NCI RAS Initiative Update

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Dhirendra Simanshu, RAS Initiative Structural Biology Lead, CRTP, FNLCR
February 3rd, 2015
Outline

• Introduction
• Structural Biology and Biophysics
• Targets and Assays
  – Biochemical screens
  – Cell-based screens
  – Multimerization and localization
  – Cell surface
• RAS Community
• Oversight and Feedback
The NCI RAS Initiative

What is RAS, and why is it so important?

<table>
<thead>
<tr>
<th>Cancer</th>
<th>KRAS Mutation</th>
<th>US - new KRAS cases/yr</th>
<th>5 yr survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal</td>
<td>45 %</td>
<td>60,000</td>
<td>45 %</td>
</tr>
<tr>
<td>Lung</td>
<td>35 %</td>
<td>45,600</td>
<td>17 %</td>
</tr>
<tr>
<td>Pancreas</td>
<td>95 %</td>
<td>32,200</td>
<td>6 %</td>
</tr>
</tbody>
</table>

137,800
Protein Biology/Biophysical Characterization

Andy Stephen

Dom Esposito
The RAS family of small GTPases

- 21 kDa small GTPases
- high homology in first 164 aa
- More than 100 members
- post-translational modifications
- membrane association
Characterization of WT KRAS and mutants –
Nucleotide binding kinetics determine KRAS signaling

- **Intrinsic GTPase**
- **GDP dissociation**
- **Rapid GTP binding**

- **Measure binding kinetics of fluorescent nucleotides (in progress)**
- **Measure binding affinity of CRAF (in progress)**
- **Measure intrinsic GTPase activity (complete)**

<table>
<thead>
<tr>
<th>Effectors</th>
<th>G12C</th>
<th>G12D</th>
<th>G12V</th>
<th>G13D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colorectal</td>
<td>6,000</td>
<td>22,000</td>
<td>12,600</td>
<td>11,250</td>
</tr>
<tr>
<td>Lung</td>
<td>22,000</td>
<td>9,520</td>
<td>11,900</td>
<td>1,190</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1,200</td>
<td>19,000</td>
<td>12,000</td>
<td>1,000</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>29,500</td>
<td>50,520</td>
<td>36,500</td>
<td>13,440</td>
</tr>
</tbody>
</table>
KRAS characterization: Intrinsic GTPase activity

Hydrolysis rate (min⁻¹)

- WT
- G12D (n=3)
- G12V (n=5)
- G12C
- G13D (n=3)
- Q61L (n=3)
- Q61R (n=3)

E. coli produced full-length KRAS 4b
MANT-GppNHpNon-hydrol dissociates 4 times faster from G13D compared with WT KRAS

\[
\text{MANT - GppNHp} \quad \text{KRAS} + \text{GDP} \rightarrow \text{GDP-KRAS} + \text{MANT - GppNHp}
\]

\[
\begin{align*}
\text{WT-KRAS} & : 1.0 \times 10^{-3} \pm 3.5 \times 10^{-6} \text{ s}^{-1} \\
\text{G12V} & : 1.9 \times 10^{-3} \pm 2.5 \times 10^{-5} \text{ s}^{-1} \\
\text{G13D} & : 4.2 \times 10^{-3} \pm 1.0 \times 10^{-4} \text{ s}^{-1}
\end{align*}
\]

E. coli produced full-length KRAS 4b
Binding of RAF Ras Binding Domain (RBD)_{1-149} to WT KRAS

RAF-RBD_{1-149} 5mM MgCl₂ 150mM NaCl

Response Units

Time (s)

KRAS-GppNHp
KRAS-GDP

K_D ~300nM

Lakshman Bindu/Karen Worthy

E. coli produced full-length KRAS 4b
Ongoing characterization

• Analysis of WT, G12C, G12D, G12V, G13D, Q61H, Q61L
  – GTP hydrolysis rate of truncated protein
  – nucleotide off-rate
  – RAF-RBD binding

• Limited analysis of “rare” KRAS oncogenic mutants:
  – Intrinsic GTP hydrolysis
  – GppNHp off-rate for subset
Fully Processed Recombinant KRAS

RAS only works when in the membrane
- Drugs that affect membrane dependent signaling?

- KRAS expressed in insect cells
- Human farnesyltransferase (FNTA/FNTB), ICMT cloned
- Triple infection of FNTA/FNTB, KRAS, and ICMT

Farnesylated KRAS

21,412 Da: farnesylated, -AAX
21,426 Da: farnesylated, -AAX, methylated
21,551 Da: unprocessed
Literature suggests poly-basic region at KRAS C-terminus interact with negatively charged lipids in the plasma membrane.

Processed KRAS – lipid interactions

**Liposomes**
- SPR to evaluate optimal lipid composition

**Tethered bilayers**
- Fluorescence fluctuation spectroscopy (Jay Groves, UC Berkeley)
- Neutron scattering (NIST)

**Nanodiscs**
- Collaboration with Steve Sligar (UI Champaign-Urbana)
- Structural biology by cryo-EM and NMR
- Next-generation HTS assays
Binding of processed KRAS to liposomes is dependent on the phosphoserine content

5μM processed KRAS binding to liposomes with variable phosphoserine content
Plans for structural and biophysical analysis of fully processed KRAS

- KRAS-lipid interactions
  - Prepare and QC liposomes
  - Prepare and QC nanodiscs

- Crystallography
- Hi-Res Cryo-EM structure (NCI)
- NMR structure KRAS-nanodisc (NMR-FAM)

- Quantitate KRAS-liposome interactions by SPR or FCS (Sligar/Groves)
- KRAS-membrane orientation by neutron reflectivity (NIST)

- Intrinsic/GAP GTP hydrolysis
- RAF-RBD binding
- Next generation screening assays
Structures available in Protein Data Bank (PDB):

- HRAS: 120 structures
- KRAS: 36 structures
- NRAS: 1 structure

Challenges to targeting RAS cancers

- No structure of KRAS mutants with any effector or regulator.
- No structural insights about how RAS activates Raf kinase.
- No structural information on full-length processed RAS.
- No structural information on full-length Raf – free or in complex with RAS.
Structural Biology Goals

• Determine structures of wild-type KRAS and oncogenic mutants in inactive (GDP-bound) and active (GTP/GMPPNP) states
  - G12C  G12D  G12V  G13D  Q61H  Q61L

• Determine structures of KRAS complexes with various effectors and regulatory proteins to aid structure-based drug design
  - Calmodulin
  - GAPs : RASA1, NF1
  - Effectors : Raf (RBD and Kinase domain, full-length), PI3-Kinase
  - Farnesyl binding : PDE6δ, smgGDS

• KRAS4a structure – Comparison with KRAS4b

• NMR efforts: processed full-length KRAS bound to nanodisc.
  *Que Van at FNLCR*
Crystal structures of KRAS in complex with GDP and GMPPNP (non-hydrolysable GTP analog)

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

Full-length-KRAS-GDP complex at 1.6 Ang

Beryllium CRO
Comparison of Switch-I conformations suggests large inherent flexibility

- KRAS-GDP complex
- KRAS-GMPPNP complex
- Allosteric HRAS-SOS complex
Structural analysis of KRAS-GDP complex

Electrostatic surface representation of KRAS-GDP complex

- Red - negative charge
- White - neutral
- Blue - positive charge

Enlarged view of the hinge region
Agni Ghosh and Steve Almo (Albert Einstein College of Medicine)
- \(\text{KRAS}^{G12D}\)-GAP complex (RASA1 and NF1)

Carla Mattos (Northeastern University)
- Calmodulin-KRAS complex

Ken Westover (UT-Southwestern)
- GDP bound structures of KRAS oncogenic mutants

National Magnetic Resonance Facility at Madison (NMRFAM)
- NMR structure of processed full-length KRAS bound to nanodisc
Targets & Assays

CRAF-RBD

KRAS

Matt Holderfield
KRAS-effector binding AlphaScreen assay

Avi-KRAS  GST-CRAF-RBD

Purified, recombinant protein used for KRAS:CRAF-RBD binding assay in vitro using AlphaScreen technology.

Binding is highly GTP dependent.

Assay is ready for pilot screening.

500 nM Avi-KRAS GTPγS
0 nM Avi-KRAS GTPγS
500 nM Avi-KRAS GDP
0 nM Avi-KRAS GDP

Avi-KRAS WT (GTP)

GST-RBD

Pete Frank, John-Paul Denson, Maria Abreu-Blanco
KRAS Nanodisc complex

Belt protein and lipids self-assemble into bilayer disk structures

Farnesylated KRAS self-associates with the lipid surface of nanodiscs

His6 tag on the belt protein is available for tag-based binding assays such as AlphaScreen
RAS-Dependent MEFs

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-OHT

In the queue:

KRAS G12V  KRAS G12A  KRAS Q61R  RB\textsuperscript{-/-}
KRAS G12C  KRAS WT  BRAF V600E  P53\textsuperscript{-/-}

Rachel Bagni, Katie Beam, Dan Soppet, Maria Abreu-Blanco, Kanika Sharma
RAS-dependent MEF Pilot screening results

MEK and ERK inhibitors are equipotent in HRAS-WT and KRAS G12D

Receptor tyrosine kinase inhibitors preferentially inhibit HRAS-WT but not KRAS-G12D

With NCATS, Ajit Jadhav, Kyle Brimacombe and Anton Simeonov
Multimerization and Localization Assays
Assays for Compounds that Disrupt KRAS Signaling

1) Membrane localization
2) Multimerization assay

Signal
Localization Assay

GFP-KRAS-G12V | Membrane | Nuclei
--- | --- | ---

Multiwell confocal imaging

Segmentation and Data Analysis

Probability Map | Mask | Segmented Boundary
--- | --- | ---

Membrane Localized GFP Signal

Z' = 0.7

Mean Membrane GFP-KRAS Intensity vs [DOX]
Goal is to develop a primary assay to screen for inhibitors of KRAS multimerization.

**Bioluminescence resonance energy transfer (BRET)**

- **Coelenterazine**
- **KRAS**
- **Rluc**
- **Venus**

- d > 10 nm: no exchange
- d < 10 nm: BRET
BRET Control Saturation Curves

Kinase domain of RAF serves as a positive control

Negative control: Kinase domain of RAF does not interact with KRAS4bG12D

Unlabeled KRAS4b disrupts BRET signal
RAS Cell Surface
Objectives and rationale

- Survey the surface of KRAS-driven cells to generate a list of proteins differentially associated with KRAS phenotype
- These KRAS associated cell surface determinants could represent new targets for
  - antibody-mediated attack
  - immune based therapy
  - nanoparticle delivery
Cell Surface Strategy

- Adapt cell surface protein labeling for mass spec proteomic analysis of KRAS cells \textit{in vitro} and \textit{in vivo}
  - In house development of “tumor-surface proteomics”
- Collaborate to use selective panning technologies (phage display) to survey the KRAS cell surface
  - Robert Rottapel and Sachdev Sidhu, Univ Toronto
  - Renata Pasqualini, Univ New Mexico (December visit to FNL)
- Use RNA seq and ER-polysome profiling to predict protein complement on KRAS cell surface
  - Martin McIntosh, FHCRC
- Bioinformatic Approaches
  - Renata Grifantini, Externautics, Italy (December visit to FNL)
- Immunotherapy Workshop
  - Elizabeth Jaffe and Bob Schrieber to help organize (at 2015 AACR)
Enabling the Community:
RAS Reference Reagents and Cancer.gov/RAS

Dom Esposito  
Jim Hartley
External reagent requests (since July 2014)

- 15 requests for RRR reagents (clone sets and other items)
- 8 approved TSAs, 2 approved MTAs, 5 others in progress
- Fred Hutch, Baylor, Munich, Stanford, MIT, Broad, Northwestern, CRUK

RAS Initiative support—344 constructs generated since July 2014

- 212 constructs for protein expression (TBU-C/Project 1)
- 79 constructs for Project 3
- 53 constructs for TBU-Z

RAS Pathway clone set underway (181 clones)

Kris Wood collaboration underway (200+ cancer toolkit clones)
Development of the RAS Initiative at the Frederick National Laboratory for Cancer Research (FNLCR)

Since the early 1970s, the National Cancer Institute (NCI) has been responsible for a contract that supports the only Federally Funded Research and Development Center (FFRDC) devoted principally to biomedical research. Located on a government campus in Frederick, MD, the FFRDC has provided a variety of laboratory services to the scientific community, performed research in response to national needs, and supervised subcontracts for the NCI for over 40 years.

In 2011, following a suggestion by the NCI's National Cancer Advisory Board, NCI Director Harold Varmus named the operational laboratory arm of the FFRDC Frederick National Laboratory for Cancer Research (FNLCR) and established an advisory committee (now called the
RAS Community feedback on RAS signaling model:

January 13, 2015 by Frank McCormick

Our original pathway diagram (posted on October 22, 2014) has been altered incorporating suggestions from the following investigators:

- Phil Stork (stork@ohsu.edu) suggested adding RASGRF1, but we left out PDZGEF1 which (I think) is a RAP1 GEF, not a RAS GEF. Any input on this would be welcome.
- Julie Irving (julie.irving@ncl.ac.uk) suggested adding FLT3 (also suggested by Kevin Shannon shannonk@peds.ucsf.edu), PTPN11 (also suggested by Ben Braun, UC San Francisco) and CBL. On Ben's advice, PTPN11 was kept vague with a positive arrow to RAS activation of RAF, without a clear target.
- Manuela Baccarini (manuela.baccarini@univie.ac.at) noted that MAP2K1 activates PTEN by sending it to the plasma membrane. RAF1 inhibits ROCK2, but was not included in our updated diagram because the drawing was getting too complicated. This may be fixed in a subsequent version.
- Mike Nickerson (nickersonml@mail.nih.gov) included negative feedback to IRS1 and 2 from the PI3 kinase pathway.
- Anne Goriely (annegoriely@imm.ox.ac.uk) suggested adding the MRAS (RIRAS3) and SHOC2.PPP2CA pathways that activate RAF kinase and ERK, an ETS-family protein that inhibits ERK1 (aka MAPK3) and 2 (MAPK1).
- Eric Collison (eric.collison@ucsf.edu) suggested adding the RHO GEF ARHGEF2, aka GEF-H1. Rob Rottoelop has recently shown this also acts on the MAPK pathway via interaction with KSR1, but I couldn't find a way of representing that in this version.
- Jim DeCaprio (james.decaprio@dfci.harvard.edu) suggested adding cyclin D2 (CCND2) and 3 (CCND3) in addition to Cyclin D1 (CCND1) as MAPK targets. Ping Lu (kl@bidmc.harvard.edu) suggested adding PIN1 as an activator of RAF1 kinase (aka RAF). It could have been added at many other sites (see Cell Res 24, 1033, 2014 for a review by Zhimin Lu and Tony Hunter).
- Michael Tainsky (mat@wayne.edu) noted that AP2 is essential for RAS transformation, but I couldn't find a direct link to the RAS pathway. Any suggestions are welcome.
- Maria Zapata-Kaye (mzappatkaye@uf.edu) suggested adding E2F target genes, including thymidine synthase (TYMS). Target genes that appear on other parts of the pathway were not listed, to keep it simple.
- Ramon Parsons (ramon.parsons@msm.mskcc.org) suggested adding the RAC GEF, PREX2.
- Shyam Biswal (shyam@bju.edu) and Carola Neumann (neumannc@upmc.edu) suggested adding NF-E2L2 (aka NFE2L2), and NOX1.
- Eric O'Neill (eric.oneill@oncology.ox.ac.uk) suggested adding a mini-version of the Hippo pathway (RASSF, MST [STK3 and 4], SAV1, YAP1).
- Joe Ramos (joeramos@hawaii.edu) and Naoto Ueno (nueno@mdanderson.org) suggested adding PEA15.
- Larry Leig (larry.leig@ufts.edu) suggested adding RASGRF1 and 2.
- Howard Crawford (crawford.howard@mayo.edu) proposed adding VAV1, ECT2 and TIAM1. Howard also suggested DOCK10, but I wasn’t sure where to put it. Suggestions welcome!
- Mike White (michael.white@utsouthwestern.edu) suggested adding EXOC1-8 connected to the RALB, B node and CNKR1, CNKR2, SHOC2 connected to the RAF node.
- Karen Cichowski (karen.cichowski@rics.bwh.harvard.edu) suggested adding DAB21P and INPP4B.
- Andrew Sharrock (a.sharrock@manchester.ac.uk) suggested adding BLK1.
- Mark Phillips (philip01@nyu.edu) suggested adding FFTA and B, RCK1 and ICMT1.
- Debbie Morrison (morrison@mail.nih.gov) suggested adding a feedback arrow from MAPK1,3 to the RAFs.
- Channing Der (cder@medunc.edu) suggested adding MYC as a MAPK1/3 substrate. He also included RGL, RGL2 and RGL3 with RALGDS as another effector that links RAS with RAL.
- Additionally, the RALGAPs, RALGAP1 and RALGAP2, and PLC61 are included as RAS effectors.
- Philippe Roux (philippe.roux@umontreal.ca) pointed out errors in the pathway as originally drawn: “The pathway should indicate that RPS6KB1 and RPS6KB2 are targets of mTOR (they are the p70 S6ks). The related p90 S6ks (RPS6KB1, RPS6KB2, RPS6KA3, RPS6KA6) are actually early targets of MAPK1/3, and should be transferred to that branch of the pathway.” Steen Hansen (steen.hansen@childrens.harvard.edu) also pointed out these errors and suggested we add FOSL1 downstream of RPS6KA1,2,3 and 6. Additionally, there are two more isoforms of EIF4EBPs, and thus the pathway should indicate EIF4EBP1-3.
- Julian Downward (julian.downward@cancer.org.uk) also noted some errors, “You should have PIK3CA, PIK3CD and PIK3CG here, but not PIK3CB as p110beta does not interact with RAS. Also, for regulatory subunits, there should be PIK3R 1, 2, 3, 5, and 6, but not 4 – this is VPS15, the regulatory partner of VPS34. PIK3R 5 and 6 are the regulatory subunits of p110gamma, so are fine to have here.”
RAS Initiative Oversight Follow-up
RAS Ad Hoc Working Group Meeting (October 31, 2014) – Action Items

• Publish protocols for production of fully processed KRAS protein and associated structural studies and assays.
  – Manuscript to be submitted by end of February 2015

• Establish process for collaboration and providing reagents to community
  – Processed KRAS will be provided to collaborators
  – Protocols and reagents provided to others

• Optimize RAS-less MEF screen: understand and eliminate sources of variation
  – Use validation inhibitor panels at NCATS
  – Evaluate conditional oncogenic KRAS MEFs (Tuveson)

• Node-knockdown-based (SiREN) approach will produce a large data set that will be discussed at the next Working Group meeting.
  – Experiments completed, data analysis ongoing
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  – Experiments completed, data analysis ongoing
RAS Initiative Postdoctoral Fellowships

• **Postdoc Program**
  
  – *Pancreatic Action Network/FNL Fellows*
    
    • **Lynn McGregor** *(Shokat lab)*
    
    • **John Hunter** *(Westover lab)*

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THE PANCREATIC CANCER ACTION NETWORK AND THE NATIONAL CANCER INSTITUTE’S FREDERICK NATIONAL LABORATORY FOR CANCER RESEARCH COLLABORATE ON TWO 2015 FELLOWSHIPS TO ADVANCE KRAS RESEARCH

A critical area of continuing research aimed at better understanding pancreatic cancer and developing new, more effective ways to treat it is focused on a genetic mutation found in most pancreatic tumors: KRAS.

And now, the Pancreatic Cancer Action Network has formed a unique partnership with the National Cancer Institute’s (NCI’s) Frederick National Laboratory for Cancer Research (FNLCR) to advance KRAS research. FNLCR is a government-owned, contractor-operated facility devoted exclusively to biomedical research and development.

Our organization and FNLCR have awarded year-long Fellowships that commenced on January 1, 2015, to John Hunter, Ph.D., and Lynn McGregor, Ph.D. Dr. Hunter is working in the laboratory of Kenneth Westover, M.D., Ph.D., at the University of Texas Southwestern Medical Center. His Fellowship is being funded by Ambassador Cynthia Stroum, the Pancreatic Cancer Action Network’s Founding Board Chair Emeritus, in memory of her father, Samuel Stroum. Dr. McGregor is conducting her postdoctoral work under the mentorship of Kevan Shokat, Ph.D., at the University of California, San Francisco (UCSF).
Interactions with the RAS Community

Seminars at FNL
- Channing Der, UNC
- Ken Westover, UTSW
- Carla Mattos, Northeastern
- Mark Philips, NYU
- Vadim Gaponenko, U Chicago
- Josh Salafsky, Biodesy
- Calvin Kuo, Stanford
- Kris Wood, Duke
- Mariano Barbacid, CNIO Madrid
- Cyril Benes, MGH
- Carolyn Buser, GSK
- Stephen Sligar, U Illinois
- Raffit Hassan, NCI
- Renata Grifantini, Externautics Siena
- Renata Pasqualini, U New Mexico
- Andrew Bradbury, Los Alamos
- Kent Rossman, U North Carolina

NCI RAS Initiative at FNL

NIH collaborators
- Ji Luo, NCI
- Anton Simeonov, NCATS
- Debbie Morrison, NCI
- Rajat Varma, NIAID

Outside collaborators
- Steve Almo, AECOM
- Jim Wells, UCSF
- Channing Der, UNC
- Ken Westover, UT Southwestern
- Carla Mattos, Northeastern
- Steve Sligar, U Illinois
- Jay Groves, UC Berkeley
- Hirsch Nanda, Susan Kreuger, NIST
- John Markley, National Magnetic Resonance Facility at Madison (NMRFAM)
- Kris Wood, UNC
- Immuno-MRM of RAS pathway
  - Mandy Paulovich, Fred Hutch
  - Steve Carr, Broad
  - John Koomen, Moffit
  - Tina Yuan, Cameron Pitt, UCSF
  - Dave Tuveson, CSH

RAS workshops
- Synthetic Lethality, January 6-7 2014
- Pathways, June 11, 2014
- Cell Surfaces, July 23, 2014
- 2015 AACR Annual Mtg, agenda pending

Information exchanges
- David Weber, U Maryland
- Hirsch Nanda, Susan Kreuger, NIST
- Amanda Altieri, U Maryland
- David Barford, ICR UK
- Bill Sellers, Novartis
- Kurt Auger, GSK
- Paul Cohen, DARPA
- Ian Prior, U Liverpool
- Said Sebti, Moffitt

Recipients of RAS Reference Reagents
- Chris Kemp, Fred Hutch
- Eric Chang, Baylor
- Silvia Thone, Munich
- Peter Jackson, Stanford
- Tyler Jacks, MIT
- Calvin Kuo, Stanford
- Bill Hahn, Broad/DFCI
- Karla Satchell, Northwestern
- Julian Downward, CRUK