Cyclin-dependent kinase 5 acts as a critical determinant of AKT-dependent proliferation and regulates differential gene expression by the androgen receptor in prostate cancer cells

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ABSTRACT Contrary to cell cycle–associated cyclin-dependent kinases, CDK5 is best known for its regulation of signaling processes in differentiated cells and its destructive activation in Alzheimer's disease. Recently CDK5 has been implicated in a number of different cancers, but how it is able to stimulate cancer-related signaling pathways remains enigmatic. Our goal was to study the cancer-promoting mechanisms of CDK5 in prostate cancer. We observed that CDK5 is necessary for proliferation of several prostate cancer cell lines. Correspondingly, there was considerable growth promotion when CDK5 was overexpressed. When examining the reasons for the altered proliferation effects, we observed that CDK5 phosphorylates S308 on the androgen receptor (AR), resulting in its stabilization and differential expression of AR target genes including several growth-priming transcription factors. However, the amplified cell growth was found to be separated from AR signaling, further corroborated by CDK5-dependent proliferation of AR null cells. Instead, we found that the key growth-promoting effect was due to specific CDK5-mediated AKT activation. Down-regulation of CDK5 repressed AKT phosphorylation by altering its intracellular localization, immediately followed by prominent cell cycle inhibition. Taken together, these results suggest that CDK5 acts as a crucial signaling hub in prostate cancer cells by controlling androgen responses through AR, maintaining and accelerating cell proliferation through AKT activation, and releasing cell cycle breaks.

INTRODUCTION

Many essential signaling pathways have been associated with prostate cancer, including alterations in growth-promoting pathways (such as phosphatase and tensin homologue [PTEN]-AKT), p53-controlled cell cycle checkpoints, and androgen receptor (AR) signaling (Eastham *et al.*, 1995; Visakorpi *et al.*, 1995; Whang *et al.*, 1998; Taplin *et al.*, 1999; Yoshimoto *et al.*, 2006). Although most localized prostate tumors respond well to prostatectomy and androgen deprivation therapy treatment, 10–20% of patients develop aggressive carcinoma to which there is currently no therapy (Jemal *et al.*, 2003). At best, clinical treatments extend the disease-free survival by months only, underscoring the acute demand for novel drugs in treating castration-resistant prostate cancer. Treatment of castration-resistant

prostate cancer is made especially difficult by the complex cross-talk and compensation occurring between multiple oncogenic signaling pathways.

Cyclin-dependent kinase 5 (CDK5) has many characteristics that distinguish it from other members of the CDK family. Although CDK5 is widely expressed in various tissues, its known actions are mainly restricted to highly differentiated cell types—mainly neurons, but also podocytes and muscle stem cells (Liebl *et al.*, 2011; Cheung and Ip, 2012). Stringent regulation of CDK5 is crucial, as its deregulation is strongly linked to Alzheimer's disease through the hyperphosphorylation of cytoskeletal substrates (Cheung and Ip, 2012). In addition, CDK5 is required for muscle differentiation, a process in which it phosphorylates and bidirectionally interacts with the cytoskeletal intermediate filament protein nestin (Sahlgren *et al.*, 2003; Pallari *et al.*, 2011). CDK5 differs from other members of the Cdk family in that, instead of cyclins, it is primarily activated by the non-cyclin activator p35. Studies that concern the function of CDK5 in regulation of the cell cycle have mainly been restricted to neuronal systems (Hamdane *et al.*, 2005; Zhang *et al.*, 2010). Recent studies have suggested, however, that CDK5 protein levels oscillate during the cell cycle (Zhang *et al.*, 2012; Nagano *et al.*, 2013), providing a novel aspect of CDK5 regulation. CDK5 activity is also recognized to contribute to other pathological conditions, including diabetes (Choi *et al.*, 2010) and inflammation (Pareek *et al.*, 2010; Berberich *et al.*, 2011), and it has been shown to be overexpressed in many cancers (Eggers *et al.*, 2011; Levacque *et al.*, 2012; Liang *et al.*, 2013).

Although a number of studies have associated CDK5 with prostate cancer, the various roles suggested for CDK5 appear somewhat contradictory. First, CDK5 has been shown to promote prostate cancer cell migration and invasion (Strock *et al.*, 2006), an observation that has been separately documented in other cancer models both in vitro and in vivo (Eggers *et al.*, 2011; Demelash *et al.*, 2012; Liang *et al.*, 2013). In addition, CDK5 was shown to promote AR phosphorylation (S81) in LNCaP cells (Hsu *et al.*, 2011), implying that CDK5 may promote tumor formation through the androgen pathway. These results are, however, contradicted by another report in which CDK5 was not found to induce S81 phosphorylation (Gordon *et al.*, 2010), whereas CDK1 and CDK9 seemingly did (Chen *et al.*, 2006, 2012b; Gordon *et al.*, 2010). On the contrary, digoxin- and retinoic acid–induced activation of CDK5 triggered apoptosis in prostate cancer cells (Lin *et al.*, 2004; Chen *et al.*, 2012a), which in turn suggests that CDK5 under some circumstances may act as a proapoptotic kinase. These results suggest that CDK5 affects several processes in prostate cancer development and that its modus operandi is likely to be highly context dependent and adaptable to the environment, as illustrated in various nonprostate cancer models. The multifaceted and sometimes incoherent functions proposed for CDK5 in prostate cancer cells are challenging for drug development strategies and the interpretation of preclinical and clinical data. Taken together, a number of studies have linked CDK5 to the progression of prostate cancer, but the underlying reasons and consequences are not clear. Thus we wanted to assess in detail how CDK5 may contribute to the behavior of prostate cancer cells and the underlying causalities.

RESULTS
CDK5 is essential for prostate cancer cell proliferation independently of AR
First we wanted to establish the extent to which CDK5 itself regulates the proliferation of different prostate cancer cells that are distinguished by distinct molecular features. To investigate how the absence of CDK5 affects prostate cancer cell behavior, we transfected androgen-dependent LNCaP cells with negative control scrambled small interfering RNA (siRNA; Scr) or a pool of CDK5-specific siRNA, yielding efficient down-regulation of CDK5 (Figure 1A). In live-cell imaging, cell populations with down-regulated CDK5 displayed a striking reduction in cell growth as compared with control cells (Figure 1A and Supplemental Videos S1 and S2). Quantification of cell confluence further highlighted the obvious cell growth retardation (Figure 1A), which was additionally confirmed with cell counting of Scr and CDK5 siRNA–transfected cells (Figure 1B). Next we sought to investigate whether LNCaP proliferation could be stimulated by expression of exogenous WT-CDK5, the transfection success being confirmed by Western blotting (Figure 1C). Measured as before, CDK5 transfection induced an increase in cell proliferation (Figure 1C and Supplemental Videos S3 and S4), which was confirmed by overexpression of CDK5 and manual counting of the cells 48 h after transfection (Figure 1D). We confirmed our observations with another prostate cancer cell line, 22Rv1, which is androgen responsive rather than strictly androgen dependent. We obtained an equally prominent response with this cell line (Supplemental Figure S1 and Supplemental Videos S5–S8).

To assess the previously suggested role of AR signaling in CDK5-mediated prostate cancer cell proliferation, we repeated the proliferation studies in the androgen-independent PC-3 prostate cancer cells. To our surprise, the AR-null prostate cancer cell line PC-3 behaved exactly the same as the androgen-dependent cells, displaying strong inhibition when CDK5 was down-regulated with CDK5-specific siRNA (Figure 1E), implying that CDK5 regulates prostate cancer cell proliferation independently of AR. Our results were corroborated in both LNCaP and PC-3 cells, with CDK5 inhibition obtained by low concentrations of roscovitine (10 μM, with previously documented minimal effects on other CDKs; Supplemental Figure S2, A–C), which is a widely used CDK5 inhibitor (Strock *et al.*, 2006; Hsu *et al.*, 2011; Chen *et al.*, 2012a). By using different types of prostate cancer cells, our results demonstrate that CDK5 is a key regulator of the proliferation of a broad variety of prostate cancer cells. This effect occurred regardless of the AR status of the cells, suggesting that the proliferation effects of CDK5 can be separated from its effects on AR regulation and signaling.

Assessing CDK5-dependent and -independent AR posttranslational modifications
To understand the apparent contradictions of our work as opposed to that of others (Lin *et al.*, 2004; Hsu *et al.*, 2011), we sought to address in detail the role of CDK5 in regulation of AR. To begin with, we observed a physical interaction between CDK5 and AR, as CDK5 could be coimmunoprecipitated with AR, and CDK5 was efficient in phosphorylating AR in vitro (Figure 2A). Furthermore, CDK5 down-regulation or exogenous CDK5 overexpression resulted in AR protein depletion and accumulation, respectively (Figure 2A), which is in line with previously suggested effects of CDK5 (Hsu *et al.*, 2011). Furthermore, we sought to examine in detail which AR phosphorylation sites would be specific for CDK5 and chose sensitive mass spectrometry as an approach. VCaP prostate cancer cells, which have abundant AR, and calyculin A–treated, AR-overexpressing HEK293 cells were used as reference for posttranslational modification (PTM) identification, for which AR was immunoprecipitated, subjected to in-gel digestion and TiO$_2$ affinity chromatography, and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). For analysis of CDK5-specific phosphorylation sites, AR was phosphorylated in vitro by recombinant CDK5/p25 in the
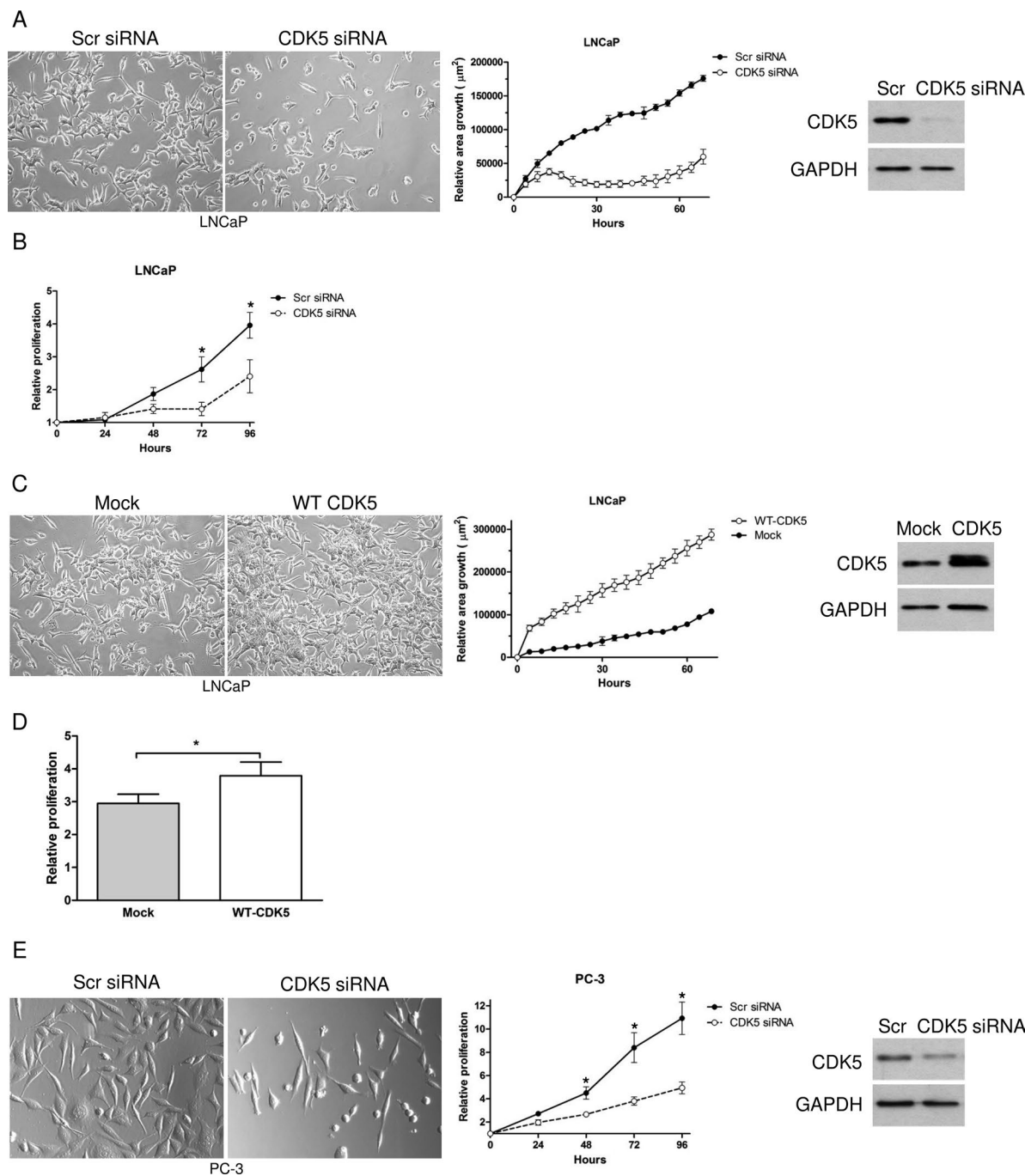
FIGURE 1: Down-regulation of CDK5 protein disturbs prostate cancer cell proliferation independently of androgen signaling. (A) Androgen-dependent LNCaP cells were transfected with indicated siRNA overnight, and three positions from a transfected well were imaged with Cell-IQ (CM Technologies) live-cell imaging platform (Supplemental Videos S1 and S2). From the video material, the cell confluency in each position was automatically quantified with Cell IQ Analyser software (CM Technologies) at fixed settings, and the relative cell confluency (relative area growth) is plotted in the graph over time. Images represent the endpoint of the experiment. A prominent change in cell morphology accompanied the loss of CDK5. Transfection efficiency was in the end validated with Western blotting. (B) LNCaP cells were transfected with indicated siRNA, and the cell population size was calculated manually at each time point. Results are plotted as relative proliferation and compared with the 0-h time point, confirming that the absence of CDK5 impairs proliferation of LNCaP cells. (C) LNCaP cells were transfected with empty plasmid (mock) or WT-CDK5 overnight, imaged with Cell-IQ (Supplemental Videos S3 and S4), and analyzed with similar settings as before. Expression of WT-CDK5 is detected as a double band in Western blots. (D) LNCaP cells were transfected as described and counted after 48 h. Cell counts were normalized to the starting time point. Indeed, cell population size was promoted after CDK5 overexpression (WT-CDK5) compared with empty vector control (mock). (E) CDK5 knockdown in androgen-independent (AR deficient) PC-3 cells causes rounding up of cells similarly to LNCaP cells. Cell counting experiments reveal that CDK5 siRNA keeps the PC-3 cell population distinctly smaller than with negative control cells (Scr). Results are plotted as mean ± SEM, *$p < 0.05$, Student's t test, $n \geq 3$).
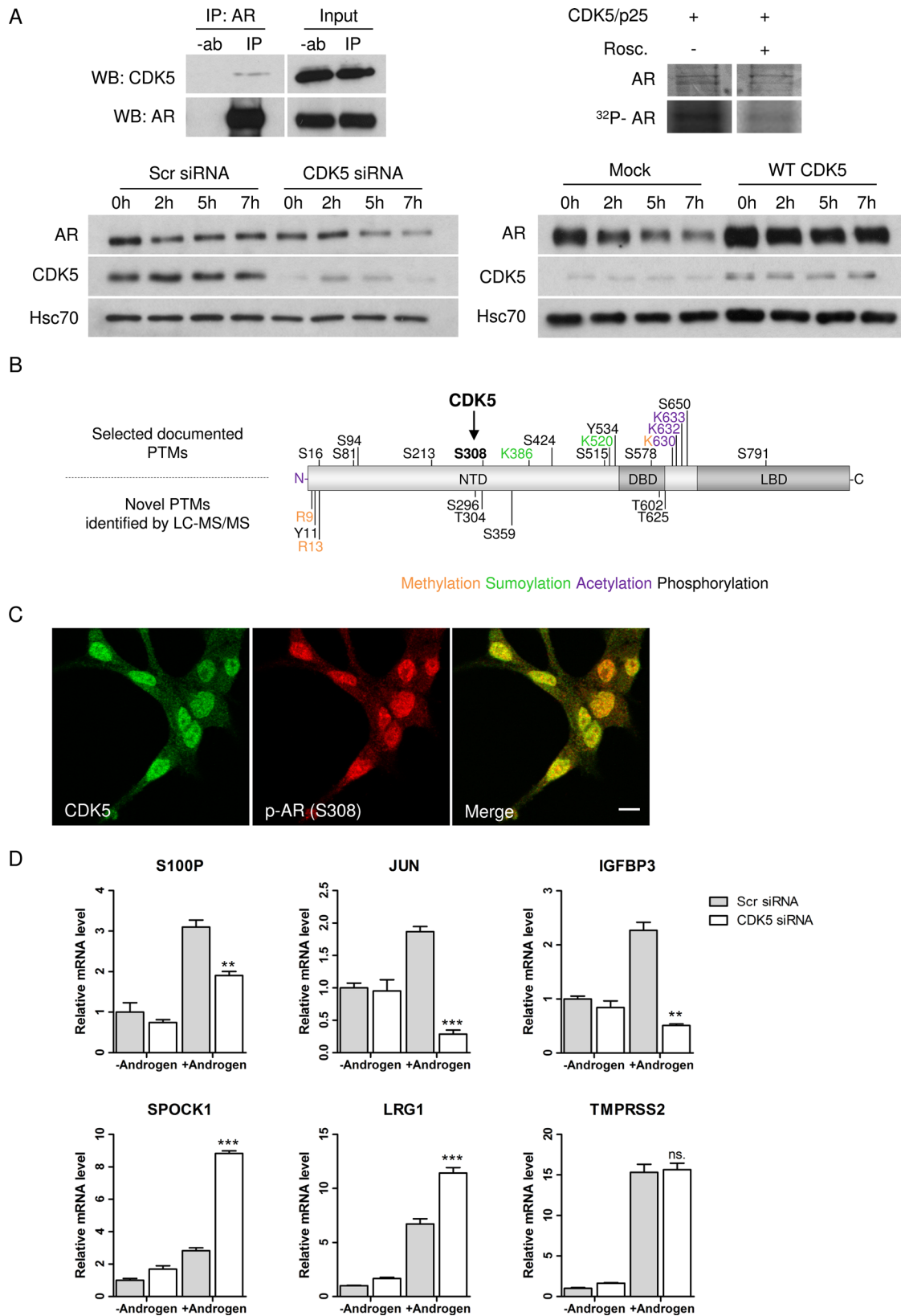
FIGURE 2: Intricate regulation of androgen receptor phosphorylation and activity by CDK5. (A) The interaction of CDK5 and AR in LNCaP cells was confirmed through immunoprecipitation of AR and WB against CDK5 (top left). Furthermore, CDK5/p25 was verified to phosphorylate AR in vitro, as detected by ^{32}P incorporation into AR, and the phosphorylation was effectively reduced by CDK5 inhibition with roscovitine (top right). In addition, CDK5 siRNA destabilized AR (bottom left), whereas overexpression of WT-CDK5 promoted AR stability (bottom right) after cycloheximide treatment, which inhibits protein synthesis. (B) Both general and CDK5-dependent AR PTMs were analyzed through mass

| Amino acids | Peptide sequence[a] | Modification | CDK5 in vitro phosphorylation[b,c] | | In vivo |
			CDK5/p25	CDK5/p25 + roscovitine	CDK5 siRNA[b,d]
10–17	VYPrPPSK	R13 (methyl)	—	—	—
	VYPrPPSK	R13 (dimethyl)	—	—	—
84–99	QQQQQQGEDGsPQAHR	S94 (phospho)	—	—	—
300–311	STEDTAEYsPFK	S308 (phospho)	Induced	Suppressed[e]	Suppressed
511–520	VPYPsPTcVK	S515 (phospho)	—	—	—
639–658	LQEEGEASSTTsPTEETTQK	S650 (phospho)	—	—	—

[a]Lowercase letters represent modified amino acid residues. c, carbamidomethylated cysteine.
[b]For phosphopeptides, quantitative results consistent before and after TiO_2 phosphopeptide enrichment were taken into consideration.
[c]AR immunoprecipitated from VCaP cells used.
[d]LNCaP cells used.
[e]Compared to CDK5-induced phosphopeptides.

TABLE 1: LC-MS/MS analysis of AR modifications regulated by CDK5.

presence of dimethyl sulfoxide (DMSO) or roscovitine and processed as before. One AR phosphorylation site (S308) was found to be specifically induced by CDK5/p25 (Table 1 and Supplemental Figure S3A). The initial in vitro analysis was confirmed in vivo by transfection of LNCaP cells with CDK5 or Scr siRNA and immunoprecipitation of AR from transfected cells, followed by LC-MS/MS analysis of the PTMs that were affected in vivo. The results show reduced AR S308 phosphorylation in CDK5 siRNA–treated cells (Table 1 and Supplemental Figure S3B), showing that CDK5 is required for AR S308 phosphorylation also in vivo. In addition, LNCaP cells were coimmunolabeled with antibodies detecting phosphorylated AR (S308) and CDK5 (Figure 2C), showing that the S308-phosphorylated pool of AR does overlap with CDK5. These results support the fact that CDK5 is able to phosphorylate AR on multiple sites, most prominently on S308 according to our mass spectrometry data. S81 phosphorylation is impossible to detect in our mass spectrometry data due to the large size of the peptide, which hampers the phosphoproteomic analysis of this peptide.

Of interest, the phosphorylation of S308 by the cyclin-D3/CDK11p58-complex has been associated with transcriptional repression of AR (Zong et al., 2007), opening the possibility that different combinations of AR-PTMs may yield specific effects on AR transactivation. To further assess the role of CDK5 in the functional regulation of AR, we analyzed the mRNA expression levels of a broad range of AR target genes by reverse transcription-quantitative PCR (RT-qPCR). Intriguingly, CDK5 was shown to have target-gene-selective effects on AR, as seen by both the promotion and suppression of specific AR-target genes in CDK5-down-regulated LNCaP cells (Figure 2D and Supplemental Figure S4), whereas some genes (TMPRSS2, FKBP5, and AR itself) remained unaffected. Although our observations showing CDK5-dependent effects regardless of the AR status of the cells demonstrated that the proliferation effects of CDK5 can be separated from its effects on AR, some of the genes affected by altered CDK5 levels will definitively be consequential for AR-dependent cell growth. In this respect, the repression of the genes coding for the growth-promoting transcription factors c-Jun (Figure 2D) and c-Myc (Supplemental Figure S4) is likely to be reflected in the retarded cell proliferation of the androgen-stimulated LNCaP cells in response to CDK5 siRNA. Because many of the AR targets analyzed here (such as S100P, IGFBP3, and SPOCK1) are involved in prostate carcinogenesis, it is clear that CDK5 does play a role in priming androgen responses to cell growth with consequences for especially the initial, AR-dependent growth of prostate cancer. Because AR PTMs are crucial for the fine-tuning of AR transactivation and specific target-gene expression (Rytinki et al., 2012), we hypothesize that the cascade of PTMs initiated by CDK5 operates well beyond the previously indicated receptor stabilization (Hsu et al., 2011) and that it instead guides differential AR activation, yielding both positive and negative effects on AR-specific gene expression. Similar to our observations, the CDK5-mediated phosphorylation of glucocorticoid receptor affects its activity in a target-gene-selective manner in neuronal systems (Kino et al., 2007).

Taken together, our cellular studies showed that CDK5 is involved in maintaining and accelerating cell growth in an AR-independent manner (Figure 1). The PTM analysis again indicates that the CDK5-specific PTM combination has a prominent effect on AR target-gene preference, which in turn will be consequential for AR functions in vivo (Figure 2), although it does not seem be essential for CDK5-mediated prostate cancer cell proliferation per se. Thus we believe that in a situation in which both proteins are present, CDK5 is important in the adjustment of AR activation

spectrometry. The model summarizes all PTM peptides identified by LC-MS/MS, highlighting identification of novel AR PTMs. S308 was found to be the major CDK5 phosphorylation site. DBD, AR DNA-binding domain; LDB, AR ligand-binding domain; NTD, AR N-terminal domain. (C) LNCaP cells were colabeled with CDK5 (green) and p-AR (S308; red) specific antibodies and analyzed by confocal microscopy, demonstrating partial overlap of the proteins and the existence of p-AR (S308) in LNCaP cells. Scale bar, 10 µM. (D) RT-qPCR analysis was conducted on RNA isolated from LNCaP cells that were transfected with either Scr or CDK5 siRNA and thereafter androgen treated for 16 h to induce activation of AR. Hormone-starved cells were used as control. The relative mRNA levels of AR-target genes from experimental triplicates are plotted as mean ± SEM; n.s., no significance; **$p < 0.01$, ***$p < 0.005$. Student's t test (Scr vs. CDK5 siRNA with androgen stimulation), $n = 3$. Results reveal a complex CDK5-dependent guidance of AR target-gene transcription.
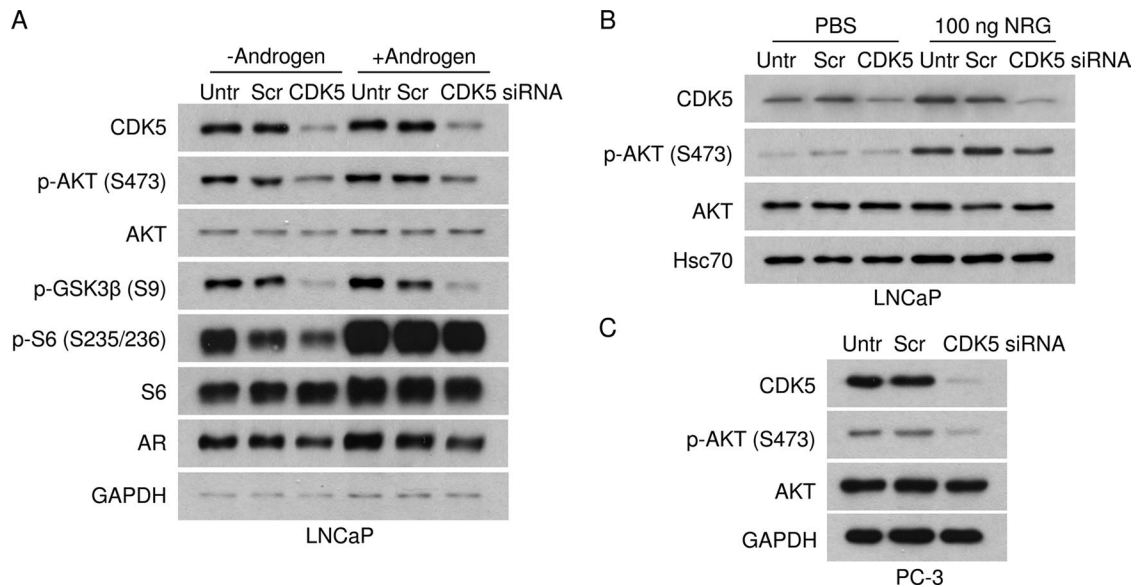
FIGURE 3: CDK5 acts upstream of the growth-promoting AKT signaling pathway. (A) LNCaP cells were transfected and either androgen starved (–androgen) or androgen stimulated (+androgen). CDK5 down-regulation decreased the expression of phosphorylated AKT (S473) regardless of the hormonal status of the cells. CDK5 siRNA also suppressed phosphorylated S6 (S235/236) compared with nonphosphorylated protein, as well as p-GSK3β (S9) expression. GAPDH was used as loading control. (B) LNCaP cells were stimulated with NRG or treated with vehicle (PBS). NRG-treated cells showed an increase in p-AKT compared with PBS-treated control cells. In the absence of CDK5, NRG-induced AKT phosphorylation is lower than in comparable controls. Hsc70 confirms equal loading. (C) CDK5-down-regulated PC-3 cells were analyzed for p-AKT expression and compared with Scr or untransfected controls.

and specificity, but this level of regulation is not necessary for CDK5-mediated growth promotion in AR-null prostate cancer cells.

It is interesting to note that we examined other plausible AR PTMs while analyzing CDK5-specific phosphorylation sites, and, to our surprise, we could detect multiple novel AR phosphorylation sites and two yet-uncharacterized methylation sites in the AR N-terminus, as well as N-terminal acetylation (Figure 2B and Supplemental Table S1). Because determining the roles of CDK5-specific PTMs was of prime interest here, characterizing the functions of these novel PTMs was well beyond the scope of this study. However, they are of great general interest, as PTM-based regulation has already been established as a prime factor determining AR functions and activation in vivo.

CDK5 acts upstream of the growth-promoting AKT pathway

Apart from AR signaling, AKT signaling has been reported as a crucial growth-promoting pathway in prostate cancer cells. Prompted by reports suggesting that CDK5 could act upstream of this critical kinase in neuronal cells (Li *et al.*, 2003), we asked whether AKT could be affected by the lack of CDK5 in prostate cancer cells. Serine 473 phosphorylation of AKT is required for activation of the kinase, and phosphorylation of this particular site is known to correlate with prostate cancer progression in patients (Ayala *et al.*, 2004; Kreisberg *et al.*, 2004). Because LNCaP cell proliferation is highly dependent on AR signaling, we conducted experiments in both androgen-starved and androgen-stimulated conditions. We observed that CDK5 down-regulation repressed AKT phosphorylation (Ser-473) without affecting total AKT protein levels in both androgen-starved and androgen-stimulated LNCaP cells (Figure 3A), suggesting that CDK5 maintains high AKT kinase activity in prostate cancer cells. To obtain further support for this assumption, we studied the responses of the well-established downstream targets of AKT, glycogen syn-

thase kinase 3β (GSK3β) and S6 ribosomal protein (S6), downstream of the growth-promoting mammalian target of rapamycin (mTOR) complex. In support of the assumed link between CDK5 and AKT, phosphorylation of the inhibitory GSK3β Ser9 was suppressed in CDK5-down-regulated samples, denoting activation of GSK3β. Phosphorylation of the regulatory Ser-235/236 S6 sites was also reduced dramatically in hormone-starved LNCaP cells lacking CDK5. Of interest, although the AR-stimulated cells showed strong amplification of S6 phosphorylation, CDK5 down-regulation was less efficient in inhibiting this effect under androgen-stimulated conditions. There is, hence, intriguing cross-talk between AKT and AR signaling in prostate cancer cells, where CDK5 seems to have the capacity to specifically promote AKT signaling, whereas the mTOR pathway in AR-stimulated cells seems also to be amplified by other means.

Furthermore, by treating LNCaP cells with the ErbB-receptor ligand neuregulin-1 (NRG; 100 ng/ml) overnight, we induced hyperactivation of the AKT signaling pathway, as shown by a significant increase in AKT Ser-473 phosphorylation (Figure 3B). In agreement with the results in unstimulated cells, siRNA-mediated down-regulation of CDK5 in NRG-stimulated cells was efficient in inhibiting Ser-473 phosphorylation (Figure 3B). Thus depletion of CDK5 attenuates receptor-mediated AKT activation. Of importance, AKT Ser-473 phosphorylation was significantly reduced in the absence of CDK5 also in AR-negative PC-3 cells (Figure 3C).

To examine the mechanism underlying the suppression of AKT activity observed after CDK5 down-regulation, we conducted coimmunoprecipitation experiments to investigate the possible direct interaction between the two proteins. Indeed, results demonstrated that endogenous CDK5 and AKT can be pulled down together (Figure 4A). The somewhat weak signal is likely to reflect that only a part of the molecules are directly interacting, as the direct interplay between the two proteins is likely to be transient, and occurring at particular cellular locations. In addition, we found that CDK5 and
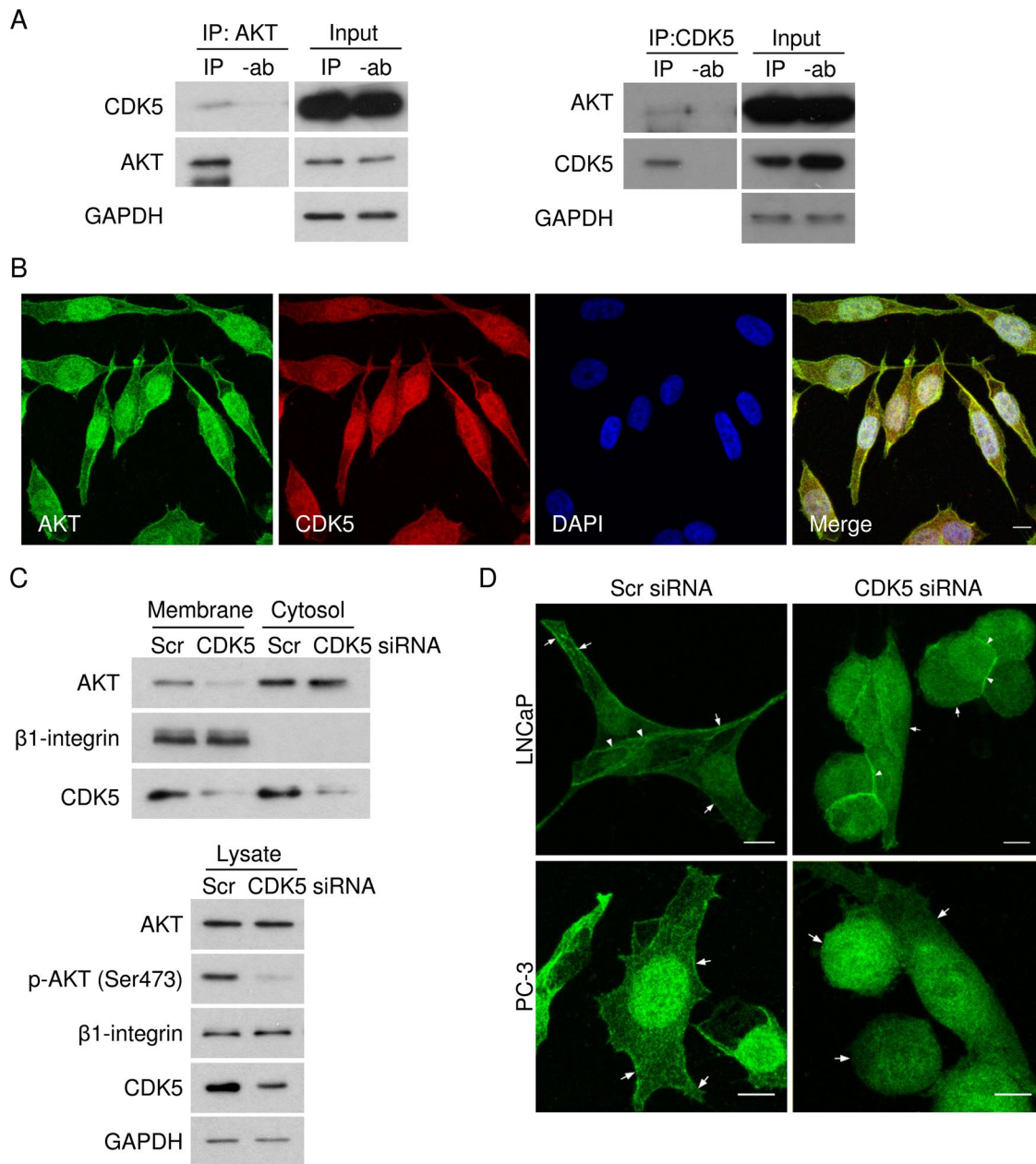
FIGURE 4: CDK5 interacts with AKT and regulates its membrane translocation. (A) Coimmunoprecipitation was performed on endogenous proteins from LNCaP cells. Left, AKT was immunoprecipitated, and samples were blotted against CDK5 to detect interaction of the two proteins. Right, CDK5 was immunoprecipitated from LNCaP lysates and probed against AKT (–ab, no-antibody control). (B) CDK5 and AKT colabeling reveals that both proteins localize to similar subcellular compartments. Similar to AKT, CDK5 can be distinguished at the plasma membrane and in the cytosol, in addition to the strong nuclear signal. (C) LNCaP cells were transfected with indicated siRNA and cells subjected to intracellular fractionation, where membrane fractions were separated from the cytosol by centrifugation. Samples were analyzed by Western blotting for AKT, revealing that CDK5-silenced samples were depleted in membrane-associated AKT. β1-Integrin was used as a marker for membrane fractions. Cell lysates demonstrate efficient down-regulation of CDK5. As expected, p-AKT (S473) was suppressed in the absence of CDK5, indicating inactivation of the AKT kinase. (D) AKT intracellular distribution was further examined by immunolabeling of AKT in Scr and CDK5 siRNA–transfected LNCaP and PC-3 cells. Cells were labeled with AKT-specific antibody and analyzed by confocal microscopy. In control cells, AKT (green) localized to cell membranes, as well as to cytoplasm and nucleus. In CDK5 siRNA–transfected cells, AKT membrane localization was altered, and distinct membrane labeling was limited to cell contact sites in LNCaP cells, whereas it remained diffuse in CDK5 down-regulated PC-3 cells. Cell membranes are marked with arrows, cell–cell contact sites with arrowheads. Scale bars, 10 μM.

AKT had similar localization pattern in PC-3 cells (Figure 4B), showing that they are present in same cellular compartments, including the cell membrane. Thus it is possible that CDK5 would affect AKT activation by directing its localization because, in response to growth factors, AKT activation through phosphorylation occurs specifically at the cell membrane. To test how the absence of CDK5 affects

AKT localization, which is crucial for its activation, we conducted a cellular fractionation assay in LNCaP cells transfected with Scr or CDK5 siRNA. Whereas AKT protein levels were constant in the cytoplasm between treatments, the cell membrane fraction in CDK5-down-regulated samples revealed a significant reduction in membrane-associated AKT (Figure 4C). To approach the CDK5-mediated AKT localization through microscopy, we transfected LNCaP and PC-3 cells with siRNA and immunolabeled them with AKT antibody. In control cells, AKT localized clearly at the cell membrane (Figure 4D). In addition, AKT could be detected in the cytoplasm and nuclei of the cells. In contrast, in LNCaP cells with siRNA-down-regulated CDK5, AKT could not be seen at the cell membrane (apart from a small pool at cell–cell contacts), implying that AKT activation does not occur normally. Instead, AKT was present mainly in the cytoplasm and nuclei of CDK5-depleted cells. In CDK5-depleted PC-3 cells, AKT-membrane localization was nearly undetectable. Inhibition of CDK5 with roscovitine in both cell types revealed a similar shift of AKT localization by microscopy (Supplemental Figure S2D). Hence we conclude that CDK5 is required to recruit AKT to cell membrane and activation of AKT at the membrane. These effects were shown to be specific for AKT, as β-catenin, which, upon its activation, shows a similar activation-dependent compartmentalization as AKT, did not show any CDK5-dependent changes in its membrane localization even though its protein levels are affected by CDK5 as a consequence of GSK3β activation (Supplemental Figure S5).

CDK5 down-regulation interferes with LNCaP cell cycle by up-regulation of the p21 cell cycle inhibitor

It is well established that p21 (CIP1/WAF1) is a protein that is affected by changes in AKT signaling and induces efficient G0/G1 cell cycle arrest through the inhibition of cyclin-CDK complexes. Active AKT inhibits p53-p21 signaling through direct phosphorylation of the MDM2 ubiquitin ligase, the activity of which targets p53 for proteasomal degradation (Zhou et al., 2001). Given the compelling effect of CDK5 siRNA on cell growth and AKT signaling, we sought to determine whether these effects were mediated by alterations in cell cycle inhibitor expression in LNCaP cells. Indeed, CDK5 siRNA caused a dramatic increase in the expression of both p53 and p21 proteins (Figure 5A), with and without androgen, demonstrating that CDK5 affects prostate cancer cell cycle inhibition independently of AR stimulation. The mRNA expression levels of p21 followed protein levels, whereas p53 seems to be mainly regulated at the protein levels (Figure 5B). Again, androgen stimulation seemed to slightly counteract the growth-inhibitory effects of CDK5 through complex cross-talk between signaling pathways in the androgen-dependent cells.

On induction, p21 is stabilized and executes its cell cycle–inhibitory functions in the nucleus. To analyze this potential effect, we immunolabeled LNCaP cells with p21 antibody and collected images with confocal microscopy. Images reveal that p21 is indeed highly up-regulated in the absence of CDK5 and localizes mainly to the nucleus (Figure 5C and Supplemental Figure S2E). DNA fragmentation could not be detected when inspecting the 4′,6-diamidino-2-phenylindole (DAPI)–labeled nuclei even in highly p21-positive cells, suggesting that the cells undergo cell cycle arrest and do not die spontaneously. We could also validate the observed effects on both AKT and p21 signaling in 22Rv1 (AR positive but androgen independent) prostate cancer cells (Supplemental Figure S6A), highlighting the generality of the phenomenon. Thus the suppression of the growth-promoting pathways AKT and AR by CDK5 down-regulation in LNCaP cells culminates in cell cycle arrest and, therefore, retarded proliferation.

Because CDK5 is an acknowledged player in apoptotic signaling and a few reports suggest that CDK5 may also be involved in induced prostate cancer cell death (Lin et al., 2004; Chen et al., 2012a), we sought to investigate apoptosis in LNCaP cells lacking CDK5. We could not detect increased cell death through Western blotting or DNA fragmentation measured with cell sorting (Figure 5D), showing that the reduced cell proliferation demonstrated in Figure 1 is a result of growth inhibition rather than accelerated cell death. Because the cells lacking CDK5 displayed an altered morphology without undergoing apoptosis per se, we analyzed cytoskeletal organization of transfected LNCaP cells by labeling of F-actin using phalloidin. Indeed, the actin cytoskeleton was markedly altered in the absence of CDK5 (Figure 5E), explaining the morphological alterations and decreased motility of the cells, phenomena that were especially conspicuous in the live-cell imaging (Supplemental Videos S1 and 2). Although CDK5 has been associated with induced prostate cancer apoptosis before (Lin et al., 2004), this occurs under specific stimuli and is not comparable to the steady-state growth of prostate cancer cells in our experimental setup. In this way, our results regarding the unchanged apoptosis are not surprising but demonstrate that the effect of CDK5 on LNCaP cell population size is not due to increased cell death but is instead a consequence of inhibited proliferation.

The proliferation defect caused by the absence of CDK5 is AKT dependent

VCaP prostate cancer cells have features that differentiate them from the other prostate cancer cell lines used; they contain AR amplification and in addition intact PTEN, leading to extremely low AKT activity and dependence (Ha et al., 2011). The molecular characteristics of the used cell lines are summarized in Supplemental Table S2. Supported by other reports, we could not detect any phosphorylated AKT (S473) in VCaP cell lysates (Figure 6A). Of interest, CDK5 down-regulation did not affect p21 expression or proliferation of VCaP cells (Figure 6, A and B), implying the possibility that prostate cancer cells that are not dependent on AKT activity are not sensitive to CDK5 down-regulation either. Of importance, CDK5 knockdown did not affect the appearance or any of the signaling pathways studied in noncancer RWPE-1 prostate epithelial cells or HEK293 cells (Supplemental Figure S6, B and C), demonstrating the specificity of the signaling pathway for cancerous growth promotion.

Finally, we sought to employ rescue experiments to confirm the AKT specificity in the effects obtained by CDK5 inhibition. To this end, we overexpressed constitutively active (CA) green fluorescent protein (GFP)–tagged AKT (Peuhu et al., 2010) in LNCaP cells that had been subjected to CDK5 down-regulation by siRNA. As control, we used CDK5-depleted cells transfected with GFP only. Western blotting (Figure 6C) confirmed transfection (the down-regulation of CDK5 under these coexpression conditions was for some reason not as efficient as in the other experiments). When LNCaP cells were counted 72 h after transfection, we observed that CA-AKT induces a slight increase LNCaP cell count (Figure 6C). The modest effect is likely to be explained by the fact that these cells have a high AKT activity to begin with and by the somewhat weak transfection efficiency. When it comes to CDK5-depleted cells, we could indeed observe that CA-AKT expression efficiently rescues the CDK5-mediated growth inhibition, which was less prominent than in the other experiments, most likely due to the less efficient down-regulation under these particular conditions.

With these results, we conclude that CDK5 is a critical kinase to maintain the AKT activity on which prostate cancer in general is

highly dependent. Inhibition of CDK5 efficiently blocks both activation and membrane sequestration of AKT, which may be released by overexpression of myristoylated and thereby membrane-bound CA-AKT.

DISCUSSION

A striking 60% of prostate cancers show loss of heterozygosity of PTEN, causing hyperactivation of the entire PI3K-AKT cascade (Morgan *et al.*, 2009). Even more remarkable is the observation of deregulated PI3K signaling in 100% of metastatic prostate cancer cases, convincingly placing the PI3K-AKT signaling pathway in a key position in prostate carcinogenesis and the development of lethal castration-resistant prostate cancer (Taylor *et al.*, 2010). The essential role of this signaling cascade can be explained by the widespread effects of the AKT kinase on numerous aspects of cancer biology; not only is AKT the key upstream activator of the growth-promoting mTOR signaling axis, but hyperactive AKT promotes cell migration and invasion, suppresses apoptotic signaling, increases cell metabolism and glucose uptake, accelerates cell cycle, and promotes a cancer-supporting microenvironment through intensified inflammation and angiogenesis. Of interest, both AKT and AR signaling regulate similar target proteins, which promote prostate cancer cell proliferation, growth, and survival. A high level of signaling complexity is achieved by synergism of these signaling pathways in prostate cancer cells (Xin *et al.*, 2006; Ha *et al.*, 2011; Thomas *et al.*, 2013). Our results imply that CDK5 is required for maintenance of adequate AKT activity in LNCaP, 22Rv1, and PC-3 prostate cancer cells, cell lines showing characteristic features of different prostate cancer stages. The observed CDK5-AKT connection was corroborated by experiments showing no differences in cell growth after CDK5 knockdown in VCaP prostate cancer cells, a prostate cancer cell line that is independent of AKT signaling (Jiang *et al.*, 2010). By regulating AKT activity and localization, CDK5 affects several downstream targets of the AKT-proliferation pathway, which seems to culminate in multifaceted cancer promotion by CDK5. In fact, CDK5-AKT cross-talk has been portrayed to occur during neuronal survival as well, where CDK5 phosphorylates the neuregulin ErbB2/3 receptors and promotes activation of the PI3K-AKT pathway (Li *et al.*, 2003). In glioblastoma cells, CDK5 phosphorylates the GTPase PIKE-A to promote AKT activation and cancer cell invasion (Liu *et al.*, 2008). Pancreatic insulin-secreting β-cells also rely on CDK5 to maintain AKT activity for survival upon apoptotic stimuli (Daval *et al.*, 2011). The results regarding CDK5-AKT interdependence in a number of cell types support our observations in prostate cancer cells. However, our results reveal that the CDK5-AKT interplay seems to occur through a direct interaction. Therefore it seems as if the context-dependent prosurvival and proproliferative pathways regulated by CDK5 in neurons (Li *et al.*, 2003; Liu *et al.*, 2008), β-cells (Daval *et al.*, 2011), and cancer cells (described in this report) may be explained by extensive effect of CDK5 on the AKT kinase. Future studies will be directed to identify the exact molecular mechanism underlying the CDK5-mediated AKT activation in our model system.

We propose a model in which CDK5 supports both AKT and AR growth-promoting pathways in parallel, with the AKT pathway being its key target in prostate cancer proliferation (Figure 7). Our study shows that in a cellular context, the CDK5-AKT pathway has a more prominent effect on cell growth than AR signaling, as the CDK5-regulated proliferation is independent of AR-mediated growth-promoting signals. There is, however, apparent cross-talk between AKT and AR, as seen by the effects of androgen treatment on AKT downstream targets (Figure 3A), and

which is also known from before to occur. Meanwhile, CDK5 was also found to have the capacity to strongly affect AR signaling, as we observed a CDK5-mediated effect on specific AR target genes and receptor stabilization. Because CDK5 in the androgen-stimulated context seems to have a priming effect on growth-promoting AR target genes, it is likely that in the in vivo situation both of the identified CDK5 targets—the AKT and the AR pathways—would act in concert to promote a common CDK5-regulated proliferative phenotype. Nevertheless, in the androgen-independent state, CDK5 can still have a strong proliferative influence through AKT only. On the basis of results obtained from our cellular experiments, we propose that CDK5 acts as a signaling hub in prostate cancer by acting as a mediator of AKT- and AR-induced proliferation.

The fact that CDK5 activity cannot be measured directly from patients in large-scale screens has hampered research studies regarding the true role of CDK5 in tumor progression. Whereas some studies have examined the expression levels of CDK5, these are not expected to reveal any relevant information, as CDK5 is a typical kinase that is primarily regulated on the posttranslational level. The effects of CDK5 in prostate cancer are likely to be related to aberrant regulation of its kinase activity and less to elevated expression levels of CDK5, as it is a kinase which is typically regulated at the posttranslational level and not through mRNA expression. This is a probable reason why CDK5 has not emerged as a hit in screens comparing nonmalignant and malignant prostate cancers (Taylor *et al.*, 2010), whereas others in turn have detected some degree of variation (Levacque *et al.*, 2012). We found that the expression of CDK5 activators showed great variation between cell lines used in this study, but the general perception was that especially the expression of p35/p25 was slightly increased in prostate cancer cells, as was CDK5 activity (Supplemental Figure S7, A and B). These results are consistent with the general notion of this study that the cancer cells showing CDK5 dependence for their growth had elevated CDK5 activity. Although the number of cell lines analyzed is too small for generalized conclusions and a broad, patient-derived analysis would be required to obtain such results, these experiments do support that the levels of active CDK5 would be increased in cancer. CDK5 has, apart from being repeatedly associated with prostate cancer, also been implicated in many other types of malignancies (such as breast, lung, ovarian, and colorectal cancer; Levacque *et al.*, 2012), many of which are distinguished by high AKT activity. The CDK5-AKT connection described here may have implications in all cancer types expressing both of these kinases, and should therefore be studied in detail in the future. Whereas the multifaceted oncogenic properties of AKT make it a highly attractive target for prostate cancer treatment, its wide-ranging functions easily yield serious adverse effects, posing obvious challenges for the use of PI3K-AKT inhibitors in the clinical context. In light of our studies demonstrating that CDK5 acts upstream of the AKT pathway, CDK5 inhibition comes across as an appealing target for treating prostate cancer and castration-resistant prostate cancer, especially in combination with other cancer drugs, as its functions in healthy tissue are relatively restricted.

To conclude, CDK5 is required for optimal prostate cancer proliferation through its multimodal action: on one hand, CDK5 is important in AR target-gene selection in those cells in which both are present, thereby affecting the notorious growth-stimulating androgen pathway in prostate cancer. On the other hand, androgen-insensitive cell lines are equally CDK5 dependent in terms of proliferation, as CDK5 was found to be a key regulator of the multifunctional oncogenic kinase AKT in both hormone-dependent and -independent
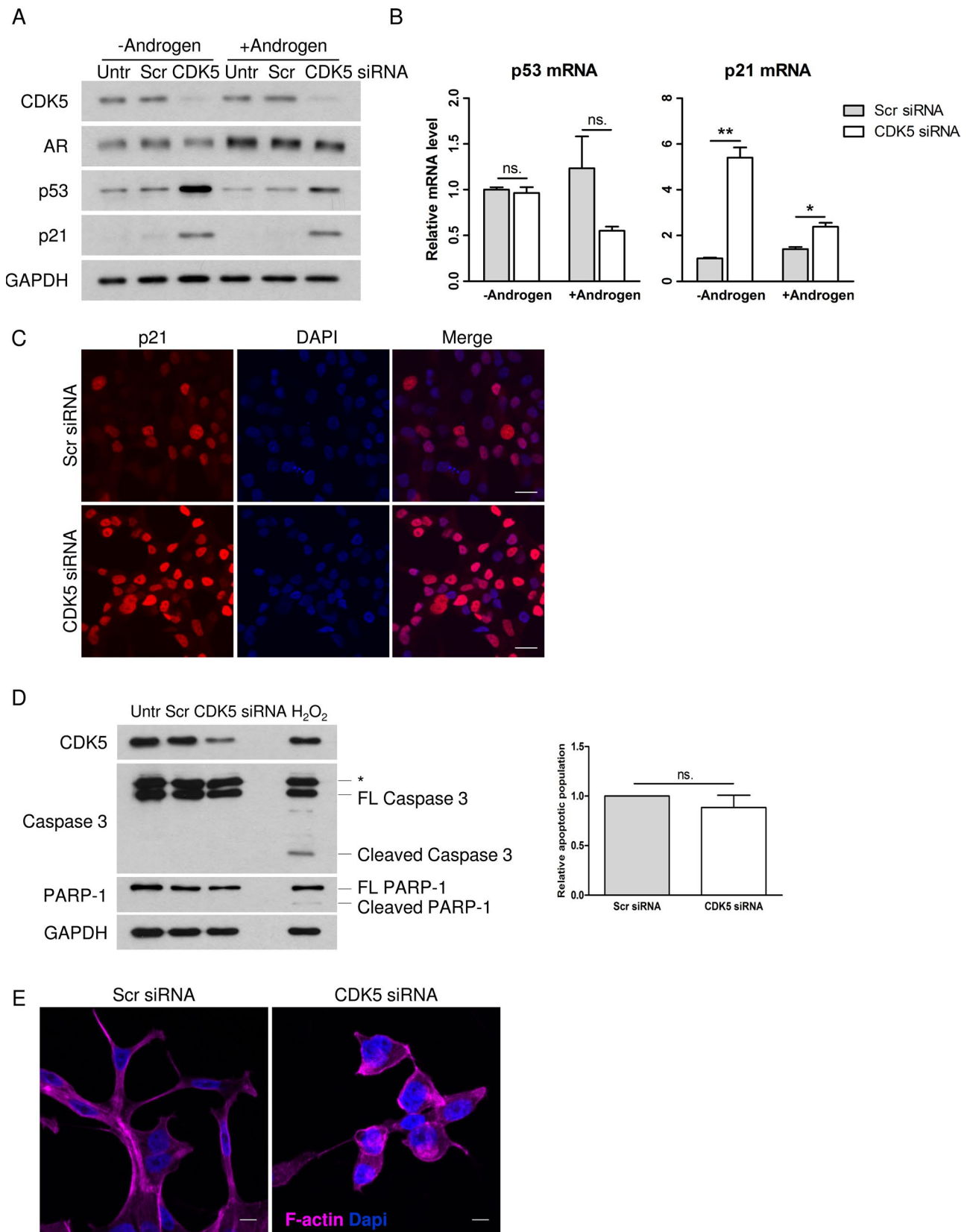
FIGURE 5: CDK5 knockdown up-regulates the expression of p53 and p21. (A) LNCaP cells were left untreated (Untr) or transfected with Scr or CDK5 siRNA. Androgen receptor stabilization after androgen addition confirmed androgen treatment efficacy. CDK5 siRNA caused up-regulation of cell cycle inhibitor p53 and p21 proteins in both conditions as determined by Western blotting. GAPDH was used to ensure equal loading. (B) p53 and p21 mRNA expression after modulation of CDK5 indicated as mean ± SEM; n.s., no significance; *$p < 0.05$, **$p < 0.01$, Student's t test, $n = 3$. (C) Behavior of p21 protein was examined by immunolabeling of p21 (red) and confocal microscopy. Images were taken
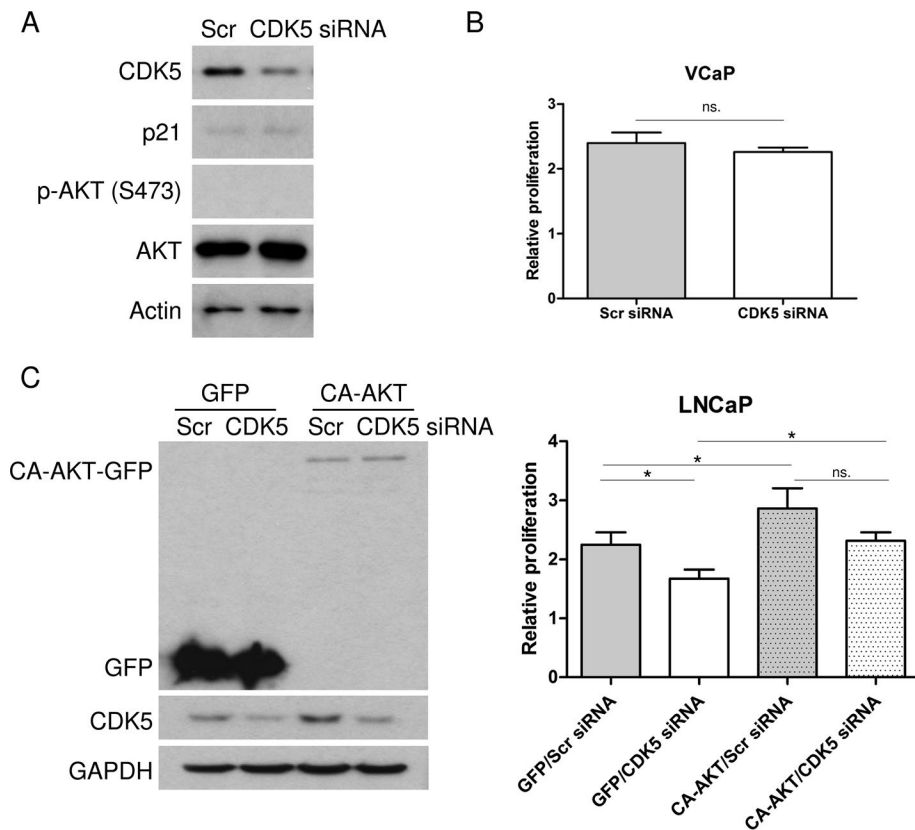
FIGURE 6: CDK5 siRNA–mediated reduction in cell proliferation is AKT dependent. (A) AKT-independent VCaP prostate cancer cells were plated and transfected with Scr or CDK5 siRNA. CDK5 down-regulation was efficient in VCaP cells, and no p-AKT could be detected in lysates. p21 levels were unchanged by CDK5 modulation. (B) VCaP cells were transfected with CDK5 siRNA, and relative cell number (compared with 0 h) was determined after 72 h. VCaP cells do not show CDK5 dependence in their proliferation (mean ± SEM; n.s., no significance; Student's t test, $n = 3$). (C) LNCaP cells were plated in equal amounts and transfected with either GFP or constitutively active GFP-tagged AKT (CA-AKT) and with Scr or CDK5 siRNA. Western blotting against GFP and CDK5 verifies efficient transfections. Relative LNCaP cell numbers were calculated for each treatment at 72 h after initiation of transfection. The CDK5-mediated growth retardation was effectively counteracted by AKT overexpression (mean ± SEM, *$p < 0.05$, n.s., no significance, one-way analysis of variance, $n = 5$).

cancer cells. The CDK5-AKT pathway is a novel revelation of a dominant and generic signaling mechanism underlying prostate cancer cell proliferation irrespective of androgen dependence.

MATERIALS AND METHODS
Materials
Caspase-3 (9662), AKT (9272), p-AKT Ser-473 (9271), S6 (2217), p-S6 Ser-235/236 (4858), p-GSK3β Ser-9 (9336), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 14C10) antibodies were purchased from Cell Signaling Technology (Danvers, MA). AR (N-20), p-AR Ser-308 (sc-26406-R), and p21 (C-19) were from Santa Cruz

Biotechnology (Dallas, TX). CDK5 antibody (DC34) was from Invitrogen (Carlsbad, CA), Hsc70 (SPA815) from StressGen (Victoria, Canada), GFP (JL-8) from Clontech (Mountain View), β-catenin from BD Biosciences (San Jose, CA), β1-integrin from Millipore (Billerica, MA), and PARP-1 (C-2-10) and actin (AC-40) from Sigma-Aldrich (St. Louis, MO). Secondary horseradish peroxidase (HRP) antibodies were from Promega (anti-rabbit; Fitchburg, WI) and GE Healthcare (anti-rat, anti-mouse; Cleveland, OH). Fluorescent secondary antibodies (Alexa Fluor 488 or 546) and Alexa Fluor 633–phalloidin were from Invitrogen.

Cell culture and treatments
LNCaP cells (clone FGC from EACC) and PC-3 (American Type Culture Collection, Manassas, VA) cells were cultured in RPMI-1640 medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated fetal calf serum (Sigma-Aldrich). LNCaP cells were supplemented with 1 mM sodium pyruvate (Sigma-Aldrich). VCaP and HEK293 cells were grown in DMEM (Sigma-Aldrich). Cells were cultured in humidified atmosphere at 37°C and 5% CO_2. Hormone depletion or stimulation was conducted by exchanging medium to 10% charcoal/dextran-treated fetal bovine serum (Thermo Fisher Scientific, Waltham, MA) in RPMI-1640 (Life Technologies, Invitrogen), without or with addition of 1 nM synthetic androgen mibolerone. LNCaP cells were treated 16 h with 100 ng/ml human neuregulin-1 β1 (R&D Systems, Minneapolis, MN) or with DMSO (Sigma-Aldrich) and 10 μM roscovitine (Calbiochem, San Diego, CA) for indicated time points. Roscovitine was replenished every 48 h. Cycloheximide (Sigma-Aldrich) was used at a concentration of 10 μg/ml.

Transfections
A pool of four siRNAs targeting CDK5 (FlexiTube GeneSolution, GS1020 for CDK5) and Scr oligonucleotide (AllStars Negative Control siRNA) were purchased from Qiagen (Hilden, Germany). Cells were transfected with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer's instructions. Medium was changed to culturing medium the following day, and cells were incubated at least 48 h before further use. For expression of GFP or

with fixed laser settings. Nuclei are labeled with DAPI (blue). Scale bar, 20 μM. (D) Left, there was no indication of apoptosis in CDK5 siRNA (CDK5)–transfected cells compared with untreated (Untr) or Scr siRNA (Scr) controls, as evaluated by Western blotting of cleaved caspase 3 or PARP-1. H_2O_2-treated cells (100 μM, 16 h) were used as positive control. FL, full length. Unspecific bands are marked with asterisk. Right, DNA fragmentation was studied by cell sorting of propidium iodine–labeled cells. Cells were analyzed for the apoptotic sub-G0/G1 population, showing no significant change in DNA fragmentation of cells (mean ± SEM; n.s., no significance; Student's t test, $n = 3$). (E) The CDK5 siRNA–induced round morphology is accompanied by a change in actin cytoskeleton. Whereas Scr siRNA cells have prominent and outspread F-actin network, CDK5 siRNA–transfected cells show a dramatic disorganized F-actin cytoskeleton. Cells were labeled with phalloidin (magenta) and DAPI (blue). Scale bar, 10 μM.
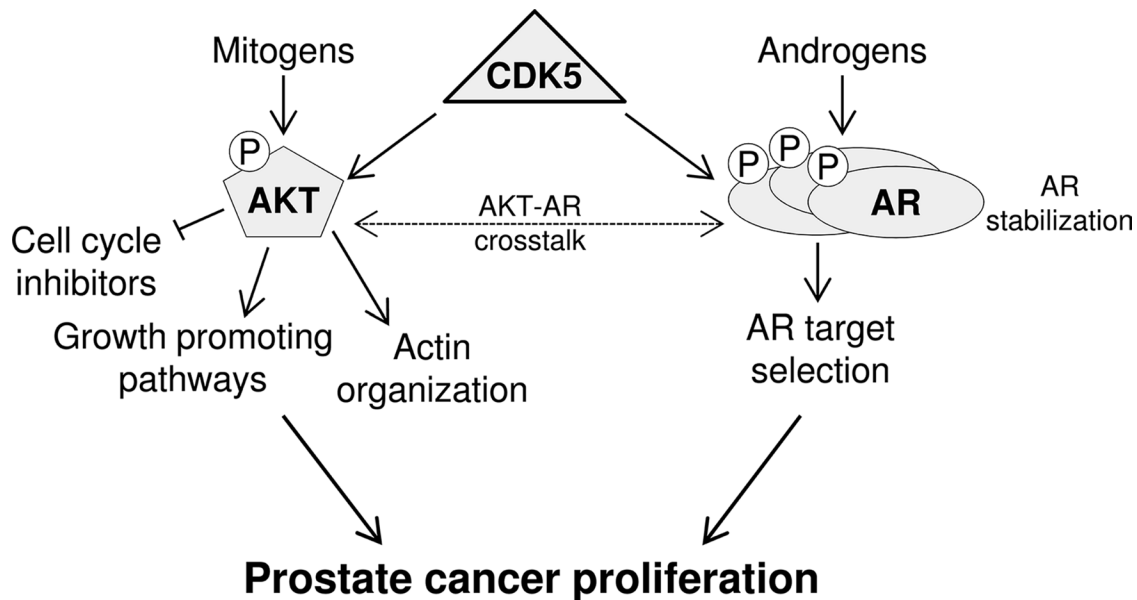
FIGURE 7: Proposed model for CDK5-mediated regulation of prostate cancer proliferation. Many prostate cancer cells are intrinsically dependent on the growth-promoting signals that derive from excessive AKT activation. We hypothesize that CDK5 affects AKT activation at the membrane, consequently repressing several of its downstream targets, all involved in a complex network regulating prostate cancer proliferation. Simultaneously, CDK5 may affect prostate carcinogenesis through modulation of AR protein stability and activity in cells in which they coexist. It thus seems that CDK5 can simultaneously affect numerous targets and promote prostate cancer cell proliferation at various stages of prostate cancer progression.

GFP-tagged CA-AKT (Peuhu *et al.*, 2010) or WT CDK5 (Addgene, Cambridge, MA), cells were transfected overnight with JetPEI (PolyPlus Transfection, Illkirch, France) following the instructions of the producer.

Cell counting
Cells were plated in equal amounts and transfected or treated with DMSO or roscovitine, and live cells were counted by trypan blue exclusion after indicated time points in a Burker chamber.

Immunoblotting
Cell lysates were collected in Laemmli lysis buffer (LLB) and separated on acrylamide gels in denaturing conditions. Proteins were transferred to polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA), after which membranes were blocked in 5% fat-free milk in phosphate-buffered saline (PBS) for 1 h and incubated in primary antibody overnight. After three washes, the membranes were incubated in HRP-conjugated secondary antibodies for 1 h at room temperature, washed three times, and visualized with an ECL detection kit (GE Healthcare).

Immunocytochemistry and imaging
Cells were plated on coverslips, transfected/treated, and incubated 48 h before they were washed and fixed in 3% paraformaldehyde. After three washes in PBS, samples were permeabilized in 0.5% Triton X-100 for 10 min. Cells were washed before blocking in 1% bovine serum albumin (BSA). Primary antibody against p21 (1:100), pS308-AR (1:50), AKT (1:100), or CDK5 (1:200) diluted in 1% BSA was incubated at room temperature for 1 h. After three washes, Alexa Fluor 488– or 546–conjugated secondary antibody (1:800) or Alexa Fluor 633–phalloidin (1:1000) was added to the samples and incubated for 1 h. Samples were then washed and mounted in

ProLong Gold Antifade Reagent with DAPI (Invitrogen). Samples were imaged with a Zeiss LSM780 confocal microscope (Carl Zeiss, Oberkochen, Germany) at the Cell Imaging Core of the Turku Centre for Biotechnology with similar settings. Images are presented as maximum projections. Cells were subjected to live-cell phase-contrast imaging with Cell-IQ platform (CM Technologies, Tampere, Finland) and analyzed with Cell-IQ Analyser software for cell confluency at fixed settings (CM Technologies).

Immunoprecipitation and cell fractionation
Cells were lysed for 30 min on ice in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and 5 mM ethylene glycol tetraacetic acid (EGTA), including protease and phosphatase inhibitor cocktails (Roche, Basel, Switzerland). For coimmunoprecipitation of CDK5 and AKT, LNCaP cells were lysed in 50 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, and 5 mM EGTA, including protease and phosphatase inhibitor cocktails (Pierce Biotechnology, Rockford, IL). Samples were centrifuged and preincubated with protein G–Sepharose beads (GE Healthcare) for CDK5, and protein A–Sepharose (Sigma-Aldrich) for AR and AKT. Samples were washed and incubated with 2 μg of CDK5, AR, or AKT antibody for 90 min. Sepharose beads were added and lysates incubated for an additional 3 h under rotation. Samples were washed three times, after which LLB was added and samples boiled.

For enrichment of membrane fractions, LNCaP cells were collected 48 h after transfection, and AKT membrane localization was analyzed according to Higuchi *et al.* (2008). Briefly, cells were lysed in 10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl$_2$, and 5 mM KCl with protease and phosphatase inhibitors for 30 min, and lysates were cleared with 720 × *g* centrifugation for 3 min. The cytosolic (supernatant) and membrane fractions (pellet) were collected after

CDK5 kinase activity and in vitro phosphorylation

CDK5 was immunoprecipitated, and samples were washed three times in kinase buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid, pH 7.2, 60 mM NaCl, 0.5 mM $CaCl_2$, 2.5 mM EGTA, and 2 mM $MgCl_2$). A mixture of ATP (Sigma-Aldrich), 3 μCi of [γ-^{32}P] ATP (final concentration, 100 μM), and CDK5 substrate histone H1 (Sigma-Aldrich) was added to the beads, and the mixture was incubated at 30°C for 30 min. The reaction was discontinued with LLB and samples boiled. Proteins were separated with SDS–PAGE and stained with Coomassie dye, and ^{32}P incorporation into histone H1 was detected by autoradiography. Alternatively, AR was immunoprecipitated from VCaP cells and probed as a CDK5 substrate (using recombinant CDK5/p25) in the aforementioned conditions.

Mass spectrometry

AR was immunoprecipitated from VCaP, LNCaP, or calyculin A–treated, AR-overexpressing HEK293 cells (Rytinki et al., 2012). In-gel digestion with trypsin, phosphopeptide enrichment by TiO_2 affinity chromatography, and analysis by LC-MS/MS were performed as described previously (Imanishi et al., 2007) with some modifications. For LC-MS/MS analysis of the digests with and without enrichment, an EASY-nLC II nanoflow liquid chromatograph coupled to LTQ Orbitrap Velos mass spectrometer and an EASY-nLC 1000 coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific) were used. Database search was performed against a concatenated forward-reverse Swiss-Prot database (Homo sapiens) using Mascot 2.4.1 (Matrix Science, Boston, MA) via Proteome Discoverer 1.4 (Thermo Fisher Scientific). Label-free quantification was performed using Progenesis LC-MS 4.1 (Nonlinear Dynamics, Durham, NC).

RNA extraction and qPCR

LNCaP cells were transfected and starved or treated with androgens for 16 h. Cells were collected, total RNA was extracted using an RNeasy kit (Qiagen), and cDNA was produced with a cDNA synthesis kit (Roche Diagnostics). RT-qPCR was conducted as described previously (Sutinen et al., 2014). Primer sequences are available upon request.

Cell sorting

Cells were trypsinized, collected, and labeled with propidium iodine (40 mM Na citrate, 0.3% Triton X-100, 50 mg/ml propidium iodine; Sigma-Aldrich) for 10 min. Samples were then analyzed in duplicates with an LSRII flow cytometer (BD Biosciences) with HTS platform at the Turku Centre for Biotechnology for DNA content.

Statistics

Student's t test or one-way analysis of variance followed by Tukey's posttest was used where appropriate to analyze statistical significance (considered significant if $p < 0.05$). Values are given as means ± SE. GraphPad Prism 5 software (GraphPad, La Jolla, CA) was used in all statistical analyses.

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