

Quantitative Cell-based Imaging Measurements of Key Targets in the mTOR and AKT Signaling Pathways

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Abstract

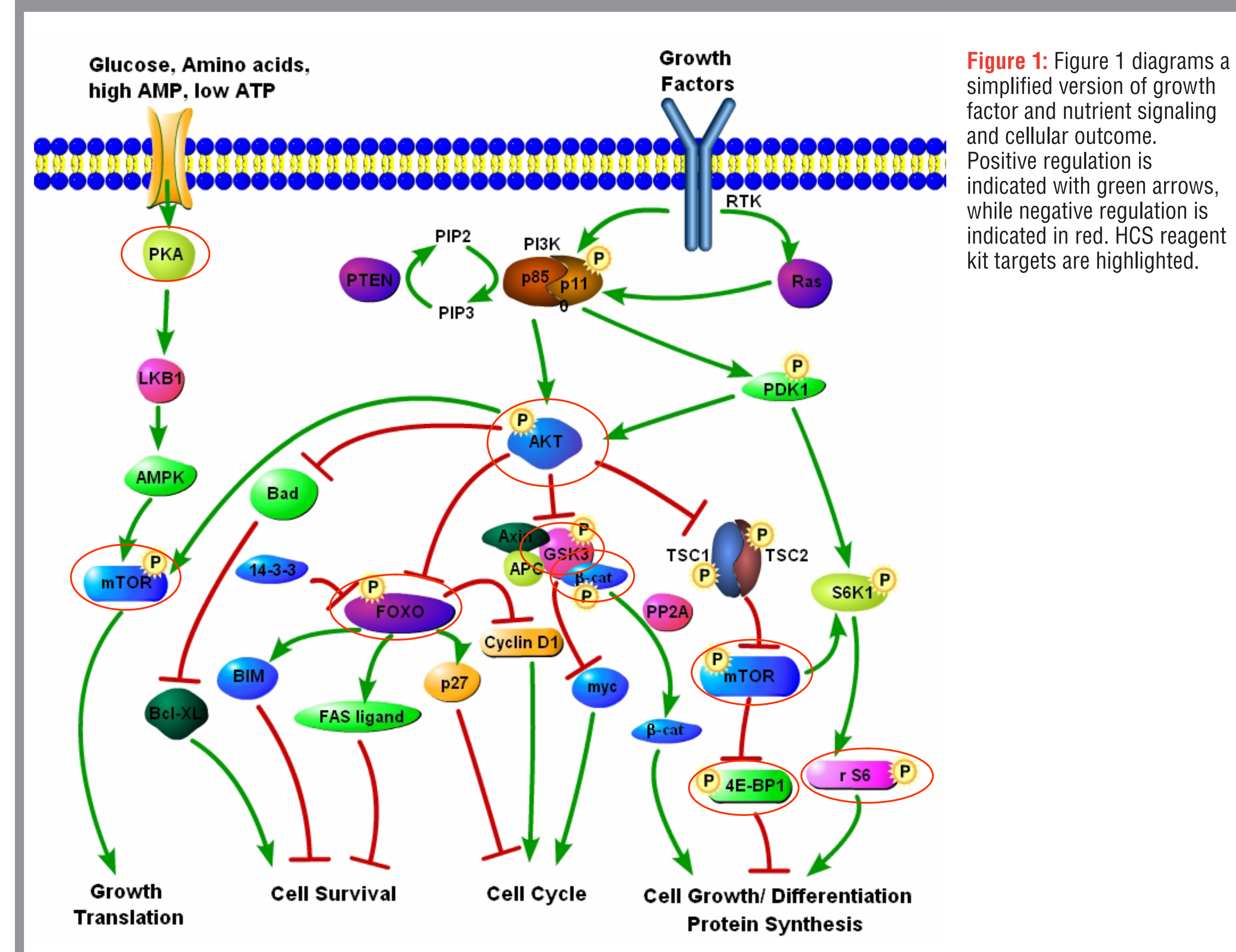
Cancer cells manipulate many intracellular pathways to enhance cancer cell growth and metastasis. Growth factors, amino acids, nutrients and metabolic intermediates serve as cellular signals to initiate the signaling pathways involved in cell growth and proliferation. These pathways often converge or intersect at AKT and mTOR to determine cell fate. The PI-3 kinase signaling pathway, responding via growth factor stimulation through receptor tyrosine kinases, can activate both AKT and mTOR. AKT phosphorylates proteins for both positive and negative regulation of cell growth, and is also coupled to translational and metabolic machinery through its ability to phosphorylate and inactivate TSC, a negative regulator of mTOR. Thus, mTOR is responsive to growth factor signaling as well as energy metabolism and nutrients to upregulate protein translation. Proteins in the PI-3 kinase pathway are frequently mutated in cancer cells to promote constitutive growth and proliferation. However, measuring changes in these pathways is challenging because of the constitutive activation of key targets that require multiple parameters to be assessed simultaneously and the rapid turnover of targets that provides only a narrow time-window for conducting the assay. Quantitative cell-based imaging assays, such as high-content analysis and screening (HCA, HCS), provide an advantage over other assay methods in that both the activation state of the target and its cellular location can be simultaneously monitored in individual cells. Here we demonstrate the power of quantitative cell-based imaging for targets in the PI-3 kinase signaling pathway. With HCA, AKT and mTOR can be visualized and quantitated, as well as subsequent positive and negative downstream effectors (FOXO1A, FOXO3A, GSK3b for AKT, and S6 and 4E-BP1 for mTOR). These intracellular targets were quantitatively assessed using a Thermo Scientific Cellomics ArrayScan HCS Reader and various Thermo Scientific Cellomics HCS Reagent Kits, thus providing a robust system for assaying challenging targets in the PI-3 kinase signaling pathway.

Background

Signaling through the PI-3 kinase pathway is critical for cell growth and proliferation. AKT regulates many targets, both positively and negatively through phosphorylation to determine cell fate. Phospho-mTOR regulates protein translation machinery. Figure 1 illustrates some elements of AKT and mTOR signaling. Alterations in these pathways are often difficult to measure. Cell-based imaging allows visualization of phosphorylation events in context to cellular location of the target protein.

Multiple Cellomics HCS Reagent kits were developed to be used with cellular-based imaging for quantitation of aspects of the AKT pathway, including kits for detecting the phosphorylation states and intracellular distributions of AKT and GSK-3, as well as the intracellular distributions of FOXO1A and FOXO3A. GSK-3 is also an important regulator of β -catenin, a transcription factor involved in cell growth and differentiation. mTOR and its downstream targets of S6 and 4E-BP1 are also quantitated using phosphorylation states. These assays were developed using fluorescent antibody staining and a Thermo Scientific Cellomics ArrayScan[®] HCS Reader for quantitation. These kits have been developed for robust performance, and are compatible with other HCS readers or microscopy-based instrumentation with similar fluorescent filter sets.

AKT and mTOR Signaling



AKT and mTOR Phosphorylation

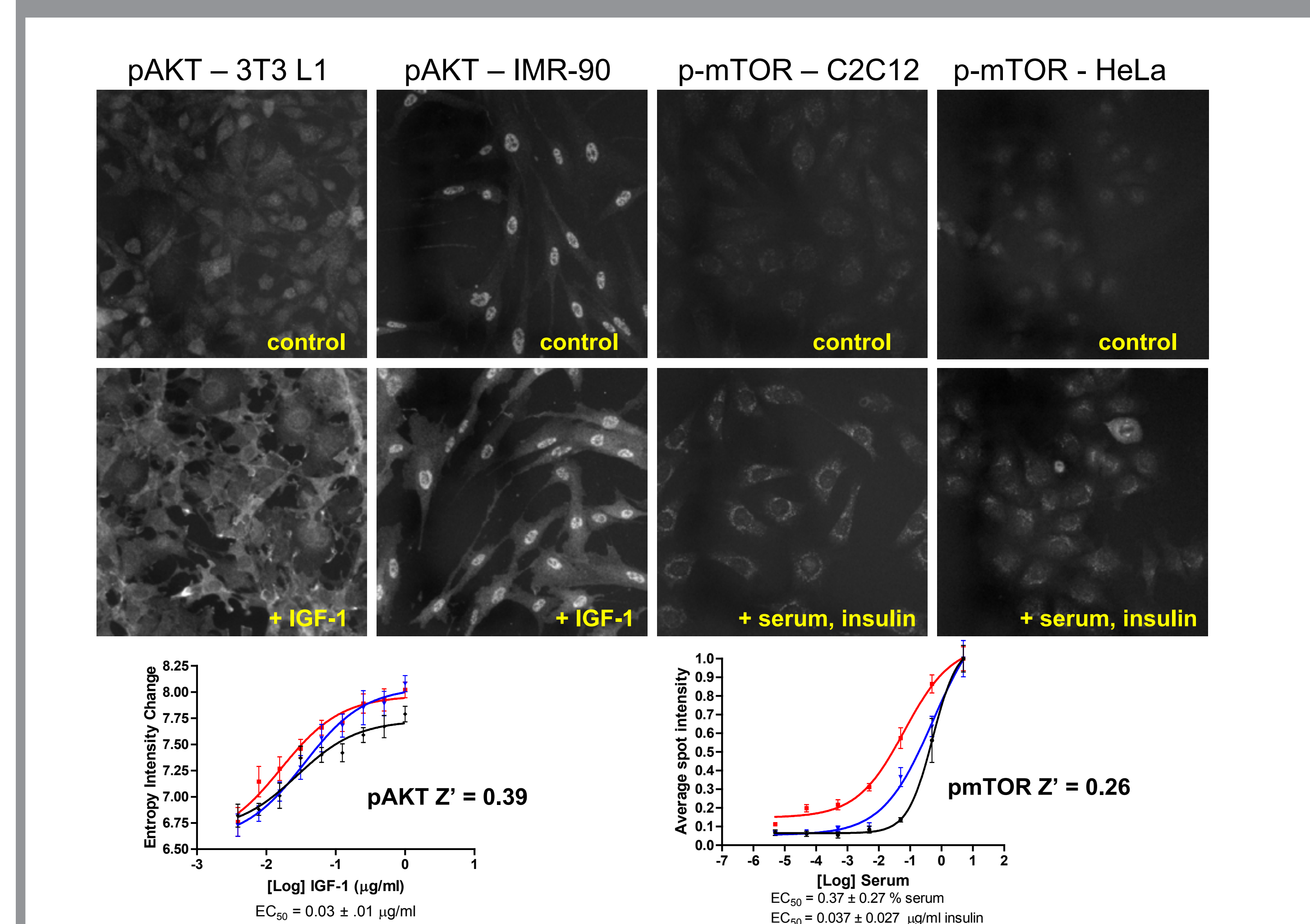


Figure 2: Images- AKT-3T3 L1 adipocytes and IMR-90 primary lung fibroblasts were incubated overnight in growth factor depleted media. Cells were then challenged with 200 ng/ml IGF-1 for ten minutes. mTOR- C2C12 and HeLa cells were serum-starved for twenty-four hours before treatment with 10% serum and 5 ng/ml insulin for 15 minutes. Top panels, non-treated; Bottom panels, treated. Dose response curves, Triplicate dose response curves are below each respective target and EC_{50} values are indicated. Z' values derived from triplicate plates are also noted.

Phospho-mTOR Activation in Alternate Cell Lines

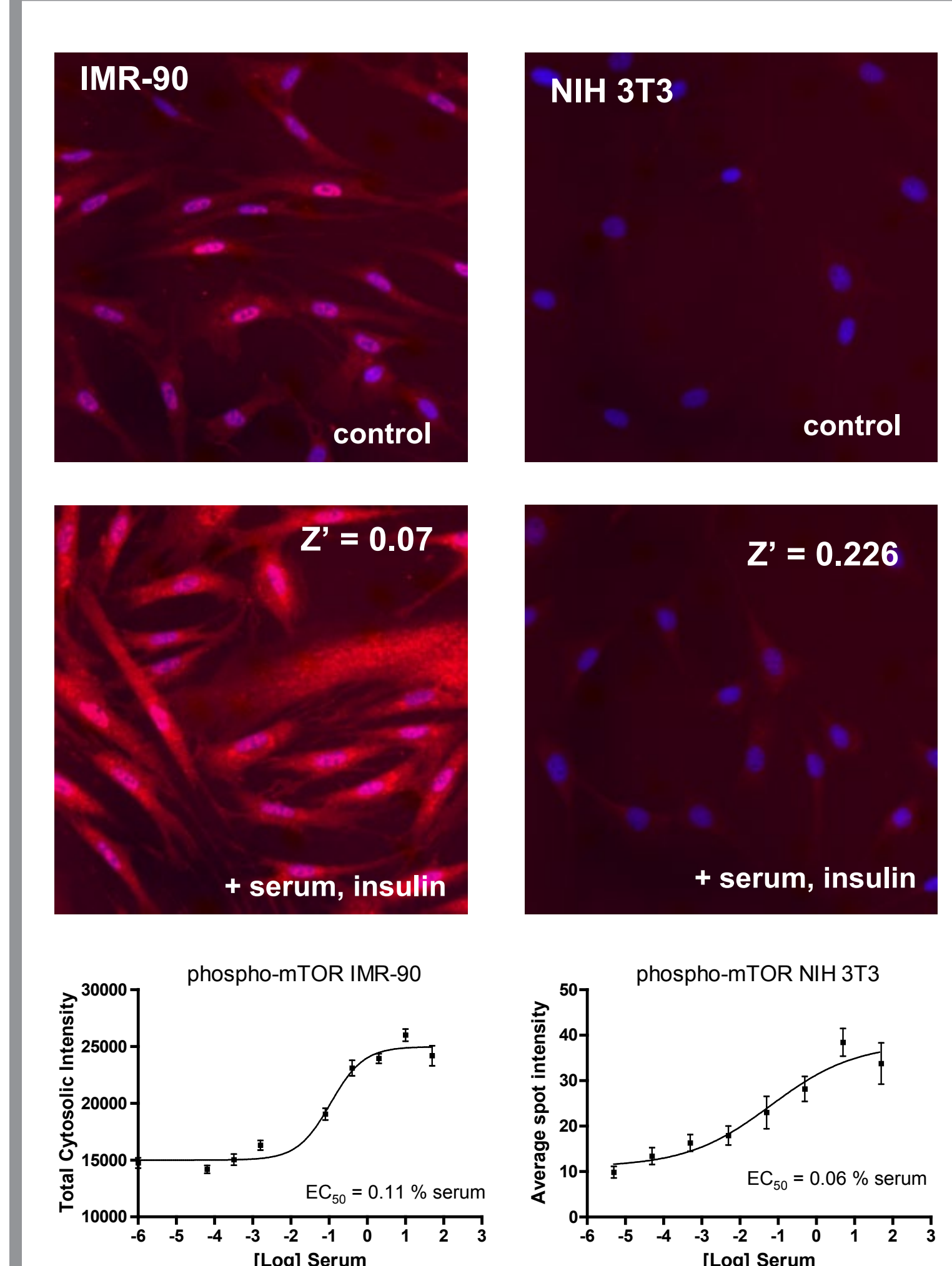


Figure 3: Top, IMR-90 cells were serum-starved for 48 hours before a 15 minute stimulation with serum and insulin. Bottom, NIH3T3 cells were serum-starved 24 hours before 15 minute treatment with serum and insulin. Z' values are noted and EC_{50} values are indicated on the respective dose response curves.

Negative Downstream Regulators of PI-3 Kinase Cell Signaling

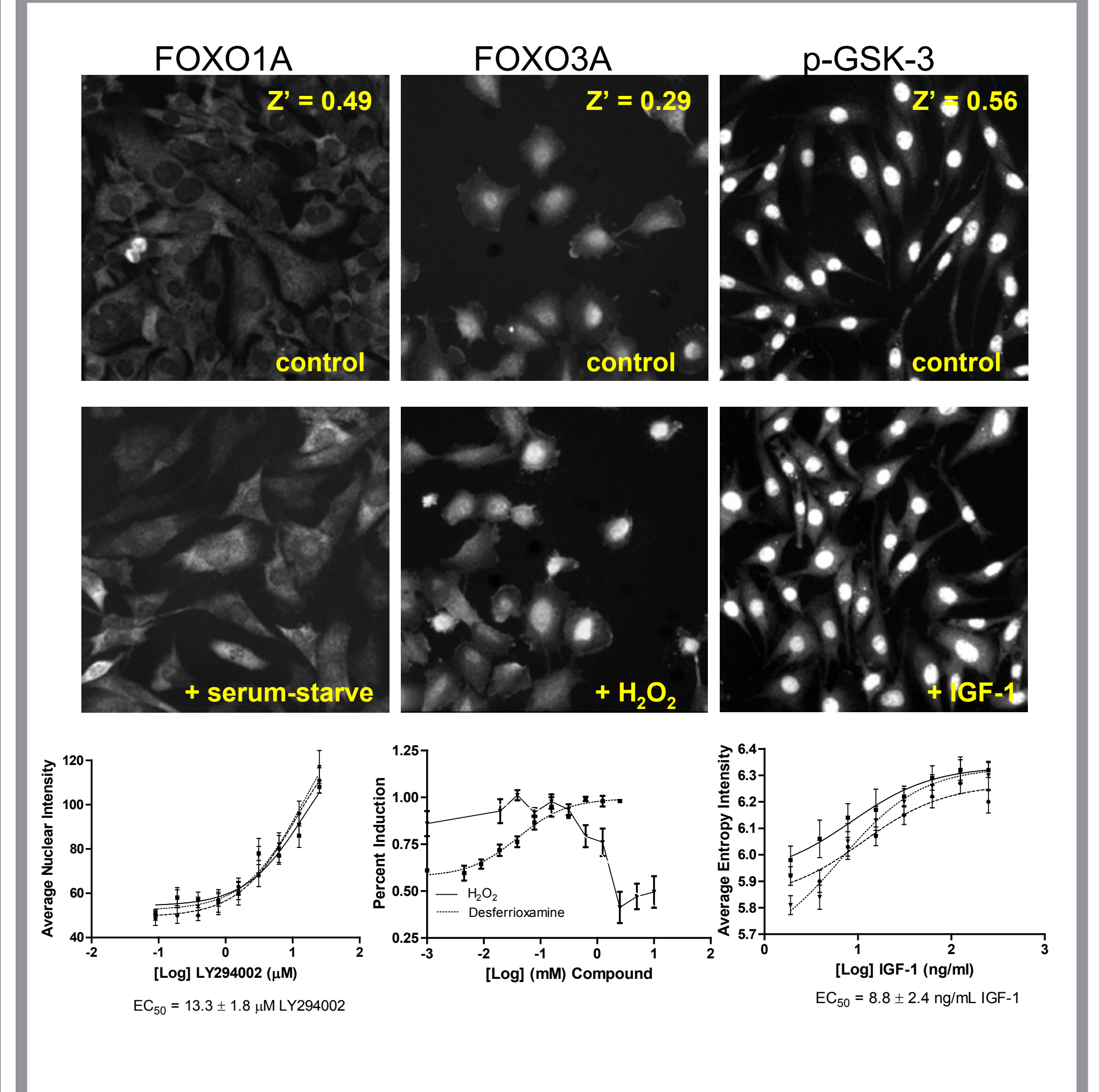
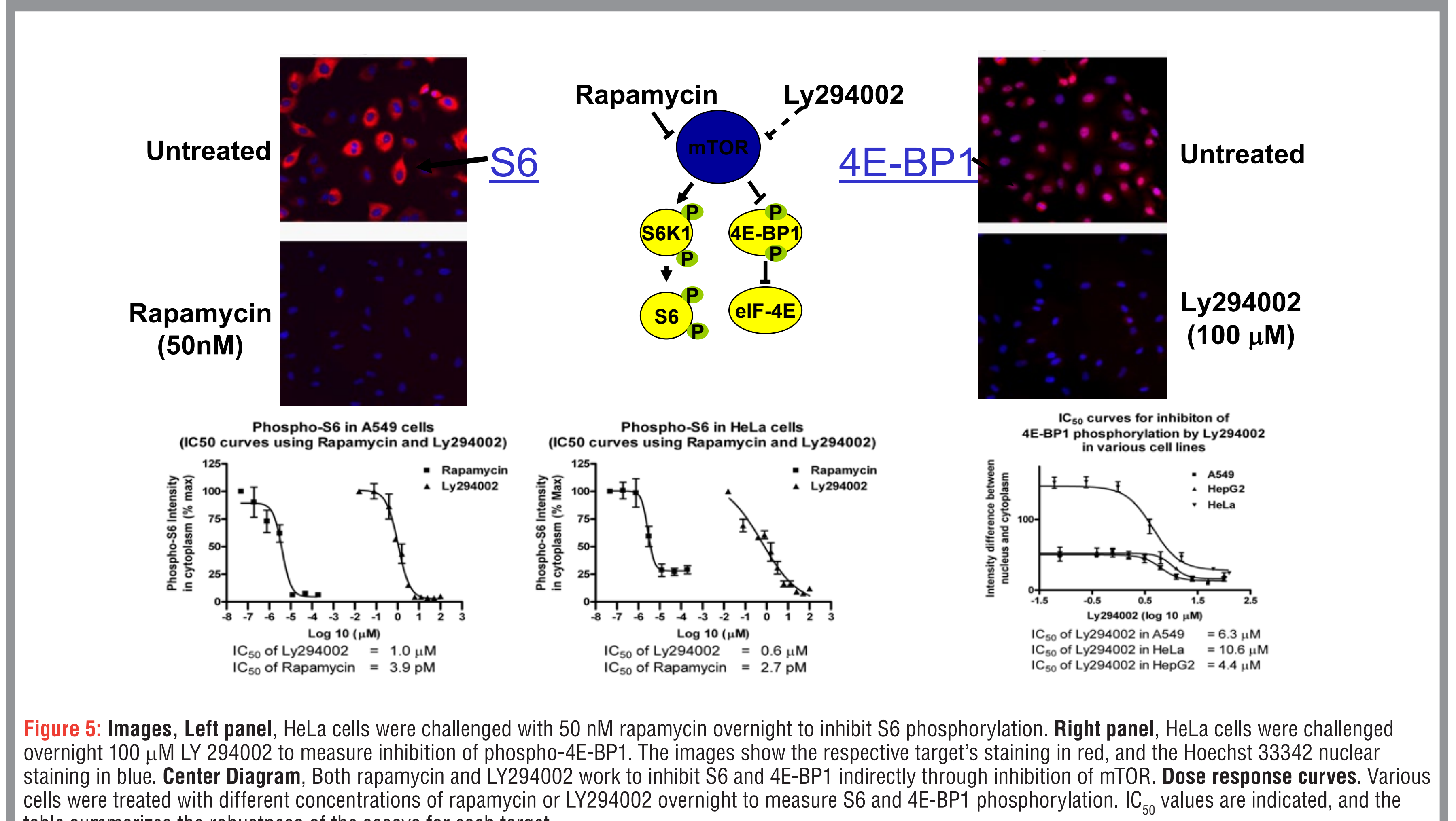


Figure 4: FOXO1A - NIH3T3 cells were serum-starved overnight, resulting in translocation of FOXO1A to the nucleus. FOXO3A - A549 cells were treated with 5 mM hydrogen peroxide to induce translocation of FOXO3A to the nucleus. GSK-3 - 3T3 L1 cells were challenged for thirty minutes with 100 ng/ml IGF-1 in growth-factor depleted media. Top panels, non-treated; Bottom panels, treated. Dose response curves are located below each respective target.

S6 and 4E-BP1 Inhibition



Co-staining of Phospho-mTOR and LAMP1 as Markers for Autophagy

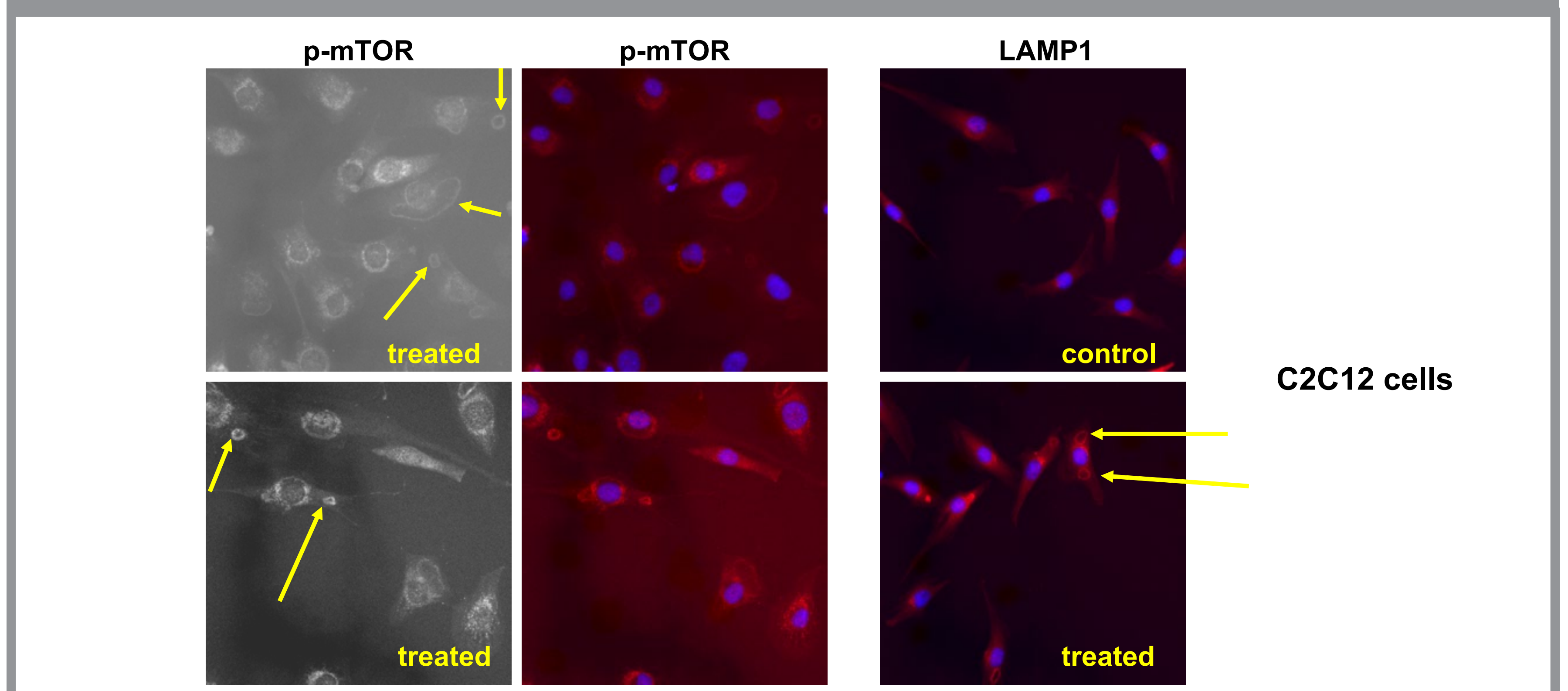


Figure 6: Staining of lysosomal structures with mTOR (left panels) and LAMP1 (right panel) in C2C12 cells after 48 hours of serum starvation. Both proteins are present on similar lysosomal structures, suggesting that these structures are autophagolysosomes that appear during autophagy. Arrows indicate lysosomal structures.

Conclusions

- New Cellomics HCS reagent kits have been developed for six targets associated with the AKT and mTOR signaling pathways, including AKT, mTOR, GSK-3, FOXO1A, FOXO3A, S6, and 4E-BP1.
- These new kits provide additional tools to examine these traditionally difficult targets in these pathways in context to cellular location.