



# $\Delta$ Np63-mediated regulation of hyaluronic acid metabolism and signaling supports HNSCC tumorigenesis

Mirco Compagnone<sup>a</sup>, Veronica Gatti<sup>b</sup>, Dario Presutti<sup>b</sup>, Giovina Ruberti<sup>b</sup>, Claudia Fierro<sup>a</sup>, Elke Katrin Markert<sup>c</sup>, Karen H. Vousden<sup>d</sup>, Huiqing Zhou<sup>e,f</sup>, Alessandro Mauriello<sup>a</sup>, Lucia Anemone<sup>a</sup>, Lucilla Bongiorno-Borbone<sup>a</sup>, Gerry Melino<sup>a,g,1</sup>, and Angelo Peschiaroli<sup>b,1</sup>

<sup>a</sup>Department of Experimental Medicine and Surgery, University of Rome "Tor Vergata", 00133 Rome, Italy; <sup>b</sup>Institute of Cell Biology and Neurobiology, National Research Council of Italy (CNR), 00015 Monterotondo (Rome), Italy; <sup>c</sup>Institute of Cancer Sciences, University of Glasgow, Glasgow, G61 1BD United Kingdom; <sup>d</sup>The Francis Crick Institute, London NW 1AT, United Kingdom; <sup>e</sup>Radboud Institute for Molecular Life Sciences, Department of Human Genetics 855, Radboud University Medical Centre, 6525 GA Nijmegen, The Netherlands; <sup>f</sup>Faculty of Science, Radboud Institute for Molecular Life Sciences, Department of Molecular Developmental Biology, Radboud University, 6525 GA Nijmegen, The Netherlands; and <sup>g</sup>Medical Research Council, Toxicology Unit, Leicester University, Leicester LE1 9HN, United Kingdom

Edited by Vishva M. Dixit, Genentech, San Francisco, CA, and approved October 31, 2017 (received for review July 3, 2017)

**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  $\Delta$ Np63 isoforms is observed in the majority of HNSCCs. Here, we unveiled a  $\Delta$ Np63-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  $\Delta$ Np63 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,  $\Delta$ Np63 directly regulates the expression of CD44, the major HA cell membrane receptor. By controlling this transcriptional program,  $\Delta$ Np63 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. Altogether, our data shed light on a  $\Delta$ Np63-dependent pathway functionally important to the regulation of HNSCC progression.**

HNSCC | p63 | hyaluronic acid

The cross-talk 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 nonsulfated, 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 HA 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-PI, 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).  $\Delta$ Np63 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 carcinomas (SCCs) of the head and neck, skin, lung, and esophagus (13). Moreover, several genetic alterations occurring in head and neck squamous cell carcinoma (HNSCC), such as *NOTCH1* mutation and *ACTL6A* amplification, favors p63 transcriptional activity (14, 15). At molecular level,  $\Delta$ Np63 isoforms, although

## Significance

The p63 isoform  $\Delta$ Np63, 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 a demonstration of a molecular and functional link between the activity of  $\Delta$ Np63 and hyaluronic acid (HA), a major component of the extracellular matrix. We unveiled a  $\Delta$ Np63-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,  $\Delta$ Np63 contributes to the activation of proproliferative and prosurvival pathway in HNSCC. Accordingly, the p63/HA pathway is a negative prognostic factor of HNSCC patient survival.

Author contributions: G.R., A.M., L.A., G.M., and A.P. designed research; M.C., V.G., D.P., C.F., E.K.M., L.A., L.B.-B., and A.P. performed research; H.Z. contributed new reagents/analytic tools; K.H.V. analyzed data; and A.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Published under the PNAS license.

<sup>1</sup>To whom correspondence may be addressed. Email: melino@uniroma2.it or angelo.peschiaroli@cnr.it.

This article contains supporting information online at [www.pnas.org/lookup/suppl/doi:10.1073/pnas.1711777114/-DCSupplemental](http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.1711777114/-DCSupplemental).

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). However,  $\Delta$ Np63 isoforms are also able to repress the transcription of several target genes by different mechanisms (19, 20).

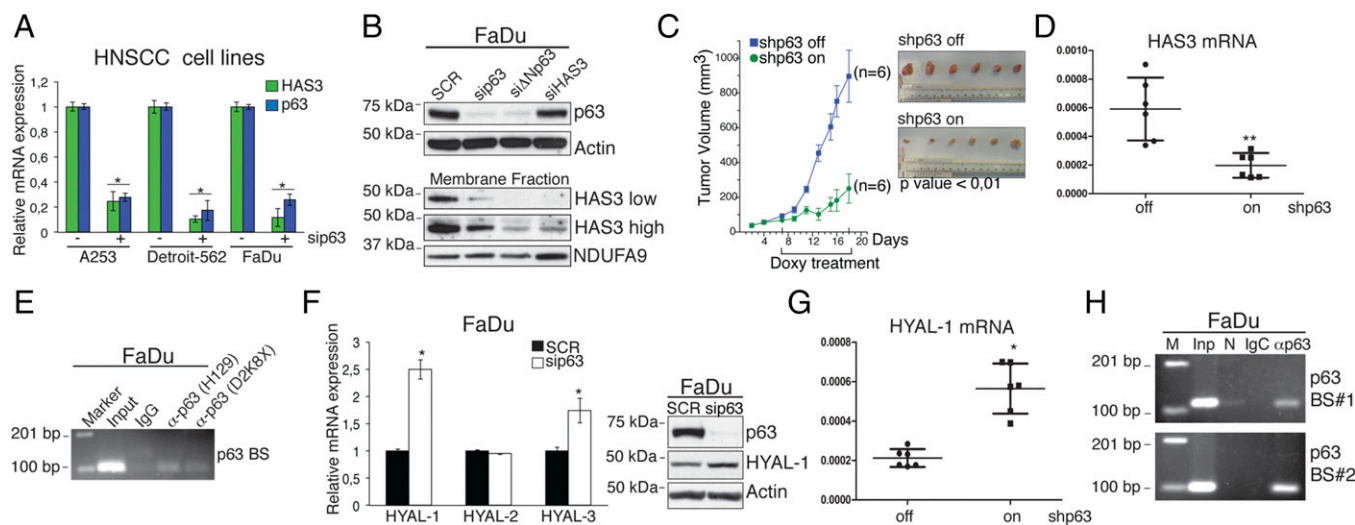
Here, we report evidence demonstrating that  $\Delta$ Np63 supports HNSCC proliferation and chemoresistance by modulating epithelial growth factor receptor (EGF-R) activation and the expression of the ABC transporter ABCC1 in a HA-dependent manner.

## Results

**$\Delta$ Np63 Regulates the Expression of HA Metabolism Genes.** During tumorigenesis, extensive remodeling of the ECM supports and enhances tumor growth. To identify novel p63 target genes potentially regulating ECM in HNSCC, we combined microarray data performed in A253 cell line upon p63 depletion (*Supporting Information*) with coexpression 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 (Fig. S14). Silencing of p63 markedly decreased HAS3 expression in A253, Detroit-562, and FaDu cells (Fig. 1A and Fig. S1B). These HNSCC cell lines mainly express the  $\Delta$ Np63 isoforms (Fig. S1C), suggesting that these p63 isoforms might regulate HAS3 expression. Accordingly, the specific depletion of  $\Delta$ Np63 isoforms efficiently decreased HAS3 expression at mRNA and protein levels (Fig. 1B and Fig. S1D). As a complementary approach, we found that the ectopic expression of  $\Delta$ Np63 $\alpha$  in SCC-9 cells, in which endogenous p63 levels are undetectable (Fig. S1E), dramatically increases HAS3 mRNA (Fig. S1F). We also confirm the p63-dependent regulation of

HAS3 expression in a xenotransplantation experiment. We inoculated FaDu cells stably expressing a doxycycline-inducible shp63 RNA in nude athymic mice. p63 silencing upon doxycycline treatment markedly decreased the proliferative capacity in vitro (Fig. S2) and the growth of xenotransplanted tumors (Fig. 1C). Importantly, the expression of HAS3 mRNA is significantly decreased in p63-silenced tumors (Fig. 1D). To further investigate the mechanism of the  $\Delta$ Np63-dependent regulation of HAS3 expression, we verified whether  $\Delta$ Np63 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) (Fig. S3A). We found that endogenous  $\Delta$ Np63 is able to occupy a p63-binding site (p63 BS) located in the HAS3 promoter site, at  $-5$  kb from the transcription start site (TSS) (Fig. 1E). Furthermore, exogenous  $\Delta$ Np63 $\alpha$  greatly enhanced the HAS3 promoter-mediated luciferase activity (Fig. S3B).

In addition to HAS3, our microarray data revealed that p63 silencing affects HYAL-1 mRNA levels (*Supporting Information*). In this case, we observed that the expression of HYAL-1 is increased upon p63 silencing at both mRNA and protein levels (Fig. 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 (Fig. 1G). HYAL-1 and HYAL-3 genes are clustered together on chromosome 3p21.3, and we found that endogenous  $\Delta$ Np63 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 (Fig. 1H and Fig. S3C). Altogether, these results indicated that the HA synthase HAS3 and the hyaluronidases HYAL-1 and HYAL-3 are bona fide  $\Delta$ Np63 transcriptional target genes in vitro and in vivo.



**Fig. 1.**  $\Delta$ Np63 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 scramble (SCR) or p63 siRNA (sip63) oligos. Bars represent the mean of three technical replicates ( $n = 3$ , PCR runs)  $\pm$  SD and are representative of two independent experiments ( $n = 2$  biological replicates).  $*P < 0.05$ . (B) Total protein lysates or purified membrane-bound proteins of FaDu cells transfected with SCR, sip63,  $\Delta$ Np63 (si $\Delta$ Np63), or HAS3 siRNA oligos (siHAS3) were subjected to immunoblotting 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 ( $n = 6$  per group). The tumor volume growth curves are shown as mean  $\pm$  SEM on the *Left*. Images of the explanted tumors are shown on the *Right*. (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.  $**P < 0.01$ . (E) ChIP analysis of endogenous  $\Delta$ Np63 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*) in FaDu cells transfected as in (A). Bars represent the mean of three technical replicates ( $n = 3$ , PCR runs)  $\pm$  SD and are representative of two independent experiments ( $n = 2$  biological replicates).  $*P < 0.05$ . In parallel, total protein lysates were analyzed by immunoblotting using antibodies to the indicated proteins (*Right*). (G) HYAL-1 mRNA levels were quantified by qRT-PCR in explanted tumors as described in (D). (H) ChIP analysis of endogenous  $\Delta$ Np63 occupancy at the p63 BS#1 and BS#2 of *Hyals* loci.

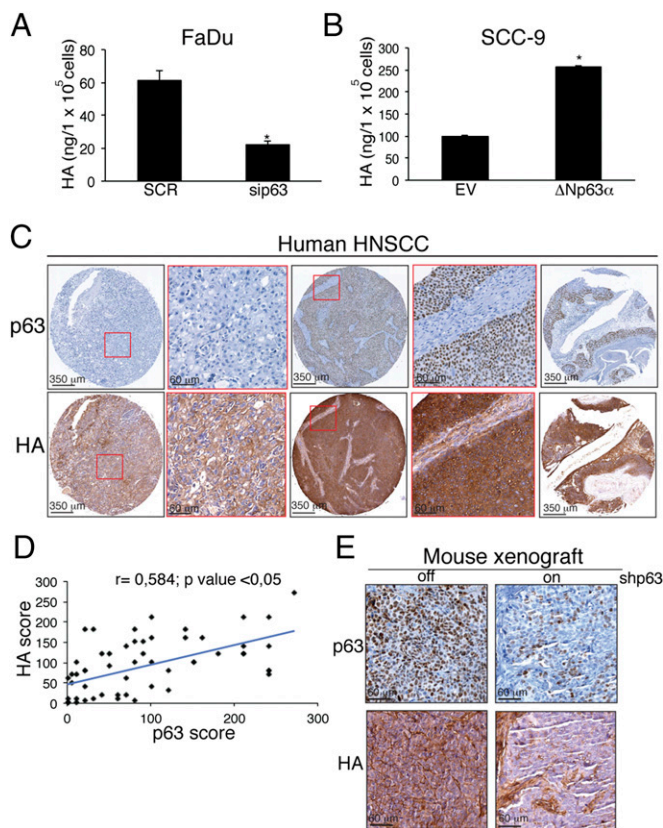
**$\Delta$ Np63 Controls Hyaluronic Acid Levels.** In light of the finding that  $\Delta$ Np63 is able to transcriptionally regulate genes involved in HA metabolism, we tested whether  $\Delta$ Np63 was capable of regulating HA levels. The HA polymer is synthesized by the HA synthases and extruded into the pericellular or the extracellular space. We stained pericellular HA by immunofluorescence using the high-affinity HA binding protein HABP, and we found that  $\Delta$ Np63 depletion decreases the levels of pericellular HA (Fig. S4A). To quantify this effect, we measured HA levels in cell medium by ELISA, and we confirmed that  $\Delta$ Np63-depleted cells display a marked reduction of HA levels in FaDu and A253 cells (Fig. 2A and Fig. S4B), similarly to the HAS3-silenced cells (Fig. S4C). Conversely, we found that the ectopic expression of  $\Delta$ Np63 $\alpha$  isoform increases HA cellular levels (Fig. 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 pretreating the section with *Streptomyces hyaluronidase* (Fig. 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 (Fig. 2C and D). In support of these data, we found that HA tumor-associated levels are markedly decreased in xenograft tumors upon silencing of p63 (Fig. 2E). Collectively, these results indicated that  $\Delta$ Np63 is able to regulate HA cellular levels, likely via transcriptional regulation of HAS3.

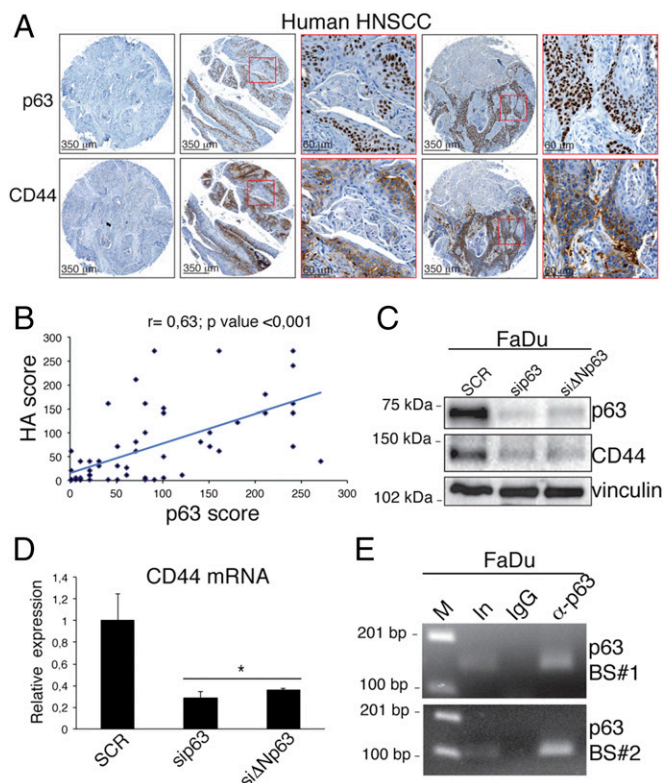
**$\Delta$ Np63 Directly Induces the Expression of the HA Receptor CD44.** CD44 is the most tumor-relevant HA receptor, and downregulation of the expression of its variant isoforms is associated with advanced diseases and chemoresistance in HNSCC (4, 21). By analyzing coexpression data in esophageal and lung squamous carcinoma datasets, we found that CD44 mRNA expression is positively correlated with that of TP63 (Fig. 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 (Fig. 3A and B). Importantly, gain or loss of function of  $\Delta$ Np63 expression markedly affected the expression of CD44 at mRNA and protein levels (Fig. 3C and D and Fig. S5B and C). Conversely, the expression of RHAMM, another well-characterized HA receptor, is not affected by  $\Delta$ Np63 depletion (Fig. S5D). These transcriptional effects are likely due to a direct binding of  $\Delta$ Np63 on the *CD44* locus. Indeed, by ChIP assay, we demonstrated the ability of endogenous  $\Delta$ Np63 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 (Fig. 3E and Fig. S5E). All together these results demonstrated that  $\Delta$ Np63 is able to directly regulate the expression of CD44 by physically recognizing two p63-binding elements located in the *CD44* locus.

**The  $\Delta$ Np63-HA Pathway Regulates EGF-R Activation.** Since the HA/CD44 interaction promotes the activation of receptor tyrosine kinases (RTKs) (22, 23), we tested whether  $\Delta$ Np63 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 (Fig. 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 (Fig. 4A and Fig. S6B). In addition to EGF-R phosphorylation,  $\Delta$ Np63 depletion reduced the phosphorylation of ErbB2, an ErbB family member whose activation is stimulated by the HA/CD44 complex (23).

To investigate whether the  $\Delta$ Np63-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 (Fig. 4B and Fig. S6C). Similarly, depletion of HAS3 gene expression by siRNA decreased EGF-R phosphorylation in HNSCC cells (Fig. 4C and Fig. S6D). In a reverse experiment, overexpression of  $\Delta$ Np63 $\alpha$  or HAS3 increased EGF-R phosphorylation (Fig. 4D and Fig. S6E). Importantly, HAS3 inhibition by 4-MU treatment reverted the ability of exogenous  $\Delta$ Np63 to enhance the activation of EGF-R, Akt, and ERK1/2 (Fig. 4E). Since EGF-R transduction pathway activates proliferative signals, we measured the proliferative capabilities of  $\Delta$ Np63 or HAS3 silenced cells by a cell counting assay. We found that the siRNA-mediated depletion of HAS3 results in a decrease of cell proliferation (Fig. 4F). All together, these observations indicated that  $\Delta$ Np63 favors the activation of oncogenic signals of EGFR by, at least in part, modulating the HA/CD44 pathway.



**Fig. 2.**  $\Delta$ Np63 controls HA levels. (A) FaDu cells were transfected with scramble (SCR) or p63 siRNA oligos (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 ( $1 \times 10^5$ ). Bars represent the mean of three technical replicates ( $n = 3$ )  $\pm$  SD and are representative of two independent experiments ( $n = 2$  biological replicates). \* $P < 0.05$ . (B) SCC-9 cells infected with empty or  $\Delta$ Np63-expressing lentiviral particles were analyzed as in A. (C) Representative images of IHC analysis of p63 and HA staining in HNSCC tissues. Red boxes indicate higher-magnification images. (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.



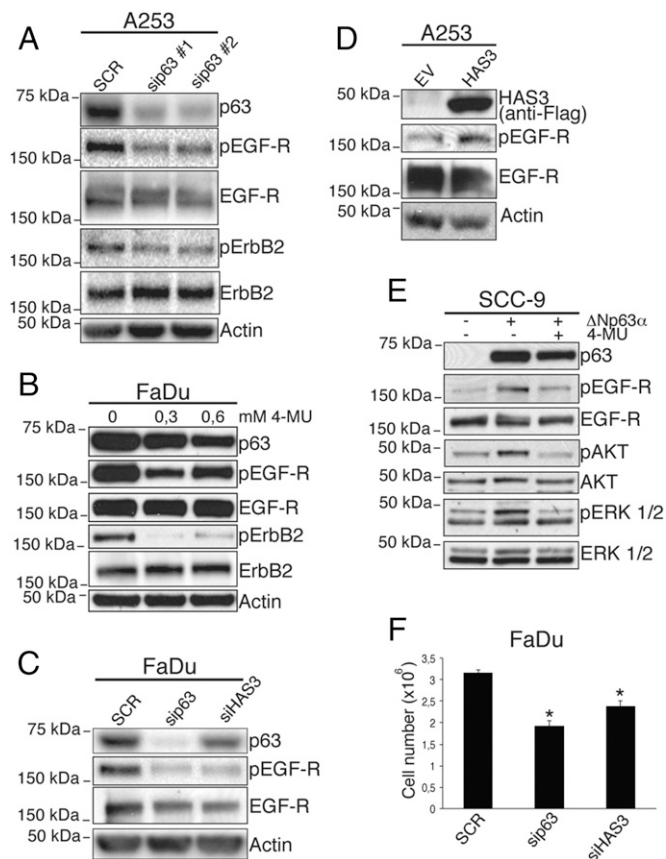
**Fig. 3.** ΔNp63 regulates the expression of the HA receptor CD44. (A) Representative images of IHC analysis of CD44 and TP63 expression in HNSCC tissues. Red boxes indicate higher-magnification images. (B) Correlation plot of p63 and CD44 H-scores in 56 human HNSCC tumors including 49 cases of squamous 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 scramble (SCR) p63 (*si*p63), or ΔNp63-specific siRNA oligos (*si*ΔNp63) 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)  $\pm$  SD and are representative of two independent experiments ( $n = 2$  biological replicates). \* $P < 0.05$ . (E) ChIP analysis of endogenous ΔNp63 occupancy at the p63 BS#1 and BS#2 in the *CD44* locus.

**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 antineoplastic agents (21). Therefore, we tested whether deregulation of the ΔNp63-HA axis might impact the chemosensitivity of HNSCC cells toward the cytotoxic effect of sublethal doses of doxorubicin and cisplatin. We found that upon chemotherapeutic agent treatment, p63 or HAS3 silencing increases the percentage of apoptotic cells and the cleavage of PARP, an apoptotic marker (Fig. 5A and Fig. S7A). A similar effect was observed in cells treated with the HAS inhibitor 4-MU (Fig. S7B). As a complementary approach, we tested whether the ectopic expression of ΔNp63 might exert a prosurvival 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 (Fig. 5B and Fig. S7C and D), suggesting that the ΔNp63-mediated prosurvival action is, at least in part, mediated by its ability to regulate HA metabolism.

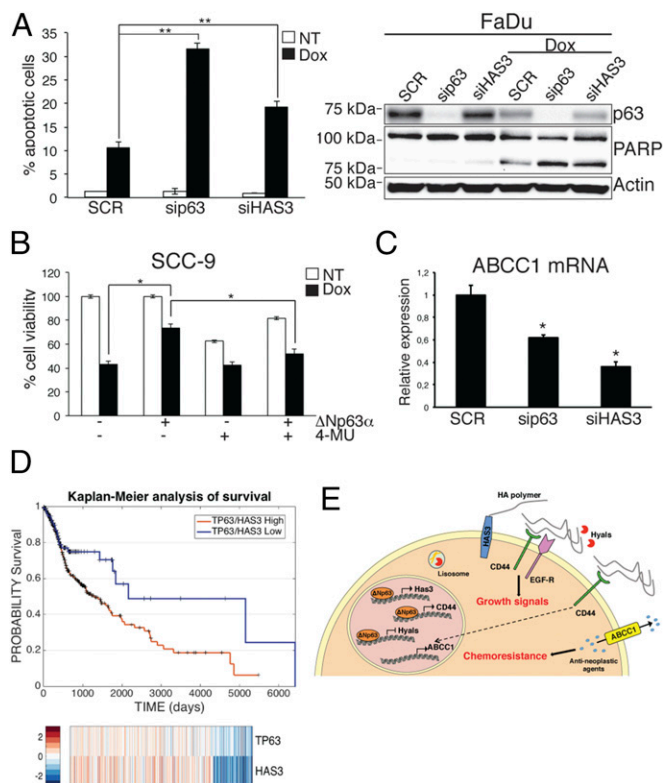
One potential mechanism underlying the chemoresistance action of the HA/CD44 interaction is through the modulation of the ATP-binding cassette (ABC) drug transporter expression (25, 26). Therefore, we screened HNSCC cell lines for the expression of different ABC transporters, and we identified ABCB1 as the

main ABC transporter expressed in HNSCC cell lines (Fig. S7E). Notably, ΔNp63 depletion, similarly to HAS3 depletion, reduced ABCB1 mRNA levels in HNSCC cells (Fig. 5C). On the contrary, the expression of ABCB3 transporter was not affected by ΔNp63 or HAS3 depletion (Fig. S7F). In agreement with these data, we found that TP63/ABCC1 and HAS3/ABCC1 coexpression is positively correlated in HNSCC dataset as well as in esophageal SCC (Fig. 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 coexpression and those with low coexpression (Fig. 5D, Lower). We found that patients displaying high p63 and HAS3 coexpression show a decrease of the overall survival compared with those with low expression (Fig. 5D). All together, these findings indicated that p63-HA pathway is a negative



**Fig. 4.** ΔNp63-HA pathway favors receptor tyrosine kinase activation. (A) A253 cells were transfected with scramble (SCR) or two distinct siRNA oligos targeting different regions of p63 mRNA (*si*p63#1 and *si*p63#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-MU for 24 h, and then protein lysates were immunoblotted for the indicated proteins. (C) FaDu cells were transfected with SCR, HAS3 (*si*HAS3), or *si*p63, and after 48 h, protein lysates were immunoblotted for the indicated proteins. (D) A253 cells were infected with Flag-tagged HAS3 expressing viral particles, and after 48 h, protein lysates were immunoblotted for the indicated proteins. (E) SCC-9 cells were infected with empty or ΔNp63 expressing lentiviral particles. Twenty-four hours after infection, cells were treated with 4-MU for 24 h and then protein lysates were immunoblotted for the indicated proteins. (F) FaDu cells were plated in 6 wells ( $8 \times 10^5$  cells per well), and after 24 h, transfected with SCR, *si*HAS3, or *si*p63. Six days after transfection, cells were counted by Trypan blue dye exclusion in triplicate. Data are shown as the mean  $\pm$  SD ( $n = 3$ ); \* $P < 0.05$ .



**Fig. 5.**  $\Delta$ Np63-HA pathway regulates HNSCC chemoresistance, and it is a negative prognostic factor of HNSCC patient survival. (A) FaDu cells transfected with scramble (SCR), p63 (sip63), or HAS3 siRNA oligos (siHAS3) were treated with doxorubicin (0.4  $\mu$ M) for 48 h. Cells were then stained with propidium iodide (PI) and fluorodiacetate (FDA), and the percentage of apoptotic cells was determined by FACS (Left). Data are shown as the mean  $\pm$  SD of three biological replicates ( $n = 3$ ).  $**P < 0.01$ . In parallel, lysates obtained from treated cells were immunoblotted for the indicated proteins (Right). (B) SCC-9 cells were lentivirally infected with empty or  $\Delta$ Np63-expressing particles. Forty-eight hours after infection, cells were concomitantly treated with doxorubicin (0.4  $\mu$ M) and 4-MU (0.3 mM) for 48 h, and then cell viability was measured by Celltiter-Glo. Data are shown as the mean  $\pm$  SD of three technical replicates ( $n = 3$ ).  $*P \leq 0.05$ . (C) A253 cells were transfected with SCR, sip63, or siHAS3, and 48 h after transfection, ABCC1 mRNA levels were measured by qRT-PCR. Data are shown as the mean  $\pm$  SD of three technical replicates ( $n = 3$ , PCR runs) and are representative of two independent experiments ( $n = 2$  biological replicates).  $*P < 0.05$ . (D) Patient samples from the TCGA cohort were clustered into two groups displaying low and high TP63 and HAS3 expression, respectively, and analyzed for survival ( $P$  value: 0.0068). Clustered expression values relative to mean expression are shown in the heat map Pearson's correlation coefficient (PCC): 0.5959;  $P$  value: 9.9639e-51. (E) Schematic model of the  $\Delta$ Np63-mediated regulation of the HA metabolism and signaling.

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 an HA-related transcriptional program directly regulated by  $\Delta$ Np63 in HNSCC, but also provide a demonstration that this program is functionally important to molecularly link  $\Delta$ Np63 activity with two critical pathways in HNSCC biology: EGF-R signaling and chemoresistance. We demonstrated that  $\Delta$ Np63 sustains HA signaling by directly regulating the expression of HAS3, HYALs, and CD44 (see proposed model, Fig. 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 that HAS3 expression is under control of the transcription factor  $\Delta$ Np63.

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 protumorigenic HA polymers (35), which are subsequently degraded by HYAL-1 in tetrasaccharides (7). Therefore, it is possible that  $\Delta$ Np63, by inhibiting the expression of HYAL-1, maintains the high levels of the 20-kDa protumorigenic fragments of HA.

In addition to regulating enzymes involved in the HA metabolic process, we also showed that  $\Delta$ Np63 directly regulates the expression of CD44, the major cell membrane HA receptor. Although several published studies have suggested a potential molecular link between  $\Delta$ Np63 activity and CD44 in breast cancer (36, 37), our data demonstrate that  $\Delta$ Np63 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  $\Delta$ Np63 activity is linked to the downregulation of EGF-R signaling (18, 38). In detail, it has been demonstrated that  $\Delta$ Np63 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  $\Delta$ Np63-dependent regulation of EGF-R transcription might be tissue-specific, as previously suggested (38). Conversely, we propose a HA-dependent mechanism, which molecularly links  $\Delta$ Np63 activity and EGF-R signaling. We found that in HNSCC cells the  $\Delta$ Np63-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,  $\Delta$ Np63-HAS3 pathway might be sustained by a positive feedback by the EGF-R signaling in HNSCC.

HA-CD44 signaling also promotes resistance to multiple antineoplastic agents, including cisplatin, methotrexate, and doxorubicin (40, 41). We found that the regulation of HA metabolism and signaling mediates, at least in part, the  $\Delta$ Np63 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  $\Delta$ Np63-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 prechemotherapy 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  $\Delta$ Np63 or HAS3 depletion decreases the expression of ABCC1. Importantly, both HAS3 and  $\Delta$ Np63 expression are positively

correlated with ABCC1 expression levels in human HNSCC primary tumors. Although we cannot rule out that the prosurvival effect of  $\Delta$ Np63 can be due to additional pathways, including the HA-dependent modulation of EGF-R signaling proposed here, our data established the existence of a functional connection between  $\Delta$ Np63 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 TMA was purchased by US Biomax, Inc (HN802a). 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 [Supporting Information](#). HNSCC tissue sample shown in [Fig. S4D](#) has been utilized under approval by the institutional review board of University Hospital "Policlinico Tor Vergata" with prior patient consent.

- Junttila MR, de Sauvage FJ (2013) Influence of tumour micro-environment heterogeneity on therapeutic response. *Nature* 501:346–354.
- Toole BP (2004) Hyaluronan: From extracellular glue to pericellular cue. *Nat Rev Cancer* 4:528–539.
- Vigetti D, et al. (2014) Hyaluronan: Biosynthesis and signaling. *Biochim Biophys Acta* 1840:2452–2459.
- Naor D, Wallach-Dayana SB, Zahalka MA, Sionov RV (2008) Involvement of CD44, a molecule with a thousand faces, in cancer dissemination. *Semin Cancer Biol* 18: 260–267.
- Törrönen K, et al. (2014) Tissue distribution and subcellular localization of hyaluronan synthase isoenzymes. *Histochem Cell Biol* 141:17–31.
- Stern R (2004) Hyaluronan catabolism: A new metabolic pathway. *Eur J Cell Biol* 83: 317–325.
- Stern R, Jedrzejak MJ (2006) Hyaluronidases: Their genomics, structures, and mechanisms of action. *Chem Rev* 106:818–839.
- Chanmee T, Ontong P, Itano N (2016) Hyaluronan: A modulator of the tumor microenvironment. *Cancer Lett* 375:20–30.
- Liu N, et al. (2001) Hyaluronan synthase 3 overexpression promotes the growth of TSU prostate cancer cells. *Cancer Res* 61:5207–5214.
- Li P, et al. (2015) Hyaluronan synthase 2 overexpression is correlated with the tumorigenesis and metastasis of human breast cancer. *Int J Clin Exp Pathol* 8: 12101–12114.
- Levine AJ, Tomasini R, McKeon FD, Mak TW, Melino G (2011) The p53 family: Guardians of maternal reproduction. *Nat Rev Mol Cell Biol* 12:259–265.
- Yang A, et al. (1998) p63, a p53 homolog at 3q27–29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol Cell* 2: 305–316.
- Rothenberg SM, Ellisen LW (2012) The molecular pathogenesis of head and neck squamous cell carcinoma. *J Clin Invest* 122:1951–1957.
- Agrawal N, et al. (2011) Exome sequencing of head and neck squamous cell carcinoma reveals inactivating mutations in NOTCH1. *Science* 333:1154–1157.
- Saladi SV, et al. (2017) ACTL6A is co-amplified with p63 in squamous cell carcinoma to drive YAP activation, regenerative proliferation, and poor prognosis. *Cancer Cell* 31: 35–49.
- Memmi EM, et al. (2015) p63 sustains self-renewal of mammary cancer stem cells through regulation of Sonic Hedgehog signaling. *Proc Natl Acad Sci USA* 112: 3499–3504.
- Giacobbe A, et al. (2016) p63 controls cell migration and invasion by transcriptional regulation of MTSS1. *Oncogene* 35:1602–1608.
- Carroll DK, et al. (2006) p63 regulates an adhesion programme and cell survival in epithelial cells. *Nat Cell Biol* 8:551–561.
- Gallant-Behm CL, et al. (2012)  $\Delta$ Np63 $\alpha$  represses anti-proliferative genes via H2A.Z deposition. *Genes Dev* 26:2325–2336.
- Ramsey MR, He L, Forster N, Ory B, Ellisen LW (2011) Physical association of HDAC1 and HDAC2 with p63 mediates transcriptional repression and tumor maintenance in squamous cell carcinoma. *Cancer Res* 71:4373–4379.
- Misra S, et al. (2011) Hyaluronan-CD44 interactions as potential targets for cancer therapy. *FEBS J* 278:1429–1443.
- Misra S, Toole BP, Ghatak S (2006) Hyaluronan constitutively regulates activation of multiple receptor tyrosine kinases in epithelial and carcinoma cells. *J Biol Chem* 281: 34936–34941.
- Ghatak S, Misra S, Toole BP (2005) Hyaluronan constitutively regulates ErbB2 phosphorylation and signaling complex formation in carcinoma cells. *J Biol Chem* 280: 8875–8883.
- Kakizaki I, et al. (2004) A novel mechanism for the inhibition of hyaluronan biosynthesis by 4-methylumbelliferone. *J Biol Chem* 279:33281–33289.

**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 [Supporting Information](#). Details of all other methods are also described in [Supporting Information](#).

**Ethics Statement.** Animals were subjected to experimental protocols (authorization no. 872/2015-PR) approved by the Veterinary Department of the Italian Ministry of Health, and experiments were conducted according to the ethical and safety rules and guidelines for the use of animals in biomedical research provided by the relevant Italian law and European Union Directive (Italian Legislative Decree 26/2014 and 2010/63/EU) and the International Guiding Principles for Biomedical Research involving animals (Council for the International Organizations of Medical Sciences, Geneva). All adequate measures were taken to minimize animal pain or discomfort.

**ACKNOWLEDGMENTS.** We thank F. Bernassola for kindly providing the pLENTI-HA- $\Delta$ Np63 $\alpha$  vector and I. Amelio for assistance in gene microarray analysis. This work has been supported by the My First AIRC (Italian Association for Cancer Research) Grant MFAG-15523 (to A.P.), Medical Research Council (United Kingdom), AIRC Grant IG-15653 (to G.M.), Project FaReBio di Qualità, and a CNR grant from the Italian Ministry of Economy and Finance (to G.R.).

- Bourguignon LY, Peyrollier K, Xia W, Gilad E (2008) Hyaluronan-CD44 interaction activates stem cell marker Nanog, Stat-3-mediated MDR1 gene expression, and ankyrin-regulated multidrug efflux in breast and ovarian tumor cells. *J Biol Chem* 283: 17635–17651.
- Misra S, Ghatak S, Toole BP (2005) Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. *J Biol Chem* 280:20310–20315.
- Sacco AG, Cohen EE (2015) Current treatment options for recurrent or metastatic head and neck squamous cell carcinoma. *J Clin Oncol* 33:3305–3313.
- Reis-Filho JS, et al. (2003) Distribution of p63, cytokeratins 5/6 and cytokeratin 14 in 51 normal and 400 neoplastic human tissue samples using TARP-4 multi-tumor tissue microarray. *Virchows Arch* 443:122–132.
- Kultti A, et al. (2014) Accumulation of extracellular hyaluronan by hyaluronan synthase 3 promotes tumor growth and modulates the pancreatic cancer microenvironment. *BioMed Res Int* 2014:817613.
- Koyama H, et al. (2007) Hyperproduction of hyaluronan in neu-induced mammary tumor accelerates angiogenesis through stromal cell recruitment: Possible involvement of versican/PG-M. *Am J Pathol* 170:1086–1099.
- Tian X, et al. (2013) High-molecular-mass hyaluronan mediates the cancer resistance of the naked mole rat. *Nature* 499:346–349.
- Itano N, et al. (1999) Three isoforms of mammalian hyaluronan synthases have distinct enzymatic properties. *J Biol Chem* 274:25085–25092.
- Tofuku K, Yokouchi M, Murayama T, Minami S, Komiya S (2006) HAS3-related hyaluronan enhances biological activities necessary for metastasis of osteosarcoma cells. *Int J Oncol* 29:175–183.
- Twarock S, et al. (2011) Inhibition of oesophageal squamous cell carcinoma progression by in vivo targeting of hyaluronan synthesis. *Mol Cancer* 10:30.
- West DC, Hampson IN, Arnold F, Kumar S (1985) Angiogenesis induced by degradation products of hyaluronic acid. *Science* 228:1324–1326.
- Di Franco S, et al. (2016)  $\Delta$ Np63 drives metastasis in breast cancer cells via PI3K/CD44v6 axis. *Oncotarget* 7:54157–54173.
- Godar S, et al. (2008) Growth-inhibitory and tumor-suppressive functions of p53 depend on its repression of CD44 expression. *Cell* 134:62–73.
- Danilov AV, et al. (2011) DeltaNp63 $\alpha$ -mediated induction of epidermal growth factor receptor promotes pancreatic cancer cell growth and chemoresistance. *PLoS One* 6:e26815.
- Bourguignon LY, Gilad E, Peyrollier K (2007) Heregulin-mediated ErbB2-ERK signaling activates hyaluronan synthases leading to CD44-dependent ovarian tumor cell growth and migration. *J Biol Chem* 282:19426–19441.
- Wang SJ, Bourguignon LY (2011) Role of hyaluronan-mediated CD44 signaling in head and neck squamous cell carcinoma progression and chemoresistance. *Am J Pathol* 178:956–963.
- Torre C, Wang SJ, Xia W, Bourguignon LY (2010) Reduction of hyaluronan-CD44-mediated growth, migration, and cisplatin resistance in head and neck cancer due to inhibition of Rho kinase and PI-3 kinase signaling. *Arch Otolaryngol Head Neck Surg* 136:493–501.
- Langer R, et al. (2007) Comparison of pretherapeutic and posttherapeutic expression levels of chemotherapy-associated genes in adenocarcinomas of the esophagus treated by 5-fluorouracil- and cisplatin-based neoadjuvant chemotherapy. *Am J Clin Pathol* 128:191–197.
- Presutti D, et al. (2015) MET gene amplification and MET receptor activation are not sufficient to predict efficacy of combined MET and EGFR inhibitors in EGFR TKI-resistant NSCLC cells. *PLoS One* 10:e0143333.
- Kouwenhoven EN, et al. (2010) Genome-wide profiling of p63 DNA-binding sites identifies an element that regulates gene expression during limb development in the 7q21 SHFM1 locus. *PLoS Genet* 6:e1001065.