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Cyclin D1 Splice Variants: Polymorphism, Risk, and Isoform-Specific Regulation in Prostate Cancer

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Abstract

Purpose: Alternative *CCND1* splicing results in cyclin D1b, which has specialized, protumorigenic functions in prostate not shared by the cyclin D1a (full length) isoform. Here, the frequency, tumor relevance, and mechanisms controlling cyclin D1b were challenged.

Experimental Design: First, relative expression of both cyclin D1 isoforms was determined in prostate adenocarcinomas. Second, relevance of the androgen axis was determined. Third, minigenes were created to interrogate the role of the G/A870 polymorphism (within the splice site), and findings were validated in primary tissue. Fourth, the effect of G/A870 on cancer risk was assessed in two large case-control studies.

Results: Cyclin D1b is induced in tumors, and a significant subset expressed this isoform in the absence of detectable cyclin D1a. Accordingly, the isoforms showed noncorrelated expression patterns, and hormone status did not alter splicing. Whereas G/A870 was not independently predictive of cancer risk, A870 predisposed for *transcript-b* production in cells and in normal prostate. The influence of A870 on overall *transcript-b* levels was relieved in tumors, indicating that aberrations in tumorigenesis likely alter the influence of the polymorphism.

Conclusions: These studies reveal that cyclin D1b is specifically elevated in prostate tumorigenesis. Cyclin D1b expression patterns are distinct from that observed with cyclin D1a. The A870 allele predisposes for *transcript-b* production in a context-specific manner. Although A870 does not independently predict cancer risk, tumor cells can bypass the influence of the polymorphism. These findings have major implications for the analyses of D-cyclin function in the prostate and provide the foundation for future studies directed at identifying potential modifiers of the G/A870 polymorphism. (Clin Cancer Res 2009;15(17):5338–49)

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Translational Relevance

This study has marked clinical and translational implications. First, these data reveal for the first time that cyclin D1b is specifically induced in prostate cancer. As cyclin D1b has distinct functions from cyclin D1a, this has important implications for interpretation of biomarker and functional analyses. Second, previous studies tentatively linked the G/A870 polymorphism to cancer risk and cyclin D1b, but until now, these postulates remained untested. Here, case-control studies showed that G/A870 is not independently predictive of risk. Third, although not sufficient for risk, functional studies identified A870 as a critical modulator of cyclin D1b in model systems and human prostate specimens. The A870 requirement is bypassed in cancer, indicating that tumor-specific events circumvent or cooperate with A870. Together, these findings identify cyclin D1b markedly enhanced in prostate cancer and reveal the effect of G/A870 for cyclin D1b production and cancer risk.

It is increasingly apparent that alternative splicing of the *CCND1* transcript results in a protein with divergent functions from full-length cyclin D1 (cyclin D1a). The alternatively spliced transcript, *transcript-b*, arises from a failure to splice at the exon 4/intron 4 boundary. The presence of a stop codon within intron 4 gives rise to a truncated protein (cyclin D1b), which harbors a unique COOH terminus that is devoid of exon 5–encoded sequences (1, 2). Functional studies showed that cyclin D1b has unique activities, distinct from cyclin D1a, with particular cancer significance. In many cell types, cyclin D1a promotes cell cycle progression through activation of cyclin-dependent kinases (CDK) 4 or 6 (3, 4). The principal substrate of cyclin D1a-CDK4/6 complexes is the retinoblastoma tumor suppressor protein (RB), whose antiproliferative capacity is weakened by cyclin D1a/CDK–mediated phosphorylation (5, 6). Although cyclin D1b binds CDK4, this variant is markedly deficient in inducing RB phosphorylation (7–10). Based on these and other observations that showed that cyclin D1a fails to alter cell cycle progression in RB-deficient cells (11–13), it might be expected that cyclin D1b is ineffective at promoting cellular proliferation and/or tumorigenesis. On the contrary, it was unexpectedly observed that cyclin D1b has enhanced oncogenic capacity compared with cyclin D1a (9, 10, 14–16).

The tumorigenic effects of cyclin D1b have been recently explored and include unique functions in different tumor types. In mouse fibroblast models, cyclin D1b (but not cyclin D1a) induced *in vitro* focus formation, *in vivo* tumor formation (9, 10), and anchorage independence (16). In breast cancer cells, cyclin D1b exclusively induced resistance to therapeutic intervention (17). The importance of differential cyclin D1b function is further underscored by observations that the alternatively spliced transcript has been detected in multiple cancers, including Ewing's sarcoma (18, 19), mantle cell lymphoma (8, 20), esophageal cancer (9), colon cancer (21, 22), B-lymphoid malignancies (7, 23), and breast cancer (24). Given the divergent and decidedly protumorigenic effects of cyclin D1b expression, it is imperative to explore cyclin D1b production and the factors that may regulate its production.

In prostate cancer (PCa), there is a particular interest in discerning the mechanisms that govern cyclin D1b production, as the D-cyclins serve specialized roles that seem to affect tumor progression. PCa is resistant to most forms of genotoxic stress (25); therefore, disseminated PCa is treated based on the androgen dependence of this tissue (26, 27). PCa cells require the androgen receptor (AR), a ligand-dependent transcription factor, for survival and progression (28, 29). Significantly, AR activation results in mammalian target of rapamycin (mTOR)–mediated induction of cyclin D1a, which impinges on CDK4 to initiate cell cycle progression (30). However, accumulated cyclin D1a seems to induce a negative feedback loop wherein cyclin D1 binds and inhibits AR transcriptional activity, thus regulating the strength and duration of AR signaling (reviewed in refs. 14, 28). The ability of cyclin D1a to regulate AR seems critical, as tumors devoid of cyclin D1a are associated with enhanced AR activity (31). Moreover, cyclin D1a can be sequestered to the cytoplasm in PCa (and other tissues; refs. 9, 32–35), indicating that aberrant regulation of cyclin D1a may be common in PCa (31). The most striking evidence of altered regulation was observed with cyclin D1b, wherein the *transcript-b* was shown to be elevated in a small sample set of PCa and precancerous prostatic intraepithelial neoplasia (PIN) specimens compared with matched nonneoplastic controls (36). The consequence of this event is of clinical relevance, as cyclin D1b is compromised in its ability to control AR-dependent signaling (36). Thus, it is hypothesized that cyclin D1b serves multiple oncogenic functions in PCa.

Despite the importance of cyclin D1b in cancer, few studies have assessed the factors that influence its production or the relative expression of each isoform in PCa. Here, for the first time, through large-scale analyses, we show that PCa specimens express elevated cyclin D1b, thus indicating the importance of delineating the factors that contribute to cyclin D1b production. Moreover, cyclin D1b is specifically and significantly induced as a function of PCa tumorigenesis. Subsequent analyses showed that cyclin D1b showed distinct expression patterns from that of cyclin D1a, which was not elevated in cancer (as compared with nonneoplastic tissue) under the conditions used. Unlike observations in breast cancer, hormone status had no detectable effect on *transcript-b* production. By contrast, the A allele of the *CCND1* G/A870 single nucleotide polymorphism (rs603965) predisposed for *transcript-b* production in a context-specific manner. Introduction of engineered *CCND1* minigenes into cyclin D1–deficient cells showed that the A allele promoted *transcript-b* and cyclin D1b production, whereas the G allele promoted *transcript-a* and cyclin D1a production. These observations were consistent with analyses of nonneoplastic prostate tissue, wherein overall *transcript-b* levels were higher in patients with an A allele. However, the requirement of the A allele for high *transcript-b* production was relieved in tumor tissue and cell lines. Moreover, analyses of two large population-based studies revealed that the A allele is not independently predictive of PCa risk. Together, these data indicate that a subset of PCa induces cyclin D1b and that the G/A870 polymorphism contributes to *transcript-b* and cyclin D1b expression. These studies show for the first time that cyclin D1b is elevated in PCa and the relevance of the G/A870 polymorphism on cancer risk and cyclin D1b production.

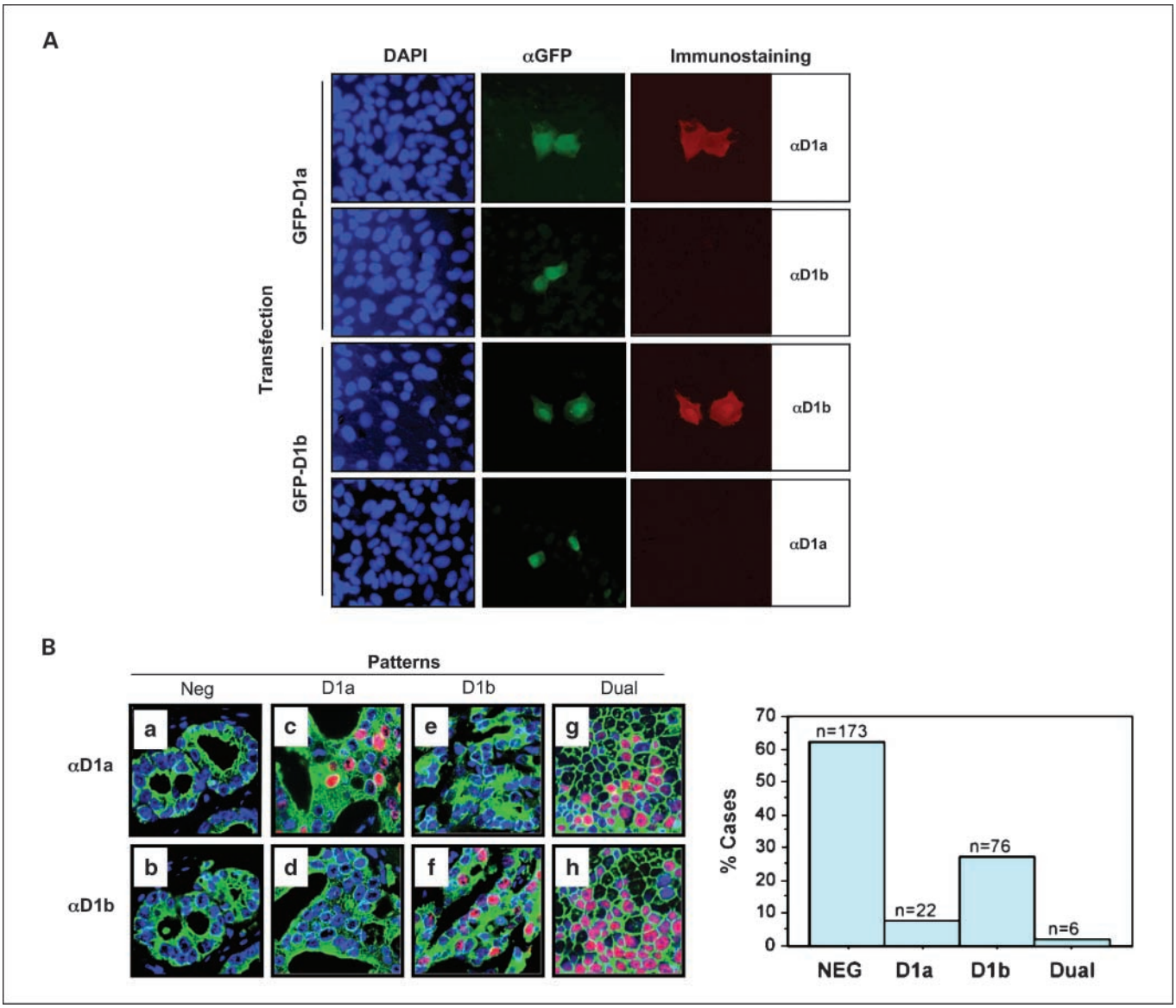
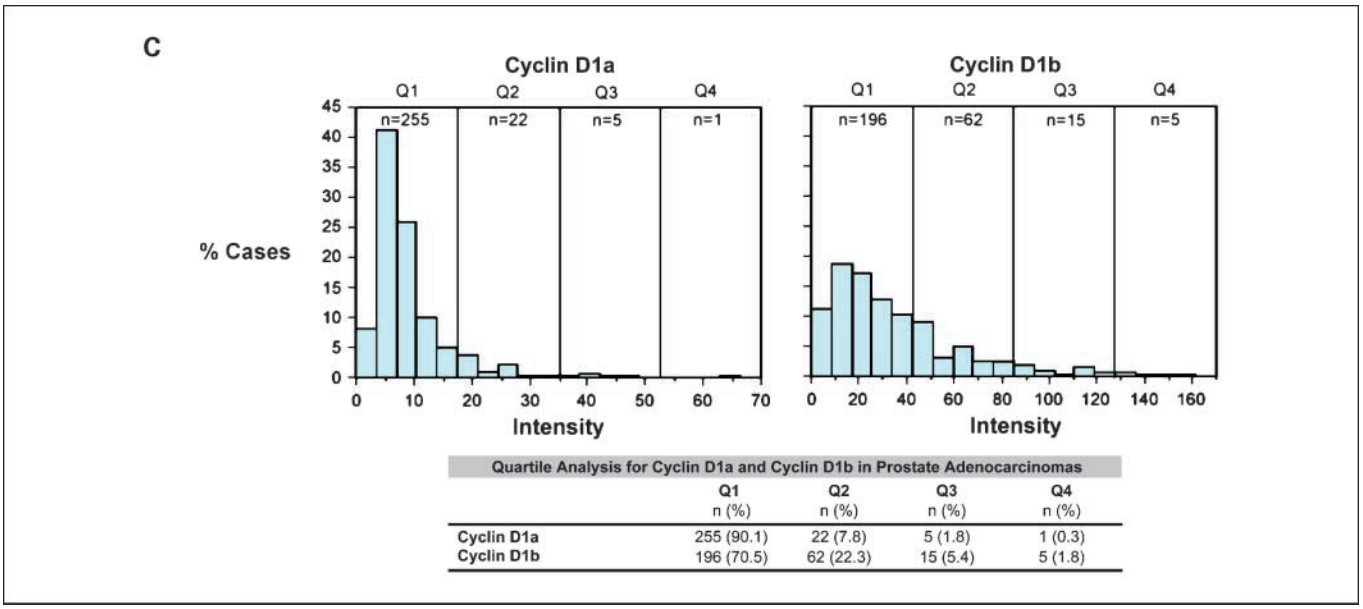


Fig. 1. The cyclin D1b isoform is frequently expressed in PCa. *A,* U2OS cells were transfected with expression plasmids encoding GFP-cyclin D1a (*top*) or GFP-cyclin D1b (*bottom*) and then immunostained with GFP-specific antisera (*middle column, green*) or isoform-specific cyclin D1 antibodies, as indicated (*right column, red*). Left column, blue, nuclei are indicated by DAPI. Representative images were captured with equal exposure times. *B,* cyclin D1a and cyclin D1b isoforms were detected in serial sections from a large PCa tissue microarray using a fluorescence-based tissue biomarker platform. Representative images (*left*) and quantification (*right*) from the tumor data set with antibodies specific to cyclin D1a (*top*) or cyclin D1b (*bottom*). Cyclin D1a and cyclin D1b are indicated by red fluorescence, cytokeratin (epithelial marker) is indicated by green fluorescence, and nuclei (DAPI) are indicated by blue fluorescence. Staining patterns are indicated: negative (*a* and *b*), cyclin D1a–positive (*c* and *d*), cyclin D1b–positive (*e* and *f*), and cyclin D1a/cyclin D1b–positive "dual-positive" (*g* and *h*).

Materials and Methods

Immunohistochemistry. Human prostate specimens organized as a tissue microarray (cohort 1) were previously described (37). A second human prostate tissue microarray (cohort 2) was purchased (US Biomax). Immunostaining was done after deparaffinization/rehydration and microwave-induced antigen retrieval with citrate buffer (DAKO). Serial sections were blocked with goat serum (10%) followed by incubation (1 h) with the following antibodies: cyclin D1b (1:50; ref. 17) or cyclin D1a (Ab-3; Lab Vision). After washing with TBS, slides were incubated with a cytokeratin antibody (AE1/AE3; DAKO). Cyclin D1 was detected using an anti-rabbit horseradish peroxidase–conjugated antibody (EnVision-Plus; DAKO) followed by incubation with Tyramide-Cy5 (TSA System; Perkin-Elmer). Cytokeratin was visualized with an anti-mouse Alexa Fluor 488–conjugated antibody (Invitrogen/Molecular Probes). Nuclear visualization was via 4′,6-diamidino-2-phenylindole (DAPI).

AQUA system analysis. Immunohistochemical sections were quantified using the unbiased automated image acquisition system AQUA/PM2000 Imaging Platform (HistoRx) as previously described (38). Briefly, immunostained sections were scanned to detect FITC/Alexa Fluor 488, Cy5, and DAPI. The cyclin D1a or cyclin D1b AQUA score (average signal intensity) was determined within epithelial compartments based on cytokeratin masking. Analysis of the cyclin D1 isoforms was done and statistical significance ($P < 0.05$) was determined using the Mann-Whitney *U* test.



Quartile Analysis for Cyclin D1a and Cyclin D1b in Prostate Adenocarcinomas

	Q1 n (%)	Q2 n (%)	Q3 n (%)	Q4 n (%)
Cyclin D1a	255 (90.1)	22 (7.8)	5 (1.8)	1 (0.3)
Cyclin D1b	196 (70.5)	62 (22.3)	15 (5.4)	5 (1.8)

Fig. 1 *Continued. C,* frequency distribution analysis of cyclin D1a (*left*) and cyclin D1b (*right*) in adenocarcinomas. Data are presented as percent cases versus staining intensity, and quartiles (Q1-Q4) are indicated by vertical line. The *n*-number for each quartile is indicated.

PCa risk associations. The Multiethnic Cohort is a large prospective study, approved by the Institutional Review Boards at the University of Hawaii and University of Southern California, and is composed of five racial/ethnic groups as previously described (39). Incident cancers and stage of disease were identified (up to April 1, 2002) using Surveillance Epidemiology and End Results cancer registries covering Hawaii and California. Controls were men without PCa before entry into the study and were matched to cases by age and ethnicity. This study consisted of 2,302 cases and 2,277 controls. Genotyping was done by the 5′-nuclease Taqman allelic discrimination assay (Applied Biosystems). Blinded duplicate samples (10%) were included to assess genotyping reproducibility.

The Australian Risk Factors for Prostate Cancer Study is a population-based case-control study including 829 cases and 739 controls predominantly of European descent and was previously described (40). Eligible cases (diagnosed and histopathologically confirmed) were notified to the State Cancer Registry during 1994 to 1997. Cases were excluded if age at diagnosis was ≥70 y or if the tumor Gleason score was <5. Controls were randomly selected from the State Electoral Rolls (compulsory to vote in Australia) and matched to cases by age group and city of residence. Informed consent was obtained from all study participants and approved by the Human Research Ethics Committee of the Cancer Council of Victoria (HREC 9500). Genotyping was done in duplicate using the MassARRAY Compact System (Sequenom) with mass spectrometry (matrix-assisted laser desorption/ionization time of flight) and 5′-nuclease Taqman allelic discrimination assay. Genotyping was done in a blinded manner and discordance was validated using amplicon-specific amplification and denaturing gradient gel electrophoresis. Allele frequency estimates and tests of deviation from Hardy-Weinberg equilibrium were carried out using standard procedures based on asymptotic likelihood theory. Tests for association between genotypes and case-control status were done under dominant, recessive, codominant, and additive models. Case-control analyses were conducted using unconditional logistic regression, and odds ratios (OR) and 95% confidence intervals (95% CI) were estimated for each genotype (relative to GG). All tests were two sided and 5% level was used as a threshold for statistical significance.

Cell culture and treatments. The androgen-dependent (LNCaP and LAPC4) and castration-resistant (PC3, 22Rv1, and DU145) PCa cell lines were maintained as described (36). The breast cancer (MCF-7) cell line was maintained as described (17). PCR-RFLP analysis of PCa cell lines was previously described (36, 41). To assess AR activity, LNCaP cells were plated on poly-L-lysine in steroid-free conditions for 48 h in 5% charcoal dextran–treated serum (HyClone) and subsequently treated for 24 h with dihydrotestosterone (1 nmol/L). Treatment (1 μmol/L for 24 h) with the antiandrogen Casodex (AstraZeneca Pharmaceuticals) or the mTOR inhibitor RAD001 (everolimus; 10 μmol/L for 4 h; LC Laboratories) was done as indicated.

CCND1 *minigenes.* The G/A870 region of *CCND1* was amplified from LNCaP (GA genotype) genomic DNA using the Expand Long Template PCR System (Roche). The amplified fragment (3,297 bp containing intron 4) and pcDNA3.1 (with bovine growth hormone polyadenylation signal) containing the coding sequence (exons 1-5) for human cyclin D1 [tagged with enhanced green fluorescent protein (EGFP) and hemagglutinin] were digested with *Bsu*36I (internal exon 4 site) and *Eco*RV (introduced site after 6 bp of *CCND1* 3′-untranslated region), purified, ligated, and screened using *Bsr*GI (internal intron 4 site) to verify insertion of intron 4–containing sequences. Positive clones were sequenced to identify those that harbor either the G or A allele. Transfection of the minigene constructs was done using the standard calcium phosphate method. Immunoblotting was done with the following antibodies: GFP (Santa Cruz Biotechnology), cyclin D1a, and cyclin D1b.

Gene expression analysis. For PCa cell lines, RNA was extracted using Trizol and cDNA was generated with SuperScript II (Invitrogen). Reverse transcription-PCR (RT-PCR) was done using GoTaq (Promega) with the following primers: KLK3/prostate-specific antigen [PSA; described previously (42)], *transcript-a*, *transcript-b*, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Supplementary Fig. S1). GAPDH and KLK3/PSA were amplified at 26 cycles. *Transcript-b* and *transcript-a* were amplified at 32 and 27 cycles, respectively. Products were resolved on agarose (2%) and visualized with ethidium bromide. Quantitative PCR was done using a StepOne machine (Applied Biosystems). Taqman assays (Applied Biosystems) were used to determine KLK3/PSA (Hs00426859_g1) and GAPDH (Hs00266705_g1). *Transcript-a*, *transcript-b*, and GAPDH (primers described above) were determined by quantitative PCR using Express SYBR (Invitrogen). Relative expression was determined as described (43). Human prostate tissue was obtained from the University of Cincinnati and Thomas Jefferson

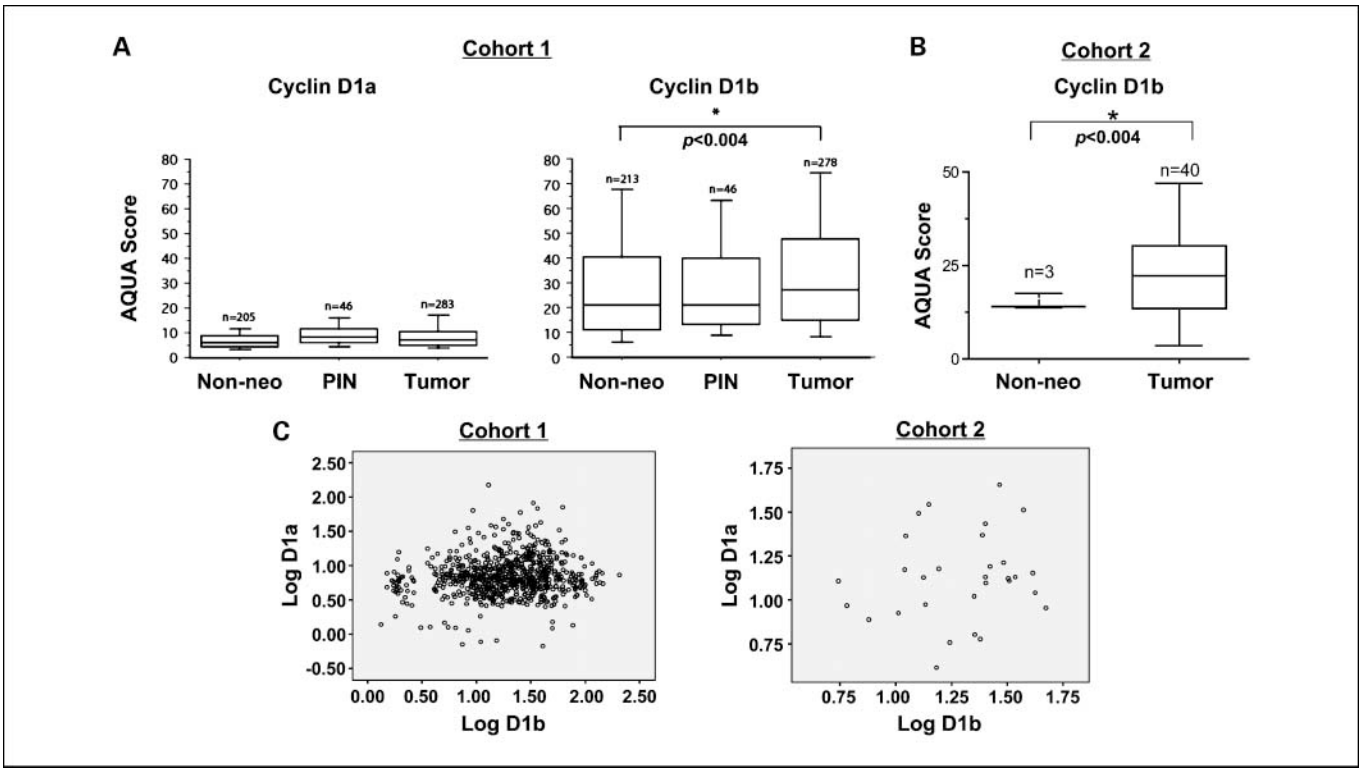


Fig. 2. Cyclin D1b is uniquely associated with tumorigenesis. *A,* immunostaining for nonneoplastic, PIN, and adenocarcinoma specimens obtained in Fig. 1 was assessed to determine the association of cyclin D1a (*left*) and cyclin D1b (*right*) with tumorigenesis. Immunostaining data (AQUA scores) are shown, and the *n*-number is indicated. Statistical significance ($P < 0.05$) was determined by Mann-Whitney *U* analysis and is indicated by an asterisk. *B,* parallel analysis was done on a second tissue array containing nonneoplastic and tumor tissue. The *n*-number is indicated. Statistical significance ($P < 0.05$) was determined by Mann-Whitney *U* analysis and is indicated by an asterisk. *C,* the relationship between cyclin D1b and cyclin D1a was determined by Pearson correlation for cohort 1 (*left*) and cohort 2 (*right*). Data are presented as log-transformed AQUA scores from individual tumor specimens for cyclin D1b and cyclin D1a. The Pearson correlations (*r*) for cohort 1 and cohort 2 were 0.106 and 0.153, respectively.

University in accordance with Institutional Review Board guidelines. OCT-embedded tissues were incubated in RNAlater-ICE (Ambion). After OCT removal, tissue was minced and homogenized in Trizol to obtain RNA (and DNA for PCR-RFLP as above). Subsequently, cDNA was generated using SuperScript VILO (Invitrogen). Cyclin D1a, cyclin D1b, and GAPDH (primers described above) expression was determined by RT-PCR in the presence of [α-^{32}P]dCTP (Perkin-Elmer), which allows accurate quantification over a large dynamic range (44, 45). Products were separated on a 6% PAGE gel and quantified with a Typhoon 9400 PhosphorImager (GE Healthcare/Amersham). Total *transcript-b* expression was determined relative to GAPDH.

Results

Cyclin D1b is specifically elevated in PCa. Despite the emerging knowledge that cyclin D1b has enhanced oncogenic functions, little is known about the frequency of cyclin D1b production and the mechanisms that regulate alternative *CCND1* splicing. Previous studies detected induction of *transcript-b* in a small subset of PIN and PCa specimens; these findings were of significance, as cyclin D1b engages in prostate-specific activities that are proposed to enhance tumorigenesis (36). Thus, it was imperative to determine the status of cyclin D1b and define the factor(s) that regulates its production in PCa. To address this, a cyclin D1b–specific antibody was used that has been extensively characterized by immunoblot and immunohistochemical approaches in multiple studies (8, 17, 19, 36). The cyclin D1b–specific antibody is directed against an epitope not present in cyclin D1a, and a recent study further validated the specificity of the cyclin D1b antisera using both fluorescence-based immunocytochemistry and immunohistochemistry (24). Although the cyclin D1b antisera are slightly more sensitive than the cyclin D1a antisera, conditions identical to those used in the present study could detect endogenous protein as well as discern tumor-associated induction of either isoform in other tumor types (17, 24). To further confirm specificity, several studies were done. First, U2OS cells [which express low levels of cyclin D1 (17)] were transfected with expression vectors encoding individual GFP-tagged cyclin D1 isoforms. As shown in Fig. 1A, immunocytochemistry revealed no cross-reactivity with the cyclin D1 isoform-specific antisera. Similar results were observed in SAOS2 cells [which express low levels of cyclin D1 (17)] transduced with adenovirus encoding either cyclin D1 isoform (Supplementary Fig. S2A), as detected by AQUA System (HistoRx) analysis. Second, immunodetection of endogenous cyclin D1b in PCa (LAPC4) cells was depleted by hormone ablation (Supplementary Fig. S2B), which blocks *CCND1* mRNA translation. Third, immunofluorescence (Supplementary Fig. S2C) and immunohistochemistry (using cells treated *in vitro*, clotted, embedded, and subsequently processed for immunohistochemistry; Supplementary Fig. S2D) showed identical profiles when detecting endogenous cyclin D1b in PCa cells in the presence of androgen or after androgen ablation. Combined, these studies validated the specificity of the cyclin D1b antibody.

Relative cyclin D1a and cyclin D1b expression was subsequently assessed in serial sections using isoform-specific antisera in a cohort of human prostate specimens (1,626 cores) containing nonneoplastic, PIN lesions, and adenocarcinomas (37). The fluorescent signal was determined using a tissue biomarker platform (AQUA System) that allows unbiased quantification of signal intensity in tissue microarrays. Quantification of cyclin D1a or cyclin D1b was done in cells of epithelial origin, as achieved through coimmunostaining for epithelial cytokeratins. Representative staining of parallel samples (*left*) and overall quantification (*right*) are shown in Fig. 1B, wherein it was apparent that distinct patterns emerged with regard to each isoform. Consistent with previous reports (9, 31, 32), a large fraction (62.5%) of tumors had low or undetectable expression of either cyclin D1 isoform. Notably, identical conditions were used in a recent study, which found significant cyclin D1a elevation in breast cancer (24), thus confirming that the conditions used are capable of detecting differences in cyclin D1a levels. These data indicate that neither protein is necessarily required for tumor maintenance in the prostate, and are congruent with a previous report that suggested that loss of cyclin D1a may facilitate AR activity (31). A second cohort showed cytoplasmic localization of cyclin D1a (data not shown), whereas nuclear cyclin D1a was detected either in isolation (7.94%) or in conjunction (2.16%) with cyclin D1b. Strikingly, a subset (27.4%) of tumors seemed to express cyclin D1b but showed minimal immunoreactivity against cyclin D1a. The differential staining patterns for cyclin D1a and cyclin D1b provide additional evidence that the isoform-specific antibodies recognize distinct epitopes, and provide the first evidence that the two cyclin D1 isoforms show distinct expression profiles in PCa. This concept was substantiated by quartile analysis, wherein comparison of frequency and intensity of immunostaining for each isoform within the adenocarcinomas was quantified (Fig. 1C). As shown, little to no cyclin D1a expression was observed in the majority of cases [quartile 1 (Q1) = 90.1%]. By contrast, cyclin D1b expression showed a larger range of intensity (Q1 = 70.5%, Q2 = 22.3%, Q3 = 5.4%, Q4 = 1.8%). Together, these data show that cyclin D1a and cyclin D1b show distinct profiles in PCa and that a significant subset of tumors expresses cyclin D1b.

Because the above data are suggestive that cyclin D1b may play a significant role in PCa, comparison of relative expression between the two variants was done using nonneoplastic and tumor tissue. As shown, no significant induction in cyclin D1a levels was observed in PIN or adenocarcinomas compared with nonneoplastic tissue (Fig. 2A, *left*). Interestingly, recent reports have shown that cyclin D1a levels can be reduced in PCa specimens (31) or xenograft models (46), and this event correlates with enhanced AR activity. By contrast, cyclin D1b levels (Fig. 2A, *right*) were elevated 1.2-fold in tumors compared with nonneoplastic tissue ($P < 0.004$). Interestingly, no induction of cyclin D1b protein was noted in PIN lesions, indicating that cyclin D1b maybe specifically associated with tumorigenesis. To expand these findings, parallel analyses were done using a second independent cohort of nonneoplastic and PCa tissue, wherein cyclin D1b status was elevated 1.5-fold in tumors compared with nonneoplastic epithelia ($P < 0.004$; Fig. 2B). The observed increase in cyclin D1b in tumors but not matched nonneoplastic tissue was also seen by immunoblot analysis from a radical prostatectomy specimen (Supplementary Fig. S2E). Combined, these data provide evidence that, different from cyclin D1a expression profiles, elevated cyclin D1b is observed in a significant fraction of tumors.

Cyclin D1b expression patterns are distinct from cyclin D1a. The above data show that cyclin D1b is induced in PCa; however, the mechanism(s) by which production of this isoform is enhanced remains unknown. Furthermore, it is likely that the mechanism(s) involves various aspects of transcription, translation, and stability. The notion that divergent pathways exist to regulate cyclin D1 isoform levels is consistent with the observation that human PCa can produce differential cyclin D1 isoform expression patterns (Fig. 1). Despite these complexities, up-regulation of cyclin D1b may be a reflection of overall cyclin D1 isoform levels. To address this possibility, Pearson correlations were determined between cyclin D1b and cyclin D1a. As shown in Fig. 2C, no relationship was observed between cyclin D1b and cyclin D1a in tumor specimens in either cohort 1 ($r = 0.016$; *left*) or cohort 2 ($r = 0.153$; *right*). Overall, these data indicate that the protein expression patterns of cyclin D1b are distinct from that of cyclin D1a and that disparate mechanism(s) may exist in PCa to regulate each isoform.

Alternative splicing occurs independent of AR activity in PCa cell model systems. Given the observation that cyclin D1b production is associated with tumorigenesis (Fig. 2A) and that cyclin D1b (unlike cyclin D1a) has a diminished capacity to negatively regulate AR function (36), the relationship between alternative splicing of cyclin D1 and AR activity was examined. These studies are imperative, as AR is the dominant signaling pathway that drives PCa progression, therapeutic failure (28, 29, 47), and androgen-dependent splicing of other factors (e.g., clusterin; ref. 48). Analyses of relative transcript expression in asynchronous cells (cultured in the presence of complete serum) revealed that *transcript-b* levels were slightly higher in AR-positive (LNCaP, LAPC4, and 22Rv1) cells compared with AR-negative (PC3 and DU145) PCa cells and an AR-positive (MCF-7) breast cancer cell line (Supplementary Fig. S3A). To directly assess the effect of AR activity on *transcript-b* levels, LNCaP cells were cultured in the absence of steroid hormones before stimulation with dihydrotestosterone. Representative transcript analyses are shown in Supplementary Fig. S3B and quantification is provided in Fig. 3A. AR activation was verified through induction of *KLK3/PSA*, a well-characterized AR target gene (Fig. 3A; Supplementary Fig. S3B, *lanes 1* and *2*, *top*). Unlike PSA (Fig. 3A, *top*), *transcript-a* levels were unchanged by the presence or absence of androgen (*middle*), consistent with the ability of the AR signaling axis to regulate cyclin D1a levels through posttranscriptional mechanisms involving mTOR (30). Likewise, *transcript-b* levels were indistinguishable between androgen-deprived and androgen-stimulated conditions (Fig. 3A, *bottom*), and similar results were observed in parallel experiments wherein an AR antagonist resulted in attenuated *KLK3/PSA* expression but no alteration in either cyclin D1 transcript. These data suggest that, in PCa cell lines, production of *transcript-b* occurs independently of hormone or AR activity.

Because these data suggested that cyclin D1b production, like cyclin D1a, may be regulated in a mTOR-dependent manner, cyclin D1b protein levels were monitored in LAPC4 cells treated with a mTOR inhibitor (RAD001). As shown in Fig. 3B and Supplementary Figs. S2C and D, cyclin D1b protein levels were suppressed by RAD001 treatment, indicating

that cyclin D1b is also regulated by mTOR. Combined, these data indicate that both cyclin D1 transcripts are regulated independently of androgen and AR transcriptional activity and that cyclin D1b is also modulated through translational mechanisms involving mTOR. The observation that both cyclin D1 transcripts are refractory to androgen is distinct from breast cancer, wherein estrogen activates cyclin D1 expression (49, 50). In the context of PCa, estrogen treatment (which activates the gain-of-function AR mutant found in LNCaP cells; refs. 42, 51) had no effect on the abundance of *transcript-b* expression (Supplementary Fig. S3C). Collectively, these data indicate that altered AR transcriptional activity cannot account for the tumor-associated induction of cyclin D1b in PCa.

The G/A870 polymorphism is not independently predictive of PCa risk. The data above indicate that cyclin D1b production must be attributed to factors independent of hormone or overall cyclin D1a levels. It has been long hypothesized that the G/A870 polymorphism could contribute to cyclin D1b production due to its location in a conserved splice donor site (15). This polymorphism is of interest, as the A allele is tentatively associated with increased risk for many cancers (15, 52). Surprisingly, this provocative hypothesis has not been tested, and the relevance of the polymorphism for PCa was not well delineated. To address these issues, the contribution of the G/A870 polymorphism to PCa risk was analyzed using two large population-based case-control studies from the Multiethnic Cohort study and the Australian Risk Factors for Prostate Cancer Study. A combined analysis among 3,131 invasive cases and 3,016 controls revealed minimal differences in the overall distribution of genotypes between cases [34.9% (GG), 48.2% (GA), and 16.9% (AA)] and controls [35.9% (GG), 46.5% (GA), and 17.6% (AA)]. Moreover, no significant association between the G/A870 polymorphism and PCa risk overall or in any ethnic population was observed (Table 1). Combined, these data indicate that the *CCND1* polymorphism is not independently predictive of PCa risk.

Minigene analyses reveal a role for the A allele in cyclin D1b production. The observation that the G/A870 polymorphism is not independently predictive of PCa risk was surprising because it has been hypothesized that the A allele supports *transcript-b* production (14, 15, 53) and that the resultant cyclin D1b protein harbors oncogenic activity (9, 10, 16, 17). To assess the contribution of the G/A870 polymorphism on *transcript-b* production, *CCND1* minigenes were generated. For these studies, the intron 4 sequence containing either the G or A allele was cloned and inserted into the full-length, EGFP-tagged cyclin D1 cDNA (Fig. 4A). Using these minigenes, the ability of G/A870 polymorphism to control *transcript-b* expression was assessed by mRNA analysis (Fig. 4B) in SAOS2 cells that express low levels of both cyclin D1 isoforms. Transfection of the A allele containing minigene resulted in 2.2-fold enhanced *transcript-b* expression when compared with the wild-type G allele minigene ($P < 0.029$). These data are consistent with published observations suggesting that the A allele correlates with *transcript-b* expression (54–57). To assess the effect on isoform production, protein analysis was done (Fig. 4C, *left*). For these studies, cotransfection with H2B-GFP was used as a control for transfection efficiency and evaluated by immunoblot. As shown, vector-transfected SAOS2 cells (*lane 1*) were devoid of detectable cyclin D1a (*top blot*) and cyclin D1b (*middle blot*), consistent with previous reports (10, 12, 17). Introduction of the *CCND1* minigenes resulted in a reciprocal relationship. As shown, a significant increase in cyclin D1a was observed with the G allele minigene (*lanes 2* and *3*, *top blot*) compared with the A allele equivalent. By contrast, the A allele minigene resulted in an approximate 3-fold increase in cyclin D1b (*lanes 2* and *3*, *top* and *middle blots*) compared with the G allele equivalent. Similar results were obtained in MCF-7 cells (Supplementary

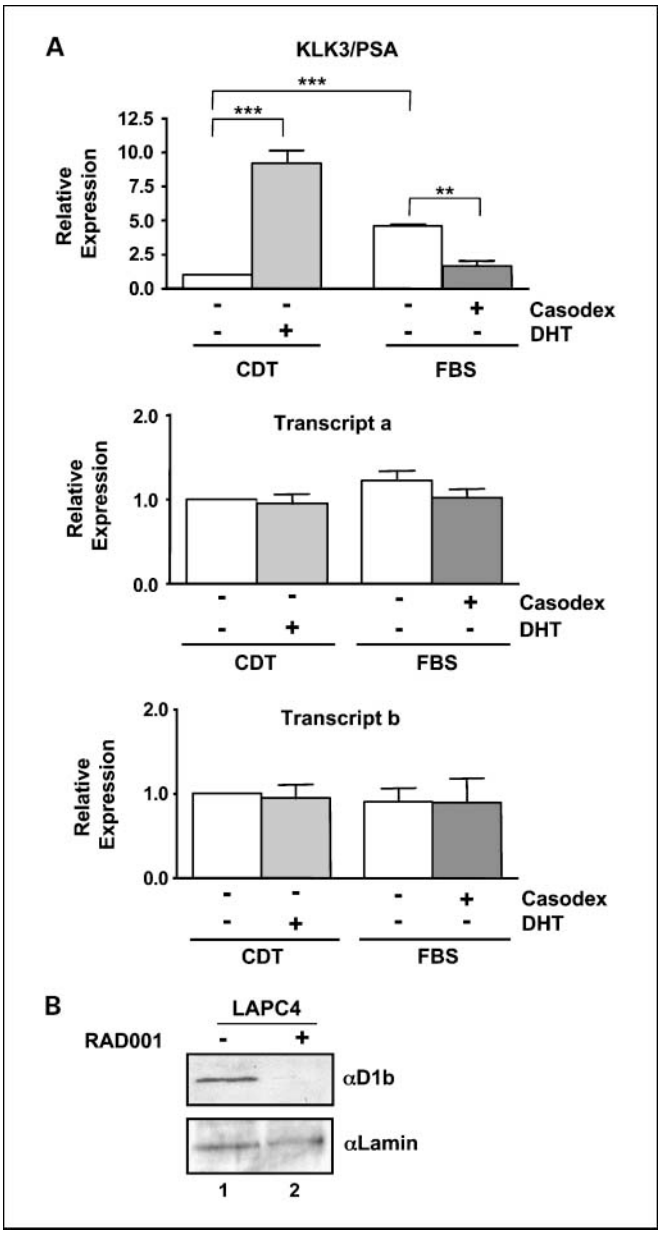


Fig. 3. *Transcript-b* levels are refractory to androgen and AR activity. *A,* to assess AR activity on cyclin D1 *transcript-a* and *transcript-b* levels, LNCaP cells were cultured either in the absence of steroid hormones [charcoal dextran–treated serum (*CDT*)] and stimulated 24 h with (or without) 1 nmol/L dihydrotestosterone (*DHT*), as indicated, or in complete serum [5% fetal bovine serum (*FBS*)] supplemented, where indicated, with 1 μmol/L Casodex. Real-time PCR for relative *KLK3/PSA* (*top*), *transcript-a* (*middle*), and *transcript-b* (*bottom*) is shown. Representative images are shown in Supplementary Fig. S3. *B,* to evaluate the contribution of mTOR-mediated posttranscriptional regulation of cyclin D1b, LAPC4 cells were treated with 10 μmol/L RAD001 for 4 h. Cyclin D1b levels were determined by immunoblot analysis using the cyclin D1b–specific antibody with lamin (control). Representative immunoblot is shown.

Table 1. Association between CCND1 G/A870 (rs603965) genotype and PCa risk

Ethnicity	*n*	Frequency A allele	Genotype							
			GG		GA		AA		Effect per allele	
			n	OR	*n*	OR (95% CI)*	*n*	OR (95% CI)*	OR (95% CI)*	*P* trend
African-Americans										
Controls	647	0.232	374		246		27			
Cases	675	0.235	387	1.0	258	1.01 (0.81-1.27)	30	1.05 (0.61-1.80)	1.02 (0.84-1.23)	0.85
Latinos										
Controls	646	0.424	214		315		117			
Cases	643	0.427	212	1.0	313	1.01 (0.79-1.29)	118	1.02 (0.74-1.41)	1.01 (0.86-1.18)	0.89
Japanese										
Controls	467	0.485	126		229		112			
Cases	457	0.480	121	1.0	233	1.08 (0.79-1.47)	103	0.96 (0.67-1.39)	0.98 (0.81-1.18)	0.86
Native Hawaiians										
Controls	68	0.588	12		32		24			
Cases	71	0.521	14	1.0	40	1.10 (0.44-2.71)	17	0.55 (0.20-1.51)	0.71 (0.43-1.18)	0.18
European Americans										
Controls	449	0.451	134		225		90			
Cases	456	0.478	117	1.0	242	1.24 (0.91-1.68)	97	1.23 (0.84-1.80)	1.12 (0.93-1.35)	0.24
Australians										
Controls	739	0.456	225		354		160			
Cases	829	0.455	241	1.0	422	1.12 (0.89-1.41)	166	0.98 (0.74-1.30)	1.00 (0.87-1.15)	0.99
All groups										
Controls	3,016		1,085		1,401		530			
Cases	3,131		1,092	1.0	1,508	1.00 (0.86-1.17)	531	1.08 (0.96-1.21)	1.01 (0.94-1.09)	0.73

*OR and 95% CI were estimated using unconditional logistic regression adjusted for age and race/country, where necessary.

Fig. S4). These results suggest that the *CCND1* minigenes are a viable means to assess *CCND1* splicing and regulation and show that the A allele can confer a selective advantage for cyclin D1b production.

AA *genotype predicts transcript-b expression in nonneoplastic but not PCa tissue.* Given the propensity for the A allele minigene to confer cyclin D1b production in a heterologous system, the influence of the G/A870 polymorphism on *transcript b* expression in human prostate specimens was determined. First, the *CCND1* genotype was determined by PCR-RFLP from nonneoplastic and tumor specimens after prostatectomy (data not shown). The genotype distribution in nonneoplastic specimens was 50% (GG), 30% (GA), and 20% (AA), and a similar distribution of 45.5% (GG), 31.8% (GA), and 22.7% (AA) was observed in tumor specimens. These data are consistent with analyses in Table 1, which show that the A allele is not overrepresented in PCa.

The relevance of the G/A870 polymorphism on *transcript-a* and *transcript-b* expression was subsequently determined using quantitative RT-PCR from available prostate specimens. As depicted in Fig. 5A, analyses of overall transcript levels as a function of genotype in nonneoplastic tissue revealed a significant (2.3-fold; $P < 0.014$) increase in overall *transcript-b* levels in tissues with the AA genotype (compared with GG). These data are consistent with the minigene analyses and suggest that A870 can influence the alternative splicing event and *transcript-b* production. Parallel analyses in a subset of tissue with sufficient material to also assess *transcript-a* levels suggest that the AA genotype may not significantly alter the *transcript-b*/*transcript-a* ratios in tissue, but analyses of a larger data set will be needed to more fully address this complex question (Supplementary Fig. S5A). Finally, overall *transcript-b* levels were analyzed in tumor tissue from radical prostatectomy (Fig. 5B). In these tissues, the AA genotype may influence the expression of cyclin D1b isoforms but does not predict overall *transcript-b* levels ($P < 0.371$). Together, these data reflect a context-specific influence of the A870 polymorphism, which can predispose for *transcript-b* expression; however, additional events may circumvent or cooperate with A870 to induce cyclin D1b production.

Discussion

It has been previously shown that the cyclin D1b splice variant harbors differential oncogenic activities and likely serves specialized functions in the prostate that promote development and/or progression of cancer (14, 15, 36). Despite these observations, little is known about the factors that control cyclin D1b production. The present study is the first to examine expression profiles for each cyclin D1 isoform and to address the mechanisms of cyclin D1b production in PCa. Consistent with our previous report, a large number of prostatic adenocarcinomas show mislocalization or are devoid of detectable cyclin D1a (31). However, a significant fraction of tumors express cyclin D1b, which showed distinct expression profiles not correlated with detected cyclin D1a. Notably, cyclin D1b was significantly enhanced in PCa compared with nonneoplastic tissue. Thus, the cyclin D1b isoform seems to be specifically induced in a significant fraction of PCa. Functional studies showed that *transcript-b* levels are refractory to androgen or AR activation, but the G/A870 polymorphism is a critical effector of cyclin D1b production. Subsequent analyses in human specimens showed that whereas the A allele predicts for enhanced overall *transcript-b* levels in prostate tissue, G/A870 is not independently predictive of PCa risk. Parallel studies revealed that the requirement of the A allele for enhanced *transcript-b* expression is relieved in tumor tissue.

Together, these data show that the cyclin D1b isoform is predominantly associated with tumorigenesis in PCa, and implicate both the polymorphism and modifiers thereof as effectors of the cyclin D1b oncogenic variant.

Only recently have the cyclin D1 isoforms been examined in human disease, and current observations suggest that cyclin D1b regulation is under stringent, tissue-specific control. First, induction of both isoforms has been documented in primary breast carcinomas (17, 24). These observations are distinct from what was observed in PCa, wherein no significant induction in cyclin D1a was observed in neoplastic disease. In breast cancer models, antiestrogen therapies enhanced cyclin D1b production, whereas cyclin D1a was reduced (17). In contrast, neither cyclin D1 transcript in the current study was influenced by antiandrogen therapy, suggesting that unique distinctions exist between these two hormone-dependent cancers. Second, like the current study, the majority of primary esophageal carcinomas expressed cyclin D1b (9). Subsequent studies revealed esophageal tumor-derived mutations that increased the oncogenic capacity of cyclin D1a in a manner synonymous to cyclin D1b (58). Finally, increased cyclin D1b (not cyclin D1a) has been associated with histologic grade in non–small cell lung carcinoma patients (32). Importantly, cyclin D1b correlated with poor survival and was found to be an independent risk factor for lung cancer development. These collective studies (summarized in Supplementary Fig. S6) highlight the importance of examining cyclin D1b levels and show the need to discern the contributing isoform in tumorigenesis. Moreover, previous studies examining cyclin D1 used reagents that can potentially detect either isoform (13, 59–61). Nonetheless, it is apparent from the present findings that cyclin D1b induction is characteristic of a subset of PCa, and ongoing studies will address the effect of this induction on clinical outcome.

Based on these observations, it is imperative to define the mechanism of cyclin D1b production. In addition to alternative splicing, it is likely that multiple mechanisms exist to regulate cyclin D1b production, including gene activation (17), translational control (30), transcriptional elongation (19), and mRNA stability (62). In breast cancer, both cyclin D1 isoforms are regulated at the transcriptional level through the actions of the estrogen receptor (49, 50). This is in marked contrast to PCa cells, wherein both transcripts were unchanged by manipulation of the AR pathway. By contrast, mTOR-mediated enhancement of translation seems to be required for cyclin D1b production, which has been established for cyclin D1a (30). Thus, current therapies directed at ablation of AR activity are not likely to alter alternative splicing but may assist in preventing translation of *transcript-b*.

Based on these findings, critical questions were addressed with regard to the induction of *transcript-b* and resultant cyclin D1b protein production. The data herein show that the *CCND1* G/A870 polymorphism is a potent effector of this event. Using novel *CCND1* minigenes that harbor a single base change (G or A allele), it was evident that the A allele predisposes for cyclin D1b production in cells. To our knowledge, this is the first report to show that the A allele, in isolation, influences cyclin D1b production. However, a noted caveat is that the minigenes lack most of the 3′-untranslated region and natural polyadenylation site that could potentially contribute to transcript stability (62), which could potentially influence the relative levels of cyclin D1a and cyclin D1b. Nonetheless, analyses of nonneoplastic prostate tissue showed that *transcript-b* levels were enhanced in tissue with the AA genotype, consistent with the minigene analyses and additional tumor types (54–57). However, functional studies show that the G allele can also produce cyclin D1b, supporting the hypothesis that the A allele is not universally required for *transcript-b* or cyclin D1b production.

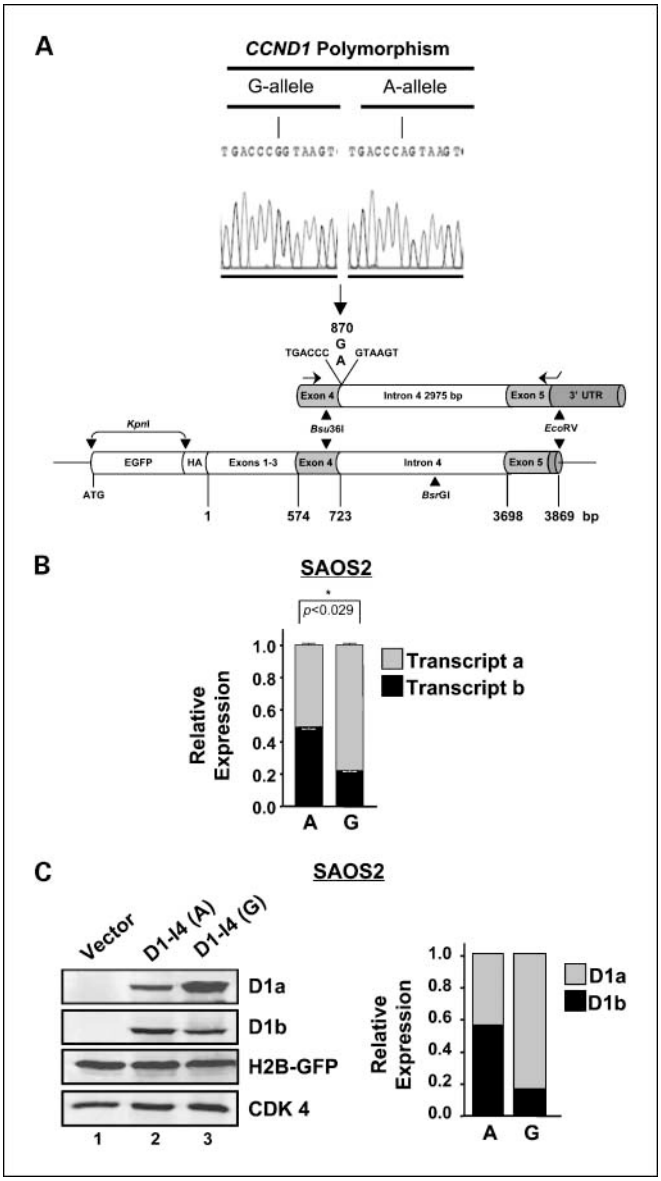


Fig. 4. The A allele predisposes for cyclin D1b production using *CCND1* minigenes. *A,* sequence analysis (*top*) and cloning strategy (*bottom*) surrounding the *CCND1* polymorphism (G/A870) from two independent constructs showing the G and A allele of human cyclin D1. Briefly, a 3.3-kb genomic PCR fragment containing either the G or A allele and the entire intron 4 was digested with *Bsu*36I and *Eco*RV and subcloned into pcDNA3.1 containing a EGFP-tagged and hemagglutinin (*HA*)–tagged human cyclin D1. *B,* quantitative PCR analyses ($n = 4$) of *transcript-a* and *transcript-b* expression in SAOS2 cells (express low levels of cyclin D1) transfected with the A and G allele containing *CCND1* minigenes [D1-I4 (*A*) or D1-I4 (*G*)]. Statistical significance ($P < 0.05$) was determined by Mann-Whitney *U* analysis and is indicated by an asterisk. *C,* left, representative immunoblots for cyclin D1a and cyclin D1b from SAOS2 cells transfected with the A allele (*lane 2*) or G allele (*lane 3*) *CCND1* minigenes and a plasmid encoding H2B-GFP (used as a loading/transfection control); right, quantification of cyclin D1a and cyclin D1b immunoblots, as a function of allele, after correcting for transfection efficiency (transfected H2B-GFP) and loading (endogenous CDK4).

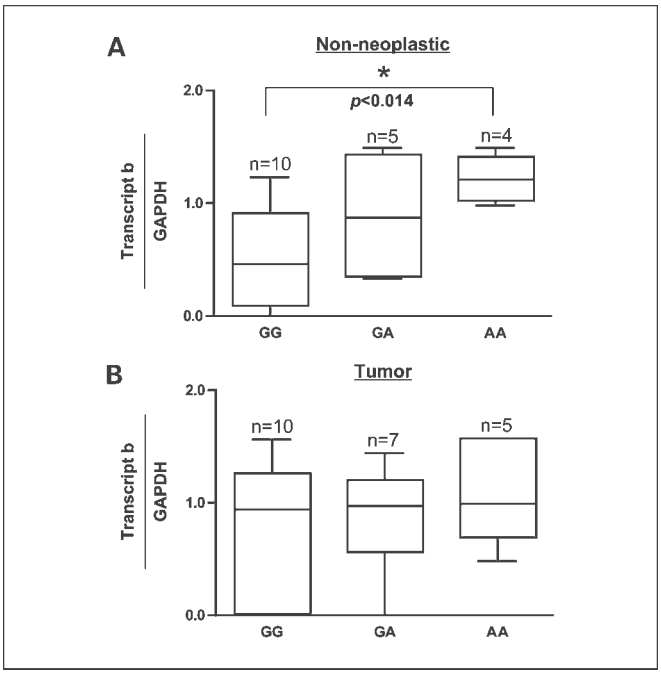


Fig. 5. The AA genotype correlates with overall *transcript-b* levels in a context-dependent manner. *Transcripts-b* levels relative to total mRNA (GAPDH) were determined by quantitative RT-PCR (described in Materials and Methods) and plotted as a function of genotype for nonneoplastic (*A*) and tumor tissue (*B*). The *n*-number is indicated. Statistical significance ($P < 0.05$) was determined by Mann-Whitney *U* analysis and is indicated by an asterisk.

Future studies will be directed at factors that influence the A allele effect and on the importance of the *transcript-b:transcript-a* ratio. Collectively, these data indicate that the A allele predisposes for *transcript-b* and cyclin D1b production in PCa but that additional factors may influence the alternative splicing of cyclin D1.

An association between the A allele and increased PCa risk (63, 64) has been suggested in prior studies of limited size (<300 cases and <300 controls) and composition. Consistent with the supposition that the A allele is not sufficient for *transcript-b* production, two population-based PCa studies (>3,000 cases and >3,000 controls) determined no independent risk association. These analyses had 80% power to detect effects as low as 1.11 and 1.17 for the A allele, assuming a minor allelic frequency of 40% (which is the average minor allelic frequency in the multiethnic sample), and a log-additive or dominant effect on risk, respectively. However, assessment of luteinizing hormone levels in the controls with the AA genotype was modestly elevated (12%, $P = 0.02$) compared with controls with the G allele, whereas other hormones (testosterone, estradiol, sex hormone binding globulin, dehydroepiandrosterone sulfate, androstenedione, androstanediol glucuronide, and prolactin) were not significantly different (data not shown). Nonetheless, the consistent lack of a significant association of the polymorphism to PCa risk, in all populations, further supports that this variant is not an independent marker.

Intriguingly, despite the relevance of the A allele for overall *transcript-b* production in nonneoplastic prostate tissue, this requirement was alleviated in tumor tissue. These observations suggest a provocative hypothesis that tumor-specific alterations may bypass or synergize with the G/A870 polymorphism to enhance total *transcript-b* production. Although the data herein clearly indicate the importance of the polymorphism, several additional factors have been suggested to influence cyclin D1b production. First, recent observations in Ewing's sarcoma cell lines and tumors suggest that chromosomal alterations between the Ewing's sarcoma oncogene and the *ets* family transcription factor (FLI1) result in up-regulation of cyclin D1b through alteration of transcript elongation (18, 19). Interestingly, chromosomal translocations of other *ets* family transcription factors are frequently observed in PCa (e.g., TMPRSS2:ERG; refs. 65, 66). Therefore, it will be of importance to characterize the role of these fusions on cyclin D1b production. Second, loss of Brahma, the core ATPase of the SWI/SNF chromatin remodeling complex, significantly increased *transcript-b* expression in colorectal cells (67). Moreover, Brahma is frequently reduced or lost in human PCa and correlates with enhanced cellular proliferation (68). Finally, recent observations show that AR is alternatively spliced, thus indicating that aberrant splicing of several critical effectors of AR activity and cellular proliferation occur in PCa (69–71). It will be intriguing whether the mechanisms that underlie the alternative splicing events are linked.

In summary, the data herein show that cyclin D1b is specifically enhanced in PCa, thus showing a distinct expression profile from the cyclin D1a isoform. Accordingly, expression of the two isoforms is likely differentially regulated, and unlike breast cancer, the transcription and splicing of *CCND1* (a or b) is independent of hormone. Rather, the A870 polymorphic allele was identified as a significant effector of cyclin D1b production, but additional events in prostate tumorigenesis may alleviate the influence of the A allele. Together, these studies highlight a unique mechanism of cyclin D1b regulation, thereby providing the basis for future studies directed at identifying modifiers of the G/A870 polymorphism and the oncogenic consequence of cyclin D1b in PCa.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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