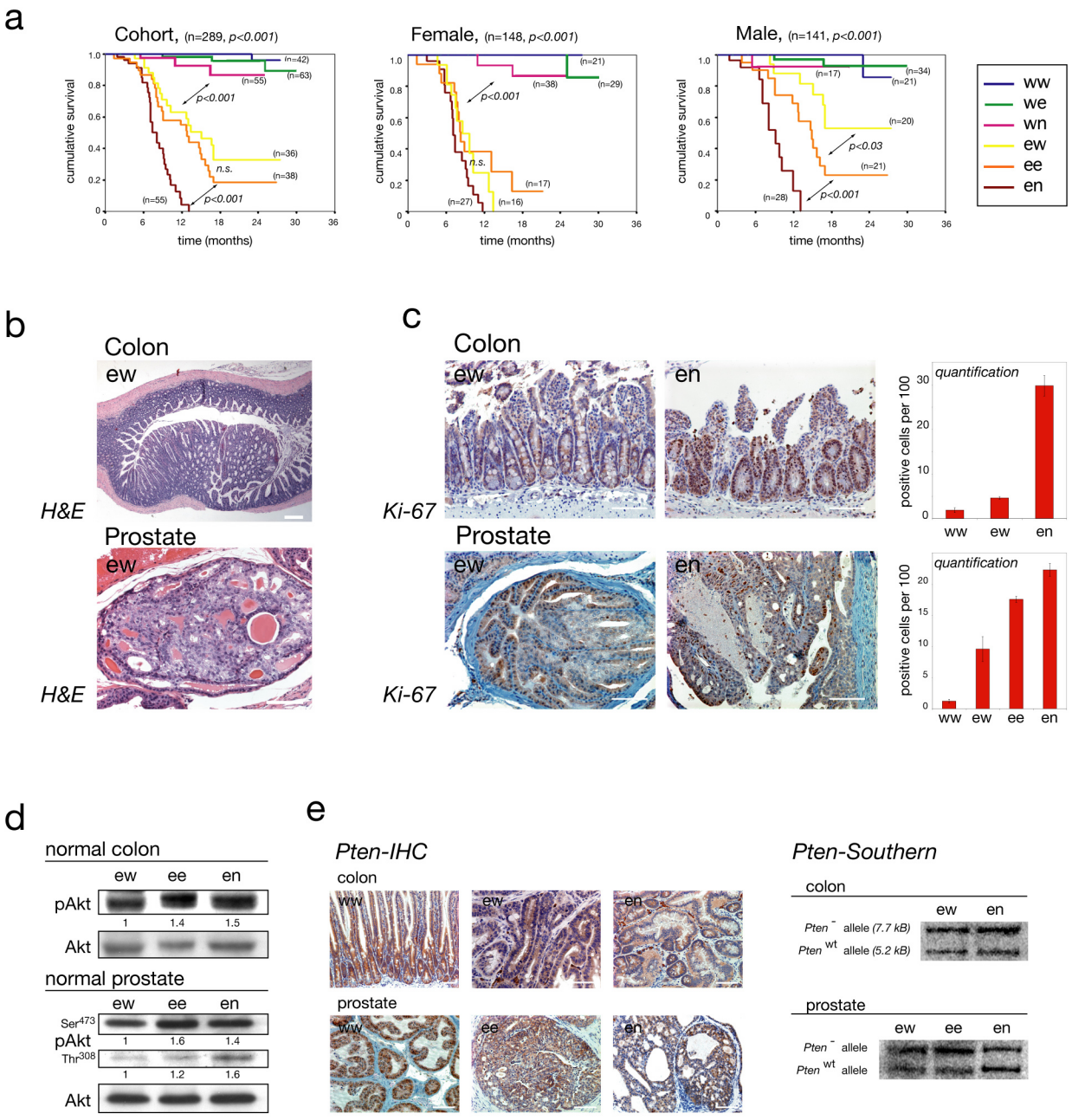
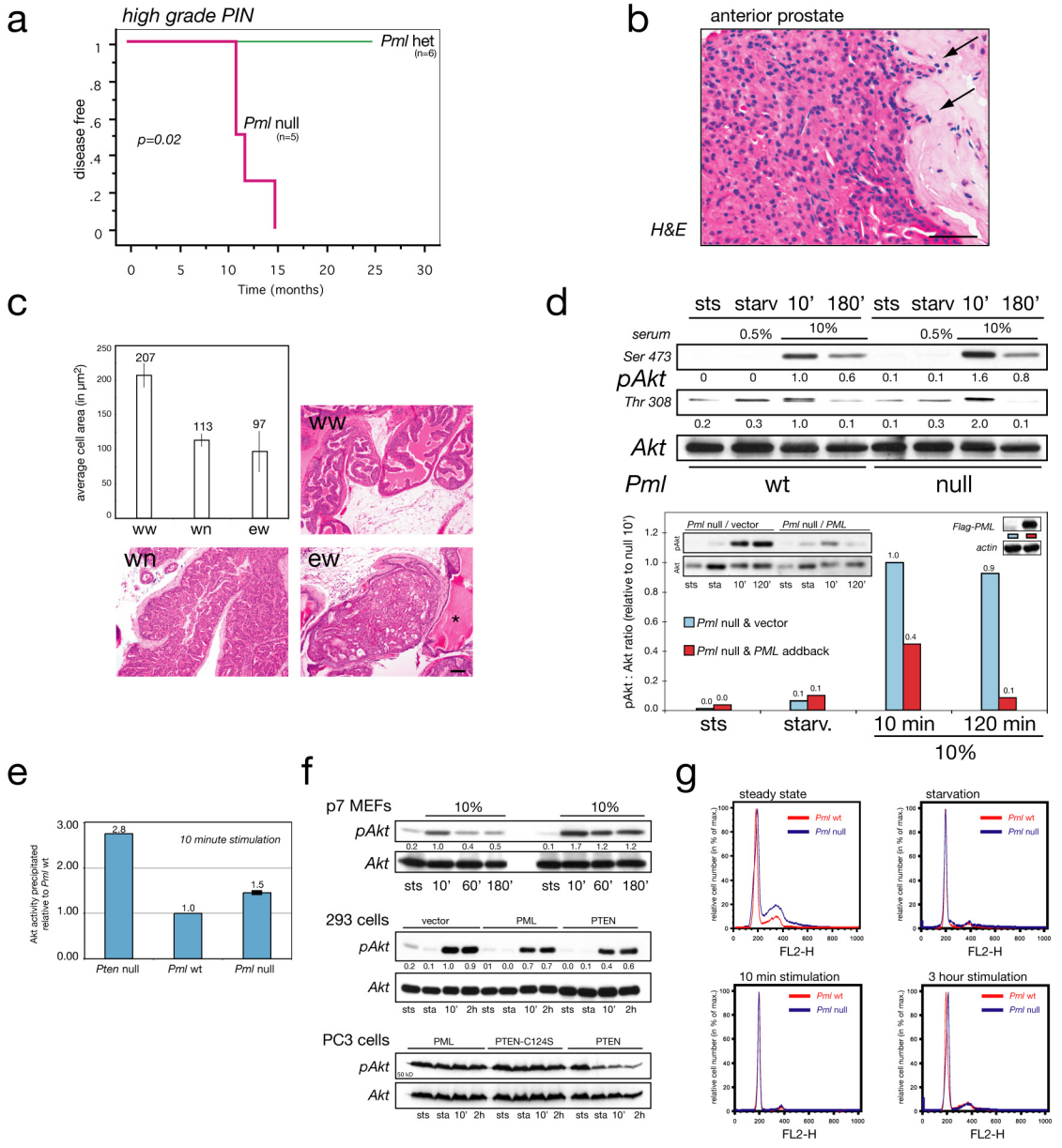
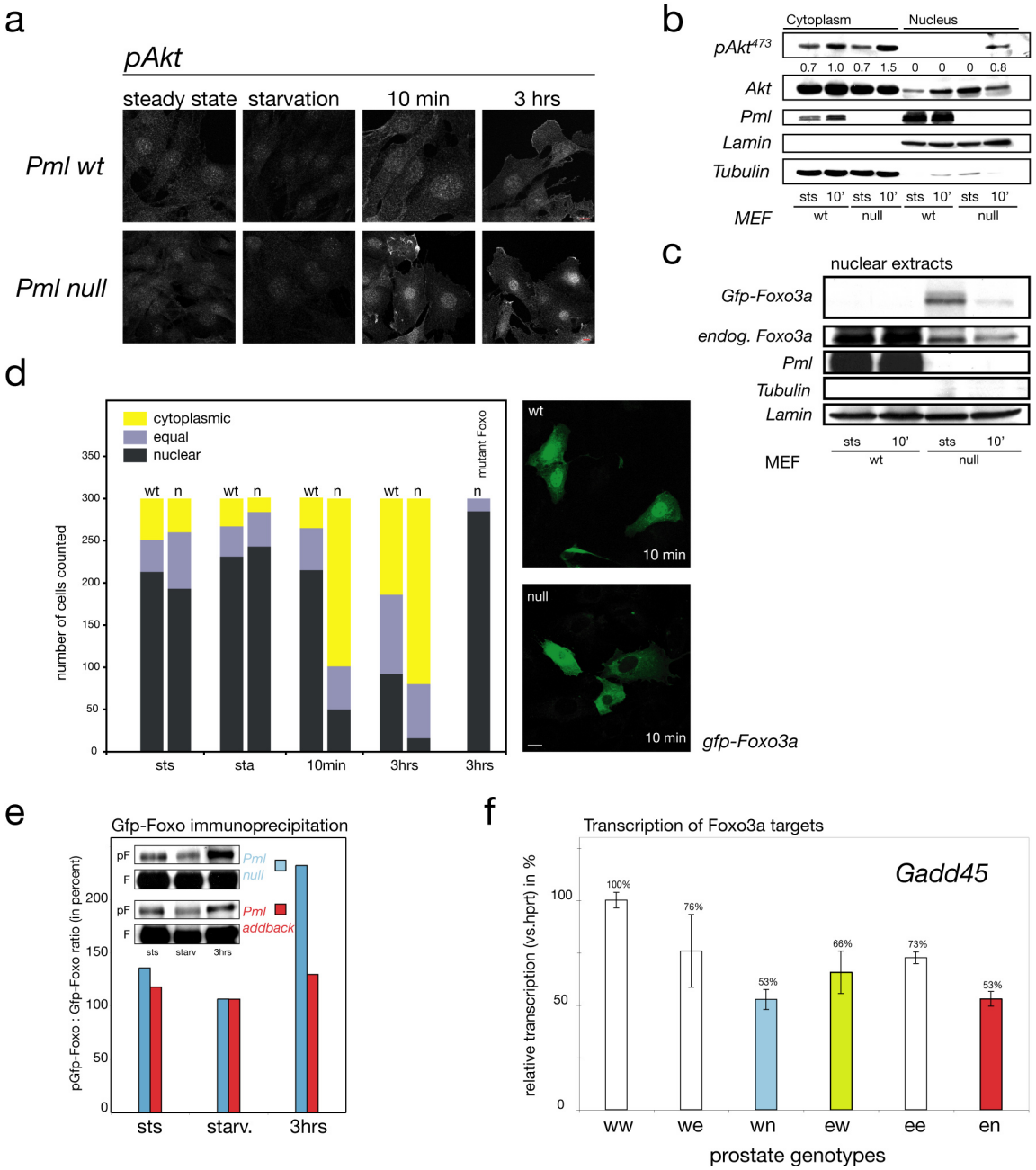


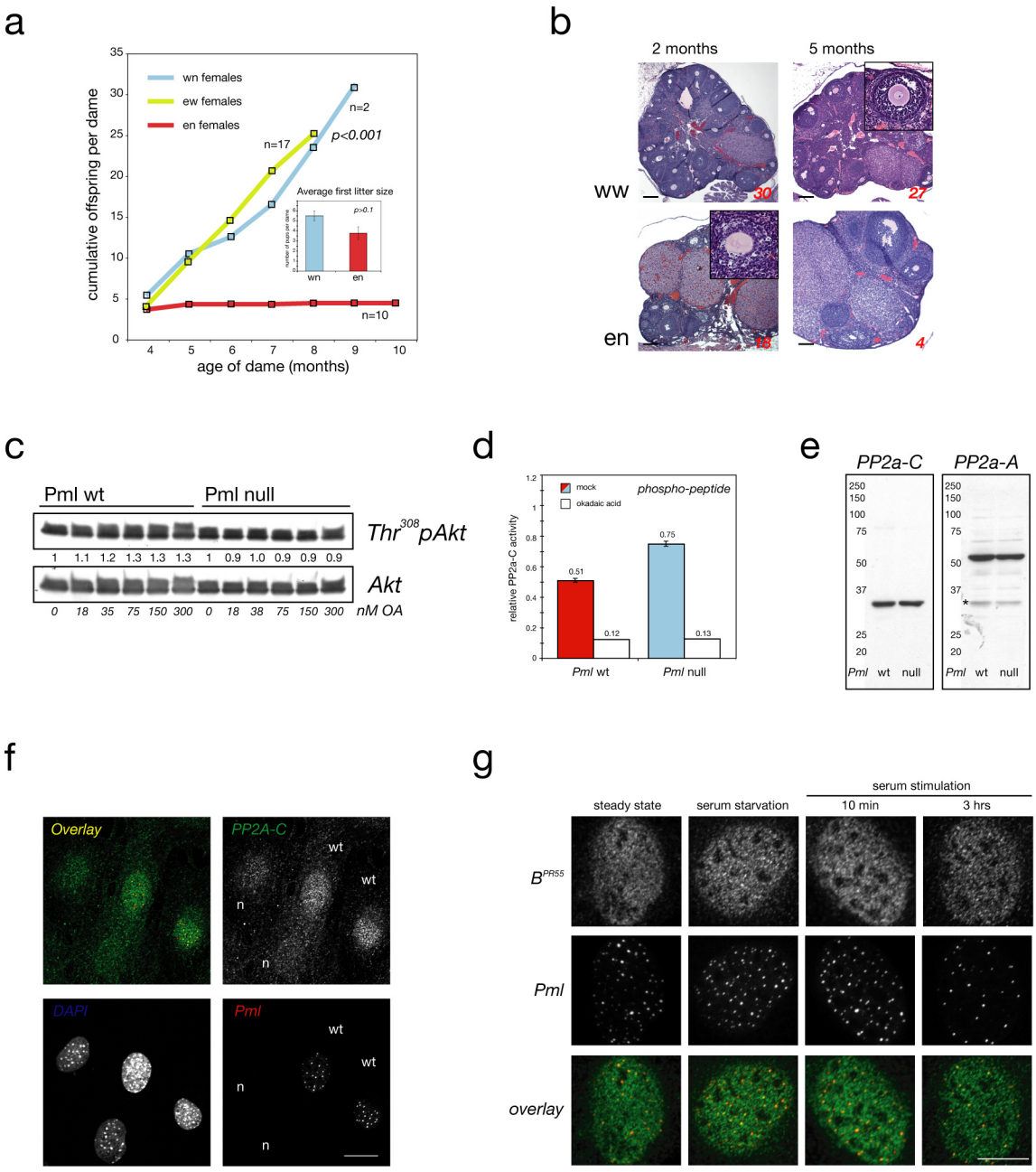
Supplementary Figure S1 | *Pml*-loss in the context of *Pten* heterozygosity. **a**, Kaplan-Meier plot for survival. Number of mice (n) and statistical significance (p) is given for overall and selected comparisons (arrows). Insignificant differences (p>0.05) are indicated (n.s.). Abbreviation code used is: single letter code for *Pten*-status followed by *Pml*-status. Code: w=wt, e=het, n=null. **b**, H&E analysis of colon and prostate tissue reveals only non-invasive lesions in *Pten* het animals (ew). Scale bars, 100 μm. **c**, Ki-67-staining for cell proliferation in normal colon and neoplastic prostate and quantification of Ki-67 positive cells per 100 (right panels) done on 3 areas of two mice per genotype. Scale bars, 100 μm. Error bars are s.d. **d**, Western blotting and quantification of pAkt: Akt ratios of lysates from indicated pre-neoplastic tissues and genotypes (see also Extended Methods below). **e**, Pten IHC-staining in colon and prostate carcinoma (left panels) and Southern blot analysis for *Pten* in tumours from indicated genotypes (right panels) demonstrating retention of the *Pten*-wt allele.



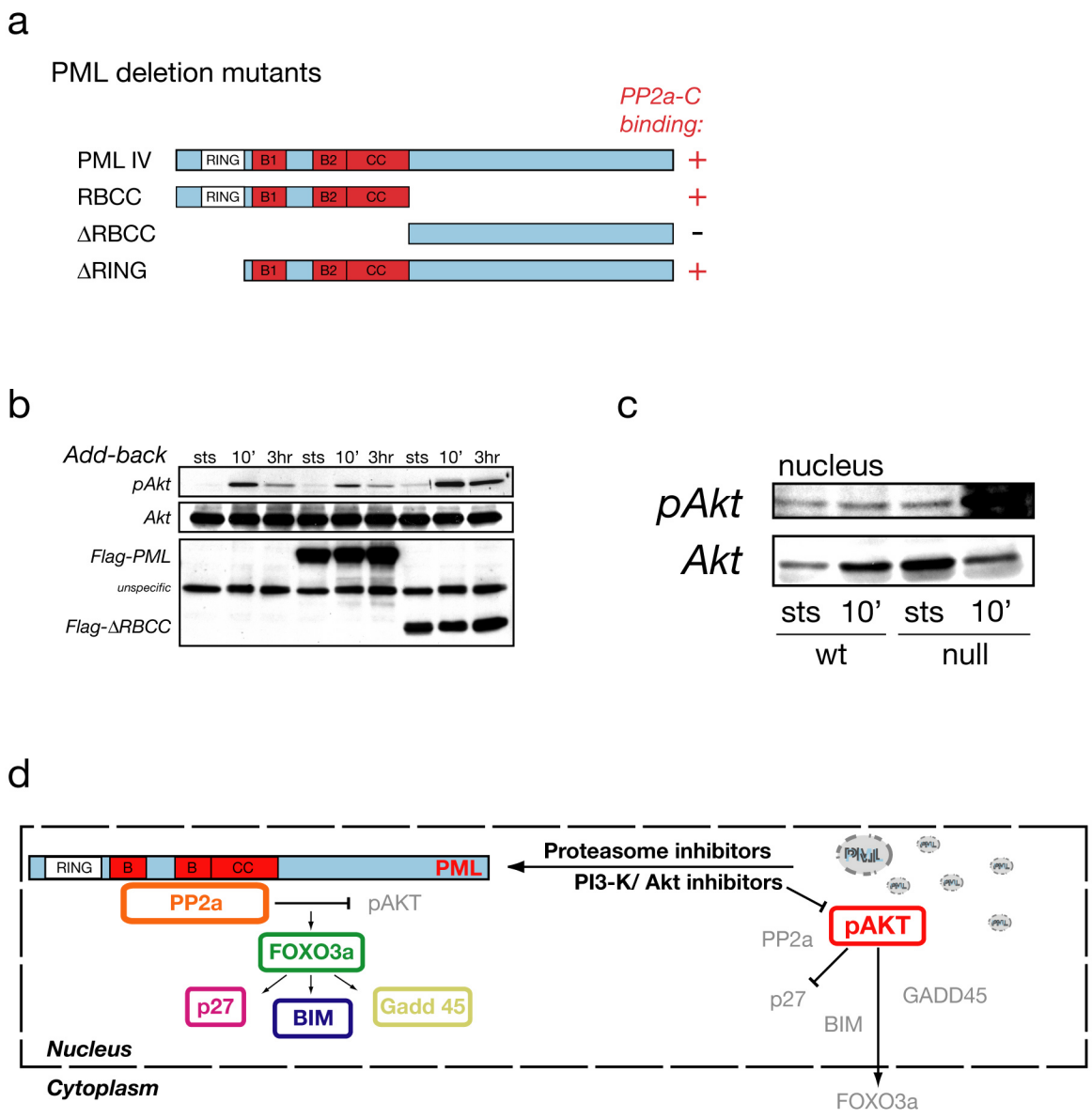
Supplementary Figure S2 | Prostate phenotype and biology of *Pml*-loss. **a**, Kaplan-Meier analysis of disease-free survival in prostate reveals PIN onset in *Pml*-null mice around one year of age. **b**, H&E-staining of anterior prostate lesion from *Pml*-null animal with early signs of epithelial cell invasion into the stroma (arrows). Scale bar, 100 μm. **c**, Calculation (bar diagram) and H&E-stainings for average cell size in prostates of indicated genotypes. Asterisk shows typical example of an enlarged gland lumen, filled with secreted fluid. Error bars are s.d. Scale bar, 100 μm. **d**, upper panels: pAkt Western blotting of littermate MEFs subjected to serum-starvation/stimulation as indicated (pAkt: Akt ratios are shown) at steady-state (sts), starvation (starv) and 10 minutes or 180 minutes post-stimulation. Lower panels: Western blotting (insert) and quantification of pAkt: Akt ratios in immortalized *Pml*-null MEFs infected with *PML* transgenic- or vector control-virus after indicated treatment. Upper right insert: *PML* expression efficiency. **e**, Kinase activity assay with immunoprecipitated Akt. Cells were serum starved and stimulated for 10 minutes. Error bar is s.d. **f**, Top panels: western blotting analysis of serum-starvation/stimulation assay with passage seven primary MEFs. Numbers indicate densitometrically determined pAkt: Akt ratio. Middle panels, overexpression of *PML* or *PTEN* in 293 cells (*PML*-wt and *PTEN*-wt) treated as indicated. Numbers indicate pAkt:Akt ratio. Bottom panels: analysis as above but in *PTEN*-deficient (*PML*-wt) PC3 cells. **g**, Cell cycle profiles of starvation-stimulation treated vector and *PML*-infected *Pml*-null MEFs.



Supplementary Figure S3 | Nuclear Akt and Foxo3a localization and function. **a**, Confocal Laser Scanning Microscopy analysis of immortalized *Pml*-wt and null MEFs stained for pAkt. **b**, Fractionation of primary MEFs treated as indicated shows nuclear Akt-activation quantitatively. Note that lysis and fractionation of adherent cells results in some discrepancies in respect to localization as compared with fixation and IF (e.g. compare Pml in fractionation with exclusive nuclear Pml-localization in immunofluorescence of Fig. 3a). **c**, Nuclear extracts of Gfp-FOXO transfected *Pml*-wt and null MEFs subjected to starvation-stimulation. Note that the low levels of Gfp-FOXO3a in the wt cells are due to lower transfection efficiency. **d**, Left panel: quantification of Gfp-FOXO3a (and Akt-insensitive mutant) localization after transfection into *Pml*-wt and null (n) cells and starvation-stimulation (see also methods section). Right panels: representative immunofluorescence examples of Gfp-FOXO3a localization after 10 minutes of serum-stimulation. Bar, 10 μm. **e**, Gfp-FOXO3a-immunopr pho-FOXO3a (pF) and total FOXO precipitated (F) after starvation-stimulation of *Pml*-null or stably *PML*-transduced cells. **f**, *Gadd45* mRNA levels in pre-neoplastic prostate determined as in Fig. 3c. Mouse genotypes are indicated.



Supplementary Figure S4 | Premature infertility and PP2a activity in *Pml*-null cells. **a**, Fertility of females of various genotypes and average size of first litters (insert). Error bars are s.d. **b,** H&E staining of ovaries from indicated genotypes. Follicle-number per ovary is given and inserts illustrate follicle morphology. Scale bars, 250 μm. **c**, Western blot and quantification of (Thr308)-pAkt-stimulation after OA treatment of wt and null cells quantified in Fig. 4a. Numbers indicate (Thr308)-pAkt: Akt ratio. **d**, MEF-extracted PP2a-C activity assayed on synthetic peptide target. Error bars are s.d. of triplicates. **e**, Specificity of PP2a A/C antibodies used in Figure 4. Asterisk denotes remaining signal from prior staining with PP2aC- antibody. Numbers are Mol. wt. in kD. **f**, CLSM of PP2a-C in *Pml*-wt and null cells 3 hours post-stimulation. **g**, Subnuclear distribution of a random PP2a B-family (PR55). Note that although NB-colocalization throughout the serum-starvation/stimulation cycle is evident, no clear NB-specific enrichment of PR55-family proteins is observed.



Supplementary Figure S5 | *PML*-deletion mutants and model for PML in pAkt inactivation. **a**, PML domains and PML deletion-mutants. 'B' denotes B-box motif and 'CC' coiled-coil domain. **b**, pAkt levels after add back of empty vector (lanes 1-3), Flag-*PML* (lanes 4-6), Flag-ΔRBCC (lanes 7-9) to *Pml*-null cells. Treatments and unspecific band are indicated. **c**, Nuclear pAkt levels at steady-state are not higher in *Pml*-null than in *Pml*-wt cells as seen in a long exposure of the nuclear pAkt fraction shown in Supplementary Fig. S3b. **d**, Model: PML regulates the activity of a tumour suppressor network opposing nuclear AKT-signaling. The three tumour suppressors Pten, Pml and PP2a thus cooperate in opposing nuclear Akt function.

Extended Methods

Mice. *Pml*-null mutants (129/Sv)[28] were crossed with wt (C57/bl6), the offspring crossed with *Pten*-het (129 Sv/C57 bl6) mice[7] and their offspring interbred to generate the 6 genotypes of the study cohort. Abbreviations used for cohort genotypes: Code letter for *Pten* status followed by code letter for *Pml* status. Code: w=wt, e=het, n=null. Genotypes produced for this study: (1) *Pten*-wt/*Pml*-wt (ww), (2) *Pten*-wt/*Pml*-het (we), (3) *Pten*-wt/*Pml*-null (wn), (4) *Pten*-het/*Pml*-wt (ew), (5) *Pten*-het/*Pml*-het (ee), (6) *Pten*-het/*Pml*-null (en). For Genotyping tail snips by PCR primers 5'-TGGGAAGAACCTAGCTTGGAGG-3' and 5'-TTCCATTTGTCACGTCCTGCAC-3' and 5'-ACTCTACCAGCCCAAGGCCCGG-3' were used for *Pten*. Primers 5'-TTTCAGTTTCTGCGCTGCC-3' and 5'-CGACCACCAAGCGAAACA-3' and 5'-TTGGACTTGCGCGTACTGTC-3' were used for *Pml*. Southern blot analysis was done as described[7] and scanned using a Storm system (Amersham). For statistical analyses the SPSS software package (v.11) was used (SPSS Inc., USA). Overall and disease-free survival curves were calculated by the Kaplan-Meier method and log rank t-tests for statistical significance.

MRI. For initial assessment of tumour locations and ages of onset, mice of all genotypes were subjected to monthly MRI screening. In brief, mice were anesthetized with 2% isofluorane, and images were obtained on a 4.7T Bruker 40 cm bore magnet (Billerica, Mass) using a commercial 7 cm inner diameter bird cage coil similar to the protocol described previously[11]. Low-resolution sagittal and axial scout images were obtained initially followed by high spatial resolution T2 weighted axial images (Repetition interval (TR) = 3800 ms, Effective echo time (TE) = 35 ms, 8 echoes per phase encoding step, spatial resolution = 1.0 mm slice thickness X 112 mm X 112 mm in plane resolution, and 4 repetitions, 8-9 minutes of data acquisition).

Western Blotting (WB), (co)-immunoprecipitation and *in vitro* kinase assays. Tissue and cell lysates were prepared as previously described[11]. Prostate and intestinal tissues were from two sets of mice of all six genotypes and samples were run at least two times for confirmation and to best match loading. Differences in p(S473)-Akt: Akt ratios between *Pml*-wt and null animals (irrespective of *Pten* status) were consistently in the range of 1.5-fold (with a margin of 10% difference) except for *Pml*-null colon which displayed greater 2-fold difference as shown. *Pml*-het (*Pten*-het) animals showed slightly higher variability (1.5 fold activation difference, with a margin of 20%). In prostate,

no significant differences between *Pml*-wt and *Pml*-het (of *Pten*-wt) were seen. Western Blotting was done using the following rabbit polyclonal antibodies from Cell Signalling (cat.#): pAkt (Ser 473, #9271 and Thr 308, #9275), Akt (#9272), pan-PP2a-C (#2038), pan-PP2a-A (#2039), PP2a-B (PR55,#4953), phospho-Foxo3a (#9465), as well as anti-Lamin B1 (Abcam, ab16048-100) and anti-Foxo3a (Upstate, 07-702) and anti-Gfp (see below). Mouse monoclonal antibodies were hsp90 (#610419, BD Transduction Laboratories), anti-α-Tubulin (Sigma, #T5168) and Pml (mAb36, a kind gift of Dr. S. Lowe, Cold Spring Harbor Laboratory). Quantification of densitometry was performed as previously described[11]. Co-immunoprecipitations (IP) were carried out without overexpression of exogenous proteins (except for Supplementary Figure S5b) by scraping cells off a 10 cm dish with lysis buffer and incubation for 30 min at 4 deg on a rocking platform. The 30 min (13 krpm) supernatants were pre-cleared with Protein-G Sepharose beads (Amersham) for 1 hr and mouse anti-Pml (1:1000, see below) was added to equal protein amounts of cleared supernatants for overnight incubation at 4 deg followed by Protein-G Sepharose co-precipitation (1 hour) and 5 consecutive washes (5 min each) at 4 deg with lysis buffer. IP-antibodies used were: monoclonal anti-PML (endogenous PP2a-C and Akt co-IP in primary MEFs), anti-Flag (Stratagene, #200471, for endogenous PP2a-C coIP in *Pml-Trp53*-null primary MEF) or rabbit polyclonal anti-Gfp (BD Biosciences, #632460, for exogenous Gfp-Foxo3a IP in *PML*- or vector-transduced *Pml*-null MEF). *In vitro* kinase assays were done with the non-radioactive Akt Kinase Kit (Cell Signaling, #9840) on primary MEFs (*Pml*-wt and null) and *Pten-Trp53* deficient MEFs[27] were used as positive control.

Cells, Plasmids and Immunofluorescence (IF). For the serum-starvation/stimulation assay, MEFs (produced as previously described[11]) were starved in 0.5% serum (1/20 of normal) in DMEM (with Antibiotics and Glutamine) for 12 hours then treated with full, temperature and pH-pre-equilibrated serum (10%) for times indicated. *Pten*-null/*Trp53*-null MEFs where prepared as described (*Pten*-null MEFs senesce prematurely and thus could not be used)[27]. For Okadaic acid (OA) sensitivity MEFs were treated with the indicated OA concentration (in fresh 10% serum) for 4 hrs and p-Thr^{308}-Akt: Akt ratios determined by Western blotting. Note that Figure 4a shows ratios for each genotype normalized to their own mock-treatment ratios while in Figure 4b the *Pml*-wt ratios are expressed relative to the ratio of the untreated *Pml*-null. PP2a activity in *Pml*-wt and null MEFs was determined on a substrate phospho-peptide using the "PP2a IP Phosphatase Assay Kit" (Upstate) according to the manufacturers instructions. For IF, cells were seeded on glass cover slips in 24-well plates and 24 hours post seeding subjected to serum-starvation/stimulation cycles where needed,

treated with 3.5 % para-formaldehyde (10 min), 50 mM NH_4Cl (10 min), 0.5% Triton X-100 (5 min), PBS (3 times wash), 10% goat serum (10 min) and primary antibodies were added for 5 hours (4 deg) in goat serum or as indicated by the manufacturer. To faithfully test differences observed between wt and null cells, both genotypes were seeded together, allowing relative quantification of staining intensity in the same field. Nucleo-cytoplasmic fractionation was carried out using the NE-PER fractionation kit (Pierce) according to the manufacturer's instructions. Quantification of nucleo-cytoplasmic distribution of wt and mutant Gfp-Foxo3a was done by fixing transfected cells as above after the indicated treatment but mounting for IF without permeabilization to best preserve structural integrity of cells. 300 cells per genotype and condition were scored for dominant nuclear, dominant cytoplasmic or even Gfp-distribution. Plasmids for Foxo3a and the Akt-phosphorylation deficient Foxo3a triple mutant[19] were kind gifts of Dr. M. Greenberg, Harvard Medical School, that were subcloned into pEGFP-C3 (Clontech). The Flag-PML IV plasmid and its derivatives (Flag-RBCC, Flag-ΔRBCC, Flag-ΔRING) were done as previously described[29]. Primary rabbit antibodies used were against pAkt (Ser473-IHC specific, Cell signalling # 9277), pan-PP2a-A and pan-PP2a-C (as above). Primary mouse antibody against Pml was mAb36. Secondary antibodies were goat anti-rabbit and goat anti-mouse Alexa 546 or 488 (Invitrogen-Molecular Probes, IMP) and DAPI was used for identification of cell nuclei as suggested by the manufacturer. Cover slips were mounted in Anti-Fade (IMP) and sealed with clear nail polish. Confocal analysis was on a Leica DMRXA2 microscope (HCX PL APO 63x water objective, 1.2 nominal aperture) using Leica Confocal Software v. 2.61. All data analysis was done on Apple Macintosh Computers.

Immunohistochemistry (IHC), Quantifications and Flow cytometry.

For IHC, tissues were fixed in 10% formalin and embedded in paraffin according to standard procedures. Gastro-intestinal tracts were carefully rinsed with PBS before macroscopic examination and fixation. Sections were processed with H&E reagents or stained for phospho-Akt (Ser 473, IHC-specific as above), Pten (Ab-2, NeoMarkers), Ki-67 (Novocastra) as previously described[11]. Quantification of Ki-67-staining was performed on 3 sections from two different mice for each genotype. For quantification of polyposis whole H&E sections of the GI-tracts of mice were analyzed. Tumour diameters were determined on H&E-stained slides using an Olympus BX40 microscope (10x objective) and a diaMax Swiss Precision Vernier caliper (Scienceware). For determination of average polyp number as seen in H&E per mouse intestine (Figure 1b, upper insert, polyposis) numbers of mice analyzed were 6, 5, 5 for ew, ee, en respectively. For average polyp size

the number of polyps found and measured are n=10, 15, 28 for ew, ee, en, respectively. Follicle counts were performed on H&E sections of Ovaries from 2 different mice each. For determination of average prostate cell size, prostate sections of three 1-year-old animals of each genotype were assayed for cell number per area of tissue. A total area containing 350 cells was scored for each genotype on images taken at 10x magnification. Cell cycle profiles were obtained by running methanol-fixed and propidium-iodide-stained cells on a FACSCalibur flow cytometer (Becton Dickenson). Data were analyzed using the FlowJo software package.

Quantitative real time PCR. RNA was isolated from tissue or cells using the Trizol method and cDNA produced from 2µg of RNA using the SuperScript III system with oligo dT primers (both Invitrogen) as suggested by the manufacturer. Quantitative real-time PCR was performed on a Roche LightCycler using the Quantitect SYBR Green PCR kit (Qiagen) and the following amplification protocol: 15 min at 95 deg, 40 cycles (15 sec at 94 deg - 20 sec at 57 deg - 20 sec at 72 deg) followed by determination/confirmation of amplicon melting temperature. Reactions were performed in triplicates, primer pairs were confirmed to yield a single amplicon band by 3% agarose gel electrophoresis and absence of amplification from non reverse-transcribed RNA was confirmed to exclude genomic DNA amplification. The following mouse primer sets used were obtained from *PrimerBank* (http://pga.mgh.harvard.edu/primerbank/index.html), see also "Wang, X. & Seed, B. A PCR primer bank for quantitative gene expression analysis. Nucleic Acids Res 31, e154 (2003)": $p27^{Kip1}$, pair 1 (PrimerBank ID: 31542372a1), Fwd1 (5'-TCAAACGTGAGAGTGTCTAACG-3'), Rev1 (5'-CCGGGCCGAAGAGATTTCTG-3'), pair 2 (ID: 31542372a2) Fwd2 (5'-TCTCTTCGGCCCGGTCAAT-3'), Rev2 (5'-GGGGCTTATGATTCTGAAAGTCG-3'), pair 3 (ID: 31542372a3) Fwd3 (5'-GGGCAGATACGAGTGGCAG-3'), Rev3 (5'-CCTGAGACCCAATTAAAGGCAC-3'). For *Bim*: (ID: 6753192a1), Fwd (5'-CCCGGAGATACGGATTGCAC-3'), Rev (5'- GCCTCGCGGTAATCATTTGC-3'). For *Gadd45* the following mouse primer pairs were used: Fwd1 (5'-AGACCGAAAGGATGGACACG-3'), Rev1 (5'- TGACTCCGAGCCTTGCTGA-3'), Fwd2 (5'-AGCAAGGCTCGGAGTCAGC-3'), Rev2 (5'-ACGTTGAGCAGCTTGGCAG-3') as previously published[30]. The following primers for *Hprt1* cDNA were used as reference standards: Fwd (5'-CACAGGACTAGAACACCTGC- 3'), Rev (5'-GCTGGTGAAAAGGACCTCT-3').