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Perspective

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# Targeting histone modifiers in bladder cancer therapy — preclinical and clinical evidence

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## Abstract

Bladder cancer in the most advanced, muscle-invasive stage is lethal, and very limited therapeutic advances have been reported for decades. To date, cisplatin-based chemotherapy remains the first-line therapy for advanced bladder cancer. Late-line options have historically been limited. In the past few years, next-generation sequencing technology has enabled chromatin remodelling gene mutations to be characterized, showing that these alterations are more frequent in urothelial bladder carcinoma than in other cancer types. Histone modifiers have functional roles in tumour progression by modulating the expression of tumour suppressors and oncogenes and, therefore, have been considered as novel drug targets for cancer therapy. The roles of epigenetic reprogramming through histone modifications have been increasingly studied in bladder cancer, and the therapeutic efficacy of targeting those histone modifiers genetically or chemically is being assessed in preclinical studies. Results from preclinical studies in bladder cancer encouraged the investigation of some of these drugs in clinical trials, which yield mixed results. Further understanding of how alterations of histone modification mechanistically contribute to bladder cancer progression, drug resistance and tumour microenvironment remodelling will be required to facilitate clinical application of epigenetic drugs in bladder cancer.

## Sections



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## Introduction

Bladder cancer is one of the most common urological malignancies, with 573,278 new diagnoses and 212,536 new deaths estimated worldwide in 2020 (ref. 1). Bladder cancer is a heterogeneous disease with various clinical outcomes[2]. The majority of newly diagnosed bladder cancers is non-muscle-invasive bladder cancer (NMIBC), including carcinoma in situ and papillary tumours restricted to the urothelium and the lamina propria[3]. Patients can be stratified based on clinicopathological risk factors into low-risk, intermediate-risk and high-risk disease categories according to the American Urological Association guidelines[4]. Approximately 25% of patients have tumours invading the detrusor muscle at first diagnosis, which is referred to as muscle-invasive bladder cancer (MIBC)[3]. MIBC is more lethal and has greater potential to spread to local lymph nodes and distant organs than NMIBC[5]. At this stage, metastases are already present in ~10% of newly diagnosed patients[6]. Management of patients with NMIBC frequently involves transurethral resection of the bladder followed by intravesical therapy based on disease risk category. The 5-year overall survival (OS) for patients with NMIBC is ~90%[2]. However, the 5-year recurrence-free survival is only 20–40%[7,8], and up to 15– 20% of NMIBC instances progress to MIBC[9,10]. Radical cystectomy (RC) with pelvic lymph node dissection is the standard of care for patients with MIBC. The 5-year OS in patients with non-metastatic MIBC is 36–48%[4]. Unfortunately, MIBC treated with surgical procedure alone has a high probability of recurrence, as ~50% of patients eventually experience recurrence at distant sites[11,12], with the 5-year OS rate dropping to 5–30%[6]. Systemic therapy combined with local therapy for MIBC can have an important role in reducing recurrence rate.

Generally, MIBC is chemotherapy sensitive. Methotrexate, vinblastine, doxorubicin and cisplatin (MVAC); dose-dense methotrexate, vinblastine, doxorubicin and cisplatin (ddMVAC); and gemcitabine in conjunction with cisplatin or carboplatin are the most commonly used therapy regimens. Cisplatin-based neoadjuvant chemotherapy has been associated with an improvement of 5–8% in the 5-year OS rate compared with RC alone[13]. However, nearly half of patients with MIBC are ineligible for carboplatin-based chemotherapy owing to comorbidities[14]. Different from many other cancers, systemic treatment for locally advanced bladder cancer and metastatic disease has not achieved substantial progress for more than 30 years, owing to a lack of public awareness and underfunding[5]. To date, cisplatin-based chemotherapy remains the standard first-line therapy for patients with advanced MIBC. Second-line therapy options have historically been limited[5]. During the past decade, several treatment strategies, including targeted therapies (fibroblast growth factor receptor (FGFR) inhibitors), immune checkpoint inhibitors (ICIs) and antibody-drug conjugates, have been successfully tested and approved in late treatment lines, as a consequence of an improved understanding of bladder cancer biology[4]. The advent of next-generation sequencing technology helped understand the molecular profiling and mutational landscape of bladder cancer, which can facilitate the identification of potential therapeutic targets[15,16].

Epigenetics refers to information carried by the genome that is not coded by DNA[17]. Epigenetic modifications include DNA methylation and histone modifications[18]. The nucleosome is the basic unit of chromatin, consisting of four core histones (H2A, H2B, H3, H4) and 147 base pairs of DNA that form an octamer[17]. Histone modifications can occur in both the flexible tails and the core regions of histones. Specifically, the N-terminal tails are the hotspots of histone modification owing to a large number and types of modifiable residues[17] (Fig. 1). Covalent modifications of histones include histone acetylation and methylation and respond to developmental and environmental changes altering chromatin structure and function; these modifications affect gene transcription, splicing, DNA repair, DNA replication and cell-cycle control[17,19,20]. Thus, epigenetic control is important for different processes including cell-type identity, cellular reprogramming and malignant transformation[20]. Traditional cancer treatment research focused on genetic alterations (such as mutations, gene rearrangements and copy number variation). Different from permanent changes in the DNA sequence, chromatin modifications have the potential to be entirely reversible, a characteristic that can be leveraged in potential epigenetic therapies[20].

Many enzymes are involved in histone modifications, and are responsible for placement (writers), removal (erasers) or recognition (readers) of these epigenetic marks (Fig. 1). Gain-of-function mutations of these histone modifiers can be targeted by direct inhibition, and many chemical inhibitors have been developed, such as histone deacetylases (HDAC) inhibitors[21], histone methyltransferases (HMT) inhibitors[22] and histone acetylation reader proteins inhibitors (for example, the BET inhibitors)[23]. Histone modifiers can act on multiple genes. Thus, inhibitors targeting histone modifiers could produce genome-wide changes, influencing different cellular processes rather than a single gene or pathway[24], which highlights the need to identify disease-related targets and improve inhibitors' selectivity. Notably, histone modifiers generally act in complexes. For example, Polycomb group (PcG) and Trithorax group (TrxG) complexes regulate chromatin state by directly methylating histones through the addition of the H3K27me3 (repressive) and H3K4me3 (activating) histone marks, respectively[19]. This interplay helps to maintain a delicate balance in the control of the chromatin state. Therapeutic strategies targeting these complexes can be based on the inhibition of an enzymatically active subunit or the disruption of complex stability, which might not necessarily yield equivalent effects[25]. With regard to loss-of-function mutations, targeting these alterations can be challenging, but the concept of synthetic lethality has provided a means of therapeutically exploiting these mutations in cancer[22]. Synthetic lethality refers to the interplay between two genes, in which the loss of either gene individually does not affect cell survival, but the concurrent loss of both genes results in cell death[22]; an example of therapies exploiting synthetic lethality is the use of poly-ADP-ribose polymerase (PARP) inhibitors to treat cancers harbouring *BRCA1* or *BRCA2* mutations. Targeting antagonistic regulators of the same process might be another therapeutic strategy to target loss-of-function mutations. For example, bladder cancer cells harbouring loss-of-function mutations in the histone lysine demethylase *KDM6A* have been shown to be vulnerable to the inhibition of the histone lysine methyltransferase enhancer of zeste homologue 2 (*EZH2*)[26].

To date, epigenetic alterations are more commonly detected in MIBC than in any other cancer type[16,27]. Results from the analysis of 131 high-grade MIBC tumours from The Cancer Genome Atlas (TCGA) project showed that 99 tumours (76%) harboured an inactivating mutation in one or more chromatin regulatory genes, and 53 tumours (41%) had at least two of these mutations, suggesting a distinctive mutational landscape in bladder cancer[28]. *KDM6A, KMT2C, KMT2D, ARID1A, CREBBP* and *EP300* are among the most commonly altered chromatin regulatory genes in bladder cancer[29]. Moreover, integrated network analyses showed that these mutations had a profound effect on the activity levels of numerous transcription factors and pathways that are known to be involved in cancer development processes[28]. String analyses showed

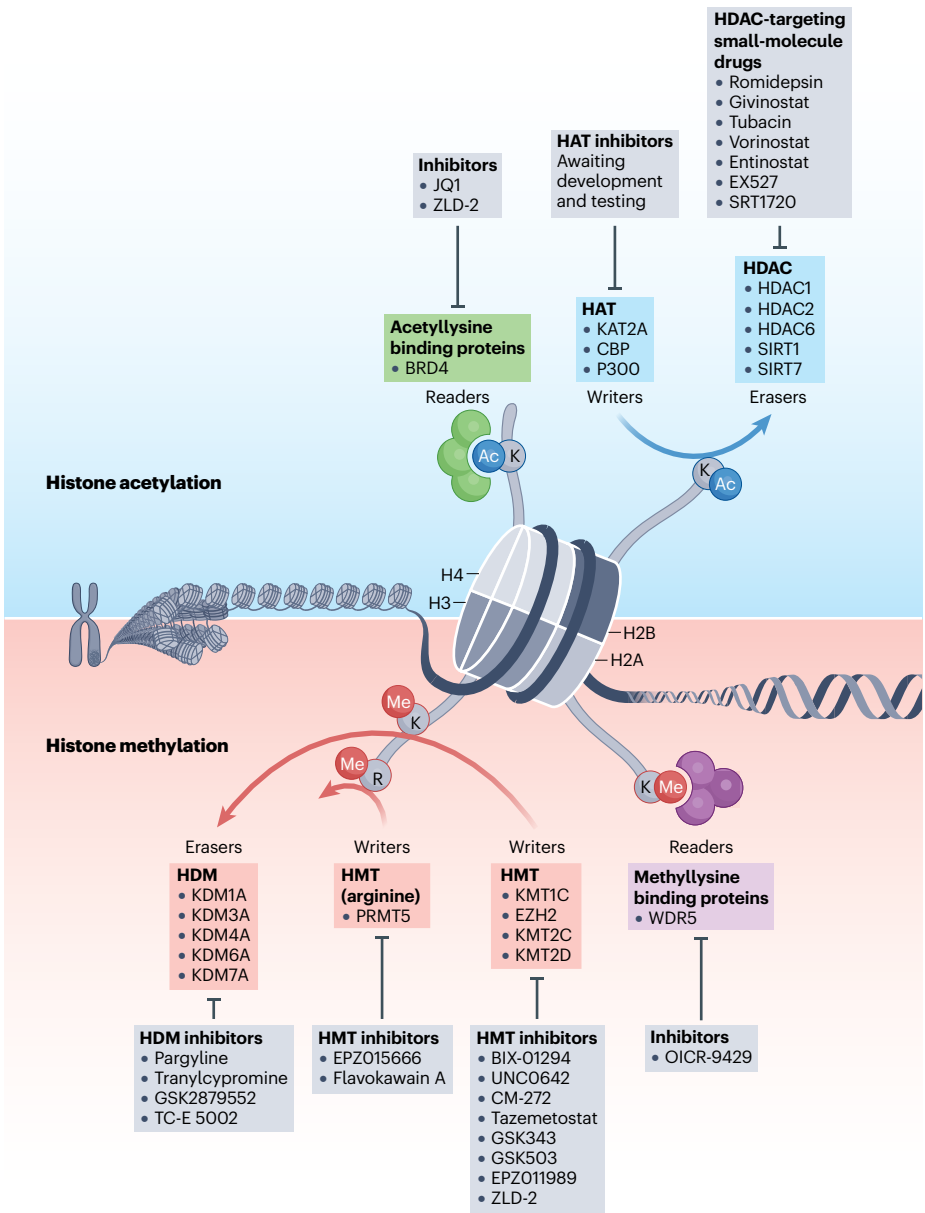


**Fig. 1 | Histone modifications potentially targetable in bladder cancer.** Double-strand DNA wraps around core histones (H2A, H2B, H3, H4) forming an octamer called nucleosome. Nucleosome is the basic unit of chromatin. Histone modifications (acetylation or methylation) can take place in the flexible 'tails' of histones, specifically in the N-terminal tails. Histone acetylases (HATs) and histone methyltransferases (HMTs) are the enzymes responsible for adding an acetyl group or a methyl group, respectively, to lysine residues on histones, and are known as 'writers'. Enzymes responsible for removing the acetyl or methyl group (histone deacetylases (HDACs) and histone-demethylases (HDMs)) are known as 'erasers'. 'Readers' are proteins that recognize methylated or acetylated lysine residues. Small-molecule drugs targeting writer, eraser and reader proteins that have been tested in bladder cancer preclinical models are shown.

that genomic changes in chromatin regulators can have implications in cell differentiation, cellular adaptability and clonal expansion, contributing to the pathogenesis of bladder urothelial carcinoma[30]. These results indicate that loss of epigenetic regulation of chromatin might be a primary driver mechanism in bladder cancer progression and might be exploited to identify therapeutic targets. In bladder cancer, HDAC inhibitors were the most widely investigated in clinical trials[24,31,32].

In this Perspective, we discuss the idea of targeting histone modifications in bladder cancer, especially well-studied modifications such as acetylation and methylation. We summarize potential targets of histone modifications and underlying mechanisms based on preclinical and translational research findings. Additionally, we provide biological rationale and feasibility for the potential combination of therapies targeting these epigenetic targets with other therapeutic strategies. Relevant clinical trials in which the therapeutic efficacy of targeting histone regulators alone or in conjunction with other approved therapies was assessed in bladder cancer are also described. Last, current challenges in targeting epigenetic regulators are briefly discussed.

## Targeting histone modifiers in bladder cancer — preclinical evidence

Currently, many histone modifiers have been found to be aberrantly expressed in bladder cancer and have essential roles in tumour initiation and progression. In preclinical studies in bladder cancer cellular or animal models, chemical or genetic inhibition of some of these enzymes have shown therapeutic effects.

## Histone acetylation

Functionally, histone acetylation in the gene body, promoters and enhancers is associated with transcriptional activation[33]. A widely accepted hypothesis for the mechanism through which histone acetylation facilitates the establishment of an active chromatin state is that adding an acetyl group to the lysine residues on histone tails might affect the compaction state of chromatin by neutralizing basic charges, reducing the electrostatic attraction between histones and DNA, in turn leading to an open chromatin state and increasing chromatin accessibility to transcription factors and other regulatory proteins[17]. Additionally, a set of non-histone proteins with enzymatic activities (for example, acetylation, methylation and helicase activities) are recruited to recognize and bind the modified residues to further modify chromatin[17]. Histone acetyltransferases (HATs), also known as lysine acetyltransferases (KATs), are enzymes that catalyse the addition of an acetyl group from acetyl-CoA cofactors to lysine residues. Conversely, HDACs are in charge of removing the acetyl group. Different enzymes involved in histone acetylation modification could be potential therapeutic targets in bladder cancer (Table 1).

**Targeting histone acetyltransferases.** Three primary HATs families exist: the Gcn5-related N-acetyltransferase (GNAT) family; the MYST family (named after the four founding members of this family: MOZ, Ybf2, Sas2 and Tip60); and the orphan family (CBP and p300, and nuclear receptors)[34]. HAT mutations can act as both tumour drivers and tumour suppressors. Results from one study in which data from TCGA database were analysed showed that the expression of two HATs from the GNAT family, general control nonderepressible 5 (GCN5, also known as KAT2A), and p300/CBP-associated factor (PCAF, also known as KAT2B) are elevated in bladder cancer. Knockdown of *GCN5, PCAF*, or both in bladder cancer cell lines showed that *GCN5* knockdown inhibited tumour cell proliferation, whereas the effect of *PCAF* knockdown on the same phenotype was negligible[35]. CBP and p300 are chromatin modifier proteins responsible for the acetylation of two lysine (K) residues on histone H3 (H3K18ac and H3K27ac)[36]. CBP and p300 are transcriptional co-activators. Results from genome-wide sequencing studies showed that 12–21% of patients with MIBC harbour loss-of-function mutations in *CBP*[15,37–39]. In 2016 (ref. 40), a novel therapeutic strategy called 'paralogue targeting' has been exploited in CBP-deficient

**Table 1 | Targeting histone acetylation modifiers in bladder cancer**

| Category | Target (common aliases) | Model | Targeting strategy | Results | Combination therapy | Refs. |
|---|---|---|---|---|---|---|
| Histone acetyltransferases (writer) | KAT2A (GCN5, hGCN5, GCN5L2, PCAF-b) | Cell lines | siRNA | Inhibition of cell proliferation | NA | 35 |
| | CREBBP (CBP, RSTS, KAT3A, MKHK1, RSTS1)/ EP300 (p300, KAT3B, MKHK2, RSTS2) | Cell lines | CRISPRi-mediated synthetic lethality | Inhibition of EP300 in CREBBP-deficient bladder cancer cells impaired cell proliferation and induced apoptosis (c-MYC) | NA | 41 |
| Histone deacetylases (erasers) | HDAC1 (HD1, GON-10, RPD3L1, KDAC1)<br>HDAC2 (KDAC2, RPD3, YAF1) | Cell lines | siRNA<br>Chemical inhibitors (romidepsin, givinostat) | Induction of cell-cycle arrest in the G1 phase (activation of p21 and non-apoptotic cell death | Combination of HDACi (SAHA) and anti-PDL1 therapy in xenograft models of bladder cancer improved tumour regression and survival; combination of selective HDACi (entinostat) and anti-PD1 therapy in xenograft models of bladder cancer led to tumour reduction and long-term immunological memory | 32,147,149 |
| | HDAC6 (HD6, JM21, CPBHM, KDAC6, PPP1R90) | Cell lines<br>Foxn1$^{nu/nu}$ mice | Chemical inhibitors (tubacin) | Decreased cell viability; Induction of DNA-damage response and apoptosis (FGFR3, MYC and cyclin D1) | | 52,53 |
| | SIRT1 (SIR2, SIR2L1, SIR2alpha) | Cell lines<br>Mouse MIBC organoid; PDX | Chemical inhibitors (EX527; cell lines)<br>Chemical activators (SRT1720; mouse MIBC organoid[#]; PDX) | Inhibition of cell proliferation and glucose uptake (GLUT1); Inhibition of bladder cancer organoids and PDX growth (HIF signalling pathway) | NA | 58,59 |
| | SIRT7 (SIR2L7) | Cell lines | shRNA | Enhancement of cell migration and invasion | NA | 57 |
| Acetyllysine binding proteins (readers) | BRD4 (CAP, MCAP, HUNK1, HUNKI, FSHRG4) | Nude mice<br>Cell lines | shRNA (cell lines)<br>injection with shRNA BRD4 cells (nude mice)<br>Chemical inhibitors (JQ1; cell lines and nude mice) | Inhibition of cell proliferation, induction of cell apoptosis (through BRD4-C-MYC–EZH2 axis)<br>Attenuation of cell migration and invasion and improvement of platinum-based chemoresistance Sonic hedgehog signalling pathway) | Dual EZH2–BRD4 inhibitor (ZLD-2) strongly inhibits proliferation of T24 cells<br>Combination therapy with HDACi (romidepsin) and JQ1 showed a synergistic effect on inducing apoptosis in bladder cancer cells | 63–66 |

CRISPRi, CRISPR interference; HDAC, histone deacetylases; PDX, patient-derived tumour xenograft; shRNA, short hairpin RNA; siRNA, small interfering RNA.

human cancers. In this study, a functional synthetic-lethality screening showed that lung cancer cells lacking *CBP* were effectively targeted and killed by short hairpin RNA (shRNA)-mediated inhibition of the paralogue *p300*. Synthetic lethality of CBP and p300 was shown to be the result of G1 arrest and apoptosis through the deregulation of the transcription of the *MYC* gene in lung cancer cells. Subsequently, in bladder cancer cells, the specific inhibition of *CBP* and *p300* expression through the CRISPR interference system was shown to impair cell proliferation and induce apoptosis of tumour cells in vitro through decreasing c-*Myc* expression[41]. Thus, the *CBP* paralogue *p300* might be a potential therapeutic target for the treatment of *CBP*-deficient bladder cancers. Currently, no small-molecule inhibitors of p300 have been tested in bladder cancer cell lines or animal models. Future preclinical studies are needed to evaluate the potential therapeutic efficacy of chemical inhibition of p300 (for example, with Lys-CoA[42]) in bladder cancer. To date, the majority of HAT-targeting research in bladder cancer is at a very preliminary stage, and no HAT targets have moved into clinical trials. HATs embrace a large enzyme family that includes multiple protein subtypes, each with different functions within the cell beyond histone acetylation; thus, designing HAT inhibitors with high selectivity, effectiveness and safety is a complex task.

**Targeting histone deacetylases.** In opposition to histone acetylation, deacetylation of histone lysine tails usually causes transcription repression and gene silencing. HDACs show variations in cellular localization and can affect various cellular processes such as cell proliferation, cell cycle, differentiation, apoptosis, and also modulate the immune system by enhancing acetylation of histones or non-histone protein substrates[24]. HDACs can be divided into four classes, based on sequence similarity: class I (HDAC1, 2, 3, 8); class II (HDAC4, 5, 6, 7, 9, 10); class III (SIRT1–7); and class IV (HDAC11). Classes I, II and IV HDACs are $Zn^{2+}$ dependent, whereas class III are dependent on nicotinamide adenine dinucleotide (NAD) for their activity[43].

Class I HDACs stand out as the most extensively studied HDACs in bladder cancer. The expression levels of HDAC1 and HDAC2 are frequently elevated in human bladder tumours[31,44,45], which increases cell proliferation, concurrently reducing apoptosis and impairing cell differentiation[24]. In bladder cancer cell lines, small interfering RNA (siRNA)-mediated double knockdown of *HDAC1* and *HDAC2* or treatment with HDAC class I inhibitors (especially the specific HDAC1 and HDCA2 inhibitors romidepsin and givinostat) substantially reduced cell proliferation by impairing G1-to-S cell-cycle phase transition through the induction of p21, and induced non-apoptotic cell death[32]. However, inhibition of *HDAC1* or *HDAC2* individually did not have the same effects[32], as the functions of these two proteins largely overlap, and compensatory mechanisms are triggered when one of the two enzymes is downregulated[46,47].

Class II HDACs such as HDAC4 and HDAC5 were found to be often downregulated in bladder cancer cell lines, suggesting a possible tumour suppressive function[48]. Overexpression of HDAC5 in bladder cancer cell lines decreased cell proliferation, impaired clonogenic potency and induced epithelial–mesenchymal transition (EMT); however, overexpression of HDAC4 exerted only weak effects on these phenotypes. This difference might occur because in bladder cancer cell lines, HDAC5, but not HDAC4, is consistently weakly expressed[49,50]. HDAC6 is well-suited to specific inhibition owing to a unique protein structure, a diverse set of substrates (for example, histones, α-tubulin, HSP90) and cellular localization (generally located in the cytoplasm)[51]. Targeting HDAC6 in bladder cancer provided conflicting results in preclinical studies. Results from one study in which 12 small-molecule HDAC6 inhibitors were tested in bladder cancer cell lines showed that 3 out of 12 HDAC6 inhibitors decreased cell viability with low IC50 values[52]. In another study, treatment with the HDAC6 inhibitor tubacin substantially reduced the growth of bladder tumours in subcutaneous xenograft Foxn1$^{nu/nu}$ mice (tumours induced by the injection of RT-112 cells overexpressing mutant FGFR3) by inhibiting the accumulation of mutant FGFR3, and caused downregulation of MYC and cyclin D1, ultimately inducing DNA-damage response and apoptosis[53]. Conversely, in another study, three HDAC6-specific inhibitors (tubacin, tubastatin A and ST-80) were tested in bladder cancer cell lines. Results showed that tubacin was the most potent, although all three inhibitors had limited efficacy in these cells lines. siRNA-mediated HDAC6 knockdown failed to induce cell-cycle arrest, inhibit cell viability or induce apoptosis in bladder cancer cells, indicating that these cells do not depend on HDAC6 expression for proliferation and survival[54]. Results from these studies indicate the need to explore the therapeutic potential of HDAC6 using in vivo models or combination treatment strategies.

In mammals, seven different SIRTs (SIRT1–7, also referred to as class III HDACs) exist, which show variations in substrate specificity, catalytic activity and cellular functions[55,56]. Results from one study in which *SIRT1–7* transcription levels were assessed in 94 human bladder cancer samples compared with bladder normal mucosa showed that *SIRT1, 2, 4* and *5* were significantly ($P < 0.0001$) downregulated in bladder cancer, whereas *SIRT6 and SIRT7* were significantly overexpressed ($P < 0.0001$)[57]. Similarly, *SIRT1* and *SIRT3* expression levels, and *SIRT6* and *SIRT7* levels, were shown to be decreased and elevated, respectively, in bladder cancer tissues in TCGA dataset[57]. In 2021, the first chemical screening of 276 epigenetic drugs was conducted on MIBC organoids established from an orthotopic bladder cancer mouse model harbouring deletion of *Rb1*, *Trp53* and *Pten*, and overexpression of *Kras* and *c-Myc*[58]. Treatment with SRT1720, a SIRT1 activator, considerably inhibited bladder cancer organoid growth both in vitro and in vivo through the repression of the HIF signalling pathway. Also, in this study, CRISPR-CAS9-mediated SIRT1 inhibition significantly ($P < 0.0001$) enhanced the growth of mouse bladder cancer organoids, further indicating that SIRT1 might act as a tumour suppressor in MIBC. Inconsistent with these findings, in many other instances, SIRT1 has been shown to have an oncogenic role in bladder cancer. In a study in which SIRT expression was assessed in 12 bladder cancer and adjacent paired non-cancerous tissue samples, SIRT1 was found to be upregulated in cancer tissue samples[59]; similarly, SIRT expression was upregulated in the bladder cancer cell lines T24 and 5637 compared with the normal uroepithelium cell line SV-HUC-1 (ref. 59). SIRT1 overexpression in bladder cancer cells was shown to promote glucose uptake by activating transcription of the glucose transporter *GLUT1*; treatment with the specific SIRT1 inhibitor EX527 could suppress cell proliferation and glucose uptake in these cell lines[59]. Results from these studies highlight that SIRT1 seems to have two faces in bladder cancer biology, and activation or inhibition of SIRT1 might be effective for treatment in different contexts. SIRT7 was shown to be overexpressed in bladder cancer tissues compared with normal tissues[57]. However, a substantial reduction in SIRT7 expression was observed in invasive bladder cancer compared with papillary bladder cancer samples. shRNA-mediated *SIRT7* knockdown in MGHU3, J82 and 5637 bladder cancer cell lines increased cell migration and invasion, with a concomitant decrease of the epithelial marker E-cadherin (*CDH1*). These results indicate that *SIRT7* might have a dual role in bladder cancer carcinogenesis and progression[57]. In summary, the biological

functions of the sirtuin family members in different stages of bladder cancer are not fully understood, also considering the heterogeneity of this disease. Thus, additional basic research is needed to explore the roles of these proteins in bladder cancer.

In current clinical trials, some inhibitors target a broad spectrum of HDACs (pan-HDACi), whereas others are specific to particular isoenzymes. Preclinical evidence suggesting that different isoenzymes vary in terms of cellular location, expression levels and biological functions in bladder cancer highlights that HDACi should ideally be tailored to inhibit a specific subset of HDACs.

**Targeting histone acetylation readers.** Histone acetylation readers are proteins that recognize and bind histone acetylated lysine residues through N-terminal tandem bromodomains (BRDs). BRDs are the most prominent and thoroughly studied histone recognition domains. BRDs are evolutionarily conserved and are present in diverse nuclear proteins including HATs (PCAF, GCN5), methyltransferases (MLL, ASH1L), ATP-dependent chromatin-remodelling complexes (BAZ1B), helicases (SMARCA), transcriptional coactivators (TIF1, p300/CBP, TAFs), nuclear-scaffolding proteins (PB1), and proteins of the bromodomains and extra-terminal (BET) family[60,61];]the BET family includes BRD2, BRD3, BRD4 and BRDt. Proteins of this family share two conserved N-terminal BRDs and a divergent C-terminal recruitment domain[23]. BRDs provide a functional link between histone acetylation and acetylation-mediated protein–protein interactions in chromatin remodelling and related gene transcription[34].

In the search for novel epigenetic drugs, BRD has been a notable druggable motif, owing to its wide occurrence. Many BRD-containing proteins also have enzymatic activities, which have been discussed elsewhere[62]. Dysfunction of BRD proteins has been linked to the development of various diseases including cancer. In bladder cancer, the BET family member BRD4 has been most frequently studied. Results from a study in which 55 primary bladder cancer and surrounding normal bladder tissue samples were compared showed that BRD4 was significantly ($P < 0.05$) overexpressed in bladder cancer tissues; moreover, BRD4 was overexpressed in bladder tumour cell lines compared with normal urothelial cell lines[63]. Additionally, BRD4 inhibition using shRNA or the chemical BET selective inhibitor JQ1 (ref. 23) blocked cell proliferation and induced apoptosis in both bladder cancer cell lines and xenograft mice by decreasing the recruitment of C-MYC to the EZH2 promoter[63]. In another study, siRNA-mediated BRD4 downregulation attenuated bladder cancer cell lines migration and invasion and helped to overcome platinum-based chemoresistance through the regulation of the sonic hedgehog pathway. Overexpression of BRD4 induced cisplatin resistance in a bladder cancer xenograft mouse model[64]. These results suggest that BRD4 might be a new therapeutic target in patients with cisplatin-resistant bladder cancer. In 2023, a dual EZH2–BRD4 inhibitor, ZLD-2, was developed and showed notable antiproliferative effects on various solid tumour cells, including the bladder cancer cell line T24 (ref. 65). ZLD-2 showed stronger antiproliferative activity than the EZH2 inhibitor GSK126 in T24 cells, but comparable antiproliferative activity with that of JQ1 or the combination of GSK126 and JQ1 (ref. 65). Additionally, JQ1 and the HDAC class I-specific inhibitor romidepsin showed a synergistic effect on inducing apoptosis in bladder tumour cells, whereas only a minor effect was observed in benign cells (less than 5% of apoptotic cells)[66]; thus, this small-molecule combination therapy could be a promising approach to testing for bladder cancer. Results from these studies suggested that targeting histone acetylation readers, especially BRD4 alone or in combination with other epigenetic inhibitors, might be promising in bladder cancer.

## Histone methylation

Methylation occurs at histone lysine and arginine residues. In this Perspective, we focused on lysine residues, which were the most extensively studied. Lysine can be monomethylated, dimethylated or trimethylated, and each of these methylation levels is likely to be associated with a different function[67]. Overall, lysine methylation influences many biological processes, including heterochromatin formation, X chromosome inactivation and transcriptional regulation[67]. Histone lysine methylation can either activate or suppress transcription depending on the situation. Generally, H3K9, H3K27 and H4K20 methylations are associated with suppression of transcription, whereas H3K4, H3K36, and H3K79 methylations are associated with transcription activation[68]. Similarly to histone acetylation, histone methylation is based on the activity of important enzymes: histone methyltransferases, histone demethylases and histone methylation-recognizing proteins.

**Targeting histone methyltransferases.** Histone methyltransferases transfer a methyl group from S-adenosylmethionine (SAM) to lysine (K) or arginine (R) residues on histones, and are known as HMTs (or KMTs) and PRMTs, respectively. To date, KMTs have been better studied than PRMTs in bladder cancer. All KMTs possess a conserved enzymatic SET (Su(var), Enhancer of zeste, Trithorax)[69] domain. An exception is DOT1 (and its homologues), which was the first non-SET domain-containing histone lysine methyltransferase identified and showed intrinsic histone methyltransferase activity targeting Lys79 of histone H3 within the nucleosome core[70]. Multiple HMTs have been investigated in bladder cancer and could be a promising target for therapy (Table 2).

Methylation of H3K4 is mainly mediated by the MLL (also named KMT2 or SET1) family of methyltransferases. Mammalian MLL family proteins include six members: MLL1 (KMT2A), MLL2 (KMT2B), MLL3 (KMT2C), MLL4 (KMT2D), SETD1A (KMT2F) and SETD1B (KMT2G)[71]. KMT2C and KMT2D are the most frequently investigated in bladder cancer. KMT2C and KMT2D are part of the complex proteins associated with Set 1 (COMPASS) complex, and are the main proteins responsible for H3K4 monomethylation, which marks the primed transcriptional enhancers[72]. Active enhancers are further marked by H3K27ac modification carried out by CBP and p300 (ref. 72). KMT2C, also known as MLL3, is commonly mutated (~18%) in MIBC[37]. In one study, including 104 patients with bladder cancer, KMT2C was shown to be downregulated in bladder tumour tissue compared with normal tissue[73]. In this study, consistently with the KMT2C known role in enhancer marking, *KMT2C* knockdown was shown to influence enhancer activity in a subset of genes in bladder cancer cell lines, including genes involved in cell adherence, extracellular organization and epithelial differentiation[73]. Additionally, KMT2C was also shown to localize at the promoter (in turn controlling promoter activation) of genes involved in the DNA-damage and repair pathways. Thus, shRNA-mediated *KMT2C* knockdown in human bladder cancer cell lines (T24, HTB9) led to extensive decreased expression of genes involved in DNA-damage response (*ATM, ATR*) and DNA repair pathway (*BRCA1, BRCA2, RAD50, RAD51*)[73]. KMT2C loss in bladder cancer cells resulted in homologous recombination pathway deficiency and, therefore, high levels of genomic instability[73]. Notably, in this study[73], KMT2C deficiency led to PARP1 or PARP2 dependence for DNA repair in vitro and in vivo, suggesting that PARP1 or PARP2

**Table 2 | Targeting histone methylation modifiers in bladder cancer**

| Category | Target (common aliases) | Catalytic activity | Model | Targeting strategy | Results | Combination therapy | Refs. |
|---|---|---|---|---|---|---|---|
| Histone methyltransferases (writers) | EHMT2 (BAT8, C6orf30, G9A, GAT8, KMT1C, NG36 | H3K9me1and H3K9me 2 | Cell lines<br>Nude mice<br>QKO mice[a] | shRNA (cell lines)<br>Chemical inhibitors (BIX-01294, UNC0642)<br>Chemical inhibitors (CM-272) | Induction of autophagy (AMPK–mTOR pathway; cell lines)<br>Induction of apoptosis (nude mice)<br>Inhibition of cell proliferation, induction of apoptosis and autophagy (QKO mice) | Combination of CM-272 and anti-PDL1 in QKO mice resulted in increased immune cell infiltrations ($CD3^+$, $CD8^+$ T and natural killer cells) and inhibition of metastasis | 87,88,90 |
| | EZH2 (KMT6A, WVS, ENX1, KMT6, WVS2, ENX-1, EZH2b) | H3K27me3 | Mouse models[b]<br>*KDM6A*-null cell lines, nude mice and PDX | Chemical inhibitors (EPZ011989; mouse models)<br>Chemical inhibitors (GSK343 GSK503 EPZ011989; *KDM6A*-null models) | Induction of an immune response (immunocompetent mouse models)<br>Inhibition of subcutaneous tumour growth (*KDM6A*-null models)<br>Delay of tumour onset; G2-to-M phase cell-cycle arrest (*KDM6A*-null models) | NA | 26,82,118 |
| | PRMT5 (HSL7, JBP1, SKB1, IBP72, SKB1Hs, HRMT1L5) | H4R3me2s | Cell lines<br>Nude mice | shRNA<br>Chemical inhibitors (EPZ015666, Flavokawain A) | Induction of apoptosis; inhibition of cell viability | NA | 92,93 |
| | KMT2C (MLL3, HALR, KLEFS2) | H3K4me1 | Cell lines | NA | shRNA-mediated *KMT2C* knockdown in bladder cancer cell lines led to extensive decreased expression of genes involved in DNA-damage response (*ATM, ATR*) and DNA repair pathway (*BRCA1, BRCA2, RAD50, RAD51*) | NA | 73 |
| | KMT2D (MLL4, ALR, KMS, AAD10, BCAHH, KABUK1, TNRC21) | H3K4me1 | Cell lines | NA | Overexpression of KMT2D effectively induced PTEN and p53 expression and repressed STAG2 expression; KMT2D induced the expression of genes involved in the maintenance of epithelial development stemness, polarity and adhesion (through inducing p63) | NA | 74,75 |
| Histone demethylases (erasers) | KDM1A (LSD1, AOF2, CPRF, KDM1, BHC110) | H3K9me<br>H3K4me | Cell lines<br>Nude mice<br>PDX | siRNA<br>Chemical inhibitors (pargyline, tranylcypromine, GSK2879552) | Suppression of proliferation and androgen-induced transcription (cell lines)<br>Downregulation of LEF1 and decreased EMT (cell lines and nude mice)<br>Suppression of tumour growth (nude mice and PDX) | NA | 97,98 |
| | KDM3A (JMJD1A, TSGA, JMJD1, JHDM2A, JHMD2A) | H3K9me1<br>H3K9me 2 | Cell lines<br>Nude mice | shRNA | Suppression of cell proliferation through cell-cycle arrest at the G1 stage (HOXA1–CCND1 pathway); (cell lines)<br>Decrease in cell proliferation, colony formation and xenograft tumour growth (inhibition of the expression of the glycolytic gene *PGK1*); (cell lines, nude mice) | NA | 99,100 |
| | KDM4A (JHDM3A, JMJD2A, TDRD14A) | H3K9me3 | Cell lines | siRNA | Inhibition of cell growth (through the regulation of the G1-to-S transition)<br>Inhibition of cell migration, invasion and EMT (through SLUG inhibition) | NA | 105,106 |

**Table 2 (continued) | Targeting histone methylation modifiers in bladder cancer**

| Category | Target (common aliases) | Catalytic activity | Model | Targeting strategy | Results | Combination therapy | Refs. |
|---|---|---|---|---|---|---|---|
| Histone demethylases (erasers) (continued) | KDM6A (UTX, KABUK2, bA386N14.2) | H3K27me3 | Cell lines; nude mice; *Kdm6a*$^{\Delta/\Delta}$ mice[c] | *KDM6A*-DNA plasmid transfection (cell lines, injection into nude mice)<br>KDM6A mRNA nanoparticles intravesical delivery<br>Targeting of antagonistic regulators (EZH2 inhibitors)<br>Chemical inhibition of CCR2 (propagermanium) combined with IL6 receptor-neutralizing antibody (MR16-1) | Inhibition of migration, invasion and metastasis through activation of *ARHGDIB* (cell lines, nude mice)<br>Inhibition of tumour growth and metastasis (nude mice)<br>Inhibition of tumour growth (nude mice) | NA | 26,114, 116,118,119 |
| | KDM7A (JHDM1D) | H3K27me1<br>H3K27me2 | Cell lines<br>Nude mice | shRNA (cell lines)<br>Injection with shRNA-KDM7A cells (nude mice)<br>Chemical inhibitors (TC-E 5002) | Inhibition of cancer cell growth and migration (cell lines and nude mice)<br>Inhibition of cancer cell growth and block of apoptosis (regulation of AR transcription activity) | NA | 123 |
| Methyllysine binding proteins (readers) | WDR5 (BIG3, SWD3, BIG-3, CFAP89) | H3K4me | NOD/SCID mice+cell lines[a] | Injection with shRNA WRD5 cells (NOD/SCID mice)<br>Chemical inhibitors (OICR-9429) | Suppression of subcutaneous tumour growth (inhibition of *cyclin B1, cyclin E1, cyclin E2, UHMK1, MCL1, BIRC3* and *NANOG* expression) (NOD/SCID mice)<br>Suppression of cell proliferation (through G1 arrest), reduced migration and invasion, increased apoptosis and chemosensitivity to cisplatin (cell lines)<br>Reduction of PDL1 expression (cell lines) | Combination of OICR-9429 and cisplatin in nude mice inhibited subcutaneous tumour growth rates compared with treatment with either agent alone | 128,130 |

EMT, epithelial–mesenchymal transition; MIBC, muscle-invasive bladder cancer; PDX, patient-derived tumour xenograft; QKO, quadruple-knockout; shRNA, short hairpin RNA; siRNA, small interfering RNA. [a]Cre-dependent inactivation of *Pten*, *Trp53* and *Rb1* in *Rbl1*-deficient mice. [b]N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced immunocompetent MIBC mice (C57BL/6) and BBN-induced MIBC immunocompromised mice (Rag1$^{-/-}$ mice). [c]*Kdm6a*$^{flox/flox}$ *UpkIICre*$^{+}$ *p53*$^{+/-}$ mice+non-obese diabetic and severe combined immunodeficiency mice.

inhibitors could be used to treat KMT2C-deficient bladder cancer. Indeed, *KMT2C* knockdown in cell lines and xenograft mice resulted in increased sensitivity to the selective PARP inhibitor olaparib[73]. KMT2D (also known as MLL4), is frequently mutated (~27%) in bladder cancer based on data from several large sequencing projects; most of these alterations are loss-of-function mutations[29]. KMT2D is responsible for H3K4 monomethylation and, in bladder cancer cells, might act as a tumour suppressor by inducing the expression of tumour suppressor genes, in turn inhibiting tumour cell growth, migration and invasion. Specifically, overexpression of KMT2D enhanced the level of H3K4me1, and effectively induced PTEN and p53 expression in bladder cancer cells[74]. Additionally, KMT2D was reported to interact with p63 and to enrich at p63 target enhancers, inducing the expression of p63 target genes including crucial genes involved in the maintenance of epithelial development stemness, polarity and adhesion[75]. Epithelial tissues depend on a meticulously orchestrated equilibrium among self-renewal, proliferation and differentiation, disruption of which might drive carcinogenesis. Thus, depletion of KMT2D might result in epithelial homeostasis disturbance and lead to carcinogenesis. In one study in which somatic clonal events were assessed in morphologically normal urothelium from patients' tumour-bearing bladders, *KMT2D* mutations were identified in 16 of the 133 regions (12.0%) examined, further indicating that *KMT2D* loss of function might be a driver mutation in the carcinogenesis process[76–78]. However, targeting loss-of-function alterations is challenging, as restoring functions can be difficult. To date, no effective methods to target KMT2D directly or indirectly are available to restore KMT2D function in bladder cancer. Results from a study in C57BL/6 mice showed that *Kmtd2* deficiency promotes myeloid leukaemia's tumorigenesis in these mice. Mechanistically, Kmt2d might regulate the expression of Ddit4, a negative regulator of mTOR. The absence of Ddit4 activates the mTOR pathway, leading to the induction of ribosome biogenesis. Consequently, Kmt2d-deficient myeloid leukaemia cells are sensitive to the chemical inhibition of ribosome biogenesis (CX-5461)[79]. In-depth investigations into the mechanisms underlying *KMT2C* and *KMT2D* deficiency-driven bladder cancer will be needed to identify potential therapeutic targets for patients with *KMT2C* and *KMT2D*-deficient bladder cancer.

Methylation of H3K27 is mainly mediated by the Polycomb repressive complex 2 (PRC2). The PRC2 complex includes at least four components: enhancer-of-zeste homologue 1or 2 (EZH1or EZH2), suppressor of zeste 12 (SUZ12), embryonic ectoderm development (EED) and retinoblastoma-associated protein 46 or 48 (RBAP46 or RBAP48)[80]; and EZH2 is the catalytic subunit of PRC2. The core trimeric complex EZH2–EED–SUZ12 mediates H3K27me2 and H3K27me3 methylation through EZH2-mediated methyltransferase activity[71]. EZH2 presents point mutations in small subsets of haematological tumours[81], whereas in most solid tumours including bladder cancer, the EZH2 wild-type form is frequently overexpressed, and exerts oncogenic roles through the H3K27me-mediated silencing of important genes involved in differentiation and cell-cycle arrest[70]. The EZH2 inhibitor (EZH2i) EPZ011989 was tested in an immunocompetent N-butyl-N-(4-hydroxybutyl)-nitrosamine (BBN)-induced MIBC mouse model that faithfully recapitulates characteristics of human tumours[82,83]. In this study[82], EZH2 catalytic inhibition could induce an immune response, including an increased number of tumour-infiltrating $CD3^+$ T cells and increased transcript levels of MHC-II genes in the EZH2i-treated group. Immunocompromised mice treated with EZH2i developed an increased number of high-grade tumours (≥T2) and a decrease in H3K27me3 levels compared with mice treated with vehicle control ($P < 0.0001$). These results indicate that EZH2i antitumour activity completely depends on an intact adaptive immune system and provides biological rationale for combination therapies of EZH2i with immune checkpoint inhibitors. Notably, results from this study prompted the start of a clinical trial to evaluate the efficacy of tazemetostat (EZH2 catalytic inhibitor) and pembrolizumab (PD1 inhibitor) in patients with locally advanced or metastatic urothelial carcinoma[84].

G9a, also known as KMT1C or EHMT2, belonging to the Su(var)3-9 family, is responsible for H3K9me1 and H3K9me2, leading to gene expression silencing[85]. G9a methylates histone tails and also has the ability to recognize this modification, serving as a scaffold for recruiting other target molecules onto the chromatin[86]. G9a was shown to be significantly overexpressed in human bladder cancer tissue samples ($P = 0.0096$)[87]. Chemical inhibition of G9a using the inhibitor BIX-01294 or genome inhibition using a shRNA targeting G9a reduced cell proliferation in bladder cancer cell lines by inducing autophagy through the activation of AMPK, which in turn inhibits the mTOR pathway[87]. Treatment with UNC0642, a small-molecule inhibitor of G9a, could also decrease cell viability and induce apoptosis in bladder cancer cell lines and block subcutaneous tumour growth in nude mice[88]. Additionally, CM-272, a reversible small-molecule dual inhibitor of G9a and DNA methyltransferase (DNMT), which had been previously discovered and tested in haematological malignancies[89], was shown to be effective in multiple bladder cancer models. Treatment with CM-272 in bladder cancer cell lines had a potent inhibitory effect on cell proliferation, and induced apoptosis and autophagy. Notably, two cell lines (J82 and 253 J) resistant to CM-272 treatment showed *PIK3CA* mutations[90]. Gain-of-function mutations in *PIK3CA* post-transcriptionally downregulate EZH2 expression and H3K27me3 levels in bladder cancer[91]. G9a collaborates with EZH2 in the regulation of H3K27me3; thus, CM-272 resistance in these cells could be related to abnormal EZH2 activity[90]. Moreover, treatment with CM-272 in nude mice bearing xenografts or in quadruple-knockout (QKO) transgenic mice (harbouring Cre-dependent inactivation of *Pten*, *Trp53* and *Rb1* carried out specifically in urothelial cells (AdK5Cre) of *Rbl1*-deficient mice) caused substantial suppression of tumour growth and metastasis, increased apoptosis and autophagy, and decreased H3K9me2 and H3K27me3 (ref. 90).

Mutations of arginine methylation have not been broadly investigated. Among PRMTs, PRMT5 was identified as a therapeutic target in bladder cancer. PRMT5 was shown to be highly expressed in bladder cancer cell lines; PRMT5 overexpression promotes proliferation and colony formation of bladder cancer cells as well as inhibits apoptosis through the inhibition of NF-κB-dependent pathways[92]. *PRMT5* shRNA-mediated knockdown induced bladder cancer cell apoptosis, and pharmacological inhibition of PRMT5 using the selective inhibitor EPZ015666 blocked bladder tumour growth in a xenograft model[92]. In another study, PRMT5 was shown to be upregulated in bladder tumour tissues and cell lines, and PRMT5 upregulation correlated with poor prognosis[93]. In this study, high-throughput screening was performed to identify small-molecule compounds that could specifically bind PRMT5. Flavokawain A was selected from this screening and showed a better response (cell viability inhibition) in bladder cancer cell lines than the other two PRMT5 inhibitors (EPZ015666 and GSK3326595). Moreover, treatment with flavokawain A reduced tumour size in subcutaneous xenograft nude mice[93].

**Targeting histone demethylases.** Histone demethylases remove methyl groups from histone residues. Histone methylation was considered irreversible until the discovery of the first histone demethylase LSD1 (ref. 94), after which, several demethylases were identified. To date, histone lysine demethyltransferase (also known as KDMs) can be divided into two groups according to distinct catalytic domains: the LSD1 domain family (including KDM1) and the Jumonji C (JmjC) domain family (including KDM2–KDM8).

LSD1 (also known as KDM1A) has been shown to be upregulated in human bladder cancer tissue samples compared with normal tissues and might have essential roles in carcinogenesis[95]. LSD1 can function as either a transcription activator — through the demethylation of H3K9 — or a transcription repressor — through the demethylation of H3K4 (ref. 94). Results from a study in prostate cancer cell lines showed that androgen receptor (AR) and LSD1 interact and form a chromatin-associated complex in a ligand-dependent manner[96]. Under stimulation with AR agonists, androgen-induced transcription is accompanied by a relief of repressive histone marks through LSD1-mediated H3K9 demethylation, resulting in the de-repression of AR target genes in prostate cancer cells[96]. The role of LSD1 and JMJD2A (a histone demethylase from the JmjC domain family) as AR coregulators was also assessed in bladder cancer. In this study, treatment with the non-specific LSD1 inhibitors pargyline and tranylcypromine or *LSD1* knockdown in bladder cancer cell lines suppressed tumour cell proliferation and androgen-induced transcription[97]. In another study, LSD1 was shown to interact with β-catenin to transcriptionally upregulate LEF1, a crucial component of the WNT signalling pathway, and in turn leading to enhanced EMT-mediated cancer progression in bladder cancer cell lines and subcutaneous xenograft nude mice[98]. Treatment with the LSD1 inhibitor GSK2879552 suppressed tumour growth in bladder cancer xenograft nude mice and PDX models[98].

JMJD1A (also known as KDM3A) is a jmjC domain-containing histone demethylase that specifically demethylates H3K9me1 and H3K9me2. JMJD1A was shown to be significantly ($P < 0.01$) upregulated in human bladder carcinoma compared with normal bladder tissue samples according to analyses of data in the public gene expression omnibus (GEO) profile dataset[99]; similar results were shown in another study in which 119 human bladder cancer and 26 normal tissue samples

were analysed ($P < 0.0001$)[100]. In human bladder cancer cell lines, results from ChIP assay showed that JMJD1A regulates the expression of HOXA1 by decreasing levels of H3K9me2 at the promoter region of HOXA1. HOXA1 is a transcription factor with unique expression patterns throughout development, and belongs to the HOX family. This transcription factor family determines the cellular fate during embryonic morphogenesis and the maintenance of adult tissue architecture[101]; HOX cellular effects are strongly cell-type dependent, and abnormal HOXA1 expression has been associated with a range of conditions, including tumorigenesis[102,103]. In bladder cancer cell lines, HOXA1 is actively involved in cell-cycle regulation by controlling the transcription of proteins such as Cyclin D1 (CCND1). CCND1 binds CDK4 or CDK6 to phosphorylate crucial substrates that are necessary for cells to move from the G1 phase into the S phase[100]. In bladder cancer cells, *JMJD1A* knockdown resulted in the suppression of cell proliferation through cell-cycle arrest in the G1 phase[100]. In another study, JMJD1A was shown to be recruited to the promoter of the glycolytic gene *PGK1* in bladder cancer cell lines; JMJD1A-mediated H3K9me2 resulted in the activation of *PGK1* expression, which promoted bladder cancer cell proliferation through enhanced glycolysis. shRNA-mediated downregulation of *JMJD1A* suppressed bladder cancer cell proliferation and colony formation in cell lines, and inhibited xenograft tumour growth in nude mice[99]. These findings suggest that JMJD1A could be a bladder tumour target, and specialized JMJD1A inhibitors could be designed as antitumour agents.

Proteins in the JMJD2 family (JMJD2A–D) primarily recognize and demethylate di- and tri-methylated H3K9 and H3K36 (ref. 104). JMJD2A (also known as KDM4A) is the most frequently studied in bladder cancer. Results from a study in which JMJD2A expression was analysed in 122 clinical bladder cancer and 25 adjacent normal tissue samples showed that JMJD2A levels were significantly ($P < 0.0001$) higher in bladder cancer tissue samples than in normal adjacent tissues[105]. In another study in which 89 pairs of clinical bladder cancer and matched adjacent normal tissue samples were collected and analysed, JMJD2A was significantly ($P < 0.05$) upregulated in bladder cancer tissue samples, and high expression of JMJD2A was associated with worse survival outcomes[106]. JMJD2A promotes bladder tumour cell proliferation through regulating the G1-to-S transition in vitro[105]. In bladder cancer cell lines, siRNA-mediated knockdown of *JMJD2A* leads to substantial suppression of tumour cell growth through G1 arrest[105]. Mechanistically, in this study, chromatin immunoprecipitation sequencing (ChIP-Seq) analysis showed that JMJD2A regulated cancer-related genes (*ADAM12, CXCL5* and *JAG1*) through H3K9me3 demethylation[105]. JMJD2A also has a role in promoting migration and invasion of bladder cancer cell lines through decreasing H3K9me2 at the promoter of *SLUG*, an EMT-related transcription factor, leading to transcriptional activation of SLUG, in turn facilitating EMT in vitro[106].

Mutations in *KDM6A* (also known as ubiquitously transcribed tetratricopeptide repeat X chromosome, *UTX*), which encodes a protein that directly promotes H3K27me3 demethylation through the JmjC domain and also contributes to the methylation of H3K4 and acetylation of H3K27 as a component of COMPASS-like complex[107,108], are most common in bladder cancer (~21%) across all cancer types, and are the most frequent mutations among all chromatin regulator genes in bladder cancer[109,110]. Results from a genomic analysis showed a higher frequency of *KDM6A* mutations in low-grade NMIBC than in high-grade MIBC patient samples[110]. Intriguingly, a strong gender bias was observed, with *KDM6A* mutations found approximately twice as frequently in women than in men[111,112]. The majority of *KDM6A* mutations in bladder cancer are truncating mutations in the JmjC domain, which are predicted to result in loss of demethylase activity of KDM6A. Thus, *KDM6A* is considered to be a tumour suppressor gene in bladder cancer[28,29,110].

KDM6A reintroduction through *KDM6A*-DNA plasmid transfection into different *KDM6A*-null cancer cells, including human bladder cancer cell lines (RT112, KU-19-19), resulted in the slowing down of cell proliferation[113]. Moreover, KDM6A was also shown to have a role in inhibiting bladder cancer cell migration and invasion in bladder cancer cell lines, and metastasis in nude mice through the demethylation of H3K27me2 and H3K27me3 at *ARHGDIB* promoter regions, which leads to the activation of *ARHGDIB*, a guanosine diphosphate dissociation inhibitor that has been reported to be a suppressor of metastasis in human bladder cancer[114,115]. Results from a study in *KDM6A*-null bladder cancer cells showed that re-expression of exogenous KDM6A through mRNA nanoparticles prevented migration and invasion in these cells[116]. In vivo, intravesical delivery of *KDM6A*-mRNA through mucoadhesive mRNA nanoparticles in mice bearing orthotopic Kdm6a-null bladder cancer substantially inhibited tumour growth[116]. The number of mice with metastasis and the number of metastatic lymph nodes were also reduced[116]. Additionally, in this study, the clinical relevance of KDM6A was assessed in a cohort of 110 patients with bladder cancer, and low expression of KDM6A was associated with an increased risk of invasiveness, metastasis and poor prognosis[116]. Results from this study provide proof-of-principle evidence for intravesical delivery of messenger RNA (mRNA) through mucoadhesive nanoparticles, and show the potential of the restoration of KDM6A expression to prevent bladder cancer invasiveness and metastasis, indicating that KDM6A could be a target for future therapy.

Notably, KDM6A antagonizes PRC2 in maintaining the dynamics of repression and activation of gene expression through H3K27 methylation[117]. In urothelial carcinoma, ChIP-seq analysis showed an enrichment of H3K27me3 at specific loci in *KDM6A*-null bladder tumour cells. In this study, loss of KDM6A caused aberrant activation of transcriptional repression regulated by EZH2, and induced EZH2-dependent cell proliferation in bladder cancer cell lines[26]. Treatment of KDM6A-null bladder cancer cells with selective EZH2 inhibitors (GSK343 and GSK503) before subcutaneous implantation in nude mice significantly ($P = 0.0314$) inhibited tumour growth. Mechanistically, EZH2 inhibitors induced cell-cycle arrest in the G1 phase by reducing H3K27me3 in the insulin-like growth factor binding protein 3 (*IGFBP3*) promoter region, leading to increased *IGFBP3* expression[26]. In this study, PDX models were also used to confirm the therapeutic vulnerability of *KDM6A*-null urothelial bladder carcinoma cells to EZH2 inhibitors; in these PDXs, the growth of *KDM6A*-null engrafted tumours was significantly ($P = 0.0066$) inhibited by treatment with GSK503, but this antitumour effect was not observed in PDX models established from *KDM6A*-wild type cells[26]. These results showed that KDM6A-null urothelial bladder carcinoma cell lines and PDX tumours are sensitive to EZH2 inhibition. Results from another study led to similar conclusions. In this study, the EZH2 inhibitor EPZ011989 was tested in three *KDM6A*-null MIBC cell lines and *KDM6A*-null cell-derived xenografts; EPZ011989 treatment decreased H3K27me3 levels and caused G2-M arrest, as well as increasing cell death, in turn blocking tumour proliferation both in vitro and in vivo. Mechanistically, EZH2 inhibition in the context of KDM6A and/or SWI/SNF mutations increased the transcript level of natural killer (NK) cell-associated signalling molecules and *IFN-γ* gene expression, and activated NK cell-mediated death. However, the

exact mechanisms of epigenetic regulation of NK cell activity by EZH2 inhibition is not fully understood[118]. Results from these studies showed the potential of EZH2 inhibitors as therapeutic options for bladder carcinoma in patients harbouring KDM6A loss.

In another study, generation of a urothelium-specific deletion of Kdm6a in mice (*Kdm6a*$^{flox/flox}$ *UpkIICre*$^{+}$ (*Kdm6a*$^{\Delta/\Delta}$) mice) showed that *Kdm6A* deficiency activates cytokine and chemokine pathways[119]. The expression levels of Kdm6A correlated negatively with IL6 and CCL2 expression. IL6 and CCL2 overexpression in response to Kdm6A deletion drove macrophage migration and M2 macrophage polarization. M2 tumour-associated macrophages constitute a substantial proportion of tumour-infiltrating cells and assist tumour cell proliferation, invasion and metastasis through promoting angiogenesis and suppressing the antitumour immunoresponse[120,121]. Treating nude mice transplanted with *Kdm6a*-deficient bladder cancer cells with propagermanium, an inhibitor of the CCL2 receptor CCR2, and MR16-1, a neutralizing antibody against the IL6 receptor significantly ($P < 0.05$) suppressed tumour growth in mice transplanted with Kdm6a-deficient bladder cancer cells. Conversely, no obvious changes in tumour growth were observed in mice transplanted with Kdm6a-wild type cells[119]. These results suggest that anti-human IL6 receptor antibody therapy combined with CCR2 inhibitors might have therapeutic potential for patients with bladder cancer harbouring *KDM6A* deficiency. Additional preclinical studies are needed to explore the feasibility of this treatment approach.

KDM7A (also known as JHDM1D) is both a demethylase for H3K9me1and H3K9me2 through the JmjC domain at the C-terminus, and a methyl-recognition protein binding H3K4me3 through a PHD domain at the N-terminus[122]. In one study in which the role of KDM7A was investigated in bladder cancer, KDM7A was shown to be upregulated in tumour tissue samples compared with normal bladder tissue samples. sh-RNA-mediated *KDM7A* knockdown in bladder cancer cell lines led to impaired cell growth and migration and attenuated orthotopic bladder cancer xenograft growth in NOD-SCID gamma (NSG) immune-deficient mice[123]. Moreover, KDM7A activated AR expression in bladder cancer cells through H3K27me2 demethylation of AR target gene promoters[123,124]. The role of androgens and AR pathway in bladder cancer progression is still contradictory; however, results from several in vitro studies provided evidence that AR has a role in promoting chemotherapy resistance in bladder cancer[125]. In this study, AR expression was increased in chemotherapy-resistant bladder cancer cells compared with parental cells. Cisplatin-resistant bladder cancer cell lines were more sensitive to treatment with the KDM7A inhibitor TC-E 5002 and/or the AR antagonist enzalutamide than cisplatin-sensitive bladder cancer cell lines, and co-treatment with these two agents produced a synergistic effect[123]. These results suggest that KDM7A could be a promising target in bladder cancer; moreover, combination therapy with KDM7A and AR inhibitors could have potential as a future therapeutic strategy to overcome cisplatin resistance.

**Targeting methyl-histone recognition proteins.** Methylation of lysine or arginine is recognized by 'reader' proteins containing specific domains such as chromo-like domains of the royal family (chromo, tudor, MBT domains) and the plant homeodomain (PHD)finger motif[17].

WD repeat domain 5 (WDR5) is a H3K4 reader protein, which recognizes H3K4 methyl residues and regulates the epigenetic state by recruiting different proteins; for example, WDR5 interacts with the H3K4 methyltransferases MLL1–MLL4, and forms histone methyltransferases complexes, in turn regulating the expression of target genes[126,127]. Results from a study in which WDR5 expression was assessed in 134 bladder cancer and 77 normal tissue samples through immunohistochemistry showed that WDR5 is upregulated in bladder cancer and correlates positively with advanced tumour stage and poor survival[128]. In bladder cancer cell lines, WDR5 overexpression promoted self-renewal, cell proliferation and chemoresistance in vitro; moreover, in NOD/SCID mice, subcutaneous injection of cells overexpressing WDR5 resulted in accelerated tumour growth compared with control cells[128]. Conversely, subcutaneous tumours derived from the injection of WDR5 knockdown cells were prominently suppressed compared with control cells. Mechanistically, WDR5 promotes H3K4me3 (which is associated with activation of gene expression) in the promoter regions of pro-proliferative genes (*cyclin B1*, *cyclin E1*, *cyclin E2*, *UHMK1*, *MCL1*, *BIRC3* and *NANOG*). Thus, WDR5-mediated H3K4me3 stimulates the expression of these genes, leading to bladder cancer cell proliferation in vitro and in vivo[128].

OICR-9429 is a high-affinity small-molecule compound that binds to the central peptide-binding pocket of WDR5 competitively blocking the interactionof WDR5 with MLL, and is under preclinical development to become a novel anticancer agent[129]. In bladder cancer cell lines, treatment with OICR-9429 suppressed cell proliferation through cell-cycle arrest in G1, and reduced migration and invasion while enhancing apoptosis and chemosensitivity to cisplatin by blocking the WDR5–MLL complex-mediated H3K4me3 in target genes[130]. Interestingly, in this study, WDR5 expression correlated positively with that of programmed cell death ligand 1 (PDL1) in bladder cancer cell lines and TCGA samples. In bladder cancer cell lines, treatment with OICR-9429 reduced the expression of PDL1 induced by IFN-γ. Mechanistically, inhibition of the MLL1–WDR5 complex using OICR-9429 decreased H3K4me3 and RNA polymerase-II levels at the *PDL1* promoter, in turn decreasing *PDL1* expression and blocking PDL1-based T cell exhaustion and immune evasion. These results indicate that OICR-9429 regulates the immune microenvironment in bladder cancer and, therefore, might be a promising drug to give in combination with immunotherapy to improve clinical outcomes. However, to date, to our knowledge, no preclinical experimental evidence investigating the efficacy of combination therapy with OICR-9429 plus ICIs is available. Thus, further research is needed in this field.

**Combination approaches.** Preclinical experimental evidence provides biological rationale for combining epigenetic therapy with other therapies, such as classical oncogenic signalling pathway inhibitors (for example, the AR pathway)[123], PARP inhibitors[73] and other epigenetic drugs[60,61] (such as a EZH2 plus BRD4 inhibitors) owing to epigenetic crosstalk[131]. However, these treatment strategies currently lack experimental and clinical evidence.

The aim of combining epigenetic agents with current standard bladder cancer therapy includes preventing or overcoming resistance of current therapy or altering the transcriptional profile in advance, in turn priming the tumour to be sensitive to the second agent[132]. To date, substantial interest has been raised in the use of epigenetic agents to enhance sensitivity to chemotherapy or enhance ICI efficacy[133–135].

In one study, bladder cancer cell lines were treated with the WDR5 inhibitor OICR-9429 in combination with various concentrations of cisplatin to assess whether this combination could enhance cisplatin antitumour efficacy[130]. OICR-9429 in combination with cisplatin enhanced the cytotoxicity of cisplatin in a dose-dependent manner. The combination index calculated with the Chou–Talalay method[136] was <1, which indicated a synergistic effect of the two drugs. Similarly,

in nude mice, combination therapy with a small dose of OICR-9429 (30 mg/kg) and cisplatin (2.5 mg/kg) significantly ($P < 0.01$) inhibited subcutaneous tumour growth rates compared with treatment with either agent alone.

The effects of combination therapy with CM-272, a dual G9a and DNMT inhibitor (5 mg/kg), plus anti-PDL1 (200 μg) was assessed in a newly developed QKO metastatic bladder cancer mouse model. Simultaneous inhibition of G9a and PDL1 resulted in extensive $CD3^+$, $CD8^+$ and NK immune cell infiltrations; moreover, at the end of treatment, the percentage of QKO animals still harbouring tumour and metastases was 75% among QKO mice treated with anti-PDL1 alone, and only <30% among mice receiving combination therapy[90]. These results suggest that CM-272 in combination with ICIs could be a promising new strategy to test in the treatment of patients with bladder cancer. Exploring this strategy will be important as a means of improving the response of patients with bladder cancer.

HDAC inhibitors have been shown to enhance immunotherapy response by modulating tumour microenvironment in many solid tumours[135,137–144] and haematological malignancies[145,146]. In bladder cancer, SAHA (vorinostat), a broad inhibitor of HDACs, was delivered locally through intratumoural or intravesical injection in combination with systemic anti-PD1 (200 μg) in C57BL/6 mice with intradermal bladder cancer cell line implants, or in an orthotopic bladder cancer model established through intravesical injection of bladder cancer cells, respectively. Mice receiving intratumoural or intravesical SAHA in combination with systemic anti-PD1 therapy had better tumour regression and survival than mice receiving PD1 antibody or SAHA alone ($P < 0.05$), and showed durable antitumour immunity against a secondary and distal tumour[147]. A highly selective HDAC1 and HDAC3 inhibitor, entinostat, was tested in immunocompetent C57BL/6 mice and immunodeficient NSG mice bearing subcutaneously implanted bladder tumours derived from a BBN-induced MIBC model[148,149]. In NSG mice, after 5 weeks of treatment, entinostat (12 mg/kg) suppressed tumour growth by 30%. However, in C57BL/6 mice, treatment with entinostat induced a 90% reduction of tumour growth, indicating that entinostat antitumour efficacy is dependent on an intact immune system. In C57BL/6 mice, entinostat efficiently reshaped the tumour's immune microenvironment to an 'inflamed' state, resulting in increasing expression of immune gene signatures. Additionally, entinostat modified the profile of the presented antigens, leading to enhanced presentation of immunogenic neoantigens. When entinostat was combined with anti-PD1 treatment, 67% of mice (6 of 9) showed a complete response characterized by a substantial reduction in tumour volume to an unmeasurable level. Conversely, only 1 of 9 mice (11.1%) showed a complete response when treated with entinostat alone. No tumour formation was observed over 8 weeks in any of the mice that experienced a complete response. Encouragingly, re-injection of bladder cancer cell lines into mice previously receiving entinostat plus anti-PD1 treatment did not lead to tumour formation, whereas in mice that had never been exposed to bladder cancer cells or drug treatments, a tumour take rate of 80% was observed ($P < 0.01$). These results indicate that combination treatment with HDAC inhibitor and anti-PD1 led to complete response and long-term immunological memory in bladder cancer mouse models[149].

## Clinical trials targeting histone modifiers in bladder cancer

A few epigenetic targets have moved into clinical trials for bladder cancer (Table 3). The most extensively investigated epigenetic drugs in bladder cancer are HDAC inhibitors. To date, four HDAC inhibitors,

**Table 3 | Epigenetic drugs in clinical trials for bladder cancer**

| Drug | Target | Drug action | Treatment | Start date | Participants | Phase | Current status | Refs. |
|---|---|---|---|---|---|---|---|---|
| Tazemetostat | EZH2 | KMT inhibitor | Pembrolizumab+tazemetostat | 2019 | Patients with locally advanced and metastatic urothelial carcinoma ($n$=12) | I/II | Active, not recruiting | 84 |
| Vorinostat | Class I, II and IV HDAC | HDAC inhibitor | Pembrolizumab+vorinostat | 2016 | Patients with locally advanced or metastatic urothelial cancer ($n$=53) | I | Completed | 154 |
| Mocetinostat | Class I and IV HDAC | HDAC inhibitor | Mocetinostat monotherapy | 2014 | Patients with previously platinum-treated advanced or metastatic urothelial carcinoma and harbouring inactivating alterations of *CREBBP* and *p300* ($n$=17) | II | Completed | 153 |
| Entinostat | HDAC1 and HDAC2 | HDAC inhibitor | Pembrolizumab+entinostat | 2020 | Patients with MIBC (cT2–T4aN0M0) who did not receive neoadjuvant chemotherapy ($n$=20) | II | Active, not recruiting | 157 |
| Vorinostat | Class I, II and IV HDAC | HDAC inhibitor | Vorinostat monotherapy | 2006 | Patients with recurrent or metastatic transitional cell carcinoma of the urothelium ($n$=14) | II | Terminated (futility) | 152 |
| Belinostat | Pan-HDAC | HDAC inhibitor | Belinostat+paclitaxel or carboplatin | 2007 | Patients with advanced urothelial carcinoma of the bladder ($n$=15) | I/II | Completed | 158 |
| Vorinostat | Class I, II and IV HDAC | HDAC inhibitor | Vorinostat+docetaxel | 2007 | Patients with advanced and relapsed solid malignancies including urothelial carcinoma ($n$=12) | I | Terminated (toxicity) | 156 |
| Vorinostat | Class I, II and IV HDAC | HDAC inhibitor | Vorinostat monotherapy | 2001 | Patients with advanced solid tumours including bladder cancer who experienced disease progression refractory to standard therapy or for whom no curative therapy exists ($n$=42) | I | Completed | 150 |
| Belinostat | Pan-HDAC | HDAC inhibitor | Belinostat monotherapy | 2006 | Patients with advanced solid tumours including bladder cancer ($n$=121) | I | Completed | 151 |

HDAC, histone deacetylases; KMT, histone methyltransferases.

including vorinostat, romidepsin, panobinostat and belinostat, have been approved by the FDA for the treatment of either relapsed or refractory cutaneous T cell lymphoma, peripheral T cell lymphoma or multiple myeloma[21]. However, to date, HDAC inhibitors have not yet been approved for solid tumours.

Vorinostat (SAHA) and belinostat (PXD101) are wide-spectrum HDAC inhibitors. Back in 2001, a phase I clinical trial was started at Memorial Sloan Kettering Cancer Center to assess the efficacy of SAHA as a treatment for patients with advanced solid and haematological tumours[150]. A total of 42 patients with advanced solid tumours including bladder cancer who experienced disease progression after standard therapy or for whom no curative therapy exists were enrolled in the study. Subsequently, in 2005, a phase I dose escalation study of PXD101 was initiated[151]; overall, 121 patients with advanced solid tumours including bladder cancer were enrolled. The primary objective of this trial was to assess the safety and maximum tolerated dose of oral PXD101. These two clinical studies are currently at the completion stage, but the results have not yet been disclosed. A phase II clinical trial[152] enrolling 14 patients with recurrent or metastatic transitional cell carcinoma of the urothelium receiving vorinostat stopped for futility at an early stage. In an open-label, single-arm, phase II study, the efficacy of mocetinostat (a class I and class IV HDACi) was assessed in patients with previously platinum-treated advanced or metastatic urothelial carcinoma (mUC) and harbouring inactivating alterations of *CREBBP* and *p300* (ref. 153). The primary end point was the objective response rate (as per Response Evaluation Criteria in Solid Tumors (RECIST), version 1.1). A total of 17 patients were enrolled, 9 of whom were evaluable for efficacy. Partial response was observed in 1 of these 9 patients (11%), and all patients experienced ≥1 adverse event primarily including nausea and fatigue. Thus, in this trial, mocetinostat as a monotherapy showed modest clinical activity and was associated with a substantial toxic effect, which is insufficient to warrant further investigation of mocetinostat as a single agent in this setting.

Considering the limited efficacy of monotherapy in most bladder cancer trials, epigenetic drugs were assessed in combination with standard-of-care drugs for anticancer therapy. The safety and efficacy of vorinostat combined with chemotherapy (docetaxel) and immunotherapy (pembrolizumab) in treating advanced urothelial cell carcinoma was investigated in different clinical trials. A phase I/Ib, open label study started in 2016 to evaluate the efficacy and safety of pembrolizumab in combination with vorinostat in patients with locally advanced or metastatic urothelial, renal and prostate carcinoma showing disease progression after previous treatments[154]. The primary end point was objective response rate and the secondary end point was the assessment of adverse events. To date, this clinical trial has been completed with a total of 53 participants ultimately enrolled, and the final release of the results is awaited. Preliminary results released in 2019 showed that 4 of 37 patients (10.8%) experienced grade 3 and 4 toxic effects in the dose expansion cohorts; the most common grade 3 and 4 toxic effects were acute kidney injury ($n = 1$), anaemia ($n = 1$), diarrhoea ($n = 1$) and hypothyroidism ($n = 1$). The median PFS for cohort A (previously treated patients with urothelial and renal cancer, anti-PD1 or PDL1-naive, $n = 15$) and cohort B (previously treated patients with urothelial and renal cancer, anti-PD1 or PDL1-resistant, $n = 14$) were 2.8 months and 5.2 months, respectively. These results indicated that the combination of vorinostat and pembrolizumab is relatively well tolerated and might be effective in a subset of patients with immune checkpoint-resistant urological malignancies[155]. Another phase I study to investigate vorinostat in combination with docetaxel in patients with advanced and relapsed solid malignancies including urothelial carcinoma has stopped early owing to toxicity[156]. HDAC inhibitor entinostat has been shown to have immunomodulatory effects in preclinical studies[149]. A phase II clinical trial[157] started in 2020 is specifically designed to observe whether the HDAC inhibitor entinostat has an immunomodulatory effect in patients with bladder cancer. Patients with MIBC (cT2–T4aN0M0) who did not receive neoadjuvant chemotherapy and are scheduled to undergo RC will receive pembrolizumab on day 1 and day 22, and oral entinostat (5 mg) or placebo on day 1, day 8 and day 15. The primary objective of this study is to assess changes to immunogenomic markers (based on mRNA sequencing) after receiving the PD1 inhibitor pembrolizumab compared with patients receiving entinostat in combination with pembrolizumab. This study is a window-of-opportunity platform study with no disease progression or survival-related end points and is currently at the active but not recruiting stage. In a phase I/II clinical trial[158], a total of 15 patients with advanced urothelial carcinoma of the bladder were enrolled and received belinostat in combination with carboplatin and paclitaxel. The primary end point was safety and the secondary end point was complete or partial response. Overall, 4 out of 15 patients (26.7%) had complete or partial response, and 7 of 15 participants (46.67%) experienced serious adverse events including chest pain, dyspnoea and pneumonia. All patients experienced non-serious general adverse events. These results indicate that the safety of combination therapy still warrants attention.

To date, inhibitors for HMTs have been tested in only one clinical trial for bladder cancer. Tazemetostat, a small-molecule catalytic inhibitor targeting EZH2, has been approved by the FDA for the treatment of epithelioid sarcoma[159]. In a phase I/II trial started in 2019, the safety and efficacy of pembrolizumab in combination with tazemetostat were assessed in patients with locally advanced and metastatic urothelial carcinoma[84]. Overall, 12 patients with cisplatin-refractory mUC received tazemetostat (800 mg) plus pembrolizumab (200 mg) every 3 weeks. In 3 patients (25%), treatment-related grade 3 and 4 adverse events including sepsis, lymphopenia, anaemia, increased alkaline phosphatase, and HSV oral infection were observed. A total of 3 patients (25%) experienced partial response and 3 patients (25%) had stable disease. Median progression-free survival (PFS) was 3.1 months (95% CI: 2.3–not available (NA), and median OS was 8.0 months (95% CI: 4.7–NA)[160]. These preliminary results indicated that this combination approach was feasible, well tolerated and resulted in durable responses in patients with poor-risk chemo-refractory urothelial carcinoma.

## Challenges of epigenetic therapies

Understanding and addressing the challenges within bladder cancer epigenetics is a multifaceted endeavour. Bladder cancer is a heterogeneous disease, with diverse genetic and epigenetic profiles observed among patients. This heterogeneity can make it difficult to identify a single target or treatment approach that is effective for all patients, which partially explains why most targeted therapies and epigenetic drugs failed in clinical trials for bladder cancer. Thus, identifying reliable biomarkers that can accurately categorize different tumour subtypes and guide personalized treatment strategies is important.

Epigenetic regulation is an intricate process involving a network of interactions among different epigenetic marks, enzymes and pathways. Our understanding of the epigenetic landscape of bladder cancer is still evolving. To date, the epigenetic landscape of bladder cancer cells has not been fully mapped on a structural, biochemical and functional level. Lack of a comprehensive understanding of epigenetic changes

in bladder cancer and underlying mechanisms introduces barriers to the identification of most disease-related targets and associated biology[161]. Moreover, epigenetic modifications can interact with each other and with genetic mutations in complex ways; thus, modulating a single epigenetic enzyme might lead to a panel of epigenetic enzymes alterations. For example, EZH2 inhibition was shown to lead to a global landscape change of histone marks[131]. EZH2 inhibitors remain unsatisfactory and limited to certain haematological malignancies. In a study in which EZHi-sensitive and EZHi-resistant pan-cancer cell lines were compared, H3K27 acetylation was shown to be greatly upregulated in EZHi-resistant cells[131]. H3K27me loss, which is expected to increase the accessibility of p300 to the H3K27 residue only caused an increase in H3K27ac in the resistant cells[131]. In EZHi-resistant cells, MLL1 is recruited as a p300 binding partner to facilitate p300-catalysed H3K27ac[131]. A switch from H3K27me to H3K27ac induces transcriptional reprogramming from a silenced to an activated state, leading to the activation of multiple onco-pathways in a cell context-dependent manner (for example, Wnt pathway activation in hepatocellular carcinoma cells). Moreover, treatment with a BRD4 inhibitor improved the efficacy of EZHi in >11 resistant cancer cell lines. These results indicated that transcriptional changes induced by EZH2 inhibition are dispensable for the H3K27me-regulated transcriptional network. Instead, MLL1–p300-dependent H3K27ac is essential to determine the ultimate transcriptional output[131]. EZH2i induces this crosstalk between H3K27me and H3K27ac and leads to oncogene activation; thus, targeting this crosstalk might expand the therapeutic benefits of EZH2 inhibitors on the basis of tumour-intrinsic MLL1 expression.

Many epigenetic enzymes do not specifically catalyse the addition of epigenetic modifications on histone substrates, but can post-translationally modify non-histone targets, such as p53 (ref. 162), STAT3 (ref. 163) and AR[164]. Additionally, some epigenetic enzymes function as an integral part of heterogeneous multimeric complexes (such as PRC2 and the COMPASS complex). One crucial point to emphasize is that inhibition of an enzymatically active subunit and disruption of complex stability might not necessarily yield equivalent effects. For example, EZH2 is an enzymatic subunit of PRC2, but in addition to a well-established function as an H3K27 histone methyltransferase and transcriptional suppressor, EZH2 has additional non-catalytic roles independent of PRC2, acting as a transcriptional coactivator and directly influencing the activity of transcription factors and other proteins[163–166]. Moreover, cancer cells with SWI/SNF mutations were shown to primarily rely on a non-catalytic function of EZH2 to stabilize the PRC2 complex, and the dependency of these cells on EZH2 histone methyltransferase activity is limited[167]. The discovery of non-enzymatic functions for EZH2 raises the possibility that the enzymatic inhibitors currently explored in clinical trials might not fully suppress its oncogenic activity. Thus, in preclinical experiments, knockdown of an epigenetic regulator can fail to predict the activity profile associated with the catalytic inhibition of this protein, suggesting that these preclinical results should be considered with caution.

Overall, discovering epigenetic crosstalk phenomena and understanding molecules and pathways driving these alterations is pivotal for the development of therapies that can target specific pathways and processes driving tumorigenesis with improved efficiency. Moreover, the presence of epigenetic plasticity in cancer cells, consisting in the ability of cancer cells to adapt and alter their epigenetic profiles to evade treatment effects, underscores the need for innovative approaches to combatting drug resistance[168]. Thus, targeting a single epigenetic modification might not be sufficient to induce long-lasting responses and reverse the complex changes associated with cancer; developing therapies that target multiple facets of the disease's epigenetic landscape, or combining epigenetic therapies with other treatment modalities, might hold better promise than monotherapies.

Epigenetic drugs might affect not only cancer cells but also tumour microenvironment cells and normal cells. Thus, adverse effects of epigenetic drugs cannot be ignored, and constitute one of the most important factors causing the failure of these drugs in some clinical trials in bladder cancer. However, many of the currently used preclinical models are either pure tumour cell lines or immunocompromised mouse models, which results in an inability to observe the effect of epigenetic drugs on the tumour microenvironment. This evidence highlights the importance of choosing appropriate models in basic or translational preclinical research. Syngeneic mouse models, genetically engineered mouse models, or humanized mouse models are needed to explore the collective interactions among epigenetic drugs, tumour cells and the tumour microenvironment. However, to date, cellular and simple mouse models are still important for evaluating the antitumour activity of monotherapies and contribute to the development of clinical strategies.

Achieving success in epigenetic therapy for bladder cancer necessitates a precision medicine approach incorporating patient-specific factors into treatment strategies, as well as well-designed clinical trials that include patient stratification and reasonable outcome assessment. Collaborative efforts and interdisciplinary research are crucial to advancing our understanding of bladder cancer biology and translating this knowledge into improved therapeutic interventions.

## Conclusions

Mutations in chromatin remodelling genes dominate the driver landscape of bladder cancer and have the potential to reverse tumorigenic modifications, in turn reversing the path that would eventually lead to cancer progression. Thus, targeting epigenetic enzymes holds promise in bladder cancer treatment. The reversibility, suitability for personalized medicine, and potential to address early events in cancer development make epigenetic treatments appealing. Many small-molecule compounds have achieved promising outcomes in preclinical studies, but only a limited number of these compounds have progressed to clinical trials. Trial results are mixed, with most trials focusing on safety evaluation, and at least some results suggesting that the safety profile is acceptable. Progress in the treatment of bladder cancer is slower than that of many solid tumours. Thus, additional targets and drugs should be assessed in clinical trials to increase therapeutic possibilities for patients. In general, combination therapy strategies showed more promising prospects than single-target therapies. Further basic and translational research are needed to understand in-depth epigenetic mechanisms and identify additional target drugs that could be tested alone or in combination with other therapies in future clinical trials.

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## Author contributions
S.Z. and X.X. researched data for the article. S.Z., T.L. and P.T. contributed substantially to discussion of the content. S.Z. wrote the article. Q.W., S.Z., C.C. and P.T. reviewed and/or edited the manuscript before submission.

## Competing interests
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