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

Direct KRAS effectors

Indirect KRAS effectors

MAPK
- ARAF
- BRAF
- RAF1
- MAP2K1
- MAP2K2
- MAPK1
- MAPK3
- RPS6KA1
- RPS6KA3
- RPS6KA2
- RPS6KA6
- RPS6KA5
- RPS6KA4
- KSR1
- KSR2

PI3K
- PIK3CA
- PIK3CB
- PIK3CD
- AKT1
- AKT2
- AKT3

RHO
- RHOC
- ROCK1
- ROCK2

RAC
- RAC1
- RAC2
- RAC3
- PAK1
- PAK2
- PAK3
- TIA1
- TIA2

RAL
- RAL
- RALB
- RALBP1
- RALGDS
- RGL1
- RGL2
- RGL3
- RHEB
- IKBKB
- Chuk
- NFkB

Metabolism
- HK1
- HK2
- HK3
- LDHA
- LDHB
- PDK1
- PDK2
- GLS
- HIF1A
- EPAS1

Autophagy
- ATG5
- ATG7
- BECN1

Stress
- MAPK8
- MAPK9
- MAPK10
- MAPK12
- MAPK13

Epigenetics
- DNMT1
- DNMT3A
- DNMT3B

Survival
- BCL2
- BCL2L1
- MCL1

→ 40 KRAS Effector Nodes = 84 genes

Christof Fellmann, Scott Lowe, Chih-Shia Lee, Ji Luo
SiREN assay for Ras dependency
Global assessment of KRAS-effector dependency

Sensitive

Resistant

Tissue Origin
- lung
- pancreas
- large_intestine
- colon

p-value = 4.842e-06

* AUC computed by Ming Yi
SiREN assay for Ras dependency
RAS Initiative Accomplishments:

*Biophysical and structural analysis*
Ras proteins

1 -166
G-domain

167-185,6
Hyper-variable region

Raf, PI 3’ kinase
RalGDS, GAPs

Raf, PI 3’ kinase
RalGDS, GAPs

Raf, PI 3’ kinase
RalGDS, GAPs

Raf, PI 3’ kinase
RalGDS, GAPs

Palmitoyl

Farnesyl

PO₄

KRAS 4B

KRAS 4A

NRAS

HRAS
Fully processed KRAS4b

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 phosphotidylserine-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

Characterize reagents

- Liposomes and tethered bilayers
- Lipid Nanodiscs
- Processed KRAS

Assays and Screens

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

Structural analysis

- Protein QC
- Biophysical properties
- Membrane interactions
- Sligar lab (U-Illinois)
- Groves lab (UC-Berkeley)
- Heinrich Lab (NIST)
- NMR (NMRFAM)
- Hi-res Cryo-EM (NCI)
- Crystallography (FNL)

Structures of KRAS and effectors on membranes
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.
Processed KRAS in complex with PDEδ

Resolution: 2.0 Ang
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

Untreated MEFs

G1 arrest (day 19*)

+4-OHT

Re-enter cell cycle

+ drugs

+ drugs

+ drugs

+4-KRAS

+HRAS

Relative Cell Growth

Dactolosib (log nM)

Tipifarnib (log nM)
HRASWT vs KRASG12D 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

Kanika Sharma (FNLCR), Kyle Brimacombe (NCATS)
KRAS-effector Inhibitor Screen
Daiichi-Sankyo Protein-Protein Interaction Library

Avi-KRAS/GST-RBD (GTP)

Z-factor = 0.65

Relative activity

Positive control
Negative control

Relative Activity

3σ

20 μM inhibitor
100 μM inhibitor

Relative Activity

3σ

Controls

20 μM inhibitor
100 μM inhibitor
Assay is highly reproducible at 50 μM
The “hit” rate at 50 μM is approximately 4%
 13/320 compounds inhibit the alpha signal >25%
KRAS4b-FME binds to CRAF-RBD on Nanodiscs

Matt Holderfield, Maria Abreu Blanco
RAS Localization Assay Overview

60 Wells

~125 Cells/location

Hit identification

8 Locations/well

Segmentation and Analysis

GFP-KRAS4b<sup>G12V</sup>

Nucleus  GFP  Membrane

Alla Brafman
NCI Developmental Therapeutics Program screening set

Primary assay: GFP-KRAS$^{G12V}$

~800 small molecules with biological activity

Reconfirmed hits
HaloTag-KRAS\textsuperscript{WT} driven-MEFs Proliferate

HRAS\textsuperscript{-/-} NRAS\textsuperscript{-/-} KRAS\textsuperscript{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*)

\(+4\text{-OHT}\)

HaloTag-KRAS4b can be imaged in live cells.

HaloTag-KRAS4b rescues RASless MEF proliferation.

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

TIRF Image: membrane

Transmitted light image

\(+\text{HaloTagKRAS}\)

Scale bar 20 µm

Nikki Fer and De Chen
Characterization of RAS molecules in live cell membranes

Jump squared displacement analysis

HaloTag-KRAS\textsuperscript{WT} driven-MEFs

<table>
<thead>
<tr>
<th>Model</th>
<th>Diffusion (\text{um}^2/\text{s})</th>
<th>Fraction Mean (\text{SDev})</th>
<th>Const. Rad. ( R_c ) (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ( \rightarrow ) Normal</td>
<td>0.73</td>
<td>0.505 (0.0193)</td>
<td>-</td>
</tr>
<tr>
<td>2 ( \rightarrow ) Constrained</td>
<td>0.1805</td>
<td>0.233 (0.021)</td>
<td>44.2</td>
</tr>
<tr>
<td>3 ( \rightarrow ) Constrained</td>
<td>0.0178</td>
<td>0.2624 (0.026)</td>
<td>1.2</td>
</tr>
</tbody>
</table>

De Chen and Prabhakar Gudla
Single molecule tracking analysis suggests three RAS states in live cell membranes.

Information extracted from individual trajectories

Hypothesis: states represent different complexes in membrane.

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

De Chen and Prabhakar Gudla
RAS Initiative Accomplishments:

Towards a “RAS Interactome”
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 cancers.

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.

The Hub & Spoke Model

NCI hopes to attack mutant RAS-driven cancers through an integrated initiative that enlist collaborations 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 engine.

The FNLCR serves as the research hub that connects to research collaborators nationally and internationally. FNLCR scientists carry out a number of multidisciplinary 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.

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 Laboratory 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 Fellowship candidates to join the RAS Initiative team. Please share this information with your trainees or other interested individuals.

NCI Fellowships Available

The NCI requests postdoctoral fellowship applications related to the goal of building effective translational approaches for RAS-driven tumors. Applicants should apply through the Parent Program Announcement (F32) due dates are April 18, August 6, 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 consortia to the Fred Hutch Cancer Research Center (Dr. Amanda Paulovich), the Moffitt Cancer Center (Dr. John Konnras), and the Broad Institute (Dr. Steven Carr). They will develop quantitative immune-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 cancer. For more details please see the blog post in RAS Central.

KRAS Post-doctoral Fellowships

In cooperation with the Frederick National Laboratory for Cancer Research (FNLCR), The Pancreatic Cancer Action Network (PanCAN) has established fellowships to support talented post-doctoral researchers, engaged in KRAS research. PanCAN recently announced two outstanding fellowship awardees: John Hunter, Ph.D., who
RAS Lab (Basecamp)

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

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.

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

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
• 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
RAS Immunotherapy Workshop Speakers Include:
Jay Berzofsky, NCI
Jamie Chaft, Sloan-Kettering
Lisa Coussens, Oregon Health Sciences
Tom Dubensky, Aduro Biotech
Victor Engelhard, University of Virginia
Gustav Gaudernack, Oslo University Hospital
Liz Jaffe, Johns Hopkins
Ira Mellman, Genentech
Isabelle Riviere, Sloan-Kettering
Steve Rosenberg, NCI
Ugur Sahin, Translational Oncology, Mainz, Germany
Bob Schreiber, Washington University
Bob Vonderheide, University of Pennsylvania

RAS Symposium Confirmed Speakers Include:
Harold Varmus, Weill Cornell Medical College
Kevan Shokat, University of California, San Francisco
Allan Balmain, University of California, San Francisco
Mariano Barbacid, Spanish National Cancer Research Center (CNIO), Madrid
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

Seminars at FNLCR
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 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
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, Wizmann
- 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 Bandopadhayay, UCSF
- Robert Chapkin, Texas A&M

**External collaborators**
- Steve Almo, Einstein
- Jim Wells, USCF
- Channing Der, UNC
- Ken Westover, UTSW
- Carla Mattos, Northeastern
- Steve Sligar, U-III
- 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, USAMR IID
- 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

**NIH collaborators**
- Ji Luo, NCI
- Anton Simeonov, NCATS
- Debbie Morrison, NCI
- Rajat Varma, NIAID
- Udo Rudloff, NCI
- Sriram Subramaniam, NCI
FNLAC RAS Working Group Recommendations
Science

• 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?*