Targeting the MLL complex in castration-resistant prostate cancer

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Resistance to androgen deprivation therapies and increased androgen receptor (AR) activity are major drivers of castration-resistant prostate cancer (CRPC). Although prior work has focused on targeting AR directly, co-activators of AR signaling, which may represent new therapeutic targets, are relatively underexplored. Here we demonstrate that the mixed-lineage leukemia protein (MLL) complex, a well-known driver of MLL fusion–positive leukemia, acts as a co-activator of AR signaling. AR directly interacts with the MLL complex via the menin–MLL subunit. Menin expression is higher in CRPC than in both hormone-naïve prostate cancer and benign prostate tissue, and high menin expression correlates with poor overall survival of individuals diagnosed with prostate cancer. Treatment with a small-molecule inhibitor of menin–MLL interaction blocks AR signaling and inhibits the growth of castration-resistant tumors in vivo in mice. Taken together, this work identifies the MLL complex as a crucial co-activator of AR and a potential therapeutic target in advanced prostate cancer.

For prostate cancer, androgen deprivation therapies are front-line treatments, in addition to surgery and radiotherapy, for patients with high-risk localized disease, and second-generation anti-androgens such as abiraterone and enzalutamide have recently been shown to benefit individuals with advanced disease1–5. However, the lack of a cure for patients who progress to the hormone-refractory castration-resistant disease results in a high mortality rate6.

AR and its downstream signaling have a crucial role in the development and progression of both localized and CRPC7. Despite androgen-ablation therapies, castration-resistant tumors restore AR signaling through several mechanisms, such as AR gene amplification and activating mutations8–10. Substantial efforts are being invested to fully understand the regulation of AR in CRPC and to discover novel ways to target the AR pathway11.

The MLL protein, a homolog of trithorax (trxG) from Drosophila melanogaster, is a component of a large SET1-like histone methyltransferase (HMT) complex that possesses inherent histone 3 lysine 4 (H3K4) methyltransferase activity12. The MLL–HMT complex consists of highly conserved core proteins including MLL, ASH2L, RBBP5, and WDR5, which are essential for the enzymatic activity of the complex13–15. Frequent translocation of the gene encoding MLL (KMT2A) in acute leukemia results in the formation of chimeric proteins with aberrant transcriptional activity12; however, these chimeric proteins depend on direct interaction with menin for their oncogenic activity16.

The 67-kDa menin protein, which binds to the N terminus of MLL, is essential for the expression of MLL target genes17–19. Small-molecule inhibitors of the menin–MLL interaction can block MLL fusion protein–mediated leukemic transformation19. The lack of a DNA binding motif in the menin protein is overcome by its direct interaction with MLL, other transcription factors such as c-MYB, or chromatin-associated proteins such as lens epithelium–derived growth factor20,21. The function of menin and its ability to coordinate oncogenic behavior in other cell types is an area of active research. For example, in breast cancer, the direct binding of menin to activated estrogen receptor (ER) facilitates MLL recruitment, thereby modulating the ER transcriptional response22. Notably, an oncogenic role of menin in ER-positive breast cancers has been previously suggested22,23, as patients with high menin expression show poor outcomes23. Similarly, high menin expression is also correlated with poor prognosis in hepatocellular carcinoma24. In addition, a recent study identified menin as a potential therapeutic target in pediatric gliomas harboring H3.3K27M mutations25, and a drug screen identified a small-molecule inhibitor of the menin–MLL interaction, MI-2 (ref. 18), as a suppressor of tumor growth. Taken together, these studies suggest an oncogenic role of menin in solid tumors.

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Here we describe a functionally important interaction between AR, menin and the MLL complex in advanced prostate cancer. We found that AR associates with the MLL–HMT complex through a direct interaction with menin. Furthermore, the MLL complex is required for AR-mediated gene expression and can be targeted with small-molecule menin–MLL inhibitors, suggesting that therapies being developed for the treatment of MLL fusion–positive leukemias may have utility for castration-resistant prostate cancer as well.

RESULTS

AR interacts with the MLL complex

By using co-immunoprecipitation (co-IP) assays in the AR-dependent prostate cancer cell line VCaP, we previously reported that AR interacts with proteins of the MLL complex26. To further study the nature of this interaction, we fractionated VCaP cell nuclear extracts by size-exclusion chromatography and measured the presence of AR and MLL-complex proteins by immunoblot analysis. AR eluted in a fraction that contained high-molecular-weight complexes, as did certain MLL-complex components, including MLL, MLL4, WDR5, ASH2L, and menin (Fig. 1a). Next we co-immunoprecipitated endogenous ASH2L, menin, and AR from VCaP and another AR-dependent prostate cancer cell line, LNCaP, to confirm the association between AR and MLL-complex proteins. IP with AR, ASH2L, and menin antibodies and subsequent immunoblot analysis for AR and MLL-complex proteins demonstrated this association (Fig. 1b,c). To test the robustness of the interaction, we performed co-IP experiments in VCaP cells under stringent conditions (350 mM NaCl), and we used a different AR antibody; in both instances, MLL-complex proteins co-immunoprecipitated with AR (Supplementary Fig. 1a,b). Confocal immunofluorescence microscopy in VCaP cells also demonstrated that both ASH2L and menin co-localize with AR in the nucleus (Fig. 1d). To corroborate this interaction in situ, we stained sections from benign, localized, and metastatic human prostate cancer tissue with antibodies against menin, MLL, and AR; AR and menin staining was predominantly nuclear in epithelial cells, with some staining observed in the stroma (Supplementary Fig. 1c,d). Likewise, most of the MLL staining was primarily limited to the nuclei of epithelial cells; however, some smooth muscle cells also showed nuclear MLL-specific staining (Supplementary Fig. 1e).

Collectively, these results show that AR physically associates with the MLL complex in prostate cancer cells and tissues.

The MLL complex is required for AR signaling and cell growth

Next we conducted knockdown experiments to study the role of the MLL complex in AR-driven transcription. Compared to VCaP cells treated with two independent siRNAs against the gene encoding the MLL subunit ASH2L, cells treated with control siRNA showed higher induction of AR-target gene expression after treatment with synthetic androgen (R1881), as revealed by microarray and gene set enrichment analysis (GSEA) with an AR gene signature (Fig. 2a,b, Supplementary Fig. 2a and Supplementary Table 1). Similar effects of ASH2L knockdown on AR signaling (both at transcript and protein levels) were observed in LNCaP cells (Fig. 2c and Supplementary Fig. 2b–e). Next, using quantitative PCR (qPCR) and immunoblotting, we assessed the role of menin on AR signaling. Analogous to what we observed with ASH2L knockdown, knockdown of MEN1 (encoding menin) resulted in a significant ($P < 0.01$) decrease in the dihydrotestosterone (DHT)-induced expression of AR target genes (Fig. 2d,e). This was further confirmed by negative enrichment of the AR target gene signature in MEN1-knockdown cells (Supplementary Fig. 2f,g).

As menin is a crucial component of the MLL–MLL4 complex but not the MLL2–MLL3 complex, we examined the effects of KMT2A and KMT2B (encoding MLL4) knockdown on AR signaling. Knockdown of either KMT2A or KMT2B using siRNA attenuated the transcription of known AR target genes in both LNCaP and VCaP cells (Supplementary Fig. 3a,b). Similar results were obtained by GSEA analysis with the AR gene signature in VCaP cells expressing

**Figure 1** Androgen receptor interacts with MLL-complex proteins. (a) VCaP nuclear lysate fractions from a Superose 6 gel-filtration column (top) immunoblotted using indicated antibodies (bottom). Box indicates fraction where all the proteins co-eluted. Arrows indicate the approximate molecular mass of eluted complexes. Distribution of cytoplasmic (actin) or nuclear proteins (histone-H3 and SNRNP70) demonstrates the efficiency of lysate fractionation (inset western blot). β-catenin is known to be present in both fractions. (b,c) VCaP (b) and LNCaP (c) nuclear lysates immunoprecipitated (IP) with antibodies specific to AR, ASH2L and menin, and immunoblotted (IB) using indicated antisera. (d) VCaP cells immunostained with antibodies specific to AR (green), ASH2L (red, bottom), or menin (red, top). Yellow, co-localization. Scale bars, 10 µm and 2.5 µm (in inset). Results are representative of three independent experiments.
KMT2B-specific shRNA (Supplementary Fig. 3c). Knockdown of both KMT2A and KMT2B did not have a synergistic effect when compared to independent knockdowns of either KMT2A or KMT2B, suggesting that both MLL and MLL4 are necessary for MLL-complex activity in this context.

Next we investigated the role of MLL in the proliferation of AR-driven prostate cancer cells. Stable ASH2L knockdown using shRNA reduced AR-mediated gene expression, as evidenced by microarray (Supplementary Fig. 3d) and decreased proliferation of VCaP cells in vitro (Supplementary Fig. 4a), supporting a potential oncogetic role for MLL. Importantly, ASH2L-knockdown VCaP cells generated smaller xenograft tumors in vivo compared to those from control VCaP cells (Fig. 2f). Similarly, knockdown of MEN1, KMT2A, or KMT2B decreased both the proliferation of prostate cancer cells in vitro (Supplementary Fig. 4b–d) and the growth of VCaP xenografts in vivo (Fig. 2g,h). Notably, inhibition of menin by shRNA also suppressed the growth of the AR-negative cell line Du145 (data not shown). Taken together, our data suggest that MLL-complex proteins are required for the AR transcriptional program and tumor growth.

The MLL complex is localized on AR target genes

Given the role of MLL-complex proteins in AR transcriptional regulation, we hypothesized that the MLL complex may co-localize with AR on a genome-wide scale. To investigate this, we identified genome-wide ASH2L binding by chromatin immunoprecipitation coupled with high throughput sequencing (ChIP-seq) in VCaP cells upon treatment with synthetic androgen (R1881) or vehicle (ethanol) and compared the data with published AR ChIP-seq data. First, we noted an overlap between ASH2L binding sites and androgen-stimulated AR binding sites (Fig. 3a,b). Next, we identified a total of 15,637 distinct genome-wide individual AR peaks (false discovery rate (FDR) < 0.05), out of which 12,243 peaks increased upon androgen stimulation (Fig. 3c). For ASH2L, we identified a total of 30,114 peaks (FDR < 0.05), out of which 2,187 showed increased binding upon androgen stimulation (Supplementary Fig. 5a). Importantly, we noted a substantial overlap of 1,410 target regions (64.4% of the ASH2L binding sites) where both ASH2L and AR were concomitantly recruited after androgen stimulation (Fig. 3c). A representative gene promoter with overlapping AR and ASH2L binding patterns is shown in
Figure 3 AR and ASH2L are recruited to the same genomic loci upon androgen stimulation. (a) A heat map representation of AR and ASH2L binding to promoter regions (2.5 kb flanking transcriptional start sites (TSS), indicated by 0) in vehicle- or R1881-stimulated VCaP cells. Gene promoters are rank-ordered by the level of AR enrichment at the TSS. IgG, control. (b) Average TSS-aligned profiles of AR (bottom) and ASH2L (top) occupancy for all annotated genes before and after vehicle (red) or R1881 (black) stimulation. (c) The overlap between R1881-induced AR and ASH2L peaks. (d) A representative gene (NDRG1) co-occupied by AR and ASH2L before and after AR stimulation. Chr8, chromosome 8; hg19, University of California Santa Cruz (UCSC) Genome Browser Homo sapiens hg19 assembly; y axis, coverage (reads per million). (e) De novo motif detection with the MEME program identified enrichment of two half androgen response elements among ASH2L binding sites. y axis, nucleotide conservation in bits calculated at each position ranging from 0 (no conservation) to 2 (100% conservation). (MEME E value, 2.1e–025 (2.1 x 10^-25)).

Figure 3d, and others are shown in Supplementary Figure 5b–e. To investigate the presence of potential cis-regulatory elements among ASH2L genomic binding regions, we performed de novo motif discovery using Multiple EM for Motif Elicitation (MEME) on the ASH2L ChIP-seq data. We identified substantial enrichment of two androgen responsive element half-sites in the ASH2L binding site, further supporting the overlap observed between ASH2L and AR binding in AR-dependent cell lines (Fig. 3e). We next examined the expression profile of genes that were within 10 kb of androgen-induced ASH2L peaks, and observed a marked decrease in their expression upon ASH2L knockdown (Supplementary Fig. 5f). Similar to that seen with ASH2L, we observed an enrichment of MLL and menin on AR target genes by ChIP-PCR (Supplementary Fig. 6a–c). Taken together, these data suggest that, upon androgen stimulation the MLL complex is co-recruited to direct AR targets, and that it modulates their transcriptional activation.

AR directly interacts with menin
Having demonstrated the recruitment of MLL-complex proteins to AR-bound chromatin regions, we sought to further characterize this interaction. We performed in vitro pull-down experiments and detected a direct interaction between AR and menin (Fig. 4a). Furthermore, we observed binding between purified, untagged menin and Halo-tagged AR (Halo-AR; Fig. 4b). IP of purified menin also pulled down Halo-AR (Fig. 4c). Next, to finely map the AR–menin interaction, we generated deletion constructs for Halo-AR (Fig. 4d), and we found that menin interacts with the N-terminal domain of AR (Fig. 4e), specifically amino acids 469–559 (Fig. 4f). We examined the effect of AR stimulation on the distribution of menin and saw no change, as compared to vehicle-treated cells (both AR and menin were mostly localized to the nucleus) (Supplementary Fig. 7a,b). Taken together, our experiments suggest a direct interaction between AR and menin.

Menin expression is elevated in human prostate cancer
Given the importance of the MLL complex in solid tumors, we examined menin expression in a set of human prostate cancer tissue samples. By using RNA sequencing (RNA-seq) we observed that MEN1 expression was associated with disease progression, with significantly elevated levels seen in metastatic prostate cancer compared to those observed in hormone-naïve prostate cancer and benign prostate (Fig. 5a). We validated this observation using prostate cancer samples from The Cancer Genome Atlas; these also demonstrated upregulation of MEN1 in prostate cancer compared to benign controls (Fig. 5b). Notably, among other members of the MLL complex, WDR5 transcript levels were elevated in metastatic prostate cancer, but those of KMT2A, KMT2B, ASH2L and RBBP5 were not different from benign controls (Supplementary Fig. 8a–e). We next analyzed the expression of MEN1 in published microarray data sets from the Oncomine database. Similarly to what we observed in the RNA-seq data, MEN1 expression was elevated in localized and metastatic prostate cancer in multiple published studies (Fig. 5c,d). Similarly, we found that menin protein levels were also elevated during prostate cancer progression, with notably higher protein levels in metastatic compared to localized disease (Fig. 5e).

To assess whether elevated MEN1 expression is associated with a poor prognosis, we used outcomes data from a large, published prostate cancer study to carry out Kaplan–Meier analysis. We found MEN1 mRNA overexpression was predictive of poor patient survival (Fig. 5f). Taken together, these data establish that menin is upregulated at both the transcript and protein levels in localized and metastatic prostate cancer and its expression is associated with poor survival.

Inhibition of the menin–MLL complex suppresses AR signaling
Because the MLL–HMT complex mediates AR signaling, and menin is a key player in recruiting the complex to AR targets, we hypothesized that inhibiting the interaction between menin and MLL would block AR signaling and tumor growth. To test this hypothesis, we used MI-136, a variant of a previously described inhibitor that can specifically inhibit the menin–MLL interaction (Supplementary Fig. 9a). AR-positive cell lines such as VCaP, LNCaP, and 22Rv1 were sensitive...
to MI-136, as assessed by in vitro cell-viability assays (Supplementary Fig. 9b). Treatment with MI-136 blocked DHT-induced cell proliferation in AR-dependent cell lines (LNCaP and VCaP) (Supplementary Fig. 9c). The effect of MI-136 on cell proliferation was similar to that of MDV-3100, a second-generation, FDA-approved anti-androgen for people with refractory prostate cancer.

Next we monitored the effect of MI-136 on the AR transcriptional program using qPCR on VCaP cells treated with MI-136, MDV-3100, or MI-nc (a non-active control). Treatment with either MI-136 or MDV-3100 inhibited DHT-induced expression of AR target genes, as compared to treatment with MI-nc (Supplementary Fig. 9d). Inhibition of prostate-specific antigen (PSA) protein expression was also observed in both VCaP and LNCaP cells treated with either MI-136 or MDV-3100 (Supplementary Fig. 9e,f). To examine the effects of menin inhibition on global AR signaling, we performed microarray analysis on DHT-stimulated VCaP cells pre-treated with MI-136. GSEA revealed that MI-136 treatment blocked the induction of AR-upregulated genes, as compared to androgen treatment (Fig. 6a,b). Treatment with MI-136 also inhibited the expression of genes that were bound to ASH2L after AR stimulation (Supplementary Fig. 9g and Supplementary Table 1). We also observed that treatment with MI-136 induced apoptosis of VCaP cells, as evidenced by PARP cleavage (Supplementary Fig. 9h).

Next we looked into the mechanism of action of MI-136. In leukemic cells, treatment with MI-136–like compounds inhibits the menin–MLL interaction19. Similarly, in prostate cancer cells, MI-136 concentrations as low as 10 μM inhibited menin–MLL interaction (Supplementary Fig. 10a), but the menin–AR interaction was retained even at MI-136 concentrations of 100 μM. Similar results were also seen in an in vitro–purified protein pull-down experiment (Supplementary Fig. 10b). Next we looked at the recruitment of the MLL complex to AR target genes in the presence of MI-136. Consistent with what was seen in the interaction data, we observed that recruitment of ASH2L (but not AR) to transmembrane protease, serine 2 (TMPRSS2) and KLK3 (encoding PSA) promoters was significantly (P < 0.05) decreased in the presence of MI-136, as compared to both MI-nc and vehicle (Supplementary Fig. 10c). Together these results suggest that MI-136 inhibits AR-mediated transcription by blocking MLL recruitment predominantly at the level of the menin–MLL interaction.

Menin–MLL inhibitor reduces tumor growth in vivo

We next examined the efficacy of MI-136 in inhibiting tumor growth in vivo using VCaP xenografts31. Treatment of VCaP tumor-bearing mice with MI-136 (40 mg/kg) led to a modest but significant (P < 0.05) reduction in tumor volume compared to vehicle treatment (Supplementary Fig. 10d) with no effect on mouse body weight (Supplementary Fig. 10e). Next we investigated the impact of MI-136 treatment in the context of mouse castration, which deprives the AR-dependent VCaP cells of circulating mouse androgens (Supplementary Fig. 10f)11,32. We castrated mice bearing VCaP xenografts, and when (~4 weeks) the tumors reached their original volume (~100 mm), these mice were treated with MI-136 (40 mg/kg) and tumor regrowth was monitored. Treatment with MI-136 led to a significant (P < 0.05) decrease in the growth of castration-resistant VCaP tumors compared to treatment with vehicle (Supplementary Fig. 10g), confirming an important role for the menin–MLL complex in the biology of hormone-refractory prostate cancers.

But as the effects of MI-136 on VCaP tumor growth were modest compared to that in vehicle, we examined the efficacy of a variant
compound (MI-503), which has a better solubility and bioavailability profile and is derived from the same scaffold as MI-136 (ref. 33) (Supplementary Fig. 11a). We first evaluated the target binding specificity of MI-503 using the cellular thermal shift assay (CETSA)34. Treatment of VCaP cells with MI-503 increased the levels of menin protein at 45 °C (Fig. 6c), whereas most menin protein precipitated at 45 °C in untreated cells, indicating that MI-503 stabilizes the menin protein upon binding. Similar thermal stability of menin was also seen in LNCaP cells (Supplementary Fig. 11b). In vitro, MI-503 had modestly lower half-maximal inhibitory concentration (IC50) values than MI-136 (Supplementary Fig. 11c). Like MI-136, MI-503 inhibited AR signaling, as determined by reduction in the expression of both PSA protein (Fig. 6d) and canonical AR-induced genes (Fig. 6e and Supplementary Fig. 11d), and interaction between menin and MLL (Supplementary Fig. 11e). MI-503 also induced apoptosis in VCaP and LNCaP cells, as determined by PARP cleavage (Fig. 6f). We next assessed the effect of MI-503 on global AR-mediated gene regulation by performing gene expression microarrays in VCaP cells. MI-503 repressed DHT-mediated gene transcription (Fig. 6f), and had a superior effect on AR target genes as compared

![Figure 5](image_url) Menin is upregulated in both localized and metastatic CRPC. (a, b) RNA-seq data of menin expression from benign (n = 38), localized (PCa; n = 118) and metastatic tumor (Met; n = 55) prostate tissues from the Michigan Center for Translational Pathology (a) and from benign (n = 37) and localized tumor (n = 137) prostate tissue samples from The Cancer Genome Atlas (b). The y axis denotes fragments per kilobase of transcript per million mapped reads (FPKM). P values are calculated using one-way ANOVA (a) or t-test (b). (c–d) MEN1 transcript expression in multiple prostate cancer microarray studies from the Oncomine database. Data sets were analyzed for menin expression in benign versus PCa (c) and PCa versus metastatic CRPC (Met) (d). Study first author, statistical significance and number of samples are indicated. P values are calculated using two-sample, one-tailed Welch’s t-test. (e) Immunoblot of menin expression in benign (n = 6), PCa (n = 5) and CRPC (n = 8) tissues. β-actin, loading control. (f) MEN1 mRNA expression correlates with poor overall survival by Kaplan–Meier analyses of prostate cancer outcome in the Nakagawa study30. Samples are divided into quartiles on the basis of MEN1 mRNA expression. Expression in the middle two quartiles is merged (26–75%). **P < 0.001; compared to low expressers on the basis of a log-rank (Mantel–Cox) test. Box plot lines (from top to bottom): maximum, 90th percentile, median, 10th percentile, minimum.
Figure 6 A menin–MLL small molecule inhibitor impairs prostate cancer growth in mice. (a) Heat map representation of the impact of 5 μM MI-136 on DHT-induced genes in VCaP cells. (b) GSEA using an AR target gene signature (Supplementary Table 1). (c) Immunoblot of soluble proteins from VCaP cells treated with either DMSO or 5 μM MI-503 and incubated at indicated temperatures. Representative blots, n = 2. (d) Immunoblot of PSA and cleaved PARP (cPARP) from VCaP, LNCaP or PNT2 cells treated with either DMSO or the indicated amount of MI-503 for 48 h. Representative blots, n = 3. (e) AR target gene expression (TMPRSS2, FKBP5, and KLK3) in VCaP cells pre-treated with either DMSO or MI-503 and subsequently stimulated with 10 nM DHT for 6 h. *P < 0.01; **P < 0.001; compared to untreated by one-way ANOVA. n = 3. (f) Heat map representation of the impact of 5 μM MI-503 on DHT-induced genes in VCaP cells. GSEA was performed using an AR target gene signature (Supplementary Table 1). (h) Heat map representation of ASH2L knockdown and MI-503 treatment on the expression of various genes. Bold letters, widely studied AR target genes. (i) Effect of MI-503 treatment on the growth of LNCaP-AR xenografts in castrated mice treated daily (after tumors reached 80–100 mm³) with vehicle (n = 20) or 60 mg/kg MI-503 (n = 18) i.p. (j) Effect of MI-503 treatment on the growth of castration-resistant VCaP xenografts in mice treated (after tumors reached 80–100 mm³) with vehicle (n = 20), MI-503 (i.p.; 75 mg/kg, n = 16), MDV-3100 (oral; 10 mg/kg, n = 20) or a combination of MI-503 and MDV-3100 (n = 20). Tumors were measured using caliper measurements taken bi-weekly. *P < 0.05, **P < 0.005; compared to vehicle by Student’s t-test. Error bars are mean ± s.e.m.
to MI-136 (Supplementary Fig. 11f). To assess whether MI-503 treatment phenocopies ASH2L knockdown, we compared the microarray data obtained from VCaP cells treated with either ASH2L siRNA or MI-503 and observed substantial overlap in AR target gene repression (Fig. 6h).

Next we investigated the effect of MI-503 treatment on the in vivo growth of an LNCaP-AR xenograft model. Once the LNCaP-AR xenografts were established in castrated mice, MI-503 (60 mg/kg) was intraperitoneally (i.p.) injected daily and tumor growth was monitored for 27 d (Supplementary Fig. 12a). Treatment with MI-503 significantly impeded tumor growth as compared to treatment with vehicle (Fig. 6i and Supplementary Fig. 12b). Additionally, we evaluated the effect of MI-503 on VCaP xenograft growth in a mouse castration model, as was done with MI-136 (Supplementary Fig. 10f,g). Treatment with MI-503 (75 mg/kg) led to a significant decrease in the growth of castration-resistant VCaP xenografts without any effect on mouse body weight as compared to vehicle (Fig. 6j and Supplementary Fig. 12c). We also examined the efficacy of co-treatment with MI-503 and MDV-3100 in this model. Although MDV-3100 alone had a less pronounced effect on tumor growth than that of MI-503, the combination treatment of MDV-3100 and MI-503 demonstrated a slightly stronger reduction in tumor growth than MI-503 alone (Fig. 6j). Next we evaluated the post-treatment status of neuroendocrine differentiation markers in both LNCaP and VCaP xenografts. No significant (P > 0.05) change in the mRNA expression of synaptophysin or chromogranin A was seen (Supplementary Fig. 13a–d). Taken together, these findings show that inhibition of the AR–menin interaction may create a new therapy for hormone-refractory prostate cancers.

DISCUSSION

Mammalian SET domain–containing proteins such as MLL1, MLL2, SET7, and SET9 mediate nuclear hormone receptor signaling through their ability to promote gene activation.15,16 Exploring our prior observation of the AR–MLL interaction,20 we now reveal a mechanism of gene regulation by AR that is mediated by MLL complex members ASH2L and menin. Specifically, our work addresses the key question of how AR signaling continues despite anti-androgen treatment, aiding in the progression of CRPC. Here we establish the key regulatory role for the MLL complex in the AR transcriptional program, thereby uncovering a potential therapeutic angle for CRPC treatment.

In this study, we find that individuals with prostate cancer characterized by menin overexpression show poor overall survival. Although menin has been extensively characterized as a tumor suppressor in multiple endocrine neoplasia type 1 (ref. 17), our data and previous literature on the estrogen receptor strongly argue that menin can facilitate oncogenic gene activation through hormone receptor signaling in a contextual manner.

Given that small molecule inhibitors targeting the menin–MLL interaction have been pursued as a potential therapy for MLL-associated leukemias (for which menin has a known oncogenic role),16,19,37, we envisioned that a similar strategy might disrupt AR-mediated signaling and cause tumor growth impairment in prostate cancer. We confirmed the utility of menin–MLL inhibition both in vitro and in vivo using AR-dependent prostate cancer cell lines and castration-resistant xenograft models.

Inhibition of menin by shRNA or small molecules suppressed the growth of the AR-negative cell line Du145, suggesting that menin might use other transcription factors in these cells to recruit the MLL complex. Although there is currently a lack of experimental evidence to support this hypothesis, we speculate that the menin inhibitors would be efficacious on neuroendocrine-type prostate cancers. More detailed studies directed toward understanding the role of menin and the MLL complex in AR-negative prostate cancer is required.

Collectively, our study proposes a model in which the binding of menin to AR recruits the MLL complex to AR target genes, modulating AR-dependent gene activation (Supplementary Fig. 13e). We therefore propose menin as a key mediator of aggressive prostate cancer, and our study provides a rationale for the refinement of small-molecule menin inhibitors as a novel therapeutic strategy for patients with advanced castration-resistant prostate cancer.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Gene Expression Omnibus: Coordinates have been deposited with accession code GSE60842.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

R.M., A.P.K. and A.M.C. conceived and designed the research. R.M. performed the experiments. R.M., A.P.K. and A.M.C. conceived and designed the research. R.M. performed the experiments. R.M. and X.W. carried out data analysis. R.M., A.P.K. and A.M.C. conceived and designed the research. R.M. performed the experiments. R.M. and X.W. carried out data analysis. M.I. performed ChIP-seq analysis. M.K.I. performed ChIP-seq analysis. M.K.I. and Y.N. performed gene expression analysis, J.E.-W., R.S. and F.Y.F. performed mouse xenograft studies. Y.-M.W. generated ChIP-seq data. M.K.I. and Y.N. performed gene expression analysis, J.E.-W., R.S. and F.Y.F. performed mouse xenograft studies. M.-W. generated ChIP-seq libraries and X.C. performed the sequencing. N.P. and L.P.K. performed IHC. A.L.N., A.K.Y. and D.M. assisted with data analysis. D.B., J.G. and T.C. provided inhibitors. R.M. and A.M.C. wrote the manuscript with help from S.M.D., I.A.A. and A.P.K. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Cell lines and lentiviral and siRNA transfections. VCaP prostate cancer cells were cultured in DMEM (Invitrogen, Carlsbad, CA) with Glutamax (Gibco), LNCaP and 22Rv1 cells in RPMI 1640 (Invitrogen), DU145 in DMEM; all the culture media were supplemented with 10% FBS (Invitrogen). LNCaP-AR cells were a gift from Dr. Charles Sawyer (Memorial Sloan-Kettering Cancer Center, New York, NY). All the cell lines were cultured in a 5% CO2 cell culture incubator. To ensure the identity, all the cell lines were genotyped at the University of Michigan Sequencing Core using Profiler Plus (Applied Biosystems) and compared with the short tandem repeat (STR) profiles of respective cell lines available in the STR Profile Database (ATCC). All cell lines were tested and found to be free of mycoplasma contamination.

Lentiviral plasmid encoding shRNA targeting ASH2L (RHS4430-98881191 and RHS4430-99881709) or control shRNA (GIPZ, RHS4346) were from Thermo Scientific. shRNA targeting MEN1 (TRCN0000338331 and TRCN0000040141) and control shRNA (SHC202) were from Sigma. shRNA targeting KMT2A (TL314662) and KMT2B (TL315696) and control shRNA (TR30021) were from Origene. Lentiviral particles were generated by the University of Michigan Vector Core. VCaP and LNCaP cells were infected with lentiviruses and stable cell lines were generated by selection with 5 µg/ml puromycin.

Knockdown of ASH2L, KMT2A and KMT2B in VCaP and LNCaP cells was accomplished by RNAi using commercially available siRNA duplexes for ASH2L (Dharmacon, cat. no. J-019831-05 and J-019831-08), KMT2A (Dharmacon, cat. no. L-009914-00-0010), and KMT2B (Dharmacon, cat. no. L-009670-00-0010). Transfections were performed with OptiMEM (Invitrogen) and Oligofectamine (Dharmacon, cat. no. J-019831-05 and J-019831-08), following manufacturer’s instructions.

Inhibitors. Discovery, chemical synthesis and characterization of MI-136 and MI-503 compounds were described previously.33

Antibodies and immunoblot analyses. Cell lysates were separated on 4–12% SDS polyacrylamide gels (Novex) and transferred onto nitrocellulose membrane (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight). The membrane was incubated for 1 h in blocking buffer (Tris-buffered saline, 0.1% Tween (TBS-T), 5% nonfat dry milk) (Novex) using wet transfer (30 V overnight).

Gel-filtration chromatography. VCaP nuclear extracts were obtained using the NE-PER nuclear extraction kit (Thermo Scientific), and dialyzed against FPLC buffer (20 mM Tris-HCl, 0.2 mM EDTA, 5 mM MgCl2, 0.1 M KCl, 10% (vol/vol) glycerol, 0.5 mM DTT, 1 mM benzamidine, 0.2 mM PMSF, pH 7.9). 5 mg of nuclear extract was concentrated to 500 µl using a Microcon centrifugal filter (Millipore) and then applied to a Superose 6 size-exclusion column (10/300 GL GE Healthcare) pre-calibrated using the Gel Filtration HMW Calibration Kit (GE Healthcare). 500 µl of eluate was collected for each fraction at a flow rate of 0.5 ml/min, and eluted fractions were subjected to SDS-PAGE and western blotting. To assess the purity of fractionation, equal amounts of cytoplasmic and nuclear fractions were analyzed by SDS-PAGE.

Immunoprecipitation. For endogenous immunoprecipitation experiments, nuclear extracts from the prostate cancer cell lines LNCaP and VCaP were obtained using NE-PER nuclear extraction kit (Thermo Scientific). Nuclei obtained were lysed in the IP buffer (20 mM Tris–HCl, pH 7.5, 1% Triton-X, 150 mM NaCl and 1x Halt Protease Inhibitor Cocktail (Fisher)). Nuclear lysates (0.5–1.0 mg) were then pre-cleaned by incubation with protein G Dynabeads (Life Technologies) for 1 h at 4 °C. 5 µg of antibody was added to the pre-cleaned lysates and incubated on a rocker at 4 °C overnight before the addition of protein G Dynabeads for 1 h. Beads were washed thrice in IP buffer, resuspended in 40 µl of 2× loading buffer and boiled at 90 °C for 10 min for separation of the protein and beads. Samples were then analyzed by immunoblotting as described above.

To assess the effect of MI-136 on the interaction between the MLL complex and AR, VCaP cells were treated with varying concentrations of MI-136 for 24 h and nuclear extracts were prepared as described above. Immunoprecipitation was performed using anti-menin antibody exactly as described above followed by immunoblotting using anti-AR and anti-MLL antibody.

Immunofluorescence analysis. VCaP cells were fixed with 3.7% paraformaldehyde and then permeabilized with 0.1% (wt/vol) saponin for 15 min. Cells were co-incubated with primary antibodies against AR and menin or ASH2L for 12 h at 4 °C, followed by incubating with appropriate Alexa Fluor–conjugated secondary antibodies for 30 min at 37 °C. Cells were washed and mounted onto glass slides using VectaShield mounting medium containing DAPI. Samples were analyzed using a Nikon A1 laser-scanning confocal microscope equipped with a Plan-Apo ×63/1.4 numerical aperture oil lens objective. Acquired images were then analyzed using ImageJ software (version 1.41o).

Gene expression microarray. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) and treated with RNase-free DNase (Qiagen). Integrity of RNA was assessed using a Bioanalyzer (Agilent). Expression profiling was performed using the Agilent Whole Human Genome Oligo Microarray (Santa Clara, CA) according to the manufacturer’s protocol. Microarrays were analyzed using Bioconductor and limma software (http://www.bioconductor.org/packages/release/bioc/html/limma.html). Briefly, raw Agilent data files were imported and normalized both within (method = “normexp”, offset = 200) and between (method = “Aquantile”). All arrays where used for between-array normalization, but log, fold changes were not quantile normalized, as differences in the magnitude of responses were expected for the various data sets. Control probes and probes for which the log, fold change could not be estimated were removed. Limma was run with default parameters. Design matrices were created to contrast the treatment (for example, drug + DHT) with vehicle (for example, vehicle + DHT). Heat maps represent log2 fold changes estimated by limma. For display purposes of replicate drug-treatment reproducibility, the mean log2 fold changes of vehicle treatment were subtracted from the log2 fold changes of drug treatment; i.e., the presented log2 fold changes are relative to the vehicle-treated log2 fold changes, which approximated the statistical modeling procedure above. For GSEA analysis, genes were ranked according to the shrunken limma log2 fold changes and the GSEA tool was used in ‘pre-ranked’ mode with all default parameters. Multiple-hypothesis correction was not necessary, as only the AR signature was used. To assess the effect of MI-136 and MI-503 on AR signaling, VCaP cells were treated with DMSO, 5 µM MI-136 or 5 µM MI-503 for 48 h. Cells were serum starved by replacing the media with DMEM containing 5% charcoal-stripped serum and MI-136 or MI-503 for 48 h. Cells were then stimulated with 10 nM DHT for 12 h and RNA was isolated and processed for expression microarrays as described above.

Quantitative RT-PCR assay. RNA was isolated from cell lysates by the RNeasy Micro Kit (Qiagen), and cDNA was synthesized from 1 µg RNA using SuperScript III (Invitrogen) and Random Primers (Invitrogen), per the manufacturer’s protocol. qRT-PCR was carried out on the ABI7900 HT Fast Real time system (Applied Biosystems) using gene-specific primers designed with Primer3 software and synthesized by IDT Technologies. qRT-PCR data were analyzed using the relative quantification method and plotted as average fold change compared to the control. GAPDH and actin were used as an internal reference. To evaluate the effect of ASH2L and KMT2A knockdown on AR signaling, cells were first hormone starved and then treated with indicated siRNAs against ASH2L, MEN1 or KMT2A. After 48 h, cells were treated with 10 nM of DHT and RNA isolation and qPCR were performed essentially as described above using Power SYBR Green Mastermix (Applied Biosystems). The primers used for qPCR are described in Supplementary Table 2b.

Cell viability assay. Cells were seeded in 96-well plates at 2,000–10,000 cells per well (optimum density for growth) in a total volume of 100 µl media containing 10% FBS. Serially diluted compounds in 100 µl of media were added to the cells 12 h later. Following 5 d of incubation, cell viability was assessed by Cell-Titer Glo (Promega). The values were normalized and the IC50 was calculated using GraphPad Prism 6 software.

Chromatin immunoprecipitation (ChIP). The ChIP assays were performed using HighCell ChIP kit (Diagenode) according to the manufacturer’s protocol. Briefly, VCaP cells were grown in charcoal-stripped serum containing media for 48 h followed by stimulation with 10 nM DHT or 100 nM R1881 for 12 h. Next, cells were cross-linked for 10 min with 1% formaldehyde. Cross-linking was
terminated by the addition of a one-tenth volume of 1.25 M glycine for 5 min at room temperature, followed by cell lysis and sonication (Bioruptor, Diagenode), resulting in an average chromatin fragment size of 300 bp. Chromatin equivalent to 5 × 10^6 cells was used for ChIP using different antibodies. ChIP DNA was isolated from samples by incubation with the antibody at 4 °C overnight followed by washing and reversal of cross-linking.

ChIP-seq library construction and sequencing analysis. DNA was purified for library preparation using the IPure Kit (Diagenode). The ChIP-seq sample preparation for sequencing was performed according to the manufacturer’s instructions (Illumina). ChIP-enriched DNA samples (1–10 ng) were converted into blunt-ended fragments using T4 DNA polymerase, *E. coli* DNA polymerase I large fragment (Klenow polymerase) and T4 polynucleotide kinase (New England BioLabs (NEB)). A single adenine base was added to fragment ends by Klenow fragment (3′ to 5′ exo); NEB) followed by ligation of Illumina adaptors (Quick ligase, NEB). The adaptor-modified DNA fragments were enriched by PCR using the Illumina Barcode primers and Phusion DNA polymerase (NEB). PCR products were size selected using 3% NuSieve agarose gels (Lonza) followed by gel extraction using QIAEX II reagents (QIAGEN). Libraries were quantified with the Bioanalyzer 2100 (Agilent) and sequenced on the Illumina HiSeq 2000 Sequencer (100 nucleotide read length). ChIP-seq data were mapped to human genome version hg19 using BW A47. The MACS program was used to generate Sequencer (100 nucleotide read length). ChIP-seq data were mapped to human genome version hg19 using BWA. The MACS program was used to generate coverage map files to visualize the raw signal on the UCSC genome browser. A hidden Markov model (HMM)-based peak-calling software program designed for the identification of protein–interactive genomic regions, was used for ChIP-seq peak determination. For enrichment plots shown in Figure 3a,b, identified peaks for each sample were centered by peak summit, and the average coverage per million was counted within 1,500 bp relative to the peak center. The overlap of AR and ASH2L enriched regions were calculated by BEDtools. The overlap of AR and ASH2L enriched regions were calculated by BEDtools. The coverage per million was counted within 1,500 bp relative to the peak center. The MAP peak enrichment was generated using Python–based script on raw data and visualized using JavaTreeView3 (ref. 52). Motif analysis was performed using MEME motif analysis.

Cell-free protein–protein interaction studies. Full-length AR and AR truncation mutants were cloned into pFN21A vectors (Promega) in accordance to the manufacturer’s instructions. After cloning, the fusion proteins were expressed using the TNT SP6 High-Yield Wheat Germ Reaction cell-free transcription and translation system (cat. no. L5030, Promega) following the manufacturer’s protocol. For each reaction, protein expression was confirmed by immunoblotting. Purified menin protein was kindly provided by Dr. Tomek Cierpicki, University of Michigan. Purified AR was purchased from Prolias Technologies (R1089-2), purified ASH2L was purchased from Cayman Chemicals (cat. no. 10946). Purified MAX and RING1B proteins were kindly provided by Dr. Xiaoju Wang, University of Michigan.

For in vitro purified pull-down experiments, 100 ng of AR was incubated with 20 ng of purified protein in binding buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 (vol/vol), 1 mM dithiothreitol and 1× Halt Protease Inhibitor Cocktail (Fisher)) and incubated at 4 °C for 1 h. AR was then immunoprecipitated and bound proteins were analyzed by western blotting as described above.

For interaction between menin and AR truncation mutants, 200–250 ng of purified menin was incubated with Halo-tagged FL-AR and truncation mutants in binding buffer. Menin was immunoprecipitated and bound proteins were analyzed by immunoblotting with anti-Halo antibody.

Cellular thermal shift assay. Cellular thermal shift assay (CETSA) was performed as previously described. Briefly, 1 × 10^6 (VCAp) or 5 × 10^6 (LNCaP) cells were plated in a 10 cm Poly-l-lysine coated plate (Corning 354469) and incubated overnight (37°C, 5% CO2). Cells were treated the following morning with DMSO or 5 μM MI-503 for 3 h at 37°C and 5% CO2. Following the incubation, cells were washed twice with PBS to remove residual drug and detached from the surface using TrypLE solution (Life Technologies). Cells were resuspended in PBS with HALT protease inhibitors (Promega) and equal amounts of cell suspensions were aliquotted into a 0.2 ml PCR plate. The plate was heated individually at different temperatures (Veriti thermal cycler, Applied Biosystems/Life Technologies) for 4 min followed by cooling at room temperature for 3 min. Cells were lysed by two cycles of freeze–thawing with liquid nitrogen and soluble fractions were centrifuged at 20,000g for 20 min at 4°C. Soluble proteins were measured by western blotting.

Prostate tumor xenograft model and drug studies. 4–6-week-old male CB17 severe combined immunodeficiency (SCID) mice were used from the University of Michigan ULM-1 Breeding Colony. Based on power calculation (http://www.biomath.info/power/index.htm), we determined that more than six mice per group were sufficient to detect significant differences in tumor volume with a high statistical power. Parental prostate cancer cells (VCAp or LNCaP-AR; 3–4 × 10^6 cells) were injected subcutaneously in 50% Matrigel (BD Becton, Becton Drive, NJ) into both sides of the dorsal flank of mice. Knockdowns of ASH2L, MEN1 and KMT2A genes using targeting shRNAs in VCAp cells were studied for their effects on tumor growth. The efficacy of small molecule menin inhibitors (MI-136 and MI-503) was similarly studied. Tumors were measured biweekly using digital calipers following the formula (π/6) (L × W^2), where L = length and W = width of the tumor. The University of Michigan University Committee on the Use and Care of Animals (UCUCA) approved all in vivo studies. All in vivo experiments were done in a blinded fashion. The person performing the measurements was blinded to the treatment groups.

To study the effect of ASH2L, MEN1 and KMT2A knockdown, VCAp cells infected with lentivirus encoding control shRNA or shRNA targeting specific genes (see ‘Cell lines and lentiviral and siRNA transfections’ section above) were injected subcutaneously into both sides of the dorsal flank of mice. Tumors were measured biweekly as described above.

To study the effect of menin inhibitors (MI-136 on tumor growth, VCAp xenografts were grown as described above. Once the tumors reached a palpable stage (80 mm^3), the animals were randomized and treated with either vehicle (15% DMSO and 25% PEG + PBS; n = 8) or 40 mg/kg MI-136 (n = 9) i.p. daily for the first 2 weeks, then 5 d a week thereafter. Before randomization, mice were excluded from the study on the basis of three criteria: i) if there was no tumor uptake, ii) if the tumor growth was static 23 d after initial uptake, and iii) if tumor size at the time of randomization was more than twice the s.d. of the mean. Tumor volumes were measured as described above.

To assess the efficacy of MI-136 treatment on the growth of castration-resistant VCAp xenografts (CRPC model), mice were injected with VCAp cells. Once the tumors were approximately 200–300 mm^3 in size, tumor-bearing mice were physically castrated and tumors were observed for regression and regrowth to approximately ~150 mm^3. Mice were then randomized (10 mice per group) and treated i.p. with vehicle or 40 mg/kg MI-136.

To assess the efficacy of MI-503, the VCAp CRPC model was generated as described above. Mice were then randomized (10 mice per group) and treated with vehicle, 75 mg/kg MI-503, 10 mg/kg MDV-3100 or a combination of 75 mg/kg MI-503 and 10 mg/kg MDV-3100. MDV-3100 was given by oral gavage and MI-503 was administered i.p. To assess the effect of MI-503 treatment on the growth of LNCaP-AR xenografts, 4-week-old male SCID mice (CR.17, SCID) were surgically castrated and allowed to recover for 2–3 weeks. LNCaP-AR cells were inoculated subcutaneously and once the tumors reached 80–100 mm^3, mice were randomized (10 mice per group) and treated i.p. with vehicle or 60 mg/kg MI-503 daily for 27 d.

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