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Original research

Identification of PRMT5 as a therapeutic target in cholangiocarcinoma

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ABSTRACT

Background Cholangiocarcinoma (CCA) is a very difficult-to-treat cancer. Chemotherapies are little effective and response to immune checkpoint inhibitors is limited. Therefore, new therapeutic strategies need to be identified.

Objective We characterised the enzyme protein arginine-methyltransferase 5 (PRMT5) as a novel therapeutic target in CCA.

Design We evaluated the expression of PRMT5, its functional partner MEP50 and methylthioadenosine phosphorylase (MTAP)—an enzyme that modulates the sensitivity of PRMT5 to pharmacological inhibitors—in human CCA tissues. PRMT5-targeting drugs, currently tested in clinical trials for other malignancies, were assessed in human CCA cell lines and organoids, as well as in two immunocompetent CCA mouse models. Transcriptomic, proteomic and functional analyses were performed to explore the underlying antitumoural mechanisms.

Results PRMT5 and MEP50 proteins were correlatively overexpressed in most CCA tissues. MTAP was absent in 25% of intrahepatic CCA. PRMT5-targeting drugs markedly inhibited CCA cell proliferation, synergising with cisplatin and gemcitabine and hindered the growth of cholangiocarcinoma organoids. PRMT5 inhibition blunted the expression of oncogenic genes involved in chromatin remodelling and DNA repair, consistently inducing the formation of RNA loops and promoting DNA damage. Treatment with PRMT5-targeting drugs significantly restrained the growth of experimental CCA without adverse effects and concomitantly induced the recruitment of CD4 and CD8 T cells to shrinking tumourous lesions.

Conclusion PRMT5 and MEP50 are frequently upregulated in human CCA, and PRMT5-targeting drugs have significant antitumoural efficacy in clinically relevant CCA models. Our findings support the evaluation of PRMT5 inhibitors in clinical trials, including their combination with cytotoxic and immune therapies.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Cholangiocarcinomas (CCAs) are very difficult-to-treat cancers, very resistant to chemotherapy and exhibit a very limited response to immune checkpoint inhibitors. The identification of new targets to improve the response to systemic therapies is much needed.

WHAT THIS STUDY ADDS

⇒ We demonstrate that the protein arginine-methyltransferase (PRMT5) is upregulated in a high proportion of CCA tissues. Pharmacological targeting of PRMT5 with clinically approved small molecules potently inhibits CCA cell growth, synergising with chemotherapeutic drugs. This study shows that treatment with PRMT5 inhibitors has antitumourigenic effects in clinically relevant mouse CCA models.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings highlight the relevance of PRMT5 as a new therapeutic target in CCA and provide experimental evidence supporting the initiation of clinical trials with PRMT5 inhibitors in patients with CCA.

INTRODUCTION

Cholangiocarcinomas (CCAs) are very aggressive cancers that can be classified according to their origin within the biliary tree as intrahepatic (iCCA), perihilar and distal CCAs, with the latter two collectively known as extrahepatic CCAs (eCCA).[1] Overall, the incidence of CCA is globally increasing, mainly due to a rise in iCCA cases.[1 2] Patients are usually diagnosed at late stages when surgical resection is not possible and chemotherapy is mostly palliative, resulting in a dramatically low 5-year overall survival between 7% and 20%.[2–4] High-throughput sequencing analyses have elucidated

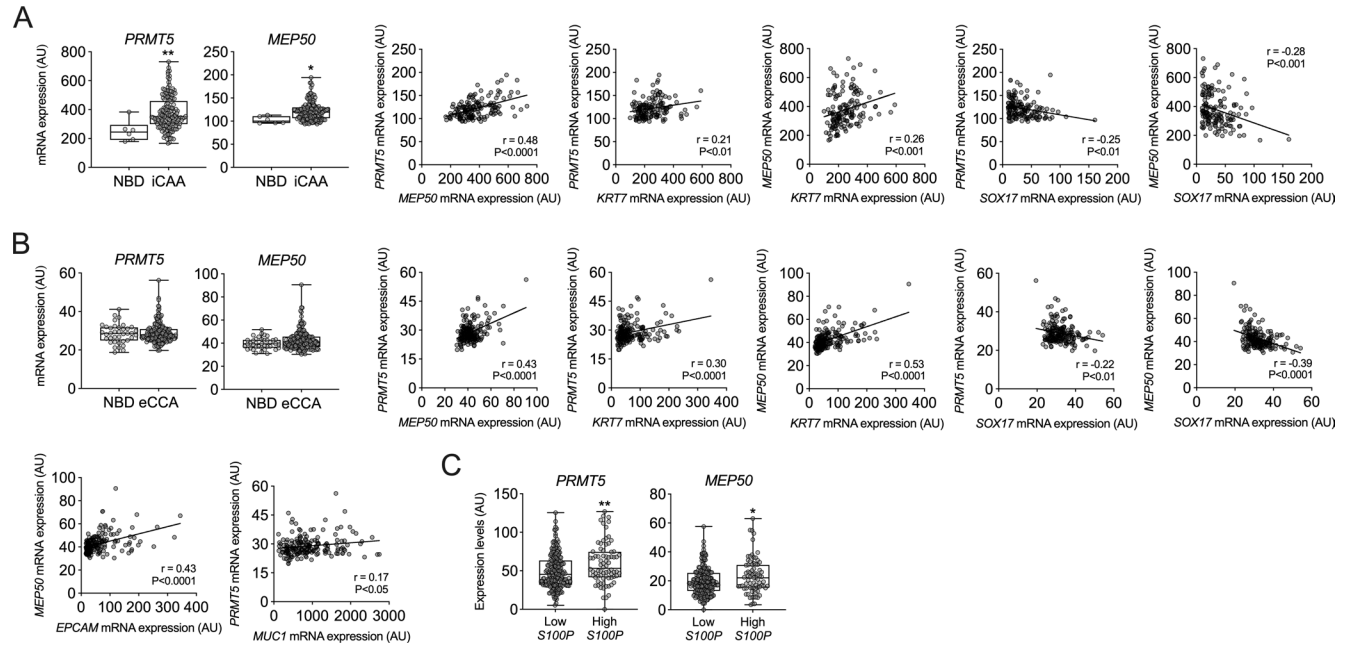

Figure 1 PRMT5 and MEP50 gene expression in human cholangiocarcinoma (CCA). (A) *PRMT5* and *MEP50* mRNA levels in intrahepatic CCA (iCCA) compared with normal bile ducts (NBDs) (transcriptomic dataset GSE32225), analysis of their mutual gene expression correlation and correlation with the indicated genes. (B) *PRMT5* and *MEP50* mRNA levels in eCCA compared with NBDs (transcriptomic dataset GSE132305), analysis of their mutual gene expression correlation and correlation with the indicated genes. (C) *PRMT5* and *MEP50* gene expression in an integrated dataset of iCCA samples (n=276) according to the levels of expression of the *S100P* gene, a marker of highly aggressive tumours. * p<0.05, ** p<0.01.

the molecular landscape of CCAs, identifying actionable alterations such as *FGFR2* fusions and *IDH1* mutations in iCCA, and *HER2* amplifications and *KRAS* mutations more frequently found in eCCAs, enabling the development of targeted therapies.[1] Encouraging signs of clinical activity are observed for drugs targeting *FGFR2* fusions and *IDH1* mutations in selected patients. However, the low prevalence of these mutations and the emergence of acquired drug resistance limits their effectiveness.[1 5] On the other hand, clinical studies of immune checkpoint inhibitors (ICIs) as monotherapy have shown limited efficacy, consistent with the low frequency of mismatch repair deficiency, microsatellite instability or tumour mutational burden in biliary cancers.[6] These outcomes have promoted the integration of cytotoxic, targeted and immune approaches in combined therapies, yielding promising results.[5 7] It has become evident that CCAs are notoriously difficult-to-treat cancers, and therefore, while the outcomes of ongoing trials testing combination strategies are eagerly awaited new therapeutic avenues should be explored.

These novel approaches may come from mechanisms emerging as the biological makeup of CCA is unravelled. In this context, accumulating evidence indicates an important role for epigenetic changes in the induction and progression of CCA.[8] These protumourigenic changes extend beyond mutations in genes involved in chromatin remodelling and include alterations in DNA methylation and histone post-translational modifications (PTMs).[8] The reversible nature of epigenetic modifications makes them attractive targets for pharmacological intervention, as recently demonstrated for DNA-methyltransferase and histone lysine-methyltransferase inhibitors in experimental models of CCA.[9–11] Arginine methylation is another type of PTM carried out by a family of nine protein arginine-methyltransferases (PRMTs) classified into three groups: type I enzymes that catalyse asymmetric arginine dimethylation (PRMT1, 2, 3, 4, 6 and 8); type II enzymes which are symmetrical dimethyltransferases (PRMT5 and 9) and a type III monomethyltransferase (PRMT7).[12] Among them, PRMT5 is highly expressed in cancer cells and tissues, and its overexpression is directly linked to tumour progression in a broad range of solid malignancies.[12–14] Together with methylosome protein 50 (MEP50), also known as WDR77, PRMT5 forms a hetero-octameric complex that catalyses the methylation of arginine residues in histone tails, leading to the repression of tumour suppressor genes and the activation of genes involved in cell proliferation and invasion.[12 13 15] Importantly, PRMT5 targets also comprise many non-histone proteins involved in key pathways critical for the growth and survival of cancer cells, including growth factor signalling, pre-mRNA splicing, protein translation and DNA damage repair.[13 15–17] These observations, together with robust preclinical studies demonstrating the efficacy of PRMT5 inhibition in therapy-refractory tumours such as pancreatic cancer and hepatocellular carcinoma,[18–21] led to the development of PRMT5 inhibitors that are showing promising preliminary results in clinical trials.[22–24] In this study, we show that PRMT5 and MEP50 are frequently overexpressed in CCAs, and we provide experimental in vitro and in vivo evidence supporting the efficacy of PRMT5 inhibition for the treatment of this malignancy.

MATERIALS AND METHODS

Human samples

CCA tissues were obtained from patients with iCCA (n=73; 43 surgical samples, 30 needle biopsies) or eCCA (n=23) that underwent biopsies or surgical resection for diagnostic or therapeutic purposes. Tumour grade was established on anatomopathological examination of tissue sections according to the WHO Classification of Tumours, Digestive System Tumours, fifth edition. Patients or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Mouse models of CCA

The $Jnk^{\Delta hepa}$ mice were previously described.[25] For CCA induction, 2-week-old male mice received 25 mg/kg (intraperitoneally)

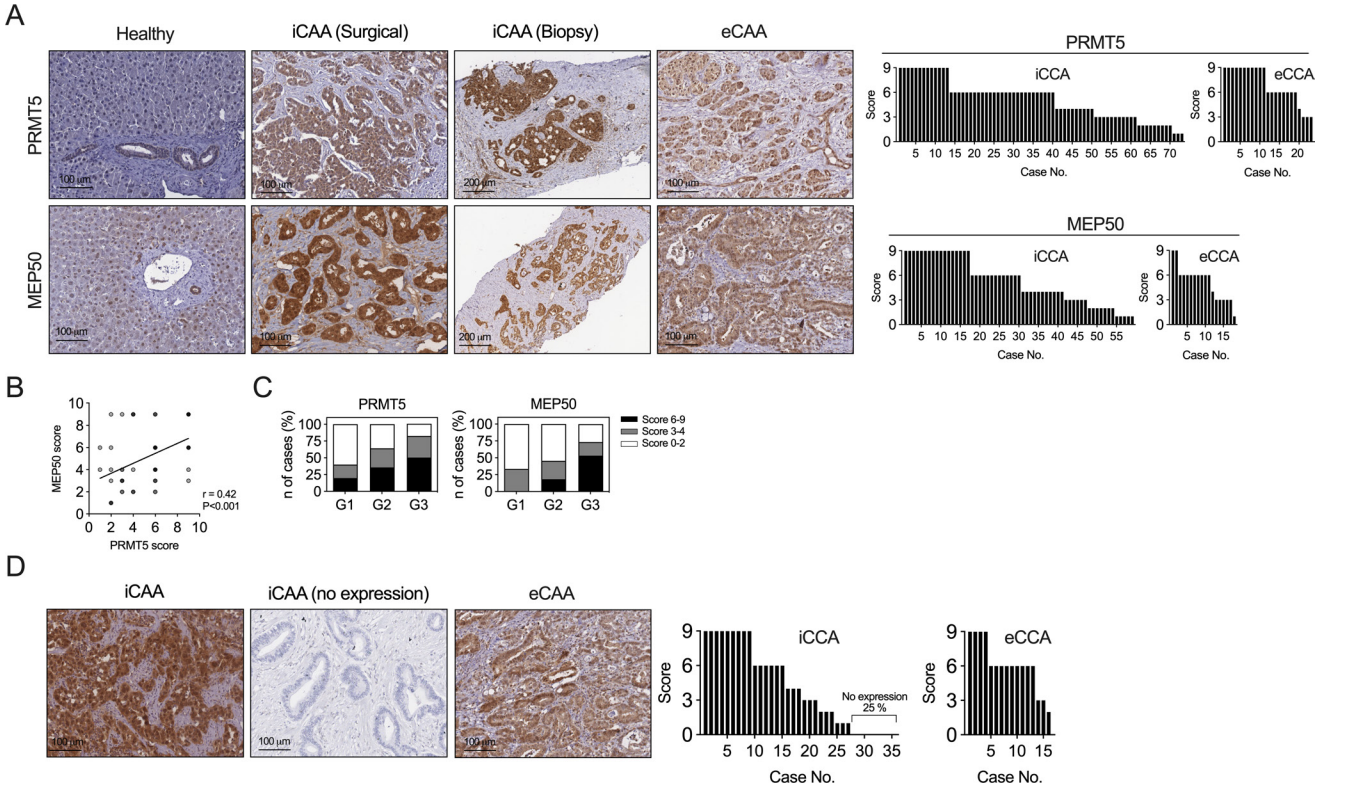

Figure 2 Immunohistochemical analysis of PRMT5, MEP50 and MTAP proteins in human cholangiocarcinoma (CCA) tissues. (A) Representative images of PRMT5 and MEP50 immunohistochemical analyses in human iCCA and eCCA tissue samples, including iCCA samples obtained by needle biopsies. Graphs show the immunohistochemical scores for each protein in intrahepatic CCA (iCCA) and extrahepatic CCA (eCCA) tissue samples. (B) Analysis of the correlation between PRMT5 and MEP50 protein levels in iCCA tissue samples. (C) Graphs showing the distribution of PRMT5 and MEP50 scores according to tumour grade (G1–G3). (D) Representative images of MTAP immunohistochemical analyses in human iCCA and eCCA tissue samples. Graphs show the immunohistochemical scores for MTAP in iCCA and eCCA tissue samples.

of diethylnitrosamine (DEN) and from week 8 to week 25 were treated with CCl_4 (0.5 mL/kg, intraperitoneally) twice per week.[9 26] From weeks 22 to 25, one group of mice (n=6) was treated with GSK3326595 (MedChemExpress, Monmouth, New Jersey, USA), at 50 mg/kg orally. daily. Control mice (n=6) received the same volume of vehicle, as described.[20] All mice were littermates.

CCA was also induced in 5-week-old C57BL/6J mice by hydrodynamic tail vein injection (HTVI) of plasmids coding for mutant TAZ (TAZS89A, 10 µg/mouse), myr-AKT (10 µg/mouse) and the sleeping beauty transposase (SB, 0.8 µg/mouse) (GenScript, Piscataway, New Jersey, USA) as described.[27] One group of mice (n=12) was treated with JNJ-64619178 (MedChemExpress)(10 mg/kg orally daily) and control mice (n=12) received the same volume of vehicle, as described.[21] As reported for other HTVI oncogene-induced CCA models treatments started 2 days after HTVI,[11] and mice were treated for 2 or 3 weeks. C57BL/6J mice (n=4) without HTVI injection nor treatment were used as healthy controls and were sacrificed at same ages as the treated groups. Animals received humane care and protocols were according to the Animal Care Committee of the University of Navarra (approval #R-CP001-15GN) and the Autonomous Region of Madrid (approval #PROEX210/18 and 125.1/20) guidelines.

Additional information is provided in online supplemental materials.

RESULTS

PRMT5 and MEP50 expression in human CCA

First, we examined the expression of *PRMT5* and *MEP50* in well-characterised transcriptomic datasets from human CCA tissues. In the cohort of Sia *et al*, including iCCAs,[28] we observed a significant upregulation of *PRMT5* and *MEP50* gene expression compared with normal bile ducts (NBDs), and a direct positive correlation between the expression levels of both genes in tumour tissues (figure 1A). Here, we also found a positive correlation in *PRMT5* and *MEP50* expression and that of the CCA marker *KRT7*, and an inverse correlation with the tumour suppressor gene *SOX17*[3] (figure 1A). In another transcriptomic dataset including eCCA samples,[29] we did not observe a statistically significant upregulation of *PRMT5* and *MEP50* in tumourous tissues versus NBDs; however, we did find a positive correlation between the expression of both genes, and with that of *KRT7*, *MUC1* and *EPCAM* (for *MEP50*), while a negative correlation with *SOX17* was observed (figure 1B). *S100P* expression has been robustly associated with iCCA aggressiveness.[30] When we analysed the integrated data from eight RNAseq studies performed in iCCA (see online supplemental materials and methods), we found that *PRMT5* and *MEP50* expression was significantly higher in tumours showing higher levels of *S100P* expression (figure 1C).

PRMT5 and MEP50 were also examined by immunohistochemistry. Both proteins were readily detected in malignant tissues, including needle iCCA biopsies (patients' and tumours' characteristics are described in online supplemental table 1), and quantitative evaluation encompassing signal intensity and the proportion of positive cells stained indicated that both iCCAs and eCCAs frequently overexpress PRMT5 and MEP50 (figure 2A) (representative images are shown in online supplemental figure 1). A positive correlation was also observed in the expression levels of both proteins in iCCAs tissues (figure 2B). Nuclear localisation of PRMT5 has been associated with worse

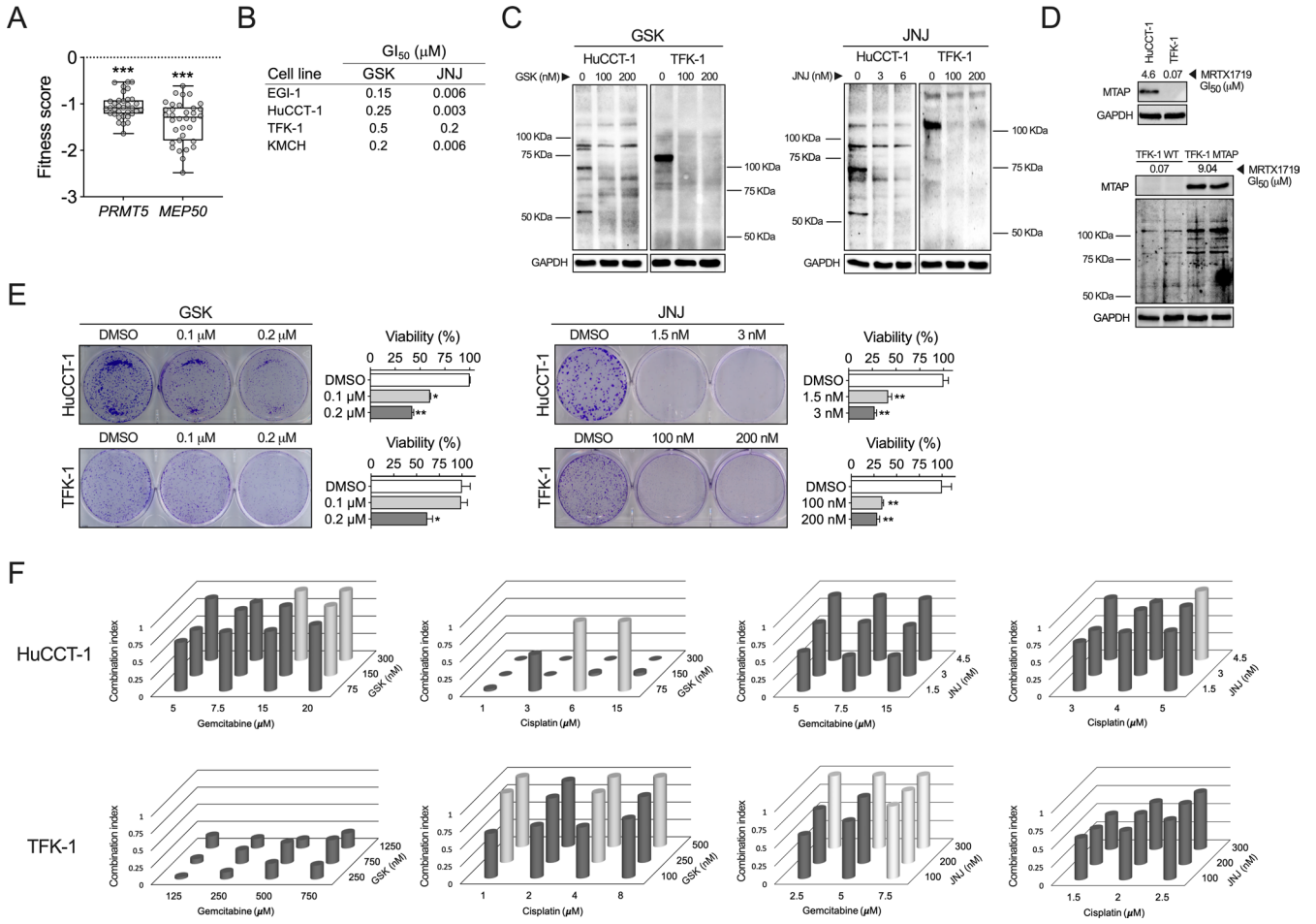

Figure 3 Impact of PRMT5 targeting on the growth of cholangiocarcinoma (CCA) cells. (A) CCA cell lines viability (fitness score) on CRISPR/Cas9 drop-out screen for PRMT5 and MEP50. Negative values indicate reduced survival on gene knockout. Data were retrieved from https://score.depmap.sanger.uk/. (B) GI_{50} values for the PRMT5 inhibitors GSK3326595 and JNJ64619178 in the indicated CCA cell lines. (C) Immunoblot analysis of PRMT5-dependent symmetric dimethylarginine (SMDA) protein marks in control and GSK3326595 or JNJ64619178 treated HuCCT-1 and TFK-1 cells at the indicated doses for 5 days. (D) Upper panel: GI_{50} values for MRTX1719 in HuCCT-1 and TFK-1 cells and analysis of MTAP protein levels by immunoblot. Lower panel: GI_{50} values for MRTX1719 in TFK-1 control cells (TFK-1 WT) and TFK-1 cells expressing MTAP (TFK-1 MTAP), and immunoblot analyses for MTAP and SMDA protein marks in these cells. (E) Colony formation assays in the indicated cell lines treated with GSK3326595 or JNJ64619178. (F) Combination studies of the growth inhibitory effects of GSK3326595 or JNJ64619178 with cisplatin or gemcitabine in HuCCT-1 and TFK-1 cells. Dark grey bars denote the existence of synergism at the indicated doses (combination index, CI, <1). CI was calculated as described in online supplemental materials and methods. *p<0.05, **p<0.01 and ***p<0.001 vs controls.

clinical outcomes in other solid tumours.[31] We observed that in 63% of iCCA samples PRMT5 was found in the cells' cytoplasm and also in 100% of cells' nuclei while this was the case for 78% of eCCA samples. Only in about 4% of samples, both iCCA and eCCA, PRMT5 was localised exclusively in the cytoplasm. Noteworthy, iCCAs with higher PRMT5 and MEP50 scores were more represented among higher-grade tumours (figure 2C).

It is known that PRMT5 methyltransferase activity is highly sensitive to the intracellular levels of methylthioadenosine (MTA),[32] a metabolite in the methionine salvage pathway and a S-adenosylmethionine competitive inhibitor of PRMT5.[16 33 34] MTA is catabolised by the ubiquitous enzyme MTA-phosphorylase (MTAP), and the *MTAP* gene is frequently deleted in solid tumours.[35] MTAP loss leads to MTA accumulation and a potential improvement in the therapeutic efficacy of PRMT5-targeting drugs.[24 33–35] Therefore, we evaluated MTAP expression by immunohistochemistry in CCA tissues. We observed that MTAP protein was undetectable in 25% of iCCA samples (9 out of 36 samples analysed) (figure 2D and online supplemental figure 1). Together, these observations highlight the frequent overexpression of PRMT5 and MEP50 in human CCA in association with a more advanced disease, and that homozygous *MTAP* loss is a relatively frequent event in iCCA.

Effect of PRMT5 targeting on the growth of human CCA cells

A first indication of PRMT5 as a relevant therapeutic target in CCA was obtained by accessing data of a CRISPR/Cas9 drop-out screen in human CCA cell lines (n=24) (https://score.depmap.sanger.uk/).[36] We found that 83% of CCA cell lines had significantly impaired growth on genetic inactivation of *PRMT5*, and a similar observation was made for *MEP50* (figure 3A). Next, we tested the effect of two clinically approved PRMT5 pharmacological inhibitors, GSK3326595 and JNJ64619178,[22] on the growth of four well-characterised CCA cell lines.[37] In all cases, GI_{50} values were in the low nanomolar range (figure 3B), and as expected both drugs significantly reduced the global levels of symmetric dimethyl arginine (SDMA) in protein extracts from CCA cells

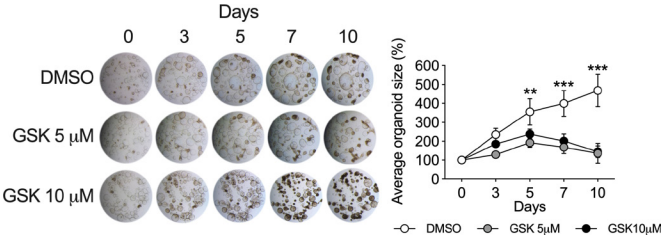

Figure 4 Pharmacological targeting of PRMT5 inhibits the growth of human CCA tumouroids. Effect of GSK3326595 on the growth of human CCA tumouroids. Representative images of control and treated tumouroids at the end of treatments are shown along the quantification of tumouroids growth in the indicated conditions. **p<0.01 and ***p<0.001 vs controls (DMSO, vehicle-treated cultures). CCA, cholangiocarcinoma; DMSO, dimethyl sulfoxide.

(figure 3C). MRTX1719 is a small molecule that selectively binds and inhibits PRMT5 complexed with MTA. It was recently developed with the aim of increasing the efficacy of PRMT5 targeting in *MTAP*-deleted cancers in which MTA levels build up, enabling a personalised therapy and potentially reducing toxicities.[24] Interestingly, when we tested MRTX1719 we found a higher GI_{50} value in the MTAP-positive HuCCT-1 cells compared with MTAP-negative TFK-1 cells (figure 3D), contrary to what we observed for GSK3326595 and JNJ64619178 (figure 3B). To test if this enhanced response to MRTX1719 could indeed be attributed to the *MTAP* status, we generated a TFK-1 cell line stably expressing MTAP. Consistent with the inhibitory effect of MTA on PRMT5 activity[24 33 34] TFK-1-MTAP cells had higher basal SDMA levels than TFK-1-WT (figure 3D). Importantly, we found that TFK-1-WT cells were more sensitive to MRTX1719 than TFK-1-MTAP (figure 3D), suggesting that patients harbouring *MTAP*-deleted CCAs could benefit from this personalised therapy. In agreement with their antiproliferative activity PRMT5 inhibitors also markedly impaired the clonogenic potential of CCA cells (figure 3E). Combination strategies are actively being explored to improve the efficacy of cisplatin and gemcitabine-based CCA chemotherapy.[5 7] We tested the effects of GSK3326595 and JNJ64619178 combined with cisplatin or gemcitabine and found a clear synergistic effect in HuCCT-1 and TFK-1 cells at most dose combinations (figure 3F). To further examine the antitumoural effects of PRMT5 inhibition, we used human CCA-derived organoids (tumouroids) that retain the histological characteristics, expression profile, genomic landscape and neoplastic behaviour of the original tumours from which they were derived.[38] We observed that treatment with GSK3326595 potently reduced tumouroid growth, with the presence of smaller organoids filled with dying (figure 4). In sum, these findings indicate that PRMT5 is required for CCA cell growth, and that its inhibition markedly enhances the sensitivity of CCA cells to chemotherapeutic agents.

Mechanisms involved in the anti-CCA effect of PRMT5 inhibition

To explore the antitumoural mechanisms of PRMT5 targeting in an unbiassed manner, we performed a transcriptomic analysis in the well-characterised HuCCT-1 iCCA cells[9 10 37] on JNJ64619178 treatment. We detected 602 upregulated and 193 downregulated genes compared with controls. As expected, gene ontology biological process (GO-BP) functional classification of differentially expressed genes identified general categories related to tumourous behaviour, such as cell cycle progression and cell migration (figure 5A). Consistent with the biological roles of PRMT5, we observed changes in genes related to chromatin regulation, mRNA splicing, DNA repair and DNA damage response.[13 15 39] Interestingly, we also found a significant enrichment of genes involved in processes like inflammation, immune response and lipid metabolism (figure 5A). PRMT5 is critical for spliceosome assembly, and tumour cells are quite dependent on a hyperactive core splicing machinery.[15 39] To broadly assess whether splicing impairment occurred on JNJ64619178 treatment, we analysed global alternative splicing (AS) changes in our RNAseq dataset. We identified 1374 AS events in protein-coding genes, with the majority being skipped exons and retained introns (figure 5B), two events that have shown PRMT5 dependency in other tumour types.[17 40] GO analysis of the altered splicing events revealed significant enrichment in genes involved in cell division, the regulation of RNA splicing, inflammatory and metabolic pathways and several categories related to DNA damage and repair (figure 5C,D). Among the aberrantly spliced genes we identified the chromatin modifiers *KAT5* and *KMT5C*, critically involved in the DNA-damage response and that undergo altered splicing in *PRMT5* knockout cells[17]; and *POLD1*, the major catalytic DNA polymerase subunit essential for DNA replication fidelity,[41] which splicing also depends on PRMT5[40] (figure 5D).

To further characterise the antineoplastic effects of PRMT5 inhibition in CCA cells, we performed complementary proteomic analyses. We identified 795 proteins that were differentially expressed (602 upregulated and 193 downregulated) in JNJ64619178-treated HuCCT-1 cells. Consistent with the RNAseq analysis, GO-BP functional classification identified enriched categories encompassing genes involved in cell cycle, chromatin regulation, RNA metabolism and splicing, DNA repair, and again immune response and lipid metabolism (figure 6A,B). We validated the effects of PRMT5 inhibition on the expression of relevant cancer-associated genes involved in chromatin remodelling, epigenetic regulation and DNA synthesis (*HELLS*, *UHRF1*, *POLD1*),[9 41 42] antigen presentation (*NLRC5*, *B2M*, *PSMB9*, *TAP1*)[43] and DNA repair (*BRCA1*, *BRCA2*, *ATR*, *ATM*, *RAD51*, *RAD51AP1*)[44] in HuCCT-1, TFK1 and KMCH cells (figure 6C and online supplemental figure 2). Noteworthy, the remarkable effects of PRMT5 inhibition on HELLS expression may also take place at the level of protein stability, as JNJ64619178-induced HELLS protein downregulation was significantly attenuated by the proteasome inhibitor MG-132 (online supplemental figure 4). Moreover, *POLD1* and *HELLS*, which are important genes in DNA replication are also highly relevant in DNA repair and genome stability.[41 42] Collectively, these observations suggest that PRMT5 activity would be critical to support mRNA splicing dynamics and DNA integrity in rapidly proliferating CCA cells, along with a potential role in tumour immune evasion.

PRMT5 inhibition promotes DNA damage in CCA cells

In view of the synergism of PRMT5 inhibitors when combined with cisplatin and gemcitabine (figure 3E), and their prominent effects on the expression of genes involved in the DNA damage response (DDR), we directly measured the levels of DNA strand breaks in a comet assay.[18] As shown in figure 7A, this analysis indicated an increased frequency of DNA breaks in JNJ64619178-treated CCA cells. This response was also accompanied by the induction of apoptosis (online supplemental figure 4). DNA damage can be triggered by the formation of RNA/DNA hybrid structures known as R-loops, which lead to the exposure of single-stranded DNA and the impairment of replication fork

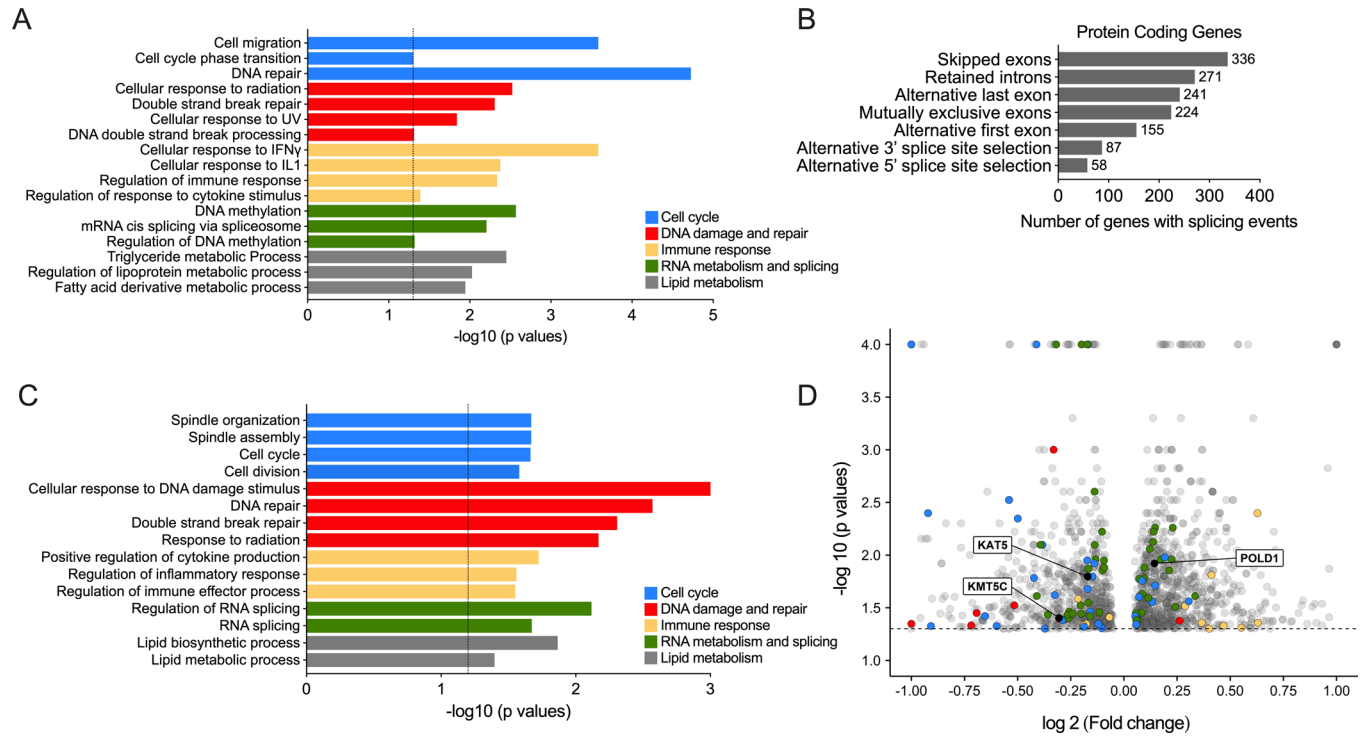

Figure 5 PRMT5 inhibition markedly alters the expression of genes involved in chromatin regulation, DNA damage repair, lipid metabolism and immune response and induces aberrant mRNA splicing in CCA cells. (A) Most relevant categories of differentially expressed genes identified by Gene Ontology Biological Process (GO-BP) functional classification in HuCCT-1 cells treated with JNJ64619178 (3 nM, 3 days treatment). (B) Number of alternative splicing events in protein-coding genes differentially affected by PRMT5 inhibition in HuCCT-1 cells. (C) GO-BP functional classification of the aberrant splicing events in HuCCT-1 cells treated with JNJ64619178. (D) Volcano plot representing all the significant ($p<0.05$) splicing events. Selected genes from the indicated GO-BP functional categories are highlighted. The chromatin modifiers and DNA replication and repair genes *KAT5*, *KMT5C* and *POLD1* are identified.

progression causing replication stress.[45] By immunofluorescence analysis with the R-loop specific S9.6 antibody we found that PRMT5 inhibition leads to R-loop formation in both HuCCT-1 and TFK-1 cells (figure 7B). On DNA damage, histone 2AX (H2AX) is phosphorylated at DNA lesion sites and critically contributes to the recruitment of repair factors.[46] Interestingly, we found that the levels of H2AX protein were significantly reduced on PRMT5 inhibition (figure 7C). These findings are consistent with the role of PRMT5 in the preservation of H2AX protein stability recently described in glioblastoma cells[47] and emphasise the role of PRMT5 in maintaining genomic stability in CCA cells.

Pharmacological targeting of PRMT5 inhibits CCA development in vivo

Next, we evaluated the antitumoural efficacy of PRMT5 inhibition in two complementary mouse CCA models using two different PRMT5 pharmacological inhibitors. In the first model, mice with hepatocellular c-Jun N-terminal kinase 1/2 (*Jnk1/2*) deletion ($Jnk^{\Delta hepa}$) treated with CCl_4 and DEN ($Jnk^{\Delta hepa}$+CCl_4+DEN) develop transplantable tumours with histological and molecular features consistent with human iCCA in a context of inflammation and fibrosis.[9 25 26] Therefore, after a single dose of DEN, 8 weeks old $Jnk^{\Delta hepa}$ mice started to receive CCl_4 twice per week until 25 weeks of age ($Jnk^{\Delta hepa}$+DEN+CCl_4 mice) (figure 8A). A second group of $Jnk^{\Delta hepa}$+DEN+CCl_4 mice were treated with GSK3326595 for 4 weeks, from week 22 when tumours were established to week 25 (figure 8A). Immunohistochemical analysis demonstrated PRMT5 and MEP50 expression in the CK19-positive CCA lesions (figure 8B). Tumour burden, as estimated by the liver-to-body weight ratio (liver index), was significantly reduced in treated mice, and macroscopic inspection of livers showed a marked reduction in the number of nodules (figure 8C). Consistently, histological analyses demonstrated significantly reduced CCA-like structures in GSK3326595-treated mice (figure 8D), and these structures displayed reduced tumour cell proliferation (Ki67), increased apoptosis (cleaved caspase-3) and increased DNA damage (p-H2AX) on immunohistochemical analyses (online supplemental figure 5). Interestingly, we detected increased $CD4^+$ and $CD8^+$ T cells infiltration in tumourous areas of GSK3326595-treated animals (figure 8E). By the end of treatments, mice receiving GSK3326595 showed a reduction in body weight. However, serum levels of hepatic enzymes, which were significantly increased in $Jnk^{\Delta hepa}$+DEN+CCl_4 mice, markedly diminished on GSK3326595 treatment (figure 8F).

The antineoplastic effect of PRMT5 inhibition was tested in a second clinically relevant mouse iCCA model induced by the hepatic expression of activated forms of TAZ and AKT by HTVI[27] (figure 9A). Here, very aggressive tumours develop rapidly after the administration of the oncogenes, replacing most of the normal parenchyma by 10 weeks postinjection, when mice die.[27] PRMT5 and MEP50 were clearly detected in the early lesions already present 2 weeks after HTVI and were markedly overexpressed in the extensive tumours found 1 week later (figure 9A). Next, we tested the effects of PRMT5 inhibition on iCCA development. To this end, mice injected with TAZ and AKT plasmids were treated with JNJ64619178, or vehicle, up to 3 weeks post-HTVI (figure 9A). According to

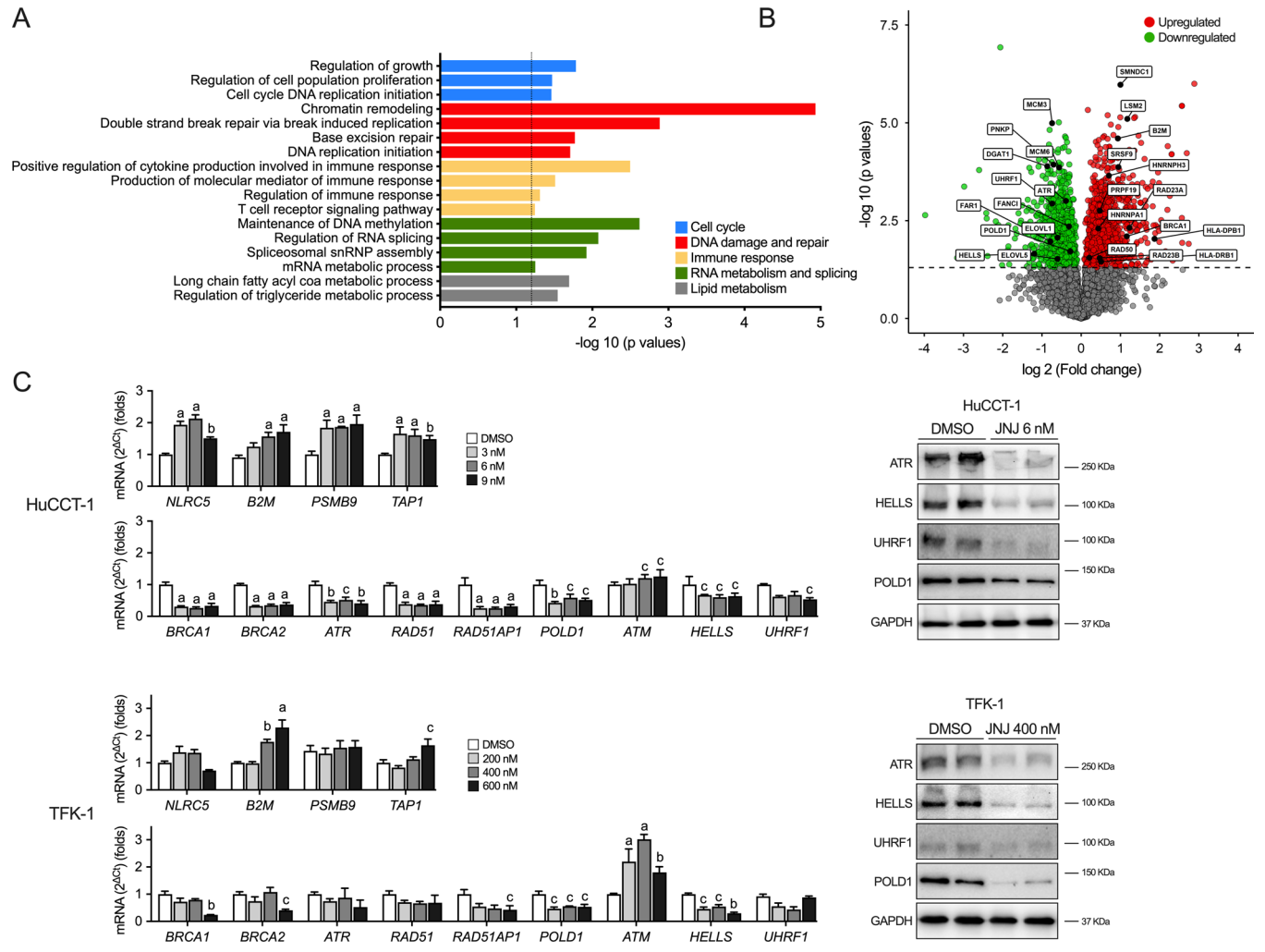

Figure 6 The effects of PRMT5 inhibition on the proteome of cholangiocarcinoma (CCA) cells are consistent with the observed transcriptional alterations. (A) Most relevant categories of differentially expressed proteins identified by Gene Ontology Biological Process (GO-BP) functional classification in HuCCT-1 cells treated with JNJ64619178 (6 nM, 4 days treatment). (B) Volcano plot representing all the significant (p<0.05) differentially expressed proteins. Selected proteins included in the indicated GO-BP functional categories are identified. (C) Validation of the effects of PRMT5 inhibition on the expression of the indicated genes and proteins identified in the transcriptomic and proteomic analyses. HuCCT-1 and TFK-1 cells were treated with the indicated concentrations of JNJ64619178 for 3 or 4 days for qPCR and Western blot analyses, respectively. a, p<0.001; b, p<0.01; c, p<0.05 vs controls.

serum transaminase levels, JNJ64619178 treatment was well tolerated, moreover, it significantly attenuated liver injury (figure 9B). At the end of treatments, extensive macroscopic tumourous lesions were appreciated in control TAZ-AKT mice while these lesions were less apparent in JNJ64619178-treated animals. The liver index confirmed a significantly reduced tumour burden (figure 9C). Consistently, histological analyses revealed a marked reduction in tumourous lesions area in mice receiving the PRMT5 inhibitor (figure 9D). These lesions also displayed reduced tumour cell proliferation (Ki67), increased apoptosis (cleaved caspase-3) and increased DNA damage (p-H2AX) (online supplemental figure 6). As observed in the $Jnk^{\Delta hepa}$+DEN+CCl_4 model, we clearly detected increased infiltration of $CD4^+$ and $CD8^+$ T cells in tumourous areas of JNJ64619178 treated mice compared with TAZ-AKT control mice (figure 9E). To better understand the antitumoural mechanisms of PRMT5 inhibition in this model, we performed transcriptomic analyses in liver tissues from TAZ-AKT mice treated with JNJ64619178 or vehicle. GO-BP functional classification of differentially expressed genes identified general categories related to cell proliferation and cell cycle progression, consistent with the observed antineoplastic effects (figure 9F). Intriguingly, the expression of genes involved in lipid metabolism was also affected, which may be in line with the recently described role for PRMT5 in liver fat metabolism.[48] In agreement with our in vitro findings, PRMT5 targeting significantly affected gene categories related to immune and inflammatory responses, mRNA splicing and the DDR (figure 9F). Interestingly, a recent report demonstrated how aberrant TAZ/YAP expression contributes to human CCA development by promoting DNA damage and chromosomal instability.[49] Consistent with this human study, and with our transcriptomic observations, we found that the expression of several key DDR genes (*BRCA1*, *BRCA2*, *RAD51*, *POLD1*, *HELLS*) was induced in the liver of TAZ-AKT mice, and this response was blunted on JNJ64619178 treatment (figure 9G). Finally, it should be noted that the expression of MTAP was preserved in both murine CCA models (online supplemental figure 7).

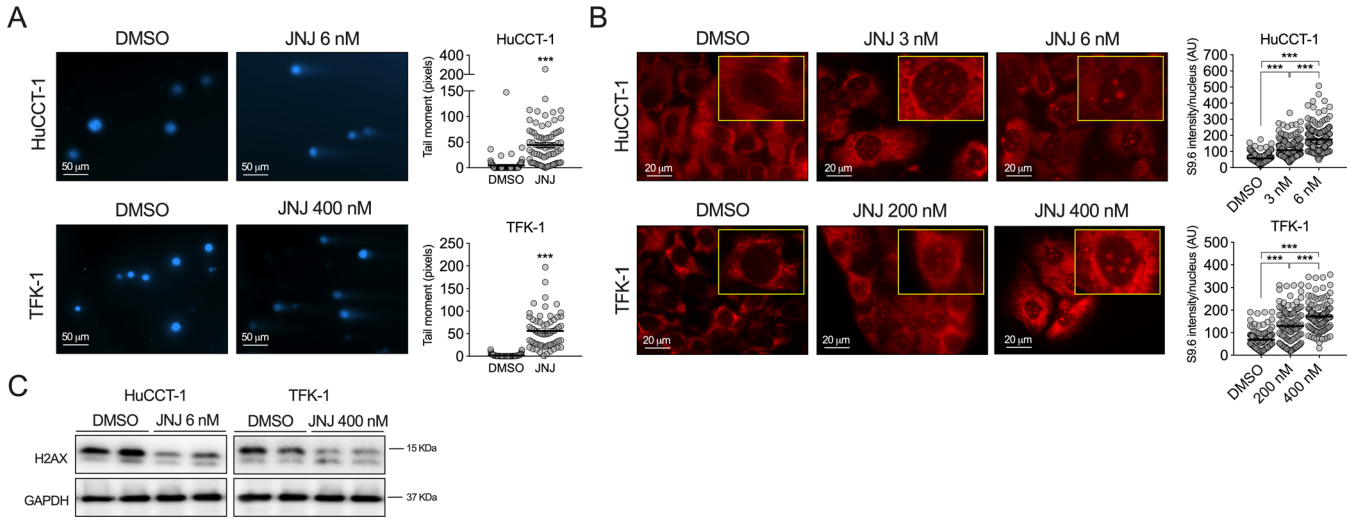

Figure 7 PRMT5 inhibition induces DNA damage in cholangiocarcinoma (CCA) cells. (A) Representative images of comet assays showing levels of overall DNA strands breaks in HuCCT-1 and TFK-1 cells treated with JNJ64619178 (3 nM) for 3 days. Graph shows the quantification of the comet tail length at the level of individual cells in the number of cells indicated. (B) HuCCT-1 and TFK-1 cells were probed for R-loops using the S9.6 antibody after 3 days of treatment with JNJ64619178. Graph shows the quantification of RNA–DNA hybrids (R-loops) per nucleus. (C) Effect of JNJ64619178 on H2AX protein levels in HuCCT-1 and TFK-1 cells. Cells were treated for 3 days with the indicated JNJ64619178 concentrations. ***p<0.001 vs controls (DMSO, vehicle-treated cultures). DMSO, dimethyl sulfoxide.

DISCUSSION

The identification of novel therapeutic strategies for CCA patients, particularly those that can be combined with chemotherapy or immunotherapies, is much needed. In this study, we provide evidence supporting that the arginine-methyltransferase PRMT5 could be a new pharmacological target for the systemic treatment of this extremely aggressive cancer. *PRMT5* gene mutations are not commonly detected in tumours; however, overexpression of this gene is frequently observed in a broad range of malignancies.[13] Here, we found that PRMT5 expression, and that of its functional partner MEP50, was upregulated in a significant proportion of CCAs of different anatomical origins in association with advanced stages of the disease. Previous experimental studies demonstrated that high PRMT5 levels directly contribute to malignant progression, and therefore, it should not be regarded as a mere cancer-related passenger alteration.[13 15] Most relevantly from a therapeutic point of view, both genetic and pharmacological approaches have demonstrated that cancer cells require 2–5 fold more PRMT5 activity than normal cells, which can survive with about 15%–20% residual PRMT5 activity.[39] Hence, a number of ongoing clinical trials are currently testing PRMT5 small molecule inhibitors in solid and haematological malignancies.[22 23 50] Interestingly, it was previously demonstrated that cancer cells with *MTAP* gene deficiency are more vulnerable to PRMT5 targeting, as *MTAP*-deleted cells accumulate the metabolite MTA, which is an endogenous PRMT5 inhibitor catabolised by MTAP.[13 35 39] Homozygous *MTAP* deletion has been observed in various solid malignancies, and a very recent genomic investigation performed in gastrointestinal cancers found that *MTAP* deletion occurred in about 15% iCCAs.[51] Our immunohistochemical analysis confirmed the loss of MTAP protein in more than 20% of iCCAs while additional studies including more samples are needed to establish the frequency of *MTAP* loss in eCCAs. Nevertheless, we show that MTAP status can be readily established in CCA tissue samples by routine IHC. This could guide the selection of patients who might respond better to PRMT5 inhibitors-based therapy, particularly to molecules like MRTX1719[24] as suggested by our in vitro observations.

Our findings demonstrate that the growth and viability of human CCA cells are highly dependent on PRMT5 expression and activity. Enhanced PRMT5 activity is increasingly recognised as a critical adaptation of cancer cells to sustain growth and survival programmes through the rewiring of epigenetic mechanisms, pre-mRNA splicing, DDR and even immune evasion.[13 15 39 43 52] In our hands, PRMT5 inhibition led to profound alterations in the transcriptome and proteome of CCA cells, suggesting that the antitumourigenic mechanisms of PRMT5 targeting are likely multifarious. We found that the expression of chromatin modifiers such as *UHRF1*, an oncogenic protein in CCA,[9] and the mRNA splicing of the histone modifiers *KAT5* and *KMT5C*,[17] was markedly impaired on PRMT5 inhibition. In agreement with the strong antiproliferative effects, we also detected a significant reduction in the expression of *POLD1* and *HELLS* genes, involved in DNA replication and chromatin remodelling, respectively. Noteworthy, HELLS protein has been recently identified as a target of PRMT5 methyltransferase activity, and HELLS arginine methylation influences its tumour-promoting activities.[53] Now, we also demonstrate that PRMT5 activity also contributes to preserve HELLS protein stability in CCA cells. *POLD1* and *HELLS* are frequently overexpressed in tumours, and it is important to note that both genes also play key functions in DNA repair and genome maintenance.[41 42] Their downregulation on PRMT5 inhibition, along with that of other important DDR genes such as *BRCA1*, *BRCA2*, *ATR*, *RAD51AP1* and H2AX protein, suggests that PRMT5 could be a master regulator of DNA repair in CCA cells as it has been recently demonstrated for other malignancies.[17 40 47 54 55] Indeed, we could show that PRMT5 inhibition leads to apoptosis, the accumulation of DNA strand breaks and the formation of R-loops, DNA-RNA hybrids that cause DNA damage.[45] These effects may underlie the synergistic cytotoxicity observed for PRMT5 inhibitors when combined with either gemcitabine or cisplatin and suggest that such combinatorial strategies may have a strong therapeutic

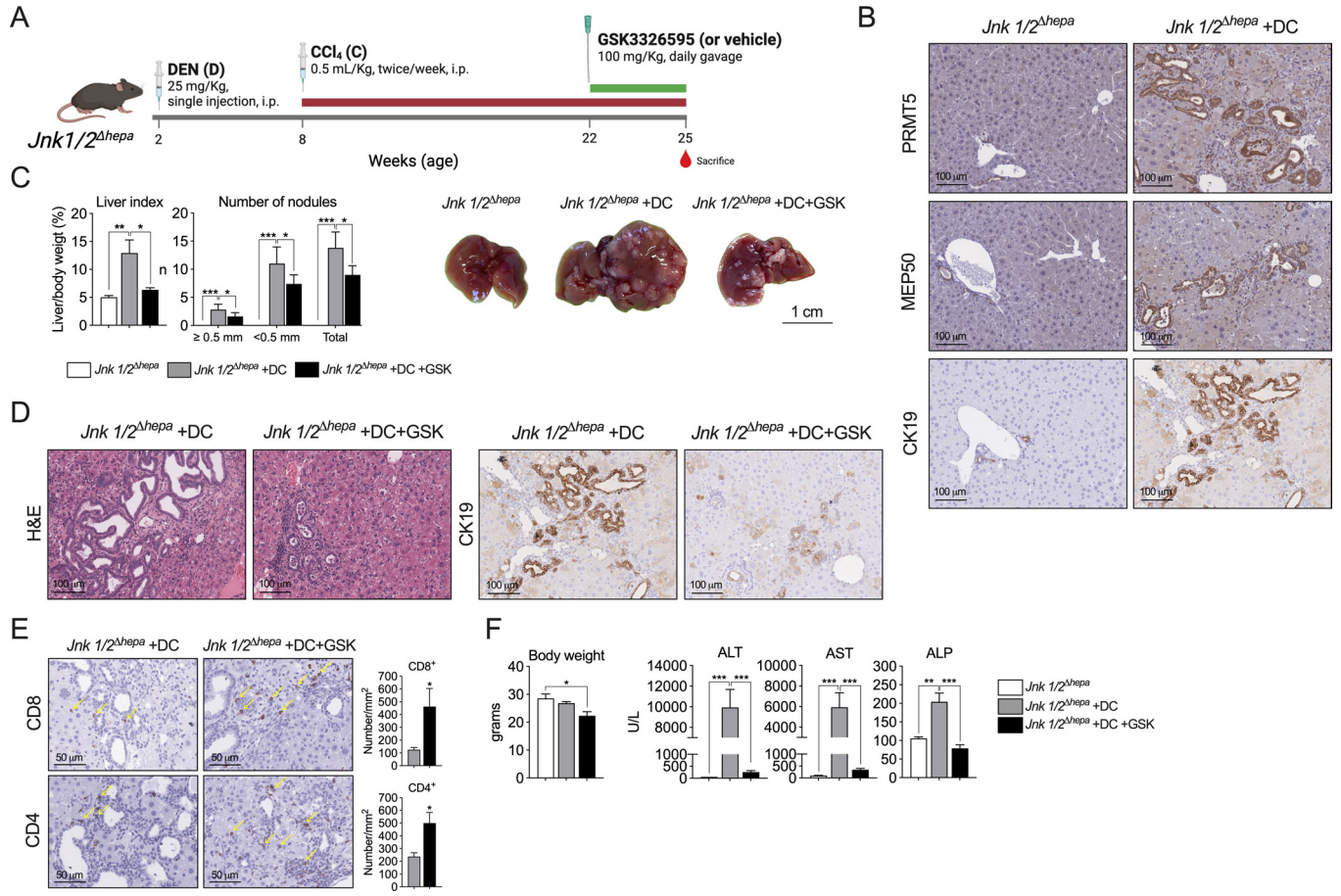

Figure 8 Antitumoural effects of PRMT5 inhibition in a mouse model of cholangiocarcinoma (CCA) development in the context of liver injury. (A) Diagram showing the Jnk$^{\Delta hepa}$+DEN+CCl$_4$ (Jnk$^{\Delta hepa}$+DC) experimental CCA model and the treatments applied (n=6 mice per group). (B) Immunohistochemical detection of PRMT5, MEP50 and CK19 proteins in liver tissue sections harbouring CCA lesions developed in this model. Representative images are shown. (C) Graphs show tumour burden, estimated as the liver-to-body weight ratio (liver index), and the quantification of nodules in the surface of the livers from the different groups of mice. Representative images of the livers of control *Jnk*$^{\Delta hepa}$+DEN+CCl$_4$, and GSK3326595-treated mice are shown. (D) Immunohistochemical analysis of CK19 and H&E stainings showing the extent of CCA lesions in control *Jnk*$^{\Delta hepa}$+DEN+CCl$_4$ and GSK3326595-treated mice. Representative images are shown. (E) Representative images showing the immunohistochemical detection of CD4$^+$ and CD8$^+$ T cells (yellow arrows), and quantification of tumour-infiltrating CD8$^+$ and CD4$^+$ T cells in control *Jnk*$^{\Delta hepa}$+DEN+CCl$_4$ and GSK3326595-treated mice. (F) Body weights and serum liver parameters (AST, ALT and ALP) in the different groups of mice at the end of treatments. *p<0.05, **p<0.01 and ***p<0.001.

index in CCA.[56] Moreover, the marked increase in R-loops accumulation induced by PRMT5 targeting may also improve the efficacy of therapeutic regimens including topoisomerase inhibitors,[5] as recently shown in a molecular subclass of glioblastoma cells prone to R-loop formation.[57] An important consequence of impaired mRNA splicing and enhanced DNA damage would be the generation of immunogenic signals and neoantigens that may induce antitumour immunity.[58] However, deficiencies in antigen processing and presentation are an independent hallmark of tumour immune evasion,[43] and although these mechanisms have not been thoroughly characterised in CCA they are likely to contribute to the low immunogenicity of biliary cancers.[6] In this context, epigenetic repression of the antigen-presenting machinery has been recently described in CCA, particularly in the predominant non-inflamed subtype.[59] Interestingly, we found that PRMT5 inhibition in CCA cells induced the expression of key genes involved in antigen presentation such as *NLRC5* and *B2M*, which is consistent with the recent description of PRMT5-mediated repression of major histocompatibility complex class I-related genes in melanoma cells.[43]

We also provide in vivo evidence of the antitumoural potential of two PRMT5 inhibitors in two complementary immunocompetent mouse models that recapitulate key molecular features of human iCCA.[9 27] We found a marked reduction in the extent of histological lesions and overall tumour burden. Importantly, no apparent signs of toxicity were observed in drug-treated animals, on the contrary, a significant attenuation in serum markers of liver damage was observed. The in vivo antitumoural mechanisms likely include interference with the overstressed DNA repair and mRNA splicing machinery characteristic of cancer cells, which to a great extent depend on PRMT5 activity.[39] However, we also noticed that PRMT5 inhibition increased lymphocyte infiltration in tumourous lesions, suggesting the activation of antitumour immunity. While this response may be related to an enhanced expression in tumour cells of key genes involved in antigen presentation, as found in vitro, we cannot discard other effects such as the inhibition of T regulatory cells as previously suggested.[13] Altogether, our study provides experimental evidence supporting the clinical evaluation of PRMT5 inhibitors in CCA patients to leverage the efficacy of

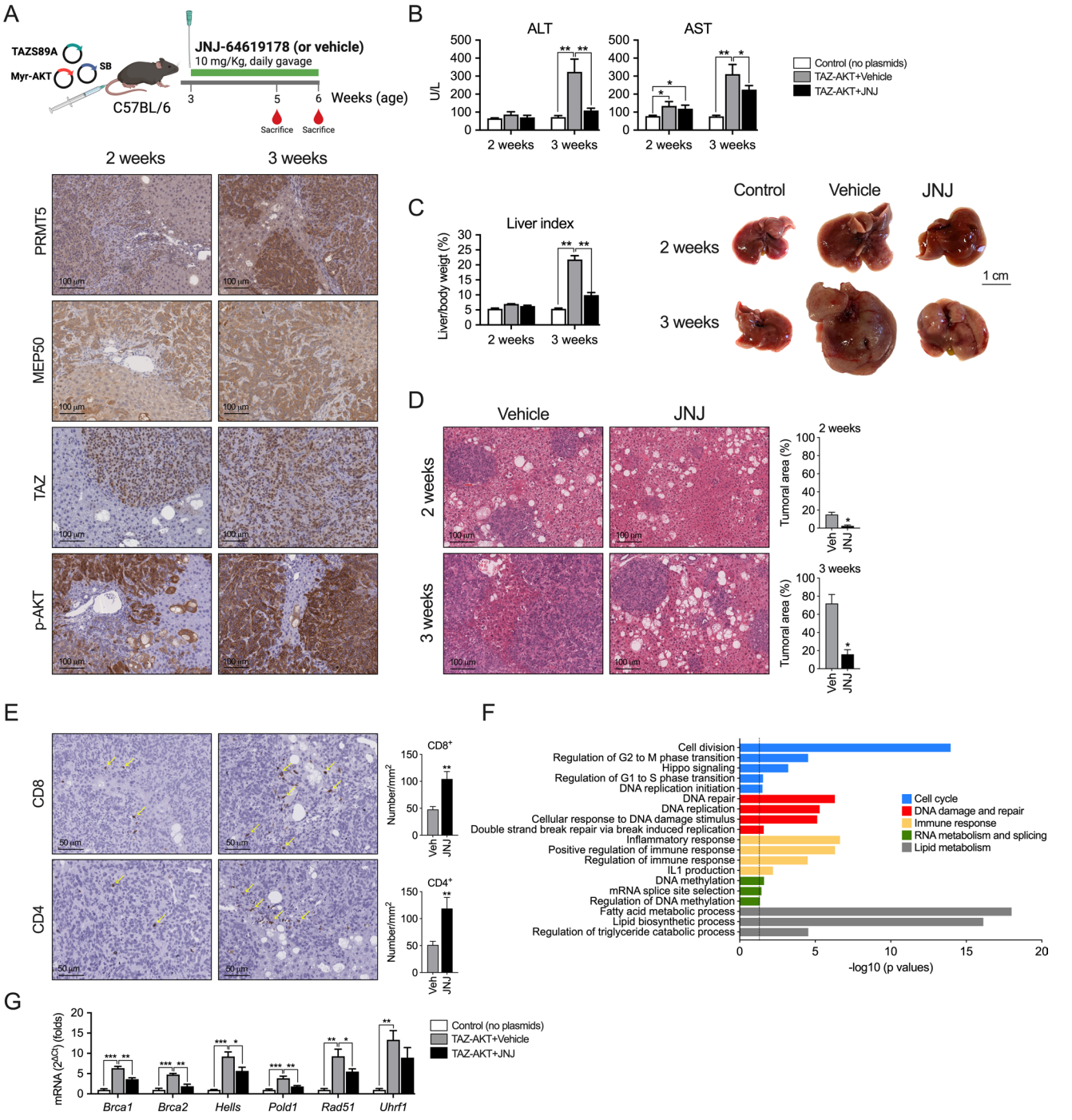

Figure 9 Antitumoural effects of PRMT5 inhibition in an aggressive cholangiocarcinoma (CCA) model triggered by hydrodynamic tail vein injection (HTVI) of activated forms of TAZ and AKT. (A) Diagram showing the TAZ-AKT model and the treatments applied (n=6 mice per group). Lower panels show representative images of the immunohistochemical analysis of PRMT5, and MEP50 in the lesions developing in TAZ-AKT mice at 2 and 3 weeks after plasmids injection, along with the demonstration of TAZ and p-AKT (Ser473) expression. Representative images are shown. (B) Serum transaminases (AST and ALT) levels in the different groups of mice. (C) Graphs show tumour burden, estimated as the liver-to-body weight ratio (liver index), and representative macroscopic images of the livers from control, TAZ-AKT mice and TAZ-AKT mice treated with JNJ64619178. (D) Representative H&E-stained liver tissue sections showing the extent of CCA lesions in TAZ-AKT and JNJ64619178-treated TAZ-AKT mice. Graph shows the quantification of tumourous areas. (E) Representative images showing the immunohistochemical detection of $CD4^+$ and $CD8^+$ T cells and quantification of tumour-infiltrating $CD8^+$ and $CD4^+$ T cells (yellow arrows) in control TAZ-AKT and JNJ64619178-treated TAZ-AKT mice. (F) Most relevant categories of differentially expressed genes identified by Gene Ontology Biological Process (GO-BP) functional classification of transcriptomic data from TAZ-AKT and JNJ64619178-treated TAZ-AKT mice livers. (G) qPCR analysis of the expression of DNA damage response (DDR)-related genes in control, TAZ-AKT and JNJ64619178-treated TAZ-AKT mice livers. *p<0.05, **p<0.01 and ***p<0.001. ALT, alanine transaminase; AST, aspartate aminotransferase.

chemotherapy or ICI-based strategies. In support of this latter possibility, a recent study demonstrated increased efficacy of ICI in combination with PRMT5 inhibitors in experimental models of breast cancer,[60] while on the other hand, the efficacy of immunotherapy was significantly lower in lung adenocarcinoma patients with high tumourous PRMT5 expression.[61]

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Competing interests MAA is editor of Gut. All other authors have no conflict of interest.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not applicable.

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