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Conflict, competition and cooperation regulates social interactions in filamentous fungi

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Abstract

Social cooperation impacts the development and survival of species. In higher taxa, kin recognition occurs via visual, chemical or tactile cues that dictate cooperative versus competitive interactions. In microbes, the outcome of cooperative versus competitive interactions is conferred by identity at allorecognition loci, so called “kind recognition”. In syncytial filamentous fungi, the acquisition of multicellularity is associated with somatic cell fusion within and between colonies. However, such intraspecific cooperation entails risks as fusion can transmit deleterious genotypes or infectious components that reduce fitness, or ‘cheaters’, that can exploit communal goods without contributing to their production. Allorecognition mechanisms in syncytial fungi regulate somatic cell fusion by operating pre-contact during chemotropic interactions, during cell adherence, and post-fusion by triggering programmed cell death reactions. Alleles at fungal allorecognition loci are highly polymorphic, fall into distinct haplogroups and show evolutionary signatures of balancing selection, similar to allorecognition loci across the tree of life.

I. Conflict, competition and cooperation regulate social behavior

1. Greenbeard genes, altruism and allorecognition

Altruism is defined as an individual acting at a cost to themselves but benefiting, directly or indirectly, another individual, without the expectation of reciprocity (self-sacrifice). Self-sacrifices include complex behaviors, such as in meerkats, that watch for predators while other members of their family forage (14) or as in bacteria, that absorb peptides that help the survival of the population (117). A gene-centered view of altruism provides an explanation for self-sacrifices: a gene can be favored in a population even if it is costly, if it provides benefits for other individuals carrying copies of that same gene (22). Thus, altruism is evolutionarily beneficial if the relatedness of the individual that profits from the altruistic act is higher than the cost/benefit ratio that this act imposes (Hamilton's rule) (57). This gene-centered view, in combination with kin recognition, can explain altruism in higher organisms, where genome-wide relatedness can be assessed based on a combination of visual, chemical and tactile cues.

The concept of kin recognition is difficult to explain when considering microbes. How can microbes assess the genealogy of other individuals without 'seeing' their surroundings? How can a microbial 'selfish gene' (22) identify copies of itself in others? Originally envisioned to explain the genetic basis of social behavior (22), organisms containing "green beards" allows for easy identification by other green beard carriers. Greenbeard genes promote altruism toward individuals who share a specific phenotypic trait controlled by a given gene; an interaction defined as kind or allorecognition (40). Multiple interaction modes between individuals using allorecognition are possible (*e.g.* cooperation *versus* antagonism). Allorecognition functions in phylogenetically diverse organisms (Fig. 1): in social bacteria *Myxococcus xanthus* (126) and *Proteus mirabilis* (43), and eukaryotic colonial species including invertebrates *Hydractinia symbiolongicarpus* and *Botryllus schlosseri* (108), the slime mold *Dictyostelium discoideum* (74), and fungi *Cryphonectria parasitica* (84; 133), *Podospora anserina* (110), and *Neurospora crassa* (20; 45).

2. Evolutionary features of allorecognition systems

Three potential drivers of allorecognition evolution in social organisms have been identified: cheaters (freeloaders), inbreeding, and disease transmission. Cheater/freeloader genotypes are named by analogy to the "tragedy of the commons" (102) and participate in an organism's social phase and receive social goods without contributing to their production (5),

increasing the cheater's relative fitness at the expense of the social group (79). Selection for cheaters is an impediment to the progression of multicellularity and a primary driver of allorecognition evolution (5), which reduces the cheater/freeloader problem by permitting organisms to limit social behaviors to genetically similar individuals (74). The chestnut blight fungus, *C. parasitica*, provides examples for two potential drivers of fungal allorecognition. First, *C. parasitica* colonies are filamentous and syncytial, a lifestyle that selects for cheaters (5) (see example, Fig. 2B). Second, *C. parasitica* can be infected with Hypoviridae mycoviruses that reduce fitness (21). Mycoviruses lack external vectors and are transmitted via somatic cell fusion between an infected and an uninfected colonies (42). Isolates of *C. parasitica* exhibit a form of allorecognition termed vegetative incompatibility that inhibits successful somatic cell fusion between genetically different strains (84; 133). Thus, disease transmission pressures may explain why some organisms have developed multiple allorecognition checkpoints that operate at various levels of intercellular intimacy.

Although kind recognition genes are not derived from common ancestors, they share evolutionary characteristics. Typically, genes encoding kind recognition systems exhibit evidence of balancing selection, including the long-term maintenance of multiple alleles at similar frequencies in well-mixed populations (104). Alleles at kind recognition loci are typically highly polymorphic with signatures of positive selection, and fall into discrete allelic classes, termed haplogroups, which often show trans-species polymorphisms, a phenomenon observed when alleles from different species are more closely related to each other than they are to other intra-species alleles (104). Kind recognition systems are often composed of multiple genes that are tightly linked, thus reducing the probability of recombination between a module's components, and resulting in coevolution of the components and allelic diversification (8). Allorecognition systems could also represent cases of exaptation, a hypothesis developed as an explanation for allorecognition systems in fungi, where anti-pathogen defense systems are potentially harnessed for the recognition of conspecifics (54; 96).

3. 'Harming' and 'helping' kind recognition

Allorecognition can be divided into "harming" and "helping" types (101). For example, bacteriocin toxins can be considered greenbeard traits of the harming type (40; 105). Some bacteria and archaea produce bacteriocins released at times of stress, with 'self' cells producing an antidote to the poison, which they keep private (105). Cells lacking the

poison/antidote genes are killed ("spite" for individuals lacking the greenbeard genes). "Helping" kind discrimination is defined by actions that provide fitness benefit to individuals that share the trait, but not to those that lack it. The tumor inducing (*Ti*) plasmid of *Agrobacterium tumefaciens* can be considered a helping greenbeard trait (40). Genes are transferred from the *Ti* plasmid to plant cells, which induces tumors and production of opines (food source), which is a public good. However, opine production is only beneficial for *Ti* plasmid bearers, because opine catabolism is also encoded on the *Ti* plasmid. In *D. discoideum*, starvation leads to the aggregation of free-living ameoboid cells to form multicellular structures composed of spores supported on a stalk (119). Stalk cells perform an act of self-sacrifice as they enable other cells to differentiate into spores for dispersal (82). Following the genetic logic above suggests that *D. discoideum* strains would be willing to sacrifice themselves only if they can help other individuals of the same genetic background to proliferate. When populations of *D. discoideum* contain a mixture of genetically different strains, the frequency of stalk formation is based on the likelihood of whether the benefit will go to members of the group that share their genes (Fig. 1); in this case, allorecognition is determined by the *tiger* genes (*tgrB1* and *tgrC1*) (63).

II. Molecular mechanisms of cooperative behavior

1. Examples of cooperation, with a focus on fungi

In nature, cooperation occurs at all levels and across taxa. For example, bacteria regulate their cooperative behavior in a process called quorum sensing: autoinducers (like acylated homoserine lactones) increase in concentration depending on cell density, enabling bacterial communities to behave as a multicellular organisms (85). *Candida albicans* also secrets quorum sensing molecules such as farnesol, tyrosol and tryptophol (76). Fungal quorum sensing is involved in many cellular processes, including morphogenesis (*e.g*. yeast to hypha transition), germination, biofilm formation, control of nutrient levels, cell death induction, antifungal activity and pathogenicity (92).

In filamentous fungi such as *N. crassa*, mycelial growth results from tip elongation and somatic cell fusion (61) (Fig. 2). This coordinated and cooperative behavior leads to the formation of an interconnected mycelial network. In many filamentous fungi, colony establishment is characterized by somatic cell fusion between genetically identical germinated spores (germlings) and hyphae that are in close proximity. Cells deficient in

somatic cell fusion show an increase in time for colony establishment (52; 67), indicating that the capacity to undergo somatic cell fusion contributes to fitness.

Filamentous ascomycete colonies contain septa that are often perforated and that allow movement of cytoplasm and organelles, including nuclei, throughout the colony (106). This syncytial lifestyle makes the products of each nucleus potential social goods, which is predicted to strongly select for cheaters (5). Hyphal anastomosis and the mycelium it generates enhance fitness by increasing colony growth rates and improving the production of asexual spores (3), by distributing resources throughout the colony (116), and increasing colony size when higher densities of spores are present (103).

2. Molecular pathways involved in cooperative somatic cell fusion in filamentous fungi

The molecular pathways required for somatic cell fusion between germlings/hyphae in filamentous fungi have recently been reviewed in detail (35). Here, we highlight pathways important for cooperative somatic cell fusion between genetically identical cells that are also implicated in allorecognition. In *N. crassa*, components of a mitogen-activated protein kinase (MAPK) signaling complex composed of NRC-1 (MAPKKK), MEK-2 (MAPKK) and MAK-2 (MAPK) and a scaffold protein HAM-5 assembles and disassembles at fusion tips of interacting cells during chemotropic interactions, with an ~8 min regularity and opposite dynamics in interacting cells (28; 39; 67) (Fig. 3A). A second protein, SOFT, also associates and disassociates at fusion tips, but with completely opposite dynamics to the MAK-2 signaling complex (39) (Fig. 3A). This so-called "ping-pong" mechanism of communication provided a hypothesis on how cells can avoid self-stimulation when undergoing chemotropic interactions with a genetically identical partner (53).

A second MAPK cascade, the cell wall integrity (CWI) MAPK pathway, is also required for somatic cell fusion in a number of filamentous fungi. The CWI MAPK pathway is composed of MIK-1 (MAPKKK), MEK-1 (MAPKK) and MAK-1 (MAPK) kinase and includes membrane-spanning sensors, such as WSC-1 and WSC-2 (35; 81). The MAK-1 complex does not show dynamic oscillation during chemotropic interactions, but once cells adhere, MAK-1 localizes to the contact zone where it remains during fusion pore formation (128). In *Sordaria macrospora*, the ortholog of SOFT (PRO40) functions as a scaffold of the MAK-1 signaling complex (120; 128). Both the MAK-1 and MAK-2 signaling pathways regulate gene transcription through the activation of the transcription factors PP-1 and ADV-1 (37); ADV-1 is a direct activator of many of the genes required for somatic cell fusion (27;

37). Upstream of the two MAPK signaling cascades, the WHI-2, CSP-6 and AMPH-1 proteins putatively function to control endocytosis, which could be involved in the perception of chemotropic signals (50). Following cell-cell contact, cell wall dissolution at the fusion spot and plasma membrane merger is necessary to complete somatic cell fusion. A number of genes encoding proteins important for membrane merger have been identified in *N. crassa* (38; 93; 94), although a fusase has not been identified.

Screening of the full genome deletion strain set available for *N. crassa* (97) revealed that ~80 genes affect or are required for somatic cell fusion, including components of signaling pathways, predicted membrane proteins, genes encoding proteins that affect secretion and a number of hypothetical proteins (35). Importantly, genes encoding the receptor or ligand involved in chemotropic interactions have not been identified or characterized. These data suggest that the genes encoding the receptor and ligand required for somatic cell fusion may have redundancy or that the receptor and ligand genes are members of the hypothetical protein gene set that have not been biochemically characterized, but that are essential for somatic cell fusion.

III. Allorecognition at distance

1. Determinants of fungal communication and chemotropic interactions

A fungal greenbeard locus that acts at distance by regulating chemotropic interactions has been characterized in *N. crassa* (59). Germlings that share compatible alleles at the Determinant Of Communication (*doc*) loci exhibit homing growth *en route* to somatic cell fusion to form a cooperative colony (Fig. 2). Within *N. crassa* populations, five communication (CGs) haplogroups have been identified and which exhibit CG-specific rearrangements, duplications, and deletions. Alleles at the linked *doc* loci, *doc-1* and *doc-2* are ~99% identical within a CG, but only <50% identical between CG haplogroups. Strains of identical CG specificity home towards each other, while strains from different CGs ignore each other (Fig. 3). Alleles at the *doc-1* and *doc-2* loci also show evidence of balancing selection and trans-species polymorphisms (59), supporting their role in mediating kind recognition in fungal populations.

Communication phenotypes of *Δdoc-1*, *Δdoc-2*, and *Δdoc-1 Δdoc-2* mutants confirmed that the *doc* locus is necessary and sufficient for CG identity (59). The DOC-1 and DOC-2 proteins function to negatively regulate chemotropic interactions as a *Δdoc-1 Δdoc-2* mutant displays a high self-communication frequency, but a complete loss of communication

and chemotropic interactions with its isogenic parental strain (59). The introduction of *doc-1* and *doc-2* alleles from a different CG (CG3) into the *Δdoc-1 Δdoc-2* mutant resulted in a switch to CG3 specificity. Localization studies showed that DOC-2 localizes to the periphery of the cell while DOC-1 co-localizes and oscillates with components of the MAK-2 complex during chemotropic interactions (59). These data suggest that DOC-1 regulates reinforcement of MAK-2 complex signaling during chemotropic interactions. When cells carry different alleles at *doc-1* and *doc-2*, reinforcement of MAK-2 signaling is prevented, resulting in a decreased frequency of communication and fusion.

A link between somatic cell fusion between genetically identical cells and allorecognition by the *doc* system was recently revealed (36). The *N. crassa Δham-11* mutant fails to undergo self-fusion, but will undergo chemotropic interactions and fusion with its wild type parent. A *Δdoc-1* mutant undergoes self-fusion and fusion with its wild type parental strain. However, when a *Δham-11 Δdoc-1* double mutant was constructed, somatic cell fusion was completely abolished (36). These data implicate DOC-1 in regulating somatic cell fusion between genetically identical cells in a parallel pathway to HAM-11.

IV. Allorecognition upon contact

1. Contact-induced allorecognition

While greenbeard genes that function at a distance offer an advantageous mechanism to recognize non-self partners, allorecognition also operates after physical contact between conspecific individuals/colonies. For example, in *P. mirabilis*, boundaries form between swarming colonies of different strains, but not between colonies of a single strain (Fig. 1) (43). Strains of *P. mirabilis* that carry incompatible alleles at identification of self, or *ids* genes induce growth arrest in interaction areas between colonies (44)(12). Growth arrest is correlated with formation of a heterotypic IdsD and IdsE complex (12). In the aggregative bacterium *M. xanthus*, contact-dependent exchange of factors that promote group motility and transition to sporulation are controlled by an allorecognition checkpoint regulated by homotypic interactions between a TraA/TraB complex (10). The *traA* gene is highly polymorphic in wild isolates of myxobacteria (11; 129).

In animal systems, the molecular basis of contact-induced allorecognition has been studied in the protochordate, *B. schlosseri*, and the hydroid, *H. symbiolongicarpus* (Fig. 1). In *B. schlosseri,* isogenic colonies fuse to form larger colonial chimeras, resulting in sharing of public goods (108). However, fusion between *B. schlosseri* colonies only occurs if both have

allelic identity at the fusion/histocompatibility (*fuhc*) locus; if incompatibility is perceived, an inflammatory response resulting in blockage of vascular interactions followed by allograft rejection is triggered (112) (Fig. 1). The *fuhc* locus contains two adjacent genes (*fuhc*^{sec} and *fuhc*^{tm}) that show evidence of balancing selection (91); the extracellular region of the FuHC protein is highly polymorphic (23). In the cnidarian *H. symbiolongicarpus*, an analogous mechanism determines fusion of tissue projections known as stolons, that arise from asexual polyps and adhere between conspecific colonies (90) (Fig. 1). In this case, allorecognition is defined by *Alr1* and *Alr2*, two highly polymorphic genes that encode transmembrane proteins (69; 90; 107).

2. Contact-dependent allorecognition in filamentous fungi

N. crassa cells/hyphae of identical CG specificity undergo chemotropic growth and reach a cell adherence stage. However, two phenotypes were revealed after adherence in otherwise genetically different strains: 1) those that completed cell fusion and exchanged cytoplasmic contents; 2) strains unable to undergo cell wall dissolution (52) (Fig 1; Fig. 3). In cells blocked in fusion, the oscillation of MAK-2 and SOFT at fusion tips was extended, suggesting that arrested cells fail to transit from chemotropic interactions to cell wall dissolution and membrane merger. Two linked loci, Cell Wall Remodeling (*cwr)-1* and *cwr-2* are necessary and sufficient to regulate cell wall dissolution. Consistent with their role in kind recognition, alleles at *cwr-1* and *cwr-2* are highly polymorphic, fall into six discrete haplogroups within *N. crassa* populations and show evidence of trans-species polymorphisms (52). The *cwr-1/cwr-2* loci segregate independently from the *doc-1/doc-2* loci. As with *doc-1* and *doc-2,* allelic differences at *cwr-1* and *cwr-2* negatively regulate somatic cell fusion, as *Δcwr-1* and *Δcwr-2* mutants are capable of undergoing both self-fusion and fusion with formerly incompatible partners.

Sequence analyses of orthologs of *cwr-1* and *cwr-2* alleles in population samples from filamentous fungal species where the two loci are linked revealed high sequence diversity in species of *Neurospora*, *Fusarium*, *Trichoderma* and *Zymoseptoria*. Allele-specific haplogroups that show trans-species polymorphism at *cwr-1* and *cwr-2* were identified among isolates of different species of *Fusarium* (*F. tricinctum, F. oxysporum, F. fujikuroi, F. graminearum, F. proliferatum* and *F. verticillioides*) (52). However, the *cwr-1/cwr-2* haplogroups identified in *N. crassa* were not conserved in the *Fusarium cwr-1* and *cwr-2* haplogroups, indicating convergent evolution and that polymorphisms at these loci can be

repeatedly lost and gained. Genomic pairs of *cwr-1* and *cwr-2* are only present in sublineages of the Pezizomycotina, one of the only two groups where complex multicellularity has arisen in fungi (72). These observations suggest that diversification of *cwr* alleles alongside with the appearance of multicellularity could be linked to formation of the syncytial fungal colonies.

The *cwr-1* locus encodes a secreted polysaccharide monooxygenase (PMOs) (52). PMOs catalyze the oxidative cleavage of glycosidic bonds in recalcitrant substrates, such as cellulose, hemicellulose and chitin (118). CWR-1 is a member of the auxiliary activity (AA) 11 family homologous to a chitin-active copper-dependent PMO from *Aspergillus oryzae* (60). Genetic analyses showed that cell fusion arrest is mediated by interactions between CWR-1 in one cell and CWR-2 from a different haplotype in the partner cell. CWR-2 contains two conserved 'domains of unknown function' and eight predicted transmembrane regions (52). These data suggest that CWR-2 may function as a membrane receptor that could interact with a haplotype-specific cell wall product produced by the activity of CWR-1. A somewhat analogous situation is observed during neural self-avoidance in *Drosophila*, where alternative splicing of *Dscam* results in thousands of distinct ectodomains with self-binding specificity (130; 131).

V. Allorecognition after somatic cell fusion

1. Allorecognition and germling-regulated death

In crosses between wild isolates, progeny that are capable of undergoing chemotropic interactions and cell wall dissolution often display rapid cell death upon fusion. At least two loci in *N. crassa* mediate germling-regulated death (GRD) (Fig. 3). GRD is controlled by allelic interactions between *rcd-1-1* and *rcd-1-2* (18) or non-allelic interactions between the antagonistic and closely linked *plp-1* and *sec-9* (58). In germling pairs, GRD occurs rapidly (~20-25 minutes) after fusion of *rcd-1* or *sec-9/plp-1* incompatible cells and is associated with massive vacuolization and cell lysis (58). Genetic differences at *rcd-1* or *sec-9/plp-1* also induce death upon hyphal fusion between incompatible colonies.

The allorecognition determinant *rcd-1* encodes a 257 amino acid protein of unknown biochemical function (18). Alleles of *rcd-1* fall into two haplogroups and are one of the most polymorphic genes in the genomes of wild *N. crassa* isolates; alleles of the two *rcd-1* haplogroups also show trans-species polymorphisms (18). Strains carrying a deletion of *rcd-1* form viable heterokaryons with formerly incompatible cells, while the co-expression of two antagonistic *rcd-1-1* and *rcd-1-2* alleles is sufficient to trigger cell death in fused germlings

and hyphae (18). *rcd-1* belongs to a large gene family in fungi, with some species, like *N. crassa*, having only one *rcd-1* locus, while other species have multiple *rcd-1* paralogs within their genomes. These observations suggest that the function of this allelic allorecognition system might be conserved throughout the fungal kingdom.

The second allorecognition system that induces GRD upon cell fusion involves the linked loci *sec-9* and *plp-1* (58). *sec-9* encodes a t-SNARE protein, which is orthologous to a protein required for secretory vesicle/plasma membrane fusion in *Saccharomyces cerevisiae* (9); *sec-9* is an essential gene in *S. cerevisiae, P. anserina* and *N. crassa*. The *plp-1* locus encodes a protein with an N-terminal patatin-like phospholipase domain, a central NB-ARC domain and C-terminal tetratricopeptide repeats. Incompatible genetic interactions between *sec-9* and *plp-1* from different haplogroups are necessary and sufficient to induce GRD. In *N. crassa*, gene genealogies revealed four long-diverged haplogroups for *sec-9* and *plp-1,* which show no recombination and are in the top 0.1% for the number of polymorphic sites and nucleotide diversity in population samples (58). As with other allorecognition loci, *sec-9* and *plp-1* alleles show signatures of balancing selection and trans-species polymorphism (58; 84). In *P. anserina* and *C. parasitica, sec-9/plp-1* also functions in hyphal incompatibility (13; 58); evolutionary analyses indicates that convergent evolution is the most strongly supported scenario for the common use of the *plp-1/sec-9* system in allorecognition in these three fungal genera (58).

In *N. crassa* and *P. anserina*, the C-terminal region of SEC-9, which includes the SNARE domains essential for protein function, is highly polymorphic between different haplogroups (58). These polymorphic SNARE domains mediates allelic specificity via interactions with incompatible PLP-1 proteins. In *N. crassa*, co-immunoprecipitation experiments showed that incompatible SEC-9 and PLP-1 from different haplogroups induces PLP-1 complex formation (58). Both the phospholipase catalytic activity of the patatin-like domain and a functional NB-ARC domain are necessary for full GRD induction. Allorecognition and cell death are dependent upon physical interaction between incompatible SNARE domains of SEC-9 and tetratricopeptide repeats of PLP-1. The tripartite architecture of PLP-1 is reminiscent of NOD (nucleotide-binding and oligomerization domain)-like receptors in plants and animals (30). NLRs are intracellular multi-domain modular sensors in plants and animals involved in innate immunity (66) and detect pathogen-associated molecular cues or danger signals to induce downstream signaling, resulting in cell death.

These observations suggest that the NLR-like protein PLP-1 monitors the essential SNARE protein SEC-9.

2. Allorecognition-induced death during hyphal fusion

In contrast to GRD, hyphal fusion incompatibilities have been assessed in a large number of fungal species (20; 51; 110) and is termed vegetative (or heterokaryon) incompatibility (HI). Despite limiting cooperation (*i.e.* resource sharing) between fungal colonies, HI prevents genome exploitation, the spread of deleterious mycoviruses and horizontal transfer of mitochondrial plasmids (25; 26; 125). Experimental evolution studies indicate that HI evolution and maintenance is probably driven by the need to counteract selection for freeloaders (3; 17).

HI-associated PCD is spatially restricted to heterokaryotic fusion cells in which cytoplasmic mixing has occurred. In many species, HI results in a barrage line that separates genetically averse strains (110). At the cellular level, HI initiates septal plugging of fusion cells, isolating them from the rest of the colony (48; 65). Heterokaryotic cells undergo extensive vacuolization, reactive oxygen species production, lipid droplet formation, cell wall thickening and hyper-septation, culminating in cell lysis and release of cellular contents to the extracellular medium (110). In fungal plant pathogens, vegetative compatibility groups (VCGs) have been used as a proxy of genetic relatedness (77), as isolates within a common VCG often share similar virulence and host-specificity functions (33; 70).

The molecular basis of HI has been investigated in three ascomycete species: *N. crassa*, *P. anserina* and *C. parasitica* (15; 87; 110). The genes controlling HI in *P. anserina* and *N. crassa* have been named *het* (*het*erokaryon), while in *C. parasitica* they are known as *vic* (*v*egetative *inc*ompatibility). In *N. crassa*, the characterized *het* loci interactions are restricted to the hyphal stage and do not cause GRD. The number of identified *het* loci in the three species varies between twelve (*N. crassa*) and six (*C. parasitica*) (15; 48). In *C. parasitica*, disruption of *vic* genes allowed the spread of virulence-attenuating mycoviruses between formerly incompatible colonies (134).

The inability to form viable heterokaryons using auxotrophic markers correlates perfectly with induction of cell death upon fusion of incompatible hyphae (6; 41). This 'forced heterokaryon' methodology has been used to identify *het* genes in other fungal species, including *Aspergillus oryzae* (86) and *Fusarium* (71). The use of strains carrying genomic rearrangements enabled the identification and genetic mapping of *N. crassa het* loci

(87; 98; 99), while more recent approaches have taken advantage of population genomics (86; 135).

As in the other allorecognition systems, genes controlling HI are highly polymorphic and multiallelic (95). The number of alleles in wild populations varies from two (*e.g.*, *het-s* in *P. anserina* (122)) to more than ten (*e.g.*, *het-c* in *P. anserina* (4)). Highly polymorphic *het* loci are frequently found in hypervariable genomic regions (135). Consistent with balancing selection, alleles with different HI specificity are found in nearly equal frequency in wild populations (4; 24; 56; 84; 132). Using a comparative population genomics approach, loci with highly polymorphic alleles that displayed trans-species polymorphism and balancing selection were used to identify candidate *het* genes in *Neurospora* populations (135).

Genetic interactions triggering HI involve two or more antagonistic alleles of the same gene (allelic HI systems) or alleles belonging to different genes (non-allelic HI systems) (20; 110). At present, three incompatibility systems are strictly allelic – the *het-S*/*het-s* system in *P. anserina* (111) and the *het-e1*/*het-e2*/*het-e3* and *rcd-1-1*/*rcd-1-2* recognition systems in *N. crassa* (18; 135). Most of the characterized non-allelic HI systems involve interactions between alleles of closely linked genes; examples are the *het-c*/*pin-c*, *het-6*/*un-24* and *sec-9/plp-1* systems in *N. crassa* (56; 58; 68; 75) and *vic-6*/*pix-6* non-allelic incompatibility in *C. parasitica* (13). In *P. anserina het-c*/*het-e* or *het-c*/*het-d* non-allelic HI systems, the genes are located on different chromosomes (31; 109).

In a number of filamentous ascomycete species, including *N. crassa*, *Sordaria brevicollis*, *Ascobolus stercorarius*, *A. heterothallicus* and perhaps the black truffle, *Tuber melanosporum*, the MAT locus functions as a *het* locus (47; 113). As with allelic differences at *het* loci, somatic cell fusion between opposite mating type hyphae results in compartmentation of the fusion cell and rapid cell death (41; 46). However, unlike most allorecognition loci, the two *mat* haplotypes in filamentous ascomycete fungi are composed of evolutionarily unrelated genes, termed "idiomorphs" (83). The mating type idiomorphs do not show variability within populations and are highly conserved between different filamentous ascomycete species (7). In *N. crassa*, mating type incompatibility only occurs in the hyphal stage and is dependent on an unlinked locus, called "tolerant" or *tol* (115). TOL, similar to predicted proteins from other *het* loci, contains a HET domain (see below). Mutations in *tol* block mating type incompatibility, but do not affect sexual fertility (88). In *C. parasitica,* the *vic-4-1*/*vic-4-2* system is also composed of idiomorphic genes, although they do not play a role in mating (13).

3. Molecular mechanisms of programmed cell death in allorecognition.

Despite the shared evolutionary signatures of *het* genes, they show little conservation between species (95; 124). Nevertheless, molecular characterization of various HI systems shows that *het* genes encode proteins with shared domains and that belong to large protein families (20; 51). A particular protein domain of unknown biochemical function, named 'HET', is encoded by more than half of HI genes (135). *In silico* analyses established a potential evolutionary relation between the HET domain and the Toll/interleukin-1 receptor/resistance (TIR) domain that plays key roles in plant and metazoan innate immune systems (30). TIR domains are involved in homotypic interactions in signaling complexes, which trigger cell death by NAD^+ depletion (64; 127). NAD^+ cleavage by TIR domains has an ancient origin (32) and represents a tempting hypothesis for the function of HET domains.

The products of multiple *het* genes (*het-e*, *het-d*, *het-r, PaPlp1*) from *P. anserina* (31), *plp-1* from *N. crassa* (58), *vic2* and *vic4-2* from *C. parasitica* (13) belong to the family of fungal NLR-like proteins (30). Remarkably, the HET and TIR domains are found similarly situated in the domain architectures of fungal NLR-like proteins and plant/metazoan NLRs (30). Thus, fungal NLR-like proteins may function similarly to NLR immune receptors in plants and animals, suggesting that proteins of this architecture are major contributors to innate immunity in all three kingdoms.

In *P. anserina*, the *het-S/het-s* system is unique in that it functions as a prion. The HET-S protein is a pore-forming toxin, targeting the plasma membrane when co-expressed with an alternate allelic variant termed HET-s (55; 114). HET-S and HET-s consist of two domains: an N-terminal globular α-helical HeLo domain (55) and a C-terminal prion-forming domain (PFD) (1). The inactivation of the HeLo domain allows the HET-s variant to propagate as a prion [Het-s] (16; 80). The transconformation of the PFD of the cytotoxic HET-S variant by [Het-s] aggregates activates the HeLo domain of HET-S, leading to the release of an N-terminal α-helix that targets the plasma membrane to induce rapid cell death (114). Evolutionary analyses linked the HeLo domain to domains controlling cell death in plants and animals, notably the 4HB domain (4-helix bundle) of the MLKL (Mixed Lineage Kinase Domain-Like) protein, which controls necroptosis (19; 62).

VI. Why do fungi have so many allorecognition mechanisms?

The relationship between protein architectures of GRD and HI determinants and proteins involved in innate immunity systems in plants and metazoans have led to the hypothesis that fungal allorecognition genes may be recruited from molecular circuits mediating broader biotic interactions in fungi, akin to a fungal immune system (123). Importantly, higher genetic relatedness appears to correlate with cooperative behaviors to avoid parasitism and to prevent the exploitation of public goods (for example, access to nutrients in a fungal colony) by cheaters (5; 29; 74). Hence, fusion between conspecific but genetically distant individuals and consequent somatic chimerization poses a dilemma. On the one hand, fusion could prove beneficial due to an enhanced ability to withstand environmental variations and eventual increase in organismal size, in turn favoring reproductive output. Moreover, heterokaryon formation in fungi can result in functional diploidy and mitotic recombination during the parasexual cycle (100). On the other hand, fusion can result in the transmission of infectious elements and in the incorporation of deleterious mitochondrial or nuclear genotypes that negatively impact fitness (2; 3; 26; 34; 133). Fungi, in particular, appear to favor the latter option, having evolved a very large number of allorecognition systems to limit genome exploitation. However, a recent study demonstrated that fusion in *N. crassa* is mutually beneficial compared to fusion blockage by allorecognition (3), suggesting a dynamic relationship between beneficial aspects of cell fusion *versus* the risks associated with it. Importantly, mechanisms regulating somatic allorecognition are suppressed during sexual reproduction. Indeed, wild isolates with allelic specificity differences at *doc, cwr, sec-9/plp-1, rcd-1* and *het* loci are able to productively mate and produce meiotic progeny, suggesting that these allorecognition systems have evolved to specifically avoid somatic cell fusion, but allowing at the same time diversification to occur through outbreeding, potentially improving adaption to new ecological niches, as shown to occur during sexual reproduction (49).

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of *B. schlosseri* and Kristin Little, Murray Tipping and Karine A. Gibbs (Harvard University) for images of *P. mirabilis*.

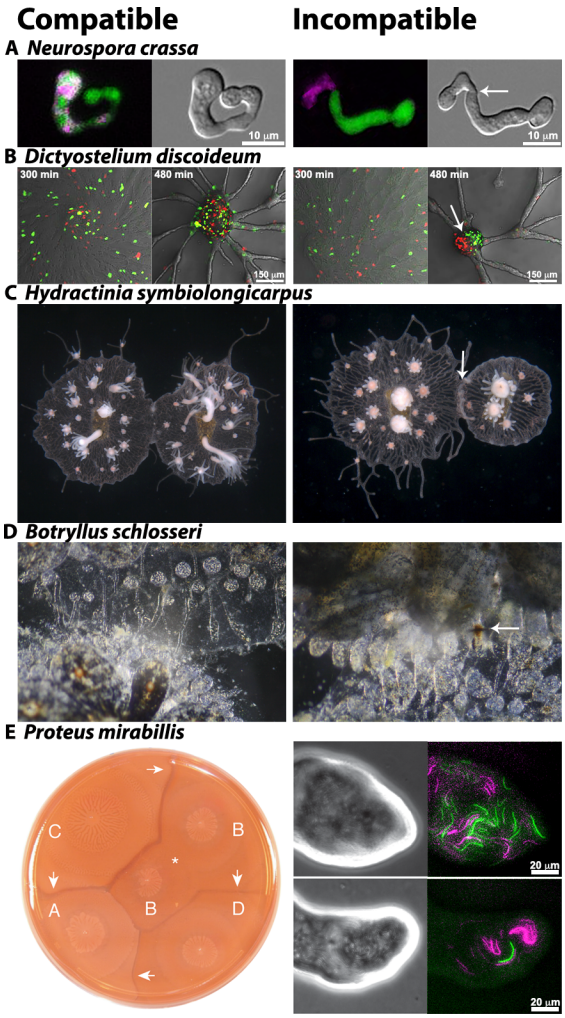

Figure 1. Allorecognition in distinct domains of life. (A) Allorecognition upon cell-cell contact in *N. crassa*. Germlings expressing cytoplasmic GFP (green) were paired with germlings stained with FM4-64 (magenta). Note fusion and cytoplasmic mixing on the left (compatible interaction) *versus* a cell fusion block upon cell-cell contact on the right (incompatible interaction) mediated by genetic differences at *cwr-1/cwr-2* (52). (B) Allorecognition during starvation-induced development in *D. discoideum*. Strain pairings in which 5% of the cells are labeled with GFP (green) and 5% with RFP (red). Panels represent time points after mixing (300 min and 480 min, as indicated). Red and green cells are intermixed regardless of their allotypes (compatible and incompatible genetic backgrounds) at 300 min due to cAMP signaling for aggregation; however, at 480 min, the red and green cells segregate from each other due to expression of allorecognition determinants TgrB1 and

TgrC1 (73). Credits: Shigenori Hirose and Gad Shaulsky, Baylor College of Medicine. (C) Allorecognition during polyp fusion in *H. symbiolongicarpus* mediated by genetic differences at Alr1 and Alr2 (69; 89). Left and right panels show compatible and incompatible (rejection reaction) at early stages of fusion, respectively. Rejection causes extensive damage to adjacent colonies and may lead to the formation of hyperplastic stolons. Credits: Matthew L. Nicotra, University of Pittsburgh. (D) Allorecognition mediated by genetic differences in *fuhc(sec)* and *fuhc(tm)* (91) during colonial chimerization in *B. schlosseri*. Images show extracorporeal vasculature of two colonies (top and bottom, respectively), showing interaction between via the ampullae (ends of the vasculature). On the left, a compatible pairing results in fusion. In incompatible pairings, a rejection response and fusion blockage occurs, as shown by the dark regions where the ampullae touch (right panel). Credits: Anthony De Tomaso, University of California, Santa Barbara. (E) Allorecognition mediated by genetic differences at *ids* genes during swarming behavior of *P. mirabilis* (121). Petri dish shows boundaries and merging colonies of *P. mirabilis*; arrows indicate boundaries between incompatible swarm colonies, whereas an asterisk marks the merging of two compatible populations. Strains A, B and C are independent wild type strains, while D lacks the *ids* self-recognition genes (44). Figure adapted from (78) with permission. Right panels show compatible (top) and incompatible (bottom) co-swarmed populations of *P. mirabilis*. The two populations have been labeled with GFP (green) or RFP (magenta), with unlabeled parental strain in the mixture. Images show the leading edge of the second swarm ring; note that fewer cells of the non-self strain (green) are present after diverting into a swarm-incompatible state (bottom). Credits: Kristin Little, Murray Tipping and Karine A. Gibbs, Harvard University. Arrows indicate the zone of interaction between incompatible pairs.

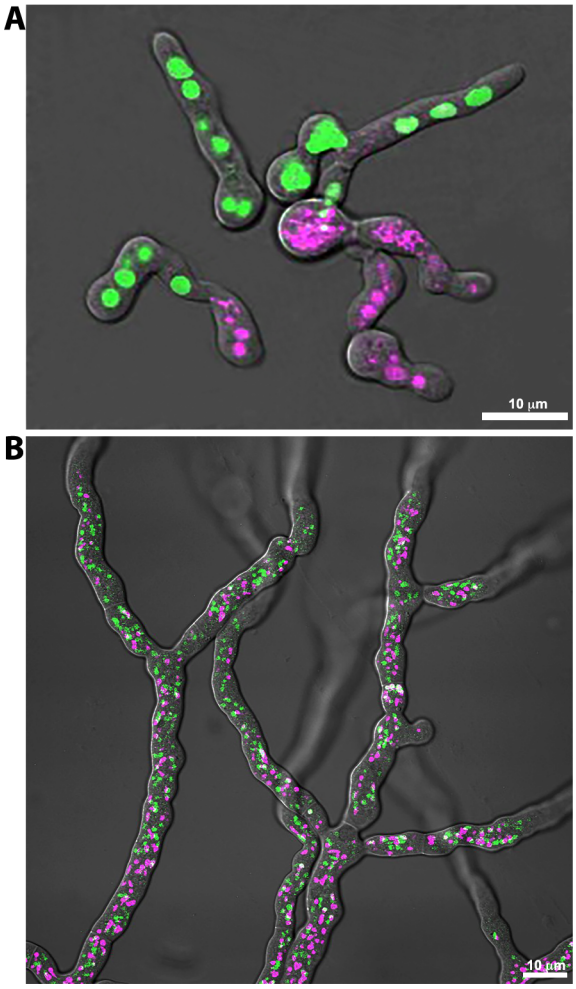

Figure 2. The syncytial lifestyle of filamentous fungi. Two genetically compatible strains of *N. crassa*, whose nuclei have been labeled with either histone H1-GFP (green) or histone H1-DsRed (magenta) were paired and allowed to fuse at different developmental stages. Germlings and hyphae that have fused to form a single colony sharing a mixture of nuclei are shown on A and B, respectively.

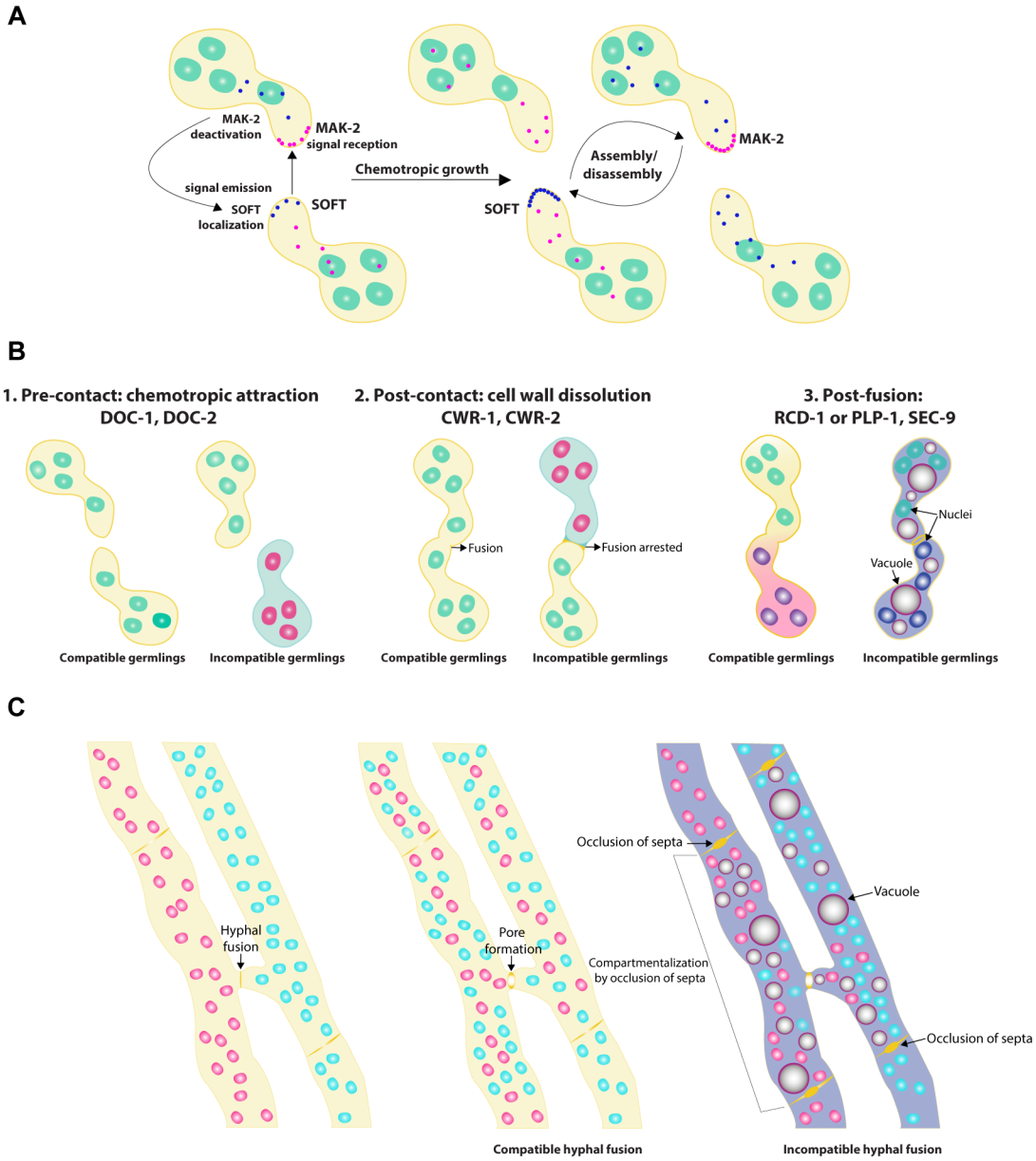

Figure 3. Somatic cell fusion and allorecognition checkpoints in *N. crassa*. (A) During chemotropic growth that precedes cell fusion, MAK-2 and SOFT are recruited to the plasma membrane of CATs (39); both cells send and receive signals generating an appropriate cellular response that culminates in cell fusion. (B) Three cellular allorecognition checkpoints (pre-contact, post-contact and post-fusion) identified during germling fusion controlled by the *doc-1*/*doc-2*, *cwr-1*/*cwr-2*, *rcd-1* and *plp-1*/*sec-9* loci, as indicated (52; 58; 59). (C) Allorecognition due to heterokaryon incompatibility occurs during hyphal fusion and is controlled by *het* genes (20; 110). Refer to the main text for details on the different phenotypes depicted in this scheme.

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