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Restoration of *PPP2CA* expression reverses epithelial-to-mesenchymal transition and suppresses prostate tumour growth and metastasis in an orthotopic mouse model

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Background: Emergence of castration-resistance in prostate cancer (PCa) is invariably associated with aggressive and metastatic disease. Previously, we reported promotion of castration-resistance upon downregulation of *PPP2CA* (encoding catalytic subunit of protein phosphatase 2A (PP2A), α -isoform); however, its role in PCa growth and metastasis remained undetermined.

Methods: *PPP2CA* was overexpressed/silenced in PCa cells by stable transfection. Gene expression was examined by reverse transcription polymerase chain reaction, immunoblot and immunofluorescence analyses, and transcriptional activity measured by luciferase-based promoter-reporter assay. Effect on PCa phenotype was studied *in vitro* and in orthotopic mouse model, and immunohistochemical/histological analyses performed to assess proliferation/apoptosis and confirm metastatic lesions.

Results: An inverse association of *PPP2CA* expression was observed with epithelial-to-mesenchymal transition (EMT) and aggressive PCa phenotype. *PPP2CA* restoration resulted in decreased nuclear accumulation and transcriptional activity of β -catenin/NF- κ B, and restitution of their activity abrogated *PPP2CA*-induced EMT reversal and suppression of PCa invasiveness. Akt mediated *PPP2CA* loss-induced nuclear accumulation of β -catenin/NF- κ B through inactivation of Gsk3- β and I κ B- α , respectively. Animal studies revealed a suppressive effect of *PPP2CA* expression on PCa growth and metastasis.

Conclusions: Our findings suggest that *PPP2CA* downregulation serves as a molecular link between gain of castration-resistance and aggressive PCa phenotype, and its restoration could be an effective preventive/therapeutic approach against the advanced disease.

Prostate cancer (PCa) is the most commonly diagnosed non-cutaneous malignancy and second leading cause of cancer-related death in American men. The American Cancer Society estimates that in 2014, there will be 233 000 new diagnoses of PCa, and nearly 29 480 patients will die of this disease (Siegel *et al*, 2014). Owing to widespread screening, most patients are now diagnosed with localised prostate tumours; however, a significant proportion still continues to present with locally advanced or metastatic

disease (Cooperberg *et al*, 2005). At the present time, there is no effective cure for the advanced disease and androgen deprivation therapy (ADT) remains the principal treatment option (Feldman and Feldman, 2001). Unfortunately, most PCa patients treated with ADT eventually acquire castration-resistant phenotype, a form, which is highly aggressive and unresponsive to other therapies (Joly and Tannock, 2004; Sridhar *et al*, 2013). Therefore, characterisation of molecular targets facilitating PCa progression

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and metastasis, and deciphering their mechanisms of action is critically important to develop effective treatment approaches.

Protein phosphatase 2A (PP2A) is the most abundant serine/threonine phosphatase in mammals and has important roles in several biological processes (Mumby, 2007; Perrotti and Neviani, 2013). It consists of a common heteromeric core enzyme, which is composed of a catalytic subunit and a constant regulatory subunit that associates with a variety of regulatory subunits (Janssens and Goris, 2001). Previously, we identified that *PPP2CA*, which encodes the alpha isoform of the catalytic subunit (PP2A α), is one of the downregulated genes in castration-resistant PCa cells (Singh *et al*, 2008). More importantly, an inverse correlation of *PPP2CA* expression with increasing Gleason grades and tumour stage was also observed (Singh *et al*, 2008). In other recent studies, we have observed that *PPP2CA* downregulation confers androgen depletion resistance to PCa cells (Bhardwaj *et al*, 2011). The data revealed that the loss of PP2A-mediated checkpoints led to activation of Akt and ERK, and partially sustained androgen receptor signalling under steroid-deprived condition (Bhardwaj *et al*, 2011). Interestingly, studies from other groups have reported loss and functional significance of additional PP2A subunits (PP2A-C β , PP2A-B γ and PP2A-A α) as well; suggesting that dysregulation of PP2A may be a frequent occurrence in PCa pathogenesis (Prowatke *et al*, 2007; Bluemn *et al*, 2013; Pandey *et al*, 2013).

As emergence of castration-resistance in PCa is invariably associated with highly aggressive and metastatic disease (Jennbacken *et al*, 2006; Srivastava *et al*, 2012), it appears that there may be a common molecular thread for these distinct phenotypes. If such an association is characterised at the molecular level, it can have significant impact on the management of PCa. Therefore, this study was aimed at determining the role of *PPP2CA* in PCa progression and metastasis. We developed 'paired' PCa cell lines, in which *PPP2CA* expression is either stably 'restored' or 'silenced' through genetic engineering approaches. Using these cell lines, we demonstrate that *PPP2CA* downregulation promotes migration and invasion of PCa cells. Furthermore, our data show that *PPP2CA* expression is associated with loss of mesenchymal and gain of epithelial characteristics. Mechanistically, we find important roles of Akt-driven β -catenin and NF- κ B activation in *PPP2CA* downregulation-induced epithelial-to-mesenchymal transition (EMT) and invasiveness of PCa cells. Finally, our data demonstrate that restoration of *PPP2CA* expression attenuates PCa growth and metastasis in an orthotopic mouse model. Thus, our findings suggest that *PPP2CA* downregulation may be a common link between castration-resistance and aggressive tumour phenotypes, and its targeting may be useful in stalling PCa progression as well as in the treatment of the advanced disease.

MATERIALS AND METHODS

Cell lines, antibodies and plasmids. All cell lines (LNCaP, C4-2 and PC3) were procured, maintained and validated intermittently as described earlier (Srivastava *et al*, 2012). Anti-PP2A α (rabbit polyclonal) and anti-cytokeratin 18 (mouse monoclonal) antibodies were purchased from Abcam (Cambridge, MA, USA). Antibodies against ERK1/2, NF- κ B/p65, Slug (rabbit monoclonal), pERK1/2, I κ B- α (mouse monoclonal) and p-I κ B- α (rabbit polyclonal) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies against Akt, p-Akt and vimentin (rabbit monoclonal) were from Epitomics (Burlingame, CA, USA). Antibodies against β -catenin, E-cadherin and N-cadherin (all mouse monoclonal) were from BD transduction laboratories (Bedford, MA, USA). Anti- β -actin (mouse monoclonal) antibody was from Sigma-Aldrich (St Louis, MO, USA). Anti-Twist (rabbit

polyclonal), anti- α -tubulin, anti-laminin (mouse monoclonal), and all HRP-, FITC- and TRITC-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). *PPP2CA*-short hairpin RNA expression plasmid (*PPP2CA*-shRNA-pGFP-V-RS), non-targeted control plasmid (NT-shRNA-pGFP-V-RS), *PPP2CA* overexpression (*PPP2CA*-pCMV6) and empty vector (pCMV6) constructs were purchased from Origene (Rockville, MD, USA). pGL4.32 (luc2P/NF-B-RE/Hygro) and pRL-TK plasmids were from Promega (Madison, WI, USA). TOPflash or FOPflash reporter plasmids were kindly provided by Dr R Samant, UAB, Birmingham, AL, USA. pcDNA3- β -catenin S33Y (plasmid number 19286), pCMV-IKK β S177E S181E (plasmid number 11105) and pcDNA3-HA PKB T308D S473D (plasmid number 14751) were from E Fearon, A Rao and J Woodgett Laboratories, respectively, and procured through Addgene (Cambridge, MA, USA).

Transfections and treatments. For overexpression (in PC3 and C4-2) and knockdown (in LNCaP) of *PPP2CA*, cells were transfected with *PPP2CA*-overexpressing or shRNA plasmids, respectively, along with their control plasmids (empty vector for overexpression and non-targeted control vector for shRNA) using FuGENE (Roche, Mannheim, Germany) as a transfection reagent following the manufacturer's instructions. Stable pooled population of transfected cells were obtained using selective antibiotic containing media (G418, 200 μ g ml⁻¹ or puromycin 2 μ g ml⁻¹). Following selection, cells were expanded and examined for stable *PPP2CA* overexpression or silencing. Cells were transiently transfected with constitutively active mutant plasmids of IKK β , β -catenin and PKB, or with respective empty vector plasmids using FuGENE as per the manufacturer's instructions. To dissect the role of the Akt signalling pathway, cells were treated with 20 mM LY294002 (PI3K inhibitor; Cell Signaling Technology) for various time intervals as described in respective figure legends.

RNA isolation and reverse transcription polymerase chain reaction (RT-PCR). Total RNA was isolated from the cultured cells using RNeasy Kit (Qiagen, Gaithersburg, MD, USA). Subsequently, complementary DNA (cDNA) was synthesised using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA). Quantitative real-time PCR was performed in 96-well plates using SYBR Green Master Mix (Applied Biosystems) on an iCycler system (Bio-Rad, Hercules, CA, USA). Specific sequences of PCR primers used in this study are listed in Supplementary Table S1. The thermal conditions for real-time PCR assays were as follows: cycle 1: 95 °C for 10 min, cycle 2 (\times 40): 95 °C for 10 s and 58 °C for 45 s. Threshold cycle (C_T) values for each were separately normalised against C_T values for GAPDH, and a relative fold change in expression with respect to a reference sample was calculated by the $2^{-\Delta\Delta C_T}$ method.

PP2A activity assay. Protein phosphatase 2A activity was determined using PP2A immunoprecipitation phosphatase assay kit as described earlier by us (Bhardwaj *et al*, 2011).

Nuclear and cytoplasmic fractionation. The preparation of cytoplasmic and nuclear extracts was performed using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA) as described previously by us (Arora *et al*, 2011).

Immunoblot analysis. Immunoblotting was performed as described earlier (Bhardwaj *et al*, 2011) using specific antibodies against various proteins. β -Actin, α -tubulin and laminin were used as loading controls for total, cytoplasmic and nuclear proteins, respectively. All the primary antibodies were used at 1:1000 dilution except antibodies against Twist, α -tubulin and laminin, which were used at 1:200 dilution. All the secondary antibodies were used at 1:2500 dilution.

Immunofluorescence assay. Actin staining was performed as described previously by us (Srivastava *et al.*, 2012). For β -catenin and NF- κ B/p65 staining, cells were fixed in ice-cold methanol, washed, blocked and incubated with respective antibodies diluted in antibody diluent (1:50) for 90 min at room temperature followed by washing. Cells were then incubated with FITC- or TRITC-conjugated goat anti-rabbit secondary antibodies (1:500) for 60 min. Thereafter, cells were washed, mounted with antifade Vectashield mounting medium (Vector Laboratories Inc., Burlingame, CA, USA) and observed under Nikon Eclipse TE2000-U fluorescent microscope (Nikon Instruments Inc., Melville, NY, USA).

TCF/LEF/ β -catenin and NF- κ B transcriptional activity assays. To examine the effect of PPP2CA modulation on transcriptional activity of LEF/TCF and NF- κ B, cells were transiently co-transfected with the luciferase promoter-reporter constructs (TOPflash or FOPflash, or pGL4.32 (luc2P/NF- κ B-RE/Hygro)) and pRL-TK (control reporter plasmid containing a *Renilla reniformis* luciferase gene downstream of the TK promoter). After 24 h of transfection, total protein was harvested in reporter lysis buffer (Promega). Firefly and Renilla luciferase activities were measured using a dual-luciferase assay kit (Promega) according to the manufacturer's instructions.

Motility and invasion assays. Effects of the modulation of PPP2CA expression on the migration and invasion ability of PCa cells were examined using non-coated or Matrigel-coated transwell chamber by following the previously described procedure (Srivastava *et al.*, 2012).

Orthotopic xenograft mouse tumour model. All animal experiments were performed in compliance with Institutional Animal Care and Use Committee (IACUC) guidelines. Immunodeficient male mice (4- to 6-week old; Harlan Laboratories, Prattville, AL, USA) were anaesthetised with intraperitoneal injection of ketamine (100 mg kg⁻¹) and xylazine (15 mg kg⁻¹). After cleaning their abdomen, a small midline incision was made to expose the prostate gland, and cells (1 × 10⁶ suspended in 50 μ l of HBSS medium) were injected into the dorsal prostatic lobe. The abdominal wound was closed in two layers and animals were monitored every alternate day. At the end point (30 days post-implantation), mice were killed by CO₂ asphyxiation and autopsied. Prostate tumours were resected, weighed and measured for their dimensions using Vernier Calipers. Tumour volume was calculated by the following formula: $(A \times B^2)/2$, where *A* is the larger and *B* is the smaller of the two dimensions. To examine the metastases, distinct organs (liver, lung, bone and lumbar lymph nodes) were dissected and fixed in Bouin's solution. Numbers of visible metastatic nodules were counted (in case of liver and lung) and total mass of collected lumbar lymph nodes was recorded.

IHC and histological analyses. Immunohistochemical (IHC) analysis was performed on deparaffinised and rehydrated tissue sections from formalin-fixed, paraffin-embedded blocks of orthotopically developed prostate tumours. In brief, 5- μ m thick tumour sections were deparaffinised using EZ-Dewax (Biogenex, Fremont, CA, USA) and incubate in methanol containing 3.0% hydrogen peroxide for 30 min to block endogenous peroxidase activity. Thereafter, antigen retrieval was achieved by using decloaking Chamber (Biocare Medical, Concord, CA, USA) according to the manufacturer's protocol. Later sections were blocked for 10 min with Background Sniper (Biocare Medical) and incubated with the following primary antibodies for 60 min at room temperature. Subsequently, sections were incubated at room temperature with recommended polymer and probe (Biocare Medical) according to the manufacturer's protocol. Immunoreactivity was visualised by using DAB Chromogen followed by haematoxylin counterstain. Negative control tissues were also incubated in all reagents

with no primary antibody. Apoptotic cells in tumour samples were identified by using DeadEnd Fluorometric TUNEL System (Promega) according to the manufacturer's instructions. Presence of a reddish brown precipitate indicated a positive reaction. The number of proliferative and apoptotic cells were counted in ten random view fields ($\times 200$ magnification) in a double-blinded manner and expressed as average number of cells per field view. Histological examination followed by haematoxylin and eosin (H&E) staining was performed on the Bouin's solution-fixed, paraffin-embedded tissue sections of the livers, lungs, lymph nodes and bones to examine the presence of metastatic tumour nodules. In case of bone, specimens were decalcified in 10% EDTA in PBS for 14 days at 4 °C before paraffin embedding.

Statistical analysis. All the experiments were performed at least three times, independently and all data are expressed as 'mean \pm s.d.' Wherever appropriate, the data were also subjected to unpaired two-tailed Student's *t*-test. $P < 0.01$ was considered statistically significant.

RESULTS

PPP2CA expression is inversely associated with migratory and invasive potential of PCa cells. To investigate the role of PPP2CA in the aggressive malignant behaviour of PCa cells, we overexpressed it in C4-2 and PC3 (low PPP2CA expressing) and silenced in LNCaP (high PPP2CA expressing) cells by stable transfection. Expression and activity of its encoded protein (PP2A α) were assessed in stable transfectants by immunoblot and malachite green-based assays, respectively. The data demonstrate significant overexpression and enhanced activity of PP2A α in C4-2-PPP2CA and PC3-PPP2CA cells as compared with their respective controls (C4-2-Neo and PC3-Neo; Figures 1A and B). Similarly, PPP2CA-knockdown LNCaP cells (LNCaP-shPPP2CA) exhibit reduced expression and activity of PP2A α in comparison with its non-targeted scrambled sequence expressing control (LNCaP-NT) cells (Figures 1A and B). Furthermore, consistent with our prior observation in transient assays (Bhardwaj *et al.*, 2011), PPP2CA-overexpressing C4-2 and PC3 cells exhibit decreased phosphorylation of Akt and ERK (PP2A substrates), while it is increased in PPP2CA-silenced LNCaP cells (Supplementary Figure S1).

We next examined the effect of PPP2CA overexpression or silencing on the migration and invasive potential of PCa cells. Our data show that PPP2CA-overexpressing C4-2 and PC3 cells have significantly ($P < 0.01$) reduced migratory (2.3- and 2.2-fold, respectively) and invasive (2.7- and 2.8-fold, respectively) potential as compared with their respective controls (Figure 1C). Similarly, we also observe a significant gain ($P < 0.01$) in migratory (2.4-fold) and invasive (3.0-fold) behaviour of PPP2CA-knockdown LNCaP cells (Figure 1C). Together, these data indicate that PPP2CA suppresses aggressive behaviour of the PCa cells.

Loss of PPP2CA facilitates EMT transition in PCa cells. Several lines of evidence indicate that increased malignant potential of cancer cells is associated with their transition from epithelial-to-mesenchymal phenotype, a process referred as EMT (Kang and Massague, 2004; Nauseef and Henry, 2011). Therefore, we investigated whether altered expression of PPP2CA had an impact on EMT process. For this, we first examined actin organisation in PPP2CA-overexpressing or PPP2CA-knockdown PCa cells considering the fact that actin-dependent membrane protrusions act as critical determinants of EMT (Shankar *et al.*, 2010). Staining of filamentous-actin with FITC-conjugated phalloidin revealed the presence of many filopodial structures in low PPP2CA-expressing (C4-2-Neo, PC3-Neo and LNCaP-shPPP2CA) cells, while they were absent or less obvious in the high

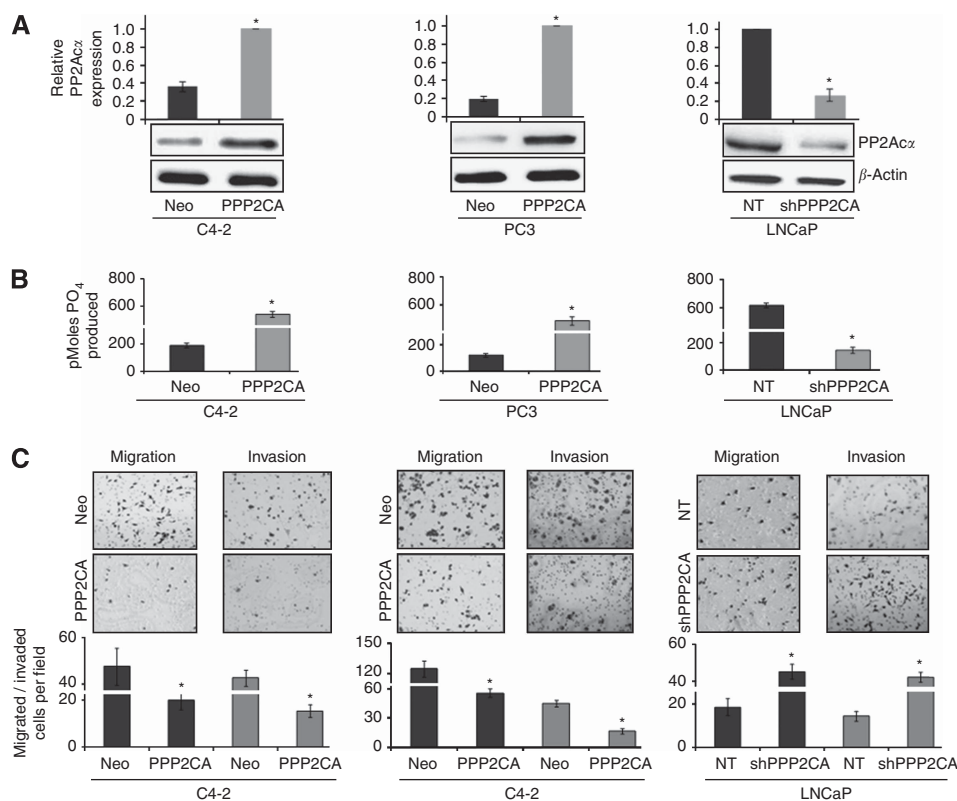


Figure 1. Overexpression of *PPP2CA* suppresses malignant behaviour of PCa cells. Expression (A) and activity (B) of PP2Acz in stable pooled populations of *PPP2CA*-overexpressing (C4-2- and PC3-*PPP2CA*) and *PPP2CA*-silenced (LNCaP-sh*PPP2CA*) PCa cells, along with their respective empty vector (-Neo) and non-targeting scrambled-shRNA (-NT) transfected control cells were examined by immunoblot analysis and malachite green-based assay, respectively. β -Actin was used as a loading control in immunoblot analysis. (C) Cells were seeded in a transwell chamber (8 μ m pore size, non-coated or Matrigel-coated) and allowed to migrate or invade overnight under chemotactic drive. After overnight incubation, the cells that did not migrate or invade through the membranes were removed and migrated/invaded cells were stained using a commercial kit. Images were taken in 10 random fields (magnification \times 100) and cell number counted. The data are presented as mean \pm s.d., (n = 3). *P < 0.01.

PPP2CA-expressing (C4-2-*PPP2CA*, PC3-*PPP2CA* and LNCaP-NT) cells (Figure 2A). To further confirm the role of *PPP2CA* on EMT, we examined the expression of markers associated with epithelial (E-cadherin and cytokeratin-18) and mesenchymal (N-cadherin, vimentin, Twist and Slug) phenotypes by qRT-PCR and immunoblot assays. Our data show that suppression of *PPP2CA* in LNCaP cells causes increased expression of mesenchymal markers, whereas those associated with epithelial phenotype are suppressed both at transcriptional (Figure 2B) and protein levels (Figure 2C). On the other hand, *PPP2CA*-overexpressing C4-2 and PC3 cells exhibit greater expression of epithelial and reduced expression of mesenchymal markers as compared with their respective controls (Figures 2B and C). Together, these findings suggest that loss of *PPP2CA* expression facilitates EMT in PCa cells.

Activation of β -catenin and NF- κ B is involved in *PPP2CA* downregulation-induced EMT and aggressive behaviour of PCa cells. To explore the mechanistic basis of *PPP2CA* loss-induced EMT, we focused on β -catenin and NF- κ B, which have earlier been shown to be aberrantly activated in PCa (Ross *et al*, 2004; Jaggi *et al*, 2005; Lessard *et al*, 2006), and implicated in transcriptional regulation of EMT markers (Min *et al*, 2008; Li *et al*, 2012, 2013). Our data from luciferase-based promoter-reporter assays show decreased transcriptional activity (>2.4-fold) of both LEF/TCF/ β -catenin and NF- κ B in *PPP2CA*-overexpressing PC3 cells as compared with that in control cells (Figure 3A). Similarly, we observe gain of transcriptional activity (>2.8-fold) of both LEF/TCF/ β -catenin and NF- κ B in *PPP2CA*-silenced LNCaP cells,

when compared with non-targeted siRNA-expressing cells (LNCaP-NT) (Figure 3A). In accordance with these findings, our immunoblot analysis reveal decreased nuclear accumulation of β -catenin and NF- κ B that correlate with their increased cytoplasmic levels in high *PPP2CA*-expressing (PC3-*PPP2CA* and LNCaP-NT) cells as compared with their low *PPP2CA*-expressing sublines (PC3-Neo and LNCaP-sh*PPP2CA*) (Figure 3B). These findings are further confirmed in immunofluorescence assay. The data show that β -catenin is predominantly localised in the membrane region in *PPP2CA*-overexpressing LNCaP (endogenous) and PC3-*PPP2CA* (exogenous) cells, whereas its nuclear staining is prominent in *PPP2CA*-silenced LNCaP or low endogenous *PPP2CA*-expressing PC3 cells. Similarly, we observe reduced nuclear localisation of NF- κ B in *PPP2CA*-overexpressing LNCaP-NT and PC3-*PPP2CA* cells as compared with low *PPP2CA*-expressing (PC3-Neo and LNCaP-sh*PPP2CA*) cells (Figure 3C).

We next investigated the involvement of β -catenin and NF- κ B in *PPP2CA*-induced EMT reversal and suppression of migration and invasion. For this, we transfected *PPP2CA*-overexpressing PC3 cells with constitutively active mutants of β -catenin (β -catenin-S33Y) and/or IKK β (IKK β -SSEE) along with their respective control vectors (pcDNA3 and pCMV6). Our data show that transfection of active β -catenin and IKK β mutants efficiently restores the β -catenin and NF- κ B transcriptional activity, respectively, in PC3-*PPP2CA* cells, while it remains suppressed in control-transfected cells as observed in promoter-reporter assay (Figure 3D). This is accompanied by enhanced nuclear

accumulation of β -catenin and NF- κ B in active mutant-transfected PC3-PPP2CA cells (Figure 3E, upper panel). Interestingly, we also observe that activation of either β -catenin or NF- κ B led to partial loss of epithelial (E-cadherin) and regain of mesenchymal (Twist) markers, while their simultaneous activation reverse PPP2CA-induced EMT completely (Figure 3E, lower panel). In addition, migration and invasive potential of PC3-PPP2CA cells is also restored upon forced activation of β -catenin and NF- κ B (Figure 3F). Together, these findings indicate that both β -catenin and NF- κ B cooperatively mediate PPP2CA loss-induced EMT and invasiveness of PCa cells.

Akt mediates PPP2CA silencing-induced activation of β -catenin and NF- κ B. Having observed important roles of β -catenin and

NF- κ B in mediating the effect of PPP2CA downregulation, we next investigated the mechanism(s) underlying their suppression in PPP2CA-overexpressing PCa cells. As Akt is a target of PP2A and its downstream signalling has been shown to promote EMT and aggressive tumour phenotype (Grille *et al*, 2003; Bhardwaj *et al*, 2011; Yoo *et al*, 2011), we examined if its activation is involved in mediating enhanced transcriptional activity of β -catenin and NF- κ B upon PPP2CA silencing. For this, PPP2CA-silenced LNCaP were treated with pharmacological inhibitors of Akt (LY294002) and their effect on transcriptional activity of β -catenin and NF- κ B was examined. The data reveal that Akt inhibition leads to abrogation of PPP2CA silencing-induced stimulation of transcriptional activity of β -catenin and NF- κ B (Figure 4A, left panel). We later confirmed this finding by transfecting the PC3-PPP2CA cells

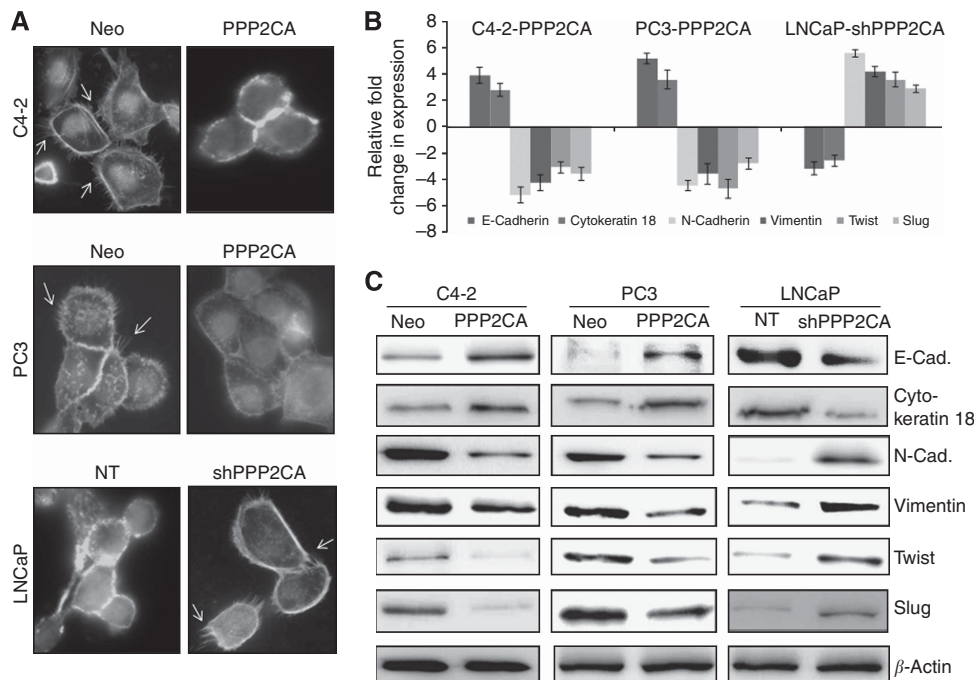


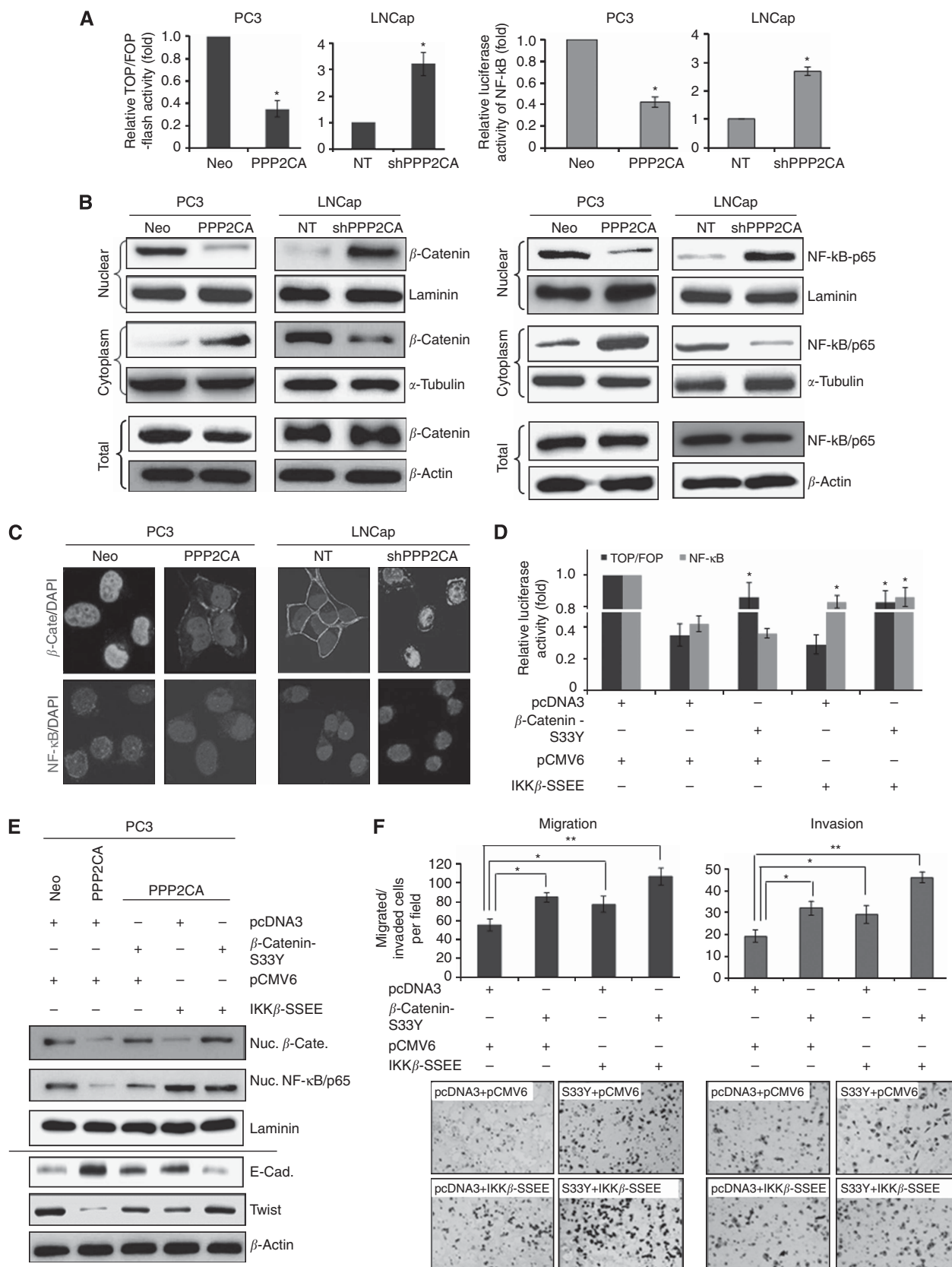
Figure 2. PPP2CA overexpression causes reversal of EMT in PCa cells. (A) PPP2CA-overexpressing or PPP2CA-silenced PCa cells were grown on fluorodish, fixed and stained with Alexa Fluor 488-conjugated phalloidin. Images were taken under confocal microscope. Data show that low PPP2CA-expressing (C4-2-Neo and PC3-Neo and LNCaP-shPPP2CA) cells exhibited several filopodia- and lamellipodia-like projections (arrows) as compared with PPP2CA-overexpressing (PC3-PPP2CA and C4-2-PPP2CA and LNCaP-NT) cells. Expression of various epithelial (E-cadherin and cyto-keratin-18) and mesenchymal (N-cadherin, vimentin, Slug and Twist) markers at (B) transcript and (C) protein levels was examined by qRT-PCR and immunoblot assays, respectively. Data are presented as fold change in expression upon PPP2CA overexpression and silencing. Bars represent mean \pm s.d., $n = 3$.

Figure 3. Suppression of β -catenin and NF- κ B is responsible for PPP2CA-mediated reversal of EMT and reduced aggressiveness.

(A) Transcriptional activities of β -catenin/LEF/TCF and NF- κ B in PPP2CA-overexpressing or PPP2CA-silenced PCa cells were measured as described in Materials and Methods section. Data are presented as fold change in normalised luciferase activity. (B) Expression level of β -catenin and NF- κ B in different cellular fractions was examined by immunoblot analysis. Laminin, α -tubulin and β -actin were used as loading controls for nuclear, cytoplasmic and total protein, respectively. (C) Cells grown on glass bottom were fixed, incubated with β -catenin or NF- κ B antibodies for 90 min at room temperature and subsequently stained using FITC-conjugated (for β -catenin) or TRITC-conjugated (for NF- κ B) goat anti-rabbit secondary antibodies for 60 min. Thereafter, cells were washed, mounted and observed under confocal fluorescent microscope. (D) PC3-PPP2CA cells were grown in six-well plate and transiently transfected with constitutively active β -catenin (β -catenin-S33Y) and IKK β (IKK β -S5EE) mutants (to activate β -catenin and NF- κ B, respectively) along with controls vectors. At 24 h post-transfection, cells were again transfected with TOPflash/FOPflash and NF- κ B luciferase promoter-reporter constructs to examine LEF/TCF and NF- κ B activity as described previously. (E) PC3-PPP2CA cells were transfected with constitutively active β -catenin and IKK β mutants or their respective control plasmids. Thereafter, nuclear (Nuc) and total protein lysates were prepared after 24 and 48 h of transfection and expression level of β -catenin and NF- κ B (after 24 h in nuclear lysate) and E-cadherin and Twist (after 48 h in total lysate) were examined by immunoblot analysis. Laminin (for nuclear fraction) and β -actin (for total protein) were used as loading controls. (F) Cells were transiently transfected with constitutive active β -catenin and IKK β mutants, trypsinised after 48 h, and re-seeded at equal density in transwell chambers (uncoated or Matrigel-coated). Numbers of migrated/invaded cells were counted in random fields following fixation and staining. Bars represent mean \pm s.d. ($n = 3$); * $P < 0.05$ and ** $P < 0.01$.

with constitutively active protein kinase B/Akt mutant or its vector only control. The data show that inhibitory effect of *PPP2CA* overexpression on transcriptional activity of TCF/LEF/ β -catenin and NF- κ B is diminished after re-activation of Akt in PC3-PPP2CA cells (Figure 4A, right panel). Accordingly, we observe an

enhanced nuclear localisation of both β -catenin and NF- κ B in PC3-PPP2CA cells upon Akt activation, whereas the opposite is observed upon Akt inhibition in LNCaP-shPPP2CA cells (Figure 4B, upper panel). In additional assays, we find an inverse association of *PPP2CA* expression with Gsk3- β and I κ B- α



phosphorylation in PCa cells (Figure 4B, lower panel). Although Gsk3- β phosphorylation leads to its inactivation, phosphorylation of I κ B- α is associated with its destabilisation as observed in our data (Figure 4B, lower panel). Together, these findings suggest a role of PP2A/Akt axis in regulation of β -catenin and NF- κ B in PCa cells.

Restored PPP2CA expression suppresses prostate tumour growth and metastasis. To examine the effect of PPP2CA restoration on tumourigenicity and metastatic potential of PCa cells, control (PC3-Neo) and PPP2CA-overexpressing PC3 cells (PC3-PPP2CA) were injected into the dorsal prostatic lobe of immunodeficient male mice (4 to 6-week old). Tumour growth was monitored by palpation and mice were killed 30 days post-injection. We observed 100% tumour incidence in both the control and PC3-PPP2CA injected mice; however, tumours in the latter group were significantly smaller. Average volume and weight of tumours in PC3-PPP2CA group were 317.1 mm³ (range from 171.5 to 490.8 mm³) and 0.31 g (range from 0.13 to 0.46 g), respectively, as compared with 1803.98 mm³ (range from 1369.9 to 2254.0 mm³) and 1.56 g (range from 1.1 to 2.11 g) in PC3-Neo group (Figures 5A and B). Immunohistochemical analyses on paraffin-embedded tumour sections revealed significant decrease (>40%; $P < 0.01$) in Ki67-positive cells, whereas a greater percentage of TUNEL-positive cells (>50%; $P < 0.01$) was observed in tumours generated from PC3-PPP2CA

cells as compared with those developed from PC3-Neo cells (Figures 5C and D).

In parallel, we also examined the effect of PPP2CA restoration on PCa metastasis. Tissues from suspected sites of metastasis (liver, lungs and lumber lymph nodes) were collected from tumour-bearing mice and fixed in Bouin's solution, and visible metastatic nodules were quantified. Data demonstrate high metastases in case of PC3-Neo mice as evident from the presence of multiple metastatic nodules in lungs and livers (Figure 6A). Furthermore, all of the tumour-bearing mice from PC3-Neo group carried enlarged lumber lymph nodes and exhibited significant differences in average weight (Figure 6A). To further confirm the presence of tumour cells in the suspected lesions, we sectioned the tissue following paraffin embedding and stained with H&E. Microscopic examination revealed the presence of tumour cell nests in stained tissue sections from PC3-Neo group as depicted in representative photomicrographs (Figure 6B). Furthermore, bone (femur) specimens from the mice were also collected, decalcified and examined for metastasis. No visible metastatic characteristics were observed in any of the bone from PC3-Neo and PC3-PPP2CA group (data not shown). Interestingly, when we examined H&E-stained tissue sections, we found presence of micro-metastatic colonies in PC3-Neo group, whereas no metastatic colonies were observed in bone specimens collected from PC3-PPP2CA group (Figure 6B, lower panel). Altogether, our data provide strong evidence for the role of PPP2CA downregulation in the progression and metastasis of PCa cells.

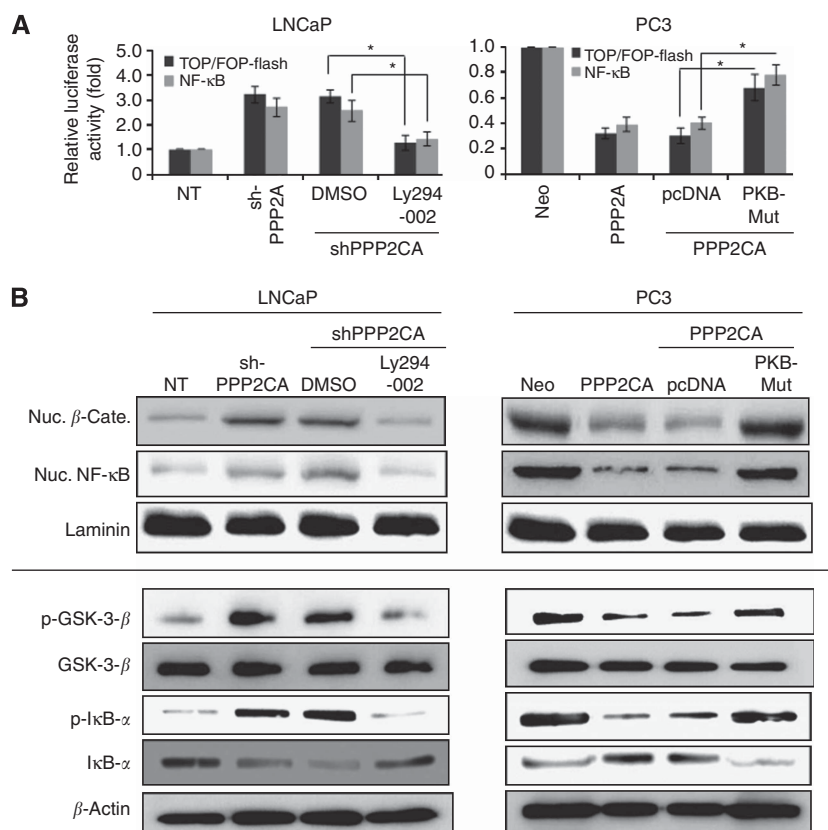


Figure 4. Role of Akt in PPP2CA overexpression-mediated suppression of β -catenin and NF- κ B. **(A)** PC3-PPP2CA cells were transiently transfected with constitutively active PKB mutant or control plasmid for 24 h, and LNCaP-shPPP2CA cells were pre-treated (for 1 h) with PI3K/Akt inhibitor (LY294002). Subsequently, cells were co-transfected with TOPflash/FOPflash/NF- κ B luciferase promoter-reporter constructs along with control plasmids for 24 h and luciferase activities were measured. Bars represent mean \pm s.d. ($n = 3$), $*P < 0.01$. **(B)** Following transfection with active PKB mutant or PI3K inhibitor treatment, nuclear and total proteins were prepared and effect on localisation of β -catenin and NF- κ B (in nuclear protein; upper panel) and on p-Gsk3- β /Gsk3- β and p-I κ B- α /I κ B- α (in total protein; lower panel) was examined by immunoblot analysis. Laminin (for nuclear protein) and β -actin (for total protein) were used as loading controls.

DISCUSSION

Several recent observations, including ours, have suggested an important role of PP2A in PCa progression and castration-resistance (Bhardwaj *et al*, 2011; Bluemn *et al*, 2013; Pandey *et al*, 2013). In the same line, this study revealed that loss of PPP2CA, a gene encoding the catalytic subunit of PP2A, promotes metastatic progression of PCa cells (Figure 7). Our data from 'gain' and 'loss' of function studies, provide compelling evidence for a role of PPP2CA in malignant behaviour of prostate tumour cells by modulating EMT process. Mechanistic studies reveal the involvement of Akt-mediated activation of β -catenin and NF- κ B in PPP2CA loss-induced EMT and potentiation of PCa cell migration and invasion. Finally, our data from *in vivo* studies establish a direct inverse association of PPP2CA expression with prostate tumour growth and metastasis.

Metastasis is a complex and multistep phenomena, which initiates and progresses as a result of several molecular alterations (Valastyan and Weinberg, 2011). The regulatory networks that control such molecular alterations in PCa cells are, however, poorly understood. Our investigations reveal a significant role of PPP2CA in reducing aggressive phenotypes, that is, invasion and migration, of PCa cells. This is highly significant considering the fact that the castration-resistant PCa cells are also highly aggressive and more metastatic than their castration-sensitive counterparts (Jennbacken *et al*, 2006; Srivastava *et al*, 2012). Epithelial-to-mesenchymal transition is an important event that aids in invasion and subsequent metastatic dissemination of tumour cells to secondary sites (Nauseef and Henry, 2011). During EMT, tumour cells acquire the expression of mesenchymal markers such as vimentin, N-cadherin, Twist and Slug, whereas epithelial markers, such as E-cadherin that promote cell-cell contact, are usually lost (Kang and Massague, 2004; Li *et al*, 2012; Srivastava *et al*, 2012). These molecular changes facilitate detachment of the tumour cell from the primary site and migration and invasion across the extracellular matrix (Valastyan and Weinberg, 2011). In

accordance with this, we observed an increased expression of epithelial and decreased expression of mesenchymal markers in PPP2CA-overexpressing cells. A similar role of PP2A in the regulation of malignant behaviour and EMT has been reported in other cancer types as well (Ito *et al*, 2000; Xu and Deng, 2006). Our *in vivo* data show that the overexpression of PPP2CA decreases the metastatic potential of PC3 cells to the various organs including liver, lung, lymph nodes and bone. Metastasis of PC3 cells to liver, lung and lymph nodes in orthotopic mouse model of PCa is widely reported (Rembrink *et al*, 1997; Wu *et al*, 2010). However, contradictory reports are available on the bone metastasis of PC3 cells upon intra-prostate implantation (Rembrink *et al*, 1997; Yang *et al*, 1999). Our findings are in consistency with the previous study published by Yang *et al* (1999), in which they observed skeletal metastases (skull, rib, pelvis, femur and tibia) of intra-prostate implanted PC3 cells.

Prostatic epithelial cells undergo EMT in response to the abnormal activation of several signalling pathways (Nauseef and Henry, 2011). Aberrant activation of Wnt/ β -catenin and NF- κ B has been linked with the high-grade PCa (Ross *et al*, 2004; Jaggi *et al*, 2005; Lessard *et al*, 2006). Moreover, multiple reports document the involvement of Wnt/ β -catenin as well as NF- κ B in the regulation of EMT and aggressive phenotypes of various tumour cells (Ganesan *et al*, 2008; Pantuck *et al*, 2010; Wu *et al*, 2012; Li *et al*, 2012, 2013). In these contexts, we investigated the involvement of β -catenin and NF- κ B in the PPP2CA-mediated regulation of EMT and aggressive phenotype of PCa cells. The data demonstrate that activation status of both β -catenin and NF- κ B is inversely correlated with the expression of PPP2CA. In general, activation of the Wnt pathway is associated with the nuclear accumulation of β -catenin, which then enhanced the transcriptional activity of LEF/TCF-responsive promoter through complex formation (Moon *et al*, 2004; Kypta and Waxman, 2012). A complex set of factors including GSK-3 β are involved in this signalling process. GSK-3 β acts as an inhibitor of β -catenin by inducing its phosphorylation-mediated proteolytic degradation (Moon *et al*, 2004; Kypta and Waxman, 2012). Similarly,

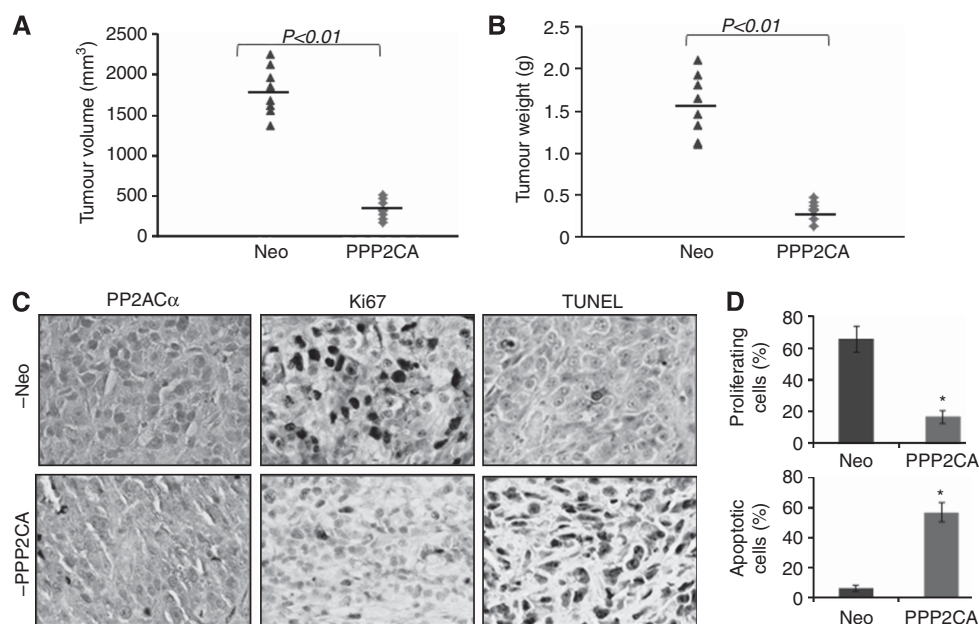


Figure 5. PPP2CA overexpression decreases tumorigenicity of PCa cells in orthotopic mice model. PC3 (control and PPP2CA-overexpressing) cells were injected into the prostate of mice ($n = 8$ per group) and killed after 30 days. (A) Volume and (B) weight of the tumours from the each animal of the control and PPP2CA-overexpressing group were calculated. Dark line indicates the median value of the respective group. (C) Immunohistological analysis of paraffin-embedded prostate tumours for PP2A α expression (left panel), Ki67 expression (a proliferative marker; middle panel) and TUNNEL assay (right panel). (D) Number of proliferating (upper panel) and apoptotic (lower panel) cells were counted in 10 random view fields (magnification $\times 100$) in a double-blinded manner and expressed as mean \pm s.d., $n = 3$, per field view. * $P < 0.01$.

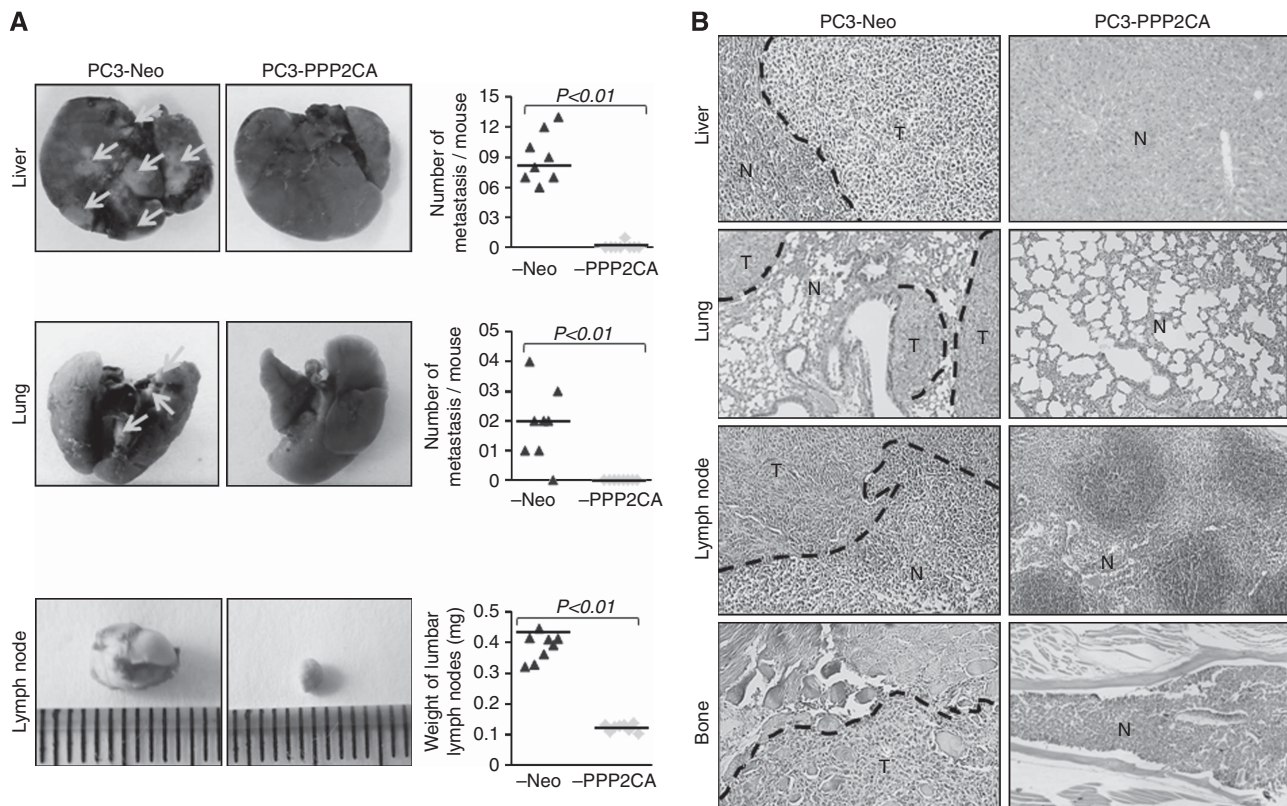


Figure 6. PPP2CA restoration decreases incidence of metastasis. (A) Liver (upper), lungs (middle) and lumbar lymph nodes (lower) from mice were excised out and fixed in Bouin's solution and number of metastatic colonies were counted (in case of liver and lungs) and total mass of all the lumbar lymph nodes was taken. Data presented as number of metastatic modules per mouse in case of liver and lungs and, total weight of the lumbar lymph nodes per mouse. Dark line indicates the median value of the group. (B) Haematoxylin and eosin staining of liver, lung, lymph node and bone sections from control and PPP2CA-overexpressing group. Mice group injected with PC3-Neo cells show metastatic deposits of cancer cells in liver, lung, lymph node and bone, whereas PPP2CA-overexpressing group had no gross evidence of deposits of cancer cells. Abbreviations: N = normal; T = tumour.

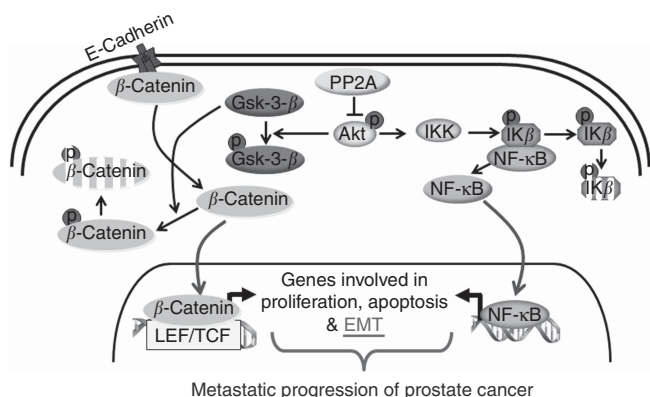


Figure 7. Diagrammatic representation of molecular pathways targeted by PP2A to suppress metastatic progression of PCa. Restoration of PP2A activity inhibits Akt phosphorylation, which then suppresses β-catenin and NF-κB through activation of GSK3β and inhibition of IKK, respectively. Inactivation of β-catenin and NF-κB leads to reversal of EMT and attenuation of tumour cell aggressiveness.

inactivation of GSK-3β leads to stabilisation of β-catenin, which subsequently enters the nucleus and promotes the expression of EMT-associated genes (Moon *et al*, 2004). On the other hand, activation of IKKβ leads to phosphorylation of IκB-α, a protein that keeps NF-κB sequestered in the cytoplasm (Gilmore, 2006). Phosphorylation of IκB-α induces its degradation and release of

NF-κB, which then translocates into the nucleus to induce gene expression (Gilmore, 2006). Our data using active β-catenin and IKKβ mutants provide convincing evidence to suggest the participation of both β-catenin and NF-κB in PPP2CA-mediated regulation of EMT and malignant properties in PCa cells.

Accumulating evidence suggests that PP2A can regulate multiple signalling pathways involved in cancer pathogenesis (Janssens and Goris, 2001; Perrotti and Neviani, 2013). In an earlier study, we also demonstrated that downmodulation of PP2A led to the activation of Akt and ERK, which sustained the growth of prostate tumour cells in androgen-depleted media (Bhardwaj *et al*, 2011). These signalling nodes were also involved in partial activation of androgen receptor signalling in a ligand-independent manner (Bhardwaj *et al*, 2011). In this study, our data reveal a role of PP2A in negative regulation of β-catenin and NF-κB through Akt inactivation. Akt-mediated regulation of NF-κB through IKK has been shown by others as well (Dan *et al*, 2008). Furthermore, a recent report suggested that carnosis acid induced growth inhibition in PCa cells involved PP2A-mediated suppression of Akt/IKK/NF-κB pro-survival signalling (Kar *et al*, 2012). In additional studies, PP2A has been shown to regulate the activity of β-catenin. Seeling *et al* (1999) reported that the regulatory subunit of PP2A (PP2A-B) interfered with the formation of APC/Axin/Gsk3β complex, an inhibitory complex of β-catenin, and thus regulated the stabilisation and nuclear translocation of β-catenin (Seeling *et al*, 1999). In addition, based on the study performed in *Drosophila*, it was proposed that PP2A55α subunit of PP2A directly interacts with β-catenin and subsequently promotes its phosphorylation and degradation (Zhang *et al*, 2009). Findings

from our work suggest that PP2A-mediated β -catenin suppression in PCa cells is mediated through GSK-3 β , which remains activated because of suppressive effect of PP2A on Akt. Similarly, Akt-mediated activation of NF- κ B through IKK is also lost, when PPP2CA expression is restored in PCa cells. These findings are suggestive of a context-dependent impact of PP2A on signalling networks, which may underlie its functional diversity in biological processes.

In summary, our findings have provided compelling support for a tumour-suppressive role of PP2A in PCa and suggest that it likely serves as a mechanistic link between castration-resistance and aggressive tumour phenotypes. In light of other recent observations, it appears that dysregulation of PP2A through altered expression of its catalytic or regulatory subunits may be a common phenomenon in advanced PCa. On these bases, it can be suggested that PP2A signalling is a key regulator of PCa progression and metastasis and its targeting may be useful in preventing the disease progression and/or treatment of the disease that has already advanced.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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