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Modulation of the Sodium/Iodide Symporter (NIS) by MEK Inhibition in MCF7 Breast Cancer Cells

Matt Flanigan
Biomedical Science

Advisor: Dr. Sissy M Jhiang, PhD
Department of Physiology and Cell Biology

Co-Advisor: Dr. Richard Burry, PhD
Director, Campus Microscopy and Imaging Facility

Committee Members:
Dr. Sissy Jhiang, PhD
Dr. Richard Burry, PhD
Dr. Ranjita Misra, PhD

ABSTRACT

The Na+/I- symporter (NIS) mediates radioiodine therapy in thyroid cancer patients. NIS is also present in many human breast tumors, suggesting that radioiodine may also be used for detection and ablation of breast cancer. Inhibition of MEK (MAPK/ERK Kinase) signaling has been shown to have anti-tumor activity in breast cancer models. Previous data from our lab shows that MEK inhibition reduces NIS protein level and function via lysosomal degradation in trans-Retinoic Acid/Hydrocortisone treated MCF7 human breast cancer cells. We investigated the effect of MEK inhibition on constitutively expressed NIS in MCF7 Cells

Western blot analysis demonstrates that MEK inhibition also reduces exogenous NIS stably expressed in MCF7 cells, suggesting that NIS downregulation by MEK inhibition occurs at the post-transcriptional level. The extent of reduction in NIS activity as determined by radioiodine uptake assay correlates with the reduction of cell surface NIS levels as analyzed by flow cytometry. Fluorescence activated cell sorting was performed to isolate a population of MCF7 cells stably expressing high levels of cell surface NIS. To investigate whether the reduction of surface NIS is due to increased endocytosis, live cell labeling of cell surface NIS was successfully performed and internalization was visualized over time. While NIS remains at the cell surface after labeling with primary antibody alone, NIS endocytosis is induced by conjugated primary and secondary antibodies, presumably due to clustering. An immunofluorescence based internalization assay was performed to determine if the degradation of NIS by MEK inhibition is via increased internalization or by degradation prior to cell surface

trafficking. However, no detectable difference in NIS levels between treated and non-treated cells was detected. Western blot analysis to determine the temporal profile of NIS degradation by MEK inhibition revealed that MEK inhibition does not lead to NIS degradation in the population of selected MCF7 cells with high surface NIS expression. Investigation of how NIS degradation is avoided in this population may help to develop strategies to prevent NIS reduction by MEK inhibition such that MEK inhibition and radioiodine therapy could be used concurrently for breast cancer treatment.

VITA

June 29, 1987…….Born – Cincinnati, OH

2009……B.S. Biomedical Science, The Ohio State University

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CHAPTER 1

PROBLEM STATEMENT

The Na^+/I^- symporter (NIS) is a transmembrane glycoprotein that mediates iodide uptake in thyroid cells for thyroid hormone synthesis. NIS-mediated iodide uptake is the molecular basis for targeted radionuclide imaging and ablation of differentiated thyroid carcinomas and their metastases. NIS expression is also found in many human breast tumors, suggesting that this symporter could be employed for radionuclide imaging and ablation of breast cancers. However, NIS expression and NIS-mediated radioiodine uptake (RAIU) activity are often low in breast cancer, such that tumors fail to concentrate sufficient levels of radioiodine for effective therapy. This makes it important to uncover signaling pathways that can upregulate NIS at several levels, from gene transcription to post-translational processing and cell surface trafficking. These investigations will eventually lead to strategies that can be used to increase functional NIS expression in breast cancer.

Previous data from our lab shows that 24 hr inhibition of the MEK (MAPK/ERK Kinase) signaling pathway by U0126 treatment reduces NIS protein level and function in *trans*-Retinoic Acid/Hydrocortisone (tRA/H) treated MCF7 cells (tRA/H treatment induces expression of endogenous NIS in MCF7 cells). We also showed that 24 hr U0126 treatment leads to lysosome-mediated NIS protein degradation in these cells as well as in MCF7 cells transiently transduced with exogenous NIS adenovirus. It was determined that this was not due to a decrease in NIS mRNA levels. To better understand the

mechanism underlying this degradation induced by MEK inhibition, we investigated the regulation of constitutively expressed NIS by MEK signaling in human breast cancer cells. Specifically, we investigated the modulation by the MEK inhibitor U0126 of exogenous FLAG-tagged NIS stably expressed in MCF7 breast cancer cells.

BACKGROUND AND SIGNIFICANCE

Breast cancer is the most common type of cancer afflicting women in the United States, and it is the second most lethal form of cancer in women. Therefore, it is vital to find ways to improve methods of prevention, detection, and treatment of breast cancer. Current treatment options include lumpectomy or mastectomy, radiation therapy, chemotherapy, and targeted therapies such as trastuzumab for HER2/neu positive breast cancers. Despite advances in treatment options, 40,930 breast cancer deaths were expected in 2008[1].

The sodium iodide symporter is an integral cell surface glycoprotein that functions to import iodide into thyroid follicular cells, utilizing the sodium concentration gradient to uptake two sodium (Na^+) ions per iodide (I^-) ion[2]. This ability to concentrate iodide is utilized clinically for imaging and ablation of thyroid carcinomas by administration of radioactive iodide after thyroidectomy[3]. NIS is expressed primarily in the thyroid gland, but is also found in the salivary glands, the gastric mucosa, and in the lactating mammary gland, where it functions to provide iodine to newborns for thyroid hormone synthesis[4].

The discovery that NIS is expressed 80% of human breast cancers suggests that it may be possible to utilize radionuclide imaging and ablation techniques for detection and

treatment of breast carcinomas[4,5]. An impediment to this prospect, however, is that cell surface NIS expression in breast tumors may be too low to accumulate sufficient amounts of radioiodine for therapy to be effective. Thus, it is pertinent to upregulate NIS expression and activity in order to realize its clinical potential for breast cancer patients[6,7].

MEK (MAPK/ERK kinase) signaling has been shown to function in cellular proliferation, differentiation, survival and metastasis, and is "aberrantly activated [with] overwhelming frequency" in cancer[8]. Inhibition of MEK signaling using the pharmacological agent CI-1040 showed anti-tumor activity in preclinical cancer models, including models of breast cancer. In one study, MEK inhibition reduced growth of tumor xenografts, reversed cellular transformation, decreased cell proliferation, and in some instances induced apoptosis[9]. Additionally, MEK inhibition has been shown to suppress estrogen-induced breast tumor growth *in vitro* and *in vivo*[10]. Therefore, the MEK signaling pathway could serve as a molecular target for cancer therapies.

It has been shown that the MEK signaling pathway modulates expression of NIS in thyroid carcinomas. Constitutively active MEK1 has been shown to reduce NIS mRNA in the thyroid, and inhibition of MEK signaling upregulates NIS in papillary thyroid cancer cells expressing the RET oncogene (RET/PTC cells), which normally have reduced NIS expression,n and function[11]. Our lab demonstrated that MEK inhibition increases NIS protein levels in RET/PTC and normal PCCl3 rat thyroid cells, but does not increase NIS-mediated radioactive iodine uptake until 24-48 hrs later[12].

Unpublished data from our lab shows that, in contrast, an indicator of MEK activation, phospho-ERK, is correlated with *increased* NIS expression in human breast

tumors (Zhang et al, manuscript in preparation). We also have data showing that MEK inhibition decreases NIS expression in several different breast cancer cell lines through lysosomal degradation of NIS protein. This was shown to be true of tRA/H induced, endogenously expressed NIS in MCF7 cells as well as transiently expressed exogenous NIS in MCF7, SK-Br-3, and T47D breast cancer cells. Treatment with the MEK inhibitor U0126 decreased NIS-mediated radioactive iodine uptake in a dose dependent manner; 24 hr treatment with 20uM U0126 decreased uptake by 27% (Zhang et al, manuscript in preparation).

These findings present a challenge, because MEK inhibition has tumor suppressant effects in breast cancer, but also decreases functional NIS expression. Therefore, the use of MEK inhibitors for breast cancer therapies is likely to be incompatible with radioiodine ablation therapies. Further investigation of this process should reveal ways to prevent the reduction in functional NIS induced by MEK inhibition such that the two therapies could be used concurrently. These studies should also provide a better understanding of NIS regulation in breast cancer. Accordingly, we investigated the modulation of NIS by MEK inhibition in MCF7 breast cancer cells stably expressing human NIS (hNIS).

OBJECTIVES

We have observed that inhibition of the MEK signaling pathway reduces NIS expression and function via lysosomal degradation in tRA/H treated MCF7 cells (endogenous NIS) and in MCF7 cells transiently transduced with exogenous NIS. These data are limited by the fact that it describes a NIS population that is newly synthesized

(induced or transiently transduced) rather than constitutively expressed, as NIS in breast cancer is. Additionally, currently available antibodies recognize only an intracellular epitope of NIS, impairing the ability to distinguish cell surface NIS from intracellular NIS. Our objective, therefore, was to investigate the effect of MEK inhibition on exogenous hNIS with a genetically fused extracellular FLAG tag (FLAGhNIS) stably expressed in MCF7 human breast cancer cells. Comparison of our results with those obtained for transiently expressed NIS should reveal whether or not NIS downregulation is restricted to newly synthesized NIS protein.

Determine the effect of MEK inhibition on the functional activity, total protein levels, and cell surface levels of stably expressed exogenous FLAGhNIS

I first sought to determine the effect of 24 hr MEK inhibition on stably expressed exogenous FLAGhNIS functional activity by analyzing NIS-mediated radioactive iodine uptake. I then investigated total protein levels after 24 hr MEK inhibition by performing western blot. Finally, I analyzed the effect of 24 hr MEK inhibition on cell surface FLAGhNIS levels by performing flow cytometry.

Investigate the temporal profile of FLAGhNIS modulation by MEK inhibition

I sought to label cell surface FLAGhNIS with fluorescent antibodies to observe the effect of MEK inhibition over time using immunofluorescent microscopy. In order to eliminate cells without FLAGhNIS expression, and to obtain higher FLAGhNIS expression to improve fluorescence signal-to-noise ratio for microscopy, live-cell Fluorescence Activated Cell Sorting (FACS) was performed to isolate “high-expression”

clones from the heterogeneous cell population. This "high-expression" population was used for all subsequent experiments. I performed live-cell labeling of cell surface FLAGhNIS with fluorescent antibodies and observed it over time. I then attempted to optimize a fluorescence-based internalization assay to determine the effect of 24 hr MEK inhibition on NIS endocytosis. Finally, I investigated the temporal profile of total FLAGhNIS protein levels after various incubation times with MEK inhibition.

CHAPTER 2

MATERIALS AND METHODS

Cell Culture

MCF7 human breast cancer cells were maintained in 44.5% Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) and 44.5% F-12 (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen). Stably transfected MCF7 FLAGhNIS breast cancer cells were maintained in MCF7 media containing 0.02% G418 (invitrogen) to maintain selection of FLAGhNIS expressing cells.

Radioactive Iodide Uptake Assay (RAIU)

MCF7 human breast cancer cells stably expressing FLAGhNIS were treated as indicated prior to RAIU, which was performed as described previously[13]. Briefly, cells were incubated with 2.0 μCi NaI^{125} in 5 μM non-radioactive NaI for 30 mins at 37°C with

5% CO_2. Cells were then washed twice with cold HBSS and lysed with 95% ethanol for 20 minutes. The cell lysate was collected and radioactivity was counted by a γ-counter (Packard Instruments). DPA test was performed as described previously[13] to determine μg DNA per well. For each sample, radioactive iodine uptake was divided by μg DNA to normalize data. Experiments were performed in triplicate.

Western Blot

MCF7 human breast cancer cells stably expressing FLAGhNIS were treated as indicated prior to harvesting. Cells were lysed and proteins were extracted. Total protein concentration was determined by Bradford protein assay, and extracted protein was denatured by adding sample buffer (Tris 150 mM, pH6.5, 4% SDS, 2% p-mercaptoethanol, 10% glycerol). Equal amounts of protein (100ug) from each cellular lysate were resolved by polyacrylamide gel electrophoresis using 10% acrylamide. The proteins were electroblotted onto a nitrocellulose membrane in transfer buffer for 1.5 hours. The membrane was blocked in TBST buffer (10mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% dry milk overnight at 4°C. FLAGhNIS was detected using M2 monoclonal mouse α-FLAG antibody (Sigma, 1:1000) or polyclonal rabbit α-hNIS antibody (1:2500). β-actin was detected using monoclonal rabbit anti-β-actin antibody (Abcam, INC, 1:2000). Phospho-ERK was detected using polyclonal rabbit α-p-ERK antibody (1:4000). Primary antibodies were detected using horseradish peroxidase (HRP)-conjugated α-mouse or α-rabbit IgG secondary antibody (Amersham, 1:2500). The signal was then detected by enhanced chemiluminescence detection reagents (Amersham). Densitometry was performed using the public domain NIH Image

program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Flow Cytometry

MCF7 cells stably expressing FLAGhNIS were treated as indicated prior to quantification of cell surface NIS levels by flow cytometry. Cells were rinsed with phosphate-buffered saline (PBS), then detached by incubation with 0.25% trypsin-EDTA (Invitrogen). Cells were suspended in cold FACS buffer (PBS containing 3% Fetal Bovine Serum, 0.02% Sodium Azide). Cells were incubated with M2 monoclonal mouse α-FLAG antibody or monoclonal mouse IgG antibody (both Sigma, 1:250). Cells were washed and incubated with FITC-conjugated α-mouse IgG secondary antibody (Sigma, 1:100). Cells were washed and resuspended in 1% Paraformaldehyde in FACS buffer. Fluorescence flow cytometry was performed at the Davis Heart and Lung Flow Cytometry core laboratory using a FACSCalibur instrument (BD biosciences). Data was analyzed using WinMDI 2.9 (Microsoft).

Fluorescence Activated Cell Sorting (FACS)

MCF7cells stably expressing FLAGhNIS were rinsed with phosphate-buffered saline (PBS), then detached by incubation with 0.25% trypsin-EDTA (Invitrogen). Cells were suspended in sterile, cold "live FACS" buffer (PBS containing 2% Fetal Bovine Serum, 0.0031% Sodium Azide). Cells were incubated with M2 monoclonal mouse α-FLAG antibody (Sigma, 1:250). Cells were washed and incubated with Alexa488-conjugated α-mouse IgG secondary antibody (Sigma, 1:250). Cells were washed and

resuspended in “live FACS” buffer. FACS was performed at the Davis Heart and Lung Flow Cytometry core laboratory using a FACSAria instrument (BD biosciences). Cells were sorted according to cell surface fluorescence and “high expression” and “low expression” populations were retrieved.

Immunofluorescence Microscopy: Optimization of Incubation Conditions

MCF7 human breast cancer cells stably expressing FLAGhNIS were seeded at 6 x 10^4 cells per well in 4-well chamber slides (Nunc Lab-tek). 24 hrs later, FLAGhNIS was labeled by incubation with M2 monoclonal anti-FLAG antibody from mouse (Sigma, 1:1000) in blocking medium for 20 min at 37°C or for 1 hr on ice. Cells were washed three times with PBS prior to incubation with anti-mouse IgG-Cy3 (Jackson Immuno, 1:500) in blocking medium for 20 min at 37°C or for 1 hr on ice. Cells were washed three times with PBS. Slides were mounted with Pro-long Gold anti-fade mounting solution (Invitrogen, 100ul), and a coverslip was added and sealed with nail-polish. Samples were viewed using a Zeiss Axiovert 200 and digital images were obtained using Axiovision software.

Immunofluorescence Microscopy: Temporal Imaging of Labeled Cell Surface FLAGhNIS

MCF7 +FLAGhNIS cells were seeded as described above. 24 hrs later, FLAGhNIS was labeled by incubation with M2 monoclonal anti-FLAG antibody from mouse (Sigma, 1:1000) in blocking medium for 20 min at 37°C. Cells were washed three times with PBS prior to incubation with anti-mouse IgG-Cy3 (Jackson Immuno, 1:500) in

blocking medium for 20 min at 37°C. Cells were washed three times with PBS prior to visualization of live cells. Cells were then incubated in media at 37°C (5% CO_2) for various times before visualization. Samples were viewed using a Zeiss Axiovert 200 and digital images were obtained using Axiovision software.

Immunofluorescence Microscopy: the Contribution of Antibody Conjugation to Induction of NIS Endocytosis

MCF7 +FLAGhNIS were seeded as described above. 24 hrs later, FLAGhNIS was labeled by incubation with M2 monoclonal anti-FLAG antibody from mouse (Sigma, 1:1000) in blocking medium for 20 min at 37°C. For sample A, cells were washed three times with PBS before incubation with anti-mouse IgG-Cy3 (Jackson Immuno, 1:500) in blocking medium for 20 min at 37°C. Cells were then washed three times with PBS and maintained in media at 37°C (5% CO_2) for 18 hrs. For samples B-D, cells were washed three times with PBS after labeling with M2 and then maintained for18 hrs (as in A). Cell surface M2:FLAGhNIS was labeled on live, non-permeabilized cells with anti-mouse IgG-Alexa488 (Sigma, 1:500) in blocking medium for 20 min at 37°C, prior to fixation. Internalized M2:FLAGhNIS was labeled after fixation and permeabilization (4% Saponin, 4% BSA, 1 hr on ice) by incubation with IgG-Cy3 (1:500). Total M2:FLAGhNIS was labeled the same way as internalized M2:FLAGhNIS, but without prior labeling of surface M2:FLAGhNIS. All fixation was performed in 4% paraformaldehyde (15 mins on ice).

Cell nuclei were labeled with Hoechst 34580 (Invitrogen, 1:10,000) in PBS for 10 min. at RT. Slides were mounted with Pro-long Gold anti-fade mounting solution

(Invitrogen, 100ul). A coverslip was added and sealed with nail-polish. Samples were viewed using a Zeiss Axiovert 200 and digital images were obtained using Axiovision software.

Immunofluorescence-based Internalization Assay with or without MEK Inhibition

MCF7 human breast cancer cells stably expressing FLAGhNIS were seeded as described above. 24 hrs later, FLAGhNIS was labeled by incubation with M2 monoclonal anti-FLAG antibody from mouse (Sigma, 1:1000) in blocking media for 20 min. Cells were fixed immediately, or maintained for 24 hrs at 37˚C (5% CO_2) with treatment as indicated before fixation in 4% paraformaldehyde (15 mins on ice). Cells were permeabilized as indicated (4% Saponin, 4% BSA, 1 hr on ice). Primary antibodies were labeled with IgG-Cy3 secondary antibody (Jackson Immuno, 1:500 in blocking media or permeabilization buffer) for 1 hr on ice. Cell nuclei were labeled with Hoechst 34580 (Invitrogen, 1:10,000) in PBS for 10 min. at RT. Slides were mounted with Pro-long Gold anti-fade mounting solution (Invitrogen, 100ul). A coverslip was added and sealed with nail-polish. Samples were viewed using a Zeiss Axiovert 200 and digital images were obtained with equal exposure times using Axiovision software. Fluorescence was quantified using MetaMorph software (kindly provided by Dr. Jon Robinson). For each sample, 30 cells and 18 background regions were selected for quantification.

CHAPTER 3

RESULTS

FLAGhNIS-mediated iodide uptake (RAIU) is reduced by MEK inhibition

Previous data from our lab shows that inhibition of the MEK signaling pathway by U0126 treatment reduces NIS function in a dose-dependent manner in *trans*-Retinoic Acid/Hydrocortisone (tRA/H) treated MCF7 cells. Specifically, treatment with 20 uM U0126 for 24 hrs decreased NIS-mediated RAIU by 27%. We hypothesized that 24 hr MEK inhibition would decrease iodide uptake in MCF7 cells stably expressing exogenous FLAGhNIS. RAIU was performed to assess FLAGhNIS function. Inhibition of the MEK signaling pathway by 20 μM U0126 treatment for 24 hrs resulted in a 26% decrease in FLAGhNIS functional activity (figure 1).

MEK inhibition reduces total FLAGhNIS protein levels

Previous data from our lab shows that 24 hr inhibition of the MEK signaling pathway by U0126 treatment reduces NIS protein level in *trans*-Retinoic Acid/Hydrocortisone (tRA/H) treated MCF7 cells. We hypothesized that 24 hr MEK inhibition would reduce total FLAGhNIS protein levels in MCF7 cells stably expressing exogenous FLAGhNIS. Western blot was performed to assess FLAGhNIS protein levels. Inhibition of the MEK signaling pathway by 20 μM U0126 treatment for 24 hrs resulted in a 20% decrease in total FLAGhNIS levels (figure 2).

MEK inhibition reduces cell surface FLAGhNIS levels

NIS must be at the cell surface in order to function, so a reduction in NIS cell surface expression would explain the observed reduction in NIS activity after 24 hr MEK inhibition. We hypothesized that 24 hr MEK inhibition would reduce cell surface FLAGhNIS levels. Flow cytometry of non-permeabilized MCF7 cells stably expressing FLAGhNIS was performed to assess cell surface FLAGhNIS levels. This method quantifies cell surface expression by recognizing fluorescent antibodies that bind the extracellular FLAG tag. Inhibition of the MEK signaling pathway by 20 μM U0126 treatment for 24 hrs resulted in a 27% decrease in cell surface FLAGhNIS levels (figure 3). Interestingly, the reduction in cell surface NIS level after 24 hr MEK inhibition seems to be directly proportional to the observed reduction in NIS activity after the same treatment.

Fluorescence-activated cell sorting of MCF7 FLAGhNIS population

Initial immunofluorescence microscopy experiments revealed that the MCF7 FLAGhNIS cell line was heterogeneous in expression of this exogenous gene (data not shown). In order to study a homogenous population, fluorescence-activated cell sorting was performed to isolate a population of "high expression" MCF7 FLAGhNIS cells (figure 4A). This population was used for all subsequent experiments and will be denoted MCF7 +FLAGhNIS. RAIU was performed to verify that this population had higher functional cell surface FLAGhNIS levels (figure 4B). Indeed, MCF7 +FLAGhNIS cells had 35% higher radioactive iodide uptake than the mixed population.

Secondary antibody conjugation induces endocytosis of FLAGhNIS

Preliminary experiments showed that cell surface FLAGhNIS clustering occurred after incubation with primary and secondary antibodies (M2 and IgG-Cy3, respectively) in MCF7 +FLAGhNIS cells. When labeling live cells with antibodies, performing incubations at physiological temperature allows cellular processes to occur during the incubation, whereas performing incubations on ice arrests cellular physiology and endocytosis. FLAGhNIS labeling was observed after incubating live cells with primary and secondary antibodies at 37°C for 20 mins or on ice for 1 hr. The temperature used for M2 primary antibody did not substantially affect the labeling pattern. However, when labeling with IgG-Cy3 secondary antibody at 37°C, clustering of FLAGhNIS occurred within 20 mins, whereas labeling with IgG-Cy3 at on ice showed a more diffuse staining pattern (figure 5). This indicates that secondary but not primary antibody conjugation induces clustering of cell surface FLAGhNIS.

We next sought to determine if this clustering during live-cell labeling would result in endocytosis of the symporter. FLAGhNIS was labeled with M2 followed by IgG-Cy3 and live cells were imaged over time. Clustering and internalization of FLAGhNIS occurred over a period of 8hrs (figure 6). The punctate staining pattern indicates that clustering occurred by 4 hrs, and it is likely that some endocytosis occurred by 4 hrs. After 8 hrs, intracellular clustering indicates that extensive internalization has occurred.

We hypothesized that this internalization was induced by clustering via conjugation of secondary antibodies. To test this hypothesis, four experimental conditions

were employed. For condition A, cell surface FLAGhNIS was labeled in live cells by primary and secondary antibodies. Cells were then maintained at physiological conditions for 18 hrs before fixation and visualization. As evident in figure 7A, FLAGhNIS was completely internalized 18 hrs after incubation with primary and secondary antibodies.

For conditions B-D, cell surface FLAGhNIS was labeled only with primary antibody before maintenance at physiological conditions for 18 hrs. In condition B, live, non-permeabilized cells were incubated with IgG-A488 secondary antibody to label M2 bound to FLAGhNIS (M2:FLAGhNIS) that remained at the cell surface over 18hrs. Cells were then fixed and visualized. Figure 7B shows that M2:FLAGhNIS remained at the cell surface (green fluorescence). In condition C, remaining cell surface M2:FLAGhNIS was labeled as in B. After fixation, cells were permeabilized and labeled with IgG-Cy3 secondary antibody to label M2:FLAGhNIS that was internalized over 18hrs. Cells were then visualized. The merged image in figure 7C shows again that M2:FLAGhNIS remained at the cell surface (green fluorescence). Additionally, the lack of red fluorescence indicates that internalized M2:FLAGhNIS was not detectable. This is either due to degradation of internalized M2:FLAGhNIS or lack of internalization. In condition D, cells were fixed and permeabilized prior to incubation with IgG-Cy3 secondary antibody to label total remaining M2:FLAGhNIS. The subcellular localization of labeled M2:FLAGhNIS in figure 7D is consistent with cell surface rather than intracellular labeling. This supports the conclusion that there is no detectable internalized M2:FLAGhNIS 18 hrs after labeling with primary antibody. Therefore, we conclude that labeling with primary antibody does not induce endocytosis of FLAGhNIS, but conjugation of secondary antibodies does.

Immunofluorescence-based internalization assay does not reveal a difference in FLAGhNIS levels after MEK inhibition in MCF7 +FLAGhNIS cells

To determine if MEK inhibition increases internalization of FLAGhNIS, an immunofluorescence-based internalization assay was performed on MCF7 +FLAGhNIS cells. FLAGhNIS was labeled with M2 primary antibody, then fixed immediately (0hr) or incubated for 24 hrs with MEK inhibition (20 uM U0126) or without treatment (DMSO). The "0hr" sample was fixed immediately and labeled with IgG-Cy3 to determine baseline cell surface FLAGhNIS levels. All other samples were fixed after 24 hr incubation with or without MEK inhibition. To label remaining cell surface M2:FLAGhNIS, cells were incubated with IgG-Cy3. To label total remaining M2:FLAGhNIS, cells were permeabilized prior to incubation with IgG-Cy3.

For each sample, images of 6 different fields of view were taken with equal exposure time. These images were then analyzed using MetaMorph software to quantify fluorescence: for each image, five cells and three regions without cells were selected for quantification. Thus, for each sample, the fluorescence of 30 cells and 18 background regions was quantified. These values were averaged, and background was subtracted from cellular fluorescence to provide the mean fluorescence above background.

We hypothesized that there would be less cell surface M2:FLAGhNIS after MEK inhibition. Surprisingly, remaining cell surface M2:FLAGhNIS and remaining total M2:FLAGhNIS levels were roughly the same for treated and untreated samples (figure

8). Therefore, we conclude that MEK inhibition did not increase the internalization or degradation of the cell surface FLAGhNIS population that was labeled by M2 primary antibody.

MEK inhibition does not reduce total FLAGhNIS levels in MCF7 +FLAGhNIS cells

24 hrs after labeling with M2 primary antibody, little M2:FLAGhNIS remains that can be detected by IgG-Cy3 secondary antibody. This makes it difficult to quantify fluorescence above background for the immunofluorescence-based internalization assay (see figure 8). We theorized that this weak signal was one reason that we were unable to quantify a difference between MEK-inhibited and untreated MCF7 +FLAGhNIS cells. If FLAGhNIS degradation by MEK inhibition occurs with shorter U0126 incubations, we would be able to shorten the incubation for the internalization assay. Therefore, we decided to investigate the temporal profile of FLAGhNIS degradation by MEK inhibition in these cells

MCF7 +FLAGhNIS cells were treated with 20 uM U0126 for 0, 8, 12, 18, and 24 hrs before harvesting for western blot. Surprisingly, we found that U0126 treatment *did not* decrease total FLAGhNIS levels (figure 9A). In fact, FLAGhNIS levels were slightly increased after 12 and 18 hrs of MEK inhibition. Densitometry was performed and FLAGhNIS band intensities were divided by their corresponding β-actin band intensities for normalization of protein loading. Probe for phospho-ERK (p-ERK) verifies that the 20 uM U0126 effectively inhibited the MEK signaling pathway. For each sample, RAIU was performed to determine functional FLAGhNIS activity. Interestingly, MEK inhibition did decrease FLAGhNIS activity in this experiment (figure 9B).

DISCUSSION

Inhibition of the MEK signaling pathway by 24 hr U0126 treatment decreases NIS function, total NIS protein levels, and cell surface NIS levels in MCF7 breast cancer cells stably expressing exogenous FLAGhNIS. Additionally, labeling of cell surface FLAGhNIS in live cells revealed that secondary antibody conjugation induces clustering and endocytosis of the symporter. Fluorescence activated cell sorting was successfully utilized to select for a subset of cells with high cell surface FLAGhNIS expression. Regrettably, the selected clones seem to have different characteristics other than increased cell surface FLAGhNIS expression. In these "high expression" clones, MEK inhibition did not result in the degradation of FLAGhNIS, although it did decrease NIS-mediated RAIU. A future direction is to investigate what makes these cells respond differently to MEK inhibition, perhaps by comparing the genetic profiles of MCF7 FLAGhNIS cells with that of the selected MCF7 +FLAGhNIS cells. This is clinically relevant, because it suggests that the use of MEK inhibitors for breast cancer therapies could be compatible with radioiodine ablation therapies in some patients.

Inhibition of the MEK signaling pathway by 24 hr U0126 treatment decreases functional activity and total protein level of FLAGhNIS in MCF7 cells stably expressing it. This is in agreement with previous data from our lab showing that MEK inhibition reduces the functional activity and total protein level of endogenous NIS protein in tRA/H treated MCF7 cells. However, tRA/H treatment induces the synthesis of endogenous hNIS. Therefore, this model allowed for analysis of the effect of MEK inhibition on the expression and activity of *newly synthesized* hNIS. Our study differs by

investigating the effect of MEK inhibition in a model of *constitutively expressed* FLAGhNIS. MEK inhibition had the same effect in both models, indicating that NIS degradation by MEK inhibition is not restricted to newly synthesized protein.

Since NIS must be at the cell surface to function as a symporter, a decrease in cell surface expression could explain the observed decrease in function. 24 hr MEK inhibition resulted in a 27% decrease in cell surface FLAGhNIS protein level as assessed by flow cytometry. It is interesting to note the concordance of this value with the reduction in function of both tRA/H induced hNIS and constitutively expressed FLAGhNIS after 24 hr MEK inhibition. In those experiments, RAIU was decreased by 27% and 26%, respectively. This suggests that the observed decrease in FLAGhNIS activity can be explained by a reduction in cell surface FLAGhNIS protein level.

We next sought to determine whether this reduced cell surface FLAGhNIS was due to increased internalization, decreased trafficking to the cell surface, or both. The first objective to this end was to label cell surface FLAGhNIS with fluorescent antibodies and to track it over time. Unlike RAIU, Western Blot, and Flow Cytometry, which are population-based assays, immunofluorescence microscopy allows for visualization and hence characterization of individual cells. Preliminary experiments revealed that the MCF7 FLAGhNIS stably transfected cell line was heterogeneous in expression level of FLAGhNIS. In fact, many cells appeared to not express FLAGhNIS at all. As we were only interested in cells that expressed cell surface FLAGhNIS at levels high enough to be detected using immunocytochemistry, a more homogenous, “high expression” population was selected for using fluorescence activated cell sorting (FACS). This technique was successfully employed to isolate a population of MCF7 FLAGhNIS with high cell surface

FLAGhNIS expression. RAIU was performed on the separated populations. As expected, the "high expression" (MCF7 +FLAGhNIS) population had the highest radioactive iodine uptake, due to higher expression of functional cell surface FLAGhNIS.

A straightforward way to investigate the effect of MEK inhibition on cell surface FLAGhNIS is to label with antibodies for indirect immunofluorescence microscopy and observe U0126 treated cells compared to non-treated cells over time. Preliminary experiments were performed to optimize live cell labeling of FLAGhNIS. As shown in figures 5-7, secondary antibodies induce clustering and internalization of FLAGhNIS. As this endocytosis is induced by antibodies and does not represent constitutive internalization, we believe that investigating the effect of MEK inhibition on this process would not be physiologically relevant. However, these data did show that labeling with primary antibody alone does not induce endocytosis of FLAGhNIS (figure 7). Since labeling of FLAGhNIS using M2 primary antibody does not seem to interfere with trafficking, this antibody can be used to pulse label a population of cell surface FLAGhNIS. This pulse labeling was used to investigate the effect of MEK inhibition on FLAGhNIS internalization by performing a fluorescence-based internalization assay.

I next sought to optimize a fluorescence-based internalization assay using MCF7 +FLAGhNIS cells. The strategy was to pulse label a population of cell surface FLAGhNIS, incubate at physiological conditions with or without MEK inhibition, and chase with one of two labeling methods: one for permeabilized cells and one for non-permeabilized cells. This determines for each treatment how much of the pulsed population (M2:FLAGhNIS) has been internalized compared to how much has remained at the cell surface. M2 primary antibody was to pulse label cell surface FLAGhNIS,

followed by 24 hr incubation with or without MEK inhibition. The pulsed population was chased with IgG-Cy3 secondary antibody to label remaining cell surface M2:FLAGhNIS in non-permeabilized cells, and to label total remaining M2:FLAGhNIS in permeabilized cells. We theorized that by comparing the two labeling patterns we would be able to determine the relative amount of internalization of M2:FLAGhNIS for each treatment.

Contrary to our hypothesis, we did not observe a more robust decrease in remaining cell surface M2:FLAGhNIS after 24 hr MEK inhibition compared to untreated cells, suggesting that MEK inhibition did not increase internalization. There was also no apparent difference in total remaining M2:FLAGhNIS between MEK inhibited and non-treated cells, suggesting that degradation was not increased. However, for both permeabilized and non-permeabilized cells, there was very little detectable M2:FLAGhNIS remaining after the 24 hr incubation (figure 8A). This made it difficult to compare treatments, as quantifiable fluorescence above background was very low (figure 8B).

We theorized that levels of remaining M2:FLAGhNIS would be higher after shorter incubations between pulse and chase labeling, such that fluorescence could be more effectively quantified and compared. However, it was unknown if the MEK inhibitor U0126 could downregulate FLAGhNIS with shorter treatment times. In order to determine the appropriate incubation time for the fluorescence-based internalization assay, the temporal profile of FLAGhNIS modulation by MEK inhibition was characterized.

To investigate the temporal profile of the effect of MEK inhibition on FLAGhNIS in MCF7 +FLAGhNIS cells, western blot was employed to quantify total FLAGhNIS

levels after 0, 8, 12, 18, and 24 hr U0126 treatment. Surprisingly, total FLAGhNIS protein levels did not decrease, and in fact were slightly higher after 12 and 18 hrs of MEK inhibition. Since U0126 inhibits phosphorylation of ERK, p-ERK was probed to ensure that the drug was effective. Indeed, p-ERK levels decreased dramatically in all U0126-treated samples. Additionally, the probe for hNIS only detected bands in MCF7 +FLAGhNIS cells, verifying that the band is specific. Therefore, we must conclude that FLAGhNIS protein levels were not decreased by MEK inhibition. Interestingly, however, FLAGhNIS-mediated RAIU did decrease in a dose-dependent manner. This could be due to downregulation of cell surface FLAGhNIS, direct inhibition of FLAGhNIS function, or it may be an artifact due to cytotoxicity.

The contradiction between the western blot results presented in figures 2 and 9 may be due to phenotypic differences between the cell populations used for the experiments. It is likely that selecting for "high expression" MCF7 FLAGhNIS cells isolated a population that differed from the mixed population in other characteristics than cell surface FLAGhNIS expression. Regrettably, we had not considered this possibility before obtaining this western blot result. These cells were also used for the fluorescence-based internalization assay, which may explain why there was no observable difference between MEK inhibited and non-treated cells in that experiment.

The conflicting western blot results reveal a major limitation of this study: results of experiments performed on MCF7 +FLAGhNIS cannot be directly compared to those of experiments performed on the mixed population, due to different properties of these populations. However, this does open the door for a novel future direction. If these two populations can be compared genetically and biochemically, we may learn why MEK

inhibition leads to NIS degradation in one population but not the other. This would be an important step towards finding a way to use radioactive iodide ablation therapy concurrently with MEK inhibition as therapies for breast cancer.

Another strategy to study the effect of MEK inhibition on NIS trafficking was pursued. A recently developed method for live cell labeling of proteins is the biarsenical-tetracysteine system, which allows for protein labeling with small, membrane permeable dyes[14]. A tetracysteine motif (-CCXXCC-) is genetically fused to the protein of interest, and serves as a small tag. This is recognized by the small biarsenical molecules FlAsH (Flourescein Arsenical Helix) and ReAsH (Resorufin Arsenical Helix), which fluoresce green and red, respectively, when bound to the tetracysteine tag. These membrane permeable dyes can be used for pulse-chase labeling of a subset of proteins to study their temporal dynamics using fluorescence microscopy.

An MCF7 human breast cancer cell line stably expressing human NIS with a tetracysteine motif fused to the intracellular C-terminus of the symporter (hNIS-TC) was established. We planned to use this system to investigate NIS modulation by MEK signaling in MCF7 cells stably expressing hNIS-TC. Initial experiments were performed to label hNIS-TC with FlAsH, but hNIS-TC labeling above the substantial background from non-specific binding was undetectable. To obtain higher expression of hNIS-TC, transient transfection of the plasmid was used in subsequent experiments. Efforts to optimize the FlAsH labeling method included using increasingly stringent wash conditions, lower FlAsH concentrations and shorter incubation times. Unfortunately, considerable non-specific binding persisted, and detection of FlAsH-labeled hNIS-TC above background was unsuccessful. Therefore, the use of this technique was abandoned.

The technology of FlAsH labeling of tetracysteine-tagged proteins was invented by Tsien et al and first reported in 1998[15]. Since then, examples of the use of this technique include observation of connexin trafficking in live cells[16], visualization of mRNA translation in live cells[17], and labeling of the reggie-1/flotillin-2 receptor[18]. However, Stroffekova et al reported that FlAsH binds not only tetracysteine motifs, but also non-specifically to endogenous cysteine-rich proteins, and concluded that FlAsH "may be useful only for labeling those recombinant proteins that express at a very high level[19]." They also found that the amount of non-specific binding varied by cell line. For example, background fluorescence was much higher in HEK293 cells than in HeLa or myocyte protein samples.

In 2005, the tetracysteine motif was optimized for higher affinity labeling. The authors found that the hairpin motif –CCPGCC– yielded higher contrast of labeled proteins above background[20]. Specifically, the sequences HRWCCPGCCKTF and FLNCCPGCCMEP resulted in a >20-fold increase in contrast. Of note, we used the latter sequence as the TC motif of hNIS-TC, but were unable to successfully label this fusion protein. A future direction of this study could be to attempt labeling of hNIS-TC expressed in other cell lines, and to further test varying wash conditions, labeling times, and FlAsH concentrations. If this labeling technique can be optimized, it will be an informative tool for investigating the effect of MEK inhibition on the temporal dynamics of NIS trafficking in live cells.

Figure 1.

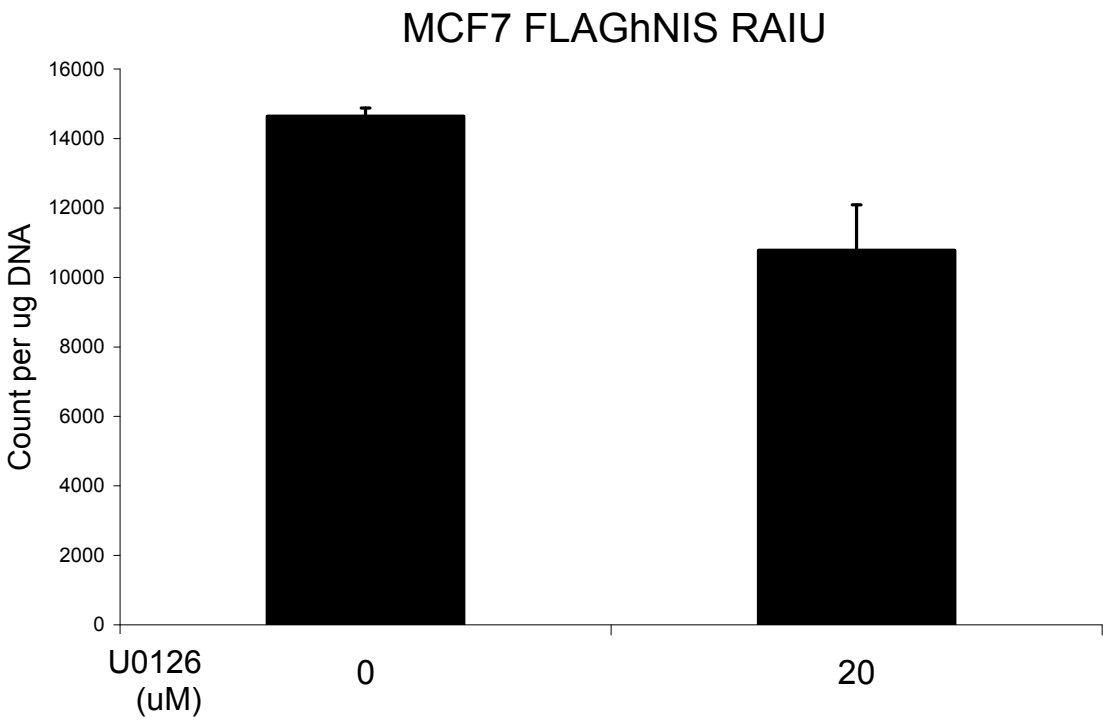


Figure 1. Reduction in FLAGhNIS function after 24 hr MEK inhibition (20 uM U0126) versus no treatment (DMSO). This corresponds to a 26% decrease in functional activity.

Figure 2.

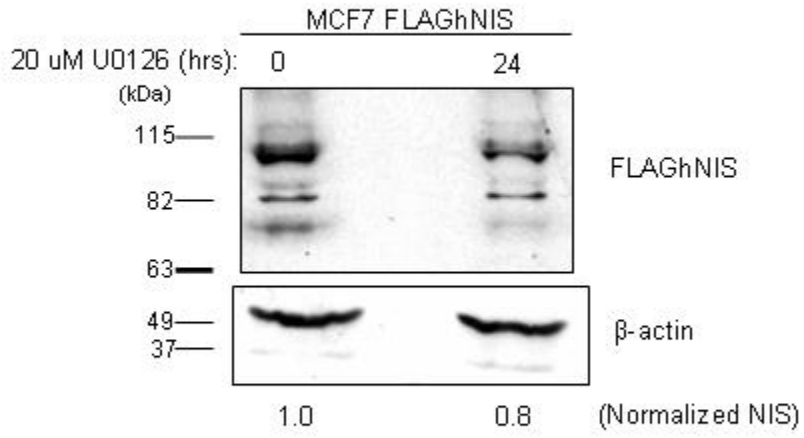


Figure 2. Reduction in total FLAGhNIS level after 24 hr MEK inhibition (20 uM U0126) versus no treatment (DMSO). Probe for β-actin allows for normalization of the amount of protein loaded per sample. This corresponds to a 20% decrease in total FLAGhNIS protein level.

Figure 3.

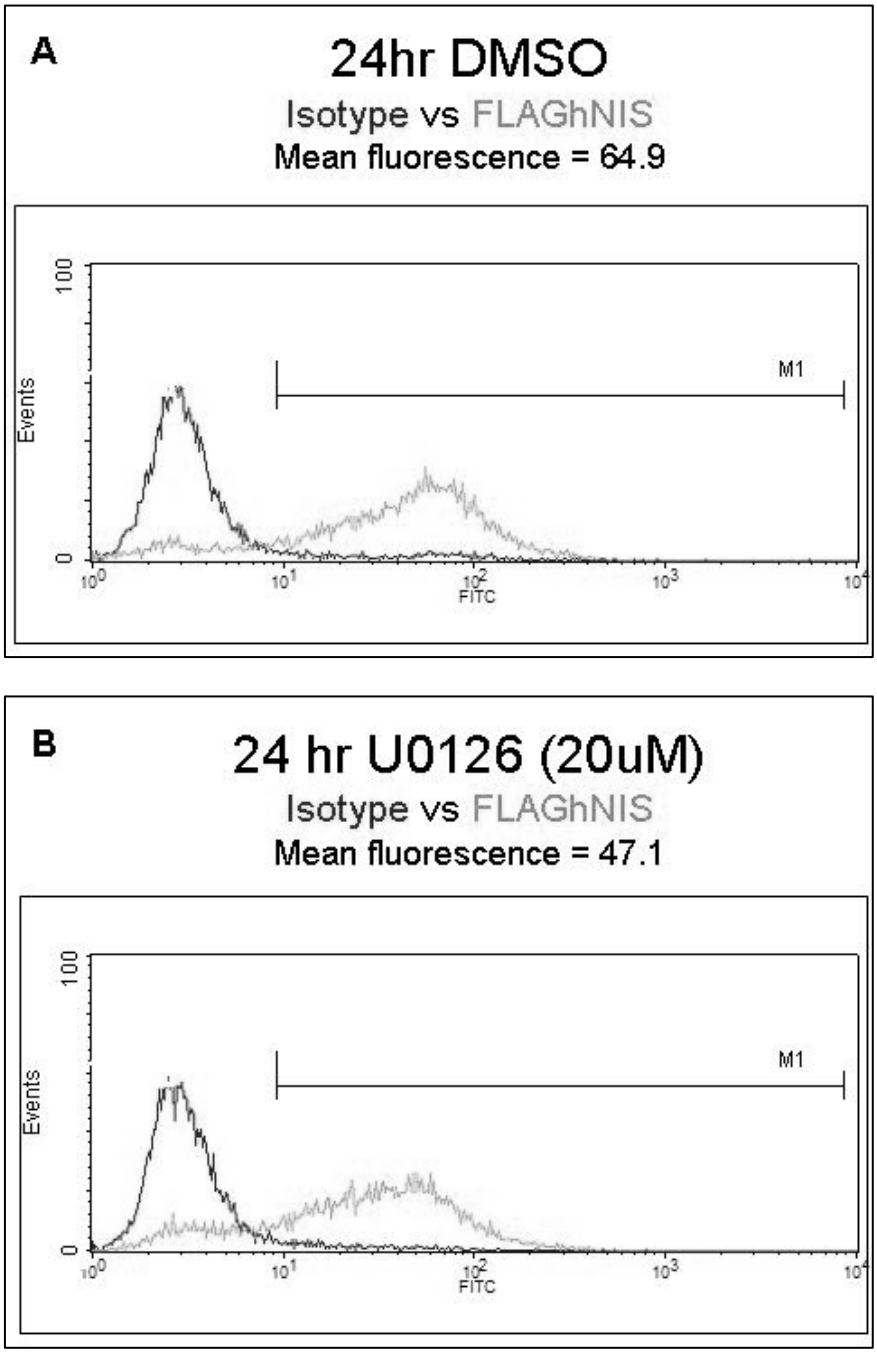


Figure 3. Reduction in surface FLAGhNIS level after 24 hr MEK inhibition (20 uM U0126) versus no treatment (DMSO). 10,000 cells were counted for each sample, and mean fluorescence was used to quantify surface FLAGhNIS level. A) DMSO treated cells yield a mean fluorescence of 64.9. B) U0126 treated cells yield a mean fluorescence of 47.1. This corresponds to a 27% decrease in surface FLAGhNIS protein.

Figure 4.

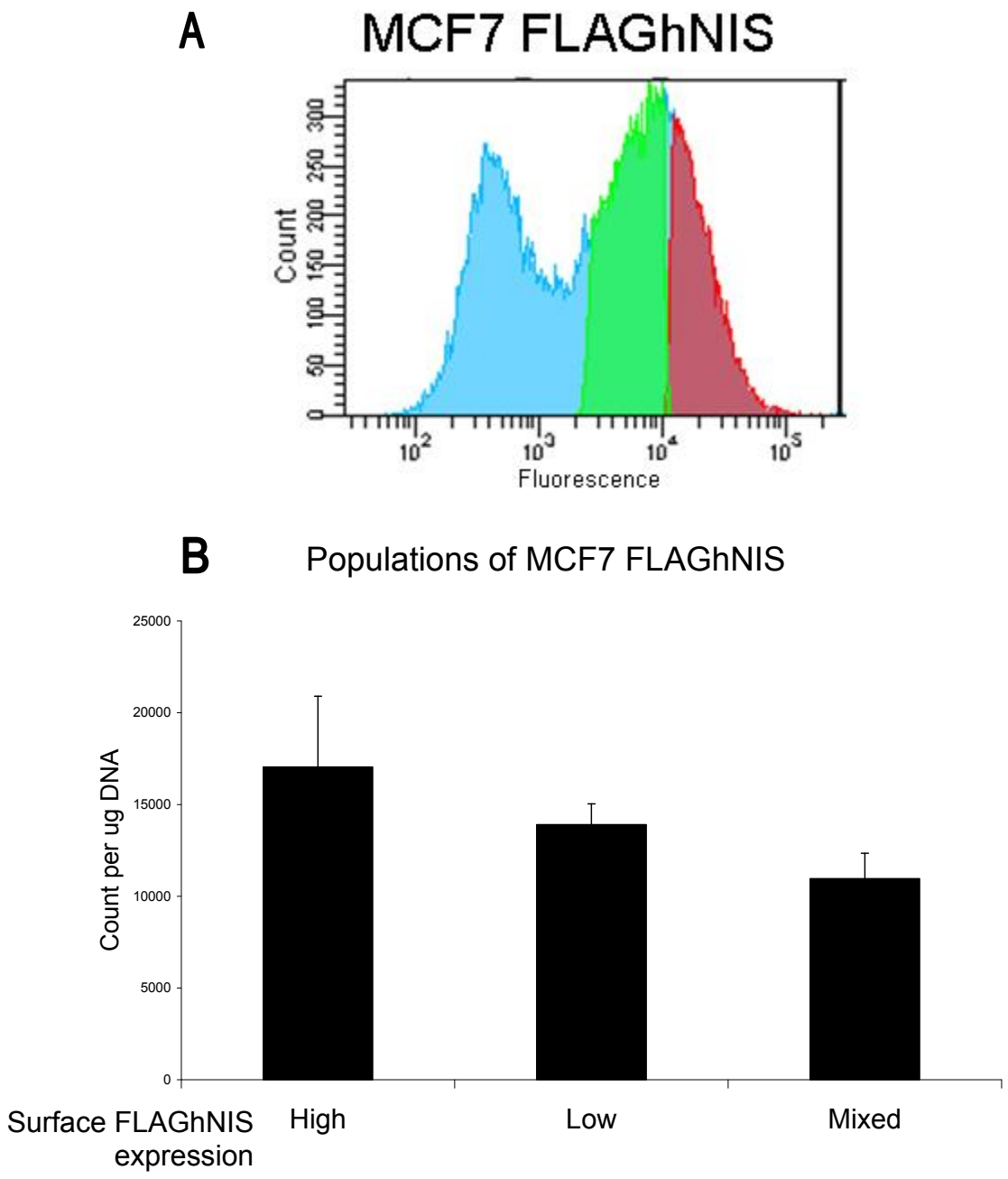

A
MCF7 FLAGhNIS
Count
0
50
100
150
200
250
300
10^2
10^3
10^4
10^5
Fluorescence
B
Populations of MCF7 FLAGhNIS
Count per ug DNA
0
5000
10000
15000
20000
25000
Surface FLAGhNIS expression
High
Low
Mixed

Figure 4. Fluorescence activated cell sorting by cell surface FLAGhNIS expression of MCF7 FLAGhNIS mixed population. A) Cells were gated by fluorescence intensity. 25,174 cells were excluded as dead or "no expression of FLAGhNIS" (blue), 10,737 cells were collected as "low expression of FLAGhNIS" (green), and 7,110 cells were collected as "high expression of FLAGhNIS" (red). B) RAIU of the different populations shows that "high expression" cells have 35% higher iodide uptake activity than the mixed population. The "high expression" clones were maintained and used for subsequent experiments.

Figure 5.

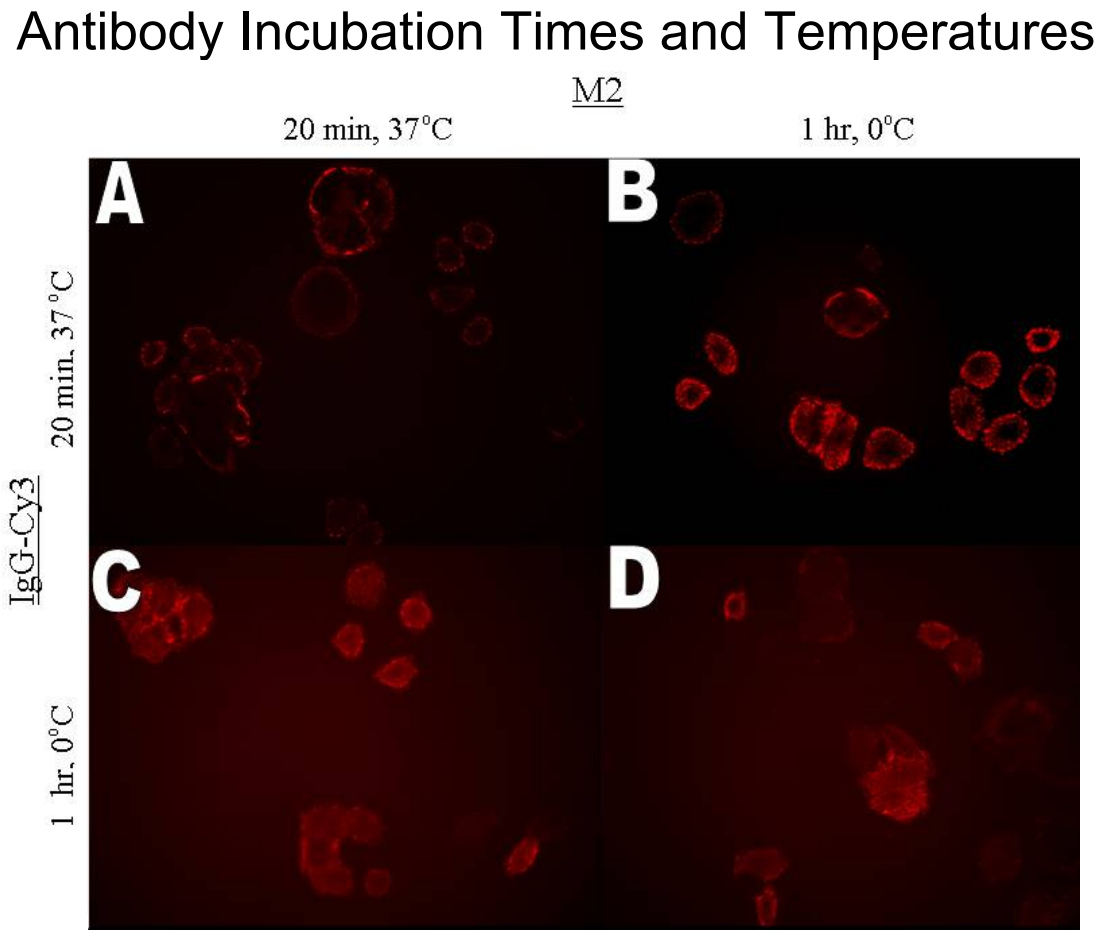


Figure 5. Secondary antibody incubation causes clustering within 20 mins when performed at physiological temperature. FLAGhNIS was labeled with M2 primary antibody (1:1000) for 20 mins at 37°C (A, C) or 1 hr on ice (B, D), followed by IgG-Cy3 secondary antibody (1:500) 20 mins at 37°C (A, B) or 1 hr on ice (C, D). Cells were fixed in 4% Paraformaldehyde, mounted, and viewed at 20x.

Figure 6.

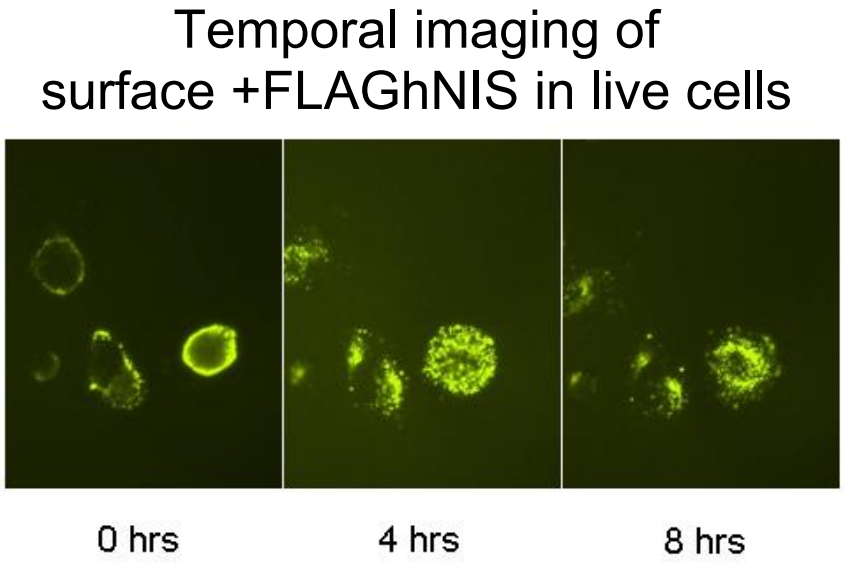


Figure 6. Internalization of FLAGhNIS after incubation with primary and secondary antibodies. Live MCF7 +FLAGhNIS cells were incubated with M2 monoclonal anti-FLAG antibody from mouse (1:1000), followed by anti-Mouse IgG-Cy3 (1:500) for indirect immunofluorescent labeling of FLAGhNIS. Cells were visualized at 20x immediately (0 hrs), at 4 hrs, and at 8 hrs. Cells were maintained at 37°C (5% CO_2) between visualizations. Images show the same field of view over time, revealing clustering and endocytosis of FLAGhNIS.

Figure 7.

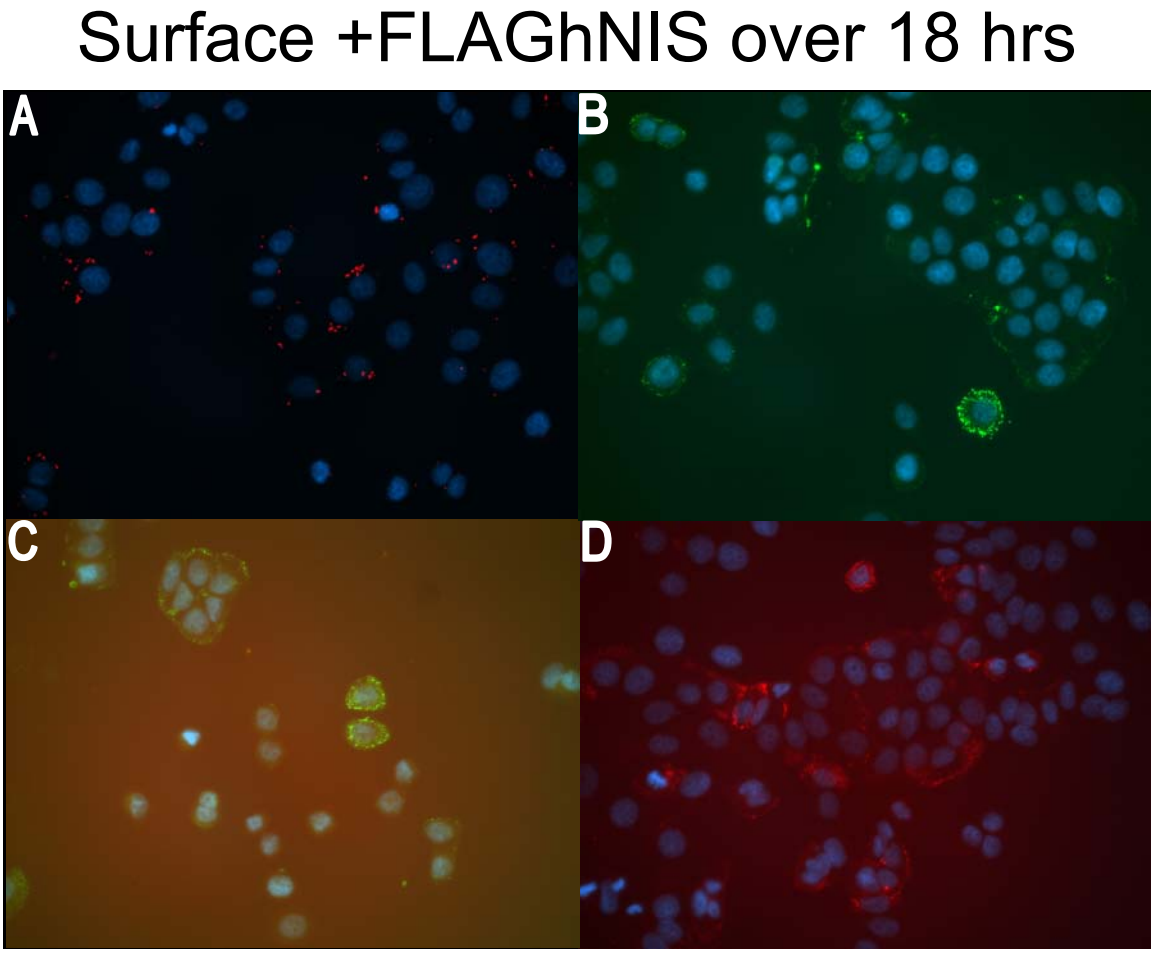
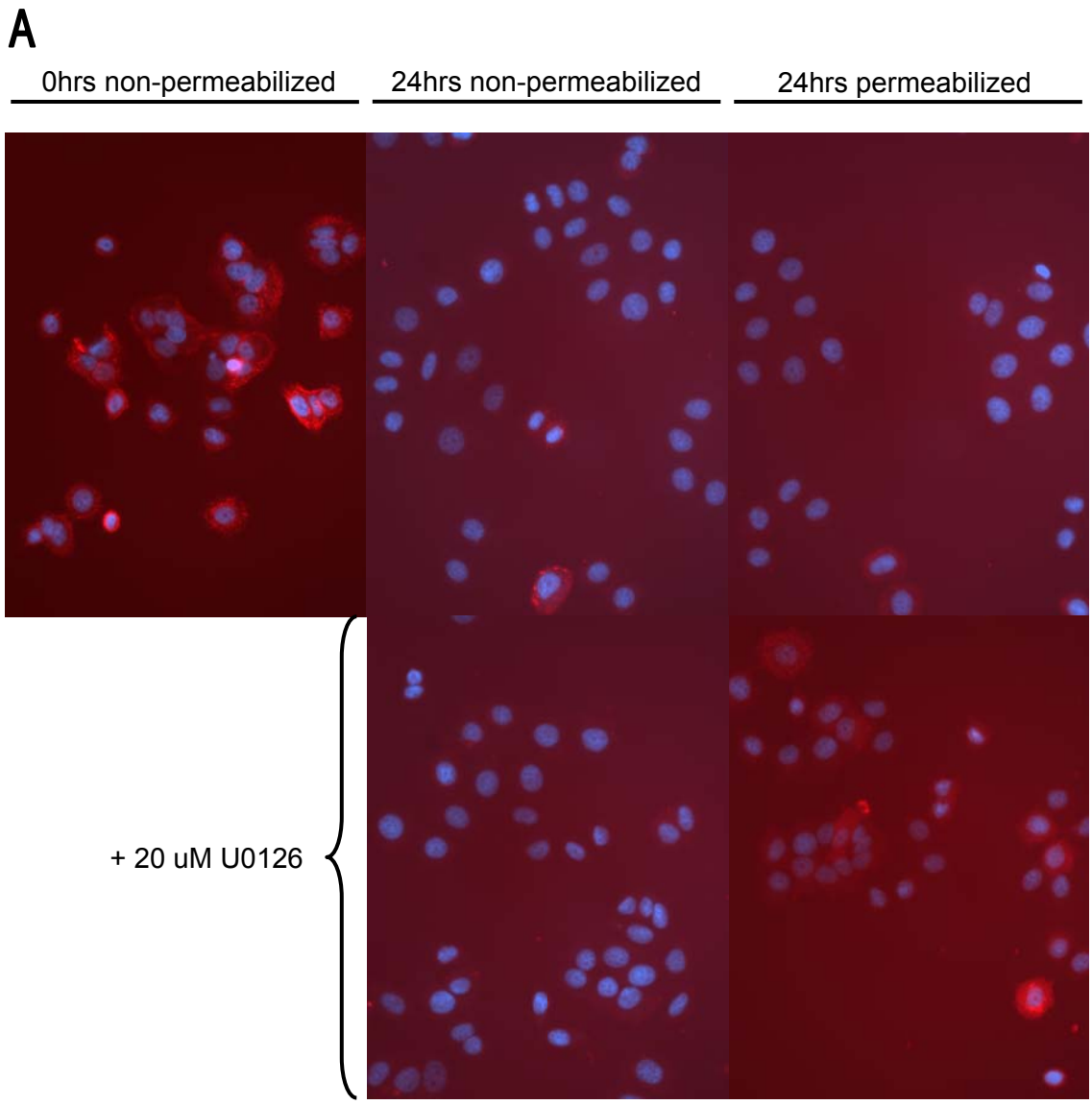


Figure 7. Incubation with primary and secondary antibodies results in endocytosis of FLAGhNIS, but incubation with primary antibody alone does not. A) Cell surface FLAGhNIS was labeled with M2 followed by IgG-Cy3 (as described in figure 6). 18 hrs later, cells were fixed in 4% Paraformaldehyde and nuclei were labeled with Hoechst 34580 (1:10,000, blue). B) Cell surface FLAGhNIS was labeled with M2. 18 hrs later, M2:FLAGhNIS that remained at the cell surface was labeled in live, non-permeabilized cells by IgG-A488 (green). Fixation and nuclear staining was performed as in A. C) Cell surface FLAGhNIS was labeled with M2. 18 hrs later, remaining cell surface M2:FLAGhNIS was labeled as in B (green). Cells were fixed and then permeabilized with 4% Saponin before incubation with IgG-Cy3 to label internalized M2:FLAGhNIS (red). Fixation and nuclear staining was performed as in A. D) Surface FLAGhNIS was labeled with M2. 18 hrs later, cells were fixed and permeabilized before incubation with IgG-Cy3 to label total M2:FLAGhNIS (red). Nuclear staining was performed as described in A. Cells were visualized at 20x.

Figure 8.


A
0hrs non-permeabilized
24hrs non-permeabilized
24hrs permeabilized
+ 20 uM U0126

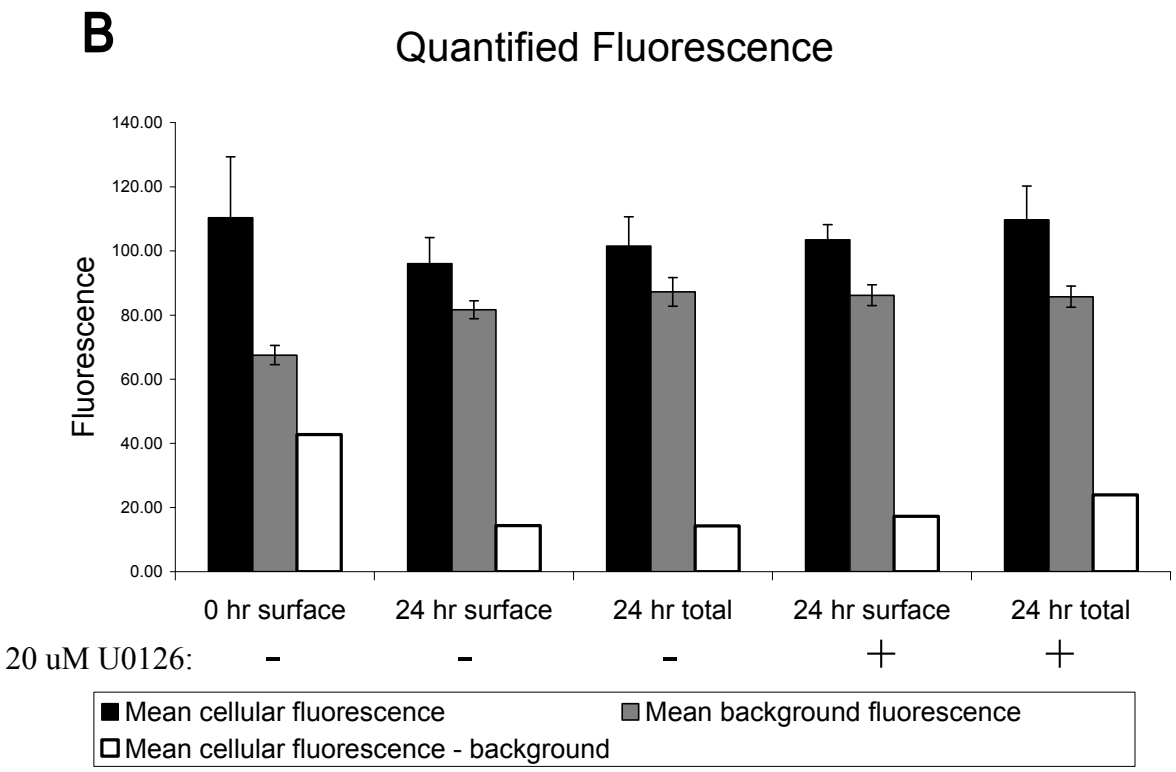


Figure 8. MEK inhibition does not increase the internalization or degradation of surface FLAGhNIS in MCF7 +FLAGhNIS cells. After labeling cell surface FLAGhNIS with M2 (1:1000), cells were fixed immediately or incubated with or without 20 uM U0126 treatment for 24 hrs. Cell surface M2:FLAGhNIS or total M2:FLAGhNIS was then labeled with IgG-Cy3. A) Selected images taken at 20x. Fluorescence from FLAghNIS is red. Nuclei were labeled with Hoechst 34580 (1:10,000, blue). B) Results of quantification. For each sample, fluorescence was quantified using MetaMorph software for 30 cells (black) and 18 background regions (grey). These values were subtracted to provide mean fluorescence above background (white).

Figure 9.

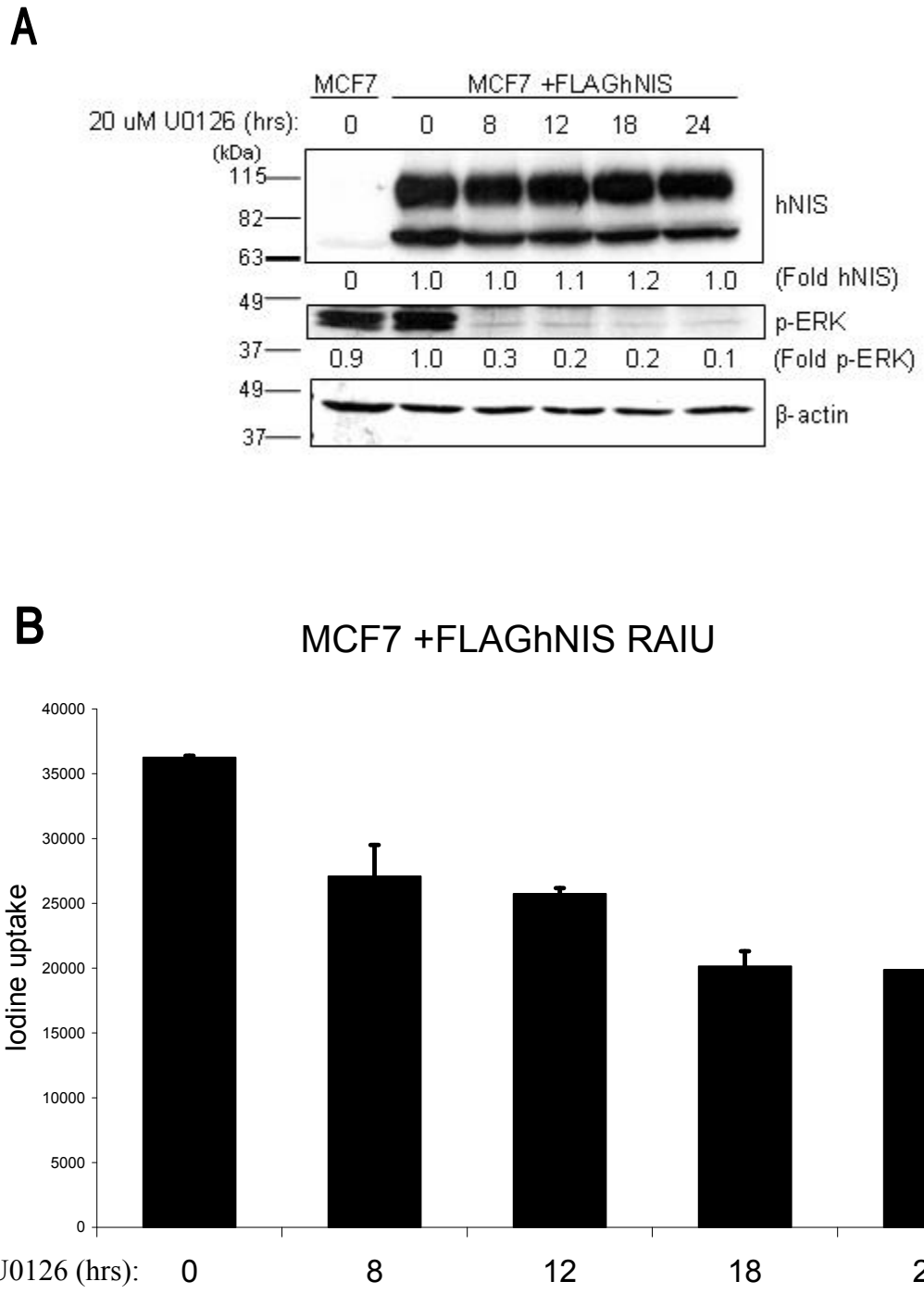

A
MCF7
MCF7 +FLAGhNIS
20 uM U0126 (hrs):
0
0
8
12
18
24
(kDa)
115
82
63
49
37
49
37
hNIS
0
1.0
1.0
1.1
1.2
1.0
(Fold hNIS)
p-ERK
0.9
1.0
0.3
0.2
0.2
0.1
(Fold p-ERK)
β-actin
B
MCF7 +FLAGhNIS RAIU
Iodine uptake
40000
35000
30000
25000
20000
15000
10000
5000
0
20uM U0126 (hrs):
0
8
12
18
24

Figure 9. MEK inhibition does not decrease total FLAGhNIS but does decrease FLAGhNIS radioiodide uptake in MCF7 +FLAGhNIS cells. A) Temporal profile of total FLAGhNIS levels after MEK inhibition. Anti-hNIS polycloncal antibody from rabbit was used to probe for FLAGhNIS. Polyclonal antibodies from rabbit were used to probe for p-ERK and β-actin. Primary antibodies were detected by anti-rabbit IgG-HRP from goat. Densitometry was performed and band intensities were divided by β-actin band intensities for normalization of protein loading. Fold hNIS and fold p-ERK are measured relative to untreated MCF7 +FLAGhNIS level. B) Temporal profile of FLAGhNIS-mediated iodide uptake after MEK inhibition. Iodide uptake was not divided by ug DNA and therefore has not been normalized for cell number.

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