RAS AND TRANSFORMATION OF THE COLONIC EPITHELIUM: FUNCTIONAL DIFFERENCES, SIMILARITIES, AND COOPERATION BETWEEN RAS FAMILY MEMBERS

By

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For my dad

Dr. D. Wayne Keller

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"So much for my abominable volume, which has cost me so much labour that I almost hate it."

--- *Charles Darwin, Letter to W.D. Fox September 23, 1859*

Such were Darwin's sentiments following completion of a preliminary version of what would become *On The Origin of Species*. Darwin had a knack, it seems, for identifying fundamental scientific truths as well as fundamental truths about doing science. Anyone who has completed a dissertation, grant or manuscript knows the difficulty of arranging and collating the facts and theories, evidence and ideas that float around, coalescing and breaking apart in your own head for years and setting them to paper. Ideas that seem rock solid can turn to gossamer when the time comes to support them in print; blindingly obvious truths materialize and force you to reconsider that which previously seemed gospel. The construction of this document represents the culmination of my graduate career, a long, difficult, frustrating, enriching, and extraordinarily educational experience that would not have been possible without the generous support of numerous people to whom I am greatly indebted.

So as I flip through the pages of my own 'abominable volume', I think as much about those who have supported this endeavor as the endeavor itself. I would like to thank all those with whom I have had the pleasure to work over the last several years. The members of the Coffey lab, the Goldenring lab, and the Means lab have provided enormously helpful input and assistance without which this work would not have been

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"And if he keeps doggedly on in his course the odds are heavy that in the end the longest lane will prove to have a turning."

--- Theodore Roosevelt

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EXPLANATION OF NOMENCLATURE

I have standardized the use of nomenclature of the Ras family of genes and proteins as follows: all references to genes are italicized (e.g. *RAS*); all references to proteins are standard font (e.g. RAS). Murine Ras genes and proteins are referred to in the lowercase consistent with the conventions described above (e.g. ras, *ras*; H-ras, *Hras*). Human Ras genes and proteins are referred to in the uppercase consistent with the conventions described above (e.g. RAS, *RAS*; H-RAS, *HRAS*). Additionally, because much of the Ras field of literature relies on the overexpression of Ras cDNA of unspecified origin, and because many of the observations about these proteins apply to human *and* mouse, I have used 'Ras' as a general term (e.g. H-Ras, K-Ras4B) when discussing general observations or findings.

CHAPTER I

GENERAL INTRODUCTION

The American Cancer Society reports that in 2005, 1.37 million new cases of cancers were diagnosed in the United States. In the same year, 570,000 Americans died of these diseases. The vast majority of these cases occur in the sixth and seventh decades of life and are caused by the accumulation of a spectrum of genetic lesions that result from myriad events, including exposure to naturally occurring and synthetic carcinogens and DNA replication errors arising during normal cell division. These events, which affect classes of genes termed either proto-oncogenes or tumor suppressors, cause loss of important regulatory or gain of signaling functions within a cell. These changes may affect any of a range of fundamental aspects of cell physiology, including loss of the ability to sense DNA damage, or loss of the ability to induce cell cycle arrest following such damage; desensitization of a cell to its extracellular environment; aberrant proliferation signals; and aberrant survival signals. The cumulative result of these changes is the dysregulated growth and/or survival of cells that compromises the normal architecture and function of a tissue, and which ultimately may breach the confines of the original site and spread to and compromise tissue functions throughout the body.

The studies I have undertaken were motivated by a desire to explore the relationship and differences among members of the Ras family of proteins. Ras proteins are small G proteins, and as such, exist in one of two conformations, one of which is biologically active, and the other inactive. The binary aspect of these proteins enables

them to function as a switch in a broad range of signaling functions, often in the transduction of extracellular signals to the interior of cells. Expression of the canonical members of this gene family is nearly ubiquitous and broadly conserved across species. The extraordinary range of upstream molecules to which they are responsive and the downstream molecules with which they interact place Ras proteins at the center of an incredible range of signaling programs within an array of cellular processes. Indicative of the centrality of the role of Ras to a broad range of basic cellular functions is the fact that they are the most commonly mutated oncogenes in human cancer. These mutations result in locking the proteins in their 'on' position, in which they constitutively signal to downstream pathways without regard to feedback mechanisms or extracellular stimuli. Many of these effector pathways are essential actors in the transformation of cells from normal to cancerous.

Ras family members were among the first oncogenes identified almost a quarter century ago, and as a result, we have amassed an extensive body of work elucidating the various aspects of their functions and how their dysregulation contributes to disease. That said, there are critical, fundamental questions that remain, and important discoveries about basic aspects of their biology continue to emerge. I have set out to explore aspects of Ras biology which have been largely ignored, but which may reveal important insights into our understanding of the role of Ras in cancer. The work described herein describes that undertaking and attempts to place our findings in the larger context of the field of Ras biology. Alas, our findings raise many questions, but hopefully help to illuminate the path forward in our understanding of the novel role these proteins play in human disease.

Biochemical Activity and Cell Biology of Ras Proteins

The canonical members of the Ras family of GTPases, the 21kDa protein products of the human *HRAS*, *NRAS*, and *KRAS2* genes, referred to here as H-RAS, N-RAS, K-RAS4A, and K-RAS4B (splice variants of the *KRAS* gene) are highly conserved proteins across species and have established roles in numerous basic cellular functions, including proliferation, differentiation, and apoptosis. H-RAS and K-RAS4B correspond to oncogenes first found in rat sarcoma virus, and N-RAS was initially identified in a human neuroblastoma (34, 115, 154, 155) (Figure 1). (An explanation of the nomenclature that I've used throughout this document to discuss Ras genes and proteins is provided on page xii of the front matter.)

Ras proteins are part of a large and diverse family of small GTPases, defined by the common ability to bind guanine triphosphate (GTP), thereby assuming an active state, and hydrolyze GTP to guanine diphosphate (GDP), returning to an inactive state (14, 45, 148). *In vivo*, this process is largely dependent on regulatory molecules, which include GTPase activating proteins (GAPs), first identified by Trahey et al. (163), and guanine nucleotide exchange factors (GEFs), first identified by Wolfman et al. (179). These factors catalyze hydrolysis of the bound guanine triphosphate nucleotide and mediate release of the guanine diphosphate following hydrolysis, enabling loading of another GTP molecule.

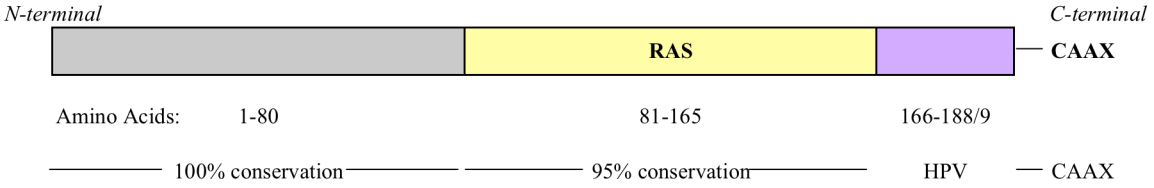
Though cycling between an active, GTP-bound state and an inactive, GDP-bound state following hydrolysis is a simple model, guanine nucleotide loading and unloading is is a highly regulated event within the cell. GAPs and GEFs are themselves subject to a separate set of regulatory events, which are not well understood. Despite the relative

abundance of GTP relative to GDP in the cytoplasm (an approximate 10-fold excess), the majority of Ras proteins exist in the GDP-bound state under most cellular conditions, due to the dramatic increase in hydrolytic activity enabled by GAPs. Specific stimulatory events increase the population of Ras molecules in the GTP-bound state by activating the exchange factors responsible for GTP-loading and/or inhibiting the GAPs responsible for facilitating hydrolysis. Stimulation of T-cells by anti-CD3 antibodies or by protein kinase C activators, for example, can rapidly increase the proportion of Ras molecules bound to GTP from 5% to 80% (36). The numerous factors that directly and indirectly influence the guanine nucleotide bound state of Ras proteins confers exquisite sophistication to their regulation and thus make them infinitely useful in nuanced signal transduction.

Ras proteins bind guanine nucleotides with high affinity in an interaction that involves at least four motifs: residues 10-18, involved in the binding to the α- and β-phosphates, residues 57-63, involved in binding to Mg^{2+} ions and to the γ-phosphate in the GTP-bound form, and regions involving residues 116-119 and 144-147, both of them important for the binding of the guanine ring. In their active, GTP-bound state, Ras proteins assume a conformation different from that of their inactive, GDP-bound state. This conformational shift enables interactions with effector molecules necessary for propagation of signals to downstream targets, and upon hydrolysis of GTP, reverts to its inactive conformation (178). These structural changes are restricted to two highly mobile regions of the protein, designated the switch I (residues 30–40) and switch II (residues 60–76) regions (108, 149). The switch I region includes the core effector domain (residues 32–40) that is critical for all effector binding and activation, as well as binding

to GAPs. GEFs, on the other hand, interact with the switch II region. The molecule therefore alternates between a GTP-bound state in which it can interact with effectors or GAPs via its switch I region, or in a GDP bound state in which the switch I region is inaccessible, but through the switch II region, can interact with GEFs. Both switch regions are close to the γ-phosphate of GTP. Thus, the conformational changes of these switch regions in the GTP cycle are involved in the binding to regulatory proteins and in the transduction of the signal to downstream effectors (61, 98). However, sequences throughout the GTPase domain might also be involved in effector interaction. When Ras is bound to GTP, the core effector domain forms an accessible loop on the surface of the protein that exhibits high-affinity binding to effector proteins (95).

Differences in the hypervariable region of the primary sequence of the Ras protein determine differences in posttranslational modification, trafficking, localization (interaction with the plasma membrane), and ultimately differences in biological activities as determined by colocalization with molecules that both regulate Ras activity such as GAPs and GEFs, as well as with upstream interactors such as receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs), and downstream effectors such as PI(3) kinase. The Ras family members, long thought to be functionally indistinct due to their high level of sequence homology, shared requirement for anchorage in plasma membrane, and biochemical affinity for common effectors *in vitro*, in truth display significant functional differences that arise from the differences in trafficking, localization, and relative activities attributable to differential processing of the proteins (57).



Differential Modification and Processing	Prenylation	Proteolysis/Methylation	Second Signal Processing
N-RAS — **CVVM**	**Farnesylation** or Geranylgeranylation	Cleavage of -AAX; methylation of C-terminal Cys	Palmitoylation of HPV Cys residue (Cys 181)
H-RAS — **CVLS**	**Farnesylation**	Cleavage of -AAX; methylation of C-terminal Cys	Palmitoylation of HPV Cys residues (Cys 181 and Cys 184)
K-RAS4A — **CIIM**	**Farnesylation**	Cleavage of -AAX; methylation of C-terminal Cys	Palmitoylation of HPV Cys residue (Cys180)
K-RAS4B — **CVIM**	**Farnesylation** or Geranylgeranylation	Cleavage of -AAX; methylation of C-terminal Cys	HPV polybasic region serves as second signal

Figure 1. Summary of Ras family homology and posttranslational modification. Canonical members of the Ras family of proteins, H-RAS, N-RAS, and K-RAS4B exhibit extensive conservation of their primary sequence but diverge significantly in their respective C-termini. Despite this divergence in a what is known as the hypervariable domain, all members share a –CAAX box at their C-termini. The hypervariable domain of these proteins undergo divergent posttranslational modifications as shown here.

Following translation on cytosolic ribosomes, all Ras family members undergo a common set of intitial posttranslational modifications as summarized in Figure 1 (92). Newly translated Ras molecules are farnesylated or geranylgeranylated on the cysteine of their C-terminal CAAX motif, targeting the proteins to the endoplasmic reticulum (ER) where they undergo proteolytic cleavage of their –AAX motif by an endopeptidase, RceI (Ras and a-factor converting enzyme), followed by carboxymethylation of the carboxy terminus of the cysteine residue (23, 27, 59, 73). Further processing takes place as the proteins transit the ER. Specifically, a second set of processing events occurs at other cysteine residues in the protein's C-terminus. In the case of N-Ras, Cys^{181} is palmitoylated; in the case of H-Ras, Cys^{181} and Cys^{184} each undergo palmitoylation (57). For H-Ras and N-Ras, these modifications serve as a "second signal" that directs further trafficking along the exocytic pathway. A summary of these processes in the context of the cell is summarized in Figure 2. H-Ras and N-Ras mutants in which these substrate cysteines are mutated to other residues arrest at the endomembranes and are not trafficked further (142). Two enzymes, ZDHHC9 and GCP16, both Golgi-associated proteins with significant homology to palmitoylatransferases identified in *Saccharomyces cerevisiae* and that possess Ras-specific palmitoyltransferase activity *in vitro,* have recently emerged as likely candidates for the mammalian palmitoyltransferase responsible for Ras modification (158). Given the near ubiquitous expression pattern of Ras proteins, the somewhat limited expression patterns of these enzymes suggest, however, that additional enzymes may possess palmitoyltransferase activity toward Ras. K-Ras4B does not undergo palmitoylation, but rather relies on a polybasic region in its

hypervariable domain consisting of a stretch of lysines for proper trafficking and membrane anchorage.

Fully processed H-Ras and N-Ras traffic via vesicles budded from the Golgi body to the plasma membrane. Under conditions in which vesicle budding from the Golgi ceases (16°C), N-Ras and H-Ras arrest at the endomembrane (22). The polybasic domain of K-Ras4B functions to direct that molecule into a separate trafficking pathway that appears to be dependent on microtubule-mediated transport (Figure 2). In support of this, regions of the K-Ras4B HPV bind microtubules, conditions impermissive to vesicle formation do not perturb trafficking of K-Ras4B, and K-Ras4B binds to taxol-stabilized microtubules *in vitro* in a manner dependent on the proper composition and processing of the polybasic domain (20). Though specifics of a microtubule-based transport system remain unclear, treatment of cells with taxol selectively mislocalizes newly synthesized K-Ras4B (161). Interestingly, the K-Ras4B splice variant K-Ras4A contains hypervariable sequence that lacks the polylysine domain of K-Ras4B but possesses a cysteine at residue 181, which is a palmitoylation substrate, and as a consequence, traffics via vesicular transport. Biologically, K-Ras4A is more similar to H-Ras in its transformational capacity and ability to induce cell migration than to the K-Ras4B molecule (170).

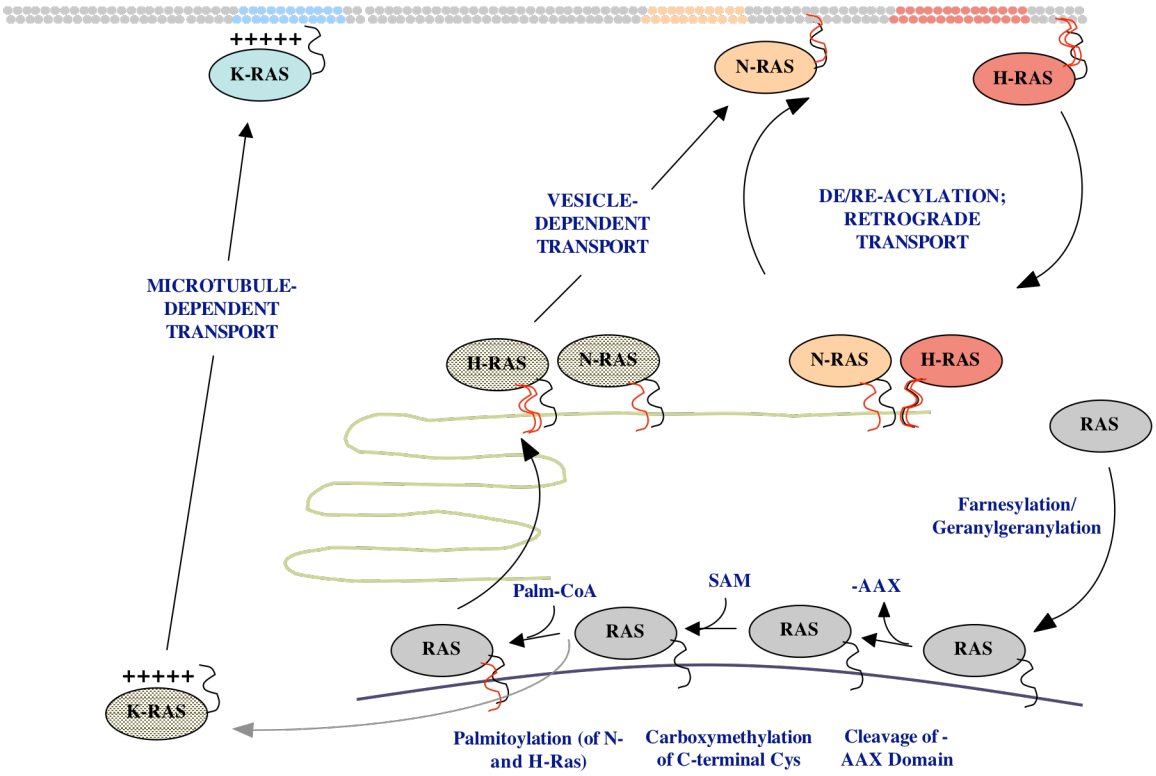


Figure 2. Summary of RAS processing, trafficking and localization within the cell. Divergence in the primary sequence of RAS family members and differential posttranslational modification result in differential processing, trafficking and localization of the RAS family members.

Differential posttranslational modification of Ras family members has a dramatic effect on how and where Ras proteins may localize within the plasma membrane. A required corollary to this emerging paradigm is a rapidly evolving understanding of the plasma membrane as a structure consisting of a dynamic complex mosaic of different lipid and protein constituents. The significance of plasma membrane heterogeneity for Ras localization, and ultimately function, is an active and novel field of study but has already proven important in determining the distinct functional identities of the various Ras family members (112, 127, 129).

The unique localizations of Ras proteins within the membrane also change depending on the GTP- or GDP-bound status of the molecule. H-Ras in its GDP-bound conformation predominantly occupies cholesterol-rich lipid rafts, as well as caveolae within lipid rafts (128). Activation of H-Ras appears to lead to off-loading of these proteins into bulk membrane regions not yet characterized by any particular lipid or protein composition. For example, the ability of H-Ras to off-load from the lipid-rafts is dependent upon the presence of the linker region of the hypervariable domain (amino acids 166-179) (70). H-Ras^{G12V} mutants lacking this linker domain are unable to migrate out of the lipid raft despite their constitutive GTP-bound state (128).

While H-Ras exits cholesterol-enriched lipid rafts upon GTP-binding, it simultaneously requires cholesterol to properly activate downstream effectors. Depletion of cholesterol from plasma membranes, either chemically (e.g. cyclodextrin) or by expression of dominant negative caveolin mutants that are incapable of proper cholesterol trafficking, increases lateral mobility of H-Ras within the membrane and disrupts

function. Though H-Ras retains the ability to recruit Raf to a cholesterol-depleted plasma membrane, H-Ras is unable to activate this substrate (141).

This observation is specific to H-Ras; K-Ras4B signals and migrates without regard to cholesterol content of the membrane. Consistent with these observations, K-Ras4B is seen to be excluded from the lipid-raft structures at which H-Ras clusters, and rather freely migrates within bulk, disordered membrane (112). N-Ras, like H-Ras, localizes to lipid rafts, but does not associate with caveolae (104). This evidence suggests two possible models of Ras association with a heterogeneous plasma membrane: (1) microdomain residence, in which Ras family members are targeted to and associate preferentially with spatially and compositionally distinct microdomains within the plasma membrane or (2) microdomain accessibility, in which Ras molecules are nonspecifically targeted to the plasma membrane but differ in the freedom with which they are able to laterally diffuse within the membrane and access the various microdomains that comprise it (141). The changing localization of H-Ras as dictated by its GTP- or GDP-bound states supports a compromise between these two models, in which the chemical compositions and conformations of Ras C-termini and their posttranslational modifications determine affinities for specific lipid and protein compositions of the membrane. In various conformations, and in association with various binding partners, these affinities may change, affording the molecule access to a region of the membrane with different chemical properties.

Evidence of membrane-localized protein-protein interactions that regulate Ras function is emerging. Interaction with galectin-1, for example, stabilizes the GTP-bound H-Ras interaction with non-raft microdomains (129). This interaction prolongs Ras

activity in response to EGF stimulation and to direct activated Ras to interact preferentially with Raf, but not PI(3) kinase (38). interacts with either H-Ras or K-Ras4A, galectin-3 participates in a similar interaction but exclusively with K-Ras4B, and attenuates Ras signaling to downstream effectors (37).

The number and residue position of the palmitoylation events confers specificity of trafficking and localization as well. For instance, wild-type H-Ras, which undergoes palmitoylation of cysteines 181 and 184, when monopalmitoylated at Cys^{181} (Cys^{184} mutated to Ser) traffics in a similar fashion to wild-type and shows strong localization at the Golgi, ER, and plasma membrane (142). Monopalmitoylation of Cys^{184} (Cys^{181} mutated to Ser), on the other hand, results in the accumulation of H-Ras at the Golgi, with minimal staining at the plasma membrane, suggesting that this prenylation event is required for either proper trafficking to or retention at the plasma membrane. Interestingly, these monopalmitoylation mutants segregate into separate microdomains at the plasma membrane: monopalmitoylation of Cys^{184} segregates in a manner similar to wild-type H-Ras, migrating laterally upon GTP-loading between cholesterol-dependent and cholesterol-independent microdomains; monopalmitoylation of Cys^{181} disrupts this lateral segregation, resembling instead the GTP-regulated microdomain interactions of N-Ras (142). Given the specificity of trafficking and localization conferred by these prenylation events, the recently described retrograde trafficking mechanism would therefore prevent Ras molecules from assuming nonspecific localization with the plasma membranes following deacylation (135).

In addition to functional Ras populations found at the plasma membrane, mature, functioning Ras molecules exist at the endomembranes of the cell, including the

endoplasmic reticulum and the Golgi body. Ras pools bind effector molecules (and presumably signal) from these endomembranes (22). How this endomembrane-resident population relates to those molecules that traffic to the plasma membrane remains unclear, though it appears that the reversibility of palmitolylation is likely a critical component in cycling Ras molecules on, off, and between membranes. Farnesylation alone has been shown to be insufficient to anchor proteins in a lipid bilayer, and a provocative model of Ras regulation that assigns central importance to the reversibility of the "second signal" palmitoylation events that N-Ras and H-Ras undergo has recently emerged (60, 135). Palmitoylated Ras proteins at the plasma membrane lose their association with the lipid bilayer following loss of palmitate residues and traffic in retrograde fashion from the plasma membrane to the Golgi, where they encounter palmitoyl transferases, recover these modifications, and reenter the exocytic pathway trafficking from the Golgi to the plasma membrane (53). Based on the different extents to which these proteins are palmitoylated (N-Ras undergoes palmitoylation of a single Cys residue whereas H-Ras undergoes palmitoylation of two), this finding raises the possibility of important differences in the functionality of these proteins. Since the rate of loss of palimoylation determines the length of residence at the plasma membrane, H-Ras proteins cycle at roughly half the rate of N-Ras proteins and possess longer dwell times at the membrane, a difference which may affect their availability to effectors and regulatory molecules following activation. Also, as these molecules lose their palmitoylation and begin retrograde transport to the Golgi, they may traffic in the GTP-bound state, or even bound to effectors, providing a possible mechanism of localizing activated Ras and active signaling complexes to endomembranes within the cell.

The polylysine domain of K-Ras4B consists of eight lysine residues in the carboxy terminus of the protein over ten residue positions confers a positive charge that enables an electrostatic interaction with the negatively charged head groups of the phospholipids which compose the plasma membrane. This interaction differs from the physical interaction seen in the case of H-Ras and N-Ras, which anchor within the plasma membrane via the palmitoyl groups of their hypervariable region, and is subject to a completely novel set of regulatory events. The other two residues in the polybasic motif of K-Ras4B, Ser^{181} and Thr^{183} serve as phosphorylation substrates for PKC (Ser181 being the principal site). The effect of phosphorylation of these residues is to largely neutralize the positive charge of the basic residues and to perturb the electrostatic interaction of K-Ras4B with the negatively charged lipid bilayer in a mechanism described as a 'farnesyl-electrostatic' switch. This results in internalization of K-Ras4B (11).

Spatial Regulation of Ras Signaling

The differential processing of Ras proteins determines important spatial organizations of Ras-mediated signaling networks within a cell. Ras signaling is spatially compartmentalized both between membranes and within membranes. One example is the recent finding that Golgi-localized H-Ras can be activated by growth factors which activate phospholipase Cγ in a Src-dependent manner, releasing Ca^{2+}, which positively regulates Ras populations localized to the Golgi complex by triggering the translocation of guanine nucleotide exchange factor RasGRP1 to the endomembrane. Simultaneously, Ca^{2+} release negatively regulates Ras on the plasma membrane by activating CAPRI, a

GTPase-activating protein (Figure 3) (10). Additionally, specific activation of Ras proteins resident at different membranes within the cell has recently been linked to the intensity of extracellular stimuli. More intense stimulation of the T-cell receptor in Jurkat cells activates both endomembranous N-Ras as well as of K-Ras4B populations at the plasma mebrane while low-grade stimulation selectively activated endomembranous N-Ras (104, 120).

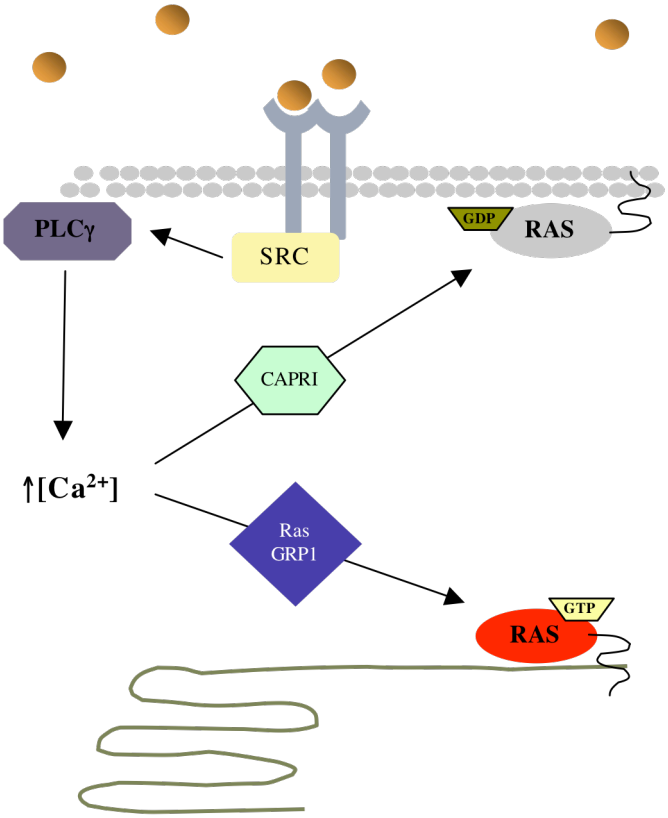


Figure 3. Spatial regulation of Ras signaling. EGF treatment of COS-1 cells activates endomembrane-localized Ras populations and inhibits plasma membrane-localized populations in a Src-dependent manner, providing a mechanism of spatial regulation of Ras activity within the cell (Adapted from Bivona et al. Nature 2003).

As mentioned earlier, the reported trafficking of deacylated Ras from the plasma membrane to the Golgi provides an alternate model of activation of Ras on endomembranes. N-Ras and H-Ras molecules that undergo depalmitoylation at the plasma memrane do not necessarily undergo hydrolysis of bound GTP molecules; indeed, activated Ras molecules at the plasma membrane may shuttle *in their active conformation* to the Golgi where they are capable of continued binding to effectors. This raises the additional possibility that these molecules may additionally bring with them effectors initially bound at the membrane such that a multiprotein signaling unit could traffic as a functional complex and may generate different signaling outputs from a novel localization within the cell. Suggestive of the idea that Ras GTP-loading may originate at these endomembranes, family members RasGRP and RasGRF, both Ras-specific guanine nucleotide exchange factors, localize to and selectively activate Ras molecules resident at the Golgi membrane (4, 17).

Additionally, the identification of Sef-1, a scaffold capable of binding MEK/ERK complexes at the Golgi and controlling nuclear import of ERK, provides yet further evidence of the emerging paradigm of subcellular compartmentalization of not just Ras molecules, but of functional Ras networks (121, 162).

Expression of a series of Ras mutants generated to exploit the determinants of trafficking and localization within the primary sequence of H-Ras in conjunction with trafficking motifs from several other well characterized proteins, demonstrates that distinct Ras localization corresponds to unique effector function (Figure 4) (105).

Distinct Biological Outcomes Resulting from Selective Targeting of Ras Proteins

	Effector Activation						
	ERK-2 Activation	Phospho Akt	Phospho ATF2	Ral GTP	**Proliferation**	**Focus Formation**	**Survival**
H-rasG12V (+)	++++	++++	++++	++++	++++	++++	++++
H-rasS17N (-)	ND	ND	ND	ND	ND	ND	ND
Palmitoylation Deficient (C184S;C186S)	++	+++	+++	++	++	++	++
Palmitoylation Deficient, ER-restricted (M1-C184S;C186S)	+++	+++	++++	+++	+++	+++	++++
Palmitoylation-Deficient, Golgi-restricted (KDELrN193D-C184S;C186S)	+	++	+++	++++	+	+	++
Palmitoylation-Deficient, Bulk Membrane-restricted (CD8-C184S;C186S)	+++	+++	++	+++	+++	++++	++
Palmitoylation-Deficient, Lipid raft-restricted (LCK-C184S;C186S)	+++	+++	++	+++	+++	++++	++

(1) Adapted from Matallanas et al., Molecular and Cellular Biology, 2006.
(2) All measurements are relative to transfection of H-rasG12V or H-rasS17N with wild-type trafficking determinants.
(3) ERK-2 activation as measured by phosphorylation of MBP in an in vitro kinase assay.
(4) Survival as measured by relative levels of cleavage of PARP following treatment with either IL-1 or adriamycin.
(5) Trafficking motifs are as follows: M1 represents amino acids 1-66 of the avian infectious bronchitis virus M protein; KDELrN193D represents a point mutant of the KDEL receptor, which is unable to recycle to the ER from the Golgi; CD8α represents the transmembrane domain of the CD8 receptor; LCK represents the palmitoylated myristoylation signal of Lck and acts as an effective lipid raft anchor. All trafficking motifs were appended to the N-terminus of the protein.

Figure 4. Summary of functional effects of domain-restricted Ras mutants. Limiting the localization of Ras proteins to various membranes within the cell that serve as signaling platforms for wild-type Ras proteins affects their functional output, supporting the notion that localization and function of Ras proteins are inextricably linked.

In addition to the emerging evidence of the complexity surrounding localization and trafficking of Ras molecules themselves, one must also consider the processing, trafficking, and localization of those molecules that interact with and regulate Ras – effectors, scaffolding proteins, guanine nucleotide exchange factors and GTPase activating proteins. For example, Ras recruitment of Raf-1 from the cytoplasm to the plasma membrane where it binds Raf-1 directly is a paradigm of Ras activity (99, 170). Raf-1 is subject to regulation beyond Ras binding, however, specifically involving four phosphorylation events to reach its full kinase activity (103). At last two of these are the result of phosphorylation of Raf-1 by Src, which itself undergoes posttranslational processing in the form of myristoylation and palmitoylation at its N-terminus. In addition to these modifications, Src possesses a polybasic domain composed of Lys and Arg residues that interacts preferentially with phosphatidylserine and phosphatidylinositol lipid species, which themselves are distributed asymmetyrically on the plasma membrane. Indeed, when taken together, the overlapping localizations and regulations of Ras proteins, effectors and regulatory molecules imply an almost infinite level of complexity of Ras biology and regulation beyond what has been conventionally appreciated.

Ras and Signal Transduction

To date, attempts to develop therapies targeted against Ras proteins have met with minimal success, and investigators have turned much of their attention to the downstream molecules that receive the signals relayed by Ras. Initial studies revealed extensive conservation of Ras signaling through a limited number of pathways, often with different

biological outcomes. Later work has demonstrated a far more extensive universe of Ras effectors and signaling pathways which is still growing and will no doubt further our understanding of the diverse range of effects Ras signaling can have within a cell (61, 138).

An effector molecule is a protein capable of strong preferential binding to the GTP-bound form of Ras and whose binding is altered by mutations in the core effector domain (133). Futher, the function of the effector protein should be modulated by interaction with Ras, for example by changes in (1) subcellular localization of the effector (recruitment); (2) intrinsic catalytic activity (allosteric regulation); or (3) interaction with other signaling components (complex formation). As well, the biological activity of Ras should depend on effector function. Several different experimental approaches have been useful in addressing this issue and include use of Ras effector domain mutants, pharmacological inhibitors of effector function, and the use of cells rendered deficient in effector expression, for example through the use of small interfering RNA (siRNA) and genetic targeting of the effector (Figure 5) (133).

Verified and putative Ras effectors are characterized by a Ras-binding domain. At least three distinct ~100 amino acid sequences have been identified as such: the Ras-binding domain (RBD) of Raf or Tiam1; the RBDs found in class I phosphoinositide 3-kinases (PI3K-RBD); and the Ras association (RA; identified initially as a sequence homology found in RalGDS and AF-6) domains found in the majority of Ras effectors (124), as well as in many proteins which have not been observed to interact with Ras. Although they do not show obvious primary sequence identity, all three domains form the same topology of an ubiquitin superfold, characterized by a βαβαβ tertiary structure

found in many proteins of different cellular functions (61). This common topology for otherwise divergent primary sequences accounts for the similar mode of interaction of Ras with effectors with different RBDs. Additionally, the topological rather than sequence conservation complicates efforts at predicting Ras binding domains bioinformatically; most effectors identified to date have been resulted from yeast-two hybrid screens or other empirical methods. It is a near certainty that the list of Ras effectors in the genome remains incomplete.

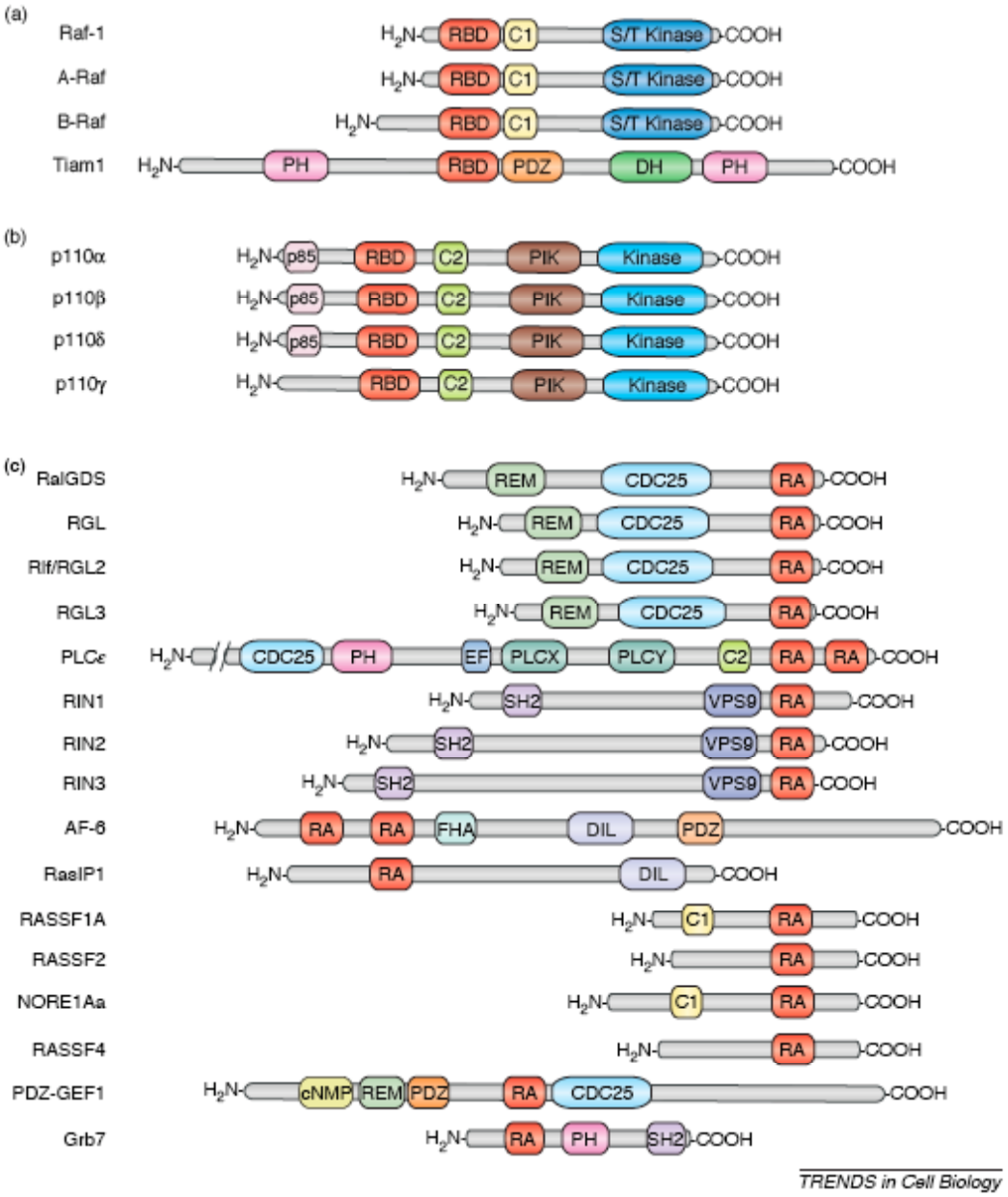


Figure 5. Topological conservation of Ras binding domains. Ras effector molecules possess Ras binding domains that can be grouped into three classes, which are typified by the Raf family of serine/threonine kinases, the PI(3)K family of seribe/threonine kinases, and the RalGDS RA domain. (Adapted from Repasky et al., 2004)

The role of mutant Ras as an oncogene as well as its regulation of normal cellular function has generated significant interest in identifying the downstream effectors through which the molecule propagates its signal. This work has utilized numerous approaches, most of which fail to faithfully recapitulate the conditions of Ras biology that take place *in vivo* and usually affect one *Ras* allele and therefore risk introduction of artifactual phenomena. Primary among these systems has been the ectopic overexpression of Ras, often in a mutant form, to study effector function. Immortalized human and murine fibroblasts have been a popular system for many of these studies for their ease of use and susceptibility to transformation. The vast majority of neoplasias arise from epithelial populations, however, and the effectors through which Ras signals almost certainly display biological differences dependent on cell context, providing a cautionary note for some of the conclusions of these efforts. Nonetheless, these approaches have proved useful in our initial forays into Ras:effector relationships and function.

The ability of constitutively active mutant Ras to drive cellular transformation has provided the basis of extensive study of numerous aspects of the signal transduction functions mediated by Ras. Ras induces numerous aspects of malignant transformation, and the assays used to study this phenomenon yield insights selectively into this process. *In vitro*, these include uncontrolled proliferation, morphological transformation, and the ability to grow in soft agar. *In vivo*, these assays include tumor formation, invasion, angiogenesis, the ability to form xenograft tumors in nude mice, and metastasis. Measuring the contribution of any individual effector to these phenomena can be difficult, given that there are very few effectors that provide a necessary and sufficient

contribution to Ras-mediated transformation. In the majority of instances, Ras signals through multiple effectors to achieve transformation, which greatly complicates effector analysis. In addition to the overexpression studies utilized historically, more sophisticated methods of investigating Ras effectors critical to Ras-mediated tumorigenesis will be discussed in greater detail in subsequent chapters.

Because the universe of Ras effectors is so expansive, the following discussion pertains only to those effector families about which the most is known and for which the evidence is strongest.

Raf Family of Serine/Threonine Kinases: The Raf serine/threonine kinases are perhaps the best-characterized and validated effectors of Ras function and provide the archetypal Ras binding domain. Although the products of the three Raf genes (*ARAF, BRAF, RAF-1*) share significant sequence similarity, including a homologous Ras-binding domain within the N-terminal regulatory region of the protein, they exhibit differential expression patterns, distinct roles in development, and significant biochemical and functional differences (100). In particular, the high basal kinase activity of B-Raf, along with minimal requirements for activation, provide the basis for the oncogenicity of BRAF mutants. A possible explanation for this difference is the fact that unlike Raf-1, which requires four phosphorylation events to assume full kinase activity, B-Raf requires only two (103). Nevertheless, these Raf molecules show redundant function in facilitating oncogenic Ras-induced activation of the MEK1/2 and ERK1/2 cascade. The importance of the Raf effector pathway in normal and neoplastic Ras function is supported by (i) the ability of constitutively activated Raf and MEK, similar to

that of Ras, to transform rodent fibroblasts (131), (ii) the occurrence of *BRAF* mutations in tumor types also characterized by Ras mutations, that occur in a mutually exclusive fashion (28); and (iii) the ability of small molecule inhibitors of Raf such as BAY-439006, to revert Ras-mediated transformation phenotypes such as growth in soft agar (94). For a more complete review of the Raf family of serine/threonine kinases, the reader is directed to (173).

Phosphatidylinositol-3-Kinase Family of Phospholipid Kinases: The phosphatidylinositol-3-kinase [PI(3)K] family of enzymes constitutes the second best-characterized family of Ras effectors, and have important roles in mediating the pro-survival and proliferative functions of Ras (168). The main activity of PI(3)K involves conversion of phosphatidylinositol (4,5)-bisphosphate [PI(4,5)P2] to phosphatidylinositol (3,4,5)-trisphosphate [PI(3,4,5)P3]. PI(3,4,5)P3 facilitates the activation of the Akt serine/threonine kinase, in addition to other proteins such as GEFs for Rho GTPases. The loss of the PTEN tumor suppressor that catalyzes the dephosphorylation of PIP3 supports the important contribution of PI(3)K hyperactivation in cancer development (168). Additionally, mutation of the *PI3KCA* gene encoding the p110a catalytic subunit of PI(3)K has been found in colorectal and other cancers (145) as have genetic mutations in other genes encoding proteins involved in the regulation of this pathway (116).

RalGEFs: RalGEFs have been repeatedly identified in yeast two-hybrid screens for Ras-binding proteins and link Ras proteins to activation of the RalA and RalB small GTPases (26). Four distinct RalGEFs have been identified as Ras effectors: RalGDS,

RGL, RGL2 (also called Rlf) and RGL3. Initial studies using NIH 3T3 mouse fibroblasts found that, unlike Raf, constitutively activated variants of RalGEFs or of their GTPase substrates failed to cause any detectable morphological or significant growth transformation *in vitro*. Co-expression of constitutively active RalA, however, enhanced Ras-mediated focus formation, whereas dominant-negative RalA impaired Ras-mediated transformation. Further, co-expression of RalGDS cooperates with activated Raf to induce synergistic focus formation, suggesting a contributory role for RalGEF in Ras-mediated transformation *in vitro* (175).

Interestingly, activated RalB was not able to mimic the effects seen with activated RalGEF. This example, in addition to others, suggests the possibility that RalGEFs might possess functions in addition to activation of Ral GTPases. Alternatively, it might reflect the distinct functional activities of the two Ral isoforms despite their 85% sequence identity. Further, the importance of RalA, but not RalB, to RAS-mediated transformation in human cancer cell lines possessing an activating Ras mutation is bolstered by a recent report showing that knockdown of RalA expression using RNAi techniques decreased tumorigenic behavior of these cell lines as measured by xenograft assays and growth in soft agar (88).

RalGDS is an essential effector of Ras-mediated transformation in an *in vivo* model of carcinogenesis (52, 175). In animals in which RalGDS was targeted by recombination (*RalGDS*$^{-/-}$), chemical induction of skin carcinogenesis (DMBA/TPA) was used to examine the importance of this effector to tumor formation. Following chemical initiation, these mice exhibited reduced tumor number as well as tumor size relative to wild-type. Further analyses of the papillomas that did arise reveal that RalGDS elevated

levels of GTP-bound RalA, which appears to regulate the phosphorylation of JNK and SAPK, well-known regulators of survival signaling. RalA activity negatively correlated with TUNEL staining in these tissues. This report complements historical mutagenesis studies that generated Ras effector domain mutants capable of binding RalGDS but not interacting with Raf or PI(3)K. These mutants, when overexpressed in cells, retained their ability to induce transformation (138).

Tiam1: Aberrant Ras signaling dysregulates the Rho family of small GTPases, including Rac1 and RhoA, though no direct association between Ras and Rho GTPases has been observed. Because aberrant Rho GTPase activation is frequently associated with promotion of tumor cell invasion and metastasis (144), effectors that facilitate Ras-mediated activation of Rho GTPases likely facilitate the role of oncogenic Ras in tumor progression. One effector that serves this role is Tiam1, a Rac GTPase-specific GEF that also contains a Raf-like RBD. The finding that mice lacking both alleles of Tiam1 are developmentally normal, but are resistant to skin tumor formation caused by carcinogen-induced mutational activation of H-ras demonstrated its importance in mediating Ras-driven tumor formation (97).

Though representative of the universe of Ras effectors we are coming to appreciate, the effector families described here are merely a sample. A more systematic and exhaustive examination of the interaction of Ras proteins with their effecto molecules has recently been described using a dual-tagged system in which both Ras protein and their extended family members along with a battery of effectors are overexpressed (138).

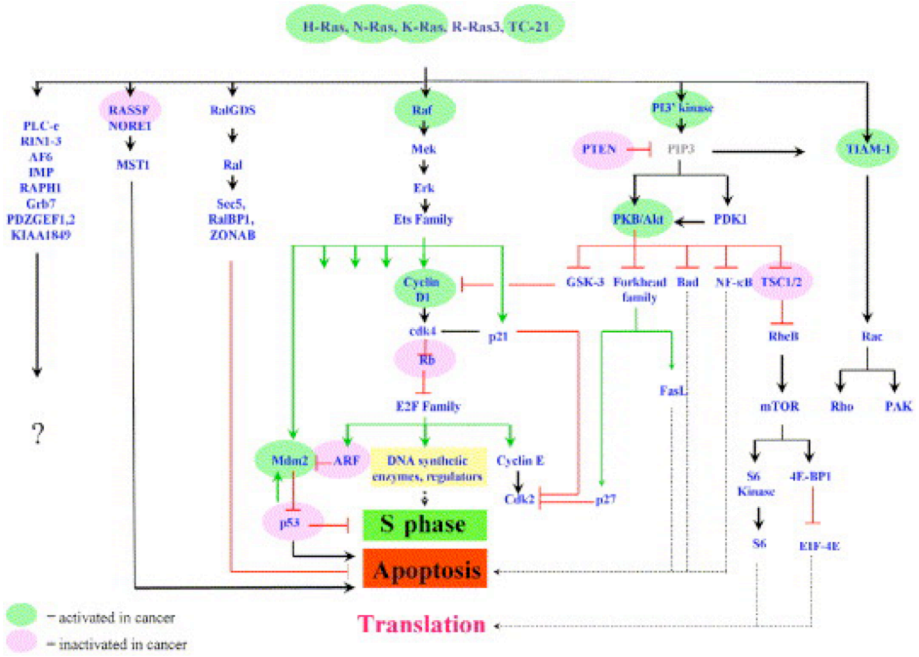


Figure 6. Partial summary of the range of effectors with which Ras interacts and the signaling pathways in which these interactions participate. (Adapted from Rodriguez-Viciana & McCormick, 2005)

The interaction of Ras molecules with effectors is not simply a matter of biochemical affinity. As discussed previously, Ras family members have unique intracellular localizations as do many of their effectors and the overlap of the these localizations likely defines much of what is possible in terms of interactions. In addition to this consideration, many of the well-studied Ras effector molecules are themselves members of larger families, which may show sequence divergence within their Ras binding motifs. Three of the best-known Ras effector families comprise multiple isoforms or distinct family members. For example, the class I family of PI(3)K has p110α, p110β, p110δ, and p110γ isoforms, and the Raf kinase family comprises Raf-1, A-Raf, and B-Raf. RalGEFs now include RalGDS, RGL, RGL2/Rlf, and RGL3. Ras molecules may differ in their abilities to regulate different isoforms or members of the same family, and these selective interactions may have important biological consequences. The sequence and structural homology between not only Ras family members, but also between Ras binding motifs, coupled with the membrane affinities of the Ras family members described above confer a remarkable range of Ras:effector selectivity and promiscuity.

Numerous reports describing biological differences associated with activities of different Ras family members toward the same effectors or ascribing functions to single members of the Ras family not shared by other members alludes to the complexity of Ras:effector interactions. For instance, it is well established that Ras family members H-Ras and K-Ras4B both interact with and differentially activate the serine/threonine kinase Raf-1 as well as the phospholipid kinase PI(3)K. In COS cells, overexpressed activated K-Ras4B is a more potent activator of Raf-1 than is H-Ras, both as measured by its

ability to increase Raf-1 activity in an *in vitro* kinase assay as well as to more efficiently recruit Raf-1 to the plasma membrane. Conversely, overexpression of activated H-Ras is associated with increased kinase activity of the PI(3)K p110 subunit (182). Similarly, when overexpressed in REF52 cells, K-Ras4B appears to more potently activate Rac1 than H-Ras as measured by Pak binding assays and cell motility (171).

Though all known effectors are thought to interact with Ras proteins through the conserved effector binding domain (aa 32-40), there are subtleties specific to each effector interaction that have been exploited to better understand Ras activation of the various effector pathways, and further, which pathways may be important to a given biological process. Specific mutations within the switch 1 region of the effector binding domain of Ras result in proteins capable of binding a limited range of effectors. The T35S mutation produces a Ras protein capable of binding only Raf-1. The Y40C mutation renders Ras•GTP capable of binding only PI(3)kinase. The E37G mutation limits Ras:effector interactions to RalGDS. Our understanding of this mutant has expanded to include interactions with Rin 1, AF-6, PLCe, and p110. The failure of each of these Ras switch 1 mutations in a G12V background to transform fibroblasts suggests that Ras must activate more than one effector pathway to generate a transformed phenotype (174, 175).

Role of Ras in Development and Disease

The extent of Ras conservation across eukaryotes suggests the critical function this family of proteins plays in developmental and homeostatic processes. Paradigms have emerged in nearly all major model systems of development involving Ras as a

critical signaling switch in fundamental developmental processes. In its most primitive form, the Ras pathway consists of two Ras genes, *RAS1* and *RAS2* in *Saccharomyces cerevisiae*. Despite the larger size than their homologues in higher eukaryotes, the primary sequence of the proteins encoded by these genes is highly conserved in the amino-terminal 180 amino acids. Deletion of both *Ras* genes is lethal; either gene is sufficient for viability alone, however (72). Mammalian Ras proteins are similarly able to sustain the growth of yeast cells in the absence of the endogenous yeast genes (33). Similarly, Ras proteins purified from yeast can transform quiescent mammalian fibroblasts (32).

In *Xenopus laevis*, Ras plays a critical role in inducing meiosis of oocytes. Injection of antibodies that block Ras function blocks a subset of stimulatory events that lead to meiotic division (9). Injection of mammalian Ras proteins, however, can stimulate meiotic division (9). In *Drosophila melanogaster*, injection of constitutively active Ras leads to reduced viability and abnormalities in the development of wings and eyes (5). Interestingly, other genes whose mutation resulted in disrupted eye formation also encode tyrosine kinase receptors. In *Caenorhabditis elegans*, the Ras gene, known as *let 60* is critical for vulva development; constitutive activation of *let 60* results in a multivulva phenotype (6). Again, *let 60* functions in vulva development downstream of *let 23*, the gene encoding a homolog of the mammalian EGF receptor (3).

In mice, independent targeted genomic disruption of the *Hras* or *Nras* loci by homologous recombination results in viable animals that develop normally (41, 67, 122, 166). Normal murine development appears to require at least one intact *Kras* transcript (4A or 4B or both) however, as targeted disruption of exons common to both the 4A and

4B transcripts results in embryonic lethality (79, 122) (K. Lane, personal communication). *Kras*$^{-/-}$ embryos appear normal until E11.5 at which point they are smaller than littermates and possess less superficially visible vasculature and smaller livers. These animals appear to suffer from anemia and deficiencies in production and/or circulation of red blood cells and die between E10.0 and E12.0. Interestingly, while *Kras*$^{+/-}$ animals are viable, *Kras*$^{+/-}$; *Nras*$^{-/-}$ animals are not, with 70% of these animals dying between E10.0 and E12.0 and the remainder dying perinatally suggesting partial functional overlap or a linear signaling relationship between K-ras and N-ras. K-ras and N-ras show broad overlapping expression spatially and temporally in the developing embryo, while H-ras is nearly ubiquitously expressed throughout development. *Kras*$^{+/-}$; *Nras*$^{-/-}$ embryos suffer severe anemia evident at E9.5, appear pale relative to littermates, and exhibit dilations of the heart and pericardial sac. These data suggest isoform-specific functions that can be partially, but not completely compensated for, by other Ras proteins (71).

Neither *Hras* nor *Nras*, alone, or in combination, are required for development (41, 67). *Nras*$^{-/-}$ animals, however, exhibit compromised function of the T-cell signaling network and are deficient in proper CD8^{+} T-cell selection. As a result, these animals exhibit decreased thymocyte proliferation and significant reduction in the production of interleukin 2 upon thymocyte activation, as well as an increased sensitivity to influenza infection (29).

With the exception of disruption of the *Kras* locus resulting in loss of both isoforms of the K-ras protein, Ras loss of function appears to be well tolerated in murine systems. Transgenic overexpression of constitutively active Ras or knock-in of an

activating mutation at an endogenous locus has a much more dramatic effect, and in numerous tumorigenesis models provides an important oncogenic contribution (165).

Though the importance of Ras proteins in developmental processes is generally appreciated, it is the oncogenic potential of constitutively activated Ras proteins that made possible their initial discovery. Ras family members were first identified as transforming genes delivered by retroviruses. Further investigation revealed cellular homologues present in bladder and lung carcinoma cell lines that corresponded to the Harvey and Kirtsten rat sarcoma viruses (31) as well as the BALB murine sarcoma virus that, like their retroviral counterparts, could transform fibroblasts upon transfection (34, 146). A greatly expanded understanding of the oncogenic contribution of Ras signaling has played a critical role in establishing a molecular basis for the pathogenesis of human cancer (63, 85, 86, 143), with activating mutations in members of the *RAS* family of genes among the most common genetic lesions in human tumors (12).

Mutations in *RAS* genes that cause a loss of the protein's ability to hydrolyze bound GTP result in oncogenic forms of the protein which are constitutively available for effector interaction. Studies that led to the initial identification of GAP proteins also revealed that Ras proteins mutant at codons 12 and 13 could not hydrolyze GTP even in the presence of the GAP-enriched fraction of cell lysates (163). Though several different mutations can render the Ras molecule incapable of hydrolysis, not all mutations do so equivalently. Impaired GTPase activity of mutants at residues 12, 13, 59, 61, and 63 have been observed, with the greatest impairment associated with mutations at 12, 13, and 61 (5, 15). Accordingly, mutations at 12, 13, and 61 are the most commonly observed in human cancer.

In earlier sections, I describe Ras effector domain mutants and the resulting restrictions they achieve in the range of effector molecules with which Ras can interact. These mutants have proved useful in parsing the effector pathways through which Ras mediates oncogenic signaling. These studies reveal the importance of three main pathways critical to Ras-driven tumorigenesis in a xenograft mouse model (Raf, RalGEF, PI(3)K). Establishing xenograft tumors requires simultaneous activation of all three of these pathways, though the requirement for these pathways changes once the tumor is established. At this stage, Ras activation of PI(3)K becomes the only critical Ras-mediated effect and appears to promote survival. Raf and RalGDS activation continue to be important, though in later stage tumors, they acquire Ras-independent means of activation. PI(3)K becomes the critical effector for survival. The importance of these three different effector pathways is further supported by work from many labs using Ras effector binding mutants, dominant-negative and constitutively active proteins downstream of Ras, and pharmacological inhibitors: Raf, PI3-kinase (PI3K), and RalGEFs (89). Activated Raf proteins initiate a MAP kinase (MAPK) signal transduction cascade that leads to transformed morphologies, anchorage-independent growth, and angiogenesis (110, 153). The PI(3)K pathway is also activated via interaction of the p85 regulatory subunit with Ras•GTP, leading to the phosphorylation of phosphoinositides, creating multiple cascades that lead to changes in cell morphology, as well as fostering angiogenesis and cell survival (93). Lastly, RalGEFs are activated via their recruitment to the plasma membrane by Ras•GTP, where they activate RalA and RalB, the former of which is required in tumorigenesis (43, 88).

The importance of the RAS signaling axis in tumor initiation and maintenance is emphasized by the prevalence not only of *RAS* mutations themselves, but also by indirect dysregulation of Ras activity as well. For example, loss of the tumor suppressor *NF1*, which encodes protein with similarities to the catalytic domain of GAP family members, results in elevated levels of RAS•GTP and is associated with syndromes characterized by benign and malignant neoplasias (24, 152, 157).

Similarly, mutations of proteins involved in downstream RAS effector pathways suggest the functional multiplicity of the Ras signaling axis in neoplasia. Oncogenic mutations in *RAS* genes and genes encoding effector proteins or downstream regulatory proteins increasingly appear to occur in non-overlapping fashion with RAS mutations suggestive of an epistatic relationship between these proteins and RAS family members. The importance of the RAS axis in specific tumor contexts is underscored by recent discoveries os activating mutations in downstream effectors. While the frequency of oncogenic *KRAS* mutations in colorectal cancer is approximately 50%, oncogenic mutations of *BRAF* mutation in colorectal adenocarcinomas are also observe in approsimately 12% of cases (28); activating mutations of the PI(3)K pathway mutations are also observed in approximately 40% of colorectal cancers (116). Similarly, *NRAS* and *BRAF* mutations are common events in the development of melanoma (25% and 66%, respectively) and occur in non-overlapping fashion. Another common event in the aetiology of melanoma is the somatic inactivation of *PTEN*, a negative regulator of the PI(3)K pathway (168). Notably, while *PTEN* mutations commonly overlap with *BRAF* mutations, both are mutually exclusive of *NRAS* mutations. This segregation is consistent with the notion of RAS proteins' ability to simultaneously stimulate multiple effector

pathways important to full transformation and supports the important but separate functions of the BRAF pathway as well as the PI(3)K pathway in melanoma aetiology (164).

To understand mutations of the Ras family and similarly oncogenic mutations of downstream effectors as epistatic is not, however, completely accurate. The characteristic of Ras that makes it such a potent oncogene is its ability to interact with multiple effectors and provide numerous independent oncogenic contributions to a cell, any of which may confer a selective advantage under the proper circumstances. This understanding explains findings which alternately disrupt by homologous recombination either an endogenous mutant *KRAS* allele or a wild-type *BRAF* allele in an cell line derived from an endometrial carcinoma (74). Disruption of *BRAF* does not revert the transformed phenotype as does disruption of the mutant *KRAS* allele. This supports the sort of 'partial epistasis' one might expect given the functional multiplicity of oncogenic Ras proteins.

For reasons that are not entirely understood, oncogenic mutations of the various Ras family members are exhibited disproportionately by different types of neoplasias. For example, activating mutations of *KRAS* are found in approximately 30% of non-small cell lung carcinoma, approximately 50% of adenocarcinomas of the colon, and 90% of adenocarcinomas of the pancreas; activating mutations of *HRAS* are found in approximately 10% of bladder carcinomas and 10% of renal cancers; and activating mutations of *NRAS* are found in 30% of melanomas, 30% of liver cancers, and 30% of acute myelogenous leukemias (35). This segregation is suggestive of either differential rates of mutagenesis according to tissue type, or isoform-specific function that accord

differential oncogenic qualities to different isoforms depending on tissue type. The former interpretation is unlikely given the extensive conservation of Ras family members, even at the nucleotide level. There are minor differences, though at the mutational hotspots of codons 12, 13, and 61, sequences are largely conserved between family members. Isoform-specific function, and therefore isoform-specific oncogenic contribution, seems a more likely possibility, though little exists to date in terms of evidence of isoform-specific molecular signatures that correlate with oncogenic contribution. This topic will be revisited in later chapters.

Because of the multiplicity of RAS function and prevalence of *RAS* mutation in human cancers, RAS has been considered a potential therapeutic target for clinical intervention. One of the earliest strategies adopted for targeting RAS function in tumors was the use of farnesyltransferase inhibitors (FTIs) which would inhibit a critical step of RAS posttranslational processing, the farnesylation of the Cys residue in the C-terminal –CAAX motif (Figure 1). Mutation of this residue to an alternate, non-farnesylable residue (Ser) arrests RAS trafficking at the endoplasmic reticulum and disrupts the transforming activity of oncogenic H-Ras (Figure 2) (19, 59, 177).

Mice expressing oncogenic H-Ras under the MMTV promoter develop mammary carcinomas. Treatment of these animals with a –CAAX peptidomimetic farnesyltrasferase inhibitor caused impressive reversion of these tumors (80). Unfortunately, these results have not been repeated clinically. The mechanism by which farnesyltransferase inhibition works is somewhat unclear, and includes the possibility that an important target is not Ras processing, but rather other farnesylated proteins within the cell, including RhoB (150). Additionally, the initial successes of FTI therapy involved

H-Ras-driven tumors. H-Ras is unique among RAS family members in that it is the only member for which geranylgeranylation cannot supplant farnesylation as a sufficient modification of the C-terminal Cys residue (69). In other words, K-Ras and N-Ras driven tumors likely simply fall back on alternate processing events to generate a functional Ras population (140). The relative frequencies of *HRAS* mutations in human tumors relative to those of *KRAS* and *NRAS* further limit the applicability of targeting farnesyltransferase alone. Inhibitors against geranylgeranyltransferases have been developed to circumvent the alternate processing events seen in the use of FTIs alone. Combinatorial therapy using these two agents has unfortunately proved highly toxic in preclinical models (91).

The history of farnesyltransferase inhibition holds interesting lessons for the design and development of therapeutics. A decade following the initial excitement over the possibilities of the FTI class of drugs, a genetically engineered mouse null at the *FT* locus which encodes farnesyltransferase (FT) was generated (107). These animals suffer from impaired embryonic development; matings between $FT^{+/-}$ animals yield no homozygous nulls at E11.5. Despite this, loss of *FT* postnatally using a floxed allele does not interfere with the formation or number of K-ras-driven adenomas or adenocarcinomas of the lung in a previously reported model (54). Perhaps surprisingly, loss of FTase in a classical initiator/promoter model (DMBA/TPA) did not prevent oncogenic activation of H-ras, the one Ras isoform that relies exclusively on farnesylation. While initiation of these lesions was comparable between FT null and wild-type animals, progression differed. The absence of FT function correlated with a decreased tumor progression and maintenance. These results provide a sound genetic basis on which to question the

premise of pharmacologic inhibition of FT as a therapeutic strategy and could have potentially avoided numerous costly, and ultimately futile, clinical trials. This example illustrates the need for a comprehensive approach utilizing all available model systems, including important mouse models, to verify strategies before committing the effort and resources required for clinical trials.

Farnesyltransferase inhibition has been largely set aside as a means of Ras inhibition for the reasons described above. Ras proteins may, however, retain their value as a therapeutic target for their multifaceted contributions as oncogenes. An exciting development has recently been reported in the search for therapeutic strategies against Ras, whoch could exploit previously unappreciated aspect of the cell biology of K-Ras4B in a therapeutic context (11). The association of K-Ras4B with the plasma membrane is the result of both the interaction of its hydrophobic farnesyl moiety and the phospholipids of the bilayer and of the electrostatic interaction between its polybasic domain and the negatively charged head groups of these phospholipids. This study reports the presence of PKC phosphorylation sites within the polybasic domain of K-Ras4B. Phosphorylation of these residues neutralizes the positive charge of the polybasic motif and results in discharge of this protein from the plasma membrane and redistribution to endomembranes within the cell, including the mitochondria. At the mitochondria, K-Ras4B interacts with Bcl-X_L in a PKC-mediated phosphorylation-dependent manner to promote apoptosis. Exploiting this fact, treatment of K-Ras transformed fibroblasts with PKC agonists such as bryostatin resulted in internalization of the mutant K-Ras protein and apoptosis. This effect was abrogated by mutation of the serine residue that serves as the primary phosphorylation substrate of PKC (11). Though these results are very

preliminary, they offer an exciting opportunity to revisit possible therapeutic strategies against one of the most common oncogenes in human tumorigenesis.

Colon as a Model System of the Role of Ras in Human Cancer

The National Cancer Institute estimates that approximately 150,000 people were diagnosed with colorectal cancer in the United States in 2005. Approximately 60,000 succumbed to this disease in the same time period, making colorectal cancer the third most common form of cancer and the third-leading cause of cancer-related mortality in this country. In addition to its importance as a public health concern, colorectal cancer makes an attractive model in which to study the molecular basis of transformation and cancer progression because of its origin in epithelial populations, the availability of tissue samples from tumor resections to such common procedures as colonoscopy, which has led to establishment and extensive characterization of colorectal cancer cell lines, and the relatively clear histological structure of the tissue which has yielded significant understanding of the natural history of colorectal cancer. For these reasons, a great deal is known about the genetic basis of this disease. Relative ease of access to tissue samples has allowed researchers to formulate a model of progression from aberrant crypt foci to adenoma to carcinoma and to assess genetic changes at the various intermediate stages. As originally shown by Fearon et al. and since expanded, there is a clear genetic progression that correlates with disease progression (42).

The human colon is arranged into discrete functional epithelial glands known as the crypts of Lieberkühn (Figure 7). During development, a continuous epithelial monolayer undergoes invagination to produce these structures. Each crypt is supported

by a single progenitor cell which is thought to reside at the base of the crypt and which is responsible for replenishing the epithelial population that lines the structure (40). Pulse 5' bromodeoxyuridine (BrdU) labeling has shown that the bulk of cellular proliferation in a given crypt occurs in the bottom 5-10 cell positions along the crypt axis. As cells migrate through this region, they collectively constitute a transit amplifying population responsible for the bulk of proliferation in any single crypt. Further along the crypt axis, cells begin to differentiate into one of at least three terminal cellular identities (goblet cells, enteroendocrine cells, and enterocytes) and as they do they lose their proliferative potential. At what point along this crypt axis cells become transformed and initiate a neoplastic lesion has been the subject of much debate and remains unresolved. The spatial and temporal linearity of these processes, ability to histologically view the entire crypt axis in a single field, rapid kinetics of this population, and frequency of neoplastic events in this tissue combine to make the colonic epithelium a highly useful model system in which to correlate molecular cell biological changes with changes in tissue morphology, proliferation, possible stromal contribution, differentiation, and disease progression.

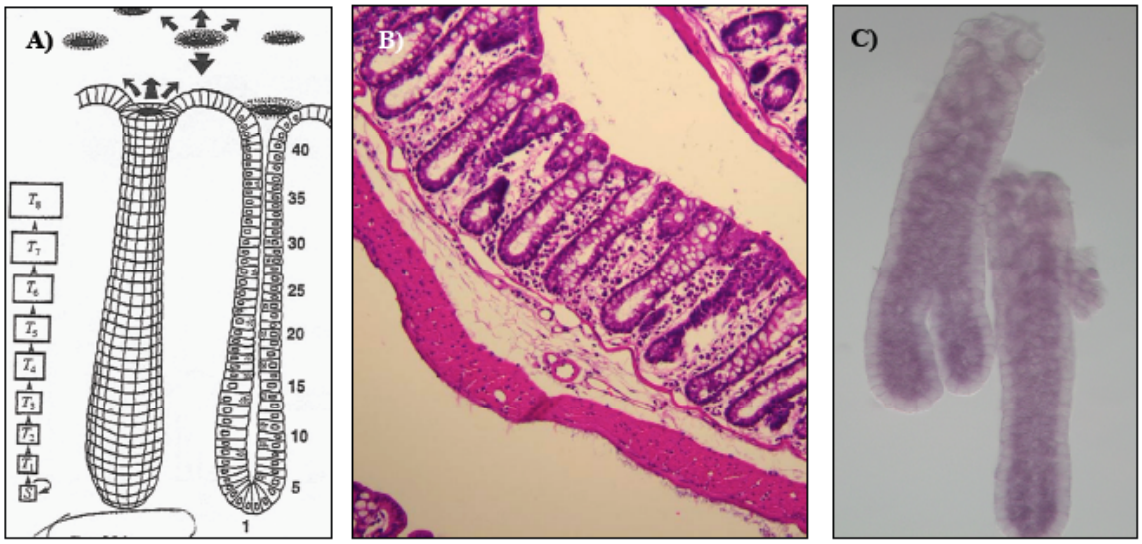


Figure 7. Colonic crypts. The small intestine and colon are arranged in functionally distinct units composed of an epithelial monolayer knowns as crypts of Lieberkühn. Proliferation occurs primarily in the bottom 5-10 cell position and as cells migrate upward, they lose proliferative capacity and assume increasingly differentiated states. A) Illustration adopted from (125); B) H&E staining of mouse colon; C) digoxygenin stain of isolated murine colon crypts. Note the bifurcated structure of the crypt on the left, which demonstrates crypt proliferation by fission.

An interesting and still unresolved observation about neoplasia in the colonic epithelium is the fact that tumors of the small intestine, an extraordinarily similar tissue in structure and biology, are exceedingly rare in humans. Why is the likelihood of tumor formation in the epithelium of the colon the third-leading site of tumor formation in humans, while the incidence of similar neoplasia in the small intestine is infinitesimally small? This question has long plagued the field. Some important observations that may partially explain this disparity are as follows: (1) in small intestine, radiolabeling studies with tritiated thymidine support the possibility that stem cells selectively sort the template DNA from the newly synthesized strand upon division, suggesting a possible mechanism for preserving an 'original copy' DNA template that is protected from potential replication errors (125); (2) in the small intestine, random errors that occur during replication trigger an apoptotic response in p53-dependent fashion (106); and (3) large scale loss of stem cells in the small intestine can result in dedifferentiation – cells in their first to third transit amplifying state can assume a multipotent progenitor phenotype (102). The colon, in contrast, relies to a greater extent on cell cycle arrest and DNA repair rather than apoptosis. In support of this, the pro-survival gene Bcl-2 is expressed at the base of the colonic crypt, but not observed in the small intestine, and is thought to abrogate the levels of apoptosis observed in the small intestine (102). The biological mechanisms used by the small intestine to deal with DNA damage and maintain a functional epithelium may be impractical in the colon where cells are presumably exposed to greater amounts of DNA-damaging agents due to the progressive concentration of luminal contents with water absorption. Apoptosis as a default response following DNA damage in the colon may result in massive, constant shedding of this

epithelial population that would outstrip the proliferative capacity of the progenitor population and may compromise the integrity and barrier function of the epithelium. Additionally, the constant regeneration required by the higher rate of DNA damage would carry with it correspondingly greater carcinogenic risk.

Choice of Experimental System for the Study of Ras: Considerations

As described previously, activating mutations of the *RAS* family of genes are among the most common genetic events in human tumorigenesis. Despite what we now understand to be unique functional identities composed of common and unique functions, Ras proteins are still biochemically very similar with highly conserved mechanisms of regulation. Though members of the GAP and GEF families of regulatory molecules display some specificity for different Ras isoforms, this is likely a function of localization within the cell rather than divergence in their regulatory mechanisms (4, 17). The switch I and II regions of the canonical members of the Ras family show complete conservation. The ability to interact with common regulatory molecules, the ability to bind common effector molecules, the conserved requirement for GTP to assume an active conformation, and the responsiveness to common upstream stimuli form the basis for our next hypothesis: that constitutive activation of one Ras family member (such as in the case of genetic mutation) may alter not only the activation state of the mutant proteins, but may also affect remaining wild-type Ras molecules. This may be the result of competition for regulatory molecules or effectors with the mutant Ras protein. Virtually nothing is known about the interaction of Ras molecules, directly or indirectly, intracellularly. Consider the various actors and actions that contribute to Ras activity:

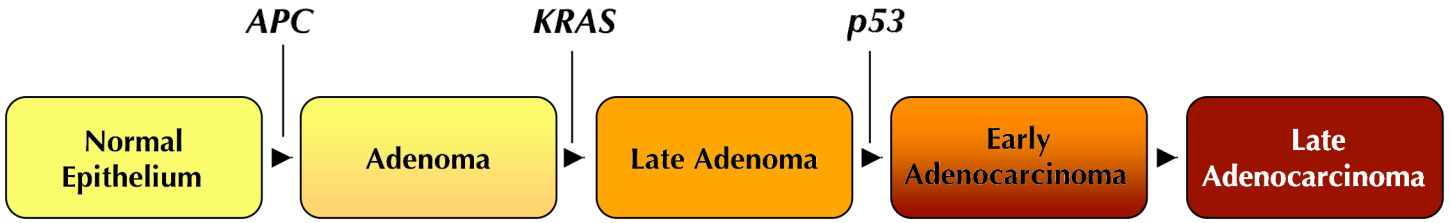
following a typical stimulus (assume ligand binding to a tyrosine kinase receptor), a GEF family member is activated and interacts with Ras•GDP and exchanges the GDP for GTP, thus causing Ras to assume an active conformation (GTP is almost certainly not limiting in the cell; estimates place [GTP]:[GDP] at 10:1 in an unstimulated cell and its association with Ras would be diffusion limited were Ras able to dissociate from GDP independent of GEF activity, A. Wolfman, personal communication). It seems likely from localization studies that numerous Ras microdomains overlap such that there are multiple Ras isoforms that respond to a common stimulus. The relative number of GEF and Ras molecules is unknown, and therefore, whether activation of an RTK results in activation of every Ras molecule in the immediate vicinity (in the case of unlimited GEFs, either through GTP exchanges on multiple Ras proteins, or more activated GEFs than inactive Ras molecules) is not known. The Ras protein thus loaded with GTP and in its active conformation is then presumably free to interact with effector molecules. These interactions may, however, depend on the interactions with adaptor proteins such as members of the galectin family. Members of this family interact with the C-termini of Ras proteins with different specificities (37, 38, 118), a fact not surprising given the divergence of Ras family members at the hypervariable region. Again, the stoichiometry of Ras and adaptor proteins such as galectins is completely unknown. Most canonical Ras effectors are activated by recruitment to the plasma membrane. Whether this is actually an active recruitment (for which no mechanism has been described) or simply diffusion of cytoplasmic proteins is not known. Similarly unknown is the stoichiometry of effectors to Ras•GTP; are all active Ras molecules able to find and bind an effector? How many different effector molecules does an active Ras molecule interact with before

hydrolysis of a bound GTP molecule? And like GEFs, how does the population and specificity of GAP family members compare to the Ras•GTP population?

The point of these speculations is to emphasize how little is really known about the multilayered kinetics of Ras activation and signaling generally, but also to illustrate that it is possible that different Ras isoforms experience common pressures in their regulation and activity. We therefore hypothesized that these dynamics may be altered by any number of factors, including constitutive activation through genetic impairment of the GTPase ability, a relatively common event in human tumorigenesis. The implications of disruption of Ras kinetics may result in altered activity of the remaining wild-type Ras isoforms, a consequence that is significant insofar as Ras isoforms are increasingly understood to possess some unique functions. These considerations raise an additional set of concerns as well considering that a significant number of the studies on Ras that have been done to date have relied on overexpression analyses, sometimes of wild-type proteins, but often of constitutively active mutants. These strategies almost certainly severely disrupt the kinetics described above, such that the conclusions from this work warrant at least serious qualification. Further complicating much of our present understanding of Ras biology is the fact that many of the studies to date rely on the use of fibroblasts, despite the fact that the majority of neoplasms arise from epithelial populations, which differ in fundamental ways from fibroblasts. These studies likely fail to quantitatively or qualitatively recapitulate the cell biology or oncogenic contribution of activated endogenous Ras.

Genetic mutation of codons 12 and 13 of the *KRAS* locus is a common and important event in tumorigenesis of the human colon, occurring in roughly half of cases.

The oncogenic contribution of activated K-RAS in this disease has been extensively studied. In two independent cell lines derived from colorectal adenocarcinomas, targeted deletion of the mutant *KRAS* allele causes loss of many of the hallmarks of the transformed phenotype, including reorganization of the actin cytoskeleton and associated changes in cell morphology, decreased rate of cell proliferation, loss of ability to form colonies in soft agar, and loss of ability to form tumors in xenograft models (156). The isogenic nature of these cell lines provides a powerful genetic tool with which to further dissect the molecular basis of the oncogenic nature of $K\text{-}RAS^{G13D}$ in cells of colonic origin. Additionally, the fact that these lines can be used in parallel provides a means of examining the conservation of mechanisms of K-RAS mediated transformation. These cells share extensive genetic similarity, though not identity, and are typical of colorectal carcinomas. Figure 8 summarizes what is known about the genomes of these cells.



Adapted from Fearon et al. Cell 1990 (61) 759-767.

Genetic Status of Colorectal Cancer-Derived Cell Lines

Cell Line	*KRAS*	*NRAS*	*HRAS*	*p53*	*p16*	*APC*	*B-catenin*	*TGF-B, type II Receptor*	Microsatellite Instability	Chromosomal Instability
HCT116	Gly13Asp *(1)*	wild-type *(2)*	wild-type *(2)*	wild-type *(3)*	methylation of one allele; G insertion at codon 23 (frameshift) *(4)*	wild-type *(5)*	three base deletion, loss of GSK3-B phosphorylation site *(6)*	inactivating mutation *(8)*	yes *(10)*	no *(11)*
DLD-1	Gly13Asp *(1)*	wild-type *(2)*	wild-type *(2)*	Ser241Phe *(1)*	Not Determined	inactivating mutation (truncation) *(5)*	wild-type *(7)*	nonfunctional; frameshift mutation and Leu452Pro missense mutation *(9)*	yes *(10)*	no *(11)*

(1) Shirasawa *et al.*, Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science 260, 85-88 (1993).
(2) J. Keller unpublished.
(3) Rodrigues *et al.*, p53 Mutations in Colorectal Cancer. PNAS 87, 9693-9697 (1990).
(4) Gayet *et al.*, Extensive characterization of genetic alterations in a series of human colorectal cancer cell lines. Oncogene 20 5025-5032 (2001).
(5) Morin *et al.*, Activation of B-Catenin–Tcf Signaling in Colon Cancer by Mutations in B-Catenin or APC. Science 287, 1787-1790 (1997).
(6) Ilyas et al., B-catenin mutations in cell lines established from human colorectal cancers. Proc. Nat. Acad. Sci. 94, 10330-10334 (1997)
(7) Lengauer *et al.*, Genetic instability in colorectal cancers. Nature 386, 623-627 (1997).
(8) Markowitz *et al.*, Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 268, 1336-1338 (1995).
(9) Parsons et al. Microsatellite instability and mutations of the transforming growth factor beta type II receptor gene in colorectal cancer. Can. Res. 55, 5548-5550 (1995).
(10) Cahill *et al.*, Mutations of Mitotic Checkpoint Genes in Human Cancer. Nature 392, 300-303 (1998).
(11) Fodde et al., Mutations in the tumour suppressor gene cause chromosomal instability. Nature Cell Biology 3, 433-438 (2001).

Figure 8. Genetic summary of DLD-1 and HCT 116 colorectal cancer cell lines. Colorectal cancer is one of the best-characterized neoplasias from a genetic perspective. Here, we compare the known genetic status of the DLD-1 and HCT 116 cell lines to our established understanding of the genetic events that drive the transformation of normal colonic epithelium to colorectal adenocarcinoma (16, 46, 50, 65, 87, 101, 109, 117, 136, 156).

Isogenic disruption of an oncogenic allele in a fully transformed cell yields a complex set of effects. Oncogenic changes accumulated during transformation are not simply additive, discrete units, but rather function in combination to achieve a cumulative effect. The order and nature of these oncogenic changes is important as well and appears to occur in particular sequences that if disrupted may result in some outcome other than transformation (130). Indeed, the context in which any particular oncogenic change occurs is crucial; most familiarly, constitutive activation of Ras in an otherwise normal fibroblast population results in cell cycle arrest (151), yet Ras activation as a midpoint genetic event in tumorigenesis may provide numerous selective advantages to a cell population in the development of numerous different tumor types.

Similarly, disruption of any particular oncogene in the context of full transformation likely triggers a complex sequence of events, and may even compromise the viability of the cell. This concept has been described as 'oncogene addiction' and connotes adaptation on the part of the cell to the constitutive set of changes resulting from genetic events so that the cell may not simply accommodate these alterations in the signaling environment, but rather may come to depend on them for their very survival (21, 44, 68, 119). These considerations influence greatly our experimental design in which we are attempting to reconstitute critical signaling events that give rise to transformation.

The isogenic nature of the DLD-1 ($KRAS^{G13D/+}$) and DKS-8 ($KRAS^{+/-}$) cell lines and of the HCT 116 ($KRAS^{G13D/+}$) and HKE-3 ($KRAS^{+/-}$) cell lines make them a uniquely useful tool in examining the contribution of constitutive RAS signaling to tumorigenesis. However, our understanding of the transformation as a specific, stepwise progression of

genetic events that presumably (fortunately) fails to result in fully transformed cell population more often than it succeeds, also requires that we confront simple disruption of an oncogenic allele as effecting a significant series of changes in the fundamental physiology of the cell rather than as a simple reversal of one genetic event, a single step backward on the journey to transformation. Even in light of this caveat, these isogenic cell lines represent possibly the best available reagent for analysis of endogenous constitutive RAS signaling in a disease-relevant cell population.

We are not the first to adopt the use of these cells for this purpose. Several studies have attempted to elucidate the oncogenic contribution of constitutive K-RAS signaling based on the behavior of these parental lines relative to their isogenic derivatives. Interestingly, these studies rely almost exclusively on HCT 116 and its isogenic derivatives and have failed to include DLD-1 and its derivatives, lines derived by many of the same investogators. Known mediators of cytoskeletal organization, Ras family members influence several other small G proteins, including Rho, Rac, and Cdc42, all of which are important regulators of the actin cytoskeleton. Not surprisingly, constitutive activation of *KRAS* changes the morphology of cells, and in both the HCT116 and DLD-1, loss of activated *KRAS* results in a reversion from a transformed morphology characterized by fibroblastic appearance, spindle-like processes, and filopodia to the more cuboidal, regular, cobblestone appearance of a non-transformed epithelial cell.

Oncogenic K-RAS4B in HCT 116 cells correlates with elevated Rho•GTP levels, though these cells seem unable to maintain stress fiber formation or focal adhesions, both of which likely explain their decreased adhesion to substrate and increased motility (123).

These characteristics are inconsistent with elevated Rho activity, which through activation of ROCK (Rho-associated coiled-coil-containing proteins kinase) leads to increased phosphorylation of cofilin and myosin light chain, which contribute to decreased actin severing and increased actomyosin contractility (96). Treatment of these cells with a small molecule inhibitor of MEK, however, results in stress fiber formation, an increase in focal adhesions, increased adhesion and decreased motility, consistent with increased Rho activity. Further investigation revealed that activated K-RAS4B in these cells constitutively stimulated competing pathways. Continuous stimulation of MEK/MAPK increased levels of activator protein-1 family member Fra-1, which is known to regulate Rho signaling and motility in cancer cells (167). Consistent with a role for K-RAS in mediating the actin cytoskeleton, constitutive K-RAS signaling regulates the expression of a novel gene known as *KRAP* (K-RAS induced actin-interacting protein) that encodes a protein that shows strong colocalization with actin filaments (66).

Ras family members also play multiple roles in the apoptotic response, and the effects of oncogenic Ras on cell death and survival vary significantly with cell type and context (25). In HCT 116 cell lines, oncogenic K-RAS4B sensitizes these cells to apoptosis induced by both treatment with the short chain fatty acid butyrate as well as to treatment with the commonly used chemotherapeutic agent 5-fluorouracil (5-FU) (76, 77). This sensitization is purportedly the result of transcriptional downregulation of gelsolin by oncogenic K-RAS signaling. First identified as a calcium-regulated actin severing and capping protein (185), gelsolin is reduced or absent in 60-90% of cancers, including those of the stomach, bladder, breast, prostate, and colon (7) and is thought to

play a role in mediating cytochrome C release during the apoptotic response as supported by its association with the voltage dependent anion channel (VDAC) (83, 84) resident on the outer mitochondrial membrane. Gelsolin is also a substrate for cleavage by caspases 7 and 9 and generates a cleavage fragment with pro-apoptotic activity (82). We revisit the purported mechanism of this sensitization in later sections.

Oncogenic RAS also contributes to angiogenesis, a role that is evident in the upregulation of vascular endothelial growth factor (VEGF) and also vascular permeability factor (VPF) in HCT 116 and DLD-1 cells relative to their isogenic derivatives. Disruption of the mutant *KRAS* allele causes a 4-5 fold drop in the mRNA levels of these genes, a finding which likely explains the inability of the isogenic derivative lines to form tumors in nude mice in part (114). (Expression of cDNAs encoding VEGF and VPF in the isogenically derived cell lines conferred only weak tumorigenic properties as measured by tumor formation in nude mice).

CHAPTER II

ANALAYSIS OF RAS EXPRESSION AND N-RAS FUNCTION IN MURINE COLONIC EPITHELIUM

Introduction

The position along the crypt axis at which tumors arise has been the subject of much debate. Cancer is increasingly thought of as a disease of 'stem cells,' and in light of the undifferentiated state and proliferative potential of the progenitor population at the base of the crypt, this possibility warrants serious consideration. This theory gives rise to the 'bottom-up' model of tumorigenesis in the colon, and relies on the thinking that stem cells residing at the base of the crypt accumulate mutations and give rise to a lesion which expands upward from the crypt base. Competing models hold that tumors arise from daughter cells that fail to undergo the normal progression seen in the colonic epithelium. For instance, a colonocyte that would normally be shed may acquire the ability to adhere; a daughter cell that would normally undergo loss of proliferative capacity may experience genetic mutations that allow proliferation to continue ectopically or may de-differentiate. This theory provides the basis for a 'top-down' model of tumorigenesis in the colon, which stipulates increased rates of mutagenesis in daughter cells that have been exposed to the carcinogenic contents of the lumen. The histological data of early stage lesions is ambiguous and is complicated by the fact that aberrant crypt foci display a compromised morphology that complicates direct comparison between morphology, genetic status, and likelihood of progression to disease.

Loss of critical regulators of the stem cell population compromise maintenance and homeostasis of the colonic epithelium. The Wnt signaling axis provides a compelling example of the consequences of dysregulation of this tissue. Loss of the Tcf4, a transcription factor that interacts with nuclear β-catenin to transduce Wnt signaling, results in complete loss of the stem cell compartments in the epithelium of the small intestine, and hence loss of the characteristic crypt/villus morphology (81). We observe here an intriguing expression pattern of *Nras* in the murine colon distinct from its closely related family members *Hras* and *Kras*, and possibly corresponding to regions of the crypt thought to harbor the long-lived pluripotent stem cell. Based on the known roles of Ras family members in wide range of cellular functions and the intriguing expression patterns we observe, we speculated that localized expression of *Nras* in the progenitor compartment may portend an important regulatory function for this protein in the intestinal epithelium.

To assess this, we chose to interrogate mice in which the *Nras* locus has been disrupted by homologous recombination resulting in a straight knockout ($Nras^{-/-}$) (166). As mentioned in the Introduction, this animal is viable and reproduces normally. Only a single, subtle phenotype has been reported to date: a deficiency in CD8 single positive thymocytes and decreased thymocyte proliferation *in vitro* (29). The morphology of the intestinal crypt is a manifestation of the equilibrium between cell proliferation and loss (either through shedding into the lumen of the intestine or apoptosis) and therefore represents a useful system in which to assess alterations to the basic functions of the progenitor compartment.

Materials and Methods

Bromodeoxyuridine Incorporation, Tissue Harvest, and Preparation

Tissue used in BrdU and histological comparisons were harvested from mice pulsed with BrdU injected intraperitoneally (16.7mg/kg solution in PBS) and chased for 2hrs. Colons were swiss-rolled, fixed in 4% paraformaldehyde, rocking at 4°C overnight, rinsed 3X30 min in PBS, then embedded in paraffin for sectioning. Five micron sections were taken and stained with anti-BrdU antibodies using Zymed BrdU kit (Invitrogen, Carlsbad, California).

Nras$^{-/-}$ Mice

Nras$^{-/-}$ mice were a kind gift from R. Kucherlapati and were generated as described (166). The primers for N-ras were LM164 (5-CCAGGATTCTTACCGAAAGCAA GTGGTG-3), LM205 (5-GATGGCAAATACACAGAGGAACCCTTCG-3), and LM166 (5CAGAGCAGATTGTACTGAGAGTGCACC-3). The LM164 and LM205 primers were specific for the *N-ras* gene (positions 4 to 31 and 121 to 148 on exon II; GenBank accession no. M12122) and amplified a fragment of 146 bp; LM166, specific for the cloning vector pUC19 (position 157 to 183), amplified a fragment of 315 bp with LM164. Oligonucleotides were used in a 50μl reaction mixture with 1 to 2μl of DNA and 1.25 U of Taq polymerase (Boehringer Mannheim, Indianapolis, Indiana). Cycling conditions were 94°C for 4 min followed by 30 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, followed by an elongation cycle of 72°C for 10 min, using a Perkin-

Elmer Thermal Cycler. Amplified products were analyzed by electrophoresis in 2.0% agarose gels.

Dextran Sodium Sulfate Treatment

DSS (molecular weight: 40,000) was purchased from ICN Biomedical, Aurora, Ohio) and dissolved in drinking water (3.5% w/v in distilled water). DSS was administered for time courses specified.

Immunoblotting and Immunostaining

For immunostaining, intact murine crypts were isolated for immunostaining per the method of Whitehead (176). Crypts were fixed in 4% paraformaldehyde for 30 min rocking at 4°C and rinsed 3X30 min in ice-cold PBS at 4°C. Crypts were then permeabilized with 0.5% Triton X-100 made in PBS for 10 min at room temperature. Crypts were then rinsed with PBS 3X 10 min and blocked with 2% BSA made in PBS for 1 h at 4°C. N-ras antibody (sc-31, Santa Cruz) was diluted 1:50 in blocking solution and added to crypts and allowed to stain overnight at 4°C. Crypts were washed 3X30 min with PBS and detected with Cy3-conjugated anti-mouse secondary at 1:500 in blocking solution (Jackson Immunosresearch Labs, West Grove, Pennsylvania) for 1 h at room temp. Crypts were then washed 3X 30 min with PBS and mounted.

For immunoblots, crypts or whole colons were washed 3X with ice-cold PBS and lysed in 25mM TRIS, pH 7.6, 1% NP-40, 2mM EDTA pH 8.0, 150mM NaCl, 5mM $MgCl_2$, 1mM DTT, proteinase inhibitors (Roche), and phosphatase inhibitor cocktails I and II (Sigma). Lysates were vortexed 5 sec, allowed to rest on ice 5 min, and

centrifuged at 13,400 rpm at 4°C. Supernatants were then transferred to fresh tubes, and protein concentration determined by BCA assay. Samples were resolved on 12% SDS-PAGE, transferred to Immobilon, blocked for 1 h with 5% nonfat milk in TBST (0.1 %Tween 20), then incubated with primary antibody overnight. N-RAS (sc-31) antibody was from Santa Cruz and were used at 1:200. Blots were detected with appropriate secondary antibodies conjugated to HRP and detected with enhanced chemiluminescence (Amersham, Uppsala, Sweden).

Results

We initially set out to understand in greater detail the biological differences between the undifferentiated, pluripotent, long-lived progenitor cells of the crypt and the differentiated terminal populations to which they give rise. To do this, we isolated crypts from adult murine colon using the method of Whitehead et al. (176). These crypts were them microdissected into top and bottom halves from which RNA was isolated, linearly amplified, and compared by microarray to examine the relative gene expression profiles of these cell populations. These experiments identified genes that are differentially expressed in the top and bottom of the crypt, and which may facilitate our understanding of the biological programs of the colonic epithelium.

Among the class of genes that were preferentially expressed in the bottom of crypts was *Nras* (bottom/top=2.7). Subsequent analyses of the expression of this gene by *in situ* hybridization supported the preferential expression of *Nras* at the base of the crypt and placed the transcript in the proliferative compartment. This expression pattern was observed in the colon as well as in the small intestine (Figure 9, panel A). *Hras* and

Kras4B appear to be expressed ubiquitously and in the bottom half of the crypt, respectively.

We must qualify this data, however, by noting that here remains a lack of consensus regarding the gene structure of murine *Nras*. At least two transcripts exist for this gene; the coding regions are identical although they utilize alternative exons in the 3'UTR. There may additionally be a third transcript. Affymetrix utilizes a cDNA identified as N-ras that maps to a region far downstream of the recognized 3'UTR. Use of this probe in *in situ* hybridization revealed expression at the base of the murine intestinal crypt. This expression pattern shows strong but not absolute correlation with the pattern of N-ras expression seen by immunohistochemistry with N-ras specific antibodies. This expression pattern contrasts with that seen in experiments utilizing a probe that recognizes both canonical transcripts, which shows near ubiquitous expression throughout the crypt axis (Figure 9).

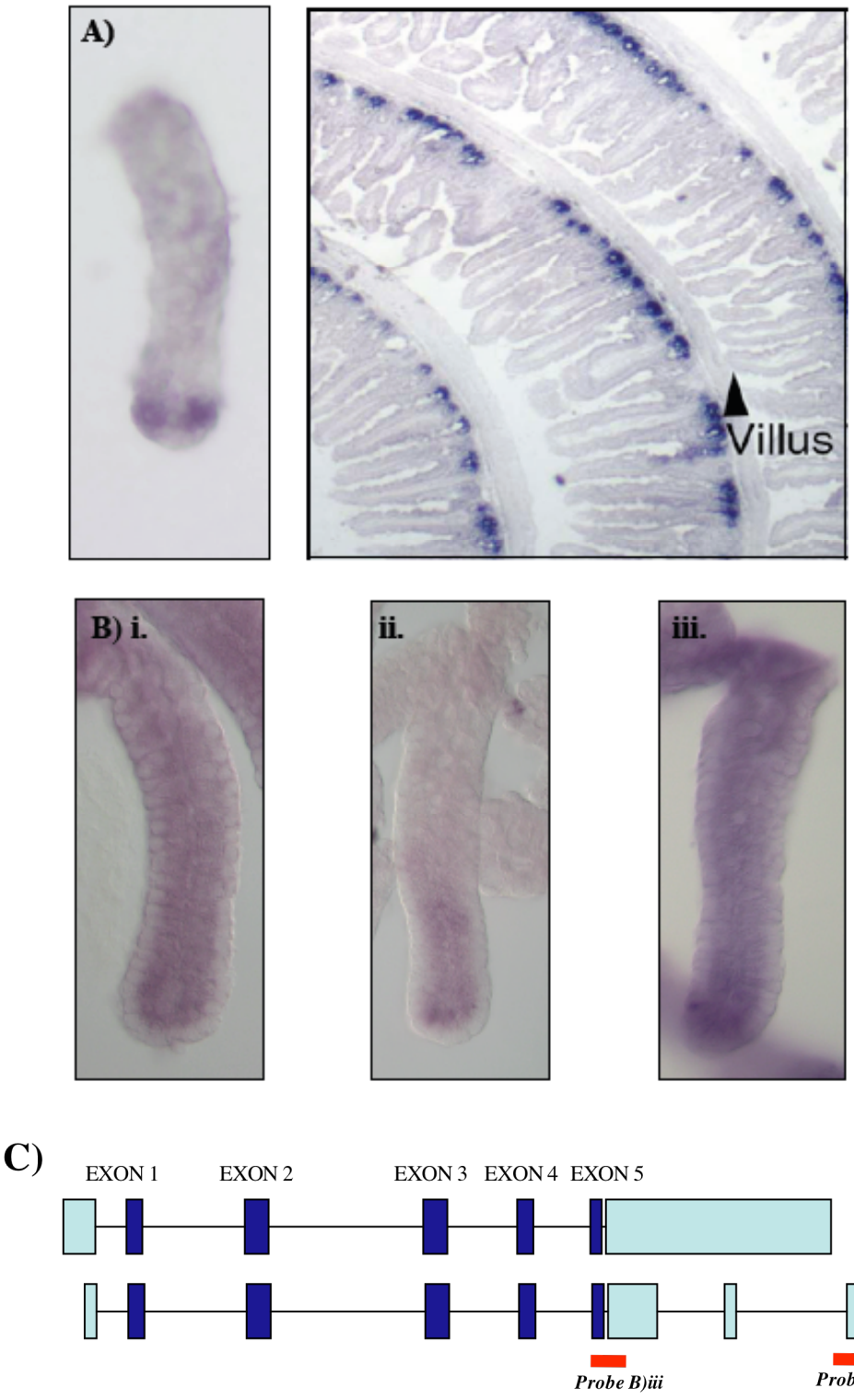


Figure 9. Expression of Ras family members in murine intestine. *In situ* hybridization using antisense digoxygenin-labeled riboprobes. A) Expression of *Nras* as detected by in isolated colonic crypt (left) as well as small intestine (right). B) Expression of *Hras* (i), *Kras4B* (ii), and *Nras* (3'UTR probe) in isolated murine crypts. C) The structure of the murine *Nras* gene remains under investigation. Structure shown here is from the ENSEMBL database.

To examine the correlation between N-ras expression and the proliferative compartment of the crypt, we pulsed mice with the thymidine analog bromodeoxyuridine (BrdU) by intraperitoneal injection (2 h chase), sacrificed the animals, harvested the crypts, and stained for BrdU and N-ras. Immunostaining for N-ras and BrdU revealed extensive overlap between proliferative cells in the base of the crypt and those that expressed N-ras. To measure whether or not N-ras expression was simply a function of proliferation, we next repeated this experiment but chased for 24 h in order to identify subsequent proliferative populations in the transit amplifying region of the crypt. In this experiment, the staining segregated, with N-ras appearing predominantly in the base of the crypt as seen previously, with the BrdU pulse moving up the crypt axis following multiple rounds of proliferation (Figure 10, panel C). We note, however, that N-ras expression as gauged by immunostaining in these experiments is not strictly delimited to the proliferative compartment; there appear to be individual cells further along the crypt axis that are positive as well that do not belong to the progenitor population.

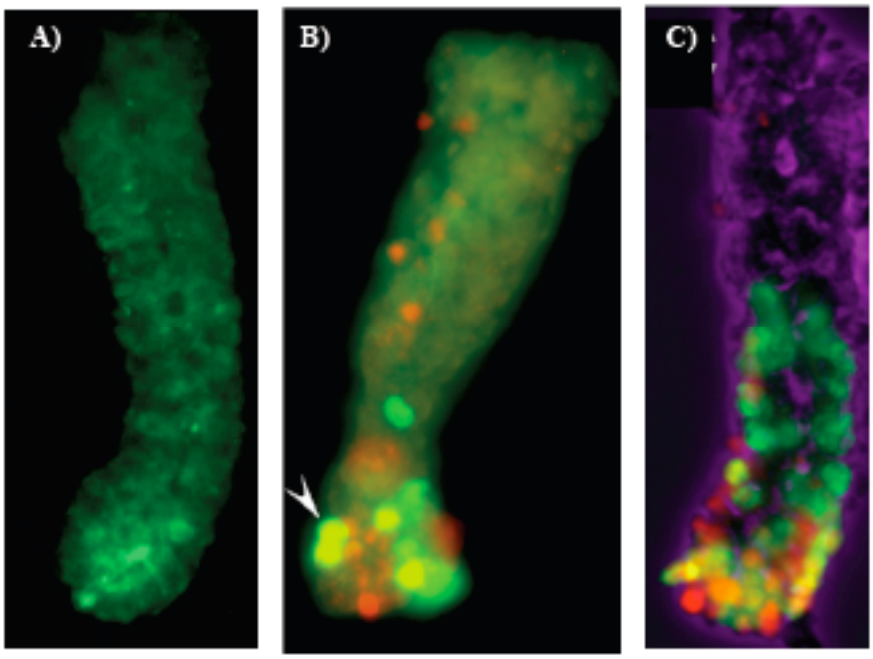


Figure 10. N-ras Immunostaining and colabeling with BrdU. A) Immunostaining using N-ras specific antisera reveals predominant localization of N-ras in the base of the crypt (green). B) Colabeling experiments staining for N-ras (red) in mice injected IP with BrdU (green) followed by 2 h chase reveals colocalization of N-ras-positive cells and dividing cells in the base of the crypt (arrowhead). C) 24 h chase following BrdU expression shows loss of colocalization between N-ras (red) and BrdU-positive cells (green). Courtesy of Dr. Jeff Franklin.

$Nras^{-/-}$ mice display colonic epithelial morphology and proliferation comparable to wild-type counterparts

We initially examined the morphology of the descending colon of $Nras^{-/-}$ animals relative to $Nras^{+/-}$ and their wild-type littermates. We injected these animals with BrdU intraperitoneally to label proliferating cells and measured both BrdU incorporation as well as crypt axis length. To standardize this survey, we made these measurements in a consistent fashion relative to a morphological landmark (a thickening of the muscularis) that is reliably found in the middle of the descending colon. Measurements of the colonic morphology and basal rates of proliferation were conducted blindly and were indistinguishable across these animals (Figure 11).

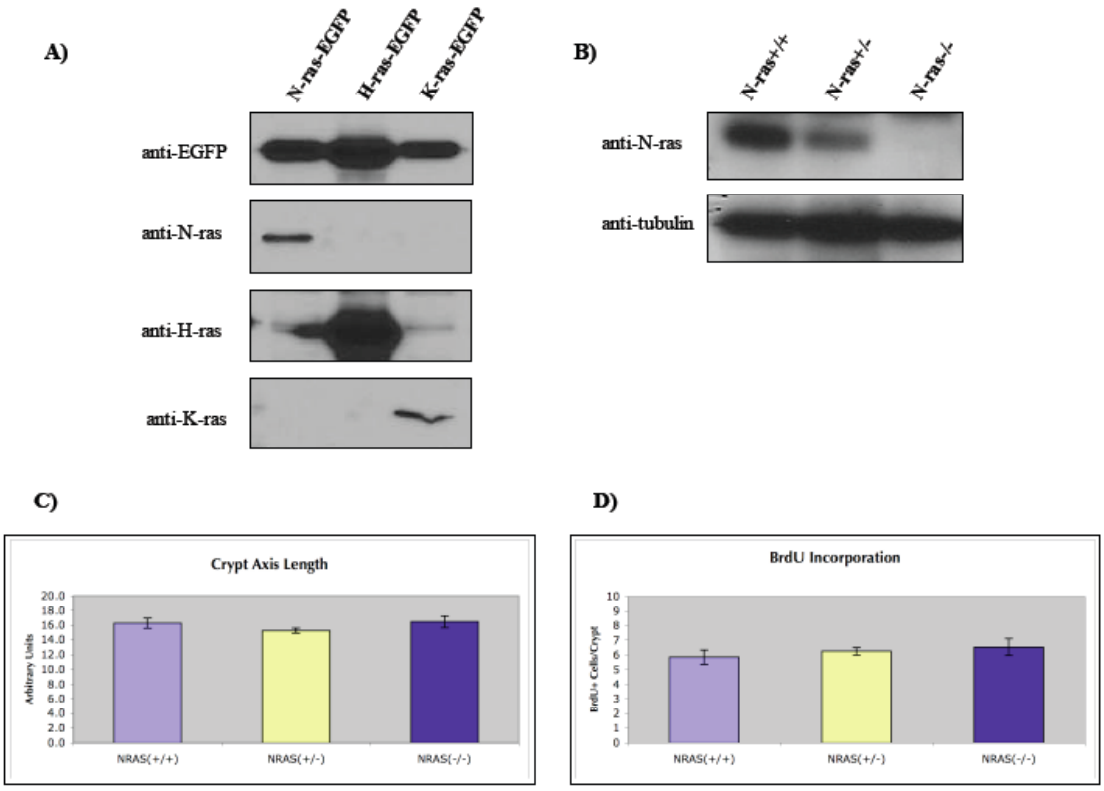


Figure 11. ***Nras*$^{-/-}$ and wild-type mice exhibit comparable morphology and baseline proliferation in the colon.** A) Demonstration of antibody specificity of various Ras specific antisera (N-ras sc-31, H-ras sc-520; K-ras4B sc-30). B) Spleen lysates from *N-ras*$^{+/+}$, *N-ras*$^{+/-}$, and *N-ras*$^{-/-}$ mice showing absence of detectable protein. C) Measurement of crypt axis length taken from 10 crypts of at least 6 mice of each genotype. D) At least six mice of each genotype injected with BrdU IP as described in Materials and Methods. Values represent the average number of BrdU-positive cells following 2 h chase from 10 crypts/animal.

$Nras^{-/-}$ mice are similar to wild-type animals in their susceptibility to ulcerative damage as well as ability to recover

Tebbutt et al. have implicated Ras function in a proliferative brake that, when dysregulated, can result in impaired wound healing in the gut, though they fail to specify which Ras family member may be involved. In an elegant series of studies, this group exploited the β-subunit of the gp130 transmembrane receptor, which transduces signals from several members of the interleukin family, including IL-6 and IL-11. The wild-type gp130 receptor has cytoplasmic binding sites for both SHP2 and STAT proteins, and through simultaneous and coordinate activation of these opposing pathways is thought to maintain epithelial homeostasis. SHP2/RAS/ERK signaling provides a proliferative brake, while STAT1/3 activation promotes proliferation (Figure 12). This is accomplished by a gene targeting strategy that knocks in mutant alleles at the *gp130* locus which are capable of preferential activation of either the SHP2/Ras/ERK pathway or STAT signaling, but not both (160). Given the demonstrated role of at least one Ras family member in providing an anti-proliferative signal in response to cytokine stimulation of the gp130 receptor, including impaired wound healing following ulceration of the gastrointestinal mucosa, we hypothesized that we may be able to provoke a phenotype from the $Nras^{-/-}$ mice by inducing ulcerative damage in the colonic epithelium. Damaging the epithelium triggers a massive proliferative response from the progenitor population in which *Nras* is disproportionately expressed. In accordance with the work described above, we predicted that loss of N-ras may result in hyperproliferation and enhanced wound healing.

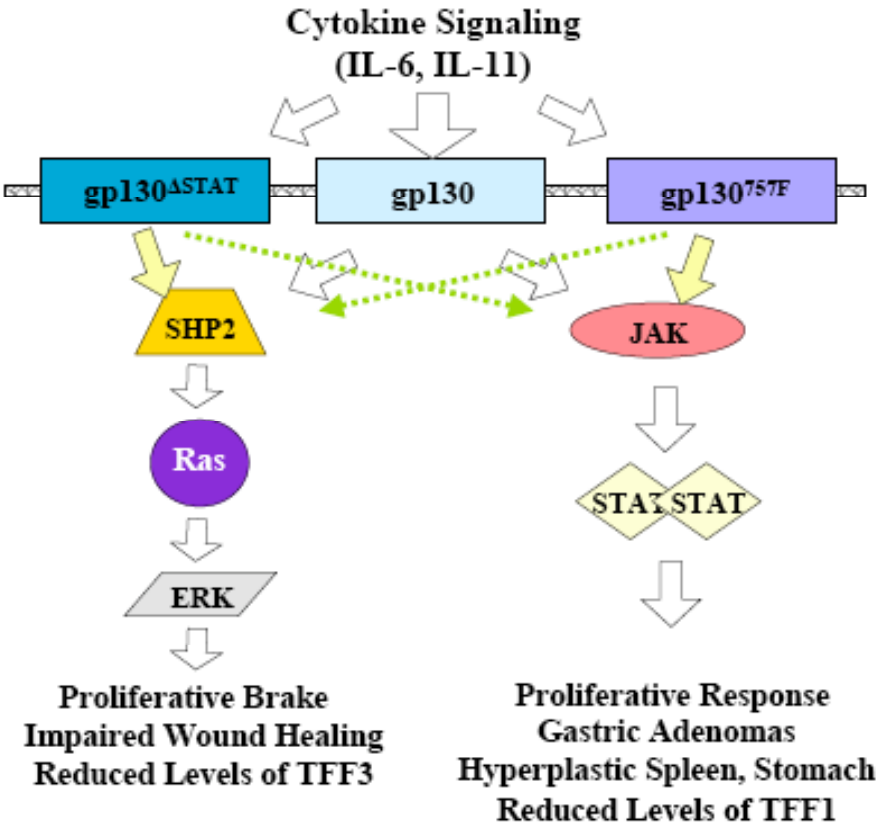


Figure 12. Cytokine signaling in the gastrointestinal tract utilizes the gp130 receptor to stimulate multiple pathways that together promote homeostasis. Adapted from Tebbutt et al., Nature Medicine 2002.

To induce damage, we administered dextran sodium sulfate (DSS) in the drinking water of these animals. The mechanism by which DSS ulcerates the epithelium of the colon is unknown, but depending on dose and duration, it can induce massive ulcerative injury, bleeding, and death. We wished to limit ulceration to a level tolerated by a wild-type animal, and then to examine the extent of damage and observe the rates of recovery. We empirically determined a treatment course of 96 h of exposure to 3.5% DSS in drinking water that induced significant levels of colonic ulceration accompanied by regions of the colon denuded of epithelium, bloody stool, and lethargy.

Close examination of these animals revealed broad variability in the severity of injury from animal to animal, with no real correlation between extent of damage, rate of recovery, and phenotype. One challenge inherent in the use of DSS-induced ulceration is that dosages cannot be controlled. Some animals showed signs of lethargy early on in the time course and likely consumed less water over the treatment than other animals that appeared relatively healthy throughout the treatment. Variability in the level of induced damage and recovery response shown may be attributable to this (Figure 13). Indeed, the DSS model has been used extensively as a colitis model, but is complicated by the lack a clear mechanism of ulceration, limited understanding of the molecular events that underlie damage susceptibility and recovery, and variability from animal to animal. Samples taken from colons collected either at 96 h of treatment, 48 h after the end of treatment, and 120 h after the end of treatment were analyzed blindly by a pathologist (K. Washington, Vanderbilt University Medical Center) and scored on the extent of ulceration and rates of recovery. No correlation was observed between extent or susceptibility of damage, extent or rate of recovery, and phenotype.

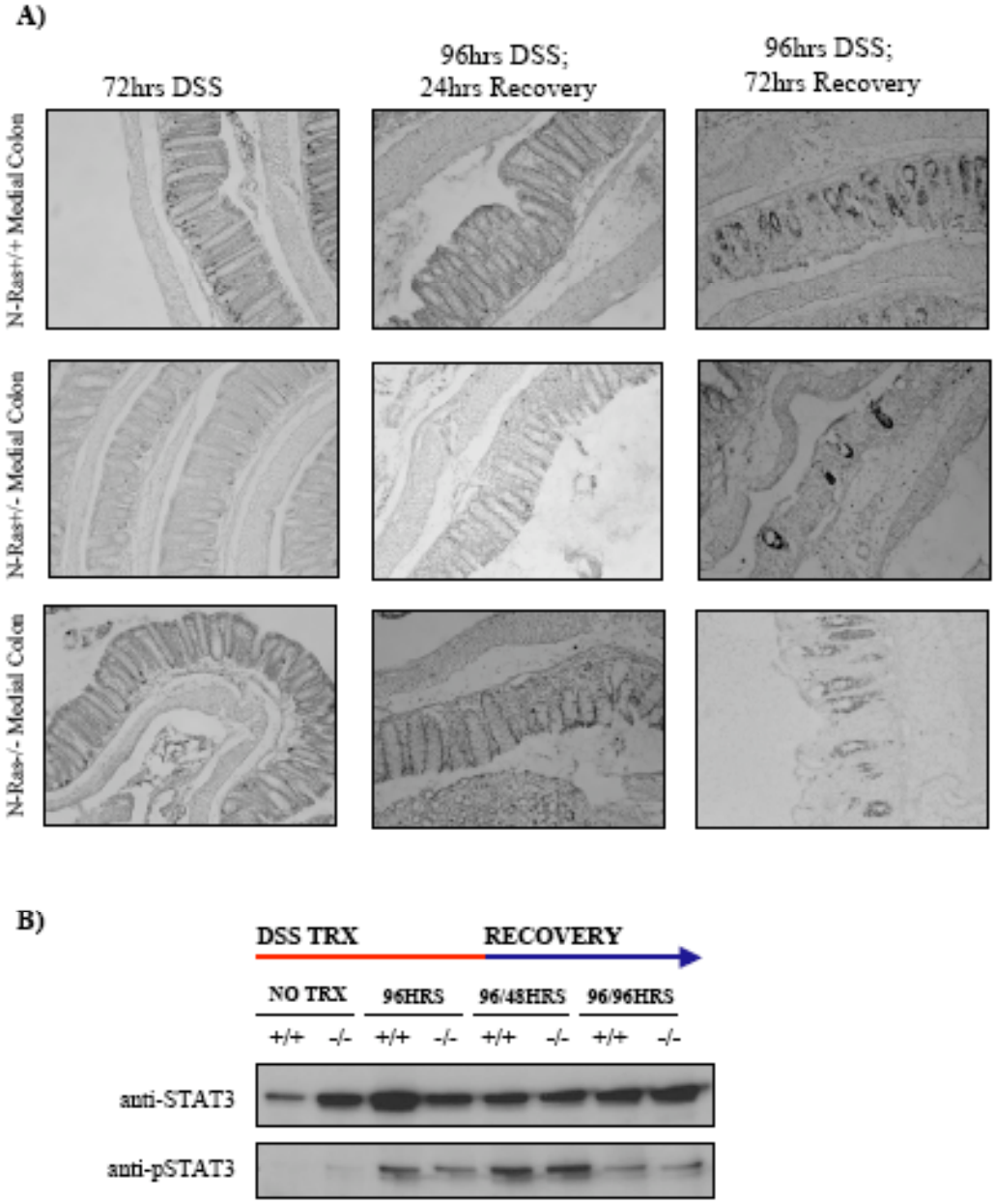


Figure 13. Dextran sodium sulfate (DSS)-induced colitis in colonic epithelium of *Nras*$^{+/+}$, *Nras*$^{+/-}$, and *Nras*$^{-/-}$ mice. A) Colonic epithelium shows a wide range of damage and differential rates of recovery following a time course of DSS without regard to genotype. Mice were injected with BrdU intraperitoneally followed by a 2 h chase before tissue harvest. A minimum of three animals of each genotype were examined at each time point. B) STAT3, a known phosphorylation target following cytokine stimulation is equivalently activated in both *Nras*$^{+/+}$ and *Nras*$^{-/-}$ animals over the time course.

Further, we analyzed levels of STAT3 activity as measured by a phospho-specific antibody in immunoblots of this tissue at various time points. This pathway is activated equivalently in the damage response in both *Nras*$^{-/-}$ and wild-type animals as indicated in Figure 13, panel B. Examination of the histology of these tissues does not support a role for N-ras in this response. We therefore discontinued these analyses in light of these finding and given the inconsistency of this model and our inability to interpret a negative result. N-ras may function in some way in the protection of this tissue from ulceration; its function may, however, be compensated for by other family members such that a phenotype would never be observed without additional gene targeting of other Ras family members. Alternately, the function of N-ras may be completely dispensable in the protection of tissue from damage or its subsequent recovery.

Discussion

Specific functions of Ras family members is strongly suggested by (1) the fact that mutations to the various members of the Ras family of genes segregate strongly by tumor type; and (2) genetic evidence suggests that though N-ras and H-ras are dispensable for normal murine development, K-ras is necessary and sufficient. We therefore hypothesized that the various Ras family members may exhibit unique functions in the colonic epithelium. Despite specific expression of N-ras in the proliferative compartment of the colonic crypt, we are unable to discern a colon phenotype in animals in which the *Nras* locus has been disrupted by gene targeting. There are several possible explanations for this observation, including: (1) N-ras function may be dispensable for normal colon physiology; (2) N-ras may participate in functions

critical to this tissue but that are not required for homeostasis and therefore may not be detected by the sort of analyses we have conducted here; (3) N-ras may participate in the homeostasis of this tissue, but its function may be compensated for by other Ras family members, thus masking any effects of its loss. This is not an exhaustive list, but it does illustrate the challenges of assigning function to genes with closely related, partially redundant family members that display overlapping expression patterns. Further investigation in the mouse using genetic approaches would require significant investments of time and labor and cannot guarantee a definitive conclusion. For these reasons, we have opted to explore the relationship of N-ras with other family members in molecular and functional studies in a different system. This work is the subject of subsequent chapters.

CHAPTER III

USE OF THE RAF-1 BINDING DOMAIN (RBD) TO GAUGE RAS•GTP LEVELS *IN VITRO* FAILS TO DISRUPT ENDOGENOUS RAS:EFFECTOR INTERACTIONS

Introduction

Members of the RAS family of small G proteins are characterized by extensive homology at the level of primary sequence and intrinsic GTPase activity. The canonical members of the RAS family (H-RAS, N-RAS, and K-RAS4B) are the archetypes of this family of proteins. To assume an active conformation wherein they are able to transduce intracellular signals by interacting reversibly with effector molecules in their conserved switch I regions, RAS proteins must bind a molecule of GTP (45, 147, 148). Hydrolysis of this nucleotide to GDP is typically assisted by GTPase activating proteins (GAPs), which have been shown to greatly increase the rate of hydrolysis over the intrinsic hydrolytic capacity of these molecules and returns the protein to an inactive conformation (149, 163).

The significance of RAS function in homeostatic and disease processes has driven efforts to identify means of examining levels of RAS activity by determining the ratio of GTP to GDP bound to RAS molecules in a given condition. The classical GTPase assay is one such method and relies on complete labeling of intracellular guanine nucleotide pools by growing cells in medium containing [^{32}P] orthophosphate followed by specific immunoprecipitation of RAS molecules, elution of bound labeled nucleotide, and resolution of GTP and GDP by thin layer chromatography as described by Downward et

al. (36). While effective, this assay requires the use of significant amounts of orthophosphate, cannot discern activity levels of specific RAS isoforms (aside from N-RAS) due to the lack of antisera capable of specific immunoprecipitation of all RAS isoforms, and is restricted to use in cell culture and *in vitro* experiments (exposure of internal tissues of animals to orthophosphate being impractical). Further, exposure of cells to such significant amounts of radiation risks additional secondary effects such as DNA breakage that may introduce variables into the experimental system. For these reasons, identifying alternative means of gauging RAS•GTP levels has long been of interest.

The RAS-binding domain (RBD) of the Raf-1 protein spans amino acid residues 81-151 (48) and serves as the archetype of one of three classes of effector binding domains that interact with RAS proteins (138). Though numerous effectors bind avidly to the RAS proteins, Raf-1, through its RBD, binds with very high affinity to the RAS effector domain (K_d=20 nM) when RAS is in its GTP-bound conformation, whereas the affinity for RAS•GDP is three orders of magnitude lower (62). Bacterially expressed recombinant RBD adopts a stable, folded conformation similar to that thought to exist in the endogenous Raf-1 protein and, as such, is able to bind to RAS•GTP directly *in vitro* (39, 47). These properties have led to the widespread use of the Raf-1 RBD as an activation-specific probe for RAS (30). The RBD fragment is typically used in two ways: (1) transiently expressed in cells such that the RBD fragment, often tagged with a fluorescent reporter for microscopic visualization, is able to access and bind RAS•GTP in a manner similar to conditions experienced by an endogenous effector; and (2) tagged with epitopes that can be biochemically purified (commonly glutathione S transferase)

and incubated with a cell lysate in which the tagged RBD fragment in excess binds RAS•GTP in a complex which can then be purified and separated by SDS-PAGE for immunoblot. The latter application presumably requires the dissociation of RAS•GTP from previously complexed effectors in addition to simply engaging RAS•GTP unbound to effector molecules.

Our use of the RBD reagent in the latter application has led us to reassess the ability of exogenous RBD to gauge accurately RAS•GTP levels in cell culture conditions relative to earlier methods. We present evidence to challenge the use of the Raf-1-binding domain-GST fusion construct *in vitro* to provide an accurate readout of RAS activation status. Specifically, we show that the RBDGST fusion fragment is unable to detect RAS•GTP within endogenous RAS:Raf-1 complexes and may even misrepresent changes in GTP-bound levels of a given RAS population. These findings are highly relevant given the expanded use of this reagent over the past several years. Indeed, many studies relying solely upon the readout of this assay may warrant reexamination in light of the data we present here.

Materials and Methods

Cell Lines, Culture Conditions and Transfections

Cell lines are maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin streptomycin, and 1% L-glutamine. Recombinant TGFα was administered at 30ng/ml. HCA-7 and DLD-1 cell lines are derived from human colorectal adenocarcinomas.

HCA-7 cells are wild-type at all *RAS* loci, whereas DLD-1 have a mutant *KRAS* allele (G13D) but are wild-type at *NRAS* and *HRAS* loci (JWK, unpublished data; (156)).

RBDGST *In Vitro* Pulldown Assays

The GST-Raf-1-RBD reagent (RBDGST) was purchased as part of the EZ-Detect RAS Activation Kit (Pierce, Rockford, Illinois) and was immediately aliquotted and stored at -70°C per manufacturer's instructions. Cells were washed three times with ice-cold PBS and lysed with supplied Lysis/Binding/Wash Buffer. Lysates were centrifuged at 16,000Xg for 15 min at 4°C, and the pellets discarded. Concentrations were quantified by BCA assay. A single SwellGel® Immobilized Glutathione Disc was placed into a spin column with a collection tube and combined with an 80μg aliquot of the GST-Raf-1-RBD reagent (RBDGST). Immediately, a 500μg aliquot of cell lysate was added and the tube was sealed and incubated for 1 h with rocking at 4°C. The spin cup with collection tube was then centrifuged at 7,200Xg for 15 sec. Depleted lysate was reserved for subsequent analysis. The resin was washed three times with supplied Lysis/Binding/Wash Buffer per manufacturer's instructions and centrifuged at 7,200Xg for 15 sec. Wash step was repeated for a total of thee washes. The spin cup was transferred to a new collection tube and 2X Laemmli (5% β-mercaptoethanol) sample buffer was added and samples heated at 100°C for 5 min. Samples were centrifuged at 7,200Xg for 2 min and eluted samples were prepared for SDS-PAGE analysis. It should be noted that a comparable reagent is also available from Upstate Cell Signaling Solutions (Lake Placid, New York). Both reagents were tested and yielded similar results.

In Situ Hybridization

Riboprobe templates were designed against 3'UTR and/or the hypervariable domains of the various Ras family members and amplified from 1st strand cDNA, then cloned into pGEM-TEasy (Promega, Madison, Wisconsin). Single-stranded RNA probes were synthesized by *in vitro* transcription of linearized vectors utilizing digoxygenin-labelled UTP (Roche, Indianapolis, Indiana). Colons were removed, flushed with PBS, and intact colonic crypts were removed by the method of Whitehead et al. (176). Crypts were sequentially fixed in 4% paraformaldehyde, rocking at 4°C overnight, rinsed 3X30 min in PBS, then fixed in several changes of -20°C MeOH for 24 h. Crypt suspensions were allowed to warm to room temperature, then washed successively 2X5 min with 50%MeOH in PBST (0.1% Tween) followed by 30%MeOH in PBST for 5 min, and finally in PBST. Quenching endogenous phosphatases, prehybridization, hybridization, stringency washes, and development were as described (The Zebrafish Book, Westerfield).

Immunoblotting and Immunoprecipitation

For immunoprecipitation, lysis conditions as described were used (1% CHAPS) (181). Protein concentrations were determined by BCA assay (Pierce, Rockford, Illinois). One hundred micrograms whole cell lysate was precleared with Protein A agarose beads (Pierce) for 2 h rocking at 4°C. N-RAS was immunoprecipitated with 50μl monoclonal antibody (Santa Cruz sc-519) rocking overnight at 4°C. Fifty microliters of Protein A bead slurry were added and incubated for 2 h rocking at 4°C. Supernatant was

removed and beads were washed 3X15 min with lysis buffer containing 0.1% CHAPS. Bound proteins were eluted with 2X Laemmli sample buffer, heated at 90°C for 5 min, and resolved by SDS-PAGE. For RAS Western blots, all cells were washed 3X with ice-cold PBS and lysed in 25mM TRIS, pH 7.6, 1% NP-40, 2mM EDTA pH 8.0, 150mM NaCl, 5mM $MgCl_2$, 1mM DTT, proteinase inhibitors (Roche), and phosphatase inhibitor cocktails I and II (Sigma). Lysates were vortexed 5 sec, allowed to rest on ice 5 min, and centrifuged at 13,400 rpm at 4°C. Supernatants were then transferred to fresh tubes, and protein concentration determined by BCA assay. Samples were resolved on 12% SDS-PAGE, transferred to Immobilon, blocked for 1 h with 5% nonfat milk in TBST (0.1 %Tween 20), then incubated with primary antibody overnight. N-RAS (sc-31) and K-RAS (sc-30) antibodies were from Santa Cruz and were used at 1:200. Raf-1 monoclonal antibody was from BD Transduction Labs (#610151) and was used at 1:1000. Blots were detected with appropriate secondary antibody conjugated to HRP and detected with enhanced chemiluminescence (Amersham, Uppsala, Sweden).

GTPase Assays and Thin Layer Chromotography

GTPase assays were as described previously (36). Briefly, cells were plated in 6-well plates and allowed to grow to confluence. Phosphate-free medium was supplemented with dialyzed fetal calf serum (10% final). [^{32}P] orthophosphate was added to a final concentration of 1mCi/ml. Cells were labeled for 4 h (18). Lysis and immunoprecipitation conditions were as described. Following immunoprecipitation, bound nucleotide was eluted by heating samples in 50µl elution buffer as described (36). Nucleotides were resolved by thin layer chromatography on PEI cellulose plates pre-

equilibrated in ddH_2O and run in 1M LiCl. Relative GDP and GTP positions were determined by loading 2μl of GTP (20μM) and 2μl of GDP (20μM) in adjacent lanes and visualizing under short wavelength fluorescence. Resolved plates were exposed to a Phosphorimager (Molecular Dynamics) and quantitated by densitometry (NIH Imager).

Results

Incubation of exogenously added RBDGST with lysates cannot detect endogenous RAS:Raf-1 complexes

As noted previously, the interaction of RAS•GTP with its best-characterized effector molecule, Raf-1, is extremely strong. While little is known about the rates of dissociation of RAS:effector complexes or half-lives of these complexes intracellularly, we hypothesized that incubation of the RBD fragment with lysates containing native RAS:effector complexes may be resistant to detection using this approach. To explore this possibility, we treated HCA-7 colon adenocarcinoma cells under serum-free conditions with recombinant human TGFα (30ng/ml) to stimulate the EGF receptor (EGFR). Following treatment, we prepared and incubated lysates from these cells with an excess of the RBDGST reagent followed by isolation using immobilized glutathione, washed the bound fraction, and eluted as described in Materials and Methods. We then separated the isolated fraction by SDS-PAGE and immunoblotted with an N-RAS specific antibody. Though we were able to detect GTP-bound N-RAS by the RBDGST assay at baseline and a slight increase at the 1 min time point, we saw no evidence of N-RAS•GTP at later time points (Figure 14, panel A). As a control to ensure that the

reagent was capable of detecting N-RAS•GTP, we also overexpressed mutant, constitutively active N-RAS (G12V) in these cells and subjected them to treatment with TGFα. Consistent with predictions, the N-RAS^{G12V} overexpressors showed elevated levels of GTP-bound RAS at 1 min relative to untransfected cells. At the 15 min time point, we detected a strong band in the N-RAS^{G12V} overexpressors in contrast to the total absence seen in the untransfected cells. Total RAS levels were unchanged over the time course as indicated by immunoblot (Figure 14, panel A).

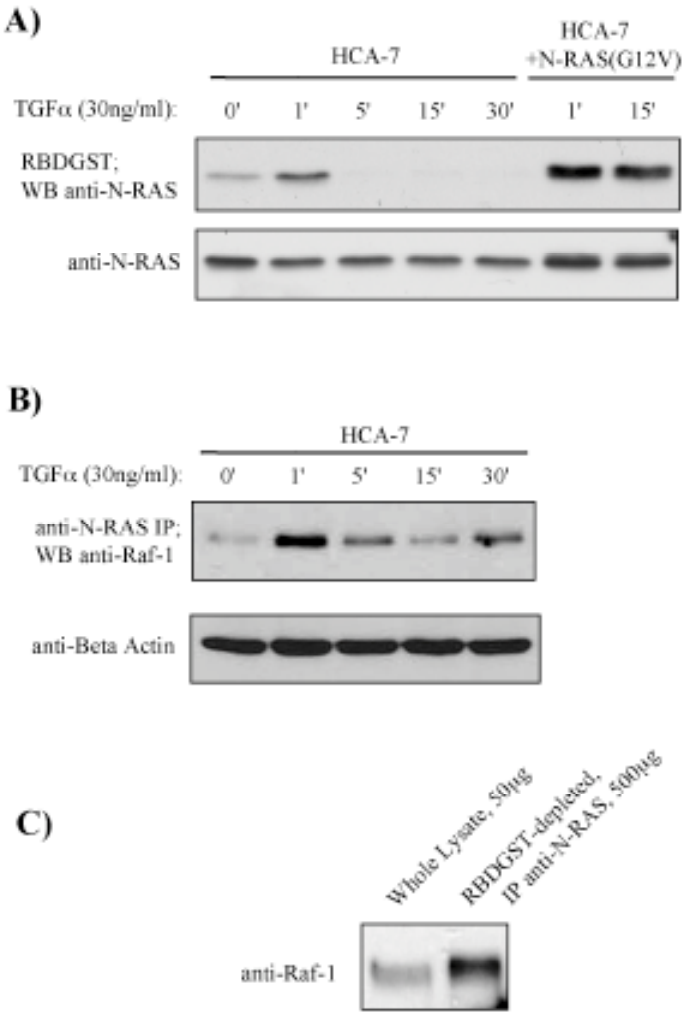


Figure 14. Comparison of RBDGST pulldown with immunoprecipitation of endogenous Ras:Raf complex. A) HCA-7 cells were treated with a 30 min time course of TGFα (30ng/ml). At time points indicated, cells were harvested and lysates prepared as described in Materials and Methods. Lysates were incubated with RBDGST reagent, resolved by SDS-PAGE, and immunoblotted with N-RAS specific antibody. HCA-7 cells expressing constitutively GTP-bound N-RAS (G12V) were subjected to same treatment and RBDGST pulldown repeated. B) Immunoprecipitation of N-RAS and immunoblotting for Raf-1 reveal increased association of N-RAS and Raf-1 over TGFα time course. C) A 500μg aliquot of whole cell lysate was incubated with an excess of RBDGST fusion protein. 'Depleted' supernatant was then immunoprecipitated for N-RAS. N-RAS immunocomplexes were then disrupted in Laemmli sample buffer, separated by SDS-PAGE and blotted for Raf-1. Results are representative of three independent experiments.

Because the RBD reagent appears capable of detecting RAS•GTP populations as indicated by the overexpression of N-RAS^{G12V}, yet shows only modest, short-lived increases in N-RAS•GTP following stimulation of the EGFR (an event known to promote RAS activation), we hypothesized that RAS•GTP molecules may be complexed with high affinity effectors such as Raf-1, effectively blocking their detection by incubation with an excess of the RBDGST reagent. To test this, we exploited the ability of N-RAS-specific antisera to immunoprecipitate endogenous N-RAS:Raf-1 complexes under comparable treatment conditions (55). We treated quiescent HCA-7 cells (serum-free) as above, and immunoprecipitated for N-RAS. We then immunoblotted for Raf-1. Consistent with the established understanding of EGFR-mediated RAS activation, we detect low levels of N-RAS:Raf-1 at baseline followed by an increase in Raf-1 associated with N-RAS immediately following treatment that subsequently decreased but was maintained at suprabasal levels thoughout the treatment time course (Figure 14, panel B). It is important to note that an N-RAS:Raf-1 complex was detected at 5 min without any significant N-RAS binding to the RBDGST. These conflicting results suggest that exogenous RBD in excess cannot detect RAS•GTP sequestered within endogenous RAS:Raf-1 complexes.

We interpret the increased amount of N-RAS•GTP detected at the 1 min time point by the RBDGST reagent as evidence of a sudden increase in N-RAS•GTP levels immediately following EGFR stimulation. N-RAS molecules newly loaded with GTP as a result of this stimulation have yet to enter into complex with effector molecules and therefore remain available for interaction with the RBDGST. Indeed, the interaction

between RAS•GTP and Raf-1 does not reach equilibrium for 2 to 5 min, suggesting the possible presence of free (detectable) RAS•GTP at the early time point (172).

To test the resistance of RAS:Raf-1 complexes to RBDGST detection, we lysed cells and incubated with an empirically determined excess of the RBDGST reagent as before. Following a 1 h incubation, we recovered the depleted lysates, and immunoprecipitated for N-RAS. We then separated this fraction by SDS-PAGE and immunoblotted for Raf-1. Confirming the existence of RAS:Raf-1 complexes resistant to the RBDGST reagent, we detected Raf-1 in complex with N-RAS in these 'depleted' lysates (Figure 14, panel C).

The RBDGST reagent yields data inconsistent with the conventional GTPase assay

Given the results described above, we sought to compare the results of the RBDGST assay to earlier methods of measuring changes in the guanine nucleotide populations bound to RAS. We have previously observed an apparent change in N-RAS activity in cell lines characterized by the presence of a constitutively activating *KRAS* mutation (DLD-1; $KRAS^{G13D/+}$) relative to isogenically derived lines in which the mutant *KRAS* allele has been disrupted by homologous recombination (DKS-8; $KRAS^{+/-}$). Use of the RBDGST reagent detected elevated levels of wild-type N-RAS•GTP in the ($KRAS^{+/-}$) environment relative to the parent line ($KRAS^{G13D/+}$) (Figure 15, panel A). In light of the findings described above, we hypothesized that the less intense N-RAS band seen in the DLD-1 cell line may actually represent an increase in N-RAS•GTP, which because of elevated levels of effector interaction, are undetectable by use of the RBD. To investigate this possibility, we conducted a classical GTPase assay on the DLD-1 and

derivative DKS-8 cell lines to assess N-RAS activity. We incubated cells in the presence of [^{32}P] orthophosphate for 4 h in dialyzed 10% fetal calf serum and lysed the cells as described in Materials and Methods. We then specifically immunoprecipitated N-RAS from these lysates, liberated the bound nucleotides, and resolved the respective populations of GDP and GTP by thin-layer chromatography (Figure 15, panel C). Densitometry of these plates revealed an increase in the levels of GTP-bound N-RAS relative to GDP-bound in the presence of the mutant K-RAS, in contrast with the results from the RBDGST assay (Figure 15, panel D). Unfortunately, there is minimal association of N-RAS and Raf-1 in these cells, thus frustrating our attempts to measure increased N-RAS:Raf-1 interaction in these lysates. Consistent with our previous findings, we do, however, observe a number of proteins visible by Coomassie stain that can be immunoprecipitated by the N-RAS antisera in the DLD-1, but not the DKS-8, lysates (Figure 18). This finding contrasts with the result achieved with the RBDGST reagent and, combined with our previous findings, suggests that the RBD *in vitro* pulldown assay misrepresents changes in the activity levels of cycling, wild-type RAS populations.

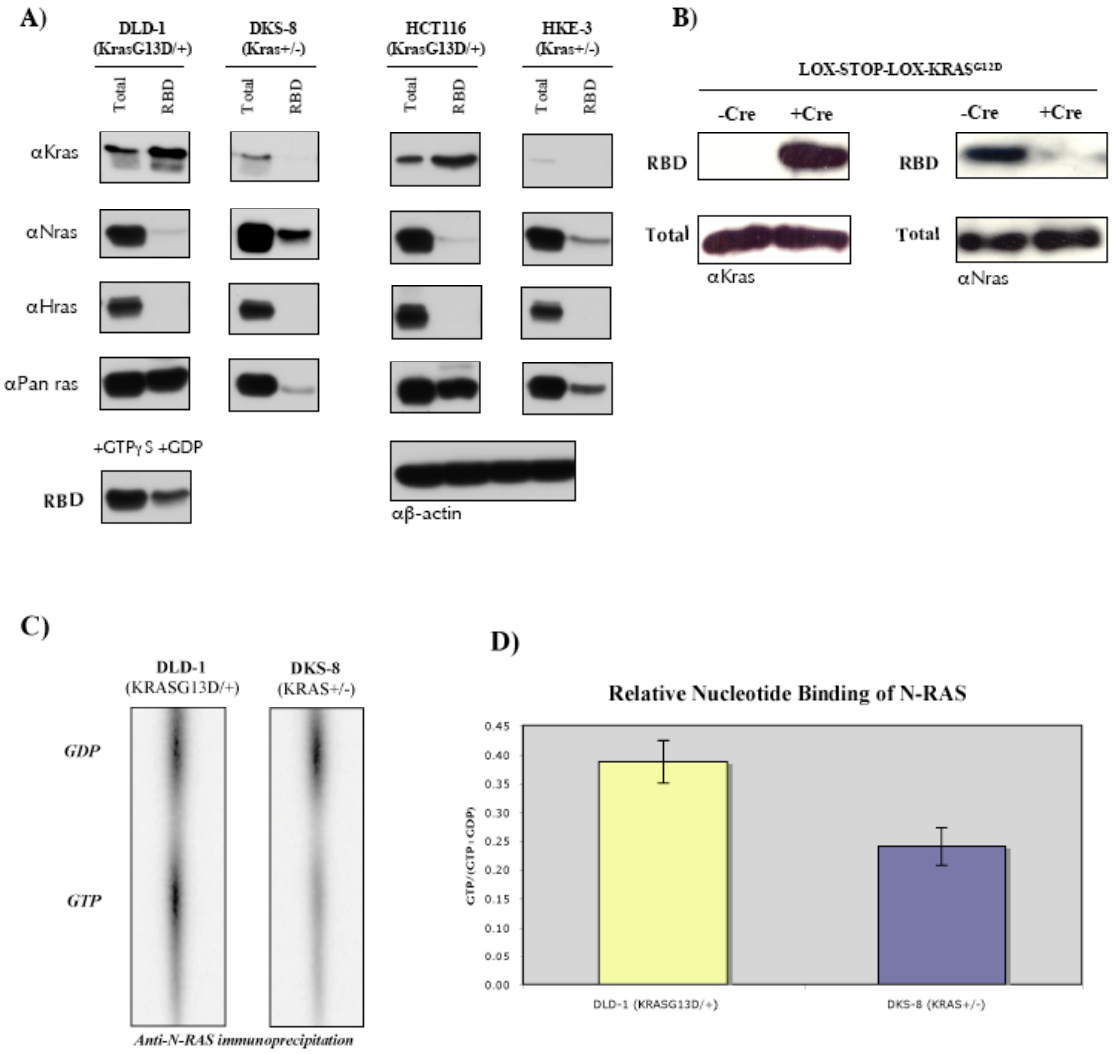


Figure 15. N-RAS activation status of HCT116 and DLD-1 cell lines relative to their isogenic derivatives. A) RBD complexes were eluted and resolved by SDS-PAGE and analyzed by Western blot for N-RAS or K-RAS. All RBDGST pulldowns performed on 500μg total lysate; 50μg aliquots were used for whole lysate samples. Loading controls were performed by immunoblotting for β–actin; RBDGST controls using GDP and GTPγS were performed according to manufacturer's instructions. B) Results of RBDGST pulldowns for colons harvested from *Kras*$^{LSL\text{-}G12D/+}$ animals and *Kras*$^{LSL\text{-}G12D/+}$; FABP-Cre animals were minced and lysates prepared as described in Materials and Methods (same as cell culture). C) GTPase assay of endogenous N-RAS in differing K-RAS environments. DLD-1 and DKS-8 cells were grown to similar density in DMEM supplemented with dialyzed fetal calf serum (10%) and ^{32}P orthophosphate (1mCi/ml) for 4 h to label intracellular guanine nucleotide pools as described in Materials and Methods. Endogenous N-RAS was specifically immunoprecipitated from DLD-1 and DKS-8 cell lysates as described previously in Experimental Procedures. Bound, labeled nucleotide was eluted from the immunoprecipitated protein and resolved by thin layer chromotography on PEI cellulose plates in 1M LiCl. Relative GTP and GDP positions determined by visualization with short wavelength fluorescence. Resolved, dried plates were exposed to Phosphorimager and quantitated. Blot is representative of three independent experiments. D) GTP eluted as a percentage of total nucleotide eluted from immunoprecipitated N-RAS. Results represent the average of three independent experiments.

Discussion

We report here that use of commercially available Raf-1-binding domain-GST (RBDGST) fusion protein fails to measure accurately total GTP-bound populations of RAS proteins in *in vitro* pulldowns. Use of the RBDGST fusion protein following stimulation of the EGFR with exogenously added TGFα in HCA-7 cells failed to detect the expected increase in GTP-bound RAS populations, although RAS association with Raf-1, a known effector that binds RAS in a GTP-dependent manner, increased during this treatment. This suggests that the RBD is unable to detect RAS•GTP complexed with endogenous effectors. The reagent is able to detect constitutively active mutant RAS during this time course, however, likely due to the abundance of uncomplexed RAS•GTP in this environment. Consistent with this finding, lysate presumably depleted of detectable RAS•GTP with an empirically determined excess of RBDGST still contains RAS in complex with Raf-1, and therefore in the GTP-bound state (62). This observations mirrors the detection of a steady-state N-ras:Raf-1 complex in quiescent murine fibroblasts (55). In this instance, the N-ras:Raf-1 complex was destabilized to utilize the RBDGST assay for the assessment of the guanine nucleotide bound to N-Ras. These findings suggest that the RBD reagent, while useful in binding free RAS•GTP molecules, such as occurs in the presence of a *RAS* mutation, is unable to detect RAS complexed with endogenous high affinity effectors. Thus, RBD may not provide an accurate indication of levels of GTP-bound RAS within a lysate. Moreover, parallel use of the RBD reagent and classical GTPase assays reveals contrasting results. Taken together, these data provide a cautionary note about the capabilities of the RBD reagent as a measure of GTP-bound RAS populations in *in vitro* pulldowns.

If the RBDGST assay does not accurately represent the true level of RAS•GTP in the cell because some portion of that population remains in complex with effectors, then what is being detected by this assay? There are two possibilities. First, the RBDGST assay might detect a population of RAS•GTP not bound to any effector, though given the number of potential binding partners and the relatively low level of Ras within cells, such a population is likely minimal in size and ephemeral (as is indicated by the slight increase we see immediately after TGFα treatment). A second possibility is that RBDGST effectively competes with RAS effectors whose interactions are characterized by lower binding affinities and more rapid turnover than that seen for the RAS:Raf-1 interaction. RAS molecules bind a broad range of effectors whose RAS binding domains can be grouped into thee categories (138). Though all utilize the effector binding domain of the RAS protein (amino acids 32-40), their own binding domains display little conservation at the level of primary sequence. Even within classes of RAS binding domains, conservation is more topological than sequence-based, and therefore likely translates into RAS-binding kinetics unique to individual effectors. It is therefore likely that some RAS:effector complexes have sufficient off-rates to generate a pool of free RAS•GTP that would be susceptible to detection using the RBD reagent. RAS:Raf-1, however, does not (Figure 16). Determining what proportion of the total RAS•GTP either of these two subpopulations represents would require extensive and detailed understanding of the kinetics of Ras activation and Ras:effector interactions that we presently do not possess.

In addition to its use in the pulldown assay, the RBD fragment, typically tagged with a fluorescent reporter, can also be transfected and expressed *in vivo* as a means of examining RAS•GTP levels and localizations within the cell (22). Our findings do not

challenge use of the RBD in this capacity because potential interacting partners are expressed *in vivo* and the RBD fragment is able to participate in the *de novo* generation of complexes with RAS•GTP. When RBD is added in excess *in vitro*, however, its interaction depends on the dissociation rates of native effector molecules bound to RAS•GTP. The latter condition would favor detection of RAS•GTP unbound to effectors at the time of lysis as well as those bound to effectors whose interaction with the RAS effector binding loop is characterized by rapid turnover. Expression of this fragment as a means of detecting intracellular interactions is supported by the fact that overexpression of the RBD fragment in cells transformed by v-H-RAS suppresses transformation, suggestive of the RBD domain's ability to bind RAS•GTP populations intracellularly (48).

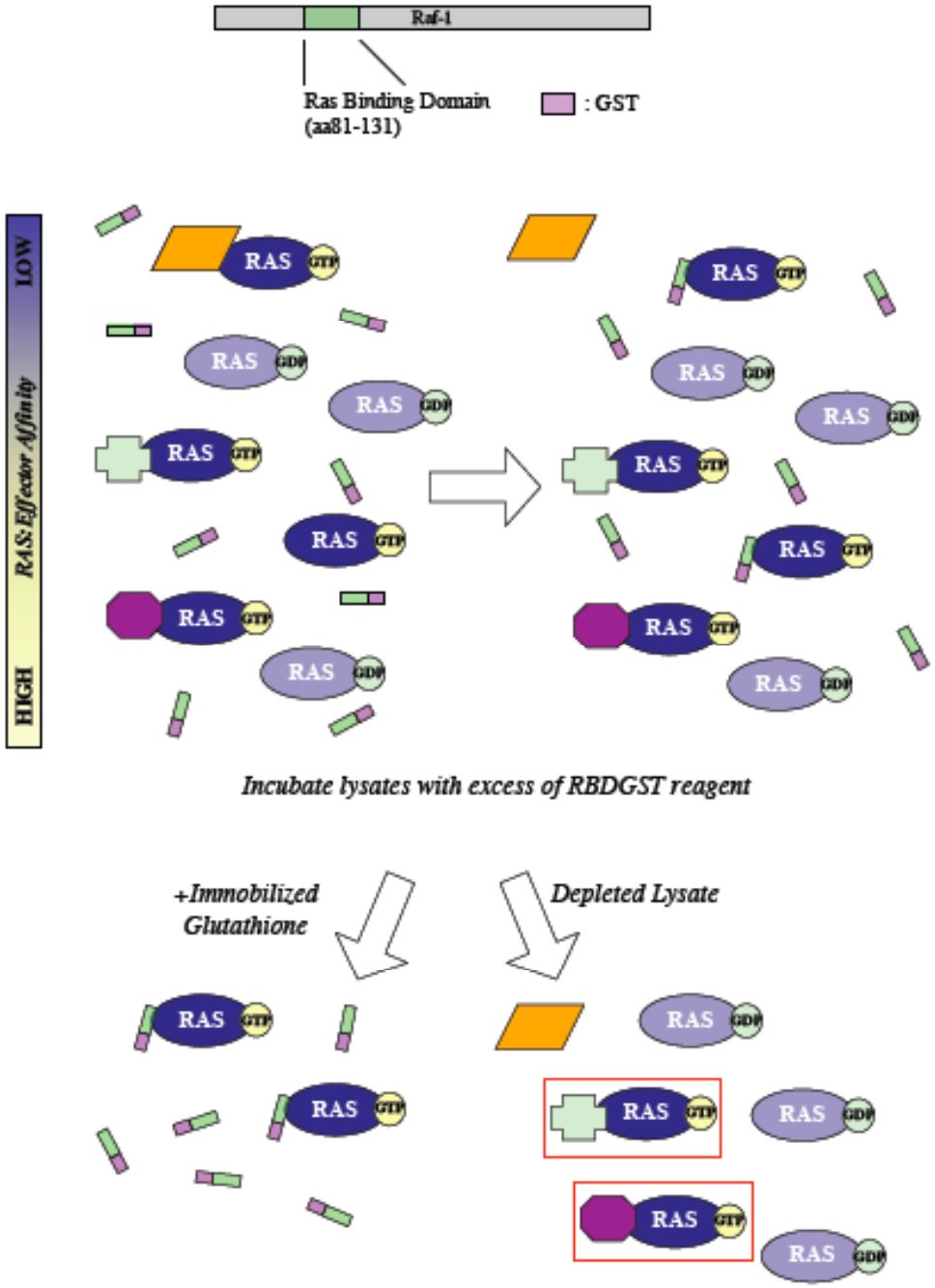


Figure 16. RBDGST pulldown assay: a more accurate picture. The RBDGST reagent is unable to fully detect the RAS•GTP population in a lysate. This is likely due to the fact that RAS:effector interactions can be extremely avid, such as that demonstrated by the interaction between RAS and Raf-1 (K_d=~20nM). In the case of interactions between RAS and effectors that are very high affinity, it is unlikely that incubation of RBDGST with the lysates results in significant turnover of these complexes and thus, RAS•GTP remains unavailable for detection the excess of RBDGST. RAS:effector interactions likely span a range of affinities and kinetics, and as such, some effectors likely demonstrate off-rates that make RAS•GTP available for detection. The kinetics of Ras:effector interactions have been described for only the RAS:Raf-1 interaction, however.

In summary, we present data here to question the accuracy of the RBD reagent for assessing RAS activation states in *in vitro* pulldown assays. We demonstrate inconsistencies between this assay and those previously established for measuring the activation states of RAS populations. While we find this reagent useful in detecting the presence of constitutively active RAS mutants, it appears unsuitable for examining changes in activation states of wild-type RAS populations in response to biological stimuli. The questions we raise here concerning the use of the RBD fragment for measuring levels of active RAS may also be applicable to the broad range of analogous reagents that have been developed for measuring the activation states of other small G proteins. These include commercially available kits for Ral, Rho, Rac, and Rap, all of which share the use of a probe derived from interacting proteins. While we have not tested these reagents, our concerns extend to their use as well.

CHAPTER IV

ONCOGENIC K-RAS SUBVERTS THE ANTI-APOPTOTIC ROLE OF N-RAS AND GELSOLIN AND ALTERS THE N-RAS:GELSOLIN INTERACTION

Introduction

The small G proteins of the RAS superfamily act as molecular switches in the transduction of a multitude of molecular signals critical to a wide range of homeostatic, developmental, and disease processes. Genetic mutations of RAS proteins that render these molecules incapable of GTP hydrolysis and thus lock them into a constitutively active conformation occur in approximately 30% of all human tumors (12). Although enormous effort has been invested in dissecting the consequences of constitutive RAS activation in general, the functional interaction of different endogenous RAS isoforms with each other has not been closely examined.

Canonical members of the RAS family (N-RAS, H-RAS, and K-RAS4B) are nearly ubiquitously expressed and exhibit both overlapping and distinct functions. Although they share extensive conservation at the level of primary sequence for approximately 90% of their respective lengths, including the effector binding loops and regulatory regions, their access to potential interacting partners is governed to large extent by their localizations within the cell (22). Isoform-specific posttranslational modifications to the divergent C-terminal hypervariable domains of these proteins provides a likely cell biological basis for isoform-specific function (57-60, 177). Recent

studies demonstrate the functional effects of these differences in processing and localization (105, 127, 129, 141, 142).

An excellent example of the functional specificity of a Ras isoform, endogenous N-ras is unique among Ras proteins in its ability to promote survival in murine fibroblasts (180, 181). Immortalized murine fibroblasts derived from *Nras*$^{-/-}$ mice exhibit loss of survival signaling through activation of the PI(3)K/Akt/Bad pathway and other mechanisms. Attempts to reconstitute the survival signal by expression of N-ras effector mutants in this system suggest that the interaction of N-ras with RalGDS is essential to this survival signal (174, 180). Consistent with an isoform-specific role for N-ras in this interaction, Ras mutants targeted to distinct intracellular microdomains demonstrate that signaling through RalGDS originates in Ras populations localized to the Golgi complex (105), a signaling platform populated exclusively by N-ras in these murine fibroblasts, since they fail to express detectable levels of H-ras (181), and K-ras does not localize to this endomembrane (1, 22, 23).

In contrast, constitutive K-RAS activity following oncogenic mutation appears to sensitize human colon cancer-derived cells to apoptosis (76, 77). These studies exploited the HCT 116 cell line and isogenic derivatives in which the mutant *KRAS* allele is disrupted by homologous recombination (156). Constitutive K-RAS activity sensitizes these cells to apoptotic stimulus through transcriptional downregulation of gelsolin, which promotes survival in other systems (82-84, 113).

Materials and Methods

Cell Lines, Culture Conditions and Transfections

HCT 116 and DLD-1 lines are derived from colorectal carcinomas and possess an activating mutation (G13D) in one *KRAS* allele. *HRAS* and *NRAS* alleles are wild-type. Isogenic derivatives have been previously described (156). Cell lines are maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% penicillin streptomycin, and 1% L-glutamine. siRNA sequences were generated with the use of pSICOLIGOMAKER software (http://web.mit.edu/ccr/labs/jacks/protocols/pSico.html) which utilizes criteria previously published for the identification of likely target sequences (134). shNRAS(3) target sequence: GGAGAAATGTCATAAATTA, and this sequence was BLASTed to confirm target specificity. For lentiviral transduction of siRNA constructs, 293T cells were plated in a 100mm dish and allowed to grow to 50-70% confluence. Cells were transfected overnight with the lentiviral vector pSICOR (5μg) as well as the Δ8.2 packaging vector (2.5μg) and the pMD26 packaging vector (2.5μg) using Fugene 6 according to the manufacturer's instructions. Media was changed and replaced with minimal volume. After 24 h, media was harvested and filtered through a 0.22μm syringe filter. Filtered viral supernatant was transferred to a plate of target cells and infection continued for 24 h. Selection was begun the following day (puromycin 7.5μg/ml) and refreshed daily for 4-6 days until a nontransfected control plate was killed completely. Cells were maintained in DMEM and selection periodically repeated. Specificity of knockdown was verified by Western blot.

Kras^{G12D} Mice

The LSL-K-rasG12D strain was interbred to FABP-Cre mice to evaluate the effects of K-rasG12D expression during development (165).

Apoptosis Induction and Lysate Harvest

Apoptosis was induced after growing cells to 70-80% confluence and initiating treatment with 1mM or 3mM sodium butyrate (Calbiochem, La Jolla, California) or 350nM staurosporine (Sigma, St. Louis, Missouri) for the time points specified. To harvest, media was collected and centrifuged to collect any non-adherent cells. These pellets were washed with ice-cold PBS and combined with lysates of adherent cells. Lysates to be used for immunprecipitation were prepared as described below (1% CHAPS), while those to be used for immunoblotting cleaved PARP were lysed with 62.5mM TRIS pH 6.8; 6M Urea; 10% glycerol (vol/vol); bromphenol blue (trace). These lysates were sonicated briefly (8 pulses, low power) to disrupt nuclei and DNA/protein interactions. Following sonication, lysates were supplemented with SDS (2% final vol/vol) and β-mercaptoethanol (5% final vol/vol) and heated at 65°C for 15 min.

Immunoblotting and Immunoprecipitation

For immunuprecipitation, lysis conditions as described previously (Hamilton 2001) were used (1% CHAPS). Protein concentrations were determined by BCA assay (Pierce, Rockford, Illinois). One hundred micrograms whole cell lysate was precleared in with Protein G agarose beads (Pierce) for 2 h rocking at 4°C. N-RAS was

immunoprecipitated with 50µl monoclonal antibody (Santa Cruz sc-31) rocking overnight at 4°C. Fifty microliters of Protein G bead slurry were added and incubated for 2 h rocking at 4°C. Supernatant was removed and beads were washed 3X15 min with lysis buffer containing 0.1% CHAPS. Bound proteins were eluted with 2X Laemmli sample buffer, heated at 90°C for 5 min, and resolved by SDS-PAGE. For Ras Western blots, all cells were washed 3X with ice-cold PBS and lysed in 25mM TRIS, pH 7.6, 1% NP-40, 2mM EDTA pH 8.0, 150mM NaCl, 5mM $MgCl_2$, 1mM DTT, proteinase inhibitors (Roche), and phosphatase inhibitor cocktails I and II (Sigma). Lysates were vortexed 5 sec, allowed to rest on ice 5 min, and centrifuged at 13,400 rpm at 4°C in a benchtop centrifuge. Supernatants were then transferred to fresh tubes, and protein concentration determined by BCA assay. Samples were resolved on 12% SDS-PAGE, transferred to Immobilon, blocked for 1 h with 5% nonfat milk in TBST(0.1%T), then incubated with primary antibody overnight. RAS specific antibodies were from Santa Cruz: H-RAS (sc-520); N-RAS (sc-31); and K-RAS (sc-30) were all used at 1:200; pan-RAS was from Sigma (RAS clone 10) used at 1:1000. Isoform specificity of RAS antibodies was verified by detection of stably expressed GFP-tagged isoforms in 293T cells. Monoclonal gelsolin antibody (Sigma) was used at 1:3000 for immunoblotting. Blots were detected with appropriate secondary antibodies conjugated to HRP and detected with enhanced chemiluminescence (Amersham, Uppsala, Sweden).

As an additional technical note, we have observed an interesting phenomenon in the use of two commercially available N-ras antibodies, both of which specifically recognize this isoform. These antibodies, sc-31 (mouse monoclonal) and sc-519 (rabbit polyclonal), both of which specifically recognize N-ras by immunoblot, appear to

recognize two separate populations of intracellular N-ras in human cells by immunoprecipitation, and possibly also by immunofluorescence. This result is intriguing given that both antibodies were raised against the C-terminal 20 amino acids of this protein. This discrepancy is evident in the ability of sc-31, but not sc-519, to immunoprecipitate immunocomplexes containing gelsolin. Conversely, sc-519, but not sc-31, is capable of immunoprecipitating complexes containing Raf-1.

GTPase Assays and Thin Layer Chromotography

GTPase assays were as described previously (36). Briefly, cells were plated in six well plates and allowed to grow to confluence. Phosphate-free media was supplemented with dialyzed fetal calf serum (10% final). [^{32}P] orthophosphate was added to a final concentration of 1mCi/ml. Cells were labeled for 4 h. Lysis and immunoprecipitation conditions were as described (36). Following immunoprecipitation, bound nucleotide was eluted by heating samples in 50ul elution buffer as described (18). Nucleotides were resolved by thin layer chromatography on PEI cellulose plates equilibrated in ddH_2O and run in 1M LiCl. Relative GDP and GTP positions were determined by loading 2μl of GTP (20μM) and 2μl of GDP (20μM) in adjacent lanes and visualizing under short wavelength fluorescence. Resolved plates were exposed to a phosphorimager and quantitated by densitometry (NIH Imager).

Soft Agar Assays

2X media was prepared as follows: 13.37g DMEM powder from Invitrogen (12100-061) (Carlsbad, California) was resuspended in 450ml water. Solution was

buffered with 3.7g $NaHCO_3$ and pH adjusted to 7.4. Media was supplemented with 2% non-essential amino acids, 2% L-glutamine, and 2% penicillin/streptomycin, and fetal bovine serum (20% final). A 1.6% solution of Seaplaque Agarose (Cambrex, Maine) was melted in a 55°C water bath and mixed 1:1 with 2X media from above (0.8% final agarose). This mixture was poured into 35mm plates (1.5ml/plate) and allowed to set up. Meanwhile, cells were trypsinized and counted, and 4X the desired number/plate were resuspended in 4ml total volume of top layer solution (1ml 2X media+2ml 1X media+1ml agarose; 0.4% final agarose) and equilibrated to 37°C. One ml of this mixture is added as the top layer and allowed to solidify at room temperature for one hour prior to being returned to a 37°C incubator. After 7-10 days, plates were chilled at 4°C and stained with 0.2% crystal violet (in ddH_2O) for 30 min at room temperature and were rinsed with successive changes of water daily until clear.

Nude Mouse Xenograft

Cells to be injected were grown to subconfluence on a 100mm dish, rinsed with PBS, and trypsinized. Cells were pelleted by centrifugation (1000Xg; 5 min), rinsed with PBS, and re-centrifuged. Cells were resuspended by gentle vortexing and counted. Desired numbers of cells were then diluted to standard volumes and injected subcutaneously into the flanks of nude mice (Jackson Laboratories, Bar Harbor, Maine) (300μl/injection). Cells were injected in a series of dilutions ($5X10^6$; $5X10^5$; $5X10^4$; $5X10^3$). Four injection sites were chosen per mouse, two on each flank at the shoulder/hip bilaterally. Mice were returned to colony and monitored for three weeks or until tumor burden exceeded the allowable mass by animal care standards.

Results

Ras proteins are expressed widely in the colonic epithelium

Immunoblot for RAS isoforms were prepared from a series of commonly used human colorectal cancer cell lines. Without exception, these cell lines show expression of all three isoforms, although levels of H-RAS and K-RAS vary and do not necessarily correlate with mutational status or oncogenic contribution. HCT 116 and DLD-1, for example, owe much of their tumorigenic phenotype to the presence of constitutively activated K-RAS (156), although the total levels of K-RAS protein are relatively low compared with other cell lines (Figure 17). Levels of N-RAS expression are fairly constant. Broad overlapping expression of RAS isoforms in this tissue allows for examination of changing activity levels of wild-type RAS isoforms in the presence of activating mutations of a single *RAS* allele.

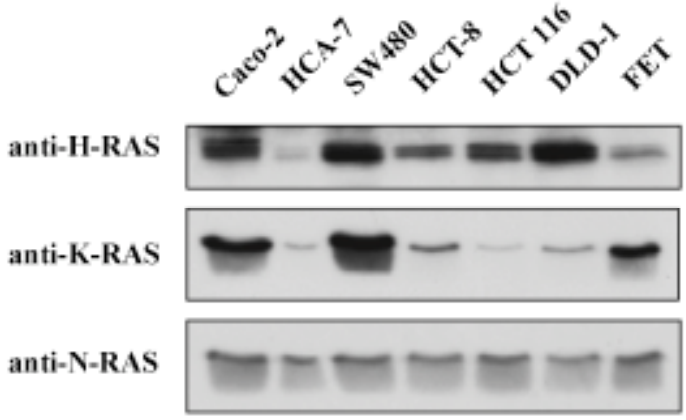


Figure 17. Protein expression of major RAS family members in representative human colorectal cancer cell lines. Lysates were prepared from a series of commonly used colorectal cancer cell lines and immunoblotted for Ras family members as described in Materials and Methods.

Constitutive K-RAS activity correlates with an increase in the levels of N-RAS•GTP

The common factors that influence the activity and signaling outputs of all Ras molecules (complete homology in effector loops and regulatory regions, ability to bind common effectors and regulatory molecules) led us to hypothesize that genetic mutation of one isoform may affect the activity of wild-type RAS populations and possibly alter their signaling outputs.

We sought to determine if oncogenic mutation of one isoform as commonly occurs in human colorectal cancer (169) altered the GTP-bound levels of other wild-type RAS isoforms. Pairwise study of the DLD-1 cell line ($KRAS^{G13D/+}$) and its isogenic derivative, DKS-8 ($KRAS^{+/-}$) allowed us to investigate this possibility in a highly controlled manner. DLD-1 and DKS-8 cell lines were cultured in the presence of [^{32}P] orthophosphate as described in Materials and Methods. We lysed these cells and selectively immunoprecipitated for N-RAS, eluted the bound, labeled nucleotide, and resolved labeled GTP and GDP by thin layer chromotography as described previously (36). The levels of N-RAS•GTP in the presence of an oncogenic *KRAS* allele were significantly higher than those observed in the absence of the mutant allele (Figure 18). Total levels of N-RAS in these cell populations are unchanged by immunoblot (Figure 20, middle panel). (Despite our reservations about this assay described in the previous chapter, this assay retains utility here insofar as *the detected change is consistent* across cell lines. Because of our reservations, we cannot conclude that this change correlates with an increase in absolute levels of N-RAS•GTP; to the contrary, we demonstrate in Figure 12 the true change in N-RAS•GTP by GTPase assay which contrass with the result shown by the RBDGST reagent.)

We repeated the GTPase assay with DKS-8 cells grown in DLD-1-conditioned media to investigate the possibility of differential growth factor secretion as an explanation for elevated levels of N-RAS•GTP and observed results comparable to those described above (data not shown). Although levels of H-RAS•GTP may also be altered in a constitutively active K-RAS environment, we were unable to explore this possibility owing to the lack of antisera capable of specific immunoprecipitation of endogenous H-RAS protein.

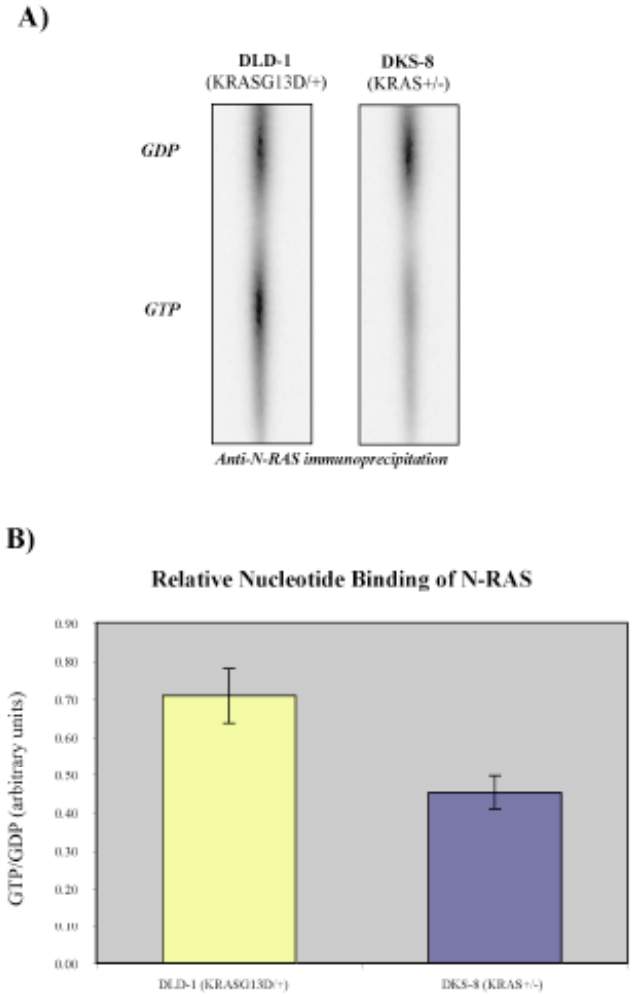


Figure 18. The presence of constitutively active endogenous K-RAS correlates with elevated levels of GTP-bound N-RAS. A) GTPase assay of endogenous N-RAS in differing K-RAS environments. DLD-1 and DKS-8 cells were grown to similar density in DMEM supplemented with dialyzed fetal calf serum (10%) and [^{32}P] orthophosphate (1mCi/ml) for 4 h to label intracellular guanine nucleotide pools as described in Materials and Methods. Endogenous N-RAS was specifically immunoprecipitated from DLD-1 and DKS-8 cell lysates as described previously in Materials and Methods. Bound, labeled nucleotide was eluted from the immunoprecipitated protein and resolved by thin layer chromotography on PEI cellulose plates in 1M LiCl. Relative GTP and GDP positions determined by visualization with short wavelength fluorescence. Resolved, dried plates were exposed to Phosphorimager and quantitated. Blot is representative of three independent experiments. B) Ratio of GTP to GDP eluted from immunoprecipitated N-RAS. Total levels of N-RAS are unchanged (Figure 20). Results represent the average of three independent experiments (+/-SEM).

Increased levels of N-RAS•GTP in mutant versus wild-type K-RAS environments translates into differential interaction with binding partners

We next hypothesized that increased levels of N-RAS•GTP may translate into increased interaction with effectors and participation in signaling complexes. To test this, we prepared lysates from both DLD-1 and DKS-8 cell lines and from the harvested colons of *K-Ras*$^{LSLG12D/+}$ and *K-Ras*$^{LSLG12D/+}$; FABP-Cre mice. We then conducted immunoprecipitations on these lysates as described in Materials and Methods, separated the bound fractions by SDS-PAGE and Coomassie stained these gels. Bands that preferentially showed up in one condition versus another were excised and submitted for identification by mass spectrometry. This assay, which consistently showed the presence of bands immunoprecipitable with N-RAS specific antisera in the presence of a mutant, but not wild-type K-RAS, was somewhat inconsistent in the specific bands that were visible from experiment to experiment (Figure 19). Individual bands were often difficult to reproduce from one experiment to another, despite standardized conditions including cell density, culture conditions, and immunoprecipitation protocol. The reason for this inconsistency were unclear, but is most likely attributable to the antibody and the suboptimal quality of individual RAS isoforms as epitopes due to their small size and homology to family members. N-RAS is a small protein and is known to participate in larger signaling complexes which may obscure the epitope from consistent detection. Further, all Ras-specifc antibodies are raised against the divergent C-termini of the proteins, regions that are posttranslationally modified with moieties that exhibit hydrophobic characteristics. These domains of the proteins interact directly with adaptor molecules, which may well occlude the epitope. In addition, the proteins we detect in

these assays may not bind Ras directly, but rather may participate in a larger complex that disassociates over the course of the immunoprecipitation. This possibility is supported by the fact that of the bands we were able to successfully identify by mass spectrometry, none have been reported as direct Ras binding partners. Despite the difficulty in reproducing specific bands, we consistently see a spectrum of bands immunoprecipitable with N-RAS in the mutant, but not wild-type, K-RAS environment, suggestive of elevated N-RAS activity in this situation. These analyses were conducted in both the DLD-1 and DKS-8 paired isogenic cell lines as well as in the *Kras*$^{LSL\text{-}G12D/+}$ and *Kras*$^{LSL\text{-}G12D/+}$; FABP-Cre colons. These systems 'genocopy' each other at the *Kras* locus and afford an additional means of analysis. Using mass spectrometry, we were able to identify some of the bands that more reproducibly appeared in this experiment.

The inconsistency observed in these immunoprecipitations is, of course, a concern. Rather than reflecting a real biological inconsistency in the system, we believe this reflects the poor quality of the reagents available for the study of endogenous Ras proteins. This is unfortunate given the constellation of proteins that appear to associate with N-Ras in these different K-Ras environments (Figure 19). The two proteins we identify by mass spectrometry in these experiments, gelsolin and the calcium chloride channel (CLCA3) both represent novel Ras interacting partners and may explain further the function of Ras in the colonic epithelium.

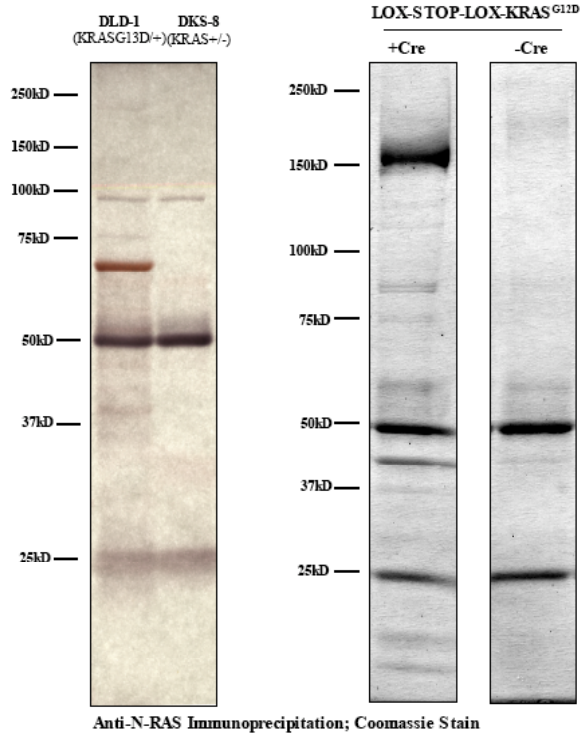


Figure 19. Immunoprecipitation of *Kras*$^{+/-}$ and *Kras*$^{*/+}$ lysates with N-ras specific antisera. N-ras was immunopreciputated from DLD-1 (*KRAS*$^{G13D/+}$) and DKS-8 (*KRAS*$^{+/-}$) lysates (left), as well as colon lysates from *Kras*$^{LSL\text{-}G12D/+}$ either crossed to FABP-Cre or not (right), resolved by SDS-PAGE, and stained by silver stain (left) or Coomassie stain (right).

N-RAS and gelsolin form a protein complex, the abundance of which correlates with elevated N-RAS•GTP levels

We next hypothesized that increased levels of N-RAS•GTP in constitutively active (*KRAS*$^{G13D/+}$) relative to wild-type (*KRAS*$^{+/-}$) K-RAS environments would translate into differential interaction with effectors. We have previously observed the association of endogenous N-RAS with gelsolin in complexes immunoprecipitated with N-RAS-specific antibodies (Figure 19). To explore the possibility of differential interactions resulting from the altered levels of N-RAS•GTP in different K-RAS environments, we lysed cells using detergent conditions favorable to preservation of protein complexes (see Materials and Methods), immunoprecipitated for N-RAS from the paired isogenic lines (DLD-1 and DKS-8; HCT 116 and HKE-3), and immunoblotted for gelsolin. Consistent with our predictions, constitutive K-RAS signaling supports not only higher levels of N-RAS•GTP, but also higher levels of N-RAS complexed with gelsolin (Figure 20).

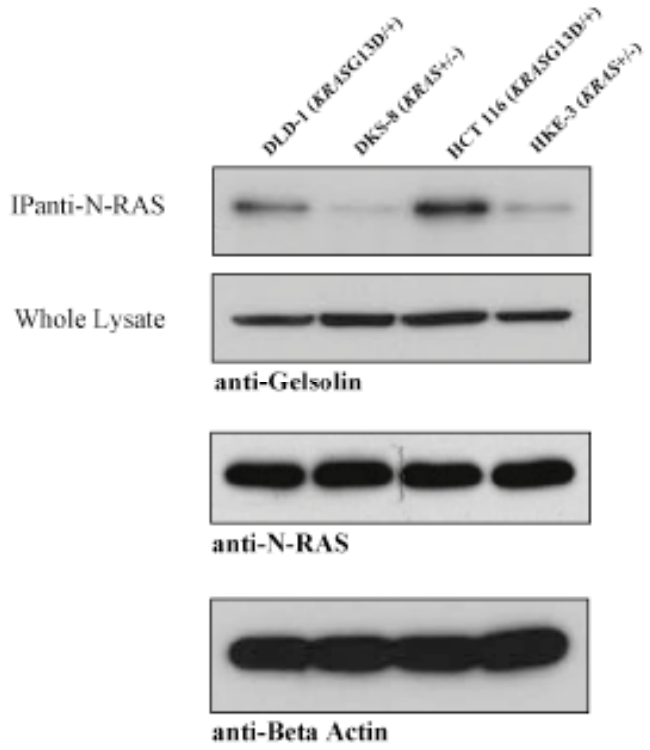


Figure 20. Constitutive K-RAS activity results in greater interaction between N-RAS and gelsolin in immunocomplexes. Lysates from DLD-1, HCT 116, and their isogenic derivatives DKS-8 and HKE-3 were prepared as described in Materials and Methods, and N-RAS was selectively immunoprecipitated. Complexes were separated and resolved by SDS PAGE (8%) and immunoblotted for gelsolin. Total gelsolin levels and N-RAS level appear equivalent in all lysates. [*Technical Note*: In co-immunoprecipitation experiments, we recognize the importance of demonstrating the ability of the antibody to immunoprecipitate its primary epitope (in this case, N-RAS). This control is complicated in the present case by the size similarity between the light chain of the antibody used (~23kDa) in the immunoprecipitation and N-RAS itself (21kDa) such that we have been unable to cleanly detect N-RAS unobscured by light chain. We take comfort in the accuracy of this result, however, from the fact that we see a consistently differential association between N-Ras and gelsolin in two sets of isogenic cell lines (above) as well as in lysates prepared from an *in vivo* model of K-Ras activation (Figure 19). It is unlikely that this genetic change precipitates changes in N-Ras distribution that so dramatically and consistently change the availability of N-Ras for immunoprecipitation.]

In addition to its actin cytoskeleton regulatory functions, gelsolin possesses pro-survival functions (82-84, 185). According to recent reports, constitutive K-RAS activity sensitizes HCT 116 (*KRAS*$^{G13D/+}$) cells to apoptotic stimuli relative to isogenic HKE-3 (*KRAS*$^{+/-}$) cells by transcriptional downregulation of gelsolin, which therefore deprives these cells of the pro-survival functions attributed to this protein (76, 77). This conclusion is based, in part, on the fact that gelsolin knockdown by siRNA sensitizes HKE-3 (*KRAS*$^{+/-}$) cells to an apoptotic stimulus to a degree comparable to parental HCT 116 (*KRAS*$^{G13D/+}$) cells. While we did not examine gelsolin mRNA levels, we consistently detect no difference in the gelsolin protein levels in the different *KRAS* environments by immunoblot (Figure 20).

The N-RAS:gelsolin protein complex is responsive to apoptotic insult and is differentially modulated in (*KRAS*$^{G13D/+}$) and (*KRAS*$^{+/-}$) environments

The observation that knockdown of gelsolin increases the sensitivity of HKE-3 (*KRAS*$^{+/-}$) cells to apoptotic stimulus and the pro-survival functions of N-ras established in other systems (180, 181) led us to hypothesize that the physical interaction we observe between these two proteins may represent a functional interaction that regulates survival.

To further explore this possibility, we treated HCT 116 (*KRAS*$^{G13D/+}$) and HKE-3 (*KRAS*$^{+/-}$) cells with sodium butyrate (3mM) over a 48 h period as an apoptotic stimulus (2). At the time points shown, we lysed these cells and immunoblotted for cleaved poly (ADP-ribosylated) protein (PARP), an apoptosis-associated marker. At baseline conditions as well as at 48 h, we immunoprecipitated for N-RAS, performed SDS PAGE, and immunoblotted for gelsolin as before to examine the status of the N-RAS:gelsolin

complex during the response to the apoptotic stimulus. The N-RAS:gelsolin complex in the constitutively active K-RAS environment (*KRAS*$^{G13D/+}$) remained constant as these cells clearly initiated an apoptotic response as measured by increased PARP cleavage. In contrast, the abundance of this complex increased dramatically in the wild-type K-RAS environment (*KRAS*$^{+/-}$) at the 48 h timepoint, well above the static levels of this complex in the mutant environment (Figure 21, panel B). This increase correlates with decreased levels of PARP cleavage consistent with a reduced apoptotic response (Figure 21, panel A). These data demonstrate a dynamic N-RAS:gelsolin complex in the presence of wild-type K-RAS signaling that correlates with increased survival. Constitutive K-RAS activity, though supporting greater levels of the N-RAS:gelsolin complex under baseline conditions, appears to compromise the responsiveness of this complex to apoptotic stimulus. Differential modulation of this complex in different K-RAS environments correlates with the differential sensitivity of these lines to apoptotic stimuli.

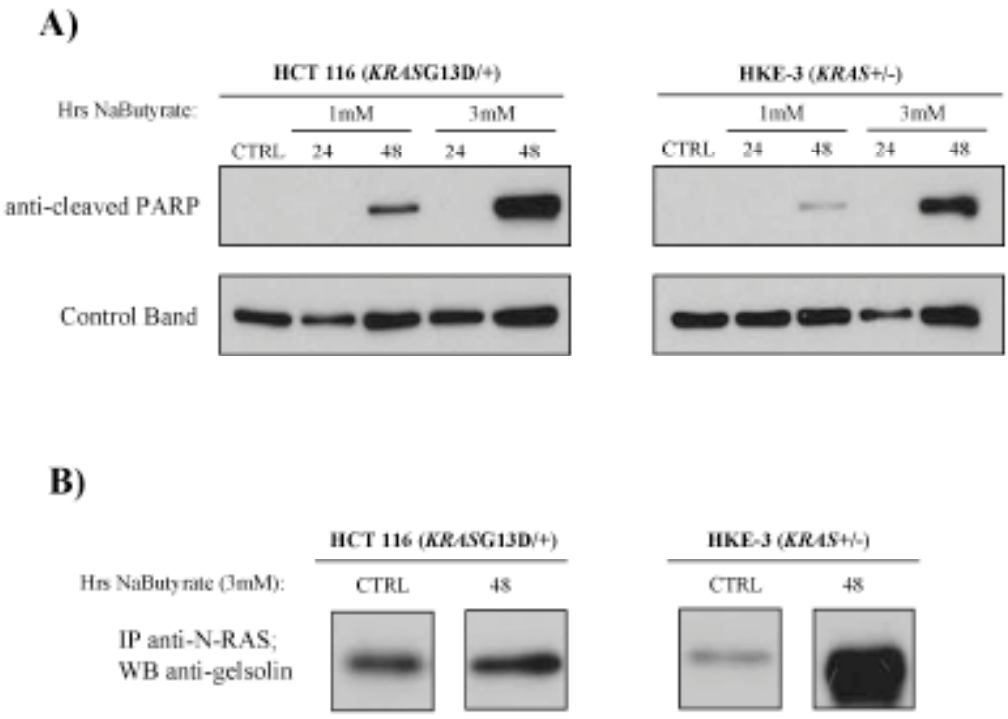


Figure 21. *KRAS* status correlates with differential response to apoptotic agent sodium butyrate. HCT 116 and HKE-3 were grown to similar densities and treated as shown. Lysates were prepared as described in Materials and Methods and separated by SDS PAGE, transferred to Immobolin and immunoblotted for cleaved PARP or gelsolin. A) Consistent with the results of Klampfer et al., HCT 116 and HKE-3 cells are differentially sensitive to apoptotic stimuli as measured by PARP cleavage. B) N-RAS:gelsolin association is differentially modulated according to *KRAS* status in response to apoptotic agents. Constitutive K-RAS signaling results in a more abundant, but static, complex containing N-RAS and gelsolin over the butyrate time course. Disruption of the mutant *KRAS* allele by homologous recombination results in lower basal levels of the N-RAS, gelsolin-containing complex but one that increases dramatically in response to apoptotic insult, corresponding to increased survival. Loading amounts were equivalent as measured by Ponceau S staining and the respective intensities of a control band.

Specific knockdown of *NRAS* selectively sensitizes cells exhibiting wild-type but not constitutive K-RAS signaling to apoptotic insult

The responsiveness of the N-RAS:gelsolin complex to apoptotic stimulus combined with the pro-survival functions previously described for each of these proteins led us to hypothesize that the N-RAS:gelsolin complex may cooperatively promote survival in these cells. We predicted that knockdown of N-RAS, like gelsolin, would compromise the survival of these cells in response to apoptotic stimuli.

We identified a siRNA sequence against *NRAS* that results in near total loss of N-RAS protein as measured by immunoblot (Figure 22, panel B) and transduced both HKE-3 ($KRAS^{+/-}$) and HCT 116 ($KRAS^{G13D/+}$) cells with lentiviral vectors expressing this hairpin to generate stable lines. We then treated these lines as well as untransfected controls and cells stably expressing the empty vector (pSICOR) with staurosporine (350nM) over a 5 h time course. We prepared lysates from these cells after 2 and 5 h and compared the apoptotic response as measured by increases in the levels of cleaved PARP (Figure 22, panel C). Consistent with a possible cooperative role between N-RAS and gelsolin in promoting survival, the N-RAS knockdown sensitized the HKE-3 ($KRAS^{+/-}$) cells to apoptosis but did not change the response of the HCT 116 ($KRAS^{G13D/+}$), which are already strongly predisposed to the initiation of the apoptotic process. These data provide direct evidence that oncogenic K-RAS compromises the ability of endogenous N-RAS to promote survival.

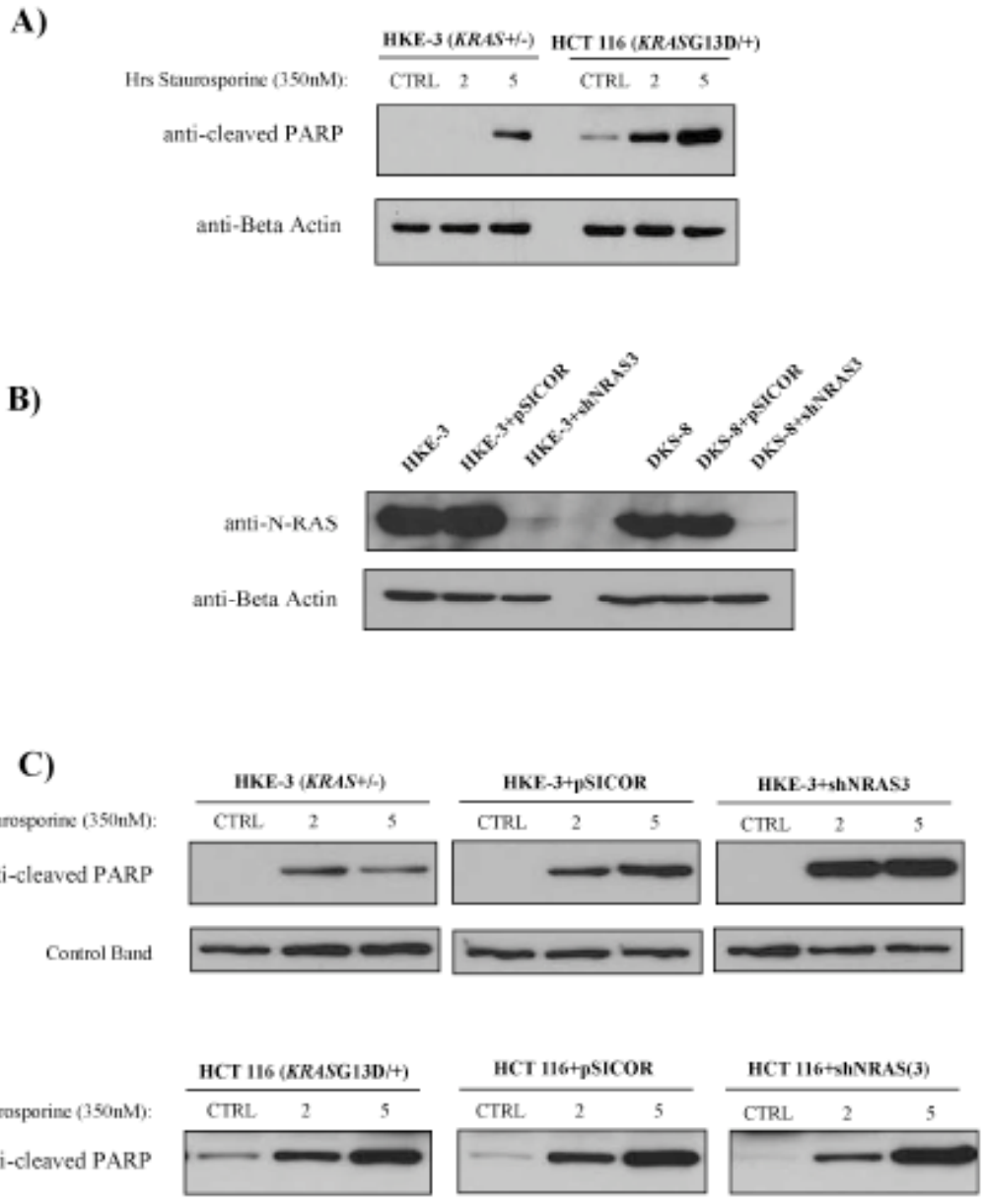


Figure 22. N-RAS-mediated survival signaling is compromised by constitutive K-RAS activity. A) HKE-3 and HCT 116 are differentially sensitive to apoptosis induced by staurosporine as measured by PARP cleavage. B) Specific knockdown of *NRAS* with stably expressed hairpin. C) Knockdown of N-RAS sensitizes HKE-3 cells, but not HCT 116, to apoptotic insult. HKE-3 cells stably expressing either pSICOR vector (empty) or a hairpin specific for *NRAS* (shNRAS3) demonstrate differential sensitivity to staurosporine (350nM) as measured by PARP cleavage.

N-RAS is required for selected aspects of K-RAS-driven oncogenic behavior

Given that constitutive K-RAS activity elevates levels of N-RAS•GTP and participation in effector complexes, we hypothesized that N-RAS may be responsible for mediating some aspects of K-RAS function, aspects perhaps important to the oncogenic contribution provided by unregulated K-RAS signaling. To test this hypothesis, we utilized the assays reported by Shirasawa et al. to examine the functional differences between cells characterized by an endogenous mutant *KRAS* allele, and those in which the mutant allele has been disrupted. These include soft agar growth, proliferation assays, and nude mouse xenograft assays. We utilized both DLD-1 and HCT 116 cell lines stably expressing short, interfering hairpins against NRAS in these assays for comparison to the parent lines and the parent lines expressing the empty vector.

Anchorage independent growth as measured by soft agar assay was unchanged in DLD-1 or HCT 116 cells expressing the empty vector versus those in which levels of N-RAS protein were knocked down due to RNA interference (Figure 23, panel C), suggesting that pathways utilized by K-RAS for promotion of anchorage independent growth are not engaged by N-RAS. In proliferation assays, DLD-1 cells and HCT 116 cells displayed some reduction in growth rate relative to cell lines expressing the vector alone. These effects were more dramatic in the cells expressing the shNRAS(3) sequence which appears to result in greater rates of knockdown than the shNRAS(2) sequence, though both reduce levels compared to control. These effects were not as great as the reduction seen in growth rate following disruption of the mutant *KRAS* allele, but were significant nonetheless. Subcutaneous injections of the N-RAS knockdown lines into the flanks of nude mice interestingly showed a dramatic effect in the HCT 116 cells, but not

the DLD-1. Decreasing dilutions ($5X10^6$; $5X10^5$; $5X10^4$; and $5X10^3$) of cells expressing either empty vector or the shNRAS(3) hairpin were injected and allowed to grow. No tumors whatsoever formed in the HCT 116 cells expressing the shNRAS(3) hairpin after 18 days of tumor growth when the control cells (vector only) demonstrated significant tumor burdens (Figure 23, panel B). The DLD-1 cells lines expressing the same vector showed no change whatsoever from cells expressing only vector, suggesting that K-RAS relies on N-RAS to a greater extent for its oncogenic signaling in the HCT 116 cells than in the DLD-1 cells, and further that the mechanisms of K-RAS mediated transformation may differ in these two cell lines. This question will be revisited in subsequent chapters.

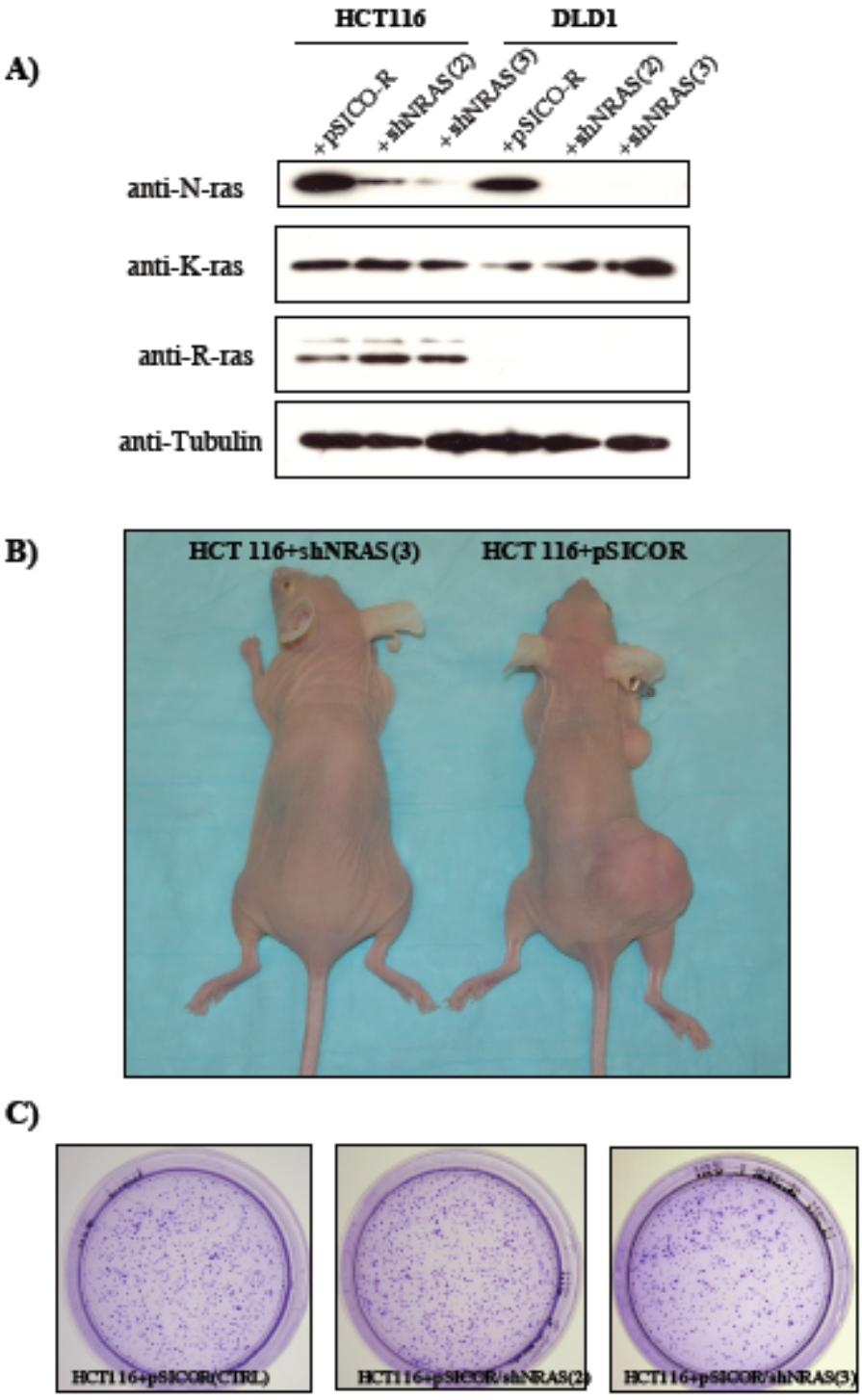


Figure 23. Selective knockdown of N-RAS affects selected tumorigenic properties of HCT 116. A) Specific knockdown of N-RAS in HCT 116 and DLD-1 cell lines. B) HCT 116 cell lines stably expressing the shNRAS(3) hairpin fail to grow in nude mouse xenografts. Mice were injected with sequential dilutions of cells as dicussed in Materials and Methods. This effect was exclusive to HCT 116; DLD-1 cell lines in which N-RAS had been knocked down formed tumors normally (n=6). C) Both HCT 116 and DLD-1 cells stably expressing the *NRAS* hairpins grow in soft agar.

A possible mechanism for this observation is the differential production of angiogenic factors such as vascular endothelial growth factor (VEGF) and vascular permeability factor (VPF) by the parent lines relative to their isogenic derivatives (114). One major difference between the tumorigenic qualities measured by nude mouse xenografts versus soft agar assays or other measures of transformation almost certainly has to do with the ability to promote angiogenesis. It is therefore possible that the K-RAS in the HCT 116 cell line, but perhaps not the DLD-1 cell line, relies on N-RAS signaling for upregulation of these pro-angiogenic factors, though this hypothesis has not been tested.

Discussion

Oncogenic mutation of *KRAS* is a common genetic event in human tumorigenesis and is observed in approximately half of colorectal cancers (169). Use of colon cancer-derived cell lines, HCT 116 and DLD-1 in combination with their isogenic derivatives has allowed for relatively direct study of the oncogenic contribution of constitutive K-RAS activity. We used this system to identify biochemical evidence of a functional relationship between RAS isoforms, K-RAS and N-RAS. Our findings establish that mutation of *KRAS* to its oncogenic form supports increased levels of N-RAS•GTP and increased interaction between N-RAS and gelsolin in a previously unrecognized complex. Knockdown of either N-RAS or gelsolin in the wild-type K-RAS environment ($KRAS^{+/-}$) compromises the survival of these cells following apoptotic challenge, suggesting a pro-survival function for each of these proteins. The parent cell line HCT 116 ($KRAS^{G13D/+}$), in contrast, is strongly predisposed to the initiation of apoptosis,

suggesting that constitutive K-RAS activity compromises the pro-survival functions of N-RAS and gelsolin.

Pro-survival functions for these proteins have been established in other systems. N-RAS mediates survival signaling by stimulation of the PI(3)K/Akt pathway and other mechanisms in murine fibroblasts (180, 181). No differences in Akt phosphorylation (Ser 473) were observed between (*KRAS*$^{G13D/+}$) and (*KRAS*$^{+/-}$) cell lines, however, suggesting that N-RAS promotes survival in these cell lines by an alternate mechanism. Gelsolin also promotes survival, possibly by regulation of mitochondrial permeability (82-84). Gelsolin overexpression inhibits apotosis in Jurkat cells, and gelsolin null (*Gsn*$^{-/-}$) neutrophils display elevated apoptosis relative to their wild-type counterparts (82, 113). Thus, the interaction of N-RAS and gelsolin raises an intriguing possibility: that the N-RAS:gelsolin complex represents a cooperative functional interaction in the promotion of survival. Under baseline conditions, we observe elevated levels of this complex in HCT 116 (*KRAS*$^{G13D/+}$) cells, which are also highly sensitized to apoptotic insult. Interestingly, in response to apoptotic stimuli, this complex is completely static in this line, with relative levels after 48 h of sodium butyrate treatment comparable to those observed at baseline. In stark contrast is the dynamic response of this complex to the same stimulus in HKE-3 (*KRAS*$^{+/-}$) cells. The responsiveness of this complex in cells expressing only wild-type K-RAS correlates with their enhanced survival relative to cells expressing oncogenic K-RAS. Together with the observed pro-survival function of these proteins, the differential modulation of the N-RAS:gelsolin complex suggests the possibility that this interaction represents physical and functional cooperation between these two proteins to promote survival.

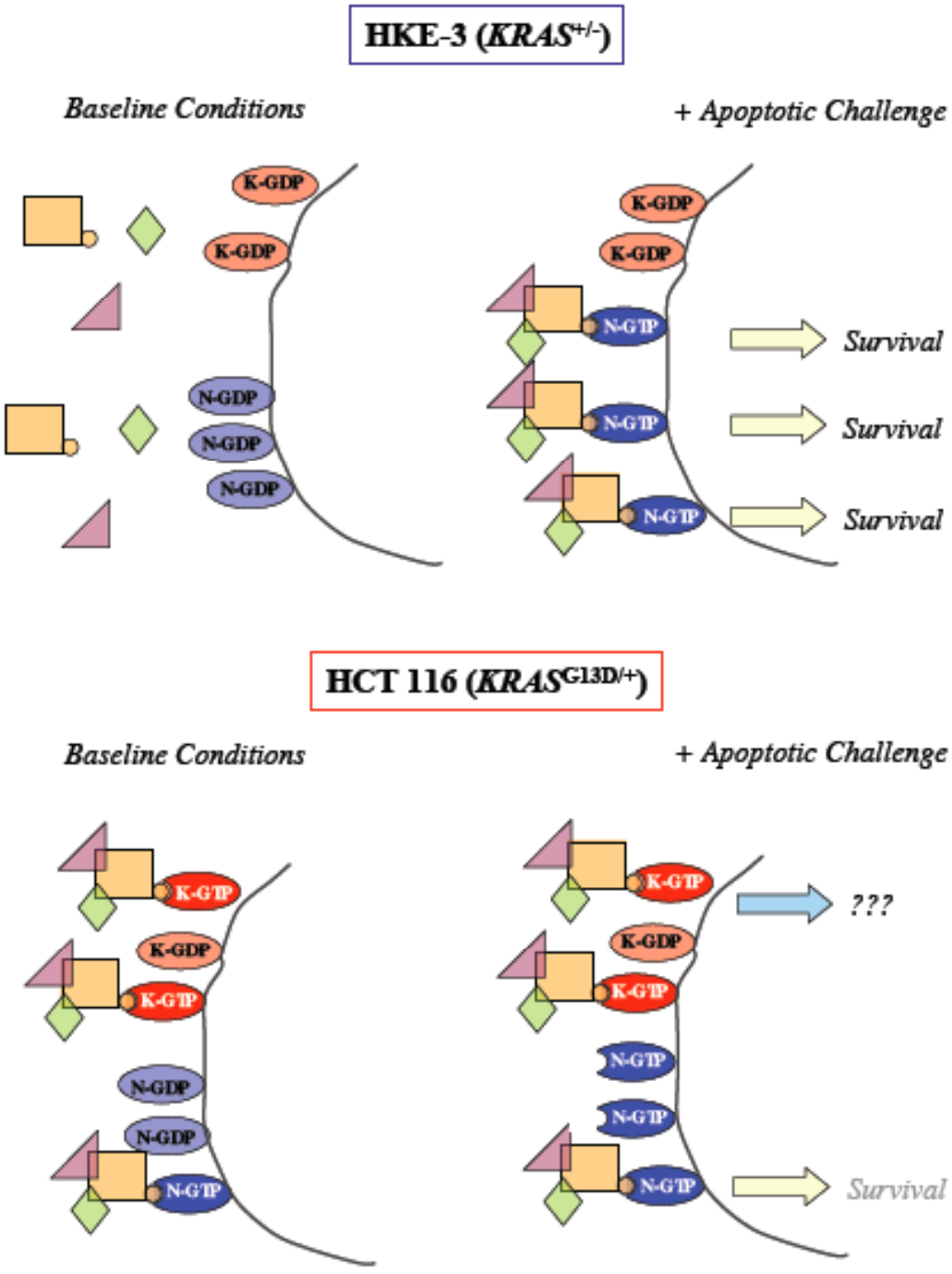


Figure 24. 'Competitive Equilibrium' model of Ras family member cross-talk. Given the overlapping localizations, ability to bind common effectors, and shared biochemical requirements of Ras family members, Ras activation may respresent an equilibrium. Oncogenic mutation of any single family member may perturb this relationship and compromise isoform-specific function.

We postulate that the differential modulation of the N-RAS:gelsolin complex is the result of a competitive equilibrium that exists between RAS isoforms. Given the partially overlapping localization of various RAS isoforms to specific membrane domains (127, 129) and the ability of different isoforms to interact with common effectors, competition for a limiting population of effectors is highly possible. The collective interaction of multiple RAS isoforms with a given effector species can therefore be thought of as an equilibrium subject to perturbation by any number of factors, including oncogenic mutation of one *RAS* allele. We postulate such an equilibrium between N-RAS and K-RAS for a limiting effector population, either gelsolin itself or an effector that N-RAS may use to dock onto a larger complex containing gelsolin and that K-RAS may use for a different (non-survival) purposes.

In a 'competitive equilibrium' such as that described above, constitutive K-RAS signaling results in elevated levels of N-RAS•GTP and higher basal levels of N-RAS interaction with gelsolin. Further increases in N-RAS•GTP levels (perhaps in response to apoptotic challenge) do not translate into increased interaction with gelsolin, owing to sequestration of limiting effectors by K-RAS, a dominant competitor in its activated form (Figure 24). Thus, in the mutant K-RAS environment, the abundance of the N-RAS:gelsolin complex cannot be increased above its baseline levels, a limitation that accounts for the impaired survival response we and others observe. The absence of effect of N-RAS knockdown in the HCT 116 ($KRAS^{G13D/+}$) cell line (Figure 22, panel B) supports this conclusion.

The mechanism by which constitutive K-RAS activity alters levels of N-RAS•GTP is unclear. In light of the effects of oncogenic mutation of one RAS isoform,

one possibility involves the availability of regulatory molecules, specifically guanine nucleotide exchange factors (GEFs), responsible for offloading GDP and onloading GTP. Oncogenic Ras proteins incapable of GTP hydrolysis likely experience vastly reduced interaction with GEF molecules. Mutation of one RAS isoform may therefore subject wild-type Ras molecules to greater rates of GTP-loading owing to decreased competition for what may have previously been a rate-limiting GEF population.

Both altered juxtacrine signaling and the perturbation of RAS:GEF stoichiometry suggest that increased levels of N-RAS•GTP are an indirect consequence of genetic activation of K-RAS. Genetic data suggests a more direct relationship between Ras isoforms, however. Among Ras isoforms, only K-ras is required for normal murine development and viability (41, 71, 79, 166). Interestingly, *Kras*$^{+/-}$ mice and *Nras*$^{-/-}$ mice are separately viable (71, 166); in combination, however, 70% of *Kras*$^{+/-}$; *Nras*$^{-/-}$ animals are embryonic lethal, with the remainder dying perinatally, suggesting some functional overlap between N-ras and K-ras in aspects of signaling critical to murine development or in which different Ras isoforms act in linear fashion to transduce a signal (71). Our data support the latter interpretation. Additionally, in overexpression studies, H-ras-mediated transformation of murine fibroblasts requires N-ras function for requisite activation of MAPK (56).

We describe here elevated levels of N-RAS•GTP as a function of constitutive K-RAS activity and, correspondingly, increased N-RAS:effector interaction. Increased interaction of N-RAS and gelsolin as a consequence of K-RAS activity is one example of what is likely a broader phenomenon, however. These findings take on added significance when considered in light of the oncogenic contribution of K-RAS in human

tumorigenesis. RAS isoforms are the most frequently observed oncogenes in human tumors, and *KRAS* is the most frequently mutated member of this family (35). It is quite possible that K-RAS derives certain aspects of its oncogenicity from its ability to constitutively signal not only to its own effectors, but also to amplify signaling through effectors exclusive to other RAS isoforms as well, including N-RAS. Indeed, preliminary findings suggest that N-RAS activity is required for the full oncogenic potential of mutant K-RAS to be realized (Figure 23).

To our knowledge, this report provides the first biochemical evidence of a functional relationship between RAS isoforms. We demonstrate that constitutively active K-RAS increases levels of N-RAS•GTP yet subverts its putative pro-survival functions. We report a novel interaction between N-RAS and gelsolin in a complex that may promote survival under normal conditions, but possibly due to altered modulation in the presence of oncogenic K-RAS, the pro-survival effects of this complex are countermanded, thus predisposing the cell to apoptosis. These findings revise and expand upon data previously reported describing the sensitization of tumor cells to apoptotic insult by constitutive K-RAS activity (76, 77) and open a novel avenue of inquiry into a critically important human oncogene.

We note in the Introduction some of the possible artifacts attributable to the cell lines used here, specifically in regard to their isogenic derivatives, and possible issues of oncogene addiction and the implications that disruption of the mutant allele may hold for the isogenic derivatives. We feel confident that the observations we make here are not artefactual results of the isogenic system, but rather are real and reproducible. Our confidence rests on the facts that (1) this phenomenon is observed in both the DLD-1 and

HCT 116 cells lines, which display some important molecular differences downstream of K-RAS to be discussed in greater detail in subsequent sections; and (2) in a non-transformed system, the K-ras$^{LSLG12D/+}$ mouse, we observe the same phenomena, specifically, differential N-ras activity in different K-ras environments as determined by both the RBDGST assay (consistent with cell line analysis) and differential interaction of N-ras with other proteins in different *Kras* environments.

CHAPTER V

CONSTITUTIVE ACTIVATION OF ENDOGENOUS K-RAS IN THE COLONIC EPITHELIUM PROVIDES A UNIQUELY POTENT ONCOGENIC CONTRIBUTION AMONG RAS FAMILY MEMBERS

Introduction

Activating mutations of the members of the RAS family of small GTPases are among the most common genetic events in human tumorigenesis. Constitutive RAS signaling contributes to the transformed phenotype by promoting proliferation, enhanced motility, survival, and loss of anchorage dependence. Activating mutations of *RAS* family members do not occur with equal frequency across tumor types, but rather segregate strongly by tissue of origin, with one isoform predominating in any particular RAS-driven tumor. For example, mutations to *KRAS* are the most frequent *RAS* family mutations, and occur in 30% of non-small cell lung cancers, 50% of colorectal adenocarcinomas, and 90% of pancreatic adenocarcinomas. Fifteen percent of melanomas and 30% of acute myelogenous luekemias display activating *NRAS* mutations. Activating mutations of *HRAS* are far less common but contribute to squamous cell carcinomas, bladder carcinomas and to renal cancers (35). This segregation suggests isoform-specific oncogenic contributions in different tissue and cellular environments.

Numerous recent studies have refuted the long-held belief that Ras family members are functionally interchangeable. Despite their extensive sequence identity,

divergence in their C-terminal 24 amino acids and a series of posttranslational modifications to this region of these proteins provides the cell biological basis for the unique functional identities of the members of this protein family.

Subsequent to a common set of posttranslational modifications, the canonical RAS family members undergo differential processing. The prenylation of cysteine residues in the C-terminus of H-RAS and N-RAS constitutes a "second signal" which further directs these proteins to platforms within the cell where they interact with regulatory and effector molecules to transduce molecular signaling events. In place of this second signal, K-RAS4B possesses a polylysine domain in its hypervariable region that governs the trafficking of K-RAS4B by a non-vesicular mechanism, allows for greater mobility within the plasma membrane, and provides both a unique mechanism of membrane anchorage and broad access to compositionally distinct regions of the lipid bilayer (111).

A broad range of specific and common functions have been attributed to the various RAS family members, including the range of effectors with which they are capable of interacting. Despite their biochemical similarities, members of the RAS family of proteins interact differentially with common effectors *in vivo* (138, 182-184). Such functional differences between isoforms may explain the observation that mutations to different RAS isoforms are observed to segregate strongly by tumor type. Because of the multiplicity of RAS function, discerning specific pathways and isolating their contributions to tumorigenesis has proved daunting. Differential requirements for RAS signaling at different stages of tumor development further magnify this complexity (21, 89).

K-RAS provides a uniquely potent oncogenic contribution among Ras isoforms as demonstrated by its frequency of mutation and its role in colorectal cancer. KRAS is not only mutated in the broadest range of tumor types, but also in the most common RAS-driven tumors. We have employed isogenic pairs of colorectal cancer cell lines that owe much of their transformed phenotype to the presence of an activating mutation (G13D) of *KRAS* (156). Using homologous recombination, the mutant allele has been disrupted to generate isogenic derivatives, which we analyzed for changes attributable to oncogenic K-RAS. To understand the downstream signaling events that may explain this, we have attempted to recapitulate the transformed phenotype of these cells by overexpressing either constitutively active forms of N-RAS or H-RAS in the isogenic derivatives in order to gauge their relative oncogenic contributions as well as to identify molecular changes that are common to all Ras isoforms and therefore unlikely to explain the oncogenic potency of constitutive K-RAS signaling in this tissue. We utilized molecular and functional readouts to compare the relative oncogenic contributions of constitutively active Ras isoforms in a genetically comparable background. We identified K-RAS-mediated changes that partially explain the dramatic transformed phenotype observed in HCT 116 and DLD-1 colorectal cancer cell lines and present evidence to challenge established perceptions about the importance of canonical Ras effector pathways to transformation. Further, we report that the molecular signatures of constitutive K-RAS activity are not necessarily conserved across cell lines derived from tumors with a common origin.

Materials and Methods

Molecular Biology

Retroviral vector LZRS-MS-EGFP (a kind gift of Garry Nolan, Stanford University) was used for cloning H-RAS, N-RAS, and K-RAS4B (Ras cDNAs were the kind gift of M. Philips). G12V mutations of Ras cDNAs as well as of Rap1 were generated using site-directed PCR-based mutagenesis. Coding sequences were tagged with SgfI and SfiI restriction sites and directionally ligated into linearized, dephosphorylated LZRS vector. Rap1 clone was purchased from ATCC. All constructs were bidirectionally sequenced to ensure accuracy. Rap1 sequences were cloned into pBABE Puro (blunt, HincII). siRNA sequences were generated with the use of pSICOLIGOMAKER software (http://web.mit.edu/ccr/labs/jacks). shRAP(2) target sequence: GAAGAACTGTTGCCTAATT; shRAP(3) target sequence: GGATGCATTTCAAATGTTA. Sequences were BLASTed to confirm target specificity. Hairpins were cloned into linearized pSICOR (XhoI/HpaI) and sequenced.

Retroviral and Lentiviral Transduction

Retroviral vectors were transfected using Fugene 6 (Roche, Indianapolis, Indiana) into Phoenix293 cells. In all cases, these cells were selected in puromycin (1μg/ml) for 24hrs, or as necessary to generate packaging lines. Recovered packaging lines were passaged and grown to a density of 50-70% in a 100mm dish. A minimal volume of media was added and the packaging cells were placed at 32°C to generate virus. After 12-24hrs, media was harvested and filtered through a 0.22μm syringe filter. Polybrene

was added to 5μg/ml final concentration and retroviral-containing media was transferred to subconfluent, actively proliferating target cells. Transductions were repeated as necessary. Successfully transduced target cells were sorted by flow cytometry (VA Flow Cytometry Core Facility, Nashville, Tennessee). For lentiviral transduction of siRNA constructs, 293T cells were plated in a 100mm dish and allowed to grow to 50-70% confluence. Cells were transfected overnight with the lentiviral vector pSICOR (5μg) as well as the Δ8.2 packaging vector (2.5μg) and the pMD26 packaging vector (2.5μg) using Fugene 6 according to the manufacturer's instructions. Media was changed and replaced with minimal volume. After 24 h, media was harvested and filtered through a 0.22μm syringe filter. Filtered viral supernatant was transferred to a plate of target cells and infection continued for 24 h. Selection was begun the following day (puromycin 7.5μg/ml) and refreshed daily for 4-6 days until a nontransfected control plate of cells was completely killed. Cells were maintained in DMEM and selection periodically repeated. Specificity of knockdown was verified by immunoblot.

Immunoblotting and Small G Protein Activation Assays

For Ras Western blots, all cells were washed 3X with ice-cold PBS and lysed in 25mM HEPES (pH 7.5), 150 mM NaCl, 1%NP40, 10mM $MgCl_2$, 1 mM EDTA 2% glycerol, proteinase inhibitors (Roche), and phosphatase inhibitor cocktails I and II (Sigma). Lysates were vortexed for 5 sec, placed on ice for 5 min, and centrifuged at 13,400 rpm at 4°C in a benchtop centrifuge for 15 min. Supernatants were then transferred to fresh tubes, and protein concentration determined by BCA assay. Samples were resolved by SDS-PAGE, transferred to Immobilon, blocked for 1 h with 5% nonfat

milk in TBST (0.1 %Tween 20), and then incubated with primary antibody overnight. RAS specific antibodies were from Santa Cruz: H-RAS (sc-520); N-RAS (sc-31); and K-RAS (sc-30) were all used at 1:200; pan-RAS was from Sigma (RAS clone 10) used at 1:1000. Antibodies against ERK1/2 (Cell Signaling 9102), pERK1/2 (Cell Signaling 9101), Akt (Cell Signaling 9272), pAkt (Cell Signaling 9271) were used at 1:1000. Monoclonal Rap1 antibody was purchased from BD Transduction Laboratories (610195) and was used at 1:500. Blots were detected with appropriate secondary antibodies conjugated to HRP and detected with enhanced chemiluminescence (Amersham, Uppsala, Sweden). Rap1 Activation kit (#17-321) and Ral Activation kit (#17-300) were purchased from Upstate (Lake, Placid, New York) and used per manufacturer's instructions.

2D DIGE Analysis

TNE buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA, pH 8.0, 1 mM Na3VO4, 1 mM leupeptin, 2 mM pepstatin, and 0.1 mM aprotinin) was added to each plate (150Mm) and cells scraped and collected in an Eppendorf tube. The suspended solution was then sonicated at 4°C (3X10 pulses, power level 4), after which NP-40 (1% v/v) and PMSF (1 mM) were added to the lysate solution. Samples were then vortexed, allowed to sit on ice for 10 min, and centrifuged at 10,000Xg for 10 min at 4°C. Supernatants were removed and concentrations determined in triplicate by bicinchoninic acid (BCA) protein assay. DTT was then added to a final concentration of 2mM, and samples were visualized on a 10% SDS-PAGE gel to confirm concentrations. Cy-dye

labeling, 2-D gel electrophoresis and imaging, DIGE analysis, in-gel digestion, and mass spectrometry were performed as described previously (49).

Results

Constitutively activated K-RAS4B^{G12V} cannot be overexpressed in cells isogenically derived from (*KRAS*$^{G13D/+}$) though H-RAS^{G12V} and N-RAS^{G12V} are tolerated

Constitutive activation of Ras can yield numerous different outcomes, depending largely on cell context. In nontransformed environment, constitutive Ras activity promotes cell cycle arrest, triggering feedbacks within the cell that under normal circumstances mitigate the potentially dangerous effects of unregulated signaling through a number of pathways (151). The same event, occurring in a cell in which these mechanisms have been impaired, however, contributes significantly to transformation, and enables focus formation, altered morphology and motility, and increased proliferation (63, 143). The importance of context, therefore, likely applies similarly to the derivation of isogenic cell lines by means of targeted disruption of an oncogenic allele. A cell that has adapted to, or even become dependent on, constitutive signaling events likely undergoes dramatic changes following their removal (21, 68).

To gain some understanding of the status of the isogenic derivatives of the DLD-1 and the HCT 116 cell lines, we initially transduced these derivatives with a retrovirus carrying constitutively active K-RAS4B^{G12V} in hopes of recapitulating the transformed phenotype of the parent cell lines. Following transduction, we sorted the pool of positively expressing cells by flow cytometry using a GFP marker under promotion of an

IRES sequence as an indicator of expression levels into high, medium, and low expressors (90). Because the level of expression of a retroviral vector can vary by ~100 fold depending on its site of integration, we have relied on pools of stable transfectants rather than individual clones to ensure comparable expression levels (90). We were able to generate three distinct and equivalent pools of Ras-overexpressing target cells for both HKE-3 and DKS-8 (high, medium, and low; H-RAS^{G12V}, K-RAS4B^{G12V}, and N-RAS^{G12V}) (Figure 25, panel A). In order to verify these differential expression levels, we immunoblotted lysates made from these cells for RAS isoforms as well as GFP. While we were able to verify three distinct levels of expression for H-RAS^{G12V} and N-RAS^{G12V} overexpressors by coordinate analysis of GFP and Ras isoform protein levels, the immunoblotting results for K-RAS4B^{G12V} did not correlate with GFP expression levels (Figure 25, panel B). The three distinct levels of GFP observed in the high, medium, and low-expressing pools of K-RAS imply the presence of transcripts, but the lack of corresponding K-RAS protein suggests some downregulation either during translation or at the level of mature protein.

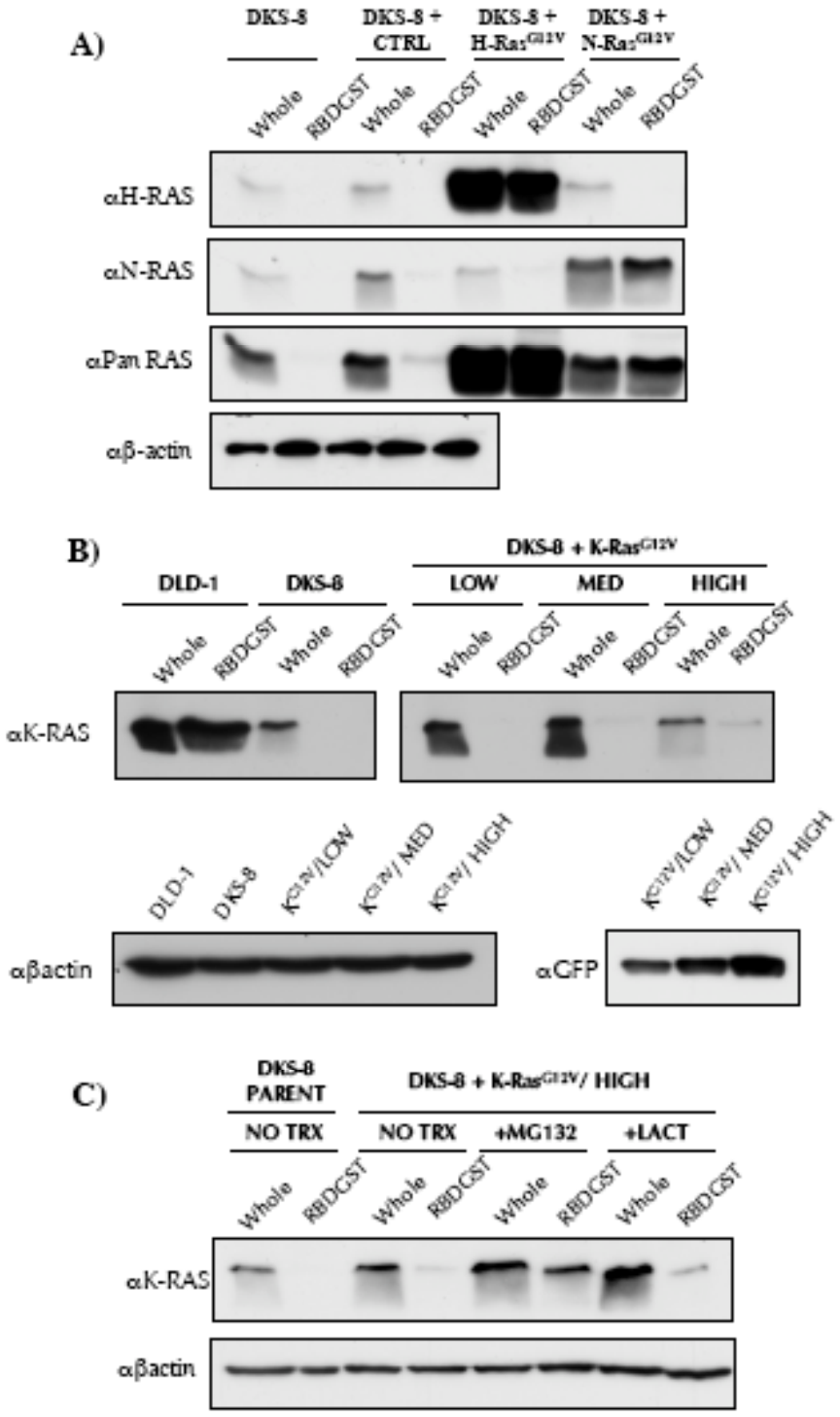


Figure 25. Overexpression of oncogenic N-RAS, H-RAS, and K-RAS4B mutants in DKS-8 cells. A) While H-RAS^{G12V} and N-RAS^{G12V} can be overexpressed in isogenic derivatives in which the mutant *KRAS* allele has been disrupted, B) K-RAS4B^{G12V} cannot. C)This appears to be the partial result of proteosomal degradation, as treatment of these cells with proteosome inhibitors MG132 and lactacystin results in partial restoration of K-RAS protein levels and activity.

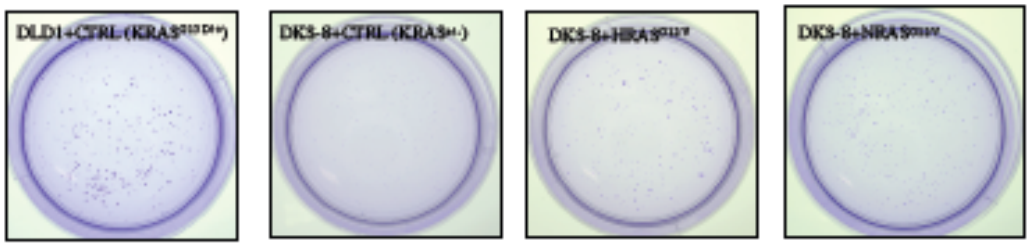
We further investigated these possibilities by treating these cells with inhibitors of proteasomal degradation MG132 and lactacystin followed by immunoblot. Interestingly, proteasomal inhibition resulted in elevated levels of K-RAS4B protein, though not complete restoration as measured by comparison with protein levels seen in the H-RAS^{G12V} and N-RAS^{G12V} overexpressors (Figure 25, panel C). This result is perhaps not surprising given that ubiquitination by E3 ubiquitin ligases which target the protein for proteasomal degradation frequently occurs on internal lysine residues, and that the distinguishing motif of K-RAS4B hypervariable domain is a polylysine motif (51). Preferential degradation of K-RAS4B^{G12V}, but not H-RAS^{G12V} or N-RAS^{G12V}, is possibly suggestive of the comparatively deleterious and potent effects of constitutive K-RAS4B signaling in cancer cells that have acquired increased mutant K-RAS4B. The inability to recapitulate the parental expression levels in the isogenic derivatives suggests that these lines have undergone changes to adapt to the loss of oncogenic K-RAS4B signaling, and that these changes may not simply be reverted by reconstitution. The tolerance of the DKS-8 and HKE-3 lines for overexpression of constitutively active mutants of H-RAS and N-RAS, but not K-RAS4B, support the possibility that K-RAS is responsible for a unique set of signaling events, which likely also are the basis of its unique oncogenic nature.

Neither overexpression of N-RAS^{G12V} or H-RAS^{G12V} can fully recapitulate the functional effects of constitutively activated endogenous K-RAS

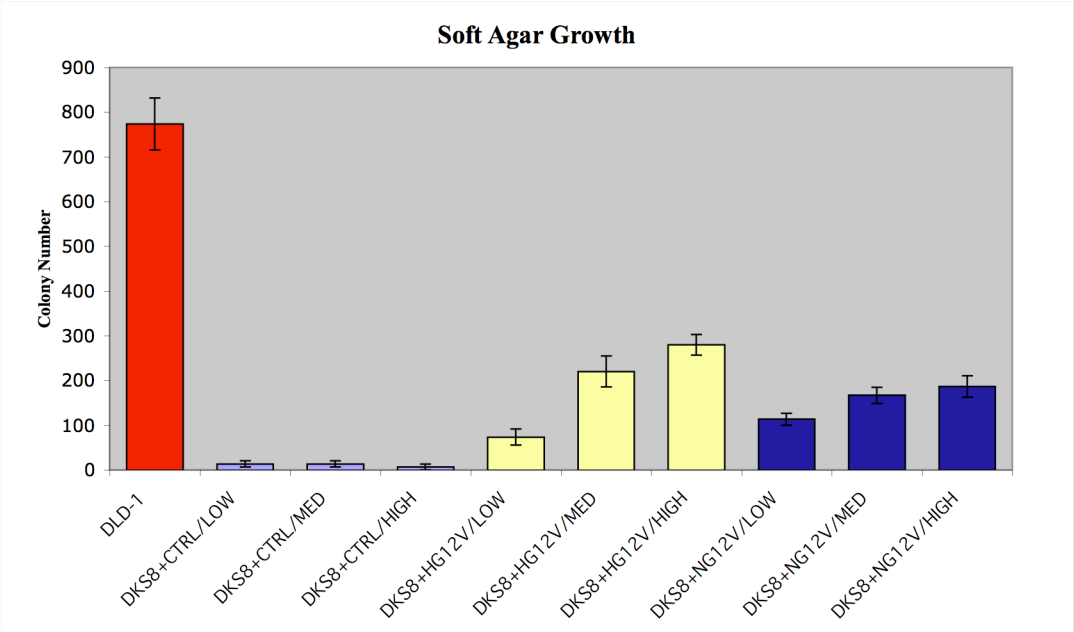
Because overexpression of K-RAS4B^{G12V} in either DKS-8 or HKE-3 (*KRAS*$^{+/-}$) cell lines does not appear to reconstitute the parental cell line as judged by protein levels and presumably signaling intensity, we have opted to compare either DKS-8 or HKE-3 overexpressing constitutively activated N-RAS or H-RAS with the respective parent lines (DLD-1 or HCT 116) expressing empty vector alone. To compare the oncogenic contribution of these Ras isoforms, we have measured the respective abilities of cells overexpressing H-RAS^{G12V} or N-RAS^{G12V} to compensate for the loss of oncogenic endogenous K-RAS in functional assays including proliferation assays as well as soft agar assays, both parameters which change dramatically following loss of the mutant endogenous *KRAS* allele (156).

In both these assays, overexpression of H-RAS^{G12V} proved to more closely recapitulate the oncogenic contribution of K-RAS than did N-RAS^{G12V}. Although both represent an increase over the baseline levels, neither approach the parental lines. Soft agar assays reveal that the respective oncogenic contributions were dose-dependent (Figure 26).

A)



B)



C)

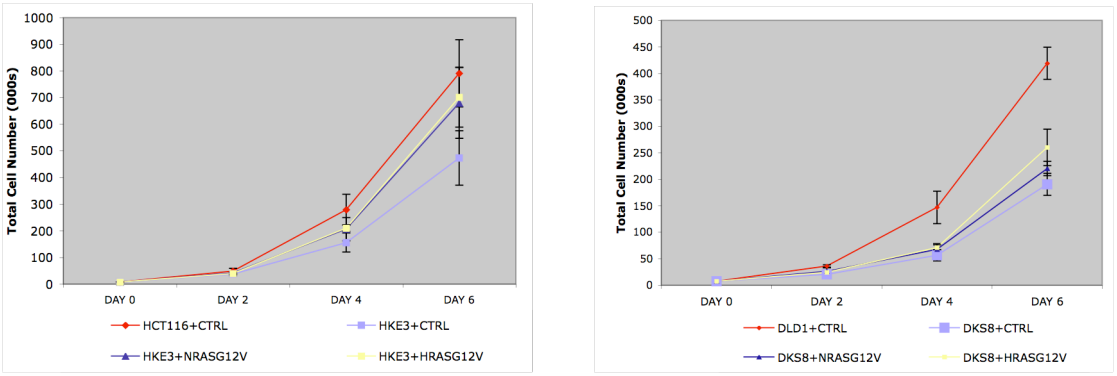


Figure 26. Overexpression of H-RAS^{G12V} and N-RAS^{G12V} cannot reconstitute the transformed phenotype of the endogenous *KRAS* allele. Reconstituted isogenic derivative lines overexpressing H-RAS^{G12V} or N-RAS^{G12V} cannot restore the functional aspects of a mutant endogenous *KRAS* allele.

Canonical Ras effector pathways are differently activated by different Ras isoforms

Three of the best-understood effector pathways of Ras proteins are Raf/MEK/ERK; PI3K/Akt; and RalGDS, all of which mediate the oncogenic contribution of Ras. Given the differences in functional assays described above, we hypothesized that endogenous K-RAS may preferentially stimulate one or more of these pathways, and in so doing, promote the transformed phenotype of the parent lines. To test this hypothesis, we conducted molecular analysis of several well-characterized effector pathways, examining either changes in phosphorylation states or other activity levels. To minimize the possibility that changes may be cell-line specific, we analyzed in parallel the DLD-1 suite (DLD-1+vector, DKS-8+vector, and DKS-8+H-RAS^{G12V}/+N-RAS^{G12V}) and the HCT 116 suite (HCT 116+vector, HKE-3+vector, and HKE-3+H-RAS^{G12V}/+N-RAS^{G12V}).

Interestingly, loss of K-RAS in DLD-1 and HCT 116 cells differentially affects the activation status of the Raf/MEK/MAPK cascade as measured by phosphorylation of ERK1/2 and MEK. In the case of DLD-1 and DKS-8, similar levels of ERK phosphorylation were observed. In contrast, a marked decrease was seen in HKE-3 relative to HCT116 (Figure 27, panel A). In both sets of cell lines, overexpression of H-RAS^{G12V} and N-RAS^{G12V} dramatically upregulate this pathway. Despite hyperphosphorylation of ERK1/2 in the H-RAS^{G12V} and N-RAS^{G12V} overexpressing cell lines, these lines failed to recapitulate either the soft agar growth or proliferation rates of the endogenous *KRAS* allele, suggesting that this pathway is insufficient for promotion of anchorage-independent growth or enhanced proliferation.

Analysis of PI(3) kinase activation as measured by phosphorylation of Akt (ser 473) revealed basal phosphorylation of Akt, but no change between the parent (*KRAS*$^{G13D/+}$) and derivative (*KRAS*$^{+/-}$) cell lines (Figure 27, panel B). Suprabasal stimulation of this pathway was isoform-specific; constitutive N-RAS activity did not alter the levels of pAkt though overexpression of H-RAS^{G12V} did, suggesting that in this cell population, basal RAS-independent activation of the PI3 kinase/Akt signaling pathway is sufficient for the maintenance of the transformed phenotype of these cell lines.

RAS mediates at least some of its oncogenic effects by signaling through RalGDS, one member of a guanine nucleotide exchange factor family specific for the Ral family of small G proteins (88, 137, 139, 174). Therefore, we utilized a Ral binding protein binding domain to immunoprecipitate RalA in its GTP-bound state. We observed no differences between the DLD-1 parent line and DKS-8, either expressing the vector alone, or overexpressing constitutively activated H-RAS or N-RAS (Figure 27, panel C).

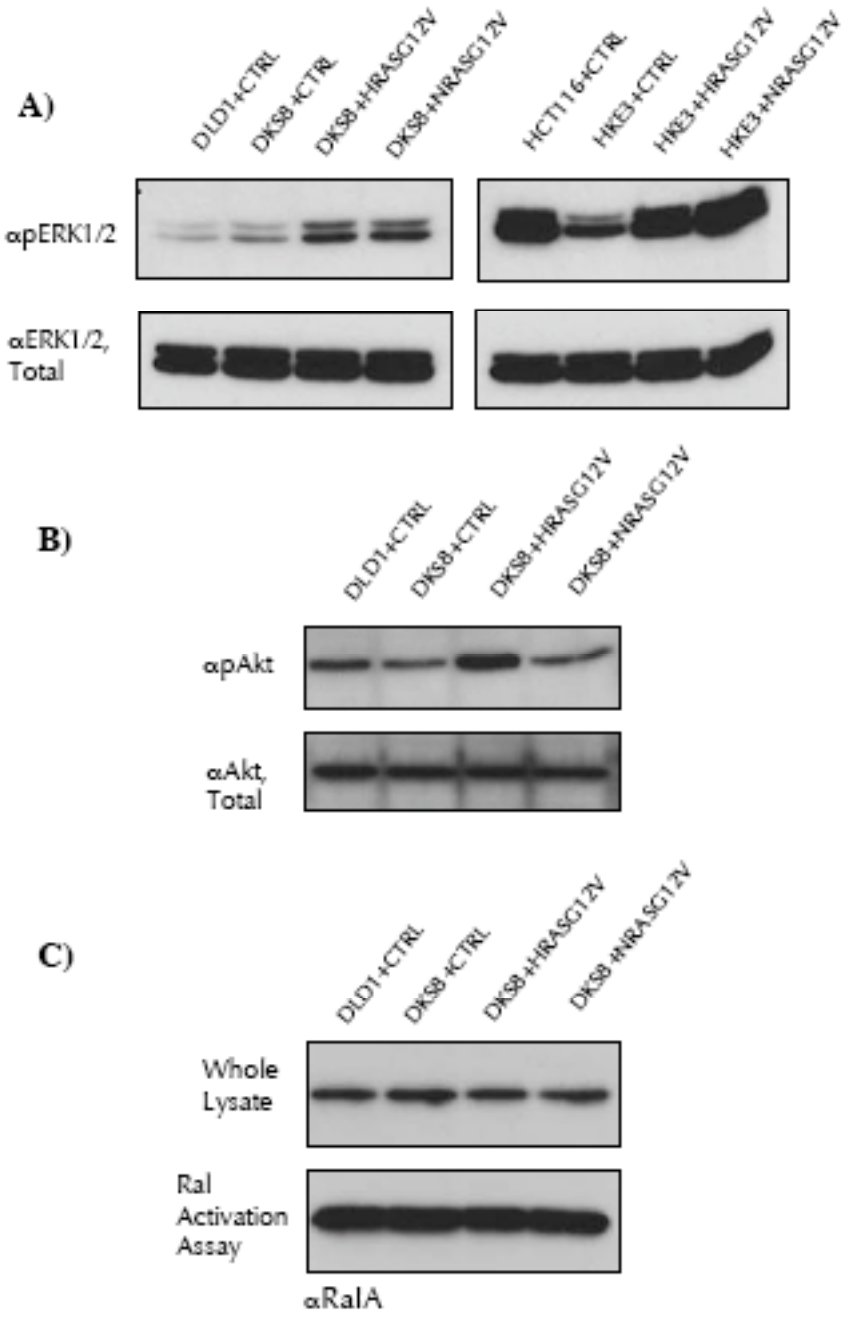


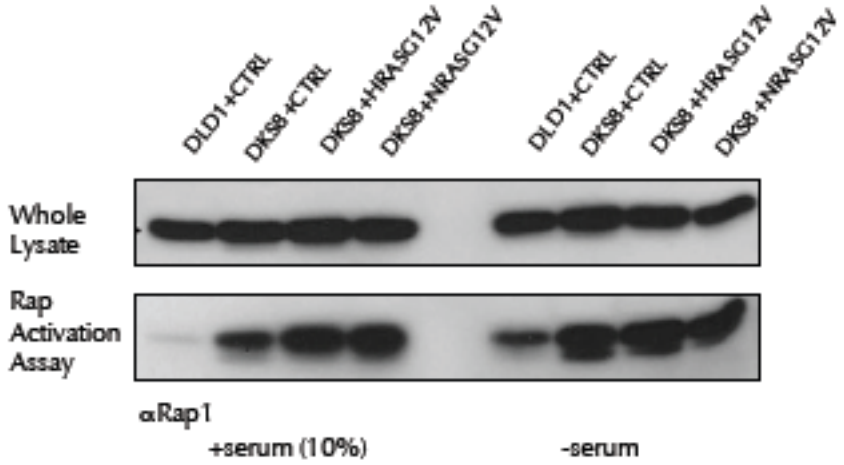
Figure 27. Status of canonical Ras effector pathways in reconstituted cell lines. A) DLD-1 and HCT 116 and their respective isogenic derivatives display differential phosphorylation of ERK1/2. Overexpression of H-RAS^{G12V} or N-RAS^{G12V} result in activation of this canonical pathway. B) Only overexpression of H-RAS^{G12V} results in elevation of levels of phospho-Akt in DKS-8 cells. C) RalA activity appears unchanged in either the parent, derivative, or reconstituted cell lines.

Oncogenic K-RAS is uniquely able promote cell motility in DLD-1 through downregulation of Rap1 activity

In further analysis of possible signaling changes that may be unique to constitutive K-RAS signaling, and which therefore may explain the highly oncogenic effects of activating mutations to this gene, we analyzed the activation state of another effector pathway, Rap1. We utilized a RalGDS Rap1 binding domain fusion protein to precipitate Rap1 in its GTP-bound state. We observed downregulation of active Rap1 as detected by this assay in DLD-1 though not in DKS-8 either expressing control vector, H-RAS^{G12V}, or N-RAS^{G12V}. Total Rap1 levels were consistent across cell lines (Figure 28).

Of the pathways tested, downregulation of Rap1 was the only one in which we observed an effect mediated by constitutively active K-RAS, but not by overexpressed constitutively activated N-RAS or H-RAS. The K-RAS-specific nature of this downregulation led us to hypothesize that Rap1 signaling is an important mediator of the tumorigenic behavior of these cells. To investigate this possibility, we generated cell lines expressing constitutively active Rap1 mutants (G12V) (activity verified by RalGDS RBD pulldown (Figure 29, panel A) as well as hairpins which specifically knockdown levels of Rap1 as confirmed by immunoblot (Figure 29, panel A)).

A)



B)

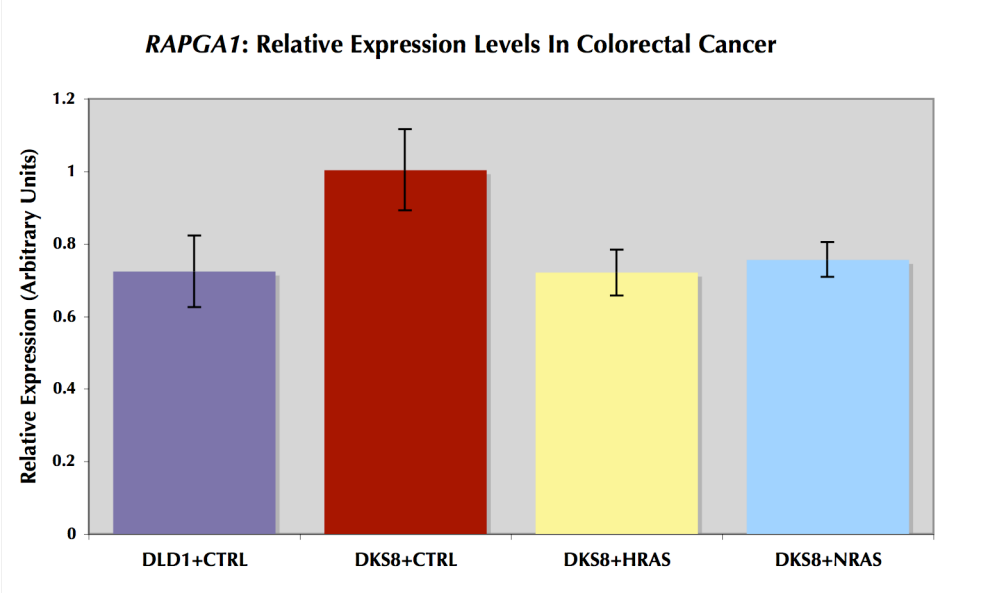


Figure 28. The presence of an endogenous mutant *KRAS* allele results in downregulation of Rap1 activity in DLD-1 cells relative to their isogenic derivatives. A) In isoform-specific fashion, an endogenous mutant *KRAS* allele suppresses activity levels of Rap1. Neither overexpression of mutant H-RAS^{G12V} or N-RAS^{G12V} can reconstitute this phenomenon. B) K-ras has been previously reported to promote this downregulation by elevating transcription of *RAPGA1*, which encodes the Rap GAP, RapGap1 (Sweet-Cordero, 2005). This is not observed in this system, however, as levels of *RAPGA1* mRNA are comparable in all samples.

Rap1 regulates integrin-mediated cell adhesion (13) and also has been shown to participate in regulation of adherens junctions through its interactions with AF-6, a protein that binds Ras as well (126, 186). Given these functional roles, we compared the motility of DLD-1 cells expressing either wild-type Rap1, constitutively activated Rap1, or cells expressing siRNA constructs specific for Rap1 in wound healing assays. We observed significantly different rates of wound closure in these various cell lines as well as changes in the morphology of the migratory edge of the monolayer. Specifically, we observed decreases in the rates of DLD-1 cells overexpressing wild-type Rap1 and, even more dramatically, in DLD-1 cell lines expressing constitutively active Rap1 (G12V) compared to control. Consistent with this, cells with decreased total levels of Rap1 resulting from stable expression of hairpins, exhibited dramatically increased motility relative to control (Figure 29, panels B and D). These results were not the result of differences in proliferation rates, as both Rap1 overexpressors and knockdown cell lines do not display significantly altered growth rates relative to control (data not shown).

We also examined the localization of E-cadherin in these various cell lines to assess the contribution of dysrregulated cell/cell adhesion to altered motility. Constitutively active Rap mutants display stronger lateral E-cadherin staining than do those cells in which Rap1 expression has been knocked down, consistent with enhanced cell/cell adhesion and reduced motility (Figure 29, panel C).

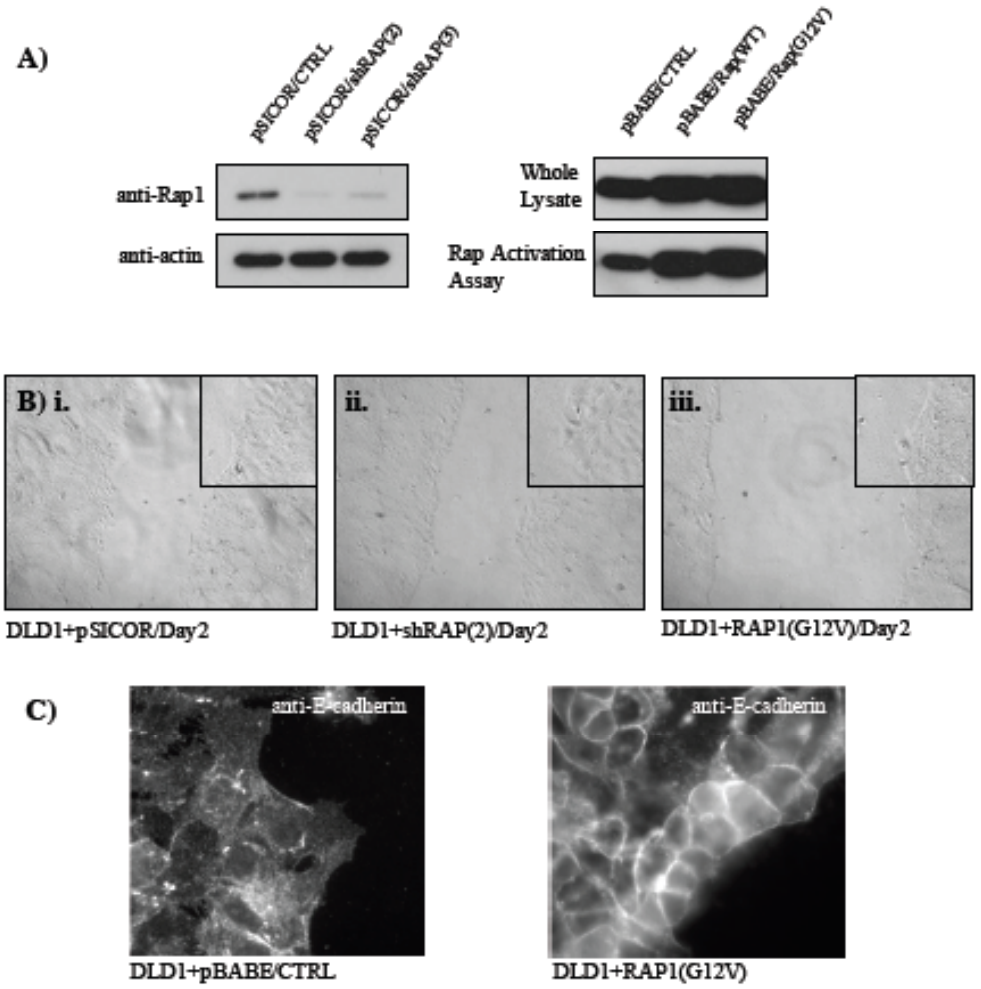


Figure 29. Suppression of Rap1 activity promotes increased motility and non-junctional E-cadherin localization in DLD-1 cells. A) Hairpins specific for Rap1 were designed and show considerable knockdown of Rap1 protein levels. B) Cells stably expressing either Rap1 hairpins or constitutively active forms of the Rap1 protein demonstrated significantly different rates of migration. Wound healing assays show a much increased rate of migration of a monolayer following wounding by cells in which Rap1 levels are reduced; those cells expressing the $Rap1^{G12V}$ mutant migrate much more slowly. C) Slower migration rates in cells expressing the constitutively active form of Rap1 correlate with junctional localization of E-cadherin. D) Relative rates of migration on cell lines

Rap1 was initially identified as an antagonist of K-Ras-driven transformation (75). Additionally, its downregulation has been previously observed in a K-ras-driven lung tumor model in the mouse (159). In this instance, downregulation of Rap1 activity was the result of increased levels of *RapGA1* mRNA, which encode a GTPase activating protein specific for Rap1 (RapGap1). We therefore postulated that similar transcriptional regulation of RapGAP1 may be a conserved mechanism for downregulation of Rap1 activity and tested this possibility by RT-PCR in the DLD-1 and DKS-8 cell lines but saw no difference in transcript abundance (Figure 28, panel B). Though abundance of this transcript appeared unchanged, RapGAP1 represents one of numerous GEF family members capable of regulating Rap1 function, such that comparable regulation of other family members may explain the observed downregulation.

Notably, the changes in Rap1 activity described here were exclusive to DLD-1 and the derivative cell lines. Similar changes in Rap1 activity were not observed in HCT 116 and its derivatives (data not shown). This observation is consistent with the cell line-specific effects of constitutive K-RAS signaling described above, specifically in regard to activation of the Raf/MEK/ERK pathway.

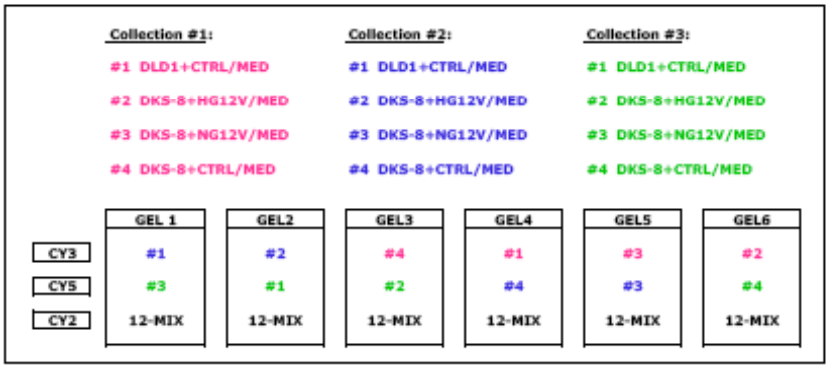
2D DIGE analysis of cells expressing activated Ras isoforms reveals commonalities in classes of proteomic changes but differences in intensity according to isoform

We next sought to conduct a more comprehensive analysis of K-RAS-mediated cellular changes by utilizing 2D DIGE technology to compare the proteomic profile of the DLD-1 ($KRAS^{G13D/+}$) parent line expressing empty vector, DKS-8 ($KRAS^{+/-}$) expressing the empty vector; DKS-8 expressing H-RAS^{G12V}; and DKS-8 expressing N-

RAS^{G12V}. We predicted that the changes that result from constitutive Ras activation might fall into two classes: those that are common to all Ras isoforms but that may differ in intensity, and those that are isoform-specific. Through this analysis, we hoped to eliminate proteomic changes that are common to all Ras isoforms and to identify proteomic changes that are unique to K-RAS and may underlie the unique oncogenicity of constitutive K-RAS signaling.

The experimental design for these studies is illustrated in Figure 30 and is based on a strategy we have previously reported (49). Briefly, lysates were prepared as described in Materials and Methods in triplicate from each of the four cell lines. Equal amounts from each of the 12 lysates were pooled to constitute an internal standard ('12-Mix'). Each of the prepared lysates was then labeled with Cy2 or Cy3 dyes, and the 12-Mix control was labeled with Cy5 dye. Any three differently labeled samples (including one 12-Mix control sample) were resolved in two dimensions on a single gel. Six gels were prepared and run in parallel in order to accommodate all samples as well as controls and to standardize conditions. Spots were blindly analyzed by intensity of Cy dye labeling using DeCyder software, and those changing significantly across lysate samples were picked, trypsin-digested in-gel, and subjected to matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) and tandem (TOF/TOF) MS to provide sensitive and accurate mass spectral data for database interrogation.

A)



B)

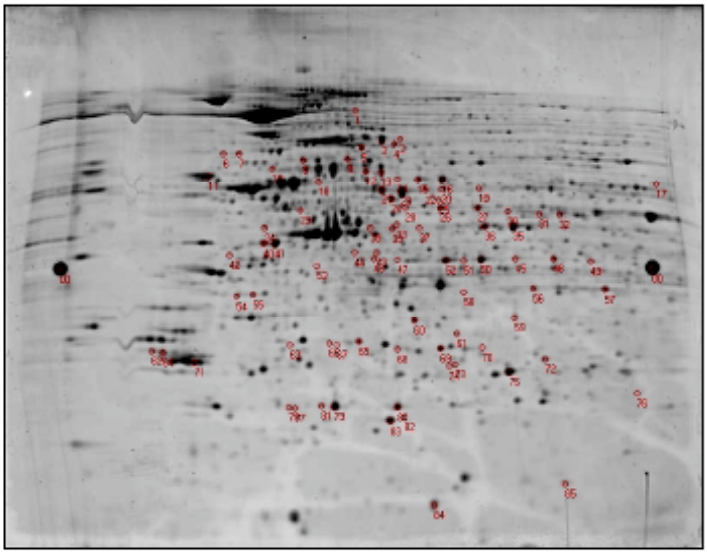
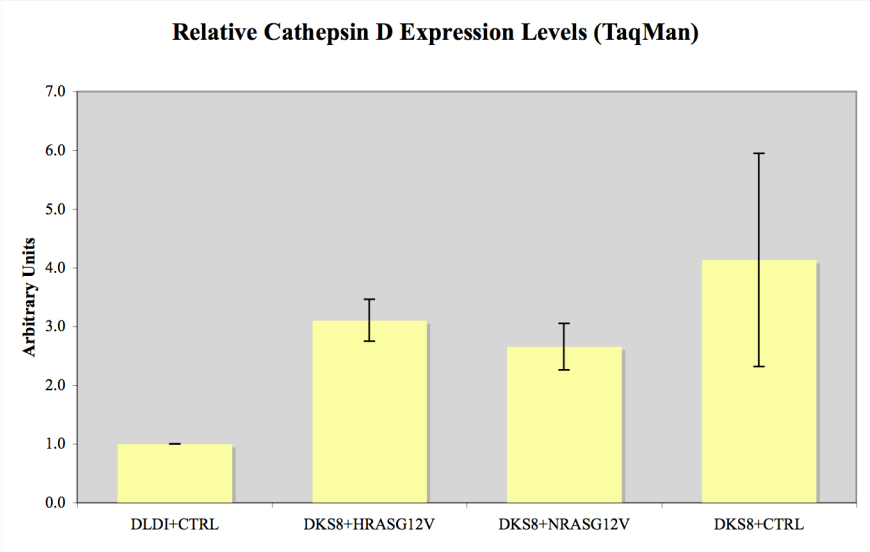


Figure 30. Schematic illustration of 2D DIGE experimental design. A) Triplicate lysate collection and silumatenous processing utilizing triplicate Cy dye labeling allows for analysis of statistical significance and minimizes variation between experiments. B) Sypro Ruby stain of typical pI 4-7 2D gel.

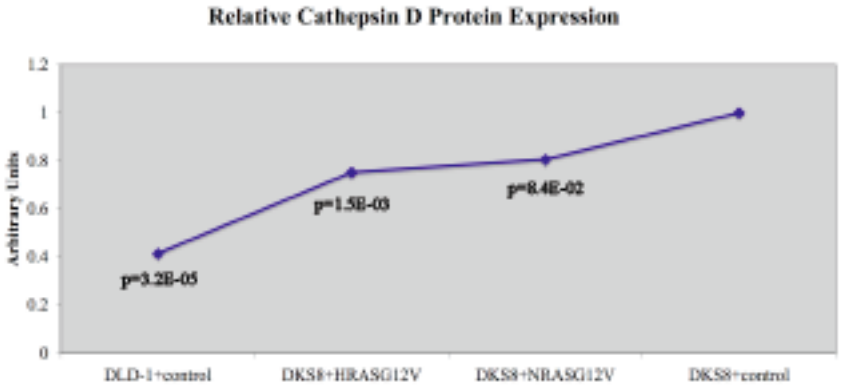
Use of the common 12-Mix internal standard on all gels allowed for standardization of all picked spots across gels, and direct comparison of any two samples from within the 12 sample set. Statistical significance of changing intensities was immediately available due to preparation and analysis of any given lysate in triplicate. These analyses were conducted on the isogenic pairs and their derivative cell lines as illustrated in Figure 30. In parallel, these analyses allowed for the examination of conservation of a given change across cell lines, as well as for identification of changes that were either isoform specific or conserved across isoforms.

Changes in the abundance of individual proteins between the lines based on DLD-1 and those based on HCT 116 showed little conservation; indeed, only one candidate, cathepsin D, showed significant conserved changes across cell lines (Figure 31). Despite this, changes to broader classes were conserved. For example, endogenous oncogenic K-RAS showed downregulation of several protein components of the ubiquitin/proteasome machinery. Similarly, proteins with a known role in cytoskeletal regulation also were downregulated more strongly by oncogenic K-RAS signaling than by H-RAS^{G12V} or N-RAS^{G12V}, or no constitutive RAS signaling at all. We present a summary of the significant proteomic changes Tables 1 and 2. Overall, a trend that appeared in the vast majority of cases was downregulation of protein levels most strongly by oncogenic K-RAS followed by either H-RAS or N-RAS, relative to the isogenic control that had no constitutive RAS signaling.

A)



B)



C)

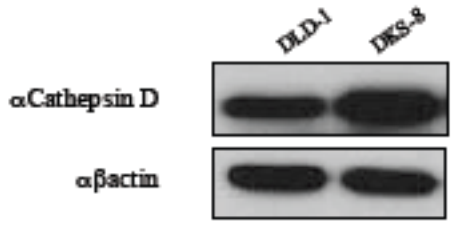


Figure 31. Downregulation of Cathepsin D is a conserved change in DLD-1 and HCT 116 cell lines relative to their isogenic derivatives and is specific to K-RAS. Constitutive activity of the endogenous *KRAS* allele correlates with decreased cathepsin D levels both at the mRNA (A) and protein levels (B and C). TaqMan expression data was standardized against mRNA levels of TBP.

Though not individually conserved across the respective sets of cell lines, other notable changes include relatively strong downregulation of cofilin by constitutive endogenous K-RAS signaling in the HCT 116 cell line. Cofilin is phosphorylated in response to Rho activity. In its phosphorylated form, cofilin is inactive and unable to sever actin filaments and correlates with increased stress fiber formation (96). Downregulation of cofilin as we observe would have a comparable effect. Increased stress fiber formation typically correlates with increased adhesion and diminished motility, effects inconsistent with the transformed phenotype. To explain this paradox, Pollock et al. have reported elevated levels of the AP-1 transcription factor family member, Fra-1 in response to constitutive K-RAS (and downstream MEK1) activity in HCT 116 cells. Fra-1 uncouples Rho activity from stress fiber formation (167) so that in this instance, constitutive K-RAS signaling relies on the simultaneous utilization of antagonistic signaling programs to achieve a selective advantage, enhanced depolymerization of the actin cytoskeleton.

Table 1. Summary of proteomic changes of DLD-1, DKS-8, and N-RASG12V and H-RASG12V overexpressors.

		DKS8 (KRAS+/-)+vector						
Protein	*Accession*	vs. DLD-1+vector		vs. DKS8+HRASG12V		vs. DKS8+NRASG12V		
Identification	*Number*	*Av. Ratio*	*T-test*	*Av. Ratio*	*T-test*	*Av. Ratio*	*T-test*	*ANOVA*
Cytokeratin 8	P05787	1.9	0.01	-1.1	0.58	1.21	0.18	0.0019
GRP 94	P14625	1.87	0.0017	1.1	0.057	1.17	0.12	0.00016
Cytokeratin 18		1.51	0.0038	1.25	0.021	1.52	0.017	0.0026
Peptidyl-propyl cis-trans isomerase	Q02790	1.51	0.0031	1.34	0.0094	1.35	0.013	0.0003
Peptidyl-propyl cis-trans isomerase	Q02790	1.5	0.0023	1.41	0.0013	1.38	0.013	0.00092
Microtubule-associated Protein	Q15691	1.48	0.00069	1.22	0.0045	1.2	0.00054	5.80E-05
Stathmin		1.36	0.017	-1.21	0.024	-1.13	0.3	0.0053
ATP-dependent helicase DDX48	P38919	1.3	0.008	1.28	0.0013	1.3	0.0011	0.00061
Tubulin b-2	P05217	1.29	0.0018	1.09	0.083	1.04	0.29	0.00012
T-complex protein 1 e-subunit		1.28	0.0049	1.18	0.032	1.16	0.019	0.0072
Stress-induced phosphoprotein 1		1.27	0.0002	1.19	0.039	1.23	0.0011	0.0028
Cytokeratin 8	P05787	1.25	0.033	1.2	0.032	1.35	0.0017	0.0075
Proliferation associated protein 2G4	P50580	1.22	0.0018	1.18	0.0093	1.2	0.0045	0.00044
Stress-induced phosphoprotein 1	P31948	1.22	0.007	1.18	0.00031	1.19	0.0093	0.0029
T-complex protein 1 g-subunit	P31948	1.16	0.047	1.15	0.0053	1.14	0.033	0.031
Tyrosyl-tRNA synthetase	P54577	1.03	0.44	1.21	0.0022	1.19	0.072	0.013
PDI A3	P30101	-1.16	0.016	-1.23	0.1	-1.19	0.0084	0.11
Glutathione-S transferase	P09211	-1.25	0.011	-1.02	0.8	-1.04	0.52	0.008
Proteosome activator complex subunit 1	P97371	-1.29	0.0042	-1.15	0.019	-1.06	0.59	0.12
Annexin 5	P48036	-1.32	0.028	1.18	0.036	1.12	0.18	0.0019
GRP 78	P11021	-1.33	0.059	1.35	0.007	1.33	0.082	0.0022
Ornithine aminotransferase (mito)		-1.34	5.40E-05	-1.04	4.70E-01	1.04	6.60E-01	0.0031
D-3-phosphoglycerate dehydrogenase	O43175	-1.41	0.015	1	0.9	-1.05	0.55	0.0024
Calponin 3	Q15417	-1.42	0.0056	1.07	0.53	-1.01	0.83	0.09
Glucose-6-phosphate-1-dehydrogenase	P11413	-1.43	0.0018	1.01	0.87	1.1	0.53	0.006
Isocitrate Dehydrogenase	O75874	-1.56	0.0016	-1.25	0.011	-1.17	0.13	0.00099
Isocitrate Dehydrogenase	O75874	-1.59	0.0024	-1.21	0.069	-1.19	0.13	0.0031
Proteosome Subunit	NP067501	-2.08	0.0026	-1.22	0.12	-1.04	0.77	0.00011
Cathepsin D	P07339	-2.12	0.0021	-1.37	0.0011	-1.26	0.084	0.00065
Cathepsin D	P07339	-2.41	3.20E-05	-1.33	1.50E-03	-1.24	8.40E-02	1.40E-05

Table 2. Summary of proteomic changes of HCT 116, HKE-3, and N-RASG12V and H-RASG12V overexpressors.

		HKE3 (KRAS+/-)+vector						
Protein	*Accession*	vs. HCT116+vector		vs. HKE3+HRASG12V		vs. HKE3+NRASG12V		
Identification	*Number*	*Av. Ratio*	*T-test*	*Av. Ratio*	*T-test*	*Av. Ratio*	*T-test*	*ANOVA*
Cytokine induced apoptosis inhibitor (CIAPIN1)	Q6FI8I	1.64	0.00095	1.13	0.58	1.43	0.25	0.14
hnRNPK	14165438	1.52	0.00057	1.31	0.014	1.14	0.044	0.00023
Mitochondrial processing b-subunit	O75439	1.46	0.013	1.12	0.23	1.02	0.74	0.0023
WD Repeat Protein	Q9gzl7	1.4	0.0052	1.03	0.6	1.06	0.41	0.0014
Proteosomal subunit (II)	O00231	1.39	0.0052	-1.09	0.47	-1.07	0.26	0.006
Elongation factor 1 gamma	P26641	1.37	0.0058	1.05	0.66	-1.06	0.36	0.0064
RevB (TATA box binding interacting protein)	Q9y230	1.27	0.0072	1.14	0.075	1.06	0.45	0.018
HSP70 (isoform)	P38646	1.27	0.00036	1.14	0.051	1.03	0.64	0.0068
t-protein complex theta protein (isoform)	P50990	1.25	0.001	1.26	0.016	1.06	0.64	0.044
Heat Shock 70kDa (HSP70)	P38646	1.24	0.0064	1.06	0.22	1.04	0.23	0.0018
hnRNPK (transforming upregulated protein)	P05091	1.23	0.0044	1.09	0.31	1.13	0.15	0.061
t-protein complex theta protein	P50990	1.22	0.01	1.3	0.0067	1.15	0.25	0.039
GDI 1	P31150	1.17	0.047	1.3	0.0079	1.07	0.54	0.04
Replication protein A	P15927	1.16	0.0019	-1.01	0.69	1.03	0.44	0.002
N-myc downstream regulated protein	Q92597	1.1	0.45	-1.12	0.52	-1.68	0.0033	0.021
Calponin 3	Q15417	1.07	0.37	-1.24	0.0039	-1.01	0.88	0.019
Ubiquitin 1	Q9UMX0	1.01	0.95	1.19	0.0091	1.07	0.44	0.14
Ubiquitin 1 (isoform)	Q9UMX0	1	0.99	1.3	0.0015	1.15	0.24	0.03
Adhesion regulator molecule 1	Q16186	-1.04	0.1	1.11	0.028	1.04	0.24	0.0059
Proteosomal subunit tat binding protein	P17980	-1.06	0.11	1.12	0.022	1.06	0.048	0.0015
Lamin B2	Q03252	-1.1	0.14	-1.37	0.00033	-1.13	0.021	0.001
Proteosome subunit	O35593	-1.1	0.067	-1.38	0.0035	-1.31	0.0079	0.00087
Eukaryotic translation elongation factor 1 delta	P29692	-1.12	0.01	1.07	0.16	1.11	0.13	0.01
HMG Coa synthatase	Q01581	-1.21	0.0012	1.08	0.26	1.08	0.073	0.0017
14-3-3 B-a	P31946	-1.21	0.0013	-1.08	0.0097	-1.04	0.08	0.0001
Periodoxin 2	P32119	-1.21	0.01	-1.06	0.11	-1.07	0.093	0.0037
Stathmin	P13668	-1.22	3.80E-06	-1.15	5.30E-02	1.09	4.10E-01	0.016
Eukaryotic translation elongation factor 1 delta	P29692	-1.22	0.00047	-1.04	0.31	1.03	0.63	0.0077
Keratin 8 (isoform)	P05787	-1.25	0.0063	-1.3	0.00072	-1.33	0.00014	0.00013
Protein disulfide isomerase	P07237	-1.25	0.02	1.01	0.84	-1.03	0.68	0.012
F-actin capping protein	P47756	-1.27	0.0068	-1.22	0.0021	-1.17	0.039	0.0051
Protein disulfide isomerase (isoform)	p30101	-1.27	0.02	-1.22	0.015	-1.16	0.084	0.017
Aldehyde dehydrogenase (isoform)		-1.28	0.0003	-1.17	0.047	-1.15	0.063	0.014
Protein disulfide isomerase (isoform)	p30101	-1.29	0.019	-1.07	0.29	-1.06	0.61	0.11
Glutathione transferase	P09211	-1.31	0.18	-3.03	0.0056	-2.11	0.024	0.0053
Ubiquitin-protein ligase	P27924	-1.31	0.0045	-1.25	0.0037	-1.06	0.18	0.0014
Keratin 8	P05787	-1.31	0.0015	-1.37	0.0095	-1.46	0.0038	0.0018
Guanidinoacetate N-methyltransferase	Q14353	-1.32	0.00098	-1.06	0.25	1.06	0.099	4.50E-05
Keratin 8 (isoform)	P05787	-1.32	0.0097	-1.38	0.002	-1.5	0.0074	0.0032
Protein disulfide isomerase (isoform)	p30101	-1.38	0.00089	-1.2	0.0032	-1.12	0.017	0.00018
14-3-3 sigma form (stratifin)	P31947	-1.4	0.0048	-1.15	0.041	-1.13	0.23	0.022
14-3-3 sigma form (isoform)	P31947	-1.45	5.00E-05	-1.15	5.50E-02	-1.24	8.70E-03	0.00068
ER 29	P30040	-1.56	0.00016	-1.31	0.00049	-1.18	0.025	2.40E-05

Table 2 (continued). Summary of proteomic changes of HCT 116, HKE-3, and N-RASG12V and H-RASG12V overexpressors.

		HKE3 (KRAS+/-)+vector						
Protein	***Accession***	**vs. HCT116+vector**		**vs. HKE3+HRASG12V**		**vs. HKE3+NRASG12V**		
Identification	***Number***	*Av. Ratio*	*T-test*	*Av. Ratio*	*T-test*	*Av. Ratio*	*T-test*	*ANOVA*
Annexin 6	P08133	-1.6	0.00051	-1.32	0.026	-1.26	0.046	0.0038
Aldehyde dehydrogenase (isoform)		-1.6	0.00028	-1.39	0.00034	-1.27	0.012	4.90E-05
Proteosome subunit	Q06323	-1.65	0.00085	-1.41	0.0064	-1.22	0.053	0.00021
Cytokeratin 19	P08727	-1.7	0.0011	-1.52	0.0042	-1.76	0.0011	4.00E-05
Cytokeratin 19	P08727	-1.75	0.00064	-1.53	0.0051	-1.68	0.0015	0.00034
Cathepsin D	P07339	-1.75	0.015	-1.61	0.00085	-1.31	0.0065	0.0023
ER 29 (isoform)	P30040	-1.76	6.40E-05	-1.37	3.90E-04	-1.13	3.20E-03	1.80E-07
N-myc interactor	Q13287	-1.9	0.0046	-1.53	0.022	-1.38	0.047	0.00051
Heat Shock Protein 27kDa (HSP27)	P04792	-2.02	0.00046	-1.62	0.00085	-1.38	0.0036	4.10E-05
Glutathione transferase (isoform)	P09211	-2.04	0.0029	-1.98	0.0035	-1.32	0.15	0.0013
Proteosome subunit (isoform)	Q06323	-2.06	0.00067	-1.56	0.00048	-1.25	0.05	7.70E-05
Proteasome (prosome, macropain) activator subunit 2 (isoform)	Q9ul46	-2.07	0.0027	-1.59	0.012	-1.28	0.15	0.00069
Proteasome (prosome, macropain) activator subunit 2	Q9ul46	-2.13	0.0024	-1.52	0.0037	-1.3	0.079	0.00099
Glutathione transferase (isoform)	P09211	-2.29	0.00029	-1.94	9.10E-05	-1.43	0.0018	1.90E-06
Glutathione transferase (isoform)	P09211	-2.35	0.0015	-2.07	0.0015	-1.33	0.18	0.0012
Glutathione transferase (isoform)	P09211	-2.49	0.00015	-2.18	0.00057	-1.35	0.08	5.30E-05
Cathepsin D (isoform)	P07339	-2.76	0.00011	-2.1	1.30E-05	-1.38	0.0012	4.20E-07
Cofilin	P18760	-2.78	0.0011	-2.35	0.0037	-1.19	0.13	0.00011
Aldehyde dehydrogenase		-2.88	0.00076	-2.14	0.0015	-1.62	0.0071	1.00E-05

Discussion

Mutation of RAS family members to their constitutively active forms is among the most common genetic events in human tumorigenesis, but mutations of the three canonical *RAS* family members segregate strongly by tissue and tumor type, suggesting specificity of function of the individual isoforms in different intracellular environments. There are several hypotheses that may explain this phenomenon, including restricted expression patterns of effectors through which specific isoforms mediate their oncogenic effects, exposure of various tissues to different classes of mutagens that result in mutation of one isoform over another, and specificity of isoform function that confers a selective advantage in the transformation process. These possibilities are not mutually exclusive. We report here the results of an extensive analysis of the specific oncogenic contribution of endogenous mutant K-RAS protein in cells derived from human colorectal tumors that inform some of these hypotheses as well as our broader understanding of the role of RAS in tumorigenesis.

Among *RAS* family members, *KRAS* is the most commonly mutated to its constitutively active form, suggesting it provides a uniquely potent oncogenic contribution. Possible explanations for the prevalence of activating mutations of *KRAS* includes greater mobility of the ubiquitously expressed K-RAS4B within the plasma membrane relative to H-RAS and N-RAS (111); the production of two mutant K-RAS isoforms (4A and 4B) from a single activating mutation (170), each of which displays distinct trafficking, and presumably affinity for separate microdomains of the plasma membrane.

We have exploited two colorectal cancer cell lines that owe much of their transformed phenotype to the presence of activating mutations of *KRAS*, and their isogenic derivatives in which this allele has been disrupted. Because the functional identity of any single RAS isoform is likely composed of common Ras functions as well as isoform-specific functions, we relied on overexpression of constitutively active H-RAS^{G12V} and N-RAS^{G12V} in the isogenic derivatives of these lines to identify molecular changes that may be attributable to general RAS activity and therefore may not illuminate the uniquely oncogenic contribution of constitutive K-RAS signaling. We note that comparison of these parent lines and their isogenic derivatives in order to dissect the oncogenic contribution of *KRAS* mutation is somewhat of an oversimplification. Oncogenic changes accumulated during transformation are not simply additive, discrete units, but rather function in combination to achieve a cumulative effect. We demonstrate that the derivative lines cannot be restored to the parental phenotype by simple overexpression of mutant K-RAS4B cDNA. This finding is consistent with previous reports that restoration of oncogenic signaling following its inactivation cannot reconstitute the transformed phenotype (68). Sudden disruption of oncogenic signaling, as happens following the homologous recombination of the mutant *KRAS* allele in these parent cells, likely triggers a range of events (including, in some instances cell death) that precipitate tumor regression and allow surviving cells to redefine a physiological equilibrium (21, 44, 119). Thus the isogenic derivatives that we study here likely differ from their parent lines beyond the mere presence of a single mutant allele. Despite these qualifications, we maintain that these cell lines represent a uniquely useful tool for studying the contribution of endogenous RAS isoforms to the transformed state of a

disease-relevant cell population, as they obviate the need for overexpression and avoid the pitfalls associated with that strategy.

Our parallel studies of these cell lines lead us to conclude that K-RAS does not promote transformation by any single event; indeed, K-RAS-mediated changes are not necessarily conserved even in cell lines derived from tumors of common origin that display similar genetic background (Figure 8). We do, however, report evidence of K-RAS–driven molecular changes that alter function and likely contribute to the transformed phenotype. Specifically, we observe downregulation of Rap1 activity as a result of constitutive K-RAS signaling. Overexpression of consititutively active Rap1 mutants combined with RNA interference studies demonstrate that Rap1 suppression results in enhanced cell motility and dysregulation of adherens junctions, both commonly observed changes in invasive carcinomas. Our findings here are consistent with those reported by other groups showing that Rap1 activity participates in the maintenance of adhesions junction (78, 126).

Comparison of proteomic changes detected by 2D DIGE analysis of DLD-1 and HCT 116 and their respective derivatives suggests K-RAS mediated proteomic changes are conserved across classes of proteins, rather than individual proteins themselves. In only one instance, the downregulation of cathepsin D levels in the presence of constitutive K-RAS signaling, do we see a strongly conserved individual change across DLD-1 and HCT 116 cell lines. More typically, we observe a pattern of downregulated expression of any given protein most strongly in the case of endogenous oncogenic K-RAS, less strongly by H-RAS^{G12V}, and least strongly by N-RAS^{G12V}. The hierarchical effects of the different RAS family members on the downregulation of a given protein is

conserved in a majority of cases in this data set. Far more rare are instances of proteins that change in the setting of constitutive activity of one isoform but not the others. Further, the hierarchy observed (K4B>H>N) is the same as is seen in the functional reconstitution assays: endogenous oncogenic K-RAS conferred the most transformed phenotype by soft agar growth assays and proliferation measurements, overexpression of H-RAS^{G12V} partially recapitulated these behaviors and N-RAS^{G12V} provided the weakest oncogenic contribution. These data imply that different RAS isoforms may not mediate their oncogenic effects so much through functions that are unique to individual isoforms as through their relative competencies in serving common functions, and maybe interactions between isoforms.

Cathepsin D, the single conserved change across data sets, is also one of the most significantly downregulated proteins observed. Human cancers frequently exhibit dysregulation of the expression of the various members of the cathepsin family of aspartyl proteases (64). Many of these family members are secreted and are thought to promote the invasive phenotype by degrading components of the extracellular matrix. Cathepsin D is not a secreted enzyme, residing instead in the lysosome. It has been identified as mediating early apoptotic pathways, specifically the activation of Bax and the efflux of apoptosis-inducing factor from the intermembrane space of mitochondria (8). Examination of 59 colorectal tumors revealed altered cathepsin D expression in the majority of samples (64). Interestingly, while approximately half of samples showed downregulation relative to normal tissue, one-third of samples showed increased expression. It is notable that the percentage of samples that show cathepsin D downregulation roughly corresponds to the frequency of *KRAS* mutation in these lesions,

although whether tumors that harbor *KRAS* mutations also downregulate cathepsin D levels is unknown (169).

Constitutive activation of *KRAS* by genetic activation has long been considered a common genetic event in colorectal tumorigenesis (169). Approximately half of colon tumors arise *without* the oncogenic contribution of constitutive K-RAS signaling, however, thus implying the existence of multiple means of transformation. It is perhaps not surprising that a specific RAS-driven pathway or set of effectors is therefore not conserved across tumor-derived cell lines. Given the extraordinary selection pressures that exist within a heterogeneous tumor cell population, it may well be the case that individual cells make differential use of selected aspects of constitutive RAS signaling. The requirements for oncogenic RAS change over the course of tumor initiation and maintenance as well, which may well explain the differences we observe here (89). Nonetheless, the lack of conservation of K-RAS mediated changes across tumor lines may frustrate efforts to assign defined functions to a given oncogene and should remind us of the highly dynamic nature of a tumor and the critical importance of context in understanding any molecular or genetic change.

We present evidence that canonical RAS effector pathways are differentially activated in DLD-1 and HCT 116 cell lines. Moreover, canonical pathways long thought to be critical downstream effectors of oncogenic RAS signaling may be differentially activated, or not activated at all, by the presence of constitutive K-RAS signaling. The Raf/MEK/MAPK pathway is the best-characterized RAS effector pathway and is considered critical to oncogenic RAS signaling. Supporting the importance of this pathway is the discovery of constitutively activating mutations of the serine/threonine

kinase *BRAF* in colorectal cancers and melanomas in a non-overlapping fashion with Ras mutations suggestive of an epistatic relationship (28). Our data, however, suggest that activation of this pathway may result from constitutive signaling of any RAS isoform, and that its activation is not sufficient to recapitulate the fully transformed phenotype. Further, the presence of constitutive K-RAS signaling does not necessarily activate this pathway. DLD-1 cells display little, if any, activation of this pathway as measured by ERK1/2 and MEK phosphorylation relative to their isogenic derivatives, yet the two lines display dramatic phenotypic differences. Similarly, 40% of colorectal cancers have some alteration (either mutation or amplification) in one of eight PI(3)K pathway genes (116) seemingly underscoring the importance of another well established effector pathway of RAS signaling. No differences in phosphorylation of downstream signaling target Akt between the parent or derivative cell lines were observed though some activation of this pathway was observed in all cases. Only overexpression of constitutively active H-RAS appeared to increase the phosphorylation of Akt, again supporting the specificity of isoform function.

Our analyses of canonical effectors Raf, PI(3)K, and RalGEF, challenge recent studies in which RAS effector mutants and constitutively active effectors themselves were used to establish that RAS activation of three of these pathways is required for tumor 'initiation' (89). Further, this study reported the requirement for oncogenic RAS signaling by an established tumor to signal through the PI(3)K pathway and promote survival of these cells. Raf and RalGEF family members continue to support the growth of the established tumor as well; their activation becomes independent of oncogenic RAS signaling, however, possibly due to release of paracrine factors in the stroma (89). These

conclusions must be qualified, however, by the fact that these studies are carried out largely in an immortalized fibroblast population, use overexpression of the least commonly mutated RAS isoform (H-RAS), and depend heavily on nude mouse xenografts. Further, the establishment of a xenograft tumor using genetically engineered human fibroblasts does not fall under the classical definition of tumor initiation. Our own results are less clear-cut, but have the advantage of studying the contribution of endogenous RAS in a cell population of epithelial origin. We cannot rule out the possible artefactual contribution of the isogenic system we used here and described extensively in preceding sections. We do demonstrate, however, that cell lines in which the mutant *KRAS* allele has been disrupted but which maintain Ral activity, phosphorylation of ERK1/2, and phosphorylation of Akt fail to effectively recapitulate the transformed phenotype of the parent cell line.

Like the segregation of mutation/isoform specificity that is observed in human cancers, our data from these analyses argue against the interchangeable isoform, 'Ras-is-Ras' approach that has plagued much of the field. It is still quite common to read reports analyzing some aspect or another of transformation or tumor biology and to encounter general references to 'Ras' with no specificity regarding isoform. Like overexpression studies, which may fail to recapitulate the true oncogenic contribution of genetic activation of endogenous Ras alleles for the simple reason that the stimulus is being provided at an almost certainly supraphysiological level, simply activating any Ras isoform in any system similarly fails to model the endogenous environment. Even relatively sophisticated genetic studies, such as those reported by Chin et al. (21) continue to rely on this type of approach. In this instance, a 'melanoma' model is driven

genetically by transgenic overexpression of constitutively active H-ras. Melanoma exhibits neither genetic amplification of Ras nor mutation of *HRAS*. Indeed, H-ras, despite being the isoform least frequently associated with human cancer, continues to be the isoform most frequently used in Ras studies. We can only speculate this is because it was the first isoform identified, though this is hardly justification for continued ignorance some 25 years later.

CHAPTER VI

FUTURE DIRECTIONS

Anticipations, Challenges, Frustrations

The work described in the preceding chapters represents an effort to step out of the typical approaches used to study Ras biology for the past 24 years. While there have no doubt been flashes of brilliance along the way, in proportion to the number of Ras studies that have been generated to date, the vast majority are uninspired and follow the all too familiar a path of overexpression, the use of non-epithelial cell lines, and the 'forced transformation' of tissues that typically do not give rise to Ras-driven tumors. Ras, to the majority of the field, is just another oncogene, used because it's a tool with which researchers have become familiar and for its undisputed ability to drive the behaviors we think of as tumorigenic. When you have a hammer, as they say, everything starts to look like a nail. At a talk at in the Cancer Center a few years back by a distinguished and early pioneer of the Ras field, one attendee was heard to remark, "It's Ras. Haven't we covered that ground?" More disheartening was the fact that the talk then digressed into a sad story of overexpressed oncogenic H-RAS in HeLa cells followed by microarray experiments (or, as described by Frank McCormick, the last resort of the intellectually bankrupt). So the anonymous attendee was right: if the extent of our Ras knowledge is simply overexpression of oncogenic mutants in all the familiar cell lines, we have covered that ground. But that is only the beginning.

The very fact that 24 years of work has piled up about this single family of oncogenes without giving us a solid grasp of what they do is reason enough for any researcher to read the latest Ras literature with a vague sense of frustration, excitement, and wonder that such a simple protein could be utilized and misutilized by a cell in so many ways. Ras is the perfect oncogene in that it's complexity is the complexity of cancer itself.

In light of this, the work I set out to do here was to approach this familiar problem of Ras-driven tumorigenesis with fresh eyes. I have insisted throughout on the study of endogenous proteins, where possible, and the use of disease-relevant cell lines in the belief that proteins as powerful and multifaceted as these can cast many false shadows when viewed in the wrong light. My work here, I believe, has opened some new aspects of how to think about Ras, and raise plenty of new questions as well. These are both frustrating and exciting. The problem that I continually encountered in my studies is the dearth of effective, specific reagents that are available at this point in time. As mentioned previously, the difficulty with simple immunoprecipitations of specific Ras proteins is an excellent example. Antibodies simply do not exist that can specifically immunoprecipitate or recognize by immunofluorescence, endogenous forms of H-Ras or K-Ras4B, and the ones that exist for N-Ras are marginal at best. My own efforts were severely frustrated by the inconsistency of the N-Ras antibodies for IP such that from one experiment to the next, standardized in every way imaginable, results differed dramatically. This is likely the result of the highly interactive nature of Ras proteins; in their active conformation, they participate in numerous complexes of unknown size and dimension. Gelsolin, for example, has never previously been described as a Ras effector

and may in fact not bind Ras directly at all (indirect interaction may well explain some of the inconsistency I describe in the earlier chapters) but rather may be a completely separate participant in a Ras-containing protein complex. The relative size (especially of isoform-specific sequence) of Ras proteins no doubt leaves little in the way of accessible epitope for antibodies to find. The tools we do not presently possess are precisely those we need; the utility of Ras overexpression is quickly fading and our efforts must be redirected toward the endogenous protein.

My ambitions for this work have somewhat outstripped the capacity (both mine and the reagents') to successfully execute it. No doubt my insistence of endogenous proteins and epithelial cells cost me; on the backside of almost six years of work, however, I often look back at the questions that I know to ask now that I did not know to ask then, how they could and should be addressed in order to advance the field. Though frustrating, this is the true hallmark of something learned. Below I discuss in slightly more depth what I consider to be the most salient findings and observations from my coruse of study, and hope that these ideas may help to inspire another set of fresh eyes.

Broader Implications of Ras to Ras Signaling

The phenomenon of one Ras isoform promoting elevated activity of another Ras isoform, and further, the potential requirement of signaling by one Ras isoform to fully promote the oncogenic effects of another isoform is an intriguing and potentially significant advance in our understanding of the role this family of proteins play in promoting human tumorigenesis. The data we describe here report such a phenomenon in cells derived from a single tumor type – colorectal adenocarcinoma. There are

numerous tumor types that exhibit *RAS* mutations, and this phenomenon may be at work far more broadly than these studies demonstrate. The important questions to be addressed are whether or not this relationship between N-RAS and K-RAS is demonstrated in other tumors which rely on *KRAS* mutation such as adenocarcinomas of the lung and pancreas, whether it works bidirectionally (i.e. does the oncogenic contribution of N-RAS in melanoma rely on elevated levels of K-RAS?), and whether or not other tumor types that rely on mutations of different *RAS* family members rely on a similar relationship (i.e. does a bladder tumor driven by oncogenic H-RAS require intact K-RAS or N-RAS function?). It is plausible that the ability of K-RAS to activate other RAS isoforms partially explains its extraordinary oncogenicity among RAS family members. Genetic data support the possibility that the relationship we describe here may be limited to K-RAS and N-RAS. Studies in the mouse suggest a possible linear relationship, or possibly functional compensation for one another, though this evidence does not extend to H-RAS. These questions warrant consideration.

In all these studies, the idea of a 'competitive equilibrium' introduced in pervious chapters is, I think, a useful model with which to approach these questions. Ras family members are different, but still share a number of commonalities in their regulation, localization, and activity that subject them to common influences within the cell. For example, the increase in N-RAS activity we describe in the presence of constitutively active K-RAS could well be the result of 'mass action' of a suddenly underutilized GEF population. Oncogenic K-RAS has no need for turnover of guanine nucleotides and therefore no need for GEFs; it is highly possible that K-RAS and N-RAS share GEF

populations and under such a scenario, N-RAS may experience dramatically increased rates of GTP-loading and thus activity.

We should note that our data is limited to a system for which we have suitable tools for this type of investigation. The isogenic derivation of cell lines by homologous recombination is not without flaw, but represents a highly useful system for the type of analysis we have undertaken here. Unfortunately, comparable cell lines have not been extensively generated for other tumor types, which would allow for expanded analysis of this phenomenon, though advances in the design of gene targeting constructs, including amplification of large homology arms should facilitate generation of these reagents.

A Possible Paradigm of RAS/RAS Biology

We demonstrate a functional effect of RAS/RAS regulation in the differential sensitivity of HCT 116 and HKE-3 cells to apoptotic stimuli, and further, the regulation of the sensitivity by the activity levels of N-RAS and K-RAS. Though we do not show localization of the N-RAS:gelsolin interaction in the cell, a reasonable guess would be that this interaction takes place at the mitochondrial membrane. Indeed, both N-RAS and gelsolin have been independently localized to this membrane, as has been K-RAS4B. Further, H-ras, N-ras, and K-ras4B proteins have been shown to interact with Bcl-2 in a mitochondrial fraction (132), and further, K-ras4B interacts with Bcl-X_L at this platform as well (11). The mitochondria, long recognized as center court for numerous aspects of apoptotic signaling events, appears now to host multiple Ras isoforms, which increasingly appear to play a role in the regulation of these events. The evidence for this is growing from circumstantial status to an accepted paradigm of Ras biology. Ras

molecules interact with multiple members of the Bcl-2 family of proteins, both pro- and anti-apoptotic. Bcl-2 family members play a critical role in the regulation of the mitochondrial permeability transition pore (mtPTP), which includes voltage-dependent anion channel (VDAC) proteins. Interestingly, VDAC proteins appear under some circumstances to be regulated by interaction with gelsolin, which itself appears to participate in complex with N-RAS that may regulate cell survival. Though complex, the molecular events that regulate and constitute the initiation of the apoptotic response may provide an interesting opportunity not only to better understand the events themselves and the role of Ras proteins in their regulation, but also to understand the relationship between Ras isoforms, given the finding we present here as well as others. Again, these studies would greatly benefit from the generation of reagents that would expand the range of possible experiments that could be done, including immunoprecipitation of the H-RAS and K-RAS isoforms.

The field of Ras biology suffers greatly from the dearth of antisera capable of isoform-specific immunoprecipitation and immunofluorescence. Given the challenges associated with immunoprecipitating epitopes in the C-termini of these proteins (the only regions which show significant divergence between family members) with antibodies presently available, it is likely that this represents a suboptimal target for interaction with endogenous proteins in complex with other molecules. Combined with the extensive homology in the rest of the sequence of these proteins, these experiments again underscore the utility of knocking in a N-terminal tagged Ras construct that can be biochemically purified along with interacting molecules.

An Incremental Approach to Modeling and Targeting Tumors

The use of human cell lines in developing therapeutic compounds has been the primary means of compound screening historically. However, compounds and dosages that are effective in arresting tumor growth as gauged by their use on tumor-derived cell lines may have adverse effects when delivered clinically. Some of the challenges associated with optimizing drug design and testing include targeting delivery, identifying maximum tolerated dose, and the possibility of unanticipated non-specific effects.

With the need for a better means of identifying efficacious compounds so pressing, the scientific community has increasingly turned to modeling human cancer in mice using genetic engineering to modify the genomes of animals to reflect the changes that occur in human cancer. Because of the evolutionary conservation between the murine and human genomes, these efforts have proved promising. The best characterized of these models for colorectal cancers is the $Apc^{Min/+}$ mouse, a naturally occurring germline mutation resulting in a premature stop codon in the *APC* allele and attendant truncation of the protein. C57/Bl6 mice carrying this mutation develop adenomatous lesions of the ileum and colon. A similar genetic mutation in *APC* allele of humans is responsible for one of two familial colon cancer syndromes known as familial adenomatous polyposis coli (FAP) which is responsible for a small percentage of colon tumors and typically presents clinically as a carpet of polyps in the late second or early third decade of life.

While the modeling of human cancer in mice has yielded promise to date, there have been challenges associated with this approach. One general challenge arises from the evolutionary divergence observed between the human and mouse genomes. Although

subtle, this divergence has real biological implications for the design of model organisms. For example, small molecule inhibitors which target the ATP-binding pocket of the EGFR family of tyrosine kinases are ineffective against the human protein. Conversely, the FDA-approved antibody C225 (Erbitux), which has proved effective in inhibition of the EGF receptor in some tumor types, does not inhibit the mouse receptor due to divergence in the respective *EGFR* alleles. This has resulted in "humanization" efforts in which the portion of the murine allele encoding the divergent primary sequence is replaced by homologous recombination with a vector carrying the analogous human sequence.

Another difficulty arises from the less easily addressed concern that the lesions arising in genetically engineered mice may not faithfully recapitulate human tumors. A nagging example from the colon cancer field is that of the aforementioned $Apc^{Min/+}$ mouse, which develops lesions primarily in the ileum, and which rarely progress beyond the adenomatous stage. Tumors of the small intestine in humans are extraordinarily rare; even in FAP cases, the vast majority of lesions occur in the colon. Similarly, existing mouse models of colon cancer fail to metastasize despite the fact that metastatic growth is common among advanced adenocarcinomas of the human colon. The natural history of human cancer has been the subject of much research, and whether or not an engineered genetic change or series of changes can faithfully replicate the processes by which tumors arise, and therefore the tumors themselves, is a significant and unresolved question. Additionally, the order and timing of the known genetic mutations may be important in the development of the disease, and at present, the available technology has not allowed for the sequential triggering of more than two genetic events in the colon.

Supporters of the mouse approach to modeling cancer argue that this is a function of the relatively limited state of genetic engineering technologies, specifically the need to sequentially trigger multiple genetic events in the same tissue over time. Detractors would argue that the inability to generate a faithful model of this disease to date given the ease of access to the tissue and our extensive understanding of the genetic lesions that contribute to the aetiology of the disease is evidence of the fundamentally misguided nature of the approach. Indeed, the continued lack of an adequate model of colorectal cancer in the mouse is the elephant in the room, representing simultaneously our best opportunity and most conspicuous failure.

Despite these challenges, colon cancer remains an excellent candidate for mouse modeling given the extensive genetic characterization of the human disease, and indeed, many of the contributing mutations that occur as colon tumors arise have been recapitulated in mice. Currently, efforts are underway to combine the known genetic events that contribute to colon tumor development in a single animal in an effort to generate a mouse model of colon cancer that replicates the progression, pathology, and lethality of the human disease. To date, however, even the combination of multiple genetic events known to provide important oncogenic contributions to the development of colorectal lesions, such as simultaneous loss of *Apc* and constitutive activation of *Kras* on a $p53^{-/-}$ background, appear to do little to change the nature of the lesions found in loss of *Apc* alone (K. Haigis, personal communication). Though the incidence of these lesions is increased, they do not display altered histology and do not appear any more invasive than do those arising in the $Apc^{Min/+}$ mouse. Gauging the fidelity of these models to the human disease will be of paramount significance. This can be approached using

numerous strategies. Expression profiling of a K-ras4B-driven mouse model of lung cancer has recently been used to evaluate the model relative to a series of transcriptional changes that constitute a hallmark of the human lesions (159).

Perhaps a more stepwise approach simultaneously combining *in vitro* study of human cell lines and mouse modeling is a more reasonable middle road. The attempt to engineer a completely faithful animal model of this disease is quixotic in some ways; how can we hope to model a disease of which we have an ever changing understanding? Can such a model be fully validated when our understanding of the human disease is constantly being challenged and reshaped? The mouse is an indispensable tool, to be sure, but may be in over its head in such a bold undertaking at this point in time.

We have preliminarily utilized the previously described HCT116/HKE3 and DLD-1/DKS8 sets of cell lines in combination with the Lox-STOP-Lox *Kras*^{G12D} mouse. These colon cancer-derived cell lines possess, and indeed owe, many of their oncogenic properties to the constitutive activation of the endogenous *KRAS* allele, and we can understand important aspects of K-RAS biology by disrupting this locus. This approach represents a reverse engineering of sorts, moving the transformed cell one step backward with the incremental loss of an individual oncogene. Although this type of analysis is complicated by a host of issues surrounding the possibility of oncogene addiction described previously, it still represents an important and useful tool.

Complementing this type of approach is the converse, the activation of an endogenous allele in an untransformed, fully functional tissue *in vivo* in order to understand the early steps toward transformation. Again, this approach alone is incomplete; we understand transformation to be the cumulative result of several genetic

and epigenetic changes within a cell and a tissue. Presently, however, our lack of understanding of the development of these lesions requires that we first learn the incremental contribution of individual genetic changes.

When used in combination, these approaches offer a potentially powerful system. The cell lines (*KRAS*$^{G13D/+}$) and their isogenic derivatives (*KRAS*$^{+/-}$) I describe here and the *Kras*$^{LSL\text{-}G12D/+}$ mouse genocopy one another at the *Kras* locus: when crossed to a Cre-expressing animal such as the FABP-Cre mouse, this mouse expresses one wild-type and one oncogenic (G12D) *Kras* allele, *Kras*$^{G12D/+}$. This state matches the genotype of the HCT 116 and DLD-1 cell lines. When not crossed to a Cre expressor, the LSL-K-rasG12D mouse expresses only a single wild-type K-ras allele (*Kras*$^{+/-}$), analogous to the recombinant isogenic derivatives of HCT 116 and DLD-1, HKE-3 and DKS-8, respectively.

Despite their limitations, we feel this sort of parallel study is a useful tool for approaching the function and oncogenic contribution of any individual genetic mutation, and may illuminate our understanding of the development and biology of a tumor. Numerous assays are available for the study of either of these systems, including histological and molecular analysis of animal model, and functional and molecular analysis of the *in vitro* system.

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