様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科研費
KAKENHI

# 科学研究費助成事業　　研究成果報告書

令和　６ 年　６ 月 １１ 日現在

機関番号：８２４０１
研究種目：若手研究
研究期間：2022～2023
課題番号：２２Ｋ１５１１２

研究課題名（和文）Identification of molecular machinery to control the selective/preferential binding of kinesin KIF5C to axonal microtubules by proximity labelling

研究課題名（英文）Identification of molecular machinery to control the selective/preferential binding of kinesin KIF5C to axonal microtubules by proximity labelling

研究代表者
Ｌｉ　Ｘｕｅ（馬場雪）（Li, Xue）

国立研究開発法人理化学研究所・生命機能科学研究センター・研究員

研究者番号：５０９３６５０７

交付決定額（研究期間全体）：（直接経費）　　3,500,000円

研究成果の概要（和文）：本研究では、KIF5C（キネシン1）の微小管への結合親和性を制御する細胞内分子機構の同定を目指した。KIF5C変異体は、大脳皮質発達の重篤な奇形や小頭症の患者で発見され、その重要性が明らかになっている。我々は、選択的結合を模倣するために数種類のKIF5C結合コンストラクトを作製し、KIF5C-TurboIDとKIF5C-APEX2法により、セミインタクトの大脳皮質神経細胞における主要なタンパク質プレーヤーを標識した。現在、質量分析法を用いた制御因子の同定を進めている。KIF5C結合の主要な制御因子が明らかになれば、関連する脳疾患の早期診断や治療に役立つ可能性がある。

研究成果の学術的意義や社会的意義
KIF5C変異体は、大脳皮質発達の重篤な奇形や小頭症の患者で発見され、その重要性が明らかになっている。本研究では、KIF5C（キネシン1）の微小管への結合親和性を制御する細胞内分子機構の同定を目指した。KIF5C結合の主要な制御因子が明らかになれば、関連する脳疾患の早期診断や治療に役立つ可能性がある。

研究成果の概要（英文）：In this study, we focus on the identification of molecular machinery in cells regulating or adjusting the binding affinity of KIF5C (Kinesin 1) to microtubules, of which the importance has been highlighted after the analysis of KIF5C mutants found in patients with severe malformations of cortical development and microcephaly. We made several KIF5C binding constructs to mimic selective binding and have established KIF5C-TurboID and KIF5C-APEX2 to label key protein players in semi-intact cortical neurons. We are now proceeding to hits identification using mass spectrometry analyses. We expect to reveal key regulators of KIF5C binding that could be potentially helpful for early diagnosis and treatment of the related brain diseases.

研究分野：Cytoskeleton, Cell biology

キーワード：KIF5C　Kinesin 1　Microtubule binding　Cortical neuron

※科研費による研究は、研究者の自覚と責任において実施するものです。そのため、研究の実施や研究成果の公表等については、国の要請等に基づくものではなく、その研究成果に関する見解や責任は、研究者個人に帰属します。

## １．研究開始当初の背景

Microtubules (MTs) are well known as highways for intracellular transport. MTs are organized by the centrosome and participate in cell polarity and mitotic spindle organization. Previous study by the applicant using STED super resolution microscopy found that the organization of MT and the inter-centrosome linker are both interdigitated in interphase to keep centrioles close and maintain cell polarity (Li et al., PNAS, 2018). The functional and stability of MTs are altered by its conformation and modification. KIF5 (Kinesin-1) is essential motor protein. KIF5 dimerizes and undergoes polarized transport due to preferentially binding to GTP-tubulin-rich MTs. In motor neurons, KIF5C dimerizes and transports various cargos along axon MTs (Figure 1). Knockout of *KIF5C* in mice leads to smaller brain size and loss of motor neurons. p.Glu237Val mutation in *KIF5C* is found to cause severe malformations of cortical development and microcephaly in patients. Interestingly, this mutation in *KIF5C* does not affect the binding ability but the affinity to MTs, besides the defect in ATP hydrolysis. It suggests that besides the ATP hydrolysis ability and MT binding ability, affinity to MTs is also critical for proper KIF5C function. Efforts have been made to study the affinity of KIF5C to MTs. Our lab showed two types of KIF5C affinity to MTs *in vitro* (Figure 2 & 3). Later, our preliminary data discovered two more types of KIF5C affinity *in vivo* (Figure 2 & 4). These lead to the questions, how does KIF5C bind to MT *in vivo* and how is the affinity of KIF5C to MTs controlled in neurons (Figure 1 & 2)?

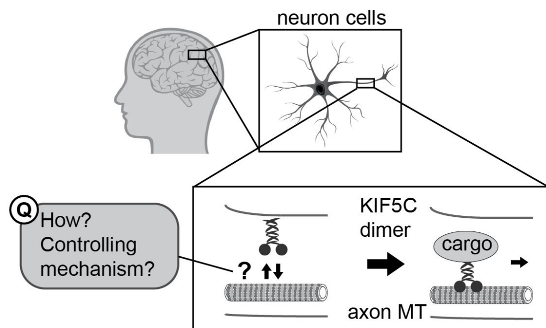



Fig 1. KIF5C transports various cargos along axon MTs in dimer. However, how does KIF5C bind to MTs and how this process is controlled are not yet well understood.

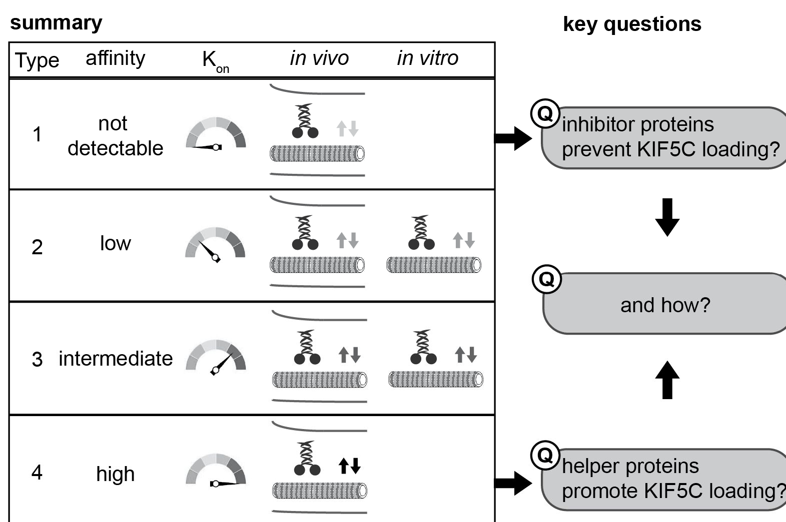



Fig 2. Summary of KIF5C affinity to MTs and the key questions of this proposal. It is found that KIF5C binds to MTs in four types of affinities *in vivo* but only two types *in vitro*. It is therefore suggested that additional proteins inhibit or facilitate KIF5C loading on MTs *in vivo*. This project is going to identify the additional players and study the mechanisms behind.

## ２．研究の目的

This study will investigate KIF5C interactional partners in mice primary cortical neuron cells. To avoid not only the low efficiency in genetic manipulation in neurons but also high background of conventional Proximity-

based TurboID protein labelling, it is planned in this project to perform TurboID in semi-intact neuron cells. Through adding purified KIF5C-TurboID dimer, biotin and AMPPNP into semi-intact neuron cells, KIF5C-TurboID dimers will be loaded and locked onto MTs, then the proteins in proximity of KIF5C will be labelled with biotin by TurboID. Unspecifically labelled proteins will be washed out. Later, the biotinylated proteins will be identified via mass spectrometry. This study will provide a feasible method of study interactors with increased efficiency in highly differentiated cells. By identify the inhibitory and helper proteins of KIF5C that alters the binding affinity to MTs, it is expected to uncover the yet unknown mechanism in controlling of KIF5C affinity to MTs in neurons. With a better knowledge of affinity control of KIF5C, it is promising to develop therapeutic treatment for patients with KIF5C defects in the future.

## ３．研究の方法

This study will construct various KIF5C TurboID constructs with different binding affinity to microtubules and perform TurboID in semi-intact neuron cells. Through TurboID-based proximity labeling under different conditions, the protein of interests will be identified in later mass spectrometry.

## ４．研究成果

(1). *In vitro* neuron cell differentiation and culture conditions for cortical neurons are optimized.

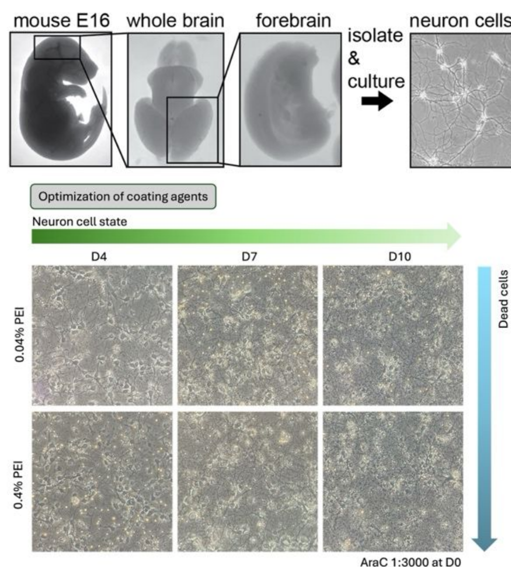



Fig 3. Optimization of *in vitro* neuron cell differentiation and culture conditions for cortical neurons. Coating agents, AraC concentration and *in vitro* culture time are optimized.

(2). Various KIF5C constructs with different binding affinity to microtubules are verified.

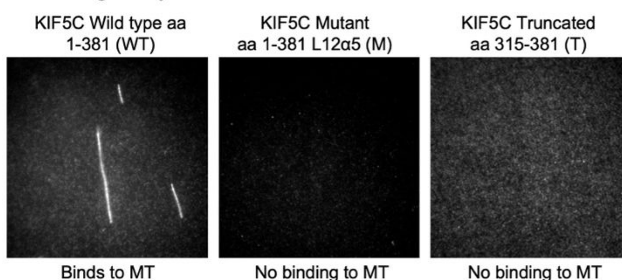



Fig 4. KIF5C Wild type without the autoinhibition area to microtubule binding, i.e., aa 1-381 (here WT), KIF5C Mutant, aa 1-381 with mutations on L12a5 (here M), and KIF5C Truncated aa 315-381(here T) are purified in *E.coli* and the different binding affinity to microtubules are verified. WT binds to MTs whereas M and T cannot bind to MTs.

(3). Various KIF5C-TurboID heterodimer constructs are purified using Äkta.

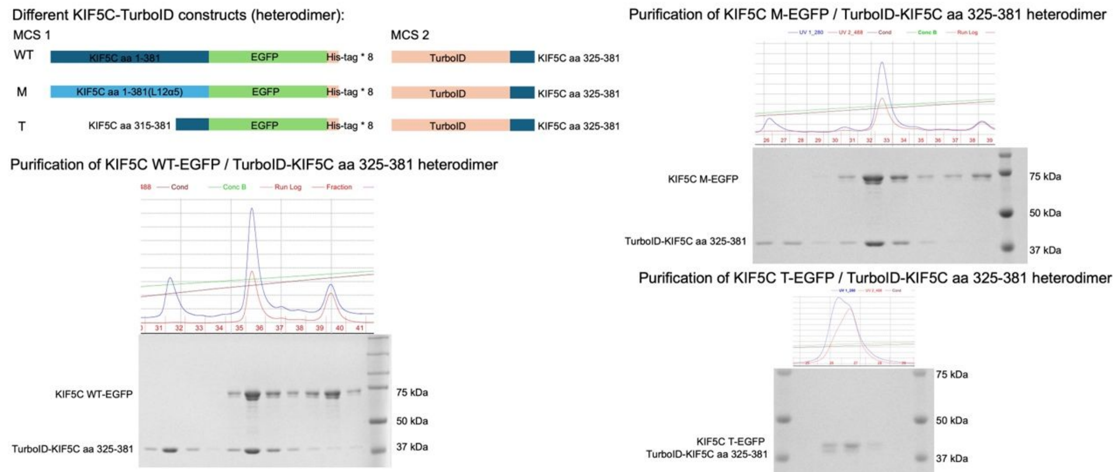



Fig 5. Various KIF5C TurboID constructs and the purifications using Äkta.

(4). The difference of binding affinity of KIF5C TurboID heterodimers to microtubules are confirmed.

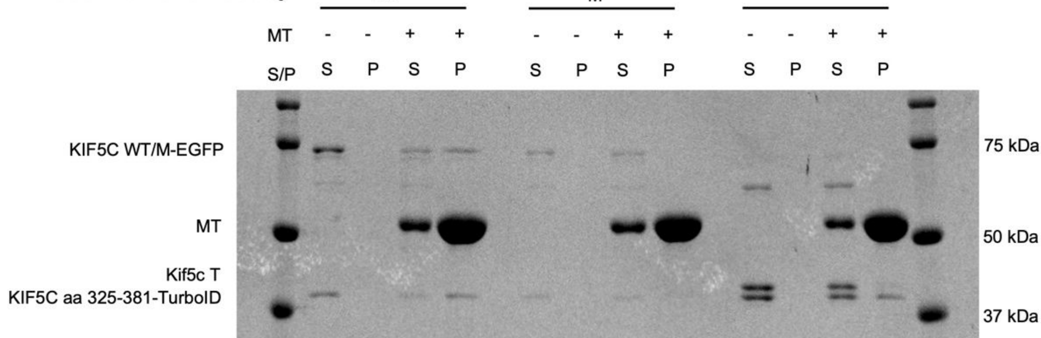
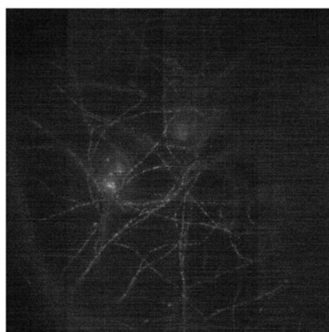



Fig 6. The difference in microtubule binding affinity of various KIF5C TurboID constructs are confirmed by MT sedimentation assay. In line with the previous MT binding assay, only KIF5C WT heterodimer binds to microtubules.

(5). The binding affinity of KIF5C WT TurboID to microtubules is excessively high, regardless of the stability of microtubules.

1 nM of KIF5CWT/TurboID loaded into semi-intact neuron cells, w/o incubation



His IP of KIF5CWT/TurboID, with biotin

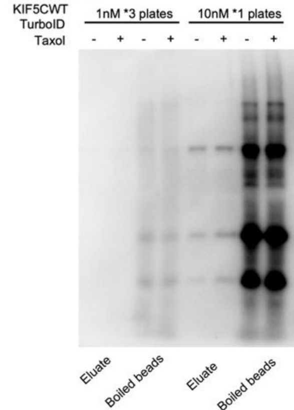



Fig 7. During the test of KIF5C TurboID in neuron cells, the microtubule binding affinity of KIF5C WT TurboID is found to be excessively high. Furthermore, differences in biotin labeling of KIF5C to stabilized microtubules and to the negative controls cannot be found due to the binding high affinity.

(6). Switch the proximity labeling method from TurboID to APEX2, for rapid and specific labeling.

Different KIF5C-APEX2 constructs (heterodimer):

Purification of KIF5C WT-EGFP / APEX2-KIF5C aa325-381 heterodimer

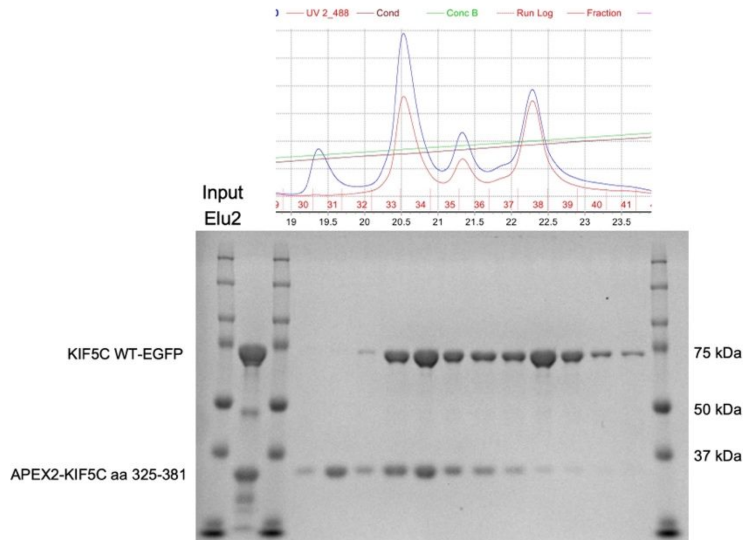



Fig 8. Constructs of KIF5C APEX2 and purification of KIF5C WT APEX2 using Äkta.

Summary:

In this study, isolated and differentiated cortical neurons from mice embryos were used. To identify the key protein players, we used proximity-based labelling method KIF5C-TurboID in semi-intact cortical neuron cells. We made several KIF5C binding constructs to mimic selective binding and established the protocol to let in vitro purified dimerized active KIF5C-TurboID proteins bind to MTs of the semi-intact cortical neurons and label the proteins in close proximity.

However, due to the highly active labelling efficiency and long required labeling time (~18h) of TurboID, it is prone to have many false-positive hits identified and is difficult to control the labelling process. Therefore, we decided to switch our system to the newly introduced proximity labeling methods APEX2. APEX2 has a lower labeling activity and a rapid labeling time (~10 min). Currently, we have optimized the labeling of KIF5C-APEX2 in semi-intact cortical neuron cells and are proceeding to the next steps.

## ５．主な発表論文等

〔雑誌論文〕　計0件

〔学会発表〕　計0件

〔図書〕　計0件

〔産業財産権〕

〔その他〕

－

## ６．研究組織

| | 氏名<br>（ローマ字氏名）<br>（研究者番号） | 所属研究機関・部局・職<br>（機関番号） | 備考 |
|---|---|---|---|

## ７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

## ８．本研究に関連して実施した国際共同研究の実施状況

| 共同研究相手国 | 相手方研究機関 |
|---|---|