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Δ Np63 α suppresses cells invasion by downregulating PKC γ /Rac1 signaling through miR-320a

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Abstract

Δ Np63 α , a member of the p53 family of transcription factors, is overexpressed in a number of cancers and plays a role in proliferation, differentiation, migration, and invasion. Δ Np63 α has been shown to regulate several microRNAs that are involved in development and cancer. We identified miRNA miR-320a as a positively regulated target of Δ Np63 α . Previous studies have shown that miR-320a is downregulated in colorectal cancer and targets the small GTPase Rac1, leading to a reduction in noncanonical WNT signaling and EMT, thereby inhibiting tumor metastasis and invasion. We showed that miR-320a is a direct target of Δ Np63 α . Knockdown of Δ Np63 α in HaCaT and A431 cells downregulates miR-320a levels and leads to a corresponding elevation in PKC γ transcript and protein levels. Rac1 phosphorylation at Ser71 was increased in the absence of Δ Np63 α , whereas overexpression of Δ Np63 α reversed S71 phosphorylation of Rac1. Moreover, increased PKC γ levels, Rac1 phosphorylation and cell invasion observed upon knockdown of Δ Np63 α was reversed by either overexpressing miR-320a mimic or Rac1 silencing. Finally, silencing PKC γ or treatment with the PKC inhibitor Gö6976 reversed increased Rac1 phosphorylation and cell invasion observed upon silencing Δ Np63 α . Taken together, our data suggest that Δ Np63 α positively regulates miR-320a, thereby inhibiting PKC γ expression, Rac1 phosphorylation, and cancer invasion.

Introduction

Δ Np63 α is a homolog of the p53 tumor suppressor gene and the dominant p63 isoform expressed in the proliferative basal layer of epithelial tissues^{1–3}. Overexpression of Δ Np63 α is frequently observed in squamous cell carcinoma (SCC) and basal cell carcinoma (BCC) where it has been shown to inhibit apoptosis and differentiation while promoting cell proliferation, thereby characterizing Δ Np63 α as a proto-oncogene^{4,5}. Δ Np63 α

has also been shown to inhibit EMT by downregulating genes that promote cell motility and mesenchymal phenotypes such as ZEB1 and Snail, thus inhibiting cell invasion^{5–8}. Loss of Δ Np63 α has been linked to increased invasiveness in SCC⁹. The contribution of Δ Np63 α to cell invasion and metastasis appears to vary by cell type, as Δ Np63 α has also been shown to increase basal-like breast cancer cell migration and invasion¹⁰. An improved understanding of Δ Np63 α -mediated regulation of cell invasion will provide potential therapeutic inroads.

p63 exists as six different isoforms arising from alternative promoter usage and differential 3' splicing. Transcription initiation from promoter 1 (P1) yields the TAp63 isoforms with a full N-terminal activation domain, while initiation from promoter 2 (P2) yields the Δ Np63 isoforms that have a truncated N-terminal domain. Moreover, alternative 3' splicing of TAp63 and Δ Np63

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leads to α , β , and γ isoforms^{11,12}. While TAp63 α and Δ Np63 α generally have opposing functions in vivo, they both suppress tumor cell invasiveness^{13,14}. Δ Np63 α is of particular interest in skin cancer because it serves as a broad regulator of microRNA (miRNA) expression, including many that inhibit cell invasion^{5,8,15–17}. miRNAs are small noncoding 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, leading to translation inhibition or mRNA degradation^{18,19}. Of particular relevance, miR-320a was previously shown to suppress colorectal cancer progression by directly binding to the 3'-UTR of the Rac1 mRNA, leading to downregulation of Rac1 protein levels²⁰.

Rac1 belongs to the Rho family of small GTPases and plays fundamental roles in cellular proliferation, adhesion, invasion, migration, and gene transcription. Altered Rac1 expression and activity are frequently observed in human cancer^{21,22}. Rac1 cycles between its active form (GTP-bound) and inactive form (GDP-bound) by the action of guanine nucleotide exchange factors that promote GTP loading and GTPase activating proteins (GAPs) that accelerate GTP hydrolysis²¹. Plasma membrane-associated active Rac1 induces actin polymerization at the edge of the cell, leading to formation of lamellipodia and promoting cell motility²³. Importantly, Rac1 localization to the plasma membrane, binding to its effector proteins, and downstream signaling are also regulated via phosphorylation by a number of kinases^{24–27}, although the specific nature of these post-translational events remains poorly understood. Rac1 activity is also regulated by protein kinase C (PKC), a family of phospholipid-dependent Ser/Thr kinases widely implicated in the control of cell proliferation, invasion, migration, and anticancer drug resistance^{28–31}. In the last years, several studies have linked PKC to the activation of Rac1 and cancer cell motility^{30–33}.

In this study, we identified miR-320a as a direct target of Δ Np63 α . We demonstrated that Δ Np63 α positively regulates miR-320a which in turn targets PKC γ 3'UTR, and thereby suppresses cell invasion. We showed that Δ Np63 α downregulates PKC γ expression and Rac1 phosphorylation through miR-320a, thus suggesting a potentially novel mechanistic link between p63 and cancer invasiveness through the regulation of the Rac1 small GTPase.

Results

Δ Np63 α induces miR-320a expression

miR-320a functions as a tumor suppressor in glioma, breast and colorectal cancers by suppressing cell migration, invasion, and proliferation^{20,34–36}. To determine if Δ Np63 α regulates miR-320 levels, we either knocked down p63 in HaCaT cells and A431 cells, which predominantly express the Δ Np63 α isoform of p63¹⁴, or overexpressed Δ Np63 α in

p63 null SW480 and H1299 cells. Both p63 knockdown and overexpression were confirmed by Western blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Fig. 1a, c). We observed that p63 knockdown led to a decrease in miR-320a transcript levels (Fig. 1b) whereas overexpression of Δ Np63 α , led to an increase in the miR-320a levels (Fig. 1d).

A previous p63 ChIP-seq study (GSE59827), demonstrated strong p63 binding site 6 kb (chr8:22,239,143–22,239,162) downstream of miR-320a gene^{37,38}. To confirm that miR-320a is a direct target of Δ Np63 α , we cloned this region containing the p63 binding site into the pGL3-promoter luciferase vector (p63-BS-luc reporter). Next, we co-transfected H1299 cells with p63-BS-luc reporter along with increasing concentrations of the expression plasmid encoding Δ Np63 α . Co-transfection of Δ Np63 α led to a dose-dependent increase in the luciferase activity of p63-BS-luc reporter (Fig. 1e). Taken together, these results demonstrate that Δ Np63 α directly regulates miR-320a transcription levels.

Δ Np63 α negatively regulates phosphorylation of Rac1 at Ser71

miR-320a has been shown to suppress colorectal cancer progression by targeting Rac1²⁰. Since we observed that Δ Np63 α positively regulates miR-320a, next we wanted to determine whether Δ Np63 α negatively regulates Rac1-expression levels. Although Δ Np63 α knockdown in both HaCaT and A431 cells led to a modest increase in Rac1 transcript levels, the total Rac1 protein levels remained unchanged (Fig. 2a). Consistently, Δ Np63 α overexpression in Δ Np63 α -deficient H1299 and SW480 cells, also did not affect Rac1 transcript and protein levels (Fig. 2b). Together these data indicate that although Δ Np63 α positively regulates miR-320a, it does not inhibit Rac1 protein levels.

Notably, despite the lack of changes in total Rac1 upon modulation of Δ Np63 α expression, Western blot analysis revealed elevated immunoreactivity using a phospho-Ser71 antibody, suggesting that the Rac1 phosphorylation status could be regulated by Δ Np63 α expression. Immunoblot analysis indicated that the knockdown of Δ Np63 α in A431 and HaCaT cells led to an increase in pRac1 (S71) levels (Fig. 2c). Conversely, ectopic expression of Δ Np63 α in Caco2 and SW480 cells resulted in a decrease in pRac1 levels, with no change in total Rac1 expression (Fig. 2d). Taken together, these results demonstrate that Δ Np63 α negatively regulates Rac1 phosphorylation without affecting total Rac1 levels.

Increased invasion observed with loss of Δ Np63 α is Rac1-dependent

We next examined whether Δ Np63 α regulates cell invasion in a Rac1-dependent manner. The effects of

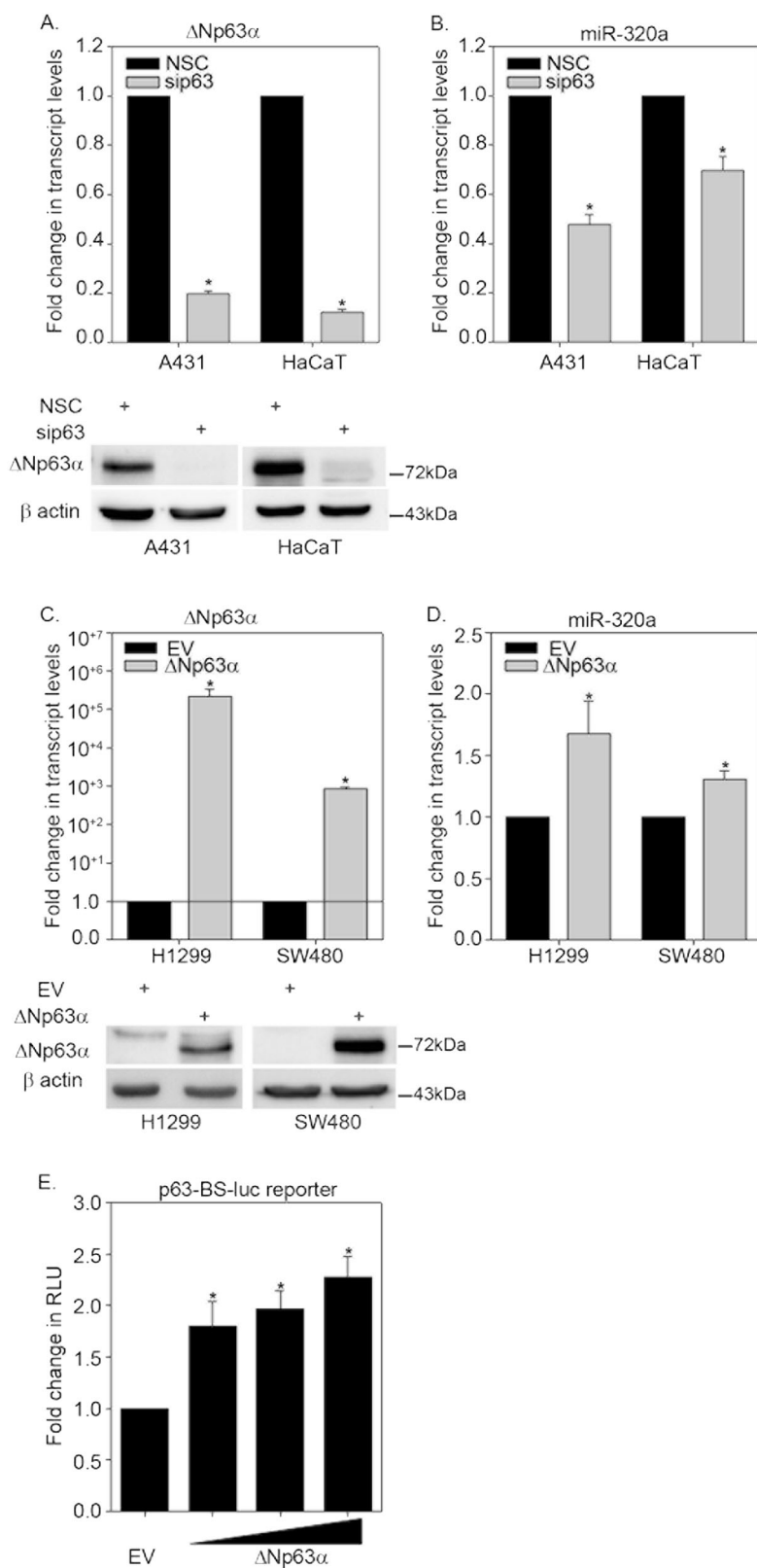


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Fig. 1 Δ Np63 α positively regulates miR-320a. **a** A431 and HaCaT cells were transfected with non-silencing control siRNA (NSC) or siRNA specific to p63. Total RNA was extracted and Δ Np63 α transcript level was measured by TaqMan based qRT-PCR. y-Axis represents the fold change in p63 transcript levels relative to NSC-transfected cells. Immunoblots of p63 in A431 and HaCaT cells transfected are shown in the bottom panels. **b** TaqMan based qRT-PCR was used to quantify miR-320a levels from the experiment described in (a). **c** H1299 and SW480 cells null for p63 were transfected with empty vector (EV) control or expression plasmid encoding Δ Np63 α . Transcripts were quantified by qRT-PCR (upper panel) while protein levels were confirmed using immunoblot analyses (lower panel). **d** Taqman based qRT-PCR was used to quantify miR-320a levels from the experiment described in (c). Immunoblot with β -actin was performed to confirm equivalent protein loading. Error bars represent standard deviation. Significant changes ($P \leq 0.05$) relative to controls are indicated with an asterisk. **e** H1299 cells were co-transfected with p63-BS-Luc reporter construct along with either empty vector or increasing concentrations of Δ Np63 α as indicated. Cells were subjected to dual luciferase assay at 24 h after transfection. The y-axis represents fold change in relative luciferase units (RLU) compared with cells transfected with empty vector. RLU values are shown as means \pm S.E.M. from $n = 3$ experiments

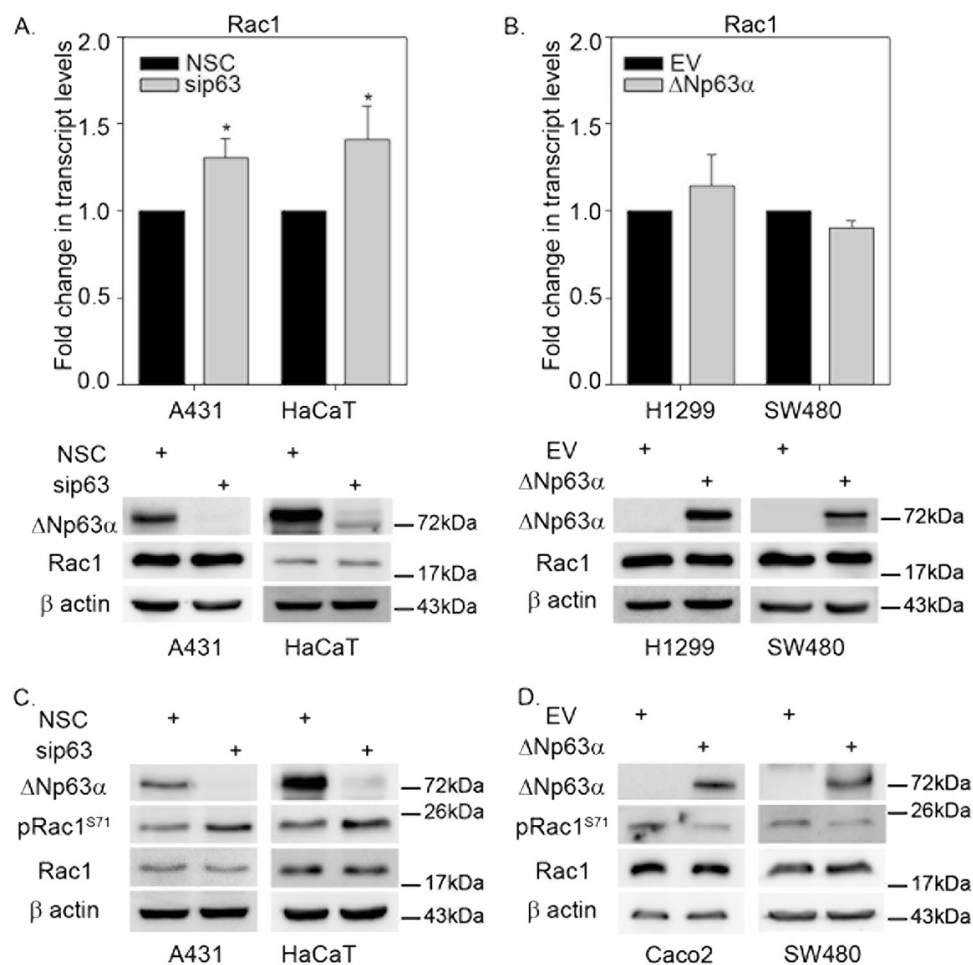
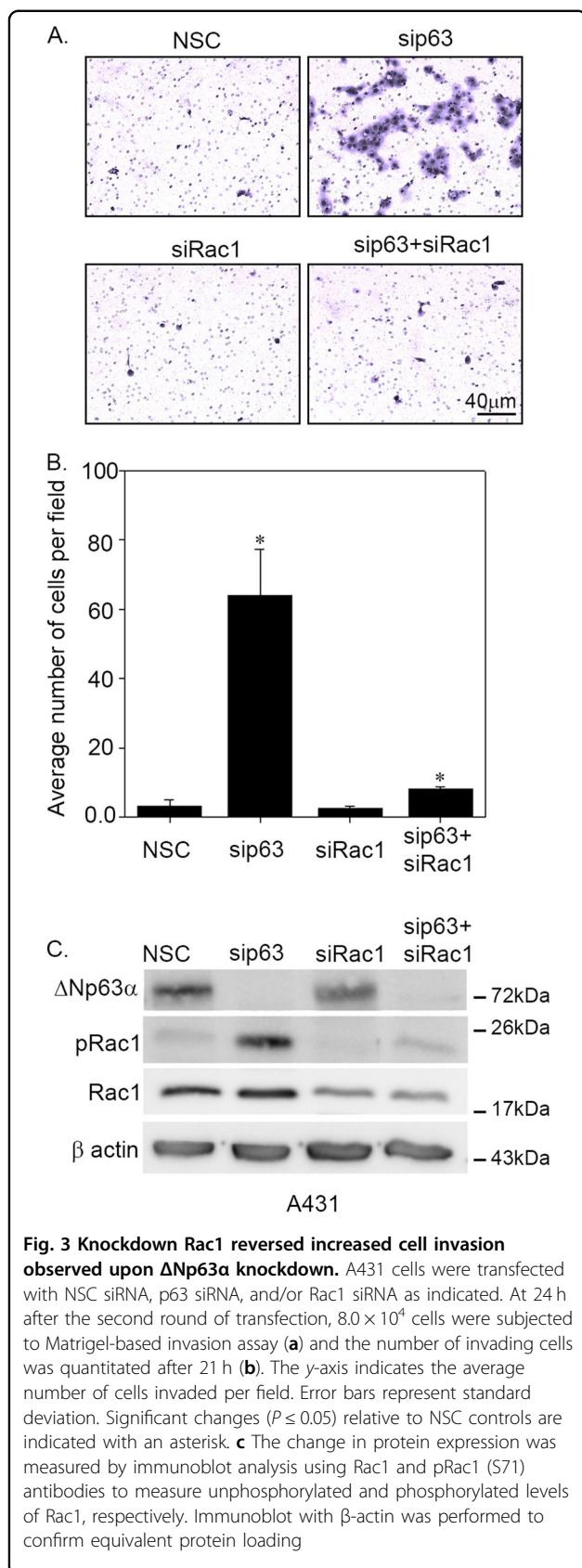


Fig. 2 Δ Np63 α negatively regulates Rac1 phosphorylation. **a** A431 and HaCaT cells were transfected with non-silencing control siRNA (NSC) or siRNA targeting p63. **b** H1299 and SW480 cells were transfected with empty vector (EV) control or expression plasmid encoding Δ Np63 α . The change in transcript and protein levels of Rac1 were measured by TaqMan based qRT-PCR (* indicates $P \leq 0.05$) and immunoblot analysis, respectively. Error bars represent standard deviation. **c** A431 and HaCaT cells were transfected with nonsilencing control siRNA (NSC) or siRNA against p63. **d** Caco2 and SW480 cells were transfected with empty vector control or Δ Np63 α and subjected to immunoblot analysis for the indicated proteins. The change in Rac1 protein expression was measured by immunoblot analysis using Rac1 and pRac1 (S71) antibodies as indicated. Immunoblot with β -actin was performed to confirm equivalent protein loading

Δ Np63 α , Rac1, or combined Δ Np63 α / Rac1 silencing on cell invasion in A431 cells were assessed using a Matrigel-based transwell assay. As previously reported¹⁴, Δ Np63 α knockdown significantly increased the number of

invading cells (Fig. 3a). Rac1 knockdown alone decreased cell invasion when compared to control cells transfected with nonsilencing control (NSC). Knockdown of both Δ Np63 α and Rac1 reversed the increased cell invasion



observed upon ΔNp63α knockdown alone (Fig. 3a, b). ΔNp63α and Rac1 knockdown was confirmed by immunoblot analysis in this cell line (Fig. 3c). As expected, silencing Rac1 significantly decreased pRac1 levels observed with ΔNp63α siRNA depletion. Thus, these results strongly suggest that ΔNp63α inhibits the cell invasion via Rac1 phosphorylation.

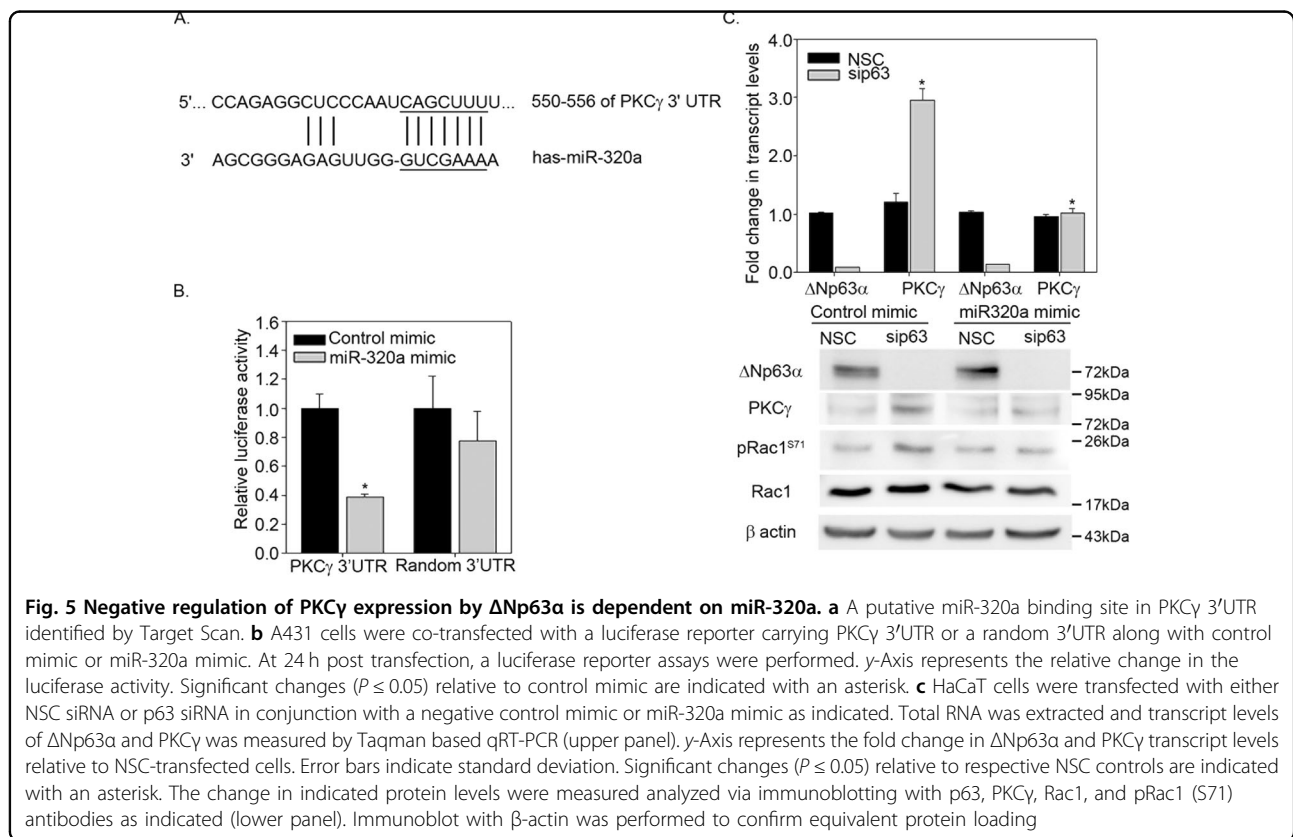
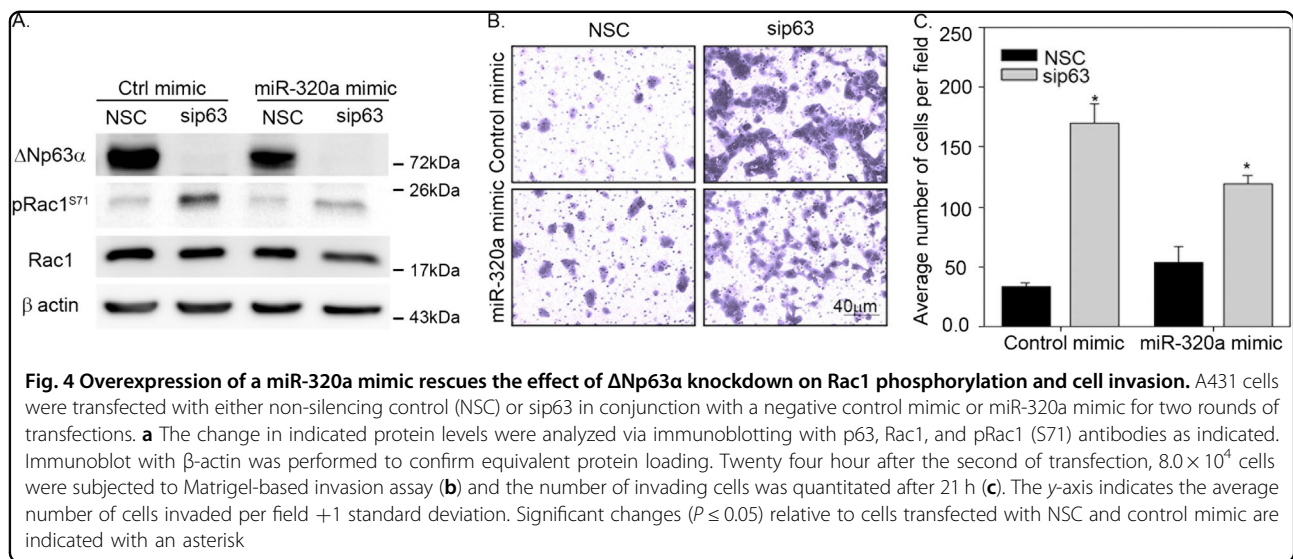
miR-320a counteracts the effect of ΔNp63α silencing on Rac1 phosphorylation and cell invasion

We next examined whether the negative regulation of Rac1 phosphorylation by ΔNp63α is dependent on miR-320a. To test this, we transfected A431 cells with siRNA against p63 or NSC in combination with miR-320a mimic or its negative mimic control. ΔNp63α knockdown led to an increase in pRac1 levels, as expected. Co-expression of miR-320a mimic reversed the increased pRac1 levels observed upon ΔNp63α knockdown to basal levels (Fig. 4a). Similar results were observed in HaCaT cells (Supplementary Fig. 1A). These results clearly demonstrate that miR-320a mimic counteracts the effect of ΔNp63α knockdown on pRac1 levels.

To further determine whether miR-320a mimic can also rescue the increased cell invasion observed upon p63 knockdown, A431 cells were transfected with control mimic or miR-320a mimic, either in presence or absence of p63 siRNA, and assessed for cell invasion. Overexpression of the miR-320a mimic in NSC control cells had only a minimal effect on cell invasion, consistent with baseline ΔNp63α-induced miR-320a levels in A431 cells. As expected, knockdown of ΔNp63α significantly increased the number of invading cells (Fig. 4b, c). Overexpression of the miR-320a mimic in cells transfected with siRNA to p63 showed a dramatic decrease in the number of invading cells when compared to cells transfected with siRNA to p63 alone (Fig. 4b, c). Similar results were observed in HaCaT cells (Supplementary Fig. 1B). Taken together, these results clearly indicate that miR-320a mimic reversed increased cell invasion observed upon ΔNp63α knockdown by targeting Rac1 phosphorylation.

PKCγ expression levels are negatively regulated by ΔNp63α and miR-320a

Next, we wished to elucidate the mechanism by which the ΔNp63α/miR-320a axis negatively regulates Rac1 phosphorylation. Therefore, we examined whether miR-320a negatively regulates pRac-1 levels by targeting a kinase. Target analysis using miRDB (www.mirdb.org) and TargetScan (www.targetscan.org)^{39,40} both identified PKCγ as a predicted target of miR-320a (Fig. 5a). To validate that PKCγ is a target for miR-320a, we co-transfected A431 cells with a luciferase reporter construct



that contains the PKC γ 3'UTR or a random control 3' UTR with either miR-320a mimic or a negative control mimic. We observed that miR-320a mimic led to a significant reduction in the PKC γ 3'UTR luciferase activity when compared to the co-transfection with the negative control mimic (Fig. 5b). No significant change in the luciferase activity was observed when the random

3'UTR luciferase reporter was co-transfected with miR-320a mimic. This confirms that PKC γ is a target for miR-320a.

Next, we wanted to determine if Δ Np63 α /miR-320a axis regulates PKC γ expression, HaCaT cells were transfected with either NSC or Δ Np63 α siRNA, along with miR-320a mimic or negative mimic control (Fig. 5c). We observed

that Δ Np63 α knockdown led to a significant upregulation in PKC γ transcript and protein levels, with a concomitant increase in pRac1 levels (Fig. 5c). Overexpression of miR-320a mimic reduced PKC γ transcript and protein to basal levels, thereby reversing the effects of p63 silencing (Fig. 5c). Taken together, these results demonstrate that Δ Np63 α negatively regulates PKC γ expression through upregulation of miR-320a.

Silencing PKC γ reverses the increased pRac1 levels and cell invasion observed upon loss of Δ Np63 α

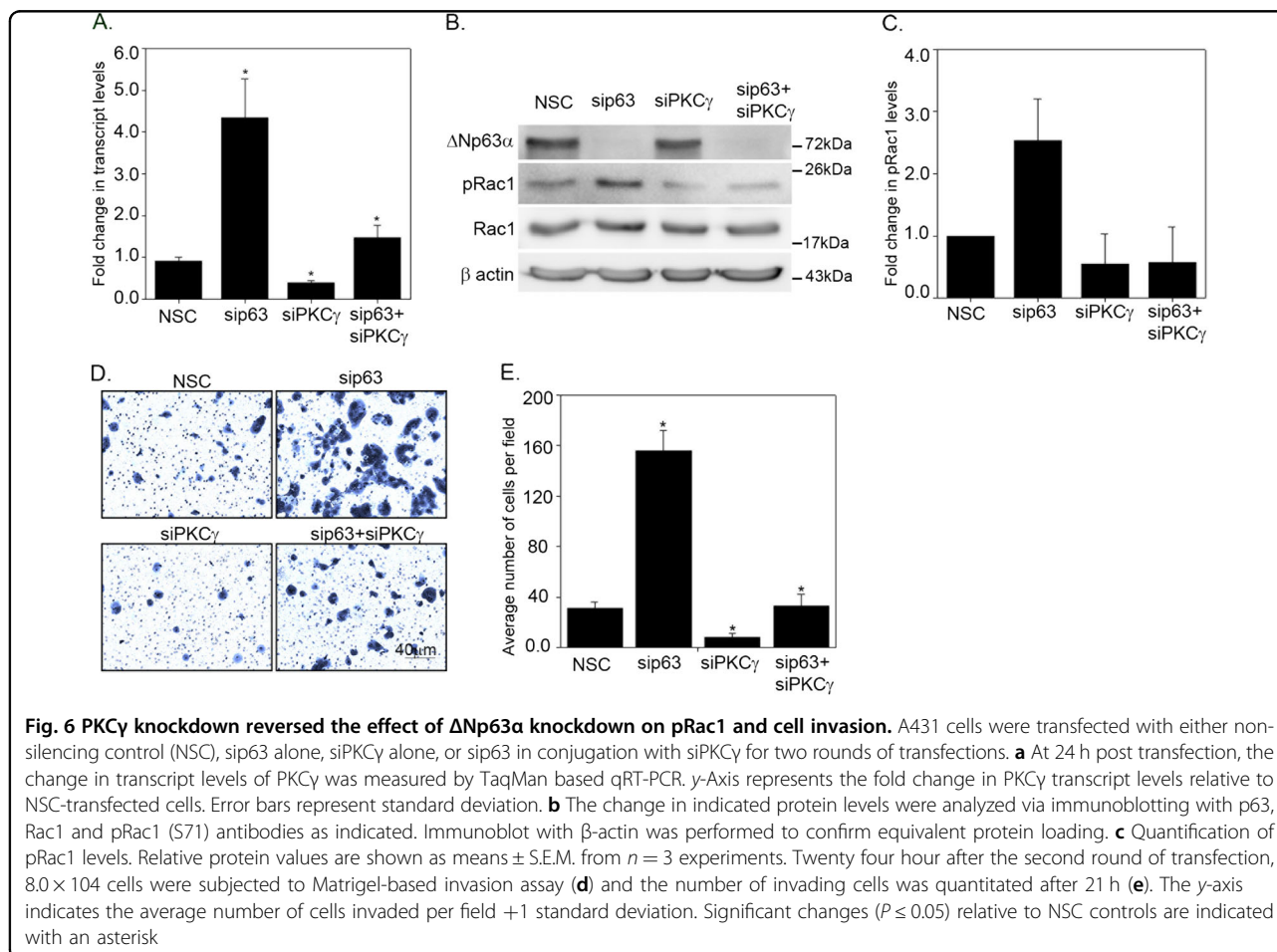
We next examined whether Δ Np63 α -mediated inhibition of Rac1 phosphorylation and cell invasion is mediated by miR-320a-PKC γ signaling. To this end, we investigated the effects of silencing p63 and/or PKC γ on pRac1 levels and cell invasion. p63 knockdown alone led to a significant increase in PKC γ transcript levels (Fig. 6a), whereas knockdown of PKC γ alone led to a further reduction in PKC γ transcript levels when compared to cells transfected with nonsilencing control. Knockdown of both p63 and PKC γ led to a reduction in the increased PKC γ transcript levels observed upon knockdown of p63

alone (Fig. 6a). Interestingly, knockdown of both p63 and PKC γ reversed the increased pRac1 levels observed upon p63 knockdown (Fig. 6b, c).

Next, we examined the effects of p63 and/or PKC γ silencing on cell invasion. As expected, p63 knockdown alone led to increased cell invasion (consistent with Fig. 4b). Knockdown of both p63 and PKC γ led to a reduction in cell invasion comparable to control cells transfected with NSC (Fig. 6d, e). Taken together, these results suggest that the inhibition in Rac1 phosphorylation and cell invasion by Δ Np63 α and miR-320a is through down-regulating PKC γ .

PKC inhibitor mimics the effect of Δ Np63 α on Rac1 phosphorylation and invasion

To further confirm that the increase in pRac1 levels upon p63 knockdown is due to PKC γ upregulation, we first tested the effect of Gö6976, an inhibitor of conventional PKC α , β and γ isoforms, on Rac1 phosphorylation in cells transfected with nonsilencing control or siRNA to p63. Consistent with PKC γ knockdown experiments (Fig. 6), we observed that the increase in pRac1 levels observed



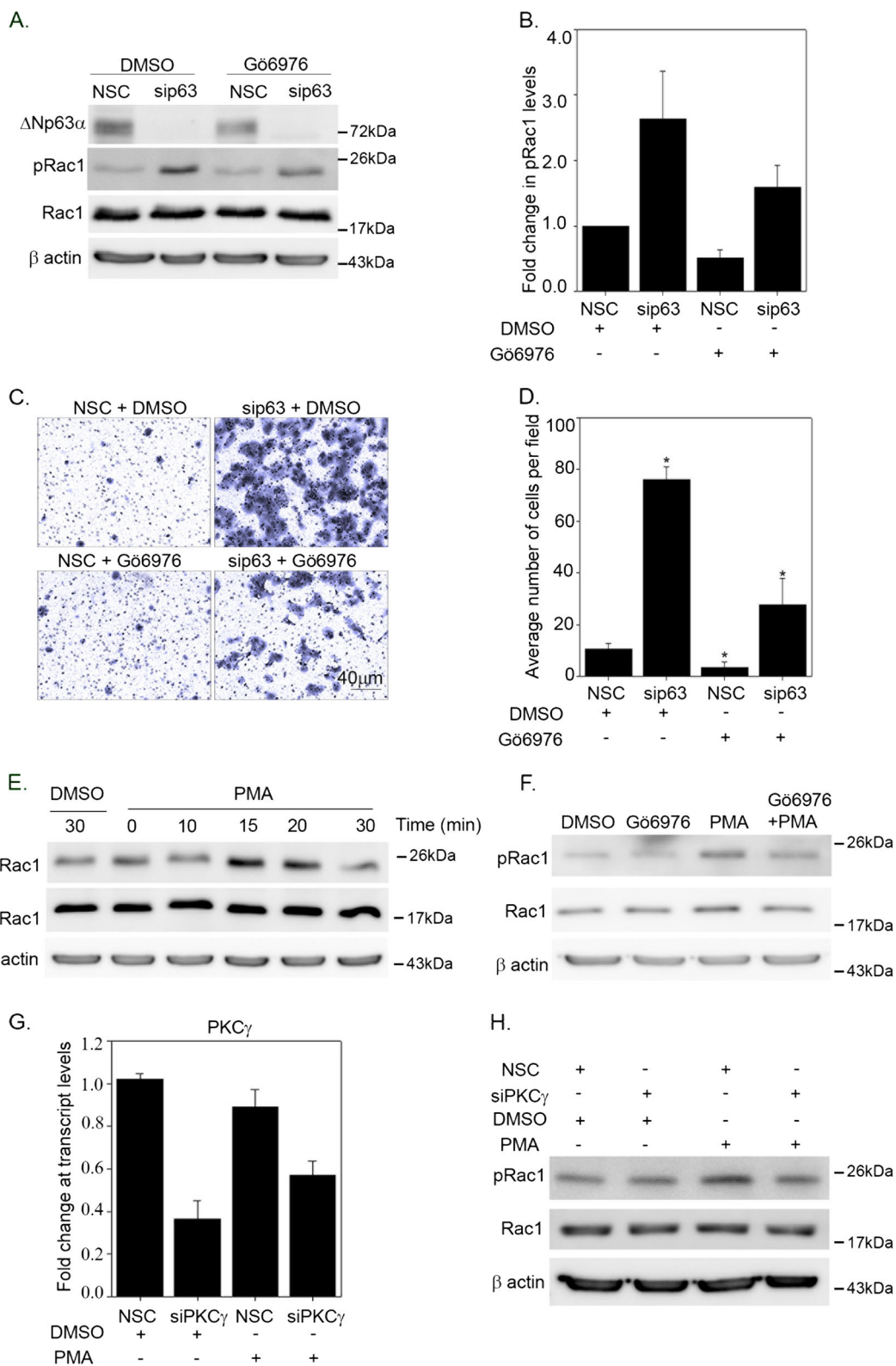


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Fig. 7 Δ Np63 α inhibits Rac1 phosphorylation and invasion by reducing PKC γ levels. A431 cells were transfected with either NSC siRNA or siRNA targeting p63 for two rounds of transfections followed by treatment with DMSO or Gö6976 for 2 h as indicated. **a** The change in indicated protein levels were analyzed via immunoblotting with p63, Rac1, and pRac1 (S71) antibodies as indicated. Immunoblot with β -actin was performed to confirm equivalent protein loading. **b** The fold change in pRac1 levels relative to NSC DMSO-treated cells levels. Relative protein values are shown as means \pm S.E.M. from $n = 3$ experiments. **c** At 24 h after the second round of transfection, 8.0×10^4 cells were subjected to Matrigel-based invasion assay (**c**) and the number of invading cells was quantitated after 21 h. **d** The y-axis indicates the average number of cells invaded per field ± 1 standard deviation. Significant changes ($P \leq 0.05$) relative to NSC controls are indicated with an asterisk. **e** A431 cells were incubated with DMSO or 100 nM of PMA for the indicated times. The change in indicated protein levels were analyzed via immunoblotting with Rac1 and pRac1 (S71) antibodies as indicated. **f** A431 cells were treated with DMSO or Gö6976 for 2 h and followed by incubation with 100 nM of PMA for 15 min. The change in indicated protein levels were analyzed via immunoblotting as indicated. **g** A431 cells were transfected with nonsilencing control siRNA (NSC) or siRNA specific to PKC γ followed by treatment with DMSO or 100 nM PMA for 15 min. Total RNA was extracted and transcript levels of PKC γ was analyzed by qRT-PCR. y-Axis represents the fold change in PKC γ transcript levels relative to NSC-transfected cells. The change in Rac1 and pRac1 levels was measured by immunoblot analysis (**h**). Immunoblot with β -actin was performed to confirm equivalent protein loading

upon p63 knockdown was reversed by treatment with Gö6976 (Fig. 7a, b). Next, we examined the effect of PKC γ inhibition on cell invasion in cells transfected with NSC or siRNA to p63. Increased cell invasion observed upon p63 knockdown was significantly reduced when cells were treated with Gö6976 (Fig. 7c, d). As a control, we also examined the effect of p63 silencing on PKC α , a ubiquitously expressed PKC that is also inhibited by Gö6976. Our results revealed that p63 knockdown (Supplementary Fig. 2a) did not significantly induce PKC α either at the transcript or protein level (Supplementary Fig. 2b, d). These results clearly indicate that Δ Np63 α inhibition of Rac1 phosphorylation is mediated through a reduction of PKC γ levels.

Next, we investigated the effect of the PKC activator phorbol 12-myristate 13-acetate (PMA) on Rac1 phosphorylation. A431 cells were treated with either 100 nM PMA or vehicle for various time intervals (0–30 min). PMA treatment led to an increase in pRac1 levels that peaked at 15 min, followed by a gradual decrease at 20 and 30 min (Fig. 7e). This effect was inhibited by Gö6976 treatment (Fig. 7f). To further confirm that the increase in pRac1 levels involves PKC γ activation, we examined the effect of silencing PKC γ and treating with PMA on pRac1 levels. Silencing PKC γ abolished the increase in pRac1 induced by PMA (compare PMA/siPKC γ with PMA/NSC in Fig. 7g, h). Taken together, these results clearly confirm that Rac1 phosphorylation is induced by PKC γ , and that Δ Np63 α and miR-320a inhibit Rac1 phosphorylation and hence cell invasion via targeting PKC γ .

Inverse correlation observed between Δ Np63 α , miR-320a expression when compared to PKC γ expression in human cancers

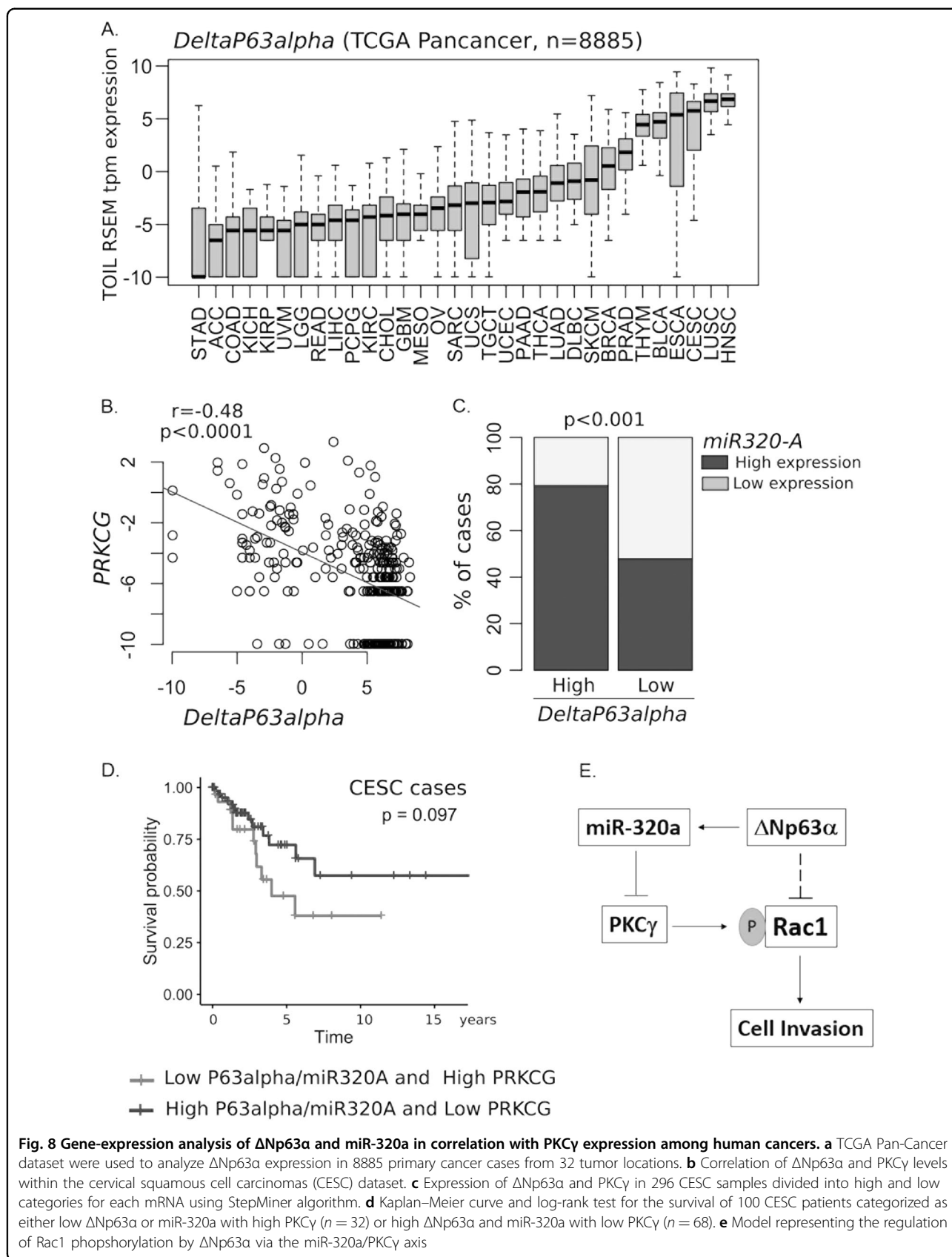
To examine the potential clinical relevance of PKC γ downregulation by Δ Np63 α /miR-320a axis, we first analyzed the level of Δ Np63 α expression among 32 different cancer types using TCGA Pan-Cancer datasets (Fig. 8a). We found the highest expression of Δ Np63 α in cervical,

lung and head and neck SCC. In silico analysis revealed a significant negative correlation between Δ Np63 α and PKC γ expressions (Fig. 8b). A significant correlation was found between the expressions of Δ Np63 α and miR-320a ($p < 0.0001$, Fisher's exact test) with 80% of cervical squamous cell carcinomas (CESC) tumors expressing high levels of Δ Np63 α also showing high levels of miR-320a (Fig. 8c). Finally, Kaplan–Meier analysis carried out in CESC revealed that patients with low p63/miR-320a levels and high-PKC γ levels have poor survival when compared with patients with high p63/miR-320a levels and PKC γ levels (Fig. 8d).

Discussion

Δ Np63 α , a master regulator of epithelial differentiation, has been shown to suppress cell invasion through downregulation of both mRNAs and microRNAs involved in cell adhesion and motility, however, a detailed molecular understanding of Δ Np63 α function in cell invasion is lacking^{11,15–17}. Here, we have identified a novel signaling mechanism by which Δ Np63 α positively regulates miR-320a to target PKC γ , resulting in suppression of Rac1 phosphorylation and thus cell invasion (Fig. 8e). Investigation into this novel mechanism could yield new therapeutic options in the treatment of metastatic cancers.

We have previously shown that increased expression of Δ Np63 α increases proliferation and is a hallmark of non-melanoma skin cancers such as BCC and squamous cell carcinoma^{4,41,42}. Interestingly, while Δ Np63 α canonically functions as an oncogene by increasing proliferation, it generally prevents tumor metastasis by suppressing invasiveness^{4,8,14}. As a result, direct therapeutic targeting of Δ Np63 α is undesirable as it would be expected to slow tumor growth while increasing invasiveness. Rather, targeting downstream effectors of Δ Np63 α involved in cell invasion pathways such as the miR-320a/PKC γ /pRac1 axis identified in this study may have therapeutic value in slowing the progression of SCC. In addition, previous studies have clearly demonstrated that Δ Np63 α plays an



important role in maintenance and proliferation of epidermal stem cells and thereby normal keratinocyte homeostasis^{43,44}. In addition, both p63 and Rac1 have been shown to be required for wound healing^{14,45}. Since no studies to date have shown an association between miR-320a, PKC γ and pRac1 in normal epidermal homeostasis or wound healing, our findings on the Δ Np63 α /miR320a/PKC γ /pRac1 axis are novel and suggest a role in both cancer cells as well as normal keratinocyte homeostasis and/or wound healing.

miR-320a, identified here as a novel Δ Np63 α -regulated miRNA, is a known tumor suppressive miRNA that is downregulated in many metastatic cancers^{20,36,46,47}. Loss of miR-320a leads to increased colon cancer cell proliferation and increased bladder carcinoma invasion^{34,48}. Further, loss of miR-320a was shown to promote invasion and metastasis in tongue SCC and breast cancer^{49,50}. Conversely, high expression of miR-320a would appear to be a good prognostic indicator in cancer, and the use of a miR-320a mimic in SCC therapy would be expected to have therapeutic value.

Our results further show that Δ Np63 α positively regulates miR-320a, which results in reduced expression of its target PKC γ . This PKC, originally thought to be expressed only in neurons, has only recently been identified as a potential mediator of colon and breast cancer invasiveness^{51,52}. PKC γ knockdown in the mouse periventricular nucleus has been shown to significantly reduce total GTP-bound Rac1 levels and the number of phospho-Rac1 positive neurons⁵³. Increased PKC γ protein levels has been shown to promote the migration of colon cancer cells whereas a reduction in PKC γ levels induces cell adhesion and proliferation⁵². Moreover, the therapeutic potential of PKC γ inhibition has been hindered by the lack of an isoform specific inhibitor, but broad spectrum PKC inhibitors such as midostaurin have been shown to suppress tumorigenesis in AML and advanced systematic mastocytosis⁵⁴.

We further show that Δ Np63 α regulates the phosphorylation of Rac1 through the miR-320a-PKC γ axis. Although miR-320a was shown to affect the expression of Rac1 in colorectal cancer cells²⁰, Δ Np63 α did not change total Rac1 levels in four different cell lines used in our study, including SW480 colon cancer cells. Although the reasons behind these differences are not known, we have clearly shown that p63 negatively regulates Rac1 phosphorylation using pRac1 specific antibodies. The role of Rac1 in the acquisition of invasive and metastatic phenotypes and thus cancer progression has been well established, and increased expression of Rac1 has been associated with poor prognostic outcome for a number of cancers^{55–57}. The effect of blocking Rac1 phosphorylation on invasion, migration and proliferation in SCC has not been explored. Thus, additional testing is needed to

determine the functional consequences and therapeutic potential of perturbing the miR-320a/PKC γ /pRac1 pathway.

In summary, the work presented in this study elucidates one arm by which Δ Np63 α functions to inhibit cell invasion. Δ Np63 α regulation of miR-320a inhibits PKC γ /pRac1 signaling leading to inhibition of cell invasion. This study strongly links Δ Np63 α to regulation of Rho small GTPases, and thus to a role in cytoskeleton rearrangement and subsequently cell motility as it pertains to invasion and migration of tumor cells.

Materials and methods

Cell culture and reagents

The SCC cell line A431, the human nonsmall cell lung carcinoma cell line H1299, and the colorectal adenocarcinoma SW480 and Caco2 cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). The nontumorigenic immortalized human keratinocyte HaCaT cell line was obtained from Dr. Nancy Bigley (Wright State University). All 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 μ g streptomycin. The PKC inhibitor Gö6976 was obtained from Sigma-Aldrich (St. Louis, MO). PMA was purchased from Cell Signaling Technology (Danvers, MA).

miRNA, siRNA, and DNA transfections

miR-320a mimic and miRNA mimic negative control were obtained from Dharmacon (Lafayette, CO, USA). A total of 40 nM of miR-320a mimic or mimic negative control was transfected into A431 or HaCaT cells using Lipofectamine RNAi-Max per manufacturer's instructions (Life Technologies, Carlsband, CA, USA). Rac1, PKC γ , 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 siRNAs used in this study were purchased from Qiagen (Valencia, CA, USA) and PKC γ siRNA was purchased from Dharmacon (Lafayette, CO, USA). The Δ Np63 α expression plasmid or the empty pcDNA3.1 control plasmid were transiently transfected into H1299, SW480 or Caco2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as reported previously⁴. Cells were harvested 24 h after transfection and cell pellets were used for immunoblot analysis and extraction of total RNA for qRT-PCR studies.

Western blot analysis

Cells were lysed in a buffer containing 50 mM Tris-HCl pH 8, 120 mM NaCl, 5 mM sodium pyrophosphate phosphatase inhibitor (NaPPi), 10 mM NaF, 30 mM paranitrophenylphosphate, 1 mM benzamide, 0.1% NP-

40, 1% Triton X-100 and 0.2 mM PMSF, 100 nM sodium orthovanadate, and supplemented with 10% protease inhibitor cocktail (Sigma, St. Louis, MO). Immunoblot analysis was carried as described previously¹⁴. Proteins were detected using the following antibodies: rabbit polyclonal anti-p63 [N2C1] (Gene Tex, Irvine, CA, USA), mouse monoclonal anti-Rac1 [23A8] (Abcam, Cambridge, MA, USA), rabbit polyclonal anti-Rac1 (C-11), rabbit polyclonal anti-phospho-Rac1 (Ser71), mouse monoclonal anti- β -actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit polyclonal anti-PKC γ (ABclonal Science, Woburn, MA, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies (Promega, Madison, WI, USA) were used for chemiluminescence detection with a Western Lightning Plus chemiluminescent kit (Perkin Elmer, Waltham, MA, USA).

Cloning and Luciferase reporter assay

The fragment of miR-320a gene enhancer region containing p63 binding site (chr8:22,239,143–22,239,162)^{37,38} was PCR amplified using the following primers: forward primer [5'-CCGGTACCGTGTGGAACTACAGGCATG-3'] and the reverse primer [5'-GCAACTCGAGCATGTAAGGGTCAAGGCGAT-3']. Amplified fragment was subcloned into pGL3-promoter luciferase vector (pGL3-promoter-Luc, Promega) via KPN1 and XHO1 sites. The resulted construct is designated as p63-BS-luciferase reporter. H1299 cells were plated on 24-well plates and transfected with p63-BS-luciferase reporter and Renilla luciferase constructs to normalize for transfection efficiency along with either an empty vector control or expression plasmid encoding Δ Np63 α . At 24 h post transfection, cells were harvested in passive lysis buffer and subjected to Dual luciferase assay as per manufacturer's protocol (Promega, Madison, WI). The relative luciferase activity was calculated as the ratio of Firefly luciferase activity to Renilla luciferase activity and normalized to empty pGL3 promoter vector transfected with increasing concentration of Δ Np63 α .

To determine whether PKC γ is a direct target of miR-320a, luciferase reporter constructs containing RenSP luciferase gene cloned upstream of either the PKC γ 3'UTR or a random control 3'UTR were obtained from SwitchGear Genomics (Carlsbad, CA, USA). A431 cells were co-transfected with either PKC γ 3'UTR or a random 3'UTR along with 100 nM of control mimic or miR-320a mimic. At 24 h post transfection, cells were assayed for the luciferase activity using LightSwitch Luciferase Assay Kit (SwitchGear Genomics, Carlsbad, CA, USA).

Cell invasion assay

Cell invasion was assessed using a two-chamber transwell system. A total of 8×10^4 transiently transfected A431 or HaCaT cells were suspended in serum-free

DMEM medium and seeded into 8 μ m pore size inserts (BD Biosciences) that were coated with 1 mg/ml Matrigel (BD Biosciences) and placed into 24-well plate. Then, DMEM containing 8% FBS was added to the bottom of each insert and cells were allowed to invade for 21 h. Cells that did not invade were removed using a cotton swab. Invading cells attached to the bottom of the transwell were fixed with 4% of paraformaldehyde and washed once with Dulbecco's Phosphate Buffered Saline. Cells were stained with crystal violet solution (0.1 g in 100 ml of H₂O) and imaged in 4–6 random fields at 10 \times magnification using a Leica CTR 6000 Microscope (Leica Microsystems, Wetzlar, Germany) and ImagePro 6.2 software (Media Cybernetics, Bethesda, MD). Cells were manually counted from these images and used to calculate the average number of cells in each field.

RNA isolation and TaqMan real-time PCR studies

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). Quantitative real-time PCR analysis was carried as previously described using the Applied Biosystem 7900HT or QuantStudio 7 Flex Real-Time PCR Systems and Assays on DemandTM (AOD) specific for the genes of interest and normalized to endogenous GAPDH (Life Technologies, Carlsbad City, CA, USA)^{14,58}. AODs used were as follows: GAPDH (4325792), Rac1 (Hs01902432_s1), PKC γ (Hs00177010), and pan-p63 (Hs00978340_ml). q-PCR reactions for each specific gene were run in triplicate.

miRNA expression: Total RNA was extracted from human cells using the EZNA RNA isolation kit according to the manufacturer's protocol (Omega Bio-Tek, Norcross, GA, USA). A TaqMan MicroRNA reverse transcription kit (Life Technologies, Carlsbad, CA, USA) was used to synthesize cDNA from 10 ng total RNA using primers specific to hsa-miR-320a (RT:002277) or RNU-48 (RT:001006) as per the manufacturer's protocol. qRT-PCR was performed using the Applied Biosystem 7900HT or QuantStudio 7 Flex Real-Time PCR Systems using TaqMan 2 \times universal master mix and miRNA specific AODs. AODs used were hsa-miR-320a (TM:002277) normalized to RNU-48 (TM:001006). q-PCR reactions for each specific gene were run in triplicate.

In silico analysis of Δ Np63 α isoform, PKC γ , and miR-320a expression among the TCGA Pan-Cancer dataset

Clinical/follow-up data and pre-processed *PRKCG* and *miR-320a* expression levels among 8885 primary cancer cases derived from 32 tumor locations were obtained from the GDC Pan-Cancer TCGA dataset at UCSC Xena browser (<https://xenabrowser.net/>). Similarly, the Δ Np63 α isoform expression levels (ENST00000354600.9 TOIL

RSEM tpm profile) were downloaded from the UCSC Toil RNAseq Recompute TCGA-Pancancer dataset at UCSC Xena browser. Univariate and bivariate (Pearson's Correlation test) expression analysis among cases were performed with the R software.

To further explore the prognostic value of the gene expression signature composed by $\Delta Np63\alpha$, *PRKCG*, and *miR-320a* in squamous carcinomas, we evaluated the CESC dataset. Briefly, a group of 296 patients with CESC and follow-up data were divided into two subgroups (Low $\Delta Np63\alpha$ /Low *miR-320a*/High *PRKCG* and High $\Delta Np63\alpha$ /High *miR-320a*/Low *PRKCG*). Discretization of the $\Delta Np63\alpha$, *PRKCG* and *miR-320a* gene expression data into low or high expression levels was performed according the StepMiner one-step algorithm of their respective profiles (<http://genedesk.ucsd.edu/home/public/StepMiner/>). These groups were then compared based on the overall survival (Kaplan–Meier curves and log-rank test) using the Survival R package. Fisher's exact test was employed to compare the distribution of CESC cases with high or low expression of $\Delta Np63\alpha$ and *miR-320a* transcripts.

Statistical analysis

Experiments were done in triplicates and values are presented as the mean \pm SD. Student's *t* test was used to analyze the statistical significant changes. Significant changes ($P \leq 0.05$) relative to controls are indicated with an asterisk.

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Authors' contributions

A.A.A.: conception and study design, execution of experiments, data collection and interpretation, preparation of figures, paper writing and approval of final paper. N.H.: conception and paper review. M.C., M.G.K. and M.C.A.: execution of experiments, data collection, and interpretation and paper writing. W.L.: conception of experiments, data interpretation, and approval of final paper. M.K.: conception and design of experiments; data interpretation, paper writing, approval of final paper; and financial support.

Conflict of interest

The authors declare that they have no conflict of interest.

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