Characterization of Ubiquitination Site of Inducible cAMP Early Repressor

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CHARACTERIZATION OF UBIQUITINATION SITE OF INDUCIBLE cAMP EARLY REPRESSOR

by

Fanaye Woldeamanuel

A Master’s Thesis Submitted to the Faculty of Montclair State University

In Partial Fulfillment of the Requirements

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ABSTRACT

Inducible cAMP Early Repressor (ICER) is a negative transcriptional regulator found in eukaryotic cells. ICER has a tumor suppressive activity and is absent in several human cancers. Cancer cells get rid of ICER by a post-translational modification known as ubiquitination. Ubiquitin molecules, which are very small regulatory proteins, attach to lysine residues of a target protein to induce either a proteolytic or a non-proteolytic pathway. Both pathways target ICER levels inside the nucleus. In the case of the proteolytic pathway, ICER gets sent to the proteasome where it gets degraded and in the non-proteolytic pathway, ICER gets sent to the cytoplasm. Thus, cancer cells take advantage of ubiquitination to either degrade ICER or change its subcellular localization to affect its activity. When ICER is reintroduced into cancer cells, it inhibits the transformed phenotype of these cells. Therefore, my research attempted to characterize the ubiquitination site(s) of ICER and determine the effect of its subcellular localization to eventually block ICER ubiquitination.

Ubiquitination occurs at lysine residues. Since ICER has eleven lysine residues, each residue was previously mutated to a relatively similar amino acid, arginine, using site directed mutagenesis to create ICER with no lysine (ICER KO). Previous studies have shown that ICER KO represses growth related genes (1) more efficiently than wt ICER. Upon testing the activity of this mutated form, eleven independent constructs were created where only one lysine residue was reintroduced at a time. All of the constructs were sub-cloned in a mammalian expression vector, transfected into eukaryotic cells and treated with proteasome inhibitor. The proteasome inhibitor blocks the pathway to the proteasome therefore the lysine residue that is being ubiquitinated could potentially be
determined by observing ICER with ubiquitin molecules attached. Subsequently, 15
ICER constructs containing hemagglutinin (HA) tag on the N-terminus and C-terminus
were constructed. These constructs were made to select the transfected samples,
excluding endogenous ICER that may interfere, when performing protein analysis.
Determining the function of the expressed ICER is important to identify ways of
inhibiting ICER ubiquitination by targeting ubiquitin from attaching to the lysine
residue(s). Blocking ubiquitin from attaching to ICER can be used as an alternate cancer
therapy that will target specific cancer cells since it will be present in the nucleus.
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Fanaye Woldeamanuel
Montclair State University
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INTRODUCTION

Cells have many thousands of proteins carrying out various tasks. They are essential in cell signaling, movement, transport, membrane fusion cell protection and regulation. Proteins such as enzymes are fundamental in catalysis for normal growth and renewal. In order to carry out these varied functions, proteins have to keep their three dimensional shape. Chaperones assist in the ATP dependent protein folding process to obtain the right three-dimensional conformation (2). Even though this process is very costly, correct protein folding is crucial to cells thus it is a necessary process. However, despite the assistance of chaperones, misfolding still occurs (2). Misfolded proteins have to get degraded in order to minimize cell destruction because the accumulation of these proteins is toxic to cells. In addition, about 5% of proteins are destroyed and replaced by new ones each day. Within 30 days, almost all molecules of proteins are recycled (2). Therefore, when there is an unwanted or a misfolded protein, it has to be degraded to continue normal function within a cell.

Ubiquitination is an energy dependent post-translational modification that occurs frequently in cells. This critical cellular mechanism attaches ubiquitin(s), an 8 kilo Dalton (kD) protein found in cells ubiquitously, on lysine residues of targeted proteins. There are two main types of ubiquitination: monoubiquitination and polyubiquitination. Monoubiquitination involves the attachment of only one ubiquitin molecule to a lysine residue on a target protein. The monomeric tagging of protein triggers internalization of cell surface proteins into the endocytic pathway and is involved in DNA replication, DNA repair and transduction (3). This posttranslational modification is also involved in protein trafficking where it dislocates a nuclear protein to the cytoplasm for example, which affects the protein’s activity to either promote or prevent protein interactions (3).
Polyubiquitination on the other hand regulates protein levels available in cells by first attaching ubiquitin molecules to a target protein and sending the target protein to the proteasome complex, where the protein gets degraded or recycled (4). Polyubiquitination provides a mechanism that removes unwanted proteins thus, keeping the cell “junk free.” The carboxyl (C)-terminus end of the ubiquitin molecule consists of a glycine residue that is critically required for its conjugation to other ubiquitin molecules and substrates, in addition to the internal lysine residues that are used in the creation of polyubiquitin chains (4). In some proteins, it has been also shown that ubiquitin can be attached to the free alpha amino (N) group of N-terminus methionine of a protein and in rare cases to a serine, threonine or cysteine residue (5,6,7).

Ubiquitination occurs in a cascade of enzymatic reactions that involve three enzymes: E1, E2 and E3. In polyubiquitination, the first step in conjugating a ubiquitin molecule to a protein involves the activation of the C-terminus of the molecule by E1, the ubiquitin-activating enzyme (8). E1 is a 110 kD enzyme that catalyzes an ATP dependent reaction that produces a ubiquitin-thioester linkage (8). This abundant enzyme produces a highly reactive form of ubiquitin due to the thioester bond (9). Once activated, the ubiquitin is transferred to a sulfhydryl group of E2 (9). E2s are small enzymes that contain a cysteine residue that forms a thioester linkage with the activated ubiquitin (10). The specificity of E2s is important because they aid in the degradation process in addition to conjugating with the key enzyme, E3 (10). E3s are the ligases that join the ubiquitin molecule with the target protein. They catalyze the transfer of the activated ubiquitin from E2 to the lysine residue of the target protein and then lysine residues in the ubiquitin (11). Upon the attachment of ubiquitin molecules (for polyubiquitination), the protein
gets sent to the proteasome since the ubiquitin acts like a signaling tag. Only one E1 has been found in mammalian cells while large number of E2s and E3s are present in cells (12). About 1000 E3s have been found in cells that attach ubiquitin to proteins in a highly regulated manner (12). Once the protein reaches the proteasome, the protein gets degraded. However, ubiquitinated molecules can be recycled by deubiquitinating enzymes (DUBs), which are proteases that cleave ubiquitin(s) after the terminal carbonyl of the last glycine residue of ubiquitin from target proteins (13,14).

Figure 1. Ubiquitination Pathway. a) Ubiquitin molecules attach to a target protein by using ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). This process is dependent on ATP. b) If four or more ubiquitin molecules attach to one residue on a target protein, the protein gets sent to a complex called proteasome. c) When a target protein reaches the proteasome, the protein gets degraded and the ubiquitins are recycled. d) If only one ubiquitin molecule attaches to one residue on a target protein, it's referred to as monoubiquitination. If two or more ubiquitin molecules attach to a target protein at independent residues, they are referred to as multiubiquitination. Both types of ubiquitinations undergo a non-proteolytic pathway such as protein trafficking (15).
The proteasome consists of six catalytic sites (16). Two sites cleave preferentially after hydrophobic amino acids; two cleave after basic residues and two after acidic ones (16). This allows for the degradation of various types of proteins. The active sites in the proteasome cleave peptide bonds by the hydroxyl group on a critical threonine residue (17). This unique proteolytic mechanism has been a target for understanding the ubiquitin-proteasome system by using proteasome inhibitors (PI) (18). These inhibitors are either synthetically synthesized or have been produced by microorganisms specifically targeting the active site (18). For example, the synthetic PI MG-132 has been used in research labs for studying the ubiquitin pathway because it acts like a peptide aldehyde that effectively blocks the proteolytic pathway of the proteasome.

The ubiquitin proteasome system is essential to life. Polyubiquitination degrades the right protein at the right time. A ubiquitin molecule does not randomly attach to any protein, it is a highly regulated and a controlled system that avoids unwanted degradation of proteins (18). Some roles of the ubiquitin-mediated protein degradation include control of cell division and signal transduction, regulation of gene expression, responses to inflammation and immunity, embryonic development and apoptosis (19). If this system fails to work, it can lead to various diseases such as Huntington's, Alzheimer's, infectious diseases, rheumatoid arthritis and cancer. In the case of Huntington's, it has been found that the proteasome complex cannot catalyze repeated sequences of glutamine. Unfortunately, polyglutamines (polyQ) within certain proteins are linked with other neurodegenerative diseases as well (20). The poor degradation of glutamine residues accounts for the accumulation of glutamine leading to toxic intracellular inclusions (20).

The ubiquitin proteasome system is involved in several types of cancer. The
malfunction of proteasomal degradation could either enhance the effect of oncoproteins or reduce the amount of suppressor proteins (19). This occurs because oncoproteins and tumor suppressor proteins were found to be targets of ubiquitination (21). Though the mechanism varies, experimental evidence indicates a possibility of using the ubiquitin proteasome system as cancer targeted therapy. Some studies have shown that inhibition of the proteasome was found to induce apoptosis, especially in neo- plastic cells and transplanted tumors (17). Since tumor suppressor proteins are usually targeted for degradation, an indirect method used to rescue tumor suppressor proteins from degradation can be used as a mode of cancer-targeted therapy.

Cancer is still one of the leading causes of death in the United States (21). Though there are cancer treatments available, their lack of specificity has made it difficult to find a universal cure. The most commonly used cancer treatments are still chemotherapy and radiation (22). Although chemotherapy is effective in targeting cells, its lack of specificity to cancer cells causes detrimental effects on a patient. Many chemotherapeutic agents such as Taxol and Vinblastine work to inhibit spindle fiber formation in mitotic cells (23). Therefore, these drugs target all cells that have a high mitotic index, such as cancer cells and gastrointestinal cells (22, 23). This results in the common side effects, such as losing hair and vomiting: making them a cytotoxic therapeutic option. One method of using targeted therapy can be the use of the nuclear tumor suppressor protein, Inducible cAMP Early Repressor (ICER), as a potential cellular target of cancer cells. ICER is a transcriptional repressor found in eukaryotic cells (24).

ICER functions as a repressor by directly binding to cAMP responsive element (CRE) within the promoter of growth-related genes such as cyclin A, cyclin D1 and c-fos and...
negatively regulating their expression (25). In cancer cells however, ICER is abnormally expressed. In several cancer cell lines including human prostate cancer tissue, ICER level is very low (26). Forced expression of ICER in cancer cells has been shown to inhibit DNA synthesis, cell growth in culture, expression of growth-related genes, anchorage-independent growth and tumor formation in nude mice (26,27). But unlike most tumor suppressors, ICER does not have a mutation. Instead, it gets post translationally modified by ubiquitination (24,25,28). Along with misfolded proteins being targeted for ubiquitination, ICER also gets ubiquitinated and is either sent to the proteasome where it gets degraded and/or gets sent to the cytoplasm (25,26,28). Previous studies have shown that mitogen-activated protein kinase (MAPKs) extracellular signal-regulated kinases 1 (Erk1) and 2 (Erk2) interact with ICER to mediate the phosphorylation of ICER on a critical serine residue 41 (Ser-41) (28). This phosphorylation was shown to be important in ICER polyubiquitination and proteasomal degradation since a mutant form of ICER where Ser-41 was substituted by an alanine was shown to have a longer half-life than wild-type ICER (28). A similar study has shown that Ser-35 is also an important phosphorylation site of ICER for a subsequent ubiquitination in the cell cycle. However, ICER phosphorylated on Ser-35 was shown to be monoubiquitinated and present in the cytoplasm (25). These outcomes prevent ICER from working as a tumor suppressor protein since it will no longer be present in the nucleus carrying out its duties as a repressor. The focus of this study is to characterize the ubiquitination sites of ICER and study the effect on subcellular localization. Identifying the ubiquitination site(s) of ICER will aid in ways of inhibiting ubiquitination as a new mode of cancer targeted therapy.
MATERIALS AND METHODS

Site Directed Mutagenesis

ICER is a 108 amino acid protein consisting of 11 lysine residues. Each lysine residue was previously mutated to another basic amino acid, arginine, by using site directed mutagenesis (25). This construct was termed ICER KO, ICER with no lysine. Eleven independent constructs were created where only one lysine residue is reintroduced at a time.

Synthesis of Constructs by Invitrogen

Fifteen different ICER constructs containing an HA tag were purchased (Invitrogen, Carlsbad, CA). The constructs were in pMS cloning vector, which has a molecular weight (MW) of 2609 bp containing Bam HI and Eco RI restriction sites. ICER HA was engineered between these two sites for each construct. Thirteen of the samples had an HA tag engineered at the N-terminus. These samples were ICER wt, ICER KO and all of the KOs containing only one lysine residue. The remaining two samples were one additional KO and one ICER wt. These two samples had an HA tag on the C-terminus. The constructs were verified by sequencing and sequence congruence within the used restriction sites was 100%.

Digestion of pMS Plasmid

ICER constructs in pMS plasmid were digested overnight with BamHI (Invitrogen, Carlsbad, CA) and EcoRI (Invitrogen, Carlsbad, CA) restriction enzymes to obtain ICER. Plasmids were reconstituted in 50 uL of deionized water. Then, the samples were vortexed and centrifuged.
Agarose Gel Electrophoresis

Digested samples were run on two 1% agarose gels (USB, Cleveland, Ohio) in 1X TAE buffer (Promega, Madison, WI). Each gel was casted with 5 uL of ethidium bromide. For detection, 5 uL of 10X loading buffer (Invitrogen, Carlsbad, CA) was added to each sample. In each gel, 10 uL of HiLo molecular weight ladder, (Minnesota Molecular Inc, Minneapolis, MN) was run along with 55 uL of each digested sample. The gels were run at 100v for 60 mins in 1X TAE buffer (Promega, Madison, WI).

Gel Extraction using QIAquick Kit

ICER DNA fragments were excised from the agarose gel with a clean scalpel and weighed in a tube. Samples were incubated at 50 °C for 10 mins with periodic vortexing to completely dissolve the agarose. After observing the pH indicating yellow color, 1 volume of isopropanol was added and samples were mixed. Upon obtaining QIAquick spin columns (QIAGEN, Valencia, CA) and placing them on 2 mL collection tube, the samples were added to allow DNA binding. The samples were centrifuged for 1 min and the flow through was discarded. To remove all traces of agarose, 0.5 mL of Buffer QG (QIAGEN, Valencia, CA) was added to the columns and centrifuged for 1 min. 0.75 mL of Buffer PE (QIAGEN, Valencia, CA) was added to the columns to wash. After discarding the flow through, the columns were centrifuged for an additional 1 min at >10,000 x g (~13,000 rpm). The QIAquick columns were then placed in clean 1.5 mL microcentrifuge tubes and 30 uL of Buffer EB (QIAGEN, Valencia, CA) was added to the center of column to elute DNA. After allowing the column to stand for 1 min, the samples were centrifuged for 1 min to obtain DNA.
**Ligation Reaction**

Each cut insert (ICER) was ligated to an expression vector, pcDNA, by using T4 DNA Ligase (Invitrogen, Carlsbad, CA). The ligation reaction was run in a 3:1 insert to vector ratio as stated in the protocol.

**Transformation: Premade Mix and Go Competent E. coli Cells**

Fifteen Mix and Go tubes containing 100 uL of Zymo 5α cells (ZYMO, Irvine, CA) were thawed on ice. Then, 2.5 uL of each plasmid DNA was added to their respective thawed tube and mixed gently for a few seconds. 50 uL of the mixture was spread onto ampicillin (US Biological, Pittsburg, PA) plates pre-warmed to 37 °C. Plates were incubated at 37 °C inverted for colonies to grow.

**Plasmid DNA Prep/ Mini Prep**

Plasmid DNA was prepared using QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA) as stated in the protocol. Colonies were selected from transformed bacterial plates to start minicultures in a glass test tube containing 3 mL of LB broth with ampicillin (ACROS, Pittsburg, PA). The tubes were placed in a shaker overnight at 37 °C and 1.5 mL of the resulting minicultures were used to collect DNA.

**Maxi Prep**

QIAprep Spin Maxi prep kit (QIAGEN, Valencia, CA) was used as stated in the protocol.

**Sequencing**

Sequence of each mini prep and maxi prep sample was performed by the sequencing facility located at the Department of Biology using a 3130 Genetic Analyzer (Life Technologies, Grand Island, NY).
Transient Transfection

HeLa cells (human epithelial cervical carcinoma cell line), JEG-3 cells (human placental choriocarcinoma cell line) AB-9 cells (primary fibroblast developed from zebra fish fin tissue) and PAC2 cells (fibroblast cell line isolated from 24-h post-fertilization zebra fish embryo) were purchased from American Type Collection (ATCC, Manassas, VA). A day before transfection, cells were subcultured to be approximately 80% confluent. HeLa and JEG-3 cells were incubated at 37 °C with 5% CO₂. AB-9 cells and PAC2 cells were incubated at 28 °C without CO₂. All transient transfections were performed using FuGene transfection reagent (Promega, Madison, WI) as stated in the protocol. Enhanced green fluorescent protein (EGFP) and green fluorescent protein (GFP) were transfected to determine transfection efficiency. All cells were cultured using Dulbecco’s Modified Eagle’s Medium as recommended by the vendor (Sigma-Aldrich, St. Louis, MO). MG-132 Proteasome Inhibitor (PI) at 2 ug/ uL (Sigma-Aldrich, St. Louis, MO) were incubated for 24 hrs.

Rapid Total Protein Extraction for SDS/PAGE

Cells were washed with 1 mL cold PBS (LI-COR Biosciences, Lincoln, NE) twice and scraped with 1 mL ice cold PBS with protease inhibitors. They were then transferred to a 1.5 mL micro centrifuge tube and centrifuged in cold for 1 min at full speed. Depending on the pellet, 100-250 uL of 2X Laemmli buffer (Bio-Rad, Hercules, CA) was added to each in a 1:1 ratio. Samples were heated for 5 min at 95 °C in a heating block and were centrifuged for 10 minutes at RT at maximum speed. The supernatant was then transferred to a clean micro centrifuge tube for SDS/PAGE analysis.
Western Blot Analysis

Each sample with or without PI treatment was run on a pre-cast SDS-PAGE gel (Bio-Rad, Hercules, CA). Into their respective well, 15 uL of the samples and 5 uL of molecular weight ladder were loaded in each gel. The voltage was set to 200v and the samples were run with a running buffer containing Tris, Glycine and SDS (LI-COR Biosciences, Lincoln, NE) for 35 mins. Then, the samples on the gel were transferred to a Nitrocellulose membrane (Novex, Salt Lake City, Utah) by using a transfer unit, transfer buffer (LI-COR Biosciences, Lincoln, NE) containing 100 mL of 10X Tris-Glycine, 200 mL methanol and 700 mL DI water, a stir rod and an ice pack. The transfer was carried out for 45 mins at 100v. Then the membranes were incubated with a blocking buffer (LI-COR Biosciences, Lincoln, NE) for an hour and treated with polyclonal Rabbit anti-HA primary antibody (Invitrogen, Carlsbad, CA) overnight. After 45 minutes wash with 0.1% Tween 20 in PBS (LI-COR Biosciences, Lincoln, NE), the membranes were incubated with a secondary antibody (LI-COR Biosciences, Lincoln, NE) for an hour. The membranes were washed again using 0.1% Tween 20 four times for five minutes each and lastly rinsed with 1X PBS to get rid of the Tween 20. Upon drying the membranes, Infrared Licor Odyssey Scanner (LI-COR Biosciences, Lincoln, NE) was used to detect the samples. Licor Image Lite Software was then used to quantify the bands for better data analysis. The same procedure was followed for all of the Western Blots carried out. What varied were the antibodies used and their dilution. All of the antibodies used were diluted using Licor's blocking buffer (LI-COR Biosciences, Lincoln, NE). For the preliminary Western Blot of samples treated with or without PI, an anti-ICER polyclonal antibody was used at 1:10,000 and the same dilution was used for the secondary antibody with IRDye 800CW (LI-COR Biosciences, Lincoln, NE). For HA samples with or
without PI treatments and the cytoplasmic and nuclear fractionations, a polyclonal anti HA antibody (Invitrogen, Carlsbad, CA) of 0.25 mg/mL was used at 1:250. An IRDye 800CW goat (polyclonal) anti rabbit secondary antibody was used at 1:10,000 (LI-COR Biosciences, Lincoln, NE). For the control, a mouse monoclonal beta actin primary antibody was used at 1:500 and the secondary antibody was used at 1:15,000 (LI-COR Biosciences, Lincoln, NE).

Immunocytochemistry

Cells were grown on slide chambers and transfected with the constructs as previously described. Culture media was removed and the cells were washed twice with PBS at room temperature. Cells were then fixed by 4% formaldehyde in PBS equal to the original volume of culture medium of 1 mL for 20 mins at RT. After removing the fixative, the cells were washed with PBS three times for five minutes each. The Antigen Retrieval Buffer (Invitrogen, Carlsbad, CA) were preheated to 95 °C and 1 mL of this buffer was added and left to incubate at 95 °C for 10 minutes. Then, the Antigen Retrieval Buffer was removed and the cells were rinsed three times with PBS. The cells were then incubated in 1 mL 0.1% Triton X-100 in PBS for 25 mins at RT. The cells were rinsed three times with 2 mL PBS and after removing all remaining PBS; the cells were incubated with a protein blocking buffer for an hour at room temperature in order to block nonspecific background staining. The cells were then washed with PBS and incubated with polyclonal Rabbit anti-HA primary antibody at 0.1 ug/mL in blocking buffer. The cells were then washed 4 times with PBS and a Biotinylated Goat Anti-Polyvalent antibody of 2 ug/mL (Invitrogen, Carlsbad, CA).
NE-PER Nuclear and Cytoplasmic Fractionation

PAC2 cells were grown on a T25-flask and transfected. The culture media was removed and cells were washed one time with 2 mL of PBS at RT. Cells were harvested by adding 1 mL of trypsin-EDTA and incubated for 3 minutes at RT. Then, 2 mL of complete cell culture media was added and pipetted up and down gently to avoid bubbles but thoroughly to collect all cells from the flask. Cells were transferred to a 15 mL tube and then centrifuged at 500 X g for 5 minutes. Cells were then washed twice with 1 mL ice-cold PBS, transferred to a 1.5 mL micro centrifuge tube and centrifuged at 500 x g for 2.5 minutes. The supernatant was removed carefully and the pellet was left to dry. Then, 100 uL of ice-cold CER I (Thermo-Scientific, Somerset, NJ) was added to the cell pellet, the tube was vortexed vigorously on the highest setting for 15 seconds. Cells were then incubated on ice for 10 mins and ice-cold CER II (Thermo-Scientific, Somerset, NJ) was added to the tube. After vigorously vortexing the tube on the highest setting for 5 seconds, the tube was incubated on ice for 1 min and vortexed again in a similar manner. The tube was then centrifuged for 5 mins at maximum speed in a microcentrifuge (~16,000 x g). The supernatant (cytoplasmic extract) was immediately transferred to a clean pre-chilled tube and stored in -80 °C. The insoluble (pellet) fraction which contained the nuclei was suspended in 50 uL ice-cold NER (Thermo-Scientific, Somerset, NJ) and vortexed for 15 seconds. Samples were incubated on ice for 10 mins and vortexed for 15 seconds repeatedly for a total of 40 mins. The tubes were centrifuged at a maximum speed (~16,000 x g) for 10 mins. Immediately, the supernatant (nuclear extract) fraction was transferred to a clean pre-chilled tube and stored at -80 °C until use.
RESULTS

*All ICER KOs treated with PI get ubiquitinated in Western Blot Analysis*

To identify the ICER mutants that were ubiquitinated, cells were transfected with the different constructs and treated with or without PI. Since the PI blocks the pathway to the proteasome, the ICER construct that gets ubiquitinated will get rescued from proteasomal degradation in cells treated with PI. If ICER is being ubiquitinated, samples treated with PI will then illustrate a shift in mobility that is not present in the untreated samples should cite previous research supporting this statement (25,28). This can be determined by adding the MW of ICER with the possible number of ubiquitins that may have attached. In order to observe possible ubiquitinated residue(s), HeLa cells were transfected with wt ICER, ICER KO and the different ICER constructs generated by site directed mutagenesis. In addition, cells were transfected with EGFP to determine transfection efficiency. They were then treated with or without PI. All of the samples were run on an SDS-PAGE and incubated with a polyclonal anti-ICER primary antibody and Licor’s goat anti-rabbit secondary antibody with IRDye 800CW for detection. As illustrated in Figure 2 A, multiple bands were observed in each lane. Samples transfected with EGFP and treated with or without PI did not show a significant difference as expected. When observing wt ICER and ICER KO, the addition of the PI shows a band that represents a form of ICER with the equivalence of five ubiquitin molecules attached, indicated by the arrows in Figure 2 (25). This is because ICER is 18 kD and the sum of five ubiquitin molecules is 42 kD, resulting in the approximately 60 kD band present in the “+PI” lanes. Modified forms of ICER are also indicated by the asterisks (*) (25). A band representing ICER observed in each sample at approximately 18 kD and a phosphorylated form of ICER (pICER) is also observed at approximately 20 kD Figure 2.
B, C and D illustrate the rest of the ICER constructs; where a strong band is present in each PI treated samples. This shows that ICER is being ubiquitinated at each construct containing only one lysine. Since ubiquitination was also observed in ICER KO, these data suggest a possible N-terminus ubiquitination.

Even though the PI treated samples demonstrated stronger bands than the untreated samples at ~60 kD, the bands were quantified using Licor’s Studio Lite software for a better quantitative analysis. As illustrated in Figure 3, the bar graph shows that the “- PI” samples were very low compared to the “+ PI” samples, excluding the EGFP samples since ICER was not transfected. This quantitative result confirms the band intensity observed in the Western blot. Overall, the ICER constructs with only one lysine residue gave similar data as ICER KO. Therefore, these data demonstrate that ICER KO and all ICER KOs containing only one lysine get ubiquitinated, strongly suggesting a possibility of an N-terminus ubiquitination.
Western Blot Analysis of with or without PI treatment

A)

B)
Figure 2. Western Blot Analysis of HeLa cells treated with PI. A) HeLa cells were transfected with Enhanced Green Fluorescent Protein (EGFP) to observe ICER background, wt ICER and ICER KO and treated with 2 ug/mL with or without MG 132 PI. Cells were collected and protein was extracted. The Western Blot was incubated with rabbit polyclonal anti-ICER primary antibody then treated with secondary antibody with IR 800 tag for detection. Each construct illustrates multiple endogenous bands. Very weak bands are observed for EGFP at approximately 60 kD compared to wt ICER and ICER KO with PI. These bands possibly represent ICER with five ubiquitins (Ub5-ICER). The asterisks (*) represent modified forms of ICER. A band representing ICER is observed in each sample at approximately 18 kD. A phosphorylated form of ICER (pICER) is also observed at approximately 20 kD. B) HeLa cells were transfected with KO -52, KO -59 and KO -65 and treated with or without 2 ug/mL MG 132 PI. Cells were collected and protein was extracted. The Western Blot was incubated with rabbit polyclonal anti-ICER primary antibody then treated with secondary antibody with an IR
800 tag for detection. Strong bands at 60 kD in the PI treated samples possibly represent ICER with five ubiquitins (Ub₅-ICER). The asterisks (*) represent modified forms of ICER. A strong ICER band is observed in each sample at approximately 18 kD. A phosphorylated form of ICER (pICER) is also observed at approximately 20 kD. C) HeLa cells were transfected with KO-71, KO-72, KO-76 and KO-90 and treated with or without 2 ug/mL MG 132 PI. Cells were collected and protein was extracted. The Western Blot was incubated with rabbit polyclonal anti-ICER primary antibody then treated with secondary antibody with an IR 800 tag for detection. Strong bands at 60 kD in the PI treated samples possibly represent ICER with five ubiquitins (Ub₅-ICER). The asterisks (*) represent modified forms of ICER. A strong ICER band is observed in each sample at approximately 18 kD. A phosphorylated form of ICER (pICER) is also observed at approximately 20 kD. D) HeLa cells were transfected with KO-90, KO-91, KO-100 and KO-106 and treated with or without 2 ug/mL MG 132 PI. Cells were collected and protein was extracted. The Western Blot was incubated with rabbit polyclonal anti-ICER primary antibody then treated with secondary antibody with an IR 800 tag for detection. Strong bands at 60 kD in the PI treated samples possibly represent ICER with five ubiquitins (Ub₅-ICER). The asterisks (*) represent modified forms of ICER. A strong ICER band is observed in each sample at approximately 18 kD. A phosphorylated form of ICER (pICER) is also observed at approximately 20 kD.

**Effect of Proteosome Inhibitor on ICER Transfected HeLa Cells**

![Graph showing the effect of proteosome inhibitor on ICER transfected HeLa cells.](image)

**Figure 3. Quantitative representation of Western blot analysis.** The Western blot band intensity of samples treated with or without proteasome inhibitor was quantified using Licor's Image Studio Lite software in pixel. The control, EGFP, illustrates that the treated and untreated samples are similar whereas the rest of the samples represent the difference in intensity, confirming the difference in band intensity observed.
Sequence of all ICER constructs with an HA tag

To study ICER ubiquitination without the interference of endogenous ICER, 15 ICER constructs with an HA tag engineered were obtained from Invitrogen. The constructs were in pMS cloning vector. Thirteen of the samples, ICER wt, ICER KO and all of the KOs containing only one lysine residue, contained an HA tag engineered at the N-terminus. The remaining two samples were one additional KO and one ICER wt which contained an HA tag on the C-terminus. As indicated in Table 1, the HA tag is represented by the underlined sequence whereas the bold “K” locates the lysine(s) present. The number present in each sample name indicates the only lysine residue present.
<table>
<thead>
<tr>
<th>ICER wt NHA</th>
<th>ICER KO -59</th>
</tr>
</thead>
<tbody>
<tr>
<td>MYPYDVDPDYAAVTGDETESA</td>
<td>MYPYDVDPDYAAVTGDETESA</td>
</tr>
<tr>
<td>TTGGMSGYQMTSPASGLSQV</td>
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**Table 1. Gene Synthesis.** All 15 forms of ICER were obtained from Invitrogen. Each construct has an HA tag at the N-terminus. An additional wt ICER and ICER KO have an HA tag at the C terminus as well. The underlined sequence represents the HA tag and the bold "K" denotes the lysine present in the sample. The number present in the sample name represents the only lysine residue present.

**ICER was obtained by digesting pMS plasmids containing ICER Constructs**

The ICER constructs were in pMS cloning vector. This vector, with a MW of 2,609 bp, contains BamH I and EcoR I restriction sites. Since the ICER constructs were in pMS plasmid, the samples were digested to obtain the different ICER constructs. Two 1% gels were run to obtain all ICER constructs. As illustrated in Figure 4, two bands were observed for each construct. The band running at approximately 2,600 bp is consistent with the MW of pMS and the band running at approximately 370 bp is
consistent with the MW of ICER HA. Since KO -59 and KO -87 illustrated in Figure 4A show weak ICER HA bands, the pMS plasmids containing these samples were digested again. As illustrated in Figure 3B, wt CHA also has a weak band thus it was digested again.

A)

![Image A]

- Figure 4. Digestion of pMS plasmid. A) pMS plasmids containing samples ICER KO -52, KO -59, KO -70, KO -71, KO -72, KO -76, KO -87, KO -90 and KO -97 were digested overnight using EcoR I and BamH I restriction enzymes to obtain each HA ICER constructs. A 1% agarose gel was run illustrating two bands. A band running at approximately 2,600 bp is consistent with the MW of pMS and a band observed at approximately 370 bp is consistent with the MW of ICER HA. KO -59 and KO -87 show weak ICER HA bands. B) pMS plasmids containing samples KO -100, KO -106, wt
NHA, wt CHA, KO NHA and KO CHA were digested overnight using EcoRI and BamHI restriction enzymes to obtain each HA ICER construct. After digestion, 1% agarose gel was run and two bands are observed for each construct. A band running at approximately 2,600 bp is consistent with the MW of pMS and a band observed at approximately 370 bp is consistent with the MW of ICER HA however, wt CHA shows a weak ICER HA bands.

**Each ICER construct was ligated to pcDNA vector**

After digestion, each ICER construct was ligated to the pcDNA expression vector (Figure 5). pcDNA expression vector contains a Human cytomegalovirus (CMV) promoter for high-level expression of protein, a T7 priming site to allow in-vitro transcription and for sequencing insert, multiple cloning site to allow insertion of gene and facilitates cloning. It also contained bovine growth hormone (BGH) polyadenylation signal for efficient transcription termination and polyadenylation, fl origin to rescue single-stranded DNA, SV40 early promoter and origin to allow efficient, high-level expression of the neomycin resistance gene, Neomycin resistance gene for selection of stable transfectants in mammalian cells, SV40 early polyadenylation signal for efficient transcription termination and polyadenylation of mRNA, pUC origin for high-copy number replication and growth in *E. coli*, Ampicillin resistance gene (Beta-lactamase) for selecting vector in *E. coli*, and Ampicillin (bla) resistance gene (Beta-lactamase) for selection of transformants in *E. coli* (Invitrogen).

![Figure 5. ICER constructs in pcDNA 3.1 + vector](image)

ICER constructs obtained from pMS cloning vector were ligated to pcDNA 3.1 vector. The different ICER constructs with HA tag in the N and C terminus was inserted between the CMV promoter and Bovine growth hormone (BGH) polyadenylation signal.
ICER HA samples treated with or without PI show consistent protein expression in different cell lines

To observe if the expression of the ICER constructs is cell specific, three cell types were chosen for transfection. PAC2 cells, AB-9 cells and JEG-3 cells were transfected with ICER NHA, ICER CHA, KO NHA and KO CHA and treated with or without PI. As illustrated in Figure 6, the Western blot probed with anti HA polyclonal antibody showed similar bands. This showed the consistency of the expressions and that this phenomenon is not cell type specific.

As illustrated in Figure 6, each construct demonstrates a band at approximately 20 kD that represents ICER HA. In the (+) wt CHA samples, a strong band that is not present in the (-) wt CHA is observed at approximately 18 kD. This might be proteolytic degradation due to proteases present in cells. The same band is present in lanes 6 and 8 but with lighter band intensity. The (+) KO NHA also illustrates a few bands at approximately 22 kD not present in the untreated samples, lane 5. Since this construct does not have lysine residues and the N-terminus is covered by the HA-tag, this is most likely the phosphorylated form of ICER (25). (+) KO NHA will probably get phosphorylated but since the N-terminus is blocked, an accumulation is observed. The same was observed for KO CHA, but with lighter band intensity. Even though the N-terminus is available, the HA tag on the C-terminus might interfere with the ubiquitination; thus, a subsequent accumulation of phosphorylated ICER is observed.
A) PAC2 Cells

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-pICER (24 kD)
-ICER (20 kD)
-ICER (18 kD)

B) AB-9 Cells

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-pICER (24 kD)
-ICER (18 kD)

C) JEG-3 Cells

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-pICER (24 kD)
-ICER (18 kD)

Figure 6. Transfection of different cells. A) PAC2 cells were transfected with wt NHA, wt CHA, KO NHA and KO CHA and treated with or without PI. The Western blot was probed with anti HA polyclonal antibody and IRDye 800 secondary antibody resulting in overall strong bands. pICER stands for phosphorylated ICER and is observed at approximately 22 kD and 24 kD. ICER is observed in each sample at 20 kD and a band present at approximately 18 kD possibly represents proteolytic degradation of ICER. B) AB-9 cells were transfected with wt NHA, wt CHA, KO NHA and KO CHA and treated with or without PI. The Western blot was probed with anti HA polyclonal antibody and IRDye 800 secondary antibody resulting in overall weaker bands. At approximately 22 kD and 24 kD, pICER is observed. ICER is observed in each sample at 20 kD and a band present at approximately 18 kD possibly represents proteolytic degradation of ICER. C) JEG-3 cells were transfected with wt NHA, wt CHA, KO NHA and KO CHA and treated with or without PI. The Western blot was probed with anti HA polyclonal antibody and IRDye 800 secondary antibody resulting in overall strong bands. At
approximately 22 kD and 24 kD, pICER is observed. ICER is observed in each sample at 20 kD and a band present at approximately 18 kD possibly represents proteolytic degradation of ICER.

All ICER HA constructs with or without PI treatment did not show a shift in mobility that would possibly result from ubiquitination

PAC2 cells were transfected with each of the different ICER constructs containing an HA tag and treated with or without PI to possibly observe ubiquitination. If a construct is ubiquitinated, a shift in mobility that accounts for the sum of ICER HA and Ub (n) molecules present in the PI treated sample will suggest ubiquitination (1, 25). In addition, cells were transfected with just GFP for control, to compare with the ICER constructs and to determine transfection efficiency. The transfection efficiency was approximately 25 % when cells were observed under the microscope, as illustrated in Figure 7. All of the samples were run on SDS-PAGE and incubated with an anti-HA primary antibody and IRDye 800 polyclonal secondary antibody. A Western blot of the same transfected samples were also run but with a monoclonal anti-Beta Actin primary antibody and IRDye 700 monoclonal antibody for normalization. The blot is presented under the PI Western blot labeled “Beta Actin” in Figure 8A-D.

As illustrated in Figure 8, the controls did not show a band because they were not transfected with ICER HA. KOs 52-106 indicated a band present at 20 kD with similar band intensity. Since each construct was transfected with ICER HA, the 20 kD band observed was expected. However, a band representing ICER with ubiquitin accumulation was not observed in the PI treated samples. This might be due to the steric hindrance from the HA tag. But if ICER gets ubiquitinated at the N-terminus, then the absence of a ubiquitinated form of ICER may be reasonable. wt ICER NHA, wt ICER CHA, KO NHA and KO CHA illustrated a consistent result as illustrated in Figure 6 since the same
samples were analyzed. Each construct demonstrates a band at approximately 20 kD that represents ICER HA. In the (+) wt CHA samples, KO NHA and KO CHA a strong band that is not present in their respective untreated samples is observed at approximately 18 kD. This is most likely proteolytic degradation.

The (+) KO NHA also illustrates a few bands at approximately 22 kD not present in the untreated samples. Since this construct does not have lysine residues and the N-terminus is covered by the HA-tag, this is most likely the accumulation of the phosphorylated form of ICER. This might occur since the N-terminus is blocked, and accumulation is observed. The same was observed for KO CHA, but with lighter band intensity. Even though the N-terminus is available, the HA tag on the C-terminus might interfere with the ubiquitination; thus a subsequent accumulation of phosphorylated ICER is observed.

Transfection Efficiency

Figure 7. PAC2 cells transfection efficiency. PAC2 cells were transfected with GFP to determine transfection efficiency. The efficiency was approximately 25%.
**Figure 8. Western Blot Analysis of ICER HA constructs with PI.**

A) PAC2 cells were transfected with a control, KO -52, KO -59 and KO -70 and treated with or without MG-132 PI. Then, protein was extracted for Western Blot analysis. The samples were run and treated with anti-HA primary antibody and polyclonal secondary antibody at 800 IR. Each sample except the control illustrates a band at approximately 20 kD that represents ICER HA. PAC2 cells were also transfected with the same constructs by using a monoclonal primary antibody to probe for Beta Actin.

B) PAC2 cells were transfected with KO -71, KO -72, KO -76 and KO -87 and treated with or without MG-132 PI. Then, protein was extracted for Western Blot analysis. The samples were run with anti-HA primary antibody and treated with polyclonal secondary antibody at 800 IR. Each sample illustrates a band at approximately 20 kD that represents ICER HA. PAC2 cells were also transfected with the same constructs by using a monoclonal primary antibody to probe for Beta Actin.

C) PAC2 cells were transfected with KO -90, KO -97, KO -100 and KO -106 and treated with or without MG-132 PI. Then, protein was extracted for Western Blot analysis. The samples were run and treated with anti-HA primary antibody and polyclonal secondary antibody at 800 IR. Each sample illustrates a band at approximately 20 kD that represents ICER HA. PAC2 cells were also transfected with the same constructs by using a monoclonal primary antibody to probe for Beta Actin.

D) PAC2 cells were transfected with wt NHA, wt CHA, KO NHA and KO CHA. Then, protein was extracted for Western Blot analysis. The samples were run with anti-HA primary antibody and treated with polyclonal secondary antibody at 800 IR. Each sample illustrates a band at approximately 20 kD that represents ICER HA. PAC2 cells were also transfected with the same constructs by using a monoclonal primary antibody to probe for Beta Actin.
polyclonal secondary antibody at 800 IR. Each sample illustrates a band at approximately 20 kD that represents ICER HA. PAC2 cells were also transfected with the same constructs by using a monoclonal primary antibody to probe for Beta Actin. D) PAC2 cells were transfected with wt NHA, wt CHA, KO NHA and KO CHA and treated with or without MG-132 PI. Then, protein was extracted for Western Blot analysis. The samples were run and treated with anti-HA primary antibody and polyclonal secondary antibody at 800 IR. At approximately 22 kD and 24 kD, pICER is observed. ICER HA is observed in each sample at 20 kD and a band present at approximately 18 kD possibly representing proteolytic degradation of ICER. PAC2 cells were also transfected with the same constructs by using a monoclonal primary antibody to probe for Beta Actin.

Most ICER constructs except KO -70, KO -90, KO -97, KO -100, KO -106 and KO CHA were mostly localized in the nucleus under Immunocytochemistry Analysis

The constructs were transfected into PAC2 cells to visualize their subcellular localization, nuclear and/or cytoplasmic. The samples were incubated with a polyclonal anti HA primary antibody. Upon examining each sample under an inverted microscope, slide images were taken at 100X TM and 400X TM. As illustrated in Figure 9, most of the samples except KO -70, KO -90, KO -97, KO -100, KO -106 and KO CHA were concentrated in the nucleus.

To confirm these data, PAC2 cells were transfected and both nuclear and cytosolic fractionations were extracted for SDS-PAGE analysis. After running the samples, the blots were incubated with a polyclonal anti HA primary antibody. As illustrated in Figure 10, all of the samples except KO -70, KO -76, KO -97 and KO -106 show a much stronger band in the nuclear extract rather than the cytosolic. Most of the results, KO -70, KO -97 and KO -106, coincided with the immunocytochemistry findings demonstrating that the constructs localized in the cytoplasm might be possible sites of internal ubiquitination, since ICER is a nuclear protein.
Immunocytochemistry

Control, 100X TM
Control, 400X TM

KO -52, 100X TM
KO -52, 400X TM

KO -59, 100X TM
KO -59, 400X TM

KO -70, 100X TM
KO -70, 400X TM
Figure 9. Immunocytochemistry. PAC2 cells were transfected with the different constructs to carry out immunocytochemistry. Samples were treated with anti HA primary antibody. They were then incubated with Biotinylated Goat Anti-Polyvalent and Streptavidin Peroxidase to observe which constructs are nuclear and/or cytoplasmic. “N” represents nuclear and “C” represents cytosol. All constructs except KO -70, KO -100, KO -106 and KO CHA are mostly localized in the nucleus.
All ICER constructs except KO -70, KO -76, KO -97 and KO -106 are localized in the nucleus in a Western Blot Analysis of Cytosolic and Nuclear Fractionations.

A)

![Image of Western Blot Analysis of nuclear and cytoplasmic extracts. A) PAC2 cells were transfected with KO -52, KO -59, KO -70 and KO -71 constructs. Both nuclear (N) and cytoplasmic (C) protein fractionations were extracted for each sample. A Western blot was run and incubated with polyclonal anti-HA primary antibody and secondary antibody with IR 800 tag for detection. In the nuclear fractions, pICER is observed at approximately 22 kD. ICER HA is observed in each sample at 20 kD. B) PAC2 cells were transfected with KO -72, KO -76, KO -87 and KO -90 constructs. Both nuclear (N) and cytoplasmic (C) protein fractionations were extracted for each sample. A Western blot was run and incubated with polyclonal anti-HA primary antibody and secondary antibody with IR 800 tag for detection. In the nuclear fractions, pICER is observed at approximately 22 kD. ICER HA is observed in each sample at 20 kD. C) PAC2 cells were transfected with KO -97, KO -100, KO -106 and KO NHA constructs. Both nuclear (N) and cytoplasmic (C) protein fractionations were extracted for each sample. A Western blot was run and incubated with polyclonal anti-HA primary antibody and secondary antibody with IR 800 tag for detection. In the nuclear fractions, pICER is observed at approximately 22 kD. ICER HA is observed in each sample at 20 kD. D) PAC2 cells were transfected with KO CHA, NHA wt, CHA wt and Control. Both nuclear (N) and cytoplasmic (C) protein fractionations were extracted for each sample. A]
Western blot was run and incubated with polyclonal anti-HA primary antibody and secondary antibody with IR 800 tag for detection. In the nuclear fractions, pICER is observed at approximately 22 kD. ICER HA is observed in each sample at 20 kD and a possible proteolytic degradation is present at 18 kD, 16 kD and 14 kD in KO NHA, KO CHA and CHA wt.
DISCUSSION

The ubiquitinated residue(s) of ICER is/are not identified to date; however, various experiments executed such as Immunocytochemistry, Western blot of PI treated samples and nuclear and cytoplasmic fractionations supports the suggested N-terminus ubiquitination of ICER in a proteolytic degradation. The data also demonstrate internal lysine ubiquitination of ICER in a non-proteolytic pathway. As illustrated in Figure 2, a Western blot was run for cells transfected with the different ICER constructs created by site directed mutagenesis. These samples were treated with or without PI to distinguish polyubiquitinated residues. A common band at approximately 18 kD was observed in each sample, which represents ICER. Since the primary antibody used for the Western blot was anti ICER, multiple bands were also observed, as depicted in Figure 2. The antibody was not specific to the samples transfected so it also probed endogenous ICER present. Nevertheless, when comparing the samples, the addition of the PI shows a band that represents a form of ICER with possibly five ubiquitin molecules attached, indicated by the top arrows in Figure 2. Surprisingly, this significant shift in mobility is present in all ICER constructs containing only one lysine and showed a similar result as ICER KO. Since ubiquitination is an ATP dependent process, it is not favorable for ubiquitination to occur on each lysine residue. In addition, a previous study has shown that ICER KO inhibits cyclin D1 more efficiently than wt ICER and that this lysine less form is also ubiquitinated (25). This suggested a ‘non-traditional’ mode of ubiquitination that occurs on the N-terminus.

N-terminus ubiquitination is a process of attaching ubiquitin molecules to the free
alpha-amino group on the N-terminal methionine. N-terminal ubiquitination is not a common mechanism, but it has been shown to target a number of proteins for proteasomal degradation including transcription factors, cell cycle regulators, and viral proteins (30). This process has been identified in the myogenic transcriptional switch protein (MyoD) (29), human papillomavirus 16 (HPV 16) oncoprotein E7 (30), latent membrane protein 1 (LMP1)/ (LMP2A) of the Epstein Barr virus (EBV) (31), the Inhibitor of differentiation 2 (Id2) developmental regulator (32) and the cell-cycle-dependent kinase (CDK) inhibitor p21 (33). In order to study a possible N-terminal ubiquitination of ICER, wt ICER, ICER KO and all ICER constructs with only one lysine residue containing an HA tag on the N and C terminus were used. This was done to exclude endogenous ICER present in cells and to observe whether ubiquitination can occur despite the tag on the N-terminus. As illustrated in Figure 8A-C, the Western blot of samples incubated with an anti HA antibody show similar band intensity of ICER around 20 kD but a shift in mobility was not observed in these samples. If ICER is indeed being ubiquitinated at the N-terminus, the shift may not be present because the HA will cover the N-terminus and subsequently block a ubiquitin from attaching, as expected.

When looking at Figure 8 D, ICER wt NHA with or without PI both show a 20 kD band as observed in the mutant samples representing ICER HA. ICER wt NHA with PI however, shows an additional band at approximately 18 kD. This is most likely ICER resulting from proteolytic degradation. wt CHA with and without PI also show an ICER HA band at approximately 20 kD and a possible proteolytic degradation of ICER at around 18 kD, but not a much higher shift in mobility representing ubiquitinated ICER. KO NHA without PI also illustrates a similar 20 kD and 18 kD bands whereas KO NHA
with PI shows these bands in addition to bands at approximately 22 kD and 24 kD. If ICER does get ubiquitinated at the N-terminus, the ubiquitination site in this construct will be blocked due to the HA tag present. Thus, ICER will get phosphorylated at serine residues 35 and/or 41 but may not be able to get ubiquitinated (25). The 22 kD and 24 kD illustrated in Figure 8 D may then be the accumulation of these phosphorylated forms of ICER. The bands present in the treated and untreated KO NHA samples are however much stronger in intensity compared to the wt NHA samples. Since ICER KO is a stronger repressor than wt ICER (1), it might be reasonable to observe stronger expression. When looking at KO CHA, similar bands as observed in KO NHA are shown. If the N-terminus of ICER is being targeted for ubiquitination, this sample should have shown a higher shift in mobility since the HA tag is on the C-terminus. However, only phosphorylated forms of ICER observed in KO NHA were observed. This led us to believe that the HA tag, even though present in the C-terminus, might be too big for an 18 kD protein, ICER. If so, ICER may get phosphorylated but because of steric hindrance resulting from the HA tag, a ubiquitin molecule may not be able to attach. Since phosphorylation is a prerequisite for ubiquitination and phosphorylated forms of ICER are observed in ICER KO constructs instead of samples containing lysine, we are speculating that ubiquitination targeted to the proteasome is occurring at the N-terminus.

Previous studies have indicated that ICER gets phosphorylated and ubiquitinated during the cell cycle (23). It was also noted that unmodified forms of ICER start to reappear in G1 phase as the phosphorylated form of ICER starts to diminish (23). Since DNA replicates in M phase, ICER would not bind to DNA during this stage: the ubiquitinated form of ICER is most likely present. In contrast, an unmodified form of
ICER is present in the G1 phase to possibly repress gene transcription and CRE controlled promoters. But at the beginning of each cycle, the phosphorylation and ubiquitination process has to start all over again as observed in other transcription factors. To determine the subcellular localization of the different constructs, immunocytochemistry was carried out. As illustrated in Figure 9, all of the samples except KO -70, KO -90, KO -100, KO -106 and KO CHA were mostly concentrated in the nucleus. To confirm these findings, a Western blot of cytosolic and nuclear extracts was run. As illustrated in Figure 10, all of the samples except KO -70, KO -76, KO -97 and KO -106 were concentrated more in the nucleus rather than cytoplasm. This data coincided with some of the immunocytochemistry results demonstrated in Figure 9, KO -70, KO -97 and KO -106. In addition, the nuclear fractionation samples showed a light band above the ICER band at approximately 22 kD. These bands were not present in the cytoplasmic extracts thus they most likely signify a phosphorylated form of ICER (29). As illustrated in Figure 9, N-HA KO shows very strong band at approximately 20 kD at both fractionations, but in addition, the nuclear fraction illustrates a possible proteolytic degradation. The same was observed in C-HA KO but with relatively lighter band intensity. If ICER does get ubiquitinated at the N-terminus but an HA tag hinders a ubiquitin from attaching, accumulated HA ICER might eventually get degraded. Though the bands weren’t as strong, the same was observed for wt CHA. However, wt NHA ICER was almost absent in the cytoplasmic fraction but present in the nuclear.

Even though KO -70, KO -97 and KO -106 were not shown to be ubiquitinated when treated with PI, their cytosolic localization can suggest ubiquitination that is not mediated by the proteasome. In p21 for example, studies have shown that a mutant form
of this protein containing no lysine was being ubiquitinated and targeted for proteasomal proteolysis through N-terminus ubiquitination but was stated that ubiquitination on internal lysine residues may have a different role in the regulation of p21 function (33). Thus, the internal lysine residues of ICER may be ubiquitinated and sent to the cytosol at different phases of the cell cycle. When looking at the residues mostly localized in the cytosol, they are actually good candidates for ubiquitination. KO -70 is found within the nuclear localization signal (NLS) sequence of ICER. If this residue gets ubiquitinated, it will block the NLS from being recognized and thus will send ICER to the cytosol. KO -106 is the DNA binding regions of ICER. If these residues get ubiquitinated, ICER can no longer bind to DNA and repress genes. ICER can then be sent to the cytosol where it may be nonfunctional.

Overall, this thesis presents data supports the suggestion that ICER ubiquitination might occur at the N-terminus. We speculate that an internal lysine ubiquitination that is not targeted to the proteasome may occur as an alternative mode of regulation. This might be involved in regulating the cell cycle. The subcellular localization of ICER KO -70, KO -97 and KO -106 were mostly in the cytosol. Since ICER is a nuclear transcriptional repressor, ubiquitinating it at the NLS and/or DNA binding region and sending it to the cytosol is plausible. Even though other studies have to be done to solidify these findings, these results give promising evidence of both N-terminus ubiquitination and internal lysine ubiquitination.
REFERENCE


