Ablation of the oncogenic transcription factor ERG by deubiquitinase inhibition in prostate cancer

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The transcription factor E-twenty-six related gene (ERG), which is overexpressed through gene fusion with the androgen-responsive gene transmembrane protease, serine 2 (*TMPRSS2*) in ∼40% of prostate tumors, is a key driver of prostate carcinogenesis. Ablation of ERG would disrupt a key oncogenic transcriptional circuit and could be a promising therapeutic strategy for prostate cancer treatment. Here, we show that ubiquitin-specific peptidase 9, X-linked (USP9X), a deubiquitinase enzyme, binds ERG in VCaP prostate cancer cells expressing TMPRSS2-ERG and deubiquitinates ERG in vitro. USP9X knockdown resulted in increased levels of ubiquitinated ERG and was coupled with depletion of ERG. Treatment with the USP9X inhibitor WP1130 resulted in ERG degradation both in vivo and in vitro, impaired the expression of genes enriched in ERG and prostate cancer relevant gene signatures in microarray analyses, and inhibited growth of ERG-positive tumors in three mouse xenograft models. Thus, we identified USP9X as a potential therapeutic target in prostate cancer cells and established WP1130 as a lead compound for the development of ERG-depleting drugs.

Prostate cancer is the most common malignancy in men and the second or third leading cause of male cancer-related death in most Western countries, including the United States (1). Advanced prostate cancer initially responds to androgen ablation therapy, but hormone-refractory prostate cancer often times recurs, which has limited treatment options. Fusions of E-twenty-six (ETS) transcription factor genes with androgen-responsive genes (2), mainly transmembrane protease, serine 2 (*TMPRSS2*), are present in up to 80% of prostate cancers. Patients with the most common ETS gene fusion *TMPRSS2*-ETS related gene (*TMPRSS2-ERG*) have a higher incidence of metastatic disease and cancer-related death compared with fusion-negative patients (3, 4), and in castration-resistant prostate cancer, TMPRSS2-ERG expression is frequently reactivated (5). In support of ERG being a key driver of prostate cancer, depletion of ERG by RNAi decreases proliferation and/or invasiveness in prostate cancer cell lines (2, 6), and ectopic expression of ERG in transgenic mice was shown to promote prostate oncogenesis in cooperation with the loss of tumor suppressors (7–12). *TMPRSS2*-driven overexpression of ERG controls a transcriptional network related to the development of prostate cancer and its progression to metastatic disease (13, 14). This crucial role of ERG and the high incidence of the *TMPRSS2-ERG* gene fusion in prostate cancer have catapulted this protein into the forefront of new targets for therapeutic intervention (3, 8). In the present study, we report the discovery of a deubiquitinase that stabilizes ERG in prostate cancer cells and demonstrate that pharmacological inhibition of this enzyme causes ERG depletion.

Results

USP9X is an ERG-Binding Protein. Proteins that interact with ERG in prostate cancer cells may modulate its activity, localization, or stability and could be harnessed as therapeutic targets. We isolated and identified such proteins from *TMPRSS2-ERG* positive VCaP cells by using "pulldown" with recombinant GST-ERG-His (GST-ERG) and immunoprecipitation of endogenous ERG (i.e., encoded by *TMPRSS2-ERG*) followed by mass spectrometry (Fig. 1 *A* and *B*). Among the putative ERG-binding proteins (*SI Appendix*, Table S1) identified by both pulldown and immunoprecipitation was a deubiquitinase enzyme, USP9X (15). We validated the physical interaction of ERG with USP9X by immunoblot analysis of coimmunoprecipitation with endogenous ERG (Fig. 1*C*), pulldown with GST-ERG (*SI Appendix*, Fig. S1*A*), and reciprocal immunoprecipitation with epitope-tagged ERG and USP9X (*SI Appendix*, Fig. S1 *B–D*). To identify the domain of ERG that is required for USP9X binding, we used coimmunoprecipitation experiments with deletion mutants of ERG. We found that deletion mutants of ERG (*SI Appendix*, Fig. S1*E*) lacking the ETS domain did not efficiently coimmunoprecipitate USP9X (*SI Appendix*, Fig. S1*F*). We verified a physical interaction between USP9X and the ETS domain of ERG by pulldown of USP9X from VCaP cell extract with recombinant GST-ERG-ETS (*SI Appendix*, Fig. S1*G*). When we tested the physical interaction between USP9X with other ETS transcription factors, we found that USP9X has the strongest affinity for members of the ERG subfamily (*SI Appendix*, Fig. S1*H*).

Significance

The transcription factor E-twenty-six related gene (ERG) is a major driver of prostate cancer, which makes this protein an interesting target for drug development. In this study, we report the discovery of an enzyme, ubiquitin-specific peptidase 9, X-linked (USP9X), which stabilizes ERG. We demonstrate that inhibition of USP9X with the small molecule WP1130 causes rapid degradation of ERG and blocked the growth of cultured prostate cancer cells and prostate tumors that express ERG. These findings suggest that inhibition of USP9X with small molecules should be explored for the development of a prostate cancer therapy that targets ERG.

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The authors declare no conflict of interest.

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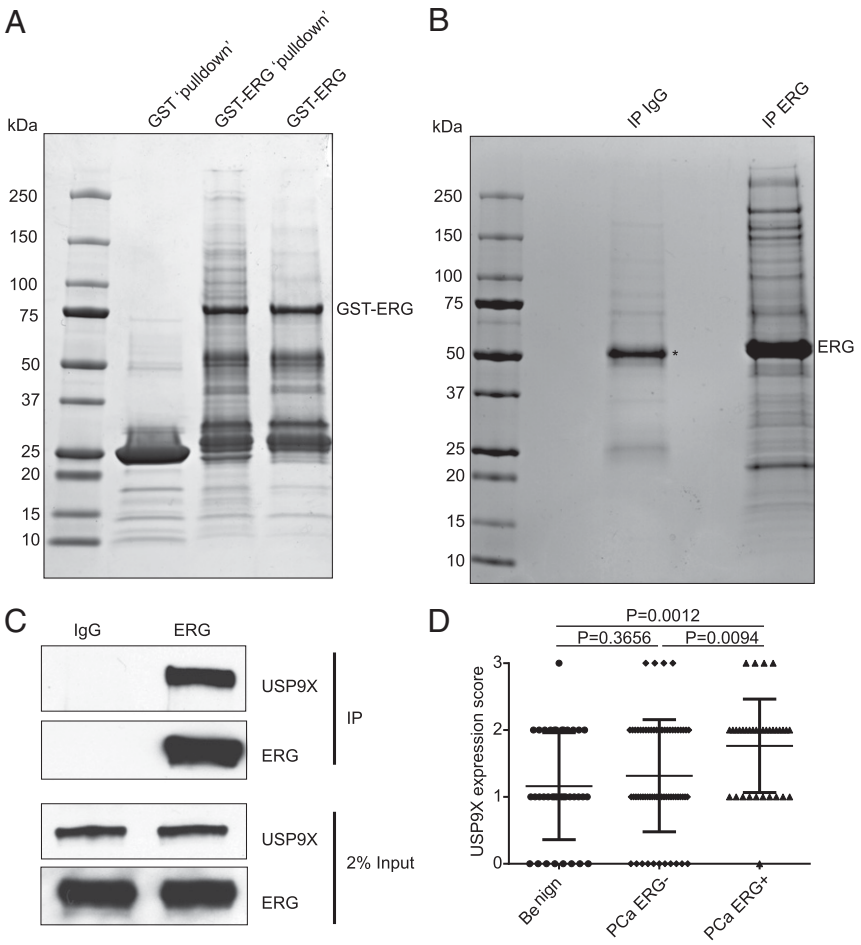

Fig. 1. Identification of USP9X as an ERG-binding protein. (*A* and *B*) Detection of ERG-binding proteins by pulldown with recombinant GST-ERG (*A*) or immunoprecipitation with an antibody against ERG (*B*) from VCaP whole cell extract. Proteins were separated by SDS/PAGE and detected by Coomassie blue staining. The asterisk indicates IgG heavy chain. (*C*) Coimmunoprecipitation of USP9X with endogenous ERG in VCaP cells with an antibody directed against ERG. Immunoblotting was performed with antibodies against ERG and USP9X. (*D*) USP9X protein expression in benign prostate (n = 37), ERG-negative (n = 63), and ERG-positive (n = 34) prostate cancer specimens (P values, Student's t test).

USP9X was found to deubiquitinate and stabilize oncogenic MCL1 in multiple myeloma (16) and, therefore, the binding of ERG to USP9X suggests that USP9X-mediated deubiquitination may be a key determinant for ERG turnover in prostate cancer. We analyzed USP9X protein expression in benign and cancerous prostate samples by immunohistochemical (IHC) analysis in 134 samples. We found that USP9X is expressed at significantly higher levels in ERG prostate tumors than in benign prostate tissue or ERG-negative prostate tumors (Fig. 1*D*). We verified a significantly higher expression of USP9X protein in ERG-positive tumors than in ERG-negative tumors by testing independent sets of 35 or 45 prostate tumors by IHC (*SI Appendix*, Fig. S2 *A*–*C*) or immunoblot analysis (*SI Appendix*, Fig. S2 *D* and *E*), respectively.

USP9X Stabilizes ERG by Deubiquitination, Which is Inhibited by WP1130. We next tested the role of USP9X as a putative ERG deubiquitinase by analyzing the impact of USP9X depletion by siRNAs on the ubiquitination of V5-tagged ERG (ERG-V5) with an ubiquitination assay (using HA-tagged ubiquitin) in HeLa cells. We found that the levels of polyubiquitinated ERG-V5 were increased in the cells transfected with the siRNAs targeting USP9X (Fig. 2*A*). When we analyzed the type of ubiquitin lysine chain linkage with HA-tagged ubiquitin mutants, we found a strong increase in polyubiquitinated ERG for K48-HA-ubiquitin (*SI Appendix*, Fig. S3*A*), which is consistent with targeting of ERG for proteasomal degradation. The increased levels of ubiquitinated ERG upon USP9X knockdown suggest that USP9X deubiquitinates ERG, and we demonstrate that affinity-purified FLAG-tagged USP9X (*SI Appendix*, Fig. S3*B*) deubiquitinates ERG in vitro (Fig. 2*B*), which depends on the catalytic activity of USP9X (*SI Appendix*, Fig. S3*C*).

Collectively, these results suggest that USP9X is an ERG deubiquitinase that may counteract proteasomal degradation of ERG. To test the role of USP9X as an ERG-stabilizing deubiquitinase, we analyzed the effect of USP9X depletion on the levels of ERG protein in VCaP cells. The knockdown of USP9X with three different siRNAs resulted in a reduction of ERG protein levels (Fig. 2*C*), which correlated with the efficiency of USP9X knockdown. We validated the specificity of this RNAi phenotype with a rescue experiment (*SI Appendix*, Fig. S3*D*). Also, USP9X knockdown did not cause a reduction of ERG mRNA levels (*SI Appendix*, Table S2). Furthermore, we found that USP9X knockdown reduced the half-life of ERG in the presence of cycloheximide from 6 to 4 h (*SI Appendix*, Fig. S3*E*). These observations suggest that inhibition of USP9X with a small molecule would result in ERG ablation in prostate tumors containing the *TMPRSS2-ERG* fusion gene. To test this hypothesis, we first examined the effect of treating HeLa cells that express ERG-V5 and HA-ubiquitin with a deubiquitinase inhibitor, WP1130 (17)

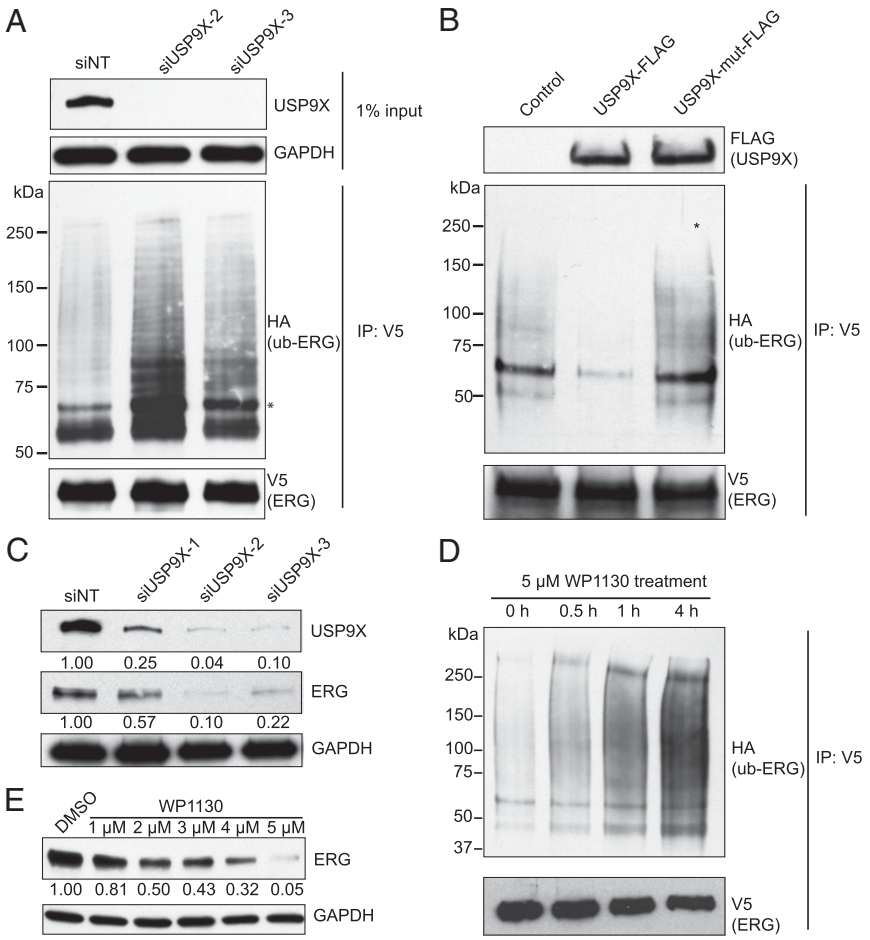

Fig. 2. USP9X stabilizes ERG by deubiquitination, which is inhibited by WP1130. (*A*) USP9X knockdown increases ERG ubiquitination. HeLa cells were transfected with siRNAs against USP9X or control siRNA (siNT); after 72 h, these cells were cotransfected with ERG-V5 and HA-ubiquitin expression constructs. After 24 h, ERG-V5 was immunoprecipitated, and immunoblotting was performed with antibodies against V5 and HA to detect ubiquitinated ERG (ub-ERG), or with antibodies against USP9X and GAPDH for input to monitor the efficiency of the USP9X knockdown. The asterisk indicates monoubiquitinated ERG (65 kDa). (*B*) USP9X deubiquitinates ERG in vitro. Ubiquitinated ERG-V5 was incubated with wild-type USP9X-FLAG, mutant USP9X-mut-FLAG, or mock (control), followed by immunoblotting. (*C*) USP9X knockdown decreases ERG levels in VCaP cells. VCaP cells were transfected with siRNAs directed against USP9X or siNT. (*D*) WP1130 increases the levels of ubiquitinated ERG. HeLa cells expressing ERG-V5 and HA-ubiquitin were treated with 5 μM WP1130 for 0–4 h before ERG-V5 immunoprecipitation. (*E*) WP1130 causes ERG depletion in VCaP cells. VCaP cells were treated with WP1130 for 24 h. Quantification of triplicate immunoblot analysis for *C* and *E* is shown in *SI Appendix*, Table S7.

that had been shown to inhibit USP9X at low micromolar concentrations (18, 19). We found that WP1130 treatment caused a rapid increase in the level of ubiquitinated ERG-V5 (Fig. 2*D*). Treatment of VCaP cells with WP1130 was associated with dose-dependent reduction in the levels of ERG protein within 24 h at a low micromolar concentration range (Fig. 2*E*), where 5 μM WP1130 caused nearly complete depletion of ERG, but did not result in decreased ERG mRNA levels (*SI Appendix*, Table S3). We found that in vitro deubiquitination of ubiquitinated ERG-V5 by recombinant USP9X was inhibited by WP1130 with an IC_{50} of ∼0.5–1.0 μM (*SI Appendix*, Fig. S3*F*). We analyzed the half-life of ERG in the presence WP1130 in VCaP cells in a time course experiment, where VCaP cells were treated with 5 μM WP1130, and observed a half-life of ∼4 h (*SI Appendix*, Fig. S3*G*). The reduction of ERG protein levels observed after WP1130 treatment was inhibited by MG132 treatment (*SI Appendix*, Fig. S3*H*) indicating that proteasomal degradation is required for WP1130-mediated ERG depletion. We next tested whether the knockdown of other deubiquitinases that are inhibited by WP1130 (USP5, USP14, UCHL5/UCH37) affects ERG protein levels. We did not observe a significant reduction in ERG levels upon siRNA-mediated knockdown of these deubiquitinases (*SI Appendix*, Fig. S3*I*), indicating that the depletion of ERG by WP1130 is caused by USP9X inhibition.

Effects of WP1130 Treatment on Prostate Cancer Cells. Next, we analyzed the effects of WP1130 on the growth of ERG-positive and ERG-negative prostate cancer cells. Because of the limited number of prostate cancer cell lines of which only VCaP cells express ERG robustly, we tested the effects of WP1130 on ERG-positive and ERG-negative primary human prostate tumors. For that purpose we used an explant culture approach (20), in which specimens of human tumors obtained after surgical resection of the cancerous prostate were cultured ex vivo for WP1130 testing (Fig. 3*A*). We found that 10 μM WP1130 reduced ERG protein levels by 72% within 24 h (Fig. 3 *B–D*). We note that the WP1130 concentration required to achieve ERG depletion was higher in the tumor explants than in VCaP cells. This is likely due to kinetic differences in WP1130 uptake of these model systems because in the explants, the uptake of a compound is affected by the rate of diffusion through a gelatin sponge and tissue. WP1130 treatment caused a marked reduction of Ki-67 in ERG-positive tumors (Fig. 3*E*), but not in ERG-negative tumors (Fig. 3*F*). Thus, WP1130 inhibits prostate cancer cell growth of

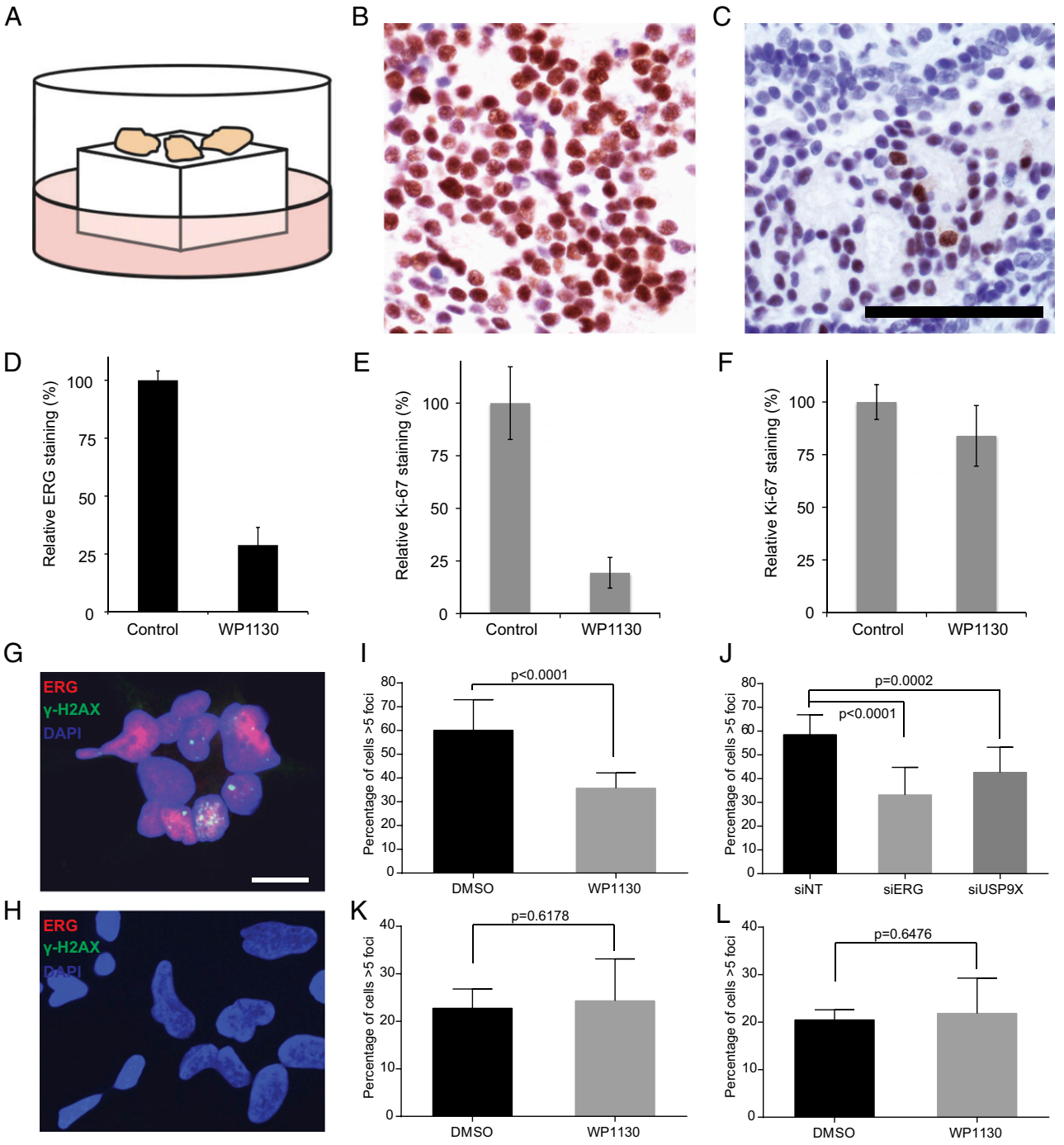

Fig. 3. Effects of WP1130 treatment on prostate cancer cells. (*A–F*) Prostate tumor explants respond to WP1130 treatment. Prostate tumor ex vivo cultures are generated from freshly resected tumor specimens and grown on gelatin sponges in culture (*A*). ERG IHC staining in ERG$^+$ prostate tumor explants following incubation for 24 h in either the presence of vehicle (DMSO) (*B*) or 10 μM WP1130 (*C*). (Scale bar: 100 μm.) Quantification of ERG levels in ERG$^+$ tumors ($n = 5$) (*D*). WP1130 treatment strongly inhibits proliferation in ERG$^+$ tumors ($n = 5$) (*E*) but not in ERG$^-$ tumors ($n = 7$) (*F*) as indicated by Ki-67 staining. Error bars represent SD. (*G–L*) WP1130 treatment reduces DNA damage. VCaP cells were treated for 48 h with DMSO (*G*) or 2 μM WP1130 (*H*) before immunofluorescence analysis of ERG, γH2AX, and DNA (DAPI). Quantification of γH2AX after WP1130/control treatment (*I*), RNAi knockdown of ERG and USP9X (*J*) in VCaP cells, or WP1130/control treatment in PC3 (*K*) and Du-145 (*L*) cells. Error bars represent SD (*P* values, Student's *t* test).

ERG-positive tumors. In cell culture experiments, we found that treatment with 5 μM WP1130 resulted in a marked increase of PARP cleavage in VCaP cells, but not in ERG-negative PC3 and Du-145 cells (*SI Appendix*, Fig. S4*A*). This treatment caused increased Caspase-3/7 activity in all three lines (*SI Appendix*, Fig. S4*B*), with the most robust increase being observed in VCaP cells. The increase in Caspase-3/7 activity upon WP1130 treatment may be due to the depletion of the USP9X target MCL1 (16), a known antiapoptotic protein, in all three cell lines (*SI Appendix*, Fig. S4*C*). These findings suggest that WP1130 robustly induces apoptosis in VCaP cells. To test whether WP1130 inhibits ERG-dependent functions that were previously demonstrated in vitro, we first tested whether WP1130 treatment reduced cell proliferation of VCaP cells at a subapoptotic WP1130 concentration (2 μM) that

achieves significant ERG depletion (*SI Appendix*, Fig. S4*D*). At this concentration, VCAP cell proliferation was significantly inhibited 3 d after treatment (*SI Appendix*, Fig. S4*E*). Likewise, USP9X knockdown with siRNA caused an inhibition of VCaP cell proliferation 5 d after siRNA transfection (*SI Appendix*, Fig. S4*F*). Next, we tested the effect of WP1130 treatment on DNA damage because ERG depletion by RNAi was shown to reduce DNA damage (21). We found a significant reduction of γH2AX foci in VCaP cells after 48 h of treatment with 2 μM WP1130 (Fig. 3 *G–I*), which mirrored the effect observed for USP9X and ERG knockdown with siRNAs (Fig. 3*J*). WP1130 treatment had no effect on the number of γH2AX foci in PC3 and Du-145 cells (Fig. 3 *K* and *L*). Next, we tested whether WP1130-mediated ERG ablation inhibited VCaP cell migration because ERG expression induces migration in prostatic epithelial cells (22). We found that treatment with 2 μM WP1130 impaired VCaP cell migration (*SI Appendix*, Fig. S4*G*). Likewise, we found that both USP9X and ERG knockdown by siRNAs resulted in reduced migration in VCaP cells (*SI Appendix*, Fig. S4*H*). WP1130 treatment had no significant effect on ERG-negative PC3 and Du-145 cells (*SI Appendix*, Fig. S4 *I* and *J*). Finally, we demonstrated that WP1130 treatment negatively affects the expression of ERG-regulated genes in VCaP cells (*SI Appendix*, Fig. S4*K* and Tables S4 and S5 and Datasets S1 and S2), which are also enriched for genes implicated in prostate tumorigenesis (*SI Appendix*, Fig. S4*L*).

Effects of WP1130 on ERG Levels and Tumor Growth in Vivo. Next, we tested whether WP1130 causes ERG depletion in vivo and inhibits the growth of ERG-overexpressing VCaP xenograft tumors in athymic nude mice. We observed that WP1130 treatment markedly inhibited the growth of VCaP tumors (P = 5e-04) (Fig. 4*A*). To validate the causative role of ERG depletion in the antitumorigenic effects of WP1130, we tested whether stable ectopic ERG expression in the ERG-negative cell line 22Rv1 (*SI Appendix*, Fig. S5*A*) confers sensitivity to WP1130 treatment. We found that the growth of 22Rv1-ERG tumors in vivo was markedly inhibited by WP1130 (P = 4e-04), but found no significant effect on the growth of the isogenic 22Rv1-vector tumors (P = 0.497) (Fig. 4*B*). WP1130 treatment resulted in marked reduction in the level of ERG protein in the VCaP and 22Rv1-ERG xenograft tumors (Fig. 4 *C* and *D*). These tumors were poorly vascularized (Fig. 4*E*) and showed a marked reduction in microvessel density (*SI Appendix*, Fig. S5 *B* and *C*). We note that the vascularization defect observed upon WP1130 treatment in ERG-positive tumors indicates impaired tumor angiogenesis, which phenocopies the effect of in vivo silencing of TMPRSS2-ERG expression in VCaP xenografts with nanoparticle-delivered siRNA (23).

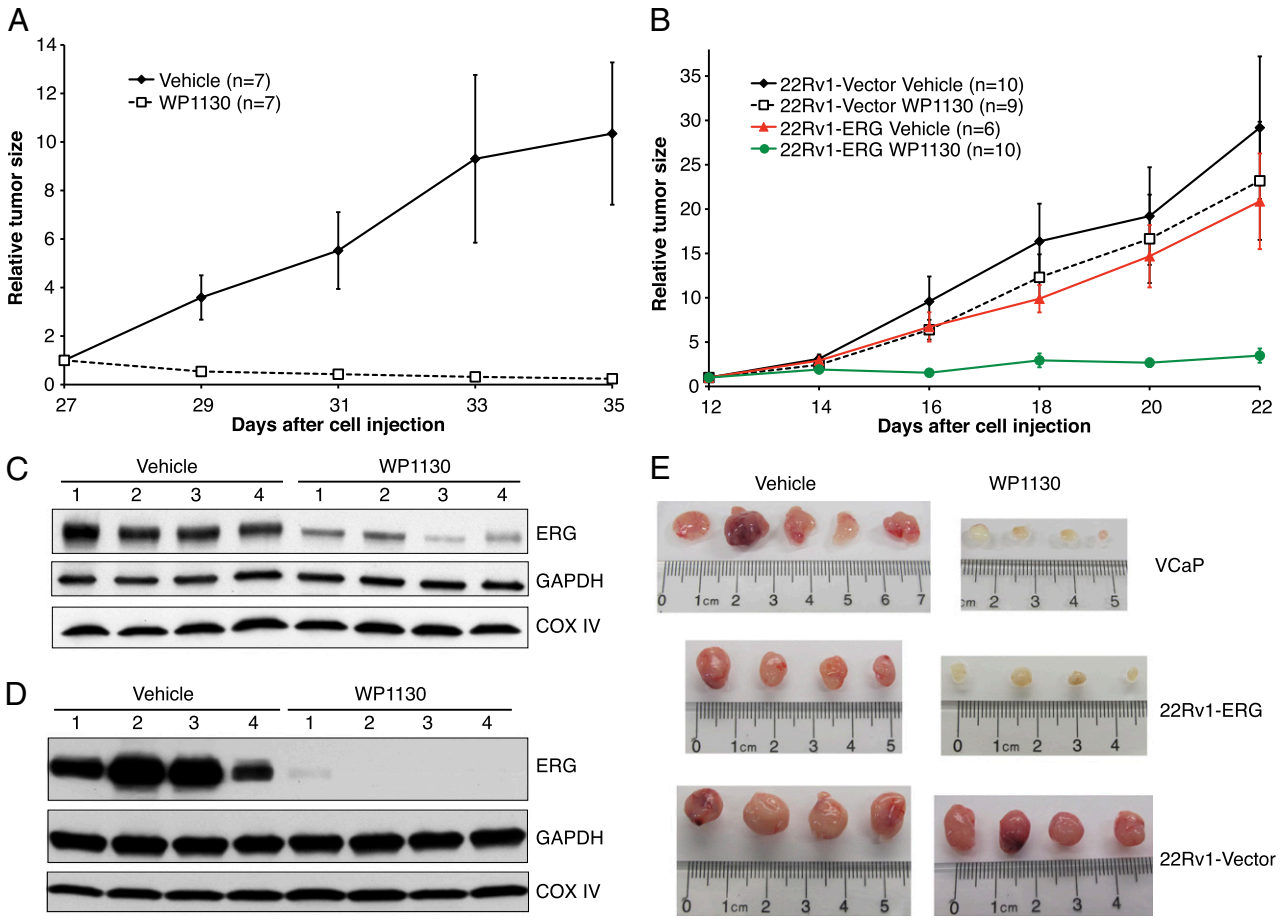

Fig. 4. Effects of WP1130 on ERG levels and tumor growth in vivo. (*A* and *B*) WP1130 inhibits the growth of ERG-overexpressing tumors in mice. Mice xenografted with ERG-overexpressing VCaP cells (*A*) or 22Rv1-ERG and 22Rv1-vector cells (*B*) were treated upon appearance of tumors with 40 mg/kg WP1130 or vehicle (i.p.) every other day beginning on day 27 (*A*) or 12 (*B*) after cell injection. Error bars represent SEM. (*C* and *D*) WP1130 causes ERG depletion in vivo. Tumors were obtained from VCaP (*C*) and 22Rv1-ERG (*D*) tumor-bearing mice 24 h after injection with vehicle or WP1130 (40 mg/kg). Immunoblotting was performed with antibodies against ERG, GAPDH, and COX IV (human protein-specific antibody). (*E*) Appearance of xenograft VCaP, 22Rv1-ERG, and 22Rv1-vector tumors at the end of the treatment course with vehicle or WP1130 (40 mg/kg). Treatment with WP1130 was not associated with any apparent toxicity (e.g., weight loss or behavioral changes).

The analysis of our gene expression data also revealed that WP1130 treatment and ERG siRNA-mediated knockdown caused the upregulation of the antiangiogenic factor ADAMTS1, which is frequently down-regulated in metastatic prostate tumors (24), where its low expression is correlated with higher microvessel density. We also tested the effect of WP1130 treatment on ERG-negative xenograft tumors (PC3 and Du-145) that express USP9X (*SI Appendix*, Fig. S5*A*) and found that WP1130 had no effect on the growth of these tumors (PC3, $P = 0.625$; Du-145, $P = 0.817$) (*SI Appendix*, Fig. S5 *D* and *E*). Collectively, these findings suggest that ERG expression is necessary for the antitumorigenic effects of WP1130 on prostate cancer cells in vivo. To further demonstrate the translational potential of deubiquitinase inhibition in a prostate cancer model that is more representative of a human tumor, we tested the effect of WP1130 treatment on LuCaP 86.2 tumors. This tumor line was derived from a metastatic site of a castration-resistant AR- and ERG-positive prostate tumor and has been continuously propagated in mice (25). WP1130 treatment recapitulated the effects observed with the VCaP and 22Rv1-ERG cell line xenografts, i.e., a marked reduction in tumor growth ($P = 0.0042$) (*SI Appendix*, Fig. S5*F*), ERG protein levels (*SI Appendix*, Fig. S5*G*), and microvessel density (*SI Appendix*, Fig. S5 *H* and *I*). Thus, we demonstrate that WP1130 causes ERG depletion and tumor growth inhibition in ERG-overexpressing prostate tumors.

Discussion

We identified USP9X as an ERG-stabilizing deubiquitinase in prostate cancer cells. We demonstrated that USP9X inhibition with the small molecule WP1130 results in ERG depletion and retards prostate tumor growth in ERG-expressing prostate tumors only. Although the latter finding cannot rule out the possibility that WP1130 may affect proteins other than ERG contributing to growth inhibition, ERG ablation appears to be necessary for the antitumorigenic effects of this compound on prostate cancer cells. In conclusion, our work provides a starting point for the development of small molecules for ERG depletion as a targeted therapy for prostate cancer. Also, the ablation of a transcription factor that was previously considered undruggable through deubiquitinase inhibition may be a promising therapeutic strategy that should be explored for other transcription factors driving cancer.

Materials and Methods

Identification of USP9X as an ERG-Binding Protein. GST-ERG was expressed in *Escherichia coli* BL21(DE3) and affinity purified. Pulldown was performed with VCaP whole cell protein extract. After SDS/PAGE of eluted proteins and Coomassie blue staining, gel slices of interest were digested with trypsin. Mass spectrometry analysis was performed to detect peptides for protein identification with Mascot. The interaction of USP9X and ERG was tested by coimmunoprecipitation in VCaP cells with an antibody against ERG, and by coimmunoprecipitation of ectopically expressed ERG-V5 and USP9X-FLAG or USP9Y-FLAG in HeLa cells with an antibody against V5.

Analysis of ERG Ubiquitination. HeLa cells were transfected with siRNAs against USP9X or a nontargeting siRNA and cultured for 72 h, cotransfected with ERG-V5 and HA-ubiquitin expression constructs, and assayed 24 h later. For the analysis of ubiquitin chain linkage, HeLa cells were cotransfected with ERG-V5 and HA-K48– or HA-K63–ubiquitin expression constructs and assayed 24 h later. For the analysis of the effects of WP1130, HeLa cells were treated with 5 μM WP1130 24 h after transfection of ERG-V5 and HA-ubiquitin expression construct.

Deubiquitination of ERG in Vitro. Ubiquitinated ERG, USP9X-FLAG, and USP9X-C1566S-FLAG were expressed in HEK293T cells and immunoprecipitated. Ubiquitinated ERG-V5 was incubated with USP9X-FLAG or USP9X-C1566S-FLAG, or mock immunoprecipitate, for 16 h at 30 °C.

Expression Profiling of WP1130 Treatment and ERG RNAi Knockdown in VCaP Cells. VCaP cells grown in DMEM with 10% (vol/vol) FBS were treated with 5 μM WP1130 or vehicle for 24 h, or grown for 96 h after transfection with two different siRNAs against USP9X and a nontargeting control siRNA. DNA-free RNA was extracted, biotin labeled, and hybridized to Human HT12v4.0 Expression Beadchips (Illumina).

Xenograft Experiments. VCaP, 22Rv1-ERG, 22Rv1-vector, PC3, and Du-145 cell suspensions were injected and LuCaP 86.2 tumor specimens were implanted s.c. into flank regions of male athymic nude mice. Tumor-bearing mice were injected with 100 μL of WP1130 suspension (40 mg/kg, i.p.) or vehicle [1:1(vol/vol), DMSO/PEG 300] every other day for a total of four or five injections.

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