Report from the RAS Ad Hoc Working Group on the FNLCR RAS Initiative

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Executive Summary

The RAS Initiative, under the expert direction of Dr. Frank McCormick, has achieved considerable success in each of its projects. The research team has uncovered new insights into RAS biology and developed reagents and assay platforms available to academics and industry researchers. The screening approaches are leading the field into uncharted biological territory and appear well positioned to serve as robust platforms for current and future collaborations and to accelerate the development of RAS-directed therapies. Recommendations:

1. The burgeoning industry collaborations that involve this RAS initiative should be used to identify lessons learned that guide development of a generalizable framework for how such activities might be extended going forward. Examples might include systematic target validation approaches, establishing avenues capable of further lead compound development, and new types of service models to engage the broader community.
2. Continuing development of large-scale approaches to both identify and validate new compounds that selectively inhibit wild-type or mutant RAS. In particular, future efforts could determine how the new (and emerging) RAS structures can inform drug screens. Of particular interest might be the expansion of in silico screens given that the DOE has a 1 billion compound library.
3. Further augmentation of the biochemical advances should be prioritized: one goal herein might be to develop an in vitro system in which RAS successfully functions in the plasma membrane to activate effectors.
4. Consider efforts to resolve RAS-effector complexes using cryo-EM.

Introduction

The FNLAC RAS Working Group was formed to advise various strategic, technical, and scientific aspects of the NCI RAS Initiative, and to present its findings and recommendations to the Frederick National Laboratory Advisory Committee (FNLAC). Specific goals included: (1) provision of feedback and suggestions to Dr. Frank McCormick and his team at FNLCR, (2) regular, candid assessments of program aspects that are working well and areas where improvements or pivots might be needed, and (3) ensuring optimal connectivity between the FNLCR RAS initiative and the extramural community. Accordingly, this working group held seven meetings between July, 2014 and August, 2016 to carry out this work.

When the RAS Initiative was established, Dr. McCormick articulated five projects that would be pursued during the initial 5-year funding period. Project 1 aimed to determine which effectors are engaged by each of the mutant proteins, solve the structures of mutant RAS proteins complexed with relevant effectors, and identify new opportunities for small-molecule therapeutics. Project 2 sought to identify KRAS-selective compounds. Project 3 focused on the development of imaging methods to identify KRAS complexes in cells and the design of screens to disrupt those complexes. Project 4 aspired to map the surface of KRAS cancer cells to identify “antigens” that might be targeted by immunotherapy, or nanoparticles for improved drug delivery. Project 5 would pursue synthetic lethal screens in advanced model systems such as three-dimensional (3-D) cultures or murine xenografts; test combinations of siRNAs, shRNAs, and/or small molecules; and incorporate new technologies in order to address the limitations of prior synthetic lethal screens.

Project 4 was initiated as a pilot proteomics study, and resulted in two manuscripts currently being prepared for publication based on this work. Thereafter, RAS Initiative efforts were consolidated around the remaining four projects based on their increasing momentum as well as decisions regarding the optimal alignment of domain expertise resident at FNLCR. Project 5 initiated with an RNAi-based effort at FNLCR to interrogate various RAS signaling nodes. The synthetic lethality avenue of this project eventually became the focus of an NCI U01 funding mechanism to engage the extramural community (RAS Synthetic Lethal Network). Thus, the focus of this report

involves the **four major areas that comprise the corpus of the RAS project: (1) biophysical characterization, (2) structural biology, (3) assay development and small-molecule screening, and (4) community interactions.**

Biophysical Characterization of KRAS

Progress in the characterization of processed full length KRAS4B and its interaction with cellular membranes stands as a highlight of the RAS Initiative to date. KRAS binds tightly to RAF kinase *in vitro*, but activates RAF kinase only at the plasma membrane *in vivo*. Thus, it is important to understand the mechanics of RAS-membrane interaction not only to clarify RAS signal transduction, but also to facilitate future drug discovery efforts directed against RAS itself. At the outset, the large-scale production of processed KRAS proteins comprised a concrete deliverable that would benefit the larger scientific community.

Using engineered baculovirus in insect cells, the RAS team synthesized fully processed KRAS (5 mg/L). They also leveraged lipid nanodiscs as membrane mimics to verify published observations that processed KRAS association with membranes is modulated in a phosphatidylserine-dependent manner. Comparisons of GTPase rates among mutant and wild-type RAS proteins showed no difference in GTPase rates between non-processed RAS and fully processed RAS except in the G13D mutant, in which processed RAS showed lower activity than its non-processed counterpart. They have developed a protocol for producing farnesylated and methylated (FMe) KRAS4B in insect cells and also have shown that the protein is active in hydrolyzing GTP and binds to RAF-RBD (RAS binding domain) on lipid nanodiscs. These data were published in the November 15, 2015 issue of *Nature Scientific Reports*. The RAS laboratory groups have also measured the intrinsic GTPase rate, the nucleotide loading rates, and the binding affinities to RAF for a panel of key RAS mutants.

Thus, a variety of biophysical tools, including an artificial nanodisc membrane, have been used to analyze the orientation of RAS in the plasma membrane. These tools also have been used to evaluate the effects of the nucleotide state, and evaluate the role of lipid composition on effector interactions, RAS orientation, and RAF activation. To maximize the value of these research findings, the FNLCR has made the reference reagents available for distribution to the RAS research community. KRAS membrane interactions provide many opportunities for drug discovery, including the identification of novel drug-binding pockets, structural analysis of KRAS on a membrane, and validation of dimer interactions.

The Biochemistry and Biophysics Group used paramagnetic relaxation enhancement–nuclear magnetic resonance (PRE-NMR) and nuclear reflectivity to study the structure of RAS on the membrane. Compared with GDP-bound protein, there were a number of key areas within the processed KRAS-GppNHp structure that have important flexibility (particularly in the switch I and II regions) in the activated state. The PRE-NMR findings at FNLCR closely match the predicted conformational changes that have been previously published. Neutron reflectivity analysis done in collaboration with NIST has revealed that in both GDP and GppNHp conformations, RAS is extended away from the bilayer and RAF does not change the orientation of RAS on the membrane. Thus, KRAS may exist in two potential RAF-binding conformations.

Ongoing studies include collaborations with the Oak Ridge National Laboratory to build PRE-NMR data into molecular dynamics simulations; collaboration with the Department of Energy (DOE) to build, refine, and test models of RAS-RAF-membrane interactions using supercomputing capabilities; identification of KRAS residues critical to binding RAF and/or membranes; and use of such biophysical methods as cryo-electron microscopy (cryo-EM) and surface plasma resonance (SPR) binding analysis to further characterize binding and orientation of KRAS-CRAF and KRAS-GDP on the membrane. These studies and techniques have provided a detailed biophysical characterization of KRAS and its effectors in biological membranes that set the stage for ongoing compound screening programs (outlined below). The depth and breadth of new biology that has been unearthed should certainly enhance the rigor and precision of these drug discovery efforts.

KRAS Structural Biology

Progress in the Structural Biology program for the RAS Initiative, led by Dr. Dhirendra Simanshu, has also been excellent. While past structural analyses of RAS have been significant, much of that work focused on HRAS rather than KRAS. Furthermore, there have been limited analyses of KRAS in complex with key regulators and effectors. RAS Initiative efforts in this regard are focused on KRAS4B, the protein encoded by the most prevalent splice variant of KRAS. Two goals of this program are to determine the structures of (1) wild-type (WT) and tumor-associated mutants of KRAS4B in their active GTP-bound states and (2) KRAS4B complexes with various effectors (e.g., CRAF) and regulatory proteins (e.g., p120 RasGAP/RASA1).

For the first goal, Dr. Simanshu reported that the crystal structures for the G-domain fragments (residues 1-169) GTP-bound WT and six mutant forms (G12C, G12D, G12V, G13D, Q61L, and Q61R) have now been solved by RAS program scientists. These structures reveal how common oncogenic KRAS mutations affect conformation of the two critical regions that impact effector and regulatory protein interactions (switch I and switch II) and perturb intrinsic and GAP-stimulated GTP hydrolysis. These observations are largely in agreement with previous studies. In WT KRAS4B, the conformation of switch 1 varies between GDP and GMPPNP with regards to the coordination of Mg. In the G12C mutant the coordination of Mg is not much different with the different nucleotides, but compared to WT KRAS, the mutation clashes with residue Y32 and impairs GAP-stimulated GTP hydrolysis through clashes with the GAP arginine finger (critical for GAP activity). The G13D mutation caused similar structural/biochemical perturbations. The G12D and G12V mutant do not clash with Y32 but do clash with the arginine finger of RASA1. The Q61L substitution is hydrophobic (and hence buried) and cannot interact with the catalytic water, disrupting GAP-stimulated GTP hydrolysis. Here, the arginine finger is positioned correctly, but since it lacks the amide group it cannot fit inside the KRAS4B catalytic pocket. While these efforts have focused on the most prevalent KRAS mutations, future analyses may extend into additional mutants. All of these structures will be made available to the scientific community.

For the second (and more challenging) goal, solving the KRAS4B structure in complex with its key effector, CRAF, has been limited by the inability to express full length CRAF. Indeed, all previous structures have been carried out using the isolated Ras-binding domain of CRAF. However, the RAS team at FNLCR has made some progress in their efforts to determine the crystal structures of KRAS4B complexes with two RasGAPs, encoded by NF1 (neurofibromin) and RASA1 (p120 RasGAP). In particular, co-structures were solved with the Q61L and Q61R mutant proteins together with RASA1. In addition, one NF1 GAP domain construct, GAP333 (1198-1530), has been designed, but extensive crystallization screening in complex with KRAS4b has not yet produced successful hits. The next steps will be to design new constructs, GAP314 (1209-1523) and GAP254 (1209-1463), and continue the work.

Furthermore, significant progress has been made toward understanding the KRAS4B interaction with phosphodiesterase delta (PDEδ). PDEδ acts as a chaperone that facilitates transit of KRAS4B from the ER to the plasma membrane, a critical location for KRAS4B effector interaction and activation. Disrupting PDEδ interaction with KRAS4B is a provocative approach for blocking KRAS4B function and recent studies have developed small molecule inhibitors of this interaction (e.g., deltarasin). Current knowledge of how PDEδ interacts and regulates KRAS4B is limited, with some conflicting observations in the literature. The RAS team successfully resolved two crystal structures (forms I and II) of fully processed, full length KRAS4B (farnesylated and carboxylmethylated (KRAS4b-FME) in complex with PDEδ. These results show that the KRAS4b/PDEδ interaction is restricted to the last six amino acids in the C-terminus. Structural analyses also reveal that in crystal form I, the FME residue C185 binds deeper inside the hydrophobic pocket of PDEδ. In addition, comparing two different crystal forms of the KRAS4b-PDEδ complex revealed two different modes of binding and provided the first view of the hypervariable region (HVR) in any RAS structure. All previous structural determinations of RAS have lacked the HVR due to its destabilization of the G-domain. Another significant finding from these studies is the importance of sequences upstream of the farnesylated cysteine residue, suggesting that PDEδ

interactions with prenylated proteins may be more selective and restricted than currently believed. This study is now accepted for publication in *PNAS*, and data for crystal forms I and II will be submitted to the Protein Data Bank.

Future directions for the Structural Biology group will include continued structural studies on KRAS4B in complex with GAPs, effectors, and other RAS-binding proteins. *In silico* screening will also be done with KRAS4B complexes to identify lead compounds in hopes of discovering small molecules that block effector interactions to force mutant KRAS to cycle back to its inactive state.

Assay Development and Screening for Small-molecule KRAS Perturbagens

The RAS program has invested considerable effort toward the development of assays that enable various small molecule screening efforts to disrupt RAS dimerization, membrane localization, and downstream effector activation. Examples include a cell-based assay that leverages the NanoLuc® technology to assess KRAS-CRAF protein-protein interactions; split NanoLuc enzymes fused to KRAS and CRAF; and isogenic mouse embryo fibroblasts (MEFs) cell lines that ectopically express wild-type HRAS or KRASG12D (or other mutant KRAS genes), which were generated using parental “RAS-less” cells from Dr. Mariano Barbacid’s laboratory. Considerable progress was also made in developing a physiological system for evaluating tagged (Halo) RAS molecules in live single cells using the step analysis of single molecule photobleaching.

These and other assays have been used in various small-molecule screens for various RAS perturbagens. For example, a proliferation screen was conducted in collaboration with the NIH National Center for the Advancement of Translational Sciences (NCATS), using a library enriched for tool compounds (i.e., chemical probes) and U.S. Food and Drug Administration (FDA)-approved drugs. Initial results suggest that oncogenic MEFs may be more sensitive to HSP90 inhibitors.

One of the most impressive successes of this RAS program project has been the extent to which multiple collaborations have been established with pharmaceutical and biotechnology companies to carry out small-molecule screens using the assays developed at FNLAC. Collaborations are being done at multiple stages of drug development: Discovery Chemistry with Daiichi Sankyo, Sanofi, Eli Lilly and the NCI; Hit-to-Lead Validations with NCATS; and Mechanism of Action studies with the Beatson Institute, Eli Lilly, and TOSK, Inc.

Some of these collaborations are quite extensive; for example, one pharmaceutical partnership involves pilot screens using oncology reference compounds for screen validation, and will progress to include an HTS of up to 1,000,000 compounds. Active hits will be screened in a larger panel using the RAS/RAF-dependent MEFs, and those with specific activity towards KRAS mutant cell lines will be returned to the FNLCR for mechanistic studies. The also exhibit considerable innovation; for example, another study involves fragment based drug screens to identify allosteric inhibitors of KRAS effector interactions. This project will use NMR and SPR to characterize KRAS binding, followed by a primary/secondary KRAS effector interaction assay and a cell based screen.

Overall, the level of interest and engagement from both the academic and biopharmaceutical sectors with the FNLCR RAS program assay development and screening efforts substantially overshoots even the most optimistic expectations held at the start of the project.

Interactions Between the RAS Initiative and the Scientific Community

The RAS Initiative has put into place a wide range of elements that constitute their community outreach program. The stated goal of this effort has been to “facilitate connections between and among academic and industry researchers, both within the US and abroad, to bring new ideas and technologies to bear on RAS.” The program involves four elements. (1) providing reference reagents to the research community; (2) establishing collaborations with the research community and (3) disseminating RAS related information through maintenance of a RAS Initiative website and by hosting workshops and meetings to promote face-to-face information exchange; and (4) identifying and promoting funding opportunities to facilitate RAS research.

Regarding the provision of reference reagents (first element), 116 individual plasmids have been sent out to 75 different laboratories (both academic and industry) spanning 23 countries on 5 continents to date. In addition, four RAS Pathway Clone Collection 2.0 kits (containing 180 genes and 360 clones) have been distributed. Moreover, reagents for producing KRAS4B-FME have been provided to 10 academic labs and to commercial entities. Ongoing efforts are focused on providing additional wild type (WT) and mutant RAS constructs and proteins to the general extramural community.

Regarding the second element (establishing formal academic and industry collaborations), the RAS program activity has been impressive, as noted earlier. Examples include formal relationships with: (1) the Pancreatic Cancer Action Network predominantly to fund trainees; (2) Defense Advanced Research Projects Agency (DARPA) Big Mechanism Program to develop novel insights into RAS signaling; and (3) Department of Energy Pilot Program to determine how RAS molecules behave at the membrane of living cells. The RAS initiative has also established formal collaborations with 53 individual scientists and companies from around the world to explore common interests in RAS signaling and biology.

The third element (promoting scientific discourse and information about RAS), has included the development and maintenance of the RAS Initiative Community Outreach Website (https://www.cancer.gov/research/key-initiatives/ras/) as well as a technical forum with over 650 members to discuss research challenges and ideas (https://basecamp.com/). Moreover, seven targeted workshops and one RAS Initiative Symposium have been hosted by the RAS program. Specifically, workshops have been conducted that focused on KRAS synthetic lethality (January 2014); RAS pathway modeling and quantitative measurements (June 2014); identifying targets for nanoparticle or immune system attack on KRAS mutant tumors (July 2014 and again in November 2015); biophysical and structural analysis of processed RAS (July 2015 and May 2016 to help researchers purify processed KRAS); and a RAS Initiative workshop for small businesses (February 2016). A major RAS Symposium was held on December 15-16, 2015. It boasted presentations by nearly all the major scientists in the RAS field and attracted approximately 550 participants.

Finally, through the NCI U01 grant mechanism, a RAS Synthetic Lethal Network (RSLN) has been established, where the RAS Initiative serves as the hub for interactions between six extramural research teams. The RSLN has held two meeting with the RAS Initiative in December 2015 and August 2016.

Overall, the RAS Initiative outreach program has been exceptional in its level of success. The individual elements have nicely matured and are now fully operational. The number of investigators who are part of the outreach program (either through establishing formal collaborations, by requesting program resources, or by participating in program sponsored events) is quite impressive. A significant amount of the work that has come out of the program has been or will soon be published and disseminated to the general research community thereby raising the level of understanding of RAS in cancer. Judging by the success of the RAS workshops and the recent Symposium, the RAS research community is vibrant, enthusiastic, and highly collaborative and this is due, in part, to the outreach efforts of the RAS Initiative. The cohesion of the members of the RAS Initiative with each other and with the community in general ensures continuity with the RAS research community and its important focus on RAS and cancer.

Conclusions and Recommendations

The FNLCR RAS Initiative has achieved considerable success in each of its major projects. Under the spectacular direction of Dr. McCormick, the RAS team has developed a multitude of structures, reagents, and assays not previously available to the RAS community; in turn, this has already yielded many new insights into RAS biochemistry that should accelerate a new wave of RAS-directed small molecule screening efforts. In parallel, the small molecule screening research is leading the field into uncharted territory for RAS biology and appears on track to serve as a robust platform for many future academic and industry collaborations. All of the data generated will be shared with the broader cancer community through publications and through other established data

and reagent sharing mechanisms. The RAS Ad Hoc Working Group offers several recommendations for consideration during the next stage of this initiative:

1. The burgeoning industry collaborations that involve this RAS initiative should be used to identify lessons learned that guide development of a generalizable framework for how such activities might be extended going forward. Examples might include systematic target validation approaches, establishing avenues capable of further lead compound development, and new types of service models to engage the broader community.
2. Continuing development of large-scale approaches to both identify and validate new compounds that selectively inhibit wild-type or mutant RAS. In particular, future efforts could determine how the new (and emerging) RAS structures can inform drug screens. Of particular interest might be the expansion of in silico screens given that the DOE has a 1 billion compound library.
3. Further augmentation of the biochemical advances should be prioritized: one goal herein might be to develop an in vitro system in which RAS successfully functions in the plasma membrane to activate effectors.
4. Consider efforts to resolve RAS-effector complexes using cryo-EM.

In summary, the successes of the FNLCR RAS initiative have been enabled by a world-class set of project objectives defined up-front; talented staff; success in increasing interest in doing RAS research; and increased awareness in the importance of RAS biology. This effort seems well positioned to bring many new biological insights and to accelerate the development of RAS-directed therapeutics in the future.

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