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EZH2 promotes E2F driven SCLC tumorigenesis through modulation of apoptosis and cell cycle regulation

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Abstract

Introduction—While EZH2 has been associated with both non small cell and small cell lung cancers, current observations suggest different mechanisms of EZH2 activation and overexpression in these lung cancer types. Globally, small cell lung cancer (SCLC) kills 200,000 people yearly. New clinical approaches for SCLC treatment are required to improve the poor survival rate. Given the therapeutic potential of EZH2 as a target, we sought to delineate the downstream consequences of EZH2 disruption to identify the cellular mechanisms by which EZH2 promotes tumorigenesis in SCLC.

Methods—We generated cells with stable expression of shRNA targeting EZH2 and corresponding controls (pLKO.1) and determined the consequences of EZH2 knockdown on the cell cycle and apoptosis by means of propidium iodide staining and fluorescence activated cell sorting, western blot, qRT-PCR as well as cell viability assessment using MTT assays.

Results—We discovered that EZH2 inhibition 1) increased apoptotic activity by up-regulating the pro-apoptotic factors Puma and Bad, 2) decreased the fraction of cells in S or G2/M phases, and 3) elevated p21 protein levels, implicating EZH2 in cell death and cell cycle control in SCLC.

Conclusions—Our findings present evidence for the role of EZH2 in the regulation of cell cycle and apoptosis, providing a biological mechanism to explain the tumorigenicity of EZH2 in SCLC. Our work points to the great potential of EZH2 as a therapeutic target in SCLC.

Keywords

SCLC; EZH2; oncogene; RB1; E2F

INTRODUCTION

Small cell lung cancer (SCLC) is a deadly malignancy requiring new clinical strategies to improve patient prognosis ¹. We recently demonstrated that deletion of miR-101, which targets EZH2 (enhancer of zeste homolog 2), is a prominent subtype specific event occurring in non-small cell lung cancer (NSCLC) ². On the other hand, disruption of the RB1/E2F pathway is a prominent feature of SCLCs ^{3,4}. RB1 is inactivated in more than 90% of cases and amplifications of E2F2 have recently been described ⁵ while proteomic profiling has revealed upregulation of E2F-regulated factors including EZH2 ⁶. These observations suggest distinct mechanisms of EZH2 activation occur in these lung cancer types. Given that (a) EZH2 activation is a major consequence of RB1/E2F pathway deregulation in SCLC, (b) EZH2 has recently been correlated with SCLC subtype of lung

cancer, and (c) the biological role of EZH2 in SCLC has not been established, we sought to investigate the functional role of EZH2 in SCLC. We studied the effects of EZH2 knockdown on apoptosis and cell cycle to elucidate the role EZH2 plays in these critical processes in SCLC.

METHODS

SCLC cell lines (HTB-175, NCI-H526, HTB-171, HTB-119, and NCI-H524) were purchased from American Type Culture Collection and had RB1 or RB1/E2F disruption^{4, 6}. RNA interference experiments were performed using lentiviral short hairpin RNA (shRNA) vectors targeting EZH2 from Open Biosystems (details at www.openbiosystems.com; RHS4533-NM_152998). RNA from cultured cells were converted to cDNA using an ABI High Capacity cDNA Archive kit and used for standard TaqMan real-time PCR for gene expression assays (Applied Biosystems): EZH2 (Hs00172783_m1), BAD (Hs00188930_m1), PUMA (Hs Hs00248075_m1), p21 (Hs00355762_m1) and HPRT1 (Hs03929098_m1). The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) assay (Trevigen) was used to assess cell viability according to the manufacturer's instructions. Cell cycle and apoptosis were quantified by flow cytometry after ethanol fixation and propidium iodide incorporation as previously described⁷. Western blot was performed as previously described⁷. All primary antibodies were purchased from Cell Signaling: GAPDH (#14C10), Pro-Apoptosis Bcl-2 Family (Kit #9942s; Bax, Bid, Bcl-2, Bak, Bik, Bim, Bad, Puma), p21 (#12D1) and EZH2 (#AC22). For all assays, p-values were calculated using the Student's t test and a one-tailed p-value <0.05 was considered significant. We calculated Pearson correlation coefficients between mRNA expression levels of EZH2 and p21 for 50 SCLC cell lines using data available from the Broad Institute Sanger Cell Line Project (broadinstitute.org/cgi-bin/cancer/datasets.cgi). P53 status for these 50 lines was available in the COSMIC database (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>) and in an online database comprised of peer reviewed literature (http://p53.free.fr/Database/Cancer_cell_lines/SCLC.htm). Only 4 out of the 50 SCLC cell lines were TP53 wild type (NCI H2081, H446, H209, and H1522).

RESULTS

shRNA knockdown of EZH2 reduces transcript and protein levels in SCLC

The involvement of EZH2 in the RB1/E2F pathway prompted us to study the function of EZH2 in the context of cell cycle and apoptosis regulation in SCLC. We assessed a panel of 5 SCLC cell lines to determine the consequence of EZH2 knockdown on the fate of SCLC cells. For each line, we generated cells with stable expression of shRNA targeting EZH2 and corresponding controls (pLKO.1). EZH2 expression levels were significantly reduced as confirmed by qRT-PCR (Supplementary Figure 1) and Western blot (Figure 1).

EZH2 is required for viability of SCLC cells

We first investigated the effect of EZH2 knockdown on SCLC cell viability using MTT assays by comparing viability of control and knockdown cells over five consecutive days. We observed a drastic reduction in viability in cells with EZH2 knockdown relative to controls for each SCLC line, although HTB-175 was less sensitive than the others (Figure 2, $p < 0.01$ in all cell lines except HTB-175 for which $p < 0.05$). HTB-171 was especially sensitive to EZH2 repression as essentially all cells died, precluding us from performing any viability studies on this line.

Knockdown of EZH2 is associated with increased rates of apoptosis and decreased proliferation

Given that EZH2 repression decreased cell viability, we next investigated whether this effect could be mediated through apoptosis or cell cycle control. We studied the effect of EZH2 knockdown on cell cycle kinetics and apoptosis rates using propidium iodide staining as previously described⁷. We observed an increase of apoptotic rates in all SCLC cells with EZH2 knockdown relative to controls ($p < 0.05$), although the magnitude of this effect was smaller in HTB-119 (Figure 3). This effect on apoptosis was coupled with a decrease in the percentage of cells in S or G2/M phase in knockdown versus control cells (Supplementary Figure 2). All knockdown cell lines, with the exception of H526, showed at least twice as many apoptotic cells than the PLKO controls. H526 cells had a high endogenous level of apoptosis but apoptotic rates were still significantly higher in EZH2 knockdown cells. HTB-171 was extremely sensitive to EZH2 knockdown, and the majority of knocked down cells were apoptotic at the time of collection ($p < 0.001$). Collectively, these results suggest that the reduction in cell viability caused by EZH2 knockdown is caused by the onset of apoptosis in SCLC cell lines with EZH2 inhibition.

EZH2 represses p21 and apoptotic factors thereby promoting cell cycling in SCLC cells

To corroborate our findings of EZH2 association with apoptosis and cell cycle regulation, we analyzed proteins involved in these two processes by Western blot (Figure 1). EZH2 knockdown caused a drastic increase in expression of the cell cycle inhibitor p21 in four of five cell lines. Moreover, repression of EZH2 increased levels of the pro-apoptotic factors, Bad and Puma, although to a lesser extent than p21. We also observed cleavage of Bax and Bid in HTB-171 upon EZH2 knockdown (Figure 1), events which are often correlated with an active stage of apoptosis⁷. These results are concordant with the high sensitivity of HTB-171 cells to EZH2 knockdown. Increases of p21, Puma and Bad after EZH2 knockdown were also observed at the mRNA level by RT-qPCR (Supplementary Figure 3).

DISCUSSION

EZH2 is a key factor in E2F-driven SCLC

Although EZH2 is highly expressed in a wide range of cancer types, over-expression is more prominent in SCLC than NSCLC⁶ which is consistent with the fact that RB1/E2F pathway disruption is more strongly associated with SCLC. However, the specific function of EZH2 in SCLC has not yet been explored. The goal of this study was to determine the effect of EZH2 on cell viability and apoptosis specifically in SCLC. It is noteworthy that E2F factors such as E2F1 may drive contradictory effects in different human cancers, promoting either proliferation or tumor suppression⁸. Considering the opposing cell responses that E2F over-activation can induce in different cell contexts, it seems that activation of EZH2, which results from E2F disruption, manifests the oncogenic role of E2F in SCLC.

Of note, Wu *et al.* also demonstrated EZH2 was a potent inhibitor of apoptosis in E2F-associated transformation in different cancer types, although not in SCLC⁹. However, mechanistic subtype differences are apparent; in NSCLC, EZH2 exerts its anti-apoptosis function through Bim repression, whereas we showed it inhibits Puma and Bad in SCLC. In addition to its effects on apoptosis, our results showed EZH2 knockdown led to increased p21 expression, which is another mechanism through which EZH2 may enhance the ability of SCLC to escape cell cycle regulation. The regulation of p21 expression by EZH2 could be due to the fact that EZH2 is known to play a role in down-regulation of RUNX3, a transactivator of p21, via H3K27 trimethylation in the promoter region¹⁰.

Additionally, it is noteworthy that transcription of EZH2 can be repressed by p53 upon p21 mediated inactivation of the RB/E2F pathway ¹¹. A correlation analysis between mRNA expression of p21 and EZH2 in cell lines with wild type TP53 revealed a strong negative correlation ($r = -0.92962$) supporting the relationship between these 2 gene expressions, while the correlation analysis in mutated cell lines did not reveal a significant relationship. Establishment of a non-malignant neuro-endocrine cell model would be required to better characterize the cell type specific interplay between EZH2 and p21 in future studies.

Taken together, our results in SCLC and those from other cancer types suggest EZH2 drives the malignant phenotype of SCLC through inhibition of apoptosis and cell cycle inhibitors (Figure 4).

EZH2 as a clinical biomarker and therapeutic target in SCLC

The utility of EZH2 as a clinical marker is currently being explored in a variety of human cancers and expression of EZH2 has recently been correlated with lung cancer subtype and metastasis ¹². Additionally, EZH2 over-expression has been shown to contribute to acquired cisplatin resistance in ovarian cancer cells ¹³. Based on these findings, there is great potential for EZH2 expression levels to serve as a predictor of drug resistance and clinical outcome in SCLC. Additionally, the biological significance of EZH2 in SCLC highlights its potential as a therapeutic target. Inhibition of EZH2 has shown promising results in lymphoma cells ¹⁴ and it synergizes with histone deacetylase (HDAC) inhibition in mice implanted with human AML ¹⁵. In fact, EZH2-mediated gene silencing was shown to be dependent on HDAC activity ¹⁶. Thus, combined inhibition of EZH2 and HDAC could generate a synergistic effect on SCLCs since they are highly sensitive to epigenetic modulators ^{7, 17}. Considering the dependence of SCLC on EZH2, patients could potentially benefit greatly from EZH2 targeting. This could be achieved by targeting the Polycomb repressor complex 2 (PRC2), for example using the S-adenosylhomocysteine hydrolase inhibitor 3-deazaneplanocin A (DZNep), or using more EZH2-specific inhibitors such as that described by Knutson et al. ¹² HDAC inhibitors (such as Valproate), which interfere with the PRC2 complex to cause global changes in chromatin modifications, should also be considered, especially in combination with these therapies.

The RB1/E2F pathway is disrupted in over 90% of SCLCs. Activation of the oncogene EZH2 is a major consequence of RB1/E2F pathway deregulation and EZH2 expression has a significant effect on SCLC viability. We demonstrated for the first time that EZH2 promotes the cell cycle and inhibits apoptosis in SCLC, favoring the oncogenic processes of the RB1/E2F pathway. Furthermore, based on the comparison of our work in SCLC and the work of others in different cancer types, EZH2 appears to regulate various apoptotic factors in a cancer specific manner. This work further confirms the oncogenic role of EZH2 in SCLC and emphasizes the great opportunity and promise for the development of novel therapeutic agents that target EZH2 for the treatment of this aggressive cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Metro G, Duranti S, Fischer MJ, et al. Emerging drugs for small cell lung cancer--an update. *Expert opinion on emerging drugs*. 2012; 17:31–36. [PubMed: 22288522]
2. Thu KL, Chari R, Lockwood WW, et al. miR-101 DNA copy loss is a prominent subtype specific event in lung cancer. *Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer*. 2011; 6:1594–1598. [PubMed: 21849855]
3. Eymin B, Gazzeri S, Brambilla C, et al. Distinct pattern of E2F1 expression in human lung tumours: E2F1 is upregulated in small cell lung carcinoma. *Oncogene*. 2001; 20:1678–1687. [PubMed: 11313916]
4. Coe BP, Lockwood WW, Girard L, et al. Differential disruption of cell cycle pathways in small cell and non-small cell lung cancer. *British journal of cancer*. 2006; 94:1927–1935. [PubMed: 16705311]
5. Peifer M, Fernandez-Cuesta L, Sos ML, et al. Integrative genome analyses identify key somatic driver mutations of small-cell lung cancer. *Nature genetics*. 2012; 44:1104–1110. [PubMed: 22941188]
6. Byers LA, Wang J, Nilsson MB, et al. Proteomic Profiling Identifies Dysregulated Pathways in Small Cell Lung Cancer and Novel Therapeutic Targets Including PARP1. *Cancer discovery*. 2012; 2:798–811. [PubMed: 22961666]
7. Hubaux R, Vandermeers F, Crisanti MC, et al. Preclinical evidence for a beneficial impact of valproate on the response of small cell lung cancer to first-line chemotherapy. *Eur J Cancer*. 2010; 46:1724–1734. [PubMed: 20451370]
8. Zacharatos P, Kotsinas A, Evangelou K, et al. Distinct expression patterns of the transcription factor E2F-1 in relation to tumour growth parameters in common human carcinomas. *The Journal of pathology*. 2004; 203:744–753. [PubMed: 15221933]
9. Wu ZL, Zheng SS, Li ZM, et al. Polycomb protein EZH2 regulates E2F1-dependent apoptosis through epigenetically modulating Bim expression. *Cell death and differentiation*. 2010; 17:801–810. [PubMed: 19893569]
10. Yoo KH, Hennighausen L. EZH2 methyltransferase and H3K27 methylation in breast cancer. *International journal of biological sciences*. 2012; 8:59–65. [PubMed: 22211105]
11. Tang X, Milyavsky M, Shats I, et al. Activated p53 suppresses the histone methyltransferase EZH2 gene. *Oncogene*. 2004; 23:5759–5769. [PubMed: 15208672]
12. Wan L, Li X, Shen H, et al. Quantitative analysis of EZH2 expression and its correlations with lung cancer patients' clinical pathological characteristics. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*. 2012
13. Hu S, Yu L, Li Z, et al. Overexpression of EZH2 contributes to acquired cisplatin resistance in ovarian cancer cells in vitro and in vivo. *Cancer biology & therapy*. 2010; 10:788–795. [PubMed: 20686362]
14. Knutson SK, Wagle TJ, Warholc NM, et al. A selective inhibitor of EZH2 blocks H3K27 methylation and kills mutant lymphoma cells. *Nature chemical biology*. 2012; 8:890–896.
15. Fiskus W, Wang Y, Sreekumar A, et al. Combined epigenetic therapy with the histone methyltransferase EZH2 inhibitor 3-deazaneplanocin A and the histone deacetylase inhibitor panobinostat against human AML cells. *Blood*. 2009; 114:2733–2743. [PubMed: 19638619]
16. Richter GH, Plehm S, Fasan A, et al. EZH2 is a mediator of EWS/FLI1 driven tumor growth and metastasis blocking endothelial and neuro-ectodermal differentiation. *Proceedings of the National Academy of Sciences of the United States of America*. 2009; 106:5324–5329. [PubMed: 19289832]
17. Kaminsky VO, Surova OV, Vaculova A, et al. Combined inhibition of DNA methyltransferase and histone deacetylase restores caspase-8 expression and sensitizes SCLC cells to TRAIL. *Carcinogenesis*. 2011; 32:1450–1458. [PubMed: 21771726]

Abbreviations

AML	acute myeloid leukemia
EZH2	enhancer of zeste homolog 2
DZNep	3-deazaneplanocin A
HDAC	histone deacetylase
PCNA	Proliferating Cell Nuclear Antigen
PRC2	polycomb Repressor Complex 2
RB1	Retinoblastoma protein
SCLC	small cell lung cancer
shRNA	small hairpin RNA

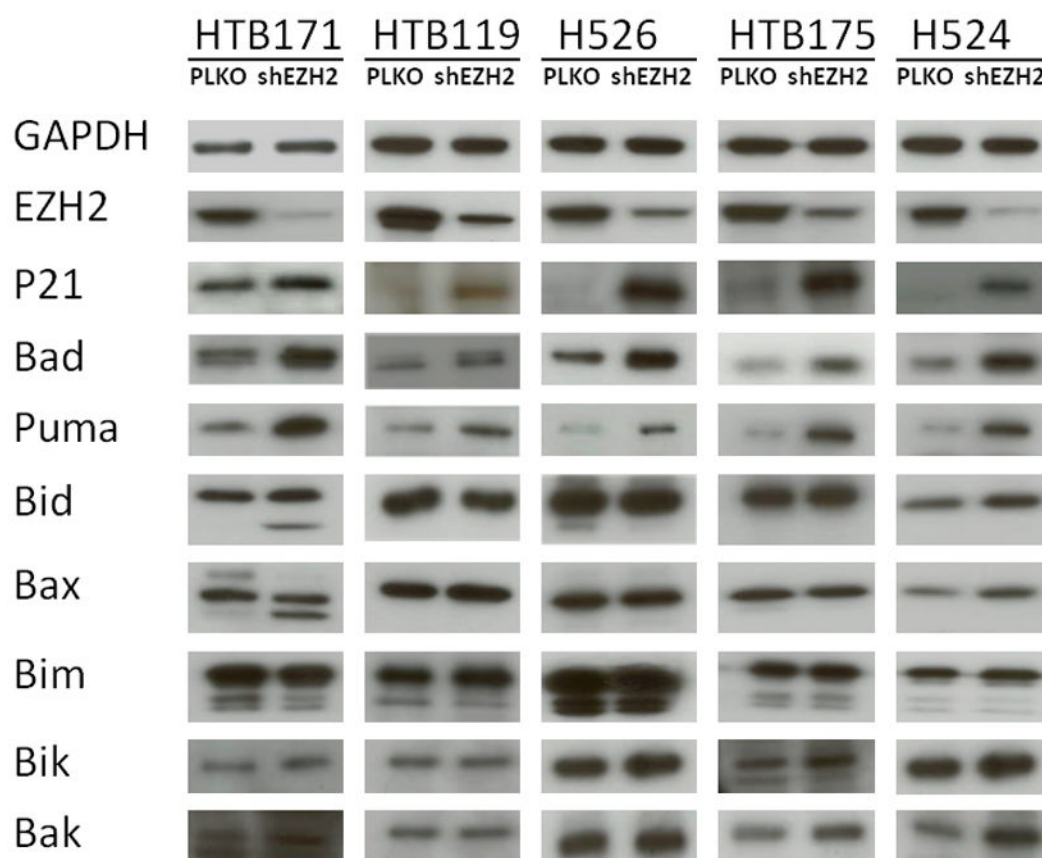


Figure 1. Knockdown of EZH2 upregulates pro-apoptotic factors and the cell cycle inhibitor, p21
 Immuno-blotting was used to determine the relative expression levels of the cell cycle inhibitor, p21, and multiple proteins of the BCL-2 family involved in the activation of apoptosis in EZH2 knockdown and control (PLKO) SCLC cells. GAPDH was used as a loading control.

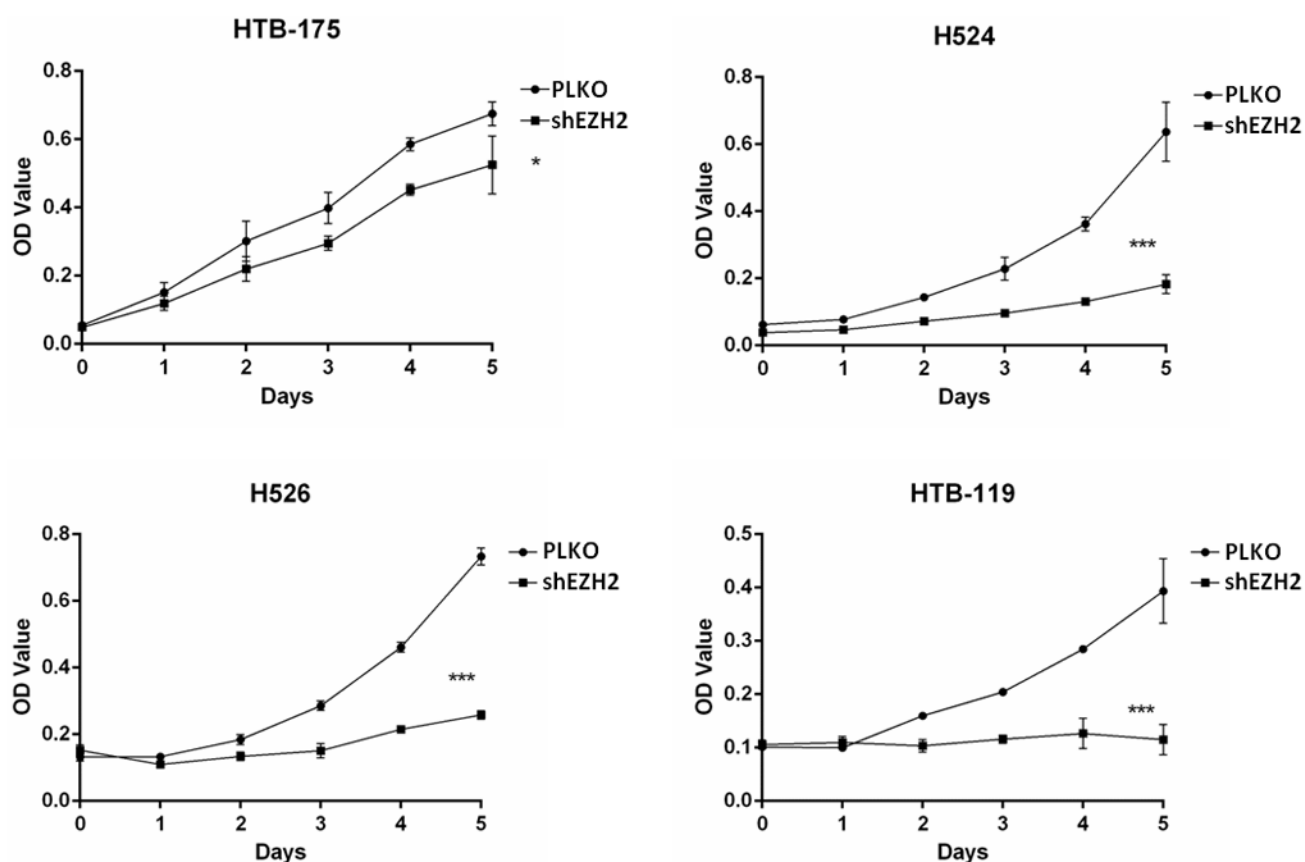


Figure 2. EZH2 knockdown reduces SCLC viability

The MTT assay was used to compare cell viability in EZH2 knockdown (EZH2) and corresponding control (PLKO) SCLC cells. Assays were performed on exponentially growing cells measured over five consecutive days. Results plotted are background subtracted mean \pm standard deviation absorbance values for triplicate wells of a representative experiment. Experiments were repeated twice. Asterisks indicate statistically significant differences in viability on day five (* p <0.05, *** p <0.001) as determined using a Student's t test.

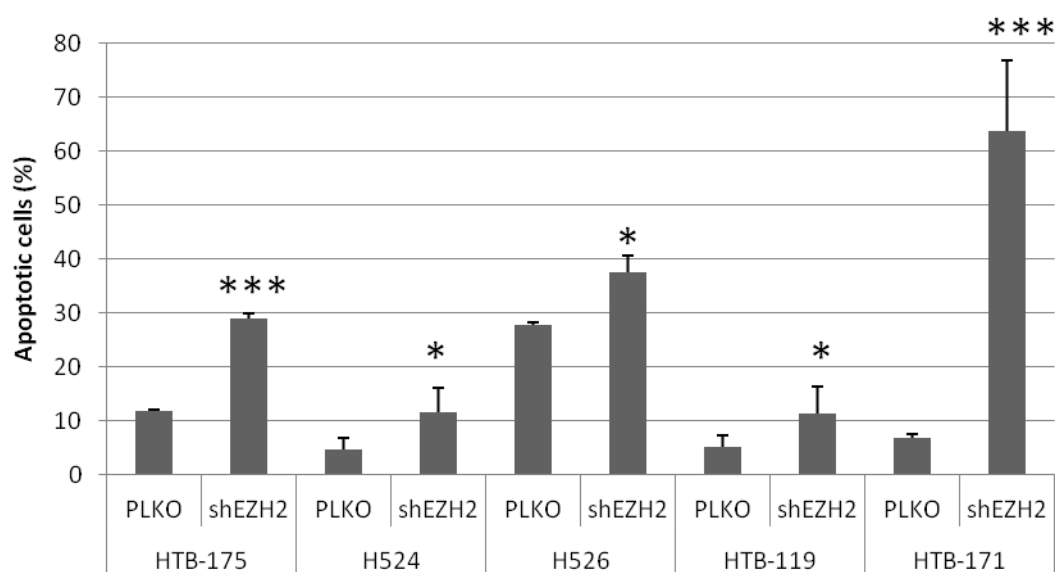


Figure 3. Apoptotic rates are elevated upon EZH2 inhibition

Levels of apoptosis for EZH2 knockdown and control SCLC cells were obtained by flow cytometry after ethanol fixation and propidium iodide incorporation. Cells in the sub-G0/1 phase (characterized by low DNA content due to loss of fragmented DNA caused by ethanol permeabilization) that were also within the gate for viable cells (forward versus side scatter dot plot) were considered apoptotic. Results plotted are mean + standard deviation of the percentage of viable cells that were apoptotic from three independent experiments. Student's *t* tests were used to compare EZH2 knockdown cells with respective PLKO controls. Asterisks indicate statistically significant differences (**p*<0.05, ****p*<0.001).

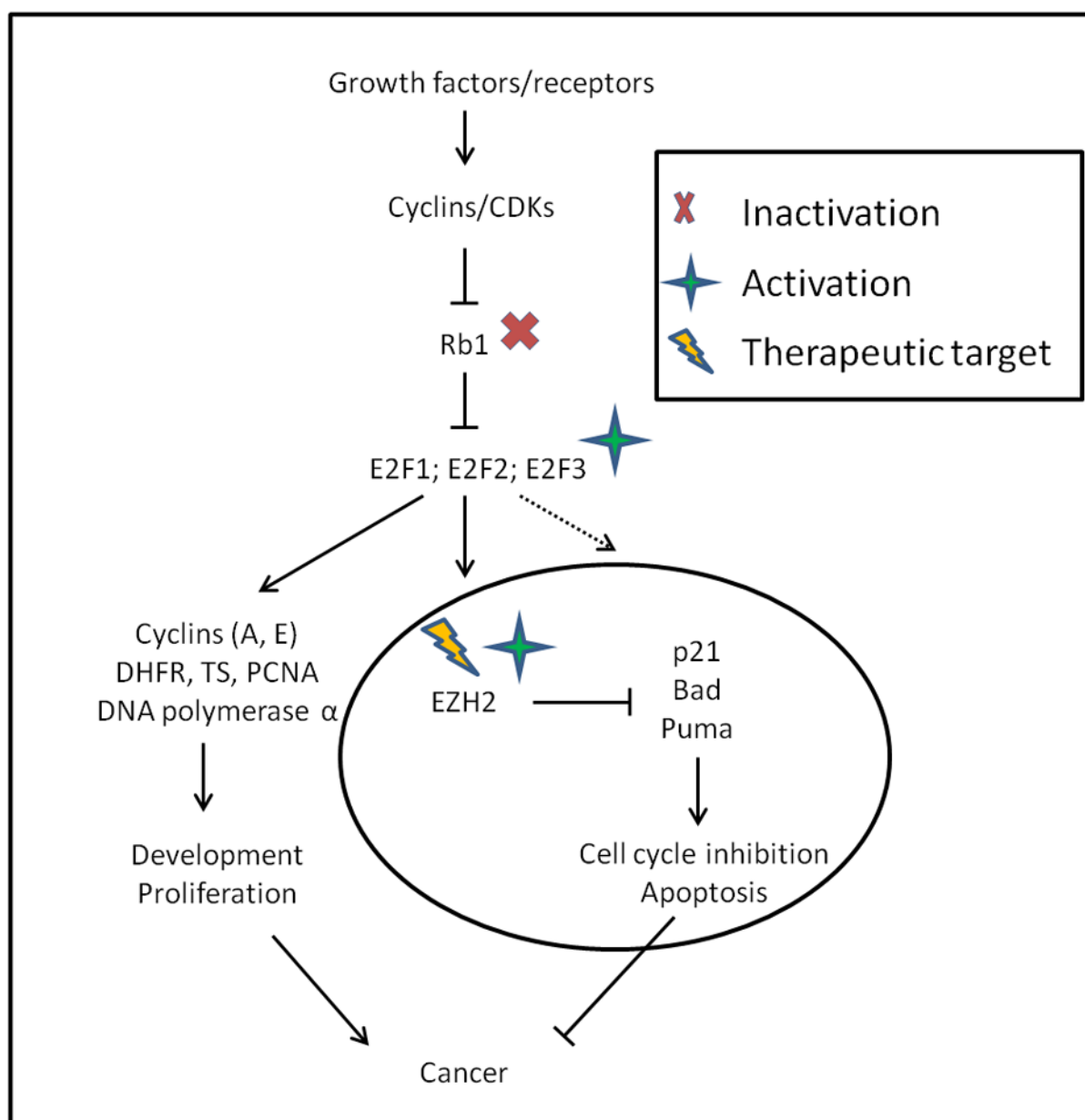


Figure 4. The postulated role of EZH2 in SCLC biology

The RB1/E2F pathway is disrupted in the vast majority of SCLCs through RB1 inactivation or E2F copy number gains. The model illustrated summarizes the different activities of E2F factors that have been described in cancer and the particular role of EZH2 that we have discovered in SCLC (circled). Besides activation of the usual cell cycle factors required for cell cycle progression, such as cyclins A and E; p107; dihydrofolate reductase; thymidine kinase; thymidylate synthetase; PCNA; or DNA polymerase α ; E2F over-activation can also lead to inhibition of the cell cycle in specific scenarios through induction of p21 and activation of apoptosis. EZH2 plays an essential role in SCLC by favoring the oncogenic processes of the RB1/E2F pathway in SCLC. Specifically, EZH2 negatively regulates the expression of the pro-apoptotic factors, Puma and Bad, as well as the cell cycle inhibitor, p21, thereby promoting SCLC tumorigenicity. Given its functions in SCLC biology, our findings suggest EZH2 is a promising therapeutic target for this cancer type.