N6-methyladenosine-modified VGLL1 promotes ovarian cancer metastasis through HMGA1/Wnt/β-catenin signaling

Jian-Chuan Xia (xiajch@mail.sysu.edu.cn)
Sun Yat-sen University Cancer Center

Han Li
Sun Yat-sen University Cancer Center

Liming Cai
Guangzhou University of Chinese Medicine

Qizhuong Pan

Xingyu Jiang
Sun Yat-sen University Cancer Center

Jingjing Zhao
Department of Biotherapy, Sun Yat-sen University Cancer Center; State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Sun Yat-sen University Cancer Center

Tong Xiang
Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, Collaborative Innovation Center for Cancer Medicine, Guangdong Key Laboratory of Nasopharyngeal Carcinoma Diagnosis

Yan Tang
Qijing Wang
Sun Yat-sen University Cancer Center

Jia He
Sun Yat-Sen University Cancer Center

Desheng Weng

Yanna Zhang
Sun Yat-sen University Cancer Center

Zhongqiu Liu
Guangzhou University of Chinese Medicine

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Abstract

The main causes of death in ovarian cancer (OC) patients are invasive lesion and the spread of metastasis. Determining the biology of cancer metastasis is important to develop novel targeted therapy. The present study aimed to explore the mechanisms that might promote OC metastasis. Here, we identified that VGLL1 expression was remarkably increased in metastatic OC samples. The role of VGLL1 in OC metastasis and tumor growth were examined by cell function assays and mouse models. Mechanistically level, METTL3-mediated N^6^-methyladenosine (m^6^A) modification contributed to VGLL1 upregulation in an IGF2BP2 recognition-dependent manner. Furthermore, VGLL1 directly interacts with TEAD4 and co-transcriptionally activates HMGA1. HMGA1 further activates Wnt/β-catenin signaling to enhance OC metastasis by promoting the epithelial-mesenchyme transition traits. Rescue assays indicated that upregulation of HMGA1 was essential for VGLL1-induced metastasis. Additionally, conditional silencing of VGLL1 using a doxycycline-induced knockdown system showed therapeutic potential against metastasis in OC. Importantly, the clinical relevance of IGF2BP2, VGLL1, HMGA1, and β-catenin was conformed in OC tissues. Collectively, these findings showed that m^6^A-induced VGLL1/HMGA1/β-catenin axis might play a vital role in OC metastasis and tumor growth. VGLL1 might serve as a prognostic marker and therapeutic target against metastasis of OC.

Introduction

Ovarian cancer (OC) is the fifth most frequent cause of cancer-related death in women worldwide and is also the most fatal gynecological malignancy among female reproductive system cancers[1]. The broadly recognized feature of OC is abdominal cavity seeding, a form of dissemination in which cancer cells shed from tumors, circulate via the peritoneal fluid, and finally implant on the peritoneal and intestinal surfaces[2]. More than 70% of patients with ovarian cancer are diagnosed at a disseminated stage, especially with colonization of the peritoneum and intestines[3]. Despite advances in debulking surgery, chemotherapy regimens, targeted therapies, and immunotherapy, the 5-year survival rate of women with widespread peritoneal metastasis is less than 45%[4]. Tumor metastasis, especially peritoneal metastasis, is the most prominent clinical obstacle to the treatment of OC[3]. However, the exact mechanisms in OC metastasis remain unclear. Further research focusing on the mechanisms and key molecules in the process of metastasis is necessary to design therapeutic strategies with the potential to control OC progression.

The biological metastatic behavior of OC is unique, differing significantly from the classic hematogenous or lymphatic vasculature metastases in most other types of cancer[5]: OC cells disseminate predominantly into the peritoneal cavity from the primary tumor, where they implant on the abdominal surfaces and organs, especially the fallopian tubes, uterus, and intestines[6]. In addition, peritoneal cavity metastasis frequently correlates with the formation of malignant ascites, which in turn further facilitates OC cell metastasis[7]. As such, malignant ascites formation and peritoneal cavity implantation are key steps in OC metastasis, ultimately contributing to recurrence[8].
Tumor relapse, mainly caused by tumor metastasis, is the major factor influencing the high mortality of patients with OC [7]. Recently, several studies have indicated that epithelial–mesenchymal transition (EMT) is one of the key factors that promotes cancer metastasis [9]. During EMT, epithelial markers containing E-cadherin and cytokeratins decrease, whereas mesenchymal markers, such as N-cadherin and Vimentin, increase [10]. Abnormal activation of the EMT process allows primary epithelial cancer cells to obtain strong mesenchymal properties, such as metastasis and chemoresistance, eventually leading to tumor cell survival in the circulatory system and subsequent colonization of distant organs [11, 12]. Additionally, the EMT process is controlled by sophisticated signaling networks including the Wnt, Notch, and Hedgehog pathways [13, 14]. Of these, Wnt/β-catenin signaling plays an important role in the development and promotion of the EMT process and tumor metastasis [15]. Therefore, EMT-based therapeutic intervention via regulation of Wnt/β-catenin signaling activity is regarded as a promising strategy for treating OC metastasis.

Here, by performing RNA-sequencing analysis, we identified that the VGLL1 is significantly elevated in OC tissues, especially in metastasis + OC tissues. The VGLL family, named VGLL1–4, interacts with TEA domain transcription factors (TEADs) via their Tondu (TDU) domain and participates in cancer genesis and metastasis [16]. Recently, VGLL1 was found to be upregulated in gastric cancer, malignant myoepithelial tumors, and triple-negative basal-like phenotype breast cancer [17–19]. Highly expressed VGLL1 interacts with TEADs to form a VGLL1-TEAD complex and then transcriptionally upregulates MMP9 to enhance the proliferation and metastasis of gastric cancer cells [18]. These studies suggest that VGLL1 might be an oncogenic protein that mediates tumor metastasis. However, the mechanism by which VGLL1 regulates OC cell metastasis remains unclear.

Cancer cells undergo genetic and epigenetic changes to obtain metastatic properties [20]. Among these modifications, the N\(^6\)-methyladenosine (m\(^6\)A) modification is frequently found in mRNA and affects the fate of RNA in various ways, such as RNA splicing, nuclear export, translation, and RNA stability [21]. m\(^6\)A RNA methylation is catalyzed by m\(^6\)A WERs (termed “writers”, such as METTL3, METTL14, and WTAP; “erasers”, such as FTO and ALKBH5; and “readers”, such as YTHDC1, YTHDC2, YTHDF1, YTHDF2, YTHDF3, and HNRNPC) [21]. Among these methyltransferases, METTL3 is the key catalytic subunit. Readers specifically identify the m\(^6\)A modification and determine the fate of m\(^6\)A-modified mRNAs [22]. Studies have shown that m\(^6\)A modification is involved in diverse processes, such as tissue development, cancer metastasis and proliferation, and stem cell self-renewal [23]. Moreover, dysregulation of m\(^6\)A modification correlates markedly with the progression of several cancers, including ovarian cancer, lung cancer, and colorectal cancer [21, 24, 25]. Nevertheless, the function of m\(^6\)A-modified mRNA in OC metastasis and its underlying mechanism are still unclear.

Herein, VGLL1 expression was observed to be upregulated significantly in OC, especially in metastatic OC, and was associated with poor survival of patients with OC.

Knockdown of VGLL1 significantly suppressed the proliferation, invasion, and metastasis of OC cells in vitro and peritoneal cavity metastasis in vivo. Mechanistically, VGLL1 is m\(^6\)A-modified by METTL3,
resulting in upregulation of \textit{VGLL1} in an insulin-like growth factor 2 mRNA binding protein 2 (IGF2BP2)-dependent manner. \textit{VGLL1} then interacts with TEAD4 to transcriptionally activate high mobility group AT-hook 1 (HMGA1)/Wnt/\(\beta\)-catenin signaling. We confirmed the important role of the \(m^6A/VGLL1/HMGA1\) axis in OC. Our findings indicated that \textit{VGLL1} could be an important biomarker for metastasis prediction in OC, which suggests a novel therapeutic strategy for OC by targeting \textit{VGLL1}.

\section*{Results}

\textbf{VGLL1 is upregulated in metastatic ovarian cancer tissues and is related to poor prognosis.}

To explore critical metastasis-related genes in OC, RNA-seq was carried out on five primary OC tissues and five paired metastatic OC nodules. The results showed a total of 30 mRNAs that were significantly altered with Log_2 \textit{Fold change (FC)} values \(>2\) and \(P\) value \(<0.05\) in metastatic OC nodules compared to primary OC tissues (Figure 1A). \textit{VGLL1} was remarkably upregulated in metastatic OC nodule OC tissues compared to primary OC tissues among the 30 mRNAs (Figure 1A). Notably, upon qRT-PCR validation in a larger cohort comprising 72 normal ovary tissues and 90 OC tissues, we found that \textit{VGLL1} was markedly overexpressed in OC tissues (Figure 1B). Additionally, we used the GEPIA 2 tool\cite{29} to analyze The Cancer Genome Atlas (TCGA) data and showed that \textit{VGLL1} was remarkably upregulated in various types of cancers, including bladder carcinoma, cervical carcinoma, ovarian carcinoma, pancreatic adenocarcinoma, uterine corpus endometrial carcinoma, and uterine carcinosarcoma (Supplemental Figure 1A), and correlated positively with poor prognosis in OC (Supplemental Figure 1B, C).

Moreover, statistical analysis showed that the \textit{VGLL1} expression level correlated markedly with metastasis status (Figure 1C) in a large cohort of patients with OC. Further analysis using qRT-PCR demonstrated upregulated \textit{VGLL1} expression in metastatic tumor cells in peritoneal metastatic nodules compared with paired primary tumors, suggesting that \textit{VGLL1} is an important factor in promoting OC metastasis (Figure 1D).

Next, we evaluated the clinical significance of \textit{VGLL1} expression in 157 ovarian cancer samples (Table S1), and we found that \textit{VGLL1} expression was remarkably correlated with FIGO stage \((P<0.001)\), intraperitoneal metastasis \((P = 0.027)\), ascites with tumor cells \((P = 0.006)\), vital status \((P<0.001)\) and tumor recurrence \((P = 0.001)\) (Table S2). However, it was not detected or was marginally detected in normal ovary epithelial tissues and primary OC tissues without metastasis (Fig. 1E). Moreover, \textit{VGLL1} levels were elevated significantly in OC cell lines compared with those in a normal human ovary epithelial cell line (HoSepiC) at both the mRNA and protein levels, as measured by qRT-PCR and western blotting (Supplemental Figure 1D, E). Consistently, \textit{VGLL1} expression was higher in primary OC tissues than in normal ovary epithelial tissues (Supplemental Figure 1F). Importantly, \textit{VGLL1} expression was higher in metastasis-positive OC tissues than in metastasis-negative OC tissues (Figure 1F). Correlation analysis revealed that a high \textit{VGLL1} level correlated markedly with patient vital status and metastatic status (Figure 1G).
Importantly, in patients with OC with high VGLL1 expression, metastasis occurred earlier, and their survival time was shorter (Figure 1H, I). Furthermore, multivariate Cox regression analysis showed that VGLL1 expression was an independent prognostic factor, along with intraperitoneal metastasis and tumor recurrence, for worse prognosis in ovarian cancer (Table S3).

Inhibition of VGLL1 suppresses ovarian cancer cell proliferation and metastasis in vitro

To further investigate the role of VGLL1 in the malignant phenotypes of OC, we established OC cell lines (A2780 and OVCAR3 cells) that were stably silenced or overexpressed VGLL1 (Supplemental Figure 2A, F).

We evaluated the function of VGLL1 in OC cell metastatic behavior. Wound healing and transwell assays showed that VGLL1 silencing markedly inhibited the migration and invasion ability of OC cells, while VGLL1 overexpression had the opposite effect (Figure 2A, Supplemental Figure 2B-E, G-L). Next, we detected the expression of EMT-related genes in VGLL1-knockdown or VGLL1-overexpressing OC cells and in corresponding control cells. Western blotting showed that silencing of VGLL1 significantly enhanced the level of E-cadherin but reduced the expression of the mesenchymal markers N-cadherin and vimentin, as well as EMT-activating transcription factors (EMT-TFs), such as SLUG and SNAIL; however, upregulation of VGLL1 had the opposite effects (Figure 2B, Supplemental Figure 3A, B). Moreover, the results of immunofluorescent staining were consistent with those of western blotting (Figure 2C, Supplemental Figure 3C, D).

Compared with that in the control cells, the MTT proliferation rate was markedly decreased in VGLL1-knockdown A2780 and OVCAR3 cells (Figure 2D, Supplemental Figure 4A). VGLL1 silencing-induced reductions in cell proliferation were further confirmed by colony formation and EdU assays. (Figure 2E, F, Supplemental Figure 4B-D). In addition, we found that VGLL1 overexpression had the opposite effect on OC cell proliferation (Supplemental Figure 4E-J).

VGLL1 enhances ovarian cancer cell metastasis in vivo

To further investigate the effect of VGLL1 on OC tumor metastasis in vivo, the ovary of BALB/c nude mice were inoculated orthotopically with control or VGLL1-knockdown OVCAR3 cells. Intraperitoneal metastasis was monitored by the in vivo imaging system (IVIS). Mice orthotopically injected with VGLL1-knockdown OVCAR3 cells formed significantly fewer metastatic implants than the control mice (Figure 2G, H). Furthermore, omental tumors generated by VGLL1-knockdown OVCAR3 cells had lower levels of Ki-67 staining (Figure 2I). In contrast, re-overexpression of VGLL1 in OVCAR3-shVGLL1#1 cells restored the above effects of VGLL1 silencing on the intraperitoneal metastasis ability (Figure 2I).

VGLL1 transcriptionally activates the HMGA1/β-catenin signaling pathway by interacting with TEAD4

To investigate the mechanism by which VGLL1 promotes OC malignant progression, we performed RNA-seq analyses in OVCAR3 cells (OVCAR3-shVGLL1#1 vs. OVCAR3 cells). After screening the transcriptional regulation prediction tool (chEA3 and hTFtarget) and the RNA-seq data, we identified 2 mRNAs regulated
by VGLL1 (Figure 3A). Next, we analyzed the clinical relevance between VGLL1 and HMGA1 and between VGLL1 and ZNF860 in OC patients. We found that the expression of VGLL1 and HMGA1 was significantly positively correlated, while the correlation between VGLL1 and ZNF860 was not statistically significant (Figure 3B, C). Moreover, we also discovered that HMGA1 was highly expressed in metastatic OC tissues compared with primary OC tissues (Supplemental Figure 5A). We analyzed the expression of these four TEADs in the OC dataset in the TCGA and GTEx databases. Interestingly, only TEAD4 was overexpressed significantly in OC tissues compared with normal ovary tissues (Supplemental Figure 5B-E).

Immunoprecipitation (IP) and immunofluorescence assays validated the interaction between VGLL1 and TEAD4 (Figure 3D, E). The TEAD family plays an important role in tumor progression; therefore, we wondered whether the VGLL1/TEAD4 complex regulates the transcription of HMGA1. Moreover, HMGA1 was upregulated in VGLL1-overexpressing A2780 and OVCAR3 cells, as detected by western blot assays (Figure 3F). These results suggested that HMGA1 might be a potential downstream target of VGLL1. Additionally, TEAD4 silencing reduced VGLL1-induced HMGA1 promoter activity (Figure 3G).

Moreover, JASPAR tool prediction based on published chromatin immunoprecipitation (ChIP-seq) data identified two potential VGLL1-TEAD4 binding sites in the HMGA1 promoter region. Luciferase reporter systems were then constructed comprising HMGA1 promoters incorporating the wild-type binding site (wild), deletion of the binding site (del), and mutation of the binding site (mut). The degree of HMGA1 promoter activation was similar between the -1765 and -361 constructs, which suggested that the -1765 binding site is not essential for the binding of the VGLL1-TEAD4 complex to HMGA1 (Figure 3H). These observations suggested that HMGA1 expression coordinates TEAD4 and VGLL1 activity. Moreover, endogenous VGLL1 could bind the putative binding site (-361 to -352 constructs) on the HMGA1 promoter region, while knockdown of VGLL1 strongly decreased the enrichment of p300, RNA polymerase II, and H3K4me3 on the HMGA1 promoter, as examined by chromatin immunoprecipitation (ChIP) assays (Figure 3I, J). These results suggested that VGLL1 serves as a cofactor of TEAD4, which binds to the -361 site in the HMGA1 promoter.

Previous studies have shown that HMGA1-mediated activation of Wnt/β-catenin signaling enhances tumor progression[30]. We found that VGLL1 elevation markedly increased β-catenin/TCF transcriptional activity. In contrast, knockdown of HMGA1 significantly repressed β-catenin/TCF transcriptional activity (Figure 3K). Next, we measured the expression of c-MYC, MMP7 and MMP9, three typical β-catenin downstream genes, using qRT-PCR and western blot analysis. c-MYC, MMP7 and MMP9 mRNA expression increased significantly when VGLL1 was overexpressed, and silencing HMGA1 expression reversed this effect (Supplemental Figure 6 A, B). These results were also verified in the indicated OVCAR3 tumors (Supplemental Figure 6 C).

**HMGA1 is essential for VGLL1-mediated metastasis in ovarian cancer**

We further investigated the role of HMGA1 in VGLL1-mediated metastasis in OC. Additionally, we also conducted several *in vitro* assays, including invasion, migration assays and EMT assays, and the results showed that knockdown of HMGA1 expression significantly inhibited the effects of
VGLL1 overexpression (Figure 4A-D). Moreover, colony formation assays and EdU assays also demonstrated that silencing HMGA1 expression obviously repressed the proliferation effects mediated by high expression of VGLL1 (Figure 4E, F). Moreover, the numbers of intraperitoneal metastatic nodules were decreased significantly in the OVCAR3-VGLL1 group upon silencing HMGA1 expression (Figure 4G, H). We also discovered that upregulation of VGLL1 increased the nuclear translocation of β-catenin in A2780 cells, and nuclear translocation of β-catenin was decreased upon silencing of HMGA1 expression, as detected by immunoblotting of nuclear and cytoplasmic cellular fractions (Figure 4I).

To further explore whether β-catenin activation was responsible for the VGLL1-mediated effects, we determined the impact of blocking the Wnt/β-catenin pathway on the metastasis of OC cells using the tankyrase inhibitor XAV-939. As shown in Figure 4J-L, VGLL1-induced invasion, migration and proliferation in OC cells were repressed by XAV939 treatment.

**M₆A modification contributes to the upregulation of VGLL1 in OC**

Recent advances have suggested that cancer progression is frequently induced by both genetic and epigenetic modifications[31]. Therefore, we explored the mechanism that induces VGLL1 upregulation in OC. First, using the “Ovarian Epithelial Carcinoma (TCGA, Provisional)” dataset from the cBioPortal database (http://www.cbioportal.org), we investigated the correlation between mRNA expression and genomic copy number of VGLL1. However, the results showed that overexpression of VGLL1 in OC was not affected significantly by genetic amplification (Supplemental Figure 7A). Emerging studies have indicated that epigenetic modifications are frequently involved in the dysregulation of mRNA. Thus, we wondered whether epigenetic modification induced VGLL1 upregulation in OC. Treatment of OC cells with a broad-spectrum HDAC inhibitor (SAHA and NaB) was used to detect the histone acetylation level of VGLL1 in A2780 and OVCAR3 cells (Figure 5A, B), and the results showed that histone acetylation did not contribute to the upregulation of VGLL1 in OC cells.

Recent advances have indicated that m₆A is the most abundant modification in mRNA, mediated by ‘writers’, inhibited by ‘erasers’ and functionally executed by readers[32]. Therefore, we explored whether m₆A modification is involved in VGLL1 upregulation. An online m₆A site predictor, SRAMP[33], showed that several m₆A sites were located in VGLL1 (Figure 5C). Moreover, the m₆A level of VGLL1 was upregulated in A2780 and OVCAR3 cells compared with that in the normal ovarian epithelial cell line Hosepic (Figure 5D). Further assessment of the expression of m₆A WERs in OC tissues obtained from SYSUCC showed significant increases in the expression levels of METTL3, YTHDC1, IGF2BP1 and IGF2BP2 in OC tissues, whereas the expression levels of other WREs showed no differences (Figure 5E-I, Supplemental Figure 7 B-G). Previous studies indicated that the IGF2BP family recognized m₆A-modified mRNAs and sustained their stability to promote cancer progression[32]. To identify the specific m₆A reader of VGLL1 and determine the m₆A-modification mechanism of VGLL1 upregulation, a streptavidin RNA pull-down assay to screen for VGLL1-related m₆A readers was conducted. We found that IGF2BP2, but not the other members of the IGF2BP family or the YTH family, directly bound to the full-length
transcripts in A2780 and OVCAR3 cells (Figure 5J, K). Moreover, RIP-PCR experiments also revealed that 
VGLL1 mRNA levels were significantly increased in RNA precipitated by METTL3 and IGF2BP2 antibodies 
compared with that precipitated by IgG in A2780 and OVCAR3 cells (Figure 5L, M, Supplemental Figure 7 
H). Furthermore, we also evaluated RNA expression after METTL3 or IGF2BP2 knockdown in OC cells. 
The mRNA level of VGLL1 was markedly decreased after METTL3 or IGF2BP2 silencing in A2780 and 
OVCAR3 cells (Figure 5N-Q).

Inducible silencing of VGLL1 abrogates OC metastasis

Finally, we evaluated whether targeting VGLL1 might abrogate metastasis in OC. A doxycycline (Dox)-
induced knockdown system was used to conditionally knock down VGLL1 in OC cells (Figure 6A). The 
migration ability, surviving colony ability and proliferation capacity were robustly impaired in VGLL1-
silenced OC cells, indicating that VGLL1 was required for these processes (Supplemental Figure 8A-H).

The therapeutic potential of VGLL1 was further assessed in vivo using xenograft models. Briefly, mice 
were randomly divided into two groups (n=6/group) and inoculated orthotopically with OVCAR3-
shVGLL1DOX cells. After 10 days of inoculation, one group of mice was fed drinking water containing Dox 
to induce VGLL1 downregulation in tumors (Figure 6B). The growth and metastasis of tumors 
were recorded. Notably, Dox-induced silencing of VGLL1 significantly reduced the growth and metastasis 
rate of tumors, suggesting that upregulation of VGLL1 was essential for OC tumor growth and 
metastasis. (Figure 6C-E).

Likewise, silencing IGF2BP2 potently impaired VGLL1 expression in OVCAR3 cells. Consistent with the 
above results, knockdown of IGF2BP2 significantly repressed the migration ability, 
colony formation ability and proliferation capacity (Supplemental Figure 9A-H). Moreover, knockdown of 
IGF2BP2 obviously repressed the growth and metastasis rate of tumors. These results indicated that 
IGF2BP2 was required for VGLL1 upregulation (Figure 6F-H).

Clinical relevance of the m^6A/VGLL1/HMGA1 axis in OC

Based on the mechanism we investigated above, we further proceeded to explore the clinical relevance 
among IGF2BP2, VGLL1, β-catenin and HMGA1 in OC specimens. IHC assays showed that IGF2BP2, 
VGLL1, β-catenin and HMGA1 expression was potently upregulated in OC specimens with metastasis 
compared with those without metastasis (Figure 6I). Notably, VGLL1 expression was positively correlated 
with IGF2BP2, HMGA1 and nuclear β-catenin in OC tissues (Figure 6J-L).

In summary, our findings showed that methylated VGLL1 was subsequently recognized 
by the m^6A “reader IGF2BP2 to maintain its mRNA stability and contribute to its upregulation. 
Increasing the VGLL1 expression level promoted OC cell metastasis by activating the VGLL1/HGMA1/β-
catenin axis (Figure 6M).

Discussion
The overall five-year patient survival rate for OC is only 40%, mainly because of its advanced stage at diagnosis and the propensity for peritoneal cavity metastasis, even when the primary tumors are small[1]. Peritoneal metastasis occurs in almost all OC patients, especially at the advanced stage, finally causing death[3]. Moreover, treatment for peritoneal cavity metastasis has limited effects in the clinic[34]. Therefore, exploration of the mechanism of peritoneal metastasis is essential to improve the clinical outcome of OC patients. Although well-established studies have indicated that m^6^A methylation modification has a broad impact on cancer progression and precision therapy[20, 24], the relationship between m^6^A methylation modification and peritoneal metastasis of ovarian cancer has not been well studied thus far.

The metastatic tropism of OC cells, by which they spread directly into adjacent organs, especially in the fallopian tubes, uterus and gastrointestinal system, is unique[2]. Hanahan et al. reported that epithelial tumor cells must acquire invasive properties to disseminate and develop new tumor nodules in the peritoneal cavity or at distant sites[35]. Tumor cells undergo EMT during the metastasis process, losing cell–cell contacts and subsequently reducing the expression of E-cadherin (an epithelial adheren junction molecule) and increasing the expression levels of several invasion and migration markers[36]. These studies emphasize the important role of EMT properties in tumor metastasis. In this study, our data shows that VGLL1 enhances EMT properties mediated by VGLL1 in OC cells to further promote metastasis.

Recently, several studies reported that high mobility group AT-hook 1 (HMGA1) contributes to tumor progression and metastatic processes in cancers via EMT[37]. However, the function and detailed understanding of the molecular mechanisms of HMGA1 in ovarian cancer cell metastasis, especially transcoelomic metastasis, are still unclear. Belton et al. reported that HMGA1 contributes to cell proliferation and polyp formation in the intestines and ultimately induces metastatic development in colon cancer cells[38]. Previous studies have shown that HMAG1 activates the Wnt/β-signaling pathway at multiple levels. First, HMGA1 upregulates the expression of Wnt agonist receptor genes and increases Wnt/TCF4/β-catenin downstream effector genes. Second, TCF4 and HMGA1 form a ‘feed-forward loop’, in which TCF4 binds to the HMGA1 promoter, and TCF4/β-catenin upregulates HMGA1, which induces Wnt/β-catenin signaling activation. Third, HMGA1 leads to inhibition of GSK3-mediated β-catenin phosphorylation and stabilization, which then induce β-catenin nuclear translocation[39]. In this study, we revealed that OC cells with high HMGA1 expression tended to metastasize and form peritoneal metastatic tumors.

VGLL1 overexpression has been shown to correlate with poorer prognosis and shorter survival in multiple types of cancer[17, 18]. VGLL1 functions as a co-transcriptional activator and a driver of metastasis and proliferation during human cancer development via interaction with the transcription factor TEAD4[40, 41]. VGLL1 interacts with TEAD4 in a similar manner to YAP/TAZ binding to TEAD proteins, which results in upregulation of IGFBP5, a proliferation-promoting protein[41]. These observations regarding VGLL1 prompted us to explore its precise role in OC metastasis. In this study, our data suggested that VGLL1 directly interacts with TEAD4 to transcriptionally activate HMGA1/Wnt/β-catenin signaling, ultimately
promoting OC cell metastasis. Therefore, we speculated that VGLL1 might serve as a valuable therapeutic target in OC.

M\textsuperscript{6}A is a widely prevalent posttranscriptional modification of RNA that controls RNA metabolism at multiple levels\cite{42}. Zhou et al. found that FTO reduced the b-catenin m\textsuperscript{6}A modification level, increased its expression, and increased ERCC excision repair 1 (ERCC1) expression. A study by Liu T et al. showed that YTHDF1 promotes ovarian cancer metastasis and progression by increasing elF3C translation in a m\textsuperscript{6}A-dependent manner\cite{21}. Similarly, our data indicated that m\textsuperscript{6}A modification caused the upregulation of VGLL1. Further exploration showed that METTL3 functions as a m\textsuperscript{6}A writer for VGLL1 and is recognized by the classic m\textsuperscript{6}A reader IGF2BP2, which has been reported to promote the stability of mRNA. Hou P et al. found that IGF2BP2 recognized m\textsuperscript{6}A-modified HTR3A to stabilize its mRNA, which promoted metastasis and proliferation of esophageal squamous cell carcinoma\cite{43}. Consistent with previous studies, we found that IGF2BP2 or METTL3 knockdown remarkably reduced the half-life of VGLL1 in an m\textsuperscript{6}A-dependent manner. In this study, we found that VGLL1 was increased in ovarian cancer with peritoneal metastasis and was correlated with poor prognosis in OC patients. Furthermore, functional assays indicated that VGLL1 knockdown inhibited metastasis and tumor growth of ovarian cancer cells. Mechanistically, upregulation of VGLL1 was mediated by m\textsuperscript{6}A modification and then induced ovarian cancer cell metastasis by forming the VGLL1/HMGA1/\beta\textsuperscript{-}catenin signaling axis.

Conclusions

In summary, our findings reveal that m\textsuperscript{6}A-modified VGLL1 is recognized by IGF2BP2, which stabilizes VGLL1 mRNA, thereby promoting tumor growth and transcoelomic metastasis of OC cells. The study uncovers a novel mechanism for transcoelomic metastasis in ovarian cancer cells. Our work also suggests that VGLL1 may serve as a promising prognostic marker and a novel therapeutic strategy against transcoelomic metastasis of ovarian cancer.

Materials And Methods

Patient information

In this study, all relevant ethical work regarding samples, including a total of ten fresh OC tissues (five primary OC tissues and five paired metastatic OC nodules) and 157 OC specimens (Paraffin-embedded tissues and liquid nitrogen preserved tissues), was performed. Seventy-two normal ovarian tissues were frozen and stored in liquid nitrogen until used. These clinical tissues were histopathologically and clinically diagnosed at Sun Yat-sen University Cancer Center from 2001 to 2020. The study protocols were approved by the Institutional Research Ethics Committee of Sun Yat-sen University Cancer Center for the use of these clinical materials for research purposes. Each patient signed a written informed consent for all the procedures according to the Declaration of Helsinki.

Cell culture
The human OC cell lines OVCAR3, SKOV3, TOV112D and CAOV3 were obtained from the American Type Culture Collection (Manassas, VA, USA). A2780 and OV90 cells were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China). COV362 and OVCAR4 were obtained from CoBioER (Nanjing, China). COV644 cells were purchased from ChuanQiu Biotechnology (Shanghai, China). HosePic cells were obtained from Jennio Biotech Co., Ltd. (Guangzhou, China).

All these cells were authenticated using short tandem repeat profiling. All the cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY) according to the manufacturer's instructions.

**Protein immunoprecipitation (IP) assays**

Lysates were prepared from A2780 cells using lysis buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 1% NP-40). Lysates were incubated with Flag affinity agarose (Sigma–Aldrich), MYC affinity agarose (Sigma–Aldrich), Flag antibody (#14793; Cell Signaling Technology), or MYC antibody (#2276; Cell Signaling Technology) with protein G agarose overnight at 4 °C. Beads containing affinity-bound proteins were washed six times using immunoprecipitation wash buffer (150 mM NaCl, 10 mM HEPES, pH 7.4, 0.1% NP-40); lysates were eluted using 1 M glycine (pH 3.0). The eluates were then mixed with sample buffer, denatured, and used for western blot analysis.

**Xenograft tumor models**

BALB/c-nude mice (female, 4-5 weeks old) were purchased and housed in barrier facilities on a 12 h light/dark cycle. In the tumor model, the indicated luciferase-expressing cells (1× 10^6 OVCAR3-sh NC, OVCAR3-shVGLL1#1, or OVCAR3-sh VGLL1#1/VGLL1 cells) were stereotactically implanted into the ovary of nude mice. Tumors were detected using a Xenogen IVIS imaging system. At the experimental endpoint, the animals were euthanized, and the tumors were excised, weighed, paraffin-embedded and sectioned for immunohistochemistry and hematoxylin–eosin (H&E) staining. Images were captured using the AxioVision Rel. 4.6 computerized image analysis system (Carl Zeiss, Oberkochen, Germany). In the in vivo target VGLL1 therapy efficiency test, mice were randomly divided into two groups (n = 6 per group). After 10 days of injection of 1× 10^6 OVCAR3-shVGLL1#1^Dox cells, one group of mice was administered Dox (2 mg/ml) in drinking water to induce VGLL1 downregulation in the tumors. Tumors were examined every 3 days. After 6 weeks of injection, animals were euthanized, and tumors were excised and weighed. Tumors were detected using a Xenogen IVIS imaging system.

The Institutional Animal Care and Use Committee of Sun Yat-sen University approved the experimental procedures.

**RNA immunoprecipitation (RIP) assays**

RIP assays were performed to detect interactions between the proteins and mRNAs in ovarian cancer cells. Briefly, cells were first starved for 24 h and then stimulated with a standard culturing medium. Cells
were harvested 8 h later and lysed in lysis buffer (20 mM Tris-Cl, pH 8.0, 10 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with RNasin (Promega). The lysates were then pulled down with anti-IGF2BP2 (Santa Cruz, sc-377014) and anti-METTL3 (Abcam, ab240595) antibodies and washed five times with lysis buffer. The retrieved pellets were then subjected to qRT-PCR analysis using the qRT-PCR primers for VGLL1. GAPDH was used as a negative control. Primers are provided in Table S4.

RNA sequencing

We extracted total RNA from five samples of nonmetastatic OC and five samples of metastatic OC using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and further purified the extracted RNA using rRNA depletion. cDNA was synthesized from the RNA, followed by PCR amplification. We then constructed RNA-seq libraries, which were sequenced on the Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA).

Immunohistochemistry (IHC)

IHC was performed on formalin-fixed, paraffin-embedded human tissue sections as described before[26]. IHC analysis was performed to determine altered protein expression in paraffin-embedded ovarian cancer tissues and peritoneal metastasis tissues with anti-VGLL1 (Proteintech, 10124-2-AP), anti-β-catenin (Proteintech, 51067-2-AP), anti-HMGA1 (Active Motif, 39615), anti-METTL3 (Proteintech, 15073-1-AP), and antibodies overnight at 4 °C. The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored separately by two independent pathologists blinded to the histopathological features and patient data of the samples. The scores were determined by combining the proportion of positively stained tumor cells and the intensity of staining. The scores given by the two independent pathologists were combined into a mean score for further comparative evaluation. Tumor cell proportions were scored as follows: 0, no positive tumor cells; 1, <10% positive tumor cells; 2, 10%–35% positive tumor cells; 3, 35%–75% positive tumor cells; 4, >75% positive tumor cells. Staining intensity was graded according to the following standard: 0, no staining; 1, weak staining (light yellow); 2, moderate staining (yellow brown); 3, strong staining (brown). The staining index (SI) was calculated as the product of the staining intensity score and the proportion of positive tumor cells. Using this method of assessment, we evaluated protein expression in normal breast tissues, breast tumor tissues and bone metastasis tissues by determining the SI, with possible scores of 0, 1, 2, 3, 4, 6, 8, 9 and 12. Samples with an SI ≥ 6 were determined to have high expression, and samples with an SI < 6 were determined to have low expression.

Mean optical density (MOD) analysis

IHC staining for protein expression of VGLL1 in OC samples with or without metastasis was quantitatively analyzed by using the AxioVision 4.6 computerized image analysis system assisted with an automatic measurement program (Carl Zeiss). The method of mean optical density (MOD) was used to determine the immunostaining intensity of each tested specimen. Briefly, the stained sections were evaluated at ×200 magnification, and 10 representative staining fields of each section were analyzed to
verify the MOD, which represents the strength of staining signals as measured per positive pixel, according to a previously reported study[27]. The MOD data were statistically analyzed by using the t test to compare the average MOD difference between different groups of tissues, and P < 0.05 was regarded as significant.

**Western blot analysis**

Western blot analyses were performed[28] using primary antibodies, including anti-VGLL1 (Proteintech, 10124-2-AP), anti-β-catenin (Proteintech, 51067-2-AP), anti-HMGA1 (Active Motif, 39615), and anti-METTL3 (Proteintech, 15073-1-AP). The membranes were stripped and re-incubated with anti-α-Tubulin as a loading control. P84 was used as a nuclear marker.

**Transwell matrix invasion assay**

Cells (1×10⁴) suspended in the upper chamber of polycarbonate Transwell filters coated with Matrigel (BD Biosciences, San Jose, CA, USA) were cultured at 37 °C for 24 hours. Then, the cells inside the upper chamber were removed with cotton swabs, and the cells that had migrated to the bottom surface of the membrane where 10% FBS was added as an attractant were fixed in 1% paraformaldehyde, stained with crystal violet and counted in five random fields of view per well. The data are shown as the mean ± SD.

**Immunofluorescence**

Cells (5×10⁴) were plated on coverslips. The cells were washed three times with PBS and treated with PBS containing 1% Triton X-100. Next, the cells were stained with a primary anti-β-Catenin antibody (Cell Signaling Technology, #8480, 1:100) for 2 hours at 4 °C according to the manufacturer's instructions. For the colonization staining of TEAD4 and VGLL1, cells were washed three times with PBS, treated with PBS containing 1% Triton X-100 and then stained with anti-TEAD4 and anti-VGLL1 antibodies. After washing three times with PBS, the cells were incubated with rhodamine-conjugated goat anti-rabbit or anti-mouse antibody (Cell Signaling Technology, 1:100) at 37 °C for 1 hour. Cells were counterstained with DAPI (Sigma–Aldrich) to visualize the nuclei. The percentage of membrane colonization of TEAD4 and VGLL1 was counted in five random fields.

**Cell proliferation assays**

MTT assays were performed according to the protocol of the MTT assay kit (Sangon Biotech Co., Ltd.). Briefly, cells were seeded into 96-well plates at a density of 2×10³ cells/well. Cell viability was assessed using the MTT assay kit at 24, 48, 72, and 96 h according to the manufacturer's protocol. After 4 h of incubation in MTT reagent at 37 °C and 5% CO₂, the medium was replaced with 150 μl dimethyl sulfoxide at room temperature (DMSO; Sangon Biotech Co., Ltd.). The absorbance of each sample was measured at 450 nm using a microplate spectrophotometer (BioTek Instruments, Inc.).

EdU assays were conducted according to the protocol of the 5-Ethynyl-2-deoxyuridine (EdU) labeling/detection kit (Ribobio, Guangzhou, China). Briefly, the cells were seeded in 24-well plates
containing sterile glass coverslips at 37 °C overnight. Then, the images were visualized by a fluorescence microscope.

**TOP/FOP flash activity assays**

The wild-type (TOP) and mutant (FOP) LEF/TCF reporters were cloned into pGL3 luciferase constructs (Promega). Twenty thousand cells were seeded in triplicate in 48-well plates and allowed to settle for 24 h. One hundred nanograms of TOP or FOP flash, plus 1 ng of pRLTK Renilla plasmid (Promega), was transfected into cells using the Lipofectamine 3000 reagent according to the manufacturer’s recommendation. Luciferase and Renilla signals were measured 24 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega) according to a protocol provided by the manufacturer. The results were calculated as the ratio of specific TOP-Flash to nonspecific FOP-Flash relative Renilla luciferase units (RLU).

**Methylated RNA immunoprecipitation (MeRIP):**

RNA immunoprecipitation was performed with Protein A/G Agarose Beads (Santa Cruz) following the manufacturer’s instructions. Briefly, Protein A/G Agarose Beads coated with 5 mg of normal antibodies against rabbit immunoglobulin G (Beyotime), Ago2 (Abcam), or m6A (Abcam) were incubated with prefrozen cell lysates or nuclear extracts overnight at 4 °C. Associated RNA–protein complexes were collected and washed 6 times and then subjected to proteinase K digestion and RNA extraction by TRIzol. The relative interaction between protein and RNA was determined by qRT–PCR and PCR and normalized to the input. Primers are provided in Table S4.

**Luciferase activity assay**

Cells (1×10^4) were seeded in 48-well plates in triplicate and allowed to proliferate to 60–80% confluence after 24 hours in culture. The indicated plasmids or luciferase reporter plasmids plus 1 ng of pRL-TK Renilla plasmid (Promega, Madison, WI) were transfected into cells using Lipofectamine 3000 reagent (Invitrogen) according to the manufacturer’s instructions. Luciferase and Renilla enzyme activity signals were measured using the Dual Luciferase Reporter Assay Kit (Promega) following the manufacturer’s protocol.

**DNA constructs and establishment of stable cell lines**

The human VGLL1 ORF was subcloned into the pSLenti-SFH-EGFP-P2A-Puro-CMV vector. To re-express VGLL1 in sh VGLL1#1 cells, the VGLL1-expressing construct was edited with the same sense mutations to prevent sh VGLL1#1-mediated downregulation. To silence endogenous VGLL1, short hairpin RNA (shRNA) oligonucleotides were cloned into the pSuper-retro-puro vector. The targeting sequences of shRNAs are provided in Table S4. Stable cells were generated from cell pools by lentivirus or retroviral infection using pSLenti-SFH-EGFP-P2A-Puro-CMV for VGLL1 overexpression and p-Super-retro-puro for silencing of VGLL1. Briefly, lentivirus or retroviral vectors were cotransfected with
packaging plasmids into 293T cells. The supernatant containing the virus was collected, and viral infections were performed serially for 3 days. Stable cell lines were selected with 1.0 μg/ml puromycin.

**Chromatin immunoprecipitation (ChIP) assay**

ChIP assays were performed as previously described[28]. The indicated cells (4×10^6) in a 100-mm culture dish were treated with a 1% final concentration of formaldehyde to cross-link proteins to DNA, and the reaction was stopped by the addition of glycine. The cell lysates were sonicated to shear the DNA to fragments of 300–1,000 bp. Chromatin supernatants were incubated with anti-H3K4me3 (#9751; Cell Signaling Technology), anti-TEAD4 (#ab58310; Abcam), anti-Flag (#14793; Cell Signaling Technology), anti-p300 (#ab14984; Abcam), anti-RNA polymerase II (#05-623; Millipore), or anti-immunoglobulin G antibody (#I8765; Sigma–Aldrich) overnight at 4 °C with rotation. After reversing the cross-linking of protein/DNA complexes to free DNA, PCR was performed using the primers listed in Table S4.

**Statistical analyses**

SPSS version 22.0 (IBM Corp., Armonk, NY) was used for all statistical analyses.

The χ^2 test, log-rank test, Spearman-rank correlation test, and Student’s t test (two-tailed) were used for data analysis. A Cox regression model was used for multivariate statistical analysis. Statistical significance of the differences was considered at P < 0.05.

**Declarations**

**Acknowledgements**

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**Conflict of Interest Statement**

The authors declare no conflict of interest.

**Author Contribution Statement**

Han Li, Liming Cai and Qiuzhong Pan carried out most of the experimental work, they collected and analyzed the data. Xingyu Jiang, Jingjing Zhao, Desheng Weng collected tissues, patient information, and conducted IHC and survival analysis. Qiuzhong Pan, Tong Xiang, Yan Tang, Qijing Wang and Jia He conducted the western blot analysis, plasmid constructions, and cell culture. Zhongqiu Liu and Jianchuan Xia raised the concept, design the experiments, wrote the manuscript, and supervised the project. All authors reviewed the manuscript.

**Ethics Statement**
Ethics approval (Approval No. SL-B2021-396-02) was obtained from the Research Ethics Committee of the Sun Yat-sen University Cancer Center. Informed consent was obtained from all patients.

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**Availability of supporting data**

The datasets used during the current study are available from the corresponding author on reasonable request.

**References**


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**Figures**

**Figure 1**

VGLL1 is upregulated in ovarian cancer tissues with metastasis and related to poor prognosis.
(A). RNA sequencing of five primary OC tissues and five paired metastatic OC nodules to screen differentially expressed mRNA. Radar chart showing dysregulated 30 mRNA in primary OC tissues and paired metastatic OC nodules. (B). qRT-PCR analysis of \textit{VGLL1} expression in a 90-cases cohort of freshly collected human OC samples and a 72-cases cohort of freshly collected human normal ovary tissues. (C). Comparison of \textit{VGLL1} expression in Me+ and Me- ovarian cancer tissues. (D). Comparison of \textit{VGLL1} expression in primary human OC tissues and paired metastatic nodules. (E). Immunohistochemistry analysis (left) and quantification (right) of VGLL1 expression in 13 normal ovarian tissues and 157 OC tissues, consist of 81 non-metastasis and 76 metastasis, and 22 metastatic OC nodules tissues (at peritoneal metastasis site). (F). Representative IHC staining images of VGLL1 in OC tissues from 81 non-metastasis and 76 metastasis patients. (G). VGLL1 strongly correlated with the status of tumor metastasis and patient survival. \(\chi^2\) statistical test was used. (H). Overall survival (OS) analysis in patients with OC stratified by low and high VGLL1 expression (n=157, log-rank test). (I). Progress-free survival (PFS) analysis in patients with OC stratified by low and high VGLL1 expression (n=157, log-rank test). Me-: without metastasis; Me+: with metastasis. ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns indicates no significance. Each error bar represents the mean ± SD of three independent experiments.
Figure 2

Inhibition of VGLL1 suppresses ovarian cancer cells metastasis

(A). Representative images of migration and invasion assays in OVCAR3 cells (Upper). Histogram analysis of migrated or invaded cell counts is shown (Bottom). (B, C). EMT-related molecules detected by western blot and immunofluorescent staining in the indicated OVCAR3 cells. (D) MTT assays assay was
performed in the indicated cells. (E). EdU assays in the indicated cells. PI indicates Propidium Iodide. Error bars represent the mean ± SD of triplicate experiments. (F) Colony formation assay was performed in the indicated cells. (G). Representative images of intraperitoneal tumor-bearing mice in the indicated groups. (H). Intraperitoneal xenograft tumor formation in nude mice in indicated groups. Number of metastatic nodules are shown (mean ± SEM). (I). IHC staining of VGLL1 and Ki-67 in the indicated group mice. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns indicates no significance. Each error bar represents the mean ± SD of three independent experiments.

Figure 3

A

B

C

D

E

F

G

H

I

J

K
Figure 3

VGLL1 transcriptionally activates HMGA1 via interacting TEAD4

(A) Flowing chart illustrates the criteria of identifying of HMGA1 as the target of VGLL1. (B) The clinical correlation between VGLL1 expression and HMGA1 expression. (C) The clinical correlation between VGLL1 expression and ZNF860 expression. (D) Interaction between VGLL1 and TEAD4. Lysates of A2780 cells that were transfected with pcDNA3.1-Flag-VGLL1 and pcDNA3.1-myc-TEAD4 were immunoprecipitated using anti-IgG, anti-Flag, and anti-Myc antibodies. Protein expression was analyzed by immunoblotting. (E) IF assay showing that VGLL1 is colocalized with TEAD4 in the nucleus. (F) HMGA1 expression levels in the indicated A2780 and OVCAR3 cells examined by western blot assay. (G) Effect of TEAD4 on VGLL1-regulated HMGA1 transcriptional activity. (H) HMGA1 promoter activities of the reporter systems containing modified binding sites were measured in OVCAR3 cells. (I) Schematic illustration of the human \textit{HMGA1} gene promoter (upper panel) and ChIP analysis of enrichment of TEAD4 on the HMGA1 promoter (lower panel). IgG was used as a negative control. The qRT–PCR amplification regions are shown in red squares. (J) ChIP assays were performed in the indicated cells using anti-p300 acetyltransferase, anti-RNA POL II (RNAP II), and anti-H3K4me3 antibodies. (K) TOP/FOP luciferase activity in the indicated cells. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns indicates no significance. Each error bar represents the mean ± SD of three independent experiments.
Figure 4

HMGA1 is essential for VGLL1-mediated proliferation and metastasis in ovarian cancer

(A). Quantification of cell invasiveness determined by transwell assays in the indicated cells. (B). Quantification of migrated cells determined by transwell assay using indicated cells. (C, D). Immunofluorescence images of EMT-associated factors, E-cadherin and vimentin using indicated cells. E-
cadherin was stained green, Vimentin was stained red and nuclei was stained with DAPI were blue. (E). Quantification of colony formation ability in the indicated cells. (F). Quantification of EdU positive cells in the indicated cells. (G, H). Quantification of the relative change in luminescence (G) and number of metastases (H) of mice from indicated groups. (I). Western blotting analysis of β-catenin expression in the cytoplasm and nucleus of the indicated cells. GADPH and p84 were used as loading controls for the cytoplasmic and nuclear fractions, respectively. (J). Quantification of cell invasiveness determined by transwell assays in the indicated cells. (K). Quantification of migrated cells determined by transwell assay using indicated cells. (L). Quantification of EdU positive cells in the indicated cells. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns indicates no significance. Each error bar represents the mean ± SD of three independent experiments.
m\(^6\)A modification contributes to the upregulation of VGLL1 in OC

(A, B). qRT-PCR analysis of VGLL1 expression in A2780 and OVCAR3 cells with or without treatment of SAHA or NaB. Transcript levels were normalized to GAPDH expression. Error bars represent the mean ± SD of triplicate experiments. (C). The putative wild-type m\(^6\)A sites and designed mutant m\(^6\)A sites in...
VGLL1. (D) m^6A RIP-qPCR analysis of VGLL1 in HosePic, A2780 and OVCAR3 cells. Error bars represent mean ± SD of triplicate experiments. (E-I) qRT-PCR analysis of m^6A WER expression in 30 OC tumor tissues and 30 normal ovary tissues. (J, K) Immunoblotting of IGF2BP2, IGF2BP1 and YTHDC1 after RNA pull down assay with cell lysate (Ly.), full-length biotinylated VGLL1 (FL), and beads only (NC) in A2780 and OVCAR3 cells. (L, M) Agarose electrophoresis and qRT-PCR analysis of RIP assays in A2780 and OVCAR3 cells showing the direct binding between the IGF2BP2 protein and VGLL1 mRNA. (N-Q) qRT-PCR analysis of VGLL1 at the indicated times after actinomycin D (5 μg/ml) treatment in A2780 and OVCAR3 cells after METTL3 inhibition (N, O), and in A2780 and OVCAR3 cells after IGF2BP2 inhibition (P, Q). ** P < 0.01, *** P < 0.001, **** P < 0.0001, ns indicates no significance. Each error bar represents the mean ± SD of three independent experiments.
Figure 6

Inducible silencing of VGLL1 abrogates metastasis of OC and Clinical relevance of m^6A/VGLL1/HMGA1 axis in OC

(A) Western blotting analysis of VGLL1 to verify that VGLL1 can be conditionally
silenced via doxycycline treatment (Dox, 100 ng/ml) in OVCAR3-shVGLL1\textsuperscript{Dox} cells. (B) Scheme for the chemotherapy mice model. (C, D) Tumors from indicated groups were shown. (E) Quantification of ovarian metastasis nodules in the peritoneal cavity.

(F) Western blotting analysis of VGLL1 and IGF2BP2 in the indicated cells. (G) Representative images of intraperitoneal tumor-bearing mice in the indicated groups. (H) Quantification of ovarian metastasis nodules in the peritoneal cavity. (I) Representative images showing high or low expression of VGLL1, METTL3, IGF2BP2 and β-catenin in OC tissues. (J-L) Correlation between VGLL1, METTL3, IGF2BP2 and β-catenin in 157 OC tumor tissues specimens. (M) Working model of the mechanism proposed in this study. * P < 0.05, *** P < 0.001, **** P < 0.0001, ns indicates no significance. Each error bar represents the mean ± SD of three independent experiments.

**Supplementary Files**

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