

# RIC8 Is a Guanine-Nucleotide Exchange Factor for G $\alpha$ Subunits That Regulates Growth and Development in *Neurospora crassa*

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**ABSTRACT** Heterotrimeric ( $\alpha\beta\gamma$ ) G proteins are crucial components of eukaryotic signal transduction pathways. G-protein-coupled receptors (GPCRs) act as guanine nucleotide exchange factors (GEFs) for G $\alpha$  subunits. Recently, facilitated GDP/GTP exchange by non-GPCR GEFs, such as RIC8, has emerged as an important mechanism for G $\alpha$  regulation in animals. RIC8 is present in animals and filamentous fungi, such as the model eukaryote *Neurospora crassa*, but is absent from the genomes of baker's yeast and plants. In *Neurospora*, deletion of *ric8* leads to profound defects in growth and asexual and sexual development, similar to those observed for a mutant lacking the G $\alpha$  genes *gna-1* and *gna-3*. In addition, constitutively activated alleles of *gna-1* and *gna-3* rescue many defects of  $\Delta ric8$  mutants. Similar to reports in *Drosophila*, *Neurospora*  $\Delta ric8$  strains have greatly reduced levels of G-protein subunits. Effects on cAMP signaling are suggested by low levels of adenylyl cyclase protein in  $\Delta ric8$  mutants and suppression of  $\Delta ric8$  by a mutation in the protein kinase A regulatory subunit. RIC8 acts as a GEF for GNA-1 and GNA-3 *in vitro*, with the strongest effect on GNA-3. Our results support a role for RIC8 in regulating GNA-1 and GNA-3 in *Neurospora*.

**E**UKARYOTIC cells sense many hormones, growth factors, neurotransmitters, and the presence of light via G-protein-signaling pathways. The G-protein heterotrimer consists of a G $\alpha$  subunit, which binds and hydrolyzes GTP, and of tightly associated G $\beta$  and G $\gamma$  subunits. G proteins interact with seven-transmembrane helix G-protein-coupled receptors (GPCRs) to regulate downstream signaling pathways (reviewed in Neves *et al.* 2002 and Wilkie and Kinch 2005). GDP-bound G $\alpha$  subunits are associated with the G $\beta\gamma$  dimer and the GPCR (Wilkie and Kinch 2005). Ligand binding to the receptor leads to exchange of GDP for GTP on the G $\alpha$ , leading to dissociation of the heterotrimer into

G $\alpha$ -GTP and G $\beta\gamma$  units, which can both interact with effector proteins to generate changes in cellular physiology (Neves *et al.* 2002). GPCRs thus act as guanine nucleotide-exchange factors (GEFs) for heterotrimeric G $\alpha$  proteins. The activation cycle is terminated by hydrolysis of GTP to GDP by the G $\alpha$  subunit and reassociation with G $\beta\gamma$  and the GPCR.

Recently a non-GPCR protein, RIC8, has been implicated as a positive regulator of G $\alpha$  proteins in several animal species, including *Caenorhabditis elegans*, *Drosophila melanogaster*, and mammalian cells (reviewed in Wilkie and Kinch 2005). Invertebrates such as *C. elegans* and *Drosophila* contain one RIC8 protein, while vertebrates contain two homologs, Ric-8A and Ric-8B (Tall *et al.* 2003). RIC8 is required for asymmetric cell division in zygotes and priming of synaptic vesicles in *C. elegans* (Miller and Rand 2000; Wilkie and Kinch 2005). In *Drosophila*, RIC8 is essential for responses to extracellular ligands and for maintenance of polarity during asymmetric cell division in embryogenesis (Hampoelz *et al.* 2005). Furthermore, RIC8 is also required for stability of a G $\alpha$  and G $\beta$  protein in *Drosophila* (Hampoelz *et al.* 2005). Of the two mammalian RIC8 proteins, only Ric-8A has demonstrated GEF activity toward the G $\alpha$  proteins G $\alpha_q$ , G $\alpha_i$ , G $\alpha_o$ , and G $\alpha_{13}$  (Tall *et al.* 2003). Ric-8A binds the

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GDP-bound  $G\alpha$  in the absence of  $G\beta\gamma$ , and GDP is then released, forming a stable Ric-8A: $G\alpha$  complex (Tall *et al.* 2003). Subsequently, GTP binds the  $G\alpha$  protein and Ric-8A is released. Similar to *Drosophila* RIC8, Ric-8B regulates the stability of a  $G\alpha$  subunit in mammalian cells (Nagai *et al.* 2010).

*Neurospora crassa* possesses three  $G\alpha$  subunits (GNA-1, GNA-2, and GNA-3), one  $G\beta$  (GNB-1), one  $G\gamma$  (GNG-1), and >25 putative GPCRs (reviewed in Li *et al.* 2007). We have previously demonstrated that loss of *gna-1* and *gna-3* or of all three  $G\alpha$  genes leads to a severe decrease in extension of both basal and aerial hyphae. The relatively subtle phenotypes of GPCR mutants in comparison to strains lacking one or more  $G\alpha$  genes (Kim and Borkovich 2004; Krystofova and Borkovich 2006; Li and Borkovich 2006; Bieszke *et al.* 2007) prompted us to explore the possibility of additional non-GPCR regulators of G proteins in *Neurospora*. It was recently reported that a single protein homologous to RIC8 is present in *Neurospora* and other filamentous fungi, but is absent from the genomes of *Saccharomyces cerevisiae* and plants (Wilkie and Kinch 2005). It has been hypothesized that RIC8 was acquired by animals and certain fungi after the evolution of G proteins and GPCRs and is therefore a fairly recent addition to G-protein regulatory pathways (Wilkie and Kinch 2005). The only other study of a RIC8 homolog in filamentous fungi was reported very recently in the fungal rice pathogen *Magnaporthe oryzae* (Li *et al.* 2010). MoRic8 was shown to bind to the  $G\alpha$  subunit MagB in the yeast two-hybrid assay and is highly expressed in the appressorium, a specialized structure that invades plant tissue during pathogenesis. MoRic8 was also found to act upstream of the cAMP pathway, which regulates appressorium formation. However, this study did not investigate possible GEF activity for MoRic8.

Here we provide evidence that RIC8 is a  $G\alpha$  GEF that regulates growth and development in *Neurospora*. Loss of *ric8* leads to several phenotypes also displayed by strains lacking the  $G\alpha$  genes *gna-1* and *gna-3*, including extremely impaired growth, short aerial hyphae, inappropriate conidiation in submerged culture, and loss of female fertility. We also demonstrate that all three *Neurospora*  $G\alpha$  proteins bind GTP and that RIC8 interacts with and has GEF activity toward GNA-1 and GNA-3 in *N. crassa*.

## Materials and Methods

### Strains and growth conditions

A list of *Neurospora* strains is provided in Table 1. Strains were maintained on Vogel's minimal medium (VM) (Vogel 1964). Synthetic crossing medium was used to induce formation of female reproductive structures (protoperithecia) (Westergaard and Mitchell 1947). All submerged liquid and VM plate cultures were inoculated with conidia and grown as described previously (Krystofova and Borkovich 2006; Li and Borkovich 2006). Where indicated, ampicillin, his-

tidine, and inositol were used at 100  $\mu\text{g/ml}$ , hygromycin at 200  $\mu\text{g/ml}$ , and phosphinothricin at 400  $\mu\text{g/ml}$ .

### Identification and characterization of the *ric8* gene

The existence of a *ric8* homologue in fungi was suggested by Wilkie and Kinch (2005). Homology searches (BLAST) (Cookson *et al.* 1997) of the *N. crassa* genome database at the Broad Institute ([http://www.broad.mit.edu/annotation/fungi/neurospora\\_crassa\\_7/index.html](http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html)) (Galagan *et al.* 2003) using the *Xenopus laevis* Ric8 protein sequence (accession no. NP\_989159) revealed that the *Neurospora* RIC8 homolog corresponds to NCU02788. A *ric8* subclone, pSM1, was generated by digesting cosmid pMOcosX G19 C10 with *EcoRI*, releasing a 12.6-kb fragment (corresponding to contig 7.7, nucleotides 24,473–37,042) that was subcloned into pGEM4 (Promega, Madison, WI). The clone was sequenced at the Institute for Integrative Genome Biology, Core Sequencing Facility, University of California, Riverside using primers T7 and SP6.

### Strain construction

A transformant containing the *ric8* deletion mutation was obtained from the *Neurospora* genome project (Colot *et al.* 2006). This  $\Delta ric8$  strain was crossed as a male to wild-type strain 74A (female), and progeny were plated on VM plates containing hygromycin to select for  $\Delta ric8::hph^+$  strains. The  $\Delta ric8$  *his-3* (R8H3) strain was isolated in a similar manner by crossing a *his-3* strain [Fungal Genetics Stock Center (FGSC) 6103] as a male to the  $\Delta ric8$  heterokaryon (female) with selection on VM-hygromycin plates containing histidine. The presence of the  $\Delta ric8$  mutation in progeny was verified by growth on hygromycin and Southern analysis, and *his-3* auxotrophs were identified by spot testing on VM medium lacking histidine.

To complement the  $\Delta ric8$  mutation *in trans*, the *ric8* open reading frame (ORF) was cloned into vector pMF272 (Folco *et al.* 2003; Freitag *et al.* 2004). pMF272 contains the *cgc-1* promoter and allows expression of an ORF with a 3' GFP fusion. The *ric8* ORF (including introns) was amplified from pSM1 using primers designed to add *XbaI* (5' end, R8 GFP fw) and *BamHI* (3' end, R8 GFP rv) sites to the *ric8* gene (supporting information, Table S1). The fragment was ligated into pMF272 digested with *BamHI* and *EcoRI*, yielding plasmid pSM2. This vector was used to target the *ric8-gfp* fusion to the *his-3* locus of the R8H3 strain (R8GFP, Table 1) (Aramayo *et al.* 1996). Transformants were plated on VM-hygromycin medium, and isolates were analyzed for integration of *ric8-gfp* at the *his-3* locus using Southern analysis. Transformants with the *ric8-gfp* Southern pattern were purified by three rounds of colony isolation from VM-hygromycin plates.

Predicted GTPase-deficient, constitutively activated alleles of *gna-1* (Q204L) (Yang and Borkovich 1999), *gna-2* (Q205L) (Baasiri *et al.* 1997), and *gna-3* (Q208L) were introduced into the  $\Delta ric8$  background to determine epistatic relationships between *ric8* and the three  $G\alpha$  genes. These

**Table 1 Strains used in this study**

Strain	Relevant genotype	Comments	Source
74A-OR23-1A	Wild type, <i>matA</i>	FGSC 987	FGSC
74a-OR8-1a	Wild type, <i>matA</i>	FGSC 988	FGSC
74-OR23-IVA	Wild type, <i>matA</i>	FGSC 2489	FGSC
ORS-SL6a	Wild type, <i>matA</i>	FGSC 4200	FGSC
Y234M723	<i>his-3 matA</i>	FGSC 6103	FGSC
<i>a<sup>m1</sup></i>	<i>a<sup>m1</sup> ad3B cyh-1</i>	FGSC 4564	FGSC
R81a	$\Delta ric8::hph^+ matA$	$\Delta ric8$ mutant	This study
R81-5a	$\Delta ric8::hph^+ matA$	$\Delta ric8$ mutant	This study
R8H3	$\Delta ric8::hph^+ his3 matA$	$\Delta ric8 his-3$ strain	This study
R8 <i>a<sup>m1</sup></i>	$\Delta ric8::hph^+ a^{m1} ad3B cyh-1$	R8H3 + <i>a<sup>m1</sup></i> heterokaryon	This study
R8GFP	$\Delta ric8::hph^+ ric8-gfp::his-3^+$	<i>ric8-gfp</i> strain	This study
R81*	$\Delta ric8::hph^+ gna-1^{Q204L}::his-3^+$	<i>gna-1<sup>Q204L</sup></i> in $\Delta ric8$ background	This study
R82*	$\Delta ric8::hph^+ gna-2^{Q205L}::his-3^+$	<i>gna-2<sup>Q205L</sup></i> in $\Delta ric8$ background	This study
R83*	$\Delta ric8::hph^+ gna-3^{Q208L}::his-3^+$	<i>gna-3<sup>Q208L</sup></i> in $\Delta ric8$ background	This study
R81*3*	$\Delta ric8::hph^+ gna-1^{Q204L}::bar^+ gna-3^{Q208L}::his-3^+$	<i>gna-1<sup>Q204L</sup></i> and <i>gna-3<sup>Q208L</sup></i> in $\Delta ric8$ background	This study
<i>mcb</i>	<i>mcb inl</i>	FGSC 7094	FGSC
R8 <i>mcb</i>	$\Delta ric8::hph^+ mcb::inl$	R8 <i>a<sup>m1</sup></i> × <i>mcb</i> progeny <sup>a</sup>	This study
3B10	$\Delta gna-1::hph^+ matA$	$\Delta gna-1$ mutant	Ivey <i>et al.</i> (1999)
a29-1	$\Delta gna-2::pyrG^+ matA$	$\Delta gna-2$ mutant	Baasiri <i>et al.</i> (1997)
43c2	$\Delta gna-3::hph^+ matA$	$\Delta gna-3$ mutant	Kays <i>et al.</i> (2000)
g1.3	$\Delta gna-1::hph^+ \Delta gna-3::hph^+ matA$	$\Delta gna-1 \Delta gna-3$ double mutant	Kays and Borkovich (2004)
noa	$\Delta gna-1::hph^+ \Delta gna-2::pyrG^+ \Delta gna-3::hph^+ matA$	$\Delta gna-1 \Delta gna-2 \Delta gna-3$ triple mutant	Kays and Borkovich (2004)

FGSC, Fungal Genetics Stock Center (Kansas City, MO).

<sup>a</sup> "X" denotes a genetic cross.

vectors will be described elsewhere (S. Krystofova, S. Won, and K. Borkovich, unpublished results). Briefly, the ORFs corresponding to the three  $G\alpha$  alleles were subcloned into vector pMF272, with eviction of the GFP gene, producing plasmids pSVK51 (*gna-1<sup>Q204L</sup>*), pSVK52 (*gna-2<sup>Q205L</sup>*), and pSVK53 (*gna-3<sup>Q208L</sup>*). These plasmids were targeted to the *his-3* locus in R8H3 as described above, generating strains R81\*, R82\*, and R83\*. Correct integration at the *his-3* locus was verified by Southern analysis.

To construct a strain containing both *gna-1<sup>Q204L</sup>* and *gna-3<sup>Q208L</sup>* alleles in the  $\Delta ric8$  background, the insert from plasmid pSVK51 (carrying *gna-1<sup>Q204L</sup>*) was introduced into a vector that would allow ectopic integration and confer resistance to phosphinothricin (Pall 1993). A 2.2-kb fragment from pSVK51 containing the *ccg-1* promoter and *gna-1<sup>Q204L</sup>* allele was released using *NotI* and *EcoRI*. This fragment was cloned into pBARGEM7-2, which contains the *bar<sup>+</sup>* gene and confers phosphinothricin resistance. The resulting vector, pSM3, was transformed into the R83\* strain using electroporation. Transformants were plated on sorbose medium containing hygromycin and phosphinothricin, and colonies were analyzed for the ectopic integration of *gna-1<sup>Q204L</sup>* using Southern analysis.

The  $\Delta ric8$  *mcb* double mutant was generated from a cross with R8 *a<sup>m1</sup>* ( $\Delta ric8::hph^+ a^{m1}$  heterokaryon, Table S1) as a female and *mcb inl* as a male. Strain *a<sup>m1</sup>* is a mating-type mutant that allows crossing of female-sterile strains, but does not passage its nuclei in a cross (Griffiths 1982). Since *mcb* is tightly linked to *inl*, inositol auxotrophy can be used to score the presence of the *mcb* mutation. Progeny were plated on VM medium containing hygromycin and inositol to isolate  $\Delta ric8$  *mcb inl* mutants (R8 *mcb*, Table 1).

### RT-PCR and Northern and Western analysis

There are two predicted introns in *ric8* (see gene structure in Figure S1A). The presence of the 3' predicted intron was confirmed by the sequence of a cDNA clone (NCW06G3T7 cDNA clone W06G3 3'; Broad Institute). The 5' predicted intron and *ric8* gene deletion were verified by semiquantitative RT-PCR (Kays and Borkovich 2004). Expression of *ric8* was analyzed using 2  $\mu$ g total RNA extracted from conidia (Puregene RNA isolation kit, Gentra Systems, Minneapolis) (Krystofova and Borkovich 2006). RT-PCR analysis was performed using the Access RT-PCR system (Promega) with gene specific primers (R8I1fw and R8I1rv) designed to flank the 5' intron (Table S1). Each reaction included 2  $\mu$ g DNase-treated RNA and both AMV reverse transcriptase and Tfl polymerase (Promega). The genomic control contained the same primers with plasmid pSM2 (genomic fragment) as the template. The RT reaction was incubated at 48° for 45 min and at 94° for 2 min. The PCR thermocycling conditions were the following: 35 cycles at 94° for 30 sec, at 50° for 1 min, and at 68° for 90 sec with a final elongation step at 68° for 7 min. The reactions were electrophoresed on an agarose gel, blotted onto a nylon membrane, and subjected to Southern analysis as described (Krystofova and Borkovich 2005), using the radiolabeled 410-bp fragment from the genomic control PCR as the probe. A control reaction for RNA quality was also run using 18S rRNA-specific primers as previously described (Bieszke *et al.* 2007).

For Northern analysis, total RNA was isolated and samples containing 20  $\mu$ g were analyzed as described (Krystofova and Borkovich 2005). Probes for Northern detection of

*gna-1*, *gna-2*, *gna-3*, and *gnb-1* were generated as described previously (Krystofova and Borkovich 2005).

For Western analysis, the particulate fraction (GNA-1, GNA-2, GNA-3, and GNB-1) or whole-cell extracts (CR-1, R8GFP) were isolated as described previously (Ivey *et al.* 1996; Kays *et al.* 2000; Krystofova and Borkovich 2005; Li and Borkovich 2006). Samples containing 100  $\mu$ g of total protein were subjected to Western analysis as described (Yang *et al.* 2002; Krystofova and Borkovich 2005). The primary polyclonal antisera for GNA-1, GNA-2, and GNB-1 were used at dilutions of 1:1000, while GNA-3 and CR-1 were diluted 1:750 and 1:5000, respectively. The primary polyclonal antibody against GFP (Abcam, Cambridge, MA) was used at a dilution of 1:2000. A horseradish peroxidase conjugate (Bio-Rad) was used as the secondary antibody at a dilution of 1:4000 (GNA-1 and GNA-2), 1:2000 (GNA-3), or 1:10,000 (CR-1), and chemiluminescent detection was performed as described previously (Krystofova and Borkovich 2005).

### Phenotypic analysis and microscopy

Plate cultures were imaged by scanning. Intracellular localization of the RIC8 protein in conidia and vegetative hyphae was achieved by microscopic observation of GFP fluorescence from a *Δric8* strain carrying a RIC8–GFP fusion protein at the *his-3* locus (see *Strain construction*). Conidia were harvested as described (Krystofova and Borkovich 2006; Li and Borkovich 2006) and imaged directly or used to inoculate 30-ml liquid cultures at a concentration of  $1 \times 10^6$  cell/ml, followed by incubation for 16 hr at 30° with centrifugation at  $200 \times g$  (for vegetative hyphae). Conidia and vegetative hyphae were viewed using differential interference contrast (DIC) and GFP fluorescence microscopy using an Olympus IX71 inverted microscope with a  $\times 60$  oil immersion objective (numerical aperture = 1.42). Images were captured using a QIClick digital CCD camera (QImaging) and analyzed using Metamorph software (Molecular Devices).

### Yeast two-hybrid assay

A *ric8* ORF clone lacking introns (pSM6) was constructed using homologous recombination of PCR fragments in *S. cerevisiae* (Colot *et al.* 2006). All DNA fragments for cDNA were amplified by PCR using Pfu turbo DNA polymerase (Stratagene) according to the manufacturer's protocols. To remove the first intron, the 5' UTR up to the first intron was amplified (574-bp product; primers R8YR11fw and R8YR11rv, Table S1) as well as the first part of the second exon (756-bp product; primers R8YR12fw and R8YR12rv), using pSM1 as a template. The second intron was removed by amplifying the last part of the second exon (727-bp product; R8YR21fw and R8YR21rv) and the third exon and 3' UTR (579 bp; R8YR22fw and R8YR22rv, Table S1).

The two sets of fragments were transformed separately into *S. cerevisiae* strain FY834 (Winston *et al.* 1995; Colot *et al.* 2006) along with vector pRS416 (Christianson *et al.*

1992; Gera *et al.* 2002) gapped with *Xba*I and *Eco*RI, with selection on SC medium lacking uracil. DNA was extracted from transformants, and plasmids were recovered by electroporation into *Escherichia coli* and plating on LB + Amp and sequenced.

PCR was used to amplify the *ric8* cDNA and introduce restriction sites for yeast two-hybrid cloning. The 5' fragment was amplified with primers R8Y2Hfw and R8YR12rv (744 bp), introducing the *Eco*RI restriction site 5' to the initiator ATG and extending to include the restriction site *Xho*I. The 3' fragment was amplified with primers R8YR21fw and R8Y2Hrv (836 bp), which include the *Xho*I site, and a *Bam*HI site was inserted in place of the TGA stop codon. These fragments were digested and ligated into *Eco*RI- and *Bam*HI-digested pGEM4 to create pSM6. pSM6 was digested using *Eco*RI and *Bam*HI, and the *ric8* insert was subsequently ligated into pGBKT7 and pGAD424 (Clontech) to yield plasmids pSM7 and pSM8, respectively.

The yeast two-hybrid assay was performed as described previously (Li and Borkovich 2006). Yeast strains containing RIC8 gene plasmids (pSM7 or pSM8) were mated to the appropriate  $G\alpha$ -vector-containing yeast to isolate diploids. Diploids were then tested on SC medium lacking leucine, tryptophan, and histidine  $\pm$  adenine to select for the expression of the *HIS3* or *HIS3* and *ADE2* reporter genes, respectively. A colony lift assay for  $\beta$ -galactosidase activity was performed according to the manufacturer's recommendations to screen for the *lacZ* reporter (Clontech).

### Expression and purification of RIC8 and $G\alpha$ proteins from *E. coli*

For amplification of the  $G\alpha$  genes, primer pairs (N $\Delta$ 13G1fw/N $\Delta$ 13G1rv, N $\Delta$ 13G2fw/N $\Delta$ 13G2rv, and N $\Delta$ 12G3fw/N $\Delta$ 12G3rv; Table S1) were used to introduce a 36 (*gna-3*) or 39 (*gna-1* and *gna-2*) nucleotide deletions at the 5' end of each gene, along with restriction sites for cloning into pET16b, which expresses a 10-histidine N-terminal fusion protein (Novagen, Gibbstown, NJ). The *ric8* primers (R8 NdeI fw/R8 BamHI rv; Table S1) were designed to clone full-length *ric8* into pET16b.

*E. coli* strain BL21 (DE3) *plysS* was used to express HIS-tagged proteins. LB medium supplemented with 100  $\mu$ g/ml ampicillin and 35  $\mu$ g/ml chloramphenicol was inoculated with 1/50 volume of an overnight *E. coli* culture and grown at 37° for 2–3 hr with centrifugation at  $225 \times g$ . Heterologous protein expression was induced by the addition of IPTG to a final concentration of 100  $\mu$ M, and incubation continued for an additional 2–3 hr at 30° with centrifugation at  $80 \times g$ . The cells were centrifuged at  $4,000 \times g$  in an Avanti J-26 XP floor centrifuge using rotor JS-4.0 for 20 min at 4°, and pellets were stored at  $-20^\circ$ .

For RIC8, 40 ml of His-Pur Lysis buffer [50 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.4), 300 mM NaCl, 10 mM imidazole (Sigma), 10% glycerol, 0.1 mM PMSF] containing 0.2 mg/ml lysozyme was added to cell pellets from a 1-liter culture, followed by incubation on ice for 20 min. The cell suspension was

then sonicated (Sonic Dismembrator, model 500, Fisher Scientific) at 30% power for 15–30 sec, two to six times with 1-min rests on ice. The extract was centrifuged at  $20,000 \times g$  using rotor JA-25.5 at  $4^\circ$  for 20 min. The supernatant was added to a 2-ml bed volume of His-Pur Cobalt beads (Thermo Scientific, Rockford, IL) in a conical tube and incubated for 1 hr at  $4^\circ$  with rotation. The slurry was centrifuged at  $700 \times g$  for 30 sec in an IEC Centra CL3 centrifuge with an IEC 243 rotor, the supernatant was removed, and the beads were transferred to a poly-prep column using 5 ml wash buffer (lysis buffer containing 20 mM imidazole). The column was washed with 10–20 ml wash buffer (until protein concentration was near zero as determined using the Bradford protein assay). Protein was eluted in 1-ml fractions with lysis buffer containing increasing amounts of imidazole: 50, 75, and 100 mM (3 ml of each concentration).

The  $G\alpha$  proteins were purified similarly to RIC8 with the few differences noted below. The  $G\alpha$  proteins were purified using Ni-NTA resin, and all buffers contained 50 mM  $\text{NaH}_2\text{PO}_4$  (pH 7.4), 500 mM NaCl, 10% glycerol, 0.1 mM PMSF. In addition, the lysis and elution buffers contained 20  $\mu\text{M}$  GDP and 1 mM  $\text{MgSO}_4$ . Lysis buffer contained 20 mM imidazole, wash buffer contained 40 mM imidazole, and protein was eluted in 4-ml fractions with lysis buffer containing increasing amounts of imidazole: 60, 80, 100, and 120 mM.

For both RIC8 and  $G\alpha$  proteins, fractions with the highest protein concentration (typically 50–75 mM imidazole for RIC8 and 80–120 mM for  $G\alpha$  proteins) were pooled and simultaneously desalted and concentrated. The protein was transferred to an Amicon Ultra 30 column (Millipore, Billerica, MA) and centrifuged for 20 min at  $4000 \times g$  in an Avanti J-26 XP floor centrifuge using rotor JS-5.3. Exchange buffer contained 200 mM HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM DTT.  $G\alpha$  exchange buffer also contained 1 mM  $\text{MgSO}_4$ , and 20  $\mu\text{M}$  GDP was added to the concentrated protein (500–1000  $\mu\text{l}$ ) in 3- to 4-ml portions and centrifuged each time for 4–10 min for a total 10–12 ml of buffer exchange. The protein was then divided into aliquots and stored at  $-80^\circ$ . The concentration of RIC8 and  $G\alpha$  proteins was determined by comparing to BSA after electrophoresis on an SDS-PAGE gel, followed by Coomassie staining. Samples containing 1–20  $\mu\text{l}$  of purified protein were subjected to Western analysis according to the manufacturer's suggestions. The hexahistidine monoclonal antibody raised in rabbit (Bethyl Laboratories, Montgomery, TX) was used at a 1:1000 dilution, and goat anti-rabbit secondary antibody (Bio-Rad) was used at 1:10,000 dilution.

GTP-binding assays were modeled after protocols described previously (Tall *et al.* 2003). Reactions were carried out in exchange buffer [200 mM HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 1 mM EDTA, and 1 mM DTT] with a total volume of 20  $\mu\text{l}$ . Each reaction contained 200 nM  $G\alpha$  protein and 0 or 200 nM RIC8. Solutions containing purified  $G\alpha$  proteins (400 nM) and 20 mM  $\text{MgSO}_4$  in a total volume of 10  $\mu\text{l}$  were prepared and kept on ice until reaction start.

Mixtures containing RIC8 (0 or 400 nM), 4  $\mu\text{M}$  GTP $\gamma\text{S}$ , and 1  $\mu\text{Ci}$  GTP $\gamma^{35}\text{S}$  (Perkin Elmer, Waltham MS) were also prepared and kept on ice until reaction start. Reactions were initiated by mixing 10  $\mu\text{l}$  of the RIC8 solution with 10  $\mu\text{l}$  of each  $G\alpha$  solution and then incubated at  $30^\circ$  for up to 60 min. Reactions were quenched using 100  $\mu\text{l}$  of cold stop solution [20 mM Tris-Cl (pH 8.0), 100 mM NaCl, 2 mM  $\text{MgSO}_4$  and 1 mM GTP]. Proteins were separated from unbound GTP $\gamma\text{S}$  by vacuum filtration using 0.45  $\mu\text{M}$  of HA mixed cellulose membranes (Millipore), and filters were washed three times with 5 ml cold wash solution [50 mM Tris-Cl (pH 8.0), 100 mM NaCl, and 2 mM  $\text{MgSO}_4$ ] using vacuum filtration. Filters were dried at room temperature and placed in vials, and scintillation fluid (Scintiverse BD cocktail, Fisher Scientific, 4–5 ml) was added prior to counting using a Beckman LS 6500 liquid scintillation counter.

## Results

### *ric8* isolation, gene structure analysis, and protein alignment

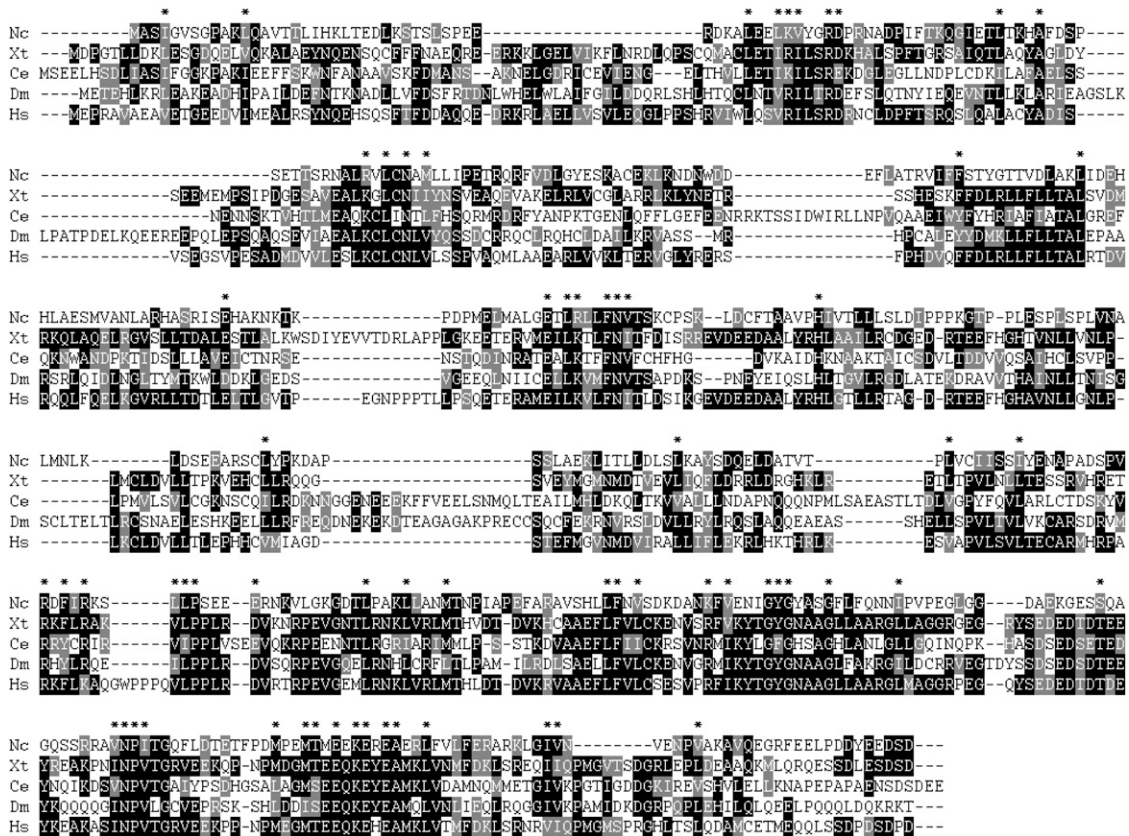
RIC8 belongs to a unique protein family that does not exhibit any conserved domains when compared to known proteins (Wilkie and Kinch 2005; Figueroa *et al.* 2009). However, analysis of the primary sequence of *Xenopus laevis* xRic-8 reveals an armadillo-type folding pattern, which is a group of three  $\alpha$ -helices folded into a triangle structure (Coates 2003; Figueroa *et al.* 2009). This armadillo fold is repeated several times in the xRic-8 protein to form a compact armadillo repeat structure. This is of interest because armadillo proteins are known to interact with several protein partners and to be involved in multiple cellular pathways (Coates 2003). These *in silico* results were corroborated by circular dichroism studies that revealed a high number of  $\alpha$ -helices in the xRic-8 protein (Figueroa *et al.* 2009).

RIC8 is found only in fungi and animals (Wilkie and Kinch 2005). In *N. crassa*, the closest homolog to animal RIC8 is a predicted 53-kDa protein designated NCU02788 ([http://www.broad.mit.edu/annotation/fungi/neurospora\\_crassa\\_7/index.html](http://www.broad.mit.edu/annotation/fungi/neurospora_crassa_7/index.html)). The predicted gene structure in the Broad Institute database was confirmed by sequence analysis and verification of the predicted introns. The presence of the 3' intron was confirmed by cDNA sequence NCW06G3T7 (cDNA clone W06G3 3'; Broad Institute), and the 5' predicted intron was verified by RT-PCR (Figure S1).

Of the animal RIC8 homologs, *Neurospora* RIC8 is most similar to *Xenopus tropicalis* Ric-8A and also exhibits significant identity to human RIC-8A (Figure 1). Although RIC8 proteins are highly conserved among filamentous fungi (Figure S2), there are no significant ( $E < 1e^{-4}$ ) homologs in the yeast *S. cerevisiae*, and RIC8 is also absent from the genomes of plants.

### Epistatic relationships between RIC8 and $G\alpha$ subunits

Vegetative growth in *Neurospora* involves apical polar growth of basal hyphae away from the point of inoculation



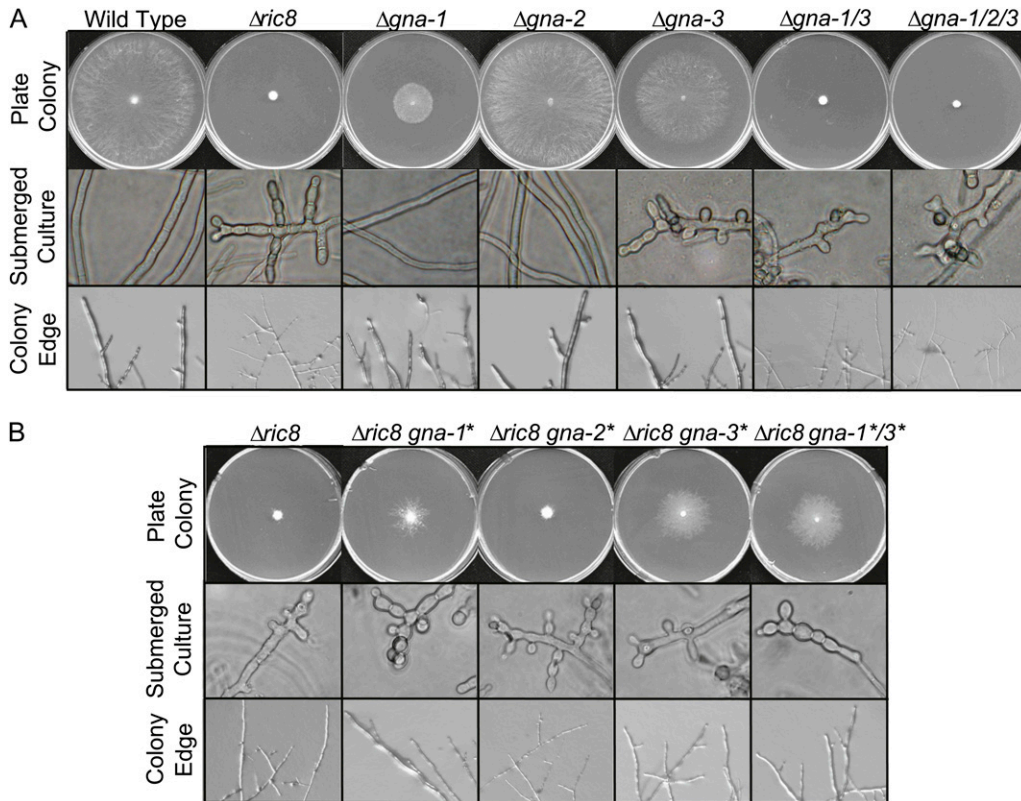
**Figure 1** RIC8 homologs. ClustalW (<http://www.ch.embnet.org/software/ClustalW.html>) was used to align RIC8 sequences from *N. crassa* (Nc; accession NCU02788.3), *Homo sapiens* (Hs; accession NP\_068751,  $E = 5e^{-8}$ ), *X. tropicalis* (Xt; accession NP\_989159,  $E = 2e^{-10}$ ), *C. elegans* (Ce; accession Q9G5X9), and *D. melanogaster* (Dm; accession Q9W358). Background indicates identical (solid) and similar (shaded) amino acid residues ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)).

and parallel to the solid medium surface, with branching occurring back from the tips of the hyphae (reviewed in Springer 1993). Under nutrient limiting conditions or in the presence of an air/water interface, specialized aerial hyphae branch perpendicularly from basal hyphae and produce asexual spore-forming structures called conidiophores, which in turn elaborate strings of asexual spores called macroconidia or conidia (Springer 1993). Once mature, conidia can be dispersed by wind currents or animals in nature. In nutrient-rich submerged cultures, wild-type *Neurospora* does not form conidiophores.

Since RIC8 has been shown to be a GEF for  $G\alpha$  proteins in other systems, we investigated a possible role for *Neurospora* RIC8 as an upstream regulator of the  $G\alpha$  subunits GNA-1, GNA-2, and GNA-3. A gene deletion mutant was created by replacement of the *ric8* gene with the *hph* gene and analyzed for growth and/or developmental phenotypes (Figure 2A). Our laboratory has previously demonstrated that simultaneous loss of the  $G\alpha$  genes *gna-1* and *gna-3* causes severe defects in vegetative growth, absence of female sexual structures (protoperithecia), and inappropriate formation of conidia in submerged cultures (Kays and Borkovich 2004). Mutation of *ric8* leads to phenotypes similar to those observed for  $\Delta gna-1 \Delta gna-3$  double mutants.  $\Delta ric8$  mutants

have thin basal hyphae that grow extremely slowly and produce short aerial hyphae, accumulate less mass, and inappropriately form conidia in submerged cultures (Figure 2A). During the sexual cycle,  $\Delta ric8$  strains do not differentiate protoperithecia and are thus female-sterile (data not shown).

The shared defects of  $\Delta ric8$  and  $G\alpha$  mutants prompted us to measure  $G\alpha$  protein levels in the  $\Delta ric8$  mutant. Loss of *ric8* causes a decrease in GNA-1, GNA-2, GNA-3, and GNB-1 protein levels (Figure 3A). In contrast, transcript amounts for the respective genes are similar in wild type and the  $\Delta ric8$  strain (Figure 3B), suggesting a post-transcriptional control mechanism. Interestingly, a similar requirement for *ric8* in maintaining normal levels of a  $G\alpha_s$  protein and an associated  $G\beta$  subunit has been observed in *Drosophila* (Hampoelz *et al.* 2005). In mammals, Ric-8B inhibits ubiquitination and proteasomal turnover of  $G\alpha_s$  (Nagai *et al.* 2010). We have previously shown that loss of the *Neurospora*  $G\beta$  gene *gnb-1* leads to decreased levels of GNA-1 and GNA-2, while GNA-3 protein levels are unaffected in submerged cultures (Yang *et al.* 2002). Although a decreased level of GNB-1 in the  $\Delta ric8$  mutant could account for low amounts of GNA-1 and GNA-2, the reduction in GNA-3 levels in  $\Delta ric8$  mutants cannot be explained by the loss of GNB-1. This suggests that GNA-3 stability is regulated by RIC8 independently of GNB-1.



**Figure 2** Epistatic relationships between *ric8* and  $G\alpha$  genes. (A) Phenotypes of  $G\alpha$  deletion and  $\Delta ric8$  strains. Strains were inoculated on VM plates and cultured for 1 day at 25° ("Plate Colony" and "Colony Edge") and in VM liquid for 16 hr at 30° with shaking ("Submerged Culture"). Strains used for analysis were wild type (FGSC 2489),  $\Delta ric8$  (R81a),  $\Delta gna-1$  (3B10),  $\Delta gna-2$  (a29-1),  $\Delta gna-3$  (43c2),  $\Delta gna-1 \Delta gna-3$  ( $\Delta gna-1/3$ , g1.3), and  $\Delta gna-1 \Delta gna-2 \Delta gna-3$  ( $\Delta gna-1/2/3$ , noa). Submerged Culture and Colony Edge photos were taken at  $\times 400$  and  $\times 560$  magnification, respectively. (B) Phenotypes after introduction of GTPase-deficient, constitutively activated  $G\alpha$  alleles (indicated by asterisks) into the  $\Delta ric8$  background. Strains used for analysis were  $\Delta ric8$  (R81a),  $\Delta ric8 gna-1^{Q204L}$  (R81\*),  $\Delta ric8 gna-2^{Q205L}$  (R82\*),  $\Delta ric8 gna-3^{Q208L}$  (R83\*), and  $\Delta ric8 gna-1^{Q204L} gna-3^{Q208L}$  (R81\*3\*). Culture conditions are as described in A.

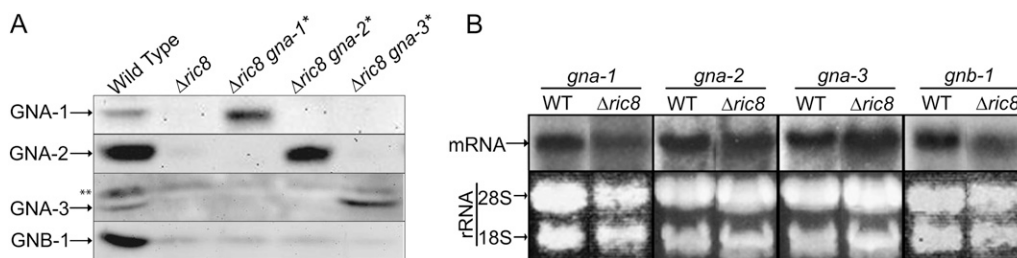
During the G-protein cycle, GTP-bound  $G\alpha$  is released from  $G\beta\gamma$  and the GPCR and is then free to interact with downstream effectors. Hydrolysis of GTP to GDP leads to reassociation of the  $G\alpha$  protein with  $G\beta\gamma$  and termination of signaling. Because of these attributes, a GTPase-deficient  $G\alpha$  allele is constitutively active and independent of  $G\beta\gamma$ . We therefore tested whether introduction of GTPase-deficient alleles of the three  $G\alpha$  genes (under control of the *ccg-1* promoter) into the  $\Delta ric8$  background would rescue  $\Delta ric8$  phenotypes.

The results show that  $\Delta ric8$  strains carrying a GTPase-deficient activated  $G\alpha$  allele restored the encoded protein to wild-type levels (Figure 3A). This suggests that overexpression and/or a constitutively GTP-bound  $G\alpha$  can override the reduced protein levels observed in  $\Delta ric8$  mutants. Transformation of the *gna-2*<sup>Q205L</sup> activated allele into the  $\Delta ric8$

background had no effect. However, the presence of the *gna-1*<sup>Q204L</sup> or *gna-3*<sup>Q208L</sup>-activated allele partially rescued the slow growth and reduced mass and thin basal hyphal phenotypes of  $\Delta ric8$  (Figure 2B), with *gna-3*<sup>Q208L</sup> having the greatest effect on growth. When both *gna-1*<sup>Q204L</sup> and *gna-3*<sup>Q208L</sup> are introduced into the  $\Delta ric8$  background, the phenotype resembles that obtained after transformation with *gna-3*<sup>Q208L</sup> alone (Figure 2B). Taken together, these results suggest that RIC8 acts upstream of and is a positive regulator of GNA-1 and GNA-3, but not of GNA-2 in *Neurospora*.

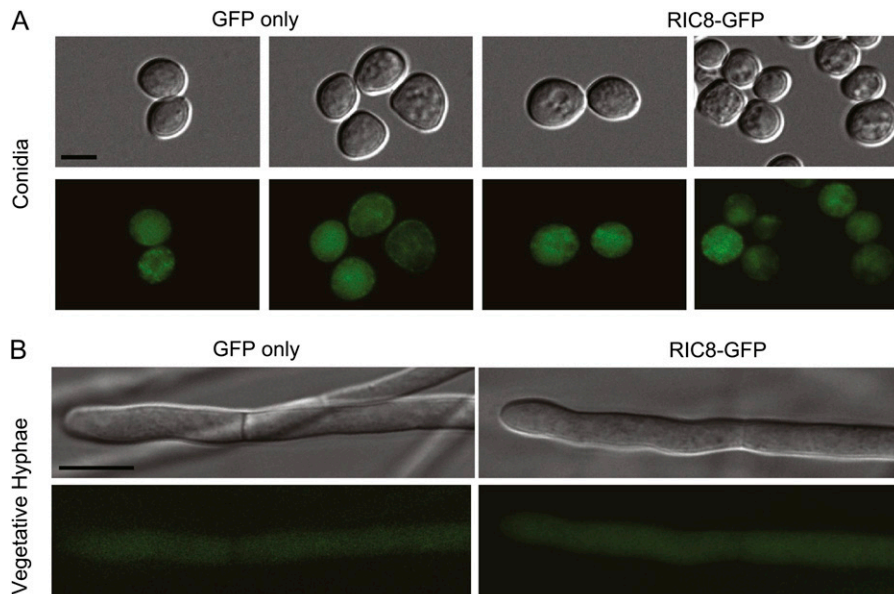
### RIC8 is cytoplasmic in conidia and vegetative hyphae

To determine the localization of RIC8 in *Neurospora*, we produced a vector containing a C-terminal fusion of GFP to RIC8. This construct complemented  $\Delta ric8$  phenotypes and restored



**Figure 3** Expression of G proteins in a  $\Delta ric8$  mutant. (A)  $G\alpha$ - and  $G\beta$ -protein levels. Samples containing 100  $\mu$ g of protein from particulate fractions isolated from 16-hr submerged cultures were subjected to Western analysis using the GNA-1, GNA-2, and GNA-3 antibodies. The asterisk (\*) denotes strains containing activated alleles of  $G\alpha$

genes. The double asterisk (\*\*) indicates a background band observed in all GNA-3 Western blots. Strains used for analysis were wild type (FGSC 2489),  $\Delta ric8$  (R81a),  $\Delta ric8 gna-1^{Q204L}$  (R81\*),  $\Delta ric8 gna-2^{Q205L}$  (R82\*), and  $\Delta ric8 gna-3^{Q208L}$  (R83\*). (B) Levels of  $G\alpha$  and  $G\beta$  transcripts. Samples containing 20  $\mu$ g of total RNA isolated from 16-hr submerged cultures were subjected to Northern analysis using gene-specific probes. Strains used for analysis were wild type (FGSC 2489) and  $\Delta ric8$  (R81a).



**Figure 4** Localization of RIC8-GFP in *Neurospora*. Cultures were grown as indicated in *Materials and Methods*. Images were obtained using an Olympus IX71 microscope with a QIClick™ digital CCD camera and analyzed using Metamorph software. (A) DIC and GFP fluorescence micrographs showing localization of RIC8-GFP in conidia. Bar, 5  $\mu$ m. (B) DIC and GFP fluorescence micrographs showing localization of RIC8-GFP in vegetative hyphae. For both A and B, GFP fluorescence images for a wild-type strain without a GFP construct were completely black. Bar, 10  $\mu$ m.

expression of the *ric8* transcript (Figure S1, B and C). Production of a full-length RIC8-GFP fusion protein was observed by Western analysis using whole-cell extracts and GFP antiserum (Figure S1D). Analysis of strains carrying the GFP fusion showed that RIC8 is cytosolic in mature conidia and vegetative hyphae; this was confirmed by comparison to a control strain expressing a cytosolic GFP protein (Figure 4). GFP fluorescence images for a wild-type strain without a GFP construct were completely black using the same settings, indicating negligible background fluorescence (data not shown).

This observation of a cytosolic location for RIC8 is consistent with observations in animals (Hampoelz *et al.* 2005) and in *M. oryzae* (Li *et al.* 2010). In higher eukaryotes, RIC8 has been shown to have cytoplasmic localization, which moves to the mitotic machinery at specific times during embryonic mitosis. In *Drosophila* embryos, RIC8 is located in the cytoplasm, with concentrated amounts at the mitotic spindle (Hampoelz *et al.* 2005). In *C. elegans* embryos, RIC8 is localized at the cortex, mitotic spindle, nuclear envelope, and around chromatin (Couwenbergs *et al.* 2004). The absence of nuclear localization for RIC8 in the two fungi analyzed could result from a number of factors, including growth conditions, time in the life cycle, or absence of an environmental trigger.

#### **Loss of *ric8* affects the cAMP-signaling pathway, which is regulated by $G\alpha$ proteins in *Neurospora***

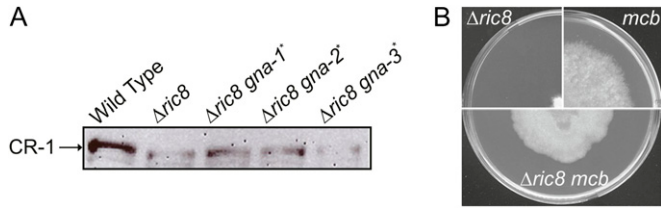
Adenylyl cyclase has been extensively characterized in eukaryotes and has been shown to be activated and repressed by G-protein signaling in various systems (reviewed in Neves *et al.* 2002). Activated adenylyl cyclase produces cAMP, which binds to the regulatory subunits of protein kinase A (PKA), releasing the catalytic subunits of PKA to phosphorylate a wide variety of target proteins (Neves *et al.* 2002). In the *C. elegans* synaptic signaling pathway, RIC8 has been linked to cAMP metabolism through the identification of mutations that sup-

press defects of *ric8* loss-of-function mutants (Schade *et al.* 2005; Charlie *et al.* 2006). These mutations include dominant activated alleles of *gsa-1* ( $G\alpha_s$ ) and *acy-1* (adenylyl cyclase) and reduction-of-function alleles of *kin-2* (regulatory subunit of PKA) and *pde-4* (cAMP phosphodiesterase). These results are consistent with RIC8 as an upstream activator of the cAMP pathway during synaptic signal transmission in *C. elegans*. In *Neurospora*, previous work from our group suggests that adenylyl cyclase (CR-1) is activated by GTP-bound GNA-1, while GNA-3 is required to maintain normal levels of CR-1 protein (Ivey *et al.* 1999; Kays *et al.* 2000). Furthermore, a mutant allele of the PKA regulatory subunit (*mcb*) that is predicted to lead to hyperactivation of the catalytic subunit is epistatic to *gna-3* and suppresses the  $\Delta$ *gna-1* mutation (Bruno *et al.* 1996; Kays and Borkovich 2004).

On the basis of the known relationship between RIC8 and cAMP metabolism in *C. elegans* as well as the genetic relationship between RIC8, GNA-1 and GNA-3 in *Neurospora*, we investigated whether cAMP signaling is affected in *Neurospora*  $\Delta$ *ric8* mutants. We first assessed levels of the CR-1 adenylyl cyclase using western analysis with a CR-1 antiserum (Figure 5A). The amount of CR-1 protein was reduced in the  $\Delta$ *ric8* background (Figure 5A), similar to results observed for  $\Delta$ *gna-3* mutants and consistent with low cAMP levels (Kays *et al.* 2000). However, expression of the activated forms of GNA-1, GNA-2, or GNA-3 did not return the CR-1 protein levels to normal (Figure 5A). These results suggest a  $G\alpha$ -independent role for RIC8 in modulation of CR-1 protein levels.

We followed up on the above finding by testing the effect of the *mcb* PKA regulatory subunit mutation described above (Table 1). The *mcb* mutation partially suppresses the  $\Delta$ *ric8* growth and conidiation phenotypes (Figure 5B). These results support a role for RIC8 as a positive regulator of the cAMP-signaling pathway through the activation of  $G\alpha$  subunits and at least in part via a  $G\alpha$ -independent pathway that influences the stability of cAMP-signaling pathway components.





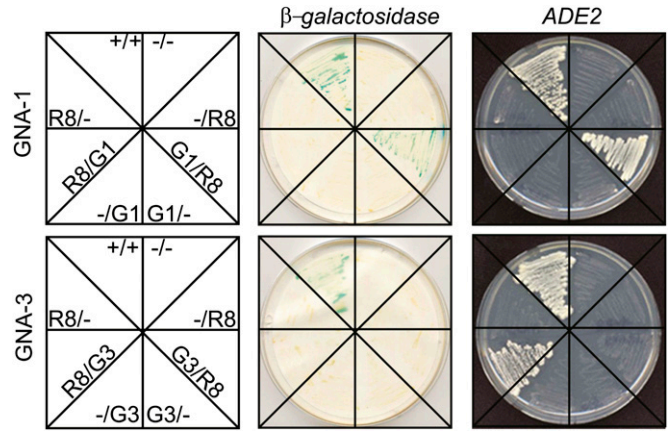
**Figure 5** Relationship to the cAMP pathway. (A) CR-1 (adenylyl cyclase) protein levels. Samples containing 100  $\mu$ g of protein from whole-cell extracts from 16-hr submerged cultures were subjected to Western analysis using the CR-1 antibody. Strains used were the same as in Figure 3A. (B) Effects of the *mcb* mutation. Strains  $\Delta ric8$  (R81a), *mcb inl*, and  $\Delta ric8 mcb inl$  were cultured on VM + inositol plates for 3 days at 25°.

### RIC8 interacts with and acts as a GEF toward $G\alpha$ subunits

Results from the genetic epistasis experiments suggest that RIC8 is a positive regulator of GNA-1 and GNA-3. We next tested for a possible physical interaction between  $G\alpha$  proteins and RIC8 using the yeast two-hybrid assay. RIC8 was observed to interact with both GNA-1 and GNA-3, but not with GNA-2 (Figure 6). Interestingly, RIC8 interacts with GNA-1 only as a Gal4p DNA-binding domain fusion and with GNA-3 only as a Gal4p activation domain fusion (Figure 6). This may indicate a different physical relationship and/or binding site for RIC8 on these two  $G\alpha$  subunits.

Having established a physical association between RIC8 and a subset of *Neurospora*  $G\alpha$  subunits using the yeast two-hybrid assay, we next performed experiments to directly test whether RIC8 can accelerate exchange of GDP for GTP on  $G\alpha$  proteins. Since  $G\alpha$  proteins had not been previously purified or assayed for GTP binding in any filamentous fungal species, we had to devise methods to facilitate expression of soluble, active proteins and their subsequent purification. A previous study reported that efficient purification of mammalian  $G\alpha$  proteins from *E. coli* and subsequent protein crystallization were facilitated either by removal of the disordered N-terminal region or by replacement with the N terminus of another  $G\alpha$  subunit (Kimple *et al.* 2002). These proteins were shown to retain normal GTP binding (Kreutz *et al.* 2006). We predicted that the first 12 (GNA-3) or 13 (GNA-1 and GNA-2) amino acids of *Neurospora*  $G\alpha$  proteins are disordered using the DisEMBL Intrinsic Protein Disorder Prediction program (<http://dis.embl.de/>). We therefore produced *E. coli* vectors expressing deca-histidine (10H) N-terminal tagged and N-terminal truncated versions of all three  $G\alpha$  subunits, termed 10H-N <sup>$\Delta$ 13</sup>-GNA-1, 10H-N <sup>$\Delta$ 13</sup>-GNA-2, and 10H-N <sup>$\Delta$ 12</sup>-GNA-3. For clarity, we refer to these proteins as GNA-1, GNA-2, and GNA-3. A vector encoding full-length 10H-tagged RIC8 was also produced for expression in *E. coli*. Proteins were overexpressed in *E. coli* and then purified using nickel or cobalt affinity chromatography (see *Materials and Methods*).

GTP $\gamma$ S-binding assays were conducted in the absence and presence of RIC8. All three  $G\alpha$  proteins bind GTP in the absence of RIC8, demonstrating that the fusions are

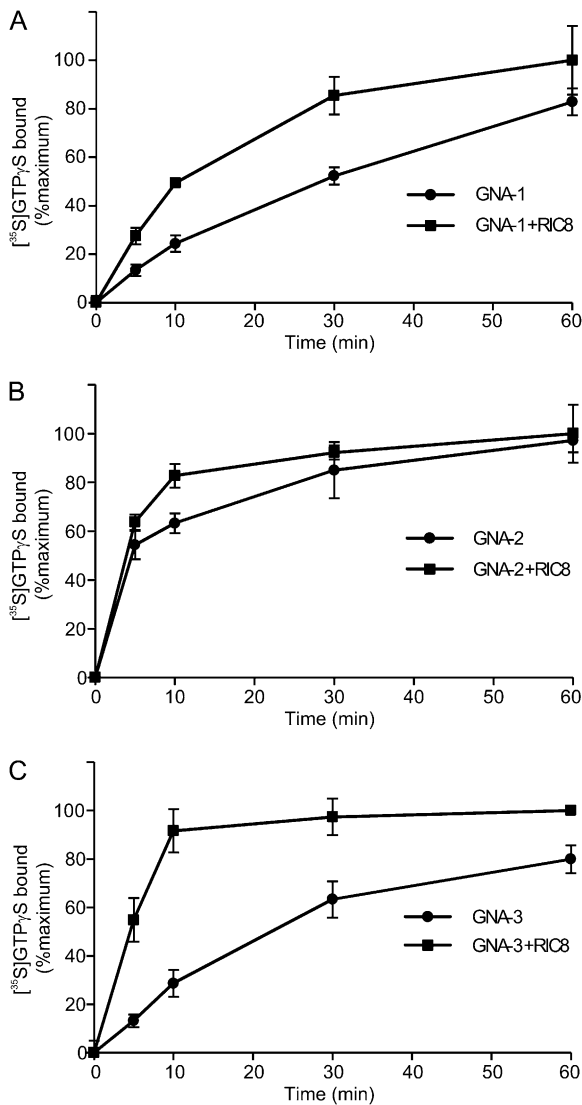


**Figure 6** Interaction between RIC8 and  $G\alpha$  subunits. Yeast two-hybrid analysis was employed using diploid strains containing the GAL4 activation domain (pGAD424) and the DNA-binding domain (pGBKT7) vectors. Labels represent inserts in the pGAD424/pGBKT7 plasmids, respectively (G1, GNA-1; G3, GNA-3; R8, RIC8). Minus sign (–), no insert in vector; plus sign (+), positive control interactors.  $\beta$ -Galactosidase activity shows strains containing vectors with the indicated genes that were cultured on medium lacking tryptophan and leucine for 3 days at 30°. Plates were scanned 24 hr after the colony lift assay for  $\beta$ -galactosidase. The adenine growth assay (*ADE2*) shows strains that were grown on medium lacking tryptophan, leucine, and adenine for 5 days.

active and confirming the identity of the three *Neurospora* proteins as G proteins (Figure 7). Addition of RIC8 had different effects on GTP $\gamma$ S binding to the three  $G\alpha$  proteins. RIC8 does not appreciably influence binding of GTP $\gamma$ S to GNA-2 (Figure 7B). However, binding of GTP $\gamma$ S by GNA-1 is increased twofold by the addition of RIC8 at early time points (5–30 min, Figure 7A), consistent with GEF activity for RIC8. A more dramatic effect is observed with GNA-3, where binding is significantly greater in the presence of RIC8 at all time points, with a maximum stimulation of more than fourfold at 5 min (Figure 7C). These results suggest that RIC8 acts as a GEF for GNA-1 and GNA-3, playing a major role in the activation of GNA-3. Importantly, the findings from the GEF assays are consistent with those obtained from the genetic epistasis analysis and yeast two-hybrid assays and support a model in which RIC8 activates GNA-1 and GNA-3, but not GNA-2, in *Neurospora*.

### Discussion

The discovery and subsequent study of RIC8 in metazoan cells has shown that this protein is required for major cellular processes, including asymmetric cell division and synaptic signaling. Here we demonstrate that RIC8 is involved in critical functions, including vegetative growth and sexual and asexual development in *Neurospora*. Mutations that activate the GNA-1 and GNA-3  $G\alpha$  proteins in the  $\Delta ric8$  background partially suppress  $\Delta ric8$  phenotypes, and RIC8 interacts with GNA-1 and GNA-3 in the yeast two-hybrid assay. Most importantly, we show that RIC8 acts as a GEF for GNA-1 and GNA-3 *in vitro*. The data suggest that RIC8



**Figure 7** GTP binding of G $\alpha$  proteins and GEF activity of RIC8. GNA-1 (A), GNA-2 (B), and GNA-3 (C) were incubated with [ $^{35}$ S]GTP $\gamma$ S in the absence (●) or presence (■) of RIC8. All protein concentrations were 200 nM. Reactions were performed in triplicate, as indicated in *Materials and Methods*. A representative result from several independent assays is shown for each protein.

participates in pathways involving GNA-1 and GNA-3 to regulate growth and development.

Loss of *ric8* leads to lower levels of G-protein subunits in *Neurospora*, *Drosophila*, *C. elegans*, and mammalian cells. In *Drosophila*, RIC8 is required for proper plasma membrane localization and normal protein stability of G $\alpha_i$ , but only correct membrane localization of G $\alpha_o$  (Hampoez *et al.* 2005). In *C. elegans*, RIC8 is essential for membrane localization and normal protein stability of G $\alpha_{16}$ , while G $\alpha_o$  does not depend on RIC8 for protein stability or localization (Afshar *et al.* 2004, 2005). In *Drosophila*, it has been proposed that effects on G $\alpha$ -protein localization and stability in the absence of RIC8 may stem from defective assembly of the heterotrimer and inhibited transport to the plasma mem-

brane (Hampoez *et al.* 2005). In mammalian cells, it has been demonstrated that Ric-8B inhibits ubiquitination of G $\alpha_s$ , thus preventing degradation of the protein (Nagai *et al.* 2010). The mechanism underlying how loss of RIC8 leads to lower levels of G $\alpha$  proteins in *Neurospora* is unknown, but may be a consequence of both mislocalization and accelerated protein turnover (A. Michkov, S. Won, and K. Borkovich, unpublished observations).

Work in *C. elegans* has demonstrated the importance of cAMP metabolism to RIC8-related functions in the synaptic signaling pathway. In addition, stabilization of G $\alpha_s$  by Ric-8B in mouse embryonic fibroblast cells is required for increased cAMP in response to isoproterenol (Nagai *et al.* 2010). The similarities between the genetically tractable *C. elegans* and *Neurospora* with regards to positive regulation of the cAMP pathway by RIC8 are particularly striking. In both systems, a mutation in the PKA regulatory subunit suppresses a *ric8* mutation. Importantly, we also demonstrate that loss of RIC8 leads to a reduced level of adenylyl cyclase protein, a finding that has not been previously reported in any system. This observation may explain why a hyperactive allele of adenylyl cyclase was recovered in a *ric8* suppressor screen in *C. elegans* (Schade *et al.* 2005). As mentioned above, RIC8 is also required for normal levels of various G proteins in *Neurospora*, *Drosophila*, *C. elegans*, and mammalian cells. Taken together, these results suggest that maintenance of normal levels of G proteins and adenylyl cyclase (and perhaps other yet-unknown regulatory components) is an important conserved function of the RIC8 protein and one that directly impacts cAMP signaling.

A prediction based on our results is that RIC8 regulates G $\alpha$  activity and subsequent cAMP production in response to a specific developmental signal. However, the mechanism by which RIC8 is regulated at the pre- or post-translational level is currently unknown. Regarding transcriptional regulation, microarray analysis indicates that *ric8* is expressed ubiquitously throughout the vegetative colony in *Neurospora* (Kasuga and Glass 2008). Results from additional microarray studies using *Neurospora* grown on medium that mimics either symbiotic or pathogenic plant relationships suggest that *ric8* expression is not induced during fungal/plant interactions (<http://bioinfo.townsend.yale.edu/browse.jsp?scope=4&tabid=1&query=ncu02788>).

Heterotrimeric G $\alpha$  proteins are activated by the exchange of GDP for GTP. This exchange is facilitated by a GEF, such as a GPCR or RIC8. Our results provide the first evidence that filamentous fungal G $\alpha$  subunits do indeed bind GTP and that RIC8 functions as an apparent GEF by increasing GDP-GTP exchange on two of the three subunits. The main target of RIC8 appears to be GNA-3, as GNA-3 is activated most strongly by RIC8 *in vitro*, accelerating the GTP binding by fourfold. This activation is comparable to the more than sevenfold activation of G $\alpha_{i1}$  by RIC8 in mammals (Tall *et al.* 2003) and to the fourfold activation of Gpa1 by another GEF, Arr4, in *S. cerevisiae* (Lee and Dohlman 2008). The twofold activation of GNA-1 by RIC8 is similar to that observed

for mammalian  $G\alpha_o$ , while mammalian  $G\alpha_s$  is not affected by RIC8 (Tall *et al.* 2003). The finding that RIC8 acts preferentially on GNA-3 is supported by the observations that the GTPase-deficient allele of GNA-3 suppresses the  $\Delta ric8$  mutation to the greatest extent and that CR-1 protein levels are reduced in both  $\Delta ric8$  and  $\Delta gna-3$  mutants.

Our results reveal important parallels between RIC8 functions during cell division, cAMP signaling, and perhaps regulated protein turnover in *Neurospora* and metazoans. *Neurospora* is a haploid organism that has a relatively short doubling time, well-developed genetic tools, and a sequenced genome. There is also a comprehensive collection of knockout mutants for most genes, and it is relatively easy to isolate and clone suppressor mutations. Future investigations will capitalize on these tools to reveal roles for known proteins, as well as to screen for novel components that participate in RIC8-regulated processes, thus illuminating conserved functions for RIC8 during growth and development in *Neurospora* and animals.

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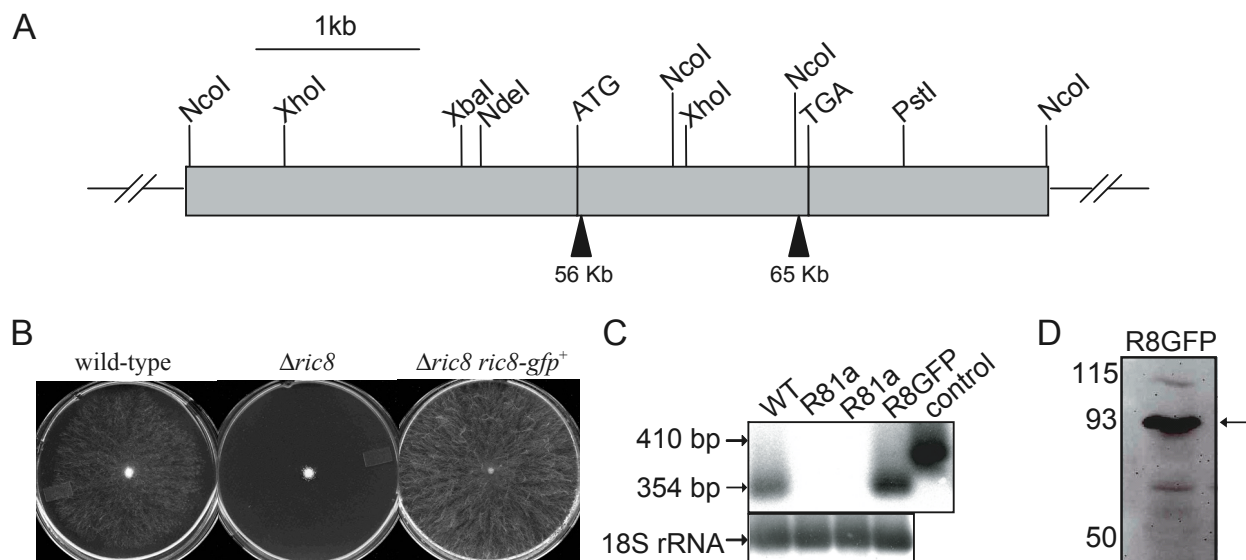
# GENETICS

Supporting Information

<http://www.genetics.org/content/suppl/2011/07/12/genetics.111.129270.DC1>

## **RIC8 Is a Guanine-Nucleotide Exchange Factor for G $\alpha$ Subunits That Regulates Growth and Development in *Neurospora crassa***

Sara J. Wright, Regina Inchausti, Carla J. Eaton, Svetlana Krystofova, and Katherine A. Borkovich



**Figure S1** *ric8* gene structure, mutant verification, and expression RIC8-GFP. A) The *ric8* genomic region from *Neurospora crassa*. Intron locations (black triangles) and restriction enzyme sites (black lines) are shown. B) Complementation of  $\Delta ric8$  by expression of a *ric8-gfp* allele. A VM plate culture is shown for wild-type (2489),  $\Delta ric8$  (R81a), and  $\Delta ric8, ric8^+::gfp^+::his-3^+$  (R8GFP). C) RT-PCR analysis of *ric8* mRNA levels. Samples containing 2  $\mu$ g of total RNA isolated from conidia of strains used in (B), with a duplicate R81a reaction, were subjected to RT-PCR with primers designed to flank the first predicted intron of *ric8* (R811fw and R811rv, TABLE S1). The predicted intron size is 56 bp, making the size of the RT-PCR fragment about 354 bp. For the control and probe DNA, a 410 bp fragment of genomic DNA was amplified by PCR from plasmid pSM2 using the same primers. Expression of the 18S rRNA gene was assessed under identical RT-PCR conditions (See methods). D) Western analysis. Protein from a whole cell extract isolated from a 16 hr submerged culture of strain R8GFP was subjected to Western analysis using anti-GFP antiserum (See methods). The approximately 93 kDa protein is indicated by the black arrow.

```

Nc -----MASTIGVSGPAKLVAVTTLHKLTEDLKSTSLSP-----ERDKALELKVYGRDPRNADPI-----FTKCGIETLTKHAFDS
Pa -----MNAKAKIPVAKLDVAKVQLLGSLEDELETSSLAQRWFIPFAALEIASNKFEIGRESLLELKIYGRDPNCAADPI-----FTKEGITLVRHAFDS
Mg -----MLNNTLTKGTDKLILDLKHNLSSQ-----ERDAALEQLKVYGRDPRDAPI-----FTKEGISTLAHSEFDG
Fg -----MALPAPAGLPTGTDKLEYIKELINDLEDLNELELDP-----DRAAVLDQKHLTRDPIINADPI-----YTBEGISTLLRHAYDK
Ss MSISSTRTSLSQCLTGEAKLADVTNIVBSLKADELNISLLPH-----ORDALLEQLKVYGRDPRADAPI-----FTREGIETLSKHAFNS
An -----MDLRILQSGEKLQOVTKLLNSLEKDLKNSLNSA-----ORTQILQLRQYGTSPDNAGPI-----YSKKGIEITLSKVCIDG
Hc -----MATPALVVEVTSCLNAEKTAAQFGSRGS-----WEQNINLEINFLGLOFQONNSR-----IRGEGKTRG
Bc MSISARTSLSQELTGAKLTDVTKIVBSLKEDELEKISLLEPH-----ORDALLEQLKVYGRDPRADAPI-----FTKEGIEITLSKHAFNS
Sj -----MPDSKSTVDLLSFERDNGSFVPTICSKINLLKPTAWELSAN-----LIEIAVSLKELRKKSRPKDACQKISNALDWTLVLAERAFVSKLRNSA
Yl -----MIGHLHLYDITYTISLLEHNPVQYHFSPPKMSKKTAYEDFQS-----EPQDPLICCALKMSLTTAQAWTCIG-----GISAADDIATHVLDG

* * *
Nc PSETTSRNALRVLGNAMLLIPETQRQRFVDLGYEAKACEKLN--DNWDEFLAARVIFSTYGTIVDLAKLIDBHHLAESVANLARHARSISEHAKNKIKPDPME
Pa TSVNTRSRGALRILCNLILLPBPETQRQRFVDLGYAAKASEKLE--DNSDDEFLVARLILISTYNTNIDLKLITQHGGLADSIANHLARHAKRISSTNR-STTANPMD
Mg SDDTTSRNALRCLANAMLLPSSRQELVDLGYHIKAKALAS--DSWDEFLVSRVLMLANARNINHEILITQHGGLAELIARLESMMRFLP----DEDSSPNTME
Fg PSTKSAADARRVLANAMVLTVTVDIFVNKGFAPNAQOGLNG--GSFDDEFLNSRILFLSTYGTIKVDLKLIDDEKLADRISENLARHAK-----ESKAKSDPMQ
Ss PSKFTSRNALRCLANALLKPDTRQTLVTLGYDAKACTLLKDRNGSVVDEFLSRIIFLITYGTTININLIDHCHLADATSHNLANHARLHNAKQKVKQLDPM
An ETTDVRHAALRCVANALLDSNMROLFVDTCGKGRLEAMLK--DSSBHEMVISRILFLSTYDININFDLNNHSLGDNINYOILRHSKQPKSGR--PLSQVD
Hc -----CHAIKIKITCOQYKNSSSFSKSVNTECDL--BNSDDEFLASRLFLFLATYDIDDFKLDIDNNLGEYINNHYRHSKRFKARKK--KLDQMD
Bc PSKFTSRNALRCLANALLKPDTRQTLVGSYDERACALLKDRIASVDEFLSRIIFLITYGTTININLIDQCHLADITSHNLANHDKLHSAKQKKTROLDPME
Sj HSLELIRLIVNVLVNPMSQRIASLFSNSAFDEESFQNSACQIALRLFLLCVGENDEDRWTFRITPEGLQALGIVVNNLSTPEVSASVLDLRLRAY
Yl SLDEASTLNLAATVSNQYFLLRQYITSATKAPKLVNWAATTPDTERGKMCRRILELCSSEIRIEGLNVATLAKGLEBLGLTDLQDLQLEKQKQKQLLLOHREIL

* * *
Nc LMAIGETLRLFFNVTSKCPKSLDCFTAAVPHIVTLLSLDIPPKKTPPLESPLSPLVNALMNLKLDSEARSCLYPKDAPSSLAELKITLLELSIKAYS-DOELD
Pa PMALBETLKLITFNVSCHSPNHLASHEPALPHIITILCSIDLPSHTKTPGPFPGPAVNALNLDLSPTAKYLYLDPSSPSSFDRLIKLISLSTIKAYK-NPDLE
Mg GMALSETLKMFPVAVLRFQGOSTRBACAGHILTLICQRPLE--AESVLDPPFGPLINALCTLDLEDEAITNIFPERDPSIVTSRLISILDRALSEKNGMDD
Fg DMALVESAKLLFNVTYCPDKSSFSATAPHLVALLLKQDIS--QTKPLDPPVGFIFINALANLVDGSDCQKSIHPEDEPKVVSRLISILDPAAMKNVP-DNQLD
Ss DMALAESLKLFLNITFCPCRNSSFSQALPHILSILDKREI--PNTPLESPISQLINLNLPLEDEPCNIVLFRANPHSHVYCKFLELLEHSHINEYK-DEELE
An ELAFTDITLKLFNIAKLFQDLAPITFSPSTPYIFKILSRIDILPLKPLDGLLGTLLNCLSTLLENKKNKPYDGNPIFFPTFNQCNVDKLNILDQAVSAYE-PSELE
Hc ELALSETLKLNFNITNBYHIVDAPSPSPHILKILSRIEITPEPLQAPVNYLINSLLNLDLEAKKSKHFCTNPLPKFDQNCNVDKLNILDQAVAMHK-POHLE
Bc DMALGS-----PNNVVEKFNILLDHSINKYK-DEELE
Sj AVGSKDKPKSSTAQLSPEFVIQLWKETGTRDPSYTSNTSWHIVNALVLPLQNLTSSSLYQITDIALTALDVLPLKGIQLLEAGNASFKVPNFASVVELTAKELE
Yl SGFVCLITVYKTVREDQEKQTSSELKQIVPHVLTIAQNP-----BLLYPCPIISCRPMAVESQKLFVYLETLEVOIKQVLCATYPTDIALA---QLL

* * *
Nc ATVIPLVCLISSIYENAPASPVRDFIRKSLLPSEERKNVKGKGDTPAKLLANMTPNPLAPBFARAVSHLLFNVSQDKANKFVENIGYGYASGFLFQNNIPV--
Pa QIVIPLYCALSTVYEHAPAS--VKQSIKCALLPTEKDRTEALGKADTLQGHLLKNWNSPEAPELCKATAHLYFDLSNRDPHKFVKNVGYGYASGFLFQNNISFTPE
Mg PTMTVLAGSLCIIIEAEP--VRISMCAQLLPTDEDRKEVLGSTELESPLSKLLKMTNMAPKSRKAIHLLFLGLSDRDAGKVFVKVGYGYASGFLFEENIPVPOS
Fg ATVSPLLGVISSVYKHAPDS--SKKYIREKLLPTEEDRKEVLGKGDALSAKLLQNFNPLAPSIVGTVITCHLLYDLSENDANKFVENVGYGFASGFLFQNNIPVPS
Ss QLVSPLLTLIRRYEYEGPSE--VHKFLQIRLLPSDKD-----HASTFVQNLGYGFASGFLFQRNIPIPEN
An TKATPLFHTLVITHEVADPG--PRKYMOWLLLEDNDRDRPTGQSDTSSKLLKLSMTHYAN-LKVAISELMFVLSCKNABSLTNIGYGFAGLLASRGMDIPKS
Hc SLAVPLLTLLRKLVSFAPEG--PKKYMFWLLLEDNDHDLPIGKSNLTLSSRLLRLSSTPVAPSLREGISALMFELSAGSDAIDFVNVNMGYGFAGFLSHDVPVPE
Bc QLVSPLLTLIRKANEIGPSE--VHOFMCIQLLPSDKDREQPVGRGESLSARLQOLSTSPMAPOVRESISALLFELSDKDASTFVENLGYGFASGFLFQHNVPIPEN
Sj VRSPLLLALLQNVKINDTO--TCRLLESRLPSEADRSASLAFSGSLPSRLLRLVIPCFLQPOQAHLIYSLCRENAKVLDTIGYGFASGILRVCCBETDST
Yl YGLAFLVPIVTTVDGABPAR---EILCRVFLPSDEDRKEALGKTKSIPSRLLKVIIEEPFQDCFLVNEAQVLCENKDKFTDAILGAYAGYLTFGAASSAS

* * *
Nc EGLCGDAKKGESSQACQSS--RRAVNPITGQFLDTEFTFDPPEMTEEEKEREAERLFLVLFERAKLGIIVNVENPVAKAQEGRFEELPDDYEDSD-----
Pa ELNKGGETVECEDEGVREI--KRFINPITGQFLDTEFVSELPEMTEDEKEREERLFLVLFERLQOTGIIDVONPVECAMREGRFEELPDDK-----
Mg ALAS-----EGASCSTMQ--GRAVNPITGQFLDAEKLSLIPEMSOEKEKEAERLFLVLFERLKKITGVVDVONPVEKAFQEGRVEELDEDDGCDENKVKVEQK
Fg ASDPG-----S--QKLVNPTGQHVDAKPVDEPEMTEEEKEREAERLFLVLFERLKNITGVVDVONPVEBAMREGRVRELKDDVEEIE-----
Ss ALEAWSKENDNDGKARSSHDSGKAVNPITGQFLHAEKIDMPEMSQEEKEREAELFLVLLK---KNGIITAKNPEBALQGRFEELDDADSD-----
An ACEAFATN-----SCLNPNINPTGQFWAAEKEDTGPMTKEEKEREAERLFLVLFERAKANGILGVENPVQALREGRVFEELPDSSE-----
Hc AKEAFSTS-----AGGLDPMNPTGQFWDAEPQDGGPEMTEEEKEREAERLFLVLFERLKATGVVDVNPVSAALQTCRFEELG-----
Bc AFEAWSTENDKNGKARPSQDSGKAVNPITGQFLHAEKIDMPEMSQEEKEREAERLFLVLFERLKNNGIITANPNPEBALQGRFEELDDADSD-----
Sj VSKDSLFSG-----LSTATADNVNPTGQFTSEKPP--VPMSMEEKEREAERLFLVLFERLKNNGIITANPNPEBALQGRFEELDDADSD-----
Yl GPASNISGFG-----YDKVITGQKLTPELQELKQEQEIS--AMSPEEKEREAELMYLNLNRMRELGIIVNFHPHRAAQESGRFEELD-----

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**Figure S2** RIC8 homologs in other fungi. ClustalW (<http://www.ch.embnet.org/software/ClustalW.html>) was used to align RIC8 sequences from *Neurospora crassa* (Nc; NCU02788.3), *Podospora anserina* (Pa; Pa\_1\_3900), *Magnaporthe grisea* (Mg; MGG\_14008), *Fusarium graminearum* (Fg; FSG\_01511), *Sclerotinia sclerotiorum* (Ss; SS1G\_02787.1), *Aspergillus nidulans* (An; AN1661.3), *Histoplasma capsulatum* (Hc; HCAG\_03335.1), *Botrytis cinerea* (Bc; BC1G\_02317.1), *Schizosaccharomyces japonicus* (Sj; SJAG\_02027), *Yarrowia lipolytica* (Yl; ACCESSION# XP\_505953). Shading indicates identical (black) and similar (grey) amino acid residues ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)).

**Table S1 Oligonucleotides used in this study**

Name	Sequence (5'-3')
R8 GFP fw	TATCTAGAATGGCCTCAATAGGAGTTTCTGGGCCAGGTC
R8 GFP rv	GGGGATCCAGTCCGAATCCTCTTCGTAGTCGTCGGG
R8I1fw	CACCATGGCCTCAATAGGAGTTTCTGGG
R8I1rv	CAGTTGTCGTTTTGAGCTTTTCGCAGGCC
R8YR11fw	GTAACGCCAGGGTTTTCCAGTCACGACCGCCTGAGATGTGTTGTATCGAGAG
R8YR11rv	CAGCTTATGAATGAGAGTGGTGACAGCTTGCAACTGGCTGGCCAGAACTCCTATTGAGG
R8YR12fw	CCAAGTTGCAAGCTGTCACTCTCATTATAAGCTGACCGAGGACCTCAAGAGCAC
R8YR12rv	GCGGATAACAATTTACACAGGAAACAGCCGGCAAGACTGGAGGGTGCCTTGGGG
R8YR21fw	GTAACGCCAGGGTTTTCCAGTCACGACCGTCCGGTCCCGCATATAGTG
R8YR21rv	CAGGGTTCTCAACATTAACGATTCCAGCTTCCTCGCCCTCTCAAACAAGACAAACAGTCTCTC
R8YR22fw	GGCGAGGAAGCTGGGAATCGTTAATGTTGAGAACCCTGTTGCCAAGGCGGTTTCAGGAG
R8YR22rv	GCGGATAACAATTTACACAGGAAACAGCGGGAGTCGTATACCTCCGCGGAGGAAGGCC
R8Y2Hfw	AATTGAATTCATGGCCTCAATAGGAGTTTCTGGG
R8Y2Hrv	AATTGGATCCTCAGTCCGAATCCTCTTCGTAGTC
NΔ13G1fw	TTAACATATGGCCCGAACGAGGAGATTGAGAAC
NΔ13G1rv	TTAAAGATCTTCAAATCAAACCGCAGAGACGCAGG
NΔ13G2fw	TTAACATATGGCCTCCCGGTCGCGCGACGTAG
NΔ13G2rv	TTAAGGATCCTCACAGGATAAGTTGTTTCAGGTTTCGCTGG
NΔ12G3fw	TTAACATATGGAGCAGAAGAAGAGGAGCCAGAAGATC
NΔ12G3rv	TTAAGGATCCTCATAGAATACCGGAGTCTTTAAGGGCG
R8 NdeI fw	AATTCATATGGCCTCAATAGGAGTTTCTGGGC
R8 BamHI rv	AATTGGATCCTCAGTCCGAATCCTCTTCGTAGTCG