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

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Introduction

Squamous cell carcinoma is a common cancer of the 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 (Simon and Lange, 2008; Chi et al., 2010). The 5-year survival rate is poorer than that of most major cancers, including melanoma and breast cancer (Todd et al., 1997; Yarbrough et al., 2006). Unfortunately, in 40 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 that trimethylates histone 3 at lysine 27 (H3K27) (Simone and Lange, 2008; Chi et al., 2010). 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 (Varambally et al., 2002; Cao et al., 2008; Chi et al., 2010). The other members of the polycomb repressive complex 2 complex are Suppressor of Zeste 12 homolog and Embryonic Ectoderm Development. The polycomb repressive complex 2 complex also serves as a recruiting platform for DNA methyltransferases, thereby linking two epigenetic repression systems, that is, histone methylation and promoter hypermethylation. The histone methyltransferase repression mark facilitates gene silencing and oncogenesis by inhibiting the binding of transcription factors to the promoter region of genes (Simon and Lange, 2008; Chi et al., 2010). 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 (Varambally et al., 2002; Kleer et al., 2003; Kidani et al., 2009).

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, b; Zheng et al., 2009; Zuo et al.,...
However, the mechanistic basis of rap1GAP downregulation 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; Reedquist et al., 1999; Hogan et al., 2004; Price et al., 2004). 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 are endogenous, noncoding RNAs that inhibit tumor suppressor genes or upregulate oncogenes, thereby promoting tumorigenesis (Vararambally 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 miR-101 promotes upregulation of EZH2 and epigenetic silencing of rap1GAP via methylation of H3K27 and via promoter hypermethylation, and establish a novel role for miR-101 and EZH2 in rap1GAP-mediated tumor progression.

Results

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

![Figure 1](image_url)
tissue (Figure 1d, left and right panels, respectively). Cancers showed overexpression of EZH2 in the proportional odds model ($P = 1.3 \times 10^{-5}$). 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 (Figure 1b). The siRNA-mediated 82% reduction in EZH2 expression in OSCC3 cells (Figure 2a) was accompanied by a decrease in both proliferation (Figure 2b, left panel) and invasion (Figure 2c). The reduction in EZH2 induced an insignificant change in the apoptotic cell population (Figure 2d).

**Figure 2** EZH2 promotes proliferation and invasion in HNSCC. (a, d) OSCC3 and UM-SCC-29 cells were transfected with small interfering RNA of EZH2 (siEZH2) or nontarget (NT) siRNA. At 48 h after transfection whole-cell lysates were electrophoresed and immunoblotted with EZH2 and actin antibodies. (b, e) Left panel: OSCC3 and UMSCC29 cells were seeded in a 24-well plate 48 h after transfection with nontarget (NT) 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 72 h and presented as percent of normal (negative-non-apoptotic) cells normalized to 100%. Negative cells, Annexin V negative/PI negative. Positive (apoptotic) cells, Annexin V positive/PI positive. (c, f) Invasion in cells transfected with NT or siEZH2. (g) HOK16B cells were infected with control adenovirus (Ad-C) or Ad-EZ. At 48 h after transfection whole-cell lysates were electrophoresed and immunoblotted with EZH2 and actin antibodies. (h) Left panel: HOK16B cells 48 h 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.
Overexpression of EZH2 in non-malignant keratinocytes with low endogenous EZH2 had the reverse effect on proliferation and invasion. There was a greater than sixfold increase in EZH2 expression in cells infected with Adeno-EZH2 (Ad-EZ) relative to control Adeno-CMV (Figure 2g). Proliferation and invasion were significantly increased in cells infected with Ad-EZH2 compared with control (Figure 2h, left panel, and Figure 2i, respectively). Overexpression of EZH2 induced a slight decrease in the apoptotic cell population (Figure 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, that is, original and dye swap) and downregulated with Ad-EZH2 were nominated as tumor suppressor genes (Figure 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 (http://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 5-aza-2'-deoxycytidine (AZA), a methylation inhibitor, and genes that were downregulated in stem cells (Figure 3a, lower panel).

We have shown that rap1GAP has an important tumor suppressor role in HNSCC (Zhang et al., 2006b; Mitra et al., 2008). Hence, subsequent studies focused on EZH2-mediated regulation of rap1GAP. Rap1GAP...
expression and role of ADRB2 as a positive control were validated by qPCR (Figure 3b). Downregulation of EZH2 induced an increase in both rap1GAP and ADRB2 in OSCC3 and UM-SCC-29 (Figure 3b, siEZ; P < 0.05). Conversely, overexpression of EZH2 in normal keratinocytes downregulated rap1GAP and ADRB2 (Figure 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 (Figure 3c, left panel) and knockdown of EZH2 in HNSCC cells, increased rap1GAP protein expression (Figure 3c, right panel). As 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 (Figure 3c, left panel). In OSCC3, siEZH2 decreased EZH2 expression by 77% (Figure 3c, right panel). This was accompanied by a greater than eightfold 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 (Supplementary Figure 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 Figure 4a, EZH2 is upregulated in 5/6 HNSCC tissue samples (sample 1, 2, 3, 4 and 6). In four of these five samples (Figure 4a, sample 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 (Varambally et al., 2008; Friedman et al., 2009; He et al., 2010). To determine whether the increase in EZH2 expression is a function of gene amplification, fluorescence in-situ hybridization and immunohistochemistry were performed on human HNSCC tissues. No gene amplification was observed in paired tumor/normal tissue samples (Supplementary Figure S2A). Upregulation of EZH2 in tumor was verified (Supplementary Figure S2B). To determine whether the increase in EZH2 in HNSCC was a function of a change in miR-101, the latter 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 (Figure 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 with the corresponding cells transfected with control pre-miR (Figure 4c, lane 4 compared with lane 3). This downregulation in EZH2 expression was similar to that observed with siEZH2 and corresponded to an increase in expression of rap1GAP (Figure 4c, lane 2 compared with 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 suberoylanilide hydroxamic acid (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 the combination SAHA plus AZA (Figure 5a, top panel). Reduction in levels of H3K27 tri-methylation was verified (Figure 5a, lower middle panel). As 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 chromatin immunoprecipitation (ChIP) of methylated H3K27 (mark of EZH2) followed by PCR with primers (Supplementary Table S2) spanning the trimethylated H3K27 binding region (Figure 5c). As shown in Figure 5b, trimethylation of H3K27 with the promoter region of rap1GAP decreased upon treatment with SAHA, AZA and the combination 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 (Figure 5c). OSCC3 cells were treated with SAHA, AZA or the combination 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 relative to methylated DNA, particularly in CpG74A and to a lesser extent in CpG74B (Figure 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 stable knockdown of EZH2 (shEZH2). Unmethylated CpG74A and CpG74B...
increased compared with corresponding methylated CpG74A and CpG74B (Figure 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 with transiently with siEZH2 (Figure 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 and control shRNA were stably selected. Whole-cell lysates were immunooblotted 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 nontarget (NT) control siRNA. Whole-cell lysates were immunooblotted with rap1GAP to verify knockdown. Right panel: OSCC3-shEZH2 cells were transfected with siRap1GAP (si5, si6) or NT siRNA. OSCC3 cells were seeded in triplicate at 48 h 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 72 h 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 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 immunooblotted 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 nontarget (NT) control siRNA. Whole-cell lysates were immunooblotted with rap1GAP to verify knockdown. Right panel: OSCC3-shEZH2 cells were transfected with siRap1GAP (si5, si6) or NT siRNA. OSCC3 cells were seeded in triplicate at 48 h 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 72 h 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).
In vivo, downregulation of EZH2 (OSCC3-shEZH2) significantly inhibited tumor growth, compared with control tumors (Figure 6c). EZH2 tumors were smaller by 354 mm³ on average, adjusted for the mouse-specific effect (95% confidence interval of 137–571 mm³; \( P = 0.033 \)) and the weight of the tumors was less by 0.15 g (95% confidence interval of 0.04–0.24 g; \( P = 0.049 \)). Similar effects of EZH2 on cell proliferation and tumor growth were observed in UM-SCC-29 (Supplementary Figure S3).

Discussion

Rap1GAP is downregulated in multiple aggressive human tumors including HNSCC, pancreatic cancer, thyroid and colon cancer (Zhang et al., 2006a; Zhang et al., 2009; Tsygankova et al., 2010) but the mechanism of downregulation is unclear. In this important and novel study, we demonstrate that silencing of rap1GAP is regulated by EZH2 that represses transcription of rap1GAP by H3K27 trimethylation and promoter hypermethylation. Moreover, reduction in miR-101 expression upregulates 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 (Figure 7). Given the crucial role of rap1GAP in aggressive tumors (Zhang et al., 2006a; 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, that is, tumor progression (Thomas and Speig, 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 (Figure 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). Although 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 (Simon and Lange, 2008; Chi et al., 2010). This histone methylation facilitates chromatin compaction and reduces gene transcription (Francis et al., 2004). Furthermore, the polycomb repressive complex 2 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 (Figure 5d), suggesting EZH2-mediated methylation on H3 and promoter hypermethylation are coordinated.

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 (Figure 4). EZH2 overexpression in HNSCC was not due to gene amplification (Supplementary Figure S2A) but was correlated with downregulation of miR-101. Furthermore, knockdown of EZH2 or overexpression of miR-101 in HNSCC cells increased the expression of rap1GAP and established a tumor suppressor role of miR-101 controlling another tumor suppressor rap1GAP. Subsequently, in in-vitro experiments overexpression of EZH2 in non-malignant keratinocytes with low endogenous 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 (Figure 6). Finally, shEZH2 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 (Varambally et al., 2002; Cao et al., 2008). 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 were 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 (Varambally et al., 2002; Kleer et al., 2003; Cao et al., 2008; Chi et al., 2010; Morin et al., 2010). 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 (Varambally et al., 2008; Friedman et al., 2009; He et al., 2010). 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., 2006a). In the present study, EZH2 promoted 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 (Zhang et al., 2006a; b; Tsygankova et al., 2007; 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.

Materials and methods

Cell culture

Human HNSCC cells, primary HOK and immortalized HOK16B were cultured as described previously (Mitra et al., 2003, 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, Rockville, MD, USA) was performed as described (Mitra et al., 2003), with affinity-purified anti-EZH2 (BD Transduction, Lexington, KY, USA). 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 variables were 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, Lafayette, CO, USA). For stable knockdown of EZH2, OSCC3 and UM-SCC-29 cells were transduced with lentiviral particles of scramble shRNA (shVSVG) and shEZH2 (Open Biosystems, Huntsville, AL, USA) according to the manufacturer’s instructions. 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 overexpression of miR-101 cells were transfected with pre-miR-101 and pre-miR negative controls (Ambion, Austin, TX, USA).

Measurement of apoptosis

Apoptosis was evaluated by double staining with FITC-labeled Annexin V and propidium iodide using FITC Annexin V apoptosis detection system (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer’s instructions. After transfection of HNSCC or infection of HOK16B, respectively, cells were plated at 2 × 10⁵ cells per well on a 12-well plate. Before 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 (Agilent, Santa Clara, CA, USA) according to the manufacturer’s protocol. The gene expression signatures were filtered and uploaded into the
Onchome 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). The gene expression data has been submitted to GEO database under accession no GSE28501 (http://www.ncbi.nlm.nih.gov/geo/info/linking.html). More information is available in Supplementary Text S1.

Chromatin immunoprecipitation
ChIP was performed with the EZ-Magna ChIP A/G (Millipore/Upstate, Billerica, MA, USA) assay system. More information is available in Supplementary Text S1.

Rap1 activation
Active rap1 was assayed with ral-GDS that binds active Rap1 activation information is available in Supplementary Text S1.

Methylation of CpG islands in the promoter region of Rap1GAP DNA was isolated with PureLink genomic DNA preparation kit (Invitrogen, Carlsbad, CA, USA). About 1 μg DNA was modified with bisulfite treatment with EpitTect Bisulfite kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instruction followed by PCR using methylation and unmethylation specific primers listed in Supplementary Table S3. More information is available in Supplementary Text S1.

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

Conflict of interest
The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)