University of Nevada, Reno

Role of COP9 signalosome subunit 3 (CSN3) phosphorylation in integrin-mediated cardiac hypertrophy

A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Biochemistry and Molecular Biology and the Honors Program

by

Denise Nicole E. Teh

Dr. Maria Valencik, Thesis Advisor

May, 2010

University of Nevada Reno, The Honors Program

We recommend that the thesis
prepared under our supervision by

Denise Nicole Esguerra Teh

entitled

Role of COP9 signalosome subunit 3 (CSN3) phosphorylation in integrin-mediated cardiac hypertrophy

be accepted in partial fulfillment of the
requirements for the degree of

Bachelor of Science in Biochemistry and Molecular Biology

Maria Valencik, Ph.D., Faculty Mentor

Mariam Ba, Graduate Student, Advisor
University of Nevada, Reno

Tamara Valentine, Ph.D., **Honors Program**

Abstract

Integrins are involved in various aspects of cell regulation, signaling, and growth and have been shown to react to environmental stimuli such as mechanical stress in cardiac myocytes. These integrin-mediated reactions may be linked to the upregulation of hypertrophic genes that result in cardiac hypertrophy, which is the focus of our lab. CSN is a highly conserved complex involved in cell regulation and a critical component to cell differentiation. Previous research has shown that the third subunit of the COP9 signalosome (CSN3) complex binds specifically to ß1D integrin, an isoform that is predominant in adult striated muscle. CSN3 is phosphorylated on serine residues 410, 421 and 423; however the role of these phosphorylation sites remains to be determined. Therefore, we hypothesize that phosphorylation at one or a combination of these sites results in the physical disassociation of CSN3 from ß1D integrin upon integrin activation. To test this hypothesis, CSN3 serine residues 410, 421 and 423 were mutated to either Alanine (to prevent phosphorylation) or Aspartic Acid (to mimic phosphorylation). The following techniques were utilized in this experiment: polymerase chain reaction (PCR), gel electrophoresis and DNA purification. All constructs were confirmed to contain both the pShuttle-IRES-hrGFP-1 vector and the CSN3 gene with the desired mutations via DNA sequencing.

Acknowledgements

I would like to thank Mariam Ba and Dr. Maria Valencik from the Department of Biochemistry and Molecular Biology at the University of Nevada Reno for all of their help and guidance on this project.

Table of Contents

Introduction:

Heart disease is a broad term referring to any pathology that impedes the normal physiological function of the heart. It can be caused by a wide array of factors such as environmental, genetics, and natural aging. One common characteristic of preclinical heart disease is the hypertrophy of the left ventricle. Similar to other muscles, when the heart encounters stress, the cells undergo changes such as an increase in size. Too much remodeling can cause structural and functional abnormalities, resulting in heart failure and death (Katz AM, 1994; Berenji K. et al., 2005). At a cellular level, pathological hypertrophy involves altered gene expression, reorganization of the cell's cytoskeleton and cell death (Diwan A et al., 2007). But the question is, how does mechanical stress lead to alternations in cellular function and structure? Studies have shown that integrins are the link between the cell's cytoskeleton and the extracellular matrix, allowing cells to react to mechanical stress (Knoll R, 2003).

Integrin is a heterodimer transmembrane protein composed of two chains, the alpha and beta subunit. These chains interact with extracellular proteins and the cell's cytoskeleton, allowing the cell to react to signals such as mechanical stress. Such interactions influence cell differentiation, shape, migration and gene expression (Hunter et al. 2008). The ß1 integrin subunit exists in four splice variants in the heart, A-D (Baudoin et al. 1996). The most prevalent forms are ß1A and ß1D. Although the two isoforms are nearly identical, ß1D is predominantly expressed in mature striated muscle and is thus the focus of my research (Belkin A.M. et al. 1997). ß1D is also known to be expressed in cardiomyocytes of infarcted myocardium, further indicating the significance

of this isoform in heart cells (Sun M. et al. 2003).

One protein that is known to bind to ß1D integrin is the third subunit of COP9 signalosome (CSN) (Hunter et al. 2008). First discovered in *Arabidopsis Thaliana*, CSN is a highly conserved protein made of 8 subunits (Deng et al. 2000; Wei et al. 1998). Previous studies have shown that CSN is involved in a number of cellular functions such as DNA repair, MAPK signaling, protein phosphorylation and other regulatory activities (Bech-Otschi et al. 2002). Recently, it was discovered that the third subunit of CSN, CSN3, binds specifically to ß1D integrin in adult heart cells (Hunter et al. 2008). In another experiment, CSN regulated gene expression under the stimulus of light (Wei & Deng 1992; Chamovitz 2009). Although it is clear that CSN plays a significant role in gene expression, its mechanism and pathway in cardiomyocytes are unknown.

My project focuses on CSN3's binding interaction with ß1D integrin. A hypothetical model for CSN3 in cardiomyocytes will now be described (Figure 1). Upon receiving stressful stimuli, ß1D integrin is activated, leading to the disassociation and activation of CSN3. Free CSN3 can either translocate into the nucleus, where it activates the transcription of hypertrophic genes via a pathway such as AP-1 or affect the phosphorylation of NF-kB, which may also translocate to the nucleus to upregulate transcription. This experiment involves the mutagenesis of three phosphorylation sites at Serine 410, 421, and 423 of CSN3. These serine residues were mutated to either Alanine (Ala), to prevent phosphorylation, or to Aspartic Acid (Asp), to mimic phosphorylation. The results of mutagenesis were analyzed by gel electrophoresis and confirmed by DNA sequencing.

The future direction of this experiment will involve using yeast two hybrid to determine if the mutated constructs interact with ß1D integrin.

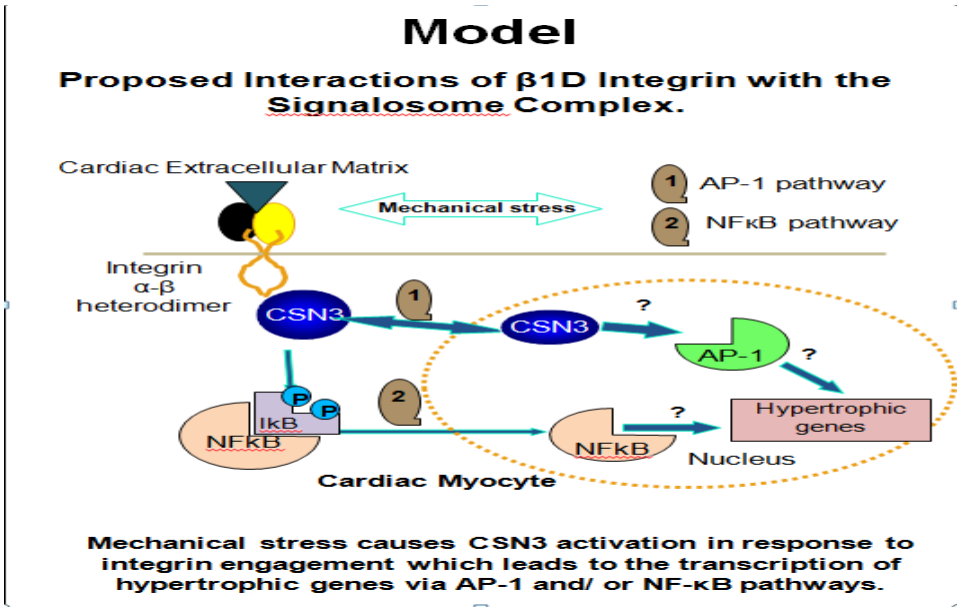

Figure 1. Proposed working model of CSN3 in relation to integrin-mediated cardiac hypertrophy.

Heart disease affects more than one in three American adults and results in over 2.4 million casualties annually (American Heart Association 2006). An estimated 81.1 million American adults have one or more types of heart disease, and with no current cure, the majority of those people will die due to complications. The results obtained from this experiment will provide insight on the binding interaction between ß1D integrin and CSN3. If CSN3 is responsible for the activation of hypertrophic genes in cardiomyocytes, preventing the disassociation and translocation of CSN3 may be crucial for the treatment and/or prevention of cardiac hypertrophy.

Methods:

Mutagenic Primer Design

Human COP9 signalosome subunit 3 (CSN3) was previously cloned into the pShuttle-IRES-hrGFP-1 (Mariam Ba, University of Nevada, Reno). The map of pShuttle-IRES-hrGFP-1 is shown in figure 2. Mutagenic primers were constructed according to the recommendations listed in the QuikChange Lightning Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, Catalog #210513-13). Site directed mutagenesis was performed on CSN3 at all three potential Serine phosphorylation sites using the following primers: Alanine-410 (5'- GTACAAAAGAGTATGGGC**G**CACAAGAAGATGATTC-3'), Aspartic acid-410 (5'-GTACAAAAGAGTATGGGC**GAC**CAAGAAGATGATTCAGG-3'), Alanine-421,423 (5'-GGAAACAAACCATCC**GC**TTAT**G**CTTGAGTCGACGCC-3'), Aspartic acid-421,423 (5'- GGAAACAAACCATCC**GA**TTAT**GA**TTGAGTCGACGCC-3') and none mutagenic primer, 5009-psh (5'-CGCAAAAAAGAAAGCACATCGTAGTCATGC-3').

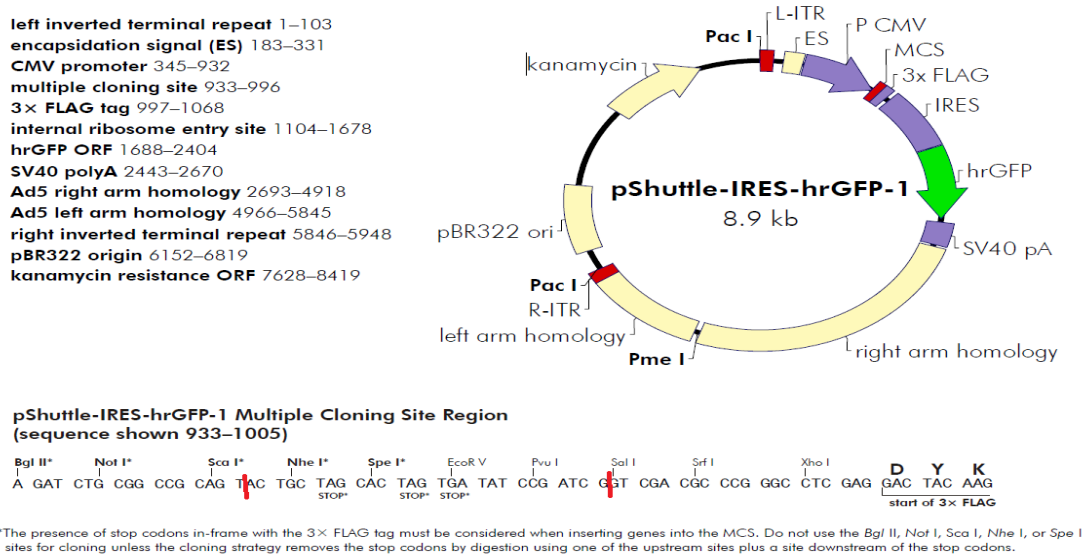

Figure 2. pShuttle-IRES-hrGFP-1. The red lines indicate the exact positioning of the CSN3 gene (between the Sal I and Sca I cut sites) in the multiple cloning site.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) was performed according to manufacture recommendations (the QuickChange Lightning Multi Site-Directed Mutagenesis kit, Stratagene, La Jolla, CA, Catalog #210513-13). Briefly, each mutagenesis reaction was composed of 2.5 ul 10x reaction buffer, 140 ng of template DNA (10 kb), 130 ng of each primer (one mutagenic and one none mutagenic), 1.5 ul of QuickSolution, 1ul odd NTP mix and 1ul enzyme blend. The final volume of each reaction was 25ul. The cycling parameters were 95°C for 2 minutes, 30cycles of 95°C for 20 seconds: 55°C for 30 seconds: 65°C for 5minutes then 65°C for 5minutes.

Digestion with Dpn I

One ul of Dpn I (provided by the Mutagenesis kit to digest the methylated parental bacterial DNA) was added to the sample at the conclusion of PCR. The sample was then

incubated at 37ºC for 5 minutes in a dry incubator.

Transformation

The transformation reaction was performed as follow: 5ul of Dpn I digested DNA was inoculated into 30 ul of XL10-Gold UltraCompetent cells, the reaction was incubated for 30 minutes on ice then heat-pulsed at 42°C for 30 seconds. 100 ul of the transformed bacteria was plated onto LB Agar plates containing kanamycin (25 ug/ml).

DNA purification from single colonies (Mini Prep)

QIAprep Spin Miniprep was used to isolate DNA and purify the. The purity and concentration of these samples were determined using a Nanodrop Spectrophotometer.

Restriction Digest

Sal I and Sca I were used to digest approximately 500 ng of each CSN3 construct. The digests were incubated at 37ºC overnight.

Gel Electrophoresis

500ng to 1ug of each construct was run on a 0.8% agarose gel. Two to three ul of ethidium bromide was added per 50 ml of gel solution to visualize the DNA bands under UV light. Loading dye (6x) was added to the cut and uncut constructs as 3x and 2x respectively. The gels were run under 110 volts for approximately 80 minutes. A 1kb ladder was used as a standard.

DNA Sequencing and Analysis

Constructs containing two bands, one at 8.9kb and 1.27kb each, were sent to the Nevada Genomics Center for sequencing. The constructs were sequenced with a 653-CSN3 (5'-GGAGGGATGATCTATACTGG-3) and a forward pShuttle-IRES (5'-

CTCACGGGGATTTCCAAGTC-3') primer. The results were analyzed using the NCBI website and constructs containing the desired mutations were stored in a 1:1 solution of glycerol for further use.

Results:

I- Mutation from Serine to Alanine at position 410

1. DNA gel of CSN3-Alanine 410pShuttleIREShrGFP-1 clones

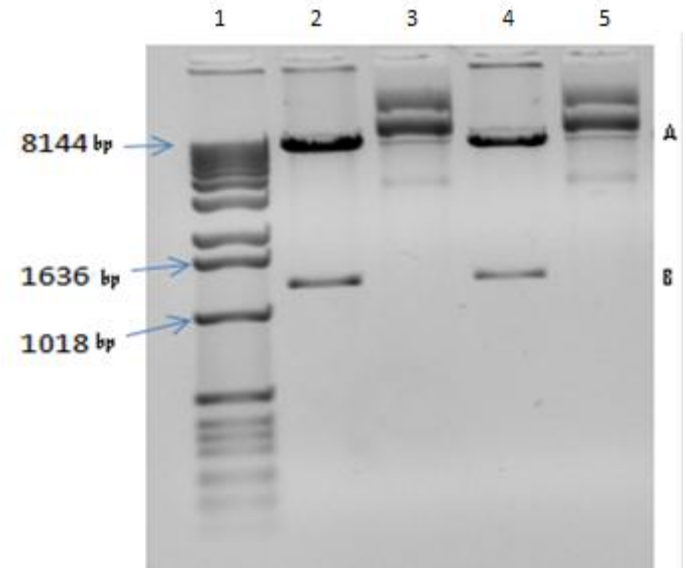

Figure 3. Gel image of CSN3 Serine 410 to Alanine 410. 500ng to 1ug DNA was run on a 0.8% agarose gel at 110 volts for 80 minutes. (A) represents the location of the pShuttle-IRES-hrGFP-1 vector at 8.9 kb. (B) represents the CSN3 gene insert (1.2kb). The DNA ladder (1kb), CSN3 Alanine 410, clone A (uncut), CSN3 Alanine 410, clone A (cut), CSN3 Alanine 410, clone B (cut), and CSN3 Alanine 410, clone B (uncut) are represented in lanes 1, 2, 3, 4 and 5 respectively. Constructs containing both fragments at A and B were sent for sequencing at the Nevada Genomics Center.

2. Sequencing results of CSN3-Alanine 410 pShuttleIREShrGFP-1, clone A

```
ref|NM_003653.2| Homo sapiens COP9 constitutive photomorphogenic homolog subunit
3 (Arabidopsis) (COPS3), mRNA
Length=1652

GENE ID: 8533 COPS3 | COP9 constitutive photomorphogenic homolog subunit 3
(Arabidopsis) [Homo sapiens] (Over 10 PubMed links)

 Score = 1205 bits (652),  Expect = 0.0
 Identities = 654/655 (99%), Gaps = 0/655 (0%)
 Strand=Plus/Plus

Query  3     ACAGGCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAA  62
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  709   ACAGGCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAA  768
```

```
Query  63    GTATATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATC  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  769   GTATATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATC  828

Query  123   TCAAATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGT  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  829   TCAAATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGT  888

Query  183   GTATTCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTT  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  889   GTATTCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTT  948

Query  243   CACTCGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAA  302
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  949   CACTCGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAA  1008

Query  303   TATTCAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGT  362
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1009  TATTCAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGT  1068

Query  363   GCAGTTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGA  422
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1069  GCAGTTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGA  1128

Query  423   GATTTTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAA  482
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1129  GATTTTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAA  1188

Query  483   ATATAATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCT  542
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1189  ATATAATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCT  1248

Query  543   GGATGAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAA  602
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1249  GGATGAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAA  1308

Query  603   GAGTATGGGCGCACAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCTTGA  657
             |||||||||  ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1309  GAGTATGGGCTCACAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCTTGA  1363
```

Figure 4. Nucleotide sequence of CSN3-Alanine-410 blasted against the human CSN3 in the NCBI database. Query represents the CSN3-Alanine-410 (clone A) and Sbjct shows the human CSN3 (NM-003653) sequence in the NCBI database. This sequence was generated by the CSN3-653 primer. The mutation located at nucleotide position 1318 (in red) is confirmed to be the expected mutation. The three nucleotides highlighted in yellow (Query line) code for Alanine while the nucleotides highlighted in green (Sbjct line) code for Serine. These results indicate a successful mutation from Serine to Alanine at position 410 in the CSN3 gene.

II. Mutation from Serine 421,423 to Alanine at position 421,423.

1. DNA gel of CSN3-Alanine 421,423 pShuttle-IRES-hrGFP-1 clones

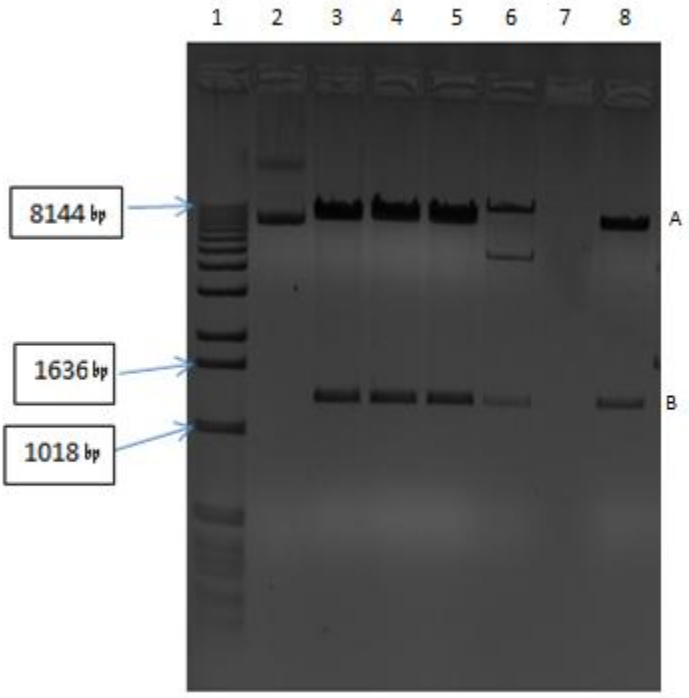

Figure 5. Gel image of CSN3 Serine 421,423 to Alanine 421,423. 500ng to 1ug DNA was run on a 0.8% agarose gel at 110 volts for 80 minutes. (A) represents the location of the pShuttle-IRES-hrGFP-1 vector at 8.9 kb. (B) represents the CSN3 gene insert (1.2kb). The DNA ladder (1kb), CSN3 Alanine 421,423 clone H (uncut), CSN3 Alanine 421,423 clone H (cut), CSN3 Alanine 421,423 clone I (cut), CSN3 Alanine 421,423 clone J (cut), CSN3 Alanine 421,423 clone K (cut), and CSN3 Alanine 421,423 clone L (cut) are represented in lanes 1,2, 3, 4, 5, 6, 8 respectively. Constructs containing both fragments at A and B were sent for sequencing at the Nevada Genomics Center.

2. Sequencing results of CSN3- Alanine 421,423 pShuttle-IRES-hrGFP-1, clone J.

ref|NM_003653.2| Homo sapiens COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) (COPS3), mRNA
Length=1652
GENE ID: 8533 COPS3 | COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) [Homo sapiens] (Over 10 PubMed links)

Score = 1011 bits (637), Expect = 0.0
Identities = 640/643 (99%), Gaps = 0/643 (0%)
Strand=Plus/Plus

```
Query  1     TACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTATATTTTAGT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  721   TACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTATATTTTAGT  780

Query  61    GTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAAATTGTGGG  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  781   GTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAAATTGTGGG  840

Query  121   TAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTATTCAACCAA  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  841   TAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTATTCAACCAA  900

Query  181   CAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACTCGCGATAA  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  901   CAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACTCGCGATAA  960

Query  241   CAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATTCAGAGGCT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  961   CAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATTCAGAGGCT  1020

Query  301   AACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAGTTGTCTGG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1021  AACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAGTTGTCTGG  1080

Query  361   ACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATTTTTGCAAG  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1081  ACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATTTTTGCAAG  1140

Query  421   TATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATATAATAACCC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1141  TATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATATAATAACCC  1200

Query  481   AGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGATGAGCGGCT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1201  AGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGATGAGCGGCT  1260

Query  541   GAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGTATGGGCTC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1261  GAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGTATGGGCTC  1320

Query  601   ACAAGAAGATGATTCAGGAAACAAACCATCCGCTTATGCTTGA  643
             ||||||||||||||||||||||||||||||||  ||||  |||||
Sbjct  1321  ACAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCTTGA  1363
```

Figure 6. Nucleotide sequence of CSN3-Alanine-421, 423 blasted against the human CSN3 in the NCBI database. Query represents the CSN3-Alanine-421,423 (clone J) and Sbjct shows the human CSN3 (NM-003653) sequence in the NCBI database. This sequence was generated by the CSN3-653 primer. The mutations located at nucleotide positions 1352, 1353 and 1358 (in red) are confirmed to be the expected mutations. The six nucleotides highlighted in yellow (Query line) code for Alanine while the nucleotides highlighted in green (Sbjct line) code for Serine. These results indicate a successful mutation from Serine to Alanine at position 421,423 in the CSN3 gene.

III. Mutation from Serine 410,421,423 to Alanine at positions 410,421,423.

1. DNA gel of CSN3-Alanine 410,421,423 pShuttle-IRES-hrGFP-1 clones

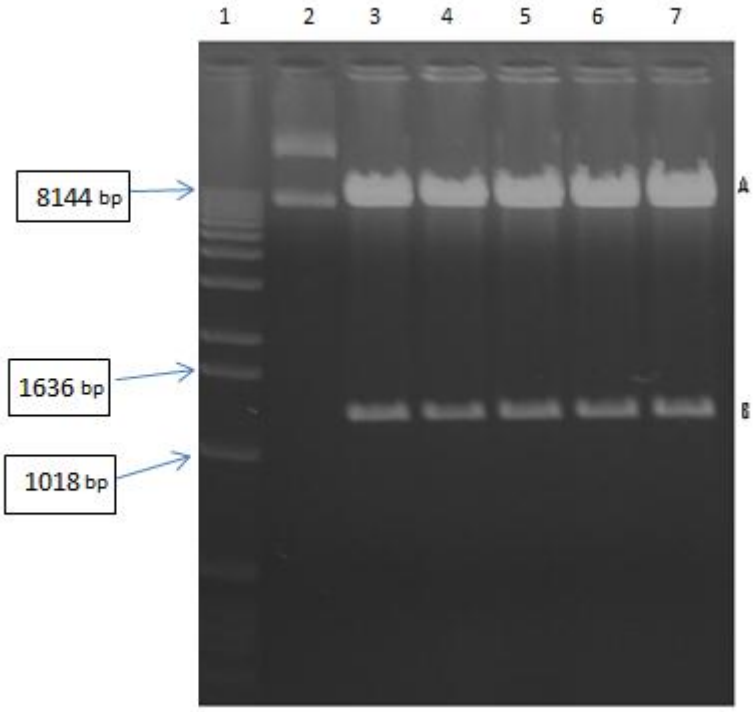

Figure 7. Gel image of CSN3 Serine 410,421,423 to Alanine 410,421,423. 500ng to 1ug DNA was run on a 0.8% agarose gel at 110 volts for 80 minutes. (A) represents the location of the pShuttle-IRES-hrGFP-1 vector at 8.9 kb. (B) represents the CSN3 gene insert (1.2kb). The DNA ladder (1kb), CSN3 Alanine 410, 421,423 clone C (uncut), CSN3 Alanine 410, 421,423 clone C (cut), CSN3 Alanine 410, 421,423 clone D (cut), CSN3 Alanine 410, 421,423 clone E (cut), CSN3 Alanine 410, 421,423 clone F (cut), and CSN3 Alanine 410, 421,423 clone G (cut) are represented in lanes 1, 2, 3, 4, 5, 6, 7 respectively. Constructs containing both fragments at A and B were sent for sequencing at the Nevada Genomics Center.

2. Sequencing results of CSN3- Alanine 410, 421,423 pShuttle-IRES-hrGFP-1, clone G.

ref|NM_003653.2| Homo sapiens COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) (COPS3), mRNA
GENE ID: 8533 COPS3 | COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) [Homo sapiens] (Over 10 PubMed links)
Length=1652

Score = 1020 bits (643), Expect = 0.0

```
Identities = 647/651 (99%), Gaps = 0/651 (0%)
 Strand=Plus/Plus

Query  1     GCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  713   GCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTAT  772

Query  61    ATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  773   ATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAA  832

Query  121   ATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  833   ATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTAT  892

Query  181   TCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  893   TCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACT  952

Query  241   CGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  953   CGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATT  1012

Query  301   CAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1013  CAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAG  1072

Query  361   TTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1073  TTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATT  1132

Query  421   TTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1133  TTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATAT  1192

Query  481   AATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1193  AATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGAT  1252

Query  541   GAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1253  GAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGT  1312

Query  601   ATGGGCGCACAAGAAGATGATTCAGGAAACAAACCATCCGCTTATGCTTGA  651
             ||||||  ||||||||||||||||||||||||||||||||  ||||  |||||
Sbjct  1313  ATGGGCTCACAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCTTGA  1363
```

Figure 8. Nucleotide sequence of CSN3-Alanine-410, 421, 423 blasted against the human CSN3 in the NCBI database. Query represents the CSN3-Alanine-410, 421,423 (clone G) and Sbjct shows the human CSN3 (NM-003653) sequence in the NCBI database. This sequence was generated by the CSN3-653 primer. The mutations located at nucleotide positions 1318, 1352, 1353 and 1358 (in red) are confirmed to be the expected mutations. The nine nucleotides highlighted in yellow (Query line) code for Alanine while the nucleotides highlighted in green (Sbjct line) code for Serine. These results indicate a successful mutation from Serine to Alanine at position 421,423 in the CSN3 gene.

IV. Mutation from Serine 410 to Aspartic Acid at position 410.

1. DNA gel of CSN3-Aspartic Acid 410 pShuttle-IRES-hrGFP-1 clones

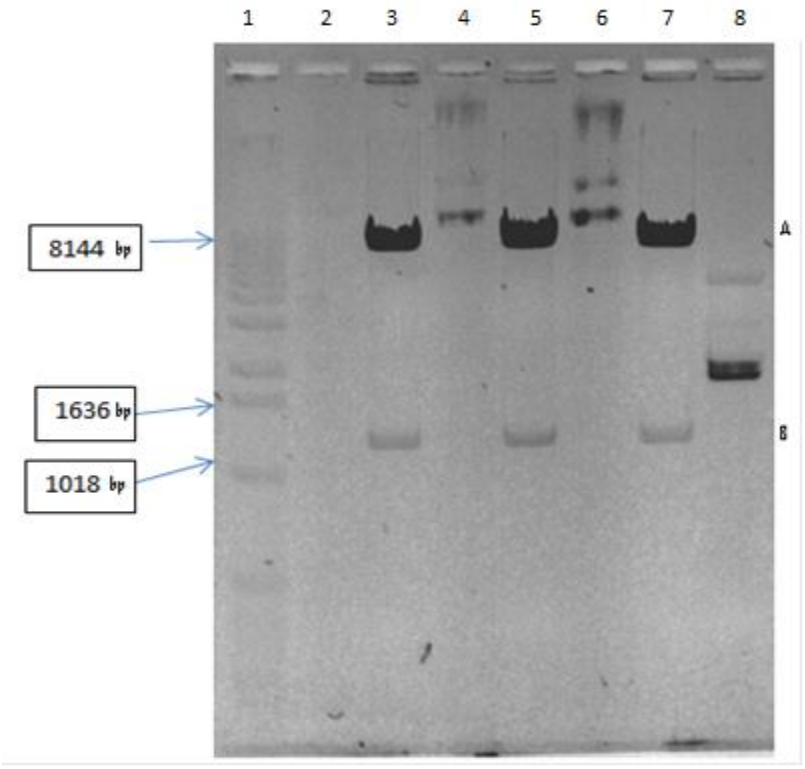

Figure 9. Gel image of CSN3 Serine 410 to Aspartic Acid 410. 500ng to 1ug DNA was run on a 0.8% agarose gel at 110 volts for 80 minutes. (A) represents the location of the pShuttle-IRES-hrGFP-1 vector at 8.9 kb. (B) represents the CSN3 gene insert (1.2kb). The DNA ladder (1kb), CSN3 Aspartic Acid 410 clone A (uncut), CSN3 Aspartic Acid 410 clone A (cut), CSN3 Aspartic Acid 410 clone B (uncut), CSN3 Aspartic Acid 410 clone B (cut), CSN3 Aspartic Acid 410 clone C (uncut), and CSN3 Aspartic Acid 410 clone C (cut), and CSN3 Aspartic Acid clone E (cut) are represented in lanes 1, 2, 3, 4, 5, 6, 7, and 8 respectively. Constructs containing both fragments at A and B were sent for sequencing at the Nevada Genomics Center.

2. Sequencing results of CSN3- Aspartic Acid 410 pShuttle-IRES-hrGFP-1, clone A.

ref|NM_003653.2| Homo sapiens COP9 constitutive photomorphogenic homolog subunit
GENE ID: 8533 COPS3 | COP9 constitutive photomorphogenic homolog subunit 3
(Arabidopsis) [Homo sapiens] (Over 10 PubMed links)
3 (Arabidopsis) (COPS3), mRNA
Length=1652

```
Score =  758 bits (654),  Expect = 0.0
 Identities = 659/663 (99%), Gaps = 1/663 (0%)
 Strand=Plus/Plus

Query  1     TTTTATG-ACAGGCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCA  59
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  701   TTTTATGAACAGGCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCA  760

Query  60    TATAAAAAGTATATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAA  119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  761   TATAAAAAGTATATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAA  820

Query  120   TATACATCTCAAATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTA  179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  821   TATACATCTCAAATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTA  880

Query  180   GCACAAGTGTATTCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGT  239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  881   GCACAAGTGTATTCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGT  940

Query  240   GAAACCTTCACTCGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTAT  299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  941   GAAACCTTCACTCGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTAT  1000

Query  300   AAGAAGAATATTCAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCA  359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1001  AAGAAGAATATTCAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCA  1060

Query  360   AGTCGTGTGCAGTTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAA  419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1061  AGTCGTGTGCAGTTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAA  1120

Query  420   GATGGTGAGATTTTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAAC  479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1121  GATGGTGAGATTTTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAAC  1180

Query  480   CCTGAAAAATATAATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGC  539
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1181  CCTGAAAAATATAATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGC  1240

Query  540   ATTGAGCTGGATGAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTT  599
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1241  ATTGAGCTGGATGAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTT  1300

Query  600   GTACAAAAGAGTATGGGCGACCAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCT  659
             |||||||||||||||||  ||||||||||||||||||||||||||||||||||||||||||
Sbjct  1301  GTACAAAAGAGTATGGGCTCACAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCT  1360

Query  660   TGA  662
             |||
Sbjct  1361  TGA  1363
```

Figure 10. Nucleotide sequence of CSN3-Aspartic Acid-410 blasted against the human CSN3 in the NCBI database. Query represents the CSN3-Aspartic Acid-410 (clone A) and Sbjct shows the human CSN3 (NM-003653) sequence in the NCBI database. This sequence was generated by the CSN3-653 primer. The mutations located at nucleotide positions 1318, 1319, and 1320 (in red) are confirmed to be the expected mutations. The three nucleotides highlighted in yellow (Query line) code for Aspartic Acid while the nucleotides highlighted in green (Sbjct line) code for Serine. These results indicate a successful mutation from Serine to Aspartic Acid at position 410 in the CSN3 gene.

V. Mutation from Serine 421, 423 to Aspartic Acid at positions 421,423.

1. DNA gel of CSN3-Aspartic Acid 421,423 pShuttle-IRES-hrGFP-1 clones

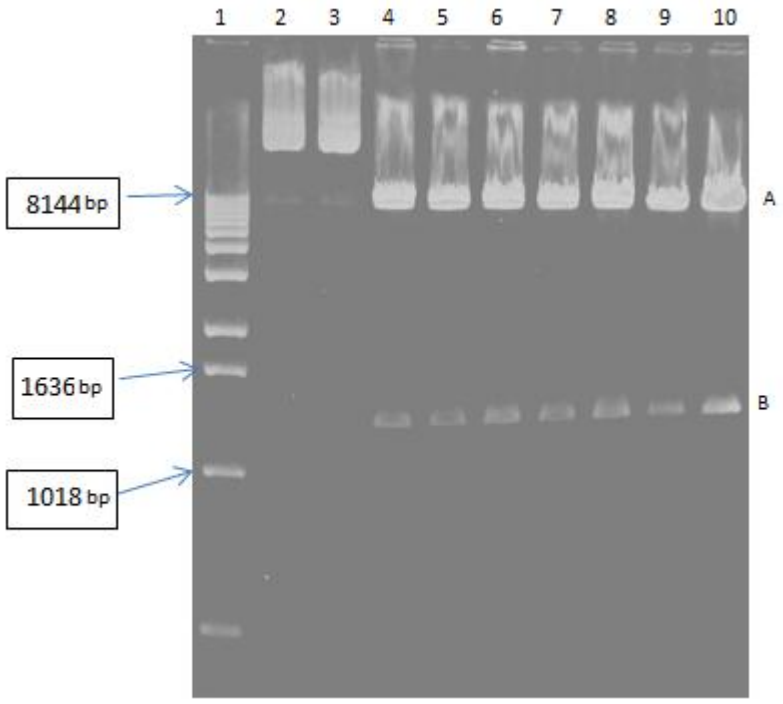

Figure 11. Gel image of CSN3 Serine 421,423 to Aspartic Acid 421,423. 500ng to 1ug DNA was run on a 0.8% agarose gel at 110 volts for 80 minutes. (A) represents the location of the pShuttle-IRES-hrGFP-1 vector at 8.9 kb. (B) represents the CSN3 gene insert (1.2kb). The DNA ladder (1kb), CSN3 Aspartic Acid 421, 423 clone I (uncut), CSN3 Aspartic Acid 421, 423 clone K (uncut), CSN3 Aspartic Acid 421,423 clone G (cut), CSN3 Aspartic Acid 421, 423 clone I (cut), CSN3 Aspartic Acid 421,423 clone J (cut), CSN3 Aspartic Acid 421,423 clone K (cut), CSN3 Aspartic Acid 421,423 clone M (cut), CSN3 Aspartic Acid 421,423 clone O (cut), and CSN3 Aspartic Acid 421,423 clone P (cut) are represented in lanes 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 respectively. Constructs containing both fragments at A and B were sent for sequencing at the Nevada Genomics Center.

2. Sequencing results of CSN3- Aspartic Acid 421,423 pShuttle-IRES-hrGFP-1, clone J.

ref|NM_003653.2| Homo sapiens COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) (COPS3), mRNA
Length=1652
GENE ID: 8533 COPS3 | COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) [Homo sapiens] (Over 10 PubMed links)

```
 Score = 1020 bits (643),  Expect = 0.0
 Identities = 647/651 (99%), Gaps = 0/651 (0%)
 Strand=Plus/Plus

Query  1     GCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  713   GCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTAT  772

Query  61    ATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  773   ATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAA  832

Query  121   ATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  833   ATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTAT  892

Query  181   TCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  893   TCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACT  952

Query  241   CGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  953   CGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATT  1012

Query  301   CAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1013  CAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAG  1072

Query  361   TTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1073  TTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATT  1132

Query  421   TTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1133  TTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATAT  1192

Query  481   AATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1193  AATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGAT  1252

Query  541   GAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1253  GAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGT  1312

Query  601   ATGGGCTCACAAGAAGATGATTCAGGAAACAAACCATCCGATTATGATTGA  651
             |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1313  ATGGGCTCACAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCTTGA  1363
```

Figure 12. Nucleotide sequence of CSN3-Aspartic Acid-421,423 blasted against the human CSN3 in the NCBI database. Query represents the CSN3-Aspartic Acid-421,423 (clone J) and Sbjct shows the human CSN3 (NM-003653) sequence in the NCBI database. This sequence was generated by the CSN3-653 primer. The mutations located at nucleotide positions 1352, 1353, 1358, and 1359 (in red) are confirmed to be the expected mutations. The three nucleotides highlighted in yellow (Query line) code for Aspartic Acid while the nucleotides highlighted in green (Sbjct line) code for Serine. These results indicate a successful mutation from Serine to Aspartic Acid at position 421,423 in the CSN3 gene.

VI. Mutation from Serine 410,421,423 to Aspartic Acid at positions 410, 421,423.

1. DNA gel of CSN3-Aspartic Acid 410, 421,423 pShuttle-IRES-hrGFP-1 clones

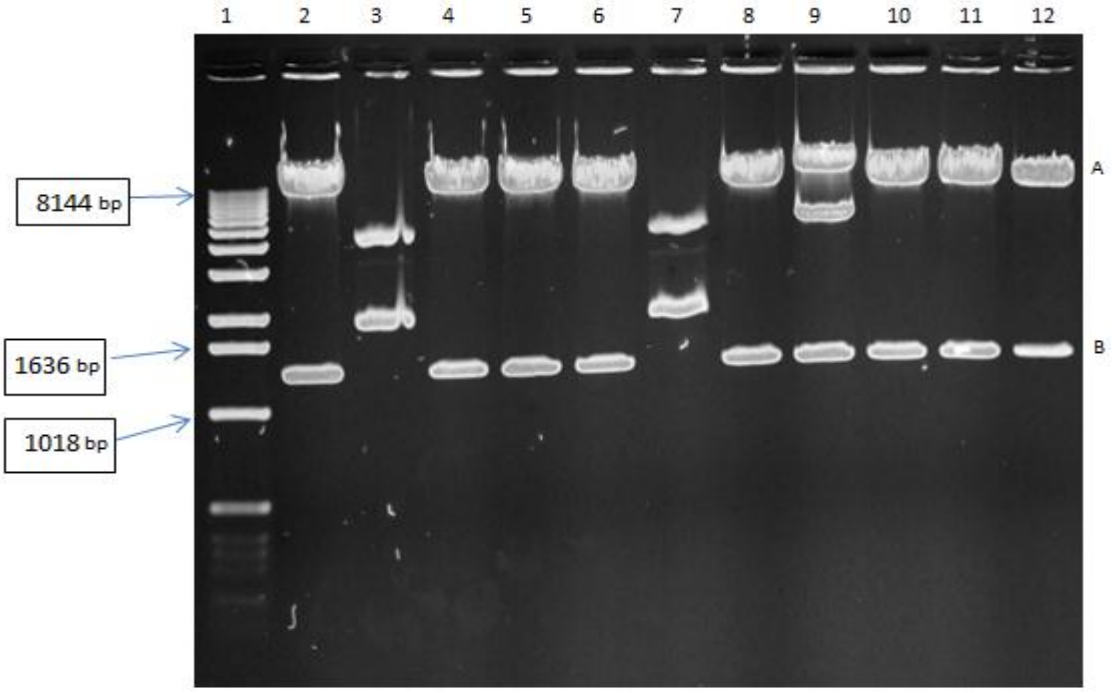

Figure 13. Gel image of CSN3 Serine 410,421,423 to Aspartic Acid 410, 421,423. 500ng to 1ug DNA was run on a 0.8% agarose gel at 110 volts for 80 minutes. (A) represents the location of the pShuttle-IRES-hrGFP-1 vector at 8.9 kb. (B) represents the CSN3 gene insert (1.2kb). The DNA ladder (1kb), CSN3 Aspartic Acid 410, 421, 423 clone A (cut), CSN3 Aspartic Acid 410, 421, 423 clone B (cut), CSN3 Aspartic Acid 410, 421, 423 clone C (cut), CSN3 Aspartic Acid 410, 421, 423 clone D (cut), CSN3 Aspartic Acid 410, 421, 423 clone E (cut), CSN3 Aspartic Acid 410, 421, 423 clone F (cut), CSN3 Aspartic Acid 410, 421, 423 clone G (cut), CSN3 Aspartic Acid 410, 421, 423 clone H (cut), CSN3 Aspartic Acid 410, 421, 423 clone I (cut), CSN3 Aspartic Acid 410, 421, 423 clone J (cut), and CSN3 Aspartic Acid 410, 421, 423 clone N (cut) are represented in lanes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12, respectively. Constructs containing both fragments at A and B were sent for sequencing at the Nevada Genomics Center.

2. Sequencing results of CSN3- Aspartic Acid 410, 421,423 pShuttle-IRES-hrGFP-1, clone A.

ref|NM_003653.2| Homo sapiens COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) (COPS3), mRNA
Length=1652

GENE ID: 8533 COPS3 | COP9 constitutive photomorphogenic homolog subunit 3 (Arabidopsis) [Homo sapiens] (Over 10 PubMed links)
Score = 1011 bits (637), Expect = 0.0
Identities = 644/651 (99%), Gaps = 0/651 (0%)
Strand=Plus/Plus

```
Query  1     GCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTAT  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  713   GCTATAACTACTCCTGCCATGGCGGTCAGTCATATCATGTTGGAATCATATAAAAAGTAT  772

Query  61    ATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  773   ATTTTAGTGTCTTTGATATTACTTGGCAAAGTACAACAGCTACCAAAATATACATCTCAA  832

Query  121   ATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTAT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  833   ATTGTGGGTAGATTCATTAAGCCTCTTAGCAATGCATACCACGAGTTAGCACAAGTGTAT  892

Query  181   TCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACT  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  893   TCAACCAACAACCCCTCAGAACTCCGAAACCTGGTGAATAAGCACAGTGAAACCTTCACT  952

Query  241   CGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  953   CGCGATAACAACATGGGGCTGGTGAAGCAATGCTTGTCATCTCTTTATAAGAAGAATATT  1012

Query  301   CAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAG  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1013  CAGAGGCTAACAAAGACCTTTTTAACTCTATCATTACAAGATATGGCAAGTCGTGTGCAG  1072

Query  361   TTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1073  TTGTCTGGACCTCAGGAGGCAGAGAAATACGTTCTGCACATGATAGAAGATGGTGAGATT  1132

Query  421   TTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATAT  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1133  TTTGCAAGTATTAACCAGAAGGACGGTATGGTCAGTTTCCATGATAACCCTGAAAAATAT  1192

Query  481   AATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGAT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1193  AATAACCCAGCCATGCTTCATAACATTGATCAGGAGATGCTGAAGTGCATTGAGCTGGAT  1252

Query  541   GAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGT  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1253  GAGCGGCTGAAAGCCATGGACCAGGAGATCACAGTGAACCCTCAGTTTGTACAAAAGAGT  1312

Query  601   ATGGGCGACCAAGAAGATGATTCAGGAAACAAACCATCCGATTATGATTGA  651
             ||||||   ||||||||||||||||||||||||||||||   ||||  ||||
Sbjct  1313  ATGGGCTCACAAGAAGATGATTCAGGAAACAAACCATCCAGTTATTCTTGA  1363
```

Figure 14. Nucleotide sequence of CSN3-Aspartic Acid-410, 421,423 blasted against the human CSN3 in the NCBI database. Query represents the CSN3-Aspartic Acid-410,421,423 (clone A) and Sbjct shows the human CSN3 (NM-003653) sequence in the NCBI database. This sequence was generated by the CSN3-653 primer. The mutations located at nucleotide positions 1318, 1319, 1320, 1352, 1353, 1358, and 1359 (in red) are confirmed to be the expected mutations. The nine nucleotides highlighted in yellow (Query line) code for Aspartic Acid while the nucleotides highlighted in green (Sbjct line) code for Serine. These results indicate a successful mutation from Serine to Aspartic Acid at positions 410,421, and 423 in the CSN3 gene.

VII. Compilation of CSN3 Mutagenic Constructs

1. DNA gel of CSN3 Final Constructs for Alanine and Aspartic Acid 410, 421,423 and 410,421,423

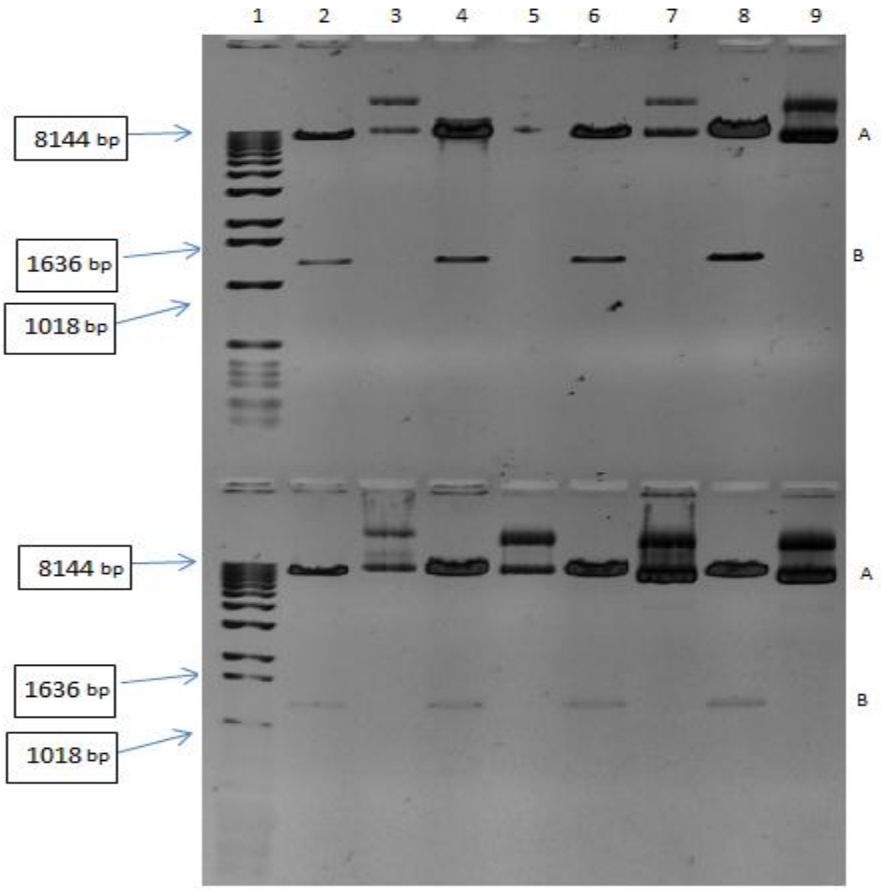

Figure 15. Gel image of CSN3 Maxiprep Constructs. 500ng of DNA was run on a 0.8% agarose gel at 110 volts for 80 minutes. (A) represents the location of the pShuttle-IRES-hrGFP-1 vector at 8.9 kb. (B) represents the CSN3 gene insert (1.2kb). In row one, the DNA ladder (1kb), CSN3 Template (cut), CSN3 Template (uncut), CSN3 Alanine 410 (cut), CSN3 Alanine 410 (uncut), CSN3 Alanine 421, 423 (cut), CSN3 Alanine 421,423 (uncut), CSN3 Alanine 410, 421,423 (cut), and CSN3 Alanine 410,421,423 (uncut) are represented in lanes 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively. In row two, the DNA ladder (1kb), CSN3 Template (cut), CSN3 Template (uncut), CSN3 Aspartic Acid 410 (cut), CSN3 Aspartic Acid 410 (uncut), CSN3 Aspartic Acid 421, 423 (cut), CSN3 Aspartic Acid 421,423 (uncut), CSN3 Aspartic Acid 410, 421,423 (cut), and CSN3 Aspartic Acid 410,421,423 (uncut) are represented in lanes 1, 2, 3, 4, 5, 6, 7, 8 and 9 respectively.

2. Multiple Sequence Alignment for CSN3 Constructs

Figure 16. CLUSTAL 2.1 multiple sequence alignment of CSN3 constructs. The sequence shown here is from nucleotides 1316 to 1363 (15 amino-acids) of the human CSN3 sequence (NM-003653). The stars indicate sequence identity and the gaps represent mismatches at amino acid residues 410, 421 and 423.

Gel Analysis

The restriction digest of each construct with Sal I and Sca I should result in the appearance of two bands, one the size of 8.9kb (pShuttle-IRES-hrGFP 1) and the other with the size of 1.27kb (CSN3). CSN3 was inserted into the multiple cloning site of pShuttle-IRES-hrGFP 1 prior to this experiment between recognized cut sites for Sal I and Sca I (Figure 2). These two bands are clearly visible in all gels and at the correct locations. Only the constructs that had these two bands present were sequenced.

DNA Sequencing Analysis

All DNA Sequencing was performed at the Nevada Genomics Center. The trimmed sequence of each result was blasted against the human CSN3 in the NCBI database (NM-003653). Expected mutations are indicated by a mismatch between the query and the subject sequences. These mutations resulted in a change from serine to Alanine or to Aspartic acid at position 410(fig.4 and fig. 10), 421-423 (fig. 6 and fig. 12) or 410-421-423 (fig. 8 and fig 14). Furthermore, although two primers (forward and CSN3 653)

were used, only the CSN3-653 primer results are shown. All good constructs did not contain any mutations or abnormalities in their forward sequences.

Discussion:

The goal of this experiment was to generate mutagenic constructs of *Homo sapien* CSN3 containing the following mutations: Serine 410, Serine 421,423 and Serine 410,421,423 to either Aspartic Acid or Alanine. These three serine sites in CSN3 are known to be phosphorylated but the effect of phosphorylation is currently unknown. As hypothesized, these sites may be crucial to the activation and disassociation of CSN3 from ß1D Integrin, and thus the focus of this project.

The first step of this project was to use mutagenic primers containing the desired mutations in a Polymerase Chain Reaction. The first attempt at doing PCR resulted in low efficiency as determined by the small number of bacterial colonies on the agar plate. The QuickChange protocol was then changed to increasing the amount of template DNA and the primers in the reaction as stated in the methods. This resulted in a nearly tenfold increase of colonies for the majority of the constructs.

As shown in the gel and sequencing results, the transformation of the mutagenic PCR products was a success. Each construct was verified to contain the correct mutations and the pShuttle-IRES-hrGFP-1 vector. Good constructs were then stored in a glycerol solution(1:1) for future use. The next step of this project will include a yeast-two hybrid technique in order to test whether the CSN3 mutants interact with ß1D Integrin . The interaction between CSN3 mutants and b1D integrin will be confirmed in mammalian cells using adenoviruses.

Heart disease is a leading killer of adults in the western world. As integrins have already been implicated in having a significant role in cellular adaptation and communication, it is critical to understand the mechanisms and pathways associated. Cardiac hypertrophy is certainly not the only form of heart disease, but it is definitely an important precursor and indicator of the severe complications to come. Discovering the mechanisms by which integrins mediate cardiac hypertrophy is of great interest to Dr. Valencik's lab, the millions of Americans affected with the disease today and the world.

References:

Baudoin C, Van der Flier A, Borradori L, Sonnenberg A, Genomic organization of the mouse beta 1 gene: Conservation of the beta 1D but not of the beta 1B and beta 1C integrin splice variants. Cell Adhes Commun, 1996. 4: 1-11.

Bech-Otschir, D., M. Seeger, and W. Dubiel, The COP9 signalosome: at the interface between signal transduction and ubiquitin-dependent proteolysis. J Cell Sci, 2002. **115**(Pt 3): p 467-73.

Belkin, A.M., et al., Muscle beta1D integrin reinforces the cytoskeleton-matrix link: modulation of integrin adhesive function by alternative splicing. J Cell Biol, 1997. **139**(6): p. 1583-95.

Deng, X.W., et al., Unified nomenclature for the COP9 signalosome and its subunits: an essential regulator of development. Trends Genet, 2000. **16**(7): p. 289.

Diwan, A. and G.W. Dorn, 2nd, Decompensation of cardiac hypertrophy: cellular mechanisms and novel therapeutic targets. Physiology (Bethesda), 2007. **22**: p. 56-64.

Hunter, C., J. Evans, and M.L. Valencik, Subunit 3 of the COP9 signalosome is poised to facilitate communication between the extracellular matrix and the nucleus through the muscle-specific beta1D integrin. Cell Commun Adhes, 2008. **15**(3): p. 247-60.

Katz, A.M., The cardiomyopathy of overload: an unnatural growth response in the hypertrophied heart. Ann Intern Med, 1994. **121**(5): p. 363-71.

Knoll, R., M. Hoshijima, and K. Chien, Cardiac mechanotransduction and implications for heart disease. J Mol Med, 2003. **81**(12): p. 750-6.

Sun, M., et al., Temporal response and localization of integrins beta1 and beta3 in the heart after myocardial infarction: regulation by cytokines. Circulation, 2003. **107**(7): p. 1046-52.

Wei, N. and X.W. Deng, COP9: a new genetic locus involved in light-regulated development and gene expression in arabidopsis. Plant Cell, 1992. **4**(12): p. 1507-18.

Supplemental data

Table 1. NanoDrop DNA Concentration and Purity Analysis. 2 ul of sample was analyzed by a Nanodrop Spectrophotometer.

DNA Concentration and Purity Analysis

Date	Sample	ng/µl	A260	A280	260/280	260/230
9/17/2010						
	Ala 410b	54.26	1.085	0.543	2	2.16
	Ala 410c	26.12	0.522	0.269	1.94	2.15
10/4/2010						
	Ala 410 A1	250.92	5.018	2.601	1.93	2.33
	Ala 410 A2	310.14	6.203	3.223	1.92	2.33
	Ala 410 A3	101.5	2.03	1.036	1.96	2.03
	Ala 410 A4	105.32	2.106	1.092	1.93	2.41
	Ala 410 A5	222.05	4.441	2.282	1.95	2.37
	Ala 410 B1	242.41	4.848	2.511	1.93	2.36
	Ala 410 B2	68.39	1.368	0.659	2.08	2.62
	Ala 410 B3	232.47	4.649	2.419	1.92	2.37
	Ala 410 B4	77.94	1.559	0.805	1.94	2.42
	Ala 410 B5	279.26	5.585	2.89	1.93	2.37
11/10/2010						
	Ala 410 664C1	95.85	1.917	1.024	1.87	2.15
	Ala 410 664C2	75.28	1.506	0.794	1.9	2.18
	CSN3 Temp 664C1	91.96	1.839	0.982	1.87	2.21
	CSN3 Temp 664C2	113.23	2.265	1.211	1.87	2.22
	CSN3 Temp 664C3	93.86	1.877	1.007	1.86	2.14
	CSN3 Temp 664C4	98.92	1.978	1.069	1.85	2.22
	CSN3 Temp 664C5	78.16	1.563	0.834	1.87	2.22
11/30/2010						
	CSN3-664C	600.42	12.008	6.657	1.8	2.22
	Ala 410 664C	737.12	14.742	8.01	1.84	2.25
Date	**Sample**	**ng/µl**	**A260**	**A280**	**260/280**	**260/230**
12/16/2010						

	Asp 410 A	124.55	2.491	1.322	1.88	2.23
	Asp 410 B	154.69	3.094	1.635	1.89	2.21
	Asp 410 C	187.75	3.755	1.997	1.88	2.26
	Asp 410 D	56.69	1.134	0.589	1.92	2.08
	Asp 410 E	72.29	1.446	0.766	1.89	2.2
1/20/2011						
	Asp 410 maxi	79.42	1.588	0.919	1.73	2.39
	Ala 421,423 A	61.52	1.23	0.682	1.8	2.15
	Ala 421,423 B	60.16	1.203	0.685	1.76	2.07
	Ala 421,423 C	46.49	0.93	0.52	1.79	2.19
	Ala 410, 421,423 A	71.44	1.429	0.794	1.8	2.17
	Ala 410, 421,423 B	30.62	0.612	0.377	1.62	2.1
	Ala 410, 421,423 C	57.81	1.156	0.633	1.83	2.11
1/24/2011						
	Ala 421,423 A	165.67	3.313	1.731	1.91	2.3
	Ala 421,423 D	65.7	1.314	0.677	1.94	2.24
	Ala 421,423 E	192.88	3.858	2.018	1.91	2.28
	Ala 421,423 F	137.03	2.741	1.427	1.92	2.3
	Ala 421,423 G	197.1	3.942	2.053	1.92	2.31
	Ala 410, 421,423 C	155.77	3.115	1.622	1.92	2.29
	Ala 410, 421,423 D	131.13	2.623	1.366	1.92	2.29
	Ala 410, 421,423 E	155.45	3.109	1.623	1.92	2.26
	Ala 410, 421,423 F	180.18	3.604	1.881	1.92	2.27
	Ala 410, 421,423 G	209.3	4.186	2.192	1.91	2.3
02/01/11						
	Ala 410,421,423 H	43.18	0.863	0.44	1.94	2.1
	Ala 410,421,423 J	11.73	0.235	0.136	1.72	1.83
	Ala 421,423 H	157.26	3.145	1.67	1.88	2.19
	Ala 421,423 I	158.76	3.18	1.67	1.9	2.26
	Ala 421,423 J	178.36	3.57	1.9	1.88	2.08
	Ala 421,423 K	45.1	0.902	0.474	1.9	2.16
	Ala 421,423 L	166.06	3.321	1.778	1.87	2.2
02/07/11						
	CSN3 temp purified maxi	126.9	2.54	1.37	1.86	2.29
	Asp 410C maxi	575.14	11.5	6.16	1.87	2.29

2/10/2011						
	Asp 421,423 A	73.66	1.47	0.79	1.87	2.27
	Asp 421,423 B	24.95	0.5	0.269	1.86	2.33
	Asp 421,423 C	49.38	0.99	0.53	1.88	2.35
	Asp 421,423 D	53.54	1.07	0.56	1.92	2.31
	Asp 421,423 E	37.67	0.753	0.398	1.89	2.28
	Asp 421,423 F	53.12	1.06	0.56	1.89	2.38
2/14/2011						
	Asp 421,423 B	140.7	2.81	1.52	1.85	2.08
	Asp 421,423 C	225.4	4.51	2.42	1.86	2.22
	Asp 421,423 D	146.78	2.94	1.57	1.87	2.15
	Asp 421,423 E	127.04	2.54	1.37	1.86	2.12
2/25/2011						
	Asp 421,423 G	41.01	0.82	0.093	8.77	2.84
	Asp 421,423 H	-30.38	-0.61	-0.62	0.98	2.11
	Asp 421,423 I	129.99	2.6	0.996	2.61	2.65
	Asp 421,423 J	76.08	1.52	0.52	2.96	2.54
	Asp 421,423 K	113.06	2.26	0.84	2.7	2.35
	Asp 421,423 L	-10.42	-0.21	-0.31	0.68	1.58
	Asp 421,423 M	56.92	1.14	0.24	4.79	2.39
	Asp 421,423 N	11.98	0.24	-0.22	-1.07	4.36
	Asp 421,423 O	162.42	3.25	1.33	2.45	2.4
	Asp 421,423 P	99.49	1.99	0.68	2.92	2.4
3/2/2011						
	Asp 421, 423 G	129.86	2.6	1.39	1.86	2.16
	Asp 421,423 H	59.77	1.2	0.64	1.86	1.77
	Asp 421,423 I	129.22	2.58	1.38	1.87	2.2
	Asp 421,423 J	141.47	2.83	1.5	1.88	2.13
	Asp 421,423 L	34.41	0.69	0.37	1.88	1.88
	Asp 421,423 M	98.37	1.97	1.06	1.86	1.89
	Asp 421,423 N	78.85	1.58	0.84	1.88	2.03
	Asp 421,423 P	115.69	2.31	1.24	1.87	2.12
3/8/2011						
	Ala 421,423 maxiA	68.61	1.37	0.76	1.8	2.24
	Ala 421,423 maxiB	273.42	5.47	2.94	1.86	2.12
3/11/2011						

	Asp 410,421,423 A	201.98	4.04	2.14	1.88	2.27
	Asp 410,421,423 C	152.45	3.05	1.6	1.9	2.3
	Asp 410,421,423 D	188.81	3.776	1.99	1.89	2.32
	Asp 410,421,423 E	166.09	3.322	1.77	1.88	2.28
	Asp 410,421,423 G	157.48	3.15	1.68	1.87	2.24
	Asp 410,421,423 I	148.85	2.98	1.59	1.88	2.28
	Asp 410,421,423 J	152.33	3.05	1.62	1.89	2.29
3/23/2011						
	Ala 421,423 maxiA	63.19	1.26	0.69	1.81	2.31
	Ala 421,423 maxiB	317.62	6.35	3.43	1.85	2.27
	Ala 410,421,423maxiA	254.52	5.09	2.86	1.78	2.28
	Ala 410,421,423maxiB	423.45	8.47	4.62	1.83	2.22
3/28/2011						
	Asp 410,421,423 maxi	473.17	9.46	5.11	1.85	2.28
	Asp 421,423 maxi	75.42	1.5	0.86	1.76	2.6

List of Reagents and Equipment:

GenePure LE Agarose 500g, E-3120-500, ISC BIO EXPRESS

Luria Broth Base, Miller, L1900-IKG, SIGMA, Batch #: 108K0128

Kanamycin, Monosulfate, Fisher Biotech, BP906-5

Maxi Prep: Endo Free Plasmid Maxi Kit, Catalog #: 12362

Mini Prep: QIAprep Spin Miniprep Kit, Catalog #: 27104

Table top centrifuge: Sorvall Biofuge Pico, Beckman Coulter, Avanti J25 Centrifuge

PCR Machine: Gradient Cycler, Gradient Engine, MJ Research PTC-200 Peltier Thermal Cycler

42ºC dry heater: Fisher Scientific, Dry Bath Incubator

37ºC plate incubator: Fisher Scientific, Isotemp Incubator

Nano drop: NanoDrop, ND-1000 Spectrophotometer

Media and plate preparation:

LB media: Add 15.5 g/L LB, add desired deionized water volume, autoclave at 121°C for 1 hour

NZY+ Broth: Instructions on how to create this are located in the mutagenesis kit

Antibiotic LB Agar plates: Instructions on how to create this are located in the mutagenesis kit. The desired concentration of kanamycin was created then filter sterilized before use.

Abbreviations

CSN: COP9 signalosome

CSN3: Third subunit of CSN

PCR: Polymerase chain reaction

Ala: Alanine

Asp: Aspartic Acid