UNCOVERING THE COMPLEXITY OF RAS SIGNALING NETWORKS

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DEDICATION

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UNCOVERING THE COMPLEXITY OF RAS SIGNALING NETWORKS

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The Raf/MEK/MAP Kinase signal transduction cascade is a key Ras effector pathway that mediates diverse cellular responses to environmental cues, and makes a major contribution to Ras-dependent oncogenic transformation. Here we describe two important aspects to understanding the signaling complexity of any multiphenotypic arrangement, namely, the identification and examination of new effectors and the direct authentication of protein-protein interactions.

We have identified a new Ras effector, IMP (Impedes Mitogenic signal Propagation) that negatively regulates MAP kinase activation by limiting formation of Raf/MEK complexes. The mechanism of inhibition appears to be through inactivation of the KSR1 adaptor/scaffold protein, demonstrating that in addition to promoting signal transmission,

scaffold proteins can function to restrict signal propagation. Ras can inactivate IMP through induction of IMP auto-ubiquitination, facilitating KSR-dependent engagement of MEK by activated Raf. These observations reveal dual Ras effector inputs to the MAP kinase cascade: induction of Raf kinase activity concomitant with derepression of KSR-dependent Raf/MEK complex formation. This relationship provides a mechanism to limit engagement of the MAP kinase cascade in the absence of Ras activation. MAP kinase activation contributes to multiple diverse cellular responses to environment. The capacity to control the amplitude of this response via molecules like IMP likely contributes to flexible and adaptive cellular behavior in the context of complex regulatory signals.

Analysis of the primary amino acid sequence of IMP indicated that it may regulated on multiple levels in different ways. We show that IMP can be phosphorylated by ERK and that this correlates with IMP translocation to the nucleus. We also demonstrate that SUMO-1 exerts negative effects on IMP inhibitory activity. Additionally, a predicted UBP-ZnF was examined for regulation of autoubiquitination. Together, these data point to the existence of multiple regulatory inputs that control IMP activity.

Finally, we discuss the importance of establishing true binding relationships between associated proteins for the characterization of signaling pathways. Using Ras and its putative effector RalGDS as an example, we describe a simple method to this end. By identifying loss-of-function mutations in both proteins that reciprocally compensate to restore a biological outcome, we confirm that RalGDS is a bona fide effector of Ras, thereby proving the validity of this technique for authenticating other protein-protein interactions.

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PRIOR PUBLICATIONS

Sharon A. Matheny, Chiyuan Chen, Robert E. Lewis, and Michael A. White, *Ras regulates assembly of mitogenic signaling complexes through the novel effector protein IMP*. Nature, 427: 457-460 (2003).

In prep. "Establishing bona fide enzyme-effector relationships using complementary point mutations" Sharon A. Matheny, Yu-chen Chien and Michael A. White.

Abstract. "Linchpin proteins in a tumorigenic platform" Michael A. White, Sharon A. Matheny, Yu-chen Chien and Latha Shivakumar. July 2003. American Association for Cancer Research 2003 Annual Meeting.

Abstract. "SAM: A RING-H2 Ubiquitin Ligase that Regulates Ras Signaling through the ERK1/2 Kinase Cascade" Sharon A. Matheny, Bing-e Xu, Robert E. Lewis, Melanie H. Cobb and Michael A. White. March 2002. Keystone Symposium "Regulation of Kinase Cascades". *Awarded Keystone Symposia Scholarship*

Abstract. "SAM: A RING-H2 Ubiquitin Ligase that Regulates Ras Signaling through the ERK1/2 Kinase Cascade" Sharon A. Matheny, Bing-e Xu, Robert E. Lewis, Melanie H. Cobb and Michael A. White. August 2002. Gordon Research Conference "Mechanisms of Cell Signaling".

Abstract. "Complexities in the Functional Organization of Ras/MAP Kinase Cascades" Anthony Anselmo, Ron Bumeister, Sharon Matheny, Latha Shivakumar and Michael A. White. April 2001. Keystone Symposium "Signaling Systems: Chemistry, Biology and Pathology".

Abstract. "Functional Organization of Ras Signaling Cascades" Michael A. White, Pingsheng Liu, Sharon A. Kelly, Dale Henry, Richard G. W. Anderson. 1999. Keystone Symposium "The Functions of Small GTPases".

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LIST OF ABBREVIATIONS

ATP	adenosine 5'-triphosphate
CNK	Connector Enhancer of KSR
CMV	cytomegalovirus
EGF	epidermal growth factor
ERK	Extracellular Regulated Kinase
GEF	guanine nucleotide exchange factor
GAP	GTPase-activating protein
GDP	guanosine 5'-diphosphate
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine 5'-triphosphate
HA	hemagglutinin
HEK293	human embryonic kidney
HOG	high osmolarity glycerol
IMP	Impedes Mitogenic signal Propagation
JIP	JNK-interacting protein
JNK	c-Jun N-terminal kinase
KSR	Kinase Suppressor of Ras
MAPK	Mitogen activated protein kinase

MEK	MAPK/ERK Kinase
$MgCl_2$	magnesium chloride
NaCl	sodium chloride
PBS	phosphate buffered saline
PC12	pheochromocytoma
PCR	polymerase chain reaction
PI3K	Phosphatidyl inositol 3' kinase
RalGDS	Ral guanine nucleotide dissociation stimulator
Ras	rat sarcoma
RING	Really interesting new gene
RKIP	Raf kinase inhibitory protein
RLIP	Ral-interacting protein
RNA	ribonucleic acid
RNAi	RNA interference
SDS	sodium dodecyl sulfate
SRE	serum response element
Ste5	Sterile 5
SUMO	Small ubiquitin-like modifier
Sur-8	Suppressor of Ras

c-TAK-1	Cdc25C-associated kinase
UBP-ZnF	Ubiquitin protease zinc finger

CHAPTER ONE

Introduction

Achieving Specificity in Molecular Signaling: A Complex Issue

In recent years, there has been an explosion of information about how cells respond to their environment; how they interpret extracellular signals into a coherent process of enzymatic reactions to produce a response that is beneficial to the unicellular animal, or the coordinated cellular responses required by the multicellular metazoan. At any one time, the cell receives multiple signals from varied sources—hormones, growth factors, environmental stress, etc—that eventually converge on experimentally testable outcomes, or observable cell phenotypes. Of great interest and importance to human disease is how cellular responses are controlled to result in the correct behavior. For instance, activation of the Ras proto-oncogene results in the activation of multiple proteins, or effectors, with which it interacts. As individual effector molecules may mediate opposing effects on cells, such as proliferation versus differentiation, it may be presumed that all Ras effectors are not activated at the same time. To understand the contribution of Ras to cell regulation, therefore, is to understand how a particular effector is chosen, in response to which extracellular stimuli, the identities and functions of its downstream partners, and the cellular consequences of such activation.

In an atomic sense, Ras effector discrimination occurs at the level of the switch regions, specific amino acid sequences that have been shown to adopt several distinct conformations in the GTP-bound state, and may afford Ras and other G-proteins the ability to distinguish between multiple ligands [1]. Yet how a particular effector is chosen

for a given signaling event is still unclear. Early models proffered random diffusion as a possible mechanism, yet this is inefficient and unlikely given the observed alacrity of signal transmission in stimulated cells. More recently has been the vague concept of "recruitment" to active Ras proteins. Recent studies indicate that there may be truth to this idea, in the form of scaffolding proteins that can simultaneously associate with several components of a specific signaling pathway. In unstimulated cells, scaffolds would hold essential proteins away from upstream activators to prevent 'leaky' signal transmission, much in the way a short circuit stops electron flow. Upon stimulation, the scaffold and its associated proteins would translocate to the activator and complete the circuit. Thus, a cellular response may be thought of as the coordinated movement of multilevel signal transduction machines whose dedicated functions and binding specificities are predetermined.

The Ras G-protein

Over twenty years ago, the Ras oncogene was identified as a protein encoded by certain retroviruses known to cause cellular transformation in animals upon infection [2]. The v-Ras gene was subsequently discovered to be a mutationally activated form of c-Ras, a cellular protein conserved throughout eukaryotic evolution, which had been acquired by the viruses long ago [2, 3]. The discovery of the c-Ras gene in human carcinomas confirmed the notion that aberrant proteins contribute importantly to human cancer [2], and precipitated the ensuing intense examination of Ras function in cellular transformation and normal biology. What emerged out of the first fifteen years of study was the description of an elegant system of signal transfer from liganded growth factor

receptors to regulatory proteins that activate Ras enzymes, which in turn activate a canon of kinases that communicate directly with transcription factors in the nucleus [3]. Yet, as one may have guessed, given the pivotal role of Ras in normal cellular regulation, the situation is much more complicated than previously thought. In fact we have only a nascent understanding of the integrated function of Ras, which we now know extends beyond the province of an activator of a single kinase cascade. Indeed, Ras occupies an established niche in signal transduction as a conveyor of stimulus-specific information from membrane-associated receptors to a variety of downstream recipients.

Ras is a small monomeric GTPase that is expressed in all cells of the body. Like all GTPases, its enzymatic activity is regulated by a cycle of guanine nucleotide binding in which association with GTP activates the protein, while binding to GDP maintains the protein in an inactive state. Determining which guanine nucleotide will be bound, and hence regulating the activity of Ras, are two classes of molecules [4]. Guanine nucleotide exchange factors (GEFs; RasGRF, SOS1/2) promote the release of nucleotide (GDP or GTP) from Ras, which inevitably results in Ras binding to GTP, as it is the most prevalent guanine nucleotide in the cell. Thus, GEFs are positive modulators of Ras activity. Conversely, GTPase-associated proteins (GAPs; p120 GAP, NF1/neurofibromin) antagonize Ras by stimulating the intrinsic GTP hydrolysis activity of Ras, which on its own is very slow, leaving Ras bound to GDP. Point mutations in Ras at residues 12, 13, or 61, which occur in a significant proportion of human cancers, severely disrupt its ability to associate with GAPs, resulting in constitutive activation of Ras independent of upstream signaling [5].

Ras is represented by four isoforms encoded by three separate genes, H-Ras, N-Ras, K-RasA, and K-RasB. H- and K-Ras are named for the murine sarcoma viral strains in which they were identified (*H*arvey and *K*irsten), and N-Ras was named for the SK-N-SH *n*euroblastoma cell line in which it was first discovered [4].

The Ras proto-oncogene

While a causal role for Ras in human cancer has not been proven, its role as a central component in the genesis and maintenance of cancer is clear. Ras is mutated in 20% of all cancers, and is the most frequently mutated oncogene in solid tumors and neoplasias [5]. Oncogenic mutations of Ras also strongly correlate with chemoresistance in malignant melanoma [6].

The most frequently mutated Ras protein is K-Ras, which is mutated in 90% of pancreatic cancers and 60% of colorectal cancers [5]. H-Ras mutations have been found in lung adenocarcinomas [7] and follicular and undifferentiated thyroid carcinomas [8]. Mutations in N-Ras are commonly found in hematopoietic cancers, being present in 60% of chronic myelomonocytic leukemias and 40% of acute myelogenous leukemias [5].

Besides its apparent complicity in carcinogenesis as an oncogene, Ras has other activities that may contribute to cellular transformation, particularly with regard to its ability to modulate the immune response. For example, Ras activation leads to downregulation of major histocompatibility class I, which impairs the ability of killer T-cells to identify aberrant cells [9]. Ras also downregulates the tumor necrosis factor receptor, which causes resistance to the cytotoxic effects of TNF [10]. Similarly, Ras-mediated downregulation of Fas can result in resistance to Fas ligand-induced apoptosis

of tumor cells [11, 12]. Finally, active Ras interferes with the cytokine dependency of certain hematopoietic cell lines, which is a characteristic of leukemic cells, and the cytokines that are induced by Ras, such as TGF-β, actively suppress T-cell activation [13].

Signal transduction through Ras

Effector proteins

The means by which extracellular signals are passed on from Ras are via direct associations with effector molecules (Fig 1.1). Ras bound to GTP selectively associates with its effectors via its switch I and II domains, effectively coupling GTP hydrolysis to signal throughput [14].

The best characterized Ras effector is c-Raf-1, one of three Raf isoforms. Raf is a serine-threonine kinase that is first in the sequence of three kinases that ends in the activation of extracellular-regulated kinase 1/2 (ERK1/2), and mediates mitogenic signaling and uncontrolled growth in the case of cellular transformation. Raf is activated by Ras-GTP and association with the plasma membrane [15]. Raf activates MEK by dual phosphorylation, which then goes on to activate ERK by phosphorylation on threonine and tyrosine. Raf has other functions that appear to be independent of MEK/ERK, including roles in ERK5 signaling [16] and activation of NFκB [17]. When present on the outer mitochondrial membrane, Raf-1 directly or indirectly induces phosphorylation of Bad, which may account in part for the apoptotic effects of Ras activation [18].

Another well-studied Ras effector is phosphatidyl inositol 3' kinase (PI3K), a lipid kinase. Ras directly associates with the p110α catalytic subunit of PI3K, which results in

enhanced lipid kinase activity and increased cellular concentrations of PI(3,4,5)P3 [19]. PI(3,4,5)P3 can bind and activate Rac guanine nucleotide exchange factors (RacGEFs) [19]. Activation of RacGEFs induces nucleotide exchange on RacGDP for GTP, thus initiating changes in cellular morphology and activation of downstream molecules important for signal convergence between the ERK1/2 and Rac pathways that results in synergistic activation of mitogenic phenotypes [20].

Finally, Ral guanine nucleotide dissociation stimulator (RalGDS) is a Ras-interacting protein that may serve as an intermediary through which Ras can regulate the activity of Ral. The exchange activity of RalGDS appears to be regulated by subcellular localization, which is altered upon Ras activation [21]. Chapter 4 discusses RalGDS in greater detail and demonstrates the biological validity of its interaction with Ras.

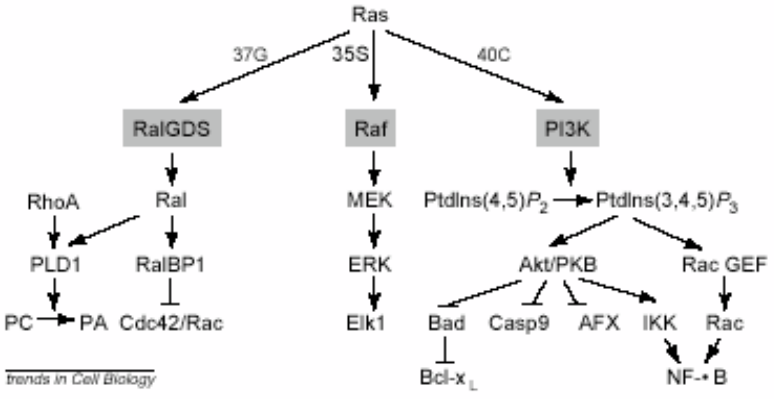

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Figure 1.1 Ras effector pathways.

ERK1/2

ERK1/2 is a mitogen-activated protein kinase (MAPK) and as such is the last of a three-kinase signaling module that is conserved throughout evolution [22]. In this

capacity, ERK receives information from its upstream kinases Raf and MEK, activated by extracellular signals passed down through Ras, and relays this information to multiple cytoplasmic, membrane and nuclear substrates [23]. These signals may come from liganded receptor tyrosine kinases, G-protein-coupled receptors, or integrins [23].

Signals are passed down in the form of phosphorylation events on specific residues, depending on the type of kinase; Raf and ERK modify serine and threonine, while MEK modifies threonine and tyrosine. A possible reason for the existence of three kinases in a common pathway is the substantial signal amplification that can be achieved. In fact, such is the amplification through the sequential activation of MEK and ERK that only 5% of endogenous Ras proteins need to be activated for complete engagement of ERK function [24].

Activation of ERK has pleiotropic effects and has been implicated in cell growth, differentiation, apoptosis, and cell migration [25]. These responses depend on cell type, cell state/fate, and stimulus. As a required element for growth in cell culture, ERK engenders multiple effects to this end, including enhancement of nucleotide synthesis, phosphorylation of immediate-early transcription factors, and formation of the active cyclin D-CDK4 complex that is rate-limiting for $G_1 \rightarrow$ S phase progression [23]. It has also been suggested to play a role in mitotic progression following cdc2 activation and mitotic exit [26]. Interestingly, ERK activation can lead to seemingly contradictory results. For example, neuronal-derived PC12 cells require ERK for both proliferation and differentiation, which are opposite cell fates. The intensity and duration of the signal correlates with the outcome, where transient ERK activation by EGF promotes proliferation and prolonged ERK activation by NGF results in differentiation [27, 28].

As these cellular functions must be tightly controlled for normal cell behavior, a high degree of specificity must be maintained to ensure appropriate responses to a given stimulus. It is curious, then, that ERK is expressed in the same cells as other highly similar MAPKs that recognize some of the same substrates [29]. ERK is 60% homologous to JNK and p38, which are involved in stress responses and cytoskeletal remodeling, respectively [23]. So how do these kinases achieve the sort of specificity necessary to mediate distinct cell functions without nonproductive effects on other pathways? Fundamentally, specificity in signaling is achieved through regulated protein-protein interactions. For kinase cascades this means regulated organization of the three-kinase module, and controlled interactions with substrates, activators and regulators.

Stimulus-appropriate responses by a given cascade are likely achieved through association with scaffolding proteins, which bind some members of the same kinase module simultaneously. In terms of signal control this makes sense, since the cellular location of a pre-assembled complex is easier to regulate than individual enzymes that must first be collected before signaling can occur. Thus, scaffolds can be locally 'assigned' to particular receptors or other activators for stimulus-specific induction of the correct pathway. (See the next section for more on scaffolds.)

ERK recognizes its cognate interactors via complementary docking sites. On the interactor these are either distinct domains, such as the D-domain, or amino-acid sequence motifs, such as the FXFP motif [30]. The D-domain comprises an area of positively charged residues that binds a patch of negatively charged residues on ERK [23]. Since all D-domain-containing interactors will recognize the same domain in ERK, binding events are mutually exclusive, thus ensuring sequential activation, substrate

recognition and deactivation [23]. Yet as with anything biological, the situation is not that simple. For example, exchanging the docking site on ERK for the site on p38 still allows MEK binding but not MKK6, the MAPKK for p38 [31]. It is more likely that a broader three-dimensional structure in addition to the docking site is required. The phosphorylation state of ERK also significantly affects the affinity of the interaction. For example, the transcription factor Elk-1 has greater affinity for the active dually-phosphorylated ERK, whereas MEK binds better to the inactive kinase [23]. Also, the position and number of FXFP motifs and D-domains on a substrate can direct ERK phosphorylation on particular serine/threonine residues and in different combinations to achieve different biological outcomes [30]. Finally, phosphatases can influence access of ERK to its interactors, not only by ERK dephosphorylation but also by obscuring its docking sites [23].

On a larger scale, specificity in ERK signaling can be achieved by its intracellular localization, which can be modulated in stimulus-specific ways. In the absence of stimulus, ERK constantly shuttles between the cytoplasm and the nucleus [32]. Upon mitogenic stimulation, active ERK rapidly accumulates in the nucleus during an initial burst of translocation, then re-accumulates after several hours of stimulation [23]. Differentiation signals cause the initial nuclear entry but not the longer-term accumulation. However, this response depends on cell fate; in cells fated to differentiate, only a differentiation signal results in both initial and secondary nuclear accumulation as with mitogens [23]. In fact, cytoplasmic retention of ERK may be important for maintaining a differentiated phenotype in some cells, as suggested by experiments

showing that deletion of the ERK regulator PEA15 increases proliferation of terminally-differentiated astrocytes [33].

Scaffolding proteins

While the epistasis of the ERK kinase cascade is firmly established, the mechanisms that regulate signal relay between kinases are only beginning to be understood. For instance, it is known that Ras activation is required for Raf to activate MEK, but it is unknown whether additional proteins are required as well. Certain observations suggest that protein adaptors, or scaffolds, are important for signal transmission through this pathway. These proteins physically connect kinases and substrates together. These connections may serve to isolate, and perhaps stabilize, kinases as a signaling module and to bring individual components into spatial proximity to each other and to sites of action in order to enhance temporal responses [29]. They may also insulate particular pathways from nonproductive crosstalk with homologous signaling constructs. Scaffolds, then may allow for more specific and/or efficient responses amongst multiple signaling events that can occur at any one time.

The prototypical scaffolding molecule is Ste5p, the first MAPK scaffold to be described. Ste5p contains multiple protein binding domains and simultaneously associates with the MAPK kinase module (Ste11p, Ste7p, and Fus3p) required for the mating response in *S. cerevisiae* [34]. Fus3 is highly homologous to another MAPK, Kss1, with which it shares the same upstream activators, Ste11 and Ste7 [23]. Yet, each kinase mediates a very different biological outcome, with Fus3 dedicated to the mating response and Kss1 involved in filamentous growth. An important difference between

these enzymes is their association with Ste5. Fus3 is activated in a scaffold-dependent fashion by transient, high concentrations of stimulus. By contrast, Kss1 responds to sustained, low levels of stimulus without requirement for a scaffold [23].

Other MAPK scaffolds have been characterized, such as PBS2, which binds the HOG kinase module for osmosensation [23]; JIP1/2, which mediates stress responses by association with the JNK kinases [35]; and MP1, which promotes MEK and ERK interactions [36]. Other putative scaffolds include spinophilin [37] and POSH [38]. It is important to note that while scaffolds provide a framework on which to build a functional signaling system, these constructs are not stably assembled. For example, during vegetative growth Ste7 and Ste11 are cytoplasmic, whereas Ste5 and Fus3 shuttle between the cytoplasm and nucleus [39]. Upon exposure to mating factor, scaffold and kinases localize to the cytoplasm. A role for nucleocytoplasmic transport has been suggested for regulation of the MEK/ERK scaffold KSR [40].

The importance of scaffolds to biology has been demonstrated by experiments examining the effects of their absence. It known that Ste5 is itself required for response to pheromone [34]. More striking is the finding that JIP1/2-/- mice are defective for responding to stress in brain [41], consistent with its suggested role in organizing the JNK module.

Of much recent interest are scaffolding proteins discovered in genetic screens in *Drosophila* and/or *C. elegans* for suppressors of activated Ras phenotypes. Most notable of these are the Raf-binding proteins KSR [42], CNK [43], and Sur-8 [44], which may function to enhance signaling between Ras and Raf or Raf and MEK. These proteins are particularly interesting because of the level of target discrimination required during

simultaneous activation of membrane receptors, with regard to multiple Ras effectors and Ras-independent signals that go through Raf; these putative scaffolds may provide that specificity. While Sur-8 and CNK are required for Raf to associate with the cell membrane in S2 cells [45], and are known to be important for Ras signaling, whether or not they serve as scaffolds has yet to be determined. However, the current data on the role of KSR in Ras signaling argue strongly for its status as a bona fide scaffolding molecule for the ERK kinase module.

Kinase Suppressor of Ras

Kinase suppressor of Ras (KSR) was discovered in a genetic screen for suppressors of activated Ras phenotypes in *Drosophila* and *C. elegans* [42]. Epistasis studies using activated components of the ERK pathway place the impact of KSR below Ras but upstream or parallel to Raf [42]. KSR is structurally related to Raf, and like Raf, binds to Hsp90, p55/CDC37, and 14-3-3 proteins [36]. However, KSR differs from Raf in three important ways [36]: 1) it does not contain the Ras-binding domain found in other Raf-like molecules; 2) the putative kinase domain (CA3 domain) has an arginine (R589) in place of lysine, which, in other kinases, is required for transfer of the phosphate group; 3) it contains a unique domain of ~40aa at its N-terminus (CA1 domain).

The scaffolding activity of KSR is suggested by its ability to directly interact with components of a common signaling cascade. It constitutively binds MEK and interacts with ERK in a stimulus-dependent fashion [46]. Both kinases bind directly via the CA3 domain, which contains a possible ERK docking site (FXFP). The function of KSR seems to be intimately linked to its ability to associate with MEK. In fact, the KSR

mutant discovered in a *C. elegans* Ras suppressor screen contains a single point mutation (C809Y in murine KSR) that prevents it from binding to MEK [47]. KSR is required for ERK activation by insulin or phorbol ester in *Drosophila* Schneider (S2) cells [45], which correlates with observations that it is required for proliferation and differentiation of *Drosophila* embryos. Interaction with 14-3-3 and Raf occurs at the CA1 domain; however, whether the association with Raf is direct has yet to be shown conclusively. Raf interaction has been suggested to depend on Ras activation, yet others have shown Ras-independent binding and indicate that it may be direct.

Consistent with a scaffolding function, KSR can become inhibitory if greatly overexpressed, due to sequestering individual signaling components away from each other. Some would argue that both kinase and scaffolding functions are required for full activation of the ERK cascade [48, 49], while others believe that KSR only functions as a scaffold, as its proposed ceramide-activated kinase activity [50] has yet to be recapitulated. Sugimoto et al suggested that KSR-mediated inhibition of Elk-1 activity depends on an intact CA3 kinase domain, since the R589M mutant was defective for Elk-1 inhibition [48]. However, rather than defective kinase activity, a more plausible explanation is that MEK association is required for KSR inhibit Elk-1.

Work by several groups [36, 47, 51] has demonstrated that enzymes of the ERK pathway exist in very high molecular weight complexes (>700kD) that disappear in the absence of KSR. In a well-controlled study, *Drosophila* KSR complexes were observed in a homologous system [36]. Using *Drosophila* homologs of KSR, MEK, and Ras in S2 cells, they showed that KSR promoted Raf-mediated MEK phosphorylation only if it could bind Raf and MEK individually. Ras activity was not required for Raf and KSR to

interact; co-localization of KSR and Raf was sufficient to activate MEK in quiescent cells and in transgenic flies expressing these proteins in the eye, as evidenced by a rough eye phenotype. Also, RNAi against Ras or MEK in flies had no effect on a KSR-Raf interaction. This is consistent with other reports in S2 cells that KSR is required for activation of Raf by phorbol ester or insulin, but not Ras [45].

Work by Morrison and colleagues [46] suggests that the subcellular localization of KSR is regulated by phosphorylation by the serine/threonine kinase c-TAK-1. Phosphorylation of serine 392 (S392) by c-TAK-1 promoted 14-3-3 binding to KSR and retained it in the cytoplasm. Upon EGF stimulation, S392 was rapidly dephosphorylated and lost association with c-TAK-1, resulting in movement of KSR to the plasma membrane, where it associated with ERK and Raf (Fig 1.2). Substitution of serine for alanine at this site maintained KSR at the plasma membrane and promoted ERK binding and Ras signaling on its own, thus sensitizing the pathway to stimulation. These data provide insight into how scaffolds may be regulated and underscore the importance of controlling the intracellular location of multiprotein complexes.

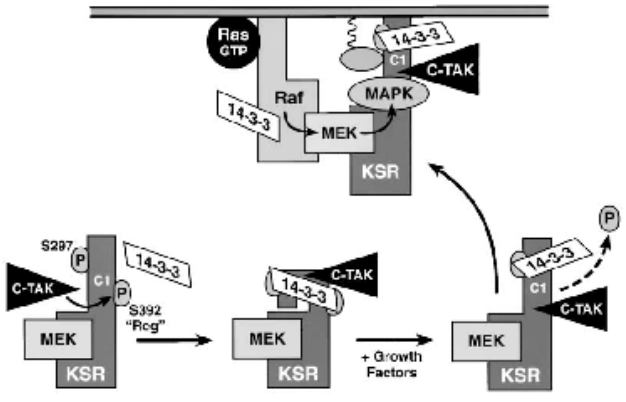

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Figure 1.2 A possible model for KSR scaffolding of the MAPK pathway.

Ubiquitin and ubiquitin-related modifiers

Ubiquitin

Ubiquitin has long been known as an oligomeric tag for proteins destined for destruction by the proteasome. Ubiquitin modification is now known to have multiple roles, in cell cycle regulation, modulation of transcription, signal transduction, and antigen presentation--and not always as a messenger for elimination [52].

Ubiquitin is an 8 kD protein that is conserved from yeast to human. It is covalently attached to substrates in a sequential three-enzyme reaction that begins with the activation of free ubiquitin (see Fig 1.3). In an ATP-dependent step, free ubiquitin is activated via a thioester bond between the C-terminus of ubiquitin and an E1 activating enzyme. Ubiquitin is passed down via thioester linkage to an E2 ubiquitin-conjugating enzyme and finally to an E3 ubiquitin ligase, which mediates modification of the target molecule. This process is repeated for ubiquitin chain elongation, with subsequent ubiquitin molecules transferred to the previous one [52].

There are two types of E3 ligase, distinguished by their enzymatic domain and mode of ligation. The HECT domain E3s receive activated ubiquitin like E1 and E2, through an isopeptide linkage that it passes on the target; HECT E3s are thus true enzymes. RING domain E3s can function as multiprotein complexes, like the SCF complex involved in cell cycle regulation, or individual enzymes such as the tumor suppressors BRCA1 or MDM2 [53]. RING domain E3s simultaneously bind the E2 and target protein and hence facilitate direct ubiquitin transfer by bringing the proteins into spatial proximity. There is another type of E3 ligase domain, the U-box, which is predicted to

form a RING-like structure and mediates untypical ubiquitin linkages [54]. In all organisms there are only one or two E1 enzymes, and tens of E2 enzymes; yet there are hundreds of E3 ligases that selectively interact with particular E2s, and thus are likely responsible for target selection.

As indicated above, the involvement of ubiquitin in various cell regulatory systems does not always end at the proteasome. Proteins to be sent to the proteasome are tagged with ubiquitin chains that are linked by the C-terminal glycine of the distal unit to lysine 48 (K48) of the proximal unit [52]. However, chains that are formed by conjugation to the proximal lysine 63 (K63) have been shown to be regulatory elements in signal transduction pathways [55]. Monoubiquitination has functions in many systems, including chromatin remodeling, endocytosis, and retroviral entry into the cell [52].

The best evidence of a role for ubiquitin in the ERK cascade comes from studies in *S. cerevisiae*, where ste11[56] and ste7 [57, 58] are ubiquitinated upon pheromone induction of the mating response pathway. The RING-containing ste5 scaffold mediates neither of these modifications, as it does not appear to be a functional E3 ligase. Rather, ubiquitination of ste7 is mediated by the SCF complex, upon ste11 phosphorylation of ste7 [58]. Intriguing work in *Dictyostelium* suggests a signal-dependent interplay between ubiquitination and sumoylation in the regulation of dDMEK [59], as described in the next section. It has also been reported that the PHD domain of MEKK1 has E3 ligase activity and can ubiquitinate ERK in manner that leads to its degradation [60]. There is an emerging story in which Raf may become ubiquitinated through its association with Bag1 and the U-box E3 ligase, CHIP [61]. Finally, the RasGAP NF1 may become ubiquitinated upon Ras activation [62]. A function for ubiquitin in Ras

signaling will be further expanded in Chapter 2 with the description of a unique RING E3 ligase we have termed Impedes Mitogenic signal Propagation (IMP).

A growing family of ubiquitin-like proteins (NEDD, Fub, UCRP, SUMO) bears many similarities to ubiquitination, such as tertiary structure, proteolytic processing and serial enzymatic transfer [63]. Yet, among other differences, substrate conjugation by these proteins does not appear to result in proteosomal degradation. Examination of these proteins continues to provide insights into structure-function relationships, discrimination between homologous enzymatic systems, and the diverse consequences of post-translational modifications. The best studied of these ubiquitin-like proteins is SUMO.

SUMO

SUMO, also known as UBL1, Sentrin, PIC1, GMP1, or SMT3c [63], has multiple regulatory roles in the cell. It is critical for nucleocytoplasmic translocation, being required for RanBP2-mediated sumoylation of nuclear-target proteins and for RanGAP to bind RanBP at the nuclear pore [64]. It is involved in the formation of intranuclear structures such as PML bodies, as well as the recruitment of many proteins (SP100, Daxx, CBP, and others) to these structures [65]. Sumoylation of c-Jun and the androgen receptor negatively regulates their transcriptional activity [66]. It may positively regulate p53 transcriptional activity [67], but downregulates p53 protein by stabilizing Mdm2 [68]. Finally, sumoylation can antagonize ubiquitin degradation of substrates, either directly, by occupying target lysines as with IκBα, or indirectly, by unknown mechanisms as with Mdm2.

The importance of SUMO has also been demonstrated in animals. The yeast homologue SMT3p is essential for cell viability, as are most of the genes involved in SMT3p conjugation and deconjugation [63]. Mutation of the SUMO-conjugating enzyme Ubc9 homologue in *Drosophila* prevents nuclear import of the transcription factor bicoid and results in embryonic defects [63].

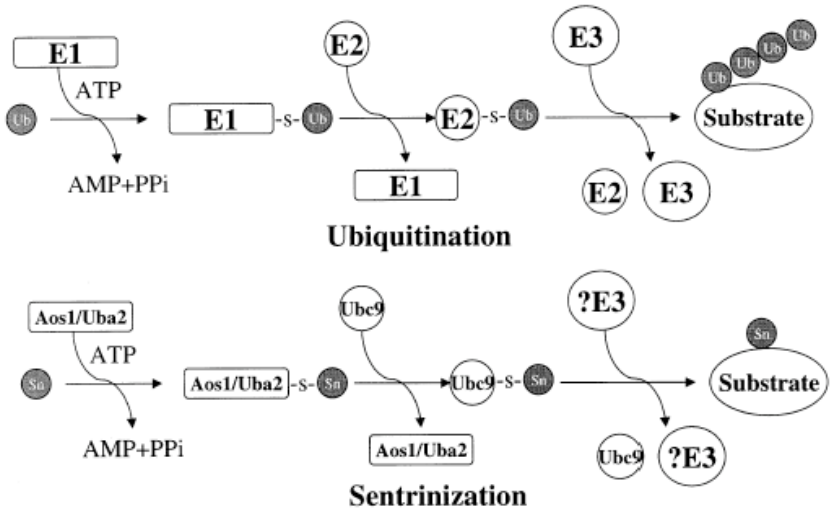
SUMO has three isoforms in mammals, in contrast to ubiquitin of which there is only one. SUMO-1 is most abundant and most studied. Like ubiquitin, it is synthesized as a precursor and must undergo proteolytic cleavage of the C-terminus to expose the necessary glycine for conjugation to lysine in the substrate. The consensus sequence for sumoylation is ΨKXE, where Ψ is a hydrophobic residue and X is any amino acid. Most, if not all, sumoylating enzymes to date have been found in the nucleus, consistent with the suggestion that both the ΨKXE motif and nuclear localization are required for sumoylation to occur [69]. For substrates of RanBP2, their modification may take place upon arrival at the nuclear pore [70].

Until recently, it was believed that sumoylation only required the activities of an E1 heterodimer (Aos1/Uba2) and E2 (Ubc9), yet recently some SUMO-1 E3s have been reported. PIAS domain-containing proteins have been shown to mediate SUMO-1 ligation [66]. The primary sequence of this domain is very similar to the RING domain, yet lacks critical cysteines. Even so, it may still facilitate small-protein transfer like a RING E3, as suggested by experiments showing that mutation of the PIAS domain compromises binding to Ubc9 but not to a substrate, p53 in this case [70]. However, RanBP2 does not contain any identifiable E3 motif and is a demonstrated SUMO-1 E3

[71]; hence, there may be other domains that possess this activity as well. HECT-like SUMO E3s have not been identified.

Importantly, SUMO-1 does not appear to form chains on substrates in cells, thus substrates are only mono-sumoylated (Fig 1.3), although a given protein may have multiple mono-sumoylations on different sites. This is in contrast with its isoforms, SUMO-2/3, with which it shares 50% identity [66]. SUMO-2/3 are 95% identical to each other and contain the ΨKXE motif, and thus can form poly-SUMO-2/3 chains [66]. Interestingly, the yeast homologue SMT3p also contains this motif, suggesting that poly-sumoylation may have been a regulatory feature before the evolution of mono-sumoylation, or that both processes can be accomplished with the same molecule.

Sumoylation is directly implicated in the regulation of the ERK pathway by work in *Dictyostelium*, in which dDMEK is alternately ubiquitinated and sumoylated [59]. These modifications were required for proper chemotaxis and resulted in nuclear translocation following stimulation (ubiquitination), followed by movement to the plasma membrane for activation by dDERK (sumoylation). This sequence for MEK activation is unusual, since in mammalian cells MEK is activated at the plasma membrane. Intriguingly, SUMO was recently shown to repress the transcriptional activity of Elk-1, and was reversed by phosphorylation of Elk-1 by ERK [72]. This is particularly interesting since SUMO as a repressor of activity had not before been demonstrated, and is also indicated in the negative regulation of IMP in Chapter 3. Finally, the pathogenic effector protein YopJ causes mass de-sumoylation in the cell, and may be the mechanism by which it inhibits MAPK signaling [73].



[66]

Figure 1.3 Enzymatic processes of ubiquitination and sumoylation.

CHAPTER TWO

Ras regulates assembly of mitogenic signaling complexes through the novel effector protein IMP

Abstract / Introduction

The Raf/MEK/MAP Kinase signal transduction cascade is a key Ras effector pathway that mediates diverse cellular responses to environmental cues, and makes a major contribution to Ras-dependent oncogenic transformation. Here we have identified a novel Ras GTPase effector protein IMP (Impedes Mitogenic signal Propagation) that modulates sensitivity of the MAP kinase cascade to stimulus-dependent activation by limiting functional assembly of the core enzymatic components. This is accomplished through inactivation of KSR, a scaffold/adaptor protein that couples activated Raf to its substrate MEK. IMP is a Ras-responsive E3 ubiquitin ligase. Upon Ras activation, IMP is modified by auto-polyubiquitination, thereby relieving inhibition of Raf/MEK complex formation. Thus, Ras activates the Raf/MAP kinase cascade through dual effector interactions: induction of Raf protein kinase activity concomitant with derepression of KSR-dependent Raf/MEK complex formation. This relationship may contribute to failsafe Ras-dependent activation of the MAP kinase cascade. Inhibition of native IMP expression results in elevated stimulus-dependent MEK activation without alterations in the timing or duration of the response. Therefore, IMP likely functions as a threshold modulator, controlling sensitivity of the cascade to stimulus, and likely providing a mechanism to allow adaptive behavior of the cascade in the context of chronic or complex signaling environments.

Results and Discussion

IMP was identified as a Ras-interacting protein from a Xenopus yeast two-hybrid screen using the Ras effector loop variant G12V/E37G (compromised for Raf interaction) as bait. The association was specific, as IMP did not interact with other effector loop variants, nor with any other Ras family G-protein tested, which included RalA, Rap1, RacD, RhoA, Cdc42, and R-Ras (Fig 2.1a and not shown). The reliability of the screen was indicated by multiple isolations of RalGDS, a likely Ras effector [74], and the *Xenopus* ortholog of JC310, a human protein isolated in a screen for suppressors of activated Ras in yeast [75]. Truncation analysis of IMP identified a 104 amino acid domain as minimally sufficient to mediate the two-hybrid interaction with Ras (not shown). Using recombinant proteins, we found that this domain binds directly to human H-Ras in a GTP-dependent manner (Fig 2.1b). The full-length human ortholog (GenBank accession # AY332222) was isolated from a Jurkat T-cell library and shown to associate with oncogenic Ras and mitogen-activated Ras in cells (Fig 2.1b,c,d). Importantly, native IMP forms a stimulus-induced complex with native Ras in human cells (Fig 2.1e), suggesting it may be a bona fide Ras effector protein.

IMP was previously reported as BRCA1-interacting protein 2 (BRAP2), based on two-hybrid analysis [76]. However, the interaction between BRAP2 and BRCA1 is not discriminatory, being mediated exclusively by the polybasic nuclear localization signal (NLS) on BRCA1 [76]. The primary amino acid sequence of IMP predicts a RING-H2 domain followed by a ubiquitin protease-like zinc finger (UBP-ZnF) and leucine heptad-repeats predicted to form a coiled-coil (SMART, Simple Modular Architecture Research

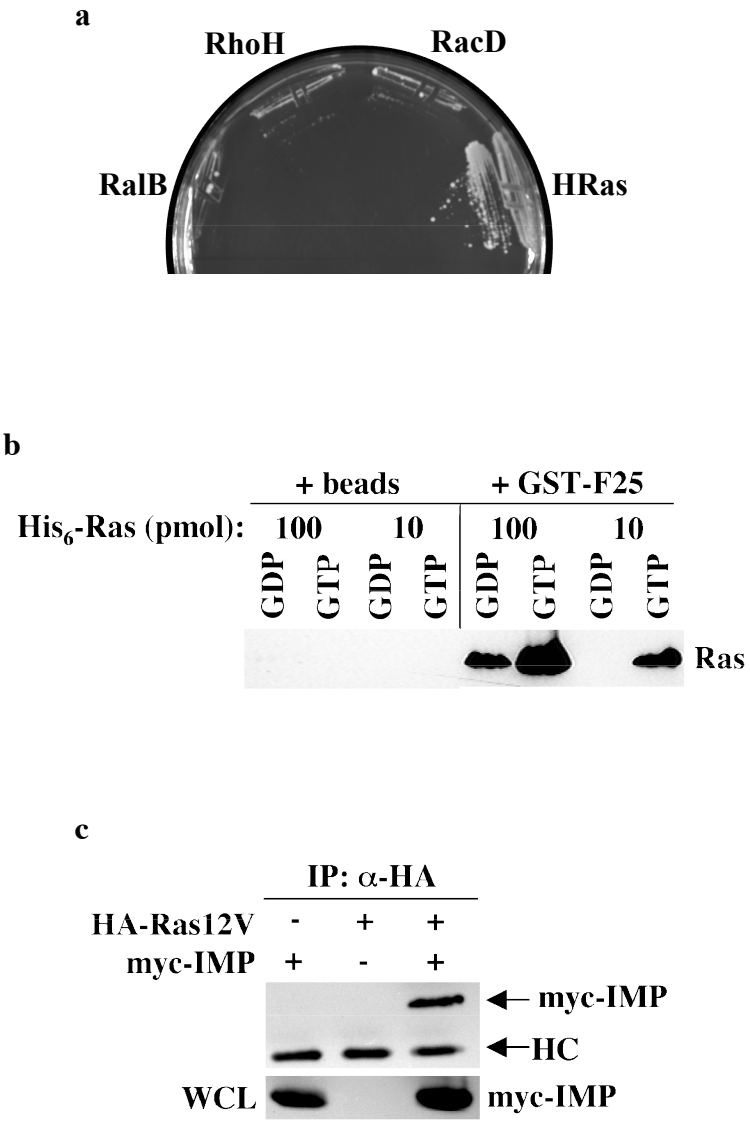


Figure 2.1 IMP is a Ras effector. **a**, IMP selectively associates with H-Ras in yeast. **b**, IMP binds directly to Ras-GTP. The indicated amount of His6-Ras was loaded with either GDP or GTPγS and mixed with GST-F25. Complexes were immobilized on glutathione-sepharose beads and analyzed with anti-Ras mAb. **c**, Co-immunoprecipitation of IMP with oncogenic Ras. Myc-IMP was transiently expressed in NIH 3T3 cells stably expressing HA- RasG12V. HA-RasG12V was immunoprecipitated with anti-HA.11 mAb conjugated to protein A/G agarose and blotted with anti-myc9E10 to detect myc-IMP.

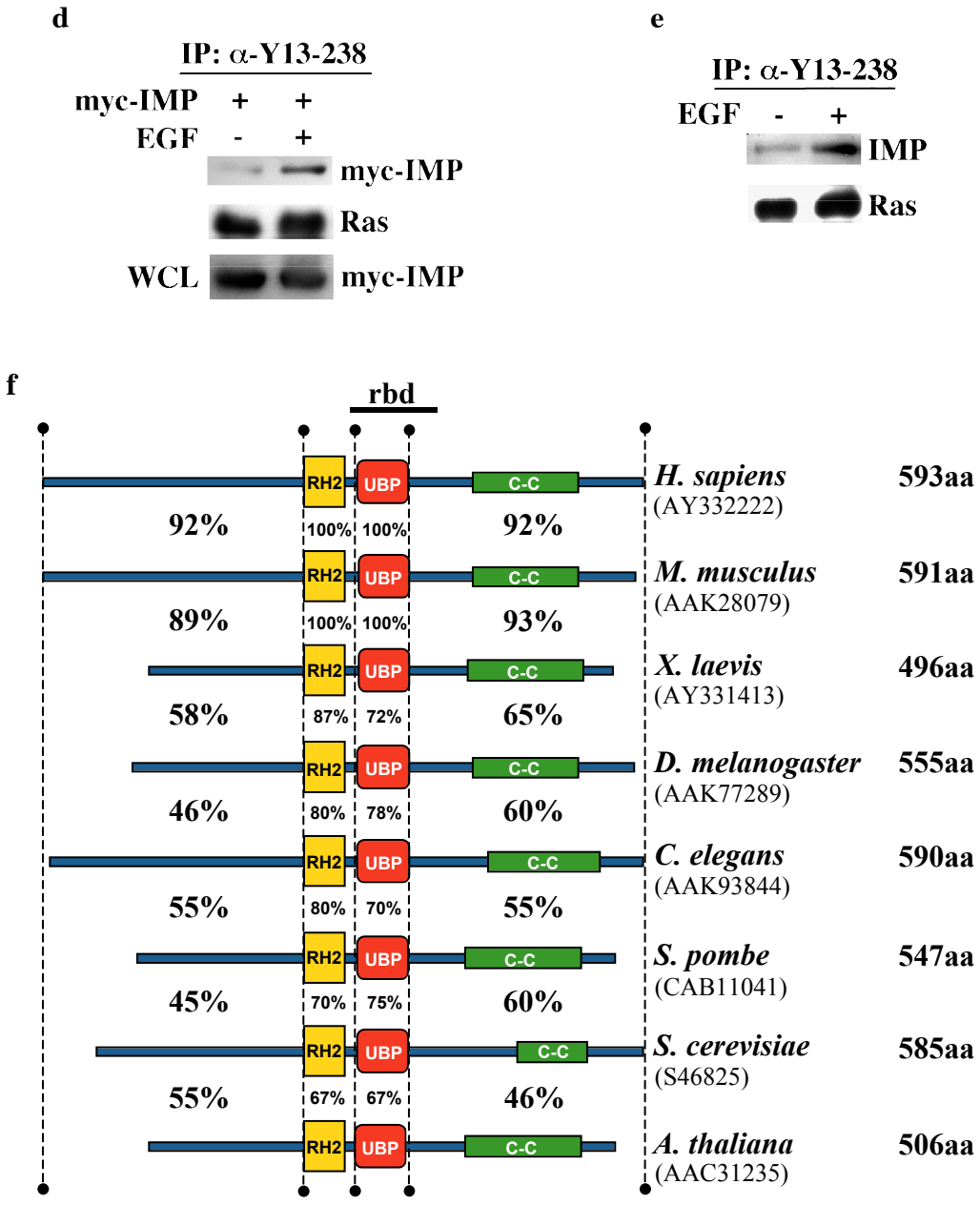


Figure 2.1 d, Stimulus-dependent association of IMP with Ras. HeLa cells were transfected with myc-IMP and stimulated with EGF (1ug/ml) for 5 min or left untreated. Endogenous Ras was immunoprecipitated with anti-Y13-238 conjugated to agarose and complexes were analyzed with anti-myc9E10 to detect myc- IMP. **e**, Stimulus-dependent association of endogenous Ras and IMP. As in panel d, HeLa cell were stimulated or not with 1ug/ml EGF for 5 min. Endogenous Ras was immunoprecipitated with anti-Y13-238 conjugated to agarose and complexes were analyzed with anti-IMP pAb to detect endogenous IMP. **f**, Domain structure of IMP orthologs. Amino acid homologies for each motif are relative e to the human sequence. Rbd indicates the minimal Ras binding domain, RH2 = RING-H2, UBP = UBP Zinc finger, C-C = coiled coil.

Tool, http://smart.embl-heidelberg.de). This domain architecture is strikingly similar to the RING B-box Coiled-Coil (RBCC) family of proteins that includes the proto-oncogenes PML and TIF-1 [77]; the difference being a UBP-ZnF in place of a B-box zinc-finger. The conserved sequential domain organization of RBCC proteins has been shown to be essential for proper enzymatic function and/or appropriate protein-protein binding events [77]. The UBP-ZnF is a motif found only in ubiquitin proteases and some histone deacetylases (in which it has been termed Polyubiquitin-Associated Zinc finger (PAZ)), where it appears to facilitate binding to polyubiquitin chains [78]. IMP is the only protein outside of these two protein families that contains a UBP-ZnF domain. IMP is highly conserved across eukaryotes, with a single ortholog present in each species (Fig 2.1f). Multiple human tissue northern blot analysis revealed broad-spectrum expression as previously described in mice [76] (and not shown).

Given that multiple Ras effector pathways can collaborate with the Raf/MAP kinase cascade to induce oncogenic transformation [79], we set out to examine the contribution, if any, of IMP to Ras signaling. We expressed full-length myc-tagged IMP together with the oncogenic Raf variant RafBXB [80] and examined the consequences on downstream signal output by a variety of measures. We found that rather than cooperating, IMP expression resulted in a dramatic inhibition of Raf-induced activation of endogenous MEK and ERK (Fig 2.2b) that likewise blocked subsequent accumulation of endogenous c-fos protein (Fig. 2.2b). IMP also inhibited focus formation of RafBXB-transformed NIH 3T3 cells (not shown). Using reporter assays for transcriptional activation of the serum response element (3X-SRE), we found that IMP suppressed signaling of a MEK

mutant activated by deletion of the nuclear export sequence (MEKΔ) (Fig. 2.2a). However, it did not interfere with signaling from a constitutively active MEK that contains the NES deletion in addition to phosphomimetic amino acid substitutions at Raf phosphorylation sites (MEKR4F) [81], nor an ERK2 variant constitutively activated by three point mutations [82] (ERKact) (Fig. 2.2a). The differential suppression of the MEK variants supports a point of impact above the level of MEK. Additionally, this inhibitory effect appears to be selective for Raf, as the activity of another MAP3K family protein kinase, MEKK3, was not affected by IMP expression (Fig. 2.2b). These results suggest that IMP negatively regulates Raf kinase signaling. While IMP inhibits the activation of MEK by Raf, it does not appear to block Raf activation. In response to EGF-stimulation, Raf1 is phosphorylated on serine 338. This is an activating phosphorylation that occurs upon Ras association and contributes to Raf kinase activity [83]. As shown in Fig. 2.2c, IMP expression does not inhibit stimulus-dependent phosphorylation on serine 338; on the contrary, the phosphorylation is enhanced. Considering that IMP blocks ERK activation, this is likely a consequence of uncoupling negative feedback inhibition of EGF signaling. Finally, we saw no inhibition of the constitutive kinase activity of the Raf1 catalytic domain (RafC') (Fig. 2.2d). However, IMP did block mitogen-induced association of Raf1 with cellular MEK1/2 (Fig. 2.2e). Thus, IMP inhibits signal propagation through Raf by uncoupling activated Raf from its downstream substrates MEK1 and MEK2. This inhibition translates to downstream cellular responses as demonstrated by the capacity of IMP to block Raf-induced immediate early gene expression (Fig. 2.2b), and Raf-induced neurite-like differentiation of PC12 cells (Fig. 2.2f). These observations suggest that IMP is a negative modulator of mitogenic signaling

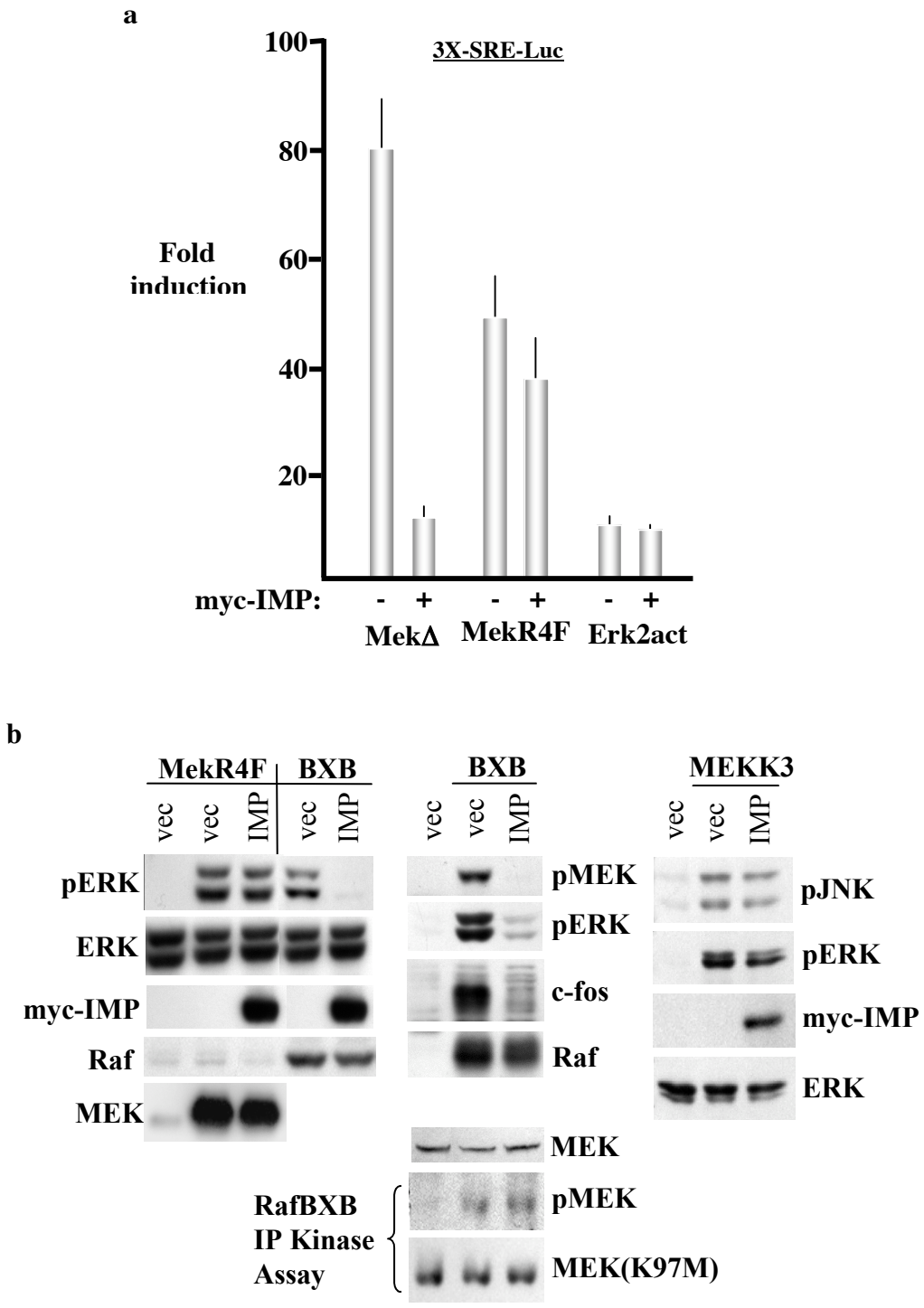


Figure 2.2 IMP impedes signal transmission from Raf to MEK. **a**, HEK293 cells were transfected with 3XSRE-luc reporter together with the indicated constructs. Reporter activation is expressed as fold induction relative to vector controls. **b**, Inhibition of endogenous MEK and ERK1/2 activation but not JNK1/2. 293 cells were transfected as indicated. Whole-cell lysates were immunoblotted for endogenous ERK1/2, phospho-ERK, phospho-MEK, c-fos, and phospho-JNK1/2 and for ectopic proteins with anti-Raf1 (C-12), anti-MEK, and anti-myc9E10.

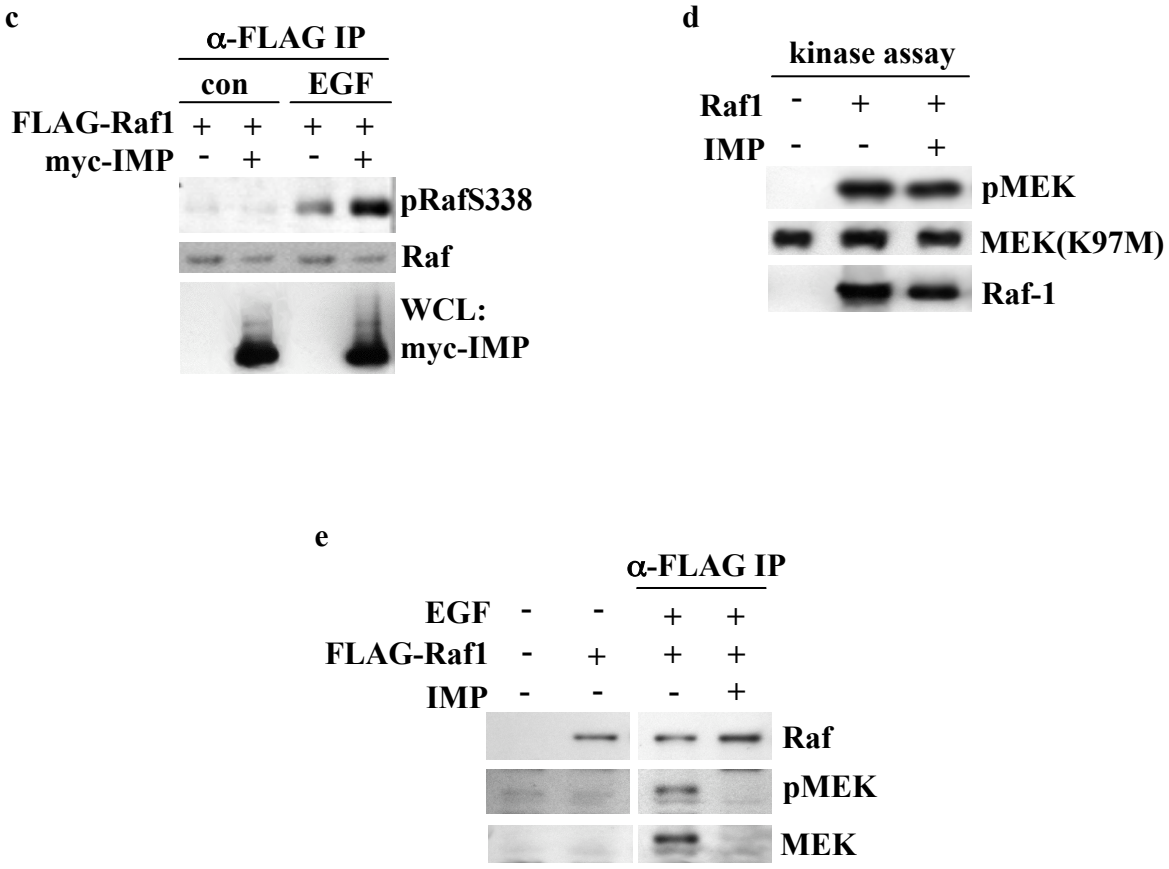


Figure 2.2 c, IMP does not inhibit activating phosphorylation of Raf. 293 cells were transfected as indicated and, following an 18 hour serum deprivation, stimulated for 5 minutes with EGF (100ng/ml). FLAG-Raf1 was immunoprecipitated with anti-FLAG agarose and immunoblotted with anti-Raf phosphoSer338, anti-Raf1 (C-12). **d**, IMP does not inhibit Raf kinase activity. The catalytic domain of Raf (RafC') was expressed in 293 cells with myc-IMP. HA-RafC' was immunoprecipitated with anti-HA agarose and assayed for kinase activity *in vitro* using recombinant kinase-dead MEK1 as substrate. Raf-dependent MEK phosphorylation was visualized with anti-phospho MEK. Recombinant MEK and HA-RafC' were detected with anti-MEK and anti-Raf (C-12), respectively. **e**, IMP blocks Raf-MEK association. 293 cells were transfected with FLAG-Raf1 and myc-IMP as shown. Cells were stimulated with EGF (100ng/ml) for 7 minutes prior to lysis. FLAG-Raf1 was immunoprecipitated with anti-FLAG agarose. Raf1 was detected with anti-Raf (C-12) and endogenous MEK and phospho-MEK were detected as shown.

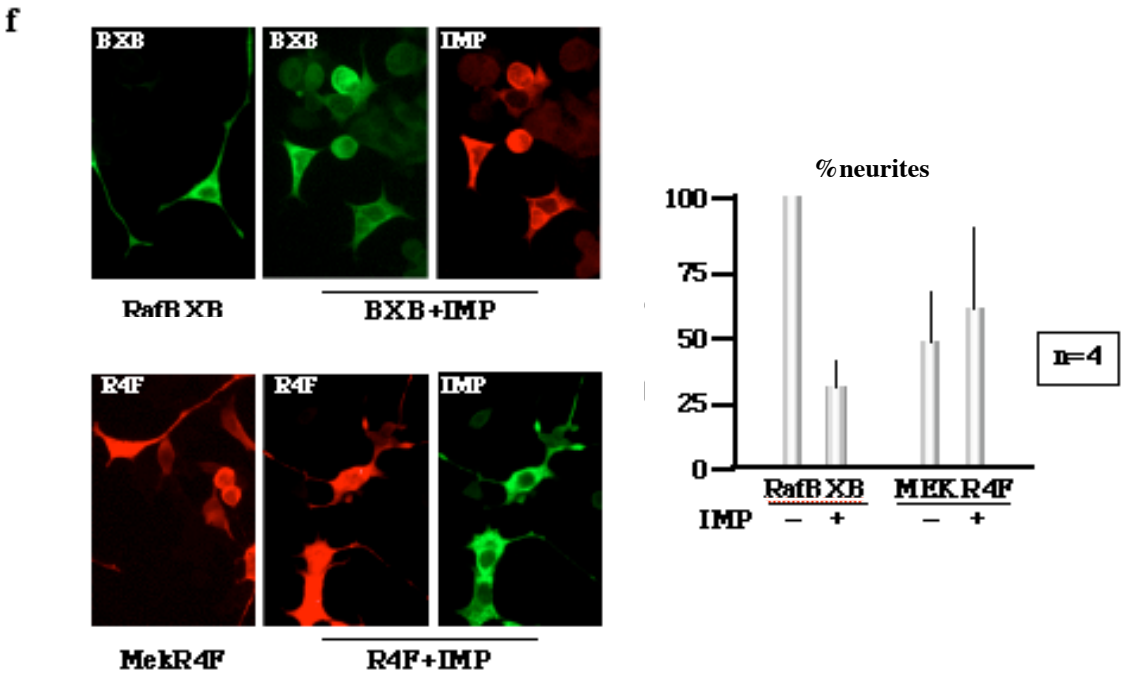


Figure 2.2 f, IMP inhibits BXB-induced PC12 neurite-like extensions but not those induced by MEKR4F. PC12 cells were assayed for extensions in response to transient expression of Raf BXB or MEKR4F by microscopic observation. RafBXB and MEKR4F were detected by anti-Raf mAb and anti-MEK1/2 pAb, respectively. Myc-IMP was detected with anti-myc9E10. The observations were quantitated and are shown in the right panel. Values are expressed as the percentage of cells displaying extensions greater than two cell bodies in length, normalized to that observed with RafBXB (arbitrarily set at 100). Bars represent standard deviation from four independent experiments.

acting on the ERK1/2 kinase pathway at the level of kinase-substrate interactions between Raf and MEK.

To validate the hypothesis that IMP is a negative regulator of Raf kinase signaling, we inhibited expression of native IMP in a variety of cell types. As shown in Fig 2.3a, inhibition of IMP expression in *Drosophila* S2 cells resulted in elevated levels of active ERK in the absence of additional stimulus. S2 cells constitutively secrete growth factors, and proliferate in culture in the absence of serum. However, ERK activity can be further stimulated by insulin in a Ras-dependent fashion [45, 84]. In human epithelial cells and

primary human fibroblasts, depletion of IMP did not elevate baseline ERK activity in serum-starved cells, but did elevate the amplitude of the response of the MAP kinase cascade to mitogen-stimulation (Fig 2.3b,c). The duration of MEK activation was not appreciably altered upon loss of IMP expression, suggesting IMP does not contribute to immediate early negative feedback control. Instead, native IMP appears to limit the absolute capacity of the system to respond to ligand stimulation. EGF signaling is not generally elevated upon inhibition of IMP expression, as activation of AKT was unaffected (Fig 2.3b). Our cumulative observations support the hypothesis that native IMP is a negative modulator of mitogenic signaling acting on the MAP kinase pathway at the level of Raf/MEK kinase substrate interactions. Thus IMP may function to add impedance or resistance to signal propagation through Raf to MEK, thereby modulating the cellular threshold of sensitivity to stimulus. Consistent with this, we found that siRNAs targeting rat IMP enhanced the sensitivity of PC12 cells to sub-optimal doses of NGF, as evaluated by morphological differentiation (Fig. 2h).

An obvious potential functional motif in IMP is the putative RING-H2 domain (Fig 2.4a). In many proteins, this domain functions to facilitate protein ubiquitination via E3 ubiquitin ligase activity [85]. To examine if IMP displays E3 ubiquitin ligase activity, immunoprecipitated IMP or an IMP variant, in which the first cysteine in the RING-H2 consensus sequence was changed to alanine (C264A), was assayed for ligase activity using a standard *in vitro* reconstitution assay. As shown in Fig 2.4b, IMP becomes polyubiquitinated *in vitro* in the presence of recombinant E1, UbcH4 (E2), and ubiquitin. This activity is dependent upon the integrity of the RING-H2 domain. Therefore IMP can function as an E3 ubiquitin ligase and, as with most E3s, IMP is a good auto-substrate *in vitro*. Attempts to identify a target substrate in the MAP kinase pathway by

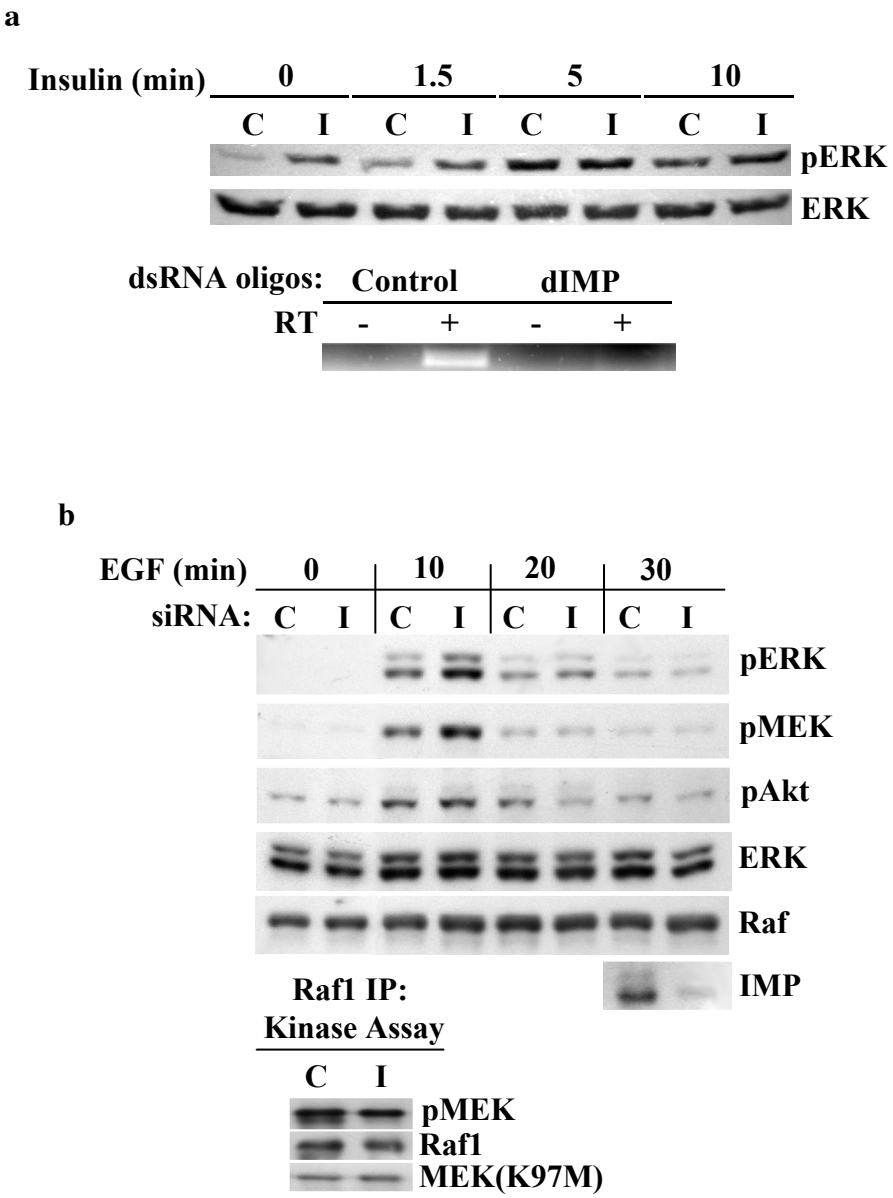


Figure 2.3 Inhibiting native IMP enhances stimulus-dependent MEK activation. **a**, Knockdown of *Drosophila* IMP in S2 cells. S2 cells were treated with dsRNA targeting dIMP or DREDD as a control. After 72h, cells were stimulated with human recombinant insulin (10ug/ml) as indicated. Whole-cell lysates were immunoblotted for endogenous dERK and phospho-dERK. RT-PCR was used to verify knockdown of dIMP mRNA. **b**, Knockdown of IMP in HeLa cells. HeLa cells were transfected with siRNA oligonucleotides targeting human IMP (I) or rat RalB as a control (C). After 72h, cells were stimulated with EGF (1ng/ml) as indicated. Whole-cell lysates were immunoblotted for the indicated proteins. Similar results were obtained with two independent siRNAs targeting IMP.

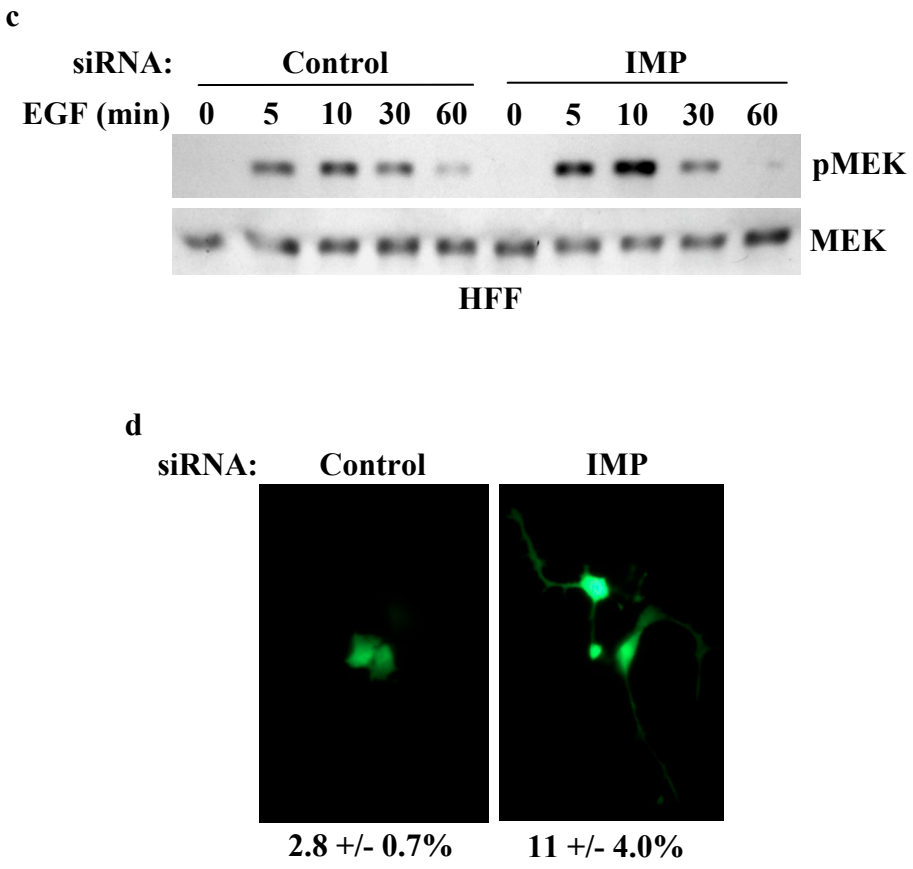


Figure 2.3c, Depletion of IMP in primary human foreskin fibroblasts (HFF). HFFs were treated as in **b**. **d**, Depletion of IMP in PC12 cells. PC12 cells were transfected siRNAs together with GFP and treated with 10ng/ml NGF. GFP positive cells were scored for the presence of neurite-like extensions as described in **2.2f**.

the candidate approach were unsuccessful, as we could not detect any IMP-dependent modification of H-Ras, Raf1, MEK1, or ERK1 (not shown). However, IMP is likely a bona fide target for its own E3 ligase activity in cells, as we noticed the appearance of a "ladder" of higher molecular weight IMP species in mitogen-stimulated cells, or cells expressing Ras12V together with IMP (Fig 2.4c). This was not observed in cells

expressing the ligase-defective C264A mutant (Fig 2.4c), and suggests that IMP E3 ligase activity is regulated by Ras. Interestingly, in contrast to RafBXB, IMP does not inhibit oncogenic Ras activation of MAP kinase. However, inactivation of IMP's E3 ligase domain reveals inhibitory activity (Fig 2.4d). Most importantly, these observations indicate that IMP inhibition of the Raf kinase cascade is Ras sensitive, and suggests that the Ras couples to the MAP kinase cascade through dual effector interactions mediating Raf kinase activation together with derepression of Raf/MEK complex formation.

We have demonstrated that IMP inhibits signal propagation through the Raf/MAP kinase pathway by interfering with Raf/MEK complex formation. This could be accomplished through steric inhibition as a consequence of competitive association of IMP with Raf and/or MEK. This mechanism of action has been suggested for RKIP (Raf kinase inhibitory protein) [86]. However, we failed to detect any substantial interaction of IMP with Raf1 or MEK1 either by yeast two-hybrid or by immunoprecipitation of ectopically expressed proteins (data not shown). KSR1 is an adaptor/scaffold protein that functions at least in part to couple Raf to MEK [36, 51, 87]. As IMP functions to uncouple Raf from MEK, we examined if IMP impacts the MAP kinase cascade at the level of KSR. IMP associates with KSR in cells, apparently through the respective amino terminal halves of the two proteins (Fig 2.5b,c) and an endogenous KSR/IMP complex can be detected (Fig 2.5d). The dynamics of KSR function in cells are not well understood; however, recent observations suggest that KSR can respond to signaling events. For example, KSR is phosphorylated on multiple sites in cells and some of these sited are modified in a mitogen-dependent manner. The serine/threonine protein kinase c-

Tak-1 phosphorylates KSR on serine 392, and this modification can inactivate the scaffolding function of KSR in cells [87]. In addition, an inactivated KSR variant (C809Y) was identified as the product of a loss of function KSR mutant in *C. elegans* [88]. This variant has lost the capacity to associate with MEK, is hyper-phosphorylated in cells, and partitions to a Triton-insoluble cell fraction [47]. We find that expression of IMP together with KSR results in a dramatic accumulation of a higher molecular weight species of KSR, mimicking the consequences of the inactivating C809Ymutation (Fig 2.5d). The IMP-induced modification of KSR is hyper-phosphorylation as demonstrated by phosphatase treatment (Fig 2.5f), and, like KSRC809Y, this form partitions to a Triton-insoluble cell fraction (Fig 2.5e). Although phosphorylation of KSR serine 392 is somewhat enhanced in the presence of IMP, a KSR variant defective for c-Tak-1 association [87] demonstrates that c-Tak1-independent phosphorylation events on KSR accumulate in the presence of IMP (Fig 2.5d). KSR-/- mouse embryo fibroblasts display reduced ERK activation in response to a variety of stimuli as compared to wild-type cells[9], which can be enhanced by low-level ectopic expression of KSR (R.L.K and R.E.L. personal communication). We used this system to examine whether IMP requires KSR to inhibit ERK activation. As shown in Figure 2.5g, IMP does not inhibit EGF- or PDGF -stimulated ERK activation in KSR-/- cells, however IMP can eliminate the ability of KSR to enhance ERK activation. Although the mechanistic consequences of the IMP-induced modifications of KSR are unknown, they are consistent with the behaviour of inactive KSR, and suggest that IMP functions to hold KSR in an inactivated state.

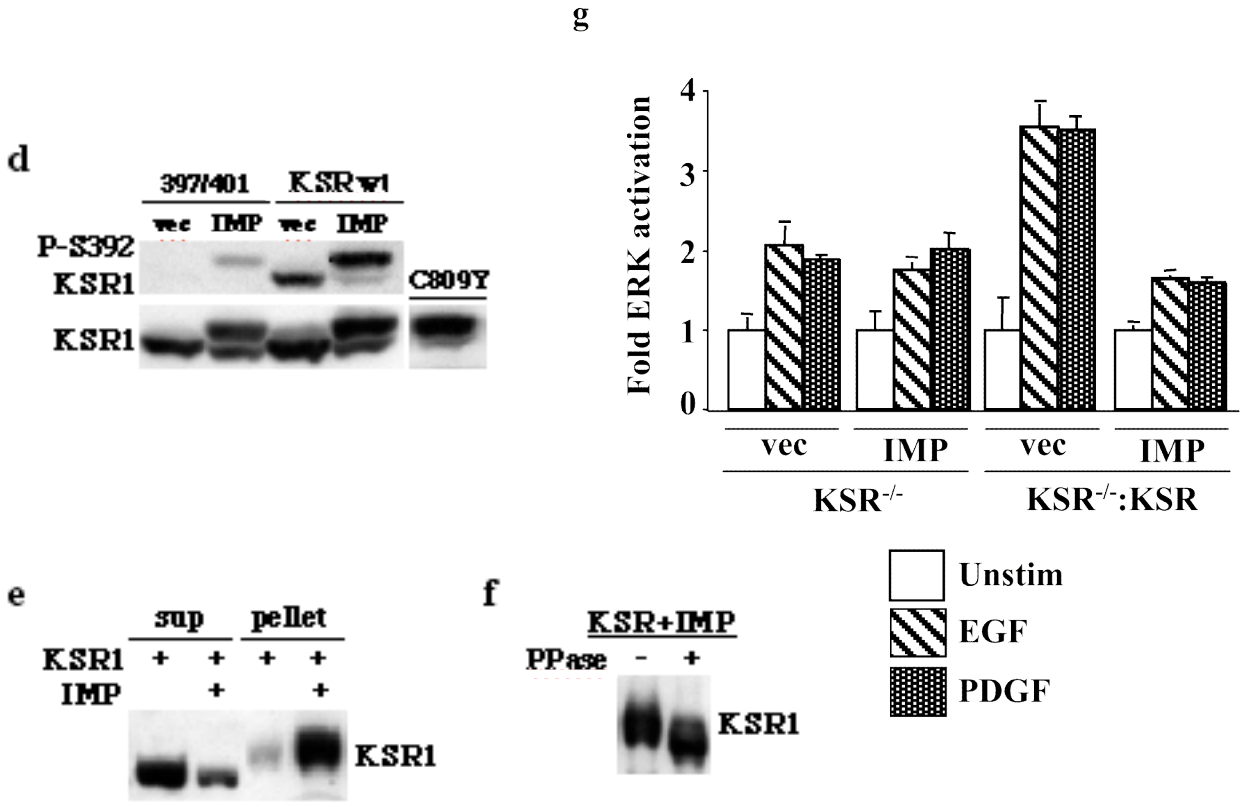


Figure 2.5 d, IMP induces mobility shift and enhances inhibitory S392 phophorylation on KSR1. 293 cells were transfected with myc-IMP and KSRwt or KSR I397A/V401A. Whole-cell lysates were immunoblotted with anti-KSR1 or anti-phosphoSer392 KSR1. **e**, High-molecular weight KSR is Triton-insoluble. 293 cells were transfected with HA-KSR1 and myc-IMP and separated into Triton-soluble and -insoluble fractions. Equal amounts of protein were loaded and immunoblotted with anti-KSR1. **f**, IMP induces hyperphosphorylation on KSR1. 293 cells were transfected with HA-KSR1 and myc-IMP. HA-KSR1 was immunoprecipitated from the detergent-solubilized pellet fraction with anti-HA agarose and subjected to lambda phosphatase treatment. **g**, IMP inhibition of ERK activation is KSR-dependent, as shown by in situ ERK activation assay.

Immunostaining shows co-localization of IMP and KSR in Triton-resistant punctate structures (Fig 2.5h), suggesting that IMP may recruit KSR to microdomains inaccessible to activators of KSR function. Expression of oncogenic Ras reverses this phenotype, further supporting the hypothesis that Ras inactivates IMP (Fig 2.5h).

Here, we have identified a new Ras effector, IMP (impedes mitogenic signal propagation) that negatively regulates MAP kinase activation by limiting formation of

Raf/MEK complexes. The mechanism of inhibition appears to be through inactivation of the KSR1 adaptor/scaffold protein, demonstrating that in addition to promoting signal transmission, scaffold proteins can function to restrict signal propagation. Ras can inactivate IMP through induction of IMP auto-ubiquitination, facilitating KSR-dependent engagement of MEK by activated Raf. These observations reveal dual Ras effector inputs to the MAP kinase cascade: induction of Raf kinase activity concomitant with derepression of KSR-dependent Raf/MEK complex formation. This relationship provides a mechanism to limit engagement of the MAP kinase cascade in the absence of Ras activation. MAP kinase activation contributes to multiple diverse cellular responses to environment. The capacity to control the amplitude of this response via molecules like IMP likely contributes to flexible and adaptive cellular behaviour in the context of complex regulatory signals.

h

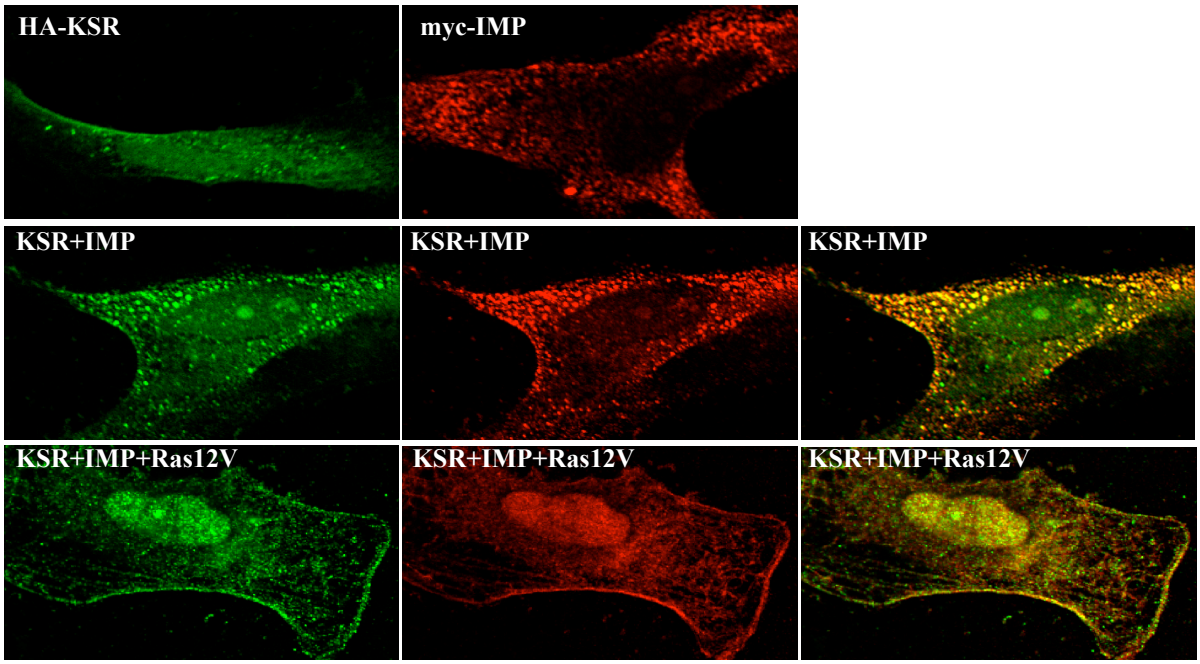


Figure 2.5 h, IMP and KSR1 colocalize in Ras-sensitive Triton-insoluble structures. HeLa cells were transfected with HA-KSR and myc-IMP. Prior to formaldehyde fixation, the cells were incubated in 0.1% Triton for 10 minutes at 4 degrees C to release soluble contents. IMP and KSR were detected with anti-mycA14/goat anti-rabbit rhodamine and anti-HA/goat anti-mouse FITC, respectively.

Methods and Materials

Expression vectors. Full-length IMP was isolated from a Jurkat cDNA library by PCR as a 1.7kD fragment, using the following primers: (forward) 5'GATCTGGAATTCA TGAGTGTGTCACTGGTT and (reverse) 5'GATCTGGGATCCTCAGGGATGTC TGTTGCT, with 5'EcoRI and 3'BamHI restriction sites for subcloning. pCMV5myc-IMP was constructed by EcoRI-BamHI digestion of pCMV5myc1 and the IMP PCR fragment. pCMV5myc1-IMP C264A was produced by PCR-mediated site directed

mutagenesis. pCMV5myc-IMP N' encodes residues 1-255. pGEX4T1-F25 comprises a portion of the *Xenopus* IMP cDNA as an EcoRI-ScaI fragment that was isolated in a yeast two-hybrid screen using Ras12V/37G/186S [79] as bait. This portion corresponds to residues 273-377 of the human IMP sequence, and is 98% identical to the human protein. The following plasmids have been previously described: His6-Ras, pGL2-3XSRE-luc [89], pDCR-HA RasG12V [79], pLNX2-FLAG Raf-1, pCGN-HA RafC' [90], pCDNA3-MEKK3, pSRα-RafBXB [79], pCDNA3-MEKΔ, pCMV5myc-MEKR4F [81], pCDNA3-HA KSR1, pCDNA3-HA KSR1 I397A/V401A [87], pCMV5 FLAG KSR1 N539 [40], and pMM9-ERK2 L73P/S151D/D319N [82].

Antibodies. All primary antibodies were obtained from Santa Cruz Biotech except the following: Anti-HA.11 (Babco), anti-Ras (Transduction Laboratories), anti-phospho-S338 Raf (Upstate Biotech), anti-phospho-T183/Y185 JNK1/2 (Cell Signaling), anti-c-fos (Upstate Biotech). Antibodies against MEK1/2, phospho-S218/S222 MEK1/2, ERK1/2, and phospho-T202/Y204 ERK1/2 were obtained from Sigma, except the MEK1/2 pAb used in Figure 3b, which was obtained from Santa Cruz. All Raf-1 immunodetection was done with anti-Raf (C-12) from Santa Cruz except in Figure 3a, which used the anti-Raf-1 mAb from Pharmingen. Anti-IMP was generated against a carboxy-terminal peptide (Biocarta). Anti-phospho S392 KSR1 (R. Lewis). All secondary antibodies were obtained from Jackson Laboratories: goat anti-mouse HRP, goat anti-rabbit HRP, goat anti-mouse FITC, and goat anti-rabbit rhodamine. Anti-HA agarose, anti-Y13-238 agarose and anti-myc9E10 agarose conjugates were obtained from Santa Cruz. Anti-FLAG M2 agarose was purchased from Sigma.

Cell culture and transfection. All reagents described below were obtained from Gibco, except where indicated. All mammalian cell lines were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM) and 0.5% penicillin/streptomycin, supplemented with serum as indicated. NIH 3T3 fibroblasts were grown in 10% calf serum, HeLa cells were grown in 10% fetal bovine serum, and HEK293 cells were grown in DMEM without sodium pyruvate supplemented with 10% fetal bovine serum. PC12 were grown in 10% HS (heat inactivated) and 5% FBS in RPMI 1640. *Drosophila* S2 cells were cultured in D-SFM media without glutamine, supplemented with 18mM L-glutamine. Plasmid DNA was transfected by calcium phosphate precipitation for NIH3T3 and HEK293 cells and by Lipofectamine 2000 (Promega) for Hela and PC12 cells. siRNAs were transfected using Oligofectamine (Promega). S2 cells were treated with dsRNA as described [84].

In vitro binding assays. GST-F25 and His6-H-Ras were purified by standard procedures from DH5αe E. coli (Gibco). GST-F25 was isolated on glutathione sepharose (Sigma). His6-H-Ras was isolated on Ni-agarose (Qiagen) and eluted with 200mM imidazole (Sigma) and concentrated through a Centricon filter (Millipore). Purified Ras was loaded with either GDP or GTPγS (Sigma) by incubating in loading buffer (50mM Hepes 7.5, 5mM EDTA, 5mg/ml BSA, 500uM nucleotide per 100pmol Ras protein) for 3 min at 30ºC. To each binding reaction, 100pmol of GST-F25 was mixed with either 100pmol or 10pmol of His6-H-Ras in binding buffer (BSA 100ug/ml, 50mM Tris 7.5, 1% Triton X-100, 100mM NaCl, 1mM $MgCl_2$) for 1 hour at RT. The proteins were washed 4X in binding buffer without BSA.

Ubiquitin ligase assays. Immunoprecipitated myc-IMP or myc- IMP C264A were added to purified assay components (5uM bovine ubiquitin (Sigma), 0.33uM E1, 13nM recombinant human UbcH4, energy regeneration mix, and ligase buffer (50mM Tris 7.5, 150mM KCl, 1mM $MgCl_2$) and mixed for 1.5 hours at 37ºC.

RNA interference. For RNAi in S2 cells, dIMP was amplified from EST clone AA817466 (Genbank) with following primers: (forward) 5'GAATAATACGACTCACT ATAGGGAGACGCTTTGGAGTTCTACA and (reverse) 5'GAATAATACGACTCAC TATAGGGAGAGCATAGTCCCATACGCTT. dsRNA was prepared according to manufacturer's instructions using the Megascript T7 kit (Ambion). The cells were treated with 15ug of annealed dsRNA, as described previously [84]. At 72 hours post-transfection the cells were stimulated with human recombinant insulin (10ug/ml) (Sigma) and lysed either with the High Pure RNA purification kit (Roche) for RT-PCR, or in boiling SDS-Tris (1% SDS, 10mM Tris 7.5) for immunoblotting. RNAi in HeLa cells was performed using the following pairs of RNA oligos: (IMP-FW1) 5'UAUAUGGUGCU GAUAAAGUdTdT and (IMP-RV1) 5'ACUUUAUCAGCACCAUAUAdTdT, (IMP-FW2) 5'GACAAAUAAGAUGACCUCCdTdT and (IMP-RV2) 5'GGAGGUCAUCUUAUUUGUCdTdT. The cells were transfected with 400pmol annealed siRNA oligos, as previously described [91]. Twenty-four hours later, the cells were stimulated with EGF (1ng/ml) (Sigma) followed by lysis in boiling SDS-Tris for immunoblotting. Immunoprecipitates from rat brain lysates were prepared as described[9] using two different anti-KSR antibodies, from Transduction Labs (Ab1) or Santa Cruz (Ab2). 'Normal' mouse IgG was used as a control. The presence of KSR and IMP in the

immunoprecipitates was detected with the Transduction Labs anti-KSR antibody and anti-IMP respectively.

Luciferase assays. Twenty-four hours post transfection, the cells were serum-starved for 18 hours then lysed in 2X assay buffer (Promega) according to manufacturer's instructions. Reporter gene expression was measured by luminescence using a TD 20/20 dual-injection luminometer (Turner Designs), with luciferin (Promega) as a substrate. Relative light units were normalized to β-galactosidase activity from CMV-LacZ, an internal transfection control.

Immunofluorescence. Cells were washed with PBS then fixed in 3.7% formaldehyde. Permeabilization, washes, and antibody dilutions were performed with 1% calf serum, 0.25% Triton X-100 in PBS. Primary antibodies were diluted 1:80. Goat anti-mouse FITC was used 1:300 and goat anti-rabbit rhodamine was used 1:1000. For the experiments in Figure 5g, the cells were washed with PBS then incubated in cold 0.1% Triton in PBS for 10 minutes at 4ºC. The cells were then fixed and immunostained as described above. All images were captured at 40X on a Leica confocal microscope.

For fluorescent *in situ* ERK activation assays (Figure 2.5g), cells were stained with anti-phospho-ERK1/2 (Cell Signalling, 1:100) and anti-ERK1 (Santa Cruz, 1:100) primary antibodies and anti-mouse Alexa Fluor 680 (Molecular Probes, 1:100) and anti-rabbit IRDye800 (Rockland, 1:100) secondary antibodies. Signal detection and quantification was performed using the Li-Cor Odyssey system as directed by the manufacturer.

In situ ERK activation assay. KSR1$^{-/-}$ cells were infected with bicistronic retroviruses encoding KSR1-IRES-GFP and IMP-IRES-YPF or control viruses as indicated and sorted for green and yellow fluorescence by FACS. Sorted cells were seeded at 1.5X10^4 cells/well in a 96 well plate 24 hours prior to analysis. Cells at 70% confluence were

deprived of serum for four hours, then treated with either 100 ng/mL EGF or 25 ng/mL PDGF for 5 minutes followed by a quantitative fluorescence *in situ* plate assay for ERK activation.

Phosphatase assays. Immunoprecipitated HA-KSR1 was added to lambda phosphatase with reaction buffer supplied by the manufacturer (New England Biolabs). The reactions were incubated 30 min at 30ºC and terminated by addition of 2X sample buffer.

Raf kinase assays. HA-RafC' was immunoprecipitated and washed 5X in NP40 buffer, then washed once each in 1X PBS and 25mM HEPES 7.5, 10mM $MgCl_{2.}$ The beads were then added to kinase assay reagents (10mM $MgCl_2$, 83uM ATP, 25mM HEPES 7.5, 50ng/ul recombinant MEK(K94A). The reaction was incubated at 30ºC for 40 min with regular mixing, and stopped upon addition of 2X sample buffer.

CHAPTER THREE

Regulation of IMP

Abstract

IMP is a newly described Ras effector that negatively regulates the ERK pathway, possibly at the level of KSR. Examination of the primary amino acid sequence reveals conserved motifs that suggest multiple modes of IMP regulation. One possible regulatory mechanism, Ras-stimulated autoubiquitination of IMP, was previously described. Here, we present data that suggests the existence of additional regulatory inputs.

Introduction

Mutations that result in a gain-of-function essentially reflect the loss of regulatory control over enzymatic activity. It is therefore just as important to understand how a protein is regulated as is its function. As a ubiquitin ligase, it was obvious to test IMP for self-modification; as discussed, we found that IMP does autoubiquitinate in the presence of oncogenic Ras (Ras12V). Analysis of the primary amino acid sequence indicated that IMP may be regulated on multiple levels in different ways. Two conserved motifs for posttranslational modification were identified: PXSP for ERK phosphorylation and ΨKXE for sumoylation. Here, we show that IMP can be phosphorylated by ERK and that this correlates with IMP translocation to the nucleus. We also demonstrate that SUMO exerts negative effects on IMP inhibitory activity. Additionally, a predicted UBP-ZnF (SMART, Simple Modular Architecture Research Tool, http://smart.embl-heidelberg.de) was examined for contribution to regulation of autoubiquitination.

Together, these data point to the existence of multiple regulatory inputs that control IMP activity.

Results and Discussion

As indicated in Chapter 2, IMP autoubiquitination may be induced upon Ras activation, resulting in downregulation of inhibition to allow signal transmission through the ERK pathway. To see if autoubiquitination could occur in response to a biological stimulus, we induced 293 cells with EGF in the presence or absence of a proteosome inhibitor (LLnL) to visualize accumulation of laddering due to poly-ubiquitination. As seen in Fig 3.1b, high molecular weight IMP species are present even without EGF in cells pretreated with LLnL, suggesting that IMP is continually ubiquitinated and degraded by the proteosome. Further, it confirms that the autoubiquitination of IMP induced by Ras12V in Chapter 2 is true to the biology of IMP in this pathway and not simply due to overexpression of an activator. The same bands accumulate in the absence of LLnL by 60 minutes of EGF exposure, indicating that the rate of IMP ubiquitination exceeds the rate of degradation, or that some poly-ubiquitinated IMP proteins are not degraded. The same experiment was performed in HeLa cells, in which similar laddering was observed (not shown).

A puzzling phenomenon in HeLas had been noted in past experiments in which EGF stimulation caused a ‘spike’ in IMP protein levels. The band would appear around 5 or 10 minutes and disappear within two minutes. Interestingly, this is the same point at which MEK and ERK approach peak activation. Immunoblotting revealed that this was likely the result of epitope masking, as an IMP antibody to the N-terminus detected IMP in all

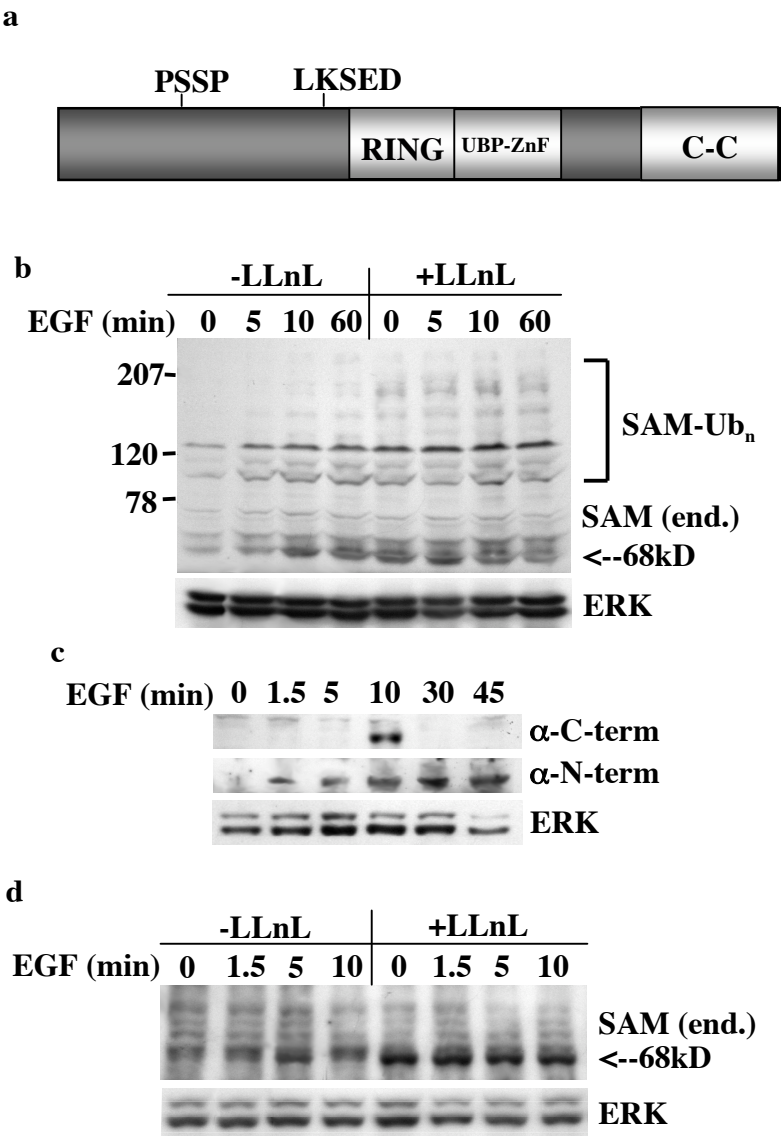


Figure 3.1 Ubiquitination of endogenous IMP. **a**, Domain structure of full-length IMP. PSSP = putative ERK phosphorylation site, LKSED = putative sumoylation site, RING = ubiquitin E3 ligase domain, UBP-ZnF = ubiquitin-specific protease zinc finger, C-C = predicted coiled-coil. **b**, IMP is polyubiquitinated in 293 cells. 293 cells were grown to confluence and deprived of serum ~18 hours, then exposed to LLnL for 30 min prior to EGF (100ng/ml) stimulation. Whole cell lysates were prepared in boiling SDS-Tris and immunoblotted with anti-IMP C-term and anti-ERK for loading control. **c**, IMP protein undergoes stimulus-dependent epitope masking. HeLa cells were grown and treated as in **c**, but without LLnL. Lysates were blotted with anti-IMP antibodies to detect the C- and N-termini. **d**, IMP protein is transiently stabilized upon EGF stimulation. HeLa cells were treated exactly as the 293 cells in **c**.

stimulated lanes, while the C-terminal antibody only revealed the spike (Fig 3.1c). We examined the behavior of IMP protein levels in HeLas exposed to LLnL to see if the epitope masking could be due to IMP ubiquitination. As expected, the IMP spike was present after 5 minutes of EGF. However, in cells pretreated with LLnL, IMP levels were the same even without stimulation, and appeared to be greater than the spike itself (Fig 3.1d). This supports the findings in the 293 cells and suggests that IMP is constantly ubiquitinated and degraded. Additionally, in HeLas this modification may be transiently stabilized to coincide with the crescendo of MEK/ERK activity, perhaps to establish a ceiling of kinase activity to prevent overstimulation of the cell, as proposed in the previous chapter.

It is an attractive notion that the functional reason for the IMP-Ras interaction is to downregulate an inhibitory input, yet it is not known whether IMP autoubiquitination is the result of Ras binding or if other signals are required as well. Phosphorylation has been shown to precede ubiquitination of several proteins. IMP has a putative ERK phosphorylation site, PSSP, at its N-terminus (Fig 3.1a). Thus, we tested whether ERK could phosphorylate IMP. We found that ERK can phosphorylate IMP in vitro, both full-length and an N-terminal portion (Fig 3.2a), indicating that ERK can bind directly to IMP. Ectopically expressed HA-ERK also enhanced IMP phosphorylation induced by Ras12V in cells (Fig 3.2b). Correlated with this was the finding that HA-ERK, in combination with Ras12V, promoted nuclear accumulation of IMP in 30-50% of cells (Fig 3.2c). With Ras12V alone, <5% of cells had nuclear IMP; this effect was not seen with HA-ERK or IMP alone. To eliminate the possibility that this was simply due to overexpression of a potent activator and a kinase, we examined the cellular location of

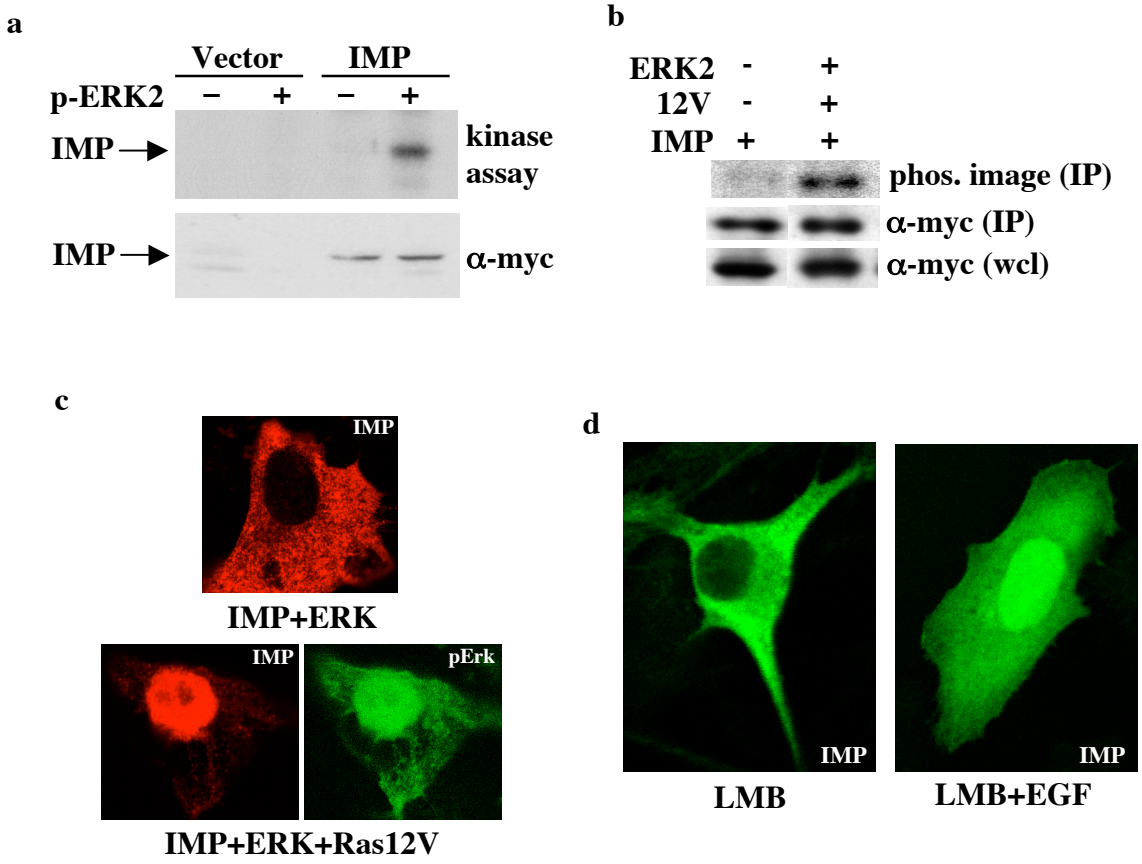


Figure 3.2 IMP is phosphorylated and translocates to the nucleus upon ERK activation. **a**, IMP is ERK substrate. Recombinant phospho-ERK was incubated with anti-myc immunoprecipitates from 293 cells expressing either myc-IMP or vector. **b**, IMP is phosphorylated in cells. Myc-IMP was expressed alone or with Ras12V and HA-ERK2 in 293 cells. The cells were serum-starved ~18 hours, then incubated in phosphate-free media for 5 min. 500uCi ^{32}P-H_3PO_4 were added and allowed to incorporate for 3 hours. The cells were lysed in NP-40 buffer and supernatants incubated with anti-myc agarose to isolate IMP. The IPs were analyzed by SDS-PAGE followed by transfer to PVDF membrane. The membrane first immunoblotted for myc-IMP, then exposed to a phosphorimager plate overnight to detect phosphorylated IMP. **c**, IMP relocates to the nucleus upon Ras12V-induced ERK activation. HeLa cells were transiently transfected as shown. After 18 hours serum-starvation the cells were fixed and immunostained for myc-IMP and phosphoERK as described in Methods. **d**, IMP cycles through the nucleus upon EGF stimulation. HeLa cells were transfected with myc-IMP. Following serum deprivation the cells were exposed to 20nM LMB for 3 hours, then stimulated with EGF (100ng/ml) for 7 min. The cells were fixed and immunostained for myc-IMP as in **c**.

IMP in starved and stimulated cells. The cells were pretreated with a nuclear export inhibitor, leptomycin B (LMB), to facilitate nuclear accumulation. As seen in Fig 3.2d, IMP did not accumulate in the nuclei of unstimulated cells treated with LMB, indicating that IMP does not continuously cycle through the nucleus as has been shown for KSR, MEK and ERK. However, IMP was observed in the nuclei of stimulated cells pretreated with LMB, demonstrating that IMP does translocate to the nucleus upon EGF stimulation. IMP was not seen in the nucleus in stimulated cells that were not exposed to LMB (not shown), suggesting that the nuclear IMP observed with Ras12V and ERK co-expression was likely the result of chronic stimulation. These data place ERK-mediated IMP phosphorylation in the same cellular context as nuclear accumulation of IMP, and suggest that the latter phenomenon may be the result of the former.

Just downstream from the RING domain, IMP contains an ubiquitin binding protein-like zinc finger (UBP-ZnF) (Fig 3.1a) that is exclusively found in many ubiquitin proteases and a few histone deacetylases. In fact, IMP is the only protein in the database outside these groups to possess this domain. The UBP-ZnF in these proteins is not a catalytic motif; rather, it seems to function as a poly-ubiquitin binding domain. We reasoned that this could be a regulatory domain for IMP auto-ubiquitination. The idea was that the UBP-ZnF would limit auto-ubiquitination, and loss of the domain would result in comparatively greater accumulation of poly-ubiquitinated IMP species. We tested two IMP C-terminal deletion mutants for autoubiquitination in vitro (Fig 3.3a). The T3 truncation comprises residues 1-344, which includes the RING domain and interrupts the UBP-ZnF; the T4 truncation comprises residues 1-456 and contains the complete UBP-ZnF. Both mutants displayed E3 ligase activity, suggesting that the C-

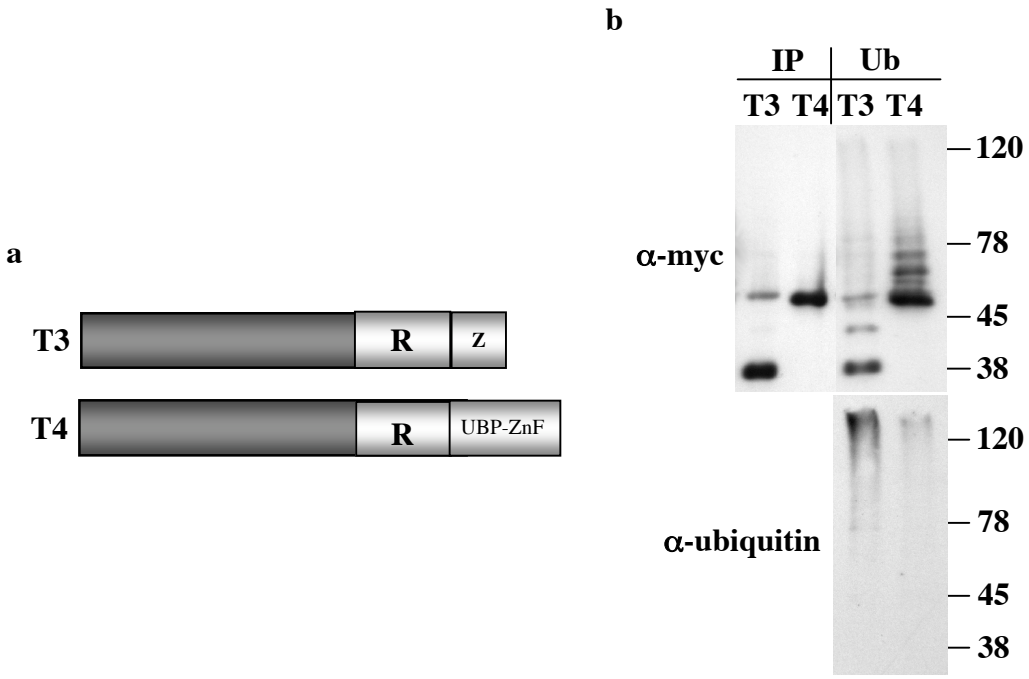


Figure 3.3 The UBP-ZnF of IMP has autoregulatory activity. **a**, Domain structure of the T3 and T4 truncation mutants. **b**, T3 is hyper-ubiquitinated. Myc-T3/T4 were expressed in 293 cells and immunoprecipitated with anti-myc agarose. A sample of each IP was saved for immunoblotting. The remaining portions were assayed for ubiquitin ligase activity as described in Methods. The IPs and assays were immunoblotted with anti-myc to detect laddering and anti-ubiquitin to detect long-chain polyubiquitination.

terminus is not critical for this function (Fig 3.3b). However, T3 accumulated poly-ubiquitinated species to a much greater extent than T4 (see anti-ubiquitin blot). Further, while the levels of immunoprecipitated T3 and T4 were similar, the amount of unmodified T3 in the assay was noticeably less than unmodified T4. This is consistent with the greater accumulation of poly-ubiquitinated T3 and suggests that depletion of the unmodified form was taking place.

In addition to the PSSP and UBP-ZnF motifs, IMP contains a consensus sequence for sumoylation (Fig 3.1a) that may have regulatory functions. To test the effect of SUMO on IMP activity, HA-SUMO was expressed with IMP and BXB. As expected, IMP inhibited BXB-induced phosphorylation of MEK and ERK; however, this activity was potently suppressed upon SUMO-1 expression (Fig 3.4a). To see if other IMP phenotypes were affected, we expressed HA-SUMO with IMP and KSR and observed IMP-induced hyperphosphorylation on KSR. Interestingly, SUMO-1 expression inhibited KSR hyperphosphorylation as well, suggesting that SUMO-1 may be a negative regulator of IMP activity. Finally, we tested IMP for direct modification by SUMO-1. IMP was co-expressed with HA-SUMO-1 and HA-SUMO QT, a variant that cannot be conjugated. We reasoned that if sumoylation downregulates IMP activity, then it is possible that this modification would only occur in stimulated cells, so the cells were either untreated or treated with EGF prior to lysis. As shown in Fig 3.4c, IMP immunoprecipitates blotted with anti-SUMO antibody show a band at approximately 20kD higher than expected for unmodified IMP (68kD). There are lighter bands approximately 40 and 60 kD higher than unmodified IMP, which may represent IMP protein with two and three modifications, respectively. This result was not apparent in

cells expressing SUMO QT (Fig 3.4c), nor in unstimulated cells (not shown). This suggests that IMP may be directly sumoylated at the LKSED site, and may have other less obvious sumoylation sequences as well. Further, this modification may occur upon activation of the ERK pathway; phosphorylation by ERK may target IMP to the nucleus, whereupon it becomes sumoylated. This additional modification may maintain IMP in an inactive state until negative feedback mechanisms have attenuated Ras and/or ERK signaling to the point where IMP can reclaim its inhibitory function. Thus, downregulation of IMP may involve proteosomal degradation for acute derepression of signaling, and sumoylation of additional IMP proteins to maintain a ready pool of threshold modulators to prevent superfluous signal amplitude.

As previously noted, sumoylation is important for the regulation of signaling by the ERK cascade. In *Dictyostelium*, modification of MEK promotes nuclear export and facilitates its interaction with ERK. The pathogenic effector YopJ inhibits MAPK pathways, apparently by mass de-sumoylation [46]. Most recently, SUMO was shown to negatively influence transcription, in that sumoylation of Elk-1 was necessary for its autoinhibitory activity. That IMP contains a consensus sumoylation sequence and that it translocates to the nucleus upon Ras/ERK activation are consistent with the notion that SUMO modification requires the ΨKXE motif and nuclear localization. Further work must be done to confirm these findings and to understand how these putative regulatory elements function to regulate the inhibitory activity of IMP.

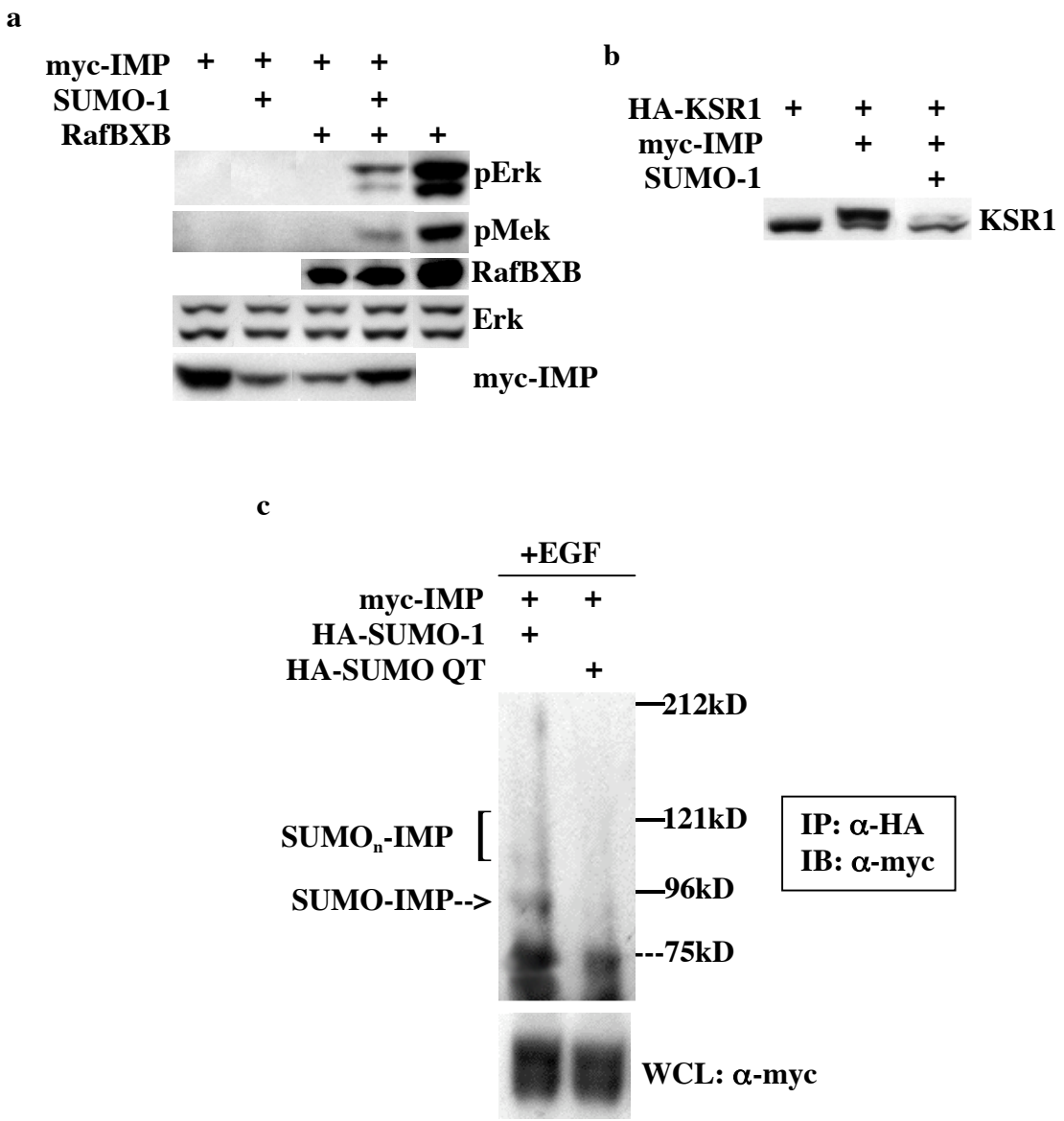


Figure 3.4 SUMO negatively regulates IMP. a, SUMO-1 blocks IMP inhibitory activity. 293 cells were transfected as indicated. The cells were serum-starved ~18 hours, then lysed in boiling SDS-Tris. Whole-cell lysates were immunoblotted for endogenous phospho-ERK, phospho-MEK, and total ERK1/2; ectopic BXB and myc-IMP were detected with anti-Raf (C-12) and anti-myc9E10, respectively. b, SUMO-1 blocks IMP-induced hyperphosphorylation on KSR. 293 cells were transfected as indicated and lysed ~36 hours later as in a. Whole-cells lysates were immunoblotted for HA-KSR with anti-KSR mAb. c, IMP is sumoylated in cells. 293 cells were transfected as indicated and serum-starved ~18 hours. The cells were either exposed to EGF (100ug/ml) for 7 min or left untreated, then lysed in boiling 4% SDS-Tris. The lysates were diluted and analyzed for IMP sumoylation as described in Methods.

Methods and Materials

Expression constructs. pCMV5myc1-IMP T3 and pCMV5myc1-IMP T4 were made by PCR using full-length IMP as a template, using the following primers: T3 RV 5'ATCACTGGATCCTCACTCTAGATTATCACACTT3', T4 RV 5'ATCACTGGATCCTCAATGTCGACTGACATACCG3', IMP FW 5'GATCTGGAATTCATGAGTGTGTCACTGGTT3' (The same forward primer was used for both). Both truncations were cloned into pCMV5myc1 as EcoRI-BamHI inserts, using restriction sites afforded by the primers. Cloning of full-length IMP and the following plasmids were described in Chapter 2: pCMV5myc1-IMP wt/C264, pDCR-HA RasG12V [79], pSRα-RafBXB [79], pCDNA3-HA KSR1 [62], pCDNA3-HA SUMOwt/QT [46]

Antibodies and cell culture reagents. Anti-myc9E10 and anti-Raf (C-12) were obtained from Santa Cruz Biotech. Anti-HA.11 was purchased from Babco. The following antibodies were obtained from Sigma: anti-phospho-S218/S222 MEK1/2, anti-ERK1/2, anti-phospho-T202/Y204 ERK1/2 and anti-ubiquitin. Anti-IMP N-term and anti-IMP C-term were made by immunizing rabbits against peptides corresponding to sequences at the amino and carboxy termini of IMP (Biogen). All secondary antibodies were obtained from Jackson Laboratories: goat anti-mouse HRP, goat anti-rabbit HRP, goat anti-mouse FITC, and goat anti-rabbit rhodamine. Anti-HA agarose and anti-myc9E10 agarose conjugates were obtained from Santa Cruz. Leptomycin B, epidermal growth factor (EGF) and LLnL were obtained from Sigma. ^{32}P-H_3PO4 for cell labeling was from Amersham.

Cell culture and transfection. All reagents described below were obtained from Gibco, except where indicated. All mammalian cell lines were cultured in Dulbecco's Modified Eagle Medium high glucose (DMEM) and 0.5% penicillin/streptomycin, supplemented with serum as indicated. HeLa cells were grown in 10% fetal bovine serum, and HEK293 cells were grown in DMEM without sodium pyruvate supplemented with 10% fetal bovine serum. Cell labeling media. Plasmid DNA was transfected by calcium phosphate precipitation for HEK293 cells and by Lipofectamine 2000 (Promega) for Hela.

Ubiquitin ligase assays. Immunoprecipitated myc-IMP T3 or myc- IMP T4 were added to purified assay components (5uM bovine ubiquitin (Sigma), 0.33uM E1, 13nM recombinant human UbcH4, energy regeneration mix, and ligase buffer (50mM Tris 7.5, 150mM KCl, 1mM $MgCl_2$) and mixed for 1.5 hours at 37ºC.

Immunoprecipitation. Cells were washed in cold PBS and lysed in NP40 buffer (1%NP40, 10mM Tris 7.5, 250uM sodium deoxycholate, 1mM MgCl, 1mM EDTA, 5mM BME, 10% glycerol, 150mM NaCl). The lysates were homogenized by rotating for 30 min at 4ºC and then cleared by centrifugation at 17000xg at 4ºC. Antibody-conjugated agarose was added to the supernatant and incubated either 3 hours or overnight at 4ºC. Beads were washed 4X in lysis buffer plus 500mM NaCl.

Immunofluorescence. Cells were washed with PBS then fixed in 3.7% formaldehyde. Permeabilization, washes, and antibody dilutions were performed with 1% calf serum, 0.25% Triton X-100 in PBS. Primary antibodies were diluted 1:80. Goat anti-mouse FITC was used 1:300 and goat anti-rabbit rhodamine was used 1:1000. All images were captured at 40X on a Leica confocal microscope.

ERK kinase assay. Performed as described in fig.

IMP sumoylation. 293 cells were transfected by $CaPO_4$ ppt. The cells were serum starved 24 hours post-transfection for 18 hours. The cells were lysed in 200uL boiling 4%SDS-10mM Tris 7.5. The lysates were sonicated 4 sec at 80% power, then centrifuged at 17,000 X g for 30 min at 4ºC. The supernatants were diluted 1:40 in NP40 buffer (see above), and centrifuged again. The supernatants were concentrated 4X through a Microcon filter MWCO 50 kD (Millipore), and incubated with anti-HA agarose overnight at 4ºC. The IPs were washed 5X 1ml in NP40 buffer + 500uM NaCl.

Cell labeling. 293 cells were plated 60-80% and transfected by $CaPO_4$ ppt. The cells were serum starved 24 hours post-transfection for 18 hours. The cells were incubated in phosphate-free DMEM w/o serum for 5 min, followed by addition of 500uCi ^{32}P-H_3PO4. The media was removed after 3 hours. The cells were washed in cold PBS, then lysed in NP40 buffer (see above). Immunoprecipitations were performed as indicated above.

CHAPTER FOUR

Using complementary compensatory mutations to establish biological relevance of protein binding events

Abstract

This paper highlights the importance of establishing true binding relationships between associated proteins for the characterization of signaling pathways. Using Ras and its putative effector RalGDS as an example, we describe a simple method to this end. By identifying loss-of-function mutations in both proteins that reciprocally compensate to restore a biological outcome, namely, activation of a downstream GTPase, we support previous suggestions that RalGDS is a bona fide effector of Ras, thereby proving the validity of this technique for authenticating other protein-protein interactions.

Introduction

The small G-protein Ras is a central figure in the regulation of multiple biological functions, including differentiation, cell growth, development, apoptosis, and cell motility [3, 14]. Mutationally activated Ras is significant to the pathology of cell transformation [92], metastasis [92], and evasion of immune recognition [93], and can itself induce growth and morphological transformation of multiple cell lines [94]. Much work has therefore focused on determining the biological role of Ras and discovery of the proteins that mediate its effects, termed Ras effectors. However, the majority of proteins that have been isolated based on their interaction with Ras have unknown biological functions. It is also unclear which Ras phenotypes, if any, many of these candidate effectors mediate.

For example, Nore1 directly binds Ras in a GTP-dependent manner and heterodimerizes with RasSF1A/C [95], yet has not been shown to be important for any known Ras function. The situation is similar for Ras interactors MEKK1, AF6, RGL3, and RIN [3, 14].

A direct way of determining the authenticity of a particular Ras interactor is to generate point mutants in both Ras and the interactor and screen for restoration of an expected Ras phenotype in cells [94]. Past approaches to verifying biologically significant relationships between proteins were either time-consuming or required genetically tractable animal systems. Two-hybrid analysis allows for rapid screening of large numbers of mutants without the need for prior knowledge of required residues or domains [94]. Rescue of the phenotype upon manipulation provides information about the capacity of the interaction to engage in a biological response.

RalGDS, a guanine nucleotide exchange factor (GEF) for RalA/B, is a putative Ras effector protein that may serve as an intermediary through which Ras can regulate the activity of Ral [96]. Many studies have suggested the validity of a RalGDS-Ras interaction with correlative data [97], yet it has not been confirmed in cells. RalGDS has been shown to elicit GTP exchange on Ral upon binding the Ras effector domain mutant 12V37G. This activity does not occur with a Ras mutant 12V40C, to which RalGDS does not bind. Thus we wished to identify a point mutation in the Ras-binding domain (rbd) of RalGDS that would allow it to bind the Ras12V40C effector domain mutant and see if this interaction was sufficient to restore GEF activity toward Ral.

Here we demonstrate a simple approach to verifying the biological significance of a given protein-protein interaction. This approach has broad implications for verifying

physiological relationships, particularly for proteins, such as Ras, with multiple binding partners.

Results and Discussion

To identify a compensatory mutation in the RalGDS rbd that would allow binding to Ras12V40C, we generated a library of rbd mutants from the minimal rbd of RalGDS by error-prone PCR with Taq polymerase. Full-length RalGDS or other parts of the protein were not used as a template for the library because we did not want to produce mutants that would effect unnatural interactions, i.e., between Ras and portions of RalGDS that are known to not facilitate binding with Ras. The library was cloned into a yeast two-hybrid vector that expresses inserts as Gal4-activation domain fusions. The library was screened for interaction with Ras12V40C expressed as a Gal4-DNA binding domain fusion, as determined by X-gal assay and growth on media lacking histidine. Two mutants, D806Vrbd and N749Yrbd, were repeatedly isolated. These mutants were tested pairwise for binding against three Ras-effector domain mutants (12V35S, 12V37G, 12V40C). While other Ras effector domain mutants exist, the variants studied here have been extensively characterized biochemically, in cell culture and in vivo. N749Yrbd interacted with all three to apparently the same degree, while D806Vrbd interacted selectively with 12V40C (Fig 4.1).

We wished to understand the binding strength of the D806Vrbd-12V40C interaction relative to the wildtype interaction. We first subcloned D806Vrbd into full-length RalGDS in place of the wildtype rbd to make pGAD-D806Vfl. We performed a quantitative β-galactosidase assay and found that the relative binding affinity between

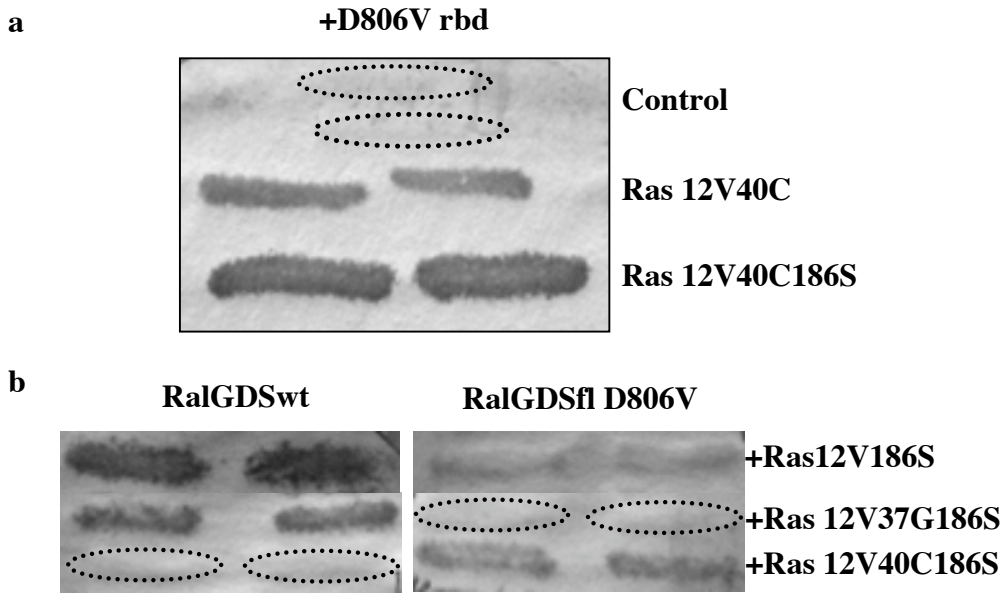


Figure 4.1 D806V interacts with 12V40C in two-hybrid binding assay. Pairwise two-hybrid interaction between pGAD-806Vrbd **(a)**, or pGAD-D806Vfl **(b)** with pLEX-Ras12V40C186S. Positive interaction was determined by growth on His- media (not shown) and blue color in X-gal assay (above).

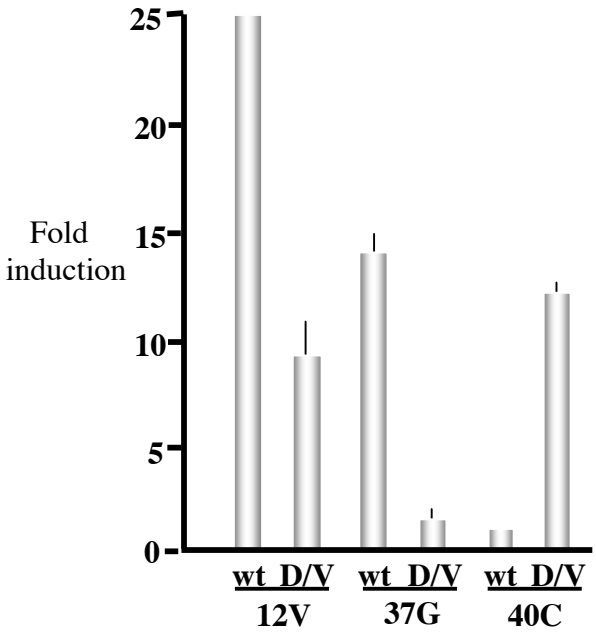


Figure 4.2 D806Vfl binds 12V40C comparably to RGDS wt with 12V37G. Quantitative β-galactosidase assay in *S. cerevisiae*. Yeast were transformed with pBTM116 plasmids encoding 12V, 12V37G or 12V40C and pGADGE plasmids encoding RalGDSwt or D806Vfl. Fold inductions are relative to OD_{600} empty vector. D/V = D806Yfl

pGAD-D806Vfl and 12V40C was the same as that for wtRalGDS and 12V37G (Fig 4.2). Also, the affinity of pGAD-D806Vfl for 12V37G was just as weak as wtRalGDS for 12V40C. These results show the isolation of an ideal candidate RalGDS mutant to test for functional binding.

We then determined whether introducing the D806Vmutation would restore a RalGDS-Ras (12V40C) interaction in cells. D806Vfl was removed from pGAD and subcloned into pCIneoFlag and was transfected into 293 cells along with HA-Ras12V40C. As seen in Fig 4.3, Flag-D806Vfl co-immunoprecipitates with HA-Ras12V40C, albeit not as well as wtRalGDS with Ras12V37G. This could be due to competition by additional cellular effectors for association 12V40C as compared to 12V37G.

Finally, we tested whether this interaction could mediate Ral activation, as measured by GTP binding. We took advantage of the fact that RLIP (*Ral I*nteracting *P*rotein), a protein of unknown function, binds selectively to Ral-GTP. In cells co-expressing RalGDS and Ras, we assayed for active Ral by incubating the supernatants with the Ral-binding domain of RLIP (RLIPrbd) immobilized on agarose. As seen in Fig 4.4, the amount of Ral pulled down was approximately two-fold greater when D806Vfl and Ras12V40C were co-expressed as compared to vector control. This indicates that the binding observed between these mutants may result in a biologically relevant outcome.

In summary, we have described the identification of a RalGDS variant that functionally compensates for a mutation in Ras that blocks binding to wildtype. Ras signaling is a good example for a multi-phenotypic construction composed of many protein interactions. Many molecules have been reported to interact with Ras in two-

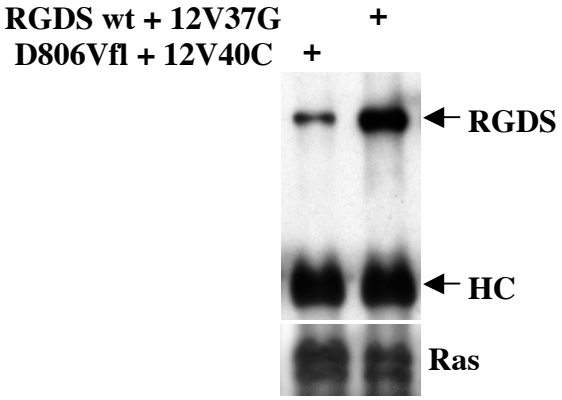


Figure 4.3 RalGDS D806Vfl associates with Ras 12V40C in cells. 293 cells were transfected as indicated and lysed 36 hours later in NP40 buffer. Supernatants were incubated with anti-Flag agarose and immunoblotted with anti-Flag M2 to detect RalGDS IP and anti-Ras to detect Ras co-IP. Ras appears as a doublet due to reactivity of both endogenous (bottom) and HA- tagged Ras (top).

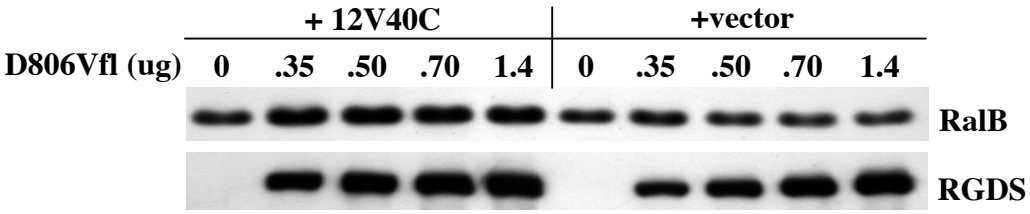


Figure 4.4 Ras 12V40C activates Ral through RalGDS D806Vfl. 293 cells were transfected with either 12V40C or vector, plus increasing amounts of D806Vfl to reveal Ral activation over background. The cells were serum-starved ~18 hours then lysed in Ral binding buffer. The supernatants were incubated with GST-RLIPrbd to bind Ral-GTP. Pull-downs were immunoblotted with anti-RalB and whole cell lysates were blotted with anti-Flag to detect D806Vfl. (Contributed by Y. C. Chien)

hybrid and in vitro, yet the biological role of many of these proteins is unclear. It is also unknown to which Ras-induced phenotypes these proteins contribute, or if even they do mediate Ras functions in cells. The approach described here can be generally applied to any protein with multiple partners to show the biological significance of interactions through complementary compensatory mutations.

With the complete sequencing of the human genome comes a groundswell of proteomic information to be deciphered and verified. Such information will likely suggest new protein families, new members of known families, implied relationships between signaling pathways, and so on. As the ensuing biochemistry will need to be validated, the described technique can provide a reassurance that the direction being pursued is likely true because the binding event requires the reconstitution of an established biological outcome. Thus, a positive result would decrease the possibility of nonspecific binding due to charged/hydrophobic patches, conserved interaction domains, and structural or sequence homology to other proteins. In addition, it is a useful way to parse the contributions of individual protein family members; to explain redundancies, establish/corroborate isoform-specific signaling, reveal points of convergence between pathways, and other issues that can be addressed by direct binding information.

Methods and Materials

Plasmids. pGADGH-RalGDSrbd library, pGADGE-D806Vfl, pCIneo Flag-D806Vfl, pGEX-RLIPrbd, pBTM116- RasG12V/Y40C/C186S, pDCR HA-Ras12V40C

Cell culture reagents. HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose w/o sodium pyruvate, supplemented with 10% calf serum and 1% penicillin/streptomycin (Gibco).

Yeast two-hybrid library screen. The S. cerevisiae strain L40 (MATa his3D200 trp1-901 leu2-3,112 ade2 LYS2::$(lexAop)_4$-HIS3 URA3:: ::$(lexAop)_4$-lacZ gal4? gal80?) was transformed by lithium acetate precipitation with the pGADGH-RalGDSrbd cDNA library (100ug) and pBTM116-RasG12V/Y40C/C186S (500ug) as bait. The transformation yielded approximately 150,000 transformants, for a sampling efficiency of 1. Transformants were selected in media lacking leucine (selects the library plasmid) and tryptophan (selects the bait plasmid). The cells were initially screened for interaction by plating on -leu-trp –his, where histidine production is a reporter for interaction. For a second round of screening, the his+ colonies were replica-plated onto filters to test for positivity in an assay for β-galactosidase acitivity, evidenced by blue colonies in the presence of X-gal substrate. Colonies that were his+ blue were subjected to pairwise two-hybrid analysis to eliminate false positives. First, the bait plasmid was cured away from the library plasmid by transformation of his+ blue yeast DNA extracts (2% Triton, 1% SDS, 100mM NaCl, 10mM Tris 8.0, 1mM Na_2EDTA and phenol/chloroform) into an E. coli strain deficient for leucine production. Miniprep DNA (~1ug) from leu+ E. coli was transformed into L40 with the bait plasmid (1ug) and retested for growth on –his plates

and blue color in β-galactosidase assay. Out of 100 original his+ blue colonies, 40 were tested pairwise, and of these, 21 remained positive. Because of the high sampling frequency, only four positive clones were sequenced. One contained a point mutation, D806V, and the other three contained the same mutation, N749Y.

Quantitative β-galactosidase assay. L40 was transformed pairwise as described above with pGADGE-RasGDS3rbd (1ug) and the indicated bait plasmids (1ug). Transformants were selected on –leu-trp plates; the number of transformants was similar between pairs. One colony from each plate was grown in liquid –leu-trp to OD_{600} ~0.7. The cells from 1ml of culture were pelleted and the media was aspirated. The cell pellet was resuspended and vortexed 15 sec in 500ul Z-buffer (60mM Na_2HPO_4, 40mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH 7) and 25ul trichloromethane (Sigma). After incubation for 10 min at 30C, 100ul ONPG (4mg/ml in H_2O) was added and incubated at 30C until a yellow color developed. The reaction was stopped with 250ul 1M sodium bicarbonate and kept on ice. The samples were analyzed by spectrophotometry at OD_{420}.

Co-immunoprecipitation. HEK293 cells were transfected by calcium phosphate precipitation on 35mm dishes with the indicated plasmids. The media was replaced 18h later. Approximately 36h after transfection, the cells were lysed in NP40 buffer, scraped into cold eppendorph tubes and homogenized by rotation at 4C for 30min. A sample of whole cell lysate was taken at this time. The lysates were clarified by centrifugation for 30min 4C. The supernatants were incubated with 5ug anti-HA .11 (Babco) 30min 4C, then 20ul protein A/G agarose was added for overnight incubation at 4C. The IPs were washed 4X 0.5ml lysis buffer, rotating 10min at 4C in buffer for each wash. After aspirating the last wash, the IPs were boiled in 20ul of 2X sample buffer.

Ral activation assay. HEK293 cells were transfected and lysed as for co-IP experiments, except for the lysis buffer (10% glycerol, 1% NP40, 50mM Tris 7.4, 200mM NaCl, 2.5mM $MgCl_2$, 1mM PMSF, protease inhibitors). The supernatants were incubated with GST-RLIPrbd on glutathione-agarose for 1 hour at 4C. The beads were washed 4X 1ml lysis buffer and analyzed by immunoblot for the presence of endogenous RalB.

CHAPTER FIVE

Discussion and Future Directions

The molecular complexity of Ras signaling is evident by the multiplicity of cellular processes over which it has influence. In directly modulating the activities of relatively few proteins, Ras effects a chain reaction of interprotein binding events that results in posttranslational modification, intracellular translocation, and eventually, changes in cell behavior. How the chain reaction is controlled to prevent crosstalk between similar pathways with shared substrates and to achieve signal-appropriate mobilization of effector proteins is accomplished, in part, by organizing members of a dedicated pathway into preassembled modules whose access to upstream activators can be readily controlled. Not only does this ensure specificity, but it also embeds 'damage control' into the system such that an aberrant protein does not (easily) impair other pathways. Described here were two important aspects to understanding the signaling complexity of any multiphenotypic arrangement, namely, the identification and examination of new effectors and the direct authentication of protein-protein interactions.

Examination of IMP revealed previously undiscovered mechanisms for regulating Ras signal transduction through ERK. IMP serves as a threshold modulator for Ras signaling, in that its overexpression prevents signal throughput between Raf and MEK, and its reduction allows hyperactivation of MEK and ERK; in other words, the amount of IMP protein limits signal transmission through this pathway. Upon Ras activation, IMP autoubiquitinates and is proteosomally degraded in response to EGF. Presumably, signal throughput occurs when IMP levels have been sufficiently decreased. In this capacity, it

may serve as a tumor suppressor; thus, it would be informative to determine IMP protein levels in a variety of cancers. Any cancers with low IMP levels can then be examined for phenomena that would impact its expression, such as promoter dysregulation, gene deletion, genetic mutation of conserved domains/motifs, and many others. The requirements for IMP autoubiquitination should also be determined, whether direct Ras binding is sufficient or if feedback mechanisms, such as ERK phosphorylation and nuclear translocation, are needed as well. Along the same line, the autoregulatory function for the UBP-ZnF needs to be further explored. Mutation of critical zinc-coordinating residues and identification of UBP-ZnF-binding partners will be important to this end. Also of significant interest is the involvement of SUMO in regulation of the ERK cascade and in suppression of IMP activity. SUMO as a positive regulator may impact the pathway at the level of MEK. This is consistent with the de-sumoylation and MAPK inhibitory activity of YopJ and suggests that IMP may be a target of YopJ, at least with regard to the ERK pathway. Conversely, SUMO is reported to support autoinhibition of Elk-1 transcriptional activity. Sumoylation of IMP may reverse the autoinhibitory function of the UBP-ZnF and help stimulate autoubiquitination. It should be straightforward to determine whether mutation of the LKSED site in IMP renders it resistant to SUMO inhibition; other, less perfect motifs should be discerned for SUMO modification. It would be particularly interesting to see if ERK-induced phosphorylation of IMP triggers its nuclear localization and subsequent sumoylation.

As stated previously, the regulated organization of protein complexes is fundamental to achieving signal specificity. IMP prevents access of Raf to MEK, apparently through modulation of KSR's phosphorylation state and intracellular location. Since IMP is not

itself a kinase, it may stabilize a KSR-kinase (c-TAK1?) interaction. IMP may also impact 14-3-3 association with KSR and/or influence the activity of RKIP. Regardless, it is certain that IMP exists in a dynamic complex that at least includes components of the ubiquitin-conjugating pathway and likely other enzymes. Additional ubiquitin ligases may be present as well, since RING E3 ligases have been shown to heterodimerize with other RING-containing proteins and even HECT E3s. Delineation of IMP complexes and its impact on those of KSR is essential to understanding the role of this unique protein in Ras signaling.

Perhaps most interesting is the discovery of dual Ras effector inputs into the same signaling pathway, whereby mitogenic Ras activation leads to induction of Raf kinase activity concomitant with derepression of KSR-dependent Raf/MEK complex formation. This relationship provides a mechanism to limit engagement of the MAP kinase cascade in the absence of Ras activation. The capacity to control the amplitude of this response via molecules like IMP likely contributes to flexible and adaptive cellular behavior in the context of complex regulatory signals.

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VITAE

Sharon A. Matheny was born Sharon Annette Kelly to Marsha and Robert Kelly in St. Louis, Missouri. She spent most of her childhood in rural Illinois, where she dreamed of becoming a paleontologist; her favorite prehistoric animal was the rhinoceros-like *Brontotherium*. She lived with her parents and two brothers in a 19th-century converted tavern surrounded by wheat fields and pig farms, in a town too small to be placed on the map. The town was virtually destroyed in 1993 by a flood of biblical proportions. Fortunately, she and her family had moved to Arizona nine years earlier. She finished her childhood and adolescence in a small but bona fide house in an unincorporated part of east Mesa, which meant that the residents had to pay taxes but the roads were not paved. There, she dreamed of becoming a professional musician and by the time she graduated high school, had won many awards for classical clarinet and jazz saxophone performance, in addition to awards in French and drama. After graduation she worked at a discount eyeglasses store, to which she drove a 1980 blue Ford Pinto that one day caught on fire as she was driving it. Feeling that she was going nowhere fast, that winter she accepted a full music scholarship to Northern Arizona University. After one semester she changed her major to psychology, thus losing the music award but gaining an academic scholarship. That same semester she met her future husband, Dave. While in college she worked as a grocery bagger, a copy girl, a burrito-roller, a sub sandwich-maker, and finally, the head teaching assistant of the organic chemistry labs. Now dreaming of a career in clinical psychology, she spent her sophomore year in an experimental psych lab that studied the interactions of people with the natural environment. The upshot of that work was that men are more destructive towards the environment than are women. Desiring more mystery in her science, she moved to the chemistry department, where she worked on receptor protein aggregation. Now fascinated with molecular biology, she applied for and was awarded a National Science Foundation Undergraduate Research Fellowship to study actin-binding proteins. For her academic endeavors she garnered the Outstanding Psychology Student award two years in a row. In 1996 she graduated magna cum laude from NAU with a bachelor of science in psychology and a minor in chemistry. She spent almost two years working as a research technician and commenced graduate school at UTSW in 1998. Her graduate experience was frustrating, painful, infuriating, enlightening, invigorating and deeply gratifying. A significant part of all these sentiments was due to her mentor, who likely found working with her just as frustrating, painful, infuriating, etc. Yet through this process she learned how to (and how not to) be a scientist, which is something not all graduate students can rightly claim. Now she looks forward to her postdoctoral training in molecular neuroscience, specifically, neural regeneration after CNS injury. In her free time, late at night, she explores the human psyche through abstract oil-collage painting. What will come next is comfortably left to wonder—the realm of human experience is vast, and she is curious.