However, these GEFs and GAPs have different cellular localization and preferential activity toward various Rap family members. Thus, Rap members are regulated via specific expression in each cell type as well as via intracellular localization. The diversity of Rap family members and regulatory proteins allow Rap to intricately control many processes in a cell and tissue specific manner. Given reported roles of Rap and its regulators in acinar polarity (*114*), gastrulation development (*155*), and angiogenesis (*156, 157*), it is likely that different Rap members have evolved to accommodate development of various organs as well as tissue homeostasis in multicellular organisms.

Here, we found that Rap1A and Rap1B activate Yap while Rap2A and 2B mildly represses Yap activity. Thus Rap1 and Rap2 might differentially regulate cell growth even though

both Rap1 and Rap2 have been shown to promote cell adhesion (*105, 109, 135, 158*). This could explain the "bad Rap/good Rap" effects where increased Rap activity was reported to both negatively and positively influence cancer progression depending on the particular circumstance (*99*). Additionally, Rap2A was recently shown to mediate brush border formation in response to LKB1 activation in intestinal cells (*135*). In this report, LKB1 promotes recruitment of PDZGEF to the cell membrane. This event specifically increased Rap2A-GTP (and not other Rap members) and triggered a cascade composed of the Rap2A effector called TRAF2 and NCK interacting kinase (TNIK), the Mst4 kinase, and Ezrin (*135*). Thus, it is possible that the loss of LKB1 decreases Rap2-GTP level while the expression of E6 increases Rap1 activity leading to cervical cancer. Our result of Rap1 promoting and Rap2 repressing Yap activity would be consistent with this idea.

CHAPTER 6. CONCLUDING REMARKS

6.1. FUTURE STUDIES

6.1.1. The dilemma of growth and death

At the turn of this century, Yap was identified as a proapoptotic factor in Cos7 (monkey kidney fibroblast) and MCF7 (human breast cancer) cell lines (*38*). Several reports subsequently showed that nuclear Yap interacted with the transcription factor p73 (p53 homolog) and promyelocytic leukemia protein (PML) (*38, 159, 160*) to promote the expression of apoptotic proteins such as Bax, p53AIP, and PUMA. This activation of Yap was in response to apoptotic signals by the stimulation of Fas ligand receptor. However, a shift away from the focus on Yap's pro-apoptotic function(s) resulted from overwhelming *Drosophila* and mouse genetic data. These studies confirmed the role of Yap (Yki in flies) in promoting cell proliferation. However, we observed that Yap activation also causes cellular toxicity. The knockdown of Yap suppressors such as NF2, LKB1, and Lats1/2 promotes cell death even though Yap downstream transcription of proliferative genes is greatly increased. Indeed, non-cancerous keratinocytes with NF2 knockdown survive less than a week. Strangely, although the knockdown of LKB1 increased growth readouts such as cell size and CTGF gene expression, cells eventually died. More interestingly, the stable knockdown of Lats1 or Lats2 by shRNAs (not transiently expressed siRNAs) lead to cell death of both cancerous HeLa cells and non-cancerous keratinocytes. It is worth noting that similar to Yap, the deregulation of other oncogenes like c-myc (*161*) and ras (*162*) also causes cell death under numerous conditions. For instance, fibroblasts with c-myc ectopic expression undergo apoptosis without the presence of survival factors in culture media (*163*). Similarly, a review by Maltese outlined various pathways that mediate Ras-induced cell death including the regulation of Bcl proteins via RASSF1A, ERK, Rac, and p53 downstream signals (*162*). Although many controversies surround oncoprotein-induced apoptosis, the most widely held view is that proliferation sensitizes cell to apoptosis and the availability of co-factors and survival signals allow cancer cells to thrive. Thus far, there are few factors like p73 and PML

that influence Yap pro-apoptotic function. Deciphering the cellular events/factors which determine the different outcomes of Yap/Taz activation is an intriguing source of ongoing investigation with important implications in cancer development and treatment.

6.1.2. The regulation of Taz

In mammalian cells, Yap and Taz are often mentioned as two identical orthologs of the *Drosophila* Yorkie. This grouping of Yap and Taz proteins is deemed acceptable due to their redundant functions as transcription co-activators. For instance, both Yap and Taz interact with transcription factors such as TEAD, RUNX, p73, PAX, TTF-1 (also known as NKX2.1), TBX5 (also known as NKX2.5), PPARγ, and SMAD family (*27*). Additionally, YAP and Taz are suppressed by phosphorylation and cytosolic retention via interactions with 14-3-3 proteins in growth disadvantaged conditions. The fact that Yap/Taz double knockout mice cease to develop beyond the 16-32 cell stage further confirms their roles in cell proliferation. However, the phenotypes of the individual gene knockouts in mice indicate there may be subtle differences in their functions. For example, mice with Yap deletion will not develop past E8.5, while Taz depletion causes lung and kidney defects that usually lead to embryonic lethality (*27*). A recent study examining the effect of stress fibers, induced by cell spreading, on Yap/Taz localization showed that stress fiber disruption preferentially affects the phosphorylation and stability of Taz protein more than Yap (*41*). Thus it is possible that these homologous proteins are differentially regulated.

Our preliminary work on Taz showed that Taz is regulated in a similar manner to Yap. As observed with Yap, E6 expression and NF2 knockdown both significantly increase endogenous Taz levels (Figure 6-1 A and B). This result is consistent with the previously observed increase in the activity of TEAD transcription factor, which binds to both Yap and Taz. Meanwhile, LKB1 expression did not affect Taz protein expression levels (Figure 6-1 B). However, this steady Taz level with LKB1 expression might represent Taz sequestration at the

junction. Thus measurement of the Taz phosphorylation sites Serine 89 and Serine 311, Taz accumulation in the nuclear fraction, or Taz turn-over would be needed to confirm whether LKB1 does not affect Taz. Most interestingly, AMPK activation via glucose starvation in the presence of LKB1 decreases Taz level (Figure 6-1 A). This result is surprising since we did not see any change in Yap phosphorylation with AMPK activation using this same method with or without LKB1 (Figure 3-8 C). AMPK regulation of Taz stability suggests that LKB1-induced growth suppression is mediated by both Yap and Taz through distinct regulators. However, no conclusion about Taz regulation can be made from these limited findings.

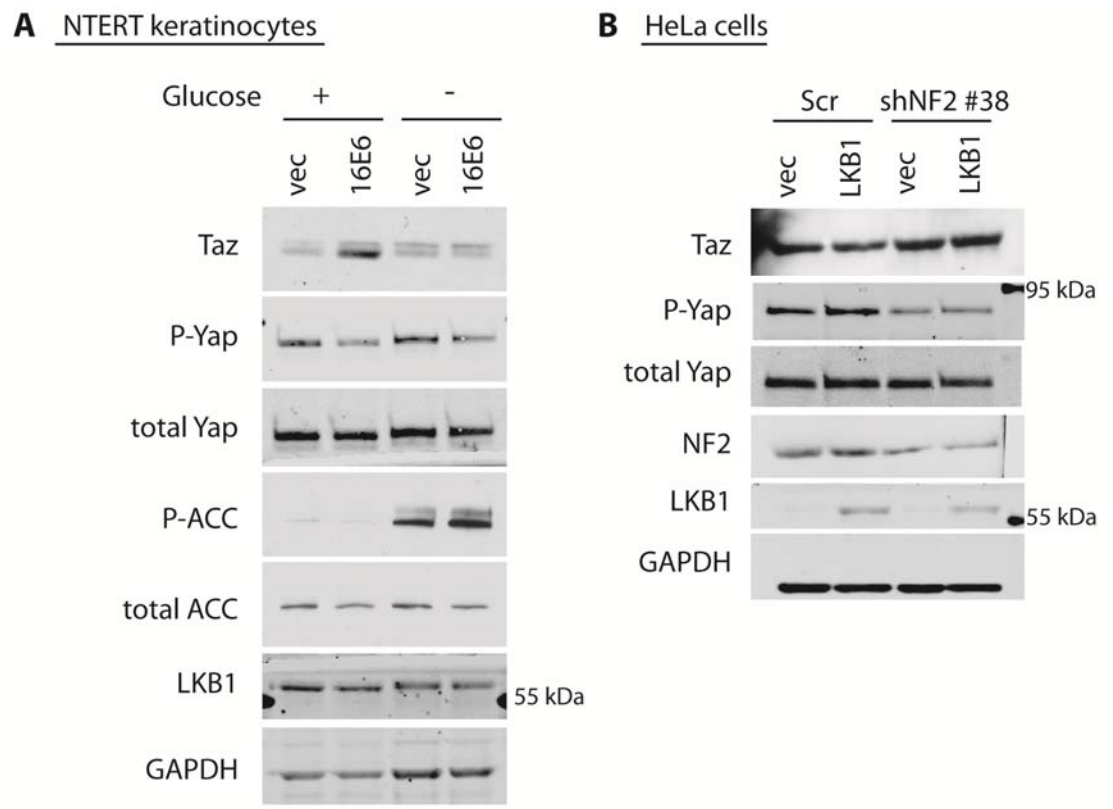

Figure 6-1. The regulation of Taz by NF2, E6, and possibly AMPK.

A. Keratinocytes stably expressing HPV-16E6 or control (as described in Figure 5-3) were incubated in glucose deficient media for 2 hours. WCL from the treated and control were immunoblotted for Taz, P-Yap, and total Yap level. P-ACC and total ACC were also measured as control for the activation of AMPK by glucose starvation. LKB1 and GAPDH were blotted to ensure equal loading.

B. HeLa cells stably expressing LKB1 in combination with shRNA targeting NF2 or control (as described in Figure 4-3 C) were plated out for 2 days. Cell lysates were immunoblotted for Taz and Yap level as in part A. NF2 level were also measured to ensure adequate knockdown.

6.1.3. The mechanism of how NF2 suppresses Yap

The scaffolding protein NF2 (Merlin) suppresses cell growth through multiple signaling pathways. The best characterized signaling event downstream of NF2 is the inactivation of Rac1-PAK which subsequently leads to activation of the MAPK pathway. This inhibition of the Rac1-PAK axis is mediated by a tight-junction-associated protein complex comprising of Amot, Patj, and Pals1. In growth suppressive condition, NF2 binds to this complex and releases Rich1, which is a GAP that deactivates Rac1 (*61, 72, 134*). Additionally, NF2 mediates contact inhibition of cell growth through the Hippo/Yap pathway. NF2 expression activates the Hippo kinases Mst and Lats (*61-63*) and could suppress Yap independent of Mst or Lats (*32, 43*). While Yap activity was not strongly affected by Rac1, PAK, or Amot in our system, reports regarding Amot inhibition of Yap (*136*) and Pals1-Patj interaction with Yap (*27*) in different cellular systems suggest a possible cross-regulation between these seemingly parallel pathways.

Since LKB1 expression promotes a drastic change in the actin cytoskeleton structure and NF2 is required for epithelial-like cell shape, we hypothesize that ERM proteins might mediate LKB1 suppression of Yap. This idea is supported by ERM proteins key role in actin regulation. Furthermore, Ezrin (an ERM protein) works downstream of LKB1 to promote formation of the brush borders of intestinal epithelial cells. Additionally, when NF2 is phosphorylated and assumes an “open” conformation, it forms heterodimers with ERM proteins. Since LKB1 restoration activates a number of kinases, it is possible that NF2 phosphorylation affects NF2-ERM proteins heterodimerization. Therefore, future investigation examining the impact of phospho-deficient S418A and phospho-mimic S418D mutants of NF2 (Ref) might implicate NF2 phosphorylation in mediating effects of LKB1. Cellular phenotypes such as Yap dependent transcription and cell growth could be measured in combination with NF2 mutants. Alternatively, NF2 and Yap proteins’ subcellular localization with LKB1 expression may help answer whether NF2 functions in a juxta-membrane complex that suppresses Yap.

6.1.4. The synergistic effect between E6 expression and LKB1 loss

An equally intriguing question not addressed in this dissertation is possible synergism between LKB1 loss and HPV infection in cervical cancer malignancy. The evidence for this synergistic effect is that patients with LKB1-deficient cervical cancer have a median survival of 13 months while others with LKB1-wild type tumors survive more than 100 months (*7*). Additionally, Peutz-Jeghers patients with LKB1 loss suffer a >15 times risk of developing malignant epithelial cancers at various sites (*164*). We speculate three possible consequences of combining LKB1 deletion with E6 incorporation. Firstly, E6 expression prevents cell death that is induced by the loss of LKB1 as discussed above. Thus keratinocytes harboring somatic mutations in LKB1 or its downstream mediator of Yap suppression could survive proliferative-stress and continue to growth. Secondly, LKB1 and E6 might affect different opposing growth regulators. For instance, LKB1 promotes activation of Rap2 (*135*) which has negative influence on TEAD gene transcription (Figure 5-5 A), while E6 activates Rap1 which increases Yap function (Figure 5-5 B). Alternatively, LKB1 depletion and E6 expression may differentially increase Yap or Taz levels (Figure 6-1). A third possibility is that epithelial structure disruption, as a result of LKB1 deficiency, allows HPV-infected cells to invade and metastasize. This hypothesis is in line with our previous discussion that LKB1 may enhance cell adhesion through the redistribution of junction proteins. This idea is also supported by numerous reports that describe LKB1's role in suppressing the metastasis and invasiveness of lung epithelial cancers, adenocarcinomas, squamous cell carcinomas, prostate epithelium, and endometrial epithelium (*49, 117, 165*). Future investigations using an E6/E7 transgenic mouse that has been crossed to enable epithelial tissue specific knockout of LKB1 might provide mechanistic insights into the development of malignant cervical cancer. Understanding the synergy between HPV oncoproteins and LKB1 loss may lead to possible treatments for these highly metastatic cervical carcinomas.

6.2. CONCLUSION

In summary, I have identified two pathways, LKB1-NF2-Yap and E6-Rap1-Yap that uncover how the structure of a cell directly influences its gene expression and proliferation. These pathways both have crucial implications in the development of cervical cancer. LKB1 and NF2 induce actin cytoskeleton organization and cell cohesion, which in turn suppress both Yap activity and cell growth. Meanwhile, the viral oncogene HPV-E6 activates the Rap1 GTPase leading to Yap nuclear localization and increased transcription of proliferative genes. These results are consistent with clinical data indicating poor prognosis of patients with combined somatic mutations in LKB1 plus E6 overexpression (*7*). Therefore, future investigations examining possible synergism between these events may provide either a diagnostic tool or new therapy for malignant cases of cervical cancer.

APPENDIX – NUCLEOTIDE SEQUENCES

CLONING PRIMERS

LKB1 PCR amplification with EcoRI/NotI overhang
Forward 5'- GAATTC atgga ggtggtggac ccgcag
Reverse 5'- GCGGCCGC TCA CTG CTG CTT GCA GGC CGA C
E6TP1 (two-steps cloning with internal HindIII)
Forward 5'- atat GGCGCGCC AT GAC CAG CTT GAA ACG GTC AC
Reverse 5'- AGG CGA ATT CAA GCT TTC TCC AGA TGT TCC CTG
KIAA1389 (two-steps cloning with internal XhoI)
Forward 5'- cact GGCGCGCC gt ccctttggg ttccctgaat
Reverse 5'- tagc CTCGAG AGA GCG CAA TCC GCC T
hSIPA (two-steps cloning with internal StuI-methylated)
Forward 5'- gctaGGATCC CCC ATG TGG GCC GGC
Reverse 5'- tagc GAATTC CCA CCC AGG CCT AGC

POLYLINKERS OF MULTIPLE CLONING SITES

v309-linker with the following restriction sites
AscI, BamHI, XhoI, EcoRI, NheI, SmaI/XmaI, PacI
GGCGCGCCGGATCCCTCGAGGAATTCGCTAGCCCCGGGTTAATTAA
v309-L2 and v309-L3 are the same modified polylinker added HindIII site with second and third reading frame, respectively.
v309-L2
GGCGCGCCAGGATCCAAGCTTCTCGAGGAATTCGCTAGCCCCGGGTTAATTAA
v309-L3
GGCGCGCCAAGGATCCCTCGAGAAGCTTGAATTCGCTAGCCCCGGGTTAATTAA
pCDH-puro and hygro with the following restriction sites
NotI, AscI, BamHI, XbaI, EcorI, PacI
GCG GCC GCG GCG CGC CGG ATC CTC TAG AGA ATT CTT AAT TAA

RT-PCR PRIMERS AND PROBES

CTGF NM_001901.2
Forward 5'- agctgacctggaagagaacatt
Reverse 5'- gctcggtatgtcttcatgctg
Probe #71 universal probe library
HDM2 NM_002392.3
Forward 5'- tctgatagtatttccctttcctttg
Reverse 5'- tgttcacttacaccagcatcaa
Probe #21 universal probe library
ACTIN (note that actin is not a good normalizing standard in many cases)
Multiplex Actin probe – HEX dye
GAPDH NM_002046.3 (standard)
Forward 5'- agccacatcgctcagacac
Reverse 5'- gcccaatacgaccaaatcc

Probe #60 universal probe library
RPLP0 NM_053275.3 (standard)
Forward 5'- tctacaaccctgaagtgcttgat
Reverse 5'- caatctgcagacagacactgg
Probe #6 universal probe library

shRNA SEQUENCES
Non-targeted scramble of luciferase (*166*)
CCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGG
Targeting LKB1
shLKB1#1 or 408 (TRCN0000000408)
CCGGGCCAACGTGAAGAAGGAAATTCTCGAGAATTTCCTTCTTCACGTTGGCTTTTT
409 (TRCN0000000409)
CCGGGATCCTCAAGAAGAAGAAGTTCTCGAGAACTTCTTCTTCTTGAGGATCTTTTT
shLKB1#2 or 411 (TRCN0000000411)
CCGGCATCTACACTCAGGACTTCACCTCGAGGTGAAGTCCTGAGTGTAGATGTTTTT
Targeting LATS
shLATS 1 (TRCN0000001777)
CCGGCACGGCAAGATAGCATGGATTCTCGAGAATCCATGCTATCTTGCCGTGTTTTT
shLATS 2 (TRCN0000000880)
CCGGCCGTCGATTACTTCACTTGAACTCGAGTTCAAGTGAAGTAATCGACGGTTTTT
Targeting NF2
shNF2 #1 or 38 (TRCN0000018338)
CCGGTAGTTCTCTGACCTGAGTCTTCTCGAGAAGACTCAGGTCAGAGAACTATTTTTG
shNF2 #2 or 74 (TRCN0000039974)
CCGGGCTCTGGATATTCTGCACAATCTCGAGATTGTGCAGAATATCCAGAGCTTTTTG
shNF2 #77 (TRCN0000039977)
CCGGCGACTTCAAAGATACTGACATCTCGAGATGTCAGTATCTTTGAAGTCGTTTTTG
Targeting RAP1
mouse shRNA Rap1A #4 (TRCN0000055269)
CCGGGCTCAGTCTACGTTTAATGATCTCGAGATCATTAAACGTAGACTGAGCTTTTTG
mouse shRNA Rap1A #5 (TRCN0000055272)
CCGGCGGGTAGTTGGCAAAGAACAACTCGAGTTGTTCTTTGCCAACTACCCGTTTTTG
mouse shRNA Rap1B #1 (TRCN0000102735)
CCGGCGCTTTGATTAACACAGCTATCTCGAGATAGCTGTGTTAATCAAAGCGTTTTTG
mouse shRNA Rap1B #2 (TRCN0000102736)
CCGGCCTACGATAGAAGATTCTTATCTCGAGATAAGAATCTTCTATCGTAGGTTTTTG
mouse shRNA Rap1B #5 (TRCN0000102739)
CCGGCAGTCGACATTTAACGACTTACTCGAGTAAGTCGTTAAATGTCGACTGTTTTTG
human shRNA Rap1A #4 (TRCN0000029787)
CCGGGCTCTGACAGTTCAGTTTGTTCTCGAGAACAAACTGAACTGTCAGAGCTTTTT
mouse shRNA Rap1B #1 (TRCN0000029174)
CCGGCCAATGATTCTTGTTGGTAATCTCGAGATTACCAACAAGAATCATTGGTTTTT
mouse shRNA Rap1B #4 (TRCN0000029177)

CCGGGCACAACAGTGTATGCTTGAACTCGAGTTCAAGCATACACTGTTGTGCTTTTT

siRNA SEQUENCES

Non-targeted si control Dharmaco ON-TARGET Plus

D-001810-10 sequence not available

siLATS1 – siGENOME SMART pool M-003865, includes these sequences

D-003865-04 GAACGAUGCCAGCGAAGGU

D-003865-03 GAUCGGUGCCUUUGGAGAA

D-003865-02 GAAAGAGUCUAAUUACAAC

D-003865-01 GUUCGGACCUUAUCAGAAA

siLATS2 - siGENOME SMART pool M-004632, includes these three sequences

D-004632-04 GAUAAAGACACUAGGAAUA

D-004632-03 GAAAUCAAGUCGCUCAUGU

D-004632-02 GCAAGUCACUCUGCUAAUU

D-004632-01 GAACCAAACUCUCAAACAA

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CURRICULUM VITAE

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EDUCATION

Bachelor of Science in Molecular Biochemistry and Biophysics
Illinois Institute of Technology (IIT), Chicago, Illinois Graduated May 2007
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Doctor of Philosophy (PhD) in Biochemistry and Molecular Biology
Indiana University, Indianapolis, IN Graduated Dec 2012

TEACHING EXPERIENCES

Teaching Assistant for Microbiology lecture and lab, Spring 2005
Dr. Douglas Cork, College of Science and Letters, IIT.
Chemistry and Biology tutor, Academic Resource Center, IIT, 2006-2007
Vietnamese Teacher for English speaking children, Indiana Church 2009-2010

RESEARCH EXPERIENCES

Team leader, Interprofessional Project 302 Synthetic Biology, IIT, 2005
- This project aimed to create and mathematically model a synthetic oscillating system of three fluorescent proteins.

Independent research, Dr. Nicholas Menhart, IIT, 2006-May 2007
- This study aimed to synchronize the oscillation plasmids in a cell group, and to bring such system into *Danio rerio* (Zebra fish).

Summer Research, Dr. Erin Adams, The University of Chicago, Summer 2006
- We used Surface Plasmon Resonance to measure binding affinity and lipid specificity of non-classical MHC composed of CD1c and single chain γ and δ TCR.

Graduate study, Dr. Lawrence Quilliam, Indiana University, 2008-2012
- Project #1: LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap trancription coactivator.
- Project #2: The loss of LKB1 tumor suppressor synergizes with Human Papilloma Virus- E6 viral oncogene to promote the development of cervical cancer.

HONORS AND AWARDS

Dean's List, IIT 2003-2007
IIT Camras/NEXT scholarship 2003-2007
Resident Advisor scholarship, IIT 2006-2007
School of Medicine BioMedical Gateway Fellowship, Indiana 2007-2008
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PUBLICATIONS

Nguyen, H.B., Babcock, J.T., Wells, C.D., and Quilliam, L.A. LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap. Oncogene (2012) doi:10.1038/onc.2012.431.

Nguyen, H.B. and Quilliam, L.A. [Electronic Book]. Chapter 274: Rap GEF Family. In *Encyclopedia of Signaling Molecules*, ed: Choi, S. Springer. (In press).

Babcock, J.T., **Nguyen, H.B.**, He, Y., Wek, R.C., and Quilliam, L.A. mTORC1 enhances bortezomib-induced death in TSC-null cells by a c-MYC-dependent induction of the unfolded protein response. J. Biol. Chem. (Under review).

Babcock, J.T., **Nguyen, H.B.**, Hendricks, J., Wells, C.D., and Quilliam, L.A. Induction of autophagy is required for endoplasmic reticulum expansion in TSC2-null angiomyolipoma. (In preparation).

PLATFORM PRESENTATIONS AND POSTERS

Nguyen, H.B., Babcock, J.T., Wells, C.D. and Quilliam, L.A. LKB1 tumor suppressor regulates AMP kinase/mTOR-independent cell growth and proliferation via the phosphorylation of Yap. *102nd AACR Annual Meeting, Chicago IL, March 31-April 4, 2012*.

Babcock, J.T., **Nguyen, H.B.**, He, Y., Wek, R.C., and Quilliam, L.A. mTORC1 enhances bortezomib-induced TSC-null cell death through c-MYC-dependent up-regulation of the unfolded protein response. *102nd AACR Annual Meeting, Chicago IL, March 31-April 4, 2012*.

Nguyen, H.B., Babcock, J., and Quilliam, L.A. LKB1 Tumor Suppressor Regulates AMPK/mTOR-Independent Cell Growth and Proliferation Via the Phosphorylation of YAP. *Platform Presentation Feb 2012 at IU Research Day.*

Nguyen, H.B., Babcock, J., Wells, D.C., and Quilliam, L.A. Novel Yap oncoprotein regulation by the tumor suppressor LKB1 independent of AMPK and mTOR. *Poster presented twice Feb 2012 and May 2012 in Biochemistry and Simon Cancer Center at Indiana University.*

Nguyen, H.B., Yan, J., He, Y., and Quilliam, L.A. High risk HPV-16E6 disturbs Ras-proximate protein 1 (Rap1) dynamic contributing to the inhibition of keratinocyte differentiation and survival. Biochemistry Research Day. *Poster presented Feb 2010 at Indiana University.*

Nguyen, H.B., Hammes, E., Allam, E., Bridgeman, B., Cadet, J.R., Cankova, Z. et.a. IPRO 302 Synthetic Biology: Engineering Novel Organisms. Interprofessional Project Day. *Presentation and poster presented Dec 2005 at IIT.*

LABORATORY TECHNIQUES AND COMPUTER SKILLS

- Cloning techniques: primer design, gene amplification, and plasmid modification.
- Protein purification, Western Blot, transfection, and other biochemical techniques.
- Tissue culture a variety of cell lines.
- Murine bone marrow isolation and primary macrophage culture.
- Lenti-virus, retro-virus, and baculo-virus production and transduction.
- Fluorescence-activated cell sorting (FACS) and other types of flow cytometry.
- Confocal Microscopy of immunofluorescence (IF) staining and live cells.
- Transgenic mouse genotyping.
- Enzyme-linked immunosorbent assay (ELISA).
- Real time RT-PCR and mRNA isolation.
- Immunoprecipitation and other pull-down techniques.
- Probability and statistics.

Microsoft: Words, Excel, PowerPoint
Adobe: Photoshop, Illustrator, Acrobat Pro
Web-design: Dreamweaver, Publisher
Scientific: Blast, ClustalW, IDT PrimerQuest, and Cloneman or pDRAW32
CN3D, DeepView (spdbv), ImageJ, LightCycler, and CellQuest/ FlowJo.

WORK AND EXTRA CURRICULUM EXPERIENCES

- Union Board Films programmer, IIT, 2004-2005 (Volunteer)
- Conference Housing Assistant, Summer 2005 (Paid)
- Resident Assistant, 2006-2007 (Paid)
- President of International Students Organization, IIT 2006-2007 (Volunteer)
- President of Vietnamese Student Association, IIT 2004-2007 (Volunteer)
- Dance teacher at IntoSalsa Indianapolis 2009-2010 (Volunteer)