

ATRX Loss Upregulates UGDH via Chromatin Remodeling to Promote SOX2 m6A Modification and Stemness Maintenance in Glioblastoma

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Keywords: UGDH, Chromatin remodeling, GSC, ATRX, m6A modification.

Posted Date: July 30th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10432576/v1>

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Additional Declarations: There is **NO** conflict of interest to disclose.

Abstract

UDP-glucose 6-dehydrogenase (UGDH) catalyzes the conversion of UDP-glucose (UDP-Glc) to UDP-glucuronic acid (UDP-GlcUA) and is implicated in tumor progression. However, the mechanisms governing UGDH expression and its potential feedback regulation in glioma stem cell (GSC) maintenance remain unclear. Here, we demonstrate that ATRX loss-induced chromatin remodeling drives UGDH upregulation, which in turn promotes malignant properties and stem-like characteristics in GSCs. Mechanistically, elevated UGDH depletes intracellular UDP-Glc, relieving its inhibition of VIRMA, a core component of the m⁶A methyltransferase complex. Consequently, VIRMA binds more efficiently to SOX2 mRNA, promoting its m⁶A modification and stabilization. Collectively, our findings establish UGDH as a critical regulator of GSC stemness and highlight its potential as a therapeutic target in glioblastoma through epigenetic modulation. Pharmacological inhibition of UGDH by the small-molecule inhibitor HY-155534 effectively suppressed GSC stemness and tumor growth *in vivo*.

Introduction

Changes in tumor metabolism can arise from various factors. A common cause is the abnormal expression of enzymes involved in pre-transcriptional regulation of gene expression. Indeed, chromatin remodeling in tumor cells is known to play a crucial role in such regulation, leading to abnormal gene expression during tumorigenesis.^[1; 2] Chromatin remodeling is a fundamental process that governs the organization of chromatin structure and its accessibility by the transcriptional machinery, directly influencing gene expression in normal and cancerous cells. The α -Thalassemia/mental retardation X-linked (ATRX), one of the top 20 most frequently mutated genes in human cancers, has been definitively shown to play pivotal roles in both the maintenance and dysregulation of 3D chromatin architecture.^[3; 4] In gliomas, mutations in ATRX and IDH1 exhibit strong co-occurrence in diffuse gliomas (WHO grades II-III),^[5; 6] but independent mutations in ATRX can also occur in glioblastoma (GBM).^[7]

Recent advances in chromatin conformation capture techniques, such as Hi-C, have provided deep insights into the three-dimensional architecture of the genome. Hi-C allows for the detection of chromatin interactions, revealing how spatial organization impacts gene regulation.^[8] In cancer cells, Hi-C studies have identified disrupted chromatin loops and topologically associating domains (TADs), which can drive aberrant enhancer-promoter interactions. These interactions often lead to the misexpression of oncogenes. One important mechanism uncovered through Hi-C is the formation and dysregulation of enhancer-promoter loops, which are essential for precise gene regulation. Such dysregulation can aberrantly activate oncogenes promoting malignant and stem-like phenotypes.^[9; 10] Understanding these processes offers potential therapeutic targets to correct epigenetic dysregulation and chromatin disorganization in tumor cells.

Recent studies have highlighted the role of metabolic enzymes in influencing a variety of tumor biological behaviors.^[11; 12] Enzymes that control the availability of metabolites like ATP, NADH and other

intermediates directly support cellular processes that sustain stem-like features in tumors.^[13] UDP-glucose 6-dehydrogenase (UGDH) is a key metabolic enzyme that facilitates the conversion of UDP-glucose into UDP-glucuronic acid, which is critical for synthesizing glycosaminoglycans, such as hyaluronic acid (HA), heparan sulfate and chondroitin sulfate. These compounds are essential components of the extracellular matrix (ECM) and contribute to cell signaling, adhesion and migration, making UGDH integral to both normal cellular and tumor cell functions.^[14] In cancer metabolism, UGDH helps maintain the structural and functional integrity of the tumor microenvironment by promoting ECM formation, which can support tumor growth and metastasis.^[15; 16] Increased UGDH activity has been associated with chemotherapy resistance in certain cancers, where it facilitates the excretion or inactivation of drugs.^[15; 17] However, the regulatory mechanisms by which UGDH enzyme activity influences tumor cell biology require further investigation. Here, we identified a strong association between UGDH and Vir-like m⁶A methyltransferase-associated protein (VIRMA), suggesting a previously unrecognized mechanism involving N6-methyladenosine (m⁶A) modification that supports the maintenance of tumor cell stemness.

The m⁶A modification, the most prevalent RNA modification in eukaryotes, plays a critical role in tumorigenesis and cancer progression.^[18–20] By influencing RNA stability, translation and splicing, m⁶A regulates key genes associated with cell proliferation, apoptosis and differentiation, making it essential in cancer cell adaptability and growth.^[20; 21] In tumors, aberrant m⁶A modifications often alter oncogene or tumor suppressor gene expression, thereby promoting stemness and treatment resistance.^[22–24] Research into m⁶A-related enzymes, including "writers" (e.g., METTL3, VIRMA), "erasers" and "readers" (e.g., IGF2BPs), continues to uncover potential therapeutic targets.^[22; 23] VIRMA (also known as KIAA1429), a major component of the m⁶A methyltransferase complex, acts as an m⁶A "writer" and has been implicated in targeting specific mRNA sites for methylation, including those within the 3' untranslated region (3' UTR) of SOX2.^[25] Specifically, m⁶A reader proteins, such as the IGF2BP family, bind to m⁶A-modified transcripts like SOX2 mRNA to enhance their stability and promote stemness in cancer stem cells (CSCs).^[26; 27] Such stemness is central to cancer cell resilience, in which a subpopulation of cancer cells, CSCs, possess the ability to escape therapeutic targeting and undergo self-renewal and differentiation to initiate tumor regrowth.^[28; 29] This stemness trait is a major driver of cancer cells resistance to treatment and its recurrence following surgical and pharmacological interventions.^[30] In glioblastoma research, we also refer to this sub-type of cells as glioma stem cells (GSCs). In GSCs, SOX2 supports stem cell-like properties by driving pathways linked to proliferation, self-renewal and survival under stress. Additionally, SOX2 interacts with other oncogenic signals, further promoting tumor initiation, invasion and recurrence.^[31; 32] Recent research has highlighted the importance of post-transcriptional regulation in sustaining SOX2 levels, particularly through m⁶A modifications that enhance the stability of SOX2 mRNA.^[26; 27] However, whether UGDH metabolic activity modulates VIRMA-mediated m⁶A modification of SOX2 mRNA remains unclear. Understanding how the

metabolic activity of UGDH and m⁶A regulatory mechanisms interact may reveal novel therapeutic targets for inhibiting GSCs maintenance and disrupting tumor growth.

Results

Chromatin remodeling enhances transcription of UGDH

The regulation of chromatin structure is related to a variety of fundamental nuclear processes. Its dysregulation contributes to tumorigenesis, tumor progression and responses to therapeutic treatment. [1; 2] ATRX, an ATP-dependent chromatin remodeling factor, is essential for chromatin architecture and is notably among the commonly mutated genes in different human cancers. To elucidate the role of ATRX in regulating three-dimensional chromatin organization and the downstream consequences of such alterations, we performed a Hi-C assay in LN229 cells, a human glioblastoma (GBM) cell line, which we used as a parental cell line for isogenic CRISPR ATRX knockout (KO) or an empty vector (EV) control and was utilized in our previous study. [33] The Hi-C data suggested that ATRX^{KO} cells have an obvious difference in the 3D conformation compared to ATRX^{EV} cells between 39.45 Mbp to 39.80 Mbp, which is upstream of the UGDH promoter (Fig. 1A). ChIP-seq profiles of H3K27ac and H3K4me3 data showed that there are two candidate enhancers located in this locus, while CTCF enrichment revealed that those two candidate enhancers and the UGDH promoter may be separated by a candidate boundary (Fig. 1B). These enhancers were looped to the UGDH gene in both ATRX^{KO} and ATRX^{EV} cells, but the interaction between the UGDH promoter and the candidate enhancers in the ATRX^{KO} cells was stronger than in ATRX^{EV} cells. We then performed qRT-PCR in LN229 and HG7 (patient-derived glioblastoma cell line [34]) cells and we found that UGDH mRNA levels were higher in ATRX^{KO} cells compared to ATRX^{EV} cells in both cell lines (Supplementary Fig. 1A). Using ChIP-qPCR for H3K27ac, we observed significantly stronger H3K27ac enrichment at candidate enhancers in ATRX^{KO} cells compared to control cells (Fig. 1C and Supplementary Fig. 1B). We found similar results for H3K4me3 by ChIP-qPCR (Fig. 1D and Supplementary Fig. 1C). And by ChIP-qPCR of CTCF we also found that its occupancy was lower in ATRX^{KO} cells, relative to control cells (Fig. 1E and Supplementary Fig. 1D).

We then performed 3C experiments in ATRX^{EV} cells and ATRX^{KO} cells. We designed twelve test primers, which were evenly distributed upstream of the UGDH promoter (Fig. 1F). [35] By 3C-qPCR we detected stronger interactions between the UGDH promoter and the candidate enhancers in ATRX^{KO} cells compared to the ATRV^{EV} cells (Fig. 1G). To further confirm the function of the candidate enhancers, we utilized the CRISPR interference (CRISPRi) system to silence each candidate enhancer. [35; 36] By Western blotting and qPCR we found that silence of candidate enhancer 1 and 2 resulted in lower UGDH expression (Supplementary Fig. 1E-F). Meanwhile, multiple metabolic enzymes have been identified as critical regulators of tumor cell stemness. [13; 37] To systematically assess the impact of metabolic enzymes on ATRX^{KO} tumor cells sphere formation ability, we used siRNA to knockdown 90 rate-limiting metabolic enzymes in ATRX^{KO} LN229 cells and detected their sphere-forming capacity. UGDH

knockdown significantly impaired tumor sphere formation (Fig. 1H). These results suggested that chromatin remodeling influenced the expression of the UGDH gene and this mechanism may be associated with tumor stemness.

To further investigate the role of ATRX in tumorigenesis and malignancy *in vivo*, we generated tumor models by inducing heterozygous deletion of the tumor suppressor genes Nf1 and Tp53.^[38; 39] This was achieved using a Cre-loxP recombination system under the control of the Nes promoter, which specifically targets neural progenitor cells. This approach allowed us to simulate tumorigenesis and assess the effects of ATRX on tumor progression and malignancy. We injected such mice and their controls (Nes-CreERT; Atrx^{fl/-} or Atrx^{fl/fl}/Atrx^{+/-} or Atrx^{+/+}; Nf1^{fl/fl}; Tp53^{fl/fl}) with tamoxifen at 4 weeks of age, which successfully induced the development of tumors.^[38] By hematoxylin and eosin (H&E) staining of the tumors we found histological features consistent with gliomas. By IF we found that the tumors demonstrated a high number of Ki67⁺ cells, indicating active proliferation. Additionally, we observed extensive expression of Pdgfr- α (oligodendrocyte precursor cell marker), Sox2 and Olig2 (stem-like markers), suggesting the presence of a significant stem-like cell population within the tumors (Supplementary Fig. 2A).^[40–42] And by IHC we found that the expression of Sox2, Olig2 and Ugdh in the tumors were higher in the Atrx conditional knockout (Atrx^{CKO}) mice compared to those in the Atrx wild-type (Atrx^{WT}) mice (Supplementary Fig. 2B-C). Notably, the Atrx^{CKO} mice show a significantly shorter survival time compared to Atrx^{WT} mice (Supplementary Fig. 2D). These findings indicated that ATRX deletion drives tumor aggressiveness and is associated with worse outcomes in primary tumor models.

Next, we generated primary cell lines from the tumor tissues of mutant mice and subsequently cultured them *in vitro*. We then transfected them with a luciferase-expressing lentivirus and stereotactically injected them into the brains of mice to construct orthotopic tumor models (Supplementary Fig. 2E). By bioluminescence imaging we found that the tumor models generated by using the Atrx^{CKO} cells exhibited faster tumor growth and resulted in a shorter survival time than the mice harboring tumors from the Atrx^{WT} cells (Supplementary Fig. 2F and Supplementary Fig. 3A-B). We also found that tumor cells derived from ATRX^{CKO} mice had lower levels of UDP-Glc than tumor cells derived from ATRX^{WT} mice (Supplementary Fig. 3C). By sphere formation and limited dilution assays we found that ATRX^{CKO} cells exhibited a stronger self-renewal capacity compared to ATRX^{WT} cells (Supplementary Fig. 3D-F). Meanwhile, UGDH expression was significantly higher in ATRX-negative GBM patient samples than in ATRX-positive tumors. (Supplementary Fig. 3G-H). These findings indicated that knockout or loss of ATRX upregulates UGDH expression through alterations in 3D chromatin architecture, and such a negative association between ATRX and UGDH protein expression is also seen in human cases of glioma. Notably, Atrx conditional knockout in mice primary tumors was associated with a higher degree of malignancy and significantly impacts the survival outcomes.

UGDH enhances the stemness, proliferation and invasion ability of tumor cells

To investigate the correlation between UGDH expression and clinical features in glioma, we analyzed data from the TCGA and CGGA databases. We found that UGDH expression is higher in WHO grade 4 gliomas than in grade 2 and 3 tumors. Moreover, UGDH levels are elevated in ATRX-mutant compared to ATRX-wild-type samples (Supplementary Fig. 4A-C). GO and KEGG pathway analyses revealed that UGDH-associated genes are enriched in pathways related to RNA degradation and stem cell regulation, and GSEA further supported the involvement of these genes in RNA-binding protein function and glioma stem cell maintenance (Supplementary Fig. 4D).

Based on our previous single-cell RNA sequencing (scRNA-seq) data of patient-derived GBM tissues,^[43] we identified significant co-expression patterns between UGDH and stemness markers (SOX2, OLIG2, NES) (Fig. 2A and Supplementary Fig. 5A), suggesting a potential role of UGDH in glioma stem cell maintenance. We next overexpressed UGDH in LN229 and HG7 cells to test what effect its elevated levels would have on tumor cell function. Using a sphere formation assay, we found that more spheres were formed by UGDH-overexpressing cells compared to the control cells (Fig. 2B and Supplementary Fig. 6A). In limited dilution assays we also found that UGDH overexpression increased the self-renewal capacity of stem-like cells (SLCs) (Fig. 2C and Supplementary Fig. 6B). Meanwhile, by immunofluorescence (IF) and qPCR of the spheres formed by UGDH-overexpression in the SLCs we found greater expression of the stem cell regulator sex-determining region Y(SRY)-box 2 transcription factor (SOX2)^[44] at the protein and mRNA levels, while there was lower expression of the astrocyte marker GFAP, than in the control cells (Fig. 2D-E and Supplementary Fig. 6C-D). In addition, we also found that the proliferation of SLCs was greater in the UGDH-overexpressing groups compared to control groups (Fig. 2F and Supplementary Fig. 6E). By a transwell invasion assay we also found that UGDH overexpression enhanced the invasive ability of tumor cells (Fig. 2G and Supplementary Fig. 6F).

To detect the effect of UGDH overexpression *in vivo*, we used UGDH-overexpressing glioma cells and control cells to establish orthotopic mouse models. By bioluminescence imaging we found that overexpression of UGDH resulted in larger tumors, as well a shorter survival time, compared to those in the control cell-bearing mice (Supplementary Fig. 7A-C). By immunohistochemistry (IHC) of tumor tissues we found that UGDH overexpression was associated with higher SOX2 protein levels (Supplementary Fig. 7D-E). We also examined the expression of various stemness markers in the tumor sphere cells in both two glioma lines compared to their respective normal cells and found higher expression of the stemness markers (SOX2, OLIG2, NES) and lower expression of GFAP (Supplementary Fig. 7F). These results indicate that overexpression of UGDH may contribute to stemness and tumor biological behaviors in GSCs.

UGDH enhances the mRNA stability of the stem cell regulator SOX2

To explore the mechanism by which UGDH affects tumor cells stemness, we examined the mRNA and protein levels of SOX2 and the mRNA levels of 5 other well-regarded stemness-associated genes and found that UGDH knockdown inhibited the expression of SOX2 and NES (the gene encoding for NESTIN)

and increased the expression of GFAP (Fig. 3A-B and Supplementary Fig. 8A-B). To further investigate whether the maintenance of stemness of tumor cells depended on UGDH, we knocked down UGDH by using short hairpin RNA (sh-UGDH). We found that such knockdown inhibited the sphere formation ability of the tumor cells while re-expression of UGDH (r-UGDH) recovered the sphere formation ability of the UGDH knockdown cells (Fig. 3C and Supplementary Fig. 8C-D). Mice injected with UGDH knockdown glioma cells, which exhibited slower growth, resulted in a lengthened survival time compared to the control group or mice injected with r-UGDH/UGDH knockdown cells (Fig. 3D-F).

Higher expression of SOX2, whose activity has been associated with the maintenance of stemness and self-renewal ability of SLCs, is associated with a poor clinical outcome.^[31; 32; 44] SOX2 can be regulated at multiple levels, including transcription, mRNA decay and protein degradation.^[27; 45] We also found that both mRNA and protein levels of SOX2 were recovered in UGDH knockdown cells with r-UGDH (Supplementary Fig. 8E). To investigate whether UGDH enhances tumor cells stemness through regulation of SOX2 expression, we overexpressed SOX2 in UGDH knockdown cells and control cells (Supplementary Fig. 8F) and found that this resulted in the recovery of the lower sphere formation of UGDH knockdown cells (Fig. 3G and Supplementary Fig. 8G, Supplementary Fig. 9A). Likewise, repletion of SOX2 expression (r-SOX2) partially restored the sphere formation ability of UGDH knockdown cells (Fig. 3H and Supplementary Fig. 9B-D).

To explore how UGDH regulates SOX2 expression, we measured promoter reporter luciferase intensity to evaluate the activity of the SOX2 promoter. We found that UGDH knockdown did not influence SOX2 promoter activity (Fig. 3I and Supplementary Fig. 9E), but in an RNA decay assay we observed that UGDH knockdown significantly reduced SOX2 mRNA stability (Fig. 3J and Supplementary Fig. 9F). In addition, knockdown of UGDH in ATRX^{KO} cells also impaired SOX2 mRNA stability (Fig. 3K and Supplementary Fig. 9G). These results reveal that UGDH regulates the self-renewal capacity of tumor cells by enhancing the stability of SOX2 mRNA. Knockdown of UGDH restored the ATRX^{KO}-enhanced SOX2 mRNA stability to levels observed in ATRX^{EV} cells. We speculated that the increased stability of SOX2 mRNA in ATRX^{KO} cells depends on elevated UGDH expression.

VIRMA interacts with UGDH to recognize SOX2 mRNA

To investigate how UGDH regulates the stability of SOX2 mRNA, we sought to identify UGDH-binding proteins by using LC-MS/MS. Among a comprehensive analysis of several UGDH-binding proteins, we focused on Vir-like m⁶A methyltransferase-associated protein (VIRMA) as a potential UGDH-binding protein for further research (Fig. 4A-B). VIRMA is a key component of the RNA m⁶A methyltransferase complex (MTC) and has been reported to play a critical role in recruiting the catalytic core components of the MTC, facilitating preferential mRNA methylation in the 3' UTR and near the stop codon.^[25] We next validated an interaction between UGDH and VIRMA by co-immunoprecipitation assay (Supplementary Fig. 10A). VIRMA is mostly located in the nucleus, and by IF and co-immunoprecipitation assays after cell fractionation we verified that UGDH interacted with VIRMA in the nucleus (Fig. 4C-D and

Supplementary Fig. 10B-C). Furthermore, in the RNA decay assay, VIRMA knockdown reduced SOX2 mRNA stability in ATRX^{KO} cells, thereby returning it to a level similar to that in ATRX^{EV} cells. (Supplementary Fig. 10D). To explore whether VIRMA plays a critical role in UGDH regulating SOX2 mRNA stability, we next knocked down VIRMA in cells with UGDH overexpression and results of RNA decay assays found that VIRMA knockdown abolished the enhancing effect of UGDH overexpression on SOX2 mRNA stability (Fig. 4E and Supplementary Fig. 10E). To detect whether UGDH regulates VIRMA binding to SOX2 mRNA, we performed RNA immunoprecipitation (RIP)-PCR assays and found a lower binding affinity in the UGDH knockdown group compared to the control cells (Fig. 4F and Supplementary Fig. 10F). We also used an anti-m⁶A antibody to perform the RIP-PCR assays to assess the m⁶A levels of SOX2 mRNA and found that knockdown of UGDH impaired these levels (Fig. 4G and Supplementary Fig. 10G). These results suggested that UGDH regulates the m⁶A modification process of SOX2 mRNA by VIRMA. These findings were further supported by streptavidin RNA pull-down assays (Supplementary Fig. 10H). Additionally, we also performed RIP-PCR assays of ATRX^{EV} and ATRX^{KO} cells transfected with sh-NT or sh-UGDH to assess the binding affinity between VIRMA or the anti-m⁶A antibody to SOX2 mRNA and found that knockdown of UGDH eliminated the enhancement of their binding affinity to this mRNA in the ATRX^{KO} cells (Supplementary Fig. 11A-B). Further, by luciferase assays we found that the activity of the SOX2 3' UTR was impaired in UGDH knockdown group (Supplementary Fig. 11C). These results demonstrated that VIRMA-mediated m⁶A modification of SOX2 mRNA is required for maintaining its stability, and this process is regulated by UGDH.

To investigate whether VIRMA regulates the stability of SOX2 mRNA via an m⁶A modification-mediated mechanism, we concentrated on two primary families of m⁶A "readers"; namely, the insulin-like growth factor-II binding protein (IGF2BP) family and the YTH family.^[21] To identify the m⁶A reader specifically binds to SOX2 mRNA, we performed streptavidin RNA pull-down assays and found that IGF2BP1 specifically bound to SOX2 mRNA (Fig. 4H and Supplementary Fig. 11D-E). As one of the m⁶A readers, IGF2BPs have been found to play critical roles in regulating the levels of methylated mRNA.^[46] Recent studies have revealed that the 3' UTR of SOX2 mRNA contains three candidate m⁶A sites (GGACH) that can be targeted by IGF2BP1 and m⁶A modification of these sites results in SOX2 mRNA stabilization.^[26; 27] By RNA decay assays we confirmed that knockdown of IGF2BP1 impaired the stability of SOX2 mRNA (Fig. 4I and Supplementary Fig. 11F). Additionally, by RIP-PCR we found that the binding affinity of IGF2BP1 to SOX2 mRNA was lower in tumor cells with UGDH knockdown compared to control cells (Supplementary Fig. 11G). Next, we aimed to determine whether the three candidate m⁶A sites are essential for VIRMA binding to the 3' UTR of SOX2 mRNA. We constructed point mutations of the three candidate m⁶A sites (Fig. 4J). Upon transfection of the WT or mutated SOX2-3' UTR constructs into the tumor cell lines, followed by RIP-PCR, we found that mutations at each of the three candidate m⁶A sites decreased the binding affinity of VIRMA and IGF2BP1 to the SOX2-3' UTR (Fig. 4K-L and Supplementary Fig. 12A-B). We also found that the m⁶A levels of SOX2 mRNA were lower in the cells transfected with any of the SOX2-3' UTR mutants compared to the WT construct (Supplementary Fig. 12C). Furthermore,

we observed greater luciferase activity in the cells harboring the WT reporter in ATRX^{KO} cells compared to ATRX^{EV} cells; however, this increase was diminished by mutation of any one of the m⁶A sites in the reporter (Supplementary Fig. 12D). By RNA decay assays, we found that each point mutation reduced the stability of the SOX2-3' UTR construct (Fig. 4M and Supplementary Fig. 12E). These results indicated that all three sites are critically involved in SOX2 m⁶A modification and UGDH plays a critical role in VIRMA-mediated regulation of m⁶A modification of the 3' UTR of SOX2 mRNA.

UGDH enhances SOX2 levels by depleting UDP-Glc

UGDH, as the key enzyme in the uronic acid pathway, catalyzes the 2-fold oxidation of UDP-glucose (UDP-Glc) to UDP-glucuronic acid (UDP-GlcUA), using NAD (P)⁺, typically NAD⁺, as the oxidizing agent. This process is essential for the biosynthesis of glycosaminoglycans, which play important roles in cell signaling and tissue remodeling.^[14] To investigate whether UGDH's role in regulating tumor cells stemness is linked to its involvement in biosynthetic processes, we transfected an expression vector harboring UGDH-ED, a C276S mutant version of UGDH that maintains its protein stability but results in a lack of its proper metabolic activity,^[47] into UGDH knockdown cells to investigate the effects of rescuing UGDH expression with a version of the protein that lacks enzyme activity (Fig. 5A-B and Supplementary Fig. 13A-C). By streptavidin RNA pull-down assays we found that UGDH-ED expression reduced the binding affinity of VIRMA to SOX2 mRNA (Fig. 5C and Supplementary Fig. 13D). Meanwhile, cells expressing UGDH-ED had lower stability of SOX2 mRNA compared to cells rescued by UGDH-WT (Fig. 5D and Supplementary Fig. 13E). These results suggested that UGDH enzymatic activity is required for maintaining SOX2 mRNA stability.

To examine the effects of UGDH substrates and products in tumor cells, we selected the solute carrier family-35D1 (SLC35D1) to facilitate our further investigation. SLC35D1 is a gene that encodes a nucleotide sugar transporter involved in the transport of UDP-Glc and UDP-GlcUA.^[48] We transfected tumor cells with Flag-SLC35D1 and by Western blotting we demonstrated that there was an elevated expression level of SLC35D1 in the transfected cells (Fig. 5E and Supplementary Fig. 13F-G). To assess intracellular UDP-GlcUA levels, we supplemented tumor cells with exogenous UDP-GlcUA, which successfully restored the diminished intracellular UDP-GlcUA levels observed in UGDH knockdown cells. However, the addition of UDP-GlcUA did not fully restore the decrease of SOX2 mRNA levels in the UGDH knockdown cells (Fig. 5F and Supplementary Fig. 13H). The RNA decay assays further indicated that the addition of UDP-GlcUA did not restore SOX2 mRNA stability in UGDH knockdown cells (Supplementary Fig. 13I). UDP-GlcUA is the critical precursor in the biosynthesis of hyaluronic acid (HA), a key glycosaminoglycan involved in various physiological processes. The addition of GlcUA fully restored the cellular HA levels (Supplementary Fig. 14A) but only slightly improved the SOX2 mRNA level of UGDH knockdown cells (Supplementary Fig. 14B). To investigate whether this slight recovery was attributed to HA biosynthesis, we supplemented different concentration of HA to the tumor cells and found that concentrations that sufficiently restored intracellular HA levels did not adequately restore sphere

formation ability (Supplementary Fig. 14C-E). These results suggested that UDP-GlcUA and its downstream metabolites are not the primary drivers of enhanced stem cell properties.

We next supplemented the tumor cells with UDP-Glc, which significantly decreased SOX2 mRNA levels and its stability (Fig. 5G and Supplementary Fig. 14F-G). UDP-Glc also inhibited tumor cells sphere formation in a dose-dependent manner (Fig. 5H-J and Supplementary Fig. 14H-I). To further investigate the mechanism of action by which UDP-Glc is involved in this process, we knocked down the expression of UGP2, the gene encoding UTP-glucose-1-phosphate uridylyltransferase, the enzyme responsible for UDP-Glc synthesis. UGP2 knockdown reduced intracellular UDP-Glc levels, which could be restored by re-UGP2 expression (Supplementary Fig. 15A). Moreover, UGP2 knockdown restored sphere formation ability that was impaired by UGDH knockdown (Fig. 5K-L and Supplementary Fig. 15B-D). Meanwhile, In RNA decay assays we found that UGP2 knockdown increased SOX2 mRNA stability (Supplementary Fig. 15E). *In vivo* bioluminescence imaging revealed that tumors in the UGP2 knockdown group exhibited greater sizes and faster growth rates than those in the control group. (Fig. 5M-N and Supplementary Fig. 15F). These results suggested that UDP-Glc negatively regulates SOX2 expression and stability, and that UGDH may modulate SOX2 mRNA levels by consuming UDP-Glc.

UDP-Glc restricts the mRNA binding of VIRMA

As metabolites have also been shown to regulate the function and activity of non-metabolic proteins, their role extends beyond traditional metabolic pathways.^[49] We employed AlphaFold Server to predict the protein–protein interaction and obtained a potential binding mode (Supplementary Fig. 15G). While further analysis using PRODIGY yielded a predicted binding energy of -7.3 kcal/mol. Co-immunoprecipitation (Co-IP) with an anti-UGDH revealed that UDP-Glc promoted the association of UGDH with VIRMA (Supplementary Fig. 15H). Therefore, we hypothesized that UDP-Glc, the substrate of UGDH, plays a critical role in maintaining the stemness of GSCs by regulating the binding of VIRMA to SOX2 mRNA. Indeed, pull-down assays revealed that UDP-Glc more strongly inhibited the binding of VIRMA to SOX2 mRNA compared to UDP-GlcUA (Fig. 6A). We then used molecular docking to predict the binding of either UDP-Glc and UDP-GlcUA to VIRMA^[50]. We were able to define the binding pocket that both UDP-Glc and UDP-GlcUA bound, which were both in the the same domain of VIRMA. We found that the residues that favor UDP-Glc binding were I483, R522, R524, E527, K528, G530, while those favor UDP-GlcUA binding were T475, S484, A519, R522, R524, K533 (Fig. 6B). We also predicted the binding strength by utilizing the DOCK 6.9^[51; 52] program (Supplementary Table 3).

To compare the interaction differences between the combinations of VIRMA-UDP-Glc and VIRMA-UDP-GlcUA, we assessed their thermodynamic properties using isothermal titration calorimetry (ITC) assays. This technique allows for precise measurement of binding affinities and enthalpic/entropic changes associated with the molecular interactions, providing insights into the relative strength and nature of these binding events. Via the ITC assays we found that VIRMA exhibited a higher affinity for UDP-Glc compared to UDP-GlcUA (Fig. 6C). This difference in binding affinity may have implications for better understanding how VIRMA regulates mRNA modifications. Guided by molecular dynamics simulation

results, we generated VIRMA mutants R524A and R522A. By pull-down assays we found that VIRMA-R522A exhibited reduced binding affinity to SOX2 mRNA in the presence of UDP-Glc, while VIRMA-R524A binding was unaffected by UDP-Glc supplementation (Fig. 6D and Supplementary Fig. 16A-B). These results suggested that the R524 mutation abolishes the inhibitory effect of UDP-Glc on SOX2-VIRMA binding. By ITC assays we confirmed that the R524A mutation disrupted the binding of VIRMA to UDP-Glc (Supplementary Fig. 16C). In cells, compared to VIRMA-WT, VIRMA-R524A exhibited a higher expression of SOX2 (Fig. 6E and Supplementary Fig. 16D-E). Additionally, by Western blotting of the pull-down assays we found that VIRMA-R524A had a higher affinity to SOX2 mRNA in both ATRX^{EV} and ATRX^{KO} cells compared to VIRMA-WT (Fig. 6F and Supplementary Fig. 16F). Notably, the VIRMA-R524A did not significantly alter its binding affinity to the mRNAs of other stemness-related genes, including GFAP, NES, OCT4, CD133 and OLIG2, in ATRX^{KO} cells (Supplementary Fig. 16G). We also found that the m⁶A levels of stemness-related mRNAs showed no significant changes except for SOX2 (Supplementary Fig. 17A). RNA decay assays revealed that SOX2 mRNA stability in ATRX^{EV} cells expressing VIRMA-R524A was significantly enhanced compared to the VIRMA-WT control, mirroring the stability observed in ATRX^{KO} cells. (Supplementary Fig. 17B).

To explore the biological role of UDP-Glc-mediated regulation of VIRMA we performed sphere formation assays and found that tumor cells expressing VIRMA-R524A exhibited greater self-renewal capacity compared to those expressing VIRMA-WT. Meanwhile, ATRX^{KO} cells expressing VIRMA-R524A didn't show a significant increase in sphere formation (Fig. 6G and Supplementary Fig. 17C). Furthermore, we established orthotopic mouse models using tumor cells expressing either VIRMA-R524A or VIRMA-WT. By bioluminescence imaging we found that mice inoculated with tumor cells expressing VIRMA-R524A had a poorer outcome compared with VIRMA-WT (Fig. 6H and Supplementary Fig. 17D-E). These findings indicate that UDP-Glc suppresses the malignant behavior of tumor cells by binding to VIRMA and preventing its interaction with SOX2 mRNA.

Identification and functional characterization of a UGDH inhibitor that blocks GSC maintenance and tumor growth

We next sought to identify small-molecule inhibitors of UGDH with potential therapeutic relevance. After virtual screening, we filtered out compounds with molecular weight > 400 and retained those with AlogP values between 2.0 to 4.0. Based on functional relevance and prior research background, we selected HY-15534 for further experimental validation (Supplementary Table 4). This compound has been shown to penetrate the blood-brain barrier without apparent neurotoxicity, but its anti-tumor efficacy remains unclear. To evaluate its inhibitory effect on UGDH and therapeutic potential, we first performed molecular dynamics simulation. The results showed that HY-15534 can stably insert into the internal binding pocket of UGDH, with its overall conformation exhibiting good spatial complementarity to the pocket shape (Fig. 7A and Supplementary Fig. 18A). The two-dimensional interaction map further reveals an extensive network of non-covalent interactions between the ligand and the protein (Supplementary

Fig. 18B). The Root Mean Square Deviation (RMSD) of the complex rose rapidly from 0 to approximately 0.25 nm at the onset of the simulation, reflecting the expected conformational relaxation of the system. It then mainly fluctuated between 0.25 and 0.32 nm, with a brief increase to nearly 0.40 nm at around 35–40 ns, suggesting possible local conformational adjustments. After returning to 0.30 nm, the RMSD remained stable for the remainder of the 100 ns trajectory. Overall, no persistent significant deviation was observed, indicating that the protein-ligand complex maintained good kinetic stability throughout the simulation (Fig. 7B and Supplementary Fig. 18C). The Root Mean Square Fluctuation (RMSF) values of protein residues remained generally low, with most residues fluctuating by less than 0.25 nm, indicating that the protein backbone maintained structural stability throughout the simulation (Supplementary Fig. 18D). During the simulation, the Radius of gyration (Rog) of the complex was predominantly distributed between 2.46 and 2.50 nm, reflecting the overall compactness of the system. A brief increase to approximately 2.55 nm was observed at around 35–40 ns, consistent with the local conformational adjustment seen in the RMSD analysis. Subsequently, the Rog decreased markedly and stabilized within 2.42–2.46 nm, suggesting that the complex adopted a more compact conformation in the middle and later stages. Notably, no sustained increase in Rog was observed at the end of the simulation, indicating that ligand binding did not destabilize the protein structure, but rather may have promoted a more tightly packed conformation (Fig. 7C). The Solvent Accessible Surface Area (SASA) of the complex fluctuated mainly between approximately 205 and 218 nm² over the 100 ns simulation, with a relatively limited overall variation. This result further supports that the protein-ligand complex maintained a stable conformational state in the aqueous environment (Supplementary Fig. 18E). The ligand-protein Center of Mass (COM) distance decreased from 0.75–0.90 nm to 0.55–0.65 nm after 25–35 ns and remained stable, reflecting adaptive binding. This tighter association did not compromise complex stability, as confirmed by RMSD and Rog, indicating successful ligand accommodation within the binding pocket (Fig. 7D). Throughout the simulation, hydrogen bonds formed dynamically between the protein and ligand, with the network exhibiting continuous rearrangement rather than remaining static. Collectively, these hydrogen bonds contributed significantly to the directional and short-range stability of ligand binding, and together with hydrophobic interactions, supported the overall stability of the complex (Supplementary Fig. 18F). Per-residue binding energy analysis revealed that most residues contributed negligibly (near 0 kcal/mol), whereas a small number of key hotspot residues accounted for the majority of the binding affinity, thereby stabilizing the complex (Supplementary Fig. 18G). The free energy landscape constructed from RMSD and Rog revealed a well-defined low-energy basin centered around RMSD of 0.23–0.30 nm and Rog of 2.42–2.47 nm. This region corresponds to the most stable and preferentially sampled conformational state, indicating that the complex favors a moderately compact binding conformation with stable RMSD throughout the simulation (Fig. 7E and Supplementary Fig. 18H). The electrostatic potential model revealed that the ligand occupies a well-defined concave region of the charge distribution, which likely facilitates hydrogen bonding and electrostatic interactions between its polar groups and nearby charged or polar residues (Supplementary Fig. 18I). Comparison of the complex conformations at 0, 50 and 100 ns revealed that the overall protein fold remained stable throughout the simulation, with no signs of unfolding or large-scale structural disruption. The ligand consistently occupied the same binding region at all three time points, indicating that it did not

dissociate from the pocket (Fig. 7F and Supplementary Fig. 18J). Collectively, these results demonstrated that the protein-ligand complex maintained favorable structural stability and binding persistence throughout the 100 ns molecular dynamics simulation.

By monitoring absorbance at 340 nm, we confirmed that HY-155534 inhibits UGDH enzymatic activity, with an IC_{50} of approximately 23.8 nM (Fig. 7G). The sphere formation assay demonstrated that HY-155534 suppresses the sphere-forming capacity of tumor cells in a dose-dependent manner (Fig. 7H and Supplementary Fig. 19A-B). The *in vivo* safety profile of the inhibitor was assessed via histopathological examination (H&E staining) of major organs and complete blood count analysis in treated mice (Supplementary Fig. 19C and Supplementary Table 5). Tumor-bearing mice were treated with daily intraperitoneal injections of HY-155534 (4 mg/kg) starting one week post-implantation. The inhibitor exerted therapeutic effects in both ATRX^{EV} and ATRX^{KO} xenograft models, with the ATRX^{KO} group showing the greatest response (Fig. 7I and Supplementary Fig. 19D-E). Together, these results not only validated the identification of HY-155534 as a UGDH inhibitor but also highlighted its potential as a targeted therapeutic agent.

Discussion

In recent years, metabolic enzymes have become a central focus of tumor research, reflecting their critical roles in cancer development and progression.^[11; 12; 53] These enzymes exert their influence through diverse pathways, including reshaping metabolic networks to meet the demands of rapidly proliferating tumor cells, modulating nutrient uptake, and supporting metabolic reprogramming to sustain growth and survival.^[11; 54] Several of them also have moonlighting functions in which they act beyond their roles in metabolism to affect tumor cells.^[55; 56] Beyond these direct effects, metabolic enzymes regulate various tumor biological behaviors by consuming specific substrates and generating metabolites that act as signaling molecules.^[57] These processes are observed in different cancer types. Gaining a deeper understanding of the influences in tumor progression caused by abnormal metabolic enzyme activity is crucial for uncovering potential therapeutic targets. By elucidating these mechanisms, the aim is to identify novel strategies to disrupt tumor metabolism, improve treatment efficacy and ultimately enhance patient outcomes.

Substrates and metabolites of enzymes significantly influence tumor progression and resistance to therapy in many ways. As a key enzyme in the hexosamine biosynthetic pathway, UGDH catalyzes the conversion of UDP-Glc to UDP-GlcUA, a precursor for glycosaminoglycans and other macromolecules. The altered enzymatic activity of UGDH has also been associated with the malignant behavior of cancer cells,^[15; 16; 58; 59] and it has emerged as a promising therapeutic target for the treatment of various cancers.^[16; 58–60] Our study here reveals a new mechanism by which UGDH plays a further role in tumor persistence; namely, in the maintenance and self-renewal capacity of GSCs. Notably, we found that chromatin remodeling in glioma cells leads to overexpression of UGDH.

Chromatin remodeling refers to the dynamic process by which the structure of chromatin is altered to regulate gene expression.^[61] In cancer, dysregulated chromatin remodeling can lead to aberrant gene expression, contributing to tumorigenesis, metastasis and resistance to therapy.^[8; 10] Given ATRX's role in maintaining 3D chromatin structure and its high mutation frequency across multiple cancer types,^[3] we analyzed the results of Hi-C of glioma cell lines with ATRX knockout or not, we observed that chromatin remodeling was induced and changes in the 3D structure of the UGDH promoter and its enhancers, which, through their close spatial proximity, facilitated the enhanced activation and transcriptional activity of the UGDH promoter. Related studies have reported the role of 3D structure of promoters and enhancers in driving oncogene expression.^[62; 63] By analyzing ChIP-seq data, we identified two H3K27ac and H3K4me3 enrichment regions in the upstream of the promoter of UGDH that were candidate enhancers. We hypothesized that the 3D structure of these two candidate enhancers of the UGDH promoter became closer to the promoter upon chromatin remodeling. These hypotheses were further corroborated by the 3C experiment. Our data suggests that these two candidate enhancers of UGDH contribute to the upregulation of its promoter expression when chromatin remodeling occurs during tumorigenesis.

To validate the role of UGDH elevated expression in the regulation of cancer stemness, we constructed UGDH overexpression tumor cell lines and found that this resulted in an upregulation of SOX2 and other tumor stemness markers, as well as enhancements in self-renewal capacity and sphere formation. Stemness is a key characteristic of CSCs that drives tumor initiation, progression and metastasis, as well as the reemergence of tumors after treatment.^[64; 65] SOX2, a transcription factor critical for maintaining stem cell properties, plays a pivotal role in regulating the self-renewal and pluripotency of CSCs in various cancers, including gliomas, breast cancer and colorectal cancer.^[64; 66; 67] Understanding the mechanisms that maintain stemness and the factors that promote CSC survival is critical for developing targeted therapies aimed at eradicating these cells and improving patient outcomes in cancer treatment.^[65] Our results here show that the high expression of SOX2 in UGDH overexpression cell lines was not a result of enhanced transcription, but rather by decreases in its RNA decay, resulting in an enhanced self-renewal capacity and stemness of the UGDH overexpression cells. However, the underlying mechanism by which abnormal UGDH expression enhances SOX2 mRNA stability in tumor cells remains unclear.

To further explore the underlying mechanisms by which UGDH upregulation affects cancer stemness, we conducted a co-immunoprecipitation followed by mass spectrometry (CoIP-MS) assay and identified VIRMA as a binding partner of UGDH. VIRMA (also known as KIAA1429) is an essential component of the m⁶A methyltransferase complex, functioning as a "writer" to facilitate the addition of m⁶A modifications to mRNA.^[68] The m⁶A modification, a crucial post-transcriptional regulation of mRNA, plays a significant role in the expression and stability of mRNAs, including those encoding stemness factors, such as SOX2, in many types of tumor cells.^[23; 26; 27; 68] To investigate whether UGDH facilitates tumor cells stemness maintenance through regulation of VIRMA binding to SOX2 mRNA, we examined the interaction between VIRMA and SOX2 mRNA by RIP-PCR and RNA pull-down assays and found that

overexpression of UGDH enhanced the binding of VIRMA to SOX2 mRNA. These findings fit with our other findings showing that the overexpression of UGDH increases the m⁶A modification level of SOX2 mRNA. And we further validated a role for m⁶A modification in UGDH-mediated stabilization of SOX2 mRNA by showing that mutation of the m⁶A sites in the 3' UTR of SOX2 mRNA blunted this effect of UGDH overexpression. Notably, these mutated m⁶A sites prevent VIRMA binding to SOX2 mRNA and reduced the m⁶A levels and the stability of SOX2 mRNA. Together, these results suggest that the binding efficiency of VIRMA to SOX2 mRNA, and thus the stability of this mRNA species, is regulated by a physical interaction with UGDH, and that this interaction is crucial for the maintenance of tumor stemness.

Metabolites within the human body have been reported to hold significant potential in cancer treatment.^[69] Interestingly, inactivating UGDH enzymatic activity also resulted in the inhibition of VIRMA binding to SOX2 mRNA. This suggests that the metabolic activity of UGDH plays a crucial role in regulating the binding of VIRMA to SOX2 mRNA and modulating its m⁶A modification. A variety of substrates or metabolites have been reported that can affect the function of non-enzymatic proteins.^[11] So we hypothesized that the substrate or product of UGDH plays a pivotal role in either inhibiting or promoting the binding of VIRMA to SOX2 mRNA. We used recombinant His-VIRMA and biotin-SOX2 to explore whether the binding of them was affected by the substrate or product of UGDH and by pull-down assays found that UDP-Glc inhibited the binding more strongly. After supplementing tumor cells with UDP-Glc, we also found an inhibition of the binding between VIRMA to SOX2 mRNA through RIP-PCR. We also observed a decrease in the m⁶A levels of SOX2 mRNA in the treated tumor cells. These results suggest that upregulation of UGDH that occurs upon chromatin remodeling results in the cellular depletion of UDP-Glc that in turn leads to less suppression of VIRMA binding to SOX2 mRNA and its m⁶A modification. By molecular docking and ITC experiments, as well as site-directed mutagenesis of key residues, we confirmed that UDP-Glc strongly binds to VIRMA, whereas UDP-GlcUA exhibited a weaker binding affinity, likely explaining how a depletion of UDP-Glc, which occurs when UGDH levels are elevated, results in greater VIRMA binding to its target mRNAs. Whether other targets of VIRMA also play a role in cancer cell stemness warrants further exploration. But even so, our findings extend the role of metabolites in cancer progression by identifying UDP-Glc as a direct modulator of epitranscriptomic machinery.

To further verify the inhibitory effect of UDP-Glc on VIRMA binding to SOX2, we knocked down UGP2, which encodes for UTP-glucose-1-phosphate uridylyltransferase, a key enzyme in the uronic pathway and one involved in the biosynthesis of UDP-Glc.^[70] After confirming that intracellular UDP-Glc levels were reduced in UGP2 knockdown cells, we further validated that the decrease of UDP-Glc led to an increased binding of VIRMA to SOX2 mRNA. This was demonstrated through RIP-PCR and RNA pull-down assays. Also we found that a reduction of intracellular UDP-Glc levels in tumor cells enhances their self-renewal capacity. This finding was further confirmed by examining the intracellular UDP-Glc levels in spontaneous tumor models using condition knock out mice harboring the same genetic mutations as those utilized at the outset of this study. We further confirmed that the knockout mice had significant

alterations in the expression levels of SOX2 and other stemness markers in their tumor tissues and showed reduced stemness function in primary cells generated from their tumors.

In addition to elucidating the mechanistic link between ATRX loss and GSC stemness, we identified HY-155534 as a small-molecule inhibitor of UGDH through virtual screening. HY-155534 exhibited potent inhibitory activity against UGDH and demonstrated stable binding in molecular dynamics simulations. Functionally, pharmacological inhibition of UGDH with HY-155534 impaired GSC sphere formation capacity and suppressed tumor growth *in vivo*, phenocopying the effects of UGDH knockdown. Notably, HY-155534 can cross the blood-brain barrier and shows no obvious neurotoxicity, underscoring its potential for clinical translation.

To conclude, this work extends our understanding of nonmetabolic biological processes regulated by intracellular enzymatic metabolic activities in tumor cells. The role of UGDH's metabolic function within this mechanism is further elucidated (Fig. 8). Notably, these findings imply an interaction between cellular metabolic activities and m⁶A methylation, highlighting the functional significance of intracellular metabolic processes in regulating RNA metabolism to maintain and to promote the function of GSCs. The clinical relevance of our findings lies in the potential targeting of UGDH-mediated metabolic pathways as a strategy to overcome or to prevent cancer cell resistance to therapeutic treatments, especially for particularly aggressive or difficult to eradicate cancers, such as gliomas.

Materials and Methods

Data collection

ChIP-seq data of CTCF, H3K27ac and H3K4me3 were obtained from the Gene Expression Omnibus database (GEO, <https://www.ncbi.nlm.nih.gov/geo/>).

Cell lines and cell culture

The human tumor cell lines LN229 were purchased from the Chinese Academy of Sciences Cell Bank. Patient-derived tumor cells HG7 were isolated from discarded glioblastoma (GBM) specimens according to the previous studies.^[71; 72]

In brief, tumor tissues were first washed and mechanically dissociated using MACS C Tubes (Miltenyi Biotec, Germany). The tissue fragments were then enzymatically digested in a solution containing 0.1% trypsin (Invitrogen, USA) and 10 U/mL DNase I (Promega, USA) at 37°C for 45 minutes. Digestion was halted by adding minimum essential medium (MEM-α) supplemented with 10% fetal bovine serum (FBS). Red blood cells were lysed using ACK lysis buffer (Beyotime, China). The resulting cell suspension was filtered through a 100 μm cell strainer to obtain a single-cell suspension. Cells were then cultured in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) supplemented with N2 and B27 supplements (Gibco, USA) and 20 ng/mL human basic fibroblast growth factor (bFGF; Sino Biological,

China). All primary cell lines were authenticated by short tandem repeat (STR) profiling (Genetic Testing Biotechnology, China).

Cell transfection

Lentiviral particles and plasmids products were purchased from GeneChem Company (Shanghai, China). Lipofectamine 2000 (Invitrogen, USA) was used for transfection according to the manufacturer's instructions. After 48 and 72 hours, the virus-containing supernatant was collected, filtered through a 0.45 µm filter, and concentrated using PEG8000 (Sigma, USA) precipitation. Target tumor cells were infected with lentivirus in the presence of 8 µg/mL polybrene (Sigma, USA) for 24 hours. Successfully infected cells were selected using puromycin (2 µg/mL) for 72 hours before further experiments.

RNA extraction and qRT-PCR

The total RNA of each cell line was extracted by using TRIzol reagent (Invitrogen, USA). The cDNAs were synthesized by using the PrimeScript RT reagent Kit (TaKaRa, Japan, cat.RR037A). In the final reaction volume of 20 µL, 2 µL cDNA were used at a concentration of 100 ng/µL. Real-time quantitative PCR was performed on triplicate samples in a reaction mix of SYBR Green (TaKaRa, Japan). Conventional PCR and qRT-PCR were conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA); The qRT-PCR data were normalized to GAPDH and analyzed by using the $2^{-\Delta\Delta C_t}$ method. The primers used in qRT-PCR were listed in (Supplementary Table 1).

Total Protein Extraction and Western Blotting

Total protein was extracted using pre-chilled RIPA buffer (Solarbio, China) supplemented with 1% protease and phosphatase inhibitor cocktails (Selleck, China). Protein concentrations were determined using the BCA Protein Quantification Kit (Sigma, USA), and the protein levels were measured using a NanoDrop 2000C spectrophotometer (NanoDrop Products) within a range of 0.5–30 µg/mL. For Western blotting, 20 µL of each protein sample was subjected to 7.5%, 10%, or 12.5% SDS-PAGE (EpiZyme Scientific). The PVDF membranes were blocked with a 5% milk-TBST solution and incubated overnight at 4°C with primary antibodies (Supplementary Table 2). After washing, membranes were incubated with HRP-conjugated secondary antibodies: mouse IgG (ZSGB-BIO, China) or rabbit IgG (ZSGB-BIO, China). Protein bands were visualized using the chemiluminescence (ECL) kit (Boster, China) and quantified with ImageLab software.

CCK8 assays

Cell viability was assessed using the Cell Counting Kit-8 (CCK-8; Dojindo, Japan) following the manufacturer's protocol. Briefly, tumor cells were seeded into 96-well plates at a density of 3×10^3 cells per well in 100 µL of complete medium and cultured under standard conditions. After the desired treatment period, 10 µL of CCK-8 reagent was added to each well and incubated at 37°C for 1 hour. The absorbance was then measured at 450 nm using a microplate reader (BioTek, USA).

Luciferase reporter assay

Tumor cells were seeded into 24-well plates and transfected with the pGL2 promoter luciferase reporter construct (Promega, USA) using Lipofectamine 3000 (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. To normalize transfection efficiency, cells were co-transfected with a Renilla luciferase control plasmid (Promega, USA). After 24–48 hours, luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA) following the manufacturer's protocol. Firefly luciferase activity was normalized to Renilla luciferase activity.

Sphere formation assays

Tumor cells were enzymatically dissociated and cultured in serum-free medium (SFM), composed of a 1:1 mixture of DMEM and F12 supplemented with 20 ng/mL epidermal growth factor (EGF) (Sino Biological, China), and 0.5× B27 and N2 supplements (Gibco, USA). Cells were plated in ultralow-attachment 6-well plates (Corning, USA) to promote tumor sphere formation. Fresh SFM was replenished every 3 days, and cultures were maintained for 14 days to allow for sphere growth.^[73; 74]

Xenograft model in vivo

All animal experiments in this study were performed in accordance with the International Guiding Principles for Animal Research and were approved by the Laboratory Animal Centre of Harbin Medical University. All the animals were housed under identical controlled specific pathogen-free (SPF) standard environmental conditions ($23 \pm 2^\circ\text{C}$, 12 h light/dark cycle) with free access to food and allowed to move freely. Four-week old female athymic BALB/c nude mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). To established PDX-GBM (LN229) models, LN229 cells were stereotactically injected into the brain at a dose of 5×10^5 cells (18 μL) per mouse (4-week-old). The intracranial tumors were measured at the 7th, 14th and 21st days after tumor implantation with bioluminescence imaging via D-Luciferin, Potassium Salt (Yeason, China) intraperitoneal injection with concentration 150 mg kg⁻¹ using an IVIS Lumina Imaging System (Xenogen, USA).^[75]

Transgenic mouse

All animal experiments in this study were performed in accordance with the International Guiding Principles for Animal Research and were approved by the Laboratory Animal Centre of Harbin Medical University. All animals were maintained under standardized specific pathogen-free (SPF) conditions, with unrestricted access to food and water, and housed in environments that allowed free movement. Transgenic mice used to induce primary tumors were purchased from Cyagen (USA). In brief, Nes-CreERT mice were established by and purchased from Cyagen (USA). Nf1^{fl/+} and Tp53^{fl/+} mice were created by Cyagen (USA) through ES genome engineering. Nf1^{fl/+} mice were mated with Tp53^{fl/+} mice to generate Nf1^{fl/fl}; Tp53^{fl/fl} mice. Nf1^{fl/fl}; Tp53^{fl/fl} mice were mated with Nestin-CreERT mice and finally generated Nestin-CreERT; Nf1^{fl/fl}; Tp53^{fl/fl} mice. Then Atrx^{fl/+} mice were mated with Atrx^{+/-} to generate Atrx^{fl/-} and Atrx^{fl/fl} mice. Then Atrx^{fl/fl} mice were mated with Nestin-CreERT mice to generate Nestin-CreERT; Atrx^{fl/fl} or Atrx^{fl/-} mice. Nestin-CreERT; Atrx^{fl/fl} were mated with Nf1^{fl/fl}; Tp53^{fl/fl} mice to generate

Nestin-CreERT; Atrx^{fl/fl} or Atrx^{fl/-}; Nf1^{fl/fl}; Tp53^{fl/fl} mice. Mice (Nestin-CreERT; Atrx^{fl/-} or Atrx^{fl/fl}/Atrx^{+/-} or Atrx^{+/-}; Nf1^{fl/fl}; Tp53^{fl/fl}) were intraperitoneally injected with tamoxifen (1 mg/d, 5d) (Rhawn, China) at 4 weeks of age to obtain mice with conditional knockout triggering primary tumors.^[76–78]

Hematoxylin-eosin (H&E) staining and immunohistochemistry (IHC)

Tumor tissues were fixed in 4% paraformaldehyde for 24 to 48 hours, followed by dehydration and embedding in paraffin according to standard protocols. For hematoxylin and eosin (H&E) staining, the slides were deparaffinized and rehydrated, then stained with hematoxylin solution (ZSGB-BIO, China) for 8 minutes and eosin-phloxine solution (ZSGB-BIO, China) for 30 seconds to 1 minute, before being mounted with xylene. For immunohistochemistry (IHC), 4 μ m paraffin sections were processed using a DAB staining kit (ZSGB-BIO, China). Briefly, formalin-fixed, paraffin-embedded tissue sections were incubated at 80°C for 15 minutes, dewaxed in xylene, and rehydrated in graded ethanol, followed by washing with double-distilled water. Antigen retrieval was performed by steaming the slides in sodium citrate buffer at 95°C for 15 minutes. The sections were then incubated overnight at 4°C with the relevant primary antibodies. After incubation, the sections were treated with HRP-labeled anti-mouse or anti-rabbit IgG (ZSGB-BIO, China) secondary antibodies for 30 minutes. Finally, the slides were stained with DAB chromogen solution for 2 minutes, rinsed with PBS, and counterstained with hematoxylin.

Quantitative evaluation was performed by examining each section using at least 10 different high-power fields with the most abundant stained cells.^[79; 80] Two independent neuropathologists reviewed and scored each stained slide in a double-blinded fashion. The proportion of stained cells counts per field was used for statistical analysis. Staining was scored using a 4-point scale from “–” to “+++” or “+” to “++++”, with “–” if there was no staining or very little staining, “+” if less than 10% of cells stained positively, “++” if 10–30% of cells stained positively, “+++” if 30–50% of cells stained positively, and “++++” more than 50% of cells stained positively.^[71; 81] The representative imaged field were determined by the average method.

Transwell migration assays

LN229 and HG7 (4×10^4) cells were replated onto the upper chamber of a Transwell filter with 8 μ m pores (Jet Biofil, China) with serum free medium. After 12h culture in a humidified atmosphere with 5% CO₂ and 95% air with 37 °C, cells were fixed with 4% paraformaldehyde in PBS. Non-migrated cells on the upper side of the filter were removed with a cotton swab, and cells on the underside of the filter were stained with 0.4% crystal violet in 10% ethanol.

UGDH activity assays

UGDH enzymatic activity was measured as previously described,^[16; 47] based on the reduction of NAD⁺ to NADH. Briefly, the assay was performed in 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM UDP-Glc and 10 mM NAD⁺. The reaction was initiated by adding the enzyme-containing sample and allowed to

proceed at room temperature for 1 minute. The enzymatic activity was quantified by monitoring the increase in absorbance at 340 nm using a spectrophotometer (Thermo Fisher Scientific, USA), which reflects the generation of NADH.

Quantification of UDP-Glc and UDP-GlcUA

The intracellular levels of UDP-Glc and UDP-GlcUA were quantified by mass spectrometry. 1×10^7 cells were harvested and centrifuged at $1\,000 \times g$ for 5 min. Cell pellets were washed once with ice-cold PBS, followed by the addition of 200 μ L of 0.5 M ice-cold perchloric acid and vigorous mixing. After incubation on ice for 2 min, samples were centrifuged at $10\,000 \times g$ for 5 min at 4°C to remove cellular debris. The resulting supernatants were added with 50 μ L of 2.5 M KOH in 1.5 M K_2HPO_4 and incubated on ice for 2 min. Precipitated potassium perchlorate was removed by a second centrifugation step, and clarified extracts were filtered through 0.2- μ m filters. Metabolite separation was achieved using an HPLC system. The UDP-Glc and UDP-GlcUA standards (MCE, China) were used to generate calibration curves for quantification. Mass spectrometric detection was performed in negative ion mode, monitoring m/z 566.30 for UDP-Glc and m/z 610.27 for UDP-GlcUA. Peak areas from experimental samples were quantified relative to standard curves to determine intracellular concentrations.

3C-qPCR

3C-qPCR assays were performed in LN229 ATRX^{EV} and ATRX^{KO} cells according to protocol.^[35; 82; 83] Briefly, ten million cells were trypsinized and resuspended in a 10% FBS/PBS buffer. The cells were fixed with 1% formaldehyde in 10 ml of 10% FBS/PBS buffer for 10 minutes at room temperature. The reaction was then quenched with ice-cold glycine. After centrifugation, the pellets were washed with cold PBS and re-suspended in lysis buffer (10 mM NaCl; 10 mM Tris-HCl, pH 7.5; 0.2% NP-40; 1x protease inhibitor). Nuclear extracts were obtained after centrifugation, and genomic DNA was digested using HindIII (Abclonal, China). Digestion efficiency was evaluated by SYBR-qPCR, and only fully digested chromatin DNA was ligated using T4 DNA ligase (Beyotime, China). After reverse crosslinking, the DNA fragments were purified by ethanol precipitation. The concentration of ligated DNA samples was measured by SYBR-qPCR, and samples were diluted to 100 ng/ μ L for qPCR analysis. The standard curve for each primer was generated by serial dilution of the control template, and results were normalized to ERCC3 as a control. All primers used in this experiment are listed in (Supplementary Table 1).

Hi-C construction and data analysis

In situ Hi-C experiments for the two cell lines were performed following the protocol (Annoroad, China).^[84] Raw Hi-C files were processed using HiCExplorer^[85; 86] (<https://hicexplorer.readthedocs.io/en/latest/>) for filtering, mapping, and generating raw contact count matrices. These matrices were then normalized using the iterative correction method.

mRNA decay assays

The mRNA decay assay was performed refer to protocol.^[87] In brief, tumor cells were pretreated with actinomycin D (Abcam, USA). Total RNA was extracted by above method mentioned. The cDNAs were synthesized by using The PrimeScript RT reagent Kit (TaKaRa, Japan). The qRT-PCR were conducted using the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The primer pairs used are listed in (Supplementary Table 1).

RNA immunoprecipitation (RIP)

Tumor cells were lysed and immunoprecipitated using an anti-VIRMA antibody (Proteintech, China), with normal rabbit IgG (ZSGB-BIO, China) used as a negative control. The mRNA bound to the anti-VIRMA antibody or normal IgG was reverse transcribed into cDNA and analyzed by qRT-PCR. The levels of precipitated mRNAs were normalized to the input RNA to account for potential variations in RNA preparation. The specific primer sequences used for PCR are listed in (Supplementary Table 1).

Chromatin Immunoprecipitation (ChIP)

A Millipore EZ-Magna ChIP kit (Sigma-Aldrich, USA) was used to perform all ChIP experiments. Briefly, 2×10^5 cells were crosslinked with 1% formaldehyde for 10 min at room temperature. Next, 10 mM glycine quenched cross-linking. Chromatin was sonicated to 200-1 000 bp in lysis buffer and ChIP DNA was extracted following the manufacturer's protocol. The specific primer sequences used for PCR are listed in (Supplementary Table 1).

Biotin pull-down assays

Pull-down assays were performed by incubating biotinylated SOX2 (Roche, Switzerland) (0.4 μ g) with cell lysates, or with purified recombinant His-tagged VIRMA (5 μ g), for 4 hours. The resulting complexes were then captured using Pierce streptavidin agarose beads, and immunoblotting was carried out using the specified antibodies.

Molecular dynamics simulation

The binding pocket was defined based on the crystal structure of VIRMA with UDP-Glc and UDP-GlcUA using Autodock 4.2.^[50] The structure of VIRMA (AF-Q69YN4-F1-v4) was downloaded from the the AlphaFold Database (<https://www.alphafold.ebi.ac.uk/>). The structures of UDP-Glc and UDP-GlcUA were downloaded from the the PubChem Database (<https://pubchem.ncbi.nlm.nih.gov/>). Molecular docking calculations were further conducted using the Dock6 protocol in Yinfo Cloud Computing Platform (<https://cloud.yinfotek.com/>). DOCK 6.9^[51] program was utilized to execute semi-flexible docking and output poses were evaluated by the Grid scoring function.

Isothermal titration calorimetry assay

Thermodynamic parameters for the binding of VIRMA or its variants to UDP-GlcUA or UDP-Glc were measured using isothermal titration calorimetry (ITC) (Bio-Lab Company, China). In brief, the binding

affinity and thermodynamic parameters between ligands (UDP-Glc or UDP-GlcUA) and protein samples (VIRMA or its variants) were measured using a MicroCal ITC200 system (Malvern Panalytical, USA) at 25°C. All proteins and ligands were dialyzed against the same buffer (20 mM HEPES, 150 mM NaCl, pH 7.4) to minimize buffer mismatch heat effects. In each experiment, 250 μ L of protein solution (0.1 mM) was loaded into the sample cell, and 1 mM ligand solution was loaded into the injection syringe. A total of 20 injections (2 μ L each) were performed at 180-second intervals, with the first injection (0.5 μ L) discarded to eliminate diffusion-related artifacts.

The raw data were integrated and analyzed using the Origin 7.0 software (MicroCal), and the binding isotherms were fitted using a single-site binding model to calculate the binding constant (K_d), enthalpy change (ΔH), entropy change (ΔS), and Gibbs free energy (ΔG).

Statistical analysis

The differences between variables in groups were assessed using Student's t-test and chi-squared test. Survival analyses were performed using Kaplan-Meier curves, the Log-rank test. Data analysis and visualization were conducted with R version 4.1.0, while statistical analyses were performed using GraphPad Prism version 9.0 or IBM SPSS Statistics 23.0.

Animal Studies Statement

All animal experiments were performed in accordance with institutional guidelines for the care and use of laboratory animals and were approved by the Animal Ethics Committee of Laboratory Animal Centre of Harbin Medical University.

Human Subjects Statement

All human tissue samples were collected with informed consent from patients or their legal guardians after the nature and potential consequences of the study had been fully explained. The study protocol was reviewed and approved by the Clinical Research Ethics Committee of Harbin Medical University.

Declarations

Author Contributions

J.Cai. and C.Jiang. designed the experiments. K.Tian. and X.Meng. performed the experiments. K.Tian., P.Sun., J.Zhong., N.Sun., X.Hou and Y.Zhao. analyzed the data. K.Tian. wrote the manuscript. W.Ma., Y.Song., D.Yu., J.Hu., H.Xuan., F.Xu., T.Wang., R.Tian., Z.Chen., J.Dong. and Y.Wang. contributed materials and analysis tools.

Acknowledgments

This study was supported by 1. National Natural Science Foundation of China (82372901, 82230086, 82272988, 82472925); 2. Youth Science and Technology Talent Team of Heilongjiang Province (CYQN24012); 3. Heilongjiang Provincial Key R & D Project (2022ZX06C18); 4. Natural Science Foundation of Heilongjiang Province (LH2022H022); 5. China Postdoctoral Science Foundation (2019M660074, 2022T150173); 6. Heilongjiang Postdoctoral Science Foundation (LBH-Z19029, LBH-TZ2218); 7. Medjaden Academy & Research Foundation for Young Scientists (MJR202310029); 8. Health China · BuChang ZhiYuan Public Welfare Project for Heart and Brain Health (HIGHER2023042).

Data Availability Statement

All relevant data supporting the findings of this study were available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

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Figures

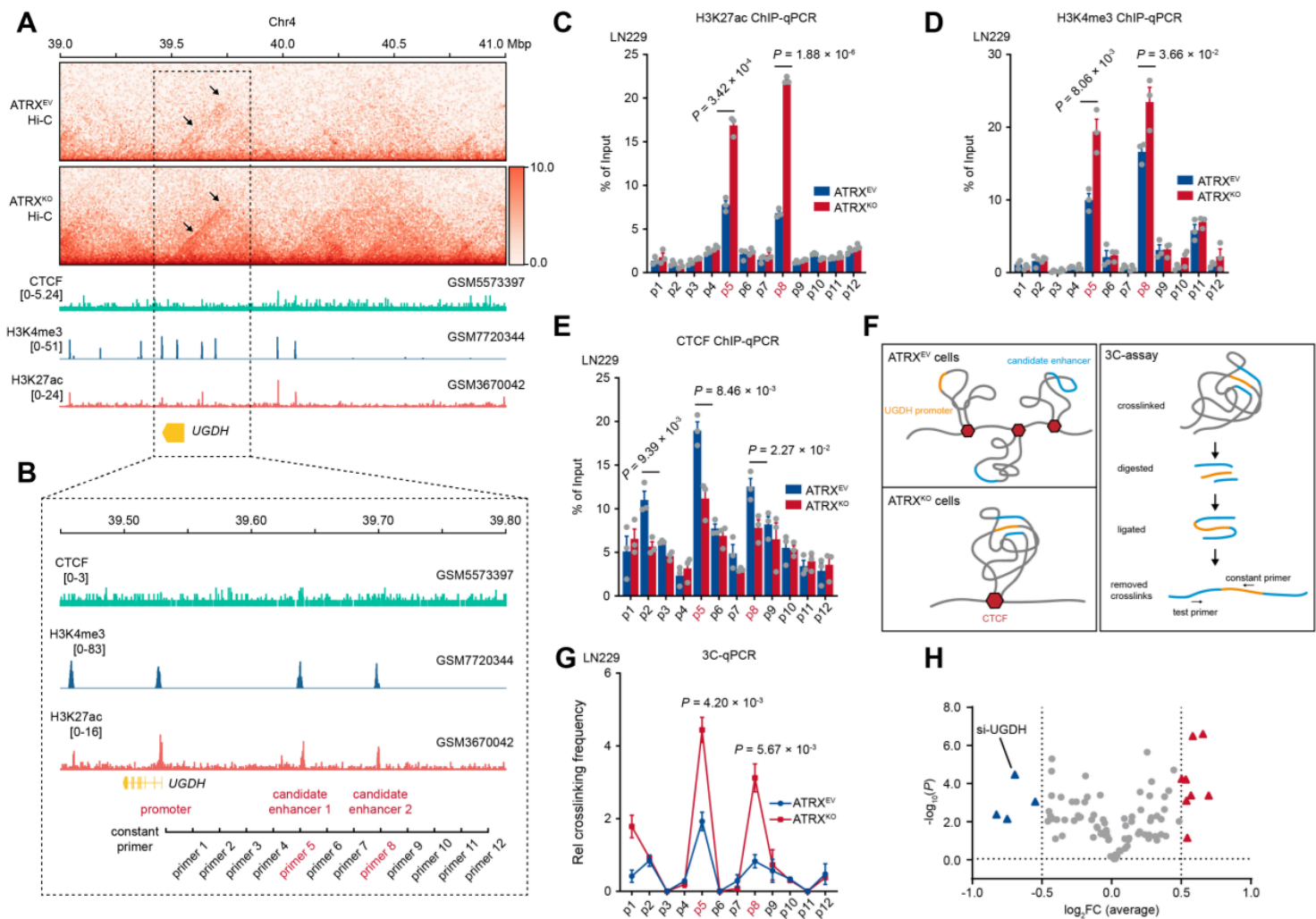


Figure 1

Chromatin remodeling facilitates transcriptional activation of the UGDH promoter.

A. Hi-C maps and histone ChIP-seq tracks surrounding the upstream region of the UGDH locus (chr4: 39 000 000-41 000 000).

B. Upper: Histone ChIP-seq tracks (CTCF; H3K4me3; H3K27ac) surrounding the UGDH locus (chr4: 39 450 000-39 800 000); Bottom: Schematic diagram shows the design of primers for 3C experiments.

C. ChIP-qPCR data of LN229 cells showing the enrichment of H3K27ac in the upstream promoter of UGDH ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

D. ChIP-qPCR data show that different enrichment of H3K4me3 in the upstream region of the UGDH promoter ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

E. ChIP-qPCR data show that different occupancy of CTCF in the upstream region of the UGDH promoter ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

F. Schematic diagram of the 3C experiment.

G. 3C-qPCR of the interaction between the upstream region of UGDH and its promoter ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

H. ATRX^{KO} LN229 cells were independently transfected with siRNAs targeting of the 90 metabolic rate-limiting enzymes, followed by sphere-formation assays. Fold changes (FC) were normalized to si-Control, with means (average) calculated from three independent experiments. P value was determined by Student's t -test.

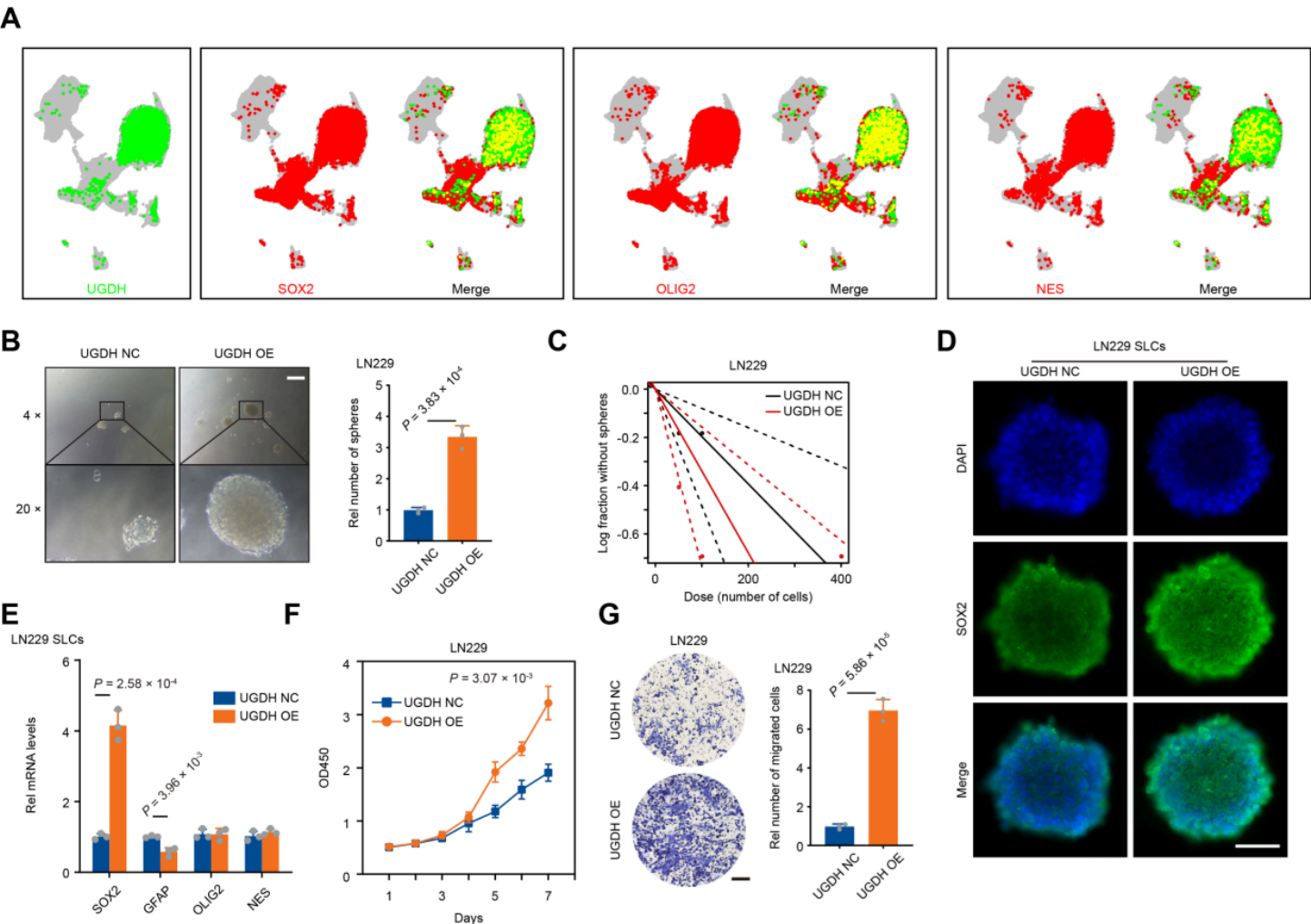


Figure 2

High expression of UGDH enhances the malignant behavior of tumor cells.

- A.** The stemness markers (SOX2, OLIG2, NES) and UGDH expression of cells in scRNA-seq data. Data analysis employed the UMAP dimensionality reduction method.
- B.** Representative images of sphere formation of LN229 cells that have either normal (NC) or overexpression (OE) of UGDH (left) and their quantitation (right). Tumor spheres with diameters > 50 μm were counted ($n = 3$). Scale bar, 200 μm . Data are presented as the mean $\pm$ S.D. *P* value was determined by Student's *t* - test.
- C.** Limited dilution assays (LDA) were used to test self-renewal ability of LN229 cells after UGDH overexpressing. Data are representative of three independent experiments.
- D.** Representative images of IF assays show SOX2 expression in LN229 cells with UGDH overexpression or not. Scale bar, 100 μm . Images are representative of three independent experiments.
- E.** qRT-PCR analysis of mRNA levels of SOX2, GFAP, OLIG2, NES in stem-like cells (SLCs) of LN229 with UGDH overexpressing or not ($n = 3$). Data are presented as the mean $\pm$ S.D. *P* value was determined by Student's *t* - test.
- F.** CCK-8 assay of LN229 cell growth following UGDH overexpression. ($n = 3$). cells were transfected with UGDH overexpression vector or empty vector control. Cell viability was measured at indicated time points using the CCK-8 reagent. Data are presented as the mean $\pm$ S.D. *P* value was determined by Student's *t* - test.
- G.** Left: Representative images of transwell assays performed by using LN229 cell, with transfected with UGDH overexpression vector or empty vector control. Right: The statistical analyses of numbers of migrated cells ($n = 3$). Scale bar, 100 μm . Images are representative of three independent experiments. Data are presented as the mean $\pm$ S.D. *P* value was determined by Student's *t* - test.

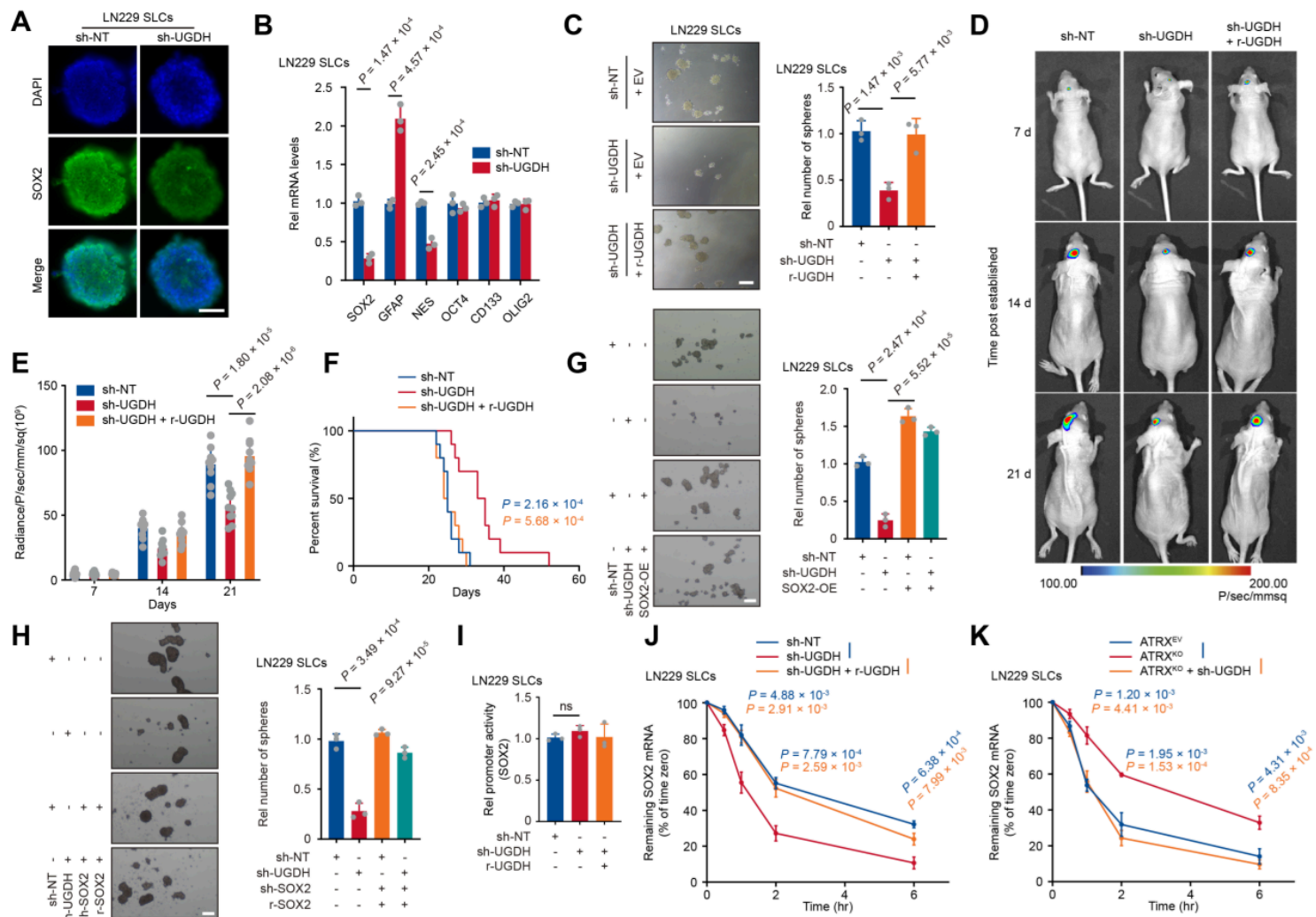


Figure 3

UGDH promotes the maintenance of tumor cell stemness by stabilizing SOX2 mRNA.

A. Representative images of IF assays show SOX2 expression in LN229 SLCs with UGDH knockdown or not. Scale bar, 100 μ m.

B. qRT-PCR analysis of the mRNA levels of SOX2, NES, OCT4, CD133, OLIG2 and GFAP after UGDH knockdown ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

C. Left: Sphere formation assays were performed in UGDH knockdown LN229 SLCs rescued with empty vector (EV) or r-UGDH; Scale bar, 200 μ m. Images are representative of three independent experiments. Right: Relative number of sphere formation are shown in histogram. Tumor spheres with diameters > 50 μ m were counted ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

D. Representative bioluminescence images of tumor-bearing mice at the indicated time points. Mice were implanted with tumor cells expressing sh-NT, sh-UGDH or sh-UGDH together with UGDH rescue construct. Bioluminescence signals reflect tumor burden in each group ($n = 10$).

- E.** Quantification of bioluminescent imaging signal intensities ($n = 10$). P value was determined by Student's t - test.
- F.** Kaplan-Meier survival curves were generated for mice bearing tumors ($n = 10$). P value was determined by Log-rank test.
- G.** Left: Sphere formation assays were performed in UGDH knockdown LN229 SLCs with or without SOX2 over-expression; Scale bar, 200 μm . Images are representative of three independent experiments. Right: Relative number of sphere formation are shown in histogram. Tumor spheres with diameters $> 50 \mu\text{m}$ were counted ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.
- H.** Left: Sphere formation assays were performed in control and UGDH knockdown LN229 SLCs with SOX2 rescue or not; Scale bar, 200 μm . Images are representative of three independent experiments. Right: Relative number of sphere formation are shown in histogram. Tumor spheres with diameters $> 50 \mu\text{m}$ were counted ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.
- I.** LN229 SLCs were transfected sh-NT and sh-UGDH with or without rescued by r-UGDH, and luciferase activity of SOX2 promoter reporter was measured ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t -test. ns (no significance).
- J.** UGDH knockdown LN229 SLCs rescued with empty vector (EV) or r-UGDH were treated with actinomycin D (1 $\mu\text{g/mL}$). The remaining SOX2 mRNA were examined by qRT-PCR at indicated time ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.
- K.** ATRX^{EV} and ATRX^{KO} LN229 SLCs with or without UGDH knockdown were treated with actinomycin D (1 $\mu\text{g/mL}$). The remaining SOX2 mRNA were examined by qRT-PCR at indicated time ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

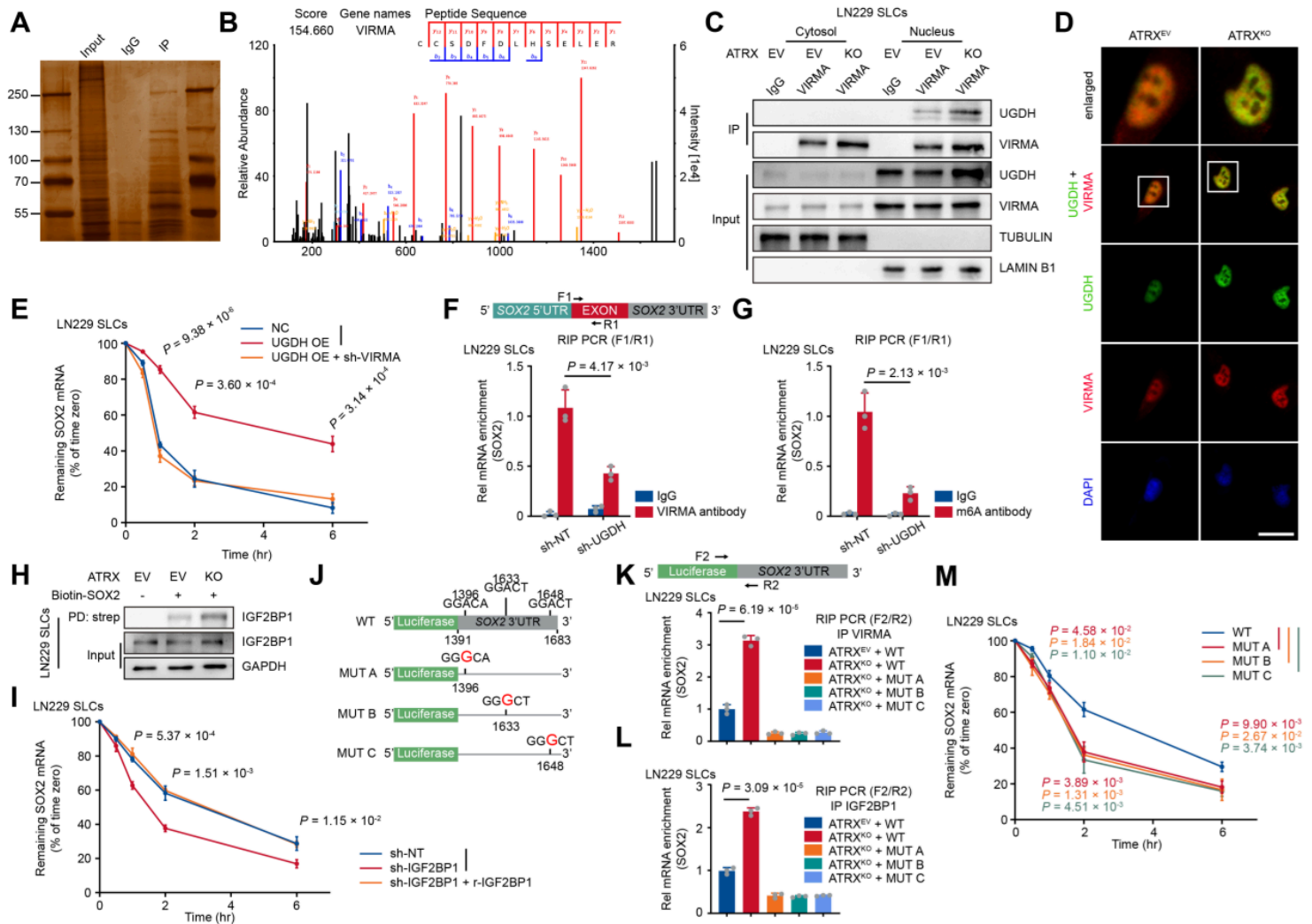


Figure 4

The interaction between UGDH and VIRMA facilitates m⁶A modification of SOX2 mRNA.

A. Silver staining assay were performed by using Co-IP samples (UGDH, IgG antibody).

B. High-resolution MS/MS spectra of cross-linked peptide pairs between UGDH and VIRMA.

C. Cytoplasmic and nuclear proteins from ATRX^{EV} and ATRX^{KO} cells were extracted separately for IP experiments. Western Blot analyses are shown.

D. Immunofluorescence (IF) were performed with anti-UGDH and anti-VIRMA antibodies in adherent ATRX^{EV} and ATRX^{KO} LN229 SLCs. Scale bar, 10 μ m.

E. LN229 SLCs transfected with UGDH OE with or without sh-VIRMA were followed by treatment with actinomycin D (1 μ g/mL). The remaining SOX2 mRNA were examined by qRT-PCR at indicated time ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

F. Top: Schematic illustration of the primer pair F1/R1 design. Bottom: RNA immunoprecipitation (RIP) assays were performed in LN229 SLCs transfected with sh-NT or sh-UGDH. The relative precipitated SOX2 mRNA levels were examined by qRT-PCR ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

G. RNA immunoprecipitation (RIP) assays were performed in LN229 SLCs transfected with sh-NT or sh-UGDH. The relative precipitated SOX2 mRNA levels were examined by qRT-PCR ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

H. Pull-down assays were performed by incubating the cell lysates of ATRX^{EV} and ATRX^{KO} SLCs with or without biotin-SOX2.

I. IGF2BP1 knockdown LN229 SLCs rescued with empty vector (EV) or r-IGF2BP1 followed by treatment with actinomycin D (1 μ g/mL). The remaining SOX2 mRNA were examined by qRT-PCR at indicated time ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

J. Schematic representation of reporters with point mutations (A to G, shown in red) at three m⁶A sites within the SOX2-3' UTR.

K. Top: Schematic illustration of the primer pair F2/R2 design. Bottom: RNA immunoprecipitation (RIP) assays were performed in ATRX^{EV} and ATRX^{KO} LN229 SLCs transfected with wild-type or mutant SOX2-3' UTR. The relative precipitated SOX2 mRNA levels were examined by qRT-PCR ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

L. RNA immunoprecipitation (RIP) assays were performed in ATRX^{EV} and ATRX^{KO} LN229 SLCs transfected with wild-type or mutant SOX2-3' UTR. The relative precipitated SOX2 mRNA levels were examined by qRT-PCR ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

M. LN229 SLCs were transfected with wild-type or mutant SOX2-3' UTR, followed by treatment with actinomycin D (1 μ g/mL). The remaining SOX2 mRNA were examined by qRT-PCR at indicated time ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

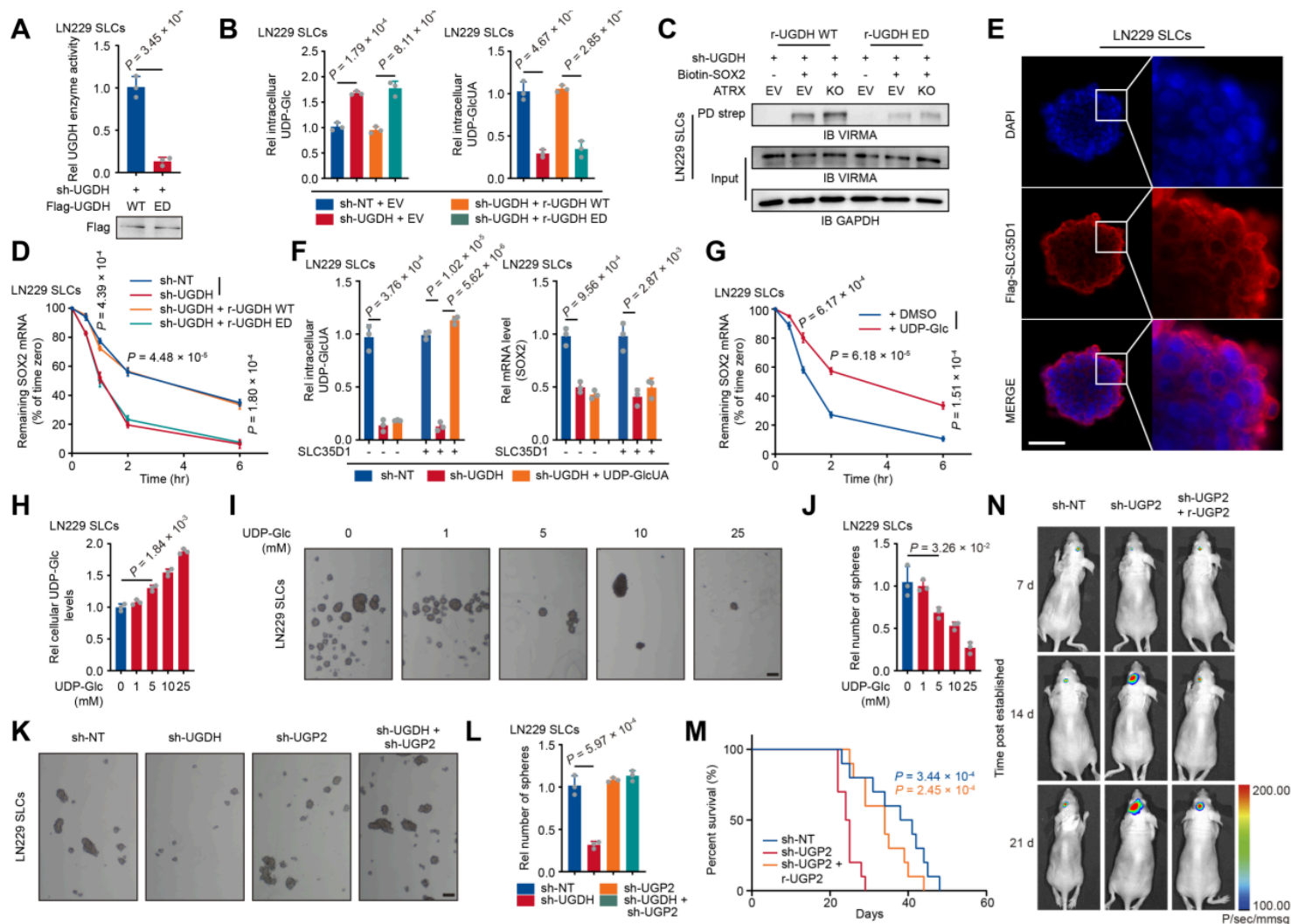


Figure 5

UGDH promotes SOX2 mRNA stability and tumor stemness by depleting UDP-Glc.

A. LN229 SLCs were transfected with sh-UGDH and rescued by Flag-UGDH (WT) or Flag-UGDH (ED), then the enzymatic activity was measured ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

B. The UDP-Glc and UDP-GlcUA intracellular concentrations of LN229 SLCs transfected with sh-NT or sh-UGDH rescued with r-UGDH (WT) or r-UGDH (ED) were measured ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

C. ATRX^{EV}/ATRX^{KO} LN229 SLCs with UGDH knockdown were rescued with r-UGDH (WT) or r-UGDH (ED). Pull-down assays were conducted by incubating the cell lysates with or without biotin-SOX2 for 4 hours.

D. LN229 SLCs were transfected with sh-UGDH and rescued by r-UGDH (WT) or r-UGDH (ED), followed by treatment with actinomycin D (1 μ g/mL). The remaining SOX2 mRNA were examined by qRT-PCR at

indicated time ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

E. Representative images of immunofluorescence (IF) assays were performed by anti-Flag to confirm Flag-SLC35D1 expression in LN229 SLCs. Scale bar, 50 μ m.

F. SLC35D1-overexpressing cells, with or without UGDH knockdown, were supplemented with UDP-GlcUA (5 mM). Then the intracellular UDP-GlcUA levels were measured ($n = 3$). Then the SOX2 mRNA levels were measured ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

G. LN229 SLCs transfected with sh-NT or sh-UGDH were supplemented with or without UDP-Glc (5 mM), followed by treatment with actinomycin D (1 μ g/mL). The remaining SOX2 mRNA were examined by qRT-PCR at indicated time ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

H. LN229 SLCs were supplemented with different dose of UDP-Glc. The concentrations of cellular UDP-Glc were determined ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

I. Sphere formation assays were performed in LN229 SLCs with different dose of UDP-Glc. Scale bar, 200 μ m. Images are representative of three independent experiments.

J. Relative number of sphere formation are shown in histogram. Tumor spheres with diameters > 50 μ m were counted ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

K. Sphere formation assays were performed in LN229 SLCs with UGDH/UGP2 knock down or both. Scale bar, 200 μ m. Images are representative of three independent experiments.

L. Relative number of sphere formation are shown in histogram. Tumor spheres with diameters > 50 μ m were counted ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

M. Kaplan-Meier survival curves were generated for mice bearing tumors ($n = 10$ mice). The mice were implanted with genetically modified LN229 cells transfected with sh-NT and sh-UGP2 with or without rescued by r-UGP2. P value was determined by Log-rank test.

N. Representative bioluminescence images of tumor-bearing mice at the indicated time points. Mice were implanted with tumor cells expressing sh-NT, sh-UGP2 or r-UGP2. Bioluminescence signals reflect tumor burden in each group ($n = 10$).

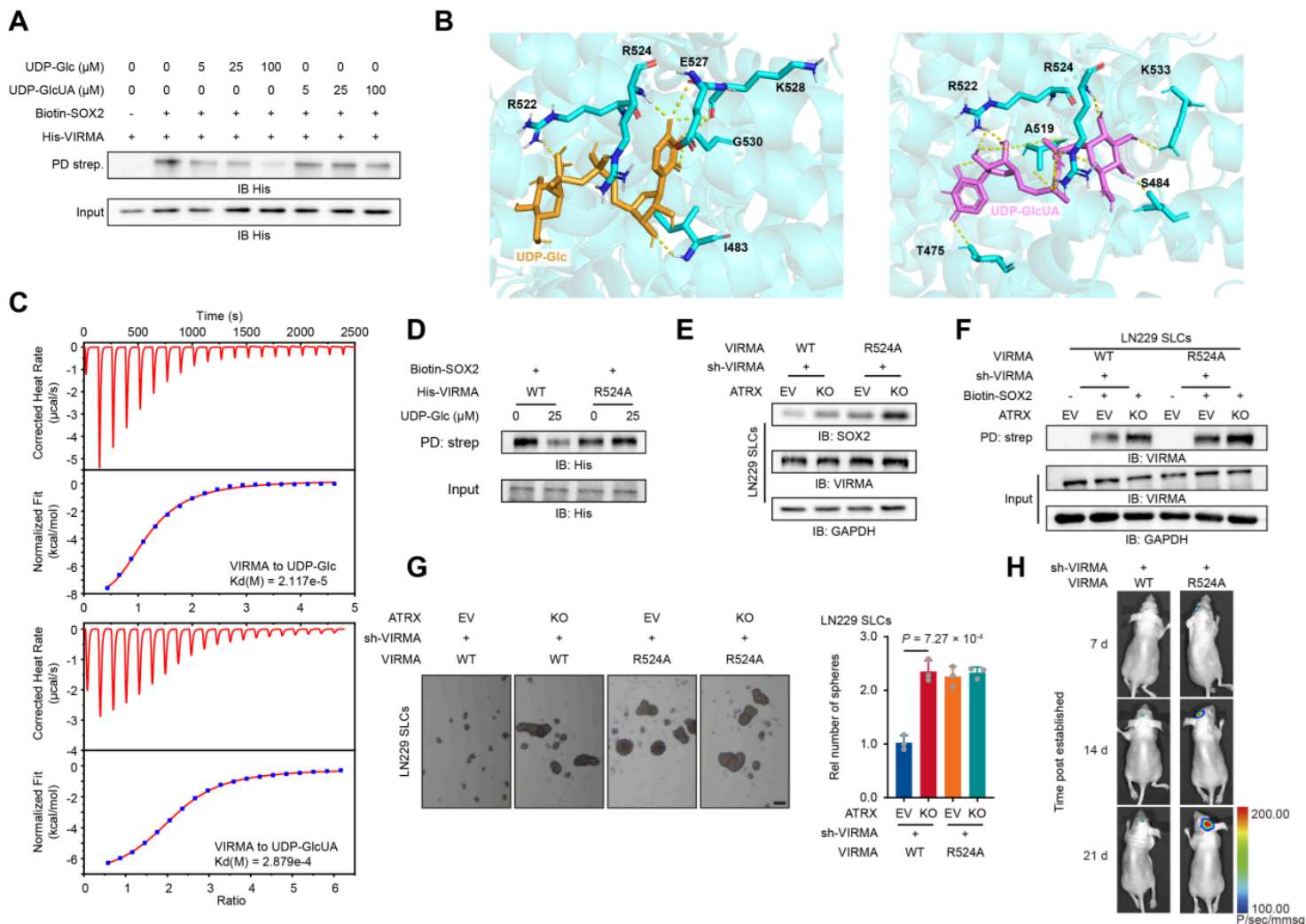


Figure 6

UDP-Glc binds to VIRMA and disrupts its association with mRNA.

A. Purified recombinant His-VIRMA (10 μg) was incubated with biotin-SOX2 and varying concentrations of UDP-Glc or UDP-GlcUA, followed by pull-down assays.

B. The binding modes of UDP-Glc (top) and UDP-GlcUA (bottom) with VIRMA are shown. The protein shown as a cyan-colored cartoon. The two ligands, UDP-Glc and UDP-GlcUA, are presented in stick representation, and the dominant residues involved in the interaction are also highlighted in stick form. This visualization emphasizes the structural details of how these ligands bind to VIRMA and the key residues that mediate their interaction.

C. Isothermal titration calorimetry assays were performed with purified recombinant His-VIRMA (0.1 mM) and UDP-Glc (1 mM) or UDP-GlcUA (1 mM). K_d , dissociation constant.

D. The recombinant His-VIRMA-WT, His-VIRMA-R524A (5 μg) was incubated with or without UDP-Glc. Then incubated with biotin-SOX2 and followed by pull-down assays.

E. ATRX^{EV} and ATRX^{KO} LN229 SLCs with VIRMA knockdown were rescued by VIRMA-WT or VIRMA-R524A. The western blot analysis confirmed the expression of VIRMA and SOX2.

F. Pull-down assays were performed by incubating the cell lysates with or without biotin-SOX2.

G. Left: LN229 ATRX^{EV} or ATRX^{KO} SLCs rescued by wild-type or mutant VIRMA, sphere formation assays were performed. Scale bar, 200 μ m. Images are representative of three independent experiments. Right: Relative number of sphere formation are shown in histogram. Tumor spheres with diameters > 50 μ m were counted ($n = 3$). Data are presented as the mean $\pm$ S.D. P value was determined by Student's t - test.

H. Representative bioluminescence images of mice derived from LN229 cells transfected with sh-VIRMA and rescued by VIRMA-WT or VIRMA-R524A ($n = 10$).

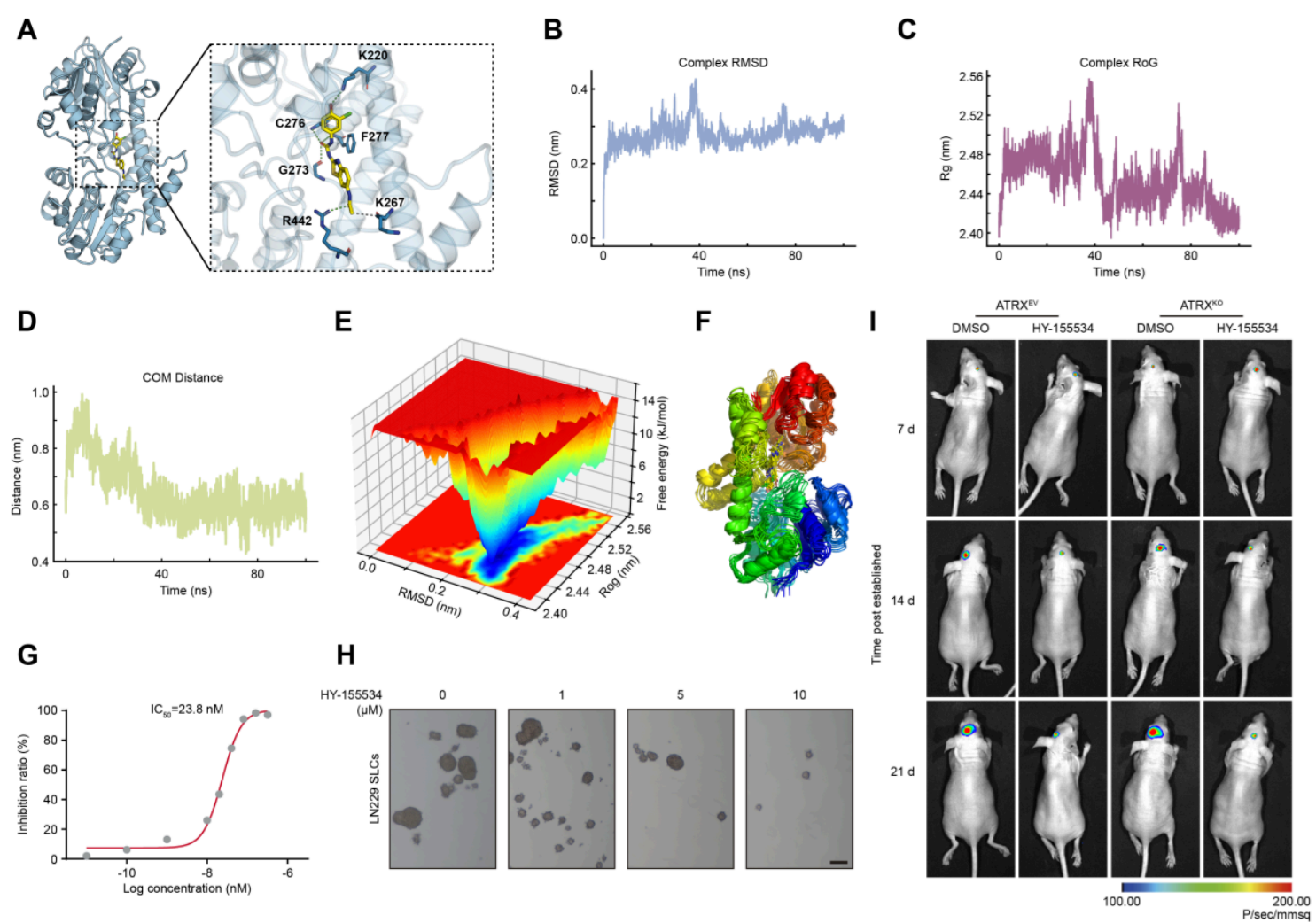


Figure 7

Treatment with the UGDH inhibitor HY-155534 suppresses tumor growth *in vivo* and impairs the self-renewal capacity.

- A.** The binding modes of HY-155534 with UGDH are shown. The protein shown as a cyan-colored cartoon. HY-155534 are presented in stick representation, and the dominant residues involved in the interaction are also highlighted in stick form.
- B.** Time-dependent RMSD variations of the protein, ligand, and complex over the 100 ns molecular dynamics (MD) simulation. RMSD was computed with respect to the starting conformation and monitored throughout the trajectory.
- C.** Time-dependent variation of the radius of gyration (Rog) of the complex over the 100 ns MD simulation. Rog values were calculated to monitor the overall compactness of the complex throughout the trajectory.
- D.** Distance between the center of mass (COM) of the ligand and that of the protein over the 100 ns MD simulation. The COM distance was monitored to evaluate the positional stability and binding tightness of the ligand within the binding pocket.
- E.** Three-dimensional free energy landscape (FEL) of the complex as a function of RMSD and Rog. FEL was constructed based on the RMSD and Rog values extracted from the 100 ns MD simulation. The low-energy basin (blue) represents the most thermodynamically favorable conformational ensemble.
- F.** Superimposed conformations of the complex at 10 ns intervals over the 100 ns MD simulation.
- G.** IC₅₀ determination of HY-155534 inhibit UGDH enzymatic activity. UGDH activity was measured in the presence of increasing concentrations of HY-155534 ($n = 3$). Data are presented as the mean $\pm$ S.D.
- H.** LN229 SLCs were treated with different dose of HY-155534 and performed sphere formation assays. Scale bar, 200 μ m. Images are representative of three independent experiments.
- I.** Representative bioluminescence images of tumor-bearing mice at the indicated time points. Mice were implanted with ATRX^{EV} or ATRX^{KO} cells. One week after tumor cell implantation, mice were treated with daily intraperitoneal injections of HY-155534 (4 mg/kg) or an equivalent volume of DMSO control ($n = 10$).

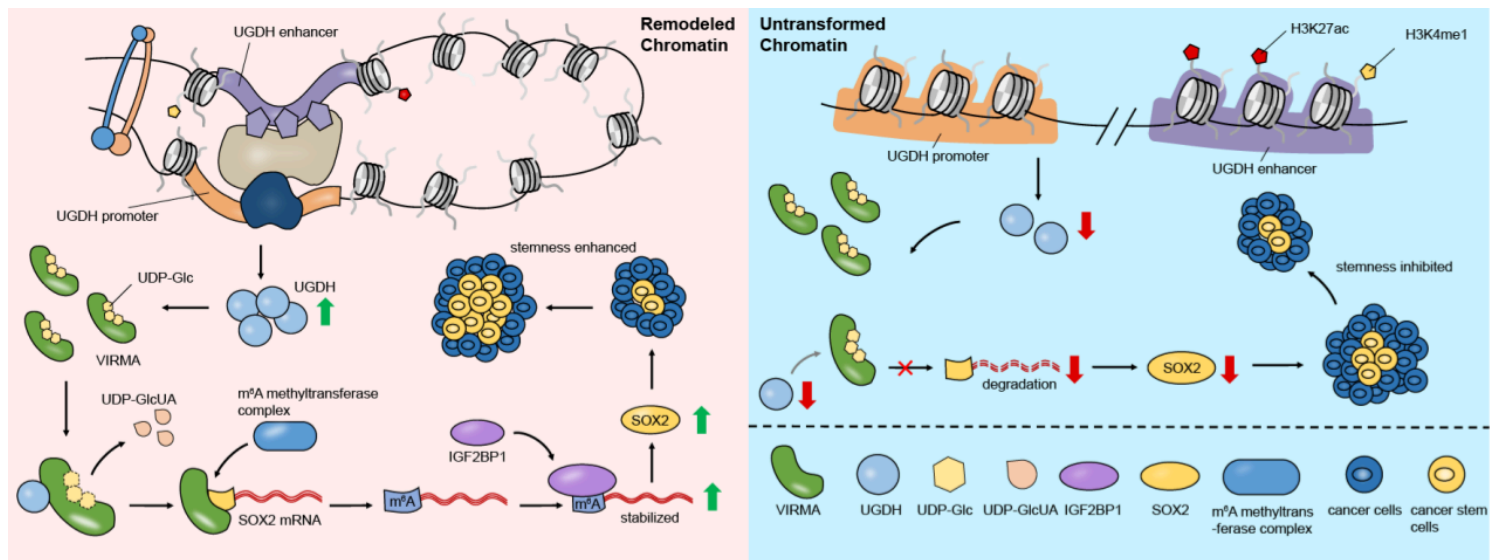


Figure 8

Chromatin remodeling promotes UGDH expression, thereby driving tumor stemness maintenance.

Figure 8. The mechanistic scheme diagram of UGDH promoting stemness maintenance in tumor cells.

Supplementary Files

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