Cover Page

TITLE Purification, Identification, and Characterization of the Diphtheria Toxin Cytosolic Translocation Factor Complex - Career Development Award for Ryan C. Ratts.

Salary Support Requested $267,285

Operational Support Requested: $50,000

Dates of Scholarship: Begin 07/01/2012 End 06/30/2015

Applicant Name Ryan C. Ratts MD/PhD

Title Assistant Professor of Medicine, Assistant Professor of Pediatrics

Department Department of Medicine, Section of Hospital Medicine

Name of Department Chair Rich Rothstein – Medicine, John Modlin - Pediatrics

Mailing address: Hospital Medicine 3B, One Medical Center Drive, Lebanon, NH 03756

Telephone No. 603-650-8380

Percent effort devoted to project

Research 0.6 FTE (60% time)

Clinical 0.2 FTE Pediatrics, 0.2 FTE Medicine

Teaching Included in clinical duties above

Mentor(s) Name	Title
Marc Ernstoff MD	Professor of Medicine; Associate Director Clinical Research, Norris Cotton Cancer Center; DHMC
John R Murphy PhD	Professor of Medicine/Microbiology, Director National Emerging Infectious Diseases Laboratory; Boston University
Cathy Costello PhD	Professor of Biochemistry, Biophysics and Chemistry; Director, Boston University Center for Biomedical Mass Spectrometry

I have read and fully understand the terms and conditions of this application.

Approved by _____
Primary Mentor

Approved by _____
Department Chair

Signature _____
Applicant

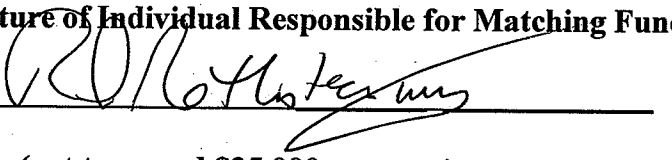
II. BUDGET SHEET

I. Salary Request Per Year

	Candidate's % effort	Candidate's salary and benefits (annual)
Total Scholarship Support	60 %	$ 133,643
Foundation Support	40 % *(two-thirds of total)*	$ 89,095 *(up to $100,000)*
Department Match	20 % *(one-third of total)*	$ 44,548 *(up to $50,000)*
Non-Scholarship Support	%	$

**note: total research fte=60% clinical fte =40% The above annual amounts are based on the 3 yr average and a Yr 1 100%fte salary rate of $158,950 and fringe rate of 35.5%. An estimated 3% salary increase per yr and .5% increase in fringe benefit rate per yr is included in our 3 yr estimate.*

Commitment and Signature of Individual Responsible for Matching Funds:

Richard Rothstein: 

II. Operational Expenses (not to exceed $25,000 per year)

Item	Year 1	Year 2	Year 3
Part-time lab technician	$ 0	$ 15,000	$ 15,000
Lab supplies	$ 0	$ 7,500	$ 7,500
Software License	$ 0	$ 500	$ 500
Conference	$ 0	$ 2,000	$ 2,000
Totals	$ 0	$ 25,000	$ 25,000

Budget Justification Materials/Facilities:

Costs for laboratory supplies during Year 1 of the proposed award in the amount of $30K are covered by a Hitchcock Foundation Pilot Grant granted 2011, and Department of Medicine Junior Faculty Developmental Award, $10K. The following justification applies to year 2 and 3.

Personnel
Lab Technician (33%FTE in yr 2 and 3)
A part-time laboratory technician is needed to perform basic experiments.

Lab Supplies ($7,500 per yr for yr 2 and 3)
Tissue culture reagents, Buffers, Chromatography supplies, Protease Inhibitors, Antibiotics, Bafilomycin A1, SDS-Page Gel electrophoresis supplies, Radioisotopes (P32) NAD for Ribosylation assay. Current costs are $323 dollars for two week supply=$8,398 per yr. HF Scholar funds will be supplemented by department of medicine junior faculty development funds to cover total expenses needed for supplies.

All biological reagents listed in this proposal are being generously donated by John Murphy. MTAs have already been signed by Boston University for all reagents and includes:

Hela cell line; Hut102/6TG cells; Hut102 Clone A4 cells; Hut102 Clone A2 cells; DT T1 motif plasmid DNA construct and transfection construct; DT T1 motif siRNA DNA construct and transfection constructs; DT protein construct; DT plasmid DNA construct; DAB389IL2 protein construct; DAB389IL2 plasmid DNA construct; DT His constructs from Ratts' thesis; Hut 102 DT His construct cell lines from Ratt's thesis (both bacterial and eukaryotic transfection vectors); DT-T1-GST plasmid DNA construct; p-Tracer CMV2 cloning transfection vector.

Upon currently pending approval by the Dartmouth Biosafety Office, all reagents will be transported to DHMC.

Software ($500 per yr for yr 2 and 3)
License fees for Mass Spec/Proteomics Data Analysis

Travel ($2,000 per yr for yr 2 and 3)
Funds for conferences will be to attend 1-2 conferences per year applicable to either: Mechanisms of Toxin/Pathogen Entry or Mass Spectrometry Proteomic Analysis

Human Subjects not applicable

Vertebrate Animals not applicable

III. Grand Total: for Hitchcock Foundation Career Development Award.

Item	Year 1	Year 2	Year 3
Salary	$ 86,151	$ 89,063	$ 92,072
Operational Expenses	$ 0	$ 25,000	$ 25,000
Totals	$ 86,151	$ 114,063	$ 117,072

III. APPLICANT INFORMATION

A. Curriculum Vitae –NIH biosketch format (2 page max):

Education and Training

UC Berkeley	BA in History	1996
	BA in Molecular and Cell Biology, with Honors	1996
Boston University	MD	1997-2006
	PhD, Molecular and Cell Biology Program	1997-2006
Harvard Combined	Internal Medicine – Massachusetts General Hospital	2006-2010
Residency Program	Pediatrics – Children's Hospital Boston	2006-2010

Positions/ Employment

2010-present	Assistant Professor of Medicine, Dartmouth Medical School, NH
2010-present	Assistant Professor of Pediatrics, Dartmouth Medical School, NH
1998	Lecturer in Organic Chemistry, University of California Berkeley, CA
1993-1997	Teaching Assistant in Organic Chemistry, UC Berkeley, Ca.
1995-1997	Undergraduate Research Assistant: Cloning of Drosophila Transcription Factor IIE at UC Berkeley
1992-1994	Summer Research Assistant in Physics at Stanford University.

Professional Memberships

2010	Board Certified, American Board Internal Medicine
2010	Board Certified, American Academy of Pediatrics
2006-present	Member, American Medical Association
2006-present	Member, American Academy of Pediatrics

Honors

2011	Excellence in Teaching Award, Internal Medicine, DHMC
2006	Henry Bakst Clinical Scholar
2006	Pauline Mitchell Pediatrics Scholar
2004	Elected Alpha Omega Alpha Medical Honor Society
2002	NIH Training Grant in Endocrinology
2001	Karin Grunenbaum Cancer Research Foundation Fellow
1998	Wotiz Family Clinical Research Fellowship
1998	American Cancer Society Stone Research Fellow
1996	UC President's Undergraduate Research Fellow
1996	Howard Hughes Medical Institute Undergraduate Research Fellow

Publications

- Ratts, R and Murphy, J.R. "Diphtheria Toxin and Cytosolic Translocation Factors". Infectious Disease. Petra Nenadic editor, InTech. In press, 2011, ISBN 979-953-307-497-8
- Trujillo C, Ratts R, Tamayo A, Harrison R, & Murphy JR. "Trojan horse or proton force: Finding the right partner(s) for toxin translocation." Neurotoxicity Res, 2006; 9: 63-71. PMID 16785102
- Ratts, R., C. Trujillo, A. Bharti, J. vanderSpek, R. Harrison, and J. R. Murphy. "A motif in transmembrane helix 1 of diphtheria toxin mediates catalytic domain delivery to the cytosol." PNAS, October 25, 2005; v.102, no. 4, 15635-15640. PMID 16230620

- Ratts, R. "Purification, Identification, and Characterization of the Diphtheria Toxin Cytosolic Translocation Factor Complex." PhD Dissertation. 1-227. Copyrighted 2004.
- Ratts, R. and J.R. Murphy. "Diphtheria toxin, diphtheria-related fusion protein toxins, and the molecular mechanism of their action against eukaryotic cells." Topics in Current Genetics. M. J. Schmitt and R. Schaffrath, editors. Springer Link, 2004. Vol 11, p. 1-20.
- Ratts, R., H. Zeng, E.A. Berg, C. Blue, M.E. McComb, C.E. Costello, J.C. vanderSpek, J.R. Murphy. "The Cytosolic Entry of Diphtheria Toxin Catalytic Domain Requires a Host Cell Cytosolic Translocation Factor Complex." Journal of Cell Biology, 2003 Mar. 31, 160(7):1139-50. PMID 12668662
- Ratts, R., and J.C. vanderSpek. "Diphtheria Toxin: Structure Function and its Clinical Applications." Chimeric Toxins. H. Lorberboum-Galski, P. Lazarovici, ed. Taylor and Francis, London, New York. 2002. p. 14-36.
- Wang, X., S. Hansen, R. Ratts, S. Zhou, W. Zehring. "Drosophila TFIIE: Purification Cloning and Functional Reconstitution." PNAS 1998, (94): 443-438. PMID 9012800

Abstracts and Lectures

- Ratts R. Diphtheria Toxin. Pediatric Grand Rounds, DHMC. 2011
- Ratts, R. Cytosolic Translocation Factor Complexes: Basic Mechanism of Entry: Invited Speaker Microbiology and Immunology Seminar Series, Dartmouth Medical School, 2011.
- Ratts, R. Diphtheria Toxin: Paradigm Mechanism of Entry: Invited speaker. COBRE, Lung Biology Group, Dartmouth Medical School, 2010
- Ratts, R. Diphtheria Toxin and Cytosolic Translocation Factors. Lecture, 104th General Meeting of the American Society of Microbiology, New Orleans 2004.
- Ratts, R. and J.R. Murphy. Diphtheria, Anthrax, and Botulinum Toxins: Studies on the Mechanism of Catalytic Domain Entry into the Cytosol of Target Cells. New England Regional Center of Excellence for Biodefense and Emerging Infectious Diseases (NIAID), Plenary Session, 2004.
- Ratts R, Zeng H, Berg EA, *et al.* In vitro translocation assay for the diphtheria toxin catalytic domain across the endosomal membrane: Implications for understanding the pathophysiology of the botulinum and tetanus neurotoxins. Naunyn-Schmiedeberg's Archives of Pharmacology 365: 108 Suppl. 2 JUN 2002.

Patents:

- Novel Compositions and Methods for Promoting, Inhibiting, and Detecting Protein Entry into Cells.
  International WO/2005/014798 filed 9/30/2005. Ratts and Murphy, co-inventors.
  US 60/459,185 (filed 3/31/2005).

B. Summary of the candidate's career accomplishments since the completion of the most recent degree/training (1 Page Max).

Upon completion of residency training in both Internal Medicine and Pediatrics in 2010, I was appointed as an Assistant Professor of Medicine and Pediatrics at Dartmouth Medical School. For the past 14 months at DHMC, I have been actively working as an attending hospitalist on both the adult and pediatric wards for 0.7 FTE (0.5 and 0.2 FTE, respectively). During this time period I have successfully completed board certification in both Internal Medicine and Pediatrics. My dual training reflects my passion for clinical medicine and brings a unique perspective to both fields.For example, I have facilitated the transition of complicated pediatric patients with congenital conditions to adult medicine. As part of my clinical duties, I precept first year medical students for their On-Doctoring course, participate in DMS Student Interest Groups regarding careers in medicine, and serve on the Internal Medicine Intern Selection Committee.

My enthusiasm for and expertise in teaching has been rewarded by outstanding evaluations from Pediatric and Medical house staff, and an Excellence for Teaching Award in Internal Medicine. Given my unique training in Med/Peds, I have also dedicated myself to training my pediatric and adult medicine colleagues about the interface of these categories by giving a seminars and didactic sessions on a wide range of conditions including adults patients with congential heart disease and diphtheria. I have used by research experience and expertise to discuss at Pediatric Grand rounds diphtheria toxin as a paradigm for intracellular protein delivery as an example of how basic science translates into clinical practice. .

My long term passion relates to molecular medicine, and I have continued to center my research efforts on the mechanism of entry of diphtheria toxin as a model for delivering proteins inside cells. Since arriving at Dartmouth, I have focused my 0.3 FTE time on building an active research laboratory. My co-discovery of a complex of proteins, or cytosolic translocation factors (CTFs), required for diphtheria toxin entry into the cell while working with Dr. Murphy forms the foundation of my research effort. Dr. Murphy and I have summarized this mechanism of entry for a diverse group of toxins and other proteins across biological membranes, and proposed a new and refined model for toxin entry in a recent chapter review. During this start up phase I have registered as a PI through the DMS lab safety office, completed my own BioRaft and biosafety protocols, and have submitted MTAs for all the reagents being generously shared by Dr. Murphy. I have also networked with established Dartmouth investigators including Richard Enlow and Marc Ernstoff and have identified Marc Ernstoff as my primary research mentor. I have continued my relationships with both Dr. Jack Murphy and Dr. Cathy Costello at Boston University.

My commitment to laboratory based research is reflected in my efforts to find additional funding to increase my protected time to 60% . I submitted three grants this year, and was successfully awarded of a Hitchcock Foundation Research Pilot Grant for $30K on the conditional basis of increasing my protected time. My two NIH grants were criticized for needing additional preliminary data. The HF Career Development award will allow me the time to generate the necessary preliminary data for successful application to external peer reviewed agency and hone my skills in in grant writing .

IV. CAREER DEVELOPMENT PLAN

A. Training (1 page max)

The primary purpose of this Award for my career development will be to effectively use my protected time to generate a significant amount of preliminary data, improve my grant writing skills, and to submit competitive grants to the NIH and other funding agencies. The secondary purpose will be to refocus my research from pure basic science and translate it into clinically relevant tools.

The training plan provides protected time for intense research which is a continuation of prior work completed during my PhD training. The research will initially focus on the development of improved protocols for the purification, identification and characterization of cytosolic translocation factor (CTF) complexes required for diphtheria toxin entry. My prior work has already resulted adoption of the experimental protocol as the standard model for the field of toxin entry. Continued collaboration with my secondary mentors, Dr. Murphy and Dr. Costello, will provide additional technical expertise needed for the proposed research plan with respect to toxin entry and proteomics, respectively.

My training plan calls for active participation in in Dartmouth research seminars and attendance to national conferences on toxin entry and organellar proteomics which is incorporated into the budget for years 2 and 3. I anticipate presenting my data at these conferences which will provide an expanded peer review of my work. In addition I will be attending Dartmouth seminars and courses on grant writing (Christopher Dant) and translational research (provide by the Clinical Trials Office) Importantly, this proposed training plan requires identifying appropriate grants (e.g. NIH K award) in year 1, writing and refining the grant application in year 2, and submitting the grant in years 2-3.

The HF Career Development Award will provide the support to realize my own long term goals of bridging bench to bedside. Accordingly, Dr. Ernstoff is a very calculated decision as my primary research mentor at DHMC. Dr. Ernstoff is a leader in translation of biological agents from bench to bedside with a deep experience in designing, conducting, and analyzing studies needed to bring therapeutics to clinical trial and practice, including fusion protein toxins such as the ricin immunotoxins. Once my active laboratory is established, I intend to expand my work on toxin entry to include the development of toxin entry inhibitors and fusion proteins based on the toxin translocation machinery to deliver surrogate cargo proteins inside specifically targeted cells. I will utilize Dr. Ernstoff's experience and guidance in developing a translational program for any potential therapeutics derived from my research.

B. Mentor's Plan and Expectations (1 page max)

In his training as a physician-scientist, Ryan has an impressive resume with respect to both his research and clinical work. Having just finished his training, he is still within the early phase of his career and seeks to make his mark establishing a basic science lab that will perform translational research leading directly to clinical trials. Given his ambition and track record he will undoubtedly eventually succeed, and with proper mentorship he will reach that goal sooner. I will assist Ryan in developing his research aims and in grant writing and submission to support his basic science work with translational clinical relevance. I am poised and prepared to assist Ryan in his goal to become an independent investigator as I have been successful myself in numerous translational grants, have successfully mentored many junior investigators, and served on multiple NIH review committees and journal editorial boards.

As his mentor, I will work closely with the Drs. Modlin and Rothstein (Department Chairs of Pediatrics and Medicine respectively) and Dr. Ed Merrens (Section Chief of Hospital Medicine) to ensure that Ryan is provided the much needed protected time, and will keep him focused on specific goals with reasonable time lines. We will meet monthly to review his data and his progress at fulfilling the aims of his research. I will assist him in preparing and submitting grants. Ryan will maintain his relationship with Dr. Murphy for the expertise in the molecular mechanisms of toxin entry and I will serve as his mentor for career development and translational research approaches. For example, the identification of geldanamycin – a specific inhibitor of chaperone Hsp 90 – as a toxin entry inhibitor has significant amount clinical data from cancer trials regarding the safety profile and the side effects and represents a target that could be fast tracked into clinical trials as a toxin entry inhibitor. We will focus his basic laboratory research effort with the specific goal to accelerate translational development consistent with Ryan's long term goal to design novel vectors using the diphtheria toxin translocation machinery to deliver proteins inside cells.

As secondary mentors, and collaborators, Dr. John R Murphy is supplying many of the biological reagents that will be used in the study, Dr. Murphy is not only a leading expert in toxin entry, but he also designed the first fusion protein toxin, and only fusion protein toxin to receive FDA approval. He is currently the Director of the National Emerging Infectious Diseases Laboratory at Boston University. Dr. Costello is the Director Proteomics Core Facility at Boston University and a leading expert in Proteomics. Her lab will be actively collaborating with Dr. Ratts for both protein identification and helping to establish an endosomal based organellar approach for identifying novel cytosolic translocation factors.

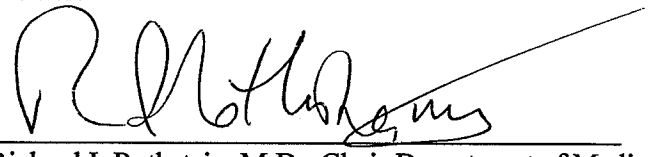
D. Institutional Commitment (1 page max)

The sponsoring Departments of Medicine and Pediatrics are committed towards the development of Ryan Ratts into a productive and independent investigator. Since his initial recruitment (10/1/2010), the Chair of Medicine, Dr. Richard Rothstein, and Section Chief of Hospital Medicine, Dr. Edward Merrens, have been committed towards ensuring that Dr. Ratts is successful in establishing a basic science research laboratory. Currently, Dr. Ratts receives 20% FTE protected time plus 10K per yr for 2 years (10/1/2010-9/30/2012) as an awardee under the Department of Medicine Junior Faculty Development Program. In addition, Dr. Merrens independently committed 0.1 FTE in section discretionary funds to bring his total dedicated research time to 30% FTE.

If Dr. Ratts receives a Hitchcock Foundation Scholars award, we will increase his protected research time to 60% FTE, and the department will provide the required salary matching of approximately 40K per yr for three years. Dr. Merrens will assure the needed release time from normal clinical duties, and the Section of Hospital Medicine will continue to support his office space and administrative support. Under the current scheduling system, Dr. Ratts' clinical duties in the proposed application will include approximately 14 weeks of clinical service, including CME time. This is the minimum amount of clinical time (0.2 FTE Medicine and 0.2 FTE Pediatrics) that Dr. Ratts feels is appropriate to maintain appropriate proficiency in each discipline for a leading academic medical center. Accordingly, the annual reappointment process for Dr. Ratts in both the Department of Medicine and Pediatrics will also be contingent on the successful progression of his research as judged by the generation of meaningful data, presentation at conferences, publication record, and establishment of independent funding within the time period of the award. The Section of Hospitalist Medicine will continue to offer a discretionary 0.1 FTE committed support if additional time is needed to further reduce clinical duties during the period of the Career Development Award.

The Department of Medicine and Pediatrics have been proactive in helping Dr. Ratts network and meet fellow researchers, providing opportunity to present his research to diverse audiences at DMS and DHMC. Dr. Enelow was helpful in the process of identifying a primary research mentor, Dr. Marc Ernstoff, at DHMC. Dr. Ernstoff has clinical experience using the diphtheria toxin fusion protein constructs in the clinical setting, and as the former Director of Immunotherapy and current Associate Director of Clinical Research at the Norris Cotton Cancer Center will be instrumental in guiding Dr. Ratts towards maintaining focus on specific research goals as well as writing and developing successful grants.

Dr. Rick Enelow, as Vice-Chair for Research Affairs in the Department of Medicine, has graciously offered laboratory space and the use of laboratory reagents and equipment, including -70 freezer space, liquid nitrogen for cell lines, tissue culture cabinet, incubators, basic centrifuge and basic laboratory supplies/equipment. Within the next 6-12 months, Dr. Ratts will be moving to the Ernstoff lab for improved logistics with working with his primary mentor.



Name: Richard I. Rothstein, M.D., Chair Department of Medicine

V. RESEARCH PLAN

A. Long term goals (1 page):

Mechanisms for intra-cellular protein delivery provides an avenue for efficient delivery of large biological molecules such as proteins or small RNA inside specifically targeted cells represents a powerful clinical tool that has eluded many researchers. Although current transfection techniques for delivery of proteins or nucleic acid work excellently in cell culture, their clinical applications are extremely limited within living subjects due to their general lack of specificity. Attempts to specifically target transfection vectors have yielded limited successes as the majority of the targeted molecules remain trapped within the endosomal system of targeted cells and never reach the cytosol. Nature has elegantly solved this problem using bacterial protein toxins, and it is not surprising that understanding the molecular mechanism of toxin entry is the focus of my research endeavors.

Bacterial protein toxins are responsible for mediating the toxic effects of many strains of bacteria. These toxins bind to specifically targeted host cells and once inside the cell, the toxins either kill the cell or manipulate the cell towards functioning in the bacteria's own interest. The identification of a complex of host cell proteins that facilitate the entry of diphtheria toxin by Ratts et al (2003, 2005) has led to insights into a fundamental mechanism of toxin entry. This mechanism is exploited by a growing list of bacterial protein toxins that includes anthrax lethal factor, iota toxin, clostridial toxins including perfingens and difficile toxins, cholera toxin, and has been hypothesized to include the botulinum neurotoxins. Likewise, the entry of HIV viral TAT protein, an important factor in HIV infection, as well as the membrane translocation of endogenous proteins, such as fibroblast growth factor, have been shown to use the same complex of host cell factors.

Importantly, inhibitors of the identified host cell factors have been shown to block toxin entry, and represent a novel class of therapeutics that protects cells against intoxication. Some of these inhibitors, already in clinical trials for alternative uses, will likely be implemented soon in the clinical setting of inhibiting toxin entry and will have a dramatic effect in reducing the worldwide burden of several epidemiologically important pathogens.

My planned studies will continue to identify the remaining factors required for diphtheria toxin entry using well established techniques, and will also implement a refined assay system to study toxin entry. This refined assay will result in a more efficient and faster method for identifying novel factors required for entry. The identification of additional factors required for toxin entry will lead to new targets for inhibiting toxin entry.

Given that multiple toxins have independently evolved to exploit this same mechanism of entry, the identification of each new factor will also reveal more clues about the fundamental role this complex plays in basic cellular biology. A better understanding of this molecular mechanism ultimately allows for the rational design of toxin based nano-machines capable of delivering large biological cargoes inside specifically targeted cells.

B. Research Proposal (4 Pages)

This research proposal is a refined version of my previously submitted and awarded Hitchcock Foundation Pilot Grant.

Abstract/Introduction:

Diphtheria toxin (DT) and the DT related fusion protein toxins entry into the cell represent a paradigm for a diverse group of A-B bacterial protein toxins entering the cytosol from the endosomal compartment. DT is synthesized as a single polypeptide chain composed of three structural domains with exclusive function: Receptor binding (R) domain, Transmembrane (T) domain, and Catalytic (C) domain (Choe et al., 1992). Intoxication by DT involves an ordered sequence of events mediated by each domain, starting with receptor binding (Naglich et al, 1992) and internalization of toxin-receptor complex by clathrin mediated endocytosis (Moya et al., 1985). Acidification of the endosomal lumen induces a conformation change in the DT T domain, promoting spontaneous membrane insertion of the T domain and the formation of a pore (Donovan et al., 1981; O'Keefe et al., 1992). Successful delivery of the DT C domain through the pore and into the cytosol of targeted cells results in the ADP-ribosylation of Elongation Factor-2 (EF-2), and the subsequent inhibition of protein synthesis results in cell death (Collier and Cole 1969).

The translocation of the DT catalytic domain across the endosomal membrane requires a host cell cytosolic translocation factor (CTF) complex that is highly conserved in eukaryotic cells (Ratts et al, 2003). Components of the DT CTF complex have also been shown to mediate the cytosolic entry of a growing list of bacterial protein toxins, viral proteins, and endogenous proteins including antigen presentation (Ratts and Murphy, 2011). Furthermore, toxin binding motifs that mediate interaction between toxin and CTFs are potential targets for the development of toxin entry inhibitors (Ratts et al., 2005). Since recombinant factors of previously identified CTFs are not sufficient to promote translocation of DT across the endosomal membrane, it is hypothesized that additional factor(s) within the CTF complex remain to be identified. The further identification of any additional required CTFs will increase both our understanding of toxin entry and the number of potential targets for developing novel toxin entry inhibitors.

While the current protocol for purifying and identifying toxin CTFs using an *in vitro* translocation assay I developed (Ratts et al. 2003) , it is also technically difficult, labor intensive, expensive, and time consuming. Thus there is a need to develop novel assay systems that are more rapid, easier to implement, and readily adaptable to other pathogens. This research project focuses on the development of two such novel methods for identifying CTFs – toxin "entry motif" affinity purification and toxin-endosomal based organellar proteomics. This project uses DT as a model since the identification of already known CTFs using these novel purification systems will allow for validation. The specific aims of this project are:

1. **Identification of novel putative CTFs using diphtheria toxin "entry motif" affinity purification.**

2. **Development of an organellar based proteomics approach to identify putative CTFs using a diphtheria toxin-endosomal pull down assay.**

3. **Validate functional roles for any putative CTFs identified in specific aims 1 and 2 using both *in vitro* translocation and cytotoxicity assays.**

Specific Aim 1: Identification of novel putative CTFs using diphtheria toxin "entry motif" affinity purification.

Hypothesis: Proteins directly binding to portions of toxin first emerging through the nascent pore are likely factors involved in translocation.

I was the first to identigy the role of a sequence specific "entry motif" for DT (Ratts et al. 2005), and the DT T1 di-lysine motif mediates direct binding to beta-COP, a known DT CTF (Trujillo et al 2010). This entry motif also mediates the binding of beta-COP to anthrax lethal factor, and is required for anthrax toxin entry (Tamayo et al., 2008). Importantly, I demonstrated that beta-COP can be affinity purified using a GST fusion polypeptide corresponding to regions of the DT C domain and T domain (residues 140-271) containing the DT T1 entry motif (Ratts et al. 2005). These residues are the first portions of the toxin that are threaded through the nascent pore and presented to the cytosolic side of the endosomal membrane (Ariansen et al. 1993, vanderSpek et al., 1994).

Several other proteins that co-purified with beta-COP are not yet identified, and these proteins represent high yield targets as potential CTFs (Ratts et al., 2005). Specific Aim 1 proposes to complete the identification of the proteins affinity purified in the GST pull-down of DT residues 140-271 (Ratts et al. 2005). Similarly, affinity purification will also be performed using several constructs of various different length DT polypeptides as shown in Figure A-1 (Appendix A). Differential patterns of protein binding will likely reveal which regions of the toxin are mediating interaction with the affinity purified factor, which could lead to the concomitant identification of novel entry motifs. Alternatively, the differential pattern may also result from interference caused by proximity of the Histidine tag to adjacent regions or non-specific binding. Affinity purified proteins would be identified by mass spectrometry sequencing as previously described (Shevchenko et al., 1996)

Summary Aim 1: Affinity chromatography immobilized portions of DT will allow for a rapid and cost effective method of identifying putative CTFs, as exemplified by the purification of beta-COP (Ratts et al., 2005). Since the genetic constructs for affinity chromatography have already been made, it is reasonable to generate sufficient quantities of affinity purified proteins for mass spectrometry sequencing within 6-12 months. Identification and data analysis would occur during year 2. Targets would then be validated as per Aim 3 during year 2 and year 3.

Specific Aim 2: Development of an organellar based proteomics approach to identify putative CTFs using a diphtheriatoxin-endosomal pull down assay.

Hypothesis: Endosomal proteins directly impact toxin translocation across the endosomal membrane, and the endosome itself can function as a platform for the affinity purification of CTFs.

In contrast to studies using toxin in artificial lipid bilayers (Ren et al. 1999, Oh et al. 1999), the physiological requirement of cellular factors for toxin entry underscores the need to study toxin translocation using appropriate biologically derived membrane systems. The ability to pellet early endosomes pre-loaded with a stable "translocation intermediate" mutant toxin will allow for purification of cytosolic proteins specifically recruited to the endosomal membrane by the presence of membrane inserted toxin. This is a relatively simple experiment followed by proteomic-informatic analysis that will be performed in collaboration with the Boston University Proteomics Core. This is an extension of a previous collaboration with Cathy Costello, the Director of the Core Proteomics Facility at Boston University, and a letter of support is included. An endosomal based "translocation intermediate" can be constructed using DT mutants containing C domains that are incapable of complete unfolding. These toxins form pores under acidic conditions, but are not unable to complete their transfer through the pore due to size

constraints (Falnes et al. 1994; Weidlocha et al. 1992). The C-terminal end of the C domain in these mutants is threaded into the pore and presented to the cytosolic side of the endosomal membrane, but the C domain remains trapped within the pore. The double cysteine mutant, as described by Falnes has been made, expressed, and is ready for loading into endosomes.

Whole organellar proteomic analysis of endosomes has been described (Wiederhold et al., 2010; Duclos et al., 2011). In the assay system proposed here – control endosomes, endosomes loaded with wild type toxin, and endosomes loaded with the double cysteine mutant will be incubated with cytosol under conditions similar to the *in vitro* translocation assay described by Ratts et al. (2003). After incubation with cytosol, the pelleted endosomes are resuspended and proteins on the cytosolic surface of the early endosome are stripped using a high salt wash and/or trypsinization (unpublished data). The endosomes are then pelleted again by repeat ultracentrifugation. The resulting supernatant fractions contain stripped proteins and peptide fragments, respectively, from the cytosolic surface of the endosome (unpublished data), and these fractions will then be sent to the Proteomics Core at Boston University for analysis.

The difference between the control groups and the two toxin groups will be any proteins that are specifically recruited to the early endosomal membrane as a result of the toxin inserted into the endosomal membrane. Importantly, the goal will not be to identify every single protein on the endosomal surface, but to generate a peptide map. Subtractive analysis of the peptide maps (Dieguez-Acuna et al. 2005) correlating to control endosomes vs toxin loaded endosomes will result in a subset of peptides that are unique to the endosomes containing membrane inserted toxin. Only those specific proteins/ peptides specifically recruited (or modified) by the presence of toxin will be identified by standard proteomics. Furthermore, a quantitative subtractive analysis between these groups will also be performed using standardized SILAC proteomic protocols (Ong et al., 2002).

This method takes of advantage of a stable toxin-endosomal "translocation-intermediate" and the ability to pellet endosomes by ultracentrifugation to purify proteins recruited by toxin to the cytosolic surface of the endosomal membrane. It is hypothesized that these specifically recruited proteins are likely CTFs required for toxin entry. It is possible that proteins recruited to the endosome may not be present in sufficient quantity to be detected compared to background, or could be recruited to the endosome but not participate actively in translocation.

Summary Aim 2: Sufficient quantities of control early endosomes, early endosomes loaded with wild type toxin, and early endosomes loaded with the double-cysteine translocation deficient toxin will be generated within 6-12 months. The endosomal pull down assay and preparation of samples for proteomic analysis will be completed within 6 months. Proteomics and data analysis will then be performed with collaborators at Boston University during year 2. Targets would then be validated as per Aim 3 during years 2 and 3.

Specific Aim 3: Validate functional roles for any putative CTFs identified in specific aims 1 and 2 using both *in vitro* translocation and cytotoxicity assays.

Hypothesis: Proteins affinity purified in specific aim 1 or recruited to the endosomal membrane by toxin in specific aim 2 likely function as CTFs.

The techniques applied in specific aim 1 and specific aim 2 will result in the affinity purification of proteins based upon protein-protein interactions. Although the identification of previously identified CTFs (e.g. Hsp 90, TrR-1, beta-COP) will validate the assay systems, a functional role for any novel putative CTFs in facilitating translocation of the C-domain across the endosomal membrane must be confirmed.

The *in vitro* translocation assay is both sensitive and specific, and employs purified early endosomes from Hut102/6TG cells pre-charged with the fusion protein toxin $DAB_{389}IL\text{-}2$ in the presence of the ATPase inhibitor bafilomycin A1 (Lemichez et al., 1997; Ratts et al., 2003). As shown in Figure A-2, the charged early endosomes are then incubated under various conditions –

e.g. +/- ATP, cytosol – to study translocation of the toxin across the endosomal membrane. The reaction is quenched on ice and ultracentrifugation separates the supernatant fluid fraction containing translocated C domain from the pellet fraction containing vesicle associated C domain. The presence of the C domain is then detected by an *in vitro* ribosylation assay performed under refolding conditions (Chung et al, 1977; Ratts et al. 2003). Importantly, the use of the *in vitro* translocation and *in vitro* ribosylation assays in series is capable of distinguishing between CTFs roles in translocation events, refolding of the C domain into an active conformation, and reduction of the DT inter-chain disulfide bond (Ratts et al. 2003). Furthermore, controls indicate that non-specific lysis and release of endosomal luminal proteins does not occur, and that the CTFs are not required for acidification of the endosomal lumen (Ratts et al., 2003), and this *in vitro* translocation assay has become the gold standard experimental platform in studying protein toxins that translocate across the endosomal membranes.

A functional role for putative CTFs can be analyzed in this system using any known specific inhibitors, immunodepletion, or the use of known mutants (Ratts et al., 2003; Ratts et al., 2005). In the situation where unknown proteins are identified as putative CTFs, specific antibodies can be generated. Alternatively, crude cytosol from cells depleted of the putative CTF by siRNA can be used. A physiologically relevant role for putative CTFs can also be confirmed in cytotoxicity assays using specific inhibitors (Ratts et al. 2003) or siRNA silencing of targeted CTFs (Tamayo et al., 2011).

Summary Aim 3: Novel CTFs from Aims 1 and 2 will be validated using well established protocols consisting of an *in vitro* translocation assay in series with an *in vitro* ribosylation assay, and cytotoxicity assays. Analysis of single CTFs can be performed in 4-6 months if known inhibitors or antibodies already exist. An additional 2-4 months will be needed for antibody generation or siRNA silencing of any unknown proteins.

Conclusion:

The impact of studying diphtheria toxin CTFs is epitomized by the growing list of proteins whose membrane translocation is facilitated by the same components of the CTF complex. The two techniques described in this proprosal – toxin "entry motif" affinity purification and toxin-endosomal based proteomics – are simple yet powerful experimental protocols that should allow for rapid purification of putative CTFs. Purified factors can then be identified in combination with respective proteomic core facilities. If successful, this cost-effective approach would be readily adaptable to other pathogens and would increase access to studying toxin translocation.

If Aims 1 and 2 above are not successful, then DT CTFs will be purified according to the standard protein chromatography protocol as described by Ratts et al. (2003) and any putative CTFs will be confirmed as above in Aim 3. This traditional purification process would most likely require 12-16 months to generate enough sample for proteomic identification.

The use of affinity purification of CTFs using toxin fragments of various lengths might allow for concomitant identification of binding motifs mediating interaction between toxin and CTFs. Furthermore, the concept of using the endosome itself to purify CTFs in a biologically relevant setting is important since studies using purified toxin and artificial lipid bilayers have failed to fully reflect the true complexity of the translocation process. Taken together, these assay systems will improve our understanding of toxin entry at the molecular level. Such insight will result in new targets for developing toxin entry inhibitors, and will also facilitate the rational rational design of toxin based vectors for the efficient delivery of proteins inside cells.

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Appendix A:

Figure A-1: Affinity purification of mammalian expressed (6x His) diphtheria toxin (DT) based polypeptides. Various DT based polypeptides containing a C-terminal (6x His) tag were expressed in HUT102/6TG cells using the pTRACER-CMV2 vector. The constructs were transfected into HUT120/6TG cells, and 48 hours later the (6x His) tagged constructs were purified using Ni^{2+} magnetic beads (Qiagen) under native conditions according to the manufacturer's directions. The purified samples were then analyzed by 4-20% SDS-PAGE and stained with colloidal Coomassie. Black arrows indicate protein bands corresponding to the tagged construct. Asteriks indicate protein bands that bind only in the presence of DT residues 230 – 272. Triangles indicate protein bands that bind only in the presence of DT residues 140 – 210. (Ratts, unpublished data, 2004).

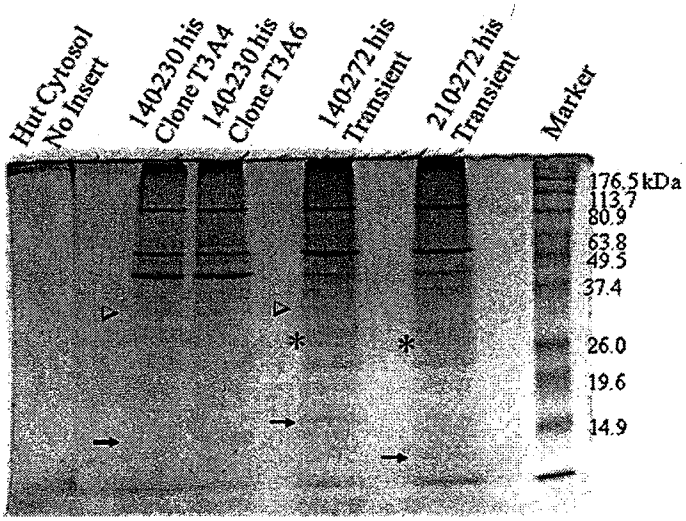
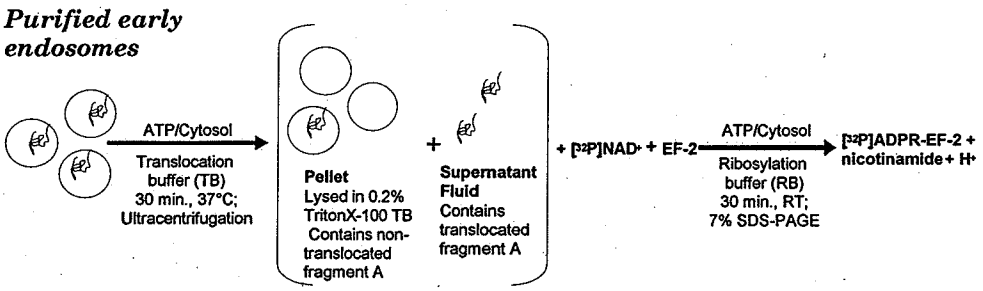


Figure A-2: Schematic diagram of the *in* vitro translocation assay coupled to the *in vitro* ADP-ribosylation assay. Early endosomes are pre-loaded with toxin in the presence of vesicular ATPase inhibitor Bafilomycin A1, which prevents acidification of the endosomal lumen, and purified as per Ratts et al., 2003.



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EDUCATION AND TRAINING

Undergraduate: 1970 - 74	Emory University Atlanta, GA	B.A.	History of Art 1974
Graduate: 1974 - 78	New York University School of Medicine New York, NY	M.D.	1978
Postgraduate: 1978 - 79	Intern, Internal Medicine Bronx Municipal Medical Center and The Hospital of The Albert Einstein College of Medicine New York, NY		
1979-81	Resident, Internal Medicine Bronx Municipal Medical Center and The Hospital of The Albert Einstein College of Medicine New York, NY		
1981	US Indian Health Service Rosebud, SD		
1981-84	Fellow in Medical Oncology Yale University School of Medicine New Haven, CT		

APPOINTMENTS AND POSITIONS

1984-1986	Assistant Professor of Medicine Yale University School of Medicine New Haven, CT
1984-1986	Director, Clinical Research Office Yale Comprehensive Cancer Center New Haven, CT
1985-1986	Acting Director, Yale Melanoma Unit Yale New Haven Hospital New Haven, CT
1986-1990	Assistant Professor of Medicine University of Pittsburgh, School of Medicine Pittsburgh, PA
1990-1991	Associate Professor of Medicine and Surgery University of Pittsburgh, School of Medicine Pittsburgh, PA
1986-1991	Director, Fellowship Program Division of Medical Oncology/Hematology University of Pittsburgh Pittsburgh, PA
1986-1991	Medical Director, Genitourinary Tumors Study Group University of Pittsburgh, Pittsburgh Cancer Institute Pittsburgh, PA
1991-1995	Associate Professor of Medicine Dartmouth-Hitchcock Medical Center Lebanon, NH
1992-1995	Director, Clinical Therapeutic Research Program Norris Cotton Cancer Center Lebanon, NH
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1996-2005	Board of Trustees The Hitchcock Foundation Lebanon, NH
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1999-2002	Chairman, Board of Trustees The Hitchcock Foundation Lebanon, NH
2004-2009	Director, Cancer Immunotherapy, Immunotherapy Center Dartmouth Medical School Lebanon, NH
2011-present	Associate Director, Clinical Research Norris Cotton Cancer Center Dartmouth-Hitchcock Medical Center Lebanon, NH

CERTIFICATION

1979	Diplomate, National Board of Medical Examiners
1981	Diplomate, American Board of Internal Medicine
1989	Diplomate, Subspecialty of Medical Oncology
1992	Fellow, American College of Physicians

LICENSURE

1979	New York (inactive – 60-138461)
1980	New Jersey (inactive - 25MA0381160)
1981	Connecticut (inactive – 022878)
1986	Pennsylvania (inactive-MD037142E)
1991	New Hampshire #8616
1992	Vermont (inactive)

PROFESSIONAL SOCIETY MEMBERSHIPS

American Association for Cancer Research 1983-present
American College of Physicians 1993
American Federation of Clinical Research 1984
American Society of Clinical Oncology 1983-present
Clinical Immunology Society 1988
International Society of Interferon Research 1983
Society for Biological Therapy 1988
Affiliate Member, American Urological Association 1992
American Society of Clinical Oncology, Melanoma Program Committee 1997
Member, WHO Melanoma Programme 1997
International Cytokine Society 1998
Collaborative Ocular Melanoma Study Group 1998
American Society of Clinical Oncology Program Committee 2000-present
Life Member of the National Registry of Who's Who 2000-present
Consultant, Immunology Devices Panel of the Medical Devices Advisory Committee, Center for Devices and Radiological Health, Food and Drug Administration 2003-2008

ACADEMIC HONORS

1972	Alpha Epsilon Upsilon, Emory University
1973	Omicron Delta Kappa, Emory University
1974	Phi Beta Kappa, Emory University
1974	Magna Cum Laude, Emory University
1974	Sigma Xi, Emory University
1975	Bernard Baruch Award, American College of Rehabilitation and Physical Medicine
1996-present	The Best Doctors in America
1997	First Steven B. Currier Clinical Oncology Scholar, endowment to support clinical melanoma research
2003-2004	Who's Who in Medicine and Healthcare, 4th edition
2004-2005	Who's Who in Science and Engineering, 7th edition
2004-present	Faculty of 1000 Medicine
2005-2006	Who's Who in American Education, 7th edition
2005-2006	The United Who's Who Registry, "Honors Edition"
2008-2009	Who's Who Among Executives and Professionals, "Honors Edition"
2009	New Hampshire Magazine Top Doctors Poll 2009
2010	New Hampshire Magazine Top Doctors Poll 2010

PROFESSIONAL ACTIVITIES

Research:

1) Peer Reviewed Grants and Contracts:

	Year	Title	Agency (Grant #)/Title/Award Amount Total
1.	1984-1986	PI	ACS/Junior Faculty Award/ $38,000
2.	1984-1986	PI	Pharmaceutical Manufacturers Association/Foundation Award for Clinical Pharmacology of Interferon/$60,000
3.	1986-1991	PI	ACS/Fellowship Training Award/$20,000
4.	1987-1988	PI	NCI/Master Agreement, Interferon alpha plus interferon gamma in patients with renal cell cancer/$148,582
5.	1988-1989	Co-PI	NCI (N01 CM-67893-02)/Phase I/II clinical evaluation of biological response modifiers (BRMs) for the treatment of cancer (A-LAK)/$706,744
6.	1988-1989	Co-PI	NCI (N01 CM-67843-01)/Phase I/II clinical evaluation of biological response (BRMs) for the therapy of cancer/$308,512
7.	1989-1990	PI	ACS/Institutional Grant, Novel assay for metallothionein in expression in human tumors/$5,500
8.	1989-1991	Co-PI	ACS/Metallothionein expression and anticancer therapy/$40,000
9.	1989-1991	PI	NIH (IT3CA9620)/Research and Training in Medical Oncology NRSA Institutional Training Grant/$391,068
10.	1990-1994	PI	NCI (1 R01 CA52166-01)/Metallothionein and Tumor Resistance to Chemotherapy/ $800,000

CV – Ernstoff: Abbreviated at page 4. Pages 5-40 available upon request.

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Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
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INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Emmanuel College, Boston, MA	A.B.	1964	Chemistry
Georgetown University, Washington, DC	M.S.	1967	Organic Chemistry
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Mass. Institute of Technology, Cambridge, MA	Postdoc	1970-73	Mass Spectrometry

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Positions and Employment

1974-1979 Research Associate, M.I.T.
1975-1995 Associate Director, Mass Spectrometry Resource, M.I.T.
1979-1988 Principal Research Scientist, M.I.T.
1980-1985 Part-time Lecturer, Depts. of Chem. & Med. Chem., Northeastern Univ.
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1994-present Founding Director. Mass Spectrom. Resource, B. U. School of Medicine
1998-present Res. Prof. of Biochemistry, B. U. School of Medicine
2002-present Founding Director, Cardiovascular Proteomics Center, B. U. School of Medicine

NIH Study Sections: Physiolog. Chem., 1993-7; Biomed. Res. Technol., 1986-9 (Chair,1989); NIH Special Study Sections, Rev. Panels, Site Visitor, 1979-; NSF Site Visitor/Reviewer, 1984-. Advisory Comm.: NRC Inst. Marine Sciences (Halifax, NS, Canada), 1990-3, 2000-; Pacific NW Nat'l Lab, MS Resource, 1998-present (Chair, 2004); Complex Carbohydr. Res. Ctr., Univ. Georgia, 2002- (Chair 2005-), Glycomics Resource, Univ. Indiana, 2006-, Proteomics Ctr., Univ. Puerto Rico, 2007-, Barnett Inst. Northeastern Univ., 2008. Boards: Human Proteomics Org.-US, 2004-; Human Glycoproteom. Initiative, 2004-; US Natl. Comm.-IUPAC, 2004-; Int'l HUPO Council, 2005-.

Professional Societies

AAAS; ACS (Councilor, 1989-; Alt. Councilor, 1986-8; Comm: Int'l. Activities, 1992- (Chair, 2004-5); C&B, 1994-9; Lecture Tours, 1974-92; NE Sect.Comm: Nom., 1982, '83, '87, '92; Publ., 1988-93 (Chair, 1990, 93); C&B Chair, 1997-; Finance, 2002-5); ASMS (V-P. Arrangements, 1985-7, V-P Programs, 2000-2; President, 2002-4); Council Sci. Soc. Pres. (2000-7); Soc. Glycobiology (Exec. Bd., 1998-2000); Int'l Mass Spectrom. Fdn. (Sec'y, 2006-). **Current Ed. Boards**: *Rapid Commun. Mass Spectrom.* (1990-), *Mass Spectrom. Revs.* (1994-), *Eur. J. Mass Spectrom.* (1995-), *Amyloid:Int. J.Prot. Fold.Disord.* (2000-), *Anal. Chem.* (2005-), *Mol. Cell. Prot.* (2006-).

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24. Mass Spectrometry of Glycoprotein Glycans: HUPO HGPI (Human Proteome Organisation Human Disease Glycomics/ Proteome Initiative) Multi-institutional Study. Wada, Y.; ... Costello, C. E.; ...Taniguchi, N. (total 26 authors) *Glycobiology*, **2007**, *17*, 411-422.

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CURRICULUM VITAE

Name: John R. Murphy

Address: 4958 Black Walnut Point Road, Tilghman, MD 21671

Social Security Number:

Date of Birth: October 7, 1941

Place of Birth: Bridgeport, Connecticut, United States of America

Education:

1965	B.A.	Zoology	University of Connecticut, Storrs, CT
1969	M.S.	Microbiology	University of Connecticut, Storrs, CT
1972	Ph.D.	Microbiology	University of Connecticut School of Medicine, Farmington, CT

Postdoctoral Training: 1972 –1974 Research Fellow of Biology, Harvard University, Cambridge, MA

Awards and Honors:

1990	Recipient, Pierce Immunotoxin Award
2000	The Moxie Trophy, Newport-Bermuda Race, First in Class Double Handed Division

Academic Appointments:

1973 – 1974	Tutor in Biology, Dunster House, Harvard University, Cambridge, MA
1974 – 1977	Assistant Professor, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA
1977 - 1984	Associate Professor, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA
1979 – 1984	Lawrence J. Henderson Associate Professor of Health Science and Technology, Harvard – Massachusetts Institute of Technology Program in Health Sciences, Technology, and Management, Boston, MA
1979 – 1984	Board of Tutors in Biochemical Sciences, Harvard University, Cambridge, MA
1984 – present	Professor of Medicine, Department of Medicine, Boston University School of Medicine, Boston, MA
1984 – 2002	Chief, Section of Biomolecular Medicine, Department of Medicine, Boston University School of Medicine, Boston, MA
1987 – present	Professor of Microbiology, Boston University School of Medicine, Boston, MA
1992 – present	Professor of Biomedical Engineering, College of Engineering, Boston University, Boston, MA

2002 – 2010	Chief, Section of Molecular Medicine, Department of Medicine, Boston University School of Medicine, Boston, MA
2011 – present	Director *ad interim*, National Emerging Infectious Diseases Laboratories, Boston University School of Medicine, Boston, MA

Other Professional Positions and Major Visiting Appointments:

1974 – 76	Research Fellow, The Medical Foundation, Inc., Boston, MA
1976 – 81	Career Development Award Recipient, United States Public Health Service, National Institute of Allergy and Infectious Diseases, Bethesda, MD (KO4 AI-00146)
1978	Fellow, Japan Society for the Promotion of Sciences, Tokyo, Japan
1978	Visiting Professor, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Japan
1979 – 1980	Short-term Consultant, World Health Organization, Geneva, Switzerland

Major Committee Assignments:

1976 – 1982	United States – Japan Cooperative Medical Sciences Program, Cholera Panel Member, National Institutes of Allergy and Infectious Diseases, Bethesda, MD
1980 – 1988	Research Advisory Committee, The Medical Foundation, Inc., Boston, MA
1980 – 1983	Program Committee, Division of Medical Sciences, Harvard Medical School, Boston, MA
1981 – 1983	Standing Committee on Admissions of Students in the Medical School, Harvard Medical School, Boston, MA
1981 – 1983	Standing Committee on Animals in the Medical School, Harvard Medical School, Boston, MA
1981 – 1983	Faculty Council in the Medical School, Harvard Medical School, Boston, MA
1984 – 1987	United States – Japan Cooperative Medical Sciences Program, Cholera Panel Member, National Institute of Allergy and Infectio0us Diseases, Bethesda, MD
1986 – 2002	Research Advisory Committee, Betty Lea Stone Fellowship, American Cancer Society, Massachusetts Division, Boston, MA
1987 – 2008	Standing Committee on Appointments and Promotions, Department of Medicine, Boston University School of Medicine, Boston, MA

Major Research Interests:

1. Regulation, secretion, and mechanism of diphtheria toxin action

2. Protein engineering: design, construction, and characterization of receptor specific targeting of chimeric toxin genes which encode fusion proteins composed of the native diphtheria toxin in which the native receptor binding domain is replaced with growth factors or peptide hormones.

Teaching Experience:

1965 – 1967	Instructor in Biology, Wilbraham Academy, Wilbraham, MA
1967 – 1968	Graduate Teaching Assistant, Department of Microbiology, University of Connecticut, Storrs, CT
1972 – 1974	Tutor in Biology, Harvard University, Cambridge, MA
1974 – 1976	Course Director, Medical Microbiology, Harvard Medical School, Boston, MA
1974 – 1981	Lecturer, Microbiology, Harvard Medical School, Boston, MA
1975 – 1982	Lecturer, Mechanisms of Microbial Pathogenesis, Harvard – M.I.T. Program in Health Sciences, Technology, and Management, Harvard Medical School, Boston, MA
1977 – 1983	Course Director, Mechanisms of Microbial Pathogenesis, Harvard – M.I.T. Program in Health Sciences, Technology, and Management, Harvard Medical School, Boston, MA

Bibliography:

Original Reports: (Limited to past 2 years)

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149. Murphy, JR. Mechanism of diphtheria toxin catalytic domain delivery to the eukaryotic cell cytosol: A possible roadmap for the development of 21st century therapeutics. *FEBS Journal*, 2011; (submitted).

Book Chapters & Reviews: ***(Limited to past 2 years)***

57. Bishai WR, & Murphy JR. Diphtheria. In *Harrison's Principles of Internal Medicine*, 19th Edition (Kasper DL, Fauci AS, Braunwald E, Hauser SL, Longo DL, Jameson JL, Loscalzo J, eds) McGraw-Hill, Medical Publishing Division, 2011 (in press).

58. Ratts, R & Murphy Diphtheria Toxin and Cytosolic Translocation Factors. In *Infectious Disease.* Petra Nenadic editor, InTech. In press, 2011, ISBN 979-953-307-497-8

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December 14, 2011

The Hitchcock Foundation
Attn: Jennifer Reining/Michael Shoob
Executive Director

RE: Career Development Award for Ryan C. Ratts: Purification, Identification, and Characterization of the Diphtheria Toxin Cytosolic Translocation Factor Complex

To the Selection Committee:

It is with great enthusiasm I write in support of Dr. Ryan Ratts' application for a Career Development award.

Dr. Ratts is one of the very few physicians I have met over my 20 years at Dartmouth with a significant background in bench research, an impressive resume for a junior investigator, and a burning desire to develop a laboratory based translational research program. He has taken a 70% position in Hospital Medicine as an environment that has no longitudinal patient care responsibilities and defined inpatient time commitment. This arrangement is conducive to establishing time for development of a translational research career which would be significantly enhanced by a Career Development Award.

Dr. Ratts is passionate about his research and has, in my opinion, the most important characteristics which define successful independent investigators: desire and creativity. He has superb training in the laboratory with John R Murphy at Boston University, an international leader in fusion proteins. He has maintained significant relationships with both Drs. Murphy and Costello at Boston University who are committed and willing to provide assistance in his research project. He has had outstanding clinical training in Internal Medicine at Massachusetts General Hospital and in Pediatrics at Children's Hospital Boston. He is now board certified in both specialties. Thus, Ryan has an exceptional array of tools to use in developing his career.

I have committed myself to assisting Dr. Ratts in his efforts to develop a career in laboratory based translational research. My position as Associate Director of Clinical Research in the Norris Cotton Cancer Center and as one of the Dartmouth leaders in our U54 Collaborative Grant with University of Vermont for an IDeA-CTR Clinical Trials Research unit puts me in a unique position to provide mentoring and oversight for Dr. Ratts' efforts. I have had significant success as an independent investigator as well as having successful mentored many junior faculty.

Dr. Ratts' proposal to identify and validate the function of novel putative cytosolic translocation factor (CTFs) will provide a foundation for developing a pathway for drug delivery which would have significant impact on creating new agents to treat illnesses. It will also expand our knowledge of toxin pathways and identify potential therapeutic points to lessen the effects of toxin exposure, an important area in medicine but a critical area for national defense from bioterrorism.

In summary, Dr. Ryan Ratts is a bright, energetic, creative and passionate physician scientist who has all the necessary components to launch a career in translational research. He has established a mentoring team who will support and guide his career and with funding from the Hitchcock Foundation will have the time to pursue the work.

Thank you for your consideration.

Sincerely,

Marc S. Ernstoff, MD
Professor of Medicine
Director, Melanoma Program, NCCC
Associate Director for Clinical Research, NCCC

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John R. Murphy, Ph.D.
Professor of Medicine and Microbiology
Director *ad interim*
National Emerging Infectious Diseases Laboratory

December 13, 2011

Attn: Michael Shoob – Executive Director

michael.shoob@hitchcock.org

Attn: Jennifer Reining – Executive Director

jennifer.reining@hitchcock.org

Hitchcock Foundation

Dartmouth Hitchcock Medical Center

One Medical Center Drive

Lebanon, NH 03756

Re. Ryan Ratts, MD, PhD – Career Development Award.

Dear Members of the Hitchcock Foundation and Selection Committee,

I am writing in enthusiastic support of Ryan Ratts, MD, PhD and his application for the Hitchcock Foundation Career Development Award. Dr. Ratts earned his PhD in my laboratory, and his research was focused on whether, or not the translocation of diphtheria toxin fragment A (catalytic domain) from the lumen of endosomal vesicles required the participation of host cell factors. Ryan was intellectually engaged on the project, and he displayed a natural gift for designing and carrying out experiments. His critical thinking skills and creativity allowed him to quickly learn, adapt, and incorporate a diverse set of experimental approaches to his project.

Dr. Ratts' research led to the publication of two important papers in the field that resolved a long standing controversy regarding the mechanism of entry of diphtheria toxin across the endosomal membrane: Ratts *et al.*, 2003 and Ratts *et al.*, 2005. The 2003 *Journal of Cell Biology* paper described the partial purification of a Cytosolic Translocation Factor complex which demonstrated that both Hsp90 and thioredoxin reductase were essential and played a direct role in the delivery of the catalytic domain from the endosomal lumen to the external medium. These observations provided the first demonstration that cellular factors played an essential role in the entry process, which is now recognized as common and basic mechanism of toxin entry. Based on this work, Ryan cleverly reasoned that if cellular factors were involved in the entry process, there must also be toxin sequences that mediated the interaction with these or other cellular factors. The 2005 *Proceeding of the National Academy* paper defined a lysine rich sequence, T1, which was also essential for catalytic domain entry. This paper also demonstrated that the T1motif is a lysine rich region on the N-terminal end of the transmembrane which specifically interacts with coatomer complex I (COPI). Although Ryan left research to complete his medical

training, he diligently followed the field and the current literature. Ryan's conceptual understanding of bacterial protein toxins and his contributions makes him one of the leading experts in the field, and he was recently asked to write and completed a chapter review with me that proposed new models for the mechanism of toxin entry.

Ryan went on to finish his medical degree at Boston University and completed his training in the Harvard Combined Internal Medicine-Pediatrics Residency Program. It has been a pleasure to both observe and participate in the development of Ryan's career, and I have eagerly awaited his return to the laboratory. When making decisions about his career following residency, I tried hard to recruit Ryan back to Boston University as a junior faculty member to start his own independent basic science laboratory. Ryan has instead accepted a position at Dartmouth, and is pursuing a career in academic medicine. He is an extremely talented young man. Without saying, I am delighted to support him in any way that I can. Toward that end, I have made available to him all the reagents and tools that we've developed since the time he left the laboratory. With adequate protected time from his clinical duties, I have evey confidence that Ryan will realize his full potential as an independent investigator.

I am excited to continue working with him as a mentor, collaborator, and as a peer. Ryan is also continuing to collaborate with Cathy Costello who is the Director of the BU center for biomedical mass spectrometry. The approach that he is taking to the continuing study of the toxin entry process is sound, and will almost certainly yield exciting new insights. His new mentor, Dr. Marc Ernstoff, will be pivotal in developing Ryan's skills to take his work performed in the laboratory and translating it into new clinical tools. The Hitchcock Foundation Career Development Award will help ensure that the best parts of his career are yet to come.

Best regards,

John R. Murphy, Ph.D.

Professor of Medicine, and

Director *ad interim*, National Emerging Infectious Diseases Laboratories