NUCLEAR LOCALIZATION OF COILED COIL DOMAIN-CONTAINING 3 PROTEIN IN BREAST CANCER CELLS

AN HONORS THESIS

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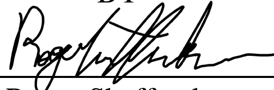

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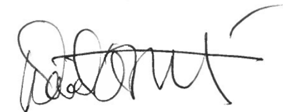

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Abstract

A preliminary study from our lab shows that Coiled Coil Domain-Containing 3 (CCDC3) or "Favine" plays a significant role as a tumor suppressor protein in breast cancer cells. This thesis aims to dig deeper into the mechanisms behind the protein's nuclear localization. Next, it pursues CCDC3's nuclear localization's role in cancer cell tumor suppression. This thesis suggests that CCDC3 may be imported into the nucleus via the importin-β pathway, suggesting that CCDC3 may contain a classical nuclear localization sequence. To better understand the mechanism behind this protein's import, research in our lab mutated human CCDC3 protein via a K233T mutation to try to inhibit nuclear localization. This single nucleotide substitution still presented nuclear localization when overexpressed in cells. Thus, this thesis took more drastic measures. Primers were designed to further mutate the CCDC3 gene via mutagenesis and transfect this mutated gene into Cal51 cells, a breast cancer cell line. It was found by cell immunofluorescence that CCDC3 mutants K233T + K234M and R232G + K233T + K234M are not imported into the Cal51 cell nucleus. These promising results expose specific amino acids required for a functional nuclear localization sequence of CCDC3. After determination of how the protein enters the cell's nucleus and what sequence is used to signal that, the next mystery was how that would affect the protein's nuclear function. This research demonstrates the fast growth of breast cancer cells when CCDC3 double or triple mutants were overexpressed. This suggests that inhibiting nuclear import disrupts the tumor suppressive function of CCDC3. These findings will deepen the understanding of the molecular mechanisms underlying the anti-breast cancer function of CCDC3 and provide new information for the future study of this protein.

Acknowledgements

I wish to express my deepest gratitude to Dr. Hyemin Lee for her unwavering patience, unyielding support, and invaluable mentorship over the past three years. She has guided me to become not only a better researcher, but also a more dedicated one. Dr. Lee has taught me extraordinary skills that I will use for the rest of my scientific career. I would also like to thank her for generating Figure 5. I would like to thank Dr. Hua Lu, who first accepted me into his lab three years ago. Though at the time I had nothing to offer but a willingness to try, he never failed to push me to dive right into the world of a researcher. From that moment, Dr. Lu has been entirely behind me, and now I am honored to have his guidance throughout the writing of this thesis. I wish to thank Dr. David A. Mullin for building my foundation of molecular understanding which allowed me to pursue this project. He has been an incredibly helpful reader and has kept me on track over the course of this year. I would also like to thank Professor Edward White for his teachings in writing, which certainly came into play in this thesis. His opinion as a reader and editor is highly regarded, and I thank him for accepting my request to be a reader for this thesis. I would also like to thank Dr. Shelya X. Zeng for generating Flag-hCCDC3(K233T)-Myc-His plasmids and providing guidance throughout the primer selection process, plasmid mutagenesis, and other experiments along the way.

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Introduction

Coiled Coil Domain-Containing Protein 3 (CCDC3) is best known for its role as a secretory protein in mature adipocytes, adipose tissues, and endothelial cells in the vascular system. Previous studies have shown that an N-terminal sequence on this protein is responsible for Golgi-mediated secretion of this factor. CCDC3 is used in adipogenesis and lipogenesis (1,2).

Breast cancer is the most common type of cancer in women worldwide. P53 is one of the most heavily studied proteins and is involved in cell cycle arrest and apoptosis. P53 is mutated in approximately 30% of all breast cancer tumors (3,4). In recent findings within an unpublished paper in our lab, CCDC3 was discovered to stabilize p53 in breast cancer cells. This immediately makes CCDC3 a protein of high interest. It was found to be transcriptionally induced by p53, have a tumor-suppressive role in breast cancer cells, and be under-expressed in these cells. Higher CCDC3 levels are correlated with higher overall survival and lower relapse rates in breast cancer patients. CCDC3 also upregulates ubiquitination of p53, but surprisingly resists its degradation by preventing its association with the 26s proteosome (a main proteolytic tool of eukaryotic cells). CCDC3 suppresses breast cancer cell proliferation by inhibiting P53 degradation (unpublished). Although this study unveiled many secrets of CCDC3, many remain unknown.

Nuclear import of proteins is essential to the cell. Proteins need to be able to enter and exit the nucleus to complete various functions required for cell life. The nuclear localization sequence (NLS) is a string of amino acids on a protein that allow other proteins to bind to it and transport it to the nucleus. Classical NLSs contain repeats of basic amino acids, particularly lysine (K) and arginine (R) (5). There are a multitude of nuclear transport mechanisms. One of the most common of these is the importin-α/β nuclear import pathway. This process imports proteins by recognizing classical NLS sites These nucleocytoplasmic

transport receptors import and export proteins through nuclear pores. Import usually begins with the formation of a ternary complex between importin-α, importin-β, and the protein cargo. Importin-β releases the protein into the nucleus through a nuclear pore when bound to Ran-GTP (6,7). Importazole, a 2,4-diaminoquinazoline, inhibits importin-β's activity, likely by blocking Ran-GTP and importin-β binding (8,9).

Since the purpose of tumor suppressor proteins is to prevent uncontrolled cancer cell growth, their functions directly impact cancer cell proliferation. When tumor suppressor proteins that normally enter the nucleus are unable to do so, this could impact their nuclear function negatively. For example, disruption of the p53 NLS has been shown to inhibit its activity. This leads to more aggressive cell growth and reproduction (10). CCDC3 was recently discovered by our lab to play a tumor suppressive role in breast cancer cells, and it is also translocated into the nucleus. This thesis aims to demonstrate that the disruption of its own NLS may inhibit nuclear import, leading to a loss of tumor suppressive function.

Methods and Materials

Importazole Treatment

Cal51 cells were seeded on 10cm plates. The next day, the cells were treated with 0μM or 10μM of importazole. A negative control received no importazole. The cells were then harvested after 16 hours.

Cell fractionation

Collection, PBS wash, and resuspension of Cal51 cells in CE buffer (10mM HEPES, 60 mM KCl, 1mM EDTA, 0.075% NP-40, 1mM DTT, and 1mM PMSF, adjusted to pH 7.6) was followed by a 5-10min incubation period on ice. Tubes were next spun down at 1500rpm for 4min and CE was pipetted into a clean tube. CE buffer without detergent was used to wash and resuspend the nuclei pellet. The pellet was again spun down at 1500rpm for 4min.

The nuclei were next lysed using NE buffer (20mM Tris/HCl, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2mM EDTA, 1mM PMSF and 25% glycerol, adjusted to pH 8.0). They were incubated on ice for 10min and then spun down at maximum speed for 10 min. The NE was transferred to a clean tube.

Western blotting

As was previously explained (11), lysis buffer consisting of 50 mM Tris/HCl (pH7.5), 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM pepstatin A and 1 mM leupeptin was used to lyse the cells. Western blot (WB) analyses were then completed using equal amounts (40µg) of the clear cell lysates.

NLS Prediction

To best predict the NLS of CCDC3, "cNLS Mapper" (12) was used. This site predicts classical NLSs that use importin-α/β pathways.

Primer Selection

The primers (Life Technologies Corporation, CA, USA) for generating human CCDC3 mutants are as follows:

CCDC3 (K233T + K234M), F: 5'- GCGCGTACGATGGGCCGCCACCTGGAGCTGG -3' and R: 5'- CCCATCTGACGCGCCTGCCGCAAGGACCTCTTGACC -3';

CCDC3 (R232G + K233T + K234M),

F: 5'- GCGGGTACGATGGGCCGCCACCTGGAGCTGGCGA -3' and

R: 5'- GCCCATCGTACCCGCCTGCCGCAAGGACCTCTTGACCTTCTTCAC -3';

Mutagenesis

Primers were selected and previously described for PCR that resulted in two mutants of the original single mutant (K233T) previously made in our lab. These were: K233T+K234M, R232G+K233T+K234M. Plasmid pcDNA3.1/Myc/His A was used to

modify CCDC3 NLS. All of the plasmids were transformed in NEB Stable Competent E. coli cells (New England Biolabs, Massachusetts). Plasmids for transfection to Cal51 cells were extracted using GeneJET Plasmid Miniprep Kit (Thermo fisher, Massachusetts). Pfu DNA polymerase, DpnI restriction enzyme, and Q polymerase were used in PCR mutagenesis. PCR cycling was carried out using a 50μL solution of 2-10ng of template DNA, 1μM primer pair, 200 μM dNTPs and 3 units of Pfu DNA polymerase. For single- and double-site mutations, the protocol of PCR reaction and transformation of E. coli cells were followed this previous study by Liu H and Naismith JH group(13). The sequences of PCR products generated using GeneJET Plasmid Miniprep Kit were checked by Genewiz, Chelmsford, Massachusetts. After mutagenesis generated successfully, the plasmids were introduced into the Cal51 cells via transfection.

Transfection

RPMI-1640 medium with added 10% fetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin were used for Cal51 cells. A CO 2 humidified atmosphere at 37 ˚ C in a 5% was used to culture cells. Using TurboFect transfection reagent following the manufacturer's protocol (Thermo Scientific), cells were seeded overnight and subsequently transfected with plasmids. 30–48 hours post-transfection, cells were collected to be used for experiments.

Immunofluorescence staining

Cal51 cells were first transfected with respective plasmids for this experiment. Next, they were incubated at 4°C for 3 hours in RPMI medium. The cells were fixed in formaldehyde. Using 0.2 % Triton X-100 and blocked with 1% BSA in PBST (PBS with 0.1 % Tween 20), the cells were permeabilized. Overnight, the cells were incubated with primary antibody at 4°C. The slides were then incubated in Alexa-488, the secondary antibody, at room temperature for one hour. For one hour at room temperature, another primary antibody was added and

incubated. This was to co-stain. The respective secondary antibody was then added and incubated at room temperature for one hour. DAPI (Sigma) was used to stain the nucleus. The slides were detected by Confocal microscopy (Nikon TiE-2, Nikon Inc., Tokyo, Japan) after mounting overnight.

Colony formation assay

800μg/mL G418 was used to treat Cal51 cells for 3 days for selection after transfection. 1,000 cells per well were seeded into a 6-well plate. Every 3 days media were changed for 10 days. Cells were fixed with formaldehyde and 0.25% crystal violet was used to stain the cells at the end of the 10 days. Images could then be taken of the cells.

Antibodies

The Flag-CCDC3-Myc-His plasmids have previously been described (2). The antibodies used were:

- Anti-CCDC3 GeneTex, GTX81055; 1:500 dilution for WB; Thermo Fisher Scientific, PA5-49634 1:500 for WB; 1:20-1:50 for IF
- Anti-p53 DO-1, Santa Cruz Biotechnology, catalogue no. sc-126, diluted 1:1,000 for WB
- Anti-Flag Sigma-Aldrich, catalogue no. F1804, diluted 1:3,000 for WB; 1:250 dilution for IF
- Anti-Myc 9E10, Santa Cruz Biotechnology, catalogue no. sc-40, diluted 1:1,000 for WB; 1:200 for IF

Results

CCDC3 is located mostly in the nucleus in Cal51 cells.

Cal51 cells express hCCDC3 on their own, and we wanted to see where exactly the endogenous CCDC3 was located within the cell. IF shows CCDC3 mostly located in the

same area as DAPI, which stains the nucleus (Fig. 1A). WB shows a strong band in the nucleus (Fig. 1B). This data supports that endogenous CCDC3 is largely imported into the nucleus from the cytoplasm in Cal51 cells.

CCDC3 enters the nucleus via an importin β dependent pathway.

We looked to find how the CCDC3 enters the nucleus. Cal51 cells were treated with importazole. Importazole inhibits importin-β's ability to bring cargo proteins into the nucleus, which disrupts the importin-α/β pathway and renders many classical NLSs useless (6,7). Cell fractionation followed by WB of the cells show no import of CCDC3 into the nucleus when treated with importazole (Fig. 2). As p53 is known to be imported into the nucleus via an importin dependent pathway, we used p53 as an indicator to prove importazole worked (14). Since CCDC3 was unable to enter the nucleus when importin-β's function was disrupted, CCDC3 is imported into the nucleus via importin-β.

CCDC3(K233T + K234M) and CCDC3(R232G + K233T + K234M) may inhibit nuclear import of the protein.

We wanted to find where the NLS was on the protein that the transport protein recognized to translocate the CCDC3. An immunofluorescence assay was used to observe this. DAPI can be seen staining the nucleus. The Alexa 488 secondary antibody, which appeared to be green, was used to detect the a-Flag primary antibody. This specifically located the mutant proteins. The Pc-DNA which had not been transfected with a CCDC3-containing plasmid, only showed evidence of the nucleus in blue (Fig. 4). This confirms that a-Flag antibodies bind specifically. Cal51 cells transfected to overexpress exogeneous WT CCDC3 (Fig. 5B) are shown to mostly import CCDC3 into the nucleus. The IF assay of the MCF7 cells transfected with Flag-hCCDC3(K233T)-Myc-His plasmids appear to have CCDC3(K233T) protein localized mostly in the nucleus, similarly to the WT CCDC3. We

mutated the Flag-hCCDC3(K233T)-Myc-His plasmid to create CCDC3(K233T+K234M) and CCDC3(R232G+K233T+K234M) mutants. After transfection, Cal51 cells overexpressed the double or triple mutant CCDC3. In the confocal images of the CCDC3(K233T + K234M) and CCDC3(R232G + K233T + K234M) overexpressing Cal51 cells (Fig. 6B, 7B), there is a large amount of CCDC3 in the cytoplasm, and very little in the nucleus. This suggests that the NLS of CCDC3 was successfully predicted and mutated. The disfunction of the NLS created a buildup of mutant CCDC3 in the cytoplasm, as it was unable to be transported into the nucleus.

Cal51 cells expressing Flag-hCCDC3(K233T + K234M)-Myc-His or Flag-hCCDC3(R232G + K233T + K234M)-Myc-His produce more colonies than GFP-Control transfected cells.

After finding that the CCDC3(K233T + K234M) and CCDC3(R232G + K233T + K234M) mutants disrupt nuclear import, we wanted to see if that had an effect on cell proliferation. The CCDC3(K233T + K234M) and CCDC3(R232G + K233T + K234M) mutant protein overexpression within Cal51 cells caused them to grow more rapidly over 10 days than the control group (Fig. 8A, 9A). More colonies were formed by the double and triple mutant overexpression cells. Graphs comparing the difference between the number of colonies quantify the rapid growth of the cells containing the mutants (Fig. 8B, 9B). It can be seen that the inability for the protein to enter the nucleus disrupted its tumor suppressive ability and subsequently induced rapid cell division.

Discussion

Little is known about CCDC3, as it is extremely understudied. Our lab has unpublished data supporting CCDC3's ability to stabilize p53. This thesis shines light on the inner workings of the protein and how it functions within the cell. The nuclear import of

CCDC3 may be crucial to its role as a tumor suppressor. First, we looked to determine what proteins were responsible for CCDC3's translocation. After Cal51 cell treatment with importazole, cell fractionation followed by WB revealed that CCDC3 utilizes an importin-β dependent pathway. This suggests that the NLS of CCDC3 may be a classical NLS. Although it seems likely that the CCDC3 NLS uses the importin-α/β pathway, more research could be done to confirm this. In the future, researchers may investigate inhibiting importin-α, other supportins, or determining if importin-β binds to the CCDC3 NLS directly.

Determining the NLS was the next step, and NLS Mapper helped to point in the right direction. The NLS was predicted (Fig 3B), primers were ordered, plasmids were mutated, and cells were transfected. The result was Cal51 cells overexpressing CCDC3(K223T + K234M) and CCDC3(R232G + K233T + K234M). These were found to disrupt the NLS function enough to suppress most of the protein's nuclear import. The double mutant was sufficient to block entrance into the nucleus, so the third mutation proved to be excess. However, the data does show that the 234th amino acid is necessary for CCDC3's nuclear import. Further study would need to be completed to determine whether a single substitution mutation at the 234th site alone would inhibit translocation. Although the NLS location has been identified, more combinations of mutants would provide a more accurate assessment of exactly which amino acids are necessary for nuclear import.

After the tremendous discovery of CCDC3's mechanism of nuclear import, the last mystery was its effect on function. The colony assay answered this question. Inhibition of importation into the nucleus of CCDC3 in Cal51 cells had a positive effect on proliferation. More Cal51 colonies were found 10 days after transfection with the mutants that inhibited nuclear import of CCDC3. This finding supports the nuclear role that CCDC3 plays on tumor suppression. It suggests that CCDC3 does not activate a cascade of proteins which then enter the nucleus to impact cell proliferation. CCDC3 itself acts within the nucleus.

Discovering the mechanism behind this protein's nuclear import will help widen the knowledge on its function within the breast cancer cell. Its interaction with p53 (unpublished) has caught the attention of researchers. As new information on this curious protein is unlocked, its true role becomes clearer, and our understanding of breast cancer at the cellular level continues to grow.

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Figures

Figure 1

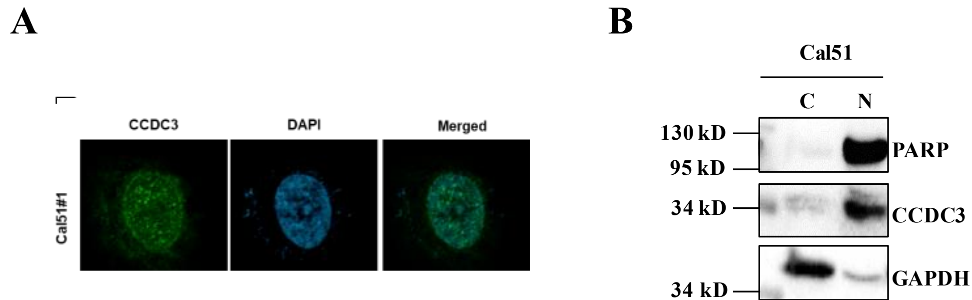

Figure 1. CCDC3 is largely located in the nucleus of Cal51 breast cancer cells. (A) Confocal image of endogenous CCDC3 localization in Cal51 cell. CCDC3 is shown to be localized mostly in the nucleus. DAPI was used to stain the nucleus. After Cal51 cells were fixed, the cells were stained with a-CCDC3 followed by secondary antibody Alexa 488. Scale bar: 10μm. Representative cell pictures are shown. (B) Cell fractionation followed by Western Blotting of Cal51 cells confirms this nuclear localization of the protein.

Figure 2

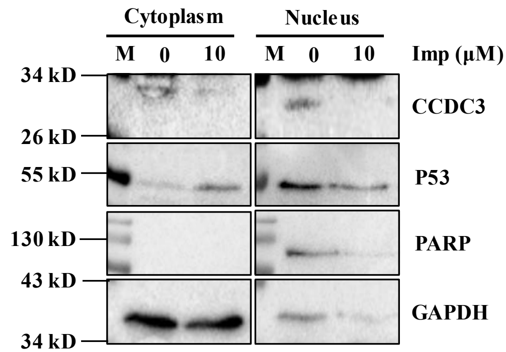

Figure 2. Importin beta inhibition may disrupt CCDC3 nuclear translocation. Cal51 cells were fractionated after non-treatment or treatment of 10μM of importazole for 16 hours. Western Blotting succeeded this process to confirm protein levels using a-CCDC3, a-DO-1 for p53, a-PARP and a-GAPDH. PARP and GAPDH were used as indicators of the nucleus and cytoplasm, respectively.

Figure 3

A

The CCDC3 amino acid sequence is:

“MLRQLLLAALCLAGPPAPARACQLPSEWRPLSEG
CRAELAETIVYARVLALHPEAPGLYNHLPWQYHA
GQGGLFYSAEVEMLCDQAWGSMLEVPAGSRLNLT
GLGYFSCHSHTVVQDYSYFFFLRMDENYNLLPHG
VNFQDAIFPDTQENRRMFSSLFQFSNCSQGQQLAT
FSSDWEIQEDSRLMCSSVQKALFEEEDHVKKLQQ
KVATLEKRNRQLRERVKKVKRSLRQARKKGRHLE
LANQKLSEKLAAGALPHINARGPVRPPYLRG”

B

The NLS software predicted the sequence to be “LRQARKKGRHL” starting at the 228th amino acid.

Figure 3. The Nuclear Localization Sequence of CCDC3 is predicted. (A) The amino acid sequence of the CCDC3 protein. (B) NLS of CCDC3 predicted using NLS Mapper software that predicts classical NLSs. The predicted sequence are amino acids 228-238.

Figure 4

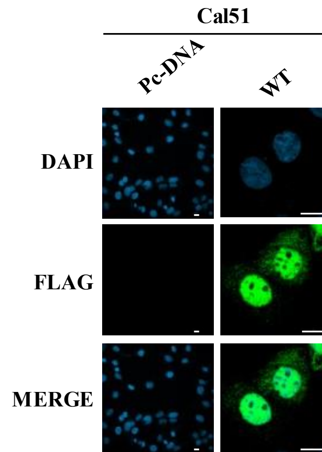

Figure 4. Exogeneous CCDC3 wild type is located in the nucleus. Pc-DNA transfected Cal51 cells as control were included to show a-Flag antibody works specifically (left). Flag-hCCDC3-Myc-His plasmid vector transfected into Cal51 cells. After transfection for 48 hours, a-Flag and secondary antibody, Alexa 488 were used to locate the exogeneous CCDC3. DAPI was used to stain the nucleus. Exogeneous WT CCDC3 can be seen mostly in the nucleus. Scale bar: 20µm.

Figure 5

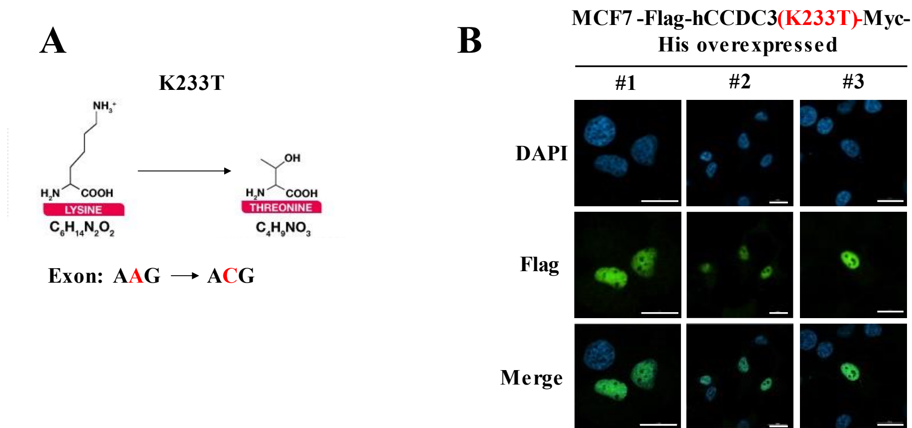

Figure 5. K233T mutation of CCDC3 does not inhibit nuclear import of the protein. (A) A visual of lysine being replaced by threonine. Below is the DNA codon single point mutation which results in the given amino acid mutation. (B) MCF7 cells were transfected with Flag-hCCDC3(K233T)-Myc-His plasmid. The cells were fixed and stained with a-Flag followed by Alexa 488 secondary antibody. DAPI was used to stain the nucleus. Confocal imaging reveals hCCDC3(K233T) mutant still mostly located in the nucleus.

Figure 6

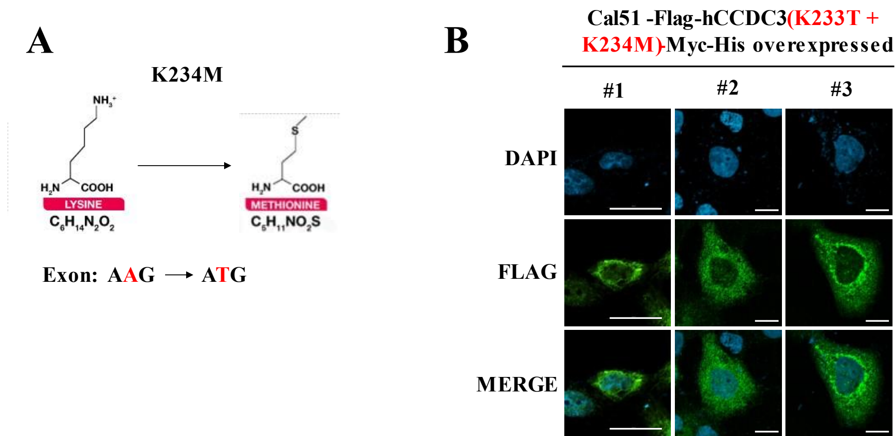

Figure 6. CCDC3(K233T + K234M)-Myc-His overexpressed Cal51 cells show large accumulation of CCDC3 in cytoplasm. (A) Visualization of methionine replacing lysine structurally. Underneath is the codon mutated during mutagenesis. (B) Confocal images shown after CCDC3(K233T + K234M) was overexpressed in Cal51 cells. The cells were fixed and stained with a-Flag and Alexa 488 antibodies. These show hCCDC3(K233T + K234M) largely located in the cytoplasm.

Figure 7

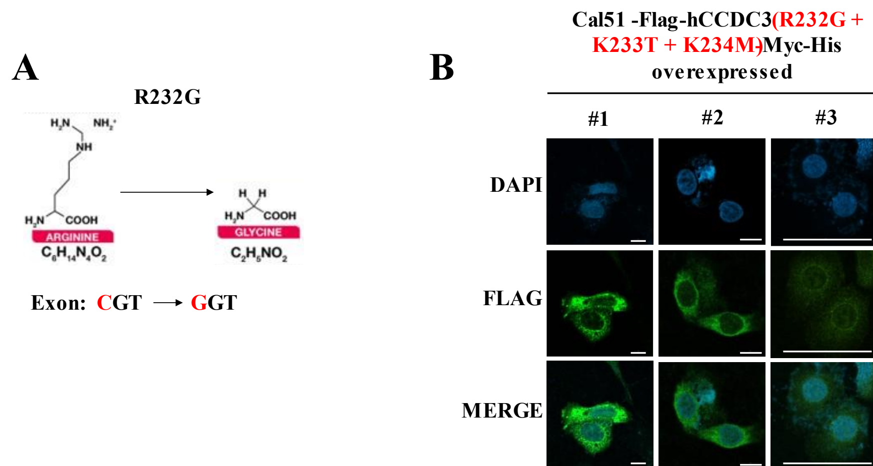

Figure 7. CCDC3(R232G + K233T + K234M) overexpressed Cal51 cells show large accumulation of CCDC3 in cytoplasm. (A) Diagram of arginine to glycine substitution. Below is the mutated exon required to produce the substitution. (B) Immunofluorescence assay using a-Flag as primary antibody and Alexa488 as the secondary antibody was performed after Cal51 cells were overexpressed with Flag-hCCDC3(R232G + K233T + K234M)-Myc-His. Confocal imaging shows the triple mutant proteins mostly located in the cytoplasm.

Figure 8

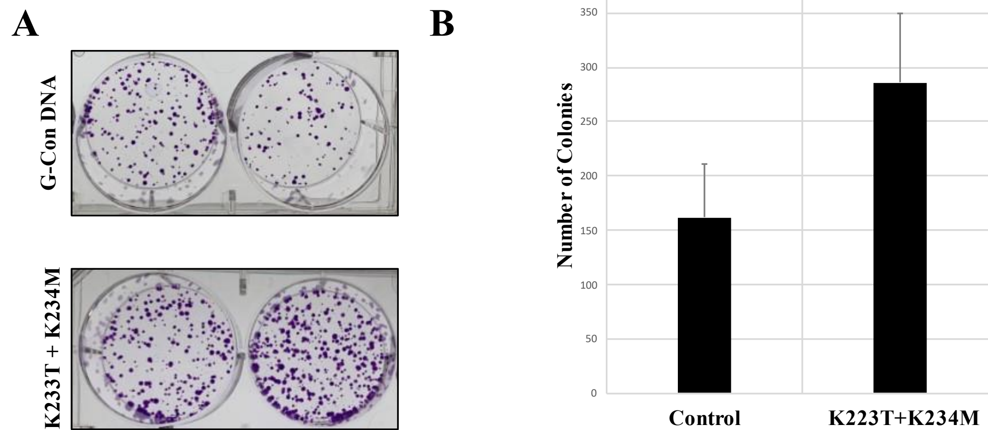

Figure 8. Cal51 Flag-hCCDC3(K233T + K234M)-Myc-His overexpressed cells proliferate more than Control Cal51 cells. (A) Cal51 cells were overexpressed with the control or Flag-hCCDC3(K233T + K234M)-Myc-His plasmid. After transfection, the cells were treated with 800µg/mL G418 for 3 days to select. 1,000 cells per each well were reseeded into 6-well plate, and fresh media were changed every 3 days for 10 days. Images were taken after cells were fixed with formaldehyde and stained with 0.25% crystal violet after 10 days. (B) Graph of number of colonies per cell type. Cal51 transfected with Flag-hCCDC3(K233T + K234M)-Myc-His produced more colonies than the Cal51 Control cells.

Figure 9

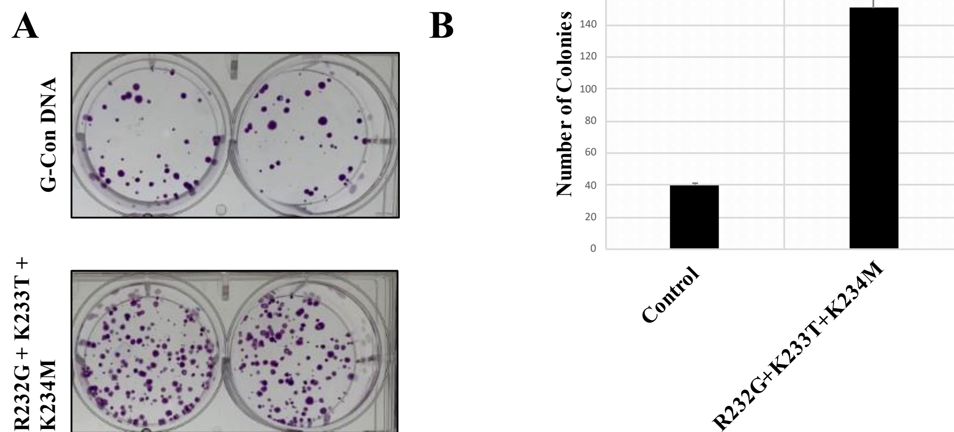

Figure 9. Cal51 Flag-hCCDC3(R232G + K233T + K234M)-Myc-His overexpressed cells proliferate more than Control Cal51 cells. (A) Cal51 cells were overexpressed with the control or Flag-hCCDC3(R232G + K233T + K234M)-Myc-His plasmid. After transfection, the cells were treated with 800µg/mL G418 for 3 days to select. 1,000 cells per each well were reseeded into 6-well plate, and fresh media were changed every 3 days for 10 days. Images were taken after cells were fixed with formaldehyde and stained with 0.25% crystal violet after 10 days. (B) Graph of number of colonies per cell type. Cal51 transfected with Flag-hCCDC3(R232G + K233T + K234M)-Myc-His had better survival from single cells than the control group.