Ras Protein/cAMP-dependent Protein Kinase Signaling Is Negatively Regulated by a Deubiquitinating Enzyme, Ubp3, in Yeast*

Yang Li and Yuqi Wang

From the Department of Biology, Saint Louis University, St. Louis, Missouri 63103

**Background:** Ras proteins are important molecular switches. RasGAP is an essential negative regulator of Ras, and its activity is controlled by ubiquitination.

**Results:** RasGAP interacts with a deubiquitinating enzyme, Ubp3. Disrupting Ubp3 activity leads to accumulation of ubiquitinated RasGAP and hyperactivation of Ras signaling.

**Conclusion:** Appropriate deubiquitination of RasGAP by Ubp3 is important for Ras signaling.

**Significance:** This study reveals a new layer of mechanism that regulates Ras.

Ras proteins and cAMP-dependent protein kinase (protein kinase A, PKA) are important components of a nutrient signaling pathway that mediates cellular responses to glucose in yeast. The molecular mechanisms that regulate Ras/PKA-mediated signaling remain to be fully understood. Here, we provide evidence that Ras/PKA signaling is negatively regulated by a deubiquitinating enzyme, Ubp3. Disrupting the activity of Ubp3 leads to hyperactivation of Ras, as evidenced by much enhanced phosphorylation of PKA substrates, decreased accumulation of glycogen, larger cell size, and increased sensitivity to heat shock. Levels of intracellular cAMP and the active forms of Ras proteins are also elevated in the ubp3Δ mutant. Consistent with the possibility that the increased CAMP is responsible for the abnormal signaling behavior of the ubp3Δ mutant, overexpressing PDE2, which encodes a phosphodiesterase that hydrolyzes cAMP, significantly relieves the cell size increase and heat shock sensitivity of the mutant. Further analysis reveals that Ubp3 interacts with a Ras GTPase-accelerating protein, Ira2, and regulates its level of ubiquitination. Together, our data indicate that Ubp3 is a new regulator of the Ras/PKA signaling pathway and suggest that Ubp3 regulates this pathway by controlling the ubiquitination of Ras GTPase-accelerating protein Ira2.

Ras proteins belong to a family of highly conserved small GTPases that act as a molecular switch to control a large variety of biological processes, including cell growth, proliferation, differentiation, and survival (1, 2). Like other GTPases, Ras proteins cycle between inactive GDP-bound (Ras-GDP) and active GTP-bound (Ras-GTP) states (3, 4). The intrinsic GTPase activity of Ras is extremely slow, and GTPase-accelerating proteins (RasGAP) are obligated for turning off Ras-GTP (5, 6). Thus, inactivation of RasGAP can cause aberrant activation of wild-type Ras and result in diseases (7–9). A good example is neurofibromatosis type 1, a disease caused by lack of RasGAP neurofibromin that has an incidence of about 1 in 3500 live births (10, 11). Clearly, a thorough understanding of the mechanisms that control Ras and Ras regulators such as RasGAP is important.

Ras proteins are highly conserved, enabling the use of model organisms such as yeast to uncover novel and fundamentally important mechanisms for their regulation (12–14). In the budding yeast *Saccharomyces cerevisiae*, Ras proteins are components of a nutrient signaling pathway that senses and responds to the availability of glucose (13, 15). In the presence of glucose, Ras proteins become activated, and the resulting Ras-GTP binds to adenylate cyclase (Cyr1) and stimulates the production of second messenger cAMP (13). An increased intracellular cAMP triggers the activation of cAMP-dependent protein kinase (PKA), which phosphorylates a spectrum of substrates, including transcriptional regulators and metabolic enzymes to regulate cell growth, differentiation, and survival (13, 16) (Fig. 1). The activation status of Ras proteins is controlled by a balance between their guanine nucleotide exchange proteins (Ras-GEF) (Cdc25 and Sdc25 in yeast) and their GTPase-activating proteins (RasGAP) (Ira1 and Ira2 in yeast). Notably, RasGAP in both yeast (Ira2) and human (neurofibromin) are regulated by ubiquitination (17, 18), a posttranslational modification that typically leads to protein degradation in the proteasome (19). In yeast, addition of glucose triggers ubiquitination and inactivation of Ira2, which, consequently, leads to an accumulation of cellular Ras-GTP and enhanced activation of the Ras/PKA pathway (18). Likewise, in mammals, growth factor stimulates ubiquitination of neurofibromin, thereby enhancing the activity of Ras (17). Thus, regulated ubiquitination of RasGAP appears to be a highly conserved mechanism that modulates Ras signaling. It is unclear whether this regulatory mechanism is reversible. In addition, the enzymes in the ubiquitination pathway that control the ubiquitination status of Ira2 or neurofibromin remain to be established clearly.

The process of ubiquitination is catalyzed by a cascade of enzymes composed of a ubiquitin-activating enzyme (E1), a
ubiquitin-conjugating enzyme (E2), and a ubiquitin-ligating enzyme or ligase (E3) (19, 20). Most substrates are modified with a polyubiquitin chain that targets the substrates for degradation by the 26 S proteasome (19). However, ubiquitination does not always lead to degradation of the substrate, and it can play a much broader role in regulating protein function by proteolysis-independent processes (21, 22). Perhaps the best studied example is ubiquitination acting as a signal for membrane protein trafficking. In yeast and in mammalian cells, ubiquitination is a necessary and sufficient signal for the internalization of many cell surface proteins into the endocytic pathway (23, 24). Ubiquitination can also regulate protein-protein interactions. One good example is ubiquitination of TRAF6, which acts to recruit and activate downstream TAK1 kinase (25, 26).

Similar to phosphorylation, the process of ubiquitination is reversible (27). Removal of conjugated ubiquitin or the ubiquitin chain from substrates is achieved by deubiquitinating enzymes (DUB) (28). In yeast, there are about 20 deubiquitinating enzymes that range in size from 54–146 kDa (29–31). Sixteen of them are also known as ubiquitin-processing proteases (Ubps). Genome analysis has predicted the existence of more than 90 deubiquitinating enzymes in humans (31). The large number and diversity of deubiquitinating enzymes suggests that they function in specific biological processes (30, 31). However, the cellular function of most Ubps is unknown (31, 32). Systematic disruption of the genes encoding Ubps in yeast reveals only minimal phenotypic abnormalities, and none have proved to be essential (30).

In this study, we focus on Ubp3, a yeast deubiquitinating enzyme that has been implicated in the regulation of nutrient signaling (33). Disrupting the UBP3 gene leads to an increased sensitivity to rapamycin, a specific inhibitor of TOR (target of rapamycin), but the molecular mechanism by which Ubp3 affects rapamycin sensitivity is unclear (33). TOR is a central component of another nutrient signaling pathway that operates in parallel with Ras/PKA (34, 35). A recent report revealed an intriguing antagonistic relationship between TOR and the Ras/PKA signaling pathways (36). In particular, it was demonstrated that enhanced sensitivity to rapamycin is often a result of hyperactivation of Ras/PKA signaling (36). Thus, it seems plausible that Ras/PKA might be hyperactive in the ubp3Δ mutants and that is why the mutant cells display increased sensitivity to rapamycin. Accordingly, we examined the activation status of Ras/PKA in the ubp3Δ mutants. We found that disrupting the activity of Ubp3 leads to an elevated level of Ras-GTP and much enhanced activation of its downstream PKA. As expected, the ubp3Δ mutants display common phenotypes associated with hyperactivation of Ras/PKA, such as enhanced sensitivity to heat shock, decreased accumulation of glycogen, and enlarged cell size. Notably, the abnormal signaling phenotypes displayed by the ubp3Δ mutants can be partially rescued by overexpressing PDE2, a cAMP-specific phosphodiesterase (37). Further analysis revealed Ubp3 interacts with Ira2 and regulates its level of ubiquitination, suggesting Ira2 as a potential target of Ubp3. Together, our study reveals a new role for Ubp3 in regulating Ras/PKA signaling.

**EXPERIMENTAL PROCEDURES**

**Strains and Plasmids**—Standard methods for the growth, maintenance, and transformation of yeast and bacteria and for the manipulation of DNA were used throughout. The yeast *S. cerevisiae* strains used in this study are BY4741 (*MATa leu2Δ met15Δ his3Δ ura3Δ*) and BY4741-derived mutants lacking *RAS1, RAS2, UBP3, IRA2*, or *TFS1* (Research Genetics, Huntsville, AL). Expression plasmids used in this study that have been described previously are pCK70-UBP3 and pCK72-UBP3C469A (33) (provided by Dr. Claudine Kraft), pPH795-MET-RAS2val19 and pPHY1107-PDE2 (36) (provided by Dr. Paul K. Herman), and pKT64-HA-IRA2 (38) (provided by Dr. Fuyuhiko Tamanoi).

**URA3**—Marked plasmids that express N-terminal FLAG-tagged full-length Ubp3 were generated by PCR amplification using pYES-UBP3 (39) as a template and subcloning it into the pYES2.1/V5-His-TOPO plasmid (2 μm of URA3, GAL1 promoter, and CYC1 terminator) (Invitrogen). The forward PCR primer was 5′-GGT ACC ATG AAC GAT TAT AAA GAT TTA TCT-3′, and the reverse primer was 5′-GGT ACC TAT TAT AAA GAT GAC GAT GAC AAG ATG CAA GAC GCT AAC AAG GAA G-3′, and the reverse primer was 5′-GGT ACC TAT TAT AAA GAT GAC GAT GAC AAG ATG CAA GAC GCT AAC AAG GAA G-3′, and the reverse primer was 5′-GGT ACC TAT TAT AAA GAT GAC GAT GAC AAG ATG CAA GAC GCT AAC AAG GAA G-3′.

**Measurement of cAMP**—The intracellular cAMP level was measured by the Amersham Biosciences cAMP Bioreagent enzyme-linked immunoassay system (GE Healthcare). Cells were grown to early log phase, and cAMP was measured using cAMP Biotrak assay kit (GE Healthcare). Cells were grown to early log phase, and cAMP was measured using cAMP Biotrak assay kit (GE Healthcare).

**Measurement of the Ras-GTP Level**—The Ras-GTP level was determined by GST-RBD pull-down assay that has been described previously (36, 40, 41). *Escherichia coli* cells carrying plasmid pPHY2640 (36) that expresses GST-RBD were grown in Luria Broth with carbencillin medium at 37 °C to an *A*~s~ of about 0.5. Expression of GST-RBD was induced by treatment with 100 μM isopropyl 1-thio-β-D-galactopyranoside for 3 h. Cells were collected by centrifugation and resuspended in buffer A (25 mM Tris-HCl (pH 7.4), 140 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF). Cells were lysed by sonication for 2 min. After centrifugation at 15,000 rpm for 20 min, the super-

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**FIGURE 1. The Yeast Ras/PKA pathway.** See text for explanations.
natant was incubated with glutathione beads at 4 °C for 1.5 h. The GST-RBD-bound beads were washed five times with washing buffer (25 mM Tris-HCl (pH 7.4), 500 mM NaCl, 0.1% Triton X-100, and 1 mM PMSF). To prepare the yeast whole cell extract for the pull-down assay, yeast cells in early log phase were washed twice with ice-cold water and once with ice-cold cell washing buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, and 0.1% Nonidet P-40). Cells were resuspended in buffer B (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 5 mM MgCl₂, 0.1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM PMSF, 10 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerol phosphate, and protease inhibitor mixture from Roche) and lysed by vortexing with glass beads. The whole cell lysates were centrifuged at maximum speed for 10 min, the protein concentration was determined, and the same amount of whole cell lysate was then incubated with GST-RBD-bound beads at 4 °C for 1.5 h. After washing with buffer B for 3 times, bound proteins were eluted with sample buffer and analyzed by immunoblotting with anti-Ras1 (Abcam) and anti-Ras2 (Santa Cruz Biotechnology, Inc.) antibodies.

**Immunoprecipitation**—The association of Ubp3 and Ira2 was examined by immunoprecipitation of FLAG-tagged Ubp3 and immunoblotting with anti-HA antibodies for HA-Ira2. Cells transformed with pYES-FLAG-Ubp3, pKT64-HA-Ira2, or empty vector were grown to early log phase, harvested by centrifugation, and resuspended in 550 μl of lysis buffer (50 mM NaPO₄ (pH 7.5), 400 mM NaCl, 0.1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, 25 mM NaF, 25 mM glycerophosphate, 1 mM sodium orthovanadate, 10 mM N-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, and 1 pellet of complete EDTA-free protease inhibitor mixture from Roche) and lysed by vortexing with glass beads. The whole cell lysates were centrifuged at maximum speed for 10 min, the protein concentration was determined, and the same amount of whole cell lysates was then incubated with GST-RBD-bound beads at 4 °C for 1.5 h. After washing with buffer B for 3 times, bound proteins were eluted with sample buffer and analyzed by immunoblotting with anti-Ras1 (Abcam) and anti-Ras2 (Santa Cruz Biotechnology, Inc.) antibodies.

**Heat Shock Assay**—Stationary phase cells were diluted to A₆₀₀ 0.002 in sterile water and treated with or without heat shock at 55 °C for 10 min. Cells (50 μl) were plated and incubated at 30 °C for 2–3 days before the images were recorded.

**Glycogen Measurement**—Cells from the storage plate were inoculated into liquid medium and grown for 10 h. About 200 μl of culture was spotted onto a filter paper. To stain for glycogen, the filter paper containing the culture was exposed to iodine vapor generated from iodine in a solid state for 1 min. Cells with a higher glycogen content display darker staining by iodine.

**Microscopy**—Early log phase cells were collected by centrifugation. Cells were transferred on glass slides and visualized by Leica DM400B-M microscope with a ×63 oil objective. Images were taken by QIMAGING. The experiments were done at least three times, and representative results are shown.

**RESULTS**

The Deubiquitinating Enzyme Ubp3 Negatively Regulates Ras/PKA Signaling—It has been reported that disrupting UBP3 leads to an increased sensitivity to rapamycin (33), a drug that specifically inhibits TORC1. However, the underlying mechanism by which Ubp3 modulates rapamycin sensitivity is unknown. A recent study indicates that there is an antagonistic relationship between the TORC1 and PKA pathways (36). Specifically, it has been demonstrated that hyperactivation of the PKA pathway often leads to increased sensitivity to rapamycin. Conversely, down-regulating TORC1 activity typically triggers elevated activation of PKA (36). Thus, it is possible that Ubp3 may affect rapamycin sensitivity via negatively regulating PKA activity. Accordingly, we compared the level of PKA activation in wild-type cells versus mutant cells that lack Ubp3.

To monitor the activation status of PKA, we made use of an antibody raised against phospho-PKA substrate (RRXS/Y**T**) (catalog no. 9624, Cell Signaling Technology, Inc.). To confirm that the immunoblotting signal detected by this antibody reflects PKA activity in yeast, we prepared whole cell extracts from yeast cells that were starved for glucose, a physiological...
condition that inhibits PKA activity in vivo (45), separated the extracts by SDS-PAGE, and probed the blot with the phospho-PKA substrate antibody. As shown in Fig. 2A, the antibody is able to detect quite a few distinct bands in extracts prepared from non-starved cells. The intensity of several bands (marked by asterisks) is diminished markedly in extracts prepared from glucose-starved cells (B), suggesting that these bands represent phosphorylated species of PKA substrates. We also examined the whole cell extracts from yeast cells that express a constitutively active allele of Ras2 (i.e. Ras2val19) (46) that locks the protein in a GTP-bound active state and, consequently, leads to elevated activation of PKA. As expected, expressing Ras2val19 leads to a clear increase in the intensity of some of the same bands that are responsive to the level of glucose (Fig. 2C). Together, this analysis indicates that immunoblotting of whole cell extract with phospho-PKA substrate antibody can be used as a convenient and reliable means to monitor the activation status of PKA.

Next, we examined the activation status of PKA in the ubp3Δ mutants using the validated phospho-PKA substrate antibody. As shown in Fig. 3A, the intensity of several bands that correspond to the activation status of PKA is increased significantly in the ubp3Δ cells. Importantly, the effect observed in the ubp3Δ mutant is indeed due to the loss of Ubp3, as it can largely be rescued by expressing the UBP3 gene from a plasmid. In addition, expression of a catalytically inactive allele of Ubp3 (i.e. Ubp3C408A) fails to prevent the accumulation of hyperphosphorylated PKA substrates in the ubp3Δ mutants (Fig. 3B), indicating that the catalytic activity of Ubp3 is required in this process. Note that it has been documented previously that the expression level of the inactive allele of Ubp3 is about the same as that of wild-type Ubp3 (47).

Mutants with high PKA activity are typically more vulnerable to heat shock (48). To examine whether disrupting UBP3 confers an increase in heat shock sensitivity, we compared this property between wild-type and the ubp3Δ mutants. As shown in Fig. 3C, after a brief heat shock at 55 °C, compared with the wild type, the ubp3Δ mutants display a substantially decreased rate of survival, indicating that the mutants are indeed more sensitive to heat shock. PKA also has an important function in cell size control, and mutants with constitutively activated PKA often display an increase in cell size (49). As would be expected
**Regulation of Ras/PKA Signaling by Ubp3**

**FIGURE 4. An elevated level of intracellular cAMP is partially responsible for phenotypes displayed by the **ubp3Δ** mutants.**

A, wild-type and **ubp3Δ** cells were grown to early log phase, and levels of intracellular cAMP were measured by ELISA. The values shown in the bar graph are from three independent experiments. The difference between the wild-type and **ubp3Δ** was statistically analyzed by Student’s t test. *, p < 0.05. B, wild-type and **ubp3Δ** cells were transformed with either an empty vector or plasmids expressing high copy phosphodiesterase 2 (PDE2). Cells were grown to stationary phase, treated or not treated by heat shock at 55 °C for 10 min, and plated. The images were taken after 2 days of incubation at 30 °C. C, cAMP sensitivity of the same cells as described in B was measured by comparing the growth of serially diluted exponentially growing yeast cells with or without the addition of 8 ng/ml of rapamycin. The data shown are representative of three independent experiments. D, images of the same cells as described in B visualized under a light microscope.

Repeatedly observed a significantly higher level of cAMP in the **ubp3Δ** mutants, and the magnitude of increase is comparable with that observed in the mutants, such as tfs1Δ, that lack known negative regulators of the Ras/PKA pathway (53). Note that even the hyperactive Ras<sup>V2019</sup> only increases cAMP 1.7-fold (54).

Next, we asked whether an elevated intracellular cAMP is responsible for the phenotypes displayed by the **ubp3Δ** mutants. In yeast, cAMP is hydrolyzed and thus removed by the action of a phosphodiesterase, Pde2 (37). If the phenotypes of the **ubp3Δ** mutants are due to the elevated intracellular cAMP, removing cAMP via PDE2 overexpression might suppress these phenotypes. To test this, we first compared the heat shock sensitivity of the wild type and the **ubp3Δ** mutants with or without PDE2 overexpression. Similar to what we observed earlier, the **ubp3Δ** mutants were much more sensitive to a brief heat shock at 55 °C than the wild type (Fig. 4B). However, the difference in heat shock sensitivity between the wild type and the **ubp3Δ** mutants was largely reduced by PDE2 overexpression (Fig. 4B). Likewise, the differences in cell size (Fig. 4C) and rapamycin sensitivity (D) between the wild type and the mutants were also diminished upon PDE2 overexpression. These data indicate that an elevated level of intracellular cAMP is partially responsible for increased heat shock sensitivity, enlarged cell size, and increased sensitivity to rapamycin of the **ubp3Δ** mutants.

**Ubp3 Modulates the Activation Status of Ras**—Our data thus far indicate that cAMP is elevated in the **ubp3Δ** mutants and that this increase is partially responsible for the abnormal signaling behavior of **ubp3Δ**. Next, we asked whether signaling steps upstream of cAMP might be altered in the **ubp3Δ** mutants. For this purpose, we compared the activation status of Ras in the wild type versus the **ubp3Δ** mutants. The activation status of Ras was measured via GST-RBD binding. RBD is a Ras-binding domain derived from Raf that specifically binds GTP-bound Ras but not GDP-bound Ras and is widely used for the measurement of the Ras-GTP level (18, 36, 40, 55). Whole cell lysates were prepared from both the wild type and the **ubp3Δ** mutants and incubated with purified GST-RBD proteins that were bound to glutathione beads. The bound samples were eluted, and the level of Ras bound to GST-RBD was revealed via immunoblotting with antibodies that recognize Ras1 and Ras2. As shown in Fig. 5, the levels of Ras1-GTP and Ras2-GTP are substantially higher in the extracts prepared from the **ubp3Δ** cells, indicating that there is more active Ras in the **ubp3Δ** mutants. These data suggest that the activation status of Ras is directly or indirectly regulated by Ubp3.

**Ubp3 Interacts with RasGAP Ira2 and Regulates Its Ubiquitination**—The well known negative regulators of yeast Ras proteins are their GTPase-activating proteins Ira1 and Ira2 (56, 57). Interestingly, the activity of Ira2 is known to be regulated by ubiquitination (18). Specifically, ubiquitination of Ira2 inactivates the protein and leads to accumulation of Ras-GTP and enhanced Ras activation (18). Given the similar phenotypes displayed by the **ubp3Δ** and **ira2Δ** mutants in accumulating Ras-GTP, we reasoned that Ubp3 may inhibit Ras/PKA signaling via regulating Ira2. To test this, we first examined whether...
there was any detectable interaction between these two proteins. For this purpose, FLAG-tagged Ubp3 and/or HA-tagged Ira2 were expressed in the ubp3Δ mutant. FLAG-Ubp3 was purified via immunoprecipitation. Copurification of Ira2 with Ubp3 was examined via immunoblotting of the purified samples with an antibody that recognizes HA-Ira2. As shown in Fig. 6A, Ira2 indeed copurifies with Ubp3, suggesting that these two proteins interact proximately.

Given that Ira2 undergoes ubiquitination and that Ubp3 interacts with Ira2, we then asked whether Ubp3 modulates the ubiquitination status of Ira2. For this purpose, we exploited an affinity purification method for isolating ubiquitin-conjugated proteins (43). Cells were transformed with a plasmid that expresses poly-His-tagged ubiquitin (8His-Ubi) from the GAL1 promoter or an empty vector as a control. Cells in early log phase were collected, and extracts from collected cell pellets were prepared for affinity purification. The purifications were conducted under denaturing conditions to dissociate Ira2 from other ubiquitinated proteins that happen to bind Ira2 via non-covalent interactions. Samples of the extracts and purified proteins were resolved by SDS-PAGE and analyzed by immunoblotting. Ubiquitinated Ira2 was revealed by immunoblotting of the affinity-purified 8His-Ubi-conjugated proteins with an antibody that recognizes HA-Ira2 (denoted with an asterisk) in the purified sample. This phenomenon has been observed previously (44).

DISCUSSION

The Ras/PKA pathway plays an essential role in coordinating cell growth and proliferation with the nutrient status. Signaling of the Ras/PKA pathway has to be regulated tightly, and its abnormal regulation can lead to deleterious consequences, such as cancer. In this work, we provide evidence that Ubp3 is a negative regulator for this important signaling pathway in yeast. Similar to other mutants, such as ira2Δ and tfs1Δ, that lack known negative regulators for this pathway, the ubp3Δ mutant displayed increased sensitivity to severe heat shock, reduced accumulation of glycogen, and an enlarged cell size. Likewise, the ubp3Δ mutants also have elevated levels of intracellular cAMP and Ras-GTP. Consistent with the notion that an elevated cAMP is responsible for the signaling behaviors of the ubp3Δ mutants, its many abnormal phenotypes can be rescued via overexpressing Pde2, a phosphodiesterase that specifically hydrolyzes cAMP.

It has been shown previously that the ubp3Δ mutants are more sensitive to rapamycin treatment, but the underlying mechanism is unclear (33). Notably, we find that the increased rapamycin sensitivity of the ubp3Δ mutants is also partially rescued by Pde2 overexpression. Thus, the increased rapamy-
Regulation of Ras/PKA Signaling by Ubp3

...cin sensitivity of the ubp3Δ mutants can be partially attributed to its hyperactivation of Ras/PKA signaling. This is consistent with a recent report that the Ras/PKA and TOR pathways work in parallel and can antagonize each other to achieve optimal growth signaling suitable to the perceived nutrient status (36).

How does Ubp3 regulate Ras/PKA signaling? The significant accumulation of Ras-GTP in the ubp3Δ mutants suggests that the target of Ubp3 lies at the step of Ras or upstream of Ras. In support of this notion, we find that the PKA activity is nearly identical in the wild-type cells and the ubp3Δ mutants once cells reach the stationary phase, at which point neither Ras1 nor Ras2 is detectable3. It is certainly plausible that Ras proteins are the relevant targets of Ubp3 because emerging evidence suggests that ubiquitination is an important mechanism for the regulation of Ras. For example, ubiquitination of K-Ras at a specific site (Lys-147) leads to persistent signaling and, thereby, promotes tumorigenesis (58). However, it has not been demonstrated clearly in yeast that Ras is ubiquitinated. Our attempts of detecting ubiquitinated Ras (Ras1 and Ras2) in either the wild type or the ubp3Δ mutants were not successful. It is possible that ubiquitination of Ras1 or Ras2 is either very transient or that the stoichiometry is extremely low, preventing its efficient detection. Although it remains possible that Ubp3 directly regulates Ras proteins, our analysis suggests that RasGAP Ira2 might be a relevant target. It is known that Ira2 undergoes ubiquitination (18). Here we find that Ubp3 interacts with Ira2 and modulates its level of ubiquitination. Despite the accumulation of ubiquitinated Ira2 in the ubp3Δ mutant, neither the abundance nor the half-life of the protein is affected by Ubp33, suggesting that ubiquitination may regulate other aspects of Ira2 properties, such as its interaction with Ras proteins. Regulation of protein-protein interaction via ubiquitination is not without precedence. In fact, recent work demonstrated that ubiquitination of Lys-147 of K-Ras severely impairs its interaction with RasGAP neurofibromatosis type 1 (55), which provides a satisfying explanation why ubiquitination of K-Ras promotes tumorigenesis. In addition, it is possible that interaction with Ubp3 could alter how Ira2 interacts and, thus, acts on its targets, Ras1/2. Future studies will investigate how ubiquitination regulates the property of Ira2 and also identify additional targets of Ubp3 in modulating its regulation of Ras/PKA pathway.

Ubp3 is found in both cytoplasmic and nuclear compartments. Which pool of Ubp3 is primarily responsible for its regulation of Ras/PKA signaling? Our preliminary studies suggest that Ubp3 in the nucleus does not have any significant role in this regulation, as a variant of Ubp3 engineered to be exclusively plasma membrane-localized (possessing a CAAX box from Ste18 at its C-terminal) is capable of rescuing the signaling phenotype of the ubp3Δ mutants3. Note that this same variant is unable to rescue the phenotype of the ubp3Δ mutants because of its loss of Ubp3 in the nucleus, such as its enhanced sensitivity to 6-AU (59). The fact that nuclear Ubp3 is not required for its regulation of Ras/PKA signaling is consistent with our model that Ubp3 acts at the step of Ras or upstream of Ras.

Ubp3 has a human homolog, Usp10, a protein implicated in tumor suppression (31, 60). Ira2 has a human homolog, neurofibromin, a tumor suppressor whose activity is controlled by ubiquitination and degradation. Abundant examples in the past have clearly demonstrated that mechanisms regulating Ras signaling are highly conserved (12–14). Thus, it is likely that Usp10 plays a similar role in humans in regulating Ras activation.

In summary, we have shown that Ubp3 negatively regulates Ras/PKA signaling, possibly at the step of Ras or upstream of Ras. Thus, the deubiquitinating enzyme joins the list of other families of proteins such as Ras-GEF, Ras-GAP, phosphodiesterase, and phosphatase as new Ras/PKA regulator. Given the crucial roles of Ras/PKA signaling in human diseases, targeting the deubiquitinating enzyme may offer a new strategy to beneficially modulate signaling by the Ras/PKA pathway.

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