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Identification of PKCζII: an endogenous inhibitor of cell polarity

Scott J Parkinson[1], J Anne Le Good[2], Richard DH Whelan, Phil Whitehead and Peter J Parker*

Protein Phosphorylation Laboratory, Cancer Research UK, London Research Institute, London, UK

A new member of the atypical protein kinase C (aPKC) family, designated PKCζII, is identified in this study. The gene contains no introns and is 98% homologous with the cDNA encoding PKCζ. The PKCζII coding region is frame-shifted with respect to the PKCζ open reading frame, resulting in expression of an aPKC regulatory domain without associated kinase activity. PKCζII mRNA is detected in various mouse tissues and an immunoreactive 45 kDa protein is present in epithelial cell cultures. PKCζII is shown to interact with the Par6 protein and functions in the development of cell polarity. HC11 epithelial cells express PKCζII and are maintained in a nondifferentiated state characterised by the absence of tight junctions and cell overgrowth. HC11 cells harbouring a PKCζII-specific RNAi, recruit ZO-1 and other tight junction markers to cell–cell boundaries and adopt a monolayer phenotype in the presence of growth factors. The data demonstrate a regulatory role for PKCζII in the maintenance of cell transformation and the development of cell polarity.

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Introduction

Cell polarisation is a fundamental property of every cell type, with symmetric and/or asymmetric distribution of cellular components being essential for the development, cell division, cell migration, and maintenance of specialised compartments within cells. Atypical protein kinase (aPKC) is implicated in the development of cellular asymmetry and contributes to astrocyte migration and epithelial cell polarity (Joberty *et al*, 2000; Lin *et al*, 2000; Etienne-Manneville and Hall, 2001; Hirose *et al*, 2002).

Epithelial cells are composed of distinct apical (lumenal) and basolateral domains separated by a specialised cellular structure known as the tight junction. The tight junction maintains a barrier that prevents diffusion of molecules across the epithelial cell layer (Dragsten *et al*, 1981). The protein components of the tight junction are beginning to be elucidated. The junctional adhesion molecules (JAMs) appear to be essential for tight junction assembly; monoclonal antibodies against JAMs inhibit junction formation following Ca^{2+} switch (Liu *et al*, 2000). These transmembrane proteins associate as dimers in trans through their extracellular domains at sites of cell–cell contact and recruit intracellular scaffolding components to the tight junction (Kostrewa *et al*, 2001). The intracellular domains of JAMs bind directly to the first PDZ domain of the cell polarity protein Par3/Asip/Bazooka (Ebnet *et al*, 2001). Par3 also associates with Par6, Cdc42, and aPKC (Joberty *et al*, 2000; Lin *et al*, 2000) all of which contribute to tight junction formation. The JAMs, as well as the tight-junction-localised proteins occludin and the claudins, also associate with the PDZ domains of the ZO family of scaffolding proteins (Furuse *et al*, 1993, 1998; Fanning *et al*, 1998; Haskins *et al*, 1998, Itoh *et al*, 1999).

aPKCs appear to be essential for the formation but not maintenance of tight junctions. MDCK cells expressing kinase-inactive aPKC mutants appear normal, however after Ca^{2+} depletion to disrupt cell–cell contacts, the localisation of the tight junction markers ZO-1, Par3, occludin, and claudin-1 is erratic following Ca^{2+} replacement (Suzuki *et al*, 2001). These and other observations led to the hypothesis that aPKCs catalyse the transition of premature cellular junctions into differentiated asymmetric structures (Suzuki *et al*, 2002).

The aPKCs are activated by protein–protein interactions within the regulatory domains of the proteins. This serves the two purposes of destabilising the pseudosubstrate site interaction with the catalytic domain permitting binding/phosphorylation of substrate and tethering the kinase activity to specific regions of the cell (Moscat and Diaz-Meco, 2000). PKCζ and PKCλ/ι are homologous proteins that interact with Par6, a protein necessary for cellular asymmetry and asymmetric cell division. Interaction of aPKCs with Par6 restricts their localisation in the cell and activates their kinase activity (Joberty *et al*, 2000; Lin *et al*, 2000).

In addition to the full-length aPKCs, there is also a PKCζ isoform (PKMζ) synthesised following transcription initiation of the PKCζ gene from a brain-specific promoter located in an intron (Marshall *et al*, 2000). This results in translation of an aPKC catalytic protein lacking the regulatory domain. The PKMζ transcript is implicated in long-term potentiation, likely as a result of its constitutive kinase activity (Sacktor *et al*, 1993, Osten *et al*, 1996).

In this study, we describe the identification and characterisation of a distinct aPKC gene, designated PKCζII. This gene has a unique chromosomal localisation and is 98% identical with the cDNA encoding PKCζ. An immunoreactive protein corresponding to PKCζII is detected in cultured cell lines and

*Corresponding author. Protein Phosphorylation Laboratory, Cancer Research UK, London Research Institute, 44 Lincoln's Inn Fields, London WC2A3PX, UK. Tel.: 020 7242 0200;
Fax: 020 7269 3094; E-mail: peter.parker@cancer.org.uk
[1]Current address: Dana-Farber Cancer Institute, Division of Cancer Biology, Harvard Medical School, Boston 02115, USA.
E-mail: Scott_Parkinson@dfci.harvard.edu
[2]Current address: ISREC, Chemin des Boveresses 155, CH 1066 Epalinges, Switzerland

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RNA can be detected in many mouse tissues. Amongst the 29 bp differences with PKCζ, a deletion at nucleotide C1146 of PKCζII shifts the reading frame and results in truncation of the protein at amino acid 376. Therefore, PKCζII is composed of an aPKC regulatory domain without a functional catalytic domain. Recombinant PKCζII is partitioned in the nucleus, however its location changes when it interacts with proteins intrinsic to development of tight junctions. Expression of endogenous PKCζII in epithelial cells disrupts the assembly of tight junctions and promotes overgrowth of cells demonstrating its role in regulating aPKC-dependent functions.

Results

Cloning and identification of PKCζII

A stringent screen of a mouse genomic library (using the 5′ end of the PKCζ cDNA as a probe) identified three hybridising clones. The three clones were identical by restriction analysis and Southern blotting. Clone 3, containing a 13 kb insert, was selected for further analysis. Sequencing revealed this clone to contain a DNA sequence homologous with PKCζ that was designated PKCζII (the genomic sequence has been annotated recently by the NCBI Annotation Project and is designated locus XM_124895). The genomic sequence of PKCζII was approximately 98% identical at the nucleotide level with the cDNA encoding 'authentic' murine PKCζ (Figure 1) and retained a 3′UTR containing AATAAA poly A addition signal and a poly A stretch (data not shown). The PKCζII genomic sequence contains 29 nucleotide divergencies with respect to the PKCζ cDNA sequence including deletion of nucleotides C1146, G1943, and a region flanking the PKCζ stop codon (C1779–G1819).

Translation of the putative coding region of PKCζII demonstrates a high degree of identity at the protein level with PKCζ (Figure 2). The start codon of PKCζ is conserved in PKCζII; however, the nucleotide deletion of C1146 in PKCζII results in a shift of the reading frame and termination of the putative coding region at nt1152 (Figure 1). Following translation, PKCζII would comprise a functional regulatory domain homologous with PKCζ. The differences between PKCζ and PKCζII in the catalytic region, however, are significant. In addition to loss of 60% of the catalytic domain, the shift in the PKCζII reading frame converts amino acid D356 to a threonine. This mutation ensures PKCζII lacks all catalytic activity since D356 is the catalytic base of PKCζ responsible for stabilising the serine/threonine oxyanion of the substrate prior to transfer of the γ phosphate from ATP (Protein Kinase Resource: www.sdsc.edu/kinases/). The frame shift also terminates the coding sequence in subdomain VIB (the catalytic loop) of the kinase domain suggesting that there is no catalytic function for this putative protein (see below).

Fluorescence *in situ* hybridisation (FISH) was performed to map the location of PKCζ and PKCζII on mouse metaphase spreads stained with specific chromosome paints (Figure 3A, A′). Probes specific for PKCζII (X7; see Materials and methods) hybridised strongly to chromosome 7(A_{2-3} region). PKCζ-specific hybridising probes (pZ-X5; see Materials and methods) hybridised with chromosome 4(E_2 region). These locations were also confirmed by analysis of the Celera mouse genome database using the nucleotide sequences in Figure 1 (J Sgouros and S Parkinson, unpublished). Thus PKCζII has a unique chromosomal location on chromosome 7.

The data indicate that a PKCζ-like gene on chromosome 7, lacking any introns, encodes a protein with an atypical PKC regulatory domain. To determine if this PKCζ-homologous region is simply a nonexpressed pseudogene, Balb/c mouse RNA was extracted from various organs in order to assess PKCζII transcription by RT–PCR (Figure 3B). Since PKCζII genomic DNA contains no introns, extreme care was taken to ensure there was no DNA contamination of the extracted RNAs. Specific primers were designed that only amplified PKCζ, PKMζ, or PKCζII, as determined in control reactions. RNA encoding PKCζ was detected primarily in muscle, heart, lung, kidney, and liver. PKMζ RNA was abundant in the brain and could not be detected in other tissues. RNA derived from PKCζII was abundant in bone, lung, kidney, and liver, however it could be detected in most of the tissues tested. This demonstrates that PKCζII is transcribed from its locus on chromosome 7.

Next, we sought to address whether the RNA detected (Figure 3B) could be translated, generating this putative novel aPKC protein. *In vitro* transcription/translation of the open reading frame of the PKCζII genomic sequence identified the expected 45 kDa protein following autoradiography (Figure 3C). Detection was dependent on the ATG homologous to the PKCζ open reading frame and, as expected, the T7-dependent product was disrupted by the presence of the 5′UTR. No product was detected from the 3′UTR only, demonstrating that the identified protein product derives from translation of the predicted open reading frame identified in Figure 2. Expression and immunoprecipitation of PKCζII or PKCζ as a positive control demonstrated conclusively that PKCζII had no intrinsic catalytic activity (Figure 3D).

In order to determine whether PKCζII was expressed *in vivo*, an antibody recognising the amino terminus of PKCζ (kindly provided by Dr T Sacktor) was used to determine the expression of PKCζII in various cell lines. PKCζII and PKCζ contain virtually identical N-termini. Therefore, detection of an immunoreactive 45 kDa band would indicate the presence of a PKCζII-related protein (Figure 3E). Various cell lines were grown to confluence, the number of cells determined and lysates analysed by Western blot using the N-terminal PKCζ antibody or C-terminal aPKC antibody. A C-terminal antibody that does not distinguish between the aPKC subtypes (ζ/ι/λ) (71 kDa; Figure 3E, top panel) gave a signal following Western blot analysis of total cell lysates. NIH and U251 cells also gave a signal following longer exposure of the film. An N-terminal PKCζ-specific antibody detected a 45 kDa band that comigrated with that detected in lysates from Cos7 cells transfected with a plasmid encoding PKCζII. Full-length PKCζ could also be detected by the N-terminal antibody in these epithelial cell lines. This data support the conclusion that the PKCζII genomic locus on mouse chromosome $7A_{2-3}$ encodes a new member of the aPKC family. An equivalent protein species is detected in human (293, Hela, MCF10A, MCF7), green monkey (Cos7), mouse (NIH3T3, HC11) and canine (MDCK)-derived cells suggesting PKCζII is expressed across mammalian species.

PKCζII interacts with Par6 and PKMζ

To determine the potential for PKCζII to interact with candidate partners, we exploited the fact that ectopic expression of tagged PKCζII in COS7 cells produced an accumulation of the fusion protein in nuclei, such that the influence

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murPKCzeta       TCCGGCGGCT GGCGGAGTGC GCCATGCCCA GCAGGACGGA CCCCAAGATG GACCGGAGCG GCGGCCGCGT CCGTCTGAAG GCGCACTACG GCGGGGACAT
murPKCzetaII     TCCGGCGGCT GGCTGAGTGC GCCATGCCCA GCAGGACGGA CCCCAAGATG GACCGGAGCG GCGGCCGCGT CCGTCTGAAG GCGCACTACG GCGGGGACAT

murPKCzeta       CCTGATTACC AGCGTGGATG CCATGACAAC ATTCAAGGAC CTCTGTGAGG AAGTGCGAGA CATGTGTGGC CTGCACCAGC AGCACCCACT CACCCTCAAG
murPKCzetaII     CCTGATTACC AGCGTGGATG CCATGACAAC ATTCAAGGAC CTCTGTGAGG AAGTGCGAGA CATGTGTGGC CTGCACCAGC AGCACCCACT CACCCTCAAG

murPKCzeta       TGGGTGGACA GTGAAGGTGA CCCTTGTACT GTGTCCTCAC AGATGGAGCT GGAGGAGGCC TTCCGCCTGG TCTGTCAGGG CAGGGACGAA GTGCTCATCA
murPKCzetaII     TGGGTGGACA GTGAAGGTGA CCCTTGTACT GTGTCCTCAC AGATGGAGCT GGAGGAGGCC TTCCGCCTGG TCTGTCAGGG TAGGGACGAA GTGCTCTTCA

murPKCzeta       TTCATGTTTT CCCAAGCATC CCAGAGCAGC CGGGCATGCC TTGTCCTGGA GAAGACAAGT CCATCTACCG CCGTGGAGCC AGAAGATGGA GGAAGCTGTA
murPKCzetaII     TTCATGTTTT CCCAAGCATC CCAGAACAGC CAGGCATGCC TTGTCCTGCA GAAGACAAGT CCATCTACCG CCATGGAGCC AGAAGATGGA GAAAGCCGTA

murPKCzeta       CCGAGCCAAC GGCCACCTCT TCCAAGCCAA GCGCTTTAAC AGGGGAGCGT ACTGCGGCCA GTGCAGCGAA AGGATATGGG GTCTCTCGAG GCAGGGCTAC
murPKCzetaII     CCGAGCCAAT GGCCACCTCT TCCAAGCCAA GCGCTTTAAC AGGGGAGCGT ACTGCGGCCA GTGCAGCGGA AGGATATGGG GTCTCTCGAG GCAGGGCTAC

murPKCzeta       AGGTGCATCA ACTGCAAGCT GCTGGTCCAT AAACGCTGCC ACGTCCTCGT CCCGCTGACC TGCAGGAGGC ATATGGATTC TGTCATGCCT TCCCAAGAGC
murPKCzetaII     AGGTGCATCA ACTGCAAGCT GCTGGTCCAT AAACGCTGCC ACGTCCTCGT CCCGCTGACC TGCAGGAGGC ATATGGATTC TGTCACGCCT TCCCAAGAGC

murPKCzeta       CTCCAGTAGA TGGCAAGAAC GATGGTGTAG ACCTTCCTTC AGAAGAAACT GATGGAATTG CTTATATTTC TTCATCTCGG AAACATGATA ATATCAAAGA
murPKCzetaII     CTCCAGTAGA TGACAAGAAC GATGGTGTAG ACCTTCCTTC AGAAGAAACT GATGGAATTG CTTATATTTC TTCATCTCGG AAACATGATA ATATCAAAGA

murPKCzeta       TGATTCTGAG GACCTTAAGC CTGTCATCGA TGGGGTGGAT GGGATCAAAA TCTCTCAGGG GCTGGGGCTG CAAGACTTCG ACCTCATCAG AGTCATCGGG
murPKCzetaII     TGATTCTGAG GACCTTAAGC CTGTCATCGA TGGGGTGGAT GGGATCAAAA TCTCTCAGGG GCTGGGGCTG CAAGACTTCG ACCTCATTAG AGTCATCGGG

murPKCzeta       CGTGGAAGCT ATGCCAAGGT CCTCCTGGTG CGGTTGAAGA AAAACGACCA GATTTACGCC ATGAAGGTGG TAAAGAAGGA GCTTGTCCAC GACGACGAGG
murPKCzetaII     CGTGGAAGGT ATGCCAAGGT CCTCCTGGTG CGGTTGAAGA AAAACGACCA GATTTACGCC ATGAAGGTGG TAAAGAAGGA GCTTGTCCAT GACGACGAGG

murPKCzeta       ATATCGACTG GGTGCAGACA GAGAAACATG TGTTTGAGCA GGCGTCCAGC AACCCCTTCC TGGTTGGCTT ACACTCCTGC TTCCAGACAA CGAGCCGGTT
murPKCzetaII     ATATCGACTG GGTGCAGACA GAGAAGCATG TATTTGAGCA GGCGTCCAGC AACCCCTTCC TGGTTGGCTT ACACTCCTGC TTCCAGACAA CGAGCCGGTT

murPKCzeta       GTTCCTGGTC ATCGAGTATG TCAATGGCGG GGACCTCATG TTCCACATGC AGAGGCAGAG AAAACTTCCA GAGGAGCATG CCAGGTTCTA TGCTGCTGAG
murPKCzetaII     GTTCCTGGTC ATCGAGTATG TCAATGGGGG GGACCTCATG TTCCACATGC AGAGGCAGAG AAAACTTCCA GAGGAACATG CCAGGTTCTA TGCTGCTGAG

murPKCzeta       ATCTGTATCG CTCTCAACTT CTTGCATGAG AGGGGGATCA TCTACCGGGA CCTAAAACTG GACAACGTCC TCCTTGATGC CGACGGACAC ATTAAGCTGA
murPKCzetaII     ATCTGTATCG CTCTCAACTT CTTGCATGAG AGGGGGATCA TCTAC.GGGA CCTAAAACTG GACAACGTCC TCCTTGATGC CGACGGACAC ATTAAGCTGA

murPKCzeta       CGGACTACGG CATGTGCAAG GAAGGTCTAG GCCCCGGTGA TACAACAAGC ACTTTTTGTG GAACCCCGAA CTATATCGCC CCCGAAATCC TGCGAGGAGA
murPKCzetaII     CGGACTACGG CATGTGCAAG GAAGGTCTAG GCCCCGGTGA TACAACAAGC ACTTTTTGTG GAACCCCGAA CTATATCGCC CCCGAAATCC TGCGAGGAGA

murPKCzeta       AGAGTACGGG TTCAGCGTGG ACTGGTGGGC ACTGGGTGTC CTTATGTTTG AGATGATGGC TGGGCGCTCC CCCTTTGACA TCATCACGGA CAACCCTGAC
murPKCzetaII     AGAGTACGGG TTCAGCGTGG ACTGGTGGGC ACTGGGTGTC CTTATGTTTG AGATGATGGC TGGGCGCTCC CCCTTTGACA TCATCACGGA CAACCCTGAC

murPKCzeta       ATGAACACTG AAGACTACCT TTTCCAAGTT ATCCTGGAAA AGCCAATTCG GATTCCCCGT TTCCTGTCTG TCAAGGCCTC ACACGTCTTA AAAGGATTTT
murPKCzetaII     ATGAACACTG AAGACTACCT TTTCCAAGTT ATCCTGGAAA AGCCAATTCG GATTCCCCGT TTCCTGTCTG TCAAGGCCTC ACACGTCTTA AAAGGATTTT

murPKCzeta       TAAATAAGGA TCCCAAAGAG AGGCTTGGCT GCCGGCCACA GACTGGGTTT TCCGACATCA AGTCTCATGC CTTCTTCCGA AGCATAGACT GGGACCTGCT
murPKCzetaII     AAAATAAGGA TCCCAAAGAG AGGCTTGGCT GCGGGCCACA GACTGGGTTT TCCGACATCA AGTCTCATGC TTTCTTCCGC AGCATAGACT GGGACCTGCT

murPKCzeta       GGAAAAGAAG CAGACCCTGC CTCCCTTCCA GCCCCAGATC ACAGATGACT ATGGCCTGGA CAACTTTGAC ACGCAGTTCA CCAGCGAGCC TGTGCAGCTG
murPKCzetaII     GGAAAAGAAG CAGACCCTGC CTCCCTTCCA GCCCCAGATC ACAGATGACT ATGGCCTGGA CAACTTTGAC ACGCAGTTCA CCAGCGAGCC TGTGCAGCTG

murPKCzeta       ACCCCAGATG ATGAGGACGT CATAAAGAGG ATCGACCAGT CCGAATTTGA AGGCTTTGAG TACATCAACC CACTTCTGCT GTCTGCTGAG GAGTCCGTGT
murPKCzetaII     ACCCCAGATG ATGAGGACGT CATAAAGAGG ATCGACCAGT CCGAGTTTGA AGGCTTTGAG TACATCAACC CGCTTCTG.. .......... ..........

murPKCzeta       GAGGCCATGA GCATCTCTGT TGTGGACACG CCTGTGAATG ACCCTGTCAC TTTACCCTTA ACTACAGCAT ATGCATGCCA GGCCGGGCAC CGAGGCTCCA
murPKCzetaII     .......... .........T TGTGGACACG CCTGTGAATG ACCCTGTCAC TTTACCCTTA ACTACAGCAT AAGCATGCCA GGCCGGGCAC CGAGGCTCCA

murPKCzeta       AGCAGCCAGG GAGGGATGCT GGCCACCAAG ACCGAAGAGG GGGCGTCCAA CAGGCACTTC TAGACAGAGC AATCTCTTGT GTCCAGGCCC CAGAGGCTGG
murPKCzetaII     AGCAGCCAGG GAGGGATGCT GGCCACCAAG ACCGAAGAGG GG.CGTCCAA CAGGCACTTC TAGACAGAGC AATCTCTTGT GTCCAGGCCC CAGAGGCTGG

murPKCzeta       CTTTGTGCTG GAGGAACCGC TTCCTGTGCA CAGAGGCCCT ACCGGAGGGT GAGACAGCCA GCCACGCCGT TTGGAAAGGT GCACATCTTC CACAGAAACA
murPKCzetaII     CTTTGTGCTG GAGGAACCGC TTCCTGTGCA CAGAGGCCCT ACCGGAGGGT GAGACAGCCA GCCACGCCGT TTGGAAAGGT GCACATCTTC CACAGAAACA

murPKCzeta       GAACTCGATG CACTGACCCG CTCCAGGAAA AGTTAGCGTG TAATGCCCTG AGGAATAAAG TGTACCGATG
murPKCzetaII     GAACTCGATG CACTGACCCG CTCCAGGAAA AGTTAGCGTG TAATGCCCTG AGGAATAAAG TGTACCGATG
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Figure 1 Nucleotide alignment of murine PKCζII genomic DNA with murine PKCζ cDNA (accession number M94632). Predicted start codons are bold and overmarked with ***. Differences in nucleotide sequences are red and underlined. Stop codons for murPKCζII (green) and murPKCζ (blue) are also underlined.

of coexpressed proteins could be investigated. Recombinant PKCζII and candidate binding partners were expressed in Cos7 cells tagged with the fluorescent proteins, CFP or YFP. YFP-PKCζII (Figure 4A; top left) was found primarily in the nucleus and could also be observed at cell contacts (Figure 4A; top left, arrow). This was in contrast to YFP-PKMζ (Figure 4A; bottom right) and YFP-PKCζ (not shown), which were located almost exclusively in the cytoplasm.

PKCζII retains the regulatory domains predicted to be required for interaction with the aPKC catalytic domain (PKMζ) and also with regulatory protein Par6.

Expressed alone, PKCζII and PKMζ are located in non-overlapping regions of the cell (Figure 4A). However, when CFP-PKCζII and YFP-PKMζ were coexpressed in Cos7 cells, CFP-PKCζII was observed in the cytoplasm in proportion to the relative PKMζ expression (Figure 4B). In high YFP-PKMζ-expressing cells (single arrowhead) CFP-PKCζII was observed in the cytoplasm. In relatively low YFP-PKMζ-expressing cells (double arrowhead) CFP-PKCζII still accumulated in the nucleus.

PKMζ and full-length PKCζ express identical catalytic domains. Despite the presence of its own regulatory domain, PKCζ could also stabilise PKCζII in the cytoplasm although this required significantly higher expression levels for PKCζ than those for PKMζ (data not shown). These data suggest that the PKCζ catalytic domain can interact with PKCζII.

Expressed alone, Par6c (Figure 4A; top right) could be detected in both the cytoplasm and nucleus of cells while, as noted above, PKCζII (Figure 4A; top left) was primarily nuclear. In low expressing CFP-Par6c cells,

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PKCzeta     MPSRTDPKMD RSGGRVRLKA HYGGDILITS VDAMTTFKDL CEEVRDMCGL
PKM         .......... .......... .......... .......... ..........
PKCzetaII   MPSRTDPKMD RSGGRVRLKA HYGGDILITS VDAMTTFKDL CEEVRDMCGL

PKCzeta     HQQHPLTLKW VDSEGDPCTV SSQMELEEAF RLVCQGRDEV LIIHVFPSIP
PKM         .......... .......... .......... .......... ..........
PKCzetaII   HQQHPLTLKW VDSEGDPCTV SSQMELEEAF RLVCQGRDEV LFIHVFPSIP

PKCzeta     EQPGMPCPGE DKSIYRRGAR RWRKLYRANG HLFQAKRFNR GAYCGQCSER
PKM         .......... .......... .......... .......... ..........
PKCzetaII   EQPGMPCPAE DKSIYRHGAR RWRKPYRANG HLFQAKRFNR GAYCGQCSGR

PKCzeta     IWGLSRQGYR CINCKLLVHK RCHVLVPLTC RRHMDSVMPS QEPPVDGKND
PKM         .......... .......... .......... ...MDSVMPS QEPPVDGKND
PKCzetaII   IWGLSRQGYR CINCKLLVHK RCHVLVPLTC RRHMDSVTPS QEPPVDDKND

PKCzeta     GVDLPSEETD GIAYISSSRK HDNIKDDSED LKPVIDGVDG IKISQGLGLQ
PKM         GVDLPSEETD GIAYISSSRK HDNIKDDSED LKPVIDGVDG IKISQGLGLQ
PKCzetaII   GVDLPSEETD GIAYISSSRK HDNIKDDSED LKPVIDGVDG IKISQGLGLQ

PKCzeta     DFDLIRVIGR GSYAKVLLVR LKKNDQIYAM KVVKKELVHD DEDIDWVQTE
PKM         DFDLIRVIGR GSYAKVLLVR LKKNDQIYAM KVVKKELVHD DEDIDWVQTE
PKCzetaII   DFDLIRVIGR GRYAKVLLVR LKKNDQIYAM KVVKKELVHD DEDIDWVQTE

PKCzeta     KHVFEQASSN PFLVGLHSCF QTTSRLFLVI EYVNGGDLMF HMQRQRKLPE
PKM         KHVFEQASSN PFLVGLHSCF QTTSRLFLVI EYVNGGDLMF HMQRQRKLPE
PKCzetaII   KHVFEQASSN PFLVGLHSCF QTTSRLFLVI EYVNGGDLMF HMQRQRKLPE

PKCzeta     EHARFYAAEI CIALNFLHER GIIYRDLKLD NVLLDADGHI KLTDYGMCKE
PKM         EHARFYAAEI CIALNFLHER GIIYRDLKLD NVLLDADGHI KLTDYGMCKE
PKCzetaII   EHARFYAAEI CIALNFLHER GIIYGT*... .......... ..........

PKCzeta     GLGPGDTTST FCGTPNYIAP EILRGEEYGF SVDWWALGVL MFEMMAGRSP
PKM         GLGPGDTTST FCGTPNYIAP EILRGEEYGF SVDWWALGVL MFEMMAGRSP
PKCzetaII   .......... .......... .......... .......... ..........

PKCzeta     FDIITDNPDM NTEDYLFQVI LEKPIRIPRF LSVKASHVLK GFLNKDPKER
PKM         FDIITDNPDM NTEDYLFQVI LEKPIRIPRF LSVKASHVLK GFLNKDPKER
PKCzetaII   .......... .......... .......... .......... ..........

PKCzeta     LGCRPQTGFS DIKSHAFFRS IDWDLLEKKQ TLPPFQPQIT DDYGLDNFDT
PKM         LGCRPQTGFS DIKSHAFFRS IDWDLLEKKQ TLPPFQPQIT DDYGLDNFDT
PKCzetaII   .......... .......... .......... .......... ..........

PKCzeta     QFTSEPVQLT PDDEDVIKRI DQSEFEGFEY INPLLLSAEE SV
PKM         QFTSEPVQLT PDDEDVIKRI DQSEFEGFEY INPLLLSAEE SV
PKCzetaII   .......... .......... .......... .......... ..
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Figure 2 Amino acid alignment of murPKCζII with murPKMζ, murPKCζ (accession number M94632). The amino acid sequences of PKCζ and PKCζII were predicted from the nucleic acid sequence in Figure 1. The PKMζ sequence was derived from sequenced RT–PCR products in Figure 3 (below). Amino acid differences are underlined. The three shadowed sequences define the three domains of the protein; in order from the amino-terminus of PKCζ these are PB1, C1, and kinase domains.

YFP-PKCζII retained its nuclear localisation (single arrowheads; Figure 4C), however in high CFP-Par6c-expressing cells YFP-PKCζII resided in the cytoplasm (Figure 4C; double arrowhead) suggesting that Par6c interacts with PKCζII *in vivo*.

The effects on cellular localisation of PKCζII were also assessed by cell fractionation (Figure 4D, E). Crude nuclear and cytoplasmic fractions were isolated from Cos7 cells expressing mycPKCζII, PKMζ or mycPKCζII, and PKMζ and the distribution of the expressed proteins was evaluated in the two fractions. Endogenous PKCλ/ι and exogenous PKMζ were primarily cytoplasmic in the presence or absence of mycPKCζII. MycPKCζII was detected primarily in the nucleus when expressed alone in Cos7 cells. However, it appeared mainly in the cytoplasmic fraction when coexpressed with PKMζ. This cell fractionation supports the fluorescent microscopy results presented in Figure 4B.

These data, exploiting the subcellular location observed on overexpression of PKCζII, indicate that PKCζII can interact with aPKCs and with aPKC regulatory proteins mutually regulating their cellular location. The pseudosubstrate site of PKCζII is a potential regulatory site that mediates interaction with the catalytic domain of the aPKCs. In addition, Par6 interacts with the N-terminal 120 amino acids of the aPKCs that includes the pseudosubstrate site. Interestingly, mutation of the PKCζII pseudosubstrate site (A119E) stabilises the protein in the cytoplasm (Figure 4A; bottom left). This indicates that the pseudosubstrate site determines the nuclear location of the overexpressed protein. To investigate this further, mycPKCζII or mycPKCζIIA119E was expressed in Cos7 cells with flagPar6c or PKMζ and their association was examined following immunoprecipitation with the anti-myc 9E10 antibody (Figure 4F). The flagPar6c and PKMζ detected in immunoprecipitates were specific for

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mycPKCζII-expressing cells. Notably, mutation of the mycPKCζII pseudosubstrate site (mycζIIA119E) had no effect on the interaction between PKCζII and PKMζ or flagPar6c (see Discussion). This indicates that while the pseudosubstrate site contributes to nuclear localisation it is not required for binding to PKCζ or Par6. It is likely however that PKCζII binding to these proteins blocks recognition of its pseudosubstrate site, leading to inefficient nuclear import.

PKCζII inhibits tight junction formation in mammary epithelial cells

PKCζII protein interacts with the cell polarity protein Par6 (Figure 4). This suggests that PKCζII could play a role in development of cellular junctions. It was previously reported that aPKC activity was required for the formation but not maintenance of tight junctions presumably via aPKC interaction with Par6 (Suzuki *et al*, 2001). PKCζII lacks any catalytic activity and could compete with aPKCs for binding to Par6 (Figure 4) inhibiting tight junction formation. Therefore, the role of endogenous PKCζII in the development of cell junctions was investigated using an RNAi approach. HC11 cells are a mouse mammary epithelial cell line endogenously expressing PKCζII (Figure 3B). They are normally maintained in an undifferentiated state in the presence of EGF and insulin. However, they form tight junctions and become differentiated in response to lactogenic hormones and produce β-casein and other differentiation markers (Stelwagen *et al*, 1999; Xie *et al*, 2002).

Preliminary experiments demonstrated that the selected RNAi sequence specifically inhibited expression of recombinant PKCζII but not PKCζ in 293T cells (data not shown). HC11 cells were infected with an ecotropic retrovirus driving transcription of this RNAi molecule complementary and specific to PKCζII. RNA was extracted from HC11 cells infected with the PKCζII RNAi retrovirus (pSRII) or control virus (pSR), and the expression of PKCζ, PKCζII, and β-actin was assessed by RT–PCR (Figure 5A). HC11 pSRII cells showed a significant loss of PKCζII message in all conditions tested while the detection of PKCζ was unaffected demonstrating the specificity of the targeting sequence. The levels of expressed proteins were also assessed in the two cell lines (Figure 5B). Western blot analysis revealed a loss of the 45 kDa immunoreactive band (PKCζII) in pSRII cells while

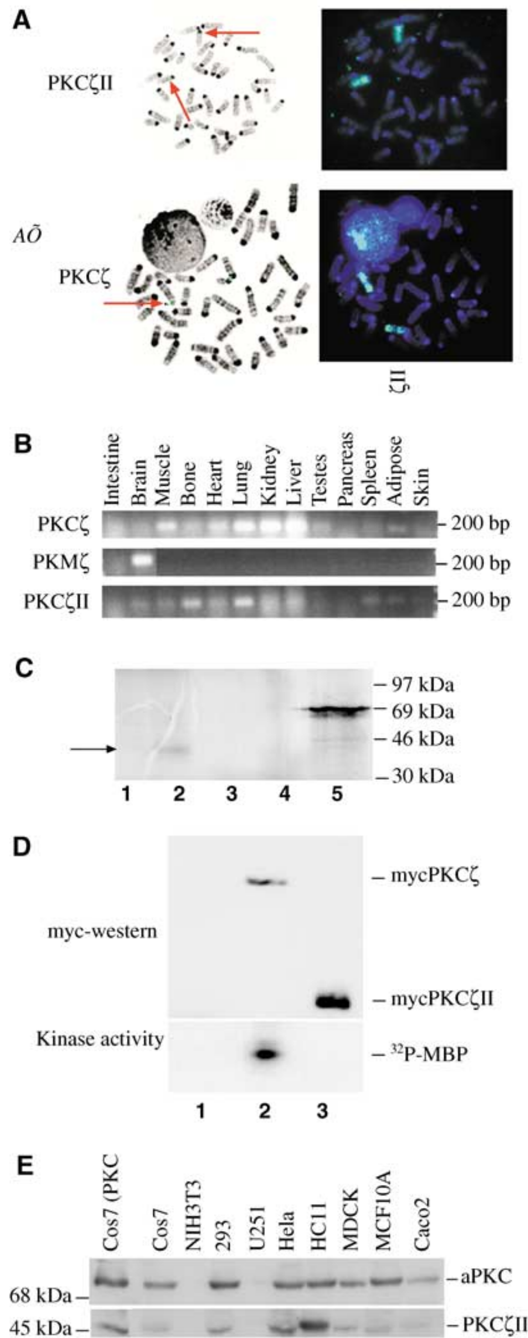

Figure 3 The gene encoding PKCζII is on mouse chromosome number 7 and is transcribed and translated in various tissues and cell lines. (**A**) Chromosome localisation of the PKCζII gene. FISH was performed with a specific probe recognising the PKCζII 3′UTR and chromosomal paints recognising chromosome 7. Hybridising regions of the PKCζII probe are indicated with red arrows and correlate with the highlighted chromosome 7s (right panel). (**A′**) Chromosome localisation of the PKCζ gene. FISH was performed with a specific probe recognising an intron sequence from the murine PKCζ gene and chromosomal paints recognising chromosome 4. Fluorescent-tagged PKCζ hybridising regions are indicated with a red arrow and correlate with the presence of chromosome 4 in the right panel. (**B**) Transcription of aPKCs detected in mouse tissues by RT–PCR. Total RNA was isolated from the indicated Balb/C mouse tissues and treated with DNAse. All RNAs were free of DNA contamination as demonstrated by a lack of visible β-actin or PKCζII product in the absence of reverse transcriptase (not shown). The specific primers used to detect PKCζ, PKMζ, and PKCζII are indicated in Materials and methods. (**C**) *In vitro* transcription/translation of isolated PKCζII genomic clone and detection of a 45 kDa protein. Genomic DNA encoding PKCζII was added to a rat reticulocyte lysate and transcribed and translated *in vitro* in the presence of ^{35}S methionine. The following templates were used: lane 1—PKCζII no ATG, lane 2—PKCζII no 5′-UTR, lane 3—PKCζII with 5′ and 3′ UTR, lane 4—PKCζII 3′UTR only, lane 5—luciferase control. (**D**) myc-PKCζ or PKCζII were expressed in Cos7 cells and immunopurified. Activity was determined against myelin basic protein (MBP) as described in Materials and methods. (**E**) Detection of endogenous PKCζII protein in various cell lines. The indicated cell lines were grown to confluence, trypsinised, counted, and lysed in sample buffer. Lysate from the equivalent of 10^6 cells was loaded on 12.5% PAGE gel, transferred to PVDF and incubated with an antibody directed against the C-terminus to detect full-length aPKCs (λ/ι/ζ) (top panel) or an antibody directed against the N-terminus to detect PKCζII (bottom panel) (see Materials and methods for specificity). Lysate from Cos7 cells expressing a PKCζII cDNA plasmid (Cos7 (PKCζII)) is included as a migration control.

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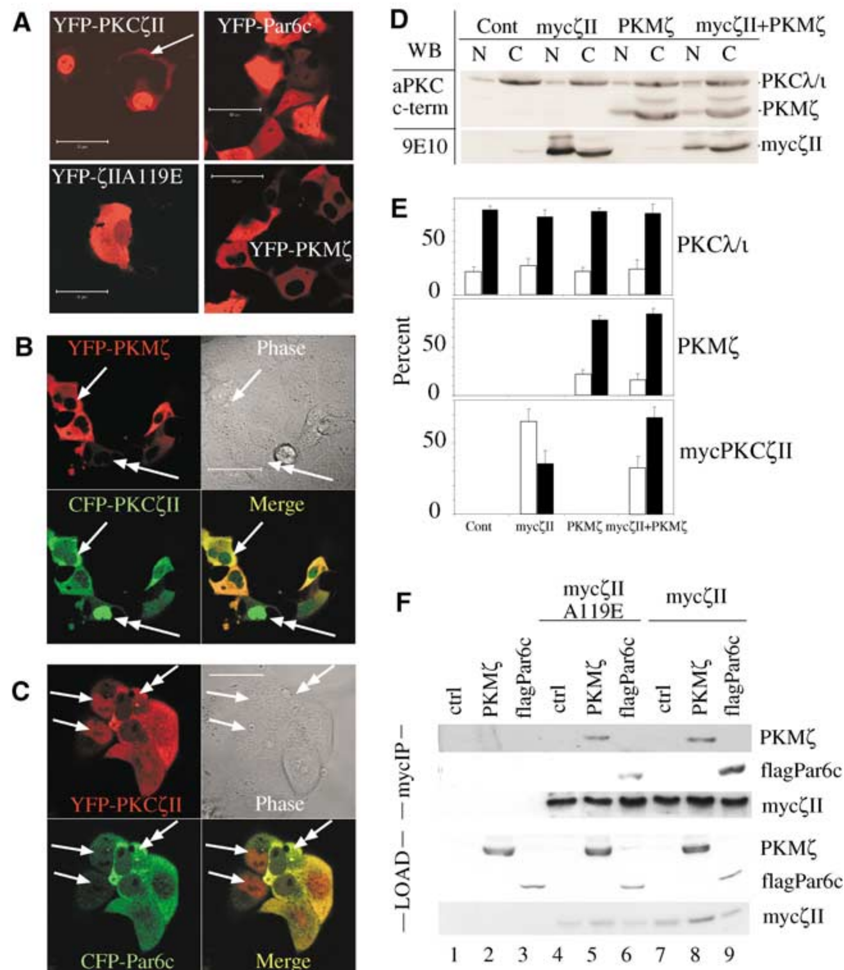

Figure 4 Characterisation of PKCζII localisation and protein interactions. (**A**) Localisation of PKCζII, PKCζIIA119E, PKCζ, and Par6c by live-cell confocal microscopy. YFP-tagged plasmids encoding PKCζII (top left), PKCζIIA119E (bottom left), PKMζ (bottom right) and Par6c (top right) were transfected into Cos7 cells and visualised (pseudocolour red) by confocal microscopy (bar = 50 μM). (**B**) PKCζII and PKMζ colocalise in the cytoplasm of coexpressing cells. Cos7 cells (phase, top right) expressing YFP-PKMζ (red) and CFP-PKCζII (green) were observed by live-cell confocal microscopy (merge, bottom right) (bar = 50 μM). (**C**) PKCζII and Par6c colocalise in the cytoplasm of coexpressing cells. Cos7 cells (phase, top right) expressing YFP-PKCζII (red) and CFP-Par6c (green) were visualised by live-cell confocal microscopy (merge, bottom right) (bar = 30 μM). (**D**) Interaction with PKMζ stabilises PKCζII in the cytoplasm. Cos7 cells (Cont) expressing mycPKCζII (mycζII), PKMζ (PKMζ) or coexpressing mycPKCζII and PKMζ (mycζII + PKMζ) were lysed and nuclear-rich fractions obtained as described in Materials and methods. Equivalent volumes of nuclear (N) or cytoplasmic (C) fractions in sample buffer were run on a 12.5% PAGE gel, transferred to PVDF and immunoblotted with 9E10 (anti-myc) or aPKC C-terminal (aPKC C-term) antibodies. PKCλ/ι is endogenously expressed in Cos7 cells. (**E**) The intensity of the indicated bands (PKCλ/ι, PKMζ, or mycζII) from Figure 4D were quantitated (NIH ImageQuant) from three independent experiments and the percentage of protein in the nuclear (open bars) or cytoplasmic (closed bars) fractions was quantified (±s.d.). (**F**) PKCζII forms a complex with Par6c or PKMζ that is not dependent on the pseudosubstrate site. Cos7 cells (lane 1) expressing PKMζ (lane 2), flagPar6c (lane 3), mycPKCζIIA119E (lane 4), mycPKCζIIA119E + PKMζ (lane 5), mycPKCζIIA119E + flagPar6c (lane 6) mycPKCζII (lane 7), mycPKCζII + PKMζ (lane 8) or mycPKCζII + flagPar6c (lane 9) were lysed and the detergent-soluble fraction was incubated with Protein G Sepharose-coupled 9E10 (αmyc) antibody. Detergent soluble (LOAD; bottom panels; 1:25 of total) and immunoprecipitates (mycIP; top panels) were run on 12.5% gels, transferred to PVDF and associated proteins were detected by probing with anti-aPKC C-terminal (PKMζ), anti-flag (flagPar6c), or anti-myc (mycPKCζII/mycζIIA119E) primary antibodies.

no significant difference in the 70 kDa band (PKCζ) was observed between pSR and pSRII HC11 lysates. These data demonstrate that the PKCζII gene identified here is expressed and further that PKCζII is translated from an mRNA distinct from PKCζ.

Using the RNAi cell lines characterised in Figure 5A and B, we investigated the role of PKCζII in the development of cell polarity.

HC11 pSR and HC11 pSRII cells were seeded on coverslips, allowed to grow to confluence, fixed and stained for various junction markers (Figure 5C). Loss of PKCζII in pSRII cells correlated with the appearance of contiguous staining of ZO-1 in the apical plane. This contrasts with pSR cells where ZO-1 staining is punctate throughout the cytoplasm and disjointed at cell junctions. A similar pattern of behaviour was observed for the tight junction marker claudin-1 (data not shown). The β-actin staining was consistent with the ZO-1 localisation. Bundled cortical actin could be observed in the apical plane of pSRII but not pSR cells. The adherens junction marker β-catenin was located at cell–cell contacts in both cell lines suggesting that PKCζII did not regulate adherens junction formation. These results suggest that PKCζII specifically inhibits the development of tight junctions in HC11 cells.

In growth media containing EGF and insulin, HC11 cells adopt a transformed phenotype. They are characteristically rounded and grow in multiple layers losing contact inhibition

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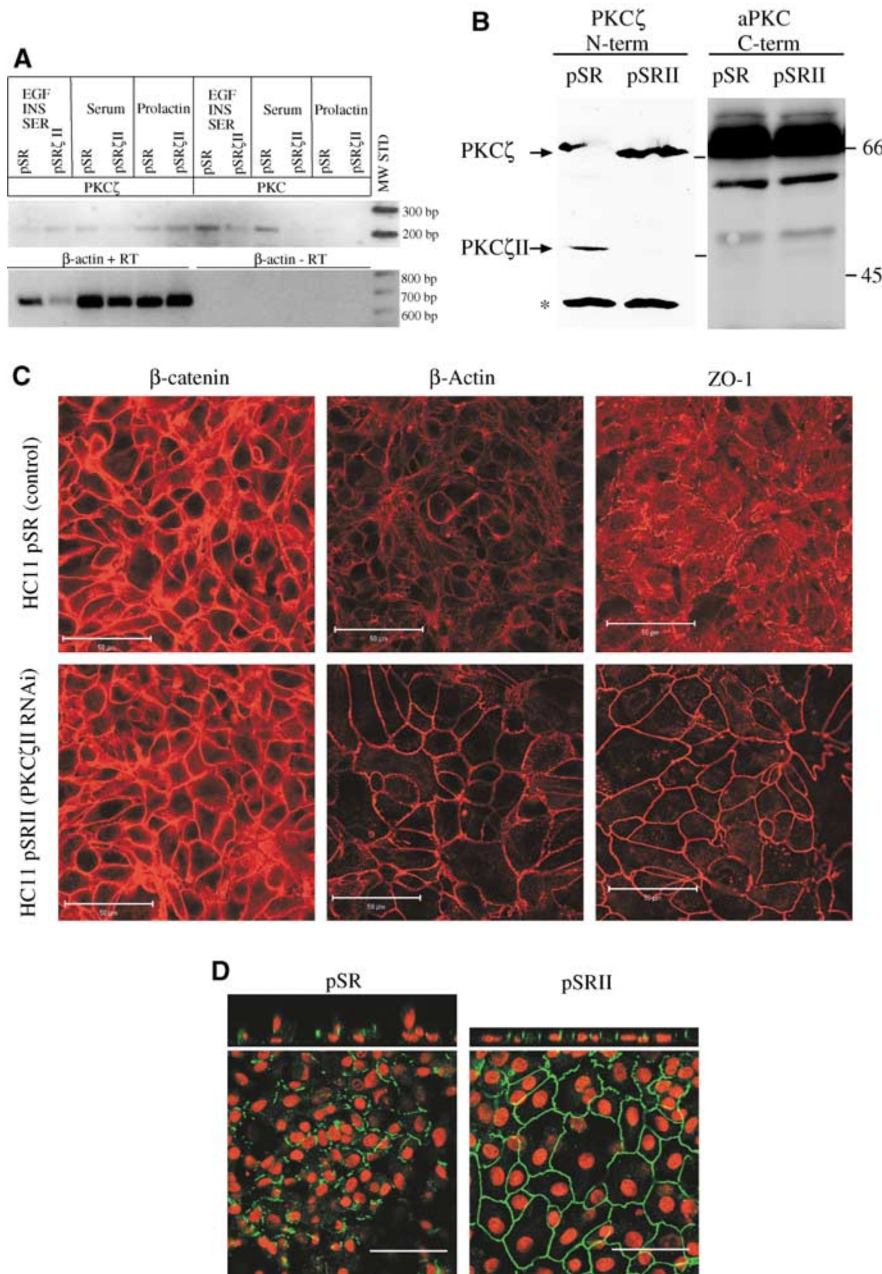

Figure 5 PKCζII inhibits formation of tight junctions in HC11 cells. (**A**) PKCζII RNAi specifically reduces PKCζII, but not PKCζ RNA. RT–PCR detection of PKCζII, PKCζ, and β-actin message in control HC11 cells (pSR) and PKCζII RNAi-expressing HC11 cells (pSRII). Cells were grown to confluence on 6 cm^2 plates under standard growth conditions (EGF, INS, and SER). Following confluence, parallel cultures were switched to 2% serum for 24 h (serum) and then treated with prolactin/hydrocortisone (prolactin) for 72 h to induce differentiation (Xie *et al*, 2002). Cells grown under these different conditions were harvested, RNA extracted, DNAse treated and the indicated message amplified with specific primers. For clarity, the images shown are negatives. Differentiation was confirmed by β-casein mRNA expression (not shown). DNA contamination was minimal as detected by the lack of amplification of β-actin in the absence of reverse transcriptase using the specified primers (β-actin – RT). (**B**) Western blot detection of PKCζ and PKCζII in HC11 pSR (control) and HC11 pSRII (PKCζII RNAi) cells. HC11 pSR and pSRII cells were grown for 72 h postconfluence and the proteins extracted in lysis buffer. Lysates were run on 10% PAGE gels, transferred to PVDF membrane and immunoblotted with rabbit antibodies against the N-terminus of PKCζ or the C-terminus of atypical PKCs. PKCζII is recognised by the N-terminal but not C-terminal antibody and is decreased in HC11 pSRII (PKCζII RNAi) versus pSR (control) cells. The asterisk denotes a nonspecific immunoreactive band. (**C**) Immunostaining of adherence and tight junction markers in HC11 pSR (control) and HC11 pSRII (PKCζII RNAi) cells. Cells were seeded on glass coverslips and grown to confluence in EGF, insulin, and 10% serum. After 72 h, cells were fixed in MeOH and stained to detect β-catenin, β-actin, and ZO-1. Single confocal sections (0.5 μm) are shown for the two cell lines (bar = 50 μm). (**D**) PKCζII is necessary for the transformed phenotype of HC11 cells. HC11 pSR or pSRII cells were grown for 72 h postconfluence and stained with propidium iodide to identify nuclei (red) and an antibody against ZO-1 (green). Z stack profiles (top, bar = 10 μm) of stained cells are shown above confocal images in the *X*–*Y* plane (bottom, bar = 50 μM).

(pSR cells, Figure 5D). However, in pSRII cells, loss of PKCζII correlates with a monolayer phenotype as demonstrated in Z stacks of cells stained with propidium iodide to label nuclei. The loss of cell overgrowth (ie monolayer growth) can also be observed in orthogonal sections of cells stained with E-cadherin and β-catenin (Figure 6). The adherens junction

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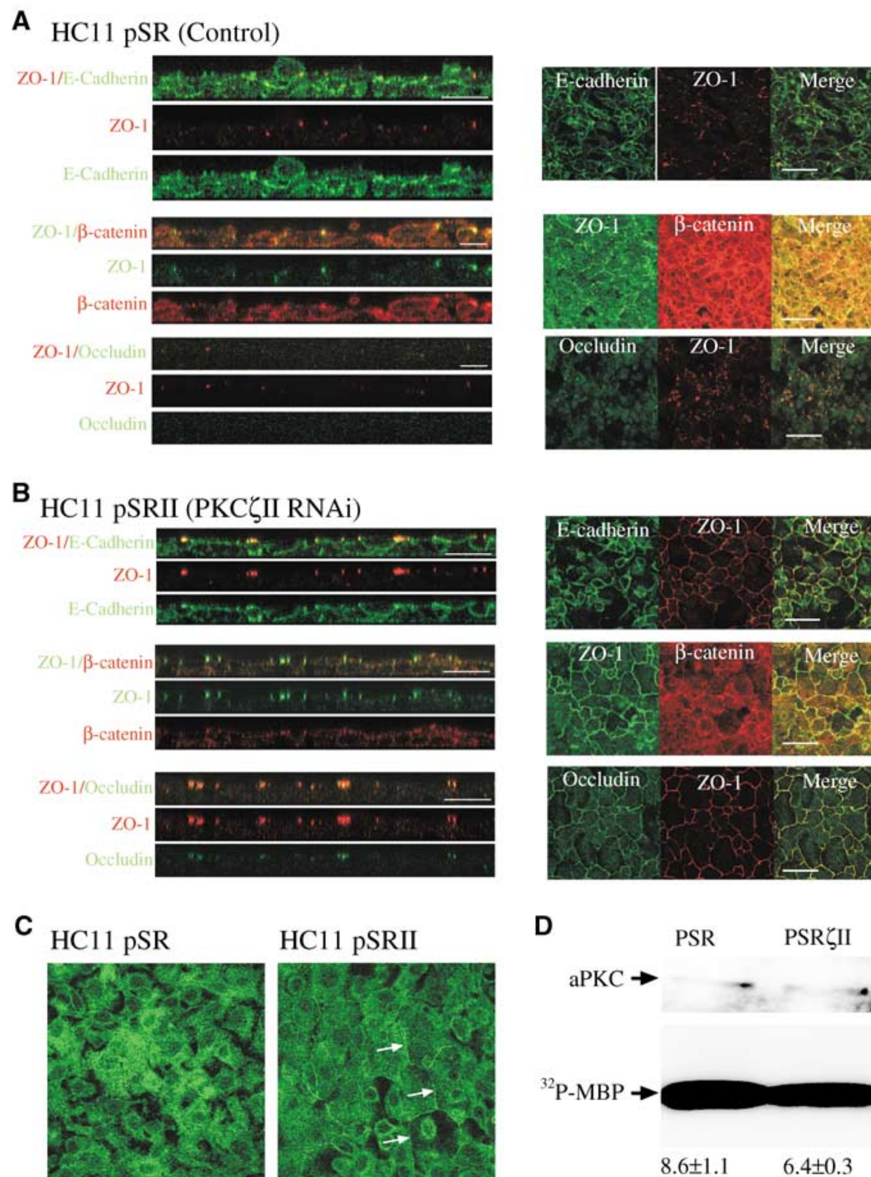

Figure 6 Characterisation of cell–cell junctions in HC11 pSR (**A**) and pSRII knockdown (**B**) cells. HC11 pSR and HC11 pSRII cells were grown for 72 h postconfluence in the presence of EGF, insulin, and serum on glass coverslips, fixed in MeOH, and stained for various junctional markers as indicated. The left panels show composite Z stack profiles of stained cells (bars = 20 μm). The right panels show confocal *X–Y* sections through an apical plane (bars = 50 μM). (**C**) aPKC localisation is junctional in pSRII cells. aPKC was immunostained in pSR and pSRII cells as indicated. Examples of the junctional location of aPKC are highlighted by the arrows. (**D**) PKCζ activity is not altered by suppression of PKCζII expression. PKCζ was immunoisolated from cells as described in the Materials and methods section. PKCζ protein and activity were determined and no change in activity was observed. Quantitation of four observations is shown under the representative western (PKCζ) and autoradiograph (^{32}P-myelin basic protein).

marker localises to areas of cell–cell contact and multiple layers of cells can be observed. Blocking PKCζII expression repressed the transformed phenotype. HC11 pSRII cells stained with E-cadherin and β-catenin were essentially monolayer and contact inhibited (Figure 6B). In pSRII cells, the tight junction marker ZO-1 is located apically to the adherens junction markers E-cadherin and β-catenin and colocalises with the tight junction marker occludin (Figure 6B).

The data presented suggest that PKCζII interacts with Par6 and with the catalytic domain of aPKCs. Both interactions could potentially inhibit cell polarity and explain the formation of tight junctions in the absence of PKCζII expression. aPKC localisation was also modified in the pSRII cells (Figure 6C; note the junctional accumulation of aPKC) while the total aPKC activity in immunoprecipitates was not stimulated following PKCζII knockdown (Figure 6D). This data supports the hypothesis that PKCζII inhibits tight junction formation by preventing aPKC accumulation and activation at tight junctions.

Discussion

The data reported here introduce a new member of the murine aPKC family (PKCζII) and implicate it in regulating aPKC-dependent cellular processes. The murine gene encoding this protein has a unique chromosome location and

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retains a high degree of identity with the PKCζ cDNA sequence. The synthesised protein has 98% amino acid identity to the regulatory domain of PKCζ but lacks any catalytic function. Despite lacking catalytic activity, domains for interaction with regulatory proteins are retained (Lin *et al*, 2000; Suzuki *et al*, 2001). It is shown that PKCζII interacts with Par6c (Figure 4), an aPKC-binding protein required for tight junction formation, and so prevents tight junction formation (Figures 5, 6). Thus *in vivo*, PKCζII functions to control aPKC signalling pathways contributing to cell polarity.

The knockdown of PKCζII protein using an RNAi approach in HC11 epithelial cells demonstrates that this aPKC homolog inhibits tight junction formation. Previous studies have identified an essential role for aPKC activity in establishing tight junction formation (Suzuki *et al*, 2001, 2002). The N-terminus of Par6 contains an aPKC-binding motif that recognises the regulatory domain of the kinase. Cdc42 binds to Par6 through its CRIB and PDZ domains and activates aPKC kinase activity (Garrard *et al*, 2003). The Par6/aPKC complex also interacts with the scaffolding protein Par3 permitting its phosphorylation; an event correlated with development of tight junctions (Hirose *et al*, 2002). In the absence of any effect on aPKC activity it is concluded that PKCζII competes for recruitment to cell junctions. This may be direct competition with Par6 or the Par6/PKCζII complex may compete with Par6/aPKC for activated Cdc42 preventing phosphorylation of Par3 and/or recruitment of other complexes required for development of tight junctions. Other factors may also contribute to the phenotype observed in Figures 5 and 6 including the possibility that PKCζII interacts with the catalytic domain of aPKCs (see Figure 4).

The data presented are consistent with a previous study that determined a PKCζ regulatory fragment inhibited tight junction formation in mammalian epithelial cells (Gao *et al*, 2002). In this study, a synthetic recombinant N-terminal fragment consisting of the first 126 amino acids of PKCζ lacking kinase activity delayed the development of mature tight junctions. This was dependent on its interaction with Par6 since mutation of the PKCζ Par6-binding site abrogated the delay in the recruitment of ZO-1 to tight junctions. This data support the idea that PKCζII inhibits development of the Cdc42–Par6–aPKC complex although it is unclear whether the Par6-binding mutant used by Gao *et al*. (2002) still interacts with aPKC catalytic domains. In addition, a PKCλ mutation identified from a genetic screen in zebrafish was found to be responsible for the *heart-and-soul* (*has*) phenotype in which the animals demonstrate a loss of normal heart, kidney, and retinal patterning (Horne-Badovinac *et al*, 2001; Peterson *et al*, 2001). The *has* phenotype results from premature termination of the PKCλ coding sequence. The *has* mutants identified lack the C-terminal 69 and 73 amino acids and completely lack any catalytic activity.

The Ca^{2+} switch assay is routinely used to investigate formation of cellular junctions. Depletion of extracellular Ca^{2+} disrupts cell–cell contacts and addition of extracellular Ca^{2+} permits junctions to form. Using this assay, previous reports demonstrated that the formation of tight junctions is dependent on aPKC activity since kinase-dead mutants disrupt their formation (Suzuki *et al*, 2001). Supporting these findings, MDCK cells overexpressing PKCζII over grow each other forming multiple layers and have disrupted ZO-1 localisation following Ca^{2+} switch (data not shown). This observation is consistent with the HC11 data, however PKCζII is poorly expressed in transfected epithelial cells complicating analysis through ectopic expression.

The presence of the PKCζII gene in mice likely represents a reverse transcriptase-dependent insertion of the PKCζ cDNA into the genome since the PKCζII genomic sequence retains a high degree of homology but lacks any introns. Spontaneous mutation of the inserted sequence resulted in the deletion of nucleic acid 1146 and synthesis of a PKCζ regulatory domain lacking catalytic function. There are several reports of similar insertions occurring in the mammalian genome however most of these events result in nonfunctional genomic sequences referred to as pseudogenes. From the data presented it appears that PKCζII was inserted into a region that permits transcription of its sequence resulting in a stable mRNA and 45 kDa protein.

Part of the analysis of the PKCζII–Par6c interaction *in vivo* exploited the distinct subcellular compartmentation of the proteins. In Cos7 cells PKCζII and a homologous fragment of PKCζ (not shown) are primarily localised in the nucleus of cells, suggesting the presence of a nuclear localisation signal (NLS) in the protein. These observations support previous studies identifying an NLS within the regulatory domain of full-length aPKCs (Perander *et al*, 2001). A pseudosubstrate site PKCζII mutant is cytoplasmic suggesting this region plays a role in nuclear retention or targeting. In contrast, an homologous pseudosubstrate site mutation in PKCλ/ι stabilises the protein in the nucleus (Perander *et al*, 2001). Differences in the nuclear trafficking of PKCλ/ι and PKCζ have been reported previously. Significantly, identical mutants deleting the N-terminus up to and including the pseudosubstrate site had completely opposite cellular locations; the PKCζ mutant was cytoplasmic while the PKCλ mutant was nuclear (Perander *et al*, 2001). The location of the N-terminal mutant of PKCζ (Perander *et al*, 2001), PKMζ, PKCζII, and PKCζIIA119E (this study) are all consistent and demonstrate a contribution of the pseudosubstrate site to nuclear localisation of PKCζ. For PKCλ/ι, there may be an additional NLS located C-terminal to the pseudosubstrate site that is exposed upon pseudosubstrate site release from the catalytic domain.

The presence of PKCζII in the cytoplasm following mutation of A119E correlates with the location of PKCζII *in vivo* when it is in a complex with Par6c or PKMζ. Therefore, PKCζII interaction with Par6c or PKMζ likely masks the NLS, of which the pseudosubstrate site appears to be a part. Furthermore, recombinant Par6c is also partially located in the nucleus when expressed on its own, however, in the presence of PKCζ or PKCζII it is primarily cytoplasmic suggesting that the interaction either masks an NLS in Par6c or exposes a nuclear export signal somewhere in the complex. In differentiated epithelial cells, Par6 has only been detected at cell junctions. This may be due to its interaction with aPKC and other proteins that limit its localisation; the distribution of endogenous Par6 in other cell types and under various conditions may be varied. The observation that recombinant Par6c is entirely cytoplasmic when coexpressed with interacting proteins but only partially in their absence suggests this might be the case and needs to be investigated further.

Synthesis of truncated proteins that regulate cellular functions is a common theme in signal transduction. Translation of these inhibitory proteins can occur from alternate splicing

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of mRNAs or from distinct genes with unique chromosomal locations. Synthesis of the focal adhesion kinase (FAK)-related protein FRNK is an example of regulation by alternate splicing of a single mRNA (Schaller *et al*, 1993). Reminiscent of PKCζII and PKCζ, FRNK is composed of the C-terminal regulatory domain of FAK but lacks any kinase activity. Another example is the soluble frizzled-related proteins (sFRPs). The sFRPs are located on distinct genes that are homologous to the frizzled receptors for the Wnts. These secreted proteins are homologous to the cysteine-rich domain (CRD) of the frizzled 7-transmembrane receptors and inhibit Wnt-dependent signalling pathways leading to stabilisation of β-catenin in the cytoplasm (Rattner *et al*, 1997). Interestingly, it appears that PKCζ signalling pathways are also regulated by multiple strategies. PKMζ is synthesised from the PKCζ gene on mouse chromosome 4. It lacks most of the regulatory domain and likely constitutively phosphorylates substrates due to lack of an inherent pseudosubstrate site. PKCζII, however, lacks all catalytic activity but can interact with regulatory proteins preventing aPKC-dependent signalling. All of the aPKC members likely contribute to form an integrated cellular response to each stimulus.

In conclusion, PKCζII is identified as a new member of the aPKC family. It serves as an endogenous inhibitor of aPKC functions through interaction with known regulatory proteins. As a result of these properties, PKCζII prevents tight junction formation in epithelial cells.

Materials and methods

Reagents

Antibodies. The following antibodies were used: rabbit anti-aPKC C-terminal antibody (Santa Cruz) detects PKCλ/ι and PKCζ (data not shown), rabbit anti-PKCζ N-terminal antibody (generously provided by Dr T Sacktor) is specific for PKCζ and does not cross-react with PKCλ/ι, mouse M2 anti-flag (Sigma), rabbit anti-ZO-1 (Zymed), mouse fitc-conjugated anti-occludin (Zymed), tritc-conjugated phalloidin (Molecular Probes), mouse fitc-conjugated E-cadherin (BD Transduction Labs), mouse anti-β-catenin (BD Transduction Labs), Cy3-conjugated anti-mouse (Jackson ImmunoResearch Laboratories), FITC-conjugated anti-rabbit (Dako).

Plasmids. PKCζII was amplified from mouse brain DNA using the following primers; zetaII24(+) (5′-AAGCTTTGGCGGAGTGCGC-CATG-3′) and zetaII2133(−) (5′-GGTACCAGA-CATCATCGGTACAC-3′). The resulting 2 kb band was ligated into PCR Blunt (Invitrogen), excised with *Hind*III and *Kpn*I and ligated into pDNA3.1(Hygro+) (Invitrogen). PcDNA3.1-PKCζ (rat) was made by subcloning PKCζ into pcDNA3.1-PKCζII cut with *Not*I/*Xba*I. PcDNA3.1-PKMζ was made by subcloning in the RT–PCR product (see RT–PCR below) cut with *Spe*I/*Cla*I (500 bp fragment) into pcDNA3.1-PKCζ cut with *Nhe*I/*Cla*I. PEYFP-PKMζ was constructed by cutting pcDNA3.1-PKCζ with *Nde*I/*Pme*I, blunting the ends with Vent polymerase and ligating the 1.2 kb fragment into pEYFP-C1 (Clontech) cut with *Sma*I. The amino acid sequence of PKMζ was predicted from the sequence of RT–PCR products using PKMζ-specific primers and RNA from mouse brain (see below). PcDNA3.1-mycPKCζII was generated by partial digestion of pcDNA3.1-PKCζII with *Nhe*I/*Ngo*MIV and ligation of oligos encoding the myc epitope. PEYFP-PKCζ was constructed by cutting pEYFP-PKMζ with *Bsp*E1/*Eco*RV and ligating in a fragment from pcDNA3.1-PKCζ cut with *Ngo*MIV/*Eco*RV. PECFP-PKCζ was constructed by subcloning pEYFP-PKCζ into the pECFP-C1 vector (Clontech). PEYFP-PKCζII was constructed by cutting pEYFP-PKCζ with *Bst*EII/*Bam*HI and ligating in the equivalent fragment from pcDNA3.1-PKCζII. PECFP-PKCζII was constructed by subcloning into the pECFP vector. Flag-tagged Par6c (pFlagPar6c) was a gift from Dr Dan Lin and Dr Tony Pawson. To create pECFP-Par6c and pEYFP-Par6c the pFlagPar6c cDNA was cut with *Eco*RI/*Pvu*II and ligated into pECFP-C1 and pEYFP-C1 cut with *Eco*RI/*Sma*I. The PKCζIIA119E mutation was generated by PCR using pcDNA3.1-PKCζII as the template and the primers; 5′-CTACCGCCATGGAGAAAG-AAGATGGAGAAAGCCG-3′ and zetaII-2133(−) (described above). The product was cut with *Nco*I/*Cla*I and ligated into pcDNA3.1-mycPKCζII to make pcDNA3.1-mycPKCζIIA119E. All DNA constructs were confirmed by sequencing. The PKCζII sequence obtained correlated with the sequence established by Celera for the mouse genome (J Sgouros and S Parkinson, unpublished).

Tissue culture. Cos7, MDCK, HELA, 293, NIH3T3, U251, and Caco2 cells were maintained in DMEM containing 10% FBS and penicillin/streptomycin. MCF10A cells were grown in DMEM containing Ham's F12, 5% horse serum, 10 μg/ml insulin, 5 μg/ml hydrocortisone, 20 ng/ml EGF, 100 ng/ml cholera toxin, and penicillin/streptomycin. HC11 cells were maintained in RPMI, 10% FBS, penicillin/streptomycin, 10 μg/ml insulin, and 20 ng/ml EGF.

Virus production. Retrovirus expressing an RNAi against PKCζII were developed using the pSuppressorRetro (pSR) system (Imgenex, San Diego). Complementary oligos (PKCζII target sequence: 5′-aagcatgtatttgagcaggcg-3′) were ligated into pSR, checked by sequencing, and cotransfected into 293T cells with the packaging vector pCL-Eco. Control virus was generated against PKCζ and a sealed pSR plasmid cotransfected with the packaging vector. Virus was collected 48 h post-transfection, filtered, added to HC11 cells and stable lines selected with neomycin. Under growth culture conditions (above), only the PKCζII viral-infected lines demonstrated the change in phenotype; the other cell lines were indistinguishable from noninfected cells. However, in other culture conditions, virus targetting PKCζ demonstrated a significant phenotype distinct from PKCζII. To avoid confusion concerning these cell lines, the 'vector only' line of pSR is shown as a control.

Cloning and chromosome localisation of murPKCζII. Using a randomly labelled ([γ-^{32}P]-dCTP) 300 bp 5′ fragment of PKCζ cDNA as a probe, a mouse genomic λ gt 10 phage library was screened. Three identical hybridising clones were isolated after primary screening and purified by two further rounds of selection. Clone 3, containing a 13 kb insert (flanking PKCζII), was selected for further analysis. The chromosome localisation of PKCζII was assessed by FISH using the following probes and chromosomal paints on mouse metaphase spreads, X7 (PKCζII 3′UTR), pZ-X5 (PKCζ intron sequence).

Alignments. Alignments were performed using Clustal X software with the sequences encoding mouse PKCζII and PKCζ (accession number M94632).

In vitro transcription/translation. In total, 1 μg of genomic PKCζII DNA composed of 5′UTR (2.5 kb), putative PKCζII coding region (1.8 kb) and 3′UTR (8.7 kb) was transcribed *in vitro* and translated in rabbit reticulocyte lysates (Promega) according to manufacturers instructions at 30°C for 2 h in the presence of ^{35}S-methionine. The entire reaction was denatured in sample buffer, separated on a 10% acrylamide gel and analysed by autoradiography.

RT–PCR. Mouse tissue total RNA was extracted using Trizol reagent (Gibco) followed by extensive treatment of the RNA fraction with DNAse. Reverse transcription was carried out using the Access RT–PCR system (Promega) with the following changes to the manufacturers protocol. Reverse transciption was performed using random 9-mers as primers. RNA was ensured to be DNA-free if β-actin was amplified using musBactI (5′-tgacggggtcacccacac tgtgccc-atcta-3′) and musBactII (5′-agtaacagtccgcctagaagcacttgcggt-3′) in the presence but not absence of reverse transcriptase. The following primers were used for amplification by RT–PCR and were specific for their designated targets as confirmed by sequencing: PKCζ-(zeta199(+) 5′-tgggtggacagtgaaggtgac-3′, zeta392(−) 5′-gtt ggctcggtacagcttc-3′), PKCζII-(zeta199(+), zetaII390(−) 5′-attggctc-ggtac-ggcttt-3′), PKMζ-(psizeta(+) 5′-ggagacccacccagcccattg-3′, zeta392(−)). reverse transcription: 48°C—60 min, 95°C—2 min. Add (+) and (−) primers and Tfl polymerase on ice. PCR: 95°C—2 min, 35 × (95°C—1 min, 62°C—1 min, 68°C—1 min), 68°C—10 min.

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Transfections. Cos7 cells (1.5×10^5) were seeded into 3 cm dishes, left overnight, and transfected by $CaPO_4$ with 1 μg of DNA. The following day the precipitate was removed, cells washed and left to recover overnight.

Immunoprecipitations. Cells (10 cm dish) were lysed in 1 ml buffer containing 25 mM HEPES (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5 mM EDTA, 5 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF, and complete protease inhibitor cocktail tablet (Boehringer Mannheim). The lysate was collected by scraping and insoluble material removed by centrifugation (100 000 *g*, 30 min). Protein G-coupled antibody was added to the soluble lysate and rotated for 2–3 h at 4°C. Immunocomplexes were washed 4 × with lysis buffer and solubilised for gel electrophoresis in 100 μl of sample buffer.

Immunocomplex activity determination. Immunoprecipitates of PKCζ or myc-tagged PKCζ and PKCζII were isolated essentially as above except in the presence of 1 μM okadaic acid, 50 mM sodium fluoride, 10 mM *p*-nitrophenylphosphate, 10 mM sodium orthovanadate and in the absence of $MgCl_2$. Immunocomplexes on protein G-Sepharose beads were incubated with shaking at 22°C in the presence of 50 mM HEPES pH 7.5, 10 μM [γ-^{32}P]ATP, 10 mM $MgCl_2$, 1 mg/ml myelin basic protein and 1 μM okadaic acid. After 15 min the reaction was stopped by the addition of sample buffer. Radiolabelled myelin basic protein was separated on a 12.5% polyacrylamide gel, identified by autoradiography, and quantified by direct Cerenkov counting.

Fractionation. Cos7 cells were lysed with 1 ml (4°C) hypotonic buffer (10 ml Tris (pH 8.0), 10 mM NaCl, 3mM $MgCl_2$, 0.25% NP-40)/10 cm^2 dish. Cells were scraped into Eppendorf tubes and passed 4 × through a 21 gauge needle. The lysate was spun for 6 s at 12 000 rpm at 4°C to isolate nuclei. The cytosolic fraction was removed and proteins precipitated with TCA. The nuclear- and cytosolic-rich fractions were resuspended in equal volumes of sample buffer and analysed by electrophoresis and Western blot.

Immunofluorescence and confocal microscopy. Live cell and fixed cell microscopy was performed on an inverted confocal laser scanning microscope (Carl Zeiss, Inc.) equipped with an 63X/1.4Plan-Apochromat oil-immersion objective and fitted with an environmental chamber. Each image represents a single two-dimensional projection. For live cell microscopy, cells were seeded on 35 mm glass bottom dishes (MatTek Corporation), and transfected with the indicated plamids the following day. Alternatively, cells were fixed on 1.5 mm coverslips with 100% MeOH (−20°C) for 10 min, and blocked in Tris-buffered saline 0.1% Triton (TBST) with 3% BSA (30% stock, Sigma) for 30 min. Primary antibodies were added in TBST 3% BSA overnight at 4°C. Cells were washed, then fluorescent-conjugated secondary antibody (see reagents) added (1:200 in TBST) for 30 min. Cells were washed in TBST, TBS, and H_2O, then mounted in Mowiol Mount (Sigma) containing DABCO (Sigma).

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