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AR intragenic deletions linked to androgen receptor splice variant expression and activity in models of prostate cancer progression

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Abstract

Reactivation of the androgen receptor (AR) during androgen depletion therapy (ADT) underlies castration-resistant prostate cancer (CRPCa). Alternative splicing of the AR gene and synthesis of constitutively-active COOH-terminally truncated AR variants lacking the AR ligand binding domain has emerged as an important mechanism of ADT-resistance in CRPCa. In a previous study, we demonstrated that altered AR splicing in CRPCa 22Rv1 cells was linked to a 35 kb intragenic tandem duplication of AR exon 3 and flanking sequences. In this study, we demonstrate that complex patterns of AR gene copy number imbalances occur in PCa cell lines, xenografts, and clinical specimens. To investigate whether these copy number imbalances reflect AR gene rearrangements that could be linked to splicing disruptions, we carried out a detailed analysis of AR gene structure in the LuCaP 86.2 and CWR-R1 models of CRPCa. By deletion-spanning PCR,

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Conflict of interest

The authors declare no conflict of interest.

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we discovered a 8,579 bp deletion of AR exons 5, 6, and 7 in the LuCaP 86.2 xenograft, which provides a rational explanation for synthesis of the truncated AR v567es AR variant in this model. Similarly, targeted re-sequencing of the AR gene in CWR-R1 cells led to the discovery of a 48 kb deletion in AR intron 1. This intragenic deletion marked a specific CWR-R1 cell population with enhanced expression of the truncated AR-V7/AR3 variant, a high level of androgen-independent AR transcriptional activity, and rapid androgen independent growth. Together, these data demonstrate that structural alterations in the AR gene are linked to stable gain-of-function splicing alterations in CRPCa.

Keywords

prostate cancer; androgen receptor variants; castration-resistant; intragenic rearrangement, AR alternative splicing

Introduction

PCa initially presents as an androgen- and AR-dependent disease. Therefore, suppressing the production or action of androgens, which inhibits AR transcriptional activity, leads to stabilization or regression of advanced PCa. The duration of response to ADT is variable with the end point typically marked by rising serum levels of prostate specific antigen (PSA), an AR transcriptional target gene, and rapid growth of PCa metastases. This transition from androgen-dependent to CRPCa is frequently due to aberrant AR re-activation despite ongoing treatment with AR-targeted therapies (1-4). Various mechanisms have been advanced to explain AR activation in CRPCa cells. These include AR gene amplification and/or AR protein overexpression (5-12), point mutations that permit promiscuous AR transcriptional responses (13-20), and intra-tumor steroid synthesis or sequestration (21-24). This knowledge has driven the clinical development of new inhibitors of androgen production and AR signaling (25), including the CYP17 inhibitor abiraterone acetate, which has been recently shown to increase overall survival in patients with metastatic CRPCa (26).

Synthesis of truncated AR variant proteins via AR alternative splicing has recently emerged as an additional mechanism of ADT resistance in PCa (27-31). These proteins lack the AR ligand-binding domain (LBD), display constitutive, ligand-independent transcriptional activity, and mediate androgen-independent growth of PCa cells in various model systems (27-31). Increased expression of the AR3 variant protein (also termed AR-V7 (29)) in PCa prostatectomy specimens is associated with biochemical recurrence following surgery (28). In addition, increased mRNA expression of alternatively-spliced AR variants in PCa bone metastases is associated with shorter survival (32). Therefore, understanding the mechanisms leading to increased synthesis of these species could provide important prognostic information, or perhaps guide more effective use of therapies that inhibit ligand-dependent AR activity.

Truncated AR variants proteins were originally discovered and functionally characterized in the CRPCa 22Rv1 and CWR-R1 cell lines (27-29), and the LuCaP 86.2 PCa xenograft (30). In 22Rv1 cells, a 35kb AR intragenic tandem duplication is linked to altered splicing of full-length AR as well as synthesis of truncated AR variants (33). However, the mechanisms

driving AR splicing alterations outside of the 22Rv1 model have remained elusive. Therefore, the purpose of this study was to investigate the link between AR gene structure alterations and enhanced synthesis of truncated AR variants in CRPCa.

Results

AR Gene Structure Complexity in CRPCa

In a previous study, we analyzed high-resolution whole-genome copy number data from CRPCa metastasis, which implicated frequent AR copy number imbalance concurrent with AR amplification at this stage of the disease (33). To investigate this phenomenon directly, we employed a multiplex ligation-dependent probe assay (MLPA) with probe sets targeted to coding exons in the AR gene (Figure 1a). This MLPA approach detected the 22Rv1 duplication involving Exon 3, as well as 20-fold amplification of the AR gene in VCaP cells (Figure 1b). Androgen-dependent PCa tissue obtained from xenografts (Figure 1c) or clinical specimens (Figure 1d) displayed one intact AR gene copy, with the exception of the LuCaP 35 xenograft, which displayed 4 copies of the AR gene. However, CRPCa tissue obtained from xenografts (Figure 1c) or autopsy specimens (Figure 1d) displayed frequent AR gene amplification and/or complex patterns of AR gene copy number imbalance. These data suggest that imbalances in AR gene copy number may be important for CRPCa progression.

To investigate this phenomenon in more detail, we focused on the LuCaP 86.2 xenograft, which expresses high levels of the truncated AR v567es variant arising from the mRNA splicing machinery skipping exons 5, 6, and 7 (30). LuCaP 86.2 displayed reduced genome copy number of these exons indicating a mixed cell population with ~50% of cells harboring an intragenic deletion (Figure 1c). Deletion spanning PCR yielded products consistent with an intragenic deletion encompassing AR exons 5, 6, and 7 (Figures 2a and b). Sequence analysis verified an 8,579bp deletion (Figure 2c) with microhomology at the 5′ and 3′ break fusion junctions, which signifies a non-homologous end joining (NHEJ) mechanism of origin (Figure 2d). Together, these data implicate focal intragenic deletion as a novel mechanism underlying synthesis of the truncated AR v567es variant in the LuCaP 86.2 xenograft.

Stable AR mRNA splicing alterations in CRPCa CWR-R1 cells

MLPA analysis indicated that the CRPCa CWR-R1 cell line does not harbor any AR gene copy number alterations, despite previous studies demonstrating that these cells express truncated AR variants (28). To confirm altered splicing in these cells, we assessed AR mRNA isoform levels using an absolute quantification RT-PCR assay. To correct for variability in AR gene dosage and rates of AR transcription, we scaled copy number for each AR mRNA isoform relative to full-length AR. This approach revealed changes in the ratios of full-length AR mRNA and alternatively-spliced AR isoforms in CRPCa CWR-R1 and 22Rv1 cells (Figure 3b). Conversely, androgen-dependent CWR22Pc, LNCaP, and VCaP cells expressed predominantly full-length AR mRNA (Figure 3b). To test for plasticity in the expression of truncated AR variant expression in CWR-R1 cells, we knocked down full-length AR mRNA using an AR Exon 7-targeted siRNA. No changes in

truncated AR protein expression were observed following 48h or 72h of knock-down, whereas an AR Exon 1-targeted siRNA completely abolished all AR protein expression in these cells (Figure 3c). Similarly, no changes in truncated AR variant protein expression were observed in CWR-R1 cells following 24h or 72h of androgen stimulation (Figure 3d). These data indicate that the altered AR mRNA splicing pattern in CWR-R1 cells is stable and is unlikely an acute cellular response to manipulations of androgen or AR levels.

A novel intragenic deletion in CWR-R1 cells identified by paired-end AR gene re-sequencing

Because MLPA only interrogates AR copy number at coding exons, which represent less than 1.5% of the 180kb AR gene, we analyzed the nucleotide sequence and structure of the entire AR locus in CWR-R1 cells using a combination of liquid-phase sequence capture and Illumina paired-end massively parallel sequencing (Supplementary Figure 1 and Supplementary Table 1). Androgen-dependent CWR22Pc and CRPCa 22Rv1 cells were sequenced concurrently. Because 49.9% of the AR locus iscomposed of repetitive DNA (34), which precludes the design of capture baits to these regions, we were unable to obtain sequence for nearly a quarter of the AR gene using this strategy (Supplementary Table 1). In particular, no sequence was obtained within three “gaps” of contiguous repetitive elements spanning >6kb each (Figure 4 and Supplementary Figures 1 and 2). Gaps 2 and 3 harbor the 5′ and 3′ breakpoints of the previously-identified 22Rv1 tandem duplication (33), and no paired-end reads representing this structural alteration were detected (Supplementary Table 2). However, sequence coverage plots revealed increased copy number within this region, with transition points located within gaps 2 and 3 (Supplementary Figures 2 and 3). No copy number imbalances in this region were observed in CWR22Pc or CWR-R1 cells (Supplementary Figure 2). Point mutations and indels were observed at various frequencies in all 3 cell lines, including the previously-identified H874Y coding mutation at position chrX:66,943,543 (Supplementary Tables 3-5). However, no prevalent sequence alterations were observed in splice donor/acceptor sequences in CWR-R1 and 22Rv1 cells (Supplementary Tables 4 and 5). Strikingly, structural variant analysis of paired-end reads using the Hydra workflow (35) identified a ~48kb intragenic deletion within AR Exon 1 in a sub-population of the CWR-R1 cell line, which was also apparent from a relative decrease in sequence coverage peak height within this region (Figure 4, Supplementary Table 2, and Supplementary Figure 4). To confirm this structural alteration in CWR-R1 cells, we performed nested PCR using primers spanning the deletion (Figures 5a and b). Sanger sequencing of cloned PCR products revealed deletion of 48,476bp from AR intron 1 (Figures 5c and d). Alignments of the 5′ and 3′ break fusion junctions demonstrated 3bp of microhomology, implicating NHEJ as the mechanism underlying this deletion (Figure 5e).

To quantify the prevalence of this ~48kb deletion, we performed MLPA with probe pairs custom-designed to query copy number at regular intervals along the length of the AR gene (Figure 6a). MLPA probe pairs targeted within this 48kb region displayed a ~20-30% decrease in copy number (Figure 6b). There were no copy number alterations in this region detected by MLPA in CWR22Pc or 22Rv1 cells (Figure 6b), and nested PCR with deletion-spanning primers did not generate products in CWR22Pc or 22Rv1 cells (Figure 6c). Similarly, only CWR-R1 cells yielded 76bp Illumina sequencing reads that could be aligned

to a 130bp template harboring this specific breakpoint sequence (Supplementary Figure 5). Together, these data demonstrate that a 48,476bp deletion within intron 1 of the AR gene is restricted to 20-40% of the cells in the CWR-R1 cell line.

Enrichment for cells harboring AR intron 1 deletion during castration

The levels of truncated AR isoforms expressed in CWR-R1 cells are markedly higher when these cells are grown as xenografts in castrated mice vs. intact mice (28). All the analyses in our study had been performed with CWR-R1 cells that had been cultured in complete medium (contains androgens) for 10-20 passages, which would allow the growth of both androgen-dependent and CRPCa cell populations. Therefore, we compared AR gene structure in early passage CWR-R1 cells (referred to as CWR-R1 early) to CWR-R1 cells that were cultured in the absence of androgens for 20 passages (referred to as CWR-R1-late). Remarkably, no copy number decrease within AR intron 1 was apparent following MLPA analysis of CWR-R1 early cells (Figure 7a), despite a positive nested PCR signal for this deletion (Supplementary Figure 6). Conversely, MLPA probe signal in this region was nearly completely lost in CWR-R1 late cells (Figure 7a) indicating that this deletion was a marker of the CRPCa cell sub-population. Consistent with this deletion underlying the AR splicing patterns in CWR-R1 cells, expression of the truncated AR 1/2/3/CE3 variant (also referred to as AR-V7 (29) or AR3 (28)) was low in CWR-R1 early cells, regardless of whether they were cultured in whole serum or steroid-depleted serum (Figure 7b). However, CWR-R1 late cells displayed high-level expression of AR 1/2/3/CE3 protein (Figure 7b). These changes at the protein level corresponded with a stable shift in splicing favoring the AR 1/2/3/CE3 AR mRNA isoform in CWR-R1 late vs. CWR-R1 early cells (Supplementary Figure 7). CWR-R1 cells cultured in the presence of androgens for 20 passages displayed an intermediate AR 1/2/3/CE3 protein expression pattern (Figure 7b). Immunostaining of cells grown under castrate conditions demonstrated increased nuclear expression of the AR NH_2-terminal domain vs. the AR COOH-terminal domain in CWR-R1 late cells, but not CWR-R1 early cells (Supplementary Figure 8). Together, these data demonstrate that cells harboring this ~48kb deletion within AR exon 1 display a splicing switch that favors stable, high-level expression of the truncated AR 1/2/3/CE3 variant.

To investigate whether there may be functional consequences to these differences in truncated AR variant expression, we examined AR transcriptional activity in CWR-R1 early and CWR-R1 late cells. AR transactivation in response to the synthetic androgen mibolerone was higher in CWR-R1 early cells than CWR-R1 late cells and knock-down of full-length AR inhibited this androgen response in both cell lines (Figure 7c). Interestingly, knock-down of AR expression with siRNAs targeted to either AR exon 1 or exon 7 inhibited androgen-independent transcriptional activity in CWR-R1 early cells, but only siRNA targeted to AR exon 1 had this effect in CWR-R1 late cells (Figure 7c). These data indicate that androgen independent AR activity in CWR-R1 early cells is dependent on full-length AR expression, whereas androgen-independent AR activity in CWR-R1 late cells is mediated by truncated AR variants through a mechanism that is independent of full-length AR. To investigate differential siRNA sensitivity and a differential role for full-length AR in more detail, we compared the androgen-independent growth of CWR-R1 early and CWR-R1 late cells transfected with AR-targeted siRNAs. Consistent with their selection under

castrate conditions, CWR-R1 late cells displayed a rapid androgen-independent growth rate which was inhibited by siRNA targeted to AR Exon 1 but not AR Exon 7 (Figure 7d). Conversely, CWR-R1 early cells grew slowly under androgen-independent conditions during this short time-course and there was limited response to AR-targeted siRNA (Figure 6d).

Discussion

The prevalence of AR gene mutations in CRPCa reported by different groups has been variable, but appears to be low at approximately 10% (13-20). These data suggest that the majority of CRPCa harbors a wild-type AR gene. However, mutations have been evaluated historically by sequence analysis of AR cDNA and/or AR coding exons, which represents only ~1.5% of the 180,245bp AR gene (36). The identification and clinical validation of truncated AR variant synthesis as an important mechanism of prostate cancer therapy resistance has raised the possibility that splicing disruptions may be due to non-coding AR gene alterations. Indeed, our previous work defined a ~35kb AR intragenic tandem duplication in 22Rv1 cells, which is a rational explanation for the altered splicing pattern in this cell line (33). In this study, we have defined two additional simple AR gene structural alterations that are linked to the pathologic AR splicing patterns in the LuCaP 86.2 and CWR-R1 models of PCa progression. These data, combined with MLPA analysis of additional CRPCa specimens, indicate that the prevalence of AR gene alterations in tumors resistant to ADT may be higher than previously anticipated. While targeted methods such as MLPA are useful for identifying deletions or duplications that involve probe binding sites, this study has illustrated that unbiased evaluation of the entire AR gene sequence and structure is a preferable approach. However, our work has also demonstrated that high-throughput approaches are challenged by substantial repeat in the AR locus. Indeed, paired-end reads diagnostic of the 35kb 22Rv1 AR intragenic tandem duplication were not obtained in our study, despite 6000-fold maximal on-target sequence coverage in these cells. This indicates that many AR gene structural alterations would also go undetected using larger-scale sequence capture or whole-genome sequencing approaches (37, 38). Therefore, in order to define the prevalence and spectrum of AR gene structure alterations that may exist in clinical CRPCa, methodology optimization must be a prerequisite.

Previous analysis of genome-wide copy number data from clinical CRPCa specimens suggestedthat complex patterns of copy number gain and copy number loss occurred along the lengthof the AR gene (33), which is supported by MLPA analysis in this study. Here, we demonstrated that large deletions involving intron 1 are associated with enhanced synthesis of the truncated AR 1/2/3/CE3 variant (also referred to as AR-V7 or AR3) and a growth advantage under castrate conditions. In the CWR-R1 model, castration-mediated enrichment for cells harboring intron 1 deletion resulted in an overall population that exhibited levels of AR 1/2/3/CE3 that were equivalent to or greater than the levels of full-length AR. This is important because a recent study of surgical specimens of CRPCa bone metastases with an antibody specific for the AR NTD demonstrated that protein expression of truncated AR variants can reach similar high levels relative to full-length AR (32). Moreover, patients with CRPCa bone metastases that displayed the highest levels of alternatively-spliced, truncated AR mRNA variants had shorter cancer-specific survival after metastasis surgery

than other CRPCa patients (32). Therefore, increased expression of truncated AR variants is an important component of clinical PCa progression. The data in this study strongly suggests that alterations in the architecture of the AR gene may underlie these disruptions in normal splicing patterns. With this in mind, it is important to point out that a true “alternative splicing” mechanism has not yet been elucidated for the AR gene. Rather, models that exhibit levels of truncated AR variant expression sufficient to drive the CRPCa phentoype (such as 22Rv1, CWR-R1, and LuCaP 86.2) have dramatic changes in the AR gene template. These AR intragenic duplications and deletions that we have defined all appear to result from homologous recombination-independent mechanisms such as microhomology-mediated break-induced replication (MMBIR) in 22Rv1 (33) and NHEJ in LuCaP 86.2 and CWR-R1. Therefore, the exact locations of breakpoints in the AR locus are unlikely to be recurrent between specimens. Intriguingly, these three models each display a unique splicing signature and repertoire of truncated AR variant protein expression. Therefore, it is tempting to speculate that different patterns of AR gene alteration may give rise to different AR splicing patterns in clinical CRPCa. This would argue that a complete understanding of the role of truncated AR variants in CRPCa progression will require that individual tumors be evaluated for splicing alterations using unbiased detection methods, rather than targeted approaches focused on known AR variants.

Overexpression of the AR 1/2/3/CE3 variant (also referred to as AR-V7 or AR3), the AR v567es variant, or a truncated AR variant of mouse origin (mAR-V4) in LNCaP cells can induce androgen-independent expression of AR target genes and growth under castrate conditions *in vitro* and *in vivo* (28, 30, 31). Interestingly, treatment of these engineered LNCaP cells with the next-generation antiandrogen MDV3100 or knock-down of full-length AR resulted in reversal of these CRPCa features (31). These data indicate that truncated AR variants require full-length AR to support a CRPCa phenotype. However, this is in opposition to our studies with CRPCa models that endogenously express high levels of truncated AR variants and harbor apparent gain-of-function structural alterations in the AR gene (27, 33). For example, in this study, knock-down of full-length AR had no effect on androgen-independent AR activity or androgen-independent growth in late-passage CWR-R1 cells. However, knock-down of AR 1/2/3/CE3 inhibited these parameters. We have also demonstrated this differential response to isoform-targeted siRNAs in the 22Rv1 cell line (27). Conversely, early-passage CWR-R1 cells displayed modest androgen-independent growth and measurable androgen-independent AR activity, which was inhibited following knock-down of full-length AR. These data demonstrate that the CWR-R1 cell line is heterogeneous and that growth conditions can have dramatic effects on the relative proportions of androgen-dependent cells and CRPCa cells, which may explain a previous report where CWR-R1 cells displayed decreased proliferation and increased apoptosis in response to full-length AR knock-down (28). With this in mind, it is also important to note that the LuCaP 86.2 xenograft tissue evaluated in this study was propagated in an intact male mouse, and MLPA data reflected an approximate 50/50 mixture of cells with either one intact AR gene copy or one AR gene copy with a 8,579bp deletion of exons 5, 6, and 7. If the cell population harboring the 8,549bp intragenic deletion is indeed the cell population which synthesizes the AR v567es variant, these cells would not be able to synthesize full-length AR and would be truly independent of full-length AR activity (30). Therefore, a more

thorough investigation of the requirement for full-length AR is warranted, as this will provide important insights to resistance mechanisms that may circumvent clinical responses to current and next-generation therapies targeting the AR LBD (25).

In summary, this study represents the first report of intragenic deletions involving coding and non-coding sequences in the AR gene in CRPCa, which we have linked to expression of truncated AR variants that support the CRPCa phenotype. Therefore, structural alterations in the AR gene may represent a widespread yet previously unanticipated mechanism of therapy resistance in PCa. Our findings provide justification for large-scale investigation of AR gene structure and splicing patterns in clinical specimens.

Materials and methods

Prostate cancer tissues

Genomic DNA samples from the LuCaP series of PCa xenografts and de-identified clinical CRPCa tissue were obtained from the University of Washington Prostate Cancer Biorepository, which was developed and managed by one of the co-authors (R.L.V.) and has been described in previous publications (30, 39, 40). De-identified prostatectomy tissue samples were obtained under the direction of the University of Minnesota BioNet tissue resource, which was developed and managed by one of the co-authors (S.C.S.). One millimeter cores of PCa tissue were obtained from archival formalin-fixed, paraffin-embedded (FFPE) prostatectomy blocks using a tissue microarrayer (Beecher Instruments, Sun Prairie, WI) and genomic DNA was isolated using a RecoverAll kit (Applied Biosystems/Ambion, Austin, TX).

Cell culture

The 22Rv1 (#CRL-2505), LNCaP (#CRL-1740), and VCaP (#CRL-2876) cell lines were obtained from ATCC and cultured according to ATCC protocol. CWR22Pc cells (41) were generously provided by Dr. Marja Nevalainen (Thomas Jefferson University) and cultured in RPMI 1640 supplemented with 10% FBS, 2.5 mM L-glutamine, and 0.8nM dihydrotestosterone (Sigma, St. Louis, MO). CWR-R1 cells (42) were a kind gift from Dr. Elizabeth Wilson (UNC Chapel Hill) and cultured in RPMI 1640 + 10% FBS. For androgen response experiments, cells were cultured in RPMI 1640 + 10% steroid-depleted, charcoal stripped serum (CSS) for 48h, treated at t=0 with 1nM DHT (Sigma) or vehicle (EtOH), and then harvested at indicated time points. For long-term culture experiments, CWR-R1 cells were cultured in RPMI 1640 + 10% CSS. Cells were trypsinized and re-seeded in the appropriate medium when flasks attained 80% confluence.

Transient transfections

The CWR-R1 cell line was electroporated with siRNAs targeted to AR Exon 7 (target sequence: 5′-GGAACUCGAUCGUAUCAUU) or AR Exon 1 (5′-CAAGGGAGGUUACACCAAA) and/or an MMTV-LUC reporter as described (27). Growth of electroporated cells was monitored by crystal violet staining as described (33). Luciferase activity was measured as described (27).

Quantitative real-time RT-PCR—RNA isolation and absolute quantification RT-PCR analysis of alternatively-spliced AR mRNA isoforms was performed as described (33). To correct for different levels of wild-type AR mRNA expression among the prostate cancer cell lines, copy numbers of AR mRNA isoforms were scaled relative to wild-type AR mRNA copy number in each cell line (set to 1). For relative quantification RT-PCR, fold change in expression levels were determined by the comparative Ct method using the equation $2^{-\Delta\Delta Ct}$.

Genomic PCR—Genomic PCR was performed as described (33) using primer pairs listed in Supplementary Table 6.

Western blot

Western blotting with AR NTD (N-20, Santa Cruz Biotechnology, Santa Cruz, CA), AR CTD (Santa Cruz C-19), ERK-2 (Santa Cruz D-2), and ARV-7 (# AG10008, Precision Antibody, Columbia, MD) antibodies was performed as described (33).

Multiplex ligation-dependent probe assay

MLPA for AR coding sequence was performed using a commercially available kit (P074, MRC Holland) as per the manufacturer's protocol. Briefly, 100ng of genomic DNA was hybridized at 60^{o}C for 18h with MLPA probemix. Hybridized probes were ligated and amplified by PCR with labeled universal primers provided with the MLPA kit. PCR reactions were diluted 1:10 in formamide containing ROX-500 size standards (Applied Biosystems, Carlsbad, CA), denatured, and resolved by capillary electrophoresis using a Genetic Analyzer 3130XL (Applied Biosystems). Electropherogram peak areas were obtained using PeakScanner software (Applied Biosystems). Peak areas for samples and calibration control (HPV-7 prostate epithelial cell genomic DNA) were block-normalized using X-chromosome p-arm controls and then normalized to HPV-7 copy number with the inference that the HPV-7 genome contains 1 copy of the AR gene. MLPA for AR intron sequences was performed using the exact same protocol with a commercially available reagent kit (EK1, MRC Holland, Amsterdam) and custom-designed oligonucleotide probes (Supplementary Table 7). Probe pairs that each displayed copy number values outside of one standard deviation from the mean copy number of all AR locus probes from two independent experiments were determined to have increased or decreased copy number at that location.

Paired-end library creation, sequence capture, and next-generation sequencing

Genomic DNA from CWR22Pc, 22Rv1, and CWR-R1 cells was fragmented using an S220 ultra-sonicator (Covaris, Woburn, MA) with Agilent SureSelect parameters. A Bioanalyzer DNA 1000 chip (Agilent, Santa Clara, CA) was used to verify DNA samples sheared with fragment peaks between 150-200 bp. Paired-end sequencing libraries were generated from sheared DNA samples using a SureSelect Library Preparation Kit (Agilent) and amplified for sequence capture as per the manufacturer's protocol. The amplified DNA libraries were hybridized and captured using overlapping, tiled SureSelect baits (Agilent) custom-designed to provide 2X coverage of non-repetitive regions of the AR locus (Supplementary Table 8). Target-enriched libraries were amplified for 16-cycles to add index tags and generate

sufficient template for flowcell clustering. Final libraries were quantified via quantitative PCR (Kapa Biosystems, Woburn, MA), normalized, and pooled prior to clustering on a single lane of a flowcell. The flowcell was loaded on a Genome Analyzer IIx (GAIIx, Illumina, San Diego, CA) for paired-end sequencing at 76 cycles (2×76 bp). Data analysis methodology is provided as Supplemental Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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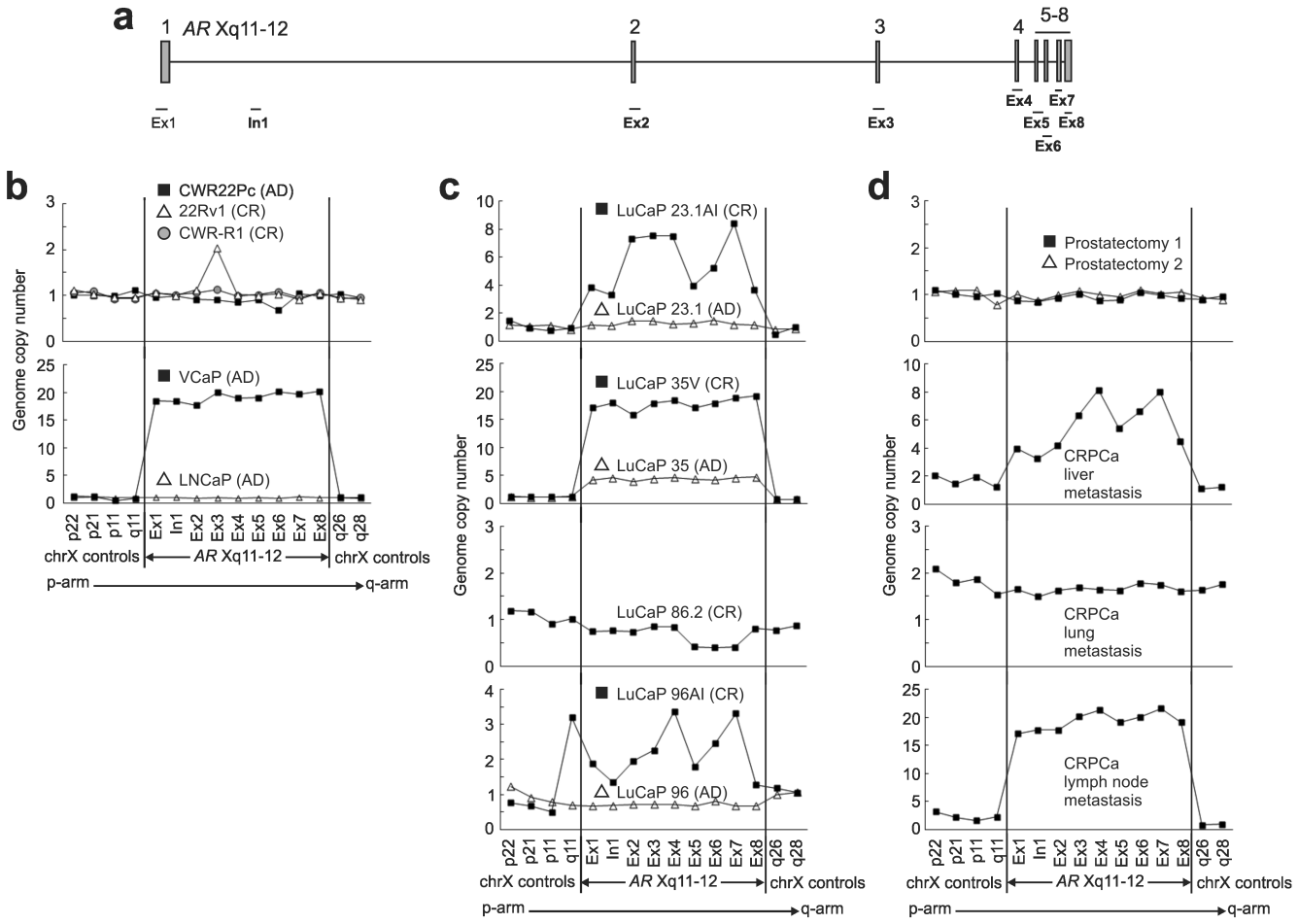

Figure 1.

Diverse and complex patterns of AR gene copy number imbalance in CRPCa. (a) Schematic of the AR gene with relative locations of multiplex ligation-dependent probe assay (MLPA) probes used for targeted copy number analysis. Genomic DNA from (b) androgen-dependent (AD) and castration-resistant (CR) PCa cell lines, (c) PCa xenografts, including AD/CR pairs propagated in intact/castrated male mice, and (d) clinical PCa was subjected to MLPA to evaluate genomic copy number across the AR locus and X chromosome.

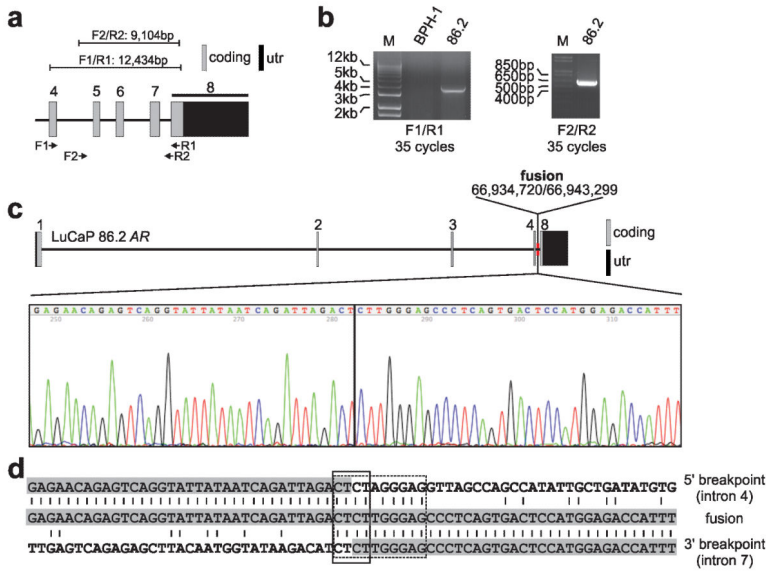

Figure 2.

Intragenic deletion encompassing AR exons 5, 6, and 7 in the LuCaP 86.2 xenograft model. (a) Relative positions of deletion-spanning PCR primers and expected PCR fragment sizes based on the hg19 build of the human genome. (b) CWR-R1 and BPH-1 genomic DNA was subjected to nested PCR using primer sets indicated in (a). (c) PCR products from (b) were cloned and sequenced using the Sanger method. The electropherogram peak trace and AR gene structure resulting from the 8,579bp intragenic deletion are shown. (d) Alignment of the 5′ deletion breakpoint, the 3′ deletion breakpoint, and the deletion fusion revealed 4bp of perfect microhomology (solid box) and 10bp of extended microhomology with 1bp mismatch (dashed box). Sequence retained in the break fusion junction is shaded in gray.

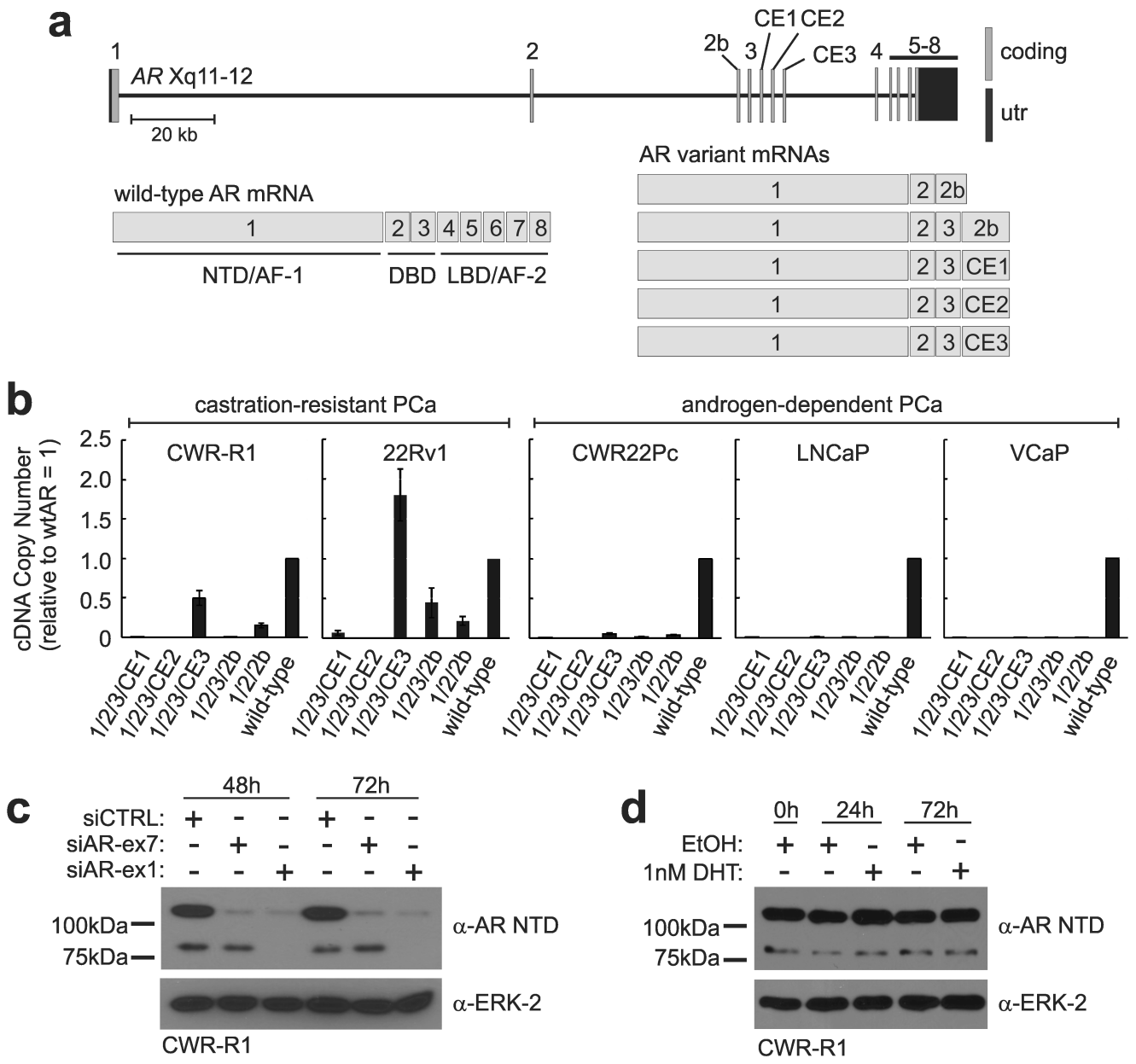

Figure 3.

Stable, high-level expression of truncated AR variants in CWR-R1 cells. (a) AR genomic organization and exon composition of alternatively spliced AR mRNA isoforms reported in cell lines derived from the CWR22 xenograft. (b) RNA from indicated PCa cells lines was subjected to quantitative RT-PCR with isoform-specific primer sets and Ct values were converted to copy number by plotting on standard curves. (c) CWR-R1 cells were electroporated with a control siRNA or siRNAs targeted to AR Exon 1 or Exon 7 and analyzed by Western blot with indicated antibodies 48h and 72h post-transfection. (d) CWR-R1 cells were treated with 1nM dihydrotestosterone (DHT) or vehicle (EtOH) for indicated times and analyzed by Western blot with indicated antibodies.

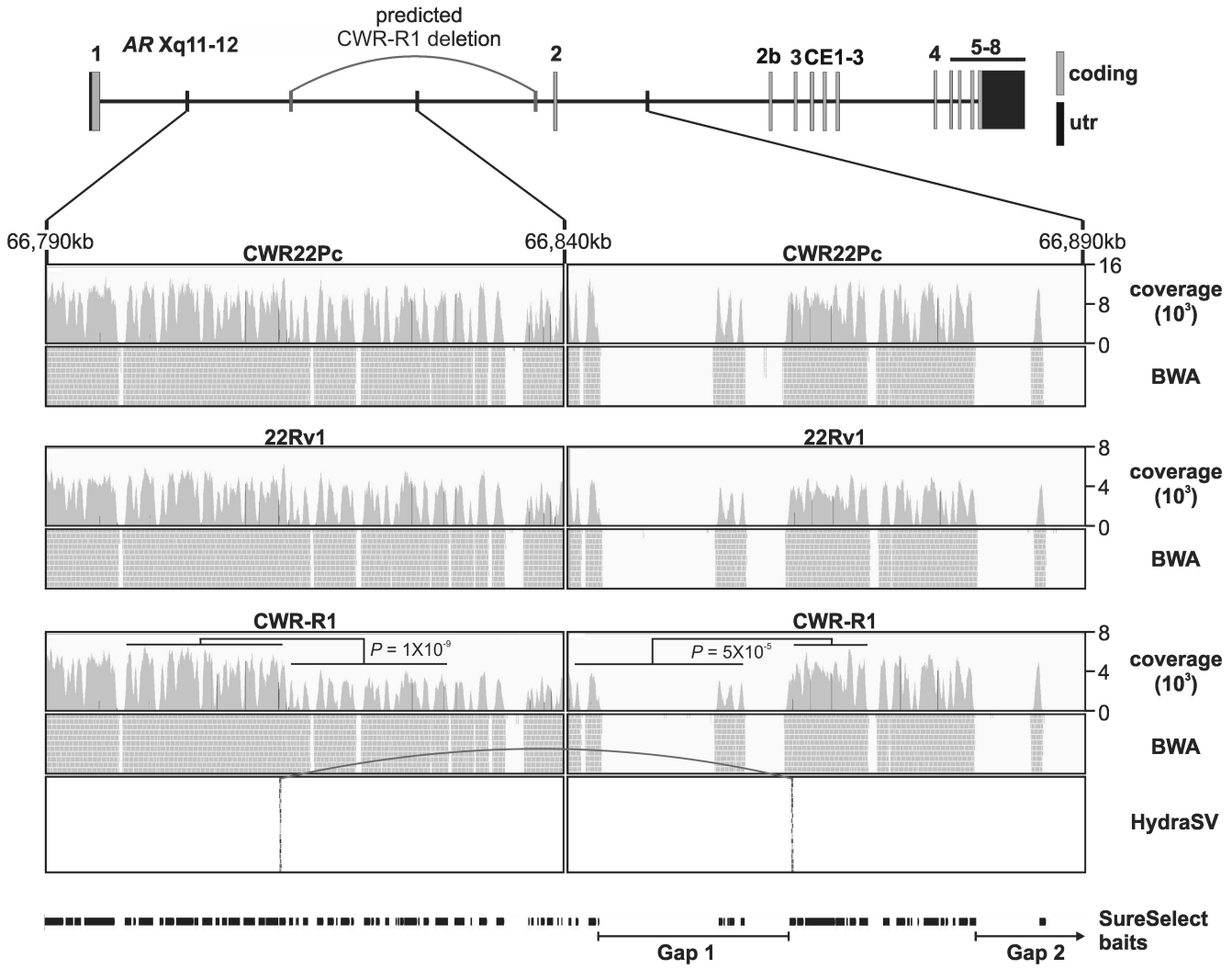

Figure 4.

CWR-R1 cells harbor an intragenic deletion in AR intron 1. A schematic of the AR locus is illustrated at the top. Paired-end sequence reads were mapped to the hg19 build of the human genome using Burrows-Wheeler Alignment (BWA) and visualized using Integrated Genomics Viewer (IGV). Two 50kb windows spanning genomic positions 66,790,000 and 66,890,000 are shown for each cell line, indicating positions and depth of coverage of BWA-mapped reads. Discordantly-mapped paired-end reads identified by the Hydra workflow are shown for CWR-R1 and the relative location of this predicted deletion is indicated on the AR locus schematic. Differences in coverage peak maxima at sites flanking the predicted deletion breakpoints were compared using t-tests. Positions of individual SureSelect baits used for AR sequence capture are tiled across the bottom. Two large gaps of extended repetitive sequence precluding design of capture baits are indicated.

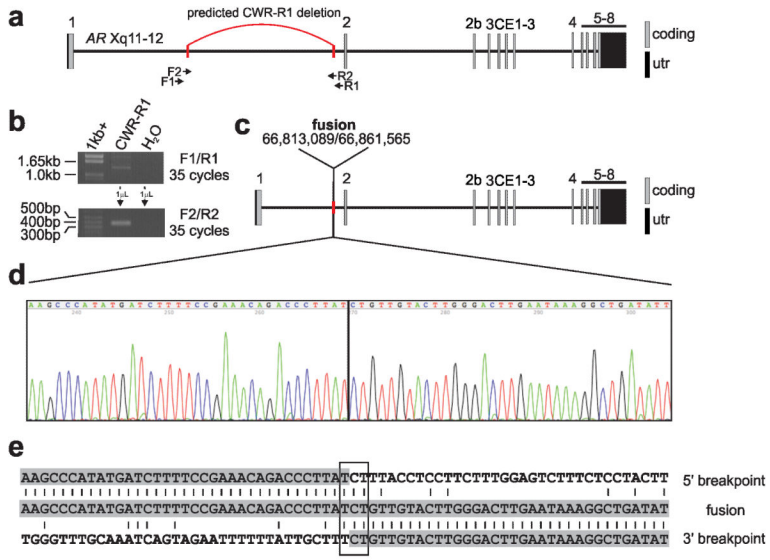

Figure 5.

Intragenic deletion involving 48,476bp of AR intron 1 in CWR-R1 cells. (a) Schematic of the AR locus with relative positions of deletion-spanning PCR primers. (b) CWR-R1 genomic DNA was subjected to nested PCR using indicated primer sets indicated in (a). (c) Schematic of the CWR-R1 AR locus harboring a 48,476bp intron 1 deletion. (d) PCR products from (b) were cloned and sequenced using the Sanger method. The electropherogram peak trace is shown. (e) Alignment of the 5′ deletion breakpoint, the 3′ deletion breakpoint, and the deletion fusion revealed 3bp of microhomology (boxed). Sequence retained in the break fusion junction is shaded in gray.

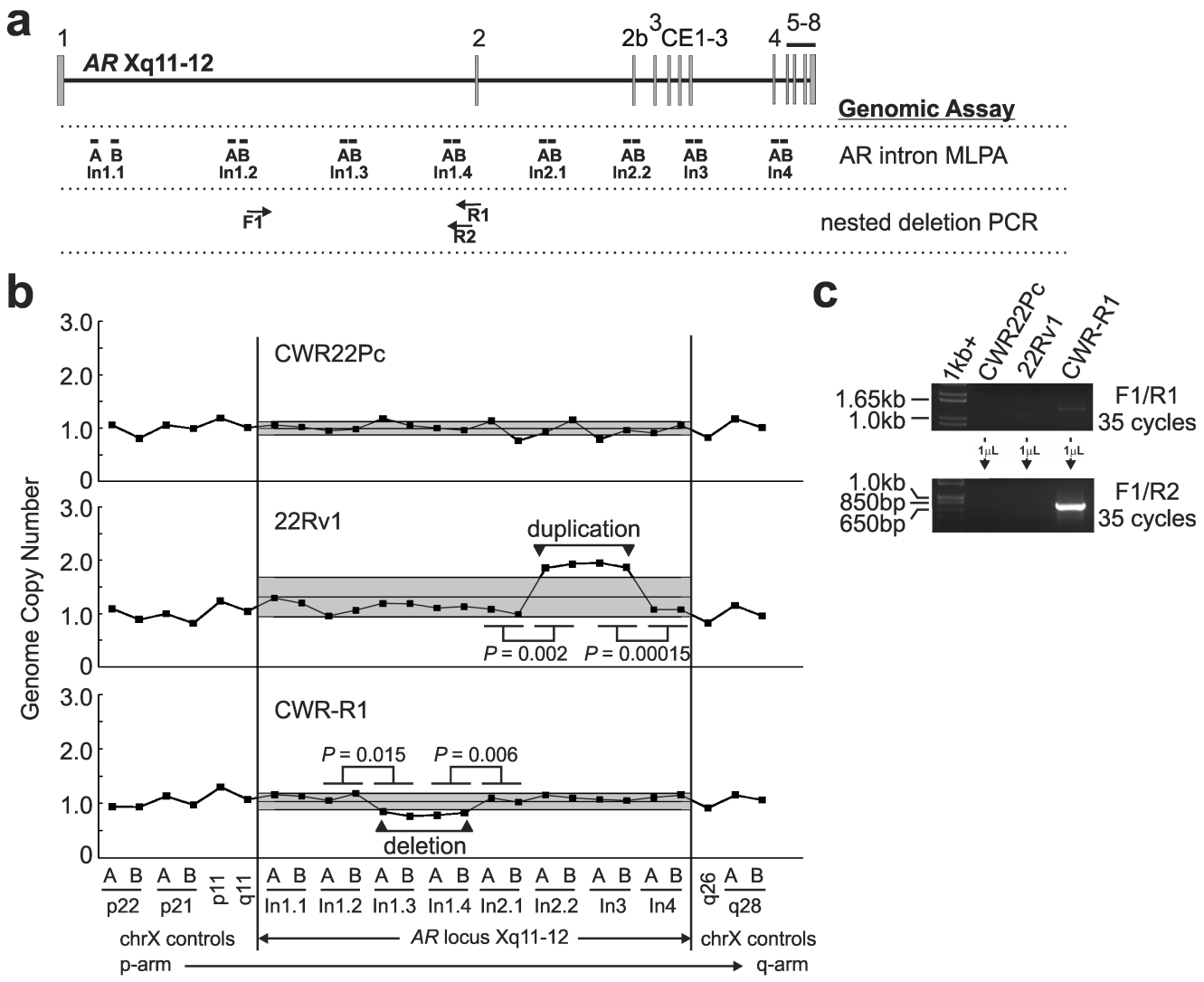

Figure 6.

Intragenic deletion is restricted to a CWR-R1 subpopulation. (a) Schematic of the AR gene with relative locations of primers and probes used for targeted genomic assays. (b) Genomic DNA was subjected to multiplex ligation-dependent probe assay (MLPA) to evaluate genomic copy number across the AR locus and X chromosome. Relative positions of AR-specific MLPA probes are indicated in (a). Gray boxes represent the mean +/− standard deviation of all AR locus probes from 2 independent experiments. Probe pairs displaying copy number greater than 1 standard deviation away from the mean AR copy number are diagnostic of duplication or deletion. Differences in copy number measured by probe pairs flanking predicted duplication or deletion breakpoints were compared with t-tests. (c) Genomic DNA was subjected to nested PCR using primer sets depicted in (a).

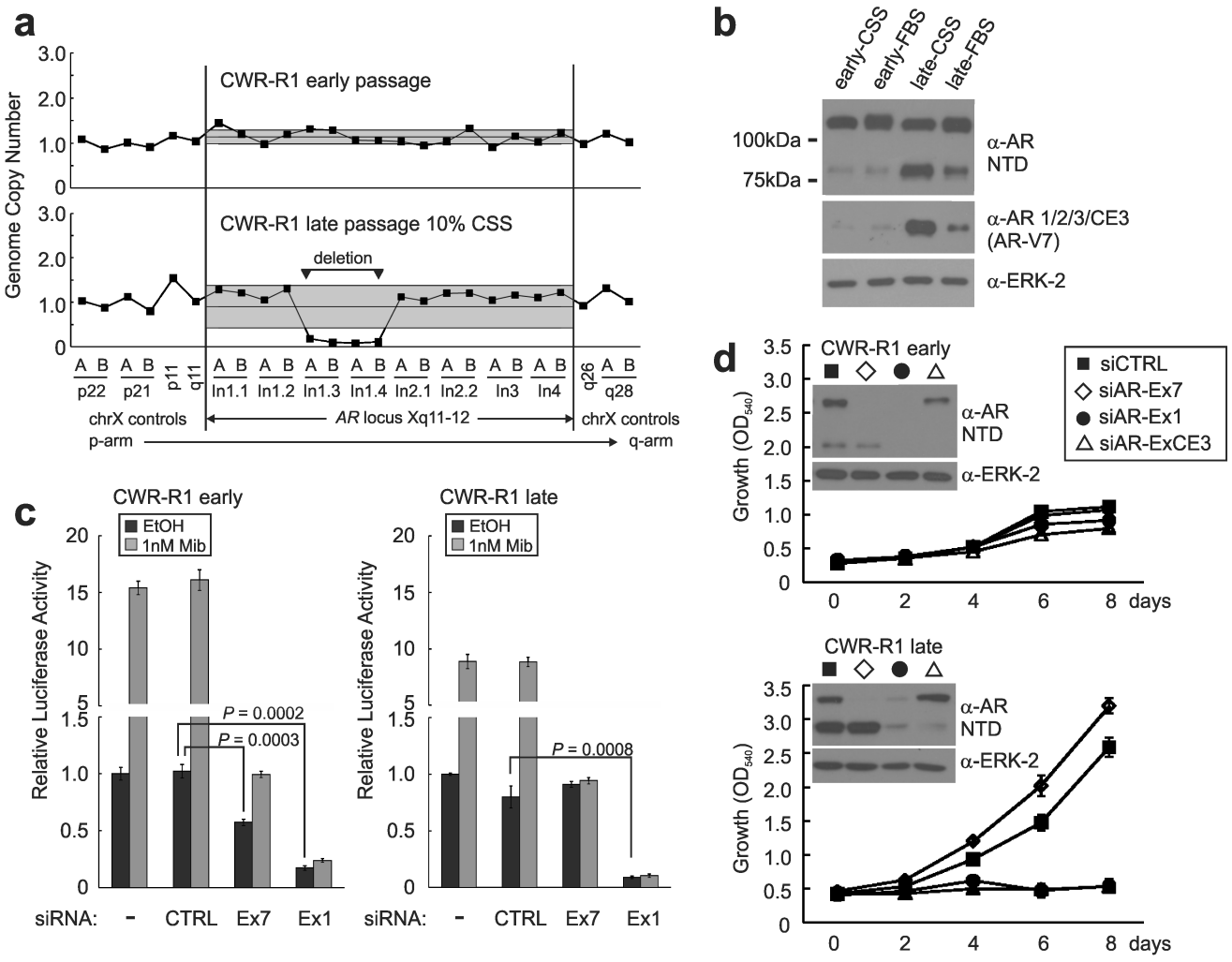

Figure 7.

CWR-R1 cells with intron 1 deletion are castration-resistant and dependent on truncated AR variant function. (a) Genomic DNA from early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions was subjected to multiplex ligation-dependent probe assay (MLPA) to evaluate genomic copy number across the AR locus and X chromosome. Gray boxes represent the mean +/− standard deviation of all AR locus probes from 2 independent experiments. Probe pairs diagnostic of deletion are indicated. (b) Lysates from early-passage CWR-R1 cells cultured in the presence (FBS) or absence (CSS) of androgens for 24h and CWR-R1 cells cultured in the presence (FBS) or absence (CSS) of androgens for 20 passages (late) were analyzed by Western blot with indicated antibodies. (c) Early-passage CWR-R1 cells and CWR-R1 cells cultured for 20 passages under castrate conditions (late) were transfected with MMTV-Luc, non-targeted control (CTRL) siRNA, or siRNAs targeted to AR Exon 1 or 7. Cells were grown 24h in serum-free medium and treated with 1nM mibolerone (synthetic androgen) or EtOH (vehicle control) for 24h. Luciferase activity was determined. Data represent the mean +/− S.E. from two independent experiments, each performed in duplicate. MMTV promoter activity without androgens and siRNAs was arbitrarily set to 1. (d) Early-passage CWR-R1 cells and CWR-R1 cells

cultured for 20 passages under castrate conditions (late) were transfected with non-targeted control (CTRL) siRNA, or siRNAs targeted to AR Exon 1 or 7. Growth of transfected cells under castrate conditions was monitored every 2 days.