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TITLE: The Importance of Autophagy in Breast Cancer Development and Treatment

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13. SUPPLEMENTARY NOTES					
14. ABSTRACT Autophagy is an evolutionally conserved process employed by cells to degrade proteins and organelles in response to metabolic stress. Cells can recycle amino acids, fatty acids and nucleotides for macromolecular biosynthesis and ATP generation, and by sequestering damaged organelles can prevent the release or accumulation of toxic substances. We described elongation factor-2 kinase (eEF-2 kinase) as a structurally and functionally unique enzyme that is activated by starvation and phosphorylates and inactivates eEF-2, thereby terminating peptide chain elongation. Since protein synthesis is a major energy-consuming process, decreasing protein elongation by activating eEF-2 kinase could be an energy-saving survival strategy. We now test the hypothesis that eEF-2 kinase plays a critical role in the ability of cancer cells to survive oxygen and nutrient deprivation. MCF-7 human breast cancer cells were transfected with a GFP-tagged LC3 expression vector to track the formation of autophagosomes. Autophagy was induced by nutrient/growth factor deprivation as manifested by autophagosome formation in GFP-LC3- transfected MCF-7 cells. Treatment with a potent and specific inhibitor of eEF-2 kinase, NH125 inhibited autophagy as indicated by a reduction in autophagosome formation. In either transient or stable MCF-7 transfectants, NH125 was 10-times more potent and more effective than 3-methyladenine, a known autophagy inhibitor. To determine the effects of blocking autophagy via inhibition of eEF-2 kinase on cellular energetics, we studied the rate and amount of ATP depletion in NH125- and vehicle-treated MCF-7 transfectants. Following nutrient deprivation, inhibition of eEF-2 kinase by NH125 resulted in a greater and more rapid reduction of cellular ATP as compared to vehicle treatment.					
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INTRODUCTION

We propose that breast cancer cells utilize autophagy (“self-digestion”) to survive during times of nutrient and/or growth factor deprivation. For example, when cells lack an adequate blood supply, or treatment with drugs that block growth factors, a percentage of cells survive by slowing energy utilization and recycling their own proteins and damaged organelles. These “hibernating” cells can subsequently be revived when nutrients become available. Therefore, by inhibiting autophagy normal breast cells will be less able to survive malignant transformation and treatment with drugs that block growth factors.

Autophagy is a survival process first uncovered in yeast and is now known to be activated in response to nutrient deprivation in plants, worms, flies, mice, and man (1). In yeast, nutrient deprivation results in two outcomes: 1) self-digestion of cytoplasm and organelles and recycling of “spare parts” (e.g., amino acids) for energy utilization and; 2) the budding of an immortal spore. This response in unicellular organisms leads to self-preservation in times of famine. Not surprisingly, autophagy finds its counterpart in multicellular organisms, as high up the animal kingdom as man, where nutrient deprivation prompts cells to exit the cell cycle, shrink, digest long-lived proteins and damaged organelles, and recycle the components to maintain cellular energy. To support autophagy, cells must limit further use of energy, and have evolved a pathway to inhibit protein synthesis, the cell’s major consumer of energy. Autophagy may thus result in cellular destruction or alternatively cellular “hibernation” until the supply of nutrients is restored.

Despite targeted therapies with selective estrogen receptor modulators (e.g., Tamoxifen), aromatase inhibitors (e.g. Arimidex), and antagonists of Her-2/neu (e.g., Herceptin), the response to these treatments are unacceptably limited. Much remains to be discovered about the mechanisms by which breast cancer cells escape the inhibitory effects of these medications. New and exciting data are emerging in model systems that suggest the possibility that breast cancer cells survive growth factor depletion/inhibition through autophagy. Furthermore, it is possible to inhibit this survival response by targeting one of several promising components of the autophagy pathway. We will test the hypothesis that breast cancer cells survive nutrient/growth factor deprivation through autophagy and that targeting a key component of the autophagy pathway will decrease breast cancer viability. One promising target is an enzyme, eEF-2 kinase, which is activated during autophagic survival to inhibit protein synthesis and conserve energy. Our rationale for targeting eEF-2 kinase is its: 1) overexpression in human breast cancer; 2) activation during autophagy; 3) ability to inhibit protein synthesis and conserve energy and; 3) unique structure, making this kinase amenable to selective inhibition. By blocking eEF-2 kinase in the face of nutrient/growth factor deprivation, protein elongation would continue and deplete critical supplies of energy resulting in cell death.

BODY

Task 1 To determine the importance of autophagy in the life and death of breast cancer cells.

During the last grant period we tested the hypothesis that eEF-2 kinase plays a critical role in the ability of cancer cells to survive nutrient deprivation. In these experiments, MCF-7 human breast cancer cells were transfected with a GFP-tagged LC3 expression vector to track the formation of autophagosomes. We observed that autophagy was induced by nutrient/growth factor deprivation, as manifested by autophagosome formation in GFP-LC3 - transfected MCF-7 cells (**Figure 1**). Treatment with a potent and specific inhibitor of eEF-2 kinase, NH125 (2), inhibited autophagy as indicated by a reduction in autophagosome formation (**Figure 2 and 3**). In either transient (**Figure 2**) or stable (**Figure 3**) MCF-7 transfectants, NH125 was 10 - times more potent and effective than 3-methyladenine, a known autophagy inhibitor. To determine the effects of blocking autophagy via inhibition of eEF-2 kinase on cellular energetics, we measured the rate and amount of ATP depletion in NH125- and vehicle-treated MCF-7 transfectants. Following nutrient deprivation, inhibition of eEF-2 kinase by NH125 (**Figure 4**) or by EF-2 kinase – targeted siRNA (**Figure 5**) resulted in a greater and more rapid reduction of cellular ATP as compared to vehicle treatment. Nutrient starvation activated EF-2 kinase in MCF-7 cells, as evidenced by increased phosphorylation of EF-2, the substrate of the kinase (**Figure 6**); treatment with NH125 blocked the activation of EF-2 kinase (**Figure 6**). The results of these studies are shown in the Appendix. These results provide additional evidence that eEF-2 kinase is activated in response to metabolic stress, and that inhibiting eEF-2 kinase may overcome the cellular attempts to survive via autophagic regeneration of ATP.

Task 2 To determine the role of autophagy in the sensitivity of breast cancer to treatment.

We have begun to determine the effect of autophagy on the sensitivity of breast cancer cells to growth factor receptor antagonists such as trastuzumab and cetuximab. Our preliminary results demonstrated that inhibition of EF-2 kinase by siRNA or small molecule inhibitor may increase sensitivity of some types of breast cancer cells to those drugs.

KEY RESEARCH ACCOMPLISHMENTS

- We demonstrated that EF-2 kinase is activated in response to nutrient/growth factor deprivation in human breast cancer cell lines MCF-7, BT-549, and MDA-MB-231.
- Nutrient/growth factor deprivation induces autophagy in MCF-7 cells.
- Induction of autophagy by nutrient/growth factor deprivation can be blocked by inhibition of EF-2 kinase.
- Inhibition of EF-2 kinase causes a greater and more rapid depletion of ATP under nutrient deprivation condition.

REPORTABLE OUTCOMES

Manuscript

None

Abstracts

Li HM, Yang JM, Jin SK, Hait WN: Targeting Autophagic Survival by Inhibiting Elongation Factor-2 Kinase in Human Breast Cancer Cells. Proc Amer Assoc Cancer Res 48: 4917, 2007.

Degree obtained that are supported by this award

None

CONCLUSIONS

Targeting autophagic survival by inhibiting EF2K, a inhibitor of peptide elongation, may render cells continue to elongate peptide, deplete critical ATP, and sensitize cancer cells to metabolic stress.

REFERENCES

1. Shintani, T. and Klionsky, D. J. Autophagy in health and disease: a double-edged sword. *Science*, 306: 990-995, 2004.
2. Arora S, Yang JM, Kinzy TG, Utsumi R, Okamoto T, Kitayama T, Ortiz PA, Hait WN: Identification and Characterization of an Inhibitor of Eukaryotic Elongation Factor 2 Kinase against Human Cancer Cell Lines. *Cancer Res* 63: 6894-6899, 2003.

Appendix

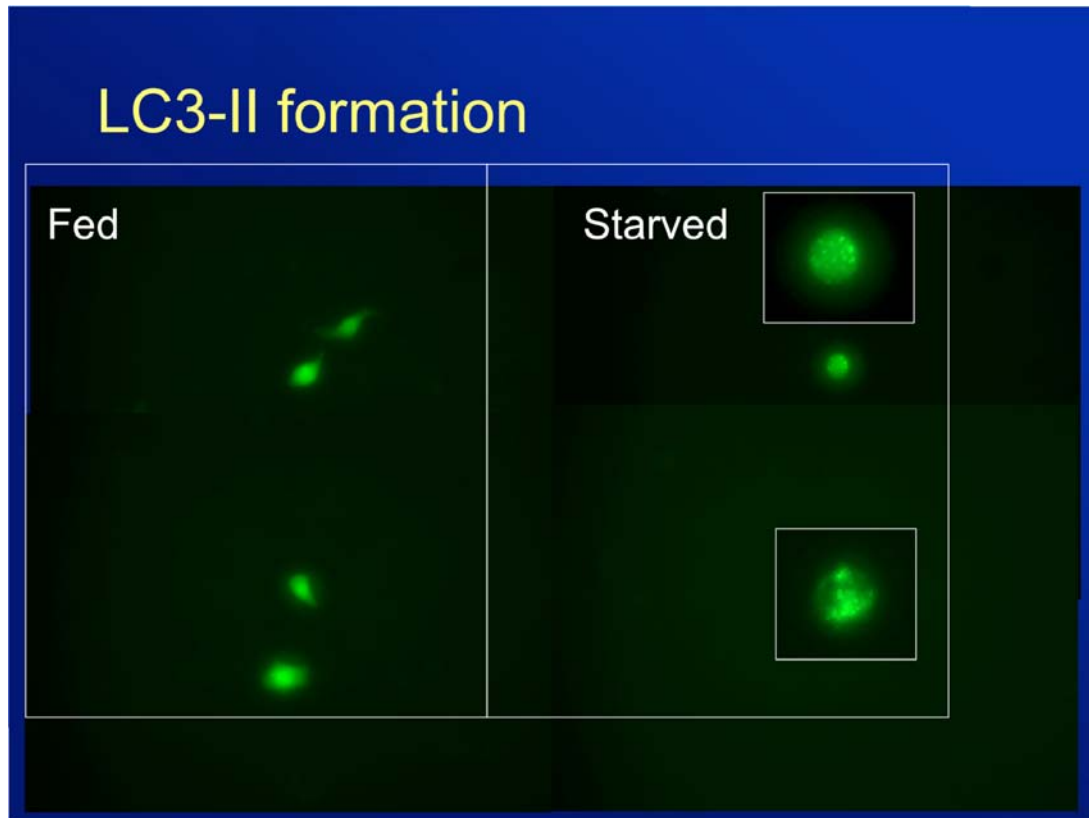


Figure 1. Starvation Induces Autophagy in Human Breast Cancer Cell Line MCF-7. GFP-LC3 – transfected MCF-7 cells were deprived of nutrient and growth factors for 24 hours, and then were observed under a fluorescent microscope. The punctuate-positive cell indicated the activity of autophagy.

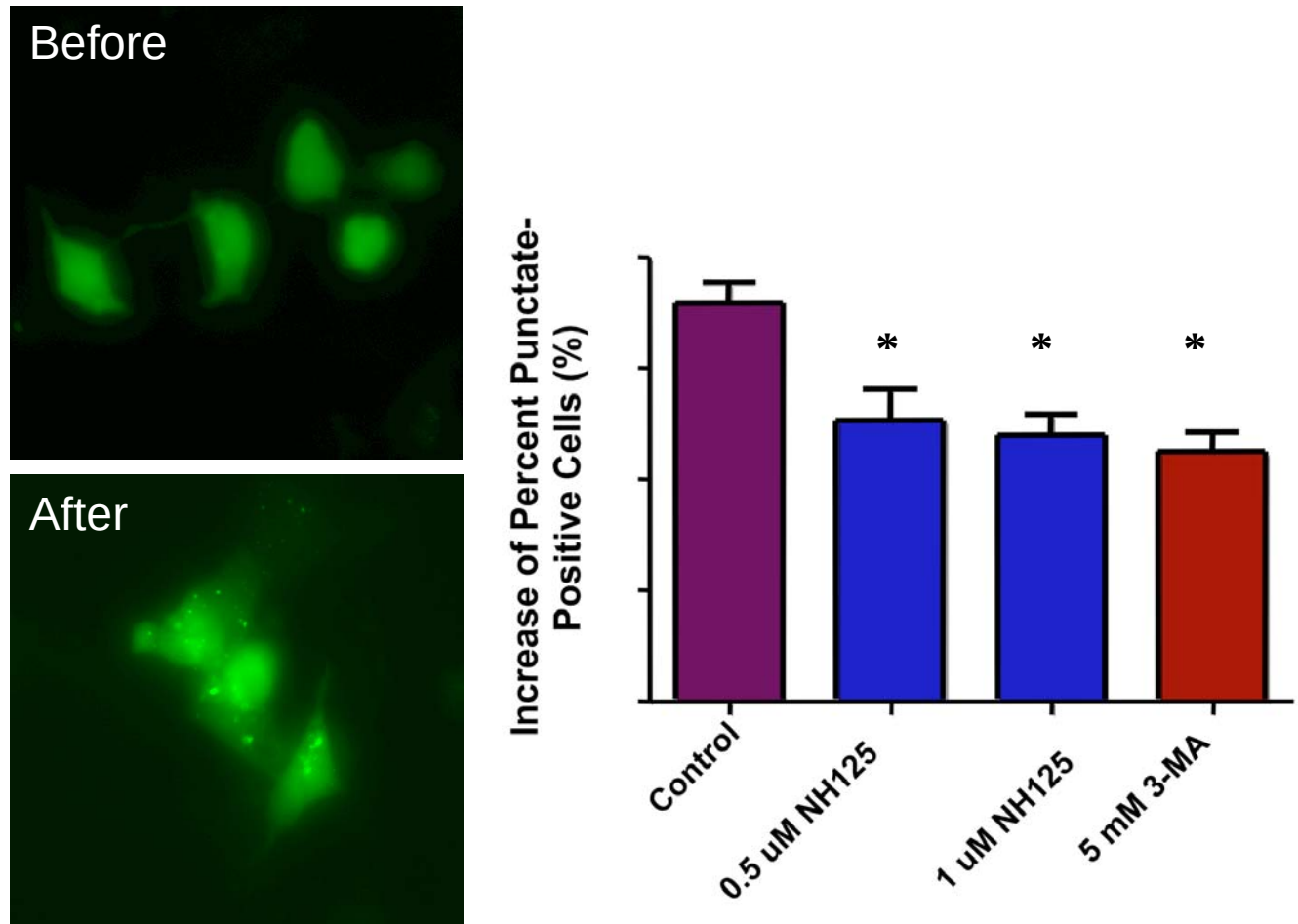


Figure 2. Inhibition of autophagy by NH125 in transient transfection assay. MCF-7 cells were transfected with GFP-LC3 expression vector, treated with NH125, 3-MA or vehicle control overnight, followed by starvation treatment. Punctate-positive and -negative transfected cells were counted before and after starvation. The increase of percent punctate-positive cell indicated the activity of autophagy.

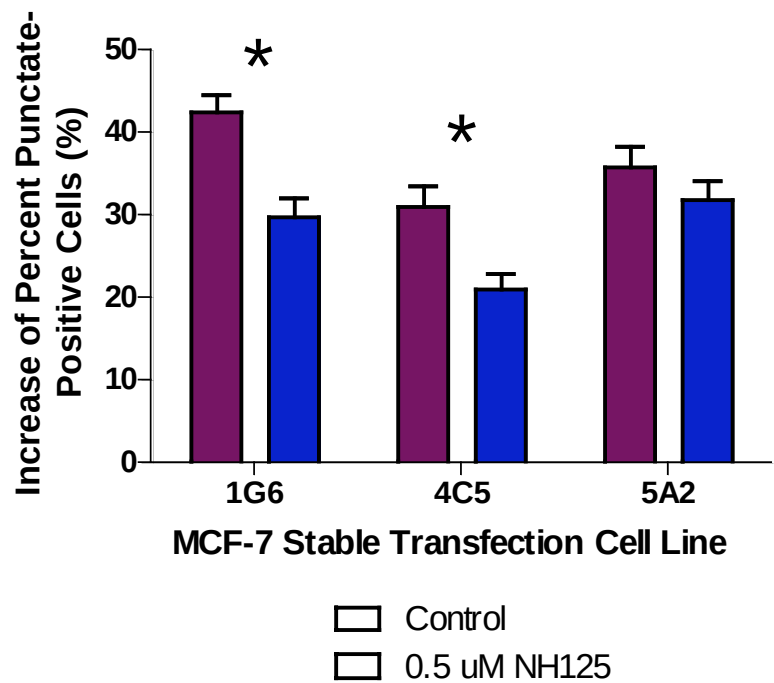
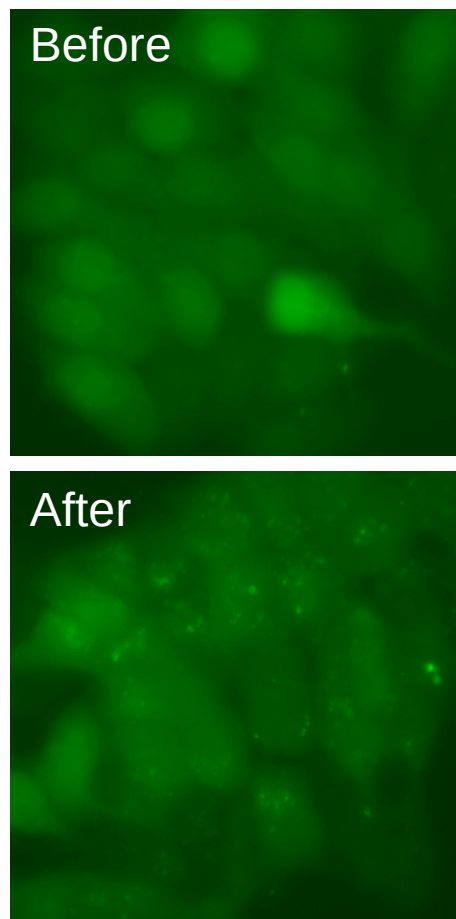


Figure 3. Inhibition of autophagy by NH125 in stable transfection cell lines. Stable transfection cell lines of MCF-7 with GFP-LC3 expression vector were selected. Three independent stable lines were used to test the inhibition effect of autophagy by NH125. The increase of percent punctate-positive cell indicated the activity of autophagy.

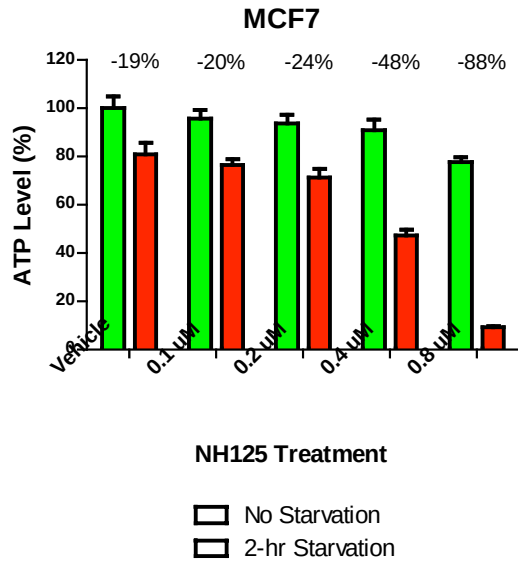
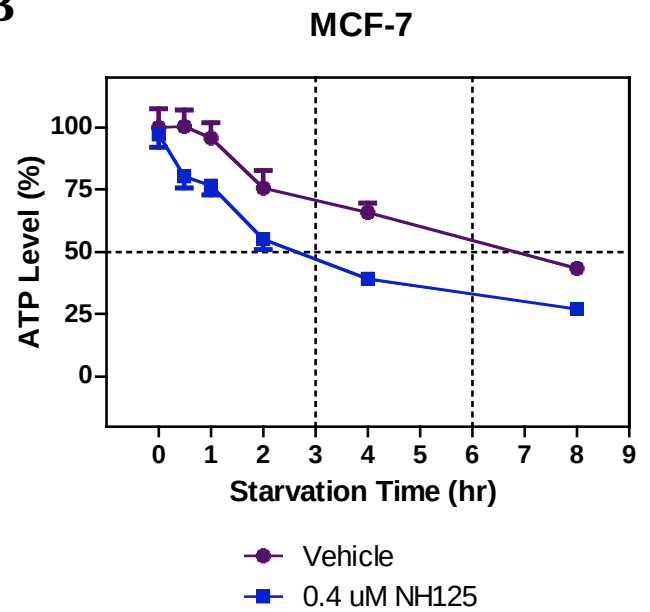
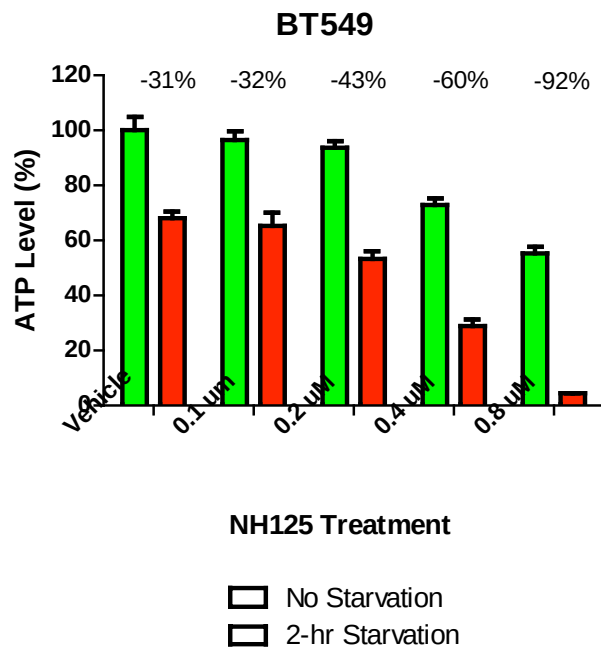
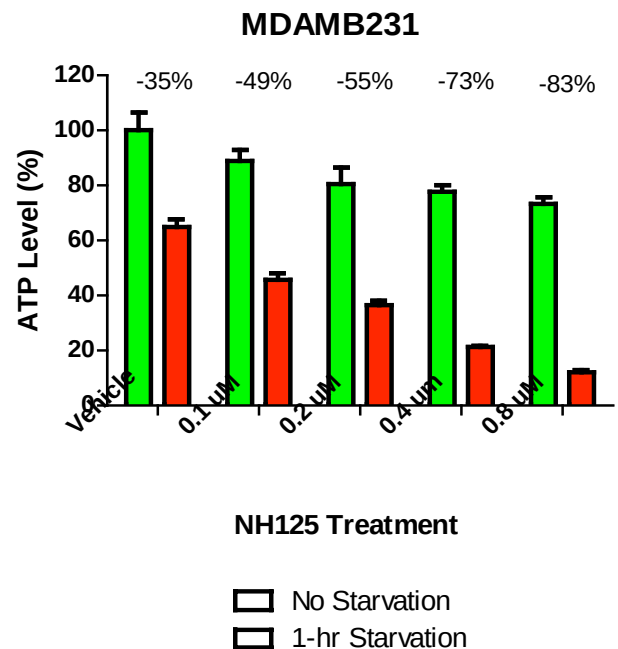
A**B****C****D**

Figure 4. Accelerated ATP Depletion by NH125 Treatment. Cellular ATP level decreased upon nutrient/growth factor deprivation. NH125 treatment resulted in greater and faster ATP reduction compared vehicle. A. Comparison of ATP level in MCF-7 cells after 2hr starvation and no starvation with different concentrations of NH125. B. Comparison of cellular ATP level in MCF-7 cells with 0.4uM of NH125 and vehicle control over a starvation time course. C. Comparison of cellular ATP level in BT549 cells after 2hr starvation and no starvation with different concentrations of NH125. D. Comparison of cellular ATP level in MDA-MB-231 cells after 1hr starvation and no starvation with different concentrations of NH125.

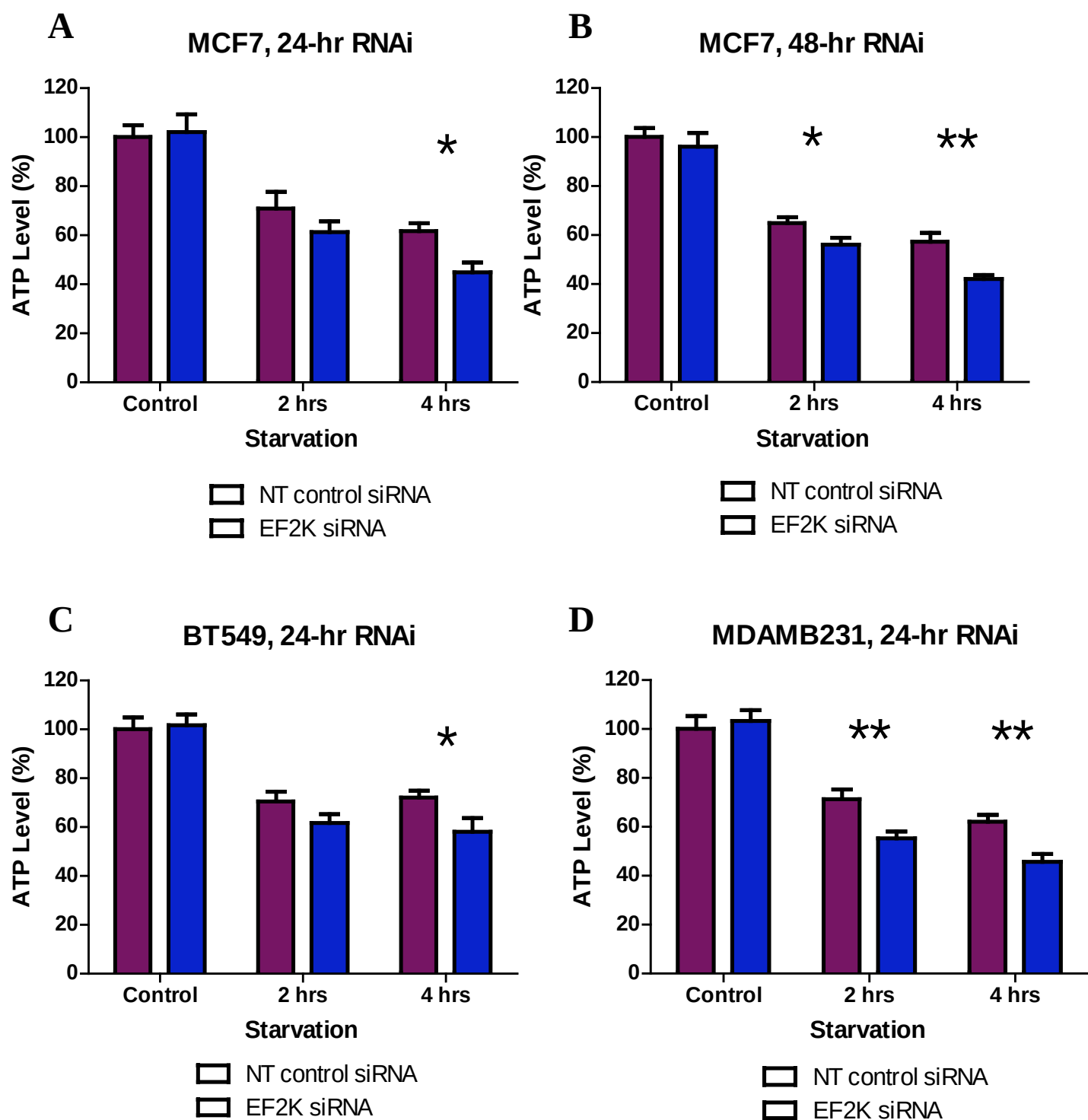


Figure 5. Accelerated ATP depletion by RNAi. Transfection with EF2K siRNA resulted in greater reduction of ATP level after starvation compared to NT control siRNA. A. MCF-7 cells with 24-hr transfection. B. MCF-7 with 48-hr transfection. C. BT549 with 24-hr transfection. D. MDA-MB231 with 24-hr transfection.

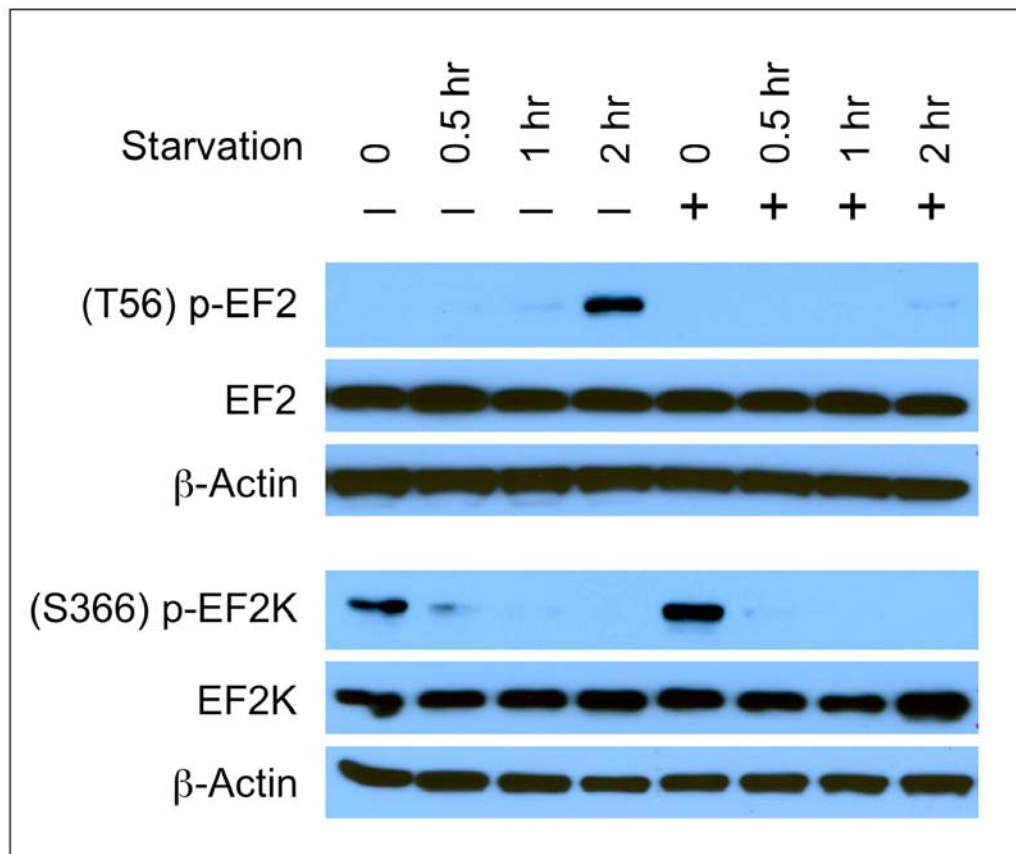


Figure 6. Activation of EF2K by nutrient starvation, and inhibition of EF2K activity by NH125. MCF-7 cells were treated with 0.2uM NH125 or vehicle control overnight. The activity of EF2K was induced by starvation treatment as shown in the control group, the p-EF2 (T56) was greatly increased after 2hr starvation. In NH125 treatment group the increase of phospho-EF2 was evidently inhibited. NH125 treatment didn't change the relief effect of starvation on p-EF2K (S366), which inactivates EF2K .