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The tumor suppressor gene rap1GAP is silenced by mir-101-mediated EZH2 overexpression in invasive squamous cell carcinoma

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Abstract

Rap1GAP is a critical tumor suppressor gene that is down-regulated in multiple aggressive cancers such as head and neck squamous cell carcinoma, melanoma and pancreatic cancer. However, the mechanistic basis of rap1GAP down-regulation in cancers is poorly understood. By employing an integrative approach, we demonstrate polycomb mediated repression of rap1GAP that involves EZH2, a histone methyltransferase in head and neck cancers. We further demonstrate that the loss of miR-101 expression correlates with EZH2 up-regulation, and the concomitant down-regulation of rap1GAP in head and neck cancers. EZH2 represses rap1GAP by facilitating the trimethylation of H3K27, a mark of gene repression, and also hypermethylation of *rap1GAP* promoter. These results provide a conceptual framework involving a microRNA-oncogene-tumor suppressor axis to understand head and neck cancer progression.

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Conflict of Interest

The authors declare no conflict of interest in this manuscript.

Supplementary information

Supplementary information is available at the Oncogene's website

Keywords

mir101; EZH2; rap1GAP; rap1; promoter hypermethylation

Introduction

Squamous cell carcinoma is a common cancer of the uterine cervix, skin, esophagus, lung and head and neck. Squamous cell carcinoma of the head and neck (HNSCC) is the sixth most common cancer in the world with more than 850,000 cases diagnosed worldwide(Chin et al 2006). The five year survival rate is poorer than most major cancers, including melanoma and breast cancer (Todd et al 1997, Yarbrough et al 2006). Unfortunately, in forty years, there have been no major treatment breakthroughs. Elucidation of the mechanism of regulation of critical mediators of cancer development, such as tumor suppressor genes, will facilitate the identification of novel therapeutic targets (Bracken and Helin 2009).

Silencing of tumor suppressor genes via methylation of histones and promoter region of DNA promotes tumor progression through essential phenotypes, such as invasion and proliferation (Simon and Lange 2008). Enhancer of Zeste Homolog 2 (EZH2), a histone methyltransferase, is the catalytic member of the polycomb repressive complex 2 (PRC2) that trimethylates histone 3 at lysine 27 (H3K27) (Chi et al 2010, Simon and Lange 2008). EZH2 is a master regulatory gene that has a significant role in cancer development via methylation-mediated repression of transcription of genes and maintain cellular homeostasis (Cao et al 2008, Chi et al 2010, Varambally et al 2002). The other members of the PRC2 complex are Supressor of Zeste 12 homolog (SUZ12) and Embryonic Ectoderm Development (EED). The PRC2 complex also serves as a recruiting platform for DNA methyltransferases, thereby linking two epigenetic repression systems i.e. histone methylation and promoter hypermethylation. The histone methylation repressive mark facilitates gene silencing and oncogenesis by inhibiting the binding of transcription factors to the promoter region of genes (Chi et al 2010, Simon and Lange 2008). Trimethylation of H3K27 is associated with inhibition of transcription of genes that maintain cellular homeostasis (Chi et al 2010). EZH2 silences tumor suppressor genes but its targets are relatively uncharacterized (Kidani et al 2009, Kleer et al 2003, Varambally et al 2002).

Rap1GAP is a critical tumor suppressor gene that is downregulated in multiple aggressive cancers such as HNSCC, melanoma, pancreatic and thyroid cancer (Zhang et al 2006a, Zhang et al 2006b, Zheng et al 2009, Zuo et al 2010). However, the mechanistic basis of rap1GAP down-regulation in cancers is poorly understood. In HNSCC, rap1GAP inhibits tumor growth by delaying the G1/S transition of the cell cycle (Zhang et al 2006b). In melanoma cells, rap1GAP inhibits extracellular signal-regulated kinase, cell proliferation, survival and migration (Zheng et al 2009). In pancreatic cancer and thyroid tumors, loss of heterozygosity of the *rap1GAP* gene occurs (Zhang et al 2006a, Zuo et al 2010). Rap1GAP inactivates GTP-bound rap1 by enhancing its endogenous GTPase activity (Takai et al 2001). Rap1 is a ras-like protein that shuttles between an inactive GDP- and an active GTP-bound form. Active, GTP-bound rap1 has a significant role in cell proliferation and cell adhesion in epithelial cells (Altschuler and Ribeiro-Neto 1998, Caron et al 2000, Hogan et al

2004, Price et al 2004, Reedquist et al 2000). The activation of rap1 is regulated by guanine nucleotide exchange factors including C3G, Epac and Dock-4 (Bos et al 2001, Stork and Dillon 2005). Inactivation of rap1 is regulated by rapGAP (Rubinfeld et al 1991, Su et al 2003).

MicroRNAs (miRNAs) are endogenous, noncoding RNAs that inhibit tumor suppressor genes or upregulate oncogenes, thereby promoting tumorigenesis (Varambally et al 2008, Yang et al 2010). MiRs regulate gene expression by repressing translation or decreasing mRNA stability thereby regulating biological processes, including differentiation, proliferation and apoptosis.

EZH2 contributes significantly to the development of solid tumors (Bracken and Helin 2009, Chi et al 2010). Further characterization of EZH2 targets and the mechanism of methylation-mediated silencing of critical tumor suppressor genes will likely facilitate the identification of novel therapeutic targets. In the present study, using integrative genomic analysis in an HNSCC model in which EZH2 expression was modulated, we nominated EZH2 target genes. Furthermore, we showed that downregulation of miR101 promotes upregulation of EZH2 and epigenetic silencing of *rap1GAP* via methylation of H3K27 and via promoter hypermethylation, and establish a novel role for mir101 and EZH2 in rap1GAP-mediated tumor progression.

Results

EZH2 expression was evaluated in human oral keratinocytes (HOK), an immortalized, non-malignant oral keratinocyte cell line (HOK16B) and in HNSCC cells. A significant upregulation in the steady state levels of EZH2 mRNA (Fig. 1A) and an increase in EZH2 protein (Fig. 1B) were observed in all HNSCC cell lines compared to normal or immortalized keratinocytes.

EZH2 was primarily in the nucleus of HNSCC tumor cells (Fig. 1C). Increased intensity and proportion of EZH2 was observed in HNSCC relative to normal tissue (Fig. 1D, left and right panels, respectively). Cancers showed overexpression of EZH2 in the Proportional Odds model ($p=1.3\times10^{-5}$). The effect size measured as log cumulative odds ratio comparing cancer versus normal, was estimated to be 2.87 with a 95% confidence interval of (1.58, 4.16).

Invasion and proliferation are phenotypes that are essential to HNSCC progression (Simon and Lange 2008). Therefore, the functional significance of EZH2 upregulation in HNSCC was verified by invasion and proliferation assays after downregulation of EZH2 expression in OSCC3 and UM-SCC-29, which exhibit strong endogenous EZH2 (Fig. 1B). The siRNA-mediated 82% reduction in EZH2 expression in OSCC3 cells (Fig. 2A) was accompanied by a decrease in both proliferation (Fig. 2B, left panel) and invasion (Fig.2C). The reduction in EZH2 induced an insignificant change in the apoptotic cell population (Fig. 2B right panel). A similar effect was noticed for UM-SCC-29 (Fig. 2D, 2E and 2F).

Overexpression of EZH2 in non-malignant keratinocytes with low endogenous EZH2 had the reverse effect on proliferation and invasion. There was a greater than six-fold increase in

EZH2 expression in cells infected with Adeno-EZH2 (Ad-EZ) relative to control Adeno-CMV (Ad-C) (Fig. 2G). Proliferation and invasion were significantly increased in cells infected with Ad-EZH2 compared to control (Fig. 2H, left panel, and Fig. 2I, respectively). Overexpression of EZH2 induced a slight decrease in the apoptotic cell population (Fig. 2H, right panel). Thus, EZH2 promotes proliferation and invasion in HNSCC.

To identify the targets of EZH2, gene expression profiling was performed. RNA isolated from OSCC3 cells transfected with siEZH2, from normal keratinocytes infected with Ad-EZH2, and from corresponding control cells, was labeled and hybridized to cDNA microarrays. Genes exhibiting a 1.5 fold or greater change in expression relative to the corresponding control (non target siRNA) and a p-value <0.003 were identified. A subset of genes that were upregulated with siEZH2 (in both replicates i.e. original and dye swap) and downregulated with Ad-EZH2, were nominated as tumor suppressor genes (Fig. 3A, top panel). Among these genes, *ADRB2* is regulated by EZH2 in prostate cancer (Yu et al 2007) whereas other nominees such as *rap1GAP*, have not been linked to EZH2, supporting the notion that the repertoire of EZH2-regulated genes varies between cancers.

To functionally classify tumor suppressor genes that were upregulated by EZH2 knockdown, we performed Molecular Concept Map analysis using literature defined molecular concepts/gene sets in the Oncomine database (www.oncomine.com). Genes that were upregulated by EZH2 knockdown in OSCC3 cells were related to genes that are upregulated by p53, an important tumor suppressor gene, genes that were upregulated by AZA, a methylation inhibitor, and genes that were downregulated in stem cells (Fig. 3A, lower panel).

We have shown that rap1GAP has an important tumor suppressor role in HNSCC (Mitra et al 2008, Zhang et al 2006b). Hence, subsequent studies focused on EZH2-mediated regulation of rap1GAP. *Rap1GAP* expression, and *ADRB2* as a positive control, were validated by qPCR (Fig. 3B). Downregulation of *EZH2* induced an increase in both *rap1GAP* and *ADRB2* in OSCC3 and UM-SCC-29 (Fig. 3B, siEZ; $p<0.05$). Conversely, overexpression of *EZH2* in normal keratinocytes downregulated *rap1GAP* and *ADRB2* (Fig. 3B, Ad-EZ; $p<0.05$).

The effects of EZH2 modulation were also observed with rap1GAP protein. Overexpression of EZH2 in non-malignant keratinocytes resulted in downregulation of rap1GAP (Fig. 3C, left panel) and knockdown of EZH2 in HNSCC cells, increased rap1GAP protein expression (Fig. 3C, right panel). Since rap1GAP inactivates rap1 due to its GTPase activity, we investigated whether downregulation of rap1GAP induced a change in GTP-bound rap1. In keratinocytes overexpressing EZH2, the decrease (44%) in rap1GAP was accompanied by a corresponding increase (45%) in active rap1 while total rap1 was unchanged. EZH2 overexpression in cells infected with Ad-EZH2 was verified (Fig. 3C, left panel). In OSCC3, siEZH2 decreased EZH2 expression by 77% (Fig. 3C, right panel). This was accompanied by a greater than 8 fold increase in rap1GAP expression. Consistent with the upregulation of rap1GAP, there was a 51% decrease of GTP-bound rap1 when normalized to total rap1. EZH2-mediated regulation of rap1GAP was also observed in a prostate cancer cell line, LnCap (Fig. S1). Thus, EZH2 downregulates the expression and function of rap1GAP.

To investigate whether EZH2 and rap1GAP expression are inversely correlated in human tissue, immunoblot analysis of tissue lysates from HNSCC and matched normal tissues was performed. As shown in Fig. 4A, EZH2 is upregulated in 5/6 HNSCC tissues (samples 1, 2, 3, 4 and 6). In four of these five samples (Fig. 4A, samples 2, 3, 4 and 6), rap1GAP is inversely correlated with EZH2.

Two recent studies in prostate and esophageal cancers showed that EZH2 is upregulated as a consequence of genomic loss of miR-101 or gene amplification, respectively (Friedman et al 2009, He et al 2010, Varambally et al 2008). To determine whether the increase in EZH2 expression is a function of gene amplification, FISH and immunohistochemistry were performed on human HNSCC tissues. No gene amplification was observed in paired tumor/normal tissue samples (Fig. S2A). Upregulation of EZH2 in tumor was verified (Fig. S2B). To determine whether the increase in EZH2 in HNSCC was a function of a change in miR-101, miR101 was quantified in the same matched normal/tumor samples. MiR-101 was downregulated in 4/5 HNSCC tissues in which expression of EZH2 was upregulated and rap1GAP was silenced relative to the paired normal tissues (Fig. 4B, samples 2, 3, 4 and 6). For confirmation of miR-101 mediated regulation of EZH2 and rap1GAP in HNSCC, OSCC3 cells were transfected with pre-miR-101. EZH2 expression was downregulated with overexpression of mir-101 compared to the corresponding cells transfected with control pre-miR (Fig. 4C, lane 4 compared to lane 3). This downregulation in EZH2 expression was similar to that observed with siEZH2 and corresponded to an increase in expression of rap1GAP (Fig. 4C; lane 2 compared to lane 1).

EZH2 methylates H3K27 to facilitate repression of tumor suppressor genes (Simon and Lange 2008). To confirm EZH2-mediated downregulation of rap1GAP is due to methylation, OSCC3 cells with high endogenous EZH2 were treated with SAHA (Histone deacetylase inhibitor), AZA (DNA methyltransferase inhibitor) or a combination of SAHA plus AZA. Expression of Rap1GAP was increased by SAHA, AZA and maximally by SAHA plus AZA (Fig. 5A, top panel). Reduction in levels of H3K27 tri-methylation was verified (Fig. 5A, lower middle panel). Since deacetylation is required for histone methylation, SAHA reduces methylation. As expected, AZA, the methyltransferase inhibitor, reduced methylation. Combined treatment with SAHA plus AZA reduced methylation synergistically.

In a complementary study to support that methylated H3K27 is associated with the promoter region of *rap1GAP*, we performed ChIP of methylated H3K27 (mark of EZH2) followed by PCR with primers (Table S2) spanning the trimethylated H3K27 binding region (Fig. 5C). As shown in Fig. 5B, trimethylation of H3K27 with the promoter region of *rap1GAP* decreased upon treatment with SAHA, AZA and SAHA plus AZA. *ADRB2* served as a positive control (Yu et al 2007). Thus, EZH2 mediated methylation of H3K27 on *rap1GAP* promoter results in its repression.

Subsequently we investigated methylation status in the CpG islands near the promoter region of *rap1GAP* (Fig. 5C). OSCC3 cells were treated with SAHA, AZA or SAHA plus AZA. Chromosomal DNA was prepared and modified by bisulfite treatment. CpG islands near the transcription initiation site (CpG24, 74A and 74B) showed a prominent decrease in

methylation as is evident from the increase in signal intensity generated with primers specific for unmethylated DNA (U) relative to methylated DNA (M), particularly in CpG74A and to a lesser extent in CpG74B (Fig. 5D, left panel, U versus M). Unmethylated CpG24 increased only with combined treatment of SAHA and AZA. To verify that methylation of these CpG islands is a function of EZH2, we performed similar experiments with downregulated EZH2 expression either transiently with siEZH2 or stably with shEZH2. Unmethylated CpG74A and CpG74B increased compared to corresponding methylated CpG74A and CpG74B (Fig. 5D, middle and right panel). However for CpG24, a remarkable increase in unmethylated CpG24 was observed only when EZH2 was downregulated stably with shEZH2 compared to transiently with siEZH2 (Fig. 5D, middle and right panel).

To determine whether EZH2 regulates proliferation via suppression of rap1GAP, we performed “rescue” experiments in OSCC3 cells transduced with shEZH2. EZH2 knockdown was verified by immunoblot (Fig. 6A). As observed earlier with siEZH2 (Fig. 3C, right panel), stable knockdown of EZH2 (shEZH2) induced upregulation of rap1GAP, downregulation of active, GTP-bound rap1 and decrease in H3K27 tri-methylation (Fig. 6A, left panel). Similar to siEZH2 (Fig. 2B, left panel), proliferation was decreased in shEZH2 transduced cells compared to control cells (Fig. 6A, right panel). For “rescue” experiments, OSCC3-shEZH2 cells were transfected with siRNA for rap1GAP and knockdown was verified (Fig. 6B). Two different siRNAs to rap1GAP si5 and si6, reduced rap1GAP expression 69% and 80%, respectively (Fig. 6B, left panel). In corresponding proliferation experiments in OSCC3-shEZH2 cells, both siRNAs significantly increased proliferation as early as 60h after transfection (Fig. 6B, right panel, $p<0.05$).

In vivo, downregulation of EZH2 (OSCC3-shEZH2) significantly inhibited tumor growth, compared to control tumors (Fig. 6C). EZH2 tumors were smaller by 354 mm^3 on average, adjusted for the mouse-specific effect (95% confidence interval of 137–571 mm^3; p=0.033) and the weight of the tumors was less by 0.15g (95% confidence interval of 0.04–0.24g; p = 0.049). Similar effects of EZH2 on cell proliferation and tumor growth were observed in UM-SCC-29 (Fig. S3).

Discussion

Rap1GAP is downregulated in multiple aggressive human tumors including HNSCC, pancreatic cancer, thyroid and colon cancer (Tsygankova et al 2010, Zhang et al 2006a, Zhang et al 2006b, Zheng et al 2009) but the mechanism of downregulation is unclear. In this important and novel study, we demonstrate that silencing of *rap1GAP* is regulated by EZH2 which represses transcription of *rap1GAP* by H3K27 trimethylation and promoter hypermethylation. Moreover, reduction in miR-101 expression up-regulates EZH2, which subsequently downregulates rap1GAP revealing a key mechanism of a tumor suppressor (miR-101) controlling an oncogene, EZH2, which downregulates another tumor suppressor gene, *rap1GAP*, thereby promoting tumor progression (Fig. 7). Given the crucial role of rap1GAP in aggressive tumors (Zhang et al 2006a, Zhang et al 2006b, Zuo et al 2010), these findings are exciting and significant in understanding the development of multiple tumors.

Although a recent study showed that EZH2 is expressed in HNSCC, neither the oncogenic role of EZH2 nor its mechanism of action was investigated (Kidani et al 2009). The present study investigated the functional relevance of upregulated EZH2 in HNSCC biology. This is significant because proliferation and detachment of keratinocytes with invasion and migration into the underlying tissues are necessary for transformation of oral pre-cancerous lesions to cancer. In existing HNSCC, migration/invasion promotes spread of tumor cells to distant sites, i.e. tumor progression (Thomas and Speight 2001). This progression of HNSCC is incompatible with patient survival. Knockdown of EZH2 in HNSCC inhibited proliferation and invasion. In contrast, overexpression of EZH2 in immortalized keratinocytes had the reverse effect (Fig. 2).

In HNSCC, methylation is an important epigenetic event. In fact, promoter hypermethylation markers facilitate detection and evaluation of tumor margins in HNSCC (Glazer et al 2009). While the mechanism of EZH2-mediated cancer initiation and progression is not well-established, it is likely via epigenetic silencing of tumor suppressor genes (Beke et al 2007, Yu et al 2007). Two main mechanisms for epigenetic silencing are via acetylation or methylation of lysine residues in histones and via hypermethylation of CpG islands in the promoter region of genes (Herceg 2007, Kondo et al 2008). In one of the most important histone repressive marks, the polycomb complex silences genes during embryonic development and carcinogenesis via methylation of H3K27 (Chi et al 2010, Simon and Lange 2008). This histone methylation facilitates chromatin compaction and reduces gene transcription (Francis et al 2004). Furthermore, the PRC2 complex provides an anchor for recruitment of DNA methyltransferases to facilitate gene silencing via DNA methylation (Vire et al 2006). Consistent with this dual role, in the present study, EZH2 facilitated histone and DNA methylation of the promoter region of *rap1GAP*. We showed by ChIP-PCR that treatment of HNSCC cells with Histone deacetylase inhibitor and or DNA methyltransferase inhibitors decreased methylation of H3 at the promoter of *rap1GAP*. Furthermore, these inhibitors and EZH2 knockdown decreased methylation of the CpG islands at the promoter region of *rap1GAP* (Fig.5D) suggesting EZH2 mediated methylation on H3 and promoter hypermethylation are co-ordinated.

Downregulation of EZH2 by siEZH2 or inhibition of histone deacetylase/DNA methylation by SAHA/AZA, induced rap1GAP expression. Consistent with these findings, in HNSCC tissues that express high EZH2, rap1GAP is downregulated relative to matched normal tissues (Fig. 4). EZH2 overexpression in HNSCC was not due to gene amplification (Fig S2A) but was correlated with downregulation of miR101. Furthermore, knockdown of EZH2 or overexpression of miR101 in HNSCC cells increased the expression of rap1GAP and established a tumor suppressor role of miR101 controlling another tumor suppressor rap1GAP. Subsequently in *in-vitro* experiments overexpression of EZH2 in non-malignant keratinocytes with low endogeneous EZH2 increased active GTP bound rap1 and when EZH2 downregulated in HNSCC cell line had the reverse effect. Active GTP bound rap1 facilitates tumor progression (Zhang et al 2006b). Importantly, the inhibitory effect of shEZH2 on proliferation in HNSCC was “rescued” by concurrent knockdown of rap1GAP supporting its significant role in HNSCC (Fig. 6). Finally, stable knockdown of EZH2 inhibits HNSCC progression in vivo.

Cancers at different sites have phenotypic similarities such as invasion, proliferation and metastasis which may be attributable to activation of proliferative and survival pathways. EZH2 has a significant role in the development of multiple cancers via repression of transcription (Cao et al 2008, Varambally et al 2002). Polycomb group target genes are well characterized in prostate cancer (Varambally et al 2002, Yu et al 2010). However, given the diversity in etiology and biology between tumors, some of these targets may be tumor specific, as suggested previously (Martinez-Garcia and Licht 2010). Consistent with this notion, gene expression studies in HNSCC nominated several molecular targets of EZH2, some of which, such as *rap1GAP*, were not identified in prostate cancer whereas others, like ADRB2 are more universal (Yu et al 2007). In contrast to breast cancer (Cao et al 2008), E-cadherin was not identified as an EZH2 target in HNSCC.

In prostate cancer, upregulation of EZH2 is associated with a more aggressive phenotype (Varambally et al 2002). Although the intensity of EZH2 staining and the proportion of EZH2 positive cells was increased in HNSCC relative to normal oral epithelium, we observed no difference between early and advanced tumor stage relative to EZH2 expression, suggesting an underlying role for EZH2 in malignant transformation.

The role of EZH2 in cancer development varies with different types of cancer (Cao et al 2008, Chi et al 2010, Kleer et al 2003, Morin et al 2010, Varambally et al 2002). Overexpression of EZH2 or downregulation/ inactivation of UTX, which removes H3K27me3 marks, promotes an oncogenic phenotype by promoting methylation in epithelial malignancies (Martinez-Garcia and Licht 2010). Recent studies in myeloid malignancies and lymphomas show that EZH2 has a tumor suppressor role (Ernst et al 2010, Morin et al 2010, Nikoloski et al 2010). Our studies show that EZH2 has an oncogenic role in HNSCC, an epithelial malignancy.

Increased expression of EZH2 in cancer may be due to gene amplification or genomic loss of miR-101 (Friedman et al 2009, He et al 2010, Varambally et al 2008). Although 54% of esophageal cancers have high EZH2, only 12% exhibit gene amplification (He et al 2010). Although we did not identify gene amplification in human HNSCC, EZH2 upregulation was associated with loss of miR-101. In 38% and 67% of early and late stage prostate cancers, respectively, loss of miR-101 promotes overexpression of EZH2 and disruption of epigenetic regulation (Varambally et al 2008).

Rap1GAP is a negative regulator of rap1, a ras-like protein. Recently, rasGAP, a negative regulator of K-ras, was shown to have a critical role in EZH2-mediated prostate cancer metastasis (Min et al 2010). These studies emphasize the importance of regulators of small-GTP binding proteins in tumor progression. Previously we showed that rap1GAP inhibits HNSCC growth by delaying the G1-S transition in the cell cycle (Zhang et al 2006b). In the present study, EZH2 promotes proliferation via inhibition of rap1GAP.

Given that recent studies also support a tumor suppressor role for rap1GAP in pancreatic cancer, melanoma and thyroid cancer, and that methylation of *rap1GAP* occurs in melanoma and thyroid cancer (Tsygankova et al 2007, Zhang et al 2006a, Zhang et al 2006b, Zheng et

al 2009, Zuo et al 2010), it is likely that miR-101 regulated EZH2 has a critical role in these tumors via disruption of rap1GAP expression.

Methods

Cell Culture

Human HNSCC cells, primary human oral keratinocytes (HOK) and immortalized human oral keratinocytes (HOK16B) were cultured as described previously (Mitra et al 2003, Mitra et al 2008).

Western blot analysis

Cells were lysed with 1% NP40 lysis buffer, followed by sonication and immunoblot analysis. All antibodies used are listed in supplementary Text S1.

Immunohistochemistry

Immunostaining on a human HNSCC tissue microarray (US Biomax) was performed as described (Mitra et al 2003) with affinity-purified anti-EZH2 (BD Transduction). Mouse IgG was used as a negative control. Interpretation and scoring were performed by a board certified pathologist, as described (Mitra et al 2008). Multivariate analysis of EZH2 activity was based on the Proportional Odds ordinal regression model. The analysis had intensity in the nucleus as the primary response variable categorized as none, low, medium, and high. Explanatory variables included cancer diagnosis (binary) and TNM stage (ordinal, 4 categories).

Cell transfection and infection

EZH2 was downregulated in HNSCC cell lines with EZH2 siRNA (Dharmacon). For stable knockdown of EZH2, OSCC3 and UM-SCC-29 cells were transduced with lentiviral particles of scramble shRNA (shVSVG) and shEZH2 (Open Biosystems) and was selected against 50μg/ml of puromycin for OSCC3 and 25 μg/ml for UM-SCC-29. After selection, cells were maintained at 20 μg/ml of puromycin. For overexpression of EZH2, cells were infected with adenovirus containing human EZH2 and control (CMV) as described (Varambally et al 2008). For over expression of miR101 cells were transfected with pre-miR101 and pre-miR negative controls (Ambion).

Measurement of Apoptosis

Apoptosis was evaluated by double staining with FITC labeled Annexin V and Propidium Iodide (PI) using FITC Annexin V apoptosis detection system (BD Biosciences) according to the manufacturer's instructions. After transfection of HNSCC or infection of HOK16B, respectively, cells were plated at 2×10^5 cells per well on a 12 well plate. Prior to flow cytometry (University of Michigan Flow cytometry core), cells were detached, stained and analyzed.

Gene expression profiling

Gene expression profiling was performed with Agilent Whole Human Genome Oligo Microarray according to the manufacturer's protocol. The gene expression signatures were filtered and uploaded into the Oncomine Molecular Concepts Map (http://oncomine.org) as molecular concepts, using all features on the Agilent Whole Human Genome Oligo Microarray as the null set as described (Varambally et al 2008). More information is available in Supplementary Text S1.

Chromatin Immunoprecipitation (ChIP)

ChIP was performed with EZ-Magna ChIP A/G (Millipore/Upstate) assay system. More information is available in Supplementary Text S1

Rap1 Activation

Active rap1 was assayed with ral-GDS which binds active, GTP-bound rap1, as described (Mitra et al 2003).

Quantification of microRNA

For microRNA qPCR, total RNA including small RNA was isolated from HNSCC matched normal and tumor tissues as described (Varambally et al 2008). The detailed method is provided in Supplementary Text S1.

Inhibition of DNA methyltransferase (DNMT) with 5-aza-2'-deoxycytidine (AZA) and Histone deacetylase with Suberoylanilide hydroxamic acid (SAHA)

HNSCC cells were treated with DMSO as control or 5μM AZA (DNMT inhibitor, Calbiochem) for 6 days. For inhibition of histone deacetylase, cells were treated with DMSO for 4 days followed by 2 days with 3μM of SAHA (BioVision). To investigate the combined effect of both these inhibitors, cells were pre-treated with 5μM AZA for 4 days followed by combined treatment with 5μM AZA + 3μM SAHA for 2 days. Media was changed every day.

Methylation of CpG islands in the promoter region of Rap1GAP

DNA was isolated with PureLink genomic DNA preparation kit (Invitrogen). About 1μg DNA was modified with bisulfite treatment with EpiTect Bisulfite kit (Qiagen) according to manufacturer's instruction followed by PCR using methylation and unmethylation specific primers listed in Table S3. More information is available in Supplementary Text S1.

In vivo studies

The effects of EZH2 on tumor growth was evaluated using the athymic nude mouse model as described (Zhang et al 2006b). OSCC3 (1×10^5) and UM-SCC-29 cells (1×10^6) stably transduced with shEZH2 and shVSVG were injected subcutaneously in mice. A linear mixed effects model was used to analyze the data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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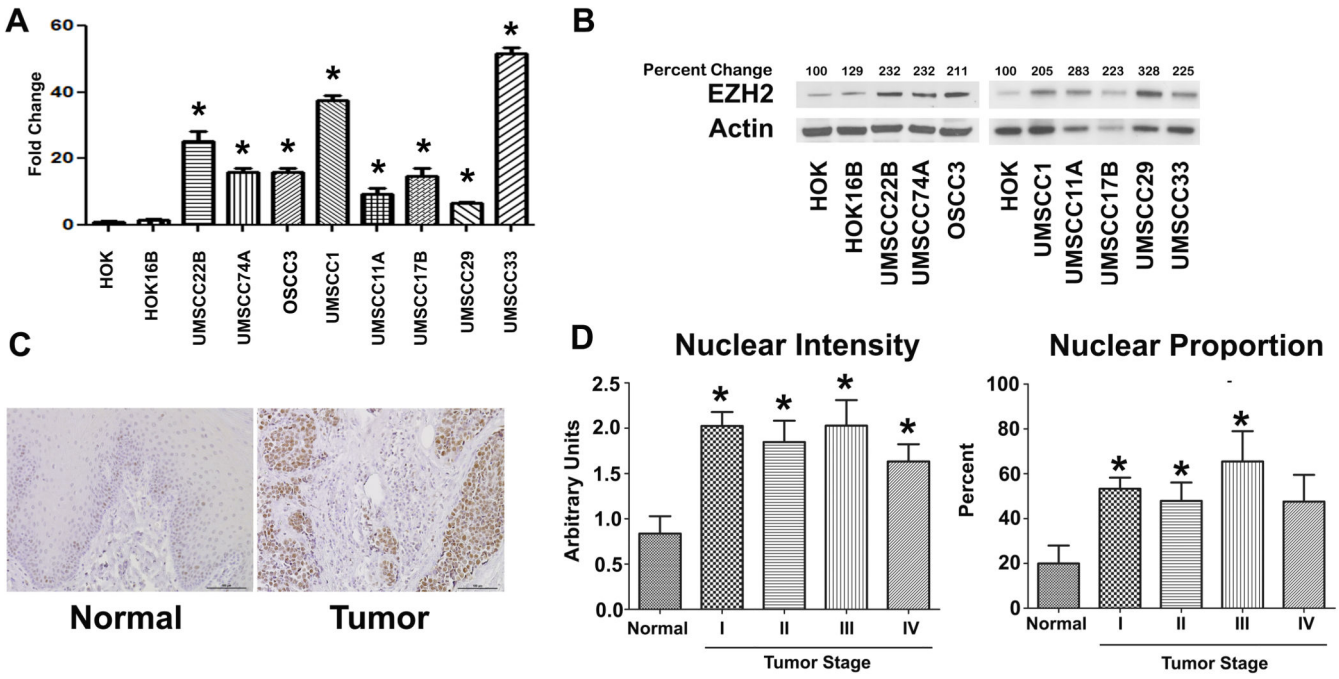

Figure 1. EZH2 is upregulated in HNSCC

A) *qPCR*. Total RNA was isolated from normal (primary, HOK) and immortalized (HOK16B) keratinocytes and HNSCC cell lines, UMSCC-(22B, 74A) OSCC3, UMSCC-(1, 11A, 17B, 29, 33). cDNAs were synthesized and qPCR was performed. Data were analyzed by relative quantification method with normalization to GAPDH and then relative to normal keratinocytes. * $p<0.009$ B) *Immunoblot analysis*. Whole cell lysates were immunoblotted with anti-EZH2. Actin was used as a loading control. Signal intensity was quantified by densitometric analysis with normalization to actin and then expressed as percent of the signal of normal keratinocytes (HOK). C) A TMA with normal and HNSCC tissues was incubated with anti-EZH2 antibody followed by DAB detection. D) The intensity and proportion were scored. Stages I, II, III and IV (n = 25, 16, 7, 10 respectively) were compared to normal (n = 11) (* $p<10^{-4}$).

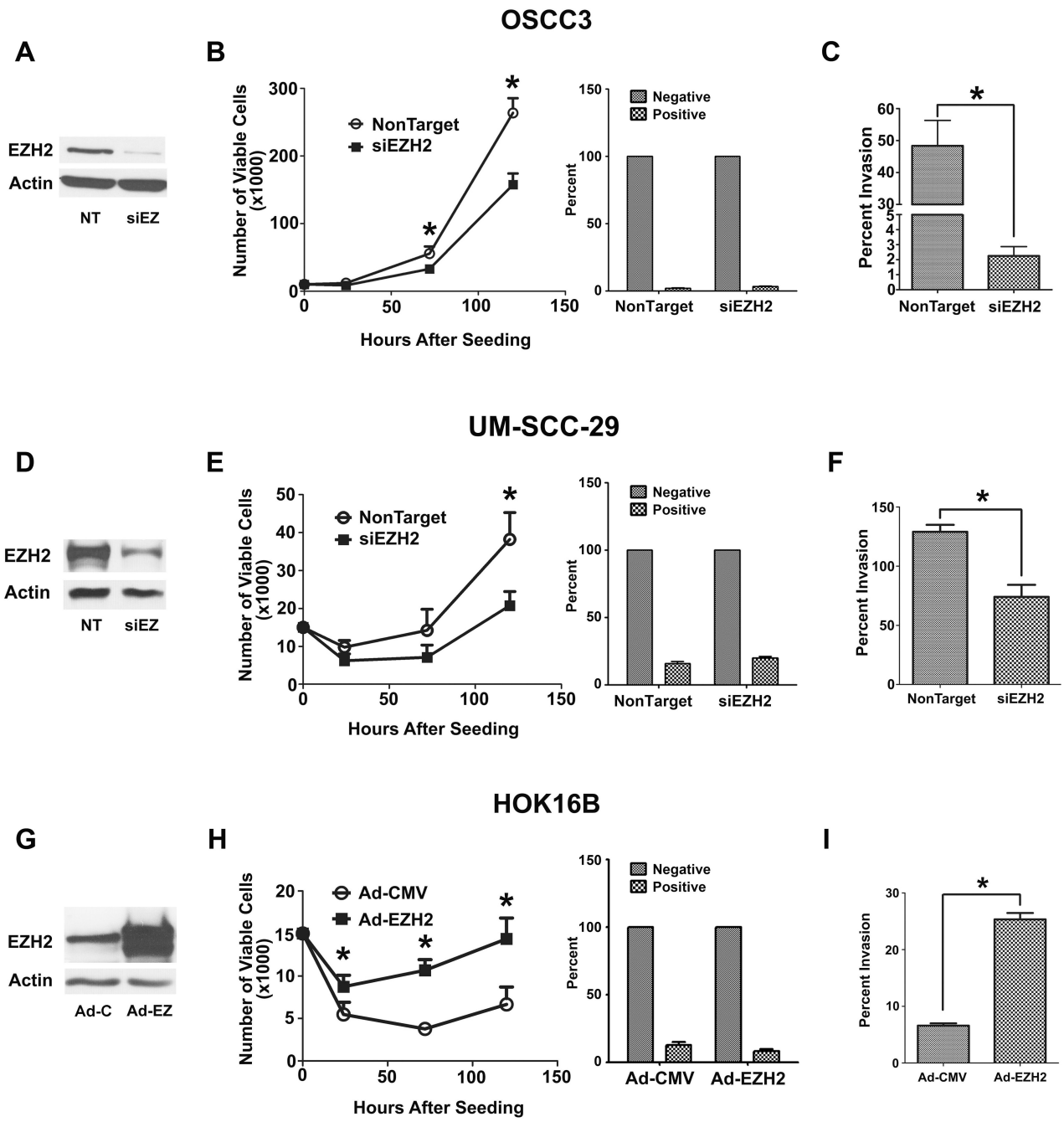

Figure 2. EZH2 promotes proliferation and invasion in HNSCC

A and D) OSCC3, and UM-SCC-29 cells were transfected with small interfering RNA of EZH2 (siEZH2) or non target (siNT) siRNA. 48h after transfection whole cell lysates were electrophoresed and immunoblotted with EZH2 and actin antibodies. B and E) *Left panel:* OSCC3 and UMSCC29 cells were seeded in a 24 well plate 48h after transfection with Non Target and EZH2 siRNA and were counted at 1, 3 and 5 days after seeding. Total viable cells are shown. *Right panel:* The apoptotic cell population was evaluated at 72hrs and presented as percent of normal (Negative) cells normalized to 100 percent. Negative cells

Annexin V negative/PI negative. Positive cells Annexin V positive/PI positive. C and F) Invasion in cells transfected with NT or siEZH2. G) HOK16B cells were infected with control adenovirus (Ad-C) or Adeno-EZH2 (Ad-EZ). 48h after transfection whole cell lysates were electrophoresed and immunoblotted with EZH2 and actin antibodies. H) *Left panel:* HOK16B cells 48 hours after infection with adenovirus were seeded in a 24 well plate and viable cells were counted at 1, 3 and 5 days after seeding (* $p < 0.05$). *Right panel:* The apoptotic cell population was evaluated as above. I) Invasion in cells with adenovirus Ad-CMV and Ad-EZH2 infected HOK16B.

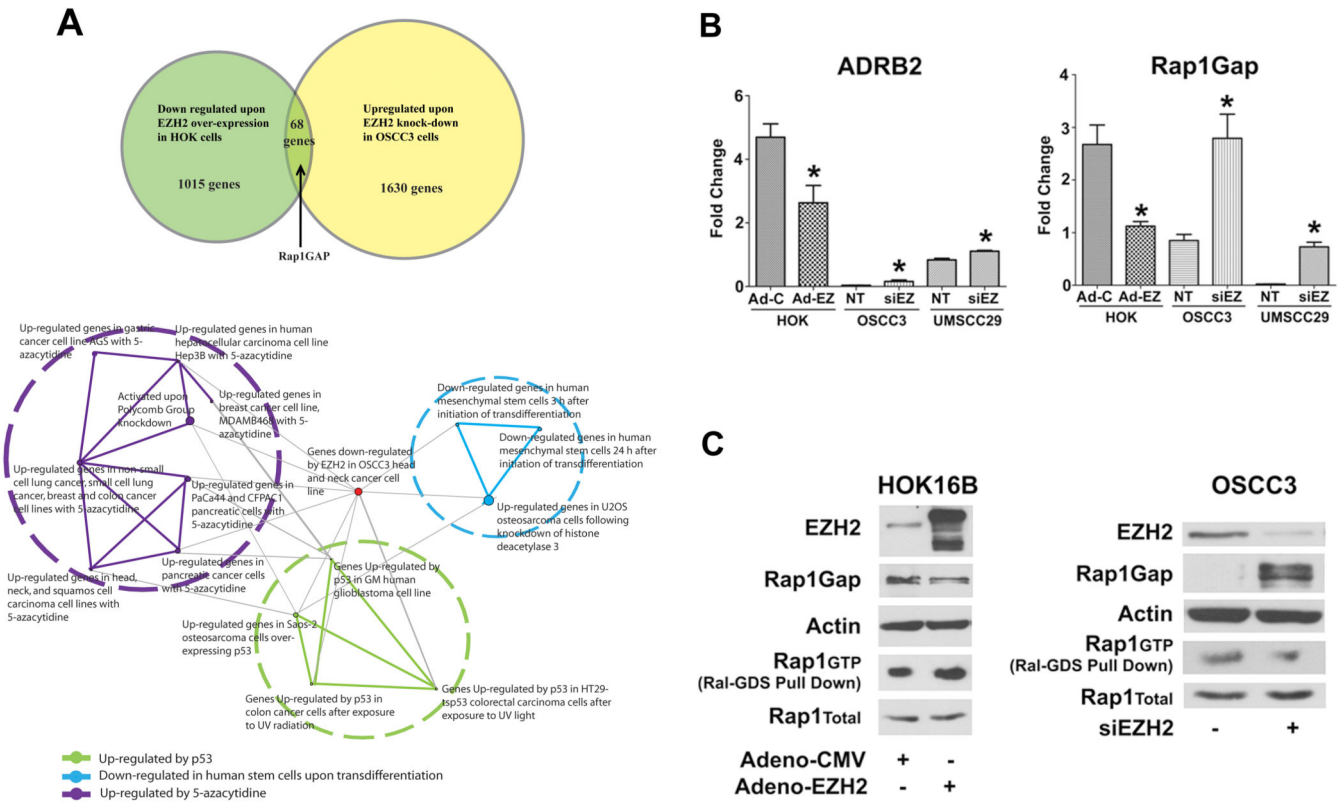

Figure 3. EZH2 downregulates rap1GAP in HNSCC cell lines

A) *Top panel.* Venn diagram showing overlap of tumor suppressor genes upregulated by EZH2 knockdown in cancer cells and dowregulated upon EZH2 overexpression in normal keratinocytes. *Lower panel.* An enrichment network linking genes up-regulated by siEZH2 (tumor suppressor genes, red node), genes up-regulated by a methylation inhibitor (purple nodes), p53 up-regulated genes (green nodes) and stem cell genes (blue nodes). Gene lists were derived from gene array data from siEZH2 in HNSCC cells and publically available data sets and analyzed by the molecular concept map module in Oncomine. Each node represents one molecular concept or gene set with node size proportional to the number of genes. Each edge represents a statistically significant ($p < 0.0001$) overlap of genes in the two linked nodes. Molecular concepts were grouped into three major clusters indicated by oval rings, the colour designated as above. B and C) RNA and whole cell lysates were isolated from normal keratinocytes (HOK) infected with Ad-EZH2 or empty vector (CMV) and from OSCC3 and UM-SCC-29 cells transfected with siEZH2 or non target siNT control siRNA. B) qPCR was performed with ADRB2 and rap1GAP primers. Data were analyzed by relative quantification method normalized to GAPDH and then relative to the corresponding control cells (* $p < 0.04$). C) Whole cell lysates from immortalized keratinocytes (HOK16B) and OSCC3 cells were immunoblotted. Data are representative of two independent experiments in each cell line.

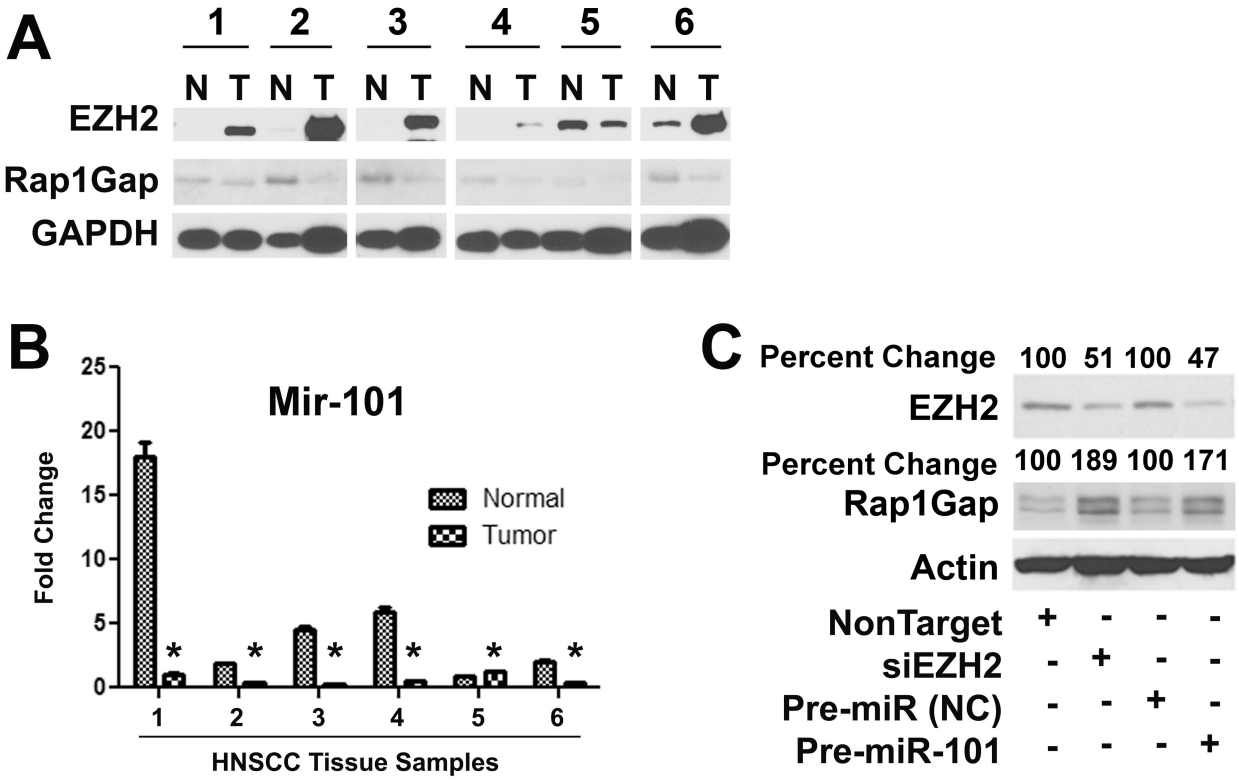

Figure 4. miR101 and EZH2 and rap1GAP are inversely correlated in human HNSCC

A) Matched normal/tumar tissue lysates were immunoblotted with anti-EZH2, anti-rap1GAP and GAPDH. B) miR-101 was quantifed in the same matched normal/tumor tissues by qPCR ($p<0.05$). C) OSCC3 cells transfected with siEZH2, non target siNT and pre-miR-101 and pre-miR negative control (NC), as indicated. Cell lysates were immunoblotted with anti-EZH2, anti-rap1GAP and anti-actin. Numbers are densitometric units (DU) with normalization to actin and expressed as percent of the corresponding control.

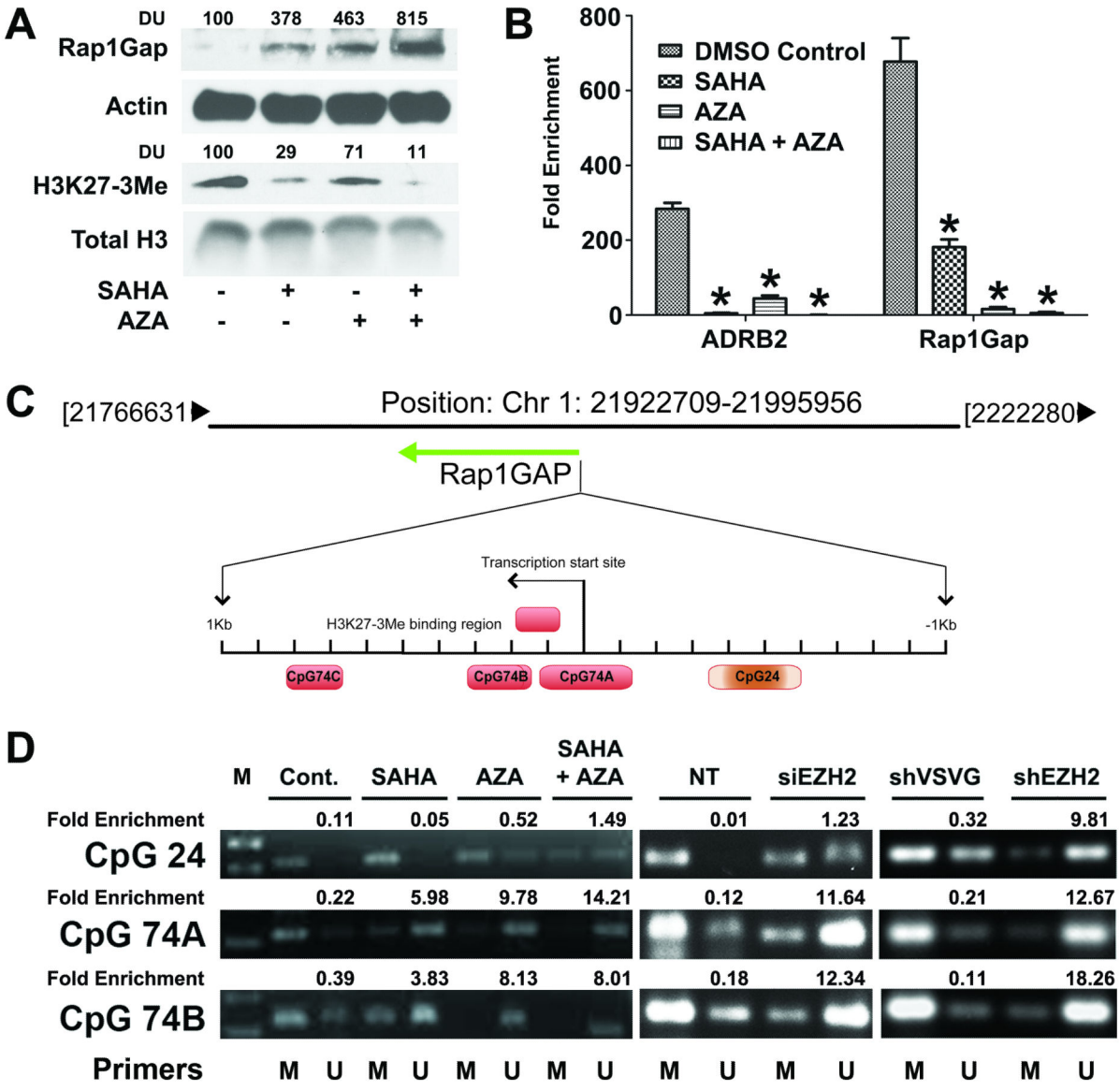

Figure 5. EZH2 silences rap1GAP via methylation

OSCC3 cells were treated with SAHA, AZA or both SAHA and AZA. A) Whole cell lysates were immunoblotted with rap1GAP, actin (loading control), methyl (H3K27-3Me) and total H3 antibodies (loading control). Densitometric unit (DU) represents values normalized to actin for rap1GAP or total H3 for H3K27-3me, and expressed as percent change relative to controls. B) qPCR was performed with ChIP eluted DNA with validated primers specific to the H3K27 binding site in the promoter region of *ADRB2* (positive control) and *rap1GAP*. C) Schematic representation of promoter region of rap1GAP showing CpG islands that were investigated for methylation and putative H3K27-3me binding region. D) OSCC3 cells were treated with SAHA, AZA or SAHA and AZA combined (left panel) as well as were transfected with siEZH2 or control non targeting siRNA (middle panel) and also transduced with shVSVG and shEZH2 (right panel). DNA was isolated and modified with bisulfite. Primers specific for methylation (M) and unmethylation (U) in three CpG islands in the

promoter region were used for PCR (Table S3). The fold enrichment with unmethylated primers relative to corresponding methylated primers is shown.

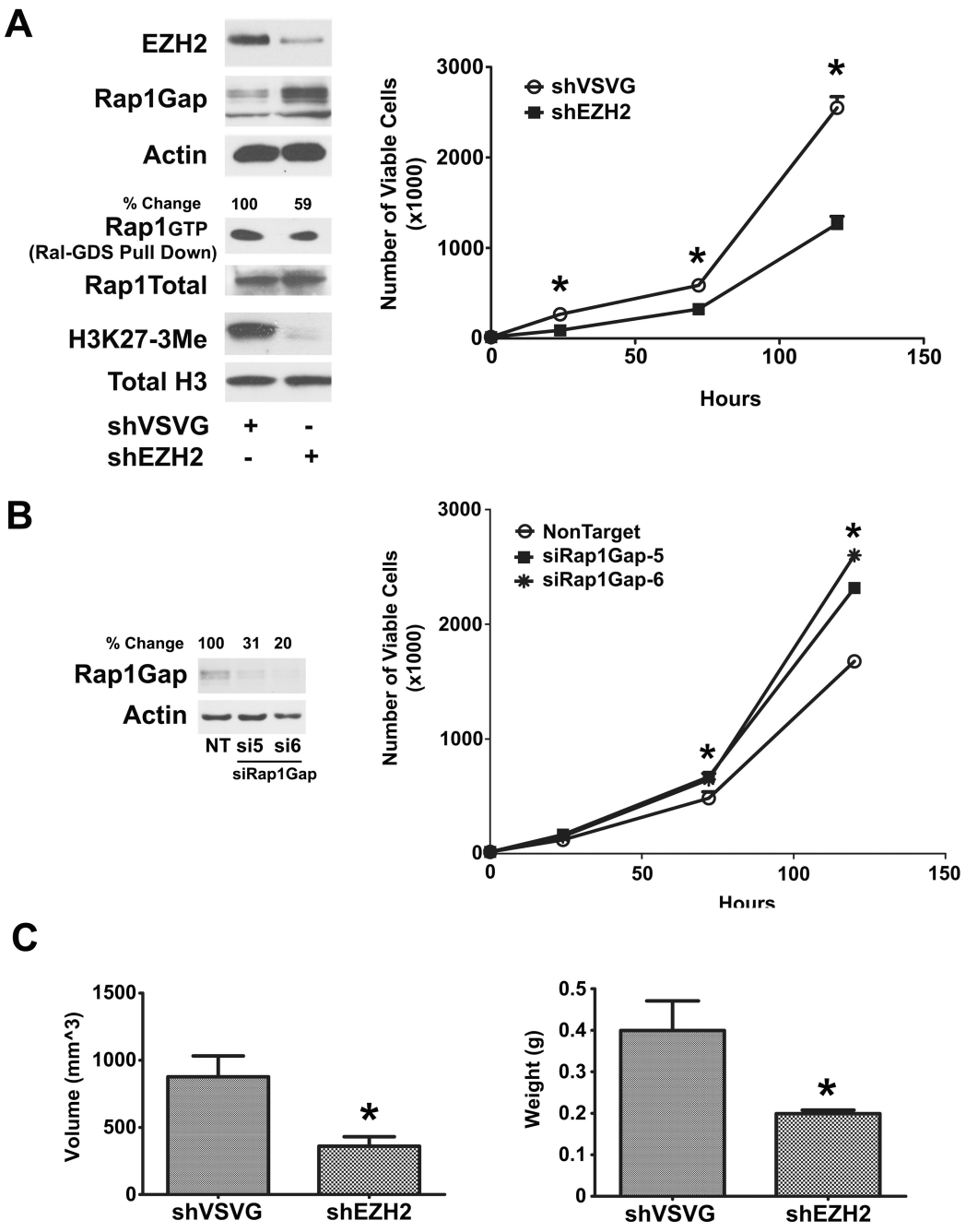

Figure 6. EZH2 promotes proliferation via inhibition of rap1GAP

A) Left panel: OSCC3 cells transduced with shEZH2 or control shRNA were stably selected. Whole cell lysates were immunoblotted with EZH2, rap1GAP, actin, rap1, H3K27-3Me and total H3 antibodies. A pull down assay for active rap1 (rap1GTP) was also performed. Right panel: OSCC3 cells stably transduced with empty vector or shEZH2 were seeded in triplicate in a 24 well plate and cells were counted at 1, 3 and 5 days after seeding. Total viable cells are shown. * $p < 0.02$. Data are representative of three independent experiments. B) Left panel: OSCC3-shEZH2 cells were transfected with siRap1GAP (si5

and si6) or non target (NT) control siRNA. Whole cell lysates were immunoblotted with rap1GAP to verify knockdown. Right panel. OSCC3-shEZH2 cells were transfected with siRap1GAP (si5, si6) or non target siRNA (NT). OSCC3 cells were seeded in triplicate at 48h after transfection in a 24 well plate and cells were counted at 1, 3 and 5 days after seeding. Total viable cells are shown. * $p < 0.05$ at 72h and $p< 0.0002$ at 120 h. The data are representative of two independent experiments. C) OSCC3 cells stably transduced with shEZH2 and shVSVG were injected subcutaneously in mice ($n=5$; $p<0.05$).

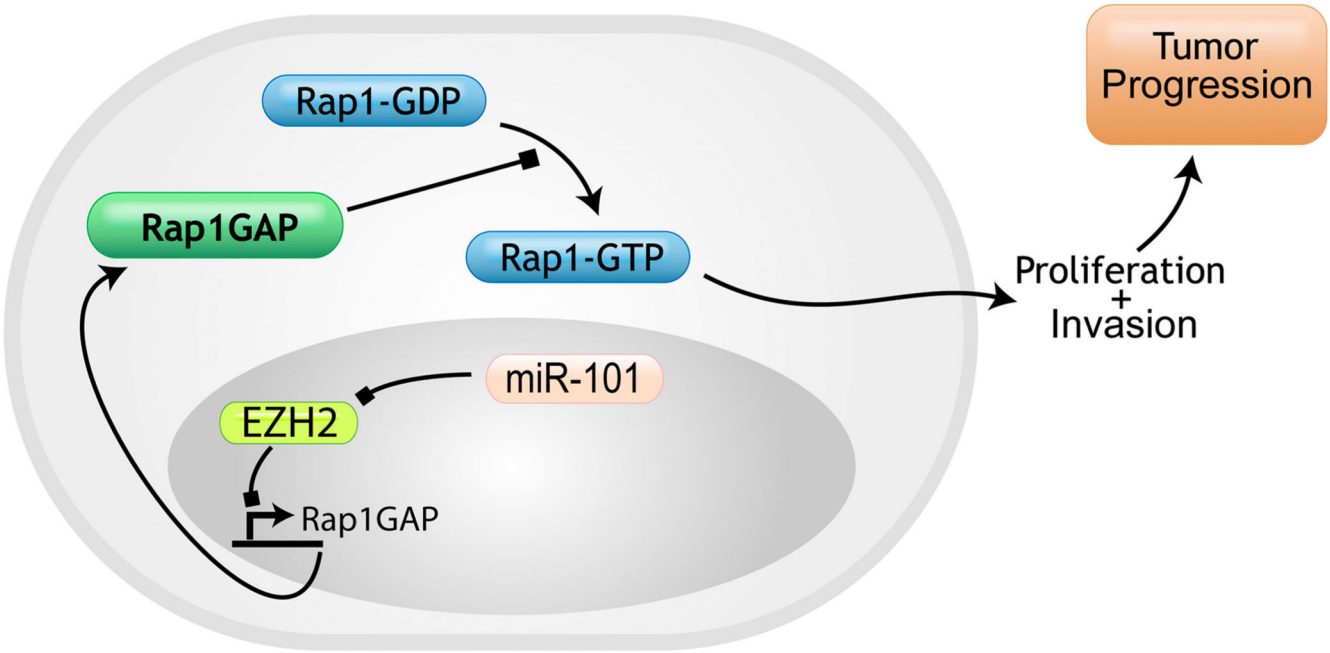

Figure 7. Proposed mechanism involving a microRNA-oncogene-tumor suppressor gene axis to understand head and neck cancer progression

Downregulation of miR-101, promotes EZH2 expression, and methylation-mediated downregulation of rap1GAP. This upregulates active rap1, thereby promoting proliferation via ERK-mediated cell cycle effects, as shown previously.