Diverse Mechanisms of G Protein Regulation by Monoubiquitination

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ABSTRACT

RACHAEL BAKER: Diverse Mechanisms of G Protein Regulation by Monoubiquitination
(Under the direction of Henrik Dohlman and Sharon Campbell)

Cell signaling pathways convert information from the extracellular environment into an intracellular response. It is essential that these pathways be turned on and off on the appropriate timescales. Post-translational modifications are one essential mechanism used to maintain proper signaling. One post-translational modification that is emerging as a key regulator of cell signaling is monoubiquitination. Monoubiquitination is dynamic and reversible, making it ideal for temporal and spatial regulation. It has recently become evident that monoubiquitination regulates G proteins, which are the molecular switches that turn signaling pathways on and off. However, the mechanisms by which monoubiquitination acts on these enzymes is not known.

We used a chemical ubiquitination approach coupled with biochemical and biophysical assays to elucidate the mechanisms by which two G proteins, the small G protein Ras and the heterotrimeric G protein Gpa1, are regulated by monoubiquitination. Monoubiquitination at one position activates K-Ras by impeding regulator-mediated hydrolysis while monoubiquitination at a distinct site activates H-Ras by increasing intrinsic nucleotide exchange. Together, these results demonstrate that monoubiquitination contributes to isoform-dependent regulation of Ras in a site-specific manner. Furthermore, we found that the site of ubiquitination on Gpa1 was in a unique

domain that is essential for trafficking but does not contribute to enzymatic activity. The G protein substrates we chose exhibited diverse mechanisms of regulation by monoubiquitination including altering protein interactions (K-Ras), intrinsic activity (H-Ras), and localization (Gpa1). In summary, our results represent the first mechanistic study of G protein regulation by monoubiquitination and contribute to understanding Ras and Gpa1 regulation specifically as well as regulation of G proteins by monoubiquitination generally. More broadly, these results illustrate the diverse roles for monoubiquitination in the regulation of cell signaling.

To my husband, who believes in me when I lack the confidence to believe in myself

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABD-F	1 mM 4-fluoro-7-aminosulfonylbenzoflurazan
Ala or A	Alanine
α	Alpha
AlF_3	Aluminum Fluoride
A. thaliana	*Arabidopsis thaliana*
Arg or R	Arginine
ATP	Adenosine Triphosphate
β	Beta
CD	Circular Dichroism
Cys or C	Cysteine
CRD	Cysteine Rich Domain
Δ	Deletion
DUB	Deubiquitinating Enzyme
D. melanogaster	*Drosophila melanogaster*
E. coli	*Escherichia coli*
E1	Ubiquitin Activating Enzyme
E2	Ubiquitin Conjugating Enzyme
E3	Ubiquitin Ligase
ERK	Extracellular Signal-Related Kinase
fQCR	Fast Quantitative Cysteine Reactivity
FRET	Fluorescence Resonance Energy Transfer

γ	Gamma
GAP	GTPase Activating Protein
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
Gly or G	Glycine
GMPPNP	Guanylyl Imidodiphosphate
GPCR	G Protein Coupled Receptor
GST	Glutathione S-Transferase
GTP	Guanosine Triphosphate
HECT	Homologous to E6-ap Carboxyl Terminus
His or H	Histidine
HSQC	Heteronuclear Single Quantum Coherence
HTPF	Homogenous Time-Resolved Fluorescence
HVR	Hypervariable Region
IKK	IB Kinase
Ile or I	Isoleucine
Leu or L	Leucine
Lys or K	Lysine
MANT-GDP	2'- / 3'- O- (N'- Methylanthraniloyl)- Guanosine Diphosphate
MD	Molecular Dynamics
Mg	Magnesium
mUbRas	Monoubiquitinated Ras
NMR	Nuclear Magnetic Resonance

PCNA	Proliferating Nuclear Cell Antigen
PDB	Protein Data Bank
PI3K	Phosphatidyl-Inositol-3-Kinase
RBD	Ras Binding Domain
RGS	Regulator of G Protein Signaling
RING	Really Interesting New Gene
RTK	Receptor Tyrosine Kinase
S. cerevisiae	*Saccharomyces cerevisiae*
SCF	Skp1-Cullin-F-Box
Ser or S	Serine
TCEP	Tris (2-carboxyethyl) Phosphine Hydrochloride
TEV	Tobacco Etch Virus
TLS	Translesion Synthesis
T_m	Melting Temperature
Ub	Ubiquitin
UBA	Ubiquitin Associated Domain
UBD	Ubiquitin Binding Domain
UD	Ubiquitination Domain
UIM	Ubiquitin Interacting Motif
UL	Ubiquitin Linker
Val or V	Valine
WT	Wild Type

CHAPTER I

INTRODUCTION[1]

When cells receive a signal, it is crucial that they respond correctly and limit the time frame of their response. The regulation of all cellular events requires careful maintenance of proper protein levels and activity, a crucial balance that is determined by the control of protein synthesis, localization, activation, and degradation. Many of these levels of control are fine-tuned by post-translational modification of proteins by mechanisms such as lipidation, glycosylation, phosphorylation, monoubiquitination, and polyubiquitination. While monoubiquitination is similar in name to polyubiquitination, it is more similar in function to conditional post-translational modifications like phosphorylation. The dysregulation of ubiquitination is implicated in many diseases including cancer, autoimmune disorders, neurodegenerative diseases, and developmental disorders. However, particularly in the case of monoubiquitination, we have yet to understand the full extent of the mechanisms by which this modification is used to regulate proper cellular signaling and response. The potential functional diversity of this signal is staggering, and our ability to understand its mechanism of action is in many cases limited only by our ability to generate enough modified substrate to study by biochemical and biophysical methods. This thesis will focus specifically on the study of monoubiquitination of key components of signaling pathways, G proteins, using a

[1] All figures contributed by Rachael Baker

chemical approach to generate monoubiquitinated substrate. This approach allows us to couple the mechanistic understanding of a protein developed from biochemical and biophysical studies to data that demonstrate the importance of monoubiquitination for maintaining proper cellular signaling *in vivo*. This introductory chapter will specifically focus on what is known about regulation by monoubiquitination and limitations in our knowledge and available resources to study monoubiquitinated proteins.

Monoubiquitination

Monoubiquitination is a dynamic, reversible post-translational modification that involves attaching the 76 amino acid protein Ubiquitin to a targeted substrate. Even though monoubiquitination involves modifying a protein with a distinct protein, it is used in a manner similar to post-translational modification by phosphorylation. Phosphorylation is central to the regulation of cell signaling pathways (*1-4*) and can even be required as a precursor for ubiquitination (*5*). Given the similarities between the ways these two post-translational modifications can be used and their effects on substrates, it is now evident that monoubiquitination is also emerging as a major player in cell signaling regulation.

Evidence for regulation by monoubiquitination is present in a number of key cell pathways, including regulation of DNA expression through modification of histones and processivity factors, regulation of signaling through endocytosis, and regulation of viruses (*6*). However, our understanding of the mechanisms by which monoubiquitination can be used in cellular regulation is still limited. Furthermore, we lack an understanding of the changes in protein structure, dynamics, and activity that

occur when a protein is monoubiquitinated and that could contribute to understanding the mechanisms through which substrates are regulated by monoubiquitination.

One challenge to advancing our understanding of the breadth of regulation by monoubiquitination is the lack of a resource that consolidates published information on substrates of monoubiquitination. As of April 2013 there have been 10,787 non-redundant substrates of ubiquitination (all species) published in the literature, and while this is less than the 207,569 non-redundant phosphorylation sites identified on 19,807 different proteins, it is a number that will continue to grow as detection methods improve and the diversity and importance of this modification is more fully appreciated (*7*). However, while there are databases that seek to document substrates of ubiquitination in general, as of the time of publication, there is no database that separately documents and categorizes substrates of monoubiquitination. In the future, considering monoubiquitination as a post-translational modification distinct from polyubiquitination will be crucial for understanding and appreciating the complex and elegant way in which monoubiquitination aids in orchestrating proper cell function.

History of Discovery of Monoubiquitination

The discovery of the first monoubiquitinated substrate pre-dates the discovery that polyubiquitination is used for the regulation of protein abundance (*8*). The first protein known to be modified by Ubiquitin was histone H2A, although in the initial publication in 1977, Ubiquitin was not mentioned by name; the authors established that histone H2A could be linked through an isopeptide linkage to a peptide of non-histone origin (*9*). The conjugate was later shown to be Ubiquitin (*10*). In the early 1980s, Ubiquitin was rediscovered in the form of polyubiquitination, a post-translational modification that led

to protein degradation (*8, 11, 12*). It was polyubiquitination and degradation that dominated the next years of Ubiquitin research, eventually leading to the Nobel Prize in 2004 (*12*). While our understanding of the regulation of protein abundance by polyubiquitination expanded, studies also began to show that monoubiquitination played a diverse role in the regulation of proper cellular function.

In 1986, a lymphocyte homing receptor was shown to be monoubiquitinated on its extracellular domain, suggesting a more general role of monoubiquitination in the non-degradive regulation of cell surface proteins (*13, 14*). There was also early evidence that plasma membrane proteins were modified with Ubiquitin and that this modification could direct proteins into the endocytotic pathway (*15, 16*). More recent reports have demonstrated the functional importance of monoubiquitination for inhibiting the ability of endocytotic adaptor proteins to bind to other monoubiquitinated proteins and regulate endocytosis (*17-19*).

The early discoveries and knowledge of the first identified functions of ubiquitination shaped our perception of the way the monoubiquitination is used to regulate proteins in the cell. It is only more recently, as techniques for the detection and study of ubiquitinated substrates have improved, that it has become apparent that monoubiquitination could be more than a binding partner for endocytotic signaling proteins and could directly modulate protein activity. These recent findings suggest a more careful study of monoubiquitinated substrates and the ways monoubiquitination is used to regulate cell functions is required. The rest of the introduction chapter will describe what is known about the process and outcomes of monoubiquitination, focusing on areas where our knowledge of this post-translational modification is still limited.

How Substrates are Monoubiquitinated

Proteins are ubiquitinated by a three component enzyme cascade that includes a Ubiquitin activating enzyme (E1), Ubiquitin conjugating enzyme (E2), and Ubiquitin protein ligase (E3) (**Figure 1.1**) (*20-24*). Ubiquitin is first activated by the E1 enzyme in an ATP-dependent reaction in which a thioester bond is formed between the c-terminal glycine of Ubiquitin and a cysteine on the E1 (*25*). After activation by the E1 enzyme, Ubiquitin is transferred to a cysteine on the E2 enzyme through trans-esterification (*26*). The E3 ligase is then used to transfer the Ubiquitin either directly or indirectly to the substrate, depending on which type of E3 is used. The final result is a substrate modified with Ubiquitin through an isopeptide linkage between the lysine side chain of the substrate and the c-terminal glycine of Ubiquitin (**Figure 1.1**).

E3 enzymes can be divided into two classes based on their mechanisms of Ubiquitin conjugation: HECT (Homologous to E6-ap Carboxyl Terminus) E3s and RING (Really Interesting New Gene) E3s, which are further subdivided depending on whether they are single-subunit or multi-subunit RING E3s (*21*). HECT domain-containing E3s first bind to the E2, and then Ubiquitin is transferred from the E2 to the active cysteine on the E3 (*27*). The E3 then binds the substrate and directly catalyzes ubiquitination through the transfer of the Ubiquitin from the active site cysteine to the lysine residue on the substrate (*21*). RING domain-containing E3s differ from HECT domain-containing E3s in that they have no enzymatic activity. They act as scaffolds to bring together the Ubiquitin-containing E2 and substrate to be ubiquitinated (*28, 29*). Single-subunit RING E3s have the RING E3 activity and substrate binding domain in one protein, while multi-

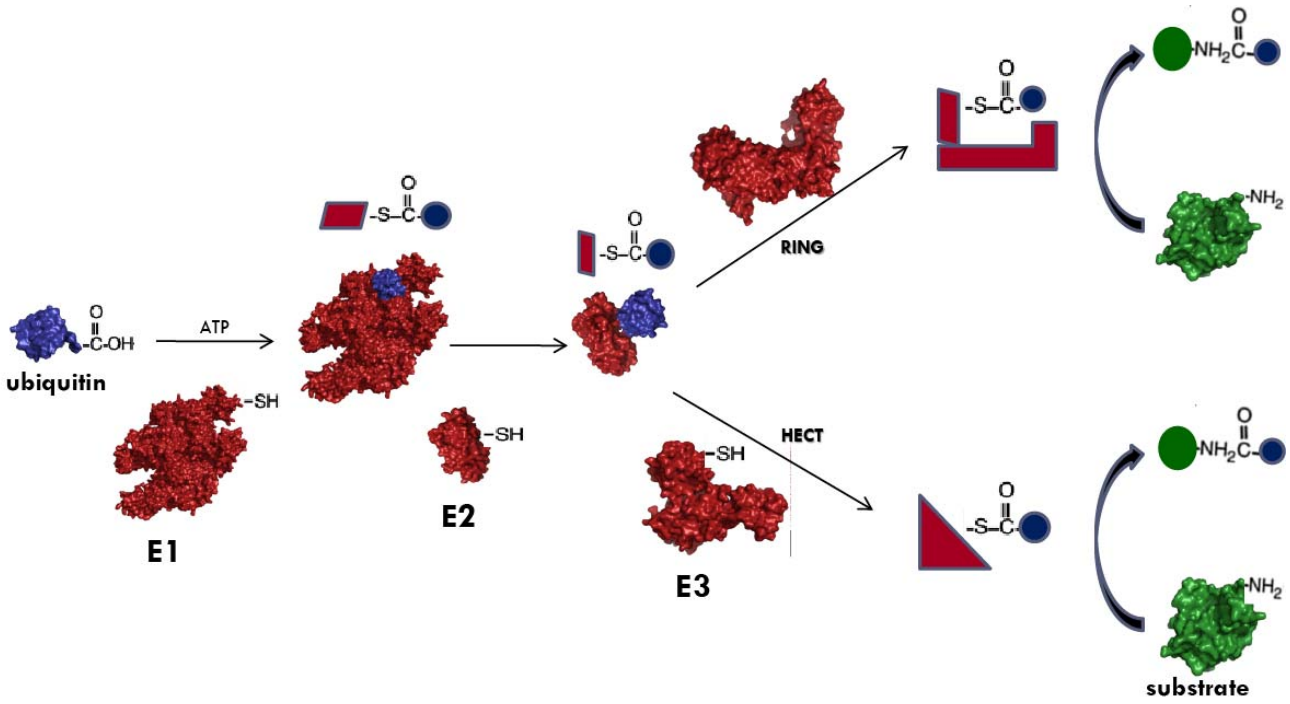

Figure 1.1. Monoubiquitination by the Ubiquitin Ligase Complex**.** Ubiquitin is activated by the E1 enzyme and then transferred to the E2 enzyme through transthiolation. If a RING Ubiquitin ligase is being used, the E2 with the activated Ubiquitin interacts with the RING E3, which provides substrate specificity, to transfer Ubiquitin to a lysine on the substrate. If a HECT Ubiquitin ligase is used, the activated Ubiquitin is first transferred directly to the E3, which both binds the Ubiquitin and contains the substrate specificity, before being transferred to a lysine on the substrate.

subunit RING E3s consist of multiple proteins, one of which contains the RING E3 and another that has the substrate binding specificity (*29*).

Ubiquitination is a specific and selective process. Monoubiquitination can target a single isoform of highly conserved proteins in a defined region of cell space. The specificity of substrate selection is primarily provided by the number of E3 ligases that exist in the cell (*30, 31*). There are 500 E3s in mammals, while there are 30 E2s and only a few E1s (*6, 30, 32-34*). Each E2 can therefore provide Ubiquitin for a number of different E3 ligases, and the E3 ligases can recognize distinct substrates, thus providing careful control over which substrates are ubiquitinated (*35*).

While the process of substrate ubiquitination has been extensively characterized, less is understood specifically about how monoubiquitinated signals (as opposed to polyubiquitinated signals) are generated. Cells adopt several strategies to ensure that a substrate is monoubiquitinated. The first mechanism involves using an E2 that only leads to monoubiquitination. For example, when the E2 Rad6 is used, histone H2B is only monoubiquitinated because this E2 does not remain associated with the E3, which is necessary for additional rounds of ubiquitination to occur (*36*). In a similar mechanism, Ubiquitin chain elongation could be restricted by coupling an E2 and E3 that do not strongly interact with each other. For example, when the E2 Cdc34 is coupled with the E3 Rag1, an unusual mode of interaction is used that does not favor re-association between the E2 and E3 after the first Ubiquitin has been transferred to the substrate (*37*). For polyubiquitination to occur, reassociation between the E2 and E3 must occur (*37*). Finally, monoubiquitination can also be achieved by linking ubiquitination and low affinity Ubiquitin binding, which is referred to as coupled monoubiquitination (*38*). An

example of this mechanism is the substrate Eps15. When Eps15 is monoubiquitinated, it undergoes a conformational change and folds back on itself, binding to Ubiquitin with its own ubiquitin-interacting motif (UIM). The conformational change that Eps15 undergoes inhibits the Ubiquitin ligase, Nedd4, from further interactions with Eps15, which are required for Ubiquitin chain elongation (*39*).

The complexity exhibited in the process of ubiquitination extends to a complex network of mechanisms that regulate ubiquitination. The activity of the Ubiquitin ligases themselves is carefully regulated. Some Ubiquitin ligases are constitutively active, but have an adaptor protein that must be recruited before the E3 can bind to the E2 (*27*). Other Ubiquitin ligase complexes are not active until they have been post-translationally modified (most often, phosphorylated). Phosphorylation often serves to release inhibitory interactions between the domains of an E3 so it can bind E2 or substrate and transfer Ubiquitin (*27*). Regulation of ubiquitination can also occur through localization of the Ubiquitin ligase complex, which ensures that only specific pools of a protein or only specific isoforms are ubiquitinated. Finally, ubiquitination can be regulated by first requiring alternative post-translational modification of the substrate. For example, many substrates of Skp1-Cullin-F-box Ubiquitin ligase complex (SCF) must be phosphorylated before they are recognized as substrates for ubiquitination (*29*).

Similar to phosphorylation, monoubiquitination is not a permanent post-translational modification. Deubiquitinating enzymes (DUBs), which are akin to the phosphatases that remove phosphorylation, can also regulate cellular processes by removing Ubiquitin from a substrate by cleaving the bond between the substrate and Ubiquitin (*5, 27*). The human genome codes about 80 DUBs, which are also involved in

recycling and processing polyubiquitin chains (*40, 41*). The large number of DUBs suggest that the act of removing Ubiquitin, similar to adding Ubiquitin, is both tightly controlled and substrate specific. The DUBs themselves are regulated by mechanisms such as conformational changes that occur when the DUB binds to a substrate, a requirement for an adaptor protein to recognize and bind to a substrate, or post-translational modification of the DUB itself, in some cases by monoubiquitination (*42*). The presence of DUBs highlights the exciting possibility that monoubiquitination can be used to transiently alter protein localization, binding partners, or even function or activity.

Substrates of Monoubiquitination

Very little is known about what makes substrates amenable to monoubiquitination. There is some general evidence that the amino acid composition and local structure surrounding the ubiquitination site on the substrate is crucial to the process of protein targeting. A recent analysis of almost 150 ubiquitination sites in yeast demonstrated that some Ubiquitin ligase complexes have a strong sequence bias for lysines surrounded by polar acidic and uncharged residues (*43*). However, the sites of ubiquitination identified in this study were primarily substrates of the HECT Ubiquitin ligase Rsp5, so this may not represent a universal observation about sites of ubiquitination. In support of amino acid sequence directing ubiquitination, a study by Sadowski et al. showed that the propensity of Ubiquitin for a lysine within a substrate can be altered by mutating residues around the known ubiquitination site (*44*). These studies demonstrate that the sequence surrounding lysines in a substrate is a determinant for

ubiquitination and can in some cases be used to predict where ubiquitination can occur (*45*).

Understanding what makes a good site for ubiquitination also requires consideration of structural elements of the substrate. A recent study by Hagai et al. showed that ubiquitination sites of a large number of substrates of monoubiquitination are targeted toward structured regions of proteins (*46*). Ubiquitination sites appear to favor a helix or a coil over a strand, and ubiquitination sites that exist on helices or strands are most often surrounded by ordered residues (*46*). Other studies focused on polyubiquitination observe a preference for ubiquitination of lysines in stretches of amino acid sequence that are likely to be disordered (*43, 45, 47-50*). One reason it may be challenging to identify conserved patterns for sites of ubiquitination is that, as noted in a recent study of the evolutionary development of ubiquitination, in many cases it appears that Ubiquitin ligases evolve to modify existing lysines rather than lysines of substrates evolving to become favorable sites of ubiquitination (*51*). Thus, preference for targeting may be specific for a particular Ubiquitin ligase complex. It is also important to note that while the studies described give information about protein sites that are amenable to ubiquitination, they fail to provide information about whether those identified sites will actually become ubiquitinated *in vivo.*

Outcomes of Monoubiquitination

It is clear from the literature that monoubiquitination is a more important and versatile post-translational modification than was initially predicted (*43*). There is evidence for monoubiquitination regulating processes as diverse as gene transcription, protein localization, and protein activity. There is also evidence of substrates, Ubiquitin

ligases, and DUBs being mutated or misregulated in disease and cancerous states. In many cases, a key determinant of the outcome of monoubiquitination is the presence of proteins or domains that recognize the post-translational modification (*5, 52*). Ubiquitin has also been shown to adopt distinct conformations depending on its binding partner, which can aid in recognition by distinct regulators of the monoubiquitinated substrate (**Figure 1.2a**) (*53*). In some cases, monoubiquitination alters interactions with pre-existing protein binding partners, but in other cases, the modified substrate is recognized by a new protein, often containing a Ubiquitin binding domain (UBD). There are sixteen different types of UBDs that mediate most of the interactions with ubiquitinated substrates and that can form interactions with multiple surfaces of Ubiquitin (*53, 54*). Most UBDs interact with a hydrophobic patch on Ubiquitin (Leu8, Ile44, and Val70) (**Figure 1.2b**) (*53*).

There are numerous reviews on the well-characterized cellular functions of monoubiquitination, and these outcomes will be only briefly discussed here (*52, 55*). A summary of the known outcomes of monoubiquitination is shown in **Figure 1.2c**. Monoubiquitination is clearly involved in three distinct cellular functions: gene and protein expression through histone and transcription regulation, endocytosis, and retroviral budding. Monoubiquitination may also control the activity of the endocytotic machinery (*15, 55, 56*). Monoubiquitination can act on these systems through mechanisms as diverse as regulating the activity of transacting endocytotic proteins (*57, 58*) and transcription factors in the nucleus (*59*), or regulating protein-protein interactions (*60*).

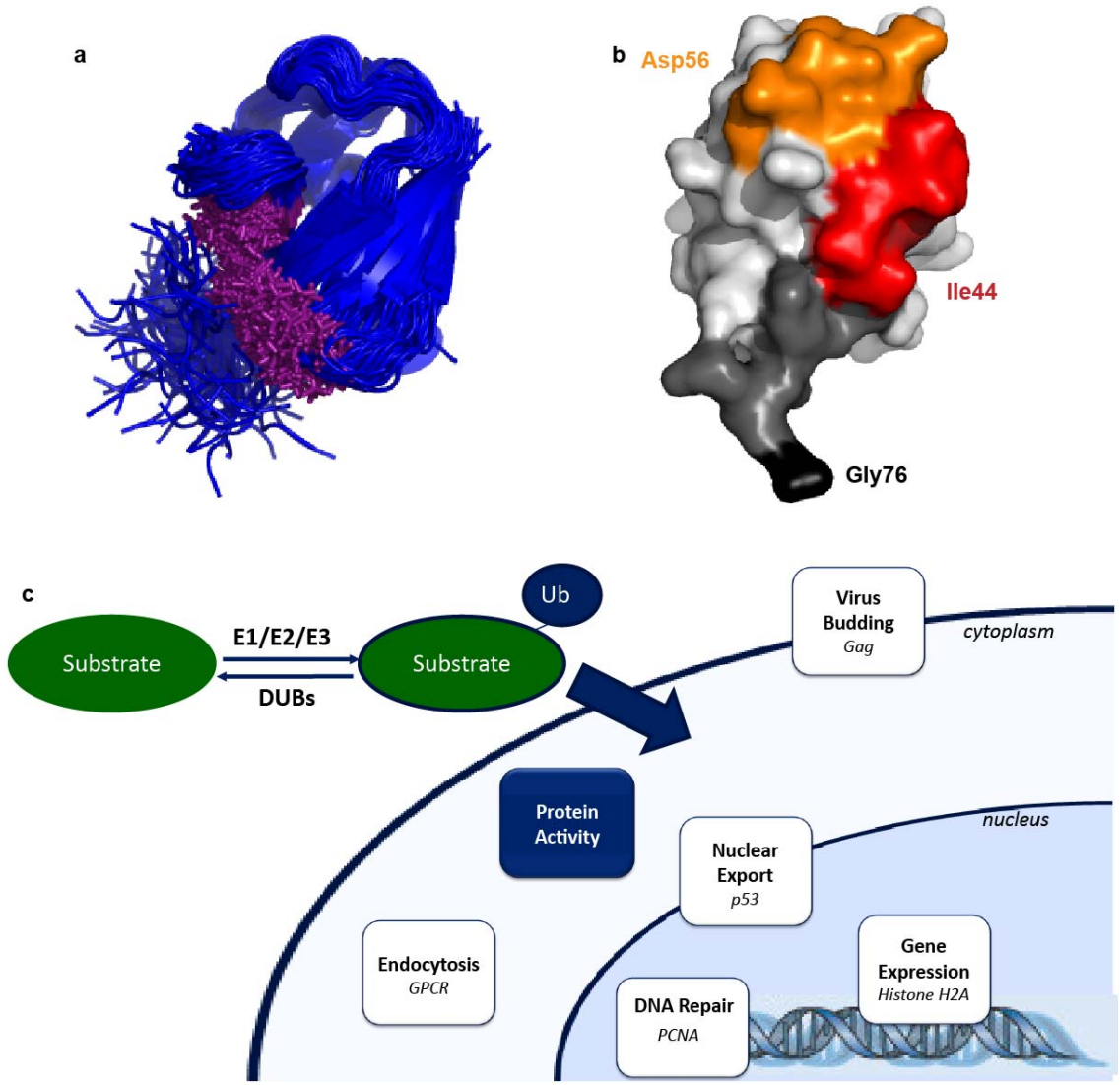

Figure 1.2. Recognition and Outcomes of Monoubiquitination**.** (**a**) When Ubiquitin (2K39) is in solution, it exhibits conformational diversity. UBDs recognize different conformations of Ubiquitin, and the side chains of the hydrophobic amino acids (Leu8, Ile44, and Val70) most frequently recognized by the UBDs are highlighted in purple. (**b**) Surface of Ubiquitin (1UBQ) with the C-terminal region where ligation to substrate occurs shown in gray, the hydrophobic patch recognized by most UBDs shown in red (centered on Ile44). In orange is a diglycine patch that is also recognized by some UBDs (centered on Asp56). (**c**) Substrate monoubiquitination leads to the regulation of a variety of processes including endocytosis, DNA repair, gene expression, nuclear export, and virus budding (examples given in italics). Monoubiquitination also regulates protein activity, although this function of monoubiquitination has not been well-characterized.

Monoubiquitination is intricately involved in the regulation of gene expression in the nucleus. This post-translational modification is one of many (including methylation and acetylation) that can modify histones and alter chromatin structure, which directly alters gene expression (*17, 59*). Furthermore, monoubiquitination regulates gene expression by targeting enzymes involved in DNA repair and transcription. When the DNA processivity factor Proliferating Cell Nuclear Antigen (PCNA) is monoubiquitinated, it recruits Translesion DNA synthesis (TLS)-specific DNA polymerases, which allow PCNA to bypass a DNA lesion (*17, 61-63*). In another example, the transcription factor NF-κB, which controls expression of genes involved in cell growth and immunity (*64, 65*), is regulated by IB Kinase (IKK), which block NF-κB inhibitors by marking them for polyubiquitination and degradation (*66*). For IKK to be activated, it must first be phosphorylated, which also makes it a substrate for monoubiquitination in chronically activated cells (*67, 68*). IKK that cannot be monoubiquitinated is resistant to chronic activation (*69*).

Many of the best characterized substrates of monoubiquitination are involved in membrane protein trafficking and receptor internalization through endocytosis (**Figure 1.3**) (*30, 58, 70*). In endocytosis, monoubiquitination is a sorting signal for Receptor Tyrosine Kinases (RTKs), G Protein Coupled Receptors (GPCRs), transporters, and ion channels (*17, 18, 58, 71*). For example, the yeast GPCR, Ste2, is ubiquitinated after binding to its ligand, pheromone, and monoubiquitination promotes its entry into endocytotic vesicles and its rapid removal from the plasma membrane (*15, 72*). Endocytosis of membrane proteins is important because it regulates signaling by quickly removing the protein from the site at which it mediates activity (*15*). Monoubiquitinated

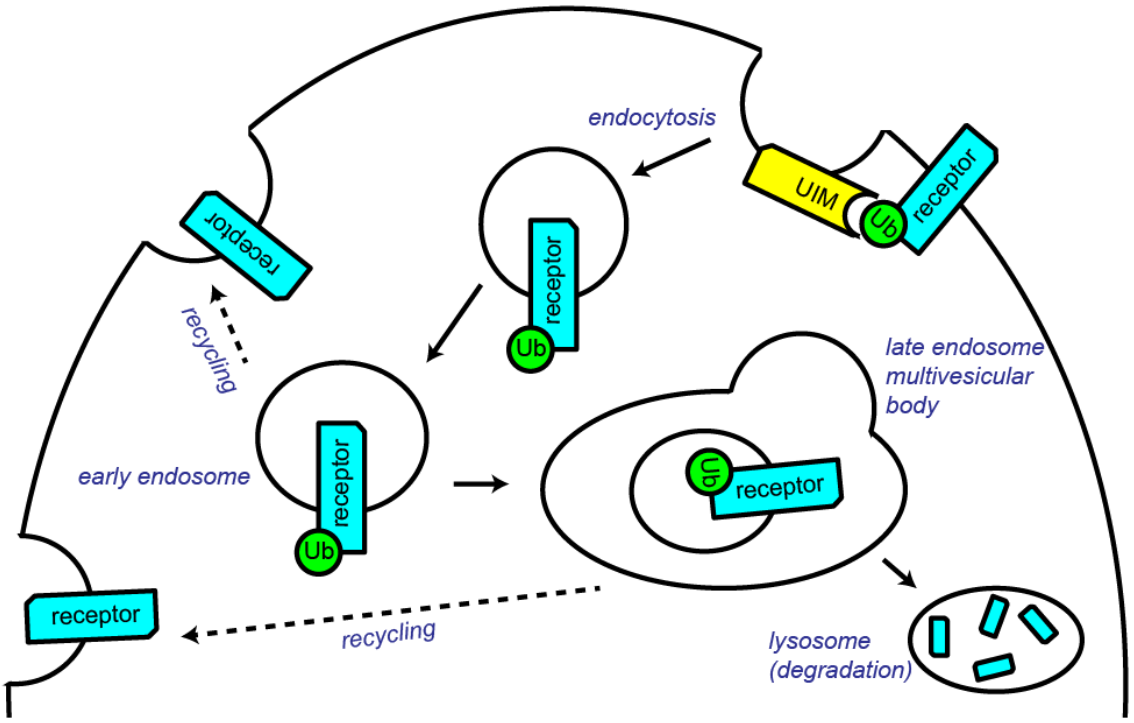

Figure 1.3. Monoubiquitination Leads to Receptor Endocytosis. When a receptor is monoubiquitinated, the Ubiquitin is recognized by a protein with a Ubiquitin interacting motif (UIM), which also binds to a clathrin-coated pit. At the early endosome, the receptor can continue through the process of endocytosis, or be recycled back to the membrane. The receptor can also be recycled back to the membrane from the late endosome. Finally, endocytosis can lead to receptor degradation at the lysosome.

proteins, like Ste2, can in some cases be degraded through the lysosomal system after endocytosis occurs (*13*). The Ubiquitin moiety fused to a membrane protein carries with it the necessary information for endocytosis facilitated by endocytotic adaptor proteins (*73, 74*). Ubiquitin binding endocytotic adaptor proteins are also themselves regulated by monoubiquitination (*17*). One example of this type of regulation is β-arrestin, which binds phospholipids and clathrin and is important for the endocytosis of activated GPCRs. β-arrestin itself is monoubiquitinated after the β2-adrenergic receptor is activated, and this monoubiquitination is required for the rapid internalization of the receptor (*75, 76*). In fact, monoubiquitination of β-arrestin is sufficient for β2-adrenergic receptor endocytosis (*77*).

Monoubiquitination is also important in the process of virus budding. Enveloped viruses exit the cell by budding from the cell membrane, and it is known that reducing cellular levels of Ubiquitin inhibits budding (*55*). One example of this regulatory process is monoubiquitination of the protein Gag, which is an essential component of retroviruses. The Gag protein has an embedded sequence, termed a late domain, which is essential for budding. This late domain is known to be an interaction motif for the Ubiquitin ligase Nedd4, leading to ubiquitination when Gag is properly localized to the membrane (*78, 79*). When Gag cannot be monoubiquitinated, viral budding does not occur (*80, 81*).

The above examples describe the well-documented uses of monoubiquitination in cell regulation. There are, however, a number of substrates that have been identified *in vivo* and *in vitro* that either do not yet have clear physiological outcomes or whose outcomes do not fit easily into one of these three primary categories (*55, 82*). For

example, monoubiquitination has been shown to directly regulate the activity of the DUB ataxin-3, leading to an enhancement of its enzymatic activity (*83*). On the other hand, monoubiquitination of dihydrofolate reductase, an enzyme involved in DNA synthesis, suppresses its enzymatic activity (*84*). Finally, monoubiquitination of the tumor suppressor p53 leads to a conformational change in the protein that exposes a previously buried nuclear export signal (*85*). Other substrates, like the small GTPase Rac1, have been identified as substrates of monoubiquitination, but the physiological role of the post-translational modification is not yet known (*86*). It is likely that as our ability to detect and study monoubiquitinated substrates improves, new cellular functions for the modification will be discovered.

One challenge that exists in understanding the role of monoubiquitination in cell regulation is that there is no online resource that consolidates information on all monoubiquitinated substrates. There are databases that list all currently identified substrates of ubiquitination, but they do not distinguish between polyubiquitination and monoubiquitination. As we have previously discussed, the outcomes of these two types of post-translational modifications are clearly distinct. Mass spectrometry and other large scale approaches are being adapted to generate large databases of monoubiquitinated proteins (*87, 88*). However, it is important not just to document occurrences of this post-translational modification, but to understand and categorize the ways this post-translational modification is being used in cellular regulation. A resource containing this type of analysis would allow continued identification of patterns and trends in the way monoubiquitination is used *in vivo*.

Ubiquitination and Disease

Understanding the diverse mechanisms by which monoubiquitination is used to regulate substrate localization, binding, and activity becomes more important as a role for ubiquitination is emerging in the study of cancer and developmental disorders. For example, sequence preferences surrounding the ubiquitination sites of Rsp5 have been identified, and many known protein mutations that lead to disease alter these potential ubiquitination sites (*43*). There is evidence that ubiquitination is also involved in sensing of neuropathic pain and its misregulation in disease (*89*). Mutations of Ubiquitin, Ubiquitin ligases, and DUBs are all found in human diseases and disorders. For example, many Ubiquitin ligases are proto-oncogenes (*90, 91*). Receptor Tyrosine Kinases that are not ubiquitinated or lack proper ubiquitination can lead to constitutive receptor signaling and carcinogenesis (*92*).

There is already some precedence for targeting similar post-translational modification for disease treatment. Targeting the post-translational phosphorylation for drug development has been successful, and there are currently over 150 drugs in various stages of clinical trials (*93*). There are many parallels between the phosphorylation and ubiquitination systems suggesting the ubiquitination may prove to be just as important a pathway to target for drug development. For example, the human genome contains more E3 Ubiquitin ligases than protein kinases (*93*). Furthermore, there is significant interplay between phosphorylation and ubiquitination that is critical for cell regulation. For example, some E3 ligases require substrate phosphorylation before ubiquitination can occur (*93*).

In fact, there are already drugs that successfully target the ubiquitination system. There are some therapies involving monocolonal antibodies that act by promoting Ubiquitin-dependent receptor degradation (*94*). However, a detailed knowledge of the mechanisms by which ubiquitination regulates these pathways is required for the design of effective inhibitors (*95*). Some of the most promising targets in the ubiquitination pathway are the E3 Ubiquitin ligases, which are crucial for ubiquitination and are also the critical point for substrate specificity (*96*). Currently, there has been some success in finding small molecule inhibitors of E3 substrate interactions, for example the interaction of the E3 MDM2 with its substrate p53 (*96*). There are also drugs already on the market that block the downstream effects of substrate ubiquitination downstream. For example, Bortezomib is a small molecule inhibitor of the 20S proteasome (*97, 98*). Bortezomib is used to treat multiple myeloma, likely by limiting cell immortality by blocking the degradation of pro-apoptotic proteins (*97, 98*). Another promising target for drug development is deubiquitinases; there are known small molecule inhibitors for some deubiquitinases already, but it remains to be seen whether they will become successful new anticancer therapies in the near future (*99*). In fact, deubiquitinases, which have a clear protein binding pocket and enzymatic activity, may represent the best targets for future drug development studies (*99*).

One of the challenges facing drug development targeting the Ubiquitin system is that there is no general approach for disrupting E3 ligase substrate interactions. While many compounds have been developed to target enzymes such as protein kinases, it is more challenging to disrupt a protein-protein interaction (*93*). Designing inhibitors to disrupt protein-protein interactions, which would lead to successful targeting of E3s and

ubiquitinated substrates, requires knowledge of the structures and interactions that are formed between E3s, Ubiquitin, and substrates. As our understanding of the structural aspects of ubiquitination improves, drug development should prove more fruitful. Success in this regard will require information about protein-protein interactions, the impact of Ubiquitin on substrate structure, and the mechanisms of deubiquitination.

However, despite extensive characterization of the outcomes of ubiquitination *in vivo,* questions remain about how specific lysines on a substrate are ubiquitinated, how ubiquitination directly affects the structure and properties of the substrate, and how changes to the structure or dynamics of the substrate may contribute to the function of different substrates (*100, 101*). The answers to these questions are essential to fully understanding the roles ubiquitination plays in proper (normal signaling) and improper (disease and cancer) cellular functions.

New Approaches to Study Ubiquitination

As discussed in the previous section, an advance in our ability to understand and target the process of ubiquitination requires a clearer structural and mechanistic knowledge of monoubiquitination, including how it may lead to changes in the structure and activity of the substrates that it modifies. While significant effort has been focused on characterizing the outcomes of monoubiquitination *in vivo*, very little has been done to understand what this modification does to the biochemical and biophysical properties of its substrates. There is, however, precedence for the value of information obtained from asking these types of questions. For example, the charge introduced by another conditional post-translational modification, phosphorylation, is known to lead to a

perturbation of the biophysical properties of a protein structure, which can lead to a conformation change that alters activity and protein-protein interactions (*52, 102, 103*).

There are some recent studies that focus on the structural and biophysical aspects of ubiquitination. Computational modeling of the ubiquitinated substrate Ubc7 suggests that ubiquitination changes the thermodynamic stability of a protein in a site-specific and modification-specific manner (*104*). Furthermore, Ubc7 was most thermodynamically destabilized by ubiquitination at the known site of polyubiquitination *in vivo (104)*. Studies of the interaction between Ubiquitin and the Ubiquitin binding domains (UBDs) of proteins that recognize monoubiquitinated substrates suggest that different UBDs recognize and stabilize slightly different conformations of Ubiquitin (*53*). In solution, Ubiquitin is a dynamic molecule, and it is possible that when a substrate is ubiquitinated it stabilizes a conformation of Ubiquitin that is recognized by UBDs. These studies illustrate that knowledge of how structure and dynamics change lead to insight into the mechanism by which a monoubiquitinated substrate is recognized. Other structural studies have shown how Ubiquitin associated (UBA) domains recognize and bind to specific hydrophobic patches on Ubiquitin (*105*). These biochemical and structural analyses of interactions between Ubiquitin and Ubiquitin-binding proteins have helped develop a mechanistic understanding of the link between the modification, the process that it regulates, and the proteins that recognize the modified substrate (*17*).

Despite insights gained using biochemical and biophysical approaches, few studies of monoubiquitinated substrates have been conducted. Two of the primary reasons are that [1] it is difficult to obtain enough natively modified substrate from cells to study by biochemical and biophysical methods and [2] many of the current synthetic

methods are highly technical and may not be accessible to a molecular and cellular biologist. This is a problem not only for the study of monoubiquitination, but for other post-translational modifications as well. There are now over 200 documented post-translational modifications, a number of which involve modifying a substrate with another protein (like Sumoylation) that have the same constraints on the ability to perform mechanistic studies *(17, 52, 63)*.

Use of Chemical Modification to Study Monoubiquitinated Substrates

One particularly promising approach to gaining a mechanistic understanding of regulation by monoubiquitination is through the use of synthetic methods to generate monoubiquitinated substrates. There are three approaches to generate ubiquitinated substrate suitable for study by biophysical methods: non-natural amino acids coupled with organic synthesis, semi-synthesis, and chemical modification that takes advantage of amino acid chemistry. The simplest approach to chemical modification is to form a disulfide bond between Ubiquitin and the substrate (*106*). The advantages and disadvantages, especially relating to ease of use, of these approaches are discussed in Chapter V of this dissertation.

Currently, there are a few examples of using chemical modification to study monoubiquitinated substrates by biophysical methods. Many of the successful studies of monoubiquitinated substrates have come from the use of either isopeptide bond surrogates, or the semi-synthesis of a monoubiquitinated substrate. Histone H2B, the first known substrate of monoubiquitination, was also one of the first proteins to be studied using one of these approaches (*107, 108*). Biochemical analysis of synthetically generated monoubiquitinated H2B showed that monoubiquitinated H2B directly activates

methylation of histone H3, demonstrating the importance of cross-talk between post-translational modifications. Studies of PCNA were also performed using multiple chemical approaches to modification (*109, 110*). By solving the crystal structure of monoubiquitinated PCNA, the authors found that monoubiquitination does not change the structure of PCNA itself, suggesting ubiquitination recruits alternative binding partners to PCNA, but that Ubiquitin does display limited conformational flexibility relative to PCNA, constraining the ways in which binding partners can interact with the protein (*111-113*). Finally, a semi-synthesis approach was also used to study α-synuclein, a protein central in the development of Parkinson's disease. Using monoubiquitinated α-synuclein, the authors directly demonstrated that ubiquitination led to the inhibition of fibril formation (*114*), which was consistent with previous *in vivo* studies suggesting that N-terminal monoubiquitination stabilizes the monomeric form of the protein. Furthermore, additional studies of this protein using cysteine mutations showed that different ubiquitination sites had different effects on the formation of fibrils (*115*).

We are currently at an exciting time in the study of monoubiquitinated substrates. New approaches to study these substrates are available as well as evidence suggesting that monoubiquitination regulates substrates through mechanisms more diverse than the three primary categories described previously. In the future, it will be important to continue to systematically study and characterize the mechanisms by which monoubiquitination regulates substrate localization, binding, and activity. Mechanistic studies, such as the ones described above, will be particularly important for substrates where the role of monoubiquitination in *in vivo* regulation is challenging to elucidate.

The recent advances in chemical ubiquitination approaches described above provide the opportunity to study monoubiquitinated substrates. Chemical ubiquitination will be especially important in the cases where the population of monoubiquitinated substrate might be too small to purify efficiently from cells or the Ubiquitin ligase is not known or cannot be reconstituted *in vitro*.

Regulation of GTPase Signaling by Monoubiquitination

One prominent family of proteins that promises to be particularly interesting for mechanistic studies of signaling regulation by monoubiquitination is GTPases. There is evidence in the literature for diverse mechanisms of GTPase regulation by ubiquitination and there are also a number of instances where monoubiquitination can regulate protein function by mechanisms other than protein localization. GTPases regulate cell signaling pathways, and are enzymes with a well-characterized guanine nucleotide binding and hydrolysis activity. These proteins are also integral in driving many types of cancer and developmental diseases. The remainder of this thesis will focus on the regulation of particular GTPases by monoubiquitination. The implications from these studies for the larger field of monoubiquitination will be considered in Chapter V.

GTPases as Regulators of Signaling Pathways

GTPases are a family of molecular switches that regulate cell signaling pathways. This family includes monomeric Ras-like GTPases and heterotrimeric Gα proteins, all of which have a conserved GTPase domain (**Figure 1.4a-b**) (*116-118*). The Ras-like GTPases were named after the founding members of this class of GTPases, H-Ras and K-

Ras, which were first discovered due to their oncogenic potential in retroviruses (*119*). Ras-like GTPases are divided into five subfamilies: Ras, Rho, Rab, Arf, and Ran (*120*). Each subfamily has different localization in the cell and different downstream effectors, leading to much of their observed functional specificity. Ras superfamily GTPases regulate a number of pathways, including cell cycle progression and gene expression (Ras), cytoskeletal rearrangement (Rho), nuclear import (Ran) and cellular trafficking (Rab and Arf) (*120, 121*). Small GTPases also have significant roles in driving cancer and, in some cases, developmental disorders. In particular, Ras is activated in over 30% of all human cancers. Germline mutations of Ras are found in Noonan syndrome, Costello syndrome, and Cardiofacio-cutaneous syndrome (*122*).

Heterotrimeric Gα proteins are coupled directly to cell-surface receptors and are responsible for receptor-mediated communication between the exterior and interior of the cell (*121*). There are four classes of heterotrimeric Gαs that are based on their homology and downstream effectors: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$ (*123*). $G\alpha_s$ proteins activate adenylyl cyclase, while $G\alpha_i$ are known to inhibit adenylyl cyclase and act in opposition to $G\alpha_s$ (*124*). $G\alpha_i$ GTPases are also coupled to taste and odor receptors, and facilitate vision through phototransduction. $G\alpha_q$ proteins activate phosphoinositide-specific phospholipase C isozymes. This leads to the generation of the second messenger signals inositol 1,4,5-trisphosphate and diacylglycerol (*124*). Finally, $G\alpha_{12/13}$ proteins regulate the GTPase RhoA from the Rho family of small GTPases (*125*). These heterotrimeric G proteins mediate signaling pathways such as protein-protein phosphorylation, gene transcription, cytoskeleton reorganization, membrane depolarization, and secretion (*126*).

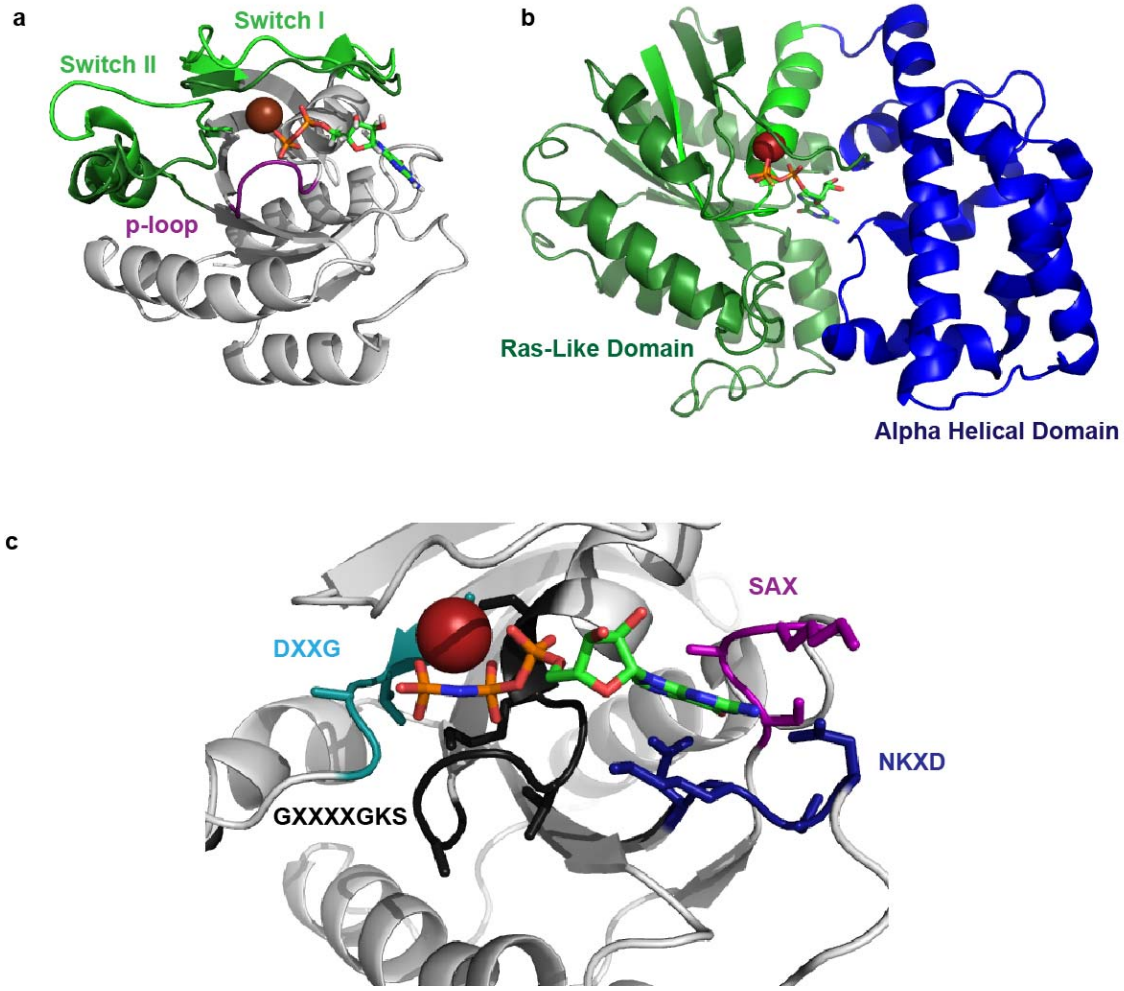

Figure 1.4. Domains of Small and Heterotrimeric GTPases. (**a**) Ribbon diagram of representative Ras domain of a small GTPase (1CRR). GDP-bound switch regions are shown in light green, while GTP-bound switch regions are shown in dark green. The p-loop is highlighted in purple. Magnesium shown in red and GDP in various colors. (**b**) Ribbon diagram of a heterotrimeric Gα protein (1GIA). Ras-like domain is shown in green and the α-helical domain is shown in blue. (**c**) Ribbon diagram of a small GTPase (5P21) showing the four binding motifs important for nucleotide coordination, DXXG, GXXXXGKS, SAX, and NKXD.

The primary mechanism by which GTPases signal is by switching between GDP- and GTP-bound states, which results in conformational changes that allow interactions with downstream effectors when the GTPase is in the GTP-bound state (*127-133*). Small GTPases bind nucleotide with a K_d in the picomolar to nanomolar range. While there are variations in their c-terminal targeting sequences, the core Ras domain is highly conserved (*119*). The Ras domain consists of an α/β Rossman fold of about 20 KDa and contains the basic function of guanine nucleotide binding and hydrolysis (*118*). There are four regions in the Ras domain that are directly involved in guanine nucleotide binding (**Figure 1.4c**) (*119*). The NKXD motif forms interactions with the nucleotide base and is crucial for nucleotide binding affinity. The other most important interaction for nucleotide binding is the GXXXXGKS motif, which forms interactions with the α, β, and γ phosphate of GDP and GTP and provides a serine or threonine for coordination with the cofactor Magnesium (*121, 134*). Specificity for guanine over other nucleotides comes from two motifs, the asparagine side chain of the DXXG motif and a main chain interaction with the alanine in the SAX motif (*118*). There are also three primary structural elements that define the protein's activity and ability to be regulated: switch I, switch II, and the phosphoryl loop (p-loop), which is also part of the GXXXXGKS motif (**Figure 1.4b**). The main conformational changes occur in the switch regions of the proteins. Ras-like GTPases have two switch regions that sense changes in nucleotide binding (*117, 132, 135*). The switch regions are conformationally dynamic in the GDP bound state, but less so (and much more conserved between GTPases) in the GTP-bound state of the protein (*118*). The slower time scale and reduced population of conformers in the GTP-bound state of the protein is due to additional hydrogen bonds that form between

key residues (G60 and T35 on Ras) and GTP (*118*). Downstream effectors recognize and bind preferentially to the GTP-bound state of the GTPases through the switch regions.

On their own, GTPases are not very good enzymes; their enzymatic activity does not occur on a timescale fast enough to allow them to respond appropriately to extracellular signals. Therefore, signaling is regulated both by Guanine Nucleotide Exchange Factors (GEFs) and GTPase Activating Proteins (GAPs) (**Figure 1.5)**. GEFs facilitate GDP release by stabilizing the nucleotide-free state of the GTPase (*118*). The reaction is driven in the forward direction by the presence of excess GTP over GDP in the cell (*136*). The GEFs interact with the switch regions of the GTPase and residues close to the p-loop and magnesium binding region, leading to structural changes in the GTPases that do not favor binding to phosphates and magnesium. The release of magnesium and additional structural disturbances in the p-loop region account for the increased rate of GDP release in the presence of the GEF (*118, 137*). The mechanism of GAP-mediated hydrolysis depends on a conserved glutamine residue located near the γ phosphate of the nucleotide. The glutamine residue facilitates the formation of the transition state interaction by priming a catalytic water for in-line nucleophilic attack on the γ phosphate (*138*) . GAPs supply an arginine finger that binds in the active site (*138, 139*). The mechanisms of regulation are highly conserved, but not universal for all small GTPases. Some small GTPases, particularly the Rho and Rab families, are also regulated by guanine nucleotide dissociation inhibitors (GDIs), which recognize a prenylated c-terminus and allows sequestration of, and recycling of, the GTPases between different cell membrane compartments (*118*).

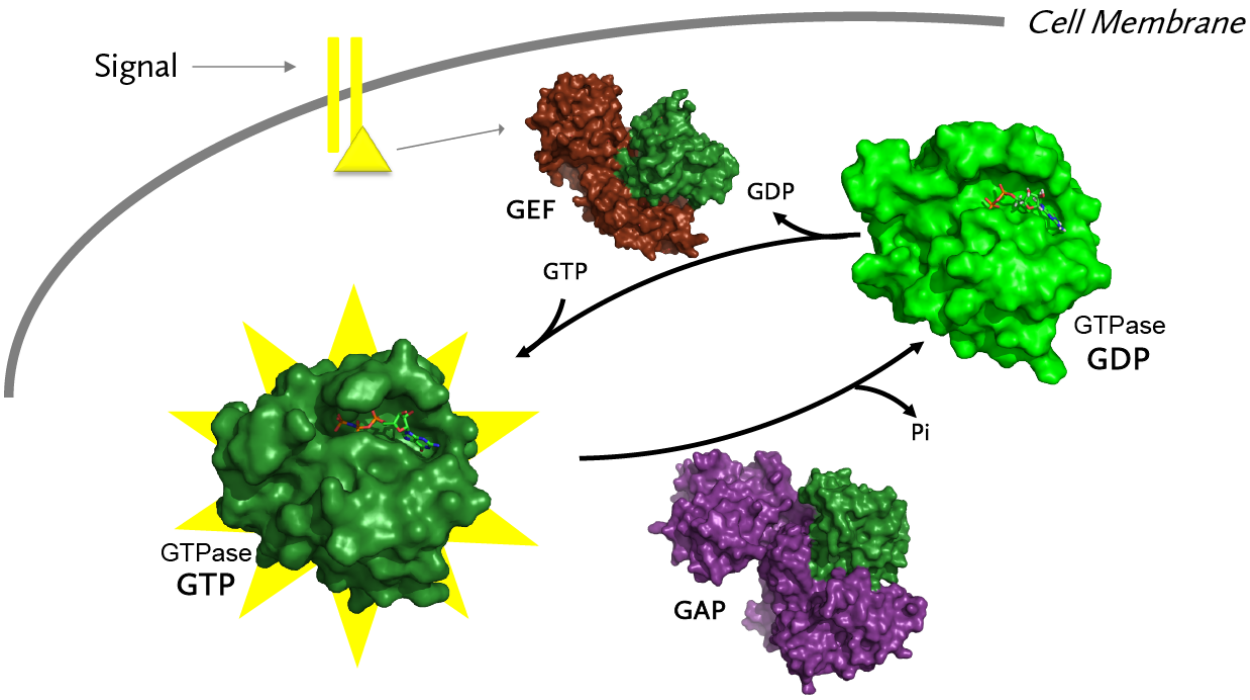

Figure 1.5. Small GTPase Enzymatic Cycle. Small GTPases cycle between a GDP-bound "off" state (light green) and a GTP-bound "on" state (dark green). The rate of GDP dissociation in increased by GEF (brown). The rate of GTP hydrolysis is increased by GAP (purple).

While in many ways the activity of heterotrimeric Gα proteins is similar to Ras-like GTPases, there are some differences (**Figure 1.6**). Nucleotide binding still occurs primarily within the Ras-like domain, but recent data have demonstrated that the α-helical domain also contributes to the activity of Gα proteins. Evidence for α-helical domain involvement includes the observation of a major displacement of the α-helical domain during receptor activation (*140*). Furthermore, recent structural analysis has also shown that the α-helical domain is highly flexible in the absence of nucleotide, suggesting that this domain undergoes a nucleotide-dependent transition to a stabilized state (*141*). Gα proteins, along with the canonical switch I and switch II regions, also have an extra structural element called switch III (*116, 118*). The switch I region also serves as one of the two connections between the Ras-like and α-helical domains (*142*). Conformational changes occur in all three switch regions upon changes in the nucleotide binding state, similar to the mechanism of small GTPases (*143*). However, the mechanism for hydrolysis is slightly different. The amino acid sequence of the Gα protein already contains the arginine finger which is provided by the GAPs for small GTPases, leading to faster observed rates of intrinsic hydrolysis (almost 100 times faster than small GTPases) (*144*). Heterotrimeric G proteins are regulated by GAPs, commonly referred to as regulators of G protein signaling (RGS) proteins (*145*). While RGS proteins do not contain an arginine finger, they do stabilize the active conformation of the transition state required for increasing the rate of hydrolysis (*146*). GPCRs serve as GEFs for Gα proteins, catalyzing GDP for GTP exchange when activated (*142*). Heterotrimeric GTPases use the β and γ subunits as their GDIs (*118, 145*). They are

coupled to Gβγ when the Gα is GDP-bound. Upon GTP binding, the βγ interaction is released, allowing signaling through both Gα and Gβγ to occur.

GTPases as Substrates of Monoubiquitination

GTPases are known to undergo a number of post-translational modifications that are crucial for their localization and proper signaling. Some examples of these include phosphorylation (*147*), myristoylation (*148*), prenylation, and palmitoylation (*147*). Ubiquitination is also important for the regulation of many GTPases, not only to control total substrate levels but to target and regulate these proteins in a temporal and spatial manner (*149, 150*). Two isoforms of Ras, H-Ras and K-Ras, are substrates of monoubiquitination, but monoubiquitination has distinct outcomes. Monoubiquitination of H-Ras in CHOK-1 cells is necessary to stabilize its association with the endosome and allow signaling to occur (*151, 152*). Monoubiquitination of K-Ras in HEK293T cells activates K-Ras and contributes to Ras-mediated tumorigenesis (*153*). Ubiquitination of Rap1B, another Ras-like GTPase, induces relocalization of the protein from the plasma membrane to a subcellular compartment, which is required for the establishment of neuronal polarity (*154*). It was also shown that Rap2A is monoubiquitinated by Nedd4, which acts as a positive regulator of dendrite development (*155*). In this case, ubiquitination disrupts Rap2A interactions with effector proteins and blocks signaling. However, the authors were not able to determine if monoubiquitination altered the biochemical activity of Rap2A. Monoubiquitination is specifically targeted to the active, GTP-bound state of Rap2A. To reduce Rap2A ubiquitination, it was necessary to mutate lysines 5, 94, 148, and 150; expression of a mutant lacking these four lysines impaired neurite development (*155*).

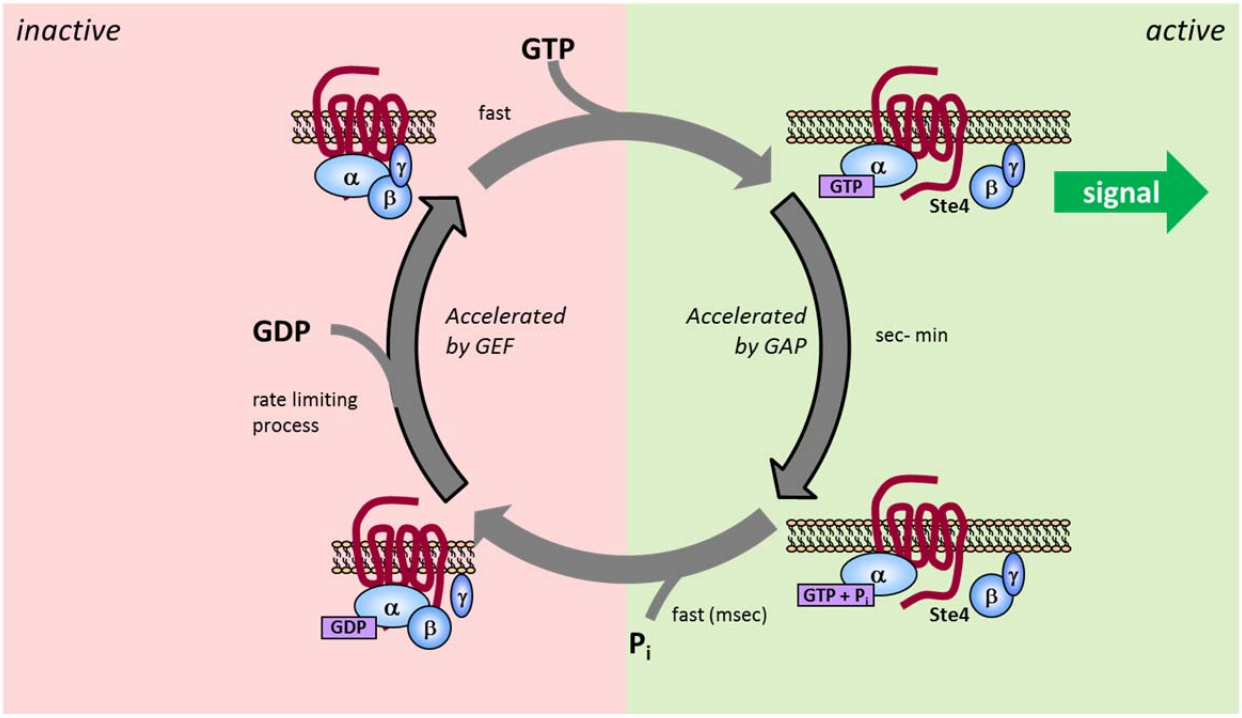

Figure 1.6. Heterotrimeric Gα Protein Enzymatic Cycle. Ligand binding to the G protein coupled receptor causes the Gα subunit to exchange GDP for GTP. When Gα is bound to GAP, it no longer interacts with Gβγ. When dissociated, both Gα and Gβγ can interact with downstream effector proteins. When Gα hydrolyzes GTP to GDP, signaling is inactivated. The duration of signaling can be regulated by RGS proteins, which act as GAPs for Gα proteins.

The small GTPases RalA and RalB are also substrates of monoubiquitination(*156*). RalA is important for anchorage-independent proliferation as well as tumor growth while RalB contributes to cancer cell survival. In this case, activation of RalA does not affect the ubiquitination state of the protein, however, monoubiquitinated RalA was significantly enriched in the GTP-bound state (*156*). RalA ubiquitination increases when a cell is detached from its substrate (*156*). No single lysine of the 21 lysines within RalA appear key for ubiquitination based on systematic single and multiple mutations of the lysines to arginines, suggesting RalA can be ubiquitinated on multiple lysines (*156*). Monoubiquitination selectively modulates RalA and RalB localization, which is critical for their differences in the roles they play in cell signaling regulation. Finally, Rac1 monoubiquitination has been observed to occur at a single lysine, K147 (*86, 157, 158*) that lies within an insert region that is conserved in the Rho family of small GTPases. Rac1 is also polyubiquitinated, but when Cav1, which regulates Rac1 polyubiquitination, is lost, a monoubiquitinated species appears. The data suggests that monoubiquitination may have a distinct role to play in Rac1 regulation, but this mechanism has not yet been pursued (*86*).

While there are multiple examples of monoubiquitination of small GTPases, less is known about how monoubiquitination regulates Gα proteins. A number of Gα proteins are known to be regulated by polyubiquitination and degradation, including $G\alpha_o$ (*159*), $G\alpha_{i3}$ (*160*), $G\alpha_{i2}$ (*161*), and $G\alpha_s$ (*162*). However, currently, the only Gα currently known to be monoubiquitination is Gpa1, a Gα in *Saccharomyces cerevisiae.* There is a long history of discoveries made in yeast that have shaped our understanding of signaling

pathways in more complex systems, suggesting that as detection techniques are refined, other examples of Gα monoubiquitination may come to light.

This brief summary focuses primarily on monoubiquitination of the GTPases themselves, when in fact regulators of GTPase signaling are also known to be monoubiquitinated (*163, 164*). There are many examples of monoubiquitination and subsequent endocytosis of GPCRs being critical in a number of pathways (*16, 58, 70, 77*). Targeting the ubiquitination or deubiquitination of these GTPases and their regulators may represent an exciting new possibility for drug development, but first requires that the mechanisms of regulation by monoubiquitination be clearly understood (*163*).

Thesis Summary

GTPases undergo multiple forms of ubiquitination that lead to a variety of different outcomes. G proteins should serve as an excellent model system to study the effect of ubiquitination on substrate structure, dynamics, and thermodynamic stability. GTPases are of interest particularly because of the key role they play in cell signaling, which makes them good candidates for drug targeting. However, while we have extensive knowledge of the role of GTPases in signaling pathways and the structural and mechanistic details that drive their *in vivo* activity, we have a very limited knowledge of the role monoubiquitination plays in their regulation. It is clear monoubiquitination is involved in the regulation of a number of these GTPases and that it may modulate signaling or localization. However, no studies beyond the *in vivo* observations of monoubiquitination have been done to understand the mechanism through which this

post-translational modification may act. In this thesis, we have employed biochemical and biophysical methods to understand the molecular basis through which GTPases can be regulated by monoubiquitination.

To be able to perform our studies of monoubiquitinated substrates *in vitro,* we first had to optimize a method to generate enough monoubiquitinated substrate. As described previously, chemical ubiquitination was the most promising approach to suit our needs. We used a simplified and optimized version of a disulfide chemistry ubiquitination approach present in the literature. Our new approach gave us the ability to drive modification of our substrate to completion, as seen in Chapters II and III. One limitation of this type of approach is that, until recently, it has not been applied to studying other ubiquitinated substrates, in part because it was not known if the differences in the linkage type would alter the behavior of the monoubiquitinated substrate. As discussed in Chapter II, we employed computational modeling to show that chemically monoubiquitinated protein accurately mimics natively ubiquitinated protein.

We chose this approach in the study of two GTPases. In Chapter II, we look at the effects of monoubiquitination on the structure and activity of K-Ras, which was recently shown to be monoubiquitinated (*153*). Using our biochemical approach, we elucidate the mechanism by which K-Ras is activated by monoubiquitination, which included a change in switch region dynamics. By collaborating with another lab to do assays in cell lysates, we verified that the observed mechanism of activation *in vitro* reflected the mechanism by which monoubiquitinated Ras is activated *in vivo.* In Chapter III, we use the power and versatility of the chemical ubiquitination approach to study isoform dependent site-specific monoubiquitination. We show that in H-Ras

monoubiquitination at a different lysine than K-Ras results in protein activation through a distinct mechanism.

Finally, in Chapter IV, we turn our attention to a heterotrimeric GTPase, the yeast Gα Gpa1. Gpa1 is an interesting protein because it is both monoubiquitinated and polyubiquitinated at the same site. There are very few known substrates where this occurs, but they include PCNA (*63*), a processivity factor, and p53, a well-known tumor suppressor (*165*). Structural studies of a substrate like this will likely offer insights into structural determinants and outcomes of monoubiquitination versus polyubiquitination. Choosing a yeast protein afforded us the ability to couple our biochemical and biophysical approach to a system where we can also perform genetic studies. Gpa1 was a challenging substrate to study, and much of our effort was focused on optimizing methods to obtain pure, stable substrate. While we have not completed the studies of ubiquitinated Gpa1, we have gained insight into the ways in which this protein has evolved to allow it be targeted for monoubiquitination

In the discussion section of Chapter V, I will highlight not only what we have learned from these studies about Ras and Gpa1, and regulation of GTPases in general, but also what implications this work has for the field as a whole. There is much to be gained by using *in vitro* approaches to study regulation by monoubiquitination, and the simple method of chemical ubiquitination we have developed should make it feasible to expand our mechanistic understanding to other substrates of monoubiquitination.

CHAPTER II

SITE-SPECIFIC MONOUBIQUITINATION ACTIVATES RAS BY IMPEDING GTPASE ACTIVATING PROTEIN FUNCTION[1,2]

Cell growth and differentiation are controlled by growth factor receptors coupled to the GTPase Ras. Oncogenic mutations disrupt GTPase activity leading to persistent Ras signaling and cancer progression. Recent evidence indicates that monoubiquitination of Ras leads to Ras activation. Mutation of the primary site of monoubiquitination impairs the ability of activated K–Ras to promote tumor growth. To determine the mechanism of human Ras activation we chemically ubiquitinated the protein and analyzed its function by NMR, computational modeling, and biochemical activity measurements. We established that monoubiquitination has little effect on Ras GTP binding, GTP hydrolysis, or exchange factor activation, but severely abrogates the response to GTPase activating proteins in a site–specific manner. These findings reveal a new mechanism by which Ras can trigger persistent signaling in the absence of receptor activation or an oncogenic mutation.

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[1] Elements of the work referenced in this chapter have been published in:
Baker, R., Lewis, S. M., Sasaki, A. T., Wilkerson, E. M., Locasale, J. W., Cantley, L. C., Kuhlman, B., Dohlman, H. G., and Campbell, S. L. (2013) Site-Specific Monoubiquitination Activates Ras by Impeding GTPase-Activating Protein Function, *Nature Strucutural and Molecular Biology 20*, 46-52.

[2] Figures contributed by:
Rachael A. Baker: 2.1, 2.3, 2.4, 2.6, 2.7, 2.8, 2.9a-b, 2.9d-f, 2.10, 2.12, 2.13b-d, 2.14a
Steven M. Lewis: 2.5, 2.9c, 2.13a, 2.15
Atsuo T. Sasaki: 2.2, 2.9c, 2.11
Emily M. Wilkerson: 2.14b

Introduction

Ras plays a central role in cell growth, differentiation, and apoptosis and is a member of a large superfamily of guanine nucleotide binding proteins whose activity is regulated by cycling between inactive GDP–bound and active GTP–bound states (*166*). Conformational changes associated with the GDP– and GTP–bound states are localized primarily to two regions, Switch I (residues 30–37) and Switch II (60–76), and these conformational changes direct specific interactions with regulators and effectors (*167, 168*). Ras effectors recognize the GTP–bound state of Ras with higher affinity than the GDP–bound state, and these effectors serve to initiate downstream signaling events. Ras has weak intrinsic GTPase activity, but it does not act alone (*138*). The guanine nucleotide state of Ras is regulated by two distinct types of protein modulators, which act in opposition to one another. Guanine nucleotide exchange factors (GEFs) facilitate exchange of GDP with GTP to promote Ras activation (*169*) whereas GTPase–activating proteins (GAPs) stimulate the hydrolysis of GTP and Ras deactivation (*121*). Ras is the most prevalent oncogene found in human cancer; about 30% of human tumors contain an activating Ras mutation (*170, 171*). Most commonly, transforming Ras mutations decrease the sensitivity of the protein to GAP–mediated regulation (*172*).

While the roles of GEFs and GAPs have been extensively characterized, it is less clear how some post–translational modifications, like monoubiquitination, contribute to Ras function and signaling. Monoubiquitination is a dynamic and reversible modification that can orchestrate cellular events including DNA repair, gene expression, endocytosis, and nuclear export (*55*). Emerging evidence suggests that monoubiquitination regulates large and small GTPases, including Ras (*86, 155, 157, 173*). Monoubiquitination of K–

Ras at position 147 has been shown to promote tumorigenesis (*153*); mutation of oncogenic K–Ras to prevent monoubiquitination (Ras^{K147L}) impaired its ability to promote tumor growth when ectopically expressed in NIH 3T3 mouse fibroblasts. These findings suggest that Ras activity and signaling are modulated by monoubiquitination, in the manner of an oncogenic mutation or receptor stimulus. Left unresolved is the mechanism by which monoubiquitination leads to activation of Ras.

Here, we set out to identify the molecular mechanism through which Ras activity is regulated by monoubiquitination. We first developed a method to chemically ubiquitinate Ras using conditions that drove post-translational modification to completion. Furthermore, we used computational modeling to validate the chemical ubiquitination approach. Using our system, we show that monoubiquitination at position 147 does not alter the intrinsic biochemical properties of Ras, but severely disrupts regulation of Ras by GAPs. This effect is specific to monoubiquitination at position 147 and is not observed when Ras is monoubiquitinated at other adjacent lysines. The loss of GAP–mediated hydrolysis accounts for the accumulation of Ras–GTP *in vivo.* Thus monoubiquitination reversibly renders the protein resistant to GAP–mediated regulation.

Results

Monoubiquitination of Ras

We conducted a series of *in vitro* studies to elucidate the mechanism of Ras regulation by monoubiquitination. These studies required fully ubiquitinated protein that was exclusively modified at Lys147 and in quantities sufficient for detailed biochemical and biophysical analysis. Recent investigations of monoubiquitinated substrates and

ubiquitinating enzymes employed multiple methods of direct chemical ligation to generate the protein–Ubiquitin linkage (*109, 110, 174-177*). In our approach, we replaced the native Ubiquitin linkage with a disulfide bond between a substituted cysteine at position 147 of Ras (Ras^{K147C}) and a cysteine at the carboxyl–terminus (c–terminus) of Ubiquitin ($Ubiquitin^{G76C}$). A surface accessible cysteine (Cys118) in Ras was replaced with serine to avoid unwanted modification (Ras^{C118S}, hereafter "Ras"). We previously showed that the C118S mutation did not alter Ras structure or biochemical properties (*178*). The chemical ligation method does not require complicated intermediate chemical or enzymatic steps but instead provides a simple, specific approach to ubiquitination. The disulfide ligation strategy, using a more complicated cysteamine intermediate, was validated in previous studies of Proliferating Cell Nuclear Antigen (PCNA), where it was shown that chemically and enzymatically monoubiquitinated PCNA exhibit identical catalytic properties (*109*). As seen in **Figure 2.1a**, we drove Ubiquitin modification of Ras at position 147 to completion by the addition of a ten–fold excess of $Ubiquitin^{G76C}$ at pH 8.0. We conducted our experiments using H–Ras (1–166), for which the biochemical and structural (NMR and X–Ray) properties are best established, but corroborated the results using K–Ras (1–166) as indicated. All three mammalian isoforms H–, K–, and N–Ras show similar biochemical properties in the absence of the hypervariable c–terminus (*128, 179*). Furthermore, we used immunoprecipitation assays to show that, in the absence of c–terminal modification, monoubiquitination still leads to an increase in the GTP–bound population of H–Ras or K–Ras in HEK293T cells (**Figure 2.2**).

Downstream effectors of Ras, like Raf, have a Ubiquitin–like fold (*180*). Thus, we considered whether Ubiquitin could bind to Ras in the manner of an effector. To this

end, we used NMR in the presence of free unlabeled Ubiquitin to determine whether Ubiquitin altered spectral features associated with Ras backbone amides. A $^{1}H–^{15}N$ 2D HSQC overlay of ^{15}N–enriched H–Ras^{K147C} in the absence and presence of Ubiquitin is shown in **Figure 2.1b**. The assignments for H–Ras (1–166) were previously determined (*181*) and we verified the shifted backbone amide resonances of H–Ras^{K147C} using 3D HNCACB data (**Figure 2.3**). Comparison of the position and intensity of the backbone amide resonances indicates that Ras is not altered by the presence of free Ubiquitin. In support of these observations, as shown in **Figure 2.4a–b**, we found that the intrinsic rate of GDP dissociation and GTP hydrolysis were unaffected by the presence of Ubiquitin.

Furthermore, the presence of Ubiquitin dimers in solutions also had no effect on measuring thermal stability, intrinsic GDP dissociation, or GTP hydrolysis, as shown in **Figure 2.4d-f.** These results indicated that Ras did not specifically interact with Ubiquitin. Therefore, for subsequent analyses, we did not separate monoubiquitinated Ras (mUbRas) from free Ubiquitin.

Monoubiquitinated Ras Retains Intrinsic GTPase Activity

Previous computational studies predicted that the stability of a ubiquitinated substrate depends on the site of ubiquitination and type of Ubiquitin–Ubiquitin linkage (*104*). To determine if monoubiquitination alters Ras, we compared the thermal stability of unmodified Ras and mUbRas. To this end, we employed the Quantitative Cysteine Reactivity (fQCR) assay (*182*), which uses a cysteine reactive dye to measure rates of protein unfolding as a function of temperature. Because Ubiquitin does not have any native cysteines, it is invisible by this method. As shown in **Figure 2.4c**, we found that monoubiquitination decreases the thermal stability of Ras by 3.5 degrees (43.1± 0.2 ^{o}C,

39.2 ± 0.3 ^{o}C, and 39.6 ± 0.2 ^{o}C for Ras, Ras^{K147A}, and mUbRas, respectively), a change that is not likely to have a substantial effect on this otherwise highly stable protein *in vivo*. These data suggest that, despite the size of Ubiquitin, monoubiquitination at position 147 does not lead to thermal destabilization of Ras.

While monoubiquitination of Ras does not substantially affect thermal stability, it could alter intrinsic activity. For example, it is possible that ubiquitination impairs guanine nucleotide binding, similar to mutations at the adjacent residue, Ala146 (*183, 184*). To measure rates of nucleotide dissociation, we equilibrated Ras and mUbRas with the fluorescent analog N–methylanthraniloyl (MANT)–GDP and measured fluorescence over time in the presence of excess unlabeled GDP. We observed a slight increase (2–3 fold) in the intrinsic rate of nucleotide dissociation for Ras^{K147C} as compared to native Ras, while the rate for mUbRas was unaltered (**Figure 2.4a**). This result suggests that ubiquitination of Ras does not have the same impact on nucleotide binding as a point mutation at the same residue.

We next sought to establish whether monoubiquitination alters the intrinsic rate of GTP hydrolysis. To this end we measured single turnover GTP hydrolysis using Flippi, a fluorescent sensor that detects free phosphate (*185*). As shown in **Figure 2.4b**, neither mutation of Lys147 nor monoubiquitination of Ras affected the intrinsic rate of GTP hydrolysis (calculated as 0.012 ± 0.002 min^{-1} for all variants). Taken together, these results indicate that monoubiquitination does not alter the activity of Ras, and that another mechanism must account for the accumulation of mUbRas in the GTP–bound state *in vivo*.

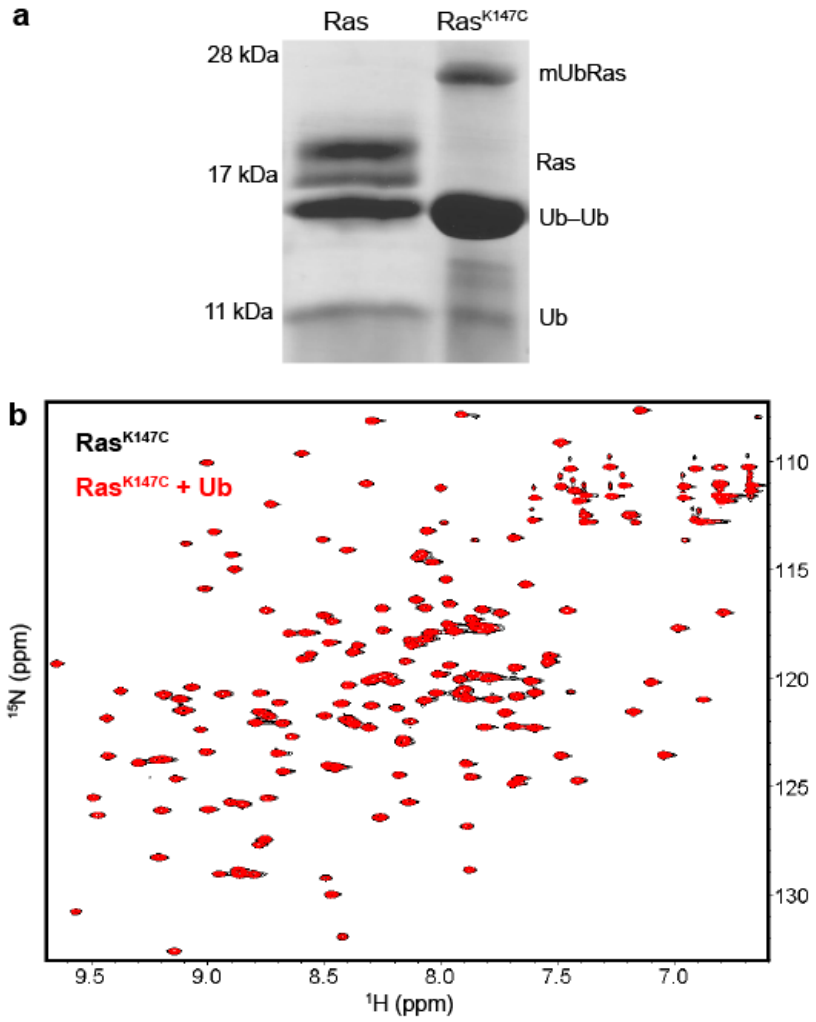

Figure 2.1. Monoubiquitination of Ras. (**a**) Reaction of Ubiquitin^{G76C} with Ras or a Ras^{K147C} mutant, under non–reducing conditions. The product of the reaction contains mUbRas, Ras, Ubiquitin–Ubiquitin dimer (Ub–Ub), and free Ubiquitin (Ub). (**b**) HSQC spectra of ^{15}N–Ras^{K147C} bound to Mg–GDP in the absence (black) and presence (red) of ten–fold excess free Ubiquitin.

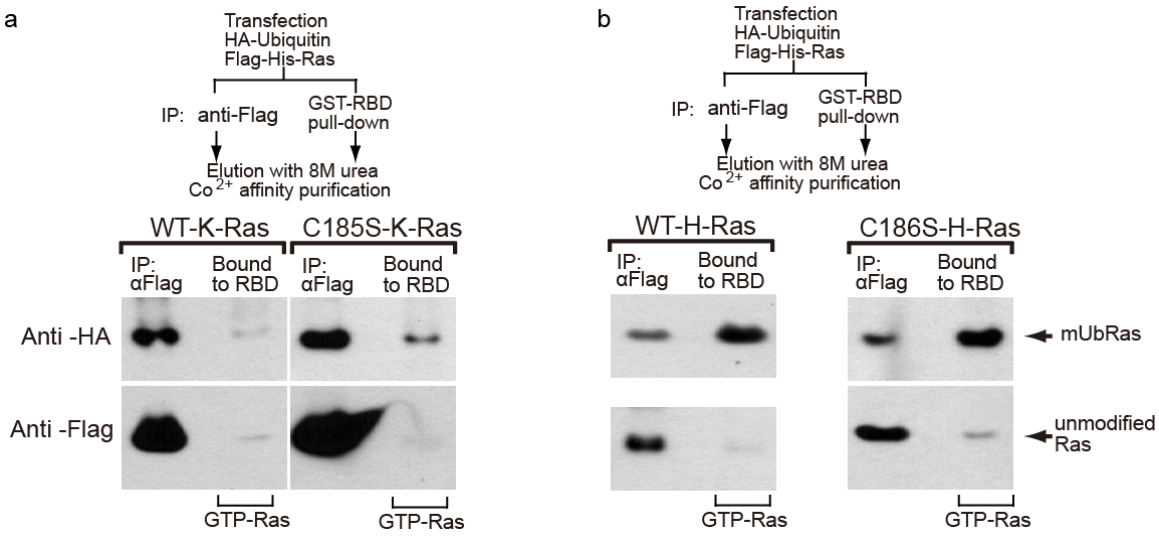

Figure 2.2. Raf-RBD Pull-Down from HEK293T Cells Indicates that Ubiquitination Increases the Fraction of GTP-Bound Ras. Either (**a**) K-Ras or (**b**) H-Ras Flag-His-Ras^{WT} or Caax-mutated-Ras mutants were co-expressed with HA-Ubiquitin in HEK293T cells. Ras proteins were either immunoprecipitated with an anti-Flag antibody or GST-Raf-RBD. Each precipitate was then dissolved in 8 M urea and further purified on a Co^{2+} affinity column to eliminate antibody and GST-Raf-RBD contamination. Western blots with anti-Flag and anti-HA antibodies revealed the relative fraction of total Ras and mUbRas.

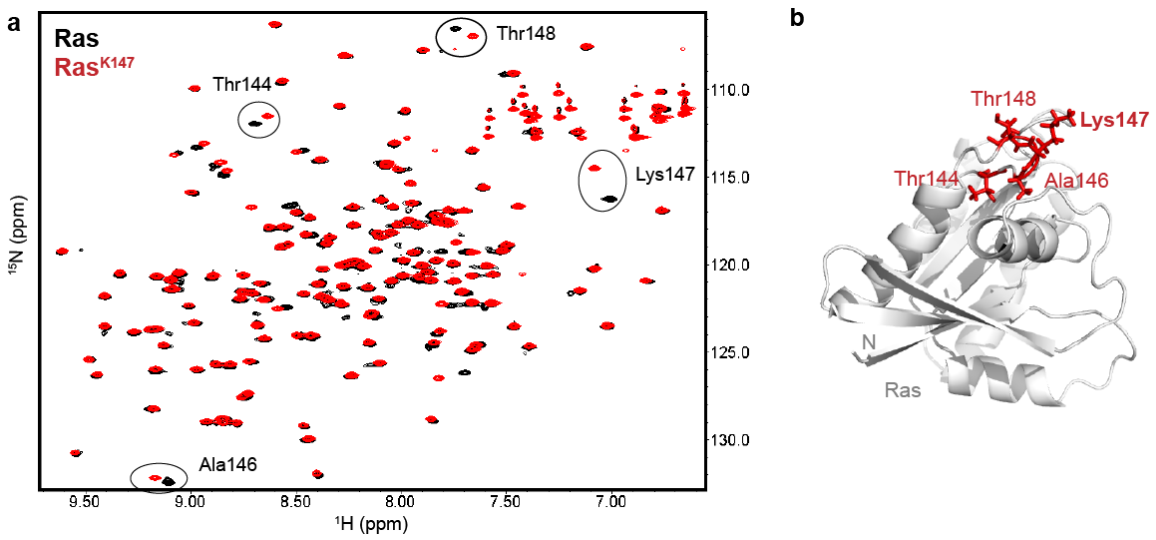

Figure 2.3. NMR Comparison of Ras and Ras^{K147C}. (**a**) HSQC overlay of ^{15}N-Ras bound to Mg-GDP (black) and ^{15}N-Ras^{C118SK147C} bound to Mg-GDP (red). Assigned residues that shift are labeled. (**b**) Ribbon representation of the structure of Ras-GDP (1CRR) showing assigned residues that shift for the K147C mutation in red.

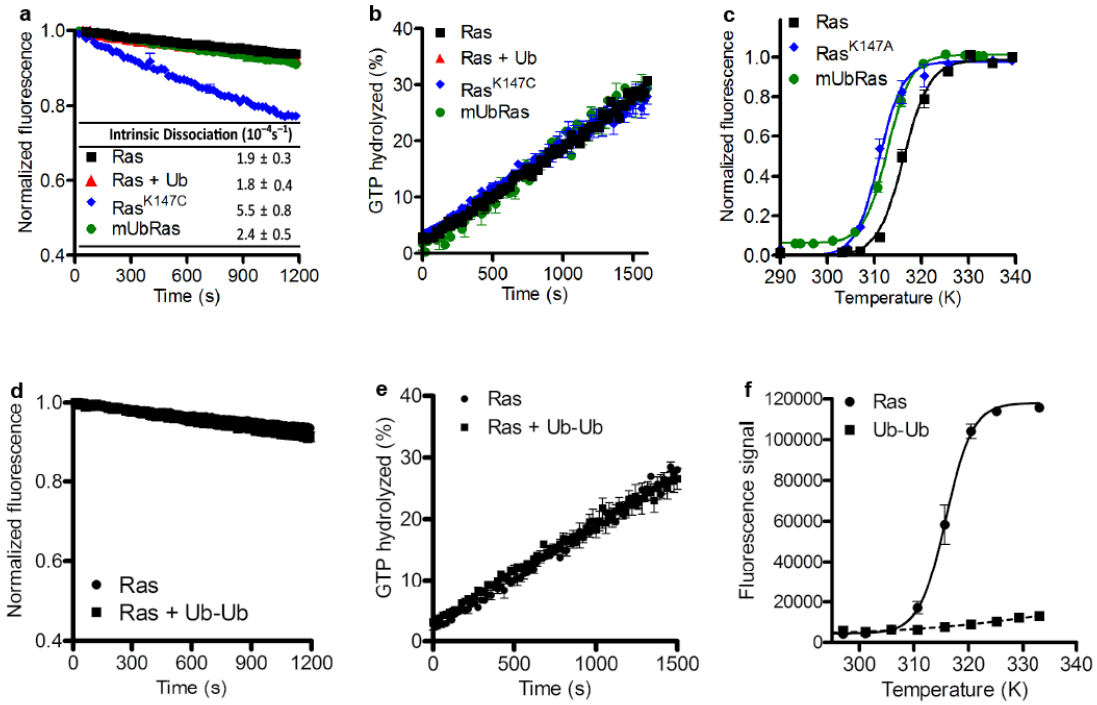

Figure 2.4. Monoubiquitinated Ras Retains Intrinsic Stability and Activity. (**a**) Intrinsic nucleotide dissociation rates for Ras, Ras^{K147C}, and mUbRas loaded with MANT–GDP. Dissociation was monitored following the addition of unlabeled GDP by the decrease in fluorescence emission over time. Data were fit to an exponential dissociation curve, and the results are the mean ± s.d. (n=4). (**b**) Intrinsic single–turnover GTP hydrolysis for Ras, Ras^{K147C}, and mUbRas. Hydrolysis was initiated by the addition of Mg^{2+} and monitored by the change in fluorescence of Flippi when bound to free phosphate. Data were converted to a phosphate concentration using a standard curve. The concentration of phosphate equal to 100% GTP hydrolyzed was determined in the presence of GAP. Results are the mean ± s.d. (n=6). (**c**) Thermal stability of Ras, Ras^{K147A}, and mUbRas measured by ABD–F incorporation as a function of temperature. The data were normalized using the maximum fluorescence intensity. Results are the mean ± s.d. (n=4). (**d**) Intrinsic nucleotide dissociation rates for Ras loaded with MANT-GDP in the absence and presence of Ub-Ub measured as described in (**a**). (**e**) Intrinsic single-turnover GTP hydrolysis for Ras in the absence and presence of Ub-Ub measured as described in (**b**). (**f**) Thermal stability of Ras and Ubiquitin dimer (Ub-Ub) alone measured as described in (**c**). Rather than normalizing the fluorescent output, the raw fluorescent signal is shown.

Chemical Ubiquitination Mimics Native Ubiquitination

We found that monoubiquitination does not alter the intrinsic biochemical activity of Ras, even though mUbRas accumulates in the GTP–bound state *in vivo*. Previous studies have shown that chemically ubiquitinated PCNA functions similarly to the enzymatically ubiquitinated protein (*109*). To further establish that chemical ubiquitination of Ras is a good mimic of native ubiquitination, we built computational models of Ubiquitin ligated to Ras. To create the model, we used a recently developed module of the Rosetta protein modeling software suite (*186, 187*) that samples the conformational space available to ligated proteins. We modified Rosetta to consider disulfide and native isopeptide ubiquitination linkages and generated model structures of mUbRas using both linkages. We generated these models without the use of experimentally–derived constraints.

Shown in **Figure 2.5a-b** are the ten lowest scoring structures of each type of linkage, sorted by Rosetta total score from populations of approximately 2000 models. Comparison of these models indicates that the two systems behave similarly; Ubiquitin samples a wide range of conformations when ligated to Ras and all Ubiquitin positions are allowed at low energy scores (**Figure 2.5c-d**). This modeling result suggests that chemical ubiquitination is a good surrogate for native ubiquitination of Ras. The data also suggest that Ubiquitin does not bind with high affinity to any single site on Ras, which is consistent with our findings by NMR that Ubiquitin does not specifically interact with Ras when the two proteins are free in solution.

Monoubiquitination Affects the Switch Regions of Ras

Results obtained from computational modeling suggest that there is no single preferred interaction between Ubiquitin and Ras. To test this prediction experimentally, we used NMR to examine spectral differences between Ubiquitin and Ras upon monoubiquitination. First, we ^{15}N–enriched Ubiquitin^{G76C} and examined the ^{1}H–^{15}N 2D HSQC spectrum of Ubiquitin when ligated to Ras^{K147C}. By this method we observed partial to complete resonance broadening of eleven backbone amides, but no substantial chemical shifts within Ubiquitin (**Figure 2.6a**). By mapping these spectral changes onto the structure of Ubiquitin in **Figure 2.6b**, it is evident that one face of Ubiquitin is primarily altered upon ligation with Ras. A possible explanation for the inability to detect a subset of Ubiquitin amide resonances is that Ras ligation restricts conformational sampling, leading to exchange broadening.

We next reversed our labeling scheme and ^{15}N–enriched Ras prior to ligation with Ubiquitin and collected a ^{1}H–^{15}N 2D HSQC spectrum (**Figure 2.7a**). Eighty–four of the 137 detectable backbone amide resonances dispersed across mUbRas exhibited multiple populations rather than a single, Lorentzian shaped peak (**Figure 2.7b-c)**. The multiple populations indicate that Ubiquitin adopts more than one position relative to Ras on a timescale detectable by NMR. We also observed a substantial number of residues that broadened and, in some cases, could no longer be detected in mUbRas (**Figure 2.7a**). The broadened peaks primarily localize to the switch regions (**Figure 2.7d**). In the NMR spectra of Ras–GTP, backbone amides associated with residues in Switch I and Switch II are not detectable because they are in intermediate exchange on the NMR timescale (*188*).

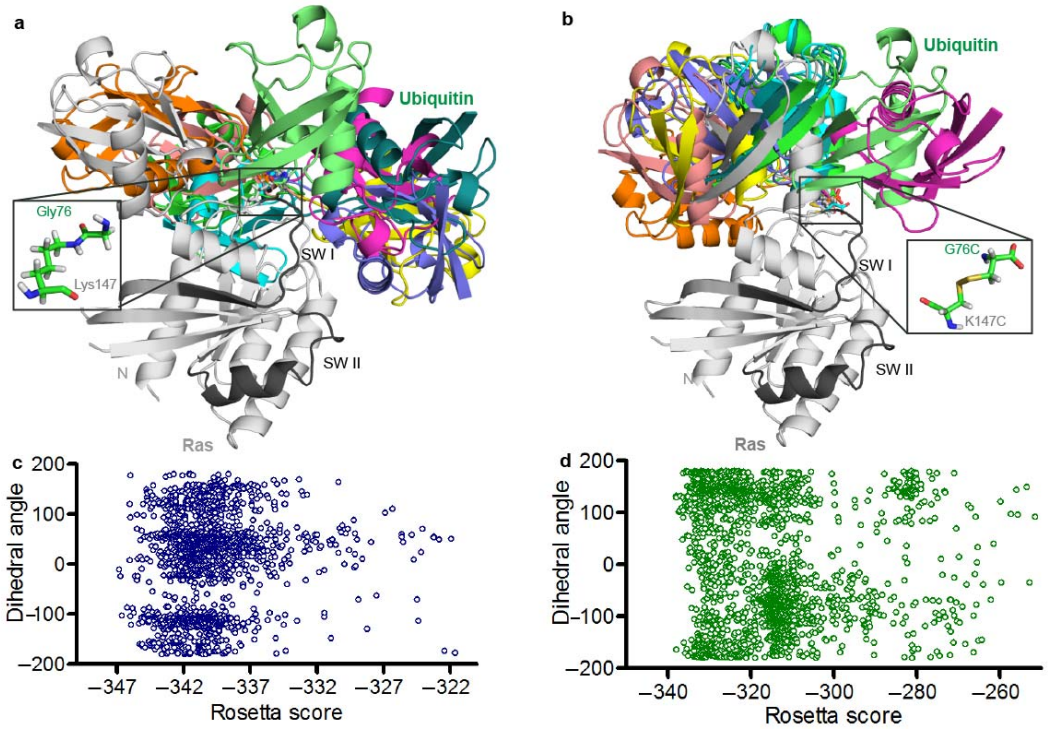

Figure 2.5. Rosetta Model of Native and Chemical Ubiquitination of Ras. (**a**) The ten lowest scoring Rosetta models of the native linkage of Ras monoubiquitination at position 147 lacking constraints to bias the model. Ras (5P21) is shown in grey with switch regions (SWI and SWII) highlighted in black. Ubiquitin (1UBQ) conformers shown in colors. Inset: native linkage between Ras Lys147 and Ubiquitin G76. (**b**) The ten lowest scoring Rosetta models of the chemical linkage of Ras monoubiquitination at position 147 lacking any constraints to bias the model. Ras and Ubiquitin colored as in panel (**a**). Inset: chemical linkage between Ras K147C and Ubiquitin G76C. (**c**) The distribution of Ubiquitin orientations relative to Ras plotted against Rosetta energy scores for the native linkage. The Y axis shows the dihedral angle, in degrees, of the torsional angle between the center of mass of Ubiquitin, the linking Ras residue (147), the center of mass of Ras and an arbitrary Ras reference atom. (**d**) The distribution of Ubiquitin orientations relative to Ras plotted against Rosetta energy scores for the chemical linkage. Axes are the same as described in panel (**c**).

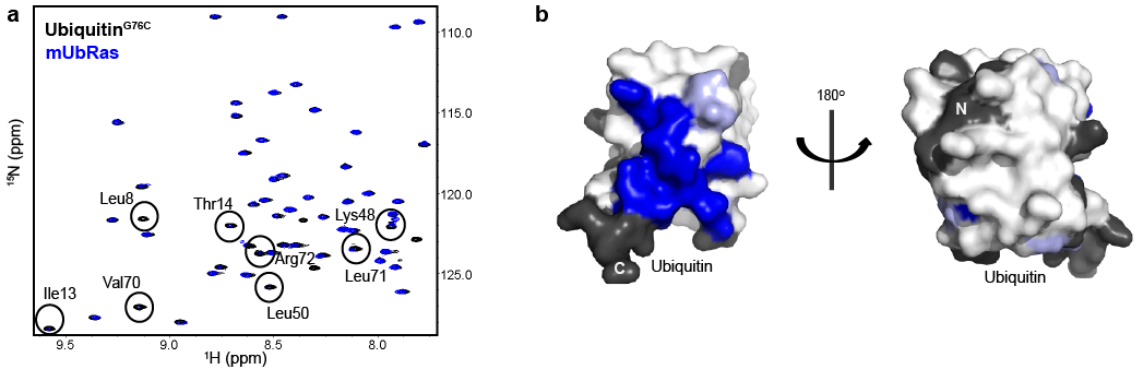

Figure 2.6. Surfaces of Ubiquitin Affected by Monoubiquitination. (**a**) HSQC spectra of ^{15}N–Ubiquitin^{G76C} free (black) or ligated to Ras^{K147C} (blue). Residues that broaden are labeled based on previous assignments (*189*). (**b**) Space filling model of the structure of Ubiquitin (1UBQ) with residues that show decreased intensity when ligated to Ras (blue). Residues with no information are colored black.

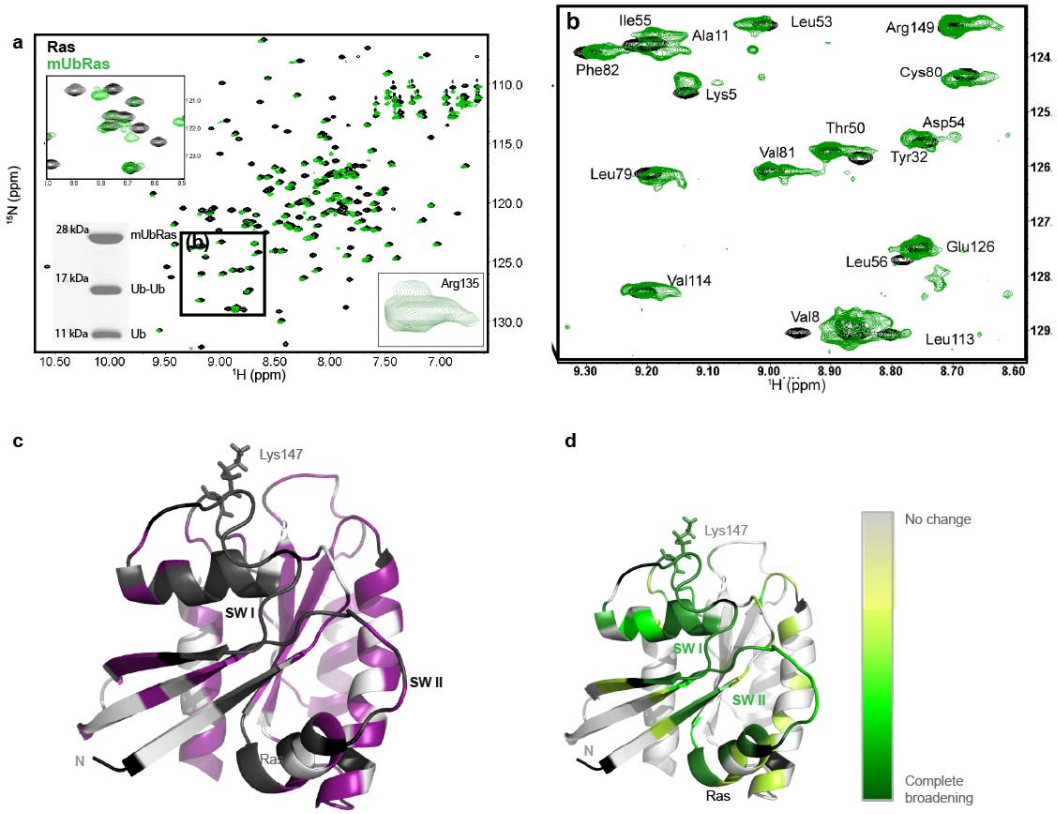

Figure 2.7. Surfaces of Ras Affected by Monoubiquitination. (**a**) HSQC spectra of ^{15}N–Ras^{K147C} bound to Mg–GDP alone (black) and when monoubiquitinated (green). Inset (Top): enhancement of one expanded region showing residues that broaden and disappear. Inset (Bottom Left): SDS–PAGE gel showing integrity of mUbRas sample after HSQC analysis. Inset (Bottom Right): close up of Arg135, which exhibits multiple populations. (**b**) Expansion of a region within the HSQC spectrum (panel **a**) highlighting the multiple populations of residues in the mUbRas spectrum (green). Assignments for Ras^{K147C} are shown in black. (**c**) Mapping of Ras backbone amides that exhibit multiple populations upon monoubiquitination onto the structure of Ras (5P21) in purple. GDP is shown as a stick and magnesium as a sphere. Residues with no information in the HSQC spectrum are colored black. (**d**) Mapping of Ras backbone amides that disappear upon monoubiquitination onto the structure of Ras. Darker green indicates more appreciable broadening (primarily in the SW I and SW II). Residues with no information are colored black.

The observed broadening in the GDP–bound state of mUbRas suggests a change in backbone dynamics, possibly due to conformational exchange or dynamic sampling of the switch regions. This hypothesis is supported by the observation that when an HSQC of mUbRas is collected on a 500 MHz spectrometer, some of the broadened residues in the switch regions and p-loop become visible, as seen in **Figure 2.8**. This suggests that the switch regions are beginning to shift from intermediate exchange back to fast exchange on the NMR timescale. Since Ras regulators and effectors interact through the switch regions, monoubiquitination could alter the population of active Ras by changing how mUbRas interacts with regulators.

Monoubiquitination of Ras Inhibits GAP–Mediated Hydrolysis

In cells, the nucleotide–bound state of Ras is regulated both by GEFs, which increase the rate of GDP dissociation, and GAPs, which enhance the rate of GTP hydrolysis. Our NMR data suggest that monoubiquitination affects the switch regions of Ras, which in turn could alter interactions with GEFs and GAPs. Thus the increased GTP–bound population of mUbRas *in vivo* could be caused by either an increased sensitivity to GEFs or decreased sensitivity to GAPs.

We first determined if the rate of GEF–mediated GDP dissociation is altered when Ras is monoubiquitinated. For these experiments, we equilibrated Ras^{K147C}, mUbRas, and Ras with MANT–GDP and measured the rate of GDP dissociation in the presence of a catalytic fragment from the Ras GEF, Sos (Sos^{cat}) (*190*). While the rate of GEF–mediated GDP dissociation was faster for Ras^{K147C} than Ras, the percent increase compared to the intrinsic rate of dissociation was the same, indicating that mutation at position 147 does not change the overall sensitivity of Ras to GEF–mediated regulation.

However, we observed a decrease in the rate of GEF–mediated nucleotide dissociation for mUbRas compared to unmodified Ras (**Figure 2.9a**).

We next considered the effect of Ras monoubiquitination on GAP–mediated hydrolysis. To this end we compared the rate of GTP hydrolysis for Ras and mUbRas in the presence of the catalytic domains of two GAPs, NF1 ($NF1^{333}$) and p120GAP (GAP–334)(*138, 191*). At a GAP–to–Ras ratio of 1:500, we observed an order of magnitude increase in the rate of GTP hydrolysis for unmodified Ras relative to the intrinsic rate of GTP hydrolysis. No increase in the rate of GTP hydrolysis was observed for mUbRas in the presence of the same GAP–to–Ras ratio (**Figure 2.9b**). Therefore, mUbRas is insensitive to GAP–mediated regulation, similar to an oncogenic Ras^{G12V} mutation (*172*). We obtained similar results using K–Ras (**Figure 2.10**), indicating that the effects of monoubiquitination on Ras are not isoform–specific when the proteins are modified at the same lysine.

To validate the use of an *in vitro* system to dissect the mechanism of Ras regulation, we measured the sensitivity of mUbRas to GAP–mediated hydrolysis in a cellular reconstitution system. We immunoprecipitated Ras from HEK293T cells and compared the sensitivity of the monoubiquitinated and unmodified fractions of Ras to regulation by GAP. As seen in **Figure 2.9c**, monoubiquitinated K–Ras is less sensitive than the unmodified protein to GAP–mediated GTP hydrolysis. These data support our *in vitro* findings that monoubiquitination increases the population of active, GTP–bound Ras through a defect in sensitivity to GAP–mediated regulation.

To determine if the reduced response to regulators is due to a change in binding affinity for mUbRas, we first measured the extent to which monoubiquitination disrupts

the interaction between Ras and Sos^{cat} (**Figure 2.9d**). Results from these analyses indicated that the binding affinity between mUbRas and Sos^{cat} is 8.3 ± 0.9 μM, which is half the observed binding affinity between Ras and Sos^{cat} (4.2 ± 0.4 μM), consistent with the small reduction in the rate of GDP dissociation observed. However, a decrease in the rate of GDP dissociation would favor the GDP–bound state of Ras. Thus, the minor differences in GEF binding do not account for the accumulation of Ras–GTP *in vivo.*

To determine whether ubiquitination also leads to a reduction in GAP binding affinity, we compared the ability of Ras and mUbRas to bind to $NF1^{333}$ in the presence of AlF_4^-. As seen in **Figure 2.9e**, in the presence of AlF_4^- almost 100% of $NF1^{333}$ bound to Ras, which was present in slight excess. In contrast, about 50% of the $NF1^{333}$ bound to mUbRas under the same conditions (**Figure 2.9f**), which suggests that the binding affinity between GAP and mUbRas is reduced relative to unmodified Ras.

While monoubiquitination affects both GEF– and GAP–mediated activity, the GAP defect has a greater influence on the enzyme kinetics and as such is predicted to have a dominant effect on the contribution to GTP–bound Ras. We created a kinetic model that measured the effects of GEF and GAP activity on the observed population of activated Ras. These data show that at the difference in GEF and GAP activity measured experimentally, the amount of GTP-bound Ras will increase relative to unmodified Ras (**Figure 2.11)**. Taken together, our data reveal a substantial reduction in GAP activity as a consequence of Ras monoubiquitination, which accounts for the accumulation of activated Ras *in vivo.*

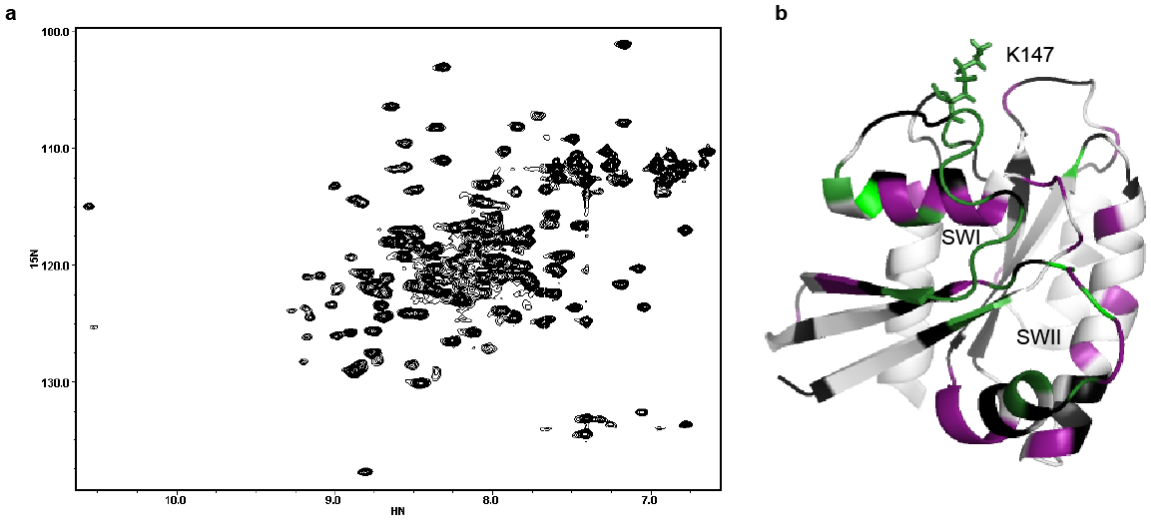

Figure 2.8. Increased Detection of mUbRas Backbone Amides at 500 MHz**.** (**a**) HSQC spectra of ^{15}N–Ras^{K147C} bound to Mg–GDP when monoubiquitinated (black). (**b**) Mapping of Ras backbone amides that disappear upon monoubiquitination onto the structure of Ras. Darker green indicates more appreciable broadening (primarily in the SW I and SW II). Residues with no information are colored black. Residues in purple represent peaks where broadening is reduced and amide resonances can again be detected at 500 MHz.

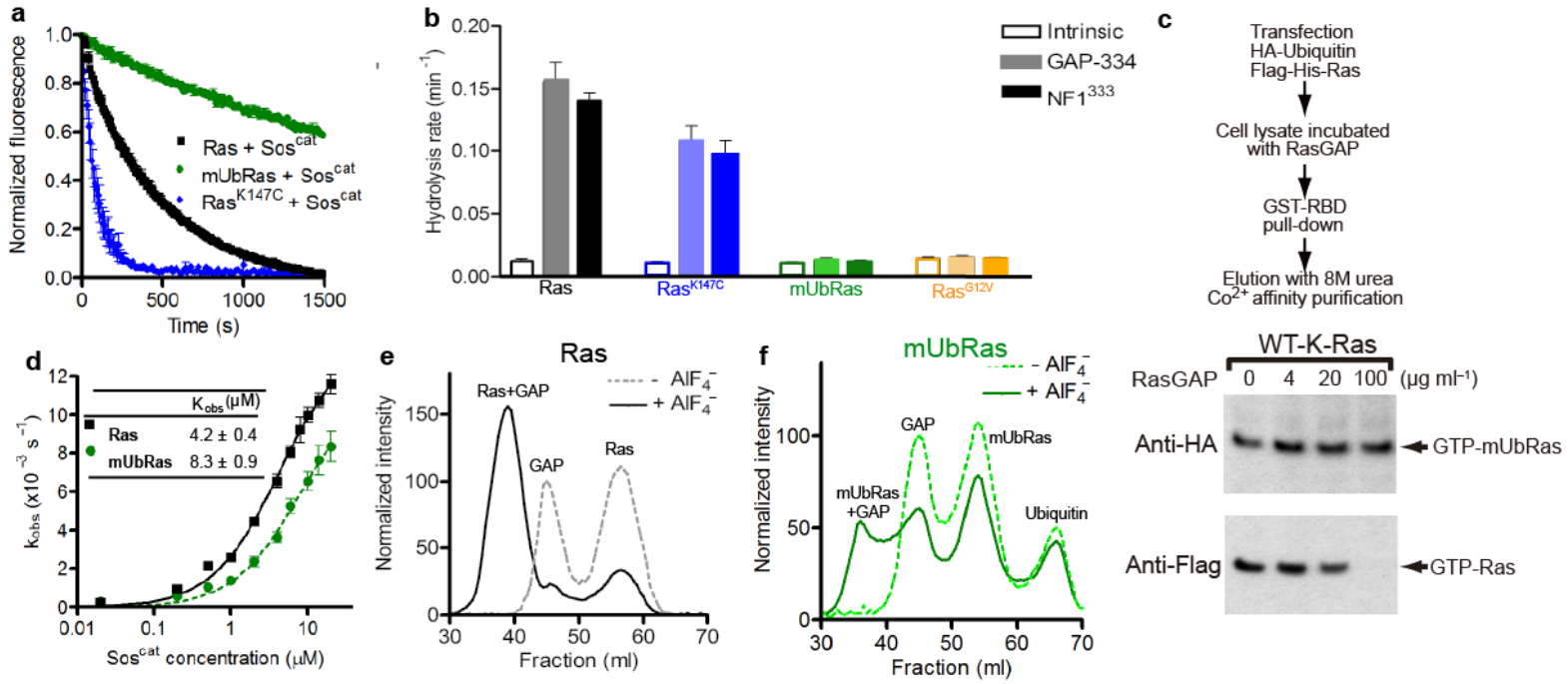

Figure 2.9. Monoubiquitination Decreases the Sensitivity of Ras to Downregulation by GAPs. (**a**) Nucleotide dissociation reaction for Ras, Ras^{K147C}, and mUbRas loaded with MANT–GDP in the presence of a 1:1 molar ratio of Ras to Sos^{cat}. Data were fit to an exponential dissociation curve, and the results are the mean ± s.d. (n=4). (**b**) Single–turnover GTP hydrolysis for Ras, Ras^{K147C}, mUbRas, and Ras^{G12V} in the presence of $NF1^{333}$ or GAP–334 at a molar ratio of 1:500 GAP:Ras. Results are the mean ± s.d. (n=6). (**c**) Immunoblotting of GTP-bound Ras and GTP-bound mUbRas in cell extract in the presence of increasing concentrations of RasGAP. Anti–Flag and anti–HA antibodies reveal the relative fraction of total Ras and mUbRas, respectively. (**d**) Titration of Ras with Sos^{cat}. Experiments were performed as described panel **a**, except the concentration of Sos^{cat} was varied while Ras was held constant at 0.2 μM. Data plotted as a function of the Sos^{cat} concentration. Results are the mean ± s.d. (n=3). (**e**) Gel filtration of Ras and $NF1^{333}$ in the absence (dotted line) and presence (solid line) of AlF_4^-. (**f**) Gel filtration of mUbRas and $NF1^{333}$ in the absence (dotted line) and presence (solid line) of AlF_4^-.

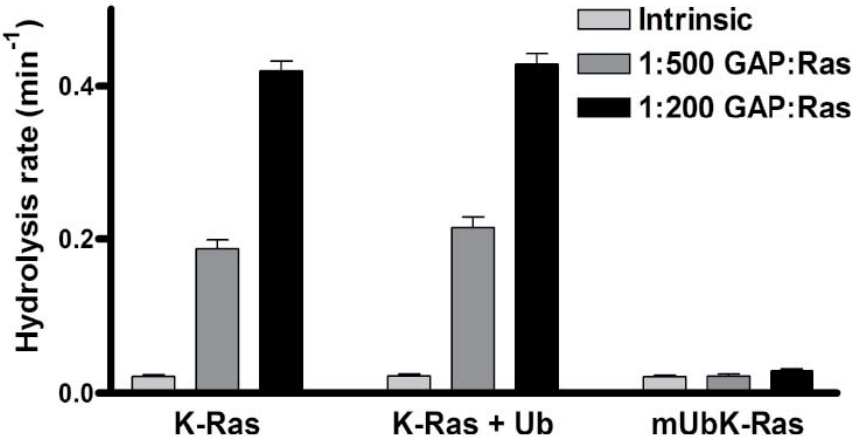

Figure 2.10. GAP-Mediated Hydrolysis of Monoubiquitinated K-Ras. Intrinsic and GAP-mediated single-turnover GTP hydrolysis of Ras in the absence and presence of GAP-334 (intrinsic, 1:500 GAP:Ras, and 1:200 GAP:Ras). Rates of GTP hydrolysis were measured for K-Ras, K-Ras with free Ubiquitin (K-Ras+Ub), and monoubiquitinated K-Ras (mUbK-Ras). Data were fit to a single exponential association curve with the maximum determined by the highest phosphate concentration reached in the presence of the GAP. Results are the mean ± s.d. (n=6).

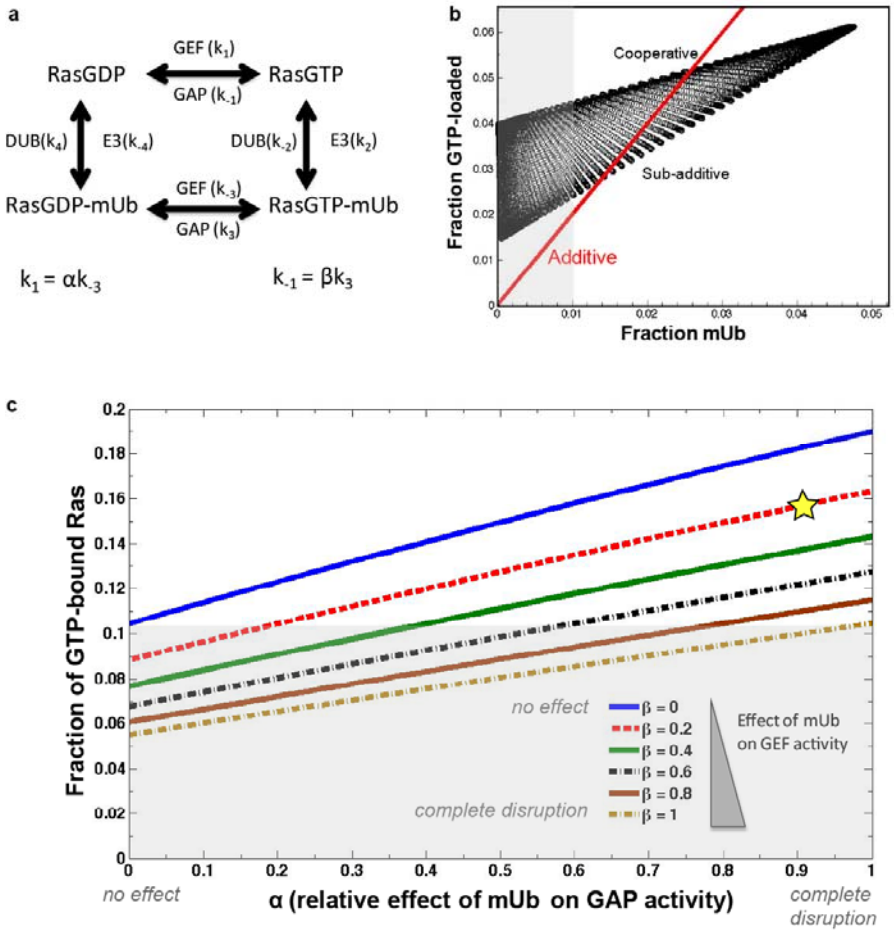

Figure 2.11. Kinetic Modeling of Changes in Ras Activity Due to Monoubiquitination. (**a**) Ras transitions through four states: GDP or GTP-loaded, unmodified, and monoubiquitinated. Each transition is determined via enzyme kinetics involving a GEF, GAP, E3 ligase (E3) and deubiquitinating enzyme (DUB). Two additional parameters, α and β, account for the relative effects of monoubiquitination on GAP and GEF activity, respectively. (**b**) The effects of monoubiquitination on the total amount of GTP-bound Ras. A scatterplot of resulting simulations is shown using 6562 log-uniform sampled parameter values varying the catalytic rate (vmax) and Michaelis Constant (Km) of the deubiquitinating enzyme over four orders of magnitude. The fraction of GTP-loaded Ras is plotted as a function of the fraction of mUbRas. The red line represents a linear (additive) effect in which each modified Ras contributes additively to the total amount of GTP-loaded Ras. For small amounts of ubiquitination (Fraction mUb < 0.01, shaded area), data points lie above the red line indicating that mUbRas contributes cooperatively to the steady state levels of total GTP-bound Ras. (**c**) Modeled effects of mUbRas on GEF and GAP activity. The fraction GTP-loaded is plotted against different values of α. Six values of β (legend) are also considered. For each case, the amount of activated Ras increases (white area) with increasing relative disruption of GAP activity. The star indicates the differences in GEF and GAP activity measured experimentally.

Modifying Ras with PDZ2 Impairs GAP–Mediated Hydrolysis

Our computational and NMR data suggest that Ubiquitin does not form a specific, high–affinity interaction with Ras. If this observation is correct, then modification of Ras with any protein similar to Ubiquitin should also impair GAP–mediated hydrolysis. As a test of this model, we chemically ligated Ras to PDZ2 (RasPDZ2), a 9 kDa protein with a Ubiquitin–like fold but no obvious sequence similarity to Ubiquitin (*192*). We replaced the unstructured c-terminal extension of PDZ2, defined as the region after the folded domain ends in the crystal structure, with that of Ubiquitin ($PDZ2^{UL}$). Therefore, all differences between $PDZ2^{UL}$ and Ubiquitin are contained in the folded regions of the two proteins.

Modeling of PDZ^{UL} on Ras shows that PDZ adopts a similar spread of possible conformations as Ubiquitin (**Figure 2.12a-b**), suggesting that it could have an impact on Ras activity that is comparable to that of Ubiquitin ligation. As seen in **Figure 2.12c**, mUbRas and $RasPDZ2^{UL}$ have identical melting temperatures, indicating that neither ubiquitination nor $PDZ2^{UL}$ ligation substantially alters the thermal stability of Ras. Similar to ubiquitination, $PDZ2^{UL}$ ligation does not alter intrinsic Ras nucleotide dissociation rates, and GEF–mediated dissociation is reduced to the same extent as for mUbRas (**Figure 2.12d**). Finally, $RasPDZ2^{UL}$ retains intrinsic GTP hydrolysis activity, but is insensitive to GAP–mediated GTP hydrolysis (**Figure 2.12e**). These data indicate that non–specific interactions between Ras and Ubiquitin are responsible for the insensitivity of mUbRas to GAPs.

In the studies described in the previous paragraph, we replaced the c-terminus of PDZ with the c-terminus of Ubiquitin. This was done so that the differences between the

two proteins would be isolated to the structured region and not due to differences in the conformational motility of the PDZ domain compared to Ubiquitin. Preliminary Rosetta models of Ras modified with PDZ with the native c-terminus (RasPDZ) suggest that the shorter and stiffer c-terminal region of the PDZ leads to changes in proximity of Ras and its modifier. As seen in **Figure 2.13a**, the PDZ domain does not appear to make contact with as much of the surface of Ras as Ubiquitin or PDZ^{UL}. If access to the switch regions is important for the mechanism by which ubiquitination regulates Ras, then modification with PDZ should not alter regulator-mediated activity to the same extent as modification with PDZ^{UL}. As seen in **Figure 2.13b**, mUbRas and RasPDZ2 have identical melting temperatures, indicating PDZ2 ligation does not substantially alter the thermal stability of Ras. Consistent with ubiquitination, PDZ2 ligation to Ras also does not alter intrinsic nucleotide dissociation or hydrolysis (**Figure 2.13c-d**). However, RasPDZ2 was more responsive to Sos^{cat} and GAP-334 than mUbRas. As seen in **Figure 2.13c**, RasPDZ2 shows a six-fold increase in the rate of GDP-dissociation in the presence of Sos^{cat}, in comparison to the three-fold increase observed for mUbRas. However, RasPDZ2 is still less responsive to Sos^{cat} than unmodified Ras, which exhibits a fourteen-fold increase in dissociation under the same conditions. Additionally, while RasPDZ2 retains GAP sensitivity, it is almost ten-fold less responsive than unmodified Ras to GAP-334 mediated GTP hydrolysis (**Figure 2.13d**). Thus, these results indicate that RasPDZ2, while not identical to mUbRas, is less sensitive than Ras to GEF- and GAP-mediated regulation.

The difference between the response of mUbRas and RasPDZ2 to GAPs was likely due to the observed differences in length and flexibility of the c-termini of

Ubiquitin and PDZ2. Therefore, we wanted to verify that the differences between the enzymatic and chemical ubiquitination linkers (seven bonds and five bonds, respectively) were not large enough to alter GAP-responsiveness. We placed an additional cysteine at the c-terminus of Ubiquitin (Ub^{C77}) thereby creating a linker slightly longer than the native linker. We measured the rate of GAP-mediated GTP hydrolysis and observed that the response of Ras ligated to Ub^{77C} is identical to Ras ligated to Ub^{G76C} (**Figure 2.13d**). These results indicate that variations in the linker length on this scale (1-2 bonds) do not influence the sensitivity of mUbRas to GAP downregulation.

Therefore, chemical ubiquitination is a good surrogate for enzymatic ubiquitination. Our data indicate that ubiquitination activates Ras by impairing GAP-mediated hydrolysis. Essential to this mechanism of activation is the ability of Ubiquitin to have access to a particular surface of Ras. The interactions between Ras and Ubiquitin are non-specific, but lead to a reduction in catalytic efficiency of Ras GAPs.

The Effect of Ubiquitination is Site–Specific

Finally, while the GAP insensitivity of modified Ras is not specific to Ubiquitin, it could be specific to modification at position 147. To address this possibility, we chose two other lysines on Ras that were not identified as sites of monoubiquitination in the mass spectrometry screen of monoubiquitinated K–Ras(*153*). We chose position 88 because it is near the switch regions of Ras, similar to position 147, and could have a similar effect as monoubiquitination at position 147. We chose position 101 because the side chain is oriented toward the opposite face of Ras and would likely be less disruptive to the Ras active site and switch regions if monoubiquitinated (**Figure 2.14a**).

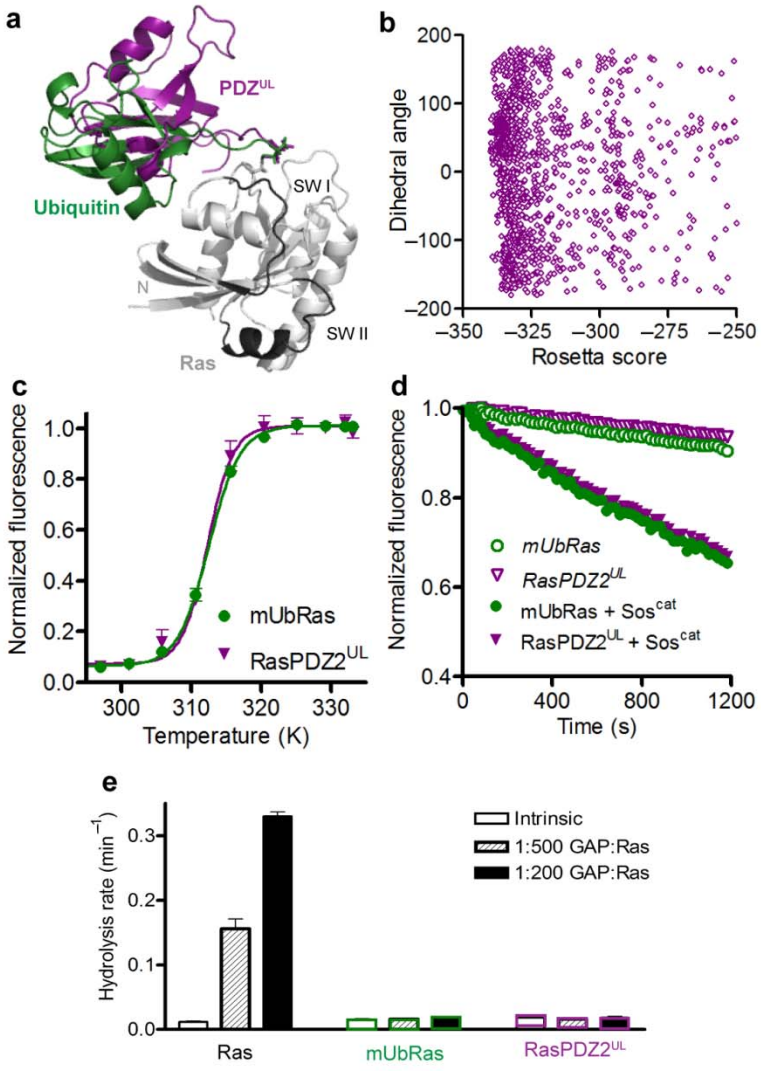

Figure 2.12. Modification of Ras with PDZ2^{UL} Resembles Modification with Ubiquitin. (**a**) Rosetta model of Ras (5P21) in grey modified at position 147 with Ubiquitin (1UBQ) in green and PDZ^{UL} (3LNX) in purple. (**b**) The distribution of PDZ^{UL} orientations relative to Ras plotted against Rosetta energy scores for the chemical linkage. This plot follows the scheme of Figure 3B. (**c**) Thermal stability of Ras and RasPDZ2 with the Ubiquitin linker (RasPDZ2^{UL}) measured by ABD–F incorporation as a function of temperature. Results are the mean ± s.d. (n=4). (**d**) Nucleotide dissociation reaction for RasPDZ2^{UL} and mUbRas loaded with MANT–GDP in the absence and presence of a 1:1 molar ratio of Ras to Sos^{cat}. Results are the mean ± s.d. (n=4). (**e**) Single–turnover GTP hydrolysis for Ras, RasPDZ2^{UL}, and mUbRas in the presence of GAP–334 at a molar ratio of 1:500 and 1:200 GAP:Ras. Results are the mean ± s.d. (n=6).

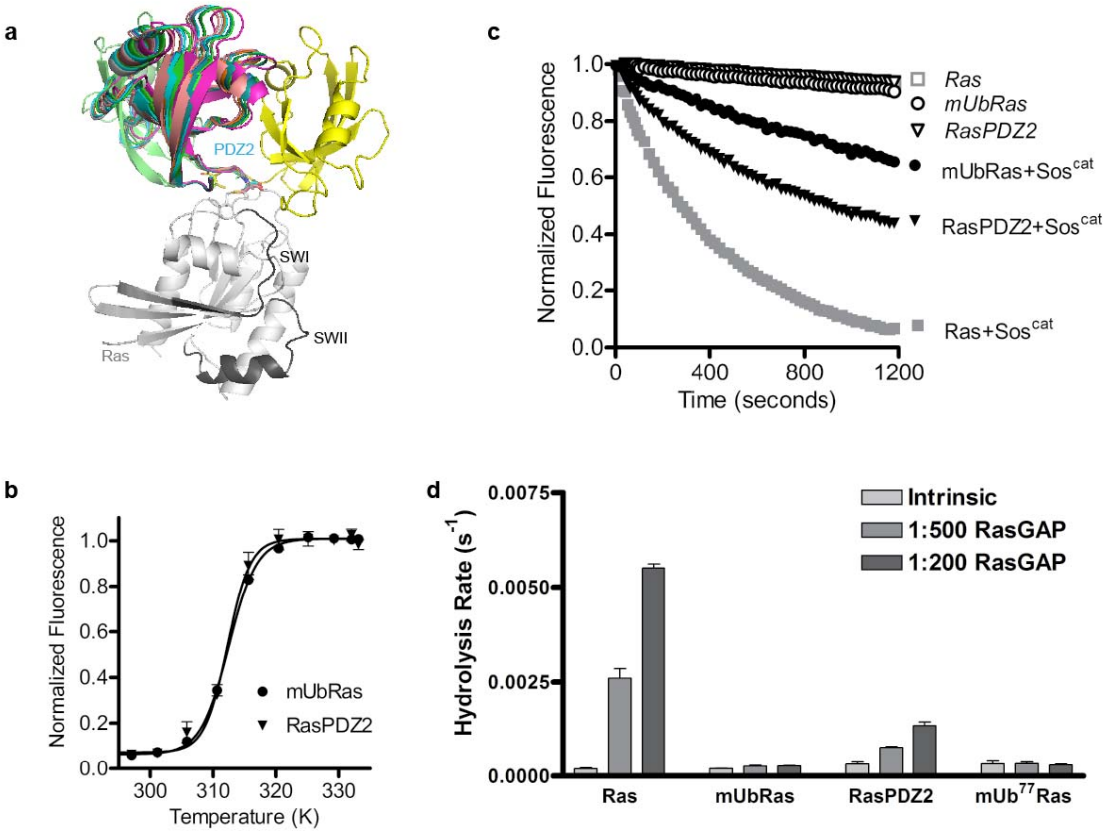

Figure 2.13. Modification of Ras with PDZ is Distinct from Modification with Ubiquitin. (**a**) The ten lowest scoring Rosetta models of the native linkage of Ras monoubiquitination at position 147 lacking constraints to bias the model. Ras (5P21) is shown in grey with switch regions (SWI and SWII) highlighted in black. PDZ2 (3LNX) conformers shown in colors. (**b**) Thermal stability of mUbRas and RasPDZ2 measured by ABD–F incorporation as a function of temperature. Results are the mean ± s.d. (n=4). (**c**) Nucleotide dissociation reaction for Ras, RasPDZ2 and mUbRas loaded with MANT–GDP in the absence and presence of a 1:1 molar ratio of Ras to Sos^{cat}. Results are the mean ± s.d. (n=4). (**d**) Single–turnover GTP hydrolysis for Ras, RasPDZ2, mUbRas, and mUbRas modified with $Ubiquitin^{C77}$ ($mUb^{77}Ras$) in the presence of GAP–334 at a molar ratio of 1:500 and 1:200 GAP:Ras. Results are the mean ± s.d. (n=6).

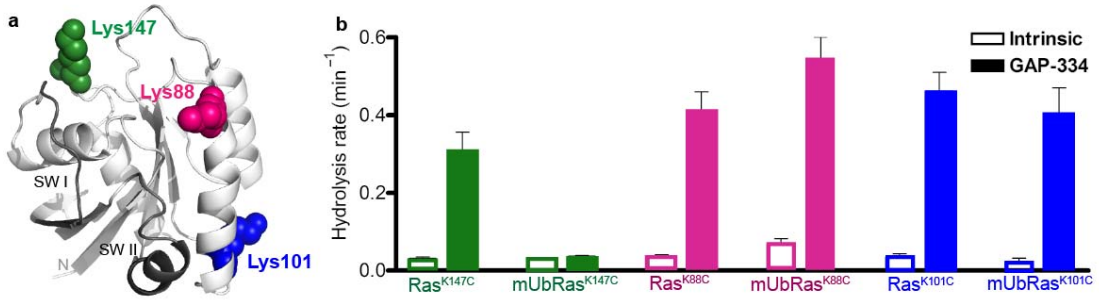

Figure 2.14. The Impaired GAP–Sensitivity of mUbRas is Site–Specific. (**a**) Ribbon diagram of Ras–GDP (1CRR) with the switch regions highlighted in black and the side chains of Lys147, Lys88, and Lys101 represented as spheres in green, fuchsia, and blue, respectively. (**b**) Single–turnover GTP hydrolysis for Ras mutated and ubiquitinated at position 147, 88, or 101 in the absence and presence of GAP–334 at a molar ratio of 1:200 GAP:Ras. Results are the mean ± s.d. (n=4).

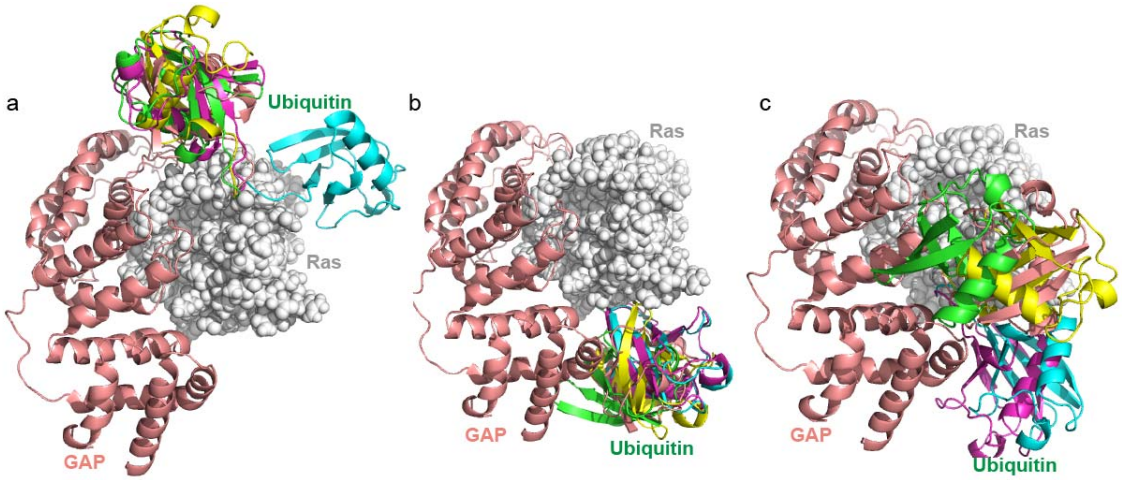

Figure 2.15. Monoubiquitination Does not Sterically Occlude GAP Binding to Ras. The five lowest energy models from the chemical ubiquitination Rosetta model of mUbRas in the presence of GAP-334 (1WQ1). Ras is represented as grey spheres and GAP-334 in salmon. Ubiquitin (1UBQ) conformers are shown in green, cyan, fuchsia, pink, and yellow. (**a**) Monoubiquitination at position 147. (**b**) Monoubiquitination at position 88. (**c**) Monoubiquitination at position 101.

Using Rosetta models, we found that monoubiquitination of Ras at position 88 or 101 does not cause steric clashes with GAP–334, similar to monoubiquitination at position 147 (**Figure 2.15**). To determine whether monoubiquitination at position 88 or 101 could affect GAP–mediated regulation, we mutated each of these residues to cysteine, modified them with Ubiquitin^{G76C}, and measured the effect of Ubiquitination on intrinsic and GAP–mediated GTP hydrolysis. As seen in **Figure 2.14b**, only monoubiquitination at position 147 impairs GAP–mediated hydrolysis, indicating that the outcome of monoubiquitination is site–specific.

In summary, we used a combination of biochemical, structural, and computational approaches to uncover the mechanism of Ras regulation by monoubiquitination. Our data indicate that ubiquitination activates Ras by impairing the catalytic efficiency of Ras GAPs. Furthermore, the most commonly ubiquitinated position *in vivo*, position 147, is the only lysine tested that impairs GAP–mediated hydrolysis. More broadly, our findings reveal how monoubiquitination promotes sustained signaling and cell transformation.

Discussion

It was established recently that monoubiquitination increases the proportion of Ras that is in the activated (GTP–bound) state, that monoubiquitination enhances association with the downstream effectors Raf and PI3–Kinase, and that mutation of the primary site of monoubiquitination impairs oncogenic Ras–mediated tumorigenesis. Here we show that monoubiquitination decreases the sensitivity of Ras to GAP–mediated hydrolysis. A major advance was our ability to easily generate mUbRas, modified at a single site, in a form suitable for detailed biophysical studies. This chemical ligation

strategy will likely be useful for the study of other monoubiquitinated proteins. Surprisingly, monoubiquitination did not alter the intrinsic activity of Ras, despite the size of the modification. Our modeling and NMR analyses indicated that Ubiquitin dynamically samples a broad surface area of Ras that alters switch region dynamics. These results led us to examine the effect of monoubiquitination on the interaction of Ras with its cognate GEF and GAPs, which also target the switch domains. The analysis revealed that monoubiquitination abrogates GAP–mediated GTP hydrolysis. All other activities, including the ability to bind regulators, were largely preserved and our kinetic modeling suggests that the GAP defect will dominate. Furthermore, this outcome was specific to monoubiquitination at position 147. Thus our work establishes an entirely new mode of Ras activation in which signaling is sustained even in the absence of hormone stimulus or oncogene mutation.

It will be interesting to determine how monoubiquitination affects other signaling proteins including other Ras–family GTPases. Known targets of monoubiquitination include K–Ras (*153*), H–Ras, and N–Ras (*173*). Monoubiquitination disrupts interactions of Rap2A with effector proteins and inhibits the ability of Rap2A to promote dendrite development (*155*). Monoubiquitination has also been observed in Rac1, although the biological consequence of this modification is not yet known (*86, 157*). Our chemical ligation strategy and multi–dimensional approach will be useful for the study of these targets, particularly in cases where the relevant ubiquitin ligase has not been identified.

Another question concerns the role of the preferred site of monoubiquitination, Lys147 (*153*). Whereas ubiquitination of this site has severe consequences for GAP function, targeted ubiquitination of two other candidate sites left GAP–mediated

hydrolysis unperturbed. Lys147 is part of the SAX motif, and a lysine at the third position in this motif is conserved in Ras proteins across species, as well as in other Ras–family GTPases including RhoA, RhoB, RhoC, Rap, Ral, Rab, Rheb and Ran (*120*). It has been shown previously that mutation of the highly conserved adjoining residue, Ala146, leads to enhanced GDP exchange, GTP loading and cellular transformation (*183, 184, 193*). In contrast, we have shown that a mutation of the ubiquitination site itself, Lys 147, has little effect on nucleotide binding or regulator–mediated activity. We speculate that the lysine has been conserved to allow regulation through monoubiquitination. It will be interesting to determine whether other members of the Ras subfamily are also ubiquitinated at this position and whether ubiquitination in such cases leads to sustained activation.

We have demonstrated that monoubiquitination of Ras impedes the function of GTPase activating proteins. Key to our analysis was the ability to generate monoubiquitinated protein, modified at a single residue, suitable for biophysical analysis. Through multi–disciplinary computational, structural and biochemical approaches we identified a novel mechanism of Ras activation, one that is independent of any oncogenic mutation or a sustained receptor stimulus. Given the established importance of Ras in the control of cell growth and differentiation, our findings may reveal opportunities for new pharmaceuticals that target the ubiquitination machinery.

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Methods

Protein Purification

The Ras domains (1–166) of H–Ras and K–Ras were expressed in the pQlinkH vector (Addgene) with a histidine purification tag in *Escherichia coli* BL21 (DE3) RIPL cells (Stratagene: La Jolla, CA). Proteins were purified following standard Qiagen nickel affinity purification procedures. The His tag was cleaved overnight with Tobacco Etch Virus. Ras proteins were further purified by removal of uncleaved protein using Ni–NTA agarose beads (Qiagen). The final product was judged > 95% pure by SDS–PAGE. Proteins were stored in 20 mM HEPES, pH 8.0, 50 mM NaCl, 500 μM TCEP, 50 μM GDP and 5 mM $MgCl_2$.

Sos^{cat} (John Kuriyan; University of California, Berkeley) was purified as previously described(*190*). The catalytic domains of p120GAP (GAP–334) (*138*) and NF1 ($NF1^{333}$)(*191*), were expressed in pQlinkH and purified as described for Ras. Purified proteins were stored in 20 mM HEPES, pH 8.0, 50 mM NaCl and 500 μM TCEP. Full length $Ubiquitin^{G76C}$ and hPTPe–$PDZ2^{ULG97C}$ (c–terminal residues KGQSPC

replaced with the Ubiquitin residues VLRLRGC)(*192*) were expressed in the pQlinkH vector system and purified as described for Ras. Proteins were stored in 20 mM HEPES, pH 8.0, 50 mM NaCl and 500 μM TCEP.

Ligation of Ubiquitin^{G76C} and PDZ2 ^{ULG97C} to Ras^{K147C}

The chemical ligation strategy used to link Ras to Ubiquitin^{G76C} or PDZ2^{ULG97C} was adapted from Merkley et al.(*174*). Briefly, a ten–fold excess of Ubiquitin^{G76C} or PDZ2 ^{ULG97C} was added to Ras^{K147C} and dialyzed into 20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 50 μM GDP at 4 ^{o}C overnight. The amount of disulfide complex formation was determined by non–reducing SDS–PAGE and considered complete by the absence of modified Ras.

Thermal Stability of Ras

The fast quantitative cysteine reactivity (fQCR) method(*182*) was employed to measure changes in Ras thermal stability. Briefly, 2 μM Ras was incubated with 1 mM 4-fluoro-7-aminosulfonylbenzofurazan (ABD–F, Anaspec) at pH 7.0 in the presence of 20 μM GDP and 2 mM $MgCl_2$ at the desired temperature for five minutes. Fluorescent intensity was measured on a PHERAstar plate reader (BMG Labtech). The data were normalized and fit to determine the temperature at which half the protein was unfolded, representing the T_m. Results are the mean ± s.d. (n=3).

Ras Nucleotide Dissociation and Hydrolysis Assays

The rate of nucleotide dissociation was measured using MANT–GDP (BioLog: San Diego, CA) as previously reported(*194, 195*). Briefly, MANT–GDP–bound Ras (2 μM) was added to 1 mL assay buffer (50 mM Tris, pH 7.4, 50 mM NaCl and 5 mM

$MgCl_2$) and exchange initiated by addition of 2 mM GDP. MANT–GDP dissociation was measured as a change in fluorescence intensity over time (excitation: 360 nm, emission: 440 nm) (LS50B Perkin–Elmer Luminescence Spectrometer). Fluorescence data were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a one–phase exponential decay curve. For GEF–mediated dissociation and binding, 200 nM Ras and 0.2 μM to 20 μM Sos^{cat} were used. The nucleotide dissociation rate was plotted as a function of Sos^{cat} concentration and fit to one site binding to determine the binding affinity between Ras or mUbRas and Sos^{cat}. Results are the mean ± s.d. (n=4).

Single–turnover GTP hydrolysis assays were performed as previously described(*196*), except that the phosphate binding protein Flippi 5U (Addgene) was used to detect inorganic phosphate released upon GTP hydrolysis(*185*). Flippi 5U was purified as previously described(*185*). All buffers were made phosphate free by dialysis with 1 U nucleoside phosphorylase (Sigma, USA) and 2 mM inosine (Sigma, USA). For GAP–mediated hydrolysis, 50 μM Ras and 0.1 μM (1:500) and 0.25 μM (1:200) $NF1^{333}$ and GAP–334 were used. The ratio of fluorescence emission was measured at 480 nm and 530 nm with an excitation of 435 nm on a SpectraMax M5 (Molecular Devices). Fluorescence ratios were converted to phosphate concentrations using a standard curve. Hydrolysis curves were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a one–phase exponential association curve. Results are the mean ± s.d. (n=6).

GAP binding was monitored as previously described(*197*). Briefly, 50 μM Ras and 40 μM $NF1^{333}$ were mixed in the presence or absence of AlF_4^- (10 mM NaF, 450 μM $AlCl_3$) on ice. The sample was run on an S75 column in 30 mM Tris, pH 7.5 and 5 mM $MgCl_2$. Data was normalized to the amount of free $NF1^{333}$ in the absence of AlF_4^-.

GST–RBD Immunoprecipitation Assay

Ras activation was measured as described previously(*153*). Flag–His–K–Ras or the c–terminal mutants Flag–His–K–Ras^{C185S} and Flag–His–H–Ras^{C186S} were co–expressed with HA–Ubiquitin in HEK293T cells. The cells were rinsed with cold PBS and lysed with Buffer A (0.5% NP–40, 40 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1 mM DTT, 1 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} pepstatin A, 100 μM AEBSF, Halt phosphatase inhibitor cocktail (Thermo Scientific), 10 mM iodoacetamide (IAA) and 5 mM *N*–ethylmaleimide (NEM)). The soluble fraction from the cell lysates were isolated by centrifugation at 13,000 rpm for 10 min, split, and subjected to anti–Flag agarose immunoprecipitation or incubated with 10 μg of GSH–Sepharose bound GST–Raf–RBD in the presence of 1 mg ml^{-1} BSA for 30 min as described previously[15]. The immunoprecipitated proteins were washed three times with Buffer A and eluted by the addition of 8 M urea. To ensure detection of mUbRas, a secondary purification on Co^{2+} Talon metal affinity chromatography beads (Clontech) was performed. Flag–His–Ras was eluted with sample buffer containing 50 mM EDTA. For the GAP sensitivity assay, bacterially produced GAP–334 was incubated with the cell lysate for 20 min at room temperature and subjected to analysis using GST–Raf–RBD.

NMR Experiments

For NMR studies, ^{15}N– and ^{15}N,^{13}C–enriched samples of Ras and Ubiquitin were produced using standard protocols in M–9 minimal media(*181*). ^{1}H–^{15}N 2D HSQC experiments were conducted on a Varian 700 MHz and a Bruker 500 MHz with a cryoprobe in 20 mM MOPs, pH 6.8, 100 mM NaCl, 5 mM $MgCl_2$, 0.01% sodium azide, 10% D_2O, 1 mM DPTA, and 2 mM GDP at 25^{o}C and with 500 μM protein.

Rosetta Modeling

The modeling strategy used was adapted from Saha et al.(*186*). The previous protocol modeled a thioester linkage between Ubiquitin Gly76 and cysteine on a Ubiquitin E2 enzyme. Modifications include altering the linkage type to disulfide or isopeptide linkages and replacing the pre–existing system–specific constraints with optional command–line defined constraints. Further modifications include the reporting of specific Ubiquitin–Ras residue pair distances and Ubiquitin–Ras positional metrics used to quantify mUbRas conformational ensembles. Also added was the ability to include arbitrary nonmoving atoms in the simulation, used to include the guanine nucleotide, magnesium ion, and in some cases, GAP during simulations. A chemically conjugated model of Ras and Ubiquitin was created and the torsion angles within the linker region were modeled while sampling side chain conformations throughout the interface. For the isopeptide linker, protocol UBQ_Gp_LYX–cterm was used. Torsions allowed to vary included: the chi angles of Lys147 of Ras (sampled from Rosetta's implementation of Dunbrack's 2002 rotamer library (*198, 199*)), the isopeptide bond, and both phi and psi for the Gly76, Gly75, and Arg74 of Ubiquitin. For the disulfide linker, protocol UBQ_Gp_CYD–CYD was used. Torsions sampled include the chi angles for K147C on Ras and G76C on Ubiquitin (from the Dunbrack library and explicit sampling of chi 2), the disulfide bond, phi of Ubiquitin G76C, and both phi and psi for Gly75 and Arg74 of Ubiquitin. Sampling was performed with a standard Rosetta Metropolis–Monte Carlo search protocol(*198*). For each combination of ligand, attachment chemistry, and Ras attachment location, the protocol was run for 2400 hours on a 2.66 MHz chip. This produces about 1500–3000 structures using 20,000 Monte Carlo cycles per trajectory.

CHAPTER III

ISOFORM-SPECIFIC DIFFERENCES IN THE REGULATION OF K-RAS AND H-RAS BY MONOUBIQUITINATION[1]

The major Ras isoforms that regulate important cell processes such as proliferation, differentiation, and survival are highly conserved, yet have distinct biological outputs. Post-translational modification of Ras contributes to the differences in isoform dependent signaling outputs through regulation of Ras activity and subcellular localization. Ubiquitination is one post-translational modification that regulates all three Ras isoforms. Monoubiquitination of H-Ras in CHOK-1 cells promotes endosomal transport and signal dampening. In K-Ras, monoubiquitination specifically at lysine 147 impairs GAP-mediated hydrolysis, leading to GTPase activation and increased signaling output. Intriguingly, the sites of monoubiquitination for H-Ras and K-Ras differ in HEK293T cells. Here, we further explore the role of site-specific monoubiquitination in isoform-specific regulation of Ras. We find that monoubiquitination of H-Ras at lysine 117 activates the protein by enhancing the intrinsic rate of nucleotide dissociation. These findings reveal that monoubiquitination activates H-Ras by a mechanism unique from K-Ras. Furthermore, the site at which monoubiquitination occurs dictates the mechanism

[1] Figures contributed by:
Rachael A. Baker: 3.1a-b, 3.1d, 3.2a-b, 3.3a-b, 3.3d, 3.4a-b, 3.4d
Atsuo T. Sasaki: 3.5
Emily M. Wilkerson: 3.1c, 3.2c, 3.3c, 3.4c

by which Ras is regulated. More broadly, these results identify a role for monoubiquitination in the regulation of isoform-specific Ras activity and signaling.

Introduction

The small GTPase Ras is a signaling switch that controls a number of cellular processes, including gene expression, cell differentiation, and programmed cell death (*168*). To control the activation of these essential pathways, Ras binds and hydrolyzes GTP. Ras is active when it is GTP-bound and becomes inactive when GTP is hydrolyzed to GDP (*127, 200*). The conformational changes that are associated with the most significant differences between the GDP- and GTP-bound states of Ras are primarily localized to the switch I and switch II regions. These switch regions are less conformationally dynamic in the GTP-bound state, and populate conformers critical for downstream effector recognition (*167*). On its own, Ras is not a very good enzyme, and requires regulators to respond to signals on an appropriate timescale. The primary regulators of Ras cycling are guanine nucleotide exchange factors (GEFs), which increase the rate of GDP dissociation (*201, 202*), and GTPase activating proteins (GAPs) that increase the rate of GTP hydrolysis (*200, 203*). These regulators also primarily interact with the switch regions of Ras. Due to its essential role in regulating cell growth and differentiation, Ras mutations are common in cancer. Over 30% of all human tumors contain an activating Ras mutation (*170, 204, 205*). Ras mutations are particularly prevalent in three of the four most common types of cancer, pancreatic (~90%), colon (~50%), and lung cancer (~30%) (*206*).

There are three distinct isoforms of Ras: K-Ras (two splice variants, K-Ras4A and K-Ras4B), H-Ras, and N-Ras. While these isoforms share a core domain that contains the enzymatic activity (>90% identical in the first 168 amino acids), there are differences in their c-terminal targeting domains. The c-terminal domain, or hypervariable region (HVR) is a short region of the protein (about 20 amino acids) that contains lipid modification sites. K-Ras is only farnesylated while N-Ras and H-Ras are also palmitoylated at one and two additional sites, respectively (*207-210*). The differential lipidation of the Ras isoforms is essential for membrane targeting, which is necessary for signaling. Post-translational modification of the Ras isoforms dictates their localization and trafficking (*211-214*). Inactive H-Ras has been identified in cholesterol rich microdomains within the plasma membrane (*214, 215*). When H-Ras is activated, it appears to move to more disordered plasma membrane regions (*216, 217*). In an opposing example, it is active, GTP-bound N-Ras that is found in cholesterol rich microdomains (*218*). K-Ras4B, on the other hand, is most often found outside of lipid rafts or sometimes in cholesterol-independent microdomains that are distinct from the lipid rafts where H-Ras is localized (*215, 219*).

Localization of Ras is important; there are distinct biological outcomes from downstream signaling pathway activation in different subcellular compartments (*220-222*). At steady state levels, H-Ras and N-Ras are localized at both the Golgi and plasma membrane, while K-Ras is only at the plasma membrane (*223*). There is evidence that these variations in compartmentalization modulate signaling outputs, in part due to differential localization of the effectors and regulators with which Ras interacts. Activation of Ras at the plasma membrane is very rapid, while activation at the Golgi

membrane is both delayed and sustained (*224*). Because K-Ras is not shuttled between multiple membranes, more protein is present at the plasma membrane, which makes K-Ras a more potent activator of the downstream effector Raf than H-Ras (*225*).

The differential localization of the Ras isoforms leads to differences in the biology regulated by these isoforms, including different patterns of mutation in various human diseases (*211-214*). K-Ras is the most commonly mutated isoform in human cancers (*206*). Activating mutations of H-Ras are common in bladder cancer, whereas mutations of K-Ras occur at high frequency in pancreatic cancers, and mutations of N-Ras occur frequently in acute myeloid leukemia (*211, 226, 227*). Moreover, activating mutations in K-Ras cause syndromes such as Noonan syndrome and cardio-facio-cutaneous syndrome (*122, 228, 229*). H-Ras mutations are common in Costello syndrome (*230*) and mutations in N-Ras lead to autoimmune lymphoproliferative syndrome (*211*). Furthermore, the Ras isoforms are not interconvertable. For example, oncogenic N-Ras mutations are not able to drive colon cancers that are commonly driven by the same activating mutation in K-Ras (*231*).

Mounting evidence indicates that post-translational modification by ubiquitination contributes to differentiation between Ras isoforms in a cell-line and isoform-specific manner. H-Ras and N-Ras have long been known to be substrates of ubiquitination (*173*). Ubiquitination of H-Ras results in the stabilization of its association with the endosomes and, therefore, a change in signaling output due to inhibited membrane recycling and increased protein accumulation in the endosome (*173*). However, it is not known whether the ubiquitinated and consequently endosome-localized Ras proteins continue to signal through downstream pathways (*226*). Evidence for a change in

signaling output consists of showing that a mutant of H-Ras that could not be ubiquitinated was more efficient at specifically recruiting Raf-1 and activating the ERK mitogen-activated protein kinase (*232*). Regulation of H-Ras by ubiquitination was observed in CHOK-1 cells and did not appear to depend on GTP loading (*173*). No K-Ras ubiquitination was observed in the same cell line (*173*). In the early studies of H-Ras and N-Ras ubiquitination, it was shown that the membrane anchoring domain of the protein was necessary and sufficient to direct monoubiquitination, but did not contain the site of monoubiquitination (*173*). For H-Ras to fail to be ubiquitinated, it must lack 8 surface exposed lysines including 5, 42, 88, 101, and 147 in the core GTPase domain (*173*). The modification appears to be conserved between *D. melanogaster* and human cell lines (*232, 233*) and represents a new way of controlling the spacial sorting of H-Ras (*234*).

We and others have more recently shown that ubiquitination also acts as a reversible mechanism of K-Ras regulation (*153, 235*). Monoubiquitination leads to activation of K-Ras through inhibition of GAP-mediated GTP hydrolysis as well as increased interactions with select downstream effectors (*153, 235*). We showed that this mechanism of Ras activation is specific to ubiquitination at K147 (*235*). In these studies, H-Ras was also identified as a substrate of monoubiquitination by mass spectrometry in HEK293T cells. K-Ras ubiquitination was not detected in CHOK-1 cells, consistent with previous studies of H-Ras ubiquitination (*153*).

There appears to be very divergent ways by which H-Ras and K-Ras are regulated by monoubiquitination. When H-Ras is monoubiquitinated in CHOK1 cells, the restricted ability of the protein to signal could be due to sequestration from particular

effectors (*211*). This is distinct from K-Ras ubiquitination, which did not appear to change the subcellular localization of the protein but did alter Ras activity. Sasaki et al. observed monoubiquitination in H-Ras as well as K-Ras in HEK293T cells. Furthermore, varying degrees of activation for H-Ras and K-Ras were observed, which suggests that the isoforms may be regulated by monoubiquitination through two distinct mechanisms (*153*). In support of this hypothesis, Sasaki et al. observed by mass spectrometry that H-Ras, but not K-Ras, could be monoubiquitinated at K117 and that there were minor instances of K-Ras ubiquitination at K104. Since we previously showed that the mechanism by which K-Ras is activated by monoubiquitination is site-specific, it is likely that ubiquitination at these other two lysines has, if any, an alternative mechanism of regulating Ras.

Here, we fully characterize two alternative sites of monoubiquitination, K104 (K-Ras) and K117 (H-Ras). We show that ubiquitination at K104 does not lead to a change in Ras activity, demonstrating that site specificity is key for the regulation of Ras by ubiquitination. Furthermore, we show that monoubiquitination at K117 upregulates H-Ras activity by increasing the intrinsic rate of nucleotide exchange, a mechanism distinct from K-Ras ubiquitination. Experiments in cell lysates exhibit a phenotype of monoubiquitinated H-Ras that is consistent with modification at K117. More broadly, our results describe an isoform-specific mechanism of Ras post-translational modification, which may play a role in isoform-dependent differences in Ras activation and signaling.

Results

Ras Monoubiquitinated at K104 Retains the Activity of Unmodified Ras

Monoubiquitination of K-Ras occurs primarily at K147 (*153*). However, monoubiquitination was also observed, albeit infrequently, at K104. We hypothesized that since K104 was a secondary site of K-Ras monoubiquitination and was not in the same proximity to the switch regions as K147, the enzymatic activity of Ras would not change upon modification at this site. To measure the effect of ubiquitination on Ras activity, we used our recently published chemical ubiquitination approach to modify the protein (*235*). Briefly, this method requires making a cysteine mutation at the site of Ras to be ubiquitinated (Ras^{K104C}) and at the c-terminus of Ubiquitin ($Ubiquitin^{G76C}$). These studies were done in a Ras^{C118S} background (hereafter, Ras) which does not alter the biochemical properties of Ras (*178*). We first measured the effect of mutation at K104 on intrinsic and regulator-mediated Ras activity. As shown in **Figure 3.1a**, while K104 is not adjacent to the switch regions, it is near the edge of the surface of Ras involved in binding to the GEF. Therefore, a change at position 104 could alter GEF-mediated dissociation. In **Figure 3.1b**, we show that the K104C mutation does not alter Ras thermal stability (T_m=51 ^oC). We also measured rates of intrinsic and GEF-mediated GDP dissociation in the presence and absence of a Ras K104C mutation. As seen in **Figure 3.1c**, Ras^{K104C} maintains the intrinsic rate of GDP dissociation of Ras ($0.13\pm0.02x10^{-3}$ s^{-1}). However, while the addition of the GEF, Sos, increases the rate of Ras GDP dissociation 10-fold ($1.3\pm0.1x10^{-3}$ s^{-1}), it only increases the rate of Ras^{K104C} dissociation 2-3 fold ($0.30\pm0.05x10^{-3}$ s^{-1}). In contrast, neither intrinsic nor GAP-mediated GTP hydrolysis was altered by the K104C mutation.

We chemically monoubiquitinated K104 on Ras ($mUbRas^{K104}$) and repeated the assays described above to determine if the effect of monoubiquitination on Ras at this position was different from a mutation at the same position (**Figure 3.2a**). As seen in **Figure 3.2b**, the thermal stability of $mUbRas^{K104}$ is similar that of Ras (T_m=50.8±0.4 ^oC), indicating that monoubiquitination does not thermally destabilize Ras. We next measured intrinsic and Sos-mediated rates of GDP dissociation and observed no difference between Ras and $mUbRas^{K104}$ (**Figure 3.2c**). It is interesting to note that, similar to modification at K147, monoubiquitination has a distinct effect on Ras activity from a mutation at the same site (*235*). Mutation of lysine to cysteine alters either intrinsic (K147) or Sos-mediated (K104) exchange, but mUbRas has the same rates of dissociation as unmodified Ras. While we have not yet measured intrinsic and GAP-mediated hydrolysis for $mUbRas^{K104}$, we do not expect any significant differences from unmodified Ras. Taken together, these data demonstrate that some sites on Ras can be monoubiquitinated without altering intrinsic or regulator-mediated activity.

Monoubiquitination at K117 Activates Ras by Increasing Guanine Nucleotide Dissociation

While K-Ras can be ubiquitinated at two distinct lysines *in vivo* (K147 and K104), only the more prevalent site of ubiquitination leads to a change in the active state of the protein. The mechanism of Ras regulation by monoubiquitination is site-specific, suggesting that monoubiquitination at K117 on H-Ras could have a distinct effect from monoubiquitination at K104 or K147. Lysine 117 is part of the NKxD motif of Ras-like GTPases that contributes to nucleotide affinity by forming interactions with the guanine nucleotide base (**Figure 3.3a**). Oncogenic mutations at K117 (K117R and K117N) are.

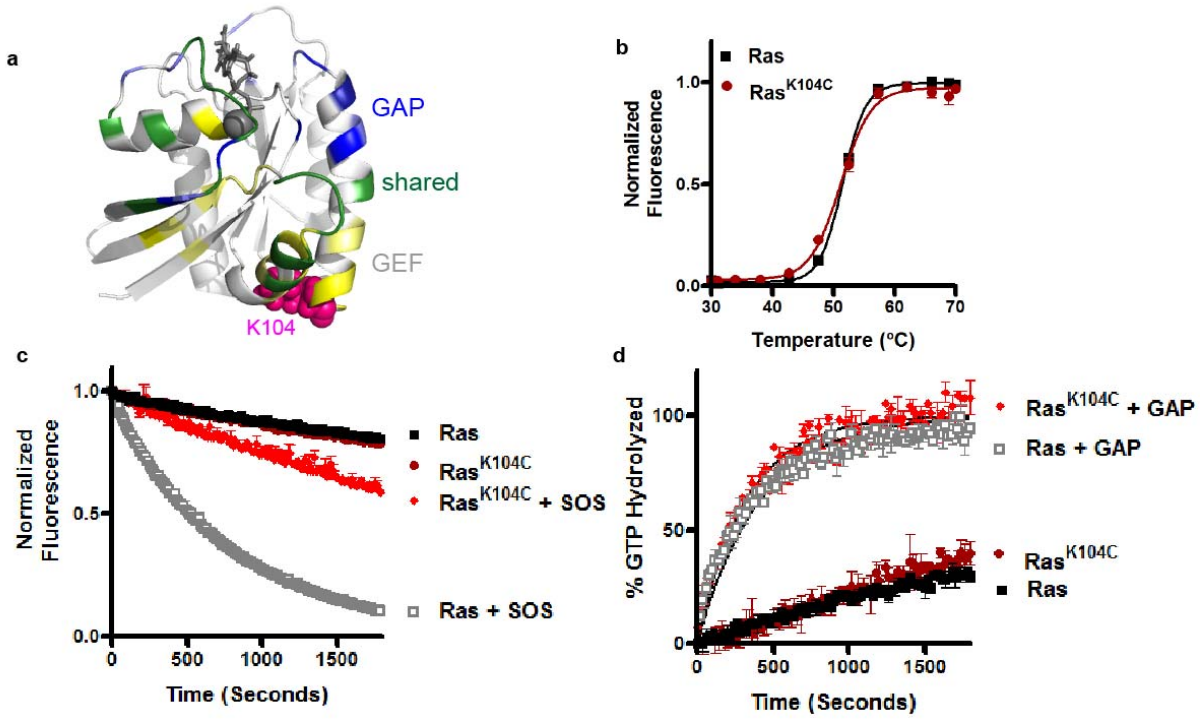

Figure 3.1. Mutation of Ras at Lysine 104. (**a**) Structure of Ras (PDB 1CRR) with nucleotide and Magnesium shown in grey and the K104 sidechain highlighted in magenta. Backbone residues in yellow make contact with GEF when it is bound, backbone residues in blue make contact with the GAP when it is bound, and backbone residues in green make contact with both the GEF and the GAP. (**b)** Thermal stability of Ras and Ras^{K104C} in the presence of GTPγS measured by ABD–F incorporation as a function of temperature. The data were normalized using the maximum fluorescence intensity. Results are the mean ± s.d. (n=4). (**c**) Intrinsic nucleotide dissociation rates for Ras and Ras^{K104C} loaded with MANT–GDP. Dissociation was monitored following the addition of unlabeled GDP by the decrease in fluorescence emission over time. Nucleotide dissociation was also measured in the presence of a 1:1 molar ratio of Ras to Sos^{cat}. Data were fit to an exponential dissociation curve, and the results are the mean ± s.d. (n=4). (**d**) Intrinsic single–turnover GTP hydrolysis for Ras and Ras^{K104C}. Hydrolysis was initiated by the addition of Mg^{2+} and monitored by the change in fluorescence of Flippi when bound to free phosphate. Single-turnover GTP hydrolysis of Ras was measured in the absence and presence of GAP-334 (intrinsic and 1:500 GAP:Ras). Data were converted to a phosphate concentration using a standard curve. The concentration of phosphate equal to 100% GTP hydrolyzed was determined in the presence of GAP. Results are the mean ± s.d. (n=6).

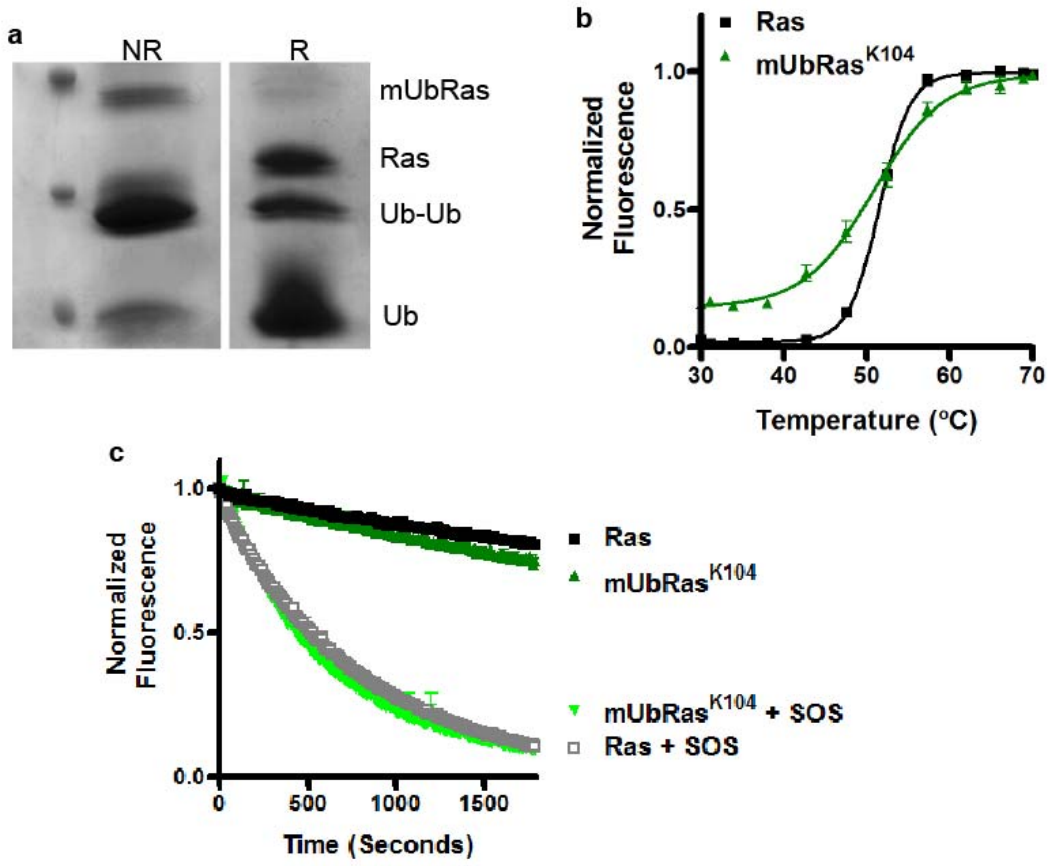

Figure 3.2. Monoubiquitination of Ras at Position 104. (**a**) SDS-PAGE gel under non-reducing (NR) and reducing (R) conditions showing the formation of monoubiquitinated Ras (mUbRas) and Ubiquitin-Ubiquitin dimers (Ub-Ub) after dialysis. (**b**) The thermal stability of Ras and mUbRas^{K104} measured by fQCR as described in Figure 3.1b. (**c**) Intrinsic and Sos-mediated GDP dissociation rates for Ras and mUbRas^{K104} measured as described in Figure 3.1c.

present in human cancers and developmental disorders (*183, 236, 237*). Mutations at K117 reduce Ras nucleotide binding affinity, resulting in increased protein activation due to increased rates of nucleotide exchange (*183, 236, 237*). Therefore, it is possible that ubiquitination at K117 could also lead to Ras activation through a similar mechanism.

We first mutated K117 to cysteine (Ras^{K117C}) and measured the effects of this substitution on thermal stability, nucleotide dissociation, and nucleotide hydrolysis. As seen in **Figure 3.3b**, mutation of K117 decreases the thermal stability of the protein by almost 10 degrees (T_m=43.8±0.9 ^oC). The intrinsic rate of GDP dissociation is also affected by the K117C mutation (11.0±0.4x10^{-1} s^{-1}), increasing 100-fold over the rate of Ras dissociation (**Figure 3.3c**). The observed increase in intrinsic hydrolysis is consistent with other mutations previously characterized at position 117 (*238*). We also measured the rate of GEF-mediated GDP dissociation in the presence of Sos. Although the intrinsic rate of GDP dissociation is at the limits of detection of our assay, making it difficult to quantify, Ras^{K117C} does appear to be GEF-responsive. Finally, we also measured the rate of intrinsic and GAP-mediated GTP hydrolysis. Due to protein instability, we were not able to perform single turnover assays, but instead measured multi-turnover rates of GTP hydrolysis in the presence of various GAP concentrations, as previously described (*238*). While Ras^{K117C} is still GAP responsive, it appears to be less sensitive to GAP-mediated regulation than Ras. The apparent binding affinity between the GAP and Ras decreases from 0.19±0.06 μM to 4.0±0.9 μM when Ras is mutated at position 117, leading to the observed decrease in sensitivity to GAP-mediated hydrolysis.

The significant change in the rate of intrinsic GDP dissociation when K117 is mutated is due to disruption of side chain interactions with the base of the guanine

nucleotide. Therefore, it is possible that monoubiquitination also activates the protein through a similar mechanism, since monoubiquitination captures the lysine side chain. We generated mUbRas^{K117} (**Figure 3.4a**) as previously described and measured changes in protein thermal stability and activity. As shown in **Figure 3.4b**, monoubiquitination of Ras at K117 does not thermally destabilize the protein, unlike a mutation at the same site (T_m=52.5±0.5 ^oC). However, the rate of intrinsic dissociation is 70-fold faster for mUbRas^{K117} compared to Ras (8.3±0.3x10^{-3} s^{-1}) (**Figure 3.4c**), very similar to mutation at the same position. This 70-fold increase in the rate of intrinsic hydrolysis would lead to increased activation *in vivo*. As seen in **Figure 3.4d**, when Ras is monoubiquitinated it is still GAP-responsive. The binding affinity between GAP and Ras decreases to 0.6±0.1 μM, a smaller change than caused by mutation at the same site. These data demonstrate that monoubiquitination at position 117 can directly alter Ras activity. The greatest change in activation will be from the increased intrinsic nucleotide dissociation rates, but the decreased sensitivity to GAP-mediated hydrolysis will also contribute. Taken together, these data suggest that *in vivo*, mUbRas^{K117} is likely more activated than unmodified Ras due to an increased rate of intrinsic nucleotide exchange.

Pull Downs with Cell Lysate Support Isoform Specificity of Ras Monoubiquitination

Our *in vitro* chemical ubiquitination system coupled with biochemical characterization studies indicate that monoubiquitination of H-Ras at K117 activates Ras through a mechanism distinct from monoubiquitination of K-Ras at K147. In the original study by Sasaki et al., mass spectrometry data identified ubiquitination of H-Ras at either K117 or K147 (*153*). Since the mechanisms of activation at these two lysines are distinct, we characterized H-Ras activity in cell lysates to determine if modification at

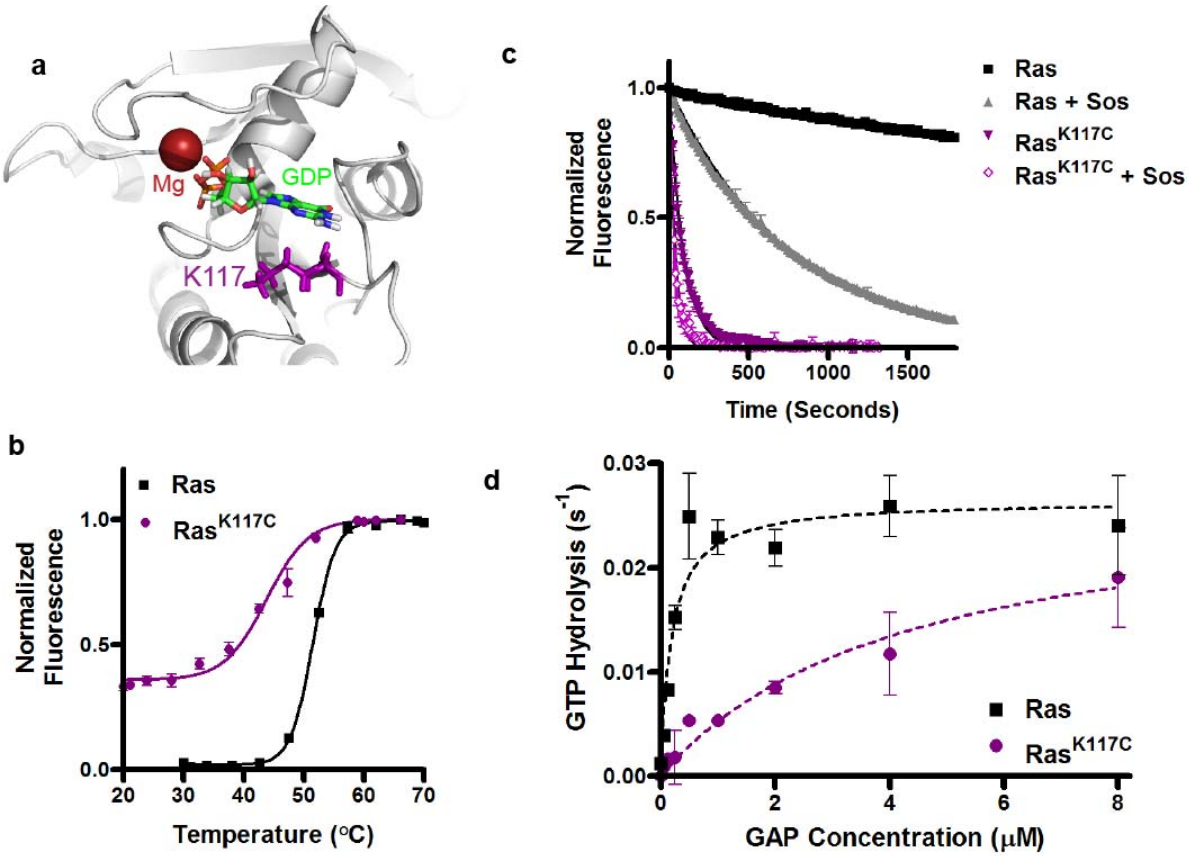

Figure 3.3. Mutation of Ras at Lysine 117. (**a**) Ribbon diagram highlighting the active site of Ras (PDB 1CRR) with nucleotide and Magnesium shown in various colors and red, respectively. The sidechain of K117 is highlighted in purple. (**b**) Thermal stability of Ras and Ras^{K117C} in the presence of GTPγS measured by ABD–F incorporation as a function of temperature. The data were normalized using the maximum fluorescence intensity. Results are the mean ± s.d. (n=4). (**c**) Intrinsic nucleotide dissociation rates for Ras and Ras^{K117C} loaded with MANT–GDP. Dissociation was monitored following the addition of unlabeled GDP by the decrease in fluorescence emission over time. Nucleotide dissociation was also measured in the presence of a 1:1 molar ratio of Ras to Sos^{cat}. Data were fit to an exponential dissociation curve, and the results are the mean ± s.d. (n=4). (**d**) Intrinsic and GAP-mediated multi-turnover hydrolysis measured by the change in fluorescence of Flippi when bound to free phosphate. Data were converted to a phosphate concentration using a standard curve. The rate of phosphate release was measured for 20 μM Ras in the presence of GAP concentrations from 0.0625 μM to 8 μM. Results are the mean ± s.d. (n=3). Data were fit to a one site binding model in GraphPad Prism to calculate the apparent binding affinity between GAP and Ras.

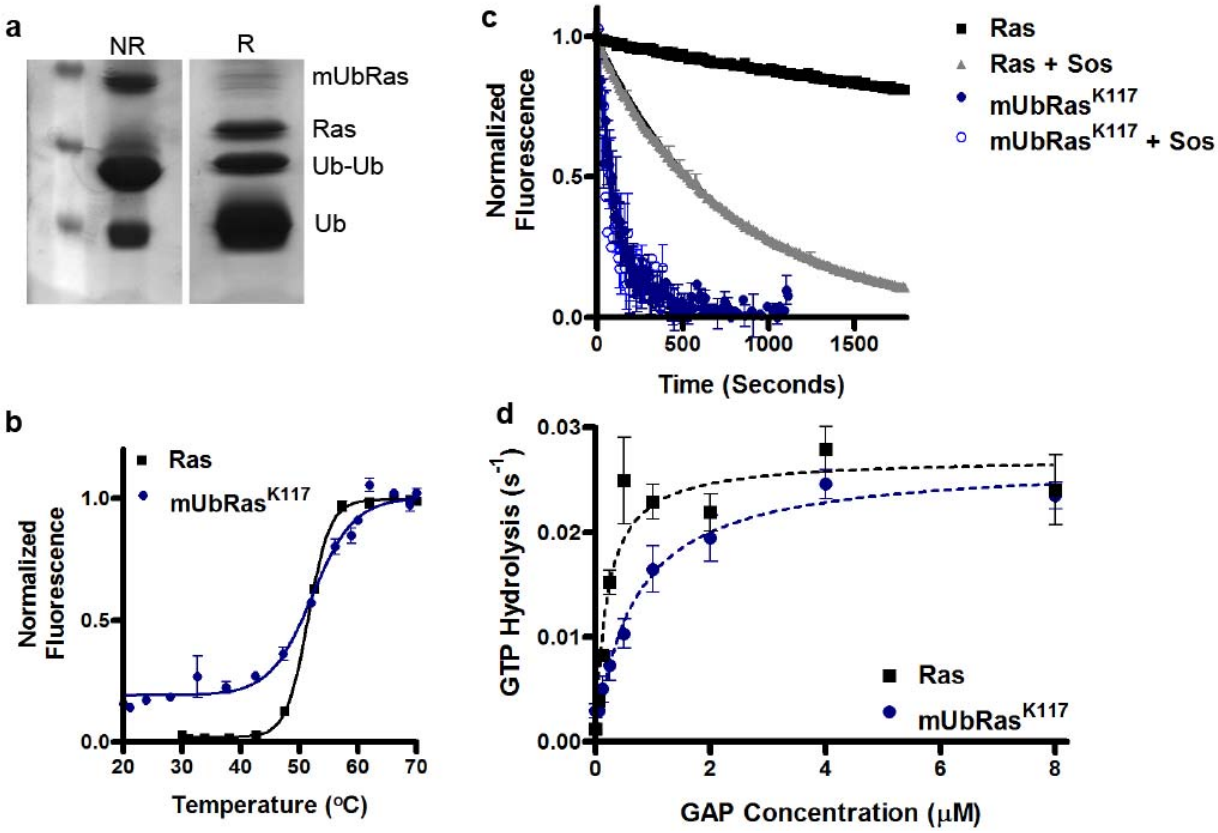

Figure 3.4. Monoubiquitination at Position 117. (**a**) SDS-PAGE gel under non-reducing (NR) and reducing (R) conditions showing the formation of monoubiquitinated Ras (mUbRas) and Ubiquitin-Ubiquitin dimers (Ub-Ub) after dialysis. (**b**) Thermal stability of Ras and mUbRas^{K117} in the presence of GTPγS measured as described for Figure 3.3b. (**c**) Intrinsic nucleotide dissociation rates for Ras and mUbRas^{K117} measured as described in in Figure 3.3c. (**d**) Intrinsic and GAP-mediated multi-turnover hydrolysis for Ras and mUbRas^{K117} measured by the change in fluorescence of Flippi when bound to free phosphate as described in Figure 3.3d.

one lysine dominated over the other. H-Ras was immunoprecipitated from HEK293T cells, and the populations of activated Ras and activated mUbRas were compared using Ras Binding Domain (RBD) pull downs. We measured the activation state of H-Ras as well as its sensitivity to GEF- and GAP-mediated regulation in a cellular reconstitution system. As seen in **Figure 3.5a**, GTP-bound mUbRas decreases more rapidly than unmodified GTP-bound H-Ras in the presence of excess GDP. The increased loss of activated mUbRas in the presence of GDP indicates that the rate of nucleotide exchange is faster when H-Ras is monoubiquitinated. This result is similar to what is observed in the Ras^{K117N} variant (**Figure 3.5a**), which is also known to increase the rate of intrinsic nucleotide exchange. *In vitro, a*n increased rate of nucleotide dissociation was observed when Ras was monoubiquitinated at K117, but not at K147. Together, these data indicate that the observed increased rate of dissociation is due to a population of the Ras that is monoubiquitinated at K117. We next determined whether the population of H-Ras that was monoubiquitinated *in vivo* was sensitive to GAP-mediated regulation. As seen in **Figure 3.5b**, the amount of both Ras and mUbRas decreases as the concentration of recombinant GAP added to the cell lysate increases. If H-Ras were monoubiquitinated at K147, it would not be sensitive to GAP-mediated hydrolysis as we previously showed with K-Ras (*235*). Finally, the amount of activated Ras was measured in the presence of increasing concentrations of Sos. While the population of GTP-bound unmodified Ras increases as the Sos concentration increases, mUbRas is either not sensitive to Sos-mediated exchange or is already fully activated. Taken together, these data indicate that H-Ras is primarily monoubiquitinated at K117 in HEK293T cells, which leads to its activation through a mechanism distinct from K-Ras ubiquitination.

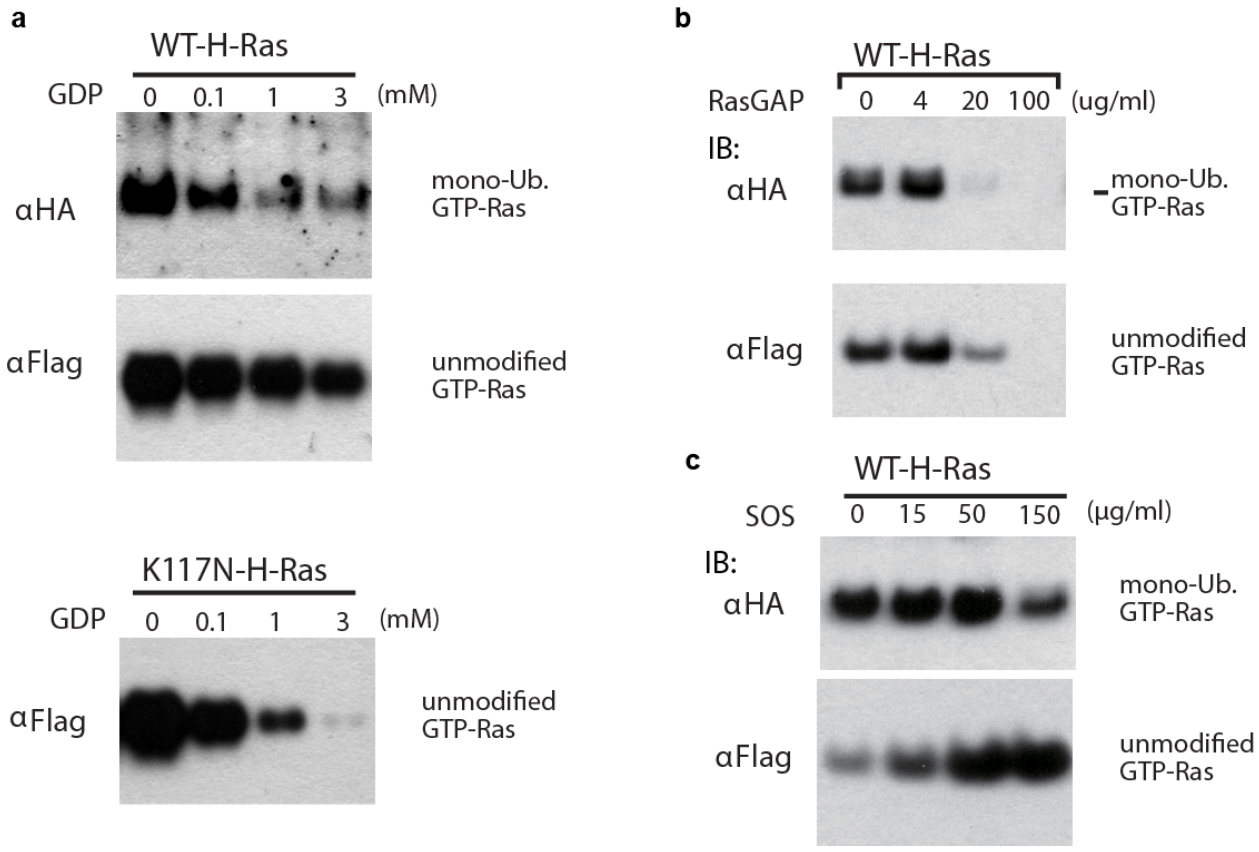

Figure 3.5. Assays of H-Ras in Cell Lysate Indicate H-Ras is Primarily Modified at K117**.** (**a**) Immunoblotting of GTP-bound Ras, GTP-bound mUbRas, and GTP-bound Ras^{K117N} in cell extract in the presence of increasing concentrations of GDP. Anti–Flag and anti–HA antibodies reveal the relative fraction of total Ras and mUbRas, respectively, for all assays. (**b)** Immunoblotting of GTP-bound Ras and GTP-bound mUbRas in cell extract in the presence of increasing concentrations of recombinant RasGAP. (**c**) Immunoblotting of GTP-bound Ras, GTP-bound mUbRas, and GTP-bound Ras^{K117N} in cell extract in the presence of increasing concentrations of recombinant Sos.

Discussion

It was established recently that both K-Ras and H-Ras are substrates for monoubiquitination in HEK293T cells and the sites of monoubiquitination were identified as K104, K117, and K147. We established that the mechanism by which monoubiquitination of K-Ras at K147 promotes Ras activation is by rendering it insensitive to GAP-mediated GTP hydrolysis. Here, we have shown that monoubiquitination of the second minor site on K-Ras, K104, does not alter the intrinsic or regulator-mediated activity of Ras. Furthermore, H-Ras is primarily monoubiquitinated on K117, a site distinct from K-Ras ubiquitination, and monoubiquitination at K117 activates H-Ras by increasing the intrinsic rate of nucleotide dissociation. Monoubiquitination of K-Ras also increases the affinity of the protein for select downstream effectors (*153*). It remains to be seen if monoubiquitination of H-Ras at K117 also alters interactions with downstream effectors, perhaps in a manner distinct from K-Ras ubiquitination.

Isoform specific ubiquitination may represent a new mechanism of dictating functional hierarchy of Ras isoforms (*173*). It is likely that the differences in Ras ubiquitination, both in the studies described here as well as the previous studies of H-Ras ubiquitination, are due to differences in E3 ubiquitin ligase and deubiquitinating enzyme expression or localization (*153*). It is these differences, as well as the differences in Ras localization, that contribute to differential modification of Ras isoforms by monoubiquitination, leading to translocation or activation of a particular Ras isoform in a particular tissue.

It is interesting to note that the secondary site of monoubiquitination (K104) does not alter Ras activity. Because both of the primary sites of ubiquitination (K117 and K147) alter Ras activity, these data suggest that the change in Ras activity are an important component for how monoubiquitination is used to regulate Ras in HEK293T cells. However, the identification of a site on Ras where monoubiquitination does not alter activity is also consistent with data from previous studies of Ras ubiquitination in CHOK-1 cells. In these previous studies, trafficking to the endosome rather than Ras activation was the end result of H-Ras monoubiquitination. The site of monoubiquitination in CHOK-1 cells was determined to be one of 8 surface exposed lysines, which include lysines 5, 42, 88, 101, and 147. Consistent with ubiquitination at K104, we have already shown that ubiquitination at K88 or K101 does not impact GAP-mediated regulation of GTP hydrolysis (*235*). Therefore, it is likely that in this case, ubiquitination of H-Ras is primarily a trafficking signal.

Lysine 104 is also a known site of other post-translational modifications; it was recently shown that Ras can be acetylated at this position (*239*). Molecular dynamics simulations suggest that acetylation at this position alters the conformational stability of switch II due to a perturbation in electrostatic interactions, which is known to be important for GEF-mediated hydrolysis (*239*). Furthermore, a conservative mutation at the same position (K140R) did not significantly impact GEF-mediated dissociation (*239*). We showed that a lysine to cysteine mutation, which also alters the charge at position 104, similarly impaired GEF-mediated dissociation. However, monoubiquitination at K104 did not alter GEF-mediated dissociation, likely because the modification does not alter charge at this position in the same manner as a mutation. While the extent to which

both acetylation and monoubiquitination of K104 contribute to Ras-mediated regulation *in vivo* remains to be seen, it has not escaped our notice that monoubiquitination at K104, which does not affect Ras activation, would exclude the possibility of acetylation at the same position. Therefore, there may be a role for K104 monoubiquitination in protection of Ras from other post-translational modifications.

Taken together, these data have implications for studying the regulation of Ras by monoubiquitination. This post-translational modification appears to be used in a site-specific, isoform-specific, and cell-line specific manner. This means that while monoubiquitination likely represents an essential component of Ras regulation *in vivo*, its effect on the protein is dependent on the lysine which is modified. Our data indicate that the site of modification by monoubiquitination is essential to understanding the role this modification plays in the regulation of Ras *in vivo*. Thus, it is crucial not only to identify when Ras is ubiquitinated, but to determine the primary sites of monoubiquitination. It is possible that monoubiquitination is used in different ways in different tissue types or in various Ras-driven cancers. Monoubiquitination of K-Ras at K147 has already been shown to play a role in Ras-driven tumorigenesis in a mouse model of cancer (*153*). Ras regulates many key pathways within the cell, and its regulation of these diverse pathways is achieved through controlling spatial and temporal localization as well as through the use of distinct Ras isoforms. Monoubiquitination represents a new mechanism through which isoform-dependent Ras activity and signaling is distinguished.

Methods

Protein Expression and Purification

The Ras domains (1–166) of H–Ras^{C118S}, H–Ras^{C118SK104C}, H–Ras^{C118SK117C} were expressed in the pQlinkH vector (Addgene) with a histidine purification tag in *E. coli* BL21 (DE3) RIPL cells (Stratagene: La Jolla, CA). Proteins were purified following standard Qiagen nickel affinity purification procedures. The His tag was cleaved overnight with Tobacco Etch Virus (TEV). Ras proteins were further purified by removal of uncleaved protein using Ni–NTA agarose beads (Qiagen). The final product was judged > 95% pure by SDS–PAGE. Proteins were stored in 20 mM HEPES, pH 8.0, 50 mM NaCl, 500 μM TCEP, 50 μM GDP and 5 mM $MgCl_2$.

The standard Ras protocol was revised for purification of Ras^{C118SK117C} to accommodate for the instability of the protein as follows. The cells were lysed by homogenization at 2000 psi (Nano DeBEE Laboratory Bench Homogenizer) and pelleted by centrifugation at 4°C (Beckman Coulter J2-HS Centrifuge). Proteins were purified using 2 mL of Ni-NTA (Qiagen) beads in a 15 mL conical equilibrated with Ras Buffer A (50 mM HEPES pH 7.75, 50 mM NaCl, 5 mM $MgCl_2$, 80 mM Imidazole, 500 μM GDP, 500μM TCEP, and 10% glycerol) at 4°C. The cell lysate was allowed to bind to the beads for 20 minutes. The beads were washed in succession with two washes with Buffer A, one wash with Buffer B (50 mM HEPES pH 7.75, 1 M NaCl, 5 mM $MgCl_2$, 80 mM Imidazole, 500 μM GDP, 500μM TCEP, and 10% glycerol), and one more wash with Buffer A. Then, TEV protein was added in Ras Buffer D (20 mM HEPES pH 7.75, 50 mM NaCl, 5 mM $MgCl_2$, 500 μM GDP, 500 μM TCEP, and 10% glycerol) to cleave the

histidine tag overnight. The protein was determined pure by an SDS-PAGE gel and stored at 4^{o}C to be used fresh within 3 days.

Sos^{cat} (John Kuriyan; University of California, Berkeley) was purified as previously described(*190*). The catalytic domain of p120GAP (GAP–334) (*138*) was expressed in pQlinkH and purified as described for Ras. Purified proteins were stored in 20 mM HEPES, pH 8.0, 50 mM NaCl and 500 μM TCEP. Full length Ubiquitin^{G76C} was expressed in the pQlinkH vector system and purified as described for Ras. Proteins were stored in 20 mM HEPES, pH 8.0, 50 mM NaCl and 500 μM TCEP.

Chemical Ubiquitination

The chemical ligation strategy used to link H-Ras^{K104C} and H-Ras^{K117C} was performed as described in Baker et al. (*235*). Briefly, a ten–fold excess of Ubiquitin^{G76C} was added to Ras^{K147C} and dialyzed into 20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 50 μM GDP at 4 ^{o}C overnight. The amount of disulfide complex formation was determined by non–reducing SDS–PAGE and considered complete by the absence of unmodified Ras. The protocol was adapted for ubiquitination of Ras^{K117C} with the addition of 10 % glycerol.

Thermal Stability of Ras

The fast quantitative cysteine reactivity (fQCR) method (*182*) was employed to measure changes in Ras thermal stability. Briefly, 2 μM Ras^{K104C}, mUbRas^{K104}, Ras^{K117C}, or mUbRas^{K117} was incubated with 1 mM ***4–fluoro–7–aminosulfonylbenzofurazan*** (ABD–F, Anaspec) at pH 7.0 in the presence of 20 μM GDP and 2 mM $MgCl_2$ at the desired temperature for three minutes. Fluorescent intensity was measured on a PHERAstar plate reader (BMG Labtech).

MANT-Nucleotide Dissociation Assay

For the Ras^{K104C} and $mUbRas^{K104}$variants, the assay was performed as previously published in Baker et al. (*235*). The assay was adapted in the following way for Ras^{K117C} and $mUbRas^{K117}$. Protein was exchanged into Mant Nucleotide Exchange Buffer (50 mM Tris pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 100 μM DPTA). To a cuvette, 0.5 μM Mant-GDP was added to 1 μM of protein. The protein was determined to be fully loaded when the fluorescence of the spectra no longer increased, approximately 500s. Unlabeled GDP (final concentration of 100 μM) was added to initiate dissociation. For Sos-mediated measurements, 1:1 molar ratio of SOS^{CAT}:H-Ras was also added. MANT–GDP dissociation was measured as a change in fluorescence intensity over time (excitation: 360 nm, emission: 440 nm) (LS50B Perkin–Elmer Luminescence Spectrometer). Fluorescence data were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a one–phase exponential decay curve. Results are the mean ± s.d. (n=4).

Single Turnover Nucleotide Hydrolysis

Single turnover nucleotide hydrolysis assays were performed as described in Baker et al. (*235*). The ratio of fluorescence emission was measured at 480 nm and 530 nm with an excitation of 435 nm on a SpectraMax M5 (Molecular Devices). Fluorescence ratios were converted to phosphate concentrations using a standard curve. Hydrolysis curves were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a one–phase exponential association curve. Results are the mean ± s.d. (n=6).

Multiple Turnover Nucleotide Hydrolysis

Due to limitations with the stability of Ras^{K117C} and $mUbRas^{K117}$, we measured rates of hydrolysis in a multiple turnover nucleotide hydrolysis assay. We also repeated

the assay under the same conditions with Ras^{C118S} for comparison. The phosphate binding protein Flippi 5U (Addgene) was used to detect inorganic phosphate released upon GTP hydrolysis (*185*). Ras was extensively exchanged into phosphate free buffer with 5mM EDTA and no magnesium. For intrinsic hydrolysis, 20 μM Ras was mixed with 60 μM GTP and hydrolysis was initiated by the addition of Magnesium. For GAP-mediated hydrolysis, a range of GAP concentrations from 0.0625 μM to 8 μM were also added to the reaction. Fluorescence ratios were converted to a phosphate concentration using a standard curve. Data from the first 10 minutes of hydrolysis were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a linear regression and the results were plotted as a function of GAP concentration. Results are the mean ± s.d. (n=3).

Mapping GEF and GAP Binding Sites on Ras

Residues were considered to be part of the interaction surface if they were less than 6 Angstroms apart. Interactions surfaces were determined from the crystals structures of Ras with Sos (PDB 1BKD) and Ras with RasGAP (PDB 1WQ1). See Appendix A for a list of all residues that interact and the distance between the interactions.

Assays in Cell Lysate

Ras activation was measured as described previously (*153*). Flag–His–H–Ras or the mutant Flag–His–H–Ras^{K117N} were co–expressed with HA–Ubiquitin in HEK293T cells. The cells were rinsed with cold PBS and lysed with Buffer A (0.5% NP–40, 40 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 1 mM DTT, 1 μg ml^{-1} leupeptin, 2 μg ml^{-1} aprotinin, 1 μg ml^{-1} pepstatin A, 100 μM AEBSF, Halt phosphatase inhibitor cocktail (Thermo Scientific), 10 mM iodoacetamide (IAA) and 5 mM *N*–ethylmaleimide

(NEM)). The soluble fraction from the cell lysates were isolated by centrifugation at 13,000 rpm for 10 min, split, and subjected to anti–Flag agarose immunoprecipitation or incubated with 10 μg of GSH–Sepharose bound GST–Raf–RBD in the presence of 1 mg ml^{-1} BSA for 30 min as described previously. The immunoprecipitated proteins were washed three times with Buffer A and eluted by the addition of 8 M urea. To ensure detection of mUbRas, a secondary purification on Co^{2+} Talon metal affinity chromatography beads (Clontech) was performed. Flag–His–Ras was eluted with sample buffer containing 50 mM EDTA. For the Sos and GAP sensitivity assays, bacterially produced protein was incubated with the cell lysate for 20 min at room temperature and subjected to analysis using GST–Raf–RBD.

CHAPTER IV

THE HELICAL DOMAIN INFLUENCES THE ENZYMATIC ACTIVITY OF THE YEAST G ALPHA PROTEIN[1]

Heterotrimeric G proteins transmit signals from cell surface receptors to intracellular effector proteins. Whereas G protein βγ subunits are largely interchangeable, the Ras-like domain of the Gα subunit confers effector binding specificity. It has recently become evident that the α-helical domain of Gα subunits also plays a crucial role in G protein-mediated signaling. In particular, biophysical characterization of Gα proteins has revealed large scale movements in the α-helical domain during receptor-mediated activation. Furthermore, the dynamic properties of the helices within the α-helical domain have been shown to influence Gα enzymatic activity. Here, we examine the function of the G protein α subunit in yeast, Gpa1. By site-directed mutagenesis we show that the α-helical domain dictates the thermal stability and intrinsic activity of the protein. Apart from the canonical Ras-like and α-helical domains, Gpa1 also contains a ubiquitination domain, a unique 109 amino acid insert within the α-helical domain, that is post-translationally modified by phosphorylation and ubiquitination. We show that the ubiquitination domain, while known to be important for Gpa1 trafficking to the endosome, does not impact catalytic function. These data suggest that while the α-helical

[1] All figures contributed by Rachael Baker

domain modulates Gα activity, the ubiquitination domain promotes Gpa1 trafficking without affecting enzymatic activity. More broadly, these data support recent evidence of the importance of the α-helical domain in Gα signaling but also establish a distinct function for the ubiquitination domain *in vivo.*

Introduction

Gα proteins are enzymatic switches that are part of a multi-component signaling complex at the cell membrane (*240*). The complex typically consists of a seven transmembrane spanning G protein coupled receptor (GPCR), a guanine nucleotide binding protein (Gα) and an associated dimer consisting of β and γ subunits (Gβγ) (*241*). Signaling is turned on and off based on the nucleotide-bound state of the Gα protein. When the Gα is GDP-bound, Gβγ is sequestered and signaling pathways are off (*145*). When the Gα releases GDP and binds GTP in response to GPCR activation, Gβγ dissociates and the signaling pathways are turned on (*242*). For the pathway to be turned off, the Gα must hydrolyze GTP back to GDP. GTP hydrolysis is facilitated by regulators of G protein signaling (RGS proteins) (*145, 243, 244*).

Small G proteins are fully functioning enzymes with only the Ras-like domain. Large Gα proteins contain a Ras-like domain as well as an independently folded α-helical domain (*116, 245*). Within this group of proteins there is a well-established role for the Ras-like domain in specifying interactions with Gβγ, effectors and RGS proteins (*145*). Furthermore, crystal structures of Gα showed that nucleotide binding was mediated by residues in the Ras-like domain (*144*). Therefore, historically, the Ras-like domain of Gα proteins was assumed to be responsible for guanine nucleotide binding and hydrolysis

activity (*116*). However, recent evidence has shown that the α-helical domain is also important for modulating signaling (*246*). For example, crystal structure determinations have revealed differences in the α-helical domain of $G\alpha_i$ when bound to GDP and GTPγS (*132*). Similarly, structure-based analysis of Gαβγ bound to a GPCR revealed that upon receptor activation, the Ras-like and α-helical domains separate, which exposes the nucleotide binding pocket (*141*). Significant α-helical domain displacement was also observed using electron-electron resonance spectroscopy (*247*) and electron microscopy (*141*). Finally, the Gα protein in *A. thaliana,* AtGpa1, requires no receptor for activation, displaying a rate of nucleotide exchange two-orders of magnitude faster than its mammalian counterparts (*248, 249*). Crystal structure analysis and molecular dynamic simulations revealed that AtGpa1 possesses a more dynamic and flexible α-helical domain than that of other Gα proteins (*249, 250*). Furthermore, the difference in the dynamics of two helices within the α-helical domain (the A/B helix) accounts for the change in observed nucleotide exchange rate (*249*). Together, these data suggest that the α-helical domain is a more important component of Gα-mediated signaling than was previously assumed.

Another potential role for the α-helical domain is in proper protein localization. Gα subunits are known to exist at the plasma membrane (*251-255*), the Golgi (*256*), endoplasmic reticulum (*251*), endosomes (*257*), and the nucleus (*258*). While it is not clear if the Gα signals from all these locations, investigators have observed stimulus-dependent movement of Gα and Gβγ to various endomembrane compartments (*259*). Direct evidence of intracellular signaling comes from work in *S. cerevisiae*, where the Gα Gpa1 was shown to transmit a signal from the endosome, and to do so by binding and

activating the phosphatidylinositol-3-kinase (PI3K) Vps15/Vps34 (*260, 261*). More recently, work by Irannejad et al. demonstrated that mammalian $G\alpha_s$ is also present at, and can signal from, endosomes (*262*).

While there are several possible functions for the α-helical domain, there are few examples of known binding partners. The α-helical domains of some Gα proteins are known to be phosphorylated or ubiquitinated. Thus at a minimum, the α-helical domain participates in binding to the appropriate ubiquitin ligases and protein kinases (as well as phosphatases and deubiquitinating enzymes) (*263-265*). Furthermore, it is known that α-helical domain contacts are formed between particular Gα proteins and their cognate RGS proteins (*266-272*).

Here we focus on the α-helical domain of the yeast G protein Gpa1 (**Figure 4.1**). Due to the complex nature of G protein mediated signaling in mammalian systems, model systems like *S. cerevisiae* are a valuable resource for studying and understanding these pathways. The key features of yeast that make it a useful model organism are (a) the ability to perform genetic manipulations including gene replacement (b) the ability to exist stably as a haploid or diploid, (c) the availability of powerful genetic tools such as libraries of knockouts or tagged proteins (*273, 274*), and (d) the strong similarities between yeast and mammalian signaling pathways (*275*). Indeed the founding members of many protein families were discovered in yeast, including the first regulator of G protein signaling (*276*).

While the Ras-like and α-helical domains of Gpa1 are highly conserved with their mammalian counterparts, Gpa1 also possesses a unique 109 amino acid insert within the α-helical domain (*277*). This insert contains the known sites of phosphorylation,

monoubiquitination, and polyubiquitination; both forms of ubiquitination occur at the same site in the ubiquitination domain, K165 (*278-280*). Given that the α-helical domain of Gα proteins modulates Gα activity it is therefore possible that the ubiquitination domain, or ubiquitination thereof, could influence Gpa1 activity. Here we show that mutations within the α-helical domain of Gpa1 influence the activity and thermal stability of the protein. In contrast, complete deletion of the ubiquitination domain is without consequence for GTP binding or hydrolysis. Thus the ubiquitination domain does not contribute to the overall enzymatic activity of Gpa1. These findings suggest that the G protein in yeast has acquired this unique domain to regulates delivery to the endosome, vacuole, or proteasome.

Results

Optimizing Gpa1 Growth and Purification for Biophysical Studies

While Gpa1 has been extensively characterized using genetics and molecular biology, few studies have been done on the biophysical properties of this substrate. A better knowledge of Gpa1 structure and biophysical properties would allow us to generate hypotheses based on structural and biochemical analysis of Gpa1 *in vitro* and test their importance on Gα signaling and regulation *in vivo*. In particular, we are interested in characterizing the role of the α-helical domain and ubiquitination in Gα-mediated signaling. Biophysical analysis and chemical ubiquitination approaches require large quantities of protein. Generating high quantities of pure Gpa1 in *E.coli* to complete these studies has been a barrier to progress in this area. To overcome this problem, we used a

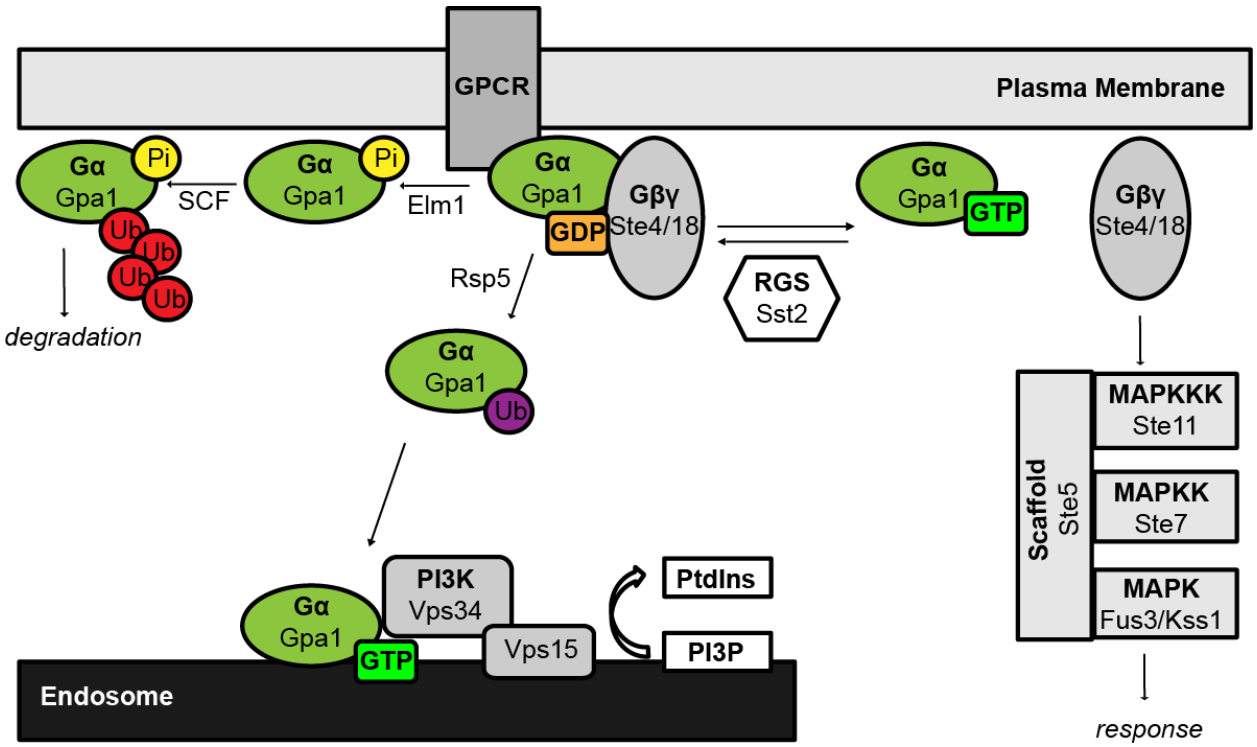

Figure 4.1. Gpa1 Activity at the Plasma Membrane and Endosome. Yeast mating response pathway is controlled by a G protein coupled system. Pheromone binding to the receptor initiates exchange of GTP for GDP on Gpa1 and dissociation of Gβγ. Gβγ activates a canonical MAP Kinase cascade. Gpa1 can also be monoubiquitinated and trafficked to the endosome, where it activates PI3K. When phosphorylated, Gpa1 is also a substrate of polyubiquitination.

number of approaches to optimize Gpa1 expression and purification. The two primary problems with the standard purification were low protein yield and impurities in the final product, which required additional purification steps. The low protein yield arose both from inefficient expression and the stability of the final product. The impurities in the purification were likely due both to the inefficient expression of Gpa1 as well as the purification process. Because Gpa1 is a yeast protein which we expressed in *E. coli,* the first step we took to overcome the inefficient expression of Gpa1 was to asses rare codon usage (*281*). We found that 46% of the codons in Gpa1 were rare *E. coli* codons, which can significantly reduce the efficiency of expression (**Figure 4.2a**) (*281*). To increase the expression of the protein in *E. coli,* we synthesized a Gpa1 construct that only uses common *E. coli* codons (**Figure 4.2b-c**). We found that by removing rare codons, we increased the final protein yield 12-15 fold. It is of note that we have also had success increasing our efficiency in expression through the use of auto-induction media (*282*).

To increase the purity and stability of our final protein product, we optimized the construct length, buffers for purification, and purification method. We chose to use a modified Gpa1 construct, $Gpa1^{\Delta N}$, which lacks the first 38 amino acids of the N-terminus. This construct was designed based on the crystal structure of $G\alpha_i$, which shows that the analogous stretch of N-terminal amino acids are unstructured (*144*). While removing these amino acids did not have a significant impact on protein expression levels, it did improve the lifetime and stability of the protein once purified, as assessed by decreased degradation and aggregation over time. We optimized the buffers for purification by using a simple phosphate buffer system with high concentrations of TCEP (reducing agent) and GDP. To speed up the process of purification and reduce expenses, Gpa1 was

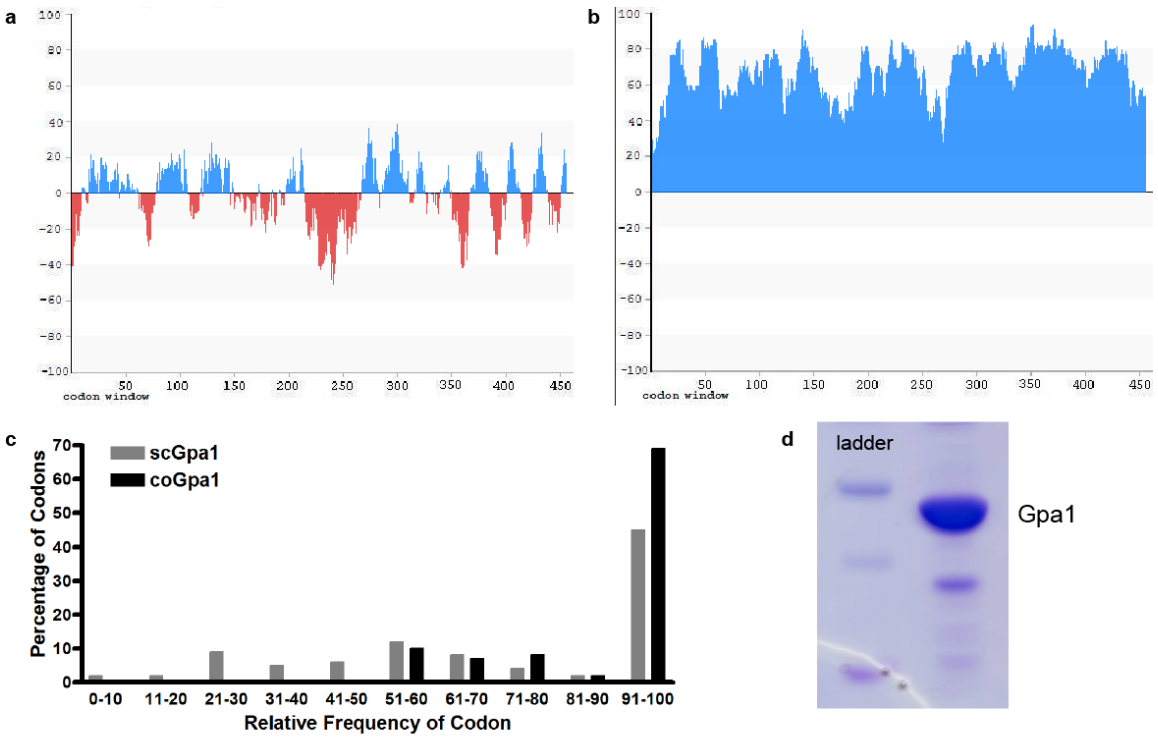

Figure 4.2. Optimization and Purification of Gpa1. (**a**) Instances of rare codons in the sequence of yeast Gpa1 (scGpa1). The x-axis is the codon window, a sliding window that averages 18 codons of the protein at a time. The y-axis is a measure of the frequency of the codon usage in *E. coli.* The higher the value, the more frequently the codon is used. Negative values (shown in red), represent instances of rare codons. (**b**) Instances of rare codons in the sequence of Gpa1 codon optimized for expression in *E. coli* (coGpa1). The x and y axes are the same as described in part (**a**) (**c**) Quantification of codons and their frequency of use for scGpa1 and coGpa1. (**d**) SDS-PAGE gel showing the result of the optimized purification of coGpa1$^{\Delta N}$.

purified using a batch purification protocol. Finally, to improve both purity and protein stability, we cloned Gpa1 into the expression vector pQlinkH, which has a Tobacco Etch Virus (TEV)-cleavable His tag. Instead of eluting the protein from the nickel beads at the end of purification, which would require imidazole as well as overnight dialysis to remove the imidazole, we added His-TEV to the beads for overnight cleavage. While this method decreased the overall efficiency of the purification (only about half of the Gpa1 is cleaved from the beads), this loss in efficiency is compensated for by obtaining pure protein that does not require further dialysis or purification (**Figure 4.2d**). Overall, our approach increased pure Gpa1 yield from 1.5 mg/L to 12 mg/L, which is sufficient for biochemical and structural studies.

The Insert Domain of Gpa1 Does Not Alter Gpa1 Activity

The site of ubiquitination on Gpa1 is on an insert, the ubiquitination domain, that is not found in other Gα proteins (*279*). Based on sequence alignment with Gα proteins of the same subtype, the ubiquitination domain of Gpa1 is likely at the end of the A/B helix in the α-helical domain, as highlighted in **Figure 4.3a**. The A/B helix is the region of the α-helical domain whose dynamics were previously shown to be important for modulating AtGpa1 activity (*250*). Therefore, it is possible that the ubiquitination domain could play a role in regulating the activity of Gpa1. To test this hypothesis, we removed the ubiquitination domain ($Gpa1^{\Delta N \Delta UD}$) determined whether the loss of this domain affected Gpa1 thermal stability and enzymatic activity. As shown in **Figure 4.3b**, removing the ubiquitination domain did not alter Gpa1 thermal stability, despite the loss of 109 amino acids. Since the structure of Gpa1 has not been solved, it is not known if the ubiquitination domain contains secondary structure or is unstructured. We

therefore used circular dichroism (CD) to determine if any secondary structure content was lost when the ubiquitination domain was removed. The CD signature of Gpa1 was the same in presence and absence of the ubiquitination domain (**Figure 4.3c**), suggesting that the insert domain does not significantly contribute to the secondary structure content of the protein and is likely unstructured.

While the thermal stability and secondary structure content of Gpa1 are the same in the absence of the ubiquitination domain, it is possible that the ubiquitination domain modulates Gpa1 activity due to its location adjacent to the A/B helix. Therefore, we measured the ability of Gpa1 to bind, exchange, and hydrolyze nucleotide in the presence and absence of the ubiquitination domain. As shown in **Figure 4.3d**, the rate of nucleotide dissociation was not altered by the absence of the ubiquitination domain. Furthermore, similar rates of nucleotide binding were obtained in the presence and absence of the ubiquitination domain (**Figure 4.3e**). The assay used to measure nucleotide hydrolysis takes advantage of a nucleotide-dependent change in the intrinsic fluorescence of Gpa1 (*283*). Although the overall intrinsic fluorescence of Gpa1 was reduced upon loss of the insert, the fluorescence changes associated with nucleotide dependent binding was retained. Finally, the observed rate of GTP hydrolysis in $Gpa1^{\Delta N\Delta UD}$ was identical to full length $Gpa1^{\Delta N}$ (**Figure 4.3f**). Together, these data suggest that the insert has evolved so its presence does not alter the enzymatic activity of Gpa1. Rather, we speculate that it functions as a site of post-translational modification for the purpose of trafficking to the vacuole (by monoubiquitination) or the proteasome (by phosphorylation and polyubiquitination).

Does Ubiquitination Alter Gpa1 Activity?

Because the ubiquitination domain does not influence Gpa1 activity, it is likely that monoubiquitination does not alter Gpa1 activity. To address this question, it is necessary to obtain enough ubiquitinated protein for biochemical analyses. We have already shown we can overcome challenges in obtaining adequate quantities of pure Gpa1. We also have the ability to generate large quantities of monoubiquitinated substrate using our previously developed chemical ubiquitination approach (*235*). This chemical ubiquitination approach requires mutation of the ubiquitination site on Gpa1 to a cysteine (K165C) and the use of Ubiquitin with a cysteine at the c-terminus (Ub^{G76C}). Gpa1 has seven native cysteines: two in the Ras-like domain, four in the α-helical domain, and one in the ubiquitination domain. Our first attempts at ubiquitination employed $Gpa1^{\Delta N}$ with a single background cysteine mutation (C208S). We chose to mutate cysteine 208 because it is the only cysteine in the ubiquitination domain. Since our previous data suggested that the ubiquitination domain lacks significant secondary structure, this cysteine is likely to be reactive. We first used the published ubiquitination reaction conditions, which include overnight dialysis of the protein in the absence of reducing agent at pH 8.5 (*235*). Chemical ubiquitination is performed at this pH because cysteines are most reactive above pH 8.0, where they are predominately in the thiolate species. Unfortunately, the pI of $Gpa1^{\Delta N}$ is close to 8.5, which leads to its destabilization under standard chemical ubiquitination conditions. We tried a number of approaches to ubiquitinate Gpa1 at a lower pH including additives that might activate the cysteine (glutathione, copper). We also tried ubiquitination reactions for shorter time periods at high pH. After one hour of dialysis at pH 8.0 we obtained 10 % monoubiquitinated

Gpa1. We tried to separate this small population of monoubiquitinated Gpa1 using gel filtration, ion chromatography, or affinity chromatography using a His-tag on Ubiquitin. Unfortunately, the yield from gel filtration or ion chromatography was not sufficient for biochemical analysis. Ubiquitination of Gpa1 with His-Ubiquitin was also not successful because His-Ubiquitin aggregates above pH 8, making the ubiquitination reaction significantly less efficient. We therefore decided to return to the full-length Gpa1 construct, which has a predicted pI of 7.5, suggesting it is much more likely to be stable at a pH above 8.0. For the first experiments, we used $Gpa1^{WT}$ with no background mutations and $Gpa1^{K165C}$. Over four hours of dialysis in the absence of reducing agent, we observed a significant loss of $Gpa1^{WT}$, but less loss of $Gpa1^{K165C}$, suggesting that a modification at that site may be stabilizing the protein (**Figure 4.4)**. However, we were unable to clearly observe modification by ubiquitination in the presence of K165C. Finally, we could not find conditions under which Gpa1 remained stable and adequate ubiquitination occurred.

Changes in the α-Helical Domain of Gpa1 Influence its GTPase Activity

It has recently come to light that the α-helical domain influences the activity of Gα proteins (*140, 250*). Direct evidence has shown that the activity of the Gα proteins in mammals and plants are influenced by dynamics of the α-helical regions (*250*). While Gpa1 has a ubiquitination domain inserted into the α-helical domain, we have already established that the presence of this domain does not alter Gpa1 activity. Therefore, we considered whether changes to the adjoining α-helical domain of Gpa1 could alter the function of the Ras domain, similar to mammalian Gα proteins. To test this hypothesis, we made point mutations in the α-helical domain of Gpa1 and measured the effect on

Gpa1 stability and activity. We chose to mutate the five cysteines in the α-helical domain since they represent a spread of the types of residues important in Gpa1 (**Figure 4.5a**). Two of these cysteines are buried and conserved within other Gα proteins in the same family as Gpa1, two cysteines are near the A/B helix whose dynamics were shown to be important in modulating the activity of plant and mammalian Gα activity, and one cysteine is proximal to the site of ubiquitination. We made a single mutation at the cysteine in the ubiquitination domain ($Gpa1^{\Delta NC208S}$) as well as a mutant that has all five cysteines replaced with serines in the α-helical domain ($Gpa1^{\Delta NC\alpha S}$). In both constructs, no changes made to the two cysteines present in the Ras-like domain. We first used fQCR to measure the thermal stability of $Gpa1^{\Delta NC208S}$ and $Gpa1^{\Delta NC\alpha S}$. As seen in **Figure 4.5b**, we found that while mutation of the single cysteine near the site of ubiquitination did not have a significant effect on protein stability, simultaneous mutation of all five cysteines in the α-helical domain significantly destabilized the protein. Furthermore, in the presence of the α-helical domain mutations, $Gpa1^{\Delta NC\alpha S}$ is no longer thermally stabilized by the addition of GTPγS (**Figure 4.5c**). This suggests a possible change in the affinity of Gpa1 for nucleotide. We next used intrinsic fluorescence to measure rates of GTPγS binding and GTP hydrolysis. While mutation of the cysteine within the ubiquitination domain alone does not alter Gpa1 activity, we observed a significant decrease in the rate of GTP hydrolysis in $Gpa1^{\Delta NC\alpha S}$ (**Figure 4.5d**). Thus, changes in the α-helical domain can influence the stability and enzymatic activity of Gpa1. Taken together, these data show that changes to the α-helical domain of Gpa1 can alter the function of the Ras-like domain of the protein.

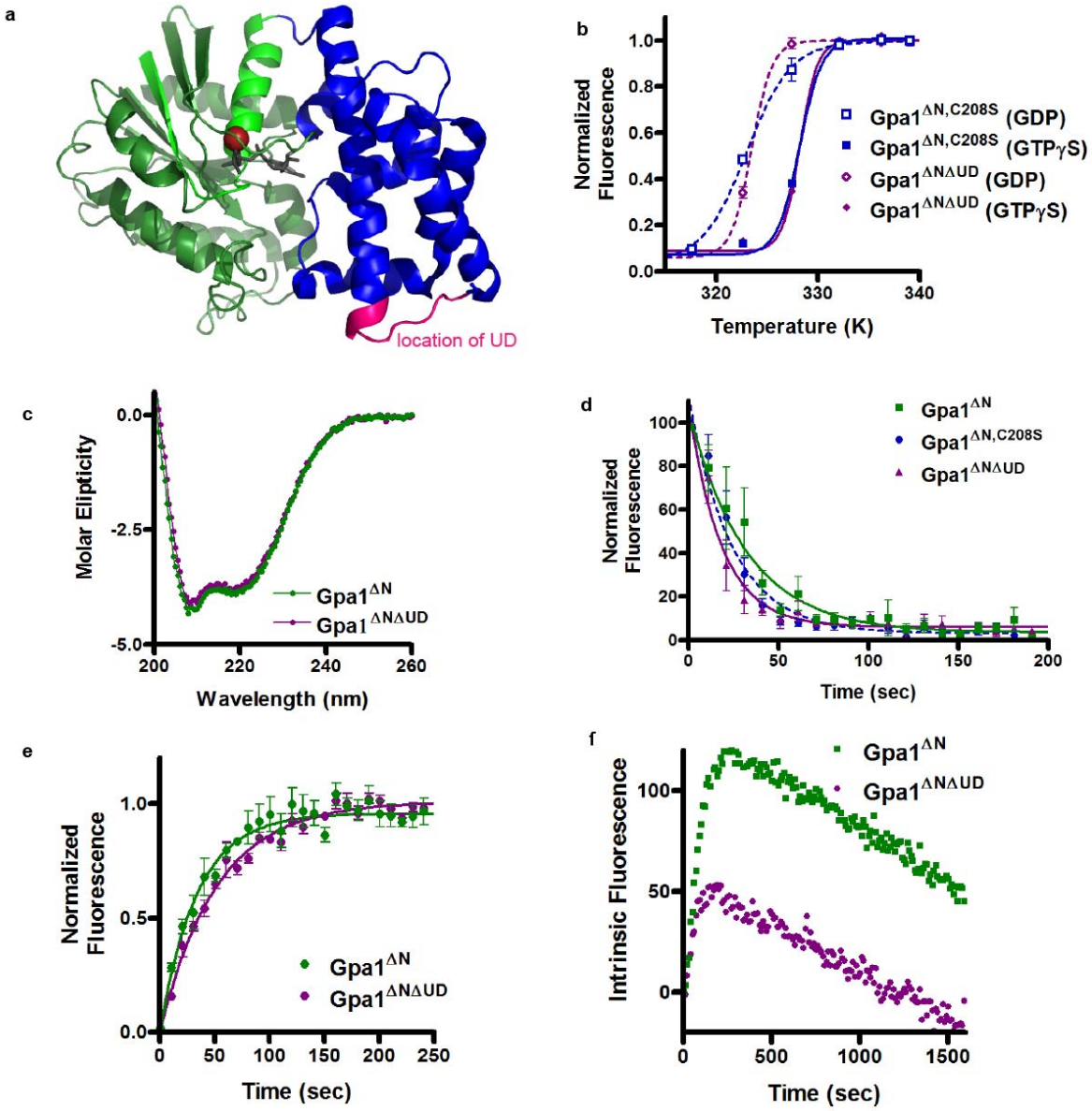

Figure 4.3. The Insert Domain of Gpa1 Does Not Alter Gpa1 Activity. (**a**) Structure of $G\alpha_i$ showing the location of the ubiquitination domain based on sequence alignment, in magenta. The Ras-like domain is shown in green and the α-helical domain is shown in blue. (**b**) Thermal stability curves showing no difference between $Gpa1^{\Delta N}$ and $Gpa1^{\Delta N\Delta UD}$. (**c**) Secondary structure content of $Gpa1^{\Delta N}$ and $Gpa1^{\Delta N\Delta UD}$ measured by circular dichroism. (**d**) Mant-GDP dissociation curves for $Gpa1^{\Delta N}$ and $Gpa1^{\Delta N\Delta UD}$. Dissociation was monitored following the decrease in fluorescence emission over time following the addition of unlabeled GDP. Data were fit to an exponential dissociation curve, and the results are the mean ± s.d. (n=4). (**e**) Mant-GDP association curves for $Gpa1^{\Delta N}$ and $Gpa1^{\Delta N\Delta UD}$. Data were fit to an exponential association curve. The results are the mean ± s.d. (n=4). (**f**) Intrinsic hydrolysis data for $Gpa1^{\Delta N}$ and $Gpa1^{\Delta N\Delta UD}$.

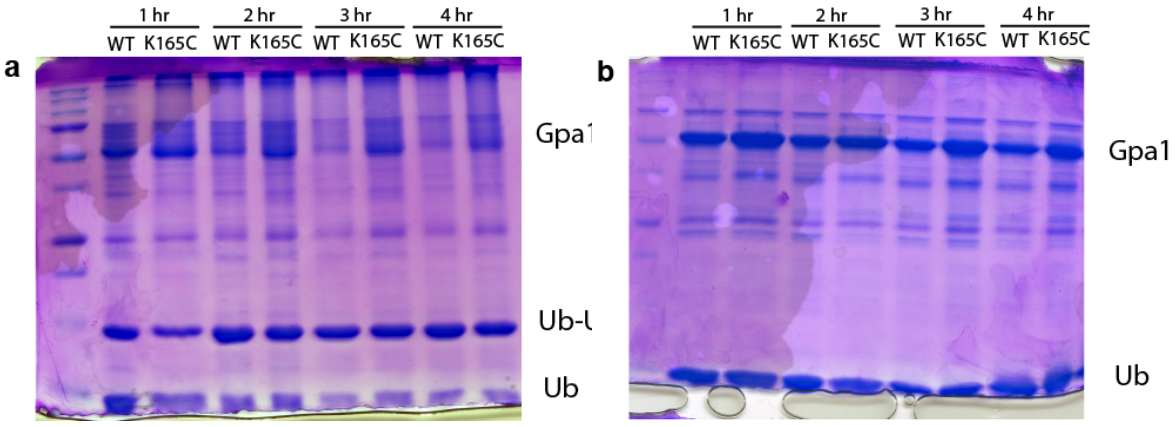

Figure 4.4. Time Course of Gpa1 Ubiquitination**.** SDS-PAGE gel of $Gpa1^{WT}$ and $Gpa1^{K165C}$ in the presence of Ubiquitin over a 4 hour time frame under (**a**) non-reducing and (**b**) reducing conditions.

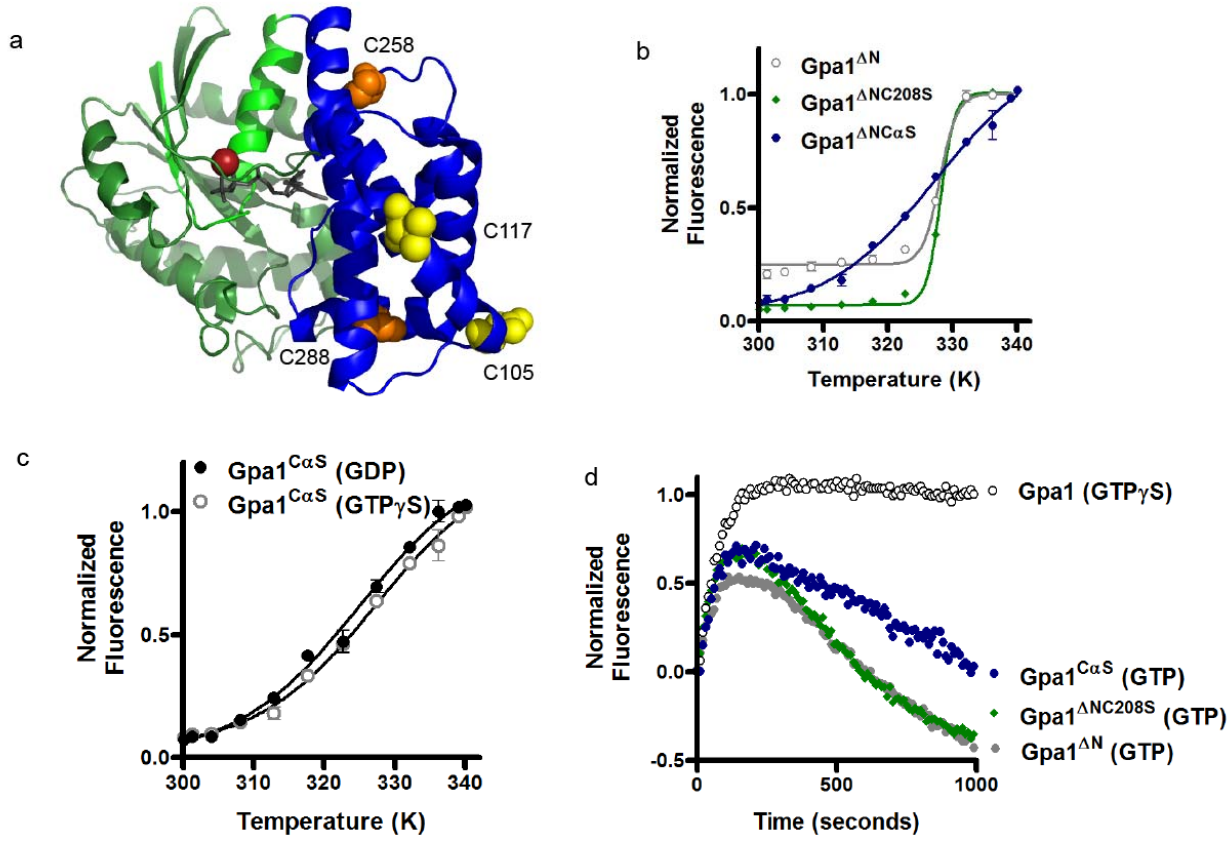

Figure 4.5. Changes in the α-Helical Domain of Gpa1 Influence its Enzymatic Activity. (**a**) Structure of $G\alpha_{i,}$ showing the location of the cysteines in Gpa1 based on structural alignment. Cysteines in orange are conserved between Gα proteins, while cysteines in yellow are present in Gpa1 but not $G\alpha_i$. (**b**) Thermal stability of $Gpa1^{\Delta N}$, $Gpa1^{\Delta NC208S}$, and $Gpa1^{\Delta NC\alpha S}$ measured by 4 –Fluoro–7–aminosulfonylbenzofurazan (ABD–F) incorporation as a function of temperature in the presence of GTPγS. The data were normalized using the maximum fluorescence intensity. Results are the mean ± s.d. (n=4). (**c**) Thermal stability of $Gpa1^{\Delta NC\alpha S}$ in the presence of GDP and GTPγS. (**d**) Intrinsic fluorescence was used to measure GTPγS binding and GTP hydrolysis in $Gpa1^{\Delta N}$, $Gpa1^{\Delta NC208S}$, and $Gpa1^{\Delta NC\alpha S}$. Data were normalized to the maximum signal achieved in each experiment upon completion of GTPγS binding.

Discussion

It has long been known that G protein signaling is highly dynamic and tightly regulated. Recent findings indicate that these properties are not confined to the Gα Ras-like domain, but may extend to the α-helical domain as well. In addition, Gpa1 has a unique domain that is subject to phosphorylation and ubiquitination. Here we used biochemical and biophysical approaches to demonstrate that the ubiquitination domain of Gpa1 functions independently of its enzymatic activity. Most strikingly, we were able to remove the ubiquitination domain without affecting nucleotide binding or hydrolysis. These results are particularly striking given that (a) the ubiquitination domain is located near a key dynamic region of the α-helical domain, (b) the ubiquitination domain is essential for transport to the endosome (*260*), and (c) there are a number of unique binding partners that specifically regulate post-translational modification of the ubiquitination domain. We conclude that the ubiquitination domain evolved to serve a unique trafficking function, and that this function is wholly separate from the regulation of G protein catalytic activity.

The ubiquitination domain is phosphorylated, polyubiquitinated, and monoubiquitinated. Recent work from our lab has identified the enzymes that add and remove these post-translational modifications (*280, 284*). One kinase responsible for phosphorylation of Gpa1 is Elm1, which had been identified by screening a panel of yeast kinase deletion strains for those necessary for proper Gpa1 phosphorylation (*285*). We have since shown that Elm1 and two related kinases, Sak1 and Tos3, also phosphorylate Gpa1 under glucose-starved conditions. Conversely, the Reg1/Glc7 phosphatase complex is needed to dephosphorylate Gpa1 (Clement, manuscript under

review). Finally, we have shown that the ubiquitin ligase SCF-Cdc4 is necessary and sufficient for polyubiquitination of Gpa1 (*280*), while the HECT ubiquitin ligase Rsp5 is responsible for monoubiquitination of Gpa1 (*284*). This list of binding partners includes only those that are known to form an interaction with the ubiquitination domain. However, we anticipate that monoubiquitination acts to regulate the binding of additional proteins that deliver Gpa1 to the endosome and vacuole.

Once at the endosome, Gpa1 transmits a signal via two subunits of phosphatidylinositol 3-kinase (Vps15 and Vps34). Thus it will be interesting to determine if these proteins also bind to the ubiquitination domain of Gpa1. In comparison, it seems unlikely that proteins involved in Gpa1 signaling at the plasma membrane, including the pheromone receptor Ste2 (*286*), Gβγ subunit Ste4/Ste18 (*287*), GTPase accelerating protein Sst2 (*288*), and non-receptor exchange factor (Get3), would be affected by Gpa1 monoubiquitination.

Given that the ubiquitination domain is found exclusively in Gpa1, information concerning its activities may not be directly relevant to other Gα proteins. Nevertheless our investigation has the potential to reveal structural determinants that promote protein ubiquitination. In particular, little is known about what makes a protein a good substrate for monoubiquitination vs. polyubiquitination. Gpa1 is a substrate of both types of ubiquitination, and these modifications are mediated through two distinct ubiquitination ligases. One distinguishing feature of polyubiquitination is that phosphorylation of the ubiquitination domain is required for SCF-Cdc4 to bind to Gpa1 (*285*). Conversely, it is likely that monoubiquitinated Gpa1 will not be recognized as a substrate for polyubiquitination. Structural analysis of the Gpa1 ubiquitination domain, before and

after phosphorylation, monoubiquitination and polyubiquitination, could reveal distinguishing features of the two processes.

Our analysis may also lead to insights into the function of unique domains in other GTPases. While no mammalian Gα proteins contain an insert similar to Gpa1, there are other families of small GTPases that are known to contain inserts within their highly conserved Ras-like domains. For example, members of the Rho family of small GTPases have a unique insert, called the Rho insert, that is not present in other small GTPases (*289*). The presence or absence of the Rho insert does not alter the intrinsic activity of these small GTPases (*290*) . When the insert is absent however, Rho can bind, but no longer activate, its downstream effector Rho kinase (*290*). There is additional evidence showing that the Rho insert participates in other effector-mediated functions such as cytoskeletal remodeling and Nox regulation (*291*). However, the mechanism through which the insert domain contributes to regulation is not known. Finally, the Rho insert in Rac1 was recently shown to be monoubiquitinated (*157*). While no function has yet been assigned to monoubiquitination of Rac1, it is possible that this modification could be involved in the mechanism by which Rho interacts with downstream effectors, similar to our proposal that Gpa1 monoubiquitination in the ubiquitination domain alters interactions with select protein binding partners.

Our understanding of the signaling and regulation of Gα proteins continues to evolve despite several decades of intensive investigation. Here, we present evidence that the α-helical domain regulates the stability as well as the enzymatic activity of the protein, and does so independently of the ubiquitination domain. More broadly our work

in vitro points to a number of potential experiments using the yeast model system to understand the impact of the α-helical domain on Gα signaling *in vivo*.

Methods

Protein Expression and Purification

Gpa1 containing a cleavable (Tobacco Etch Virus (TEV)) N-terminal His tag was expressed in the pQlinkH vector (Addgene) in *E. coli* BL21 (DE3) RIPL cells (Stratagene: La Jolla, CA). Cells were lysed by homogenization (NanDeBee) at 1000 psi in 25 mM potassium phosphate buffer with 300 mM KCl and 250 μM TCEP. After clarification by centrifugation, the lysate from 500 ml of cells was bound to 1 ml Ni-NTA agarose bead slurry (Qiagen) for 20 min at 4^{o}C. The beads were washed three times with lysis buffer, then three times with 25 mM phosphate buffer, pH 7.0, 100 mM KCl, 100 μM GDP, and 500 μM TCEP. Gpa1 was cleaved from the beads overnight by incubation with TEV. The final product was judged > 95% pure by SDS–PAGE. Ubiquitin^{G76C} was expressed in the pQlinkH vector system and purified following standard Qiagen nickel affinity purification procedures. The His tag was cleaved overnight with TEV. Ubiquitin was further purified by removal of uncleaved protein using Ni–NTA agarose beads (Qiagen). The final product was judged > 95% pure by SDS–PAGE. Proteins were stored in 20 mM HEPES, pH 8.0, 50 mM NaCl and 500 μM TCEP.

Thermal Stability of Gpa1

The fast quantitative cysteine reactivity (fQCR) method (*182*) was employed to measure changes in Gpa1 thermal stability. Briefly, 2 μM protein was incubated with 1

mM 4-fluoro-7-aminosulfonylbenzoflurazan (ABD–F, Anaspec) at pH 7.0 in the presence of 20 μM GDP or GTpγS and 2 mM $MgCl_2$ at the desired temperature for three minutes. The reaction was quenched with HCl and ABD-F fluorescence was measured on a PHERAstar plate reader (BMG Labtech, excitation at 400 nm and emission at 500 nm). The data were normalized and fit to determine the temperature at which half the protein was unfolded, representing the melting temperature (T_m).

Circular Dichroism

Circular Dichroism (CD) experiments were performed from 190 nm – 260 nm on a Chirascan plus CD spectrometer. Spectra of 5 μM protein were recorded in 25 mM Potassium Phosphate buffer, pH 7.0, 100 mM KCl, 50μM GDP, 50 μM $MgCl_2$, and 550 μM TCEP at 25^{o}C using a 1 mm quartz cell. Buffer background was subtracted from the spectra.

MANT-Nucleotide Association and Dissociation Assay

Gpa1 was exchanged into 25 mM potassium phosphate buffer, pH 7.0, 100 mM KCl, 50 μM $MgCl_2$, and 100 μM GDP. To initiate association, 1 μM Mant-GDP was added to 1 μM protein. Gpa1 was determined to be fully loaded when the fluorescence intensity reaches a maximum at approximately 250 sec. Association was measured as a change in fluorescence intensity over time (excitation: 360 nm, emission: 440 nm) (LS50B Perkin–Elmer Luminescence Spectrometer). MANT-GDP dissociation was initiated by the addition of 500 μM unlabeled GDP. Fluorescence data were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a one–phase exponential association or decay curve. Results are the mean ± s.d. (n=4).

Intrinsic GTP Binding and Hydrolysis

Purified Gpa1 (200 nM) was equilibrated in a cuvette with 25 mM phosphate buffer, pH 7.0, 100 mM KCl, and 50 μM $MgCl_2$. GTP or GTPγS at a final concentration of 200 nM was added to the cuvette, and either GTPγS binding or GTP hydrolysis was monitored by the change in intrinsic fluorescence of Gpa1 that occurs upon rearrangement of the tryptophan near the nucleotide binding region (excitation at 284 nm and emission at 340 nm). Data was collected on a Perkin Elmer Luminescence Spectrometer and analyzed using GraphPad Prism (GraphPad Software; San Diego, CA).

Chemical Ubiquitination

The chemical ligation strategy used to link Gpa1 to Ubiquitin^{G76C} was adapted from Baker et al (*235*). Briefly, a ten–fold excess of Ubiquitin^{G76C} was added to Gpa1^{K165C} and dialyzed into 20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM $MgCl_2$, and 50 μM GDP at 4 ^{o}C for 4 hours. The amount of disulfide complex formation was determined by non–reducing SDS–PAGE.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS[1]

The regulation of cell signaling is essential for maintaining normal, healthy cells. Key to the proper function and specificity of signaling pathways is the use of post-translational modifications to regulate protein abundance, localization, and activity. Monoubiquitination is one dynamic and reversible post-translational modification that is emerging as an important regulator of signaling pathways. Regulation by monoubiquitination acts on the level of protein trafficking, gene expression, and in some cases protein activity. Because the diverse roles of monoubiquitination in cell signaling have only recently been recognized, very little work has been done to pursue the mechanisms by which monoubiquitination regulates substrates. However, preliminary data suggest that biochemical and biophysical approaches, when coupled with *in vivo* data, can be a powerful tool for understanding how monoubiquitination fine-tunes cell maintenance and signaling.

In this dissertation, we report the development of a validated chemical approach to study monoubiquitinated substrates. This approach was optimized not only to completely modify the substrate of interest, eliminating the need for additional purification steps, but also to be a simple and accessible tool for use by scientists who

[1] All figures contributed by Rachael Baker

already study monoubiquitination by other methods. We used our chemical ubiquitination system to study the mechanism by which monoubiquitination regulates multiple isoforms of the small GTPase Ras. We showed that monoubiquitination at K147 activates K-Ras by impeding GAP-mediated GTP hydrolysis and that monoubiquitination at K117 activates H-Ras by increasing the rate of intrinsic nucleotide exchange. These studies demonstrated not only that the effects of ubiquitination on a substrate can be site-specific, but that two isoforms of Ras are differentially monoubiquitinated within the same cell. Therefore, monoubiquitination represents both a new mechanism of Ras signaling regulation as well a new mechanism by which Ras isoforms are potentially differentiated. Finally, we turn our attention to a heterotrimeric GTPase, Gpa1, which is a substrate of both polyubiquitination and monoubiquitination. Using biochemical approaches, we show that this protein has uniquely evolved a domain for post-translational modification by ubiquitination, and that this domain does not interfere with α-helical domain modulation of Gpa1 activity. Here, monoubiquitination is likely important for trafficking but not the direct regulation of Gpa1 activity.

The studies described in this dissertation highlight three unique cases in which a GTPase is regulated in a unique manner by monoubiquitination. Furthermore, this work highlights the importance of using multiple approaches to understand the role of post-translational modifications in protein regulation. In this last chapter, I will relate the work in this dissertation to what is already known about the regulation of both Ras and Gpa1 and discuss its implications. I will also present some preliminary studies that highlight the future directions the research on each of these proteins may take. Finally, I will discuss possibilities for the future direction of monoubiquitination research in general.

Chemical Ubiquitination

One of the challenges of studying monoubiquitinated substrates is that it requires modifying a protein with a second protein. This makes the study of ubiquitination difficult for two reasons. First, the native ubiquitination process, involving multiple enzymatic steps, is challenging to reconstitute *in vitro* and is often much less efficient and site-specific than it would be *in vivo*. This could potentially complicate the results of biochemical and biophysical studies since site-specificity is often important for the mechanism of regulation by ubiquitination. Furthermore, biochemical studies require significant amounts of protein, which is difficult to generate through native ubiquitination approaches. Second, there are a number of biophysical approaches, like CD, that give readouts of the total protein population and these approaches cannot be used to specifically observe the effects of monoubiquitination on the substrate itself. Other approaches, like NMR and crystallography, allow specific observation of the substrate separate from the Ubiquitin modifier, but if the ubiquitination reaction is inefficient, the costs of generating enough pure, properly labeled (NMR) substrate may be prohibitive.

Chemical ubiquitination is a powerful approach to study ubiquitinated substrates and can overcome a number of the challenges described in the previous paragraph. The chemical ubiquitination reaction is site-specific since it occurs at a site modified through mutagenesis (*235*). The reaction is also very efficient, in some cases leading to complete substrate modification. This approach decreases the cost of generating monoubiquitinated substrates and makes it feasible to do experiments that require large quantities of protein.

In the work described here, we took advantage of a chemical ubiquitination approach coupled with powerful methods to study ubiquitinated substrates. For example, we selectively ^{15}N-labeled Ras and monoubiquitinated it with unlabeled Ubiquitin as well as ^{15}N- enriched Ubiquitin and used it to monoubiquitinate unlabeled Ras. These approaches allowed us to observe the effects of monoubiquitination on the structure and dynamics of Ubiquitin and Ras individually. We also took advantage of the unique properties of fast quantitative cysteine reactivity (fQCR) to measure substrate thermal stability. The fQCR assay gives information about protein thermal stability by labeling accessible cysteines as a function of temperature. Because Ubiquitin does not have any cysteines (except the one we introduced to form the disulfide bond in our chemical ubiquitination system), this assay specifically measures the thermal stability of the substrate itself. A chemical ubiquitination approach coupled with these types of biochemical and biophysical experiments make it possible to elucidate mechanisms by which substrates are regulated by monoubiquitination.

Chemical Ubiquitination Strategies

There are numerous published approaches to chemically ubiquitinating a substrate. Here I will discuss three primary strategies used as well as their disadvantages and advantages. For a comprehensive review of the chemistry behind each approach, see one of the following reviews (*106, 292*). There are: chemical approaches for generating an isopeptide bond linkage, synthesis or semi-synthesis of ubiquitinated substrates, and the use of isopeptide bond surrogates. For a summary of the types of linkages generated in these various approaches, see **Figure 5.1**. First, there are numerous examples of using synthetic and organic chemistry to generate the same isopeptide bond that is formed in

enzymatic ubiquitination (**Figure 5.1a**). Many of these approaches require the use of non-natural amino acids coupled with intein-activated ubiquitin (*293, 294*) to protect the site of ubiquitination. In other approaches, native protein is used, and the lysine is chemically modified after protein expression (*295-297*). These novel approaches have all been developed recently and have the advantage of precisely mimicking enzymatic ubiquitination (*298-300*). However, the disadvantage of these approaches is that the chemistry is very slow, in some cases taking a week to complete (*106*). Furthermore, the multiple steps in many of the chemical synthesis approaches leads to low yields of modified substrate (*292*).

The second approach to generating monoubiquitinated substrate involves either the synthesis or semi-synthesis of the final modified protein. A number of the biophysical studies of ubiquitinated substrates discussed in the introduction were done through these methods, including studies of monoubiquitination of histone H2B (*107, 108*), PCNA (*109, 110*), and α-synuclein (*114*). In these approaches, the simplest strategy is to express the substrate in two separate pieces, with one piece being expressed tandem to Ubiquitin (**Figure 5.1b**). In more complex approaches, expression of the split substrate is coupled with the organic synthesis approaches above to generate the final ubiquitinated substrate (*106*). This approach generates large quantities of stable substrate for study by biochemical approaches. However, it is a viable approach for only a limited number of substrates. For this approach to work, substrate structure must be known, the substrates must be amenable to expression in two separate pieces, and methods to validate the correct reassembly of the monoubiquitinated substrate are necessary. Furthermore, additional studies with histone H2B showed that not all semi-synthesis

approaches are successful. While one approach did lead to generation of monoubiquitinated H2B that accurately mimicked natively modified protein, this approach was very inefficient and time consuming (*107, 108*). When the authors tried to adopt more efficient strategies to generate monoubiquitinated H2B, the native structure of the protein was compromised (*293, 301*).

The third strategy for generating monoubiquitinated substrate is to use isopeptide bond surrogates. The primary goal of these types of strategies is to simplify highly technical synthesis approaches while still producing stable monoubiquitinated substrate. In some cases, a non- natural amino acid is used, often a pyrrolysine analog, on the substrate, and the Ubiquitin is linked through intein-based approaches or another genetically encoded non-natural amino acid (**Figure 5.1c**) (*301-303*). However, this approach does not always successfully recapitulate the *in vivo* effects of ubiquitination (*301*). In other cases, chemical synthesis of the lysine or cysteine after protein expression is used to link the protein to processed Ubiquitin (**Figure 5.1d**) (*304*). One common form of this approach is called click chemistry and requires an alkylated cysteine residue and intein-Ubiquitin that has been processed with aminolysis (**Figure 5.1e**) (*305*). One of the simplest approaches, however, is to use a disulfide bond as a replacement for the chemical bond (**Figure 5.1f**) (*109, 301, 306*). In many of these examples, the Ubiquitin is expressed through an intein based approach and organic molecules are used to make the linker closer in length to the enzymatic linker (**Figure 5.1g**) (*307*). The advantage of these types of approaches, especially the disulfide chemistry, is that they are faster and easier than chemically synthesizing the enzymatic linkage. However, little work has been

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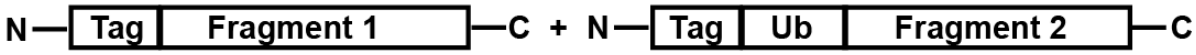
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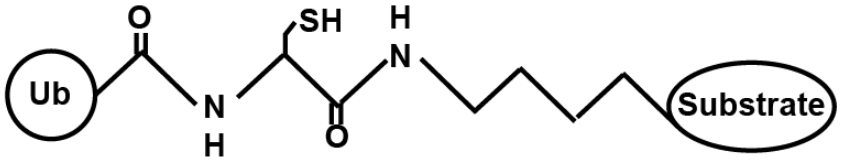
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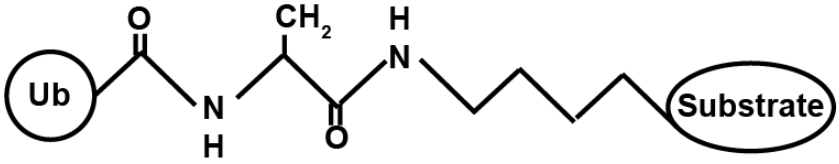
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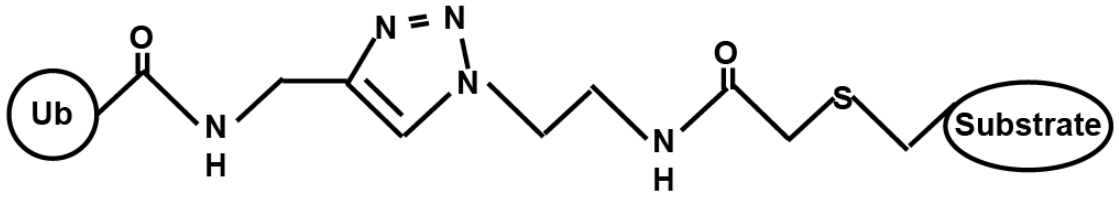
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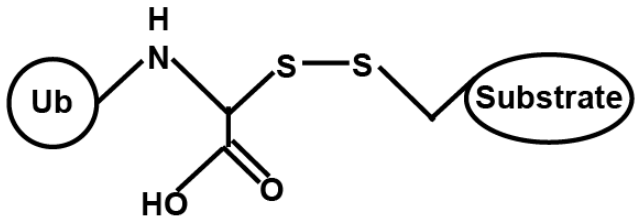
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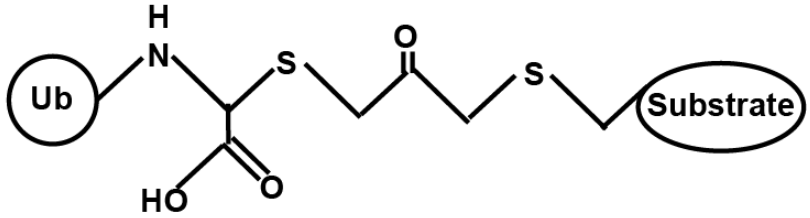
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Figure 5.1. Native and Chemical Ubiquitination Linkages**.** (**a**) Ubiquitin (Ub) substrate linkage generated through enzymatic ubiquitination or a chemical method for constructing an isopeptide bond. (**b**) An example of one straightforward strategy for the semi-synthesis of monoubiquitinated substrate. (**c**) Isopeptide bond surrogate obtained from a genetically encoded pyrrolysine analog on the substrate and intein-Ubiquitin. (**d**) Isopeptide bond surrogate obtained from chemical synthesis of the substrate. (**e**) Isopeptide bond surrogate obtained from substrate with an alkylated cysteine and intein-Ubiquitin that has undergone aminolysis. (**f**) Isopeptide bond surrogate generated from cysteine mutation on the substrate and Ubiquitin. (**g**) Isopeptide bond surrogate obtained from a cystine mutation on the substrate processed with dichloroacetone and intein-Ubiquitin.

done to validate how successfully these individual approaches mimic the behavior of substrate modified by the native ubiquitination linkage.

While each approach for chemical ubiquitination has advantages and disadvantages, the most successful approaches will likely involve the isopeptide bond surrogates. This ubiquitination approach does not require the advanced chemical knowledge of the more involved synthetic approaches and provides higher substrate yields than possible through the synthesis of an isopeptide bond. Isopeptide bond surrogates are also more likely than the semi-synthesis approaches to work on a broad array of substrates. We were particularly interested in the disulfide chemistry approaches because they represented the simplest and fastest way to generate monoubiquitinated substrate (**Figure 5.1f**). However, even within this disulfide approach there are discrepancies in the methods used and the final outcome. Representative studies that use a basic disulfide approach are described in **Table 5.1** (*174, 177, 235, 308*). As summarized in the table, we improved on existing methods by eliminating the need for additives like copper and driving substrate modification to 100% using excess Ubiquitin and a pH of 8.5, which fully activated the cysteines. This simple and efficient approach is accessible to anyone who has the ability to purify proteins. Furthermore, as described in Chapter II, using assays in cell lysate as well as computational modeling, we validated that the differences in linkage length and rotational preferences did not have a significant effect on the biochemistry of monoubiquitination (*235*).

However, as evidenced by the work with Gpa1 in Chapter IV, there are limitations to this method. There were two main reasons why our chemical ubiquitination approach was not successful for Gpa1. The problems were [1] that Gpa1

Substrate	Disulfide Bond Strategy	Buffer	pH	Additional Notes	Amount Modified	Publication
Ubc1	Cys mutation on substrate and Ubiquitin	100 mM HPO_4 100 mM NaCl 10 μM $CuCl_2$	7.5	Substrate pre-reduced with TCEP	100%	Merkley et al. (2005) Ubiquitin Manipulation by an E2 Conjugating Enzyme Using a Novel Covalent Intermediate, *J Biol Chem 280*, 31732-31738.
UbcH7	Cys mutation on substrate and Ubiquitin	100 mM HPO_4 100 mM NaCl 10 μM $CuSO_4$	8.0	Substrate pre-reduced with βME	> 50%	Purbeck et al. (2009) Kinetics of the Transfer of Ubiquitin from UbcH7 to E6AP, *Biochemistry 49*, 1361-1363.
PCNA	Cys mutation on substrate and Ubiquitin, linked with 1,3-Dichloroacetone	200 mM sodium borate	8.6	Substrate pre-reduced with βME	60%-70%	Carlile et al. (2009) Synthesis of Free and Proliferating Cell Nuclear Antigen-bound Polyubiquitin Chains by the RING E3 Ubiquitin Ligase Rad5, *Journal of Biological Chemistry 284*, 29326-29334.
Ras	Cys mutation on substrate and Ubiquitin	20 mM Tris 50 mM NaCl	8.5	Substrate pre-reduced with TCEP 10-fold excess Ubiquitin	100%	Baker et al. (2013) Site-specific monoubiquitination activates Ras by impeding GTPase-activating protein function, *Nature Structural and Molecular Biology 20*, 46-52.

Table 5.1. Summary of Disulfide Chemical Ubiquitination Approaches**.** First column describes the substrate modified. Second column summarizes the strategy used to generate the disulfide bond. Third column has the buffer components under which the disulfide formation reaction was performed, with the pH listed in column four. The fifth column describes any other key differences between the ubiquitination approaches. The efficiency of the reaction is summarized in column six, and column seven contains the original publication in which the experiment was performed.

had a number of cysteines, and mutation of a significant number of cysteines altered the biochemical activity of the protein and [2] due to its predicted pI of pH 8.5 Gpa1 was not stable at pH 8-8.5, where cysteines are most reactive. Further plans for modifying Gpa1 will be discussed in the section specifically dedicated to Gpa1. It is important to note, however, that the reasons that Gpa1 was not amenable to our chemical ubiquitination approach would also make it a poor candidate for many of the other chemical methods described above. This result highlights that while chemical ubiquitination will likely be a widely successful approach for studying monoubiquitinated substrates, it is not a universal solution to the challenge of studying these substrates *in vitro*.

Regulation of Ras by Monoubiquitination

In Chapters II and III, we used a combination of computational, structural, and biochemical approaches to show that when K-Ras is monoubiquitinated at K147, but not K104, it is activated by impairing GAP-mediated hydrolysis (*235*). Furthermore, when H-Ras is monoubiquitinated at K117, it is activated due to an increased rate of intrinsic nucleotide dissociation, distinct from the mechanism of K-Ras activation. Together, these data describe a new mechanism for the regulation of Ras activity through reversible post-translational modification by monoubiquitination. This mechanism is distinct for two Ras isoforms, H-Ras and K-Ras, which may contribute to the differences between these isoforms *in vivo*. We also showed that the mechanism of activation is site-specific, similar to the ubiquitination of α-synuclein (*114*) but distinct from ubiquitination of PCNA (*110*). Finally, our use of NMR as well as PDZ modification of Ras showed that

the effects of monoubiquitination at K147 are mediated by low-affinity, non-specific interactions between Ras and Ubiquitin rather than a specific, high-affinity interaction.

Future Questions and Experiments for K-Ras Ubiquitination at K147

There are four questions that directly arise from the studies of K-Ras monoubiquitination at K147. First, the mechanism by which Ubiquitin regulates Ras through non-specific interactions is not clear. There may be a surface on Ras where Ubiquitin spends a significant amount of time. Second, while we demonstrated that monoubiquitination alters the dynamics of Ras in the GDP-bound state, we do not know if it also alters switch dynamics in GTP-bound state and how that difference might contribute to its mechanism of activation. Third, we do not know the mechanism by which GAP-mediated catalysis is impaired. We showed that GAP still binds to Ras, but we do not know if the binding affinity is reduced or if the GAP binds Ras in the proper orientation. Furthermore, the same GAP deficiency is not observed with the shorter and stiffer PDZ linker. Finally, we have not explored how monoubiquitination of Ras at K147 increases the affinity of GTP-bound mUbRas for select downstream effectors.

To answer the first question about how Ubiquitin regulates Ras through non-specific interactions, we need to know more about how Ubiquitin dynamically samples conformational space surrounding Ras. We also need to further quantify the observed changes in the switch region dynamics. Our evidence that Ras does not form specific interactions with Ubiquitin comes from the work we did with NMR showing no specific shifts are observed when Ras is monoubiquitinated as well as the data showing that ligation of PDZ^{UL} to Ras can recapitulate the effects of monoubiquitination in terms of impairing GAP-mediated hydrolysis. The first step to address these questions would be

to more extensively characterize GDP-bound mUbRas by NMR. The types of experiments that would help us understand the behavior of Ubiquitin relative to Ras include hydrogen exchange (*308*) and Cleanex (*309*), relaxation experiments (*310*), and cross correlation relaxation experiments (*311-313*). The information we could obtain from each of these experiments individually is summarized in **Table 5.2.** Overall, these experiments will provide information about the specific surface on Ras that can come in contact with Ubiquitin and about whether Ubiquitin and Ras act as a single unit or move independently of one another.

Pursuing the differences between Ubiquitin and PDZ ligation will also aid in understanding regulation of Ras by monoubiquitination. Specifically, we would like repeat our HSQC NMR experiments with Ras ubiquitinated with Ubiquitin with the PDZ linker and Ras modified with PDZ with the native linker to determine if we still observe altered switch region dynamics. If switch region dynamics change similar to mUbRas, it would suggest that this is a side effect, rather than the central mechanism, through which monoubiquitination regulates Ras. If we do not observe changes in switch region dynamics, it would suggest that even though Ubiquitin and Ras do not form high affinity binding interactions, the ability of Ubiquitin to physically access a specific surface on Ras is necessary for its modulation of switch region dynamics in the GDP-bound state of the protein.

In conjunction with our experimental approaches, we are also pursuing molecular dynamic simulations of mUbRas in collaboration with Brenda Temple at UNC. While we have only done preliminary 20 ns and 200 ns simulations of mUbRas (without constraints), we observed that Ubiquitin spends a significant amount of time near the

NMR Approach	Information Obtained	Contribution to Mechanistic Knowledge
Hydrogen Exchange	determine which amide resonances show enhanced protection when Ras is ubiquitinated	may indicate interaction sites within or outside of the switch regions that become less accessible to the solvent in mUbRas, indicating a region where Ubiquitin spends a significant amount of time
Cleanex	measure exchange rates for residues that may be too fast to be detected by hydrogen exchange	same information as obtained from hydrogen exchange
Relaxation	(a) overall rotational tumbling time (b) the order parameter (a measure of rigidity) (c) internal motion for each observable backbone amide resonance	The rotational correlation time of Ras with and without ubiquitination will be used to determine if the two proteins tumble as independent domains or if their motions are correlated. Comparing relaxation parameters will provide additional quantification of differences in the backbone dynamics when Ras is ubiquitinated on the picosecond/nanosecond as well as milisecond timescales.
Cross Correlation Relaxation	torsion angle restraints at the site of Ubiquitin linkage to Ras	Parameters to be used for computational modeling of mUbRas

Table 5.2. Proposed NMR Experiments for the Study of mUbRas**.** All experiments would be done with ^{15}N-Ras and ^{15}N-Ras ubiquitinated with unlabeled Ubiquitin. First column lists the type of experiment (references provided in main text). Second column described the information that can be obtained from doing the experiment with unmodified and monoubiquitinated. The final column describes how the information obtained will contribute to our understanding of how Ras is regulated by monoubiquitination.

surface of Ras (**Figure 5.2**).Sometimes Ubiquitin is found interacting with the switch I region of Ras and sometimes with residues opposite the switch regions. These preliminary findings that Ubiquitin forms transient interactions with Ras are consistent with the broadening we observed near the site of ubiquitination and on the surface of Ubiquitin itself through NMR. Furthermore, in our preliminary studies of ^{15}N-enriched Ubiquitin, we observed that few Ubiquitin backbone amides were altered by monoubiquitination, which suggests that Ubiquitin undergoes only local perturbations upon ligation. The same residues that were broadened on Ubiquitin by NMR are the residues that make contact with the surface of Ras during the MD simulations. These data suggest that these MD simulations may aid in understanding how the experimental constraints determined by our proposed NMR experiments contribute the behavior of Ubiquitin on Ras.

The second question relates to whether monoubiquitination alters the dynamics of the switch regions of Ras in the GTP-bound state of the protein. When Ras binds GTP, it restricts conformational sampling of the diverse possible positions of the switch regions (*118, 139*). If ubiquitination limits dynamics of the switch regions in the GDP-bound state, it does not mean that the same changes will be observed in the less conformationally dynamic GTP-bound state of the protein. NMR is again an ideal method to use for this characterization because it provides site-specific dynamic and structural information. We have optimized a procedure to load mUbRas with a non-hydrolyzable GTP analog (GMPPNP) so that we can characterize the GTP-bound state of mUbRas by NMR. We will use the same types of approaches described above

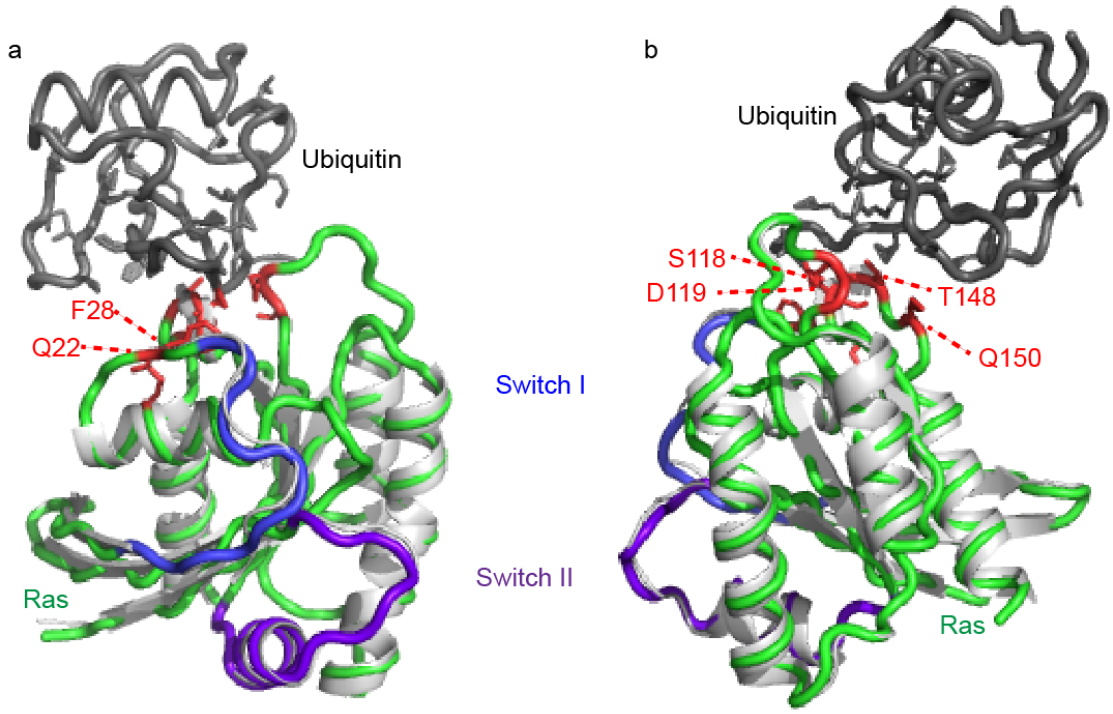

Figure 5.2. Preliminary Molecular Dynamics Simulations of mUbRas. Summary of 200 ns MD simulation for GDP-bound Ras chemically ligated to Ubiquitin. (**a**) Ras is shown in green with a cartoon of the secondary structure overlaid in light gray. Ubiquitin is shown in dark gray. The switch regions are shown in blue (Switch I) and purple (Switch II). Residues shown in red make contact with Ubiquitin when it comes close to the surface of Ras. (**b**) 180 degree rotation around the vertical axis of the image shown in (**a**).

and in Chapter II to determine if monoubiquitination alters the dynamics of switch regions in the GTP-bound state of Ras. These approaches will also provide information about whether the interaction surface of Ubiquitin is altered when Ras is bound to GTP. If we have trouble visualizing the switch regions, another NMR approach to consider is ^{31}P-NMR. There are a number of studies of Ras that observe resonances on the phosphates of GTP analogs by ^{31}P-NMR (*314, 315*). In these studies, the authors demonstrate that even in the less conformationally dynamic GTP-bound state of Ras, there are multiple conformations that the switch regions can adopt that give distinct peaks by ^{31}P NMR (*316*). Interestingly, only one conformation of the switch regions that is detected in these NMR studies is recognized efficiently by downstream effectors (*316*). We could use ^{31}P-NMR to determine if monoubiquitination of Ras alters the population of the protein into the active or inactive GTP-bound switch region conformations. These data would provide insight not only into switch regions dynamics, but also potentially into how monoubiquitination alters interactions of Ras with downstream effectors. Finally, knowing whether dynamics in the GTP-bound state of Ras change or not will contribute to answering the next two questions about the mechanisms of mUbRas interactions with GAPs and downstream effectors.

The third question to address is the mechanism by which GAP-mediated hydrolysis is impaired when Ras is ubiquitinated. While our modeling and gel filtration data suggest that ubiquitination does not impair the ability of GAP to bind to Ras, it does not mean that the GAP is adopting the proper conformation to allow catalysis of hydrolysis to occur. Furthermore, the gel filtration studies were done with AlF_3, which is transition state mimetic and may be different than studying the initial ability of GAP to

bind to Ras-GTP (*138, 197*). GAP-mediated hydrolysis is known to proceed through two steps (*317*). In the first step, the switch I region of Ras transitions from an ordered off state to an ordered on state due to GAP binding, which is essential for both GAP-mediated and intrinsic hydrolysis (*130*). The second step of hydrolysis involves the movement of the arginine finger of the GAP into the proper catalytic position (*118*). The arginine finger interacts with a water molecule at the active site and stabilizes the transition state of GTP for phosphate cleavage (*318*). It is possible that these structural rearrangements that must occur after GAP binds are not possible when Ras is monoubiquitinated. Furthermore, it is possible that the switch regions do not adopt the proper positions when bound to GAP due to altered dynamics when Ras is monoubiquitinated.

We would first like to quantify any changes in binding affinity between Ras and GAP when Ras is monoubiquitinated. One promising approach to address this question is the Homogenous Time-Resolved Fluorescence (HTRF) assay (*319*)**.** For this assay, we will load His-Ras (1-166) with GTPγS and add Flag-RasGAP^{334}. After addition of His-d2 and Flag-Eu antibodies, the FRET signal will be measured. By varying the amount of Ras or RasGAP, the binding affinity can be derived from changes in the FRET signal. These data will allow us to quantify how much monoubiquitination impairs the ability of GAP to bind to Ras.

Measuring the binding affinity of GAP for mUbRas does not provide information about whether the GAP is bound in the proper conformation. An ideal method to determine how GAP binds to mUbRas is crystallography. In Chapter II we have already shown that we can isolate the GAP-mUbRas complex, and we could use this complex to

set up crystallization trials. Another approach to visualize differences in binding when Ras is monoubiquitinated is through NMR. We will use Ras bound to a non-hydrolyzable GTP analog to look for differences in the interaction surface formed when mUbRas binds GAP as compared to unmodified Ras. This approach would provide information about whether the GAP bound to the proper surface on mUbRas. We could also ^{15}N-enrich the GAP itself and see if the surface altered upon interaction with Ras changes when Ras is monoubiquitinated. Furthermore, we could ^{15}N-enrich Ubiquitin, ligate it to Ras, and use NMR to see if there were any major shifts in the presence of GAP, which would indicate a change in the way Ubiquitin interacted with either GAP or Ras. For these experiments, further use of the Rosetta models described in Chapter II may aid in understanding how interactions between the two proteins are altered when Ras is monoubiquitinated. We could incorporate the constraints derived from the NMR studies described earlier and determine if the constraints lead to an occlusion of the GAP binding site on Ras. Rosetta modeling data used in conjunction with our experimental approaches should provide insight into the mechanism by which GAP-mediated hydrolysis is impaired. For example, if monoubiquitination of Ras appears to weaken GAP binding affinity and the Rosetta models suggest partial occlusion of the GAP binding site, we would expect the NMR data to show that GAP is not adopting the proper position on Ras to make the structural changes necessary for GAP-mediated hydrolysis. In this case, we would predict that if we ubiquitinated Ras with Ubiquitin that had the shorter, stiffer PDZ linker, it would remove the interference of Ubiquitin with GAP binding and restore GAP-mediated hydrolysis.

Our final question is related to how monoubiquitination increases the affinity of activated Ras for select downstream effectors. Sasaki et al. showed that monoubiquitination of Ras enhances its affinity for the downstream effectors Raf, PI3K, and RalGDS (*320*). Each of these effectors contains a Ras Binding Domain (RBD) or Ras Association (RA) domain that possess a ubiquitin-like fold and forms an extended beta sheet network with the switch I region of Ras-GTP (*321*). Binding of Ras to one of these domains locks the dynamic switch regions of Ras into a conformation that facilitates detection of the switch resonances (*316*). It is therefore possible that since monoubiquitination alters the dynamics of the switch regions, effector binding could be altered as well (*316*). Binding affinity between the RBD or RA domains of downstream effectors and mUbRas can also be measured using the HTFR assay described above for the measurement of GAP binding. However, particularly in the case of Raf, increased binding affinity for mUbRas was only observed if the cysteine rich domain (CRD) of Ras, which is adjacent to the RBD domain, was present (*153*). It is therefore possible that the increased affinity for select downstream effectors could be due to an additional binding interaction between Ras and the effector in the presence of Ubiquitin rather than changes in the switch region dynamics of Ras, which we have not yet characterized in the GTP-bound state of the protein. Measuring the binding affinity in the presence and absence of the CRD for both Ras and mUbRas will clarify if this is the case. If the CRD is important for the increased binding affinity, NMR can be used to determine in the additional interaction is formed between the CRD and Ras itself or the CRD and Ubiquitin.

Future Questions and Experiments for H-Ras Ubiquitination at K117

While many of our future mechanistic studies are focused on modification of K-Ras at K147, there are additional questions raised by the studies performed with H-Ras showing distinct, site-specific mechanisms of regulation. Primarily, it will be important to determine whether monoubiquitination of H-Ras at K117 also alters (either positively or negatively) the interactions of downstream effectors with mUbRas. To answer this question, the most successful approach would be to use our collaboration with Sasaki et al. to repeat the *in vivo* effector interaction assays with H-Ras. We will also repeat the assays described above for measuring interactions of effectors *in vitro* with $mUbRas^{K117}$. If monoubiquitination at K117 alters interactions with downstream effectors, particularly if it is in a manner distinct from monoubiquitination at K147, it would further support our hypothesis that monoubiquitination is one of the mechanisms by which Ras isoforms are distinguished *in vivo*.

It is interesting to note that modification of K104 does not lead to changes in intrinsic or regulator-mediated Ras activity. Besides our example of monoubiquitination at K104, we also have data that monoubiquitination at K88 or K101 has no effect on GAP-mediated Ras activity (*235*). These are two of the five lysines that had to be removed in the core domain of Ras in the previous studies of H-Ras ubiquitination leading to endosomal trafficking (*173*). This observation is consistent with our hypothesis discussed in Chapter III that in CHOK-1 cells, ubiquitination is primarily as a marker for transport rather than a direct regulator of Ras activity. Furthermore, this data raises the question of why specific sites on Ras lead to changes in activity when monoubiquitination occurs while others do not. One hypothesis is that access to the

switch regions, in particular switch I, which is most important for hydrolysis and effector recognition, is essential for the mechanism by which monoubiquitination regulates Ras activity. If that is the case, of the lysines studied only K147 and K117 appear to be close enough to this region, as seen in **Figure 5.3**. To test this hypothesis, we could use Rosetta modeling to find residues on Ras (not necessarily lysines) that provide varying degrees of access to the switch I region of the protein. We could then make the appropriate cysteine mutations and determine the extent to which ubiquitination altered the activity of the protein from each of these chosen locations. If the ability of monoubiquitination to alter Ras activity was correlated to proximity to Switch I, it would support our hypothesis, as well as our preliminary MD simulations and PDZ ligation data that it is access to Switch I that drives the mechanism by which monoubiquitination activates Ras.

Finally, our experiments with monoubiquitination at K104 also highlight an interesting phenomenon; monoubiquitination of Ras does not always have the same effect as mutation at the same site. Mutation at K147 increased intrinsic exchange slightly, but monoubiquitination did not. Mutation at K117 thermally destabilized the protein, but monoubiquitination did not. Mutation of K104 decreased sensitivity to Sos-mediated dissociation, but monoubiquitination did not. The explanations for the differences between mutations and monoubiquitination at K147 and K117 are likely connected to the mechanisms by which monoubiquitination modulate or possibly stabilize switch dynamics. However, ubiquitination at K104 does not provide the same access to the switch regions as K147 and K117. While K104 does not have the same proximity to the switch regions, it is in proximity to but not on the Sos binding interface. As seen in

Figure 5.4, the residue sits at the edge of the Sos binding interface. Molecular dynamics simulations of acetylation at K104 suggested that acetylation at this position alters the conformational stability of the switch II region due to a perturbation in electrostatic interactions, which is known to be important for GEF-mediated hydrolysis (*239*). In Chapter III, the only mutation we made at this site was a K104C mutation. It is possible that the properties of the cysteine side chain adversely effected Sos-mediated dissociation in a way that removing the side chain (ubiquitination) does not. To test this hypothesis, we should repeat our experiments with a K104G mutation, which would more closely mimic removal of the lysine sidechain, similar to monoubiquitination. If our hypothesis is correct, this mutation will not lead to the same disruption of Sos-mediated dissociation. Together, these examples show the importance of measuring the effect of monoubiquitination on a substrate. The outcome cannot always be accurately predicted from knowing the effect of a mutation at the same site

Future Directions for the Study of Ras Monoubiquitination

These studies of the regulation of Ras by monoubiquitination have also raised larger questions about how this mechanism contributes to the role of Ras in normal cell maintenance and tumorigenesis. Monoubiquitination of K-Ras was shown to be important for Ras-driven tumorigenesis, but that does not mean that it is only important in cancer progression. In cancer, cells pervert normal signaling pathways. By observing what is misregulated in cancer, we see an exaggerated picture of pathways that are important in normal cell maintenance. Therefore, even though the easiest role to detect for monoubiquitination of K-Ras is in tumorigenesis, it is very likely that it also plays an important role in normal cell maintenance. For example, it is possible that

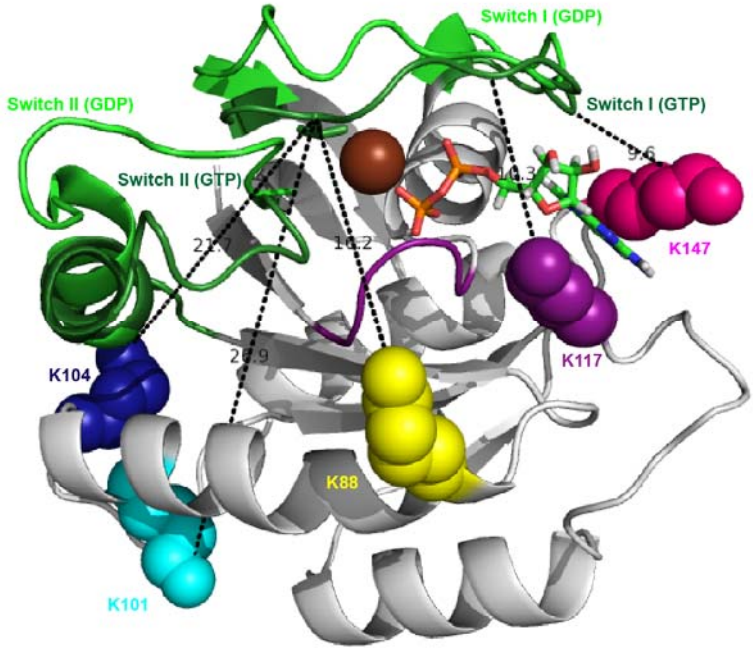

Figure 5.3. Proximity of Ubiquitination Sites to Switch I**.** Ribbon diagram of Ras shown in grey (PDB 5P21) with the switch regions highlighted in light green (GDP-bound) and dark green (GTP-bound). Mg^{2+} represented as a sphere in brown, and GDP is shown in multiple colors. Sidechains of lysines that have been chemically ubiquitinated are shown as spheres in various colors: pink (K147), purple (K117), yellow (K88), cyan (K101), and dark blue (K104). Distances are measured from the sidechain to the nearest point on switch I and are: 9.6 Angstroms (K147), 10.3 Angstroms (K117), 16.2 Angstroms (K88), 26.9 Angstroms (K101) and 23.7 Angstroms (K104). Note that proximity to the p-loop (shown in purple) is not a predictor of which residues will alter intrinsic or regulator-mediated Ras activity.

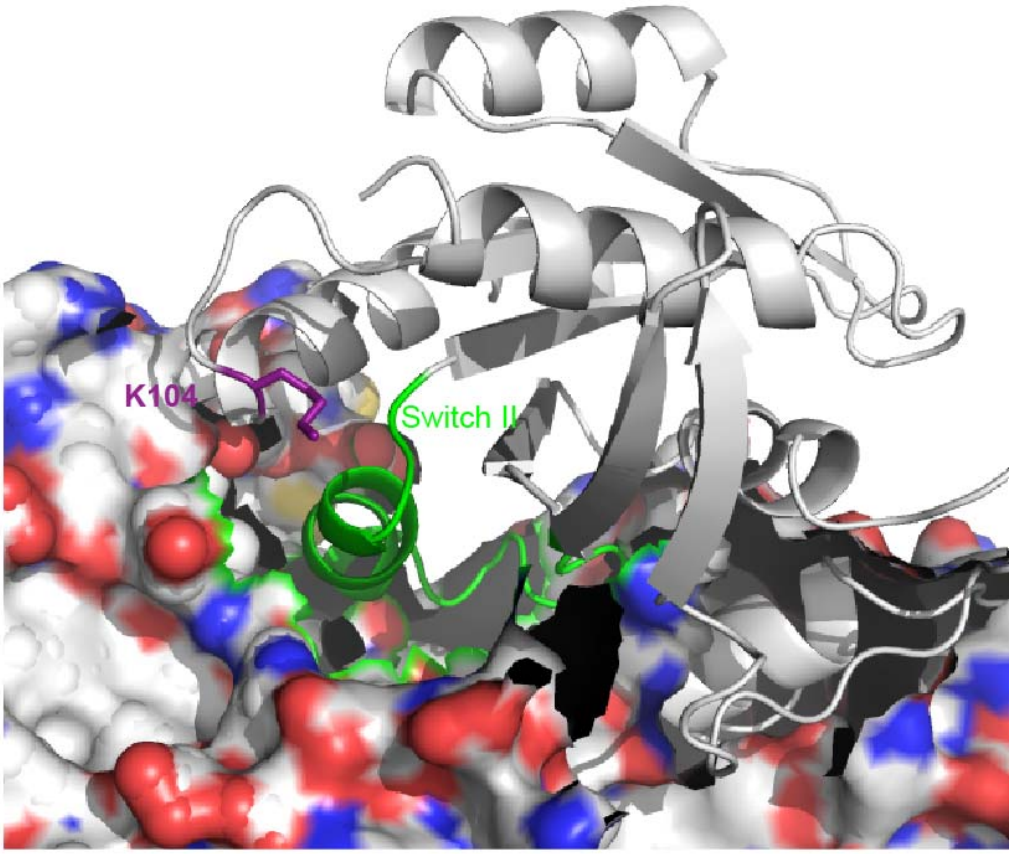

Figure 5.4. Proximity of K104 to Surface of Sos Interface with Ras**.** Crystal structure of Ras bound to Sos (PDB 1BKD) with Ras represented as a ribbon diagram in grey and Sos represented as a surface colored by the properties of the amino acid sidechain. The Lysine 104 sidechain is shown in spheres.

monoubiquitination is one of the mechanisms that lead to specificity in the interactions of particular Ras isoforms with distinct downstream effectors.

Future studies are required to address: [1] the role of K-Ras monoubiquitination in normal cell proliferation. In this case, a good initial approach may be to use mice models lacking the site of ubiquitination. [2] The role of monoubiquitination in mutant K-Ras effector signaling and cancer growth. This effect may depend on the type of cancer being considered. For example, in some cancers, mutant K-Ras (G12D and G12C) is fully activated, therefore any role of monoubiquitination would likely be due to altered interactions with downstream effectors (*322, 323*). However, in other cancers, like colorectal cancer, the mutant K-Ras (G13 and A146) is not fully activated, and in this case the increase in GTP-bound Ras when Ras is monoubiquitinated may be important (*323*). [3] The role of monoubiquitination in tumor growth for mutant H-Ras and N-Ras. Our data show differences in regulation of Ras by monoubiquitination between cell lines, suggesting that it may also be tumor-type dependent. The best place to begin these studies would be common tumor types for each isoform, which are bladder carcinomas (H-Ras) and melanoma (N-Ras) (*322, 324*).

Currently, no effective inhibitors that directly target mutant Ras proteins have successfully been developed. The first approach to developing a Ras inhibitor was to target mutant Ras that is persistently GTP-bound. However, Ras binds GTP with a high affinity (60 pM), which makes it difficult to develop competitive inhibitors that would be analagous to protein kinase competitive inhibitors of ATP binding (low μM binding affinity) (*325*). Because of its high affinity binding to GTP, Ras is considered "undruggable", and most current efforts have sought to indirectly target Ras, either

through inhibiting membrane association or downstream effector signaling (*326*). While there are over 40 inhibitors currently under clinical evaluation, none have shown significant anti-tumor activity against K-Ras mutant driven cancers (*326*). The recent findings that monoubiquitination contributes to driving oncogenesis may represent a new set of targets for developing Ras inhibitors.

Gpa1 as a Substrate for Monoubiquitination

In Chapter IV, we used biochemical approaches to study the yeast Gα protein Gpa1. Key for our ability to study this protein was the preliminary work we did optimizing the DNA sequence, expression, and purification of Gpa1. By removing the ubiquitination domain of the protein, we showed that this domain has most likely evolved for post-translational modification without influencing the biochemical or enzymatic properties of Gpa1. This finding is important because the ubiquitination domain is not present in other Gα proteins. However, its lack of influence on enzymatic activity suggests that Gpa1 may still serve as a model for the study of Gα proteins. Toward this end, we showed that mutations to the α-helical domain of Gpa1 altered the enzymatic activity of the protein, similar to what is now known for mammalian and plant Gα proteins as well.

The advantages of studying Gpa1 lie in the fact that it is a yeast protein. Thus, it is possible to couple our biochemical and biophysical studies with genetic and molecular biology studies. By this integrated approach we can better understand how the changes we observe in the protein *in vitro* contribute to its mechanisms of activity and regulation *in vivo*. There are two primary avenues of research that we would like to pursue with

Gpa1. The first relates to the study of Gpa1 as a substrate of monoubiquitination. It is intriguing that this protein possesses a domain that appears to be present only for modification by ubiquitination. We would like to know more about how this ubiquitination domain may interact with the rest of the protein, whether ubiquitination influences the biochemical properties of Gpa1, and if there are any known binding partners of Gpa1 that interact with the ubiquitination domain. The second avenue of research we would like to pursue relates to using Gpa1 as a model system to understand the contributions the α-helical domain makes to Gα signaling. Using the powerful genetics of yeast coupled with our biochemical and biophysical approaches, we are in a unique position to alter Gpa1 activity through mutation *in vitro* and then measure the effects these changes have on Gpa1 signaling *in vivo.* Our proposed experiments are described below.

First, we would like to learn more about how the ubiquitination domain may interact with the rest of the protein. Based on the preliminary data in Chapter IV showing that there is no change in Gpa1 when the ubiquitination domain is removed, we expect that it does not form significant interactions with the rest of Gpa1. It is not known, however, whether the domain itself contains any protected sites or possible secondary structure features. The two experimental approaches we would like to use are limited proteolysis and NMR. The use of limited proteolysis would give us information about whether there are any regions of the insert that are structure, and therefore protected, or whether the insert is primarily unstructured, making all residues available for proteolysis. We would also like to use NMR to observe the ubiquitination domain of Gpa1. Because Gpa1 is a large protein that is not amenable to long, three dimensional NMR experiments,

we propose to do a ^{15}N-HSQC in the absence and presence of the ubiquitination domain and compare the spectra. These experiments will provide information about whether the resonances that appear in the presence of the ubiquitination domain are primarily in the unstructured region of the spectra. They would also reveal whether there are any significant changes in the rest of the NMR spectra in the presence of the ubiquitination domain, which would suggest that the ubiquitination domain did form interactions with other regions of Gpa1.

We have already done significant work to optimize the conditions for NMR of Gpa1 without the ubiquitination domain. The thermal stability of Gpa1 is pH dependent and a significant increase in thermal stability of the GTP-bound state of the protein is observed at pH 6.0 as compared to pH 7.0 (**Figure 5.5a**). Decreasing the pH below pH 6.0 did not significantly increase thermal stability (data not shown). Using fQCR, we also showed that not all GTP analogs lead to the same increase in the thermal stability of Gpa1. AlF_3, although it has successfully been used for NMR studies of mammalian Gα (*327*), is not as thermally stabilizing as GTPγS (**Figure 5.5b**). In **Figure 5.5c-d**, the spectra obtained at pH 7.0 and pH 6.0 are shown. We can detect over 200 resonances in the Gpa1 spectrum lacking the ubiquitination domain at pH 6.0. These data suggest it will be feasible to observe changes in Gpa1 when the ubiquitination domain is present.

Next, we would like to determine if ubiquitination alters the biochemical activity of Gpa1. This requires obtaining enough monoubiquitinated Gpa1 to perform the necessary assays. In Chapter IV, we describe a number of approaches that we tried to make Gpa1$^{\Delta N}$ amenable to chemical ubiquitination. As described earlier in this chapter, there are limitations in the protein itself that made these attempts at optimization

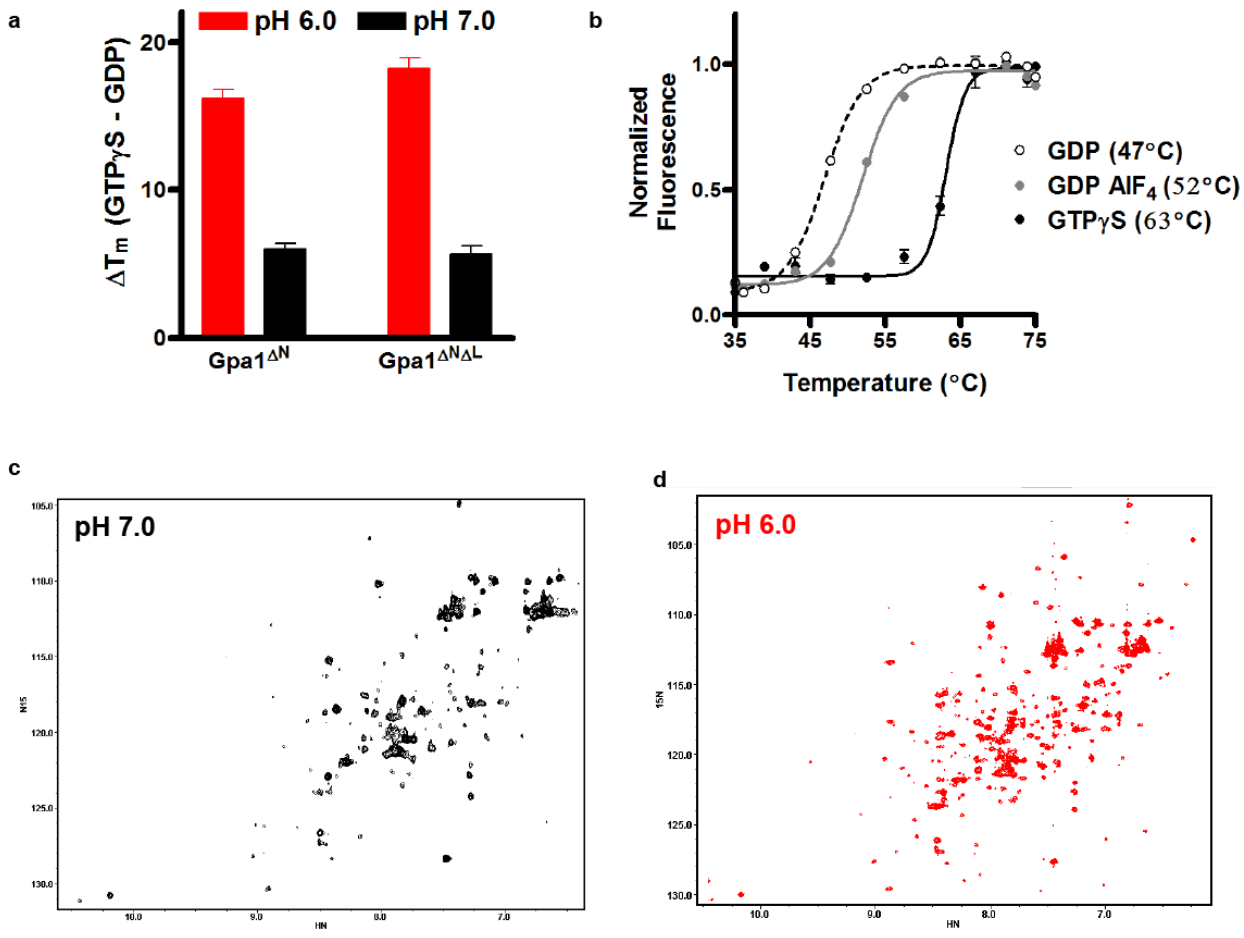

Figure 5.5. Optimization of Parameters for NMR of Gpa1. (**a**) The thermal stability of Gpa1 in the presence of GDP and GTPγS was measured by fQCR at pH 7.0 and pH 6.0. Results are plotted as the change in thermal stability between the GTPγS and GDP bound states at each pH for $Gpa^{\Delta N}$ and $Gpa1^{\Delta N\Delta L}$. (**b**) Thermal stability of Gpa1 at pH 6.0 in the presence of GDP, GDP-AlF_4, and GTPγS. (**c**) HSQC spectrum of ^{15}N-$Gpa1^{\Delta N\Delta L}$ at pH 7.0 in the presence of GTPγS. (**d**) HSQC spectrum of ^{15}N-$Gpa1^{\Delta N\Delta L}$ at pH 6.0 in the presence of GTPγS.

unsuccessful. We have a few options to generate monoubiquitinated substrate in the future. First, it is possible that ubiquitination may still work in $Gpa1^{WT}$ if the proper background cysteine mutations are made to stabilize the protein in the absence of reducing agent. Second, the protein could be ubiquitinated using the native ubiquitination complex, which has been previously reconstituted in the Dohlman lab (*284*). The problem with this approach is that the protein will not be fully modified and additional purification steps would be required to separate the modified and unmodified forms of the protein. Furthermore, Ubiquitin ligase complexes are not as specific *in vitro* as they are *in vivo*, which means Gpa1 may be monoubiquitinated at more than one lysine. Finally, there are alternative chemical ubiquitination approaches that may be successful for ubiquitinating Gpa1. One approach is to use non-natural amino acids. Recent advances in the optimization of the click chemistry approach described above have created a system where, once the proteins are expressed and purified, modification can occur in as little time as one hour in the presence of reducing agent (*328*).

Third, we would like to determine if there are binding partners of Gpa1 that recognize the ubiquitination domain. While Gpa1 may not be an ideal model for the study of Gα ubiquitination, since ubiquitination occurs on a unique insert, it is still a very interesting substrate of ubiquitination. The information obtained about this domain is relevant beyond the study of Gpa1 and G proteins. Therefore, we would like to ask not only if ubiquitination alters the activity of Gpa1, but if there are binding interactions other than those between Gpa1 and the ubiquitin ligase and deubiquitinase that utilize the ubiquitination domain. We propose to do a mass spectrometry screen of proteins that pull down with three variants of Gpa1: Gpa1, Gpa1 without the ubiquitination domain,

and Gpa1 with a lysine-less ubiquitination domain. This approach would elucidate whether there are interacting partners of Gpa1 that require the ubiquitination domain as well as whether there are interacting partners that require ubiquitination of this same domain. We predict that signaling proteins that interact with Gpa1 at the plasma membrane would not recognize the ubiquitination domain, while proteins responsible for trafficking and endosomal signaling might bind the ubiquitinated form of insert. The binding partners of Gpa1 that we would look for in our mass spectrometry screen include the pheromone receptor Ste2 (*286*), Gβγ subunit Ste4/Ste18 (*287*), GTPase accelerating protein Sst2 (*288*), non-receptor exchange factor (Get3), two subunits of phosphatidylinositol 3-kinase (Vps14 and Vps34) (*260*). Other proteins and enzymes involved in post-translational modification of Gpa1 include the N-myristoyltransferase Nmt1 (*329*), palmitoyltransferase (*330*), de-palmitoylating acyl-protein thioesterase Apt1 (*331*), Ubiquitin ligase Rsp5 (*284*), and Ubiquitin ligase SCF Cdc4 (*280*). There is precedence in other Gα proteins for another post-translational modification, phosphorylation, leading to changes in interactions with known binding partners. Gα phosphorylation leads to a loss of interaction with Gβγ (*263*), loss of interaction with RGS (*264*), loss of guanine nucleotide binding (*265*), receptor desensitization (*332, 333*), and loss of interaction with downstream effectors (*334*).

Finally, we would like to use the yeast system to understand how changes in the α-helical domain impact Gpa1 signaling *in vivo*. *S. cerevisiae* is an ideal model system for understanding mechanisms of signaling pathway output and regulation. We have already established that changes in the helical domain change the activity of Gpa1 *in vitro* and that the unique ubiquitination domain of Gpa1 does not significantly contribute to its

activity or biophysical properties. Together, these data indicate that despite the extra ubiquitination domain, Gpa1 can be used as a representative Gα for studies of α-helical domain modulation of Gα activity. We propose to test two types of Gpa1 mutants: [1] the cysteine mutations we have already characterized *in vitro* and [2] α-helical domain chimeras of the A/B helix, similar to the ones published in Jones et al. (*250*). After incorporating these mutants into yeast, we will measure pheromone pathway activation (Fus3/Kss1), gene transcription (beta-gal), endosomal localization, and possibly morphological changes or mating efficiency. We expect to see a phenotype consistent with Gpa1 activation or inactivation, depending on how the mutants or chimeras were shown to alter Gpa1 properties *in vitro*. These data would represent the first *in vivo* evidence that changes made to the α-helical domain influence the activity of the Gα.

Conclusions and Future Directions

While the studies in Chapters II, III, and IV have contributed to our knowledge about the regulation of the GTPases Ras and Gpa1 specifically, they have also contributed to our general understanding of GTPase regulation. We first see that regulation by monoubiquitination is not only site-specific, but protein specific even within the same classes of proteins or isoforms of the same GTPase. Our work highlighting the differences between mutation and monoubiquitination of Ras at K147 may explain why K147 is conserved across a number of small GTPases. There are over 150 identified GTPases, and 70 of them contain a lysine at the equivalent of position 147 (**Table 5.3**) (*120*). We mutated K147 to a number of different residues and did not observe any significant change in intrinsic or regulator-mediated Ras activity (**Table 5.4**).

The adjacent Serine (145) and Alanine (146) in the SAX motif have validated roles in coordinating the nucleotide and contributing directly to Ras activity (*183, 184*). However, the mutation to the lysine does not significantly contribute to Ras activity, suggesting it may be conserved for regulation by monoubiquitination. However, further experiments are required to determine the role of monoubiquitination in the regulation of other small GTPases. Often, it appears to be a small population of a given protein that is monoubiquitinated at a particular time. As highlighted by our kinetic modeling in Chapter II, however, a small population of an active GTPases can have a large impact on signaling. Therefore, monoubiquitination may be more prevalent in GTPase regulation than we currently know. Our mechanistic studies suggest it may be worthwhile to determine if other small GTPases that have an insert domain or lysine at the equivalent of position 147 are substrates of monoubiquitination.

Furthermore, our work has contributed to the understanding of cellular mechanisms of regulation by monoubiquitination in general. We used chemical ubiquitination approaches to mechanistically show that monoubiquitination can directly enhance the activity of its substrate upon modification. While there are more advanced methods to chemically ubiquitinate proteins that use either the enzymatic linker or a similar linker, we demonstrate that the linker generated through the formation of a disulfide accurately mimics native ubiquitination. These data should be applicable for the study of the biochemistry of other ubiquitinated substrates as well. It is also of note that this approach will be useful in characterizing the differences between regulation by monoubiquitination and other small protein modifiers such as ISG15, Nedd8, and SUMO

K147-Containing GTPases						
Di-Ras	Rab10	Rab21	Rab33A	Rab3D	Rab8A	Rap2B
Di-Ras2	Rab12	Rab22A	Rab33B	Rab41	Rab8B	Rap2C
E-Ras	Rab13	Rab22B	Rab35	Rab5A	Rab9A	RasD1
H-Ras	Rab14	Rab24	Rab35	Rab5B	Rab9B	RasD2
K-Ras2B	Rab17	Rab26	Rab36	Rab5C	RalA	RhoA
Miro1	Rab18	Rab28	Rab37	Rab6A	RalB	RhoB
Miro2	Rab19	Rab2A	Rab38	Rab6B	Rap1A	RhoC
M-Ras	Rab1A	Rab2B	Rab39A	Rab6C	Rap1A	R-Ras
Noey2	Rab1B	Rab30	Rab3B	Rab7A	Rap1B	RRP22
N-Ras	Rab1B	Rab32	Rab3C	Rab7B	Rap2A	TC21

Table 5.3. Small GTPases Containing a Lysines in the Conserved SAX motif. The table lists, in alphabetical order, all small GTPases that contain a lysine at the equivalent of Ras K147 within the SAX motif.

	Intrinsic Dissociation ($x10^{-4}$ s^{-1})	Fold increase with Sos	Intrinsic Hydrolysis (10^{-4} μM Pi/s)	Fold increase with GAP
Ras	1.2 ± 0.2	11.5	1.9 ± 0.3	12.6
Ras^{K147A}	5.4 ± 0.6	15.3	1.8 ± 0.4	10.1
Ras^{K147L}	1.9 ± 0.2	13.5	2.5 ± 0.4	11.2
Ras^{K147R}	3.1 ± 0.1	9.1	2.5 ± 0.5	10.8
Ras^{K147C}	5.5 ± 0.8	15.5	1.9 ± 0.2	10.2

Table 5.4. Mutation of K147**.** Summary of dissociation and hydrolysis data for various mutations at position 147. Results are the mean ± SE (n=4).

whose functions may, in some cases, be distinct from monoubiquitination (*5*). Furthermore, our work highlights the advantages of coupling observations of monoubiquitination *in vivo* with computational and biophysical studies on a particular substrate *in vitro*.

One of the next steps in advancing the study of monoubiquitination is the development of a database that treats monoubiquitination as a post-translational modification distinct from polyubiquitination and that recognizes that the outcomes of ubiquitination are substrate dependent. As studies that address the mechanisms by which substrates are regulated by monoubiquitination become more prevalent, new trends may emerge not only in the outcomes of monoubiquitination, but in the mechanisms by which Ubiquitin acts on its substrates. While a few small databases have begun to recognize the importance of differentiating between monoubiquitination and polyubiquitination (UbiProt), the largest databases do not include these details but primarily focus on listing substrates that are known to be monoubiquitinated (hUbiquitome and PhosphoSitePlus). On the other hand, databases of phosphorylated substrates, like PhosphoSitePlus, catalog proteins that are phosphorylated, listing both the sites of phosphorylation as well as what type of process the site-specific phosphorylation regulates *in vivo*. A similar resource for monoubiquitinated substrates would aid in elucidating patterns in mechanisms of regulation by monoubiquitination.

Understanding the diverse mechanisms by which monoubiquitination is used to regulate substrate localization, binding, and activity becomes more important as a role for ubiquitination is emerging in the study of cancer and developmental disorders. There is already evidence in the literature for the role of ubiquitination and its misregulation in

disease (*43, 89-92*). However, successful drug development studies targeting monoubiquitinated substrates will likely be case-specific, rather than designed based on general principles. This is due to differences in the way each E3 ligase interacts with its substrates as well as the fact that monoubiquitination does not act on its substrates through a single, conserved mechanism. With the ability to perform structural studies on monoubiquitinated substrates coupled with a knowledge of the mechanisms by which monoubiquitination acts, inhibitors of high priority targets could be developed using fragment-based inhibitor design approaches (*335*). Interest in understanding more about monoubiquitination is not only relevant for drug development and disease related purposes, but for understanding normal cell maintenance and functioning as well. Small populations of a substrate may be modified at any time, meaning that knowing common mechanisms of regulation by ubiquitination as well as the sites of ubiquitination will contribute to identifying potential candidate proteins for regulation by ubiquitination. As our knowledge about this post-translation modification increases, so will appreciation for the complex and elegant manner in which monoubiquitination fine tunes cell signaling regulation.

Methods

Thermal Stability of Gpa1

The fast quantitative cysteine reactivity (fQCR) method (*182*) was employed to measure changes in Gpa1 thermal stability. Briefly, 2 μM protein was incubated with 1 mM 4-fluoro-7-aminosulfonylbenzoflurazan (ABD–F, Anaspec) at pH 7.0 in the presence of 20 μM GDP or GTpγS and 2 mM $MgCl_2$ at the desired temperature for three

minutes. When the assays were performed at pH 6.0, 2 mM ABD-F was used. For assays with AlF_4, 10 mM NaF and 30 μM $AlCl_3$ was added to the reaction in the presence of 20 μM GDP. The reaction was quenched with HCl and ABD-F fluorescence was measured on a PHERAstar plate reader (BMG Labtech, excitation at 400 nm and emission at 500 nm). The data were normalized and fit to determine the temperature at which half the protein was unfolded, representing the melting temperature (T_m).

NMR Experiments with Gpa1

For NMR studies, ^{15}N– enriched samples of $Gpa1^{\Delta N\Delta L}$ were produced using standard protocols in M–9 minimal media (*181*). ^{1}H–^{15}N 2D HSQC experiments were conducted on a Varian 700 MHz (pH 7.0) and a Bruker 700 MHz (pH 6.0) with a cryoprobe in 20 mM Phosphate Buffer, 100 mM NaCl, 5 mM $MgCl_2$, 10% D_2O, 1 mM TCEP , and 2 mM GTPγS at 25°C and with 100 μM protein.

Intrinsic and Regulator-Mediated Ras Activity

The rate of nucleotide dissociation was measured using MANT-GDP (BioLog: San Diego, CA) as previously reported (*194, 195*). Briefly, MANT-GDP-bound Ras (2 μM) was added to 1 mL assay buffer (50 mM Tris, pH 7.4, 50 mM NaCl and 5 mM $MgCl_2$) and exchange was initiated by addition of 2 mM GDP. The rate of MANT-GDP dissociation was measured as a change in fluorescence intensity over time (LS50B Perkin-Elmer Luminescence Spectrometer) at an excitation wavelength of 360 nm and an emission wavelength of 440 nm. Fluorescent nucleotide dissociation curves were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a one-phase exponential decay curve. For GEF-mediated dissociation and binding, 200 nM Ras and 0.2 μM to 20 μM Sos^{cat} were used. The nucleotide dissociation rate was plotted as a function of Sos^{cat}

concentration and fit to one site binding in Prism to determine the binding affinity between Ras or mUbRas and Sos^{cat}. Results are the mean ± SE (n=4).

Single-turnover GTP hydrolysis assays were done as previously described (*196*), except that the phosphate binding protein Flippi 5U (Addgene) was used to detect inorganic phosphate released upon GTP hydrolysis (*185*). Flippi 5U was purified as previously described (*185*). All buffers were made phosphate free by extensive dialysis with 1 U nucleoside phosphorylase and 2 mM inosine (Sigma, USA). For GAP-mediated hydrolysis, 50 μM Ras and 0.1 μM (1:500) GAP-334 was used. The ratio of fluorescence emission was measured at 480 nm and 530 nm with an excitation of 435 nm on a SpectraMax M5 (Molecular Devices). Fluorescence ratios were converted to phosphate concentrations using a standard curve. Hydrolysis curves were fit in GraphPad Prism (GraphPad Software; San Diego, CA) to a one-phase exponential association curve as shown in Figure S2. Results are the mean ± SE (n=6).

APPENDIX A

INTERACTION SURFACES BETWEEN RAS AND RAS-GEF OR RAS-GAP

Ras Residue	GEF Residue	Distance (Angstroms)
5-LYS	910-ASP	5.52
5-LYS	911-HIS	5.67
17-SER	942-GLU	3.87
17-SER	938-LEU	5.42
17-SER	939-LYS	5.76
21-ILE	939-LYS	4.38
21-ILE	942-GLU	5.18
25-GLN	944-ASN	5.86
30-ASP	945-PRO	4.83
31-GLU	944-ASN	4.36
31-GLU	963-LYS	5.89
32-TYR	944-ASN	5.41
32-TYR	939-LYS	5.52
33-ASP	963-LYS	5.6
33-ASP	940-THR	5.77
34-PRO	939-LYS	4.04
34-PRO	936-ASN	4.85
34-PRO	944-ASN	5.55
34-PRO	967-ILE	5.9
34-PRO	940-THR	5.98
35-THR	936-ASN	5.25
35-THR	916-LEU	5.59
37-GLU	913-LYS	5.72
40-TYR	911-HIS	4.91
40-TYR	910-ASP	5.78
40-TYR	939-LYS	5.96
40-TYR	913-LYS	5.97
41-ARG	910-ASP	5.06
41-ARG	911-HIS	5.96
54-ASP	911-HIS	3.81
54-ASP	910-ASP	5.63
56-LEU	911-HIS	5.02
57-ASP	939-LYS	5.66
58-THR	935-THR	5.98
59-ALA	939-LYS	5.18
59-ALA	935-THR	5.85

59-ALA	942-GLU	5.95
59-ALA	938-LEU	5.98
61-GLN	935-THR	4.68
61-GLN	934-LEU	4.99
61-GLN	932-ILE	5.21
61-GLN	912-TYR	5.8
61-GLN	929-PHE	5.94
62-GLU	810-THR	5.52
62-GLU	809-TRP	5.59
62-GLU	938-LEU	5.9
63-GLU	825-ILE	4.17
63-GLU	826-ARG	5.17
63-GLU	815-GLU	5.44
63-GLU	814-LYS	5.91
63-GLU	829-THR	5.96
63-GLU	809-TRP	5.97
63-GLU	822-LEU	5.99
64-TYR	828-THR	4.28
64-TYR	934-LEU	4.74
64-TYR	825-ILE	4.86
64-TYR	821-LEU	5.42
64-TYR	929-PHE	5.81
64-TYR	824-MET	5.83
64-TYR	829-THR	5.84
65-SER	829-THR	4.48
65-SER	1002-GLU	4.79
65-SER	833-LEU	5.71
66-ALA	832-THR	3.88
66-ALA	829-THR	4.72
66-ALA	836-GLU	4.85
66-ALA	833-LEU	5.01
66-ALA	876-SER	5.72
67-MET	876-SER	4.11
67-MET	912-TYR	5.29
67-MET	872-LEU	5.35
67-MET	832-THR	5.46
67-MET	828-THR	5.76
67-MET	929-PHE	5.99
68-ARG	1002-GLU	4.92
69-ASP	881-SER	4.37
69-ASP	880-SER	5.14
69-ASP	882-PRO	5.67
69-ASP	1006-THR	5.71

69-ASP	836-GLU	5.81
70-GLN	879-ASN	3.96
70-GLN	876-SER	4.72
70-GLN	875-VAL	4.95
70-GLN	905-HIS	5.17
70-GLN	912-TYR	5.33
70-GLN	908-SER	5.5
70-GLN	872-LEU	5.64
71-TYR	929-PHE	5.06
71-TYR	912-TYR	5.43
71-TYR	932-ILE	5.67
73-ARG	884-TYR	5.44
73-ARG	881-SER	5.49
73-ARG	879-ASN	5.95
95-GLN	1007-ASP	5.08
95-GLN	1003-LYS	5.28
95-GLN	1006-THR	5.96
98-GLU	1003-LYS	5.75
99-GLN	1002-GLU	5.75
102-ARG	881-SER	4.52
102-ARG	1006-THR	5.2
102-ARG	1011-ASN	5.31
102-ARG	1007-ASP	5.38
102-ARG	1003-LYS	5.45
102-ARG	1010-PHE	5.95
103-VAL	881-SER	3.29
105-ASP	1019-ARG	4.45

Ras Residue	GAP Residue	Distance (Angstroms)
11-ALA	790-ALA	4.89
11-ALA	791-THR	5.78
17-SER	949-LYS	4.49
21-ILE	949-LYS	4.58
25-GLN	949-LYS	4.9
25-GLN	948-ALA	4.91
27-HIS	948-ALA	5.54
29-VAL	949-LYS	5.26
30-ASP	785-THR	4.76
31-GLU	785-THR	5.65
31-GLU	944-VAL	5.91
31-GLU	894-ARG	5.92
31-GLU	948-ALA	5.93
32-TYR	902-LEU	4.78
32-TYR	785-THR	5.5
32-TYR	942-ASN	5.59
32-TYR	894-ARG	5.62
32-TYR	897-SER	5.62
32-TYR	789-ARG	5.65
32-TYR	788-PHE	5.83
32-TYR	938-GLN	5.94
33-ASP	949-LYS	3.83
33-ASP	944-VAL	4.92
33-ASP	942-ASN	5.51
33-ASP	938-GLN	5.69
33-ASP	939-ASN	5.83
34-PRO	902-LEU	3.53
34-PRO	942-ASN	4.82
34-PRO	789-ARG	5.11
34-PRO	938-GLN	5.26
35-THR	949-LYS	5.05
35-THR	789-ARG	5.93
36-ILE	938-GLN	4.01
36-ILE	906-CYS	4.31
36-ILE	902-LEU	4.41
36-ILE	935-LYS	5.1
36-ILE	910-LEU	5.24
36-ILE	934-ALA	5.46
36-ILE	931-ILE	5.87
37-GLU	928-ARG	5.1

37-GLU	931-ILE	5.36
37-GLU	935-LYS	5.5
38-ASP	935-LYS	5.1
38-ASP	950-GLU	5.72
38-ASP	949-LYS	5.95
39-SER	950-GLU	4.09
39-SER	935-LYS	5.65
39-SER	928-ARG	5.67
39-SER	831-SER	5.82
40-TYR	950-GLU	5.73
40-TYR	951-PRO	5.86
40-TYR	949-LYS	5.97
41-ARG	833-SER	5.44
61-GLN	902-LEU	5.52
61-GLN	791-THR	5.74
61-GLN	789-ARG	5.79
61-GLN	903-ARG	5.96
62-GLU	750-THR	4.8
62-GLU	799-GLU	4.88
62-GLU	791-THR	4.89
62-GLU	796-THR	5.63
62-GLU	903-ARG	5.73
62-GLU	749-ARG	5.99
63-GLU	907-PRO	4.59
63-GLU	903-ARG	5.04
63-GLU	795-SER	5.21
63-GLU	799-GLU	5.36
63-GLU	802-MET	5.46
63-GLU	803-LYS	5.83
64-TYR	902-LEU	4.59
64-TYR	907-PRO	4.84
64-TYR	910-LEU	5.34
64-TYR	906-CYS	5.69
64-TYR	938-GLN	5.81
64-TYR	903-ARG	5.83
66-ALA	911-ASN	4.79
67-MET	910-LEU	5.17
67-MET	911-ASN	5.76
70-GLN	927-ALA	5.18
85-ASN	782-ASP	5.64
86-ASN	790-ALA	4.33
88-LYS	790-ALA	3.29
88-LYS	792-THR	4.01

88-LYS	791-THR	4.62
88-LYS	749-ARG	5.14
91-GLU	749-ARG	5.83
92-ASP	749-ARG	4.97
92-ASP	791-THR	5.22
92-ASP	790-ALA	5.92
95-GLN	749-ARG	5.8
117-LYS	786-THR	5.31
120-LEU	783-GLU	5.76

APPENDIX B

NMR BACKBONE ASSIGNMENTS FOR RAS^{K147A}

Ras Residue	^{1}H (ppm)	^{15}N (ppm)	Peak Intensity
4	8.77	121.94	1.63
5	9.13	124.59	1.41
6	9.45	126.27	0.95
8	7.17	112.41	1.58
9	9.17	120.66	1.52
10	7.13	107.55	1.57
11	8.76	121.57	1.37
12	8.61	106.28	1.65
13	10.57	115.01	0.95
14	7.67	113.46	1.69
15	8.57	109.51	1.08
16	10.57	125.40	0.71
17	9.34	120.48	1.32
18	9.49	125.41	1.26
19	9.06	120.44	1.90
20	7.71	116.93	1.49
21	8.90	120.66	1.47
22	7.91	120.69	1.41
23	7.67	120.63	1.58
25	9.00	115.88	1.10
26	7.95	116.48	1.56
27	6.79	111.82	4.71
28	8.59	122.51	1.26
29	7.83	126.48	1.02
30	7.81	122.25	1.59
31	7.67	119.30	2.11
32	8.82	125.69	1.41
33	7.86	128.81	1.09
35	8.98	109.94	0.99
36	6.84	120.92	1.01
37	8.41	131.92	0.87
38	8.16	124.44	1.63
39	8.39	114.01	1.38
40	9.09	121.39	1.87
41	8.38	120.23	1.63
42	8.66	122.01	1.59

43	8.93	128.95	1.21
44	8.68	121.06	1.17
45	8.05	120.92	2.15
46	8.11	125.61	1.61
47	9.55	130.74	0.78
48	8.30	133.99	-1.48
49	7.67	122.14	2.26
50	8.89	125.68	1.82
51	8.69	123.46	2.75
52	8.85	128.99	1.91
53	8.99	123.31	1.38
54	8.73	125.51	1.39
55	9.17	123.65	1.71
56	8.76	127.57	1.02
57	8.47	129.17	0.77
58	6.89	110.32	2.34
59	7.88	120.43	1.26
60	8.28	108.07	2.45
61	8.58	119.01	2.10
62	8.76	120.60	2.25
63	8.29	120.02	2.96
64	8.28	121.23	1.75
65	7.91	120.03	1.66
66	8.00	120.62	1.97
67	8.23	117.73	1.90
68	7.82	123.79	1.75
69	8.11	118.40	2.13
70	7.82	117.49	3.19
71	8.23	119.78	1.67
72	8.54	118.82	1.13
73	7.96	115.36	1.58
74	7.90	107.76	1.28
75	7.98	111.20	1.23
76	9.01	122.33	0.91
77	7.16	131.95	-1.69
78	8.16	121.31	1.06
79	9.17	125.96	1.36
80	8.66	124.19	1.58
81	8.98	125.97	1.51
82	9.27	123.83	1.46
83	8.85	128.78	1.48
84	8.24	126.32	2.20
85	7.84	117.18	1.52

86	7.95	119.30	1.95
88	8.43	124.04	2.32
89	8.08	114.29	2.13
90	7.39	124.65	1.39
91	8.47	121.64	1.66
92	8.44	117.31	2.03
93	7.57	120.55	1.98
94	7.78	116.89	1.45
95	7.45	116.65	2.20
96	7.53	119.20	1.79
97	8.36	118.79	1.75
98	7.97	117.45	1.87
100	7.76	119.94	1.40
102	7.78	117.65	2.21
103	8.35	118.43	1.44
104	8.04	116.70	1.53
106	7.47	109.07	2.64
107	8.35	122.01	2.39
108	8.40	121.08	2.68
109	7.58	122.20	3.59
113	8.79	129.04	0.85
114	9.18	128.21	1.36
115	8.02	114.57	1.12
116	8.71	121.61	1.58
117	9.10	120.87	1.89
118	7.09	114.51	1.44
119	8.72	116.74	1.36
120	7.75	120.94	1.87
121	8.16	122.92	2.07
122	7.71	121.53	2.87
123	7.99	119.74	2.45
124	9.08	113.76	0.65
125	7.65	124.59	1.28
126	8.73	127.35	1.72
127	9.41	121.77	1.61
128	8.63	117.86	1.85
129	6.77	116.90	2.08
130	7.03	123.48	1.96
131	8.56	117.83	2.19
132	7.84	119.73	1.92
133	7.47	123.52	2.15
134	8.39	121.83	2.01
135	8.46	118.27	2.31

136	7.92	117.75	2.18
137	7.60	120.07	1.92
138	8.30	110.93	1.57
139	8.04	113.04	1.77
141	8.19	120.10	2.39
142	8.45	129.93	1.47
143	7.85	124.44	1.65
144	8.29	122.24	2.91
145	8.05	118.16	1.92
146	8.15	122.75	1.84
147	8.02	117.70	1.70
148	7.74	118.74	1.14
149	8.64	111.53	1.28
150	7.36	112.40	1.18
151	8.83	114.65	1.02
152	7.09	120.24	2.15
153	8.24	116.70	1.97
154	8.10	116.32	2.49
155	8.50	124.04	2.20
156	7.15	112.78	0.76
157	9.61	119.23	1.49
158	8.51	117.02	1.54
159	7.16	121.47	1.81
160	7.50	118.85	1.65
161	8.13	119.16	1.44
162	8.12	118.21	1.57
163	8.11	121.94	1.16
164	9.41	123.50	1.72
165	7.61	115.59	2.36
166	7.67	124.84	3.40

APPENDIX C

NMR BACKBONE ASSIGNMENTS FOR RHOA

RhoA Residue	Amino Acid	^{1}H (ppm)	^{15}N (ppm)	Peak Intensity
2	ALA	8.27	125.05	1.45
3	ALA	8.19	123.46	1.67
4	ILE	8.25	122.16	0.85
5	ARG	8.54	128.19	0.62
6	LYS	8.82	123.68	0.50
7	LYS	10.57	125.08	0.29
8	LEU	9.45	133.75	0.32
9	VAL	8.01	125.80	0.39
10	ILE	8.85	123.58	0.39
11	VAL	9.18	130.47	0.36
12	GLY	7.44	113.49	-0.06
13	ASP	8.78	121.45	0.26
14	GLY	8.86	107.38	0.33
15	ALA	10.34	124.28	0.14
20	CYS	8.32	110.14	0.18
23	ILE	6.98	113.71	0.21
25	PHE	8.79	113.75	0.36
26	SER	7.63	113.73	-0.21
27	LYS	8.53	122.48	0.14
29	GLN	6.55	113.46	0.71
30	PHE	9.07	128.76	0.46
32	GLU	8.09	121.70	0.69
33	VAL	7.97	116.83	0.35
34	TYR	7.89	120.31	0.59
35	VAL	7.85	127.20	0.67
40	GLU	8.74	121.14	0.15
41	ASN	8.96	115.11	0.45
42	TYR	7.75	125.14	0.26
43	VAL	7.51	126.51	0.33
44	ALA	8.99	130.81	0.39
45	ASP	8.29	121.11	0.70
46	ILE	9.08	122.73	0.64
47	GLU	8.16	127.06	0.95
48	VAL	8.53	123.56	0.58
49	ASP	9.26	125.30	0.54
50	GLY	8.79	133.38	-0.49

51	LYS	8.19	121.71	0.83
52	GLN	8.29	122.22	0.72
53	VAL	9.14	126.69	0.55
54	GLU	8.92	127.29	0.43
55	LEU	9.33	128.19	0.47
56	ALA	9.40	129.93	0.47
57	LEU	8.96	123.66	0.23
62	GLY	7.90	115.66	0.19
64	GLU	9.08	119.52	0.45
65	ASP	8.56	120.18	0.33
66	TYR	8.07	116.58	0.47
67	ASP	8.82	124.00	0.28
68	ARG	8.54	112.17	0.37
69	LEU	7.78	111.75	0.38
70	ARG	6.92	112.21	0.14
73	SER	8.14	113.73	0.42
74	TYR	6.67	118.68	0.35
76	ASP	8.87	117.06	0.30
77	THR	7.40	116.52	0.69
78	ASP	9.00	122.61	0.30
79	VAL	7.86	116.58	0.52
80	ILE	6.53	115.56	0.55
82	MET	8.25	125.77	0.50
83	CYS	7.80	116.37	0.49
84	PHE	9.13	114.77	0.32
85	SER	9.30	113.70	0.29
86	ILE	8.79	111.96	0.42
87	ASP	8.32	115.00	0.26
88	SER	7.92	110.25	0.60
90	ASP	9.02	118.49	0.66
91	SER	8.35	117.48	0.45
92	LEU	7.14	124.48	0.42
93	GLU	7.86	119.84	0.45
94	ASN	7.53	112.93	0.51
95	ILE	8.41	123.68	0.40
97	GLU	8.74	116.26	0.24
98	LYS	8.80	115.58	0.33
99	TRP	7.67	125.88	0.38
100	THR	7.97	117.41	0.07
102	GLU	6.92	116.50	0.48
103	VAL	8.59	117.02	0.43
104	LYS	8.67	116.96	0.52
105	HIS	7.73	116.82	0.27

106	PHE	7.48	113.30	0.29
107	CYS	8.73	117.46	0.41
109	ASN	9.13	127.28	0.39
110	VAL	7.09	122.16	0.69
113	ILE	9.20	127.36	0.47
114	LEU	8.43	130.39	0.36
115	VAL	9.08	128.37	0.31
116	GLY	8.44	112.16	0.24
119	LYS	8.74	117.00	0.39
120	ASP	7.96	118.11	0.40
121	LEU	7.11	121.23	0.69
122	ARG	9.20	130.03	0.36
123	ASN	8.48	119.67	0.65
124	ASP	7.56	117.16	0.13
125	GLU	8.12	118.27	0.23
126	HIS	7.38	118.03	0.62
127	THR	8.33	117.83	0.43
128	ARG	8.27	118.91	0.51
129	ARG	8.39	120.92	0.63
130	GLU	8.39	120.08	0.55
131	LEU	8.46	119.54	0.55
132	ALA	8.13	122.31	0.73
133	LYS	7.49	119.60	0.78
134	MET	7.40	116.19	0.98
135	LYS	8.02	114.73	0.50
136	GLN	7.90	116.16	0.63
137	GLU	7.69	111.55	0.34
142	GLU	9.53	115.03	0.51
143	GLU	7.27	118.94	0.47
145	ARG	7.68	121.15	0.59
146	ASP	7.95	118.78	0.53
147	MET	7.60	120.26	0.57
148	ALA	8.21	121.60	0.66
149	ASN	7.80	116.04	0.81
150	ARG	7.57	120.43	0.51
151	ILE	7.91	109.00	0.42
152	GLY	7.64	110.53	0.43
153	ALA	8.57	122.05	0.47
154	PHE	9.76	125.04	0.30
155	GLY	7.31	129.46	-0.52
156	TYR	8.28	119.95	0.68
159	CYS	8.09	120.46	0.55
160	SER	7.78	112.33	0.40

162	Lys	8.24	123.12	0.15
164	LYS	7.81	118.15	0.39
165	ASP	7.92	121.98	0.75
166	GLY	9.14	115.43	0.44
167	VAL	7.39	120.09	0.67
168	ARG	8.70	119.12	0.40
169	GLU	9.39	118.39	0.39
170	VAL	7.86	121.33	0.58
171	PHE	7.03	116.54	0.43
172	GLU	9.04	124.23	0.49
173	MET	7.98	119.65	0.56
174	ALA	8.21	119.13	0.75
175	THR	7.84	114.49	0.42
176	ARG	8.21	120.77	0.52
177	ALA	8.02	120.56	0.65
178	ALA	7.83	119.05	0.61
179	LEU	7.84	116.08	0.91
180	GLN	7.43	118.14	1.21
181	ALA	7.71	130.00	2.21
sidechain		10.05	128.26	0.53
Sidechain		10.19	117.02	0.11
sidechain		6.70	117.02	0.19
sidechain		7.64	114.18	0.62
sidechain		7.88	113.15	0.88
sidechain		7.80	112.84	0.69
sidechain		7.59	112.79	0.64
sidechain		6.98	114.18	0.64
sidechain		7.00	113.15	1.04
sidechain		6.92	112.80	0.57
sidechain		6.88	112.84	0.81
sidechain		7.55	112.35	1.56
sidechain		7.62	112.19	1.72
sidechain		7.69	112.02	1.70
sidechain		7.55	111.87	0.31
sidechain		7.23	109.38	0.44
sidechain		6.83	112.34	1.31
sidechain		6.75	112.20	1.46
sidechain		7.01	112.02	1.57
sidechain		6.83	111.86	0.24
sidechain		6.75	109.37	0.33

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