Effect of Disease-related Mutations on Tubulins on Microtubules Properties and on Neuronal Development and Differentiation

Lau Wei Sin

School of Biological Sciences, The University of Hong Kong

Supervised by:

Jeff Ti Shih-Cheih

School of Biomedical Sciences, The University of Hong Kong

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Abstract

Microtubules are non-covalent polymers formed from heterodimers of α- and β- tubulin molecules. These polymers play a significant role in cell function, such as the formation of the mitotic spindle for chromosome segregation, the formation of flagella and cilia to allow movement and are one of the main components of the cell cytoskeleton, which gives cells their shape and allow intracellular transport of organelles. Numerous disease-associated point mutations have been found present on different tubulin genes causing a spectrum of neurodevelopmental disorder diseases, such as lissencephaly (smooth brain), polymicrogyria, and cortical malformations, collectively known as tubulinopathies (Cushion et al., 2023). Due to the difficulty of generating pure recombinant mutant tubulins, a knowledge gap still exists in the acting mechanism of these disease-causing mutations. In this research, we will purify and characterise two disease-related mutations, L286F on α-tubulin, related to lissencephaly (smooth brain) and D417H mutation on β-tubulin, causing ocular motility disorder CFEOM3, using MEC-12 and MEC-7 tubulins found in mechanosensory neurons of C. elegans. We successfully generated recombinant MEC-12L286F and MEC-7D417H using the insect cell-based protein expression system. The next step is to purify the mutant tubulins, which will

allow *in vitro* assays to study the microtubule dynamics and the interactions with microtubule-associated proteins and motors, leading to possible future *in vivo* studies.

Introduction

Microtubules are non-covalent polymers formed from heterodimers of α- and β-tubulin molecules. These polymers play a significant role in cell function, such as the formation of the mitotic spindle for chromosome segregation, the formation of flagella and cilia to allow movement and are one of the main components of the cell cytoskeleton, which gives cells their shape and allow intracellular transport of organelles. To date, 9 α and 10 β-tubulin isotypes have been identified to be present in the human body (Chew & Cross, 2023). Numerous disease-associated point mutations have been found present on different tubulin genes, causing a spectrum of neurodevelopmental disorder diseases, such as lissencephaly (smooth brain), polymicrogyria, and cortical malformations, collectively known as tubulinopathies (Cushion et al., 2023).

However, the mechanism of how a single point mutation on one isotype of tubulin can lead to neuronal maldevelopment is not well and extensively studied. Microtubules found in different parts of an organism undergo different and heavy posttranslational modifications suited to their function, such as acetylation, tyrosination, and detyrosination, making it impossible to isolate unmodified microtubules. Biochemical tools and methods that can generate pure recombinant microtubules were only recently developed in the past decade, hence allowing the study of many more microtubule properties. We are now able to purify affinity tag-free recombinant tubulin from insect cells (Ti et al., 2020).

It is found that mutations in human α-tubulin TUBA1A can cause microlissencephaly, a congenital brain disorder with both microcephaly (small head) and lissencephaly (smooth brain). Eight patients diagnosed with malformation of the central nervous system were

identified with de novo missense mutations on TUBA1A (Poirier et al., 2007). L286F is identified in patients diagnosed with severe lissencephaly and is located on the M-loop of tubulin, which is responsible for intra-protofilament lateral interaction and the binding site of the cancer-targeting drug paclitaxel (Poirier et al., 2007; Nogales et al., 1998). Previous *in vitro* studies suggest that L286F mutation on TUBA1A may disrupt cortical formation due to failure to interact with microtubule-associated proteins or motors while having no obvious effect on heterodimer formation or dynamics (Tian et al., 2010).

Clinical studies in human and knock-in mouse models have found that human β-tubulin isotype III (TUBB3) is required for axon guidance. Eight heterozygous missense mutations on TUBB3 are found to cause ocular motility disorder CFEOM3 in which all subjects with D417H and R262H are diagnosed with congenital wrist and finger contractions, suggesting maldeveloped spinal motor neurons (Tischfield, 2010). Previous *in vitro* studies have shown that R262H and D417H, mutations on the external surface near the kinesin-binding site on TUBB3, decrease the catastrophe frequency of TUBB3 and affect the polymerization frequency at both ends of microtubules. D417H mutation on TUBB3 is found to have a lower affinity to TOG domains and colchicine, both of which preferentially bind to the curved conformation of microtubules, indicating that D417H-mutated microtubules favour a straight conformation. This might be the reason why longer-lasting end-stabilising caps are found on both ends of TUBB3 with D417H, and hence a lower catastrophe frequency (Ti et al., 2016).

Here, we study two disease-related point mutations, L286F on α-tubulin and D417H on β-tubulin. Using *C. elegans* as a model organism, we generate mutated sequences of MEC-12^{L286F} and MEC-7^{D417H} from molecular cloning method and use Bac-to-Bac system to generate recombinant baculovirus which will infect insect cells to produce tag-free recombinant *C. elegans* tubulins MEC-12^{L286F} and MEC-7^{D417H} which are α- and β- tubulins

needed for the polymerization of mechanosensory neurons in *C. elegans* (Ti et al., 2020; Fukushige et al., 1999; Savage et al., 1989). These *C. elegans* tag-free recombinant tubulins can then be purified and used for *in vitro* studies, using TIRF microscopy to observe microtubule dynamics, and possible *in vivo* studies in *C. elegans*.

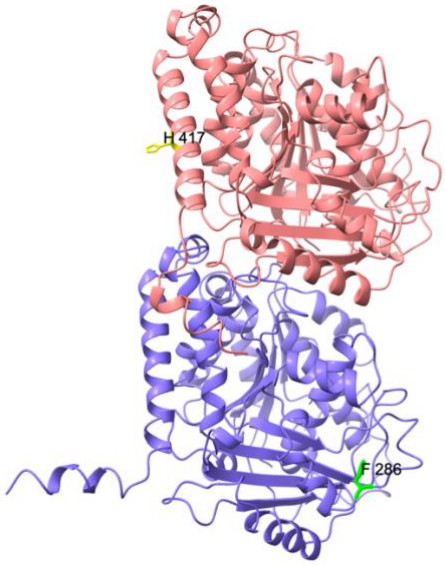

Figure 1. Structure of MEC-12^{D417H} (top) and MEC-7^{L286F} (bottom) generated by Alpha Fold 3

Results

Generation of Mutated Sequence

To generate MEC-7^{D417H} and MEC-12^{L286F}, point mutation was introduced into MEC-7 and MEC-12 (Figure 2A & 2B) encoding pFastBac Dual Vector with His and Strep-tag using the IVA cloning method (García-Nafría et al., 2016). Plasmids are then transformed into DH5α *E. coli* cells and grown on agar containing LB and gentamicin at 37 °C overnight. Single colonies are picked and cultured in LB and gentamicin medium at 37 °C overnight. Plasmid products are then extracted, size checked using restriction enzymes (Figure 2D), and sequence confirmed by Sanger sequencing.

Mutated MEC-12^{L286F} and MEC-7^{D417H} sequences on plasmids were then extracted using restriction enzymes. MEC-12 ^{L286F} encoding plasmid was digested using SpeI-HF and HndII and MEC-7^{D417H} encoding plasmid was digested using XhoI and KpnI-HF at 37 °C overnight. Digested products are then incubated with CIP at 37 °C for 3 hours. DNA gel electrophoresis is then performed on digested plasmids and wild type plasmids (pFastBac Dual-His-MEC-12-MEC-7-Strep that has not undergone PCR). Target band is then cut from the gel on the UV box and digested to retrieve wanted DNA. Insert (digested product) and vector are then ligated together to generate plasmid with targeted point mutation only. Product sequence is then confirmed by Sanger sequencing.

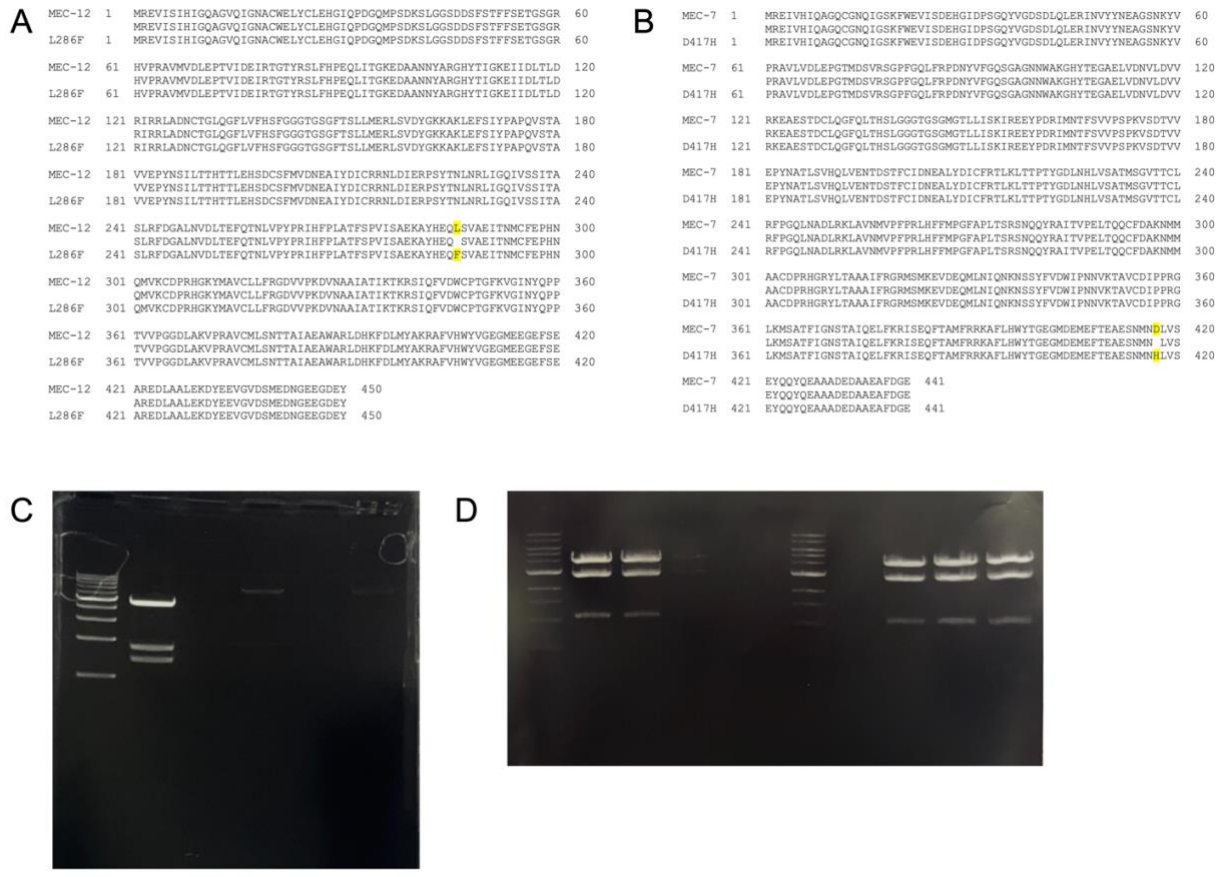

Figure 2. (A) Sequence alignment of MEC-12 and MEC-12^{L286F} (B) Sequence alignment of MEC-7 and MEC-7^{D417H} (C) PCR product check using gel electrophoresis (D) Plasmid size check using restriction enzyme

Preparation of Bacmid

The plasmid containing mutated sequences are then transformed into DH10EMBacY (*E. coli*) competent cells using the heat shock method and plated (5%) on LB agar containing kanamycin, gentamycin, tetracycline, X-gal, and IPTG at 37 °C overnights. White single colonies, which signify plasmids are successfully translocated into bacmid, are picked and streaked on new X-gal plates with a blue colony (plasmids failed to be translocated into bacmid) as control and incubated at 37 °C. White single colonies are then picked and cultured in LB with kanamycin, gentamycin, and tetracycline at 37 °C overnights. The expression bacmid is then isolated from DH10EMBacY strains, checked by gel electrophoresis (Figure 3), and concentration checked by measuring absorption at 260nm.

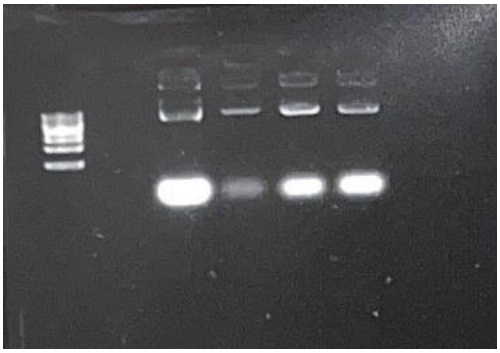

Figure 3. Bacmid with MEC-12^{L286F} (first 3 samples from the left) and MEC-7^{D417H} size check using gel electrophoresis

Generation of Baculovirus

Purified bacmid is diluted in serum and antibiotic-free SF-900 II medium mixed with Cellfectin II reagent and then added to plated Sf9 cells. Cell culture is then incubated at 27 °C for 5 hours. Medium is then changed to SF-900 II medium supplemented with 10% FBS and 1% antibiotic-antimycotic and then incubated for 72 hours at 27 °C. After 72 hours, cells are resuspended and centrifuged at 100x r.c.f. for 5 minutes at 4 °C. P1 virus stock is harvested by filtering the supernatant through a sterilised 25 mm diameter syringe filter with a 0.45 μm pore size polyethersulfone membrane. Sf9 cells are checked for fluorescence that signifies successful transfection from the YFP tag present in bacmid of DH10EMBacY (Figure 4A & 4B). P2 virus stock is then generated from P1 and harvested with the above method. Tubulin expression is checked with western blot (Figure 5). P3 virus is generated with the same method and used for tubulin purification.

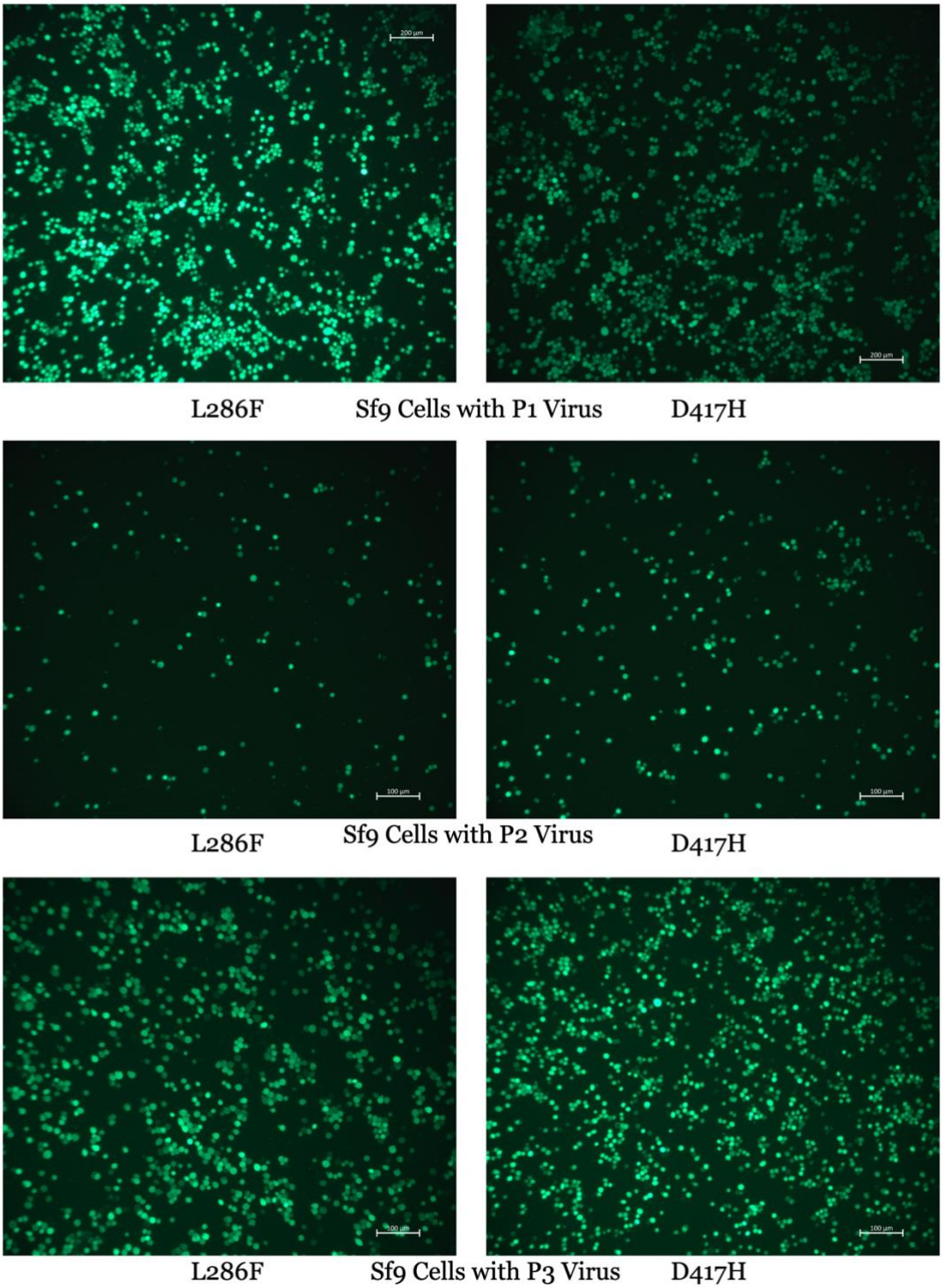

Figure 4. Fluorescent microscope images of SF9 cells with P1, P2 and P3 virus with MEC-12^{L286F} (left) and MEC-7^{D417H} (right)

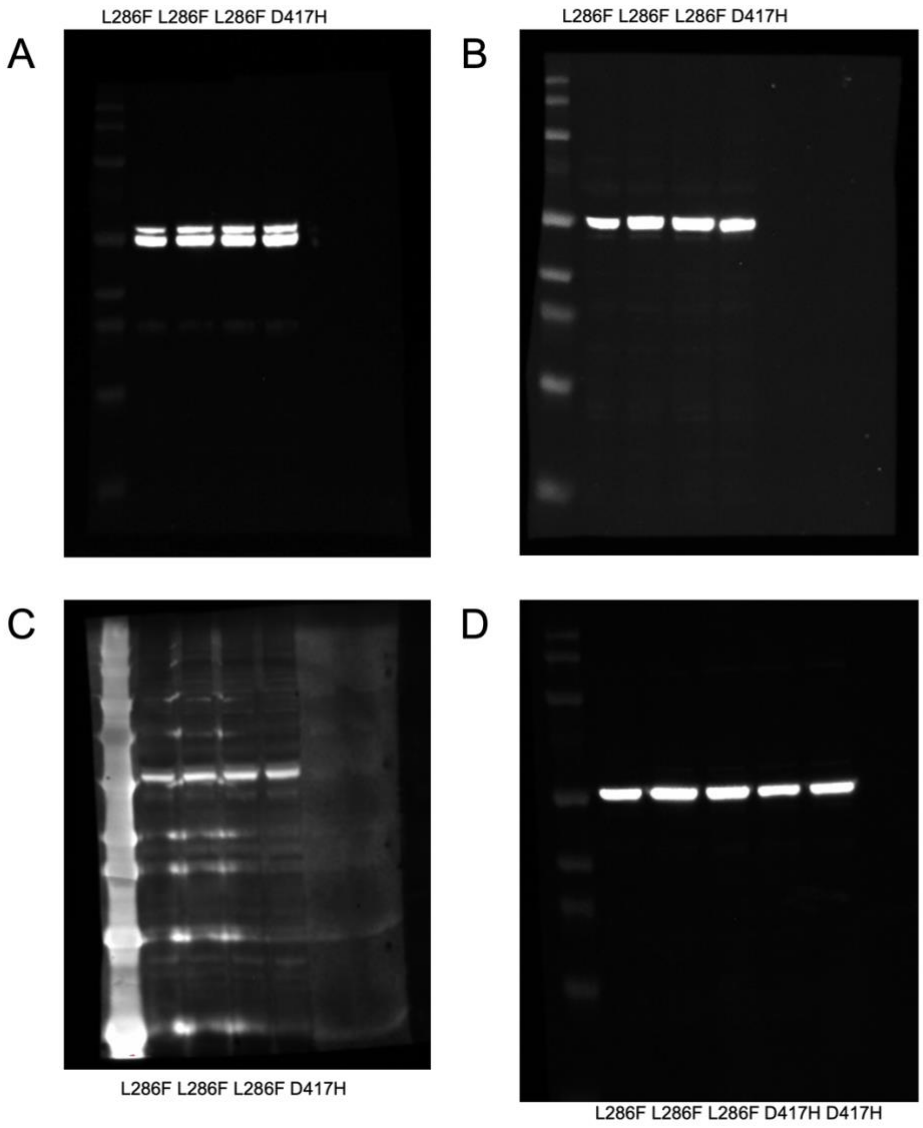

Figure 5. Western Blot image of (A) Alpha (B) Beta (C) His-Tag (D) Strep-Tag

Discussion

Our western blot results show that the generation of recombinant tubulins was successful. The next step of this project would be to purify the recombinant tubulins using an affinity tag-based strategy to generate tag-free pure recombinant MEC-12^{L286F} and MEC-7^{D417H} (Ti et al., 2020). *In vitro* biochemical and biophysical assays can then be done to study the dynamic stability of mutant microtubules polymerized from the mutant tubulins compared to wild-type MEC-12 and MEC-7, respectively. With pure recombinant *C. elegans* tubulins, it is possible to study the effects of these mutant mechanosensory tubulins on the nervous system of *C. elegans* as an animal model in the future.

Based on previous studies, D417H on TUBB3 alters microtubule dynamics in a similar way with regulatory proteins and microtubule stabilising drugs, but at both plus (more dynamic) and minus end, which is not extensively studied yet. D417H and L286F are both hypothesised to affect microtubule cellular functions due to association with microtubules related proteins, such as kinesins (Ti et al., 2016; Tian et al., 2010). Further studies on the interaction between kinesins or other microtubule-associated proteins and these mutant microtubules are still needed to know the exact mechanism of how neuronal disorders are developed from a single point mutation in one isotype of microtubules.

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Reflection

Although I have already stretched out the project duration from 6 weeks to 2 months, the biggest problem I faced was still having very limited time. I was not able to plan my project timeline wisely due to my inexperience in research, not knowing how long each procedure takes time and having unrealistic expectations for the project. Thankfully for this project, I learnt a lot about time management when it comes to doing research. Research is not rush-able, but scientific publishing is competitive, delaying your experiments would mean that you will not be able to publish papers first. Other than that, it is better to be slow and safe than to be sorry, errors in fundamental steps such as cloning would lose you a month of time if you rushed through it.

There are times where I didn't know what has to be done next being very unfamiliar with experiment procedures and the topic. It is crucial to think about the purpose of every procedure done and the purpose of the project to know what direction I want to go. In addition, there were procedures that were too complicated for me to carry out as a freshman, requiring postgraduate student's help to do them.

If I was to repeat the project, I would join the lab earlier to observe how experiments are done to be more familiar with carrying out experiments, as well as read more review articles to understand the subject deeper in order to have a better chance to complete the project further.