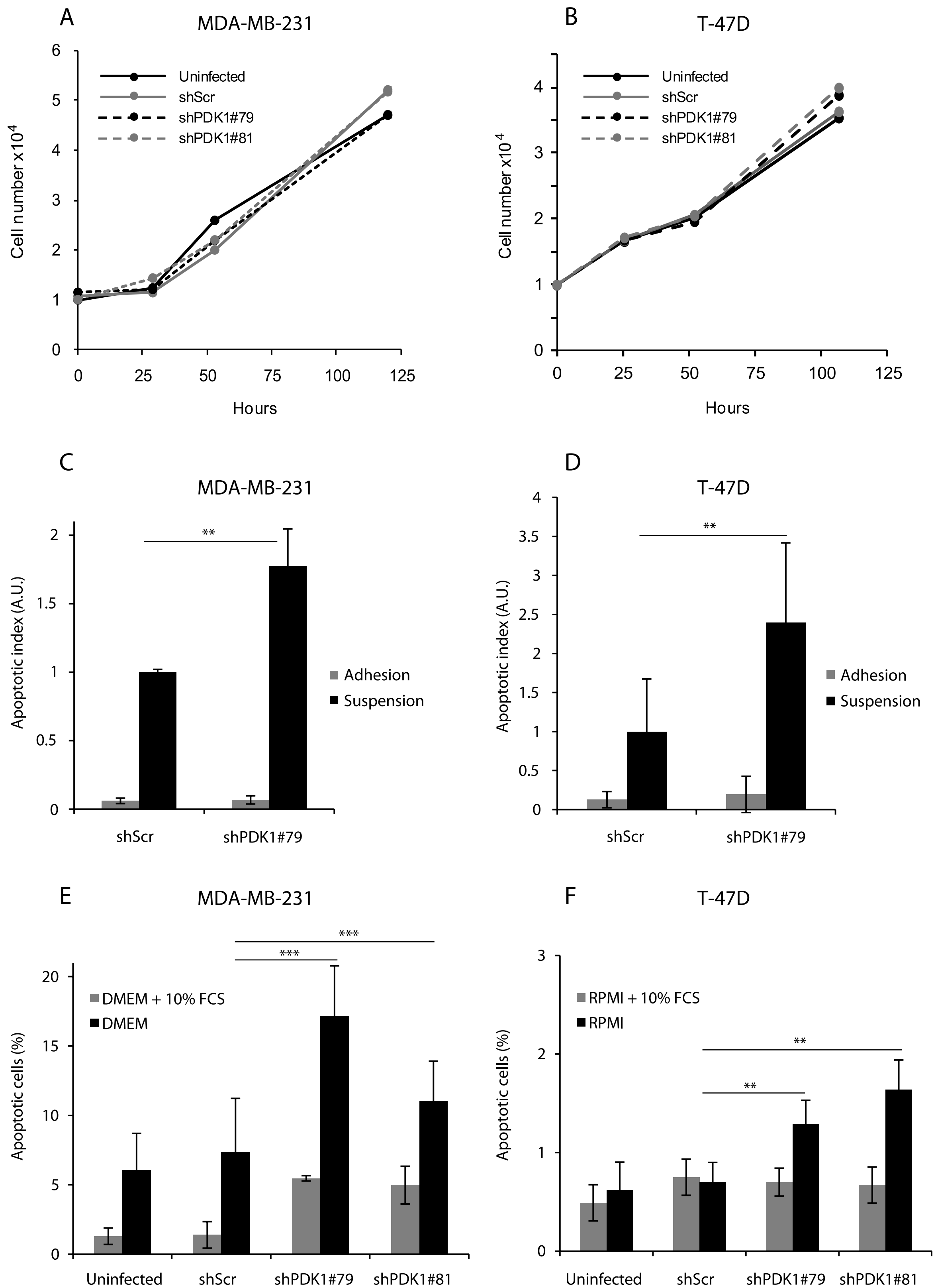

Figure W1. PDK1 knockdown does not affect proliferation of breast cancer cells but increases apoptosis induced by the absence of anchorage. (A and B) Proliferation of the indicated cells plated on plastic dishes was evaluated as cell numbers at the indicated time points. MDA-MB-231 (A) and T-47D (B) cells were transduced with a scramble shRNA (shScr), two separate shRNAs targeting PDK1 (shPDK1#79 and shPDK1#81), or left untransduced (uninfected). (C and D) Apoptosis index of the indicated cells cultured on plastic dishes or maintained in suspension and evaluated by quantifying mononucleosomes and oligonucleosomes. Cells were transduced with a scramble shRNA (shScr), normalized to 1 and compared to cells transduced with shPDK1#79. (E and F) Apoptosis assay of MDA-MB-231 and T-47D transduced as indicated above. Cells were cultured for 48 hours in the presence or absence of fetal calf serum. The percentage of apoptotic cells was evaluated by analyzing the presence of cytokeratin 18 fragment. *P < .05, **P < .01, ***P < .001.

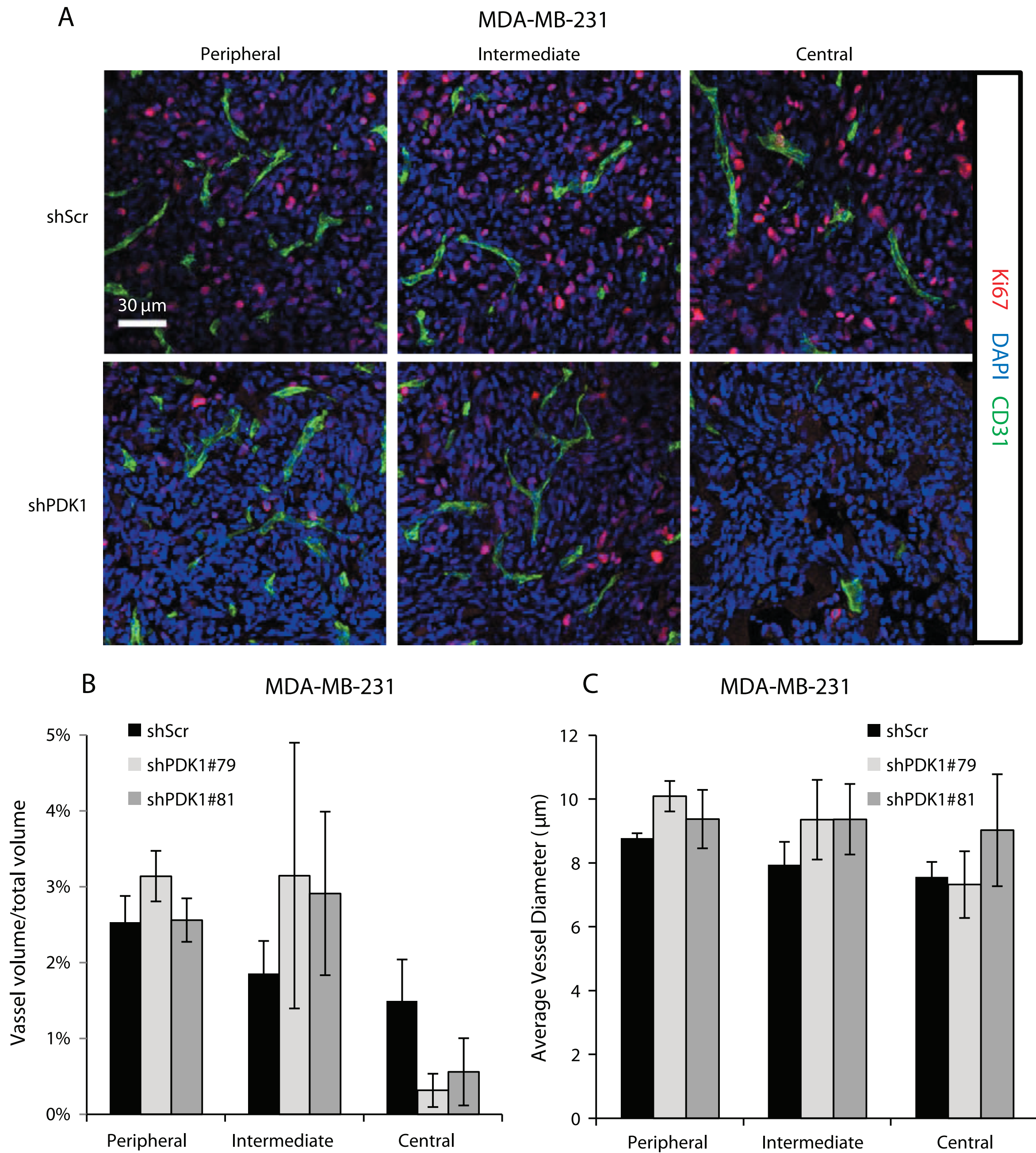

Figure W2. Angiogenesis is not impaired in tumors originating from PDK1-silenced MDA-MB-231 cells. (A) MDA-MB-231 transduced with shRNA targeting PDK1 (shPDK1) or a scramble sequence (shScr) were injected subcutaneously in immunodeficient mice. Tumors were evaluated 4 weeks after injection. Cell proliferation and angiogenesis in the peripheral, intermediate, and central regions of tumors were evaluated in immunofluorescence using an anti–Ki-67 (red) or an anti-CD31 (green) antibody, respectively. Pictures are representative of four different tumors. (B and C) Quantification of angiogenesis in three different tumor regions by calculating the vessel volume compared with the overall tumor mass (B) and by measuring the mean vessel diameter (C).

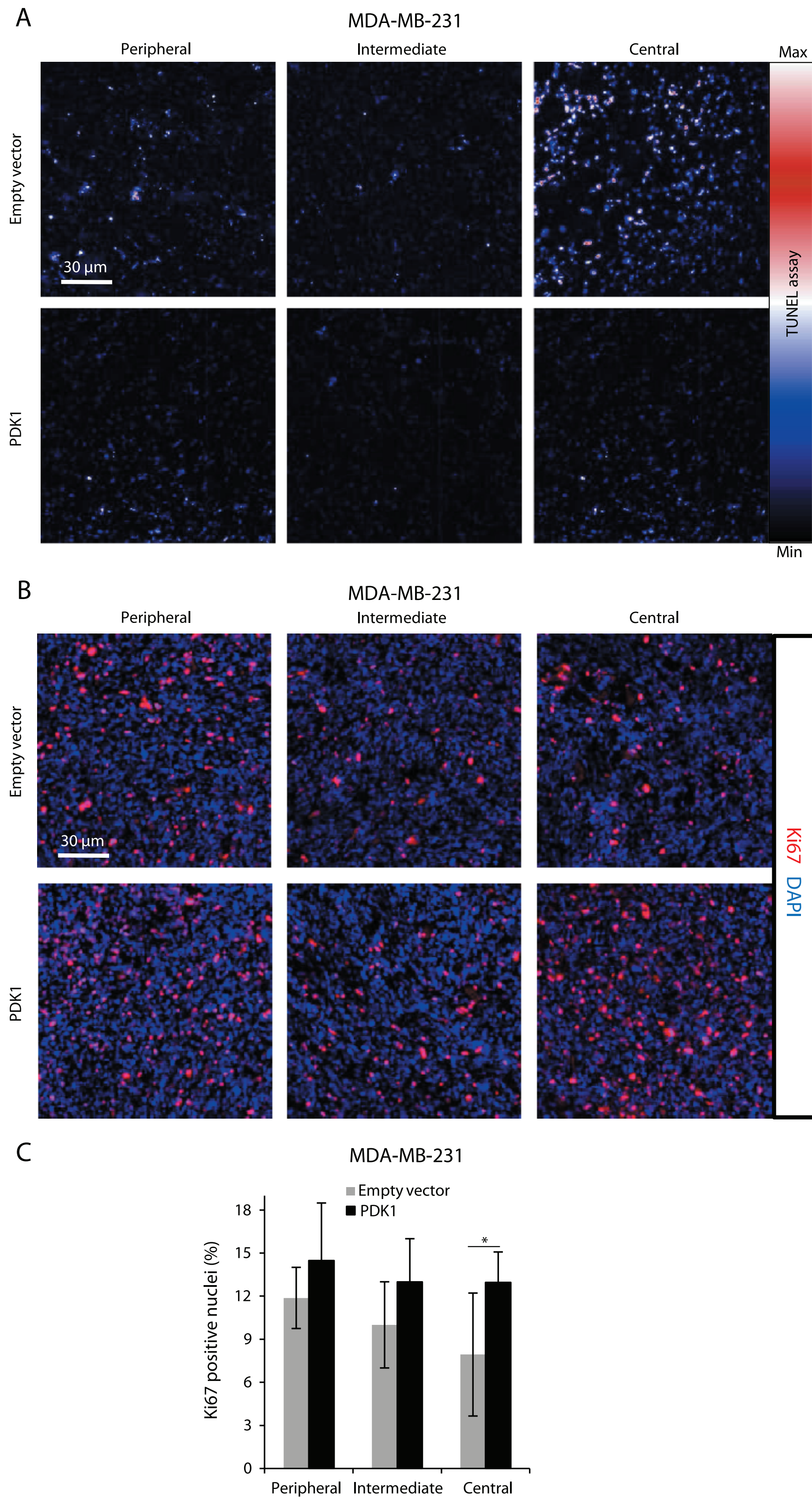

Figure W3. PDK1 overexpression reduces apoptosis and increases cell proliferation in the experimental tumor. (A) Apoptosis within tumors originating from MDA-MB-231 transduced with PDK1 or empty vector was evaluated with TUNEL assays. Representative pictures of three different tumor regions are shown in false colors: black/blue, low fluorescence intensity; white, high intensity. (B) Cell proliferation in the peripheral, intermediate, and central regions of tumors was evaluated in immunofluorescence using an anti–Ki-67 (red) and (C) quantified as Ki-67–positive cell percentage. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$.

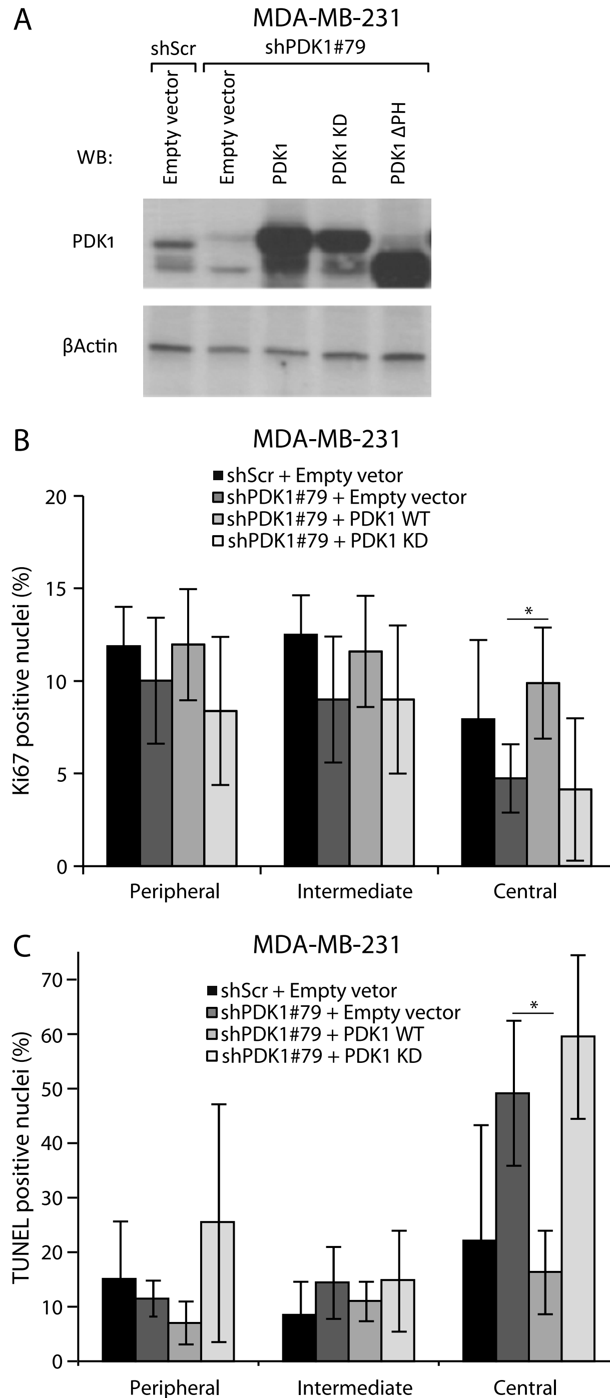

Figure W4. PDK1 regulates proliferation and apoptosis in experimental tumors in a kinase-dependent manner. (A) The expression of PDK1 was evaluated in PDK1-knockdown cells (shPDK1#79) transduced with vectors carrying PDK1 wild-type (PDK1), PDK1 kinase-dead (PDK1 KD), PDK1 PH domain–deleted (PDK1 ΔPH), PDK1 mutated in the PIF-pocket (PDK1 L155E), PDK1 with deletion of 50 C-terminus amino acids (PDK1 Δ50) or empty vector by immunoblot. Tubulin was used as a loading control. (B and C) Quantification of cell proliferation and apoptosis within tumors originating from MDA-MB-231 transduced with scramble shRNA (shScr) or with shRNAs targeting PDK1 (shPDK1#79) and retransduced with PDK1 wild-type (PDK1), PDK1 kinase-dead (PDK1-KD), or empty vector. The percentage of Ki-67–positive (B) or TUNEL-positive cells (C) was reported.

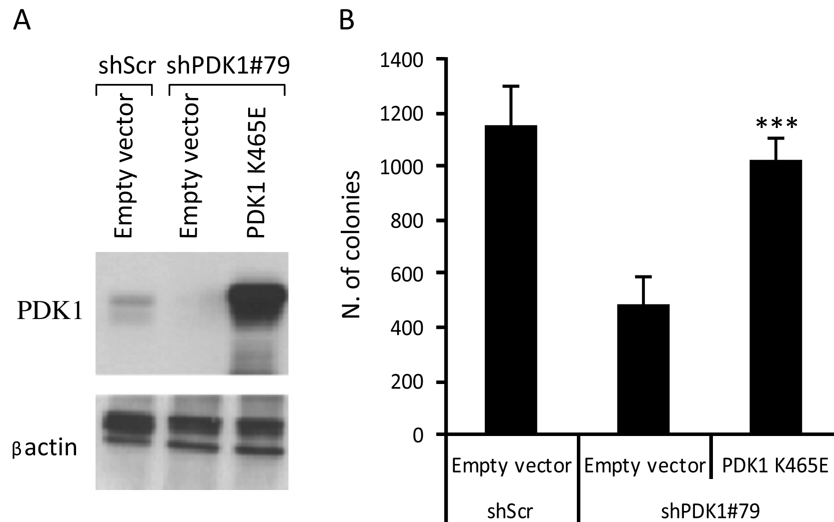

Figure W5. PDK1 mutant in the PH domain rescues anchorage-independent growth. MDA-MB-231 PDK1-knockdown cells (shPDK1#79) as in Figure 1 were transduced with vectors carrying PDK1 mutant in the PH domain (PDK1-K465E) or empty vectors. (A) PDK1 expression levels are shown by immunoblot analysis. (B) Soft agar colony formation assay was performed with the indicated cell lines. Colonies larger than 100 μm diameter were counted. $^{***}P < .001$.

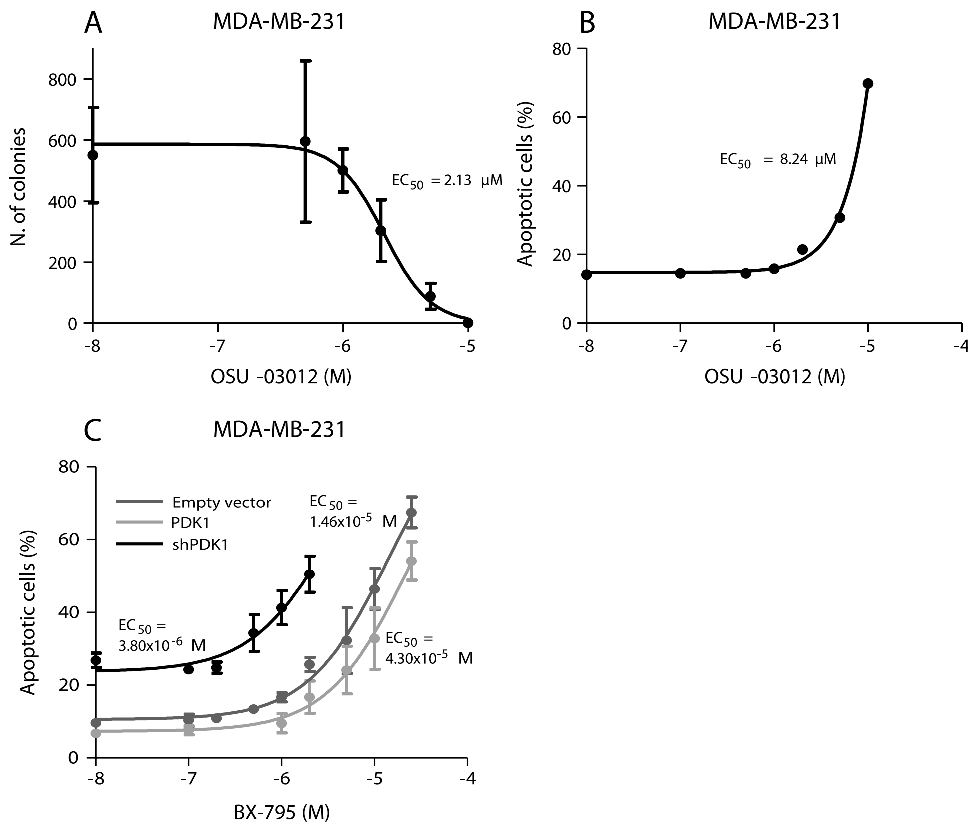

Figure W6. OSU-03012 blocks anchorage-independent growth of MDA-MB-231 and promotes anoikis. (A) Soft agar colony formation assay of MDA-MB-231 treated with different concentrations of PDK1 inhibitor OSU-03012. EC_{50} is indicated. (B) Apoptosis assay of MDA-MB-231 treated with different concentrations of PDK1 inhibitor, OSU-03012. The number of apoptotic cells was evaluated using anti–cytokeratin 18 fragment antibody, and EC_{50} is reported. (C) Apoptosis assay of MDA-MB-231 transduced with empty vector, PDK1, or shPDK1 and treated with different concentrations of PDK1 inhibitor, BX-795. The number of apoptotic cells was evaluated using anti–cytokeratin 18 fragment antibody, and EC_{50} is reported.

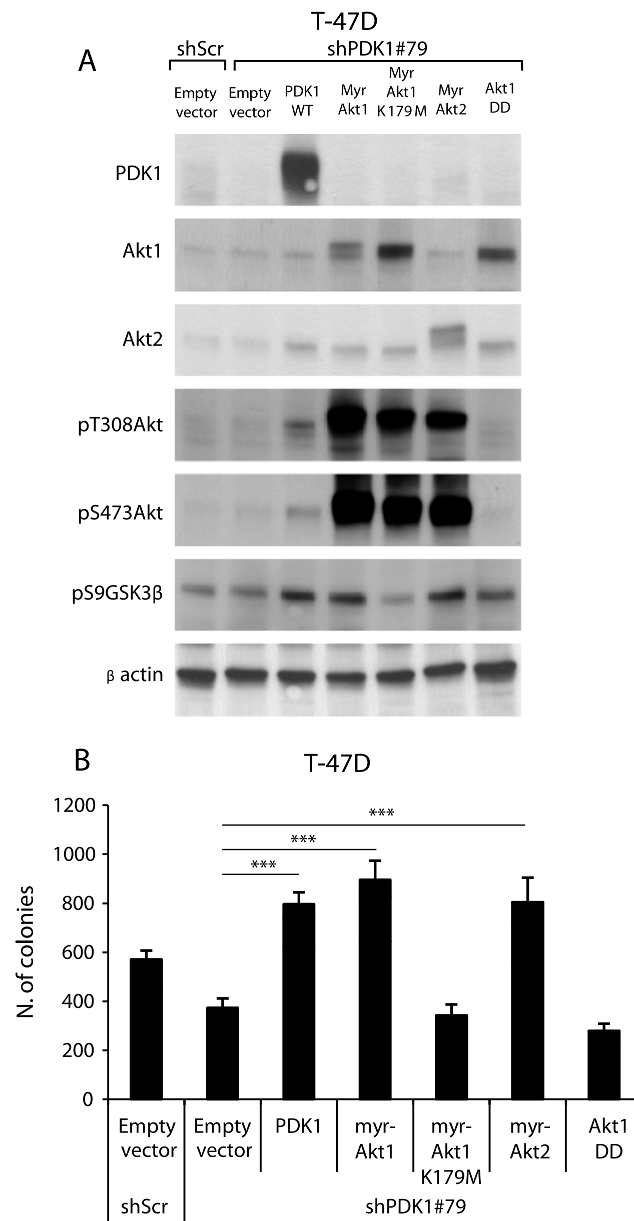

Figure W7. Myr-Akt expression rescues anchorage-independent growth of PDK1 knockdown in T-47D cells. (A) Immunoblot analysis of lysates of T-47D cells transduced with scramble shRNA (shScr) or with PDK1-targeting shRNAs (shPDK1#79) and retransduced with PDK1 wild-type (PDK1), different active mutants of Akt (myr-Akt1, myr-Akt2, Akt1DD), inactive mutant of Akt (myr-Akt1 K179M), or empty vectors. (B) Soft agar colony formation assay performed on T-47D cells transduced as indicated. Colonies larger than 100 μm diameter were counted. ***$P < .001$.