BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Heldwein, Ekaterina

eRA COMMONS USERNAME (credential, e.g., agency login): EHELDWEIN

POSITION TITLE: American Cancer Society (Massachusetts Division) Professor of Molecular Biology

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
Lomonosov Moscow State University, Moscow	MS	06/1994	Chemistry
Oregon Health & Sciences University, Portland, OR	PHD	06/1999	Biochemistry
Oregon Health & Sciences University, Portland, OR	Postdoctoral	07/2000	Structural Biology
Children's Hospital & Harvard Medical School, Boston, MA	Postdoctoral	08/2004	Structural Biology

A. Personal Statement

I am a structural virologist who uses the combined powers of structural biology, biochemistry, and cell biology to understand at the atomic level the molecular mechanisms by which herpesviruses enter and escape from the host. Structural and mechanistic studies of herpesvirus cell entry and glycoproteins are a major focus of my research. We were the first to determine the structures of key herpesviral penetration proteins, gB and gH/gL [1, 2], identifying gB as the fusogen, or “the business end”, and gH/gL as a uniquely folded regulator of gB function. These landmark studies established a new paradigm for how herpesviruses enter cells. In addition to structural studies, my laboratory branched out into functional cell-based studies, and more recently, work with membrane proteins and viruses. We have also characterized the structure and the function of the cytoplasmic domain of HSV-1 gB and proposed that it forms a novel membrane-dependent “clamp” that controls the fusogenic function [3]. Although much of our previous work focused on HSV-1 glycoproteins, we have also been pursuing HCMV gB and determined the structure of its postfusion form [4]. The current proposal is built upon exciting preliminary data and leverages our expertise in structural and functional studies of herpesviral glycoproteins.

1. Heldwein E.E., Lou H., Bender F.C., Cohen G.H., Eisenberg R.J., and Harrison S.C. (2006). Crystal structure of glycoprotein B from Herpes Simplex Virus 1. Science 313, 217-220.

2. Chowdary T.K., Cairns T.M., Atanasiu D., Cohen G.H., Eisenberg R.J., and **Heldwein E.E.** (2010). Crystal structure of the conserved herpesvirus fusion regulator complex gH–gL. Nat. Struct. Mol. Biol. 17, 882-888. PMCID: PMC2921994.

3. Cooper RC, Georgieva ER, Borbat PP, Freed JH, and **Heldwein EE** (2018). Structural basis for membrane anchoring and fusion regulation of the Herpes Simplex Virus fusogen gB. Nat. Struct. Mol. Biol. 25, 416-424. PMCID: PMC5942590.

4. Burke HG and **Heldwein EE** (2015). Crystal structure of the human cytomegalovirus glycoprotein B. PLOS Pathog. 11:e1005227. PMCID: PMC4617298.

Ongoing Research Support

R01 AI164698 Heldwein (PI) 06/08/2021-05/31/2026
NIH/NIAID
Title: Structure, antigenicity, and function of HCMV fusogen gB

The goal of this proposal is to stabilize glycoprotein gB – a molecular machine that enables HCMV entry and spread – in its biologically-active, prefusion form, characterize its structure and antigenicity, and isolate prefusion-specific neutralizing antibodies from HCMV-seropositive donors.

R01 AI147625 Heldwein (PI) 06/05/2019-05/31/2024
NIH/NIAID
Biophysical and structural analysis of the herpesviral nuclear budding machinery
The goal of this project is to systematically dissect the NEC-mediated formation of the negative membrane by characterizing essential protein/protein and protein/membrane interactions, budding intermediates, and regulatory inputs.

R21 AI160821 Heldwein (PI) 03/17/2021-02/28/2023
NIH/NIAID
In-vitro analysis of HSV-1 membrane fusion mechanism
The goal of this exploratory proposal is to reconstitute HSV-1 fusion *in vitro* and to characterize it at a single-virion level by imaging fusion of individual virions with fluid, supported lipid bilayers using total internal reflection microscopy.

R21 AI145272A1 Heldwein (PI) 09/03/2020-08/31/2022
NIH/NIAID
Single-particle analysis of HSV-1 membrane fusion mechanism
The goal of this exploratory proposal is to develop single-particle imaging of HSV-1 fusion with supported lipid bilayers to visualize different stages in fusion, measure their kinetic parameters, identify kinetic intermediates, and correlate them with structural rearrangements in gB.

HHMI Faculty Scholar, #55108533 Heldwein (PI) 11/01/2016-10/31/2022 (NCE)
Howard Hughes Medical Institute
Molecular Mechanisms of host manipulation by herpesviruses
The goal of this project is to dissect the mechanisms of herpesvirus entry and membrane fusion.
The HHMI Faculty Scholar Award is a 5-year grant with a $150,000/year budget.

Pew Innovation Fund, #34472 Heldwein (PI) 11/01/2020-10/30/2022
The Pew Charitable Trusts
Blasting a new window into “the great nuclear escape” of herpesviruses using cryo-FIB/ET
The goal of this project is to apply cryo-FIB/ET to visualize the nuclear egress process in intact cells infected with a prototypical Herpes Simplex virus (HSV)

B. Positions and Honors

Positions and Employment

2018-present	American Cancer Society (Massachusetts Division) Professor of Molecular Biology, Tufts University School of Medicine, Dept. of Molecular Biology and Microbiology, Boston, MA
2017-present	Professor, Tufts University School of Medicine, Dept. of Molecular Biology and Microbiology, Boston, MA
2011 - 2017	Associate Professor, Tufts University School of Medicine, Dept. of Molecular Biology and Microbiology, Boston, MA
2011 - 2013	Associate Professor, Tufts University School of Medicine
2006 - 2011	Assistant Professor, Tufts University School of Medicine, Dept. of Molecular Biology and Microbiology, Boston, MA
2004 - 2006	Instructor, Harvard Medical School
2000 - 2004	Postdoctoral Fellow, Harvard Medical School, HHMI, Children's Hospital, Boston, MA
1999 - 2000	Postdoctoral Fellow (AHA), Oregon Health & Science University, Dept. of Biochemistry and Molecular Biology, Portland, OR
1994 - 1999	Graduate Research Assistant, Oregon Health & Science University, Lab of Richard Brennan, Dept. of Biochemistry and Molecular Biology, Portland, OR

Other Experience and Professional Memberships

2022-present	Editorial Board Member, MDPI Viruses
2020-2024	Committee member, Editorial Committee of the Annual Review of Virology
2019-2022	Member, American Society for Microbiology Basic Research Awards Selection Committee

2018-present	Member, American Society for Cell Biology
2018-present	Member, American Society for Biochemistry and Molecular Biology
2017-present	Member, American Society for the Advancement of Science
2017-present	Director, Graduate Program in Molecular Microbiology, Graduate School of Biomedical Sciences, Tufts University School of Medicine, Boston, MA
2016-2022	Review panel member, VIRA Study Section
2016-present	Editorial Board Member, Virology
2016-present	Editorial Board Member, Journal of Virology
2015-present	Guest Associate Editor, PLOS Pathogens
2014-2015	Ad hoc reviewer, Medical Research Council (UK), LSU COBRE Pilot grant program, NSF
2013-2016	Ad hoc reviewer, NIH, Study Sections ZRG1 IDM S, VIRA, and BBM
2012	Review panel member, Tufts CTSI Pilot Studies Program
2010	Review panel member, Russo Family Charitable Foundation Trust
2010	Ad hoc reviewer, Israel Science Foundation
2009-2012	Review panel member, Charlton Fund Research Awards
2006-present	Reviewer for Advances in Anatomy, Cell, Cell Reports, Current Cancer Drug Target, Current Opinion in Structural Biology, EMBO Journal, FEBS Journal, Journal of Morphology, Journal of Virology, mBio, Nature Communications, Nature Reviews Microbiology, Nucleic Acids Research, Oncotarget, PNAS, PLOS One, PLOS Pathogens, Science, Scientific Reports, Structure, Trends in Microbiology, Virology, Virology Journal, Viruses
2008-present	Member, American Society for Microbiology
2004-present	Member, American Society for Virology

Honors

2021	Semi-finalist in the 2021 HHMI Investigator Competition
2020-2021	Top preclerkship lecturer, Tufts University School of Medicine
2019	Fellow of the American Academy of Microbiology
2016-2017	Outstanding lecturer, Tufts University School of Medicine
2017	Dean's Award for Excellence in Basic Science Teaching, Tufts University School of Dental Medicine
2016	HHMI Faculty Scholar
2015-2016	Outstanding lecturer, Tufts University School of Medicine
2014	Priscilla Schaffer lecturer, 39th Annual International Herpesvirus Workshop
2014	Milton O. and Natalie V. Zucker Prize, Tufts University School of Medicine
2011	Finalist of the Vilcek Prize for the Creative Promise in Biomedical Sciences, The Vilcek Foundation
2011	Investigators in the Pathogenesis of Infectious Disease Award, Burroughs Welcome Fund
2010	Digital LabLife Award (awarded to the Heldwein Laboratory), Lablife
2010	Merck Irving S Sigal Award, American Society of Microbiology
2008	Young Investigator Award, ICAAC
2007	Pew Biomedical Scholar, The Pew Charitable Trust
2007	New Innovator Award, National Institutes of Health
2007	Zucker Center Award, Tufts University School of Medicine
1999	Postdoctoral Fellowship, American Heart Association (AHA)
1998	Best Speaker Award, Oregon Health & Science University Student Research Forum
1997	Dean's Student Travel Award, Oregon Health & Science University
1996	Fellowship, Oregon Health & Science University Tartar Trust
1994	Red Diplom, for undergraduate academic excellence, Lomonosov Moscow State University

C. Contributions to Science

1. Structural studies of the conserved herpesvirus fusogens, gB and gH/gL.

We have determined the structures of conserved components of the herpesvirus cell entry machinery, gB and gH/gL, from Herpes Simplex Virus (HSV). The dogma was that both were non-redundant mediators of membrane fusion, in contrast to single-protein fusion mechanisms of other enveloped viruses. On the basis of structures, we identified gB as "the business end" – the fusogen – and gH/gL as a uniquely folded regulator. This changed the existing paradigm of herpesviruses entry and established a new roadmap that has enabled more mechanistic exploration of herpesvirus entry. We have also determined the structure of a gB homolog from human cytomegalovirus, which illustrated the structural plasticity of gB that may accommodate virus-specific functional requirements and showed how extensive glycosylation of the gB ectodomain influences antibody recognition. More recently, we determined the structure of full-length HSV-1 gB, which revealed the structural basis of membrane anchoring and fusion regulation.

a. **Heldwein EE**, Lou H, Bender FC, Cohen GH, Eisenberg RJ, and Harrison SC (2006). Crystal structure of glycoprotein B from Herpes Simplex Virus 1. Science 313, 217-220. (Cited over 350 times).
b. Chowdary TK, Cairns TM, Atanasiu D, Cohen GH, Eisenberg RJ, and **Heldwein EE** (2010). Crystal structure of the conserved herpesvirus fusion regulator complex gH–gL. Nat. Struct. Mol. Biol. 17, 882-888. PMCID: PMC2921994. (Cited over 150 times).
c. Burke HG and **Heldwein EE** (2015). Crystal structure of the human cytomegalovirus glycoprotein B. PLOS Pathog. 11:e1005227. PMCID: PMC4617298.
d. Cooper RC, Georgieva ER, Borbat PP, Freed JH, and **Heldwein EE** (2018). Structural basis for membrane anchoring and fusion regulation of the Herpes Simplex Virus fusogen gB. Nat. Struct. Mol. Biol. 25, 416-424. PMCID: PMC5942590.

2. Contributions of the cytoplasmic tails of gB and gH to membrane fusion.
The fusogenic activity of the conserved herpesvirus fusogen gB is negatively regulated by its cytoplasmic domain (CTD). But how the CTD can regulate the function of the ectodomain on the opposite side of the membrane was unclear. We showed that the HSV-1 gB CTD achieves its fully folded conformation only in the presence of membrane and discovered that hyperfusogenic mutations perturbed the native membrane-dependent conformation of the CTD. We also showed that the cytoplasmic tail (CT) of HSV-1 gH positively regulates fusion from the opposite side of the membrane and discovered an interplay between the HSV-1 gB CTD and the gH CT, which lead us to propose the novel "clamp-and-wedge" model of fusion regulation.
The fusogenic activity of the conserved herpesvirus fusogen gB is negatively regulated by its cytoplasmic domain (CTD). But how the CTD can regulate the function of the ectodomain on the opposite side of the membrane was unclear. We showed that the HSV-1 gB CTD achieves its fully folded conformation only in the presence of membrane and discovered that hyperfusogenic mutations perturbed the native membrane-dependent conformation of the CTD. We also showed that the cytoplasmic tail (CT) of HSV-1 gH positively regulates fusion from the opposite side of the membrane and discovered an interplay between the HSV-1 gB CTD and the gH CT, which lead us to propose the novel "clamp-and-wedge" model of fusion regulation.

a. Chowdary TK and **Heldwein EE** (2010). Syncytial phenotype of HSV-1 gB is associated with diminished membrane interactions. J. Virol. 84, 4923-4935. PMCID: PMC2863819.
b. Silverman JL, Greene NG, King DS, and **Heldwein EE** (2012). Membrane requirement for the folding of the HSV-1 gB cytodomain suggests a unique mechanism of fusion regulation. J. Virol. 86, 8171-8184. PMCID: PMC3421659.
c. Rogalin HB and **Heldwein EE** (2015). The interplay between the HSV-1 gB cytodomain and the gH cytotail during cell-cell fusion. J. Virol. 89:12262–12272. PMCID: PMC4665236.
d. Pataki Z, Sanders EK, and **Heldwein EE** (2022). A surface pocket in the cytoplasmic domain of the herpes simplex virus fusogen gB controls membrane fusion. PLOS Pathog. 18:e1010435. PMCID: PMC9275723.

3. Use of VSV pseudotyped with HSV entry glycoproteins to elucidate HSV entry mechanism.
HSV requires at least four entry glycoproteins, gB, gD, gH, and gL, for entry into target cells in addition to a cellular receptor for gD, which makes it more complex than entry of most enveloped viruses. But, the dissection of the HSV-1 entry mechanism is further complicated by the presence of over a dozen proteins on the viral envelope. To investigate HSV-1 entry requirements in a simplified system, we generated VSV virions pseudotyped with HSV-1 essential entry glycoproteins gB, gD, gH, and gL but lacking the native VSV fusogen G. These VSVΔG-BHLD virions infected a cell line expressing a gD receptor, demonstrating for the first time that the four essential entry glycoproteins of HSV-1 are not only required but also sufficient for cell entry. This was the first time the VSV pseudotyping system was successfully extended beyond two proteins. Entry of pseudotyped virions required a gD receptor and was inhibited by anti-HSV-1 neutralizing antibodies, which suggests that membrane fusion during the entry of the pseudotyped virions shares common requirements with

the membrane fusion involved in HSV-1 entry. The HSV pseudotyping system presents a novel platform for systematic exploration of the HSV entry and membrane fusion mechanisms.

a. Rogalin HB and **Heldwein EE** (2016). Characterization of VSV pseudotypes bearing essential entry glycoproteins gB, gD, gH, and gL of Herpes Simplex virus Type 1. J. Virol. 90:10321-10328. PMCID: PMC5105666.
b. Hilterbrand AT, Daly RE and **Heldwein EE** (2021). Contributions of the four essential entry glycoproteins to HSV-1 tropism and the selection of entry routes. mBio. 12:e00143-21. PMCID: PMC8092210.

4. Structural basis of herpesvirus-mediated nuclear budding.

We discovered that the herpesvirus nuclear egress complex (NEC), essential for the exit of nascent capsids from the nucleus, has an intrinsic ability to mediate budding and scission, which we showed by reconstituting the budding process in vitro using purified NEC and synthetic liposomes. We further showed that the NEC forms hexagonal coats on the inner surface of the budded vesicles, resolved the interactions forming the NEC coat at the atomic level, and proposed that oligomerization of the NEC drives the budding process. These results showed for the first time that the NEC is a complete virus-encoded membrane-budding machinery that operates by a novel mechanism. In addition, the NEC is the only viral or cellular machinery currently known to mediate budding of the nuclear, as opposed to cytoplasmic, membranes. These exciting results have transformed the way we think about how herpesviruses manipulate host membranes.

a. Bigalke JM, Heuser T, Nicastro D, and **Heldwein EE** (2014). Membrane deformation and scission by the HSV-1 nuclear egress complex. Nat. Commun. 5, 4131. PMCID: PMC4105210. (This article was highlighted in the Editors' Choice section of Science 345: 6193).
b. Draganova EB, Zhang J, Zhou ZH, and **Heldwein EE** (2020). Structural basis for capsid recruitment and coat formation during HSV-1 nuclear egress. eLife. 9:e56627. PMCID: PMC7340501.
c. Thorsen MK, Lai A, Lee MW, Hoogerheide DP, Wong GCL, Freed JH, and **Heldwein EE** (2021). Highly basic clusters in the HSV-1 nuclear egress complex drive membrane budding by inducing lipid ordering. mBio. 12:e0154821. PMCID: PMC8406295.
d. Thorsen MK, Draganova EB, and **Heldwein EE** (2022). The nuclear egress complex of Epstein-Barr virus buds membranes through an oligomerization-driven mechanism. **PLOS Pathog.** 18:e1010623. PMCID: PMC9299292.

5. Structural studies of herpesvirus tegument proteins.

The tegument layer of herpesviruses contains over a dozen proteins that play important structural roles in virion morphogenesis as well as facilitate early stages of viral replication. To provide a blueprint for mechanistic exploration of herpesvirus tegument proteins, we determined the crystal structures of PRV UL37 and HSV-1 UL21. UL37 is conserved among all 3 subfamilies of herpesviruses and is thought to control the intracellular trafficking of the viral capsids. The structure of UL37 revealed an unanticipated similarity to the components of eukaryotic multi-subunit tethering complexes (MTCs), which direct vesicular trafficking in cells by tethering vesicles to their target organelles, which pinpoints UL37 as the first viral MTC component. This novel hypothesis is framing our current efforts toward a full understanding of the mechanisms through which UL37 controls intracellular traffic in infected cells, including neurons, in culture and within infected animals. UL21 is conserved among alphaherpesviruses and has been implicated in cytoplasmic budding, intracellular trafficking, and nuclear egress. The crystal structure of HSV-1 UL21 revealed a novel protein fold along with several surface regions of potential functional importance for targeting by mutagenesis.

a. Metrick CM and **Heldwein EE** (2016). The novel structure and unexpected RNA-binding ability of the C-terminal domain of HSV-1 tegument protein UL21. J. Virol. 90:5759-5769. PMCID: PMC4886797. (This article was spotlighted in this issue of J. Virol.).
b. Koenigsberg AL and **Heldwein EE** (2017). Crystal structure of the N-terminal half of the traffic controller UL37 from Herpes Simplex virus Type 1. J. Virol. 91:e01244-17. PMCID: PMC5625493.
c. Koenigsberg AL and **Heldwein EE** (2018). The dynamic nature of the conserved tegument protein UL37 of herpesviruses. J. Biol. Chem. 293, 15827-15839. PMCID: PMC6187633.
d. Metrick CM, Koenigsberg AL, and **Heldwein EE** (2020). Conserved outer-tegument component UL11 from herpes simplex virus type 1 is an intrinsically disordered, RNA-binding protein. mBio. 11:e00810-20. PMCID: PMC7403781.

Complete List of Published Work in My Bibliography:
http://www.ncbi.nlm.nih.gov/myncbi/ekaterina.heldwein.1/bibliography/40887489/public/?sort=date&direction=ascending

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. DO NOT EXCEED FIVE PAGES.

NAME: Gonzalez-Del Pino, Gonzalo Luis

eRA COMMONS USER NAME (credential, e.g., agency login): GONZALO

POSITION TITLE: IRACDA Postdoctoral Scholar

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE (if applicable)	END DATE MM/YYYY	FIELD OF STUDY
University of Miami	BS	05/2012	Biochemistry and Molecular Biology
University of Miami, Miami, Florida	BA	05/2012	French
Harvard University	MA	06/2015	Molecular and Cellular Biology
Harvard University, Cambridge, Massachusetts	PHD	05/2020	Biochemistry and Molecular Biology
Tufts University School of Medicine, Boston, Massachusetts	Postdoctoral Fellow	present	Herpesvirus glycoprotein structural biology

A. Personal Statement

For the entirety of my scientific career my interests have centered on the form and function of molecular machines. From my first research experience in high school through the end of my doctoral work, I found myself specifically attracted to cancer research, because one of the clearest ways of discovering how a machine works is studying what happens when it is broken – in this case by mutations in the gene that codes for it. Throughout my PhD in Professor Michael Eck's group, I focused on the protein machinery responsible for translating messages from the rest of the body into a language that cells can interpret and act on. Although I worked on projects characterizing epidermal growth factor receptor signal transduction across the plasma membrane and how adaptor proteins mediate receptor interactions with downstream signaling cascades, my thesis and published work in Nature (6th author) and PNAS (first co-first author) focused on newly solved structures of the mitogen activated protein kinase (MAPK) complexes responsible for relaying these signals within the cell. My structural and biochemical characterization of these kinase complexes extended our knowledge of basic biological processes, what goes wrong when mutations perturb these processes, and how we can better design drugs to combat signal dysregulation diseases. For my postdoc, I have transitioned to structural and functional characterization of herpesvirus surface glycoproteins necessary for viral entry into host cells. I specifically work on two machines essential to host entry: glycoprotein B (gB), the viral fusogen, and gH/gL, a heterodimeric regulator of the fusion process. Viral proteins are fascinating to me because viruses are under such tremendous selection and drift pressure that their genome sequences churn rapidly, but successful tertiary protein structures remain relatively stable: the conservation of form of herpesvirus gB and gH/gL machines is a perfect example of this phenomenon. In Professor Katya Heldwein's lab at Tufts University School of Medicine, I have used the structural and biochemical expertise I developed during my PhD to solve structures of Herpes Simplex Virus (HSV) entry glycoproteins in complex with neutralizing fragments of antibody binding (Fabs) and use this information to better understand the mechanisms of membrane fusion processes. As a trained X-ray crystallographer, I obtained X-ray diffraction data for two HSV gH/gL-Fab complexes. These datasets, however, have not yielded molecular replacement models for reasons we cannot yet explain. Given these difficulties, I established a long-term collaboration with Dr. Richard Walsh, a senior scientist at the Harvard Cryoelectron Microscopy Center. Using my Institutional Research and Career Development Award (IRACDA) funding, I learned how to prepare samples and collect and analyze data for one of my gH/gL-

Fab complexes. Within six months of beginning these efforts, I have generated a sub-8Å electron density map that continues to improve. Obtaining data collection time at the New York Structural Biology Center's NCCAT facility is crucial for me to finish solving structures of the remaining four gH/gL-Fab complexes to further understand how gH/gL activates gB to effect membrane fusion.

1. Gonzalez-Del Pino GL, Heldwein EE. Well Put Together-A Guide to Accessorizing with the Herpesvirus gH/gL Complexes. Viruses. 2022 Jan 30;14(2) PubMed Central PMCID: PMC8874593.
2. Gonzalez-Del Pino GL, Li K, Park E, Schmoker AM, Ha BH, Eck MJ. Allosteric MEK inhibitors act on BRAF/MEK complexes to block MEK activation. Proc Natl Acad Sci U S A. 2021 Sep 7;118(36) PubMed Central PMCID: PMC8433572.
3. Park E, Rawson S, Li K, Kim BW, Ficarro SB, Pino GG, Sharif H, Marto JA, Jeon H, Eck MJ. Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. Nature. 2019 Nov;575(7783):545-550. PubMed Central PMCID: PMC7014971.

B. Positions, Scientific Appointments and Honors

Positions and Scientific Appointments

2020 -	IRACDA Postdoctoral Scholar, Tufts University School of Medicine, Dr. Katya Heldwein, Boston, MA
2014 - 2020	Graduate Research Assistant, Harvard University, Dr. Michael Eck, Boston, MA
2012 - 2013	Postbaccalaureate Scholar, University of Miami School of Medicine, Dr. Ralf Landgraf, Miami, FL
2009 - 2012	Undergraduate Research Assistant, University of Miami School of Medicine, Dr. Ralf Landgraf, Miami, FL
2008 - 2009	Undergraduate Research Assistant, Johns Hopkins University, Dr. Richard McCarty, Baltimore, MD
2006 - 2007	High School Research Assistant, University of Miami School of Medicine, Dr. Bob Hsia, Miami, FL

Honors

2013 - 2020	Harvard Graduate Prize Fellowship, Harvard University
2018 - 2019	Harvard Bok Center Certificate of Teaching Excellence, Harvard University
2012 - 2013	NIH Research Supplement for Post-baccalaureate Research, NIH-NCI
2020	NIH K12 Institutional Research and Career Development Award, NIH and Tufts University
2017	Harvard BCMP Departmental Retreat Poster Prize, Harvard University
2016	Harvard Bok Center Certificate of Teaching Excellence, Harvard University
2014	Harvard Bok Center Certificate of Teaching Excellence, Harvard University
2012	Dean's List, University of Miami
2012	Ann Colbert Memorial Award for Excellence in French, University of Miami
2011	Provost's Honor Roll, University of Miami
2011	STEP-UP Summer Research Program, NIH-NIDDK
2010	Dean's List, University of Miami
2010	STEP-UP Summer Research Program, NIH-NIDDK

C. Contribution to Science

1. Structural and mechanistic analysis of the regulation and pharmacology of BRAF and MEK1

   My graduate work focused on the mitogen-activated protein kinase (MAPK) cascade. This pathway is currently represented in textbook diagrams as a linear sequence of activation events – often the transfer of a phosphate from ATP to a downstream kinase – which turn on each subsequent protein in

the series. We made a discovery that changes the way we see and potentially treat dysregulation of the MAPK pathway. Using a combination of cryo-electron microscopy and X-ray crystallography, we determined the structure of an inactive BRAF:MEK1 complex bound to a 14-3-3 dimer. In a recent Nature article, we proposed a model in which the MAPK pathway is tightly regulated in healthy cells by the mutual autoinhibition of the RAF and MEK kinases in this complex. Crystal structures of the BRAF:MEK1 kinase module I solved in collaboration with Dr. Kunhua Li afforded us a higher-resolution view of the amino acid residue-level interactions that enforce the “off” state of this kinase dimer. This has further informed how oncogenic mutations can destabilize this inactive complex. For example, the most common oncogenic mutation in the MAPK pathway introduces an acidic residue in BRAF that disrupts a van der Waals network keeping an alpha-helix from swinging inward and completing the catalytic site. We have also solved the structures of the BRAF:MEK1 kinase dimer in complex with a panel of eight clinical-stage and preclinical MEK inhibitors. These structures, combined with a thorough biochemical characterization of the complex, suggest that the actual pharmacological target of MEK inhibitors is not simply the MEK kinase freely diffusing in the cytoplasm. Binding and activity experiments paint a more complex picture, where different MEK inhibitors act on the MAPK pathway at distinct stages: some inhibitors prevent phosphorylation of MEK by RAF proteins, others prevent the release of phosphorylated MEK from the BRAF-chaperone complex, while yet another class of inhibitors prevents active MEK from phosphorylating ERK. These insights into the regulation and dysregulation of the MAPK pathway highlight the importance of understanding proteins in a relevant molecular context rather than as linear circuits. Contrary to how most signal transduction diagrams portray them, cellular pathways are not simple cascades where A activates B, which activates C, and so forth. The wealth of knowledge unlocked by structural studies can be leveraged to design more effective anticancer drugs: now that we know that MEK by itself is not the only pharmacological target of MEK inhibitors, we can rationally improve on their initially serendipitous mechanisms of action for the treatment of disease. The results of our coupled BRAF:MEK:ERK activity assays and the BLI-based binding assays may suggest more effective adjuvant therapy combinations. Currently, vemurafenib and cobimetinib, as well as dabrafenib and trametinib, are being used in tandem to treat melanoma. Combining MEK and RAF inhibitors that both increase the affinity of MEK for BRAF would seem to be more effective at shutting down aberrant ERK signaling than treating patients with inhibitors that work at cross purposes to each other. Furthermore, the discovery of the autoinhibited BRAF:MEK1 complex provides a new way of inhibiting the MAPK pathway at the top of the cascade: “gluing” MEK to BRAF, either by using a PROTAC-based strategy or by extending a covalent warhead from the MEK allosteric site to ARAF, BRAF, or CRAF. This approach demonstrates that understanding how a machine’s form dictates its function can be directly applied to improving human health.

a. Gonzalez-Del Pino GL, Li K, Park E, Schmoker AM, Ha BH, Eck MJ. Allosteric MEK inhibitors act on BRAF/MEK complexes to block MEK activation. Proc Natl Acad Sci U S A. 2021 Sep 7;118(36) PubMed Central PMCID: PMC8433572.
b. Park E, Rawson S, Li K, Kim BW, Ficarro SB, Pino GG, Sharif H, Marto JA, Jeon H, Eck MJ. Architecture of autoinhibited and active BRAF-MEK1-14-3-3 complexes. Nature. 2019 Nov;575(7783):545-550. PubMed Central PMCID: PMC7014971.

2. Structural and mechanistic analysis of HSV gH/gL, an essential membrane fusion regulator

Herpesviruses infect >90% of the human population for life. Herpes Simples Viruses (HSV) cause oral and genital mucocutaneus lesions and can sometimes cause severe encephalitis in young or immunocompromised patients. There are three surface glycoproteins required for host cell entry across all herpesviruses: gB, the viral fusogen, and gH/gL, a heterodimeric machine whose function has not been fully elucidated. This protein takes cues from a variety of viral and host cell inputs and somehow engages and activates gB. Elucidating this process will facilitate development of prophylactic and reactive treatments by targeting a conserved and essential step of herpesvirus entry. Although my work on this is currently ongoing, Professor Heldwein and I have published a review

compiling all current structural information of human herpesvirus gH/gL and its complexes with other viral glycoproteins, host cell receptors, and neutralizing antibodies.

a. Gonzalez-Del Pino GL, Heldwein EE. Well Put Together-A Guide to Accessorizing with the Herpesvirus gH/gL Complexes. Viruses. 2022 Jan 30;14(2) PubMed Central PMCID: PMC8874593.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Walsh Jr., Richard Michael

eRA COMMONS USER NAME (credential, e.g., agency login): RWALS2

POSITION TITLE: Senior Cryo-EM Scientist

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Georgia, Athens, GA	B.S.	05/2011	Biochemistry and Molecular Biology
University of Texas Southwestern Medical Center, Dallas, TX	Ph.D.	08/2018	Molecular Biophysics/ Neuroscience

A. Personal Statement

I am currently a Senior Cryo-EM Scientist in the newly established Harvard Cryo-Electron Microscopy Center for Structural Biology at Harvard Medical School (HMS) under the directorship of Dr. Stephen Harrison. My expertise covers all aspects of single particle cryo-electron microscopy (cryo-EM), including structure determination and refinement[a-d]. During my graduate work, in the lab of Ryan E. Hibbs at The University of Texas Southwestern Medical Center (UTSW), I pioneered efforts to establish cryo-EM in the Hibbs laboratory, training in single-particle cryo-EM under Dr. Wah Chiu at Baylor College of Medicine in Houston, TX and subsequently training in the new state-of-the-art facility at UTSW under the directorship of Dr. Daniela Nicastro. The course of this effort led to a publication in *Nature* revealing principles governing subunit assembly of the two stoichiometries of the α4β2 nicotinic receptor, the principal pharmacological target of nicotine and a key driver in nicotine addiction, and structural differences in nicotine binding at high and low affinity binding sites[a]. My subsequent work, both in graduate school and now here at Harvard, has led to a number of other important discoveries, including the structural basis of how intravenous anaesthetics and benzodiazepines modulate $GABA_A$ receptor signaling using overlapping mechanisms [c], the mechanism of reversal by $GABA_A$ receptor antagonists such as flumazenil[b-c] and structural differences between the full length SARS-CoV-2 spike protein and the solubilized ectodomain that could have important consequences for vaccine design[d].

My collaboration with the Heldwein lab serves to efficiently determine structures of important herpesvirus glycoproteins and to provide a path to independence in cryo-electron microscopy through mentorship and training. The long history of the Heldwein lab in utilizing X-ray crystallography, which has more demanding requirements for protein homogeneity and stability, has made pivoting to sample preparation for cryo-EM experiments a straightforward endeavor. Over the past six months I have worked closely with Dr. Gonzalo Gonzalez-Del Pino to assist, advise and train him in all aspects of structure determination by cryoEM, from the optimization of sample preparation to data collection, processing, reconstruction, and structure refinement. These efforts have thus far produced a 7Å reconstruction of the ~185 kDa Herpes Simplex Virus gH/gL complexed with a neutralizing antibody fragment. My strong background in cryo-EM, in addition to passion for training the next generation of microscopists, makes me a well-suited candidate to continue to support the technical and scientific demands in this proposal.

a. **Walsh, R.M. Jr.**#, Roh S.H.#, Gharpure, A., Morales-Perez, C.L., Teng, J. and Hibbs, R.E. "Structural principles of distinct assemblies of the human α4β2 nicotinic receptor." Nature 2018: 557(7704):261- 265. PMCID: PMC6132059 #Equal contributions
b. Zhu, S., Noviello, C.M., Teng, J., **Walsh, R.M. Jr.**, Kim, J.J. and Hibbs, R.E. "Structure of a human synaptic GABAA receptor." Nature 2018: 559(7712):67-72. PMCID: PMC6220708
a. Kim, J.J., Gharpure, A., Teng, J., Zhuang, Y., Howard, R.J., Zhu, S., Noviello, C.M., **Walsh, R.M. Jr.**, Lindahl, E. and Hibbs, R.E. "Shared structural mechanisms of general anesthetics and benzodiazepines." Nature 2020: 585(7824):303-308. PMCID: PMC7486282
c. Cai, Y., Zhang, J., Xiao, T., Peng, H., Sterling, S.M., **Walsh, R.M. Jr.**, Rawson, S., Rits-Volloch, S., Chen, B. "Distinct conformational states of SARS-CoV-2 spike protein." Science 2020: 25;369(6511):1586-1592. PMCID:PMC7464562

B. Positions, Scientific Appointments, and Honors

Positions and Employment

2010 – 2011	Undergraduate Research Assistant, University of Georgia, Biochemistry and Molecular Biology, Athens, GA
2011 – 2013	Research Technician, University of Georgia, Biochemistry and Molecular Biology, Athens, GA
2013 – 2018	Graduate Research Assistant, University of Texas Southwestern Medical School, Biophysics, Dallas, TX
2018 -	Cryo-EM Specialist/ Senior Scientist, Harvard Medical School, Biological Chemistry and Molecular Pharmacology, Boston, MA

Other Experience and Professional Memberships

Honors

2011	B.S. awarded with *magna cum laude*, University of Georgia
2014-2015	Cell and Molecular Biology T32 Training Grant
2015-2018	Sara and Frank McKnight Predoctoral Fellowship
2016	Graduate Student Organization at UT Southwestern Travel Award
2018	Best Oral Presentation, UT Southwestern Biophysics Program Department Retreat

C. Contributions to Science

A full list of my publications can be found in PubMed at:
https://www.ncbi.nlm.nih.gov/myncbi/richard.walsh.1/bibliography/public/

1) Neurotransmitter gated ion channel structure and function

Nicotinic acetylcholine receptors are pentameric ligand-gated cation channels. These neurotransmitter-gated cation channels facilitate excitatory neurotransmission in the central and peripheral nervous systems. Nicotinic receptors are intricately involved in learning and memory, reward, sensory processing, pain and neuroprotection. Dysregulation of these receptors is linked to neurodegenerative diseases and mental illnesses, including epilepsy, Alzheimer's disease, Parkinson's disease and schizophrenia. Through my dissertation work, in the lab of Ryan E. Hibbs, I utilized cryo-EM to investigate the structural properties of nicotinic receptors. I developed optimized sample preparation procedures to obtain high densities of receptor molecules in random orientations over the sample holes of a cryo-EM grid. I also developed a Fab labeling strategy to facilitate the determination of both structures of the α4β2 nicotinic receptor from a mixed population. This strategy simultaneously overcame problems imposed by compositionally heterogeneous samples and the pseudo-symmetric nature of heteromeric proteins. This allowed me to determine the high-resolution structures of both physiologically relevant stoichiometries of the α4β2 nicotinic receptor from a single sample. Comparison of these structures revealed principles governing subunit assembly and why there are only two

possible arrangements of α4 and β2 subunits, structural features that govern ion conductance and permeation properties, differences in agonist binding at high and low sensitivity binding sites and putative cholesterol binding sites. This work provides a foundation to further design and refine future treatments for neurodegenerative diseases and mental illnesses, and more broadly, a general approach for resolving the structural biology of heterogeneous assemblies[e]. I subsequently applied this technical cryo-EM expertise developed working on the α4β2 nicotinic receptor to the determination of the structure of the α3β4 nicotinic receptor and the α1β2γ2 $GABA_A$ receptor[f-g]. The α3β4 nicotinic receptor study revealed that a less compact neurotransmitter binding site contributes to agonist selectivity and lower affinity for ligand of the α3β4 receptor compared to other nicotinic receptor subtypes[g]. Studies of the α1β2γ2 $GABA_A$ receptor revealed the structural basis of how intravenous anaesthetics and benzodiazepines modulate $GABA_A$ receptor signaling using overlapping mechanisms[h] and the mechanism of reversal by $GABA_A$ receptor antagonists such as flumazenil[f,h]. My involvement in the microscopy of α1β2γ2 $GABA_A$ receptor has continued as part of an ongoing collaboration in my new role as Cryo-EM Specialist[h].

b. **Walsh, R.M. Jr.**#, Roh S.H.#, Gharpure, A., Morales-Perez, C.L., Teng, J. and Hibbs, R.E. "Structural principles of distinct assemblies of the human α4β2 nicotinic receptor." Nature 2018: 557(7704):261- 265. PMCID: PMC6132059 #Equal contributions
c. Zhu, S., Noviello, C.M., Teng, J., **Walsh, R.M. Jr.**, Kim, J.J. and Hibbs, R.E. "Structure of a human synaptic GABAA receptor." Nature 2018: 559(7712):67-72. PMCID: PMC6220708
d. Gharpure, A., Teng, J., Zhuang, Y., Noviello, C.M., **Walsh, R.M. Jr**., Cabuco, R., Howard, R.J., Zaveri, N.T., Lindahl, E. and Hibbs, R.E. "Agonist selectivity and ion permeation in the α3β4 ganglionic nicotinic receptor." Neuron 2019: 104(3):501-511. PMCID: PMC6842111
e. Kim, J.J., Gharpure, A., Teng, J., Zhuang, Y., Howard, R.J., Zhu, S., Noviello, C.M., **Walsh, R.M. Jr.**, Lindahl, E. and Hibbs, R.E. "Shared structural mechanisms of general anesthetics and benzodiazepines." Nature 2020: 585(7824):303-308. PMCID: PMC7486282

2) Structural and Functional Analysis of Proteasome Core Particle Biogenesis.

In collaboration with John Hanna's group at Brigham Women's Hospital, we have developed and optimized a robust framework for structurally investigating the maturation process of the proteasome 20S core particle (CP): by pairing Cryo-EM with a novel strategy for enriching proteasome core particle assembly intermediates, which are normally fleeting in abundance. My role has been in guiding biochemical preparation to optimize cryosample preparation, preparing the cryo-EM samples, collecting data, and building and refining the atomic models. Thus far we have structurally characterized the 13S (3.6 Å) and pre-15S (3.2 Å) proteasome assembly intermediates[a]. The long-term goal of this work is to generate a comprehensive structural atlas of the pathway of the Proteasome core particle assembly, something which would represent a major contribution to cell biology.

a. **Walsh, R.M. Jr.**#, Rawson, S.,#, Schnell, H.M.#, Kaur, M., Bhanu, M.K., Tian, G., Prado, M.A., Guerra-Moreno, A., Paulo, J.A., Gygi, S.P., Roelofs, J., Finley, D. Hanna, J. "Structures of chaperone associated assembly intermediates reveal coordinated mechanisms of proteasome biogenesis." Nature Structural & Molecular Biology 2021. PMCID: PMC8160580 #Equal contributions

C3) Structure, evolution and activity in nucleotide-sugar modifying enzymes

Covalent attachment of xylose to a serine hydroxyl is the first step in the production of most proteoglycans, a core structural component of connective tissues such as the extracellular matrix, cartilage and bone. Since disruptions in proteoglycan biosynthesis can attenuate tumor growth and progression; understanding the mechanism and regulation of the enzyme UDP-xylose synthase (UXS) which produces the sugar substrate UDP-xylose, is a biomedically relevant goal. In the Laboratory of Zachary A. Wood, I led the study on the structural investigation of a mutant UXS that disrupts proteoglycan biosynthesis, resulting in severe craniofacial defects[i]. Through structural and kinetic investigation, I determined that the displacement of the catalytic tyrosine prevented the final reduction step in the production of UDP-xylose. Pairing X-ray crystallography with sedimentation velocity experiments I demonstrated this mutation induces the formation of a new hexameric

species into the normal dimer-tetramer equilibrium. This hexameric species resembles distantly related bacterial enzymes, exposing the evolutionary transition from the oligomeric state of distantly related bacterial enzymes. I was a co-author on two other UXS studies[j-k] that identified: i) a bifurcated catalytic mechanism and ii) the formation of a tetramer that is important for activity. The identification of a bifurcated mechanism explained the long-standing observation that exogenous NAD+ stimulated UXS activity *in vitro*. We demonstrated that in addition to the productive catalytic path that retains oxidized NAD+ cofactor and releases the final product UDP-xylose, there is an abortive pathway wherein NADH and a ketone sugar intermediate are released leaving inactive apo enzyme. Thus, the previously observed stimulatory effect was from rescuing the inactive apo enzyme population. I performed kinetic analysis on the productive catalytic pathway and demonstrated that crowding conditions promoted formation of the tetrameric species; which has greatly enhanced catalytic activity and a reduced percentage of enzyme undergoing the abortive catalytic cycle. In another structure/function project, I performed analytical ultracentrifugation experiments to investigate the relationship between the allosteric mechanism and the oligomeric state of human UDP-glucose 6-dehydrogenase[l].

a. **Walsh, R.M. Jr**., Polizzi, S.J., Kadirvelraj, R., Howard, W.H., and Wood, Z.A. *Man o' War* Mutation in UDP-α-d-Xylose Synthase Favors the Abortive Catalytic Cycle and Uncovers a Latent Potential for Hexamer Formation. *Biochemistry* (2015) **54**, 3, p807.
b. Polizzi, S.J., **Walsh Jr., R.M.,** Peeples, W.B., Lim, J.M., Wells, L, and Wood, Z.A. Human UDP-α-D-xylose synthase and *E. coli* ArnA conserve a conformational shunt that controls whether xylose or 4-keto-xylose is produced. *Biochemistry* (2012) **51**, 44, p8844.
c. Polizzi, S.J., **Walsh, R.M. Jr.,** Le Magueres P., Criswell A.R. and Wood, Z.A. Human UDP-α-d-xylose Synthase Forms a Catalytically Important Tetramer That Has Not Been Observed in Crystal Structures. *Biochemistry* (2013) **52**, p3888
d. Kadirvelraj, R., Custer, G.C., Keul, N.C., Sennet, N.C., Sidlo, A.M., **Walsh, R.M. Jr.,** and Wood, Z.A. Hysteresis in Human UDP-Glucose Dehydrogenase Is Due to a Restrained Hexameric Structure That Favors Feedback Inhibition *Biochemistry* (2015) **53**, 51, p8043

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: KRASILNIKOV, MARIA

eRA COMMONS USER NAME (credential, e.g., agency login): MKRASILNIKOV

POSITION TITLE: Graduate Student

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of California Berkeley Berkeley, CA	BA	05/2019	Molecular and Cell Biology, Immunology and Pathogenesis
Tufts University, Boston, MA	PhD		Molecular Microbiology

A. Personal Statement

My interest in microbiology was first kindled during my second year of college when I joined Dr. Daniel Portnoy's lab at UC Berkeley. After joining, I was instantly enamored by all the research questions asked, and that members of the lab employed techniques from biochemistry, genetics, immunology, and even inorganic chemistry to answer them. This, in combination with my organic chemistry teaching experience, made me realize I wanted to pursue microbial biochemistry research in graduate school. Following the completion of my project in the Portnoy Lab, I put my scientific abilities to the test by teaching scientific capacity-building workshops at Makerere University in Kampala, Uganda with Berkeley's Center for Emerging and Neglected Diseases. My unique experience with Bay Area and Ugandan scientists fundamentally changed the way I view scientific communication and collaboration. I have always valued scientific collaboration, but after participating in capacity building workshops, I know first-hand how on both an international and individual level it has the power to propel science forward. After working at Makerere, I wanted to strengthen my scientific abilities by immersing myself in an unfamiliar field. Hoping to diversify my perspective as a microbiologist, I decided to increase my understanding of mammalian cell biology. This led me to Dr. Tien Peng's lab in the pulmonary division at UCSF. Starting in Dr. Peng's lab was challenging, not only because of the new set of techniques I had to learn, but also because I was exposed to an entirely foreign biological discipline. In the first year of my PhD, I joined Dr. Katya Heldwein's lab as it was a perfect match for me in terms of project and mentoring style. In the Heldwein Lab I have continued to expand on my scientific interests and have added virology and structural biology to my repertoire.

I am applying for experiment and microscope time at the New York NCCAT facility to advance my career as a scientist and achieve my long-term goal of running my own laboratory in an academic setting studying host-virus interactions. I believe my background and past experiences make me well-suited for this opportunity. First, my research and teaching experiences have given me a wide scientific background from cell biology to structural biology and have taught me how to approach scientific problems and find optimized solutions, a skill that has helped me design and troubleshoot experiments throughout my research career. Second, my past experiences in biomedical research laboratories have exposed me to a wide range of techniques, including techniques that I will directly use with this funding and resource support, such as transmission electron microscopy (EM), negative stain electron microscopy, and cryo-EM. By investigating the structure of codon-stabilized prefusion HCMV gB and its interaction with neutralizing antibodies, I can pursue my interest in host-pathogen interactions, while

learning electron microscopy, a new field to me. Finally, the Molecular Microbiology program is extremely collegial, with frequent constructive feedback from all faculty, making it a great environment to learn how to become a scientist. The program gave me the opportunity to be a Biochemistry teaching assistant to first-year PhD students in my second year. This teaching experiences reemphasized my long-term desire to mentor young scientists in the future, which consolidated my wish to work in academia. Receiving this electron microscopy time and training at the NY NCAAT facility will be a critical step towards achieving my long-term career goals and will allow me to uncover new neutralizing antibodies for HCMV gB and their mechanism of action.

B. Positions and Scientific Appointments

Positions and employment:

2017-2019: Undergraduate Researcher, Portnoy Lab, University of California Berkeley, Berkeley, CA
2017-2018: Undergraduate Student Instructor, Organic Chemistry, College of Chemistry, University of California Berkeley, Berkeley, CA
2019: Alliance for Global Health and Science Intern, Center for Emerging and Neglected Diseases, Berkeley, CA
2019-2021: Research Associate, Peng Lab, University of California San Francisco, San Francisco, CA
2022: Teaching Assistant, Graduate Biochemistry, Graduate School of Biomedical Sciences, Tufts University, Boston, MA

C. Contributions to Science

1. TLR2 and endosomal TLR-mediated secretion of IL-10 and immune suppression in response to phagosome-confined *Listeria monocytogenes*

As part of its life cycle, *Listeria monocytogenes* (Lm) relies on two canonical virulence factors, ActA and LLO, to successfully infect host cells. Lm utilizes ActA to harness host actin and propel itself from cell-to-cell while LLO allows Lm to escape from the phagosome after phagocytosis into the host-cell by forming pores in the phagosome. Previously in the Portnoy Lab, it was shown that Lm lacking the ability to harness host actin and spread from cell-to-cell (Δ*actA*) can be used as a potent cancer vaccine vector strain. Given this finding, we hypothesized that deletion of LLO (Δhly) would result in a secondary or even safer vaccine strain. However, upon further investigation, we showed that phagosome-confined Lm results in a minimally effective vaccine strain by mediating immune suppression through cellular IL-10 secretion. Although this finding excluded Δ*hly* as a vaccine vector candidate, we decided to investigate *why* this strain was mediating IL-10 secretion since this was a previously uncharacterized phenomenon.

We determined that a strain lacking both LLO and prolipoprotein diacylglyceryl transferase (Δ*hly*Δ*lgt*) vaccinated mice at an efficacy rate similar to Δ*actA* and did not induce host IL-10. Lgt is responsible for anchoring Lm lipoproteins to the bacterial cell surface and following this discovery, I decided to investigate whether or not all lipoproteins contributed equally to this phenomenon. Since many of these lipoproteins are essential to Lm metabolism, I focused my efforts on the surface anchor of each protein rather than the protein itself. Mutating the individual lipoprotein anchors allowed for the lipoproteins to still be produced, but they would no longer be expressed on the bacterial surface and could not interact with any immune signaling molecules, such as Toll-Like Receptors (TLRs). My hypothesis was that the quantity of surface proteins expressed would affect IL-10 levels, rather than the specific proteins themselves. After creating over 40 individual lipoprotein-anchor mutants and testing them with in vitro infections, I concluded that all lipoproteins need to be removed from the bacterial cell wall in order to significantly affect IL-10 levels. Investigating further, I showed that removal of the most abundant lipoproteins allowed less-abundant proteins to populate a greater portion of the cell wall surface. As a result, the total quantity of lipoproteins expressed on the surface had little variation even when specific proteins were removed. I had to opportunity to present my findings through a senior thesis and an oral presentation at UC Berkeley's Summer Undergraduate Research Fellowship symposium. My research contributed to a larger investigation of how phagosome-confined Lm mediates immune suppression, which is now published in PLOS Pathogens. These findings will aid in understanding the interactions of intracellular pathogens with endosomal TLRs, potentially allowing for more bacteria to be used as vaccine vectors.

a) Nguyen BN*, Chávez-Arroyo A*, Cheng MI, **Krasilnikov M**, Louie A, Portnoy DA. TLR2 and endosomal TLR-mediated secretion of IL-10 and immune suppression in response to phagosome-confined Listeria monocytogenes. *PLoS Pathog*. 2020 Jul 7;16(7):e1008622. doi: 10.1371/journal.ppat.1008622. PMID: 32634175; PMCID: PMC7340287. (**Publication)**

*Indicates co-first authors

b) **Krasilnikov M**, Nguyen BN, Chávez-Arroyo A, Portnoy DA. Surface lipoproteins mediate immune suppression in phagosome-confined Listeria monocytogenes. Summer Undergraduate Research Fellowship Symposium. Berkeley, CA, August 2018. **(Oral Presentation)**

2. Workshop-based learning and networking: a scalable model for research capacity strengthening in low- and middle-income countries.

In 2019, I taught and developed laboratory technique workshops at Makerere University in Kampala, Uganda with Berkeley's Center for Emerging and Neglected Diseases. My role as the Global Alliance Intern involved writing and performing protocols for the three "wet lab" workshops: Protein Purification of Cas9, Basic Techniques in Immunology, and Molecular Cloning. I independently traveled to Kampala before the workshops began and adapted our protocols to the equipment available on the university campus. Along with scientists from UC Berkeley, UC San Francisco, and the Chan Zuckerberg Biohub, I instructed students on immunological techniques and gave a lecture on Listeria monocytogenes virulence genes. My unique experience with Bay Area and Ugandan scientists fundamentally changed the way I view scientific communication and collaboration. After surveying our students, we determined that in some cases over 90% were satisfied with their training and a vast majority were able to apply their training to complete their respective graduate degrees. I had the opportunity to communicate these findings and the workshop design through a publication with all my collaborators in Global Health Action so that in the future these capacity-building workshops will be more common throughout international scientific community.

a) Perier C, Nasinghe E, Charles I, Ssetaba LJ, Ahyong V, Bangs D, Beatty PR, Czudnochowski N, Diallo A, Dugan E, Fabius JM, Fong Baker H, Gardner J, Isaacs S, Joanah B, Kalantar K, Kateete D, Knight M, **Krasilnikov M**, Krogan NJ, Langelier C, Lee E, Li LM, Licht D, Lien K, Lyons Z, Mboowa G, Mwebaza I, Mwesigwa S, Nalwadda G, Nichols R, Penaranda ME, Petnic S, Phelps M, Popper SJ, Rape M, Reingold A, Robbins R, Rosenberg OS, Savage DF, Schildhauer S, Settles ML, Sserwadda I, Stanley S, Tato CM, Tsitsiklis A, Van Dis E, Vanaerschot M, Vinden J, Cox JS, Joloba ML, Schaletzky J. Workshop-based learning and networking: a scalable model for research capacity strengthening in low- and middle-income countries. Glob Health Action. 2022 Dec 31;15(1):2062175. doi: 10.1080/16549716.2022.2062175. PMID: 35730550; PMCID: PMC9225690. **(Publication)**

b) **Krasilnikov M**, Nguyen BN, Stanley S. Introduction to Listeria monocytogenes virulence genes and immune response. Workshop on tissue culture and immunological techniques, Makerere University, Kampala, UG. **(Oral Presentation)**

3. Sentinel p16INK4a+ cells in the basement membrane form a reparative niche in the lung

Previous literature has hinted at a relationship between the expression of p16, a cyclin-dependent kinase inhibitor that targets CDK4 and is a canonical senescence marker, and fibrosis, but little is known about the involved mechanisms. We hoped that by understanding the role p16 plays in idiopathic pulmonary fibrosis (IPF) we could not only identify the specific cell types involved but tease out the cell signaling mechanism to potentially understand *why* these cells were involved. Our hypothesis was that a greater degree of fibrosis would be associated with an increase in p16 expression. To test this hypothesis, we developed a novel p16-EGFP fluorescent reporter that would allow for in vivo detection of p16, which was previously unattainable. Using this reporter, I was able to establish that fibrotic injury caused an increase in p16 expression in vivo - both in the murine lung and liver. The increased p16 expression upon injury drove fibroblasts into expressing smooth-muscle markers, helping to explain how sentinel cells contribute to the fibrosis phenotype. I later validated my in vivo research with in vitro and ex vivo experiments, which included the use of a "Tet-On" p16-overexpression lentivirus system of my own creation.

While characterizing the role of p16 in fibrosis, I realized that research in the senescence field had long been hindered by a lack of tools, specifically the inability to isolate senescent cells without a reporter. To address this issue, I used our novel p16 reporter to develop a method of isolating senescent cells in vivo. Using the GFP-tagged senescent cells, I worked backwards to understand and characterize how their size, cell cycle phase, and susceptibility to various fluorescent stains were different from their non-senescent counterparts.

Using this information, I established a way of isolating senescent cells without the GFP-tag. My technique also isolated pathogenic cells involved in murine IPF. I demonstrated that upon fibrosis-inducing injury the expression of senescent genes drives healthy lung fibroblasts to become collagen-expressing myofibroblasts *in vivo* and *in vitro*. My research is now published in Science, with me as co-author.

a) Reyes N, **Krasilnikov M**, Allen NC, Lee JY, Hyams B, Zhou M, Ravishankar S, Cassandras M, Wang C, Khan I, Matatia P, Johmura Y, Molofsky A, Matthay M, Nakanishi M, Sheppard D, Campisi J, Peng T. Sentinel p16INK4a+ cells in the basement membrane form a reparative niche in the lung. *Science*, 2022, In Press. **(Publication)**
b) **Krasilnikov M**, Reyes N, Peng T. p16INK4A+ cells modulate myofibroblast transformation in idiopathic pulmonary fibrosis. Meeting of the Stem Cell. San Francisco, CA, August 2020. **(Oral Presentation)**

4. Group 2 innate lymphoid cells suppress innate type 3/17 responses in hepatic fibrosis

Type 2 immunity can promote physiologic tissue remodeling, yet excessive activation can also drive fibrotic disease. Group 2 innate lymphoid cells (ILC2s) are a dominant organizer of this immune flavor, but how ILC2 topography and local interactions dictate these responses are unknown. Using several mouse models of liver fibrosis, we used quantitative 3D imaging to define fibrosis-associated portal and periductal ILC2 accumulation in proximity to an expanded IL-33-producing fibroblast subset. However, ablation of IL-33 or IL-4/IL-13 had no impact on hepatic fibrosis. As an expert on development of murine fibrosis models, I collaborated with the primary author on this project to develop a hepatic liver fibrosis model and read-out. Unexpectedly, I found that constitutive or inducible loss of ILC2s worsened carbon tetrachloride (CCl4)- or bile duct ligation-induced liver fibrosis. Mechanistically this occurred in part via suppression of innate IL-17A-producing lymphocytes, which also accumulated in periportal regions during fibrosis. Collectively, these data identify a novel role for ILC2s in the liver portal tracts as a negative regulator of the innate type3/17 immune response to hepatic damage and suggests resident lymphocyte topographic crosstalk may be a critical determinant of liver health and disease. A manuscript for this project has just been resubmitted to Nature Immunology, with me as a co-author.

a) Sbierski-Kind J, Cautivo M, Wagner J, **Krasilnikov M**, Mroz N, Lu Gan A, Dahlgren M, Matatia P, Taruselli M, Chang A, O'Leary C, Kotas M, Caryotakis S, Mattis A, Locksley R, Peng T, Molofsky AB. Group 2 innate lymphoid cells suppress innate type 3/17 responses in hepatic fibrosis. Cytokines 2022: 4th International Conference on Innate Lymphoid Cells, September, 2022. **(Poster Presentation)**

5. Development of a Dual-Fluorescent-Reporter System in Clostridioides difficile Reveals a Division of Labor between Virulence and Transmission Gene Expression.

The bacterial pathogen *Clostridioides difficile* (Cd) causes gastroenteritis by producing toxins and transmits disease by making resistant spores. Toxin and spore production are energy-expensive processes that are regulated by multiple transcription factors in response to many environmental inputs. While toxin and sporulation genes are both induced in only a subset of Cd cells, the relationship between these two subpopulations remains unclear. To address whether Cd coordinates the generation of these subpopulations, I aided in developing a dual-transcriptional-reporter system that allows toxin and sporulation gene expression to be simultaneously visualized at the single-cell level using chromosomally encoded mScarlet and mNeonGreen fluorescent transcriptional reporters. I then contributed to adapting an automated image analysis pipeline to quantify toxin and sporulation gene expression in thousands of individual cells under different medium conditions and in different genetic backgrounds. These analyses revealed that toxin and sporulation gene expression rarely overlap during growth on agar plates, whereas broth culture increases this overlap. The results suggest that certain growth conditions promote a "division of labor" between transmission and virulence gene expression, highlighting how environmental inputs influence these subpopulations. Our data further suggest that the RstA transcriptional regulator skews the population to activate sporulation genes rather than toxin genes. Given that recent work has revealed population-wide heterogeneity for numerous cellular processes in Cd, we anticipate that our dual-reporter system will be broadly useful for determining the overlap between these subpopulations. My results helped contribute to the completion of this project, now published in mSphere.

a) Donnelly ML, Shrestha S, Ribis JW, Kuhn P, **Krasilnikov M**, Alves Feliciano C, Shen A. Development of a Dual-Fluorescent-Reporter System in Clostridioides difficile Reveals a Division of Labor between Virulence and Transmission Gene Expression. mSphere. 2022 Jun 29;7(3):e0013222. doi: 10.1128/msphere.00132-22. Epub 2022 May 31. PMID: 35638354; PMCID: PMC9241537. **(Publication)**