ARTICLE
Cellular and Molecular Biology

Synergistic antitumour activity of HDAC inhibitor SAHA and EGFR inhibitor gefitinib in head and neck cancer: a key role for ΔNp63α

Simona Citro¹, Alice Bellini¹, Claudia Miccolo¹, Lavinia Ghiani¹, Thomas E. Carey² and Susanna Chiocca¹

BACKGROUND: Epidermal growth factor receptor (EGFR) overexpression is associated with the development of head and neck cancer (HNC) and represents one of the main therapeutic targets for this disease. The use of EGFR inhibitors has limited efficacy due to their primary and acquired resistance, partially because of increased epithelial to mesenchymal transition (EMT). The HDAC inhibitor SAHA has been shown to revert EMT in different tumours, including HNC. In this study, we investigated the cooperative role of SAHA and the EGFR tyrosine kinase inhibitor gefitinib in both HPV-positive and HPV-negative HNC cell lines.

METHODS: A panel of 12 HPV-positive and HPV-negative HNC cell lines were screened for cell viability upon treatment with SAHA, gefitinib and the combination of the two. Epithelial/mesenchymal marker expression, as well as activation of signalling pathway, were assessed upon SAHA treatment. ΔNp63α silencing with shRNA lentiviral particles was used to determine its role in cell proliferation, migration and TGFβ pathway activation.

RESULTS: We found that both SAHA and gefitinib have antitumour activity in both HPV-positive and HPV-negative HNC cell lines and that their combination has a synergistic effect in inhibiting cell growth. SAHA treatment reverts EMT and inhibits the expression of the transcription factor ΔNp63α. Suppression of ΔNp63α reduces EGFR protein levels and decreases cell proliferation and TGFβ-dependent migration in both HPV-positive and HPV-negative HNC cell lines.

CONCLUSIONS: Our results, by giving a clear molecular mechanism at the basis of the antitumour activity of SAHA in HNC cell lines, provide a rationale for the clinical evaluation of SAHA in combination with gefitinib in both HPV-positive and HPV-negative HNC patients. Further knowledge is key to devising additional lines of combinatorial treatment strategies for this disease.

British Journal of Cancer (2019) 120:658–667; https://doi.org/10.1038/s41416-019-0394-9

BACKGROUND
Head and neck cancer (HNC) includes malignant squamous lesions arising in the oral cavity, oropharynx, larynx or hypopharynx, and is the sixth leading cancer by incidence worldwide, with approximately 500,000 new cases annually and only 40–50% 5-year survival rate.¹ The use of tobacco and excessive alcohol consumption are the most important risk factors identified and they also seem to have a synergistic effect. A subgroup of HNC, particularly those of the oropharynx, is caused by infection with high-risk types of human papillomavirus (HPV). Although in the past decade the incidence of HNC has been slowly declining in the western world, probably due to a decrease in the prevalence of smoking, oral tongue and particularly oropharyngeal cancers are becoming more prevalent. This may be related to an increase in oropharyngeal HPV infections and recent studies revealed that HPV16 in particular is involved in these tumours. HPV-positive tumours form a distinct group within HNC with different aetiopathological factors. These tumours are different at the molecular level, changing the clinical outcome and presenting a better prognosis.² Despite their diversities, HPV-positive and HPV-negative HNC patients are treated using the same therapies, leading the scientific and medical community to reassess the current treatment protocols, in order to develop less toxic strategies while maintaining good oncological outcomes. Recently, the anti-PD-1 antibodies nivolumab and pembrolizumab have been approved as targeted therapies for the treatment of HNC,³ as well as cetuximab. Cetuximab, an anti-epidermal growth factor receptor (EGFR) monoclonal antibody, has been approved for the treatment of HNC in patients with locally advanced tumours in association with radiotherapy and in patients with recurrent or metastatic diseases in combination with cisplatin-based chemotherapy.⁴,⁵ Small-molecule tyrosine kinase inhibitors (TKIs) such as gefitinib, which prevent the binding of ATP to the receptor and thereby inactivate EGFR, have also been investigated for HNC in preclinical and clinical settings, showing tumour growth delay and enhancement of apoptosis⁶ and single-agent activity in recurrent and metastatic disease.⁷ Although overexpression of EGFR has been observed in about 90% of HNC specimens and correlates with poor prognosis, advanced disease and reduced

¹Department of Experimental Oncology, IEO, European Institute of Oncology IRCCS, Via Adamello 16, 20139 Milan, Italy and ²Department of Otolaryngology-Head and Neck Surgery, Department of Pharmacology, Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI, USA
Correspondence: Simona Citro (simona.citro@ieo.it) or Susanna Chiocca (susanna.chiocca@ieo.it)
Lead author: Susanna Chiocca

Received: 31 July 2018 Revised: 15 January 2019 Accepted: 18 January 2019
Published online: 15 February 2019

© Cancer Research UK 2019
survival, cetuximab treatment has yielded only modest clinical outcomes as a monotherapy.\textsuperscript{17} Taken together, this suggests that primary and acquired resistance mechanisms considerably limit the clinical benefit of cetuximab in HNC,\textsuperscript{8} which translates in a clear need to understand the molecular mechanisms driving cetuximab resistance to maximise the treatment response by patient selection and to establish new treatment options to overcome resistance.

Epithelial to mesenchymal transition (EMT) is known to be deeply involved in cancer progression and metastasis. EMT is characterised by the loss of proteins involved in cell junctions such as E-cadherin, and the expression of mesenchymal markers such as vimentin.\textsuperscript{8} Acquisition of EMT features has also been associated with chemoresistance acquired after standard chemotherapy.\textsuperscript{21} Activation of both transforming growth factor β (TGFβ) signalling and receptor tyrosine kinase plays critical roles in EMT either through the downstream SMAD signalling or through a SMAD-independent pathway, such as activation of ERK MAP kinases, Rho GTPases and the PI3 kinase/Akt pathway.\textsuperscript{11} Cancer cells can increase their production of active TGFβ during development of EGFR-TKI resistance.\textsuperscript{12} TGFβ triggers EMT and allows the cells to become invasive. EMT seems to also play a role in establishing the resistance to EGFR TKIs in HNC.\textsuperscript{13}

In the last years, histone deacetylase inhibitors (HDACi) have been described as valid tools to overcome EMT in many different tumours,\textsuperscript{12,14,15} including HNC,\textsuperscript{16} whereas in other types of cancer the use of HDACi has been shown to increase EMT.\textsuperscript{17,19} HDACs are typically overexpressed in cancers, such as HNC,\textsuperscript{20} altering gene transcription and enhancing cell proliferation. To date, several HDACi have been developed and used in clinical trials for the treatment of cancers, and have been shown to induce differentiation, growth arrest or apoptosis in tumour cells.\textsuperscript{21,22} Currently, the US Food and Drug Administration has approved four types of HDACi for cancer therapy, including vorinostat (SAHA, Zolinza)\textsuperscript{23} for the treatment of refractory cutaneous T-cell lymphoma (CTCL), romidepsin (Istodax)\textsuperscript{24} for the treatment of CTCL and peripheral-cell lymphoma (PTCL), belinostat (Beleodaq) for the treatment of PTCL\textsuperscript{25} and panobinostat (Farydak) for the treatment of multiple myeloma.\textsuperscript{26,27} Recently, HDACi have been shown to reduce ΔNp63α protein stability in two cutaneous squamous cell lines.\textsuperscript{28} The p63 isoform ΔNp63α, member of the p53 family, is a transcription factor essential for terminal differentiation of stratified epithelia.\textsuperscript{29} ΔNp63α is the predominant p63 isoform expressed in normal squamous epithelia and squamous carcinomas and its expression is upregulated in up to 80% of primary HNC tumours.\textsuperscript{30} Many studies have highlighted the oncogenic potential of ΔNp63α in HNC promoting squamous epithelial proliferation, migration and inflammation\textsuperscript{31,32} and regulating EMT in primary human keratinocytes and in HNC cell lines in a TGFβ-dependent manner.\textsuperscript{33,34} Moreover, HDACi, alone or in combination, have been shown to reduce EGFR expression in HNC cell lines.\textsuperscript{16,35}

In this study we investigated the molecular mechanisms at the basis of the antitumour activity of the HDAC inhibitor SAHA and EGFR inhibitor...
RESULTS

Antiproliferative effect of SAHA and gefitinib and their synergistic activity in both HPV-positive and HPV-negative HNC cell lines

We screened the effect of both SAHA and gefitinib on cell viability in a panel of 12 HNC cell lines, 6 of them deriving from HPV-positive patients (Table S1). As shown in Table 1, cells were differentially sensitive to SAHA and gefitinib independently of the HPV status. In particular, the UPCI:SCC-90 and UD-SCC-2 cell lines responded differently upon drug treatment, despite they are both HPV-positive and have a mesenchymal phenotype as shown by the E-cadherin and vimentin expression levels (Figure S1A). Moreover, treating the cell lines most resistant to gefitinib, upon combination of SAHA and gefitinib, we could clearly appreciate a synergistic effect of the two drugs together, independently from the HPV status (Table 2, CI index). Thus, we showed that SAHA and gefitinib have an inhibitory and synergistic activity in HNC cell lines, which seems neither related to the HPV status of HNC cell lines nor to their epithelial/mesenchymal phenotype.

SAHA treatment reverts EMT in both HPV-positive and HPV-negative HNC cell lines, inhibits TGFβ pathway activation and decreases the expression of ΔNp63α

To understand the molecular mechanisms triggering the inhibitory effect of SAHA on HNC cell lines, we tested the ability of this drug in reverting the EMT phenotype, as already described in HNC HPV-negative cell lines. We confirmed these data also in HPV-positive cell lines (Fig. 1a, b), showing that SAHA was able to significantly increase the epithelial marker E-cadherin, both at mRNA and protein level, partially decreasing the protein expression of the mesenchymal marker vimentin. Moreover, as shown in figure S1B, SAHA inhibited the activation of two main proliferative and migratory signalling pathways, such as PI3K and ERK1/2. SAHA was also able to decrease protein expression of the most abundant p63 isofrom in these cell lines, ΔNp63α, in a post-transcriptional way (Fig. 1a, b), independently of the HPV status. As shown in Fig. 1a, b, UM-SCC-47 cell line does not express full-length ΔNp63α, due to the multiple integration of HPV16 at the TP63 locus, leading to the expression of a truncated 25-kDa protein at the carboxyl terminus of p63. We then further investigated the role of SAHA in reverting EMT by stimulating HNC cell lines with TGFβ, which pathway is known to be upregulated during EGFR inhibition resistance. As shown in Fig. 1, SAHA was able to attenuate the effect of TGFβ by both reducing the activation of one of the main players of the TGFβ pathway, SMAD2

| Table 1. Half maximal inhibitory concentration values for SAHA and gefitinib (µM) |
|-----------------|-----------------|-----------------|
| Cell lines      | IC50 SAHA       | IC50 gefitinib  |
| UMC-SCC-4       | 2.23            | 2.51            |
| UMC-SCC-6       | 3.46            | 0.04            |
| UMC-SCC-10A     | 4.74            | 0.47            |
| UMC-SCC-18      | 0.84            | 0.0089          |
| UMC-SCC-19      | 2.44            | 0.98            |
| UMC-SCC-23      | 1.04            | 2.57            |
| UPCI:SCC-90     | 3.75            | 8.43            |
| UPCI:SCC-90     | 1.55            | 0.37            |
| UPCI:SCC-90     | 1.84            | 0.058           |
| UPCI:SCC-154    | 2.44            | 14.3            |
| UD-SCC-2        | 5.28            | 6.96            |
| 93-VU147T       | 3.23            | 0.13            |

Mean of at least three different experiments done in duplicate

IC50 half maximal inhibitory concentration, HPV human papillomavirus
SAHA exerts its inhibitory activity through the reduction of Np63α and EGFR expression.

Since SAHA was able to reduce ΔNp63α expression in these cells, we further analysed whether this effect triggers the inhibitory effect of SAHA. We thus conducted a quantification analysis calculating the percentage of inhibition of p63 protein expression in all the cell lines (Fig. 2a) and compared it to the SAHA IC50 (Table 1), finding a great inverse correlation between the two values (Fig. 2b). These data were then confirmed by showing that in the cell line most sensitive to SAHA, UM-SCC-18, SAHA significantly decreased ΔNp63α expression at lower concentrations compared to the more resistant cell line UD-SCC-2, in which a higher concentration of SAHA was needed to considerably inhibit ΔNp63α expression (Fig. 2c). Indeed, in UM-SCC-4 cell line with a moderate sensitivity to SAHA the effect on ΔNp63α expression was halfway (Fig. 2c). Moreover, SAHA treatment was able to decrease the expression of the EGFR receptor both at mRNA and protein level (Fig. 2d and 2c), independently of the HPV status. In relation to this, we observed a great correlation between SAHA-mediated inhibition of both ΔNp63α and EGFR protein expression (Fig. 2e), together with an inverse correlation between SAHA IC50 and EGFR inhibition (Fig. 2f), suggesting that SAHA exerts its activity through the inhibition of the expression of both ΔNp63α and EGFR. In particular, these data were reinforced by the overexpression of EGFR in UM-SCC-4 cells, which caused a substantial increase in the IC50 of SAHA, gefitinib and the combination of the two drugs (Fig. 2g).

HDACi are able to decrease gene expression of both wild-type (wt) and mutant p53. Mutant p53 can control EGFR activity and increase its expression, while wt p53 loss increases EGFR expression. Thus, we checked the effect of SAHA on p53 expression in our cell lines. As shown in Figure S2A, SAHA is able to decrease p53 expression in HPV-negative cell lines with wt p53 (UM-SCC-47, UPCI:SCC-90, UM-SCC-104, UPCI:SCC-154 and UD-SCC-2), and also in 93-VU147T cells, which present a heterozygous mutation of p53 (Table S1). Only few HPV-negative cell lines express p53, among them UM-SCC-10A and UMSCC-23 cells, which mutated p53, also showed decreased expression of p53 upon SAHA treatment (Figure S2B and Table S1). We can conclude that in HNC cell lines SAHA decreases the expression of both wt and mutant p53; thus, the decreased expression of EGFR in our cell lines does not seem to be dependent on p53 expression and on its mutation status.

p63 knockdown significantly decreases HNC cell line proliferation and migration.

We then silenced p63 protein in both HPV-positive and HPV-negative cell lines using a lentiviral vector (Fig. 3a) and assessed cellular proliferation and migration. As shown in Fig. 3b, c respectively, lack of p63 expression consistently reduced cell proliferation and significantly decreased cell migration. With these data we confirmed a dominant role of p63 as a mediator of both proliferative and migratory pathway in HNC cell lines.

DISCUSSION

HNCs are a biologically heterogeneous group of cancers, originating from different subsites, mucosa of the oral cavity, nasopharynx, oropharynx, hypopharynx and larynx and with different aetiological factors, among them high-risk HPV infection and transformation. Thus, in our study we decided to collect different HNC cell lines, from different subsites, sex and HPV status, to have a better vision of the potential diversity of response to therapy. The cell lines that we obtained were not clearly clustered in subgroups by their expression of epithelial/mesenchymal markers or to the activation of different kinases important in the transduction of signalling pathways, confirming the high heterogeneity of these tumours. Although one of the signatures of these cancer is the overexpression of the EGFR, the inhibition of EGFR as main therapy has very modest efficacy due to intrinsic or acquired resistance. The HDACi SAHA was already shown to potentially overcome EGFR-TKI resistance in a small panel of HPV-negative HNC cell lines. In this study we decided to better investigate the efficacy of SAHA alone or in combination with gefitinib in a larger and heterogeneous panel of HNC cancer cell lines and to characterise the molecular mechanisms underlying its activity. We found that both SAHA and gefitinib were able to inhibit HNC cell proliferation in both HPV-positive and HPV-negative cell lines, with different sensitivity independently from either their HPV status or their epithelial/mesenchymal phenotype. Moreover, the combination of the two drugs had a

| Table 2. Combination index and dose reduction index values for SAHA and gefitinib combination (μM) |
|-----------------|-----------------|-----------------|-----------------|
| Cell lines      | CI50            | DRI50           | r               |
| UM-SCC-4        | 0.40981         | Gefitinib: 4.9226SAHA: 4.83860 | 0.94028         |
| UM-SCC-23       | 0.21784         | Gefitinib: 14.0835SAHA: 6.81026 | 0.96417         |
| UPCI:SCC-154    | 0.26621         | Gefitinib: 29.9400SAHA: 4.29528 | 0.92685         |
| UD-SCC-2        | 0.11638         | Gefitinib: 13.5178SAHA: 23.5849 | 0.96664         |

Mean of at least three different experiments done in duplicate. CI and DRI values computed at 50% of cell death. CI < 1, CI = 1 or CI > 1 generally indicate synergistic, additive or antagonistic effect. DRI values represent the order of magnitude (fold) of dose reduction obtained for IC50 (DRI50) in combination setting compared with each drug alone. r is the coefficient of correlation for the fitting between CIs and fractional effects.

CI combination index, DRI dose reduction index.
Fig. 1  SAHA treatment reverts epithelial to mesenchymal transition in human papillomavirus (HPV)-negative and HPV-positive head and neck cancer (HNC) cell lines and inhibits transforming growth factor β (TGFβ) activity. a HNC cell lines were treated with 5 μM SAHA or vehicle for 24 h, lysed and analysed by immunoblotting (IB) with the indicated antibodies. b Total RNAs from HNC cell lines treated with 5 μM SAHA or vehicle for 24 h were isolated for RT-qPCR. ΔNp63α, E-cadherin expression was normalised to ribosomal phosphoprotein (RpPO) and expressed as means ± SD of at least three independent experiments. *P < 0.05; **P < 0.01 (unpaired t test). c HNC cell lines were serum-deprived (0.5% foetal bovine serum) for 24 h and then treated with 5 μM SAHA, 5 ng/ml TGFβ or the combination of the two. At 24 h, cells were lysed and analysed by IB with the indicated antibodies. d Total RNAs from HNC cell lines treated with 5 μM SAHA, 5 ng/ml TGFβ or the combination of the two, were isolated for RT-qPCR. PA-1, Jun, ITGB6 and vimentin expression was normalised to RpPO. Graphs show means ± SD of fold changes of expression of TGFβ- and TGFβ-SAHA-treated cells compared to vehicle-treated cells and SAHA-treated cells, respectively. *P < 0.05; **P < 0.01 (unpaired t test). Means are at least from three independent experiments.
ΔNp63α downregulation is required for the inhibitory activity of SAHA, which treatment decreases epidermal growth factor receptor (EGFR) expression. 

**a** Optical density (OD) analysis of the expression of ΔNp63α from three independent western blot experiments of head and neck cancer (HNC) cell lines treated with 5 μM SAHA or vehicle for 24 h was performed. Results were normalised to loading control (GAPDH) and expressed as percentage of inhibition (means ± SD). 

**b** Percentage of ΔNp63α inhibition results obtained in **a** were correlated to SAHA IC50, Pearson correlation coefficient r = −0.6 (P < 0.05).

**c** HNC cell lines were treated with 2 or 5 μM SAHA or vehicle for 24 h, lysed and analysed by immunoblotting (IB) with the indicated antibodies.

**d** HNC cell lines were treated with 5 μM SAHA or vehicle for 24 h, lysed and analysed by IB with the indicated antibodies.

**e** OD analysis of the expression of EGFR from three independent western blot experiments of HNC cell lines, shown in **d**, treated with 5 μM SAHA or vehicle for 24 h was correlated to OD results of percentage of p63 inhibition mediated by SAHA (**e**) or SAHA IC50 (**f**). Pearson correlation coefficient r = 0.78 (P < 0.005) and t = −0.78 (P < 0.005) respectively. 

**g** UM-SCC-4 cell line was transduced with EGFR-encoding viral particle; puromycin-selected cells were treated with SAHA, gefitinib and the combination of the two drugs, and IC50 was assessed using CellTiter-Glo® Luminescent Cell Viability Assay.

---

**Fig. 2** ΔNp63α downregulation is required for the inhibitory activity of SAHA, which treatment decreases epidermal growth factor receptor (EGFR) expression. 

- **a** Optical density (OD) analysis of the expression of ΔNp63α from three independent western blot experiments of head and neck cancer (HNC) cell lines treated with 5 μM SAHA or vehicle for 24 h was performed. Results were normalised to loading control (GAPDH) and expressed as percentage of inhibition (means ± SD).
- **b** Percentage of ΔNp63α inhibition results obtained in **a** were correlated to SAHA IC50, Pearson correlation coefficient r = −0.6 (P < 0.05).
- **c** HNC cell lines were treated with 2 or 5 μM SAHA or vehicle for 24 h, lysed and analysed by immunoblotting (IB) with the indicated antibodies.
- **d** HNC cell lines were treated with 5 μM SAHA or vehicle for 24 h, lysed and analysed by IB with the indicated antibodies.
- **e** OD analysis of the expression of EGFR from three independent western blot experiments of HNC cell lines, shown in **d**, treated with 5 μM SAHA or vehicle for 24 h was correlated to OD results of percentage of p63 inhibition mediated by SAHA (**e**) or SAHA IC50 (**f**). Pearson correlation coefficient r = 0.78 (P < 0.005) and t = −0.78 (P < 0.005) respectively.
- **g** UM-SCC-4 cell line was transduced with EGFR-encoding viral particle; puromycin-selected cells were treated with SAHA, gefitinib and the combination of the two drugs, and IC50 was assessed using CellTiter-Glo® Luminescent Cell Viability Assay.
Synergistic antitumour activity of HDAC inhibitor SAHA and EGFR inhibitor. S Citro et al.

Synergistic effect on both HPV-positive and HPV-negative HNC cell lines. Intriguingly, we found that SAHA treatment was able to increase epithelial marker and partially reduce mesenchymal marker in HNC cell lines, independently of the HPV status. This confirms the role of this drug in reverting EMT also in HPV-positive cell HNC lines, a role that was reinforced by the ability of SAHA to inhibit the activation of the TGFβ pathway, one of the pathways responsible for EMT that is hyperactivated during the acquisition of gefitinib resistance. EGFR and ΔNp63α are two key markers in HNC since their expression is upregulated in 90% and 80% respectively of HNC primary tumours. They are both involved in the promotion of proliferation and migration of HNC cell lines, cooperating with the TGFβ pathway or independently, activating pathways such as PI3K and MAPK. Upon SAHA treatment, we found a remarkable decrease in the expression of both ΔNp63α and EGFR in both HPV-positive and HPV-negative HNC cell lines, together with the inhibition of PI3K and ERK1/2 pathways, and,
Fig. 4  p63 knockdown decreases epidermal growth factor receptor (EGFR) expression and inhibits transforming growth factor β (TGFβ)-induced epithelial to mesenchymal transition (EMT) in human papillomavirus (HPV)-positive and HPV-negative head and neck cancer (HNC) cell lines. a p63- and control short hairpin RNA (shRNA)-transduced HNC cell lines were lysed and analysed by immunoblotting (IB) with the indicated antibodies. For the EGFR IB both the low and high exposures are shown. b p63- and control shRNA-transduced HNC cell lines were serum-deprived (0.5% foetal bovine serum) for 24 h and then treated with 5 ng/ml TGFβ. At 24 h, cells were lysed and analysed by IB with the indicated antibodies. c Total RNAs from UM-SCC-4 cell line transduced with p63 and control shRNA treated with 5 ng/ml, were isolated for RT-qPCR. PA-1, Jun, ITGB6 and vimentin expression was normalised to ribosomal phosphoprotein and expressed as means ± SD. *P < 0.05; **P < 0.01; ns not statistically significant compared to shCTR (multiple comparison one-way analysis of variance). d Schematic representation of the action of SAHA in reverting EMT process that causes gefitinib resistance. Gefitinib treatment may induce tumour-promoting TGFβ signals, which initiate EMT through activation of SMADs and upregulation of mesenchymal transcription factors. SAHA treatment decreases ΔNp63α, and subsequently downregulates EGFR expression and prevents the upregulation of TGFβ-dependent mesenchymal transcription factors by inhibiting SMAD activation.
Synergistic antitumour activity of HDAC inhibitor SAHA and EGFR inhibitor... S. Citro et al.

as mentioned above, decreased activation of the TGFβ pathway. Thus, our results suggest that the inhibition of both EGFR and ΔNp63α expression is responsible for the inhibitory activity in cell proliferation and migration mediated by SAHA. This evidence is reinforced by the remarkable inverse correlation between SAHA sensitivity and SAHA-mediated inhibition of EGFR and ΔNp63α. This behaviour also mirrors the positive correlation between ΔNp63α and EGFR inhibition by SAHA and the less efficacy of SAHA in cells overexpressing EGFR. In particular, ΔNp63α is known to regulate EMT in primary human keratinocytes and in HNC cell lines in a TGFβ-dependent manner33,34 and its downregulation reduces EGFR expression in triple-negative basal-like breast cancer cells and in pancreatic cancer cells promoting cell growth and chemoresistance.35,36

Thus, we showed for the first time that p63 silencing reduced EGFR expression in both HPV-positive and HPV-negative cell lines, showing also that SAHA-mediated inhibition of EGFR greatly correlates with ΔNp63α inhibition. Taken together, these data suggest that SAHA-mediated EGFR downregulation is p63-dependent. Moreover, since lack of p63 consistently reduced proliferation and migration of both HPV-positive and HPV-negative cell lines, interfering with the activation of the TGFβ pathway, our results imply a key role for p63 in the SAHA-dependent regulation of proliferation and migration of these cell lines. In conclusion, we found that not only the HDAC1 SAHA synergises with gefitinib to decrease HNC cell lines viability, but it is also able to reduce EMT and inhibit TGFβ pathway activation, which are responsible for the induction of gefitinib resistance. A key regulator of this process is the transcription factor ΔNp63α, whose downregulation by SAHA treatment appears to be the major inhibitory activity of this drug, decreasing cell growth, cell migration and TGFβ pathway activation. Thus, this study uncovers a novel molecular mechanism underlying the efficacy of SAHA in the treatment of both HPV-positive and HPV-negative HNC tumours in combination with gefitinib. Gefitinib efficacy in the treatment of these tumours is limited due to the acquired resistance induced by EMT, which we demonstrated can be overcome by the concomitant use of SAHA. These results show that the combination of SAHA with specific inhibitors of EGFR, such as gefitinib, improves the antitumour efficacy of these drugs in both HPV-positive and HPV-negative HNC and should be further explored clinically.

ACKNOWLEDGEMENTS
We thank Dr. Simona Polo and Dr. Sara Sigismund (IFOM, Milano) for EGFR antibody and plasmid, Dr. Leif W. Ellisen and Dr. Sinivas Vinod Saladi (Harvard Medical School) for DNp63α constructs and Dr. Giuseppe Dafieria (IEO, Milano) for antibodies. We are very grateful to Dr. Susanne Gollin (University of Pittsburgh), Dr. Martin Rooimans and Dr. Josephine Dorsman (VU University, Amsterdam), and Dr. H. Bier (University of Munchen) for cell lines and/or advice and to G. Giardina for cell lines validation. This study was funded by Associazione Italiana per la Ricerca sul Cancro (AIRC) to S. Chiocca (IG 2015 ld.16721). S. Chiocca has been a FUV (Fondazione Umberto Veronesi) fellow and is currently a recipient of a Fondazione IEO-CCM fellowship.

AUTHOR CONTRIBUTIONS
Conceptualization: S. Citro and S. Chiocca; data acquisition and analysis: S. Citro, A.B., C.M., L.G. and S. Chiocca; writing and editing and review: S. Citro, A.B., C.M., L.G., T.E.C. and S. Chiocca.

ADDITIONAL INFORMATION
Supplementary information is available for this paper at https://doi.org/10.1038/s41416-019-0394-9.

Competing interests: The authors declare no competing interests.

Data availability: All relevant data are within the paper and its Supporting Information files.

Note: This work is published under the standard license to publish agreement. After 12 months the work will become freely available and the license terms will switch to a Creative Commons Attribution 4.0 International (CC BY 4.0).

Publisher’s note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

REFERENCES
1. Mandal, R. et al. The head and neck cancer immune landscape and its immuno-therapeutic implications. JCI Insight 1, e98929 (2016).

2. Fakhy, C. et al. Improved survival of patients with human papillomavirus-positive head and neck squamous cell carcinoma in a prospective clinical trial. J. Natl Cancer Inst. 100, 261–269 (2008).

3. Hoy, J. D., Moskovitz, J. M. & Ferris, R. L. Biological mechanisms of immune escape and implications for immunotherapy in head and neck squamous cell carcinoma. Eur. J. Cancer 76, 152–166 (2017).

4. Harari, P. M. & Huang, S. Radiation combined with EGFR signal inhibitors: head and neck cancer focus. Semin. Radiat. Oncol. 16, 38–44 (2006).

5. Santuray, R. T., Johnson, D. E. & Grandis, J. R. New therapies in head and neck cancer. Trends Cancer 4, 385–396 (2018).

6. Cohen, E. E. et al. Erlotinib and bevazucimab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck: a phase II/III study. Lancet Oncol. 10, 247–257 (2009).

7. Vermorken, J. B. et al. Open-label, uncontrolled, multicenter phase II study to evaluate the efficacy and toxicity of cetuximab as a single agent in patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck who failed to respond to platinum-based therapy. J. Clin. Oncol. 25, 2171–2177 (2007).

8. Cooper, J. B. & Cohen, E. E. Mechanisms of resistance to EGFR inhibitors in head and neck cancer. Head Neck 33, 1086–1094 (2009).

9. Klymkovsky, M. W. & Savagner, P. Epithelial-mesenchymal transition: a cancer researcher’s conceptual friend and foe. Am. J. Pathol. 174, 1588–1593 (2009).

10. Iwatsuki, M. et al. Epithelial-mesenchymal transition in cancer development and its clinical significance. Cancer Sci. 101, 293–299 (2010).

11. Xu, J., Lamouille, S. & Derynck, R. TGF-beta-induced epithelial to mesenchymal transition. Cell Res. 19, 156–172 (2009).

12. Jakobsen, K. R., Demuth, C., Sorensen, B. S. & Nielsen, A. L. The role of epithelial to mesenchymal transition in resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. Transl. Lung Cancer Res. 5, 172–182 (2016).

13. Frederick, B. A. et al. Epithelial to mesenchymal transition predicts gefitinib resistance in cell lines of head and neck squamous cell carcinoma and non-small cell lung carcinoma. Mol. Cancer Ther. 6, 1683–1691 (2007).

14. Sakamoto, T. et al. A Histone deacetylase inhibitor suppresses epithelial-mesenchymal transition and attenuates chemoresistance in biliary tract cancer. PLoS ONE 11, e0145985 (2016).

15. Ruscetti, M. et al. HDAC inhibition impedes epithelial-mesenchymal plasticity and suppresses metastatic, castration-resistant prostate cancer. Oncogene 35, 3781–3795 (2016).

16. Bruzzese, F. et al. HDAC inhibitor vorinostat enhances the antitumor effect of EGFR inhibitors in head and neck cancer. Head Neck 33, 1691–1695 (2011).

17. Feng, J. et al. Histone deacetylase inhibitor valproic acid (VPA) promotes the epithelial mesenchymal transition of colorectal cancer cells via up regulation of Snail. Cell Adh. Migr. 9, 495–501 (2015).

18. Ji, M. et al. HDAC inhibitors induce epithelial-mesenchymal transition in colon carcinoma cells. Oncol. Rep. 33, 2299–2308 (2015).

19. Kieweler, N. et al. The histone deacetylases HDAC1 and HDAC2 are required for the growth and survival of renal carcinoma cells. Arch. Toxicol. 92, 2227–2243 (2018).

20. Kumar, B., Yadav, A., Lang, J. C., Teknos, T. N. & Kumar, P. Suberoylanilide hydroxamic acid (SAHA) reverses chemoresistance in head and neck cancer cells by targeting cancer stem cells via the downregulation of nanog. Genes Cancer 6, 169–181 (2015).

21. Lane, A. A. & Chabner, B. A. Histone deacetylase inhibitors in cancer therapy. J. Clin. Oncol. 27, 5459–5468 (2009).

22. Marks, P. A. & Xu, W. S. Histone deacetylase inhibitors: potential in cancer therapy. J. Cell. Biochem. 107, 600–608 (2009).

23. Kelly, W. K., Marks, P. & Richon, V. M. CCR 20th anniversary commentary: vorinostat-gateway to epigenetic therapy. Clin. Cancer Res. 21, 2198–2200 (2015).
24. Grant, C. et al. Romidepsin: a new therapy for cutaneous T-cell lymphoma and a potential therapy for solid tumors. Expert. Rev. Anticancer Ther. 10, 997–1008 (2010).

25. Zwergel, C., Valente, S., Jacob, C. & Mai, A. Emerging approaches for histone deacetylase inhibitor drug discovery. Expert Opin. Drug Discov. 10, 599–613 (2015).

26. Singh, A. K., Bishayee, A. & Pandey, A. K. Targeting histone deacetylases with natural and synthetic agents: an emerging anticancer strategy. Nutrients 10, 731 (2018).

27. Sivaraj, D., Green, M. M. & Gasparetto, C. Panobinostat for the management of multiple myeloma. Future Oncol. 13, 477–488 (2017).

28. Napoli, M. et al. DeltaNp63/DGCR8-dependent microRNAs mediate therapeutic efficacy of HDAC inhibitors in cancer. Cancer Cell 29, 874–888 (2016).

29. Chakravarti, D. et al. Induced multipotency in adult keratinocytes through down-regulation of DeltaNp63 or DGCR8. Proc. Natl Acad. Sci. USA 111, E572–E581 (2014).

30. Sniezek, J. C., Matheny, K. E., Westfall, M. D. & Pietenpol, J. A. Dominant negative p63 isoform expression in head and neck squamous cell carcinoma. Laryngoscope 114, 2063–2072 (2004).

31. Yang, X. et al. DeltaNp63 versatilely regulates a Broad NF-kappaB gene program and promotes squamous epithelial proliferation, migration, and inflammation. Cancer Res. 71, 3688–3700 (2011).

32. Rocco, J. W., Leong, C. O., Kuperwasser, N., DeYoung, M. P. & Ellisen, L. W. p63 mediates survival in squamous cell carcinoma by suppression of p73-dependent apoptosis. Cancer Cell 9, 45–56 (2006).

33. Oh, J. E., Kim, R. H., Shin, K. H., Park, N. H. & Kang, M. K. DeltaNp63alpha protein triggers epithelial-mesenchymal transition and confers stem cell properties in normal human keratinocytes. J. Biol. Chem. 286, 38757–38767 (2011).

34. Rodriguez Calleja, L. et al. DeltaNp63alpha silences a miRNA program to aberrantly initiate a wound-healing program that promotes TGFbeta-induced metastasis. Cancer Res. 76, 3236–3251 (2016).

35. Stauter, R. H. et al. A combination of a ribonucleotide reductase inhibitor and histone deacetylase inhibitors downregulates EGFR and triggers BIM-dependent apoptosis in head and neck cancer. Oncotarget 3, 31–43 (2012).

36. Brenner, J. C. et al. Genotyping of 73 UM-SCC head and neck squamous cell carcinoma cell lines. Head Neck 32, 417–426 (2010).

37. Ballo, H. et al. Establishment and characterization of four cell lines derived from human head and neck squamous cell carcinomas for an autologous tumor-fibroblast in vitro model. Anticancer Res. 19, 3827–3836 (1999).

38. Steenbergen, R. D. et al. Integrated human papillomavirus type 16 and loss of heterozygosity at 11q22 and 18q21 in an oral carcinoma and its derivative cell line. Cancer Res. 55, 5465–5471 (1995).

39. Tang, A. L. et al. UM-SCC-104: a new human papillomavirus-16-positive cancer stem cell-containing head and neck squamous cell carcinoma cell line. Head Neck 34, 1480–1491 (2012).

40. White, J. S. et al. The influence of clinical and demographic risk factors on the establishment of head and neck squamous cell carcinoma cell lines. Oral Oncol. 43, 701–712 (2007).

41. Ragan, C. C., Reshami, S. C. & Gollin, S. M. Mapping and analysis of HPV16 integration sites in a head and neck cancer cell line. Int. J. Cancer 110, 701–709 (2004).

42. Chou, T. C. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacol. Rev. 58, 621–681 (2006).

43. Zhao, M. et al. Assembly and initial characterization of a panel of 85 genomically validated cell lines from diverse head and neck tumor sites. Clin. Cancer Res. 17, 7248–7264 (2011).

44. Akagi, K. et al. Genome-wide analysis of HPV integration in human cancers reveals recurrent, focal genomic instability. Genome Res. 24, 185–199 (2014).

45. Schafer, C. et al. Class I histone deacetylases regulate p53/NF-kappaB crosstalk in cancer cells. Cell. Signal. 29, 218–225 (2017).

46. Muller, P. A. et al. Mutant p53 drives invasion by promoting integrin recycling. Cell 139, 1327–1341 (2009).

47. Yallowitz, A. R. et al. Mutant p53 Amplifies Epidermal Growth Factor Receptor Family Signaling To Promote Mammary Tumorigenesis. Mol. Cancer Res. 13, 743–754 (2015).

48. Bheda, A., Creek, K. E. & Pirisi, L. Loss of p53 induces epidermal growth factor receptor promoter activity in normal human keratinocytes. Oncogene 27, 4315–4323 (2008).

49. Saladi, S. V. et al. AC176A is co-amplified with p63 in squamous cell carcinoma to drive YAP activation, regenerative proliferation, and poor prognosis. Cancer Cell 31, 35–49 (2017).

50. Holcakova, J. et al. DeltaNp63 activates EGFR signaling to induce loss of adhesion in triple-negative basal-like breast cancer cells. Breast Cancer Res. Treat. 163, 475–484 (2017).

51. Danilov, A. V. et al. DeltaNp63alpha-mediated induction of epidermal growth factor receptor promotes pancreatic cancer cell growth and chemoresistance. PLoS ONE 6, e26815 (2011).