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► To cite this version:

Joanne Durgan, Peter J Parker. Regulation of the tumour suppressor Fbw7 α by PKC dependent phosphorylation and cancer associated mutation. Biochemical Journal, 2010, 432 (1), pp.77-87. 10.1042/BJ20100799 . hal-00529106

HAL Id: hal-00529106

<https://hal.science/hal-00529106>

Submitted on 25 Oct 2010

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**Regulation of the Tumour Suppressor Fbw7 α by
PKC Dependent Phosphorylation and Cancer Associated Mutation**

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SYNOPSIS

F-box WD40 Protein 7 (Fbw7) is a major tumor suppressor, which mediates the degradation of several potent oncogenes. Protein Kinase C (PKC) is a serine/threonine kinase which 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 Fbw7 α are phosphorylated in a PKC-dependent manner, both *in vitro* and in mammalian cells (S10 and S18). Mutational analyses reveal that phosphorylation of Fbw7 α at S10 can regulate its nuclear localisation. Cancer associated mutations in nearby residues (K11R, +P16) influence Fbw7 α localisation in a comparable manner, suggesting that mislocalisation of this protein may be of pathological significance. Together these data provide evidence for both physical and functional interactions between the PKC and Fbw7 families, and yield insights into the isoform specific regulation of Fbw7 α .

Short title: Fbw7 α is a novel PKC substrate

Keywords: PKC, Fbw7, Fbxw7, hCdc4, nuclear localisation

Abbreviations: CPD, Cdc4 phospho-degron; Fbw7, F-box WD40 Protein 7; NLS, nuclear localisation signal; PMA, phorbol 12-myristate 13-acetate; SBD, substrate binding domain; SCF, SKP-Cullin-F-Box.

INTRODUCTION

Fbw7 is a conserved F-box WD40 protein, also known as Fbw6 in mice [1], archipelago in flies [2], SEL10 in worms [3] and Cdc4 in yeast [4]. Fbw7 functions as the substrate recognition component of a SKP-Cullin-F-box (SCF) ubiquitin ligase, binding specifically to a conserved phospho-motif, termed the Cdc4 Phospho-Degron (CPD), in all reported targets [5]. The CPD sequence comprises: L/I-L/I/P-pT-P-<R/K>4, where <> refers to disfavoured residues [6]; the central threonine is usually phosphorylated by GSK3 [5]. Through CPD recognition, Fbw7 targets an impressive collection of proteins for ubiquitination and proteasomal degradation, including cyclin E [2, 7, 8], c-myc [9] and c-jun [10, 11]. In this way, Fbw7 regulates diverse cellular functions, including cell growth, proliferation and differentiation [5]. Furthermore, since several of its substrates are potent oncogenes, Fbw7 represents a major tumour suppressor [12], and is mutated in 6% of human cancers [13].

Mammalian Fbw7 comprises a family of three differentially spliced isoforms: Fbw7 α , Fbw7 β and Fbw7 γ [14]. In certain contexts, these proteins function co-operatively. For instance, Fbw7 α and Fbw7 γ operate sequentially to degrade cyclin E in 293 cells [15]. However, abundant evidence also exists for isoform specificity within the Fbw7 family. For example, Fbw7 α is primarily responsible for targeting cyclin E, c-myc and SREBP-1 for degradation in Hct116 cells [16]. The three Fbw7 family members localise to distinct subcellular compartments, which are determined by isoform-specific, N-terminal regulatory sequences [14]. Fbw7 α is predominantly nuclear, Fbw7 β cytosolic, and Fbw7 γ resides in the nucleolus; the differential distribution of these proteins is likely to underlie important differences in their profiles of substrate degradation. For instance, Fbw7 γ specifically mediates the degradation of c-myc in the nucleus to control U2OS cell size [14]. In light of the critical roles performed by Fbw7 proteins, it will be important to further characterise the mechanisms through which they are regulated.

PKC (Protein Kinase C) is a phospholipid dependent, serine/threonine kinase which plays a central role in signal transduction [17]. In mammals, there are 13 isoforms, which can be divided into four subgroups based on different co-factor requirements: classical (cPKC α , β /II, γ), novel (nPKC, δ , ϵ , θ , η), atypical (ι / λ , ζ) and PKN (1, 2, 3) [18]. As a major cellular receptor of the tumour promoting phorbol esters, PKC has long been associated with cancer [19]. Although only a limited number of PKC mutations have been identified in tumour samples, dysregulation of this kinase activity has been widely linked with disease [20]. For instance, PKC ϵ [21] and PKC ι [22] are both oncogenes, prognostic markers and potential therapeutic targets in cancer. While the regulatory inputs that control PKC activity have been extensively characterised [23], our understanding of how PKC-mediated phosphorylation regulates specific substrates remains limited.

In the present study, we explored the relationship between the Fbw7 and PKC protein families. We show that PKC phosphorylates Fbw7 α in an isoform-specific manner and can inhibit its nuclear localisation. Furthermore, cancer associated mutations proximal to the PKC phosphorylation sites influence Fbw7 α in a comparable manner. We conclude that Fbw7 α represents a novel PKC substrate, whose exclusion from the nucleus may be of pathological significance.

EXPERIMENTAL

Materials

All reagents were purchased from Sigma-Aldrich unless otherwise indicated. Yeast SD and tissue culture media were prepared by CR-UK Research Services, Optimem was from Gibco and Foetal Calf Serum was from PAA laboratories. Pharmacological inhibitors were obtained from Calbiochem; BIM1 and MG132 were reconstituted in DMSO, Calyculin A was dissolved in ethanol. All peptides were prepared by the CR-UK Peptide Synthesis Laboratory. Purified, recombinant PKC α and PKC ζ were obtained from Mr. Phil Whitehead (CR-UK, London), PKC δ (human) and PKC ϵ (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 PKC ϵ cDNA and GFP-PKC ϵ were described previously [24]. PKC ϵ was cloned into pGBKT7 (Clontech) between Nco I/BamH I (F: 5'-cagcaccatggcaatggtagttcaatggccttc-3' & R: 5'-gtgacagatctctcagggcatcaggtcttcacc-3'; Bgl II ends are compatible with BamH I). Full length PKC ϵ (PKC ϵ F: 5'-gaacacacccgggatggtagtgttcaatggccttc-3' & PKC ϵ R: 5'-catctatggtaccggggcatcaggtcttcaccaa-3'), its regulatory (PKC ϵ R; PKC ϵ F & 5'-catataaggtaccgctggcctggcgggacttc-3') and catalytic domains (PKC ϵ C; 5'-catattaccgggatgggaattgccaaagtctgctg-3' & PKC ϵ R) were cloned into pMT2-GST (kindly provided by Dr Dick Schaap) between Xma I/Kpn I. GFP-PKC α and GFP-PKC ζ constructs (wild type and constitutively active) were obtained from Dr. Scott Parkinson (CR-UK, London) and GFP-PKC δ was described previously [25]. Wild type and constitutively active aPKC ι constructs were a kind gift from Songhai Shi (MSKCC, New York). The substrate binding domain of mouse Fbw7 (Fbw7-SBD; encodes amino acids 283-629 from AF391192) was recovered from the pACT2 vector (see below) and subcloned into pEGFP-C1 (Kpn I/Xma I) using primers complementary to the flanking vector sequence (F: 5'-aatacaggtaccgggtcatatggccatggaggcc-3' & R: 5'-ataagaaccgggtcccagttatctacgattcatagatctctcg-3'; Age I ends are compatible with Xma I). pCDNA3.1-FLAG was kindly provided by Dr. Sylvie Lachmann (CR-UK, London), and pcDNA3-FLAG-Fbw7 by Professor Shigetsugu Hatakeyama. FLAG-tagged Fbw7 α and Fbw7 α ^{ABD1} were generous gifts from Dr. Bruce Clurman (compared to NM_033632, these constructs contain an amino acid substitution, A626T, and also lack the 2 C-terminal residues). These sequences were subcloned into pEGFP-C1 (Bgl II/Sal I; F7 α F: 5'-attataagatctatgaatcaggaactgctctctgtgg-3' & F7 α R: 5'-taatgtgcgactcattccacatcaaagtcacg-3'). Fbw7 α /Fbw7 α ^{ABD1} phosphorylation site mutants were generated by site directed mutagenesis, using a two-step PCR approach. The following primers, and their reverse complements, were employed: S10A: 5'-gctctctgtggcgccaaaagacgacg-3', S10D: 5'-gctctctgtggcgacaaaagacgacg-3', S18A: 5'-cgaactggaggcgctctgagagg-3', S18D: 5'-cgaactggaggcgatctgagagg-3'; double mutants were prepared with sequential rounds of mutagenesis. K11R and +P16 mutants were generated by one-step mutagenesis using Pfu Turbo (Stratagene), the following primers and their reverse complements: K11R: 5'-ctctctgtggcgacgacagaagacgacgaactgg-3', K11R: 5'-ctctctgtggcgacgacagaagacgacgaactgg-3'. All constructs were validated by complete DNA sequencing (CR-UK Sequencing Facility).

Yeast 2-Hybrid Analysis

Yeast 2-hybrid analysis was performed using the MATCHMAKER GAL4 System 3, the AH109 yeast strain and a mouse brain cDNA library (in pACT2), according to the manufacturer's guidelines (Clontech). Full length mouse PKC ϵ was used as the bait (pGBKT7-PKC ϵ). Basic yeast media was prepared using SD (0.67% yeast nitrogen base, 2% glucose, pH 5.8, +/- 2.2% agar), 0.59g/l Complete Supplementary Dropout Medium (CSM)-Ade-His-Leu-Trp-Ura (Bio

101) and 20mg/l uracil. This was supplemented with 40mg/l adenine (A), 20mg/l histidine (H), 100mg/l leucine (L), 50mg/l tryptophan (T) and/or 20µg/ml X-α-Gal (X; Clontech), as indicated. Yeast transformations were carried out using a lithium acetate/single-stranded carrier DNA/polyethylene glycol protocol (Agatep, <http://research.bmn.com/tto>). pGBKT7-PKCε expression in AH109 was confirmed by western blotting and shown not to autoactivate the *HIS3*, *ADE2* or *MEL1* reporter genes. AH109 were co-transformed with pGBKT7-PKCε/pGADT7 as negative controls, or pGBKT7-p53/pGADT7-T antigen as positive controls (Clontech). For the screen, yeast were transformed sequentially, first with pGBKT7-PKCε and then with the library; a total of 1.4×10^5 cfus were analysed. Transformed yeast were subjected to three rounds of selection. Transformants were first plated on medium stringency, nutritionally selective agar plates (CSM-T/L/H) at 30°C for 5 days. Positive clones were restreaked onto more stringent selective plates (CSM-T/L/H/A) for a further 3 days, and then onto the highest stringency selective media (CSM-T/L/H/A/X) for 5 days. Plates were imaged by the CR-UK Photographic Department. Library vectors were rescued from positive clones and sequenced using the following primers: 5'-taccactacaatggatg-3' (pACT2 5153-5137, sense) and 5'-agatgtgcacgatgcacag-3' (pACT2 4939-4920, antisense).

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 the following: 10µM MG132, 100nM Calyculin A (cells detach and were collected in suspension from the media), 400nM PMA. All untreated controls were performed using an equal volume of DMSO.

RNA interference

RNAi was performed using siRNA duplexes from Dharmacon. siLamin A/C (M-013859-00) was used as a control; siFbw7 targets all 3 members of the mammalian Fbw7 family and has been described previously [15]. 293FT cells were seeded at 2×10^5 cells/6-well, incubated overnight and then transfected with 50nM siRNA using Lipofectamine 2000, as recommended by the supplier (Invitrogen). Cells were treated +/- 400nM PMA as indicated and harvested 3 days post-transfection, directly into LDS Sample buffer (see below). Samples were boiled for 5mins and briefly sonicated prior to analysis by SDS-PAGE and western blotting.

GST-Pulldowns and Immunoprecipitations

GST-pulldowns and immunoprecipitations were performed as described previously [24]. For FLAG immunoprecipitations, anti-FLAG M2-agarose affinity gel (Sigma) 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 (3E1, CR-UK), 1:1000; rabbit anti-GST (sc-459, Santa Cruz), 1:2000; mouse anti-PKC alpha (MC5, CR-UK), 1:1000; rabbit anti-PKC alpha pT638 (9375, Cell Signaling), 1:1000 + 5% BSA; rabbit anti-PKC epsilon (sc-214, Santa Cruz), 1:5000; rabbit anti-atypical PKC (C20, Santa Cruz), 1:5000; mouse anti-cyclin E (HE12, sc-247, Santa Cruz), 1:1000. The following phospho-specific antibodies were raised against Fbw7α (see below) and used at 1:1000 with 5% BSA and 1µg/ml of the corresponding, blocking dephosphopeptide: pS10 (PPA-574) and pS18 (PPA-527). The western blots presented are each representative of data obtained from at least 3 separate experiments.

In Vitro Kinase Assays

The following peptides were prepared for use as PKC substrates: Fbw7 α wt 7-14: N-SVGSKRRR-C; Fbw7 α S10A 7-14: N-SVGAKRRR-C; Fbw7 α wt 11-22: N-KRRRTGGSLRGN-C; Fbw7 α S18A 11-22: N-KRRRTGGALRGN-C (Peptide Synthesis Facility, CR-UK). Reactions were performed using 50 μ l of the following kinase assay buffer: 20mM Tris pH 7.5, 5mM MgCl₂, 1mg/ml Phosphatidylserine (Lipid Products), 0.2% Triton X-100, 1ng/ μ l PMA, 50 μ M ATP, 5 μ Ci [γ ³²P]-ATP (Amersham), with 0.2mM peptide substrate and 12.5ng PKC ϵ . Assays were started with the addition of ATP, incubated at 30°C for 10mins, and stopped by spotting 15 μ l onto P81 paper (Whatman) and washing 3x5mins in 30% acetic acid. P81 strips were analysed by Cerenkov counting using a Beckman LS6000IC scintillation machine to quantitate ³²P incorporation.

Preparation of Phosphospecific Antibodies

Rabbit polyclonal antisera were raised using the following phosphopeptides as antigens: Fbw7 α pS10, N-SVGpSKRRR-C; Fbw7 α pS18, N-RTGGpSLRGN-C (corresponding blocking dephosphopeptides were also synthesized). Each phosphopeptide was coupled to Keyhole Limpet Hemocyanin using glutaraldehyde and immunised in triplicate (Harlan Sera-Lab). The resulting sera were characterised by ELISA and found to exhibit both site- and phospho-specificity: anti-pS10 (PPA-574); anti-pS18 (PPA-527). An additional peptide was used to determine whether serum PPA-574 could recognise pS10 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 1mg/ml RNase (Qiagen)/PBS for 15mins, followed by 0.25 μ g/ml propidium iodide/PBS for a further 10 mins. Alternatively, 1 μ g/ml Hoechst 33342 (Invitrogen)/PBS was used for 10 mins to stain DNA. Coverslips were washed 3x10mins in PBS, once in water and mounted onto 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 63x/1.4 Plan-Apochromat Oil Ph3 objective. Individual channels were scanned sequentially, line by line, with averaging set at 8. Each image (12 bit, 1024) represents a single 1.0 μ m 'Z' optical section with a pixel size of approximately 0.1 μ m. Epifluorescent images were acquired using an Imager.A1 upright microscope (Zeiss) equipped with a Plan-Apochromat 63x/1.4 Oil objective and an Orca-ER Digital Camera (Hamamatsu), using Axiovision software (Release 4.6.3). All images presented are representative of data obtained from at least three independent experiments.

Quantitation of Fbw7 α Nuclear Localisation

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 signal; the GFP-Fbw7 α signal was then quantitated both inside and outside of this region. Nuclear localisation was expressed as the ratio of mean integrated GFP signal intensity outside/inside the nucleus. At least 20 cells were analysed per experiment to obtain a mean ratio, and the standard error of the mean (SEM) was determined across 3 separate experiments. T-tests were performed to determine statistical significance using Prism software.

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), *Macaca*

mulata (XP_001084190), *Rattus norvegicus* (EDM00812), *Mus musculus* (AAH85184), *Gallus gallus* (XP_420447), and *Xenopus laevis* (NP_001089186). The accession number for human Cyclin E is (NP_476530) and the different isoforms of human PKC are as follows: alpha (NP_002728), beta I (NP_997700), beta II (NP_002729), delta (NP_997704), epsilon (NP_005391), zeta (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 substrate binding domain of Fbw7

PKC ϵ regulates a diverse range of cellular processes, including proliferation, survival, migration and invasion [24]. Aberrant PKC ϵ 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 PKC ϵ , we performed a yeast 2-hybrid screen to identify novel binding partners.

Full length PKC ϵ bait was used to screen a mouse brain cDNA library, yielding 28 in-frame, positive clones, including a known PKC ϵ -interacting protein, Nell2 [26], which validated the approach. Among the other hits, 'clone 19' was identified as the C-terminal, Substrate Binding Domain (SBD) of the F-box WD40 Protein 7 family (Fbw7; Figures 1a and b). Fbw7 is a tumor suppressor, which mediates the degradation of several potent onco-proteins 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 PKC ϵ and Fbw7 in mammalian cells, GST pull downs were performed (Figure 1c). Cells were co-transfected with the Fbw7-SBD (GFP) and full length, or truncated PKC ϵ (GST). While pulling down with a GST control or the regulatory domain of PKC ϵ recovers only background amounts of GFP-Fbw7 SBD, significantly elevated levels are recovered with either full length PKC ϵ 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 PKC ϵ associates with the substrate binding domain 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 Fbw7 (FLAG) and a panel of different PKC isoforms (GFP) and analysed by coimmunoprecipitation (Figure 1d). Since PKC represents a potential substrate for the SCF^{Fbw7} ubiquitin ligase, immunoprecipitations were performed in the presence or absence of MG132, a proteasome inhibitor which would protect any ubiquitinated PKC from degradation. Fbw7 was immunoprecipitated using anti-FLAG, and immunoprecipitates were probed for associated PKC using anti-GFP. All PKC isoforms tested bound to Fbw7; the strongest interaction detected was with aPKC ζ . Total levels of PKC (lysate) do not change upon Fbw7 co-expression, or MG132 treatment, suggesting that PKC is not degraded under these conditions. The increase in immunoprecipitated PKC following MG132 treatment is explained by the stabilisation of Fbw7 itself (which is known to undergo autoubiquitination).

The data above suggest that PKC is unlikely to be a substrate for Fbw7. However, it is striking that the CPD motif, recognised in all Fbw7 targets, shares significant homology with the 'TP'/turn motif of PKC (Figure 1e), one of three critical 'priming' phosphorylation sites required for its activation [23]. To test directly whether endogenous Fbw7 can regulate the degradation of PKC, an RNAi-based approach was employed. For these experiments, we reproduced conditions established by van Drogen *et al*, who designed an siRNA duplex to target the Fbw7 common region, and demonstrated co-depletion of the 3 mammalian isoforms from 293T cells, by QPCR (endogenous) and western blotting (exogenous tagged-Fbw7) [15]. To validate the siRNA duplex, we sequentially transfected cells, first with siControl or siFbw7, and then with GFP alone

or GFP-Fbw7 α . As expected, the siFbw7 duplex led to a specific depletion of GFP-Fbw7 α (Figure 1f).

Using these conditions, cells were co-depleted of all 3 Fbw7 isoforms and subjected to western blotting for endogenous PKC isoforms, with cyclin E as a positive control (Figure 1g). Lysates were also probed with a phospho-specific antibody against the PKC α 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 phorbol ester (PMA), which stimulates the activation induced down-regulation of classical (e.g. PKC α) and novel (e.g. PKC ϵ) PKC isoforms [27]. As shown in Figure 1g, depletion of Fbw7 leads to a clear stabilisation of its substrate cyclin E. However, levels of both total and phospho-PKC are unchanged by Fbw7 knock-down, suggesting that none of these isoforms represent Fbw7 substrates. Phorbol ester treatment induces the complete degradation of PKC α , and a partial reduction in PKC ϵ levels after 20hrs, however, neither of these events is inhibited by depletion of Fbw7, indicating that Fbw7 is not responsible for PMA-stimulated PKC 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.

PKC phosphorylates S10 and S18 within the unique N-terminal domain of Fbw7 α *in vitro* and in mammalian cells

We next addressed whether Fbw7 may be a substrate for PKC mediated phosphorylation. First, Fbw7 protein sequences were analysed using a predictive database for PKC phosphorylation, MPR PKC Scan [28]. Two of the highest scoring sites identified are Fbw7 α residues S10 and S18, which are in the top 0.06 and 0.2 percentiles respectively for PKC ζ phosphorylation. These residues lie within the isoform specific, N-terminal region of Fbw7 α , which plays an important role in determining the unique nuclear localisation of this isoform; a nuclear localisation signal (NLS1) lies nearby between residues 11-14 (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 Fbw7 α , *in vitro* kinase assays were performed (Figure 2b). Peptides were synthesized corresponding to residues 7-14, to test for S10 phosphorylation, and residues 11-22, for S18 analysis; in control peptides the candidate site was substituted with a non-phosphorylatable alanine. Peptide kinase assays were carried out using purified PKC ϵ and [γ ³²P]-ATP; incorporation of ³²P was detected by Cerenkov counting. Both wild type peptides are efficiently phosphorylated compared to the corresponding alanine mutants, confirming the prediction that Fbw7 α residues S10 and S18 can be phosphorylated directly by PKC. Similar results were obtained using recombinant cPKC α , nPKC δ or aPKC ζ (data not shown), suggesting that Fbw7 α is a common substrate for PKC superfamily members.

To determine whether Fbw7 α is phosphorylated in mammalian cells, phosphospecific antibodies were raised. Cells were transfected with GFP-Fbw7 α constructs and analysed by western blotting (Figure 2c). As a broad stimulus, cells were treated with or without Calyculin A, an inhibitor of PP1 and PP2A phosphatases; this compound protects sensitive sites from dephosphorylation, and may also upregulate certain signalling pathways by preserving activating phosphorylations. As revealed by the phospho-specific antisera, phosphorylation of both S10 and S18 is induced by

Calyculin A treatment in all constructs, except for the corresponding alanine mutant. These data confirm that Fbw7 α residues S10 and S18 can be phosphorylated in mammalian cells, in a mutually independent fashion, and also serve to illustrate the site specificity of the antisera.

To test whether Fbw7 α phosphorylation is dependent upon PKC in a cellular context, a pharmacological approach was taken. Since Fbw7 α can be phosphorylated by multiple PKC isoforms, we employed a pan-PKC inhibitor, Gö6983. Upon pre-treatment with Gö6983, the Calyculin A-induced phosphorylation of S10 and S18 is decreased by roughly 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 Fbw7 α phosphorylation under these Calyculin A triggered conditions.

To investigate whether PKC activity is sufficient to promote the cellular phosphorylation of Fbw7 α , we tested the effect of co-expressing wild-type, or constitutively active, PKC α or PKC ϵ . Importantly, the overexpression and overactivation of PKC α [29] and PKC ϵ [22] [30] is observed in range of human cancers, so these experiments effectively model the status of certain tumours [20]. Expression of wild type PKC α or PKC ϵ induces Fbw7 α phosphorylation at both S10 and S18 (Figures 2e-f), and this is dramatically increased by the constitutively activated kinases; similar results were also observed using PKC ζ (see Figure 2g). Together, these data confirm that PKC activity is able to drive Fbw7 α phosphorylation in mammalian cells.

Finally, to establish whether nearby, cancer-associated mutations may influence these phosphorylation events, GFP-Fbw7 α K11R and +P16 mutants were analysed as described above. Fbw7 α +P16 is phosphorylated by aPKC ζ at both S10 and S18, as efficiently as the wild type protein. Fbw7 α K11R is also phosphorylated at S18. The phosphorylation of Fbw7 α K11R at S10 could not be monitored, as this mutation impairs the recognition of phospho-S10 by our serum (demonstrated by ELISA; data not shown). However, since the K11R substitution preserves a basic charge at this residue, it is unlikely to affect PKC-mediated phosphorylation; in fact, S10 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 phosphorylates residues S10 and S18 in the isoform specific N-terminal region of Fbw7 α , both *in vitro* and in mammalian cells. These phosphorylation events are not mutually dependent or affected by the cancer-associated mutations K11R and +P16.

Phosphorylation of S10 can regulate Fbw7 α nuclear localisation

We next sought to examine the functional consequences of Fbw7 α phosphorylation at S10 and/or S18. These residues lie in close proximity to a nuclear localisation signal (NLS1), and we reasoned that phosphorylation may thereby influence Fbw7 localisation (Figure 2a). Regulation of Fbw7 localisation is of particular interest because it is intimately linked to its profile of substrate downregulation, determining which of many potential targets the ubiquitin ligase is exposed to. Fbw7 α contains 2 nuclear localisation signals, NLS1 in the isoform specific N-terminus, and NLS2 in the shared SBD (Figure 3a). Either of these signals can confer nuclear localisation and both must be lost to shift Fbw7 α to the cytosol in U2OS cells [14]. Thus, to

analyse regulation of NLS1 by phosphorylation, we employed a mutant lacking NLS2 (Δ NLS2); the nuclear localisation of this protein is completely dependent on a functional NLS1, and any regulatory inputs should manifest clearly.

Cells were transfected with GFP-Fbw7 α constructs in which S10 and/or S18 sites were mutated to either non-phosphorylatable alanine, or phospho-mimetic aspartate, in the presence or absence of an intact NLS2. GFP-Fbw7 α localisation was then analysed by confocal microscopy (Figure 3b and data not shown). Quantitative analyses were performed by measuring the GFP signal intensity inside and outside of the nucleus, as judged by colocalisation with DNA staining; the ratio between these values represents Fbw7 α localisation (Figure 3c). Consistent with previous reports, wild type Fbw7 α is localised almost exclusively in the nucleus (the same pattern of localisation is observed using FLAG-Fbw7 α , data not shown and [14]). None of the phospho-mutants show an altered pattern of localisation in an otherwise wild type context (NLS2wt constructs). The Δ NLS2 mutant is also predominantly nuclear, although a weak cytosolic signal is detected, suggesting that NLS1 alone is not completely sufficient for proper localisation of the protein (the difference between the WT/WT and WT/ Δ NLS2 localisations is statistically significant, P value = 0.001). In the context of the Δ NLS2 protein, the phospho-mimetic aspartate mutation of S10, but not S18, leads 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 Δ NLS2 mutant is significantly reduced when S10 is mutated to alanine; S10A/ Δ NLS2 mutants localise more like the wild type protein (Figure 3c). This observation suggests that endogenous phosphorylation of S10 may underlie the reduced efficiency of NLS1 as a single nuclear localisation signal.

We conclude that phosphorylation of Fbw7 α at S10 strongly inhibits the activity of its isoform-specific nuclear localisation 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 Fbw7 α nuclear localisation

Since the cancer associated Fbw7 α mutations, K11R and +P16, lie close to NLS1, we reasoned that these modifications may also regulate nuclear localisation. To test this, GFP-Fbw7 α constructs harbouring these mutations, in the presence or absence of NLS2, were transfected and visualised by epifluorescent microscopy (Figure 4a). Both mutants localise to the nucleus in the presence of an intact NLS2 (NLS2wt). However, in the absence of NLS2 (Δ NLS2), these mutations relocalise the protein; both K11R and +P16 mutants are distributed throughout the cell and resemble the S10D mutant with respect to localisation.

We conclude that mutations in the isoform-specific N-terminus of Fbw7 α (K11R and +P16) can regulate the activity of NLS1, in a manner comparable to phosphorylation of S10. Furthermore, since these mutations were identified in tumours, relocalisation of Fbw7 α 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 which may play a role during transformation.

Fbw7 was identified as a PKC ϵ binding partner by yeast 2-hybrid analysis, indicating that these proteins interact directly. In mammalian cells, the interaction was mapped to the catalytic domain of PKC and the substrate binding domain (SBD) of Fbw7, an unusual 8-bladed β -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 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, Fbw7 β was detected by mass spectrometry, following immunoprecipitation of endogenous PKC ζ from T84 cells [32]. These data 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 downregulation and this may contribute to transformation under certain conditions [27]. However, to date, only two ubiquitin ligases have been identified which can mediate degradation of selected PKC isoforms, VHL [33] and RINCK [34]. The PKC 'turn'/TP motif bears striking homology to the CPD sequence recognised by Fbw7 in its substrates, consistent with the notion it may represent a novel target. However, in spite of 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 stabilise PKC expression, although it should act as a dominant negative with respect to the SCF^{Fbw7} ubiquitin ligase; iii) expression of full length Fbw7 does not downregulate PKC; iv) most importantly, depletion of endogenous Fbw7 does not protect PKC from either basal, or activation induced, downregulation.

It is important to note that our data do not completely exclude Fbw7 as a possible PKC ubiquitin ligase. Firstly, interfering with Fbw7 function does not always manifest in detectable changes in steady state substrate levels; for instance, although c-myc turnover is compromised in an Fbw7 α 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, Fbw7 γ degrades nucleolar c-myc in U2OS cells [14], but not in Hct116 [16], and Fbw7 degrades c-jun in 293T cells [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 Fbw7.

We have identified Fbw7 α as a novel PKC substrate. Two residues, S10 and S18, are phosphorylated in a PKC-dependent manner, both *in vitro* and in mammalian cells. *In vitro*, Fbw7 α 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 cPKC α or aPKC ι is sufficient to drive robust Fbw7 α phosphorylation. Since the overexpression and overactivation of PKC α [29] and PKC ι [35] is observed in a range of human tumours, we conclude that Fbw7 α phosphorylation may be elevated in certain cancers. Notably, PKC ι 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 Fbw7 α phosphorylation contributes to the pathophysiological function of PKC ϵ .

The two novel Fbw7 α phosphorylation sites identified in this study, S10 and S18, lie within the isoform specific N-terminus of Fbw7 α ; this 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 Fbw7 α influences its dimerisation [36], assembly into an SCF complex, ubiquitylation of itself [37] or other substrates. In the present study, we investigated the possibility that Fbw7 α phosphorylation may influence its localisation.

S10 is adjacent to NLS1, one of two NLS motifs in Fbw7 α which confer nuclear localisation to the protein. It is well established that phosphorylation can influence the activity of an NLS, for instance, PKC catalysed-phosphorylation proximal to the aryl hydrocarbon receptor (AhR) NLS inhibits its nuclear import [38]. Consistent with this, mutation of S10, but not S18, to a phosphomimetic aspartate residue severely inhibits the activity of NLS1. Strikingly, cancer-associated mutations in nearby residues (K11R, +P16) disrupt Fbw7 α NLS1 function in a comparable manner. Although the K11R substitution preserves a basic charge, structural and thermodynamic studies confirm that a lysine 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 Fbw7 α by PKC mediated phosphorylation may also contribute to transformation under certain conditions.

As noted above, Fbw7 α bears two redundant nuclear localisation 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 3 distinct mechanisms through which NLS1 can be inhibited: phosphorylation of S10 by PKC, substitution of residue K11 for arginine, and insertion of proline at position 16 (Figure 6b). Under conditions where NLS2 activity is suppressed, these regulatory inputs result in a dramatic relocalisation of Fbw7 α to the cytosol. Further work will be required to explore whether NLS2 is also subject to regulation, either 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 localise to the cytosol in 293T cells, even in the absence of other alterations [13]. Based on this data, we hypothesised 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 Fbw7 α in 293T cells, so could not explore this further possibility. Nevertheless, these data suggest that conditions exist in which NLS1 is solely responsible for Fbw7 α nuclear localisation, and that the regulatory inputs which influence NLS2 activity warrant further investigation.

The mammalian Fbw7 family members are localised to different subcellular compartments by regulatory signals in their isoform-specific N-termini [14]. This suggests that each isoform performs a unique function in a distinct region of the cell. Indeed, Fbw7 γ is localised to the nucleolus, and in this subcellular compartment degrades a specific pool of c-myc to control U2OS cell size [14]. Furthermore, Fbw7 α degrades full length cyclin E in the nucleus, but does not

down-regulate certain low molecular weight forms that are localised in the cytoplasm [42]. Mislocalisation of Fbw7 α to the cytosol, a compartment usually occupied by the much less abundant Fbw7 β 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-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 onco-proteins, relocalisation of Fbw7 α 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 PKC ϵ occurs concurrently with a substantial upregulation of cyclin E in certain ovarian tumours [35] and it will be interesting to explore whether mislocalisation of Fbw7 α through S10 phosphorylation contributes to this phenotype.

Our current findings integrate Fbw7 α 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 glycogen synthase kinase-3 (GSK3). GSK3 is the kinase responsible for phosphorylating most, if not all, of the CPD motifs recognised by Fbw7 in its substrates [5], while 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 S243 [44], the central phosphoserine of the c-jun CPD [11]. Thus, we could conceive of two possible mechanisms through which PKC activity may antagonise Fbw7 function: i) indirectly, by inhibiting the GSK3 mediated phosphorylation events which target substrates for Fbw7 recognition; and ii) directly, by phosphorylating Fbw7 α itself, to relocalise the protein and protect nuclear substrates from ubiquitination and degradation. Thus, stabilisation of Fbw7 substrates may partly underlie the tumor promoting properties of PKC activity.

In conclusion, our current data provide evidence for both physical and functional interactions between PKC and Fbw7 α , controlling the function of NLS1 in the latter. Future work will address the regulation of Fbw7 α NLS2, in order to define the combinatorial events which may control its nuclear uptake.

ACKNOWLEDGEMENTS

We thank Professor Shigetsugu Hatakeyama and Drs Dick Schaap, Scott Parkinson, Sylvie Lachmann, Songhai Shi and Bruce Clurman for kindly 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, Annett Jandke and members of the Parker lab for helpful discussions, and to Professor Alan Hall for supporting this work. JD was funded by the MRC and CR-UK.

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FIGURE LEGENDS

Figure 1 Fbw7 interacts with, but does not down-regulate, multiple members of the PKC superfamily. a) Yeast 2-Hybrid Analysis. Yeast were co-transformed with PKC ϵ bait vector and either Fbw7-SBD prey, or an empty pGADT7 control vector (-). p53/T-Antigen co-transformants were used as positive controls (+). Yeast were plated onto media selective 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 2-hybrid clone, 19, are represented to scale with the isoform specific N-termini, the F-box domains and the substrate binding domains highlighted [31]. c) GST-Pull Downs. COS7 cells were co-transfected with GFP-Fbw7-SBD and a panel of GST-PKC ϵ constructs (ϵ , full length PKC ϵ ; ϵ R, PKC ϵ regulatory domain; ϵ C, PKC ϵ catalytic domain). 24hrs post-transfection, lysates were prepared, subjected to GST pull down and analysed by Western blotting. d) Fbw7/PKC coimmunoprecipitations. COS7 cells were co-transfected with FLAG-Fbw7 β and a panel of GFP-tagged human PKC isoforms. 32hrs post-transfection, cells were treated +/- 10 μ M MG-132 for a further 16hrs. Lysates were prepared and immunoprecipitations were performed using anti-FLAG; samples were analysed by Western blotting. e) Sequence alignment of human PKC isoforms and a cyclin E Cdc4 phosphodegron (CPD). The 'TP'/turn motif is highlighted with a filled box and the central phospho-residue is numbered in brackets. The +4 residue is indicated with an unfilled box; in a CPD, this position is typically occupied by a phospho-serine or a negatively charged amino acid. f) Fbw7 depletion. 293FT cells 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 cotransfected with empty GFP vector and GFP-Fbw7 α and incubated for a further 16hrs. Total cell extracts were prepared and analysed by western blotting. g) Fbw7 depletion stabilises cyclin E but not PKC. 293FT cells were transfected with either control or Fbw7-specific siRNA, incubated for 3 days and treated +/- 400nM PMA as indicated. Total cell extracts were prepared and analysed by western blotting.

Figure 2 PKC phosphorylates the isoform specific, N-terminus of Fbw7 α *in vitro* and in cells. a) Amino acid sequence of the isoform specific, N-terminus of human Fbw7 α . Candidate phosphorylation sites (S10 and S18) and cancer-associated mutations (K11R, +P16) are indicated with arrows, NLS1 (11-14) is shown in bold and boxed. b) *In vitro* phosphorylation of Fbw7 α S10 and S18 by PKC. Peptide kinase assays were performed using purified PKC ϵ , the indicated peptides and [γ - 32 P]-ATP for 10mins; 32 P incorporation was quantitated by Cerenkov counting. Reactions were analysed in triplicate and error bars denote mean $\pm$ SD. c) Phosphorylation of Fbw7 α S10 and S18 in mammalian cells. HeLa cells were transfected with a panel of GFP-Fbw7 α constructs (WT, S10A or S18A), incubated for 24hrs and then treated +/- 100nM Calyculin A for a further hour. Lysates were prepared and immunoprecipitations were performed to enrich for Fbw7 α using anti-GFP. Samples were analysed by Western blotting. d) Pharmacological inhibition of Fbw7 α phosphorylation. HeLa cells were transfected with GFP-Fbw7 α and incubated for 24hrs. Cells were pretreated +/- 6 μ M Gö6983 (a pan-PKC inhibitor) for 15mins, and then stimulated +/- 100nM Calyculin A for a further 45mins. Samples were processed as in c. e and f) PKC-dependent Fbw7 α phosphorylation. HeLa cells were transfected with either e) GFP-Fbw7 α +/- aPKC ι (wild type, WT; or constitutively active, CA), or f) FLAG-Fbw7 α +/- GFP-PKC α (wild type, WT; or constitutively active, CA). 24hrs post-transfection, lysates were prepared and analysed as described in c. g) HeLa cells were co-transfected with a GFP-Fbw7 α construct (WT, K11R or +P16) and a myc-PKC ζ construct (wild type, ζ ; or

constitutively active, CA). 24hrs post-transfection, lysates were prepared and analysed as described in c.

Figure 3 S10 Phosphorylation Can Regulate Fbw7 α Nuclear Localisation

a) Schematic representation of human Fbw7 α , with 2 nuclear localisation signals indicated (NLS1/2). Both signals can confer nuclear localisation in U2OS cells, as summarised in the table [14]. b) GFP-Fbw7 α localisation. HeLa cells were seeded on coverslips and transfected with a panel of GFP-Fbw7 α constructs, in which S10 was either wild type (S10wt) or phosphomimetic (S10D), and NLS2 was either present (NLS2wt) or absent (Δ NLS2). 24hrs post-transfection, cells were fixed and stained for DNA using propidium iodide. Coverslips were mounted and analysed by confocal microscopy; these images comprise single 1.0 μ m 'Z' optical sections. Scale bars represent 10 μ m. c) HeLa cells were transfected with a panel of GFP-Fbw7 α constructs and processed as described above. Nuclear localisation was determined by measuring the GFP signal outside/inside the nucleus (defined by colocalisation with DNA). At least 20 cells were analysed per experiment. Error bars denote the mean $\pm$ SEM from 3 separate experiments. WT/ Δ NLS2 was compared to all other Δ NLS2 mutants using a t-test. *, P value < 0.025; **, P value < 0.001; no asterisk, P value > 0.05, not significant.

Figure 4 Cancer-Associated Mutations Can Regulate Fbw7 α Nuclear Localisation

a) GFP-Fbw7 α mutant localisations. HeLa cells were seeded on coverslips and transfected with a panel of GFP-Fbw7 α constructs, containing the cancer-associated mutations, K11R or +P16, in the presence (NLS2wt) or absence (Δ NLS2) of an intact NLS2. 24hrs post-transfection, cells were fixed and stained for DNA using Hoechst 33342. Coverslips were mounted and analysed by epifluorescent microscopy.

Figure 5 Model of Fbw7 α Regulation

a) Evolutionary Conservation of S10 and S18. Sequence alignment of the Fbw7 α N-termini from different species. The symbols denote the degree of conservation: *, identical residue; :, conservative substitution; ., semi-conservative substitution. b) Regulatory inputs that modulate Fbw7 α localisation. The schematic represents the 2 nuclear localisation signals in Fbw7 α with surrounding residues indicated and intervening residues represented by dots (not to scale). The phosphorylation events and mutations that impair the function of NLS1 are shown. It remains to be determined whether NLS2 is also subject to regulation.

a)

	S10	S18
<i>Homo sapiens</i>	MNQELLSVGSKRRRTGGSLRGNPSSSQVDE	
<i>Macaca mulatta</i>	MNQELLSVGSKRRRTGGSLRGNPSSSQVDE	
<i>Rattus norvegicus</i>	MNQELLSVGSKRRRTGGSLRGNASSSQVDE	
<i>Mus musculus</i>	MNQELLSVGSKRRRTGGSLRGNASSSQVDE	
<i>Gallus gallus</i>	MNQELLSVGSKRRRTGGSLRANASSSQVDE	
<i>Xenopus laevis</i>	MNQELLSVGSKRRRTGGSVRGNASSSQADE	
	*****:*.*.****.*	

b)

Isoform specific N-terminus	Common Region (SBD)
MNQELLSVGSKRRRTGGSLR...TKTTKMKRKL	
NLS1	NLS2
↓	↓
pS10	?
K11R	
+P16	