THE ROLE OF ELONGATOR COMPLEX IN THE REGULATION OF NEURONAL EXOCYTOSIS

A Thesis

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In Partial Fulfillment of the Requirements for the Degree of

Master of Science

by

Seunghye Han

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ABSTRACT

Familial dysautonomia is a neurodegenerative disease characterized by defects in the development and survival of autonomic and sensory neurons. The main cause of this disease is known to be the mis-splicing of Elp1, which is the IKB-complex-associated protein. Elp1, Elp2, Elp3 were identified as regulators of Sec2, which is a guanine nucleotide exchange factor for the Rab protein Sec4. Sec4 is a key Rab protein that regulates exocytosis in yeast, and therefore, Elp proteins are considered to be playing a role in the regulation of exocytosis in yeast. On top of this finding, we expect that they also play a role in exocytosis of neuronal cells such as neurotransmission and synaptogenesis. Here, I am investigating the hypothesis that the Elp proteins play an important role in synaptogenesis during development in neuronal cells.

Elp proteins are components of a six-subunit (Elp1p-Elp6p) elongator complex and this elongator complex is composed of two biochemically separable subcomplexes, Elp1/2/3p and Elp4/5/6p. The Elp1/2/3p subcomplex has clear structural motifs that suggest scaffolding and enzymatic activities, while the role of the Elp4/5/6p subcomplex remains unclear. Here we try to identify how elongator subunits interact with each other and downstream players to regulate secretion. Also, to study the neuronal exocytosis in vertebrate system, zebrafish are advantageous as their embryos develop outside of the mother and are transparent, allowing experiments and analysis on the living embryo during early neurogenesis. In the future, we plan to investigate the roles that Elp proteins play in neurogenesis by observing their effects on zebrafish embryos.

BIOGRAPHICAL SKETCH

Seunghye Han was born March 11, 1989 in Seoul, Korea. She received her Bachelor of Life Science and Biotechnology degree in Underwood International College from Yonsei University at Korea in 2011. She worked in Protheon, a company that is supervised by Professor Baiklin Seong in Yonsei University from 2009 to 2011. In 2011 she joined Biochemistry, Molecular and Cell Biology program at Cornell University in Ithaca. She has served as a Research Assistant and Teaching Assistant in the Department of Molecular Biology and Genetics at Cornell University.

ACKNOWLEDGMENTS

First and foremost I would like to thank my supervisor, Dr Ruth Collins, who has supported me throughout my research with her patience and knowledge whilst allowing me the room to work in my own way. She was friendly and her encouragement and effort guided me throughout this thesis.

In my daily work I have been blessed with friendly and cheerful lab members. Duane Hoch provided me with good ideas and help with using the equipments in the lab. He always gave a great discussion and ideas when an obstacle was met. Dante Lepore gave a lot of fresh ideas and was always cheerful, giving a laugh to the lab.

Dr Joseph Fetcho gave useful guidance in the use of the zebrafish that could be done in my future research. I have been aided in running the equipments by Brian Miller, a fine technician who taught me how to deal with the zebrafish and their eggs, and injecting the DNA into the embryos. All the lab members of Fetcho lab were friendly and kind enough to help me with using the equipments from the lab.

Dr Sylvia Lee has offered much advice on the thesis and also on my life so I can get through the hard times. My studies have been funded by the Program in Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca and the Department of Molecular Medicine This work was supported by a grant from the National Institutes of Health (5R01GM069596) to R.N.C.

Finally, I thank my parents for supporting me throughout all my studies and my life of studying abroad.

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CHAPTER Ⅰ

Introduction

Familial dysautonomia is a disorder of the autonomic nervous system which affects the development and survival of neurons in the autonomic and sensory nervous system. Its symptoms include insensitivity to pain, inability to produce tears, poor growth, and labile blood pressure etc. FD does not affect intelligence, but people with FD have frequent vomiting crises, pneumonia, problems with speech and movement, difficulty swallowing, inappropriate perception of heat, pain, and taste, as well as unstable blood pressure and gastrointestinal dysmotility. They also show a defect during development when they grow.

The common FD-causing mutation is on IKBKAP gene, which is the homolog of Elp1. On normal transcription, exon 19 and exon 20 is fully transcribed into the mRNA, producing a wild-type, functional IKAP protein. In Familial dysautonomia patients, the exon 20 is excised out, resulting into a mutant IKAP protein that is not functional. The inability to produce the functional IKAP protein leads to the phenotype of the disease. IKAP gene is the homolog of Elp1 gene and therefore, the fact that mis-splicing of Elp1 leads to a neurodegenerative disease suggests that Elp complex may be playing a role in neuronal cells.

Elongator complex proteins were first identified when they were pulled down with RNA polymeraseII. Therefore, it was first thought to be playing a role in the transcription process inside the nucleus, interacting with RNA polymeraseII. However, further studies on these proteins showed that these proteins were localized more in the cytoplasm rather than in the nucleus. Moreover, efforts to identify the interaction of these proteins with other transcription machinery proteins didn’t yield much result, but more studies have been done with the cytoplasmic role of the proteins.

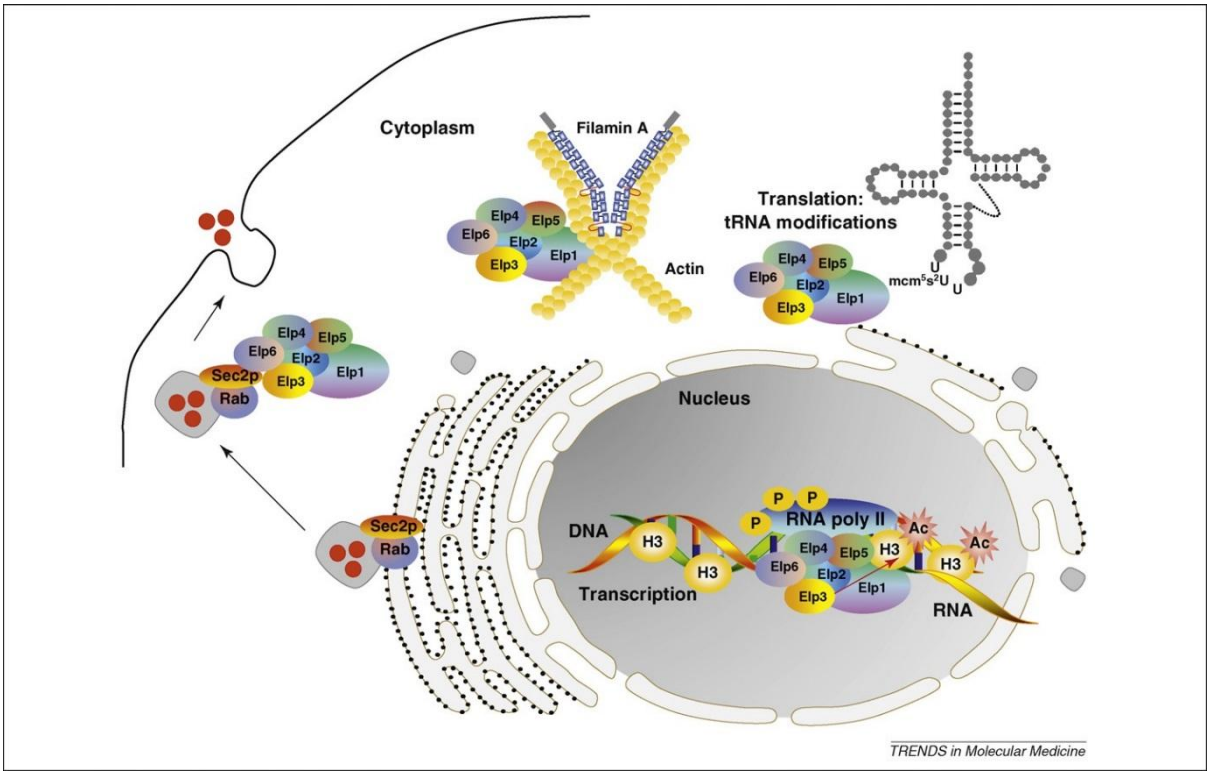


Figure 1.1 The biological roles played by Elongator complex.
The Elongator complex was originally thought to play its major roles in the nucleus, associating with hyperphosphorylated RNA polymerase II and acetylating across the transcribed regions of multiple genes. However, its cytoplasmic role started to be widely studied and it is found that the Elongator complex also plays a role in the regulation of actin cytoskeleton organization, negative regulation of exocytosis, tRNA modification regulation. (Nguyen, Humbert, Saudou, & Chariot, 2010)

One of the roles that Elp proteins were found to have was regulation of exocytosis. In previous study done by the collins lab, Elongator complex subunits Elp1,2,3 were identified as negative regulators exocytosis. They made a temperature sensitive mutant yeast strains of sec2, which were unable to localize sec2 so that they are defective in secretion at restrictive temperature. Knocking out Elp1, or Elp2, Elp3 showed suppression of the temperature sensitive mutants and cells were able to grow even on the restrictive temperature. The yeast Rab protein sec4 regulates exocytosis of post-Golgi secretory vesicles, by binding to the exocyst complex, and targets the vesicle to the bud tip. Sec4 activation is regulated by sec2, which acts as a GEF for sec4. Thus, the fact that Elp 1,2,3 regulates sec2 implies that the Elp proteins play a role in the regulation of exocytosis.

As Elp proteins were found to be playing a role in regulating exocytosis in yeast, we expect that they also play a role in exocytosis of neuronal cells such as neurotransmission and synaptogenesis. Therefore, I hypothesize that the Elp proteins play an important role in synaptogenesis during development in neuronal cells.

In yeast, Elp proteins form a six-subunit (Elp1p-Elp6p) elongator complex by putting two biochemically separable subcomplexes, Elp1/2/3p and Elp4/5/6p together. The Elp1/2/3p subcomplex has clear structural motifs that are known to be playing a role in scaffolding and enzymatic activities. However, the Elp4/5/6p subcomplex and their function remains unclear. Therefore, here we try to investigate the structure and function of the Elp4/5/6p subcomplex.

To investigate the role of Elp proteins in development of neuronal cells, we wish to use zebrafish as the model organism. Zebrafish is a model organism that is commonly used for both neurobiology and developmental biology due to its advantages such as fully sequenced genome, easily observable and testable developmental behavior etc. Its embryonic development is very rapid and occurs outside of their mother which makes it easier to observe the development. The embryo of zebrafish is transparent, and a transparent mutant strain is readily available, making it even more advantageous for research. Therefore, here we try to investigate if the Elp4/5/6p zebrafish orthologs also form a subcomplex and how they interact with each other. This study will enable further studies on what roles they play in neuronal exocytosis in zebrafish.

CHAPTER Ⅱ

Study of Sec2 Structure and its Functionality

Introduction

Rab GTPases are critical factors that regulate vesicular trafficking in eukaryotic cells. Rab GTPases function as a nucleotide dependent molecular switch, and this GTP-GDP switch is highly regulated. GTPase-activating protein, called GAP, facilitates the hydrolysis of the GTP. Guanine Nucleotide Exchange factors, called GEF, trigger the GDP release and thus promote GDP-GTP exchange, which will activate the Rab GTPase.

The yeast Rab protein sec4 regulates exocytosis of post-Golgi secretory vesicles, by binding to the exocyst complex, and targets the vesicle to the bud tip. Sec4 activation is regulated by sec2, which acts as a GEF for sec4. Thus, the regulation of sec2 impacts the regulation of sec4.

In order to identify the how sec2 functions, we tried to find out the important domains of the sec2 protein. And one of the domains we got interested was the coiled-coil domain within sec2 because previous studies showed that this domain is critical for the proper functioning of its GEF activity. Therefore, we tried to replace a portion of this coiled-coil domain with coiled-coil domains of other known proteins to test whether the protein would be still functional.

The coiled-coil domain of sec2 was replaced with that of Myo2, GCN4, and Human Rabin3. These proteins were selected because similar to sec2, they also have a coiled-coil domain at the N-terminus, and show high sequence similarity to sec2 especially after the coiled-coil domain.

Results

We constructed mutant Sec2 plasmids based on pRS315 vector. These plasmids were transformed into the yeast strain that has its genomic Sec2 gene deleted. The strain was still able to survive because it carried the pRS426 plasmid that contains wildtype Sec2 gene and URA3 gene instead. The URA3 encodes for uracil which can turn 5FOA into 5-fluorouracil, a toxic compound. The transformed yeasts were grown on 5FOA plate so they would select the wildtype Sec2 plasmid out due to the uracil produced by the plasmid.

As shown in Fig. 2.1, Myo2 mutant transformant grew as poorly as vector alone on 5FOA plate, indicating that this mutant was not able to function as Sec2. However, GCN4 mutant transformant was able to grow in a slow rate compared to wildtype Sec2 (Fig 2.1), indicating that GCN4 mutant Sec2 may be partially functional. Rabin3 mutant transformant was able to grow almost in a same rate as wildtype Sec2 (Fig 2.2), indicating that this mutant Sec2 is almost fully functional.

From this experiment, we were able to find out that Myo2 mutant Sec2 could not substitute wildtype Sec2, while GCN4 mutant were able to partially function as a Sec2. Rabin3 mutant was able to fully substitute the wildtype Sec2, indicating that this mutant Sec2 is fully functional. The functionality of these mutants may be explained by their structural similarities. It may be interesting to find out if the Rabin3 insert has more structural similarity to the wildtype Sec2 compared to the GCN4 or Myo2 insert by superimposition of its 3D structure with the wildtype Sec2.

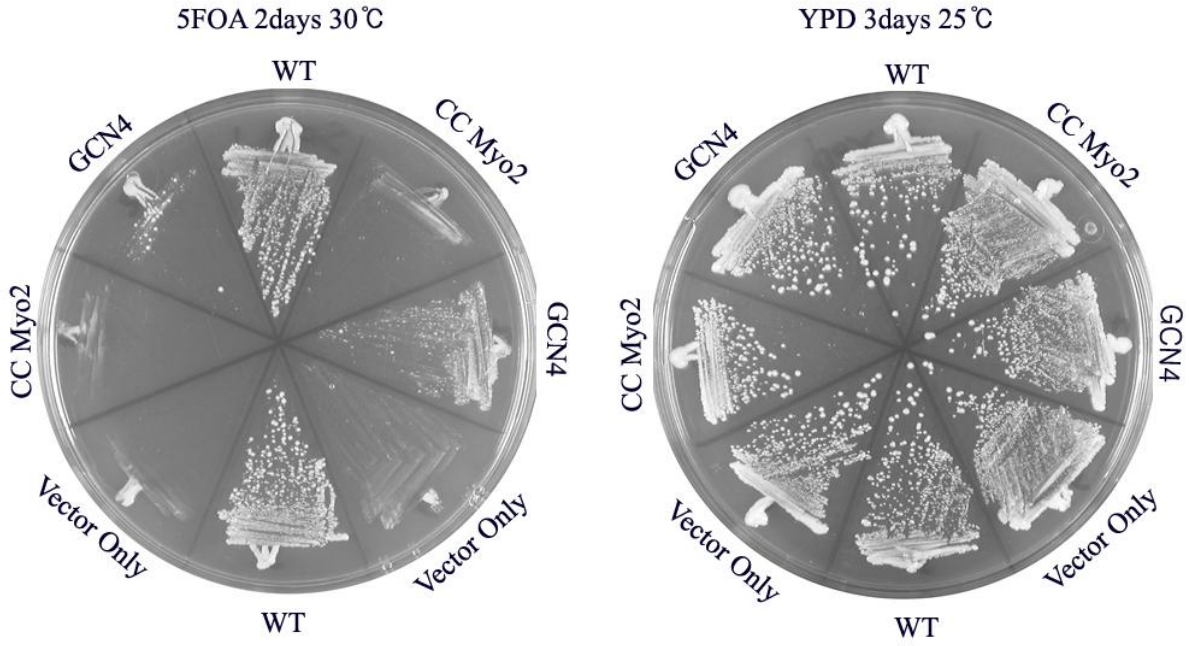


Figure 2.1 Growth of Sec2 mutants Myo2 and GCN4 on 5FOA plates.
The yeast transformed with myo2 mutant Sec2 cannot grow in 5FOA plate indicating that the mutant Sec2 is not functional. The yeast transformed with GCN4 mutant Sec2 is able to grow on 5FOA plate. Compared to wildtype, its growth is weak, indicating that the mutant Sec2 may be partially functional.

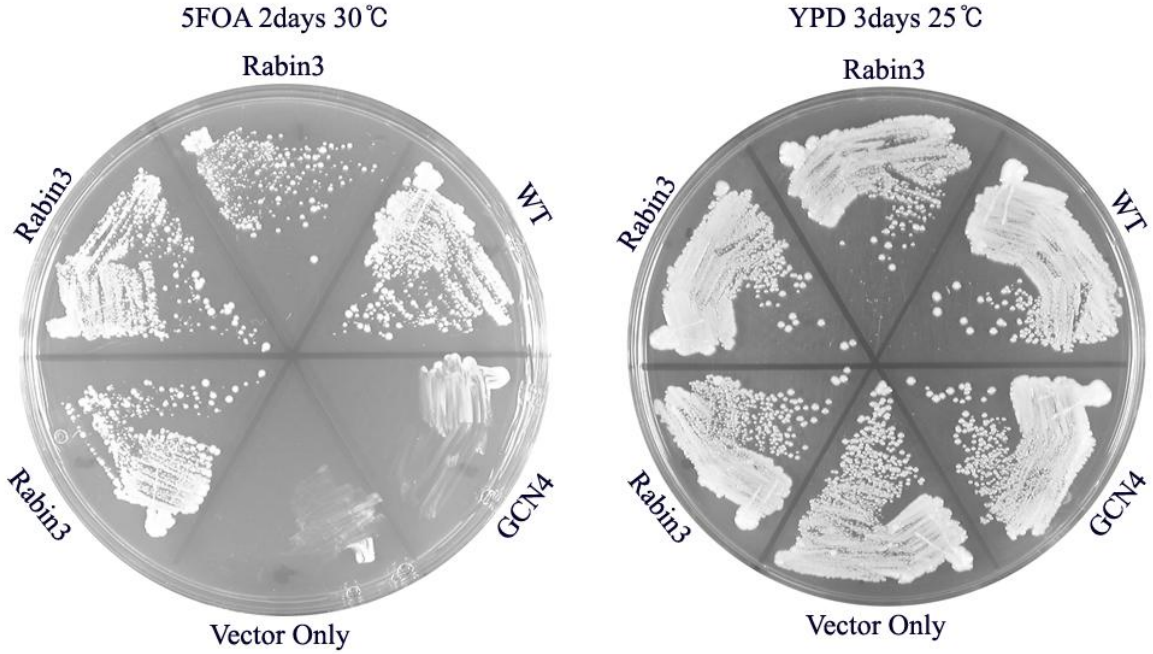


Figure 2.2 Growth of Sec2 mutants Rabin3 and GCN4 in 5FOA plates.
The yeast transformed with Rabin3 mutant Sec2 grows well on 5FOA plate, comparably to the wildtype Sec2, indicating that the mutant Sec2 is highly functional.

Tables of Plasmid and Strain List

Yeast Strain
Sec2ΔKan^r Ura3-52, Leu2-3, 112, HisΔ200 [YCP50 Sec2]

Plasmid

Vector	RCB No.	Mutant	Description	Sequencing Order No.
pRS315	RCB540		Vector only	
	RCB667		GFP-Sec2 Wild type	
	RCB736	Myo2	Myo2 CC domain inserted between amino acids 58 and 101 of Sec2p. Insert 942 VIELT QNLAS KVKEN KEMTE RIKEL QVQVE ESAKL QETLE NM 988	1027880
	RCB2800	GCN4	GCN4 CC domain inserted between amino acids 58 and 101 of Sec2p. Insert 243 RARKL QRMKQ LEDKV EELLS K 262	1027880
	RCB5296 A~C	Rabin3	Rabin3 CC domain inserted between amino acids 59 and 108 of Sec2p. Insert 159 ERLKE ELAKA QRELK LKDEE CERLS KVRDQ LGQEL EELTA S 199	
pRS426	RCB153	Rabin3	Rabin3 CC domain inserted between amino acids 59 and 108 of Sec2p. Insert 159 ERLKE ELAKA QRELK LKDEE CERLS KVRDQ LGQEL EELTA S 199	1027880

The insert sequence of RCB5296 is taken from RCB153 and Yeast Transfomation Protocol from Collins lab book was used. RCB736, RCB2800, RCB153 was made before me.

Materials and Methods

Transformed yeasts were selected and grown on 5FOA plates and YPD plates starting at the same time. 5FOA plates were grown at 30℃ and YPD plates were grown at 25℃. pRS315-Wildtype Sec2 was used as positive control and pRS315 vector only was used as negative control.

CHAPTER Ⅲ

Study of Zebrafish Elongator Protein 4/5/6 Subcomplex Structure

Introduction

Elongator complex consists of two subcomplexes, Elp1, Elp2 and Elp3 subcomplex and Elp4, Elp5 and Elp6 subcomplex. Elp1, Elp2 and Elp3 subunits have been widely studied and their function is relatively more known. Elp1 function as a scaffolding protein while Elp3 plays a key catalytic role of acetylation. However, compared to the Elp1, Elp2 and Elp3 subcomplex, the role of Elp4,5 and 6 subcomplex is relatively unknown and therefore, here we tried to study the function of elp4,5 and 6 subcomplex.

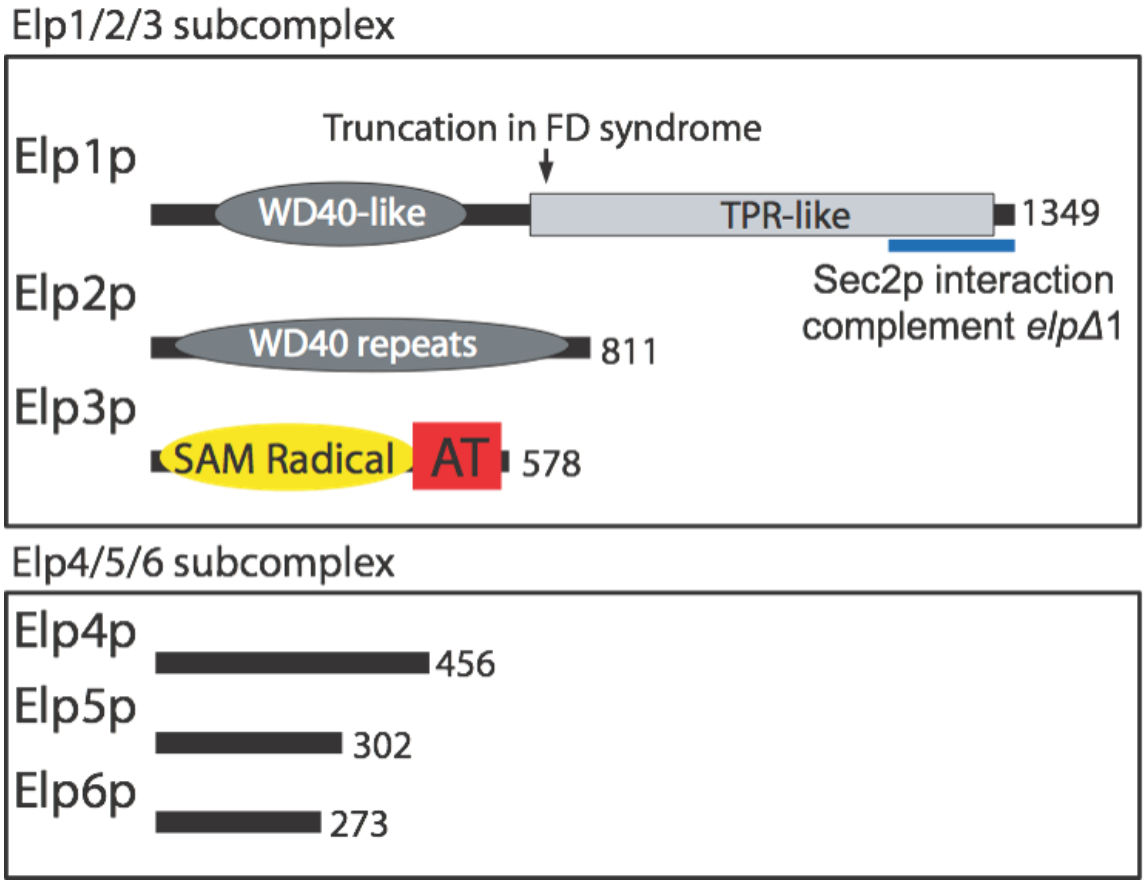


Figure 3.1 Elp1, Elp2 and Elp3 subcomplex and Elp4, Elp5 and Elp6 subcomplex
Elp1, Elp2 and Elp3 subcomplex have been widely studied and functions of each subunit are known. However, Elp4, Elp5 and Elp6 subcomplex and function of each subunit are relatively less studied.

Previous studies have shown that *Saccharomyces cerevisiae* Elp4, Elp5, and Elp6 forms a complex and its crystal structure was also identified. In order to find out if the *Danio rerio* Elp4, Elp5, and Elp6 (abbreviated as drELP) we are using in this project are the correct orthologs of the Saccharomyces cerevisiae Elp4, Elp5, and Elp6, we investigated if the *Danio rerio* ones were also able to form a complex.

To test if drELP4, drELP5, and drELP6 interact with each other, we designed an experiment where we can express the three proteins together at once and pull down one of the proteins to find out if the other proteins are pulled down together. The pull down was done by tagging with His. If the proteins other than the tagged protein were pulled down, it would indicate that these proteins are interacting with each other to form a complex.

In the study of *Saccharomyces cerevisiae* Elp4, Elp5, and Elp6, it was identified that the three proteins interacted with each other in 1:1:1 ratio to form a complex. To find out if this is also true for the *Danio rerio* Elp4, Elp5, and Elp6, we tried size-exclusion chromatography, so that we can first estimate the size of the complex and compare it to the expected molecular weight of the complex.

Results

We constructed drELP4, drELP5 and His-drELP6 to be expressed together by one vector. The pull down of drELP4,5,His-6 clearly yielded three bands A, B and C (Fig. 3.2 drELP4,5,6 lanes) suggesting a successful formation of the complex. However, because drELP5 and his-tagged drELP6 were supposed to have a similar molecular weight (drELP5 33kD, drELP6-His 32kD), we were not sure if the band B actually consists of two bands and the lower band is a non-specific binding to the complex. Also, band B shown on the gel had a higher molecular weight compared to the known molecular weight of drELP5. Therefore, to confirm if the band B is drELP5, we tagged drELP5 with EGFP. Here, we were able to see the bandshift (Fig 3.2 band B and B') by the EGFP tag, suggesting that the band B is drELP5 and the band C may be the drELP6. However, the band size of drELP6 was much smaller than what we expected, so we headed on for western blotting.

In Fig. 3.3, we performed a western blot of the pull-down gel shown in Fig. 3.2. As drELP6 was the only protein tagged with His in this construct, we used anti-His to find out where the drELP6 is shown on the gel. Here, we were clearly able to see that the band C on Fig 3.2 does have the his-tag, indicating that it must be the drELP6 being expressed. It seemed like the protein was having some degradation issues, because we were able to see another smaller band below. If it were being degraded, we assumed that it must occur on the C-terminus because the His tag was on the N-terminus of drELP6. To find out if the protein was being degraded, we made a new construct that had drELP6 C-terminally tagged with His.

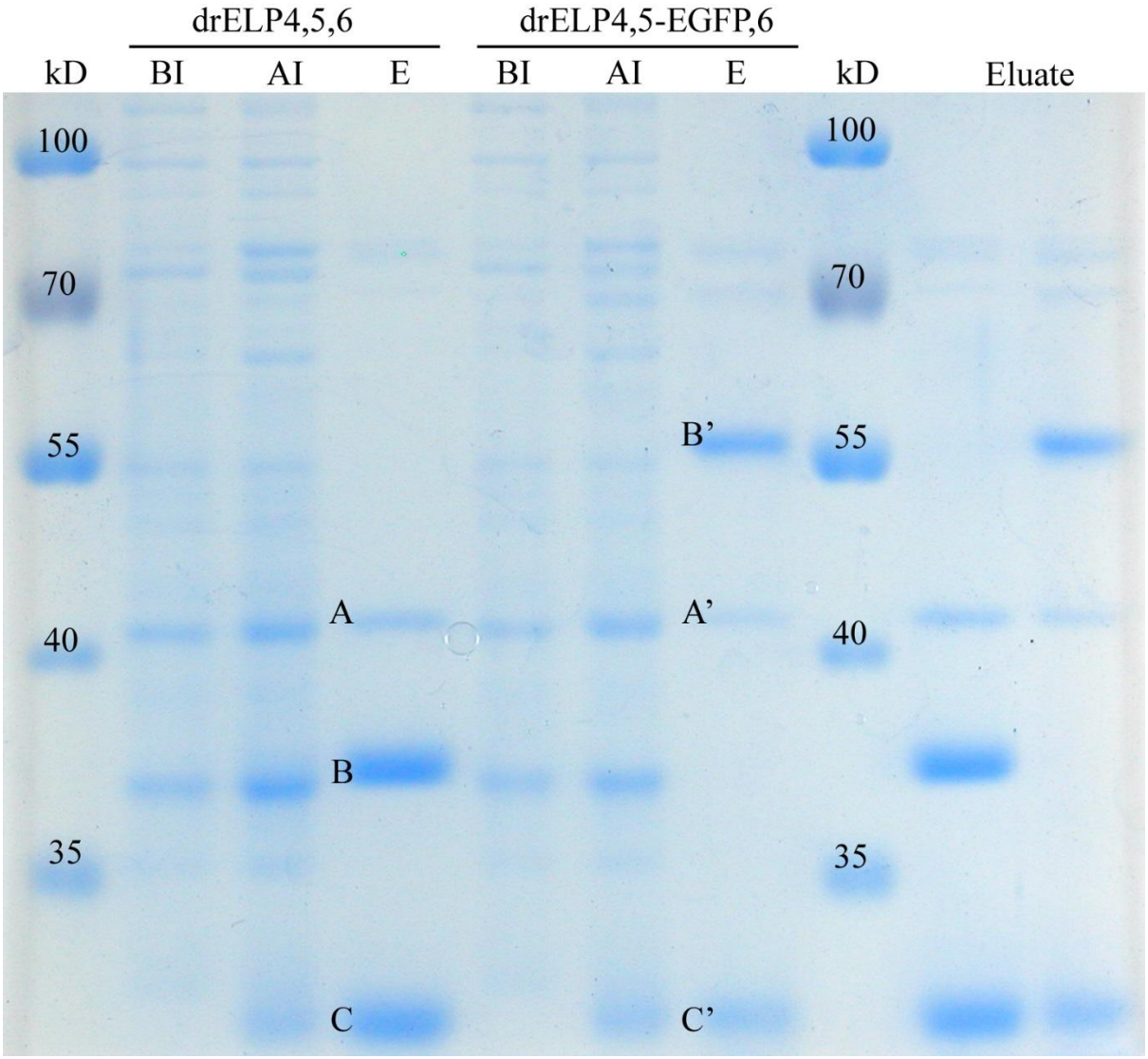


Figure 3.2 Expression of drELP4,5,His-6 and drELP4,5-EGFP,His-6
DrELP4,5,His-6 and drELP4,5-EGFP,His-6 were expressed in BL21(DE3) RIL and pulled down by cobalt resin. BI indicates 'Before Induction', AI indicates 'After Induction', and E indicates 'Eluate'. 1st and 8th lanes are size marker. Bands A and A' indicate drELP4, bands C and C' indicate drELP6, band B indicates drELP5 and band B' indicates drELP5-EGFP.

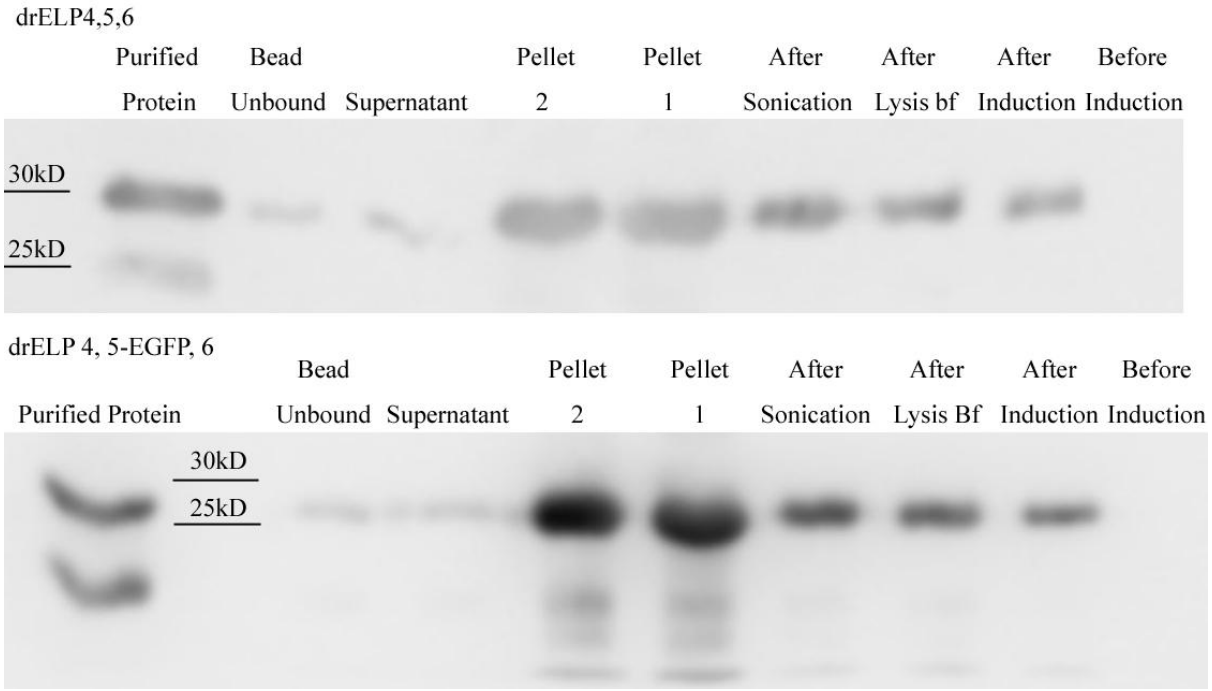


Figure 3.3 Western Blot of drELP4,5,6 and drELP4,5-EGFP,6 by His tag.
DrELP4-5-6 and drELP4-5-EGFP-6 was expressed in BL21(DE3) RIL and pulled down, western blotted with anti-His. Only drELP6 is tagged with His, so the bands shown may indicate drELP6 fragments that contain His tag.

In Fig. 3.4, we can see the N-terminally tagged drELP6 pulling down the drELP4/5/6 subcomplex (lanes 2-4), and the similar band pattern showing up with the C-terminally tagged drELP6 (lanes 5-7). The shorter band size on the C-terminally tagged drELP6 is due to the short linker between the protein and the tag compared to the N-terminal tagged drELP6. This result indicates that drELP6 is not being degraded in the C-terminus when forming the drELP4/5/6 subcomplex. We are still not sure why drELP6 shows a band with smaller molecular weight than expected, and this is a subject of further study.

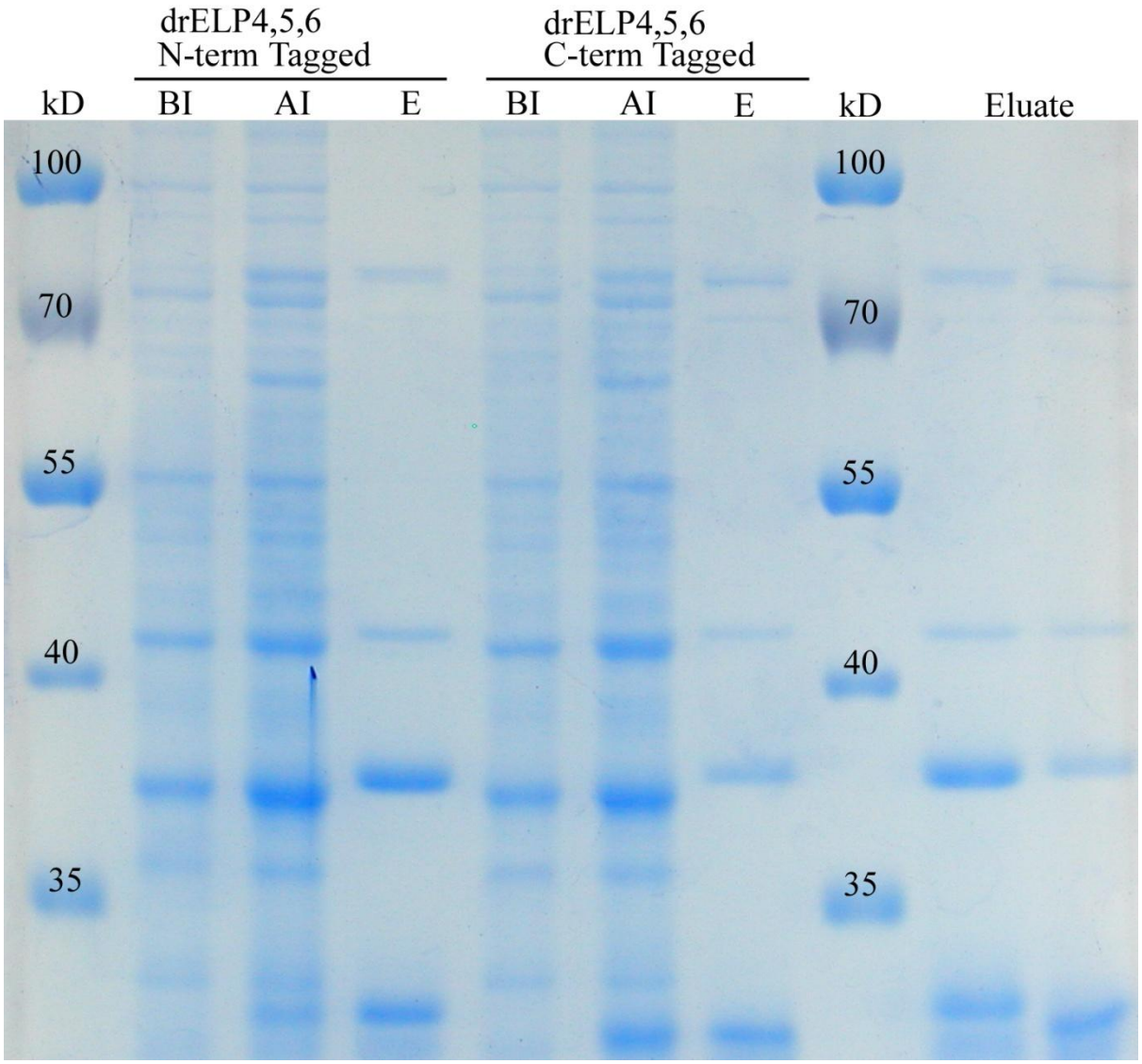


Figure 3.4 Expression of drELP4,5,His-6 and drELP4,5,6-His
DrELP4,5,His-6 and drELP4,5-EGFP,6-His were expressed in BL21(DE3) RIL and pulled down by cobalt resin. BI indicates 'Before Induction', AI indicates 'After Induction', and E indicates 'Eluate'. 1st and 8th lanes are size marker.

In order to find out the structure of the drELP4/5/6 subcomplex, we wanted to first find out how large the complex is. In Fig. 3.5, we purified drELP4/5/6 subcomplex using the His tag on drELP6 and ran the complex on a size-exclusion chromatography. We were able to see three separate peaks on 280nm absorbance, indicating that there may be two different sizes of drELP4/5/6 subcomplex that are formed. To estimate the size of the proteins that were separated

by the column, we ran another run with proteins of known molecular weights on the same column (Fig. 3.6).

In Fig. 3.6 B and C, the estimated molecular weight of the protein from first peak is more than 2000kD, indicating that it is most likely to be aggregated proteins. The estimated molecular weight of the protein from second peak is around 521kD, and third peak around 129kD. The estimated size of the drELP4/5/6 subcomplex consisting of one of each subunit is around 112kD, suggesting that the peak 3 may be this subcomplex. Peak 2 suggest that multiple drELP4/5/6 subcomplexes could interact with each other, forming a larger complex, and to investigate this possibility, methods such as X-ray crystallography could be used.

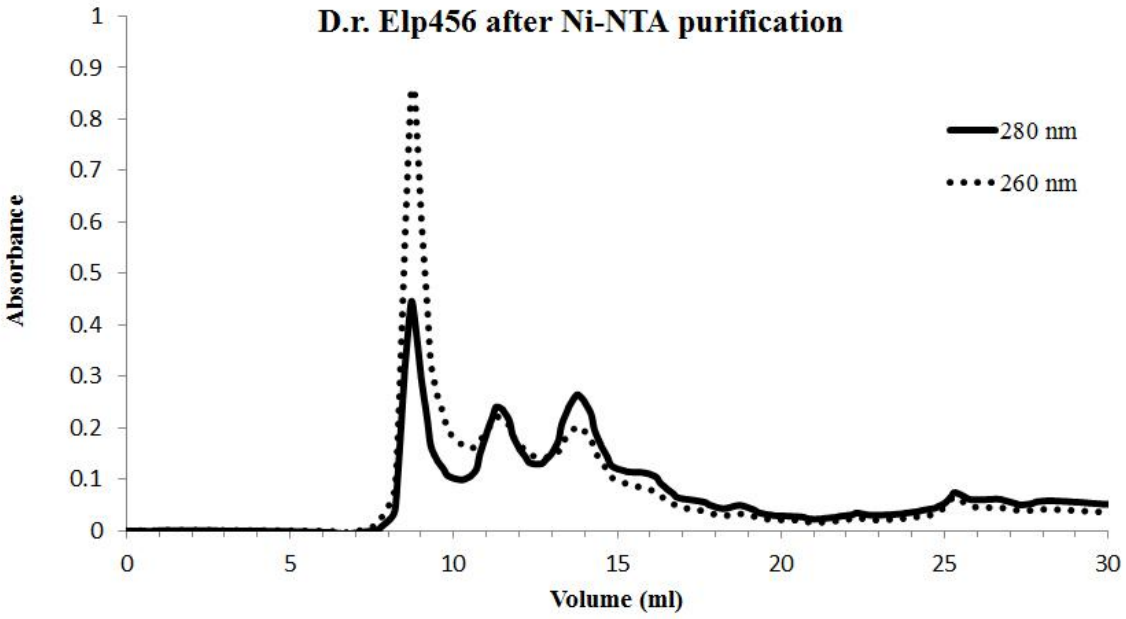
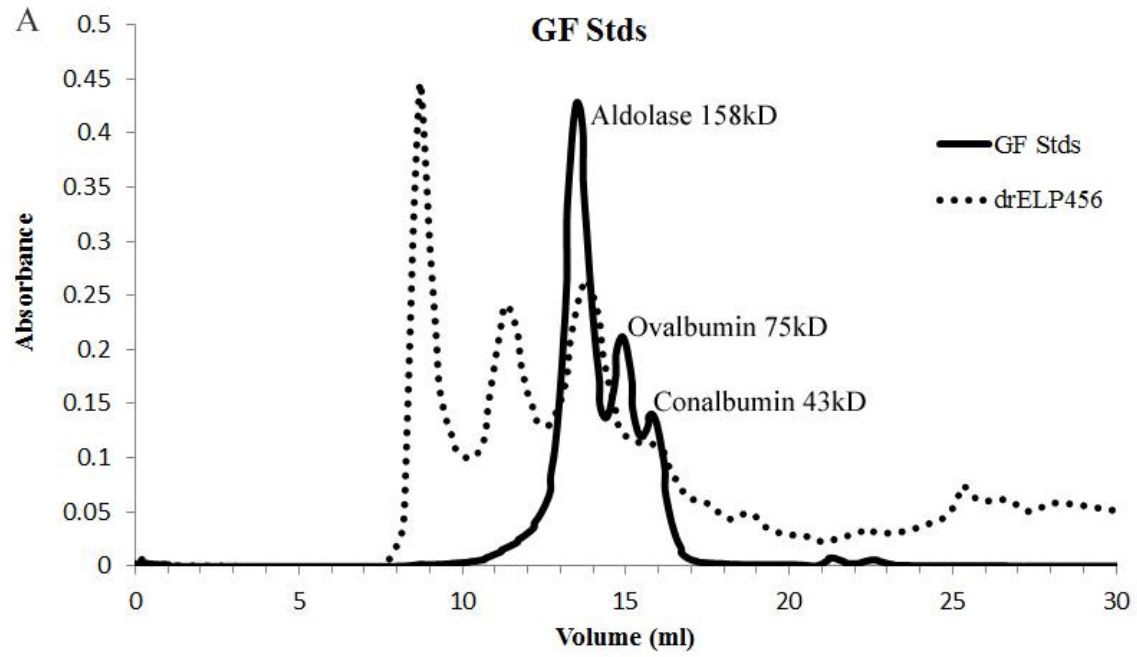
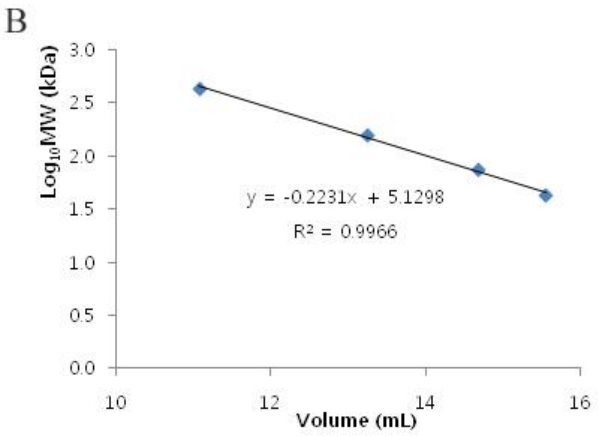


Figure 3.5 Absorbance curve of size-exclusion chromatography of drELP4,5,6
Size-exclusion chromatography was done with purified drELP4/5/6 complex. Peaks on the curve show the elution of the complex. 280nm absorbance curve represents the existence of any protein while 260nm absorbance curve represents the existence of any DNA.





C

Peak #	MW (kD)	Vol (mL)	Log10MW
Peak1	2296.8	8.75	3.36
Peak2	521.7	11.4	2.72
Peak3	128.9	13.9	2.11

Figure 3.6 Absorbance curve of size-exclusion chromatography of drELP4,5,6 compared to standard calibration curve

Size-exclusion chromatography was done with purified drELP4/5/6 complex. In order to find out how large size each peak represents, proteins with known molecular weight were run on the same column to give a standard curve so that the size of the drELP4/5/6 complex can be estimated. The first peak (Fig. A) at volume 9-10 is estimated to be more than 2000 kD (Fig. C), most likely to be aggregated proteins. The second peak (Fig. A) at volume 12 is estimated to be around 500 kD (Fig. C), and the third peak at volume 14 around 100 kD (Fig. C).

Tables of Plasmid and Strain List

Bacteria Strain
BL21 (DE3) RIL (RCB3069)

Plasmid

Vector	Host Strain	SHB No.	Insert	Sequencing Order No.
pGEM-T-Easy	TG1	SHB1H	DrELP4 PCR product #3 10/26/12 Primers SH22, SH23 Template RCB5336	10282651 10282922
		SHB3C	DrELP5 PCR product #2 10/26/12 Primers SH24, SH25 Template RCB5337	10282701
		SHB2C	DrELP6 PCR product #6 10/26/12 Primers SH26, SH27 Template RCB5338	10282651
		SHB25A	DrELP6-6x His PCR product 2/4/2013 Primers SH48, SH49 Template SHB2C	Not sequenced
		SHB16F	EGFP PCR product 11/20/12 Primers SH45, SH46 Template RCB3223	10284381

These vectors were used to get the inserts for the plasmids below.
Followed the pGEM-T-Easy Protocol.

Vector	Host Strain	SHB No.	Insert	Description
pET28a	TG1	SHB11B	DrELP4: Cut from SHB1H (EcoRI, HindIII digest) DrELP6: Cut from SHB2C (NdeI, EcoRI digest) Vector cut from RCB5340F (NdeI, HindIII digest)	pET28a-drELP4-6xHis-drELP6 Confirmed by HindIII digest
		SHB13 A,C	DrELP5: Cut from SHB3C (NotI, XhoI digest) Vector cut from SHB11B (contains drELP4, drELP6) (NotI, XhoI digest)	pET28a-drELP4-drELP5-6xHis-drELP6 Confirmed by HindIII, BamHI digest
		SHB19A	EGFP: Cut from SHB16F (XhoI, BamHI digest)	pET28a-drELP4-drELP5-EGFP-6xHis-drELP6

			Vector cut from SHB13A (contains drELP4, drELP5, drELP6) (XhoI, BamHI digest)	Confirmed by XhoI, BamHI digest
		SHB26A	DrELP6: Cut from SHB25A (NcoI, EcoRI digest) Vector cut from SHB13C (contains drELP4, drELP5) (NcoI, EcoRI digest)	pET28a-drELP4-drELP5-EGFP-drELP6-6xHis Confirmed by NcoI, EcoRI digest

These plasmids were used to get the transformed expression strain below.

Host Strain	**SHB No.**	**Plasmid**	**Description**	**Sequencing Order No.**
BL21 (DE3) RIL	RCB3642	pET28a	Vector only (Control)	
	SHB18A	SHB13A Minipreped	pET28a-drELP4-drELP5-6xHis-drELP6	10286833
	SHB24A	SHB19A Minipreped	pET28a-drELP4-drELP5-EGFP-6xHis-drELP6	Not sequenced
	SHB27A	SHB26A Minipreped	pET28a-drELP4-drELP5-EGFP-drELP6-6xHis	10288064

Final strains used for expression of drELP4, drELP5, drELP6 in the experiment.

Materials and Methods

The transformed bacteria are grown in 5ml LB with kanamycin (Kan) and chloramphenicol (Cam), for 15hrs. 500ml LB (Kan+, Cam+) is inoculated with 0.5ml of previously grown culture at 37℃ until OD_{600} reaches 0.4 - 0.6. When OD_{600} reaches 0.4 - 0.6, 500µl of culture is taken and pelleted, resuspended in TE pH 7.5 for 'Before Induction' sample. The temperature is shifted to 15℃ for 20min, and then 0.05mM IPTG is induced and the culture is grown at 15℃ for 15-20 hrs. 250µl of the culture is taken for 'After Induction' sample. Cells are collected by centrifugation at 4,000RPM for 20min at 4℃. The collected cells are resuspended in lysis buffer (50mM Tris pH 8.0, 150mM NaCl) with protease inhibitors (1mM PMSF, 1mM Benzamidine, 1µg/ml Pepstatin). 20µl of the solution is taken for 'After Lysis Bf' sample. Cells are lysed by sonication using maxi tip, 4 rounds of 30sec cycle, 1.5 sec pulse, and 0.5 sec rest. 20µl is taken for the 'Sonication' sample. The solution is then centrifuged for 10min at 4,000 RCF at 4°C (20µl taken for 'Pellet 1' sample), the supernatant is taken to further centrifuge another 30min at 7,169 RCF (20µl taken for 'Pellet 2' sample). The supernatant is collected (20µl taken for 'Supernatant' sample) and incubated with 100µl of 50% cobalt bead slurry 20min at 4℃, end-over-end. The resin is collected by centrifugation at 1,000 RCF, 2min at 4℃ and washed 4x with 1ml lysis buffer with protease inhibitors (20µl taken from the first supernatant for 'Unbound' sample). Elution is done by 200µl lysis buffer with 200mM immidazole, 5 min on ice (40µl taken for 'Eluate' sample). 10. Prepare samples (Before induction, After induction, After Lysis buffer, After sonication, Pellet1, Pellet2, Supe, Bead Unbound) by adding 60µl TE pH7.5 and 20µl 5X SDS Sample Buffer. Boil at 100˚C for 10min. 'Eluate' sample is prepared by adding 10µl 5X SDS Sample Buffer and incubation at 37˚C for 5min. The samples are run on SDS-PAGE gel.

For size-exclusion chromatography, the 500ml culture is incubated at 15˚C for 40min before 0.2mM IPTG induction. After collecting the ‘Pellet2’ sample, further centrifugation is done at 15,000rpm, 30min at 4˚C. Triton and immidazole is added to make the final concentration 0.2% triton and 10mM immidazole and the proteins are purified by Ni-NTA column.

CHAPTER Ⅳ

Discussion

Study of Sec2 structure and its functionality

Rab GTPases are nucleotide dependent molecular switches that are highly regulated. The yeast Rab protein sec4 regulates exocytosis of post-Golgi secretory vesicles, and its activation is regulated by sec2. To investigate how sec2 functions, we tried to find out how the coiled-coil domain within sec2 works. Replacement of the coiled-coil domain with that of Myo2, GCN4, and Human Rabin3 was performed. The results suggest that Myo2 coiled-coil domain is very different to sec2 coiled-coil domain, while the coiled-coil domain of GCN4 is relatively more similar. Rabin3 coiled-coil domain was able to fully substitute the wildtype Sec2 coiled-coil domain, indicating that the two may function in a very similar way. Superimposition of the 3D structure may yield further insight on structural similarity of the coiled-coil domains of wildtype Sec2 and Rabin3.

Study of zebrafish Elongator Protein 4/5/6 Subcomplex Structure

In this study, we found the correct orthologs of Elp4, Elp5, Elp6 proteins for zebrafish. We had the three protein cloned in one vector so that all three proteins can be expressed together in a same bacteria. SDS-PAGE gel analysis showed three bands that represents drELP4, drELP5, and drELP6. This result implied that the three proteins form a complex because only drELP6 was tagged with His which was used to purify the expressed proteins. Due to the similar molecular weight of drELP5 and His-tagged drELP6, we tried tagging the drELP5 with EGFP to verify that it was being expressed, and was the band shift clearly showed that drELP5 was being expressed and pulled down together with the other proteins. In SDS-PAGE gel, drELP6 seemed to have a

smaller molecular weight than expected so we went on for western blot analysis to prove that the band does represent the drELP6. We assumed that drELP6 may be having a degradation issue at the C-terminus and that may explain the smaller molecular weight and went on for His-tagging at the C-terminus and comparing the size with the N-terminal tagged drELP6, but the SDS-PAGE gel analysis showed the same result indicating that degradation may not be the appropriate explanation for the smaller band size. It is still not known why the drELP6 seemed to have a smaller molecular weight than the expected molecular weight and it needs more research to be done. Still, the results suggest that we have found the correct orthologs of the *Danio rerio* Elongator proteins and this will allow for further research on its role in neuronal exocytosis in zebrafish.

Size-exclusion chromatography gave us more information about the complex. We were able to estimate the size of the complex by comparing the peaks to the standard run and the result implied that drELP4, drELP5, and drELP6 may interact in 1:1:1 ratio to form a complex. A bigger molecular weight sized complex was also found, showing a possibility of multiple complexes interacting with each other. Further research can be done by X-ray crystallography to find out how each subunit interacts with each other.

The results show that we have found the correct orthologs of Elp proteins for zebrafish and therefore it enables us to research further on its role in exocytosis in neuronal cells. We would be able to express the proteins in zebrafish embryos and investigate their functions by using morpholinos and knockdowns.

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