Molecular Cell 22, 819–830, June 23, 2006 ©2006 Elsevier Inc. DOI 10.1016/j.molcel.2006.05.011

The Kelch Proteins Gpb1 and Gpb2 Inhibit Ras Activity via Association with the Yeast RasGAP Neurofibromin Homologs Ira1 and Ira2

Toshiaki Harashima,[1,3] Scott Anderson,[2] John R. Yates III,[2] and Joseph Heitman[1,*]
[1] Department of Molecular Genetics and Microbiology
Duke University Medical Center
Durham, North Carolina 27710
[2] The Scripps Research Institute
La Jolla, California 92037

Summary

The G protein-coupled receptor Gpr1 and associated Gα subunit Gpa2 govern dimorphic transitions in response to extracellular nutrients by signaling coordinately with Ras to activate adenylyl cyclase in the yeast *Saccharomyces cerevisiae*. Gpa2 forms a protein complex with the kelch Gβ mimic subunits Gpb1/2, and previous studies demonstrate that Gpb1/2 negatively control cAMP-PKA signaling via Gpa2 and an unknown second target. Here, we define these targets of Gpb1/2 as the yeast neurofibromin homologs Ira1 and Ira2, which function as GTPase activating proteins of Ras. Gpb1/2 bind to a conserved C-terminal domain of Ira1/2, and loss of Gpb1/2 results in a destabilization of Ira1 and Ira2, leading to elevated levels of Ras2-GTP and unbridled cAMP-PKA signaling. Because the Gpb1/2 binding domain on Ira1/2 is conserved in the human neurofibromin protein, an analogous signaling network may contribute to the neoplastic development of neurofibromatosis type 1.

Introduction

Molecular switches composed of G protein-coupled receptors (GPCRs) and associated heterotrimeric G proteins transduce extracellular stimuli to intracellular signaling molecules, including the ubiquitous second messenger cAMP. Canonical heterotrimeric G proteins consist of α, β, and γ subunits. Ligand binding induces conformational changes in the receptor, stimulating GDP to GTP exchange on the associated Gα subunits, leading to dissociation of the receptor-Gα subunit complex and release of the Gβγ dimer. Liberated Gα, Gβγ, or both signal via downstream effectors. Signal transduction is attenuated by either intrinsic or RGS-stimulated GTP hydrolysis followed by reassociation of Gα-GDP with the Gβγ dimer (Cabrera-Vera et al., 2003; Dohlman et al., 1991; Ross and Wilkie, 2000).

The budding yeast *Saccharomyces cerevisiae* deploys two distinct GPCR-G protein signaling modules to sense pheromones and nutrients, respectively (Harashima and Heitman, 2004). One is haploid and mating-type specific and involves the pheromone receptors Ste2/3 coupled to the Gα subunit Gpa1 in a canonical heterotrimeric complex with the Gβγ subunits Ste4/18. In response to pheromone, the Ste4/18 dimer dissociates from Gpa1 and activates the pheromone-responsive MAP kinase pathway to enable mating.

The second yeast GPCR signaling cascade involves the GPCR Gpr1, which is expressed in both haploid and diploid cells and activates the associated Gα subunit Gpa2 in response to glucose and structurally related sugars (Lemaire et al., 2004; Lorenz et al., 2000; Xue et al., 1998; Yun et al., 1998). Activated Gpa2 stimulates cAMP production by adenylyl cyclase and engages the PKA signaling pathway (Colombo et al., 1998; Lorenz and Heitman, 1997). In contrast to canonical Gα subunits, Gpa2 is unable to form a heterotrimeric G protein with the known Gβγ subunits Ste4/18. Instead, Gpa2 associates with two kelch proteins, Gpb1 and Gpb2, which are functionally redundant, share ~35% sequence identity, and each contain seven kelch repeat motifs. In a striking example of convergent evolution, both the WD-40 repeat-based Gβ subunits and the kelch repeat enzyme galactose oxidase are known to fold into seven bladed β propeller structures that are essentially superimposable (Harashima and Heitman, 2004).

Mutants lacking the Gpr1 receptor or the coupled Gpa2 subunit are defective in glucose-induced cAMP production and filamentous growth, whereas *gpb1,2* double mutants exhibit increased PKA phenotypes, including enhanced filamentous growth, sensitivity to nitrogen starvation and heat shock, and impaired glycogen accumulation and sporulation (Batlle et al., 2003; Harashima and Heitman, 2002). Introduction of *gpa2* mutations only partially attenuates these *gpb1,2* mutant phenotypes, providing evidence that Gpb1/2 negatively regulate cAMP signaling by inhibiting Gpa2 and an as yet unidentified second target. Mutation of the gene encoding one of the three PKA catalytic subunits, Tpk2, largely suppresses the elevated PKA phenotypes of *gpb1,2* mutants, indicating that this second target may be a component of the cAMP signaling pathway itself (Harashima and Heitman, 2002). Our recent studies provide evidence that Gpb1/2 are recruited to and function at the plasma membrane in a Gpa2-dependent manner, suggesting that the unidentified second target may be membrane-associated (Harashima and Heitman, 2005).

In *S. cerevisiae*, the cAMP-PKA signaling cascade is essential for cell viability. Loss of either adenylyl cyclase (Cyr1) or all three PKA catalytic subunits (Tpk1,2,3) is lethal (Toda et al., 1988; Toda et al., 1987b). On the other hand, elevated PKA activity as a consequence of mutations in the PKA regulatory subunit Bcy1 results in a growth defect (Toda et al., 1987a). Therefore, cAMP signaling must be strictly controlled in response to extracellular cues. Two distinct Gpr1-Gpa2 and Ras-mediated pathways converge on Cyr1. Notably, *ras2* mutants fail to produce cAMP in response to glucose, similar to *gpr1* and *gpa2* mutants, and *gpr1 ras2* and *gpa2 ras2* mutants exhibit a synthetic growth defect, suggesting that Gpr1-Gpa2 and Ras2 play a shared role in glucose-induced cAMP production (Bhattacharya et al., 1995; Kübler et al., 1997; Xue et al., 1998).

*Correspondence: heitm001@duke.edu
[3] Present address: Division of Molecular Cell Biology, National Institute for Basic Biology, Nishigonaka 38, Myodaiji, Okazaki 444-8585, Aichi, Japan.

S. cerevisiae expresses two Ras proteins: Ras1 and Ras2 (Powers et al., 1984). Although Ras1 and Ras2 are functionally redundant for cell growth, Ras2 plays the predominant role in cAMP signaling in response to glucose. Ras activity is controlled positively by the guanine nucleotide exchange factors Cdc25 and Sdc25 (GEFs) and negatively by the GTPase activating proteins Ira1 and Ira2 (GAPs) (Crechet et al., 1990; Munder and Furst, 1992; Tanaka et al., 1990a, 1991). Ras2 is known to bind to and activate Cyr1, yet how Ras2 regulates Cyr1 in response to glucose is not understood at a molecular level (Field et al., 1988; Mintzer and Field, 1994). Previous studies have implicated Cdc25 in responses to glucose (Gross et al., 1999; Munder and Kuntzel, 1989; Portillo and Mazon, 1986). On the other hand, Ira1 has been shown to interact with Cyr1 and may promote its membrane localization (Mitts et al., 1991). *ira1,2* double mutant cells exhibit constitutively elevated Ras2-GTP levels and are unable to further mount a Ras2-GTP increase in response to glucose (Colombo et al., 2004). Therefore, both Cdc25 and Ira1/2 may coordinately activate Ras2 and adenylyl cyclase in response to glucose.

The RasGAP Ira1/2 proteins are large (~350 kDa) proteins that are conserved from yeast to humans (Tanaka et al., 1989, 1990b). In humans, the RasGAP activity of the Ira1/2 homolog neurofibromin is implicated in one of the most common genetic diseases, neurofibromatosis type I (NF1) (Ballester et al., 1990; Cawthon et al., 1990; Viskochil et al., 1990; Wallace et al., 1990; Xu et al., 1990a). Although the *IRA1*, *IRA2*, and *NF1* genes were cloned more than a decade ago, how the RasGAP activity of these proteins is controlled is largely unknown. Here, we identified the yeast neurofibromin homologs Ira1/2 as targets of the kelch Gβ mimic subunits Gpb1/2. Gpb1/2 bind to a conserved C-terminal domain, stabilize Ira1/2, and thereby serve to govern cAMP-PKA signaling by constraining Ras2-GTP excursions. These findings have profound potential implications for our understanding of NF1 functions in normal cell growth control and its dysregulation in individuals with NF1.

Results

Kelch Gβ Mimic Proteins Act Upstream of the PKA Pathway

We hypothesized that the second target of the kelch Gβ mimic Gpb1/2 proteins might be either an early or a later component of the PKA pathway. Here, epistasis analysis was used to pinpoint the site of Gpb1/2 action. In models in which Gpb1/2 function downstream of Cyr1, *gpb1,2* mutations would be predicted to rescue the growth defect of *ras2 gpa2* double mutant cells. To test this hypothesis, two diploid mutants (*gpa2/gpa2 ras2/RAS2* and *gpb1,2/gpb1,2 gpa2/GPA2 ras2/ras2*) were constructed, sporulated, and dissected. As shown in Figure 1A, *gpb1,2 ras2 gpa2* cells were as growth impaired as *ras2 gpa2* cells, providing evidence that Gpb1/2 instead act early in the pathway via Cyr1 or one of its regulatory elements such as Ras1/2, Cdc25, or Ira1/2.

To examine genetic interactions between *gpb1,2* and *ras2* mutations, the *RAS2* gene was deleted in *gpb1,2* cells and the resulting *gpb1,2 ras2* cells were tested for filamentous growth, *FLO11* expression, sensitivity to nitrogen starvation, and glycogen accumulation (Figures 1B–1F). Consistent with our previous findings, *gpb1,2* cells exhibited elevated pseudohyphal and invasive growth, increased *FLO11* expression, sensitivity to nitrogen starvation, and reduced glycogen accumulation. Introduction of a *gpa2* mutation partially suppressed these *gpb1,2* mutant phenotypes (Figure 1 and Harashima and Heitman [2002]). On the other hand, introduction of a *ras2* mutation more completely suppressed these *gpb1,2* mutant phenotypes. Steady-state and glucose-induced cAMP levels were also determined. As shown previously (Harashima and Heitman, 2002), an increased basal level of cAMP was observed in *gpb1,2* cells, and this elevated cAMP level was restored to the wild-type level by a *ras2* mutation (Figure 1G). Introduction of a *ras1* mutation was unable to suppress any of the *gpb1,2* mutant phenotypes (data not shown). Taken together, these genetic studies support the hypothesis that Gpb1/2 act directly on Ras2 or one of its regulators such as the RasGEF Cdc25 or the RasGAP Ira1/2 proteins.

RasGAP Proteins Ira1/2 Interact with the Kelch Gβ Mimic Subunits Gpb1/2

Possible targets of Gpb1/2 were identified by mass spectrometry analysis of the Gpb1/2 native protein complex. For this purpose, the FLAG epitope tag was fused to the carboxy terminus of Gpb1 and to the amino terminus of Gpb2. These FLAG-Gpb1/2 proteins were expressed from an attenuated *ADH1* promoter on a 2 μm plasmid. Expression of the FLAG-tagged proteins restored wild-type filamentous growth of the *gpb1,2* double mutant strain, indicating that both fusion proteins are functional (data not shown). Because endogenous Gpa2 and Gpb1/2 may compete for Gpb1/2 with other targets, the FLAG-Gpb1/2 proteins were expressed in *gpa2 gpb1,2* triple mutant cells. Crude cellular extracts were prepared, and Gpb1/2 and interacting proteins were coimmunoprecipitated by using an anti-FLAG affinity matrix. The native protein complexes were eluted with FLAG peptide, and the eluted proteins were analyzed by mass spectrometry (see Experimental Procedures).

This analysis revealed a number of candidate Gpb1/2-interacting proteins. Importantly, the list of Gpb2-interacting proteins included Ira1 and Ira2, and no other components of the cAMP-PKA signaling cascade (including Cdc25, Ras1/2, Cyr1, Pde1/2, Bcy1, Tpk1/2/3, Flo8, or Sfl1) were identified (data not shown). Because Gpb1 and Gpb2, and also Ira1 and Ira2, represent partially redundant protein pairs, we hypothesized that Gpb1 and Gpb2 might bind to both Ira1 and Ira2. To address this possibility, Gpb1 was N-terminally tagged and expressed, and Gpb1/2-Ira1/2 interactions were examined in cells that also expressed a functional version of the Ira1 or Ira2 protein fused with three copies of the hemagglutinin epitope tag (3HA) (Figure 2). Importantly, this coimmunoprecipitation analysis revealed that Gpb1 and Gpb2 both interact with both Ira1 and Ira2 (Figures 2A and 2B).

Gpb1/2 contain a unique N-terminal domain and a C-terminal domain containing seven kelch repeats. Our previous studies revealed that the kelch domains of Gpb2 bind to Gpa2 but the unique N-terminal domain does not (Harashima and Heitman, 2002). To examine which domain(s) is required for the Gpb1/2-Ira1/2

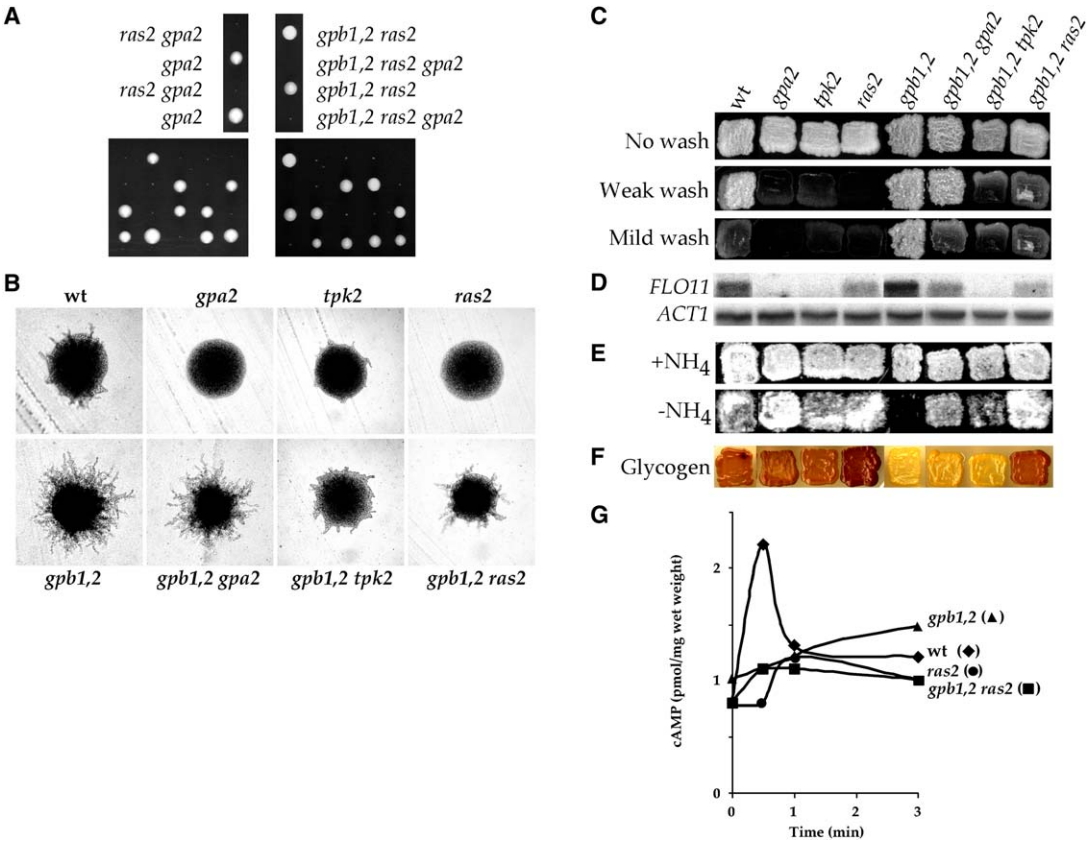

Figure 1. Genetic Interactions between *gpb1,2* and *ras2* Mutations

(A) *gpb1,2* mutations are unable to suppress the synthetic growth defect of *gpa2 ras2* mutant cells. Diploid *gpa2::G418/gpa2::hph ras2::nat/RAS2* (left) and *gpb1,2::loxP/gpb1,2::loxP gpa2::loxP-G418/GPA2 ras2::nat/ras2::nat* (right) cells were sporulated and dissected.

(B–F) *ras2* mutations alleviate increased PKA phenotypes associated with *gpb1,2* mutations, including enhanced pseudohyphal growth (B), hyperinvasive growth (C), increased *FLO11* expression (D), sensitivity to nitrogen starvation (E), and reduced glycogen accumulation (F).

(G) Glucose-induced cAMP production was examined. Glucose was added to glucose-starved cells, and at the indicated time points, cells were collected and cAMP levels were determined.

interaction, the FLAG tag was fused to either the N terminus of the N-terminal unique domain (FLAG-Gpb2N) or to the C-terminal kelch domain (FLAG-Gpb2C), and the resulting fusion proteins and FLAG-Gpb2 were coexpressed with the Ira1-3HA protein in vivo (Figure 2C). In contrast to the Gpa2-Gpb1/2 interaction, neither the Gpb2 N-terminal nor the C-terminal domain alone was sufficient to bind to Ira1. Therefore, both Gpb2 domains are required for interaction with Ira1. We note that the unique N-terminal and the C-terminal kelch domains are both essential for Gpb1/2 function in vivo (Harashima and Heitman, 2002).

Because the *gpb1,2* mutant phenotypes were suppressed by *ras2* mutations and Gpb1/2 bind to the RasGAP proteins Ira1/2, Gpb1/2 could associate with Ira1/2 and function via Ras2. However, Gpb1/2 interacted with Ira1/2 in the absence of Ras2 as strongly as in the presence of Ras2 (Figure 2D). Therefore, Ras2 is dispensable for the Gpb1/2-Ira1/2 interactions, and Gpb1/2 may control Ras activity through direct interaction with Ira1/2.

Gpb1/2 Are Genetically Implicated in Both Gpa2- and Ras-Mediated Signaling

ras2 mutants are defective in filamentous growth, whereas wild-type cells expressing a dominant active *RAS2*^{G19V} allele that lacks intrinsic GTPase activity exhibit elevated filamentous growth. Consistent with these findings, cells lacking Ira1, Ira2, or both Ira1 and Ira2 were also hyperfilamentous (Figure 3A). Importantly, *ira1,2* cells expressing Gpb1/2 and *ira1,2 gpb1,2* quadruple mutant cells lacking Gpb1/2 were morphologically indistinguishable from each other. These findings support models in which the hyperfilamentous phenotype conferred by the *gpb1,2* mutations may be exerted via Ira1/2.

Expression of the dominant active *GPA2*^{Q300L} and *RAS2*^{G19V} alleles dramatically enhances filamentous growth of wild-type cells (Figure 3B). In contrast, little if any further effect was observed when these dominant active mutant alleles were expressed in the hyperfilamentous *gpb1,2* double mutant, supporting models in which Gpa2 and Ras2 are activated by the *gpb1,2* mutations and Gpb1/2 function to negatively control the activity of both (Figure 3B).

If Gpb1/2 regulate Ira1/2, and the hyperfilamentous phenotype of the *gpb1,2* mutant is due to reduced Ira1/2 RasGAP activity, increased expression of the *IRA2* gene should suppress the *gpb1,2* mutant phenotype. In fact, overexpression of the *IRA2* gene attenuated pseudohyphal differentiation of the *gpb1,2* mutant (Figure 3C).

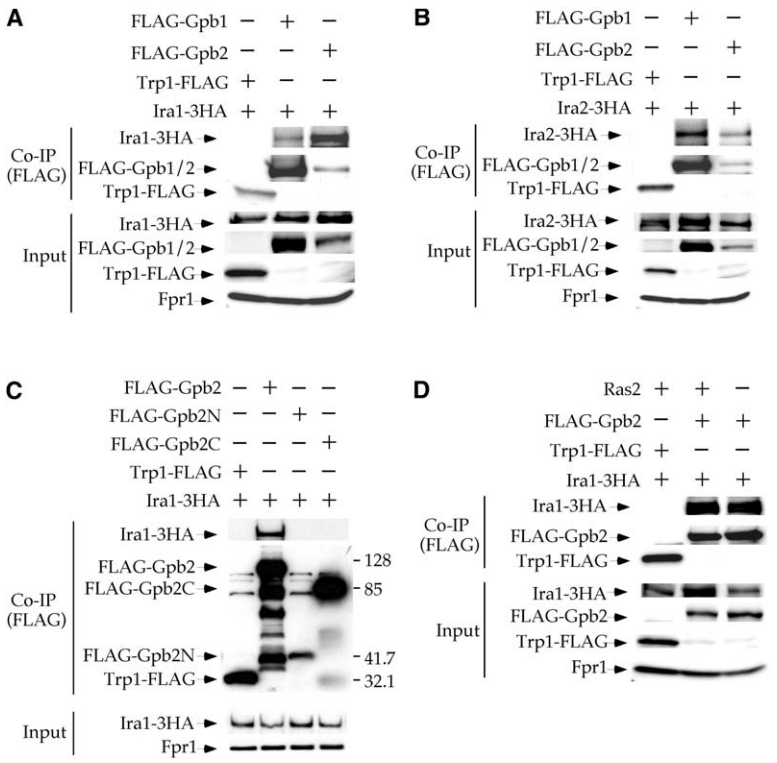

Figure 2. Kelch Gβ Mimic Subunits Interact with RasGAP Ira1/2

(A and B) Ira1 (A) and Ira2 (B) physically bind to Gpb1/2 in vivo. The N-terminally FLAG-tagged Gpb1 (pTH111) and Gpb2 (pTH88) proteins were expressed in yeast cells that also express C-terminally 3HA-tagged Ira1 or Ira2 and immunoprecipitated.

(C) Gpb2 requires both the unique N-terminal and the C-terminal kelch domains to interact with Ira1. The N-terminally FLAG-tagged Gpb2 N-terminal region (FLAG-Gpb2N), C-terminal kelch domains (FLAG-Gpb2C), or full-length Gpb2 (FLAG-Gpb2) was coexpressed with the Ira1-3HA protein in vivo. Positions of molecular marker (128, 85, 41.7, and 32.1 k) are indicated to the right of the panel.

(D) Ras2 is dispensable for the Gpb2-Ira1 interaction. Gpb2-Ira1 interactions were examined in the presence and absence of Ras2 by using cells that express FLAG-Gpb2.

Note that the first lane in the Co-IP panels of (A) and (B) was spliced to eliminate a space and that the data in each panel are directly comparable.

In (A)–(D), protein complexes were captured on anti-FLAG affinity gel and detected with anti-FLAG or anti-HA. Input levels of Ira1/2 were captured on anti-HA agurose beads, eluted, and analyzed by Western blot using anti-HA antibody (see the Experimental Procedures for details).

Consistently, neither loss of the *IRA1/2* genes nor introduction of the *RAS2*^{G19V} gene exaggerated mutant phenotypes (including hyperinvasion, nitrogen starvation sensitivity, and decreased glycogen) associated with an elevated PKA activity in *gpb1,2* cells (Figures 3D–3F). On the other hand, overproduction of Ira2 was able to alleviate these *gpb1,2* mutant phenotypes (Figures 3D–3F). In addition, the increased basal and glucose-induced cAMP levels in *gpb1,2* cells were significantly attenuated by Ira2 overproduction (Figure 3G).

In summary, these genetic data provide evidence that Gpb1/2 negatively control cAMP signaling via Ira1/2.

Gpb1/2 Control Ira1/2 RasGAP Activity

Biochemical and genetic data indicate that Ira1/2 represent secondary targets of Gpb1/2 and that Gpb1/2 function to enhance Ira1/2 activity. To investigate this at a mechanistic level, we quantified Ras2-GTP levels by measuring Ras2 protein binding to the Raf1 kinase that specifically interacts with Ras-GTP (Colombo et al., 2004). A low copy number plasmid carrying the wild-type *RAS2* or dominant active *RAS2*^{G19V} gene was introduced into wild-type, *gpb1,2*, *ira1*, *ira2*, and *ira1,2* cells. Transformants were grown in synthetic medium to mid-logarithmic growth phase, and crude cell extracts were prepared to assess the steady state levels of Ras2-GTP.

As shown previously, the Ras-GTP level was increased ~5-fold in *ira1* and *ira2* cells, (Figure 4A and Tanaka et al. [1990a]). Similarly, *gpb1,2* cells also exhibited an ~5 fold increase in Ras2-GTP levels, indicative of reduced RasGAP activity (Figure 4A). The Ras-GTP level in *ira1,2* cells was further increased and comparable to that in wild-type and *gpb1,2* cells expressing the *RAS2*^{G19V} gene, in which an ~25-fold increase in Ras-GTP was observed (Figure 4A). These observations are in accord with the previous finding documenting that Ras2-GTP levels were indistinguishable between wild-type and *ira1,2* cells when the *RAS2*^{G19V} gene was expressed (Tanaka et al., 1990a). In summary, loss of Gpb1/2 results in an elevation of Ras2-GTP, possibly by reducing, but not eliminating, the RasGAP activity of Ira1/2.

Gpb1/2 Control Protein Levels of Ira1/2

To elucidate how Gpb1/2 control Ira1/2 RasGAP activity, we investigated the levels of the Ira1/2 proteins as well as Ira1/2-Ras2 interactions in the presence and absence of Gpb1/2. To examine protein levels, the functional Ira1/2-3HA proteins were expressed. Neither Ira1 nor Ira2 was detectable by Western blot analysis using crude cell extracts because of low expression levels (data not shown). The Ira1/2-3HA proteins were therefore enriched by immunoprecipitation using anti-HA-conjugated agarose beads, which also enabled an examination of the levels of the Ras2 protein bound to the Ira1/2-3HA affinity captured proteins (Figure 4B).

As shown in Figure 4B, loss of Gpb1/2 resulted in a marked decrease in the levels of both Ira1 and Ira2 and a concomitant loss of Ras2 as an Ira1/2-interacting protein. Reintroduction of the *GPB1* and *GPB2* genes complemented this defect and restored the levels of Ira1/2 to the wild-type levels, indicating that Gpb1/2 govern the stability of Ira1/2.

To confirm this model, protein stability of Ira1/2 was examined in the presence and absence of Gpb1/2 by a pulse-chase analysis (Figures 4C and 4D). In wild-type cells, the Ira1/2 and Fpr1 proteins were stable over time, and the half life ($t_{1/2}$) of these proteins was more

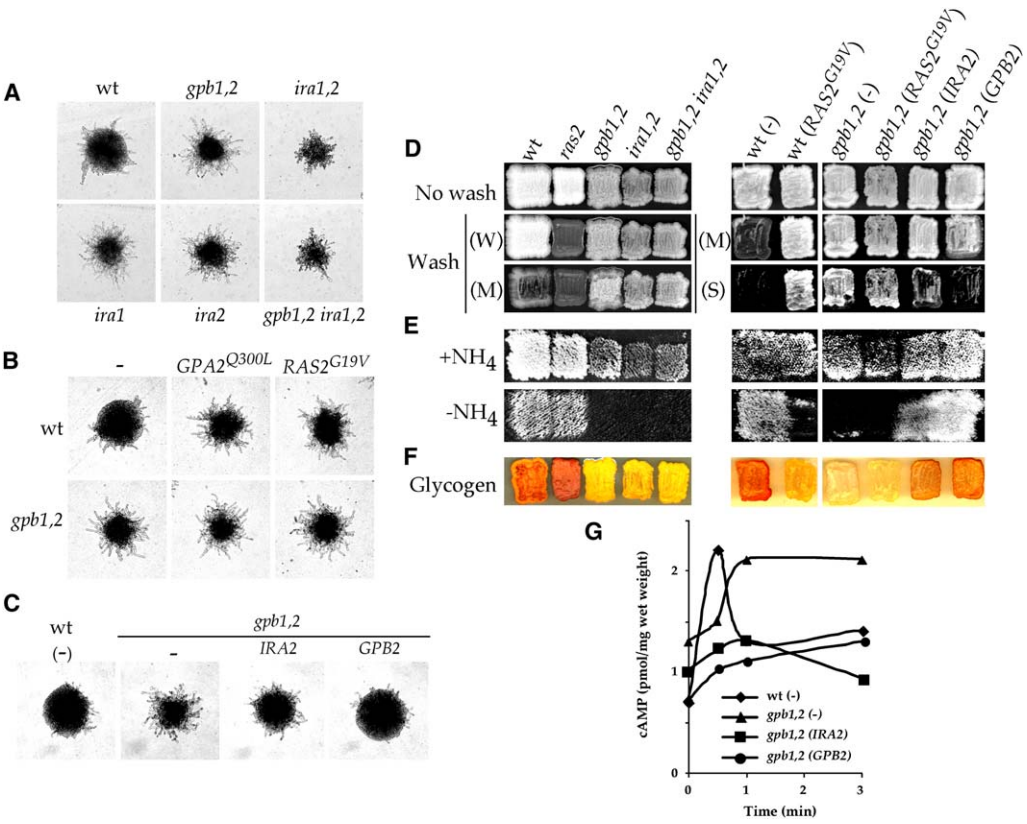

Figure 3. Genetic Interactions between *gpb1,2* and *ira1,2* Mutations

(A) Isogenic diploid strains indicated were assayed for pseudohyphal growth.

(B) The dominant active $GPA2^{Q300L}$ and $RAS2^{G19V}$ alleles were introduced into diploid wild-type and *gpb1,2* cells and tested for filamentous growth.

(C) Ira2 overproduction suppressed the increased filamentous phenotype of *gpb1,2* cells.

(D) Haploid cells indicated were tested for invasive growth. Cells were grown on YPD at 30°C for 5 days and photographed after weak (W), mild (M), or strong (S) washing.

(E) Cells were grown on YPD at 30°C for 2 days and replica plated onto nitrogen-replete ($+NH_4$) and no nitrogen ($-NH_4$) media to test for nitrogen starvation sensitivity. After 6 (left) or 10 (right) days at 30°C, cells were replica plated onto YPD again and incubated under the same conditions.

(F) Glycogen levels of cells grown on YPD at 30°C for 2 days were determined by using iodine vapor.

(G) cAMP levels were determined in response to glucose readdition as described in the legend of Figure 1.

than 4 hr ($t_{1/2} > 4$ hr, Figures 4C and 4D and data not shown). Similarly, the Fpr1 protein in *gpb1,2* cells was as stable as in wild-type cells (Figures 4C and 4D and data not shown). However, levels of the Ira1/2 proteins decreased rapidly, and the half-life of Ira1 and Ira2 was reduced to ~30 and 25 min, respectively (Figures 4C and 4D). Therefore, we conclude that Gpb1/2 bind to and stabilize Ira1/2 and that loss of Gpb1/2 leads to reduced Ira1/2 protein levels.

Gpb1/2 Stabilize Ira1/2 by Binding to a C-Terminal Domain

To establish how Gpb1/2 control stability of the Ira1/2 proteins, the Gpb1/2 binding domain on Ira1/2 was identified. For this purpose, deletions were created in the endogenous Ira1 C terminus by inserting the 3HA epitope and expressing these deletion derivatives in an otherwise wild-type background (Figure 5). Plasmids expressing the FLAG-Gpb1/2 proteins were then introduced into the resulting Ira1 deletion mutant cells, and Gpb1/2-Ira1 interactions were examined by FLAG-mediated coimmunoprecipitation (Figures 5A and 5B).

By Western blot analysis, the level of the C-terminally truncated 1–2925 aa Ira1 protein was significantly reduced and the shorter 1–2714 aa and 1–2432 aa Ira1 proteins were undetectable in this assay (Figure 5A and "Input" panel in Figure 5B). Because Gpb1/2 control the stability of the Ira1/2 proteins, deletion of a Gpb1/2 binding site should result in a decrease in Ira1/2 protein levels. Thus, we hypothesized that the Gpb1/2 binding site might be present in the C-terminal region of Ira1 necessary for Ira1 stability. In fact, Gpb1/2 bound to the Ira1 deletion protein retaining 1–2925 aa, but not to the derivative containing only 1–1257 aa (Figure 5A and "Co-IP" panel in Figure 5B). Furthermore, two N-terminal deletion derivatives that retain amino acid residues 2433–3092 and 2715–3092 were stably expressed and both associated with Gpb1/2 (Figure 5C). A C-terminal region spanning 2715–2925 aa of Ira1 also bound to Gpb1/2 (Figure 5D), and loss of this region resulted in instability of this Ira1 deletion derivative in accord with the role of Gpb1/2 in Ira1 protein stability (Figure 5A and data not shown). Taken together, these results reveal that the Gpb1/2 binding domain (GBD) maps between

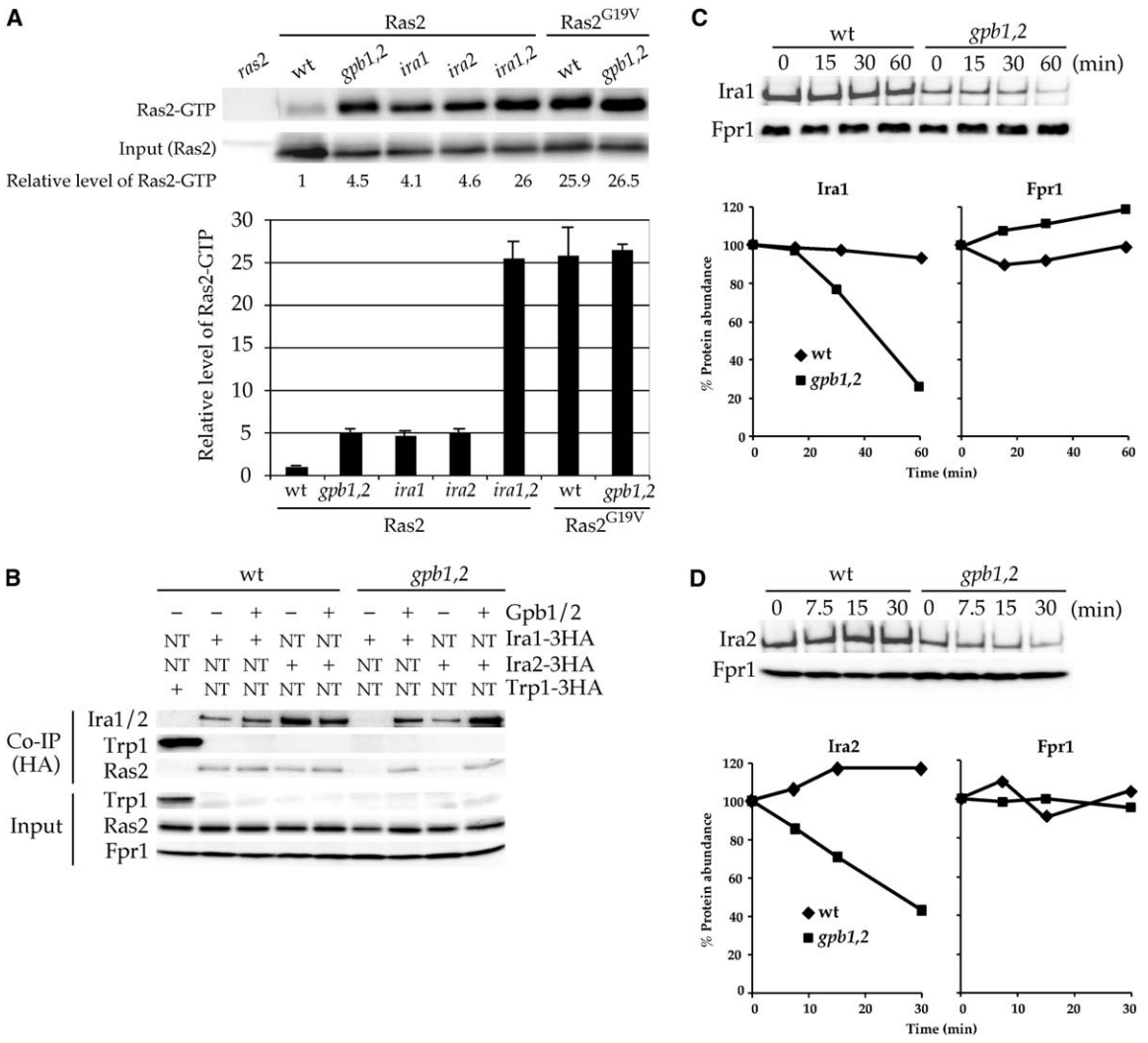

Figure 4. Gpb1/2 Stabilize Ira1/2

(A) The relative increase in Ras2-GTP was examined in haploid cells that express the wild-type or dominant active (G19V) *RAS2* gene. Representative data are shown in the top panel. Purified Ras2-GTP from *ira1,2* double mutant cells expressing the wild-type Ras2 protein and wild-type and *gpb1,2* mutant cells that express the dominant active $Ras2^{G19V}$ protein was 5-fold diluted prior to Western analysis, as the levels of Ras2-GTP in these cells were higher than those in the other cells. Levels of Ras-GTP and total cellular Ras protein ("Input") were densitometrically quantified. Ras2-GTP levels were then normalized to "Input" Ras2 levels and shown as a relative level to Ras2-GTP in wild-type cells in the bottom panel. The values shown in the bottom panel are the means of two or three independent experiments with the standard error of the mean.
(B) The *GPB1/2* genes were introduced into wild-type and *gpb1,2* cells expressing either *IRA1-3HA* or *IRA2-3HA* to examine protein stability of Ira1/2 and the Ras2-Ira1/2 interactions. "NT" indicates the nontagged, wild-type Ira1, Ira2, or Trp1 protein. Based on densitometric analysis, the steady-state protein levels of Ira1/2 were reduced in *gpb1,2* cells by at least 2- to 10-fold compared to wild-type cells.
(C and D) Protein stability of Ira1/2 was investigated by cycloheximide-chase assay in the presence and absence of Gpb1/2. Levels of Ira1/2-3HA and Fpr1 were densitometorically quantified, and the percentage of protein abundance of Ira1/2 and Fpr1 at "Time 0" is shown in the bottom panel. Note that the first and the last lanes in the "Ras2-GTP" and "Input" panels in (A) were spliced to eliminate a space or a lane and that the data in each panel are directly comparable.

amino acids 2715 and 2925 of Ira1. Significantly, this region is conserved in homologs of Ira1, including the human neurofibromin protein (see Discussion).

Equivalent deletions were also introduced into Ira2 (Figure S1 available in the Supplemental Data with this article online). The level of the Ira2 C-terminal deletion protein retaining 1–2922 aa was reduced, and this Ira2 deletion derivative was still able to interact with Gpb1/2 (Figures S1A and S1B). An Ira2 variant that preserves 1–2702 aa, but not the GBD, was now undetectable (Figure S1B). These findings are similar to the equivalent Ira1 deletion proteins (1–2925 aa and 1–2714 aa) (Figure 5). Both Gpb1 and Gpb2 bind to Ira2 deletion derivatives retaining amino acids 2703–3079 and 2703–2922 that are homologous to the regions spanning the corresponding amino acids 2715–3092 and 2715–2925 of Ira1 (Figures S1C and S1D). These results provide evidence that the GBD maps to the corresponding regions of both Ira1 and Ira2.

The C Terminus of Ira1/2 Is Required for Function

Biochemical studies reveal that deletion of the C-terminal 167 amino acids, and deletion of the C-terminal 378 amino acids or the GBD in the Ira1 protein reduce and

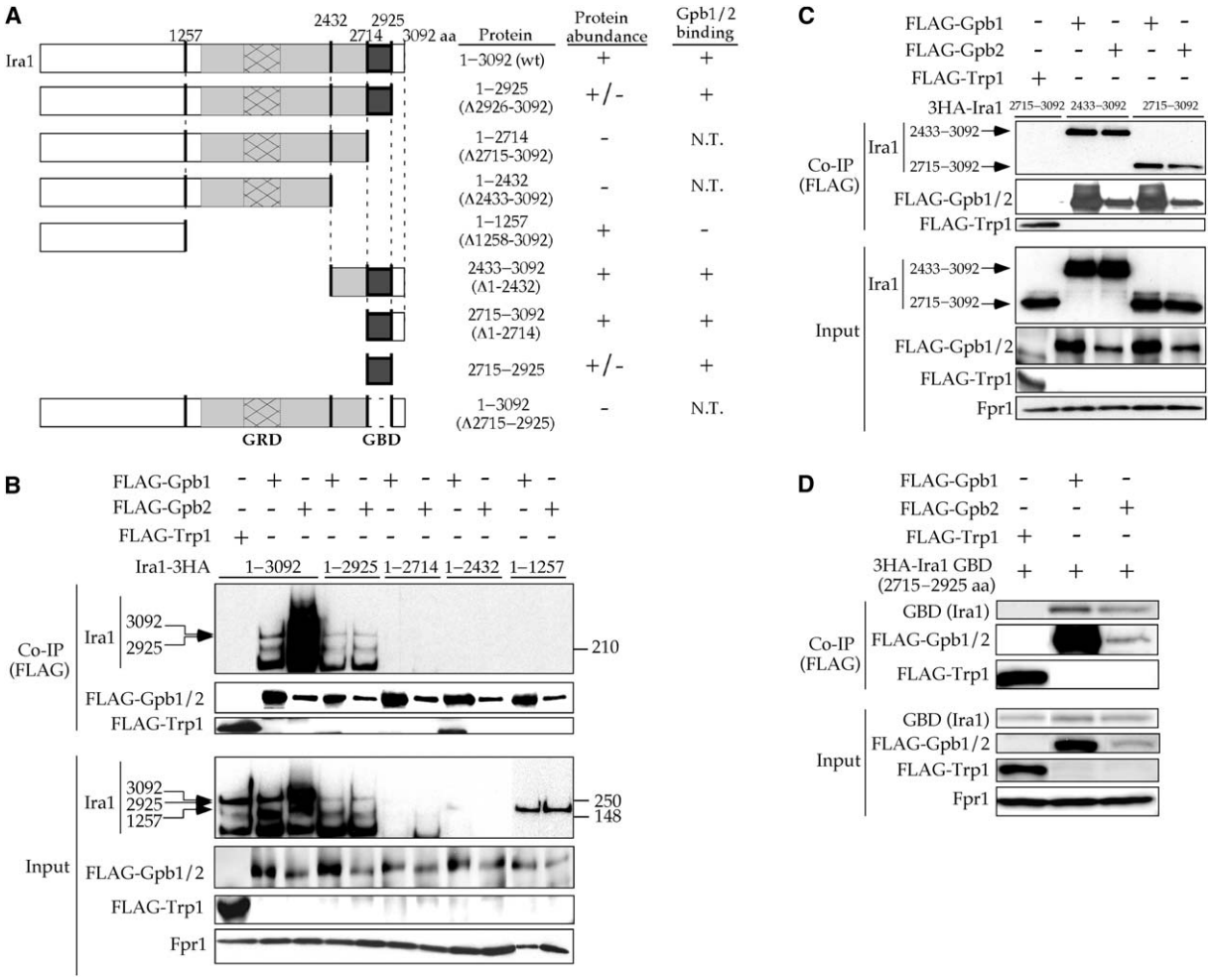

Figure 5. Gpb1/2 Bind to the C Terminus of Ira1

(A) Schematic of Ira1 deletion proteins created and summary of results obtained from assays of protein abundance (Input) and Gpb1/2 binding (Co-IP) as below. Positions of deletions created in Ira1 are shown and numbered. A conserved region between Ira1/2 and human neurofibromin is shaded in gray. The GAP-related domain (GRD) and the Gpb1/2 binding domain (GBD) are shown as hatched and dark gray rectangles, respectively.

(B) Protein interactions were investigated by using crude cell extracts from cells expressing the 3HA-tagged full-length Ira1 (1–3092 aa) or Ira1 C-terminal deletion variants. Positions of full-length wild-type Ira1 and deletion variants (1–2925 and 1 ~ 1257 aa) are indicated to the left of the panel. Positions at which molecular weight markers (250, 210, and 148 k) migrated are indicated to the right of the panels. The deletion of 167 amino acids leads to reduced protein levels of Ira1 from 3- to 7-fold in comparison of the full-length Ira1 protein level, and the further deletion (378 aa) results in undetectable levels ("Input"). Note that some smaller Ira1-3HA species were also detected via the C-terminal HA tag, indicating that these are proteolysis products lacking N-terminal regions. This further supports the assignment of the GBD to the C-terminal region of Ira1.

(C) N-terminal deletion Ira1 variants were tested for interaction with Gpb1/2. Positions of the Ira1 deletion variants (2433–3092 and 2715–3092 aa) are indicated to the left of the panel.

(D) A putative GBD of Ira1 spanning 2715–2925 aa was examined for Gpb1/2 interactions. Note that the last two lanes in (B) and the first lane in (D) were spliced to remove a space and that the Western data in each panel are directly comparable.

abolish protein stability, respectively. To test for a physiological relevance of these results, homozygous diploid cells that express these Ira1 C-terminal deletions (Δ2926–3092, Δ2715–3092, and ΔGBD) were constructed and assessed for pseudohyphal differentiation (Figure 6A). Haploid cells that carry these Ira1 C-terminal deletions were also tested for invasive growth, nitrogen starvation sensitivity, and glycogen accumulation (Figures 6B–6D). Cells expressing the truncated Ira1 derivative that contains 1–2925 aa and also includes the GBD exhibited significantly increased filamentous growth and sensitivity to nitrogen starvation and decreased glycogen (Figure 6). Cells expressing the shorter Ira1 derivatives that lack the GBD (1–2714 aa or ΔGBD) were markedly hyperfilamentous and phenotypically indistinguishable from *gpb1,2* or *ira1* null mutant cells, suggesting that these two Ira1 deletion derivatives are nonfunctional (Figure 6). This is consistent with instability of these deletion proteins (Figure 5). Therefore, the GBD and the extreme C-terminal region are both involved in protein stability and physiological functions of the Ira1 protein.

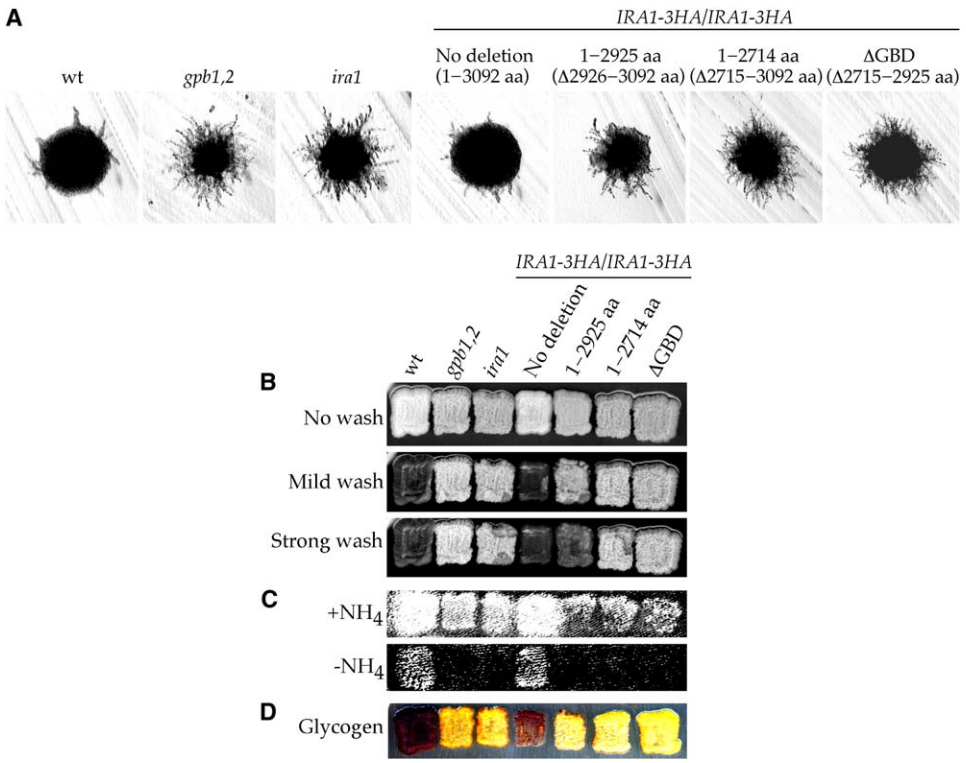

Figure 6. The C Terminus of Ira1/2 Is Necessary for Function

Homozygous diploid cells were tested for filamentous growth (A). Invasive growth (B), nitrogen starvation sensitivity (C), and glycogen accumulation (D) were examined by using isogenic haploid cells. To assay for nitrogen starvation sensitivity, cells were replica plated onto YPD after 7 days on nitrogen replete or depleted medium.

Discussion

Kelch Gpb1/2 Proteins Stabilize the RasGAP Proteins Ira1/2

The central finding of our study is the discovery that the activity of the yeast RasGAP neurofibromin homologs Ira1/2 is controlled by two components of the GPCR-Gα signaling module: Gpb1 and Gpb2. Our studies provide evidence that Gpb1/2 bind to and control the stability of Ira1/2 and thereby affect intracellular Ras-GTP levels. In conjunction with their role in binding the Gpa2-GDP complex and inhibiting receptor-Gα coupling, Gpb1/2 serve as potent molecular brakes to constrain signaling via the PKA signaling pathway during both vegetative growth and dimorphic transitions (Figure 7).

Deletion analysis enabled the definition of two C-terminal domains involved in the protein stability of Ira1/2. Namely, the Gpb1/2 binding domain (GBD) spanning 2715–2925 aa in Ira1 and the corresponding region in Ira2 (2703–2922 aa) and the more extreme C-terminal region of Ira1/2 that is unique to the yeast proteins (Figure 5 and Figure S1). The two domains have an additive effect, because the Ira1/2 C-terminally truncated proteins lacking the yeast-specific domain were still detectable, yet deletions eliminating both domains or the GBD alone resulted in undetectable levels of the Ira1/2 C-terminal deletion variants (1–2714 aa Ira1, Ira1ΔGBD, and 1–2702 aa Ira2). Consistent with this, Ira1/2 protein levels were significantly reduced in *gpb1,2* cells compared with those in wild-type cells (Figures 4B and 5 and Figure S1). Therefore, two distinct mechanisms appear to govern the stability of Ira1/2. Importantly, Ira1 was found to be ubiquitinated in a proteomic analysis of membrane-associated proteins (Hitchcock et al., 2003). This finding indicates that Ira1/2 protein stability might be controlled by a ubiquitin/proteasome-dependent mechanism as is neurofibromin (see below), and Gbp1/2 could inhibit Ira1/2 ubiquitination or interactions with the proteasome and thereby stabilize Ira1/2. Further studies will be required to elucidate in further detail the molecular mechanisms by which the yeast neurofibromin homologs Ira1/2 are stabilized. These would also shed light on how RasGAP activity of neurofibromin is controlled in response to extracellular stimuli.

Interestingly, in previous studies, a transversion mutation resulting in a premature nonsense codon at the 2700th amino acid was identified in Ira2, truncating the penultimate 222 amino acids, including the Gpb1/2 binding domain, and resulting in a loss of Ira2 function (Halme et al., 2004). These studies provide complementary support for our finding that the C-terminal domain of Ira1/2 is critical for biological function. Importantly, the role of the GBD is also likely to be conserved in human neurofibromin because the GBD is conserved among the yeast and mammalian neurofibromin homologs. Therefore, our findings should shed light on how the GAP activity of the neurofibromin homologs is controlled and its dysregulation in the ontogeny of NF1.

Ira1 and Ira2 share ~45% sequence similarity and are functionary redundant, yet each may also play specific

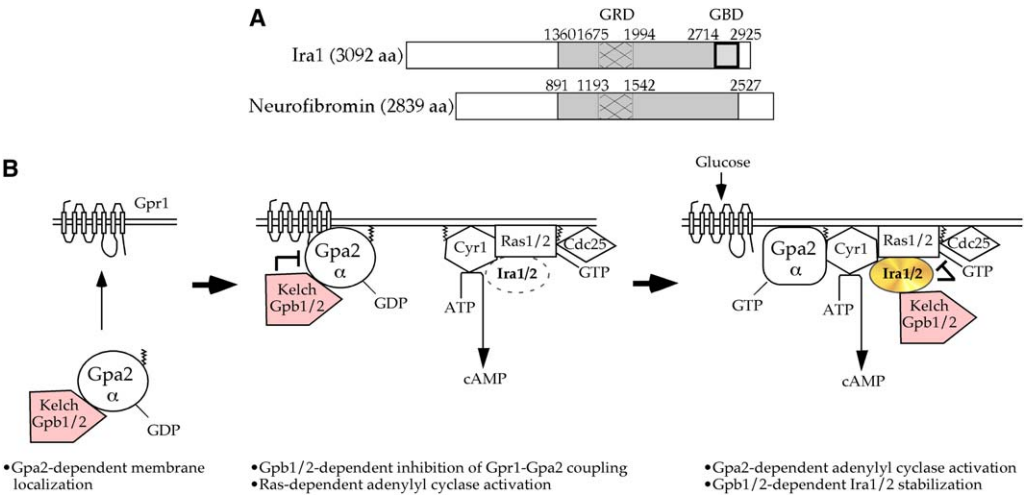

Figure 7. A Dual Role of the Kelch Proteins Gpb1/2 as Molecular Brakes on cAMP Signaling

(A) A schematic of the yeast neurofibromin homolog Ira1 and human neurofibromin proteins. A conserved region including the GRD and the GBD is shown in gray. GRD, hatched rectangle; GBD, bold rectangle.

(B) A model for how the kelch Gβ mimic proteins Gpb1/2 control cAMP signaling. See details in the text.

roles in Ras regulation. First, *ira1,2* double mutants exhibit more severe phenotypes compared to each single mutant. For instance, *ira1,2* double mutants exhibit an increased sensitivity to heat shock, enhanced filamentous growth, and increased Ras-GTP levels compared to *ira1* or *ira2* single mutants (Figure 3; [Tanaka et al., 1990a, 1990b]). Second, overproduction of the *IRA2* gene is able to suppress the heat shock sensitivity of *ira1,2* cells, but overexpression of the *IRA1* gene does not (Tanaka et al., 1990b). Similarly, we note that overexpression of *IRA2* suppresses the *gpb1,2* double mutant phenotype, whereas overexpression of *IRA1* does not (data not shown, Figure 3). Third, *ira1* mutations suppress the lethality of *cdc25* mutations more efficiently than *ira2* mutations (Tanaka et al., 1990b). Therefore, Ira1/2 may be controlled by both common and specific regulators. Indeed, Tfs1, which is a member of the phosphatidylethanolamine binding protein family and known as a cytoplasmic inhibitor CPY, specifically binds to and inhibits Ira2, although the mechanisms by which Tfs1 controls RasGAP activity of Ira2 remain unclear (Chautard et al., 2004).

Gpb1/2 Link Signaling from the Gα Subunit Gpa2 to Ras

Our studies demonstrate that a central molecular link between the GPCR Gpr1-Gα Gpa2 signaling module and Ras is exerted via the RasGAP Ira1/2. Our recent studies also demonstrate that Gpb1/2 preferentially bind to the GDP bound form of Gpa2 and Gpb1/2 are recruited to the plasma membrane in a Gpa2-dependent manner and function to inhibit coupling between the Gpr1 receptor and Gpa2 (Harashima and Heitman, 2002, 2005). We hypothesize that the following molecular events transpire when the ligand glucose binds to the Gpr1 receptor (Figure 7). First, GDP-GTP exchange occurs on Gpa2 and Gpb1/2 dissociate from Gpa2-GTP. Second, Gpa2-GTP stimulates cAMP production via adenylyl cyclase and the liberated Gpb1/2 subunits then interact with and stabilize the Ira1/2 proteins. On the other hand, Ras is also activated in response to glucose by a Cdc25-mediated GDP-GTP exchange reaction. The Gpb1/2 bound and stabilized Ira1/2 proteins now bind to Ras-GTP and stimulate GTP hydrolysis, attenuating Ras-mediated activation of Cyr1. These mechanisms provide the cell with an elaborate and balanced regulatory network that constrains cAMP signaling within a tightly controlled physiological range. In this model, Gpb1/2 play a dual inhibitory role to inhibit receptor-Gα coupling and to extinguish signaling by Ras-GTP via their action to stabilize and thereby promote the RasGAP activity of Ira1/2.

A recent report has suggested that Gpb1/2 might act late in the PKA pathway to regulate signaling, possibly via direct actions on the PKA catalytic or regulatory subunit or on a protein phosphatase that impinges on PKA (Lu and Hirsch, 2005). Although such a model is conceivable, no direct evidence linking Gpb1 or Gpb2 to either PKA subunits or candidate regulators was presented. Instead, our genetic and physical evidence presented here support a model in which Gpb1/2 directly impinge upon Ira1/2 in the PKA signaling pathway. In addition, simultaneous loss of Gpb1/2 was unable to suppress the growth defect of *gpa2 ras2* cells (Figure 1A). However, given the complex nature of the PKA signaling cascade, one idea in which these two apparently conflicting models might be reconciled would be to consider that the signaling cascade might exist as a supramolecular complex, in which it might be difficult to assign a strictly linear signaling pathway. Given the size of the Ira1/2 proteins, it would not be surprising if these were to act as scaffolds for PKA signaling via interactions with Ras, Gpb1/2, and other signaling components. In such a model, Gpb1/2 might exert regulatory roles at multiple steps in the cascade. Further studies will be required to elucidate the potent inhibitory action of Gpb1/2 on the PKA signaling pathway.

A Yeast Model for NF1

NF1 is an autosomal-dominant disorder that occurs in approximately one in every 3500 newborn infants.

Mutations in the *NF1* gene result in pleiotropic manifestations that include learning disabilities, small stature, bony abnormalities, and benign neurofibromas involving peripheral nerves. In some cases, NF1 patients present with malignant tumors involving peripheral nerve sheath tumors, optic gliomas, or the hematopoietic system (Zhu and Parada, 2002).

The human Ira1/2 homolog neurofibromin is a large protein (~300 kDa) that shares sequence identity with members of the RasGAP family, including p120GAP, and *Drosophila* NF1 (Buchberg et al., 1990; Cawthon et al., 1990; Marchuk et al., 1991; Wallace et al., 1990; Xu et al., 1990b). The RasGAP activity of neurofibromin has a pivotal role in Ras-dependent NF1 development because expression of the GAP-related domain (GRD) of neurofibromin can alleviate these $NF1^{-/-}$-deficient phenotypes (DeClue et al., 1992; Hiatt et al., 2001). In addition to regulating Ras activity, neurofibromin also governs G protein-mediated adenylyl cyclase activity in the fruit fly *Drosophila melanogaster* to control learning and memory, neuropeptide responses, and regulation of body size (Guo et al., 1997, 2000; Hannan et al., 2006; The et al., 1997; Tong et al., 2002). Expression of a human *NF1* gene complements the phenotypes in $NF1^{-/-}$ flies associated with an adenylyl cyclase defect (Tong et al., 2002). Similarly, neurofibromin controls adenylyl cyclase activity in response to the neuropeptide PACAP in mammals (Dasgupta et al., 2003; Tong et al., 2002). Therefore, neurofibromin governs adenylyl cyclase activity not only in yeast but also in flies and in mammals. Importantly, Ira1 binds to Cyr1, and this interaction plays a crucial role in Cyr1 activation (Mitts et al., 1991). Because heterologous expression of the GRD from mammalian neurofibromin rescues yeast *ira1* and *ira2* mutant phenotypes (Ballester et al., 1989; Ballester et al., 1990; Martin et al., 1990; Tanaka et al., 1990a; Xu et al., 1990a), Ira1/2 are structural and functional counterparts of mammalian neurofibromin and play key conserved roles in regulating both Ras and adenylyl cyclase.

Our studies identified the GBD in the C-terminal region of the yeast Ira1/2 proteins; importantly, this region is conserved in the fly and mammalian homologs (2247–2417 aa in human NF1, Figure 7). Analysis of the mutational spectra in the *NF1* gene from NF1 patients reveals that many mutations lie downstream of the GRD and many missense, frameshift, nonsense, and splice site mutations map near or even within the GBD homologous region (Ars et al., 2003; Fahsold et al., 2000; Origone et al., 2002). These downstream mutations presumably leave the GRD functional but may affect protein stability of neurofibromin and lead to the development of NF1. Importantly, neurofibromin stability is controlled via proteolysis by a ubiquitin/proteasome system (Cichowski et al., 2003). Mammalian cells express a myriad of kelch repeat proteins, and most of these remain to be characterized at a functional level. In many previous examples, signaling precedents established first in yeast were later found to also operate in multicellular eukaryotes. Our studies suggest that kelch repeat proteins related to Gpb1/2 may play an analogous role in controlling neurofibromin stability and signaling in flies and humans and might therefore provide clues to understand how NF1 develops and stimulate the development of therapeutic interventions.

Experimental Procedures

Strains, Media, and Plasmids

Media and standard yeast experimental procedures were as described (Sherman, 1991). A heterozygous diploid *gpb1,2::loxP/gpb1,2::loxP gpa2::loxP-G418/GPA2 ras2::nat/ras2::nat* strain was isolated after a cross between stains THY387a and THY389α. Yeast strains and plasmids used in this study are summarized in Tables S1 and S2.

Phenotypic Analysis

Pseudohyphal and invasive growth assays, sensitivity to nitrogen starvation, glycogen accumulation, Northern analysis, and cAMP assay were conducted as described previously (Harashima and Heitman, 2002). In cAMP assay data shown in Figures 1G and 3G, the values shown are the mean of two independent experiments.

Preparation of Crude Cell Extracts

Total cell extracts from yeast cells that were grown to midlog phase ($OD_{600} \cong 0.8$) in YPD or synthetic drop-out media were prepared in lysis buffer (50 mM HEPES [pH 7.6], 120 mM NaCl, 0.3% CHAPS, 1 mM EDTA, 20 mM NaF, 20 mM β-glycerophosphate, 0.1 mM Na-orthovanadate, 0.5 mM DTT, protease inhibitors [Calbiochem, cocktail IV], and 0.5 mM PMSF) by using a bead beater. Because the levels of the full-length Ira1/2 and Ira1/2 N- and C-terminal deletion variant proteins in crude extracts were too low to detect by Western blot, the full-length and deletion Ira1/2 proteins were immunoprecipitated by using anti-HA agarose beads, eluted, subjected to Western analysis, and examined for protein stability and indicated as "Input" in Figures 2, 4, and 5 and Figure S1.

Immunoprecipitation and Western Blot Analysis

HA-tagged Ira1/2 proteins were captured by using anti-HA agarose beads (F-7, Santa Cruz Biotechnology). The beads were then washed three times with lysis buffer, once with PBS, and once with elution buffer (50 mM HEPES [pH 7.6], 100 mM KCl, 1 mM EDTA, 20 mM β-glycerophosphate, 0.5 mM DTT, protease inhibitors [Calbiochem, cocktail IV], and 0.5 mM PMSF) for 5 min each. After washing, the immunoprecipitated proteins were eluted by the addition of HA peptide (Roche) at a final concentration of ~800 μg/ml in elution buffer with incubation for 30 min at 30ºC. FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 affinity gel (SIGMA), the beads were washed, and protein complexes were then eluted by incubating for 30 min at room temperature with FLAG peptide (SIGMA) at a final concentration of ~500 μg/ml in elution buffer. The eluted proteins were subjected to Western analysis with anti-HA (F-7 or Y-11, Santa Cruz Biotechnology) and anti-FLAG M2 antibodies (SIGMA). Endogenous Ras2 protein levels were analyzed with an anti-Ras2 antibody (yC-19, Santa Cruz Biotechnology). The Fpr1 protein served as a loading control and was examined with a polyclonal anti-Fpr1 antibody (Harashima and Heitman, 2002).

Mass Spectrometry

The eluted protein samples were TCA precipitated, washed with acetone, resuspended in Tris buffer, 8 M urea, pH 8.6, reduced with 100 mM TCEP, and alkylated with 55 mM iodoacetamide. Trypsin digestion was performed in the presence of 1 mM $CaCl^2$ to enhance specificity. Peptide mixtures were analyzed as described by Washburn et al. (2001). A FLAG-tagged GFP protein (pTH100) served as a mock control.

Ras-GTP Detection

Total cellular extracts were prepared as above and employed for coimmunoprecipitation by using a GST-fused Ras binding domain (RBD) from the Raf1 kinase that preferentially binds to Ras-GTP (EZ-DETECT Ras activation kit, PIERCE Biotechnology). Experimental procedures were followed per the manufacturer's instructions.

Cycloheximide-Chase Assay

Cycloheximide (CHX) was added to exponentially growing cells at a final concentration of 50 μg/ml to inhibit de novo protein synthesis. At the time points indicated, cells were collected and washed. Total cell extracts were prepared and subjected to immunoprecipitation for Ira1/2 or SDS-PAGE for Fpr1 as above.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, Supplemental References, one figure, and two tables and can be found with this article online at http://www.molecule.org/cgi/content/full/22/6/819/DC1/ or will be provided upon request from the authors.

Acknowledgments

We thank Akio Toh-e for providing a plasmid and Cristl Arndt and Emily Wenink for assistance. We also thank Julian Rutherford, Chaoyang Xue, and Yong-Sun Bahn for critical reading and Andy Alspaugh, Henrik Dohlman, Bob Lefkowitz, and Pat Casey for encouragement. We are indebted to Yoshinori Ohsumi for his tremendous support during the revision of this manuscript. This study was supported by the Neurofibromatosis program of the Department of Defense (W81xwh-04-01-0208). T.H. was supported by a fellowship from the Children's Tumor Foundation. J.H. was an investigator of the Howard Hughes Medical Institute. Funding from the National Institute of Health (P41 RR11823-10) to J.R.Y. is gratefully acknowledged.

Received: August 6, 2005
Revised: March 14, 2006
Accepted: May 8, 2006
Published: June 22, 2006

References

Ars, E., Kruyer, H., Morell, M., Pros, E., Serra, E., Ravella, A., Estivill, X., and Lázaro, C. (2003). Recurrent mutations in the *NF1* gene are common among neurofibromatosis type 1 patients. J. Med. Genet. *40*, e82.

Ballester, R., Michaeli, T., Ferguson, K., Xu, H.P., McCormick, F., and Wigler, M. (1989). Genetic analysis of mammalian GAP expressed in yeast. Cell *59*, 681–686.

Ballester, R., Marchuk, D., Boguski, M., Saulino, A., Letcher, R., Wigler, M., and Collins, F. (1990). The *NF1* locus encodes a protein functionally related to mammalian GAP and yeast *IRA* proteins. Cell *63*, 851–859.

Batlle, M., Lu, A.L., Green, D.A., Xue, Y., and Hirsch, J.P. (2003). Krh1p and Krh2p act downstream of the Gpa2p Gα subunit to negatively regulate haploid invasive growth. J. Cell Sci. *116*, 701–710.

Bhattacharya, S., Chen, L., Broach, J.R., and Powers, S. (1995). Ras membrane targeting is essential for glucose signaling but not for viability in yeast. Proc. Natl. Acad. Sci. USA *92*, 2984–2988.

Buchberg, A.M., Cleveland, L.S., Jenkins, N.A., and Copeland, N.G. (1990). Sequence homology shared by neurofibromatosis type-1 gene and *IRA-1* and *IRA-2* negative regulators of the *RAS* cyclic AMP pathway. Nature *347*, 291–294.

Cabrera-Vera, T.M., Vanhauwe, J., Thomas, T.O., Medkova, M., Preininger, A., Mazzoni, M.R., and Hamm, H.E. (2003). Insights into G protein structure, function, and regulation. Endocr. Rev. *24*, 765–781.

Cawthon, R.M., Weiss, R., Xu, G.F., Viskochil, D., Culver, M., Stevens, J., Robertson, M., Dunn, D., Gesteland, R., O'Connell, P., et al. (1990). A major segment of the neurofibromatosis type 1 gene: cDNA sequence, genomic structure, and point mutations. Cell *62*, 193–201.

Chautard, H., Jacquet, M., Schoentgen, F., Bureaud, N., and Bénédetti, H. (2004). Tfs1p, a member of the PEBP family, inhibits the Ira2p but not the Ira1p Ras GTPase-activating protein in *Saccharomyces cerevisiae*. Eukaryot. Cell *3*, 459–470.

Cichowski, K., Santiago, S., Jardim, M., Johnson, B.W., and Jacks, T. (2003). Dynamic regulation of the Ras pathway via proteolysis of the NF1 tumor suppressor. Genes Dev. *17*, 449–454.

Colombo, S., Ma, P.S., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J.H., Gorwa, M.F., Colavizza, D., and Thevelein, J.M. (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast *Saccharomyces cerevisiae*. EMBO J. *17*, 3326–3341.

Colombo, S., Ronchetti, D., Thevelein, J.M., Winderickx, J., and Martegani, E. (2004). Activation state of the Ras2 protein and glucose-induced signaling in *Saccharomyces cerevisiae*. J. Biol. Chem. *279*, 46715–46722.

Crechet, J.B., Poullet, P., Mistou, M.Y., Parmeggiani, A., Camonis, J., Boy-Marcotte, E., Damak, F., and Jacquet, M. (1990). Enhancement of the GDP-GTP exchange of Ras proteins by the carboxyl-terminal domain of Scd25. Science *248*, 866–868.

Dasgupta, B., Dugan, L.L., and Gutmann, D.H. (2003). The neurofibromatosis 1 gene product neurofibromin regulates pituitary adenylate cyclase-activating polypeptide-mediated signaling in astrocytes. J. Neurosci. *23*, 8949–8954.

DeClue, J.E., Papageorge, A.G., Fletcher, J.A., Diehl, S.R., Ratner, N., Vass, W.C., and Lowy, D.R. (1992). Abnormal regulation of mammalian $p21^{ras}$ contributes to malignant tumor growth in von Recklinghausen (type 1) neurofibromatosis. Cell *69*, 265–273.

Dohlman, H.G., Thorner, J., Caron, M.G., and Lefkowitz, R.J. (1991). Model systems for the study of seven-transmembrane-segment receptors. Annu. Rev. Biochem. *60*, 653–688.

Fahsold, R., Hoffmeyer, S., Mischung, C., Gille, C., Ehlers, C., Kücükceylan, N., Abdel-Nour, M., Gewies, A., Peters, H., Kaufmann, D., et al. (2000). Minor lesion mutational spectrum of the entire *NF1* gene does not explain its high mutability but points to a functional domain upstream of the GAP-related domain. Am. J. Hum. Genet. *66*, 790–818.

Field, J., Nikawa, J., Broek, D., MacDonald, B., Rodgers, L., Wilson, I.A., Lerner, R.A., and Wigler, M. (1988). Purification of a *RAS*-responsive adenylyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. Mol. Cell. Biol. *8*, 2159–2165.

Gross, A., Winograd, S., Marbach, I., and Levitzki, A. (1999). The N-terminal half of Cdc25 is essential for processing glucose signaling in *Saccharomyces cerevisiae*. Biochemistry *38*, 13252–13262.

Guo, H.F., The, I., Hannan, F., Bernards, A., and Zhong, Y. (1997). Requirement of *Drosophila* NF1 for activation of adenylyl cyclase by PACAP38-like neuropeptides. Science *276*, 795–798.

Guo, H.F., Tong, J., Hannan, F., Luo, L., and Zhong, Y. (2000). A neurofibromatosis-1-regulated pathway is required for learning in *Drosophila*. Nature *403*, 895–898.

Halme, A., Bumgarner, S., Styles, C., and Fink, G.R. (2004). Genetic and epigenetic regulation of the *FLO* gene family generates cell-surface variation in yeast. Cell *116*, 405–415.

Hannan, F., Ho, I., Tong, J., Zhu, Y., Nurnberg, P., and Zhong, Y. (2006). Effect of neurofibromatosis type I mutations on a novel pathway for adenylyl cyclase activation requiring neurofibromin and Ras. Hum. Mol. Genet. *15*, 1087–1098, Published online March 2, 2006.

Harashima, T., and Heitman, J. (2002). The Gα protein Gpa2 controls yeast differentiation by interacting with kelch repeat proteins that mimic Gβ subunits. Mol. Cell *10*, 163–173.

Harashima, T., and Heitman, J. (2004). Nutrient control of dimorphic growth in *Saccharomyces cerevisiae*. In Topics in Current Genetics, *Vol. 7*, J.G. Winderickx and P.M. Taylor, eds. (Heidelberg, Germany: Springer-Verlag), pp. 131–169.

Harashima, T., and Heitman, J. (2005). Gα subunit Gpa2 recruits kelch repeat subunits that inhibit receptor-G protein coupling during cAMP-induced dimorphic transitions in *Saccharomyces cerevisiae*. Mol. Biol. Cell *16*, 4557–4571.

Hiatt, K.K., Ingram, D.A., Zhang, Y., Bollag, G., and Clapp, D.W. (2001). Neurofibromin GTPase-activating protein-related domains restore normal growth in *Nf1−/−* cells. J. Biol. Chem. *276*, 7240–7245.

Hitchcock, A.L., Auld, K., Gygi, S.P., and Silver, P.A. (2003). A subset of membrane-associated proteins is ubiquitinated in response to mutations in the endoplasmic reticulum degradation machinery. Proc. Natl. Acad. Sci. USA *100*, 12735–12740.

Kübler, E., Mösch, H.U., Rupp, S., and Lisanti, M.P. (1997). Gpa2p, a G-protein α-subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. J. Biol. Chem. *272*, 20321–20323.

Lemaire, K., Van de Velde, S., Van Dijck, P., and Thevelein, J.M. (2004). Glucose and sucrose act as agonist and mannose as

antagonist ligands of the G protein-coupled receptor Gpr1 in the yeast *Saccharomyces cerevisiae*. Mol. Cell *16*, 293–299.

Lorenz, M.C., and Heitman, J. (1997). Yeast pseudohyphal growth is regulated by GPA2, a G protein α homolog. EMBO J. *16*, 7008–7018.

Lorenz, M.C., Pan, X.W., Harashima, T., Cardenas, M.E., Xue, Y., Hirsch, J.P., and Heitman, J. (2000). The G protein-coupled receptor Gpr1 is a nutrient sensor that regulates pseudohyphal differentiation in *Saccharomyces cerevisiae*. Genetics *154*, 609–622.

Lu, A., and Hirsch, J.P. (2005). Cyclic AMP-independent regulation of protein kinase A substrate phosphorylation by Kelch repeat proteins. Eukaryot. Cell *4*, 1794–1800.

Marchuk, D.A., Saulino, A.M., Tavakkol, R., Swaroop, M., Wallace, M.R., Andersen, L.B., Mitchell, A.L., Gutmann, D.H., Boguski, M., and Collins, F.S. (1991). cDNA cloning of the type 1 neurofibromatosis gene: complete sequence of the *NF1* gene product. Genomics *11*, 931–940.

Martin, G.A., Viskochil, D., Bollag, G., McCabe, P.C., Crosier, W.J., Haubruck, H., Conroy, L., Clark, R., O'Connell, P., Cawthon, R.M., et al. (1990). The GAP-related domain of the neurofibromatosis type 1 gene product interacts with *ras* p21. Cell *63*, 843–849.

Mintzer, K.A., and Field, J. (1994). Interactions between adenylyl cyclase, CAP and RAS from *Saccharomyces cerevisiae*. Cell. Signal. *6*, 681–694.

Mitts, M.R., Bradshaw-Rouse, J., and Heideman, W. (1991). Interactions between adenylate cyclase and the yeast GTPase-activating protein Ira1. Mol. Cell. Biol. *11*, 4591–4598.

Munder, T., and Kuntzel, H. (1989). Glucose-induced cAMP signaling in *Saccharomyces cerevisiae* is mediated by the Cdc25 protein. FEBS Lett. *242*, 341–345.

Munder, T., and Furst, P. (1992). The *Saccharomyces cerevisiae CDC25* gene product binds specifically to catalytically inactive ras proteins in vivo. Mol. Cell. Biol. *12*, 2091–2099.

Origone, P., De Luca, A., Bellini, C., Buccino, A., Mingarelli, R., Costabel, S., La Rosa, C., Garrè, C., Coviello, D.A., Ajmar, F., et al. (2002). Ten novel mutations in the human neurofibromatosis type 1 (NF1) gene in Italian patients. Hum. Mutat. *20*, 74–75.

Portillo, F., and Mazon, M.J. (1986). The *Saccharomyces cerevisiae* start mutant carrying the *cdc25* mutation is defective in activation of plasma membrane ATPase by glucose. J. Bacteriol. *168*, 1254–1257.

Powers, S., Kataoka, T., Fasano, O., Goldfarb, M., Strathern, J., Broach, J., and Wigler, M. (1984). Genes in *S. cerevisiae* encoding proteins with domains homologous to the mammalian ras proteins. Cell *36*, 607–612.

Ross, E.M., and Wilkie, T.M. (2000). GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. Annu. Rev. Biochem. *69*, 795–827.

Sherman, F. (1991). Getting started with yeast. Methods Enzymol. *194*, 3–21.

Tanaka, K., Matsumoto, K., and Toh-e, A. (1989). *IRA1*, an inhibitory regulator of the *RAS*-cyclic AMP pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *9*, 757–768.

Tanaka, K., Nakafuku, M., Satoh, T., Marshall, M.S., Gibbs, J.B., Matsumoto, K., Kaziro, Y., and Toh-e, A. (1990a). *S. cerevisiae* genes *IRA1* and *IRA2* encode proteins that may be functionally equivalent to mammalian *ras* GTPase activating protein. Cell *60*, 803–807.

Tanaka, K., Nakafuku, M., Tamanoi, F., Kaziro, Y., Matsumoto, K., and Toh-e, A. (1990b). *IRA2*, a second gene of *Saccharomyces cerevisiae* that encodes a protein with a domain homologous to mammalian *ras* GTPase-activating protein. Mol. Cell. Biol. *10*, 4303–4313.

Tanaka, K., Lin, B.K., Wood, D.R., and Tamanoi, F. (1991). *IRA2*, an upstream negative regulator of RAS in yeast, is a RAS GTPase-activating protein. Proc. Natl. Acad. Sci. USA *88*, 468–472.

The, I., Hannigan, G.E., Cowley, G.S., Reginald, S., Zhong, Y., Gusella, J.F., Hariharan, I.K., and Bernards, A. (1997). Rescue of a *Drosophila NF1* mutant phenotype by protein kinase A. Science *276*, 791–794.

Toda, T., Cameron, S., Sass, P., Zoller, M., Scott, J.D., Mcmullen, B., Hurwitz, M., Krebs, E.G., and Wigler, M. (1987a). Cloning and characterization of *BCY1*, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. *7*, 1371–1377.

Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987b). Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. Cell *50*, 277–287.

Toda, T., Cameron, S., Sass, P., and Wigler, M. (1988). *SCH9*, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. Genes Dev. *2*, 517–527.

Tong, J., Hannan, F., Zhu, Y., Bernards, A., and Zhong, Y. (2002). Neurofibromin regulates G protein-stimulated adenylyl cyclase activity. Nat. Neurosci. *5*, 95–96.

Viskochil, D., Buchberg, A.M., Xu, G., Cawthon, R.M., Stevens, J., Wolff, R.K., Culver, M., Carey, J.C., Copeland, N.G., Jenkins, N.A., et al. (1990). Deletions and a translocation interrupt a cloned gene at the neurofibromatosis type 1 locus. Cell *62*, 187–192.

Wallace, M.R., Marchuk, D.A., Andersen, L.B., Letcher, R., Odeh, H.M., Saulino, A.M., Fountain, J.W., Brereton, A., Nicholson, J., Mitchell, A.L., et al. (1990). Type 1 neurofibromatosis gene: identification of a large transcript disrupted in three NF1 patients. Science *249*, 181–186.

Washburn, M.P., Wolters, D., and Yates, J.R., 3rd. (2001). Large-scale analysis of the yeast proteome by multidimensional protein identification technology. Nat. Biotechnol. *19*, 242–247.

Xu, G.F., Lin, B., Tanaka, K., Dunn, D., Wood, D., Gesteland, R., White, R., Weiss, R., and Tamanoi, F. (1990a). The catalytic domain of the neurofibromatosis type 1 gene product stimulates *ras* GTPase and complements *ira* mutants of *S. cerevisiae*. Cell *63*, 835–841.

Xu, G.F., O'Connell, P., Viskochil, D., Cawthon, R., Robertson, M., Culver, M., Dunn, D., Stevens, J., Gesteland, R., White, R., et al. (1990b). The neurofibromatosis type 1 gene encodes a protein related to GAP. Cell *62*, 599–608.

Xue, Y., Batlle, M., and Hirsch, J.P. (1998). *GPR1* encodes a putative G protein-coupled receptor that associates with the Gpa2p G_α subunit and functions in a Ras-independent pathway. EMBO J. *17*, 1996–2007.

Yun, C.W., Tamaki, H., Nakayama, R., Yamamoto, K., and Kumagai, H. (1998). Gpr1p, a putative G-protein coupled receptor, regulates glucose-dependent cellular cAMP level in yeast *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. *252*, 29–33.

Zhu, Y., and Parada, L.F. (2002). The molecular and genetic basis of neurological tumours. Nat. Rev. Cancer *2*, 616–626.