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Article *in* Nature Reviews Genetics · October 2002

DOI: 10.1038/nrg889 · Source: PubMed

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THE ART AND DESIGN OF GENETIC SCREENS: FILAMENTOUS FUNGI

Lorna Casselton and Miriam Zolan‡*

In the 1940s, screens for metabolic mutants of the filamentous fungus *Neurospora crassa* established the fundamental, one-to-one relationship between a gene and a specific protein, and also established fungi as important genetic organisms. Today, a wide range of filamentous species, which represents a billion years of evolutionary divergence, is used for experimental studies. The developmental complexity of these fungi sets them apart from unicellular yeasts, and allows the development of new screens that enable us to address biological questions that are relevant to all eukaryotes.

CONDITIONAL LETHAL
A mutation that inhibits growth under some conditions, such as high or low temperature or in the absence of a specific growth supplement, but allows growth under other conditions.

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doi:10.1038/nrg889

The description of metabolic mutants in the filamentous fungus *Neurospora crassa* in 1941 by George Beadle and Edward Tatum[1,2] changed the face of genetics. Here was an organism that could be grown on a simple defined medium and that, when mutagenized, produced mutants that had lost the ability to make or use a specific metabolite. These experiments showed that the function of genes was not as complex as was previously thought: a clear relationship could be seen between the activity of a single gene and a single enzyme. In the following years, mutants were exploited to determine individual steps in metabolic pathways, and the numerous genes identified in this way provided a framework for constructing genetic maps. The simple principle of developing CONDITIONAL-LETHAL screens on the basis of metabolic requirements made other fungi accessible to genetic analysis and also paved the way for genetic studies with bacteria.

Many filamentous fungi are scavengers, and subsequent studies, particularly in the 1970s, turned to exploring the general features of metabolic regulation. These studies led to the discovery of interconnected networks of genes that enable the fungus to use different substrate resources to their best advantage and to synthesize enzymes only when needed. They also contributed to a general understanding of gene-regulatory mechanisms, specifically to defining the gene promoter and the proteins that bind to it. Significant as these studies continue to be, the more rapid development of molecular techniques for the budding yeast *Saccharomyces cerevisiae* has made this the model of choice for research in this area. However, unlike yeasts, filamentous fungi show an astonishing array of morphological complexity in structures that are specialized for vegetative growth, reproduction and infection (FIG. 1), and so they lend themselves to the examination of many questions that are relevant to eukaryotic cell biology. Davis and Perkins[3] have recently described the significant contributions made to eukaryotic genetics by studies with *N. crassa* over more than a century; these include studies of recombination, circadian rhythm, gene silencing and DNA methylation. Other filamentous species have proved particularly good models for studying the genetics of multicellular growth, such as nuclear movement and cellular differentiation (a list of some model fungal species is given in TABLE 1). Biological complexity lends itself to the development of some exciting genetic screens. We review several of the biological processes listed above and describe the screens that have been devised in filamentous fungi to uncover the genetic mechanisms that underlie them.

Life cycles of filamentous fungi

The filamentous species that are described in this article belong to two major fungal groups, the ascomycetes and the basidiomycetes. Published estimates of the time of divergence of these two groups range from 390 million years (REF. 4) to about 1.2 billion years (REF. 5) ago. So, it is

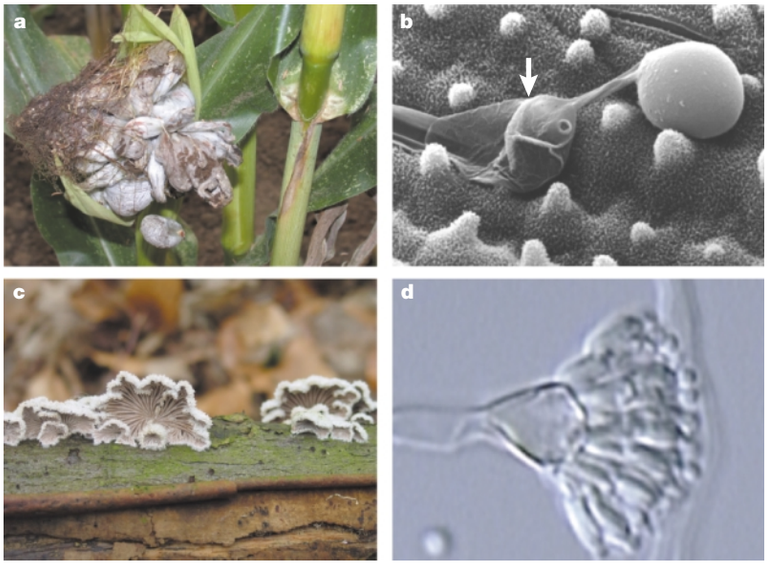


Figure 1 | **Morphological variation of filamentous fungi. a** | *Ustilago maydis* infection of a corn cob (courtesy of Regine Kahmann and Jörg Kämper, Max-Planck Institute for Terrestrial Microbiology, Marburg). **b** | Scanning electron micrograph of a *Magnoporthe grisea* appressorium (arrow), a cell used by the fungus to infect its host (courtesy of Nick Talbot, University of Exeter) (diameter of the appressorium, 8.0 μm). **c** | Fruiting body of *Schizophyllum commune* (courtesy of Luis Lugones and Han Wosten, University of Utrecht). The fruiting body is the structure that produces the sexual spores. **d** | Conidiophore of *Aspergillus nidulans* (courtesy of Michelle Momony, University of Georgia). The conidiophore is the specialized structure on which asexual spores develop.

not surprising that studies of diverse fungi reveal both conserved and divergent mechanisms in cell biology and development. One feature that distinguishes these groups is the cell in which karyogamy (nuclear fusion) and meiosis take place during sexual reproduction. In ascomycetes, which include the important models *N. crassa*[6] and *Aspergillus nidulans*[7,8], this cell is known as an ascus, and the sexual spores, or ascospores, are formed in it (BOX 1). The asci are encased in a larger structure, which then bursts to release many thousands of ascospores. The life cycle of the basidiomycetes, which include the mushroom fungi, is illustrated in BOX 2 by *Coprinus cinereus*. This group is characterized by large, distinctive fruiting bodies that produce many thousands of sexual spores, called basidiospores. Unlike in ascomycetes, the spores that contain the four meiotic products are suspended individually on the outside of the cells (basidia) that produce them.

Ascomycete and basidiomycete species that have both sexual and asexual cycles make the best model systems. The sexual cycle is essential for classical genetic analysis and gene mapping. Particularly important in this respect is the fact that the products of a single meiosis can be recovered together. In *N. crassa* and *Sordaria* spp., the asci are long, thin cells that restrict the orientation of spindles during successive meiotic and mitotic divisions to give predictable spore orders (BOX 3). The analysis of spores from a single ascus (a method known as tetrad analysis) can provide valuable information on centromere position and gene order. This genetic tool allowed classical screens for GENE CONVERSION to be carried out, on which some of the first molecular models for genetic recombination were based[8–10]. The resolving power of meiosis is immense; a single fruiting body of *A. nidulans* can contain 10^4 asci, and the mushroom of *C. cinereus* has some 10^7 basidia, with each separate meiotic event deriving from a single nuclear pair. By contrast, the asexual cycle provides a wonderful source of identical haploid cells, which can be used for mutagenesis and for DNA-mediated transformation. Heterokaryons, dikaryons and somatic diploids all provide ways of making genes heterozygous for complementation and epistasis tests. The life cycles of fungi are short and completed in the laboratory within two to three weeks. BOX 3 summarizes these and many other advantageous features of filamentous fungi as model genetic organisms.

Mating types. Sexual crosses between genetically different strains are facilitated by the fact that most ascomycete and basidiomycete fungi are self-sterile (heterothallic). Strains of heterothallic ascomycetes exist in two mating types; these are distinguished as *MatA* and *Mata* in *N. crassa* and as *mat*$^+$ and *mat*$^-$ in *Podospora anserina*, for example. The genes that determine mating type reside at a single locus so that, in any one cross, mating type segregates in a 1:1 ratio. By contrast, heterothallic basidiomycete species can have several mating types; *C. cinereus*, for example, has more than 12,000. The mating type genes of these fungi are multiallelic and, for crosses to be fertile, strains must have different alleles of the genes. The mating type genes can reside at a single locus, or be separated at two unlinked loci (*A* and *B* in mushroom species, *b* and *a* in *Ustilago maydis*)[11]. Mutations in the basidiomycete mating-type genes have proved particularly useful in that they can lead to constitutive activation of sexual development and bypass the need to mate. As we shall describe later, such mutations can be exploited to develop screens for recessive mutations that affect sexual development and meiosis.

Traditional screening approaches

Classical genetic screens. The traditional techniques of fungal genetics rely on screens for mutant phenotypes among survivors of radiation or chemical mutagenesis. Beadle and Tatum used X-irradiation in their classic experiments with *N. crassa*, but ultraviolet (UV) irradiation is probably the most commonly used mutagen today. For metabolic mutants, conditional-lethal screens can be adapted to isolate mutants in which a metabolic pathway of interest has been disrupted (reviewed in REF. 12). Survivors of the mutagenesis treatment are recovered on a medium that is designed to support the growth of the desired mutants, followed by tests on media that lack, or force the use of, the metabolite of interest. For model species that produce asexual spores, the screening procedure can be optimized by a filtration-enrichment procedure[13]. This involves growing spores in a liquid medium: non-mutants germinate into a mycelial mass that is removed by several filtration

GENE CONVERSION
A non-reciprocal recombination process that results in an alteration of the sequence of a gene to that of its homologue.

Table 1 | **Model fungal species**

Species name	Interest to geneticists	Genome project	URL
Ascomycetes			
Neurospora crassa	Used by Beadle and Tatum[1] to show the relationship between genes and enzymes. *N. crassa* continues to be the most important model for the genetic analysis of filamentous fungi	Sequence completed (but unpublished) and freely available	http://www.genome.wi.mit.edu/annotation/fungi/neurospora/
Sordaria spp.	Historically favoured for tetrad analysis and studies on gene conversion because of its linear asci and ascospore colour mutants. Also important for studies on mating-type genes, sexual development and meiosis	No sequencing programmes at present. However, most genome information can be deduced from that of *N. crassa*, as *Sordaria* spp. and *N. crassa* are closely related	
Podospora anserina	Introduced by Rizet in the 1940s[114] to study genes that prevent heterokaryosis. It is also a model for studying mating type, sexual development and senescence	No sequencing programmes at present	
Ascobolus immersus	Used for studies on gene conversion and silencing of genes by methylation	No sequencing programmes at present	
Aspergillus nidulans	The alternative model to *N. crassa* as a species for wide-ranging genetic studies. Introduced by Guido Pontecorvo in the 1950s[115]. Closely related species are the asexual *A. fumigatus* (human pathogen), *A. niger* (used for industrial production; for example, citric acid), *Penicillium chrysogenum* (producer of penicillin) and *Fusarium* spp. (plant pathogens)	Completely sequenced by Cereon Genomics. Access available to the academic community on request. Plans for a new sequencing programme so that data can be made freely accessible. Sequencing of other species, such as *A. fumigatus*, is planned	http://microbial.cereon.com/
Magnaporthe grisea (rice blast fungus)	Pathogen of rice and a model system for studying the genetics of pathogenicity	Sequence completed (but unpublished) and freely available	http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/ http://www.riceblast.org http://plpa2linux.tamu.edu/
Basidiomycetes			
Coprinus cinereus	Model species for basidiomycete genetics, particularly important for studies on mating-type determination, sexual development and meiosis	Sequencing planned	http://www-genome.wi.mit.edu/seq/fgi/
Schizophyllum commune	Another model for studying mating-type determination	No sequencing programmes at present	
Cryptococcus neoformans	Opportunist pathogen of humans that causes meningitis in immunocompromised hosts	Available sequence is freely accessible	http://www.tigr.org/tdb/e2k1/cna1/new.shtml
Ustilago maydis (corn smut)	Pathogen of maize and genetic model for studying pathogenicity	Complete sequence obtained by LION Bioscience and Bayer, but with limited access. Now being sequenced, so that data can be made freely available	http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map00?taxid=5270

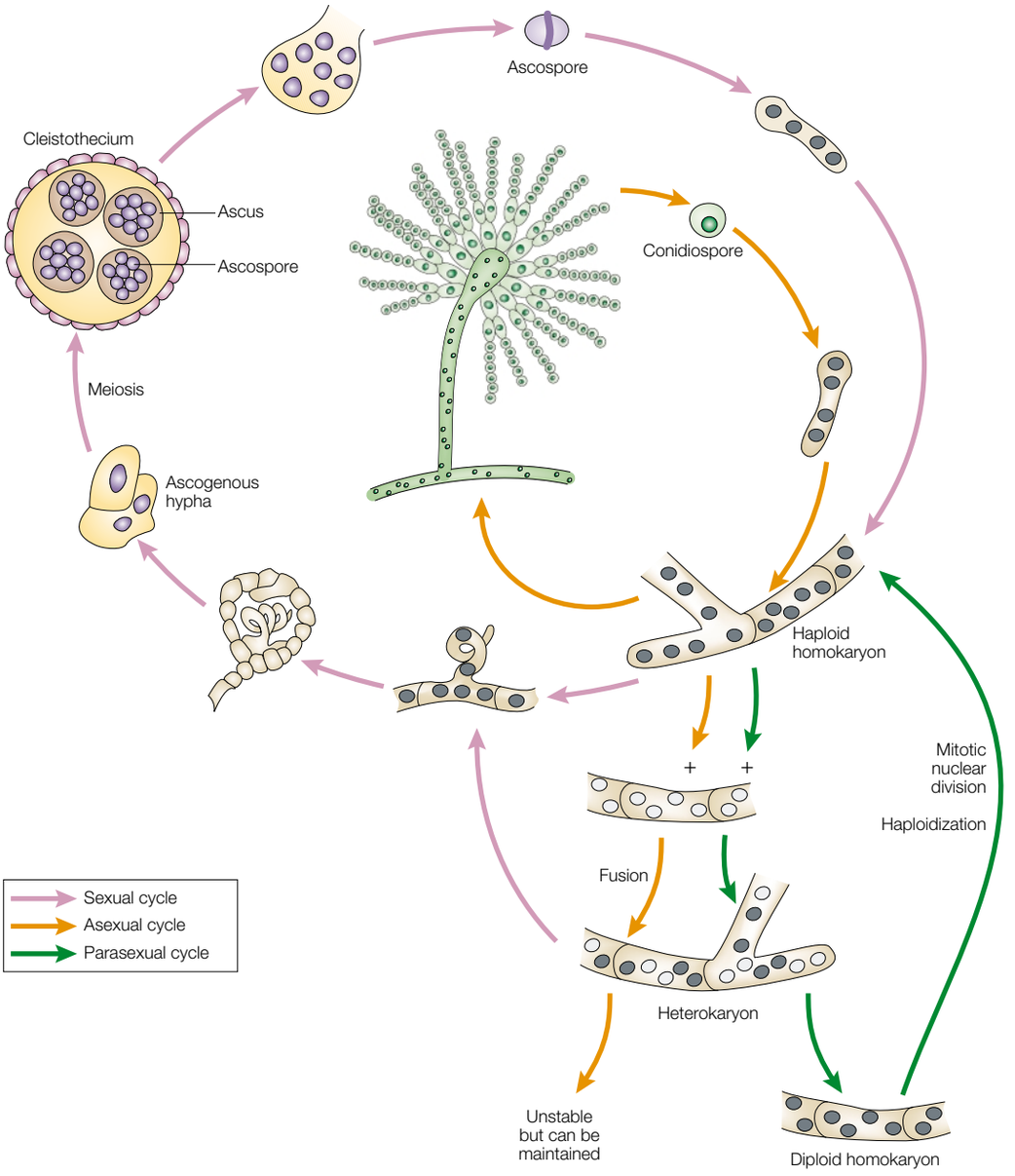
The links provided in this table give details of other sequencing projects that have been planned and that are in progress, such as that of the basidiomycete white rot fungus *Phanerochaete chrysosporium*. See Online link to fungal genome-sequencing programmes.

steps, whereas mutants are concentrated in the ungerminated spores, which are recovered at the end of the enrichment procedure when they are plated onto an appropriate selective medium.

In early studies of metabolic regulation, many successful screens were based on selections for resistance to toxic metabolites. For example, the metabolism of an amino-acid analogue causes the repression, by feedback inhibition, of the biosynthesis of the natural amino acid. However, because the analogue cannot functionally replace the normal amino acid, the cell will die. Resistance to lethality can be achieved by relaxing the normally tight regulation of the pathway by which the amino acid is synthesized, such that it is overproduced and therefore dilutes the intracellular concentration of its toxic analogue. Such screens provide a way to identify genes the activities of which regulate the rate at which the amino acid is synthesized in response to normal environmental cues[12]. Many other toxic analogues that are used in genetic screens are toxic because they act as substrates for a metabolic pathway and are converted to a toxic intermediate. In these cases, resistance can be achieved by mutations that inactivate an enzyme that is necessary for synthesis of the toxic intermediate. For example, chlorate resistance can be used to select for mutants that are defective in nitrate reductase activity (albeit only in species that can use inorganic nitrogen sources)[14], and

fluoroacetate resistance selects for mutants that lack acetyl CoA synthetase activity[15,16]. The value of these systems is that they allow two-way selection for loss- and regain-of-function mutations in the same gene. Mutants that are selected for resistance to fluoroacetate, for example, can no longer use acetate — the natural substrate for acetyl CoA synthetase — as the sole carbon source. Mutants that have regained enzyme function can then be selected by their ability to grow on acetate.

Box 1 | **Life cycle of *Aspergillus nidulans***

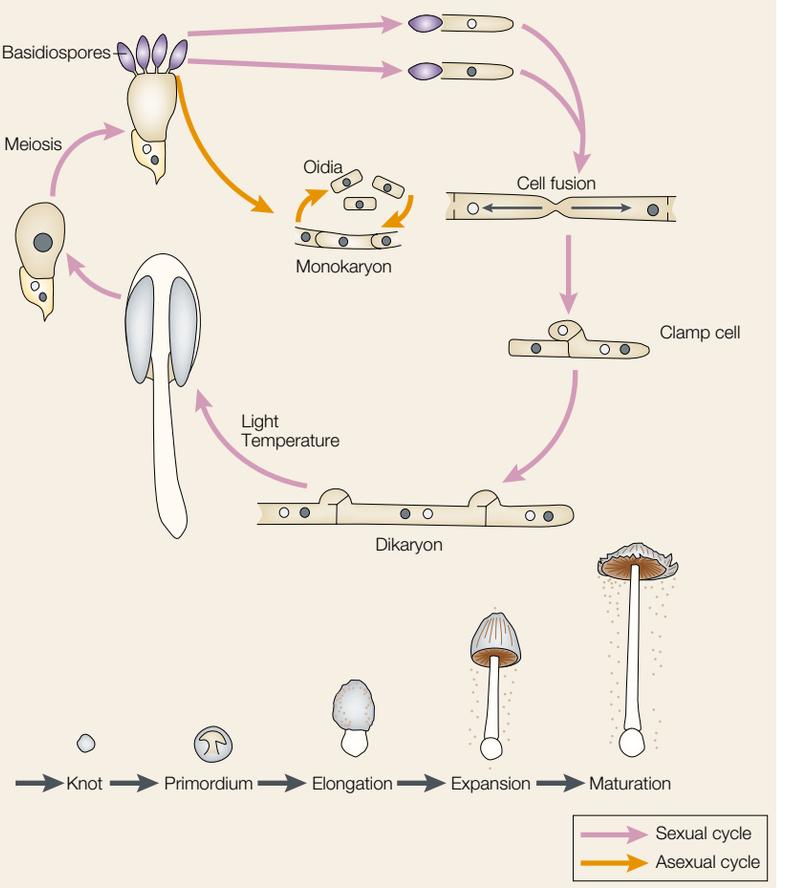


The fungal mycelium of *A. nidulans* is a web of branched filaments (hyphae) of connected compartments or cells, which each contain several nuclei (see centre figure). This mycelium, or homokaryon, which develops from a single haploid spore, differentiates many identical asexual spores known as conidia or conidiospores (see the asexual cycle in the figure). *A. nidulans* is homothallic, which means that it is self-fertile, but crosses can be initiated by hyphal fusions between homokaryons with genetically different nuclei (shown by white and dark green nuclei). The resulting heterokaryons are not stable, but can be forced to maintain a balanced ratio of the component nuclei by including complementing auxotrophic mutations in the parental nuclei and forcing growth without the corresponding supplements.

A. nidulans can also reproduce sexually (see the figure). In the fruiting body, which produces the sexual spores, a pair of nuclei that is destined for meiosis divides in synchrony to form a mass of cells known as the ascogenous hypha. These hyphae are highly branched and each tip cell becomes an ascus (a specialized cell) in which the two haploid nuclei fuse. The diploid nucleus undergoes meiosis followed by a post-meiotic mitosis, which results in the formation of eight haploid ascospores. The fruiting body, called the cleistothecium, can hold tens of thousands of ascospores, which are released into the environment when the cleistothecium bursts open.

In addition to an asexual cycle and sexual cycle, a parasexual cycle offers the genetic benefits of meiosis achieved through a mitotic route[93]. The parasexual cycle is initiated when haploid nuclei fuse in the vegetative cells of a heterokaryon and continue to divide mitotically. Crossing over might occur between homologues and random chromosome loss restores the haploid chromosome number, which is eight in the case of *A. nidulans*. These events can be used to map gene orders and assign new genes to the eight linkage groups. Many closely related fungi of economic or medical importance, such as *A. niger*, *A. fumigatus*, *Fusarium oxysporum* and *Penicillium chrysogenum*, have no sexual cycle but are exploited experimentally or genetically using technologies developed for *A. nidulans*[94].

Box 2 | **Life cycle of *Coprinus cinereus***



The hallmark feature of the basidiomycete fungal life cycle is illustrated here by *C. cinereus*. There are two distinct mycelial stages: the asexual homokaryon (or monokaryon, as it is more commonly called), which produces uninucleate asexual spores, and the fertile dikaryon, on which fruiting bodies develop and which forms when monokaryons of compatible mating types fuse.

In the sexual cycle, nuclei from each mate remain paired and divide synchronously in each dikaryotic tip cell and are partitioned equally at cell division. In *C. cinereus*, but not all species, this results in the formation of a structure known as the clamp cell, which then grows backwards to fuse with the subterminal cell to form a clamp connection. These are the characteristic structures seen at each septum on the dikaryon. Karyogamy occurs in specialized cells (basidia) that lie on the under surface of the mushroom and the four meiotic spores develop (basidiospores) on the outside of each basidium. Although there is no highly developed parasexual cycle in *C. cinereus*, it is possible to generate somatic diploids experimentally[95]. The lower diagram illustrates the stages in the development of the fruiting body.

Basidiomycetes are unique in having multiallelic mating-type genes, and a fascination with these genes was the initial drive for developing the genetics of this fungus[96]. In *C. cinereus*, the mating-type genes map to two complex polymorphic loci known as *A* and *B*, but in many other species the genes map to a single locus.

The basidiomycetes also include other important model fungi. Among them are: the mushroom *Schizophyllum commune*; the corn smut *Ustilago maydis*, in which mating causes a dimorphic switch from yeast-like SAPROPHYTIC cells to a dikaryotic mycelium that is an obligate pathogen[97]; and *Cryptococcus neoformans*, an opportunist pathogen that causes meningitis in humans[98] (see also TABLE 1).

Mutants in which developmental pathways have been blocked are generally screened visually for obvious defects, but specific stages can be targeted when an appropriate mutagen or selection system is available. The first fungal tubulin gene was identified as a result of selection for resistance to benzimidazole agents, such as benomyl, which prevent or disrupt the polymerization of tubulin. This gene, *benA* (*benomyl-resistant A*), was found in *A. nidulans* and encodes β-tubulin[17]. Benomyl-resistant mutations have had a wide application because they can be dominant and can be used to develop transformation systems for species without well-developed genetics. Incorporation of a dominant benomyl-resistance gene into the vector used for transformation provides an easily selectable phenotype for transformants that are expressing the gene. It also obviates the need to develop special strains for transformation, that is, strains with specific genetic mutations that can be complemented by the available cloned genes. Benomyl-resistant mutants have also been important for implicating microtubule functions in mitosis and in nuclear migration in fungi[18].

Suppressor screens. Suppressor screens are part of the general repertoire of mutant-detection methods that have been developed for all genetic organisms. A suppressor mutation is one that, in some way, reverses or

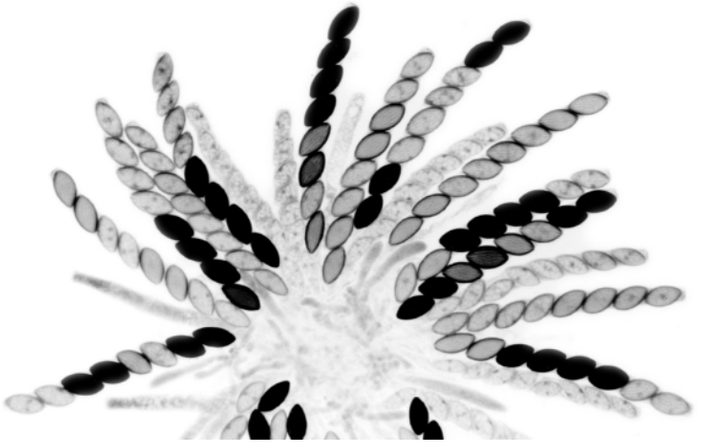
SAPROPHYTIC
An organism that obtains nutrition from dead or decaying plant or animal tissue.

Box 3 | **Tools for the fungal geneticist**

Fungi have many features that make them useful as experimental genetic organisms. Practical details, including information about how to obtain fungal stocks, how to work with filamentous fungi and how to store them are found on the excellent web site of the Fungal Genetics Stock Center (see Online links box).

Many of the fungi that are commonly used for genetic studies have most or all of the following features:

- A haploid phase for mutagenesis and screening.
- A dikaryon or heterokaryon phase for complementation and epistasis analysis.
- The ability to grow on defined media for selection and screening of nutritional and drug-resistance markers.
- The ability to grow at wide temperature ranges, which facilitates the isolation of conditional lethal mutants.
- Heterothallic strains for outcrossing.
- Homothallic derivatives to allow haploid screens for mutants that are defective in fundamentally diploid processes (for example, meiosis).
- Small genomes (12–50 Mb) to facilitate mutant recovery and gene cloning.
- The ability to carry out tetrad analysis, in which all four meiotic products are held together in an ascus (in ascomycetes; BOX 1) or on the apex of a basidial cell (in basodiomycetes; BOX 2). This allows the direct examination of reciprocal meiotic recombination events, and was crucial in the discovery of gene conversion and the development of models for genetic recombination[3,10,99,100]. An example of a *Neurospora crassa* rosette of tetrads is shown here. Reproduced with permission from REF. 101 © (1980) Urban & Fischer. Courtesy of N. Raju, Stanford University.



lessens the mutant phenotype that was caused by an initial mutation. A suppressor mutation can occur in the same gene as the mutation that it suppresses (an intragenic suppressor or second-site reversion); in this case, it causes a compensating substitution in the protein sequence that leads to at least partial restoration of protein function. When the suppressor mutation occurs in another gene, it generates a suppressor gene. This can function in one of several ways, as described in an article by Susan Forsburg in this series of articles on genetic screens in model organisms[19]. For example, nonsense and missense suppressor genes encode mutant transfer RNAs (tRNAs) that correct a mutation at the level of mRNA translation by inserting a wild-type or acceptable amino acid at the mutant site in the protein. Bypass suppressors activate an alternative pathway to the wild-type pathway. An interaction suppressor reveals other proteins with which the starting protein interacts. The rationale here is that a defect in one protein can be counteracted by a compensating defect in a protein that interacts with it[20]. After the characterization of *benA* mutants, Ron Morris, Margaret Lai and C. Elizabeth Oakley[21] identified α-tubulin as a protein that interacts genetically with β-tubulin, by selecting for mutations that allowed temperature-sensitive (*ts*) *benA* mutants to grow at the non-permissive temperature. As expected, one such mutant, *tubA1* (α1-tubulin), also had a mutant phenotype — it was hypersensitive to benomyl. In another suppressor screen, Weil *et al.*[22] used the most sensitive *ts benA* mutant available, *benA33*, and identified a cold-sensitive allele of *mipA* (microtubule-interacting protein A), which encoded a previously unknown member of the tubulin superfamily, designated γ-tubulin[23]. This protein is now known to nucleate microtubule assembly at microtubule-organizing centres[23]. This was a remarkable discovery and led to the identification of γ-tubulin in the centriole (the homologue of the fungal spindle pole body) of *Drosophila* and *Homo sapiens*[24].

Screens for cellular and developmental mutants

Screens for mitotic mutants. Screens of this type, which have been devised largely by Morris and his associates, have focused on detecting aberrant nuclear behaviour in germinating conidiospores of *A. nidulans*. Cells that cannot go through mitosis will die, so mitotic defects can be isolated only as temperature-sensitive conditional-lethal mutants. In 1976, Morris[25] carried out the classical microscopic screen (BOX 4), in which the morphology of the germinating conidiospore was used as the identifying phenotype. As the conidiospore germinates, the germ tube elongates to generate the first hyphal compartment, into which the conidiospore nucleus moves and divides. Mutants that were identified as having a defect in mitosis were those in which nuclear division, but not cell elongation, was affected at the restrictive temperature. Three mutant classes were isolated in this screen: *nim* (*never in mitosis*) mutants were blocked before mitosis; *bim* mutants were '*blocked in mitosis*'; and *nud* (*nuclear distribution*) mutants could complete nuclear division, but were unable to position their nuclei properly along the length of the cell. Forty-five mutants were identified in this screen, and subsequent molecular analysis has led to some remarkable insights into mitosis, cell-cycle regulation and nuclear migration (reviewed in REF. 26). The NimA protein, for example, is a kinase that controls entry into mitosis[27], BimC was the first fungal KINESIN to be identified[28], and NudA was found to be the DYNEIN heavy chain[29]. One of the serendipitous discoveries of this screen was that NudF is a homologue of human LIS1, which is required for neuronal migration during development of the human cerebral cortex[30].

Not all *nudA* mutations are lethal, which means that there are other proteins with overlapping functions to those of dynein. These should be apparent as mutations that have little effect on their own, but are lethal when dynein is missing. Efimov and Morris[31] used the synthetic-lethal screen illustrated in BOX 4, panel b, to look for

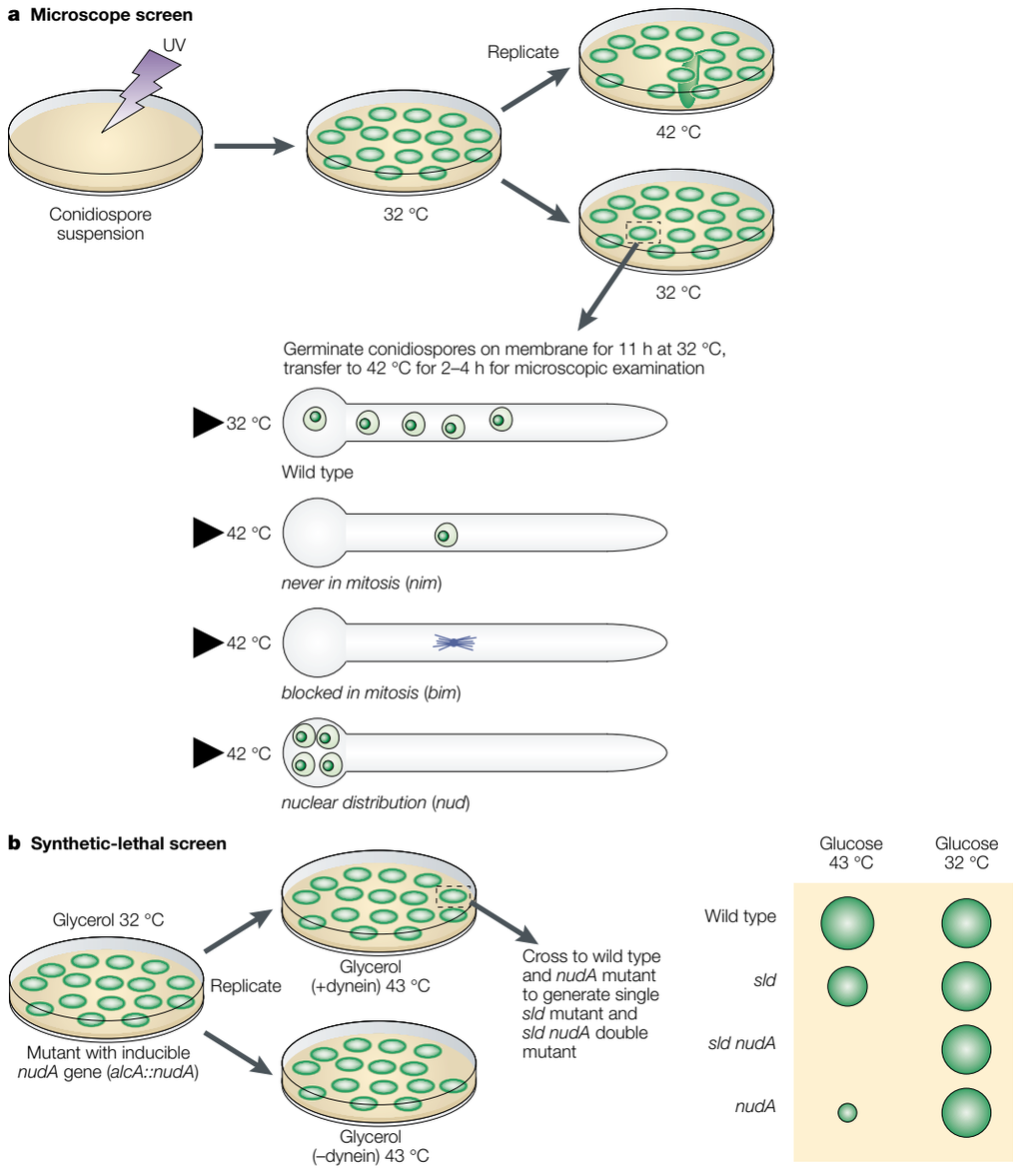
KINESIN
A motor protein that is involved in organelle transport towards the plus end of microtubules.

DYNEIN
A multisubunit motor enzyme that is involved in the transport of organelles to the minus end of microtubules.

Box 4 | **Screens for mitotic mutants of *Aspergillus nidulans***

The screen used by Ron Morris[25], in which he identified temperature-sensitive, mitotic mutants of *A. nidulans*, is shown in panel **a**. After ultraviolet (UV) mutagenesis, conidia were incubated at 32 °C to allow colonies to form. The colonies were then tested for their ability to grow at 42 °C, which is the restrictive temperature. The conidia from colonies that were unable to grow at high temperature were germinated on membranes over agar medium for several hours to allow a germ tube (hypha) to develop. The membranes were then transferred to 42 °C for 2–4 h to allow the block in growth to manifest. Microscopic observation was then used to identify defects in mitosis and nuclear migration. Three classes of mutants were isolated in this screen: *nim* (*never in mitosis*) mutants were blocked before mitosis, as shown by the fact that chromatin in the nuclei was uncondensed; *bim* mutants were *blocked in mitosis*, with condensed chromatin and divided spindle pole bodies (the fungal equivalents of centrosomes); and *nud* (*nuclear distribution*) mutants could complete nuclear division but were unable to position their nuclei properly along the length of the cell.

Panel **b** illustrates the synthetic-lethal screen for mitotic mutants[31] that was designed to detect genes with functions that overlap those of dynein, which is encoded by the *nudA* gene. The *nudA* gene was put under the control of the alcohol dehydrogenase (*alcA*) promoter so that its expression could be switched on or off by the carbon source in the growth medium. *nudA* expression will be off when glucose is present but it will be active on glycerol medium. The screen was therefore designed to isolate *ts* mutants that can grow on glycerol (no dynein) but not on glucose. The new *synthetic lethal without dynein* mutations (*sld*) that were identified in this way were then crossed with a wild-type or *nudA* mutant to expose the phenotype that is conferred by the *sld* mutation alone and to reconstruct the double-mutant phenotype in a non-inducible background.



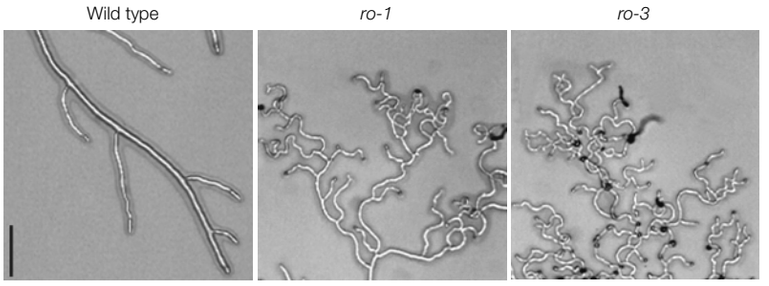


Figure 2 | ***ropy* mutants of *Neurospora crassa*.** Wild-type hyphae of *N. crassa* and those of two strains that are mutant for two *ropy* genes (*ro-1* and *ro-3*) are shown. *ro-1* encodes the heavy chain of cytoplasmic dynein, whereas *ro-3* encodes a protein of the dynactin complex. Note the curved hyphae of the mutants compared with the wild type. Reproduced with permission from REF. 104 © (2000) Society for General Microbiology.

such mutations. Among the new genes that were identified were two mutants (synthetic lethal without dynein; *sldA* and *sldB*) that are homologous to *S. cerevisiae BUB1* and *BUB3* (budding uninhibited by benzimidazole), which encode mitotic checkpoint proteins.

Screens for molecular motor proteins. Wild-type conidia of *N. crassa* germinate on agar medium to produce spreading colonies, which result from continuous tip growth and hyphal branching. Mutants with defects in hyphal tip growth are easily recognized because, in contrast to wild type, they form compact (colonial) colonies. The *cot-1* (*colonial temperature sensitive-1*) mutant was identified as having a temperature-sensitive defect in hyphal tip growth: it produces colonial colonies at 32 °C, but normal wild-type colonies at 25 °C (REF. 32). A screen for partial suppressors of the *cot-1* phenotype identifies mutants that have another well-characterized *N. crassa* phenotype, one that is displayed by ropy (*ro*) mutants. These mutants are particularly distinctive in producing slow-growing, curving hyphae (FIG. 2). Mutations in *ro* are therefore partial suppressors of *cot-1*. Other *cot-1* suppressors can be selected at the non-permissive growth temperature, in which *cot-1* mutants form colonies of only ~0.1-mm diameter and *ro cot-1* double mutants form colonies of 1–5-mm diameter. Because the *cot-1* mutant phenotype is not expressed at 25 °C, the *ro* phenotype is fully expressed when double mutants are grown at this temperature. The discovery that *ro* mutations can be isolated as partial suppressors of *cot-1* has led to large screens for new *ro* genes. As most *ro* mutants are recessive, standard complementation tests can be carried out at 25 °C to determine how many *ro* genes have been identified in the screen. Some 1,000 mutants that have been isolated in this way have helped to identify 23 *ro* genes[33].

As well as having distinctive hyphal morphology, *ro* mutants resemble *nud* mutants of *A. nidulans* in that their nuclei are poorly distributed along the length of the hyphae. Molecular analyses have shown that, like *nudA*, *ro-1* encodes the heavy chain of cytoplasmic dynein, but that two other mutants, *ro-3* and *ro-4*, encode proteins of the DYNACTIN complex. *ro* mutants therefore potentially provide a way of identifying many proteins of the dynein and dynactin complexes, which should be of general biological relevance.

Screens that exploit complexity. Filamentous fungi produce many cell types and this developmental complexity lends itself to a variety of screens. Asexual spores are a convenient source of cells for mutagenesis. In addition, their very appearance has provided a phenotypic screen for some exciting mutants. Two screens that were developed with *N. crassa* illustrate this point, one designed to identify genes that maintain circadian rhythms and the other to examine post-translational gene silencing.

A circadian rhythm is an oscillation in a biochemical, physiological or behavioural function that, in the absence of environmental time cues, has a periodicity of ~24 h. Rhythmicity is driven by an internal timekeeper/oscillator or clock. The clock can be reset by environmental signals, but the period of the rhythm remains unchanged over a physiologically relevant temperature range. In nature, light and temperature cycles entrain the clock and therefore the rhythms have a 24-h periodicity. Simplistically, the circadian system requires several key factors: one or more oscillators (its central time-keeping mechanism); input pathways that feed environmental cues, such as light, to the oscillator; and output pathways that regulate the genes and proteins that manifest the rhythm. The formation of asexual spores in *N. crassa* is under clock control (see recent reviews in REFS 34,35): when the fungus is growing on an agar medium, the clock signals the production of conidiospores once a day. This rhythm is emphasized by growing cultures in 'race tubes' (long glass tubes in which fungal growth can be monitored). Strains that contain the *band* (*bd*) mutation are typically used, as this mutation makes cells insensitive to the build-up of CO_2, which would otherwise suppress the circadian cycle of conidiation.

The race-tube assay (FIG. 3) is the classical screen for identifying mutants with altered day lengths, but it is a brute-force screen. After the mutagenesis of conidiospores, survivors are inoculated at one end of the race tubes, and variations in circadian day length (period) are detected by measuring the distance between the bands of conidia. Several genes that affect circadian rhythmicity, including *frequency*, the *period* (*prd*) genes and *chrono* (*chr*) were identified in this way. Other genes that affect the clock were discovered when the race-tube assay was used to screen the behaviour of strains that were previously isolated as nutritional or morphological mutants. The mutants that are illustrated in FIG. 3 have different alleles of *frequency* (*frq*)[36], which is one of the best-studied genes. Mutants in *frq* have period lengths of 16–29 h instead of the 22 h seen in the wild type. The *frq-9* mutant has the most extreme phenotype and is arrhythmic. Two other genes — the white collar genes *wc-1* and *wc-2* — were initially identified because they are required for light perception and, like *frq*, are also well characterized. When grown in the dark, *N. crassa* produces pigmented spores, but the mycelium is white because the enzymes that are

DYNACTIN
A multisubunit complex that is required for activating cytoplasmic dynein.

required to synthesize the mycelial carotenoid pigment are induced by light. *wc-1* and *wc-2* mutants are 'light blind' and unable to induce the enzymes for carotenoid biosynthesis, even in the light. The name white collar refers to the characteristic appearance of these mutants when grown on solid medium slants in test tubes, in which pigmented conidiospores are supported on a 'collar' of white mycelium.

The FRQ, WC-1 and WC-2 proteins are components of a circadian oscillator that is composed of two transcription–translation feedback loops. WC-1 and WC-2 are also responsible for light input into the clock. Because fungal cultures can maintain their rhythmicity in liquid media, it is possible to identify genes the expression levels of which change in mRNA populations at different time intervals, and therefore this rhythmicity provides a means to identify more genes[37]. The *N. crassa* clock is a generally relevant model for clock studies. FRQ shares a similar function with the oscillator clock proteins Period (Per) and Timeless (Tim) of *Drosophila melanogaster*, and CRY1 (cryptochrome) and CRY2 of mammals. Moreover, WC-1 and WC-2 are PAS DOMAIN PROTEINS[38] and have their counterparts in the *D. melanogaster* Clock (Clk) and Cycle (Cyc) proteins, and in the mammalian CLOCK and BMAL1 proteins[39].

The bright orange colour of *N. crassa* conidiospores has been exploited to screen for genes that are required for quelling — a fungal post-translational silencing mechanism that is related to CO-SUPPRESSION in plants and RNA INTERFERENCE in *Caenorhabditis elegans* (reviewed in REF. 40). In all of these organisms, RNA that is transcribed from a transgene leads to the degradation of the mRNA that is derived from the homologous, endogenous gene. Romano and Macino[41] transformed wild-type *N. crassa* with a strain that had the *albino* genes (*al-1* and *al-3*), which encode enzymes for carotenoid biosynthesis. They discovered quelling by observing a reduction or complete loss of colour of the conidiospores. The reverse colour change from white to orange was then used as a powerful screen for mutants in which the quelling pathway was defective. As the quelled state is normally unstable, Cogoni and Macino first screened for a stably quelled *al-1* transformant, to be sure that recovery of spore pigmentation would be due to gene mutations rather than a reversal of quelling. Three *quelling defective* (*qde*) genes were uncovered in this way. *qde-1* encodes an RNA-dependent RNA polymerase[42] and *qde-3* a RecQ DNA helicase[43]. *qde-2* encodes a protein that might be a component of a small-RNA-directed ribonuclease complex that is involved in sequence-specific mRNA degradation[44]. Homologues of *qde* genes are implicated in post-translational silencing in *C. elegans* and *Arabidopsis thaliana* and the process is thought to represent an ancient natural defence system against viruses and transposons[45].

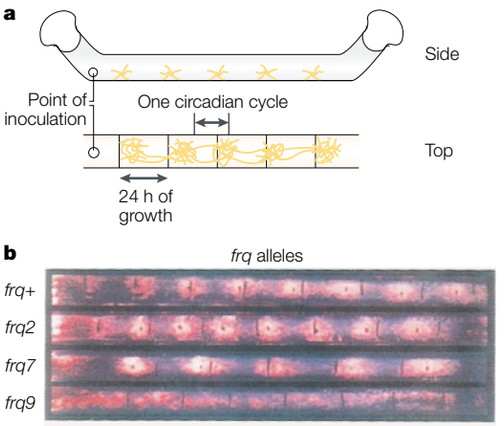


Figure 3 | **Race-tube assay to detect circadian rhythm mutants in *Neurospora crassa*. a** | The race tube is a 30–40-cm long glass tube that contains agar medium. A culture is inoculated at one end of the tube and, after a period of 24 h of growth in the light, the tube is transferred to constant darkness, which sets the clock to a particular point (dusk) in the circadian day. Thereafter, a band of conidia is produced at a time that corresponds to early morning and is followed by a period of undifferentiated aconidial growth. The fungus grows at a constant rate; so, the time taken to produce each band of conidia is the length of a circadian cycle. The period can be calculated as the time between two identical phases of the rhythm (that is, the peak of one band to the peak of the next). **b** | The wild-type fungus (*frq+*) has a circadian day length of 22 h; *frequency* (*frq*)*-2* and *frq-7* are short- and long-day mutants, and *frq-9* is arrhythmic (see main text for details). Original diagram based on a figure provided by Jennifer Loros, Dartmouth Medical School, New Hampshire, USA.

Exploring development. Because of their developmental complexity, filamentous fungi offer many opportunities to study the genes that control development. Mutations that affect conidiospore development are readily distinguished by their phenotype and have been characterized in both *A. nidulans*[46] and *N. crassa*[47]. For example, *bristle A* (*brlA*) mutants of *A. nidulans* develop only the conidiophore stalk, *abacusA* (*abaA*) mutants fail to differentiate conidiospores, and *wet-whiteA* (*wetA*) mutants form defective conidia. The molecular characterization of *brlA*, *abaA* and *wetA* genes defined a network of transcription factors that regulate each other's transcription and are required to turn on the sequentially regulated genes that bring about conidiospore morphogenesis (reviewed in REF. 48).

Many ascomycete fungi are phytopathogens and cause severe crop losses worldwide. Species such as *Magnoporthe grisea* produce a special infection structure known as an appressorium (shown in FIG. 1), and the development of this specialized cell is relevant to understanding how fungi infect their hosts. The appressorium has a strong melanized wall and accumulates high concentrations of glycerol that draw water into the cell. This allows very high internal pressures (measured at up to 8 MPa)[49] to build up. This pressure is converted into a mechanical force, which enables a peg-like infection hypha to break through the cuticle of the plant to access the underlying leaf cells. Several genes that are involved in appressorial development have been identified by using a combination of conventional mutagenesis and an insertional mutagenesis technique known as REMI (restriction-enzyme-mediated integration) (see section on 'Gene cloning' below and BOX 5), and by using differential

PAS DOMAIN PROTEINS
A family of proteins that are related by the presence of a conserved 300 amino-acid sequence that promotes dimerization. PAS is an acronym for the *Drosophila melanogaster* and mammalian proteins PER, ARNT and SIM that originally defined this family of transcriptional regulatory proteins.

CO-SUPPRESSION
The phenomenon whereby an endogenous plant gene is silenced owing to the presence of a homologous transgene.

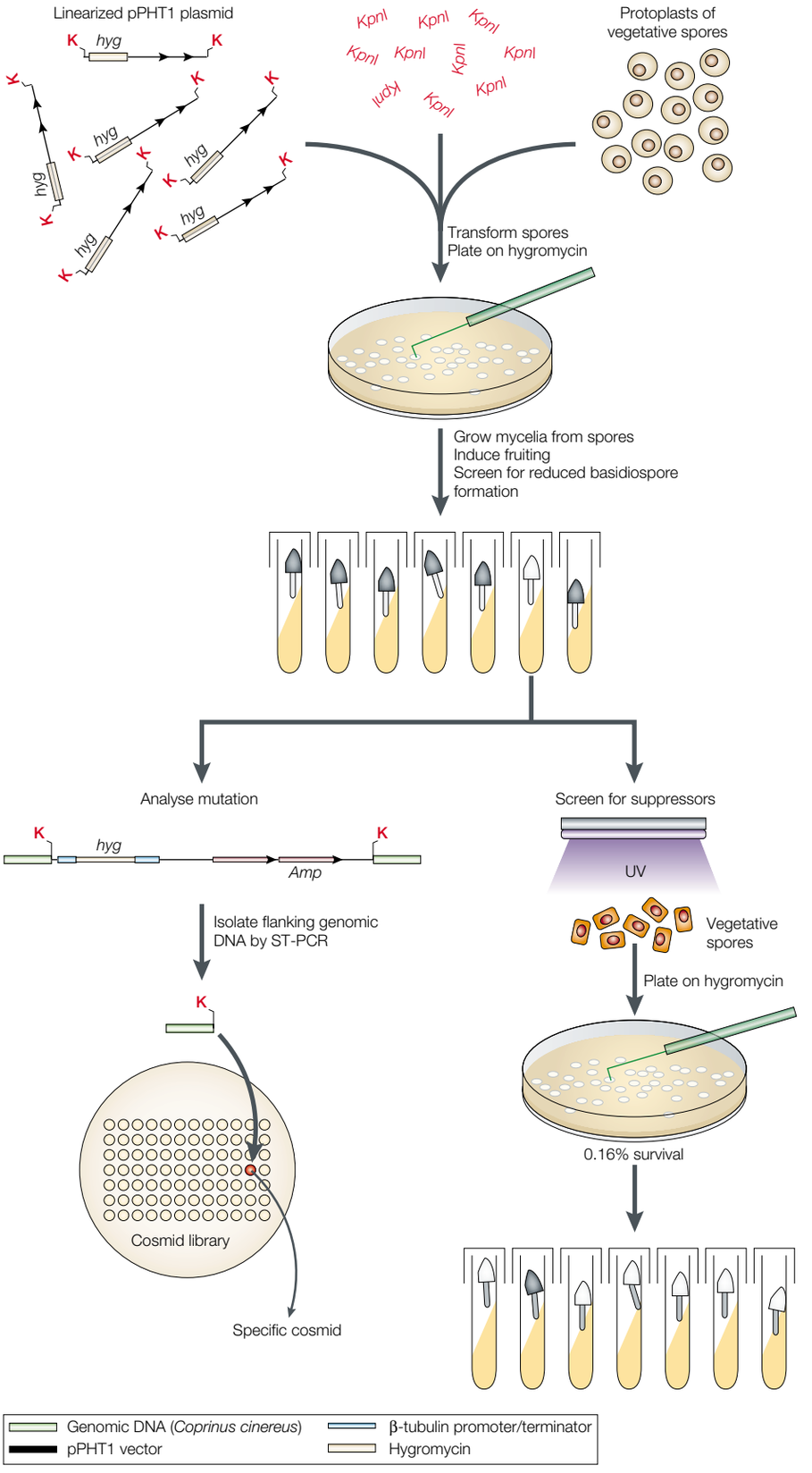
RNA INTERFERENCE
(RNAi). The process by which double-stranded RNA specifically silences the expression of homologous genes through degradation of their cognate mRNA.

Box 5 | **Cloning genes by insertional mutagenesis in *Coprinus cinereus***

The restriction enzyme integration (REMI) technique was first developed for *Saccharomyces cerevisiae*[86], and involves transforming fungal protoplasts with a linearized plasmid together with the restriction enzyme used to linearize it. Transforming DNA usually inserts at breaks created by the enzyme. In the screen illustrated here[87], the pPHT1 plasmid was constructed using the *C. cinereus* β-tubulin promoter and terminator to drive expression of the bacterial hygromycin resistance gene (*hyg*)[87]. An *AmutBmut* strain (which activates the sexual pathway without the need to mate) was transformed using REMI with *Kpn*I (indicated as 'K' on the plasmids) as the added restriction enzyme. This yielded 29 strains (out of 2,321 hygromycin-resistant transformants) that reproducibly produced white-capped fruiting bodies, which is an indication of greatly reduced spore formation. Nine of the mutants were tested to determine whether the sporulation mutations were tagged, which means that the hygromycin resistance co-segregated with the sporulation defect.

Two of the six potentially tagged genes have been identified. For both of these, SEMI-RANDOM, TWO-STEP PCR (ST-PCR)[102] was used to isolate genomic DNA that flanks the plasmid insertion, and this was then used to screen a cosmid library. One gene encodes the *C. cinereus* homologue of Spo11, a topoisomerase-like protein that is required for meiotic recombination[87,103]. The second encodes Msh5, a recombination-specific homologue of the bacterial MutS protein, which in *C. cinereus* is also required for pre-meiotic DNA replication (M. Celerin *et al.*, unpublished data).

Because sporulation-defective mutants can form fruiting bodies that are strikingly white, suppressors can be readily isolated in screens for restoration of some spore production; that is, the formation of a grey mushroom cap instead of a white one. The screen illustrated here was used to identify eight mutations that suppress the sporulation defect of the REMI-generated *C. cinereus spo11* mutant (M. Celerin *et al.*, unpublished data). Original drawing by Martina Celerin, Indiana University. UV, ultraviolet.



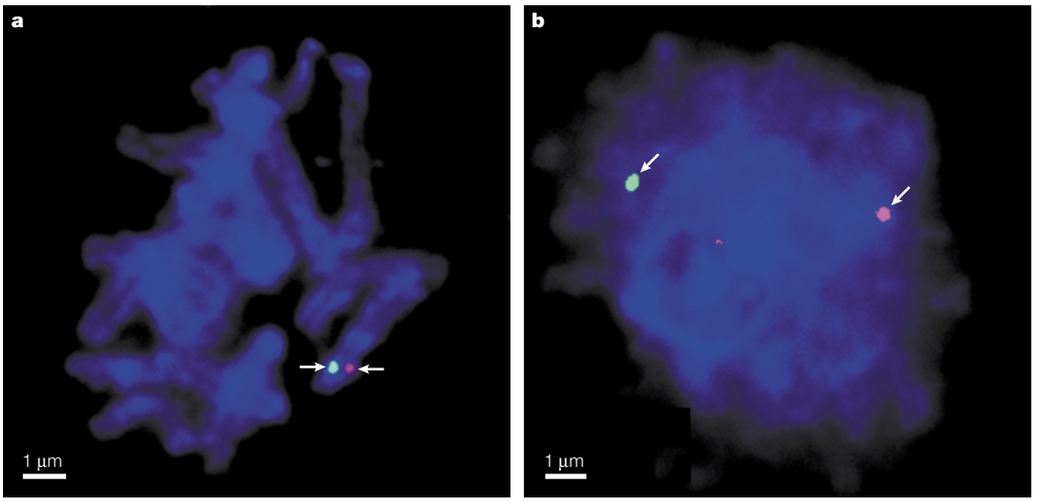


Figure 4 | **Fluorescence *in situ* hybridization analysis of meiotic mutants of *Coprinus cinereus*.** The basidiomycete *C. cinereus* is particularly amenable to cytogenetic analysis because its meiotic nuclei are in natural meiotic synchrony[66]. Meiotic chromosomes have been analysed using light and electron microscopy, by squashes of intact basidia[57], by analysis of single hymenial layers[105,106], by serial sectioning[107] and by surface spreads of meiotic nuclei[108]. A relatively recent addition to the array of cytogenetic tools for *C. cinereus* is the use of fluorescence *in situ* hybridization (FISH) to analyse meiotic homologue pairing[109]; the technique is based on those developed for other organisms[110–112]. Illustrated here is a new twist on FISH; probes are made from DNA of the *B* mating-type loci of the two parental nuclei; these sequences are allelic in chromosomal position but dissimilar in DNA sequence[113] and therefore serve as homologue-specific probes. Panel **a** shows a wild-type nucleus at the pachytene stage of prophase in meiosis I; panel **b** shows a nucleus that carries a mutant allele of *rad-9*, *rad-9-1* (*radiation-sensitive-9-1*). The separation of the two signals in **b** means that homologues are unpaired for this locus and that *rad-9* is required for wild-type levels of homologue pairing. A similar approach has been used recently to show that mutants for the *rad-9-1* allele are defective in meiotic homologue pairing and sister-chromatid cohesion (W. J. Cummings *et al.*, unpublished data). Images provided by Dan Maillet, Indiana University.

mRNA expression and targeted gene disruptions (reviewed in REF. 50). The screen for mutants that are defective in pathogenesis is straightforward: broadly speaking, mutants are identified because they are no longer pathogenic and cannot induce characteristic disease lesions on the host plant. More specifically, mutants that have their appressorial formation blocked can be identified by inoculating conidiospores onto the surface of a leaf or onto an artificial hydrophobic surface, such as Teflon or plastic. Wild-type spores germinate to produce a short germ tube that swells at its tip to develop the melanized appressorial cell. A block in appressorial function can be monitored microscopically by allowing mutagenized conidiospores to germinate on the surface of an onion skin, where it is possible to see whether the infection hyphae can penetrate the plant cell cuticle.

Numerous mutants that are blocked in fruiting and meiosis have also been described, particularly in *Sordaria macrospora*[51], *N. crassa*[52] and *C. cinereus*[53–56]. In particular, *C. cinereus* has been the focus of several mutant screens because this developmental pathway is extremely complex in this fungus, where it is regulated by light and temperature and, most importantly, it offers a uniquely accessible system for studying meiosis. As first shown by Lu[57], meiosis in *C. cinereus* lasts a long time and is naturally synchronous. In addition, its meiotic chromosomes are readily examined by light- and electron-microscopy techniques (FIG. 4). Takemaru and Kamada[53,54] mutagenized a dikaryotic mycelium and were able to assign developmental blocks in more than 2,000 mutants to one of the several distinct stages in the development of the fruiting body shown in BOX 2. Mutant alleles that are phenotypically expressed in a dikaryon are, of necessity, dominant, because each cell contains genetically different nuclei. Later screens overcame this limitation of only being able to identify dominant mutations by exploiting dominant, constitutive mutations in the mating-type genes (*AmutBmut*[58]), which activate dikaryon development and the entire fruiting pathway without the need to mate. These strains are effectively homothallic; they produce asexual spores and mate with non-mutant monokaryons. This means that REMI procedures and formal genetic analyses can be carried out with any mutants that are obtained. Inada *et al.*[59] have recently identified *clampless1* (*clp1*), a gene that is required to initiate the entire dikaryotic pathway. Importantly, *AmutBmut* strains have, as hoped, yielded good mutants with meiotic and sporulation defects (see below).

Screens for meiotic mutants. Two basic types of screen have been used to identify mutants that are defective in meiotic DNA metabolism (reviewed in REFS 52,55,60). In the first, haploid isolates are screened for sensitivity to mutagens that specifically interfere with DNA metabolism, and the identified mutants are then assayed for sporulation defects. Some of the genes that are identified in this manner are those that encode orthologues of proteins that are found in all organisms. Examples of these are *C. cinereus mre11* (*meiotic recombination*) and

SEMI-RANDOM, TWO-STEP PCR (ST-PCR). A procedure that is used to isolate unknown genomic DNA that flanks a known insert. One primer that binds to the known sequence and a degenerate primer with a non-degenerate 5′ end are used to amplify products. A second round of PCR uses a second primer in the known sequence and a primer to the non-degenerate 5′ end of the degenerate primer. This process is repeated until a single PCR product is obtained.

rad50 (*radiation sensitive*), and *A. nidulans rad51* (REFS 61–63; see REF. 64 for an excellent recent review of the DNA damage response in filamentous fungi). The advantage of this type of screen is that both the mutagenesis and initial screen are carried out in the haploid phase, and therefore mutagen-sensitive strains are identified without further genetic manipulation. However, to screen for meiotic defects, the primary isolates must be outcrossed to place the mutations into compatible mating-type backgrounds.

The second type of screen for meiotic mutants seeks sporulation defects without making assumptions about their underlying cause. Although, traditionally, this approach requires that each isolate be outcrossed for the construction of homozygous mutant strains, its advantage is that both meiosis-specific and pleiotropic genes can be identified. One landmark investigation of this type was a screen of wild-collected isolates of *N. crassa*[65] (see also REFS 52,66). In this study, 74 out of 99 wild-collected isolates were found to contain one or more recessive mutations that affect fertility.

The use of a homothallic organism, or homothallic mutants of a normally heterothallic species, is in many ways the best of both worlds, as mutations can be induced in a haploid nucleus but then tested directly for defects in the fundamentally diploid process of meiosis. For *S. macrospora*, this has facilitated the isolation of the *spo* (*sporulation*) mutants (reviewed in REF. 51), including *spo76*, which encodes a conserved protein that is important in both mitotic and meiotic sister-chromatid cohesion and chromosome compaction[67–69]. The *A. nidulans* BimD protein is orthologous to Spo76, and has also been shown to be a negative regulator of cell-cycle progression; the *bimD6* mutant cycles faster than wild-type strains[69], and overexpression of *bimD* leads to G1- or early S-phase arrest[70]. The human orthologue of BimD, AS3, is a tumour-suppressor protein[71], which fits with the characterized role of BimD[69].

Kanda *et al.*[56,72] and Pukkila[55] used UV mutagenesis of the *C. cinereus AmutBmut* strain to identify collections of sporeless mutants (termed *spo* and *bad* (*defective in basidiospore development*), respectively). The *spo* mutants were classified according to their stage of meiotic arrest[56] and whether they had defects in pre-meiotic DNA replication[72]. The *bad* mutants have been analysed, using electron microscopy, for defects in meiotic synapsis[55]. Pukkila *et al.*[55,73] confirmed the pre-meiotic DNA replication defects of some of the *spo* mutants, and also characterized meiotic chromosome synapsis in one replication-defective strain, *spo22-1*. Their finding of normal synapsis in diploid but not triploid nuclei confirmed the DNA replication defect that is caused by the *spo22-1* mutation. The *spo22* gene was recently cloned; it encodes the *C. cinereus* orthologue of Msh5 (M. Celerin *et al.*, unpublished data). This was a surprising result, because *msh5* is usually considered to be a late-recombination gene and is not known to function in DNA replication in other systems (for example, REFS 74,75). Recently, the use of *AmutBmut* has been combined with the REMI approach to facilitate the isolation and characterization of genes that are necessary for spore formation (BOX 5).

REPLICATIVE PLASMID
A plasmid molecule that contains in its sequence an origin for DNA replication and can replicate autonomously after transformation into host cells.

Gene cloning

Genes of interest that are recovered from genetic screens are generally cloned by phenotypic complementation of a wild-type gene sequence that is present in a genomic library. Therefore, cloning largely depends on efficient DNA-mediated transformation systems. Unlike *S. cerevisiae*, in which REPLICATIVE PLASMIDS make screening and recovery of a gene a one-step operation, attempts to develop replicative vectors for filamentous fungi have met with less success; they exist only for the pathogenic basidiomycete *U. maydis*[76], and the ascomycetes *A. nidulans*[77] and *Podospora anserina*[78]. A more successful, if more complicated, route[79] is to recover a rescuing plasmid — derived from a genomic library — containing a sequence that complements the mutation. Unlike in *S. cerevisiae*, a transformed plasmid integrates into the fungal genome, from where it can be recovered. To do this, genomic DNA of the transformant is digested with a restriction enzyme that does not cleave in the integrated plasmid, and therefore should create DNA fragments that contain the plasmid, plus some amount of flanking genomic DNA on each side. The digested DNA is then ligated as a dilute solution, to favour intramolecular events. These ligations are transformed into bacteria, and a drug-resistance marker on the plasmid allows selection of plasmid-genomic hybrid clones. More generally, gene identification requires sib-selection through ordered genomic libraries. For example, several thousand cosmid clones might be stored individually in 96-well microtitre dishes. Pooled DNAs from the 96 clones in each dish are assayed by transformation for their ability to complement a mutant phenotype. Once a DNA sample has been identified as having the gene of interest, the pool is successively subdivided and tested until the single cosmid clone that replicates the gene is positively identified. Although it can be time consuming, this method continues to be widely used. The use of chromosome-specific libraries (for example, see REFS 59,80) has, in some cases, greatly facilitated this process, because the drop in library complexity means that fewer clones must be tested. Total genomic and chromosome-specific libraries have also been used for efficient positional-based cloning (for example, see REFS 61,79,81).

Because DNA preferentially integrates randomly into the genome of filamentous fungi, it is potentially mutagenic. An attractive goal is therefore to disrupt genes by DNA insertion and to use the inserted sequence as a tag to recover adjacent gene sequences.

At present, the REMI technique, recently reviewed by Riggle and Kumamoto[82] and Mullins and Kang[83] (see also REFS 84,85) is the best-tried system (BOX 5). The technique, which was first developed for *S. cerevisiae*[86], involves transforming fungal cells (usually PROTOPLASTS) with a linearized plasmid together with the restriction enzyme that was used to linearize it. It is assumed that the enzyme enters the nucleus and cuts chromosomal DNA at its specific recognition site. The plasmid DNA, which has the correct complementary ends, can then be ligated into these sites *in vivo*. REMI increases the

PROTOPLAST
A cell from which the cell wall has been removed by enzymatic digestion.

frequency of stable transformation in all species tested and can lead to high levels of conservative integrations (integrations in which the enzyme sites are reconstituted on either side of the integrated DNA), although the reported frequencies vary from 50% to 100% (reviewed in REF. 87). Integration into any particular gene requires that its DNA sequence contain a site for the added restriction enzyme, so it is necessary to use several enzymes in REMI screens and to test for the optimal levels of enzyme to use, as these can vary considerably. Not all REMI-induced mutations are tagged, presumably because the enzymes create breaks in the DNA that are repaired without plasmid integration, but the technique has been used to isolate a wide variety of genes that are involved in pathogenicity (reviewed by Kahmann and Basse[84]).

Alternative tools are being developed for insertional mutagenesis. The T-DNA from *Agrobacterium tumefaciens*, which is important for transforming transgenes into plants, also causes insertional mutations in *Arabidopsis*[88]. T-DNA has been used to transform filamentous fungi, with the added advantage that it is not necessary to make protoplasts of the fungus to introduce DNA[83]. Much effort is also being put into the development of active transposons that might insert randomly into genes[89,90].

For sequenced genomes, a 'candidate-gene' approach can also be taken for gene recovery. For example, Selker and colleagues[91] isolated a mutant that was defective in cytosine methylation, and the newly identified locus was designated *dim-5* (*defective in methylation 5*). The *dim-5* gene was mapped to a region of linkage group IV between the *trp-4* and *leu-2* loci. Using the *N. crassa* genome database (see Online links box), the locations of *trp-4* and *leu-2* were identified on the basis of their similarity to *S. cerevisiae* homologues (*LEU1* and *TRP4*, respectively). Examination of the *N. crassa* genome in the 80-kb interval between these two loci revealed a putative histone methyltransferase gene, which was established definitively as *dim-5* in further experiments. This was an important result, because it showed the dependency of DNA methylation on histone methylation.

By combining genomics and insertional mutagenesis, Hamer *et al.*[92] developed a system they term TAGKO (transposon-arrayed gene knockouts). In TAGKO, pools of cosmids are mutagenized *in vitro* with a transposon; individual cosmid clones are then annotated by DNA sequencing using the transposon insertion as a priming site. Selected cosmids are transformed into fungi, and transformants are analysed to find those in which the endogenous gene had been replaced by the tagged transgene; these are then assayed for phenotype. Although any type of genomic clone would be suitable for TAGKO, cosmids were used in the *M. grisea* and *M. graminicola* systems that were tested, because longer tracts of homologous DNA were found to increase greatly the frequency of targeted gene replacements. This procedure rapidly provides both DNA sequence and functional analysis of genes, and could be applied to any fungus for which reasonable levels of gene replacement can be obtained.

Concluding remarks

The diversity of fungal lifestyles is breathtaking and presents many opportunities to study interesting facets of eukaryotic biology. In this review, we have focused on topics of general relevance, but there are many more that are specifically fungal, and of interest to industry, agriculture and medicine. There will always be a need for clever genetic screens based on traditional genetic approaches, but the explosion of new techniques that is afforded by the post-genomics era means that a lack of formal genetics is no barrier to isolating genes that are involved in any pathway of interest. The filamentous fungi have had a long history as important tools for genetics, and a productive future now seems assured.

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Acknowledgements

We thank numerous colleagues for figures, preprints and advice, and we apologize to those whose work was not cited due to the breadth of this topic and our space limitations. We thank B. Metzenberg, R. Davis, R. Morris, J. Hamer, J. Clutterbuck, S. Crosthwaite, D. Bell-Pedersen, S. Gold, S. Gurr, M. Riquelme, A. Radford and R. Aramayo for valuable discussions; E. Selker for sharing unpublished results; and R. Morris, M. Celerin, D. Maillet, J. Loros, M. Riquelme, R. Kahmannn, J. Kämper, N. Talbot, M. Momony, H. Wosten, L. Lugones and N. Raju for figures. Work in the Zolan lab is supported by the National Institutes of Health, and the research of L.A.C. is supported by the Biotechnology and Biological Sciences Research Council.

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