ARTICLE

Non-canonical activation of hedgehog in prostate cancer cells mediated by the interaction of transcriptionally active androgen receptor proteins with Gli3

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Abstract

Hedgehog (Hh) is an oncogenic signaling pathway that regulates the activity of Gli transcription factors. Canonical Hh is a *Smoothened-* (*Smo-*) driven process that alters the post-translational processing of Gli2/Gli3 proteins. Though evidence supports a role for Gli action in prostate cancer (PCa) cell growth and progression, there is little indication that *Smo* is involved. Here we describe a non-canonical means for activation of Gli transcription in PCa cells mediated by the binding of transcriptionally-active androgen receptors (ARs) to Gli3. Androgens stimulated reporter expression from a Gli-dependent promoter in a variety of AR + PCa cells and this activity was suppressed by an anti-androgen, Enz, or by AR knockdown. Androgens also upregulated expression of endogenous Gli-dependent genes. This activity was associated with increased intranuclear binding of Gli3 to AR that was antagonized by Enz. Fine mapping of the AR binding domain on Gli2 showed that AR recognizes the Gli protein processing domain (PPD) in the C-terminus. Mutations in the arginine-/serine repeat elements of the Gli2 PPD involved in phosphorylation and ubiquitinylation blocked the binding to AR. β-TrCP, a ubiquitin ligase that recognizes the Gli PPD, competed with AR for binding to this site. AR binding to Gli3 suppressed its proteolytic processing to the Gli3 repressor form (Gli3R) whereas AR knockdown increased Gli3R. Both full-length and truncated ARs were able to activate Gli transcription. Finally, we found that an AR-binding decoy polypeptide derived from the Gli2 C-terminus can compete with Gli3 for binding to AR. Exogenous overexpression of this decoy suppressed Gli transcriptional activity in PCa cells. Collectively, this work identifies a novel pathway for non-canonical activation of Hh signaling in PCa cells and identifies a means for interference that may have clinical relevance for PCa patients.

Introduction

Hedgehog (Hh) is a cell signaling pathway that regulates complex morphogenic processes during embryonic development [1]. Its actions are needed for the development of

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the central nervous system [2, 3], cranial-facial structures [4], limbs/peripheral appendages [5] and steroidogenic tissues [6], amongst others. Canonical Hh signaling functions to activate cellular transcription from the Gli family of transcription factors (Gli1, Gli2, and Gli3) [7, 8]. Gli proteins recognize a shared concensus DNA binding element, 5′-G-A-C-C-A-C-C-C-A-3′ [9], and other permutations of [10]. Classical canonical signaling is initiated by secreted peptides, referred to as hedgehogs, that recognize a cell surface receptor protein, *Patched*, on target cells [11, 12]. Ligand binding initiates a de-repression of *Smoothened* (*Smo*) and, once activated, *Smo* alters the phosphorylation and proteolytic degradation patterns of Gli2 and Gli3 leading to their transformation from a repressor state to a transcriptional activator state. In turn, transcriptionally active Gli2 and Gli3 induces expression of Gli1 that further augments the active Gli transcriptional state [13, 14].

Hh is also a proliferative stimulus [15] and hyperactive Hh in adult tissues can be oncogenic [16–18]. Basal cell

carcinoma (BCC) and medulloblastoma (MB), in particular, are sometimes driven by activating or inactivating mutations in upstream Hh signaling components that enable constitutive Gli transcription [19, 20]. Other solid tumors, including pancreatic, lung, breast, and prostate cancer (PCa) are also thought to be influenced by Hh, though the types of mutations found in BCC or MB are not detected with any significant frequency in these latter tumors. Crosstalk from other oncogenic signaling pathways (MAPK/ras, PI-3 kinase/AKT, TGF-β, and others), however, may provide a means for non-classical activation of Gli [21–23] in some tumors. Here we show that there is a novel non-canonical pathway for activation of Hh in PCa cells that is mechanistically driven by the direct binding of transcriptionally active androgen receptor (AR) proteins to Gli proteins.

PCa develops and progresses under the influence of androgenic steroids [24]. This influence identifies the importance of the AR protein as a critical effector of PCa cell survival and growth [25]. Indeed, AR pathway inhibitors provide the foundation for contemporary treatment of metastatic PCa. Unfortunately, hormonal therapeutics used in advanced PCa patients are only palliative and patients eventually recur with disease that is resistant to androgen deprivation (castration resistant prostate cancer or CRPC) as well as the potent anti-androgens now used to treat CRPC. For the most part, however, resistant CRPC cells continue to express AR and remain addicted to it [26]. In these tumors, AR continues to signal to survival and growth endpoints despite the lack of hormonal stimulation. Mechanistically, AR hyperactivity in CRPC has been linked to acquired intratumoral steroidogenesis, overexpression of full length (AR-FL) or C-terminally truncated ARs (t-ARs) or overexpression of AR co-activator proteins [27, 28]. Hh pathway activity may effect more than one of these AR aberrations.

Indeed, we have already shown that a paracrine Hh signaling microenvironment, induced by androgen deprivation, drives steroidogenesis from benign stromal cell elements within a prostate tumor [29]. In this setting, a *Smo* antagonist was able to suppress the development of CRPC in a PCa cell xenograft by reducing endogenous androgen levels in the tumor. Additionally, we previously described tumor cell autonomous activities of Gli mediated by the direct binding of Gli proteins to ARs [30]. Gli proteins recognize the AR N-terminal tau5 transactivation domain (TAD) that regulates ligand-independent activity of AR-FL and is shared by t-ARs [31]. We showed that Gli1 and 2 could co-activate both unliganded and liganded AR-FL as well as t-ARs [30, 31]. Finally, we found that exogenous overexpression of Gli1 or Gli2 was sufficient to drive androgen-independent in vitro growth of androgen growth-dependent PCa cells and we attributed this activity to the AR co-activation functions of Gli proteins [30].

We previously localized the AR binding domain on Gli2 to a 270 amino acid region of the Gli2 C-terminus (Gli2aa628-897) [31]. This region shares significant homology with Gli3 (Gli3aa678-936) and it encompasses critical phosphorylation sites that are also targets of β-TrcP ubiquitination. For Gli3, this region also contains a site of proteolytic cleavage that generates the truncated Gli3-repressor (Gli3-R) protein in the absence of Hh signaling.

As a corollary to our studies of the effects of Gli on the transcriptional activity of AR, we explored here the converse idea that AR affects Gli transcriptional activity in PCa cells. Our results show that ARs act as a co-activators of Gli-mediated transcription and that transcriptionally-active AR engagement with the protein processing domain (PPD) on Gli3 regulates its proteolytic processing and transcriptional outputs. Furthermore, we show that interference with the intracellular AR-Gli interaction using a decoy peptide can diminish the ability of AR to act as a Gli transcriptional activator.

Results

Transcriptionally active ARs upregulates Gli transcriptional activity in PCa cells

Using a panel of five AR + PCa cell lines (parental [LNCaP]; androgen growth-independent [LNCap-AI and LN95]), LAPC4 and a CWR1 cell variant, R1-D567, that expresses only a C-terminal truncated AR (AR-e567), we tested whether androgen (R1881) affected expression of a luciferase reporter under the control of a Gli-responsive promoter (Gli-luc). Each of the LNCaP variants and LAPC4 upregulated reporter expression in the presence of R1881 (Fig. 1a). Addition of enzalutamide (Enz), an AR antagonist, blocked this upregulation. While androgen did not affect reporter expression in R1-D567 cells, they had higher overall basal Gli reporter activity compared to cells that express AR-FL. Transfection of a constitutive-active truncated AR variant, AR-V7, into AR$^-$ (PC3) or AR$^+$ (LNCaP) PCa cells also upregulated Gli reporter in the presence or absence of androgen. (Figs. 1b, c). For LNCaP cells, that only express AR-FL, the AR-V7-enhanced reporter activity was not diminished by co-transfection with AR-FL siRNA, in fact, AR-FL knockdown further enhanced the ability of AR-V7 to induce Gli reporter activity (Fig. 1c). For androgen-responsive LNCaP variants, knockdown of AR-FL blocked the upregulation of Gli-reporter expression induced by androgen (Fig. 1d). We also tested whether androgens affected the expression of endogenous Gli-target genes, Gli1 and Ptch1 in AR$^+$ PCa cells. Androgen significantly upregulated the expression of Gli1 and Ptch1 at the mRNA and protein levels (Figs. 1e, f) in
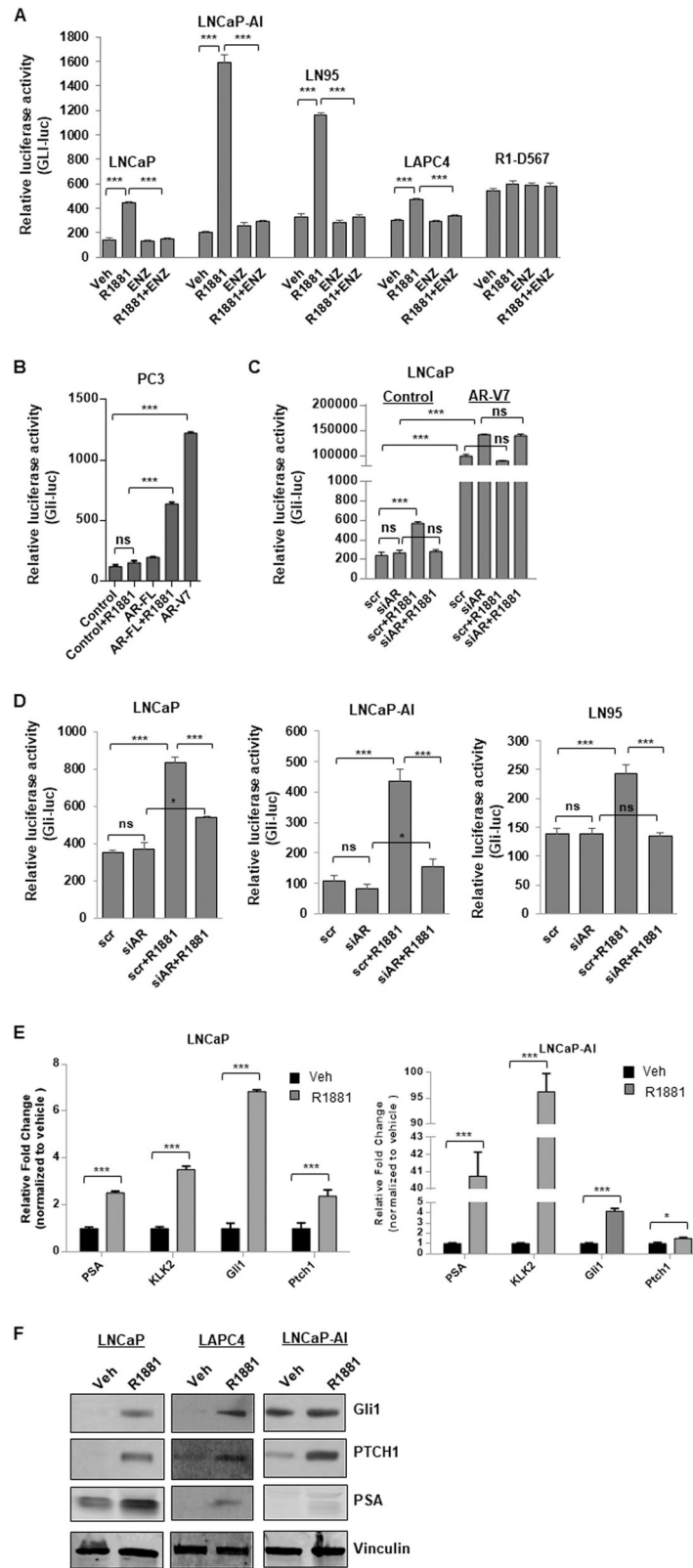
◀ **Fig. 1** Transcriptionally-active androgen receptors upregulate Gli transcriptional activity in PCa cells. A panel of AR+ PCa cells were transfected with Gli-luc reporter along with pCMV-eGFP, and (**a**) cultured in CS-FBS media in the presence of vehicle (Veh), R1881 (1 nM), Enzalutamide (ENZ, 10 µM) or both; or (**b**) AR- PC3 cells were co-transfected with Gli-luc reporter and control (empty) vector with AR-FL or ARV7. R1881 was added where indicated. Bars indicate relative luciferase activities. **c** LNCaP cells were co-transfected with Gli-luc and control vector or AR-V7 vector with a scrambled siRNA (scr) or siRNA targeting AR-FL (siAR). R1881 was added where indicated. Bars indicate relative luciferase activity. **d** Cells indicated were co-transfected with Gli-luc and control (scr) siRNA or siAR in the presence or absence of R1881, as indicated. **a–d** Luciferase activities were measured in cell protein extracts and normalized to GFP fluorescence intensities. **e** LNCaP or LNCaPAI cells were cultured in CS-FBS in the presence or absence of R1881. The mRNA levels of endogenous AR targets (PSA, KLK2) and Gli targets (Gli1, Ptch1) were determined through qPCR and bars indicate the relative fold-change compared to vehicle-treated specimens. **a–e** Bars represent mean ± SD; *$P < 0.05$; ***$P < 0.001$; ns not significant. **f** Changes in expression of endogenous Gli target proteins were also confirmed in LNCaP, LAPC4 and LNCaP-AI cells treated with R1881. Cell lysates were prepared for SDS-PAGE, followed by western blot analysis using indicated antibodies

LNCaP and its androgen growth-independent variant LNCaP-AI. LAPC4 cells showed similar upregulation of Gli target proteins (Fig. 1e) on treatment with androgen.

It was of further interest to compare Smo-driven Hh activity to AR-driven activity, however LNCaP and other AR + PCa cells are unresponsive to Smo agonists. Using Smo-responsive mouse MC3T3-E1 cells instead, we compared Gli-driven luciferase reporter activity following treatment with SAG, a small molecular Smo agonist, to transient transfection with AR-FL or AR-V7. While SAG treatment approximately doubled expression of the Gli reporter, co-transfection with AR-FL (in the presence of R1881) or AR-V7 (in the absence) increased Gli reporter expression by 100 to 200-fold (Supplementary Figure 1).

Androgen promotes the interaction of AR with Gli3 in PCa cells

In order to characterize the Gli landscape of PCa cells, we surveyed a broad variety of PCa cell lines for the relative expression of Gli1, Gli2, and Gli3. Analysis of mRNA levels by qPCR showed that Gli3 was the predominant Gli homolog expressed in each; often at levels > 100-fold higher than Gli1 or Gli2 (Supplementary Figure 2). To determine whether androgen affects the intracellular interaction between AR and Gli3, we first evaluated whether androgen influenced the co-immunoprecipitation of Gli3 with AR from extracts of LNCaP cells exogenously overexpressing myc-tagged Gli3. Western blots of AR-immunoprecipitates showed that androgen increased the Gli3 content of AR-pulldowns in a dose-dependent manner

(Fig. 2a). We also used the Proximity Ligation procedure that identifies proteins in close contact in situ, to detect and relatively quantify Gli3-AR complexes in PCa cells. Treatment of LNCaP cells with R1881 significantly increased the number of Gli-AR complexes (Fig. 2b). These complexes were overwhelmingly localized to the nucleus. Addition of Enz significantly reduced the Gli-AR complexes detected in the presence of R1881 (Fig. 2b).

The AR binding site on Gli encompasses critical phosphorylation sites involved in Gli proteolytic processing

In order to better understand functions of Gli that might be affected by AR binding, we further refined the AR binding site on Gli2 using GST-pulldown in conjunction with site-specific mutations. Previously we showed that a 270 aa polypeptide from the Gli2 C-terminus (Gli2aa$_{628-897}$) had AR binding activity. Whereas deleting back the C-terminal end of Gli2aa$_{628-897}$ to aa836 had little effect on its ability to bind AR-tau5, further deletions back to aa805 or even aa820 resulted in complete loss of binding to AR-tau5 (Fig. 3a). Deletion of the Gli2aa$_{628-897}$ N-terminal region to aa764 had no effect on binding. These results show that a 73aa polypeptide (Gli2 aa$_{764-836}$) is sufficient to bind to AR-tau5 and further suggest that a 16 peptide region (Gli2aa$_{820-836}$) is needed for Gli2 binding to AR-tau5. This small region encompasses an arginine, serine-repeat involved in the regulation of Gli2/Gli3 processing (Fig. 3a). Referred to as the PPD [32, 33], it is a target for phosphorylation that is then recognized by the ubiquitin ligase, βTrcP [34]. Ubiquitinylation of full-length Gli3 (Gli-FL) promotes a site-specific cleavage within the C-terminus to generate the Gli repressor (Gli3-R) [33]. Indeed, when these repeats were mutated (S-R-R-S-S → S-D-D-S-S), the Gli2 aa$_{628-897}$ polypeptide was no longer able to bind to AR-tau5 (Fig. 3a). Moreover, these site-specific mutations, when incorporated into the full C-terminal domain of Gli2 (Gli2-C), blocked the ability of Gli2-C to co-activate AR as determined by its effects on a luciferase reporter from the androgen-responsive PGC promoter (Supplementary Figure 3). Using a GST-pulldown, we further found that β-TrcP protein competes with the N-terminal domain of AR for in vitro binding to Gli2 aa$_{764-836}$ (Fig. 3b). We also found that increased exogenous expression of β-TrcP in LNCaP cells displaced AR from Gli3 immunoprecipitates (Fig. 3c).

These findings suggested that binding of AR to Gli3 might affect its ability to be ubiquitinylated and, ultimately, its post-translational proteolytic processing. LNCaP cells grown in androgen-depleted medium for 48 h have a low ratio of Gli-FL to Gli3-R (Fig. 4a). Treatment of androgen growth-dependent LNCaP cells with increasing doses of

Fig. 2 Androgen promotes the interaction of AR with Gli3 in PCa cells. **a** LNCaP cells were cultured in CS-FBS in the presence or absence of 1 nM R1881. Co-IP confirmed the increase of Gli3-FL in AR immuno-precipitates in dose-dependent manner. **b** LNCaP cells were seeded on coverslips in CS-FBS and treated with vehicle (Veh), R1881, Enzalutamide (Enz), or both. Proximity ligation assay was performed as described in Materials and Methods. Representative images (Top) were taken and processed using a Zeiss 780 M confocal microscope. Photomicrographs are shown at 40X, oil. Scale bar, 20 μm. PLA signals (Below) were randomly grouped into 4 groups and counted by two different investigators (JM, ST) from representative fields [vehicle, 8 fields (95 cells); R1881, 15 fields (80 cells), ENZ, 10 fields (141 cells), ENZ + R1881, 14 fields (52 cells)]. $*P < 0.05$, $***P < 0.001$
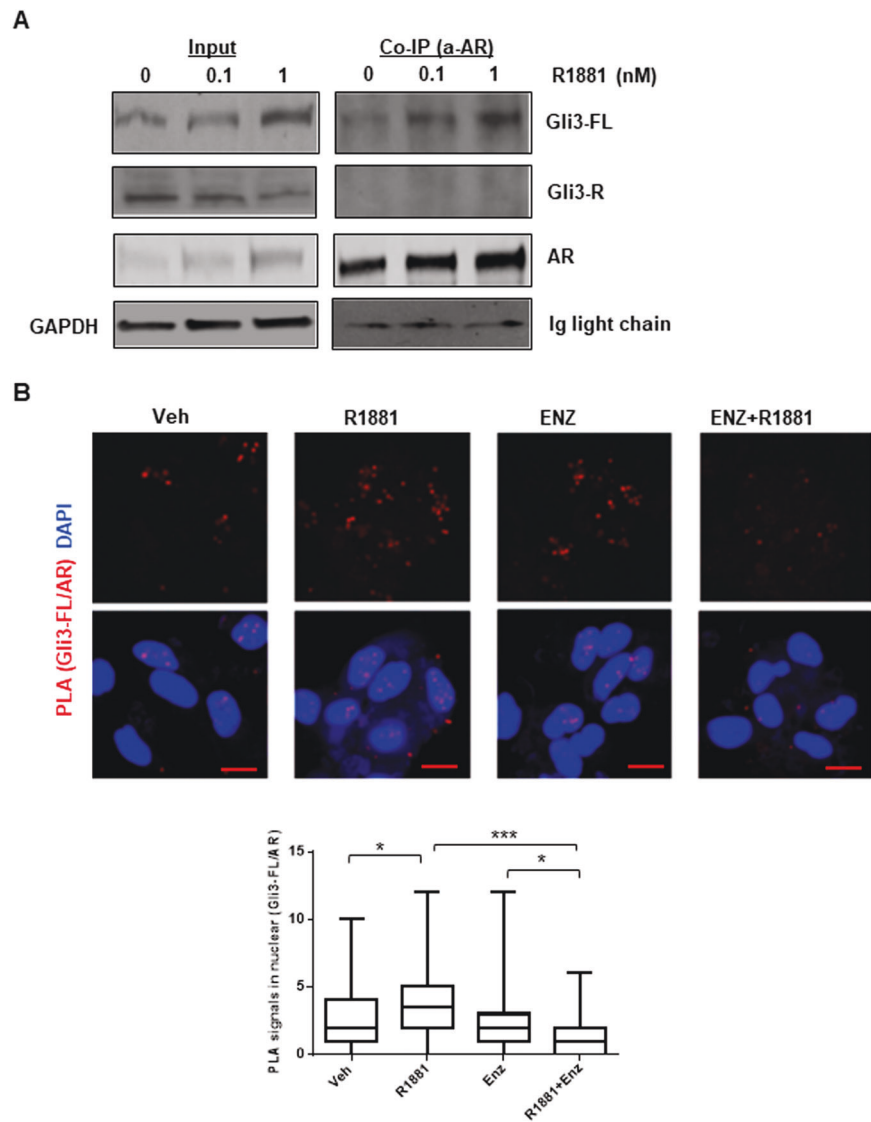

R1881 showed a progressive increase in the ratio of Gli3-FL to Gli3R (Fig. 4a, b) (top panel) that corresponded with an increased intensity of Gli-dependent luciferase reporter expression (bottom panel). Androgens increased the Gli3-FL/Gli3R ratios in other PCa cell lines (Fig. 4c) whereas knockdown of AR-FL with siRNA reduced Gli-FL/Gli3R ratios. Furthermore, we found that AR knockdown increased the relative degradation rate of Gli-FL (Fig. 4d). Finally, transfection of a t-AR (AR-V7) into LNCaP or PC3 cells also stabilized Gli3-FL (Supplementary Figure 4). It was also notable that LNCaP-AI cells, an androgen growth-independent variant of LNCaP, had a comparatively high endogenous ratio of Gli3-FL/Gli3R (Supplementary Figure 5). Even exogenous myc-tagged Gli3 was differentially processed between LNCaP and LNCaP-AI cells (Supplementary Figure 5).

Decoy peptides from the Gli2 C-terminal domain compete with Gli3 for AR binding and inhibit Hh signaling/Gli activity in PCa cells

With the AR binding site on Gli2 localized, we tested whether a 270aa truncated polypeptide derived from Gli2, containing the AR binding site, might serve as a decoy that could interfere with the AR-Gli interaction in vivo. This polypeptide (Gli2aa$_{628-897}$ or Gli-DP) co-immunoprecipitated with AR following exogenous over-expression in LN95 cells (Fig. 5a) and it displaced myc-tagged Gli3 from AR co-immunoprecipitates. It also decreased the number of in situ Gli-AR complexes when it was overexpressed in LNCaP-AI cells as was shown by a significant decrease in the Gli3-AR PLA index (Fig. 5b). Finally, Gli-DP reduced expression of the Gli-reporter in LNCaP cells in the presence of R1881 (Fig. 5c) and it

A

B

C
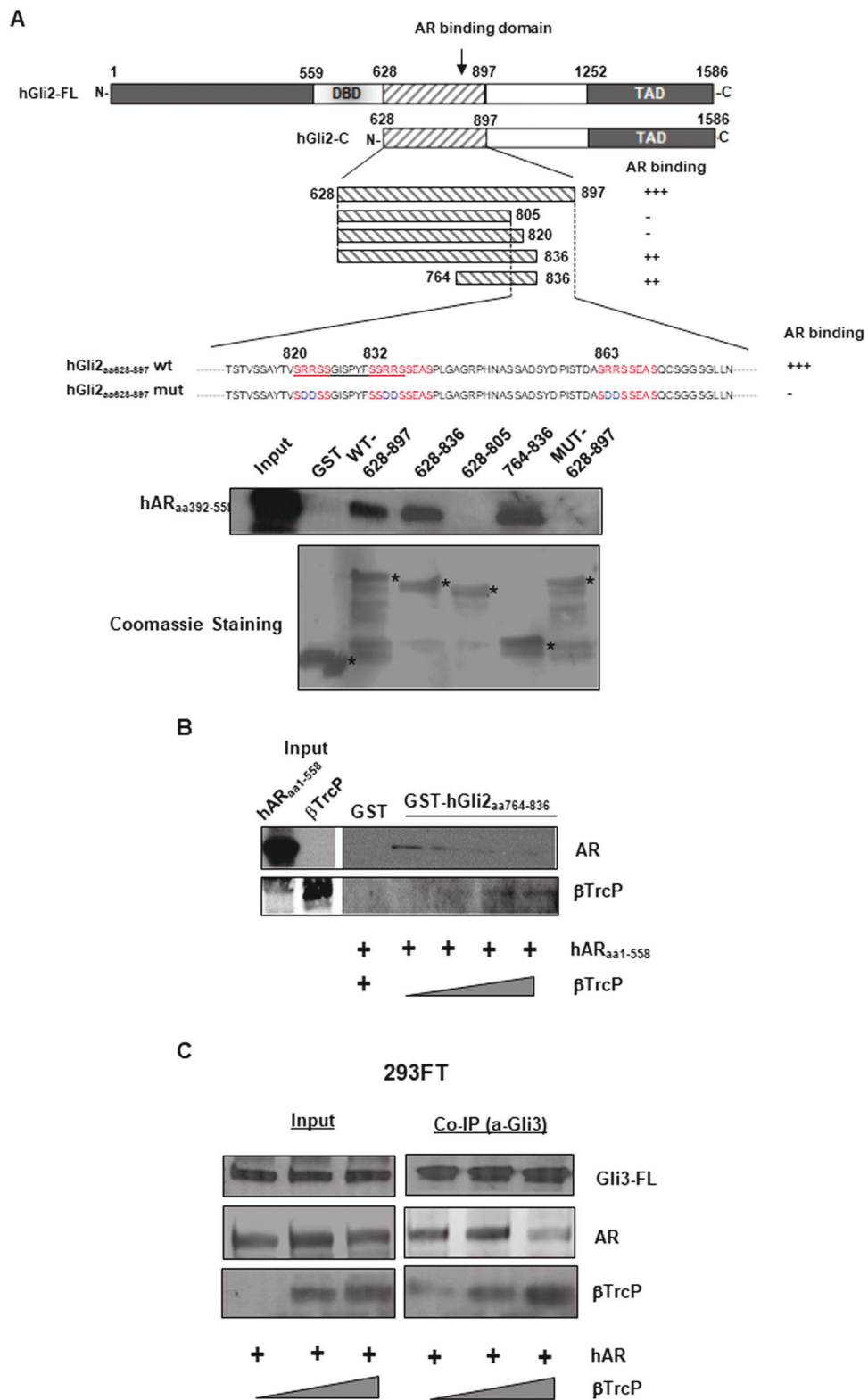
Fig. 3 The AR binding site on Gli2 encompasses phosphorylation and βTrCP binding sites within the C-terminal domain. **a** (Upper), Schematic diagram of Gli2 protein domains and various sub-fragments (below) tested for binding to AR-tau5 (hARaa$_{392-558}$) in GST-pulldowns with binding outcomes indicated on the right. (Middle) Partial sequence of Gli2aa$_{805-897}$ showing phosphorylation site (−S−R−S− repeats [red]) and below, sequence of mutated Gli2aa$_{805-897}$, as was described. (Bottom) Retention of his-tagged AR-tau5 polypeptide (Upper) on recombinant GST-tagged Gl2 polypeptides affixed to glutathione beads confirmed the minimum AR binding domain at Gli2aa$_{764-836}$. Eluates were resolved on SDS-PAGE and western blots were probed for AR-tau5 using an his antibody as described. (Lower) Gli2 recombinant proteins were visualized under Commassie staining (* indicates expected size) after SDS-PAGE. **b** Competition of βTrCP for AR binding site on Gli2. GST-tagged Gli2aa$_{764-836}$ polypeptide was captured on glutathione beads and incubated with AR N-terminus (hARaa$_{1-558}$) (fixed concentration) in the presence of increasing concentration of recombinant βTrCP (βTrCP/AR ratio of 0, 1/4, 1/2, 1/1). Eluates were separated on SDS-PAGE then probed with anti-AR or anti-βTrCP antibody as described. **c** Extracts of 293FT cells co-transfected with a fixed amount of AR-FL vector and βTrCP (at ratios of 0, 1x, 2x AR) were immunoprecipitated with anti-Gli3 antibody. Immunoprecipitates were resolved on SDS-PAGE and western blots were probed for Gli3-FL (anti-Gli3 antibody), AR (anti-AR antibody) or βTrCP (anti-βTrCP antibody)

suppressed the expression of Gli1 and Ptch1 mRNAs in LNCaP and LNCaP-AI cells (Fig. 5d).

Discussion

The androgen and Hh signaling pathways are intimately intertwined in PCa cells. Androgens regulate the expression and release of hedgehog proteins from them and androgen deprivation can convert AR + PCa cells into virtual hedgehog factories [35, 36]. In turn, hedgehogs released from androgen deprived PCa cells can stimulate intratumoral steroidogenesis from benign stromal cells in the tumor microenvironment [29, 37], promoting tumor growth after castration. Our continuing research also reveals a unique inter-relationship between the two key transcriptional mediators of these signaling pathways in PCa cells, the AR and the Gli transcription factors. AR is a fundamental regulator of prostate development and an effector of prostate disease states. It is needed for transcription of genes that distinguish the differentiated phenotype of prostate epithelial cells. AR also regulates the growth and invasiveness of PCa cells [38] and, as such, represents a principal therapeutic target for control of metastatic PCa. Glis regulate the expression of genes involved in development, cell growth, cell motility and steroidogenesis and are oncogenic when hyperactive in adult cells/tissues. Clearly, the functional gene networks regulated by these two different transcription systems overlap in PCa and this is consistent with the long-standing interest in Gli function in PCa cells [39–41].

Gli proteins bind directly to AR [31] and this binding co-activates unliganded as well as liganded AR [30]. Glis recognize the N-terminal AR tau5 TAD [31] that is involved in anomalous unliganded AR-FL activity and this explains the ability of Glis to co-activate t-ARs as well as AR-FL. While we previously suggested that Gli co-activation of AR was an enabler of androgen-independent of PCa cells, our results here shows that AR binding to Gli proteins serves to activate Gli transcription through a non-canonical signaling process in PCa and other human cells (293FT). Gli is an effector of prostate cancer cell growth [40]. In the future, we need to consider further the extent to which this non-canonical activity contributes to cell growth control in PCa.

For PCa cells that express AR-FL, we found that androgen drives inducible expression of an exogenous Gli-dependent reporter gene as well as expression of endogenous Gli target genes (Gli1 and Ptch1). This did not occur in a PCa cell line (R1-D567) that is insensitive to androgen because it only expresses a truncated AR variant protein. The presence of Enz, an AR antagonist ligand, or knock-down of AR with siRNA abrogated this response, showing that it was dependent on the presence of transcriptionally active AR. We also found that a constitutively-active t-AR, AR-V7, induced Gli reporter and endogenous target genes when exogenously expressed in both AR^{+} and AR^{-} PCa cells. It was notable that knockdown of AR-FL in LNCaP further enhanced the ability of AR-V7 to activate Gli. These responses were recapitulated in Hh-responsive mouse 3T3 cells where we found that transfection of full-length or truncated AR provided far more substantial activation of Gli signaling activity than a small molecule *Smo* agonist. This showed that AR is a relatively robust Gli activator.

Based on an assessment of the relative expression of Gli homologs across a broad panel of PCa cell lines, we concluded that Gli3 is the dominant homolog. Use of a co-immunoprecipitation procedure and proximity ligation assays allowed us to show that androgens quantitatively increased the formation of AR-Gli3 complexes in PCa cells that express AR-FL whereas Enz suppressed Gli-AR complex formation. These outcomes suggest that transcriptionally-active (liganded) AR-FL selectively forms complexes with Gli3 and this is also consistent with the Gli promoting effects of t-AR (in the absence of ligand) that has constitutive transcriptional activity.

While we reported earlier that AR binds to a 270aa polypeptide in the Gli C-terminal domain, further refinement of this binding site on Gli2 (down to a 73aa polypeptide) showed that the serine, arginine-rich repeats within the Gli PPD are necessary for AR binding. Indeed, we found that an AR-tau5 polypeptide competed with βTrCP for binding to Gli2. This finding suggested the possibility that, mechanistically, AR binding to Gli3 protein might
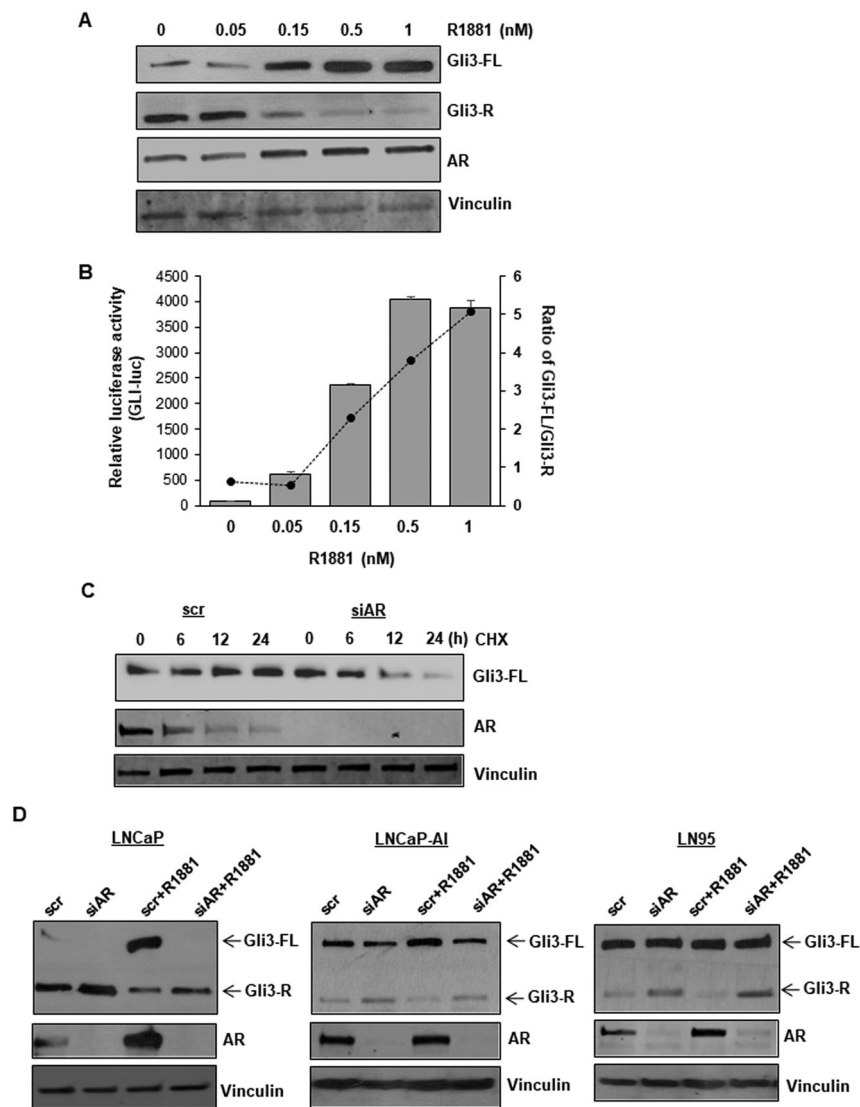
Fig. 4 Transcriptionally active AR stabilizes Gli3-FL. **a** LNCaP cells were cultured in CS-FBS in the presence of increased doses of R1881, as shown. Western blot analysis confirmed R1881 treatment progressively increased the ratio of Gli3-FL to Gli3R, along with AR protein expression. **b** In parallel, LNCaP cells stably expressing a Gli luciferase reporter were treated with increased doses of R1881. Luciferase activity was measured and shown in the bar graph. The ratios of Gli3-FL to Gli3R were determined by densitometry using Image J software, presented in the dotted line. **c** Western blot of LNCaP-AI extracts following a cycloheximide chase (20 µg/ml for hours shown) showed that AR knockdown destabilized Gli3-FL. **d** Androgen stabilized Gli3-FL whereas AR-FL knockdown destabilized Gli3-FL. LNCap, LNCaP-AI or LN95 cells were transfected with scramble (scr) siRNA or siRNA against AR-FL (siAR) and then treated with 1 nM R1881, as described in Materials and Methods. Cell lysates were resolved on SDS-PAGE and western blot was done with indicated antibodies

mask the PPD from the post-translational modifications and proteolysis involved in Gli3-R generation and stabilize Gli-FL in its active conformation. This was confirmed by our studies showing that the Gli3-FL/Gli3-R ratio increased in PCa cells treated with androgens or transfected with a t-AR. Conversely, knockdown of AR reduced Gli-FL/Gli3-R regardless of the presence of androgen and this was consistent with the concomitant loss of Gli signaling. We have, so far, been unable to determine whether AR-FL/t-AR affects Gli2 in our cell models as it is expressed at much lower levels than Gli3 and the anti-Gli2 antibodies we have

tested are inconsistent in detection of this protein by Western blot. But we will continue this effort in the future using PCa cells that appear to express higher levels of Gli2 (VCaP and LAPC4).

We also tested the idea that a limited Gli2-derived peptide that encompasses the AR binding site might serve as an in vivo decoy to engage AR in PCa cells and displace Gli-FL. This activity was confirmed by the loss of Gli3 from AR immunoprecipitates in the presence of Gli-DP as well as by a significant decrease of in situ Gli3-AR complexes formed in the presence of Gli-DP. The biological
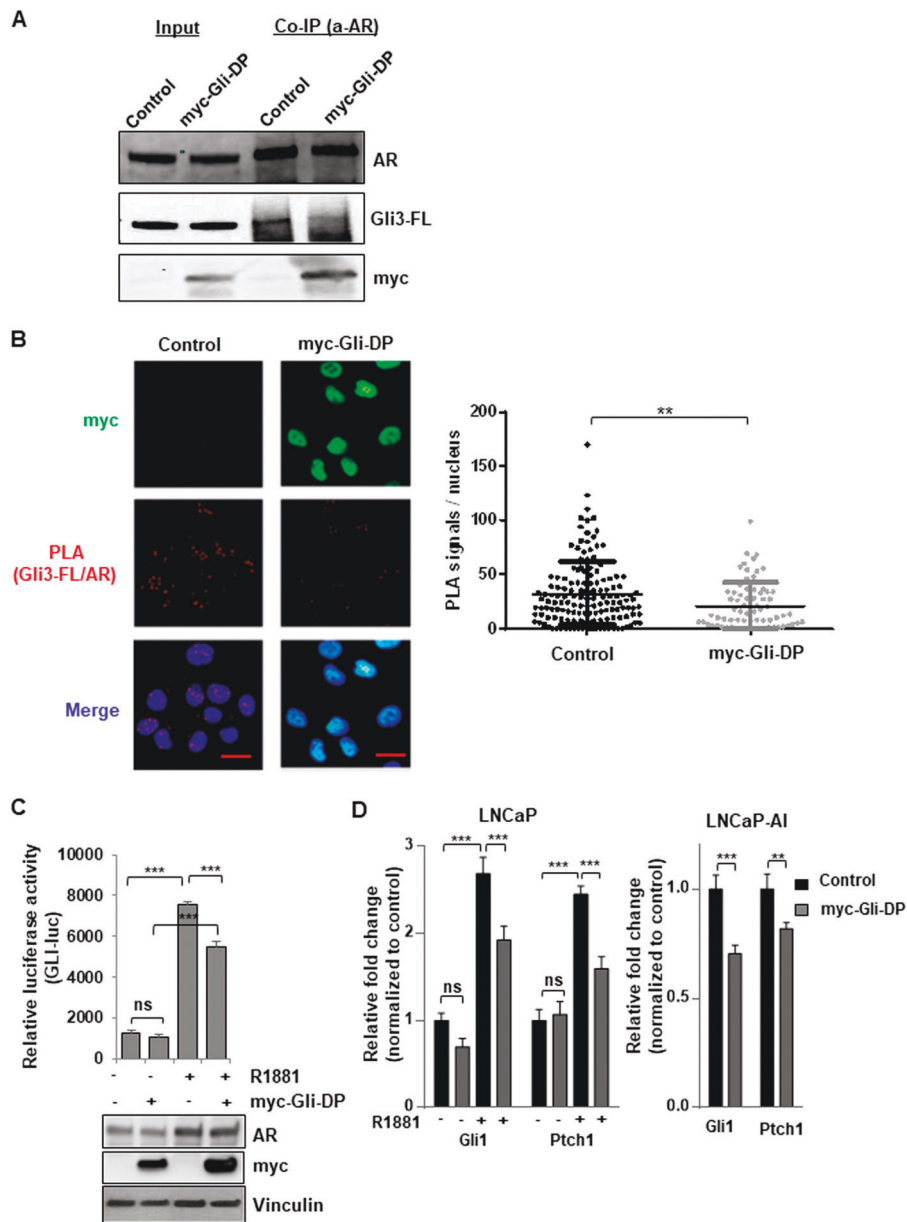
Fig. 5 A decoy peptide (myc-Gli-DP) competes with Gli3 for AR binding and inhibits Gli transcriptional activity in PCa cells. **a** LN95 cells transiently transfected with control (empty vector) or myc-Gli-DP (hGli2aa$_{628-897}$) vector. Lysates were immunoprecipitated with anti-AR antibody and immunoprecipitates were resolved on SDS-PAGE. Western blots were probed with anti-myc antibody (Gli-DP) or with anti-Gli3 antibody or anti-AR antibody. Results confirmed exogenous myc-Gli-DP interaction with AR in vivo and its displacement of endogenous Gli3-FL from AR immuno-precipitates. **b** LNCaP-AI cells overexpressing Dox-inducible myc-Gli-DP or control vector were seeded onto coverslips and cultured in CS-FBS in the presence of 0.2 μg/ml Dox for 48 h. The samples were then processed for Proximity Ligation Assay as described in Materials and Methods, followed by myc staining to confirm the expression and cellular localization of myc-Gli-DP. PLA signals were observed under the Zeiss LSM 780 Confocal Laser Microscope and representative images are shown (Left), Objectives, 40x oil. Scale bar, 20 μm. (Right) PLA quantification was done with the Duolink® ImageTool software.tool,. 147 cells (control) and 72 cells (myc-Gli-DP) were counted. **c** LNCaP cells were co-transfected with Gli-luc and pCMV-eGFP, along with control (−) and myc-Gli-DP (+) vectors. Cells were further cultured in CS-FBS in the presence or absence of R1881. Protein extracts were assayed for luciferase and normalized to GFP fluorescence intensity. **d** Endogenous mRNA levels of Gli targets (Gli1 and Ptch1) were determined in Dox-primed LNCaP or −AI cells stably expressing Dox-inducible myc-Gli-DP. Control, Dox-inducible control vector. *ns* not significant; **P < 0.01, ***P < 0.001

consequences of this decoy peptide-mediated disruption was a drop in Gli reporter activity as well as decreased expression of endogenous Gli target genes. These observations may have potential clinical relevance for therapeutic development as Hh/Gli is known to regulate genes that promote cell proliferation and EMT that is associated with tumor cell invasion and metastasis. Indeed, though not shown here, we do have preliminary evidence that this 270aa Gli2 peptide is an inhibitor of even Enz-resistant PCa cell growth.

There are other questions raised by this finding, especially as to whether other steroid receptors might have similar activities. If so, it could explain the sources of Hh/Gli activities in other steroid receptor-driven tumors. However, even if this Gli-promoting activity is restricted to AR, it could still be relevant to certain breast, endometrial and ovarian cancers where androgens sometimes have a role in disease establishment or progression. These are also questions that we will be addressing in future work.

Materials and methods

Cell lines and culture

Human prostate carcinoma cell lines, LNCaP (<Passage 40, ATCC, Manassas, VA), PC3 (ATCC), LAPC4 (obtained from Dr. Charles Sawyer, MSKCC), and the human embryonic kidney cell line, 293FT (Thermo Fisher, Carlsbad, CA, USA), were maintained in RPMI-1640 or DMEM media supplemented with 10% FBS (HyClone, Inc., Logan, UT, USA) with streptomycin/penicillin. Androgen-independent LNCaP-AI [30], LN95 (from Dr Stephen Plymate, University of Washington) and CWR1-derived R1-D567 cells (from Dr. Scott Dehm, University of Minnesota) were cultured in RPMI supplemented with 10% charcoal-stripped FBS (CS-FBS, Hyclone) and streptomycin/penicillin. LNCaP cells were authenticated by short tandem repeat profiling (IDEXX, Inc., Markham, ON, Canada).

Gene constructs and vectors

AR-FL was provided by Paul Rennie (Vancouver Prostate Centre) and AR-V7 was from Jun Luo (Johns Hopkins Medical Center). The vector expressing Gli3-FL was previously described [31]. Gli luciferase reporter was generated by subcloning an 8X repeat Gli binding concensus sequence [30] into KpnI and BglII sites of pGL4.48 (Promega, Inc., Madison, WI). A Gli2 decoy peptide was generated by subcloning N-terminal myc-tagged Gli2aa$_{628-897}$ into EcoRI and XbaI sites of pLenti4-TO (Thermo Fisher), followed by an in-frame C-terminal 3X repeats of a nuclear localization sequence (NLS; forward, 5′-CT AGA GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG AGA AAG GTA GAT CCA AAA AAG AAG AGA AAG GTA TAG T-3′, reverse, 5′-CT AGA CTA TAC CTT TCT CTT CTT TTT TGG ATC TAC CTT TCT CTT CTT TTT TGG ATC TAC CTT TCT CTT CTT TTT TGG ATC T-3′, inserted into XbaI sites.

Plasmids/siRNA transfection/lentiviral transduction

For co-immunoprecipitation studies and luciferase reporter assays, above plasmids, as described, were transiently co-transfected into PCa or 293FT cells using lipofectamine 3000 (Thermo Fisher). siRNA targeting AR-FL (AR silencer select, Thermo Fisher, S1539) was transfected into PCa cells using Lipofectamine RNAiMax (Thermo Fisher) by reverse transfection. For generation of doxycycline-inducible LNCaP-AI-myc-Gli2$_{628-897\_3xNLS}$, two steps of lentiviral transduction were performed as previously described [31]. Briefly, stable LNCaP-AI cells expressing the Tet-Repressor (TR) were generated by transducing lentivirus (pLenti6/TR vector, Thermo Fisher) and Vira-Power™ Lentiviral Packaging Mix (Thermo Fisher). Cells expressing TR were selected in 10 μg/ml Blasticidin and subsequently transduced with lentivirus made from either pLenti4-TO (empty Control) or pLenti4-TO_myc-Gli2$_{628-897\_3xNLS}$ and were selected under 100 μg/ml Zeocin.

Other recombinant vectors

Coding sequences of GST-tagged Gli2 fusion-polypeptides, aa628–897, 628–836, 628–805, 764–836 and mutant 628–897 were subcloned into EcoRI and SalI sites of pGEX-5X-3 vector (Clonetech, Mountain View, CA). QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was utilized to introduce Aspartate (D) for Arginine (R) residues of the respective Gl2 phosphorylation sites [32]. Mutagenesis primers used were, amino acid 821 R/D and 822 R/D, 5′-C ACC GTG AGC GAC GAC TCC TCC GGC ATC TC-3′; amino acid 833 R/D and 834 R/D, 5′-TAC TTC TCC AGC GAC GAC TCC AGC GAG GCC-3′; amino acid 864 R/D and 865 R/D, 5′-ACG GAC GCG TCG GAC GAC TCG AGC GAG GCC-3′.

Antibodies and reagents

Mouse anti-human AR (441) antibody (sc-7305) was from Santa Cruz Biotech. (Santa Cruz, CA); rabbit anti-human Gli1 (2553), Ptch1 (2468), PSA (5877), β-TrCP (4394), and GAPDH (5174) antibodies were from Cell Signaling (Danvers, MA); rabbit anti-human Gli3 antibody (GTX26050) was from GeneTex Inc., Irvine, CA; mouse anti-Vinculin (V9131) from Sigma-Aldrich (Oakville, ON,

Canada) and mouse anti-myc antibody (05–724) was from EMD Millipore (Etobicoke, ON, Canada). Enz was purchased from Chemexpress Co., Ltd (Shanghai, P.R. China). R1881 was from Perkin Elmer, Shelton, CT and doxycycline was from Sigma, Aldrich and was used at 200 ng/ml where indicated.

GST pull-down assays

GST-tagged Gli2 polypeptides were isolated from transfected *E. coli* BL21 (DE3) pLysS cells (Promega, Inc) using glutathione-sepharose 4B beads (Amersham, Piscataway, NJ) and were visualized after SDS-PAGE with Coomassie blue staining. Human AR truncates (aa1-558, aa392–558) [31] or βTrCP (p4489 Flag-βTrCP, Addgene #10865 [42]) proteins were prepared by Quick Coupled T7 T_NT in vitro transcription /translation kit (Promega) in the presence of methionine. These proteins were pre-cleared with GST-bound glutathione beads prior to incubation with GST-tagged recombinant proteins. Immune complexes captured on glutathione beads were eluted for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blot analysis with appropriate antibodies. For controls, in vitro translated AR truncates and β-TrCP proteins were incubated with GST beads alone and eluates were analyzed as above.

Western blot and co-immunoprecipitation

Cell extracts were prepared in 1% NP-40 lysis buffer as described [31] with 50 mM Na F, 1 mM Orthovanadate, 10 mM Iodoacetamide, 1 mM EDTA, 0.25% Na Deoxycholate, 1 mM PMSF, and protease inhibitor cocktail (Roche Diagnostics, Laval, QC, Canada). For Co-Immunoprecipitation, 1.5–3 mg of whole cell lysates were pre-cleared with isotype matching IgG for 1 h at room temperature, prior to incubation with 4–6 µg (per mg lysates) rabbit anti-Gli3 / mouse anti-AR antibodies overnight at 4 °C. Immune-complexes were precipitated with 30 µl 50% slurry of Protein A/G-Agarose (Thermo Fisher) for an additional 4 h. For SDS-PAGE, 30–50 µg of cell protein extracts and immunoprecipitates were resolved in pre-cast 4–15% gradient gels (BioRad, Mississauga, ON, Canada), followed by western blot with appropriate antibodies and detection using ECL (Amersham Biosciences).

Proximity ligation in situ assay (PLA) and confocal microscopy

PLA was performed with Duolink® In Situ Detection Reagent Fluorescence Kit (Sigma-Aldrich). Briefly, cells were grown on glass coverslips and fixed with 4% PFA for 30 min, followed by permeabilization with 0.5% Triton-X in PBS for 5 min. Cells were blocked with manufacturer-provided blocking solution for 30 min at 37 °C and incubated overnight at 4 °C in a humidified chamber with a mixture of rabbit anti-Gli3 and mouse anti-AR antibodies. Samples were then incubated for 1 h at 37 °C with the corresponding PLA probes (anti-rabbit Plus and anti-mouse Minus, provided in kit) conjugated to specific oligonucleotides. The PLA oligonucleotides were hybridized and circularized by ligation (30 min at 37 °C) and amplification (100 min at 37 °C). The visualization of PLA signals was on a Zeiss LSM 780 Confocal Laser Scanning Microscope (Carl Zeiss, Germany). Quantification of PLA staining was done with Duolink® ImageTool softwear (Sigma-Aldrich).

Quantitative real-time PCR (qPCR)

Forty-eight or seventy-two hours after siRNA transfection, total RNA was extracted using TRIzol (Thermo Fisher), according to manufacturer's instruction. 1 µg of total RNA was used to synthesize cDNA as previously described [31]. Primer sequences employed for PCR amplification are as follows: PSA, forward, 5′-AGT GCG AGA AGC ATT CCC AAC-3′; reverse, 5′-CCA GCA AGA TCA CGC TTT TGT T-3′; KLK2, forward, 5′-GCT GCC CAT TGC CTA AAG AAG-3′, reverse, 5′-TGG GAA GCT GTG GCT GAC A-3′; Gli1, forward, 5′-GGC TCG CCA TAG CTA CTG AT-3′, reverse, 5′-CCA GCG CCC AGA CAG AG-3′; Ptch1, forward, 5′-CTG GGA GAA GAC GGA GGA GC-3′, reverse, 5′-CAC AGC TCC TCC ACG TTG GT-3′; 18 s, 5′-TTG ACG GAA GGG CAC CAC CAG-3′, reverse, 5′-GCA CCA CCA CCC ACG GAA TCG-3′. qPCR was done with SYBR GREEN PCR Master Mix (Roche Diagnostics.) and analyzed in ABI PRISM 7900 HT Fast Real-Time System (Applied Biosystems, Streetsville, ON, Canada). Real-time cycle condition were 2 min at 50 °C, followed by 10 min at 95 °C then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. 18 s gene was used as an internal control, and relative fold changes were calculated based on $2^{(-\Delta\Delta Ct)}$ method (http://docs.appliedbiosystems.com/pebiodocs/00105622.pdf).

Luciferase reportor assays

PCa cells were transiently co-transfected with pGL4.28-Gli-luc and pCMV-eGFP, along with control or AR-V7 vector. Scrambled siRNA or siRNA against AR-FL was applied 24 h after plasmids transfection, when used. Luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Inc.) on the Tecan infinite 200 Pro (Tecan Group Ltd., Männedorf, Switzerland). Values were normalized to relative GFP intensity.

Statistical analysis

All values in the figures are presented as mean $\pm$ SD with $n \geq 3$ independent observations. Statistical analyses were performed using Student's t test or Kruskal–Wallis test followed by Dunn's correction with GraphPad Prism (GraphPad Software, Inc. Version 6.0.1, La Jolla, CA). A P-value < 0.05 was considered statistically significant.

Compliance with ethical standards

Conflict of interest All authors declare that they have no competing interests.

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