Research Plan

A) Rationale

The coiled-coil domain-containing protein 11 (CCDC11) is known through past studies to play a key part in cytokinesis, including viral replication and cell division. CCDC11 is mainly concentrated in the centrosomes and functions by transporting proteins from the centrosomes to the cilia. Due to its tendency to precipitate when in solution, CCDC11 is studied when attached to a Maltose-Binding Protein (MBP) fusion tag. Since the entire CCDC11 protein is relatively large and easy to disturb, the first two coils of the protein (CC1-2) or the first three coils (CC1-3) will be experimented with. The MBP tag is linked to the protein with a TEV strip, which could be removed by TEV protease.
MBP-CCDC11 was found to interact with another protein MBP-Cby, though it is unknown whether the two proteins touch directly, or interact indirectly via another protein. By isolating the coiled-coil domains CC1-2 or CC1-3, the shape can be determined through crystallization trials, which may potentially lead to antiviral drug design and infection treatments.

B) Hypothesis

Recent studies suggest CCDC11 plays a key role in cytokinesis during cell division and viral replication. Although studies use CCDC11 constructs in tandem with MBP fusion tag, we hypothesize that CCDC11 constructs are better expressed and soluble in water without the MBP tag.

Research Questions

i) Will CCDC-11 CC1-2 constructs remain soluble when the MBP tag is cleaved?
ii) How does MBP-CCDC-11 interact with MBP-Cby? Directly or indirectly?
    (1) Co ip with cby
iii) Does the MBP fusion tag enhance the expression of CCDC11 CC1-2 or CC1-3 constructs?
iv) What is the optimal duration of time for TEV cleavage, ensuring maximum cleavage with minimal precipitation?

Engineering Goals:

We aim to successfully isolate CCDC11 CC1-2 constructs from the MBP fusion tag. Next, we will undergo crystallization trials to determine the structure of the constructs. Then, we will investigate potential blocks or mimics for CCDC11 due to similar structure. The overall goal is to determine the structure of CCDC11 without the MBP tag, thus potentially leading to the controlled expression of CCDC11, which could lead to the prevention of viral budding.

Expected Outcomes

We expect that cleaving the MBP tag from CCDC11 CC1-2 constructs in the appropriate buffer solution will result in minimal precipitation. We also expect that future research would entail performing the cleavage with the CCDC11 CC1-3 constructs.

C) Procedure, Risk and Safety, Data Analysis

a) Procedure:

Bacterial Cell Culture

Bacterial strains including ArcticExpress, BL21 DE3, and Origami will be used. The Arctic bacteria is a strain that thrives at -15°C.

Bacterial Transformation

Four DH5-Alpha Cells will be used as expression vectors to propagate the plasmid. DH5-Alpha Cells are E.coli cells engineered to maximize transformation efficiency. They are defined by three mutations recA1, endA1 which help plasmid insertion. 50-100 µL alloquates were placed in 15 mL falcon tubes of chemically competent bacterial strains ArcticExpress, BL21 DE3, and Origami. These will be subject to a 42°C water bath for 60-70 seconds. The heat shock above body temp allows the DNA to slip in through porous membranes. They then will be placed back 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 and placed at 37°C for 1-1.5 hours. At this point the bacteria will be transferred into 1.5 mL eppendorf tubes and spun down at 3,000 RPM for 1 minute. Once the supernatant is discarded, the pellet will be resuspended in 100 uL of fresh LB, plated onto appropriate plates (LB AMP), and incubated at 37°C for 16 hours.

Preparation of Cultures and Incubation

Dab glycerol stock and streak onto LB/AMP plate. The ampicillin will select for bacteria containing the protein of interest, CCDC11-CC1-2, CCDC11-CC1-3, or CCDC11-FL. This will be incubated overnight at 37°C.

Induction at Specific Optical Densities (OD)

Overnight cultures of Arctic bacterial cells containing CCDC11-CC1-2 and CCDC11-CC-1-3 constructs will be incubated at 37°C for approximately 3-3.5 hours, until an optical density range of 0.1-0.3 is reached. At which point the cultures will be subject to a decrease in temperature from 37°C to 16°C, for an additional hour. Samples will be taken periodically from the cultures until the desired optical densities are reached. Once an optical density of range of 0.5-0.6 is reached, cultures will be induced with 750 µl of IPTG.

Centrifusion

ArcticExpress cultures will be incubated overnight at 15°C to be harvested the following morning. The cultures will be spun down at 4000 RPM for 10 minutes at 4°C and then resuspended in MBP-column buffer. All resuspended cultures will

be frozen at -80°C over the weekend.

Sonication

The induced cultures containing bacterial strains BL21 and ArcticExpress will be thawed and sonicated after addition of 1 μM of phenylmethylsulfonyl fluoride (PMSF). The samples will be sonicated on ice with a microtip at 40% output for 5 rounds of pulses, each lasting 15 seconds and separated by 2 minutes of rest in between. The sonicated lysate will be spun down at 12,000 RPM for 20 minutes to remove pellet formation. The supernatant lysate will then be removed and applied to an MBP column.

Elution using a Gravity Column

Gravity columns are designed for fast purification of proteins. Amylose beads create a bed on the bottom of the column to separate the MBP tag from the protein. First the column itself will be washed with MBP-Fusion Column Buffer. MBP-Fusion Column Buffer will be created with 20 mM TRIS, 200mM NaCl, and 1 mM EDTA. Once the column is washed, the supernatant solution containing the protein will be run through the column. This will be referred to as the Flow Through (FT). Then elution will start. Elution is the process of extracting one material from another by washing with a solvent. MBP-Fusion Elution Buffer will be created to contain 20mM TRIS, 200mM NaCl, 1 mM EDTA, and 10 mM maltose. The protein supernatant solution will be run through the column with the MBP-Fusion Elution Buffer 5 times and fractions will be collected during each flow.

SDS-PAGE Gel

To prepare the 10% separation gel, the following will be mixed respectively: 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 will polymerize quickly, so these reagents will be added when ready to pour. The gel will be poured, leaving ~2 cm below the bottom of the comb for the stacking gel. The top of the gel will be 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 will be completely polymerized, as which point the isopropanol will be removed and washed out with distilled water.

Stacking Gel

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 will be poured on top of the separation gel, followed by combs to make wells. In ~30 min, the stacking gel should become completely polymerized. Clamp the gel into apparatus, and both buffer chambers will be filled with gel running buffer according to the instructions for the specific apparatus. At this point, the samples will be loaded into wells for separation by gel electrophoresis.

b) Risk and Safety:

i) Human Subjects: N/A

ii) Vertebrate Animals: N/A

iii) Potentially Hazardous Biological Agents (PHBA): Bacterial strains including ArcticExpress, BL21 DE3, and Origami will be used. When handling, proper safety equipment will be worn at all times, including goggles, gloves, and lab coats. Contaminated pipette tips and gloves will be placed in red biological hazard containers. Contaminated instruments and containers will be sanitized using a bleach solution.

iv) Hazardous Chemicals/Activities/Devices: When handling chemicals, appropriate attire will be used. These include gloves, goggles, aprons, closed-toed shoes, and long pants. No loose clothing will be worn and hair will be tied back. Hazardous materials will be properly disposed of in designated bins. Reagents will be handled with care as described below. Proper supervision will be provided at all times.

(1) LB Broth Powder has level zero health hazards, zero flammability, and zero reactivity. It may cause eye or skin irritation. Inhalation and ingestion should be avoided. Water is the recommended cleansing agent for disposal. One should wear safety goggles, gloves, and protective clothing.

(2) Glycerol may cause mild eye and skin irritation. Ingestion may cause irritation of the digestive tract. Inhalation may cause respiratory tract irritation. One should wear protective clothing, chemical splash goggles, and protective gloves. It is stable under normal temperature and pressure conditions. It should be disposed of in a manner consistent with federal, state, and local regulations.

(3) Glucose has level zero health hazards, level 1 flammability, and level zero reactivity. There are no health effects anticipated for eye contact, skin contact, or inhalation. Ingestion of large amounts may cause gastrointestinal disturbances. When it burns, it can release toxic vapors. For empty containers that held glucose, rinse three times with an appropriate solvent then dispose of it as normal trash.

(4) Active Dry Yeast has level zero health hazard, level zero flammability, and level zero physical hazard. Prevent it from reaching drains, sewers, or waterways. Minimize dust generation and accumulation. Store away from strong acids or bases and oxidizing agents.

(5) Ampicillin Sodium Salt has a level two health hazard, level one flammability, and level zero reactivity. It may cause an allergic skin reaction and breathing difficulties if inhaled. Wear protective gloves, use only in a well ventilated area, and do not breathe in the dust. Dispose of contents to an approved waste disposal plant.

(6) Hydrochloric Acid (HCl) may cause irreversible eye damage and severe burns to the eyes. Skin contact with liquid is corrosive and will cause burns. Ingestion causes severe digestive tract burns with vomiting, abdominal pain, and possible death. May be fatal if inhaled. It is stable under normal temperatures and pressures. Wear protective gloves, clothing, eyewear, and facewear. Wash hands thoroughly after using. Do not get in eyes, on skin, or on clothing. One must consult state and local hazardous waste regulations for proper disposal.

(7) Sodium Chloride (NaCl) may cause eye irritation, skin irritation, digestive tract irritation, and respiratory tract irritation. May be combustible at high temperatures. Use with adequate ventilation and try to minimize dust accumulation. It is hygroscopic which means that it absorbs water from the air. For disposal purposes, chemical waste generators must determine whether it classifies as a hazardous waste.

(8) Ethylenediaminetetraacetic acid (EDTA) is a hazardous chemical. It is acute oral toxicity category 4 if swallowed. It is in skin corrosion/irritation category 2, serious eye damage category 2A, and specific target organ toxicity Category 3. One should wear safety goggles, gloves, and a dust/aerosol mask. Avoid storing it in extremely high or low temperatures. Avoid breathing in dust. Avoid disposing by releasing into the environment.

(9) Tris(hydroxymethyl) aminomethane hydrochloride- (TRIS) is a hygroscopic substance level 2 health hazard, level 1 flammability hazard, and level 1 instability hazard. Avoid contact with skin and eyes. Avoid breathing in dust, ingestion, and inhalation of this substance. Do not empty TRIS into drains. Chemical waste generators must decide if a discarded chemical is classified as hazardous.

(10) Maltose is a level 1 health hazard substance, level 1 flammability, and level 0 physical hazard. One should wear gloves, eye protection, and protective clothing. If inhaled, move the exposed individual to fresh air and give artificial respiration if needed. If swallowed, rinse the mouth thoroughly. After skin contact, wash hands and exposed skin with soap and water. After eye contact, flush the exposed eye with water for 15-20 minutes. Contact a licensed professional waste disposal service to dispose of this substance.

(11) Isopropyl β- d-1-thiogalactopyranoside (IPTG) is classified as Acute Oral Toxicity Level 4, Acute Dermal Toxicity Level 4, and Acute Inhalation Toxicity Level 4 . If the substance is inhaled, move the patient to fresh air, keep the patient warm and at rest. If symptoms persist, obtain medical attention. After ingestion, rinse the mouth and obtain medical assistance if needed. After skin

contact, remove affected clothing and wash all exposed skin with mild soap and water, followed by warm water rinse. Obtain medical attention if needed.Wear protective gloves, safety goggles with side-shields, and long-sleeved protective clothing. Dispose of it in a safe manner in accordance with local/national regulations.

(12) PMSF= Serine Protease inhibitor may cause respiratory system irritation, eye burns, and skin burns. If inhaled, remove to fresh air. If contacted with skin, wash immediately with soap and water for at least 15 minutes. Use safety goggles, lab coat, and chemical resistant gloves for protection. Dispose of this substance in accordance with local, state, and federal regulations.

(13) Tris-glycine SDS Running Buffer is a level two health hazard, level zero flammability, and level one reactivity hazard. It may cause skin irritation, eye irritation, and may be harmful if inhaled or ingested. If inhaled, move to an area of fresh air. If ingested, rinse the mouth with water and seek medical attention. After skin exposure, wash skin with mild soap and water. After eye exposure, rinse eyes with water for 15 minutes minimum. Seek medical attention when needed. Wear chemical resistant gloves and work under a general exhaust system. Dispose of this substance according to federal, state, and local regulations.

(14) Ladder- contains components glycerol which was previously addressed and sodium dodecyl sulphate. Sodium dodecyl sulphate is harmful if swallowed, causes skin irritation, causes eye irritation, and is toxic when in contact with skin. Wear protective clothing, safety glasses, and synthetic protective gloves when dealing with this substance. Do not allow it to enter any sewers or the groundwater. Dispose of Sodium dodecyl sulphate in accordance with official regulations. Water is the recommended cleansing agent.

(15) Amylose- The binding beads used in the gravity column were treated with amylose. Amylose may cause serious eye irritation.

(16) TEV Protease- Although this product does not contain any known hazard ingredients, usual precautions are to be taken when handling. Follow Good Hygiene practices in handling. Avoid contact with eyes, skin, and clothing. Avoid ingestion. Wear appropriate protective gloves and clothing to prevent skin exposure. Dispose according to local, state and federal regulations.

c) Data Analysis:

i) <u>SDS Page Analysis of the Elution Fractions Gels</u>

As shown by the gel electrophoresis samples using arctic bacteria, there is no good expression with the full length CCDC11, therefore we will not focus on it. However, there is in fact robust expression with CCDC11-CC1-2 and some expression presented by CCDC11-CC1-3,

therefore the experimentation will continue with focus on specifically CC1-2 and CC1-3. The most robust expression of the CCDC11 protein is found when grown in Arctic bacteria. Testing with the MBP column proved to be inconclusive since the maltose was so tightly bound to the protein itself. A His column will be used to determine if this column provides better purification. Protein purification is not completely successful, as the MBP Tag remains in solution with CCDC11, therefore we remain unsure of the protein's specific function due to the unknown structure.

D) Bibliography

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NO ADDENDUM EXIST