NF-YA Underlies EZH2 Upregulation and Is Essential for Proliferation of Human Epithelial Ovarian Cancer Cells

Azat Garipov1,4, Hua Li1, Benjamin G. Bitter1, Roshan J. Thapa2, Siddharth Balachandran2, and Rugang Zhang1,3

Abstract
Epithelial ovarian cancer (EOC) accounts for more deaths than any other gynecologic malignancy in the United States. Enhancer of zeste homolog 2 (EZH2), which silences gene expression through generating trimethylation on lysine 27 residue of histone H3 (H3K27Me3), is often overexpressed in EOCs and has been suggested as a therapeutic target. However, the mechanism underlying EZH2 overexpression in EOCs is unknown. Here, we show that EZH2 is upregulated at the transcription level, and two CCAAT boxes in the proximal regions of the human EZH2 gene promoter are critical for its transcription in EOC cells. Indeed, NF-YA, the regulatory subunit of the CCAAT-binding transcription factor NF-Y, is expressed at higher levels in human EOCs than in primary human ovarian surface epithelial (HOSE) cells. In addition, there is a positive correlation between expression of NF-YA and EZH2 in EOCs. Notably, high NF-YA expression predicts shorter overall survival in patients with EOCs. The association of NF-YA with the promoter of the human EZH2 gene is enhanced in human EOC cells compared with primary HOSE cells. Significantly, knockdown of NF-YA downregulates EZH2, decreases H3K27Me3 levels, and suppresses the growth of human EOC cells both in vitro and in a xenograft mouse model. Notably, NF-YA knockdown induces apoptosis of EOC cells and ectopic EZH2 expression partially rescues apoptosis induced by NF-YA knockdown. Together, these data reveal that NF-Y is a key regulator of EZH2 expression and is required for EOC cell proliferation, thus representing a novel target for developing EOC therapeutics.

Introduction
Epithelial ovarian cancer (EOC) accounts for more deaths than any other gynecologic malignancy in the United States (1). EOCs are classified into distinct histologic types including serous, mucinous, endometrioid, and clear cell. The most common histology of EOC is serous (~60% of all cancers; ref. 2). Recently, an alternative classification has been proposed, in which EOC is broadly divided into 2 types (3). Type I EOC includes mucinous, low-grade serous, low-grade endometrioid, and clear cell carcinomas, and type II EOC includes high-grade serous carcinomas, which is the most lethal histotype (3). Enhancer of zeste homolog 2 (EZH2) is a histone methyltransferase that mediates gene silencing by catalyzing trimethylation of lysine 27 residue of histone H3 (H3K27Me3; ref. 4). EZH2 is often expressed at higher levels in human EOCs, and its expression positively correlates with cell proliferation (5). Further underscoring the importance of EZH2 in EOCs, EZH2 knockdown triggers apoptosis of human EOC cells (5). These findings identify EZH2 as a putative target for developing EOC therapeutics. Thus, it is important to elucidate the mechanism underlying EZH2 upregulation in EOCs to gain insights into the biology of the disease.

Gene amplification contributes to EZH2 upregulation in several types of cancers, including malignancies of the breast and prostate (6). However, on the basis of the newly released cancer genome atlas (TCGA) ovarian database (http://tcga-data.nci.nih.gov/; ref. 7), EZH2 gene amplification (>4 copy) is rare (~2%) in EOCs, suggesting that additional mechanisms make more significant contributions to EZH2 upregulation in EOC cells.

NF-Y is a transcription factor that specifically binds to the CCAAT consensus site (8). NF-Y is a heterotrimer, consisting of 3 subunits NF-YA, NF-YB, and NF-YC. NF-YA is the regulatory subunit that is differentially expressed, whereas NF-YB and NF-YC are constitutively expressed (9–11). As a result of differential splicing, NF-YA has 2 isoforms, namely short and long (12). Both isoforms bind DNA and are equivalently active in transcriptional activation (9). NF-Y acts as a transcriptional activator by recruiting p300 histone acetyltransferase, which promotes gene expression by generating acetylation epigenetic marker on histone H3

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(13, 14). Clinically, upregulated NF-Y target genes convey a poor prognosis in multiple cancers including those of the breast and lung (15). However, the role of NF-Y in EOCs has never been investigated.

Here, we show that EZH2 is upregulated at the transcriptional level, and 2 CCAAT sites at the proximal region of the human EZH2 gene promoter play a key role in regulating its transcription. NF-YA, the regulatory subunit of NF-Y transcription factor that binds to CCAAT sites, is upregulated in human EOCs compared with normal human ovarian surface epithelial (HOSE) cells. In addition, ectopic NF-YA upregulates EZH2 in normal HOSE cells. Importantly, there is a positive correlation between expression of NF-YA and EZH2 in human EOCs, and a high level of NF-YA predicts poor overall survival in patients with EOCs. Chromatin immunoprecipitation (ChIP) analysis revealed that the interaction between NF-YA and the promoter of human EZH2 gene is enhanced in human EOC cells compared with normal HOSE cells. Knockdown of NF-YA downregulates EZH2, decreases the levels of H3K27Me3, and suppresses the growth of human EOC cells both in vitro and in a xenograft mouse model. Mechanistically, we find that NF-YA knockdown triggers apoptosis of human EOC cells and ectopic EZH2 expression partially rescues the apoptosis induced by NF-YA knockdown. Together, these data show that NF-Y plays a key role in regulating EZH2 transcription and is essential for proliferation of human EOC cells.

Material and Methods

Cell culture

Normal HOSE cells were cultured as previously described (5). Human EOC cell lines (PEO1, SKOV3, and OVCAR5) were cultured according to American Type Culture Collection in RPMI-1640 media supplemented with 10% FBS and as previously described (5). EOC cell line identification was further confirmed by DNA Diagnostic Center (Fairfield, OH).

Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and immunoblotting

RNA from cultured normal HOSE cells or human EOC cell lines was isolated using TRizol (Invitrogen) according to the manufacturer’s instructions. For qRT-PCR, TRizol-isolated RNA was further purified using the RNeasy Kit (Qiagen) following the manufacturer’s instructions. Primers used for qRT-PCR were purchased from Applied Biosystems. Expression of the housekeeping gene β2-microglobulin mRNA was used to normalize mRNA expression. The antibodies used for immunoblotting analysis were from the indicated suppliers as following: anti-NF-YA (Santa Cruz), anti-EZH2 (BD Bioscience), anti-H3K27Me3 (Abcam), anti-cleaved PARP p85 fragment (Promega), anti-cleaved caspase-3 (Cell signaling), and anti-histone H3 (Millipore).

Luciferase promoter activity and mutagenesis analysis

Indicated fragments of the proximal human EZH2 promoter were generated by PCR and cloned into the pGL2 basic luciferase reporter plasmid (Promega) following standard molecular cloning protocols. The NF-YA-binding site single basepair substitution mutant was generated by PCR using standard molecular cloning protocols. Luciferase reporter plasmids were transfected into SKOV3, PEO1, and OVCAR5 EOC cells or normal HOSE cells using Lipofectamine2000 (Invitrogen) following the manufacturer’s instructions. A luminescent β-galactosidase plasmid (Clontech) was included as an internal control to normalize the variation in transfection efficiency.

Chromatin immunoprecipitation (ChIP) analysis

ChIP was conducted using a kit from Millipore following the manufacturer’s instructions. Briefly, SKOV3, PEO1, OVCAR5 EOC cells, and normal HOSE cells were fixed with 1% formaldehyde. After cell lysis, gDNA was sheared into 300 to 500 bp fragments by sonication. Sheared chromatin was incubated with anti-NF-YA (Santa Cruz), anti-p300 (Santa Cruz), anti-histone H3 (Millipore), or anti-acetylated histone H3 (Millipore) antibodies overnight at 4°C. After reversing the cross-link, DNA was extracted using the phenol–chloroform method. The immunoprecipitated DNA was then amplified by PCR. The primers used for PCR amplification were: for distal EZH2 gene promoter: forward primer: 5’-GTCGGGAGTTCCAGAC-3’ and reverse primer: 5’-GTCGGGCTCAGTGTGTTG-3’; and for proximal EZH2 gene promoter: forward primer: 5’-CTGGATTGGAGCGGC-3’ and reverse primer: 5’-ACTCGCGTTGTCCTCC-3’.

Inducible EZH2 overexpression, short hairpin RNA, lentivirus packaging, and infection

Inducible EZH2 expression was achieved by using the Retro-X Tet-On Advanced Inducible Expression System (Invitrogen) following the manufacturer’s instructions. Lentivirus packaging was conducted using the Virapower Kit (Invitrogen) according to the manufacturer’s instructions. Briefly, cells at 40% to 50% confluence were infected with lentivirus-expressing short hairpin RNA (shRNA) to the human NF-YA gene (shNF-YA) or vector control. The infected cells were drug-selected with 3 μg/mL puromycin. The mature sense sequences of the 3 individual shNF-YA are: 5’-CCATCATGTGCAATACACCTT-3’, 5’-TTCTGTTCTCTGTAGTAAAGGGC-3’, and 5’-CGAGCTAATAGGCCAGAA-3’. pLV-CMV-H4-puro lentiviral vector (kindly provided by Dr. Alexey Ivanov, West Virginia University, Morgantown, WV) was used to produce the lentivirus-expressing NF-YA.

Anchorage-independent soft agar assay

Soft agar assays were conducted as previously described (5). Briefly, 2,500 cells were resuspended in 0.35% low melting point agarose dissolved in RPMI-1640 medium supplemented with 10% FBS and inoculated on top of 0.6% low melting point agarose base in 6-well plates. After 2 weeks in culture, the plates were stained with 0.005% crystal violet, and the number of colonies was counted.
Annexin V staining for detecting apoptotic cells (Guava assay)

Phosphatidylethanolamine externalization was detected using an Annexin V staining kit (Millipore) following the manufacturer’s instructions. Annexin V–positive cells were detected using the Guava System and analyzed with the Guava Nexin software Module (Millipore).

Xenograft tumorigenesis assay

The protocol was approved by the Institutional Animal Care and Use Committee (IACUC). A total of 6 × 10^5 cells in PBS (pH 7.4) were injected subcutaneously into 6-week-old female nude athymic mice. The mice were sacrificed 30 days postinoculation. Tumor size was measured, and the tumor volume (mm^3) was calculated using the following formula: tumor volume (mm^3) = a^2 × b/2, where a is the smaller diameter and b is the larger diameter.

Data sets

Gene expression microarray data sets for 53 cases of laser capture and microdissected high-grade serous EOCs and 10 individual isolations of normal HOSE cells were obtained from Gene Expression Omnibus (geo; http://www.ncbi.nlm.nih.gov/geo/; GEO accession number: GSE18521). Gene expression data from 24 cases of laser capture microdissected primary nonmalignant fallopian tube epithelium and 13 cases of high-grade serous EOCs (GEO accession number: GSE10971) is also used in our analysis.

Statistical analysis

Quantitative data are expressed as mean ± SD unless otherwise stated. ANOVA with Fisher least significant difference (LSD) was used to identify significant differences in multiple comparisons. Spearman test was used to measure statistical dependence between EZH2 mRNA levels and NF-YA mRNA levels. For all statistical analyses, the level of significance was set at 0.05.

Results

EZH2 is upregulated at the transcriptional level, and two CCAAT sites in the proximal region of the human EZH2 gene promoter are critical for its transcription in human EOC cells

It has been shown that EZH2 protein is overexpressed in EOC cells (5). To determine whether the upregulation occurs at the transcriptional level, we examined the mRNA level of EZH2 in human EOC cell lines and in normal HOSE cells by qRT-PCR. Compared with normal HOSE cells, the EZH2 mRNA level was significantly higher in human EOC cell lines (Fig. 1A). This suggests that EZH2 is upregulated at the transcriptional level in human EOC cells. In agreement, a luciferase reporter driven by a 1,152 bp (−1,011 to +141 bp) proximal fragment of the human EZH2 gene promoter was highly active in human EOC cell lines than in normal HOSE cells (Supplementary Fig. S1A). The 1,152 bp EZH2 promoter is sufficient to upregulate EZH2 in normal HOSE cells. Toward this goal, 2 independent isolates of normal HOSE cells were infected with a lentivirus encoding NF-YA, and EZH2 expression was examined by immunoblotting analysis. Indeed, ectopic NF-YA expression induced EZH2 expression in normal HOSE cells (Fig. 2B). On the basis of these results, we conclude that NF-YA is expressed at higher levels in human EOC cells and ectopic NF-YA expression drives EZH2 expression in normal HOSE cells

NF-YA is expressed at higher levels in human EOC cells and ectopic NF-YA expression drives EZH2 expression in normal HOSE cells

NF-Y is a transcription factor that binds specifically to the CCAAT consensus site (17). We thus sought to determine whether NF-YA, the regulatory subunit of NF-Y (8), is upregulated in human EOC cells. Indeed, NF-YA is expressed at higher levels in human EOC cells than in normal HOSE cells (Fig. 2A and Supplementary Fig. S2). The anti-NF-YA antibody we used here can recognize both short and long isoforms of the NF-YA (Fig. 2A and Supplementary Fig. S2). Consistently, both EZH2 and H3K27Me3 are expressed at higher levels in human EOC cells than in normal HOSE cells (Fig. 2A). However, NF-YA upregulation is not a consequence of cell transformation, as NF-YA is not upregulated in normal HOSE cells expressing hTERT, SV40 early region (s/L antigen) and oncogenic H-RASG12V (Supplementary Fig. S3), which has previously been shown to be sufficient for transforming normal HOSE cells (18). We next sought to determine whether NF-YA is sufficient to upregulate EZH2 in normal HOSE cells. Toward this goal, 2 independent isolates of normal HOSE cells were infected with a lentivirus encoding NF-YA, and EZH2 expression was examined by immunoblotting analysis. Indeed, ectopic NF-YA expression induced EZH2 expression in normal HOSE cells (Fig. 2B). On the basis of these results, we conclude that NF-YA is expressed at higher levels in human EOC cells than in normal HOSE cells, and ectopic NF-YA is sufficient to upregulate EZH2 in normal HOSE cells.
NF-YA expression positively correlates with the expression of EZH2 in human EOC cells and a high level of NF-YA predicts shorter overall survival in EOC patients

We next examined whether expression of NF-YA and EZH2 is positively correlated in human EOCs. In addition to EOC cells, EZH2 is also upregulated in ovarian tumor-associated stromal cells such as endothelial cells (19). To limit the confounding effects of EOC-associated stromal cells, we sought to correlate the expression of NF-YA and EZH2 in specimens from laser capture and microdissected (LCM) high-grade serous tumors, which account for a majority of EOC-associated mortalities (3). Toward this goal, we examined the expression of NF-YA and EZH2 in a published microarray database, which compares the gene expression profile in 53 cases of LCM high-grade serous EOC and 10 individual isolations of normal HOSE cells (20). Notably, NF-YA expression is significantly higher in human EOCs than in normal HOSE cells (Fig. 2C). Consistent with previous reports (5, 19), EZH2 was expressed at significantly higher levels in human EOCs than in normal HOSE cells (Fig. 2D). Indeed, there was a significant positive correlation between expression of NF-YA and EZH2 in a Spearman statistical analysis of the cases including EOC alone or both EOC and primary HOSE cells (Fig. 2E, \( P < 0.001 \)). Next, we determined whether there is an association between NF-YA expression and the survival of patients with EOCs. Upon dividing EOC cases into NF-YA high or low based on the median NF-YA expression, we found that a high level of NF-YA expression was significantly associated with shorter overall survival (Fig. 2F, \( P = 0.0388 \)). Notably, there is recent evidence to suggest that a proportion of serous histosubtype EOCs may arise from distant fallopian tube epithelium (21, 22). Using a published microarray analysis (23), we found that NF-YA is also expressed at higher levels in serous ovarian carcinomas than in primary nonmalignant fallopian tube epithelial cells (Fig. 2G, \( P < 0.001 \)). Together, we conclude that NF-YA is expressed at higher levels in serous ovarian carcinomas than in primary nonmalignant fallopian tube epithelial cells, and ectopic NF-YA is sufficient to drive EZH2 expression in normal HOSE cells. Significantly, expression of NF-YA positively correlates with expression of EZH2 in high-grade serous EOCs, and high levels of NF-YA predict shorter overall survival in patients with EOCs.
The association of NF-YA with the proximal promoter of the human EZH2 gene is enhanced in human EOC cells

Next, we determined whether the association of NF-YA with the human EZH2 gene promoter is enhanced in human EOC cells compared with normal HOSE cells by ChIP analysis using an anti-NF-YA antibody. An isotype-matched IgG was used as a negative control and an antibody to the core histone H3 was used as a positive control in ChIP analysis. To further limit potential nonspecific effects, we chose a distal region of the human EZH2 gene promoter that does not have NF-YA–binding sites as a genomic region negative control (Fig. 3A). There is evidence to suggest that NF-YA activates its target gene expression by recruiting p300 histone acetyltransferase (24, 25). Thus, we also examined the association of p300 with the human EZH2 gene promoter using an anti-p300 antibody. Indeed, both NF-YA and p300 showed an enhanced association with the proximal region of the human EZH2 gene promoter in human EOC cells compared with normal HOSE cells (Fig. 3B). In contrast, NF-YA and p300 did not associate with the distal region of the human EZH2 promoter that lacks NF-YA–binding sites (Fig. 3B). Notably, NF-YA is associated with a known NF-YA target gene CCNA2 that has NF-YA–binding sites (CCAAT) in its promoter but not with negative control genes such as α-tubulin, RPS19, and YBL1 that do not have NF-YA–binding sites in their promoters. Indeed, there was an increase in acetylated histone H3 in the proximal promoter of the human EZH2 gene where NF-YA and p300 bind in human EOC cells compared with normal HOSE cells (Fig. 3C). On the basis of these results, we conclude that there is an association of NF-YA and p300 with the proximal promoter of the human EZH2 gene in human EOC cells, and this association correlates with increased levels of acetylated histone H3.

NF-YA knockdown suppresses EZH2 expression and inhibits the growth of human EOC cells in vitro and in a xenograft EOC model

We next sought to determine the effects of NF-YA knockdown on expression of EZH2 in human EOC cells.
NF-YA Underlies EZH2 Upregulation in EOC

To this end, we used 3 individual shRNAs to the human NF-YA gene (shNF-YA). The knockdown efficacy of shNF-YAs was examined by immunoblotting (Fig. 4A and Supplementary Fig. S2A and S2B) and two shNF-YAs (#1 and #2) were found to efficiently reduce NF-YA levels, whereas a third (#3) was largely ineffective and used as a non-silencing negative control. Notably, shNF-YA that efficiently knocked down NF-YA also suppressed the expression of EZH2 (Fig. 4A and Supplementary Fig. S2A and S2B). Consistently, the levels of H3K27Me3, the product of EZH2’s methyltransferase activity, were also decreased by shNF-YA that knocks down NF-YA (Fig. 4A and Supplementary Fig. S5 and S6A–B). In addition, similar observations were made in lung cancer cell line H1299 and breast cancer cell line MCF7, suggesting that this is not unique in EOC cells (Supplementary Fig. S5). We observed the same effects using 2 individual shNF-YAs (Fig. 4A and Supplementary Fig. S5 and S6A–B), suggesting that this is not due to off-target effects. In contrast, levels of H3K9Me3, which are generated by different histone methyltransferases such as Suv39H1 and SETDB1 (31), were not affected by NF-YA knockdown (Fig. 4A). As a negative control, the shNF-YA #3 that does not efficiently knockdown NF-YA also failed to overtly decrease the EZH2 and H3K27Me3 levels in human EOC cells (Fig. 4A).

Expression of EZH2 positively correlates with expression of markers of cell proliferation (5), and NF-YA knockdown suppresses the expression of EZH2 (Fig. 4A). Thus, we wanted to determine the effects of NF-YA knockdown on the growth of human EOC cells. Compared with controls, 2 individual shNF-YAs that efficiently knocked down NF-YA suppressed both the anchorage-dependent and -independent growth of human EOC cells (Fig. 4B and C). In contrast, one shNF-YA (#3) that does not efficiently knockdown NF-YA failed to suppress the growth of human EOC cells (Fig. 4B and C). Similar effects of NF-YA knockdown on cell growth were observed in multiple EOC cells (Fig. 4 and Supplementary Fig. S6), showing that this is not cell line–specific. On the basis of these results, we conclude that NF-YA knockdown suppresses the growth of human EOC cells in vitro in both an anchorage-dependent and -independent conditions.

Next, we examined the effects of NF-YA knockdown on the growth of human EOC cells in vivo in a xenograft mouse model. We subcutaneously injected control or shNF-YA–expressing human EOC cells into the immunocompromised (athymic nude) mice. Consistent with our in vitro findings, we found that compared with controls, shNF-YAs that efficiently knocked down NF-YA expression suppressed the growth of xenografted human EOC cells (Fig. 4D and E). In contrast, the shNF-YA #3 that does not efficiently knockdown NF-YA also failed to significantly suppress the growth of implanted human EOC cells (Fig. 4D and E). Thus, we conclude that NF-YA knockdown suppresses the growth of human EOC cells in vivo in a xenograft EOC mouse model.

NF-YA knockdown triggers apoptosis of human EOC cells

We next determined the mechanism by which NF-YA knockdown suppresses the growth of human EOC cells. As NF-YA regulates EZH2 expression (Fig. 2) and knockdown of EZH2 induces apoptosis of EOC cells (5), we examined the effects of NF-YA on apoptosis of human EOC cells. We observed a significant increase of apoptosis markers in NF-YA knockdown cells. These markers include an increase in Annexin V detection as measured by the Guava Nexin assay (Fig. 5A and B) and an increase of cleaved caspase-3 and cleaved PARP p85 (Fig. 5C). HRK, a pro-apoptotic gene, has recently been shown as an H3K27Me3 target gene and plays
a key role in mediating apoptosis induced by decreasing H3K27Me3 levels (32). As NF-YA knockdown suppresses EZH2 expression and decreasing H3K27Me3 levels, we sought to determine the effects of NF-YA knockdown on the expression of HRK. Indeed, HRK expression is significantly upregulated in NF-YA knockdown cells (Fig. 5D). Together, we conclude that NF-YA knockdown induces apoptosis of human EOC cells.

As NF-YA knockdown suppresses the expression of EZH2, we examined whether downregulation of EZH2 contributes to apoptosis induced by NF-YA knockdown. Toward this goal, we ectopically expressed an inducible EZH2 in EOC cells. We examined the expression of markers of apoptosis in cells expressing shNF-YA with or without inducible EZH2 expression. Compared with shNF-YA only cells, in which EZH2 is downregulated, ectopic EZH2 induction partially rescued the apoptosis induced by NF-YA knockdown as evidenced by decreased levels of apoptotic markers such as cleaved PARP p85 and cleaved caspase-3 (Fig. 5E). Together, we conclude that apoptosis induced by NF-YA knockdown is, at least in part, due to suppression of EZH2 expression.

Discussion

We showed that ectopic NF-YA expression is sufficient to drive EZH2 upregulation in normal HOSE cells (Fig. 2B). Interestingly, we observed cell death in normal HOSE cells with NF-YA ectopic expression (data not shown), which prevented us from further analyzing these cells. Consistent with our observation, a previous report showed that ectopic NF-YA promotes p53-mediated apoptosis in normal mouse embryonic fibroblasts and inactivation of p53 partially suppresses the apoptosis induced by NF-YA expression (33). Indeed, we also found that p53 is activated upon NF-YA expression in normal HOSE cells (data not shown). These data suggest that p53 inactivation is necessary for the survival of NF-YA–expressing cells. Notably, p53 is often inactivated in EOCs. For example, p53 is inactivated in virtually all high-grade serous EOCs (7). Thus, it is possible that inactivation of p53 may cooperate with NF-YA in EOC cells. In addition, recent evidence suggests that a proportion of high-grade serous EOCs may arise from the distal fallopian tube epithelial cells (21, 22). Thus, it will be interesting to examine whether ectopic NF-YA expression is sufficient to
drive EZH2 expression in fallopian tube epithelial cells once these cells become more accessible.

We showed that NF-YA is upregulated in EOCs. However, TCGA gene copy number analysis indicates that NF-YA gene (6p21.3) amplification occurs rare in EOCs (1% specimens show >4 copy of NF-YA gene; http://cancergenome.nih.gov/). This suggests that additional mechanisms contribute to NF-YA upregulation in human EOCs. In the future, we will elucidate additional mechanisms that contribute to NF-YA upregulation in human EOCs. In addition, it will critical to further validate the correlation between NF-YA expression and survival of patients with EOCs in independent cohorts.

We discovered that the expression of NF-YA and EZH2 positively correlates in human EOCs (Fig. 2C–E), further supporting the premise that NF-YA plays a key role in regulating EZH2 expression. However, the Spearman coefficient $r$ was 0.56 for EOC only and 0.64 for EOCs plus normal HOSE cells (Fig. 2E). This result is consistent with the idea that other factors, such as E2F and Elk-1 (Fig. 1B; refs. 6, 16), also play a role in the expression of EZH2.

We showed that EZH2 is a direct NF-YA target gene (Fig. 3). EZH2 knockdown induces apoptosis in human EOC cells (5). Consistently, knockdown of NF-YA also triggers apoptosis of human EOC cells (Fig. 5). However, ectopic EZH2 expression can only partially rescue the apoptosis induced by NF-YA knockdown and these cells will ultimately undergo apoptosis (Fig. 5E and data not shown). These results suggest that other NF-YA target genes also contribute to the apoptosis induced by NF-YA knockdown. Indeed, the anti-apoptotic genes such as Bcl-xl and Bcl2 are also directly controlled by NF-Y transcription factor (34). Further studies are warranted to identify additional NF-YA target genes that contribute to the apoptosis of human EOC cells induced by NF-YA knockdown. Indeed, the anti-apoptotic genes such as Bcl-xl and Bcl2 are also directly controlled by NF-Y transcription factor (34). Further studies are warranted to identify additional NF-YA target genes that contribute to the apoptosis of human EOC cells induced by NF-YA knockdown. Indeed, the anti-apoptotic genes such as Bcl-xl and Bcl2 are also directly controlled by NF-Y transcription factor (34). Further studies are warranted to identify additional NF-YA target genes that contribute to the apoptosis of human EOC cells induced by NF-YA knockdown. Indeed, the anti-apoptotic genes such as Bcl-xl and Bcl2 are also directly controlled by NF-Y transcription factor (34). Further studies are warranted to identify additional NF-YA target genes that contribute to the apoptosis of human EOC cells.

Figure 5. NF-YA knockdown induces apoptosis of human EOC cells. A, SKOV3 EOC cells were infected with lentivirus encoding the indicated shNF-YAs or control. Guava Nexin assay was conducted at the indicated time points. B, quantification of (A). Mean of 3 independent experiments with SD. * $P < 0.05$ compared with either control or shNF-YA #3. C, same as (A) but examined for expression of apoptosis markers, cleaved caspase-3, and cleaved PARP p85 by immunoblotting. D, same as (A) but examined for $HRK$ gene expression by qRT-PCR. * $P < 0.05$ compared with either control or shNF-YA #3. E, SKOV3 EOC cells were engineered to express a Tet-inducible EZH2. These cells were infected with a lentivirus shNF-YA with or without simultaneous induction of EZH2 expression. Drug-selected cells were examined for expression of NF-YA, EZH2 and indicated apoptosis markers by immunoblotting. Expression of core histone H3 was used as a loading control.

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In summary, we show that 2 NF-Y–binding CCAAT sites within the proximal region of the human EZH2 gene promoter play a key role in regulating EZH2 expression in human EOC cells. NF-YA, the regulatory subunit of NF-Y transcription factor, is upregulated in human EOC cells compared with normal HOSE cells. In addition, ectopic NF-YA drives EZH2 expression in normal HOSE cells. The expression of NF-YA positively correlates with the expression of EZH2 in primary EOCs, and a high level of NF-YA expression predicts shorter overall survival in patients with EOCs. Consistently, NF-YA knockdown suppresses EZH2 expression and inhibits the growth of human EOC cells in vitro and in a xenograft mouse model. NF-YA knockdown triggers apoptosis of human EOC cells, and downregulation of EZH2, at least in part, contributes to apoptosis induced by NF-YA knockdown in these cells. These results establish that NF-YA underlies EZH2 upregulation and is essential for proliferation of human EOC cells. We believe that these studies provided important insights into the biology of EOC and identified NF-YA as a potential target for intervention of EOCs.

Disclosure of Potential Conflict of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Garipov, R.J. Thapa, R. Zhang
Development of methodology: A. Garipov, R.J. Thapa
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Garipov, B.G. Blixer, R.J. Thapa, S. Balachandran
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A. Garipov
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