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Expressing GFP-Coilin from its Endogenous Promoter in *Drosophila melanogaster*

by

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Undergraduate honors thesis under the direction of

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Abstract

The protein coilin is required for Cajal Body (CB) formation in eukaryotic nuclei. CBs coordinate the assembly and modification of small RNA-protein complexes used throughout the nucleus and nucleolus, where ribosome biogenesis occurs. In the fruit fly, *Drosophila melanogaster*, deletion of Nopp140, a ribosome assembly factor, causes nucleolar stress leading to p53-independent apoptosis or autophagy, depending on the cell type. Nopp140 is the closest *D. melanogaster* homologue to the human Treacle protein, another nucleolar ribosome assembly factor which when mutated, causes the Treacher Collins syndrome, a ribosomopathy leading to craniofacial birth defects.

By comparing coilin protein levels in wild type, Nopp140 knockout, and coilin null mutation larvae, we show upregulation of coilin expression in Nopp140 knockout flies, suggesting an unexplored regulatory crosstalk mechanism between the nucleolus and CB. To determine the gene expression stage of coilin upregulation, a recombinant DNA plasmid including the endogenous *coilin* gene promoter, its 5' untranslated region (UTR), the cDNA encoding the coilin protein, and the DNA encoding the green fluorescent protein (GFP) was constructed using sticky end ligation and Gibson Assembly, but then edited by Q5 site-directed mutagenesis. After confirming the final assembly by DNA sequencing, the plasmid was injected into *Drosophila* embryos. After establishing transgenic fly lines, research will examine GFP-coilin upregulation in Nopp140 knockout flies and the transgene's interaction in genetic crosses with the coilin null mutation in combination with the Nopp140 deletion. Further elucidation of coilin expression as influenced by loss of Nopp140 function should aid our understanding of Treacher Collins syndrome and other ribosomopathies.

Introduction

The nucleolus serves as the site of many cellular processes including ribosome biogenesis. Ribosomal transcription, processing, assembly, and maturation are all organized within the nucleolus. The rDNA presents in tandem repeats on the X and Y chromosomes in *Drosophila melanogaster*. RNA polymerase I (RNA Pol I) transcribes pre-rRNA transcripts which can then be processed and assembled into mature ribosomal subunits (Boulon et al., 2010).

During transcription of the initial 7,000 nucleotide pre-rRNA, processing machinery including the small subunit (SSU) processome, a U3 snoRNP particle, locates to the 5' end of the 90S pre-rRNA molecule. The SSU processome contains U3-binding proteins, the U3 snoRNA, and other proteins which introduce methylation and pseudouridylation modifications. The SSU processome also participates in 90S pre-rRNA cleavage at A_0 and A_1 sites into pre-60S and pre-40S subunits after which it dissociates from the pre-rRNA. The pre-40S subunit only associates with a few factors before leaving the nucleus and entering the cytoplasm. Here, Dim1p, a methyltransferase, dimethylates two adjacent adenines at the 20S pre-rRNA 3' end, and XRN1 exonuclease processing converts the 20S rRNA into the 18S rRNA of the mature 40S small ribosomal subunit. The pre-60S subunit is cleaved at the A_2 site after which it associates with other factors including RNA helicases which remove snoRNPs and proteins, GTPases which are involved in quality control checkpoints, AAA-ATPases which aid in correct folding, and export proteins. As it is transported out of the nucleus through a nuclear pore complex, many factors dissociate leaving the 5.8S and 28S rRNA combined with ribosomal proteins to form the 60S large ribosomal subunit (Tschochner & Hurt, 2003).

The rRNA also undergoes processing and modification by small nucleolar ribonucleoprotein particles (snoRNPs) under the guidance of the snoRNAs. The box H/ACA snoRNP and box C/D snoRNP classes chemically modify specific nucleotides by pseudouridylation and 2'-O-methylation, respectively. The snoRNP complexes contain proteins and enzymes which carry out these modifications. In yeast the box C/D snoRNPs associate with Nop1p, Nop5/58p, and Sik1/Nop56p, and the box H/ACA snoRNPs associate with Cbf5p, Gar1p, Nhp2p, and Nop10p. Nopp140, a nucleolar phosphoprotein 140 kDa in size, transiently interacts with both the box C/D snoRNPs and the box H/ACA snoRNPs acting as a chaperone in the association and transport of snoRNPs. Nopp140 moves between the nucleolus and the Cajal body suggesting its function in facilitating snoRNP transport between the two. Evidence also suggests that it participates in snoRNP biogenesis and function in rRNA processing (Yang et al., 2000).

The homeostasis of a cell relies on successful ribosome biogenesis, and when this is disrupted, diseased states called ribosomopathies result. Mutations of the ribosomal proteins that associate with the mature ribosome as well as mutations of nucleolar proteins that function in ribosome biogenesis induce a state of nucleolar stress. A cell enters a state of nucleolar stress when disruptions of ribosomal assembly, processing, and modification occur such as inhibition of RNA Pol I transcription, pre-rRNA processing, or export of ribosomal subunits (Narla & Ebert, 2010). The ribosomopathy, Treacher Collins Syndrome, results in congenital craniofacial birth defects due to a mutation in the human TCOF1 gene that codes for treacle. Due to treacle's role in ribosome biogenesis as a nucleolar phosphoprotein, its loss of function causes p53-mediated apoptosis and, therefore, the expression of this ribosomopathy (Sakai & Trainor, 2009) (He and DiMario 2011).

Nopp140 is a homologue of the human treacle protein, and its deletion models the nucleolar stress caused by treacle mutations in Treacher Collins Syndrome. Nopp140-True and Nopp140-RGG are the two isoforms encoded by the *Drosophila* Nopp140 gene. Nopp140-RGG contains a C-terminus group of Arg-Gly-Gly (RGG) motifs, but Nopp140-True is the closest homologue of human treacle (Waggener & DiMario, 2001). Nopp140 and treacle both function in chaperoning of box C/D snoRNP 2'-O-methylation of rRNA and snoRNP assembly. Due to their similar roles, comparable effects occur when either are knocked out; deletion of Nopp140 in flies causes nucleolar stress leading to p53-independent apoptosis or autophagy. However, treacle only localizes to the nucleolus, while Nopp140 localizes to both the nucleolus and the Cajal body (He and DiMario 2011).

The Cajal body (CB) is a non-membrane bound body in the nucleus first described by the neurobiologist Ramón y Cajal (Liu et al., 2009). Even though an abundance of rRNA biogenesis molecules have been found to associate with the CB, CBs have not been proven to contribute specifically to ribosome biogenesis. A class of snoRNAs called small CB-specific RNAs (scaRNAs) present in CB regulate the modification of snRNAs implicating CBs' function in the modification of snRNAs. CBs have also been shown to be involved in spliceosome assembly by interactions with the U4/U6 complex (Handwerger & Gall, 2006).

The marker protein for CBs was identified as coilin in 1991. Although it is not an essential protein for gamete viability, successful CB formation and maintenance depends on the presence of coilin (Liu et al., 2009). The C-terminus of coilin helps regulate the number of CBs present in the nucleus per cell by phosphorylation of serine residues in the C-terminus; the C-terminus also helps coilin localize to the CB (Shpargel et al., 2002).

Nopp140 knockout lines in *D. melanogaster* induce nucleolar stress due to the failure to successfully accomplish ribosome biogenesis (He and DiMario 2011), and preliminary data shows upregulation of coilin in these flies. The upregulation of coilin upon Nopp140 deletion was confirmed, and in order to determine the mode of upregulation, a construct tagging coilin with GFP expressed from its endogenous promoter was assembled. Fly lines made from this construct will be used to examine whether transcription or translation holds the most weight in regulation of coilin expression. Due to the unique relationship between coilin and Nopp140, study of coilin's interaction with Nopp140 will bring about a further understanding of this ribosome assembly factor.

Materials and Methods

Translational Expression Analysis

Fly Stocks: Fly stocks were maintained as described (James, 2015) and (He et al., 2014).

Protein Sample Preparation: *Nopp140* knockout, *Coilin 199* null, and WT (w^{1118}) larvae were collected and homogenized in a solution of Laemmli Sample Buffer, 0.1 M PMSF (in isopropanol), 5% Protease inhibitor cocktail (P-8340, Sigma-Aldrich), and 5% β-mercaptoethanol. The samples were boiled for 3 mins and then centrifuged for 5 min.

SDS-PAGE and Western Blot: An 8% SDS-PAGE gel was run with 1X Laemmli SDS-PAGE Running Buffer at 150-200 volts until sample dye ran off the gel. Using a nitrocellulose membrane, a Western blot was run with the gel using a semi-dry transfer buffer. The blot was then washed in Tris Buffered Saline containing 0.1% Tween 20 (TTBS pH 7.4) for 45 minutes.

The blot was blocked with a 3% NFDM (non-fat dry milk solution) containing a guinea pig anti-coilin GP3 primary antibody at a 1:10,000 dilution overnight (gift from Joe Gall), washed with TTBS pH 7.4 for 30 minutes, treated with an anti-guinea pig Horse Radish Peroxidase (HRP) secondary antibody (Cell Signaling) at a 1:5,000 dilution for 2 hours, and washed with TTBS pH 7.4 for 30 minutes. The blot was then treated with a 1:1 ratio of HRP Peroxide Buffer and Luminol Enhancer for 5 minutes and rinsed with TTBS pH 7.4 to visualize the blot with chemiluminescence. The blot was exposed to film and the film was developed using an automatic film processor.

Recombinant Plasmid Construction

HiFi DNA Assembly: A recombinant plasmid containing the endogenous *Drosophila coilin* gene promoter, *coilin*'s 5'UTR, *coilin* cDNA, and the DNA encoding GFP was assembled using NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs, Cat. No. E5520S). The plasmid pEGFP-N3 was digested with the restriction enzyme, SalI, to prepare it for the HiFi DNA assembly reaction. Then, PCR was used to amplify the *coilin* promoter and 5'UTR from fly genomic DNA as template with the forward P/5'UTR primer and the reverse P/5'UTR primer (Table 1). During the HiFi assembly, the *coilin* promoter and 5'UTR insert was in 2-fold molar excess to the pEGFP-N3 plasmid with the total amount of DNA not exceeding 0.2 pmols. This assembly was performed at 50°C in a thermocycler for 1 hour. The recombinant plasmid was then transformed into chemically competent XL1 Blue *E. coli* cells by heat shock at 42°C for 1 minute in LB media. LB agar plates containing 50 µg/mL kanamycin were used to select colonies containing the plasmid, while LB agar plates containing 100 µg/mL ampicillin were used for the positive control vector provided by the NEBuilder HiFi DNA Assembly Cloning

Kit. The presence of the *coilin* promoter and 5'UTR was confirmed by colony PCR and then a PCR of mini-prep DNA from the colonies.

The pEGFP-N3 plasmid containing the *coilin* gene promoter and 5'UTR was then digested with the restriction enzyme, KpnI, to prepare it for a second HiFi assembly reaction. The *coilin* cDNA fragment was amplified by PCR using the forward cDNA primer and the reverse cDNA primer (Table 1), and then the PCR product was gel extracted using the New England BioLabs Monarch DNA Gel Extraction Kit (Cat. No. T1020G). Phenol/chloroform extraction and ETOH precipitation was used to purify the gel extracted *coilin* cDNA fragment. A second HiFi assembly was performed with the same conditions as previously stated. After the HiFi assembly reaction, the plasmid was transformed into XL1 Blue *E. coli* cells by heat shock at 42°C for 1 minute in LB media. LB agar plates containing 50 µg/mL kanamycin were used to select for colonies containing the plasmid while LB agar plates containing 100 µg/mL ampicillin were used to select for the positive control. DNA was extracted from the resulting colonies using mini prep procedure. The presence of *coilin* cDNA was verified by a restriction digest with EcoRI and PCR amplification.

While performing HiFi assembly reactions previously described, we realized the *coilin* cDNA PCR product contained a stop codon which was included in the reverse primer that was used to amplify *coilin* cDNA. If left undeleted, the presence of this stop codon would have affected the protein expression of C-terminal GFP-tagged coilin. To simultaneously eliminate the stop codon from the *coilin* cDNA and change the platform from the pEGFP-N3 vector to pattB, a fly transformation vector, a third HiFi assembly reaction was performed. In this assembly, a DNA segment containing the *coilin* promoter, 5'UTR, and cDNA without a stop codon was combined with DNA encoding GFP and introduced into pattB. The previous construct in

pEGFP-N3 was used as a template for PCR amplification. The *coilin* promoter, 5'UTR, and cDNA segment was amplified by PCR with the forward P/5'UTR/cDNA primer and the reverse P/5'UTR/cDNA primer (Table 1). The GFP segment was amplified by PCR with the forward GFP primer and the reverse GFP primer (Table 1). The pattB vector was prepared by restriction digest with KpnI. The two segments were gel purified using New England BioLabs Monarch DNA Gel Extraction Kit and then subjected to ETOH precipitation. The HiFi assembly included both segments in 2-fold molar excess to the pattB vector with exactly the same assembly conditions as previously stated. The recombinant plasmid was then transformed into XL1-Blue *E. coli* cells by heat shock at 42°C for 1 minute in LB media. Screening occurred on LB agar plates containing 100 μg/mL ampicillin. DNA was extracted from selected colonies by mini-prep. PCR amplification was then performed on the isolated DNA to check for both GFP and the segment containing *coilin* promoter, 5'UTR, and cDNA.

Q5 Site Directed Mutagenesis: Sequencing revealed 6 extra nucleotides in between the *coilin* 5'UTR and the *coilin* cDNA, left over from previous HiFi assembly into the pEGFP-N3 vector. Using New England BioLabs' Q5 Site-Directed Mutagenesis Kit (Cat. No. E0554S), 6 extra nucleotides in the *coilin* 5'UTR and the *coilin* cDNA junction were deleted. Using Q5 HF DNA polymerase in PCR, we amplified the entire plasmid excluding the extra nucleotides using 1 ng of template plasmid, the forward Mutagenesis primer, and the reverse Mutagenesis primer (Table 1). Then a KLD (Kinase, Ligase, DpnI) treatment phosphorylated the PCR product, ligated the product to make a circular plasmid, and digested the original *E. coli*-generated template DNA with DpnI at room temperature for 5 minutes. The PCR-generated plasmid was transformed into 5-α Competent *E. coli* cells by heat shock at 42°C for 1 minute in SOC media. Selection

occurred on ampicillin LB agar plates. Deletion of the six extra nucleotides was confirmed by sequencing.

Sample Purification and Preparation for Sequencing: Colonies were selected and inoculated in LB broth with 50 μg/mL ampicillin at 37°C overnight. A mini prep was then performed on the cells. The extracted DNA was then subjected to treatment with 10 mg/mL RNase A, phenol/chloroform extraction, and ethanol precipitation. Polyethylene glycol (PEG) precipitation followed by ethanol precipitation was used to further purify the plasmid DNA. Sequencing reactions contained 10 ng of purified plasmid DNA and 3.2 μM of primer. The junctions between the *coilin* promoter/5'UTR and cDNA as well as between the *coilin* cDNA and GFP were sequenced. The sequencing of the *coilin* promoter/5'UTR and cDNA junction was performed in both 5' and 3' directions by using the forward P/cDNA Junction primer and the reverse P/cDNA Junction primer (Table 1). The sequencing of the *coilin* cDNA and GFP junction was performed using the forward cDNA/GFP Junction primer and the reverse cDNA/GFP Junction primer (Table 1).

Purification, Injection, and Crosses: The Thermo Scientific GeneJET Plasmid Midiprep Kit (Cat. No. K0481) Protocol A (for plasmid DNA purification using low speed centrifuges) was used to purify the plasmid DNA. DNA concentration was measured using Nanodrop. The DNA was then diluted to a concentration of 0.4 μg/μL and sent to GenetiVision for injection into *D. melanogaster* embryos for genome insertion using the PhiC31 integrase recombination site attP18(X)6C12 on the X chromosome. 480 embryos were injected with the plasmid. These embryos were progeny of attP18 line males with the platform on the X chromosome and nos-

PhiC31 line females with the integrase on the X chromosome. Injected females were selected for a cross with w^{1118} males. Progeny with red eyes due to the mini-white gene (w^{+}) will be selected to create a homozygous fly line.

Table 1. Primers

Forward P/5'UTR	5'-CAAGCTTCGAATTCTGCAGGTCTTATCGCTATCGGTCG-3'
Reverse P/5'UTR	5'-CGGGCCCGCGGTACCGTTTTGACTTAGTTTCTTATGATTAAAATG-3'
Forward cDNA	5'-GAAAACTAAGTCAAAAATGCAACACTCCAGCATGAAG-3'
Reverse cDNA	5'-TCCCGGGCCCGCGGTACATCAGTCAATTGTGGCTACAATG-3'
Forward P/5'UTR/ cDNA	5'-CGGCCGCGGCTCGAGGGTACGTCTTATCGCTATCGGTC-3'
Reverse P/5'UTR/ cDNA	5'-GCTCCTCGCCCTTGCTCACCATGTCAATTGTGGCTACAATGATTTTCGC-3'
Forward GFP	5'-GAAAATCATTGTAGCCACAATTGACATGGTGAGCAAGGGCGAGGAG-3'
Reverse GFP	5'-CGACACTAGTGGATCTCTAGAGTTACTTGTACAGCTCGTCCATGC-3'
Forward P/cDNA Junction	5'-CGGCCGCGGCTCGAGGGTACGTCTTATCGCTATCGGTC-3'
Reverse P/cDNA Junction	5'-CACTTTGATAGACTCCCTCGG-3'
Forward cDNA/GFP Junction	5'-GGCACAACCGAATTTGTCGC-3'
Reverse cDNA/GFP Junction	5'-GAAGTCGTGCTGCTTCATGTGGTC-3'
Forward Mutagenesis	5'-ATGCAACACTCCAGCATGAAGGTGG-3'
Reverse Mutagenesis	5'-TTTTGACTTAGTTTCTTATGATTAAAATGATTTTAAATGTGCTAGAGATGGAA-3'

These primers were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Results

Protein Expression Analysis

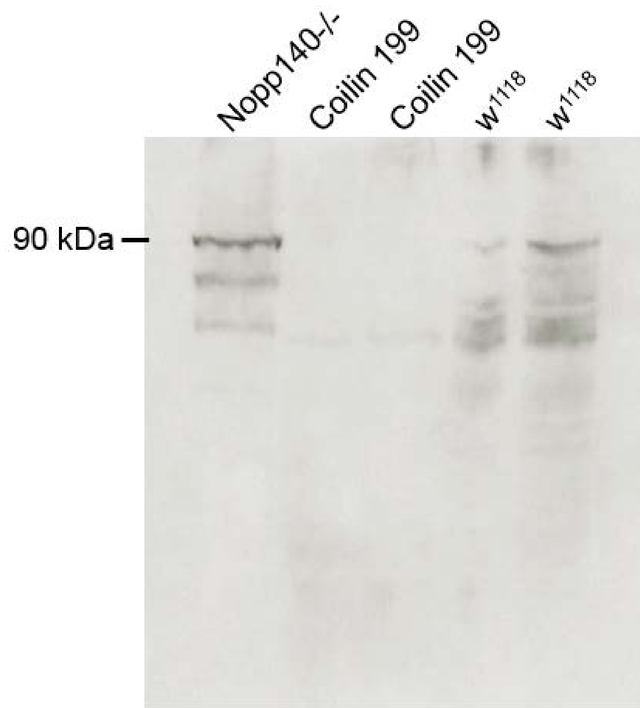

Figure 1. SDS-PAGE and Western Blot. Coilin expression in *Nopp140-/-* knockout larvae, *Coilin 199* null mutation larvae, and w^{1118} wild type larvae.

Coilin expression in *Nopp140-/-* knockout larvae, *Coilin 199* null mutation larvae, and w^{1118} wild type larvae was measured by SDS-PAGE and Western Blot analysis. The blot showed upregulation of the coilin protein in *Nopp140-/-* compared to wild type as seen in Figure 1. The upregulation occurred in the 90 kDa isoform of coilin with other splice variants of coilin present in the *Nopp140-/-* and w^{1118} larvae but with all isoforms absent in the *Coilin 199* null mutation larvae. This confirms a previous unpublished result shown by a previous graduate student.

Recombinant Plasmid Construction

HiFi Assembly

Construction of a recombinant plasmid containing the endogenous *coilin* gene promoter, 5'UTR, the *coilin* cDNA, and a C-terminal GFP tag was assembled in three HiFi assembly

reactions. The first HiFi assembly introduced the *coilin* promoter and 5'UTR into the pEGFP-N3 vector. This vector contained GFP on the C-terminal end of the multiple cloning site in order to allow C-terminal tagging of any inserted protein, in this case, coilin. This HiFi assembly was confirmed first by a colony PCR to check for the *coilin* promoter and 5'UTR segment (P/5'UTR). All ten colonies that were checked for the *coilin* P/5'UTR segment contained the 375 base pair insert as shown in Figure 2A. A mini prep followed by PCR was also conducted to confirm this assembly product in the pEGFP-N3 vector (P/5'UTR/pEGFP-N3). Two colonies were checked and were found to contain the *coilin* P/5'UTR segment as shown in Figure 2B.

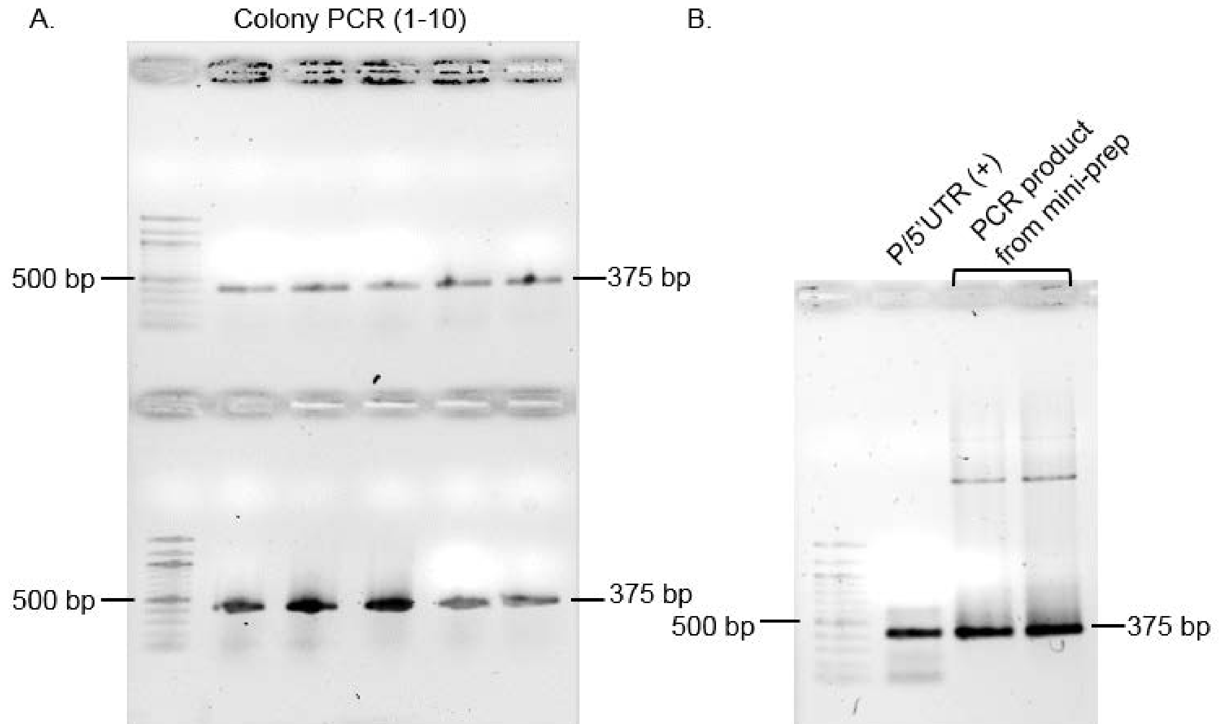

Figure 2. HiFi Assembly 1. A: Colony PCR from 10 selected colonies after transformation checking for P/5'UTR (375 bp) in pEGFP-N3. B: Mini Prep and PCR of 2 colonies after transformation checking for P/5'UTR (375 bp) in pEGFP-N3.

A second HiFi assembly was conducted to introduce the *coilin* cDNA into the P/5'UTR/pEGFP-N3 construct. The HiFi assembly was confirmed first by restriction digest using EcoRI to linearize the DNA isolated from two *E. coli* colonies. The expected size of the

linearized assembly product was a 7 kb band which was observed in one colony as shown in Figure 3A. Linearization of the P/5'UTR/pEGFP-N3 construct was performed as a control with a band size of 5.1 kb. To confirm the presence of *coilin* cDNA in P/5'UTR/pEGFP-N3 (P/5'UTR/cDNA/pEGFP-N3), a mini prep and PCR was conducted. The presence of *coilin* cDNA was confirmed in one colony with a size of 1.9 kb as shown in Figure 3B.

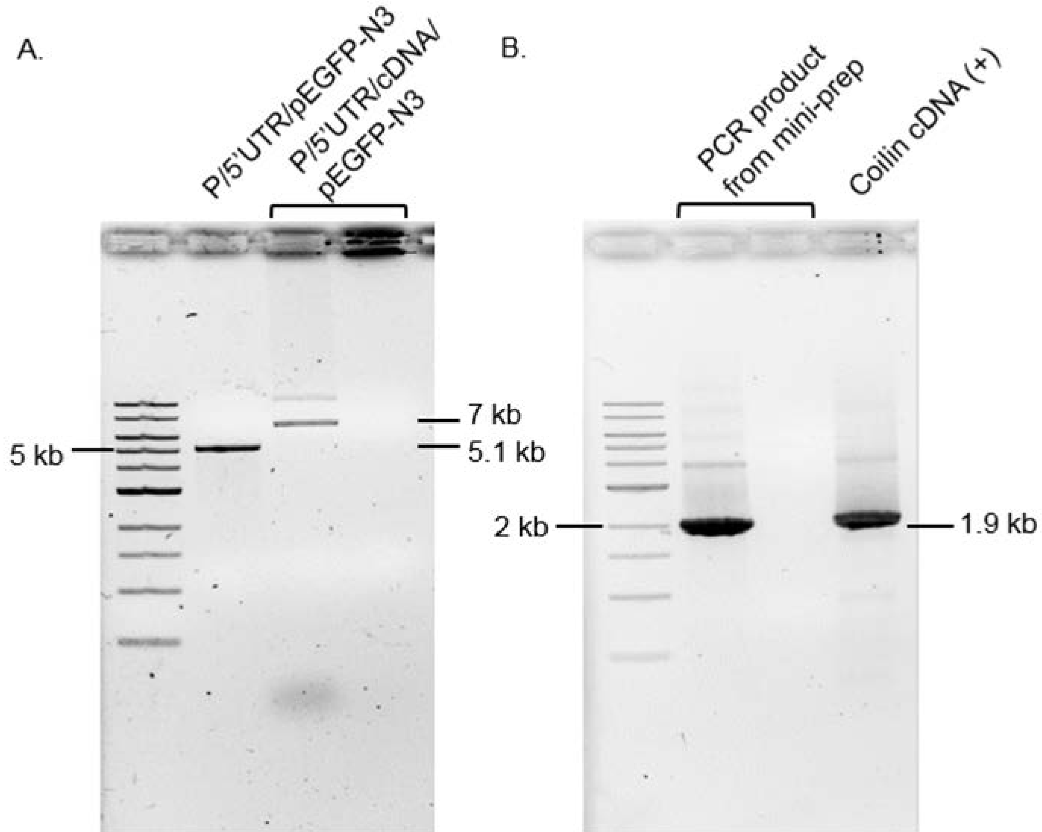

Figure 3. HiFi Assembly 2. A: Restriction digest with EcoRI of colony DNA checking for presence of *coilin* cDNA in P/5'UTR/pEGFP-N3. Total expected vector size is 7 kb. Control vector (P/5'UTR/pEGFP-N3) size is 5.1 kb. B: Mini Prep and PCR of 2 colonies after transformation checking for *coilin* cDNA (1.9 kb) in P/5'UTR/pEGFP-N3 with *coilin* cDNA control.

A third and final HiFi assembly was performed in order to remove the stop codon from the *coilin* cDNA segment and move all segments into the pattB transformation vector. A mini prep and PCR was used to confirm the incorporation of the P/5'UTR/cDNA segment and the GFP segment into pattB (P/5'UTR/cDNA/GFP/pattB). All three colonies checked for GFP

contained the 767 base pair segment as shown in Figure 4A, but only two of the three colonies contained the 2.3 kb P/5'UTR/cDNA segment as shown in Figure 4B.

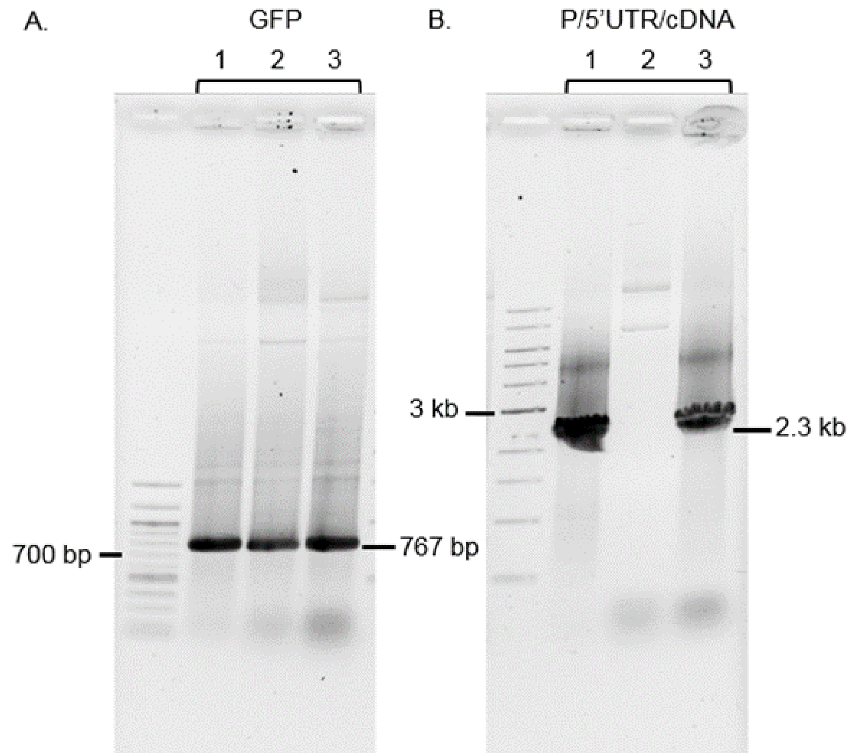

Figure 4. HiFi Assembly 3. Mini Prep and PCR of colonies 1, 2, and 3 to check for correct assembly of GFP and P/5'UTR/cDNA into pattB vector. A: The expected GFP fragment is 767 bp. B: The expected P/5'UTR/cDNA fragment is 2.3 kb.

Q5 Site Directed Mutagenesis

The P/5'UTR/cDNA/GFP/pattB construct was sequenced to confirm the removal of the *coilin* cDNA stop codon, correct assembly of the *coilin* P/5'UTR and cDNA junction, and correct assembly of the *coilin* cDNA and GFP junction. Sequencing confirmed the removal of the remaining *coilin* cDNA stop codon and the correct *coilin* cDNA and GFP junction DNA sequence; however, the *coilin* P/5'UTR and cDNA junction contained six extra nucleotides (CGGTAC) in between the two segments. In order to use this construct in the future to assess the effects that the endogenous *coilin* gene promoter and 5'UTR have on coilin expression, removal

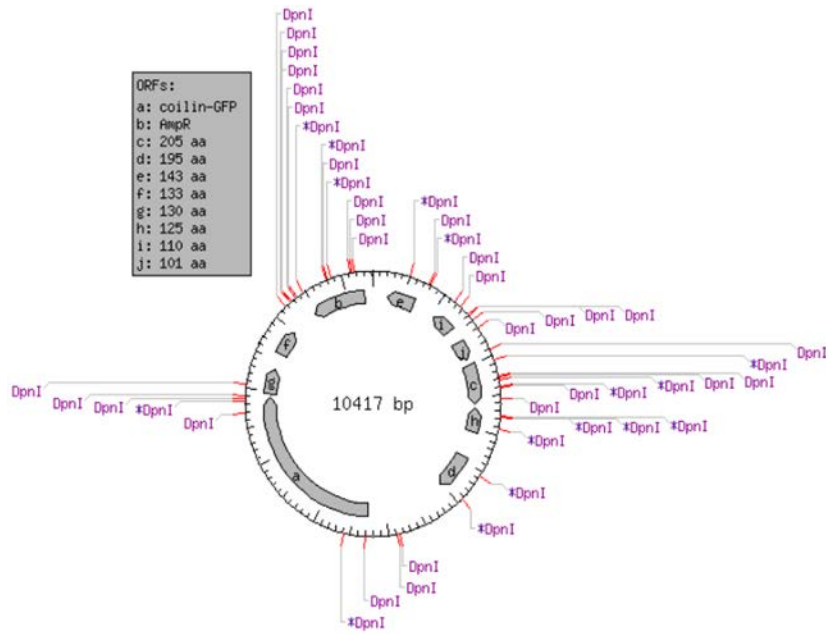

Figure 5. KLD Reaction DpnI digestion. The digestion of template DNA from the PCR reaction in the KLD reaction is accomplished by the restriction enzyme DpnI.

of these nucleotides was necessary. A site directed mutagenesis deletion of the nucleotides was used to correct the junction's DNA sequence. After the mutagenesis PCR reaction was performed, a KLD reaction circularized the mutagenesis PCR product and removed the methylated template DNA by DpnI restriction enzyme digestion as shown in Figure 5. Sequencing revealed successful deletion in sample 1 as shown in Figure 6 resulting in the correct final construct P/5'UTR/cDNA/GFP/pattB. This final construct was then purified and sent to

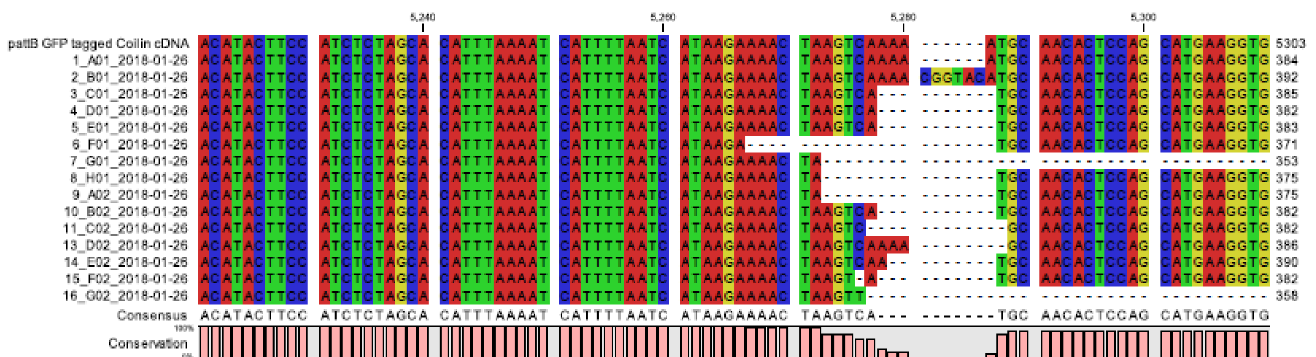

Figure 6. Site Directed Mutagenesis Deletion. Six extra nucleotides (CGGTAC) were deleted from the construct. Sample 1 was the only construct where successful deletion of only the CGGTAC sequence was accomplished. The desired sequence is labeled pattB GFP tagged Coilin cDNA.

GenetiVision for injection into fly embryos using the PhiC31 system. Successful injection of the plasmid is currently in the process of being confirmed.

Discussion

Gibson Assembly

In the Gibson assembly, DNA fragments are made by PCR amplification with homologous sequences that overlap one another and the vector. The vector can either be linearized by restriction digestion or amplified by PCR. Each fragment is made with overlapping, homologous end segments designed into the primers so that the PCR product will contain a portion of identical DNA sequence to whatever fragment should be next to it. At each junction between fragments, at least one side must be a PCR fragment in order to introduce the homologous overlaps.

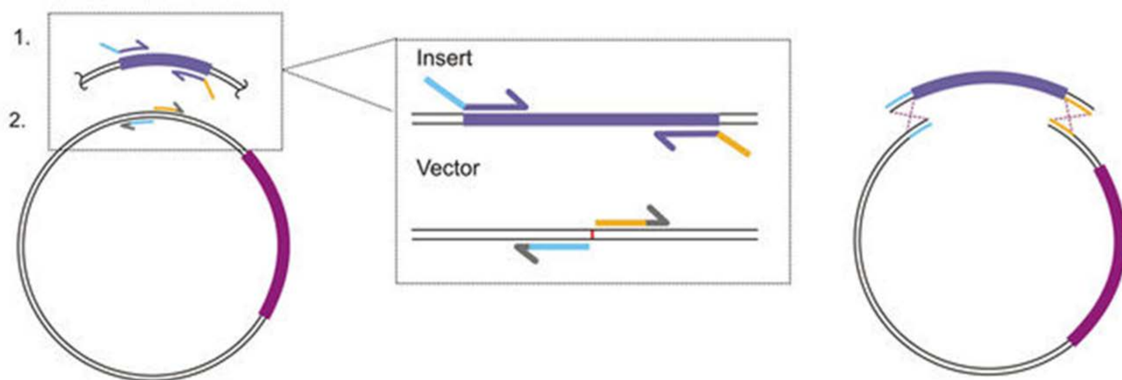

Figure 7. (from García-Nafría, Watson, & Greger, 2016)

During the assembly reaction, overlapping homologous sequences anneal to each other connecting the fragments in the desired orientation and order to create the complete plasmid as shown in Figure 7. First, a 5' exonuclease eats away a portion of each DNA fragment's 5' ends. The homologous sequences are then exposed as single-stranded 3' overhangs. Overhangs have a long stretch of homologous sequence, about 15-20 bases of overhang, making the annealing very

specific in comparison to ligation of DNA that has undergone restriction digestion leaving 3-4 nucleotide overhangs. Homologous sequences of neighboring fragments anneal to one another, and DNA polymerase fills in any gaps. Finally, DNA ligase seals the breaks. All these steps occur in a thermocycler at 50°C for just 1 hour as shown in Figure 8. This experiment used a modified version of the Gibson Assembly called the HiFi assembly marketed by New England BioLabs. This assembly used a high-fidelity DNA polymerase for all PCR reactions.

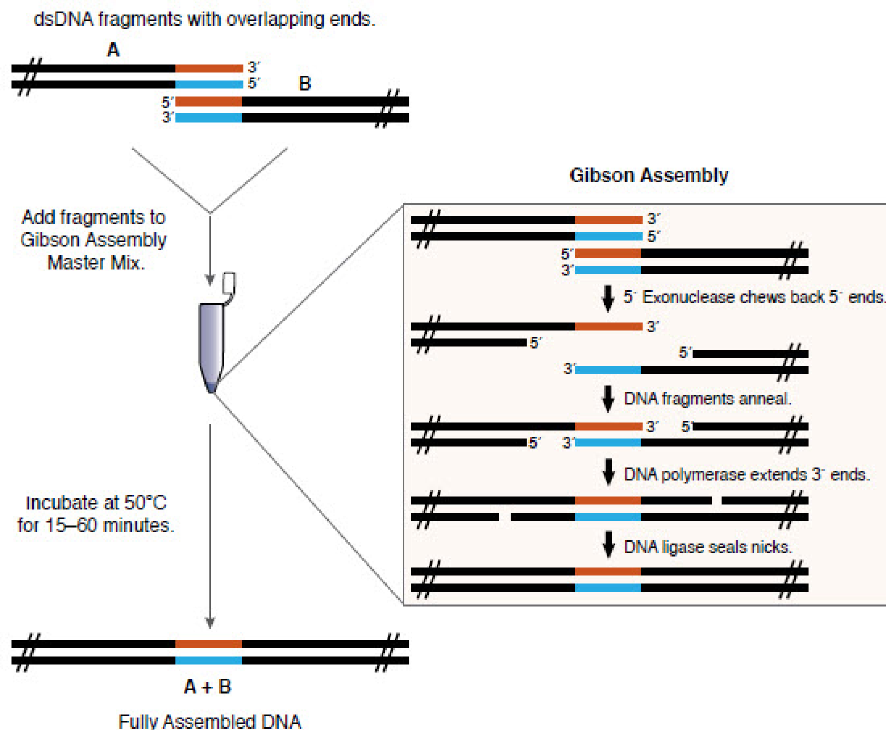

Figure 8. Gibson Assembly® Cloning Kit. Retrieved from https://www.neb.com/products/e5510-gibson-assembly-cloning-kit#Product%20Information

Q5 Site Directed Mutagenesis

In Q5 Site Directed Mutagenesis, the plasmid was edited to delete 6 extra nucleotides in between the 5'UTR and the coilin cDNA. The entire 10.4 kb plasmid was amplified in PCR with Q5 Hot Start HF DNA Polymerase. The deletion was introduced through flanking primers that excluded the 6 nucleotide region from amplification. Then in a 5 min KLD reaction at room

temperature, the plasmid is phosphorylated at its 5' ends and circularized by DNA ligase. The restriction enzyme DpnI selectively digests and removes the methylated template DNA leaving only non-methylated PCR product. This mixture can then be transformed into competent cells as shown in Figure 9.

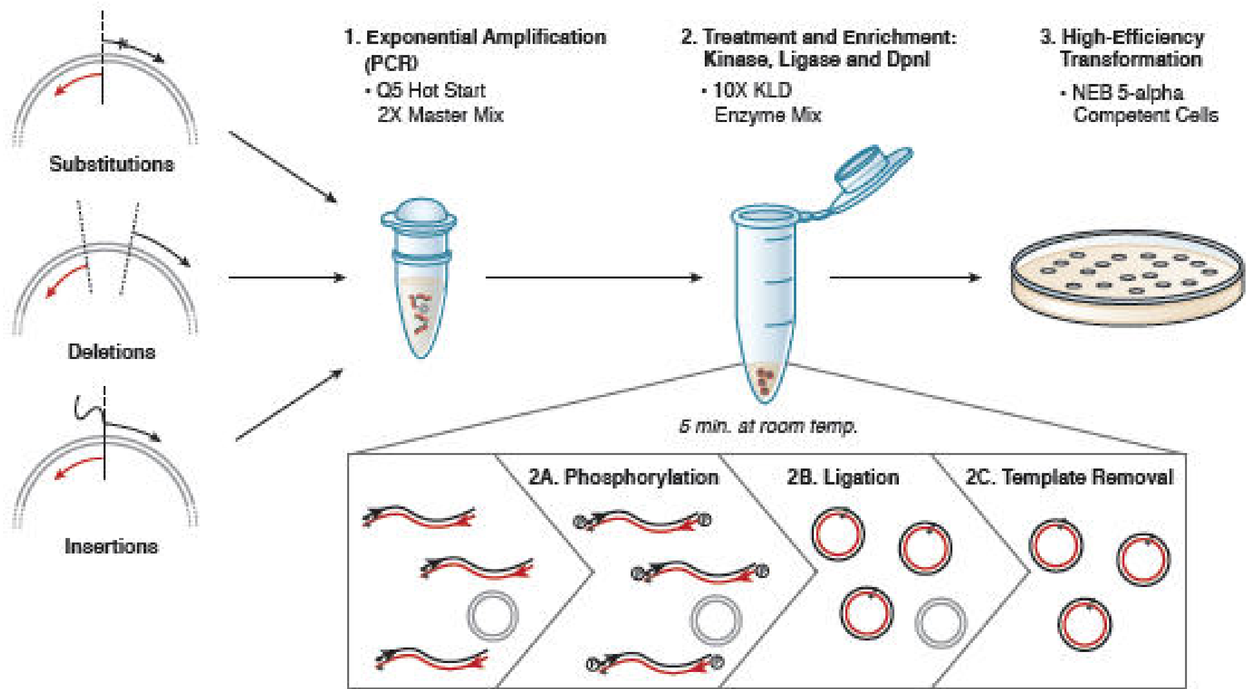

Figure 9. Q5® Site-Directed Mutagenesis Kit. Retrieved from https://www.neb.com/products/e0554-q5-site-directed-mutagenesis-kit#Product%20Information

Substitutions and insertions can also be introduced into a plasmid by this technique. For a substitution to occur, a forward primer is designed with the substitution surrounded by complementary sequences about ten base pairs in length. The reverse primer lies adjacent to the forward primer to incorporate the entire plasmid into the PCR product. To introduce an insertion, the 5' ends of either one or both primers contains the extra sequence to be incorporated.

PhiC31 Integration

The PhiC31 system was used to establish a transgenic fly line with GFP-tagged coilin by injection of a recombinant pattB plasmid. This system uses a bacteriophage integrase to insert the transgene of interest into the genome of a fly embryo. Bacteriophages are a type of virus that infect bacteria and reproduce using the bacteria's cellular structures to express its genome. They typically kill the cell by lysis to allow infection of more bacterial cells, but in unfavorable conditions, they can also incorporate their DNA into the bacterial genome to be maintained until favorable conditions. The incorporation of viral DNA is accomplished by phage integrases. These enzymes are produced from the viral genome by host cell structures and integrate DNA by

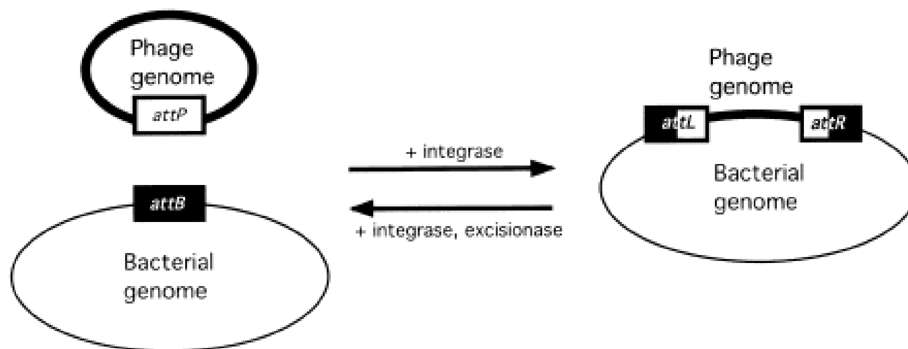

Figure 10. Phage genome integration into and excision from the bacterial genome via *attP* and *attB* sites (from Groth & Calos, 2004).

recombination between recognition sites specific to each integrase. The integrase recognizes a phage attachment site called *attP* and a bacterial attachment site called *attB*. Integration of the viral DNA results in the *attL* and *attR* sites bordering the integrated DNA. Each site contains half of the original *attP* site and half of the original *attB* site as shown in Figure 10. The *attL* and *attR* sites are recognized by another enzyme called an excisionase which can excise the viral DNA from the bacterial genome (Groth & Calos, 2004).

Phage integrases either belong to the tyrosine family of site-specific recombinases or the serine family of site-specific recombinases based on the amino acid composition of their

catalytic sites (Groth & Calos, 2004). PhiC31 is a serine phage integrase that only catalyzes the integration of DNA, not the excision of DNA (Bischof et al., 2007). PhiC31 integrase has similar binding affinity for the *attP* and *attB* sites even though the sites have different sequences. During recombination, two serine integrase monomers covalently bind to each *att* site. Serine's hydroxyl group in each integrase attacks the DNA phosphate backbone binding the 5' ends and exposing the 3' OH groups. All four strands of the DNA are broken and will ligate together after a 180° rotation of the viral DNA resulting in the integration of the viral DNA (Groth & Calos, 2004).

Exploitation of the PhiC31 system allows recombination to occur at mapped *attP* sites present in the *Drosophila* genome either naturally or introduced manually. Plasmids containing the *attB* site can then be integrated into the fly's genome after injection into embryos. These embryos come from fly lines containing pre-mapped *attP* sites called platforms as well as established PhiC31 integrase genes under the regulation of *vasa* or *nanos* (*nos*) genes. The *attP* sites have also been engineered to contain the majority of the mini white gene (w^+). This gene causes dark red pigmentation of the eyes of the adult fly. In order to allow for selection of only transgenic flies, a small portion of the w^+ gene was removed from the w^+ present at the *attP* site and then encoded on the transformation vector; therefore, only flies with integrated vector DNA will exhibit the phenotype of red eyes (Bischof et al., 2007).

Transcriptional vs. Translational Regulation of Coilin Expression

With the assembly of GFP-coilin expressed under its endogenous promoter and the resulting fly line (w^+ :: Coilin-GFP/w^{1118}), future projects will elucidate the relationship between Nopp140 deletion and coilin upregulation using this new construct. First, a new Nopp140 knockout line called J11 created by CRISPR disruption of the gene will be crossed with w^+ ::

Coilin-GFP/w^{1118} to compare its coilin protein levels with a w^{+} :: Coilin-GFP/w^{1118} cross with w^{1118} wild type. As shown in the analysis of coilin expression in the Nopp140 knockout line and wild type line, higher coilin concentration would be expected in the J11; w^{+} :: Coilin-GFP/w^{1118} line. Next, to determine coilin's level of regulation, CRISPR would be used to modify either the promotor or the 5'UTR to see the effects on coilin protein expression in transcription and translation, respectively. Modifications of these regions would provide insight into how these regions affect the expression of coilin and which holds greater importance when Nopp140 is deleted. Lastly, examination of the molecular cross-talk interaction between the Cajal body and the nucleolus would be of interest. Nopp140 travels between the two components, but coilin remains in the Cajal body.

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