Maltose-binding Protein (MBP) Fusion Tag Enhances Expression and Solubility of CCDC11 Constructs

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ABSTRACT

Through discoveries made in past research, CCDC11 is a protein known to be an integral part of cytokinesis, including cell division and viral replication. The highest concentrations of this protein are found around the centrosomes. CCDC11 functions by transporting proteins to and from the centrosomes and cilia. The purpose of MBP tagging CCDC11 constructs was to determine a way to purify the protein so that its structure can be concluded after performing crystallization. Protein purifying is a series of processes intended to isolate a protein from a complex mixture. This is vital for the characterization of the function, structure, and interactions of our proteins of interest, CCDC11-CC1-2 and CCDC11-CC1-3. The arctic bacteria strain yielded the greatest abundance of protein. These bacteria containing CC1-2 and CC1-3 constructs were grown overnight and induced at their peak optical densities. Samples taken from the supernatant were run through an amylose column containing column buffer followed by an elution buffer. Additional samples were collected at various times and run through a protein gel. CC1-2 revealed better expression than its larger counterpart, CC1-3. The solubility of CCDC11-CC1-2 remains questionable, however, if it continues to be stable, steps may be taken to attempt crystallization of this protein. This will enable the determination of its structure, which will then allow the synthetic formation of CCDC11 for uses in tumor-fighting drugs.

Introduction

Prior Characterization of CCDC11

Coiled-Coil Domain Containing 11 (CCDC11) protein is a 62-kDa polypeptide known to be associated with centriolar and ciliary structures, and is linked to ciliogenesis. It transports proteins from the centrosomes to the cilia. CCDC11 is vital to the left-right lateral asymmetry of internal organs. A decreased concentration or absence of CCDC11 can be seen in patients with laterality disorders associated with defective cilia.

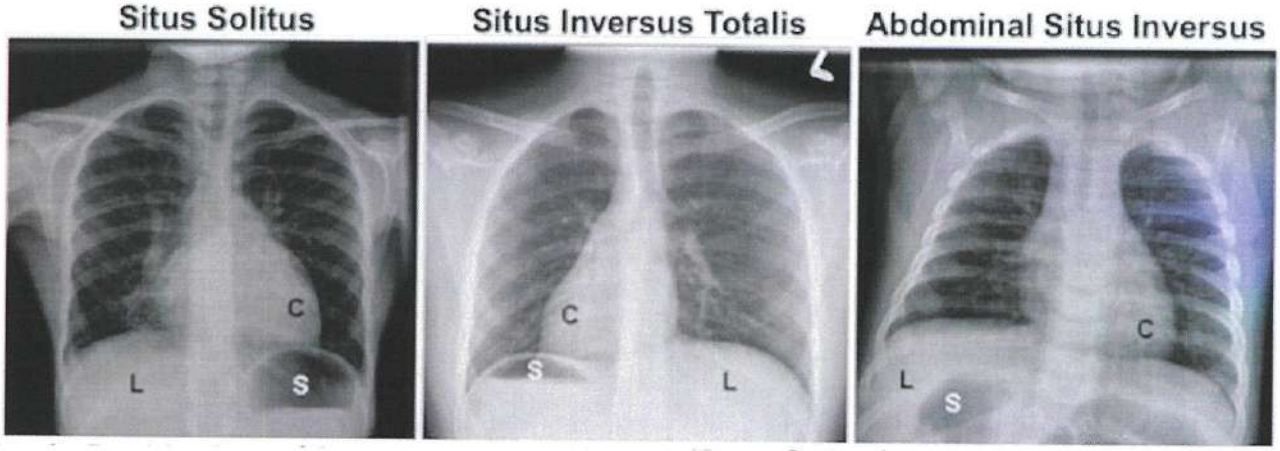

Fig. 1. X-ray scans illustrating the positioning of human organs in various individuals. From the leftmost to the rightmost image, normal (Situs Solitus) shows the correct positioning of organs, and images of two laterality disorders associated with defective cilia follow. Situs Inversus Totalis is the complete reversal of organs. Abdominal Situs Inversus (heterotaxia) is a potentially fatal disorder due to the partial reversal of organs. C, cardiac apex; L, liver; S, stomach. Source: Modified from Shapiro et al. 2014.

CCDC11 is also known to play a role in cytokinesis, including viral replication and cell division, due to prior findings that CCDC11 localizes in the midbody region during cell division. Knocking down CCDC11 in dividing cells caused multinucleation within cells by interfering with cytokinesis. While CCDC11 is associated with a variety of processes, the protein's exact function is unknown, providing a rationale to determine the structure. By determining the

structure, steps can be taken to develop potential blocks or mimics, which may eventually lead to the prevention of viral infections by inhibiting viral budding.

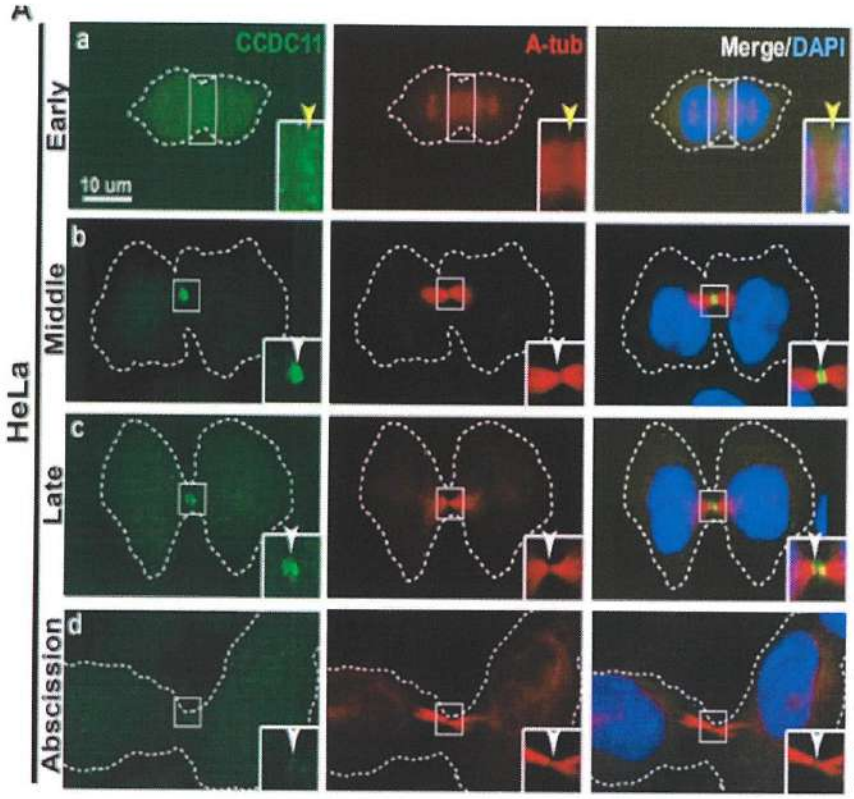

Figure 2: Endogenous CCDC11 localizes to the midbody region during cytokinesis in HeLa and U2OS cells. (A-B) HeLa and U2OS cells were co-immunostained with antibodies for CCDC11 (green) and acetylated alpha-tubulin (A-tub, red) to visualize endogenous proteins. Nuclei were stained using DAPI (blue). (A) In HeLa cells during early cytokinesis (a), CCDC11 was slightly enriched at the cleavage furrow (yellow arrowheads). During the middle (b) and late stages (c) of cytokinesis, CCDC11 was observed at the midbody (dark zones, white arrowheads) and flanking regions, respectively. During abscission (d), CCDC11 levels were severely reduced or lost in the midbody region, suggesting its dissociation from the midbody region prior to completion of cytokinesis. (B) In contrast to HeLa cells, CCDC11 signals were detectable only during abscission in U2OS cells as it localizes to two cortical regions adjacent to the midbody (white arrowheads). Dashed lines represent cell boundaries. Source: Ahmed et al. 2016.

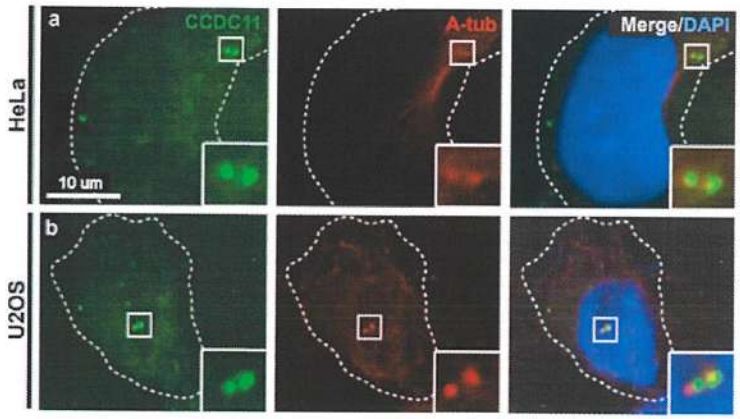

Figure 3: Endogenous CCDC11 localizes to centrioles in HeLa and U2OS cells. HeLa (a) and U2OS (b) cells were co-labelled with antibodies for CCDC11 (green) and A-tub (red). Nuclei were stained using DAPI (blue). Dashed lines represent cell boundaries, and two centrioles are enlarged in the insets. CCDC11 localized to both mother and daughter centrioles in U2OS and HeLa cells.
Source: Ahmed et al.2018

CCDC11 tends to easily get aggravated and precipitate in solution, so it is usually studied when attached to a Maltose-Binding Protein (MBP) fusion tag via a TEV sequence, which can be removed with TEV protease.

Optimal Construct Length

The full length of CCDC11 is relatively large, difficult to produce in large enough quantities by bacterial strains, and easily aggravated. As a result, a condensed version of CCDC11 would allow for a greater amount of protein production, but an appropriate construct must be determined to avoid compromising the function of the protein. By testing the localization of differing construct lengths of CCDC11, including coils 1 and 2 (CC1-2), coils 2 and 3 (CC2-3), and coil 1 (CC1), it was determined that CC1-2 was the minimal domain necessary for the function of CCDC11. Both CCDC11 Full Length and CC1-2 were observed in the midbody and centrioles of U2OS cells, while the concentration of CC2-3 and CC1 alone in those locations were dramatically decreased or non-existent.

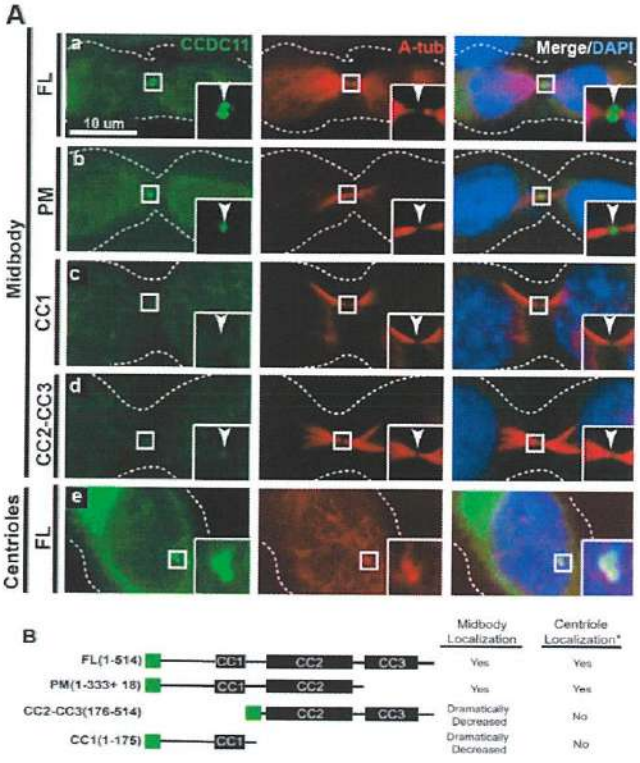

Figure 4. Ectopically expressed CCDC11 localizes to the midbody region in U2OS cells. (A) U2OS cells were transiently transfected with expression plasmids for myc-tagged CCDC11 full-length (FL), patient mutation (PM), CC2-CC3, or CC1 as illustrated in (B) and co-stained using anti-myc antibody (green) and anti-A-tub antibody. Nuclei were detected using DAPI (blue). Both CCDC11-FL (a) and CCDC11-PM (b) localized to the midbody, whereas the midbody localization of CC2-CC3 and CC1 was dramatically reduced. (c) Clear centriolar localization was observed for CCDC11-FL but not for CCDC11-PM (data not shown). (B) Schematic diagram of different CCDC11 deletion mutants used in this study and summary of the results from (A). Green boxes indicate myc-tag at N-terminus. * Centriolar localization results from (1).
Source: Ahmed et al. 2018

Constructs of CCDC11 must contain at least the first and second coils to retain the function of the full length protein, providing rationale for experimentation with CCDC11 CC1-2 and CC1-3.

Interaction with Cby

Through past projects, Cby was found to interact with CCDC11. Cby was targeted with anti-Cby antibodies and pulled down in solution. When the area was probed with anti-FLAG antibodies, CCDC11, which was tagged with FLAG, was present as well. This indicates that CCDC11 and Cby interact directly or indirectly via another protein. If there is direct interaction, CCDC11 and Cby might be able to form a complex, through which the structure could be found.

Purpose of the study

The purpose of the study is to isolate CCDC11 CC1-2 and CC1-3 constructs without the MBP tag with minimal precipitation. Then, crystallization trials will ensue, allowing for determination of the protein's structure and ultimately its exact function. Furthermore, the study includes identifying the nature of interaction between MBP-Cby and MBP-CCDC11 and includes determining whether Cby and CCDC11 are able to form a soluble complex without the MBP fusion tags. Finally, TEV cleavage results in CCDC11 aggravating because it is no longer attached to the MBP tag, so the study includes finding the optimal time for TEV cleavage with minimal precipitation.

Materials and Methodology

Protein Purification

In order to mass produce the protein of interest, three separate strains of bacteria were grown in overnight cultures: ArcticExpress, BL21 DE3, and Origami. Four DH5-Alpha Cells, which are E. coli cells used to maximize transformation efficiency, were used to propagate the plasmids containing the code for either CCDC11 CC1-2 or CC1-3. One of each plasmid was placed into each of the three bacterial strands. 50-100 µL alloquates were placed in 15 mL falcon tubes of chemically competent bacterial strains ArcticExpress, BL21 DE3, and Origami. These were subject to a 42°C water bath for 60-70 seconds, allowing the DNA to slip in through porous membranes. Then, the tubes were placed on ice for 1 minute, followed by an addition of 1 mL of Lysogeny broth (LB), a nutritionally rich medium primarily used for the growth of bacteria. Then, they were placed at 37°C for 1-1.5 hours, allowing them . At this point the bacteria were transferred into 1.5 mL eppendorf tubes and spun down at 3,000 RPM for 1 minute. Once the supernatant was discarded, the pellet was resuspended in 100 uL of fresh LB, plated onto appropriate plates (LB AMP), and incubated at 37°C for 16 hours.

After incubation, the bacteria were plated onto LB/AMP plate. The ampicillin selected for the bacteria that successfully took up the plasmid. The Origami and BL21 DE3 cultures were incubated at 37°C at 150 RPM for about 3.5-4.5 hours until an optical density (OD) of 0.5-0.6 was reached. The Arctic cultures were incubated at 37°C for 3-3.5 hours until an OD of about 0.2 was reached. The temperature was then reduced from 37°C to 12°C. In all cultures, the samples were induced with 750 µL of 0.5mM IPTG when the optical density value was in the 0.5 to 0.6 range.

Measuring Optical Densities		
Time	Sample Number	OD Value
12:30	1	0.101
12:32	2	0.100
12:50	3	0.150
12:52	4	0.117
12:54	5	0.149
12:55	6	0.158
12:57	1	0.177
1:11	1	0.240
1:14	2	0.240
1:15	2	0.259
1:17	4	0.189
1:40	1	0.409
1:41	2	0.434
1:43	6	0.396
2:05	1	0.576
2:07	2	0.562
2:10	3	0.550
2:15	5	0.555
2:18	6	0.562
2:21	4	0.442
2:36	4	0.586

Measuring Optical Densities		
Time	Sample Number	OD Value
10:54	1	0.282
10:58	2	0.215
11:00	3	0.112
11:02	4	0.188
11:04	5	0.159
11:06	6	0.178
11:16	1	0.384
11:41	1	0.632
11:45	2	0.512
11:48	4	0.375
12:08	4	0.417
12:10	6	0.415
12:35	4	0.512
12:42	6	0.438
12:44	5	0.734
12:47	6	0.517
12:52	3	0.265
12:54	3	0.312
1:18	3	0.281
1:23	3	0.313

Figure 5A&B. Highlighted data points are the times at which the specific sample number was induced with IPTG. In Figure 4A, the fastest to slowest to reach and optical density between 0.5 and 0.6 are listed as follows, respectively: samples 1, 2, 6, 3, 5, 4, 2. In figure 4B, the fastest to slowest to reach and optical density between 0.5 and 0.6 are listed as follows, respectively: samples 1, 2, 4, 6, 5, 3.

The cultures were then spun down at 4000 RPM for 10 minutes to pellet cells (Arctic were spun at 4°C and resuspended in the MBP-Column Buffer.

The Arctic and BL21 induced cultures were thawed and 1mM of PMSF, a protease inhibitor to limit protein degradation, was added. The cultures were then sonicated on ice with a

microtip at 40% output for 15 second pulse followed by 2 minute rest five times. The sonicated lysate was spun down at 12,000 RPM for 20 minutes to remove pellet and the supernatant was removed and applied to MBP Column.

The gravity column contained 3mL of amylose beads, which have an attraction for MBP. It is used to separate the protein of interest from any other impurities. First, the column itself was washed with MBP-Fusion Column Buffer. MBP-Fusion Column Buffer was created with 20 mM TRIS, 200mM NaCl, and 1 mM EDTA. Once the column was washed, the supernatant solution containing the protein was run through the column and collected as the Flow Through (FT). After two more washes, with the flows collected as wash1 and wash2, the elution began. Elution is the process of extracting one material from another by washing with a solvent. MBP-Fusion Elution Buffer was created with 20mM TRIS, 200mM NaCl, 1 mM EDTA, and 10 mM maltose. The MBP-Fusion Elution Buffer ran through the column 5 times and the fractions were collected during each flow.

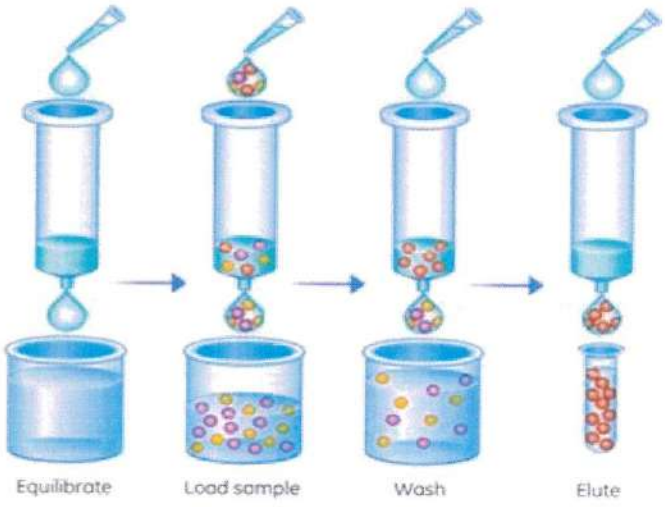

Figure 6A. Illustrates the procedure followed for the MBP columns. This would enable purifying MBP-tagged proteins a simple and quick procedure. However, no clear conclusions were determined, most likely due to the maltose being too tightly bound to the protein, thus did not combine to the column.

Anion Exchange

After producing large quantities and isolating the protein of interest, the constructs were taken to undergo Anion Exchange. Anion Exchange separates substances due to differential charges. The packing is positively charged, so it retains negative charges through coulombic interaction. CCDC11 CC1-2 and CC1-3 are acidic proteins (pI = 5.45 and 5.51 respectively), thus they have a net negative charge in a neutral pH. The goal was to separate the charged constructs from the MBP tag.

Interaction with Cby

MBP-Cby and MBP-CCDC11 were discovered to interact with one another. To determine whether there was a direct interaction, isolated MBP-Cby was mixed with MBP-CCDC11 CC1-2 and MBP-CCDC11 CC1-3in two separate dialysis tubes, each placed in a low salt buffer. After leaving the bags overnight, the samples were collected and run on a gel to view whether complex formation occurred.

TEV Cleavage

TEV cleavage requires time to complete by adding TEV protease to a sample of MBP-CCDC11. The optimal time allowed for TEV cleavage with minimal precipitation was determined by taking samples of MBP CC1-2 and CC1-3 every two hours after TEV protease was added until 12 hours, and then another sample at 24 hours. The samples were run on a gel to determine maximum cleavage.

Post-TEV Cleavage

After the TEV sequence was removed, the remaining sample was put through a HIS column, which contained positively charged beads. The beads should attract the TEV sequence and separate it from CCDC11 constructs. Then, the remaining sample was passed through an MBP column, ideally causing solely CCDC11 to flow through and be collected in its own tube.

Analysis

After each protein purification and major experimentation, a 4% stacking gel was created on top of a 10% separation gel to record data and analyze the results. To prepare the 10% separation gel, the following were mixed: 2.1 mL of water, 3.3 mL of Acrylamide/bis (30% 37.5:1, Bio-Rad), 2.5 mL of Tris-HCL (1.5 M, pH 8.8), 100 µL of SDS, 10%, 10 µL of tetramethylethylene-diamine (TEMED) (Bio-Rad), and 32 µL of Ammonium persulfate (APS), 10%. After the addition of TEMED and APS to the SDS-PAGE separation gel solution, the gel polymerizes quickly, so these reagents were added when ready to pour. The gel was poured, leaving ~2 cm below the bottom of the comb for the stacking gel. The top of the gel was layered with isopropanol to help remove bubbles at the top, as well as keep the polymerized gel from drying out. In ~30 min, the gel was completely polymerized, at which point the isopropanol was removed and washed out with distilled water.

To prepare the 4% stacking gel, the following will be mixed in the following order: 6.1 mL of water, 1.3 mL of Acrylamide/bis (30%, 37.5:1), 2.5 mL of Tris–HCl (0.5 M, pH 6.8), 100 µL of SDS, 10%, 10 µL of TEMED, and 100 µL of Ammonium persulfate (APS), 10%. The stacking gel was poured on top of the separation gel, followed by combs to make wells. In ~30

min, the stacking gel became completely polymerized. The gel was clamped into the apparatus, and both buffer chambers were filled with gel running buffer according to the instructions for the specific apparatus. At this point, the samples were loaded into wells for separation by gel electrophoresis.

Results

Protein Purification

The Arctic strain of bacteria yielded the highest concentration of CC1-2 and CC1-3. CC1-2 was produced in the highest concentrations, while the CCDC11 full length was produced in relatively low concentrations. Arctic bacteria was then used for any further protein production.

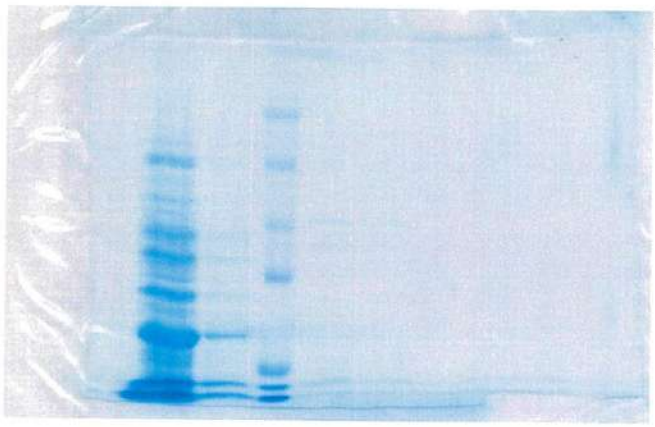

Figure 7A: Protein electrophoresis gel depicting the length of the full length CCDC11 protein when tagged with MBP.

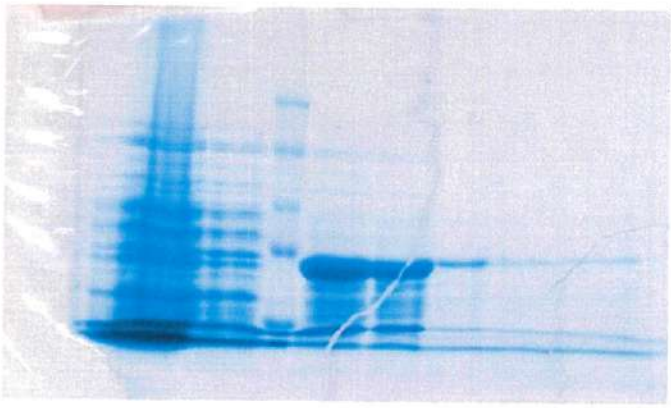

Figure 7B: Protein electrophoresis gel depicting the separation of domains 1 through 2 of CCDC11 protein when grown in the Arctic bacteria.

MBP Gravity Column

After the elutions, MBP-CCDC11 constructs were able to pass through the column bed into the elution tubes, creating samples of isolated constructs. The highest concentration of protein can be found in elution fragments 2 and 3.

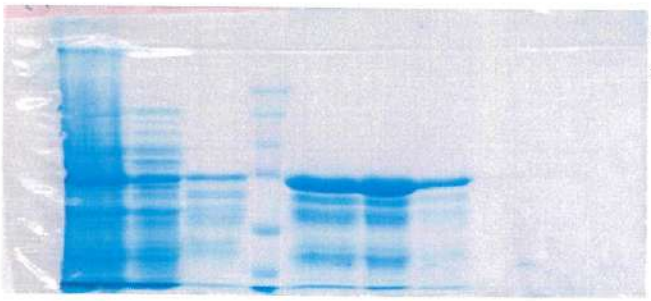

Figure 8: Protein electrophoresis gels showing the flow through and elution fractions 1-5 of the MBP-CCDC11-CC1-2

Anion Exchange

Large amounts of protein precipitated out of solution, rendering it unusable. By trying to separate the MBP from the CCDC11 constructs, the constructs were aggravated and solubility decreased.

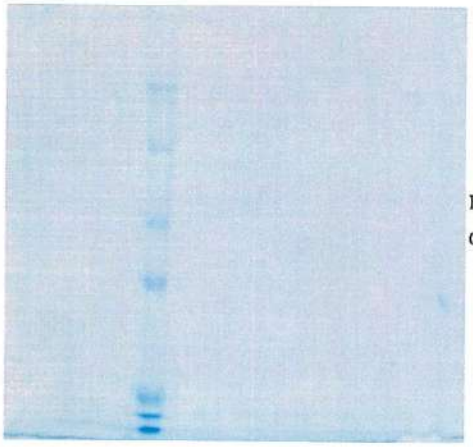

Figure 9: Protein electrophoresis gel showing the anion fractions of MBP-CCDC11 CC1-2 construct. From left to right is flow through to D3

A test was done by combining the anion fragments with MBP-Cby to determine if there was any soluble protein available, but fractions were not soluble enough after Anion Exchange.

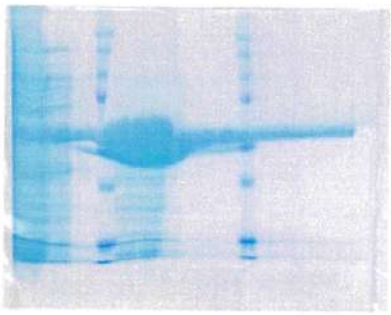

Figure 10: Protein electrophoresis gel depicting the results of MPB-Cby gravity column fractions when combined with CCDC11-CC1-2 anion fractions. Performed to determine if any of the anion exchange fractions were soluble enough to proceed with.

Interaction with Cby

Unable to determine complex formation from the gel, so there are inconclusive results on the nature of interaction. A great deal of precipitation still occurred from mixture, leading to the conclusion that solubility is not enhanced by mixing the two proteins.

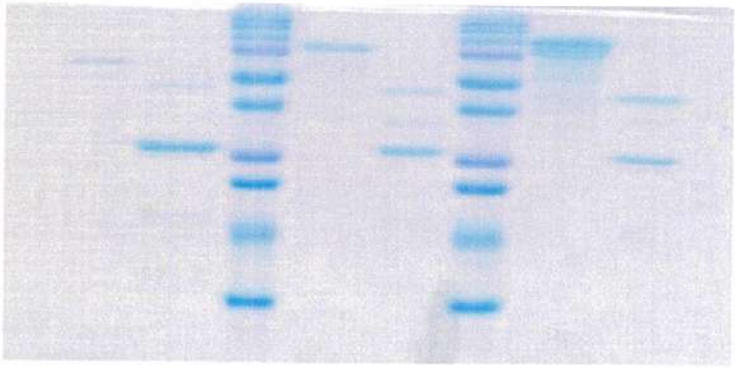

Figure 11: Protein electrophoresis gel showing MBP-Cby combined with CCDC11. Each sample is separated by a well with the ladder. From left to right, Cby only followed by Cby mixed with CC1-2 followed by Cby mixed with CC1-3. Student generated figure.

TEV Cleavage

The optimal time for TEV cleavage was determined to be 4 hours. After 4 hours, precipitation begins to occur in large amounts. At 24 hours, most of the sample crashed out of solution. 4 hours was used as a new baseline for TEV cleavage.

Post-TEV Cleavage

The TEV fragments were successfully removed post-HIS, but separated MBP could still be found in the elution tubes along with the CCDC11 constructs after run through the MBP column. This indicates that MBP was tightly bound to maltose from previous column, reducing its affinity for amylose beads within new column. Thus, MBP passed through easily into the FT.

As a result, MBP gravity column is not the right method to proceed at this point. Ion Exchange is a potential alternative.

Discussion

CCDC11 is attached to MBP via a TEV sequence. For CC1-2, MBP causes the construct to remain soluble in solution, making it easier to work with. CC1-3 likely is less soluble with the MBP tag due to its greater size.

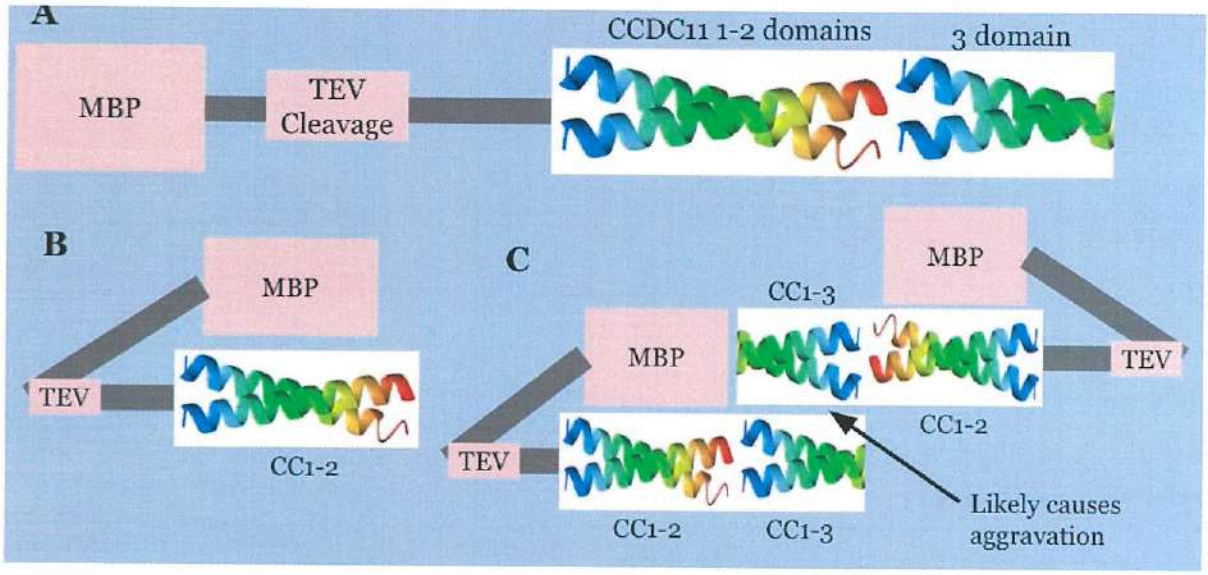

Figure 12: (A) Linear diagram of CCDC11 Protein coiled coil domains 1-3 showing the MBP Tag and site of cleavage by TEV Protease. When TEV is cleaved off, the protein remains soluble but only before 24 hours passes or else it starts to aggregate. (B) Likely arrangement of MBP and C1-2, allowing CC1-2 to stay stable in solution with the MBP tag. (C) Likely arrangement of MBP with CC1-3, showing why CC1-3 is more easily aggravated. Source: Student generated

In other research works, protein CCDC11 may be referenced as its alias, Cilia- and flagella-associated protein 53 (FAP53). FAP53 has been found on the inner surface of microtubules. Taken together, it appears that CCDC11 (FAP53) plays a large structural role in the formation of microtubules due to its specific location. An analogy to understand this relationship is the scaffolding on a renovated building, or perhaps a portion of the interior frame of a house that supports the structure. Essentially, it can be imagined as a long end-to-end vertical structure that provides support with a periodic horizontal strut or brace for additional structural integrity.

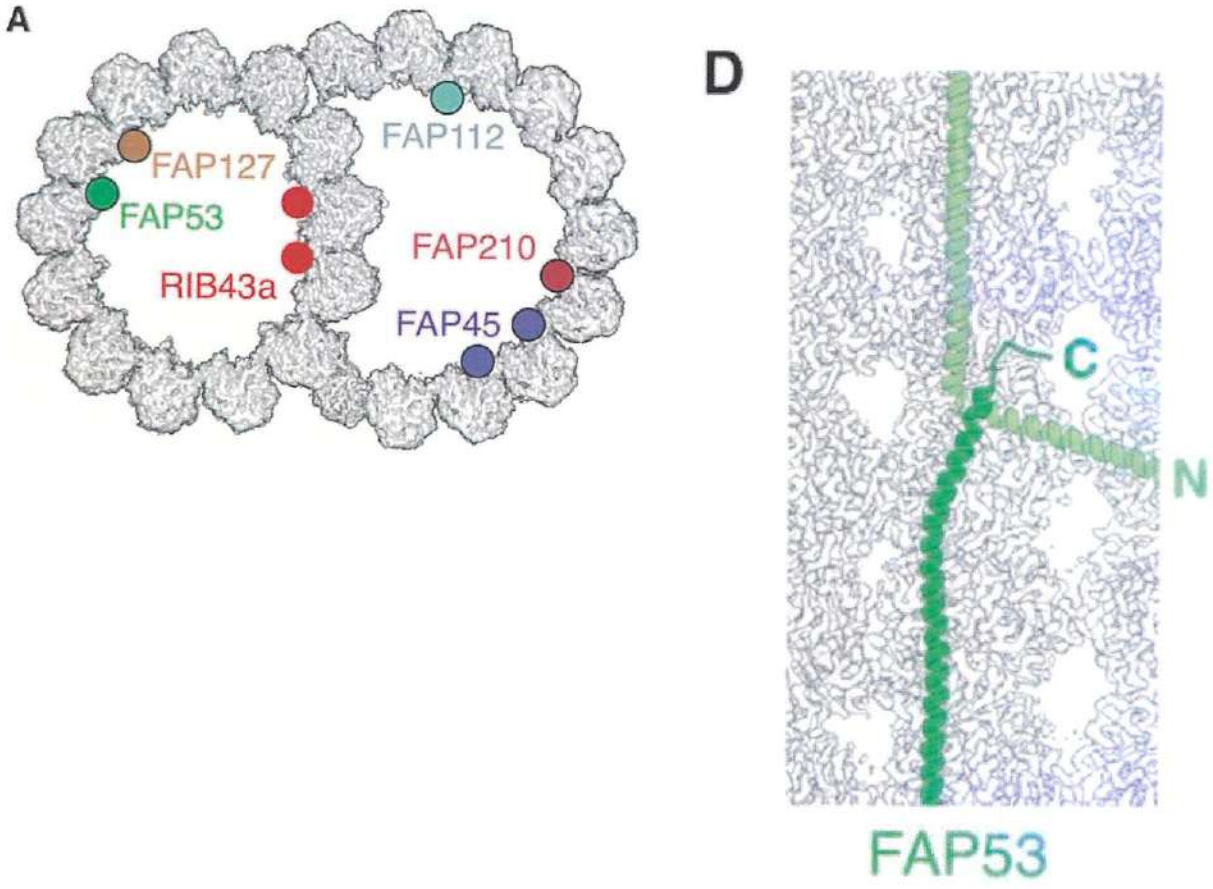

Figure 13: Maintenance of Longitudinal Periodicity through End-to-End Self-Association. (A) The positions of the fMIPs bound to the 48-nm doublet microtubule. There are two copies of RIB43a and FAP45. Diagram shows the specific location (green dot) of longituidnal FAP53 protein which runs along the interior of the "A" tubule of the doublet associated with subunits A6 and A7. (B) Diagram shows the details of the long coiled structure of FAP53 and end-to-end interaction of 2 individual FAP53 protein chains illustrated in light green and dark green. Source:Ma, Meisheng, et al. 2019

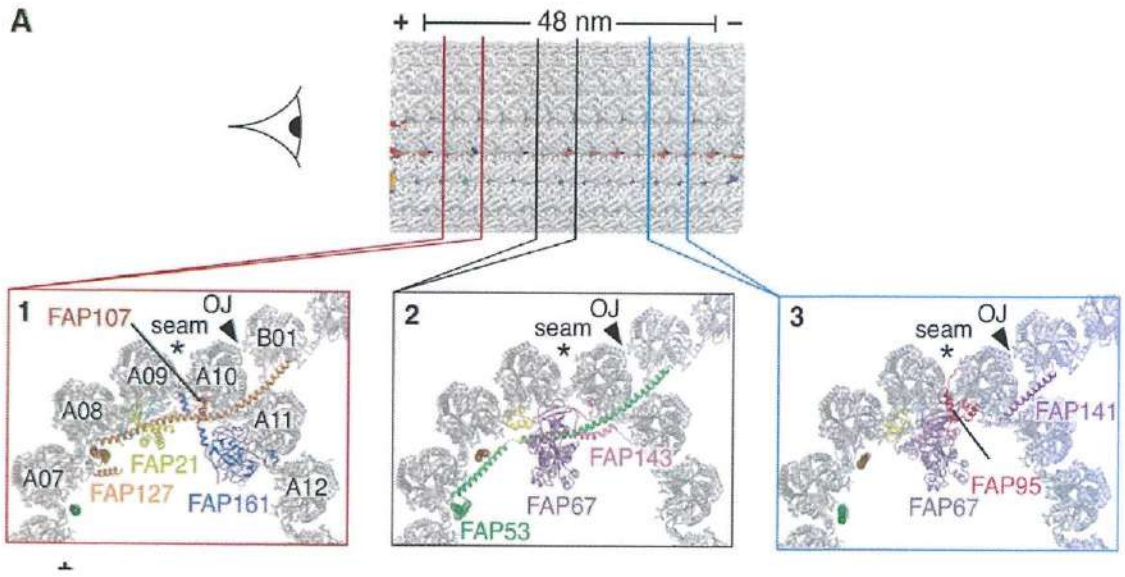

Figure 14: MIPs at the Junctions between Tubules (A) Three slabs (labeled 1–3) through the doublet microtubule showing the locations of MIPs at the seam and outer junction (OJ). The OJ is penetrated by three MIPs: FAP127 (shown in slab 1), FAP53 (slab 2), and FAP141 (slab 3). The seam is recognized by several MIPs including the kinase FAP67. Slab 2 shows that CCDC11 (FAP53) in one region extends across the seam or outer junction of the doublet starting from A6/7 and extending across to A10/B01 where the doublet tubules fuse together. Source: Ma, Meisheng, et al. 2019

Conclusion

The most robust expression of CCDC11 protein is found when grown in Arctic bacteria cultures. CC1-2 is the optimal length of protein to test because it can be produced in larger quantities, it is the most difficult to aggravate, and it is small without compromising the function of CCDC11 full length. Protein purification was not completely successful because much degradation product can be seen within each gel. When run under Anion Exchange, a great deal of protein precipitated, demonstrating aggravation upon removing the MBP tag. There is an interaction between Cby and CCDC11 proteins, but there is not sufficient evidence of a complex formation between Cby and CCDC11. The MBP Tag remains in solution with CCDC1 after

cleaving the TEV sequence and running the samples under HIS and Maltose gravity columns. Our results shed light on the optimal construct length, bacteria strain, and time for TEV Cleavage needed for continued research on CCDC11. Finally, we still remain unsure of the protein's specific function due to the unknown structure.

Future Work

Future research would entail taking steps to ensure complete purification of CCDC11-CC12 constructs by minimizing degradation product produced. Finding a better buffer to keep CCDC11 soluble after MBP tag removal, potentially by going to a higher salt concentration, may permit decreased aggravation. By continuing with isolated CC1-2 constructs, crystallization trials of CC1-2 can occur once steps are taken to remove MBP from the sample. Continuing the overall process with Cc1-3 and ultimately the full length will lead to a determination of the structure of CCDC11 and then its exact function. By finding the structure, mimics or blockers can be developed and the synthetic production of CCDC11 can be used in anti-viral or tumor-fighting drugs.

Future research may also include determining if CCDC11 either directly or indirectly interacts with protein Cby or with the protein Chmp2a, which was newly found to interact with CCDC11. The coexpression of CCDC11 constructs with Chmp2a may alleviate the solubility issue and thus allow for crystallization trials to determine the structure.

References

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