DJ-1, a novel regulator of the tumor suppressor PTEN

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Summary

The phosphatidylinositol 3′ kinase (PI3′K) pathway, which regulates cell survival, is antagonized by the PTEN tumor suppressor. The regulation of PTEN is unclear. A genetic screen of *Drosophila* gain-of-function mutants identified DJ-1 as a suppressor of PTEN function. In mammalian cells, DJ-1 underexpression results in decreased phosphorylation of PKB/Akt, while DJ-1 overexpression leads to hyperphosphorylation of PKB/Akt and increased cell survival. In primary breast cancer samples, DJ-1 expression correlates negatively with PTEN immunoreactivity and positively with PKB/Akt hyperphosphorylation. In 19/23 primary non-small cell lung carcinoma samples, DJ-1 expression was increased compared to paired nonneoplastic lung tissue, and correlated positively with relapse incidence. DJ-1 is thus a key negative regulator of PTEN that may be a useful prognostic marker for cancer.

Introduction

Cells stimulated by growth factors such as epidermal growth factor (EGF) activate the phosphatidylinositol-3 kinase (PI3′K) signaling pathway (Burgering and Coffer, 1995). Activated PI3′K catalyzes the phosphorylation of phosphatidylinositol (4,5)-bisphosphate (PIP2) to phosphatidylinositol (3,4,5)-trisphosphate (PIP3) (Fruman et al., 1998). PIP3 in turn recruits PIP3-dependent kinase (PDK), which phosphorylates and activates the survival kinase, protein kinase B (PKB/Akt) (Alessi et al., 1997; Stokoe et al., 1997). The PI3′K pathway is negatively regulated by the dephosphorylation of PIP3 mediated by the lipid phosphatase activity of the tumor suppressor PTEN (Maehama and Dixon, 1998; Myers et al., 1998; Stambolic et al., 1998; Sun et al., 1999).

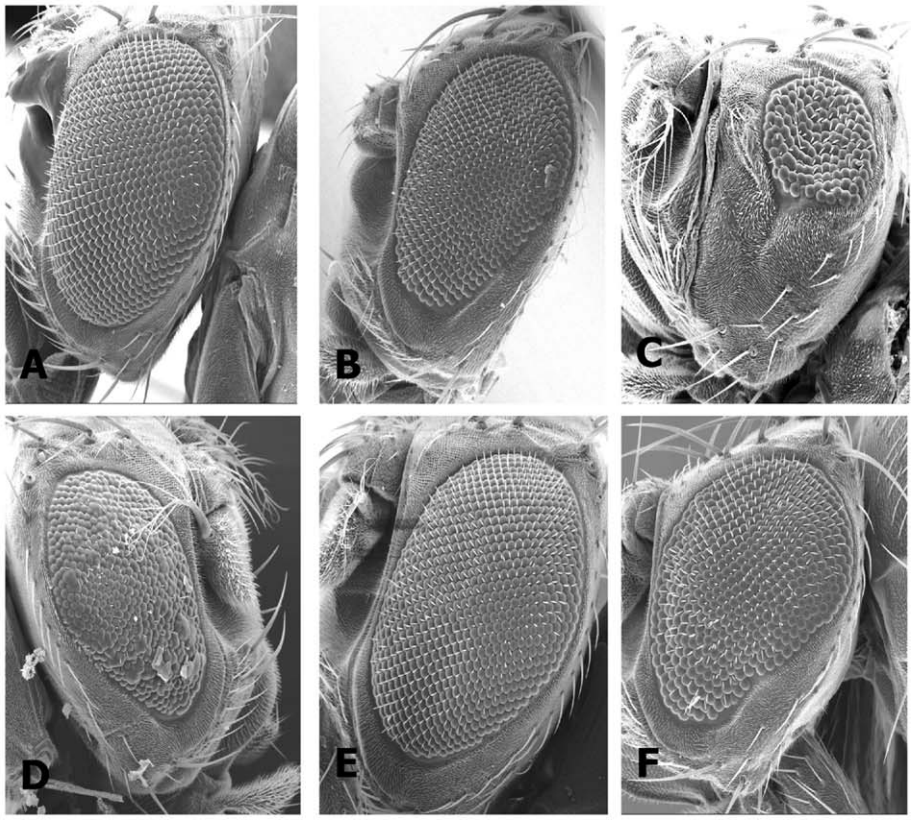
PTEN (MMAC/TEP1) is one of the most frequently mutated tumor suppressor genes in human cancer (Cantley and Neel, 1999). Germline mutations of PTEN result in Cowden syndrome (Liaw et al., 1997), Bannyan-Zonana syndrome (Marsh et al., 1997), or Lhermitte-Duclos disease (Iida et al., 1998), disorders characterized by the development of benign hamartomas and an increased risk of thyroid, breast, uterine, and skin neoplasms (Eng and Peacocke, 1998). Pten$^{+/-}$ mice are highly susceptible to tumor development in various tissues (Di Cristofano et al., 1998; Podsypanina et al., 1999; Stambolic et al., 2000; Suzuki et al., 1998). Fibroblasts from Pten$^{-/-}$ mice are resistant to various apoptotic stimuli and exhibit hyperphosphorylation and activation of PKB/Akt (Stambolic et al., 1998; Sun et al., 1999). These data indicate that PTEN exerts its tumor suppressor activity by inhibiting PKB/Akt-mediated cell survival.

The PI3′K, PKB/Akt, and PTEN signaling pathways are conserved throughout evolution (Bohni et al., 1999; Cassada and Russell, 1975; Gottlieb and Ruvkun, 1994), a fact that allowed us to use a *Drosophila melanogaster* model system to study PTEN regulation. We identified genes with modulatory effects on PTEN signaling using a gain-of-function screen based on the UAS-GAL4 system (Brand and Perrimon, 1993). One of the genes that emerged from this screen encoded the *Drosophila* homolog of mammalian DJ-1.

DJ-1 (CAP1/RS/PARK7) was originally cloned as a putative oncogene capable of transforming NIH-3T3 cells in cooperation with H-*ras* (Nagakubo et al., 1997), a protein expressed in sperm (Wagenfeld et al., 1998), and a regulator of RNA-protein interactions (Hod et al., 1999). DJ-1 has also been isolated as a gene associated with autosomal early-onset Parkinson's disease (PD) (Bonifati et al., 2003b). To date, mutations of the DJ-1 locus, such as a deletion of the first five exons (Bonifati et al.,

SIGNIFICANCE

Our work is the first evidence that DJ-1 functions in the PI3′K survival pathway as a negative regulator of PTEN-mediated tumor suppression. Breast cancer samples with high levels of PKB/Akt phosphorylation also show elevated DJ-1 expression, and lung cancer patients with high levels of DJ-1 have a poor prognosis. DJ-1 may thus be useful as a prognostic marker for cancer patients, and may represent a valuable new target for cancer therapy.



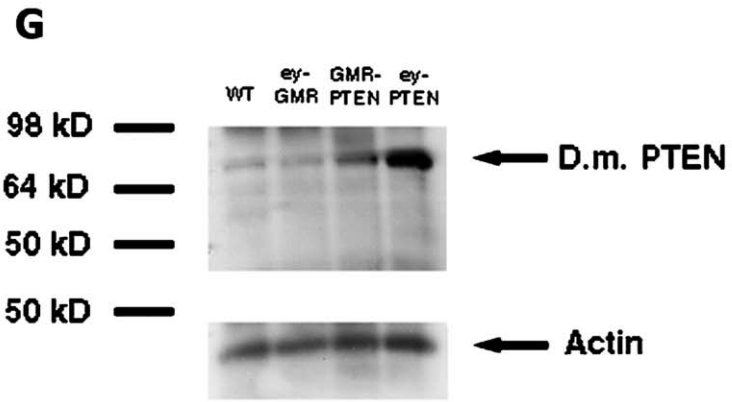


Figure 1. Interaction between PTEN and DJ-1 in the *Drosophila* eye

A–C: Scanning electron micrographs (SEMs) showing the normal eye disc of an adult WT fly (**A**), and the smaller, rougher eye discs of flies overexpressing dPTEN controlled by either the GMR promoter (**B**) or the eyeless promoter (**C**).

D: SEM of the eye of progeny of a cross of GMR-PTEN flies to P element line l(2)k04204. Increased roughness and a slight decrease in eye size are observed.

E: SEM of fly eye resulting from overexpression of human DJ-1 controlled by the eyeless promoter (ey-GAL4/UAS-DJ-1). No gross eye abnormalities are apparent.

F: SEM of fly eye resulting from coexpression of PTEN and DJ-1 controlled by the eyeless promoter (ey-GAL4-UAS-dPTEN/UAS-DJ-1). The PTEN-induced phenotype is almost completely rescued.

G: Western blot of endogenous or transgenic expression of dPTEN in 3rd instar larvae of WT flies or the indicated transgenic flies.

2003b), a mutation (L166P) which occurs in the C-terminal helix of DJ-1 (Bonifati et al., 2003b; Tao and Tong, 2003), and mutations in exon 1, intron 4, and exon 5 (Tan et al., 2004), have been identified in various PD patients.

X-ray crystallographic examination of DJ-1 structure indicates that it exists as a dimer (Tao and Tong, 2003) containing domains found in heat shock protein chaperones (Lee et al., 2003; Quigley et al., 2003) and ThiJ/PfpI proteases (Honbou et al., 2003; Huai et al., 2003; Lee et al., 2003; Tao and Tong, 2003; Wilson et al., 2003). Functionally, DJ-1 has been implicated in fertilization (Okada et al., 2002; Wagenfeld et al., 2000; Yoshida et al., 2003), the regulation of androgen receptor signaling (Niki et al., 2003; Takahashi et al., 2001), and oxidative stress (Canet-Aviles et al., 2004; Mitsumoto and Nakagawa, 2001; Mitsumoto et al., 2001; Takahashi-Niki et al., 2004). In addition, DJ-1 may be involved in apoptosis induced by TRAIL (Hod, 2004).

Several lines of evidence suggest that DJ-1 plays a role in human tumorigenesis. First, breast cancer patients have elevated levels of circulating DJ-1 and anti-DJ-1 autoantibodies compared to healthy and non-breast cancer patients (Le Naour et al., 2001). Secondly, DJ-1 protein is increased in primary non-small cell lung carcinoma samples (MacKeigan et al., 2003). Thirdly, treatment of cells from the human lung cancer cell line NCI-H157 with paclitaxel and MEK inhibitor U0126 leads to a decrease in DJ-1 protein expression (MacKeigan et

al., 2003). Nevertheless, despite these many studies, the physiological role of DJ-1 is largely unknown. Here we demonstrate that DJ-1 modulates the PI3′K survival pathway by negatively regulating the function of the tumor suppressor gene PTEN.

Results and discussion

DJ-1 is an antagonist of PTEN function in the *Drosophila* eye

In *Drosophila*, overexpression of dPTEN results in decreased eye size due to a reduction in both cell size and cell proliferation (Gao et al., 2000; Goberdhan et al., 1999; Huang et al., 1999). In our genetic screen, GMR-GAL4-mediated expression of dPTEN (GMR-GAL4/UAS-dPTEN) in the eye imaginal disc resulted in a 30% decrease in eye size compared to controls (compare Figures 1A and 1B). Higher levels of dPTEN expression directed by the stronger eyeless promoter (ey-GAL4/UAS-dPTEN) caused an 80% reduction in eye size (Figure 1C). The expression levels of dPTEN in wild-type (WT) flies and overexpressing mutants were confirmed by Western blotting of larval protein lysates (Figure 1G). While dPTEN was more markedly enhanced in ey-GAL4/UAS-dPTEN flies than in GMR-GAL4/UAS-dPTEN flies, the former exhibited high lethality such that we used the latter for the genetic screen. GMR-GAL4/UAS-dPTEN flies were crossed to a collection of 1145 P element lines, each harboring a heterozygous deletion of a single gene via P element insertion (Spradling et al., 1999). F1 progeny from these crosses were generated, and the size and morphology of their eyes were compared to those of the parental GMR-GAL4/UAS-dPTEN flies. In this way, individual genes disrupted by P element mutations were evaluated for their ability to modify the phenotype caused by dPTEN overexpression.

When GMR-GAL4/UAS-dPTEN was crossed to P element l(2)k04204, a smaller, rougher eye was observed (Figure 1D; compare with 1B), indicating that the gene mutated by this P element was an antagonist of PTEN function. The gene disrupted in P element line l(2)k04204 was identified as the *Drosophila* homolog of DJ-1. While no abnormal eye phenotype was observed when human DJ-1 was overexpressed in flies using the eyeless promoter (ey-GAL4/UAS-DJ-1) (Figure 1E), overexpression of human DJ-1 cDNA in the germline driven by the armadillo promoter (Vincent et al., 1994) rescued the homozygous lethality of the P element line (data not shown), confirming the identity of the P element mutation. To investigate the genetic interaction between DJ-1 and PTEN in *Drosophila*, ey-GAL4/UAS-DJ-1 flies were crossed to ey-GAL4/UAS-dPTEN flies. Coexpression of PTEN and DJ-1 resulted in suppression of the dPTEN-induced eye phenotype (Figure 1F; compare with 1C). These results suggest that DJ-1 functions as an antagonist of dPTEN suppressor gene function in the fly eye.

DJ-1 rescues PTEN-induced cell death and increases the expression of PI3′K downstream effectors

Transient transfection of human PTEN cDNA in NIH-3T3 fibroblasts results in decreased cell viability, indicating that the suppression of cell survival caused by PTEN is sufficient to induce cell death (Stambolic et al., 1998). To evaluate the ability of DJ-1 to alter PTEN-induced cell death, we cotransfected NIH-3T3 cells with human PTEN and DJ-1 and assessed the viability of these cells using a β-galactosidase staining method. As

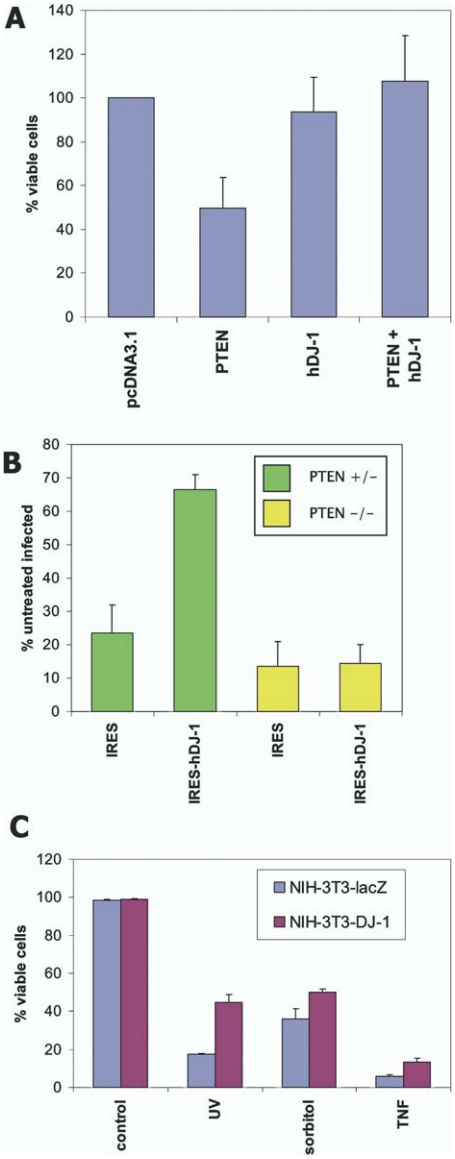


Figure 2. DJ-1 rescues PTEN-induced cell death

A: Restoration of viability. NIH-3T3 cells were transfected with constructs expressing the indicated proteins in the presence of trace amounts of a β-galactosidase expression construct. At 48 hr posttransfection, cells were fixed and stained with X-gal, and surviving blue cells were counted. Data are expressed as % viability relative to the control transfection and represent the mean ± SE of 5 independent experiments.

B: Requirement for PTEN. $Pten^{+/-}$ (green) and $Pten^{-/-}$ (yellow) MEFs were infected with retrovirus bearing either empty IRES vector or IRES-DJ-1. After 48 hr, the infected cells were treated with 5 μM staurosporine. After 24 hr, the viability of treated, infected cells was determined by 7-AAD staining. Results are expressed as the % viability relative to untreated control infectants and represent the mean ± SE of 3 independent experiments.

C: Resistance to apoptotic stimuli. NIH-3T3-lacZ (blue) and NIH-3T3-DJ-1 (red) cells were treated with the indicated apoptotic stimuli as described in the Experimental Procedures. Cell viability (%) was determined 24 hr later by 7-AAD staining and normalized to the untreated control (5% FCS). Results shown are the mean ± SE of 3 independent experiments.

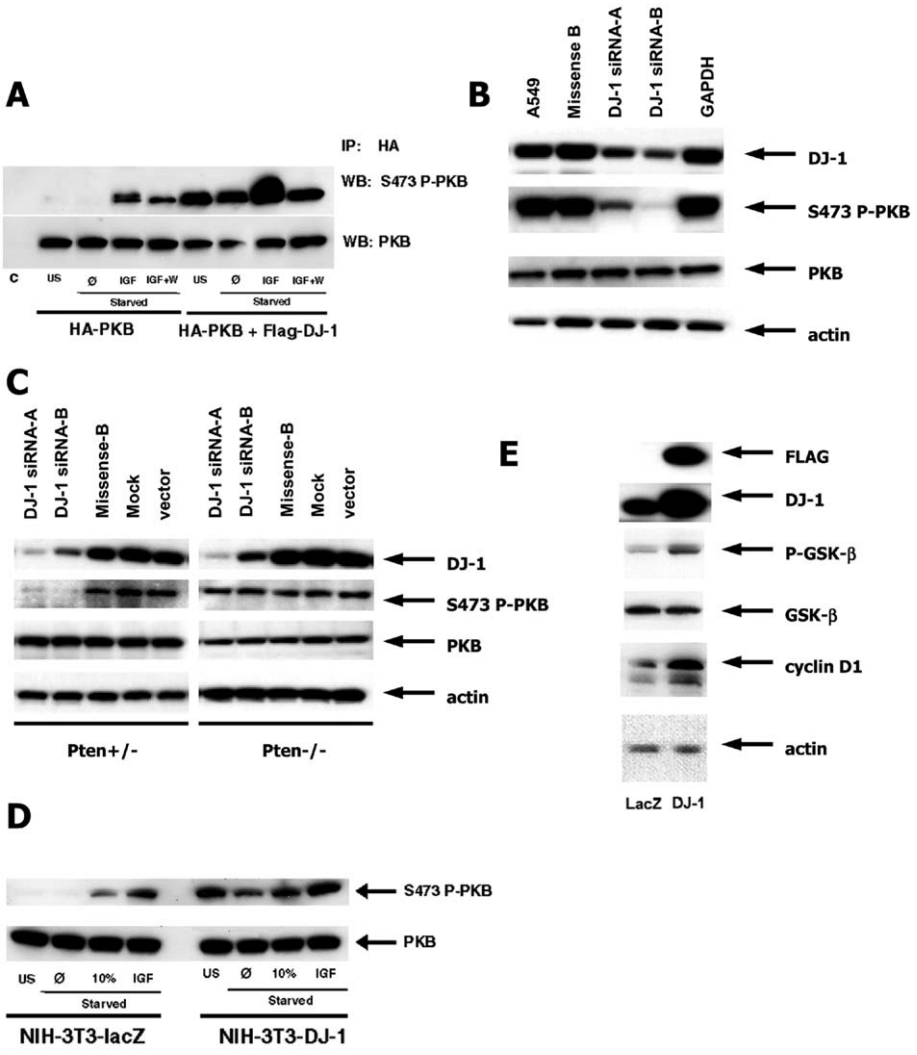


Figure 3. DJ-1 increases the activation of PI3′K downstream effectors

A: Increased phosphorylation of overexpressed PKB/Akt. Western blot analysis of lysates of COS7 cells transiently transfected with HA-PKB/Akt in the absence or presence of FLAG-hDJ-1. Transfected cells were serum-starved (0.5% FCS) or left unstarved (US), and either left untreated or treated with IGF-1 (50 ng/ml) with or without Wortmannin (W; 100 nM) as described in the Experimental Procedures. Antibodies recognizing total PKB/Akt (PKB) and PKB/Akt phosphorylated on S473 (S473 P-PKB) were used.

B: Decreased phosphorylation of endogenous PKB/Akt in response to DJ-1 knockdown. Western blot analysis of lysates of A549 cells, or A549 cells stably transfected with either a missense control of DJ-1-specific siRNA B, the DJ-1-specific siRNA sequences A or B, or control GAPDH siRNA (see Experimental Procedures). Antibodies specific for the indicated proteins were used, with washing and reprobing as necessary.

C: Unchanged endogenous PKB/Akt phosphorylation in the absence of PTEN. Western blot analysis of lysates of $Pten^{+/-}$ or $^{-/-}$ MEFs stably infected with either DJ-1-specific siRNAs A or B, or a missense control for B. Other controls included a mock transfection (no vector), and a transfection of empty vector (pSuper-retro-puro). Antibodies specific for the indicated proteins were used, with washing and reprobing as necessary.

D: Increased phosphorylation of endogenous PKB/Akt. Western blot of lysates of stably transfected NIH-3T3-DJ-1 cells (right) or control NIH-3T3-lacZ cells (left) starved, treated, and analyzed as in **A**.

E: Upregulation of cyclin D1 and GSK-3-β. Western blots of lysates of the NIH-3T3-lacZ and NIH-3T3-DJ-1 cells in **D** analyzed with antibodies specific for the indicated proteins, with washing and reprobing as necessary.

expected, expression of PTEN alone resulted in decreased cell viability, and expression of DJ-1 alone had no effect on cell survival (Figure 2A). However, coexpression of DJ-1 and PTEN completely rescued PTEN-induced cell death.

Various cell types from $Pten^{-/-}$ mice show decreased sensitivity to a broad range of apoptotic stimuli (Stambolic et al., 1998). To determine the effect of DJ-1 on apoptosis, $Pten^{-/-}$ and $^{+/-}$ cells (Stambolic et al., 1998; Suzuki et al., 1998) were retrovirally infected with human DJ-1 and treated with 5 μM staurosporine. $Pten^{+/-}$ infectants expressing DJ-1 showed a 60% increase in cell survival compared to treated infected $Pten^{+/-}$ cells lacking DJ-1 (Figure 2B). However, in the absence of PTEN ($Pten^{-/-}$ DJ-1 infectants), no significant rescue of cell survival was observed. We extended these observations by generating stable NIH-3T3 lines overexpressing FLAG-tagged human DJ-1 (NIH-3T3-DJ-1 cells), or a lacZ control (Tang et al., 2000) (NIH-3T3-lacZ cells). The response of NIH-3T3-DJ-1 cells to a range of cellular insults was then assessed using viability dye staining. NIH-3T3-DJ-1 cells exhibited a 40%–50% reduction of apoptosis (compared to control NIH-3T3-lacZ cells) in response to treatment with UV irradiation, sorbitol, or TNF-α (Figure 2C). These results indicate that overexpression of DJ-1 renders cells resistant to various apoptotic stimuli in a PTEN-dependent fashion.

We next investigated the effect of DJ-1 on PKB/Akt activation. PKB/Akt is highly phosphorylated in the absence of PTEN function (Di Cristofano et al., 1998; Stambolic et al., 1998; Sun et al., 1999; Suzuki et al., 1998). We found that transient cotransfection of DJ-1 plus PKB/Akt into COS-7 cells caused hyperphosphorylation of PKB/Akt under resting (10% FCS) and serum-starved (0.5% FCS) conditions (Figure 3A). Stimulation of COS-7 cells with insulin-like growth factor (IGF) resulted in a significant increase in PKB/Akt phosphorylation in the presence of DJ-1, but only a moderate increase in cells transfected with PKB/Akt alone. Pretreatment of starved cells with the PI3′K inhibitor Wortmannin reduced the IGF-induced increase in PKB/Akt phosphorylation in the absence or presence of DJ-1. This increase in phosphorylated PKB/Akt was reversed in A549 cells stably expressing either of two DJ-1-specific siRNAs that decreased DJ-1 expression (Figure 3B). Similarly, as shown in Figure 3C, a decrease in phosphorylated PKB/Akt was seen

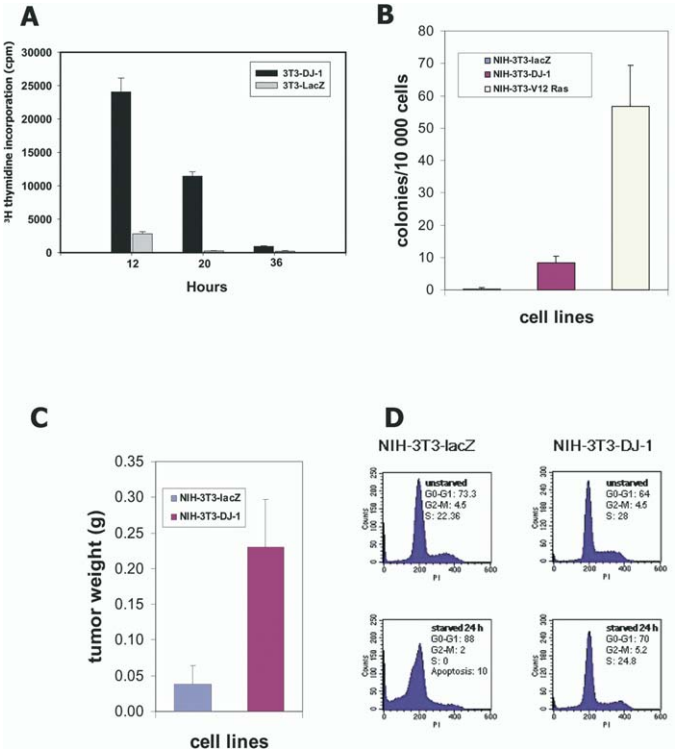


Figure 4. DJ-1 stimulates oncogenesis and alters cell cycle arrest

A: Increased proliferation. NIH-3T3-lacZ and NIH-3T3-DJ-1 cells were pulsed with [^{3}H] thymidine for 18 hr prior to measurement of [^{3}H] thymidine incorporation in extracts. Results shown are the mean ± SE of 4 independent experiments.

B: Increased growth in soft agar. NIH-3T3-lacZ or NIH-3T3-DJ-1 cells were seeded in 0.6% agar and colonies were scored 14 days later. Results shown are mean ± SE of 3 independent experiments.

C: Enhanced tumorigenesis in nude mice. Nude mice (5 per group; 4–6 weeks old) were injected with either NIH-3T3-lacZ or NIH-3T3-DJ-1 cells (1.0×10^6), and tumor weights were determined 9 days later. Results shown are the mean tumor weights (g) ± SE.

D: Altered cell cycle arrest. NIH-3T3-lacZ cells (left panels) or NIH-3T3-DJ-1 cells (right panels) were cultured for 24 hr with (top panels) or without (bottom panels) 0.5% FCS. Cell cycle distribution was analyzed by propidium iodide (50 μg/ml) staining. The percentages of cells in the G0/G1, S, or G2/M phase and in apoptosis were determined by flow cytometry and are indicated within each panel.

when murine DJ-1 expression was decreased by siRNA in Pten$^{+/-}$ immortalized mouse embryonic fibroblasts (MEFs) (Stambolic et al., 1998). Interestingly, when this protocol was applied to Pten$^{-/-}$ MEFs (Stambolic et al., 1998), the decrease in phosphorylated PKB/Akt was not observed (Figure 3C). This result indicates that the function of DJ-1 requires the presence of PTEN. When we analyzed phosphorylated PKB/Akt levels in the NIH-3T3 stable transfectants, we found that expression of DJ-1 (but not lacZ) induced high levels of endogenous phosphorylated PKB/Akt (Figure 3D). Stimulation of NIH-3T3-DJ-1 cells with IGF or 10% serum caused a further increase in phosphorylated PKB/Akt (Figure 3D). Consistent with this elevation in phosphorylated PKB/Akt, NIH-3T3-DJ-1 cells showed markedly higher levels of phosphorylated GSK-3-β (van Weeren et al., 1998) and cyclin D1 (Diehl et al., 1998) compared to control cells (Figure 3E). Taken together, these results indicate that DJ-1 suppresses PTEN function and thereby profoundly affects downstream targets of PTEN such as PKB/Akt.

DJ-1 is an oncogene

The oncogenic potential of DJ-1 was investigated by subjecting the stable NIH-3T3 transfectants to several indicative tests. Compared to NIH-3T3-lacZ cells, NIH-3T3-DJ-1 cells showed increased proliferation as measured by ^{3}H-thymidine incorporation (Figure 4A), and increased colony formation in soft agar (Figure 4B). When NIH-3T3-DJ-1 cells were subcutaneously injected into nude mice, significantly more tumor growth occurred than in animals injected with NIH-3T3-lacZ cells (Figure 4C). Because oncogenesis can also result from dysregulation of cell cycle arrest mechanisms, and ectopic PTEN expression can induce cell cycle arrest (Furnari et al., 1998; Li and Sun, 1998), we examined the effect of DJ-1 on the cell cycle. While NIH-3T3-lacZ cells underwent total G1 arrest upon 24 hr serum starvation (Figure 4D, left panels), NIH-3T3-DJ-1 cells were completely resistant to serum starvation and retained a normal cell cycle profile (Figure 4D, right panels). Our results are consistent with the previous identification of DJ-1 as a putative oncogene that cooperates with H-*ras* (Nagakubo et al., 1997), and suggest that DJ-1 can transform cells by increasing both cell proliferation and resistance to cell cycle arrest.

DJ-1 plays a role in the pathogenesis of primary breast cancer

Analyses of PTEN in primary breast carcinoma specimens by various groups have indicated that mutations of the PTEN gene are not a major factor in the development of sporadic breast cancers (Feilotter et al., 1999; Freihoff et al., 1999; Rhei et al., 1997). Primary ductal adenocarcinomas that are hemizygous for PTEN deletion, but with no structural alterations of the remaining allele, do not show positive immunostaining for PTEN. This finding suggests that an epigenetic phenomenon is affecting PTEN in these cases (Perren et al., 1999; Shi et al., 2003). Others have observed that DJ-1 levels are increased in the serum of breast cancer patients, and have suggested that this protein can be used as a prognostic marker (Le Naour et al., 2001). To investigate whether DJ-1 functions as a negative epigenetic regulator of PTEN, we examined the expression of DJ-1 protein, phosphorylated PKB/Akt, and PTEN in serial histologic sections from 73 patients with lymph node-negative breast cancer (Supplemental Table S1). We have previously shown that levels of PTEN protein and phosphorylated PKB/Akt are negatively correlated in these same samples (Shi et al., 2003). In this study, DJ-1 expression was generally observed in the cytoplasm of invasive breast cancer cells (Figure 5A), while PTEN expression was absent (Figure 5B), and phosphorylated PKB/Akt levels were elevated (Figure 5C). Among the 28 samples which were PTEN negative, 79% (22/28) were DJ-1 positive. However, only about half (51%) of 45 PTEN-positive samples were DJ-1 positive (Figure 5D, $p = 0.02$), suggesting that PTEN and DJ-1 are inversely associated. We note, however, that these findings do not preclude the existence in the PTEN-positive samples of mutations in other PI3′K genes such as PIK3CA (Lee et al., 2005; Samuels et al., 2004), which has recently been shown to contribute to oncogenesis (Kang et al., 2005). In support of a negative regulatory effect of DJ-1 on

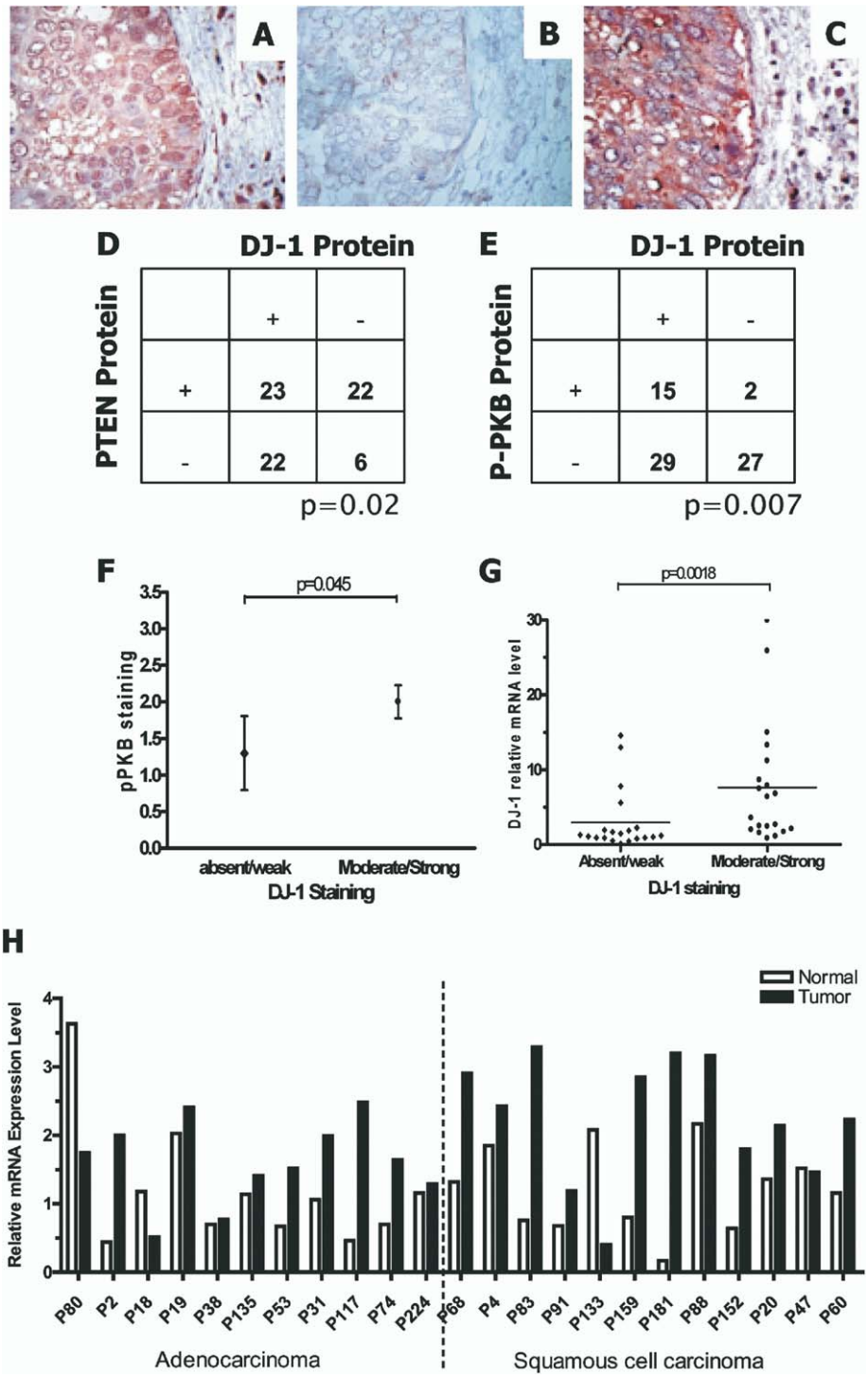


Figure 5. DJ-1 expression in primary cancers

A–C: Representative immunostaining analyses of serial sections from a single patient's surgical tumor specimen showing DJ-1 protein expression in the cytoplasm of the invasive breast cancer cells (**A**), the absence of PTEN expression (**B**), and high levels of phosphorylated PKB/Akt protein (**C**) (400× magnification).

D and E: Results from all 73 breast cancer specimens were analyzed in 2 × 2 tables. There was an inverse correlation between DJ-1 and PTEN (**D**), and a positive correlation between DJ-1 and phosphorylated PKB/Akt (**E**); χ^2 test.

F: Mean plot analyzing DJ-1 and phosphorylated PKB/Akt immunoreactivity score in 40 lung cancer specimens (± SEM). There was a positive correlation between DJ-1 and phosphorylated PKB/Akt/; unpaired t test.

G: Scatter plots of DJ-1 immunoreactivity compared to DJ-1 mRNA levels measured by real-time RT-PCR. DJ-1 immunoreactivity correlated positively with relative mRNA levels; unpaired t test.

H: Real-time RT-PCR analysis of DJ-1 mRNA in 23 paired normal and lung cancer specimens. Results shown are the relative ratio of ΔC_T(DJ-1 tumor):ΔC_T(DJ-1 mean of normal lung values). DJ-1 was significantly increased in tumors compared to normal tissues; p = 0.001, Wilcoxon sign rank test.

PTEN, we found evidence for a positive relationship between DJ-1 and phosphorylated-PKB. Fully 88% (15/17) of the phosphorylated PKB/Akt positive samples stained positively for DJ-1, but only 52% of the phosphorylated PKB/Akt negative samples were DJ-1-positive (Figure 5E, p = 0.007). In these latter cases, we speculate that other mutations and signaling pathways have come to dominate in the transformed cells, and that DJ-1 is no longer the driving force behind tumorigenesis.

No correlation between DJ-1 expression and patient age, clinical status, primary tumor size, ploidy, ER or PR status, or % S phase was found (data not shown). Our results suggest a mechanism by which DJ-1 might play a tumorigenic role in cases where PTEN mutations are not frequently observed, such as primary breast cancers. DJ-1 may modulate PTEN function such that PKB/Akt becomes hyperphosphorylated and promotes abnormal cell survival.

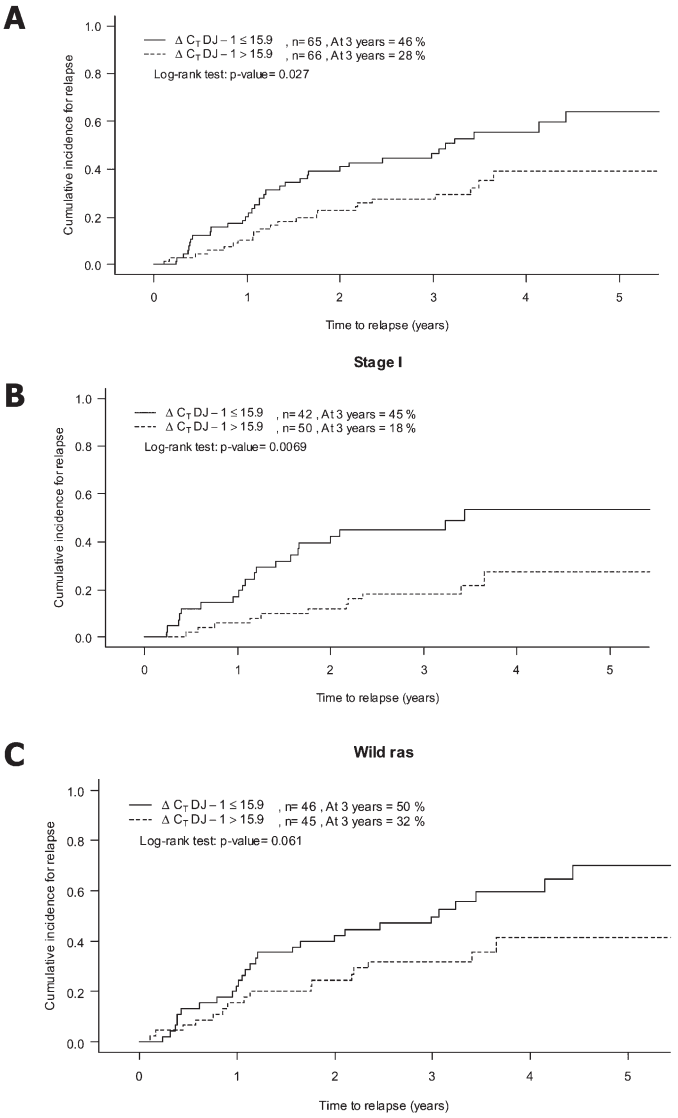


Figure 6. DJ-1 may be a prognostic marker for primary lung cancer

Cumulative incidence of risk recurrence in lung cancer patients categorized by their level of DJ-1 mRNA (ΔC_T) expression. Higher ΔC_T corresponds to lower DJ-1 expression. Tumors were dichotomized using the median ΔC_T value as the cutoff for high and low DJ-1 expressors (see Experimental Procedures). The time to relapse was evaluated for all lung cancer patients (**A**), for patients with Stage I disease (**B**), and for patients whose tumors exhibited the wild-type *Ras* genotype (**C**).

DJ-1 is a prognostic marker for primary lung cancer

To determine if elevated DJ-1 expression is a factor in other types of cancers, we examined DJ-1 and phosphorylated PKB/Akt levels in primary lung cancer samples. It was recently shown that DJ-1 protein expression is increased in primary non-small cell lung carcinomas (MacKeigan et al., 2003). Tissue blocks from 40 primary lung cancer patients (Zhu et al., 2004) (Supplemental Table S2) were analyzed by immunohistochemistry for DJ-1 and phosphorylated PKB/Akt. Consistent with our breast cancer findings, we found a significant correlation between DJ-1 and phosphorylated PKB/Akt immunoreactivity (Figure 5F, p = 0.045). Furthermore, DJ-1 immunoreactivity was correlated positively with DJ-1 mRNA levels as measured by real-time RT-PCR (Figure 5G, p = 0.0018). To further investigate the contribution of DJ-1 to lung cancer pathogenesis, we performed real-time RT-PCR on RNA samples from paired normal and malignant tissues from 23 patients with non-small cell lung carcinoma (Zhu et al., 2004). In 19/23 samples, expression of DJ-1 mRNA was increased in the tumor compared to the corresponding non-neoplastic and grossly normal lung tissue (Figure 5H; p = 0.001); nine samples had a greater than 2-fold increase.

We next investigated the prognostic significance of DJ-1 mRNA expression levels in the 23 tumor samples analyzed above plus 108 additional non-small cell lung carcinoma samples (Zhu et al., 2004) (Supplemental Table S2). Two clinical outcome measures were used: disease-free survival and incidence of relapse. Overall, analysis of the association between DJ-1 expression and the time to relapse suggested that having a higher expression level (lower ΔC_TDJ-1, see Experimental Procedures) of DJ-1 was an adverse prognostic factor. When the cumulative incidence of relapse in all patients was examined, patients who had high levels of DJ-1 expression had a significantly higher rate of relapse than patients with lower levels of DJ-1 (Figure 6A, p = 0.027). The 3-year risk of relapse for the group with high DJ-1 expression was 46% versus 28% for the group with low DJ-1. A similar observation was made when DJ-1 was evaluated as a continuous variable (data not shown, p = 0.022), suggesting a linear relationship between DJ-1 expression and the hazard ratio (relative risk) for relapse. Indeed, the hazard ratio increased by 1.2-fold for each unit increase in DJ-1 expression (data not shown).

When DJ-1 expression was analyzed by stage of cancer, we found that the median DJ-1 expression level was higher in Stage II and III patients compared to Stage I patients (ΔC_TDJ-1 of 15.56 versus 16.01, p = 0.032 by Wilcoxon nonparametric test). In fact, DJ-1 appeared to have its greatest effect in Stage I tumors, where the incidence of relapse was 45% for patients with high DJ-1 levels as opposed to 18% for patients with lower levels (Figure 6B, p = 0.0069). The increased proportion of patients with higher DJ-1 levels in the Stage II and III groups may explain the loss of prognostic significance of DJ-1 for these patients. Alternatively, DJ-1 may have greater impact in early stage lung cancers, before additional genetic aberrations accumulate during tumor progression. To explore this hypothesis, we analyzed DJ-1 expression levels in relation to another putative lung cancer prognostic marker gene, Ki-*ras* (Iyengar and Tsao, 2002). Indeed, DJ-1 was not prognostic for patients whose tumors harbored Ki-*ras* mutations (data not shown). However, for patients whose tumors had the wild-type Ki-*ras* genotype, a higher level of DJ-1 correlated positively with an increased risk of relapse (Figure 6C, p = 0.061). These data indicate that DJ-1 upregulation in lung cancer may be a useful prognostic marker, particularly for patients with wild-type *Ras* and Stage I disease. Our findings also suggest that mutation of DJ-1 may have the same biological impact as the presence of the Ki-*ras* oncogene in early stage non-small cell lung cancers.

Our study is the first demonstration that the oncogenic properties of DJ-1 are due to its effects on the PI3′K cell survival pathway. However, the many possible pathways in which DJ-1 and PTEN could interact remain to be clarified. Loss-of-func-

tion mutations in DJ-1 have been identified in Parkinson's disease (Bonifati et al., 2003a), and neuronal loss in Parkinson's patients is mainly attributed to oxidative stress (Jenner, 2003). Significantly, DJ-1 is known to be involved in apoptosis due to oxidative stress (Taira et al., 2004). PTEN has also been implicated in oxidative stress, where its function was modulated by exposure to peroxide (Leslie et al., 2003); however, PTEN's role in the oxidant-dependent pathway remains to be fully characterized. It is possible that DJ-1 exerts a redox effect on PTEN or another upstream regulator through either DJ-1's putative protease activity (Wilson et al., 2003) or its redox-sensitive chaperone activity (Shendelman et al., 2004).

In conclusion, our results demonstrate that DJ-1 negatively regulates PTEN function, and that this cell survival control mechanism is conserved among various tissues and species. We identified DJ-1 as a suppressor of PTEN function in the fly eye and extended these observations to the mammalian system. Knockdown of DJ-1 results in a decrease in phosphorylated PKB/Akt, while overexpression of DJ-1 is associated with hyperphosphorylation of PKB/Akt and upregulation of downstream effectors that promote cell survival. We have also shown that DJ-1 expression negatively correlates with PTEN staining and positively correlates with phosphorylated PKB/Akt expression in a group of primary breast cancer patients, a population that does not normally harbor mutations in PTEN. Furthermore, we have demonstrated that DJ-1 is highly expressed in primary lung cancers, that phosphorylated PKB/Akt correlates positively with DJ-1 immunoreactivity in these tumors, and that elevated DJ-1 is associated with a poor prognosis. Taken together, our data suggest that overexpression of DJ-1 can modulate PTEN tumor suppression to the point where oncogenesis may result from upregulated PKB/Akt-mediated cell survival.

Experimental procedures

Generation of transgenic flies

Oligonucleotide primers flanking the putative *Drosophila* PTEN open reading frame (Gao et al., 2000; Goberdhan et al., 1999; Huang et al., 1999) were designed (sense primer: BamHI Kozak 5′-GGA TCC ACC ATG GCC AAC ACT ATT TCG TTA ATG TCC-3′; antisense XhoI Stop primer: 5′-CTC GAG TCA CTC CGA TTC CCA ATC TTC CTC GCC ATC-3′). These primers were used to amplify a 1.53 kb product from 18 hr *Drosophila* embryos using the QUICK-Clone cDNA kit according to the manufacturer's protocol (Clontech, Palo Alto, CA). *Drosophila* PTEN and human DJ-1 cDNAs were cloned into the pUAST vector (Brand and Perrimon, 1993) and injected into w1118 (Brand and Perrimon, 1993) *Drosophila* embryos to generate transformants. For PTEN transgenic flies, UAS-dPTEN flies were crossed to those bearing GMR-GAL4 or ey-GAL4 (Peters et al., 2002). For adult fly rescue experiments, UAS-DJ-1 was crossed to flies bearing arm-GAL4 (Vincent et al., 1994). The arm-GAL4/UAS-DJ-1 transgene resulted in a rescue of viability of 23%. Rescue was scored as the percentage of live flies with a homozygous P element allele (the expected number is zero).

Drosophila Western blots

Protein extracts of 3rd instar *Drosophila* larvae were prepared as previously described (Peters et al., 2002). Extracts were subjected to Western blotting using polyclonal antibody directed against human PTEN (Stambolic et al., 1998).

Transfections and retroviral infections

Transient transfections were carried out using Lipofectamine Plus (Invitrogen). For stable transfections, FLAG-hDJ-1 was cloned into pBABE-puro. For siRNA stable transfections, A549 cells were transfected with pSilencer (Ambion) vectors containing DJ-1-specific siRNAs (clone A target, TCT GGG CGC ACA GAA TTT; clone B, GTA AAG TTA CAA CAC ACC) or control GAPDH RNA. Stable transfectants were selected for 14 days in 400 μg/ml hygromycin. For murine Pten$^{+/-}$ and Pten$^{-/-}$ infectants, FLAG-hDJ-1 was cloned into pLPC-puro-IRES-GFP, and DJ-1-specific siRNAs (clone A target, AGG CGC GGC TGC AGT CTT TAA; clone B, GTA GCC GTG ATG TAA TGA TTT) were cloned into pSuper-retro-puro (Oligoengine). All retroviruses were packaged within the ΦNX-Eco cell line.

Analyses of cell death

For viability staining, NIH-3T3 cells were transfected with cDNAs encoding PTEN and/or DJ-1 in the presence of trace amounts of a β-galactosidase expression construct as described previously (Stambolic et al., 1998). At 48 hr posttransfection, cells were fixed and stained with X-gal. Numbers of surviving transfected cells (blue) were counted in 10 randomly selected fields by two researchers in a blinded fashion.

For flow cytometry, NIH-3T3 cells (1.0×10^6) were plated and treated the next day with the following apoptotic stimuli: UV (780 J/m^2), sorbitol (0.4 mM), TNF-α (10 ng/ml) plus cycloheximide (5 μg/ml), or staurosporine (5 μM). Treated cells were stained 24 hr later with 7-amino-actinomycin D (7-AAD). For Pten$^{+/-}$ and $^{-/-}$ MEFs, infected cells were gated on GFP. Negatively staining cells were quantified in a FACScan flow cytometer (Becton Dickinson).

COS7 cells and serum starvation

COS7 cells were transiently transfected with HA-PKB/Akt (Stambolic et al., 1998) plus or minus human FLAG-DJ-1. Cells were either not starved or starved for 18 hr in DMEM containing 0.5% serum. Starved and unstarved cells were then either left untreated, treated for 20 min with IGF-1 (50 ng/ml), or treated for 30 min with Wortmannin (100 nM), followed by a 20 min incubation with IGF-1 (50 ng/ml). HA-PKB/Akt was immunoprecipitated using an anti-HA antibody (12CA5) and analyzed by Western blotting.

Western blotting

Cells were lysed in 0.5% CHAPS buffer and subjected to Western blotting using rabbit polyclonal anti-phosphoS473 PKB/Akt, anti-PKB/Akt, anti-phospho-GSK-3-β, or mouse anti-cyclin D1 antibodies (all from Cell Signaling); mouse anti-GSK-3-β (Transduction Labs); rabbit anti-actin, or mouse anti-FLAG clone M5 (Sigma-Aldrich). Bound antibodies were detected by enhanced chemiluminescence (ECL, Amersham).

Cell proliferation

Cells (1.0×10^4) were plated in quadruplicate and incubated for 12, 20, or 36 hr. For the last 12 hr of incubation, 1 μCi ^{3}H-thymidine was added to measure ^{3}H-thymidine incorporation.

Growth in soft agar

Dishes (35 mm) were prepared with a lower layer of 0.6% agar (BiTek, DIFCO). The overlay was a 0.3% agar solution containing 1.0×10^4 NIH-3T3-lacZ, NIH-3T3-DJ-1, or NIH-3T3-V12-*Ras* (positive control) cells. After 14 days growth, colonies larger than 0.1 mm in diameter were scored as positive.

Cell cycle distribution

Cells were stained with propidium iodide (50 μg/ml) and analyzed for phase of the cell cycle by flow cytometry according to standard procedures.

Injections of nude mice

Athymic, Balb-c/nu/nu mice of 4–6 weeks of age were injected with 1.0×10^6 NIH-3T3-lacZ cells (n = 5) or NIH-3T3-DJ-1 cells (n = 5) in 100 μl PBS. Tumor weights were measured at 9 days postinjection.

Anti-DJ-1 antibody

Rabbit antiserum was raised against purified Trx-huDJ-1 protein (Antibodies, Inc., Davis, CA) and purified by preadsorption on Trx-coupled CNBr-Sepharose 4B followed by affinity purification on GST-huDJ-1 fusion protein coupled to CNBr-Sepharose 4B. Low-affinity antibody was eluted in a pH 5.0 buffer and stored. The high-affinity DJ-1-specific antibody used in this study was eluted from the affinity column with 0.1M glycine (pH 2.5).

Immunohistochemistry

Archival formalin-fixed, paraffin-embedded tumor sections from primary surgical specimens of breast cancer patients (Shi et al., 2003) and lung

cancer patients (Zhu et al., 2004) were stained with antibodies recognizing PTEN (Zymed, 1:80), phosphoS473-PKB (New England Biolabs, 1:70), or DJ-1 (1:300) as previously described (Shi et al., 2003). After immunostaining overnight, the slides were incubated with biotinylated secondary (anti-rabbit) antibodies followed by Streptavidin-HRP enzyme complex (Signet Laboratories, Dedham, MA). Cells were visualized using AEC as the chromogen, and counterstained with hematoxylin to define cellular morphology. For lung cancer specimens, DJ-1 staining was evaluated as absent/weak or moderate/strong and phosphorylated Akt/PKB immunoreactivity was scored as absent (0), weak (1), moderate (2), or strong (3) by a blinded pathologist.

Real-time RT-PCR quantitation of mRNA

DJ-1 mRNA expression levels in 23 paired samples of primary non-small cell lung cancer cells and corresponding nonneoplasic lung tissues, and in 108 unpaired primary non-small cell lung cancer samples (Zhu et al., 2004), were measured using quantitative RT-PCR as previously described (Zhu et al., 2004). Primers used to amplify DJ-1 transcripts and give a 90 bp product were: DJ-1 forward, GGA GAC GGT CAT CCC TGT AGA T; DJ-1 reverse, GCT ACA CTG TAC TGG GTC TTT TCC A. Levels of mRNA in each sample were normalized using the calculation $\Delta C_T = [C_T(\text{DJ-1}) - C_T(18S)]$ (Zhu et al., 2004). High expressors of DJ-1 were defined as $\Delta C_T\text{DJ-1} \leq 15.9$ and low expressors were defined as $\Delta C_T\text{DJ-1} > 15.9$, where $\Delta C_T\text{DJ-1} = 15.9$ was the median of all samples. Relative mRNA transcript levels in tumor tissues and paired nonneoplastic lung tissues were calculated as previously described (Wang et al., 2002).

Statistical methods

Statistical analyses were carried out using SAS v8.2 and S-plus software. For analysis of immunohistochemical categorical data, the χ^2 test was used. For analysis of lung cancer immunohistochemical data, the unpaired t test was used. The Wilcoxon sign rank test was used to analyze mRNA levels in paired lung tissue samples and to compare DJ-1 levels between cancer stages. The testing of the DJ-1 mRNA level as a continuous variable was performed using the Cox proportional hazards model. The incidence of relapse was estimated using the cumulative incidence approach (Kalbfleisch and Prentice, 1980), and the difference between the low DJ-1 and high DJ-1 groups was evaluated using the log rank test.

Supplemental data

Supplemental data for this article can be found at http://www.cancercell.org/cgi/content/full/7/3/263/DC1/.

Acknowledgments

The authors are grateful to Damu Tang for stable transfectants, Ni Liu for real-time RT-PCR, and Vuk Stambolic, Jim Woodgett, Jim Rutka, Scott Pownall, and Lea Harrington for helpful discussions. We also thank Mary Saunders for scientific editing. M.-S.T. is supported by the Canadian Cancer Society (Grant 15184) and the National Cancer Institute of Canada. R.H.K. is supported by the Frank Fletcher Memorial Fund, David Rae Scholarship, and Paul Starita Fellowship, and Canadian Institutes of Health Research.

Received: October 31, 2004
Revised: January 27, 2005
Accepted: February 22, 2005
Published: March 14, 2005

References

Alessi, D.R., James, S.R., Downes, C.P., Holmes, A.B., Gaffney, P.R., Reese, C.B., and Cohen, P. (1997). Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr. Biol. *7*, 261–269.

Bohni, R., Riesgo-Escovar, J., Oldham, S., Brogiolo, W., Stocker, H., Andruss, B.F., Beckingham, K., and Hafen, E. (1999). Autonomous control of cell and organ size by CHICO, a Drosophila homolog of vertebrate IRS1–4. Cell *97*, 865–875.

Bonifati, V., Rizzu, P., Squitieri, F., Krieger, E., Vanacore, N., van Swieten, J.C., Brice, A., van Duijn, C.M., Oostra, B., Meco, G., and Heutink, P. (2003a). DJ-1(PARK7), a novel gene for autosomal recessive, early onset parkinsonism. Neurol. Sci. *24*, 159–160.

Bonifati, V., Rizzu, P., van Baren, M.J., Schaap, O., Breedveld, G.J., Krieger, E., Dekker, M.C., Squitieri, F., Ibanez, P., Joosse, M., et al. (2003b). Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science *299*, 256–259.

Brand, A.H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Burgering, B.M., and Coffer, P.J. (1995). Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. Nature *376*, 599–602.

Canet-Aviles, R.M., Wilson, M.A., Miller, D.W., Ahmad, R., McLendon, C., Bandyopadhyay, S., Baptista, M.J., Ringe, D., Petsko, G.A., and Cookson, M.R. (2004). The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc. Natl. Acad. Sci. USA *101*, 9103–9108.

Cantley, L.C., and Neel, B.G. (1999). New insights into tumor suppression: PTEN suppresses tumor formation by restraining the phosphoinositide 3-kinase/AKT pathway. Proc. Natl. Acad. Sci. USA *96*, 4240–4245.

Cassada, R.C., and Russell, R.L. (1975). The dauerlarva, a post-embryonic developmental variant of the nematode Caenorhabditis elegans. Dev. Biol. *46*, 326–342.

Di Cristofano, A., Pesce, B., Cordon-Cardo, C., and Pandolfi, P.P. (1998). Pten is essential for embryonic development and tumour suppression. Nat. Genet. *19*, 348–355.

Diehl, J.A., Cheng, M., Roussel, M.F., and Sherr, C.J. (1998). Glycogen synthase kinase-3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. *12*, 3499–3511.

Eng, C., and Peacocke, M. (1998). PTEN and inherited hamartoma-cancer syndromes. Nat. Genet. *19*, 223.

Feilotter, H.E., Coulon, V., McVeigh, J.L., Boag, A.H., Dorion-Bonnet, F., Duboue, B., Latham, W.C., Eng, C., Mulligan, L.M., and Longy, M. (1999). Analysis of the 10q23 chromosomal region and the PTEN gene in human sporadic breast carcinoma. Br. J. Cancer *79*, 718–723.

Freihoff, D., Kempe, A., Beste, B., Wappenschmidt, B., Kreyer, E., Hayashi, Y., Meindl, A., Krebs, D., Wiestler, O.D., von Deimling, A., and Schmutzler, R.K. (1999). Exclusion of a major role for the PTEN tumour-suppressor gene in breast carcinomas. Br. J. Cancer *79*, 754–758.

Fruman, D.A., Meyers, R.E., and Cantley, L.C. (1998). Phosphoinositide kinases. Annu. Rev. Biochem. *67*, 481–507.

Furnari, F.B., Huang, H.J., and Cavenee, W.K. (1998). The phosphoinositol phosphatase activity of PTEN mediates a serum-sensitive G1 growth arrest in glioma cells. Cancer Res. *58*, 5002–5008.

Gao, X., Neufeld, T.P., and Pan, D. (2000). Drosophila PTEN regulates cell growth and proliferation through PI3K-dependent and -independent pathways. Dev. Biol. *221*, 404–418.

Goberdhan, D.C., Paricio, N., Goodman, E.C., Mlodzik, M., and Wilson, C. (1999). Drosophila tumor suppressor PTEN controls cell size and number by antagonizing the Chico/PI3-kinase signaling pathway. Genes Dev. *13*, 3244–3258.

Gottlieb, S., and Ruvkun, G. (1994). daf-2, daf-16 and daf-23: genetically interacting genes controlling Dauer formation in Caenorhabditis elegans. Genetics *137*, 107–120.

Hod, Y. (2004). Differential control of apoptosis by DJ-1 in prostate benign and cancer cells. J. Cell. Biochem. *92*, 1221–1233.

Hod, Y., Pentyala, S.N., Whyard, T.C., and El-Maghrabi, M.R. (1999). Identification and characterization of a novel protein that regulates RNA-protein interaction. J. Cell. Biochem. *72*, 435–444.

Honbou, K., Suzuki, N.N., Horiuchi, M., Niki, T., Taira, T., Ariga, H., and Inagaki, F. (2003). The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease. J. Biol. Chem. *278*, 31380–31384.

Huai, Q., Sun, Y., Wang, H., Chin, L.S., Li, L., Robinson, H., and Ke, H. (2003). Crystal structure of DJ-1/RS and implication on familial Parkinson's disease. FEBS Lett. *549*, 171–175.

Huang, H., Potter, C.J., Tao, W., Li, D.M., Brogiolo, W., Hafen, E., Sun, H., and Xu, T. (1999). PTEN affects cell size, cell proliferation and apoptosis during Drosophila eye development. Development *126*, 5365–5372.

Iida, S., Tanaka, Y., Fujii, H., Hayashi, S., Kimura, M., Nagareda, T., and Moriwaki, K. (1998). A heterozygous frameshift mutation of the PTEN/MMAC1 gene in a patient with Lhermitte-Duclos disease—only the mutated allele was expressed in the cerebellar tumor. Int. J. Mol. Med. *1*, 925–929.

Iyengar, P., and Tsao, M.S. (2002). Clinical relevance of molecular markers in lung cancer. Surg. Oncol. *11*, 167–179.

Jenner, P. (2003). Oxidative stress in Parkinson's disease. Ann. Neurol. *53*, S26–S36.

Kalbfleisch, J., and Prentice, R. (1980). The Statistical Analysis of Failure Time Data (New York: John Wiley & Sons).

Kang, S., Bader, A.G., and Vogt, P.K. (2005). Phosphatidylinositol 3-kinase mutations identified in human cancer are oncogenic. Proc. Natl. Acad. Sci. USA *102*, 802–807.

Le Naour, F., Misek, D.E., Krause, M.C., Deneux, L., Giordano, T.J., Scholl, S., and Hanash, S.M. (2001). Proteomics-based identification of RS/DJ-1 as a novel circulating tumor antigen in breast cancer. Clin. Cancer Res. *7*, 3328–3335.

Lee, J.W., Soung, Y.H., Kim, S.Y., Lee, H.W., Park, W.S., Nam, S.W., Kim, S.H., Lee, J.Y., Yoo, N.J., and Lee, S.H. (2005). PIK3CA gene is frequently mutated in breast carcinomas and hepatocellular carcinomas. Oncogene *24*, 1477–1480.

Lee, S.J., Kim, S.J., Kim, I.K., Ko, J., Jeong, C.S., Kim, G.H., Park, C., Kang, S.O., Suh, P.G., Lee, H.S., and Cha, S.S. (2003). Crystal Structures of Human DJ-1 and Escherichia coli Hsp31, Which Share an Evolutionarily Conserved Domain. J. Biol. Chem. *278*, 44552–44559.

Leslie, N.R., Bennett, D., Lindsay, Y.E., Stewart, H., Gray, A., and Downes, C.P. (2003). Redox regulation of PI 3-kinase signalling via inactivation of PTEN. EMBO J. *22*, 5501–5510.

Li, D.M., and Sun, H. (1998). PTEN/MMAC1/TEP1 suppresses the tumorigenicity and induces G1 cell cycle arrest in human glioblastoma cells. Proc. Natl. Acad. Sci. USA *95*, 15406–15411.

Liaw, D., Marsh, D.J., Li, J., Dahia, P.L., Wang, S.I., Zheng, Z., Bose, S., Call, K.M., Tsou, H.C., Peacocke, M., et al. (1997). Germline mutations of the PTEN gene in Cowden disease, an inherited breast and thyroid cancer syndrome. Nat. Genet. *16*, 64–67.

MacKeigan, J.P., Clements, C.M., Lich, J.D., Pope, R.M., Hod, Y., and Ting, J.P. (2003). Proteomic profiling drug-induced apoptosis in non-small cell lung carcinoma: identification of RS/DJ-1 and RhoGDIalpha. Cancer Res. *63*, 6928–6934.

Maehama, T., and Dixon, J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J. Biol. Chem. *273*, 13375–13378.

Marsh, D.J., Dahia, P.L., Zheng, Z., Liaw, D., Parsons, R., Gorlin, R.J., and Eng, C. (1997). Germline mutations in PTEN are present in Bannayan-Zonana syndrome. Nat. Genet. *16*, 333–334.

Mitsumoto, A., and Nakagawa, Y. (2001). DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic. Res. *35*, 885–893.

Mitsumoto, A., Nakagawa, Y., Takeuchi, A., Okawa, K., Iwamatsu, A., and Takanezawa, Y. (2001). Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic. Res. *35*, 301–310.

Myers, M.P., Pass, I., Batty, I.H., Van der Kaay, J., Stolarov, J.P., Hemmings, B.A., Wigler, M.H., Downes, C.P., and Tonks, N.K. (1998). The lipid phosphatase activity of PTEN is critical for its tumor supressor function. Proc. Natl. Acad. Sci. USA *95*, 13513–13518.

Nagakubo, D., Taira, T., Kitaura, H., Ikeda, M., Tamai, K., Iguchi-Ariga, S.M., and Ariga, H. (1997). DJ-1, a novel oncogene which transforms mouse NIH3T3 cells in cooperation with ras. Biochem. Biophys. Res. Commun. *231*, 509–513.

Niki, T., Takahashi-Niki, K., Taira, T., Iguchi-Ariga, S.M., and Ariga, H. (2003). DJBP: A novel DJ-1-binding protein, negatively regulates the androgen receptor by recruiting histone deacetylase complex, and DJ-1 antagonizes this inhibition by abrogation of this complex. Mol. Cancer Res. *1*, 247–261.

Okada, M., Matsumoto, K., Niki, T., Taira, T., Iguchi-Ariga, S.M., and Ariga, H. (2002). DJ-1, a target protein for an endocrine disrupter, participates in the fertilization in mice. Biol. Pharm. Bull. *25*, 853–856.

Perren, A., Weng, L.P., Boag, A.H., Ziebold, U., Thakore, K., Dahia, P.L., Komminoth, P., Lees, J.A., Mulligan, L.M., Mutter, G.L., and Eng, C. (1999). Immunohistochemical evidence of loss of PTEN expression in primary ductal adenocarcinomas of the breast. Am. J. Pathol. *155*, 1253–1260.

Peters, M., DeLuca, C., Hirao, A., Stambolic, V., Potter, J., Zhou, L., Liepa, J., Snow, B., Arya, S., Wong, J., et al. (2002). Chk2 regulates irradiation-induced, p53-mediated apoptosis in Drosophila. Proc. Natl. Acad. Sci. USA *99*, 11305–11310.

Podsypanina, K., Ellenson, L.H., Nemes, A., Gu, J., Tamura, M., Yamada, K.M., Cordon-Cardo, C., Catoretti, G., Fisher, P.E., and Parsons, R. (1999). Mutation of Pten/Mmac1 in mice causes neoplasia in multiple organ systems. Proc. Natl. Acad. Sci. USA *96*, 1563–1568.

Quigley, P.M., Korotkov, K., Baneyx, F., and Hol, W.G. (2003). The 1.6-Å crystal structure of the class of chaperones represented by Escherichia coli Hsp31 reveals a putative catalytic triad. Proc. Natl. Acad. Sci. USA *100*, 3137–3142.

Rhei, E., Kang, L., Bogomolniy, F., Federici, M.G., Borgen, P.I., and Boyd, J. (1997). Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinomas. Cancer Res. *57*, 3657–3659.

Samuels, Y., Wang, Z., Bardelli, A., Silliman, N., Ptak, J., Szabo, S., Yan, H., Gazdar, A., Powell, S.M., Riggins, G.J., et al. (2004). High frequency of mutations of the PIK3CA gene in human cancers. Science *304*, 554.

Shendelman, S., Jonason, A., Martinat, C., Leete, T., and Abeliovich, A. (2004). DJ-1 is a redox-dependent molecular chaperone that inhibits alpha-synuclein aggregate formation. PLoS Biol. *2*, e362. 10.1371/journal.pbio.0020362

Shi, W., Zhang, X., Pintilie, M., Ma, N., Miller, N., Banerjee, D., Tsao, M.S., Mak, T., Fyles, A., and Liu, F.F. (2003). Dysregulated PTEN-PKB and negative receptor status in human breast cancer. Int. J. Cancer *104*, 195–203.

Spradling, A.C., Stern, D., Beaton, A., Rhem, E.J., Laverty, T., Mozden, N., Misra, S., and Rubin, G.M. (1999). The Berkeley Drosophila Genome Project gene disruption project: Single P-element insertions mutating 25% of vital Drosophila genes. Genetics *153*, 135–177.

Stambolic, V., Suzuki, A., de la Pompa, J.L., Brothers, G.M., Mirtsos, C., Sasaki, T., Ruland, J., Penninger, J.M., Siderovski, D.P., and Mak, T.W. (1998). Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell *95*, 29–39.

Stambolic, V., Tsao, M.S., Macpherson, D., Suzuki, A., Chapman, W.B., and Mak, T.W. (2000). High incidence of breast and endometrial neoplasia resembling human Cowden syndrome in pten+/– mice. Cancer Res. *60*, 3605–3611.

Stokoe, D., Stephens, L.R., Copeland, T., Gaffney, P.R., Reese, C.B., Painter, G.F., Holmes, A.B., McCormick, F., and Hawkins, P.T. (1997). Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science *277*, 567–570.

Sun, H., Lesche, R., Li, D.M., Liliental, J., Zhang, H., Gao, J., Gavrilova, N., Mueller, B., Liu, X., and Wu, H. (1999). PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc. Natl. Acad. Sci. USA *96*, 6199–6204.

Suzuki, A., de la Pompa, J.L., Stambolic, V., Elia, A.J., Sasaki, T., del Barco

Barrantes, I., Ho, A., Wakeham, A., Itie, A., Khoo, W., et al. (1998). High cancer susceptibility and embryonic lethality associated with mutation of the PTEN tumor suppressor gene in mice. Curr. Biol. *8*, 1169–1178.

Taira, T., Saito, Y., Niki, T., Iguchi-Ariga, S.M., Takahashi, K., and Ariga, H. (2004). DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep. *5*, 213–218.

Takahashi, K., Taira, T., Niki, T., Seino, C., Iguchi-Ariga, S.M., and Ariga, H. (2001). DJ-1 positively regulates the androgen receptor by impairing the binding of PIASx alpha to the receptor. J. Biol. Chem. *276*, 37556–37563.

Takahashi-Niki, K., Niki, T., Taira, T., Iguchi-Ariga, S.M., and Ariga, H. (2004). Reduced anti-oxidative stress activities of DJ-1 mutants found in Parkinson's disease patients. Biochem. Biophys. Res. Commun. *320*, 389–397.

Tan, E.K., Tan, C., Zhao, Y., Yew, K., Shen, H., Chandran, V.R., Teoh, M.L., Yih, Y., Pavanni, R., Wong, M.C., et al. (2004). Genetic analysis of DJ-1 in a cohort Parkinson's disease patients of different ethnicity. Neurosci. Lett. *367*, 109–112.

Tang, D., Lahti, J.M., and Kidd, V.J. (2000). Caspase-8 activation and bid cleavage contribute to MCF7 cellular execution in a caspase-3-dependent manner during staurosporine-mediated apoptosis. J. Biol. Chem. *275*, 9303–9307.

Tao, X., and Tong, L. (2003). Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. J. Biol. Chem. *278*, 31372–31379.

van Weeren, P.C., de Bruyn, K.M., de Vries-Smits, A.M., van Lint, J., and Burgering, B.M. (1998). Essential role for protein kinase B (PKB) in insulin-induced glycogen synthase kinase 3 inactivation. Characterization of dominant-negative mutant of PKB. J. Biol. Chem. *273*, 13150–13156.

Vincent, J.P., Girdham, C.H., and O'Farrell, P.H. (1994). A cell-autonomous, ubiquitous marker for the analysis of Drosophila genetic mosaics. Dev. Biol. *164*, 328–331.

Wagenfeld, A., Gromoll, J., and Cooper, T.G. (1998). Molecular cloning and expression of rat contraception associated protein 1 (CAP1), a protein putatively involved in fertilization. Biochem. Biophys. Res. Commun. *251*, 545–549.

Wagenfeld, A., Yeung, C.H., Shivaji, S., Sundareswaran, V.R., Ariga, H., and Cooper, T.G. (2000). Expression and cellular localization of contraception-associated protein. J. Androl. *21*, 954–963.

Wang, K.K., Liu, N., Radulovich, N., Wigle, D.A., Johnston, M.R., Shepherd, F.A., Minden, M.D., and Tsao, M.S. (2002). Novel candidate tumor marker genes for lung adenocarcinoma. Oncogene *21*, 7598–7604.

Wilson, M.A., Collins, J.L., Hod, Y., Ringe, D., and Petsko, G.A. (2003). The 1.1-Å resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. Proc. Natl. Acad. Sci. USA *100*, 9256–9261.

Yoshida, K., Sato, Y., Yoshiike, M., Nozawa, S., Ariga, H., and Iwamoto, T. (2003). Immunocytochemical localization of DJ-1 in human male reproductive tissue. Mol. Reprod. Dev. *66*, 391–397.

Zhu, C.Q., Blackhall, F.H., Pintilie, M., Iyengar, P., Liu, N., Ho, J., Chomiak, T., Lau, D., Winton, T., Shepherd, F.A., and Tsao, M.S. (2004). Skp2 gene copy number aberrations are common in non-small cell lung carcinoma, and its overexpression in tumors with ras mutation is a poor prognostic marker. Clin. Cancer Res. *10*, 1984–1991.