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Supplementary Materials for

Akt and ERK Control the Proliferative Response of Mammary Epithelial Cells to the Growth Factors IGF-1 and EGF Through the Cell Cycle Inhibitor $p57^{Kip2}$

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The PDF file includes:

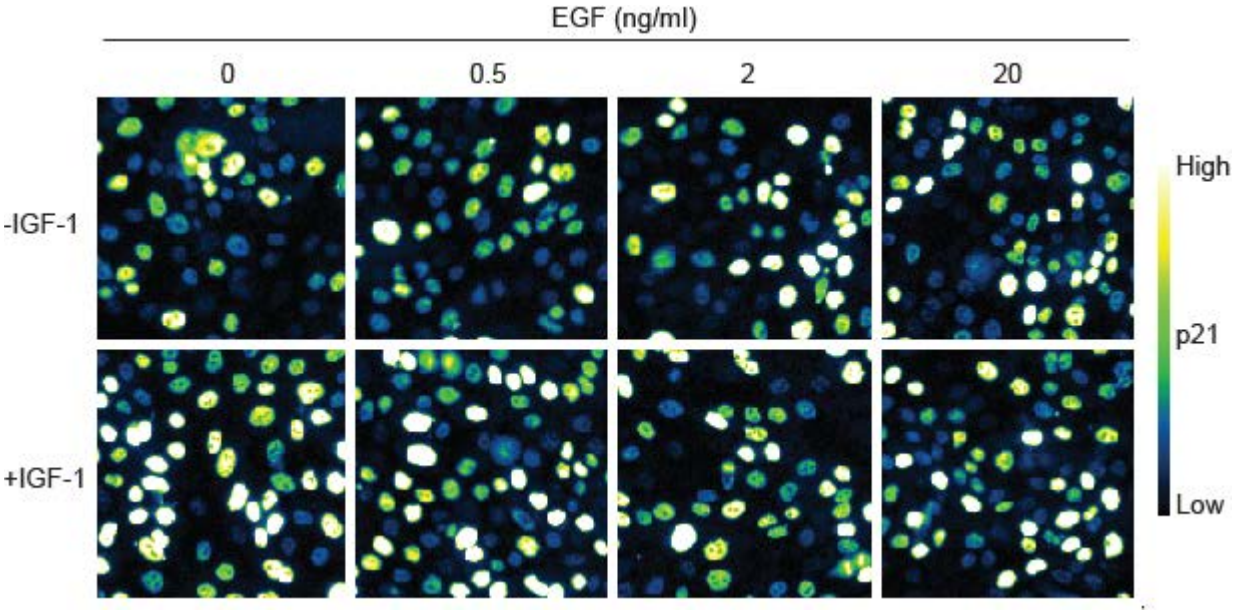

Figure S1. HCIF images of p21 under various growth factor conditions. Cells were treated and images collected as described in Fig. 2. Population distributions and quantitation of p21 are shown in Fig. 2C, D, and E. Results shown are from an individual experiment representative of three independent replicates.

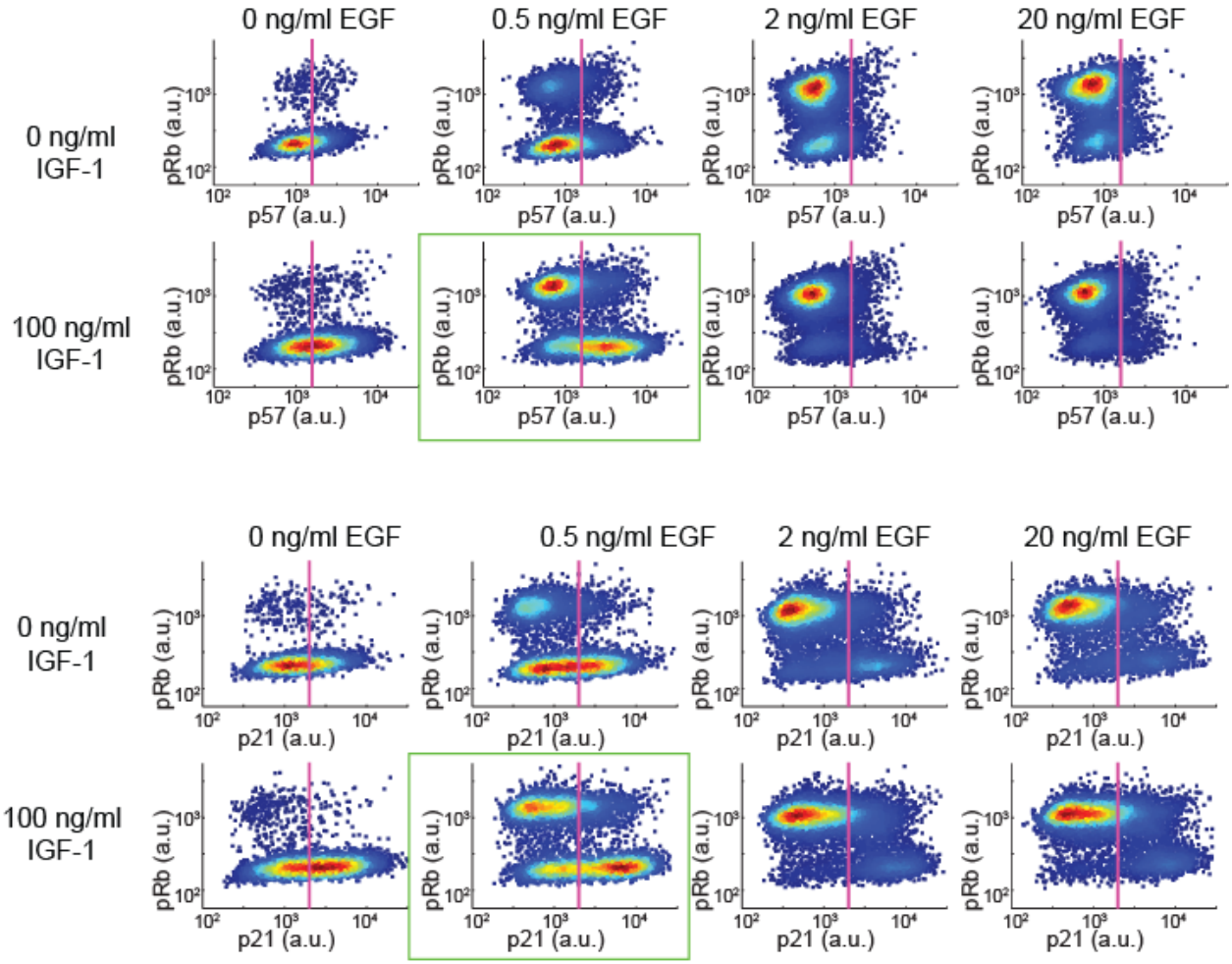

Figure S2. Covariate single-cell analysis of p57/p21 and pRb. MCF-10A cells were cultured in the indicated conditions for 24 hours, and analyzed for p21, p57, and pRb by HCIF. The conditions shown in Fig 2D are indicated by green boxes. Pink vertical lines represent the threshold amount of p21 or p57 across which pRb-positivity rapidly declines. For both p21 and p57, this critical amount remains in a similar position across other growth factor conditions - cells to the right of the pink line (high p57 or p21) are generally negative for pRb, while cells to the left of the pink line (low p57 or p21) are mostly positive for pRb. Note that under 0 ng/ml EGF, cells on either side of the pink line are negative for pRb, reflecting the fact that MCF-10A cells will not proliferate in the absence of a positive pro-growth signal, irrespective of the abundance of cell cycle inhibitors. Results shown are from one experiment representative of three independent replicates.

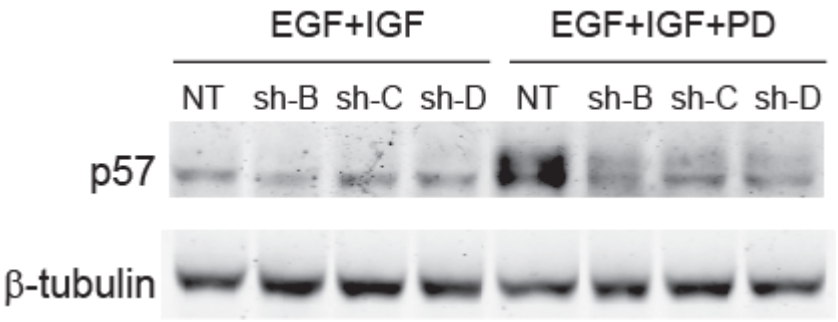

Figure S3. Immunoblot analysis of p57 depletion. MCF-10A cells were grown in the presence of EGF and IGF-1, with or without the MEK inhibitor PD325901 (PD), for 24 hours. Cells were then lysed in RIPA buffer and analyzed for p57 protein by immunoblot. β-tubulin is used as a loading control.

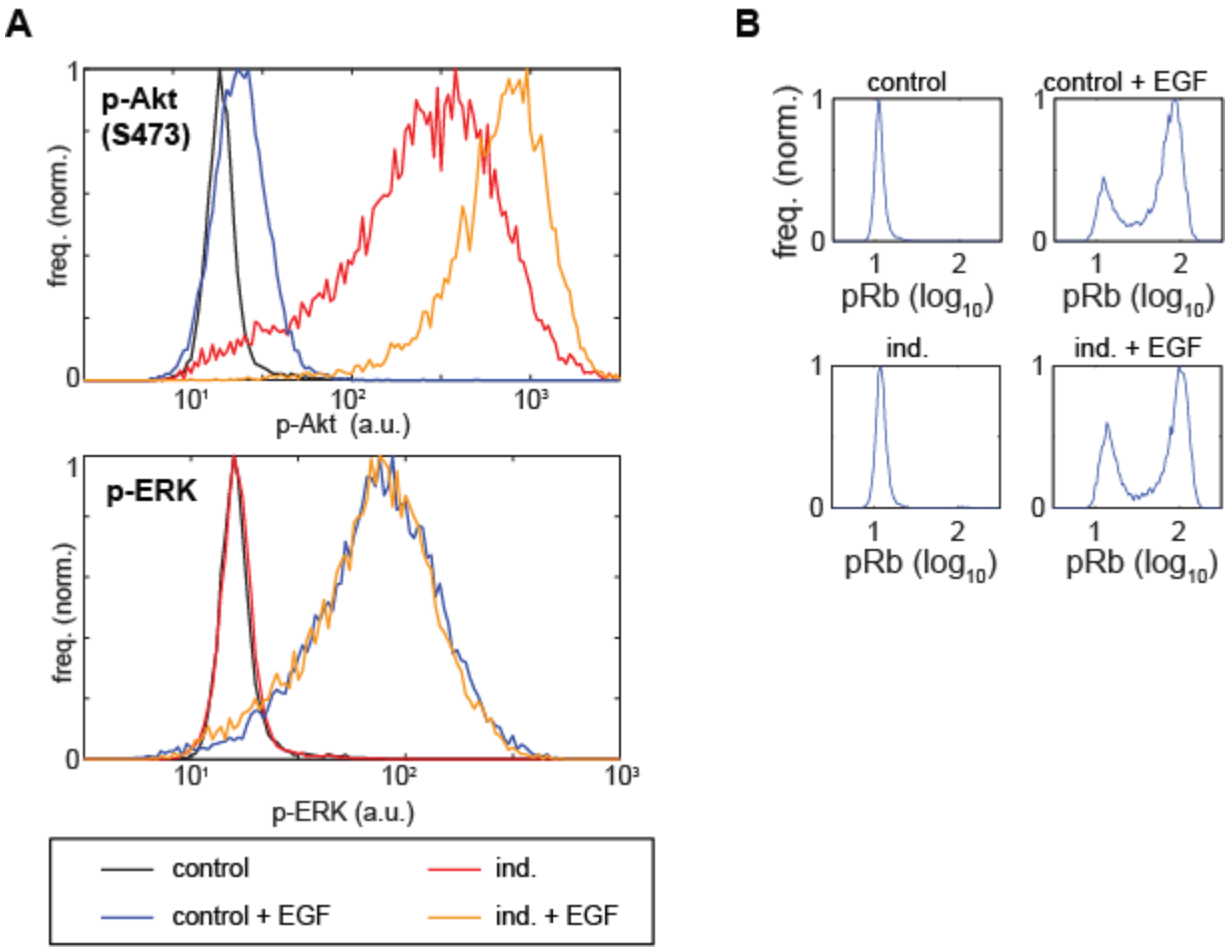

Figure S4. Proliferative and signaling response to Akt induction.MCF-10A cells stably expressing inducible Akt were cultured in the absence of growth factors for 48 hours and then stimulated with inducer (4-hydroxy tamoxifen) or vehicle (ethanol) in the presence or absence of 20 ng/ml EGF for 24 hours. pRb, pAkt, and pERK were measured by HCIF. Analysis of pAkt and pERK (A) demonstrates strong induction of pAkt but no detectable activation of pERK. Analysis of pRb (B) indicates that Akt induction alone is not capable of stimulating proliferation. Results shown are from one experiment representative of two independent replicates.

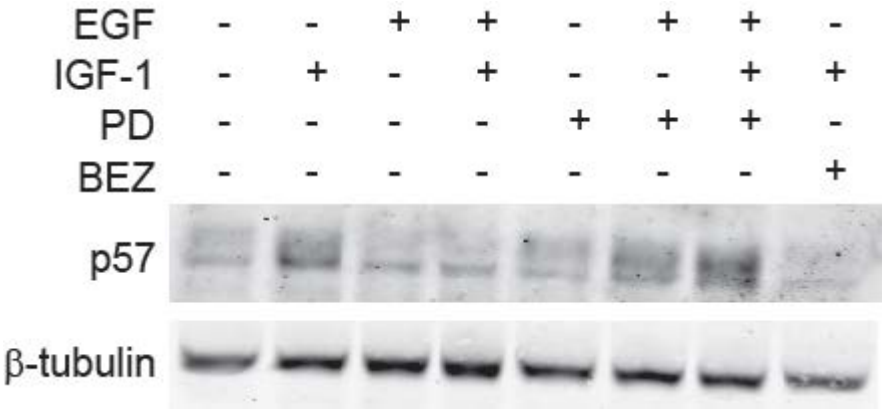

Figure S5. Immunoblot analysis of changes in p57 protein abundance. MCF-10A cells were grown in the presence of 20 ng/ml EGF, 100 ng/ml IGF-1, 1 mM MEK inhibitor PD325901 (PD), or 1 mM PI3K/mTOR inhibitor BEZ-235 (BEZ) as indicated for 24 hours. Cells were then lysed in RIPA buffer and analyzed for p57 protein by immunoblot. β-tubulin is used as a loading control. The changes in p57 observed by immunoblot correspond closely to those observed by HCIF, confirming that total cellular p57 abundance is modulated by the ERK and PI3K pathways.

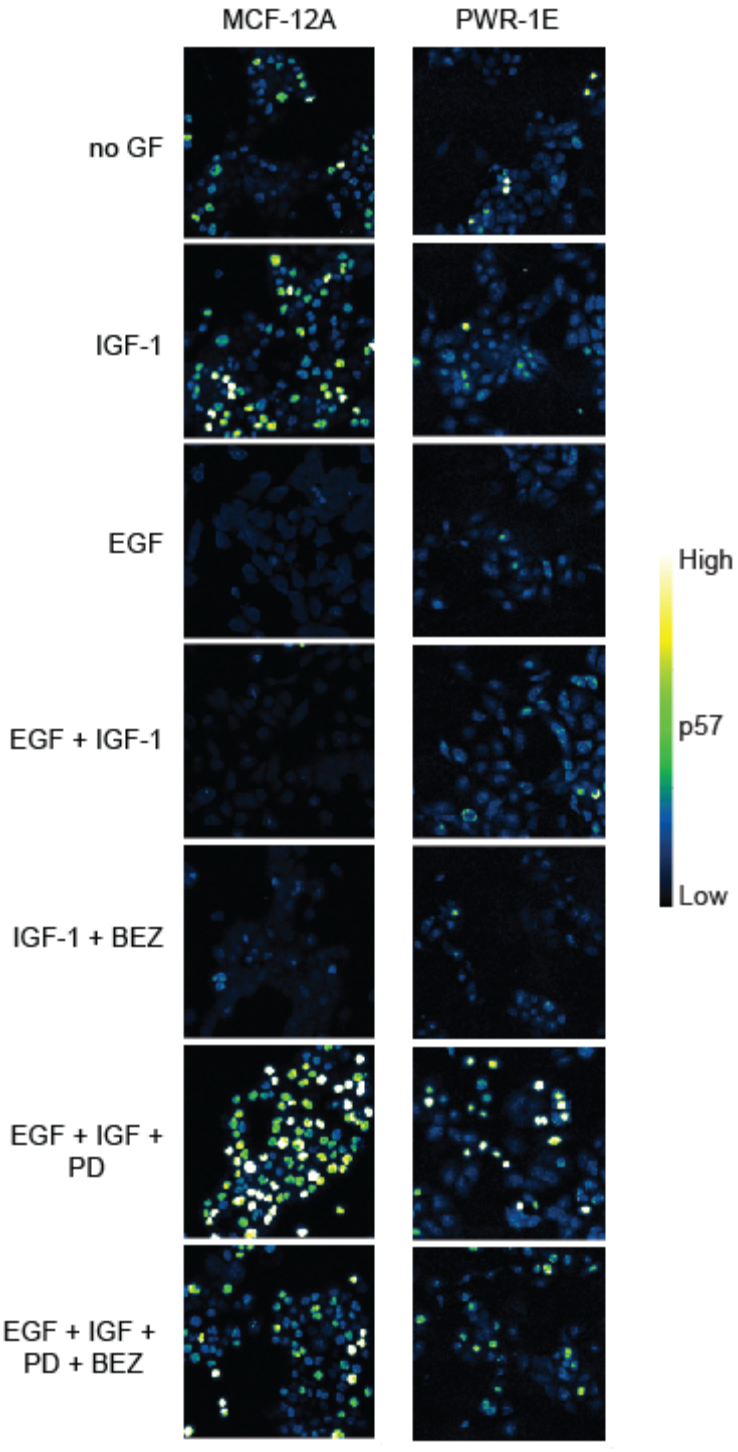

Figure S6. HCIF images of p57 in MCF-12A and PWR-1E epithelial cell lines. MCF-12A and PWR-1E cells were grown in the presence of the indicated growth factors, the MEK inhibitor PD325901 (PD), or the PI3K/mTOR inhibitor BEZ-235 (BEZ) for 24 hours. Cells were then fixed and p57 analyzed by HCIF. Quantitation of these images is shown in Fig. 6A. Results shown are from one experiment representative of three independent replicates.

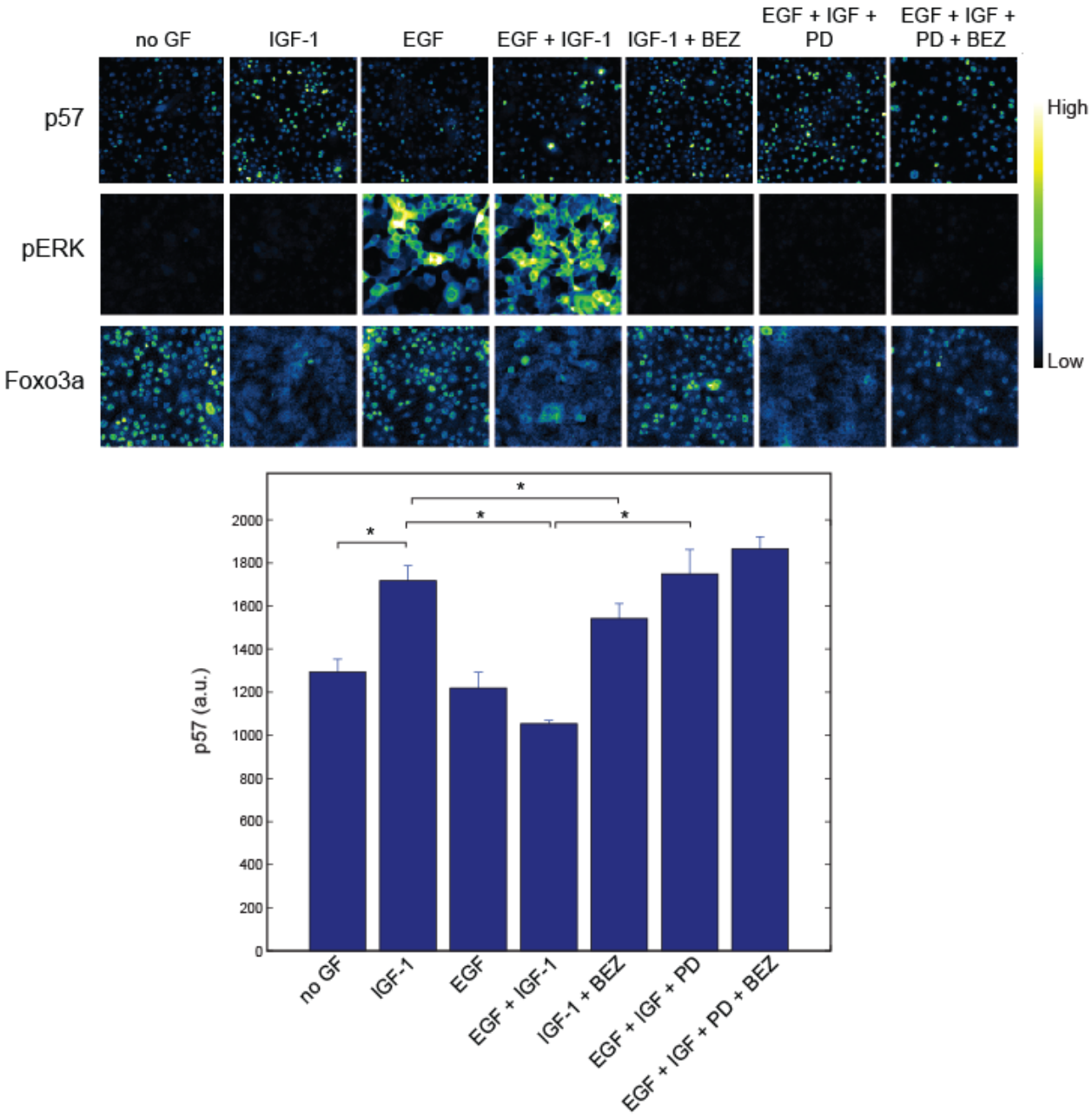

Figure S7. HCIF analysis of p57 regulation in 184A1 mammary epithelial cells. 184A1 cells were grown in the presence of the indicated growth factors, the MEK inhibitor PD325901 (PD), or the PI3K/mTOR inhibitor BEZ-235 (BEZ) for 24 hours. Cells were then fixed, and p57, pERK, and Foxo3a were analyzed by HCIF. Bar graph represents the mean, and error bars the standard deviation of triplicate wells. Results are representative of three independent experiments. The changes in p57 closely resemble those seen in MCF-10A, MCF-12A, and PWR-1E cells, with the exception that BEZ treatment does not reduce the abundance of p57 in EGF+IGF+PD-treated cells. However, this discrepancy can be explained by the observation that BEZ does not fully inhibit Akt pathway activity in this context, as indicated by residual cytoplasmic Foxo3a staining. Results shown are from one experiment representative of three independent replicates.

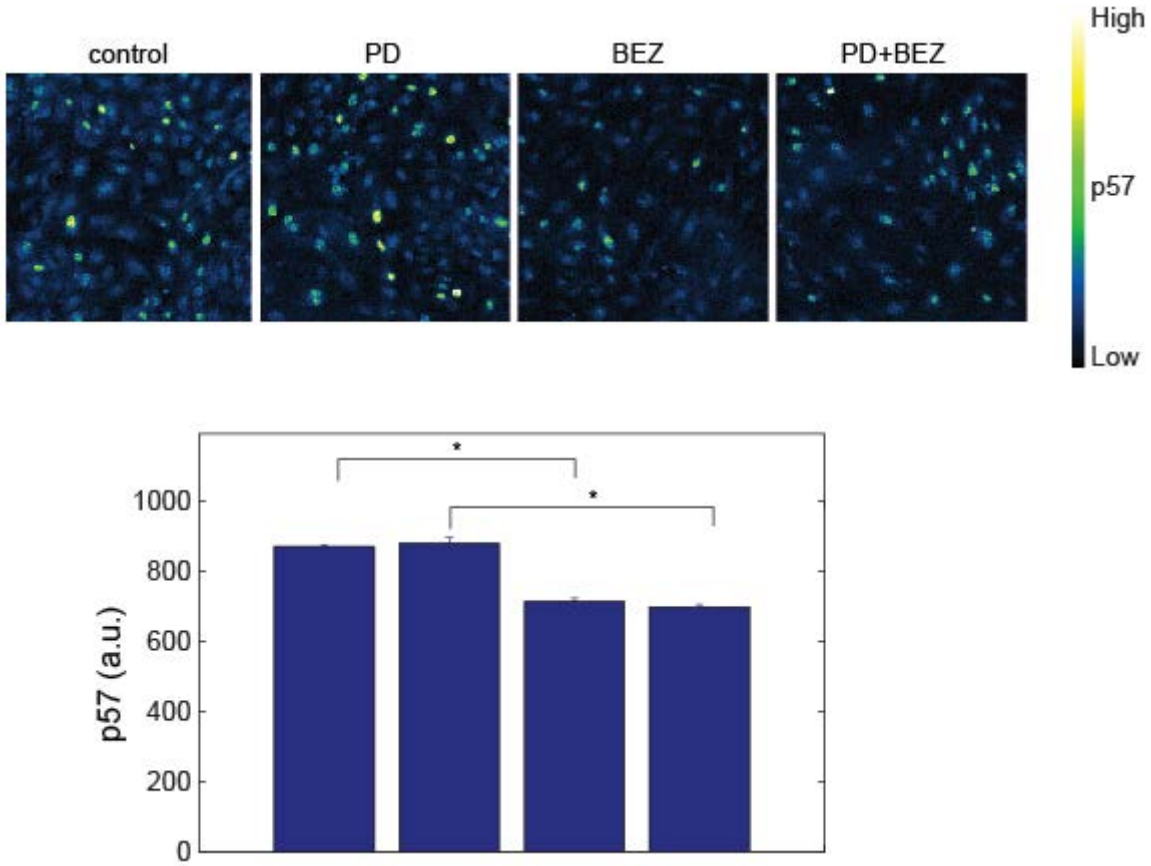

Figure S8. HCIF analysis of p57 in human umbilical vein endothelial cells (HUVECS). HUVECs were treated with the indicated inhibitors (PD-325901, BEZ-235) for 25 hours, fixed, and analyzed for p57 by HCIF. Bar graph represents the mean, and error bars SD of triplicate wells. Results are representative of four independent experiments.

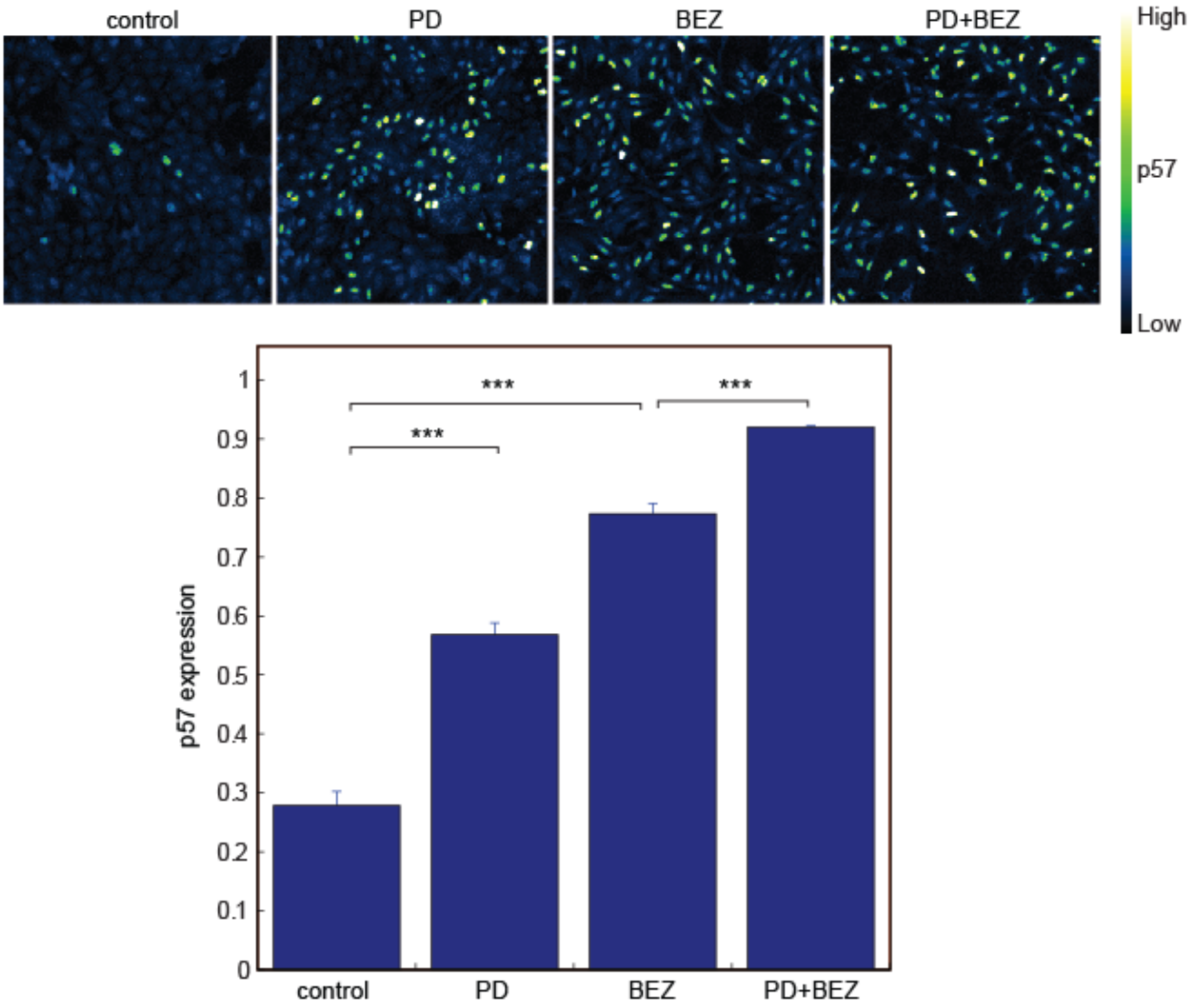

Figure S9. HCIF analysis of p57 regulation in U2OS osteosarcoma cells. U2OS cells were treated with the indicated inhibitors (PD-325901 and/or BEZ-235) for 24 hours, fixed, and analyzed for p57 by HCIF. Bar graph represents the mean, and error bars the standard deviation of triplicate wells. Results are representative of two independent experiments.