Inhibition of Twist1 mediated invasion by Chk2 promotes premature senescence in p53 defective cancer cells

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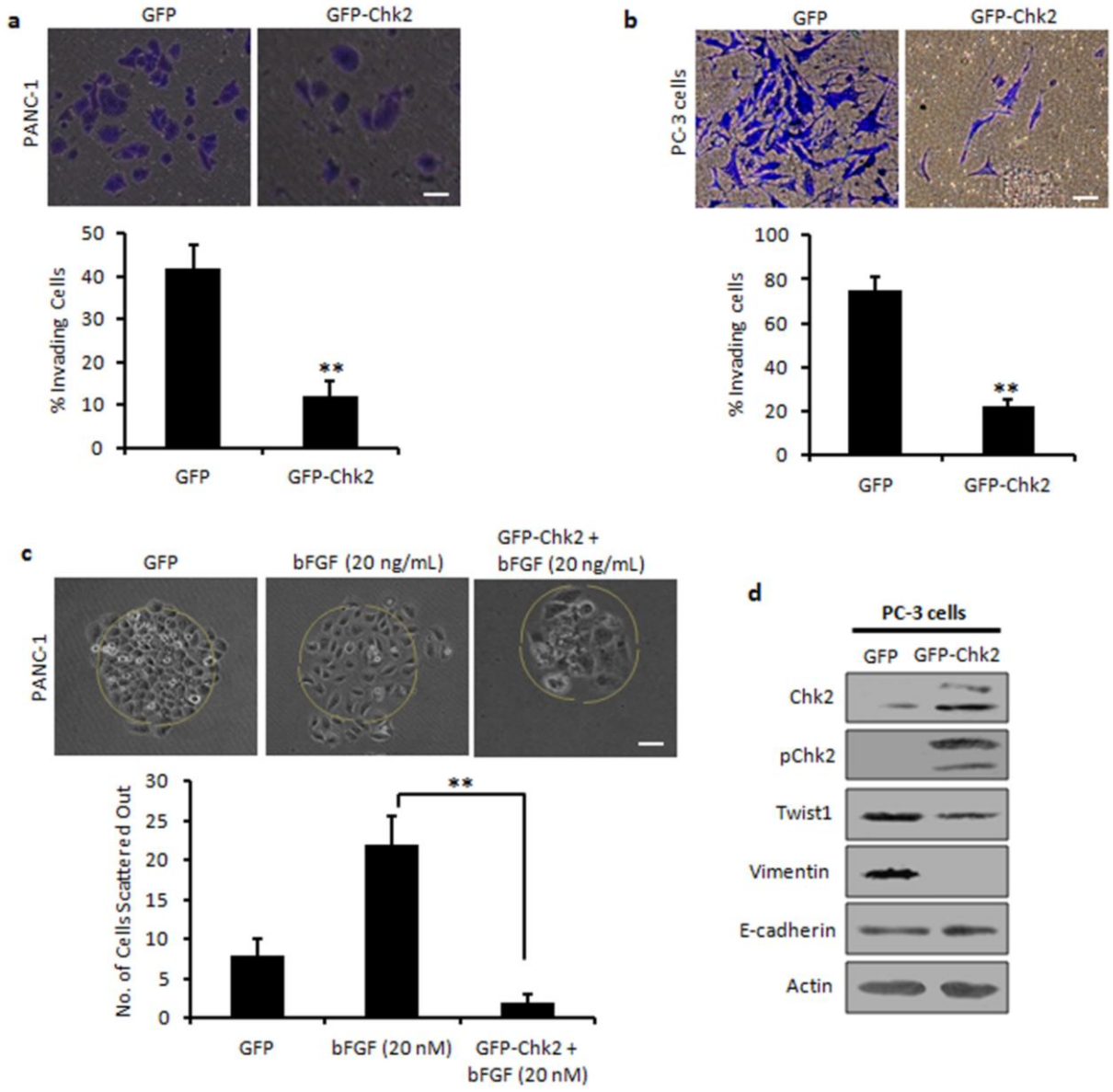

Figure S1. Effect of Chk2 expression on the invasion and scattering of PANC-1 and PC-3 cells. (**a, b**) PANC-1 and PC-3 cells were transfected with either GFP or GFP-Chk2 construct and subjected to matrigel invasion assay. Images were taken at 20x magnification. Scale bar: 20 μm. Bar graph showing the percent invading cells (n = 3, error bars indicate ± s.d.). ***P*<0.01. (**c**) PANC-1 cells were allowed to form distinct colonies, transfected with either GFP and/or GFP-Chk2 and then stimulated in presence of bFGF (20 nM). Cell scattering was observed under an inverted microscope 48 h of post transfection (original magnification 20x). Scale bar: 20 μm. Bar graph represents the number of cells scattered out of the colonies (n =

3, error bars indicate ± s.d.). **P<0.01. (**d**) PC-3 cells were transfected transiently with GFP and GFP-Chk2 construct for 48 h and whole cell lysates prepared were subjected to western blotting for the expression of Chk2, pChk2, Twist1, vimentin, E-cadherin and actin.

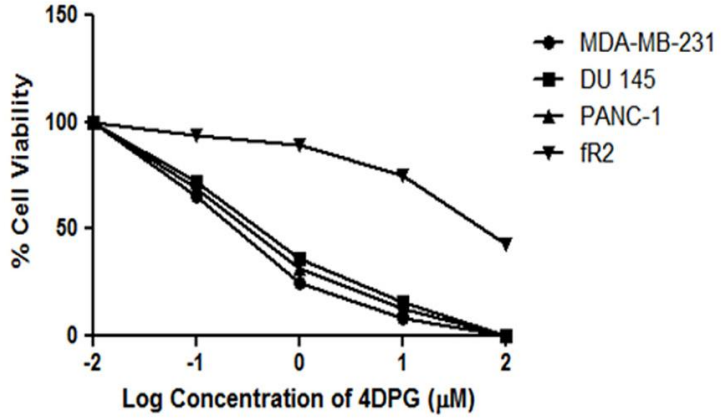

Figure S2. Cytotoxicity of 4DPG. Graph showing percent cell viability against logarithmic concentrations of 4DPG (µM) in various invasive cancer cell lines (MDA-MB-231, DU 145 and PANC-1) and normal breast epithelial (fR2) cells as determined by MTT assay.

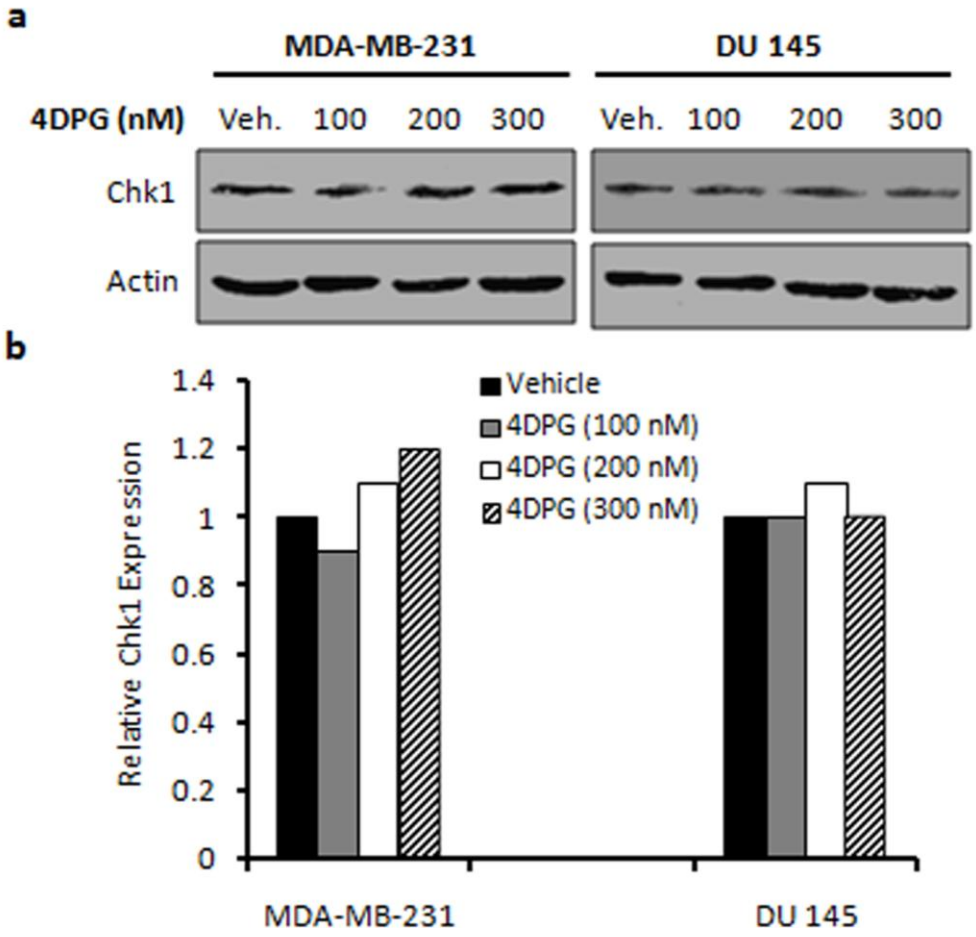

Figure S3. Effect of 4DPG on Chk1 expression. (**a**) MDA-MB-231 and DU 145 cells were treated with 4DPG (100, 200 and 300 nM) for 48 h. Whole cell lysates were prepared and employed for immunoblotting of Chk1. Actin was taken as a loading control. (**b**) Bar graph showing the densitometric analysis of bands obtained from above blots.

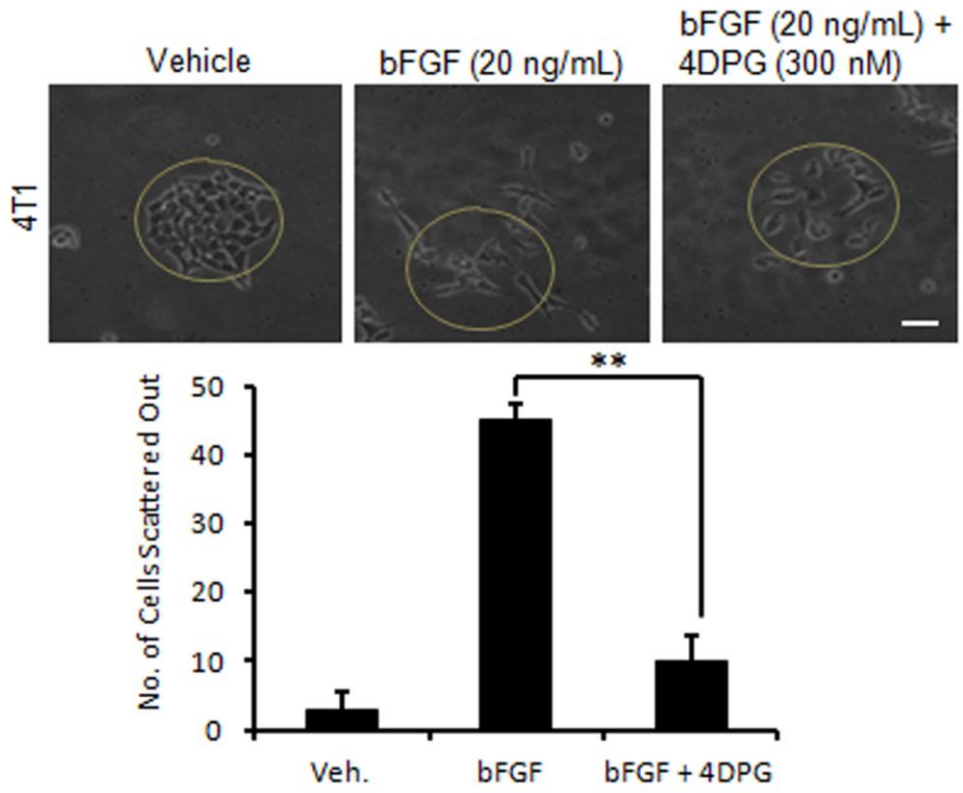

Figure S4. Effect of 4DPG on cell scattering of mouse mammary carcinoma (4T1) cells. Cells were treated with either vehicle or 4DPG (300 nM) in the presence of bFGF (20 ng/mL). Cell scattering was observed 48 h of post treatment under an inverted microscope at 20x magnification. Scale bar: 20 μm. Bar graph represents the number of cells scattered out of the colonies in each case (n = 3, error bars indicate ± s.d.). $^{**}P<0.01$.

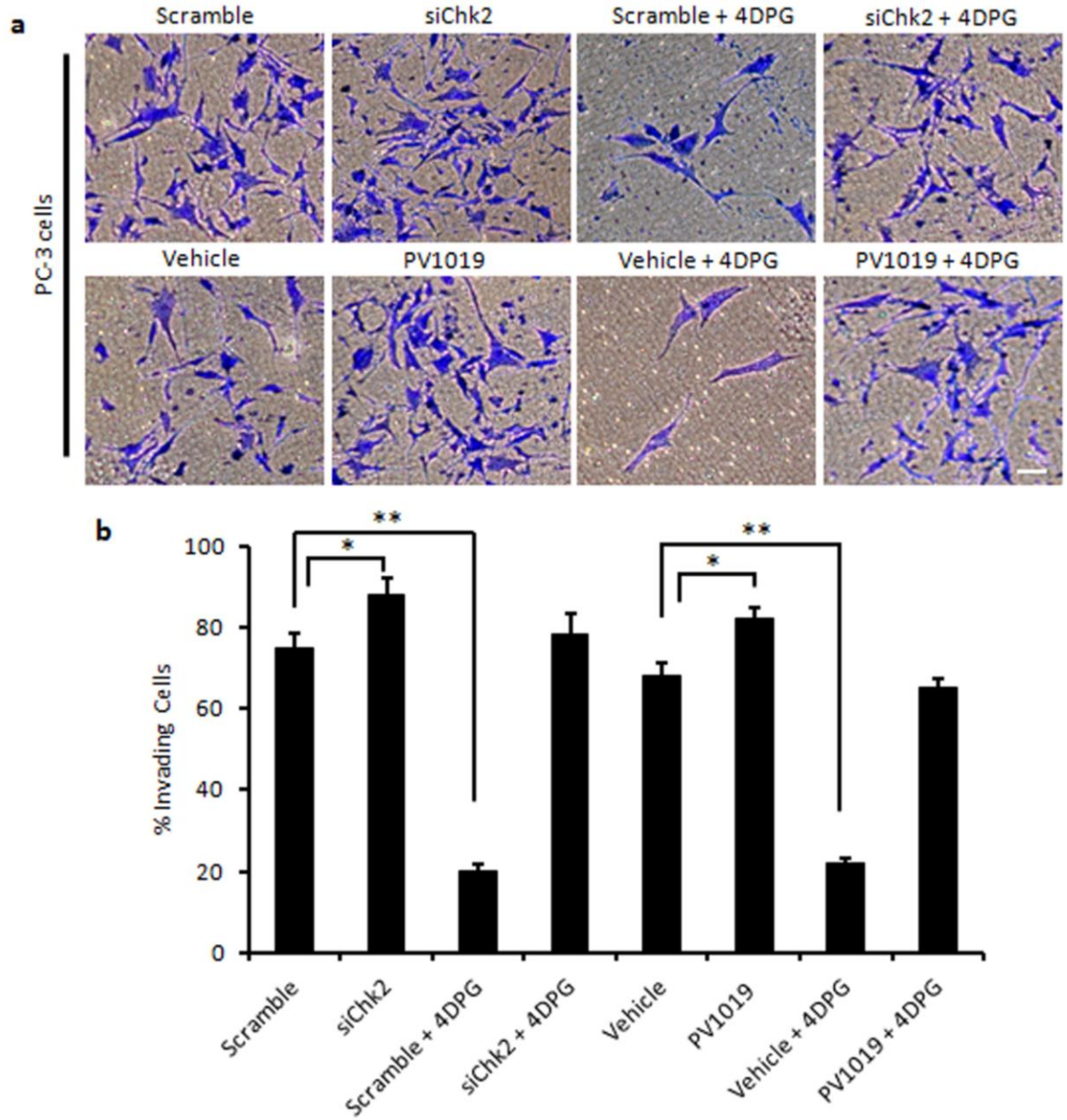

Figure S5. Effect of decreased Chk2 expression on invasion of PC-3 cells. (**a**) PC-3 cells were either transfected with scramble, siChk2 or treated with vehicle, 4DPG (300 nM), PV1019 (1.0 μM) alone each and combination of scramble plus 4DPG, siChk2 plus 4DPG and PV1019 plus 4DPG for 48 h. Cells were then checked for their ability to invade through the matrigel with the help of Boyden chamber invasion assay system. Images were captured

under an inverted microscope at 20x magnification. Scale bar: 20 μm. Bar graph showing the percent invading cells (n = 3, error bars indicate ± s.d.). *$P<0.05$, **$P<0.01$.

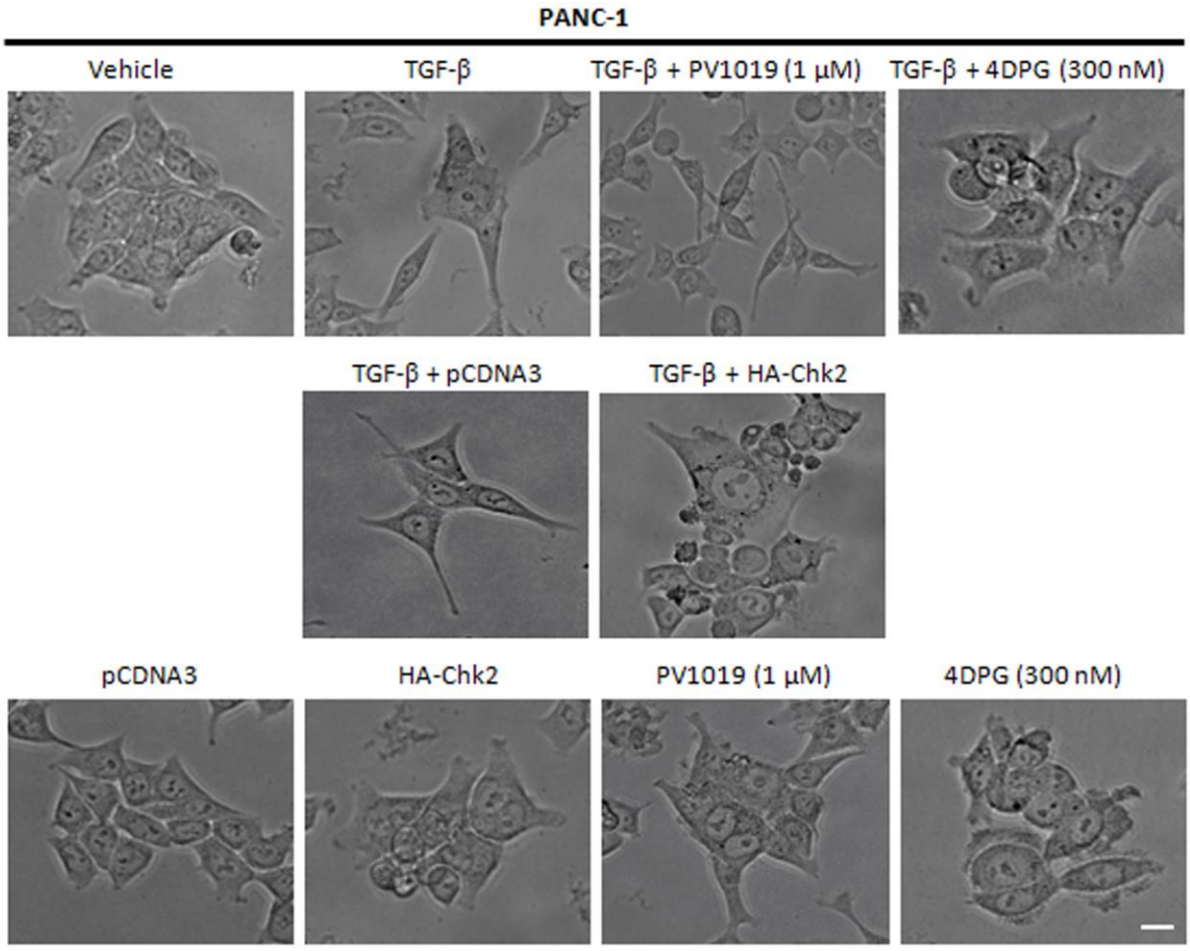

Figure S6. Effect of Chk2 expression on TGF-β induced EMT and morphology of invasive cells. PANC-1 cells were either stimulated with inducer of EMT, TGF-β and/or treated with vehicle, 4DPG (300 nM), Chk2 inhibitor PV1019 (1 μM) or transfected with pCDNA3 and HA-Chk2 for 48 h. Morphological observations were performed with the help of Floid Cell Imaging Station at 20x magnification. Scale bar: 20 μm.

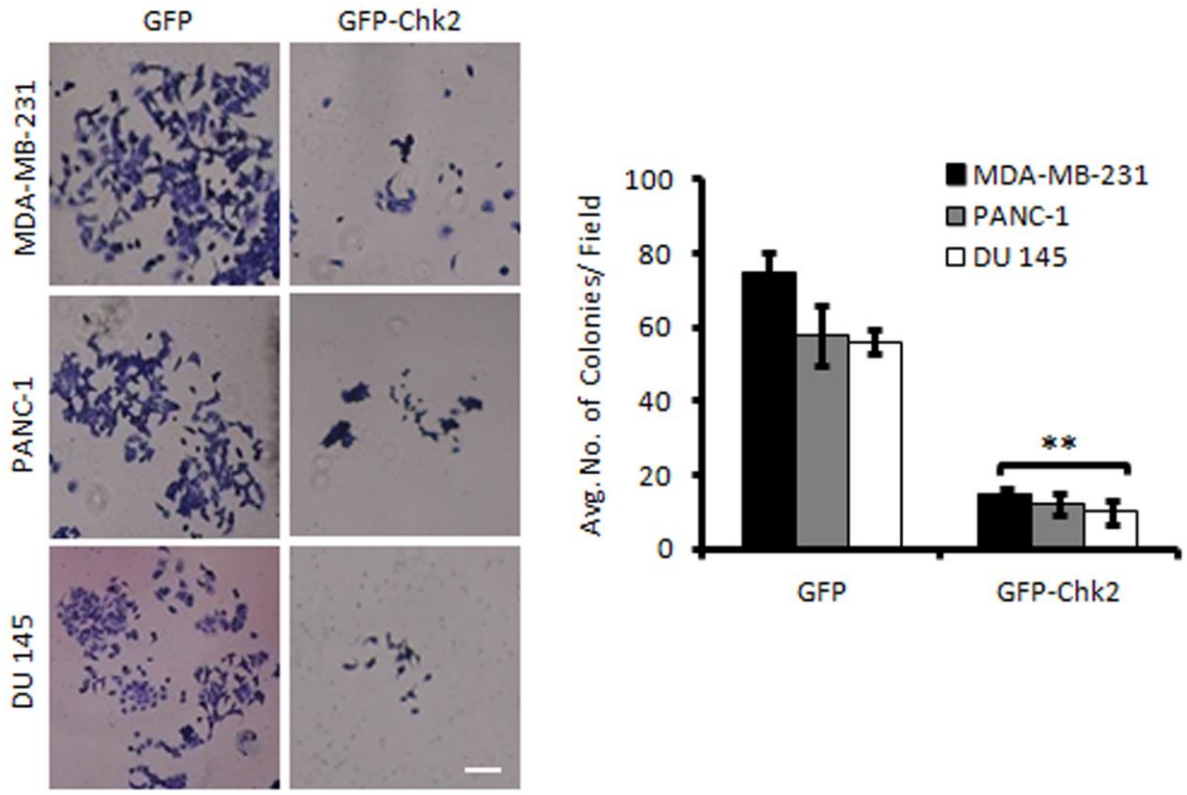

Figure S7. Effect of Chk2 expression on colony formation ability of invasive cells. MDA-MB-231, PANC-1 and DU 145 cells were transfected transiently with GFP and/or GFP-Chk2 construct and employed for colony formation assay to check the effect on cell proliferation (original magnification 4x). Scale bar: 20 μm. Bar graph represents average number of colonies/ field (n = 3, error bars indicate ± s.d.). $^{**}P<0.01$.

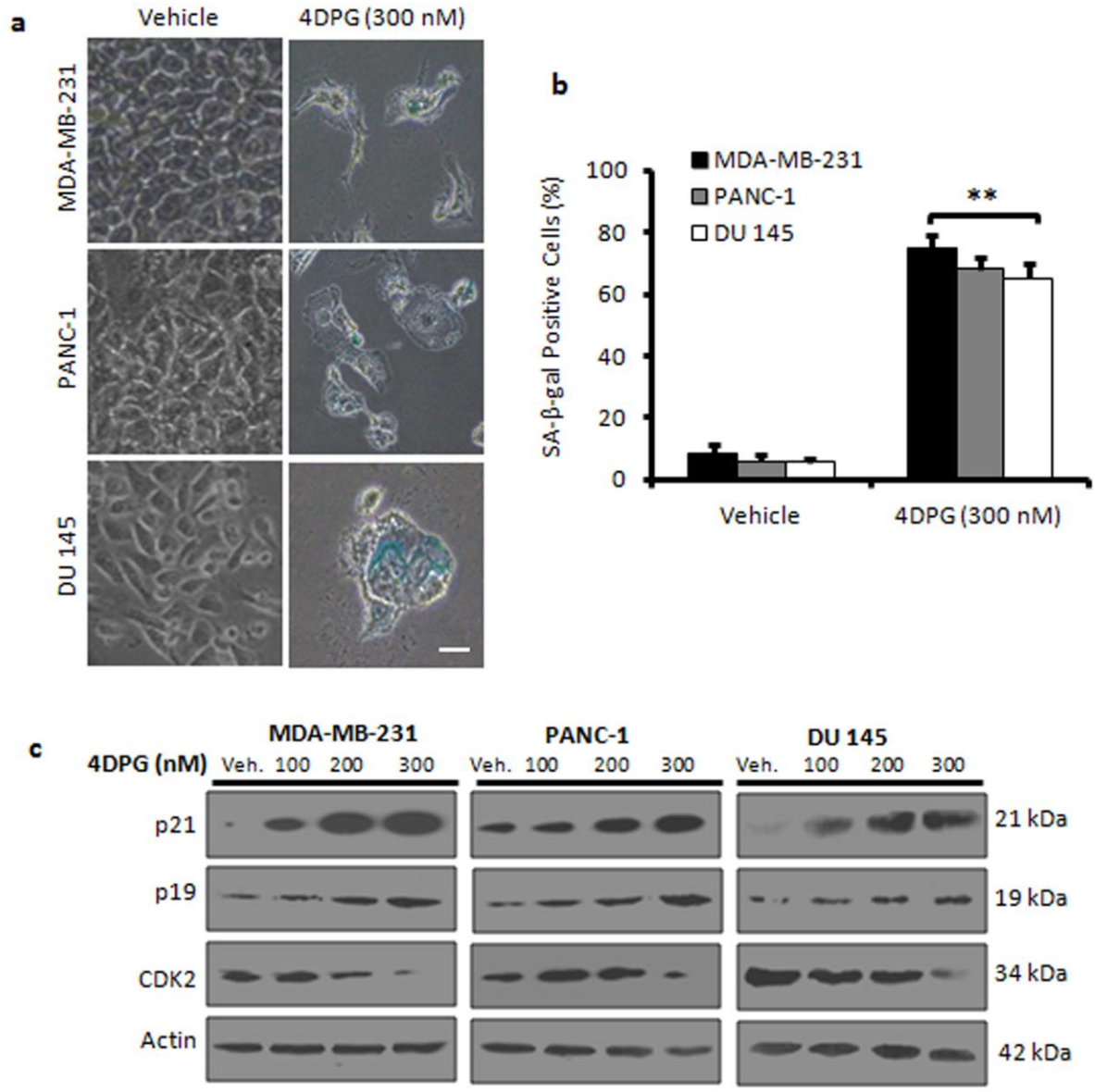

Figure S8. Effect of 4DPG on induction of premature senescence in invasive cancer cells. (**a**) MDA-MB-231, PANC-1 and DU 145 cells were treated with 4DPG (300 nM) for 72 h and then subjected to X-gal staining for SA-β-gal activity. Senescent cells were photographed under an inverted microscope at 20x magnification. Scale bar: 20 μm. (**b**) Bar graph indicates quantification of SA-β-gal positive cells (n = 3, error bars indicate ± s.d.). $^{**}P<0.01$. (**c**) MDA-MB-231, PANC-1 and DU 145 cells were treated with increasing concentrations of 4DPG (100, 200 and 300 nM) for 48-72 h. Whole cell lysates were prepared and employed for western blot analysis of p21, p19 and CDK2.

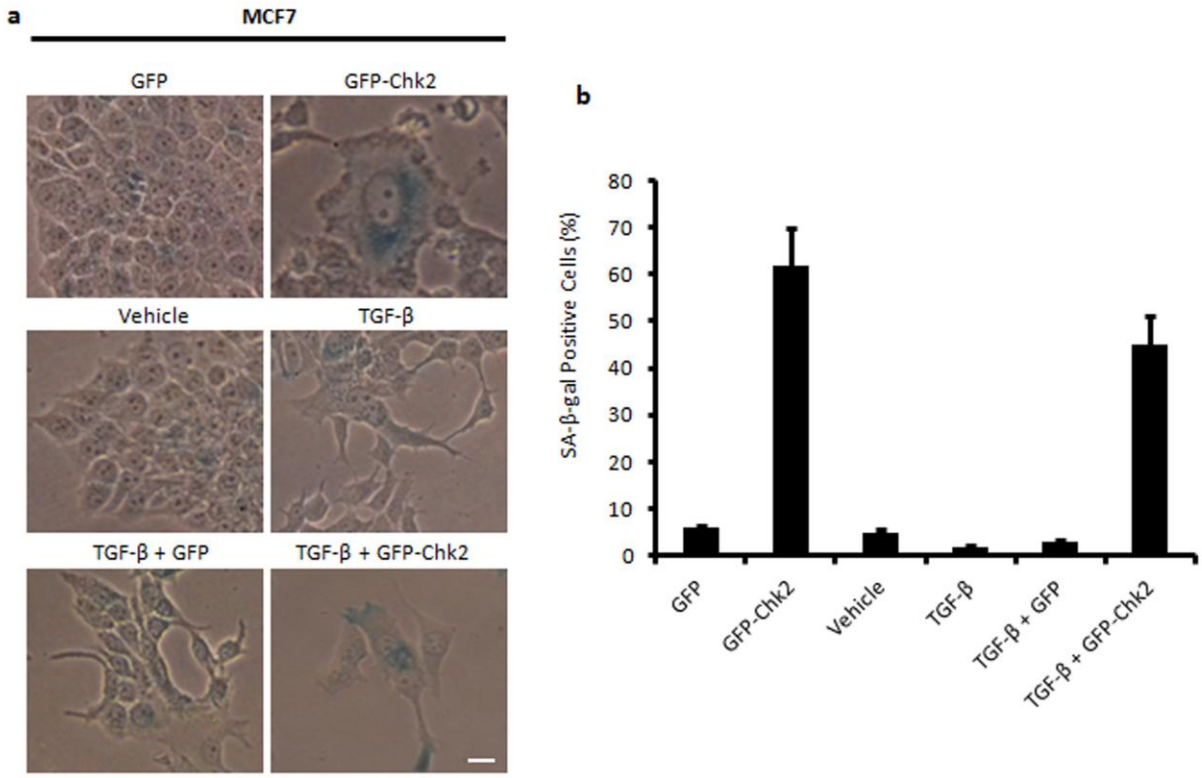

Figure S9. Effect of Chk2 activation on induction of premature senescence in p53 intact MCF7 cells. MCF7 cells were either transfected with GFP and/or GFP-Chk2 or treated with vehicle, TGF-β, GFP plus TGF-β and GFP-Chk2 plus TGF-β for 48 h. Cells were subjected to SA-β-gal activity assay. Cells were then photographed under an inverted microscope at 20x magnification. Scale bar: 20 μm. (**b**) Bar graph indicates quantification of SA-β-gal positive cells (n = 3, error bars indicate ± s.d.).

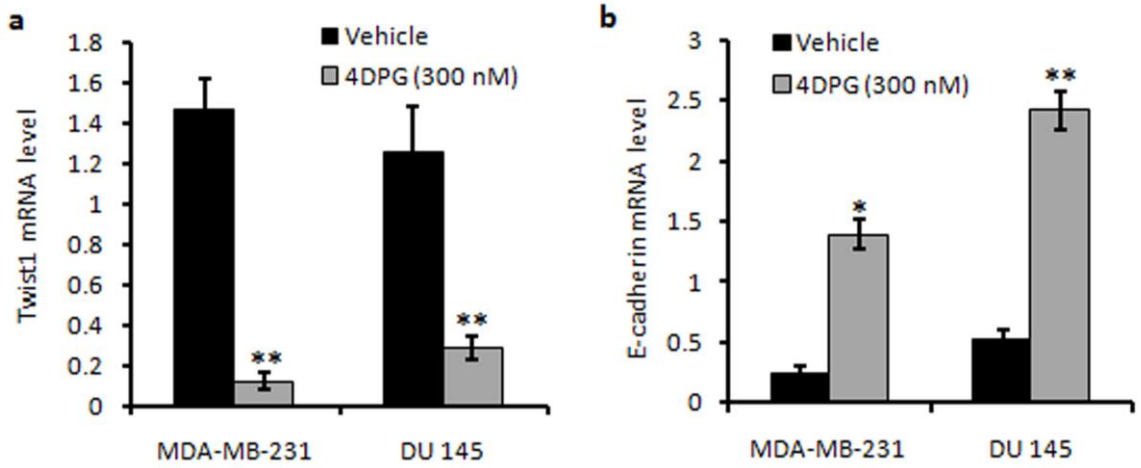

Figure S10. Effect of 4DPG on the mRNA level of Twist1 and E-cadherin in invasive cells. (**a, b**) MDA-MB-231 and DU 145 cells were treated with vehicle or 300 nM of 4DPG for 48 h. Total mRNA level of Twist1 and E-cadherin was quantified with the help of RT-PCR analysis (n = 3, error bars indicate ± s.d.). *P<0.05, **P<0.01.

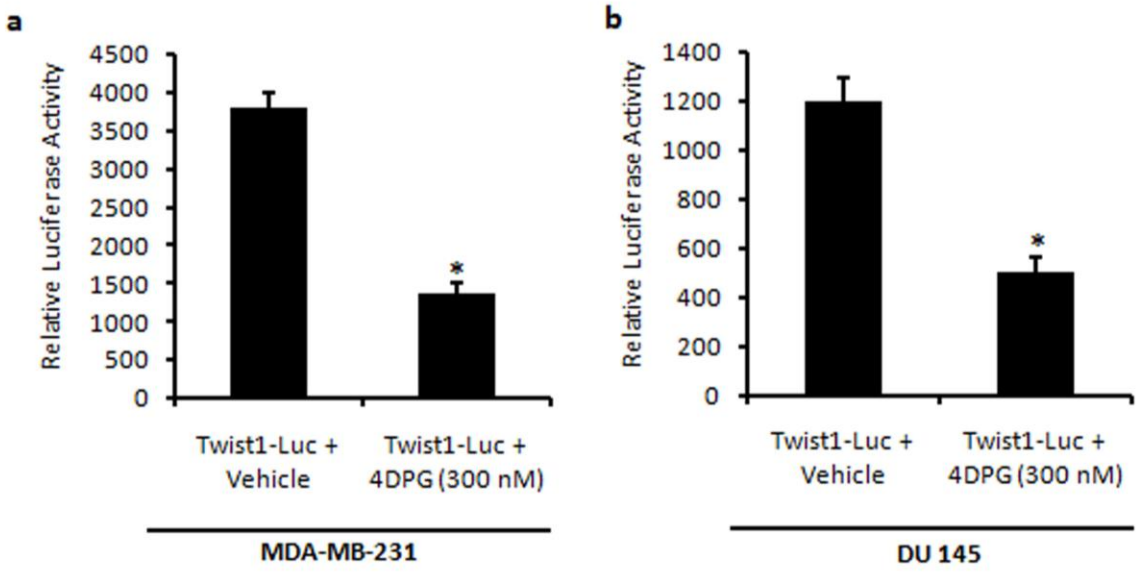

Figure S11. Effect of 4DPG on Twist1 promoter activity. (**a, b**) MDA-MB-231 and DU 145 cells were transfected with Twist1 promoter containing luciferase activity (Twist-Luc) and then treated with 4DPG (300 nM) for 48 h. Luciferase activity was measured with the help of a Dual-Glo Luciferase assay system (Promega). Normalization was done with luciferase activity of PGL3 in each condition (n = 3, error bars indicate ± s.d.). **P*<0.05.

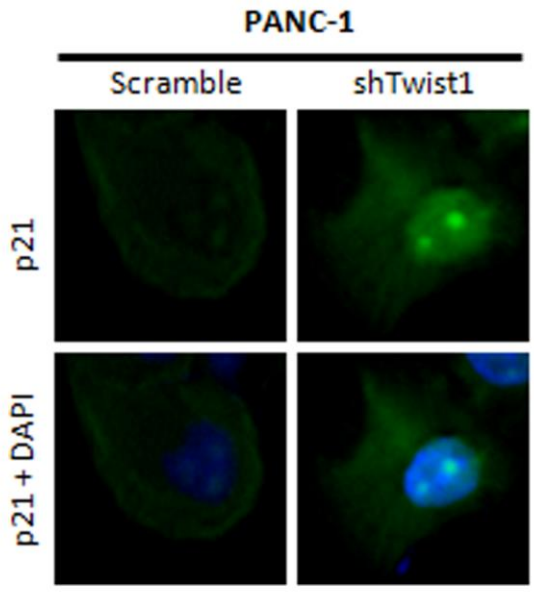

Figure S12. Effect of Twist1 stable knockdown on p21 expression. Stable clones of shTwist1-PANC-1 were subjected to immunocytochemistry for the expression of p21. Cells were photographed at 20x magnification.

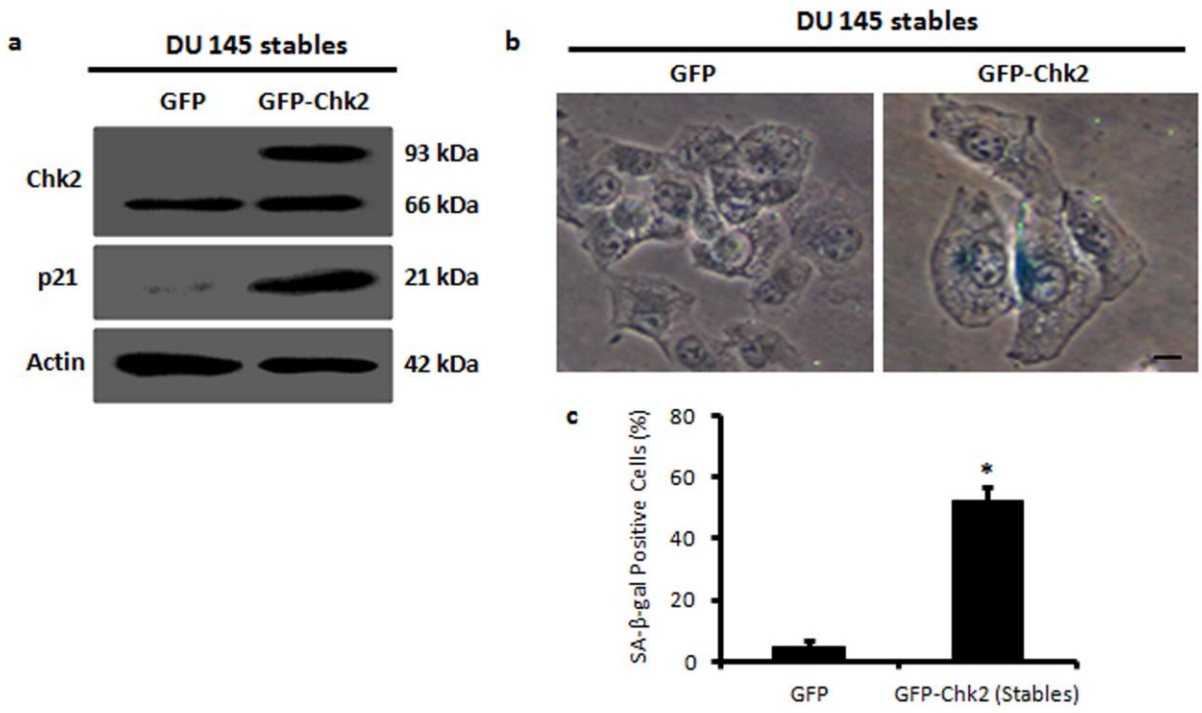

Figure S13. Effect of sustained overexpression of Chk2 on p21 expression. (**a**) Whole cell lysates from stable clones of Chk2-DU 145 were employed for immunoblotting for the expression of Chk2, p21 and actin. (**b**) The cells from above conditions were subjected to X-gal staining for the SA-β-gal activity. Images were captured at 20x magnification. Scale bar: 20 μm. (**c**) Bar graph showing quantification of the number of SA-β-gal positive cells. (n = 3, error bars indicate ± s.d.). *P<0.05.