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Original Research

Neddylation inactivation represses androgen receptor transcription and inhibits growth, survival and invasion of prostate cancer cells

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Abstract

Androgen receptor (AR) and its constitutively active variants (AR-Vs) have been extensively implicated in the progression and recurrence of prostate cancer, making them attractive targets in the treatment of this disease. Whether and how neddylation modification regulates AR, and the therapeutic implications of this potential regulation, are relatively unexplored areas of investigation. Here we report that neddylation inactivation by the pharmacological inhibitor MLN4924 or Lenti-shRNA-based genetic knockdown of neddylation activating enzyme (NAE) selectively suppressed growth and survival of prostate cancer cells with minor, if any, effect on normal prostate epithelial cells. MLN4924 also significantly suppressed the invasive capacity of prostate cancer cells. Furthermore, compared to monotherapy, the combination of MLN4924 with AR antagonist or castration significantly enhanced growth suppression of prostate cancer cells *in vitro*, and tumor growth in an *in vivo* xenograft model. Mechanistically, MLN4924 repressed the transcription of AR/AR-V7 and its downstream targets, and blocked MMP2 and MMP9 expression. Taken together, our study reveals that the neddylation pathway positively regulates AR/AR-V7 transcription, and that the neddylation inhibitor MLN4924 has therapeutic potential for the treatment of aggressive prostate cancers.

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Keywords: Neddylation, MLN4924, Androgen receptor, Prostate cancer, Targeted therapy

Introduction

Prostate cancer (PCa) is the most common non-cutaneous malignancy and the third leading cause of cancer death in men [1]. Androgen deprivation therapy (ADT), which includes using an androgen receptor (AR) antagonist to block AR-androgen interaction and/or a gonadotropin-releasing hormone (GnRH) agonist to block androgen synthesis [2], remains a cornerstone of treatment for metastatic or locally advanced disease [3]. Initial cancer control by ADT is observed in most cases. However, castration-resistant prostate cancer (CRPC) frequently develops following this initial period of response [4] with AR alterations representing a key driver of treatment failure [5,6]. Indeed, AR amplification [7], mutation [8], splicing variants [9] and aberrant activation via growth factors [10], receptor tyrosine kinases [11,12], AKT pathway [13] and long noncoding RNA-dependent mechanisms [14] have all been implicated in the acquisition of castration resistance. Thus, new approaches to actively block AR signaling pathway are urgently needed.

The ubiquitin–proteasome system (UPS) is a major protein degradation system responsible for the maintenance of protein homeostasis in cells [15]. Elevated UPS activity results in excessive degradation of tumor suppressors and is associated with many human cancers, making it an

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attractive target for anti-cancer therapy [16]. More specifically, Cullin-RING ligases (CRLs) comprise the largest family of E3 ubiquitin ligases and are responsible for ubiquitylation of 20% of all cellular proteins, including many key signaling/regulatory proteins for proteasomal degradation [17]. CRLs are often activated during tumorigenesis and serve as a valid anti-cancer target [18].

Protein neddylation is a process of attaching ubiquitin-ligase protein NEDD8 to a lysine residue on cullins, catalyzed by three enzymes, including NEDD8 activating enzyme (NAE), an NEDD8 conjugating enzyme, and an NEDD8 ligase. While cullins with 8 family members are physiological substrates of neddylation, and cullin neddylation is required for CRL activation [18], there are a number of biologically significant proteins that are also subjected to neddylation regulation [19]. MLN4924 is a small molecule compound discovered in 2009 that acts as a potent and selective inhibitor of neddylation activating enzyme (NAE), thus inhibiting the entire process of neddylation [17]. By doing so, MLN4924 inactivates all members of CRLs, as well as neddylation of all non-cullin substrates to suppress the growth and survival of various cancer cell lines in preclinical settings [20]. Currently, MLN4924, also designated as pevonedistat, is in several Phase I/II clinical trials for anti-cancer applications [20].

The effect of MLN4924 on prostate cancer cells has been studied previously. It was reported that MLN4924 suppressed the growth of various prostate cancer cell lines [21–23] by causing accumulation of tumor suppressive substrates of CRLs, including p21, p27, WEE1, IκBα, CDT1 and ORC1 [23]. MLN4924 also enhanced IR-induced G2 cell-cycle arrest and sensitized prostate cancer cells to radiation via inducing accumulation of WEE1/p21/p27 [24]. It has also been shown that MLN4924 promotes migration of PC3 cells through enhanced caveolin-1 phosphorylation [25]. However, whether and how MLN4924 affects expression of AR and its variants, along with AR downstream targets remain largely elusive.

In this study, we report that MLN4924 selectively inhibited growth of prostate cancer cells, with much lesser effect on normal prostate epithelial cells. In AR-positive prostate cancer cells (22RV1 and LNCaP), MLN4924 significantly reduced the levels of full length AR (110 kDa) and truncated AR variants (AR-Vs, 75–80 kDa), the splicing variants that contribute to the progression of castration-resistant prostate cancer (CRPC) [26], in a dose- and time-dependent manner. Mechanistic studies revealed that MLN4924 did not change the protein half-life of AR or AR-Vs, but inhibited transcription of both full length AR and AR-V7 (80 kDa), leading to suppression of AR downstream target genes. We also found that MLN4924 significantly sensitized prostate cancer cells to Enzalutamide (a 2nd generation of AR antagonist) *in vitro*, and castration *in vivo*. Thus, blockade of neddylation may represent a novel approach for prostate cancer therapy.

Materials and methods

Cell lines

All prostate cell lines used in this study were obtained from the American Type Culture Collection (Manassas, VA, USA) or Merck KGaA (Darmstadt, Germany). PNT2 cells were cultured in RPMI1640 (Gibco) supplemented with 10% FBS (Lonza) and 2 mM Glutamine (Gibco). RWPE2 cells were cultured using Keratinocyte Serum Free Medium (K-SFM kit, Gibco) supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml human recombinant epidermal growth factor (both provided in K-SFM kit). LNCaP, C42B, 22RV1 and LN95 cells were cultured in RPMI1640 supplemented with 10% FBS, 25 mM HEPES (Gibco), 50 units/ml penicillin + 50 μg/ml streptomycin (Gibco). VCaP cells were cultured in DMEM supplemented with 10% FBS, 25 mM HEPES, 50 units/ml penicillin and 50 μg/ml streptomycin. All lines were mycoplasma-free after testing by Mycoalert Mycoplasma Detection Kit (LT07-318, Lonza).

Compounds, antibodies, Lenti-shRNA and transfection

MLN4924 was obtained from Takeda Pharmaceuticals, Inc. and dissolved in DMSO. The antibodies used include: androgen receptor (rabbit polyclonal, EMD Millipore), Cul1 (rabbit polyclonal, Abcam), UBA3/NAEβ (rabbit monoclonal, Cell Signaling), actin (Santa Cruz). The Lenti-virus in pLKO-1 backbone was generated to knockdown UBA3, the catalytic subunit of NAE, the target of MLN4924. For Lenti-UBA3, the forward oligo: 5′-CCGGAAGCTTCTCTGCAAAT GAAATCTCGAGATTT CATTTGCAGAGAAGCTTTTTTTG-3′. The reverse oligo: 5′-AATTCAAAAAAAGC TTCTCTGCAAATGAAA TCTCGAGATTTCATTTGCAGAGAAGCTT-3′. For Lenti-Cont, the sequence is LT-CONT-01: 5′-ATTGTATGCGATCGCAGACTTTT CAAGAG AAAGTCTGCGATCGCATACAATTTTTTGT-3′; and LT-CONT-02: 5′-CTAGACA AAAAATTGTATGCGATCGCAGAC TTTCTCTTGAAAAGTCTGCGATCGCATAC AAT-3′.

Cell proliferation assay

Cells were plated in a 96-well plate and treated with various drugs for up to 72 h unless otherwise indicated. Cell growth was assessed by CellTiter-Glo Luminescent Cell Viability Assay (Promega) or 1-step ATPLite assay (PerkinElmer) according to the manufacturer's protocol.

Clonogenic assay

Cells were plated at 400 cells per 60-mm dish and treated with various concentration of MLN4924, along with DMSO control. After growth in culture for 14 days, cells were fixed and stained by a mixture of 6.0% glutaraldehyde and 0.5% crystal violet. Clones were counted using cell counter on Image J.

Cancer cell invasion assay

C42B or 22RV1 cells ($2–5 \times 10^4$) suspended in 100 μl of serum free medium were plated in an 8.0-mm, 24-well plate matrigel-coated chambers (Corning Life Sciences, catalog no. 354483), with medium containing 10% fetal bovine serum (FBS) at the bottom of the insert. Cells were incubated for 12–24 h, and then fixed with 4% paraformaldehyde for 5 min. After washing with PBS for three times, cells were stained with 0.5% crystal violet blue for 5 min, and then washed with double distilled water. Cells on the upper surface of the insert were removed with a cotton swab. The positively stained cells were examined under the microscope [27].

Quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed with 200 ng of RNA using CFX-connect RT-PCR detection system with iTaq Universal SYBR One-Step Kit according to manufacturer's protocol. The primer sequences were: Full length androgen receptor: forward primer, CAGCCTATTGC-GAGAGAGCTG; reverse primer, GAAAGGATCTT GGGCACTTGC; AR V7: forward primer, CCATCTTGTCGTCTTCGGAAATGTTA; reverse primer, TTTGAATGAGGCAAGTCAGCCTTTCT; KLK3: forward primer, ACGCTGGACAGGGGGCAAAAG; reverse primer, GGGCAGGGCACATGGTTCA CT; FKBP5: forward primer, TCTC ATGTCTCCCCAGTTCC; reverse primer, TTCTGGCTTTCACGTC TGTG; NKX3: forward primer, CCGCTTCCAAAGACCTA GAGGA; reverse primer, ACCGTCGTCCTCGGTCCTTGG; MMP2: forward

primer, GCTGGCCTAGTGATGATGTTAGGCA; reverse primer, CCTTGGGGCAGCCATAG AAGGT; MMP9: forward primer, GGGACGCAGACATCGTCATC; reverse primer, TCGTCATCGTCGAAATGGGC; GAPDH: forward primer, CCATCACCATCTTCC AGGAGCGA; reverse primer, GGTGGTGAAGACGCCAGTGGA.

Western blotting analysis

Cell lysates were prepared after various treatment and subjected to PAGE, and blotted with various antibodies as described previously [28].

Xenograft assay

All animal procedures were approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC). Animal care was provided in accordance with the principles and procedures outlined in the National Research Council Guide for the Care and Use of Laboratory Animals. Male 4–6 week old CB17/SCID mice were procured from our breeding colony that is maintained by the Unit for Animal Medicine (ULAM) at the University of Michigan. Xenografts were established by subcutaneously injecting 1×10^6 VCaP cells resuspended in 200 µl of a 1:1 mixture of Matrigel (BD Biosciences, San Jose, CA) and PBS bilaterally in the flanks. Tumor volume was monitored by caliper measurement using the formula $L \times W^2 \times \pi/6$. Grouping and treatment is detailed in results section.

Immunohistochemistry staining

Nude mice tumor tissues harvested from all four experimental groups were fixed, imbedded, sectioned and stained with antibodies against Ki67 and cleaved caspase-3, as described previously [29,30].

Statistical analysis

Student t tests were performed using GraphPad Prism 7. Data was represented as mean þ SEM from at least three independent experiments. Not significant (NS): $p > 0.05$; $*0.01 < p < 0.05$; $**0.005 < p < 0.01$; $***p < 0.005$.

Results

MLN4924 selectively inhibits growth of prostate cancer cells with minimal effect on normal prostate epithelial cells

It has been previously reported that the growth of prostate cancer cells can be suppressed by blockage of neddylation, by either MLN4924, a potent and selective inhibitor of NAE or Lenti-virus based shRNA knockdown of UBA3, a catalytic subunit of NAE [21–23]. However, effect of MLN4924 on normal prostate epithelial cells has not been reported. We used three prostate cancer lines and two lines of prostate normal epithelia cells and found that MLN4924 caused a dose-dependent inhibition of cancer lines with the IC_{50} value of 7, 36 or 140 nM for C42B, an androgen receptor (AR) positive CRPC cell line, as well as LNCaP and 22RV1, two prostate cancer cell lines more responsive to androgen modulation, respectively (Fig. 1A, Supplemental Fig. S1A–C). MLN4924 caused obvious morphological changes in these cancer cells with cell shrinkage and round-up (Fig. 1B, top three panels). On the other hand, normal prostatic epithelial cells, RWPE2 and PNT2, were much more resistant to MLN4924 with IC50′s of over 1200 nM and 1700 nM, respectively (Fig. 1A, Supplemental Fig. S1D&E). Furthermore, unlike cancer cells, MLN4924 had no obvious effect on the morphology of normal cells (Fig. 1B, bottom two panels).

To rule out the possible off-target effect of small molecule compound, we constructed a Lenti-virus based shRNA vector specifically targeting UBA3 (the catalytic subunit of NAE). Comparing with Lenti-shRNA control infected cells, UBA3-knockdown significantly suppressed growth of 22RV1 cells ($p = 0.0321$, Fig. 1C), and C42B cells ($p = 0.0169$, Fig. 1D), although the growth inhibitory effect by UBA3 knockdown appears to be independent of cellular response to androgen. In contrast, UBA3 knockdown has no effect on the growth of RWPE2 cells (Fig. 1E), indicating a selective growth suppression against cancer cells by targeting neddylation E1 NAE.

MLN4924 inhibits the survival and invasion of prostate cancer cells

We next determined the effect of targeting neddylation E1 on clonogenic survival of prostate cancer cells. Pharmacological inactivation of NAE by MLN4924 caused a dose-dependent inhibition of colony formation in both C42B and 22RV1 cells (Fig. 2A&B), while Lenti-virus based shRNA knockdown of UBA3 also significantly suppressed colony formation (Fig. 2C&D, $p < 0.005$). We also determined the potential effect of MLN4924 on the invasive ability of prostate cancer cells and found that MLN4924 nearly completely blocked cell invasion in both C42B and 22RV1 cells ($p < 0.005$, Fig. 2E&F). Given that invasive prostate cancer was previously shown to be associated with elevated expression of matrix metalloproteinases (MMPs), which are responsible for extracellular matrix (ECM) remodeling to promote cell invasion [31,32], we determined the effect of MLN4924 on MMP mRNA levels, and found a significant reduction in both MMP2 and MMP9 mRNA expression in C42B and 22RV1 cells, following MLN4924 treatment ($p < 0.005$, Fig. 2G&H).

MLN4924 reduces the protein levels of AR and AR-Vs without affecting their protein half-lives

The observation that proliferation, clonogenicity and invasiveness of AR-positive human prostate cancer cells were significantly inhibited following pharmacological inactivation of NAE or genetic knockdown of UBA3, regardless of the status of androgen dependency, triggered us to test whether MLN4924 would affect the levels of AR or its variants. Using an antibody targeting the modulation region (epitope: MEVQLGLGRVYPRPPSKTYRG) within the N-terminus domain (NTD) of AR that recognizes all major forms of AR splicing variants (AR-Vs) [33], we found that MLN4924 treatment of 22RV1 and LnCaP cells reduced the levels in both a dose- and time-dependent manner of full length AR (110 kDa), as well as AR-Vs within 75 ~ 80 kDa, which included AR-V7, the most significant AR-Vs contributing to aberrant activation of AR signaling that leads to castration-resistance [34] (Fig. 3A). A similar reduction of AR and AR-V7 levels was observed upon UBA3 knockdown in 22RV1 cells (Fig. 3B), indicating it is a neddylation-dependent effect.

The reduced levels of AR and AR-Vs could result from reduced mRNA transcription or enhanced protein degradation. Given the effect of MLN4924 occurs in general at the levels of protein ubiquitylation and degradation by inactivating Cullin RING ligases, we first measured the protein half-lives of AR and AR-Vs. Specifically, we treated cells with cycloheximide (CHX) to block new protein synthesis in combination of MLN4924 for a variety of time points, followed by Western blotting. The results clearly showed that MLN4924 had no effect on protein half-lives of either full length AR or AR-Vs (Fig. 3C&D).

MLN4924 reduces the mRNA levels of AR, AR-V7 and AR target genes

We then measured the effect of MLN4924 on the mRNA levels of AR, AR-V7, along with three major AR downstream targets, including KLK3

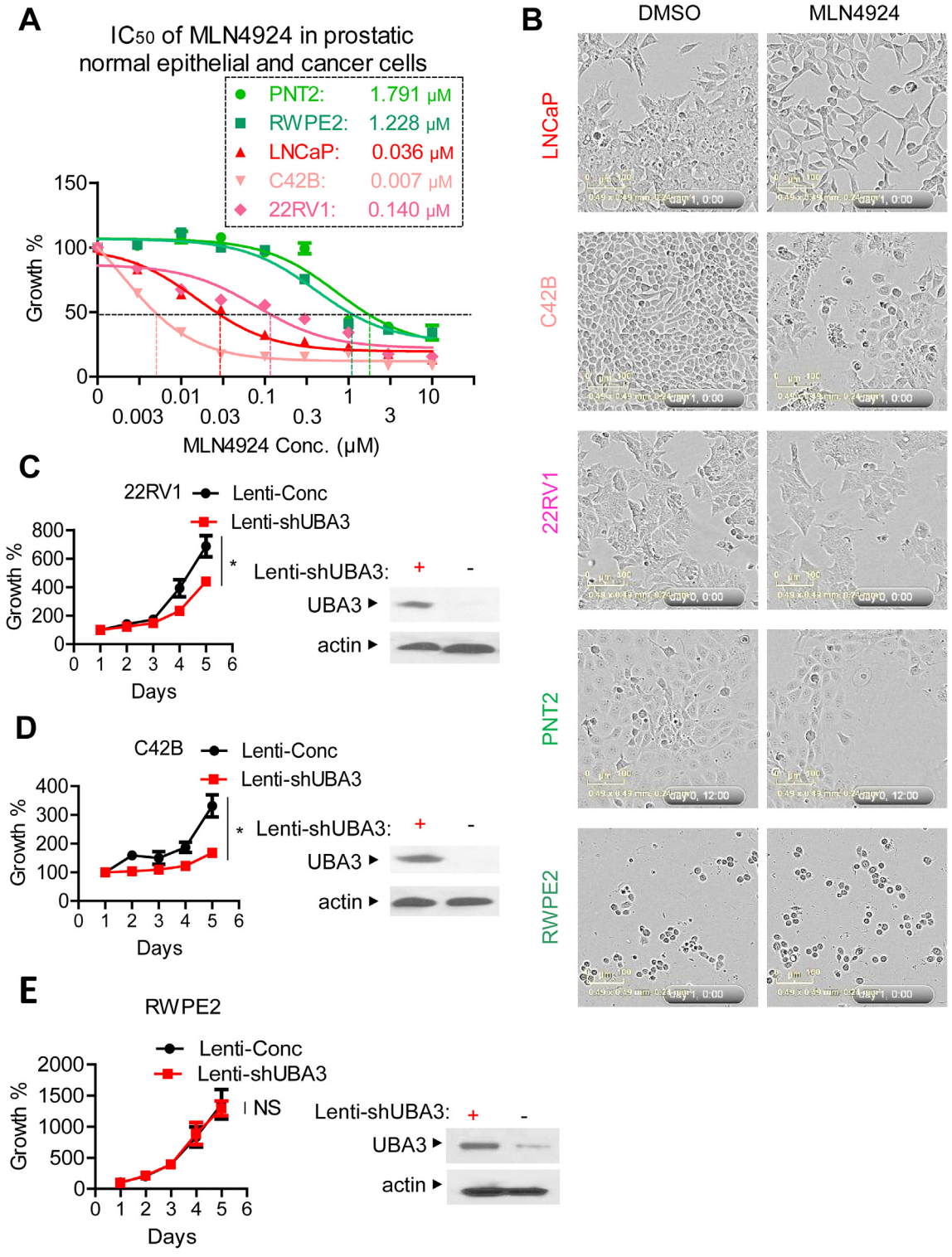


Fig. 1. Blockage of neddylation effectively inhibits growth of prostate cancer cells with much less effect on normal prostatic epithelial cells. (A&B) Growth suppression of prostate cancer cells and normal prostatic epithelial cells by MLN4924. Prostate cancer cells (C42B, LnCaP, 22RV1) or normal prostatic epithelial cells (RWPE2, PNT2) were seeded in 96-well plates in triplicates and treated with indicated concentrations of MLN4924 for 72 h. Cell growth were assessed by ATP-Lite assay, and the inhibition of growth rate was plotted with IC50 values calculated by Graphpad Prism (A). All cells were treated with vehicle or 0.1 μM MLN4924 for 72 h before representative pictures were taken (B). Scale bar: 100 μm. (C–E) Growth suppression of prostate cancer cells by UBA knockdown. Lenti-virus based shRNA targeting UBA3, the catalytic subunit of NAE, were infected into 22RV1 (C), C42B (D) and RWPE2 (E) cells, along with scramble or GFP control. 48 h post infection, one portion of cells was subjected to western blotting with indicated antibodies, whereas the second portion was plated in 96-well plates. Cell proliferation was assessed at indicated time points.

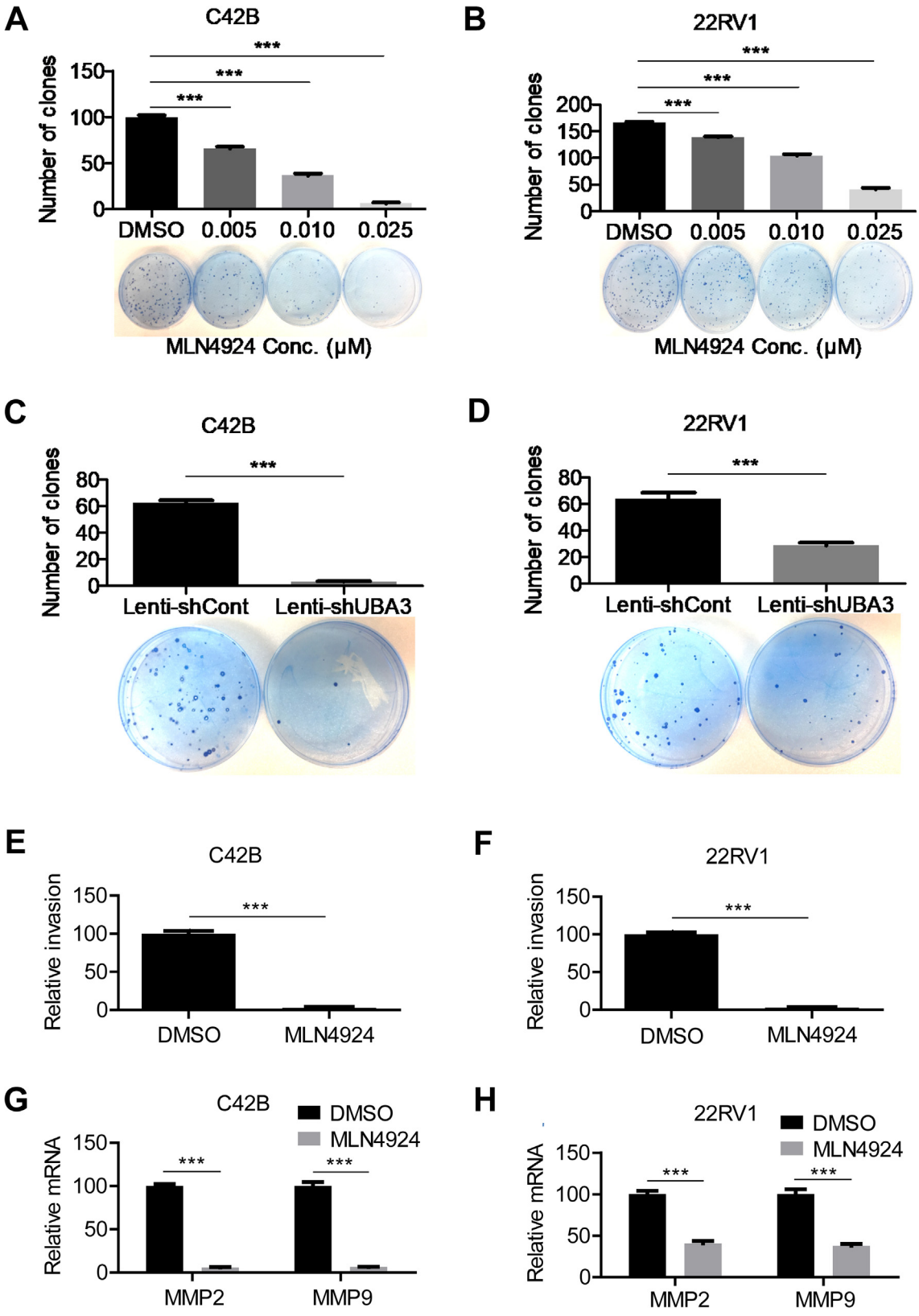


Fig. 2. Blockage of neddylation effectively inhibits clonal survival of prostate cancer cells. (A&B) 22RV1 and C42B cells were plated at clonal density and treated with vehicle or indicated concentrations of MLN4924 for 9 days, followed by the staining. The number of clones were counted and plotted against dose with photos of dishes taken and shown. (C&D) The Lenti-shUBA3 targeting the catalytic subunit of NAE, was infected into 42B and 22RV1 cells, along with Lenti-shCont. 48 h post infection, cells were plated at clonal density and grown for 9 days. (E&F) 22RV1 and C42B cells were seeded in matrigel-coated invasion chamber in the presence of vehicle or 0.25 μM MLN4924, respectively, and invasion capacity measured as described in M&M. (G&H) 22RV1 and C42B cells were treated vehicle or 0.25 μM MLN4924, respectively, for 24 h before harvested for MMP2 and MMP9 mRNA expression by qRT-PCR. The mRNA expression levels were calculated by arbitrarily setting vehicle value as 100.

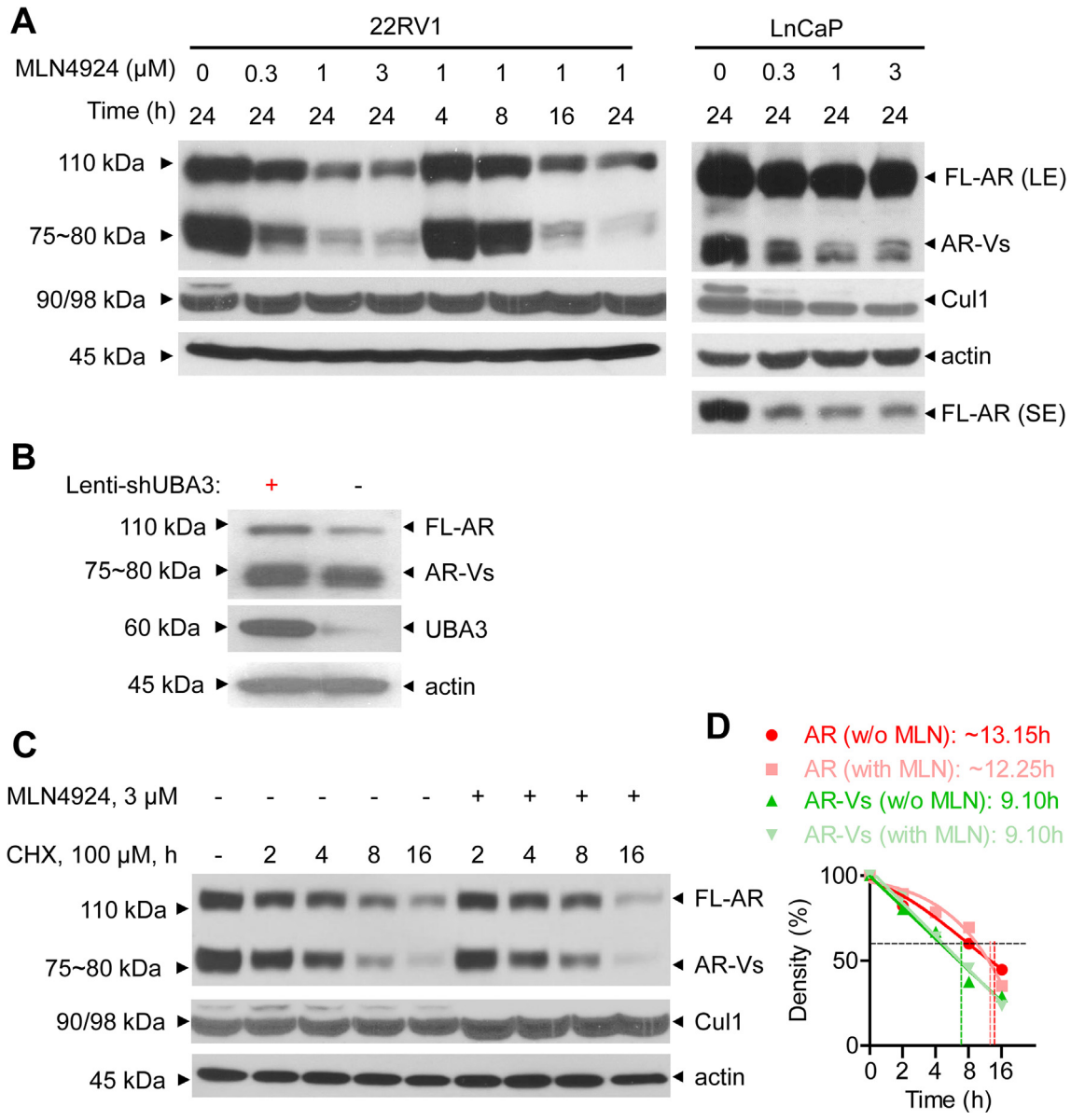


Fig. 3. MLN4924 reduced the levels AR and AR-Vs proteins in prostate cancer cells. (A) 22RV1 and LnCaP cells were treated with vehicle or indicated concentrations of MLN4924 for up to 24 h, before being harvested and subjected to western blotting using PG-21, an antibody raised against the N-terminus of AR that recognizes full length AR (FL-AR) and truncated AR (AR-Vs), along with other indicated Abs. (B) 22RV1 cells were infected with Lenti-shUBA3, along with scramble control (Lenti-shCont), followed by western blotting with indicated Abs. (C&D) 22RV1 cells were treated with vehicle or 3 μM MLN4924 and 100 μM CHX for 2, 4, 8 and 16 h, before being harvested and subjected to western blotting using indicated Abs (C). The band density was quantified by AlphaEaseFC, and plotted. The half-lives of AR and AR-Vs were calculated using Graphpad Prism 7 (D).

(Kallikrein Related Peptidase 3, also known as PSA, prostate-specific antigen), FKBP5 (FK506 binding protein 5), and NKX3 (NK3 Homeobox 1). In 22RV1 cells, MLN4924 reduced mRNA levels of all five genes in a dose- and time-dependent manner (Fig. 4A&B). In VCaP cells, expressing endogenously amplified wild-type AR, MLN4924 reduced the mRNA levels of AR and three downstream targets, again in a dose and time dependent manner (Fig. 4C&D). In LN95 cells, a LnCaP derivative line that was androgen independent but highly expressing AR-V7, we found that MLN4924 reduced AR-V7 mRNA levels again in a dose- and time-dependent manner (Fig. 4E&F). Suppression of the mRNA expression by MLN4924 was found to be statistically significant ($p < 0.005$) in all cases. Taken together, MLN4924 inhibits transcription of AR/AR-V7 and their downstream target genes, leading to blockage of AR signaling pathways.

MLN4924 sensitizes prostate cancer cells to AR antagonist or castration.

Ligand-dependent or aberrant activation of AR signaling plays a vital part in promoting tumorigenesis of AR positive prostate cancer cells. AR antagonist has been historically used as the first-line drug in hormone therapy for treatment-naive prostate cancer patients [35]. Given that blockage of neddylation by MLN4924 represses the expression of AR, AR-V7, and AR downstream targeting genes, and significantly inhibits proliferation and invasion in both androgen-sensitive and resistant prostate cancer cells, we next determined whether MLN4924 would sensitize 22RV1, an AR-positive and androgen-sensitive prostate cancer cells to AR antagonist in cell culture setting. Cells were treated with MLN4924 (30 nM) or MDV3100/Enzalutamide (10 μM), a

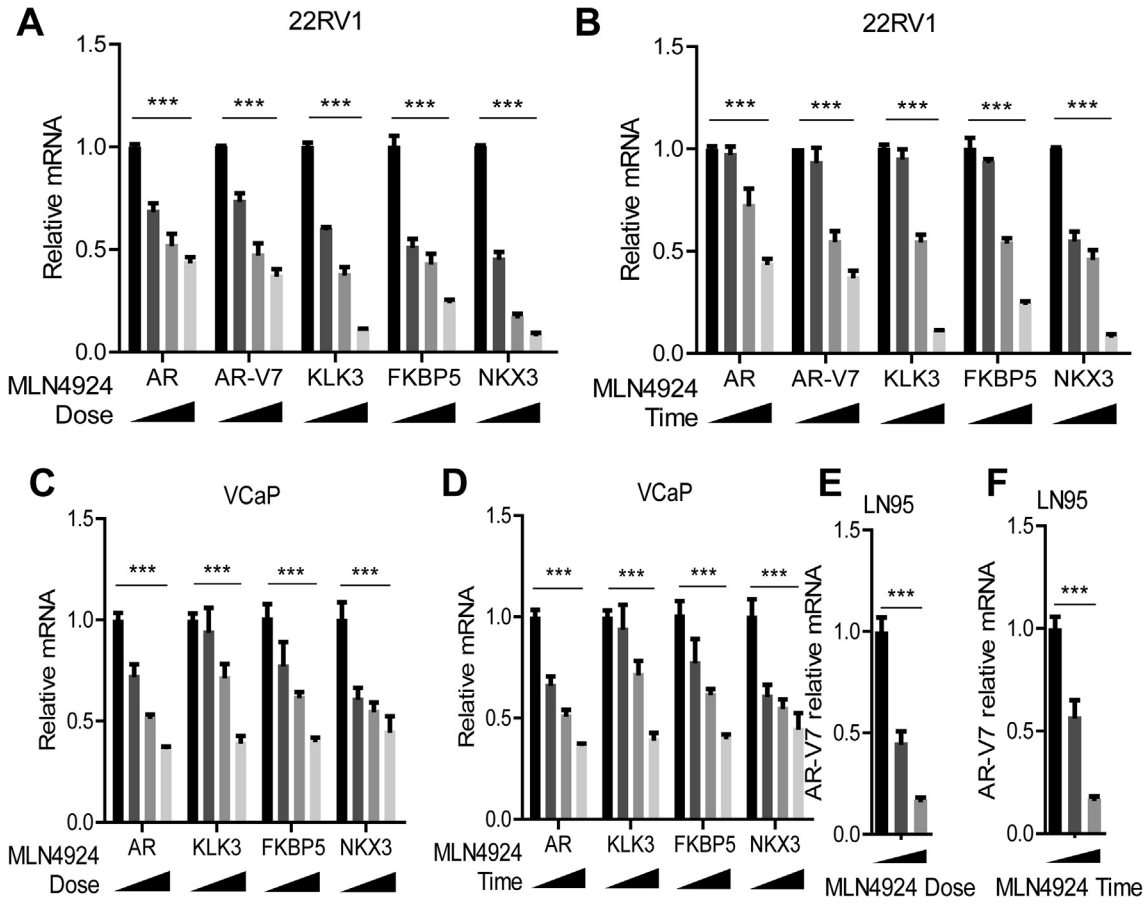


Fig. 4. MLN4924 downregulates mRNA expression of AR and AR-V7 in prostate cancer cells. (A-D) 22RV1 cells (A&B) or VCaP cells (C&D) were treated with vehicle, 0.1, 0.3 or 1 μM of MLN4924 for 24 h (A&C) or 1 μM of MLN4924 for 3, 6 or 24 h (B&D) before being harvested and subjected to RT-qPCR measurement of mRNA levels of AR, AR-V7 and AR targets KLK3, FKBP5, and NKX3. (E&F) LN95 cells were treated with vehicle, 0.25 or 1 μM of MLN4924 for 24 h (E), or 1 μM of MLN4924 for 6 or 24 h (F) being harvested and subjected to RT-qPCR measurement of mRNA levels of AR-V7. Fold change in mRNA expression was calculated by arbitrarily setting vehicle value as 1.

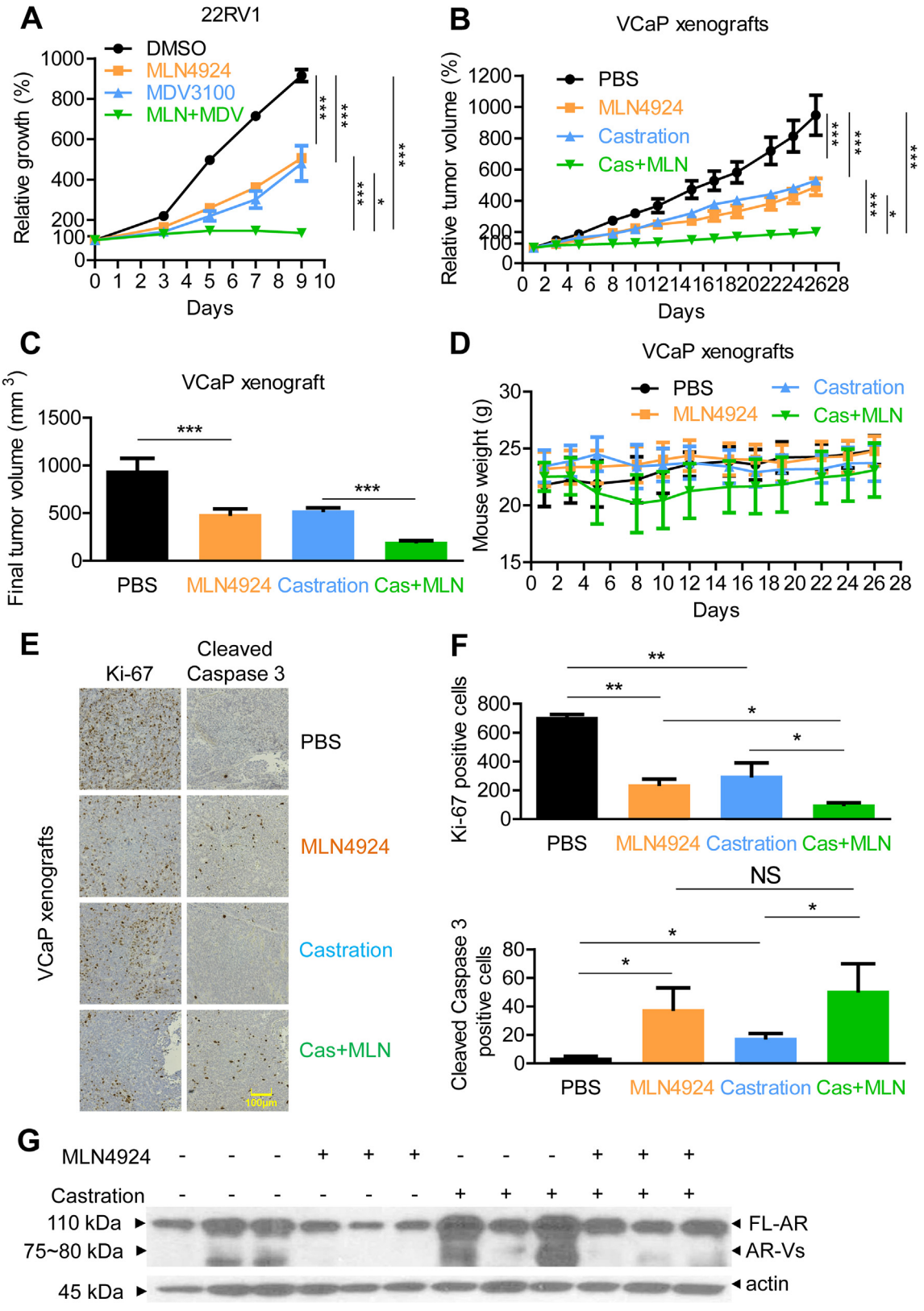
second-generation AR antagonist, alone or in combination for a period up to 9 days. While each single agent treatment indeed caused significant growth inhibition ($p < 0.005$), the combination completely suppressed growth, which is statistically significant when compared to each single agent treatment ($p < 0.005$ compared to MLN4924) or ($p = 0.017$ for MDV3100) (Fig. 5A). Thus, MLN4924 indeed sensitizes prostate cancer cells to AR antagonist.

We next used a prostate cancer xenograft model to assess MLN4924 sensitization of castration. VCaP cells were injected subcutaneously in the flank on both sides in male CB17/SCID mice. For non-castrated mice, once tumor volumes averaged 100 mm^3, vehicle or 45 mg/kg/day MLN4924 was administered via subcutaneous injection once per day, 5 days per week for a total of 4 weeks. For castrated mice, castration was performed when tumors reached an average of 200 mm^3. Treatment with vehicle or the same dose of MLN4924 was given once all tumors grew back to an average of 200 mm^3. As expected, comparing with non-castrated vehicle treated mice, the mice with castration alone had significantly reduced tumor growth ($p < 0.005$). Similar growth suppressive effect was observed in non-castrated mice with treatment of MLN4924 ($p < 0.005$). Strikingly, similar to the synergistic effect of MLN4924 and AR antagonist, the combination of castration and MLN4924 nearly completely blocked the tumor growth ($p < 0.005$, Fig. 5B). All mice were sacrificed and tumors were retrieved at the end of experiment and weighed. The results were in agreement of tumor growth with a maximum suppression seen in combination group (all $p < 0.005$, Fig. 5C). Mouse body weight remained consistent throughout the experiment (Fig. 5D), suggesting a low toxicity of the treatments.

We then performed immunohistochemistry staining of tumor tissues harvested from these four groups of mice (three tumors per group) with Ki67 (for proliferation) and cleaved caspase-3 (for apoptosis). Consistent with tumor growth rate, either MLN4924 treatment or castration caused suppression of cell proliferation and induction of apoptosis with maximal effect seen in combination group of MLN4924 and castration (Fig. 5E&F). Finally, we performed western blot analysis on AR and AR-Vs levels in VCaP xenografts tumors (3 independent tumors from each group), and found that castration increased the levels of both AR and AR-Vs, whereas MLN4924 treatment reduced the levels of both AR and AR-Vs in tumors derived from both control and castrated mice (Fig. 5G), indicating that suppression of AR/AR-Vs expression by MLN4924 seen the *in vitro* cell culture model can be extended to *in vivo* xenograft tumor model.

Discussion

Here we report a novel finding that neddylation inhibitor MLN4924 effectively and selectively inhibits the growth and survival of prostate cancer cells via repressing the transcriptional expression of AR and its variants.



MLN4924 also suppressed invasion of prostate cancer cells via blockage of transcription of MMP2 and MMP9. Importantly, MLN4924 sensitizes prostate cancer cells to the AR antagonist Enzalutamide *in vitro*, and castration *in vivo* (Fig. 6).

Regulation of AR protein turnover by the ubiquitin proteasome system (UPS) via non-cullin based E3 ligases, such as MDM2 and CHIP, has been recently reported [36,37]. How neddylation regulates AR transcription or AR protein turn-over has not been previously studied systemati-

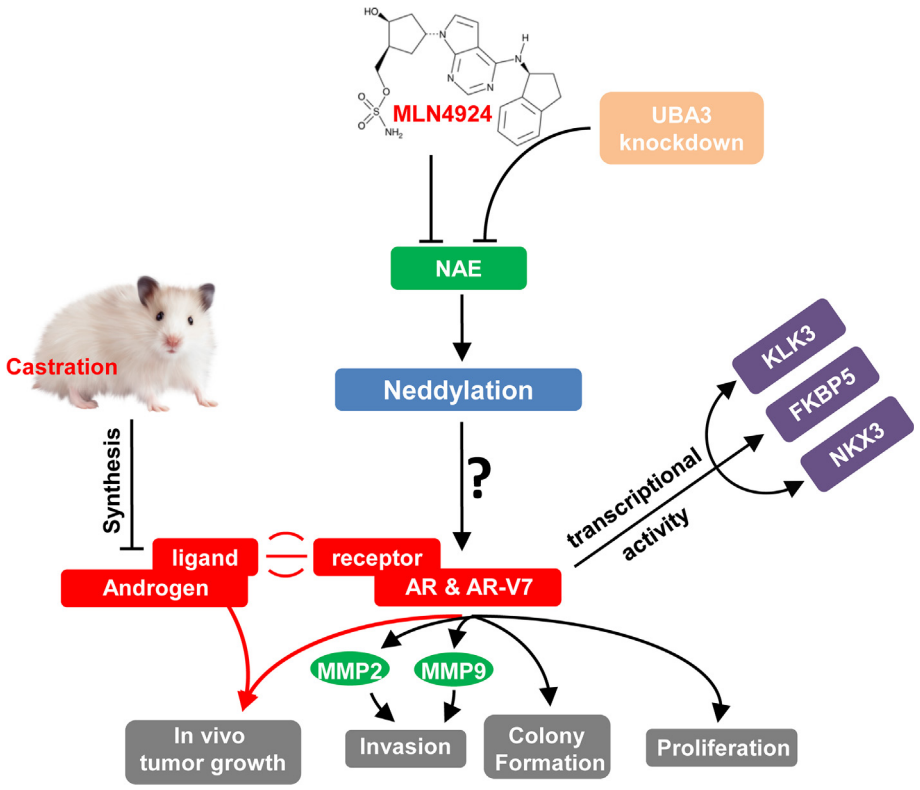


Fig. 6. Mechanism of action. MLN4924 treatment or UBA3 knockdown blocks neddylation by inactivation of NAE, leading to suppression of AR and AR-Vs, along with AR target genes, KLK3, FKBP5, and NKX3, by a yet-to-be-defined mechanism. Down-regulation of AR/AR-Vs and its targets results in suppression of growth and survival of prostate cancer cells as well as inhibition of invasion via MMP2/9 downregulation. The combination of MLN4924 with AR antagonist enzalutamide *in vitro*, and the castration *in vivo* significantly suppressed growth of prostate cancer.

cally. We found that the neddylation inhibitor MLN4924 indeed reduces the protein levels of AR and AR-Vs. However, this effect does not occur at the posttranslational levels, since MLN4924 did not change protein half-lives of AR and AR-V7. Instead, MLN4924 effectively reduced the transcription of the AR gene as well as its downstream target genes in a time- and dose-dependent manner. Among three AR target genes, which are also downregulated by MLN4924, KLK3 is a typical prostate biomarker for diagnosis and prognosis [38], FKBP5 plays a role in cancer etiology and chemoresistance [39], whereas NKX3, an androgen-regulated homeodomain transcription factor, appears to act as a tumor suppressor in prostate cancer [40], which may also be involved in resistance to castration [41]. MLN4924-induced downregulation of these genes may contribute to its anti-prostate cancer activity both seen *in vitro* and *in vivo* models. MLN4924 effect on AR expression was reported by a previous study, although AR was not the focus [22]. Nevertheless, two discrepancies were found between that study and ours: First, in their study, MLN4924 did not significantly change the level of AR; while we saw a dose (0, 0.3, 1, 3 μM)-dependent decrease in the protein levels of AR as well as AR-Vs. Second, they reported that at 50 nM MLN4924 caused a significant increase in the downstream targets of AR, including PSA, while at 500 nM MLN4924 suppressed the transcripts of PSA. In contrast, we observed a dose (0, 0.1, 0.3, 1 μM) dependent decrease in several AR

Fig. 5. MLN4924 suppresses growth of prostate cancer cells alone or in combination of hormone therapy *in vitro* and *in vivo*, respectively. (A) 22RV1 cells were treated with DMSO vehicle, 30 nM of MLN4924, 10 μM of MDV3100 or a combination of 30 nM of MLN4924 and 10 μM of MDV3100 for up to 9 days. Cells were harvested at indicated time points and assessed by ATP-Lite for growth. (B-D) 1×10^6 VCaP cells mixed with Matrigel and PBS were injected subcutaneously in the flanks of male CB17/SCID mice on each side. When all tumors reached an average of 100 mm^3, mice were then randomly placed in 2 groups: castration and non-castration group. In non-castration group ($n = 16$), vehicle (number of mice/number of tumors: 8/15) or 45 mg/kg MLN4924 (8/15) was administered via s.c. once a day, 5 days per week for 4 weeks. In castration group ($n = 15$), castration was performed when all tumors reached an average size of 200 mm^3; vehicle (7/14) or the same dose of MLN4924 (8/16) was given on the same schedule when all tumors grew back to an average of 200 mm^3. Tumor volume and mouse weight was measured every other day and plotted by arbitrarily setting the tumor volume measured on the day of initial drug injection as 100% against time (B). All mice were sacrificed at end of 4 weeks, and tumors were retrieved and weighed. The final tumor volume (mm^3) in each groups were plotted (C). Each mouse during 28-day period of experimental time was weighed and average body weight against time were plotted (D). Tumors were fixed in 10% formalin and embedded in paraffin. Each sample was stained for Ki67 and cleaved caspase-3 with representative images shown (E). Positive cells were counted from at least 5 randomly selected microscopic fields in each group with three independent tumors (F). Scale bar: 100 μm. Statistical analysis was performed using GraphPad Prism 7. $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$. (G). A total of 12 tumor tissues from four group ($n = 3$ per group) were subjected to IB analysis, using indicated antibodies.

downstream targets including PSA. These discrepancies could be derived from the use of different prostate cancer cell lines, which was not defined in their study. Another study [42] showed that UBA3, a catalytic subunit of neddylation activating enzyme inhibited transactivation by AR, but without revealing any mechanism of action.

Given the fact that a most recent comprehensive study on global site-specific neddylation profiling failed to detect AR [43], it is unlikely that AR itself is a neddylation substrate or subjected to neddylation modification. While the exact mechanism of MLN4924 inhibition on AR transcription is unknown at the present time, it is likely via an indirect effect on an AR transcription repressor, which is subjected to CRL regulation. An analogic case is our recent finding that MLN4924 suppresses SOX2 transcription via inactivating FBXW2 E3 ligase, which promotes the ubiquitylation and degradation of MSX2, a known repressor of SOX2 transcription, thus establishing a negative cascade of MLN4924, FBXW2, MSX2 and SOX2 [44,45]. Alternatively, it is also possible that a AR regulatory protein is subjected to neddylation regulation, abrogation of its neddylation by MLN4924 would suppress AR expression. The future investigation is directed to elucidate this interesting underlying mechanism.

In addition, our study showed that MLN4924 suppresses invasion of prostate cancer cells, which appears to result from down-regulation of MMP2 and MMP9 expression. It has been previously reported that finasteride, a 5α-reductase inhibitor that represses AR signaling by reducing the formation of the potent androgen dihydrotestosterone from its precursor testosterone, was also shown to downregulate MMP2 and MMP9 in prostate cancer cells [46]. Thus, downregulation of MMP2 and MMP9 by MLN4924 could also be mediated via inhibition of AR transcription to block AR signaling.

Activated AR signaling, naturally or aberrantly, has been extensively implicated in the initiation, progression and recurrence of prostate cancer, making it the most important target of medical therapy for prostate cancer management [35]. Androgen deprivation therapy (ADT) has been historically recognized as the fundamental treatment for advanced prostate cancer, which has been classified into two categories [47]: (1): targeting the ligand to reduce testosterone production by surgical removal of both testes, or the use of luteinizing hormone-releasing hormone (LHRH) agonist or antagonists, or cytochrome p450 17A (Cyp17) inhibitors; (2) targeting the receptor by the use of nonsteroidal antiandrogens (NSAAs) acting as antagonists of AR to block the AR signaling. Approaches to modulate proteostasis of AR and its constitutively active variants to further repress AR signaling are gaining increasing attention recently. For examples, inhibition of HSP70 was shown to reduce AR-V7 expression and improve enzalutamide treatment [48], whereas ARD-69, a newly discovered compound acting as a degrader of AR, was shown to inhibit AR-positive prostate cancer growth with >100-fold potency than AR antagonists [49].

In summary, we showed in this study that combination of neddylation inhibitor, MLN4924 with enzalutamide, an AR antagonist, or with castration, significantly increases the efficacy in growth suppression of prostate cancer cells either in *in vitro* cell culture or *in vivo* xenograft models, respectively. Our mechanism study revealed that this is most likely achieved via blockage of transcription of AR or AR-Vs (Fig. 6). Our study provides a mechanism-based proof-of-concept for a rational combinational therapy approach for prostate cancer, and might pave the foundation for future development of MLN4924 as a novel class of agent for prostate cancer treatment.

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Author contributions

Conceived and designed the experiments: YS, FF, XZ; performed the experiments: XZ, SH, KW-R, GS, HZ, XL, MT; analyzed the data: XZ, YS; supervised the study: YS, FF, GW; wrote the paper: XZ, YS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neo.2020.02.002.

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