Cell Biology
2019; 7(1): 1-9
http://www.sciencepublishinggroup.com/j/cb
doi: 10.11648/j.cb.20190701.11
ISSN: 2330-0175 (Print); ISSN: 2330-0183 (Online)

Different Functionals Domain of Spastin^{M87V} Affect Cellular Microtubule Cutting

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To cite this article:
Yang Yuhao, Zhang Guowei, Ji Zhisheng, Cai Zhenbin, Liu Qiuling, Li Shaojin, Lin Hongsheng. Different Functionals Domain of Spastin^{M87V} Affect Cellular Microtubule Cutting. *Cell Biology*. Vol. 7, No. 1, 2019, pp. 1-9. doi: 10.11648/j.cb.20190701.11

Received: January 14, 2019; **Accepted**: February 25, 2019; **Published**: March 20, 2019

Abstract: The research is aimed to explore the effect of different functional domains of Spastin^{M87V} on microtubule cutting of Hela cells. First, the Spastin gene was extracted and its mutants were constructed and identified. Then, Spastin and its mutants were transfected into Hela cells and their cutting effects on microtubules were observed. The results showed that Spastin^{M87V} and its truncated prokaryotic plasmids, including GST-Spastin^{N197}, GST-Spastin$^{\Delta AAA}$, GST-Spastin$^{\Delta N1}$ and GST-Spastin$^{\Delta N2}$ were constructed, and the corresponding fusion proteins were expressed in vitro. In addition, Spastin^{M87V} and its truncated eukaryotic plasmids including GFP-Spastin^{N197}, GFP-Spastin$^{\Delta AAA}$, GFP-Spastin$^{\Delta N1}$ and GFP-Spastin$^{\Delta N2}$ were successfully constructed and introduced into Hela cells. At last, the microtubules of Hela cells could be cut into small fragments by Spastin^{M87V} and Spastin$^{\Delta N1}$. Furthermore, the fluorescence intensity of the microtubules of Hela cells in the Spastin^{M87V} and Spastin$^{\Delta N1}$ groups were significantly weaker than in the control group. In this way, Spastin is mainly involved in cell microtubule cutting, and the cutting function by Spastin^{M87V} must contain complete microtubule binding- domain (MTBD) and cutting domain (AAA domain).

Keywords: Spastin^{M87V}, Functional Domain, Protein Expression, Microtubule Cutting

1. Introduction

Spastin (SPG4) is the most common mutated gene protein in hereditary spastic paraplegia. It consists of 616 amino acids. It is part of the ATPases associated with various cellular activities (in the AAA) family of proteins [1, 2]. The AAA protein family is a class of proteins that can utilize the energy generated by ATP hydrolysis to perform the corresponding functions. AAA protein family has a highly conserved AAA functional domain consisting of 200 to 250 amino acids. The seventh sub-family contains three proteins: spastin, katanin and fidgetin [3-5]. At present, two kinds of microtubule cutting proteins, katanin and spastin, have been studied widely. These two proteins are mainly bound to stable microtubules, which are primarily regulated by acetylated or poly-glutamylated post-translational modified tubulin [6-8]. Fidgetin mainly combines with unacetylated unstable microtubules [9, 10]. Spastin contains two subtypes of spastin^{M1V} (68 kDa) and spastin^{M87V} (60 kDa). Here, 68 kDa subtypes were distributed in cytoplasm, and 60 kDa subtypes were distributed in nucleus and cytoplasm, and the distribution of cytoplasm was dotted and around nucleus [11, 12]. The SPG4 reading frame has two start codons, and the weak Kozak sequence of the first start codon leads to the missed reading of the first AUG. The second AUG has a strong Kozak sequence and generally preferentially reads from the second start codon. As a result, the distribution of Spastin^{M87V} in tissues is wider than that of Spastin^{M1V} [13, 14]. Spastin consists of a tubulin interaction and transport region (MIT), a microtubule binding domain (MTBD), an ATP binding region (AAA), two nuclear localization sequences (NLS), a nuclear output signal sequence (NES), and a transmembrane sequence (TM) functional domain [15]. The crystal structure of the Spastin protein is a hollow hexamer, six "arms" around a central ring, can be combined with microtubules, a hollow structure and the entrance to the outside world has a large number of microtubule cleavage proteases, through the identification of microtubule protein C terminal for cutting. Spastin is mainly involved in cell division, differentiation, neuron growth and development, and signal transduction within cells [16-18]. Its functional domains play

different roles in cellular physiological processes. At present, the function of cell microtubule cutting in different functional domains is not very clear. In this study, we will illuminate the effect of different functional domains of Spastin^{M87V} on microtubule cutting of Hela cells.

2. Materials and Methods

2.1. Materials

Hela Cells purchased from the Shanghai Cell Bank. The types and manufacturers of materials purchased are as follows: Dulbecco's Modified Eagle Medium (GIBCO), B-27 Supplement (GIBCO), Fetal bovine serum (GIBCO), Poly-d-lysine (BD Biosciences), COVERSLIPS, 12MM (GIBCO), Rabbit polyclonal antibody to GFP (Abcam), Rat monoclonal antibody to tubulin (Abcam), Alexa Fluor488 Donkey anti Rabbit (Life), Alexa Fluor 549 Donkey anti Rat (Abcam).

2.2. Method

2.2.1. Construction and Identification of Plasmids

Different truncated fragments are designed according to different functional domains of Spastin (Figure 1) and primers are designed (table 1). These recombinant plasmids included Spastin^{M87V}, Spastin^{N197}, Spastin$^{\Delta AAA}$, Spastin$^{\Delta N1}$ and Spastin$^{\Delta N2}$.

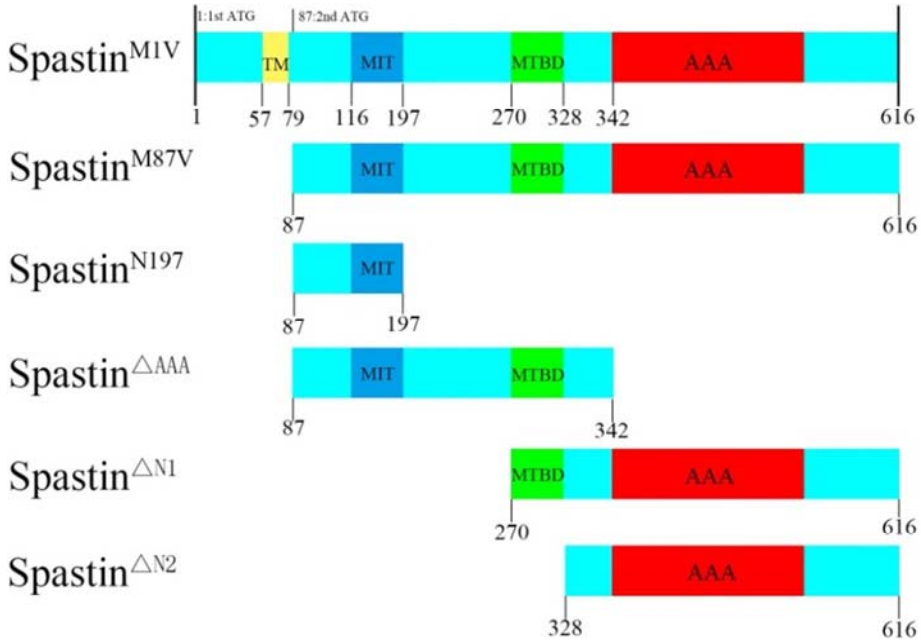


Figure 1. *Spastin^{M87V} and its truncated fragment. TM: Transmembrane sequence; MIT: Tubulin interaction and transport region; MTBD: Tubulin binding domain; AAA.*

Table 1. *Primers of constructed plasmids.*

Plasmid	Sequence	
GST-Spastin^{M87V}	Forward	5'-ATTCGTCGACATGGCCGCCAAGAGGAGC-3'
	Reverse	5'ATTGCGGCCGCTTAAACAGTGGTGTCTCC 3'
GST-Spastin^{N190}	Forward	5'ATTCGTCGACATGGC CGCCAAGAGGAGC 3'
	Reverse	5'ATTCGCGGCCGCTTGTAAACGGTCCTTAGC 3'
GST- Spastin$^{\Delta AAA}$	Forward	5' ATTCGTCGACATGGCC GCCAAGAGGAGC 3'
	Reverse	5' ATTCGCGGCCGCTTTAACAGCTGTCCCATT 3'
GST-Spastin$^{\Delta N1}$	Forward	5'ATTC GTCGACCTCCTAGAAAGTGGAGCA 3'
	Reverse	5' ATTCGCGGCCGCTTAAACAGTGGTGTCTCC3'
GST-Spastin$^{\Delta N2}$	Forward	5' ATTCGTCGACTTGATGATATAGCTGGTCA 3'
	Reverse	5'ATTCGCGGCCGCTTAAACAGTGGTGTCTCC 3'
GFP-spastin^{M87V}	Forward	5'ATTCAGATCTATGGCCGCCAAGAGGAGC 3'
	Reverse	5'ATTCGTCGACTTAAACAGTGGTGTCTCC 3'
GFP-Spastin^{N190}	Forward	5' ATTCAGATCTATGGCCGCCAAGAGGAGC 3'
	Reverse	5' ATTCGTCGACTTGTAAACGGTCCTTAGC 3'
GFP- Spastin$^{\Delta AAA}$	Forward	5' ATTCAGATCTATGGCCGCCAAGAGGAGC 3'
	Reverse	5' ATTCGTCGACTTTAACAGCTGTCCCATT 3'
GFP-Spastin$^{\Delta N1}$	Forward	5' ATTCAGATCTCTCCTAGAAAGTGGAGCA 3'
	Reverse	5' ATTCGTCGACTTAAACAGTGGTGTCTCC 3'
GFP-Spastin$^{\Delta N2}$	Forward	5' ATTCAGATCTTTGATGATATAGCTGGTCA 3'
	Reverse	5' ATTCGTCGACTTAAACAGTGGTGTCTCC 3'

2.2.2. Protein Expression and Purification

The target plasmid GST- Spastin^{M87V} and its mutant were transformed into the BL21 strain of *Escherichia coli* (Invitrogen). Fusion proteins were induced by incubation with 0.5 mmol/L Isopropyl β-D-Thiogalactoside (IPTG) for overnight at 25°C. *Escherichia coli* were spun down and resuspended with a cock-tail of protease inhibitors (Life). Ultrasound was performed 10 times at 50% intensity, 5s×5s, the cell debris was removed by centrifugation (15,000 g for 30 min). The supernatant, with 1% Triton X-100, was used for the purification of the GST fusion proteins using glutathione-Sepharose beads overnight at 4°C. The next day, centrifugal at 4°C for 5 minutes at a speed of 500g, supernatant were poured and added 1 ml Lysate, mixed solution were transferred it to a new 1.5 mL EP tube and washing it six times. At last, purification of the GST fusion proteins were used to run gel electrophoresis and performed Coomassie brilliant blue staining.

2.2.3. Transfection

The density of the transfected Hela cells was 0.5-2×10^5 / well, and the density was about 80%. Lipo 2000 reagent 2 μ l and 0.8 μg target plasmid DNA were diluted with 50 μ l MEN in each well for 5 min. The serum-containing medium was changed after 4-6 h cultured, and the cells were fixed with 4%PFA for 12 h.

2.2.4. Immunocytochemical Staining

Hela cell were *grown* on cover-slips (Fisher) and processed for immunofluorescence according to the standard protocol. Cells were fixed with 4% (w/v) paraformaldehyde (Sigma) for 5 min at room temperature and permeabilized with 0.1% Triton X-100 in PBS for 20 min. The cells were blocked in 3% normal donkey serum in TBS + 0.1% Triton X-100 for 1 h at room temperature and incubated with rabbit anti-GFP antibody at 4°C overnight. The cells were washed 3 times for 10 min with PBS + 0.1% Tween 20, and incubated with fluorescent second antibody for 2 h at room temperature. After three washes, cells were mounted on glass slides with Fluoro Gel II containing DAPI. Microscopy and image analysis were carried out using the same optical slice thickness for every channel using a confocal microscope (Carl Zeiss, Germany).

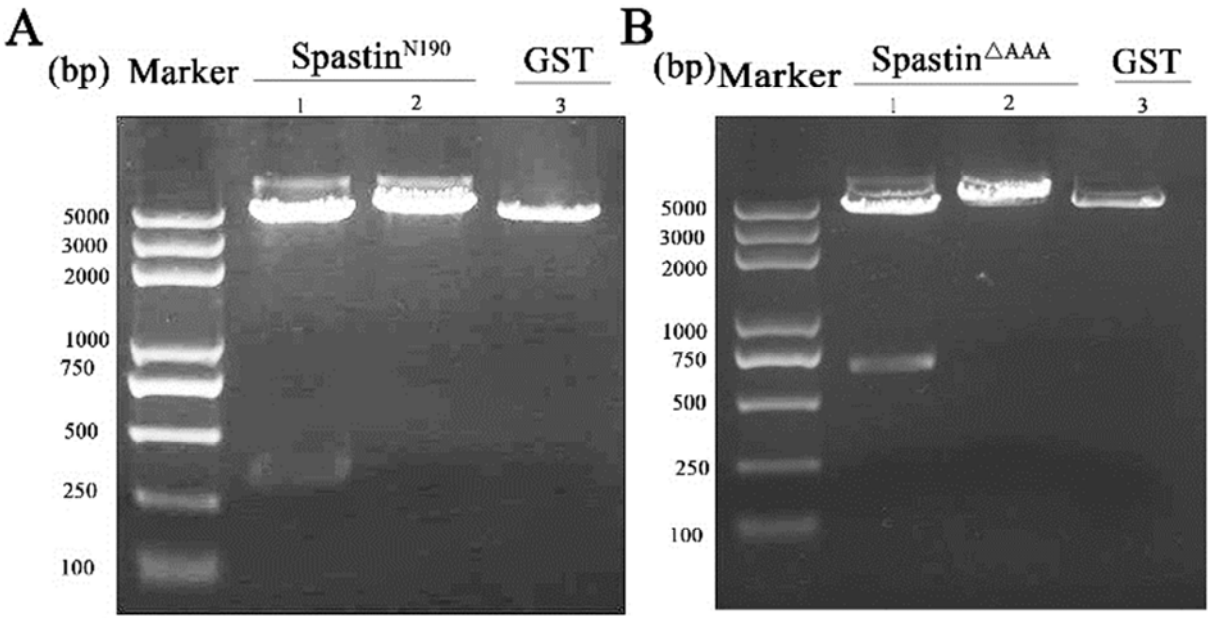
2.3. Statistical Analysis

The images of Hela cells were collected by laser confocal microscope, and the images of Hela cells were transferred into Image J for processing. Each group was set up with double blindness and repeated three times independently. At least 40 cells were counted in each group of Hela cells, and all the data were expressed in mean ±SEM. SPSS 22.0 software and One-way ANOVA method were used to carry on the correlation statistical analysis, * representing $P < 0.05$, the difference was statistically significant.

3. Results

3.1. Construct Prokaryotic Plasmid of Spastin^{M87V} and Its Mutants

According to the different functional domains of Spastin, we designed five truncated fragments of GST-Spastin^{M87V}, GST -Spastin^{N190}, GST-Spastin$^{\Delta AAA}$, GST-Spastin$^{\Delta N1}$, and GST-Spastin$^{\Delta N2}$ (Figure 1). The positive monoclonal culture was selected from the culture plate containing ampicyl antibiotic and incubated over-night. The plasmids were extracted by enzyme digestion (Figure 2) and sequenced to ensure that the recombinant plasmids were not mutated, deleted or mislocated.



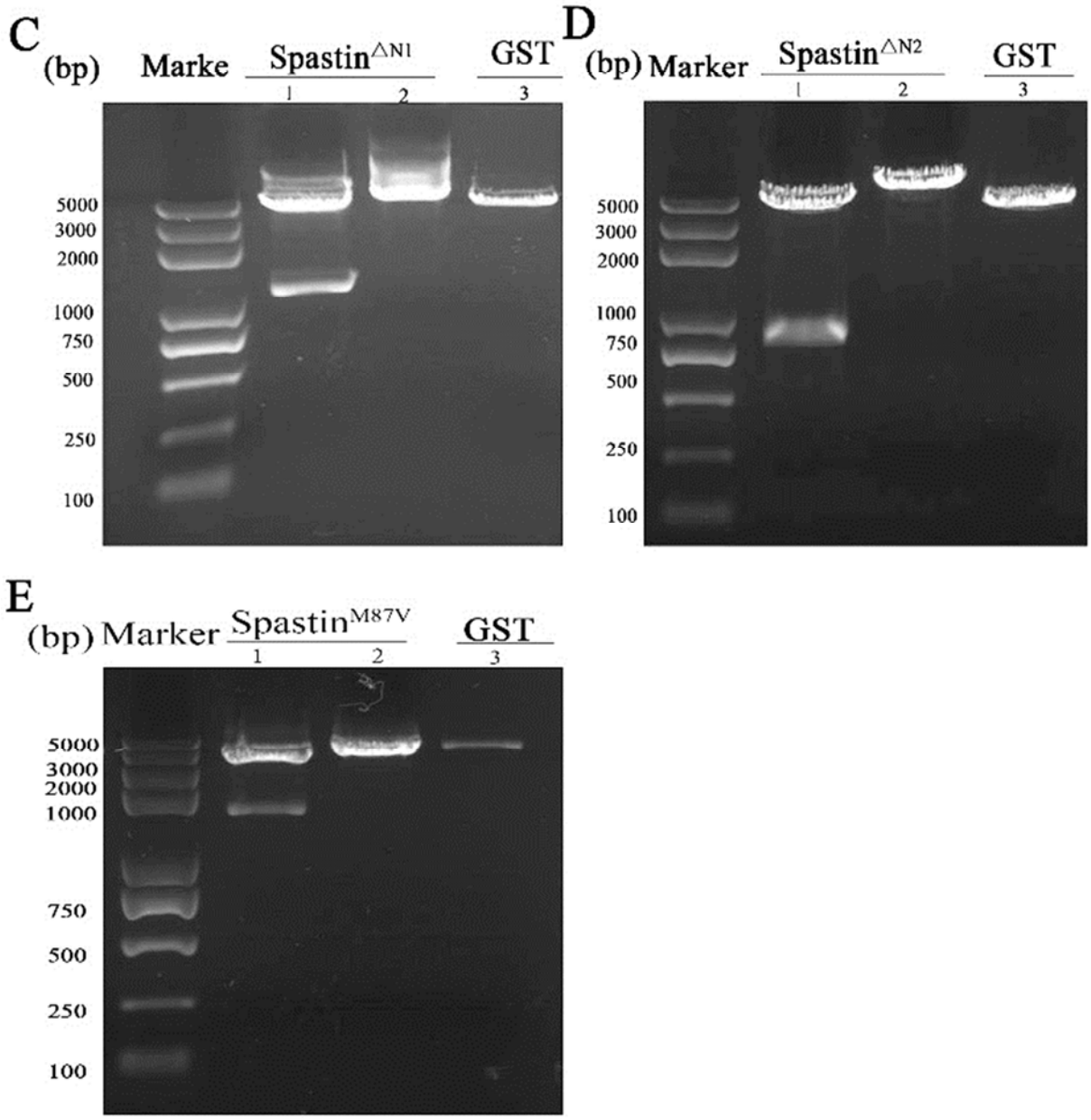


Figure 2. *Identification of prokaryotic plasmids of SpastinM87V and its mutants.*

A: GST-Spastin^{M87V} Recombinant plasmid; B: GST-Spastin$^{\Delta AAA}$ Recombinant plasmid; C: GST-Spastin$^{\Delta N1}$Recombinant plasmid; D: GST-Spastin$^{\Delta N2}$ recombinant plasmid; E: GST-Spastin^{M87V} recombinant plasmid; Lane 1: DNA Marker; Lane 2: Results of double enzyme digestion; Lane 3: Single enzyme digestion; Lane 4: Empty plasmid digestion.

3.2. Express and Purification of GST-Spastin^{M87V} and Its Mutants

The constructed GST-Spastin^{M87V} and its truncated prokaryotic plasmids were transformed into *E. coli*. The monoclonal bacteria was cultured in ampicillin resistant bacterial culture medium. When the OD value was 0.5- 0.6, the recombinant plasmid was induced to express overnight at 25°C of 0.5 mM IPTG. After induction, Coomassie brilliant blue staining was observed after SDS-PAGE electrophoresis (Figure 3 A, B). At the same time, the fusion protein was purified under suitable induction conditions and run through Coomassie brilliant blue staining (Figure 3C).

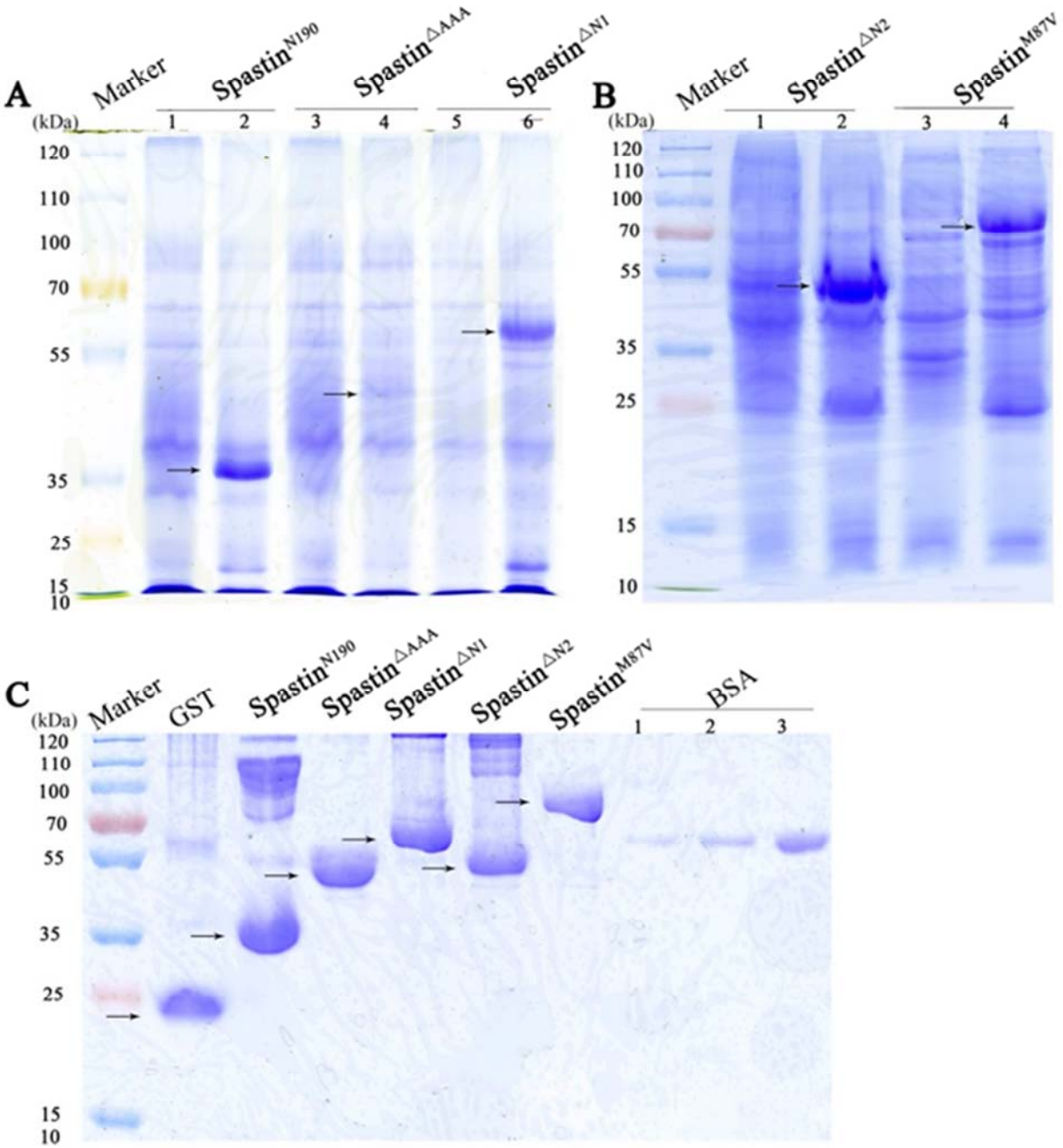


Figure 3. *Express and purification of GST-Spastin^{M87V} and its mutants.*

(A, B) Protein induced expression of Coomassie brilliant blue staining glue. (A) Lane Marker: protein Marker; Lane 1: GST-Spastin^{N190} Uninduced; Lane 2: GST-Spastin^{N190} induced; Lane 3: GST-Spastin$^{\Delta AAA}$ Uninduced; Lane 4: GST-Spastin$^{\Delta AAA}$ induced; Lane 5: GST- Spastin$^{\Delta N\ 1}$ Uninduced; Lane 6: GST-Spastin$^{\Delta N1}$ induced; (B): Marker: protein Marker; 1: GST-Spastin$^{\Delta N2}$ Uninduced; 2: GST-Spasti$^{\Delta N2}$ induced; 3: GST- Spastin^{M87V} Uninduced; 4: GST-Spastin^{M87V} induced; (C) Purified protein for Coomassie brilliant Blue staining: Lane 1: Marker; Lane 2: GST; Lane 2: GST-Spastin^{N190}; Lane 3: GST-Spastin$^{\Delta AAA}$; Lane 4: GST-Spastin$^{\Delta N1}$; Lane 5: GST-Spastin$^{\Delta N2}$ and Lane 5: GST- Spastin^{M87V}. BSA standard protein were 1 μg, 2 μ g and 4 μg respectively.

3.3. Construct Eukaryotic Plasmids of SpastinM87V and Its Mutants

Similarly, according to the different functional domains of Spastin, we designed five truncated fragments of GFP-Spastin^{M87V}, GFP -Spastin^{N190}, GFP -Spastin$^{\Delta AAA}$, GFP -Spasti$^{\Delta N1}$, and GFP -Spastin$^{\Delta N2}$ (Figure 1). The positive monoclonal culture was selected from the culture plate containing kanamycin antibiotic and incubated overnight. The plasmids were extracted by enzyme digestion (Figure 4) and sequenced to ensure that the recombinant plasmids were not mutated, deleted or mislocated.

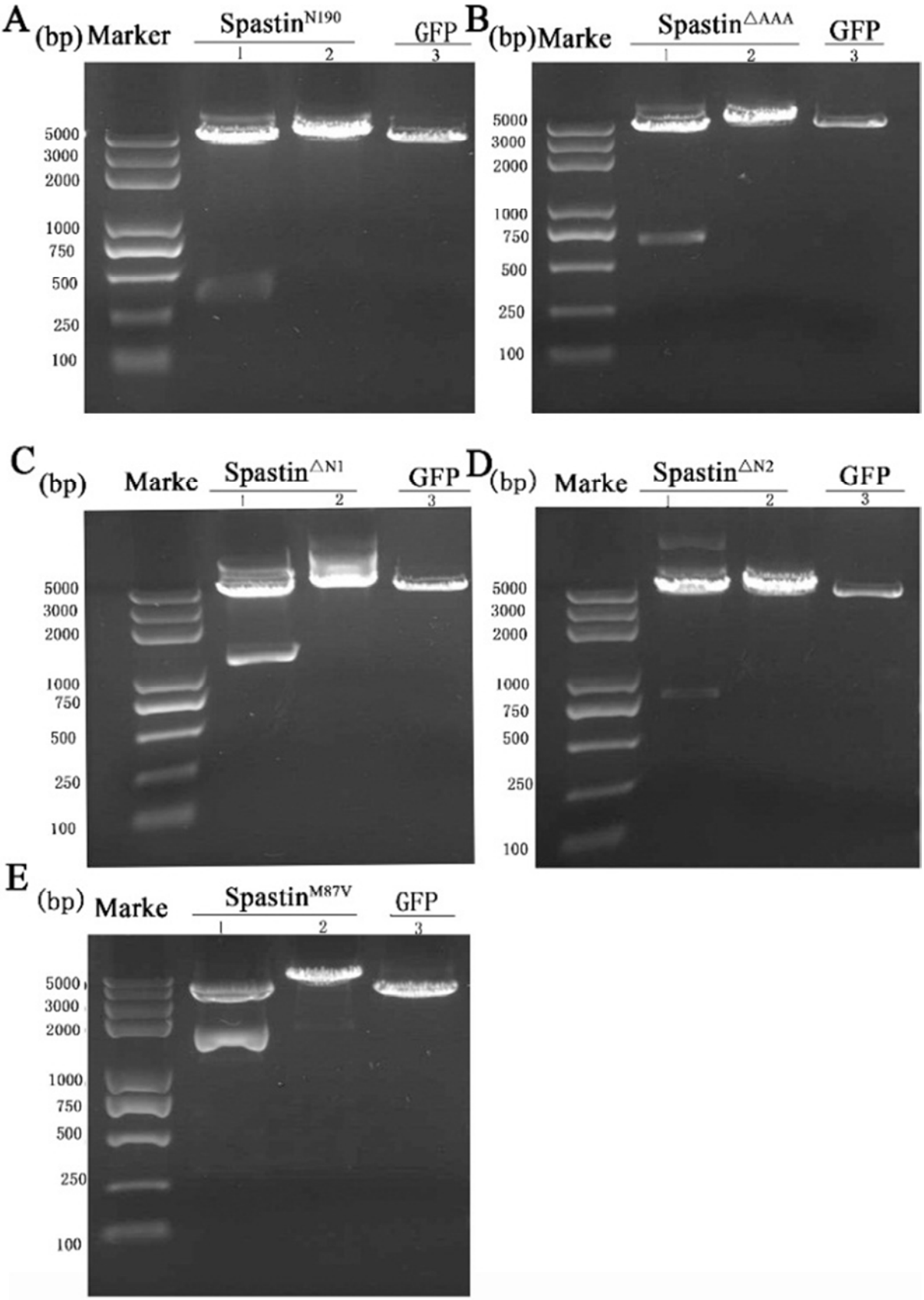


Figure 4. *Construct eukaryotic plasmids of SpastinM87V and its mutants.*

A: GFP- A: GFP-Spastin^{M87V} recombinant plasmid; B: GFP-Spastin$^{\triangle AAA}$ Recombinant plasmid; C: GFP-Spastin$^{\triangle N1}$ Recombinant plasmid; D: GFP -Spastin$^{\triangle N2}$ recombinant plasmid; E: GFP -Spastin^{M87V} recombinant plasmid; Lane 1: DNA Marker; Lane 2: Results of double enzyme digestion; Lane 3: Single enzyme digestion; Lane 4: Empty plasmid digestion.

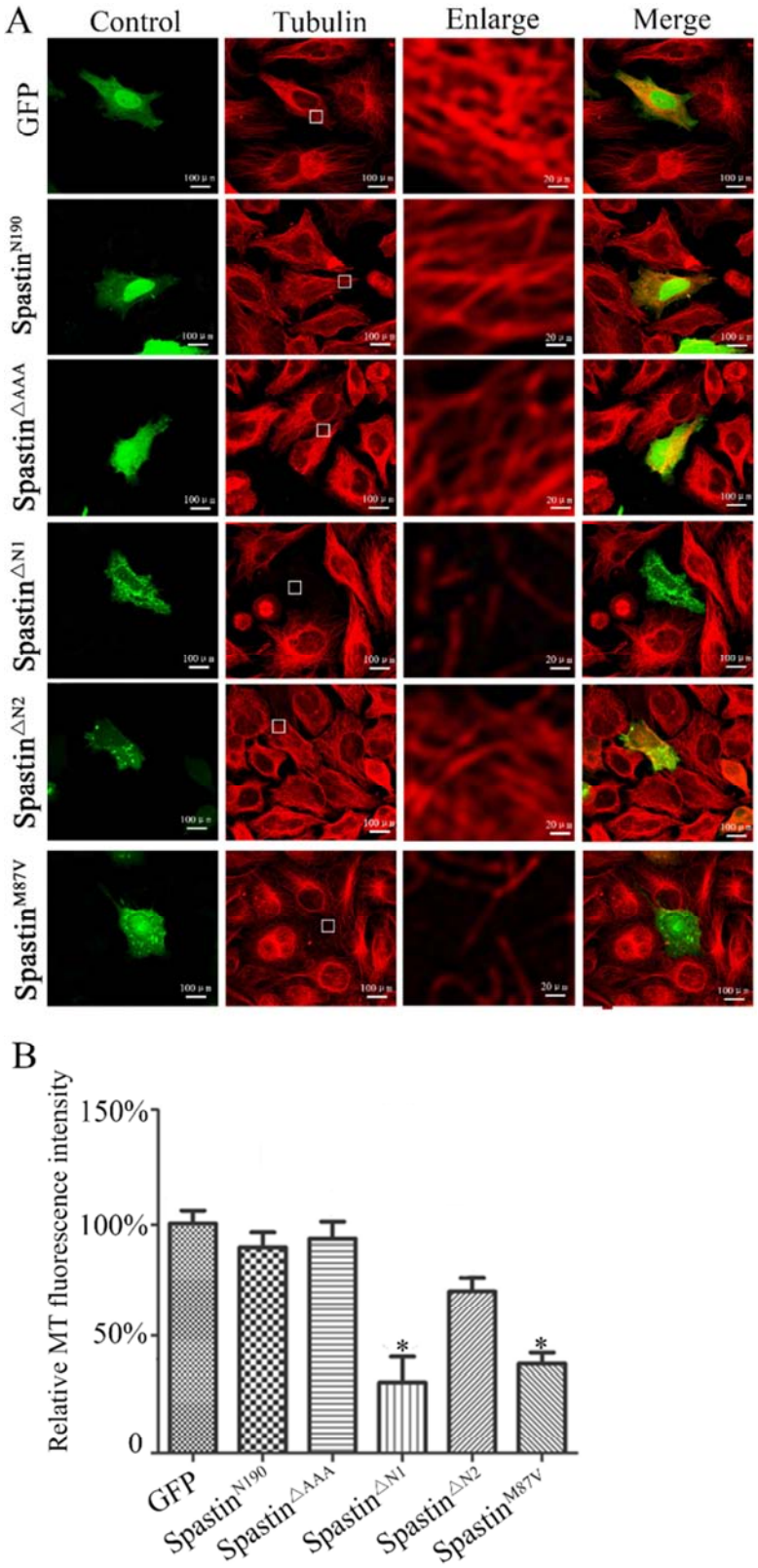


Figure 5. *Microtubule cutting of Hela cells by different functional domains of Spastin^{M87V}.*

A: Spastin (green), Tubulin (red), merge (yellow); and enlargement is local magnification. The scale is 20 μ m or 100 μ m. B: The relative fluorescence intensity of microtubules was measured by complete random analysis of variance (ANOVA). The results were expressed as mean ±SEM ($P < 0.05$). * represents statistical significance.

3.4. Microtubule Cutting of Hela Cells by Different Functional Domains of Spastin^{M87V}

To observe the microtubule cutting efficiency of Hela cells on different functional domains of Spastin^{M87V}, we introduced Spastin^{M87V} and its truncated fragments into Hela cells, cultured them for 12h, and then stained them with immunofluorescence, and observed them under a confocal laser microscope. The microtubules of Hela cells in the experimental group of Spastin^{M87V} and Spastin$^{\Delta N1}$ were depolymerized and cut into small pieces, and other groups were not. In addition, the fluorescence intensity of the microtubules of the Hela cells in the Spastin^{M87V} and Spastin$^{\Delta N1}$ groups were significantly weaker than those in the GFP control group. The results showed that overexpression of Spastin^{M87V} and Spastin$^{\Delta N1}$ had a remarkable microtubule cutting effect on Hela cells (Figure 5).

4. Discussion

The effects of different functional domains of Spastin^{M87V} on the microtubule cutting of Hela cells were investigated. Spastin is a microtubule-cutting protein that can participate in many physiological functions, such as cytoskeleton movement, the mitotic cell cycle, protein vesicle transport and synthesis of different organelles [19, 20]. The Spastin protein is a hollow hexamer crystal structure, forming a central ring of six “arms” that binds to the microtubules, and gathers a large number of tubulin-cutting enzymes at the junction of the hollow structure and the surrounding area, which can identify the C-terminal of the tubulin. Spastin maintains the dynamic balance of the microtubules by its multiple functional domains [8, 21].

First, we designed truncated Spastin fragments according to the three main functional domains of Spastin, including tubulin interaction domain (MIT), tubulin binding domain (MTBD), and ATP binding domain (AAA): Spastin^{N190} (amino acids 87–190). GST-spastin$^{\Delta AAA}$ (amino acids 87–305), GST-spastin$^{\Delta N1}$ (amino acids 191-616), GST-spastin$^{\Delta N2}$ (amino acids 306-616). In addition, Spastin^{N190} contains the MIT region, Spastin$^{\Delta AAA}$ contains MTBD, Spastin$^{\Delta N1}$ contains both MTBD and the AAA region, and Spastin$^{\Delta N2}$ mainly contains the AAA region. Through the selective prokaryotic expression of the corresponding target protein, we selected the BL21 receptive state for protein expression because BL21 *Escherichia coli* is a high expression vector, which is regulated by T7 promoter. T7 lysozyme neither directly reduces the purity of the target gene expression nor interferes with the expression of the target protein induced by IPTG. The expression of the target protein of this kind of vector is fast, and it does readily form insoluble inclusion body granules. After many experiments, we found that low temperature, low speed and low concentration can reduce the formation of inclusion bodies, and good experimental conditions can ensure the high quality of the fusion protein, which is conducive to further research [22].

Spastin has the strongest ability to cut microtubules of any protein in the AAAfamily. It can bind tubules by tubulin binding function domain (MTBD). The positively charged AAA ATP ring holes can destroy the palisade structure of microtubules and bend microtubules. It can break the stable functional regions of microtubules and form more microtubule fragments. Furthermore, glutamine acylation at the C-terminal of tubulin can significantly promote the cutting activity of Spastin [15, 16, 23]. Five truncated recombinant vectors of Spastin^{M87V}, Spastin^{N190}, Spastin$^{\Delta AAA}$, Spastin$^{\Delta N1}$, and Spastin$^{\Delta N2}$ were designed according to the different functional domains of Spastin. The Lipo-fectamine2000 carrying truncated recombinant vectors were introduced into Hela cells, and we found that the fluorescence intensity of Hela cell microtubules in Spastin^{M87V} and Spastin$^{\Delta N1}$ groups was to be significantly less intense than in the GFP control group. Since the Spastin$^{\Delta N1}$-truncated fragment contained microtubule binding domain (MTBD domain) and the cutting functional domain (AAA domain), while Spastin$^{\Delta N2}$ only contains the AAA region. It indicated that Spastin^{M87V} must have a complete microtubule binding domain (MTBD) and cutting function domain specific to microtubule (AAA). Spastin can bind to the stable functional domain of microtubules, anchor the sidewall of microtubules by MIT, bind to the C-terminal of tubulin with positively charged AAA ATP ring holes, destroy the palisade structure of microtubules and cuase long microtubules to bend and break into small segments of microtubules [3, 8].

5. Conclusion

In conclusion, we confirmed the effect of different functional domains of Spastin^{M87V} on the microtubule cutting of Hela cells. The cutting of microtubules by Spastin must involve a complete microtubule binding domain (MTBD domain) and cutting domain (AAA domain). These findings provide a new theoretical basis for further examination of the involvement of different functional domains of Spastin in cell-related physiological activities. However, the specific mechanism underlying the interaction between different functional domains of Spastin and intracellular proteins remains to be further explored.

Acknowledgements

The authors declare no conflict of interest. This work was supported by National Natural Science Foundation of China (81771331), Natural Science Foundation of Guangdong Province, China (2017A030313595), and Guangzhou science and technology project (201605130849016).

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