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Ubiquitin on Ras: Warden or Partner in Crime?

Cathie M. Pfleger*

Department of Oncological Sciences, The Mount Sinai School of Medicine, New York, NY 10029, USA

Abstract

Signal transduction through Ras translates extracellular signals into biological responses, including cell proliferation, cell survival, growth, and differentiation. For these reasons, dysregulating Ras can have dramatic effects at the cellular and organismal levels. Germline mutations that increase Ras signaling disrupt development, whereas mutational activation of Ras in somatic cells can cause cancer. Thus, identifying additional mechanisms that positively or negatively regulate Ras could have profound implications for treating human diseases. New evidence identifies K-Ras monoubiquitination as a previously unknown means to potentiate Ras signaling.

A single Ras isoform in *Drosophila* and *Caenorhabditis elegans* expands into a family of Ras proteins in humans—N-Ras, H-Ras, and differently spliced forms of K-Ras, K-Ras4A, and K-Ras4B (referred to here as K-Ras). Mammalian Ras proteins share almost 100% identity in the first 165 amino acids, and all isoforms demonstrate the ability to transform cells in culture. However, they show less than 15% amino acid identity in the C-terminal 24 amino acids, and they demonstrate key differences including compartmental localization, trafficking, post-translational modifications, and pattern of mutations in human disease (1–18).

Activating germline mutations in K-Ras occur in Noonan syndrome and cardio-faciocutaneous syndrome, and similar mutations in H-Ras give rise to Costello syndrome and in N-Ras, to autoimmune lymphoproliferative syndrome (1, 2). Mutational activation in cancer also results in different tumor spectra. Whereas mutational activation of K-Ras occurs in >90% of all pancreatic cancers (11–13), N-Ras is the most commonly mutated Ras in acute myeloid leukemia (14, 15) and is also frequently mutated in melanoma (16, 17), and mutation of H-Ras is common in bladder cancer (18).

Given the importance of Ras, we would expect cells to use multiple mechanisms to potentiate and attenuate Ras activity. Ras becomes active in the guanosine triphosphate (GTP)–bound state and inactive in the guanosine diphosphate (GDP)–bound state. Guanine nucleotide exchange factors (GEFs) promote exchange of GDP for GTP to activate Ras. In contrast, guanosine triphosphatase (GTPase)–activating proteins (GAPs) promote hydrolysis of GTP on Ras to GDP, which inactivates Ras (5). Various posttranslational modifications—including farnesylation, proteolysis, methylation, palmitoylation, and depalmitoylation—

*cathie.pfleger@mssm.edu.

modulate the membrane affinity and localization of Ras to define exposure to downstream effectors. However, the identity of proteins other than GEFs and GAPs that directly target Ras to alter its activity has remained elusive.

Normal protein turnover mechanisms regulate the abundance of all proteins; indeed, proteasomal degradation of polyubiquitinated Ras proteins regulates their stability, a process mediated by the ubiquitin ligase SCF/β-TRCP (β-transducin repeat–containing protein) (19). In addition, Wnt signaling decreases β-TRCP–induced polyubiquitination of Ras (19). Several studies also identified a nonproteasomal fate for ubiquitinated Ras. Mono- and diubiquitination of *Drosophila* Ras and mammalian H-Ras and N-Ras, but not K-Ras, restrict the ability to signal to ERK (extracellular signal–regulated kinase) (20, 21), which suggests a conserved role for ubiquitination in decreasing the activity of Ras. Experiments with mammalian cells transfected with an H-Ras–ubiquitin fusion or an H-Ras mutant that could not be ubiquitinated demonstrated that ubiquitination promotes endosomal localization or retention (or both) of H-Ras (21), which suggests that the restricted ability to signal resulted from sequestration from certain effectors. Subsequent work revealed that the ubiquitin ligase *Rabex-5* promotes ubiquitination of *Drosophila* Ras (22) and mammalian H-Ras, but not K-Ras (23), in a negative feedback mechanism (20–24).

Whereas previous studies reported that mono- and diubiquitination restrict the activity of H-Ras and of N-Ras, but not that of K-Ras, Sasaki *et al*. (25) report the monoubiquitination of K-Ras and demonstrate that K-Ras ubiquitination promotes its activity. Sasaki *et al*. found monoubiquitinated K-Ras predominantly in the GTP-loaded state and unconjugated K-Ras predominantly in the GDP-loaded state and, therefore, propose that ubiquitination may enhance GTP-loading of K-Ras. They identified the major site of K-Ras ubiquitination as Lys^{147}, with a minor site at Lys^{117}. Similarly, they identified H-Ras sites of ubiquitination as Lys^{117}, Lys^{147}, and Lys^{170}. Moreover, they showed that ubiquitinated K-Ras demonstrates increased affinity for Raf, PI3K (phosphatidylinositol 3-kinase), and RalGDS in binding assays and that it activated Raf and PI3K more efficiently than unconjugated Ras in in vitro kinase assays. In contrast, a $Lys^{147}\rightarrow$Leu (K147L) substitution in an activated $Gly^{12}\rightarrow$Val (G12V) K-Ras mutant showed reduced binding to PI3K but not to Raf. Unlike H-Ras ubiquitination, which promotes endosomal localization (21), K-Ras ubiquitination did not change its apparent subcellular localization (15). Compellingly, Sasaki *at al.* establish an in vivo role for ubiquitination of Lys^{147} in promoting the oncogenic functions of K-Ras. They observed decreased tumorigenicity of K147L substitution mutants of oncogenic G12V K-Ras in terms of tumor weight and tumor volume in nude mice. K147L/G12V K-Ras tumors that formed exhibited reduced phospho-S6 and a trend of reduced AKT activation compared with G12V K-Ras tumors. This suggests an exciting model in which ubiquitination of K-Ras at Lys^{147} normally acts to preferentially increase the strength of signaling through select downstream pathways.

The seemingly divergent means by which conjugation to ubiquitin regulates H- or N-Ras (20–23) and K-Ras (25) raise various exciting questions. First, do distinct ubiquitin ligases (specificity components of the ubiquitin pathway) regulate H- or N-Ras and K-Ras, or does the same ubiquitin ligase target all three isoforms? Previous (20–23) and current findings (25) (Fig. 1) suggest that different ubiquitin ligases target H- or N-Ras and K-Ras. For

example, GTP-loading affects Ras conformation. Sasaki *et al*. (25) demonstrated ubiquitination of a form of K-Ras that is mutated at Thr^{35} and is locked in a GDP-bound conformation, consistent with a model that ubiquitination may precede GTP-loading, whereas a previous study (21) reported that the G12V form of H-Ras (which is locked in a GTP-bound conformation that is distinct from the GDP-bound conformation) remained a suitable substrate for ubiquitination, consistent with ubiquitination occurring after GTP-loading. Targeting disparate conformations suggests that ubiquitination of K-Ras and H-Ras may occur by distinct mechanisms. Also, although formally possible that K-Ras ubiquitination was below detection limits, Xu *et al*. showed that Rabex-5 promoted ubiquitination of H-Ras but not K-Ras in a purified system and in various cell lines (23).

Second, does Rabex-5 promote ubiquitination at a lysine residue in H-Ras other than Lys^{147}, or does Lys^{147} ubiquitination play a different role in H-Ras than in K-Ras? Ubiquitinated H-Ras localizes to the endosomes (21, 23), which compromises signaling through ERK and potentially limits access to other effectors. Sasaki *et al*. (25) showed that even a low amount of ubiquitination of K-Ras at Lys^{147} could translate into meaningful outputs through PI3K, which suggests that endosomal H-Ras localization may either not limit access to PI3K, or that increased signaling through PI3K may offset any limited access. Because trafficking of H-Ras and K-Ras exposes each protein to different aspects of the cellular milieu, in terms of downstream effectors and ubiquitin-interacting proteins, modifying the same lysine residue could translate into distinct biological outputs. Alternatively, Sasaki *et al*. reported modification of both Lys^{117} and Lys^{170} in H-Ras (25), and Jura *et al*. (21) replaced eight lysines to make a form of H-Ras that could not be ubiquitinated, which suggests that modification of another lysine residue could underlie restriction of H-Ras activity.

Third, did Ras ubiquitination arise with a single function in an ancestor with only one Ras? The conserved role of Ras ubiquitination (from *Drosophila* to mammals) to restrict signaling through ERK indicates that Ras ubiquitination may have originated as an inhibitory mechanism. Subsequent evolutionary expansion with multiple Ras forms could have enabled ubiquitination of distinct Ras proteins to assume different roles depending on tissue type, compartmentalization, and other determinants. The potentiating role of K-Ras ubiquitination reported by Sasaki *et al*. (25) may reflect a recent adaptation borne of the specialization of each Ras species.

Alternatively, an activating role for Ras ubiquitination has not been evaluated in *Drosophila*. Previous studies showed that nonspecifically reducing overall ubiquitination promoted increased activation of ERK, but not that of AKT, in whole larvae (20) and that Rabex-5–mediated Ras ubiquitination restricted *Drosophila* Ras signaling to ERK (22). However, these findings do not preclude a role for Ras ubiquitination to increase signaling through PI3K—for example, in specific tissues or developmental periods not examined in these studies.

Finally, ubiquitination is reversible; deubiquitinating enzymes (DUBs) remove ubiquitin from substrates. Does a DUB attenuate K-Ras signaling specific effector pathways by removing ubiquitin from Lys^{147}? Such a DUB could add a further level of intricacy to Ras regulation and play an important role in fine-tuning signaling outputs through Ras effectors.

In light of the in vivo tumor studies performed by Sasaki *et al*. (25), a DUB acting on K-Ras Lys^{147} could represent a tumor suppressor that could act on oncogenic K-Ras, a departure from Ras inhibitors such as GAPs.

In identifying that ubiquitination of K-Ras at Lys^{147} promotes Ras signaling, Sasaki *et al*. (25) have emphasized that modification of even a small percentage of total Ras can lead to meaningful biological consequences and adds another layer of complexity to our understanding of Ras regulation. Taken together with the current state of the field, these findings implicate various mechanisms by which different cell types and tissues could establish distinct physiological outcomes in specific contexts by expressing different combinations of K-Ras and H- or N-Ras ubiquitin ligases, DUBs, or ubiquitin-interacting trafficking factors. The as-yet-unidentified ubiquitin ligase targeting K-Ras joins the ranks of GEF proteins in its ability to promote K-Ras activity and a K-Ras Lys^{147} DUB could join GAPs in inhibiting K-Ras.

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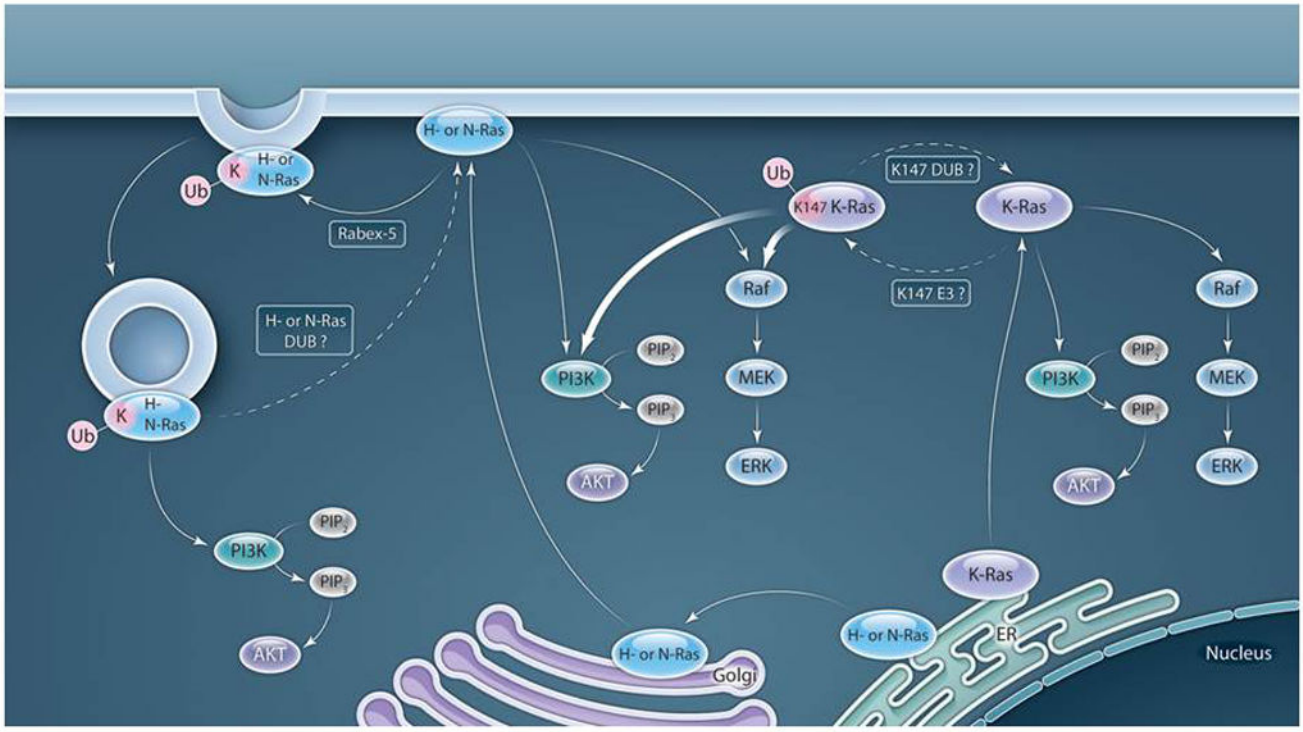

Fig. 1.

Ubiquitination differentially regulates K-Ras and H- or N-Ras. Previous studies demonstrated that mono- or diubiquitination of H- or N-Ras at an unidentified lysine (K) promotes endosomal localization or retention (depicted here as relocalization), which restricts the ability to signal to ERK, but not to AKT (20–23). Presumably, a DUB could act to remove ubiquitin from H- or N-Ras, and so enable a return to the membrane and restore the ability to signal through ERK. In contrast, Sasaki *et al*. (25) demonstrated that monoubiquitination of K-Ras at Lys^{147} (K147) by an unknown ubiquitin E3 ligase increases the ability to signal through PI3K and possibly Raf and does not alter K-Ras trafficking. Removal of the ubiquitin added to Lys^{147} by a DUB could restore activation of PI3K by K-Ras to normal amounts. Dashed lines indicate putative steps.