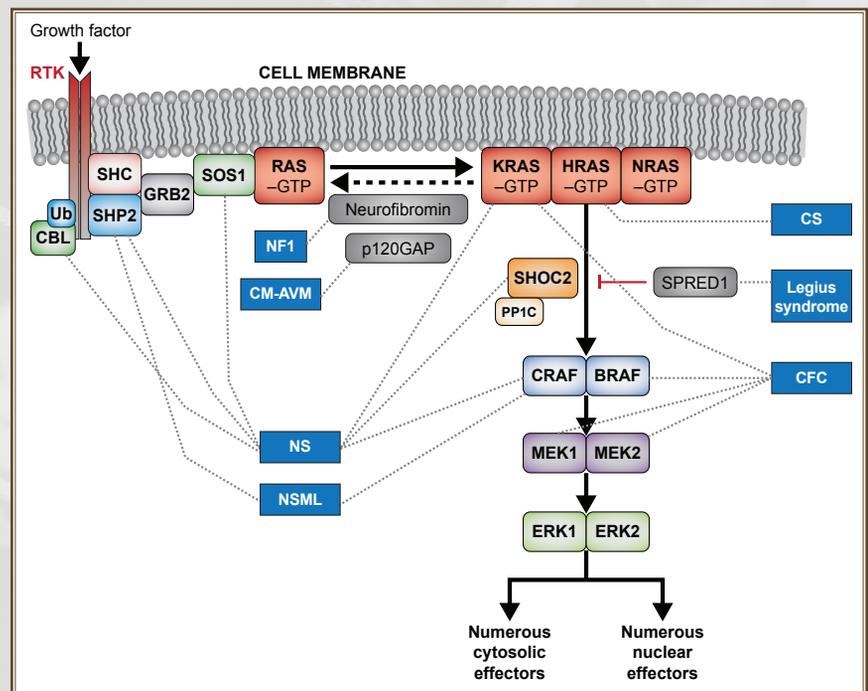


# RAS Takes Center Stage

*Wreaking cellular havoc in approximately one-third of all cancers, oncogenic RAS signaling has been extensively studied in the 30 years since the gene first associated with rat sarcoma virus was identified in human tumors. But, devising anticancer drugs that target RAS proteins has remained frustratingly elusive. RAS molecular structures lack obvious pockets for small molecule disruption and early attempts to inhibit an enzymatically driven modification of RAS (farnesylation) thought to be necessary for its translocation to the cellular membrane led to disappointing failure in clinical trials. As scientists have continued to focus on the details of RAS signaling and the extensive molecular network under its control, however, their persistence is beginning to pay off: new therapeutic approaches are once again on the horizon.*

Sitting in the office of Debbie Morrison, Ph.D., Chief of CCR's Laboratory of Cell and Developmental Signaling, it is hard not to notice the 3-D molecular structure etched in glass that is prominently displayed on her desk. "This is the first structure of the B-RAF catalytic domain," explained Morrison. U.K. scientists Richard Marais, Ph.D., and David Barford, D.Phil., first described the structure at a FASEB meeting on protein kinases and protein phosphorylation. "Dr. Marais gave me the structure as a fellow colleague who has spent her career studying the RAF kinases."

RAF kinases are key effectors of RAS signaling; RAF is the initiating kinase in the RAF-MEK-ERK cascade that regulates cellular growth in a variety of biological and pathological contexts. Using biochemical and proteomic approaches, Morrison has delved into the molecular mechanisms that regulate the RAF kinases and their response to RAS activation. Much of her work has been in the context of normal growth factor signaling



RAS is activated by growth factor receptor signaling at the cellular membrane to initiate the RAF-MEK-ERK cascade.

which relies on these signaling pathways for healthy proliferation of cells in the developing organism.

Of the components in this cascade, RAF regulation is the most complex, including negative regulation and feedback loops. RAF

also exists as multiple subtypes (A-, B-, and C-), with different properties and functional contexts. "For many years, we knew that RAFs interacted, but because there are so many other components and interactions involved in the

(Image: Courtesy of Scientific Publications, Graphics & Media)

RAF activation process, it wasn't clear how important the RAF-RAF interaction was and whether it reflected direct dimerization of the RAFs," said Morrison.

Then, a series of new findings piqued Morrison's interest, and that of her colleague, Postdoctoral Fellow Alyson Freeman, Ph.D. [See "In Conversation," *CCR connections*, Vol. 7, No. 2]. First, the 3-D structure published by Marais and Barford revealed that the B-RAF catalytic domain formed side-to-side dimers and that the dimer interface was in close proximity to the ATP-binding pocket. "Then, there were a series of papers looking at the use of ATP-dependent RAF inhibitors in melanoma," explained Freeman. "The inhibitors hampered disease progression in melanomas expressing a mutant B-RAF kinase, but in cells that contained wild-type RAF, there was a paradoxical activation of the ERK pathway that apparently involved RAS-dependent RAF dimerization."

"We decided it was really important to look at the endogenous proteins, rather than overexpressed proteins, and by studying homo- and heterodimerization of the different RAF subtypes, we discovered not only that dimerization is critical to RAF activation but that the dimer interface might be a target for therapeutic intervention," said Morrison.

Freeman, Morrison, and their colleagues went on to show that using a peptide to block the dimer interface, they could effectively silence RAF signaling in many contexts, including when RAS is activated by a mutation.



(Photo: R. Bauer)

Daniel Ritt, Debbie Morrison, Ph.D., and Alyson Freeman, Ph.D.

Interestingly, they also found that the most prevalent oncogenic mutation of RAF, V600E-B-RAF, rendered the kinase independent of dimerization and that the peptide was not effective when RAF activation was dimerization independent. "So if we can block RAF dimerization clinically, we will need to determine what the specific mutations are in a cancer to know if blocking RAF dimerization would be an effective treatment. Given the resistance that develops to current RAF inhibitors, a dimer blocking agent may also help as a combination therapy to prolong disease-free survival."

"Understanding the details of RAF activation explains a lot of what is seen in clinical treatment—why some therapies are working or not working," said Morrison. She pointed out that certain other anticancer drugs can also promote the paradoxical activation of RAF. "The ATP binding site is a very conserved region, and some ATP-competitive kinase inhibitors can have off-target effects on RAF,

such as those directed against BCR-ABL and p38. Thus, while you're trying to suppress BCR-ABL or p38 signaling, your drug may actually be binding quite well to RAF and inducing RAF dimerization. You may be trying to inhibit one pathway and be successful, but at the same time, you might be upregulating ERK signaling."

In June 2013, NCI Director Harold Varmus announced a \$10 Million initiative to develop new ways to block oncogenic RAS signaling. Morrison participated in a workshop in advance of the announcement and is enthusiastic that the time is right for a concerted attack on RAS and that a more nuanced view of therapeutic mechanisms is emerging.

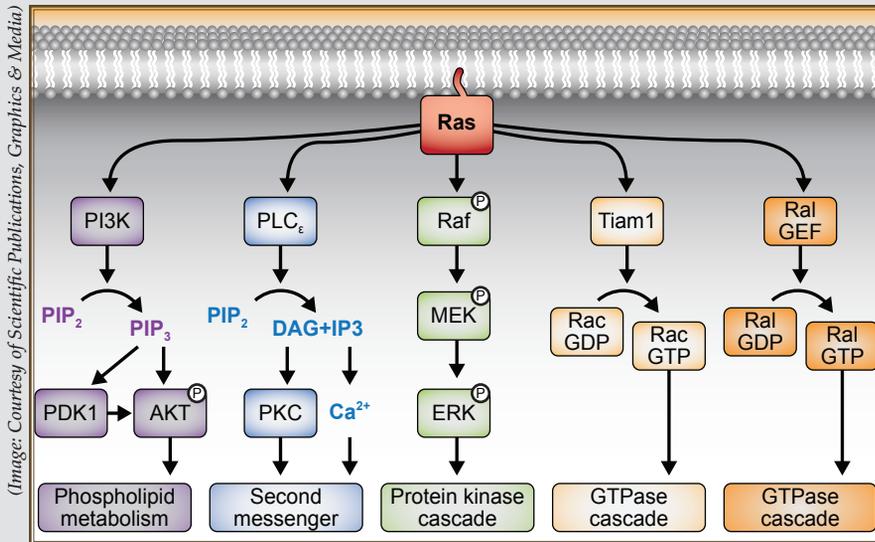
"If an inhibitor of RAF dimerization came out, I'd feel thrilled that we had contributed to it," said Morrison.

## Synthetic Lethality

Ji Luo, Ph.D., Tenure-Track Investigator in CCR's Laboratory of Cancer Biology and Genetics, agrees that the field is gaining critical momentum. "We know enough about the biology of RAS and have enough new molecular and genetic tools that we can revisit the issue of targeting RAS pathways."

Luo's laboratory is taking a multipronged approach to targeting

"We discovered not only that dimerization is critical to RAF activation but that the dimer interface might be a target for therapeutic intervention."



RAS signaling pathways in cancer

oncogenic KRAS, one of the three canonical RAS family members (H-, K-, and N-). At the heart of his approach is the concept of synthetic lethality, which comes from genetics, and refers to the impact of multiple genetic mutations on viability. Mutations in *KRAS* not only do not kill cells, they endow them with their invasive and proliferative advantages. But such oncogenic mutations achieve tumorigenesis at a cost: oncogenic stress. Cells experience increased apoptotic signals, metabolic stresses, and genomic instability, which must be

ameliorated by the expression of supporting molecular factors.

Using RNA interference (RNAi) screens to inhibit expression of individual genes, Luo and his colleagues are searching for pathways that are required for the survival of cells which express mutant *KRAS*, but not wildtype *KRAS*. Among the genes that they have thus far explored, RNA splicing factors have come to the forefront. These factors are involved in editing mRNA transcripts to produce selected gene products and until recently, have not been strongly linked with cancer.

“We think RNA splicing factors may be controlling key genes that maintain survival and growth; we have a number of candidates that we are investigating,” said Luo.

Another pathway that has received scant attention from cancer researchers is a pathway that modifies proteins after they have been translated with the addition of small ubiquitin-related modifier (SUMO) proteins. A highly dynamic, regulated process, sumoylation affects diverse properties, including protein localization, activity, and stability. Luo’s team has found that sumoylation is important for the ability of cells with *KRAS* mutations to thrive unanchored *in vitro* (the classic assay for oncogenic transformation). Furthermore, inhibition of protein sumoylation both *in vitro* and in xenograft mouse models suppresses the cancerous phenotype. “We have identified the E2 ligase UBC9 as central,” said Luo. “Its enzymatic activity is important for *KRAS*-driven transformation.” As a result, Luo is collaborating with Jay Schneekloth, Ph.D., a Tenure-Track Investigator in CCR’s Chemical Biology Laboratory [See “Putting Peptides to Work,” *CCR connections*, Vol. 7, No. 2], who uses structural approaches to design inhibitors against E2 ligases, including UBC9. “We have been going back and forth, combining my lab’s expertise in genetics with his lab’s chemical expertise to explore UBC9 as a druggable target,” said Luo.

Cognizant of the failures to target *KRAS* with small molecules, Luo is excited about the possibilities of using small interfering RNAs (siRNAs) for targeted interference with gene translation. “We’ve developed very potent siRNAs to knock down *KRAS* at low nanomolar concentrations,” said Luo, even while acknowledging that delivery is a major therapeutic challenge. Knowing of several nanoparticles to package siRNAs that are under development in academic



Chih-Shia Lee, Ph.D., Ji Luo, Ph.D., Changwoo Lee, Valentin Giroux, Ph.D., Hueyjong Shih, Joseph Carver, and Bing Yu, Ph.D.

and industrial settings, Luo is optimistic. “*In vitro*, it’s magnificent. The beauty of siRNAs as a therapeutic is that they all work the same way, you just have to change the sequence. So, it’s easy to do combinations and you can target anything.”

Luo also points out that RAS has over 50 downstream effectors, if you count all the gene isoforms. And as is increasingly the case for all cancers, RAS-driven cancers are likely to be defeated ultimately with combinatorial approaches, whether by small molecules, biologics, or RNAi. “So far, we don’t have a drug against every RAS effector, but we do have potent siRNAs, so we can develop screens to address the combinatorial issues up front. It gives us a rational path for drug discovery.”

### All in the Family

RAS is a small GTPase, meaning it is active when bound to GTP and inactive when the GTP hydrolyzes to GDP. RAS GTPases are among the most well studied, but they

are also just part of a superfamily which includes other well-known actors in cancer signaling including RHO and RAB.

“I work on the forgotten subfamily, in terms of cancer research,” said Paul Randazzo, M.D., Ph.D., Senior Investigator in CCR’s Laboratory of Cellular and Molecular Biology. “The Arf subfamily is known to regulate membrane trafficking and actin. We had the idea that it may be an important regulator of cell adhesions which are critical for survival, proliferation, migration... all things that are critical in cancer.”

It has proved challenging to purify chemically useful amounts of native RAS and RHO because they have extensive lipid modifications. Arf, by comparison, has a simple lipid modification, which allowed Randazzo and his colleagues to prepare sufficient amounts of the native protein to study its catalytic and regulatory mechanisms.

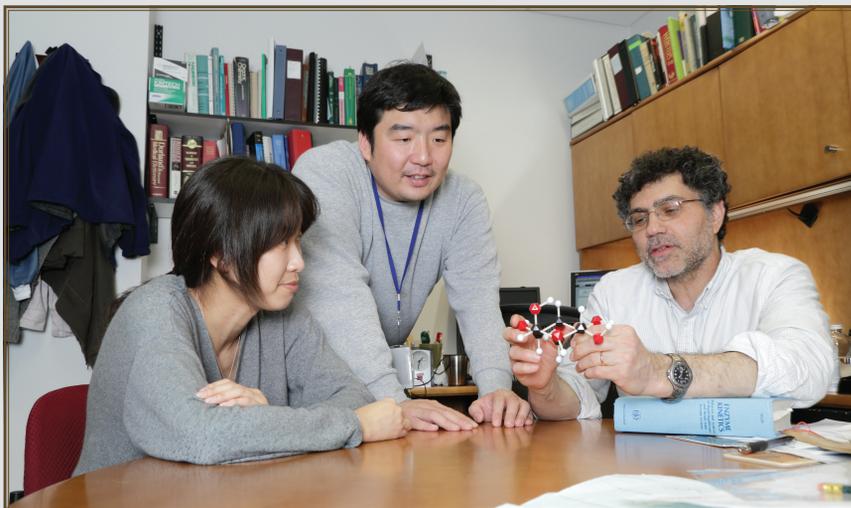
Many proteins that regulate RAS superfamily members contain

Pleckstrin Homology (PH) domains. The standard dogma is that PH domains recruit the regulatory proteins to membranes on which the RAS protein resides. Randazzo’s data indicate that in fact, lipid binding exerts a conformational change that opens up the catalytic pocket for more efficient interaction with the RAS superfamily protein. Membrane attachment itself is not required. “If you want to disrupt the function of one of these proteins, the PH domain may be appropriate as a therapeutic target.” As their research continues, Randazzo and his colleagues find that other laboratories are also beginning to question whether the membrane recruitment paradigm applies to all PH domains. “People pigeonholed protein domains based on the first discovered function—maybe the functions are a bit broader and more variable.”

Randazzo has also purified ASAP1, an Arf GTPase activating protein (GAP), regulated by phosphatidylinositols, Src, and focal adhesion kinase (FAK). They have found that ASAP1 regulates invadopodia, which, as their name implies, are invasive protrusions of the cellular membrane. The gene for ASAP1 is amplified in 50 percent of uveal melanomas, a very aggressive cancer that metastasizes to the liver. It is also amplified in 40 percent of ovarian carcinomas, as well as in 20 percent of breast and 20 percent of hepatocellular cancers. A group in Japan has recently shown with a mouse orthotopic xenograft model that elevated ASAP1 expression accelerates invasion and metastasis of breast cancer. “At this point it is part of the machinery that is necessary for malignancy, but I don’t think it’s a driver like RAS,” said Randazzo. “My goal is to acquire solid data that can be used to understand these important processes that contribute to human disease.”

“People pigeonholed protein domains based on the first discovered function—maybe the functions are a bit broader and more variable.”

(Photo: B. Brantson)



Peng Zhai, Ph.D., Pei-Wen Chen, Ph.D., and Paul Randazzo, M.D., Ph.D.

## Paving Paths to Translation

Terry Van Dyke, Ph.D., Senior Investigator in CCR's Mouse Cancer Genetics Program came to CCR six years ago to create a program that would give researchers with therapeutic hypotheses the means to put them through rigorous preclinical testing. The resulting Center for Advanced Preclinical Research (CAPR) works in partnership with researchers around the world to conduct and analyze experiments in a variety of cancer models, with an emphasis on genetically engineered mouse models.

"We set up the center as a hybrid between a rigorous research institute and an industry infrastructure... it's an efficient way to have a completely integrated set of expertise," said Van Dyke.

Several of CAPR's collaborations are centered on RAS. For example, CAPR is working with Glenn Merlino, Ph.D., Chief of the Laboratory of Cancer Biology and Genetics, and a melanoma consortium from around the country, which will involve testing an immunotherapy approach in an NRAS-driven model, among other projects. Whereas almost all other work is in the primary tumor domain, Merlino is including a rare mouse model of metastasis.

Merlino and his colleagues devised a scheme for engineering tractable preclinical mouse models by transplanting rare metastatic tumors from genetically engineered cancer models into recipient immunocompetent mice. Together, the Merlino lab and CAPR have successfully turned that concept into a preclinical model of metastasis and have begun to evaluate treatment strategies. "Early results indicate the utility in such models," said Van Dyke. "For example, in one case the primary and metastatic tumors have had distinct responses to the same therapeutic. This panel will be a valuable resource for drug and

(Photo: R. Baer)



Terry Van Dyke, Ph.D.

"CAPR has honed the ability to test combination therapies rapidly and efficiently."

biomarker development for what is now a deadly disease." The same scheme is currently being utilized to generate metastatic NRAS- and BRAF-driven melanoma models.

A newly launched partnership between the Lustgarten Foundation and CAPR is focused on preclinical development of therapeutics for pancreatic cancers, 95 percent of which are driven by RAS. The mouse model at the heart of this collaboration is one that has been engineered to develop pancreatic cancer that is extremely similar to the human disease, both at the genetic level and at the biological level. Notably, the notorious difficulty of penetrating human pancreatic tumors with administered drugs is recapitulated in the mouse model. The so-called KPC model is derived from multiple genetic events: mutations in *KRAS* and *p53* are conditionally driven and tissue specific. "The mice are completely normal until you feed them tamoxifen," said Van Dyke, "And then the oncogenes are specifically activated in pancreatic cells." Disease modeling is, of course, not limited to animals. CAPR is working with a European partner on developing organoid cultures—living tissue slices—including a

model of RAS-driven lung cancer to test potential therapeutics at a more preliminary, higher-throughput stage. CAPR also works with the National Center for Advancing Translational Sciences (NCATS) on screening drug combinations. "Combination therapies are key to treating smart tumors," said Van Dyke. "CAPR has honed the ability to test combination therapies rapidly and efficiently."

Given the complexities of RAS signaling networks and the "cleverness" of tumors in evading the impact of individual drugs, equally clever approaches to combinatorial therapeutics will likely play a crucial role in defeating RAS-driven tumors.

"Good translation always has to come from a very solid and detailed understanding of molecular mechanisms," said Luo. "Nothing is quick and painless, especially in the RAS field. Viral Ras was discovered around the time I was born, and human RAS genes were cloned when I was a kid. But we do have new technologies now and every time that happens, an 'impossible' problem becomes accessible to new therapeutic strategies."