The histone methyltransferase EZH2, an oncogene common to benign and malignant parathyroid tumors

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Abstract

Primary hyperparathyroidism (pHPT) resulting from parathyroid tumors is a common endocrine disorder with incompletely understood etiology. In renal failure, secondary hyperparathyroidism (sHPT) occurs with multiple tumor development as a result of calcium and vitamin D regulatory disturbance. The aim of this study was to investigate a potential role of the histone 3 lysine 27 methyltransferase EZH2 in parathyroid tumorigenesis. Parathyroid tumors from patients with pHPT included adenomas and carcinomas. Hyperplastic parathyroid glands from patients with HPT secondary to uremia and normal parathyroid tissue specimens were included in this study. Quantitative RT-PCR, western blotting, bisulfite pyrosequencing, colony formation assay, and RNA interference were used. EZH2 was overexpressed in a subset of the benign and in all malignant parathyroid tumors as determined by quantitative RT-PCR and western blotting analyses. Overexpression was explained by EZH2 gene amplification in a large fraction of tumors. EZH2 depletion by RNA interference inhibited sHPT-1 parathyroid cell line proliferation as determined by tritium–thymidine incorporation and colony formation assays. EZH2 depletion also interfered with the Wnt/β-catenin signaling pathway by increased expression of growth-suppressive AXIN2, a negative regulator of β-catenin stability. Indeed, EZH2 contributed to the total level of aberrantly accumulated transcriptionally active (nonphosphorylated) β-catenin in the parathyroid tumor cells. To our knowledge EZH2 gene amplification presents the first genetic aberration common to parathyroid adenomas, secondary hyperplastic parathyroid glands, and parathyroid carcinomas. This supports the possibility of a common pathway in parathyroid tumor development.

Key Words
- Axin2
- β-catenin
- EZH2
- gene amplification
- hyperparathyroidism
- PRC2

Introduction

Parathyroid disease with hypersecretion of parathyroid hormone and generally also hypercalcemia occurs in primary hyperparathyroidism (pHPT), due to growth regulatory disturbance in the parathyroid glands. pHPT is caused by a single, benign adenoma in ~85% of cases and parathyroid hyperplasia or multiple adenomas in ~15%, and very rarely by parathyroid carcinoma (<1%). In renal failure, secondary hyperparathyroidism (sHPT) occurs with multiple tumor development as a result of calcium and vitamin D regulatory disturbance (Marx 2000, Åkerström & Hellman 2004, Westin et al. 2009, Sharretts & Simonds 2010). Overexpression of the gene cyclin...
D1 (CCND1) has been demonstrated in pHPT tumors (20–40%), due to gene translocation and oncogene activation (8%) and to other unknown mechanisms. Also secondary hyperplastic parathyroid glands (31%) and parathyroid carcinomas (90%) have been shown to over-express cyclin D1 (Westin et al. 2009, Sharrett & Simonds 2010). CCND1 constitutes a target gene for the Wnt/β-catenin signaling pathway and in the absence of gene translocation, overexpression may in part be explained by pathway activation through aberrant accumulation of transcriptionally active (nonphosphorylated) β-catenin in the cytoplasm/nucleus (Shtutman et al. 1999, Lin et al. 2000, Björklund et al. 2007a, Westin et al. 2009). By immunohistochemical analysis, accumulation of total β-catenin has been observed in one study, as a frequent event in parathyroid adenomas and secondary hyperplastic parathyroid glands (Björklund et al. 2007b). Such accumulation has not been observed applying several other protocols, as recently summarized in Costa-Guda & Arnold (2013). Using western blotting analysis and a specific antibody (van Noort et al. 2002), the transcriptionally active (nonphosphorylated) form of β-catenin was found to be accumulated in parathyroid adenomas and secondary hyperplastic parathyroid glands, strongly implying activation of the Wnt/β-catenin signaling pathway (Björklund et al. 2007b, Clevers & Nusse 2012).

The accumulation of active β-catenin involved an aberrantly spliced internally truncated Wnt coreceptor LRP5. siRNA to LRP5 was shown to reduce the non-phosphorylated active β-catenin level, transcriptional activation by β-catenin, and inhibited parathyroid tumor cell growth in vitro and in a xenograft SCID mouse model (Björklund et al. 2007c). The Wnt/β-catenin signaling pathway has been shown recently to be activated also in parathyroid carcinomas, by aberrant accumulation of nonphosphorylated active β-catenin. This was caused by inactivation of the APC tumor suppressor gene, likely by promoter DNA methylation (Svedlund et al. 2010). Furthermore, inactivating somatic hereditary hyperparathyroidism type 2 (HRPT2 (CDC73)) gene mutations are common in parathyroid carcinoma and may contribute to cyclin D1 overexpression (Woodard et al. 2005, Westin et al. 2009, Sharrett & Simonds 2010).

Somatic inactivation of the multiple endocrine neoplasia type 1 (MEN1) tumor suppressor gene is frequently observed (~35%) in parathyroid adenomas (Lemos & Thakker 2008, Cromer et al. 2012, Newey et al. 2012).

Recently, it was demonstrated that the HIC1 tumor suppressor gene was generally underexpressed in parathyroid adenomas and secondary hyperplastic parathyroid glands and in parathyroid carcinomas (Svedlund et al. 2012). It was suggested that this involved repressive histone modification H3K27me2/3 rather than DNA methylation. Enhancer of zeste homolog 2 (EZH2) is the enzymatic part of the polycomb repressive complex 2 (PRC2), which is involved in many processes such as cell differentiation, proliferation, cell identity, and stem cell plasticity. PRC2 consists of several components, among them EED and SUZ12 promote PRC2 assembly and contribute to the H3K27 methyltransferase activity of EZH2 (O’Meara & Simon 2012). EZH2 is highly expressed in many solid tumors, it is considered to be an oncogene and amplification of EZH2 has been observed in several cancers (Bracken et al. 2003, Saramaki et al. 2006). In lymphoma, heterozygous missense mutations at amino acid Y641 caused higher catalytic efficiency. In contrast, inactivating EZH2 mutations have been found in myeloid neoplasms, supporting a tumor suppressor function (Chase & Cross 2011, Margueron & Reinberg 2011, Chang & Hung 2012). Recently, a link was established between EZH2 and Wnt/β-catenin signaling in the development of hepatocellular carcinoma (Cheng et al. 2011). EZH2 was shown to mediate epigenetic repression of several Wnt antagonists, including the growth-suppressive Axin2 which is known to negatively regulate the level of cytoplasmic β-catenin in the absence of Wnt ligand (Cheng et al. 2011, Clevers & Nusse 2012). EZH2 may also interact directly with β-catenin as has been demonstrated in breast cancer (Shi et al. 2007, Li et al. 2009). In this study, we investigated a possible role of EZH2 in parathyroid tumorigenesis.

Materials and methods

Tissue specimens

Parathyroid carcinomas (n=5), adenomas (n=28), and hyperplastic parathyroid glands secondary to uremia (n=16) from patients with pHPT and sHPT were acquired from patients diagnosed and operated upon in the clinical routine at Uppsala University Hospital and Martin Luther University of Halle-Wittenberg. Normal parathyroid tissue was obtained from glands inadvertently removed in surgery for goiter or follow-up. All tissues were intraoperatively snap frozen and cryosections were used in the analyses. Informed consent and approval of ethical committee was achieved.
Bisulfite treatment and pyrosequencing

Total DNA was extracted using AllPrep DNA/RNA Mini Kit (Qiagen) and bisulfite treatment was performed on 400 ng DNA using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. Before pyrosequencing, a PCR was carried out with EZH2 promoter primers: F, 5'-GTATT-TTGAGTGGGTTGGTT-3'; R, 5'-CCCCCAACCCCAATCAA-3' (biotin labeled); sequencing primer, 5'-GTTGAGG-GTTTGTGATTGGA-3'; sequence analyzed, YGGGYGTTT-TYGGTGYTTGGTGA-3'). The PCR included HotStarTaq Plus Master Mix (Qiagen), 10 pmol of each primer and bisulfite-treated DNA as template. PCR was carried out with an initial denaturation at 95 °C for 15 min, followed by 45 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and completed at 72 °C for 10 min. Pyrosequencing was done using 20 µl PCR product with the PyroMark Q24 system (Qiagen) according to manufacturer’s instructions. The assay was validated to distinguish equal amplification of unmethylated and methylated DNA. This was done by analyzing a dilution series of bisulfite-treated CpGenome Universal Methylated DNA (Millipore, Billerica, MA, USA; 100% methylated) and placenta DNA (~0% methylated).

RNA extraction and quantitative RT-PCR

DNA–free total RNA was extracted from pHPT tumors (n=28), sHPT tumors (n=16), parathyroid carcinoma (n=5), and normal parathyroid tissue (n=5) using AllPrep DNA/RNA Mini Kit and RNase-Free DNase Set (Qiagen) according to the manufacturer’s instructions. Successful DNase-treatments were established by PCR analysis of all RNA preparations. RT of total DNA–free RNA was carried out with random hexamer primers using the RevertAid First, strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. Quantitative RT-PCR was carried out on StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA, USA) using Maxima Probe/ROX qPCR Master Mix (Thermo Scientific) and Applied Biosystems TaqMan assays for EZH2 (Hs01016789_m1), SUZ12 (Hs00248742_m1), EED (Hs00537777_m1), LRP5 (Hs.00182031_m1), AXIN2 (Hs_00610344_m1), CYCLIN D1 (Hs00765553_m1), and GAPDH (Hs02758991_g1). Each cDNA sample was analyzed in triplicate.

Copy-number variation

Total DNA was extracted using AllPrep DNA/RNA Mini Kit (Qiagen). Copy-number variation was determined using Applied Biosystems TaqMan copy-number assays for EZH2 (Hs02508122_cn and Hs02941342_cn) and RNase P reference (4401631) together with Maxima Probe/ROX qPCR Master Mix (Thermo Scientific) according to the manufacturer’s instructions. The samples were run on StepOnePlus Real-Time PCR Systems and analyzed using CopyCaller v2.0 (Applied Biosystems). Each DNA sample was analyzed in quadruplicates with multiple runs and EZH2 amplification was considered when both assays demonstrated this. DNA from placenta and normal parathyroid tissue were used as reference. Both EZH2 PCR assays correctly detected four copies of chromosomal DNA from a control cell line with four EZH2 gene copies (NA12519; Coriell Institute for Medical Research, Camden, NJ, USA).

Cell culture and transfection

sHPT-1 parathyroid tumor cells (2×10^5) were distributed onto 35-mm dishes in DMEM/10% fetal bovine serum (Sigma) and transfected in triplicates using Interferin siRNA transfection reagent (Polyplus Transfection; SAS, Illkirch, France). EZH2 siRNA (HSS103462, Invitrogen), LRP5 siRNA (sc-43900, Santa Cruz), and control nonsilencing siRNA (Qiagen) were transfected using 20 pmol. The cells were harvested after 120 h and RNA/protein was extracted. The cells were transfected for 48 h and then transfected again and further incubated for 72 h (Svedlund et al. 2012).

Cell viability, 3H-thymidine incorporation, and apoptosis

sHPT-1 parathyroid tumor cells were transfected with siRNA to EZH2 (see above). The cell viability was measured after 2 h of incubation with the cell-proliferation reagent WST-1 (Roche Molecular Biochemicals), according to the manufacturer’s instructions. For 3H-thymidine incorporation, 2 µl 3H-thymidine/well were added 12 h before harvest (NET027250UC from PerkinElmer, Norwalk, CT, USA). The medium was then aspirated and unspecifically incorporated 3H-thymidine was precipitated by adding 500 µl 10% trichloroacetic acid for 20 min with the plates on ice. The precipitated material was discarded and the procedure was repeated twice before lysing the cells with 500 µl 0.5 M NaOH and 0.5% Triton X-100/well. Radioactivity was monitored in a β-counter. Apoptosis was detected by using the Cell Death Detection ELISA Kit (Roche Molecular Biochemicals), according to the manufacturer’s instructions. Alternatively, apoptosis was analyzed by poly(ADP-ribose) polymerase (PARP) expression and cleavage was detected by western blot.
analysis. As a positive apoptosis control cells were incubated with 0.1 μg/ml camptothecin.

**Colony formation assay**

sHPT-1 parathyroid tumor cells (2 × 10^5) were distributed onto 35-mm dishes in DMEM/10% fetal bovine serum (Sigma) and transfected in triplicates using FuGENE 6 transfection reagent (Roche Diagnostics) with 1 μg of control vectors or EZH2 shRNA vectors (Fussbroich et al. 2011). After 24 h, cells were seeded at 4 × 10^3 in six-well plates and the following day 60 μg/ml Hygromycin B were added to the culture medium. After 8 days, the selected cells were fixed with 10% acetic acid/10% methanol and stained with 0.4% crystal violet and the visible colonies were photographed.

To monitor the effects of the shRNAs, the vectors were transiently transfected and protein extracts were prepared after 72 h followed by western blotting analysis.

**Western blotting**

Protein was extracted using Cytobuster protein extraction reagent (Merck Millipore) supplemented with Complete mini protease inhibitor cocktail tablets (Roche Diagnostics). Primary antibodies used were anti-EZH2 mouse MAB (17-662, Millipore) or EZH2 rabbit monoclonal D2C9 (5246, Cell Signaling Technology, Danvers, MA, USA), anti-active-β-catenin (van Noort et al. 2002; Upstate, Lake Placid, NY, USA, #05-665), anti-PARP rabbit polyclonal antibody (AB16661, Millipore), and anti-β-tubulin rabbit polyclonal antibody (sc-9104, Santa Cruz). After incubation with the appropriate secondary antibody, bands were visualized using the enhanced chemiluminescence system (GE Healthcare).

**Immunohistochemistry**

Paraffin-embedded specimens were stained as described (Björklund et al. 2007a) using an anti-EZH2 rabbit MAB (Cell Signaling Technology, Inc., EZH2 (D2C9), catalog no. 5246).

**Statistical analysis**

All data are presented as arithmetical mean ± S.E.M. Paired and unpaired t-test and Spearman’s rank correlation were used.
used for statistical analysis. Statistical calculations were made in IBM SPSS Statistics, version 21. *P* < 0.05 was considered significant.

**Results**

**EZH2 is overexpressed and the gene amplified in a subset of parathyroid tumors regardless of hyperparathyroid disease state**

In order to analyze whether EZH2 was differentially expressed in parathyroid tumors compared with normal parathyroid tissue specimens, quantitative RT-PCR was carried out. As shown in Fig. 1A, EZH2 was generally significantly overexpressed in sHPT (3.9 ± 0.4) tumors and most apparent in parathyroid carcinoma (19.4 ± 4.5), when compared with normal parathyroid tissues (1.8 ± 0.4). For parathyroid adenomas, the mean value did not reach significance (pHPT: 3.5 ± 0.5), but 11 tumors showed higher mRNA levels than the highest expressing normal tissue. EZH2 was significantly overexpressed in the parathyroid carcinomas compared with the adenomas and secondary hyperplastic parathyroid glands. No correlations between EZH2 mRNA expression level and gland weight, serum PTH, serum calcium, or serum creatinine were found. Furthermore, western blotting analysis showed prominently increased protein expression of EZH2 in parathyroid tumors with relatively high mRNA levels (Fig. 1B, upper panel). EZH2 protein expression could also be detected in some pHPT and sHPT tumors with the relative EZH2 mRNA levels in the normal range (Fig. 1B, lower panel). Immunohistochemistry analysis of EZH2 revealed prominent cytoplasmic and nuclear immuno-reactivity. An evenly stained level for most cells in all areas was observed in the adenoma that appeared more heterogenous in the carcinoma (Fig. 1C). mRNA levels were also determined for the PRC2 components EED and SUZ12. Only SUZ12 showed significantly different expression, with a generally reduced level in secondary hyperplastic parathyroid glands (Fig. 2). The three parathyroid carcinomas with highest EZH2 expression also showed overexpression of EED, and the two parathyroid adenomas with the highest EZH2 showed highest expression of SUZ12. The very low expression of EZH2 in the normal parathyroid tissue specimens was not associated with DNA methylation of the EZH2 CpG island, as determined by quantitative bisulfite pyrosequencing analysis (data not shown).

Real-time quantitative PCR revealed relative amplification of the EZH2 gene (four gene copies) in 29% of the pHPT tumors, 50% of the sHPT tumors, and 60% of the parathyroid carcinomas (Table 1). Thirteen out of the 19 tumors with gene amplification displayed overexpression of EZH2 mRNA and the three tumors analyzed by western blotting showed prominently increased protein expression (Fig. 1B, upper panel). Two tumors included in the western blotting analysis with gene amplification, and EZH2 mRNA expression within the normal range showed weakly detectable or undetectable EZH2 protein level respectively compared with normal tissue (Fig. 1B, lower panel). Some of the benign tumors and two of the carcinomas showed higher EZH2 mRNA expression level without gene amplification. No correlations between EZH2 gene copy number and gland weight, serum PTH, serum calcium, or serum creatinine were found.
Maintained expression of EZH2 is required for parathyroid tumor cell growth

Next, we investigated whether EZH2 could control growth of parathyroid tumor cells. siRNA to EZH2 significantly inhibited proliferation and reduced cell viability of the human parathyroid tumor cell line sHPT-1, as determined by $^3$H-thymidine incorporation and WST-1 incubation (Fig. 3A). The sHPT-1 cell line expresses parathyroid hormone and was established from a hyperplastic parathyroid gland removed at operation of a patient with sHPT (Björklund et al. 2007a). Colony formation assay was then performed to further evaluate a growth regulatory role of EZH2. sHPT-1 cells were stably transfected with EZH2 shRNA expression vectors or control vectors (Fussbroich et al. 2011). The results showed a prominent reduction in the colony formation capacity in EZH2 shRNA-expressed cells compared with control shRNAs, only a few very small colonies were seen in EZH2 shRNA-expressing cells (Fig. 3C). Thus, EZH2 depletion blocked the growth of parathyroid tumor cells. No apparent effect on apoptosis was detected after depletion of EZH2 with siRNA or shRNA expression vectors, as determined by quantifying cytoplasmic histone-associated-DNA-fragments or by examining PARP protein expression and cleavage (Fig. 3B and D).

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Number of tumors with EZH2 amplification*</th>
<th>Total number of tumors analyzed</th>
<th>Percentage of tumors with EZH2 amplification (%)</th>
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<tr>
<td>pHPT</td>
<td>8</td>
<td>28</td>
<td>29</td>
</tr>
<tr>
<td>sHPT</td>
<td>8</td>
<td>16</td>
<td>50</td>
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<tr>
<td>Carcinomas</td>
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<td>5</td>
<td>60</td>
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</table>

*(All displayed four gene copies.)

Table 1 Copy-number variation of EZH2 in parathyroid tumors

Figure 3

Cellular proliferation, viability, colony formation, and apoptosis on EZH2 knockdown. Transient transfection of siControl and siEZH2 to the sHPT-1 parathyroid tumor cell line. Efficient knockdown of EZH2 was obtained at the mRNA (4C) and protein level (Svedlund et al. 2012). (A) Effects on cell proliferation and cell viability were determined by $^3$H-thymidine incorporation (left panel) and WST-1 incubation (right panel). (B) Effects on apoptosis were analyzed by quantifying cytoplasmic histone-associated-DNA-fragments (left panel) and induction of PARP expression and cleavage (right panel). (C) Colony formation assay. sHPT-1 cells were stably transfected with plasmids expressing two different shRNAs to EZH2 (pCEP-shEZH2-1, pCEP-shEZH2-2) or two control shRNAs (pCEP-shluc, pCEP-shcontrol-1). (D) Western blotting after transient transfection of the shRNA vectors showing EZH2 expression (upper panel) and induction of PARP expression and cleavage (lower panel).
EZH2 contributes to Wnt/β-catenin signaling in parathyroid tumor cells

It has been reported that EZH2 can regulate Wnt/β-catenin signaling indirectly by epigenetic repression of several Wnt antagonists, including the growth-suppressive Axin2, leading to enhanced level of transcriptionally active (nonphosphorylated) β-catenin (Cheng et al. 2011). In sHPT-1 parathyroid tumor cells, AXIN2 expression was indeed increased under conditions with decreased EZH2 level, and expression of CYCLIN D1, known to be regulated by β-catenin in parathyroid cells (Björklund et al. 2007a), was significantly reduced (Fig. 4A). Furthermore using an antibody specific for transcriptionally active (nonphosphorylated) β-catenin (van Noort et al. 2002), a reduced level of active β-catenin (15%) was observed in siEZH2-transfected sHPT-1 cells (Fig. 4B and C) compared with siControl (100%). As expected (Björklund et al. 2007c), siRNA to LRP5 also reduced the active β-catenin level (30%) and furthermore β-catenin was undetectable after siEZH2+siLRP5 cotransfection. Thus, EZH2 contributed to the total level of active β-catenin together with LRP5 in the parathyroid tumor cells.

Discussion

EZH2 gene amplification was observed in 19 out of 49 analyzed parathyroid tumors and was related to EZH2 mRNA overexpression in 13 of these 19 tumors (Fig. 5, for summary and possible mechanisms). Importantly, western blotting analysis showed that it was possible to detect EZH2 expression also in tumors with or without gene amplification and with an EZH2 mRNA expression level within the one for normal tissues. This may imply tight regulation of EZH2 mRNA levels and perhaps relatively long half-life for EZH2 proteins in a subset of tumors. Some of the benign tumors and two of the carcinomas showed higher EZH2 mRNA expression level without gene amplification, suggesting the involvement of other more indirect mechanisms. The c-Myc oncoprotein has recently been shown to positively regulate EZH2 expression in early prostatic neoplasia, both directly as a transcriptional activator and indirectly by repression of miR-26a and miR-26b, that regulate EZH2 mRNA at the post-transcriptional level (Koh et al. 2011). This mechanism may apply to the parathyroid as c-Myc overexpression has been reported for a subset of parathyroid adenomas and hyperplastic parathyroid glands from patients with HPT secondary to uremia (Björklund et al. 2007b). To our knowledge, EZH2 gene amplification presents the first genetic aberration...
Figure 5
Summary of the results and possible mechanisms. EZH2 protein overexpression caused by EZH2 gene amplification and increased mRNA level or by indirect mechanisms such as EZH2 protein stability and by direct or indirect regulation of EZH2 gene expression by the c-Myc oncoprotein (Björklund et al. 2007b, Koh et al. 2011). Deregulated increased EZH2 protein level could result in activation of the Wnt/β-catenin signaling pathway by repression of AXIN2 (Cheng et al. 2011) and accumulation of transcriptionally active (nonphosphorylated) β-catenin, with increased expression of the β-catenin target gene CYCLIN D1 (Björklund et al. 2007a) and proliferation. Repression of the HIC1 tumor suppressor gene in parathyroid tumors involved EZH2 and H3K27 methylation (Svedlund et al. 2012).

EZH2 may play a crucial role in metastatic parathyroid disease, as the gene was found to be significantly more expressed in the carcinomas in comparison with the benign parathyroid tumors.

Furthermore, it will be of interest to investigate whether MEN1 gene inactivation and EZH2 overexpression is functionally redundant or if both events contribute to parathyroid tumor development. It has been demonstrated that MENIN, encoded by the MEN1 gene, can repress transcription through H3K27 methylation, in suppression of lung adenocarcinoma (Gao et al. 2009).

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

observed for LRPS depletion and reduced level of non-phosphorylated active β-catenin (Björklund et al. 2007c). This in line with recent results showing reduced proliferation of primary parathyroid tumor cells and sHPT-1 cells by the global histone methyltransferase inhibitor 3-deazaneplanocin A (Miranda et al. 2009, Svedlund et al. 2012), which affects several different repressive histone modifications but also depletes EZH2 (Fiskus et al. 2009). Overexpression of EZH2 may result in deregulated expression of a large number of target genes, some of which contributes to parathyroid tumorigenesis. The tumor suppressor gene HIC1 likely presents such a target gene since the aberrant underexpression of HIC1, observed in both benign and malignant parathyroid tumors, involved repressive H3K27me3 modifications and EZH2 (Svedlund et al. 2012).

Downregulation of EZH2 inhibited proliferation and growth of sHPT-1 parathyroid tumor cells, as previously
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