ΔNp63-mediated regulation of hyaluronic acid metabolism and signaling supports HNSCC tumorigenesis

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide and several molecular pathways that underlie the molecular tumorigenesis of HNSCC have been identified. Among them, amplification or overexpression of ΔNp63 isoforms is observed in the majority of HNSCCs. Here, we unveiled a novel ΔNp63-dependent transcriptional program able to regulate the metabolism and the signaling of hyaluronic acid (HA), the major component of the extracellular matrix (ECM). We found that ΔNp63 is capable of sustaining the production of HA levels in cell culture and in vivo by regulating the expression of the HA synthase HAS3 and two hyaluronidase genes, HYAL-1 and HYAL-3. In addition, ΔNp63 directly regulates the expression of CD44, the major HA cell membrane receptor. By controlling this transcriptional program, ΔNp63 sustains the epithelial growth factor receptor (EGF-R) activation and the expression of ABCC1 multidrug transporter gene, thus contributing to tumor cell proliferation and chemoresistance. Importantly, p63 expression is positively correlated with CD44, HAS3 and ABCC1 expression in squamous cell carcinoma datasets and p63-HA pathway is a negative prognostic factor of HNSCC patient survival. All together, our data shed light on a novel ΔNp63-dependent pathway functionally important to the regulation of HNSCC progression.

Significance Statement

The p63 isoform ΔNp63, a master regulator of epithelial biology, is overexpressed/amplified in the majority of head and neck squamous cell carcinoma (HNSCC), the sixth most common cancer worldwide. Here, we provide the first demonstration of a molecular and functional link between the activity of ΔNp63 and hyaluronic acid (HA), a major component of the extracellular matrix. We unveiled a novel ΔNp63-dependent transcriptional program involving genes regulating the metabolism (HAS3 and HYAL proteins) and the signaling (CD44) of HA. By directly controlling the expression of these HA-related genes, ΔNp63 contributes to the activation of pro-proliferative and pro-survival pathway in HNSCC. Accordingly, the p63/HA pathway is a negative prognostic factor of HNSCC patient survival.

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Introduction

The crosstalk between cells and the components of the extracellular matrix (ECM) is fundamental in multicellular organisms and regulates several biological processes in both physiological and pathological conditions (1). Hyaluronic acid (HA), the major component of the ECM, is a non-sulfated, linear glycosaminoglycan (GAG), which not only contributes to tissue hydration and proper tissue architecture, but also plays important biological functions, including control of cell proliferation, cell motility, cell adhesion, survival and inflammation (2, 3). These cellular events are mainly mediated by HA binding to specific cell-surface receptors, including CD44, the most common and ubiquitous expressed receptor (4). In mammals, HA synthesis occurs on the cellular plasma membrane by means of three hyaluronan synthase isoenzymes (HAS1, HAS2, and HAS3) that extrude the growing HA polymer into the pericellular or the extracellular space (3, 5).

HA synthesis is counteracted by a degradative pathway that clears HA by endocytic uptake and/or HA hydrolysis (6). In humans, there are six hyaluronidase (*HYAL*) genes (HYAL-1-4, HYAL-P1 and the sperm-specific PH-20). Among them, HYAL-1 and HYAL-2 are the best characterized HYALs and they can have different catalytic properties (7).

Based on its role in important biological processes it is not surprising that deregulation of HA metabolism and signaling are common events in several pathological conditions such as tumor development (8). Deregulation of HA production during tumor progression is often associated with alterations of the enzymes that regulate HA synthesis and degradation. Overexpression of either HAS2 or HAS3 is associated with higher malignancy or metastasis in several tumor types, such as breast and prostate carcinomas (9, 10). Although these data indicate that deregulation of HA production is tightly linked with tumor progression, our knowledge of the molecular mechanism(s) linking oncogenes and HA metabolism has not yet been elucidated.

p63 is a transcription factor belonging to the p53 family and it is expressed as distinct protein isoforms (11, 12). ΔNp63 isoforms are the most expressed variants in the basal cells of stratified and glandular epithelia and its overexpression and/or genomic amplification is observed in up to 80% of squamous cell carcinoma (SCC) of the head and neck, skin, lung and esophagous (13). Moreover, several genetic alterations occurring in head and neck squamous cell carcinoma (HNSCC), such as NOTCH1 mutation and ACTL6A amplification, favours p63 transcriptional activity (14, 15). At molecular level, ΔNp63

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isoforms, although lacking a canonical transcriptional activation domain, are endowed with an alternative activation domain, that enable them to stimulate the expression of several target genes involved in the regulation of stemness, cell migration, invasion and cell adhesion (16-18). On the other hand, ΔNp63 isoforms are also able to repress the transcription of several target genes by different mechanisms (19, 20).

Here, we report evidence demonstrating that ΔNp63 supports HNSCC proliferation and chemoresistance by modulating epithelial growth factor receptor (EGR-R) activation and the expression of the ABC transporter ABCC1 in a HA-dependent manner.

Results

ΔNp63 regulates the expression of HA metabolism genes

During tumorigenesis extensive remodeling of the ECM supports and enhances tumor growth. In order to identify novel p63 target genes potentially regulating ECM in HNSCC, we combined microarray data performed in A253 cell line upon p63 depletion (see Supplementary data) with co-expression studies in human primary SCC datasets, searching for ECM-related genes whose expression is linked with that of p63. By cross-analyzing these data, we focused our attention on the HA synthase HAS3 and the hyaluronidase HYAL-1. We found that HAS3 expression is positively correlated with that of TP63 in HNSCC, esophageal carcinoma and lung squamous cancer datasets (Figure S1A). Silencing of p63 markedly decreased HAS3 expression in A253, Detroit-562 and FaDu cells (Figures 1A and S1B). These HNSCC cell lines mainly express the ΔNp63 isoforms (Figure S1C), suggesting that these p63 isoforms might regulate HAS3 expression. Accordingly, the specific depletion of ΔNp63 isoforms efficiently decreased HAS3 expression at mRNA and protein levels (Figures 1B and S1D). As a complementary approach, we found that the ectopic expression of ΔNp63α in SCC-9 cells, in which endogenous p63 levels are undetectable (Figure S1E), dramatically increases HAS3 mRNA (Figure S1F). We also confirm the p63 dependent regulation of HAS3 expression in a xenotransplantation experiment. We inoculated FaDu cells stably expressed a doxycycline inducible shp63 RNA in nude athymic mice. p63 silencing upon doxycycline treatment markedly decreased the proliferative capacity *in vitro* (Figure S2) and the growth of xenotransplanted tumors (Figure 1C). Importantly, the expression of HAS3 mRNA is significantly decreased in p63-silenced tumors (Figure 1D). To further investigate the mechanism of the ΔNp63-dependent regulation of HAS3 expression, we verified whether ΔNp63 is able to bind to the *HAS3*

locus. To this aim, we performed a ChIP experiment in FaDu cells relying on ChIP-seq data performed on human primary keratinocytes (NHEK) (Figure S3A). We found that endogenous ΔNp63 is able to occupy a p63-binding site (p63 BS) located in the *HAS3* promoter site, at -5 kb from the TSS (Figure 1E). Furthermore, exogenous ΔNp63α greatly enhanced the HAS3 promoter-mediated luciferase activity (Figure S3B).

In addition to HAS3, our microarray data revealed that p63 silencing affects HYAL-1 mRNA levels (see Supplementary data). In this case, we observed that the expression of HYAL-1 is increased upon p63 silencing at both mRNA and protein levels (Figure 1F). p63 depletion also increased the mRNA levels of HYAL-3, another member of the hyaluronidases family. Notably, HYAL-1 expression is also augmented in explanted tumors upon p63 silencing (Figure 1G). HYAL-1 and HYAL-3 genes are clustered together on chromosome 3p21.3 and we found that endogenous ΔNp63 is able to bind to two p63-binding sites, p63 BS#1 and BS#2, located in the HYAL-3 promoter site and in the 3'-end of HYAL-1 gene, respectively (Figures 1H and Figure S3C). All together, these results indicated that the HA synthase HAS3 and the hyaluronidases HYAL-1 and HYAL-3 are *bona fide* ΔNp63 transcriptional target genes *in vitro* and *in vivo*.

ΔNp63 controls hyaluronic acid levels

In light of the finding that ΔNp63 is able to transcriptionally regulate genes involved in HA metabolism, we tested whether ΔNp63 was capable of regulating HA levels. The HA polymer is synthesized by the HA synthases and extruded into the pericellular or the extracellular space. We stained pericellular HA by immunofluorescence using the high affinity HA binding protein HABP and we found that ΔNp63 depletion decreases the levels of pericellular HA (Figure S4A). To quantify this effect, we measured HA levels in cell medium by ELISA assay and we confirmed that ΔNp63-depleted cells display a marked reduction of HA levels in FaDu and A253 cells (Figures 2A and S4B), similarly to the HAS3 silenced cells (Figure S4C). Conversely, we found that the ectopic expression of ΔNp63α isoform increases HA cellular levels (Figure 2B). To further validate the correlation between p63 and HA levels, we performed an immunohistochemistry (IHC) staining of p63 and HA in a HNSCC tissue microarray (TMA). We first confirmed the specificity of HABP probe in tumor tissues by pre-treating the section with the *Streptomyces hyaluronidase* (Figure S4D). Although HA can be produced by stromal and tumor cells, we observed a correlation between the expression of p63 and the tumor-associated HA content in 57 tumor samples (Figures 2C and 2D). In support of these data, we found that HA-tumor associated

levels are markedly decreased in xenograft tumors upon silencing of p63 (Figure 2E). Collectively, these results indicated that ΔNp63 is able to regulate HA cellular levels, likely via transcriptional regulation of HAS3.

ΔNp63 directly induces the expression of the HA receptor CD44

CD44 is the most tumor-relevant HA receptor and deregulation of the expression of its variant isoforms is associated with advanced diseases and chemoresistance in HNSCC (4, 21). By analyzing co-expression data in esophageal and lung squamous carcinoma datasets, we found that CD44 mRNA expression is positively correlated with that of Tp63 (Figure S5A). To validate these data in human HNSCC tissues, we analyzed p63 and CD44 protein levels by IHC in the HNSCC TMA. We observed a positive correlation between p63 and CD44 expression (Figure 3A and 3B). Importantly, gain or loss of function of ΔNp63 expression markedly affected the expression of CD44 at mRNA and protein levels (Figures 3C, 3D, S5B and S5C). Conversely, the expression of RHAMM, another well characterized HA receptor, is not affected by ΔNp63 depletion (Figure S5D). These transcriptional effects are likely due to a direct binding of ΔNp63 on the *CD44* locus. Indeed, by ChIP assay, we demonstrated the ability of endogenous ΔNp63 to bind two p63-binding sites, p63 BS#1 and p63 BS#2 located in the promoter region and in the first intron of CD44 gene, respectively (Figures 3E and Figure S5E). All together these results demonstrated that ΔNp63 is able to directly regulate the expression of CD44 by physically recognizing two p63-binding elements located in the *CD44* locus.

The ΔNp63-HA pathway regulates EGF-R activation

Since the HA/CD44 interaction promotes the activation of receptor tyrosine kinases (RTKs) (22, 23), we tested whether ΔNp63 is capable of favoring RTKs activation in a HA-dependent manner. By using a RTK signaling antibody array, we found that among several RTKs and signaling nodes, tyrosine phosphorylation of EGF-R and the activation of some downstream effectors, including Akt, ERK1/2 and S6 ribosomal protein, are markedly decreased upon p63 silencing in A253 and FaDu cells (Figure S6A). We validated these data using two different p63 targeting siRNA oligos, which are both able to reduce EGF-R phosphorylation in several HNSCC cell lines (Figures 4A and S6B). In addition to EGF-R phosphorylation, ΔNp63 depletion reduced the phosphorylation of ErbB2, an ErbB family member whose activation is stimulated by the HA/CD44 complex (23).

To investigate whether the ΔNp63-HA pathway might affect the activation of the EGF-R, first we tested the effect of 4-methylumbelliferone (4-MU), a chemical inhibitor of the enzymatic activity of the HAS synthases (24), on EGF-R phosphorylation. We found that 4-MU treatment markedly reduces the phosphorylation of EGF-R and ErbB2 in A253 and FaDu cells, concomitantly with a decrease of the pericellular HA amount (Figures 4B and S6C). Similarly, depletion of HAS3 gene expression by siRNA decreased EGF-R phosphorylation in HNSCC cells (Figures 4C and S6D). In a reverse experiment, overexpression of ΔNp63α or HAS3 increased EGF-R phosphorylation (Figures 4D and S6E). Importantly, HAS3 inhibition by 4-MU treatment reverted the ability of exogenous ΔNp63 to enhance the activation of EGF-R, Akt and ERK1/2 (Figure 4E). Since EGF-R transduction pathway activates proliferative signals, we measured the proliferative capabilities of ΔNp63 or HAS3 silenced cells by cell counting assay. We found that the siRNA-mediated depletion of HAS3 results in a decrease of cell proliferation (Figure 4F). All together, these observations indicated that ΔNp63 favors the activation of oncogenic signals of EGFR by, at least in part, modulating the HA/CD44 pathway.

ΔNp63/HA axis regulates ABC drug transporter expression and it is a negative prognostic factor for HNSCC patient survival

The HA/CD44 interaction modulates the chemosensitivity of cells to various anti-neoplastic agents (21). Therefore, we tested whether deregulation of the ΔNp63-HA axis might impact the chemosensitivity of HNSCC cells toward the cytotoxic effect of sub-lethal doses of doxorubicin and cisplatin. We found that upon chemotherapeutic agents treatment p63 or HAS3 silencing increases the percentage of apoptotic cells and the cleavage of PARP, an apoptotic marker (Figure 5A and S7A). A similar effect was observed in cells treated with the HAS inhibitor 4-MU (Figure S7B). As a complementary approach, we tested whether the ectopic expression of ΔNp63 might exert a pro-survival effect in cells exposed to doxorubicin. We found that the ectopic expression of ΔNp63α in SCC-9 cells inhibits the doxorubicin-mediated apoptosis, an effect which is partially suppressed by the inhibition of the HAS3 activity by 4-MU (Figures 5B, S7C and S7D), suggesting that the ΔNp63-mediated pro-survival action is, at least in part, mediated by its ability to regulate HA metabolism.

One potential mechanism underlying the chemoresistance action of the HA/CD44 interaction is through the modulation of the ATP-binding cassette (ABC) drug transporters

expression (25, 26). Therefore, we screened HNSCC cell lines for the expression of different ABC transporters, and we identified ABCC1 as the main ABC transporter expressed in HNSCC cell lines (Figure S7E). Notably, ΔNp63 depletion, similarly to HAS3 depletion, reduced ABCC1 mRNA levels in HNSCC cells (Figure 5C). On the contrary, the expression of ABCB3 transporter was not affected by ΔNp63 or HAS3 depletion (Figure S7F). In agreement with these data, we found that p63/ABCC1 and HAS3/ABCC1 co-expression is positively correlated in HNSCC dataset as well as in esophageal SCC (Figure S7G).

Based on these results, we can postulate that the p63/HA axis would act as a negative prognostic factor in patients with HNSCC. We therefore analyzed the overall patient survival of two groups of HNSCC patients: those displaying high p63/HAS3 co-expression and those with low co-expression (see Figure 5D, lower panel). We found that patients displaying high p63 and HAS3 co-expression showed a decrease of the overall survival compared to those with low expression (Figure 5D). All together, these findings indicated that p63-HA pathway is a negative prognostic factor of patient survival and it might be functionally important for HNSCC progression.

Discussion

HNSCCs are characterized by high mortality rate and intrinsic chemoresistance (13, 27). Overexpression and/or amplification of the *Tp63 locus* is observed in the majority of invasive HNSCC (28). Here, we not only identify a novel HA-related transcriptional program directly regulated by ΔNp63 in HNSCC, but also provide the first demonstration that this program is functionally important to molecularly link ΔNp63 activity with two critical pathways in HNSCC biology: EGF-R signaling and chemoresistance. We demonstrated that ΔNp63 sustains HA signaling by directly regulating the expression of HAS3, HYALs and CD44 (see proposed model, Figure 5E). In human tumors, HA is pivotal for various aspects of tumors pathobiology (29, 30) and the biological outcome of tumor-associated HA accumulation vary and likely depends not only on its levels but also on the size of HA polymers (31). Intriguingly, HAS3 synthesizes low molecular weight chains of HA that have been associated with activation of proliferative signals (32). Accordingly, the overexpression of HAS3 in several tumors is associated with higher malignancy (33) and HAS3 knockdown negatively impacts esophageal tumor growth *in vivo* (34). Although these data identify HAS3 as a regulator of tumor growth, there are no data unveiling the molecular mechanism(s) regulating its expression in tumor cells. Our data unveils for the first time that HAS3 expression is under control of the transcription factor ΔNp63.

Regarding the HA catabolic genes, the role of HYAL proteins during tumorigenesis is controversial, acting as tumor promoters or suppressors, likely reflecting the different biological activities exerted by the HA catabolic products. Interestingly, HYAL-2 produces highly angiogenic and pro-tumorigenic HA polymers (35), which are the subsequentially degraded by HYAL-1 in tetrasaccharides (7). Therefore, it is possible that ΔNp63, by inhibiting the expression of HYAL-1, maintains the high levels of the 20-kDa pro-tumorigenic fragments of HA.

In addition to regulating enzymes involved in the HA metabolic process, we also showed that ΔNp63 directly regulates the expression of CD44, the major cell membrane HA receptor. Although several published studies have suggested a potential molecular link between ΔNp63 activity and CD44 in breast cancer (36, 37), our data demonstrate for the first time that ΔNp63 directly controls the expression of CD44 in HNSCC and unveil the biological significance of the p63-CD44 axis. We focused our attention on two closely interconnected pathways, which are functionally related to the HNSCC pathobiology: the EGF-R signaling and the regulation of the expression of multidrug transporter genes. EGF-R is overexpressed in more than 90% of HNSCCs and its expression has been correlated with poor outcome (13). Previous reports suggested that ΔNp63 activity is linked to the deregulation of EGF-R signaling (18, 38). In detail, it has been demonstrated that ΔNp63 transcriptionally induces EGF-R transcription in prostatic cancer cells (38). However, we did not observe any change of the EGF-R protein levels in HNSCC cells upon p63 silencing, suggesting that the ΔNp63-dependent regulation of EGF-R transcription might be tissue specific, as previously suggested (38). Conversely, we propose a novel HA-dependent mechanism, which molecularly links ΔNp63 activity and EGF-R signaling. We found that in HNSCC cells the ΔNp63-dependent effect on EGF-R signaling relies, at least in part, on its ability to regulate HA metabolism. Interestingly, EGF-R expression is positively correlated with that of HAS3 in human esophageal tumors (34) and that the activation of EGF-R led to an induction of HAS3 expression in ovarian and lung tumor cells (39). Therefore, ΔNp63-HAS3 pathway might be sustained by a positive feedback by the EGF-R signaling in HNSCC.

HA-CD44 signaling also promotes resistance to multiple anti-neoplastic agents, including cisplatin, methotrexate and doxorubicin (40, 41). We found that the regulation of HA metabolism and signaling mediates, at least in part, the ΔNp63 pro-survival action and reduce the cytotoxic effect of cisplatin and doxorubicin in HNSCC. This effect is likely mediated by the regulation of the expression of the ABCC1 transporter (also known as

MRP1) by the ΔNp63-HA pathway. Elevated ABCC1 expression levels have been detected in many hematopoietic and solid tumors and a significant statistical association of high ABCC1 protein levels with poor response in pre-chemotherapy biopsies of esophageal adenocarcinoma patients has been reported (42). However, the molecular mechanisms regulating ABCC1 expression in human tumors are not well known. We found that ΔNp63 or HAS3 depletion decreases the expression of ABCC1. Importantly, both HAS3 and ΔNp63 expression are positively correlated with ABCC1 expression levels in human HNSCC primary tumors. Although we can not rule out that the pro-survival effect of ΔNp63 can be due to additional pathways, including the HA-dependent modulation of EGF-R signaling proposed here, our data established for the first time the existence of a functional connection between ΔNp63 activity, HA metabolism and drug fluxes that can enhance the intrinsic chemoresistance of HNSCC. Alteration of this circuit would impinge on the capacity of tumor cells to respond to chemotherapy and therefore should impact the survival rate of HNSCC patients. Accordingly, we found that in HNSCC patients the p63-HAS3 axis is a negative prognostic factor of patient survival and it might be thus functionally important to regulate tumor progression.

Materials and Methods

Human HNSCC tumor tissues

HNSCC tissue microarray (TMA) was purchased by US Biomax, Inc (HN802a, Rockville, MD, USA). TMA slide includes 61 cases of squamous cell carcinoma, 7 adenoid cystic carcinoma, 1 each of adenocarcinoma and mucoepidermoid carcinoma, plus 10 normal tissues. Score of each tumor samples was calculated as described in detail in the SI. HNSCC tissue sample shown in Figure S4D has been utilized under approval by the institutional review board of University Hospital "Policlinico Tor Vergata" prior patient consent.

Cell lines

HNSCC and 293T cells were purchased from ATCC and routinely tested for mycoplasma contaminations. Culture condition, drug treatment, siRNA transfection, lentiviral infection, ChIP-PCR, RNA analysis, luciferase assay, apoptosis detection, immunofluorescence, HA measurement and Western blotting techniques are all described in SI. Details of all other methods are also described in SI section.

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Figure Legends

Figure 1. ΔNp63 directly induces the expression of the HA-related genes. (A) HAS3 and p63 mRNA levels were quantified by qRT-PCR in the indicated HNSCC cancer cell lines transfected with scrambled (SCR) or p63 siRNA (sip63) oligos. Bars represent the mean of three technical replicates (n=3, PCR runs) ± SD and are representative of two independent experiments (n=2 biological replicates). * p value <0,05. (B) Total protein lysates or purified membrane-bound proteins of FaDu cells transfected with scrambled (SCR), p63 siRNA (sip63), ΔNp63 siRNA (siΔNp63) or HAS3 siRNA (siHAS3) oligos were subjected to IB

using antibodies to the indicated proteins. (C) FaDu cells stably infected with doxycycline inducible shp63 expression particles (polyclonal population) were injected into the dorsal flank region of athymic female nude mice (n=6/group). The tumor volume growth curves are shown as mean ± SEM on the left panel. Images of the explanted tumors are shown on the right panels. (D) Total RNA extracted by untreated (shp63 off, n=6) or doxycycline treated mice (shp63 on, n=6) was utilized for qRT-PCR analysis of the absolute expression of HAS3 mRNA. ** p value < 0,01. (E) ChIP analysis of endogenous ΔNp63 occupancy at the p63 binding site of *HAS3* locus. (F) HYAL-1, HYAL-2 and HYAL-3 mRNA levels were measured by qRT-PCR (left panel) in FaDu cells transfected as in B). Bars represent the mean of three technical replicates (n=3, PCR runs) ± SD and are representative of two independent experiments (n=2 biological replicates). * p value <0,05. In parallel, total protein lysates were analyzed by IB using antibodies to the indicated proteins (right panel). (G) HYAL-1 mRNA levels was quantified by qRT-PCR in explanted tumors as described in E). (H) ChIP analysis of endogenous ΔNp63 occupancy at the p63 BS#1 and BS#2 of *Hyals* loci.

Figure 2. ΔNp63 controls HA levels. (A) FaDu cells were transfected with scrambled (SCR) or p63 siRNA (sip63). Forty-eight hours after transfection growth medium of transfected cells was utilized to quantitatively measure the levels of extracellular HA by Elisa assay. HA intracellular levels were normalized against the number of cells (1×10^5). Bars represent the mean of three technical replicates (n=3) ± SD and are representative of two independent experiments (n=2 biological replicates). * p value < 0,05. (B) SCC-9 cells infected with empty or ΔNp63 expressing lentiviral particles were analyzed as in (A). (C) Representative images of IHC analysis of p63 and HA staining in HNSCC tissues. (D) Correlation plot of p63 and HA H-scores in 57 human HNSCC tumor samples including 50 cases of squamous cell carcinoma and 7 adenoid cystic carcinomas. Pearson's correlation coefficient (r) and the p value of the correlation study are reported. (E) Representative images of IHC analysis of p63 and HA staining in mouse xenograft tumor tissues upon p63 depletion.

Figure 3. ΔNp63 regulates the expression of the HA receptor CD44. (A) Representative images of IHC analysis of CD44 and TP63 expression in HNSCC tissues. (B) Correlation plot of p63 and CD44 H-scores in 56 human HNSCC tumors including 49 cases of squamous cell carcinoma and 7 adenoid cystic carcinomas. Pearson's correlation coefficient
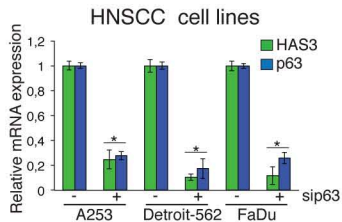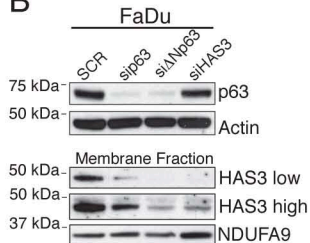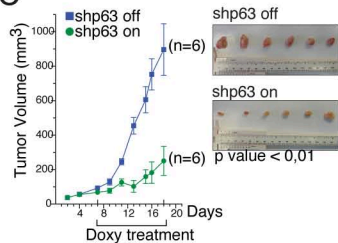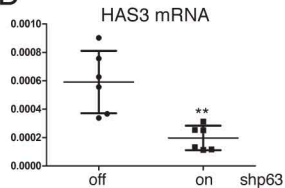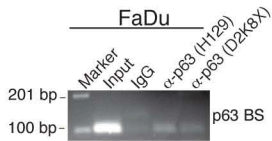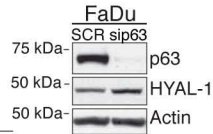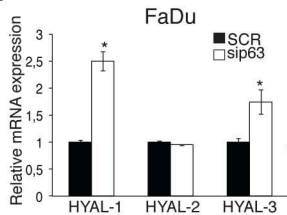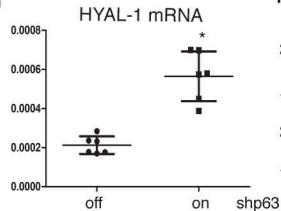
(r) and the p value of the correlation study are indicated. (C) FaDu cells transfected with scrambled (SCR), p63 siRNA (sip63) or ΔNp63 specific siRNA oligos (siΔNp63) were analyzed by immunoblotting using the antibodies for the indicated proteins. (D) FaDu cells treated as in C) were analyzed for the expression of CD44 by qRT-PCR. Bars represent the mean of three technical replicates (n=3, PCR runs) ± SD and are representative of two independent experiments (n=2 biological replicates). * p value < 0,05. (E) ChIP analysis of endogenous ΔNp63 occupancy at the p63 BS#1 and BS#2 in FaDu cells.

Figure 4. ΔNp63-HA pathway favors receptor tyrosine kinase activation. (A) A253 cells were transfected with scrambled (SCR) or two distinct siRNA oligos targeting different regions of p63 mRNA (sip63#1 and sip63#2). Protein lysates of transfected cells were immunoblotted utilizing the antibodies for the indicated proteins. (B) FaDu cells were treated with the indicated concentration of 4-methylumbelliferone (4-MU) for 24 hrs and then protein lysates were immunoblotted for the indicated proteins. (C) FaDu cells were transfected with scrambled (SCR), HAS3 (siHAS3) or p63 siRNA (sip63) oligos and after 48 hrs protein lysates were immunoblotted for the indicated proteins. (D) A253 cells were infected with Flag-tagged HAS3 expressing viral particles and after 48 hrs protein lysates were immunoblotted for the indicated proteins. (E) SCC-9 cells were infected with empty or ΔNp63 expressing lentiviral particles. 24 hrs post-infection, cells were treated with 4-MU for 24 hrs and then protein lysates were immunoblotted for the indicated proteins. (F) FaDu cells were plated in 6 well (8 x 10^5 cells/well) and after 24 hrs transfected with scrambled (SCR), HAS3 siRNA (siHAS3) or p63 siRNA oligos (sip63). Six days after transfection, cells were counted by trypan blue dye exclusion in triplicate. Data are shown as the mean ± SD (n=3); * p value < 0,05.

Figure 5. ΔNp63-HA pathway regulates HNSCC chemoresistance and it is a negative prognostic factor of HNSCC patient survival. (A) FaDu cells transfected with scramble (SCR), sip63 or siHAS3 siRNA oligos were treated with doxorubicin (0,4 μM) for 48 hrs. Cells were then stained with PI and fluorodiacetate (FDA) and the percentage of apoptotic cells was determined by FACS (left panel). Data are shown as the mean ± SD of three biological replicates (n=3). ** p value < 0,01. In parallel, lysates obtained from treated cells were immunoblotted for the indicated proteins (right panel). (B) SCC-9 cells were lentivirally-infected with empty or ΔNp63 expressing particles. 48 hrs post-infection, cells

were concomitantly treated with Doxorubicin (0,4 μM) and 4-MU (0,3 mM) for 48 hrs and then cell viability was measured by Celltiter-Glo. Data are shown as the mean ± SD of three technical replicates (n=3). * p value ≤ 0,05. (C) A253 cells were transfected with scrambled (SCR), p63 siRNA (sip63) or HAS3 siRNA (siHAS3) oligos and 48 hrs post-transfection ABCC1 mRNA levels were measured by qRT-PCR. Data are shown as the mean ± SD of three technical replicates (n=3, PCR runs) and are representative of two independent experiments (n=2 biological replicates). * p value <0,05. (D) Patient samples from the TCGA cohort were clustered into two groups displaying low and high p63 and HAS3 expressions, respectively, and analyzed for survival (p value: 0,0068). Clustered expression values relative to mean expression are shown in the heat map PCC: 0.5959; p value: 9,9639e-51. (E) Schematic model of the ΔNp63-mediated regulation of the HA metabolism and signaling.

A HNSCC cell lines

HAS3 / p63 relative mRNA expression in A253, Detroit-562, and FaDu cell lines with and without sip63. * indicates significance.

B FaDu — SCR, sip63, siΔNp63, siHAS3. p63 (75 kDa), Actin (50 kDa). Membrane Fraction: HAS3 low (50 kDa), HAS3 high (50 kDa), NDUFA9 (37 kDa).

C Tumor Volume (mm³) vs Days Doxy treatment. shp63 off (n=6), shp63 on (n=6). p value < 0,01.

D HAS3 mRNA. off vs on shp63. **

E FaDu — Marker, Input, IgG, α-p63 (H129), α-p63 (D2K8X). p63 BS. 201 bp, 100 bp.

F FaDu — SCR, sip63. HYAL-1, HYAL-2, HYAL-3 relative mRNA expression. * p63 (75 kDa), HYAL-1 (50 kDa), Actin (50 kDa).

G HYAL-1 mRNA. off vs on shp63. *

H FaDu — M, Inp, N, IgC, αp63. p63 BS#1 (201 bp, 100 bp), p63 BS#2 (201 bp, 100 bp).

A FaDu

HA (ng/1 × 10⁵ cells) — SCR, sip63 *

B SCC-9

HA (ng/1 × 10⁵ cells) — EV, ΔNp63α *

C Human HNSCC

p63

HA

350 μm, 60 μm, 350 μm, 60 μm, 350 μm

D

r= 0,584; p value <0,05

HA score vs p63 score

E Mouse xenograft

off on shp63

p63

HA

60 μm

A Human HNSCC

p63

CD44

B

r= 0,63; p value <0,001

HA score

p63 score

C FaDu

SCR sip63 siΔNp63

75 kDa — p63

150 kDa — CD44

102 kDa — vinculin

D

CD44 mRNA

Relative expression

SCR sip63 siΔNp63

*

E FaDu

M In IgG α-p63

201 bp —

100 bp —

p63 BS#1

201 bp —

100 bp —

p63 BS#2

A A253

75 kDa — p63
150 kDa — pEGF-R
150 kDa — EGF-R
150 kDa — pErbB2
150 kDa — ErbB2
50 kDa — Actin

SCR sip63 #1 sip63 #2

B FaDu

0   0,3   0,6   mM 4-MU

75 kDa — p63
150 kDa — pEGF-R
150 kDa — EGF-R
150 kDa — pErbB2
150 kDa — ErbB2
50 kDa — Actin

C FaDu

SCR sip63 siHAS3

75 kDa — p63
150 kDa — pEGF-R
150 kDa — EGF-R
50 kDa — Actin

D A253

EV   HAS3

50 kDa — HAS3 (anti-Flag)
150 kDa — pEGF-R
150 kDa — EGF-R
50 kDa — Actin

E SCC-9

ΔNp63α   -   +   +
4-MU   -   -   +

75 kDa — p63
150 kDa — pEGF-R
150 kDa — EGF-R
50 kDa — pAKT
50 kDa — AKT
50 kDa — pERK 1/2
50 kDa — ERK 1/2

F FaDu

Cell number (x10^6)

SCR   sip63*   siHAS3*

A

B SCC-9

C ABCC1 mRNA

D Kaplan-Meier analysis of survival

E