Regulation of the tumour suppressor Fbw7α by PKC-dependent phosphorylation and cancer-associated mutations

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INTRODUCTION

Fbw7 (F-box WD40 protein 7) is a major tumour suppressor, which mediates the degradation of several potent oncogenes. PKC (protein kinase C) comprises a serine/threonine kinase family that can promote transformation when dysregulated. In the present study, we investigated the relationship between Fbw7 and PKC. Multiple members of the PKC superfamily interact with the substrate-binding domain of Fbw7. However, we find no evidence for Fbw7-mediated degradation of PKC. Instead, we demonstrate that Fbw7 is a novel substrate for PKC. Two residues within the isoform-specific N-terminus of Fbw7α are phosphorylated in a PKC-dependent manner, both in vitro and in mammalian cells (Ser10 and Ser18). Mutational analyses reveal that phosphorylation of Fbw7α at Ser10 can regulate its nuclear localization. Cancer-associated mutations in nearby residues (K11R and the addition of a proline residue at position 16) influence Fbw7α localization in a comparable manner, suggesting that mislocalization of this protein may be of pathological significance. Together these results provide evidence for both physical and functional interactions between the PKC and Fbw7 families, and yield insights into the isoform-specific regulation of Fbw7α.

Key words: cancer-associated mutation, F-box WD40 protein 7 (Fbw7), isoform-specific regulation, nuclear localization, protein kinase C (PKC).

Abbreviations used: CPD, Cdc4 phosphodegron; CR-UK, Cancer Research UK; CSM, complete supplementary dropout medium; Fbw7, F-box WD40 protein 7; GFP, green fluorescent protein; GS3k, glycogen synthase kinase 3; GSK3, glycogen synthase kinase 3; GST, glutathione transferase; HEK, human embryonic kidney; HEK-293T, HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40); NCBI, National Center for Biotechnology Information; NLS, nuclear localization signal; PKC, protein kinase C; RNAi, RNA interference; SBD, substrate-binding domain; SCF, Skp1/cullin/F-box; SD, synthetic-derived; siRNA, small interfering RNA.

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mutations proximal to the PKC phosphorylation sites influence Fbw7α in a comparable manner. We conclude that Fbw7α represents a novel PKC substrate, whose exclusion from the nucleus may be of pathological significance.

**EXPERIMENTAL**

**Materials**

All reagents were purchased from Sigma–Alrich unless otherwise indicated. Yeast SD (synthetic-derived) and tissue culture medium were prepared by CR-UK (Cancer Research UK) Research Services, Opti-MEM® was from Gibco and FBS (fetal bovine serum) was from PAA laboratories. Antibodies were obtained from Calbiochem; BIM1 and MG132 were reconstituted in DMSO; calcyclin A was dissolved in ethanol. All peptides were prepared by the CR-UK Peptide Synthesis Laboratory. Purified recombinant PKCα and PKCε were obtained from Mr Philip Whitehead (London Research Institute, CR-UK, London, U.K.). PKCδ (human) and PKCε (human) were purchased from Calbiochem.

**DNA constructs and site-directed mutagenesis**

Molecular biology was performed using standard procedures. All subcloning was achieved by PCR. Mouse PKCε cDNA and GFP (green fluorescent protein)–PKCε were as described previously [24]. PKCγ was cloned into pGBK7 (Clontech) between the NcoI and BamHI sites (primers F, 5′-CAGCACCA TGGCAA TGGTAGTGTTCAA TGGCCTTC-3′ and R, 5′-GTGACAGATCTCTAGGCGATCACGTCTCTACC-3′; BglII ends are compatible with BamHI). Full-length PKCε (primers PKCε/F, 5′-GAACACACCCCGAGATGTTGATGT- TCAATGCTCTCC-3′ and PKCε/R, 5′-CATCTATGGTACC-GGCGCATCGATCTCTACCAA-3′), and its regulatory (primers PKCε/R, PKCε/F and 5′-CATATACATCCCGTGAGG- CCTTGCCGGACTCC-3′ and catalytic domains (primers PKCγ/C, 5′-CATATTACCAGGTGGATGATTTGCAAAATGTG- CTGCTGCT-3′, and PKCγ/R) were cloned into pMT2-GST between the XmaI and KpnI sites. Wild-type and constitutively active GFP–PKCε and GFP–PKCγ constructs were obtained from Dr Scott Parkinson (CR-UK, London, U.K.) and GFP–PKCδ was as described previously [25]. Wild-type and constitutively active PKC constructs were a kind gift from Songhai Shi (MSKCC, New York, U.S.A.). The SBD (substrate-binding domain) of mouse Fbw7 (Fbw7-SBD; encodes amino acids 283–629 from GenBank® accession number AF391192) was recovered from pACT2 vector (see below) and subcloned into pEGFP-C1 (between the KpnI and XmaI sites) using primers complementary to the flanking sequence 5′-AATACAGGTACCGG-TCAATAGCCATGAGGCCC-3′ and 5′-ATAGAAGCCGG- TCCCACTATCTACGATGATCTCTCG-3′; AgeI ends are compatible with XmaI), pCDNA3.1-FLAG was kindly provided by Dr Sylvie Lachmann (CR-UK, London, U.K.), and pCDNA3-FLAG-Fbw7 by Professor Shigetsugu Hatakeyama (Hokkaido University, Japan). FLAG-tagged Fbw7α and Fbw7αBDI were generous gifts from Dr Bruce Clurman (Fred Hutchinson Cancer Research Center, Seattle, U.S.A.) with [24]. 293FT cells were obtained from Invitrogen and maintained according to their guidelines. Transfection of plasmid DNA was performed using Lipofectamine™ 2000 (Invitrogen; COS7) or FuGene 6 (Roche; HeLa) as recommended by the manufacturers. Cells were stimulated with 10 μM MG132, 100 nM calyculin A (cells detach and are collected in suspension from the medium) and 400 nM PMA. All untreated controls were performed with an equal volume of DMSO.

Япония. FLAG-tagged Fbw7α and Fbw7αBDI were generous gifts from Dr Bruce Clurman (Fred Hutchinson Cancer Research Center, Seattle, U.S.A.) with [24]. 293FT cells were obtained from Invitrogen and maintained according to their guidelines. Transfection of plasmid DNA was performed using Lipofectamine™ 2000 (Invitrogen; COS7) or FuGene 6 (Roche; HeLa) as recommended by the manufacturers. Cells were stimulated with 10 μM MG132, 100 nM calyculin A (cells detach and are collected in suspension from the medium) and 400 nM PMA. All untreated controls were performed with an equal volume of DMSO.

**Yeast two-hybrid analysis**

Yeast two-hybrid analysis was performed using the MATCH-MAKER GAL4 System 3, the AH109 yeast strain and a mouse brain cDNA library (pACT2), according to the manufacturer’s guidelines (Clontech). Full-length mouse PKCα was used as the bait (pGBK7-PKCα). Basic yeast medium was prepared using SD medium [0.67 % yeast nitrogen base, 2 % (w/v) glucose, pH 5.8, with or without 2.2 % (w/v) agar], 0.59 g/l CSM (complete supplementary dropout medium; – Ade, – His, – Leu, – Trp and – Ura) (Bio 101) and 20 mg/l uracil. This was supplemented with 40 mg/l adenine, 20 mg/l histidine, 100 mg/l leucine, 50 mg/l tryptophan and/or 20 μg/ml X-α-gal (designated X; Clontech), as indicated. Yeast transformations were carried out using a lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol (Agape, http://research.bmn.com/to). pGBK7-PKCα expression in AH109 was confirmed by Western blotting and was shown not to auto-activate the HIS3, ADE2 or MEL1 reporter genes. AH109 were co-transformed with pGBK7-PKCα and pGADT7 as negative controls, or pGBK7-P53/pGADT7-F antigens as positive controls (Clontech). For the screen, yeast were transformed sequentially, first with pGBK7-PKCα and then with the library; a total of 1.4×10^12 cfu (colony-forming units) were analysed. Transformed yeast were subjected to three rounds of selection. Transformants were first plated on medium stringency nutritionally selective agar plates (CSM – Trp, – Leu and – His) at 30 °C for 5 days. Positive clones were restreaked on to more stringent selective plates (CSM – Trp, – Leu, – His and – Ade) for a further 3 days, and then on to the highest stringency selective media (CSM – Trp, – Leu, – His, – Ade and – X) for 5 days. Plates were imaged by the CR-UK Photographic Department. Library vectors were rescued from positive clones and sequenced using the primers 5′-TACCATAGTACCGG-CTCATATGCCCATGGAGGGCC-3′ and 5′-ATAAGCCGG-TCCCACTATCTACGATGATCTCTCG-3′; AgeI ends are compatible with XmaI), pCDNA3.1-FLAG was kindly provided by Dr Sylvie Lachmann (CR-UK, London, U.K.), and pCDNA3-FLAG-Fbw7 by Professor Shigetsugu Hatakeyama (Hokkaido University, Japan). FLAG-tagged Fbw7α and Fbw7αBDI were generous gifts from Dr Bruce Clurman (Fred Hutchinson Cancer Research Center, Seattle, U.S.A.) with [24]. 293FT cells were obtained from Invitrogen and maintained according to their guidelines. Transfection of plasmid DNA was performed using Lipofectamine™ 2000 (Invitrogen; COS7) or FuGene 6 (Roche; HeLa) as recommended by the manufacturers. Cells were stimulated with 10 μM MG132, 100 nM calyculin A (cells detach and are collected in suspension from the medium) and 400 nM PMA. All untreated controls were performed with an equal volume of DMSO.

**Cell culture, transfection and treatment**

COS7 and HeLa cells were cultured as described previously [24]. 293FT cells were obtained from Invitrogen and maintained according to their guidelines. Transfection of plasmid DNA was performed using Lipofectamine™ 2000 (Invitrogen; COS7) or FuGene 6 (Roche; HeLa) as recommended by the manufacturers. Cells were stimulated with 10 μM MG132, 100 nM calyculin A (cells detach and are collected in suspension from the medium) and 400 nM PMA. All untreated controls were performed with an equal volume of DMSO.
RNAi (RNA interference)

RNAi was performed using siRNA (small interfering RNA) duplexes from Dharmacon. siLamin A/C (M-013859-00) was used as a control; siFbw7 targets all three members of the mammalian Fbw7 family and has been described previously [15]. 293FT cells were seeded at 2 × 10^5 cells on six-well plates, incubated overnight and then transfected with 50 nM siRNA using Lipofectamine™ 2000, as recommended by the supplier (Invitrogen). Cells were treated or not with 400 nM PMA, as indicated and harvested at 3 days post-transfection directly into LDS Sample buffer (see below). Samples were boiled for 5 min and briefly sonicated (power setting 5, Microson sonicator; Misonix) prior to analysis by SDS/PAGE and Western blotting.

GST (glutathione transferase) pull-downs and immunoprecipitations

GST pull-downs and immunoprecipitations were performed as described previously [24]. For FLAG immunoprecipitations, anti-FLAG M2–agarose affinity gel was employed.

SDS/PAGE and Western blotting

SDS/PAGE and Western blotting were performed as described previously [24]. Primary antibodies were diluted as follows: mouse anti-FLAG (M5), 1:1000; mouse anti-GFP antibody (3E1, CR-UK), 1:1000; rabbit anti-GST antibody (sc-459, Santa Cruz), 1:1000; anti-phospho-Ser10 antiserum (PPA-574) and phospho-Ser10 antibody (sc-214, Santa Cruz), 1:2000; mouse anti-FLAG antibody (M5, CR-UK), 1:1000; rabbit anti-PKCα antibody (MC5, CR-UK), 1:1000; rabbit anti-PKCα-phospho-Thr638 antibody (9375, Cell Signaling), 1:1000 plus 5% (w/v) BSA; rabbit anti-PKCε antibody (sc-214, Santa Cruz), 1:5000; rabbit anti-atypical PKC antibody (C20, Santa Cruz), 1:5000; and mouse anti-cyclin E antibody (HE12, sc-247, Santa Cruz), 1:1000. Two phosphospecific antibodies were also raised against Fbw7α (see below) and used at 1:1000 with 5% (w/v) BSA and 1 μg/ml of the corresponding, blocking dephosphopeptide: anti-phospho-Ser10 antisemur (PPA-574) and anti-phospho-Ser18 antisemur (PPA-527). The Western blots presented are each representative of results obtained from at least three separate experiments.

In vitro kinase assays

The following peptides were prepared for use as PKC substrates: Fbw7α wild-type 7–14, N-SVGSKRRR-C; Fbw7α-S10A 7–14, N-SVGAKRRR-C; Fbw7α wild-type 11–22, N-KRRRTGGSRLGN-C; and Fbw7α-S18A 11–22, N-KRRRTGGALRGC (Peptide Synthesis Facility, CR-UK). Reactions were performed using 50 μl of the following kinase assay buffer: 20 mM Tris/ HCl, pH 7.5, 5 mM MgCl2, 1 μg/ml phosphatidylserine (Lipid Products), 0.2% Triton X-100, 1 ng/μl PMA, 50 μM ATP, 5 μCi [γ-32P]ATP (Amersham), with 0.2mM peptide substrate and 12.5 ng of PKCε. Assays were started with the addition of ATP, incubated at 30°C for 10 min, and stopped by spotting 15 μl on to P81 paper (Whatman) and washing three times for 5 min each in 30% acetic acid. P81 strips were analysed by Cerenkov counting using a Beckman LS6000IC scintillation machine to quantify 32P incorporation.

Preparation of phosphospecific antibodies

Rabbit polyclonal antisera were raised using the following phosphopeptides as antigens: Fbw7α phospho-Ser10α, N-SVGpSKRRR-C; and Fbw7α pS18, N-RTGGpSLRGN-C (corresponding blocking dephosphopeptides were also synthesized). Each phosphopeptide was coupled to KLH (keyhole limpet haemocyanin) using glutaraldehyde and immunized in triplicate (Harlan Sera-Lab). The resulting sera were characterized by ELISA and found to exhibit both site- and phospho-specificity against phospho-Ser10α (serum PPA-574) or phospho-Ser18α (serum PPA-527). An additional peptide was used to determine whether serum PPA-574 could recognize phospho-Ser10α in the context of a K11R mutation (pS10 K11R, N-SVGpSRRRR-C).

Epifluorescent and confocal microscopy

Immunofluorescence was performed as described previously [24]. For propidium iodide staining of the nuclei, coverslips were incubated with 1 μg/ml RNase (Qiagen)/PBS for 15 min, followed by 0.25 μg/ml propidium iodide/PBS for a further 10 min. Alternatively, 1 μg/ml Hoechst 33342 (Invitrogen)/PBS was used for 10 min to stain DNA. Coverslips were washed three times for 10 min each in PBS and once in water, and mounted on to glass slides using Mowiol 4–88 (Calbiochem) or Aqua Poly/Mount (Polysciences). Confocal images were acquired using a confocal, laser scanning microscope (LSM 510, Zeiss) equipped with a 63×/1.4 Plan-Apochromat Oil Ph3 objective. Individual channels were scanned sequentially, line by line, with averaging set at eight. Each image (12 bit, 1024 pixels) represents a single 1.0 μm optical z-section with a pixel size of approx. 0.1 μm. Epifluorescent images were acquired using an Imager.A1 upright microscope (Zeiss) equipped with a Plan-Apochromat 63×/1.4 Oil objective and an Orca-ER Digital Camera (Hamamatsu) using Axiovision software (Release 4.6.3). All images presented are representative of results obtained from at least three independent experiments.

Quantification of Fbw7α nuclear localization

Confocal images were captured as described above except that complete z-stacks were acquired for each field of view. Each z-stack was compressed and analysed using Metamorph. The nuclear compartment was defined using the propidium iodide staining of the nuclei, coverslips were incubated with 1 μg/ml RNase (Qiagen)/PBS for 15 min, followed by 0.25 μg/ml propidium iodide/PBS for a further 10 min. Alternatively, 1 μg/ml Hoechst 33342 (Invitrogen)/PBS was used for 10 min to stain DNA. Coverslips were washed three times for 10 min each in PBS and once in water, and mounted on to glass slides using Mowiol 4–88 (Calbiochem) or Aqua Poly/Mount (Polysciences). Confocal images were acquired using a confocal, laser scanning microscope (LSM 510, Zeiss) equipped with a Plan-Apochromat 63×/1.4 Oil objective and an Orca-ER Digital Camera (Hamamatsu) using Axiovision software (Release 4.6.3). All images presented are representative of results obtained from at least three independent experiments.

Sequence alignments

Fbw7 amino acid sequences from a variety of species were retrieved from the NCBI Entrez-Protein Database using the following accession numbers: Homo sapiens, NP_361014; Mucaca mulata, NP_01084190; Rattus norvegicus, EDM00812; Mus musculus, AAH85184; Gallus gallus, XP_420447; Xenopus laevis, NP_001089186. The accession number for human cyclin E is NP_476530 and the different isoforms of human PKC are as follows: PKCα, NP_002728; PKCβI, NP_997700; PKCβII, NP_002729; PKCδ, NP_997704; PKCζ, NP_005391; and PKCζ, NP_002735. Sequence alignments were performed using ClustalW software, with default settings, according to EMBL-EBI recommendations.
RESULTS

The catalytic domain of PKC interacts with the SBD of Fbw7

PKCζ regulates a diverse range of cellular processes, including proliferation, survival, migration and invasion [24]. Aberrant PKCζ activity is associated with a transformed phenotype and its expression is a biomarker for aggressive breast cancer [21]. To gain further insights into the physiological and pathophysiological functions of PKCζ, we performed a yeast two-hybrid screen to identify novel binding partners.

Full-length PKCζ bait was used to screen a mouse brain cDNA library, yielding 28 in-frame positive clones, including a known PKCζ-interacting protein, Nell2 [26], which validated the approach. Among the other hits, ‘clone 19’ was identified as the C-terminal SBD of Fbw7 (Figures 1a and 1b). Fbw7 is a tumour suppressor that mediates the degradation of several potent oncoproteins to control cell division, growth, differentiation and transformation [5]. As such, Fbw7 was selected as an interesting candidate for further analysis.

To verify and map the interaction between PKCζ and Fbw7 in mammalian cells, GST pull-downs were performed (Figure 1c). Cells were co-transfected with the GFP–Fbw7-SBD, and full-length or truncated PKCζ–GST. Pulling down with a GST control or the regulatory domain of PKCζ recovers only background amounts of GFP–Fbw7-SBD, whereas significantly elevated levels are recovered when using either full-length PKCζ or its catalytic domain. Since the latter two constructs are more weakly expressed, the efficiency of these pull-downs is particularly striking. We conclude that the catalytic domain of PKCζ associates with the SBD of Fbw7 in mammalian cells.

The catalytic domain is highly conserved among PKC superfamily members, raising the possibility that Fbw7 may also interact with other isoforms. To test this, cells were co-transfected with full length FLAG–Fbw7 and a panel of different GFP-tagged PKCζ isoforms, and analysed by co-immunoprecipitation (Figure 1d). Since PKCζ represents a potential substrate for the SCP2-mediated ubiquitin ligase, immunoprecipitations were performed in the presence or absence of MG132, a proteasome inhibitor, to protect any ubiquitinated PKCζ from degradation. Fbw7 was immunoprecipitated using anti-FLAG antibody, and immunoprecipitates were probed for associated PKC using anti-GFP antibody. All the PKCζ isoforms tested bound to Fbw7; the strongest interaction detected was with the atypical PKCζ, Total levels of PKC (in cell lysate) did not change upon Fbw7 co-expression or MG132 treatment, suggesting that Fbw7 is not responsible for PMA-stimulated PKCζ degradation.

For these experiments, we reproduced conditions established by van Drogen et al. [15], who designed an siRNA duplex to target the Fbw7 common region, and demonstrated co-depletion of the mammalian isoforms from HEK-293T cells [HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40)], by qRT-PCR (quantitative real-time PCR) (for endogenous Fbw7) and by Western blotting (for exogenous tagged Fbw7). To validate the siRNA duplex, we sequentially transfected cells, first with siControl or siFbw7, and then with GFP alone or GFP–Fbw7ζ.

As expected, the siFbw7 duplex led to a specific depletion of GFP–Fbw7ζ (Figure 1f).

Using these conditions, cells were co-depleted of all three Fbw7 isoforms and subjected to Western blotting for endogenous PKCζ isoforms, with cyclin E as a positive control (Figure 1g). Lysates were also probed with a phosphospecific antibody against the PKCζ turn motif (if this site acts as a CPD, it may be expected to provide a more sensitive marker of degradation). Finally, to analyse both basal and regulated degradation, these experiments were performed in the presence or absence of phosphoribosyl pyrophosphate (PMA), which stimulates the activation-induced down-regulation of classical PKCs (e.g. PKCζ) and novel PKCs (e.g. PKCε) isoforms [27]. As shown in Figure 1(g), depletion of Fbw7 led to a clear stabilization of its substrate cyclin E. However, levels of both total and phospho-PKCζ were unchanged by Fbw7 knockdown, suggesting that none of these isoforms represent Fbw7 substrates. PMA treatment induced the complete degradation of PKCζ, and a partial reduction in PKCζ levels after 20 h; however, neither of these events was inhibited by depletion of Fbw7, indicating that Fbw7 is not responsible for PMA-stimulated PKCζ degradation.

We conclude that Fbw7 interacts with multiple members of the PKC superfamily but does not target them for either basal or activation-induced degradation.

PKCζ phosphorylates Ser10 and Ser18 within the unique N-terminal domain of Fbw7ζ in vitro and in mammalian cells

We next addressed whether Fbw7 may be a substrate for PKC-mediated phosphorylation. First, Fbw7ζ protein sequences were analysed using a predictive database for PKC phosphorylation, MPR PKC Scan [28]. Two of the highest scoring sites identified were Fbw7ζ residues Ser10 and Ser18, which are in the top 0.06 and 0.2 percentiles respectively for PKCζ phosphorylation. These residues lie within the isoform-specific N-terminal region of Fbw7ζ, which plays an important role in determining the unique nuclear localization of this isoform; NLS1 (nuclear localization signal 1) lies nearby, between residues 11 and 14 (Figure 2a). Furthermore, this region is altered in certain cancer samples; the mutations K11R and +P16 have been reported [13]. We reasoned that PKC-dependent phosphorylation within this regulatory region may be of functional significance and sought to study these candidate sites further.

To test whether PKC can phosphorylate Fbw7ζ, in vitro kinase assays were performed (Figure 2b). Peptides were synthesized corresponding to residues 7–14, to test for Ser10 phosphorylation, and residues 11–22, for Ser18 analysis (in control peptides the candidate serine residue was substituted with a non-phosphorylatable alanine residue). Peptide kinase assays were carried out using purified PKCζ and [γ-32P]ATP and incorporation of 32P was detected by Cerenkov counting. Both wild-type peptides were efficiently phosphorylated compared with the corresponding alanine mutants, confirming the prediction that Fbw7ζ residues Ser10 and Ser18 can be phosphorylated directly by PKC. Similar results were obtained using recombinant PKCζ, PKCδ or PKCζ (results not shown), suggesting that Fbw7ζ is a common substrate for PKC superfamily members.

To determine whether Fbw7ζ is phosphorylated in mammalian cells, phosphospecific antibodies were raised. Cells were transfected with GFP–Fbw7ζ constructs and analysed by Western blotting (Figure 2c). As a broad stimulus, cells were treated with or without calyculin A, an inhibitor of the PP1 and PP2A phosphatases (this compound protects sensitive sites from dephosphorylation, and may also up-regulate certain
signalling pathways by preserving activating phosphorylations).

As revealed by the phosphospecific antisera, phosphorylation of both Ser10 and Ser18 was induced by calyculin A treatment in all constructs, except for the corresponding alanine mutants. These results confirm that Fbw7α residues Ser10 and Ser18 can be phosphorylated in mammalian cells in a mutually independent fashion, and also serve to illustrate the site specificity of the two antisera.

Figure 1  Fbw7 interacts with, but does not down-regulate, multiple members of the PKC superfamily

(a) Yeast two-hybrid analysis. Yeast were co-transformed with PKCε bait vector and either Fbw7-SBD prey (19), or an empty pGADT7 control vector (−). p53/T-antigen co-transformants were used as positive controls (+). Yeast were plated on to selective medium for the vectors (−T/L), medium stringency interaction (−T/L/H) or high stringency interaction (−T/L/H/A/X), incubated at 30°C for 5 days and photographed. T, tryptophan; L, leucine; H, histidine; A, adenine; X, X-α-gal. (b) Human Fbw7 isoforms (α, β and γ) and the positive yeast two-hybrid clone (19) are represented to scale with the isoform-specific N-terminal region, the F-box domain and the SBD highlighted [31]. (c) GST pull-downs. COS7 cells were co-transfected with GFP–Fbw7-SBD (GFP-19) and a panel of GST–PKCε constructs (ε, full-length PKCε; εR, PKCε regulatory domain; and εC, PKCε catalytic domain). At 24 h post-transfection, lysates were prepared, subjected to GST pull-down and the presence of the indicated proteins was analysed by Western blotting. (d) Fbw7/PKC co-immunoprecipitations. COS7 cells were co-transfected with FLAG–Fbw7β and a panel of GFP-tagged human PKC isoforms. At 32 h post-transfection, cells were treated with or without 10 μM MG-132 for a further 16 h. Lysates were prepared and immunoprecipitation (IP) was performed using anti-FLAG antibody. Samples were analysed by Western blotting (IB). (e) Sequence alignment of human PKC isoforms and a cyclin E CPD. The 'TP'/turn motif is highlighted with grey shading and the central phosphoresidue is numbered in parentheses. The +4 residue is indicated with a box; in a CPD, this position is typically occupied by a phosphoserine or a negatively charged amino acid. (f) Fbw7 depletion. HEK-293T cells (293FT) were sequentially transfected with siRNA and DNA to assess the efficacy of the siFbw7 duplex. Cells were first transfected with either no (−), control (C) or Fbw7-specific siRNA and incubated overnight. Cells were then co-transfected with empty GFP vector and GFP–Fbw7α and incubated for a further 16 h. Total cell extracts were prepared and analysed by Western blotting. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (g) Fbw7 depletion stabilizes cyclin E but not PKC. HEK-293T cells (293FT) were transfected with either control or Fbw7-specific siRNA, incubated for 3 days and treated with or without 400 nM PMA as indicated. Total cell extracts were prepared and the levels of the indicated protein analysed by Western blotting.

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Figure 2  PKC phosphorylates the isoform-specific N-terminal region of Fbw7α in vitro and in cells

(a) Amino acid sequence of the isoform-specific N-terminus of human Fbw7α. Candidate phosphorylation sites (Ser10 and Ser18) and cancer-associated mutations (K11R, +P16) are indicated with arrows. NLS1 (11–14) is shown in bold and boxed. (b) In vitro phosphorylation of Fbw7α Ser10 and Ser18 by PKC. Peptide kinase assays were performed by incubating purified PKCε, the indicated peptides and [γ-32P]ATP for 10 min; 32P incorporation was quantified by Cerenkov counting. Reactions were analysed in triplicate and results are means ± S.D. (c) Phosphorylation of Fbw7α Ser10 and Ser18 in mammalian cells. HeLa cells were transfected with a panel of GFP–Fbw7α constructs [wild-type (WT), S10A or S18A], incubated for 24 h and then treated with or without 100 nM calyculin A for a further 1 h. Lysates were prepared and immunoprecipitation (IP) was performed to enrich for Fbw7α using an anti-GFP antibody. Samples were analysed for the indicated protein by Western blotting (IB). (d) Pharmacological inhibition of Fbw7α phosphorylation. HeLa cells were transfected with GFP–Fbw7α and incubated for 24 h. Cells were pretreated with or without 6 µM Gö6983 (a pan-PKC inhibitor) for 15 min, and then stimulated with or without 100 nM calyculin A for a further 45 min. Samples were processed as in (c). (e and f) PKC-dependent Fbw7α phosphorylation. HeLa cells were transfected with either (e) GFP–Fbw7α with or without wild-type (WT) or constitutively active (CA) PKCa, or (f) GFP–PKCa with or without wild-type or constitutively active PKCε. At 24 h post-transfection, lysates were prepared and analysed as described in (c). (g) HeLa cells were co-transfected with a GFP–Fbw7α construct (WT, K11R or +P16) and a wild-type (ζ) or constitutively active Myc–PKCζ construct. At 24 h post-transfection, lysates were prepared and analysed as described in (c).

To test whether Fbw7α phosphorylation is dependent upon PKC in a cellular context, a pharmacological approach was taken. Since Fbw7α can be phosphorylated by multiple PKC isoforms, we employed the pan-PKC inhibitor Gö6983. Upon pretreatment with Gö6983, the calyculin A-induced phosphorylation of Ser10 and Ser18 decreased by approx. 20% and 40% respectively, consistent with a partial requirement for endogenous PKC activity (Figure 2d). We note that calyculin A will tend to cause phosphorylation to ratchet up inexorably, such that the observed response to inhibition of the upstream kinase(s) may be compromised, given that inhibition is never complete. Notwithstanding this caveat, because these phosphorylation events are only partially inhibited by Gö6983, it is possible that multiple kinase activities may converge on Fbw7α phosphorylation under these calyculin-A-triggered conditions.

To investigate whether PKC activity is sufficient to promote the cellular phosphorylation of Fbw7α, we tested the effect of co-expressing wild-type, or constitutively active, PKCa or PKCi. Importantly, the overexpression and overactivation of PKCa [29] and PKCi [22,30] is observed in range of human cancers, so these experiments effectively model the status of certain tumours [20]. Expression of wild-type PKCa or PKCi induced Fbw7α phosphorylation at both Ser10 and Ser18 (Figures 2e and 2f), and this was dramatically increased by the constitutively activated kinases; similar results were also observed using PKCζ (Figure 2g). Together, these results confirm that PKC activity is able to drive Fbw7α phosphorylation in mammalian cells.

Finally, to establish whether nearby cancer-associated mutations may influence these phosphorylation events, GFP–Fbw7α-K11R and +P16 mutants were analysed as described...
Fbw7α is a novel PKC substrate

Phosphorylation of Ser10 can regulate Fbw7α nuclear localization

We next sought to examine the functional consequences of Fbw7α phosphorylation at Ser10 and/or Ser18. These residues lie in close proximity to NLS1 (Figure 2a) and we reasoned that phosphorylation may thereby influence Fbw7 localization. Regulation of Fbw7 localization is of particular interest because it is intimately linked to its profile of substrate down-regulation, determining which of many potential targets the ubiquitin ligase is exposed to. Fbw7α contains two NLSs, NLS1 in the isoform-specific N-terminus and NLS2 in the shared SBD (Figure 3a). Either of these signals can confer nuclear localization and both must be lost to shift Fbw7α to the cytosol in U2OS cells [14]. Thus, to analyse regulation of NLS1 by phosphorylation, we employed a mutant lacking NLS2 (ΔNLS2) so that the nuclear localization of this protein is completely dependent on a functional NLS1, and any regulatory inputs should manifest clearly.

above. Fbw7α +P16 was phosphorylated by the atypical PKCζ at both Ser10 and Ser18, as efficiently as the wild-type protein. Fbw7α-K11R was also phosphorylated at Ser18 (Figure 2g). The phosphorylation of Fbw7α-K11R at Ser10 could not be monitored, as this mutation impairs the recognition of phospho-Ser10 by our antiserum (demonstrated by ELISA; results not shown). However, since the K11R substitution preserves a basic charge at this residue, it is unlikely to affect PKC-mediated phosphorylation; in fact, Ser10 scores even more highly as a candidate phosphorylation site in a K11R mutant (0.05 percentile) than in the wild-type protein (0.06 percentile) according to the MPR PKC Scan database.

We conclude that PKC phosphorlates residues Ser10 and Ser18 in the isoform-specific N-terminal region of Fbw7α, both in vitro and in mammalian cells. These phosphorylation events are not mutually dependent or affected by the cancer-associated mutations K11R and +P16.
Cells were transfected with GFP–Fbw7α constructs in which Ser10 and/or Ser18 were mutated to either non-phosphorylatable alanine or phosphomimetic aspartate residues, in the presence or absence of an intact NLS2. GFP–Fbw7α localization was then analysed by confocal microscopy (Figure 3b and results not shown). Quantitative analyses were performed by measuring the GFP signal intensity inside and outside of the nucleus, as judged by co-localization with DNA staining; the ratio between these values represents Fbw7α localization (Figure 3c). Consistent with previous reports, wild-type Fbw7α was localized almost exclusively in the nucleus (the same pattern of localization was observed using FLAG–Fbw7α; results not shown and [14]). None of the phosphomutants showed an altered pattern of localization in an otherwise wild-type context (NLS2wt constructs). The ΔNLS2 mutant was also predominantly nuclear, although a weak cytosolic signal was detected, suggesting that NLS1 alone is not completely sufficient for proper localization of the protein (the difference between the wild-type/wild-type and wild-type/ΔNLS2 localization is statistically significant; $P = 0.001$). In the context of the ΔNLS2 protein, the phosphomimetic aspartate mutation of Ser10, but not Ser18, led to a dramatic cytosolic redistribution of the protein, indicating that this modification severely impairs the activity of NLS1. Furthermore, the modest cytosolic signal detected with the ΔNLS2 mutant was significantly reduced when Ser10 was mutated to an alanine residue; S10A/ΔNLS2 mutants localize more like the wild-type protein (Figure 3c). This observation suggests that endogenous phosphorylation of Ser10 may underlie the reduced efficiency of NLS1 as a single nuclear localization signal.

We conclude that phosphorylation of Fbw7α at Ser10 strongly inhibits the activity of its isoform-specific nuclear localization signal, NLS1. However, for this modification to result in a cytosolic redistribution of the protein, NLS2 activity must also be suppressed.

**Cancer-associated mutations can regulate Fbw7α nuclear localization**

Since the cancer-associated Fbw7α mutations, K11R and +P16, lie close to NLS1, we reasoned that these modifications may also regulate nuclear localization. To test this, GFP–Fbw7α constructs harbouring these mutations, in the presence or absence of NLS2, were transfected and visualized by epifluorescent microscopy (Figure 4a). Both mutants localized to the nucleus in the presence of an intact NLS2 (NLS2wt). However, in the absence of NLS2 (ΔNLS2), these mutations relocalized the protein; both K11R and +P16 mutants were distributed throughout the cell and resembled the S10D mutant with respect to localization.

We conclude that mutations in the isoform-specific N-terminal domain of Fbw7α (K11R and +P16) can regulate the activity of NLS1 in a manner comparable with phosphorylation of Ser10. Furthermore, since these mutations were identified in tumours, relocalization of Fbw7α may be of pathophysiological significance.

**DISCUSSION**

In the present study, we explored the relationship between two protein families, Fbw7 and PKC, whose dysregulation has been associated with cancer. Through a series of biochemical and cell-based assays, we uncover a physical and functional relationship between these proteins that may play a role during transformation.

Fbw7 was identified as a PKCζ-binding partner by yeast two-hybrid analysis, indicating that these proteins interact directly. In mammalian cells, the interaction was mapped to the catalytic domain of PKC and the SBD of Fbw7, which is an unusual eight-bladed $\beta$-propeller composed of WD40 repeats [31]. The PKC kinase domain is highly conserved, and consistent with this, all isoforms tested are able to interact with Fbw7. In the absence of an effective anti-Fbw7 antibody, we were unable to confirm the interaction between endogenous proteins. However, we note that in a large-scale study of tight-junction components, Fbw7β was detected by MS following immunoprecipitation of endogenous PKCζ from T84 cells [32]. These results support the conclusion that PKC and Fbw7 interact under physiological conditions.

We were initially intrigued by the possibility that Fbw7 may target PKC for proteasomal degradation. PKC is well known to undergo activation-induced down-regulation and this may contribute to transformation under certain conditions [27]. However, to date, only two ubiquitin ligases have been identified that can mediate degradation of selected PKC isoforms, VHL (von Hippel–Lindau protein) [33] and RINCK (RING-finger protein that interacts with C kinase) [34]. The PKC TP/‘turn’ motif bears striking similarity to the CPD sequence recognized...
by Fbw7 in its substrates, consistent with the notion it may represent a novel target. However, despite this, several lines of evidence suggest that PKC does not represent an Fbw7 substrate: (i) all isoforms of PKC interact with Fbw7, although only some possess a well-conserved CPD; (ii) expression of the Fbw7 SBD does not stabilize PKC expression, although it should act as a dominant-negative with respect to the SCD ubiquitin ligase; (iii) expression of full-length Fbw7 does not down-regulate PKC; and (iv), most importantly, depletion of endogenous Fbw7 does not protect PKC from either basal, or activation-induced, down-regulation.

It is important to note that our results do not completely exclude Fbw7 as a possible PKC ubiquitin ligase. First, interfering with Fbw7 function does not always manifest in detectable changes in steady-state substrate levels; for instance, c-Myc turnover is compromised in an Fbw7α-null Hct116 cell line, protein levels do not change, due to a compensatory decrease in mRNA levels [16]. Secondly, the profile of Fbw7 substrate down-regulation also exhibits some cell-type specificity, such that PKC may be a substrate under different conditions to those tested. For instance, Fbw7γ degrades nuclear c-Myc in U2OS [14], but not in Hct116 cells [16]; and Fbw7 degrades c-Jun in HEK-293T [11], but not in Hct116 cells [16]. Nevertheless, in the absence of clear evidence for Fbw7-mediated PKC degradation, we focussed our attention on the reciprocal relationship and investigated whether PKC can phosphorylate Pbw.

We have identified Fbw7α as a novel PKC substrate. Two residues, Ser10 and Ser18, are phosphorylated in a PKC-dependent manner, both in vitro and in mammalian cells. In vitro, Fbw7α can be phosphorylated by multiple PKC isoforms, suggesting it is a common substrate for the PKC superfamily. In mammalian cells, the overexpression of either active classical PKCα or atypical PKCε is sufficient to drive robust Fbw7α phosphorylation. Since the overexpression and overactivation of PKCα [29] and PKCε [35] is observed in a range of human tumours, we conclude that Fbw7α phosphorylation may be elevated in certain cancers. Notably, PKCε is a known oncogene [22] and its amplification is one of the most common somatic alterations in human cancer [30]. As such, it will be important to investigate whether aberrant Fbw7α phosphorylation contributes to the pathophysiological function of PKC.

The two novel Fbw7α phosphorylation sites identified in the present study, Ser10 and Ser18, lie within the isoform-specific N-terminus of Fbw7α, which is the most abundant Fbw7 family member and bears primary responsibility for the degradation of several critical substrates [16]. Importantly, both of these phosphorylation sites are evolutionarily conserved (Figure 5a), suggesting that they may be of functional significance. Future work will aim to determine whether phosphorylation of Fbw7α influences its dimerization [36], assembly into an SCF complex, auto-ubiquitylation [37] or ubiquitylation of other substrates. In the present study, we investigated the possibility that Fbw7α phosphorylation may influence its localization.

Ser10 is adjacent to NLS1, one of the two NLS motifs in Fbw7α that confer nuclear localization to the protein. It is well-established that phosphorylation can influence the activity of an NLS, for instance, PKC-catalysed phosphorylation proximal to the AhR (aryl hydrocarbon receptor) NLS inhibits its nuclear import [38]. Consistent with this, mutation of Ser10, but not Ser18, to a phosphomimetic aspartate residue severely inhibits the activity of NLS1. Strikingly, cancer-associated mutations in nearby residues (K11R and +P16) disrupt Fbw7α NLS1 function in a comparable manner. Although the K11R substitution preserves a basic charge, structural and thermodynamic studies confirm that a lysine residue is strictly required in the +1 position of a monopartite classical

NLS [39]. The +16 insertion of a rigid proline residue would yield a significant change in local structure and so it is perhaps not surprising that this mutation also interferes with the function of NLS1. Importantly, since these mutations were identified from somatic events in tumours, these observations indicate that aberrant function of NLS1 may be of pathological significance. By extension, we surmise that dysregulation of Fbw7α by PKC-mediated phosphorylation may also contribute to transformation under certain conditions.

As noted above, Fbw7α bears two redundant nuclear localization signals (NLS1 and NLS2) and it is necessary to suppress the activity of both to shift the protein outside of the nucleus [14]. We have identified three distinct mechanisms through which NLS1 can be inhibited: phosphorylation of Ser10 by PKC, substitution of residue Lys11 for an arginine residue, and insertion of a proline residue at position 16 (Figure 5b). Under conditions where NLS2 activity is suppressed, these regulatory inputs result in a dramatic relocalization of Fbw7α to the cytosol. Further work will be required to explore whether NLS2 is also subject to regulation, by mutation, post-translational modification or masking by protein–protein interactions [40]. Since it lies within the conserved SBD, an interesting possibility is that engagement of certain substrates may mask NLS2. Related to this, we note that the +P16 mutant, which is nuclear in HeLa cells (Figure 4), is reported to localize to the cytosol in HEK-293T cells, even in the absence of other alterations [13]. On the basis of these results, we hypothesized that the presence of large T-antigen, which binds competitively to the SBD [41], may ameliorate the activity of NLS2 in these cells. However, we have been unable to detect cytosolic +P16 Fbw7α in HEK-293T cells, so could not explore this possibility further. Nevertheless, these results suggest that conditions exist in which NLS1 is solely responsible for Fbw7α nuclear localization, and that the regulatory inputs that influence NLS2 activity warrant further investigation.

The mammalian Fbw7 family members are localized to different subcellular compartments by regulatory signals in their
isoform-specific N-terminal regions [14]. This suggests that each isoform performs a unique function in a distinct region of the cell. Indeed, Fbw7γ is localized to the nucleolus, and in this subcellular compartment degrades a specific pool of c-Myc in order to control U2OS cell size [14]. Furthermore, Fbw7α degrades full-length cyclin E in the nucleus, but does not down-regulate certain low-molecular-mass forms that are localized in the cytoplasm [42]. Mislocalization of Fbw7α to the cytosol, a compartment usually occupied by the much less abundant Fbw7β isoform [14,16], is therefore expected to have significant effects on its pattern of substrate down-regulation. Under these conditions, full-length cyclin E and other nuclear substrates may be spared from degradation, while cytosolic targets, such as Notch, may be exposed to higher levels of the ubiquitin ligase. Future work will be aimed at investigating such changes by differential proteomics, for instance by employing a SILAC (stable isotope labelling by amino acids in cell culture)-based strategy. The combined effects of elevated nuclear and decreased cytosolic substrate expression would be expected to have profound consequences for cell proliferation, growth and differentiation. Furthermore, since many nuclear Fbw7 substrates are potent oncoproteins, relocalization of Fbw7α is also likely to drive transformation, ultimately with a similar outcome to loss or mutation of the protein. Related to this, we note that overexpression/activation of PKCι occurs concurrently with a substantial up-regulation of cyclin E in certain ovarian tumours [35] and it will be interesting to explore whether mislocalization of Fbw7α through Ser18 phosphorylation contributes to this phenotype.

Our current findings integrate Fbw7α into a novel PKC-dependent signal transduction pathway. In light of this, it is interesting to consider the place of Fbw7 within a wider network of signalling events. For instance, we note the potential for interplay between PKC, Fbw7 and GSK3. GSK3 is the kinase responsible for phosphorylating most, if not all, of the CDK motifs recognized by Fbw7 in its substrates [5], and phosphorylation of GSK3 by PKC has been shown to inhibit its activity [43]. As such, it is plausible that PKC activity may inhibit GSK3-mediated CPD phosphorylation, thereby protecting potential substrates from Fbw7 recognition. Notably, PKC activation results in reduced phosphorylation of c-Jun by GSK3 in the region of Ser32 [44], the central phosphoserine of the c-Jun CPD [11]. Thus we can conceive two possible mechanisms through which PKC activity may antagonize Fbw7 function: (i) indirectly, by inhibiting the GSK3-mediated phosphorylation events which target substrates for Fbw7 recognition; and (ii) directly, by phosphorylating Fbw7α itself, to relocalize the protein and protect nuclear substrates from ubiquitination and degradation. Thus stabilization of Fbw7 substrates may partly underlie the tumour-promoting properties of PKC activity.

In conclusion, our current results provide evidence for both physical and functional interactions between PKC and Fbw7α, controlling the function of NLS1 in the latter case. Future work will address the regulation of Fbw7α NLS2, in order to define the combinatorial events which may control its nuclear uptake.

**AUTHOR CONTRIBUTION**
Joanne Durgan and Peter Parker conceived the experiments and wrote the manuscript; Joanne Durgan performed all of the experiments.

**ACKNOWLEDGEMENTS**
We thank Professor Shigetsugu Hatakeyama, Dr Songhai Shi and Dr Bruce Clurman for providing us with plasmids. We are grateful to Phil Whitehead for preparing purified recombinant PKCs, and to Peter Jordan and Alastair Nichol for their assistance with confocal microscopy and image analysis respectively. We also express thanks to Axel Behrens, Annette Jandke and members of the Parker laboratory for helpful discussions, and to Professor Alan Hall for supporting this work.

**FUNDING**
This work was supported by a PhD fellowship from the Medical Research Council (to J.D.) and by CR-UK.

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