



New paradigms in Ras research

Ras is a family of genes encoding small GTPases involved in cellular signal transduction. If their signals are dysregulated, Ras proteins can cause cancer. [Dr Sharon Campbell](#) explains her lab's research into a novel mechanism for regulation of Ras proteins by reactive free radical species

Can you explain a little about the background of your research into Ras, its aim and where the concept came from?

We had been working on Ras for some time. In the mid-90s there was a group that published an observation that Ras could be activated by nitric oxide (NO[•]), which is a small, highly reactive, radical that turned out to be the molecule of the year a couple of years back because it regulates numerous cellular processes. So, we followed up because they had observed this phenomenon *in vitro* as well as in cultured cells. We decided to pursue those observations by testing whether we could reproduce it and if so, whether we could understand the mechanism by which it occurred. So we did that and we were able to repeat the experiment, although the mechanism that was proposed was different from what we found it to be. It turned out to be a pretty complex electron transfer mechanism involving radical chemistry rather than a simpler NO-modification. We then proposed that other redox agents could work by a similar mechanism, and indeed found that Ras could be modified and regulated by a host of reactive oxygen and nitrogen species.

What in your opinion is the wider impact of this research?

Once activated, Ras can regulate numerous and complex pathways that control cellular growth. However, the activation state of Ras was previously thought to be regulated solely by protein modulatory factors. What we found is that the regulation is probably more complicated than previously envisioned and that reactive oxygen or nitrogen species can also regulate Ras proteins. From a therapeutic/health standpoint, there have been numerous efforts to target Ras as they are the most prevalent proteins mutated in human cancer. So far, therapeutic intervention efforts to target Ras have not been successful. Part of the problem with the protein is that the

activated form and inactivated form of Ras don't look all that much different from a structural standpoint. Although inhibitors that prevent Ras proteins from associating with the membrane initially appeared promising, it was later found that these inhibitors were not specific for Ras. More recent efforts have focused on how other proteins modulate Ras and targeting those has become an area of interest. The impact here is that now we've found a whole host of regulatory factors that are distinct from protein modulatory factors that we can also consider as alternative strategies to target Ras. As the altered redox environment in cancer cells may make Ras activity particularly sensitive to redox agents, approaches that target redox regulation of Ras activity may provide new directions for drug discovery efforts. In fact, findings highlighted in a recent Nature article⁷ indicate that a pathway which links Ras to enzymes that generate nitric oxide, plays an important role in Ras-mediated cancer initiation and progression.

So initially we started with Ras and also looked at related proteins, and found that other members within this GTPase superfamily can be regulated by redox agents. We have become particularly interested in Rho GTPases, as they are dysregulated in disease states such as cancer, vascular disease and neurological disorders. Elucidating how reactive oxygen and nitrogen species regulate these Ras-related proteins may aid in identifying novel therapeutic approaches that target these GTPases. While our initial funding supported our studies on Ras proteins, our most recent grant is focused on redox regulation of Rho GTPases.

Have there been any major challenges so far?

We've contributed to the discovery of a new paradigm in the field and initiated research efforts to investigate this further. It's hard to break through dogma and this is a new area of regulation so that has been a challenge.



MIKE DAVIS

I think the complications are that these redox species are highly reactive and the chemistry is complex. We initiated our early studies by trying to carefully manipulate the chemistry so that we could really understand what was going on with single proteins, because these reactions are fast and can generate multiple different species. Our longer term goal is to detect reactive GTPase intermediates and elucidate how redox agents regulate GTPase-mediated processes in living cells. In order to react in cells, reactive species need to be generated and localized spatially with the target GTPases. Moreover, the redox chemistry is highly complex. As they can react with a multitude of biomolecules, there may be multiple ways and types of reactive species that regulate GTPase-mediated cellular processes. Furthermore, these radical intermediates are very difficult to detect, so I think where the field needs to go is better detection methods of these highly reactive, very fast intermediates. Elucidating how they regulate GTPase activity and downstream pathways, will help us better understand how they modulate both physiological and pathophysiological processes. So those are the challenges and that's really where we've put a lot of our energy, developing better tools for detection and better understanding of the complexities of the chemistry so that we can better interpret how these agents regulate GTPase-mediated cellular processes.

Alternative mechanisms for regulating Ras Activity

Ras proteins have been a focus of intense investigation for close to 30 years, as they are frequently mutated in human cancer. [Dr Sharon Campbell](#) explains how the protein can be deregulated and the impact this has on pathways responsible for cell growth

THEY ARE CLASSIFIED as GTPases because they possess high affinity and specificity for guanine diphosphate (GDP) and guanine triphosphate (GTP) and weak intrinsic GTP hydrolysis activity. Ras is considered to be biologically active when bound to GTP and inactive when GDP-bound. The guanine nucleotide bound state and consequently the activity of Ras proteins is modulated in the cell by other proteins. Guanine Nucleotide Exchange Factors (GEFs) bind to Ras and promote exchange of GDP for GTP, leading to an active state of the protein, whereas GTPase Activating Proteins (GAPs) have the opposite function and promote the inactive state by facilitating hydrolysis of GTP to GDP (Figure 1). Cycling between active GTP- and inactive GDP-bound states allows Ras to act as a “molecular switch” that regulates numerous signaling pathways involved in cell growth, differentiation, and apoptosis (programmed cell death)². Abnormal cell growth and loss of apoptosis are considered hallmarks of cancer development. Many of the mutations linking Ras to cancer alter guanine nucleotide cycling by stabilizing the active GTP bound form of Ras. This leads to chronic activation, or deregulation of the protein, which in turn deleteriously impacts pathways responsible for cell growth.

While the central dogma of Ras regulation involves protein modulatory factors (e.g. activity regulation by GEFs and GAPs), an alternative mechanism for regulation of Ras activity entails interactions between Ras and free radicals. Free radicals are generally short-lived, highly reactive molecules that are generated from both endogenous enzymes (i.e. Nitric Oxide Synthase, NADPH Oxidase) and external sources (UV radiation). Antioxidants, biomolecular scavengers, or other free radicals can typically eliminate radical species in the body. However, sometimes free radicals react with DNA or proteins and alter their function. The effect of naturally occurring free radicals on Ras was first described by Lander *et al.* when they demonstrated both *in vitro* and in

cultured cells, that activation of Ras can occur in the presence of nitric oxide (NO^\bullet) radicals³. Importantly, Lim *et al.* later performed studies directly tying an enzyme that generates nitric oxide, endothelial Nitric Oxide Synthase (eNOS), with Ras activation and tumor initiation, growth, and maintenance⁷. As tumors often possess an altered redox environment⁴, regulation of Ras proteins by redox agents may represent an important mechanism for regulation of Ras activity in cancer cells as well as other cellular states (vascular disease, etc.) where the redox environment is altered.

While a direct connection between free radical chemistry and Ras activity regulation existed at the time, the mechanism by which activation occurred remained a mystery. Early studies by Lander *et al.* indicated that redox regulation of Ras activity requires reaction of a cysteine residue in Ras with either NO^\bullet gas or NO^\bullet -generating agents⁵. The Campbell laboratory worked with the Lander laboratory to identify the redox active cysteine in Ras as Cys-118⁶. This cysteine is located in one of the conserved guanine nucleotide binding motifs. As cysteine thiol-nitrosation has been shown to alter

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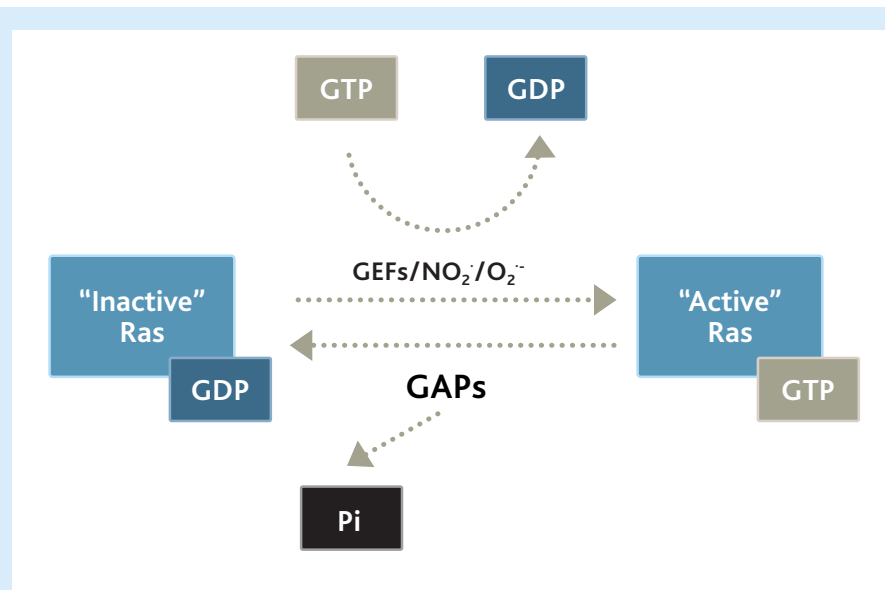


FIGURE 1. GUANINE NUCLEOTIDE CYCLING IN RAS IS MEDIATED BY PROTEIN MODULATORY FACTORS (GEFS AND GAPs) AND REACTIVE OXYGEN AND NITROGEN SPECIES (NO_2^- , O_2^-).

structure and activity of proteins, much like protein phosphorylation does, it was postulated that covalent modification by nitric oxide may alter Ras structure and activity. However, in 2003, Williams and coworkers in the Campbell laboratory found that S-nitrosation of Cys-118 does not alter either the structure or activity of Ras⁷. The Campbell laboratory then went on to further investigate the mechanism by which nitric oxide regulates Ras activity, and found that it was the actual process or mechanism of NO[•] addition that led to regulation of Ras activity⁸, not the endpoint of the reaction (e.g., S-nitrosation). While NO[•] itself is not considered highly reactive,

it combines with O₂ in solution to produce nitrogen dioxide (NO₂[•]), a very powerful oxidizing agent. NO₂[•] radicals are believed to perform a one electron oxidation of Cys-118 in Ras which generates a radical on the cysteine residue. Transfer of the radical at Cys-118 to the bound guanine base is believed to initiate the release of oxidized GDP⁹, and much like the action of GEFs, promote GDP exchange for GTP, which can lead to Ras activation in cells.

The Campbell laboratory has also demonstrated that the redox chemistry governing Ras activation is not limited solely to the Ras GTPases. In fact,

Ras proteins comprise a superfamily of GTPases that regulate a host of cellular processes including cellular growth control, motility and transport. In particular, like Ras proteins, Rho family GTPases such as RhoA, Cdc42, and Rac1 regulate cellular growth. However, in contrast to Ras, they also regulate distinct processes such as cellular motility and oxidant production². Intriguingly, Rac GTPases can co-localize, directly interact with enzymes that produce reactive nitrogen/oxygen species and regulate the production and interconversion of these oxidants^{9,10}. In fact, the Campbell laboratory demonstrated that the activity of the Rac1 GTPase, as well as RhoA and Cdc42, can be regulated by NO[•] as well as other reactive nitrogen or oxygen species¹¹, but via a distinct redox active cysteine residue from

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that of Ras. The significance of this discovery is three fold. First, the difference in redox architecture of the Ras and Rho families may imply different mechanisms of oxidation. While Ras proteins can react with redox agents to indirectly oxidize the bound guanine via electron transfer from Cys-118, the guanine nucleotide binding properties of certain Rho GTPases can be modulated by direct cysteine oxidation, thereby providing interesting differentiation in the redox mechanism (Figure 2). Secondly, it illustrates the presence of redox regulation in Rho family members that directly localize and interact with enzymes such as Superoxide Dismutase and NADPH Oxidase. Both of these enzymes are critical in generation and removal of superoxide free radicals (O₂^{•-}), an oxidant also implicated in the enhancement of GDP exchange¹². Finally, it implies that several of the Ras superfamily GTPases may be directly regulated by reactive nitrogen and oxygen species, thereby altering their activity and affecting numerous pathways that regulate both physiological and disease states.

The challenge for the future lies in improving techniques to detect these radical events in cells. Collaborations with Ron Mason and Keith Burridge, world experts in protein radical detection and cell adhesion, respectively,

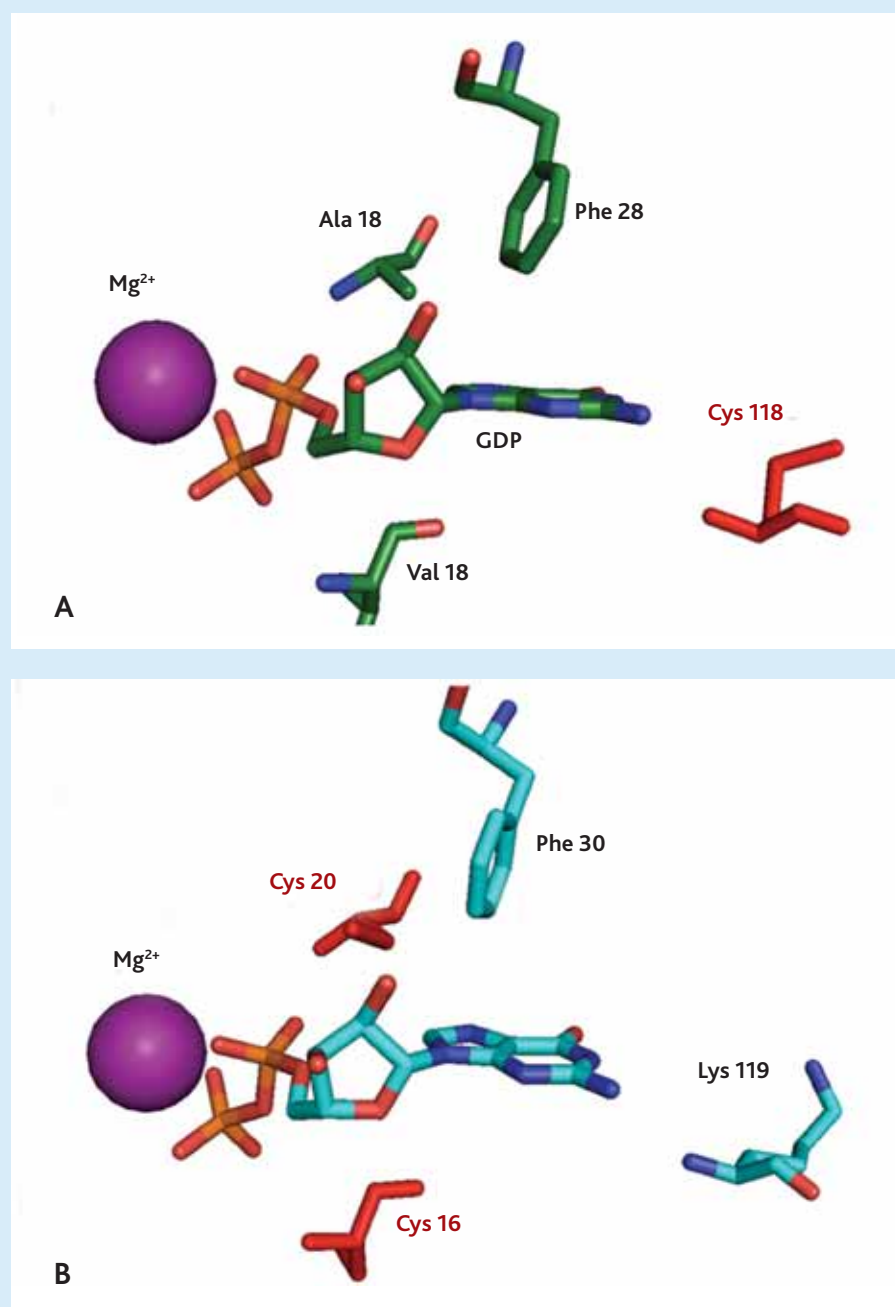


FIGURE 2. DIFFERENCES IN THE REDOX ACTIVE ARCHITECTURE BETWEEN A) RAS GTPASES (H-RAS) AND B) THE RHOA GTPASE.

PROJECT OBJECTIVES

Elucidate mechanisms by which Ras and Rho GTPases are regulated by reactive oxygen and nitrogen species (ROS, RNS). Develop both *in vitro* and *in cell* methods to detect the effect of ROS and RNS on redox active Ras and Rho GTPase structure, activity and signaling properties.

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are being utilized to advance *in vivo* detection of GTPase radical intermediates and determine how their generation alters GTPase-mediated cellular processes. One approach involves using spin traps which bind to, and facilitate detection of protein radicals. Moreover, recent development of specific antibodies against the spin trap 5,5-dimethylpyrroline-N-oxide (DMPO) as pioneered by the Mason lab, has allowed for a fast, efficient test for radical formation in proteins over traditional ESR spectroscopy¹³. A great advantage of the immunospin trapping approach is the ability to translate these detection methods to *in vivo* studies. Generation and subsequent detection of DMPO adducts in cultured cells would allow for assessment into the significance of protein radical formation on the activation state of the GTPase. Additional studies, relating GTPase activity with effects on downstream signaling events will aid in elucidating how redox agents modulate GTPase-mediated cellular processes. Assessing the impact of Ras superfamily activation via redox based mechanisms in living cells will help advance our knowledge of how this novel mechanism relates to development of cancer and other disease states, such as vascular and neurological disorders.

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