Par-4 inhibits Akt and suppresses Ras-induced lung tumorigenesis

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The atypical PKC-interacting protein, Par-4, inhibits cell survival and tumorigenesis *in vitro*, and its genetic inactivation in mice leads to reduced lifespan, enhanced benign tumour development and low-frequency carcinogenesis. Here, we demonstrate that Par-4 is highly expressed in normal lung but reduced in human lung cancer samples. We show, in a mouse model of lung tumours, that the lack of Par-4 dramatically enhances Ras-induced lung carcinoma formation *in vivo*, acting as a negative regulator of Akt activation. We also demonstrate in cell culture, *in vivo*, and in biochemical experiments that Akt regulation by Par-4 is mediated by PKC ζ , establishing a new paradigm for Akt regulation and, likely, for Ras-induced lung carcinogenesis, wherein Par-4 is a novel tumour suppressor.

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Introduction

Inactivating mutations and deletions of tumour suppressor genes along with gain-of-function mutations in proto-oncogenes give rise to cells that can grow independent of external proliferation and survival cues and result in tumour transformation and cancer (Hanahan and Weinberg, 2000). Among the proto-oncogenes most frequently altered in human tumours are the small GTPases of the Ras family, which have been found to be mutated in at least 25% of human lung adenocarcinomas (Bos, 1989). This is of particular interest because lung neoplasia is the leading cancer-related cause of

death in the United States, with an estimated incidence of 213 380 new cases and 160 390 deaths in 2007 (<http://www.cancer.gov/cancertopics/wyntk/lung>). Mouse lung cancer models are available that reproduce the human disease relatively faithfully, thus allowing the study of the cellular and molecular basis of this neoplasia at an organismal level (Fisher *et al*, 2001; Meuwissen and Berns, 2005). The two main types of lung cancer are small-cell lung cancer and non-small-cell lung cancer (NSCLC). Among the latter, the most prevalent type is the adenocarcinoma, which accounts for more than 40% of all lung cancers (<http://www.cancer.gov/cancertopics/wyntk/lung>). Current treatments do not lead to a cure for most patients with lung cancer. Targeted anti-tumour therapies are likely to prove more effective, but their development will require a better understanding of the signalling cascades involved in the initiation and progression of this type of tumour. In this regard, Ras oncogenes trigger a myriad of signalling pathways, of which only a few have been characterized in detail, such as the Raf-MEK and PI3 Kinase-Akt signalling cascades (Downward, 2003; Malumbres and Barbacid, 2003). With regard to tumour suppressors, we have reported the reduction of Par-4 (also known as PAWR) levels in Ras-transformed cells (Barradas *et al*, 1999). In the case of cultured fibroblasts, this Par-4 reduction is a required event for Ras to manifest its full transforming potential (Barradas *et al*, 1999). However, its role in more physiological cancer models is still unclear. Par-4 was initially identified in an *in vitro* differential screen for pro-apoptotic genes in human carcinoma cell lines (Sells *et al*, 1994). The *Par-4* gene maps to chromosome 12q21, a region frequently deleted in certain malignancies (Johnstone *et al*, 1998), and encodes a protein (38 kDa) containing a leucine zipper domain in the COOH-terminal region, which interacts with a variety of proteins (Moscat and Diaz-Meco, 2003), including the atypical protein kinases (aPKCs), PKC ζ and PKC $\lambda/1$ (Diaz-Meco *et al*, 1996). Among the mechanisms by which Par-4 triggers apoptosis, the best established one, which is supported by studies in knockout (KO) mice, is through inhibition of the aPKCs and the ensuing downmodulation of NF- κ B and its prosurvival transcriptional targets, such as X-linked inhibitor of apoptosis (XIAP) (Garcia-Cao *et al*, 2003, 2005; Lafuente *et al*, 2003). These previous studies all point to a link between *Par-4* downregulation and cancer. Indeed, we have recently shown that *Par-4*-null mice develop spontaneous benign prostate neoplasias and endometrial carcinomas, thus implicating Par-4 deficiency in the development of tumours (Garcia-Cao *et al*, 2005). In addition, our recent data also demonstrate, using cDNA arrays, quantitative reverse-transcription PCR, and by immunohistochemistry (IHC), that Par-4 is downregulated in approximately 40% of human endometrial carcinomas (Moreno-Bueno *et al*, 2007).

Lungs, along with prostate (see below) and endometrium (Moreno-Bueno *et al*, 2007), are the tissues that exhibit the highest levels of Par-4. We reasoned that if Par-4 is a tumour

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suppressor, its loss in the lung would lead to increased tumorigenicity. In this study we provide compelling evidence that Par-4 deficiency in the lung leads not only to enhanced NF- κ B but also to increased Akt activity, thus making Par-4-deficient lungs more sensitive to Ras-induced oncogenesis. We demonstrate that the negative actions of Par-4 on Akt are cell autonomous and mediated by the ability of PKC ζ to phosphorylate Akt at Ser124, which impacts the phosphorylation status of Ser473 and Thr308, two critical residues for its enzymatic activity (Cantley, 2002). Akt's Thr308 is phosphorylated by PDK1 in response to activation of PI 3-kinase, which generates PIP₃, which by binding the pleckstrin homology domain of Akt and PDK1, makes that residue accessible to the action of PDK1 (Manning and Cantley, 2007). Ser473 has been shown to be phosphorylated by an mTOR-Rictor complex, termed as TORC2, which in contrast to the TORC1 complex, composed of mTOR and raptor that phosphorylate the Ser396 of S6K1, is insensitive to rapamycin (Sarbasov *et al*, 2005; Bhaskar and Hay, 2007). Our observations reported here unveil a novel role for Par-4 and PKC ζ and reveal a novel mechanism of action for the regulation of Akt involving PKC ζ phosphorylation of Ser124, which is negatively regulated by Par-4 and which is likely important in lung cancer *in vivo*.

Results

Tissue distribution of Par-4 and loss in human tumours

Our previous observations have demonstrated that Par-4 KO mice have decreased lifespan and display higher tumour incidence with ageing. These tumours are mainly prostate intraepithelial neoplasias (PIN) and endometrial carcinomas, and low-frequency lung adenocarcinomas (Garcia-Cao *et al*, 2005). This, together with the fact that Par-4 is absent in about 58% of human prostate cancers (our unpublished observations) and in about 40% of human endometrial carcinomas (Moreno-Bueno *et al*, 2007), indicates that Par-4 can be considered as a tumour suppressor. Thus, it is of interest to determine whether Par-4 also has tumour-suppressive activity in other organs. We reasoned that Par-4 will most likely have an important function as a tumour suppressor in those organs that exhibit the highest levels of Par-4 expression. Therefore, we analysed, by immunoblotting with a specific anti-Par-4 antibody, the levels of this protein in different normal mouse tissues. Interestingly, the Par-4 protein is highly expressed in the lung, with levels comparable with those of prostate (Figure 1A). Prostate displays the highest Par-4 mRNA levels, whereas lung, liver and kidney also expressed high levels as compared with other tissues such as brain and intestine (Figure 1B). On the basis of the high Par-4 protein levels in the lung in mouse samples, we determined the distribution of Par-4 in human and mouse lung sections by IHC. The data of Figure 1C show that Par-4 is expressed mostly in the epithelial cells of the airways both in human and mouse lung, as well as in the alveoli.

We hypothesized, then, that the loss of Par-4 in the lung would be instrumental for lung cancer initiation and progression. To begin addressing this possibility, we next determined Par-4 expression levels in tissue microarrays of human NSCLC. Interestingly, 47% ($n = 133$) of NSCLC were negative for Par-4 as determined by IHC (Figure 1D). Importantly, there is a clear correlation between the loss of Par-4 and the type of tumour. That is, 41% of the adenocarcinomas were

negative for Par-4 expression, whereas only 6% of squamous cell carcinomas show negative staining for Par-4. Also, when the adenocarcinomas are stratified by grade, it is clear that 74% of grade III tumours have lost Par-4 expression, whereas 59% of grade I-II are negative for Par-4. In summary, Par-4 levels are reduced in a significant number of human lung malignancies, strongly suggesting that this protein may have an important function in lung cancer prevention.

Par-4 deficiency enhances Ras-induced lung cancer *in vivo*

Par-4 deficiency leads to only benign tumorigenicity in prostates, and to low-frequency lung carcinomas (Garcia-Cao *et al*, 2005). As Par-4 is reduced most significantly in adenocarcinomas, which are the type of lung tumours that best correlate with the expression of mutant oncogenic Ras (Bos, 1989), we next wanted to test the hypothesis that loss of Par-4 favours the tumorigenic actions of this oncogene. To determine whether Ras-induced lung cancer may be influenced by Par-4, we asked whether *in vivo* genetic ablation of Par-4 would affect tumour development in lungs. To do this, we used a mouse model of pulmonary adenocarcinoma in which oncogenic Ras was introduced by a knock-in strategy and is inducibly expressed in an endogenous manner (Guerra *et al*, 2003). This model leads to lung adenomas and adenocarcinomas in which the likely target cell is the type II pneumocyte, evidenced by the observation that the resulting tumour cells express surfactant protein C, a marker of type II pneumocytes (Guerra *et al*, 2003; Tuveson *et al*, 2004). This is a physiologically relevant model for human cancer, as it has been reported that, in addition to Clara cells, type II pneumocytes are the most likely precursors of human lung adenocarcinomas (Fisher *et al*, 2001; Tuveson *et al*, 2004; Meuwissen and Berns, 2005).

To address whether the role of Par-4 is to physiologically restrain Ras-induced lung tumorigenesis, we tested the hypothesis that expression of oncogenic Ras in a Par-4^{-/-} background would increase tumour burden as compared with that in Ras-expressing WT mice. Therefore, Par-4 KO mice were bred to the mice expressing oncogenic Ras in the lung. In this model, a limited percentage of oncogenic Ras-expressing lung broncho-alveolar cells undergo malignant transformation leading to adenomas and adenocarcinomas (Guerra *et al*, 2003). Therefore, this is an ideal *in vivo* model to determine whether the loss of Par-4 under these conditions would lead to increased tumorigenicity in the lung. Results in Figure 2A demonstrate that the tumour burden in oncogenic-Ras-expressing WT lungs is approximately 18% of the total lung tissue, whereas that parameter is significantly higher in Par-4 KO mice (about 75%). Tumour incidence in the Ras-expressing Par-4 KO lungs was significantly higher as compared with the Ras-expressing WT controls (Figure 2B). The lifespan was dramatically reduced in Ras-expressing Par-4 KO mice as compared with the other genotypes (Figure 2C). The loss of Par-4 also accelerated the progression of Ras-initiated lung adenocarcinomas, as the percentage of high-grade tumours was significantly higher in the Par-4 KO mice as compared with their corresponding WT controls (Figure 2D). Consistent with increased tumorigenesis in the Par-4 KO lungs, proliferation was also enhanced, as determined by increased Ki67 staining compared with WT controls, both in the normal alveolar tissue (Figure 2E,

Figure 1 Par-4 is highly expressed in the lung and is downregulated in human lung tumours. (A) Par-4 protein expression in normal mouse tissues. (B) Par-4 mRNA levels in normal mouse tissues. (C) Par-4 distribution in human and mouse tissues, determined by IHC. (D) Commercial tissue microarrays of NSCLC human samples ($n = 133$) were stained with anti-Par-4 antibody. Par-4 expression was analysed in adenocarcinoma and squamous cell carcinoma tumours compared with normal lung tissue. A representative example of positive and negative tumour samples and normal control for Par-4 staining is shown at two magnifications ($\times 2.5$ and $\times 20$). Scale bar = 50 µm.

upper panels) and in the tumours (Figure 2E, lower panels) of Ras-expressing lungs. We have reported previously *in vitro* downregulation of Par-4 in cultured, oncogenic Ras-expressing fibroblasts (Barradas *et al*, 1999). Surprisingly, the levels of Par-4 were similar in normal lung of WT mice and in cancer tissues of the oncogenic Ras-expressing mice (Figure 2F). Therefore, our observation that the loss of Par-4 dramatically enhances Ras tumorigenic potential in lung (Figure 2A and B) is consistent with: (1) oncogenic Ras expression not being sufficient to downregulate Par-4 *in vivo*; and (2) the ability of Par-4 inactivation in tumours to facilitate the development of Ras full-transforming potential. Globally, these data strongly suggest that Par-4 is a physiologically relevant negative regulator of lung tumorigenesis.

Signalling alterations in Par-4-deficient lungs

To gain understanding of the molecular and cellular mechanism(s) whereby Par-4 restrains lung tumorigenesis, we next

analysed the state of different signalling pathways in lungs from WT and Par-4-deficient mice under basal conditions. We have recently demonstrated in cell cultures of Par-4-deficient EFs, and in Par-4 KO prostates and uteri, that the loss of Par-4 produces enhanced NF- κ B activation, which leads to the increased expression of XIAP, a well-established anti-apoptotic downstream target of NF- κ B (Garcia-Cao *et al*, 2003, 2005). Consistent with this, our analysis of lung extracts from Par-4 KO mice revealed increased levels of XIAP as compared with lung extracts from WT controls (Figure 3A). Par-4 constitutively inhibits aPKCs, as demonstrated *in vivo* by increased phospho-PKC ζ staining in lung sections from Par-4 KO mice as compared with WT controls, both in the alveoli and in the airways (Supplementary Figure 1A). Consistently, PKC ζ activity in lung extracts is also increased in the Par-4 KO mice, as determined in an *in vitro* enzymatic assay in PKC ζ immunoprecipitates (Supplementary Figure 1B).

Figure 2 Par-4 cooperates with Ras-induced tumorigenesis in the lung. (A) H&E staining of lungs from WT and Par-4 KO mice crossed with K-ras^{+/V12}, RERT2^{T/T} mice and analysed 5 months after activation of the K-rasV12 allele by injection of 4-hydroxytamoxifen ($n = 5$ per genotype). Overall tumour burden was determined by quantification of the tumour area as a percentage of total area of H&E-stained tissue (right panel). (B) Number of total tumours per mouse in WT and Par-4 KO mice. (C) Survival of mice of different genotypes represented as percentage of total; the cause of death was asphyxiation. (D) Loss of Par-4 leads to increased Ras-induced high-grade lung adenocarcinomas. (E) Representative sections and quantification of proliferation index measured as percentage of positive nuclear staining for Ki67 in lung sections from normal alveolar or tumour tissue from WT and Par-4 KO Ras-expressing mice. (F) Par-4 expression levels in the lung from different mouse genotypes. **, $P < 0.01$; ****, $P < 0.0001$.

Figure 3 Increased levels of XIAP and activation of RelA and Akt in lungs of Par-4 KO mice. (A) The levels of XIAP, phospho-Akt-S473, Akt, Par-4 and actin were determined in lung extracts from WT and Par-4 KO mice. These are representative experiments where there were at least two others with similar results. (B, C) Sections of alveolar lung tissue from WT and Par-4 KO mice were stained by IHC with anti-RelA (B) or anti-phospho-Akt-S473 (C) antibody and scored for the number of cells with nuclear staining. Results are the mean $\pm$ s.d. of 10 different fields per mouse, with a total of five mice for each condition. Scale bar = 50 μ m. **, $P < 0.01$; ***, $P < 0.003$.

As PKC ζ is required for the nuclear translocation of NF- κ B in lung (Leitges *et al*, 2001), we next determined by IHC analysis whether the loss of Par-4 in the lung leads to enhanced nuclear levels of RelA in this tissue. The percentage of cells staining positive for nuclear RelA (also known as p65) was significantly increased in Par-4 KO lungs as compared with WT controls (Figure 3B). Therefore, the loss of Par-4 enhances basal NF- κ B activity in lungs, which most likely accounts for the increased XIAP levels (Figure 3A). These results are consistent with the increased proliferation observed in Par-4 KO lungs, as enhanced NF- κ B would result in higher cell survival during the oncogenic transformation process (Karin, 2006a,b). This observation provides *in vivo* confirmation of our previous observations on the effect of Par-4 deficiency in EFs, prostate and uterus (Garcia-Cao *et al*, 2003, 2005), and extends their relevance to the lung. When the activation of other signalling molecules related to cell survival and proliferation was determined in lung extracts, we observed that the lack of Par-4 correlated with increased Akt phosphorylation of Ser473 in lung extracts (Figure 3A). Ser473 phosphorylation is a bona fide marker of Akt

activation (Cantley, 2002), indicating that the Par-4 KO lungs have increased levels of Akt activity under basal conditions. These results were confirmed by IHC analysis of lung sections, which demonstrated increased phospho-Akt-Ser473 staining *in vivo* in Par-4 $^{-/-}$ lung alveolar (Figure 3C) and bronchiolar (not shown) cells, as compared with WT controls. Importantly, levels of phospho-Akt-Ser473 were also significantly augmented in Ras-expressing Par-4 KO tumours as compared with WT tumours (Figure 4A and B). Surprisingly, nuclear RelA levels were not detectable in Ras-expressing WT or Par-4 KO tumours (Figure 4C), although the Par-4 KO cells in those tumours showed enhanced cytosolic staining for RelA (Figure 4C). However, increased RelA nuclear translocation was observed in the Par-4 KO, oncogenic Ras-expressing alveolar normal and hyperplastic tissues as compared with WT controls (Supplementary Figure 2A). Phospho-Akt-Ser473 levels in the Par-4 KO mice were increased as compared with WT controls in all oncogenic Ras-expressing lung tissues analysed (Supplementary Figure 2B). These results suggest that increased levels of Akt activity, but probably not of NF- κ B, correlate with Par-4 KO-enhanced Ras

Figure 4 Increased activation of Akt but not RelA in Ras-expressing Par-4 KO lung tumours. Sections of lung tumours from WT and Par-4 KO mice expressing Ras were stained by IHC with anti-phospho-Akt-S473 (A) or anti-RelA (C) antibody. (B) Quantitation of cells showing positive nuclear staining for phospho-Akt-S473. Results are the mean $\pm$ s.d. of 10 different fields per mouse, with a total of five mice for each condition. Scale bar = 50 μ m. ***, $P < 0.001$.

tumorigenesis. These are important observations because they establish Par-4 as a novel critical negative regulator, in the lung, of two essential prosurvival cascades, NF- κ B and Akt. The mechanism whereby Par-4 regulates NF- κ B is well established and most likely involves PKC ζ (Leitges *et al*, 2001; Garcia-Cao *et al*, 2003). However, the results of this article unveil an unexpected regulatory role of Par-4 as a negative modulator of Akt that deserves further analysis.

Cell-autonomous inhibition of Akt by Par-4

To investigate how Par-4 influences Akt Ser473 phosphorylation, we first determined whether the upregulation of Akt activity by Par-4 deficiency is cell autonomous, and if it can be reverted in KO cells by ectopically reintroducing Par-4. To address these two important issues, we used anti-Par-4 and anti-XIAP antibodies to perform immunofluorescence analysis of EFs from WT and Par-4-deficient mice. Both types of fibroblasts were co-cultured on the same coverslips so that the staining conditions for the different types of cells (WT and KO) would be identical for the different antibodies. Results in Figure 5A (upper panel) show that KO cells (which are easily identified because they stain negatively for Par-4) give a much stronger signal for XIAP, consistent with Par-4 being a negative regulator of XIAP expression (Garcia-Cao *et al*, 2003, 2005). As the antibodies to detect phospho-Akt-Ser473 are not compatible with those that detect Par-4, but are compatible with those for XIAP, we assessed phospho-Akt levels in cells with elevated XIAP levels (thus identified as *Par-4*^{-/-}). The lower panel of Figure 5A shows a representative experiment. Interestingly, cells that were *Par-4*^{-/-} displayed higher phospho-Akt-Ser473 levels, demonstrating a clear inverse correlation between Par-4 and Akt activity. Furthermore, when EFs were made quiescent by serum starvation and subsequently activated by serum, the levels of phospho-Akt were more potently stimulated in the Par-4 KO cells than in the WT controls, in both time- and dose-dependent manners, and in both the cytoplasm and the nucleus (Figure 5B and C). Importantly, when Par-4 was re-expressed in KO EFs, the activation of Akt was severely compromised (Figure 5D). These results support the notion that Par-4 is a cell-autonomous negative regulator of Akt in the lung and EFs. To test whether this is also true in human cells, we used a Par-4 siRNA to deplete endogenous Par-4 levels in human 293 cells and in the A549 human lung adenocarcinoma cell line. Cells were treated with control or Par-4-specific siRNAs, after which they were kept for 24 h in serum-free medium conditions and then stimulated with serum. Data in Figure 5E and F clearly demonstrate that the knockdown of Par-4 provokes enhanced serum-activated phospho-Akt-Ser473 levels in A549 and 293 human cells, respectively. These data strongly indicate that the extent of phospho-Akt-Ser473 activation is linked to Par-4 levels in several cell systems, including human lung cancer cells.

Thr308 is also critical for Akt activity and function and is phosphorylated by PDK1 (Manning *et al*, 2002). To test whether the loss of Par-4 impacts not only Akt's Ser473 but also Thr308, we determined by immunoblotting with a specific anti-phospho-Akt-Thr308 antibody the levels of this phosphorylation in EFs from Par-4 KO and WT controls that have been either serum-starved or restimulated for 10 and 30 min with serum. We also tested in these extracts the phosphorylation levels of GSK3 β and FOXO3, two direct substrates of Akt. From the data of Figure 5G, it is apparent that the lack of Par-4 leads to increased levels of phospho-Akt-Thr308, phospho-GSK3 β and phospho-FOXO3. The enhanced phosphorylation of the two Akt substrates is consistent with the enhanced phosphorylation of Akt at its two critical residues and reflects increased Akt activity in the Par-4 KO cells.

The fact that Akt's Thr308 phosphorylation is also enhanced in the mutant cells is puzzling and suggests that Par-4

Figure 5 Par-4 deficiency induces increased nuclear phospho-Akt *in vivo*. (A) Confocal immunofluorescence on WT and Par-4 KO EFs seeded on the same coverslip, double-stained for Par-4 and XIAP (upper panels), or XIAP and phospho-Akt-S473 (lower panels). WT and Par-4 KO EFs stimulated with serum (FCS) for different durations or a dose-response for 15 min (C) in total extracts (C, D) or in nuclear and cytosol extracts (B). Reconstitution of Par-4 KO EFs with Par-4 restored phospho-Akt-S473 levels to basal levels (D). (E, F) A549 or 293 cells treated with a control siRNA or with Par-4-specific siRNA were stimulated with serum for different times and the levels of phospho-Akt-S473 were determined. Knockdown of Par-4 was confirmed by immunoblot. (G) WT and Par-4 KO EFs stimulated with serum were analysed by immunoblot for phospho-Akt-S473 and phospho-Akt-T308 levels as well as the Akt substrates, Gsk3 β -S9 and Foxo3-T32. (H) Knockdown of Akt blocks the increased cell proliferation induced by knockdown of Par-4 in A549 cells. Cell number was determined by trypan blue exclusion. Knockdown of Par-4 and Akt was analysed by immunoblot. These are representative experiments where there were at least two others with similar results.

might be controlling directly or indirectly the phosphorylation of both sites or that their phosphorylation might be interconnected. Akt activation lies downstream of PI 3-kinase whose stimulation can be detected by increased production of PIP₃ (Manning *et al*, 2002). A potential explanation for the fact that Akt phosphorylation at Ser473 and Thr308 is enhanced in Par-4 KO cells would be a similarly activated PI 3-kinase activity in the mutant cells. However, when PIP₃ levels were measured in Par-4 KO and WT cells, we did not detect an increase in Par-4 KO cells but, on the contrary, serum-induced PIP₃ levels were reduced in Par-4 KO cells. These results suggest that an increase in PI-3 kinase activity could not

account for the hyperactivation of Akt in Par-4-deficient cells, and that feedback loop mechanisms may take place in an attempt to control a deregulated and constitutive Akt activation in these cells (Supplementary Figure 3).

Collectively, these results demonstrate that Par-4 deficiency leads to the activation of the Akt pathway *in vivo* and in several cell systems, including human lung cancer cells, which may offer a biochemical explanation for the increased tumorigenicity observed in Par-4 KO lungs. This previously unrecognized link between Par-4 and Akt is functionally relevant as the knockdown of Akt significantly reduces the increased cell proliferation produced by the knockdown of Par-4 (Figure 5H).

Role of PKC ζ in the negative regulation of Akt by Par-4

As others and we have reproducibly detected a specific interaction between PKC ζ and Par-4 (Diaz-Meco *et al*, 1996; Goswami *et al*, 2005), which leads to the inhibition of PKC ζ activity *in vitro* and *in vivo* (Diaz-Meco *et al*, 1996), we reasoned that Par-4 regulation of Akt might be mediated by PKC ζ . Results of Supplementary Figure 1 demonstrate increased PKC ζ activation in Par-4 KO lung extracts, confirming and extending to an *in vivo* lung system our previous observations in EFs (Garcia-Cao *et al*, 2003). We thought it conceivable that PKC ζ could be an Akt kinase and that its regulation by Par-4 could account for the role of this tumour suppressor as a negative modulator of Akt activity *in vitro* and *in vivo*. To address this possibility, we first transfected 293 cells with Par-4 along with a plasmid control or a PKC ζ expression vector. We reasoned that if Par-4 reduces Akt's Ser473 phosphorylation through its ability to inhibit PKC ζ , the overexpression of PKC ζ should impair Par-4 inhibitory actions on Akt's Ser473 phosphorylation. The data in Figure 6A show that this is, indeed, the case. That is, the expression of Par-4 impairs phospho-Akt-Ser473 phosphorylation in serum-activated 293 cells, whereas the cotransfection of PKC ζ with Par-4 completely reverses that effect (Figure 6A), strongly suggesting the existence of a Par-4/PKC ζ /Akt cascade. The results of Figure 6B demonstrate that there is a specific and mostly constitutive interaction between Akt and PKC ζ . We next tested the hypothesis that Akt could be a direct substrate of PKC ζ . For this, we incubated recombinant pure PKC ζ with recombinant purified His-Akt *in vitro* in a kinase assay using γ P³²-ATP. As shown in Figure 6C, PKC ζ in addition to undergoing autophosphorylation was able to directly phosphorylate Akt, with a stoichiometry of 0.4 ± 0.1 moles of phosphate per mole of Akt. Part of the kinase reaction was analysed by immunoblotting and revealed that PKC ζ was capable to induce the phosphorylation of Akt's Ser473 and Thr308. Note that the anti-phospho-Thr308 antibody cross-reacts in this *in vitro* assay with activated PKC ζ , which is phosphorylated at Thr410, the equivalent to Akt's Thr308 in their activation loops (Figure 6C). These results demonstrate that PKC ζ is capable of directly phosphorylating Akt at both critical residues. To determine whether or not this is due to a hypothetical increase in autophosphorylation of Akt, we performed an *in vitro* kinase assay with recombinant pure PKC ζ and either WT Akt or a kinase-inactive (KD) version of that enzyme. Interestingly, PKC ζ phosphorylated both Akt forms with the same efficiency (Figure 6D). The same results were obtained when the levels of phospho-Akt-Thr308 were determined in a similar experiment (not shown). Phosphopeptide analysis of Akt phosphorylated with PKC ζ as shown in Figure 6C reveals three major peptides that, according to a theoretical map, correspond to phosphorylation of Ser124 (Figure 6E). This is consistent with the phospho-amino-acid analysis of these peptides that gives Ser as the predominantly phosphorylated amino acid. Mass-spectrometry analysis of PKC ζ -phosphorylated Akt demonstrates that Ser124, Ser473 and Thr308 were phosphorylated (Figure 6F), Ser124 phosphopeptide being the most abundantly detected. Analysis of these sites in untreated non-phosphorylated Akt by mass spectrometry revealed that Ser473 and Thr308 were both absent in the untreated control; however, we detected Ser124 phosphopeptide. To quantify the level of phosphorylation in Ser124 upon

PKC ζ incubation, we used the spectral total ion count (TIC) method, which is a label-free quantitation method that relies on spectral counting and TIC to calculate the relative intensities of phosphorylation (Asara *et al*, 2008). We observed a spectral TIC ratio of threefold for S124 phosphopeptide in PKC ζ -phosphorylated Akt compared with untreated Akt. Phosphorylation of Ser124 has previously been shown to be constitutive and to regulate the extent of Akt activation (Manning *et al*, 2002). These results could be interpreted as that the loss of Par-4 leads to increased PKC ζ activity, which by phosphorylation of Ser124 modulates the extent of Akt activation. However, the fact that PKC ζ directly phosphorylates Akt at Thr308 and Ser473 could suggest that it could have an important function on Akt activation. However, a more plausible explanation to these data is that, although PKC ζ phosphorylates Ser473 *in vitro*, it does it inefficiently *in vivo* in the presence of the TORC2 complex, its role being limited to phosphorylate Ser124, which nonetheless will impact the total Ser473 phosphorylation by the TORC2 complex. If this is the case, we predicted that the knockdown of Rictor should abolish Akt phosphorylation and Ser473 even in the absence of Par-4. Results of Figure 6G demonstrate that this is actually the case. Also, this model is consistent with the fact that Ser124 phosphopeptide was most abundantly detected in the mass-spectrometry analysis and in the phosphopeptide map of Figure 6E.

Collectively, these results indicate that PKC ζ is an upstream regulator of Akt activation by phosphorylating Ser124. To demonstrate that PKC ζ is actually required *in vivo* for the phosphorylation of Akt's Ser473 and Thr308, we analysed these parameters in EFs WT and KO for PKC ζ either untreated or stimulated with different doses of serum. Of relevance, the lack of PKC ζ severely impairs Akt phosphorylation at both critical residues (Figure 7A). The analysis of lung extracts from PKC ζ -deficient mice supports a critical role for this kinase in Akt activation *in vivo* (Figure 7B). To demonstrate that PKC ζ is actually required for Par-4 regulation of Akt phosphorylation at its two critical residues, we generated a Par-4/PKC ζ double KO (DKO) mice cell line. We then analysed the levels of phospho-Akt-Ser473 and phospho-Akt-Thr308 in lung extracts from WT, Par-4 KO and DKO mice. Data of Figure 7C show that the increased phosphorylation in these two residues observed in the Par-4 KO lung extracts is abolished in lung extracts of DKO mice. Also, FOXO3 phosphorylation that is increased in the Par-4 KO lung extracts is similarly inhibited in the DKO mice (Figure 7C). Similar results were obtained when phospho-Akt-Ser473 and phospho-Akt-Ser124 levels were analysed by IHC of lung sections of mice with different genotypes (Figure 7D and E).

Collectively, all these results establish the Par-4/PKC ζ cassette as an important regulator of Akt activation, most likely through the ability of PKC ζ to directly phosphorylate Akt's Ser124 and its subsequent impact in the phosphorylation of residues Ser473 and Thr308, which are critical for the regulation of Akt activity and function.

Discussion

The balance between oncogenic and tumor-suppressive signals is central to the control of tumour initiation and progression (Hanahan and Weinberg, 2000). Our laboratory identified *Par-4*, a gene previously discovered in a screen

Figure 6 PKC ζ directly interacts and phosphorylates Akt. (A) Overexpression of Par-4 in 293 cells inhibited serum-induced phospho-Akt-S473 phosphorylation and was reversed by PKC ζ co-expression. (B) Serum-stimulated 293 cells extracts were immunoprecipitated with anti-Akt or IgG control antibody and the immunoprecipitates were analysed by immunoblot with anti-PKC ζ or anti-Akt, as a loading control. (C) *In vitro* phosphorylation of recombinant Akt by recombinant PKC ζ with γ P³²-ATP. Part of the assay reaction was also immunoblotted with anti-phospho-Akt-Ser473, anti-phospho-Akt-T308 and anti-Akt antibodies. (D) Immunoprecipitates of WT or KD Akt were phosphorylated *in vitro* by PKC ζ and phospho-Akt-Ser473 levels were determined. (E) Phosphopeptide map of *in vitro* PKC ζ phosphorylated-Akt (left panel). Phospho-amino-acid analysis of peptides A, B, C and the total reaction. (F) MS/MS spectra of the identified phosphopeptides corresponding to sites S124, S473 and T308 phosphorylated in Akt by PKC ζ . (G) A549 cells treated with a control siRNA, Par-4-siRNA, Rictor-siRNA or both siRNAs were stimulated with serum and the levels of phospho-Akt-S473 were determined. Knockdown of Par-4 and Rictor was confirmed by immunoblot. These are representative experiments where there were at least two others with similar results.

for genes upregulated in cells undergoing apoptosis (Sells *et al*, 1994), as a critical negative regulator of aPKCs (Diaz-Meco *et al*, 1996). In overexpression experiments, Par-4 has also been reported to interact with other potential partners (Johnstone *et al*, 1996; Page *et al*, 1999; Kawai *et al*, 2003; Roussigne *et al*, 2003), but whether these interactions actually have an important function *in vivo* is still unclear. Subsequent studies demonstrated, *in vivo* and under physiological conditions, that PKC ζ and Par-4 KO mice display opposite phenotypes (Moscat and Diaz-Meco, 2003; Moscat *et al*, 2006), lending support to the notion that Par-4 is a

negative regulator of PKC ζ . In addition, from those studies, it is clear that the Par-4/PKC ζ cassette is relevant for NF- κ B activation during cell survival (Garcia-Cao *et al*, 2003; Duran *et al*, 2004).

Par-4 is reduced in a significant number of endometrial (Moreno-Bueno *et al*, 2007), prostate (our unpublished observations) and lung (this study) human carcinomas. Furthermore, loss of Par-4 in mice leads to the spontaneous appearance of PIN and endometrial tumours (Garcia-Cao *et al*, 2005), and the expression of oncogenic Ras in fibroblast cultures leads to a significant reduction in Par-4 levels

Figure 7 Activation of Akt by Par-4 deficiency is dependent on PKC ζ *in vivo*. (A) WT and PKC ζ KO EFs stimulated with serum were analysed by immunoblot for phospho-Akt-S473 and phospho-Akt-T308 levels. (B) Lung extracts from WT and PKC ζ KO were immunoblotted with XIAP, phospho-Akt-S473 and phospho-Akt-T308 antibodies. (C–E) Phosphorylation of Akt in Par-4 KO is reverted in DKO (Par-4/PKC ζ). Phosphorylation of Akt and its substrate FOXO3 were determined in lung extracts of the different KO mice (C). Lung sections were stained for phospho-Akt-S473. Quantitation of cells showing positive nuclear staining for phospho-Akt-S473 (D). Results are the mean $\pm$ s.d. of 10 different fields per mouse with a total of five mice for each condition. (E) Lung sections were stained for phospho-Akt-S124. Negative control was performed on Par-4 KO sample with no primary antibody. These are representative experiments where there were at least two others with similar results. Scale = 50 μ m.

(Barradas *et al*, 1999). All these studies suggest that Par-4 exerts tumour-suppressive effects in human cancer and in cell culture systems, although its ability to restrain oncogenesis in physiologically relevant model systems has not been demonstrated yet. The data shown here demonstrate that, although Par-4 levels are reduced in fibroblast cell cultures expressing oncogenic Ras (Barradas *et al*, 1999), Par-4 levels remain unaltered in normal and tumour-derived lung tissues from mice expressing the oncogenic form of Ras. This suggests that, *in vivo*, inactivating mutations or deletions of Par-4 could be instrumental for Ras-induced tumour transformation. Here we addressed this important question by determin-

ing the ability of Ras to induce lung adenocarcinomas in Par-4 KO mice. The rationale for this experiment is that if Par-4's role is to suppress tumorigenicity, Ras-induced lung cancer would be enhanced by the lack of Par-4. The data shown here clearly support that hypothesis in that the tumour burden induced by Ras was significantly higher in Par-4 KO lungs than in the WT controls. Also, the loss of Par-4 resulted in a higher percentage of Ras-induced high-grade lung adenocarcinomas, indicating that the absence of Par-4 promotes lung tumour progression. These are important observations that, coupled with our data that Par-4 is absent in a significant proportion of human NSCLC, mostly in adenocarcinomas,

strongly suggest that Par-4 is a relevant tumour suppressor in lung cancer.

From a mechanistic point of view, the data presented here unveil an unexpected and important role of Par-4 as a negative regulator of Akt that exerts an effect by controlling the phosphorylation of Akt's Ser473 and Thr308, two critical events in the activity of this kinase (Cantley, 2002). Our findings have to be put in the context of other observations demonstrating that the proto-oncogene Akt is activated in many human cancers, mostly due to loss of the *PTEN* tumour suppressor. Interestingly, in tumorigenesis, *PTEN* synergizes with a network of other tumour suppressors, all of them modulating Akt activation through different molecular mechanisms. For example, the tumour suppressor PML cooperates with *PTEN* inside the nucleus through the recruitment of Akt itself and the phosphatase PP2a into the PML nuclear bodies. This leads to the efficient inactivation of Akt by dephosphorylation (Trotman *et al*, 2006). On the other hand, the tumour suppressor NEP inhibits Akt by direct interaction with, and stabilization of, *PTEN* (Sumitomo *et al*, 2004). We show here that the effect of Par-4 on Akt activation is cell autonomous and is observed not only in mouse tissues and EFs, but also in human lung adenocarcinoma cells, thus supporting its physiological relevance. In this study, we confirm and extend our views through genetic evidence gathered in both *in vivo* and *in vitro* studies that Par-4 not only impinges on the PKC ζ signalling pathway leading to NF- κ B activation (Leitges *et al*, 2001; Garcia-Cao *et al*, 2003; Duran *et al*, 2004), but also on Akt activity. Akt Thr308 phosphorylation is mediated by PDK1 and activated as a consequence of the stimulation of PI 3-kinase (Manning *et al*, 2002). The kinase responsible for Akt phosphorylation at Ser473 has been the subject of intense research. The Sabatini laboratory has found that a Rictor-containing mTOR complex is responsible for this phosphorylation (Sarbasov *et al*, 2005). However, recent data from Tarakhovskiy's laboratory show that a PKC isoform, PKC β , accounts for Ser473 phosphorylation *in vivo*, in at least B cells (Patke *et al*, 2006). We show here that PKC ζ overexpression suppresses Par-4's inhibitory effect on Akt phosphorylation at residues Ser473 and Thr308, as well as that both phosphorylation events are dramatically reduced in the lungs of PKC ζ KO mice. We also demonstrate that the enhanced Akt activation produced by Par-4 ablation is almost completely reduced to WT levels in Par-4/PKC ζ DKO lungs and cells. Interestingly, *in vitro* assays demonstrate that PKC ζ directly interacts and phosphorylates Akt, supporting the existence of a Par-4/PKC ζ cassette that directly targets Akt. Mass-spectrometric, peptide mapping, immunoblotting and phospho-amino-acid analyses of the PKC ζ -phosphorylated Akt reveal that although recombinant pure PKC ζ directly phosphorylates WT and kinase-dead Akt at Ser473, it also targets Thr308 and to a larger extent Ser124. The fact that PKC ζ can directly phosphorylate Ser473 would be consistent with previous observations that PKC β can also fulfil such a role (Patke *et al*, 2006). However, our data that inactivation of the TORC2 complex by the knockdown of Rictor in the Par-4 KO cells totally abolishes Akt's Ser473 phosphorylation clearly indicate that PKC ζ cannot be the most relevant regulator of Ser473 *in vivo* in Par-4-inactivated cells and that TORC2 is essential also for that function. Interestingly, our experiments indicate that the Akt residue more abundantly phosphorylated by PKC ζ is

Ser124. This is very important because this residue along with Thr450 is constitutively phosphorylated and is necessary for an efficient phosphorylation of Ser473 and Thr308 and the subsequent full activation of Akt (Alessi *et al*, 1996; Bellacosa *et al*, 1998; Conus *et al*, 2002). These are relevant findings because the identity of the Ser124 and Thr450 kinases remained unknown. Previous data suggest that JNK is the Thr450 kinase (Shao *et al*, 2006). The results presented here establish that the Ser124 kinase is PKC ζ .

Collectively considered, these results lead us to propose a model whereby the Par-4/PKC ζ complex modulates the extent of Akt activation by directly targeting Ser124. This is relevant, as overactivation of Akt in Par-4-deficient cells could be an important contributor to the pro-neoplastic phenotype of Par-4-deficient mice. In this regard, and taking into account the essential role of Akt in Ras transformation (Malumbres and Barbacid, 2003), our data suggest that the mechanism whereby the loss of Par-4 enhances the Ras-transforming potential in lung cancer *in vivo* will likely be accounted for by increased Akt activation.

Materials and methods

Mice

Par-4 KO and PKC ζ KO mice were described previously (Leitges *et al*, 2001; Garcia-Cao *et al*, 2003). Par-4 KO and PKC ζ KO were crossed to generate DKO. K-ras^{+/V12}, RERT2n^{T/T} mice (Guerra *et al*, 2003) were crossed with Par-4 KO mice. The KRasV12 allele was activated when mice were 1 month old by a single intraperitoneal injection of 500 μ g of 4-hydroxytamoxifen dissolved and sonicated in olive oil. All mice were born and kept under pathogen-free conditions. Animal-handling protocols conform to institutional guidelines. All genotyping was done by PCR.

Cell culture

Wild-type, Par-4 KO and PKC ζ KO primary EFs were derived from E13.5 embryos (Garcia-Cao *et al*, 2003). Cells were maintained in DMEM (Gibco BRL) supplemented with 10% (v/v) fetal calf serum (FCS), 1% glutamine and 1% penicillin/streptomycin (Gibco-Invitrogen) in an atmosphere of 95% air and 5% CO₂, and immortalized by retroviral infection with pBabeT-Ag followed by puromycin selection (1 μ g/ml). The established cell lines represent pools of at least 100 independent clones. The HEK293-derived virus-packaging cell line 293T, and 293 and A549 cells were cultured in DMEM with 10% FCS. For knockdown of Par-4 and Rictor, two different human siRNAs were obtained from Qiagen with the following target sequences: Par-4: AAGTGGGTTCCTAGATATAA and CACAGCCGTTTGAATATATT; Rictor: AAACAAGGCTGTGATATTCTA and AAAGACTACAGCAACAAGAA; Akt: AAGCACCTTCATTGGCTACAA and AAGGAGGGTTGGCTGCACAAA.

Similar results were obtained with both siRNA sequences. AllStars Negative Control siRNA (Qiagen) was used as the negative control. Transfection of siRNAi was performed by the calcium phosphate method. For Par-4 reconstitution experiments, the retroviral expression vector pWZL-Par-4 was used. Retroviruses were produced in 293T cells by transient transfection using Lipofectamine 2000 (Invitrogen). Culture supernatants were collected 24, 48 and 72 h post-transfection, filtered (0.45 μ m) and then supplemented with 4 μ g/ml polybrene. EFs were infected with three rounds of viral supernatants and selected with hygromycin (75 μ g/ml). The following expression plasmids were used: CMV-6-hPar-4, pCDNA3-HA-PKC ζ , pSG-HA-Akt and pSG-HA-Akt-KD. PIP₃ levels were measured by ELISA, using PIP₃ ELISA kit (Echelon).

Confocal analysis

Wild-type and Par-4 KO EFs seeded on coverslips were fixed with 4% formaldehyde and permeabilized with 0.1% Triton X-100. Free aldehyde groups were quenched with 50 mM NH₄Cl. Endogenous peroxidase activity was quenched by treatment with 3% H₂O₂ in methanol for 15 min. Fixed cells were treated with blocking

solution. Cells were incubated with monoclonal anti-XIAP and polyclonal anti-Par-4 or anti-phospho-Akt for 1 h at 37 °C. Anti-rabbit-Alexa-594 and anti-mouse-Alexa-488 were used as secondary antibodies. Glass cover slips were mounted on Mowiol and examined with a Zeiss LSM 510 Meta confocal system.

Western blotting

Cell extracts for western blot were prepared in RIPA buffer (1 × PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenyl methyl sulphonyl fluoride and protease inhibitors). Lysates were separated by SDS-PAGE and transferred to nitrocellulose-ECL membranes (GE Healthcare) and the immune complex was detected by chemiluminescence (GE Healthcare). The following primary antibodies were used for western blot: phospho-Akt-S473, phospho-Akt-T308, phospho-FOXO3-T32, phospho-GSK3β-S9, PKCζ and phospho-PKCζ-T410/403 from Cell Signalling; Akt, actin, HA and Par-4 antibodies from Santa Cruz, and XIAP from BD. All antibodies were used according to the manufacturers' instructions.

References

- Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA (1996) Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* **15**: 6541–6551
- Asara JM, Christofk HR, Freemark LM, Cantley LC (2008) A label-free quantification method by MS/MS TIC compared to SILAC and spectral counting in a proteomics screen. *Proteomics* **8**: 994–999
- Barradas M, Monjas A, Diaz-Meco MT, Serrano M, Moscat J (1999) The downregulation of the pro-apoptotic protein Par-4 is critical for Ras-induced survival and tumor progression. *EMBO J* **18**: 6362–6369
- Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng J, Tsichlis P (1998) Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* **17**: 313–325
- Bhaskar PT, Hay N (2007) The Two TORCs and Akt. *Dev Cell* **12**: 487–502
- Bos JL (1989) Ras oncogenes in human cancer: a review. *Cancer Res* **49**: 4682–4689
- Cantley LC (2002) The phosphoinositide 3-kinase pathway. *Science* **296**: 1655–1657
- Conus NM, Hannan KM, Cristiano BE, Hemmings BA, Pearson RB (2002) Direct identification of tyrosine 474 as a regulatory phosphorylation site for the Akt protein kinase. *J Biol Chem* **277**: 38021–38028
- Diaz-Meco MT, Municio MM, Frutos S, Sanchez P, Lozano J, Sanz L, Moscat J (1996) The product of par-4, a gene induced during apoptosis, interacts selectively with the atypical isoforms of protein kinase C. *Cell* **86**: 777–786
- Downward J (2003) Targeting RAS signalling pathways in cancer therapy. *Nat Rev Cancer* **3**: 11–22
- Duran A, Rodriguez A, Martin P, Serrano M, Flores JM, Leitges M, Diaz-Meco MT, Moscat J (2004) Crosstalk between PKCζeta and the IL4/Stat6 pathway during T-cell-mediated hepatitis. *EMBO J* **23**: 4595–4605
- Fisher GH, Wellen SL, Klimstra D, Lenczowski JM, Tichelaar JW, Lizak MJ, Whitsett JA, Koretsky A, Varmus HE (2001) Induction and apoptotic regression of lung adenocarcinomas by regulation of a K-Ras transgene in the presence and absence of tumor suppressor genes. *Genes Dev* **15**: 3249–3262
- Garcia-Cao I, Duran A, Collado M, Carrascosa MJ, Martin-Caballero J, Flores JM, Diaz-Meco MT, Moscat J, Serrano M (2005) Tumour-suppression activity of the proapoptotic regulator Par4. *EMBO Rep* **6**: 577–583
- Garcia-Cao I, Lafuente M, Criado L, Diaz-Meco M, Serrano M, Moscat J (2003) Genetic inactivation of Par4 results in hyperactivation of NF-κB and impairment of JNK and p38. *EMBO Rep* **4**: 307–312
- Goswami A, Burikhanov R, de Thonel A, Fujita N, Goswami M, Zhao Y, Eriksson JE, Tsuruo T, Rangnekar VM (2005) Binding and phosphorylation of par-4 by akt is essential for cancer cell survival. *Mol Cell* **20**: 33–44
- Guerra C, Mijimolle N, Dhawahir A, Dubus P, Barradas M, Serrano M, Campuzano V, Barbacid M (2003) Tumor induction by an

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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- endogenous K-ras oncogene is highly dependent on cellular context. *Cancer Cell* **4**: 111–120
- Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**: 57–70
- Johnstone RW, See RH, Sells SF, Wang J, Muthukkumar S, Englert C, Haber DA, Licht JD, Sugrue SP, Roberts T, Rangnekar VM, Shi Y (1996) A novel repressor, par-4, modulates transcription and growth suppression functions of the Wilms' tumor suppressor WT1. *Mol Cell Biol* **16**: 6945–6956
- Johnstone RW, Tommerup N, Hansen C, Vissing H, Shi Y (1998) Mapping of the human PAWR (par-4) gene to chromosome 12q21. *Genomics* **53**: 241–243
- Karin M (2006a) NF-kappaB and cancer: mechanisms and targets. *Mol Carcinog* **45**: 355–361
- Karin M (2006b) Nuclear factor-[kappa]B in cancer development and progression. *Nature* **441**: 431–436
- Kawai T, Akira S, Reed JC (2003) ZIP kinase triggers apoptosis from nuclear PML oncogenic domains. *Mol Cell Biol* **23**: 6174–6186
- Lafuente MJ, Martin P, Garcia-Cao I, Diaz-Meco MT, Serrano M, Moscat J (2003) Regulation of mature T lymphocyte proliferation and differentiation by Par-4. *EMBO J* **22**: 4689–4698
- Leitges M, Sanz L, Martin P, Duran A, Braun U, Garcia JF, Camacho F, Diaz-Meco MT, Rennert PD, Moscat J (2001) Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. *Mol Cell* **8**: 771–780
- Malumbres M, Barbacid M (2003) RAS oncogenes: the first 30 years. *Nat Rev Cancer* **3**: 459–465
- Manning BD, Cantley LC (2007) AKT/PKB signaling: navigating downstream. *Cell* **129**: 1261–1274
- Manning BD, Tee AR, Logsdon MN, Blenis J, Cantley LC (2002) Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberlin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol Cell* **10**: 151–162
- Meuwissen R, Berns A (2005) Mouse models for human lung cancer. *Genes Dev* **19**: 643–664
- Moreno-Bueno G, Fernandez-Marcos PJ, Collado M, Tendaro MJ, Rodriguez-Pinilla SM, Garcia-Cao I, Hardisson D, Diaz-Meco MT, Moscat J, Serrano M, Palacios J (2007) Inactivation of the candidate tumor suppressor par-4 in endometrial cancer. *Cancer Res* **67**: 1927–1934
- Moscat J, Diaz-Meco MT (2003) Par-4 keeps the atypical PKCs at bay. *Cell Cycle* **2**: 71–72
- Moscat J, Rennert P, Diaz-Meco MT (2006) PKCζeta at the crossroad of NF-kappaB and Jak1/Stat6 signaling pathways. *Cell Death Differ* **13**: 702–711
- Page G, Kogel D, Rangnekar V, Scheidtmann KH (1999) Interaction partners of Dlk/ZIP kinase: co-expression of Dlk/ZIP kinase and Par-4 results in cytoplasmic retention and apoptosis. *Oncogene* **18**: 7265–7273
- Patke A, Mecklenbrauker I, Erdjument-Bromage H, Tempst P, Tarakhovsky A (2006) BAFF controls B cell metabolic fitness through a PKC beta- and Akt-dependent mechanism. *J Exp Med* **203**: 2551–2562

- Roussigne M, Cayrol C, Clouaire T, Amalric F, Girard JP (2003) THAP1 is a nuclear proapoptotic factor that links prostate-apoptosis-response-4 (Par-4) to PML nuclear bodies. *Oncogene* **22**: 2432–2442
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM (2005) Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science* **307**: 1098–1101
- Sells SF, Wood Jr DP, Joshi-Barve SS, Muthukumar S, Jacob RJ, Crist SA, Humphreys S, Rangnekar VM (1994) Commonality of the gene programs induced by effectors of apoptosis in androgen-dependent and -independent prostate cells. *Cell Growth Differ* **5**: 457–466
- Shao Z, Bhattacharya K, Hsich E, Park L, Walters B, Germann U, Wang YM, Kyriakis J, Mohanlal R, Kuida K, Namchuk M, Salituro F, Yao YM, Hou WM, Chen X, Aronovitz M, Tsichlis PN, Bhattacharya S, Force T, Kilter H (2006) c-Jun N-terminal kinases mediate reactivation of Akt and cardiomyocyte survival after hypoxic injury *in vitro* and *in vivo*. *Circ Res* **98**: 111–118
- Sumitomo M, Iwase A, Zheng R, Navarro D, Kaminetzky D, Shen R, Georgescu MM, Nanus DM (2004) Synergy in tumor suppression by direct interaction of neutral endopeptidase with PTEN. *Cancer Cell* **5**: 67–78
- Trotman LC, Alimonti A, Scaglioni PP, Koutcher JA, Cordon-Cardo C, Pandolfi PP (2006) Identification of a tumour suppressor network opposing nuclear Akt function. *Nature* **441**: 523–527
- Tuveson DA, Shaw AT, Willis NA, Silver DP, Jackson EL, Chang S, Mercer KL, Grochow R, Hock H, Crowley D, Hingorani SR, Zaks T, King C, Jacobetz MA, Wang L, Bronson RT, Orkin SH, DePinho RA, Jacks T (2004) Endogenous oncogenic K-ras(G12D) stimulates proliferation and widespread neoplastic and developmental defects. *Cancer Cell* **5**: 375–387