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14. ABSTRACT

The goal of the current proposal was to develop a novel "split and splice" approach for highly selective targeting and elimination of NSCLC cells. We have optimized $DnaE_{Npu}$ intein for Stx2A-specific intracellular delivery mechanism by replacing four lysine residues in its C-terminal part with arginines and confirming that the resulted mutated intein retains its trans-splicing activity *in vivo*. We have confirmed that fusion of the C-terminal $DnaE_{Npu}$ split intein does not interfere with cellular delivery of bacterial toxins; whereas the N-terminal $DnaE_{Npu}$ intein slows down the delivery. The work is in progress to optimize properties of the N-terminal $DnaE_{Npu}$ intein to allow for more efficient translocation of the desired toxins by removing two cysteine residues and affecting thermodynamic properties of this part of the split intein.

The most significant hindrance that we have observed is low solubility of the C-terminal Stx2A split intein constructs and their inability to form a complex with Stx2B subunits. Currently we are employing several parallel strategies to optimize solubility of the above protein constructs via co-expression and refolding/solubilization strategies.

15. SUBJECT TERMS
Split and splice approach, cancer cell targeting, intein based protein trans-splicing, bacterial toxins, Shiga toxin, Anthrax toxin

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INTRODUCTION

The major goal of the current proposal is to overcome a hindrance of insufficient selectivity in therapeutic targeting of NSCLC tumors via development of a novel, split intein based approach, which we called "Split-and-splice" approach. Briefly, the idea is to split a highly toxic bacterial toxin into to benign parts and to deliver them to the cytoplasm of cancer cells via two independent toxin delivery pathways over-represented on the surface of cancer cells as compared to normal human cells. The effector toxin of choice was selected to be Shiga toxin (Stx2A subunit) and the independent delivery systems are the Stx2A/2B hetero-complex and Antharax toxin Protective Antigen/ N-terminus of Lethal Factor (LF_N) fused with the effector of interest. A successful development and implementation of the "split-and-spice" approach required optimization of many independent parameters, which were addressed in parallel during the funded period. The following goals were set as the priorities: 1) optimization of split positions on Stx2A toxin and optimization of expression/purification protocols for obtaining soluble and trans-splicing competent products (Fig. 1); 2) selection and optimization of inteins of choice for optimal performance in trans-splicing assays with the selected effectors; 3) optimization of the intein N- and C-terminal parts for efficient delivery of the effector domains to the cytoplasmic domain of cancer cells. The experimental details and major achievements on each of the indicated topics are provided below.

KEY WORDS

cancer targeting, NSCLC, lung cancer, shiga toxin, Stx2A, intein trans-splicing

The following Specific Aims were proposed:

Aim 1. To create recombinant split-variants of the Stx toxin capable of efficient *trans*-splicing *in vitro (months 1-8)*.
1A. To design constructs with various split positions in the context of several most effective inteins (months 1-2).
1B. To optimize expression and purification procedures in order to gain stable and soluble split-toxins with high *trans*-splicing efficiency in experiments *in vitro (months 1-8).*
Aim 2. To verify the feasibility of the "split and splice" approach for targeting human NSCLC tumor cell lines in culture and prepare the optimized toxins for their future testing in human xenograft nude mice models (months 3-12).
2A. To confirm that the individual split-components of the toxin are not toxic for cultured cells (months 2-8).
2B. To test the efficiency of cell targeting by the toxin variants reconstituted *in vitro (months 3-6).*
2C. To validate the efficiency of the proposed "split and splice" approach against human NSCLC-derived cells (months 6-12).

ACCOMPLISHMENTS

Aim 1. To create recombinant split-variants of the Stx toxin capable of efficient *trans*-splicing *in vitro (months 1-8)*.
1A. To design constructs with various split positions in the context of several most effective inteins (months 1-2).
1B. To optimize expression and purification procedures in order to gain stable and soluble split-toxins with high *trans*-splicing efficiency in experiments *in vitro (months 1-8).*

Summary: The goals of this Aim were only partially accomplished due to the difficulties with solubility of the C-terminal parts of the Stx2A subunit of Shiga Toxin. *Although much more work that initially planned was*

accomplished to overcome this problem and many more constructs were produced, expressed and purified, the problem has not yet been completely solved. The following wteps were undertaken in this regard:

Genomic material of the Strain EDL933 of enteropathogenic *E.coli* was acquired from BEI Resources and the sequences corresponding to the Stx2A and Stx2B Shiga toxin subunits were amplified by PCR.

Optimization of split positions on Stx2A toxin and optimization of expression/purification protocols for obtaining soluble and trans-splicing-competent products.

1A.1 DnaB intein constructs. Our first intein of choice was DnaB intein optimized for low extein context dependence and acquired from Dr. Liu (Sun et al., 2005). Coding DNA sequence for Stx2B subunit was cloned into two different vectors to provide flexibility for a potential co-expression with the Stx2A constructs. Three split locations were selected within toxin Stx2A to satisfy the following desired parameters: 1) Catalytic domain of Stx2A must be split between two split exteins (N- and C-terminal parts of Stx2A effector toxin; 2) all three regions are located in loop regions of Stx2A to allow conformational flexibility and stability of individual split parts of the toxin; 3) Ser residues are located at the N-terminal region of Stx2A-C-extein bordering the DnaB Intein-C as a requirement for efficient trans-splicing; 4) after initial finding that DnaB does not provide a desired trans-splicing efficiency, the residues at the C-termini of the N-intein parts of the Stx2A were mutated to glycines to provide optimal conditions for trans-splicing. The three N-terminal Stx2A constructs fused to the DnaB N-intein were cloned into anthracis toxin LF_N-containing pET15b plasmid; whereas the three C-terminal constructs were cloned into the same plasmid without LF_N (Fig. 2). All six Stx2A-containing proteins expressed well, but the longest N-terminal construct of Stx2A (N3) as well as all three C-terminal Stx2A constructs were insoluble and precipitated from solution upon dialysis from 8 M urea whether in the absence or presence of the Stx2B toxin subunit. ***Moreover, trans-splicing that normally occurs successfully in as high as 2M urea, failed to proceed between any of the N- and C-terminal constructs of Stx2A.***

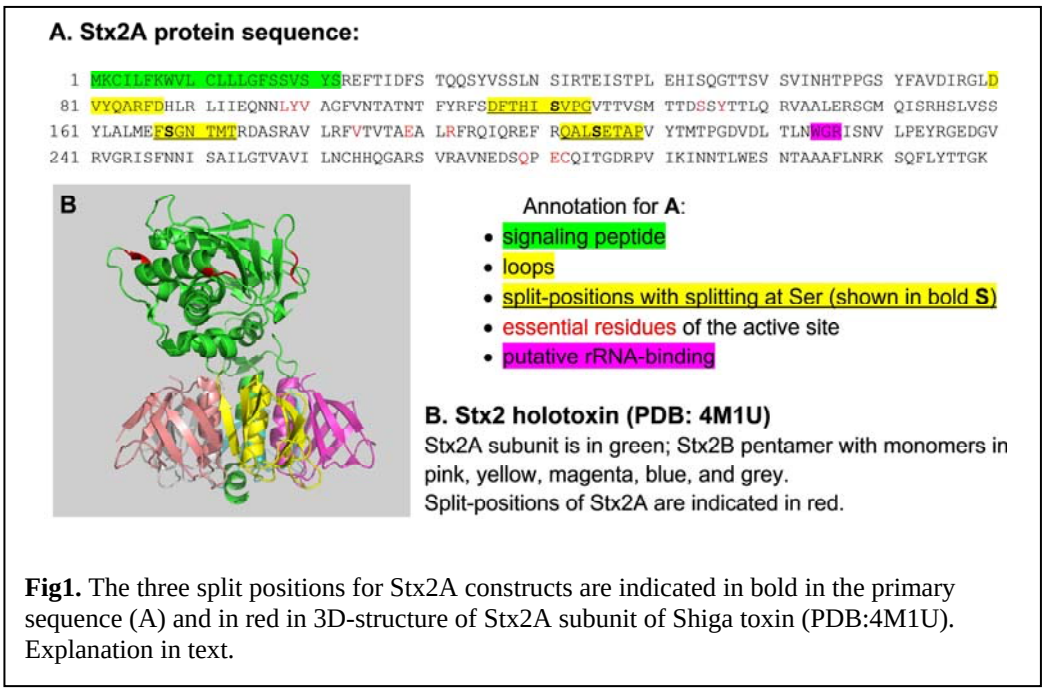

Fig1. The three split positions for Stx2A constructs are indicated in bold in the primary sequence (A) and in red in 3D-structure of Stx2A subunit of Shiga toxin (PDB:4M1U). Explanation in text.

1A.2 DnaE intein constructs. At this stage we decided to switch to a potentially more efficient naturally occurred split DnaE intein from *Nostoc punctiforme* ($DnaE_{Npu}$), acquired from Dr. Henning Mootz (Muenster University, Germany) (Shah et al., 2012). Being ultrafast and more efficient than other known inteins, $DnaE_{Npu}$ is not directly applicable for *in vivo* trans-splicing of Stx2A split toxin due to the presence of four lysines in the C-terminal part of the intein, which can be recognized by host ubiquitin system and targeted for degradation. Therefore, we first tested whether the replacement of the Lys residues with four Arg residues would affect trans-splicing efficiency of the intein. To this end, we applied multi-site mutagenesis to replace the lysines and tested the resulted mutated intein using split-GFP complementation approach (Fig. 3). We found that split GFP fusion products with both wt-$DnaE_{Npu}$ and 4R-$DnaE_{Npu}$ efficiently completed trans-splicing of N- and C-GFP parts, whereas $DnaE_{Npu}$ with the removed essential CFN amino acids failed to do so upon co-expression of the split products in *E.coli* cells (Fig. 3).

Therefore, we re-cloned all the Stx2A split constructs as fusion products with DnaE$_{Npu}$ and inserted them into a modified pColdI plasmid with FactorXa protease recognition region replaced by a consensus recognition motive of more effective TEV-protease. The rationale behind using the pCold-I vector was being operated by a cold-shock promoter it allows expression at low temperature (15^{o}C) and provides more soluble and overall cleaner proteins. We expressed and purified all three Stx2A N- and C-terminal pair of constructs fused to split intein DnaE$_{Npu}$, but once again the C-terminal parts were insoluble and precipitated after dialysis from 8M Urea.

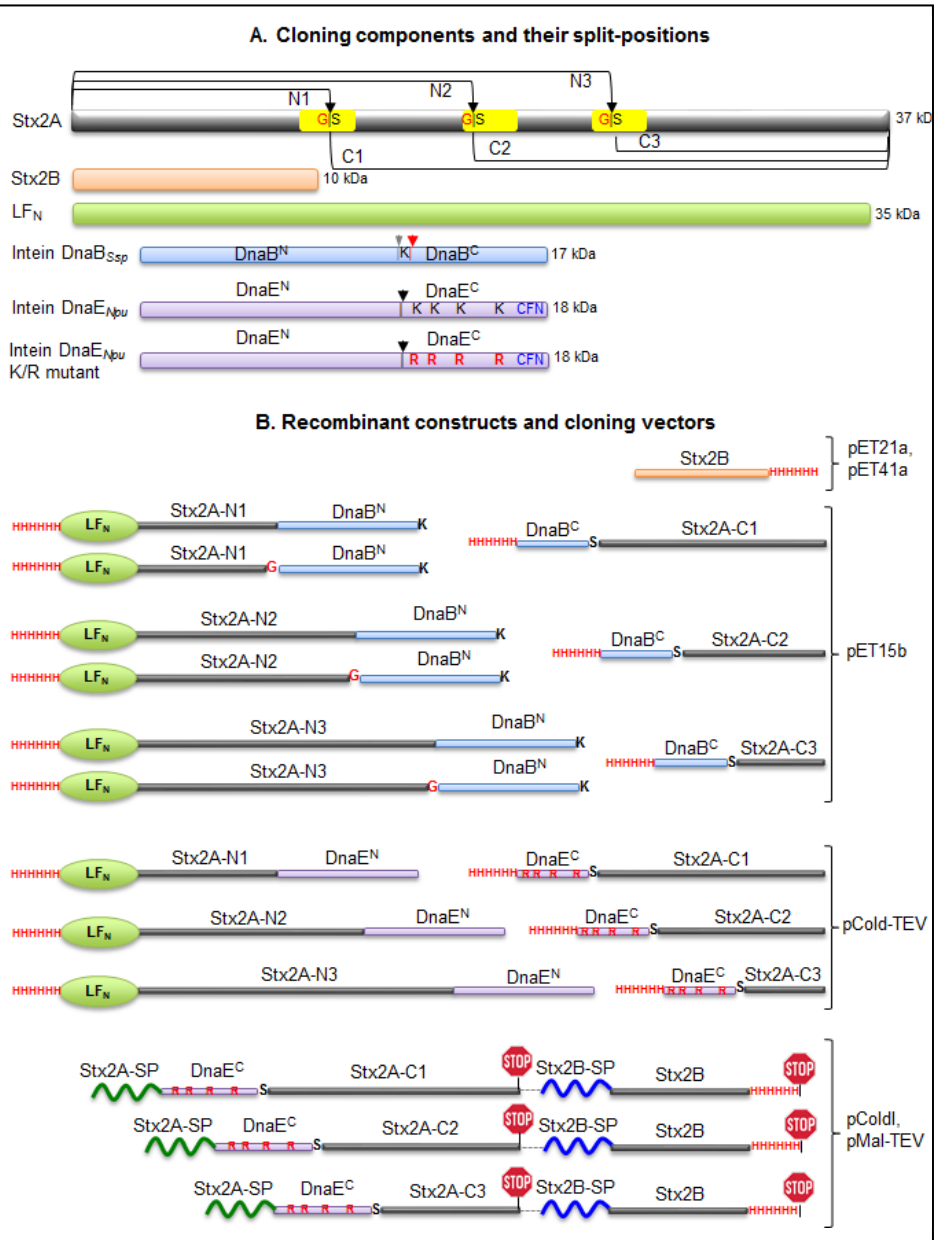

Fig.2. Stx2A and Stx2B constructs used in this study. Explanations in text

Aim 1B. Strategies for solubilization of the C-terminal Stx2A constructs.

1B.1. Since the C-terminal part of Stx2A is intrinsically hydrophobic due to the need to establish contacts with the inner hydrophobic core of the Stx2B homo-pentamer, we attempted to co-express the two parts of the toxin. To this end, the Stx2A-C-intein constructs were cloned upstream of the Stx2B gene in their original orientation (Tu et al., 2009) with the Stx2A signaling peptide for periplasmic secretion localized at the N-terminus of the construct (Fig. 2). To further improve overall hydrophilicity and solubility of the products, additional constructs were created with Maltose Binding Protein (MBP) added as a tag sequence at the N-terminus of Stx2A-C constructs (Fig. X). MBP is well-known for its ability to improve solubility and promote proper folding of difficult proteins. Unfortunately, expression of these constructs did not produce measurable amounts of either Stx2A mutants or Stx2B subunits.

Our immediate plan in this regard is to contact Alison Weiss, who is an expert in biochemistry and pathophysiology of Stx toxin from Cincinnati University, to hopefully solve this problem with their help.

1B.2. In parallel, we are currently utilizing another strategy to improve solubility of the C-terminal fragments of Stx2A by using a semi high-throughput refolding screen based on fractional dilution of the protein of interest in solubilization buffers in a 96-well format (Dechavanne et al., 2011).

1.B.3. The above high throughput refolding protocol will be followed up by reconstitution of the Stx2A/2B hetero-hexameric protein complex either by including five molar excess of Stx2B subunit into original screen, or by adding it later at the reconstitution dialysis step.

Aim 2. To verify the feasibility of the "split and splice" approach for targeting human NSCLC tumor cell
lines in culture and prepare the optimized toxins for their future testing in human xenograft nude mice
models (months 3-12).

2A. To confirm that the individual split-components of the toxin are not toxic for cultured cells (months 2-
8).

We have tested all soluble constructs of split- intein constructs on HeLa and IEC18 cells as well as Stx2B subunit alone and found them to be non-toxic in the entire range of tested concentrations (1nM – 0.2 μM). The C-terminal constructs were not tested because of their low solubility in physiological buffers.

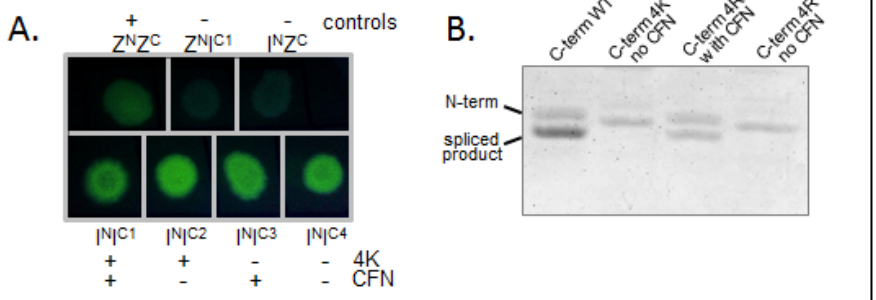

Fig. 3. Substitution of all 4 lysines in the C-terminal intein part by arginines preserves its trans-splicing activity in vivo as revealed by using split-GFP constructs. Removal of essential CFN triad from C-terminal intein construct abolishes its activity.

A. In vivo trans-splicing in E.coli: Z^NZ^C – positive control (split-GFP with interacting Z- protein sequences); I^C – split GFP C-terminus fused with wild type or mutated C-terminal $DnaE_{Npu}$:(C1-4K/CFN, C2-4K/noCFN, C3-4R/CFN, C4-4R/no CFN); I^N – split GFP N-terminus fused with N-terminal $DnaE_{Npu}$

B. Efficient *in vivo* trans-splicing of DnaE intein- GFP constructs confirmed by SDS-gel analysis of His6-purified protein products.

***2B.* To test the efficiency of cell targeting by the toxin variants reconstituted *in vitro (months 3-6)*.**

2B.1 Trans-splicing of DnaE intein constructs. *In vitro* trans-splicing of the N-terminal Stx2A constructs with their C-terminal counterparts, as well as the N-terminal Stx2A constructs with the C-terminal intein constructs of ACD toxin as a positive control clearly showed that desired products are formed, but predominantly in 2M urea as low solubility of the C-terminal constructs of Stx2A in physiological buffers precluded efficient trans-splicing under physiological conditions (Fig. 4). *Therefore, we succeeded in creating trans-splicing capable products of split Stx2A toxin, the important experimental problem that remains to be solved and that we are currently working on is to increase solubility of the C-terminal parts of the constructs.*

***2C.* To validate the efficiency of the proposed "split and splice" approach against human NSCLC-derived cells (months 6-12).**

The accomplishment of this central part of the proposal was greatly delayed due to the described above solubility issues with the C-terminal parts of the Stx2A split toxins.

***2D.* Membrane permeability of intein-containing split toxins.**

Although the solubility of the Stx2A C-terminal split constructs is our most significant current concern, in parallel we focus our attention on other experimental challenges that need to be solved to ensure prompt development of the desired technology. In the process of conducting experiments described above in Aims1A-2B, we realized that another experimental challenge that requires our attention is membrane permeability of the designed split toxins.

One such anticipated technical difficulty is a membrane permeability of the split intein constructs.

2D.1. $DnaE_{Npu}$ C-intein. We and others have demonstrated that bacterial toxins evolved to have marginal thermodynamic stability (Kudryashova et al., 2014). This quality allows an effortless transition from fully

folded to partially or even fully unfolded states required for efficient passage across the membranes of host cells. On other hand, inteins do not necessarily possess this property as they are not known to cross membranes. A crystal structure of *Npu* split intein has been recently solved demonstrating that the C-terminal intein fragment is fully disordered before binding to its N-terminal counterpart (Shah et al., 2013). This suggests that the C-intein should not humble membrane penetration abilities of bacterial toxins in fusion constructs. To directly assess that, we created a construct of *V.cholerae* ACD toxin (soluble and potent) fused with the anthracis LF_N and with the C-intein of $DnaE_{Npu}$ and evaluated the specific cell-rounding toxicity of this construct with the one without C-intein addition. Our data unambiguously demonstrate that the addition of C-intein N-terminally to the LF_N-ACD construct does not affect the efficiency of this toxin (data not shown).

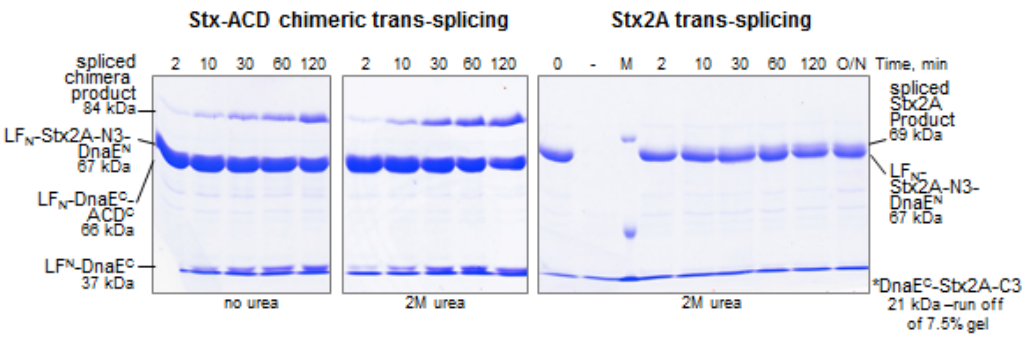

Fig. 4. In vitro trans-splicing of a split Stx2A (right panel) and chimeric Stx2A-N/ACD-C (left panels) under native and denaturing conditions. Notice, that the N-Stx2A construct and the trans-splicing product of full length Stx2A are similar in size and therefore not well separated on the gel.

2D.2 $DnaE_{Npu}$ N-intein. In contrast, the N-terminal part of $DnaE_{Npu}$ split intein is partially folded in its uncomplexed state. More importantly, this part contains three cysteine residues, only one of which is essential for the intein's specific catalytic activity. Membrane penetrating bacterial toxins are under strong evolutional pressure to contain few cysteines as possible due to the ability of the latter to spontaneously form disulfide bonds preventing efficient transition through the membrane. Indeed, we found that LF_N-ACD construct containing the N-terminal DnaE split intein part inhibits, but does not abolish, the cell toxicity of this toxin. The mutagenesis is on the way to eliminate unnecessary cysteine residues to allow more efficient delivery of the toxins.

TRAINING AND PROFESSIONAL DEVELOPMENT

Two graduate students, David Heisler (Ohio State Biochemistry Program) and Christopher Schwebach (Molecular, Cellular, and Developmental Biology Program), **were trained in** advance cloning and protein solubilization techniques by the PI and the Research Scientist Elena Kudryashova. In addition, several undergraduate students, namely Mubarik Mohammad, Lauren Strange, and Mathew Saab, were trained to conduct basic biochemical and molecular biology experiments by the above graduate students.

All graduate and undergraduate students mentioned above were involved in **professional development** activities via participation in laboratory meetings (twice a week). Also, David Heisler has participated in several Departmental and University wide conferences and was selected as a plenary speaker for IGP (OSU Molecular Life Sciences Interdisciplinary Graduate Programs) symposium in Spring 2014.

IMPACT

Impact on the development of the principle disciplines of the project

Our results obtained in the course of the current proposal have positively solved some of the technical difficulties of the proposed innovative "split and splice" approach for selective targeting cancer cells. We have also identified other difficulties that remain to be solved and delineated the approaches to address them.

1) Particularly, we have demonstrated that DnaE$_{Npu}$ intein is tunable for the specific, Lys-intolerant StxA delivery machinery without substantial loss of its trans-splicing activity. We have optimized DnaE$_{Npu}$ intein for Stx2A-specific intracellular delivery mechanism by replacing four lysine residues in its C-terminal part with arginines and confirming that the resulted mutated intein retains its trans-splicing activity *in vivo and in vitro (Figs*. 3 and 4).

2) We have confirmed that fusion of the C-terminal DnaE$_{Npu}$ split intein does not interfere with cellular delivery of bacterial toxins via Anthrax toxin-based delivery system. We also identified a previously under-appreciated problem that fusion of the N-terminal DnaE$_{Npu}$ intein can slow down the delivery of fused toxins. In this regards, we outlined the ways to overcome this difficulty via mutagenesis of cys residues on N-intein-DnaE$_{Npu}$.

Therefore, we have established a solid ground for application of intein technology for independent delivery of potent toxins split to benign components. In perspective, our major accomplishments in this area will enable applicability of the "split and splice" approach for delivery of various toxins for highly specific targeting of specific cancers, while employment of modified delivery systems (e.g. tuned to specific cancer receptors) should allow targeting many different cancers. We anticipate also that this approach will be useful for advanced studies of organelle-specific processes via specific sub-cellular targeting of split-toxins.

Impact on other disciplines, technology transfer, and society

Nothing to report

CHANGES/PROBLEMS

All problems are described in detail in Accomplishments section. Briefly, the major challenge to be solved is the low solubility of the C-terminal parts of Stx2A in physiological buffers. We are currently using several independent approaches to address this issue.

Most changes are directly connected to the identified problems. The accomplishment of Aim2C and partly Aim2A-B were delayed because of the above problem. In addition, we formulated a new Aim (2D) to address potential problems with delivery of intein-tagged split toxins. This Aim has been partially addressed, and in part it will be further addressed during a "no cost" extension of this proposal.

PRODUCTS

There are no products to report

PARTICIPANTS AND OTHER COLLABORATING ORGANIZATIONS

(1) PI: Dmitri Kudryashov

Name:	Dmitri Kudryashov
Project Role:	PI
Researcher Identifier	
Nearest person month worked:	1.5 months
Contribution to project:	Overall administration
Funding support:	University provided salary

(2) Other persons:

Name:	Elena Kudryashova
Project Role:	Research Scientist
Researcher Identifier	NA
Nearest person month worked:	4 months
Contribution to project:	Direct participation in cloning and purification of all constructs, solubilization, trans-splicing analysis, and cell related experiments
Funding support:	American Heart Association Innovative Grant

(3) Other persons:

Name:	Barry Golestany
Project Role:	Visiting Scholar
Researcher Identifier	NA
Nearest person month worked:	2.5 months
Contribution to project:	Optimization of protein expression, trans-splicing and solubilization analyses.
Funding support:	None

(4) Other persons:

Name:	David Heisler
Project Role:	Graduate Student
Researcher Identifier	NA
Nearest person month worked:	7 months
Contribution to project:	Optimization of protein expression, trans-splicing analysis, protein solubilization, assessing the role of intein fusion constructs in cell permeability of bacterial toxins.
Funding support:	Teaching Assistance

(5) Other persons:

Name:	Chris Schwebach
Project Role:	Graduate Student
Researcher Identifier	NA
Nearest person month worked:	3 months
Contribution to project:	Optimization of intein-N-DnaE thermodynamic plasticity via multi-site and point mutagenesis.
Funding support:	MCDB program

CONCLUSIONS AND FUTURE DIRECTIONS

Although, we have accomplished a substantial progress in each of the prioritized goals, the overall goal of the project has not been achieved due to solubility issues of Stx2A split intein constructs. We believe that we will be able to solve the technical problems described above. The “no cost extension” for this proposal has been requested and granted.

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