Yves Brun,
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Dear Dr. Brun and Search Commitee:

I am applying for the advertised tenure track position in the Department of Biology at Indiana University. My primary research interest is in understanding the molecular mechanisms of membrane traffic, with a current concentration on clathrin dependant traffic between the *trans*-Golgi network (TGN) and endosomes with the yeast *Saccharomyces cerevisiae* as a model organism. My current and future studies focus on the identification of new protein components and the discovery new chemical inhibitors of membrane traffic for probing these pathways in yeast and other eukaryotes.

Chemical genetics is a growing field with the potential to revolutionize investigations in various disciplines. In particular, the ability to immediately inhibit cellular processes may become central in systems biology. At UCLA, I was the first screener at our chemical screening facility and have since been an active member of a growing chemical biology group, discussing screening ideas and problems with many new users. I envision becoming an active member of a similar interactive culture at the Indiana University. My study of membrane traffic adds to the diversity of cell biology studied in the Department of Biology and will synergize with the department's existing strengths in the cytoskeleton.

Included is a description of my recent research identifying a novel inhibitor of membrane traffic and the use of this compound in whole genome analysis which discovered new phenotype for probing membrane traffic. I am finalizing experiments examining the effects of this compound on mammalian cells as I prepare the manuscript for publications. I have also included a description of my unpublished results dealing with the clathrin adaptor, Ent5p, and the interactions it makes within the clathrin coat. As described in my research proposal, I plan to expand both projects as an independent scientist. Both of my projects provide ample starting material with which to start a new lab, and I have obtained funding for each in the past.

Letters of recommendation from Professor Greg Payne, my current mentor at UCLA, and Professors David G. Drubin and Jeremy Thorner from U.C. Berkeley should be arriving separately.

Thank you for your time and consideration.

Sincerely,

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Research Statement:

My primary interest is in understanding the molecular mechanisms of membrane traffic, with a current focus on transport between the *trans*-Golgi network (TGN) and endosomes. Membrane traffic is central to all biological signaling processes and understanding the mechanisms will provide insights into how the extra-cellular signals are integrated with growth control, an issue that underlies normal development and abnormal cell proliferation during cancer. My postdoctoral studies have applied standard genetic, cell biological and biochemical techniques as well as novel chemical genetic approaches using yeast as a model organism. These strategies have identified new genes and led to new mechanistic insights into traffic between the TGN and endosomes. My future studies will employ these approaches not only to understand the mechanisms of membrane traffic in yeast but also to discover new chemical inhibitors of membrane traffic for probing these pathways in other eukaryotes.

To date my work has led to one paper in Nature Cell Biology. The chemical screening project is nearly complete and I am in the process of investigating effects in mammalian cells before submitting the work for publication.

Background: Membrane traffic, the complex movement of membrane bounded carriers between intracellular organelles, depends on cytosolic proteins for the proper assembly and targeting of the carriers. These carriers contain the lumenal and transmembrane protein cargoes destined for the target organelle. The heteromeric complex clathrin, which coats a subset of vesicular carriers, is thought to play a largely structural role in endocytosis and traffic between the TGN and endosomes. Clathrin function at each step in these pathways requires additional cytosolic factors termed adaptors, which help to recruit clathrin to membranes and also concentrate the trans-membrane cargoes into forming clathrin coated vesicles. Defects in the Golgi and endocytic pathways are associated with a wide variety of human diseases ranging from cancer to inherited neurological defects [1, 2]. An emerging literature suggests adaptor proteins are key targets of a variety of cellular pathogens including HIV [3]. Recent results also indicate regulation of adaptor function plays important roles in maturation of certain immune cells [4]. The identification and characterization of novel cytosolic components of membrane traffic has been an active field of study in recent years and has contributed to our understanding of both the endomembrane system and its function in the larger context of cellular biology. My work has focused in two areas. The first, identification of novel proteins involved in TGN-endosome traffic, resulted in the discovery of two new adaptors at the TGN and endosomes and the characterization of conserved binding sites for adaptors involved in membrane traffic [5]. Our findings helped establish lipid-binding adaptors as a fundamental element of clathrin coated vesicle biogenesis [6]. In a second study, I developed techniques to identify small molecules that inhibit membrane traffic and applied the compounds to identify new cargoes of traffic between the TGN and endosomes.

Identification of novel proteins: At the outset of my postdoctoral studies, there was a clear deficiency in our knowledge of the proteins involved in membrane traffic between the TGN and endosomes, thus I determined to identify new proteins involved in these

pathways. I used a 2-hybrid screen to identify a new set of adaptor proteins at the TGN and endosomes [5]. These proteins termed Ent3p and Ent5p are required for the proper function of clathrin dependant traffic between the TGN and endosomes. Ent3p and Ent5p belong to a large family of conserved traffic proteins containing ENTH or ANTH type lipid binding domains; many known to function in clathrin mediated endocytosis. Identification of Ent3p and Ent5p as key players in TGN-endosome traffic firmly established this family of proteins as central to clathrin mediated traffic in general. Our studies defined highly conserved motifs in Ent3p and Ent5p that mediate interaction with other clathrin adaptors Gga1p, Gga2p (Ggas) and the AP-1 clathrin adaptor complex. Such motifs are found in a number of mammalian proteins which interact with the mammalian Gga proteins and AP-1, and we were the first to characterize this conserved mechanism for adaptor binding. Identification of this motif has clarified one aspect of the network of protein interactions acting in clathrin dependant traffic and has been used by other researchers to identify novel components of clathrin dependant traffic[7].

Recent findings: Clathrin coats are built by assembly of a network of relatively low affinity binding motifs and their target domains within the coat components. My recent results investigate the relative role of different motifs of Ent5p *in vivo*. This study has generated several important findings; the most important of which is that clathrin binding *in vivo* is cooperative between Ent5p and the other clathrin adaptors. Presumably, this requirement provides a fail-safe mechanism to ensure that clathrin is not recruited until the full adaptor machinery is assembled. I am currently pursuing this hypothesis.

Chemical inhibition of membrane traffic: Small molecule inhibitors such as Brefeldin A have been essential in investigating the dynamic and adaptable steps of membrane traffic. In the course of my studies of Ent3p and Ent5p, I became interested in the possibility of generating small molecule inhibitors of specific events in membrane traffic. Until recently, identification of inhibitors was an arduous proposition and usually relied on purification of toxins from natural sources. Recent advances in chemical genetics and the growing availability of small molecule libraries have revolutionized approaches to identify compounds with novel and specific effects. Such specific small molecule inhibitors may become indispensable additions to the cell biologists tool-kit as well as potential starting points for therapeutic development.

In order to identify inhibitors of membrane traffic to use in my studies, I developed generalizable novel screening techniques to identify chemical inhibitors of cellular processes *in vivo*. The approach is based on synthetic genetic lethality- the strong reduction in growth when viable mutations in different genes are combined. I postulated that a chemical that inhibits a pathway should display the same spectrum of synthetic lethality as deletions of genes encoding the proteins of that pathway. Therefore, starting with cells lacking the Gga adaptors, which require AP-1 mediated pathways for viability, I screened a library of ~30,000 compounds from Chembridge for chemicals that inhibited the growth of the gga mutant cells and not wild-type cells. I was successful in identifying three related compounds which show specific defects in membrane traffic that mimic deletions of AP-1. By screening the ~6000 strains in the yeast knockout collection for deletions that are sensitive to my compounds, I identified the tryptophan permease Tat2p as a new cargo of AP-1 dependant traffic. Deletion of AP-1 redistributes Tat2p, this is the first phenotype of AP-1 visible without additional mutations or compound

treatment. This new phenotype is an important new handle for the will further investigation of AP-1 dependant traffic. By combining the newly identified chemical inhibitors with whole genome analysis, I have established a stepwise procedure for the characterization of biological pathways in yeast.

Currently, I am investigating the effects of my identified compounds on AP-1 dependant traffic in mammalian cells. Also, in collaboration with the Jung group in the Chemistry and Biochemistry Department at UCLA, I am generating derivatives of my compounds with the aim of affinity purifying the direct target of these compounds. In addition, my original chemical screen identified 5 unrelated compounds which seem to have more general effects on membrane traffic. In the future, I plan to fully characterize these chemicals.

The new screening techniques I developed should be adaptable to identify inhibitors of diverse cellular functions in different cell types and organisms. The screens are inexpensive, facile and specific using yeast, but similar approaches should be relatively straightforward in tissue culture by combining RNAi or knockout cell lines with chemical inhibition.

Proposed research plan:
My future research plans can be broken down into three specific aims:

1. Characterize novel chemical inhibitors of membrane traffic
2. Screen for inhibitors of GGA dependant traffic
3. Characterize proteins and interactions important for TGN-endosome traffic.

Aim 1: Characterize novel chemical inhibitors of membrane traffic. The value of a chemical inhibitor, even in a genetically tractable organism like yeast, is illustrated by the use of the actin polymerization inhibitor latrunculin A[8, 9]. This chemical was instrumental in defining role for actin polymerization in cell polarity, mitotic spindle orientation, and endocytosis. Current inhibitors of membrane traffic remain limited and novel inhibitors of different stages of membrane traffic may provide insight impossible with other methods. In my initial screen for chemical inhibitors of AP-1 dependant membrane traffic, I identified five unrelated compounds that had growth inhibitory profiles which suggest these compounds may target clathrin dependant traffic more generally than my currently characterized compounds.

Using techniques I have developed for investigating AP-1 inhibitors, I will probe the effects of these compounds with the goal of developing new tools for studying membrane traffic. We will investigate the effect of these compounds on endocytosis, traffic between the TGN and endosomes, and traffic between the TGN and vacuole. A panel of strains containing GFP-tagged proteins will be used to assess the effects of the compounds on localization of proteins involved in membrane traffic. We will screen the yeast deletion collection for sensitive strains in order to better define the pathways inhibited. Once we have identified the target pathway, we will investigate the effect of these compounds on the analogous pathway in mammalian cells or other eukaryotic systems such as *D. melanogaster* cells or *C. elegans*. Finally, depending on the potency and difficulty of synthesis, we will perform affinity chromatography with the compounds or multi-copy suppressor screens in an effort to define the molecular target of the compounds.

Aim 2: Screen for inhibitors of GGA dependant traffic. Controversy still surrounds the relative roles of AP-1 and GGA proteins in membrane traffic. In mammalian cells AP-1 and Gga proteins are closely associated at the TGN and have been demonstrated to physically interact [10]. Nonetheless, AP-1 and Ggas clearly display some independent functions as demonstrated by phenotypic analysis in yeast [11]. A rapid and reversible inhibitor of Gga dependant traffic may have advantages over other approaches to illuminate the roles of Ggas and AP-1.

My whole genome analysis using AP-1 traffic inhibitors revealed aspects of AP-1 dependant traffic that had not come to light using other methods. While other approaches (such as systematic synthetic genetic arrays) might have revealed the same features, the chemical sensitivity analysis was easy, rapid and reproducible. Considering Gga-dependent traffic, there are two redundant *GGA* genes, making it difficult to apply existing systematic synthetic genetic approaches to analyze the pathway. A chemical inhibitor of the Gga-dependent pathway would allow an immediate screen of the yeast gene deletion collection for synthetic interactions. Furthermore, depending on the mode of action, inhibitors may be used in temporal studies that would be impossible with other approaches. For instance, Gga proteins are proposed to be the main cargo recruitment

mechanism for clathrin dependant traffic from the TGN in mammalian cells[10], an inhibitor that rapidly and reversibly blocks this step might allow a compelling test of this hypothesis.

The approaches I developed for the identification of inhibitors of AP-1 dependant traffic can be used for the identification of inhibitors of Gga dependant traffic. In this case, diverse chemical libraries will be screened for compounds which inhibit the growth of cell lacking AP-1 subunits but do not inhibit the growth of wild-type cells. If on site screening is not available, such screens can be performed for a small fee at screening facilities around the country, or because my screening techniques require only standard laboratory equipment, the screens can be performed in house if appropriate libraries are available.

In order to determine which of the initial hits target GGA traffic, we will examine the growth effects of on cells carrying the *chc1-ts* allele or lacking GGA proteins. Compounds that inhibit Gga dependant traffic should inhibit *chc1-ts* cells but not cells lacking Gga proteins. In addition to the strong growth defect, cells lacking AP-1 and GGA proteins together secrete precursor yeast mating pheromone α-factor because of the mislocalization of a TGN processing enzyme. I have developed small volume techniques to analyze the secretion of unprocessed α-factor, these techniques will be used to determine whether candidate compounds increase the secretion of unprocessed α-factor of cells lacking AP-1 subunits. Compounds with the appropriate growth profile and which cause secretion of unprocessed α-factor will be further analyzed for effects on GGA dependant membrane traffic using pulse chase and other phenotypic analyses that are routinely applied to study this pathway. Follow-up studies will be similar to those in Aim 1.

Aim3: Characterize proteins and interactions important for TGN-endosome traffic. Results from several proteomic analyses of clathrin coated vesicles indicate that we have yet to characterize many of the proteins involved in TGN-endosome traffic [12, 13]. In endocytic coats a variety of associated kinases and other enzymes are thought to regulate coat formation or removal, few such proteins are known to be associated with TGN-endosome traffic. Additionally, the multiple interactions between proteins of the TGN-endosome coat suggest a mechanism to coordinate activity. Accordingly, I plan to identify novel proteins involved in TGN-endosome traffic and also characterize the requirements for interactions between proteins involved in membrane traffic with a goal of understanding how vesicle formation is coordinated and regulated.

In the course of my postdoctoral work, in addition to identifying Ent3p and Ent5p by 2-hybrid screening, I identified proteins which I thought were good candidates to be involved in membrane traffic based on mining databases from high throughput screens. Two such candidates have been demonstrated by research in the Payne lab to be involved in membrane traffic. I plan to identify new candidate proteins using systematic screens such as yeast 2-hybrid or mass-spectrometry analysis of complexes containing TGN-endosome adaptors or by database analysis. In the initial phases, projects investigating the role of candidate proteins in membrane traffic are straightforward due to the ease of genetic manipulations in yeast. Genes encoding the candidate protein will be deleted. Growth and α-factor maturation will be examined in the deletion strains and in double mutant combination with the *chc1-ts* alleles or deletions in AP-1 and GGA genes. In addition, the candidate proteins will be epitope tagged. These epitope tagged alleles will

be used to determine whether the proteins are incorporated into clathrin coated vesicles or co-localize with clathrin *in vivo*. The epitope tagged proteins will be immuno-precipitated and probed with antibodies to known membrane trafficking proteins. Genes/proteins that show traffic phenotypes, co-localization or co-immuno-precipitate other membrane traffic proteins will be pursued further. Depending on the identity of the proteins, this may include site-directed mutagenesis or 2-hybrid analysis to identify binding partners.

In a complementary approach, one project in my group will address whether adaptor-adaptor interactions are required for efficient interactions between clathrin and the GGAs and AP-1 adaptors. My work investigating *ENT5* mutants has revealed a requirement for adaptor binding in efficient clathrin binding. This leads directly to the question of whether the same is true of GGAs and AP-1 adaptors. I previously defined the surfaces on Gga2 involved in Ent3p and Ent5p interaction and work in the field has identified regions required for interaction between GGAs and AP-1 [10, 14]. As was done for Ent5p, alleles encoding point mutations of Ggas or AP-1 will be generated that abolish the relevant interactions. We will assay these alleles for interaction with clathrin *in vivo* by immunoprecipitation and the function of these alleles will be tested using phenotypes known to be associated with the deletions of the genes. If like Ent5p, GGAs and AP-1 require interactions with adaptors for clathrin interaction, this may suggest a new paradigm for the field that adaptor-adaptor interaction in general provides a mechanism to restrict clathrin recruitment to adaptor networks rather than individual adaptors.

Future work will investigate the nature of the requirement for adaptor-adaptor interaction in clathrin binding. This requirement could be due to intra-molecular inhibition that is relieved by adaptor binding or could reflect increased avidity of the adaptor-adaptor complex. These possibilities will be addressed using site-directed mutagenesis, combined with chimeric molecules and *in vitro* biochemistry.

1. Crosetto, N., R. Tikkanen, and I. Dikic, *Oncogenic breakdowns in endocytic adaptor proteins.* FEBS Letters, 2005. **579**(15): p. 3231-3238.
2. Nixon, R.A., *Endosome function and dysfunction in Alzheimer's disease and other neurodegenerative diseases.* Neurobiology of Aging, 2005. **26**(3): p. 373-382.
3. Morita, E. and W.I. Sundquist, *RETROVIRUS BUDDING.* Annual Review of Cell and Developmental Biology, 2004. **20**(1): p. 395-425.
4. Santambrogio, L., et al., *Involvement of caspase-cleaved and intact adaptor protein 1 complex in endosomal remodeling in maturing dendritic cells.* Nat Immunol, 2005. **6**(10): p. 1020-1028.
5. Duncan, M.C., G. Costaguta, and G.S. Payne, *Yeast epsin-related proteins required for Golgi-endosome traffic define a gamma-adaptin ear-binding motif.* Nat Cell Biol, 2003. **5**(1): p. 77-81.
6. Duncan, M.C. and G.S. Payne, *ENTH/ANTH domains expand to the Golgi.* Trends Cell Biol, 2003. **13**(5): p. 211-5.
7. Burman, J.L., et al., *Aftiphilin is a component of the clathrin machinery in neurons.* FEBS Letters, 2005. **579**(10): p. 2177-2184.

8. Ayscough, K.R., et al., *High rates of actin filament turnover in budding yeast and roles for actin in establishment and maintenance of cell polarity revealed using the actin inhibitor latrunculin-A.* J Cell Biol, 1997. **137**(2): p. 399-416.
9. Valdivia, R.H., et al., *The yeast clathrin adaptor protein complex 1 is required for the efficient retention of a subset of late Golgi membrane proteins.* Dev Cell, 2002. **2**(3): p. 283-94.
10. Doray, B., et al., *Cooperation of GGAs and AP-1 in Packaging MPRs at the Trans-Golgi Network.* Science, 2002. **297**(5587): p. 1700-1703.
11. Valdivia, R.H., et al., *The Yeast Clathrin Adaptor Protein Complex 1 Is Required for the Efficient Retention of a Subset of Late Golgi Membrane Proteins.* Developmental Cell, 2002. **2**(3): p. 283-294.
12. Blondeau, F., et al., *Tandem MS analysis of brain clathrin-coated vesicles reveals their critical involvement in synaptic vesicle recycling.* PNAS, 2004. **101**(11): p. 3833-3838.
13. Harasaki, K., et al., *Sorting of Major Cargo Glycoproteins into Clathrin-Coated Vesicles.* Traffic, 2005. **6**(11): p. 1014-1026.
14. Bai, H., B. Doray, and S. Kornfeld, *GGA1 Interacts with the Adaptor Protein AP-1 through a WNSF Sequence in Its Hinge Region.* J. Biol. Chem., 2004. **279**(17): p. 17411-17417.

Teaching Statement:

My love of science started early with a third grade trip to the local university's entomology department, from that point on I knew I wanted to be a scientist. A key part of what I think being a scientist is includes passing on the knowledge and skills I have gained. For me instruction has two important functions, the first of which is to educate the average citizen about science. All scientific knowledge is irrelevant if it never leaves academic departments, by instructing future medical doctors and business leaders we ensure that scientific knowledge will get applied to solve problems in everyday life. In addition, there is a growing mistrust of biological science in the culture which I think is largely due to ignorance and misunderstanding. Effective undergraduate instruction is one way to fight this mistrust. The second goal of instruction is to train the next generation of scientists. Of course, like many I would like to be able to look back on our instructional careers and point to influential scientists I have trained.

I have taken opportunities to hone my teaching skills. Starting as a high school senior, I was a teacher's assistant for the freshman biology lab where I helped set up the lab materials, demonstrated techniques and was available during class time for questions and assistance. In graduate school, in addition to the two required teaching assistanceships, I took the opportunity to design a literature seminar course for undergraduate students. The literature seminar introduced me to the challenges of teaching solo but also gave the feeling of reward when my students seemed to embrace the topic.

I am well versed and excited about genetics in general and would be eager to teach an intro Genetics course. I am also well prepared to teach Cell Biology or Biochemistry at either the graduate or undergraduate level.