Supplemental Methods

Cells. Du145 PCa cells were obtained from the American Type Culture Collection (Rockville, MD) and were maintained on plastic in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), l mM sodium pyruvate, lx penicillin-streptomycin, 0.l mM nonessential amino acids, 2 mM L-glutamine, and lx vitamin solution (Invitrogen, Carlsbad, CA), at 37°C in 5% CO_2-95% air. Du145 were transduced with lentiviral constructs to produce a stable pool of cells expressing a non-targeting shRNA control or the p21 shRNA 888 (Open Biosystems, Huntsville, AL). Murine bone marrow stromal ST-2 cells were obtained from RIKEN Cell Bank (Ibaraki, Japan) and maintained in α-MEM supplemented with 10% FBS, l mM sodium pyruvate, lx penicillin-streptomycin, and 2 mM L-glutamine. All cells were shown to be free of *Mycoplasma* by the PlasmTest mycoplasma detection method (Invivogen, San Diego, CA).

***In vitro* attachment dependent growth assay.** Cellular proliferation was determined as previously described (5). Briefly, cells were plated in 96 well plates at a density of $1.5x10^3$ cells per 0.2 ml/well in complete medium in triplicate. The total number of viable cells on one plate was determined every 24 hours by the addition of a final concentration of 1 mM MTT (3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide). The formazan product was dissolved in DMSO and absorbencies were read at 570 nm on a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA).

Attachment independent survival assay. Control shRNA and DKK-1 shRNA 796 cells ($2.5x10^5$/2ml) were plated to Corning Ultra-low attachment tissue culture plates (Corning, Lowell, MA) and incubated for 72 hours at 37 C. The total number of viable cells on one plate was determined every 24 hours by the addition of a final concentration of 1 mM MTT. The

formazan product was dissolved in DMSO and absorbencies were read at 570 nm on a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA).

β-catenin reporter assay. On day 0, $1.2x10^5$ ST-2 cells/2 ml complete medium were plated to 6 well plates and allowed to attach 24 hours. The following day (day 1), the cells were co-transfected with the TOP-FLASH β-catenin reporter plasmid and a pRL Renilla reporter vector, the later to serve as an internal control. A β-catenin cDNA expression vector was also transfected to one well as a positive control for TOP-FLASH activity. Twenty four hours later (day 2), the media was removed and replaced with complete medium, 50 ng/ml recombinant Wnt3a (R&D Systems, Minneapolis, MN), or a 72 hour conditioned medium prepared from PC-3 control shRNA or DKK-1 shRNA 796-transduced cells. On day 3, whole cell lysates were prepared and evaluated by luciferase expression using the dual luciferase reporter assay (Promega, Madison, WI).

Measurements of steady-state and nascent mRNA synthesis. PC-3 control shRNA or DKK-1 shRNA 796-transduced cells were incubated in media containing 2 mM bromouridine (BrU) (Sigma-Aldrich, St. Louis, MO) for 60 minutes at 37°C to label nascent RNA. After the labeling, the cells were washed in PBS followed by detachment in trypsin. The cells were washed once in PBS and total RNA isolated using TRIzol according to manufacturer's instruction (Invitrogen). A portion of the RNA was set aside for steady-state determinations. To specifically isolate the BrU-labeled nascent RNA, we used magnetic beads (Dynabeads, Goat anti-Mouse IgG, Invitrogen) that had been conjugated with anti-BrU antibodies (BD Biosciences). The RNA samples (100 μg from each sample) were heated to 80°C for 10 min and added to 200 μl of anti-BrU antibody-conjugated bead slurry and the samples were incubated on a rocking platform at room temperature for 1 hour. The beads were washed three times in PBS

containing 0.1% BSA, and re-suspended in 50 µl DEPC-treated water. Finally, the suspensions were boiled for 10 minutes and the magnetic beads were captured, leaving the nascent RNA in the solution. The isolated steady-state RNA and the nascent RNA samples were processed at the Microarray Core at the University of Michigan Comprehensive Cancer Center and expression levels were determined using real-time PCR (Cell Cycle RT-PCR array, SABiosciences). The values obtained were normalized to 5 housekeeping genes on the array and expressed as fold difference in expression between the DKK-1 knocked down cells an the control cells.

Western blot for analysis. Equal amounts of protein (30 µg/sample) were resolved using 12% SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were transferred onto 0.45 µm PVDF membranes (Millipore, Bedford, MA). The filters were blocked with 5% milk in TBS-T and probed with rabbit anti-JNK pAb or anti-phospho-JNK pAb (both 1:1000, Cell Signaling, Danvers, MA). Protein bands were visualized using the ECL detection system (Cell Signaling, Beverly, MA). To normalize for differences in loading, the blots were stripped and reprobed with mouse anti-α-tubulin mAb (1:1000, Sigma, St. Louis, MO).

***In vivo* animal model of bone metastasis.** Performed as described in the Materials and Methods section of the main text.

PCR analysis. The expression of p21, DKK-1, and β-actin was evaluated by quantitative PCR as described in the Materials and Methods section of the main text.

Supplemental Figure Legends

Figure 1. DKK-1 knock-down reduces PCa cell proliferation. A) Parental PC-3 or B) Du145 PCa cells were transiently transfected with a control siRNA oligo or a DKK-1 366 targeting siRNA. After 24 hours, cells were harvested and plated in quadruplicate to four, 96 well plates ($1.5x10^3$ cells/0.2ml/well). The total number of viable cells was determined every 24 hours for four days with the addition of 1mM MTT, n=2. C) Aniokis assay. Control shRNA-transduced or DKK-1 shRNA 796-transduced cells ($2.5x10^5$ cells/well) were plated in duplicate to 3, Corning Ultra-low attachment 6 well plates. Every 24 hours, total number of viable cells was determined every 24 hours for three days with the addition of 1mM MTT.

Figure 2. DKK-1 knock-down regulates p21 expression at the post-transcriptional level and is independent of canonical Wnt signaling. A) At Day 0, ST-2 bone marrow stromal cells were transiently transfected with the β-catenin reporter construct TOP-FLASH and a Renilla control reporter vector. On Day 1, cells were fed with complete medium, 50 ng/ml rWnt3a, or 72 hour condition medium from control shRNA-transduced or DKK-1 shRNA 796-transduced cells. On day 3, the cells were lysed and luciferase activity measured by Dual-luciferase assay, n=5. B) Control shRNA-transduced or DKK-1 shRNA 796-transduced cells were evaluated for the basal JNK expression and activity. Shown is a representative western blot, n=3. C) Measurements of p21 steady-state and nascent mRNA synthesis. Steady-state and nascent RNA transcripts were isolated from control shRNA-transduced or DKK-1 shRNA 796-transduced cells and evaluated using PCR arrays. The values obtained were normalized to 5 housekeeping genes on the array and expressed as fold difference in expression between the DKK-1 shRNA 796-transduced and control shRNA-transduced cells.

Figure 3. Characterization of Du145 p21 shRNA transduced cells. Parental Du145 PCa cells were transduced with lentivirus carrying a non-targeting control shRNA or a p21 specific shRNA that targets nucleotide 888. A) RT quantitative-PCR analysis of transduced cells for DKK-1 or p21, mean±standard deviation of duplicate experiments; *$p<0.005$ compared to control shRNA cells by t-test. B) *In vitro* proliferation assay. Du145 control shRNA and p21 shRNA 888 cells were harvested and plated in quadruplicate to four, 96 well plates ($1.5x10^3$ cells/0.2ml/well). The total number of viable cells was determined every 24 hours for four days with the addition of 1mM MTT, n=2.

Figure 4. Knock-down of p21 alone was not sufficient to increase PCa cell proliferation. A) DKK-1^+ control shRNA-transduced or p21shRNA-transduced Du145 cells ($1x10^6$ cells/50 µl) were directly injected into the tibiae of anesthetized male nude mice (5 mice/group). Tumors were allowed to grow for 3 weeks at which time the mice were sacrificed and bones radiographed and processed for histological evaluation. Shown are representative H&E sections and corresponding x-ray of injected tibia. Key: normal trabeculae (arrow), marrow (M), tumor (T), and growth plate (arrowhead). B) Bones were subjected to DEXA to quantify bone mineral density. Data are presented as mean±standard error within each group.