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# ΔNP63α SUPPRESSES CELL INVASION BY TARGETING RAC1 THROUGH MIR-320A

A thesis submitted in partial fulfillment
of the requirements for the degree
of Master of Science

By

AMJAD AHMED ALJAGTHMI
B.Sc., King Abdul Aziz University, 2012

2017
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

July 24, 2017

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Amjad Ahmed Aljagthmi ENTITLED ΔNp63α suppresses cell invasion by targeting Rac1 through miR-320a BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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## ABSTRACT

Aljagthmi, Amjad Ahmed. M.S. Department of Biochemistry and Molecular Biology, Wright State University, 2017. ΔNp63α suppresses cell invasion by targeting Rac1 through miR-320a

ΔNp63α, a member of the p53 family of transcription factors, is overexpressed in a number of cancers and known to play a role in proliferation, differentiation, migration and invasion. ΔNp63α has been shown to regulate several microRNAs that play a role in both development and cancer, but to date there has not been a global analysis of p63-regulated miRNA. Using next-generation sequencing of small RNA from wild type and sip63 transfected HaCaT cells, our laboratory recently identified a number of ΔNp63α-regulated miRNAs by RNA-Seq studies which may serve as biomarkers of cancer progression. We identified a novel miRNA, miR-320a which is positively regulated by p63. Previous studies have shown that miR-320a is downregulated in colorectal cancer and targets Ras-related C3 botulinum toxin substrate 1 (RAC1), leading to a decrease in non-canonical WNT signaling and EMT and thereby a corresponding decrease in tumor metastasis and invasion. We hypothesize that ΔNp63α decreases cell invasion through down-regulation the activity of Rac1 via miR-320a. We showed that knockdown of ΔNp63α in HaCaT and A431 cell lines lead to a decrease in miR-320a levels and a corresponding increase in the phosphorylation of Rac1 at Ser71, while overexpression of ΔNp63α in SW480 and Caco2 cells led to a decrease in the S71 phosphorylation of

Rac1. We also showed that ΔNp63α effect the GTP activity of Rac1. Knockdown of ΔNp63α showed significant increase in Rac1 GTP levels and subsequent increase the activity of its effector PAK1. Finally, we showed that the increase observed upon knockdown of ΔNp63α is reversible by overexpressing miR-320a. Taken together, our data suggest that ΔNp63α-mediated increase in miR-320a levels has potential implications for cancer migration and metastasis.

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To the memory of my father, Ahmed Aljagthmi

And to my mother Saleha Aljagthmi

You mean the world to me

# I. INTRODUCTION

## A. ΔNp63α

ΔNp63α is a homolog of the p53 tumor suppressor gene and the dominant p63 isoform expressed in the proliferative basal layer of epithelial tissues (Mills et al., 1999; Shimada et al., 1999; Yang et al., 1999). Like p53, p63 contains three functional domains: a transactivation domain, a DNA binding domain (DBD), and an oligomerization domain (Yang et al., 1998). Unlike p53, p63 exists as 6 different isoforms arising from alternative promoter usage and differential 3' splicing (**Figure 1**). The p63 gene contains two promoters. Transcription initiation from promoter 1 (P1) yields the TAp63 isoforms that have a full N-terminal activation domain, while initiation from promoter 2 (P2) yields the ΔNp63 isoforms that have a truncated N-terminal domain. The TAp63 isoforms generally function similarly to p53, while the ΔNp63 isoforms generally oppose p53 function (Kommagani, Caserta, & Kadakia, 2006; Marinari et al., 2009; Ortt, Raveh, Gat, & Sinha, 2008; Osada et al., 2005; Senoo, Matsumura, & Habu, 2002). Moreover, alternative 3'splicing of TAp63 and ΔNp63, leads to the α, β and γ isoforms (**Figure 1**).

ΔNp63α is involved in many cellular processes including cellular differentiation, proliferation, adhesion and cell survival (Mills et al., 1999; Shimada et al., 1999; Yang et al., 1999). A previous study showed that p63 plays a crucial role in the development of ectodermal structures during the early development stages (Mills et al., 1999). Mice lacking p63 are born without a stratified epithelium, their limbs are truncated and they die shortly

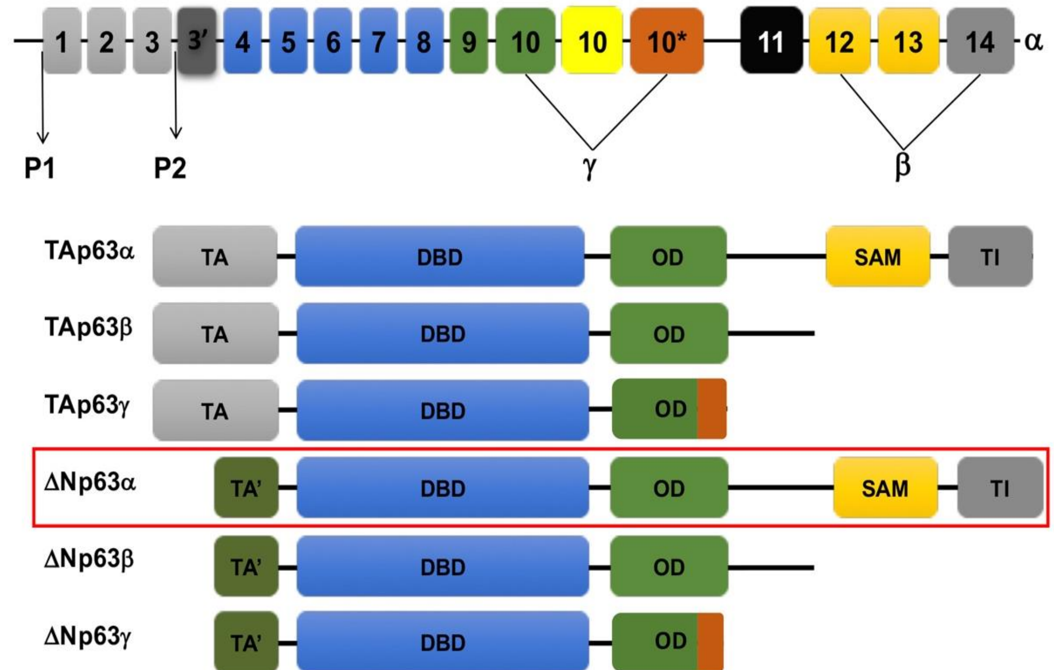



**Figure 1: p63 isoforms**. Schematic of the p63 gene comprised of the two promoter sites, 3' splicing segments, resulting in six main p63 isoforms. The p63 domains are as follows: transactivation domain (TA), DNA-binding (DBD), oligomerization domain (OD), sterile alpha motif (SAM) and transactivation inhibitory domain (TI).

after birth due to dehydration (Mills et al., 1999; Yang et al., 1999). In addition, they lack mammary glands, hair follicles and teeth (Mills et al., 1999).Furthermore, the loss of ΔNp63, but not TAp63, induces cell detachment and it has been shown that ΔNp63 modulates many key proteins involved in cell adhesion (Carroll et al., 2006). ΔNp63α is the most abundantly expressed and physiologically relevant isoform of p63 (Koster, Kim, Mills, DeMayo, & Roop, 2004).

### B. ΔNp63α and human cancer

ΔNp63α is known for its oncogenic role in squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) where it is overexpressed, and recent evidence suggests that ΔNp63α can modulate cellular processes critical for the spread of tumors, metastasis and epithelial-mesenchymal transition (EMT). Interestingly, invasive cancers such as colorectal cancer (CRC) have low levels of ΔNp63α expression (Finlan & Hupp, 2007). ΔNp63α expression is found to be decreased as cancer becomes more aggressive which suggests that ΔNp63α can act as an oncogene in the early stages of cancer, however, it plays a role in inhibiting cancer migration and metastasis in the late stages (Bergholz et al., 2014; Danilov et al., 2011; Finlan & Hupp, 2007; Kommagani et al., 2009). Metastasis, the spread of cancer cells from the site they first formed through the blood or lymph system to other parts of the body, frequently occurs in lung, liver and brain and that serves as the primary cause of mortality of cancer patients (Vatandoust, Price, & Karapetis, 2015). The epithelial-mesenchymal transition (EMT) is the primary driver of tumor invasion and migration, and is one of key cellular programs activated during cancer metastasis (Cao, Xu, Liu, Wan, & Lai, 2015). Previous studies from ours and other laboratories have shown that ΔNp63α dramatically inhibits cell migration and invasion, potentially by regulating

signaling pathways that induce the EMT (Bergholz et al., 2014; Danilov et al., 2011; Finlan & Hupp, 2007; Kommagani et al., 2009; Leonard et al., 2011). ΔNp63α has been shown to inhibit EMT through miRNA-mediated regulation (Lin et al., 2015; Ratovitski, 2014; Stacy, Craig, Sakaram, & Kadakia, 2017; Tucci et al., 2012). A number of microRNAs (miRNAs) that are regulated by ΔNp63α have been implicated in EMT. ΔNp63α was shown to inhibit EMT by upregulating miR-205 which suppresses Zeb1 and Zeb2 expression, EMT-related transcription factors (Chu et al., 2013; Tran et al., 2013; Tucci et al., 2012).

### C. microRNAs

microRNAs (miRNAs) are small non-coding RNA molecules of 18-24 nucleotides in length. They regulate gene expression post-transcriptionally by binding to complementary sequences in the 3' untranslated region (UTR) of their target mRNA. This binding can lead to translation inhibition or mRNA degradation (Finnegan & Pasquinelli, 2013; Kloosterman & Plasterk, 2006). miRNA synthesis begins with transcription of the primary miRNA (pri-miRNA) by RNA polymerase II (RNA POL II) (**Figure 2**). The pri-miRNA is then processed by Drosha/DGCR8 in the nucleus to yield a hairpin structured precursor miRNA (pre-miRNA) which is then exported to cytoplasm through Exportin 5. The pre-miRNA is processed in cytoplasm by Dicer to produce mature double-stranded miRNA (**Figure 2**). A single strand is selected from the double-stranded miRNA molecule for loading into Argonaute (AGO) to form RNA-induced silencing complex (RISC), whereas the second strand is degraded (Lin et al., 2015; Wahid, Shehzad, Khan, & Kim, 2010).

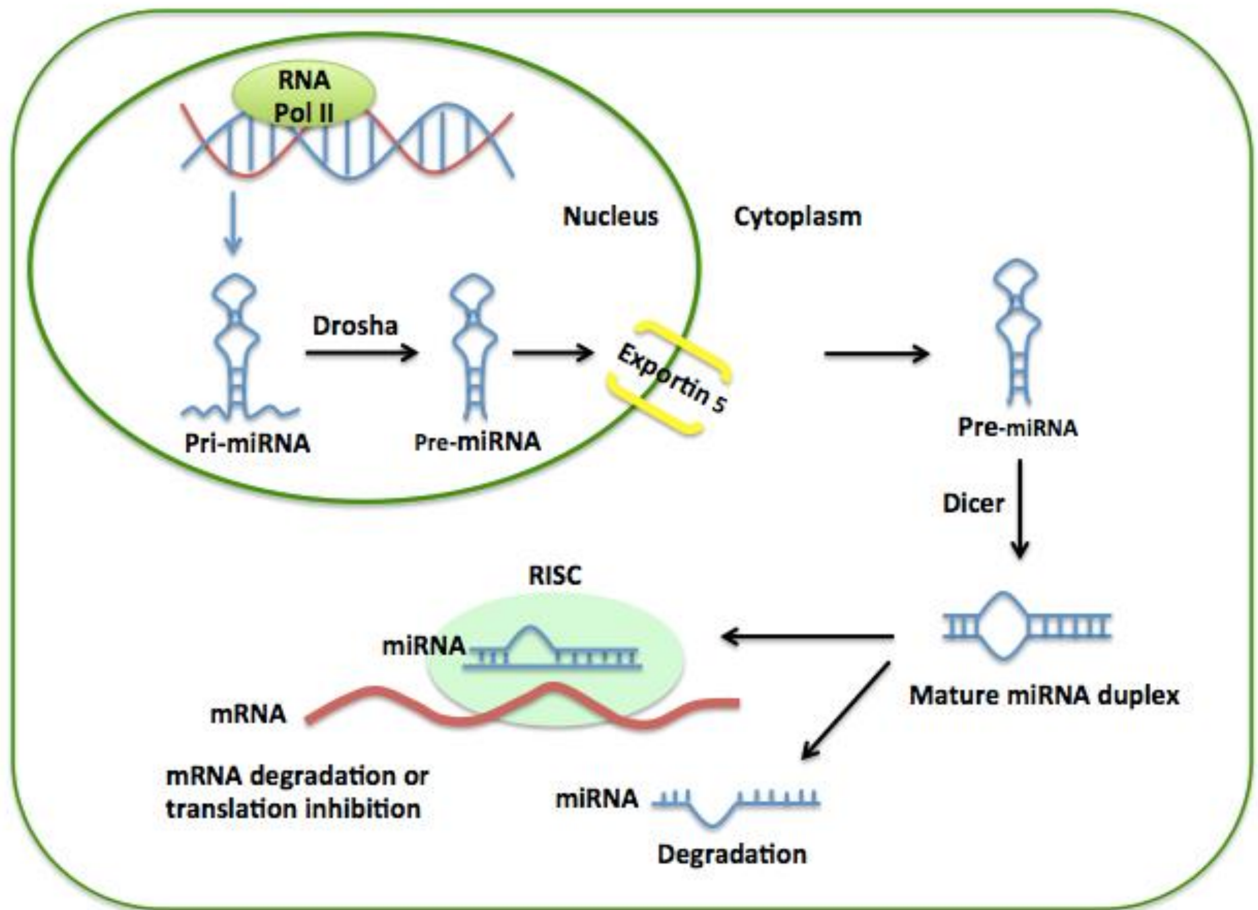



**Figure 2: miRNA biosynthesis.** The synthesis of microRNA starts with transcribing gene into primary miRNA (pri-miRNA) by the action of RNA Pol II. pri-miRNA is then processed by Drosha to generate miRNA precursor (pre-miRNA) which is exported to the cytoplasm by Exportin 5. The loop region in the pre-miRNA is removed by an endonuclease complex containing Dicer to generate a mature double-stranded miRNA. One strand of the duplex miRNAs is degraded and the other strand is loaded on Argonaute (AGO) to form RISC which then binds to mRNA of the target gene and leads mRNA degradation or translation inhibition.

The miRNA-loaded RISC then binds to the target mRNA and inhibits translation or leads to mRNA degradation (**Figure 2**).

A single miRNA may regulate multiple mRNAs and a single mRNA may be targeted by multiple miRNAs, thus the dysregulation of miRNAs can have strong consequences on the dysregulation of genes. miRNAs have been implicated in multiple cellular processes including cell development, differentiation, proliferation and apoptosis (Andersen, Duroux, & Gazerani, 2014). Dysregulation in miRNAs expression is greatly involved in tumor initiation and progression, drug resistance and other cancer pathogenesis (Ratovitski, 2014).

**D. miRNA in cancer**

miRNAs control the expression of proteins involved in cancer biology (Hayes, Peruzzi, & Lawler, 2014). They can enhance or suppress the pathogenesis of cancer by regulating the expression of tumor suppressors and oncogenes, respectively, or by directly functioning as oncogenes or tumor suppressors (Zhang et al., 2012). Importantly, each tumor type has a unique miRNA signature that distinguishes it from normal tissues and other cancer types (Hayes et al., 2014). Thus, miRNAs can be used as diagnostic and prognostic biomarkers for cancers especially since they are stable and abundant in biological fluids such as serum, urine and saliva (Alemar, Gregorio, & Ashton-Prolla, 2015; Hayes et al., 2014).

miRNA are dysregulated in many cancers through different genetic mechanisms such as promoter methylation, gene amplification or deletion, and regulation by transcription factors(Hayes et al., 2014). In addition, mutations in the miRNA binding

sites of the target mRNA is yet another mechanism observed in cancer cells which makes mRNA insensitive (Hayes et al., 2014).

### E. ΔNp63α regulation of miRNAs

Several studies showed that many proteins involved in cell death, survival, and tumor development are controlled by ΔNp63α through miRNA-mediated regulation (Lin et al., 2015; Ratovitski, 2014; Stacy et al., 2017). In fact, ΔNp63α can control miRNA expression through regulating several steps in miRNA synthesis. It can decrease the binding of RNA POL II to the promoter of the miRNA host gene leading to transcriptional inhibition (Tran et al., 2013). It can also influence pri-miRNA processing through transcriptionally regulating the expression of DGCR8 (Chakravarti et al., 2014). In addition, p63 can control the processing of pre-miRNA by regulating the expression of DICER (Boominathan, 2010; Huang et al., 2011). Finally, p63 can also regulate transcription factors that control miRNA expression levels. For instance, p63 can indirectly upregulate the expression of miR-630 and miR-885-3p by upregulating the transcription factors CARM1, KAT2B and TFAP2A that can bind to the promoter of the of these miRNA genes and induce their expression.

Recent studies in our laboratory sought to examine the effects of ΔNp63α on global miRNA regulation. Therefore small RNA sequencing studies were performed to determine microRNAs regulation by ΔNp63α. miR-320a was identified as a novel microRNA regulated by ΔNp63α.

### F. miR-320a

miR-320a generally functions to repress tumor metastasis. Accordingly, it is strongly downregulated in many metastatic cancer types such as Salivary adenoid cystic

carcinoma (SACC), colorectal cancer (CRC), non-small cell lung cancer (NSCLC) and breast cancer (L. Sun et al., 2015; J. Yu et al., 2016; Zhang et al., 2012; Zhao et al., 2014). miR-320a has been shown to inhibit breast cancer metastasis *in vitro* and *in vivo* by directly targeting metadherin (MTDH) (J. Yu et al., 2016). miR-320a also suppresses NSCLC growth and invasiveness through downregulation of insulin-like growth factor 1 (IGF-1) receptor (L. Sun et al., 2015). In addition, a number of oncogenic proteins that are upregulated in CRC are known targets for miR-320a. miR-320a inhibits colorectal cancer growth by directly targeting SOX4, FOXM1, and FOXQ1 (Vishnubalaji et al., 2016). Moreover, it also inhibits colorectal cancer cell migration and invasion by targeting Rac1 (Zhao et al., 2014).

### G. Small GTPase-Rac1:

The Ras homology (Rho) GTPases family is subfamily of Ras small GTPases that are small G proteins with molecular weight of ~21 kDa. The members of this family are signaling molecules that control cellular responses to stimuli by regulating a variety of cellular processes including actin organization, cell cycle progression, activation of protein kinases and transcriptional regulation (Hartman & Spudich, 2012). Ras-related C3 botulinum toxin substrate 1 (Rac1) belongs to the Rho family and plays fundamental roles in cellular proliferation, adhesion, migration and gene transcription. Rac1 is a plasma membrane associated small GTPase encoded by *RAC1* gene that also produces another splice variant of the Rac1 protein, Rac1b (Matos & Jordan, 2006). Like other small GTPases, Rac1 fluctuates between its active form (GTP-bound) and inactive form (GDP-bound) (**Figure 3**). Rac1 also has GTPase activity which facilitates the hydrolysis of GTP. This molecular switch is regulated by three types of regulatory molecules including

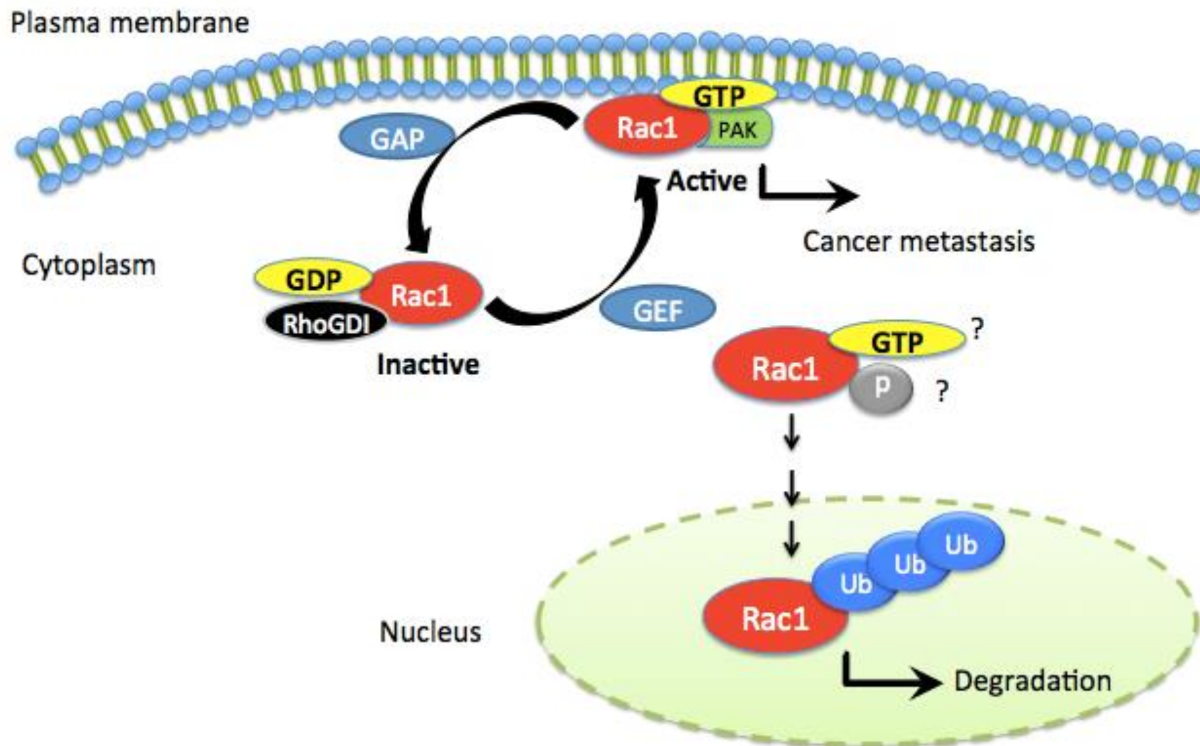



**Figure 3 Rac1 molecular switch.** Rac1 is a plasma membrane associated small GTPase that cycles between its active form (GTP-bound) and inactive form (GDP-bound) by the action of Guanine nucleotide exchange factor (GEF) and GTPase-activating protein (GAP), respectively. GTP-Rac1 is localized to the plasma membrane, whereas GDP-Rac1 associated with Rho GDP-dissociation inhibitor (RhoGDI) localized to the cytoplasm. GTP-Rac1 is also present in the nucleus where it upregulates gene transcription.

Guanine nucleotide exchange factor (GEF), GTPase-activating protein (GAP) and Rho GDP-dissociation inhibitor (RhoGDI) (Bos, Rehmann, & Wittinghofer, 2007). GEFs activate Rac1 by facilitating the exchange of GDP with GTP, whereas GAPs facilitate the hydrolysis of GTP to GDP and converting Rac1 to its inactive conformational state. RhoGDI has been shown to downregulate Rac1 activity by preventing the exchange of GDP with GTP and by sequestering Rac1 in the cytoplasm (Bos et al., 2007). Moreover, the carboxyl (C-) terminus of Rac1 undergoes post-translation modification by prenylation (i.e. addition of a lipid tail) that facilitates the attachment of Rac1 to plasma membrane. RhoGDI prevents the association of Rac1 to cellular membrane and localizes it to the cytoplasm through sequestrating this lipid tail (**Figure 3**) (ten Klooster, Leeuwen, Scheres, Anthony, & Hordijk, 2007).

The subcellular localization of Rac1 is essential to its function in the cells. In addition to its association to the plasma membrane and cytoplasm, Rac1 can also be localized to the early endosomal compartment, the nuclear envelope and the nucleoplasm (Navarro-Lerida et al., 2015). In fact, the subcellular compartmentalization of Rac1 depends on its activation state. When Rac1 is bound to GTP, it is detected at the plasma membrane where it is involved in inducing the membrane ruffling and lamellipodia, structures which are associated with cell movement and are indicative of metastasis, through coordinating the dynamics of the actin cytoskeleton (ten Klooster et al., 2007). On other hand, inactive GDP-Rac1 is associated with RhoGDI which is a cytoplasmic protein and localized to the cytoplasm (ten Klooster et al., 2007).

While the cytoplasmic and plasma membrane functions of Rac1 are well investigated, little is known about Rac1 nuclear localization and function. Lanning et al., (2004) identified a molecular mechanism by which Rac1 is imported to nucleus (Lanning, Daddona, Ruiz-Velasco, Shafer, & Williams, 2004). They found that the C-terminal polybasic region (PBR) within Rac1 is essential to promote the interaction of Rac1 with the nucleocytoplasmic shuttling proteins such as SmgGDs. The C-terminal poly basic region (PBR) of Rac1 consists of a series of basic residues, lysines and arginines (Lanning et al., 2004). The PBR contains a specific amino acid sequence called the nuclear localization signal (NLS) that is required for shuttling Rac1 to nucleus. Other Rac isoforms, Rac2 and Rac3, lack NLS (Sandrock, Bielek, Schradi, Schmidt, & Klugbauer, 2010). It is thought that Rac1 nuclear importing induces its proteasome-mediated degradation. GTP-Rac1, but not GDP-Rac1, interacts with the anaphase-promoting complex (APC/C) that ubiquitinates Rac1 and targets it for degradation by 26 S proteasome. Interestingly, only Rac1 is found to be subjected to proteasomal degradation among Rac isoforms. This could be a result of the lack of an NLS in Rac2 and Rac3. When Rac1 PBR is replaced by Rac2 PBR or Rac3 PBR, Rac1 is protected from degradation (Lanning et al., 2004).

Sequestering Rac1 to the nucleus may prevent it from interacting with cytoplasmic proteins and inhibit its membrane ruffling induction that is driven by the assembly of actin filaments and essential for epithelial cells to be motile (Koster et al., 2004). The fact that Rac1 is degraded in the nucleus does not eliminate its other nuclear functions. In fact, active nuclear Rac1 has been reported and was attributed to binding to exchange factors such as Dock180 (Wong & Isberg, 2005). It is thought that Rac1 shuttling to nucleus occurs in a cell cycle-dependent manner. Rac1 is not detectable in the nucleus during G1, however, it

has been shown to accumulate in the nucleus in G2 phase. The presence of Rac1 in the nucleus is believed to accelerate the cell cycle and protect cells from apoptosis, however, the underlying mechanism is not understood (Michaelson et al., 2008). In addition, Rac1 participates in a number of nuclear processes that facilitate its degradation (Lanning et al., 2004). Thus, there is a critical need for further investigation of nuclear Rac1 function(s) and the mechanism that regulates its activation in the nucleus.

The C-terminal domain of Rac1 has been shown to bind to its downstream effectors leading to activation of Rac1 signaling (ten Klooster et al., 2007). Active Rac1, GTP-Rac1, can activate signaling pathways through binding to PAK (p21-activated kinase), IQ-GAP, POSH, POR1, WASP, p67PHOX and Sra-1 (Rane & Minden, 2014). Among Rac1 effectors, p21-activated kinases (PAKs) are the best characterized and the prime mediators of Rac1 signaling in the cells. PAKs are serine/threonine kinases that consist of 6 isoforms (PAK1-6) and are classified into two groups based on their biochemical structures (Rane & Minden, 2014). Group I consists of PAK1, 2 and 3, while group II consists of PAK4, 5 and 6. Both groups have an N-terminal regulatory domain and a carboxyl terminal kinase domain, however the structure of the regulatory domains is completely different between the two groups resulting in distinct activation mechanisms (Rane & Minden, 2014). In addition, they share only 50% identity to Cdc42 Rac interactive binding (CRIB) domain (Rane & Minden, 2014).

PAK1 is the most extensively studied member of PAKs and a major mediator of Rac1 signaling. In its inactive state, PAK1 is present as homodimers in a trans-autoinhibitory conformation in which the autoinhibitory domain (AID) in one monomer binds to the kinase domain in the second monomer preventing it from autophosphorylation

(Kumar, Sanawar, Li, & Li, 2017). GTP-Rac1 binds to the CRIB domain overlapping by the AID in PAK1 leading to disruption of the homodimerization structure, and subsequent auto-phosphorylation of PAK1 at Ser21, Ser144 and T423 in the activation loop of the catalytic domain in addition to other residues in other sites (Parrini, Lei, Harrison, & Mayer, 2002; Zenke, King, Bohl, & Bokoch, 1999). Upon activation, PAK1 can phosphorylate or interact with downstream effectors leading to regulation of a variety of cellular processes including cytoskeleton rearrangement, survival, proliferation and cell motility.

### H. Rac1 in cancer

The dysregulation of signaling pathways regulated by Rac1 can promote various aspects of tumorigenesis including anchorage-independent growth, cell transformation, survival, and invasion (Navarro-Lerida et al., 2015). Rac1 expression or activity has been shown to be frequently altered in human cancers. This alteration can be at the transcript or protein levels of Rac1. Rac1 overexpression has been reported in multiple types of cancer such as myeloma, head and neck squamous cell carcinomas, colorectal, pancreatic, breast, and testicular cancers and in leukemia (Davis et al., 2013; Tong, Li, Ballermann, & Wang, 2013). Rac1 has been shown to be strongly involved in colorectal adenocarcinoma initiation and progression (Espina et al., 2008; Matos et al., 2000). Moreover, Rac1b, a highly activated splicing variant of Rac1 with increased expression in colorectal tumors, was also shown to promote cell transformation and epithelial-mesenchymal transition (Matos et al., 2000). Although, mutation in Rac1 is rarely detected, mutations in its upstream regulators such as GEFs, GAPs or RhoGDI, or mutation in its downstream effectors, e.g. PAK1, have been reported to alter Rac1 activity. For instance, upregulation

of PAK1 activity is associated with the malignancy of breast cancer (Holm et al., 2006). PAK1 also found to promote the progression of colorectal cancer (Huynh, Liu, Baldwin, & He, 2010).

### I. miRNA regulation of Rac1

Recent studies have uncovered an additional regulatory mechanism by which miRNA can alter Rac1 expression and activity. For example, miR-320a suppresses colorectal cancer progression by directly binding to the 3'-UTR of Rac1 and downregulating its protein levels (Zhao et al., 2014). Moreover, miR-124 is reported to negatively regulate Rac1 by changing its localization to nucleus, without affecting its protein levels, where it participates in signaling pathways that target its degradation (**Figure 3**) (Lanning et al., 2004; J. Y. Yu, Chung, Deo, Thompson, & Turner, 2008). miRNA can also indirectly regulate Rac1 activity through targeting GEFs or GAPs upstream of Rac1. miR-512-3p is found to inhibit migration and invasion in non-small cell lung cancer (NSCLC) through down regulating Rac1-GEF, DOCK3 (Zhu et al., 2015). Taken together, these studies demonstrate both direct and indirect regulation of Rac1 signaling by miRNA.

### J. Phosphorylation of Rac1

The function of Rac1 may also be modulated via protein phosphorylation. Rac1 has been shown to be phosphorylated at multiple sites (**Table 1**). Although the phosphorylation of other Rho family members RhoA and CDC42 limits their functions and increases their binding to RhoGDI, the effect of Rac1 phosphorylation on its activity is not fully understood (Forget, Desrosiers, Gingras, & Beliveau, 2002). ERK1/2 directly interacts with Rac1 through an ERK D site that is present in the C-terminus of Rac1. This

interaction results in phosphorylation of Rac1 at T108 by ERK1/2 (Tong et al., 2013). Rac1 phosphorylated at T108 showed less binding to PAK and accumulated in the nucleus. It is suggested that this accumulation isolates Rac1 from the cytoplasmic GEFs that are not localized to nucleus. However, phospho-Rac1 T108 is still able to interact with other molecules in the nucleus and regulates different cellular process. Thus, phosphorylation of Rac1 at T108 may inhibit the migratory function of Rac1 by isolating it to nucleus, however, it retains its activity in terms of cell growth and proliferation.

Y64 in Rac1 is shown to be phosphorylated by Focal Adhesion Kinase (FAK) and Src kinase (**Table1**). The phosphorylation at this site inhibits cell spreading and decreases Rac1 binding to PAK. It also increases the binding of Rac1 to RhoGDI (Chang, Lemmon, Lietha, Eck, & Romer, 2011). Thus, phosphorylation of Rac1 at Y64 is thought to decrease its activity. By contrast, FAK has been reported before to upregulate Rac1 activity by phosphorylating PIX (PAK-interacting exchange factor) and increasing its binding to Rac1. PIX targets Rac1 to focal adhesion and leading to an upregulation in cell spreading and migration (Chang et al., 2011).

AKT can phosphorylate Rac1 at S71 (**Table 1**). Phosphorylation at S71 decreases Rac1 binding to GTP since this phosphorylation site is located in Switch II domain (Rac1 residues 57–75), where the GTP can bind (Kwon, Kwon, Chun, Kim, & Kang, 2000; Worthylake, Rossman, & Sondek, 2000). Another study showed that Rac1 phosphorylation does not affect the activity of Rac1, but rather leads to a decrease in Rac1 binding to specific effectors and a shift in specificity toward other effectors (Schwarz et al., 2012) . Another study showed that phosphorylation of Rac1 interferes with its interaction with RhoGDI, while pRac1 still binds to PAK1 even though the phosphorylation decreases

**Table1: Known phosphorylated residues in Rac1**

| Rac1 residue | Kinase | References |
|---|---|---|
| T108 | ERK1/2 | (Tong et al., 2013) |
| Y64 | Src | (Chang et al., 2011) |
| Y64 | FAK | (Chang et al., 2011) |
| S71 | Akt | (Kwon et al., 2000) |

its GTP binding (Schoentaube, Olling, Tatge, Just, & Gerhard, 2009). Thus, the impact of S71 phosphorylation on Rac1 activity and function is controversial and poorly understood and more investigation is needed.

Rac1 phosphorylation at T108, Y64 and S71 occurs in an EGF-dependent manner. Rac1, but not CDC42 or RhoA, has been shown to be required for EGF-stimulated migration (Dise, Frey, Whitehead, & Polk, 2008). EGF stimulates SRC and PI3K resulting in an upregulation of Rac1 activity (Dise et al., 2008). Phosphorylation of related GEFs has not been identified. These two kinases work in parallel to activate Rac1 and the inhibition of one of them does not block the activity of the other (Dise et al., 2008).

### K. Significance

Determining the mechanisms by which p63 regulates migration and invasion is critical for understanding its proto-oncogenic functions and thereby the appropriate conditions for its use as chemotherapeutic agent. Previous studies and our data indicate that ΔNp63α plays an important role in inhibiting invasion and migration. ΔNp63α is known for its pro-proliferative oncogenic role in Squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) where it is overexpressed (Bircan, Candir, Kapucoglu, & Baspinar, 2006; Lo Muzio et al., 2005; Reis-Filho, Torio, Albergaria, & Schmitt, 2002). The mechanisms by which ΔNp63α negatively regulates tumor invasion and migration are not fully understood. Based on previous studies and our data, we hypothesize that ΔNp63α positively regulates miR-320a resulting in reduced migration and invasion through modulation of Rac1 activities. The proposed study will advance our understanding of the role played by ΔNp63α in regulating EMT and thereby cancer progression. Our goal is to elucidate the mechanisms by which ΔNp63α inhibits invasion and metastasis, and to

determine if ΔNp63α elicits these functions through its regulation of miR-320/Rac1. Our purposed studies will fill a gap in understanding of ΔNp63α-mediated inhibition of cancer migration and invasion.

**L. Rationale**

ΔNp63α expression is downregulated in a number of invasive cancers (Finlan & Hupp, 2007). EMT represents one of cellular programs that are activated during cancer metastasis and can drive tumor invasion and migration. Multiple markers associated with EMT are shown to be affected by ΔNp63α (Olsen et al., 2013; Stacy et al., 2017; Tran et al., 2013). ΔNp63α can act as an oncogene in the early stages of cancer, however, it plays a role in inhibiting cancer migration and metastasis in the late stages (Bergholz et al., 2014; Danilov et al., 2011; Finlan & Hupp, 2007; Kommagani et al., 2009). Previous studies and data from our laboratory showed that ΔNp63α inhibits cell migration and invasion, potentially by inhibiting signaling pathways that induce EMT (Bergholz et al., 2014; Danilov et al., 2011; Finlan & Hupp, 2007; Kommagani et al., 2009; Leonard et al., 2011).

miR-320a is downregulated in many cancer types such as colorectal cancer (CRC) and non-small cell lung cancer (NSCLC). Small RNA sequencing studies from our laboratory indicated that mi-R320a is positively regulated by ΔNp63α. miR-320a is known to downregulate a number of oncogenic proteins such as SOX4, FOXM1 and Rac1 that are involved in EMT (Vishnubalaji et al., 2016; Zhao et al., 2014). Chief among these is Rac1, a key protein that modulates actin cytoskeletal dynamics including cell adhesion and motility (Zhao et al., 2014). Rac1 activity is upregulated in several different tumor types and correlate with aggressive malignant characteristics (Bid, Roberts, Manchanda, &

Houghton, 2013). Our preliminary data showed that ΔNp63α negatively regulates Rac1 activity and changes its subcellular localization to nucleus, thus, suggesting a potential mechanistic link between p63 and cancer invasiveness through the regulation of Rac1. Therefore, in this study we tested whether ΔNp63α could potentially inhibit the activities of Rac1 through upregulating miR-320a levels.

# II. II. MATERIALS AND METHODS

## A. Cell culture and Reagents

The squamous cell carcinoma cell line A431, the human non-small cell lung carcinoma H1299, the colorectal adenocarcinoma SW480 and the colorectal adenocarcinoma Caco2 were purchased from American Type Culture Collection (Manassas, Virginia, USA). The non-tumorigenic immortalized human keratinocyte HaCaT cell line was obtained from Dr. Nancy Bigley (Wright State University). The five cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 8 % fetal bovine serum (FBS) and 250 U penicillin and 250 μg streptomycin.

## B. microRNA and siRNA transfection

miR-320a mimic and miRNA mimic negative control were obtained from Dharmacon (Lafayette, CO, USA). The mature sequence for miR-320a mimic is 5'-AAAAGCUGGGUUGAGAGGGCGA-3' and the mature sequence for miRNA mimic negative control is 5'-UCACAACCUCCUAGAAAGAGUAGA-3'. A total of 40 nM of miR320a mimic or mimic negative control was transfected into A431 or HaCaT cells using Lipofectamine RNAi-Max as per manufacturer's instructions (Life Technologies, Carlsband, CA, USA). miR-320a inhibitor and miRNA inhibitor negative control were also obtained from Dharmacon (Lafayette, CO, USA). The precursor sequence for miR-320a inhibitor is 5'-GCUUCGCUCCCCUCCGCCUUCUCUUCCCGGUUCUUCCCGG AGUCGGGAAAAGCUGGGUUGAGAGGGCGAAAAAGGAUGAGGU-3' and the mature sequence for miRNA inhibitor negative control is 5'-

UCACAACCUCCUAGAAAGAGUAGA-3'. A total of 40 nM of miR-320a inhibitor or inhibitor negative control was transfected into A431 using Lipofectamine RNAi-Max. Rac1 and p63 knockdown studies conducted in HaCaT and A431 cells were performed by two rounds of siRNA transfection using Lipofectamine RNAi-Max. Rac1 and p63 siRNA used in this study were purchased from Qiagen (Valencia, CA, USA). A pool of four siRNAs was used to target Rac1: 5'-ATGCATTTCCTGGAGAATATA-3', 5'-CAGCACGTGTTCCCGACATAA-3', 5'-ACGAAGTGGAGATTTACACTA-3' and 5'-ACAAGCCTTCTTAAAGCCTTA -3', and the siRNA sequence used for p63 was 5'-CACCCTTATAGTCTAAGACTA-3'.

### C. DNA constructs and transient transfections

EGFP-tagged wild type Rac1, dominant-negative Rac1 (T17N) and constitutive active Rac1 (Q61L) constructs were purchased from Addgene (Cambridge, MA, USA). EGFP-tagged Rac1 S71A was generated commercially by site-directed mutagenesis (GenScript, Piscataway, NJ), and the mutant sequence was verified by sequencing the entire coding region. ΔNp63α, Rac1-WT, T17N Rac1, Q61L Rac1 or S71A Rac1 expression vectors or the empty vector control, pcDNA3.1, was transiently transfected into H1299, SW480 or Caco2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Briefly, cells were trypsinized and plated onto a six-well plate at a density of $3.0 \times 10^5$ cells / well for H1299 and $5.0\times 10^5$ cells / well for SW480 and Caco2 cells per well in 2 ml of DMEM plus 8% FBS with 250 U penicillin and 250 μg streptomycin for 24 hours. 1μg of plasmid DNA in 250 μl of Opti-MEM (Invitrogen, Carlsbad, CA, USA) was mixed with 2 μl of Lipofectamine 2000 in 250 μl of Opti-MEM, incubated for 20 minutes and added to cells in DMEM media supplemented with 8% FBS without antibiotic

for overnight incubation. The following day, media was changed to DMEM plus 8% FBS with 250 U penicillin and 250 μg streptomycin. Cells were harvested 24 hours after transfection and cell pellets were used for immunoblot analysis and extraction of total RNA for qRT-PCR studies.

**D. Immunoblot analysis**

Whole cell lysates were prepared by lysing cells in phosphatase inhibitors containing buffer (50mM Tris-HCl pH 8, 120 mM NaCl, 5mM sodium pyrophosphate phosphatase inhibitor [NaPPi], 10mM NaF, 30 mM paranitrophenylphosphate, 1mM benzamidine, 0.1% NP-40, 1% Triton X-100 and 0.2 PMSF, 100nM sodium orthovanadate) supplemented with 10% protease inhibitor cocktail (Sigma, St. Louis, MO). Total protein concentrations were determined by BCA assay (Thermo Fisher Scientific Inc., Fremont, CA, USA). Equivalent concentrations of protein were resolved on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. Proteins were detected using rabbit polyclonal anti-GFP (FL) and mouse monoclonal anti β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) used at 1:1,000 and 1:10,000, respectively. β-actin was used as a loading control. Rabbit polyclonal anti-p63 [N2C1] (Gene Tex, Irvine, CA, USA). Mouse monoclonal anti-Rac1 (Abcam, Cambridge, MA, USA) was used at 1:1,000 to 1:2,000. Rabbit polyclonal anti-Rac1 (C-11) and rabbit polyclonal anti-phospho-Rac1 (Ser71) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used at1:500 and 1:1,000, respectively. Rabbit polyclonal anti-PAK1 and rabbit polyclonal anti-pPAK1/2 (Cell Signaling Biotechnology, Cambridge, MA, USA) were used at 1:500. Appropriate horseradish peroxidase-conjugated secondary antibodies

(Promega, Madison, WI, USA) were used for chemiluminescence detection with Western Lightning Plus chemiluminescent kit (Perkin Elmer, Waltham, MA, USA).

### E. Rac1 activation assay

A Rac1 pull-down activation assay (Cytoskeleton, BK035, Denver, CO) was used to measure Rac1-GTP activity. Whole cell lysates were prepared by lysing cells in ice-cold lysis buffer (50mM Tris pH 7.5, 10mM MgCL2, 0.5M NaCL, and 2% Igepal) containing 1X Protease Inhibitor Cocktail and protein concentrations were determined by BCA assay (Thermo Fisher Scientific Inc., Fremont, CA, USA). Equivalent concentrations of protein (500 μg) were added to 10 μl of p21-activated kinase-protein binding domain (PAK-PBD) beads and rotated at 4°C on a tube rotator for 1 hour. Next, the PAK-PBD beads were pelleted by centrifugation at 5,000 x g at 4°C for 1 minute. After removing about 90 % of the supernatant, the pellet was washed one time with 500 μl of wash buffer. GTP-Rac1 bound to PAK-RBD beads were run on an SDS-PAGE (10% gradient gel), electrotransferred to a polyvinylidene difluoride membrane (Sequi-Blot PVDF membranes; BIO-RAD). After blocking with 5 % milk, membranes were probed with a rabbit polyclonal anti-Rac1 (C-11) to detect total Rac1 and rabbit polyclonal anti-phospho-Rac1 (Ser71). Subsequently, the membranes were probed with a mouse monoclonal anti-Rac1 (Abcam) which detects both pRac1 and total Rac1. Antibodies dilutions used as described previously in section D.

### F. Cell Immunofluorescence Assay

H1299 cells were grown on sterile glass coverslips and transiently transfected with the following plasmids: GFP-Rac1 WT, GFP-Rac1 S71A, GFP-Rac1 T17N and GFP-Rac1 Q61L alone or along with overexpression of ΔNp63α. At 24 hours post transfection, cells

were fixed with 2% paraformaldehyde for 15 min. After three consecutive washes with PBS, cells were permeabilized with 0.2% Triton X-100 diluted in PBS for 5 min. Cells were washed and blocked with 0.5% normal goat serum in PBS (PBS-NGS) three times 5 min each before incubating with rabbit polyclonal anti-p63 (H129) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) primary antibody used at 1:100 dilution for 1 h at room temperature. Excess primary antibody was removed with three consecutive 5 min washes in PBS-NGS followed by incubation with AlexaFluor goat anti-rabbit 568 used at 1:500 for 1 h at room temperature. Excess secondary was removed with three consecutive 5 min washes in PBS-NGS and one wash in PBS prior to mounting with Vecta-Shield plus DAPI Mounting Media (Vector Laboratories, Burlingame, CA, USA). Cells were visualized and captured with a Leica CTR 6000 Microscope (Leica Microsystems, Wetzlar, Germany) using a 63X objective and analyzed using ImagePro 6.2 software (Media Cybernetics, Bethesda, MD). Rac1 subcellular localization was quantified by imaging at least 100 cells per condition with the same exposure parameters. The localization of Rac1 in plasma membrane, cytoplasm or nucleus was determined manually by evaluating each single cell.

### G. Migration and Invasion assay

Cell migration and invasion was assessed using a two-chamber transwell system. For migration assays, a total of 9 x $10^4$ stable transfected SW480 cells, 8 x $10^4$ transient transfected A431 or HaCaT cells were suspended in 200 µl of serum-free DMEM medium and seeded into 8 µm pore size inserts (BD Biosciences) and placed into 24-well plate. Then, 600 µl of DMEM containing 8% FBS was added to the bottom of each insert. Cells were incubated at 37 °C and allowed to migrate for 18 hours for SW480 and 21 hours for

A431 and HaCaT cells following. Cells that did not migrate were removed with cotton swab and migrated cells which had attached to the bottom of the transwell were fixed with 4% of paraformaldehyde for 20 min and washed once with Dulbeco's Phosphate Buffered Saline . Next, cells were stained with 600 μl of crystal violet solution (0.1g in 100 ml of $H_2O$) for 10 min and then washed with water. Cells were visualized and imaged in four to six random fields at magnification of 40X using a Leica CTR 6000 Microscope (Leica Microsystems, Wetzlar, Germany) and ImagePro 6.2 software (Media Cybernetics, Bethesda, MD). Cells were counted manually from these pictures and average was taken to calculate the standard deviation. Invasion assay was performed using the same protocol except that transwell inserts were coated with 1 mg/ml Matrigel (BD Biosciences) to measure cell invasion. A total of 1.4 x $10^5$ stable transfected SW480 cells were suspended in 200 μl of serum-free DMEM medium and seeded into 8 μm pore size inserts and allowed to invade for 21 hours at 37 °C.

### H. Quantitative Reverse transcription PCR for gene expression

*mRNA expression:* Total RNA was extracted from human cell lines using the EZNA RNA isolation kit according to the manufacturer protocol (Omega Bio-Tek, Norcross, GA, USA). A TaqMan reverse transcription kit (Life Technologies, Carlsbad, CA USA) was used to synthesize cDNA from 1μg of total RNA. Quantitative real-time PCR was performed using the Applied Biosystem 7900HT or QuantStudio 7 Flex Real-Time PCR Systems using using Assay on Demand (AOD) specific for the genes of interest and normalized to endogenous GAPDH for human genes (Life Technologies, Carlsbad City, CA, USA) (Kommagani et al., 2009; Pfaffl, 2001). AODs used were GAPDH (4325792), RAC1 (Hs01902432_s1) and pan-p63 (Hs00978340_ml).

*miRNA expression-* Total RNA was extracted from human cells using the EZNA RNA isolation kit according to the manufacturer protocol (Omega Bio-Tek, Norcross, GA, USA). TaqMan MiroRNA reverse transcription kit (Life Technologies, Carlsband, CA, USA) was used to synthesize cDNA from 10 ng of total RNA with primers specific to hsa-miR-320a (RT:002277) or RNU-48 (RT:001006) as per manufacturer protocol. Quantitative real-time PCR was performed using the Applied Biosystem 7900HT or QuantStudio 7 Flex Real-Time PCR Systems using TaqMan 2X universal master mix and miRNA specific assays on demand. Assays on demand used were hsa-miR-320a (TM:002277) normalized to RNU-48 (TM:001006). qRT-PCR was done in triplicate for each specific gene of interest for each sample.

# III. RESULTS

## A. ΔNp63α positively regulates miR-320a

EMT, a process in which epithelial cells lose their characteristics and gain a mesenchymal-like phenotype, is a fundamental process in embryonic development and during wound healing (Kalluri & Weinberg, 2009). In carcinogenic context, EMT is upregulated which allows cancer cells to lose their cell-cell adhesion and gain migratory and invasive properties. ΔNp63α plays an important role in inhibiting EMT by upregulating genes involved in cell adhesion, while loss of ΔNp63α upregulates genes that promote cell motility and mesenchymal phenotypes (Tucci et al., 2012; Yoh et al., 2016). ΔNp63α is a known regulator of miRNA, including many which have been shown to regulate EMT (Tran et al., 2013; Tucci et al., 2012). Thus, small RNA sequencing was performed by Dr. Natasha Hill and Suraj Sakaram to study the regulation of miRNA by ΔNp63α. ΔNp63α was silenced in three biological replicates of HaCaTs, a non-tumorigenic keratinocyte cells which express ΔNp63α. ΔNp63α protein and transcript levels were significantly reduced by 80% in cells transfected with siRNA specific to p63 relative to non-silencing (NSC) controls (**Figure 4A**) (representative data shown). Following confirmation of p63 knockdown by immunoblot and qRT-PCR analysis, small RNA was sequenced using the Ion Torrent Platform. Differential gene expression analysis was performed using Partek Flow and PGS which led to the identification of several miRNAs potentially regulated by the knockdown of ΔNp63α. Table 2 represents a subset of 7 microRNAs significantly regulated by ΔNp63α. Of these, let-7d-5p was previously

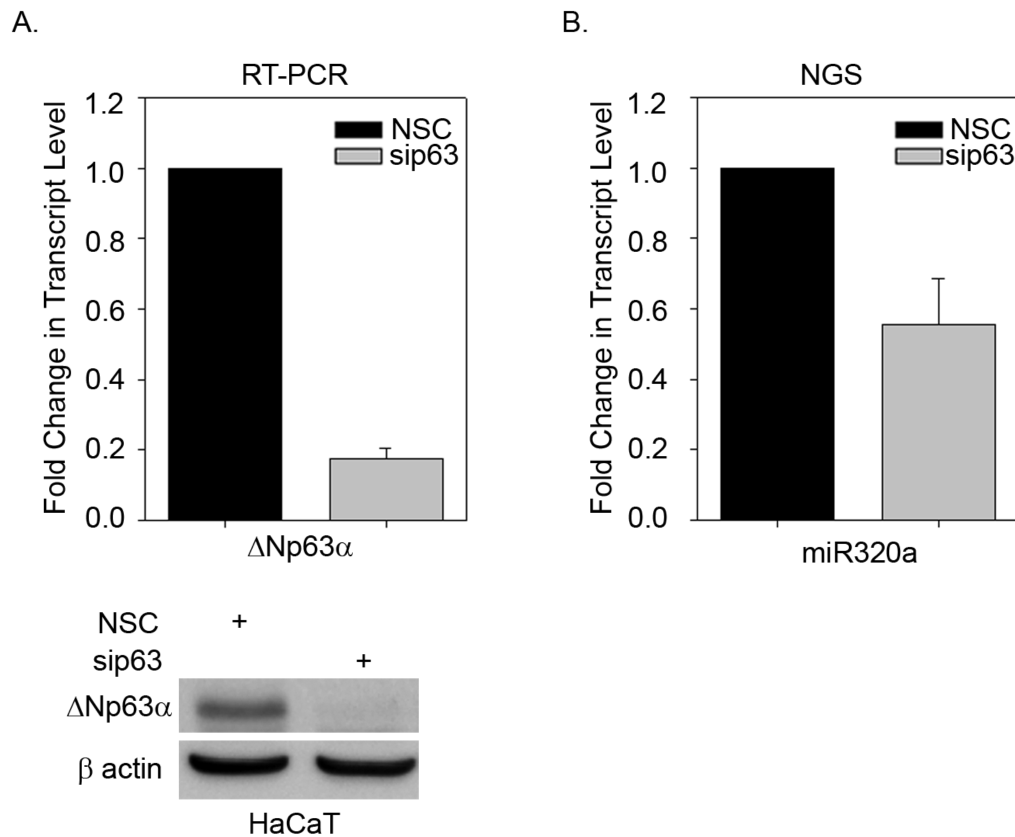



**Figure 4: ΔNp63α knockdown in HaCaT cells leads to a reduction in miR-320a levels.** (A) HaCaT cells were transfected with non-silencing control siRNA and siRNA against p63. At 24 h post-transfection, the change in transcript levels of ΔNp63α was measured by Taqman based qRT-PCR. In the bottom panel, immunoblot analysis was performed with the indicated antibodies. Immunoblot with β-actin was performed to confirm equivalent protein loading. (B) miR-320a transcript levels in NSC and sip63 HaCaT samples following small RNA sequencing on HaCaT cells transfected with the indicated siRNA. Error bars indicate +1 standard deviation.

| miRNA | p-Value | Fold Change |
|---|---|---|
| hsa-let-7d-5p* | $8.68x10^{-231}$ | -1.68 |
| hsa-miR-141-3p+ | $2.42x10^{-14}$ | -1.19 |
| hsa-miR-23a-3p+ | $2.09x10^{-132}$ | -1.19 |
| hsa-miR-24-3p+ | $9.33x10^{-24}$ | -1.31 |
| hsa-miR-31-5p+ | $7.77x10^{-263}$ | -1.38 |
| hsa-miR-320a+ | $3.92x10^{-87}$ | -1.42 |
| hsa-miR-9-5p+ | $5.79x10^{-18}$ | 1.45 |

**Table 2: Knockdown of p63 identified putative p63-regulated miRNAs involved in EMT.** Small RNA was isolated from three biological replicates of HaCaT cells transfected with non-silencing control or p63 siRNA and sequenced on the IonTorrent platform. Partek Flow was used to identify miRNA with significant changes in expression ($p \leq 0.05$) and to identify differentially expressed miRNA with known roles in EMT. * indicate known p63 Targets; + indicate novel p63 targets.

shown to be regulated by ΔNp63α (Boominathan, 2010), and the remaining 6 were novel miRNAs. In this study we only focused on miR-320a. miR-320a is a metastatic repressor that is known to inhibit EMT via targeting Wnt pathway and which has not previously shown to be regulated by p63 (J. Y. Sun et al., 2012; Zhao et al., 2014). ΔNp63α knockdown led to a concomitant decrease in miR-320a levels (Fold Change = -1.42, p = $3.92x10^{-87}$) (**Table 2**, **Figure 4 B**). Therefore miR-320a is positively regulated by ΔNp63α.

In order to validate miR-320a is positively regulated by ΔNp63α, we silenced ΔNp63α in HaCaT cells, the same cell line used in the RNA sequencing study which identified miR-320a as a putative p63-regulated miRNA. ΔNp63α was also silenced in A431 squamous cell carcinoma cells that have ΔNp63α as the only expressed form of p63 (Kommagani et al., 2009). HaCaT and A431 cells were transfected with siRNA against ΔNp63α resulting in a greater than 80% reduction in ΔNp63α transcript in both cell lines and no detectable p63 protein in sip63 whole cell lysates by immunoblotting, thus p63 knockdown (**Figure 5A**). Knockdown of ΔNp63α led to a significant reduction in miR-320a transcript levels in A431 (FC= 0.47 ± 0.04) and in HaCaT (FC= 0.69 ± 0.05) cell lines (**Figure 5B**), thus validating the NGS data (**Table 2**).

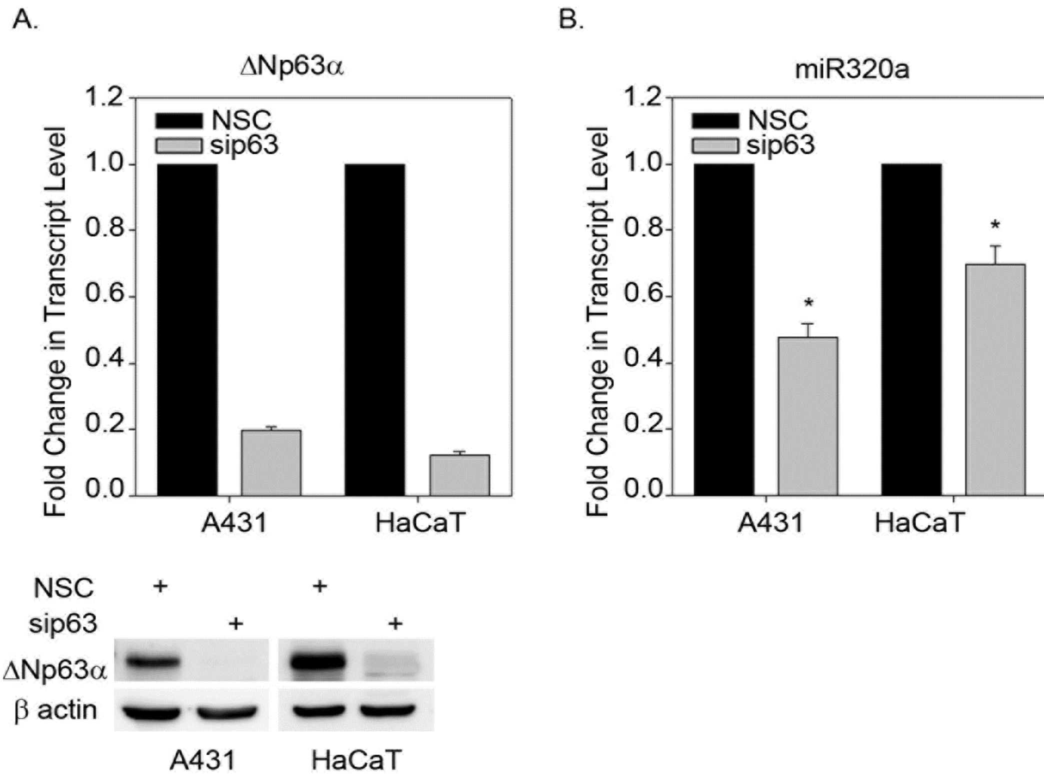



**Figure 5: ΔNp63α knockdown leads to a reduction in miR-320a transcript levels.** (A) A431 and HaCaT cells were transfected with nonsilencing control siRNA (NSC) or siRNA specific to p63. The change in mRNA level and protein expression of ΔNp63α were measured by Taqman based qRT-PCR and immunoblot analysis. Immunoblot with β-actin was performed to confirm equivalent protein loading. (B) Taqman based qRT-PCR was used to quantify miR-320a levels from the experiment described in (A). Error bars indicate +1 standard deviation. Significant changes with $p \leq 0.05$ are indicated with an asterisk.

To further verify that ΔNp63α regulates miR-320a, we examined the effects of ΔNp63α overexpression on miR-320a in SW480, a colorectal adenocarcinoma cell line, and H1299, a human non-small cell lung carcinoma cell line. Both of these cell lines are highly invasive and do not express ΔNp63α. H1299 and SW480 cells were transfected with a plasmid encoding ΔNp63α or the corresponding empty plasmid vector (EV) as a control. At 24 hours post-transfection, p63 transcript and protein levels were examined by qRT-PCR and immunoblotting, respectively. As shown in Figure 6A we observed a significant increase in both p63 transcript and protein levels confirming ΔNp63α overexpression in both cell lines. Endogenous ΔNp63α was not detected in cells transfected with EV for either cell line, while robust overexpression was shown in cells transfected with ΔNp63α plasmids (**Figure 6A, bottom**). miRNA qRT-PCR quantitation of miR-320a levels in these cells indicated that miR-320a was upregulated in cells overexpressing ΔNp63α compared to cells transfected with EV. miR-320a transcript levels increased significantly in both H1299 (FC= 1.68 ±0.27) and in SW480 (FC= 1.3 ±0.16) ($p \leq 0.05$) (**Figure 6B**). Taken together, these results along with the knockdown experiments confirm that ΔNp63α positively regulates miR-320a.

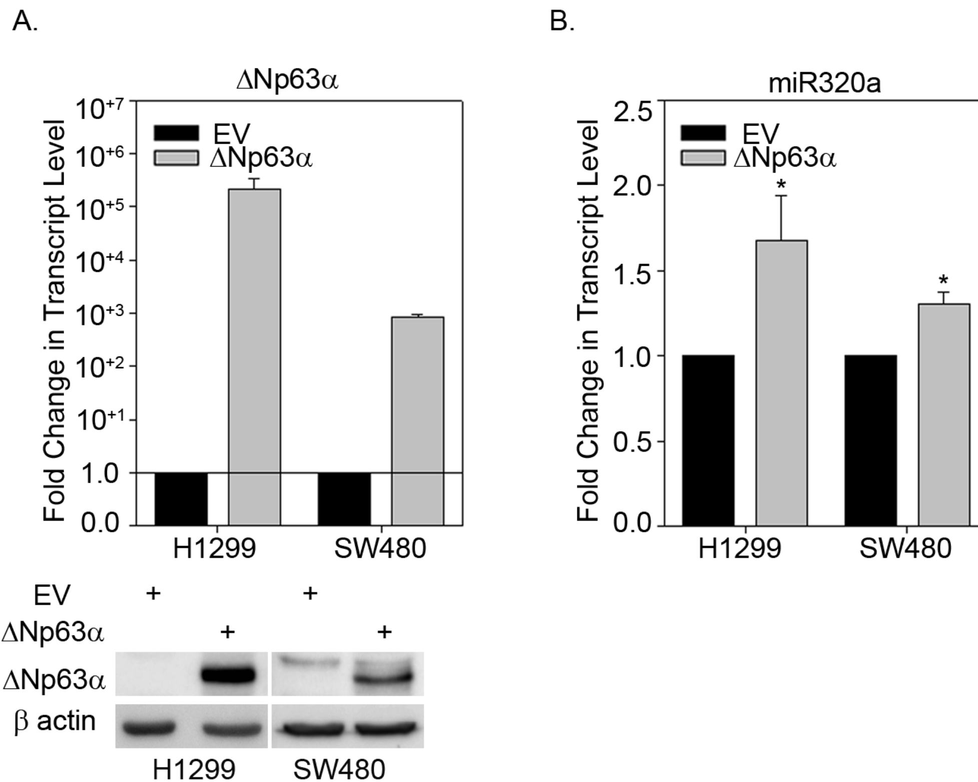



**Figure 6: ΔNp63α overexpression leads to an increase in miR-320a transcript levels.** (A) H1299 and SW480 cells were transfected with empty vector (EV) control or expression plasmid encoding ΔNp63α. Transcripts were quantified by qRT-PCR (upper panel) while protein levels were confirmed using immunoblot analyses (lower panel). Immunoblot with β-actin was performed to confirm equivalent protein loading. (B) Taqman based qRT-PCR was used to quantify miR-320a levels from the experiment described in (A). Error bars indicate +1 standard deviation. Significant changes with $p \leq 0.05$ are indicated with an asterisk.

### B. ΔNp63α does not regulate the total protein levels of Rac1

miR-320a exerts its metastatic suppressive function through targeting genes involved in EMT (J. Y. Sun et al., 2012; Zhao et al., 2014). Using TargetScan 7.1 and miRDB.org, Rac1 was identified as a putative target for miR-320a. In addition, a previous study showed that miR-320a suppresses EMT in colorectal cancer through targeting Rac1 (Zhao et al., 2014). Since we validated that ΔNp63α positively regulates miR-320a, we next sought to determine if regulation of Rac1 by ΔNp63α could be detected in H1299 and SW480 cells. We overexpressed ΔNp63α in H1299 and SW480 cells and measured the change in Rac1 transcript and protein levels. ΔNp63α overexpression in H1299 and SW480 cells was confirmed by immunoblotting with p63 specific antibody (**Figure 7A**). ΔNp63α overexpression did not significantly affect the transcript levels of Rac1 in H1299 (FC= 1.14 ± 0.18) and in SW480 (FC= 0.90 ±0.04) cells (**Figure 7A upper**). Furthermore, no change in total protein levels in Rac1 was observed in both those cell lines (**Figure 7A bottom**). Conversely, we silenced ΔNp63α in HaCaT and A431 cells. The loss of ΔNp63α protein was confirmed by immunoblot (**Figure 7B**). Although ΔNp63α knockdown led to a modest increase in Rac1 transcript levels in A431 (FC= 1.31 ±0.11) and HaCaT (FC= 1.41 ±0.20), there was no change in total protein levels of Rac1 (**Figure 7B**). These data suggest that ΔNp63α does not regulate Rac1 protein and transcript levels and Rac1 in our studies is not affected by miR-320a levels.

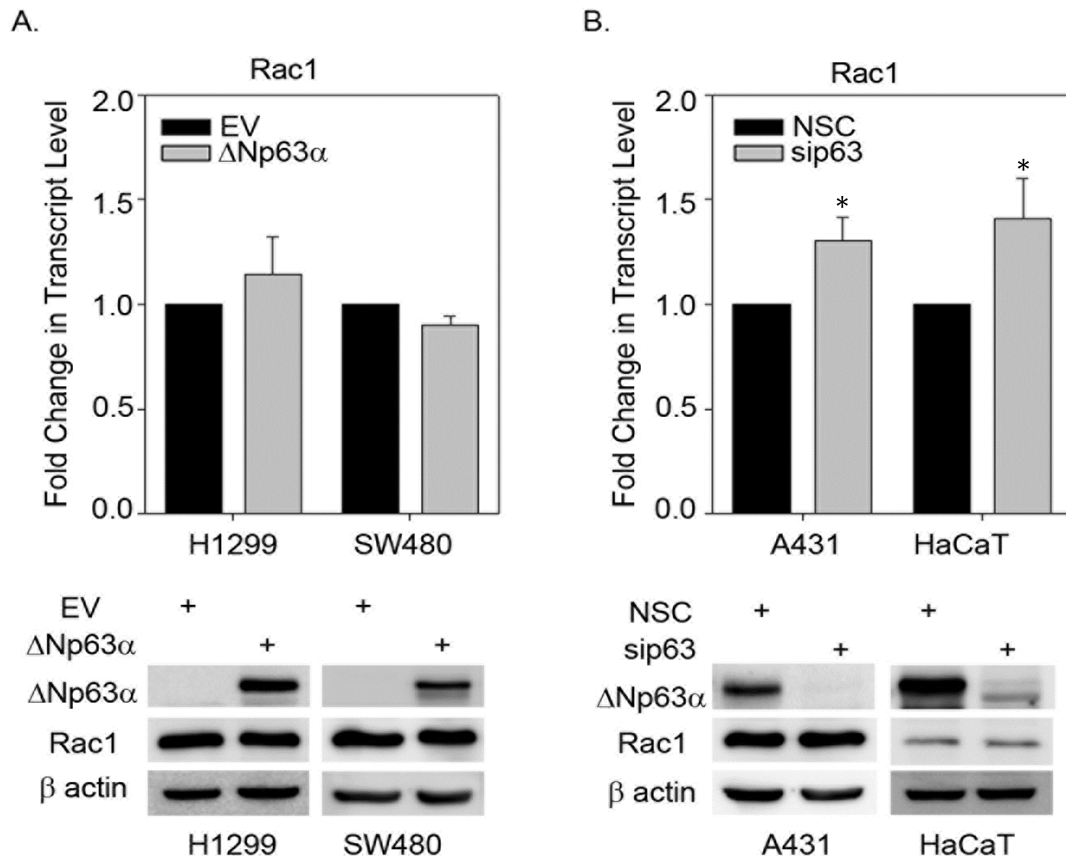



**Figure 7: ΔNp63α does not regulate the Rac1 protein levels.** (A) H1299 and SW480 cells were transfected with empty vector (EV) control or expression plasmid encoding ΔNp63α. ΔNp63α transcript levels were quantified by qRT-PCR while protein levels were confirmed using immunoblot analyses using p63 specific antibody. Rac1 was detected with mouse anti-Rac1 antibody from Abcam. (B) A431 and HaCaT cells were transfected with nonsilencing control siRNA (NSC) or siRNA specific to p63. The change in transcript and protein levels of Rac1 were measured by TaqMan based qRT-PCR and immunoblot analysis, respectively. Immunoblot with β-actin was performed to confirm equivalent protein loading. Error bars indicate +1 standard deviation. Significant changes with $p \leq 0.05$ are indicated with an asterisk.

### C. ΔNp63α negatively regulates phospho-Rac1 at S71

Interestingly, the mouse monoclonal anti-Rac1 antibody obtained from Abcam that we used to probe for Rac1 protein levels detects two bands close to the expected 21kDa molecular weight of Rac1. Although we did not see change in the band that appeared at 21 kDa which was assumed to be total Rac1, the higher 26 kDa band showed a significant increase with the knockdown of ΔNp63α (**Figure 8A**) and a significant decrease with the overexpression of ΔNp63α (**Figure 8B**). Interestingly, this observation happened in every experiment and in all cell lines except H1299 cells (**Figure 8A**). Rac2 and Rac3 isoforms have the same molecular weight of Rac1, thus eliminating the possibility that the upper band could be either of these Rac isoforms. Rather, it appeared likely that the 26 kDa band observed might be phosphorylated Rac1 as phospho-Rac1 at S71 was is detected at 26 kDa . To determine if the upper band was indeed phosphorylated Rac1, we obtained two other Rac1 antibodies: rabbit polyclonal anti-Rac1 (C-11) that detects total Rac1 and rabbit polyclonal anti-phospho-pRac1 which detects phospho-Rac1 at Ser 71 (**Table 3**). We overexpressed ΔNp63α in two colorectal adenocarcinoma cell lines, SW480 and Caco2, and confirmed overexpression of ΔNp63α at the protein levels in both cell lines (**Figure 9A**). Using the phosphospecific antibody for pRac1 S71, a single band was detected at 26 kDa which showed a significant decrease in cells overexpressing ΔNp63α (**Figure 9A**). Anti-Rac1 C-11 antibody was used to detect total Rac1 that was observed at 21 kDa. To further confirm these findings, we also knocked down ΔNp63α in A431 and HaCaT and we observed that pRac1 S71 significantly increased with ΔNp63α silencing (**Figure 9B**). Thus, these findings suggest that the band at 26 kDa detected earlier using the Abcam mouse monoclonal anti-Rac1 antibody was pRac1, and further that ΔNp63α

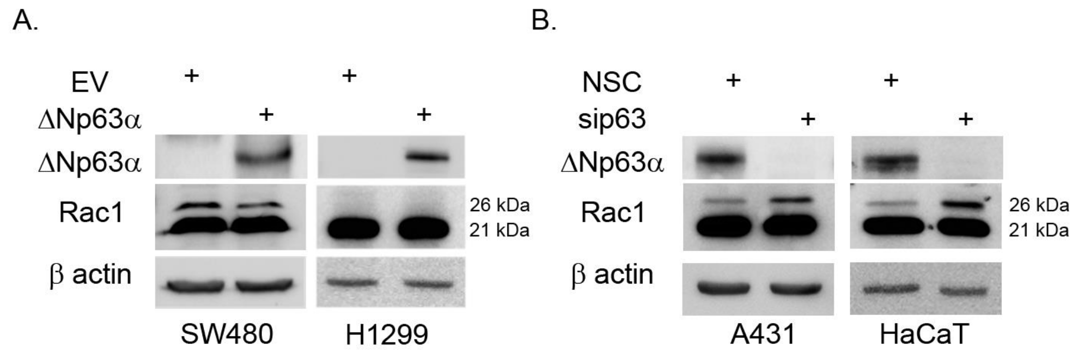



**Figure 8: ΔNp63α negatively regulates an p-Rac1 reactive 26 kDa protein.** (A) H1299 and SW480 cells were transfected with empty vector control or expression plasmid encoding ΔNp63α Overexpression of ΔNp63α was confirmed using immunoblot analyses. (B) A431 and HaCaT cells were transfected with nonsilencing control siRNA (NSC) or siRNA specific to p63. The change in ΔNp63α and Rac1 protein levels were measured by immunoblot analysis as indicated. Immunoblot with β-actin was performed to confirm equivalent protein loading.

**Table 3: Rac1 antibodies used to detect protein levels of Rac1.**

| **Antibody** | **Bands detected** |
|---|---|
| Mouse monoclonal anti-Rac1-Abcam | Detects total Rac1: unphosphorylated and phosphorylated Rac1 |
| Rabbit polyclonal anti-phospho-Rac1 (Ser71)-Santa Cruz | Detects only pRac1 at S71 |
| Rabbit polyclonal anti-Rac1 (C-11) -Santa Cruz | Detects only unphosphorylated Rac1 |

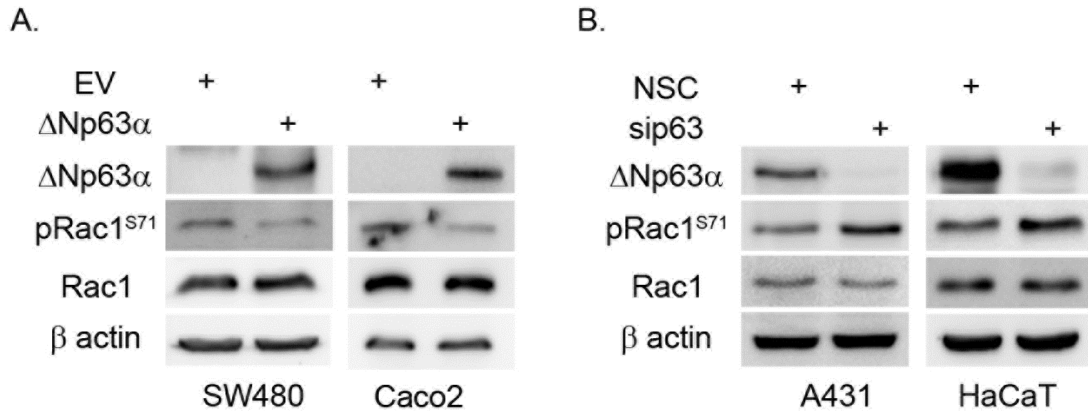



**Figure 9: ΔNp63α negatively regulates Rac1 S71 phosphorylation.** (A) SW480 and Caco2 cells were transfected with empty vector control or expression plasmid encoding ΔNp63α protein levels were confirmed using immunoblot analyses. (B) A431 and HaCaT cells were transfected with nonsilencing control siRNA (NSC) or siRNA specific to p63 and the change in protein expression was measured by immunoblot analysis. Immunoblot with β-actin was performed to confirm equivalent protein loading.

negatively regulates phosphorylation of Rac1 at S71, but not total Rac1 levels. However, further investigation was needed to confirm the specificity of the pRac1 antibody.

### D. Phospho-Rac1 antibody is specific

Until recently, there was no antibody available that could detect the phosphorylation of Rac1 at S71. Previous studies that investigated the phosphorylation of Rac1 used antibodies that could not distinguish between pRac1 and pCdc42 S71 and were therefore limited to use in cells lacking Rac1 gene (Kwon et al., 2000; Schoentaube et al., 2009; Schwarz et al., 2012). To confirm that the phosphospecific antibody was specific for pRac1 S71, we silenced either ΔNp63α alone, Rac1 alone, or both ΔNp63α and Rac1 in HaCaT cells. Immunoblot analysis confirmed the successful knockdown of ΔNp63α and Rac1 in these cells (**Figure 10, lanes 2 and 3 respectively**). The rabbit polyclonal anti-Rac1 C-11 antibody was used here to confirm the silencing of Rac1. As expected, the 26 kDa band increased significantly with the silencing of ΔNp63α alone and disappeared when Rac1 was silenced (**Figure 10, compare lanes 2 and 3 to lane 1**). Interestingly when both ΔNp63α and Rac1 were silenced, the 26 kDa band was not observed (**Figure 10, lane 4**), thus confirming that anti-phospho-Rac1 antibody is specific and detects phosphorylated Rac1. Taken together, this experiment confirm phospho-Rac1 antibody specificity and indicate that the lower 21 kDa band is total Rac1 and the upper 26 kDa band is pRac1 Ser71.

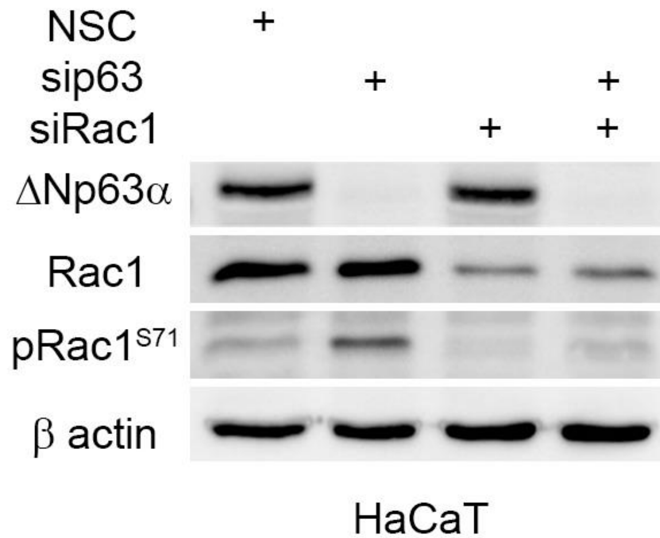


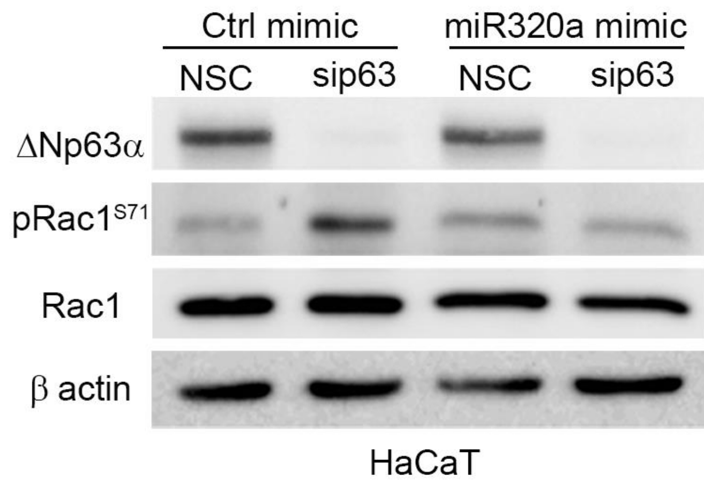

**Figure 10: The Santa Cruz anti-pRac1 (S71) antibody is specific for pRac1 S71.** HaCaT cells were transfected with nonsilencing control siRNA (NSC), siRNA specific to p63 and/or Rac1 as indicated. The change in protein expression was measured by immunoblot analysis using p63, Rac1 and pRac1 antibodies as indicated. Immunoblot with β-actin specific antibody was performed to confirm equivalent protein loading.

### E. ΔNp63α negatively regulates pRac1 S71 via miR-320a

Since we showed that ΔNp63α positively regulates miR-320a and negatively regulates pRac1 S71, we next wanted to investigate if ΔNp63α negative regulates pRac1 S71 via upregulating miR-320a. We used miRNA gain or loss of function experiments using a miR-320a mimic that functions like endogenous miR-320a or a single-stranded miR-320a inhibitor designed to bind to and inhibit endogenous miR-320a. Both HaCaT and A431 cells were co-transfected with siRNA against p63 or NSC along with negative control mimic or miR-320a mimic. HaCaT cells have endogenous ΔNp63α that suppresses pRac1S71 levels; thus, pRac1 S71 was detected at the basal levels when NSC and negative control mimic were co-transfected (**Figure 11A**). Silencing ΔNp63α upregulated the levels of pRac1 S71 as observed early (**Figure 11A, lane 2**). Overexpressing miR-320a significantly decreased pRac1 to the basal levels when ΔNp63α was silenced in both HaCaT cells (**Figure 11A, lane 4**) and A431 cells (Figure **11B**). These results clearly demonstrate that miR-320a rescued the effect of ΔNp63α silencing on pRac1 S71 protein levels

Next we wanted to examine the effect of miR-320a inhibitor on pRac1 levels in presence or absence of ΔNp63α. A431 cells with endogenous ΔNp63α were transfected with negative control inhibitor or miR-320a inhibitor. The miR-320a inhibitor significantly increased pRac1 S71 levels in the presence of endogenous ΔNp63α (**Figure 12**). Together, these results demonstrate that ΔNp63α negatively regulates the levels of pRac1 through upregulation of miR-320a. It is likely that miR-320a may target an upstream regulator that induces the phosphorylation of Rac1.

A




B.

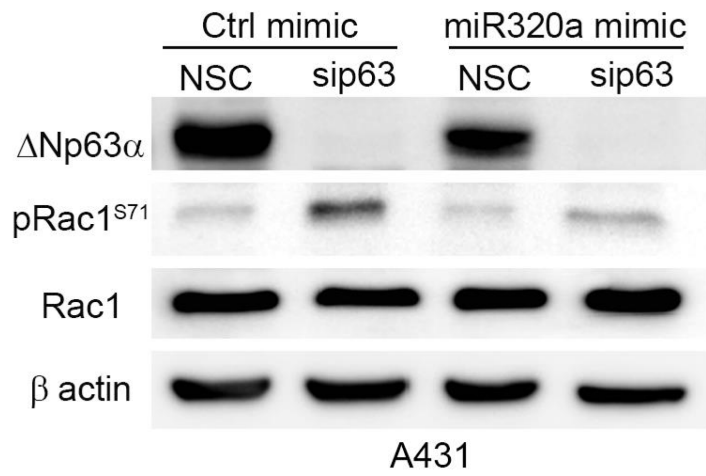


**Figure 11: Overexpression of a miR-320a mimic counters the effect of ΔNp63α knockdown on Rac1 phosphorylation.** HaCaT (A) and A431 (B) cells were transfected with either non-silencing control (NSC) or siRNA specific for p63 in conjunction with a negative control mimic or miR-320a mimic. The change in indicated protein levels were measured analyzed via immunoblotting with p63, Rac1 and pRac1 antibodies as indicated. Immunoblot with β-actin was performed to confirm equivalent protein loading.

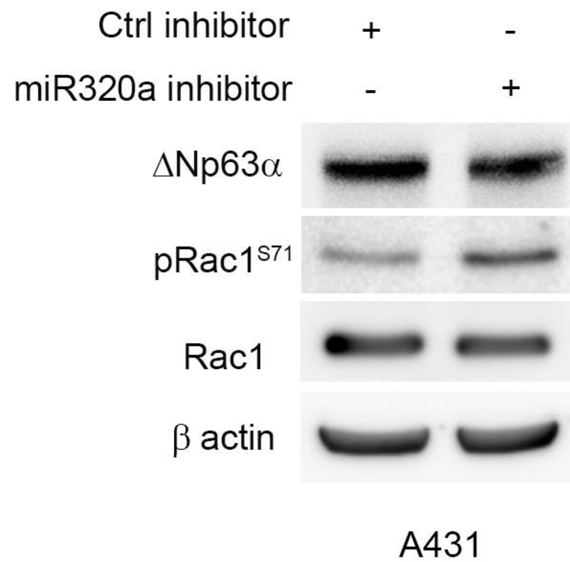



**Figure 12: Inhibition of miR-320a increases pRac1 S71 levels.** A431 cells were transfected with a negative control inhibitor or miR-320a inhibitor. The change in protein levels were analyzed via immunoblot as indicated. Immunoblot with β-actin was performed to confirm equivalent protein loading.

### F. ΔNp63α negatively regulates GTP-Rac1 levels

Rac1 is considered in its active state when it is bound to GTP. GTP-bound Rac1 has been shown to control cell shape, adhesion and mobility (Hartman & Spudich, 2012). It is shown that the phosphorylation of Rho small GTPases inhibits their activity by increasing their interaction with RhoGDI (Forget et al., 2002). However, there are multiple contradicting reports about the effect of phosphorylation on Rac1 activity. Phosphorylation of Rac1 at S71 by AKT kinase was shown to inhibit its GTP-binding activity (**Table 1**) (Kwon et al., 2000). However, pRac1 S71 was also shown to bind to the PAK-CRIB domain indicating that phosphorylation of Rac1 at S71 does not significantly affect GTP binding function, but instead modulates its downstream signaling by inhibiting its interaction with some effectors and increasing its interaction with others (Schwarz et al., 2012). Thus, we wanted to investigate if Rac1 GTP-binding activity is modulated by ΔNp63α. To this end, we silenced ΔNp63α in A431 cells and measured the level of endogenous GTP-bound (active) Rac1 by pulling down active Rac1 with purified p21-activated protein kinase protein binding domain (PAK-PBD). Immunoblotting was performed to analyze the whole cell lysates and lysates immunoprecipitated with the GST-tagged PAK-PBD. Knockdown of ΔNp63α significantly increased the levels of pRac1 in the whole cell lysates and total Rac1 showed no change (**Figure 13, compare lanes 1 and 3**). GST pull down of GTP-bound total Rac1 showed that the ΔNp63α knockdown significantly increased the level of GTP-Rac1 (**Figure 13, lane 4**). pRac1 S71 was also detected in the immuneprecipitated material and it was also significantly increased with the knockdown of ΔNp63α. The activity of Rac1 can also be assessed by measuring the

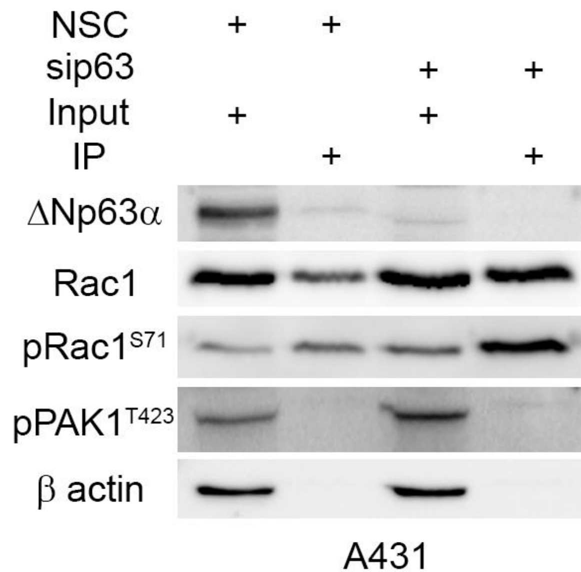



**Figure 13: ΔNp63α negatively regulates GTP-binding activity of Rac1.** A431 cells were transfected with non silencing control siRNA (NSC) or siRNA specific to p63. Whole cell lysates were subjected to pull down using PAK-PBD beads which specifically bound only GTP-bound active Rac1. Immunoblot analysis was performed with the indicated antibodies. The amount of Rac1 visualized by immunoblot represents the amount of GTP-bound Rac1. Immunoblot for the downstream effector pPAK1 was included as an additional readout of Rac1 activity.

Phosphorylation of its target effector protein PAK1. We probed for pPAK1 at Threonine 423. Knockdown of ΔNp63α significantly increased the phosphorylation of PAK1 at T423 suggesting that increased active Rac1 levels in sip63 cells led to increased pPAK1 levels. Our results showed that the knockdown of ΔNp63α increased GTP-Rac1 levels as observed early (**Figures 9**). Moreover, pRac1 S71 was also detectable in the activation assay indicating that pRac1 S71 does not affect the binding of Rac1 to its effector PAK1.

### G. Overexpression of ΔNp63α decreases Rac1 localization to the plasma membrane and induces its nuclear localization

Rac1 subcellular localization plays critical role in its function. Rac1 is associated with the plasma membrane when it is GTP-bound and localized to the cytoplasm with RhoGDI in its inactive state, GDP bound. However, the effect of Rac1 phosphorylation on its localization is not well investigated. Having demonstrated that ΔNp63α decreases pRac1 S71 and GTP-Rac1, we wanted to study the effect of ΔNp63α on Rac1 localization. We overexpressed GFP-Rac1 WT, GFP-Rac1 S71A, GFP-Rac1 T17N and GFP-Rac1 Q61L (**Table 4**) in H1299 cells by transient transfection. GFP-Rac1 WT or mutants were transfected alone or in conjunction with ΔNp63α and the localization of was assessed by immunofluorescence microscopy through measuring the intrinsic fluorescence of GFP without the need for an anti-Rac1 antibody. We first overexpressed ΔNp63α alone as positive control for p63 overexpression. ΔNp63α was robustly overexpressed in the nucleus (**Figure 14A**). Rac1 WT was mainly localized to the plasma membrane when it was transfected alone (**Figure 14B and Figure 15**) (representative images shown). ΔNp63α overexpression decreases Rac1 WT localization to plasma membrane and targets

it to the nucleus (**Figure 14B and Figure 15**). Overexpressing Rac1 S71A mutant (**Table 4**) decreased the localization of Rac1 to plasma membrane (48/100) in comparison to the wild-type Rac1 (80/100), Moreover, localization of Rac1 S71A mutant to nucleus was higher than observed with Wild type Rac1 (16/100 for Rac1S71A and 5/100 for Rac1 WT) when expressed alone (**Figure 14C and Figure 15**). Consistent with wild type Rac1, co-overexpression of ΔNp63α with Rac1 S71A also decreased Rac1 localization to the plasma membrane and induced its nuclear localization (**Figure 14C and Figure 15**). Dominant negative Rac1 T17N and the constitutively active Rac1 Q61L (**Table 4**) were used as negative and positive controls for Rac1 activity, respectively. Rac1 T17N was primarily localized either to plasma membrane (33/100) or nucleus (22/100) or in some cases distributed over cells (**Figure 14E and Figure 15**). Co-expression of ΔNp63α led to a decrease in Rac1T17N localization to plasma membrane (19/100) and a modest increase its localization to nucleus (43/100) (**Figure 14D and Figure 15**). Interestingly, constitutively active Rac1 Q61L mutant showed increased localization to both the plasma membrane and nucleus and in many cases was localized to both plasma membrane and nucleus simultaneously (plasma membrane= 43/100, nucleus= 3/100 to and plasma membrane and nucleus= 46/100). Furthermore, co-overexpression of ΔNp63α did not affect Rac1 Q61L localization (**Figure 14E and Figure 15**). These results suggest that ΔNp63α inhibits the activity of Rac1 as it decreases its localization to the plasma membrane and targets it to nucleus.

**Table 4: Rac1 mutants used to study the subcellular localization of Rac1.**

| Rac1 mutants | | | |
|---|---|---|---|
| **Position** | **Wild Type** | **Mutant** | **Description** |
| **71** | **S** | **A** | Phospho-deficient at S71 |
| **17** | **T** | **N** | Constitutively active (CA) (GTP-bound) |
| **61** | **Q** | **L** | Dominant negative (DN) (GDP-bound) |

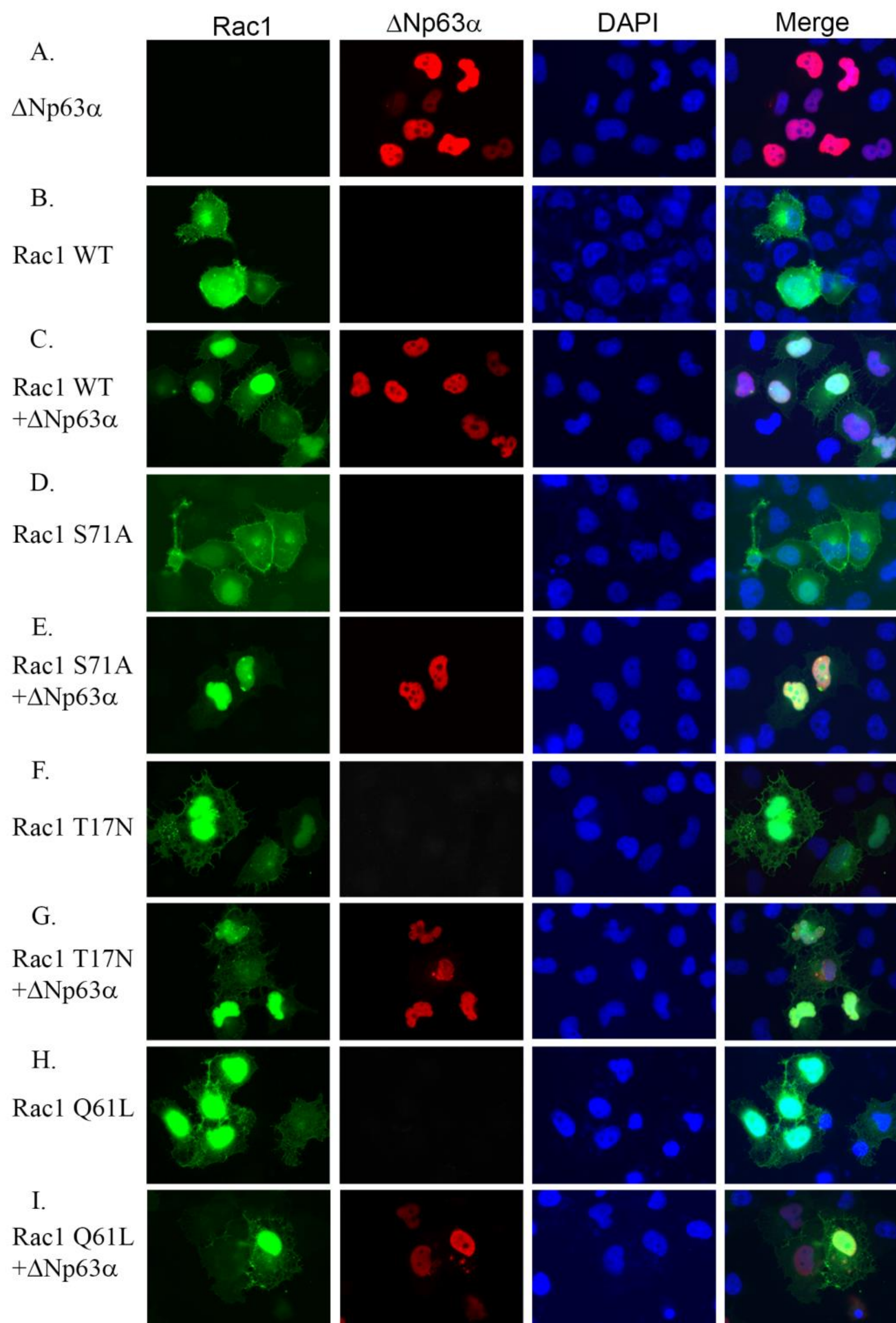

Rac1
ΔNp63α
DAPI
Merge
A.
ΔNp63α
B.
Rac1 WT
C.
Rac1 WT
+ΔNp63α
D.
Rac1 S71A
E.
Rac1 S71A
+ΔNp63α
F.
Rac1 T17N
G.
Rac1 T17N
+ΔNp63α
H.
Rac1 Q61L
I.
Rac1 Q61L
+ΔNp63α

**Figure 14: Overexpression of ΔNp63α decreases Rac1 localization to the plasma membrane and induces its nuclear localization.** H1299 cells were transfected with ΔNp63α alone, Rac1 WT alone, Rac1 S71A alone, Rac1 T17N alone, Rac1 Q61L alone or along with ΔNp63α as indicated in the panels above. The localization of Rac1 WT or mutants was examined by fluorescence microscopy following immunostaining for

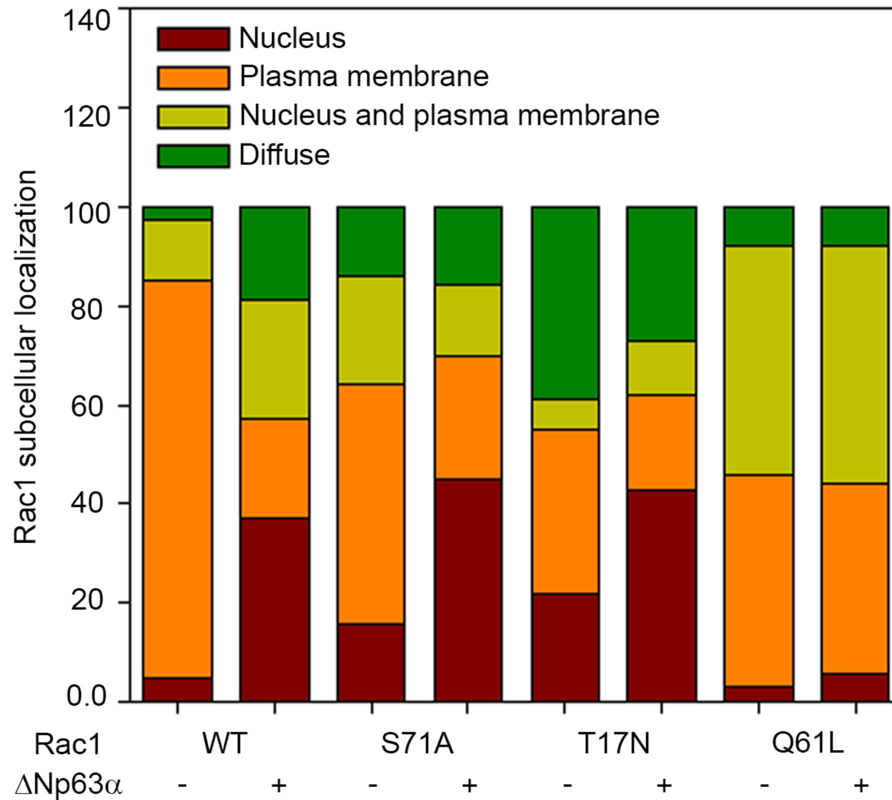



**Figure 15: Overexpression of ΔNp63α decreases Rac1 localization to the plasma membrane and induces its nuclear localization.** Quantification of the immunofluorescence analysis for Figure 14. Rac1 subcellular localization was quantified by imaging at least 100 cells per condition with the same exposure parameters. The localization of Rac1 in plasma membrane, cytoplasm or nucleus was determined manually by evaluating each single cell. Cells were grouped into four categories based on Rac1 localization: (Red) cells that have Rac1 mainly localized to plasma membrane, (Orange) cells that have Rac1 mainly localized to nucleus, (Yellow) cells that have Rac1 localized to both nucleus and cell membrane, and (Green) cells that have Rac1 distributed over cells without main localization to either plasma membrane or nucleus.

### H. miR320a mimic rescues the effect of ΔNp63α silencing on invasion

ΔNp63α is known to inhibit cell invasion by negatively regulating genes involved in EMT (Bergholz et al., 2014; Danilov et al., 2011; Finlan & Hupp, 2007; Kommagani et al., 2009). miR-320a was also shown to inhibit cell invasion through targeting Rac1 (Zhao et al., 2014). In our results, we confirmed that miR-320a is positively regulated by ΔNp63α. In addition, ΔNp63α negatively regulates Rac1 activity and this regulation is mediated by miR-320a. Thus, we hypothesized that ΔNp63α may inhibit invasion through targeting Rac1 activity via miR-320a. To this end, we transfected HaCaT cells with control mimic or miR-320a mimic along in presence or absence of ΔNp63α silencing and assessed cell invasion using a transwell invasion assay. Knockdown of ΔNp63α dramatically increased the number of invading cells (**Figure 15**), consistent with the expected role of p63 and our hypothesis. Overexpression of the miR-320a mimic in sip63 cells significantly decreased the number of invading cells although ΔNp63α was knocked down (**Figure 15**). This experiment was repeated in A431 cells, a more invasive cell line, and same results were obtained (**Figure 16**). We concluded that the increase in invasion observed upon ΔNp63α knockdown is reversed by miR-320a mimic, potentially through targeting Rac1 activity.

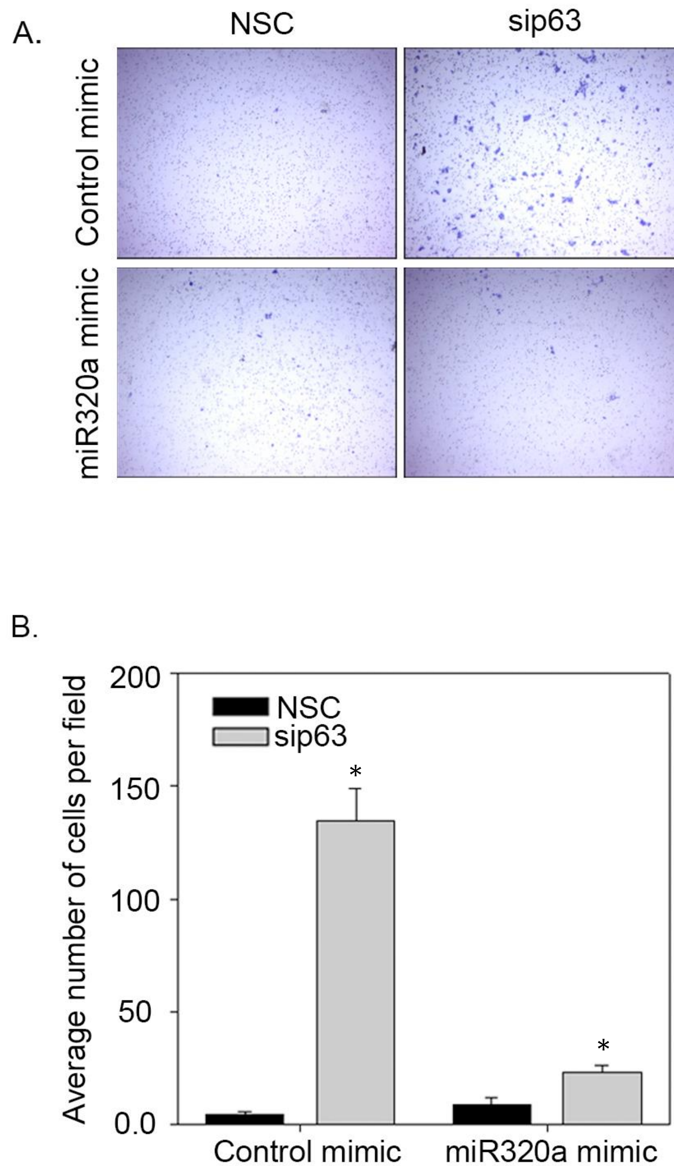



**Figure 16: Overexpression of a miR-320a mimic counters the effect of ΔNp63α knockdown on invasion in HaCaT cells.** HaCaT cells were transfected with either non-silencing control (NSC) or sip63 in conjunction with a negative control mimic or miR-320a mimic for two rounds of transfections. 24 hours after the second of transfection, $8.0 \times 10^4$ cells were subjected to Matrigel-based invasion assay (A) and the number of invading cells was quantitated after 21 hours (B). The y-axis represents the number of cells invaded.

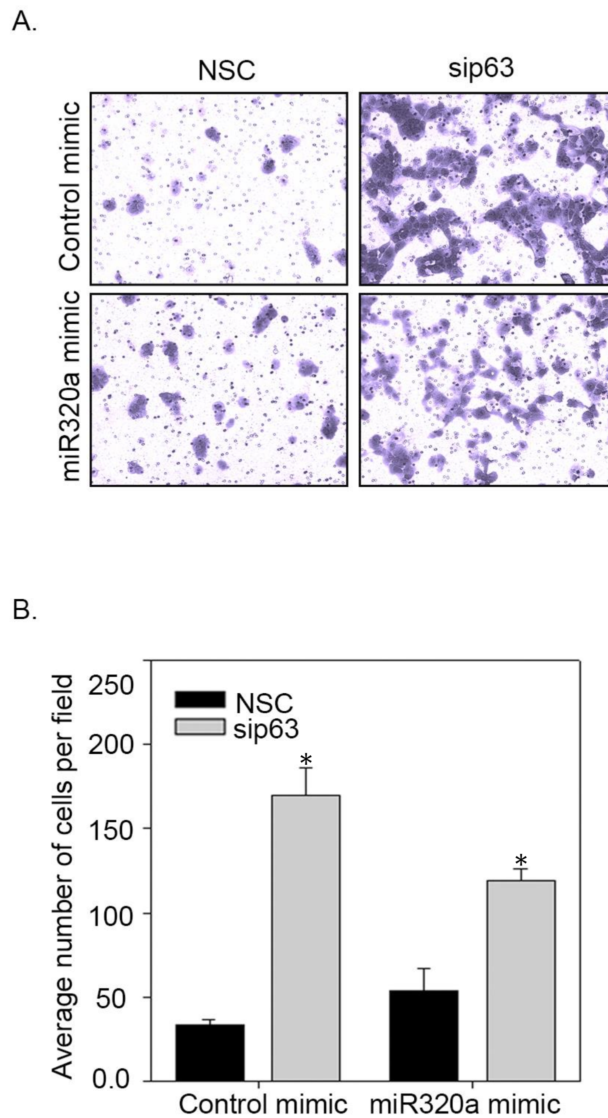



**Figure 17: Overexpression of a miR-320a mimic counters the effect of ΔNp63α knockdown on invasion in A431 cells.** A431 cells were transfected with either non-silencing control (NSC) or sip63 in conjunction with a negative control mimic or miR-320a mimic for two rounds of transfections. 24 hours after the second of transfection, $8.0 \times 10^4$ cells were subjected to Matrigel-based invasion assay (A) and the number of invading cells was quantitated after 21 hours (B). The y-axis represents the number of cells invaded.

# IV. Discussion

Cancer invasion has remained a focus of research for many years. Invasion is the main phenomena of tumor progression. EMT, a process in which epithelial cells lose their characteristics and gain a mesenchymal-like phenotype, is a fundamental process in embryonic development and during wound healing. In a carcinogenic context, EMT is upregulated which allows cancer cells to lose their cell-cell adhesion and gain migratory and invasive properties. ΔNp63α is the most abundantly expressed isoform of p63 and considered the master regulator of epithelial differentiation (Mills et al., 1999; Yang et al., 1998). It was previously shown that ΔNp63α suppresses cell invasion through downregulating genes involved in EMT, however, the detailed molecular regulation of ΔNp63α in EMT is not fully understood (Chu et al., 2013; Tran et al., 2013; Tucci et al., 2012). ΔNp63α has been shown to regulate several miRNAs many of them play a role in EMT. Thus, small RNA sequencing study was performed previously in our laboratory to investigate the regulation of global miRNAs by ΔNp63α.

miR-320a, a tumor suppressive miRNA that is downregulated in many metastatic cancers, was shown in our RNA-Sequencing data set to be positively regulated by ΔNp63α (L. Sun et al., 2015; J. Yu et al., 2016; Zhang et al., 2012; Zhao et al., 2014). We confirmed the regulation of miR-320a by ΔNp63α through performing RT-PCR validating the small RNA sequencing results. Here, we examined the effect of ΔNp63α loss or gain on one of miR-320a targets, Rac1, shown to play a critical role in cell motility. We found a novel mechanism by which ΔNp63α can regulate the activity of Rac1. ΔNp63α suppresses Rac1 phosphorylation through upregulating miR-320a levels. We also showed that the negative

regulation of pRac1 S71 by ΔNp63α correlates with Rac-1-GTP-levels potentially through miR-320a. This is the first study which demonstrates a mechanistic link between p63 and Rho small GTPase family.

Rac1 belongs to the Rho family and plays a fundamental role in cellular proliferation, adhesion, migration and gene transcription (Hartman & Spudich, 2012). The role of Rac1 in the acquisition of invasive and metastatic phenotypes and thus cancer progression has been well known. In the present study, we first sought to examine the direct regulation of Rac1 by miR-320a since it was previously shown that miR-320a directly binds to the 3'-UTR of Rac1 and reduces its expression (Zhao et al., 2014). Although we saw a modest increase in Rac1 transcript levels upon ΔNp63α knockdown, we did not observe any change in total Rac1 protein levels. These results were further confirmed by ΔNp63α overexpression in two cell lines, SW480 and H1299 which are null for ΔNp63α and showed no change in Rac1 transcripts or protein levels. In line with our results, the study by Zhao et al (2014) also showed no change in Rac1 transcripts with the modulation of miR-320a levels. Although they did see a change in total protein levels of Rac1 when miR-320a was overexpressed, we argue here this change could be in phosphorylated Rac1 but not total Rac1 protein levels since the antibody they used detects both total Rac1 as well as pRac1 (**as shown in Figure 8**).

Rho small GTPases share high homology thus it is likely that regulation of one member of Rho family is also observed with other Rho family members. Protein phosphorylation is thought to negatively affect RhoA and CDC42 activities, however, this is not totally true in the context of Rac1 activity (Forget et al., 2002). In this study we focus

on studying the only available phospho Rac1-S71 antibody which detects the phosphorylated Rac1. We found that Rac1 phosphorylation is greatly inhibited by ΔNp63α. This pattern of regulation was observed in every experiment we have done in all cell lines except in H1299 cells where we do not see phosphorylation of Rac1. This suggests that the effector molecular that modulates Rac1 phosphorylation and is a target for miR-320a is missing in H1299 cells.

One essential tool for investigating Rac1 phosphorylation is using a phosphospecific antibody. Until recently, there was no antibody available that could detect the phosphorylation of Rac1 at S71. Thus, previous studies that investigated the phosphorylation of Rac1 used antibodies that could not distinguish between pRac1 and pCdc42 S71 and were therefore limited its use in cells lacking Rac1 gene (Kwon et al., 2000; Schoentaube et al., 2009; Schwarz et al., 2012). In the present study we used two Rac1 antibodies that can recognize the phosphorylated form of Rac1. We confirmed the specificity of the anti-phospho-Rac1 (S71) antibody by knockdown of Rac1 and ΔNp63α and monitor the change in total and pRac1 levels.

Rac1 phosphorylation is poorly understood and there are contradicting reports regarding whether phosphorylation of Rac1 at S71 affects its activity. For example, one study showed that phosphorylation of Rac1 at S71 by Akt kinase decreases Rac-1 GTP levels and hence its activity (Kwon et al., 2000). In contrast, another study showed that pRac1 still binds to PAK-PBD in pull down assay indicative of active Rac1-GTP (Schoentaube et al., 2009). Our results in this study were was consistent with the latter study wherein we found that ΔNp63α knockdown led to a dramatic increase in both pRac1

S71 Rac1 GTP levels. Moreover, our observations confirmed that pPAK1 levels were increased upon ΔNp63α knockdown, a downstream effector of active Rac1. A recent study showed that Rac1 phosphorylation leads to a decrease in Rac1 binding to specific effectors, such as PAK1, and a shift in specificity toward other effectors without affecting its activity (Schwarz et al., 2012). This could be interpreted as the reduction in the interaction between Rac1 and PAK1 due to Rac1 phosphorylation may lead to a decrease in the cell invasion since PAK1 is one of major mediators for Rac1 activity in cell migration and invasion. In contrast, the increase we see in pPAK1 upon knockdown of ΔNp63α is more likely through upregulating Rac1 activity which means the phosphorylation of Rac1 at S71 does not affect its binding to PAK1 in our experiments.

Based on our results it is likely that upregulation of miR-320a by ΔNp63α leads to inhibition of a kinase upstream of Rac that is a target for miR-320a and can phosphorylate Rac1 at S71. We exclude Akt-mediated phosphorylation in our study since our laboratory previously showed that ΔNp63α positively regulates Akt kinase (Leonard et al., 2011). In silico analysis led to the identification of a number of putative kinases associated with Rac1 activity and are likely to be targets of miR-320a. However none of those have been shown to phosphorylate Rac1. Further studies are therefore essential to determine which of the upstream effector(s) of Rac1 mediate its phosphorylation.

Targeting Rac1 to different subcellular compartments is an important mechanism to regulate Rac1 activity. Our study clearly demonstrated that ΔNp63α targets Rac1 to the nucleus. We showed that the overexpression of ΔNp63α decreases the localization of Rac1 to the plasma membrane and induces its nuclear localization. It was previously shown that

Rac1 accumulates in nucleus during G2 phase and induces cell division (Michaelson et al., 2008). Moreover, Rac1 is shown to affect the transcription machinery by directly binding to STAT3 which plays significant roles in upregulating cell proliferation (Simon et al., 2000). Thus, targeting Rac1 to nucleus by ΔNp63α may upregulate cell proliferation which is known oncogenic role of ΔNp63α. Moreover, the decrease in GTP-Rac1 levels in cells co-transfected with ΔNp63α could be as a result of Rac1 degradation in nucleus. It was previously shown that GTP-Rac1, but not GDP-Rac1, is subjected to proteasomal degradation in nucleus (Lanning et al., 2004). Moreover, we observed decreased plasma membrane localization of Rac1 S71A mutant when compared to WT Rac1. This mutation also did not increase Rac1 localization to nucleus. These results highlight the importance of Rac1S71 phosphorylation in both the stability of Rac1 GTP activity to the plasma membrane and the kinase that phosphorylates it is a plasma membrane kinase although this remains to be experimentally validated. It is likely that when Rac1 activated through binding to GTP is localized to the plasma membrane and interacts with a plasma membrane kinases that phosphorylates it back and stabilizes its binding to plasma membrane. We also found that the overexpression of ΔNp63α also targets Rac1 S71A to nucleus. The mechanism by which ΔNp63α affects Rac1 nuclear localization is unclear and could be a third mechanism by which ΔNp63α can regulate Rac1 activity.

The main goal of this study is to elucidate the mechanism by which ΔNp63α can inhibit cell invasion. We hypothesized that ΔNp63α inhibits cell invasion though targeting Rac1 activity via miR-320a. Our study demonstrated that increased invasion observed upon ΔNp63α knockdown can be reversed by miR-320a mimic Cell invasion was significantly

reduced after knockdown of ΔNp63α and overexpressing miR-320a. This notion is supported by previous data demonstrating that miR-320a greatly suppresses cell invasion through targeting Rac1.

# V. Conclusion

ΔNp63α and Rac1 play central roles in cancer through modulation of cell invasion. The work presented in this dissertation elucidates one arm by which ΔNp63α functions to inhibit cell invasion as shown in our model (**Figure 17**). We showed that ΔNp63α positively regulates miR-320a levels. Furthermore, we showed that ΔNp63α downregulates Rac1 activity leading to inhibition in cell invasion. ΔNp63α significantly inhibits Rac1 phosphorylation at S71 and this regulation occurs through miR-320a (**Figure 17**). Further experiments are needed to identify the miR-320a target that modulates Rac1 phosphorylation. GTP-Rac1 level is also inhibited by ΔNp63α (**Figure 17**). The mechanism by which ΔNp63α regulates GTP-Rac1 levels is not fully revealed. ΔNp63α may indirectly regulates GTP-Rac1 through either downregulating a GEF or upregulating a GAP that induces or inhibits Rac1 GTP activity, respectively. We further showed that the activity of PAK1 is negatively affected by ΔNp63α, emphasizing the tight and effective regulation of Rac1 by ΔNp63α. Moreover, we found that ΔNp63α reduces Rac1 localization to plasma membrane while promoting its nuclear localization. These findings are consistent with the negative regulation of GTP-Rac1 levels by ΔNp63α since GTP-Rac1 is bound to plasma membrane.

The interplay between ΔNp63α and Rac1 will not only allow us to understand the anti-invasive role of ΔNp63α but may also explain some of other known biological roles of ΔNp63α. In addition, Rho small GTPases works in signaling network to regulate cytoskeleton rearrangement and subsequently cell motility (Sadok & Marshall, 2014). The

regulation of one member may easily impact the regulation of other members. Thus, the presented study strongly links ΔNp63α to regulation of Rho small GTPases.

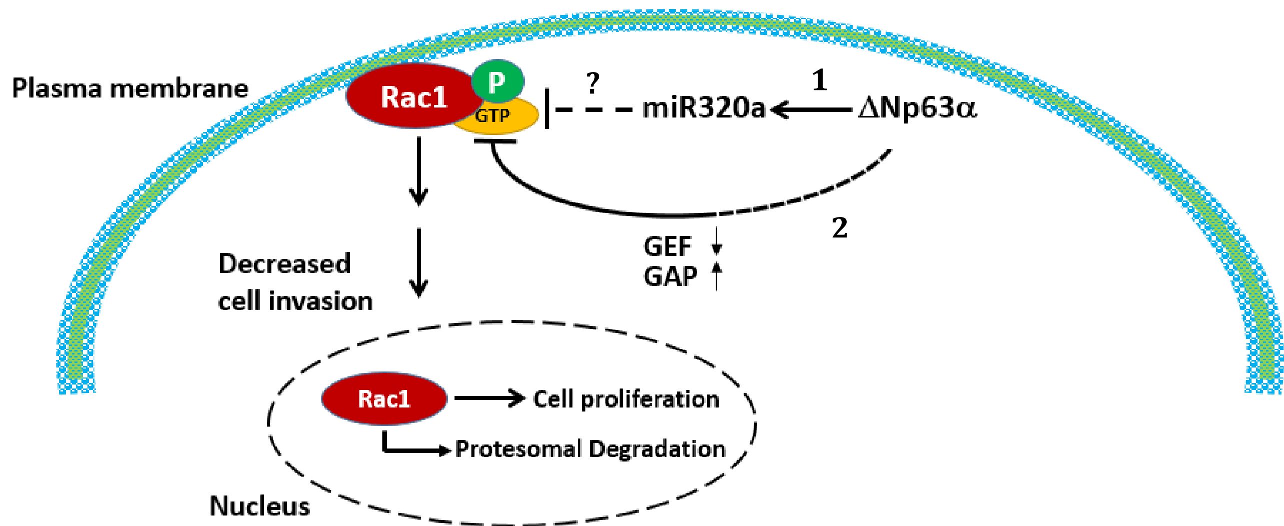



**Figure 18: ΔNp63α inhibits Rac1 activity through upregulating miR-320a.** ΔNp63α upregulates the levels of miR-320a which targets a kinase that phosphorylates Rac1 (1). GTP-Rac1 is also regulated by ΔNp63α through targeting a GEF or GAP that works upstream of Rac1 (2).

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