E Pluribus Unum: The Fungal Kingdom as a Rosetta Stone for Biology and Medicine

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THE Genetics Society of America's (GSA's) Edward Novitski Prize recognizes a single experimental accomplishment or a body of work in which an exceptional level of creativity, and intellectual ingenuity, has been used to design and execute scientific experiments to solve a difficult problem in genetics. The 2019 recipient is Joseph Heitman, who is recognized for his work on fungal pathogens of humans and for ingenious experiments using yeast to identify the molecular targets of widely used immunosuppressive drugs. The latter work, part of Heitman's postdoctoral research, proved to be a seminal contribution to the discovery of the conserved Target of Rapamycin (TOR) pathway. In his own research group, a recurring theme has been the linking of fundamental insights in fungal biology to medically important problems. His studies have included defining fungal mating-type loci, including their evolution and links to virulence, and illustrating convergent transitions from outcrossing to inbreeding in fungal pathogens of plants and animals. He has led efforts to establish new genetic and genomic methods for studying pathogenesis in *Cryptococcus* species. Heitman's group also discovered unisexual reproduction, a novel mode of fungal reproduction with implications for pathogen evolution and the origins of sexual reproduction.

Genetics strives to explain biological diversity by identifying and analyzing variants in form and function. Solving biological questions requires creativity and ingenuity, and for some, this requires little more than agar medium, petri dishes, toothpicks, and the right organism. Genetics began with the analysis of crosses formulated by Mendel, and came of age with the DNA double helix, solution of the genetic code, and molecular biology. Integral to genetics is a focus on model systems, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila*, *Arabidopsis*, and *Caenorhabditis elegans*. We now have access to abundant sequence data, and novel approaches (*e.g.*, clustered regularly interspaced short palindromic repeats, CRISPER) open doors to study virtually any organism. Our focus is on model and pathogenic fungi addressing fundamental biological questions, and unmet medical needs in transplantation and infectious diseases. Our experimental palette is the fungal kingdom, and with several million species, opportunities abound. These reflections are in homage to Edward Novitski, on occasion of the GSA 2019 Novitski Prize (Figure 1).

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Discovery of TOR via Yeast Genetics

As an European Molecular Biology Organization fellow with Mike Hall at the Biozentrum in Basel, Switzerland, we discovered targets of immunosuppressants in *S. cerevisiae* (Heitman *et al.* 1991a,b). At that time, cyclosporine was a gold-standard immunosuppressant for transplant patients, while FK506 and rapamycin were still experimental drugs. All three are natural antimicrobial products produced by soil microbes, likely to inhibit competitors in the environment. With our collaborator Rao Movva at Sandoz Pharmaceuticals, the FKBP12 protein (which binds FK506 and rapamycin) was purified and sequenced, and we cloned and deleted the gene via transformation, and homologous recombination (Figure 1). At the time, many thought FKBP12 would not be involved in the activity of FK506 or rapamycin, due to the protein's high abundance and ubiquity across tissues of the body. Remarkably, yeast mutants lacking FKBP12 were viable and completely resistant to rapamycin. This result produced by simple genetics experiments proved FKBP12 is required for drug action. Because the drug is toxic to the cell, but FKBP12 is not essential, we hypothesized there was a target of the FKBP12–rapamycin complex. To identify this target, we isolated rapamycin-resistant mutants defining three genes: *FPR1* encoding FKBP12, and two novel genes we named

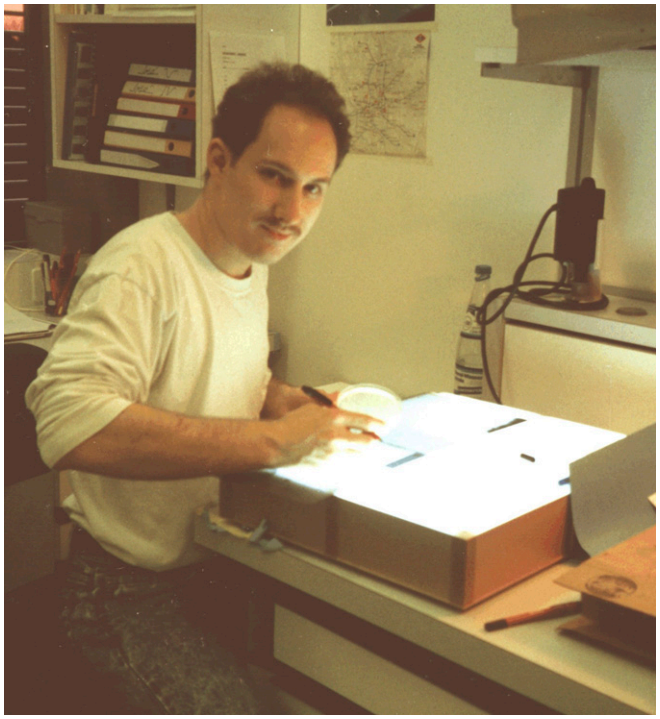

Figure 1 At the Biozentrum of the University of Basel, Switzerland, cloning the yeast gene encoding FKBP12 in early 1990.

Figure 2 With Mike Hall and Rao Movva at the Lasker Award reception in September 2017, at which Mike Hall received the Lasker Award for the discovery of TOR (Target of Rapamycin) as a globally conserved nutrient sensor that controls cell growth.

TOR1 and *TOR2*. We proposed these novel yeast genes would have human homologs, which were later found by others (mTOR, for mammalian TOR). We now know the TOR signaling cascade is conserved from yeast to humans and senses nutrients to control cell growth. Drugs targeting TOR are widely used in transplantation, cancer chemotherapy, and interventional cardiology, and TOR inhibitors may find further indications, such as in aging (Figure 2).

Calcineurin as a Globally Conserved Eukaryotic Microbial Pathogen Virulence Factor

A year after I started my laboratory at Duke, John Perfect, the Infectious Diseases Division Chief, called (Figure 3). John studies *Cryptococcus* and was seeking a yeast geneticist to collaborate with on a conundrum: the immunosuppressant cyclosporine exhibited robust antifungal activity against *Cryptococcus in vitro* and some efficacy in mouse infection models (Mody *et al.* 1988, 1989), yet patients receiving cyclosporine remained at high risk for cryptococcal meningitis. Following our meeting, I returned to the laboratory with a strain (H99) and a few reprints.

Initially, we struggled to repeat previous *in vitro* findings of cyclosporine antifungal activity under standard conditions (YPD medium, 30°), until we realized that Mody and colleagues had incubated their cultures at 35°. Then, we found that both cyclosporine and FK506 exhibited potent activity, but only at higher temperatures, like 37°. We hypothesized their target, the phosphatase calcineurin, would be required for growth at 37°, and mutating genes encoding the phosphatase catalytic or regulatory subunits would confer temperature-sensitive growth. Following genetic disruption of the *CNA1* gene, transformants exhibited temperature-sensitive growth, and reintroducing the gene restored 37° growth. In an animal model, calcineurin mutants were avirulent, and reintroducing the gene restored virulence, fulfilling Falkow's molecular virulence postulates and establishing calcineurin as a molecular virulence determinant (Odom *et al.* 1997). Subsequently, we and others showed calcineurin is globally required for pathogenicity of human fungal pathogens (*e.g.*, *Candida spp.*, *Aspergillus fumigatus*, *Mucor circinelloides*), plant fungal pathogens, and eukaryotic parasite pathogens (Cruz *et al.* 2002; Bader *et al.* 2003; Blankenship *et al.* 2003; Sanglard *et al.* 2003; Steinbach *et al.* 2006, 2007; Lee *et al.* 2013; Park *et al.* 2019).

The answer to the original conundrum of why cyclosporine is active *in vitro* and in experimental animal lung infection but not in humans against *Cryptococcal meningitis* is that cyclosporine does not cross the blood–brain barrier, and therefore is ineffective against brain infections in patients. Our ongoing studies have two goals: (1) define downstream transcriptional and post-transcriptional signaling branches (Park

Figure 3 With Arturo Casadevall and John Perfect at the Association of American Physicians induction meeting in April 2006.

et al. 2016; Chow *et al.* 2017) and (2) develop FK506 analogs as novel therapeutics for fungal infections (Juvvadi *et al.* 2019).

Mating-Type Locus of Pathogenic Fungi

Our research on pathogenic yeast expanded to focus on the mating-type locus (*MAT*), which orchestrates sexual reproduction. My interest in *MAT* was piqued by serving on the Ph.D. thesis committee for Tim James, who cloned multiple *MAT* loci by linkage to the *MIP* gene (James *et al.* 2004, 2006). We were motivated to study *MAT* because previous studies found: (1) mating type was linked to virulence (Kwon-Chung *et al.* 1992), (2) one mating type (α) predominated, and (3) sexual reproduction produced infectious spores. Early work by Edman revealed a small, *MAT*-specific region (Moore and Edman 1993) that served as a probe for June Kwon-Chung (Karos *et al.* 2000) to identify a larger ∼50-kb *MAT* region. This revealed that the pathogenic yeast *MAT* locus was unusual compared to smaller *MAT* loci of budding yeast and other fungi.

Our studies on *MAT* pursued two complementary directions. Our first goal was to identify genes encoding hypothesized homeodomain factors that were not present in the defined 50-kb interval. These studies, conducted by a fellow in the laboratory, Christina Hull, identified the *SXI1* gene from the *MAT*α locus (sex inducer 1, also known as "sexy one") and *SXI2* from the *MAT***a** locus ("sexy two") (Hull *et al.* 2002, 2005). Concomitantly, a graduate student, Rob Davidson, sought to delete the 50-kb *MAT* interval, but he was unable to isolate deletions in a haploid, suggesting the presence of essential gene(s) in the interval. Undaunted, Rob set out to delete this region in an α/**a** diploid and succeeded, but the mystery deepened; the resulting Δ/**a** mutants were self-filamentous and sporulated like the wild-type α/**a** parent. Spore dissection isolated only **a** progeny, in accord with essential gene(s) in the region. For 24 hr, we thought there was a problem with the experiment, until we realized this meant the entire *MAT* locus had not been deleted and that unknown regions lay beyond the borders of the defined interval.

Following this, a fellow in the laboratory, Klaus Lengeler, cloned and sequenced the entire *MAT*α and *MAT***a** mating-type alleles using BAC libraries, and robotic Sanger sequencing with new Applied Biosystems instruments collaborating with Fred Dietrich. Klaus's studies established that *MAT* spans > 100 kb and encompasses ∼25 genes (Lengeler *et al.* 2002). He found that the left *MAT* border mapped earlier was within rather than flanking *MAT*. The second approach resolved the structure of *MAT* by mapping the *SXI1* gene, which turned out to be the first gene in the locus, ∼50 kb away from the previously mapped left border.

Subsequent work followed on *MAT* evolution throughout the *Cryptococcus* pathogenic species complex (Fraser *et al.* 2004, 2005; Byrnes *et al.* 2011), on how the derived inbreeding bipolar system of the pathogenic species arose from nonpathogenic species with a more complex tetrapolar outbreeding mating-type system (Hsueh *et al.* 2008; Findley *et al.* 2012; Sun *et al.* 2017), and on the identification of *MAT* in other model and pathogenic fungi including *Candida* species, dimorphic human fungal pathogens, dermatophytes, *Malassezia*, *Phycomyces*, *Mucor*, and *Rhizopus* (Fraser *et al.* 2007; Idnurm *et al.* 2008; Lee *et al.* 2008; Reedy *et al.* 2009; Gryganskyi *et al.* 2010; Li *et al.* 2010, 2013; Metin *et al.* 2010; Gioti *et al.* 2012; Guerreiro *et al.* 2013). These studies, and those discussed below on unisexual reproduction, led to a model that there was an evolutionary epoch in which sexual reproduction occurred before there were mating types or sexes (Heitman 2015).

Unisexual Reproduction: Implications for Microbial Pathogen Evolution and Origins of Sexual Reproduction

Our second foray into fungal sexual reproduction stemmed from discussions with Arturo Casadevall (Figure 3). In his terms, much of what we labor to accomplish is map-making until an "Aha!" moment in which one realizes how the pieces fit together as part of a greater whole, what Arturo calls "the tectonic plate moment," analogous to the realization that the continents fit together as pieces of an older, single continent. His insight ignited our efforts on fungal sexual reproduction, which he said had the potential to transcend fields and be of lasting significance.

June Kwon-Chung originally described the sexual cycles of *Cryptococcus neoformans* and *C. gattii* (Kwon-Chung 1975, 1976a,b). Jef Edman and colleagues also described an unusual developmental process whereby some α strains produce hyphae, basidia, and spores without the requirement of a mating partner. Instead, an α strain undergoes development all on its own, which they termed haploid or monokaryotic fruiting, and they concluded was a strictly mitotic process leading to spore production (Wickes *et al.* 1996).

We considered an alternative hypothesis: that this might represent homothallic, self-fertile sexual reproduction. A talented fellow in the laboratory, Xiaorong Lin, characterized the process in detail, discovering ploidy changes (from haploid to diploid to haploid), necessary key meiotic regulators (*DMC1* and *SPO11*), and the occurrence of independent chromosomal assortment and meiotic recombination (Lin *et al.* 2005). Her studies revealed that this is a novel type of fungal homothallic sexual reproduction that we named unisexual reproduction. Unisexual reproduction also occurs in other pathogenic and nonpathogenic fungi, and possibly also in parasites (Alby *et al.* 2009; Heitman 2010; Wilson *et al.* 2015).

Our studies revealed that unisexual reproduction commonly occurs in nature, impacting the population and genomic structure of *C. neoformans* (Lin *et al.* 2006, 2007, 2009; Bui *et al.* 2008; Phadke *et al.* 2014; Desjardins *et al.* 2017). Unisexual reproduction might (1) explain why the α mating type predominates in the environment and patients (> 99%), (2) be the process that produces infectious spores, and (3) drive inbreeding, and yet enable sufficient genetic diversity and exchange to reap the benefits of sexual reproduction (Ni *et al.* 2013) (Figure 3).

Fungal Genomics and Karyotype Reorganization

Our entry into fungal genomics began with a cryptic voicemail message: "Joe, this is Gerry Fink. Please call me." Gerry assembled a small forum at the Whitehead Institute to discuss a Fungal Genome Initiative to sequence genomes of pathogenic and model fungi. *C. neoformans* was an early focus (Loftus *et al.* 2005; D'Souza *et al.* 2011; Janbon *et al.* 2014; Farrer *et al.* 2015). Several sequencing centers (the Whitehead Institute, later the Broad; Stanford; TIGR; and UBC Vancouver) and additional key collaborators (Guilhem Janbon, Pasteur and James Fraser, Brisbane) were enlisted. The field rapidly advanced and there are now > 500 sequenced and publicly available genomes for the globally distributed serotype A *C. neoformans* species (Desjardins *et al.* 2017; Rhodes *et al.* 2017). These resources enabled development of large-scale gene deletion collections (Suzanne Noble and Hiten Madhani), revealed RNA interference loss in the Pacific Northwest outbreak species, and traced evolutionary genomic trajectories (with Kaustuv Sanyal) (Liu *et al.* 2008; Feretzaki *et al.* 2016; Yadav *et al.* 2018).

These fungal genome projects revealed striking diversity across the kingdom, with repeated, independent evolution of virulence (Butler *et al.* 2009; Floudas *et al.* 2012; Martinez *et al.* 2012; Corrochano *et al.* 2016; Persinoti *et al.* 2018). Stimulated by conversations with Rytas Vilgalys, we also focused on a group of closely aligned species encompassing pathogenic and nonpathogenic species in the *Cryptococcus* genus, and the sibling genus *Kwoniella*, discovering new species and novel forms of self-fertility, and illustrating *MAT* evolutionary trajectory (Findley *et al.* 2009, 2012; Metin *et al.* 2010; Rodriguez-Carres *et al.* 2010; Guerreiro *et al.* 2013; Sun *et al.* 2017; Passer *et al.* 2019).

Our studies revealed two forces driving karyotypic change. First, chromosomal translocations occurred frequently involving intercentromeric recombination driven by retrotransposons shared between large regional centromeres (Janbon *et al.* 2014; Sun *et al.* 2017; Yadav *et al.* 2018; Passer *et al.* 2019). These translocations cause chromosome arm exchanges without unstable dicentric or acentric intermediates, and are implicated in the transition from outbreeding tetrapolar mating types to inbreeding fused bipolar mating types of the pathogens (Sun *et al.* 2017). Second, our collaborator Christina Cuomo first appreciated that some *Kwoniella* species have unusual giant chromosomes. Our studies with Christina, Marco Coelho, Marcia David Palma, and Sheng Sun revealed that pathogenic *Cryptococcus* species all have 14 chromosomes, as does a basal *Kwoniella* species, yet other species have as few as 3. The chromosomes appear to have fused together into a progressively larger and larger chromosome, resulting in a giant chromosome (15–18 Mb). This seems a finding Ed Novitski would have found interesting.

These genomic studies bring us full circle in paying homage to Ed Novitski and his pioneering studies of *Drosophila* chromosome structure. Ed was a gifted, creative geneticist who isolated his first *Drosophila* mutant as a high-school student (Novitski and Rifenburgh 1938). He had a highly distinguished career (Crow *et al.* 2006) and was a consummate geneticist. His research focused on chromosome structure and dynamics, including giant chromosomes (Lindsley and Novitski 1959; Novitski *et al.* 1981). It is an honor to be associated with his legacy and a testament to being surrounded by legions of talented students, fellows, laboratory staff, collaborators, and colleagues who made this possible. To them, I am grateful, and to the colleagues who make the fungal genetics community collegial.

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