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Report

FKBP51 Affects Cancer Cell Response to Chemotherapy by Negatively Regulating Akt

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SUMMARY

Akt is a central regulator of cell growth. Its activity can be negatively regulated by the phosphatase PHLPP that specifically dephosphorylates the hydrophobic motif of Akt (Ser473 in Akt1). However, how PHLPP is targeted to Akt is not clear. Here we show that FKBP51 (FK506-binding protein 51) acts as a scaffolding protein for Akt and PHLPP and promotes dephosphorylation of Akt. Furthermore, FKBP51 is downregulated in pancreatic cancer tissue samples and several cancer cell lines. Decreased FKBP51 expression in cancer cells results in hyperphosphorylation of Akt and decreased cell death following genotoxic stress. Overall, our findings identify FKBP51 as a negative regulator of the Akt pathway, with potentially important implications for cancer etiology and response to chemotherapy.

INTRODUCTION

The serine/threonine kinase Akt (also called PKB) is a central module in cell signaling downstream of a variety of stimuli (Manning and Cantley, 2007). Akt is a major kinase downstream of phosphatidylinositol 3-kinase (PI3K). PI3K converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3), which in turn recruits Akt and PDK1 to the plasma membrane (Brazil and Hemmings, 2001). PDK1 then phosphorylates the activation loop of Akt at Thr308 (Alessi et al., 1997). Akt is also phosphorylated at Ser473, and the phosphorylation of both Ser473 and Thr308 is required for the full activation of Akt (Alessi et al., 1996) The kinase that phosphorylates Ser473 remained elusive until mTOR complex 2 (mTORC2) was found to directly phosphorylate Akt at Ser473 (Sarbassov et al., 2005). By contrast, the phosphatase PHLPP (PH domain leucine-rich repeat protein phosphatase) was shown to specifically dephosphorylate the hydrophobic motif of Akt (Ser473 in Akt1) and inhibit Akt activity (Brognard et al., 2007; Gao et al., 2005). However, how Akt is targeted to PHLPP is not clear.

Akt has been shown to inhibit apoptosis and promote cell survival, activities that contribute to its oncogenic potential (Manning and Cantley, 2007). Several mechanisms underlie Akt's antiapoptotic effect. First, Akt phosphorylates the proapoptotic protein BAD, preventing binding to its target protein (Datta et al., 1997; del Peso et al., 1997). Second, Akt phosphorylates FOXO transcription factors, resulting in their export from the nucleus and the downregulation of FOXO target genes (Biggs et al., 1999; Brunet et al., 1999; Kops et al., 1999; Rena et al., 1999), including the proapoptotic BH3-only protein BIM. Third, Akt phosphorylates MDM2, resulting in its translocation to the nucleus, and facilitating MDM2's inhibition of p53 (Mayo and Donner, 2001; Zhou et al., 2001). Finally, Akt phosphorylates and inhibits GSK isoforms (Cross et al., 1995) that play a proapoptotic role by inhibiting antiapoptotic protein MCL-1 (Maurer et al., 2006). Therefore, Akt activity needs to be tightly regulated

SIGNIFICANCE

Resistance to chemotherapy is a major hurdle for successful cancer therapy. Therefore, an improved understanding of the mechanisms responsible for chemoresistance will be helpful in the development of strategies to sensitize cancer cells to chemotherapy. We have found that decreased expression of FKBP51 results in resistance to chemotherapy. Mechanistically, we found that FKBP51 negatively regulates Akt through an apparent scaffolding function. Our studies identified FKBP51 as an important determinant for cancer cell response to chemotherapy and revealed one mechanism by which cancer cells are resistant to chemotherapy.

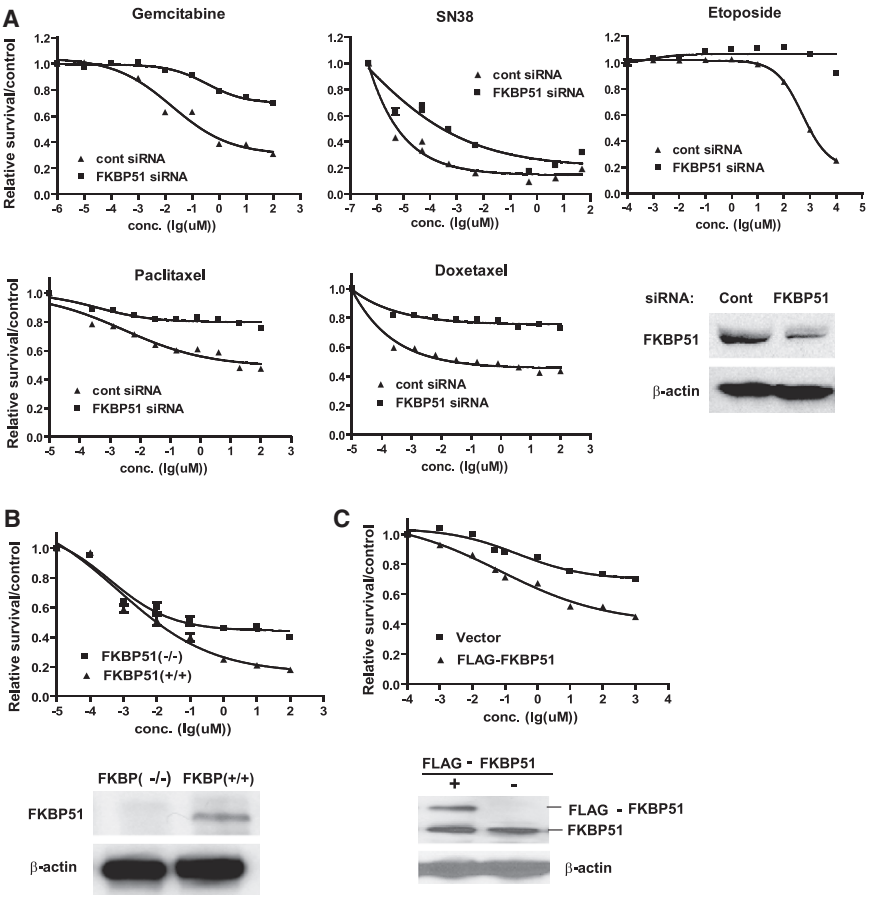

Figure 1. FKBP51 Regulates Cellular Response to Genotoxic Stress

(A) SU86 cells were transfected with control or FKBP51 siRNA and then treated with the indicated drugs. Cell survival was determined as described in Experimental Procedures.

(B) FKBP51$^{+/+}$ or FKBP51$^{-/-}$ cells were treated with gemcitabine, and cell survival was determined as in (A).

(C) SU86 cells were transfected with vector or constructs encoding FLAG-FKBP51. Transfected cells were treated with gemcitabine, and cell survival was determined as in (A). Points show mean values for three independent experiments; error bars represent standard error of the mean (SEM).

in cells, and hyperactivation of Akt has been linked to cancer predisposition and cancer cell resistance to chemotherapy.

We recently identified FKBP51 (also called *FKBP5*) as a regulator of cell death in response to gemcitabine (Li et al., 2008); downregulation of FKBP51 results in decreased cell death in response to gemcitabine and Ara-C treatment. FKBP51 is a member of the FK506-binding protein (FKBP) family (Baughman et al., 1995). Like other family members, it contains peptidyl-prolyl *cis/trans* isomerase (PPIase) activity and FKBP-C domains (Harding et al., 1989). It is well established that FKBP51 regulates steroid receptor activation (Cheung and Smith, 2000). In addition, FKBP51 has been shown to be required for IKK (IκB kinase) activation (Bouwmeester et al., 2004). However, this action of FKBP51 on NFκB activation cannot explain the increased chemoresistance in cells with decreased FKBP51 expression, because downregulation of FKBP51 should inhibit IKK activation and lead to increased cellular sensitivity to chemotherapy. This suggests the existence of other mechanisms by which FKBP51 regulates cell survival.

RESULTS

To examine whether FKBP51 regulates cellular response to multiple classes of chemotherapeutic drugs, we used microtubule stabilizers and topoisomerase I and II inhibitors to treat the pancreatic cancer cell line SU86. Downregulation of FKBP51 with two different siRNAs resulted in increased resistance to these treatments (Figures 1A and S1A available online). Downregulation of FKBP51 also resulted in resistance to these drugs in the lung cancer cell line A549 and the breast cancer cell line MDA-MB-231 (Figure S2). Furthermore, loss of FKBP51 expression resulted in increased resistance of mouse embryonic fibroblasts (MEFs) to gemcitabine (Figure 1B). In contrast, overexpression of FKBP51 resulted in hypersensitivity to gemcitabine (Figure 1C). Overall, these results have established an important role of FKBP51 in regulating cellular response to a wide range of clinically important antineoplastic agents in both transformed and nontransformed cells.

This raised the question, how does FKBP51 regulate cellular response to these therapeutics? We found that overexpression of FKBP51 resulted in a reduced phosphorylation of Akt at Ser473, but had no effect on the phosphorylation of Thr308 (Figure 2A). There was no visible difference in Akt phosphorylation with or without gemcitabine treatment. However, downregulation of FKBP51 resulted in increased Ser473 phosphorylation, with no effect on Thr308 phosphorylation (Figures 2B and S1A). Furthermore, hyperphosphorylation of Ser473 was observed in FKBP51$^{-/-}$ MEFs (Figure 2C). These results suggest that FKBP51 negatively regulates Akt phosphorylation, which might account for its effects on cell survival.

To further confirm that FKBP51 regulates Akt activity, we examined the phosphorylation of downstream substrates of Akt, such as GSK-3β and FOXO1. We found that overexpression of FKBP51 decreased the phosphorylation of GSK-3β (pSer9 GSK-3β) and FOXO1 (pThr24 FoxO1) (Figure 2A, lower panels), which was consistent with decreased Akt phosphorylation. In contrast, downregulation of FKBP51 significantly increased the phosphorylation of GSK-3β and FOXO1 (Figure 2B, lower panels). These results confirmed that FKBP51 inhibits Ser473 phosphorylation and Akt activity.

Because FKBP51 specifically regulates Ser473 phosphorylation, but not Thr308 phosphorylation, it is likely that FKBP51 regulates signaling events that directly control Akt phosphorylation

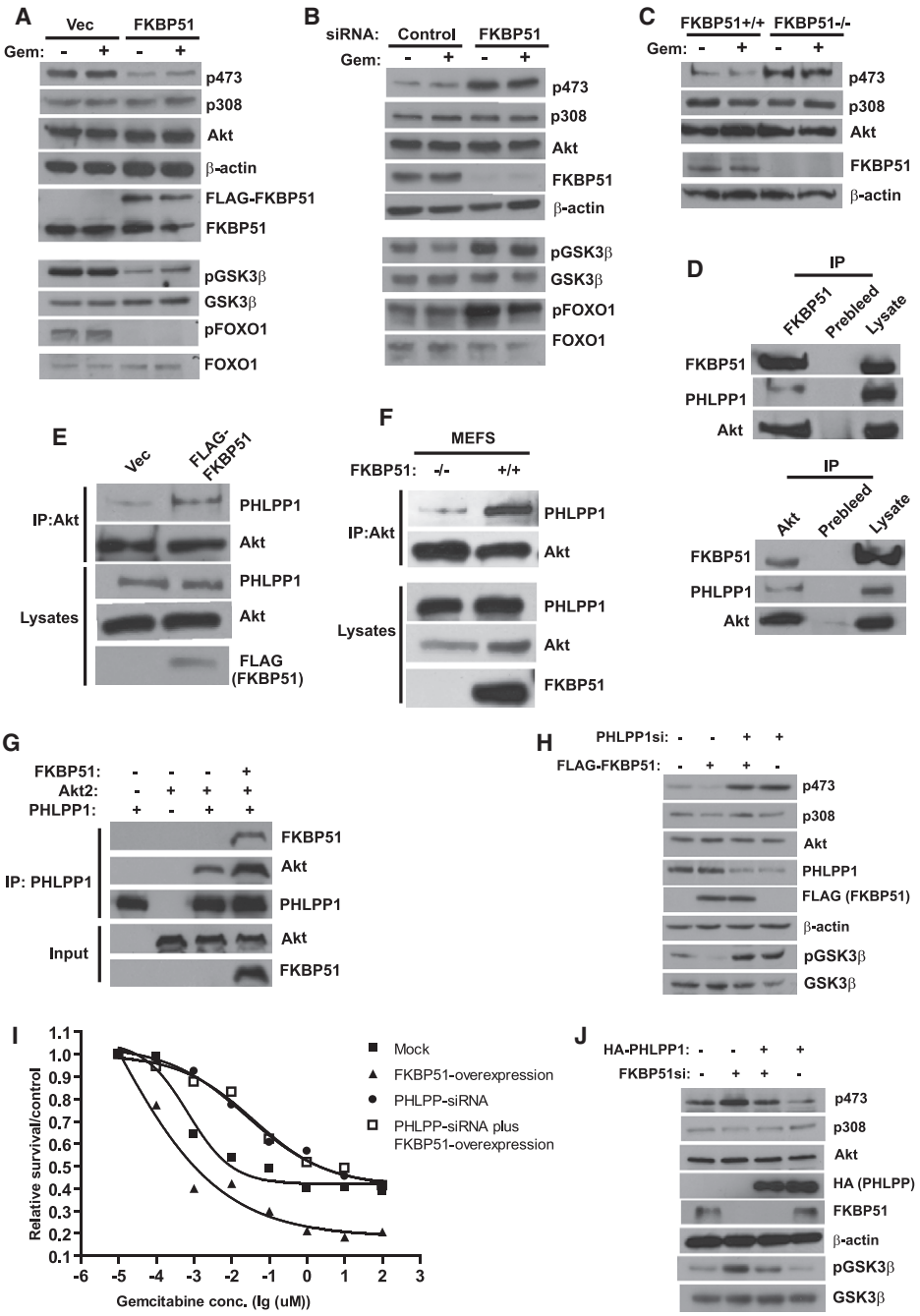

Figure 2. FKBP51 Regulates Akt Phosphorylation at Ser473 by Promoting Akt-PHLPP Interaction

(A) SU86 cells were transfected for 48 hr with indicated constructs. Cells were treated with dimethyl sulfoxide or gemcitabine (Gem, 20 nM, 12 hr), and the phosphorylation of Akt, FOXO1, and GSK-3β in cell lysates was detected by western blot.

(B) SU86 cells were transfected with indicated siRNA. Cells were then treated and harvested as in (A).

(C) The phosphorylation of Akt in cell lysates from FKBP51$^{+/+}$ or FKBP51$^{-/-}$ cells was examined.

(D) The coimmunoprecipitation of Akt, PHLPP, and FKBP51 was examined.

(E) 293T cells were transfected with indicated constructs, and the interaction between Akt and PHLPP was examined.

(F) The PHLPP-Akt interaction was examined in FKBP51$^{+/+}$ and FKBP51$^{-/-}$ cells.

(G) Purified recombinant Akt, PHLPP1, and FKBP51 were incubated in vitro as indicated. The Akt-PHLPP interaction was then examined by coimmunoprecipitation.

(H and I) SU86 cells were transfected with FLAG-FKBP51 and/or PHLPP siRNA as indicated. The phosphorylation of Akt and GSK-3β, and sensitivity to gemcitabine, were then examined. Points show mean values for three independent experiments; error bars represent ± SEM.

(J) SU86 cells were transfected with HA-PHLPP and/or FKBP51 siRNA as indicated. The phosphorylation of Akt and GSK-3β was then examined.

at Ser473. The Ser473 of Akt is specifically phosphorylated by mTORC2 and dephosphorylated by PHLPP phosphatases (PHLPP1 and PHLPP2) (Brognard et al., 2007; Gao et al., 2005; Sarbassov et al., 2005). Another FKBP family member, FKBP38, has previously been shown to bind and inhibit mTORC1 activity (Bai et al., 2007). However, we could not detect any interaction between FKBP51 and mTOR. Instead, we found that PHLPP1 and Akt were coimmunoprecipitated with FKBP51 (Figure 2D, upper panels). Similarly, FKBP51 and PHLPP1 were coimmunoprecipitated with Akt (Figure 2D, lower panels). These results suggest that FKBP51, Akt, and PHLPP could exist as a complex in cells.

The findings that FKBP51 interacts with PHLPP and Akt led us to hypothesize that FKBP51 acts as a scaffolding protein that promotes the interaction between Akt and PHLPP, thereby enhancing the dephosphorylation of Akt. To test this hypothesis, we overexpressed FKBP51 and found that the interaction between PHLPP and Akt increased in cells with FKBP51 overexpression (Figure 2E). Furthermore, there was less interaction between Akt and PHLPP1 in FKBP51$^{-/-}$ cells, than in FKBP51$^{+/+}$ cells (Figure 2F). To test whether FKBP51 directly promotes the Akt-PHLPP interaction, we expressed and affinity purified FKBP51, PHLPP, and Akt. As shown in Figure 2G, FKBP51 can increase the interaction between Akt and PHLPP in vitro.

In the absence of FKBP51, Akt becomes hyperphosphorylated at Ser473 due to inefficient binding of PHLPP to Akt, which might contribute to the chemoresistance observed in cells depleted of FKBP51. Indeed, cells expressing AktS473D, which mimics Ser473 phosphorylation, became resistant to gemcitabine (Figure S1B). Similarly, depletion of PHLPP rendered cells resistant to gemcitabine (Figure S1C). These results support the hypothesis that FKBP51 regulates chemoresistance through the Akt pathway.

To further confirm that FKBP51 regulates Akt Ser473 phosphorylation through PHLPP, we overexpressed FKBP51 while downregulating PHLPP. As we demonstrated earlier, overexpression of FKBP51 alone decreased Akt Ser473 phosphorylation and downstream GSK-3β phosphorylation. However, these effects were reversed by reducing PHLPP (Figures 2H and S1D). Overexpression of FKBP51 did not have further effect on Akt Ser473 phosphorylation in cells depleted of PHLPP. Consistent with these observations, although FKBP51 overexpression alone sensitized cells to gemcitabine treatment, downregulation of PHLPP reversed this sensitizing effect (Figure 2I). Furthermore, PHLPP overexpression blocked the effects of FKBP51 knockdown on Akt Ser473 and GSK-3β phosphorylation (Figure 2J). These results establish that FKBP51 regulates Akt Ser473 phosphorylation through PHLPP.

It is possible that FKBP51 not only increases the Akt-PHLPP interaction but also enhances the dephosphorylation of Akt by stimulating PHLPP activity. FKBP51 has peptidylprolyl isomerase activity thus could affect PHLPP activity. However, overexpression of the FKBP51 mutant FD67/68DV, which lacks peptidylprolyl isomerase activity (Barent et al., 1998), had similar effect on Akt Ser473 phosphorylation as wild-type (WT) FKBP51 (Figure S3A), suggesting that FKBP51 regulates Akt phosphorylation in a peptidylprolyl isomerase-independent manner. Although it is possible that the binding of FKBP51 to PHLPP directly stimulates PHLPP activity, we found that overexpression or depletion of FKBP51 did not affect PHLPP phosphatase activity in vitro (Figures S3B and S3C). PHLPP has been shown to regulate the levels of PKC βII by dephosphorylating the hydrophobic motif of PKC βII, resulting in rapid degradation of PKC βII (Gao et al., 2008). However, FKBP51 did not interact with and did not significantly affect the phosphorylation or levels of PKC βII, (Figures S3D and S3E). These results further confirm that FKBP51 regulates Akt Ser473 phosphorylation mostly through its scaffolding function.

Akt has three isoforms (Akt1, Akt2, and Akt3) (Manning and Cantley, 2007). The antibodies that we used in previous experiments recognize all three isoforms. In addition, PHLPP has two isoforms (PHLPP1 and PHLPP2) (Brognard et al., 2007; Gao et al., 2005). Previous studies have established that both PHLPP1 and PHLPP2 dephosphorylate the same hydrophobic phosphorylation motif on Akts (Ser473 on Akt1), but they inhibit Akt signaling differently by interacting with distinct Akt isoforms (Brognard et al., 2007). PHLPP1 specially regulates Akt2 and Akt3, and PHLPP2 regulates Akt1 and Akt3. We next examined whether the isoform-specific effects of PHLPP on Akt are regulated by FKBP51. Consistent with previous studies (Brognard et al., 2007), Akt1 and Akt3 were coimmunoprecipitated with PHLPP2, whereas Akt2 and Akt3 were coimmunoprecipitated with PHLPP1 (Figures 3A and 3B). Overexpression of FKBP51 increased the interaction between both PHLPP isoforms with their corresponding Akt isoforms (Figure 3A), whereas downregulation of FKBP51 decreased these interactions (Figure 3B). These results suggest that FKBP51 facilitates isoform-specific interaction between Akt and PHLPP.

To investigate how FKBP51 enhances the Akt-PHLPP interaction, we generated a series of deletion mutants of FKBP51. As shown in Figure 3C, we found that deletion of either the FKBP1 (residues 1–138) or FKBP2 (138–251) domain abolished the interaction between FKBP51 and Akt, suggesting that both domains are required for this interaction. In addition, we found that the C-terminal TPR domain of FKBP51 was essential for the binding of FKBP51 to PHLPP. These results suggest that FKBP51 binds PHLPP and Akt using distinct domains, consistent with our hypothesis that FKBP51 acts as a scaffolding protein to promote the Akt-PHLPP interaction. If so, we expected that FKBP51 deletion mutants that could not bind either Akt or PHLPP would not enhance the Akt-PHLPP interaction. Indeed, deletion of the FKBP1 domain or the TPR domain abolished the ability of FKBP51 to enhance the Akt-PHLPP interaction (Figure 3D).

To further confirm that the scaffolding function of FKBP51 is important for the regulation of Akt phosphorylation and cell survival, we reconstituted FKBP51$^{-/-}$ MEFs with wild-type FKBP51 (FK), FKBP51 deleted of the FKBP1 domain (ΔN2) or FKBP51 deleted of the TPR domain (ΔC2). As shown in Figure 2, pSer473, pGSK-3β, and pFOXO1 levels were higher in FKBP51$^{-/-}$ cells than in FKBP51$^{+/+}$ cells (Figure 3E). Reconstitution of the WT FKBP51 in FKBP51$^{-/-}$ cells returned the phosphorylation levels of AktSer473, GSK-3β, and FOXO1 to those observed in FKBP51$^{+/+}$ cells, whereas reconstitution with either mutant had no effect. This observation correlated with a decreased Akt-PHLPP interaction in the absence of WT FKBP51 (Figure 3E). Importantly, reconstitution of WT FKBP51 in FKBP51$^{-/-}$ MEFS restored cell sensitivity to

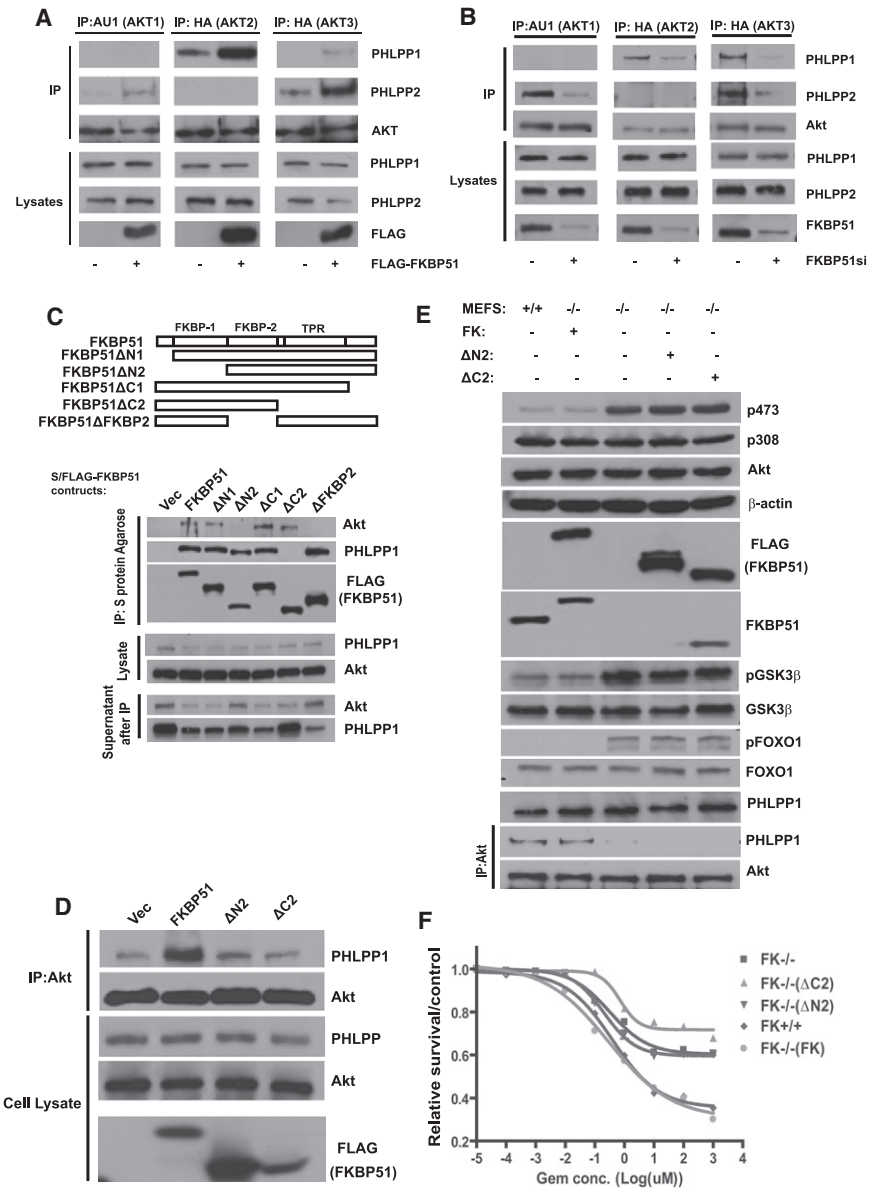

Figure 3. FKBP51 Scaffolding Function Regulates Akt Phosphorylation and Cell Survival

(A) 293T cells or 293T cells stably transfected with FKBP51 were transfected with AKT isoforms (lanes 1 and 2, AU1-AKT1; lanes 3 and 4, HA-AKT2, lane5, 6 HA-AKT3). Cells were lysed, and lysates were subjected to immunoprecipitation with indicated antibodies. PHLPP1, PHLPP2, and AKT in the immunoprecipitates or cell lysates were detected by immunoblotting.

(B) SU86 cells were transfected with control or FKBP51 siRNA together with AKT isoforms. The interaction between Akt isoforms and PHLPP isoforms was then examined as in (A).

(C) 293T cells were transfected with different S/FLAG-tagged FKBP51 truncated mutants. Lysates from transfected cells were subjected to immunoprecipitation with S protein agarose, PHLPP1, and Akt in the immunoprecipitates were then detected by immunoblotting.

(D) 293T cells were transfected with WT FKBP51 or FKBP51 truncation mutations. Transfected cells were then lysed, and lysates were subjected to immunoprecipitation with anti-Akt antibodies. PHLPP1 and Akt in the immunoprecipitates or cell lysates were detected by immunoblotting.

(E and F) FKBP51$^{+/+}$, FKBP51$^{-/-}$, or FKBP51$^{-/-}$ MEFs stably expressing WT or mutant FKBP51 were used to examine Akt, GSK-3β, and FOXO1 phosphorylation as well as the Akt-PHLPP1 interaction (E). Cells were examined for gemcitabine sensitivity using the MTS assay (F). Points show mean values for three independent experiments; error bars represent ± SEM.

gemcitabine to a level similar to that of FKBP51$^{+/+}$ MEFS (Figure 3F), whereas neither mutants had this rescue effect. These results confirmed that the scaffolding function of FKBP51 is important for the regulation of Akt phosphorylation and cell survival.

Because decrease or loss of FKBP51 expression results in Akt hyperactivation, which has been observed in many cancers, it is possible that FKBP51 expression is downregulated in cancer cells. Consistent with this notion, we found that FKBP51 expression is lost or significantly decreased in a high percentage of the pancreatic cancer cell lines and breast cancer cell lines that we examined (Figure 4A). Reconstitution of FKBP51 in Miapaca2 or BxPC3 cells decreased Akt phosphorylation at Ser473 (Figure 4B), and sensitized these cells to Ara-C (Figure 4C), supporting the hypothesis that loss of FKBP51 expression renders these cells resistant to chemotherapy. Furthermore, overexpression of FKBP51 in a pancreatic cancer cell line (Panc0403) having high endogenous levels of FKBP51 did not affect Akt phosphorylation (Figure 4B).

These results suggest that cancer cell lines having high FKBP51 expression might depend less on the PI3K-Akt pathway to survive. To test this possibility, we treated Miapaca2 (low levels of FKBP51 expression) and Panc0403 (high levels of FKBP51 expression) with the PI3K inhibitor wortmannin, together with gemcitabine. As shown in Figure S4A, Miapaca2 cells are more sensitive to PI3K inhibition, consistent with the notion that cells expressing low levels of FKBP51 might be more dependent on the PI3K-Akt pathway. However, these two cell lines have different genetic backgrounds, so factors other than FKBP51 expression might contribute to their different responses to the PI3K inhibitor.

Our results also imply that FKBP51 might function as a tumor suppressor. As an initial step to test this hypothesis, we performed microarray analysis using RNA isolated from 36 pancreatic tumor and 19 normal tissue samples. These were fresh-frozen samples obtained during surgical procedures. The microarray data revealed that expression levels of *FKBP51* were significantly lower in pancreatic tumor tissue than in normal pancreatic tissue (Figure 4D). The comparison of expression profile between normal and tumor tissues identified genes expressed significantly differently ($p < 10^{-6}$) between the two (Figure S4B). Network analysis using Ingenuity Pathway analysis

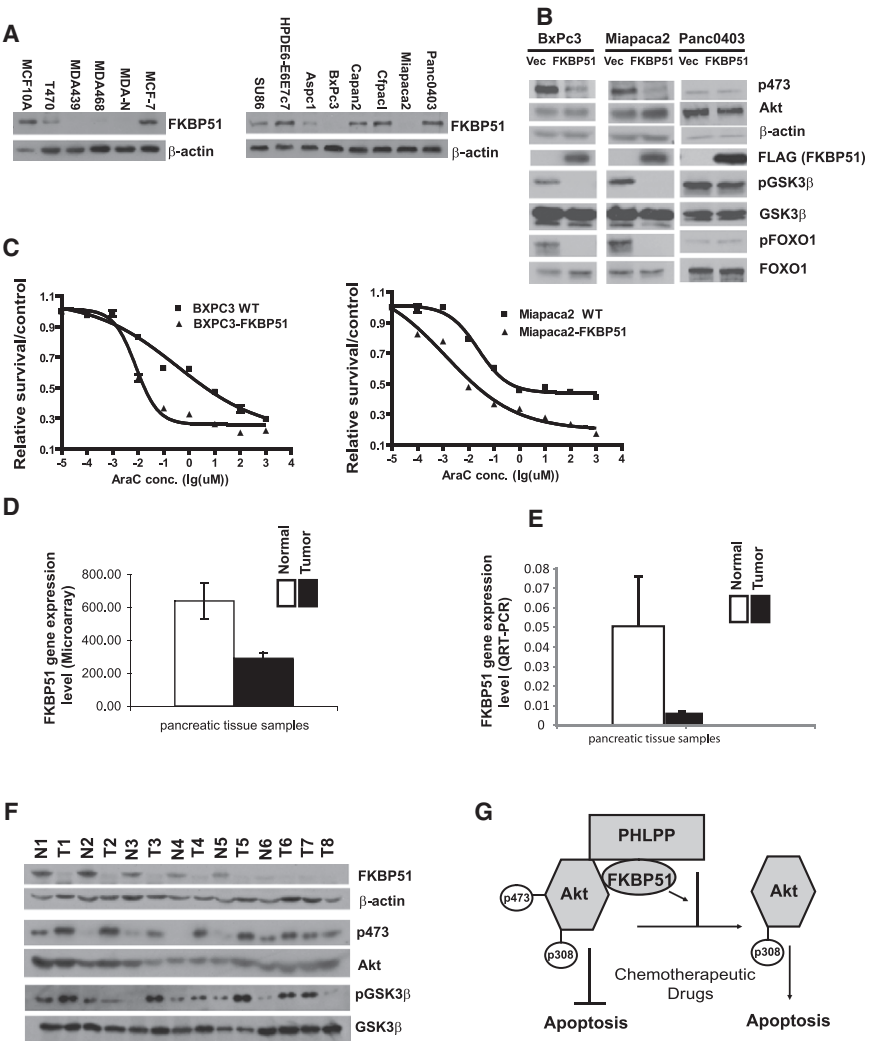

Figure 4. Loss of FKBP51 Expression in Cancer Cells and Tissues

(A) Cell lysates from pancreatic and breast cancer cell lines were blotted with FKBP51 antibodies. Lysates from normal breast (MCF10A) and pancreatic (HPDE6-E6E7c7) epithelial cells were used as controls.

(B and C) Miapaca2, BxPC3, or Panc0403 cells were reconstituted with FKBP51, and Akt phosphorylation and sensitivity to genotoxic stress were then determined. Points show mean values for three independent experiments; error bars represent ± SEM.

(D and E) FKBP51 gene expression in tumor and normal pancreatic tissues was determined using microarray analysis (D, 19 normal pancreatic and 36 tumor tissue samples, $p = 0.0092$) or real-time quantitative RT-PCR (E, 25 pancreatic cancer samples and 12 normal pancreatic tissues, $p = 0.001$). Error bars represent ± SEM.

(F) Western blot of lysates from a subset of tumor and normal tissues. T, tumor; N, normal pancreatic tissue. Numbers 1–8 indicate patient number.

(G) A model illustrates how FKBP51 regulates cell survival through the Akt pathway.

software of the most differentially expressed genes showed that a network surrounding Akt was the top network (Figure S4C). We also performed real-time quantitative reverse-transcriptase polymerase chain reaction (QRT-PCR) to validate the microarray results and found these results to be similar with the correlation coefficient between the QRT-PCR and microarray data (approximately 0.8, $p < 0.0001$) (Figure 4E). Furthermore, lower or loss of FKBP51 protein levels were found in selected pancreatic cancer samples, many of which had increased Akt/GSK-3β phosphorylation (Figures 4F, S4D, and S4E). Decreased expression levels of FKBP51 were also found in ovarian, head and neck, seminoma, leukemia, and prostate cancer tissues based on expression data obtained through the Oncomine (www.oncomine.org). Overall, our results suggest that FKBP51 negatively regulates Akt activation through its scaffolding function and that hyperactivation of Akt caused by the loss of FKBP51 might contribute to tumorigenesis and cancer cell resistance to chemotherapy.

DISCUSSION

Resistance to chemotherapy represents a major challenge for cancer therapy. Therefore, the identification of biomarkers for chemoresistance and understanding mechanisms of chemoresistance will reveal possible strategies to overcome this problem. We have identified FKBP51 as an important determinant of cancer cell response to a wide range of clinically important chemotherapeutic agents. Decreased expression of FKBP51 caused resistance to chemotherapy in cancer cell lines. Mechanistically, FKBP51 acts as a scaffolding protein for Akt and PHLPP, a phosphatase that specifically dephosphorylates Akt Ser473 (Gao et al., 2005), thereby enhancing the phosphatase activity of PHLPP toward Akt.

Because Akt is a major signaling node within the cell, its activity needs to be tightly regulated. Misregulation of the Akt pathway can disrupt the balance between cell survival and death, affecting cancer development and therapy. Indeed, the PI3K-Akt pathway has been linked to resistance to a variety of chemotherapeutics, such as gemcitabine, irinotecan, etoposide, and Taxol (West et al., 2002). Hyperphosphorylation of Akt has also been linked to poor prognosis in a variety of cancers (West et al., 2002). Based on our studies, we expect that lower expression of FKBP51 will also associate with poor prognosis in clinical settings. This hypothesis remains to be validated in the future.

Many regulators of the Akt pathway, such as PI3K, PTEN, and Akt, are mutated in cancers. It is plausible that downregulation or mutation of FKBP51 contributes to not only chemoresistance but also tumorigenesis. We found that FKBP51 expression is lost in many cancer cell lines and pancreatic cancers, supporting a possible role of FKBP51 in tumor suppression. However, the causal role of FKBP51 in tumor suppression remains to be determined.

Our findings will have a significant impact on the dissection of components in the pathway controlling Akt activity. Furthermore, because dysregulation of the Akt pathway is frequently linked to cancer predisposition and poor prognosis, our findings might also have important implications for cancer etiology and response to therapy.

EXPERIMENTAL PROCEDURES

Phosphatase Assay

PHLPP1 was immunoprecipitated with PHLPP1 antibody from the indicated cells. HA-Akt2 was expressed and purified from 293T cells with hemagglutinin (HA) tag antibody and subsequent HA peptide elusion. Immunoprecipitated PHLPP1 was incubated in phosphatase buffer (50 mM Tris [pH 7.4], 1 mM DTT, and 5 mM $MnCl_2$) with purified phosphorylated Akt at 30°C for 0–10 min as previously described (Gao et al., 2005).

MTS Assay

AraC, paclitaxel, and docetaxel were purchased from Sigma-Aldrich (St. Louis, MO) and gemcitabine was provided by Eli Lilly (Indianapolis, IN). Assays with 10-fold diluted concentrations of drugs were performed in triplicate with the CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay (Promega Corporation, Madison, WI). After incubation with drugs for 72 hr, the plates were measured in a Safire2 microplate reader (Tecan AG, Switzerland).

Expression Array Data

Total RNA was extracted from pancreatic tumor tissue samples using QIAGEN RNeasy Mini kits (QIAGEN Inc. Valencia, CA). These samples were obtained during clinically indicated surgical procedures and were consented for experimental purposes. The present study was reviewed and approved by Mayo Clinic Institutional Review Board. RNA quality was tested using an Agilent 2100 Bioanalyzer, followed by hybridization to Affymetrix U133 Plus 2.0 GeneChips (Affymetrix, Inc., Santa Clara, CA). Expression levels were normalized by GCRMA.

Pancreatic Tissue Sample Preparation for Western Blot

Proteins were extracted from portions of the selected fresh-frozen pancreatic tumor and normal tissue, samples that were also used to perform microarray and real time RT-PCR. These samples were consented for research use and the present study was reviewed and approved by Mayo Clinic Institutional Review Board. To extract proteins from these tissues, we froze the tissue samples in liquid nitrogen and cut them into small fragments, followed by incubation on ice in NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40) containing 50 mM glycerophosphate and 10 mM NaF, with a protease inhibitor cocktail. The tissues were milled in a blender for 5 min and lysed on ice for 30 min. The loading buffer for the SDS-PAGE was added and boiled for 20 min. Samples were then centrifuged before loading.

Statistical Analysis

Gene signatures between tumor and normal tissues were determined with the Student's t test for each probe set. Ingenuity Pathway Analysis was performed with the most differentially expressed genes ($p < 10^{-6}$) between normal and tumor tissues by calculating the p values for the probability of finding a set of genes within a given pathway. Fischer's exact test was used to calculate the p values.

ACCESSION NUMBERS

Coordinates have been deposited in the GEO database with the accession code GSE16515.

SUPPLEMENTAL DATA

Supplemental Data include four figures and can be found with this article online at http//www.cell.com/cancer-cell/supplemental/S1535-6108(09)00251-7.

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