Identification and analysis of essential *Aspergillus nidulans* genes using the heterokaryon rescue technique

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In the heterokaryon rescue technique, gene deletions are carried out using the *pyrG* nutritional marker to replace the coding region of target genes via homologous recombination in *Aspergillus nidulans*. If an essential gene is deleted, the null allele is maintained in spontaneously generated heterokaryons that consist of two genetically distinct types of nuclei. One nuclear type has the essential gene deleted but has a functional *pyrG* allele (*pyrG*$^+$). The other has the wild-type allele of the essential gene but lacks a functional *pyrG* allele (*pyrG*$^-$). Thus, a simple growth test applied to the uninucleate asexual spores formed from primary transformants can identify deletions of genes that are non-essential from those that are essential and can only be propagated by heterokaryon rescue. The growth tests also enable the phenotype of the null allele to be defined. Diagnostic PCR can be used to confirm deletions at the molecular level. This technique is suitable for large-scale gene-deletion programs and can be completed within 3 weeks.

INTRODUCTION

One of the most fundamental pieces of information regarding the function of any gene is whether the gene is essential or not. Also of great importance are the defects associated with deletion of an essential gene. In model yeast systems, propagation of essential null alleles is accomplished by making the deletion in a diploid strain. Allowing the diploid to undergo meiosis to generate spores allows isolation of the null allele in a haploid state, and the phenotypes caused by the deletion can be defined. Although deletion of essential genes can be accomplished in diploids of the model filamentous fungus *Aspergillus nidulans*, vegetative diploids of this species are unable to complete the sexual cycle. Instead, the diploid can be broken down via the parasexual cycle[1] to generate haploid strains. If the null allele is not recovered in the haploids generated from the heterozygous diploid, this is taken as evidence that the deleted gene is essential. There are two problems with this approach. The first problem is that the conclusion that a gene is essential comes from negative data. The second problem is that, when using this approach, the phenotype of the null allele cannot be determined. To circumvent these problems, the heterokaryon rescue technique was developed[2]. This technique positively identifies essential genes and allows the phenotypic characterization of the null allele. It has previously been used extensively[3–6].

Overview of the heterokaryon rescue technique

To delete a gene, we typically use the *pyrG* gene from *Aspergillus fumigatus*[7], which encodes orotidine-5′-phosphate decarboxylase and complements the uridine and uracil auxotrophy that is caused by the *pyrG89* mutation. The *pyrG* gene is targeted to the locus to be deleted using a linear DNA-deletion cassette, which is generated using fusion PCR[8,9] consisting of *pyrG* flanked by targeting sequences. Following transformation into a *pyrG89* auxotrophic strain, homologous recombination occurs between the targeting domains and the genome. This replaces the target gene with *pyrG* and, at the same time, generates uridine and uracil prototrophy (see Fig. 2b in ref. 9). Transformants (which are *pyrG*$^+$) are selected for by growth on selective media lacking uridine and uracil. When a non-essential gene is deleted, the resulting strains are able to grow normally after streaking (**Fig. 1b**) on selective media (lacking uridine and uracil) or non-selective media (with uridine and uracil). However, if an essential gene is deleted, rather than causing death, the null allele will be rescued by spontaneous generation of a heterokaryon (see below). This phenomenom forms the basis of the heterokaryon rescue technique. To better understand the heterokaryon rescue technique, it is important to understand some basic biology of *A. nidulans* (**Fig. 2**).

Similar to most filamentous fungi, *A. nidulans* cells can maintain many nuclei in a common cytoplasm. Furthermore, *A. nidulans* has the capacity to maintain two genetically distinct types of nuclei in this common cytoplasm. Such strains are called heterokaryons. During the heterokaryon rescue technique, the heterokaryotic state is imposed when an essential gene is deleted because, during the deletion procedure, the deletion-DNA cassette is transformed into protoplasts that are multinucleate. It is possible to generate *pyrG*$^+$ *geneX*$^-$ nuclei within a background of *pyrG*$^-$ *geneX*$^+$ nuclei in a common cytoplasm (**Fig. 2a**). This heterokaryotic state is selected for only when *geneX* is essential. This is because when such protoplasts are regenerated on selective media, both types of nuclei are selected for. The *pyrG*$^+$ *geneX*$^-$ nuclei require *geneX* function and the *pyrG*$^-$ *geneX*$^+$ nuclei require *pyrG* function. Therefore, on *pyrG*$^+$ selective media, the *pyrG*$^+$ *geneX*$^-$ nuclei provide *pyrG* function and the *pyrG*$^-$ *geneX*$^+$ nuclei provide *geneX* function. In this way, heterokaryons are generated that undergo normal asexual development to form uninucleate spores (**Figs. 2a–c, 3a**).

Because asexual *A. nidulans* spores (conidia) contain a single nucleus, the heterokaryotic state is broken during sporulation (**Fig. 2c**). Some spores from the heterokaryotic colony have *pyrG*$^-$ *geneX*$^+$ nuclei, whereas others contain *pyrG*$^+$ *geneX*$^-$ nuclei. If the mixed spores from the heterokaryon are streaked on non-selective media (YAGUU), the *pyrG*$^-$ *geneX*$^+$ spores grow and divide normally but the *pyrG*$^+$ *geneX*$^-$ spores only grow to the extent possible without the essential *geneX* function (**Fig. 2e**). Therefore, spores streaked from a *pyrG*$^-$ *geneX*$^+$/*pyrG*$^+$ *geneX*$^-$ heterokaryon

on non-selective plates will seem to grow normally, although it is only the *pyrG*$^{-}$ *geneX*$^{+}$ spores that grow to form colonies (for example, **Fig. 3b**, YAGUU). When streaked on *pyrG*$^{+}$ selective media (YAG), the *pyrG*$^{-}$ *geneX*$^{+}$ spores cannot grow, whereas the *pyrG*$^{+}$ *geneX*$^{-}$ spores again grow to the extent possible without *geneX* function. Therefore, on selective YAG plates, the mixed spores that are streaked from a *pyrG*$^{-}$ *geneX*$^{+}$/*pyrG*$^{+}$ *geneX*$^{-}$ heterokaryon will not form colonies (five streaked colonies in **Fig. 3b**, YAG, for example). Note that on selective or non-selective media, the *pyrG*$^{+}$ *geneX*$^{-}$ spores will grow to the extent possible without the essential gene function, and it is possible to define the phenotypes caused by the deletion of essential genes (**Fig. 3d,e**). This can be carried out by processing the germinated spores from the *pyrG*$^{-}$ *geneX*$^{+}$/*pyrG*$^{+}$ *geneX*$^{-}$ heterokaryons to visualize subcellular components (for example, **Fig. 3e** shows defects in nuclear division that is revealed by DNA staining with DAPI).

The first gene deletion to be rescued in a heterokaryon[2] was *bimE*^{APC1}, which encodes an essential subunit of the anaphase-promoting complex/cyclosome[10]. The deletion of *bimE*^{APC1} was expected to cause an arrest of mitotic progression with condensed mitotic chromatin. Both phenotypes were confirmed using DAPI staining to visualize DNA of germinated spores from the rescued Δ*bimE*^{APC1} heterokaryon. It is also possible to use indirect immunoflourescence to image any protein for which a specific antibody is available. Finally, by completing the deletion in strains that have specific proteins tagged with GFP, or similar fluorescent tags, it is possible to determine the effects that a deletion has on specific cellular processes using live cell imaging[6,11].

Applications of heterokaryon rescue

The heterokaryon rescue technique should be applied whenever the function of an unstudied gene is to be determined. If the gene is non-essential, then a strain will be generated that has the null allele. This strain can be used for subsequent analysis and genetic manipulations. If the gene is essential, then this fact can be proven and the terminal phenotype of the null allele can be defined. As the essential null allele is rescued in a heterokaryon, the allele can be stored indefinitely for later analysis. In addition, because the deletion cassette DNA can be stored indefinitely, or remade when required, the deletion can be repeated in other strain backgrounds to further study the defects caused by the deletion. Finally, the heterokaryon rescue technique should be applicable to any filamentous fungus that can be transformed with DNA that will integrate at some frequency by homologous recombination. Another requirement is that the fungus must be able to undergo asexual spore formation and that the spores be uninucleate. Because the heterokaryotic state must be broken during spore formation for the heterokaryon rescue technique to work, fungi forming multi-nucleated spores are not suitable candidates for the heterokaryon rescue technique.

Jung *et al.*[12] developed a variation on heterokaryon rescue that allows determination of whether or not an allele of a gene created by *in vitro* mutagenesis is lethal. In this study, the γ-tubulin gene, which is essential, was deleted and the deletion was maintained in a heterokaryon. Protoplasts from the heterokaryon were transformed with a plasmid carrying a γ-tubulin allele produced by *in vitro* mutagenesis and a selectable marker that complemented a mutation carried by the nuclei with the γ-tubulin deletion. Nuclei with the wild-type γ-tubulin gene from the heterokaryon carried another mutation that prevented growth under selective conditions. If the mutant allele that was created *in vitro* was lethal, no viable homokaryotic transformants were obtained, whereas they were obtained if the allele was viable.

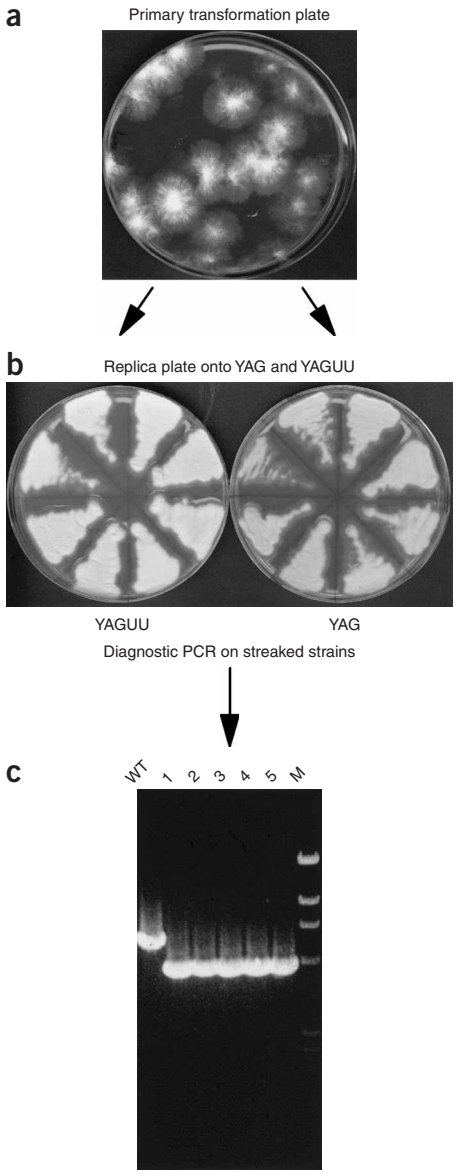

Figure 1 | Example of anticipated results when a non-essential gene is deleted. (**a**) Primary transformation plates grown for 3 d at 32 °C on selective YAG sucrose plates. (**b**) From the primary transformant colonies, spores were replica streaked onto YAGUU and YAG plates. Because, in this example, a non-essential gene has been deleted, the spores from eight primary transformation colonies grow equally well on selective YAG plates and non-selective YAGUU plates. (**c**) DNA was prepared from five of the deleted strains (after they had been streaked to a single colony three times on selective media) and subjected to diagnostic PCR, along with wild-type control DNA. The resulting PCR products were run on an agarose gel. The expected sized band is amplified from the wild-type (WT) control DNA. However, in the five deleted strains (1–5), the wild-type sized band is not amplified, but instead a smaller sized band of the predicted size is amplified. This demonstrates that the wild-type target gene has been replaced with the smaller *pyrG* gene. This result proves that the deleted allele does not cause lethality. M, Lambda DNA/HindIII Marker.

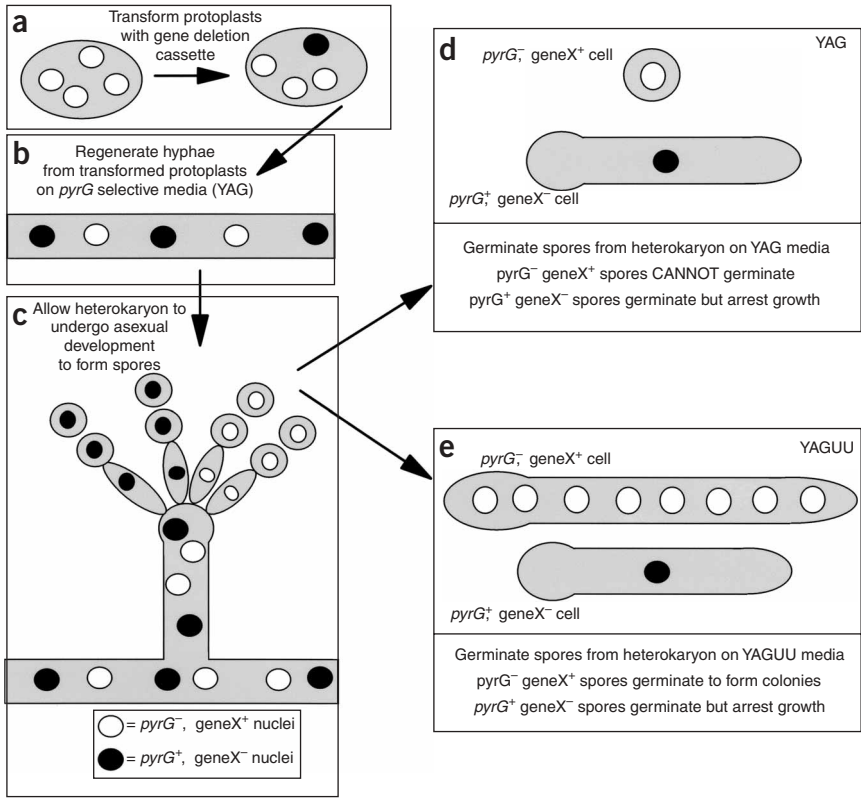

Figure 2 | Outline of heterokaryon rescue. (**a**) Protoplasts containing multiple nuclei are transformed with a deletion cassette to generate *pyrG*$^{+}$ *geneX*$^{-}$ nuclei in a common cytoplasm with parental *pyrG*$^{-}$ *geneX*$^{+}$ nuclei. (**b**) Protoplasts are regenerated on *pyrG*$^{+}$ selective YAG plates. (**c**) Colonies are allowed to undergo asexual spore formation to produce both *pyrG*$^{+}$ *geneX*$^{-}$ and *pyrG*$^{-}$ *geneX*$^{+}$ spores on aerial structures called conidiophores, the structure of which is idealized here for simplicity. (**d**,**e**) Spores from heterokaryon colonies are replica streaked onto *pyrG*$^{+}$ selective YAG (**d**) and *pyrG* non-selective YAGUU plates (**e**). On selective YAG plates (**d**), *pyrG*$^{-}$ *geneX*$^{+}$ spores cannot grow due to lack of *pyrG*. The *pyrG*$^{+}$ *geneX*$^{-}$ spores are able to grow as they have *pyrG* function but will arrest due to lack of *geneX* function. In the example represented, a cell-cycle gene has been deleted. The spores can germinate and undergo short-term growth but stop with a single, cell-cycle arrested, nucleus (compare the cell-cycle arrested cell in **Fig. 3e** with the normal cell in **Fig. 3f**). On non-selective YAGUU plates (**e**), *pyrG*$^{-}$ *geneX*$^{+}$ spores can grow and complete the cell cycle. With time, cells would grow beyond the eight nuclear stage shown and form normal sporulating colonies. The *pyrG*$^{+}$ *geneX*$^{-}$ spores are able to grow, but will arrest due to lack of *geneX* function and will have the same terminal phenotype as revealed on YAG media.

Alternatives to heterokaryon rescue

An alternative approach that can be used to study essential genes in *A. nidulans* is promoter replacement. In this approach, the promoter of the target gene is replaced with a regulatable promoter. By turning off expression from the regulatable promoter, it is possible to run down the target gene expression and hence study the effects of loss of gene function. In *A. nidulans*, this approach was first implemented using 3′ truncated plasmid constructs to place the expression of essential genes under the control of the inducible/repressible *alcA* promoter[13,14]. Fusion PCR could also be used to generate a promoter replacement cassette (see **Fig. 2c** in ref. 9). One advantage of the regulatable promoter run-down approach is that cells can be grown with the essential gene being expressed. The expression of the essential gene can then be turned off under defined conditions. This enables biomass to be generated for biochemical analysis. This cannot be done using the heterokaryon rescue approach. One drawback of the promoter replacement approach is that the *alcA* promoter is not switched off completely on repressing media[15]. Therefore, some genes cannot be down-regulated enough to cause loss of function[16].

Advantages and limitations of heterokaryon rescue

The heterokaryon rescue technique is extremely powerful and robust and provides direct evidence regarding whether a gene is essential or not. The terminal phenotype that is caused by deletion of essential genes can also be defined. However, there are two instances in which the utility of the approach is limited. On occasion, we have observed that although the null allele of an essential gene can be rescued in heterokaryons, the null allele is not propagated efficiently through to the asexual spores. For example, this was observed after deletion of *nimA*, which encodes a cell-cycle-regulated protein kinase that is degraded at each mitosis[17]. This limits the number of spores generated from the heterokaryon that carry the null allele, thus making phenotypic analysis difficult. In addition, if the null allele causes a tight growth defect, it can be challenging to distinguish spores carrying the null allele from those that do not germinate due to the *pyrG89* mutation[6]. We have not encountered any essential gene that does not form a heterokaryon when it is deleted (>20 genes). As a consequence, we do not yet know whether or not there are any types of essential genes that cannot be rescued in heterokaryons.

In the deletion protocol described, we have replaced the target genes with *A. fumigatus pyrG*, which complements the *pyrG89* mutation of *A. nidulans*. Any other nutritional marker could be used as long as the marker causes a tight growth defect on selective media. It is advisable to use an heterologous marker to prevent targeting to the marker site in *A. nidulans*[8] and to utilize strains with the Δ*nkuA* mutation (Δ*ku70*) to enhance the frequency of correct gene targeting[9,11]. In addition, for the transformation protocol described, asexual spores (called conidia) are germinated and the small germlings (called 'smoo' cells) that are generated are converted to protoplasts (**Fig. 4**) using cell-wall-degrading enzymes. We have found that white spores formed from *wA3*-containing strains are more susceptible to cell-wall removal and, therefore, we suggest using strains with this color marker for the protocol described here. (See ref. 9 for an alternative method using non-*wA3* strains.)

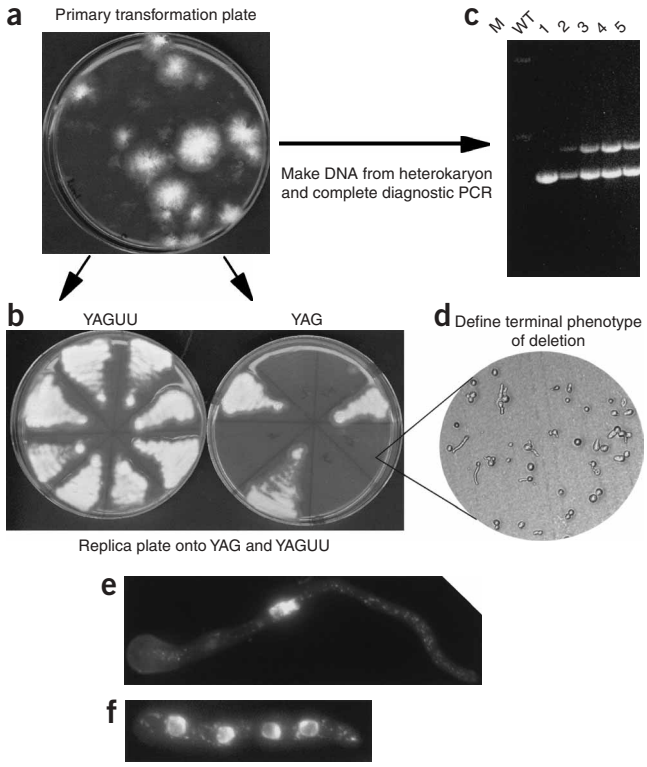

Figure 3 | Example of anticipated results when an essential gene is deleted. (**a**) Primary transformation plates grown for 3 d at 32 °C on selective YAG sucrose plates. Note that it is not possible to distinguish between the normal haploid transformants and heterokaryotic transformants. (**b**) From the primary transformants, spores are replica streaked onto YAGUU and YAG plates. During asexual spore formation, heterokaryons carrying both *pyrG*⁻ *geneX*⁺ parental nuclei and deleted *pyrG*⁺ *geneX*⁻ nuclei will produce uninucleate spores with either type of nuclei. When these mixed spores are inoculated onto non-selective YAGUU media, the *pyrG*⁻ *geneX*⁺ spores germinate and grow into colonies. The *pyrG*⁺ *geneX*⁻ spores will germinate but arrest growth due to lack of *geneX* function. However, the same mixed spores from the heterokaryon streaked on selective YAG media are unable to form colonies because the *pyrG*⁻ *geneX*⁺ spores cannot grow without UU and the *pyrG*⁺ *geneX*⁻ spores cannot form colonies without *geneX* function. Spores from 5 of the 8 transformant colonies tested in **b** have the growth characteristics of YAGUU (growth) and YAG (no growth), indicating that the deleted gene is essential. DNA is prepared from the putative heterokaryons and subjected to diagnostic PCR. In the example shown (**c**), the wild-type band (lower) is amplified from control DNA. In addition to the wild-type band, all four heterokaryons that were tested amplified a larger band of the size predicted following integration of the deletion cassette. The growth phenotype on YAG and YAGUU, in combination with the diagnostic PCR result producing both the wild-type and deleted allele bands, confirms that the deleted gene is essential. Note that it is possible to generate the same diagnostic PCR result from a heterozygous diploid. However, unlike the heterokaryon, the diploid will produce one type of nuclei that will grow and form colonies on both YAG and YAGUU plates. In Δ*ku70*-deleted strains, this is the likely reason why not all eight colonies tested have the growth characteristics of heterokaryons. In normal strains, ectopic integration of the deletion cassette will lead to many colonies that generate spores that are able to grow on both YAG and YAGUU plates. (**d**) A low-magnification image of the germinating spores growing on YAG plates is shown. The round cells that are unable to form a germ tube are the *pyrG*⁻ *geneX*⁺ spores, whereas the small germlings are the *pyrG*⁺ *geneX*⁻ cells. (**e**) An example of a DAPI-stained cell carrying a deletion of An-*cdc31* (ref. 6). A single large nucleus is present within the large germling, indicating the expected cell-cycle arrest without An-*cdc31* function. Compare with **f**, which is an An-*cdc31*⁺ cell that has grown for a shorter period of time but has already gone through two normal cell cycles to produce a germling with four nuclei.

MATERIALS

REAGENTS

- *A. nidulans* strain carrying the *wA3*, *pyrG89* and Δ*nkuA* (Δ*ku70*)[11] mutations; two suitable strains are SO451 and SO452, which are available from the Fungal Genetics Stock Center (http://www.fgsc.net/)
- Deletion cassette DNA, made using fusion PCR[8,9]
- Yeast extract (Bacto; Fisher Scientific, cat. no. 212750)
- Glucose (Dextrose, Fisher Scientific, cat. no. D16-3)
- Agar (Lab Scientific, cat. no. A466)
- Uracil (USB, cat. no. 23020)
- Uridine (Sigma, cat. no. U3750)
- $MgSO_4$ (Sigma, cat. no. M1880)
- NaCl (Fisher Scientific, cat. no. S271-10)
- Ammonium sulfate (Fisher Scientific, cat. no. A702-3)
- Citric acid (Fisher Scientific, cat. no. A940-500)
- Tween-80 (Fisher Scientific, cat. no. BP338-500)
- KOH (Fisher Scientific, cat. no. P250-1)
- Sucrose (sugar from grocery stores)
- Carbowax PEG 8000 (Fischer Scientific, cat. no. P156-500)
- Calcium chloride (Fisher Scientific, cat. no. BP510-500)
- Potassium chloride (Fisher Scientific, cat. no. P217-500)
- Tris Base (Sigma, cat. no. T6066)
- MOPS base (Sigma, cat. no. M1254)
- Bovine serum albumin (BSA; Sigma, cat. no. A7906)
- Driselase (Sigma, cat. no. D9515)
- Vinoflow FCE (cat. no. 3459923, Gusmer Enterprises, Inc.)
- Na azide (Sigma, cat. no. S2002) **! CAUTION** Highly toxic; do not ingest.
- EDTA (Fisher Scientific, cat. no. 02793-500)
- Na fluoride (Sigma, cat. no. S1504)
- Gel Slick Solution (Fisher Scientific, cat. no. BMA50640)
- YAGUU plates (see REAGENT SETUP)
- YGUU media (see REAGENT SETUP); this liquid media is used to germinate *pyrG89* strains during the transformation procedure
- YG media: as YGUU media without uracil or uridine — autoclaved; this liquid media is selective for *pyrG*⁺ and is used to grow strains with non-essential gene deletions and also heterokaryons carrying essential gene deletions
- YAGUU top agar: as YAGUU but with 7.5 g l^{-1} agar
- YAG plates: as YAGUU plates but without uridine or uracil
- YAG sucrose plates: as YAG but with 1 M sucrose
- YAG sucrose top agar: as YAG sucrose plates but with 7.5 g l^{-1} agar
- Tween solution: 0.2% Tween-80 in water; autoclaved
- Spore suspension solution (see REAGENT SETUP)
- Solution 1 (see REAGENT SETUP)
- Solution 2 (see REAGENT SETUP)
- Solution 3 (see REAGENT SETUP)
- Solution 4 (see REAGENT SETUP)
- Solution 5 (see REAGENT SETUP)
- Lytic solution for protoplast generation (see REAGENT SETUP)
- Stop buffer (see REAGENT SETUP)
- Mini Prep kit (Promega, cat. no. A7510)
- Expand Long Template PCR System (Roche, cat. no. 11681842001)

EQUIPMENT

- Test tubes (Fisher Scientific, cat. no. 14-958D)
- 250 ml flasks (Fisher Scientific, Pyrex cat. no. 4980)
- Filter unit (Fisher Scientific, cat. no. 09-740-21B)
- 150 ml beaker (Fisher Scientific, Pyrex cat. no. 1000)
- Mira cloth (Calbiochem, cat. no. 475855)
- Thermo IEC Centra CL2 bench-top centrifuge

- Standard and epifluorescence microscope
- Lyophilizer
- Suitable PCR machine; we have used an Applied Biosystems GeneAmp PCR system 9700 or an Eppendorf Mastercycler ep

REAGENT SETUP

Primer design for diagnostic PCR As described previously[8], it is critical to design primers for the diagnostic PCR that are anchored outside the limits of the flanking regions of the deletion cassette construct. Primers should be 18–21 bp in length and have a Tm of 60–70 °C and a GC content of 50–55%. For details regarding the design of primers to generate the deletion cassette using fusion PCR, refer to ref. 9.

Trace element solution 1.0 g l^{-1} $FeSO_4$•$7H_20$ (Sigma, cat. no. 7002), 8.8 g l^{-1} $ZnSO_4$•$7H_2O$ (Fisher Scientific, cat. no. Z68-500), 0.4 g l^{-1} $CuSO_4$• $4H_2O$ (Fisher Scientific, cat. no. C493-500), 0.15 g l^{-1} $MnSO_4$•$4H_2O$ (Fisher Scientific, cat. no. M113), 0.10 g l^{-1} $Na_2B_4O_7$•$10H_2O$ (Fisher Scientific, cat. no. S248-500), 0.05 g l^{-1} $(NH_4)_6MO_7O_{24}$• $4H_2O$ (Sigma, cat. no. A7302). Dissolve each in order and add 5 ml of chloroform.

YAGUU plates 5 g l^{-1} yeast extract, 10 g l^{-1} glucose, 15 g l^{-1} agar, 1.12 g l^{-1} uracil, 1.2 g l^{-1} uridine, 10 mM $MgSO_4$, 1 ml l^{-1} trace elements. Autoclave at 10 psi for 20 min.

YGUU media 5 g l^{-1} yeast extract, 10 g l^{-1} glucose, 1.12 g l^{-1} uracil, 1.2 g l^{-1} uridine, 10 mM $MgSO_4$, 1 ml l^{-1} trace elements. Autoclave at 10 psi for 20 min.

Spore suspension solution 0.85% sodium chloride, 0.02% Tween-80; autoclaved.

Solution 1 0.8 M ammonium sulfate, 100 mM citric acid, pH to 6 with KOH. Autoclave at 10 psi for 20 min and store at 4 °C.

Solution 2 10 g l^{-1} yeast extract, 10 g l^{-1} sucrose, 20 mM $MgSO_4$. Autoclave at 10 psi for 20 min and store at 4 °C.

Solution 3 0.4 M ammonium sulfate, 1% sucrose, 50 mM citric acid, pH to 6 with KOH. Autoclave at 10 psi for 15 min. Store at 4 °C.

Solution 4 25% Carbowax PEG 8000, 100 mM calcium chloride, 0.6 M potassium chloride, 10 mM Tris-Cl at pH 7.5. Heat to 100 °C to dissolve PEG. While still hot, filter-sterilize. Aliquot in 15 ml tubes. Due to the high viscosity of this solution, the filtration rate is slow. Store at –20 °C.

Solution 5 0.6 M potassium chloride, 50 mM calcium chloride, 10 mM MOPS. Make 50 ml at a time, pH to 6.0 with KOH. Filter-sterilize and store at 4 °C.

Lytic solution for protoplast generation 25 ml Solution 1, 25 ml Solution 2, 80 mg BSA, 400 mg Beta-D Glucanase, 50 mg Liticase, 250 mg Driselase. Add all ingredients and mix at room temperature for 10 min. Spin for 1 min in a bench-top centrifuge. Filter-sterilize the supernatant directly into a sterile flask. Suspend germlings in this mix for protoplasting. (Alternate protocol): The supplier, Interspecs, of the lytic enzymes recently terminated their business. We have had equally good results using 10 mg ml^{-1} Vinoflow FCE + 1 mg ml^{-1} Driselase from Gusmer Enterprises, Inc. and Sigma, respectively. (Although Vinoflow FCE will generate transformable protoplasts from germinated conidia, addition of Driselase improves the yield of protoplasts.)

Stop buffer 0.9% NaCl, 1 mM Na azide, 10 mM EDTA at pH 7.2, 50 mM Na fluoride. ! **CAUTION** Na azide is highly toxic and should not be ingested by any means!

EQUIPMENT SETUP

Siliconizing 250 ml flasks Add 5 ml of Gel Slick solution to flask and rotate to coat the entire internal surface of the flask. Pour solution out and allow flask to air-dry before using.

PROCEDURE

Generate conidial stock of *A. nidulans*

1| Inoculate 4 ml molten (47 °C) YAGUU top agar with ~1 × 10^7 conidia, vortex and pour onto a YAGUU plate. Inoculate six such plates. Once the top agar has solidified, incubate at 32 °C for 30 h. This method provides immature conidia, which germinate synchronously if used within 7 days of harvesting. The synchronous germination aids in the conversion of the germlings to protoplasts as ungerminated spores are resistant to protoplasting. It is necessary to use six plates to generate enough spores for the protoplast procedure as described.

2| Harvest spores from the first plate into 10 ml 0.2% Tween-80 by placing the solution onto the surface of the plate and scraping the entire plate surface with a bacterial cell spreader that has been kept wet in the 0.2% Tween-80. We typically use Pasteur pipettes that are bent to the shape of a spreader, using a bunsen burner to weaken the thin end of the pipette at two points before bending by gravity to the shape desired. Once the spores from a single plate are collected from the surface, the 0.2% Tween-80 spore solution can be poured from the first plate onto a second plate to harvest its spores. This step is then repeated for a third plate. The spore solution is transferred into a 15 ml centrifuge tube. The other three plates are harvested in a similar fashion using another 10 ml of 0.2% Tween-80.

3| Vigorously vortex each tube for 10 s and spin both tubes at top speed for 2 min in a bench-top centrifuge. Discard supernatant.

4| Remove the top white layer of spores, taking care not to disturb the darker material below the white layer of spores. This is done by repeated pipetting of 1 ml of spore suspension solution onto the top white layer to dislodge the spores, but leaving the darker layer. The dark layer contains conidiophores and agar debris, which can interfere with the protoplasting and transformations steps. Once the white spores are in suspension away from the darker debris, they are placed into fresh tubes. Bring the volume to 10 ml with the spore suspension solution, vortex and spin for 2 min at top speed in a bench-top centrifuge. Discard supernatant. Remove the white spore layer as before and repeat the wash step two more times by repeated re-suspension and centrifugation.

5| Quantify conidia using a hemocytometer. At least 1 × 10^9 are required, which should be free of conidiophores and agar debris. This can be monitored when counting the spores under the hemocytometer.

■ **PAUSE POINT** Spores can be kept for 7 d at 4 °C.

Generate protoplasts for transformation

6| Inoculate 1 × 10^9 fresh conidia from Step 5 above into 50 ml YGUU in a siliconized (use Gel Slick Solution) 250 ml Erlenmeyer flask. Incubate at 250 rpm and 32 °C for 4–6 h.

▲ CRITICAL STEP Use fresh conidia within 7 d of harvesting to ensure that germination is synchronous. This will aid conversion of all germinated spores to protoplasts. The use of a siliconized flask is important to prevent the germinating spores from binding to the surface of the flask.

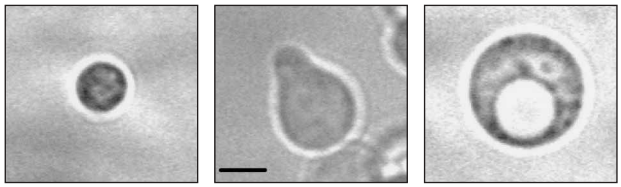

Figure 4 | Examples of the cell types used in the heterokaryon rescue protocol. The starting cells for the transformation protocol are asexual spores (called conidia), which are small, dormant uninucleated cells. During the transformation protocol, the conidia are germinated until they have swollen and started to send out a germ tube. Such cells are called 'smoo' cells. The cell walls of the smoo cells are removed by enzymatic digestion to generate protoplasts, which are round and contain a distinctive large vacuole that is clear (shown as a white circle within the example protoplast). Scale bar, 5 μm.

7| Monitor the germination of conidia under a microscope to determine when the majority of the spores have just started to send out a germtube ('Smoo' cells, typically 4.5–5.5 h). Compare the size and shape of conidia with germinated smoo cells, as in **Figure 4**. Harvest cells by centrifugation using a bench-top centrifuge at top speed for 2 min. Discard supernatant.

8| Resuspend in 50 ml of lytic solution. Incubate at 32 °C and 200 rpm in a siliconized 250 ml Erlenmeyer flask. At 30 min intervals, vigorously pipette solution in and out of a 10 ml pipette 10–15 times. This procedure aids the release of protoplasts. There is a common misconception that protoplasts are highly susceptible to mechanical stress. However, protoplasts that are formed in the buffer system used are remarkably resistant to mechanical stress.

9| Monitor for protoplast conversion under the microscope. Protoplasts are readily identified as, unlike germinated spores, they are round and contain a large visible vacuole under phase-contrast microscopy (**Fig. 4**).

10| When conversion is complete (typically within 2–3 h), collect protoplasts by centrifugation in a bench-top centrifuge at top speed for 2 min. Discard supernatant.
▲ CRITICAL STEP From this point on, keep the protoplasts at 4 °C.

11| Resuspend protoplasts in ice-cold Solution 3. This is done by repeated pipetting up and down with 1 ml Solution 3 using a Gilson Pipetman. As mentioned in Step 8, protoplasts are remarkably resistant to mechanical stress and can withstand one cycle of pipetting per second. Once resuspended, the volume is made up to 50 ml using Solution 3. Centrifuge at top speed for 2 min. Discard supernatant.

12| Repeat Step 11 and then resuspend protoplasts in 1 ml of Solution 5. Store on ice.
■ PAUSE POINT Protoplasts can be kept on ice in a cold room for 2 d before transformation with no loss in the number of transformants obtained.

Transform deletion cassette into protoplasts

13| Place ~1–4 ug of deletion cassette DNA into a microfuge tube in less than 10 μl H_2O.
▲ CRITICAL STEP It is important to include a negative 'no DNA' control transformation and a positive control transformation using a plasmid containing the selection marker, which in the example followed here would be an *A. nidulans pyrG*-containing plasmid.

14| Add 100 μl of protoplasts and mix by pipetting on ice.

15| Add 50 μl of room temperature Solution 4 to the DNA/protoplast blend and mix using pipette.

16| Leave on ice for 20 min.

17| Add 1 ml of room temperature Solution 4 and incubate at room temperature for 20 min. The samples are now ready for plating.

18| Plate 10 μl, 50 ul and 100 μl of the transformation mix on single plates. The remaining volume should be split equally between two plates. Plate by pipetting the transformation mix into 4 ml YAG + 1 M sucrose top agar at 47 °C, vortex briefly and overlay on YAG sucrose plates. The sucrose provides osmotic stabilization. Once solidified, incubate plates at 32 °C for 3 d to allow transformed colonies to grow and form conidia (**Figs. 1a, 3a**).
▲ CRITICAL STEP After plating, no colonies should grow on the 'no DNA' control plates but many colonies (>50 colonies per μg DNA) should grow on the positive transformation control plates.

Test transformants for heterokaryon formation

19| From the primary transformation plates (Step 18), carefully remove conidia from the surface of eight colonies using a flamed loop that has been immersed in 0.2% Tween-80. Avoid conidiophores as much as possible, as these structures, which

are multinucleated, can allow re-growth of the heterokaryon. Replica streak on YAG and YAGUU plates and incubate at 32 °C (**Figs. 1b, 3b**).

20| After 24 h, observe plates and score for growth on YAGUU and YAG. If the deleted gene is non-essential, then growth will occur on both the YAG and YAGUU plates (**Fig. 1b**). If the deleted gene is essential, then the null allele will be rescued by formation of a heterokaryon containing *pyrG*$^-$ *geneX*$^+$ nuclei and *pyrG*$^+$ *geneX*$^-$ nuclei in a common cytoplasm. These nuclei are segregated into individual conidia during asexual spore formation, forming a mixture of *pyrG*$^-$ *geneX*$^+$ and *pyrG*$^+$ *geneX*$^-$ conidia on the surface of heterokaryon colonies. When this mixture is streaked on the YAG (*pyrG*$^+$ selective) plates, the *pyrG*$^-$ *geneX*$^+$ conidia cannot grow because they are *pyrG*$^-$ and the *pyrG*$^+$ *geneX*$^-$ conidia cannot form colonies because they lack essential *geneX* function. Thus, no colonies are formed on the YAG streaked plates if the original colony is a heterokaryon in which a null allele of an essential gene is rescued (**Fig. 3b**, YAG). When streaked on the YAGUU non-selective plates, the *pyrG*$^+$ *geneX*$^-$ conidia still cannot grow due to lack of *geneX* function. However, the *pyrG*$^-$ *geneX*$^+$ conidia can grow because the *pyrG*$^-$ marker is complemented by the uridine and uracil in the YAGUU media and these conidia have *geneX* function. The *pyrG*$^-$ *geneX*$^+$ conidia therefore germinate and grow to form colonies on the YAGUU media (**Fig. 3b**, YAGUU).

21| After 48 h, score again for growth on YAGUU and YAG. If the deleted gene is non-essential, spores from all primary transformants will form colonies equally well on both YAG and YAGUU plates (**Fig. 1b**). If the deleted gene is essential, spores from more than half of the primary transformants will not form colonies on YAG but will do so on YAGUU (**Fig. 3b**). This is because, in some transformants, the essential null allele is rescued in heterokaryons, as described in Step 19. It is possible to also generate heterozygous *pyrG*$^-$ *geneX*$^+$/*pyrG*$^+$ *geneX*$^-$ diploids during the transformation and these will grow equally well on YAG and YAGUU. This is the reason that, in Δ*ku70* strains, not all colonies that have an essential gene deleted become rescued in heterokaryons (3 of 8 in the example shown in **Fig. 3b**).

▲ **CRITICAL STEP** As just mentioned, null alleles of essential genes can propagate as diploids as well as heterokaryons. Unlike heterokaryons, spores from diploids will grow and form colonies equally well on YAG and YAGUU plates. It is therefore important to confirm that the heterokaryotic state is maintained using the YAG–YAGUU replica plate test when heterokaryons are propagated. Heterokaryons can be maintained and stored as described in **Box 1**.

22| Streak strains with non-essential gene deletions three times to single colony. This is done by carefully removing conidia from the surface of a colony from the primary transformation plate using a flamed loop that has been immersed in 0.2% Tween-80. Streak these conidia from side to side, from the top to the bottom, of a YAG plate. Incubate at 32 °C for 2–3 d to allow well-separated sporulating colonies to form. Streak conidia from a well-separated colony to a single colony again. Repeat one more time. Streaking to a single colony three times will ensure that the resulting strain is clonal. Prepare DNA and carry out diagnostic PCR, as described in Steps 24–31 below.

23| Confirm heterokaryon status by preparing DNA and carrying out diagnostic PCR, as described in Steps 24–31 below. Phenotypic analysis can be carried out as described in Steps 32–34 below.

Growth of strains with non-essential deletions and heterokaryons for DNA preparation

24| To grow strains containing deletions of non-essential genes, follow option A. To grow heterokaryons carrying deletions of essential genes, follow option B.

(A) Strains with deletions in non-essential genes

(i) After streaking three times to a single colony (Step 22), inoculate a loop-full of conidia into 30 ml of YG in a Petri dish. Grow overnight until mycelia fill the plate but are not significantly conidiating.
(ii) Harvest mycelia by filtration through Miracloth.
(iii) Wash briefly with Stop buffer and immediately freeze in liquid nitrogen.
(iv) Lyophilize samples until totally dry, usually overnight.

(B) Heterokaryons with deletions of essential genes

(i) To propagate heterokaryons, it is important to transfer mycelia from the primary transformation plates onto YAG plates. Remove a small portion (~1 mm^2 is sufficient) of the growing edge of a colony using flamed, sterile, curved forceps. Place onto the center of a YAG plate and incubate at 32 °C for 3–4 d.
Cut around the growing edge of the resulting colony (about 2 cm long), being careful to take just the top growing surface 5 mm from the leading edge.
(ii) Using tweezers, cut the growth into ~50 pieces and inoculate into 30 ml of YG *pyrG*$^+$ selective media in a Petri dish. Grow overnight until mycelia fill the plate but are not significantly conidiating.
(iii) Harvest mycelia by filtration through Miracloth.
(iv) Wash briefly with Stop buffer and immediately freeze in liquid nitrogen.
(v) Lyophilize samples until totally dry, usually overnight.

Extracting DNA for PCR analysis

25| Grind dry mycelium from Step 24 using a sterile tooth pick and move ~20–30 mg of ground mycelium to a microfuge tube.

26| Add 200 µl of cell lysis solution from the Promega Mini Prep kit and mix for 5 s using a vortex that is at a high setting.

27| After 5–10 min, add an equal volume of Neutralizing Solution from the kit and mix for 5 s using a vortex that is at a high setting.

28| Centrifuge for 10 min in a microcentrifuge. Transfer the supernatant to purification columns, and proceed as described in the DNA purification protocol of the kit. After elution from the column, the final volume of DNA is 50 µl. This protocol removes contaminants from the sample and rapidly provides enough DNA for at least 10 PCR amplifications.

Diagnostic PCR

29| Set up a PCR reaction for each transformant using the Expand Long Template PCR System, as follows:

Component	Amount
DNA from Step 28	5 ul
10 uM Primer A	0.75 ul
10 uM Primer B	0.75 ul
10 mM dNTPs	1.25 ul
10 x Buffer 3	2.5 ul
Polymerase mix	0.375 ul
d.H_2O	To 25 ul

▲ **CRITICAL STEP** It is important to include a wild-type control DNA sample to confirm that both the DNA purification and the PCR reactions are working. This reaction will also provide a control wild-type sized PCR product for comparison with the size of the deleted allele PCR product (**Figs. 1c, 3c**).

30| Amplify the template using the following PCR program:

Cycle number	Denature	Anneal	Extend
1	94 °C, 2 min		
2–11	94 °C, 10 sec	60 °C*, 30 s	68 °C, X** min
12–26	94 °C, 10 sec	60 °C*, 30 s	68 °C, X** min + 20 s each cycle
27			68 °C, 8 min
28			4 °C

*Check your primers for the best annealing conditions. **Extension times are: 2 min for up to 3 kb, 4 min for 6 kb, 8 min for 10 kb and 15 min for 20 kb.

31| Run 2–5 µl PCR product on 0.8% agarose gel in TAE buffer. After deletion of a non-essential gene, only the band corresponding to the deleted allele is amplified and not the wild-type sized allele (**Fig. 1c**). **Figure 3c** provides an example of expected data from rescue heterokaryons, with bands corresponding to both wild-type and deleted alleles that have been amplified from the heterokaryotic DNA.

Phenotypic analysis of null alleles rescued in heterokaryons

32| Streak conidia from putative heterokaryons grown as described in **Box 1**, Propagation of heterokaryons, onto YAG media and incubate at 22 °C.

BOX 1 | PROPAGATION AND STORAGE OF HETEROKARYONS

Propagation of heterokaryons

To propagate heterokaryons, it is important to transfer mycelia from the primary transformation plates onto YAG plates. Remove a small portion (~1 mm^2 is sufficient) of the growing edge of a colony using flamed, sterile, curved forceps. Place onto the center of a YAG plate and incubate at 32 °C for several days. To confirm that the heterokaryon has been maintained, replica plate on YAG and YAGUU from several places around the edge of the subcloned colony.

Storage of heterokaryons

To maintain heterokaryons, it is necessary to store mycelial plugs. Remove sections (~5 mm × 5 mm) of the leading edge of heterokaryotic colony, including the agar in which the colony is growing. Place into a sterile microfuge tube. Place tube into a –80 °C freezer. Under these conditions, heterokaryons are stable for at least 1 year, and probably longer. To re-propagate, remove tube from the freezer, thaw between fingers and place plug onto the center of a YAG plate and incubate at 32 °C. Confirm heterokaryon by replica streaking spores on YAG and YAGUU.

33| Record growth at 24 h and at 48 h. Because *pyrG*$^{-}$ *geneX*$^{+}$ conidia are unable to elaborate a germ tube, whereas *pyrG*$^{+}$ *geneX*$^{-}$ conidia are often able to send out a germ tube, conidia with the null allele are typically easy to distinguish (**Fig. 3d**, for example).

34| Record the degree of growth defects and any effect on germination by photography. Further characterization using DAPI staining (**Fig. 3e,f**, for example) and immunofluorescence is possible using standard protocols[18]. In addition, by completing the deletion in appropriately GFP-tagged strains, the effects of deletions on any cell-biological process can be defined using live cell imaging[6,8].

● TIMING

Steps 1–5: 1 h preparation time and 2 d incubation.
Steps 6–12: 1 d.
Steps 13–18: 1–5 h, then 3 d incubation.
Steps 19–21: 1–2 h, then 2 d incubation.
Step 22: 6 d.
Step 24: 1–3 h, then 2 d incubation.
Steps 25–31: 2 d.
Steps 32–34: 2 d.
Propagation and storage of Heterokaryons (**Box 1**): 1–2 h.

? TROUBLESHOOTING

Problem: No transformants.
Solution: If the positive control DNA yields no transformants, this indicates that the control DNA used is defective or that the protoplasts or the plates used on which to regenerate them are defective. It is advisable to generate a large stock of control DNA, stored in aliquots, to always use as the positive control. This removes one variable. If the quality of the control DNA is known to be OK but no transformants are obtained, it is advisable to re-make all solutions and plates, making sure to avoid contamination with detergents. In addition, remake the conidial stock, taking care to remove agar debris and conidiophores, as described in Step 4. Use strain SO451 as the recipient strain and make sure that a homologous plasmid control gene is used when utilizing Δ*ku70* strains such that there is some homologous DNA to target integration into the genome. If the control DNA yields transformants but the deletion cassette does not, then the cassette should be remade, ensuring that the construct has been designed correctly.

If the strain being transformed does not have the *wA3* spore color mutation and the transformation frequencies are low, use the protocol given by Szewczyk *et al.*[9] to generate protoplasts from hyphae.

ANTICIPATED RESULTS

As an example of anticipated results, we have recently carried out deletion analysis of 30 nuclear transport genes using the heterokaryon rescue technique[6]. Each targeted gene was successfully deleted. Of the 30 deleted genes, 12 were shown to be non-essential and 18 to be essential. In addition, the effects of the essential deletions on nuclear structure and number were defined using DAPI staining of germinated spores containing the null allele. **Figures 1** and **3** provide examples of expected data at key points in the heterokaryon rescue protocol.

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1. Pontecorvo, G., Roper, J.A., Hemmons, L.M., MacDonald, K.D. & Bufton, A.W. *Advances in Genetics* (ed. Demerec, M.) 141–238 (Academic Press, New York, 1953).
2. Osmani, S.A., Engle, D.B., Doonan, J.H. & Morris, N.R. Spindle formation and chromatin condensation in cells blocked at interphase by mutation of a negative cell cycle control gene. *Cell* **52**, 241–251 (1988).
3. O'Donnell, K.L., Osmani, A.H., Osmani, S.A. & Morris, N.R. bimA encodes a member of the tetratricopeptide repeat family of proteins and is required for the completion of mitosis in *Aspergillus nidulans*. *J. Cell Sci.* **99**, 711–719 (1991).
4. Martin, M.A., Osmani, S.A. & Oakley, B.R. The role of gamma-tubulin in mitotic spindle formation and cell cycle progression in *Aspergillus nidulans*. *J. Cell Sci.* **110**, 623–633 (1997).
5. Prigozhina, N.L. *et al.* Gamma-tubulin plays an essential role in the coordination of mitotic events. *Mol. Biol. Cell* **15**, 1374–1386 (2004).
6. Osmani, A.H., Davies, J., Liu, H.L., Nile, A. & Osmani, S.A. Systematic deletion and mitotic localization of the nuclear pore complex proteins of *Aspergillus nidulans*. *Mol. Biol. Cell* **17**, 4946–4961 (2006).
7. Chaveroche, M.K., Ghigo, J.M. & d'Enfert, C. A rapid method for efficient gene replacement in the filamentous fungus *Aspergillus nidulans*. *Nucleic Acids Res.* **28**, E97 (2000).
8. Yang, L. *et al.* Rapid production of gene replacement constructs and generation of a green fluorescent protein-tagged centromeric marker in *Aspergillus nidulans*. *Eukaryot. Cell* **3**, 1359–1362 (2004).

9. Szewczyk, E. *et al.* Fusion PCR and gene targeting in *Aspergillus nidulans*. *Nature Protocols* DOI: 10.1038/nprot.2006.405 (2006).
10. Peters, J., King, R.W., Hoog, C. & Kirschner, M.W. Identification of BIME as a subunit of the anaphase-promoting complex. *Science* **274**, 1199–1201 (1996).
11. Nayak, T. *et al.* A versatile and efficient gene targeting system for *Aspergillus nidulans*. *Genetics* **172**, 1557–1566 (2006).
12. Jung, M.K., Prigozhina, N., Oakley, C.E., Nogales, E. & Oakley, B.R. Alanine-scanning mutagenesis of *Aspergillus* gamma-tubulin yields diverse and novel phenotypes. *Mol. Biol. Cell* **12**, 2119–2136 (2001).
13. Lu, K.P., Rasmussen, C.D., May, G.S. & Means, A.R. Cooperative regulation of cell proliferation by calcium and calmodulin in *Aspergillus nidulans*. *Mol. Endocrinol.* **6**, 365–374 (1992).
14. McGoldrick, C.A., Gruver, C. & May, G.S. myoA of *Aspergillus nidulans* encodes an essential myosin I required for secretion and polarized growth. *J. Cell Biol.* **128**, 577–587 (1995).
15. Som, T. & Kolaparthi, V. Developmental decisions in *Aspergillus nidulans* are modulated by ras activity. *Mol. Cell. Biol.* **14**, 5333–5348 (1994).
16. Bussink, H.J. & Osmani, S.A. A cyclin-dependent kinase family member (PHOA) is required to link developmental fate to environmental conditions in *Aspergillus nidulans*. *EMBO J.* **17**, 3990–4003 (1998).
17. Ye, X.S., Fincher, R.R., Tang, A., Osmani, A.H. & Osmani, S.A. Regulation of the anaphase-promoting complex/cyclosome by $BIMA^{APC3}$ and proteolysis of NIMA. *Mol. Biol. Cell* **9**, 3019–3030 (1998).
18. Oakley, B.R. & Osmani, S.A. in *The Cell Cycle, a Practical Approach* (eds. Fantes, P. & Brooks, R.) 127–142 (Oxford University Press, New York, 1993).

Erratum: Identification and analysis of essential *Aspergillus nidulans* genes using the heterokaryon rescue technique

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In the version of this article initially published, the black ball in **Figure 2c** was incorrectly described as representing a *pyrG*$^{-}$, geneX^{+} nuclei. This ball represents *pyrG*$^{+}$, geneX^{-}. The error has been corrected in all versions of the article.