Example TP1 review for reference

TrainingProposals

These proposals are a different category than instrumentation access. We can take on a limited number of people who want to shadow our staff as they operate a national facility and center to learn best practices and our workflow. The proposal below probably should be treated more like a TP1 (embedded training) rather than a TP2 (facility manager training)

Project ID: NCCAT-TP1-SP181214
Project Name: Single-particle analysis of the HOPS complex
Primary User Name: Sarah Port
eRA Commons User Name: SAPORT
Institution: Princeton University
Submission Date: 12/14/18

Averaged URC scores:
(i) training goals: 2
(ii) training plan: 2
(iii) resources requested:2.5
(iv) user EM background and history: 1.25
(v) geographical demographics or need: 1.75
Raw average score: 1.9

Comments:

Reviewer 1:

The scientific objective is the 660-kDa multisubunit tethering complex HOPS. a major organizer of membrane tethering and fusion within the endo-lysosomal system of all eukaryotes. The applicant has had some experience with negative stain EM and cryo sample preparation. She is the first in her crystallography group to move towards EM and Princeton has invested in a Titan Krios, but lacks a training facility. The proximity to NYSB makes it feasible for the applicant to commute to the NYSB facility. The applicant could be a means of expanding the training experience at Princeton following her training at NYSBC.

Reviewer 2:

This proposal is aimed at training one of the first cryo-EM users at Princeton. With a background in X-ray crystallography, and preliminary negative stain data of a challenging complex, the application is well positioned to take full advantage of training at the facility, and this will have a strong impact on her home institution. The training goals are quite broad, and the training plan does not have clearly defined milestones, somewhat lowering enthusiasm for the application.

Reviewer 3:

The goal of this proposal to obtain an additional training in operation of a Titan Krios microscope. Dr. Sarah Port had experience in cryo-EM sample preparation, training on Krios from her home institution, Princeton University, and she already obtained cryo-EM data related to the proposed project. Princeton has one experienced cryo-EM researcher and I would think this faculty was hired to help/train others in cryo-EM. In addition, their microscope could not be fully occupied. It is not clear in this proposal why Dr. Port really needs to come to NCCAT for this long training, as I would assume she can easily get full training in Princeton facility and get access to cryo-EM expert there.

Reviewer 4:

The candidate is a postdoc at Princeton and is requesting 6-8 weeks. Preliminary 2D class averages of sample but issues with aggregation and stability. Previous experience in negative stain, freezing grids and screening on a Krios. Interested in learning alternative sample prep methods, scope alignment and introduction to data processing. In a X-ray crystallography lab and interested in training others in cryo-EM at Princeton.

Project ID: NCCAT-TP1-SP181214
Project name: Single-particle analysis of the HOPS complex
Primary username: Sarah Port
eRA Commons username: SAPORT
Institution: Princeton University
Submission date: 12/14/18

Summary statement:
I want to receive training in single-particle cryo-EM - from sample preparation to data processing. The training will be directly applicable to my research and in addition will help in developing the recently acquired biological cryo-EM resources on our campus.

Training Goals:
I am applying to receive training in sample preparation, microscope operation, data acquisition, and data processing.

Training Plan:
The electron microscopes at Princeton University are housed in a central facility that, until quite recently, was mainly used for material science. A Titan Krios for biological cryo-EM and cryo-ET is a relatively recent addition. Consequently, the expertise for training new users for biological EM applications is still limited. From initial observations, my sample is prone to aggregation, and the stability of the complex seems to vary with ice thickness. Therefore, I am interested in sample preparation options and sample optimization strategies. I would like to develop expertise in assessing whether the microscope is properly aligned and in judging the quality of samples and deciding whether it is worth collecting data on a grid. Because my protein complex may have multiple conformations, I am interested in determining how much data needs to be collected to model different conformational states. For the same reason, introduction to data processing strategies would be very advantageous.

Resources requested:
The training could take 6-8 weeks, during which I would be at NCCAT 3-5 days per week. I would bring my own research/ protein complex. Access to different types of sample preparation equipment as well as screening time would be helpful.

Background and history:
I have performed negative-stain EM and recently started using cryo-EM. I have frozen grids with a Vitrobot and screened samples with EPU on a Titan Krios with K2 camera and Volta phase plate. I have limited experience using RELION.
Recently, I have been studying the online training videos provided by the Jensen lab (em-learning.com) to develop a theoretical background and begin picking up strategies for sample preparation and screening.

Geographical Demographics:
I am the first member of a protein crystallography lab to transition to single particle cryo-EM, as well as one of the first researchers using cryo-EM for biological applications in Princeton. In addition, I am involved in developing the EM resources on campus as part of the EM user committee and owner of the departmental structural biology mailing list. After proper training, I will be able to help other interested members of the lab and department get started with cryo-EM. Furthermore, as our department is still in the process of setting up its cryo-EM, insight into the infrastructure and proceedings in a National Center would be help us organize our resources in the most beneficial way.
As Princeton is well connected to New York City via express trains, a daily commute is entirely feasible.

Single-particle analysis of the HOPS complex

The 660-kDa multisubunit tethering complex HOPS is a major organizer of membrane tethering and fusion within the endo-lysosomal system of all eukaryotes. The misregulation or mutation of human HOPS can lead to developmental defects, neurodegenerative diseases, and cancer. HOPS interacts with SNARE proteins, Rab GTPases, specific lipids and vesicle coats. To understand the molecular mechanisms underpinning HOPS function, we want to determine its structure and characterize its interactions with other key components of the membrane trafficking machinery.

Previous negative-stain EM studies of the Saccharomyces cerevisiae HOPS complex yielded mutually inconsistent low-resolution structures (Fig. 1). We have therefore chosen the HOPS complex from the thermotolerant fungus *Chaetomium thermophilum* for single-particle cryo-EM, complemented with X-ray crystallographic analysis of individual subunits or domains thereof, with the goal of determining a structure at atomic or near-atomic resolution. The optimization of the protein sample preparation is ongoing. Currently, we can purify large amounts of individual subunits and subcomplexes of HOPS after expression in *E. coli*, and small amounts of the fully assembled complex after expression in insect cells (Fig. 2). Recently, we started analyzing subcomplexes of HOPS with negative-stain and cryo-EM (Fig. 3).

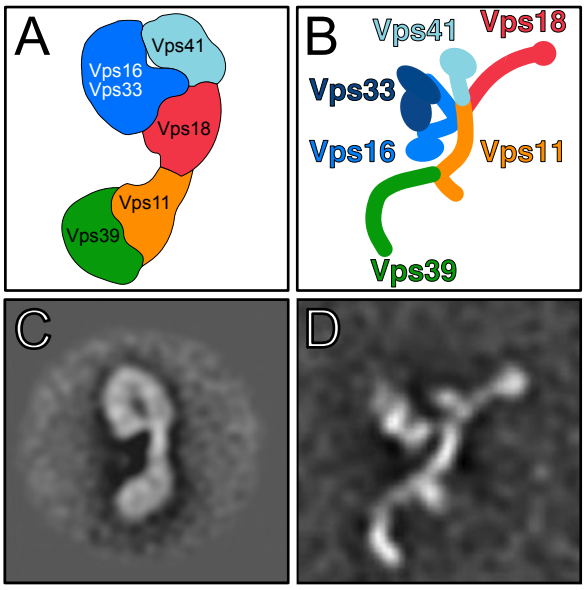

Fig. 1 Proposed architecture of the HOPS complex. (A,B) Schematic drawings of the proposed subunit organization of HOPS and (C,D) selected class averages derived from negative-stain EM (modified from [1,2], respectively). (A,C) show samples that were cross-linked with glutaraldehyde.

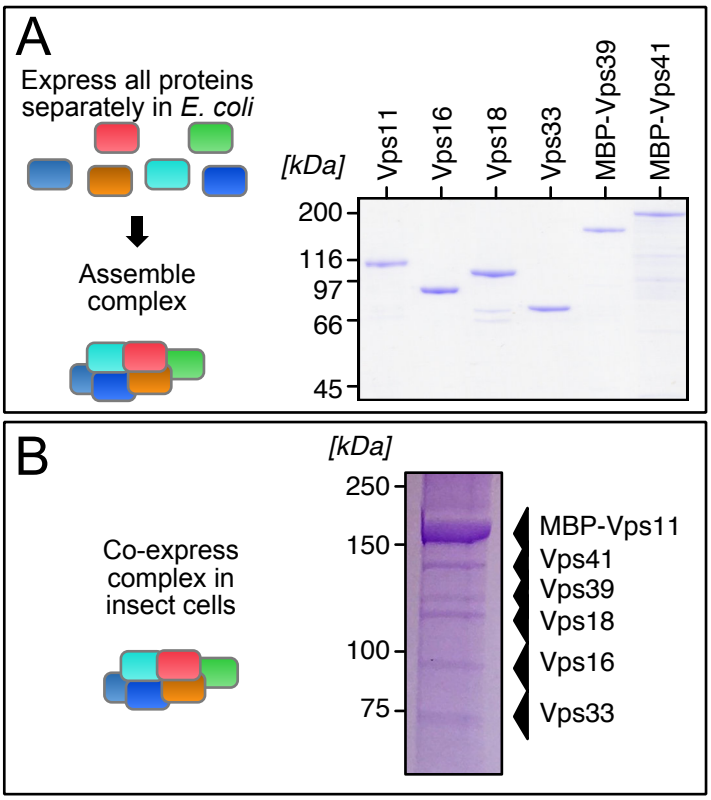

Fig. 2 Purification strategies for the HOPS complex. (A) HOPS subunits can be purified after recombinant expression in *E. coli* and assembled into a complex (B) or the entire HOPS complex can be purified after recombinant co-expression in insect cells.

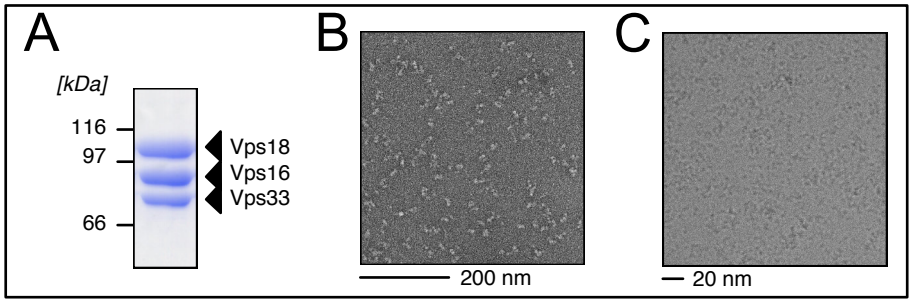

Fig. 3 Initial EM studies of a subcomplex of HOPS. (A) A complex containing three HOPS subunits was purified after recombinant expression in *E. coli*. (B) The complex was visualized by negative-staining with uranyl acetate and (C) cryo-EM. Due to aggregation, potential conformational heterogeneity and complex dissociation as well as low contrast, the identification of individual particles in the cryo-EM micrographs is challenging.

References

[1] Bröcker C et al. (2012) Proc Natl Acad Sci U S A, 109(6):1991-1996.

[2] Chou HT et al. (2016) Nat Struct Mol Biol, 23:761-763.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
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NAME: Port, Sarah Alexandra

eRA COMMONS USER NAME: SAPORT

POSITION TITLE: Postdoctoral Researcher

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Technische Universität München, Germany	BS	09/2008	Molecular Biotechnology
Technische Universität München, Germany	MS	12/2010	Molecular Biotechnology
Georg-August-Universität Göttingen, Germany	PhD	09/2015	Molecular Biology
Georg-August-Universität Göttingen, Germany	postdoc	04/2016	Molecular Biology
Princeton University, USA	postdoc	present	Structural Biology

A. Personal Statement

My long-term research interest is to understand the mechanisms underlying the specificity and efficiency of intracellular transport on a molecular level. For my graduate studies, I focused on the molecular interactions of transport complexes and the nuclear pore complex during nucleocytoplasmic transport. We collaborated closely with experts to apply cross-linking mass spectrometry, single particle cryo-EM and X-ray crystallography methods to a complex of nuclear transport receptor, transport cargo and a fragment of the nuclear pore complex. This work culminated in the first crystal structure of a nuclear transport complex bound to a component of the nuclear pore complex, thereby showing that the phenylalanine-glycine repeats of the nucleoporin interact with hydrophobic pockets on the surface of the transport receptor.

For my postdoc, I joined the Hughson lab at Princeton University to conduct postdoctoral studies on vesicle trafficking, with a focus on the molecular interactions of proteins involved in membrane tethering and fusion. My graduate research has provided a strong foundation in many of the areas of expertise needed for my project. Specifically, I have extensive experience in cloning, protein expression and purification and the analysis of protein interactions *in vitro* and *in vivo*, as well as substantial exposure to crystallographic and cryo-EM-based structural studies of macromolecular complexes. I am also expert in immunoprecipitation, Western blotting, fluorescence and confocal microscopy, and flow cytometry.

B. Positions and Honors

Positions and Employment

2015-2016 Postdoctoral Researcher, Department of Molecular Biology, Universitätsmedizin Göttingen, Germany
2016- Postdoctoral Researcher, Department of Molecular Biology, Princeton University, USA

Other Experiences and Professional Memberships

2016-2017 American Heart Association
2017- Gesellschaft für Biochemie und Molekularbiologie (German Society for Biochemistry and Molecular Biology)
2017- American Association for the Advancement of Science
2017- American Society for Cell Biology

Honors

2015 *Summa cum laude* (PhD thesis), Georg-August-Universität Göttingen

C. Contributions to Science

For my graduate research as well as my first year of postdoctoral research, I worked on nucleocytoplasmic transport. The sole gateways for transport between the cytoplasm and nucleus are the nuclear pore complexes (NPCs). A subset of the nucleoporins that make up the NPCs contain hydrophobic and intrinsically disordered stretches of phenylalanine-glycine (FG-) repeats, which form a permeability barrier. As a result, most molecules cannot diffuse through the NPC, but need to be actively transported by interaction with nuclear transport receptors. The major receptor for nuclear export is CRM1, which upon binding of the small GTPase Ran loaded with GTP, exports cargoes by interacting with their nuclear export signals (NES). The nucleoporin with the longest FG-repeat domain is Nup214.
For my postdoc, I study the structure and function of the multisubunit tethering complex HOPS, a 660 kDa hetero-hexameric complex involved in the tethering a fusion of late endosomes. In a first step, we investigated the functional interactions of the HOPS subunit Vps33 with SNARE proteins using single-molecule force microscopy.

1. Structural and functional characterization of the interaction of the nuclear export receptor CRM1 with the nucleoporin Nup214.

We generated a crystal structure of a FG-repeat-containing fragment of Nup214 bound to a CRM1 export complex. This was the first time the interaction of any transport receptor with a FG-nucleoporin had been visualized at high resolution. The interactions our structure revealed were verified using biochemical and cell-based assays and cross-linking mass spectrometry. Combining the insights in the molecular mechanisms of the interaction between nucleoporins and transport receptors with observations of the localization and mobility of two leukemogenic Nup214-fusions proteins in the cell, revealed the molecular basis of how nucleocytoplasmic transport can be disturbed in disease.

a) Port, S.A., Monecke, T., Dickmanns, A., Spillner, C., Hofele, R., Urlaub, H., Ficner, R., and Kehlenbach, R.H. (2015) Structural and Functional Characterization of CRM1-Nup214 Interactions Reveals Multiple FG-Binding Sites Involved in Nuclear Export. *Cell Reports*, 13(4):690-702, PMID: 26489467

b) Monecke T, Dickmanns A, Weiss,MS, Port SA, Kehlenbach RH, Ficner R (2015) Combining dehydration, construct optimization and improved data collection to solve the crystal structure of a CRM1-RanGTP-SPN1-Nup214 quarternary nuclear export complex. *Acta Crystallographica Section F,* 71(Pt 12):1481-1487, PMCID: PMC4666476

c) Port SA, Mendes A, Valkova C, Fahrenkrog B, Kaether C, and Kehlenbach RH (2016). The oncogenic fusion proteins SET-Nup214 and SQSTM1-Nup214 form dynamic nuclear bodies and inhibit nuclear protein- and mRNA export, *The Journal of biological chemistry*, 291(44):23068-23083, PMCID: PMC5087727

2. Development of assays to monitor CRM1-mediated nuclear export and quantify the involved protein interactions.

Our original assay recapitulating CRM1-dependent nuclear export made use of a stable cell line expressing GFP-NFAT and was therefore not widely available to other researchers. To fix this situation, we extended the assay using cells transiently transfected with various cargo proteins and a transfection marker. CRM1-, Ran- and energy-dependent nuclear export was reconstituted in digitonin-permeabilized cells and quantified by flow cytometry. This simplified assay can be applied for the in vitro analysis and characterization of any potential CRM1 cargo without the need for a stable cell line.
Furthermore, we developed a bead-based, semi-quantitative assay to analyze the interaction of Cy3-labeled CRM1 with a variety of cargoes and transport factors by flow cytometry. Compared to conventional gel-based pulldown assays, the power of the bead–based assay lies in the analysis of protein-protein interactions of very different strengths, covering a detection range of three orders of magnitude. The assay therefore allowed for the comparison of CRM1 binding to very different cargoes and nucleoporin fragments, and should be extendable to the analysis of other proteins.

a) Kehlenbach RH and Port SA (2016) Analysis of CRM1-dependent Nuclear Export in Permeabilized Cells. *Methods in Molecular Biology*, 1411:489-501, PMID 27147061

b) Thakar K, Karaca S, Port SA, Urlaub H, Kehlenbach RH (2013) Identification of CRM1-dependent Nuclear Export Cargos Using Quantitative Mass Spectrometry. *Molecular & Cellular Proteomics*, 12:664-678, PMCID: PMC3591659

c) Landry-Voyer AM, Bilodeau S, Port SA, Rouleau C, Boisvert FM, Kehlenbach RH, and Bachand F. (2016) Human PDCD2L is an export substrate of CRM1 that associates with 40S ribosomal subunit precursors, *Molecular and cellular biology*, 36(24):3019-3032, PMCID: PMC5126290

3. Templating of the SNARE complex by the SM protein Vps33.

To test and extend our model that the SM protein Vps33 acts as a molecular chaperone to template the formation of productive SNARE complexes, we used single-molecule force microscopy. Data are consistent with the model that the SM protein Vps33 catalyzes the step-wise assembly of the four SNARE motifs into a four-helix bundle via a defined pathway. First, the ‘half-zippered’ template complex forms. Second, the Qb- and Qc-SNAREs recognize and bind to the template complex. Finally, full zippering of the SNARE complex displaces the SM protein template. Analogous experiments using the neuronal SM protein Munc18-1 and the SNAREs required for neurotransmitter release lead to a similar conclusion, suggesting that the templating mechanism is conserved among SM proteins.

a) Jiao J, He M, Port SA, Baker RW, Xu Y, Qu H, Xiong Y, Wang Y, Jin H, Eisemann TJ, Hughson FM, Zhang Y (2018) Munc18-1 catalyzes neuronal SNARE assembly by templating SNARE association, *eLife* 2018;7:e41771

Conference contributions

11/2018 2018 Purdue Cryo-EM Symposium
07/2018 Princeton-Nature Conference "The Frontiers in Electron Microscopy for the Physical and Life Sciences", Princeton, NJ, USA (Poster, Talk)
12/2017 ASCB-EMBO meeting, Philadelphia, USA (Poster)
07/2017 Gordon Conference on Molecular Membrane Biology, Andover, NH, USA (Poster)
06/2017 FEBS/ EMBO Advanced Lecture Course “Molecular Architecture, Dynamics and Function of Biomembranes”, Cargèse, France (Poster)
09/2015 EMBO meeting, Birmingham, UK (Poster)
08/2014 Nuclear Organization and Function, Cold Spring Harbor, USA (Talk)
07/2014 Nuclear Envelope “Life at the Edge”, Potsdam, Germany (Poster)
02/2013 Molecular machines in RNA processing, translation and transport, Göttingen, Germany (Poster)

Complete List of Published Work in MyBibliography:

https://www.ncbi.nlm.nih.gov/sites/myncbi/1Xy_tr6VnSX5N/bibliography/48341369/public/?sort=date&direction=descending

D. Additional Information: Research Support and/or Scholastic Performance

Ongoing Research Support

5/2017-4/2019 Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) research fellowship PO 2195/1-1

Completed Research Support

NA.