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Estrogen receptor–negative (ER-negative) breast cancers are extremely aggressive and associated with poor prognosis. In particular, effective treatment strategies are limited for patients diagnosed with triple receptor–negative breast cancer (TNBC), which also carries the worst prognosis of all forms of breast cancer; therefore, extensive studies have focused on the identification of molecularly targeted therapies for this tumor subtype. Here, we sought to identify molecular targets that are capable of suppressing tumorigenesis in TNBCs. Specifically, we found that death-associated protein kinase 1 (DAPK1) is essential for growth of p53-mutant cancers, which account for over 80% of TNBCs. Depletion or inhibition of DAPK1 suppressed growth of p53-mutant but not p53-WT breast cancer cells. Moreover, DAPK1 inhibition limited growth of other p53-mutant cancers, including pancreatic and ovarian cancers. DAPK1 mediated the disruption of the TSC1/TSC2 complex, resulting in activation of the mTOR pathway. Our studies demonstrated that high DAPK1 expression causes increased cancer cell growth and enhanced signaling through the mTOR/S6K pathway; evaluation of multiple breast cancer patient data sets revealed that high DAPK1 expression associates with worse outcomes in individuals with p53-mutant cancers. Together, our data support targeting DAPK1 as a potential therapeutic strategy for p53-mutant cancers.

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Death-associated protein kinase 1 promotes growth of p53-mutant cancers

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Estrogen receptor–negative (ER-negative) breast cancers are extremely aggressive and associated with poor prognosis. In particular, effective treatment strategies are limited for patients diagnosed with triple receptor–negative breast cancer (TNBC), which also carries the worst prognosis of all forms of breast cancer; therefore, extensive studies have focused on the identification of molecularly targeted therapies for this tumor subtype. Here, we sought to identify molecular targets that are capable of suppressing tumorigenesis in TNBCs. Specifically, we found that death-associated protein kinase 1 (DAPK1) is essential for growth of p53-mutant cancers, which account for over 80% of TNBCs. Depletion or inhibition of DAPK1 suppressed growth of p53-mutant but not p53-WT breast cancer cells. Moreover, DAPK1 inhibition limited growth of other p53-mutant cancers, including pancreatic and ovarian cancers. DAPK1 mediated the disruption of the TSC1/TSC2 complex, resulting in activation of the mTOR pathway. Our studies demonstrated that high DAPK1 expression causes increased cancer cell growth and enhanced signaling through the mTOR/S6K pathway; evaluation of multiple breast cancer patient data sets revealed that high DAPK1 expression associates with worse outcomes in individuals with p53-mutant cancers. Together, our data support targeting DAPK1 as a potential therapeutic strategy for p53-mutant cancers.

Introduction
Breast cancer is the second most frequent cancer (excluding non-melanoma skin malignancy) and second most common cause of cancer-related death among women in the United States (1). Clinically, breast cancers are subtyped according to their estrogen receptor (ER) status. The ER-negative subtype accounts for 30% to 40% of all breast cancers and is typically associated with worse prognosis (2, 3). To date, few effective targeted treatments are available for ER-negative breast cancer, and in particular, cancers that are both ER-negative and progesterone receptor–negative (PR-negative) and Her2-negative (triple receptor-negative breast cancer [TNBC]). Multiple large-scale sequencing efforts have demonstrated that p53 is the most commonly mutated gene in TNBCs, with up to 80% carrying mutations, predominantly nonsense and frame-shift mutations (4–6). To identify novel molecular targets for ER-negative breast cancer, particularly the more aggressive TNBC, we previously conducted a human kinome screen to identify kinases differentially expressed in ER-positive and ER-negative breast cancers (7). Four subtypes of ER-negative disease were defined: cell-cycle checkpoint, MAPK, immunomodulatory, and S6 kinase groups. Of these 4 groups, the S6 kinase group of breast cancers has the worst prognosis. The death-associated protein kinase 1 (DAPK1) is one of the kinases most upregulated within the S6 kinase group. Because upregulation of a cell death–inducing gene was paradoxically associated with ER-negative cancers, this gene was selected as the focus of the current study.

DAPK1 belongs to a family of kinases that includes DAPK2, DAPK3, DAP kinase–related apoptosis-inducing protein kinase 1 (DRAK1), and DRAK2 (8). DAPK1 is a calcium/calmodulin–regulated (CaM-regulated) protein kinase that activates death signaling in response to IFN-γ, TNF-α, and TGF-β, among others (9–11). Recent studies have shown that DAPK1 can transduce death signaling through p53-dependent pathways (12). Proteins such as p21 and p53 have been shown to serve as substrates for DAPK1 (13). In response to stimuli (e.g., apoptotic inducers, oncogenes), DAPK1 expression is increased, the protein is activated by desphosphorylation of Ser308, and activation of p53 occurs through the p14/ p19ARF pathway, ultimately resulting in apoptosis (12, 14). In addition to regulating apoptosis, DAPK1 has also been reported to be involved in autophagy, immune response to inflammatory signals (15, 16), and even proliferative signaling (17). However, the specific role of DAPK1 in ER-negative and, particularly, in p53-mutant breast cancer has not been previously studied. We hypothesize that in the p53-WT setting, DAPK1 serves as a death-inducing factor, while in the p53-mutant background, this protein switches roles to function as a critical growth promoter.

Results
DAPK1 expression is significantly increased in ER-negative breast cancer. To determine the spectrum of DAPK1 expression across breast cancers, we first compared DAPK1 RNA and protein levels in cell lines and in patient breast tumor expression data sets. As shown in Figure 1, A and B, ER-negative breast cancer cells tended to express higher levels of DAPK1 than ER-positive cell lines. In 4 publicly
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Figures:

A. mRNA expression of DAPK1 in different cell lineages.

B. Protein expression of DAPK1 and β-Actin.

C. DAPK1 expression in ER-positive and ER-negative samples.

TCGA dataset:
Sample: 368 samples (95-ER neg, 273-ER pos)

Curtis dataset:
Sample: 1944 samples (440-ER neg, 1504-ER pos)

E. DAPK1 tissue array IHC

Fisher’s exact P = 0.03

10% negative
90% negative
36% negative
64% positive

p53-positive tumor
p53-negative tumor
of DAPK1 in the regulation of cell growth, we performed siRNA suppression that depends upon p53 status. The quantification of DAPK1 expression levels in noninvasive, ER-positive, and ER-negative cell lines is shown in the right panel, with results reported as average ± SEM. Two-tailed Student’s t test was used for statistical analysis. (C) mRNA expression of DAPK1 in ER-positive compared with ER-negative breast tumors in TCGA data set (368 samples) (4). (D) mRNA expression of DAPK1 in ER-positive compared with ER-negative in the Curtis data set (1944 samples) (20). See also Supplemental Figure 1. DAPK1 expression data were grouped according to clinical receptor status annotations, and DAPK1 expression was compared using box-and-whisker plots and presented on a log2 scale. Two-tailed Student’s t test was used for statistical analysis. (E) Immunohistochemical detection of DAPK1 and p53 in human patient tissue microarray tumor cores and a comparison of the proportion of patients with or without p53 staining associated with DAPK1 levels (right panel). Original magnification, ×40. Comparison of immunohistochemical staining was evaluated using Fisher’s exact test.

Figure 1. DAPK1 is differentially expressed in ER-negative and ER-positive breast cancers. mRNA and protein expression of DAPK1 in ER-positive and ER-negative breast cell lines and tumors. Fifteen human breast cell lines and 2 data sets were used to analyze the expression of DAPK1 in breast tumors. (A) mRNA expression of DAPK1 in 15 human breast cell lines. Each cell line was done in triplicate, and 2-tailed Student’s t test was used for statistical analysis. (B) Protein expression of DAPK1 in 15 human breast cell lines. The quantification of DAPK1 expression levels in noninvasive, ER-positive, and ER-negative cell lines is shown in the right panel, with results reported as average ± SEM. Two-tailed Student’s t test was used for statistical analysis. (C) mRNA expression of DAPK1 in ER-positive compared with ER-negative breast tumors in TCGA data set (368 samples) (4). (D) mRNA expression of DAPK1 in ER-positive compared with ER-negative in the Curtis data set (1944 samples) (20). See also Supplemental Figure 1. DAPK1 expression data were grouped according to clinical receptor status annotations, and DAPK1 expression was compared using box-and-whisker plots and presented on a log2 scale. Two-tailed Student’s t test was used for statistical analysis. (E) Immunohistochemical detection of DAPK1 and p53 in human patient tissue microarray tumor cores and a comparison of the proportion of patients with or without p53 staining associated with DAPK1 levels (right panel). Original magnification, ×40. Comparison of immunohistochemical staining was evaluated using Fisher’s exact test.
We next investigated whether DAPK1 regulates anchorage-independent growth of breast cancer cell lines using inducible DAPK1 knockdown MDAMB 231, MDAMB 468, HCC1143, and MCF7 cells (Figure 4B). The addition of Doxy significantly suppressed anchorage-independent growth of HCC 1143, MDAMB 468, and MDAMB 231 cells (all p53-mutant), by 70%, while anchorage-independent growth of MCF7 (p53-WT) cells remained unaffected (Figure 4B). Soft agar assays of vector control cell lines were also performed to eliminate potential effects of the vector backbone (Supplemental Figure 2, A and B, right panels). These results, combined with those shown in Figure 4A, indicate that suppression of DAPK1 inhibits growth of p53-
DAPK1 inhibitor specifically suppresses p53-mutant breast cancer growth. To further assess whether DAPK1 is a critical growth regulator of p53-mutant breast cancer cell lines, we blocked DAPK1 signaling in a panel of breast cancer cell lines using a DAPK1-specific inhibitor and measured cell growth. We had tested the specificity of this inhibitor using Invitrogen’s LanthaScreen TR-FRET Kinase Assay to determine the concentration of drug required for inhibition of 40 kinases. We found that this DAPK inhibitor is relatively specific for DAPK1, with an IC_{50} of 218 nM. Other enzymes inhibited by higher concentrations of this inhibitor include BRAF (IC_{50} 668 nM), KDR (VEGFR2) (IC_{50} 926 nM), and DAPK3 (IC_{50} 1060 nM). The p53-mutant TNBC cell lines (HCC1143, HCC1937) showed dose-response suppression, with more than 50% growth suppression being observed with the addition of 1 μM DAPK inhibitor (Figure 4C). Several other p53-mutant breast cancer cell lines (HCC1954, MDAMB 231, and MDAMB 468) displayed a similar dose response (Supplemental Figure 2E). In contrast, p53-WT cell lines (MCF7, MDA175-VII) showed no growth suppression following addition of DAPK1 inhibitor (Figure 4C).

Inhibition of DAPK1 suppresses p53-mutant but not p53-WT breast cancer growth in vivo. Since suppression of DAPK1 inhibits both anchorage-dependent and -independent growth of p53-mutant breast cancer cell lines, we investigated whether suppression of DAPK1 inhibits xenograft growth in vivo using a nude mouse model. For these experiments, we used inducible DAPK1 knockdown cell lines (MCF7, MDAMB 231, MDAMB 468) and their corresponding vector controls. Mice injected with the MCF7 cell line received estrogen pellets to support tumor growth. After tumors reached 20 to 50 mm^3 in volume, mice were randomized into 2 groups, with one group receiving Doxy water and the other group (the control group) receiving Doxy-free water. As shown in Figure 4D, where tumor size is depicted as a function of time, comparison of tumor growth rates with and without Doxy in inducible DAPK1 knockdown MDAMB 231 and MDAMB 468 xenografts showed strong suppression associated with Doxy.
associated with loss of DAPK1 (Figure 4D). Conversely, growth rates of vector control MDAMB 231, MDAMB 468, and MCF7 cell lines were not affected by Doxy treatment (Supplemental Figure 2, F–H, respectively). There was no significant difference in tumor growth rates observed in inducible DAPK1-knockdown MCF7 cells with or without Doxy (Figure 4D). These results are consistent with our in vitro studies and demonstrate that suppression of DAPK1 inhibits growth of p53-mutant breast tumors in vivo.

To assess the tumor-suppressive effect of the DAPK1 inhibitor in vivo, we utilized the p53-mutant MDAMB 231 xenograft model in nude mice. After injection of MDAMB 231 cells, mice were randomized to receive vehicle or DAPK1 inhibitor (20 mg/kg) by i.p. injection when tumors reached 50 mm³. As shown in Figure 4E, comparison of tumor growth in vehicle and DAPK1 inhibitor (20 mg/kg) groups showed a significant reduction in tumor growth rate upon DAPK1 inhibition by the pharmacological inhibitor. This result is consistent with our in vitro kinase assays demonstrating that in vitro–synthesized DAPK1 is capable of phosphorylating itself and its known substrate MBP, while DAPK1 inhibitor blocks this phosphorylation (Supplemental Figure 2I).

DAPK1 inhibition suppresses growth of other cancer types that carry p53 mutations. Given the evidence that DAPK1 plays an
essential role in the growth regulation of p53-mutant breast cancers, we next investigated whether DAPK1 is critical for the growth of other p53-mutant cancers, including pancreatic and ovarian cancers. Two p53-mutant cancer cell lines (PANC-1 [pancreatic] and SKOV-3 [ovarian]) and 1 p53-WT cancer cell line (H1650 [lung]) were used in this study to determine the effect of DAPK1 inhibition on growth. Two different strategies were used to inhibit DAPK1 activity: siRNA knockdown and inhibition by a small molecule inhibitor. We found that the growth of p53-mutant cell lines (PANC-1 and SKOV-3) was suppressed by DAPK1-targeting siRNAs (Figure 5, B and C, respectively), while growth of the p53-WT cell line (H1650) was unaffected (Figure 5A). We also treated cancer cells with the DAPK1 inhibitor and measured cell growth. The p53-mutant cancer cell line PANC-1 showed dose-response growth suppression with a GI50 (half maximal inhibitory concentration) of 1 μM (Figure 5E). Conversely, the growth of the p53-WT cell line (H1650) was not suppressed by DAPK1 inhibitor (Figure 5D). These results are generally correlated with the levels of DAPK1 expression across the cell lines (Figure 5F) and extend our previous findings in breast cancers, indicating a similar role for DAPK1 function in p53-mutant pancreatic and ovarian cancers.

Collectively, these data show that different types of p53-mutant cancer cells are uniquely sensitive to DAPK1 inhibition, suggesting that DAPK1 is a promising target for the treatment of multiple cancer types that carry mutant p53.

DAPK1 suppression does not affect apoptosis in p53-mutant breast cancer cells. Given the evidence that depletion of DAPK1 suppresses growth of p53-mutant breast cancer cells, we investigated the mechanism by which DAPK1 regulates cell growth. To determine whether inhibition of growth was due to increased apoptosis of p53-mutant cancer cells upon suppression of DAPK1, we performed FACS analysis on the apoptotic rate of p53-mutant inducible knockdown cell lines (MDAMB 231, MDAMB 468, and HCC1143) before and after Doxy treatment. Our results showed no difference in apoptotic rates following DAPK1 suppression in all tested cell lines (Supplemental Figure 3), indicating that growth inhibition induced by the DAPK1 suppression in p53-mutant breast cancer cells is not a result of increased apoptosis. Due to its known role in activating autophagy (16), we also investigated whether DAPK1 could be acting as a negative regulator of autophagy in the setting of TNBC. However, knockdown of DAPK1 in these cells did not alter autophagy marker (LC3-II) levels in TNBC cells (data not shown). Since DAPK1 responds to death-inducing factors under normal conditions, we sought to determine whether p53 mutation alters cellular sensitivity to such agents. After 48 hours of exposure to the DAPK1 inducer, TNF-α, p53-WT MCF7 cells robustly activated an apoptotic program (which is reversed by DAPK1 knockdown), while p53-mutant HCC143 cells did not (Figure 6I), indicating that p53-mutant cells have uncoupled the DAPK1-associated death-signaling pathway from its growth-promoting function.

Reverse-phase protein array identifies downstream proteins regulated by DAPK1. We conducted reverse-phase protein array (RPPA) analysis using the inducible knockdown cell lines (MCF7 inducible DAPK1 MDAMB 231 inducible DAPK1, MDAMB 468 inducible DAPK1, and HCC1143 inducible DAPK1) before and after addition of Doxy to identify changes in proteins and phospho-proteins resulting from DAPK1 suppression (Supplemental Figure 4A and Supplemental Table 4). MCF7 was used as a DAPK1 inhibition–resistant control. The expression of 161 protein and phospho-proteins before and after Doxy addition in MCF7, HCC1143, MDAMB 231, -
and MDAMB 468–inducible knockdown cell lines was plotted as hierarchical clusters (Figure 6A). To compare changes in protein expression and signaling in breast cancer cells upon DAPK1 knockdown, we calculated the change in expression for each protein affected by Doxy treatment (depicted as a hierarchical cluster, Figure 6A). Those proteins whose expression significantly changed upon DAPK1 depletion in p53-mutant HCC1143 cells that were not changed (or inversely changed) in p53-WT MCF7 cells are listed in Supplemental Table 5.

DAPK1 regulates p53-mutant breast cancer tumorigenicity through the mTOR pathway. The phosphorylation of several proteins was found to be downregulated after DAPK1 depletion in p53-mutant breast cancer cells. These included phospho-S6 (at S235/236 and S240/244), phospho-S6K (at T389), and phospho-4EBP1 (at S65), all critical proteins in the mTOR-signaling pathway. Western blot detection of phospho-S6 and phospho-S6K in HCC1143 cells. (B) Level of phospho-S6 at Ser235/236 and Ser240/244 and of phospho-S6K at T389 before and after DAPK1 knockdown in HCC1143 cells. (E) Expression of phospho-S6 at Ser235/236 and Ser240/244 and of phospho-S6K at T389 before and after DAPK1 knockdown in MCF7 cells. (D) Growth and levels of phospho-S6 at Ser235/236 in HCC1143 ctrl and DAPK1 overexpression cell lines. Western blot analysis was done 3 times. A representative IP Western blot and quantification of TSC2/TSC1 ratio is shown. (F) Co-IP of TSC1/TSC2 complex using TSC1 antibody in HCC1143 before and after DAPK1 knockdown. This experiment was done 3 times. A representative IP Western blot and quantification of TSC2/TSC1 ratio are shown. (G) Co-IP of TSC1/TSC2 complex using TSC1 antibody in HCC1143 before and after DAPK1 overexpression. This experiment was done 3 times. A representative IP Western blot is shown, and quantification of the TSC2/TSC1 ratio is shown. (H) Phosphorylation of TSC2 by DAPK1 in vitro kinase assay. (I) Level of phospho-TSC2 at Ser939 in HCC1143 control and HCC1143 DAPK1 overexpression cell lines. All lysates were collected 4 days after treatment. Western blot was performed 3 times. A representative blot and quantification of phospho-TSC2/TSC2 are shown. (I) Fold induction of apoptosis in HCC1143 (p53-mutant) and MCF7 (p53-WT) cells treated with 50 ng/ml TNF-α for 48 hours, measured by FAC5 analysis of annexin V/PI staining. Cell lines were either shEmpty vector control or shDAPK1-induced, as indicated. Each data point represents 3 technical replicates, with results reported as average ± SEM. *P < 0.01; **P < 0.001, 2-tailed Student’s t test.

High DAPK1 expression is associated with worse prognosis in patients with breast cancers that carry p53 mutations. Due to the high level of DAPK1 expression observed in ER-negative breast tumors, we investigated whether DAPK1 is a prognostic marker. We performed survival analyses in multiple breast tumor data sets for which both overall survival and DAPK1 RNA expression are available. Using the van de Vijver (n = 295) (20) and Desmedt (n = 198) (19) data sets and grouping individuals based on tumor DAPK1 expression (with high and low DAPK1 groups reflecting expression above or below the median level, respectively), we investigated whether DAPK1 expression was associated with clinical outcome. As shown in Figure 7A, patients exhibiting high DAPK1 expression...
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in their tumors showed significantly lower overall survival times compared with those with low DAPK1 expression. We next performed a subset analysis to determine whether DAPK1 expression correlated with prognosis specifically within ER-negative breast cancer patients. As shown in Supplemental Figure 5, A and B, ER-negative tumors with high DAPK1 expression carried a worse prognosis. However, the small sample size of ER-negative tumors within each data set did not allow the difference between the 2 DAPK1 expression groups to reach statistical significance (n = 69 and n = 64 for the van de Vijver and Desmedt data sets, respectively; refs. 19, 20). We also performed survival analyses of the publicly available Sørlie (n = 66) (23) and Curtis (n = 755) (18) data sets by stratifying the patients into 4 groups: p53-WT/low DAPK1, p53-WT/high DAPK1, p53-mutant/low DAPK1, and p53-mutant/high DAPK1. For each data set, we performed Kaplan-Meier analyses to determine the association between DAPK1 expression and p53-mutational status with clinical outcome. As shown in Figure 7B, patients with p53 mutations and high DAPK1 expression had worse prognoses compared with p53-mutant/low DAPK1 group or p53-WT cases, suggesting that DAPK1 is a strong prognostic indicator only for p53-mutant breast cancers. Since DAPK1 activates mTOR signaling through S6K, we similarly examined the correlation between survival and a stratification of patients based on combined DAPK1 and S6K expression in the Curtis et al. data set (18) and found that tumors with high S6K/DAPK1 levels also resulted in a poor prognosis (Supplemental Figure 5C).

Discussion

The identification of targeted therapies for the treatment of ER-negative breast cancers, particularly TNBCs, has been a focal point of breast cancer research for years, yet progress has been limited. This is largely due to the heterogeneous mutational landscape of this subtype. Only 2 genes, TP53 and PIK3CA, have mutational frequencies above 6% (4, 5). In this study, we have discovered an unexpected role of DAPK1 in altering the growth of breast cancer cells in a p53-dependent context. In p53-WT cells, we confirm that DAPK1 increases apoptosis in a p53-dependent manner (12). However, in p53-mutant breast cancer cells where DAPK1-induced apoptosis is compromised, DAPK1 increases growth of tumor cells by redirecting its activity through the mTOR/S6 pathway. Our results establish a link between p53-mutation status and cellular sensitivity to DAPK1 inhibition. Pharmacologic inhibition of DAPK1 in p53-mutant, but not WT, cancer cells decreases their growth in mouse xenografts, and DAPK1/p53-mutational status is an independent prognostic indicator in breast cancer patients, suggesting that DAPK1 should be explored as a target for the treatment of p53-mutant cancers, including TNBCs.

DAPK1 has gained notoriety as an established tumor suppressor, in part by acting as the prototypical gene for promoter DNA methylation-induced silencing. Indeed, DAPK1 expression is downregulated in multiple cancer types (24, 25), and it is known to be an important mediator of death-inducing signals (8, 26–28). In particular, TNF-α and IFN-γ activate DAPK1-mediated cell death, possibly through inhibition of NF-κB (29). DAPK1 is reported to both directly transactivate p53 (30) and to be a transcriptional target of p53 (31). This cumulative evidence would suggest that DAPK1 serves as a growth-limiting factor; however, we have shown that in the setting of p53 mutation, DAPK1 has growth-promoting function. Our findings are supported by prior data in MCF7-matched p53-WT/p53-mutant cell lines showing that resistance to TNF-α-induced cell death is associated with p53 mutation (32). Additional studies have convincingly demonstrated that DAPK1 expression is capable of activating the survival functions of the mTOR pathway (17, 33); however, the specific
cellular context (i.e., p53 mutation) was not described. Here, we have established the links among apoptotic initiators, DAPK1-mediated survival, and p53 mutation.

TP53 is a frequently mutated tumor-suppressor gene in human breast cancer (34, 35), as evident from a recent TCGA report demonstrating that 37% of breast tumors harbor p53 mutations. However, the mutation rate of p53 in TNBCs is enriched up to 80% (4). The majority of TNBC-associated p53 mutations are nonsense or frame-shift mutations. Therefore, therapeutic approaches that take advantage of a loss in p53 function are most likely to be effective and the development of such targeted options is critically needed. The low frequency of DAPK1 mutations in TNBC (<1%), our findings that DAPK1 expression is selectively elevated in p53-mutant breast cancers, and the fact that this overexpression correlates with growth and tumorigenicity of p53-mutant breast tumors. How- ever, the mechanism of DAPK1 upregulation remains unknown. The dysfunction of p53 in these cells prevents DAPK1 from inducing apoptosis. A potential caveat is that ER-positive cells appear less sensitive to the synthetic lethal effects of DAPK1 and p53 knockdown for unclear reasons, but this may be due to the known activation of the PI3K and mTOR pathway mediated by ER (37). Our study shows that elevated expression and activity of DAPK1 are critical for the growth and tumorigenicity of p53-mutant breast tumors. However, the mechanism of DAPK1 upregulation remains unknown. Our results inZR-75-1, ER-positive cells suggest that p53-WT may be repressing DAPK1 expression. In any case, elevation of DAPK1 should be explored as a synthetic lethal therapeutic target in p53-mutant breast cancer. Furthermore, a majority of all cancers have p53 pathway alterations. Thus, the clinical impact of the observation that p53 mutation rewires DAPK1 signaling to promote tumor growth, as opposed to death, is greatly expanded and suggests that a significant percentage of all cancer patients would benefit from DAPK1-targeting pharmaceuticals.

**Methods**

**Reagents and plasmids.** Primary antibodies for phospho-S6 (S235/236, catalog 2211), phospho-S6 (S240/244, catalog 2215), S6 (catalog 2317), phospho-S6K (T389, catalog 9205), S6K total (catalog 9202), TSC1 (catalog 6935), TSC2 (catalog 3990), phospho-TSC2 (S939, catalog 3615),

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**Figure 8. Proposed functional switch model of DAPK1 in p53-WT and p53-mutated breast cancer cells.** DAPK1 can be activated by various factors, such as cellular stress and growth signaling. In p53-WT cells, DAPK1 can activate p53, leading to p53 target gene transcription and apoptosis under death-related signaling. Simultaneously, DAPK1 can regulate cell growth by modulating TSC1/TSC2 complex formation in the mTOR pathway. Phosphorylation of TSC2 at Ser939 by DAPK1 reduces interaction between TSC1 and TSC2, leading to increased cell growth and protein synthesis and thereby maintaining a homeostatic balance between survival and death signaling. In contrast, DAPK1 expression is elevated in p53-mutant cells. The dysfunction of p53 in these cells prevents DAPK1 from inducing apoptosis, thereby resulting in a shift of function from apoptosis toward activation of the growth pathways. Therefore, in p53-mutated cells, suppression of DAPK1 strongly suppresses tumor cell growth.
phospho-TSC2 (S1387, catalog 5584), and phospho-TSC2 (T1462, catalog 3611) used in Western blot experiments were purchased from Cell Signaling Technology Inc. Antibody for DAPK1 (catalog 610290) was purchased from BD Transduction Laboratories. Antibody specific for β-actin (catalog A-5441) and DAPK1 (D1319) was purchased from Sigma-Aldrich. Antibodies for TSC2 (37-0500, Life Technology) and TSC1 (MAB4379, R&D Systems) were used in IP experiments. Anti-mouse (catalog NA931V) and anti-rabbit (catalog NA934V) secondary antibodies were obtained from GE Healthcare. IHC antibodies included DAPK1 (catalog SAB4500620, Sigma-Aldrich) and p53 (catalog sc-126, Santa Cruz Biotechnology Inc.). Recombinant human TNF-α (catalog PHC3015) was purchased from Life Technologies, and the DAPK inhibitor was obtained from EMD Millipore (catalog 324788).

Individual pGIPZ lentiviral shRNAs for DAPK1 and p53 were purchased from Open Biosystems Inc. The shRNA oligonucleotide ID is provided in Supplemental Table 1. For the inducible RNAi experiments, shRNAs were subcloned individually into a pTRIPZ lentiviral expression system (Open Biosystems Inc.). For the inducible overexpression experiments, red fluorescent protein (RFP) was replaced with DAPK1 cDNA to generate an inducible overexpression system.

**Cell culture.** The MCF7 (HTB-22), MDAMB 231 (HTB-26), MDAMB 361 (HTB-27), MDAMB 415 (HTB-128), and MDAMB 468 (HTB-132) cell lines were purchased from ATCC and cultured in DMEM (Cellgro by Mediatech Inc.) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. The ZR-75-1 (CRL-1500), HCC1143 (CRL-2321), HCC1937 (CRL-2336), and HCC1954 (CRL-2338) (ATCC) cell lines were cultured in RPMI-1640 (Cellgro by Mediatech, Inc.) supplemented with 10% FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. MCF10A cells (CRL-10317, ATCC) were cultured in DMEM/F12 (Cellgro; Mediatech Inc.) supplemented with 10 μg/ml insulin, 20 ng/ml human EGF, 0.5 μg/ml hydrocortisone, 100 ng/ml cholera toxin (Sigma-Aldrich), 5% horse serum (Gibco Laboratories), 100 U/ml penicillin, and 100 mg/ml streptomycin. Human mammary epithelial cells (HMECs) (Lonza Walkersville Inc., CC-2551) were cultured in mammary epithelial cell growth medium (MEGM) (Lonza Inc.) supplemented with bullet kits. Pancreatic cancer cell line PANC-1 and ovarian cancer cell line SKOV-3 were gifts from Xiangwei Wu, Qiang Shen, and Anil Sood (University of Texas MD Anderson Cancer Center). All cell lines were incubated at 37°C in 5% CO₂. Cells were verified by morphology and DNA fingerprinting with the Cell Line Core Facility (38) and were tested for mycoplasma using PCR.

**Virus production and generation of stable cell lines.** Stocks containing lentiviral particles were generated by cotransfection using FuGENE 6 Transfection Reagent (Roche Applied Science) of HEK293T cells with lentiviral constructs and helping plasmids encoding VSV-G, Gag, Pol, and Tat. Medium overlaying the cells was harvested twice, at 48 and 72 hours after transfection, and was filtered through a 0.45-μm MEF filter. PEG precipitation was used to concentrate the virus. Concentrated virus was aliquoted and stored at −80°C. Stable p53 knockdown cell lines were generated by lentiviral infection using shRNA against p53 followed by puromycin selection. Stable cell lines expressing inducible shRNAs and cDNAs were generated by lentiviral infection using the pTRIPZ lentiviral expression system in the presence of 4 μg/ml polybrene, followed by puromycin selection (2 μg/ml). All pTRIPZ cell lines were maintained in media with Tet-safe Serum (Clontech Laboratories Inc.). Stable cell lines continuously expressing DAPK1 cDNA were generated by retroviral infection using the pBABE-Hygro expression system (Cell Biolabs Inc.) and were followed by hygromycin selection (500 μg/ml).

**Western blot analysis.** Cells lysates were prepared as previously described (39). From 30 to 50 μg total protein extract was run on an 8% to 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare). Membranes were blocked in TBST with 5% BSA for phospho-specific antibodies and with 5% nonfat milk for other primary antibodies. Primary antibodies were used at recommended dilutions according to the manufacturer’s instructions. After incubation with a horseradish peroxidase–conjugated secondary antibody (1:5,000 dilution), detection was performed using a Chemiluminescence Assay Kit (GE Healthcare). Band intensities were quantified by Bio-Rad Quantity One 1-D Analysis Software (Bio-Rad Life Sciences). See complete unedited blots in the Supplemental Material.

**siRNA transfection and cell growth assays.** siRNAs for DAPK1 were purchased from Sigma-Aldrich and pooled. Catalog numbers and sequences are given in Supplemental Table 2. siRNA transfection was performed at a final concentration of 20 nM using DharmaFECT Transfection Reagent (Dharmacon Inc.), according to the manufacturer’s protocol. Transfected cells were plated in 48-well plates at 5,000 cells/well. Cell growth was measured by manual counting using a hemocytometer. Each data point represents 3 technical replicates, with results reported as average number ± SEM.

**Drug treatment.** For experiments using the DAPK1 inhibitor, cells were plated in 48-well plates at 5,000 cells/well and then treated with DAPK1 inhibitor at concentrations ranging from 1 to 1,000 nM (in media with 0.1% DMSO). Media alone with 0.1% DMSO was used as vehicle control. For experiments using the DAPK1 inhibitor and mTOR inhibitor, 1 μM DAPK1 and 10 nM everolimus were used alone or in combination. Cell growth was measured by manual counting using a hemocytometer after 5 days of drug treatment. Vehicle-treated cells were set at 100% growth, and other groups were normalized to vehicle control as percentage of growth of vehicle. Each data point represents 3 technical replicates, with results reported as average number ± SEM.

**Cell growth assay of inducible knockdown cell lines.** Cells were seeded in 6-well plates with or without treatment with Doxy for 2 days. Addition of Doxy induces DAPK1 shRNA expression. Cells were then plated in 48-well plates at 5,000 cells/well with or without Doxy. Cell growth was measured by manual counting. Each data point represents 3 technical replicates, with results reported as average number ± SEM.

**RNA preparation and qRT-PCR.** Total RNA was isolated using the RNeasy Kit (QIAGEN Inc.). A TaqMan assay was designed for DAPK1, and qRT-PCR was performed as previously described (40). Primers and probes are listed in Supplemental Table 3. Cyclophilin was used as an endogenous control. Data were reported as normalized quantity ± SEM.

**Anchorage-independent growth assay.** Anchorage-independent growth assays were performed as previously described (41). Briefly, 10⁴ cells were suspended in 0.375% SeaPlaque GTG Agar (FMC) in DMEM or RPMI-1640 supplemented with 10% FBS and 1% penicillin-streptomycin. Suspended cells were layered over 0.75% agar base in the same medium. Colonies were counted 3 to 4 weeks after plating by GelCount (Oxford Optronix). All experiments were performed in triplicate and repeated 3 times. Data reported represent average colony number ± SEM.

**Nude mouse xenograft experiments.** Xenograft experiments were performed as previously described (42). Briefly, 10 mice per group of BALB/c nude mice (Harlan Teklad) were used for xenograft experi-
ments. Estrogen pellets (Innovative Research of America) were injected into animals to stimulate growth of MCF7 xenografts. The next day, mice from each group were injected in the fat pad with approximately 5 × 10^6 cells of one of a range of cell lines (MCF7 ind-shDAPK1, MDAMB 231 ind-shDAPK1, MDAMB 468 ind-shDAPK1, MCF7 vector control, MDAMB 231 vector control, or MDAMB 468 vector control). After tumors developed and reached the size of 20 to 50 mm^3, mice were randomized to receive Doxy-containing (200 μg/ml) or Doxy-free water to induce or suppress the expression of DAPK1 shRNA, respectively. For inhibitor study, 20 mice were injected with 2 × 10^6 MDAMB 231 cells. After tumors reached the size of 50 mm^3, mice were randomized to receive vehicle (sesame oil) or DAPK1 inhibitor (dose: 20 mg/kg) by i.p. injection. Tumor sizes were measured twice a week as previously described (42). Tumor growth rates of the different groups were compared by linear regression of log-transformed tumor volumes over time using 2-tailed Student’s t test. Mice were sacrificed and tumors harvested in 0.5% NP40 lysis buffer. Precleared extracts were incubated with overnight incubations of the DAPK1 antibody (1:400 dilution) and the p53 antibody (1:200 dilution). For our analyses, the 15 noninvasive cases were excluded. An additional 2 invasive carcinomas were excluded due to nonstaining by any immunohistochemical antibody (including those performed by the manufacturer, such as Ki-67). For the remaining 58 cases (116 cores) only, the first core was utilized for further analysis, although the second core was compared for concordance of staining. DAPK1 was scored by assigning a proportion and an intensity of staining, as outlined by Allred et al. (43), and a score threshold of greater than 2 was considered DAPK1 positive (Supplemental Figure 1F). Staining with p53 was used as a surrogate for p53 mutational status (44) and was scored using a simplified threshold value of 50% positively staining epithelial nuclei as p53 positive.

**Data sets used for DAPK1 expression and survival analysis.** Four publicly available human breast tumor data sets were used for these studies: TCGA (4), Curtis (18), Desmedt (19), and van de Vijver (20). DAPK1 expression was grouped according to clinical receptor status annotations, and groups were compared using 2-tailed Student’s t test. For overall survival analysis, the log-rank (Mantel-Cox) test or Kaplan-Meier analysis was used to determine the statistical differences among stratified groups in Curtis (18), Desmedt (19), van de Vijver (20), and Sorlie (23) data sets. A value of P < 0.05 was considered statistically significant.

**Statistics.** Statistically significant differences between groups of anchorage-independent growth were also determined by 2-tailed Student’s t test. Since tumor growth in vivo was approximately exponential, we compared tumor growth rates among different treatment groups by linear regression of log-transformed tumor volumes over time and compared these growth rates by Student’s t test. Immunohistochemical staining resulted in breast cancer patients segregating into 1 of 4 groups. Comparison of the group proportions was evaluated using Fisher’s exact tests.

**Study approval.** Humane animal use procedures were approved by the University of Texas MD Anderson Institutional Animal Care and Use Committee under the protocol numbers 10-09-12432/06-13-05831 and overseen by the University of Texas MD Anderson Office of Research Administration.

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