Regulation of large and small G proteins by ubiquitination

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ABSTRACT
Many sensory and chemical signal inputs are transmitted by intracellular GTP-binding (G) proteins. G proteins make up two major subfamilies: “large” G proteins comprising three subunits and “small” G proteins, such as the proto-oncogene product RAS which contains a single subunit. Members of both subfamilies are regulated by post-translational modifications, including lipidation, proteolysis and carboxyl methylation. Emerging studies have shown that these proteins are also modified by ubiquitination. Much of our current understanding of this post-translational modification comes from investigations of the large G protein α subunit from yeast (Gpa1) and the three RAS isotypes in humans, NRAS, KRAS and HRAS. Gα undergoes both mono- and polyubiquitination, and these modifications have distinct consequences for determining the sites and mechanisms of its degradation. Genetic and biochemical reconstitution studies have revealed the enzymes and binding partners required for addition and removal of ubiquitin, as well as the delivery and destruction of both the mono- and polyubiquitinated forms of the G protein. Complementary studies of RAS have identified multiple ubiquitination sites, each having distinct consequences for binding to regulatory proteins, shuttling to and from the plasma membrane, and degradation. Here, we review what is currently known about these two well-studied examples, Gpa1 and the human RAS proteins, which have revealed additional mechanisms of signal regulation and dysregulation relevant to human physiology. We also compare and contrast the effects of G protein ubiquitination with other post-translational modifications of these proteins.

A variety of sensory and chemical signals are detected by receptors at the cell surface. In many cases these inputs are transmitted by intracellular GTP-binding proteins (Figure 1). Generally speaking, large G proteins are activated by 7 transmembrane segment receptors, also known as G protein-coupled receptors (GPCRs). When these receptors bind to an agonist ligand, the G protein α subunit releases GDP, binds to GTP, and dissociates from the Gβγ subunit dimer. GTP-bound Gα, Gβγ, or both go on to activate intracellular effectors that transduce the signal. Similarly, many small G proteins are activated by growth factor receptors and their associated guanine nucleotide exchange factors (GEFs). As with GPCRs, GEFs promote the exchange of GTP for GDP and subsequent activation of downstream effectors. For both large and small G proteins, signaling persists until GTP is converted to GDP, and this inactivation step is accelerated by binding to GTPase activating proteins (GAPs). Thus, GEFs and GAPs work in opposition to one another to regulate the activity of their cognate G proteins, large and small. Here we review newer mechanisms of G protein regulation by ubiquitination, with a focus on a large G protein from yeast and three small G proteins from humans: NRAS, KRAS and HRAS. We compare and contrast the effects of ubiquitination with that of other post-translational modifications, where such comparisons have proven to be instructive.

Small (~21 kD) G proteins are part of a large superfamily of GTPases that contains over 170 members in humans. In addition to RAS, these include members of the RAB, RHO, ARF, and RAN subfamilies [reviewed in (1)]. The mammalian RAS proteins – comprised of HRAS, NRAS and two alternatively spliced forms of KRAS (KRAS4A and KRAS4B) – play key roles in regulating a host of cellular processes including cell growth, differentiation, morphology, motility and survival. These RAS proteins show high (~90%) sequence identity within the core guanine nucleotide binding (G)-domain but show substantial sequence divergence in the C-terminal hypervariable region. In addition, several post-translational modifications occur within the hypervariable region including prenylation, proteolysis and carboxyl methylation [reviewed in (2)]. These modifications facilitate membrane association needed for RAS protein function.

Prenylation is the transfer of either a farnesyl (C15) or geranyl-geranyl group (C20) to the C-terminal Cys of a target protein. Correspondingly, farnesylation is common to all three RAS proteins, and is the first step required for membrane association. Following the prenylation step, three C-terminal amino acids are cleaved,
and the new C-terminus is carboxyl methylated. HRAS, NRAS and KRAS4A further undergo palmitoylation (2,3), which refers to the reversible thioester linkage of palmitic acid (C16:0) to cysteine residues. Whereas lipidation is required for membrane association, sequence differences in the hypervariable region give rise to distinct and dynamic partitioning of the RAS isoforms between various endomembranes and the plasma membrane. This in turn, contributes to isoform-specific localization and signaling (2).

Large G proteins undergo many of the post-translational modifications reported for RAS, and these processes play a particularly important role in the life cycle of yeast *Saccharomyces cerevisiae*. Yeast can exist as one of two haploid cell types (α and α) that secrete specific peptide pheromones (α factor and α factor), which bind to cell-surface receptors that activate a heterotrimeric G protein. This initiates events necessary for mating, or the fusion of α and α cells to form an α/α diploid, including new gene transcription, morphological changes, cell-cycle arrest and cell-cell adhesion. In this instance the Gbg subunit dimer (Ste4/18) binds to effector proteins, which include a MAPK scaffold (Ste5) and a GEF for the small G protein Cdc42 (Cdc24) [reviewed in (4)]. In addition, the Ga subunit Gpa1 activates the phosphatidylinositol 3-kinase Vps34, located predominantly at endosomes (5).

Proper localization of the heterotrimeric G protein complex depends on post-translational modification. Gpa1, like most other Ga proteins (6,7), is myristoylated (8,9) and palmitoylated (10,11) [reviewed in (12)]. Myristoylation refers to the covalent attachment of myristic acid (C14:0) to the N-terminal glycine of proteins, a process that occurs co-translationally and is considered irreversible. The Gβγ dimer is anchored to the plasma membrane through prenylation (introduced above) and palmitoylation of the γ subunit, Ste18 (11,13,14).

While past efforts have focused on protein lipidation, our focus here is on regulation by ubiquitination. Ubiquitination and lipidation share the ability to regulate protein-protein interactions and to direct modified proteins to specific compartments within the cell. Our discussion is centered on two of the best characterized substrates for ubiquitination, RAS and Gpa1. In this context it is worth noting the important role of yeast in the identification of the enzymes that modify these G proteins. Yeast genetic screens coupled with biochemical analysis, using RAS as a test substrate, revealed the first enzymes responsible for protein palmitoylation (15-17) as well as farnesylation, proteolysis and carboxymethylation [Reviewed in (18)]. The first myristoyltransferase was isolated from yeast and initially characterized using Gpa1 as a substrate (19-22). The first farnesyltransferase inhibitors, used subsequently to inhibit RAS oncogenesis, were identified in yeast (18) and with Gbg signaling as a functional readout of inhibitor activity (13). Below we describe the identification of enzymes that ubiquitinate G proteins, and the functional consequences of this modification for yeast Gpa1 and mammalian RAS.

**Gα regulation by mono- and poly-ubiquitination: lessons from yeast.**

Given their role as transducers between cell surface receptors and intracellular effectors, G proteins are well positioned to serve as targets of regulation. Recent attention has focused on the regulation of G protein signaling by ubiquitination [reviewed in (23)] (Figure 2). Although originally considered a tag for the irreversible destruction of protein substrates, ubiquitination is now recognized to serve as a reversible modification that affects a wide array of cellular processes, including enzyme activity, subcellular localization and protein-protein interaction.

Ubiquitination is the process by which the ubiquitin polypeptide is covalently attached to a lysine residue in a given substrate. This three-step process is mediated by distinct enzymes (E1, E2 and E3), the last of which defines substrate specificity and the timing of ubiquitination. The E1 activating enzyme transfers ubiquitin to the E2 conjugating enzyme, which in conjunction with the E3 ligase transfers ubiquitin to lysine residues on the substrate. These are subdivided into two major families known as HECT
family ligases and RING (Really Interesting New Gene) family ligases (Figure 2). Ubiquitin can itself be ubiquitinated at any of 7 internal lysine residues, resulting in various branch-chain forms of poly-ubiquitin. Generally speaking, polyubiquitinated substrates are recognized and degraded by the proteasome protease complex. More rarely, proteins are mono-ubiquitinated and subsequently transported to the lysosome (or vacuole in yeast) where they are degraded by resident proteases [Reviewed in (24-26)]. In that regard, ubiquitination, like myristoylation and prenylation, has the ability to direct G proteins to specific compartments within the cell. Whereas myristoylation and prenylation are required for delivery to the plasma membrane, ubiquitination is required for delivery to the site of degradation. As detailed below, ubiquitination of RAS promotes degradation but also affects binding to activators, regulators, and effectors.

While myristoylation and prenylation occur within well-defined consensus sequences, ubiquitination does not. This has created challenges to investigations of the biological role of ubiquitination. Although it is possible to systematically replace lysine residues and monitor the presence or absence of ubiquitin-modified protein, this approach may be impractical for proteins with a large number of these residues (there are 43 lysines in Gpa1). Moreover, such attempts are often futile since mutation of a ubiquitinated lysine can lead to the modification of a second non-physiological site, possibly affecting the function of the protein (27,28). Given that the mutagenesis approach can be misleading, an unbiased approach is needed to identify the native site of ubiquitination for each protein before it can be characterized functionally. A breakthrough in these efforts has been the implementation of mass spectrometry methods to map ubiquitination sites, as first reported for Gpa1 (29). This advance required several technical refinements necessary to obtain sufficient quantities of material, particularly since the ubiquitinated species represents a very small proportion of the total protein and, once modified, is usually destined for rapid degradation.

The example of Gpa1 is illustrative of the approach, which was later used to map more than 19,000 unique sites of ubiquitination in human cells (30) and an additional 100,000 + sites currently entered in PhosphoSitePlus (www.phosphosite.org). These efforts have revealed over 400 ubiquitination sites for all 16 subtypes of Ga in mammalian cells and >180 sites in RAS superfamily proteins. Furthermore, these advances have facilitated the development of powerful new bioinformatics tools designed to predict sites of modification (23,31).

As originally described (29), His6-tagged Gpa1 was used because it will bind to metal affinity resins even in the presence of denaturing agents such as urea. The purified protein was analyzed by electrospray ionization tandem mass spectrometry and the result spectra scanned for peptides corresponding to the predicted tryptic cleavage products of ubiquitin ligated to Gpa1 (29). In particular, trypsin cleavage separates the C-terminal Gly-Gly dipeptide from the preceding Arg residue of ubiquitin. Alternatively, the di-Gly motif can be detected with specific antibodies (30).

The next objective was to determine the function(s) of ubiquitinated Gpa1. As noted above, Gpa1 is an unusual example of a protein that undergoes both mono- and poly-ubiquitination. Other prominent examples include the human tumor suppressor p53 (32) and the proliferating cell nuclear antigen (33). Such dually-modified proteins afford the opportunity to compare regulatory mechanisms and functional consequences for each type of ubiquitin linkage, for a given substrate. Gpa1 is particularly amenable to such analysis, given the large number of quantitative functional readouts of G protein signaling and the sophisticated genetic tools available in yeast.

Initial efforts at functional characterization revealed that Gpa1 abundance is elevated in mutants deficient in either proteasomal (cim3) or vacuolar protease (pep4) function (34). Whereas the vacuolar protease mutants accumulate monoubiquitinated Gpa1, proteasome-defective mutants accumulate polyubiquitinated Gpa1. Whereas monoubiquitinated Gpa1 becomes
concentrated within the lumen of the vacuole, polyubiquitinated Gpa1 exhibits a punctate expression pattern in the cytoplasm. In cells lacking the deubiquitinating protease Ubp12, Gpa1 accumulates in both mono- and polyubiquitinated forms, and is enriched in both the cytoplasm and vacuole. Thus, mono- and poly-ubiquitination represent important targeting signals, both of which lead to the eventual degradation of Gpa1. The yeast Gβ (Ste4) and G protein-coupled receptor (Ste2) are likewise monoubiquitinated (35,36). In contrast to Gpa1 however, monoubiquitination of Ste4 does not lead to internalization and delivery to the vacuole. Rather monoubiquitination occurs in response to pheromone stimulation and promotes proper cell polarization and pheromone gradient detection (36). 

After identifying the enzymes responsible for monoubiquitination of Gpa1, the next challenge was to establish the machinery used for delivery of modified protein to the vacuole. Critical to this process is recognition by ubiquitin-binding domain (UBD)-containing proteins [reviewed in (37)]. Numerous UBDs have been defined and are annotated in the protein descriptions found in the Saccharomyces Genome Database (SGD; www.yeastgenome.org). Although structurally diverse, UBDs share the ability to bind non-covalently to ubiquitin-conjugated substrates and to transport monoubiquitinated proteins through the various stages of endocytosis to their final destination [reviewed in (38)]. With 39 known UBD-containing proteins as a starting point, gene deletion mutants were systematically screened for any with defects in the vacuolar delivery of GFP-tagged Gpa1 (39). Because Gpa1 and Ste2 are targeted for internalization by the same modification, the two proteins were tracked side-by-side. This analysis revealed seven ubiquitin binding domain-containing proteins required for constitutive internalization of Gpa1. Of these, four were required for Gpa1 but not Ste2, thereby demonstrating that endocytosis of the G protein and receptor are distinct processes regulated by distinct binding partners. The question remained, was mono- and poly-ubiquitination mediated by the same or distinct ubiquitin ligases?

Given that the receptor Ste2 and Ga protein Gpa1 are both monoubiquitinated and, given that both are eventually delivered to the vacuole, it seemed likely that both proteins were modified by the same ligase. To the best of our knowledge, a single enzyme (Rsp5) is responsible for most, if not all, monoubiquitination in yeast. Indeed early genetic analysis had shown that monoubiquitination of Ste2 requires Rsp5 (NEDD4 in humans) (40) and it was subsequently shown that monoubiquitination of Gpa1 likewise requires Rsp5 (41). To determine if Rsp5 is sufficient as well as necessary for Gpa1 monoubiquitination, the proteins were purified and shown to reconstitute the mono-ubiquitination reaction in vitro (41).

Notably, while monoubiquitination was diminished in the absence of Rsp5, there was a concomitant increase in Gpa1 poly-ubiquitination as well as a marked redistribution of protein from the vacuolar compartment to puncta within the cytoplasm, possibly representing aggregates or association with the proteasome protease complex (41). Thus, while removal of Rsp5 could prevent monoubiquitination, it was evident that a second enzyme was responsible for Gpa1 poly-ubiquitination. The identity of that second enzyme was revealed as part of a separate effort to identify new regulators of the mating pathway, through a systematic analysis of yeast essential genes (42). In that endeavor, nearly every essential gene was placed under the control of a tetracycline-regulatable promoter (TetO7 promoter), which allows for precise control of gene expression with no change in protein sequence or function. A total of 870 TetO7 strains were transformed with a pathway-specific transcription reporter and exposed to a range of pheromone concentrations. Of 92 genes required for proper signaling, a disproportionate fraction were involved in protein degradation. These included gene products that either form (Cdc53 and Cdc34) or function with (Cdc4) the SCF (Skp1, Cullin, F box protein) ubiquitin ligase (43,44). Knock down of CDC4, CDC34, or CDC53 genes all resulted in diminished activation of the effector MAPK as well as the transcription response. The functional similarity of the mutants, and the fact that the proteins exist as a complex in cells, suggested that SCF/Cdc4
had a particularly important role in G protein signal regulation. Since SCF is a negative regulator of protein stability, the reduction in signal output indicated that the likely target itself had an inhibitory function in signaling, most likely Gpa1. In support of the hypothesis, reconstitution of purified SCF and Cdc4 was sufficient for the ubiquitination of Gpa1. Conversely, ubiquitination was abrogated by knockdown of SCF or by removing the ubiquitination site within Gpa1 (42). Together these results indicate that SCF is necessary and sufficient to polyubiquitinate Gpa1. Loss of SCF stabilizes the Ga protein and limits signaling, presumably by sequestering free Gβγ.

In a follow up analysis it was shown that, whereas the F-box protein Cdc4 selectively targets Gpa1, other F-box proteins target downstream components of the pheromone signaling pathway: Dia2 selectively limits pheromone-induced vacuolar targeting, Ucc1 selectively limits MAPK induction and Pfu1 is needed for proper mating morphogenesis and the disassembly of unproductive mating projections (45).

To summarize, our current understanding is that mono- and poly-ubiquitination are catalyzed by different ubiquitin ligases with different functional consequences (Figure 2). Whereas mono-ubiquitinated Gpa1 is targeted to the vacuole, poly-ubiquitinated Gpa1 is directed to the proteasome. There may be some important benefits to having two ubiquitinating pathways that can target the same protein. For example, degradation could be triggered in response to different signals and/or functional states of the protein. In support of this model, SCF (but not Rsp5) is thought to recognize only substrates that are phosphorylated [reviewed in (46)]. A subsequent search, done through a comprehensive screen of 109 kinase deletion mutants, revealed that Elm1 is sufficient and necessary to phosphorylate Gpa1, at least under nutrient-rich conditions (47). Elm1 is expressed predominantly during the G2-M phase of the cell cycle, and this expression pattern was reflected in a cell cycle–dependent pattern of Gpa1 phosphorylation and polyubiquitination. Elm1 was already known to be one of three protein kinases that phosphorylate and activate the ADP-activated protein kinase Snf1 under low glucose conditions, where ADP accumulates (48,49). Phosphorylation of Snf1 is reversed by the protein phosphatase comprised of Reg1 and Glc7 (50,51). Gpa1 is likewise phosphorylated under low glucose conditions and is regulated by the same kinases and phosphatase as those that act on Snf1. When all three kinases are deleted, pheromone signaling is amplified. Deletion of the phosphatase has the opposite effect (52).

The opposing functional effects of the kinase and SCF mutants are somewhat paradoxical given that phosphorylation is needed for ubiquitination by SCF. We speculate that phosphorylation has additional functional effects on Gpa1 activity apart from its role in directing ubiquitination and degradation. Regardless, current evidence indicates that cell cycle progression and the glucose-sensing pathway act to directly modulate the pheromone signaling pathway and do so through the combined effects of phosphorylation and polyubiquitination of the G protein. In this context it is important to consider the important role of G proteins and GPCRs in regulating cellular growth and metabolism. Classical studies, performed over the past half century, revealed that G proteins and second messengers – most notably cAMP – lead to the mobilization of glucose from glycogen stores (53). More recent studies have implicated G proteins and GPCRs in tumorigenesis [Reviewed in (54,55)].

Ubiquitination regulates RAS localization, activity and stability.

RAS is the founding member of an expansive superfamily of small GTPases. Like Ga, RAS functions as a molecular switch, cycling between active GTP- and inactive GDP-bound states to regulate signaling pathways important for cellular growth control. The four mammalian RAS proteins (HRAS, NRAS, and KRAS 4A,B), contain a highly conserved core guanine nucleotide binding (G)-domain and a
hypervariable carboxyl terminal region. The core G-domain dictates high affinity and specificity for guanine nucleotides (GDP and GTP) as well as protein binding partners. The hypervariable region specifies post-translational modifications that facilitate membrane association. Both of these regions contain sites of ubiquitination, as detailed below.

There are many parallels in the activation cycle for large and small G proteins. In an unstimulated cell, RAS is populated in the ‘inactive’ GDP-bound conformation, despite the high ratio of intracellular GTP/GDP. Exchange factors bind and upregulate RAS activity by promoting association with GTP, whereas RAS GAPs accelerate the intrinsic rate of GTP hydrolysis to downregulate RAS activity. Binding of either GDP or GTP promotes distinct conformational changes in two key regions, termed switch I and II. The active GTP-bound conformation is recognized by downstream targets and culminates in downstream signaling. The best characterized RAS effectors are RAF kinases, phosphatidylinositol 3-kinases (PI3K) and Ral exchange factors (RalGEFs). All three effectors contain a conserved RAS binding domain, have well characterized roles in RAS-mediated tumorigenesis and have been extensively targeted for therapeutic drug treatment [Reviewed in (56)].

RAS mutations are commonly found in cancer and development disorders (e.g., RASopathies). Notably, RAS is the most frequently mutated oncogene, with ~30% of human tumors containing an activating RAS mutation. Of the three isoforms, KRAS is the most highly mutated, particularly in pancreatic (~90%), colon (~50%), and lung (~30%) cancers. These mutations chronically activate RAS and promote dysregulated cell proliferation. Point mutations in RAS can lead to chronic cellular activation in two ways, by increasing the rate of GDP dissociation or by inhibiting the rate of GTP hydrolysis; either of these mutational effects populates RAS in its biologically active GTP-bound form. The subset of RAS mutations that accelerate guanine nucleotide exchange are located in two motifs (SAK and NKXD), which form key interactions with the guanine nucleotide. Mutations within these motifs perturb nucleotide binding and promote GTP binding, given the high GTP/GDP ratio in cells. However, most oncogenic KRAS mutations are found at glycine 12 and glycine 13, and less frequently glutamine 61. These mutations populate the active GTP-bound form of RAS by interfering with GTP hydrolysis [Reviewed in (57)].

In addition to mutations that permanently activate RAS, there is growing interest in protein modifications that suppress or mimic the effects of such mutations. Understanding the molecular basis of these modifications, and how they might be regulated pharmacologically, would complement wide scale efforts to generate agents that antagonize aberrant RAS function. For example, early efforts to develop anti-RAS therapies relied on developing inhibitors of RAS farnesylation, but these drugs proved ineffective against KRAS as an alternative lipid modification, geranylgeranylation, can occur instead (58) [reviewed in (59-62)]. More recently, compounds have been developed that chemically modify a cysteine unique to the oncogenic KRAS G12C variant, which is particularly prevalent in lung cancer, resulting in inhibition of KRAS activity (63-67) [reviewed in (68)]. These covalent inhibitors are now in phase I clinical trials (Clinical Trial number: NCT03600883; NCT03785249). Although, these inhibitors represent an exciting breakthrough, there is a need to develop inhibitors that recognize other oncogenic RAS mutants. Understanding novel mechanisms of RAS regulation may prove helpful in identifying new therapeutic approaches for targeting RAS-driven tumors and developmental disorders.

RAS proteins are also substrates for ubiquitination. This modification targets multiple lysine residues and has been shown to alter RAS function in at least three distinct ways, by (i) altering its subcellular localization, (ii) altering protein-protein interactions and (iii) promoting its degradation (Figure 3). Below, we consider these three mechanisms in turn. For each we highlight important differences in the ubiquitin machinery responsible, the specific residues that are modified, and the RAS isoforms affected.
The earliest studies examining the effects of ubiquitination on RAS were conducted in mammalian and Drosophila cells. In both studies, Rabex-5 was identified as a key regulator of RAS function (69,70). Rabex-5 is a multi-domain protein that contains an exchange factor domain that promotes activation of RAB5 GTPases during endocytosis. It also contains a ubiquitin binding and E3 ubiquitin ligase domain that catalyzes mono- and di-ubiquitination of H- and NRAS, but not KRAS. Similar to Gpa1, ubiquitination of either H- or KRAS protein results in their endosomal membrane localization and a reduction in MAPK activation. An HRAS variant with several lysine residues mutated was more efficient at recruiting RAF1 and activating the MAPK cascade. While these observations provide a link between RAS ubiquitination, membrane localization and downregulation of RAS activity, several questions remain. First, what are the sites of ubiquitination that drive endosomal localization? The membrane anchoring hypervariable domain of HRAS was postulated to contain modification sites, however the exact sites of ubiquitination were not identified. Indeed, the mutant also contained several lysine substitutions (including the strictly conserved K117) in the G-domain that could affect localization and activity independent of ubiquitination. It is also unclear whether mono-versus diubiquitination have distinct functional effects on H- and NRAS and whether signaling pathways other than the MAPK (e.g. PI3K, RALGEF) are modulated by endosome localization. Another question lies in the isoform specificity associated with Rabex-5 mediated ubiquitination. Is the ability of Rabex-5 to selectively ubiquitinate H- and NRAS but not KRAS in CHO-K cells due to specific colocalization of Rabex-5 complexes with these RAS isoforms or is it through another mechanism? Future studies directed at identifying the key sites and sites of modification, for example by mass spectrometry, may aid in clarifying the role of ubiquitination in RAS endosome localization/retention and signaling.

While these pioneering studies were the first to demonstrate that RAS signaling is modulated by mono- and diubiquitination, other factors that may link these two processes have been identified. More recently, a deubiquitinase, OTUB1, was identified that facilitates removal of mono- and di-ubiquitin from RAS proteins, and appears to promote retention of RAS proteins at the plasma membrane in a nucleotide-independent manner (71). Consistent with findings that OTUB1 may upregulate RAS function, overexpression of OTUB1 promotes MAPK signaling and is commonly observed in non-small cell lung carcinomas that contain WT KRAS (71).

While the studies described above reveal a role of ubiquitination in RAS localization and suppression of RAS function, it was recently shown that mono-ubiquitination of KRAS alters protein-protein interactions and upregulates KRAS activity. In one landmark study, mass spectrometry was used to identify sites of ubiquitination of K- and HRAS isolated from HEK293T cells, with lysine 147 identified as the most frequent site of KRAS monoubiquitination. Compared with unmodified RAS, the ubiquitinated subpopulation was predominantly in the activated GTP-bound state, and showed increased association with the downstream effectors RAF, PI3K, and RalGEF. Consistent with these observations, mutation of KRAS to prevent ubiquitination at lysine 147 impaired tumor growth in a mouse xenograft model system (72). As lysine 147 is part of the conserved SAK motif important for coordinating the guanine nucleotide base of GTP or GDP, it was initially hypothesized that monoubiquitination upregulates KRAS activity by promoting guanine nucleotide exchange. However, our subsequent studies, detailed below, showed that monoubiquitination at lysine 147 does not significantly alter nucleotide exchange, but rather impairs GAP-mediated downregulation in vitro (73). These findings are significant because they indicate that ubiquitination can activate RAS-mediated signaling and tumorigenesis. The activation mechanism is similar in nature to GAP-defective oncogenic RAS mutations. Thus, monoubiquitination of KRAS at lysine 147 represents a third means of stimulating RAS signaling.

To investigate the mechanism of activation, large quantities of fully ubiquitinated substrate are
often required, which is difficult to obtain by enzymatic reactions. Accordingly, KRAS was monoubiquitinated at position 147 using a chemical biology approach (73). In our approach, we replaced the native ubiquitin linkage with a disulfide bond between a substituted cysteine at position 147 of RAS and another cysteine at the carboxyl–terminus of ubiquitin (G77C). A surface accessible cysteine (C118) in RAS was replaced with serine to avoid unwanted modification. Ubiquitin modification of RAS at position 147 was driven to completion by the addition of a ten–fold excess of ubiquitin (G77C) at pH 8.0. In contrast to previously described methods (74–79), our 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 was validated in previous studies, using a more complicated cysteamine intermediate in Proliferating Cell Nuclear Antigen (PCNA), where it was shown that chemically and enzymatically monoubiquitinated PCNA exhibit identical catalytic properties (75).

Using this disulfide ligation strategy, we showed that KRAS147 monoubiquitination impedes GAP-mediated GTP hydrolysis and effector interactions in vitro and in cell lysates (73). This activation mechanism appears site specific, as monoubiquitination at three other sites did not alter GAP regulation. Intriguingly, ubiquitin does not make specific interactions with RAS, but rather dynamically samples conformational space so as to occlude a subset of RAS conformers, thus generating a distinct and more compact RAS conformational ensemble that impairs recognition by GAP proteins. Consistent with these observations, GAP activity was dependent on the size of the modification and linker length (80). These findings have broader impact beyond RAS regulation, as larger protein modifications like sumoylation could modulate a variety of systems via a similar mechanism.

Whereas lysine 147 was the predominant site of KRAS monoubiquitination identified in HEK293 cells, lysine 117 is the major ubiquitination site in HRAS (81). Notably, this lysine (117) is strictly conserved in the RAS superfamily of GTPases and it makes key contacts with the bound nucleotide. Mutations at this residue cause fast exchange, enhanced GTP loading and RAS activation. Consistent with these observations, ubiquitin modification at lysine 117 in HRAS promotes RAS activation due to faster nucleotide cycling, rather than by disrupting GAP interactions (81). Although the side chain makes contacts with the bound nucleotide, it is possible that ubiquitination occurs when RAS is in complex with factors that expose this site, such as GEFs which stabilize the nucleotide free state of RAS. Another residue that is a minor site of KRAS ubiquitination is lysine 104 (72). However, unlike lysine 117 and 147, ubiquitin modification of lysine 104 does not appear to modulate nucleotide cycling, and thus may primarily serve as a recognition site for ubiquitin-binding proteins. Notably, lysine 104 and 147 in KRAS have also been shown to undergo other post-translational modifications, e.g. acetylation (82–84). Although the role of acetylation in RAS function is controversial, these observations suggest that other lysine post-translational modifications may compete with ubiquitination. Moreover, the ubiquitin machinery may be cell-specific, as N- and HRAS mono- and diubiquitination was observed in CHO-K cells, yet KRAS ubiquitination was not. This is perhaps not surprising, as H-, N- and KRAS isoforms undergo distinct spatial/temporal localization, which likely contributes to different mechanisms of ubiquitin regulation.

The studies described above support a role of mono- and di-ubiquitination in the regulation of RAS localization and protein-protein interactions. In addition, and more classically, polyubiquitination can alter RAS function through degradation. Proteasomal degradation of HRAS has been reported to occur in response to activation by the Wnt/β-catenin signaling pathway (85–87). This polyubiquitin-dependent degradation occurs upon recruitment of β-TrCP-E3 ligase after HRAS phosphorylation by glycogen synthase kinase 3β. Conversely inhibition of this pathway by aberrant Wnt/β-catenin signaling enhances RAS expression levels and RAS-induced colorectal tumorigenesis. In an effort to develop anticancer drugs targeting RAS, small molecules were
identified that induce the degradation of RAS and β-catenin through the Wnt/β-catenin pathway (86,88). These compounds act by enhancing formation of the β-catenin destruction complex and subsequent recruitment of β-TRCP E3 ligase, leading to polyubiquitination-dependent proteasomal degradation of RAS. Subsequent studies identified more specific compounds, which degrade RAS without affecting the β-catenin levels. Results from these studies indicate that downregulation of RAS signaling is mediated through degradation of RAS (89,90). In a separate study, the Smad ubiquitination regulatory factor 2 (SMURF2), was shown to monoubiquitinate its E2 ubiquitin conjugating enzyme (UBCH5) to form an active E3:E2 complex. This complex polyubiquitinates and degrades β-TrCP1 E3 ligase that modulates KRAS protein stability. Of note, oncogenic KRAS mutant levels appear more sensitive than wild type KRAS to SMURF2-mediated protein stability regulation (91). Degradation appears dependent on lysosomal proteolysis, as a lysosome inhibitor protected KRAS from SMURF2 whereas proteasomal inhibition was ineffective. Moreover, degradation by this route is enhanced by treatment with the estrogen antagonist 4-hydroxy-tamoxifen (91). These findings are consistent with previous observations that KRAS can undergo lysosomal degradation (92). Thus, both proteasome and lysosome-mediated degradation mechanisms appear to modulate RAS levels and do so in a stimulus-dependent manner.

More recently, a new player that regulates RAS protein levels was identified. LZTR1, or leucine zipper-like transcription regulator 1, associates with the Cullin 3 E3 ubiquitin ligase and facilitates ubiquitination of all RAS isoforms. Recent work by three groups showed that LZTR1 is mutated in human cancers and developmental diseases, and attenuates RAS signaling (93-95). Correspondingly, reduced expression or loss of LZTR1, in mammalian cells or Drosophila, result in constitutive RAS signaling. Consistent with these observations, LZTR1 haploinsufficiency in mice result in a phenotype resembling Noonan syndrome, a development disorder that arises from enhanced RAS and MAPK signaling. Moreover, a loss of LZTR1 in Schwann cells promotes differentiation and proliferation. These disease-associated LZTR1 mutations disrupt either formation of the LZTR1-CUL3 complex or its interaction with RAS proteins (94).

While ubiquitination by LZTR1 was initially proposed to modulate RAS function by a non-degradative mechanism (94), a more recent study showed LZTR1-dependent proteolysis of RAS was prevented by treatment with a proteasome inhibitor, suggesting that LZTR1 facilitates polyubiquitination and degradation of RAS via the ubiquitin-proteasome pathway (95). Although LZTR1 drives multiple sites of RAS ubiquitination, lysine 170 in the hypervariable region of HRAS appears to be key, as loss of ubiquitination at this site leads to dissociation of RAS from the plasma membrane (94). Conversely, the hypervariable region and farnesylation of the C-terminal cysteine 186 of KRAS4A is required for LZTR1-mediated ubiquitination (94). These findings, taken together, suggest a mechanism whereby LZTR1-mediated RAS ubiquitination down regulates RAS function. While these studies identify a new regulatory mechanism, several questions remain unresolved. Is LZTR1-mediated ubiquitination dependent on the activation state of RAS? As several lysines undergo ubiquitination, are there sites other than lysine 170 that contribute to downregulation of RAS signaling?

The advances noted above have created new opportunities for potential therapies. In particular, efforts to promote association with ubiquitinating (E2, E3) enzymes may provide a unique strategy to specifically destroy persistently activated KRAS mutants, and thus ablate RAS-driven tumorigenesis. In support of this concept, an engineered E3 ubiquitin ligase was generated to target the KRAS oncoprotein for ubiquitination and proteasomal degradation (96). In this study, a fusion was generated between a U-Box–based chimeric E3 ligase, a RAS binding (RBD) and a membrane localization domain (CRD), to target activated KRAS for ubiquitination and degradation. Expression of this fusion in pancreatic cancer cells resulted in reduced KRAS expression, diminished MAPK signaling and pancreatic cancer cell growth in
vitro and in vivo. These same investigators have also developed a method to target proteins for degradation without ubiquitin-driven degradation, one that relies on fusion of the substrate to ornithine decarboxylase, an enzyme that plays a key role in polyamine biosynthesis. Interaction with a natural cellular inhibitor of ornithine decarboxylase (antizyme) exposes the proteasome-binding site and promotes proteolytic degradation. A fusion that links ornithine decarboxylase to a domain in RAF-1, which recognizes activated RAS, leads to diminished expression and RAS-mediated growth in pancreatic cancer cells lines that have been co-transfected with the antizyme (97).

Finally, an emerging and particularly powerful strategy is to use bivalent ligands known as PROTACS (PROteolysis TArgeting Chimeras) for inducing target protein degradation. These bifunctional small molecules combine a small molecule that binds to the target (RAS in this case), a linker and a small molecule that binds and recruits an E3 ligase, enabling selective target ubiquitination and subsequent protein degradation. This novel technology has led to first ‘degrading’ drug that is targeted to the androgen receptor for prostate cancer treatment, and is now in phase 1 clinical trials [reviewed in (98,99)].

Unlike kinases which bind weakly to nucleotides, RAS binds GDP and GTP with picomolar affinity. Hence, it is very difficult to generate inhibitors that target the nucleotide binding site with selectivity and affinity in this range. As RAS proteins lack other druggable pockets, it has been challenging to identify high affinity compounds that specifically recognize activated RAS or an oncogenic mutant. PROTACS that target RAS for degradation may be a particularly attractive anti-cancer strategy. However, this approach not only requires identification of bivalent ligands that engage both RAS and a specific E3 ligase, but also the formation of a complex between the E3 ligase and RAS in order to catalyze addition of ubiquitin molecules onto specific lysine residues. Moreover, the polyubiquitinated substrate must be recognized by the proteasome machinery. For RAS, optimization may also require generation of PROTACS with appropriate cellular distribution and activity in a cancer cell type dependent manner. There are now several reports of small molecules that bind to RAS proteins with high affinity, which should open up new avenues for design of PROTACS [reviewed in (100)].

Concluding remarks

Our hope is that this review will be instructive to a broad group of readers, including those who may be knowledgeable about G proteins but not about their ubiquitination, as well as readers who know about ubiquitination but not about G proteins. The advances summarized here reveal how (i) a single protein (yeast Gpa1) is targeted for two different forms of ubiquitination, each having profoundly distinct consequences for targeting and degradation of the substrate (Figure 2), and (ii) how a single protein (mammalian RAS) is targeted at different sites, each having unique consequences for protein-protein interactions and pathway activation (Figure 3). A major technological breakthrough was the ability to map sites of ubiquitination directly, through mass spectrometry. Perhaps the biggest conceptual advance is the realization that RAS proteins can be activated by ubiquitination, as they are by growth factor stimulation and oncogenic mutations.

Gpa1 and RAS proteins share similar structures, mechanism of activation and cellular effectors (Figure 1). Moreover, investigations of Gpa1 in yeast led to similar studies of other ubiquitinated proteins including RAS. Indeed, studies in yeast revealed many of the proteins and processes required for RAS activation, post-translational modifications, membrane anchoring, and inactivation.

If history is any guide, the application of the yeast model will continue to inform advances in cancer biology. Conversely, the complexity of mammalian cancer biology will continue to inspire new questions that may be addressed more easily in yeast. Given the ubiquity of G proteins as signal transducers and of ubiquitination as a mechanism of cell regulation, the extension of genetic, biochemical, and chemical biology approaches employed for the
studies described here should prove useful in the study of other important targets of ubiquitination.
CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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FOOTNOTE

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The abbreviations used are: GAP, GTPase Activating Protein; GEF, Guanine nucleotide Exchange Factor; GPCR, G Protein Coupled Receptor; PROTACS, PROteolysis TArgeting Chimeras; MAPK, Mitogen-activated Protein Kinase;
**Figure 1.** Large and small G proteins. G protein signaling is initiated by GDP release and GTP binding, which is accelerated by an agonist-bound receptor (left) or guanine nucleotide exchange factor (GEF, right). GTP-bound G proteins activate effector enzymes and ion channels within the cell (boxes). G protein signaling is concluded by GTP hydrolysis to GDP and phosphate (Pi), which is accelerated by a Regulator of G protein Signaling (RGS, left) or GTPase activating protein (GAP, right). Components of the large G protein signaling complex from yeast are noted. GIRK, G protein-coupled inwardly-rectifying potassium channels; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; PI3K, phosphatidylinositol 3-kinase; TIAM1, T-cell lymphoma invasion and metastasis-inducing protein, RAF, Rapidly Accelerated Fibrosarcoma.
Figure 2. Ubiquitination controls trafficking of a Gα in yeast. Ubiquitination is mediated by three distinct enzymes (E1, E2 and E3), the last of which defines substrate specificity and the timing of ubiquitination. A protein complex containing the E3 Rsp5 is necessary and sufficient for mono-ubiquitination of the G protein α subunit Gpa1. The modified protein is internalized by a cascade of ubiquitin-binding domain proteins and degraded in the vacuole. A protein complex containing the E3 Cdc53 and F-box adapter Cdc4 is necessary and sufficient for poly-ubiquitination of Gpa1, resulting in degradation by the proteasome protease complex. The ubiquitin protease Ubp12 is required for Gpa1 de-ubiquitination.
Figure 3. Ubiquitination controls multiple interactions of RAS. A. RAS activity is regulated by ubiquitination. Primary sites of monoubiquitination occur at residues 147 in KRAS, whereas in HRAS it is at 117. Monoubiquitination of KRAS at 147 upregulates RAS activity through a GAP defect leading to enhanced MAPK activation. In contrast, monoubiquitination of HRAS at 117 induces fast exchange and activates RAS in a GEF-independent manner. B. RAS localization is regulated by ubiquitination. Rabex-5 promotes mono- and diubiquitination of HRAS and NRAS resulting in endosome localization and reduced MAPK signaling. The deubiquitinase, OTUB1, removes ubiquitin from RAS and promotes plasma membrane localization and MAPK signaling. C. Ubiquitination by LZTR1, β-TrCP1 and SMURF2 promote RAS degradation through proteasome and autolysosomes resulting in reduced MAPK signaling.
Regulation of large and small G proteins by ubiquitination
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