

Frederick National Laboratory for Cancer Research



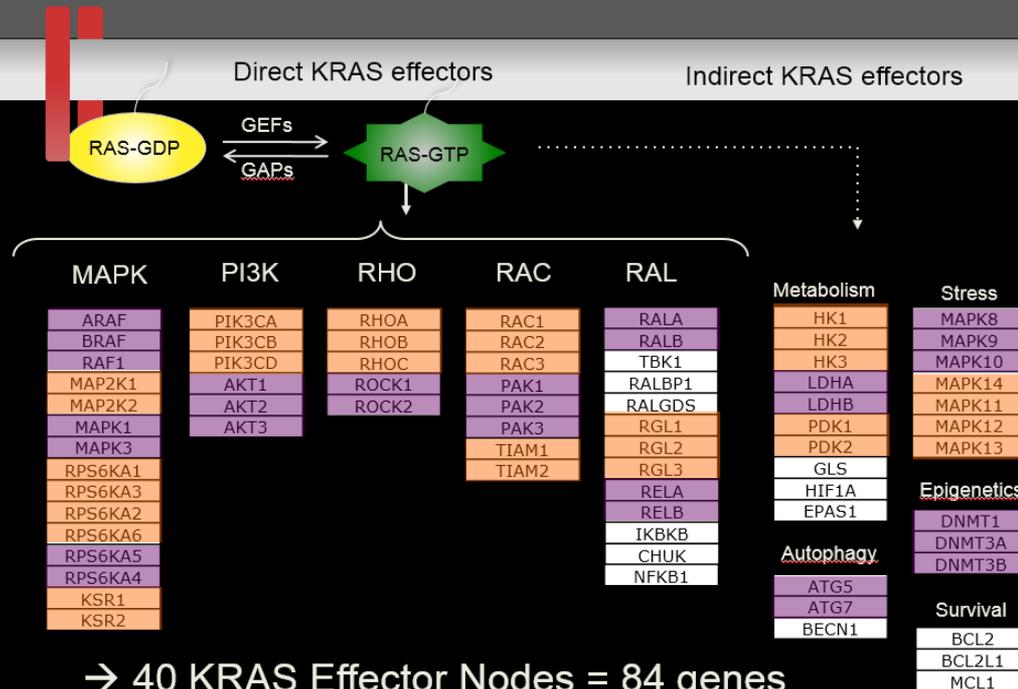
Ras Initiative Update

Frank McCormick and Levi Garraway

RAS Initiative Accomplishments: *Evaluating Ras dependency*

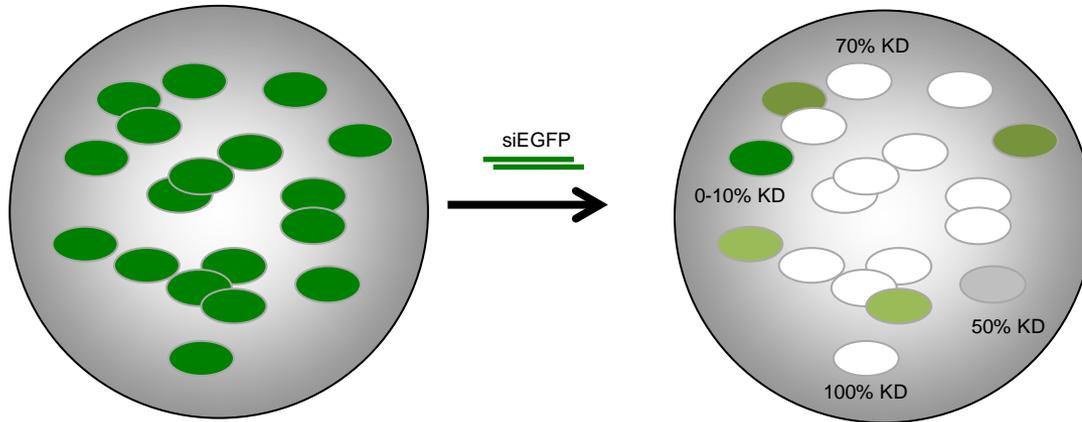
SiREN assay for Ras dependency

✧ Complete NODE knockdown: compensatory activation by redundant isoforms masks the importance of many nodes

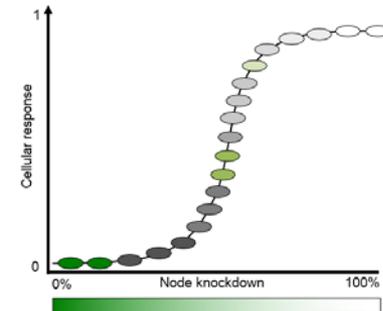
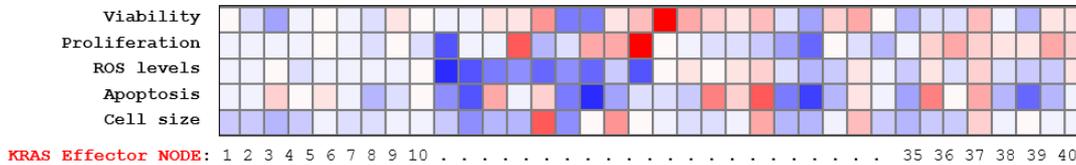


Christof Fellmann, Scott Lowe, Chih-Shia Lee, Ji Luo

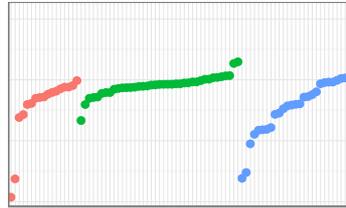
SiREN assay for Ras dependency



Effector Dependency Profile



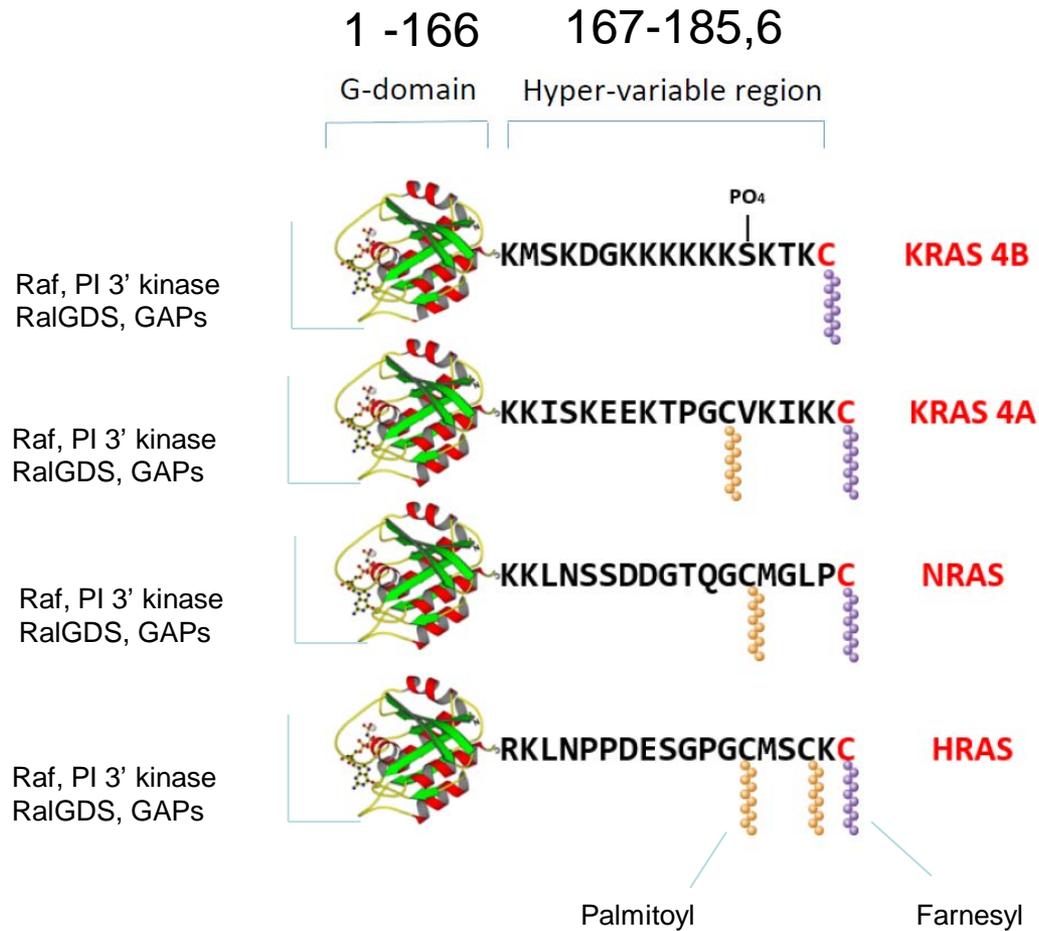
SiREN assay for Ras dependency



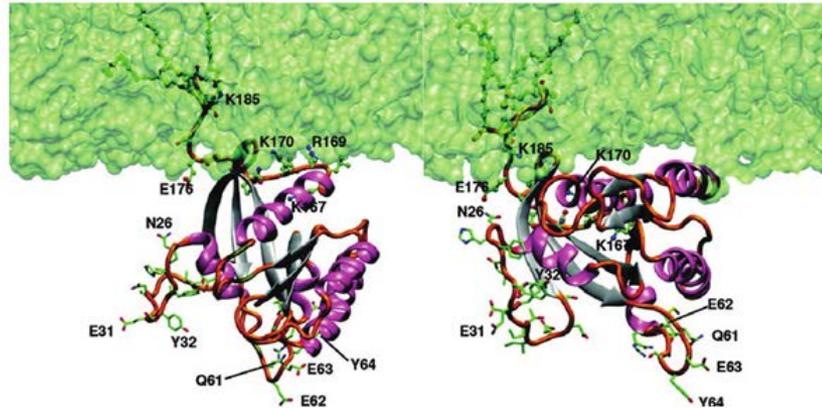
stine
Lung_NSCLC
Pancreas

RAS Initiative Accomplishments:
Biophysical and structural analysis

Ras proteins



Fully processed KRAS4b



A. Gorfe, U-Texas Houston

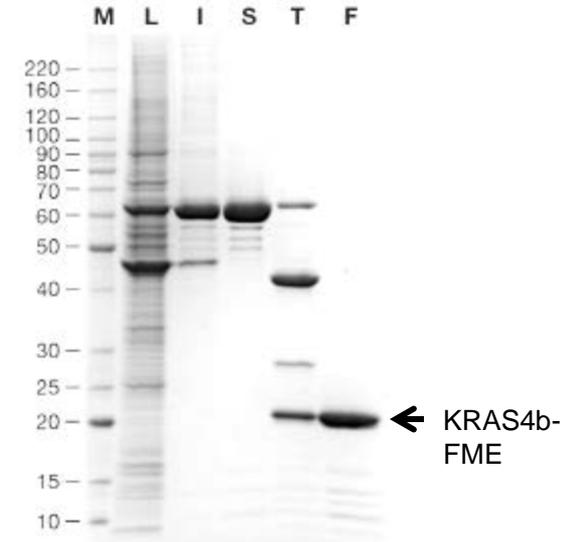
Engineering baculovirus for improved production of processed KRAS

- recombineering used to insert FNTA/FNTB genes into the baculovirus genome
- eliminated issues with coinfection of multiple viruses
- maltose-binding protein (MBP) fusion for greater yield and solubility
- *Trichoplusia ni* (Hi5) insect cells for increased yield

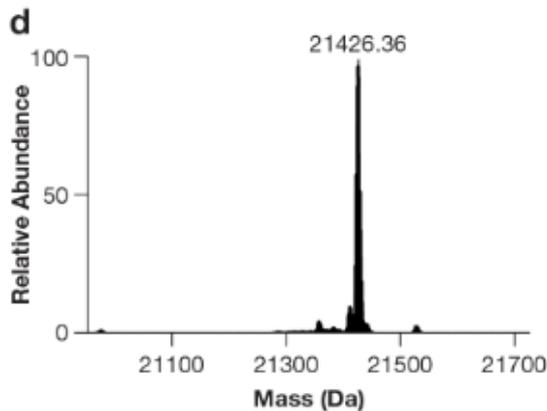
Processed KRAS4b characterization

- **Extensive protein characterization**

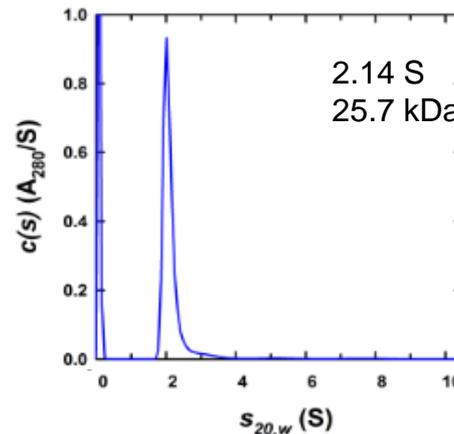
- Purified to homogeneity; yield >7mg/L
- Intact mass
- Predominantly monomeric
- Secondary structure equivalent to non-processed KRAS4b
- Lower thermal stability



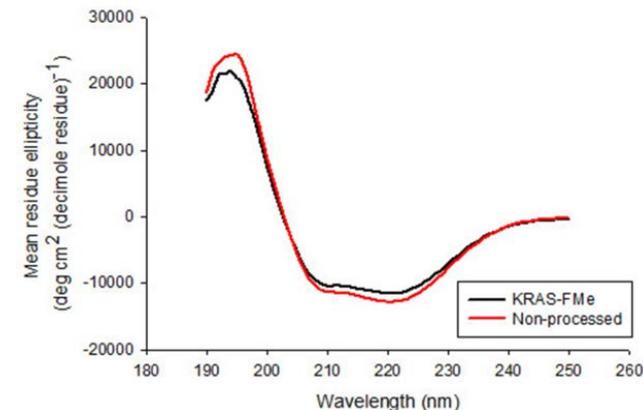
Intact mass analysis



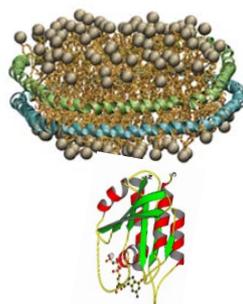
Analytical ultracentrifugation



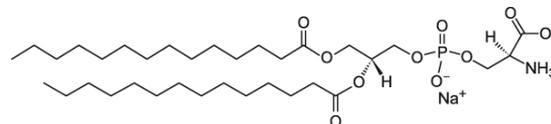
Secondary structure by CD



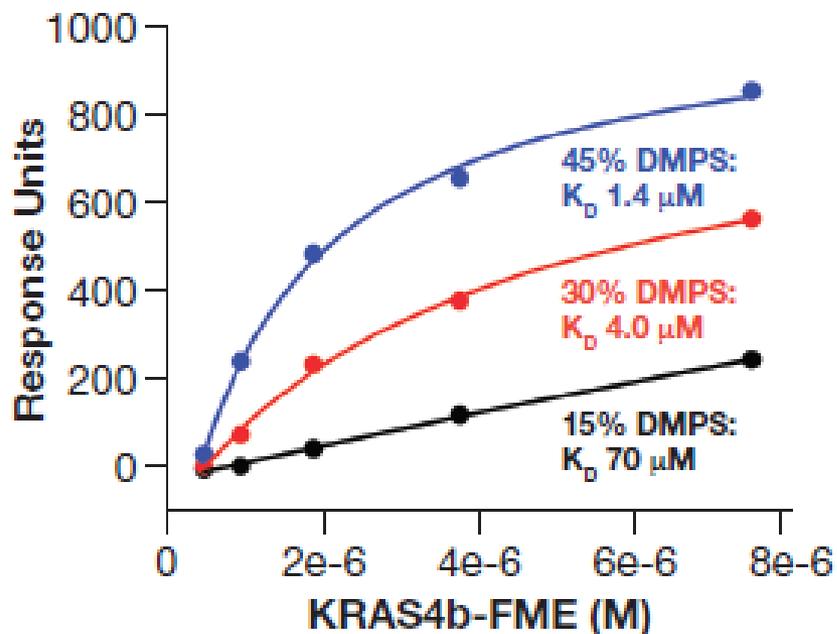
Processed KRAS4b binds to Nanodiscs in a phosphatidylserine-dependent manner



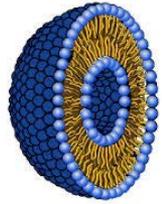
Nanodiscs containing DMPS



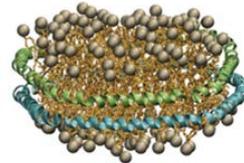
1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine



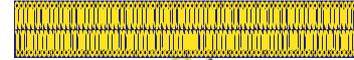
Processed KRAS enables assays, screens and structural analysis in the context of membrane



Liposomes and tethered bilayers



Lipid Nanodiscs



Processed KRAS

Characterize reagents

Assays and Screens

Structural analysis

Structures of KRAS and effectors on membranes

Protein QC
Biophysical properties
Membrane interactions

Sligar lab (U-Illinois)
Groves lab (UC-Berkeley)
Heinrich Lab (NIST)

Intrinsic GTP hydrolysis
GAP-stimulated hydrolysis
Effector binding
RBD-KRAS Alpha assays

NMR (NMRFAM)
Hi-res Cryo-EM (NCI)
Crystallography (FNL)

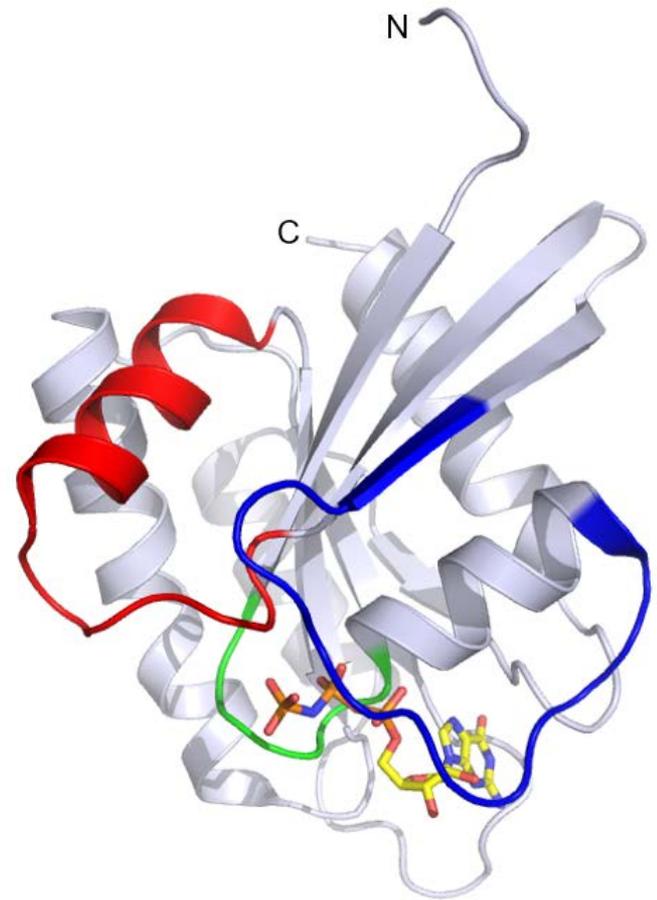
Processed KRAS4b workshop – collaborative opportunities

- **Frank Heinrich** ([Neutron reflectivity of protein/membranes](#); Carnegie Mellon University)
 - Determine orientation of KRAS4b-FME GDP/GMP-PNP on membrane
 - Impact of Calmodulin on HVR
- **Alemayehu Gorfe** ([Modeling of RAS on membranes](#); University of Texas Health Science Center)
 - Interest in testing predicted dimer interactions
 - Molecular dynamics analysis of FNL extended switch 1 KRAS4b-GDP structure
- **Mitsuhiko Ikura** ([NMR of KRAS on membranes](#); University of Toronto)
 - Analysis of processed KRAS4b by NMR
- **Jeff Perry** ([Small angle X-ray scattering](#); University of California Riverside)
 - Screening of crystallography conditions of challenging targets
- **Vadim Cherezov** ([Crystallography of membrane proteins](#); University of Southern California)
 - Attachment of transmembrane helix for anchoring of processed KRAS4b for crystallography
- **Jay Groves** ([RAS on tethered bilayers](#); University of California at Berkley)
 - Effector interactions
 - Development of screenable assays on tethered bilayers

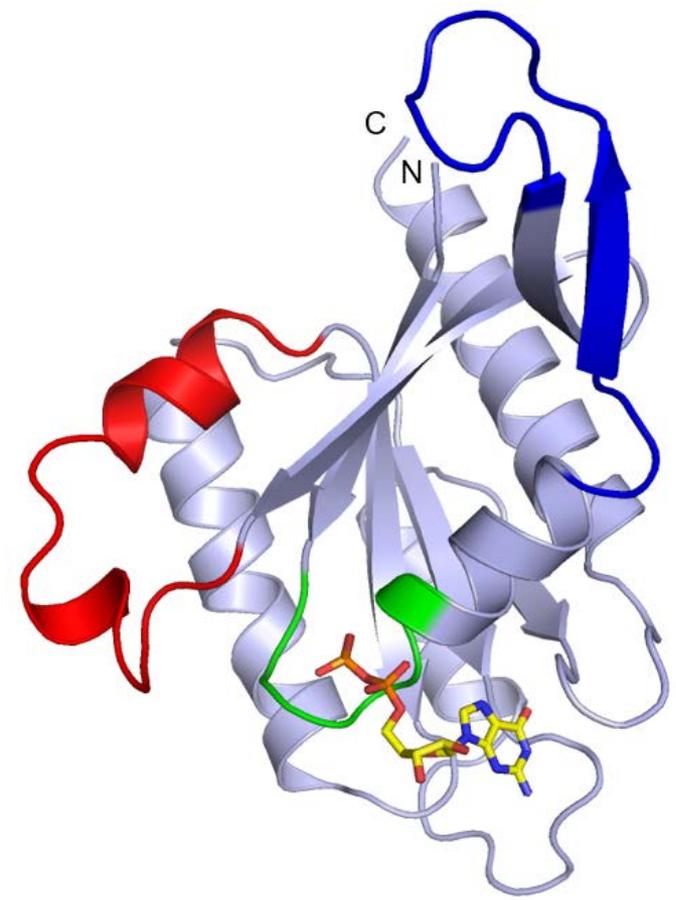


Full-length KRAS in complex with GDP

Wild-type KRAS(1-166)-GMPPNP complex at 1.35 Ang



Full-length Wild-type KRAS-GDP complex at 1.6 Ang

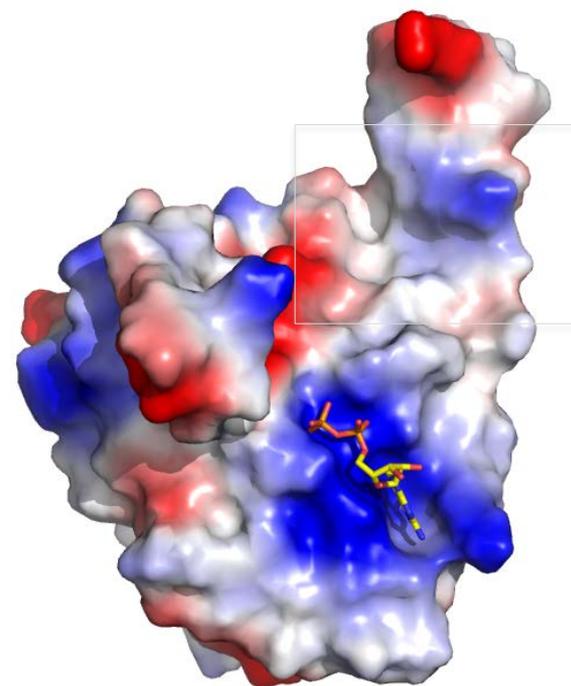


Switch-I
Switch-II
P-loop

Extended switch-I conformation in KRAS

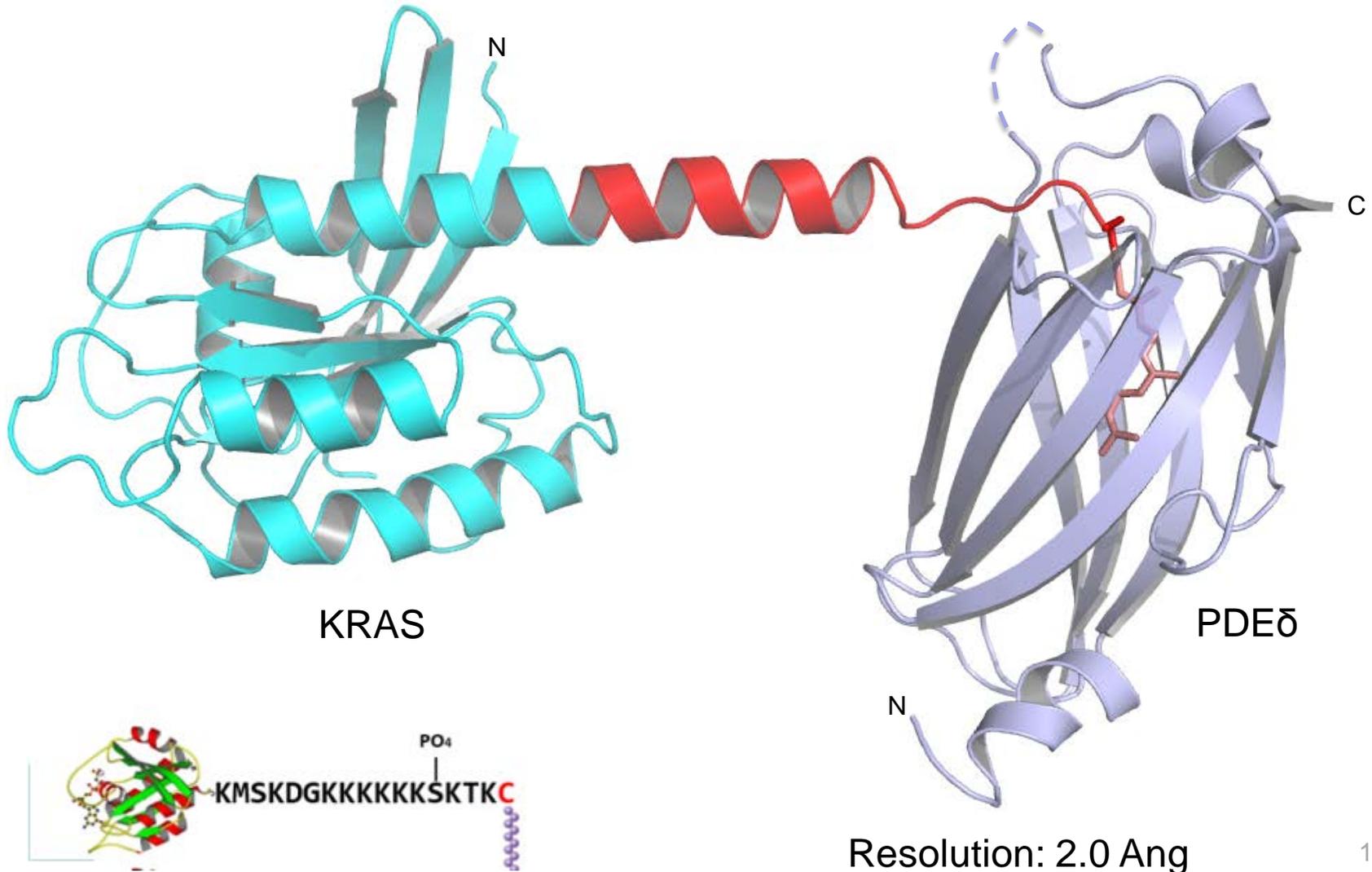
- **Validate presence of extended switch-I conformation in solution by NMR.**
 - Dynamic studies in collaboration with National Magnetic Resonance Facility at Madison.
Que Van at FNLCR
 - High-pressure NMR studies in collaboration with Dr. Kalbitzer, University of Regensburg, Germany.
- **Virtual compound screening to target the groove present at the base of switch-I region**
 - in collaboration with Dr. Brian Shoichet's group at UCSF.

Electrostatic surface



Red - negative charge
White - neutral
Blue - positive charge

Processed KRAS in complex with PDE δ

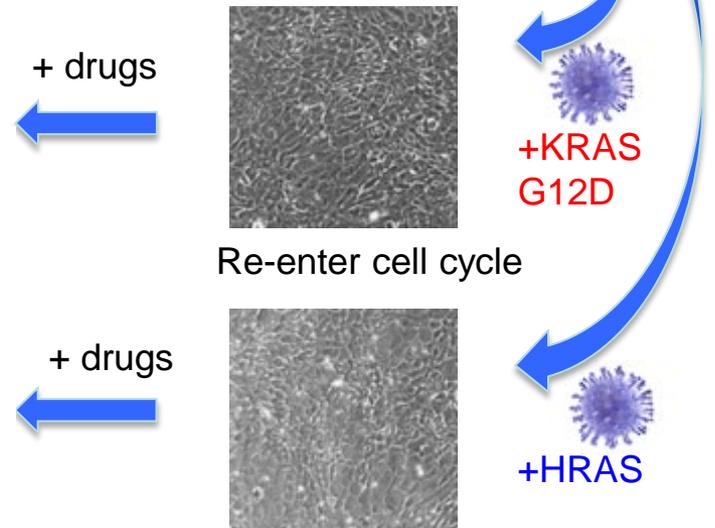
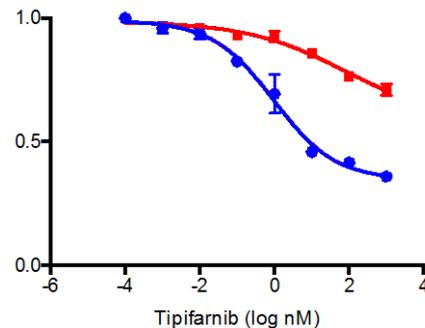
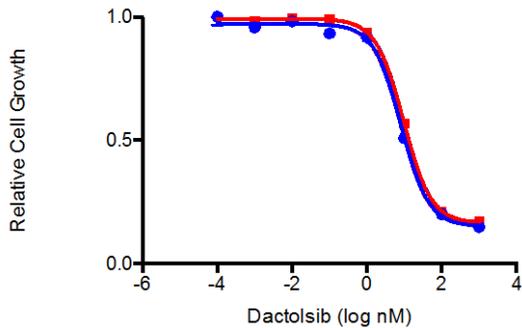
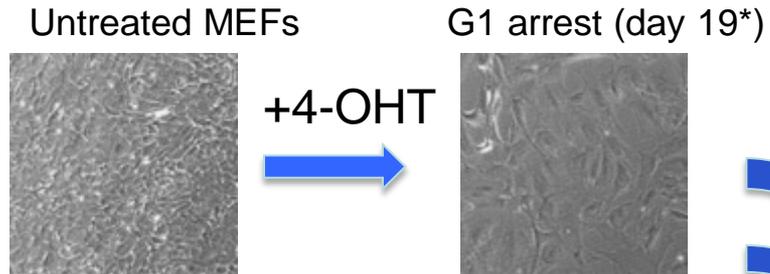


RAS Initiative Accomplishments: *Inhibitor Screens and Assays*

Isogenic Screen for RAS Selective Inhibitors

HRAS^{-/-} NRAS^{-/-} KRAS^{lox/lox} MEFs

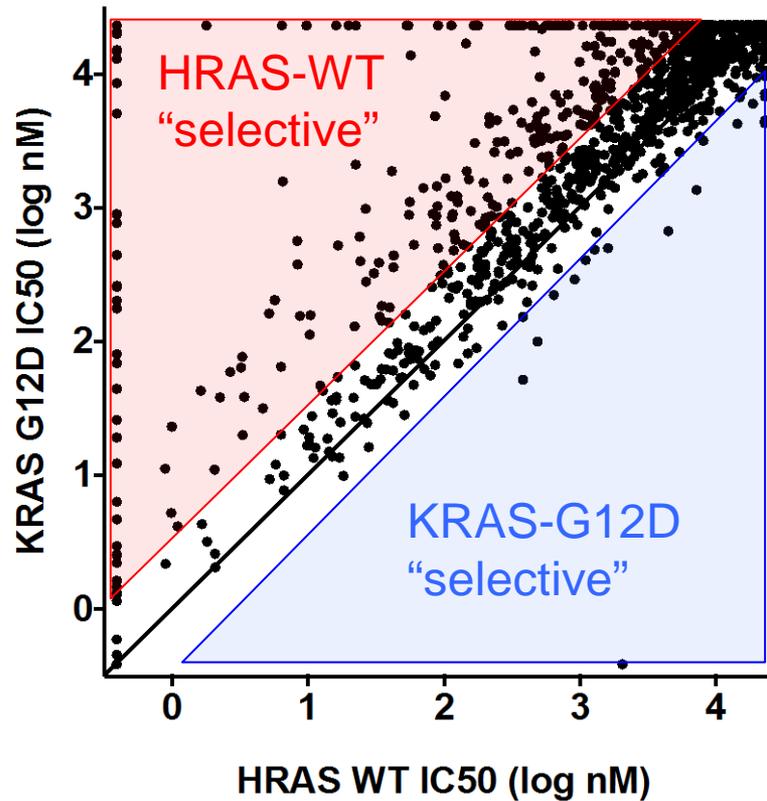
Drosten M, Dhawahir A, Sum EY, Urosevic J, Lechuga CG, Esteban LM, Castellano E, Guerra C, Santos E, **Barbacid M.** EMBO J. 2010



+ drugs

+ drugs

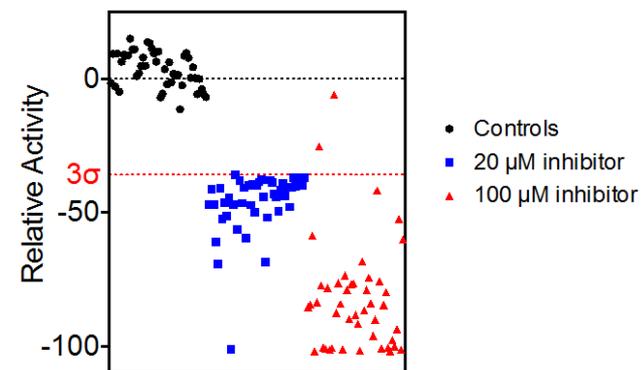
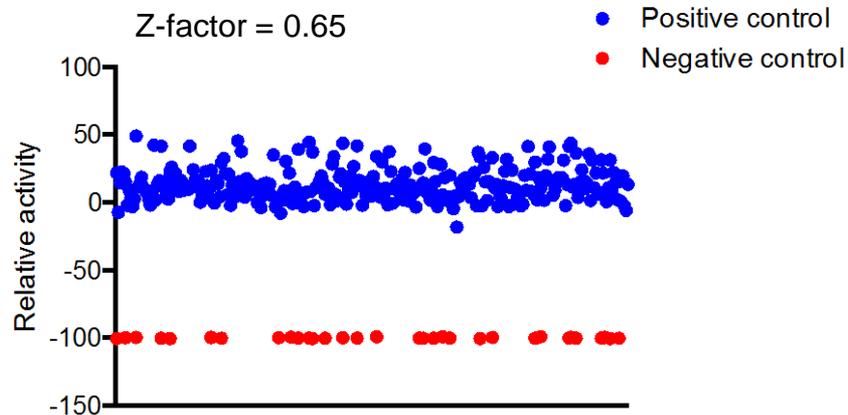
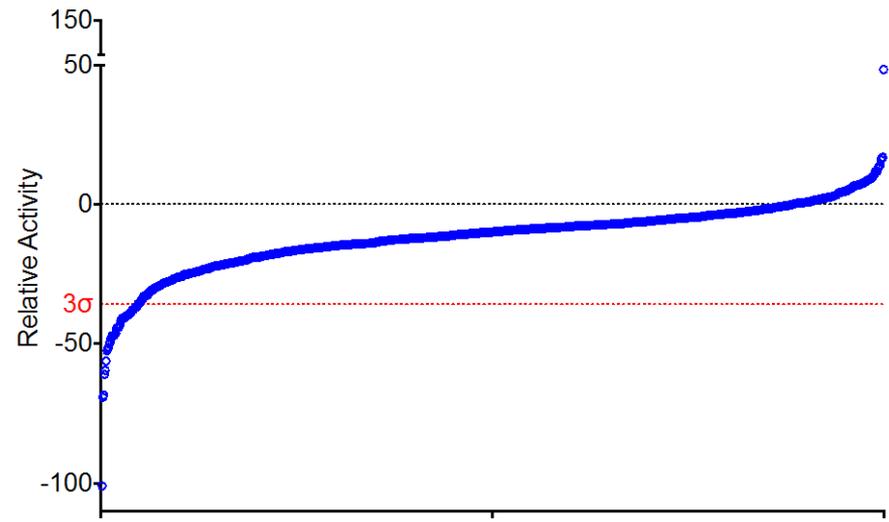
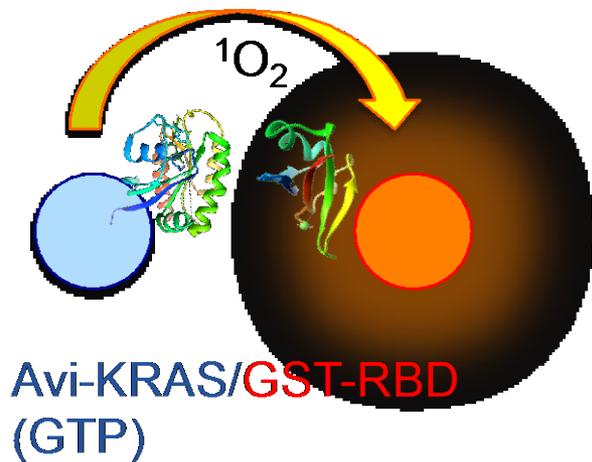
HRAS^{WT} vs KRAS^{G12D} Pilot Screen



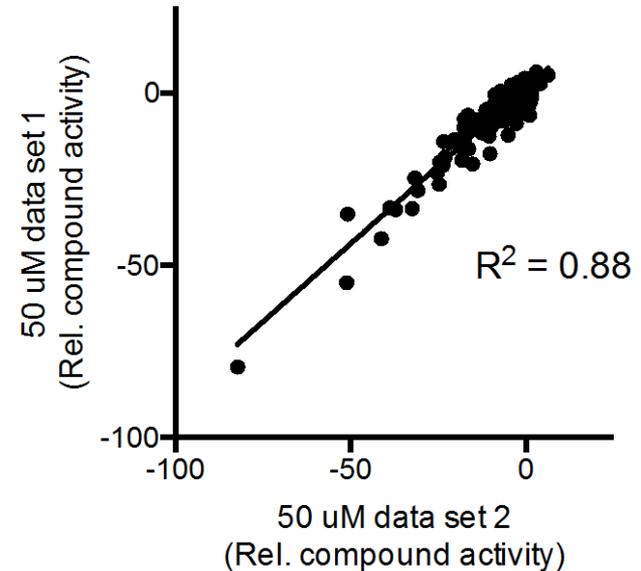
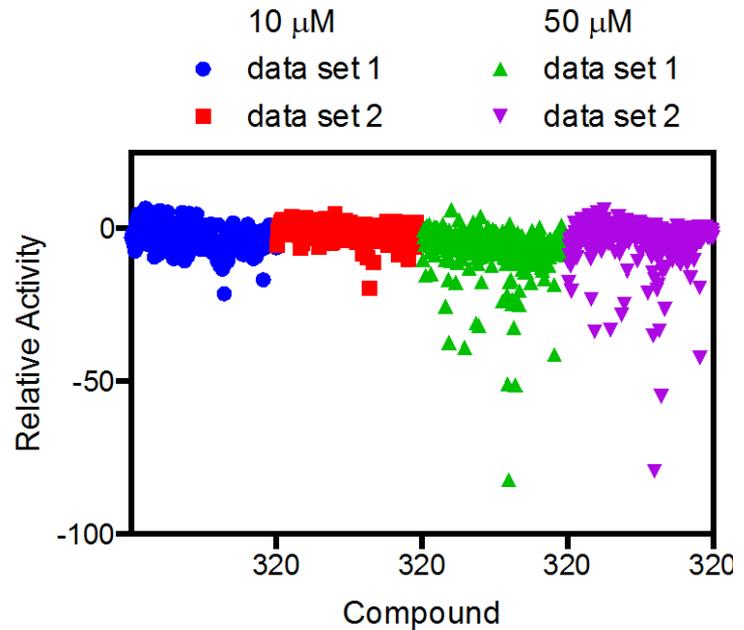
- Compound library was provided by NCATS (National Center for the Advancement of Translational Sciences)
- The library is enriched for “tool” compounds, but also contains FDA approved drugs

KRAS-effector Inhibitor Screen

Daiichi-Sankyo Protein-Protein Interaction Library



Reproducibility of KRAS-RBD screening assay

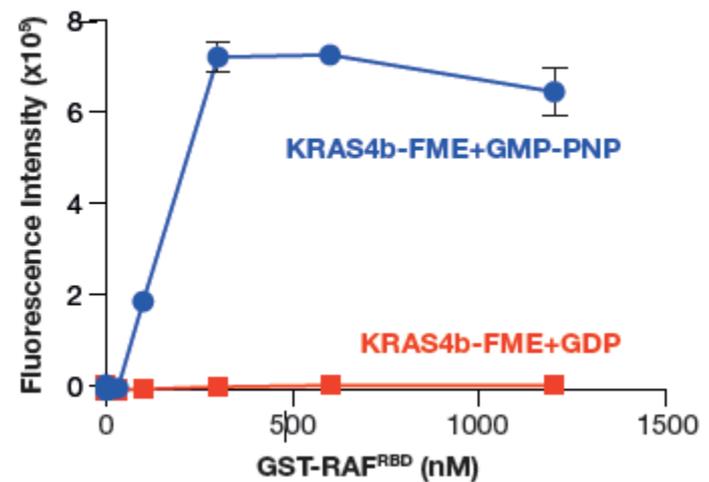
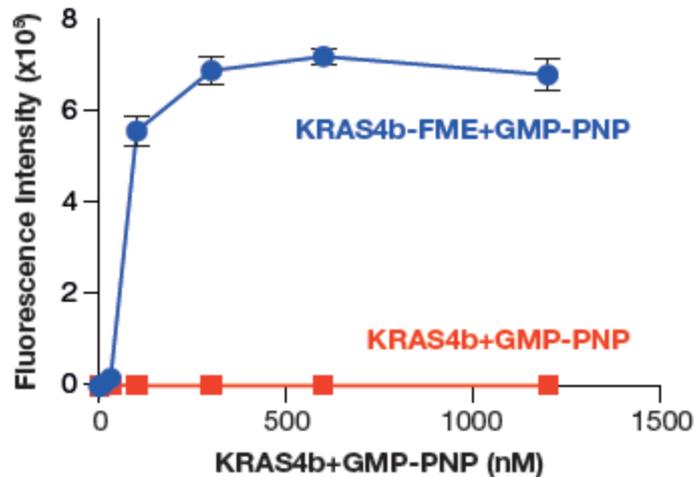
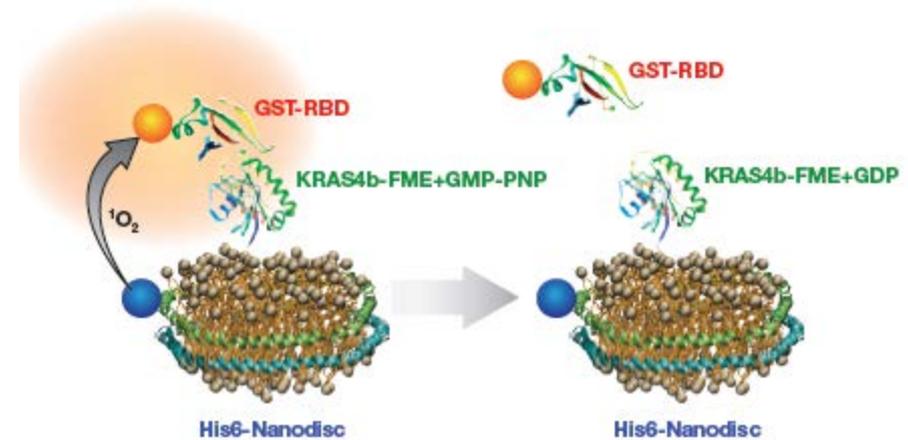
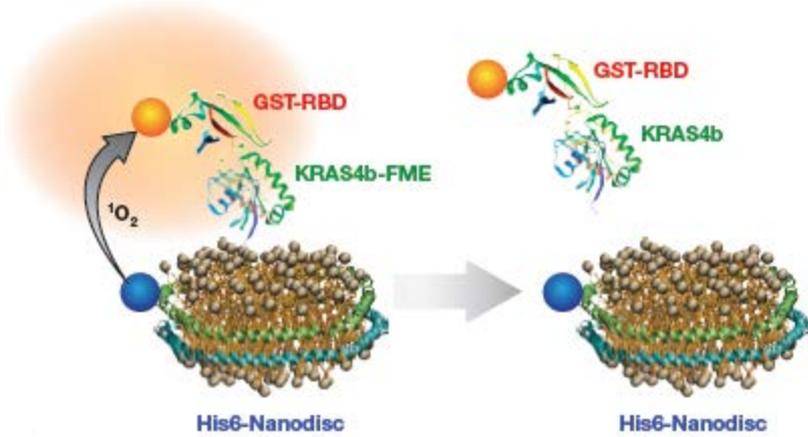


Assay is highly reproducible at 50 μ M

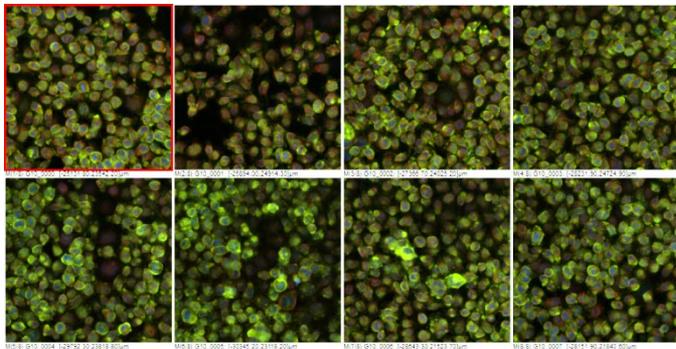
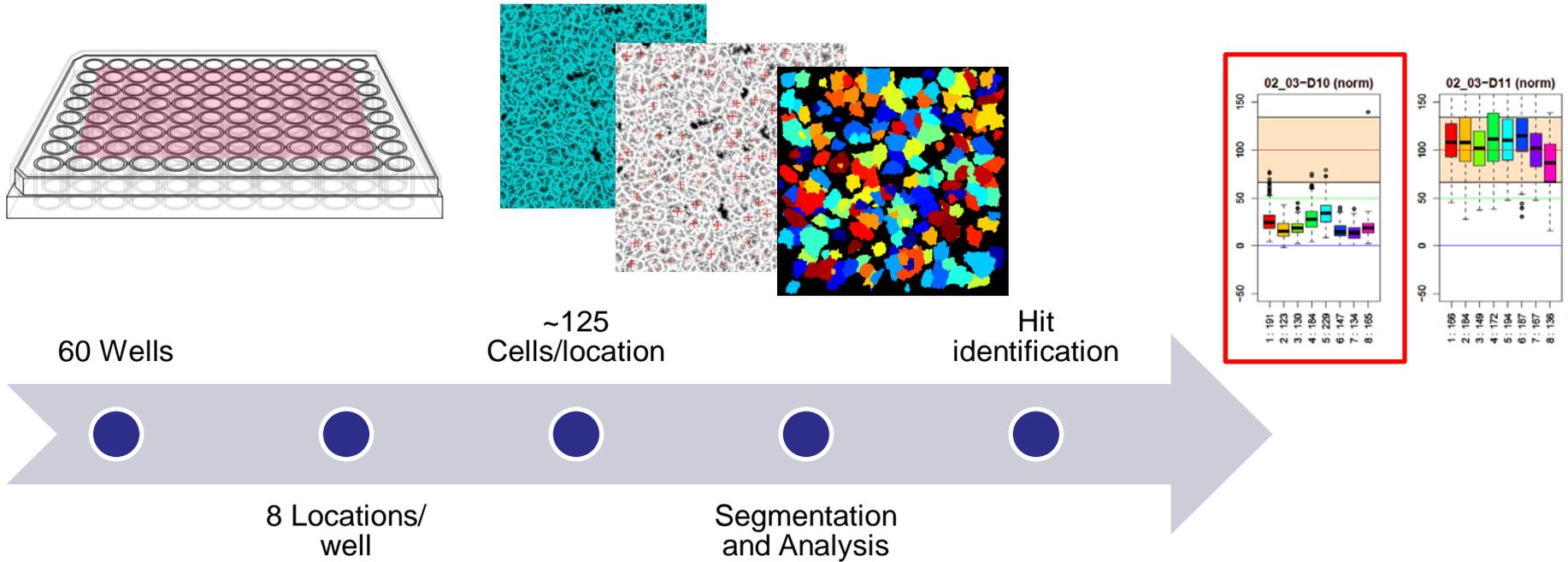
The “hit” rate at 50 μ M is approximately 4%

13/320 compounds inhibit the alpha signal >25%

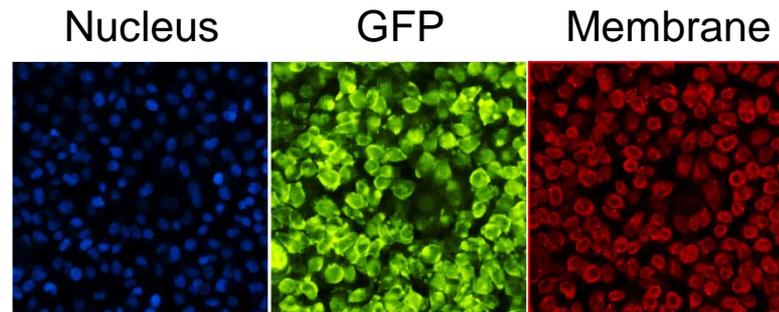
KRAS4b-FME binds to CRAF-RBD on Nanodiscs



RAS Localization Assay Overview

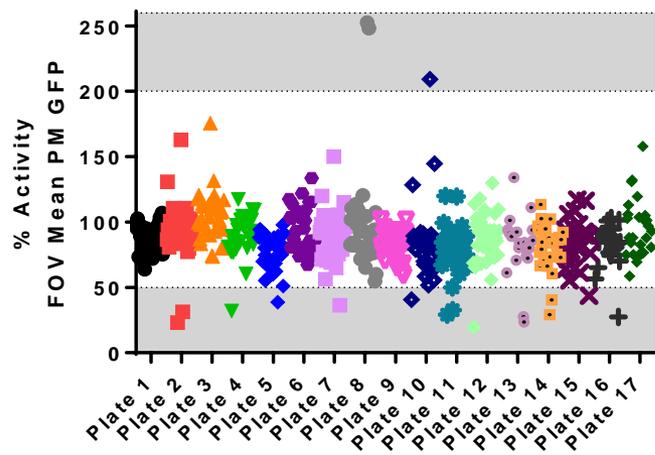


GFP-KRAS4b^{G12V}



NCI Developmental Therapeutics Program screening set

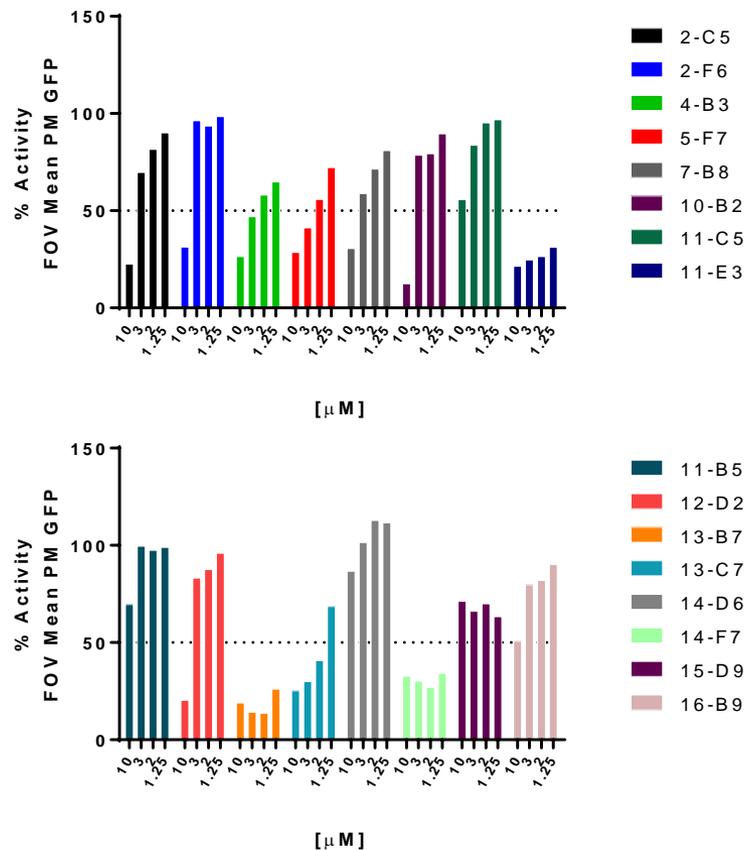
Primary assay: GFP-KRAS^{G12V}



~800 small molecules with
biological activity

- Plate 1 (Z' = 0.84)
- Plate 2 (Z' = 0.77)
- ▲ Plate 3 (Z' = 0.89)
- ▼ Plate 4 (Z' = 0.80)
- ◆ Plate 5 (Z' = 0.79)
- Plate 6 (Z' = 0.68)
- Plate 7 (Z' = 0.64)
- Plate 8 (Z' = 0.84)
- ▼ Plate 9 (Z' = 0.74)
- ◆ Plate 10 (Z' = 0.73)
- Plate 11 (Z' = 0.83)
- ◆ Plate 12 (Z' = 0.74)
- Plate 13 (Z' = 0.83)
- Plate 14 (Z' = 0.79)
- ✕ Plate 15 (Z' = 0.88)
- Plate 16 (Z' = 0.74)
- ◆ Plate 17 (Z' = 0.88)

Reconfirmed hits

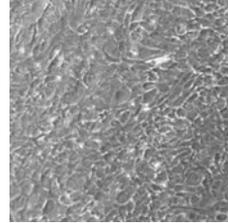


HaloTag-KRAS^{WT} driven-MEFs Proliferate

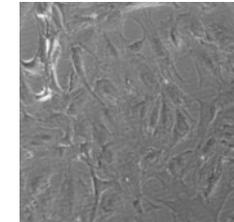
HRAS^{-/-} NRAS^{-/-} KRAS^{lox/lox} MEFs

Drosten M, Dhawahir A, Sum EY, Urosevic J,
Lechuga CG, Esteban LM, Castellano E,
Guerra C, Santos E, **Barbacid M.**
EMBO J. 2010

Untreated MEFs



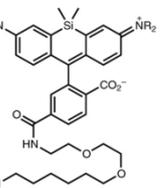
G1 arrest (day 19*)



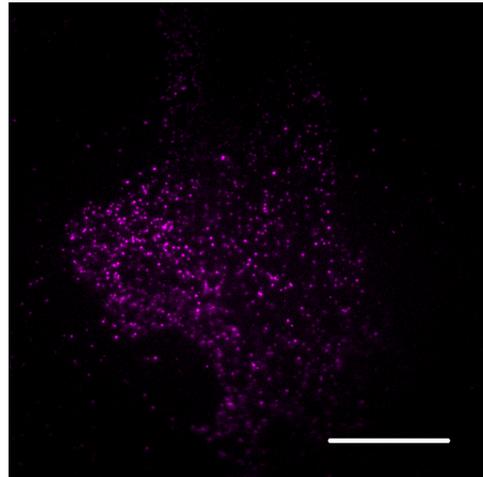
+4-OHT
→



+HaloTagKRAS

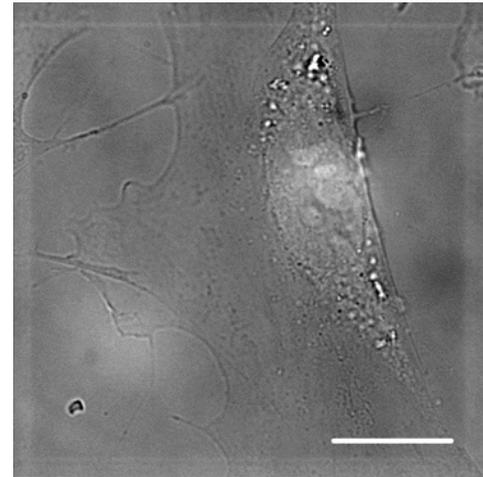


HaloTag-KRAS4b can be imaged in live cells.



TIRF Image: membrane

HaloTag-KRAS4b rescues RASless MEF proliferation.



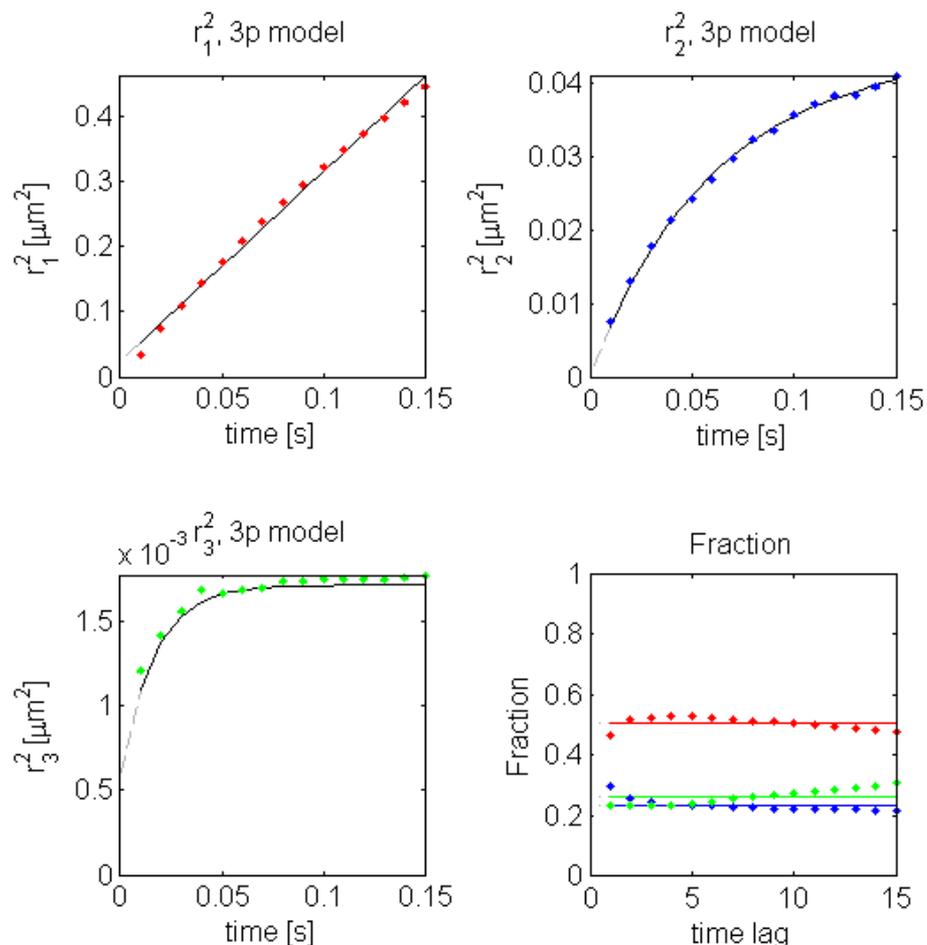
Transmitted light image

Scale bar 20 μm

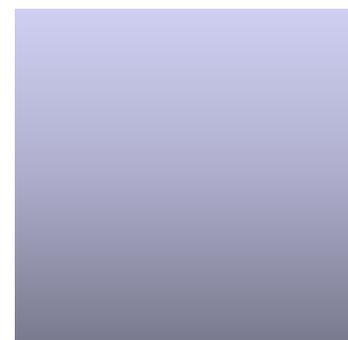
Cell permeant,
super bright,
fluorescent Halo
ligand from
Janelia Farms

Characterization of RAS molecules in live cell membranes

Jump squared displacement analysis



HaloTag-KRAS^{WT} driven-MEFs

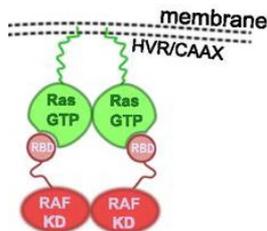


Three components

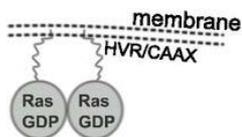
Model	Diffusion ($\mu\text{m}^2/\text{s}$)	Fraction Mean (SDev)	Const. Rad. R_c (nm)
1 \rightarrow Normal	0.73	0.505 (0.0193)	-
2 \rightarrow Constrained	0.1805	0.233 (0.021)	44.2
3 \rightarrow Constrained	0.0178	0.2624 (0.026)	1.2

Single molecule tracking analysis suggests three RAS states in live cell membranes.

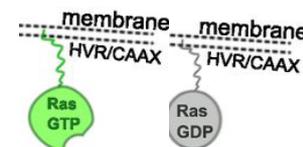
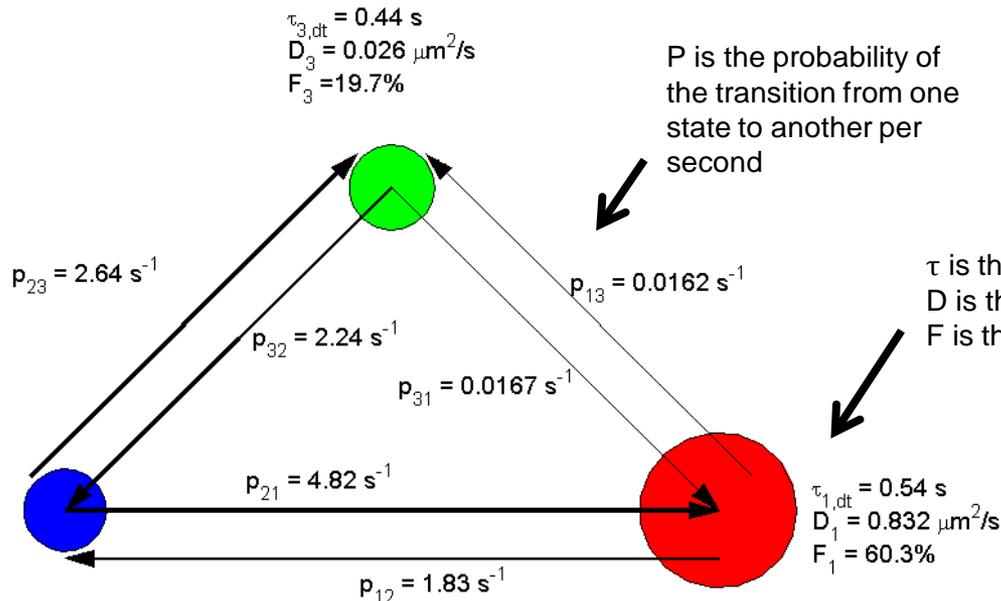
Information extracted from individual trajectories



Hypothesis: states represent different complexes in membrane.



$\tau_{2,dt} = 0.13$ s
 $D_2 = 0.183 \mu\text{m}^2/\text{s}$
 $F_2 = 20.0\%$



RASless-MEFs, HaloTag-wtKRAS4b [JF646]=50pM, Serum Starved, 37°C, 22,325 trajectories and average trajectory length 12 frames.

RAS Initiative Accomplishments: *Towards a “RAS Interactome”*

THE RAS INITIATIVE

The Problem

A Collaborative Solution

RAS Initiative at FNLCR

RAS Projects at FNLCR

RAS Laboratory Groups

Oversight

Spokes/Funding

RAS Central

A Collaborative Solution

During a series of NCI-led workshops in 2013, researchers presented data suggesting that it may now be feasible to target mutant RAS proteins directly or target other unique features of RAS-driven tumors.

Subsequently, NCI received unanimous endorsement from both the National Cancer Advisory Board and the NCI Board of Scientific Advisors to launch the RAS Initiative. Dr. Frank McCormick, a renowned expert in the field of RAS biology, joined the team at the Frederick National Laboratory for Cancer Research (FNLCR) as a consultant to lead the initiative.



Former NCI Director Harold Varmus, M.D., and National RAS Initiative Advisor Frank McCormick, Ph.D., explain the rationale for and the discussions that led to the formation of the RAS Initiative.

The Hub & Spoke Model

NCI hopes to attack mutant RAS-driven cancers through an integrated initiative that enlists collaborators from all sectors of the research community. This approach is called a "hub and spoke" model. The RAS Hub at the FNLCR and the larger community of RAS researchers around the world are now working together to find new ways to approach the RAS enigma.

The Hub - FNLCR

The FNLCR serves as the research hub that connects to research collaborators nationally and internationally. FNLCR scientists carry out a number of interlinked projects that employ the extensive infrastructure established by NCI in the areas of protein chemistry and biophysics, imaging, and genetics and genomics.

Spokes - Opportunities for Collaboration to Attack RAS

The RAS Initiative seeks to facilitate connections between and among researchers, bringing new ideas and technologies to bear on RAS. A guiding principle for these collaborations is close coordination with the activities at the FNLCR Hub, so that new efforts can leverage each other.



THE RAS INITIATIVE

The Problem

A Collaborative Solution

RAS Projects at FNLCR

RAS Laboratory Groups

Oversight

Spokes/Funding

RAS Central

RAS Spokes/Funding

The RAS Initiative seeks to facilitate connections between and among researchers, bringing new ideas and technologies to bear on RAS. A guiding principle for these collaborations is close coordination with the activities at the Frederick National Lab for Cancer Research (FNLCR) Hub, so that new efforts can have a maximum amount of leverage on each other.

Funding

RAS Initiative Postdoc Available

The Frederick National Laboratory for Cancer Research is currently seeking Postdoctoral Fellow candidates to join the RAS Initiative team. Please share this information with your trainees or other interested individuals.



NCI Fellowships Available

The NCI requests post-doctoral fellowship applications related to the goal of building effective therapeutic approaches for KRAS-driven tumors. Applicants should apply through the Parent Program Announcement (F32); due dates are April 8, August 8, and December 8, 2015. For more information and how to apply, see the published Notice.

Spokes

Contract Awardees for RAS Pathway Assays

The NCI Clinical Proteomic Tumor Analysis Consortium has awarded contracts to the Fred Hutchinson Cancer Research Center (Dr. Amanda Paulovich), the Moffitt Cancer Center (Dr. John Koomen), and the Broad Institute (Dr. Steven Carr). They will develop quantitative immuno-multiple reaction monitoring (MRM) assays to measure important peptides and phospho-peptides in the RAS pathway. Data from these assays will provide a valuable link between phenotypes and genotypes in cancers. For more details please see the blog post in RAS Central.



From left to right: Dr. Amanda Paulovich, Dr. John Koomen, Dr. Steve Carr

KRAS Post-doctoral Fellowships

In



Dr. Lynn McGregor, University of California, San Francisco and Dr. John Hunter, University of Texas Southwestern Medical Center

RAS Lab (Basecamp)

- Private, by invitation only
- All posts and comments publish immediately
- Supports uploads of documents, 1:1 interactions

[New features](#) [Account](#) [Upgrades](#) [Sign out](#)



Basecamp

[Projects](#) [Calendar](#) [Everything](#) [Progress](#) | [Everyone](#) [Me](#)

🔍 Jump to a project, person, label, or search...

RAS Lab ★

[Invite more people](#)
88 people on this project

[Catch up](#)
on recent changes

Welcome to RAS Lab! This is where we can discuss what's new in the literature or troubleshoot problems in our bench work. Thanks for being part of our community.

[36 Discussions](#) [25 Files](#)

Add the first: [To-do list](#) [Text document](#) [Event](#)

Latest project updates

- Aug 3** bob s. commented on [DARPA-funded Big Mechanisms project aims to read and interpret RAS ...](#)
- Aug 3** You commented on [DARPA-funded Big Mechanisms project aims to read and interpret RAS ...](#)
- Aug 3** bob s. posted a message: [DARPA-funded Big Mechanisms project aims to read and interpret RAS ...](#)

[See all updates](#)

Discussions

[Watch a quick video about Discussions](#)



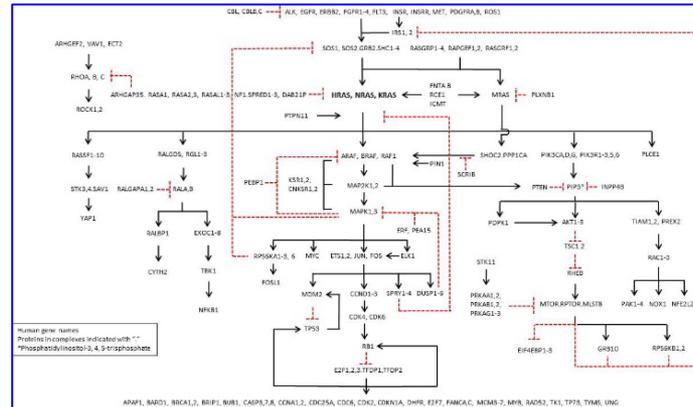
bob s.

[DARPA-funded Big Mechanisms project aims to...](#) - Hi Jim, here is a link to a recent article by the project's manager: <http://iopscience.iop.org/1478-3975/12/4/045008>

Aug 3

2

RAS Reagents *Pathway 2.0 clone collection*



- Generate stop and nostop Gateway Entry clones for all genes
- Isoforms chosen by bioinformatic analysis (R. Stephens)
 - most common transcript observed across different cell lines
 - many are NOT “isoform 1” or “longest isoform”
- 180 genes - 360 total constructs (stop/nostop)
 - 17 not available commercially in correct isoform
 - 32 additional not available without mutations
- 98% completion

Collaboration with the RAS Community

RAS events

Synthetic Lethality Workshop, [January 6-7 2014](#)

RAS Pathways Workshop, [June 11, 2014](#)

Cell Surfaces Workshop, [July 23, 2014](#)

AACR Annual Meeting, [April 21, 2015](#)

RAS Structures Workshop, [July 21-22, 2015](#)

RAS Immunotherapy Workshop, [November 3, 2015](#)

RAS Symposium, [December 15-16, 2015](#)

Seminars at FNLCCR

Channing Der, [UNC](#)

Ken Westover, [UTSW](#)

Carla Mattos, [Northeastern](#)

Mark Philips, [NYU](#)

Vadim Gaponenko, [U-Chicago](#)

Josh Salafsky, [Biodesy, Inc.](#)

Calvin Kuo, [Stanford](#)

Kris Wood, [Duke](#)

Mariano Barbacid, [CNIO, Madrid](#)

Cyril Benes, [Mass General](#)

Carolyn Buser, [GlaxoSmithKline](#)

Jay Groves, [UC-Berkeley](#)

Stephen Sligar, [UI-Champagne Urbana](#)

Raffit Hassan, [NCI](#)

Renata Grifantini, [Externautics Spa, Siena](#)

Renata Pasqualini, [U-New Mexico](#)

Andrew Bradbury, [Los Alamos](#)

Kent Rossman, [UNC](#)

Shiva Malek, [Genentech](#)

RAS Symposium Confirmed Speakers I

Harold Varmus, Weill Cornell Medical College

Kevan Shokat, University of California, San Diego

Allan Balmain, University of California, San Diego

Mariano Barbacid, Spanish National Cancer Research Centre

James Bradner, Dana Farber, Harvard University

Karen Cichowski, Brigham and Women's Hospital, Harvard University

Channing Der, University of North Carolina, Chapel Hill

Stephen Fesik, Vanderbilt University

Jay Groves, University of California, Berkeley

John Hancock, University of Texas, Houston

Frank McCormick, University of California, San Francisco and the RAS Initiative

Deborah Morrison, National Cancer Institute

Mark Philips, New York University

David Sabatini, Whitehead Institute, Massachusetts Institute of Technology

Kevin Shannon, University of California, San Francisco

David Tuveson, Cold Spring Harbor Laboratory

Michael White, University of Texas, Southwestern

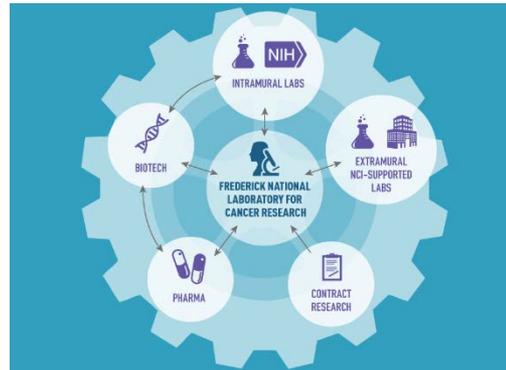
Matthew Vander Heiden, Koch Institute, Massachusetts Institute of Technology



Collaboration with the RAS Community

RAS Reference Reagents

Chris Kemp, Fred Hutch
 Eric Chang, Baylor
 Silvia Thöne, Munich
 Peter Jackson, Stanford University
 Tyler Jacks, MIT
 Calvin Kuo, Stanford
 Bill Hahn, Broad / Dana Farber
 Karla Satchell, Northwestern
 Julian Downward, Cancer Research UK
 Daniel Abankwas, University of Turku
 Said Sebti, Moffitt Cancer Center
 Ian Prior, Liverpool
 Muller Fabbri, Children's Hospital LA
 Faraz Bishehsari, Rush
 Amy Lee, USC
 Yosef Yarden, Weizmann
 Richard Klemke, UCSD
 Saidul Chowdhury, U-Texas Arlington
 Christian Gocke, JHMI
 Tobias Baumgart, U-Penn
 Emil Lou, U-Minnesota
 Ron Bose, Wash U
 Neil Kelleher, Northwestern
 Sourav Bandyopadhyay, UCSF
 Robert Chapkin, Texas A&M



NIH collaborators

Ji Luo, NCI
 Anton Simeonov, NCATS
 Debbie Morrison, NCI
 Rajat Varma, NIAID
 Udo Rudloff, NCI
 Sriram Subramaniam, NCI

Outside collaborators

Steve Almo, Einstein
 Jim Wells, USCF
 Channing Der, UNC
 Ken Westover, UTSW
 Carla Mattos, Northeastern
 Steve Sligar, U- Ill
 Jay Groves, Berkeley
 Hirsch Nanda, Susan Kreuger, NIST
 John Markley, NMRFAM, UW-Madison
 Paul Cohen, DARPA
 Kris Wood, Duke
 David Weber, U-Maryland
 Tina Yuan, Broad
 Cameron Pitt, UCSF
 Krishna Kota, USAMRIID
 Sotirios Koutsopoulos, MIT
 Fred Wittinghofer, Dortmund University
 Lynn McGregor, UCSF (PanCan postdoc)
 John Hunter, UTSW (PanCan postdoc)
 Saori Sato, Daiichi-Sankyo
 Walter Englaro, Sanofi-Aventis
 Kirk Staschke, Lilly
 Gad Getz, Mass Gen /Broad
 Matt Meyerson, Dana Farber
 Immuno-MRM of RAS pathway

- Amanda Paulovich, Fred Hutch
- Steve Carr, Broad Institute
- John Koomen, Moffitt Cancer Center

 Andreas Gosberg, Lilly

FNLAC RAS Working Group Recommendations

- **Pursue a detailed understanding of Processed KRAS4b**
 - Unique reagent that shows promise as a new tool compound and thus illustrates the potential of the RAS Initiative
 - Biophysical and structural analysis of RAS on membranes
 - Dedicate staff to make processed KRAS4b for research community
 - In-house workshops for reagent generation
- **Biochemistry and structural biology (e.g., Cryo-EM) of RAS complexes and RAS:membrane interactions is a priority**
 - Collaborative effort with CCR and Sriram Subramaniam
- **The overall set of structures and reagents should represent a concrete and useful set of deliverables from the RAS effort**
- **Other efforts were more exploratory and open-ended; some are being scaled back or phased out**

Strategy

- **Develop a plan to augment industry partnership (see next slide)**
- **Implement a framework for tool/lead compound development**
 - Need path to validation and optimization not dependent on pharma
 - Define and plan for medicinal chemistry needs
- **De-convolution assays might emerge as an area of specialty**
 - Synergize biophysics/biochemistry with assays and screens
 - Develop an assay cascade for validation of hits/leads
- **Step-up awareness and dissemination efforts**
 - Package reagents, assays and capabilities for presentation to academia and pharma
 - Publicity/marketing
 - Develop additional next-generation assays
- **Develop plan for renewal phase**
 - Present to FNLAC as part of renewal

Interactions with Pharma

- **Pharma participation is a big plus**
 - Pharma brings credibility and resources
 - Roadshows and marketing to increase participation
- **Be creative when thinking about partnering possibilities**
 - Preferred partner(s)
 - Separate company that holds IP?
 - Venture philanthropy?
 - Develop strategy for prosecuting IP
- **Blueprint for bringing all projects to successful completion**
 - Define metrics for success up-front
 - Framework for division of labor during follow-up phase

Outreach & Resources

- **Websites**

- “Interactome” that engages the community may have value Provide additional information
 - Protocols ; cell line mutation and RAS dependence

- **Compound collection and reagent distribution**

- Resource for internal efforts and extended RAS community
- Level of effort and source of compounds
- Validate compounds with “assay cascade”
- Track distribution and the experience/coaching needs of the recipients

- **Possibilities**

- *Manuscript on test compounds with assay cascade?*
- *De-bunk inaccurate claims?*
- *RAS pathway proteomic studies?*