SUPPLEMENTARY INFORMATION SECTION

PKN3 is required for malignant prostate cell growth downstream of activated phosphatidylinositol 3-kinase.

By

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Cell culture. HeLa, PC-3 and COS-7 cells (American Type Culture Collection) were cultured and transfected as described (Czauderna et al, 2003b; Klippel et al, 1998; Sternberger et al, 2002). 4-hydroxytamoxifen (4-OHT) was used at 200 nM. Stably transfected pools of HeLa cells were selected in 200 µg/ml Geneticin. MCF-10A cells were cultured as described (Debnath et al, 2003). Stably transfected MCF-10A populations were propagated in 200 µg/ml Geneticin, double-stables in Geneticin plus 2.5 µg/ml Blasticidin. shRNA expression was induced in the presence of 0.5-1 µg/ml Doxycycline (Dox). PC-3 cells stably expressing inducible p110α shRNA molecules have been described previously (Czauderna et al, 2003b) and cells stably expressing inducible PKN3 shRNAs were established accordingly. shRNA expression was induced in the presence of 0.1 µg/ml (Dox).

3D cultures using matrigel or collagen gels. PC-3 cells were pre-treated or not with GBs for 48 to 72 h. Alternatively, shRNA expression was induced in stably transfected cell populations in the presence of Doxycycline for 48 h. The cells were seeded on 250 µl solidified matrigel basement membrane matrix (Becton Dickinson) in duplicate 24-wells (typically 100.000 to 150.000 cells per well). After settling the cells were overlayed with 1 ml medium plus additives and incubated for the indicated times.

MCF-10A breast epithelial cells are typically analyzed in collagen gels (Grunert et al, 2003; Schulze et al, 2001; Weaver et al, 2002). For collagen gel assays duplicate samples of cells were embedded into rat tail type I collagen (Roche), after neutralization with 1/10 volume of 10x EMEM medium and 0.8 M $NaHCO_3$. Typically 60.000 to 100.000 cells were mixed with 300 µl collagen gel/24-well and plated on a foot consisting of matrigel. After gelling at 37°C cells were overlayed with 1 ml medium plus additives and incubated for the indicated times.

Plasmid constructions. Expression vectors for constitutively active PI3K (M-p110*), its kinase-defective control (M-p110Δkin), constitutively active Akt (M-Akt) and its inactive control (AktΔkin) have been described (Klippel et al, 1996; Kulik et al, 1997). The 4-hydroxytamoxifen (4-OHT) inducible form of constitutively active PI3K, M-p110*-ER, was decribed in (Klippel et al, 1998). The coding region was ligated via XhoI/BamHI ends into the retroviral expression vector pLXIN (Clontech). For stronger expression a SalI/BamHI fragment was isolated from the original vector and cloned into XhoI/BamHI ends of pLXIN, so that M-p110*-ER expression is controlled by the SRα promoter (Takebe et al, 1988). The vector expressing RasV12 in a inducible manner will be described elsewhere.

PKN3 cDNA (Oishi et al, 1999) was cloned in three portions: The C-terminus overlapping the catalytic domain was amplified by PCR using PfuTurbo DNA polymerase from a HeLa cDNA (Marathon-Ready; Clontech) with 5'-phosphorylated primer PKN3-C'-NdeI (5' p-CGT CCC CAT ATG GAG CCT AGG A) overlapping nucleotides 1594 to 1615 of the coding strand and 5'-phosphorylated primer PKN3-C'-BamHI (5' p-<u>TAG GAT CCT CAG CCG GCG CCC CCG AGC TC</u>G GGT TCC AGG AAT CGC TCT GAC ACA AAG) overlapping nucleotides 2640 to 2667 of non-coding strand of the PKN3 cDNA (A of the start codon is designated nucleotide 1; nucleotides that are changed with respect to the wt sequence are underlined). This extended the coding region by a sequence encoding amino acids ELGGAG as a glycine-rich hinge region overlapping restriction sites (SacI/Ecl136II and

SfoI/NarI and NaeI/NgoMIV)) and by a stop codon preceeding the restriction site for BamHI. The middle portion was amplified using primer PKN3-m-870 (5' p-CTG TGA ACA GTT GCT GAC AGC CGT) overlapping nucleotides 861 to 884 of the coding strand and primer PKN3-m-NdeI (5' p-CTC CAT ATG GGG ACG TTT GGT) overlapping nucleotides 1588 to 1608 of the non-coding strand. The N-terminal fragment was amplified by RACE-PCR: In the first step primer AP1 (Clontech) and primer PKN3-m-NdeI were used. In a second step a nested PCR fragment was amplified by touch-down PCR using primer PKN3-N'-NcoI (5' p-ACC ATG GAG GAG GGG GCG CCG CGG CA) overlapping nucleotides 1 to 23 of the sense strand in combination with primer PKN3-N'930 (5' p-CTT GGT CCG AAG CCA GCC CTC GGA) spanning nucleotides 925 to 948 of the non-coding strand. All three PKN3 fragments were ligated into the SmaI site of pBluescript II KS (Stratagene), and the identity of the fragments was confirmed by DNA sequence analysis.

The C-terminal PKN3 fragment encompassing the kinase domain was fused to an HA-tag at its 3' end via NdeI-SfoI ends and ligated into a mammalian expression vector (pBJ) that directs expression from the SRα promoter (Takebe et al, 1988), generating pBJ-KD-HA. The same fragment was fused to a Myc-tag at its 3' end via NdeI-SacI ends in the same vector background generating pBJ-KD-Myc. Mutations were introduced into the ATP-binding site by exchanging an invariant lysine residue at position 588 to arginine (KR588) or to glutamic acid (KE588) using the QuickChange mutagenesis kit (Stratagene); the following primers were used: KR588-s (5 'p-TAC TAC GCC ATC AGA GCA CTG AAG AAG), KR588-a (5' p-CTT CTT CAG TGC TCT GAT GGC GTA GTA); KE588-s (5' p-AAA TAC TAC GCG ATC GAA GCA CTG AAG AAG), KE588-a (5' p-CTT CTT CAG TGC TTC GAT CGC GTA GTA TTT). The KE588 mutation also introduced a PvuI site. KR588 and KE588 derivatives behaved undistinguishable with respect to expression level and loss of activity (not shown). The phosphorylation site in the activation loop was mutated by exchanging the threonine at position 718 to alanine (TA718) using primers TA718-s (5' p-GGA CCG GAC

TAG CGC TTT CTG TGG CAC C) and TA718-a (5' p-GGT GCC ACA GAA AGC GCT AGT CCG GTC C), which also introduced an Eco47III site. The respective mutations were introduced into the HA- and Myc-tagged kinase domain fragments of PKN3 and verified by sequence analysis.

pBJ-ΔN-HA and pBJ-ΔN-Myc vectors, expressing a truncated form lacking the PKN3 N-terminal domain, were generated by fusing the middle portion to the C-terminal fragment via a shared NdeI site in the coding regions in pBJ-KD-HA/Myc expression vectors; a start ATG in a consensus sequence providing for improved translational initiation (Matthias et al, 1989) was introduced via ligation of an oligonucleotide at the N-terminal XbaI site. Full-length PKN3 in pBJ-PKN3-HA/Myc was reconstituted by ligating an N-terminal fragment via XbaI and a shared NotI site in the coding regions into pBJ-ΔN-HA/Myc vectors. This N-terminal fragment was amplified using primer PKN3-N'Xba/Nco/Ase (5' p-CTT CTA GAA CCA TGG CTA TTA ATA TGG AGG AGG GGG CGC CGC GGC AGC CT) spanning nucleotides -2 to 27 of the coding strand and primer PKN3-N'930. The mutations in the catalytic domain described above were incorporated into the full length context by substituting the wt kinase domain region at the internal NdeI site and a ScaI site in the vector with the corresponding fragments isolated from the mutant KD vectors.

His-tagged PKN3 was generated after annealing primers 9His-s (5' p-C GGG GGC GCC GGC CAT CAT CAC CAT CAC CAT CAT CAC CAT GGC CAG CT) and 9His-a (5' p-G GCC ATG GTG ATG ATG GTG ATG GTG ATG ATG GCC GGC GCC CCC GAG CT) into the SacI site of pBJ-PKN3-Myc. This extended the PKN3 coding region at the C-terminus with nine histidine residues followed by a Myc-tag.

To generate a 4-OHT regulatable PKN3 derivative, PKN3 was fused to the hormone binding domain of mouse estrogen receptor (ER; amino acids 281 to 599) containing mutation GR525 (Littlewood et al, 1995). The ER fragment was isolated as SacI/BamHI fragment from the M-p110*-ER vector to replace the Myc-tag at the C-terminus of PKN3 in pBJ-PKN3-

Myc. PKN3-ER was then cloned via XhoI/BamHI into pLXIN. PKN3Δkin-ER was constructed accordingly from PKN3(KE588)-Myc.

Plasmids directing constitutive or inducible expression from a U6tetO promoter of p110α and p110β shRNA molecules have been recently described (Czauderna et al, 2003a; Czauderna et al, 2003b). Vectors for inducible expression of PKN3 shRNA were generated accordingly by cloning annealed oligonucleotides (see below) linkers into two BsmBI sites: shRNA PKN3-1 (1: c cgt GTG GAG CTG AAG GTG AAA CAG AAA AAA AAA AAA CTG TTT CAC CTT CAG CTC CAC, 2: a aaa GTG GAG CTG AAG GTG AAA CAG TTT TTT TTT TTT CTG TTT CAC CTT CAG CTC CAC); shRNA PKN3-2: (1:ccgt TAC TAC GCC ATC AAA GCA CTG AAA AAA AAA AAA CAG TGC TTT GAT GGC GTA GTA, 2: aaaa TAC TAC GCC ATC AAA GCA CTG TTT TTT TTT TTT CAG TGC TTT GAT GGC GTA GTA). Correct insertion of the respective sequences was confirmed by DNA sequence analysis.

A bacterial expression vector for overproducing a fragment comprising the 281 C-terminal amino acids of PKN3 was generated by ligating a into the BamHI site of pET23d. Vectors with the correct orientation, in which the PKN3 coding region is fused to the T7-tag at its N-terminus, were isolated.

Sequences of primers and probes used for Taqman analysis.

human PKN3

forward primer 5'-CACTTTGGGAAGGTCCTCCTG-3'

reverse primer 5'-CCTCCTGCTTCTTCAGTGCTTT-3'

Taqman probe 5'-Fam-TTCAAGGGGACAGGGAAATACTACGCCA-Tamra-3'

human PKN1:

forward primer 5'-ACATGGCCAGCGACGC

reverse primer 5'-GCCAGGCCCAGCTGCT

Taqman probe 5'-Fam-AGCCTCGCAGCTGGTCCCTGC-Tamra-3

human PKN2:

forward primer 5'-CCACTTGGGGAAGGCTAGTAAGA

reverse primer 5'-TGGCACTGTAGTAGGCACAGGA

Taqman probe 5'-Fam-TAAATCATTCTGGCACCTTCAGCCCTCA-Tamra-3

human p110α:

forward primer 5'-CAGGTGGAATGAATGGCTGAA-3'

reverse primer 5'-ACAGAGCAAATGGAAAGGCAAA-3'

Taqman probe 5'-Fam-ATTCCTGATCTTCCTCGTGCTGCTCG-Tamra-3'

GeneBloc antisense oligonucleotides. GBs represent gapmers, whereby a,c,g,u represent 2'-O-methyl ribonucleotides and A,C,G,T phosphorothioate-linked deoxyribonucleotides. Details of the synthesis and structure of the 3rd generation GB antisense molecules have been described, also the GBs for inhibiting expression of PTEN, p110α and p110β including their respective mismatch controls (Sternberger et al, 2002). Additional GB molecules used here and their corresponding mismatch (mm) control oligonucleotides have the following sequences (5' to 3'); the respective mismatch positions are underlined:

PKN3	GB1	caacacGGTTGTCCAccuuua
	GB2	ucagugCTTTGATGGcguagu
	GB3	cuucucGCAGTACAGgcucuc
	mm1	caagacGCTTGTGCAcguuua
	mm2	ucagagCTTAGTTGGcguugu
	mm3	cuugugGCACTAGAGccucuc
PKN1	GB1	uuucugCTGCACCATuguauc
	GB2	ccugacTGAGGAGTAucaggc
	GB3	ucucgaGCCACAATAucuccu
PKN2	GB1	cauugcTGTAGGTCTggauca
	GB2	ctgacuTCACTGGTGuucucg
	GB3	ggucagAGGTGACTTccucag
Akt1	GB1	accgugGAGAGATCAucugag
	GB2	gucuugATGTACTCCccucgu
	mm1	agcgugGACAGATGAucucag
	mm2	guguugATCTAGTCCccuccu

Akt2	GB1	uccuugTACCCAATGaaggag
	GB2	aguugaTGCTGAGGAagaacc
	mm1	ucguugTAGCCAATCaacgag
	mm2	acuugaTGGTGAGCAagaagc

Determination of the relative amounts of RNA levels by Taqman analysis and Northern blotting. RNA from cells was isolated and purified using the Invisorb spin cell RNA mini kit (InVitek GmbH, Berlin). RNA preparation of tumor tissue was performed using RNAzol (WAK Chemie). Relative levels of PKN3 mRNA expression were quantified by real time RT-PCR (Taqman) analysis as described (Sternberger et al, 2002). Expression of PKN3 shRNA in primary PC-3 cell prostate tumors isolated from mice was detected by Northern-blotting essentially as previously described (Czauderna et al, 2003a; Czauderna et al, 2003b). A 5'-^{32}[P]-labelled oligonucleotide (5'-TGTTTTTTTTTTTTCAGTGCTTTGATGGCGTAGTA-3') overlapping the PKN3(2) shRNA sequence was used as a probe.

In vitro PKN3 catalytic activity assay. Cell extracts were incubated with the indicated antibodies and protein A/G agarose beads. Immune complexes were washed in 50 mM Tris-HCl (pH 7.5), 500 mM LiCl and 0.5% NP-40, twice with PBS, and once with 30 mM Tris-HCl, 4 mM $MgCl_2$, all containing 25 mM β-glycerolphosphate. One half of the beads was analyzed by immunoblotting, the second half was subjected to an in vitro protein kinase activity assay. The assay was performed in 50 µl reactions containing 30 mM Tris-HCl, 4 mM $MgCl_2$, 2 µg/ml PKA inhibitor peptide, 10 mM β-glycerolphosphate, 5 mM DTT, 200 µg/ml MBP (Upstate), 25 to 100 µM ATP and 2.5 µCi [χ-^{32}P]ATP/reaction. The mixtures were incubated at room temperature for 5-10 min and separated by 16% SDS-PAGE. The incorporation of radiolabeled phosphate into MBP was detected by autoradiography.

In vivo phosphorylation of PKN3. COS-7 cells were transiently transfected with the indicated expression vectors. After 36 h cells were starved in serum-free and phosphate-free medium for 2 h and metabolically labeled in phosphate-free medium supplemented with 1

mCi of [32P]orthophosphate (8500 to 9000 Ci/mmol) per 10 cm dish for 12 h. PKN3-His was precipitated from cell extracts using limiting amounts of anti-His antibody and after a series of stringent washes (see above) analyzed by SDS-PAGE and autoradiography. Half of the precipitates were analyzed by immunoblotting to test for T-loop phosphorylation and for protein levels after reprobing with PKN3 antibody.

Immunohistochemistry, in situ hybridization and immunofluorescence analysis. For immunohistochemical analysis paraffin sections were deparaffinized, rehydrated and incubated in 3% hydrogen peroxide to block endogenous peroxidase. For antigen retrieval, sections were immersed in sodium citrate buffer (10mM sodium citrate, pH 6), boiled in a pressure cooker on a hot plate for 10 minutes and allowed to cool down. After blocking of non-specific antigen-binding sites in TBS with 10% normal goat serum, PKN3 antiserum or pre-immune serum were added at 1:2000 dilution. After incubation with a peroxidase-labeled secondary antibody, signals were developed via streptavidin-coupled peroxidase and the Vector NovaRed^{TM} kit (Vector Labs, Burlingame, CA). Sections were counterstained with hematoxilin, dehydrated and cover-slipped with mounting medium.

In situ hybridization was performed as described by (Schaefer and Day, 1995). Briefly, ^{35}S-UTP and ^{35}S-CTP-labelled antisense and sense probes were generated by in vitro transcription of PKN3 cDNA from T7/T3 promoters. The probes were subjected to mild hydrolysis in sodium carbonate buffer (80 mM $NaHCO_3$, 120 mM Na_2CO_3; pH 10.2) at 60°C to yield fragments of less than 250 bp. Deparaffinized sections were rehydrated and and permeabilized by proteinase K treatment. After refixing in 4% formaldehyde, sections were were acetylated in 0.1 M triethanolamine (pH 8) containing 0.25% acetic anhydride for 10 min. After a series of washes with increasing ethanol concentration, the samples were air-dried and then incubated with the cRNA probes in hybridization buffer (50% formamide, 10% dextran sulfate, 0.6 M NaCl, 10 mM Tris-HCl (pH 7.4), 1x Denhardt's solution, 100µg/ml sonicated salmon sperm DNA, 1 mM EDTA, 10 mM DTT) at 5 x 10^4 dpm/µl at 58°C for 16

h. The samples wer washed twice in SSC (0.3 M NaCl, 0.03 M sodium citrate;pH 7.0) and then treated with a mix of RNAse A and RNAse T1. Successive washes followed in 1x, 0.5x, and 0.2x for 20 minutes each and in 0.2x SSC at 60°C for 1 h. The dried samples were exposed to X-ray film for 2 days. For microscopic analysis, sections were dipped in Kodak NTB2 nuclear emulsion and stored at 4°C for 4 weeks. Sections were developed in Kodak D19 and fixed in Kodak Rapid Fix. Samples were digitized under darkfield illumination and pictures converted using Adobe Photoshop.

For indirect immunofluorescence analysis cells grown on chamberslides were fixed and permeabilized using Fix & Perm solutions (Caltag). Samples were incubated in blocking solution (10% FCS, 2% BSA, 0.1% TritonX-100, 0.025% Tween 20 in PBS) and then incubated with anti-tubulin antibody 1:200 diluted in blocking solution. After washing twice in PBS containing 0.1% TritonX-100 the slides were incubated with a 1:1000 dilution of Alexa-488 labelled secondary antibody (Molecular probes). After a series of washes, the slides were mounted and analyzed using a confocal LSM510 Meta microscope (Zeiss).

Overexpression and purification of the PKN3 C-terminal fragment for immunization. pET23d-PKN3-C' was introduced into BL21::DE3/pLysS. Cultures were grown to 0.6 OD_{600} and induced for 3 h in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C. Bacterial pellets were lysed (Klippel et al, 1994) and incubated for 10 min the presence of Benzonase (Merck) to reduce viscosity of the extract. The extract was cleared by centrifugation at 16,000x g for 10 min to harvest the inclusion body pellet consisting of insoluble PKN3 protein fragment. The inclusion bodies were solubilized after addition of 1% sodium dodecyl sulfate (SDS) and 2 mM dithiothreitol (DTT) and fractionated by preparative 12% SDS-PAGE. The protein band was excised from the gel, and the gel was crushed by pushing through a 5 ml syringe. After addition of 5 to 10 ml running buffer, the gel structure was further destroyed by freezing and thawing. The protein was eluted by shaking overnight at 37°C. After filtration using a Centrex MF-25 device (Schleicher & Schuell), the

recombinant protein was concentrated in a Ultrafree-4 centrifugal filter unit (Millipore). The isolated amount was quantified by SDS-PAGE relative to protein standards.

DNA array expression profiling. RNA from cells grown on matrigel was isolated using RNAzol and further purified using the Invisorb spin cell RNA mini kit (InVitek GmbH, Berlin). RNA sample collection, generation of biotinylated cRNA probe and hybridization to Affymetrix Human Genome U95 Set (HG U95A-E) was carried out essentially as described in the standard Affymetrix (Santa Clara, CA) protocol. Absolute analysis of each chip and comparative analysis of samples were carried out by using the Affymetrix software (Microarray Suite, version 5.0). Pairwise comparison was performed between samples with a hyperactivated PI3K pathway versus samples, in which PI3K signaling was inhibited. Filter criteria were applied (Schulze et al, 2001): Only probe sets with a "difference call" (decrease or increase) by the GeneChip software were allowed to pass. To account for high noise at low signal intensities, probe sets which were called "absent" were eliminated.

Supplementary References

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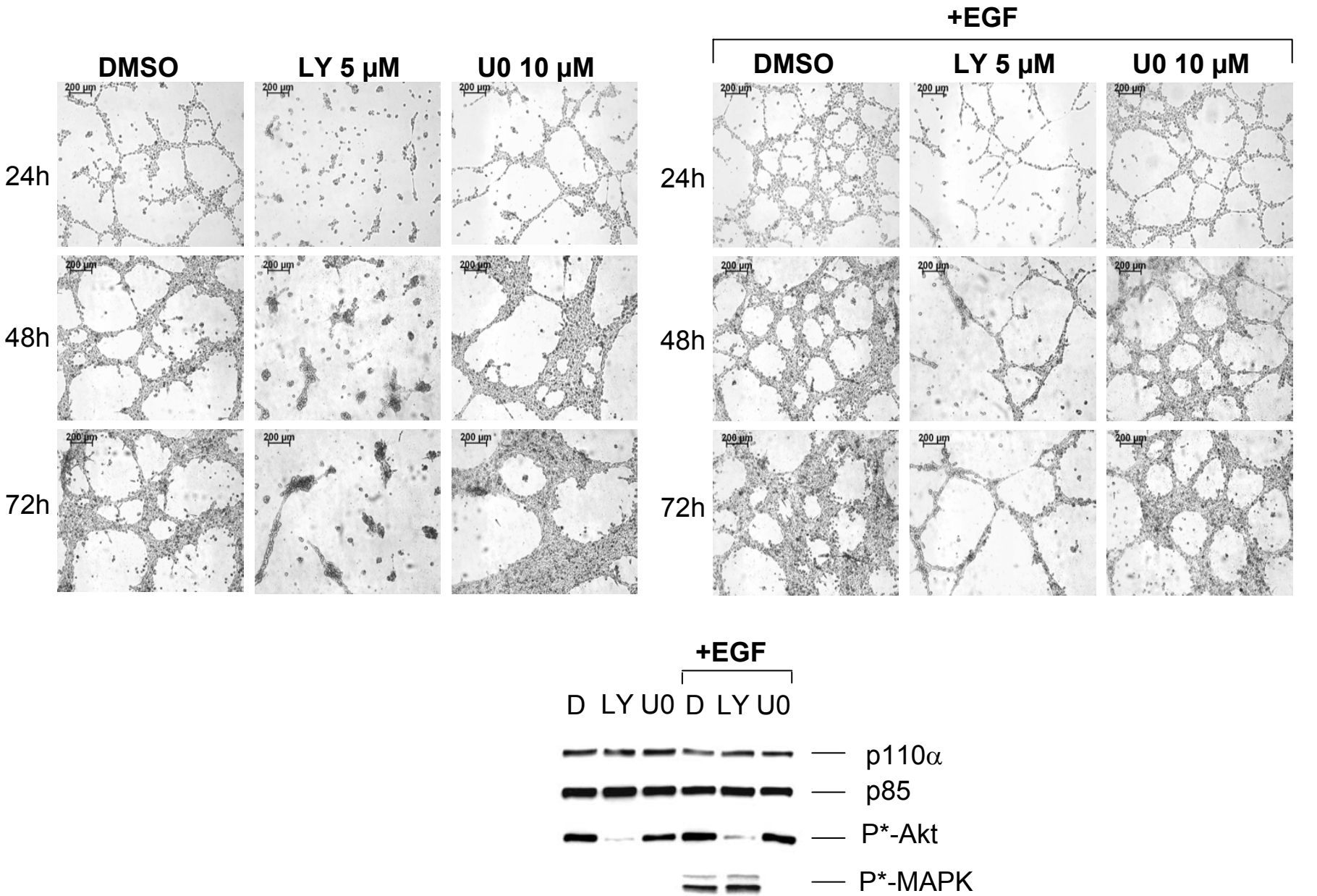
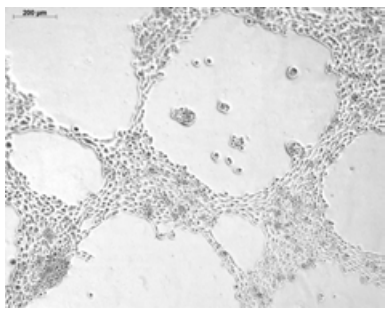
Supplementary
Figure 1



Supplementary
Figure 2A

PC-3 cells on extracellular matrix

DMSO



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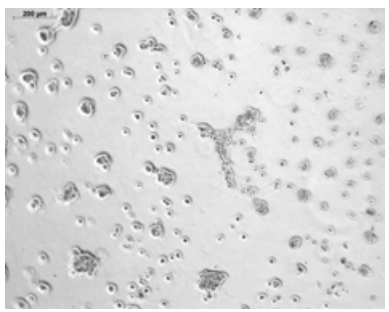
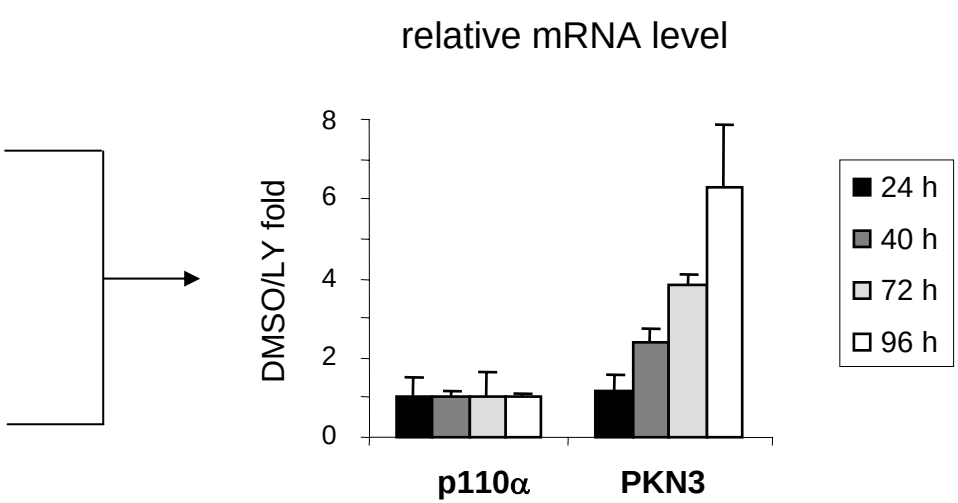
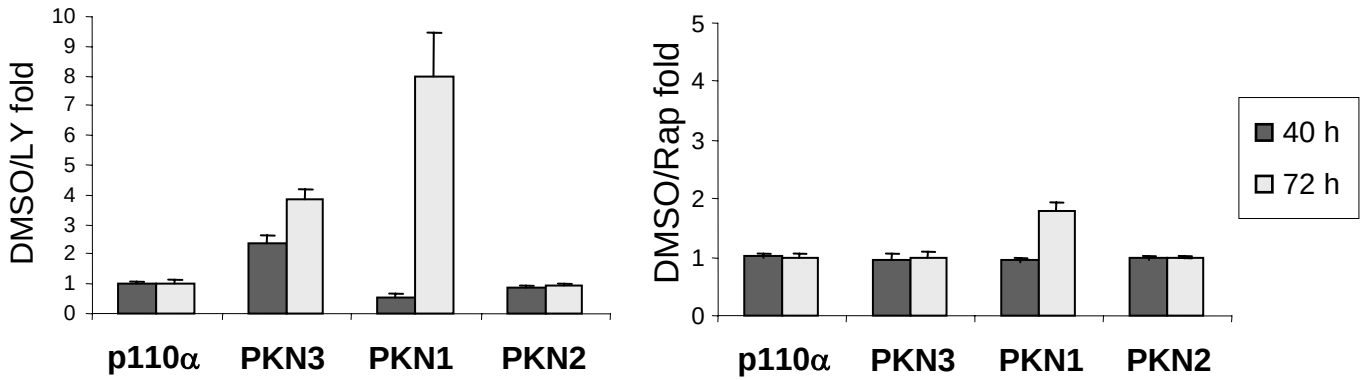
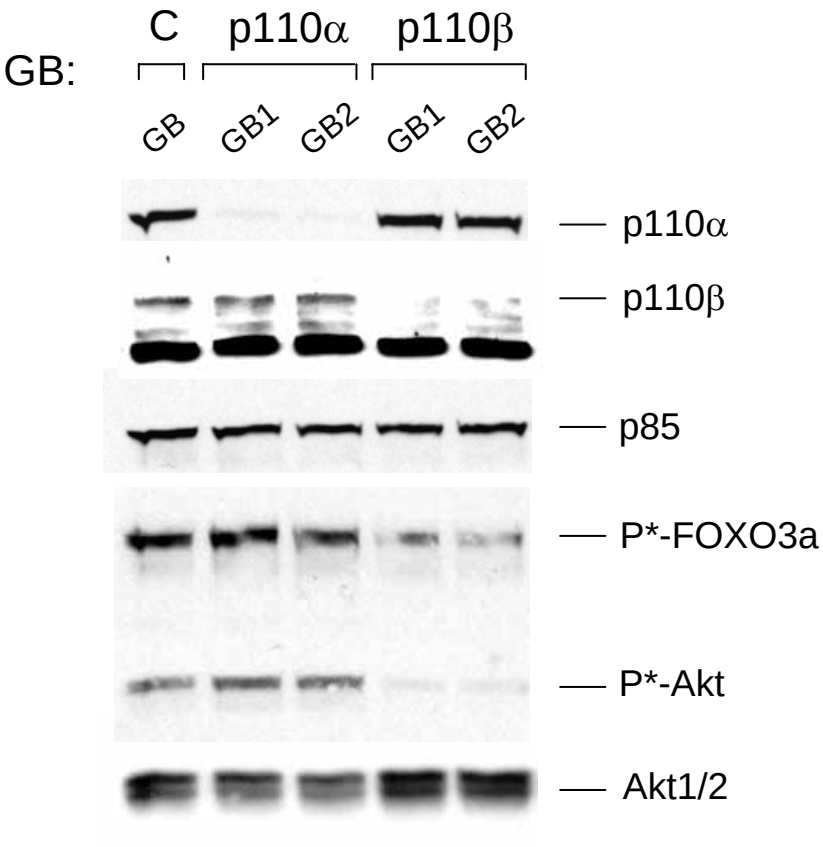


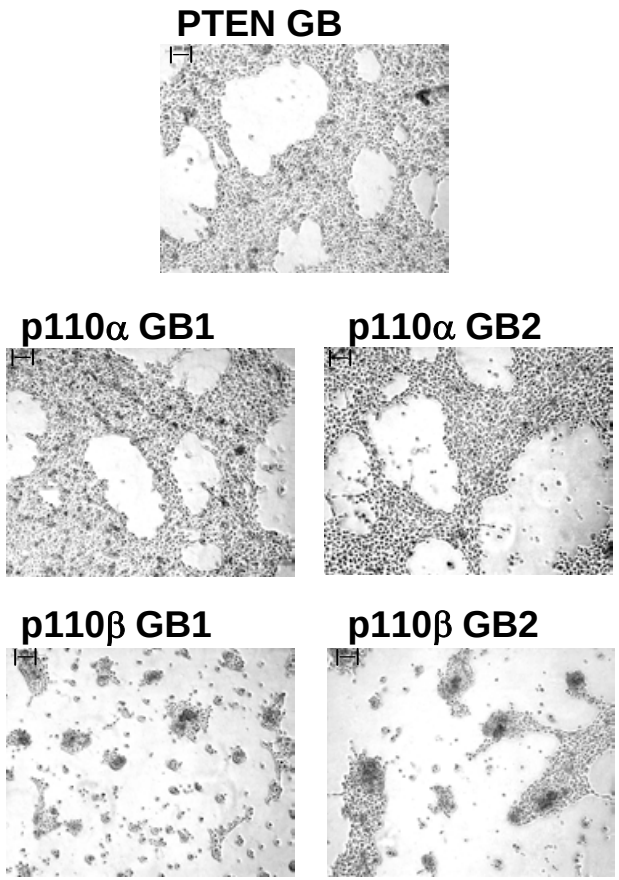


Supplementary
Figure 2B

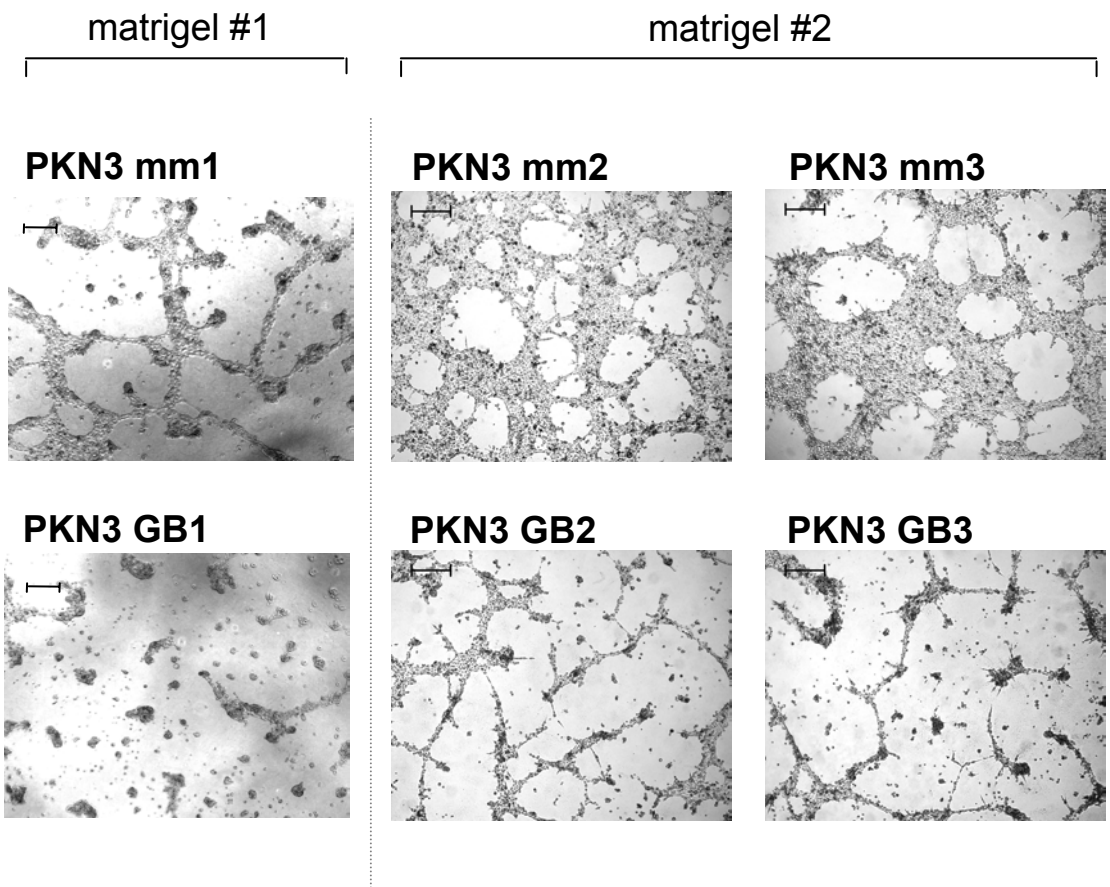


Supplementary
Figure 3





Supplementary
Figure 4A



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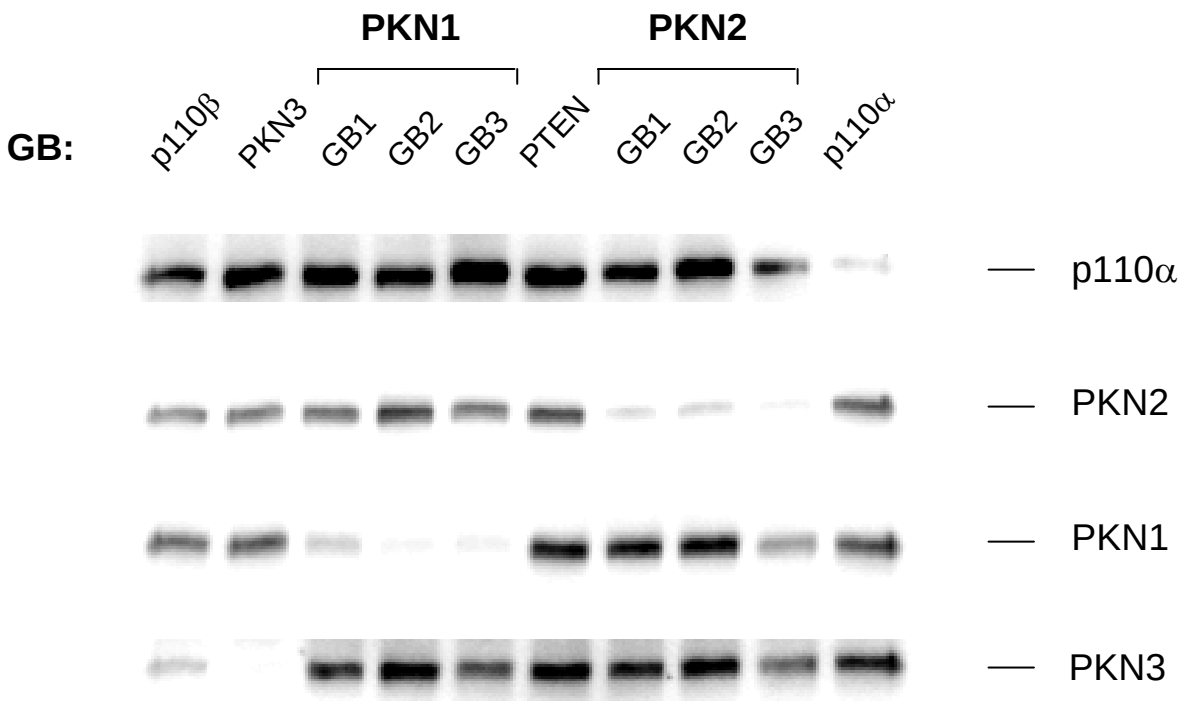

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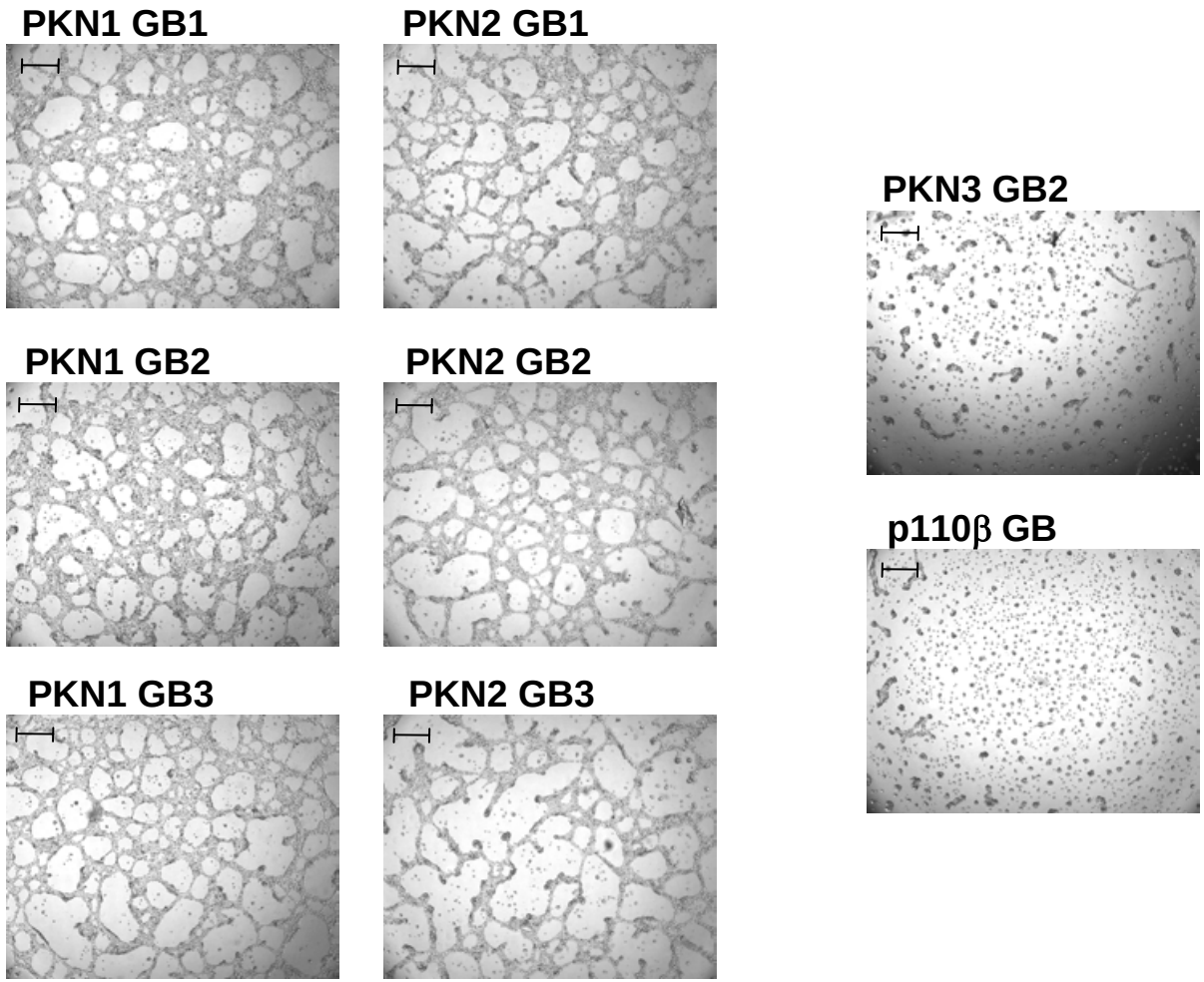
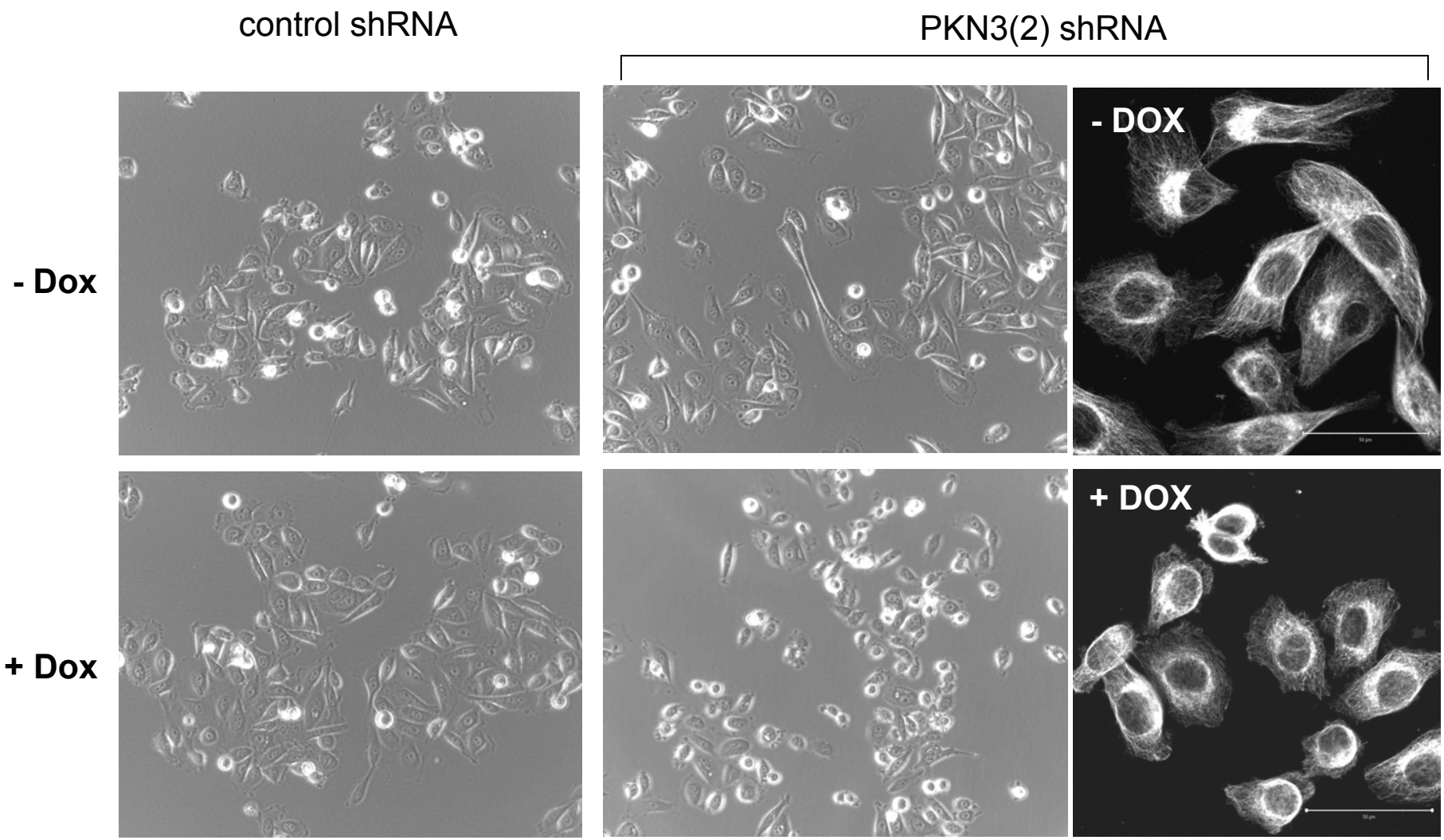
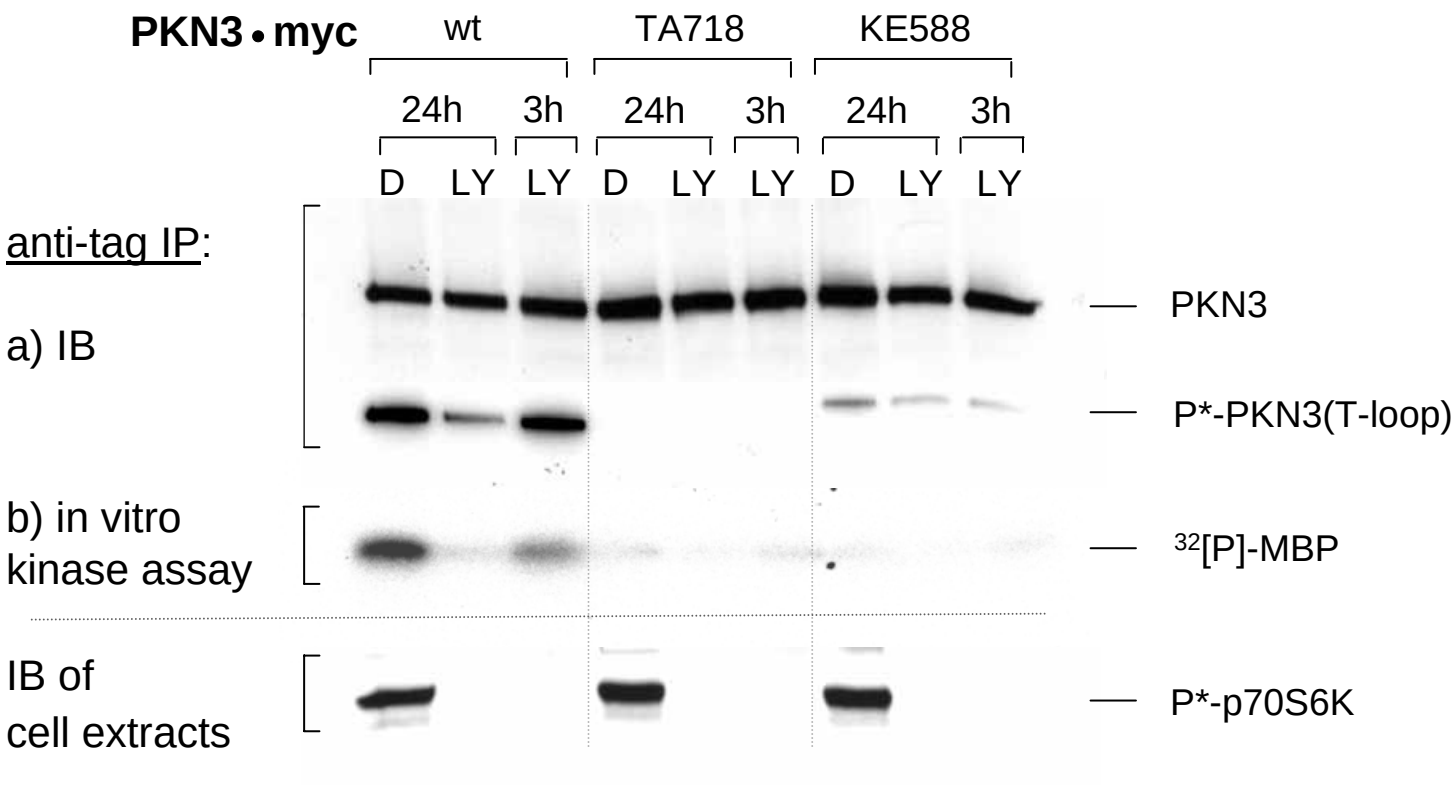
Figure 4C



Supplemetary
Figure 5



Supplementary
Figure 6



Legends to Supplementary Figures and Video Clips:

Supplementary Figure 1 Comparison of the contributions of PI 3-kinase (PI3K) or MEK-MAP kinase signaling for growth of PC-3 cells on matrigel. Left panels: PC-3 cells were seeded on matrigel matrix in 24-wells and grown in the presence of solvent (DMSO), 5 μM LY294002 (LY), a small molecule inhibitor of PI3K or 10 μM U0126 (U0) (in DMSO), an inhibitor of MEK. Photographs were taken at 5x magnification at the indicated times; size bars: 200 μm. Right panels: Since PC-3 cells do not exhibit detectable MAP kinase phosphorylation under regular growth conditions, 20 ng/ml EGF was added to stimulate the MEK-MAP kinase pathway (see lower panel) and to analyze its effect on matrigel growth. EGF-induced activation of MEK-MAP kinase signaling did partially compensate for the LY-mediated growth inhibition. U0-treatment had no effect under regular growth conditions in the absence of EGF additive. Lower panel: Parallel samples were analyzed by immunoblotting to monitor effective inhibitor treatment and EGF-induced MAP kinase phosphorylation. The experiment was performed twice; representative pictures are shown.

Supplementary Figure 2 (A) To verify PI3K-dependent expression of PKN3 PC-3 prostate carcinoma cells were grown on matrigel in 24-wells in the presence or absence of LY294002 (LY) for 72h; size bars: 200 μm. Real-time PCR (Taqman) analysis on RNA isolated at various time points was performed for expression of PKN3 mRNA relative to p110α mRNA in DMSO versus LY treated samples; the results represent triplicates ± SEM. PKN3 mRNA levels did not change in cells grown on 2D plastic surfaces (not shown). **(B)** Differential control of PKN1, PKN2 and PKN3 mRNA expression in PC-3 cells grown on matrigel. PC-3 cells were seeded on matrigel matrix in 24-wells and grown in the presence of solvent (DMSO), 3 μM LY294002 (LY) or 100 nM Rapamycin (Rap) in DMSO. Rapamycin inhibits mTOR-p70S6K signaling downstream of PI3K. RNA was isolated at the indicated times and subjected to Taqman analysis for expression of PKN3, PKN1 and PKN2 mRNA relative to

p110α mRNA in DMSO versus LY-(left panel) or DMSO versus Rap-treated samples (right panel); the results represent triplicate samples ± SEM.

Supplementary Figure 3. p110β is the predominant isoform of the PI 3-kinase catalytic subunit in PC-3 cells. PTEN$^{-/-}$ PC-3 cells were transfected in duplicate samples with the indicated GB molecules for 72 h. One set of cells was analyzed by immunoblotting (left panel), the second set was seeded on matrigel for 72 h (right panels); size bars: 200 μm. Left: Despite a significant knockdown of p110α expression, phospho(S473)-Akt (P*-Akt) and phospho(T32)-FOXO3a (P*-FOXO3a) levels remained unchanged compared to the PTEN GB control. However, knockdown of p110β strongly interfered with PI 3-kinase signaling. In agreement with this, inhibition of p110β expression, but not of p110α, inhibited PC-3 cell growth on matrigel. The experiment was repeated in several independent transfections; representative pictures are shown.

Supplementary Figure 4. (A) PKN3-specific GBs inhibit PC-3 cell growth on matrigel. PC-3 cells were transfected with three different GBs against PKN3; each GB was controlled by its four nucleotide mismatch (mm) derivative. GB-treated cells were seeded on matrigel and grown for 48 h; size bars equal 200 μm. The photographs were taken from two independent experiments, in which different matrigel lots were used. The speed of growth and the network-like structures formed depend on the respective matrigel lot. **(B)** Analysis of PKN1 or PKN2 protein knockdown. PC-3 cells were grown in duplicate samples in the presence of 30 nM of three different GBs specific for either PKN1 or PKN2, including the indicated controls. One set of cells was lysed for protein analysis (B) and the second set was seeded on matrigel (C). Cell lysates were analyzed using the indicated antibodies. **(C)** PKN1 and PKN2 are not required for PC-3 growth on matrigel. GB-treated cells were grown on matrigel; size bars: 200 μm.

Supplementary Figure 5. Increased PKN3 shRNA expression caused changes in cell shape and cytoskeletal structures. Parallel samples of inducible PKN3(2) and control shRNA PC-3

cells were grown for 48 h in the presence or absence of Dox and photographed at 10x magnification. PKN3 shRNA expressing cells grown on coverslips were fixed and incubated with an anti-tubulin antibody followed by an Alexa-488-conjugated secondary antibody. The cells were mounted and tubulin staining analyzed by confocal microscopy at 40-fold magnification (right panels); size bars: 50 μm.

Supplementary Figure 6. LY294002 interferes with PKN3 activation. Myc-tagged PKN3 derivatives were transiently expressed in COS-7 cells. The cells were treated with 10 μM LY or solvent DMSO (D) for the indicated times. Inhibition of PI3K signaling was confirmed by immunoblotting (IB) of cell extracts for the p70S6K phosphorylation status at T389 (P*-p70S6K; bottom panel). Anti-Myc immune precipitates (IP) were analyzed in parallel by a) IB and b) autoradiography for in vitro kinase activity (upper part). The presence of comparable amounts of kinase molecules in the precipitates was confirmed using anti-PKN3 antibody (top). Three hour LY treatment had little effect on T718 phosphorylation of wt PKN3, but interfered strongly with its catalytic activity as measured by the level of radio-labelled MBP. Prolonged inhibition of PI3K reduced PKN3 activity further to basal level.

Supplementary video clips. Inhibition of PKN3 expression interferes with cell motility and the ability to form network-like structures on matrigel. PC-3 cell populations stably expressing shRNA specific for p110α (control cells) and PKN3 (assayed cells) were analyzed by time-lapse-video-microscopy after seeding on matrigel. Knockdown of the respective protens was confirmed in parallel samples (not shown). For better comparison by parallel viewing of both movie clips, please open both and start them simultaneously.