Ras Project Progress Report

Frank McCormick
# Ras Mutations in Cancer

<table>
<thead>
<tr>
<th>Primary Tissue</th>
<th>KRAS</th>
<th>HRAS</th>
<th>NRAS</th>
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<tbody>
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# K-Ras Mutations in Four Major Cancers

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<th>G12C</th>
<th>G12D</th>
<th>G12V</th>
<th>G13D</th>
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<td><strong>Total new cases/yr</strong></td>
<td>137,800</td>
<td>29,700</td>
<td>53,700</td>
<td>39,100</td>
<td>15,300</td>
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Ras Proteins

G-domain

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

KRAS 4B

KRAS 4A

NRAS

HRAS
Expression of KRAS 4A & 4B in Colorectal Cancers

TCGA COAD Samples sorted by 4a/4b ratio

Bob Stephens
Colorectal Cancers by 4A/4B Ratio (TCGA)

Blue=alive; red=dead
Project Zero: Validating KRAS, Identifying KRAS dependent cells

Validate KRAS and Downstream Signaling Nodes
Pancreatic Cell Lines

Potent multi-node siRNA knock-down

Multi-parameter analysis

Cell line intake QC, Characterization

Mechanisms of Resistance

Ablate KRAS using CRISPR

Characterize resistant cells

Tina Yuan UCSF, Ji Luo NCI
Scott Lowe CSHL, Cyril Benes, Harvard/MGH

Frederick National Laboratory for Cancer Research
Project Zero: Progress

Assays

- Cell-based assays standardized
  - Viability/proliferation, apoptosis, anchorage independence (agar vs polyhema), migration/invasion

Cell Lines

- >30 cell mutant KRAS cell lines fully characterized
  - Growth, GE, protein expression, anchorage independence, migration/invasion

Reagents

- Validation of KD/KO reagents ongoing
  - CRISPR, inducible lentivirus
- Novel cell lines developed (P2, P3), validation ongoing

Frederick National Laboratory for Cancer Research
1. Validate KRAS and downstream signaling nodes
   - Collaboration with Tina Yuan, UCSF and Cyril Benes (Harvard, MGH) has been initiated
     - Reagent, cell lines and protocol transfer ongoing
   - Pancreatic cell line characterization using this method expected to be complete within 4-5 months

2. Mechanisms of resistance
   - Validating CRISPR method
   - Cell line panel is being generated using characterization data
   - Full method will be on-line within 2-3 months
Informatics approach to KRAS Dependency

- Cell line selection for the different RAS program projects benefits from full genomics assessment of the genetic background of the cells.
- Previous reports have identified panels of KRAS-dependency genes associated with various tumorigenic indicators such as EMT.
- This information can be combined with our project derived data to aid in interpretation of results (responder/non-responder etc.)

Cadherin expression across all ccle cell lines

Density plot for cdh1/expr_bygene/score

N = 1036  Bandwidth = 0.547
K-Ras dependent cells and EMT

![Plot for CDH1 lengths=8,8](image)

![Plot for ZEB1 lengths=8,8](image)
Project One: Overall goals

• Identify new pockets for drug binding, potential for affecting GTP hydrolysis, allosteric regulators, binding assays

• Structural and biophysical analysis of 4 KRAS mutants bound to key effectors and regulators (Raf, PIK, RalGDS and GAP)

• Structure of KRAS4B bound to calmodulin

• Structure of full length Raf bound to Ras

• Processed Ras bound to synthetic membrane
Project One: KRAS Structural and Biophysical Analysis

- Structural/biochemical analysis of KRAS mutants.
  - Pilot crystallization for KRAS variants (WT KRAS, G12D, G12V, G12C, and G13D)
  - Analysis of GTPase activity, GDP release, effector binding (IP or SPR)

- Analysis of the KRAS-Calmodulin interactions.
  - Crystals of KRAS-Calmodulin complex
  - Analysis of the binding determinants/affinity using SPR, IP, fluorescence polarization
  - Effect of oncogenic mutations/GTP-GDP state on the binding interaction
Protein Production

- Expressed in E. coli and insect cells as His6-tev-, and His6-MBP-tev-
  - New constructs screened by micro-scale purification
  - Scale up to 2 or 15 liter scale
- Purification process: IMAC/TEV digestion/IMAC/SEC
- Yields from His6-MBP- constructs (~100+ mg/liter) are 10-fold better than His6- constructs
- Optimization of insect expression ongoing

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<tr>
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<th>His6-tev</th>
<th>His6-MBP-tev</th>
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<td>G12D</td>
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Quantitative Analysis of KRAS by Amino Acid Analysis and HPLC

RAS calibration by HPLC; 280 nm

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<th>RAS; ug (aaa)</th>
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<td>ras, G12D</td>
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Column: Poros R2
UV 280 nm

Oleg Chertov and Protein Core AIDS Cancer Vaccine Program, FNL
Determination of GXP Concentration in G12D RAS Prep

RAS GDP loading = 26%
(no added GDP)

10 µM, G12D prep
2 µM, GXP mix
5 µM, GXP mix
Addition of GDP increases the Stability of WT KRAS.
MALDI-TOF MS of KRAS 4B (wt) Expressed in Insect Cells

This m/z could correspond to C-terminal peptide cleaved by Glu-C confirming that Major form of KRAS expressed in insect cells is Truncated at C-terminus

E.KMSKDGGKKKKKKSKTK
Expected m/z 1878.168
KRAS : Calmodulin Interaction

Calmodulin Binds to K-Ras, but Not to H- or N-Ras, and Modulates Its Downstream Signaling

PRIAM VILLALONGA,1 CRISTINA LÓPEZ-ALCALÁ,1 MARTA BOSCH,2 ANTONIO CHILOECHES,2 NATIVITAT ROCAMORA,3 JOAN GIL,4 RICHARD MARAIS,2 CHRISTOPHER J. MARSHALL,2 ORIOL BACHS,1 AND NEUS AGELL18

KRAS 4B

KEKMSKDGGKYYYYYKSKTKC

Diagram of proteins and molecular structures.
KRAS-G12D-CaM Complexes by SEC and Western Blot

![Graph showing SEC fractions and Western blot results](chart.png)
Binding kinetics of CaM to WT-KRAS

Time (s) 0 200 400 600
Binding (Response Units) 0 200 400 600 800 1000 1200 1400 1600
CaM concentration (M) 0 2e-5 4e-5 6e-5 8e-5 1e-4

K_D 11uM

100uM 33uM 11uM 3.6uM 1.2uM

Lakshman Bindu
Binding Kinetics of CaM to WT-KRAS

K_D 11uM

CaM concentration (M)

Binding Kinetics

Time (s)

K_D 11uM

GDP “loaded”

GTP “loaded”

Lakshman Bindu
CaM binding is Ca\textsuperscript{2+} Dependent

Time (s)

Binding (Response Units)

0 200 400 600

0 200 400 600 800 1000

Ca\textsuperscript{2+}

No Ca\textsuperscript{2+}

Lakshman Bindu
Project Two: Cell-based Screens for Compounds that Target K-Ras

RAS-less MEFs

Drosten M, Dhawahir A, Sum EY, Urosevic J, Lechuga CG, Esteban LM, Castellano E, Guerra C, Santos E, **Barbacid M**. EMBO J. 2010
Work with NCATS to Develop 4A/4B Screen

• Presentation of RAS Program to NCATS Dec 19, 2013
• Developed 4A/4B screen strategy and workplan; presented to NCATS on January 17, 2014
• Discussions lead to strategy that meets RAS Program goal to find hits that target unique KRAS features
  – 1st identify compounds that differentially effect mutant KRAS 4b relative to wt HRAS
  – then focus on the difference between KRAS 4a and KRAS 4b hypothesis is that the unique KRAS carboxy-terminus contributes to KRAS dependent oncogenesis
Iterative Assay Development

• Proximity of NCATS to ATRF imparts advantages to assay optimization and qualification

• Division of Labor:
  – Ras Program
    • Develop cell lines
    • Proof of principle two color growth readout
    • Confirm response to FTIs
    • Develop secondary screens
      – Cellular, biophysical, high-content imaging
  – NCATS:
    • Provide advice and expertise for assay development
    • Run assay against select libraries to assess performance
    • Assess full-scale screen
Several iterations of assay optimization expected before full library can be screened by NCATS.
4A/4B Assay Flow Diagram

**Bins**

1. Hits that show differential in primary and counter screens with multiple family members and reasonable dose response

2. Hits that show differential in primary and counter screens with restricted family members and reasonable dose response

3. Hits that show differential in primary and one counter-screen
Optimizing the First Screen

Limit of detection ~200-400 cells
Cell number determined by counting at high dilution

Fluor ratio is reasonably linear as a function of cell density in mixed cultures

RPZ 123 percentage in 123/125 cell mix

Results with manual washing/automated plate washing dislodges cells

Ratio mCherry to eGFP for 123 diluted in 125 cells
Project Three: Disrupting K-Ras complexes
Oncogenic signaling driven by KRAS is mediated by KRAS dimers and higher order structures in the cell membrane.

Disruption of these complexes will attenuate the oncogenic signaling and therefore represents a target of drug discovery.
PANC-1 cells Expressing GFP-HRas

DMSO

12.5 µM

25 µM

FTI-276
Micropatterns

Cells on micropatterns are more homogenous in the localization of cellular structures vastly improving ability to quantify changes.

Surface of cell when spread out on micropattern @1600 µm²
RAS-less MEFs on Micropatterns

non-micropatterned cells

micropatterned cells

actin

nucleus
Development of a Ras biosensor

FRET signal in PANC-1 cells expressing Rac biosensor
Techniques like PALM and FCS provide detailed, and perhaps actionable, single molecule information about the location and persistence of KRAS complexes in cell compartments.

PALM image of PA-mCherry-HRAS-G12V-MEF

Bright field and TIRF image

Scale bar 2 μm

30 nm resolution

250 nm resolution
Project Four: surface proteins on KRAS Cancers

- Goal: identify proteins on the surface of KRAS cancer cells for targeting nano-particles or immunotherapy, or for use as biomarkers

- Approaches:
  
  Mass spec analysis of proteins on KRAS cancer cells

  Phage display (Jim Wells et al)

  Validate candidates from literature

  Bioinformatics
Method 1: Cell Surface Proteome Mapping - Fractionation

1. Whole proteome MCF10A cells
   Cell lysis - sonication - SCX

2. Microsomal fraction
   Ultracentrifugation - SCX

3. Plasma membrane
   Sucrose gradients - SCX

4. Lipid rafts
   Detergent resistant fraction - SCX

LC-MS
Method 2: Chemical Tagging of Cell Surface Proteins on Live Cells

- sodium periodate
- biocytin hydrazide
- cell lysis
- digestion
- LC-MS analysis
- peptide release
- glycosidase treatment
- affinity enrichment

## LC-MS analysis of MCF10A Cell Surface Proteins isolated using CSC

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<th>Gene</th>
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<th># Peptides</th>
<th># PSMs</th>
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</table>
Method 3: Cell Surface Protein Labeling, Isolation and Identification

MCF10 + Sulfo-NHS-SS-Biotin → Labeling

Lysis → Isolation

Trypsin → DTT → SH
Informatics approach

• *In-silico* identification of genes potentially differentially expressed on the surface of KRAS mutant cells relative to normal cells derived from the same tissue

• Additional potential to identify novel KRAS-associated targets and also help guide/support clinical decision making

• Approach: scan TCGA lung tumor data available for matched tumor normal pairs for differentially expressed genes
Differentially Expressed Genes from Lung Adenocarcinoma Matched Tumor Normals

(n=45)
Jan 8/9
Workshop at ATRF

Organized by Ed Harlow
Jim Hartley, Sara Hook et al

Outcome: Two potential PARS for new synthetic lethal screens
• Previous whole-genome RAS synthetic lethality (SL) screens were substantially underpowered

• From early results, CRISPR is probably superior to either RNAi technology (shRNA or siRNA)

• Heterogeneity matters, the more cell lines, the better

• There are indications that selections in 3D yield hits that are substantially different from 2D

• in vivo (i.e., in mice) screens require cells that form tumors very efficiently, this imposes selective pressure

• Combining knockdown or knockout of genes with inhibition of specific (druggable) pathways can reveal new susceptibilities

• Some pathway nodes are comprised of multiple redundant proteins, components of such nodes may not yield lethality or sickness that is synthetic with mutant RAS.
• Pooled screens, in which all targeting agents are applied as a mixture to millions of cells for selection in a single container, cannot detect SL based on loss of secreted molecules.
  - This suggests that arrayed screens (target one gene at a time) using pre-validated knockdown reagents is a better approach.

• There are SL interactions that we do not understand. Cautionary to only following hits that "make sense".

• If even one KRAS mutant allele in a major human cancer could be targeted as the result of a new SL screen, thousands of lives could be saved
• support internal FNL/CRTP Ras projects with qualified and standardized reagents
• generate high-quality reference reagents for the Ras extramural community (national reference reagents)

DNA clones  
Cell lines  
Viruses  
Proteins  
Antibodies
RAS Reference Reagents

**CRISPR detailed plans**

- Initial test constructs in pX330 (GFP, NRAS, HRAS, KRAS)
- Generate Gateway-CCP compatible cassettes
  - Inducible promoters, tissue-specific promoters, cell-line specific promoters
  - CAS9 with T2A-GFP or T2A-Ab, or separate markers with their own promoters
  - CAS9 wt and nickase versions
  - FNLCR “self-inactivating” cgRNA
RAS Reference Reagents

• 109 fully sequence validated Ras Entry clones
  – Most clones are in multiple formats (ATG-closed, ATG-open, tev-closed)
  – KRAS4b (wt, G12C, G12D, G12V, G13D, 17 other mutants, 1-166)
  – KRAS4a (wt, 7 mutants)
  – HRAS (wt, 3 mutants)
  – NRAS (wt, 3 mutants)

• 36 fully sequence validated RAS pathway and RAS-related gene Entry clones
  – Open and closed full-length clones
  – for structural studies, assay development, validated clone collections

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<th>Completed</th>
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<th>Completed</th>
<th>In Progress</th>
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<td>KNDC1</td>
<td>RASAL1</td>
<td>RGF (RalGDS)</td>
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<td>RASGRP3</td>
<td>RASGEP1a</td>
<td>CRAF (RAF1)</td>
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</table>
Materials generated for other RAS Projects

• Project Z
  – lentiviral constructs for wt and GFP tagged KRAS, HRAS, and NRAS
  – allele-specific shRNA constructs including inducible designs
  – materials for AAV-based gene editing

• Project 1
  – 107 protein production clones for E. coli and insect cell production
  – clones for biotin-tagged Ras for biophysical studies

• Project 3
  – mEos2/mDendra fusions of KRAS, HRAS, and NRAS for PALM
  – 3xFLAG and Halo fusions of KRAS4b and mutants for localization and P-P interaction
  – KRAS biosensors
### FNLCR/CRTP Ras Program Web Page Index

(11/08/2013 08:05:01)

<table>
<thead>
<tr>
<th>URL</th>
<th>Description</th>
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<tbody>
<tr>
<td>cell_lines.php</td>
<td>screen/filter through COSMIC and CCLE cell lines (file based)</td>
</tr>
<tr>
<td>cell_lines2.php</td>
<td>advanced screen/filter through COSMIC and CCLE cell lines (file based)</td>
</tr>
<tr>
<td>ccle_bygene.php</td>
<td>get expression, copy number and mutation data across cell lines (file based)</td>
</tr>
<tr>
<td>2genes.php</td>
<td>Select CCLE Cell Lines with Mutant Gene Pairs</td>
</tr>
<tr>
<td>copynum_bychrom.php</td>
<td>get CNVs by chromosome and cell line</td>
</tr>
<tr>
<td>copynum_bychrom.php</td>
<td>get CNVs by chromosome and cell line</td>
</tr>
<tr>
<td>copynum_bygene.php</td>
<td>get CNV cell lines by gene</td>
</tr>
<tr>
<td>copynum_byline.php</td>
<td>get CNV genes by cell_line</td>
</tr>
<tr>
<td>expir_bychrom.php</td>
<td>get expression by chromosome and cell line</td>
</tr>
<tr>
<td>expir_bygene.php</td>
<td>get expression cell lines by gene</td>
</tr>
<tr>
<td>expir_byline.php</td>
<td>get expression genes by cell_line</td>
</tr>
<tr>
<td>density_bygene.php</td>
<td>plot expression/copynum distribution</td>
</tr>
<tr>
<td>boxplot_bygene.php</td>
<td>plot expression/copynum by tissue</td>
</tr>
<tr>
<td>density_bygene_multi.php</td>
<td>plot expression/copynum dist. by tissue (all genes on same plot)</td>
</tr>
</tbody>
</table>

**Density plot for cdh1/expr_bygene/multi**

**Plot for CDH1 length/88**

**Lung_Adenocarcinoma_TCGA_KRAS_MutDataOnly_pairG**

**KRAS_MutType**

- 01:G
- 01:D
- 01:C
- 02:H
- 02:V
- 02:T
- 02:G
- 02:A
- 02:R
- 02:Q
- 02:W
- 02:K
- 02:R
Additional IFX Applications