รายงานวิจัยฉบับสมบูรณ์

การศึกษาบทบาทของเอนไซม์แอสพาราจีนซินทีเทส ในกระบวนการแบ่งเซลล์

โดย ฉลองรัตน์ โนรี

ธันวาคม 2560

สัญญาเลขที่ TRG5880187

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ฉลองรัตน์ โนรี
สถาบันชีววิทยาศาสตร์โมเลกุล
มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย และ
มหาวิทยาลัยมหิดล

Abstract

Asparagine synthetase (ASNS) is a metabolic enzyme, re-discovered from screening the yeast GFP collection (Invitrogen) that, in addition to its enzymatic function in asparagine biosynthesis, it is able to assemble into visible cytoplasmic structures (foci/filaments). While investigating if ASNS assembly is evolutionarily conserved in mammalian cells using immuno-staining techniques, I ran into dividing cells with ASNS, seemingly, lining up with the mitotic spindles. Pilot experiments have been performed to confirm that the observation was not resulted from cross-reaction between anti-ASNS antibody and microtubules. According to the data from indirect immunofluorescence and human cell lines expressing ASNS-EGFP, ASNS is, indeed, localized to the mitotic spindles during mitosis. This finding might have a great impact on the field of cell and cancer biology as ASNS is one of the key markers for cancer diagnosis. My hypothesis is that ASNS might moonlight in cell division process as a mitotic spindle associated protein. Misregulation of ASNS expression or mutation of ASNS might cause abnormality in spindle formation and chromosome segregation, therefore leading to production of aneuploid cells. These cells could eventually become cancerous. This project has 2 main goals which are (1) establish ASNS as a novel mitotic spindle associated protein, and (2) study the effect of asparaginase, an enzyme used to lower asparagine levels in acute lymphoblastic leukemia patients, and the effect of nocodazole, a microtubule formation inhibitor, on the relative expression between ASNS to tubulin.

Project Code:

TRG5880187

Project Title:

Asparagine Synthetase, A Novel Mitotic Spindle Associated Protein

Investigator:

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Project Period:

July 2015 – December 2017

Objectives:

(1) Establish ASNS as a novel mitotic spindle associated protein.

(2) Study the effect of asparaginase (an enzyme used to lower asparagine levels in acute lymphoblastic leukemia patients) and the effect of nocodazole (a microtubule formation inhibitor) on the relative expression between ASNS and tubulin.

Experimental Procedure:

Cell Lines and Cell Culture

CCRF-CEM, MOLT-4, RPE1 cells were provided by A. Shiau (Small-Molecule Discovery Unit, Ludwig Institute for Cancer Research, University of California, San Diego). CCRF-CEM and MOLT-4 suspension cells were cultured in RPMI-1640 medium supplemented with 10% FBS at 37°C with 5% CO_2. RPE1 adhesion cells were cultured in DMEM/Ham's F12 50/50 supplemented with 10% FBS at 37°C with 5% CO_2. The cells were counted, passaged, and maintained using a standard protocol.

Indirect immunofluorescence

5x10^4 cells were seeded into each well of 6-well plates with 2-3 sterile coverslips (pre-treated with poly-L-lysine if suspension cells were used) of 6-well plates. Cells were grown for 2 days, washed once with sterile 1xPBS, fixed with 4% paraformaldehyde (in 1xPBS) for 15 min, followed by another 3 washes (5-min incubation each) with 1xPBS. The coverslips having cultured cells attached were then transferred from the culture plates into a dark moisture chamber for immunostaining. Cells on the coverslips were treated with permeabilization solution (1xPBS, 1% BSA, and 0.1% Triton-X100) for 1 hour. After aspirating off the permeabilization solution, cells were treated with blocking solution (1% BSA in 1xPBS) for 5 min. The cells were then immuno-stained. Purified rabbit anti-ASNS antibody (CA5498; test bleed#2) (1:500) and Alexa Fluro® 568 goat anti-rabbit IgG, Invitrogen) (1:500) were used to stain ASNS. Mouse anti-alpha tubulin (12G10, DHSB, University of Iowa) (1:1,000) and Alexa Fluro® 488 goat anti-mouse IgG (Invitrogen) (1:500) were used to label the microtubules. Mouse anti-aurora A kinase (35C1, Invitrogen) (1:333) and Alexa Fluro® 488 goat anti-mouse IgG (Invitrogen) (1:500) were used to label the spindle poles. DAPI (2 μg/ml final concentration) was used to label the nucleus. Immunostaining with each primary antibody was performed for 1 hour at room temperature, or overnight at 4°C. Washing with blocking solution was performed at least 3 times (5-min incubation each) to remove the solution with primary antibody. After incubation with secondary antibody for 1 hour

at room temperature, cells on the coverslips were washed 4 times with 1xPBS (DAPI was applied during the third wash) before mounting the slides with Vectashield (Vector Laboratories).

Drug Treatment and Western Blot Analysis

RPE1 cells ($2x10^5$ cells) were seeded into each well of 6-well plates, and cultured in DMEM/Ham's F-12 50/50 medium supplemented with 10% FBS for 2 days at $37^{\circ}C$ with 5% CO_2. To inhibit microtubule formation, nocodazole (100 ng/ml final concentration) was added to the culture for 16 hours. Cells were washed once with sterile 1xPBS prior to protein sample preparation. Cells were scrapped off and collected with RIPA buffer (100 μl/well), and transferred into a microfuge tube. 2x SDS-PAGE sample buffer (100 μl) and sterile glass beads (50 μl) were added to the tube. Samples were vigorously vortexed for 1 min, boiled for 5 min, placed on ice for 5 min, and centrifuged at 10,000 rpm for 1 min. SDS-PAGE and Western blotting were performed with a standard protocol (20 μl/sample; 8% SDS-PAGE). ASNS expression levels were detected using rabbit anti-ASNS antibody (CA5498; test bleed#2, purified) (1:500) and HRP conjugated donkey anti-rabbit IgG (1:5,000). Tubulin expression levels were detected using mouse anti-alpha tubulin (12G10, DSHB) (1:5,000) and HRP conjugated sheep anti-mouse IgG (1:2,500). Expression levels of ASNS and alpha-tubulin were quantitated with ImageJ (NIH).

To test the effect of asparaginase treatment, CCRF-CEM and MOLT-4 were cultured in RPMI-1640 supplemented with 10% FBS with or without 2U/ml final concentration of asparaginase for 2 days. For each treatment, $1x10^6$ cells were collected to prepare the protein sample. Protein sample preparation and Western blotting were performed as mentioned above. In case of RPE1 cells, $2x10^5$ cells were seeded into each well of 6-well plate, cultured in DMEM/Ham's F-12 50/50 medium containing 10% FBS, supplemented with or without 2U/ml (final concentration) of asparaginase for 2 days. The RPE1 cells were not counted, but instead used from the whole well for sample preparation as the expression level of ASNS was normalized by that of alpha-tubulin before the comparison of the asparaginase effect between treated and non-treated conditions was made.

Confocal Imaging

Images were acquired using spinning disk Carl Zeiss Axiovert 200M microscope with a Plan-Apochromat 100X/1.40 Oil objective lens, and Micro-Manager operation software version 1.4.17. Z-stack images of each sample were taken every 0.25 microns over 3-10 microns, deconvolved, and then compressed into a single image.

Results and Discussion:

Asparagine synthetase (ASNS) is one of the metabolic enzymes found to be capable of supramolecular assembly, in yeast **(1-3)**. In order to investigate whether this enzyme could exhibit the same assembly property in humans, immunostaining of Jurkat cells, an immortalized line of human T lymphocyte cells, with a customized anti-ASNS antibody was performed during my summer research (2013) at MBL, Woods Hole, MA, USA. Surprisingly, not forming cytoplasmic foci/filaments as found in yeast, anti-ASNS antibody, instead, showed a staining pattern resembling the structure of mitotic spindles within the human dividing cells. This has never been reported before as the main focus of asparagine synthetase is mostly for catalyzing asparagine biosynthesis in biochemistry **(4, 5)**, or being a biomarker for cancer diagnosis in the field of cancer biology **(6, 7)**. To link its role and function, especially in the unprecedented aspect of its subcellular localization and supramolecular structure formation, with cell division is therefore beyond the current knowledge.

To ensure that the staining pattern by anti-ASNS antibody can perfectly line up with the mitotic spindles of dividing cells, co-immunostaining of CCRF-CEM, MOLT-4, and RPE1 cells with anti-ASNS and anti-alpha tubulin (tubulins are building blocks of microtubules and mitotic spindles) was performed **(Figure 1-3)**. As expected, the results revealed the co-localization of ASNS and alpha tubulin.

Anti-aurora A kinase, a marker for spindle poles, was also used together with anti-ASNS to show that ASNS heavily clustered around spindle poles in all cell lines tested, both before and during the mitosis **(Figure 4-7)**.

Since they show co-localization, it is possible that their relative expression levels of ASNS and tubulins might be associated with the regulation of cell growth and division. Nocodazole is a drug known to inhibit polymerization of tubulins into microtubules **(8)**. We found that cells treated with 100 ng/ml nocodazole for 16 hours were in mitotically arrested stage. The relative expression levels of ASNS to alpha-tubulin of the nocodazole-treated cells was about 2-fold increase when compared to the non-treated cells **(Figure 8)**.

Asparaginase (ASNase), a drug used to treat acute lymphoblastic leukemia (ALL) patients to lower intracellular asparagine and inhibit cancer cell growth **(9-11)**, showed a similar result. ASNase (2 U/ml) could induce the protein expression of ASNS relative to that of alpha-tubulin at about 5-10 folds, when compared to the control **(Figure 9-11)**.

Both drug treatments obviously affected cell growth **(Figure 8A, 9A, 10A)** and also raised ASNS to alpha-tubulin expression ratio **(Figure 8B-C, 9B-C, 10B-D, and 11)**. The availability of intracellular amino acids and tubulins could be biosensors to signal the promotion or inhibition of cell division process. ASNS, demonstrated here, might be an enzyme representative to tightly coordinate with microtubules, a cytoskeleton protein, for keeping cellular integrity. Further studies should be conducted for providing more direct evidence.

Conclusion:

We are able to confirm that asparagine synthetase (ASNS) localizes to the mitotic spindle as shown by co-immunostaining of ASNS and alpha tubulin. Moreover, we could observe the dense cluster of ASNS around the spindle poles in the cells which are about to proceed to mitotic stage. All of these findings suggest the possible role of ASNS in cell division, serving as a mitotic spindle associated protein.

In order to reveal the importance of having ASNS on the mitotic spindle, we therefore design to treat the cells with nocodazole, a chemical known to disrupt microtubule formation. As a result, the expression ratio of ASNS: α-tubulin has been found to be increased. Similarly, when treating cells with asparaginase (ASNase), the expression ratio of ASNS:α-tubulin is also significantly higher than that of the untreated group. Asparaginase is commonly used to treat the patients with acute lymphoblastic leukemia, whose asparagine levels are abnormally high. ASNS might be required by cancer cells to compensate for the loss of intracellular asparagine after ASNase treatment. Thus, ASNS is enormously upregulated in order to boost the asparagine level up to support nonstop growth and cell division of the cancer cells.

For further study, we would like to analyze how ASNS levels affect the cell cycle. By treating cells with asparaginase, the distribution of cell populations in each cell cycle stage can be monitored. Perhaps, we might find out the association between expression levels of ASNS and cell cycle progression.

References:

1. Shen QJ, Kassim H, Huang Y, Li H, Zhang J, Li G, et al. Filamentation of Metabolic Enzymes in *Saccharomyces cerevisiae*. Journal of genetics and genomics 2016 Jun 20;43(6):393-404.

2. Narayanaswamy R, Levy M, Tsechansky M, Stovall GM, O'Connell JD, Mirrielees J, et al. Widespread reorganization of metabolic enzymes into reversible

assemblies upon nutrient starvation. Proceedings of the National Academy of Sciences of the United States of America. 2009 Jun 23;106(25):10147-52.

3. Noree C. Polymerization and Partitioning: A Novel Mechanism for Regulating Metabolic Enzyme Activity in *Saccharomyces Cerevisiae*. ProQuest: University of California, San Diego; 2013.

4. Dang VD, Valens M, Bolotin-Fukuhara M, Daignan-Fornier B. Cloning of the ASN1 and ASN2 genes encoding asparagine synthetases in *Saccharomyces cerevisiae*: differential regulation by the CCAAT-box-binding factor. Molecular microbiology. 1996 Nov;22(4):681-92.

5. Jones GE. L-Asparagine auxotrophs of *Saccharomyces cerevisiae*: genetic and phenotypic characterization. Journal of bacteriology. 1978 Apr;134(1):200-7.

6. Lorenzi PL, Weinstein JN. Asparagine synthetase: a new potential biomarker in ovarian cancer. Drug news & perspectives. 2009 Jan-Feb;22(1):61-4.

7. Sircar K, Huang H, Hu L, Cogdell D, Dhillon J, Tzelepi V, et al. Integrative molecular profiling reveals asparagine synthetase is a target in castration-resistant prostate cancer. The American journal of pathology. 2012 Mar;180(3):895-903.

8. Jordan MA, Thrower D, Wilson L. Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. Journal of cell science. 1992 Jul;102(Pt3):401-16.

9. Pieters R, Hunger SP, Boos J, Rizzari C, Silverman L, Baruchel A, et al. L-asparaginase treatment in acute lymphoblastic leukemia: a focus on Erwinia asparaginase. Cancer. 2011 Jan 15;117(2):238-49.

10. Ertel IJ, Nesbit ME, Hammond D, Weiner J, Sather H. Effective dose of L-asparaginase for induction of remission in previously treated children with acute lymphocytic leukemia: a report from Childrens Cancer Study Group. Cancer research. 1979 Oct;39(10):3893-6.

11. Richards NG, Kilberg MS. Asparagine synthetase chemotherapy. Annual review of biochemistry. 2006;75:629-54.

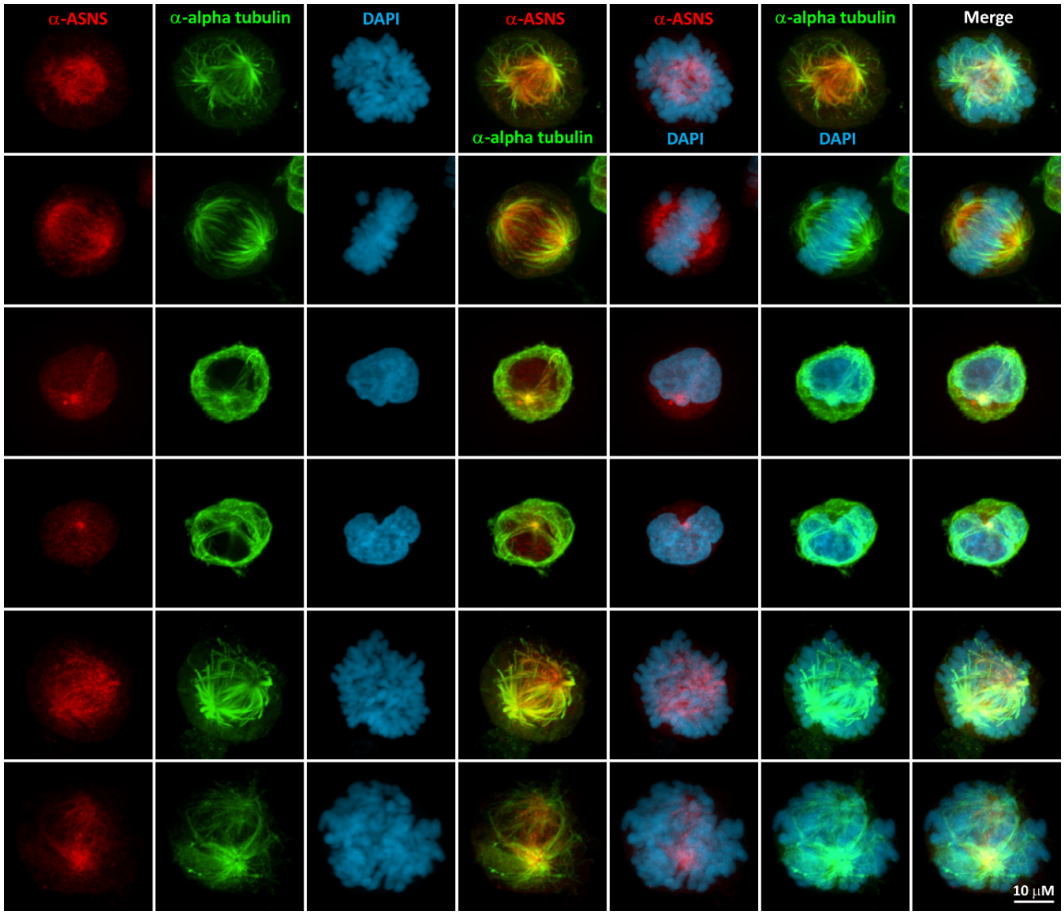


Figure 1 Co-localization of ASNS and microtubules in MOLT-4 cells. ASNS **(red)**, microtubules **(green)**, and nucleus **(blue)**.

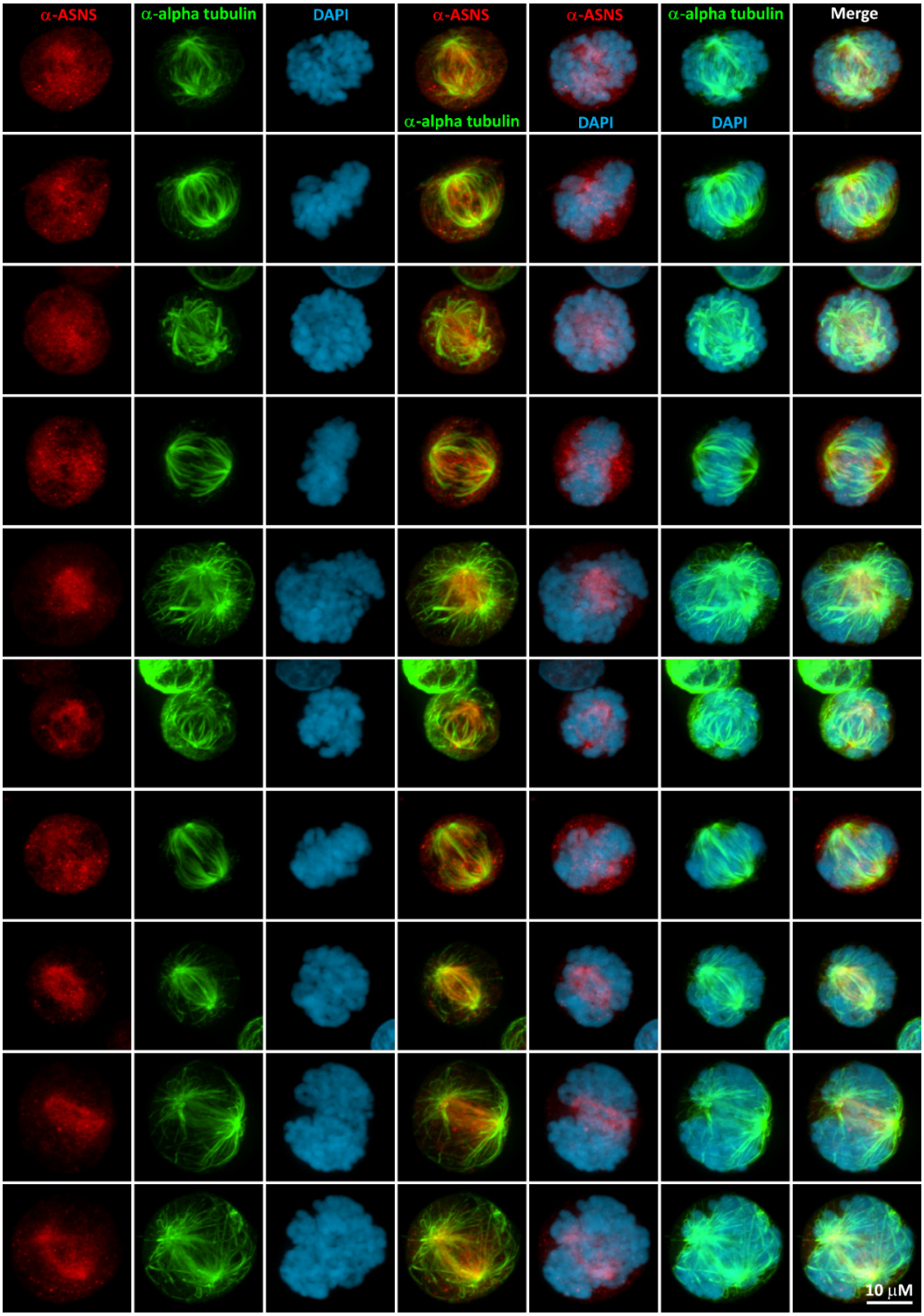


Figure 2 Co-localization of ASNS and microtubules in CCRF-CEM cells. ASNS **(red)**, microtubules **(green)**, and nucleus **(blue)**.

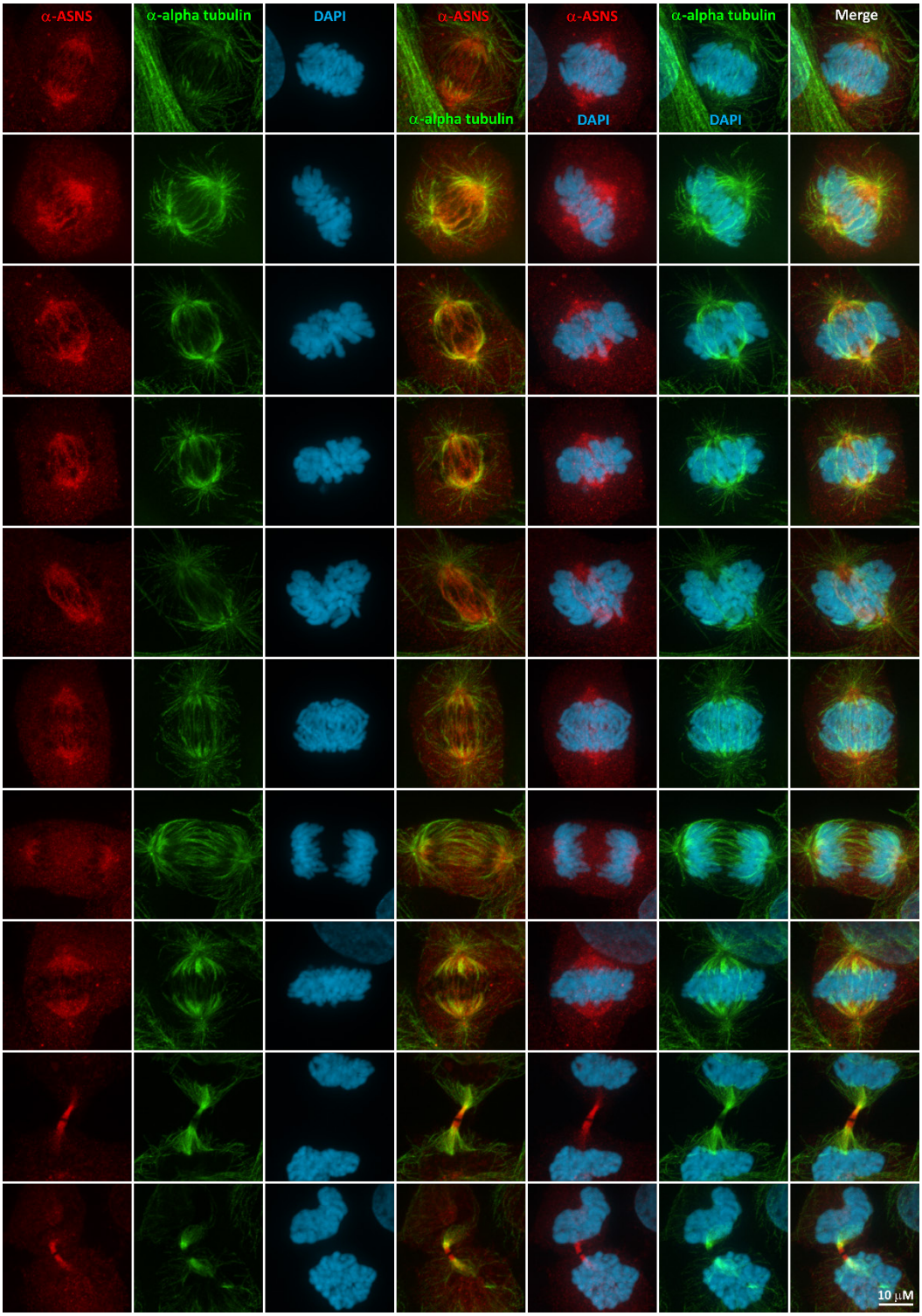


Figure 3 Co-localization of ASNS and microtubules in RPE1 cells. ASNS **(red)**, microtubules **(green)**, and nucleus **(blue)**.

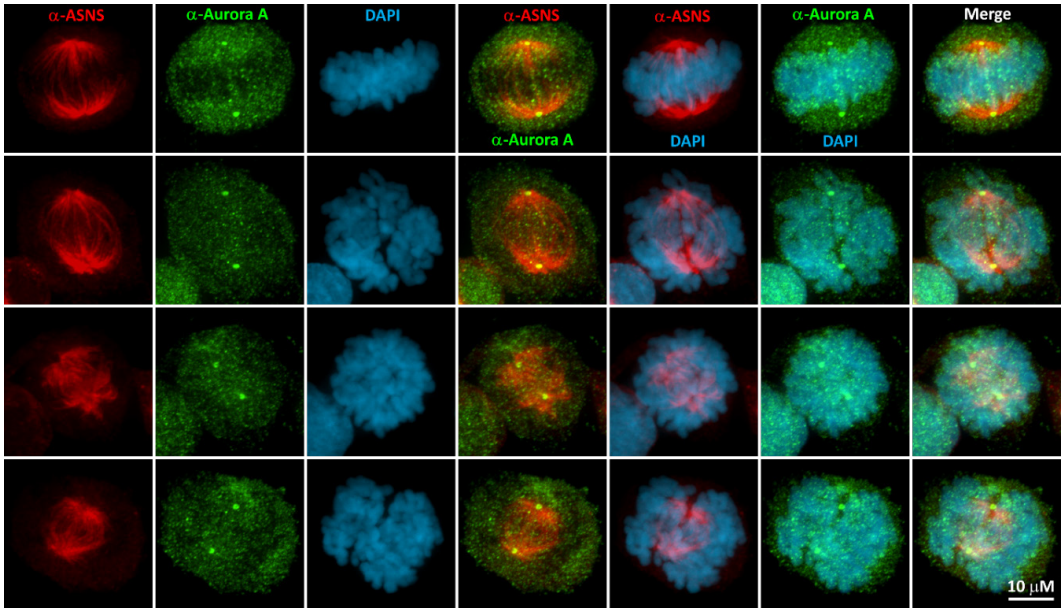


Figure 4 Localization of ASNS and spindle poles in MOLT-4 cells. ASNS **(red)**, spindle poles **(green)**, and nucleus **(blue)**.

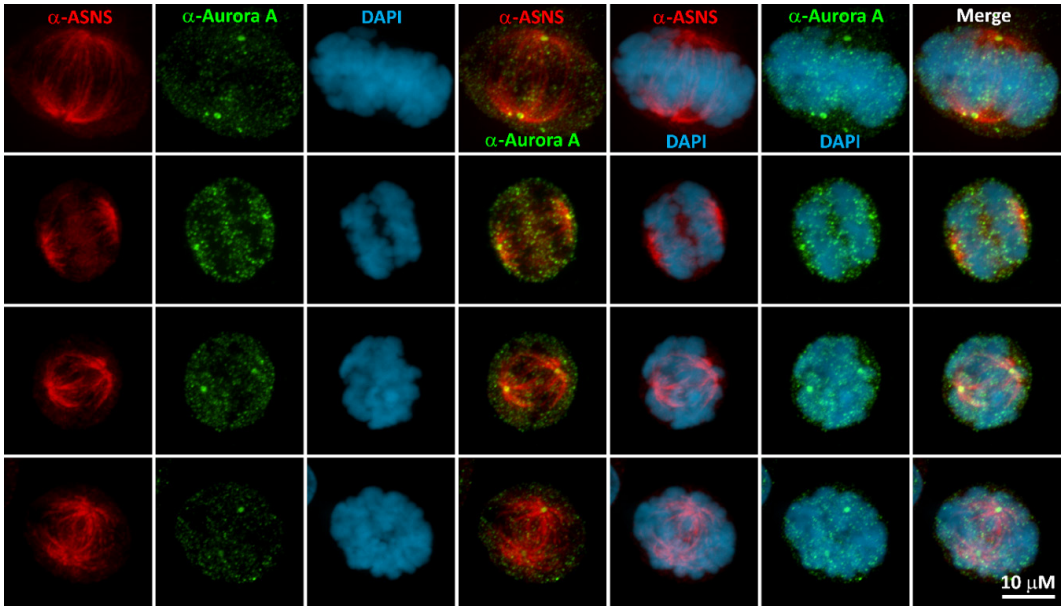


Figure 5 Localization of ASNS and spindle poles in CCRF-CEM cells. ASNS **(red)**, spindle poles **(green)**, and nucleus **(blue)**.

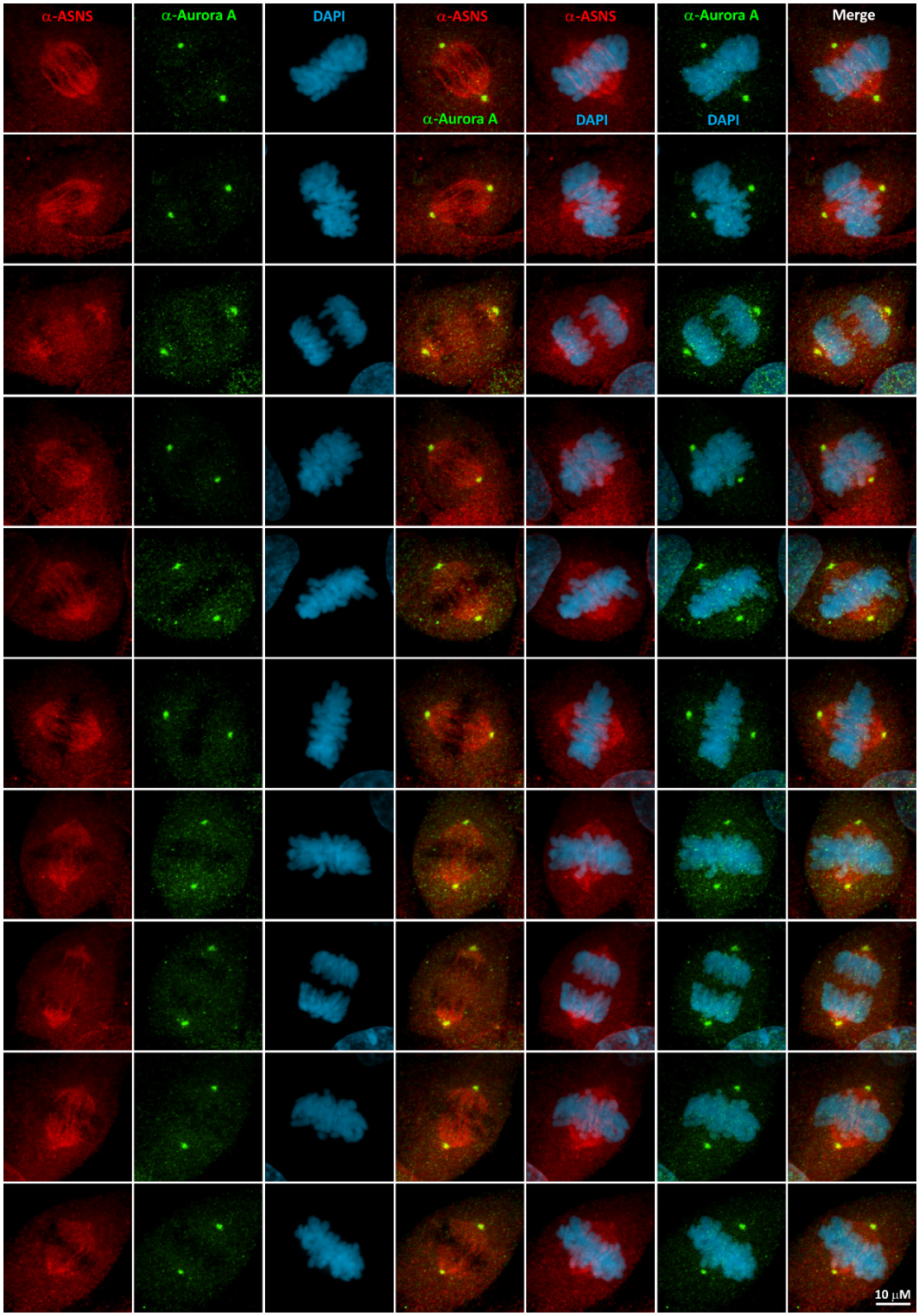


Figure 6 Localization of ASNS and spindle poles in RPE1 cells. ASNS **(red)**, spindle poles **(green)**, and nucleus **(blue)**.

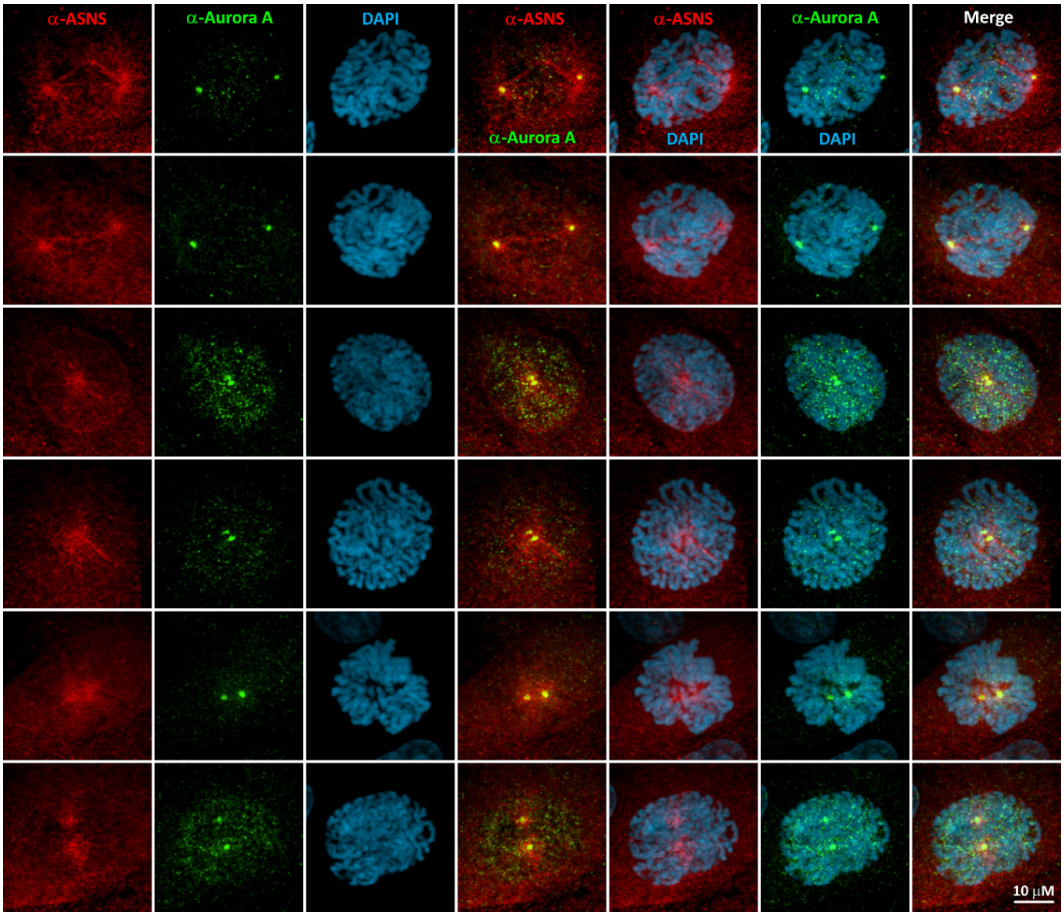
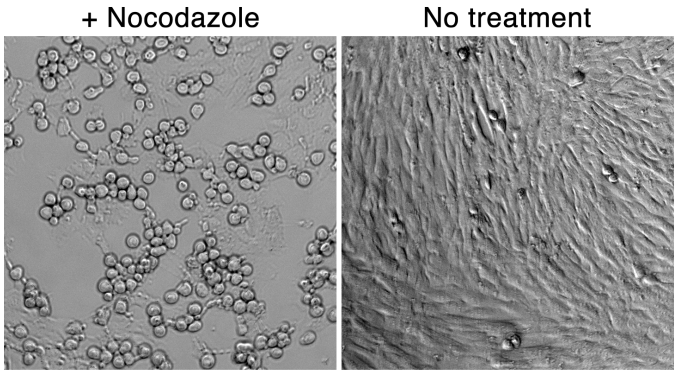


Figure 7 Clustering of ASNS around spindle poles in RPE1 cells. ASNS **(red)**, spindle poles **(green)**, and nucleus **(blue)**.

A



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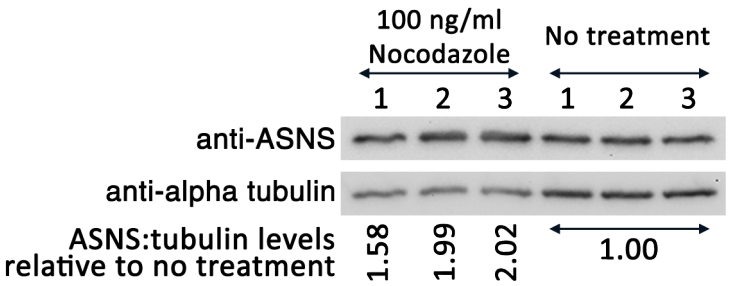

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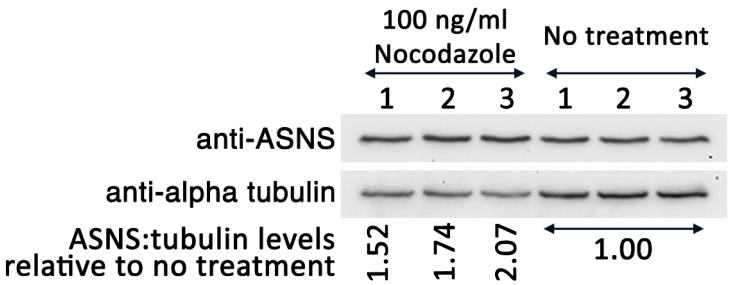
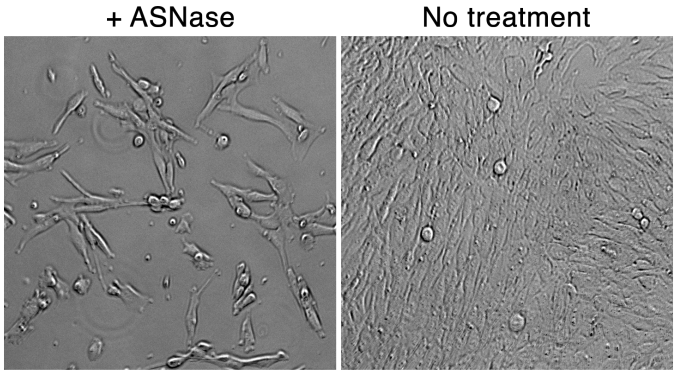


Figure 8 Approximately 2-fold increase of ASNS levels in RPE1 cells when treated with 100 ng/ml nocodazole for 16 hours. **(A)** Mitotic arrest found in cells treated with nocodazole, **(B)** and **(C)** two independent experiments performed for Western blot analysis. ImageJ was used for band intensity quantification.

A



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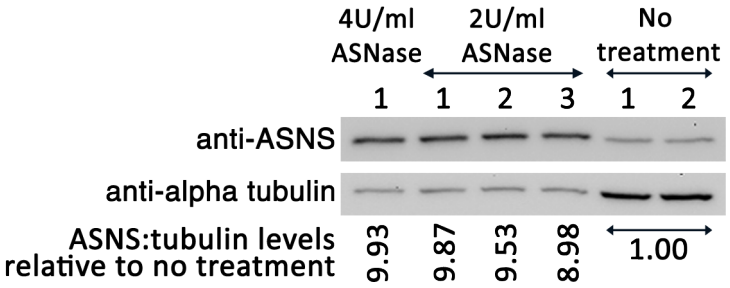

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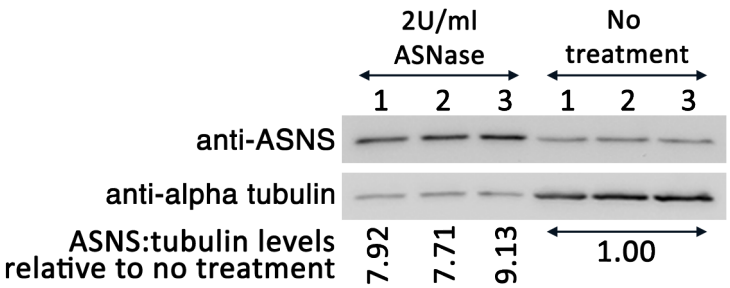


Figure 9 Approximately 9-fold increase of ASNS levels in RPE1 cells when treated with 2 U/ml asparaginase (ASNase) for 2 days. **(A)** Growth arrest found in cells treated with asparaginase, **(B)** and **(C)** two independent experiments performed for Western blot analysis. ImageJ was used for band intensity quantification.

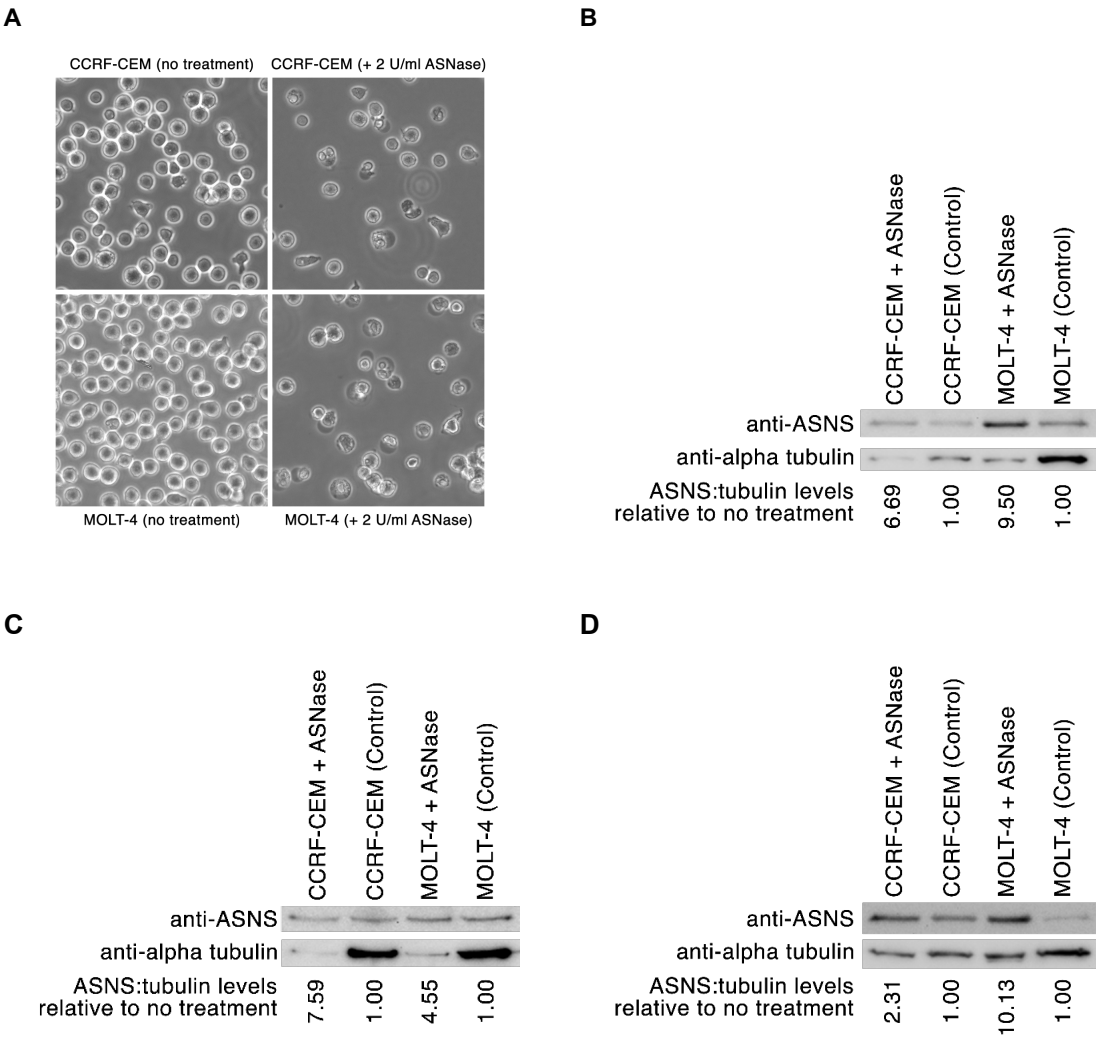
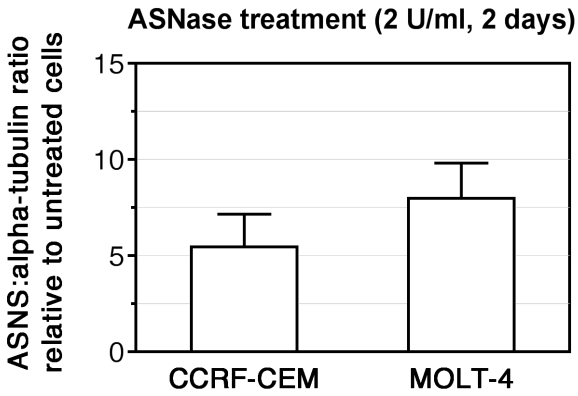


Figure 10 Approximately 5-fold and 8-fold increase of ASNS levels in CCRF-CEM and MOLT-4 cells, respectively, when treated with 2 U/ml asparaginase (ASNase) for 2 days. **(A)** Growth retardation found in cells treated with asparaginase, **(B)**, **(C)**, and **(D)** three independent experiments performed for Western blot analysis. ImageJ was used for band intensity quantification.



	ASNS:alpha-tubulin of ASNase-treated relative to untreated cells (Average ± SEM)
CCRF-CEM	5.53 ± 1.63
MOLT-4	8.06 ± 1.76

Figure 11 Ratio of Asparagine synthetase to alpha-tubulin levels of CCRF-CEM or MOLT-4 cells treated with 2 U/ml asparaginase for 2 days, relative to untreated cells. Data were obtained from 3 independent experiments.

Future Directions:

Direct interaction between asparagine synthetase and microtubules should be investigated using immunoprecipitation assay and mass spectrophotometry. Quantitation of intracellular asparagine levels should be monitored after treating cells with nocodazole and ASNase to check whether the evaluated relative expression of ASNS to tubulin contributes to the activity of ASNS. Also, cell cycle analysis might provide the information about the effect of drug treatments on cell cycle profile.

Keywords: asparagine synthetase; mitotic Spindles; cell division; asparaginase; nocodazole

Output:

Manuscript in preparation (expected to submit to Cell Reports by May 2018).