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ΔNp63α Positively Regulates ERK3 Expression in Non-Melanoma Skin Cancer

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Eid Salem Alshammari ENTITLED ΔNp63α Positively Regulates ERK3 Expression in Non-Melanoma Skin Cancer BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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Abstract

Alshammari, Eid Salem. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2019. ΔNp63α Positively Regulates ERK3 Expression in Non-Melanoma Skin Cancer.

Non-melanoma skin cancer (NMSC) is a group of skin cancer that includes basal cell carcinoma of the skin (BCC), squamous cell carcinoma of the skin (SCC), actinic keratoses, a precursor to SCC, and other rare cutaneous carcinomas. p63, a member of the p53 gene family, is an important regulator for epithelial tissue growth and development. ΔNp63α, a main isoform of p63, is highly expressed in NMSC and plays essential roles in NMSCs development. Extracellular signal-regulated kinase 3 (ERK3) is an atypical member of the MAPK family. It possesses a single phosphorylation site (serine 189) in its activation loop, which makes it different from the conventional MAPKs. Similar to ΔNp63α, the expression level of ERK3 is upregulated in cutaneous SCC and actinic keratoses. While ERK3 has been shown to promote invasiveness of squamous cell lung cancer, little is known about ERK3's role in NMSCs. In addition, how ERK3 expression is upregulated in NMSCs remains largely unknown. Given that the expression levels of both ΔNp63α and ERK3 are upregulated in NMSCs, we wanted to test whether ΔNp63α, as a transcriptional factor, regulates ERK3 expression in SCCs and if ERK3 acts as a downstream mediator of ΔNp63α in controlling NMSCs cell growth and invasiveness. We showed that ΔNp63α and ERK3 are co-overexpressed and there was a positive correlation between their expression in the skin from normal humans, and patients with actinic keratosis, squamous cell carcinomas, and basal cell carcinoma. We further showed that while silencing ΔNp63α reduced ERK3 expression level in HaCaT keratinocytes and A431 squamous cell carcinoma cells, overexpression of ΔNp63α increased ERK3 expression level in H1299. Therefore, ΔNp63α positively regulates ERK3 expression. Moreover, silencing either ΔNp63α or ERK3 greatly enhanced A431 cell migration. Importantly, restoration of ERK3 expression rescued the increased cell migration observed upon

silencing ΔNp63α. Knockdown of ERK3 in squamous cells does not show a significant effect on cell proliferation. This study demonstrates that ERK3 is positively regulated by ΔNp63α and mediates ΔNp63α's roles in controlling cell migration in NMSC.

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For Salem, Aisha, Safa, and Bakr

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I-Introduction

A. Non-melanoma skin cancer

Skin cancer, also known as cutaneous carcinoma, is a common type of cancer worldwide (Flohil, de Vries, Neumann, Coebergh, & Nijsten, 2011). Non-melanoma skin cancer is a group of skin cancer that includes basal cell carcinoma of the skin (BCC), squamous cell carcinoma of the skin (SCC), actinic keratoses, a precursor to SCC, and other rare cutaneous carcinomas, such as Merkel-cell carcinomas (Madan, Lear, & Szeimies, 2010). Unlike Melanoma, there is no enforceable legal obligation on healthcare providers to report the new cases of NMSC to the national cancer registries, which leads to an indefinite number of the new cases of NMSC each year (American Cancer Society, 2019). However, a study estimated that approximately 5.4 million cases of NMSC are in the United States in 2012 (Rogers, Weinstock, Feldman, & Coldiron, 2015) The incidence rates of NMSC are higher in women before age 50 and among the light-skinned population (Madan et al., 2010).

Normal skin is composed of two essential layers, the dermis, and the epidermis. The latter consists of four sublayers represent the maturation stages of the dividing keratinocytes, which are the stratum basale (the deepest), the stratum spinosum, the stratum granulosum, and the stratum corneum (Khiao In et al., 2019). These layers provide physical protection as well as physiologically protection functions. For example, the pigment melanin is produced by melanocytes that reside beneath the basal layer in the epidermis and protects the skin against ultraviolet (UV) radiation (Gledhill et al., 2015). The site of keratinocytes that make up the layers of epidermis determines the different types of NMSC. While the basal cell carcinoma originates from the stratum basale (the basal layer), squamous cell carcinoma arises in the middle and outer layer in the epidermis of the skin (Boukamp, 2005; Nakayama, 2010). BCC is the most common

type of the NMSC and accounts for 70-80% of the cases; however, it is slow-growing, rarely invasive, and highly curable if detected in the early stages (Linares, Zakaria, & Nizran, 2015). On the other hand, cutaneous SCC, as the second common NMSC, comprises up to 30 % of the NMSC cases and has a higher probability than BCC to metastasize to adjacent organs and lymph nodes, particularly if the lesions locate to the facial area (Craythorne & Al-Niami, 2017; Gordon, 2013). The actinic keratosis, also known as SCCs in situ, are precursor lesions to SCC due to UV-induced DNA damage, and they are associated with high risk to progress to invasive SCC without proper treatment (Gupta, Paquet, Villanueva, & Brintnell, 2012). Based on the alteration in the histologic features and the localization of the keratinocytes in the epidermis, the actinic keratosis is categorized into several subtypes of precancerous actinic keratosis, such as Acantholytic Actinic Keratosis (AA), Bowenoid Actinic Keratosis (BA) and Hyperplastic Actinic Keratosis (HA) (Goldberg, Joseph, & Tschen, 1994; Ratushny, Gober, Hick, Ridky, & Seykora, 2012; Rigel & Stein Gold, 2013).

Ultraviolet (UV) radiation is the main exogenous risk factor for NMSC and associated with 90% of the NMSC cases (Kim & He, 2014; Saladi & Persaud, 2005; Surdu et al., 2013). Several studies have demonstrated that a cumulative solar UV exposure, particularly during occupational activities, is clearly correlated with the NMSC (Bauer, Diepgen, & Schmitt, 2011; Fartasch, Diepgen, Schmitt, & Drexler, 2012; Schmitt, Seidler, Diepgen, & Bauer, 2011). Although exposure to solar UV radiation is essential in vitamin D production in the skin (T. C. Chen et al., 2007), prolonged exposure to the UV radiation brings about damaging effects. Excessive exposure to the solar UV and other forms of UV radiation, especially ultraviolet-B (290-315 nm), can induce immunosuppression effects and reactive oxygen species (ROS) production in the skin, resulting in DNA damage and interruption in the cellular functions (Fartasch et al., 2012; Rastogi, Richa,

Kumar, Tyagi, & Sinha, 2010). As a result, the epidermal damage eventually leads to NMSC. Sun protection behavior and the personal awareness about the UV radiation harmful effects as well as the use of sunscreen products have been shown to contribute to a reduction in the incidence rate of NMSC (Larese Filon, Buric, & Fluehler, 2019; Ulrich et al., 2009). The endogenous risk factors that contribute to NMSC development include the skin color phenotype, a family history of cutaneous cancers, and some inherited diseases, such as basal cell naevus syndrome (Craythorne & Al-Niami, 2017). Additional high-risk factors of NMSC include prolonging use of some immunosuppressive medication, particularly in the organ transplant recipients, and the viral infection with an oncogenic virus, such as human papillomavirus (HPV) (Baez et al., 2019; Traboulsi et al., 2019).

Excessive exposure to the UV radiation can induce the activation of many oncogenes as well as mutating critical tumor suppressor genes, which leads to various types of DNA damage that affect major pathways in the proliferating keratinocytes. For example, p53, a tumor suppressor gene and an important regulator for cell proliferation, DNA repair, and apoptosis, has been shown to be frequently mutated in cutaneous SCC (Chitsazzadeh et al., 2016). Similarly, phosphatase and tensin homolog (PTEN) plays a critical role in the regulation of UV-induced DNA damage repair mechanisms, such as nucleotide excision repair (NER) (Kim & He, 2014). PTEN has been shown to be downregulated in skin cancer (Ming et al., 2011), likely by ΔNp63α (Leonard et al., 2011). Yang et al., reported that several Nrf2-mediated anti-oxidative stress genes involving in the PTEN pathway were also regulated upon UV irradiation (Y. Yang et al., 2019). Another study has revealed that the depletion of glutathione S-transferase polymorphisms (GSTP), an important antioxidant against the effect of ROS, induced skin cancer development (Marshall et al., 2000). Moreover, it has been demonstrated that several pathways and genes, including mitogen-activated

protein kinases (MAPKs), tumor necrosis factor receptor (Tnfr), epidermal growth factor receptor (EGFR) and matrix metalloproteinases (MMPs) genes were activated due to UV-induced DNA damage and ROS generation in the skin (Ujfaludi et al., 2018; Y. Yang et al., 2019).

B. ΔNp63α and its physiological roles

p63 is a transcriptional factor and a member of the p53 family that also includes p53 and p73 (Candi, Agostini, Melino, & Bernassola, 2014). p63 is a vital proliferative factor for morphogenesis of the stratified epithelia where it is strongly expressed (Senoo, Pinto, Crum, & McKeon, 2007). p63, particularly TAp63 isoform, shares many structural as well as functional similarity with the tumor suppressor p53 (Pagano et al., 2013). The general domain architecture of p63 comprises three essential parts which are an amino-terminal transactivation domain (TAD), a central DNA binding domain (DBD), and a carboxy-terminal oligomerization domain (OD) (A. N. Yang et al., 1998). p63 is expressed as six main isoforms because of the carboxy-terminal splicing events and the usage of the alternative promoter (A. N. Yang et al., 1998). Isoforms of p63 (TAp63) that contain the full-length TA domain are transcribed from the P1 promoter located at 5' end of exon 1 whereas transcription of the isoforms that contain a truncated TA domain (ΔNp63) is initiated using the internal P2 promoter located in intron 3. Because of the alternative splicing at the carboxy-terminus, ΔNp63 & TAp63 are further subclassified into full length (α), and truncated (β) or (γ) isoforms **(Figure 1)** (A. N. Yang et al., 1998). The full length (α) isoform of p63 contains additional domains at the carboxy-terminus which are a putative protein-protein interaction domain known as a sterile alpha motif (SAM) as well as a transactivation inhibitory domain (TI) (Scoumanne, Harms, & Chen, 2005).

Like p53, TAp63 isoforms are involved in the cell cycle arrest, apoptosis, and transactive p53 target genes. ΔNp63 isoforms, however, block these processes by negatively regulating p53

and TAp63 (Benard, Douc-Rasy, & Ahomadegbe, 2003; Gressner et al., 2005; A. N. Yang et al., 1998). TAp63 is substantially expressed in oocytes, protecting the genomic integrity and promoting the epithelial differentiation in the early stages of development (Levine, Tomasini, McKeon, Mak, & Melino, 2011; Livera et al., 2008; Suh et al., 2006). In contrast, ΔNp63 is highly expressed in proliferating epithelial cells (Koster, Dai, & Roop, 2007; Mikkola, 2007; Senoo et al., 2007). It has been shown that ΔNp63α expression is 200-fold higher than Tap63 isoforms in head and neck squamous cell carcinoma (Gatti, Fierro, Annicchiarico-Petruzzelli, Melino, & Peschiaroli, 2019). Similar observations have also been reported in squamous cell lung carcinoma (Compagnone et al., 2017; Rocco, Leong, Kuperwasser, DeYoung, & Ellisen, 2006).

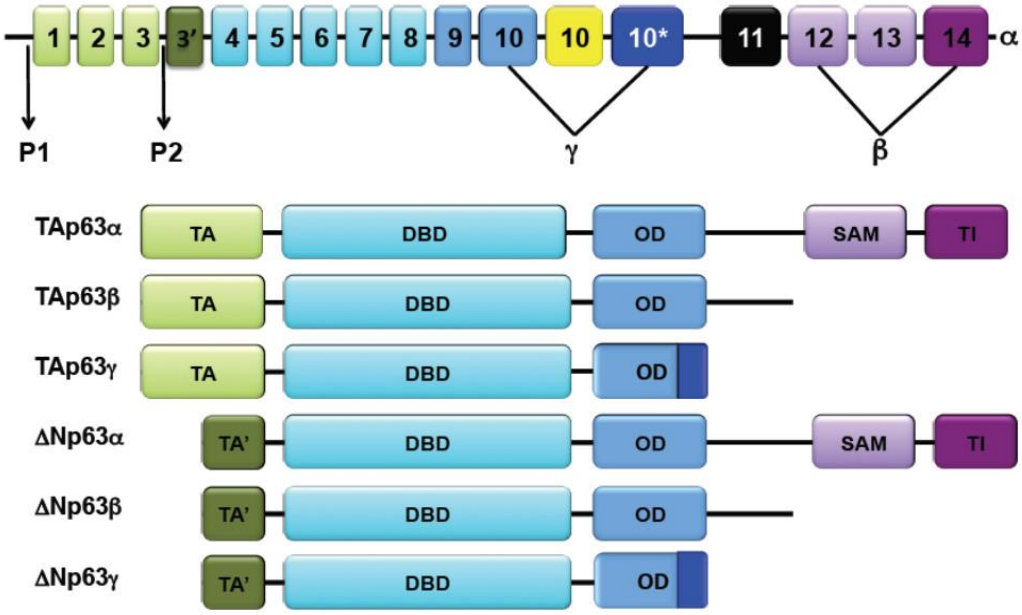


Figure 1: p63 isoforms. Schematic of the human p63 gene structure showing the six main isoforms of p63, the P1 and P2 promoter sites, and the 3' splicing segments. The structural domains of p63 consist of a transactivation domain (TA), a DNA-binding domain (DBD), and an oligomerization domain (OD). α isoform of p63 contains additional domains which are a sterile alpha motif (SAM) and a transactivation inhibitory domain (TI) (A. N. Yang et al., 1998).

ΔNp63α is a predominant and physiologically significant isoform of p63 highly expressed in the proliferating epithelial cells, including keratinocytes, in the basal layer of the epidermis (Mills et al., 1999; Romano et al., 2012; A. Yang et al., 1999). As the keratinocytes of the basal layer differentiate into the outer layers of the epidermis, the expression level of ΔNp63α gradually declines **(Figure 2)** (Koster, Kim, Mills, DeMayo, & Roop, 2004). Previous animal studies have shown that p63 knock out mice were born without normal skin, mammary glands, and hair follicles and died shortly after birth (Mills et al., 1999; A. Yang et al., 1999). Another study investigated more specifically the significance of ΔNp63α on epithelial development showing that induction of moderate levels of ΔNp63α induced normal development of the basal layer in epidermal transgenic mice whereas p63-null mice failed to develop normal skin, suggesting that ΔNp63α plays a substantial role in maintaining the integrity and morphology of the epithelial tissue of the skin (Candi et al., 2006; Dotsch, Bernassola, Coutandin, Candi, & Melino, 2010). In addition, the depletion of ΔNp63α led to cell detachment, indicating that ΔNp63α is a crucial regulator for cell adhesion (Carroll et al., 2006). ΔNp63 not only sustains the epithelial integrity but is also involved in multiple critical physiological functions in the body. A recent study pointed out to a pivotal role of ΔNp63α in regulating the progression of cell cycle and cell growth (Wang, Xia, Chen, & Xiao, 2019). ΔNp63α also positively regulates hexokinase 2 (HK2) which plays an essential role in glucose metabolism (Viticchie et al., 2015). Furthermore, expression of some important anti-oxidant proteins, such as glutathione peroxidase 2 that converts hydrogen peroxide to water, relies on the regulatory effect of ΔNp63α (Yan & Chen, 2006). Several autosomal dominant developmental disorders and ectodermal syndromes, such as Ectrodactyly-Ectodermal dysplasia cleft lip/palate (EEC) syndrome, linked to mutations in the TP63 locus (Brunner, Hamel, & Bokhoven Hv, 2002).

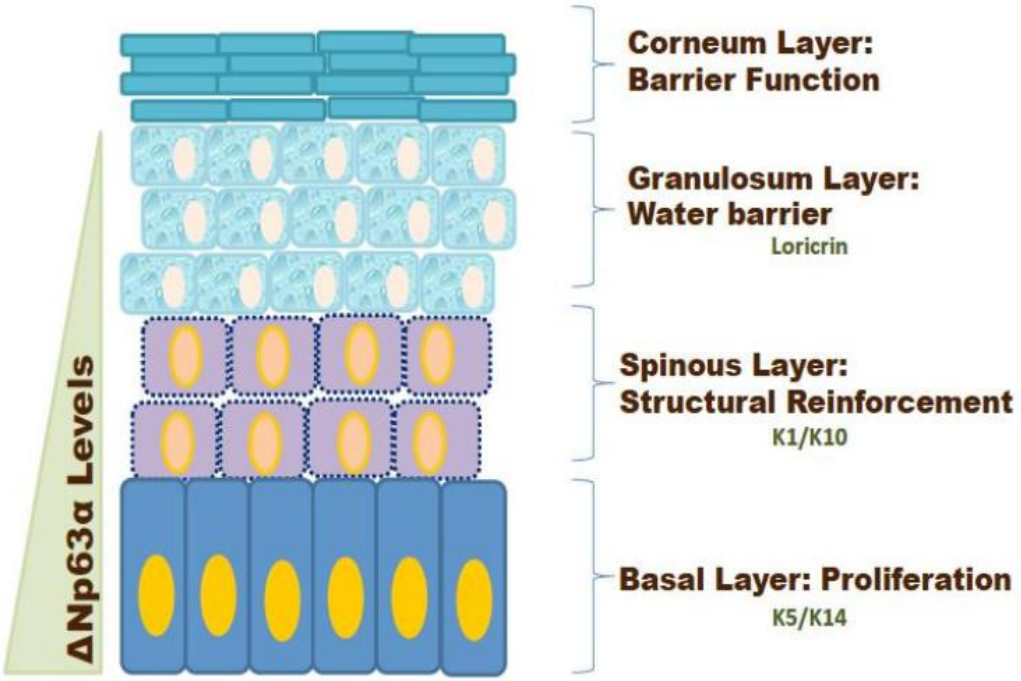


Figure 2: The expression level of ΔNp63α in the four layers of the human skin epidermis. The epidermis comprises four layers as follows: the basal layer, the spinous layer, the granulosum layer, and the corneum layer. Markers that are used to define the individual layers are indicated. ΔNp63α is highly expressed in the basal layer and gradually decreases as the cells begin to differentiate into the outer layers of the epidermis (Koster et al., 2004).

C. Role of ΔNp63α in human cancer

As ΔNp63α is a critical regulator in epithelial development, alterations in its expression and functions are implicated in tumor development. In most human cancers, ΔNp63α is considered to be proto-oncogenic. While its protein expression varies in a different type of tumors, it is highly expressed in squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) of diverse tissue origins, particularly in the skin and lung (Di Como et al., 2002). ΔNp63α exhibits complex functions in tumorigenesis, and many studies have investigated its fundamental roles in epithelial development and its involvement in different types of cancer (Dotsch et al., 2010). ΔNp63α is shown to be a biomarker for non-invasive epithelial cancers with high expression level whereas it is undetectable in the invasive adenocarcinoma of the prostate, breast, and colon (Candi et al., 2017; Finlan & Hupp, 2007).

ΔNp63α has been shown to promote tumor initiation and progression in the early stages of cancer development by activating signaling pathways involved in cell survival. For example, ΔNp63α promotes the activation of AKT pathway, which in turn enhances cell proliferation of pancreatic cancer (Danilov et al., 2011; Leonard et al., 2011) and squamous cell carcinoma of the lung (Abraham et al., 2018). Premalignant lesions and epidermal cysts were observed in the basal layer of the epidermis upon ΔNp63α induction, indicating an oncogenic role for ΔNp63α in the initiation of SCC tumorigenesis (Devos et al., 2017). Nevertheless, an inhibitory effect of ΔNp63α on cancer cell survival has been reported in several other cancers, such as breast, bladder, and prostate cancer (Di Como et al., 2002; Medina-Franco et al., 2002). These findings indicate the complexity of ΔNp63α involvement in diverse types of cancer, and therefore further studies need to be done to elucidate its regulation and functions in different tumors.

Many studies have demonstrated that ΔNp63α inhibits cancer cell migration and invasion, suggesting an inhibitory role of ΔNp63α in late stages of SCC development (Barbieri, Tang, Brown, & Pietenpol, 2006; Senoo, Matsumura, & Habu, 2002). Accumulative evidence suggests a mechanism by which ΔNp63α regulates cancer cell motility is that ΔNp63α downregulates the expression of multiple genes that contribute to the activation of the epithelial-to-mesenchymal transition (EMT) (Danilov et al., 2011; Kommagani et al., 2009; Wu et al., 2014). ΔNp63α was shown to be the only isoform of p63 to reverse the effect of increased cell migration after depletion of p63 by promoting the transcription of Mitogen-Activated Protein Kinase Phosphatase 3 (MKP3) (Bergholz et al., 2014). MKP3 dephosphorylates and thus inhibits extracellular signal-regulated protein kinases 2 (ERK2) which is highly expressed in invasive cancers and promotes tumor progression by promoting cancer cell growth and invasiveness (Bergholz et al., 2014). As such, ΔNp63α negatively regulates ERK2 signaling and ERK2-mediated cancer development. Despite the diverse important functions of ΔNp63α in the regulation of many signaling pathways and cellular functions in both physiological and pathological conditions, little is known about the downstream targets of ΔNp63α that is directly involved in cell motility and invasiveness.

D. Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases are a family of kinases that phosphorylate their substrates on serine (the most common phosphorylation site) or threonine residue. MAPKs convert a variety of extracellular physical or chemical signals into cellular responses inside the cell (Bind et al., 2004; Coulombe & Meloche, 2007). Various cellular functions, including proliferation, differentiation, and motility, are regulated by the activities of MAPKs (Dhillon, Hagan, Rath, & Kolch, 2007). The cellular activities of MAPKs are tightly controlled in the healthy cells; however, alterations in the expression and/or functions of MAPKs result in a variety of human diseases,

including cancer (H. C. Cheng, Qi, Paudel, & Zhu, 2011; Johnson, 2009). In mammalian cells, there are six groups of MAPKs **(Figure 3)**, such as Extracellular signal-regulated kinases (ERK)-1/2 and ERK3/4, (Dhillon et al., 2007). MAPKs are classified into conventional and atypical MAPKs based on the structural feature and the regulatory role (Coulombe & Meloche, 2007).

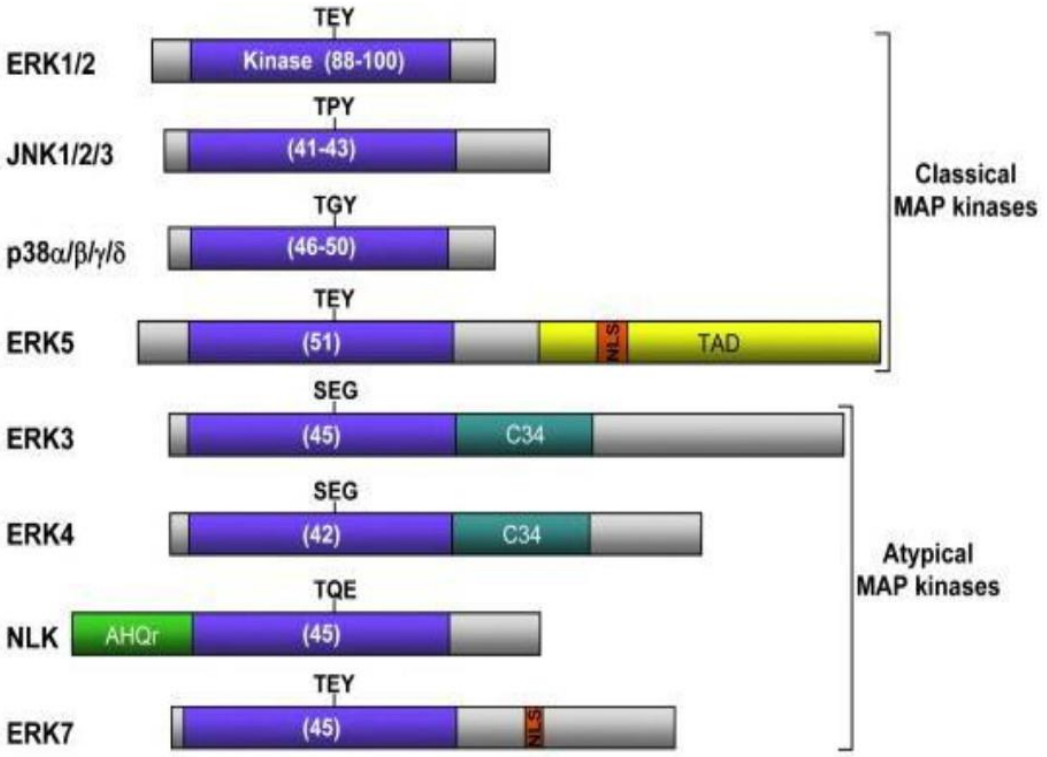


Figure 3: Structures of the conventional and atypical MAPKs. All MAPKs have a kinase domain (in blue) flanked by N- and C-terminal extensions of variable lengths. NLS: nuclear localization sequence; TAD, transactivation domain; C34: a conserved region in ERK3/4; AHQr: alanine (A), histidine (H) and glutamine (Q) rich domain. Numbers in the kinase domains indicate the percentage of homology each MAPK has in its kinase domain relative to that of ERK1 (Coulombe & Meloche, 2007).

The conventional MAPKs include ERK1/2, p38s, JNKs, and ERK5. MAPKs are activated upon the phosphorylation of a conserved and critical Thr-Xaa-Tyr (TXY) activation motif in MAPKs activation loop (Coulombe & Meloche, 2007). This activation is a result of sequential phosphorylation events carried by the kinase activity of the upstream MAPK kinase kinases (MAP3K), such as RAF, and MAPK kinases (MAP2K), such as MEK1/2 **(Figure 4)** (Kostenko, Dumitriu, & Moens, 2012). Atypical MAPKs, on the other hand, comprise ERK3, ERK4, ERK7, and Nemo-like kinase (NLK). Unlike the conventional MAPKs, this non-classical group of MAPKs have a distinct phosphorylation motif which lacks tyrosine residue; instead, it possesses glycine or glutamate and a single phosphor acceptor site. To date, the stimulation of signaling cascades as well as the regulation of atypical MAPKs are still mostly unknown (Cargnello & Roux, 2011; Coulombe & Meloche, 2007; Deleris et al., 2008).

E. ERK3 and its physiological functions

Extracellular signal-regulated protein kinases 3 (ERK3), also known as MAPK6, is a typical MAPKs member (Coulombe & Meloche, 2007). ERK3 was first identified in the early 1990s by an ERK1-derived probe (Boulton et al., 1991), followed by cloning of human ERK3 cDNA a few years later (Meloche, Beatty, & Pellerin, 1996; Zhu, Zhao, Moller, & Flier, 1994). ERK3 is expressed in most human tissues with variable levels. However, brain, skeletal muscle, and gastrointestinal tract are the parts of the body that have the highest expression of ERK3 mRNA (Turgeon, Saba-El-Leil, & Meloche, 2000). ERK3 level is also shown to peak in the early embryogenesis (Turgeon et al., 2000). However, the regulations of ERK3 gene expression and protein kinase activation are largely unknown.

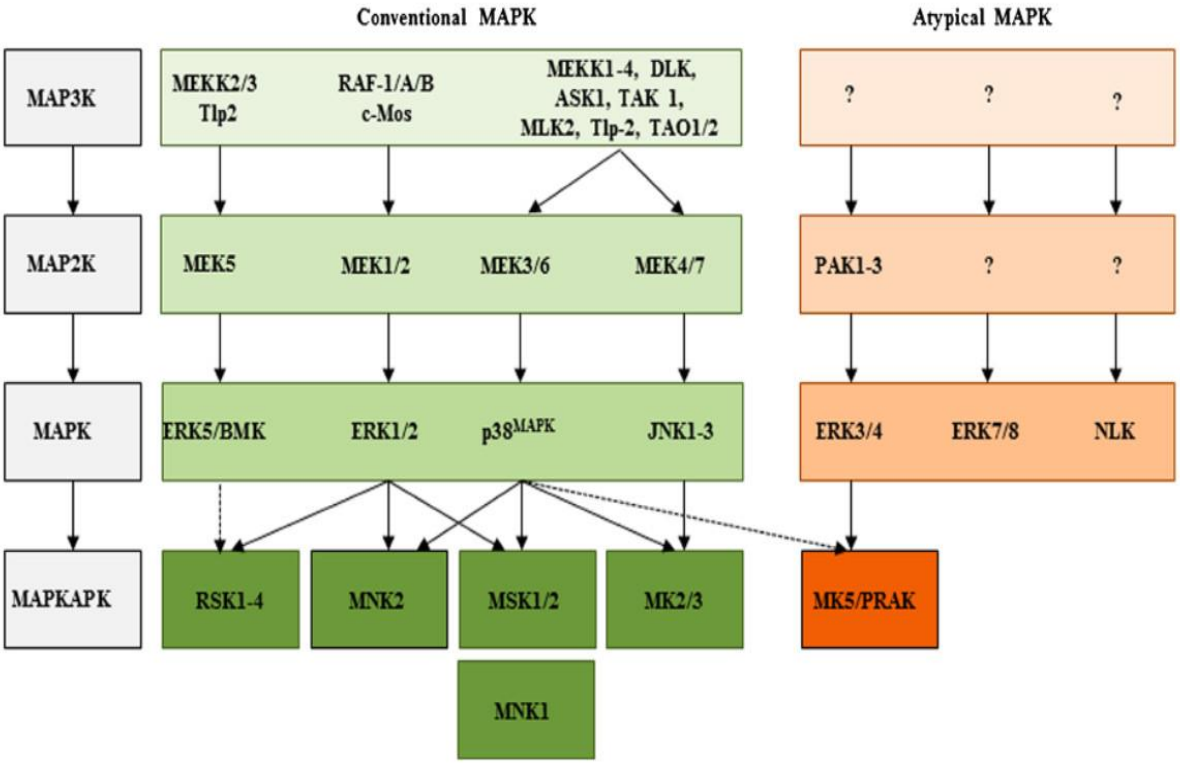


Figure 4: Schematic of MAPKs signaling pathways in the human cell. The conventional MAPKs, such as ERK1/2, are activated in response to extracellular stimuli, such as growth factors, resulting in receptor phosphorylation and recruitment of proteins that activate a small GTP-binding protein such as Ras. The latter binds and activate RAF (belongs to MAP3Ks) which in turn induces sequential phosphorylation-mediated activation of the MEKs and ERK1/2 which are examples of MAP2K and MAPK, respectively. Unlike conventional MAPKs, atypical MAPKs including ERK3/4 may not follow a similar fashion of activation since the extracellular stimulation that triggers atypical MAPKs activation is still unknown (Kostenko et al., 2012).

The general structure of ERK3 protein consists of a kinase domain at the amino-terminus, a central conserved C34 domain, and a long carboxy-terminus domain **(Figure 3)** (Coulombe & Meloche, 2007). A total of 721 amino acids makes up the ERK3 protein with a molecular weight of about 100 kDa (Turgeon et al., 2000). ERK3 and ERK4 share approximately 73% amino acid identity, but they have distinct functions (Rousseau et al., 2010). Protein structure of ERK3 also shares high similarity with ERK1/2 regarding the catalytic domain, yet the activation loop of ERK3 that lies from amino acids 174 to 216 possess a single phosphor acceptor site serine189 (S189) within S-E-G motif rather than the conserved T-X-Y motif found in conventional MAPKs. Phosphorylation of Ser 189 on the activation loop of ERK3 is not affected by the common cellular stimuli, cellular stress or mitogenic factors (M. Cheng, Boulton, & Cobb, 1996; Coulombe & Meloche, 2007; Coulombe, Rodier, Pelletier, Pellerin, & Meloche, 2003; Deleris et al., 2008). In addition, phosphorylation of ERK3 on Ser 189 can occur *in trans* by an upstream kinase and *in cis* by autophosphorylation in order to activate ERK3 (Coulombe & Meloche, 2007). For instance, ERK3 was shown to be *in trans* phosphorylated on activation loop Ser 189 by the group I p21-activated kinases (PAKs) (De la Mota-Peynado, Chernoff, & Beeser, 2011; Deleris et al., 2011). The cellular functions of the C34 domain are still ambiguous. The unique carboy-terminus of ERK3 that is not conserved in the conventional MAPKs and ERK4 is highly phosphorylated during mitosis, leading to an increase of ERK3 protein stability (Coulombe & Meloche, 2007; Turgeon et al., 2000).

ERK3 is an unstable protein with a half-life of 30–60 minutes in proliferating cells (Mathien, Deleris, Soulez, Voisin, & Meloche, 2017). Localization of ERK3 was shown to distribute in the nucleus, cytoplasm as well as plasma membrane (Al-Mahdi et al., 2015; M. Cheng et al., 1996; Julien, Coulombe, & Meloche, 2003). The cellular distribution of ERK3 was not

affected with the presence of common cellular stress and mitogenic factors or with phosphorylation of its S189 residue (M. Cheng et al., 1996; Julien et al., 2003; Seternes et al., 2004). The mechanism underlying the nucleocytoplasmic translocation of ERK3 is not fully understood. However, Exportin 1 (CRM1) was shown to mediates the nuclear export ERK3 (Julien et al., 2003).

Unlike the numerous substrates of each conventional MAPKs, ERK3 has a limited number of substrates due to its high specificity (M. Cheng et al., 1996; Coulombe & Meloche, 2007). Two studies have demonstrated that ERK3 was able to phosphorylate myelin basic protein (MBP) and histone H1 *in vitro* (Brand et al., 2012; Zhu et al., 1994). MAPK-activated protein kinase 5 (MK5), a member of the MAPKAPK family, is a well-studied ERK3 substrate (Schumacher et al., 2004; Seternes et al., 2004). The interaction of ERK3 with MK5 led to the nucleocytoplasmic shuttle of both proteins and was also associated with ERK3 stabilization by MK5 (Schumacher et al., 2004; Seternes et al., 2004). ERK3 interacts with MK5 through a distinct MAPK binding domain, also known as FRIEDE motif located in the kinase domain of ERK3, rather than the well-known CD domain of the conventional MAPKs (Aberg et al., 2009; Cargnello & Roux, 2011). However, MK5 phosphorylation by ERK3 remains elusive regarding whether Thr182 of MK5 is a direct phosphorylation site by ERK3 or its autophosphorylation at Thr182 is facilitated by ERK3 interaction (Schumacher et al., 2004; Seternes et al., 2004). Interestingly, co-overexpression of MK5 and ERK3 led to the phosphorylation of ERK3 on S189 residue independent of MK5 kinase activity, suggesting that an unknown kinase might be recruited upon the physical interaction of ERK3 and MK5 to phosphorylate ERK3 (Deleris et al., 2008). Besides MK5, recent studies revealed previously unknown substrates for ERK3. Steroid receptor coactivator 3 (SRC-3), an oncogenic protein involved in cancer progression, was shown to be phosphorylated at S857 by

ERK3 (Lei, Wang, Mei, & Wang, 2014; Long et al., 2012). Moreover, ERK3 was shown to phosphorylates tyrosyl-DNA phosphodiesterase 2 (TDP2), a critical regulator for DNA damage and cell death (Bian et al., 2016). ERK3 also can be regulated on gene expression and protein stability (Coulombe & Meloche, 2007). ERK3 expression is reported to be upregulated by BRAF in melanoma (M. Chen, Myers, Markey, & Long, 2018; Hoeflich et al., 2006) and by oncoprotein BMI1 in head and neck cancers (Elkhadragy, Chen, Miller, Yang, & Long, 2017). Protein stability of ERK3 was shown to be regulated by ubiquitination-dependent proteasomal degradation (Coulombe, Rodier, Bonneil, Thibault, & Meloche, 2004; Coulombe et al., 2003).

ERK3's functions in organogenesis, particularly in the lung, have been demonstrated in animal studies. Mice with global knockout of ERK3 were born with morphological and functional defects in vital organs including lung, heart, and liver and died shortly after birth. These findings suggest a fundamental role of ERK3 in neonatal organ development and survival (Klinger et al., 2009). ERK3 also plays essential roles in other cellular processes. Recent studies have revealed that ERK3 is an important player in immunity as the enzymatic activity of ERK3 enhances T-cell activation, fosters the survival of $CD4^+$ $CD8^+$ thymocytes, and promotes thymic positive selection (Marquis et al., 2014a; Marquis et al., 2014b; Sirois et al., 2015). In addition, the depletion of ERK3 led to a reduction in glucose-stimulated insulin secretion (Anhe et al., 2006). Moreover, a recent study suggested a role for ERK3 in endothelial cell migration and proliferation via upregulation of the vascular endothelial growth factor receptor 2 (VEGFR2), a crucial regulator for endothelial cell functions (Lei et al., 2014). ERK3 promotes the interaction of steroid receptor coactivator 3 (SRC-3) with transcriptional factor SP-1 and CREB binding protein in forming a transcriptional complex that activates VEGFR2 gene transcription (Lei et al., 2014). On the contrary, ERK3 exhibited a cell proliferation-inhibiting role in a hepatocarcinoma cell line (Xiang,

Wang, & Xiang, 2014). It also has been reported that cell cycle progression was inhibited upon the overexpression of ERK3 in human fibroblasts and squamous cell carcinoma line (Crowe, 2004; Julien et al., 2003). Interestingly, ERK3 showed little effect on lung cancer cell proliferation (Long et al., 2012). Differential mechanisms were identified for the regulation of ERK3 expression in different types of cells. Prolactin hormone is shown to upregulate ERK3 expression in pancreatic cells (Anhe et al., 2006). Also, ERK3 expression is shown to be downregulated in liver cells through microRNAs, such as miR499a and miR133a-5p (Hao et al., 2017; Xiang et al., 2014).

F. Role of ERK3 in human cancer

ERK3's role in tumorigenesis has gained the interest recently. Many studies have reported the upregulation of ERK3 in several types of cancer. A study by Long and colleagues showed that ERK3 mRNA level in squamous cell lung carcinoma is about 7-fold higher than healthy lung tissue. Also, the protein level of ERK3 is upregulated in 65% of non-small cell lung carcinomas of tumor tissue microarrays with higher expression in squamous cell lung carcinoma than lung adenocarcinoma. They also reported cell migration/invasion-promoting role for ERK3 through phosphorylating SRC-3, which enhances its activity in promoting transcription factor PEA3-mediated transcription of matrix metallopeptidases (MMPs) (Long et al., 2012). In oral squamous cell carcinoma, the average of ERK3 mRNA level was 5-fold higher than the normal mouth tissue (Rai, Mahale, & Saranath, 2004). Another study showed a 4-fold increase in ERK3 protein level in gastric cancer tissue (Liang et al., 2005). A recent study revealed a distinct role of ERK3 in promoting breast cancer cell migration by regulating cell morphology and spreading in a kinase-independent manner (Al-Mahdi et al., 2015). Furthermore, ERK3 expression also has been shown to be upregulated in melanoma by B-Raf kinase through MEK1/2/ERK1/2 pathway. On the

contrary to the oncogenic role of BRAF in melanoma, however, ERK3 inhibits melanoma cell proliferation and migration/invasion (M. Chen et al., 2018; Hoeflich et al., 2006).

In line with its role in melanoma, ERK3 exhibited an inhibitory role on cell migration/invasion in squamous cell carcinoma line by regulating the level of Rac1 which is involved in the lamellipodia extension and facilitates cell migration/invasion (Crowe, 2004; W. H. Yang et al., 2012). ERK3 did not affect cell migration in hepatocarcinoma cell lines although it inhibited cell proliferation (Xiang et al., 2014). Similarly, ERK3 suppressed cell growth and survival in intrahepatic cholangiocarcinoma by blocking the activation of mammalian target of rapamycin complex (mTORC1) which is an essential player in cell survival (Ling et al., 2017). Accumulative evidence has showed that ERK3 is also involved in chemoresistance of cancer cells by decreasing the sensitivity of cancer cells to the anti-cancer topoisomerase II inhibitors (e.g., doxorubicin) that induce DNA damage and cell death, by phosphorylating TDP2 at S60 (Bian et al., 2016; J. Y. Yang, Ha, Yang, & Kim, 2010).

Unlike ΔNp63α whose regulation and role in non-melanoma skin cancers including the cutaneous squamous cell carcinoma and the cutaneous basal cell carcinoma have been investigated by many research groups, the involvement of ERK3 signaling in NMSC has not been studied. In an attempt to find data about ERK3 regulation in non-melanoma skin cancer, we searched ONCOMINE, a cancer gene expression database, for ERK3 gene expression in non-melanoma skin cancer samples. By reviewing this database, we found that ERK3 gene expression is upregulated in the squamous cell carcinoma of the skin from two independent studies (Nindl et al., 2006; Riker et al., 2008) **(Figure 5)**. Even though the sample size of these studies is relatively small, they raise an intriguing possibility that ERK3 is upregulated and may play a role in non-

melanoma skin cancer. To test this, more studies need to be conducted to investigate the regulation and functions of ERK3 in non-melanoma skin cancer.

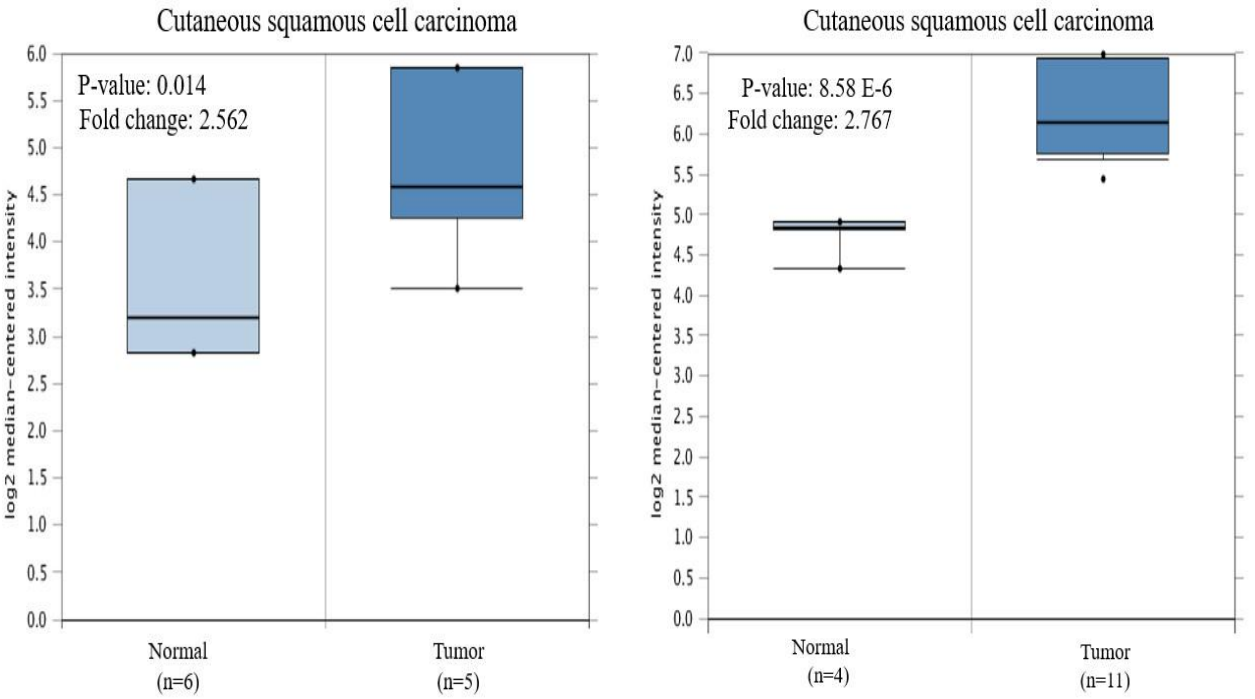


Figure 5: ERK3 gene expression is significantly upregulated in cutaneous squamous cell carcinoma. ERK3 gene expression data (RNA sequencing analysis) were retrieved from two independent studies (Nindl et al., 2006; Riker et al., 2008) on ONCOMINE, a cancer gene expression database. The statistical significance on ERK3 gene expression between cutaneous squamous cell carcinoma samples and normal skin tissue samples was conducted by student's *t*-test. "n" refers to the total number of samples.

G. Hypothesis and specific aims

ΔNp63α, the principal isoform of p63, has been shown by many studies to be upregulated in several types of cancer, including non-melanoma skin cancer, in which it regulates the expression of downstream target genes and controls cellular functions. While ΔNp63α promotes cell proliferation, it inhibits cell migration/invasion in non-melanoma skin cancer. ERK3 is also upregulated in various cancers, including lung cancer, melanoma and squamous cell carcinoma of the skin (Figure 5, Oncomine data). ERK3's role in cancers varies depending on cancer types. For instance, ERK3 shows cell migration-promoting role in breast cancer cells and squamous cell lung carcinoma whereas it inhibits migration of melanoma cells. Little is known, however, about the roles of ERK3 in cell proliferation and migration/invasion of NMSC. In addition, how ERK3 expression is upregulated in NMSC is unclear. As ΔNp63α is a master regulator of gene transcription and cellular functions of NMSC, we hypothesize that ΔNp63α as a transcriptional factor positively regulates ERK3 in non-melanoma skin cancer and that ERK3 mediates ΔNp63α's role in regulating cellular functions. To test this hypothesis, we proposed three specific aims: **(1)** To determine whether there is a positive correlation between the expression of ΔNp63α and ERK3 in non-melanoma skin cancer tissue microarrays; **(2)** To determine whether ERK3 is positively regulated by ΔNp63α; **(3)** To determine whether ERK3 mediates ΔNp63α's cellular functions of NMSC.

II. Materials and Methods

A. Cell culture and reagents

The squamous cell carcinoma cell line A431 was originally purchased from American Type Culture Collection (Manassas, Virginia, USA) while the non-tumorigenic immortalized human keratinocyte HaCaT cell was obtained from Dr. Nancy Bigley (Wright State University). A431 and HaCaT cell lines were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics Penicillin-Streptomycin (pen-strep) All the culture media and supplements were purchased from Gibco/ThermoFisher Scientific.

B. Generation of stable cell pools expressing shRNA by lentiviral transduction

A431 and HaCaT stable cell lines were generated by lentiviral transduction of a short hairpin RNA (shRNA) to target ERK3 mRNA for stably knockdown of endogenous ERK3 (shERK3) in the presence of polybrene (5μg/mL). As a control, cells with stable expression of the non-targeting shRNA (shGIPZ) were used. Cells were split and selected two days post-transduction by puromycin (0.8 μg /mL for A431, 1 μg /mL for HaCaT) for 14 days. The knockdown was confirmed by Western blotting analysis and RT-qPCR.

C. Transient transfection

Transfection with plasmids were performed by Lipofectamine 2000/3000 Reagent (Invitrogen/ThermoFisher Scientific, Carlsbad, CA, USA) or FuGENE HD transfection reagent (Active motif, USA), whereas siRNA transfection was conducted using Dharma-FECT transfection reagent (Dharmacon, Lafayette, CO, USA) or Lipofectamine RNAi-Max (ThermoFisher Scientific, Carlsbad, CA, USA), following the manufacturer's instructions. The

expression plasmid DNA (a total of 2 μg for 6 well plate) of ΔNp63α or the empty vector control, pcDNA3.1, (1:2 DNA to Lipofectamine 2000 in Opti-MEM solution) (Leonard et al., 2011) were used to transfect cells for ΔNp63α overexpression studies while the mammalian expression plasmid of ERK3 with the HA-tag at the N-terminus (pSG5-HA-ERK3) was utilized for generating ERK3 plasmids to overexpress ERK3. The lentiviral wild type pCDH-myc6-ERK3 or empty vector (generated in our lab) were also used for overexpression of ERK3 in cell migration assays. siRNA against p63 (sip63-1,2,3) or non-silencing control (NSC) (Qiagen) were used for knockdown of ΔNp63α in A431, HaCaT cells. siRNA targeting human ERK3 (siERK3, cat # 4390824, assay ID s11148) and the Silencer Negative Control # 1 (siCtrl, cat # 4390843) were purchased from Ambion/ThermoFisher Scientific and used for ERK3 knockdown studies.

D. Immunoblot analysis

Cells were briefly washed with cold PBS and lysed with EBC lysis buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP-40, 1 mM complete protease inhibitors (Roche Diagnostics) and 1 mM phosphatase inhibitor cocktail III (Sigma-Aldrich)]. Protein lysates were mixed with 5X Laemmli sample buffer, boiled for 3 minutes and then resolved on 12% SDS-PAGE gels. Proteins separated by SDS-PAGE electrophoresis were transferred onto nitrocellulose membranes, followed by blocking in 5% non-fat milk in phosphate-buffered saline with tween 20 (PBST) for 45 minutes. Afterward, the membranes were incubated overnight with the primary antibodies at 4°C and 1-hour incubation with the appropriate secondary antibodies (HRP-conjugated goat anti-mouse [170-6516, Biorad] or anti-rabbit [170-6515, Biorad]) at room temperature. The Western blot was visualized by chemiluminescence (Thermo Scientific). The following primary antibodies were used in Western blotting: Mouse monoclonal anti-pan-p63 (4A4, 1:3000, Cell Signaling), rabbit monoclonal anti-ERK3 (ab53277, 1:1000, Abcam), and mouse monoclonal anti-ß-actin

(A5316, 1:20,000, Sigma-Aldrich) antibodies were used to detect ΔNp63α, ERK3, and β-actin, respectively. ß-actin was used as a loading control.

E. RNA extraction and RT-qPCR

Total RNA was extracted from cells 24-hours post-transfection for gene expression analysis using Trizol reagent (Ambion). SuperScript VILO Master Mix (Invitrogen) was used for reverse transcription to generate cDNA as per the manufacturer's instructions. Quantitative Polymerase chain reaction (qPCR) was performed using TaqMan® Universal Master Mix (Applied Biosystems), designed Roche Universal primers and Universal Probe (Roche Diagnostics) on the 7900HT Fast Real-Time PCR Systems (Applied Biosystems). GAPDH was used as internal control, and the relative gene expression level was calculated by the ΔΔCT method (Elkhadragy et al., 2017; Kommagani et al., 2009).

F. Two-chamber transwell cell migration assay

Cell migration was analyzed using a modified two chamber transwell system (8.0 μm pore, BD Biosciences Falcon) according to the manufacturer's protocol. Cells were detached by trypsin/EDTA, washed once with serum-free medium, and then resuspended in serum-free medium. 0.6 mL of complete culture medium with 10% FBS was added to each bottom well. A total of 0.2 mL of cells suspension per well was added in transwell inserts and cells allowed to migrate for 18-20 hours in a 37°C cell incubator. Using cotton swabs, cells which failed to migrate in the upper surface of the transwell were removed. The migrated cells attached on the undersurface were fixed with 10% formalin for 15 min, stained with crystal violet solution (0.5% in water) for 15min, followed by quick washes with distilled water or PBS. Migrated cells were

then photographed under a microscope at 10 X magnification, and five images per condition were taken to count the migrated cells by ImageJ 1.52 software.

G. Cell proliferation assay

Cell proliferation was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay Kit (Promega), following the manufacturer's instructions. Stable A431 or HaCaT cells were plated in five 96-well plates and allowed to grow at 37°C in the incubator. At specific time points, MTS-containing reagent was added to the cells. After 2 hours incubation, the absorbance was measured by Synergy H1 microplate reader (BioTek) at 490 nm.

H. Tissue immunofluorescence staining

Formalin-fixed, paraffin-embedded human skin tissue microarrays as described earlier (Hill et al., 2015) were used for co-immunostaining studies. Human tissue samples consisted of normal skin (N=53), actinic keratosis (AK), a precursor to SCC, (N=66), squamous cell carcinoma of the skin (SCC)(N=59), basal cell carcinoma of the skin (BCC) (N=57). Skin tumor and non-tumor skin tissue slides were co-stained for p63 and ERK3. The immunofluorescence staining was carried out by four 10-minute deparaffinization washes in Histo-Clear (National Diagnostics, Atlanta, GA), followed by rehydrated through graded series of alcohols and a final wash in distilled water. The antigen retrieval processes were performed by a heat-based antigen retrieval method by boiling the slides in a pressure cooker for 12 minutes with a citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0), followed by three washes in PBS. Tissue sections were circled with a PAP pen and blocked for 1-hour with 5% normal goat serum at room temperature followed by overnight incubation with anti-p63 (4A4, 1:800, Cell Signaling), and anti-ERK3 (ab53277, 1:50, Abcam) primary antibodies at 4 C. Three washes in PBS was performed to remove the excess

primary antibodies prior incubation for 1-hour at room temperature with fluorescent secondary antibodies. AlexaFluor 488 anti-mouse (1:500; Molecular Probes) and 568 anti-Rb (1:500; Molecular Probes) were used to visualize p63 and ERK3, respectively. Slides were then washed three times in PBS and mounted with Vecta Shield plus DAPI Mounting Media (Vector Laboratories, Burlingame, CA). Tissue sections were visualized and imaged using a Leica CTR 6000 Microscope (Leica Microsystems, Wetzlar, Germany) and ImagePro 6.2 software (Media Cybernetics, Bethesda, MD). Three pictures were taken under a microscope at 20 X magnification for each tissue sample to measure the mean fluorescence intensity (MFI). Background intensity was normalized and nine measurements, all of the same size, were taken of the epidermal tissue or tumor tissue for each tissue sample. Average mean fluorescence intensity was calculated using ImageJ 1.52 software.

I. Statistical analysis for stained tissue

Adjusted mean fluorescence intensity and standard error of mean (SEM) levels of p63 and ERK3 from all type of skin tissue (normal skin samples, basal cell carcinoma samples, squamous cell carcinoma samples, and actinic keratosis samples were plotted. A mixed effects (ANOVA) tests were used to determine the correlation between the two response variables (MFI of p63 and ERK3) in nine measurements per sample. Post-hoc multiple comparison methods using Dunnett's test were performed to compare between mean MFI values of p63 or ERK3 between control samples (i.e., normal skin samples) and all other samples (i.e., AK, SCC, BCC) (Dunnett, 1955, 1980). PROC MIXED procedure (SAS/STAT®, Ver 9.4, SAS Institute Inc., Cary, NC) was used for analyses (Hamlett, Ryan, & Wolfinger, 2004; Irimata, Wakim, & Li, 2018). A *P* value of less than 0.05 was considered statistically significant.

III. Results

A. Both ΔNp63α and ERK3 are upregulated in NMSCs

p63α is essential for normal epidermal devolvement. Some studies showed that p63-null mice have a high mortality rate after birth (Mills et al., 1999; A. Yang et al., 1999). ΔNp63α is the principal isoform of p63 that is expressed in the basal layer of the epithelium of the skin and plays an essential role in regulating the epithelial maturation and morphology (Carroll et al., 2006; Orzol, Nekulova, Vojtesek, & Holcakova, 2012; Yao & Chen, 2012). Besides its role in regulating genes important for cell proliferation and survival, ΔNp63α also controls some genes involved in epithelial-to-mesenchymal transition (Tucci et al., 2012; Yoh et al., 2016). However, p63's role in skin cancer is still largely unknown. While ERK3 is shown to be upregulated in lung squamous cell carcinoma and head and neck squamous carcinoma (Elkhadragy et al., 2017; Long et al., 2012), little is known about the involvement of ERK3 in skin squamous cell carcinomas. The preliminary data mined from Oncomine (Figure 5), albeit on the basis of a small number of samples, suggests that ERK3 might be altered in NMSCs. Thus, we attempted to investigate the association of ΔNp63α and ERK3 expression levels with NMSC progression. We determined their expression levels in NMSCs including squamous cell carcinoma of the skin (SCCS) and basal cell carcinoma of the skin (BCCS), along with actinic keratosis (AK, a precursor to SCC) and normal skin (NS) tissues as well. First, we optimized the co-immunofluorescent staining condition for ΔNp63α and ERK3 proteins in normal skin and squamous cell carcinoma of the skin (SCCS) tissue sections. Cutaneous SCC tissue sections were co-stained for ΔNp63α and ERK3 using anti-p63 and anti-ERK3 antibodies. Additional tissue sections were stained with fluorescent secondary antibodies alone and used as a control. Both ΔNp63α and ERK3 proteins are highly expressed in the stratified epithelium of the skin. ΔNp63α expression is localized to the nuclei of the cells

whereas ERK3 shows cytoplasmic localization **(Figure 6)**. Afterward, we examined the expression levels of ΔNp63α and ERK3 proteins in formalin-fixed, paraffin-embedded (FFPE) human skin tissue microarray sections. Skin tissue microarrays used in this study include normal skin (N=53), squamous cell carcinoma of the skin (SCCS) (N=59), basal cell carcinoma of the skin (BCCS) (N=57) and actinic keratosis (N=66) sections **(Table 1)** (Hill et al., 2015). Three pictures were taken per sample, and three sites were used per picture, resulting in nine observations per sample to measure the mean fluorescence intensity (MFI) for ΔNp63α and ERK3 in these tissue sections. ΔNp63α is mainly localized to the nuclei of the cells in the epidermal skin. In line with a previous report (Hill et al., 2015), ΔNp63α is expressed in all types of skin tissues. In fact, there is a significant upregulation of ΔNp63α in BCC skin tissues, SCC skin tissues, and AK skin tissues compared with normal skin tissues **(Table 2, Figure 7 and Figure 8A)**.

ERK3 protein also is expressed in all four types of skin sections and mainly localized to the cytoplasm of the cells. Similar to the expression status of ΔNp63α, ERK3 has significantly upregulated skin SCC and AK skin tissues as compared to normal skin tissues. However, there is no statistically significant difference in ERK3 expression between skin BCC and normal skin. **(Table 2, Figure 7 and Figure 8B)**.

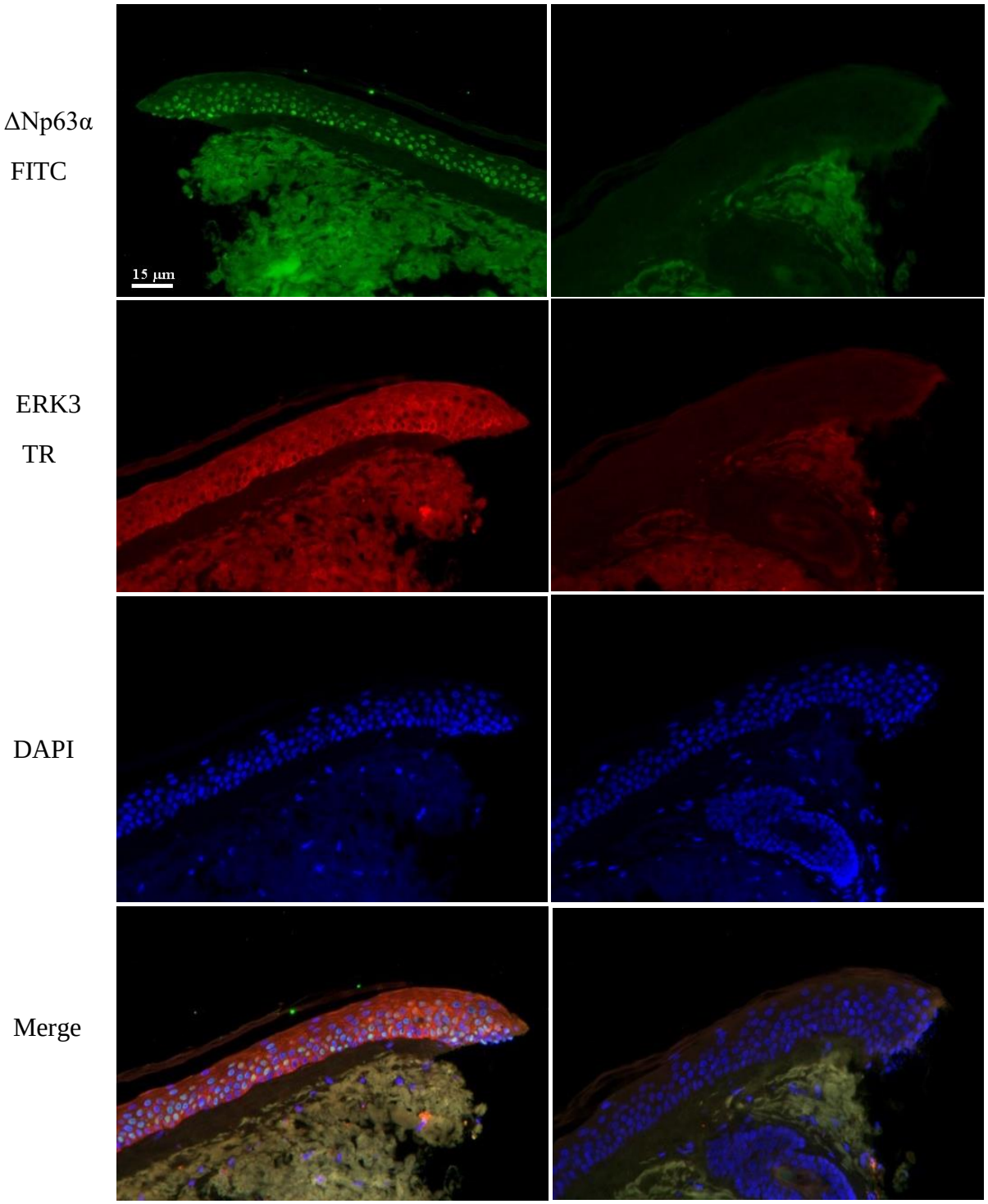

ΔNp63α + ERK3
Negative Control
ΔNp63α
FITC
15 μm
ERK3
TR
DAPI
Merge

Figure 6: ΔNp63α and ERK3 are expressed in the skin epithelium. Representative images of SCC of the skin sections were co-stained for ΔNp63α and ERK3 using anti-63 and anti-ERK3 antibodies to optimize the immunofluorescence staining. Additional tissue sections were stained with florescent secondary antibodies alone and used as a control. The images were taken under a microscope at 20 X magnification. The top panel shows ΔNp63α is expressed and localized to nuclei of the cells (in green). ERK3 is mainly expressed in the cytoplasm of the cells (in red) compared with control in the second panel. The last panel shows the merge of ΔNp63α and ERK3 for these tissue sections.

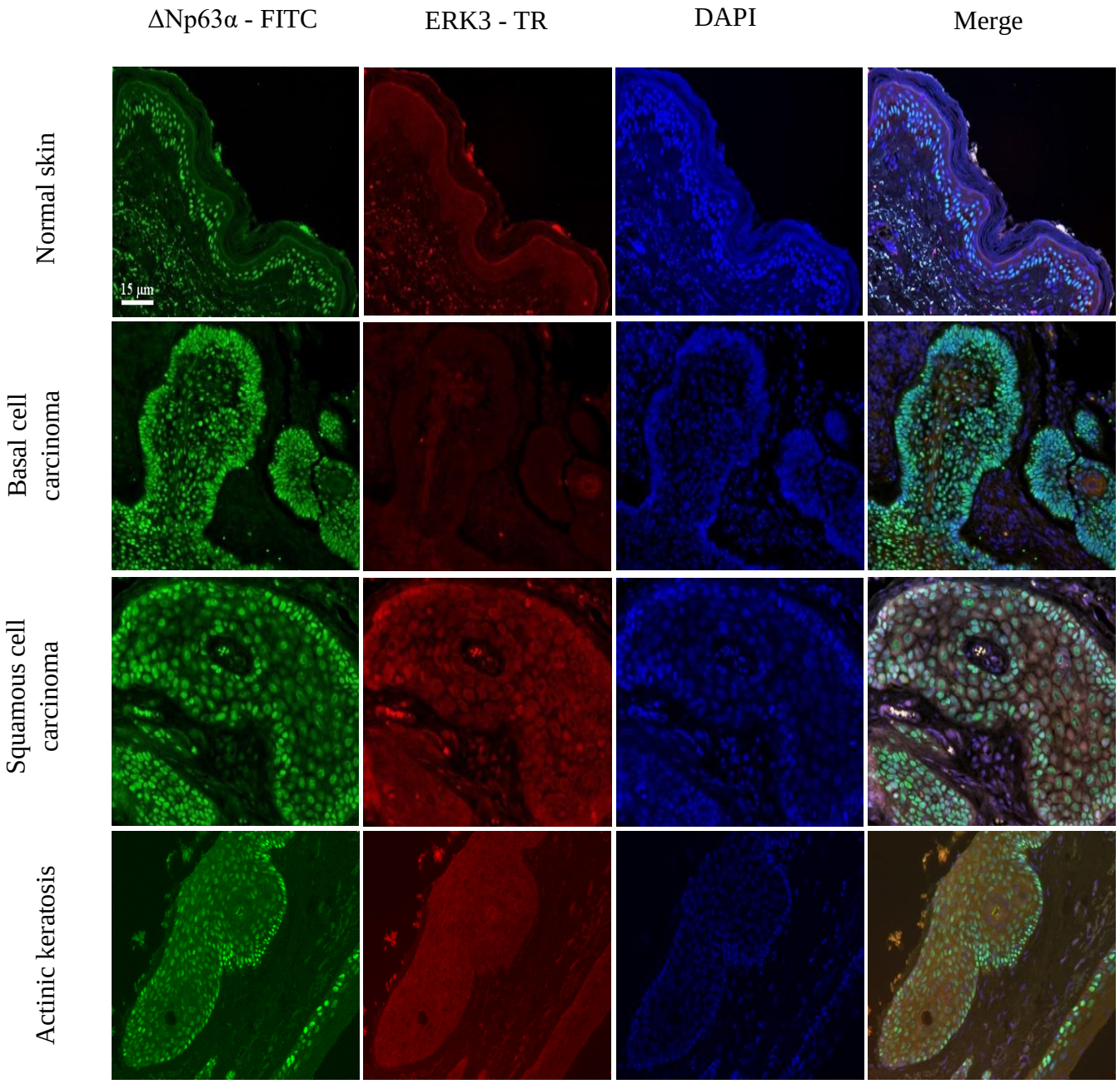

ΔNp63α - FITC
ERK3 - TR
DAPI
Merge
Normal skin
Basal cell carcinoma
Squamous cell carcinoma
Actinic keratosis
15 µm

Figure 7: ΔNp63α and ERK3 are highly expressed in non-melanoma skin cancer tissue. Representative images of formalin-fixed, paraffin-embedded human skin tissue microarrays were co-immunostained for ΔNp63α and ERK3 in normal skin (N=53), squamous cell carcinoma of the skin (SCCS) (N=59), basal cell carcinoma of the skin (BCCS) (N=57), and precursor to SCC, actinic keratosis (N=66) sections to determine the mean fluorescence intensity of ΔNp63α and ERK3. The images were taken under a microscope at 20 X magnification. ΔNp63α is expressed and localized to nuclei of the cells (in green). ERK3 is mainly expressed in the cytoplasm of the cells (in red).

ΔNp63α mean florescence intensity (MFI)

Tissue Type	*Samples(n)*	*Obs (n)*	*Least Squares Mean*	*Standard Error*
Normal	53	474	31.49	2.00
BCC	57	507	52.58	1.93
SCC	59	531	41.19	1.89
AK	66	576	58.68	1.79

ERK3 mean florescence intensity (MFI)

Tissue Type	*Samples (n)*	*Obs (n)*	*Least Squares Mean*	*Standard Error*
Normal	53	474	23.34	1.62
BCC	57	507	26.95	1.56
SCC	59	531	31.21	1.54
AK	66	576	39.81	1.45

Table 1: Descriptive statistics for ΔNp63α and ERK3 co-immunofluorescence staining in normal skin and non-melanoma cancer tissue microarrays. Normal skin (N=53), squamous cell carcinoma of the skin (SCCS) (N=59), basal cell carcinoma of the skin (BCCS) (N=57), and precursor to SCC, actinic keratosis (N=66) tissue microarrays sections were immunostained for ΔNp63α and ERK3. "*n Samples*" refers to the number of tissue samples whereas "*n Obs*" refers to the total number of observations. The means are given as least squares means that control for an imbalanced sample size (not all samples have nine observations). The least squares mean with standard error bars were plotted **(Figure 8)**.

ΔNp63α mean florescence intensity (MFI)

Comparison Change	*Difference*	*Adj. P-value*	*Adj. 95% Confidence Interval*	*Fold*
BCC – Normal	21.09	< 0.0001	(14.54, 27.64)	1.67
SCC – Normal	9.70	0.0015	(3.21, 16.19)	1.31
AK – Normal	27.19	< 0.0001	(20.86, 33.52)	1.86

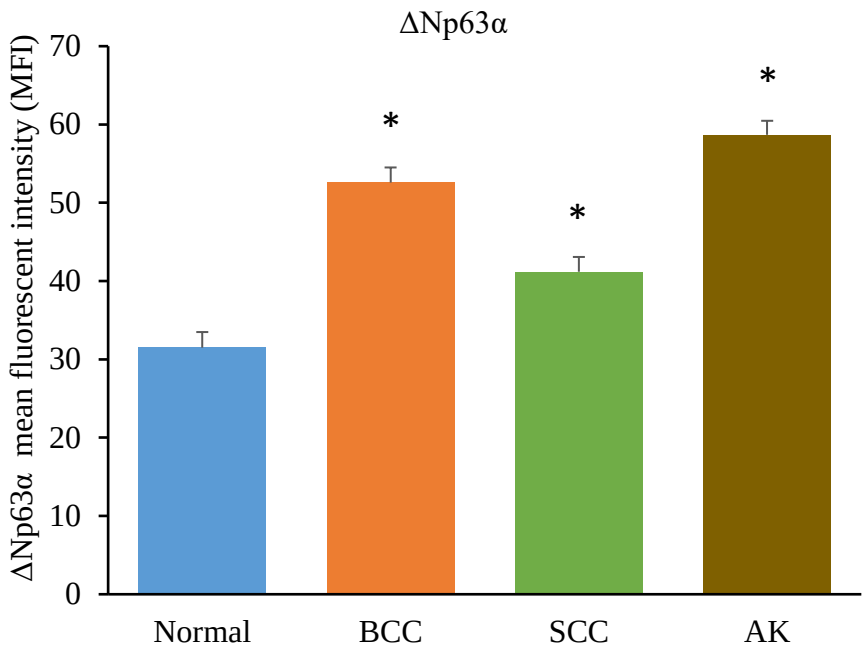
ERK3 mean florescence intensity (MFI)

Comparison Change	*Difference*	*Adj. P-value*	*Adj. 95% Confidence Interval*	*Fold*
BCC – Normal	3.61	0.25	(-1.70, 8.92)	1.15
SCC – Normal	7.87	0.0015	(2.60, 13.14)	1.34
AK – Normal	16.47	< 0.0001	(11.33, 21.61)	1.71

Table 2: Dunnett's test for ΔNp63α and ERK3 co-immunofluorescence staining in normal skin and non-melanoma cancer tissue microarrays. Based on *P*-values (are less than alpha=0.05), there is strong evidence to suggest that the mean MFI for ΔNp63α is significantly different between normal skin tissue and BCC of the skin tissue, normal skin tissue and SCC of the skin tissue, and normal skin tissue and AK of the skin tissue (*P*-values of < 0.0001, 0.0015, and < 0.0001, respectively). Since all the estimated differences are positive, we can infer that ΔNp63α is upregulated in BCC, SCC, and AK of the skin tissue relative to normal skin tissue. The estimated mean differences were 21.09 MFI higher for BCC [95% confidence interval of (14.54, 27.64)], 9.70 MFI higher for SCC [95% confidence interval of (3.21, 16.19)] and 27.19 MFI higher for AK [95% confidence interval of (20.86, 33.52)]. The observed fold changes relative to Normal were 1.67 times higher for BCC, 1.31 times higher for SCC, and 1.86 times higher for AK.

Similarly, there is a significant difference in the mean MFI for ERK3 between normal skin tissue and SCC of skin tissue, and normal skin tissue and AK of the skin tissue (*P*-values of < 0.0015, and < 0.0001, respectively). The estimated mean differences in the data were 7.87 MFI higher for SCC [95% confidence interval of (2.60, 13.14)], and 16.47 MFI higher for AK [95% confidence interval of (11.33, 21.61)]. The observed fold changes relative to the healthy tissue were 1.34 times higher for SCC and 1.71 times higher for AK. However, there is no significant difference in the mean MFI for ERK3 between BCC of the skin tissue and normal skin tissue (*P*-value = 0.25). Therefore, based on this data we can infer that ERK3 is upregulated in SCC and AK of the skin tissue relative to healthy skin tissue. The least squares mean with standard error bars were plotted **(Figure 8)**. The statistical analysis was done by Mike Bottomley form the Statistical Consulting Center at Wright State University.

A.


ΔNp63α
ΔNp63α mean fluorescent intensity (MFI)
70
60
50
40
30
20
10
0
*
*
*
Normal
BCC
SCC
AK

B.

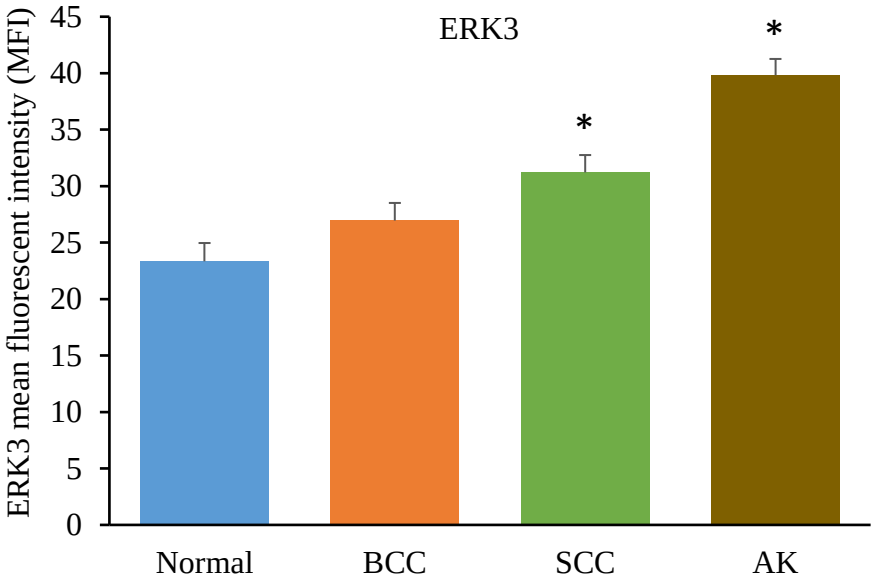

ERK3
ERK3 mean fluorescent intensity (MFI)
45
40
35
30
25
20
15
10
5
0
*
*
*
Normal
BCC
SCC
AK

Figure 8: ΔNp63α and ERK3 are co-expressed in normal skin and non-melanoma cancer tissue microarrays. (A) A bar plot of least squares means with standard error bars shows a significant difference for ΔNp63α MFI between normal skin tissue and BCC of the skin tissue, normal skin tissue and SCC of the skin tissue, and normal skin tissue and AK of the skin tissue (*P*-values of < 0.0001, 0.0015, and < 0.0001, respectively). (B) Similarly, a significant difference was also observed for ERK3 MFI between normal skin tissue and SCC of the skin tissue, and normal skin tissue and AK of the skin tissue (*P*-values of < 0.0015, and < 0.0001, respectively). ERK3 MFI in BCC of the skin tissue arrays does not show a significant difference relative to the normal skin. An asterisk indicates a significant difference with $P < 0.05$ by Student's t-test.

B. ΔNp63α expression is positively correlated with ERK3 expression in normal skin, Actinic keratosis, and non-melanoma cancer tissues.

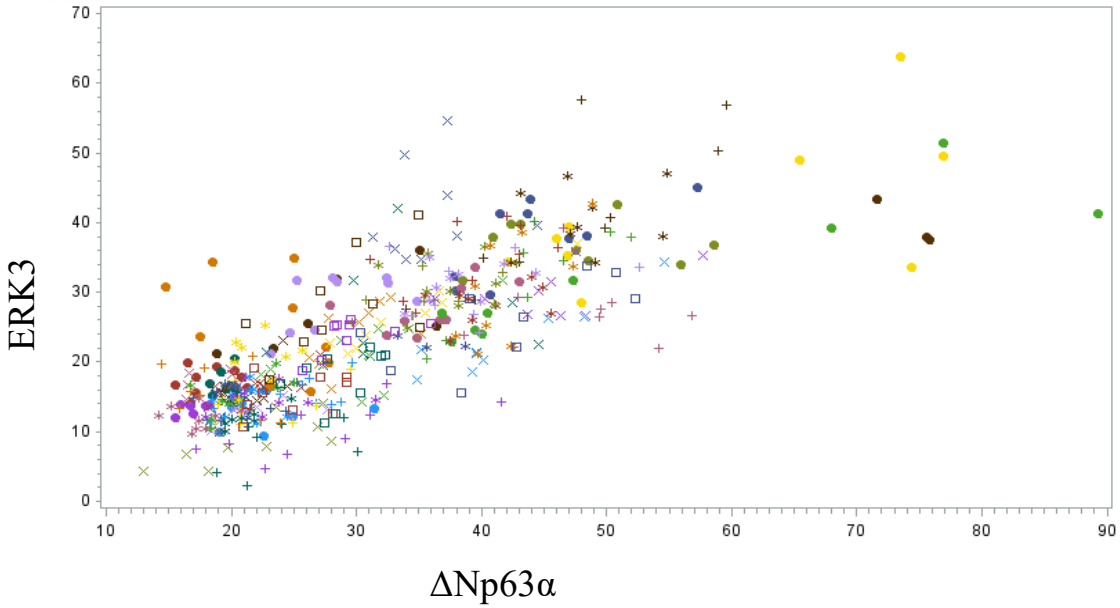
As both ΔNp63α and ERK3 are shown to be upregulated in skin SCC and actinic keratosis of skin compared to normal skin tissues **(Figure 8)**, we then asked whether their expression is correlated in non-melanoma skin cancers. Indeed, there is a significant positive correlation between ΔNp63α and ERK3's expression in each of the four tissue types of skin (all *P*-values are < 0.0001) **(Table 3, Figure 9)**. This suggests that ΔNp63α might positively regulate ERK3 in normal skin and non-melanoma skin cancers.

Type	*Estimate*	*Standard Error*	*95% Confidence Interval*	*P-value*
Normal	0.79	0.04	(0.72, 0.86)	< 0.0001
BCC	0.63	0.05	(0.52. 0.73)	< 0.0001
SCC	0.78	0.04	(0.71, 0.85)	< 0.0001
AK	0.74	0.03	(0.67, 0.81)	< 0.0001

Table 3: Correlation for the mean fluorescence intensity of ΔNp63α and ERK3 in each skin tissue type. Based on *P*-values < 0.0001, it is obvious that ΔNp63α and ERK3 are positively correlated with each other in four type of the skin tissues. The estimated correlation coefficients are 0.63, 0.74, 0.78, and 0.79 for BCC, AK, SCC of the skin, and normal skin tissue, respectively.

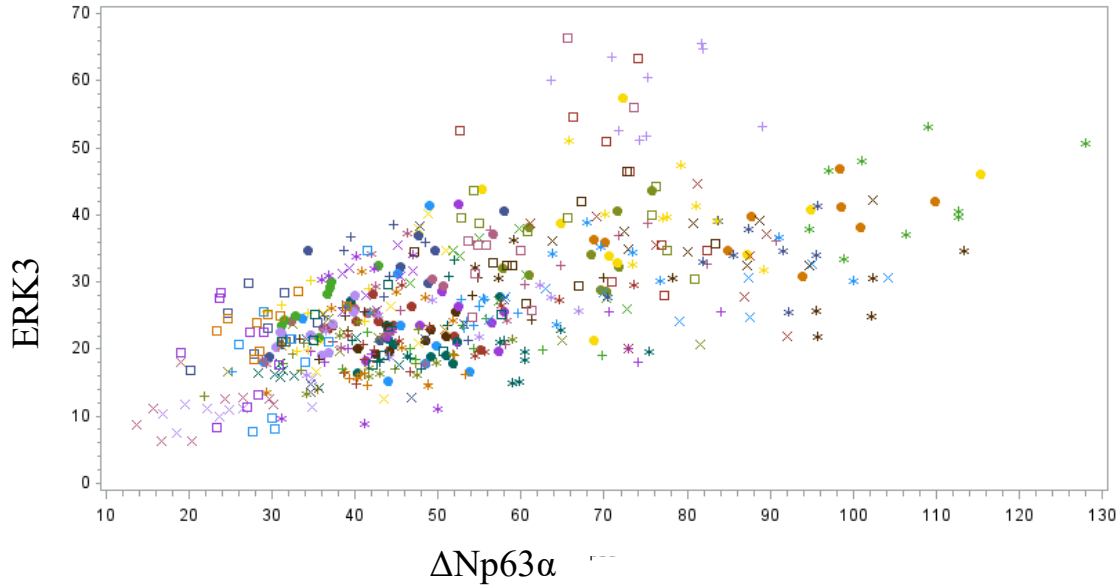
A.

Normal skin tissue



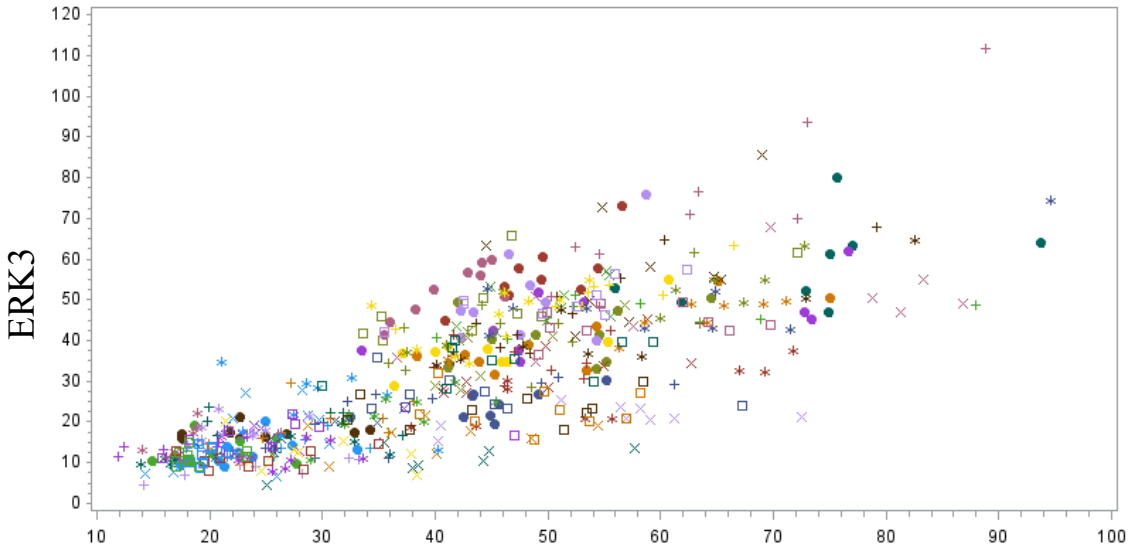
B.

Skin BCC tissue



C.

Skin SCC tissue



D.

Skin AK tissue

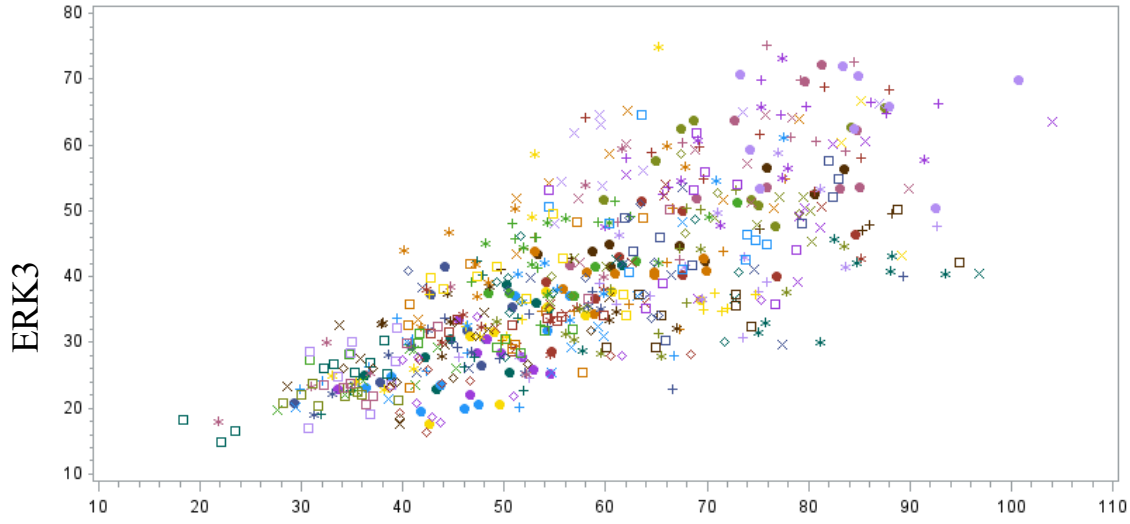


Figure 9: A significant positive correlation between the expression of ΔNp63α and ERK3 in normal skin and non-melanoma skin cancer microarrays. The mean fluorescence intensity (MFI) of ΔNp63α and ERK3 were plotted for each skin tissue type. The x-axis refers to the MFI for ΔNp63α whereas y-axis refers to the MFI for ERK3. (A) Scatterplots of MFI for ΔNp63α and ERK3, showing a positive linear correlation between their expression in normal tissue sections. (B) A significant positive correlation between ΔNp63α and ERK3's MFI in BCC of the skin. (C) ΔNp63α is positively correlated with ERK3 in the SCC of the skin tissue. (D) Similarly, AK of the skin also has a significant positive correlation for ΔNp63α and ERK3 as measured by the mean fluorescence intensity. The statistical analysis was done by Mike Bottomley form the Statistical Consulting Center at Wright State University.

C. ΔNp63α positively regulates ERK3 expression

Since both ΔNp63α and ERK3 are upregulated and positively correlated with each other in non-melanoma skin cancer tissue, we aimed to investigate whether ΔNp63α regulates ERK3 expression in squamous cell carcinoma of the skin. Therefore, we silenced ΔNp63α in HaCaT non-tumorigenic keratinocyte cells and A431 squamous cell carcinoma cells, both of which primarily express the ΔNp63α isoform of p63 (Kommagani et al., 2009). A431 and HaCaT cells were transfected with either a siRNA against ΔNp63α or a non-silencing siRNA (NSC). Transfection of ΔNp63α siRNA resulted in a significant reduction of ΔNp63α at both protein and mRNA levels **(Figure 10)** in A431 and HaCaT cells. Importantly, ERK3 transcript level was significantly reduced upon the knockdown of ΔNp63α in A431 and HaCaT cell lines **(Figure 10A and 10B, respectively)**. Consistent with the change in mRNA transcript level, immunoblot analysis shows that ERK3 protein expression was also substantially decreased when ΔNp63α is knocked down **(Figure 10C and 10D and 10E)** in both A431 and HaCaT. These findings suggest that ΔNp63α positively regulates ERK3 mRNA transcript level in both normal skin and skin SCC cells.

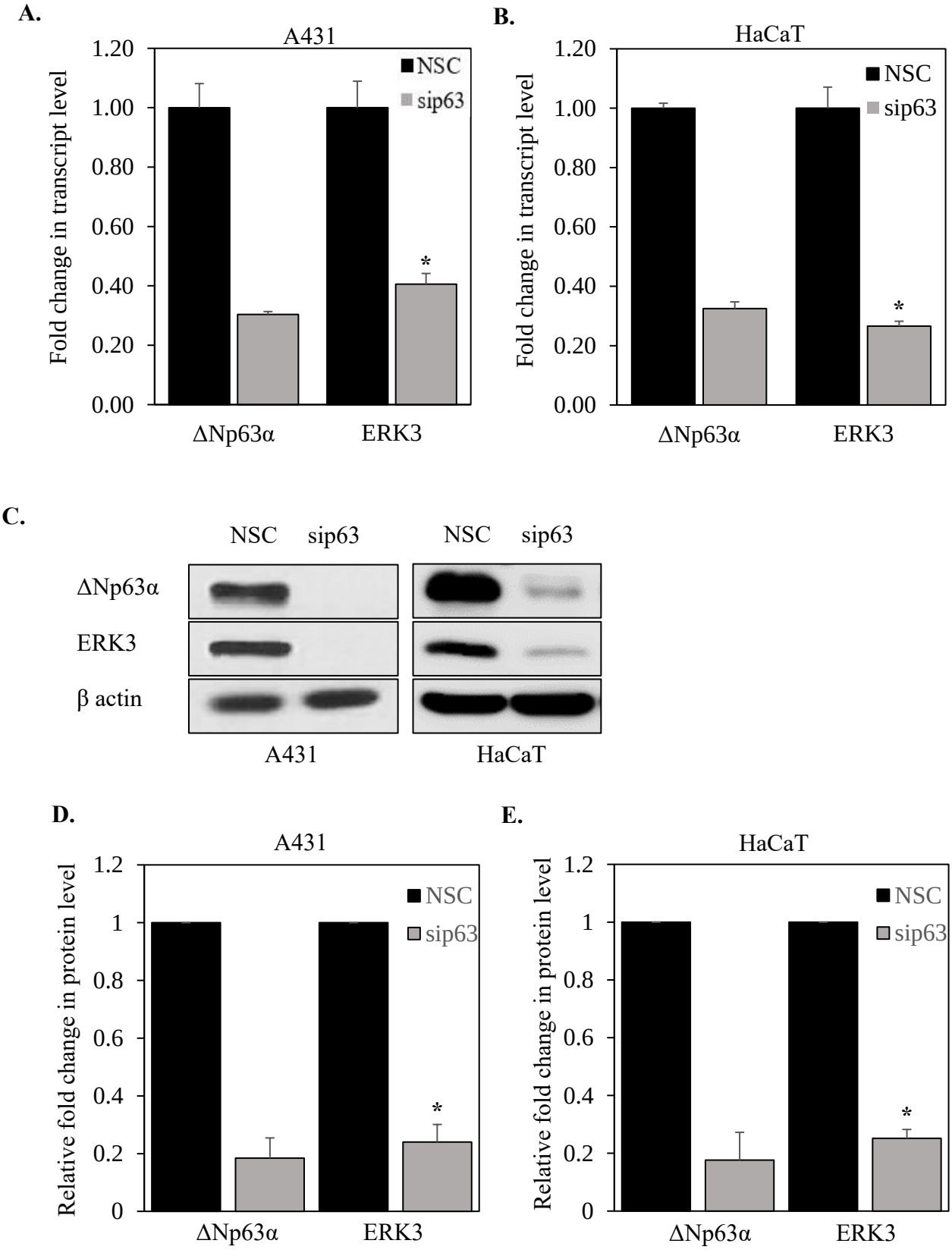

A.
A431
Fold change in transcript level
1.20
1.00
0.80
0.60
0.40
0.20
0.00
NSC
sip63
*
ΔNp63α
ERK3
B.
HaCaT
Fold change in transcript level
1.20
1.00
0.80
0.60
0.40
0.20
0.00
NSC
sip63
*
ΔNp63α
ERK3
C.
NSC
sip63
NSC
sip63
ΔNp63α
ERK3
β actin
A431
HaCaT
D.
A431
Relative fold change in protein level
1.2
1
0.8
0.6
0.4
0.2
0
NSC
sip63
*
ΔNp63α
ERK3
E.
HaCaT
Relative fold change in protein level
1.2
1
0.8
0.6
0.4
0.2
0
NSC
sip63
*
ΔNp63α
ERK3

Figure 10: The depletion of ΔNp63α decreases the expression level of ERK3. A431 and HaCaT cells were transiently transfected in two rounds with either non-silencing control siRNA (NSC) or ΔNp63α siRNA (Sip63). Cells were harvested 48 hours post-transfections. (A) and (B) RNA extracted from cells was used in the qRT-PCR analysis of ΔNp63α and ERK3 gene expression in A431 and HaCaT, respectively. The relative expressions of ΔNp63α and ERK3 were normalized to the endogenous housekeeping gene GAPDH. (C) Protein expression was analyzed by immunoblotting using ΔNp63α and ERK3 antibodies, and β-actin was included as a loading control. (D) and (E) Quantitative analysis of the fold change in protein levels of ΔNp63α and ERK3 in A431 and HaCaT, respectively. Values represent mean ± S.D of three experiments. An asterisk indicates a significant difference with $P < 0.05$ by Student's t-test.

To confirm that ΔNp63α upregulates ERK3 expression, we overexpressed ΔNp63α in a human non-small cell lung carcinoma H1299 cell line which does not express ΔNp63α. H1299 cells were transfected with plasmids encoding either ΔNp63α or its corresponding empty vector (EV). Protein levels of ΔNp63α and ERK3 were analyzed by immunoblot analysis. ΔNp63α transcript and protein levels were highly expressed in H1299 cells transfected with ΔNp63α plasmid while cells transfected with corresponding empty vehicle did not show detectable amount of endogenous ΔNp63α **(Figure 11A and 11C and 11D)**, confirming the exogenous overexpression of ΔNp63α in H1299 cells **(Figure 11)**. Importantly, ERK3 transcript level was significantly increased upon the overexpression of ΔNp63α **(Figure 11B)**. In addition, immunoblot analysis shows that ERK3 protein expression was also remarkably increased when ΔNp63α is overexpressed in H1299 cells **(Figure 11C and 11E)**. Therefore, these results further confirm that ΔNp63α positively regulates ERK3 expression.

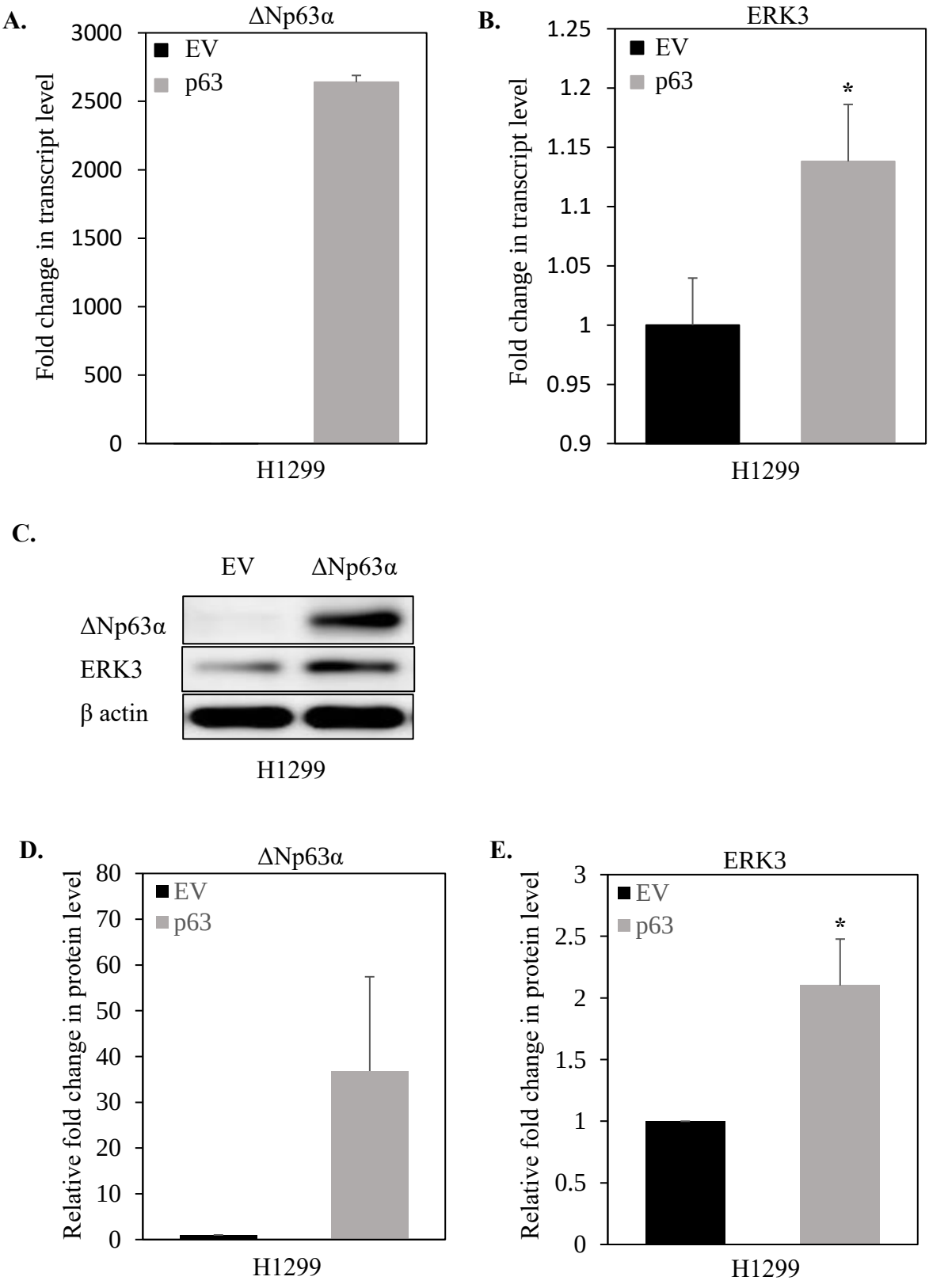

A.
ΔNp63α
EV
p63
Fold change in transcript level
3000
2500
2000
1500
1000
500
0
H1299
B.
ERK3
EV
p63
*
Fold change in transcript level
1.25
1.2
1.15
1.1
1.05
1
0.95
0.9
H1299
C.
EV
ΔNp63α
ΔNp63α
ERK3
β actin
H1299
D.
ΔNp63α
EV
p63
Relative fold change in protein level
80
70
60
50
40
30
20
10
0
H1299
E.
ERK3
EV
p63
*
Relative fold change in protein level
3
2.5
2
1.5
1
0.5
0
H1299

Figure 11: Overexpression of ΔNp63α increases the expression level of ERK3. H1299 cells were transiently transfected with ΔNp63α plasmid or its corresponding empty vector (EV). Cells were harvested 48 hours post-transfections. (A) and (B) RNA extracted from cells was used in the qRT-PCR analysis of ΔNp63α and ERK3 gene expression in H1299. The relative expressions of ΔNp63α and ERK3 were normalized to the endogenous housekeeping gene GAPDH. (C) Protein expression was analyzed by immunoblotting using ΔNp63α and ERK3 antibodies, and β-actin was included as a loading control. (D) and (E) Quantitative analysis of the fold change in protein levels of ΔNp63α and ERK3 in H1299. Values represent mean ± S.D of three experiments. An asterisk indicates a significant difference with $P < 0.05$ by Student's t-test.

D. The depletion of ΔNp63α or ERK3 enhances cell migration

ΔNp63α has an inhibitory role on cell migration as it downregulates several genes that promote epithelial-to-mesenchymal transition and cancer cell invasion/metastasis (Barbieri et al., 2006; Bergholz et al., 2014; Danilov et al., 2011; Kommagani et al., 2009). On the other hand, ERK3 is shown to promote cancer cell migration in lung and breast cancer (Al-Mahdi et al., 2015; Long et al., 2012). However, the inhibitory effect of ERK3 on cell migration and invasion in squamous cell carcinoma cell line by downregulating Rac1 level was also reported (Crowe, 2004). Since our results demonstrate that ΔNp63α upregulates ERK3, it was reasonable to hypothesize that ΔNp63α may control cell migration through the regulation of ERK3. To confirm the inhibitory role of ΔNp63α in skin cancer cell migration, cells were transfected with siRNA targeting ΔNp63α or its corresponding non-silencing control in both A431 and HaCaT cell lines, and cell migration was analyzed by transwell migration assay. In line with the previous studies, the depletion of ΔNp63α led to a remarkable increase in the migration ability of both A431 and HaCaT cell lines **(Figure 12 and Figure 13, respectively)**. Likewise, silencing ERK3 by siRNA transfection or lentiviral shRNA transduction led to a significant increase in A431 cell migration **(Figure 14 and Figure 15, respectively)**. Taken together, these results suggest that both ΔNp63α and ERK3 suppress cancer cell migration of skin SCC cells.

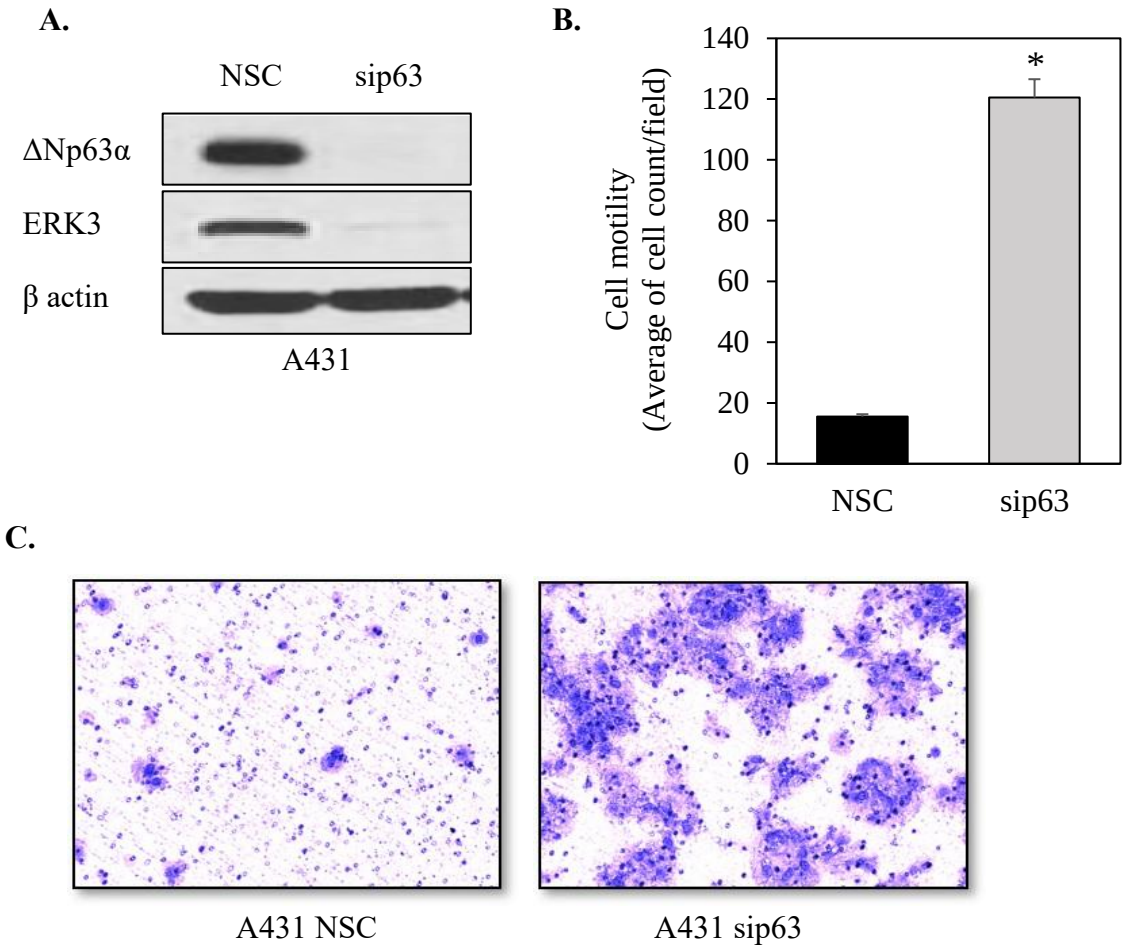


Figure 12: Knockdown of ΔNp63α dramatically increases the migration ability of A431 cells. A431 cells were transiently transfected for two rounds with either non-silencing control siRNA (NSC) or ΔNp63α siRNA (sip63) as indicated. Two days after the 2nd round transfection, one set of cells were harvested, and the change in proteins level was analyzed by immunoblot analysis (A). The other set of cells were used for the transwell cell migration assay, and the number of migrated cells was quantitated after 18 hours (B). The average number of migrated cells per well is presented in the y-axis of the bar graph. Values represent mean ± S.D. An asterisk indicates a significant difference with $P < 0.0001$ by Student's t-test. (C) Representative images of migrated A431 cells with crystal violet staining.

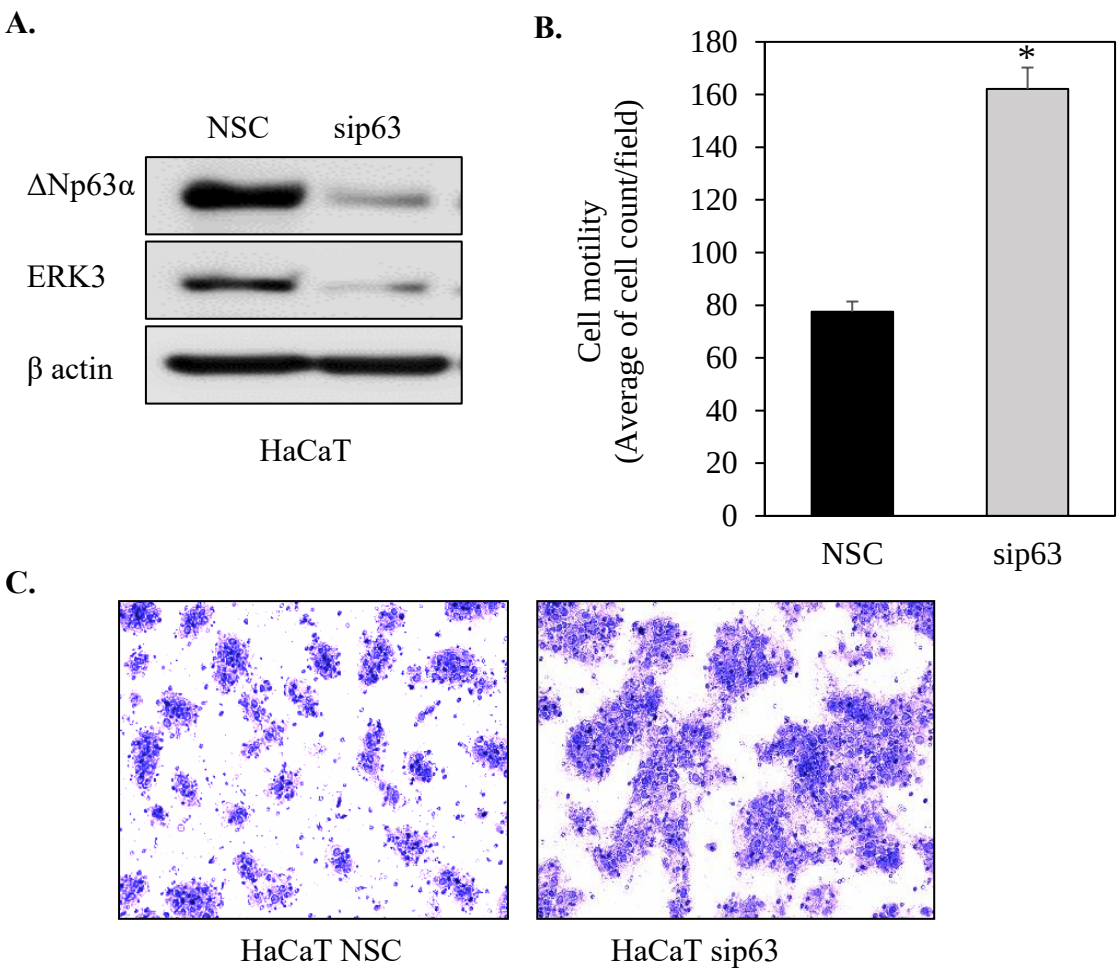


Figure 13: Knockdown of ΔNp63α increases the migration ability of HaCaT cells. HaCaT cells were transiently transfected for two rounds with either non-silencing control siRNA (NSC) or ΔNp63α siRNA (sip63) as indicated. Two days after the 2nd round transfection, one set of cells were harvested, and the change in proteins level was analyzed by immunoblot analysis (A). The other set of cells were used for the transwell cell migration assay, and the number of migrated cells was quantitated after 18 hours (B). The average number of migrated cells per well is presented in the y-axis of the bar graph. Values represent mean ± S.D. An asterisk indicates a significant difference with $P < 0.0001$ by Student's t-test. (C) Representative images of migrated HaCaT cells with crystal violet staining.

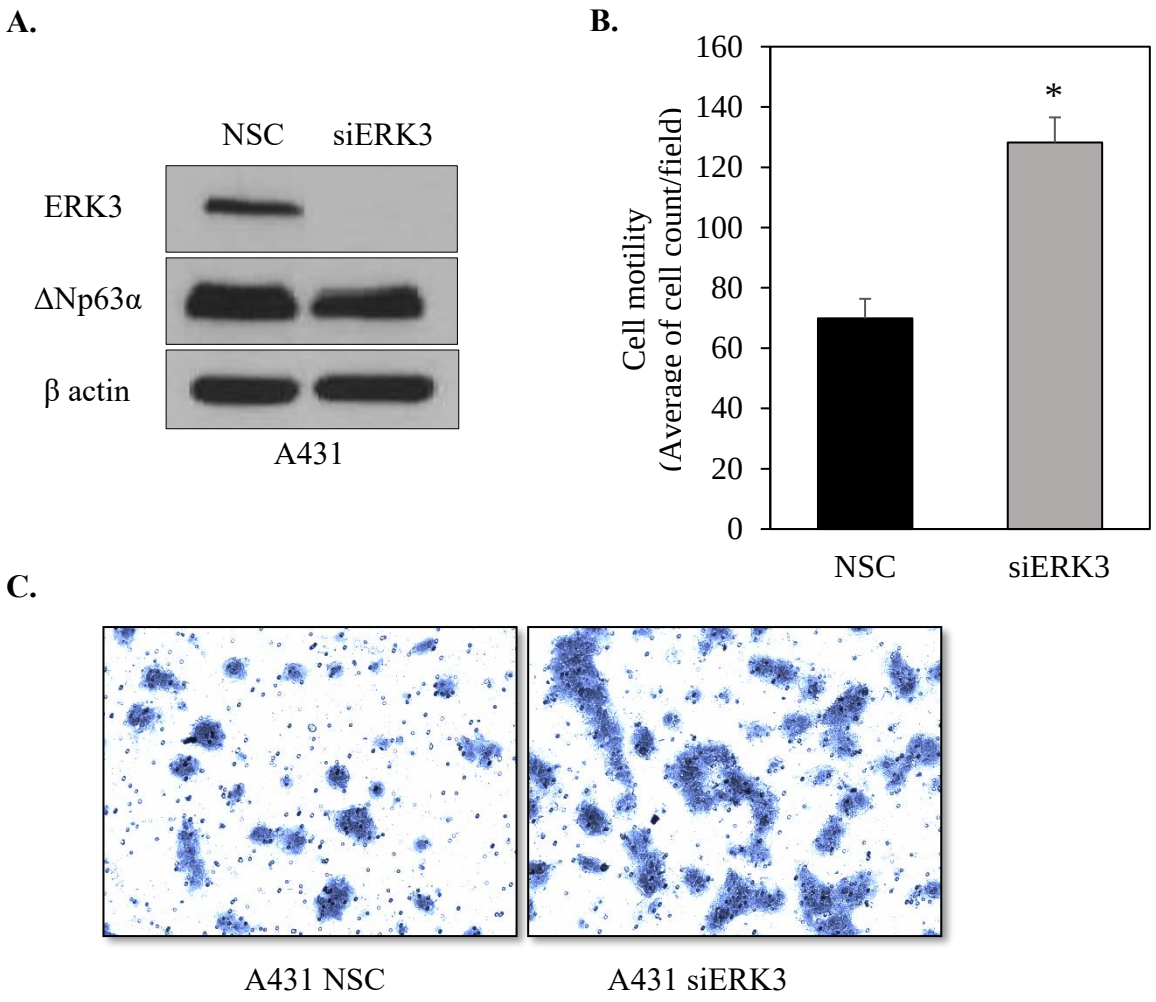


Figure 14: Silencing ERK3 enhances cell migration of A431 cells. A431 cells were transiently transfected with either non-silencing control siRNA (NSC) or ERK3 siRNA (siERK3) as indicated. Two days post-transduction, one set of cells were harvested, and the change in proteins level was analyzed by immunoblot analysis (A). The other set of cells were used for the transwell cell migration assay, and the number of migrated cells was quantitated after 18 hours (B). The average number of migrated cells per well is presented in the y-axis of the bar graph. Values represent mean ± S.D. An asterisk indicates a significant difference with $P < 0.0001$ by Student's t-test. (C) Representative images of migrated A431 cells with crystal violet staining.

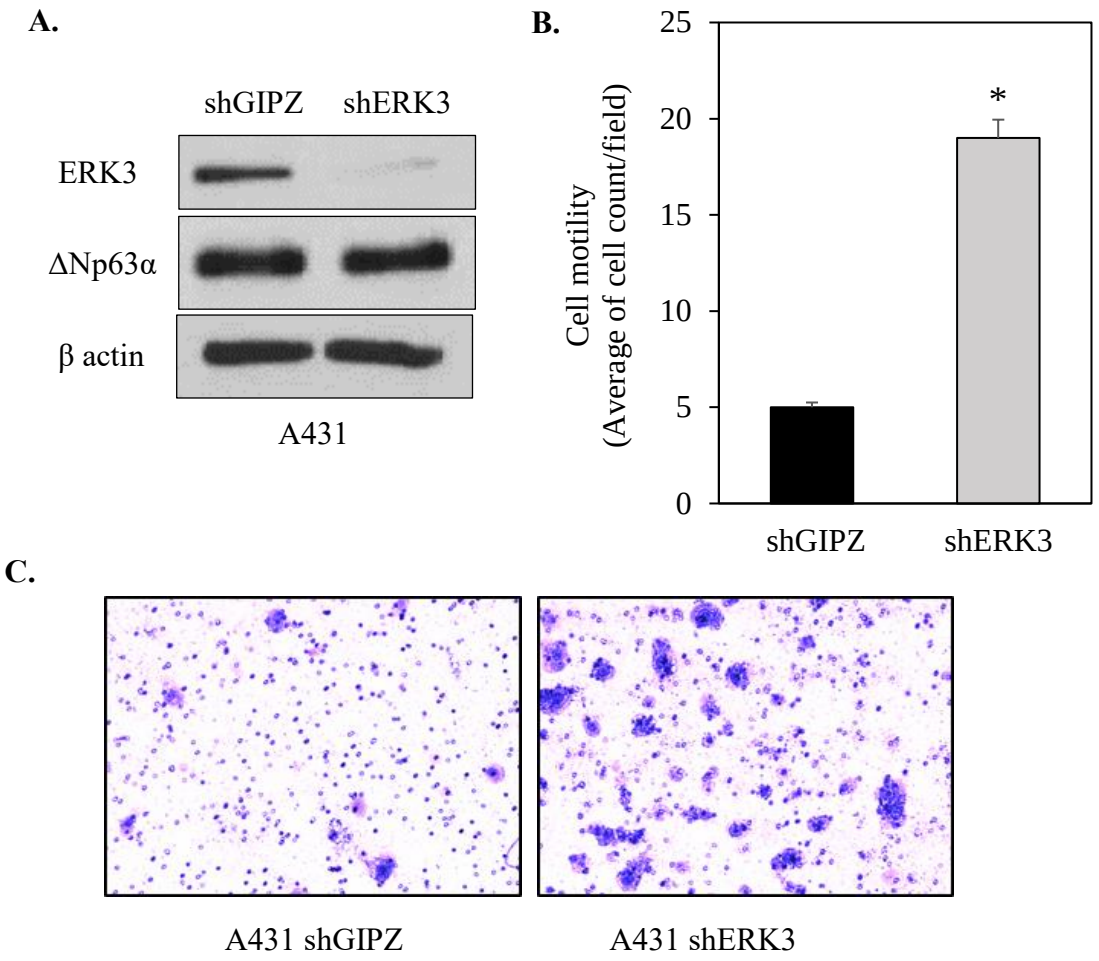
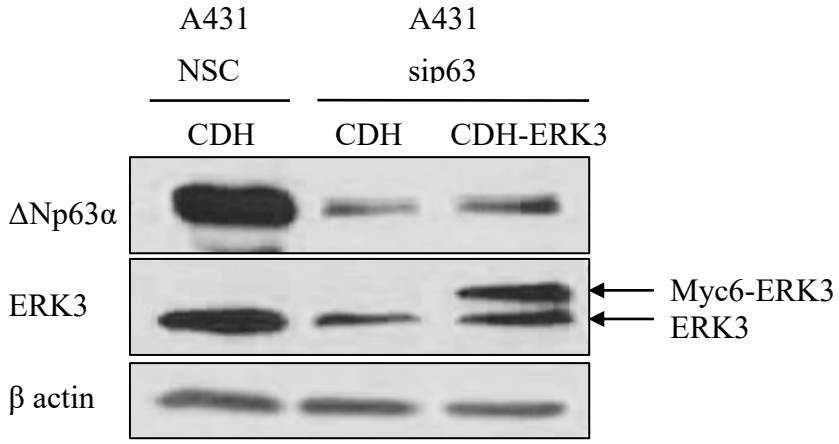


Figure 15: Silencing ERK3 by lentiviral transduction promotes A431 cell migration. A431 cells were transduced with lentiviral shRNA specifically targeting ERK3 (shERK3) or a non-targeting control shRNA (shGIPZ) as indicated. Two days post-transduction, one set of cells were harvested, and the change in proteins level was analyzed by immunoblot analysis (A). The other set of cells were used for the transwell cell migration assay, and the number of migrated cells was quantitated after 18 hours (B). The average number of migrated cells per well is presented in the y-axis of the bar graph. Values represent mean ± S.D. An asterisk indicates a significant difference with $P < 0.0001$ by Student's t-test. (C) Representative images of migrated A431 cells with crystal violet staining.

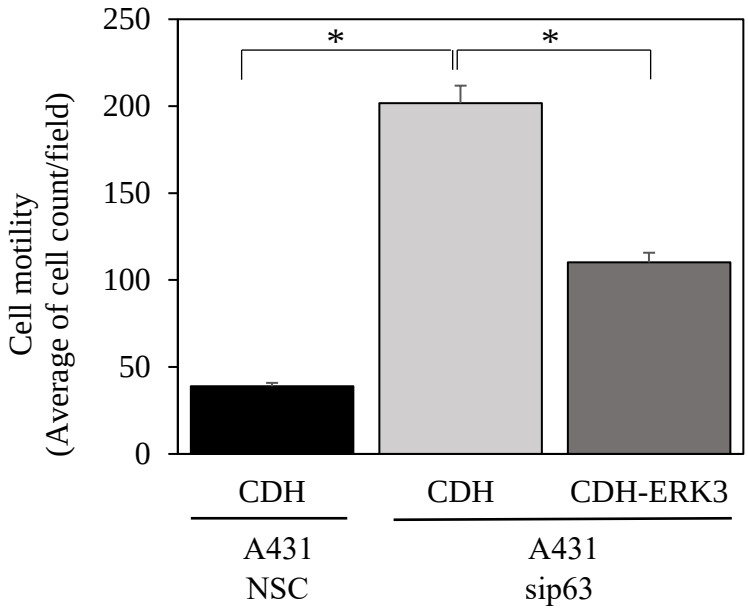
E. ERK3 may mediate the inhibitory role of ΔNp63α in suppressing cancer cell migration

As we have found that ΔNp63α upregulates ERK3 expression and that both ΔNp63α and ERK3 suppress cell migration of A431 cells, we were interested in investigating whether ERK3, as a downstream target of ΔNp63α, mediates the latter's role in this cell process. To test this, we silenced ΔNp63α in squamous cell carcinoma A431 cells with siRNA against ΔNp63α concomitant with ERK3 expression rescue by lentiviral expression of ERK3 cDNA (CDH-ERK3). As expected, ΔNp63α knockdown greatly decreased the protein level of ERK3 **(Figure 16A)** and led to a remarkable increase in the number of migrated cells (sip63/CDH versus NSC/CDH, **Figures 16B and 16C**). Importantly, restoration of ERK3 expression by lentiviral CDH-ERK3 **(Figure 16A)** rescued the increased cell migration observed upon p63 silencing (sip63/CDH-ERK3 versus sip63/CDH, **Figures 16B and 16C**). These findings suggest that ERK3 is an important downstream mediator of ΔNp63α in suppressing A431cell migration.

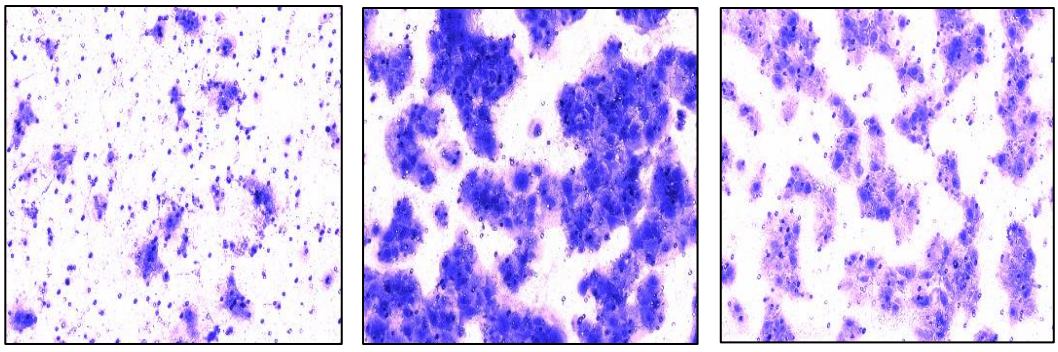
A.



B.



C.



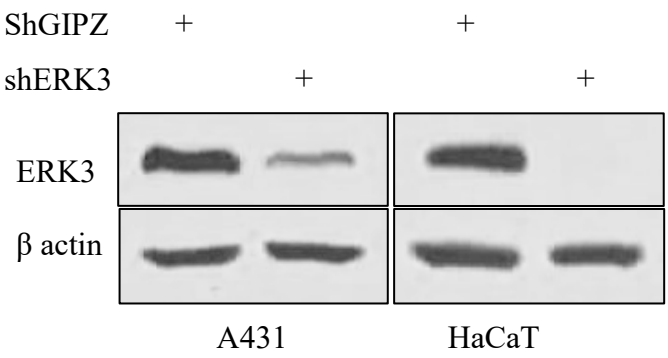
A431 NSC/CDH A431 sip63/CDH A431 sip63/CDH-ERK3

Figure 16: ERK3 restoration partly rescues the inhibitory role of ΔNp63α on cell migration in A431 cells. A431 cells were transiently transfected for two rounds with non-silencing control siRNA (NSC) or ΔNp63α siRNA (sip63) as indicated. Along with the 2nd sip63 transfection, cells were also transduced with lentiviral empty vector pCDH CMV-MCS-EF1-Puro (CDH) or pCDH-Myc6-ERK3 (CDH-ERK3) as indicated. Twenty-four hours after lentivirus transduction, one set of cells were harvested, and ERK3 and ΔNp63α proteins level were analyzed by immunoblot analysis (A). Exogenously expressed ERK3 protein with 6 myc-tags (the upper bands) and endogenous ERK3 protein (lower band) were indicated by arrows. The other set of cells were subjected to transwell cell migration assay and the number of migrated cells was quantitated after 18 hours (B). The average number of migrated cells per well under each condition is presented in the y-axis of the bar graph. Values represent mean ± S.D. An asterisk indicates a statistically significant difference with $P < 0.0001$ by Student's t-test. (C) Representative images of migrated A431 cells with crystal violet staining.

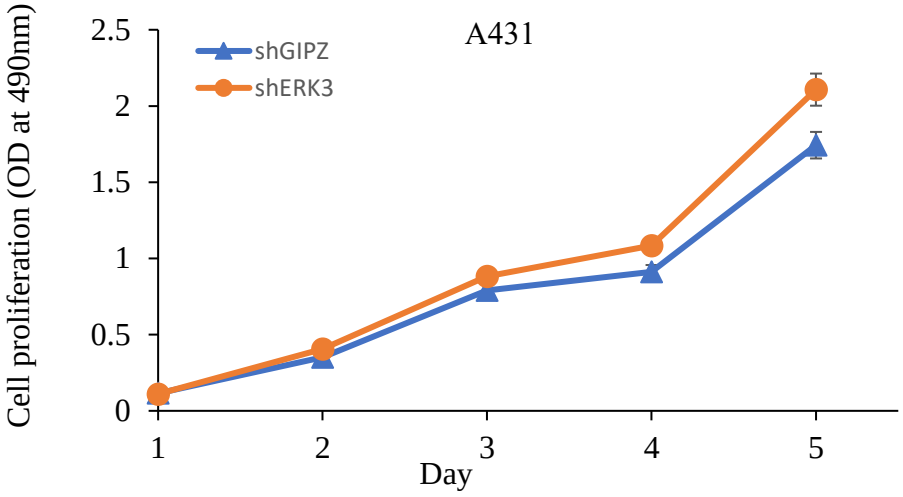
F. The depletion of ERK3 does not affect the cell proliferation of A431 or HaCaT

ERK3 was reported to inhibit cell proliferation in multiple cancer cell lines, including a squamous cell carcinoma, hepatocarcinoma, and melanoma cell lines (M. Chen et al., 2018; Crowe, 2004; Xiang et al., 2014). In lung cancer cells, however, ERK3 does not impact cancer cell growth (Long et al., 2012), indicating that ERK3's role in cell growth is cancer-type dependent. Hence, we wanted to investigate whether ERK3 plays a role in cell growth in non-melanoma skin cancer cells. For this purpose, we generated cell lines (A431 and HaCaT) with stable knockdown of ERK3 by lentiviral transduction of shRNA against ERK3. Non-targeting lenti-GIPZ shRNA was used as a control. As shown in Figure 17, silencing ERK3 did not cause a significant difference in proliferation between cells stably expressing shERK3 and cells expressing shGIPZ control in both A431 and HaCaT, suggesting that ERK3 does not affect cell proliferation in non-melanoma skin cancer cells.

A.


ShGIPZ + +
shERK3 + +
ERK3
β actin
A431
HaCaT

B.


A431
shGIPZ
shERK3
Cell proliferation (OD at 490nm)
2.5
2
1.5
1
0.5
0
1
2
3
4
5
Day

C.

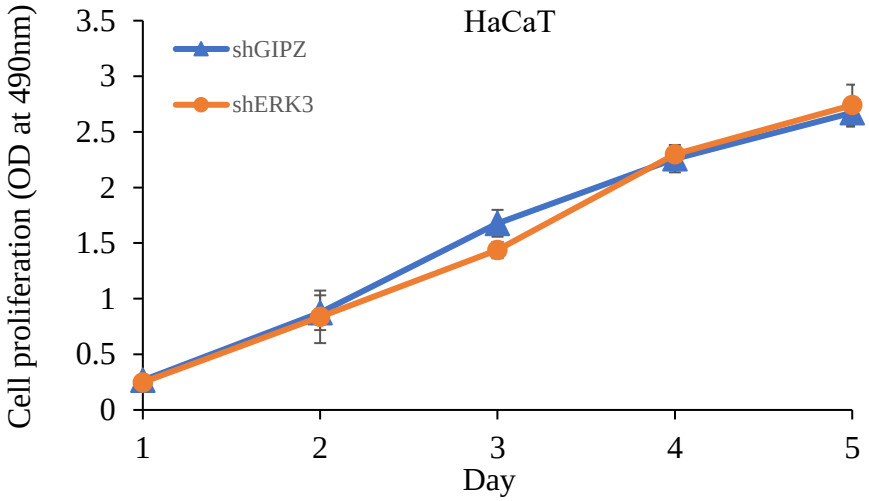

HaCaT
shGIPZ
shERK3
Cell proliferation (OD at 490nm)
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
Day

Figure 17: ERK3 knockdown does not affect cell proliferation. A431 and HaCaT cells were stably transduced with lentiviruses expressing shRNA specifically targeting ERK3 (shERK3) or a non-targeting control shRNA (shGIPZ) as indicated. (A) The change in proteins level was analyzed by immunoblot analysis after fourteen days of cells selection by puromycin. (B) and (C) MTS cell proliferation assay was performed by measuring the number of viable cells daily for five days. The relative cell growth (indicated by optical density $(OD)_{490}$) of A431 cells (B) or HaCaT cells (C) expressing shERK3 or shGIPZ at different days are presented in the y-axis. Values represent mean ± S.D. The statistical analysis shows no significant difference between the shERK3 and the shGIPZ control in both cell lines as analyzed by Student's *t*-test.

IV. Discussion

ΔNp63α is the most abundant and physiologically significant isoform of p63. It is highly expressed and plays important roles in the development of various epithelia structures including skin, lung and mammary gland (Candi et al., 2008; Mills et al., 1999; Soares & Zhou, 2018). ΔNp63α has been shown to be overexpressed in many human cancers, such as skin cancer, head and neck cancers, lung cancers, and esophageal squamous cell carcinomas (Agrawal et al., 2012; Hill et al., 2015; Reis-Filho et al., 2003; Tonon et al., 2005). ΔNp63α is not only involved in cell growth and differentiation but also affects cell invasiveness. In squamous cell carcinoma line, ΔNp63α exhibited inhibitory role on cell migration/invasion (Barbieri et al., 2006; Danilov et al., 2011; Kommagani et al., 2009; Senoo et al., 2002; Wu et al., 2014), possibly by downregulating various cellular processes and pathways, including EMT (Bergholz et al., 2014), Akt pathway (Leonard et al., 2011). Hence, ΔNp63α'role in cancer development and progression is complex and seems to be tissue-type dependent. The underlying molecular mechanisms by which ΔNp63α plays different roles in different cancers are still largely unknown and need to be further explored.

ERK3, an atypical MAPK member, has a single SEG phosphorylation site in its activation loop rather than TXY motif in conventional MAPKs. Unlike the signaling cascades of the well-studied conventional MAPKs, little is known about ERK3 signaling. Recently, ERK3 has been shown to be an essential player in organogenesis and cancer cell growth and invasiveness (Coulombe & Meloche, 2007; Klinger et al., 2009). ERK3 overexpression has been found in several human cancers, including squamous cell lung carcinoma (Long et al., 2012), oral squamous cell carcinoma (Rai et al., 2004), gastric cancer (Liang et al., 2005), breast cancer (Al-Mahdi et al., 2015) and melanoma skin cancer (Hoeflich et al., 2006). In addition, ERK3 gene mutations, albeit at a relatively low frequency were found in colon cancer, lung cancer and skin cancer

(COSMIC database; Alsaran, Elkhadragy, Shakya, & Long, 2017). ERK3 promotes cancer cell migration and invasion but has little effect on the proliferation of several types of human cancer cells, including those of lung cancer, breast cancer, and head and neck cancers. However, in a recent study ERK3 was reported to inhibit melanoma cell migration and proliferation (M. Chen et al., 2018). Hence, like ΔNp63α, ERK3 plays different roles in different cancers. Although ERK3 mRNA level was found to be upregulated in SCC of the skin according to ONCOMINE data **(Figure 5)**, little is known about the regulation of ERK3 and its role in this type of squamous cell carcinomas.

In this study, we showed that ERK3 protein is expressed in normal skin and non- melanoma skin tissues, including cutaneous squamous cell carcinoma, cutaneous basal cell carcinoma, and the precursor to SCC; actinic keratosis. Consistent with the findings of previous studies including those from our own laboratory (Hill et al., 2015; Leonard et al., 2011), ΔNp63α expression level is elevated in actinic keratosis, cutaneous squamous cell carcinoma, and cutaneous basal cell carcinoma. Importantly, we found that ERK3 protein level is also upregulated in skin tissues of actinic keratosis and cutaneous squamous cell carcinoma compared to normal skins. To our best knowledge, this is the first report that ERK3 protein is upregulated in skin SCCs. Unlike ΔNp63α, ERK3's expression in cutaneous BCC did not show a significant difference from that of the normal skin tissue. More importantly, our results demonstrate a highly significant positive correlation between ΔNp63α's expression and ERK3's expression in normal skin and non-melanoma skin cancer tissues. These findings raise an intriguing possibility that ΔNp63α may regulate the expression of ERK3 in skin tissues and suggest a cooperative role of ΔNp63α and ERK3 in promoting the initiation and development of skin SCC.

ΔNp63α is a transcriptional factor that regulates the transcription of many target genes. Given the strong correlation between ERK3 expression and ΔNp63α expression in skin tissues, we postulated that ΔNp63α might positively regulate ERK3 gene expression. We found that both ERK3 mRNA and protein expression were greatly reduced upon ΔNp63α knockdown. On the contrary, overexpression of ΔNp63α resulted in a significant increase in the transcript and protein level of ERK3. Taken together, these results proved our hypothesis that ΔNp63α upregulates ERK3 expression. However, we have not yet determined the exact mechanism by which ΔNp63α regulates the transcript level of ERK3. It is unclear whether ERK3 is a direct gene target of ΔNp63α or ΔNp63α regulates ERK3 gene expression through intermediator(s). We will investigate these in our future work.

The inhibitory role of ΔNp63α on cell migration is attributed to its function in sustaining the epithelial integrity by negatively blocking pathways that promote EMT. The exact underlying mechanisms, however, are unclear (Chu et al., 2013). Interestingly, similar to ΔNp63α, ERK3 is also involved in cell migration/invasion and exhibits different roles in different types of cancer. While it promotes migration of lung cancer cells and breast cancer cells (Al-Mahdi et al., 2015; Long et al., 2012), it inhibits migration of melanoma cell lines (M. Chen et al., 2018). In this study, we found that knockdown of ΔNp63α or ERK3 greatly increased the migration of HaCaT keratinocyte and A431 skin SCC cells, suggesting that they both have inhibitory roles in skin cell migration. Importantly, restoring ERK3 expression in A431 cells counteracted the increase of cell migration due to the depletion ΔNp63α. Taken together, these findings demonstrate that ERK3 is a downstream mediator for ΔNp63α's inhibitory role on cell migration.

ERK3 may regulate cell motility by multiple mechanisms. First, ERK3 is shown to phosphorylates SRC-3 and positively SRC3-mediated MMP gene expression, thereby promoting

cell migration and invasion (Long et al., 2012). In addition, p21-GTPases (Rac1 and cdc42) activated kinases (PAKs) were shown to interact with and phosphorylate ERK3 at S189 (De la Mota-Peynado et al., 2011; Deleris et al., 2011). Therefore, given the critical roles of RAC1/PAKs signaling pathway in cell migration, it would be important to investigate the effects of ERK3 on RAC1 phosphorylation and PAKs' activity in A431 cells.

Like its role in cell migration, ERK3 also has differential roles in cell growth in different types of cells. We also investigated the role of ERK3 on the growth of A431 and HaCaT cells. Unlike ΔNp63α's promoting role, ERK3 depletion did not show a significant effect on the proliferation of these cells. These results suggest that ERK3 acts as a downstream mediator of ΔNp63α in controlling cell migration, but not in cell growth.

In summary, we have found that ERK3 is upregulated in skin SCCs and its expression level is positively correlated with ΔNp63α. In line with these clinic findings, ERK3 mRNA transcript level is positively regulated by ΔNp63α, and ERK3 acts as an important downstream mediator of ΔNp63α in regulating cell migration. Future work will be focused on understanding the molecular mechanisms underlying the regulation of ERK3 by ΔNp63α and the inhibitory role of ERK3 by ΔNp63α/ERK3 axis in skin SCC cell migration. In addition, as both ΔNp63α and ERK3 are upregulated in lung squamous cell carcinomas, it would be interesting to investigate the interplay between ΔNp63α and ERK3 in this type of cancer.

V. References

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