Final Progress Report for Research Projects Funded by Health Research Grants

Instructions: Please complete all of the items as instructed. Do not delete instructions. Do not leave any items blank; responses must be provided for all items. If your response to an item is "None", please specify "None" as your response. "Not applicable" is not an acceptable response for any of the items. There is no limit to the length of your response to any question. Responses should be single-spaced, no smaller than 12-point type. The report **must be completed using MS Word**. Submitted reports must be Word documents; they should not be converted to pdf format. Questions? Contact Health Research Program staff at 717-783-2548.

1. **Grantee Institution:** University of Pittsburgh-of the Commonwealth System of Higher Education

2. **Reporting Period (start and end date of grant award period):** 01/01/09 – 12/31/12

3. **Grant Contact Person (First Name, M.I., Last Name, Degrees):** Margaret C. McDonald, PhD

4. **Grant Contact Person's Telephone Number:** 412-383-7474

5. **Grant SAP Number:** 4100047655

6. **Project Number and Title of Research Project:** 1 - Small Molecule Inhibitors of HIV Nef Signaling

7. **Start and End Date of Research Project:** 01/01/09 –3/31/2011

8. **Name of Principal Investigator for the Research Project:** Thomas E. Smithgall, PhD

9. **Research Project Expenses**.

   9(A) Please provide the total amount of health research grant funds spent on this project for the entire duration of the grant, including indirect costs and any interest earned that was spent:

   $ $1,365,561

   9(B) Provide the last names (include first initial if multiple individuals with the same last name are listed) of **all** persons who worked on this research project and were supported with health research funds. Include position titles (Principal Investigator, Graduate Assistant, Post-doctoral Fellow, etc.), percent of effort on project and total health research funds expended for the position. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project	Cost
Coyne, C.	Assistant Professor	25% Yr 2	$22,969
Han, D.	Postdoctoral Associate	15% Yr 2	$6,333
Hellwig, S.	Research Associate	25% Yr 1, 100% Yrs 2-3	$90,736
Liu, H.	Assistant Professor	25% Yr 2	$25,000
Meyn, M.	Research Assistant Prof	3% Yr 3	$2,256
Moroco, J.	Grad Student Researcher	50% Yrs 1-2	$26,704
O'Reilly, L.	Research Associate	25% Yr 1, 50% Yr 2, 75% Yr 3	$85,115
Pene Dumitrescu, T.	Postdoctoral Associate	50% Yrs 1-2	$37,000
Shu,S.	Postdoctoral Associate	33% Yr 2	$12,500
Zhang, X.	Grad Student Researcher	25% Yr 1, 50% Yr 2, 66% Yr 3	$35,629

9(C) Provide the names of **all** persons who worked on this research project, but who *were not* supported with health research funds. Include position titles (Research Assistant, Administrative Assistant, etc.) and percent of effort on project. For multiple year projects, if percent of effort varied from year to year, report in the % of Effort column the effort by year 1, 2, 3, etc. of the project (x% Yr 1; z% Yr 2-3).

Last Name	Position Title	% of Effort on Project
Smithgall, T.	Professor, PI	< 1%

9(D) Provide a list of **all** scientific equipment purchased as part of this research grant, a short description of the value (benefit) derived by the institution from this equipment, and the cost of the equipment.

Type of Scientific Equipment	Value Derived	Cost
General Electric Akta Explorer	This is a state-of-the-art, computer-controlled system for recombinant protein purification. It is essential for all of the work done as part of our project and saves enormous amounts of time and reagent costs.	$110,000
Li-COR Odyssey System Premium	Enables high-resolution quantitative analysis of protein levels on immunoblots using IR detection	$52,702
Applied Biosystems PCR system	Enables high-throughput parallel analysis of mRNA levels in cell and tissue samples	$63,168
VWR 72 cubic foot refrigerator	Self-explanatory	$5,217
Fisher Scientific -80 freezer	Ultralow temperature freezer essential for preservation of biological samples	$12,681
Fluoview microscope	State-of-the-art high-resolution four-laser confocal microscope enables multicolor imaging of cells	$303,181

Nanodrop 2000 with cuvette	Essential for quantitation of nucleic acid concentrations in very small biological samples	$10,035
Sanyo ultra-low freezer	Ultralow temperature freezer essential for preservation of biological samples	$9,330
Synergy 2 microplate reader	Multi-function reader enables detection of biological endpoints in high-density formats by absorbance, fluorescence, and chemiluminescence	$29,658
Fly incubator chambers	Used to culture Drosophila models of human development and disease	$22,844
Agilent Technologies Bioanalyzer	Enables high-resolution quality control determinations of DNA, RNA and protein sampled prior to high-throughput analysis of gene expression (e.g. microarray analysis) and protein levels (e.g. proteomics)	$30,837

10. Co-funding of Research Project during Health Research Grant Award Period. Did this research project receive funding from any other source during the project period when it was supported by the health research grant?

Yes _____ No X___

11. Leveraging of Additional Funds

11(A) As a result of the health research funds provided for this research project, were you able to apply for and/or obtain funding from other sources to continue or expand the research?

Yes X_____ No_____

If yes, please list the applications submitted (column A), the funding agency (National Institutes of Health—NIH, or other source in column B), the month and year when the application was submitted (column C), and the amount of funds requested (column D). If you have received a notice that the grant will be funded, please indicate the amount of funds to be awarded (column E). If the grant was not funded, insert "not funded" in column E.

Do not include funding from your own institution or from CURE (tobacco settlement funds). Do not include grants submitted prior to the start date of the grant as shown in Question 2. If you list grants submitted within 1-6 months of the start date of this grant, add a statement below the table indicating how the data/results from this project were used to secure that grant.

A. Title of research project on grant application	B. Funding agency (check those that apply)	C. Month and Year Submitted	D. Amount of funds requested:	E. Amount of funds to be awarded:
Evaluation of HIV-1 Nef Antagonists in a Mouse Model of AIDS	☐NIH ☐ Other federal (specify:_____) X Nonfederal source (specify: Johnson and Johnson)	April 2012	$100,000	$100,000
Structural Biology of HIV-1 Nef with Host Effectors and Small Molecule Inhibitors	X NIH ☐ Other federal (specify:_____) ☐ Nonfederal source (specify:_)	Sept 2012	$ 1,920,316	$ 1,728,285

11(B) Are you planning to apply for additional funding in the future to continue or expand the research?

Yes X_____ No_____

If yes, please describe your plans:

We are planning a Program Project grant submission (P01) to the NIH to extend the work described herein. This application will be designed to better understand the mechanism of action of the HIV-1 Nef antagonists that we have discovered over the past few years and push them closer to clinical trials.

12. Future of Research Project. What are the future plans for this research project?

The major goals of our future work involve structural studies of our compounds with the HIV Nef protein, which will be funded by the NIH later this year. In addition, we will continue to synthesize analogs of our compounds and begin testing them in animal models of HIV disease (supported in part by new funding from Johnson and Johnson).

13. New Investigator Training and Development. Did students participate in project supported internships or graduate or post-graduate training for at least one semester or one summer?

Yes X_____ No_____

If yes, how many students? Please specify in the tables below:

	Undergraduate	Masters	Pre-doc	Post-doc
Male			1	3
Female			1	3
Unknown				
Total			**2**	**6**

	Undergraduate	Masters	Pre-doc	Post-doc
Hispanic				
Non-Hispanic			2	3
Unknown				3
Total			**2**	**6**

	Undergraduate	Masters	Pre-doc	Post-doc
White				3
Black				
Asian			2	3
Other				
Unknown				
Total			**2**	**6**

14. Recruitment of Out-of–State Researchers. Did you bring researchers into Pennsylvania to carry out this research project?

Yes _____ No X___

If yes, please list the name and degree of each researcher and his/her previous affiliation:

15. Impact on Research Capacity and Quality. Did the health research project enhance the quality and/or capacity of research at your institution?

Yes _____ No X___

If yes, describe how improvements in infrastructure, the addition of new investigators, and other resources have led to more and better research.

16. Collaboration, business and community involvement.

16(A) Did the health research funds lead to collaboration with research partners outside of your institution (e.g., entire university, entire hospital system)?

Yes X_____ No_____

If yes, please describe the collaborations:

Investigations of the mechanism of action involved collaborations with biophysicists at Northeastern University, Boston MA (laboratory of Dr. John R. Engen, professor of chemistry and chemical biology).

16(B) Did the research project result in commercial development of any research products?

Yes ___ No X

If yes, please describe commercial development activities that resulted from the research project:

16(C) Did the research lead to new involvement with the community?

Yes _____ No X

If yes, please describe involvement with community groups that resulted from the research project:

17. Progress in Achieving Research Goals, Objectives and Aims.

List the project goals, objectives and specific aims (as contained in the grant agreement). Summarize the progress made in achieving these goals, objectives and aims for the period that the project was funded (i.e., from project start date through end date). Indicate whether or not each goal/objective/aim was achieved; if something was not achieved, note the reasons why. Describe the methods used. If changes were made to the research goals/objectives/aims, methods, design or timeline since the original grant application was submitted, please describe the changes. Provide detailed results of the project. Include evidence of the data that was generated and analyzed, and provide tables, graphs, and figures of the data. List published abstracts, poster presentations and scientific meeting presentations at the end of the summary of progress; peer-reviewed publications should be listed under item 20.

This response should be a DETAILED report of the methods and findings. It is not sufficient to state that the work was completed. Insufficient information may result in an unfavorable performance review, which may jeopardize future funding. If research findings are pending publication you must still include enough detail for the expert peer reviewers to evaluate the progress during the course of the project.

Health research grants funded under the Tobacco Settlement Act will be evaluated via a performance review by an expert panel of researchers and clinicians who will assess project work using this Final Progress Report, all project Annual Reports and the project's strategic plan. After the final performance review of each project is complete, approximately 12-16 months after the end of the grant, this Final Progress Report, as well as the Final Performance Review Report containing the comments of the expert review panel, and the grantee's written response to the Final Performance Review Report, will be posted on the CURE Web site.

There is no limit to the length of your response. Responses must be single-spaced below, no smaller than 12-point type. If you cut and paste text from a publication, be sure symbols print properly, e.g., the Greek symbol for alpha (α) and beta (ß) should not print as boxes (□) and include the appropriate citation(s). DO NOT DELETE THESE INSTRUCTIONS.

Project Overview

The HIV-1 accessory protein Nef is essential for high-titer viral replication and AIDS progression. Nef has no known catalytic activity, and functions by interacting with multiple host cell signaling proteins, including the Src family of kinases, Hck, Lyn, and c-Src. Recently, we established *in vitro* and cell-based screens to identify small molecules that target the Nef:Hck complex. These screens identified two classes of drug-like compounds with potent activity against HIV replication *in vitro*, providing the first demonstration that small molecules targeting an HIV accessory protein-host cell protein complex have anti-HIV activity. This project pursued study of these compounds based on their activity against a broad range of HIV strains, including strains resistant to current antiretroviral drugs. We also explored synergy of these Nef-directed compounds with established anti-HIV therapeutics and investigated their molecular mechanism of action. Finally, we tested the hypothesis that the Nef oligomerization interface is a rational target for Nef-directed anti-HIV therapy. Previous studies have established that Nef oligomerization contributes to host cell kinase activation as well as viral and immune receptor downregulation. Small molecule inhibitors of Nef oligomerization may interfere with these critical Nef functions *in vivo*, providing new scaffolds for anti-HIV drug development.

The project's specific aims were:

Aim 1: Explore the utility and mechanism of action of newly discovered diphenylfuro-pyrimidine (DFP) and benzenesulfonamide diaminoquinoxaline compounds as Nef-directed anti-HIV agents .

Aim 2: Test the hypothesis that the Nef oligomerization interface is a rational target for anti-HIV therapy.

Substantial progress has been made on both aims and has led to seven publications and two new grants to date. Each of these studies is detailed below. Although some of these publications appeared after 3/2/2011, the work was initiated/ongoing during the funding period.

Summary of Research Completed

Studies related to Aim 1:

As described above, Nef is an HIV-1 accessory protein essential for AIDS progression and an attractive target for drug discovery; however, lack of a catalytic function makes Nef difficult to assay in chemical library screens. To circumvent this problem, we developed a high-throughput assay for inhibitors of Nef function by coupling the protein to one of its host cell binding partners, the Src-family kinase (SFK) Hck. Hck activation is dependent upon Nef in this assay,

providing a direct readout of Nef activity *in vitro*. Using this assay to screen a small, kinase-biased library, we identified DFP analogs as strong inhibitors of Nef-dependent Hck activation. These compounds also exhibited remarkable antiretroviral effects, blocking Nef-dependent HIV replication in cell lines in which HIV replication is highly dependent on Nef. These studies identifed the first examples of lead compounds directed against HIV-1 Nef and one of its host cell binding partners that exhibit antiretroviral activity. These findings were published in *ACS Chemical Biology* in 2009; *acknowledgement of CURE funding was inadvertently omitted.*

We next extended these pilot studies to include a series of primary Nef proteins derived from HIV-1 isolates representative of all major HIV-1 subtypes responsible for the AIDS pandemic. First, we showed that Nef proteins from HIV-1 subtypes A1, A2, B, C, F1, F2, G, H, J, and K strongly activate Hck and the other Src-family members Lyn and c-Src. These results demonstrate, for the first time, that SFK activation is a highly conserved property of primary M-group HIV-1 Nef isolates. Hck activation by each of the Nef proteins was also inhibited by the DFP compounds. To determine whether DFPs also exhibited broad-spectrum Nef-dependent antiretroviral activity, we constructed chimeric forms of the HIV-1 strain NL4-3 expressing the same 10 primary Nef alleles. The infectivity and replication of these Nef chimeras was indistinguishable from that of wild-type NL4-3 in three distinct cell lines (MT2, U87MG, and CEM-T4). Importantly, the DFP compounds potently inhibited replication of all 10 chimeric forms of HIV-1 in both U87MG and CEM-T4 cells in a Nef-dependent manner. These results demonstrate that activation of Hck, Lyn, and c-Src by Nef is highly conserved among all major clades of HIV-1 and that selective targeting of this pathway uniformly inhibits HIV-1 replication. These findings were published in *PLoS One* in 2012.

As described above, we identified a series of kinase inhibitors that preferentially inhibit Hck in the presence of Nef. These compounds also block Nef-dependent HIV replication, validating the Nef-SFK signaling pathway as an antiretroviral drug target. Our findings also suggested that by binding to the Hck SH3 domain, Nef indirectly affects the conformation of the kinase active site to favor inhibitor association. To test this hypothesis, we engineered a "gatekeeper" mutant of Hck with enhanced sensitivity to the pyrazolopyrimidine tyrosine kinase inhibitor, NaPP1. We also modified the RT (arginine-threonine) loop of the Hck SH3 domain to enhance interaction of the kinase with Nef. This modification stabilized Nef:Hck interaction in solution-based kinase assays, as a way to mimic the more stable association that likely occurs at cellular membranes. Introduction of the modified RT loop rendered Hck remarkably more sensitive to activation by Nef and led to a significant decrease in the K_m for ATP as well as enhanced inhibitor potency. These observations suggest that stable interaction with Nef may induce Src-family kinase active site conformations amenable to selective inhibitor targeting. This study was published in *BMC Chemical Biology* in 2012.

We have also completed our studies of the novel Nef antagonists identified in a yeast screening assay. This assay used a yeast-based system to identify small molecules that target the Nef-Hck complex. Nef-Hck interaction is faithfully reconstituted in yeast, resulting in kinase activation and growth arrest. Yeast cells expressing the Nef-Hck complex were used to screen a library of small heterocyclic compounds for their ability to rescue growth inhibition. The screen identified 2-quinoxalinyl-3-amino-benzenesulfonamide (QBS) analogs as potent inhibitors of Nef-dependent HIV replication and MHC-I downregulation. Docking studies predict that the QBS

pharmacophore interacts directly with Nef at multiple conserved sites. The docking results are consistent with the submicromolar potency of QBS analogs against the replication of HIV NL4-3 chimeras expressing Nef alleles representative of all major HIV-1 subgroups. These data demonstrate that coupling Nef to a host cell target protein enabled discovery of a novel class of antiretroviral compounds directed against this critical viral factor. A manuscript describing these data has been submitted for publication to *BMC Retrovirology*.

Studies related to Aim 2:

We recently adapted our Nef-dependent Hck activation assay for automated, high-throughput screening and screened a diverse chemical library of more than 220,000 compounds. Active compounds were then re-screened against Hck alone to eliminate Nef-independent kinase inhibitors. The resulting compounds were then assayed for antiretroviral activity by HIV p24 ELISA in the cell lines CEM-T4 and U87MG/CD4/CXCR4, where HIV-1 replication is Nef-dependent. Compound activity against Nef-dependent HIV-1 infectivity was also tested using the TZM-bl reporter cell line. Our screening strategy identified 62 small molecules that blocked Nef-dependent Hck kinase activity with low micromolar potency and at least a three-fold preference for the Nef-Hck complex versus Hck alone. Five compounds blocked HIV replication by at least 50% at 1 μM in both host cell lines. Four of five compounds also inhibited Nef-dependent HIV infectivity in the low micromolar range. The most potent of these compounds, termed B9, blocked HIV-1 NL4-3 replication in CEM-T4 cells in the nanomolar range and was broadly active against a panel of ten HIV-1 chimeras with Nef alleles representative of all major clades of HIV-1. Surface plasmon resonance (SPR) studies showed that B9 binds directly to Nef with nM Kd values, and computational docking predicts an energetically favorable binding pocket for B9 in the Nef dimerization interface. Mutagenesis of Nef Asn126, predicted by docking to play a key role in B9 binding, completely blocked Nef interaction with the compound by SPR. Coupling of the viral accessory factor Nef to a host cell effector kinase amenable to high-throughput screening (Hck) enabled identification of direct chemical inhibitors of HIV-1 Nef function. The Nef-directed compounds identified in this screen represent useful chemical probes for Nef function as well as potential lead compounds for future antiretroviral drug development. These studies were recently published in the *Cell* press journal, *Chemistry and Biology* (January 2013); *acknowledgement of CURE funding was inadvertently omitted.*

In related work, we have solved the X-ray crystal structure of HIV-1 Nef in complex with the Hck SH3-SH2 region for the first time. The Nef:SH3-SH2 complex crystallized as a dimer of complexes, with the conserved Nef PxxPxR motif engaging the Hck SH3 domain. A new intermolecular contact was found between SH3 E93 and Nef R105. Mutagenesis of Hck SH3 E93 interfered with Nef:Hck complex formation and kinase activation in cells. The Hck SH2 domains impinge on the N-terminal loops of Nef and reorient the Nef dimer through four distinct interfaces not observed in earlier structures. Our results suggest that, in addition to serving as a kinase effector for Nef, Hck binding may reorganize the Nef dimer for functional interaction with other signaling partners. In addition, this new Nef dimer structure will help guide rational design of next-generation Nef inhibitors. A manuscript describing this study has been submitted for publication to the *J. Biol. Chem*. Figures 1 and 2 highlight critical aspects of this new structure and its significance in terms of Nef interaction with endocytic machinery important for downregulation of MHC-1.

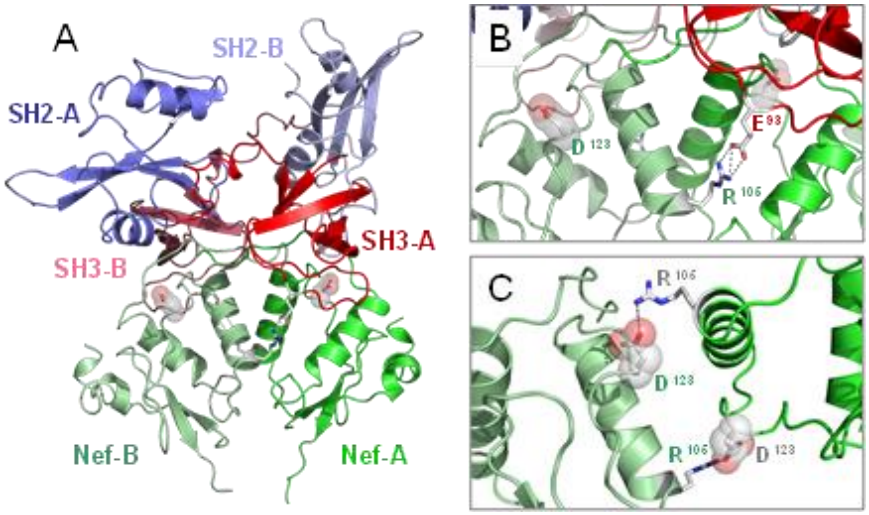

Figure 1. X-ray crystal structure of Nef-SF2:Hck-SH3-SH2 complex. A) Overview of the structure, which packs as a dimer of Nef:SH3-SH2 complexes. B) Close-up of new contact of Hck SH3 E93 with Nef R105; Nef D123 (space filled) is repositioned away from the dimer interface to the structure surface. C) Close-up of the Nef dimer interface from previous Nef:SH3-only structure, 1EFN. Here D123 and R105 form reciprocal ion pairs that flank the helical dimer interface. Comparison of B and C shows the reorganization of the dimer which results from Hck SH3-SH2 binding; the biological significance is shown in Fig. 2.

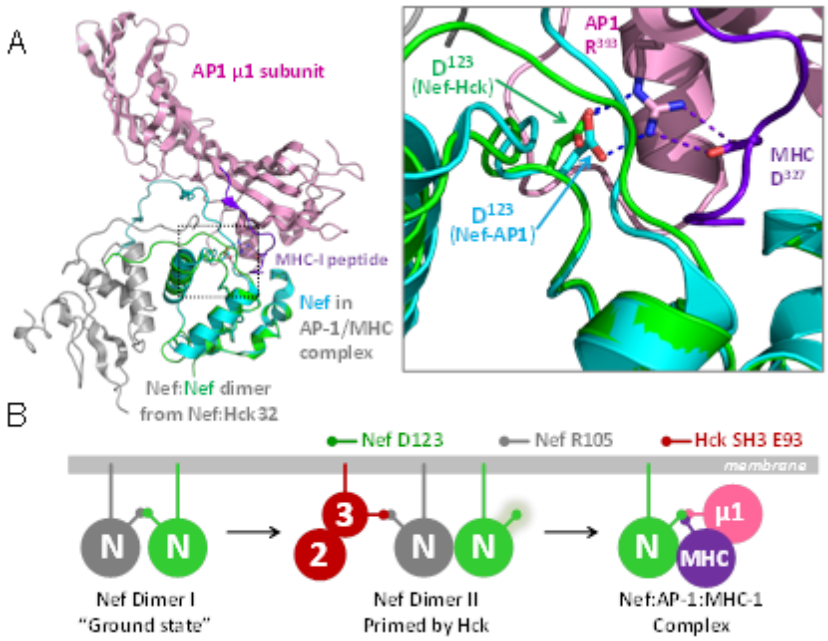

Figure 2. Alignment of X-ray crystal structures of our Nef-SF2:Hck-SH3-SH2 complex with that of Nef and AP1/MHC-1. A) Recent crystal structure of the Nef core in complex with an MHC-I peptide and the clathrin adaptor protein AP-1 (μ1 subunit), with Nef rendered in blue (PDB: 4EN2). Alignment of Nef in this complex with our new Nef:SH3-SH2 structure showed a remarkable degree of structural similarity in the Nef core (our Nef in green). A close-up view of the electrostatic network between Nef D123, AP1 R393, and MHC-I D327 is shown on the right (boxed area from overall structure). Note that D123 is positioned very similarly in both structures. B) Model of conformational changes in Nef resulting from Hck binding. In the "ground state," Nef dimers form at the membrane, with D123 pairing with R105 (as per Figure 2C). Hck binding results in reorganization of the Nef core, with Nef R105 contacting Hck SH3 E93, exposing D123 for association with AP1:MHC-I. These interactions occur in reciprocal fashion in the context of the dimer; single interactions are shown for clarity.

Finally, we have developed a high throughput-compatible screening assay for direct identification of Nef inhibitors. This cell-based assay for HIV-1 Nef dimerization was developed using bimolecular fluorescence complementation (BiFC) of Nef-YFP (yellow fluorescent protein) fusion proteins and the Cellomics ArrayScan II automated cellular imaging platform. The assay involves co-expression of non-fluorescent, complementary fragments of YFP fused to Nef in 293T cells. Dimerization of Nef leads to juxtaposition of the YFP fragments and reconstitution of the fluorophore. The coding regions of both Nef-YFP fusions, as well as an independent transfection marker (mRFP), were coupled in a single expression vector separated by picornavirus '2A' peptide sequences to permit translation of all three proteins from a single transcript. The ArrayScanII system simultaneously records information about BiFC (dimerization) and mRFP (transfection efficiency) intensities in a 384-well plate format. Validation studies revealed a critical role for gating on the mRFP signal to identify the subpopulation of transfected cells. BiFC signals from wild-type Nef versus a dimerization-defective mutant were very clearly separated, with Z'-factors consistently in the 0.6-0.7 range. An automated pilot screen of 1,597 compounds (NIH Diversity Set III) flagged five active compounds, three of which were confirmed in subsequent dose-response assays. The antiretroviral activity of these compounds is currently under investigation. Coupling bimolecular fluorescence complementation of Nef-YFP with the ArrayScanII platform enables cell-based, high-throughput screening of chemical libraries for direct identification of small molecules that interfere with Nef dimerization. A manuscript describing this assay is currently in preparation and will be submitted to the *Journal of Biomolecular Screening*. The principle of the assay is illustrated in Figure 3.

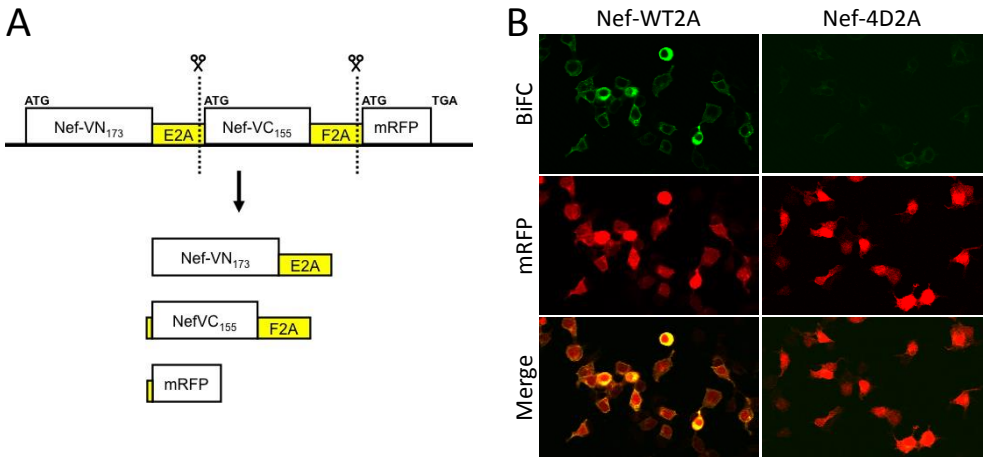

Figure 3. Design of the Nef-WT2A BiFC plasmid system for cell-based high-throughput screening for inhibitors of HIV-1 Nef dimerization. A) Cartoon of the Nef-WT2A transcript and subsequent 2A-mediated cleavage products. As indicated, each coding region contains its respective start codon (ATG) while a single stop codon is present at the end (TGA). The co-translational break points are indicated, yielding three independent proteins. These include the two Nef-BiFC fusions (Nef-VN and Nef-VC) as well as the RFP reporter. Similarly, a negative control 2A-plasmid was generated containing the dimerization-deficient Nef mutant, 4D. B) Expressed in 293T cells, the WT2A plasmid exhibits a strong BiFC signal localized to the cell periphery, while the negative control (4D2A) showed remarkable reduction in BiFC despite comparable levels of protein expression (RFP channel). These results are consistent with our previous findings under a dual-plasmid BiFC system.

18. Extent of Clinical Activities Initiated and Completed. Items 18(A) and 18(B) should be completed for all research projects. If the project was restricted to secondary analysis of clinical data or data analysis of clinical research, then responses to 18(A) and 18(B) should be "No."

18(A) Did you initiate a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
X_____ No

18(B) Did you complete a study that involved the testing of treatment, prevention or diagnostic procedures on human subjects?

_____Yes
X_____No

If "Yes" to either 18(A) or 18(B), items 18(C) – (F) must also be completed. (Do NOT complete 18(C-F) if 18(A) and 18(B) are both "No.")

18(C) How many hospital and health care professionals were involved in the research project?

_____Number of hospital and health care professionals involved in the research project

18(D) How many subjects were included in the study compared to targeted goals?

_____Number of subjects originally targeted to be included in the study
_____Number of subjects enrolled in the study

Note: Studies that fall dramatically short on recruitment are encouraged to provide the details of their recruitment efforts in Item 17, Progress in Achieving Research Goals, Objectives and Aims. For example, the number of eligible subjects approached, the number that refused to participate and the reasons for refusal. Without this information it is difficult to discern whether eligibility criteria were too restrictive or the study simply did not appeal to subjects.

18(E) How many subjects were enrolled in the study by gender, ethnicity and race?

Gender:
_____Males
_____Females
_____Unknown

Ethnicity:
_____Latinos or Hispanics
_____Not Latinos or Hispanics
_____Unknown

Race:
_____American Indian or Alaska Native
_____Asian
_____Blacks or African American
_____Native Hawaiian or Other Pacific Islander
_____White
_____Other, specify:_____
_____Unknown

18(F) Where was the research study conducted? (List the county where the research study was conducted. If the treatment, prevention and diagnostic tests were offered in more than one county, list all of the counties where the research study was conducted.)

19. Human Embryonic Stem Cell Research. Item 19(A) should be completed for all research projects. If the research project involved human embryonic stem cells, items 19(B) and 19(C) must also be completed.

19(A) Did this project involve, in any capacity, human embryonic stem cells?
_____Yes
X_____No

19(B) Were these stem cell lines NIH-approved lines that were derived outside of Pennsylvania?
_____Yes
_____ No

19(C) Please describe how this project involved human embryonic stem cells:

20. Articles Submitted to Peer-Reviewed Publications.

20(A) Identify all publications that resulted from the research performed during the funding period and that have been submitted to peer-reviewed publications. Do not list journal abstracts or presentations at professional meetings; abstract and meeting presentations should be listed at the end of item 17. **Include only those publications that acknowledge the Pennsylvania Department of Health as a funding source** (as required in the grant agreement). List the title of the journal article, the authors, the name of the peer-reviewed publication, the month and year when it was submitted, and the status of publication (submitted for publication, accepted for publication or published.). Submit an electronic copy of each publication or paper submitted for publication, listed in the table, in a PDF version 5.0.5 (or greater) format, 1,200 dpi. Filenames for each publication should include the number of the research project, the last name of the PI, the number of the publication and an abbreviated research project title. For example, if you submit two publications for PI Smith for the "Cognition and MRI in Older Adults" research project (Project 1), and two

publications for PI Zhang for the "Lung Cancer" research project (Project 3), the filenames should be:

Project 1 – Smith – Publication 1 – Cognition and MRI
Project 1 – Smith – Publication 2 – Cognition and MRI
Project 3 – Zhang – Publication 1 – Lung Cancer
Project 3 – Zhang – Publication 2 – Lung Cancer

If the publication is not available electronically, provide 5 paper copies of the publication.

Note: The grant agreement requires that recipients acknowledge the Pennsylvania Department of Health funding in all publications. Please ensure that all publications listed acknowledge the Department of Health funding. If a publication does not acknowledge the funding from the Commonwealth, do not list the publication.

Title of Journal Article:	Authors:	Name of Peer-reviewed Publication:	Month and Year Submitted:	Publication Status (check appropriate box below):
1. Nef Alleles from all major HIV-1 clades activate Src-family kinases and enhance HIV-1 replication in an inhibitor sensitive manner	Narute, P. and Smithgall, T.E.	PLoS ONE 7: e32561	Sept. 2012	☐Submitted ☐Accepted X Published
2. HIV-1 Nef Interaction Influences the ATP-binding site of the Src-Family Kinase Hck	Pene-Dumitrescu, T., Shu, S., Wales, T., Alvarado, J.J., Shi, H., Narute, P., Moroco, J.A., Yeh, J.I., Engen, J.R. and Smithgall, T.E.	BMC Chem. Biol. 12: 1, 2012.	Sept. 2011	☐Submitted ☐Accepted X Published

20(B) Based on this project, are you planning to submit articles to peer-reviewed publications in the future?

Yes X____ No_____

If yes, please describe your plans:

The following mauscripts have been written and will be submitted before the end of March 2013:

Alvarado, J.J., Tarafdar, S., Yeh, J.I., and Smithgall, T.E. Interaction with the Src-family kinase Hck SH3-SH2 region reorients the HIV-1 Nef dimer for kinase activation and effector recruitment. Submission planned to *Nature Structural and Molecular Biol.*

Poe, J.A., Vollmer, L., Smithgall, T.E., and Vogt, A. Development and validation of a high-content bimolecular fluorescence complementation assay for small molecule inhibitors of HIV-1 Nef dimerization. To be submitted to the *Journal of Biomolecular Screening*.

Trible, R.P., Narute, P., Atkins, K., Thomas, L., Yanamala, N, Emert-Sedlak, L., Korotchenko, V., Day, B., Thomas, G. and Smithgall, T.E. Discovery of a diaminoquinoxaline benzenesulfonamide antagonist of HIV-1 Nef function using a yeast-based phenotypic screen.Submitted for publication to *BMC Retrovirology*.

21. Changes in Outcome, Impact and Effectiveness Attributable to the Research Project. Describe the outcome, impact, and effectiveness of the research project by summarizing its impact on the incidence of disease, death from disease, stage of disease at time of diagnosis, or other relevant measures of outcome, impact or effectiveness of the research project. If there were no changes, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

None

22. Major Discoveries, New Drugs, and New Approaches for Prevention Diagnosis and Treatment. Describe major discoveries, new drugs, and new approaches for prevention, diagnosis and treatment that are attributable to the completed research project. If there were no major discoveries, drugs or approaches, insert "None"; do not use "Not applicable." Responses must be single-spaced below, and no smaller than 12-point type. DO NOT DELETE THESE INSTRUCTIONS. There is no limit to the length of your response.

Work supported by this grant has enabled development of new technology for antiretroviral drug discovery. In addition, we have discovered several lead compounds that will be further developed to improve their *in vivo* pharmacology as a next step towards clinical translation.

23. Inventions, Patents and Commercial Development Opportunities.

23(A) Were any inventions, which may be patentable or otherwise protectable under Title 35 of the United States Code, conceived or first actually reduced to practice in the performance of work under this health research grant? Yes____ No X

If "Yes" to 23(A), complete items a – g below for each invention. (Do NOT complete items a - g if 23(A) is "No.")

a. Title of Invention:

b. Name of Inventor(s):

c. Technical Description of Invention (describe nature, purpose, operation and physical, chemical, biological or electrical characteristics of the invention):

d. Was a patent filed for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____

If yes, indicate date patent was filed:

e. Was a patent issued for the invention conceived or first actually reduced to practice in the performance of work under this health research grant?
Yes_____ No____
If yes, indicate number of patent, title and date issued:
Patent number:
Title of patent:
Date issued:

f. Were any licenses granted for the patent obtained as a result of work performed under this health research grant? Yes_____ No____

If yes, how many licenses were granted?_____

g. Were any commercial development activities taken to develop the invention into a commercial product or service for manufacture or sale? Yes___ No____

If yes, describe the commercial development activities:

23(B) Based on the results of this project, are you planning to file for any licenses or patents, or undertake any commercial development opportunities in the future?

Yes X_____ No_____

If yes, please describe your plans:

We hope to file a US Patent on the novel compounds directed to HIV-1 Nef as described in the research report summary.

24. Key Investigator Qualifications. Briefly describe the education, research interests and experience and professional commitments of the Principal Investigator and all other key investigators. In place of narrative you may insert the NIH biosketch form here; however, please limit each biosketch to 1-2 pages.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person.

NAME	POSITION TITLE
Thomas E. Smithgall	William S. McEllroy Professor and Chair Microbiology and Molecular Genetics
eRA COMMONS USER NAME tsmithga	

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
University of Pennsylvania, Philadelphia, PA	B.A.	1981	Biochemistry
University of Pennsylvania, Philadelphia, PA	Ph.D.	1986	Pharmacology
University of Pennsylvania, Philadelphia, PA	Postdoc	1986-87	Pharmacology
National Cancer Institute, NIH, Bethesda, MD	Postdoc	1987-90	Molecular Biology

Positions and Honors

Positions and Employment

1990-91 Research Assistant Professor, Dept. Pharmacology, Georgetown University, Washington, DC
1991-98 Asst./Assoc. Professor, Eppley Cancer Institute, U. Nebraska Medical Center, Omaha, NE
1998-01 Assoc. Prof., Micro. Mol. Genetics, Univ. Pittsburgh School of Medicine, Pittsburgh, PA
2001- Professor, Micro. Mol. Genetics, Univ. Pittsburgh School of Medicine, Pittsburgh, PA
2009- Chair, Microbiology and Mol. Genetics, Univ. Pittsburgh School of Medicine, Pittsburgh, PA

Selected Honors, Awards and Service:

1983-1986 Pharmaceutical Manufacturers Association Foundation Advanced Predoctoral Fellowship
1987-1989 Pharmacology Research Associate Training (PRAT) Fellowship, NIH, Bethesda, MD
1989-1990 Biotechnology Training Program Fellowship, NCI, NIH, Bethesda, MD
1993-1995 Junior Faculty Research Award, American Cancer Society
1995-1996 NIH, Cellular Biology and Physiology 2 (CBY-2) Study Section
2002 Outstanding Investigator Award, Micro. Mol. Genetics, University of Pittsburgh
2005, 2006 NIH Cancer Molecular Pathobiology (CAMP) Study Section
2008 NIH, NIGMS, Human Embryonic Stem Cells Program Project Grant Review
2006-2008 American Cancer Society, Tumor Biology and Endocrinology Study Section Member
2008, 2009 NIH Molecular Oncology (MONC) Study Section
2009- William S. McEllroy Endowed Professorship, University of Pittsburgh
2010 Distinguished Mentor Award Finalist, Biomedical Graduate Student Association University of Pittsburgh

Recent Peer-reviewed Publications (of 113 total):

Lerner, E.C. and Smithgall, T.E. SH3-dependent stimulation of Src family kinase autophosphorylation without tail displacement from the SH2 domain *in vivo*. **Nature Structural Biology** 9**:** 365-369, 2002.

Choi, H.-J. and Smithgall, T.E. HIV-1 Nef promotes survival of TF-1 macrophages by inducing Bcl-$_{XL}$ expression in an Erk-dependent manner. **J. Biol. Chem.** 279: 51688-51696, 2004.

Meyn, M.A. III, Schreiner, S.J., Dumitrescu, T.P., Nau, G.J., and Smithgall, T.E. Src family kinase activity is required for murine embryonic stem cell growth and differentiation. **Mol. Pharmacol.** 68: 1320-1330, 2005.

Delfino, F.J., Stevenson, H.M., & Smithgall, T.E. Colon cancer-associated mutations inhibit Fes tyrosine kinase activity and suggest a novel role as a tumor suppressor. **J. Biol. Chem.** 281: 8829-8835, 2006.

Trible, R., Emert-Sedlak, L. and Smithgall, T.E. HIV-1 Nef selectively activates Src family kinases Hck, Lyn, and c-Src through direct SH3 domain interaction. **J. Biol. Chem.** 281: 27029-27038, 2006.

Meyn III, M.A., Wilson, M.B., Abdi, F.A., Fahey, N., Schiavone, A.P., Wu, J., Hochrein, J.M., Engen, J.R., and Smithgall, T.E. Src family kinases phosphorylate the Abl SH3-SH2 region and modulate Bcr-Abl transforming activity. **J. Biol. Chem.** 281: 30907-30916, 2006. Recommended by the Faculty of 1000/Biology.

Trible, R.P., Emert-Sedlak, L., Wales, T., Ayyavoo, V., Engen, J.R. and Smithgall, T.E. Allosteric Loss-of-function Mutations in HIV-1 Nef from a Long-Term Non-Progressor. **J. Mol. Biol.** 374: 121-129, 2007. PMCID: PMC2117379

Dumitrescu, T.P., Peterson, L.F., Donato, N.J., and Smithgall, T.E. An inhibitor-resistant mutant of Hck protects CML cells against the anti-proliferative and apoptotic effects of the broad-spectrum Src-family kinase inhibitor, A-419259. **Oncogene** 27: 7055-7069, 2008.

Iacob, R.E., Dumitrescu, T.P., Zhang, J., Gray, N.S., Smithgall, T.E., and Engen J.R. Conformational disturbance in Abl kinase upon mutation and deregulation. **Proc. Natl. Acad. Sci., U.S.A.** 106: 1386-1391, 2009. PMCID: PMC2635808

Poe, J.A. and Smithgall, T.E. Bimolecular fluorescence complementation reveals residues essential for HIV-1 Nef oligomerization, CD4 downregulation and viral replication. **J. Mol. Biol.** 394: 329-342, 2009.

Emert-Sedlak, L., Kodama, T., Lerner, E.C., Trible, R.P., Dai, W., Foster, C., Day, B., Lazo, J.S., and Smithgall, T.E. Chemical library screens targeting an HIV-1 Nef Accessory Factor/host cell kinase complex identify novel anti-retroviral compounds. **ACS Chemical Biol.** 4: 939-947, 2009. PMCID: PMC2861989

Meyn III, M.A. and Smithgall, T.E. Chemical Genetics Identifies c-Src as an Activator of Primitive Ectoderm Formation in Murine Embryonic Stem Cells. **Science Signaling 2**: ra64, 2009. Cover Feature. Cited as Must Read by the Faculty of 1000/Biology. Podcast.

Pene-Dumitrescu, T.P. and Smithgall, T.E. Expression of a Src-Family Kinase in CML Cells Induces Resistance to Imatinib in a Kinase-dependent Manner. **J. Biol. Chem.** 285: 21446-21457, 2010. PMCID: PMC2898387

Hellwig, S., Miduturu, C.V., Kanda, S., Zhang, J., Filippakopoulos, P., Salah, E., Deng, X., Choi, H.G., Zhou, W., Hur, W., Knapp, S., Gray, N.S. and Smithgall, T.E. Small Molecule Inhibitors of the c-Fes Protein-tyrosine Kinase. **Chem. Biol.** (Cell Press) 19: 529-40, 2012.

Narute, P. and Smithgall, T.E. Nef Alleles from all major HIV-1 clades activate Src-family kinases and enhance HIV-1 replication in an inhibitor sensitive manner. **PLoS One** 7: e32561, 2012. PMCID: PMC3290594

Pene-Dumitrescu, T., Shu, S., Wales, T., Alvarado, J.J., Shi, H., Narute, P., Moroco, J.A., Yeh, J.I., Engen, J.R. and Smithgall, T.E. HIV-1 Nef Binding Induces Allosteric Changes in the ATP-binding site of the Src-Family Kinase Hck. **BMC Chem. Biol**. 12: 1, 2012.

Panjarian, S., Iacob, R.E., Chen, S., Wales, T.E., Engen, J.R., Smithgall, T.E. Enhanced SH3:linker Interaction Overcomes Abl Kinase Activation by Gatekeeper and Myristic Acid Binding Pocket Mutations and Increases Sensitivity to Small Molecule Inhibitors. **J. Biol. Chem.**, in press.

Emert-Sedlak, L.A., Narute, P., Shu, S.T., Poe, J.A., Shi, H., Yanamala, N., Alvarado, J.J., Lazo, J.S., Johnston, P.A., and Smithgall, T.E. Effector Kinase Coupling Enables High-Throughput Screens for Direct HIV-1 Nef Antagonists with Anti-retroviral Activity. **Chem. Biol.** (Cell Press) 20: 82-91, 2013.