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Neurospora 2010 Plenary Session Abstracts

Abstract
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NEUROSPORA 2010 PLENARY SESSION ABSTRACTS

Session I:

Two *Neurospora crassa* NDR kinases (DBF2 and COT1) perform distinct functions in regulating hyphal morphology.

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The nuclear Dbf2-related (NDR) protein kinases are comprised of two subgroups (represented in *Neurospora crassa* by DBF2 and COT1). In *N. crassa*, DBF2, (a component of the HIPPO pathway in higher eukaryotes) is involved in cell cycle regulation, glycogen biosynthesis and both sexual and asexual reproduction. Defects in other DBF2 and glycogen metabolism pathway components (*mob-1, ccr-4, mst-1* and *gsk-3*) share similar phenotypes such as mitotic arrest, massive hyphal swellings, hyphal tip bursting, glycogen leakage and impaired conidiation, suggesting that DBF2 functions as a link between the DBF2 and glycogen metabolism pathways. Dysfunction of the second *N. crassa* NDR kinase, COT1, leads to cessation of tip extension and massive induction of new branches. COT1 function is dependent on interactions with other proteins (e.g., POD6 and MOB2a/b) and its phosphorylation state. Analyses of point-mutated *cot-1* strains (mimicking non and constitutively-phosphorylated states of conserved NDR residues) indicate the involvement of COT1 phosphorylation in the regulation of hyphal morphology and asexual development by altering cell wall integrity and actin organization. Furthermore, COT1 regulates elongation and branching in an independent manner, which is determined by the phosphorylation states of COT1's activation segment (at Ser417) and the C-terminal hydrophobic motif (at Thr589). Taken together, DBF2 and COT1 share structural similarity and associate with MOB family proteins, yet they perform distinct functions in governing hyphal morphology.

Regulation of COT1 activity and function by interacting proteins and pathways

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Ndr kinases are important for cell differentiation and morphogenesis in various organisms, yet the regulation of their activity and their integration into a cellular signaling context is still fragmentary. The Ndr kinase COT1 is essential for hyphal elongation in *Neurospora crassa* and important for fungal pathogenicity and differentiation in related organisms. The genetic and biochemical amenability of *N. crassa* has facilitated the identification of an intricate signaling network consisting of protein kinase A, MAP kinase cascades and COT1-associated proteins that coordinately regulate vegetative growth and conidiation, hyphal fusion and sexual development. COT1 requires the association with two MOB2-type proteins and phosphorylation of two conserved residues in the activation segment and the hydrophobic motif of the kinase, respecitvely, for their function. We will discuss a model of COT1 activation that involves autophosphorylation of the COT1 dimer in cis at Ser417 in the activation segment and phosphorylation of Thr589 in the hydrophobic motif by the germinal centre kinase POD6. Interestingly, *in vitro* activity of COT1 does not mirror the *in vivo* functionality of COT1. This discrepancy may be explained by a conformational change induced by the two phosphorylation events and altered localization of the COT1-MOB2 complex.

Directed growth in *Neurospora crassa*

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Polarized hyphal growth is a hallmark of filamentous fungi. At certain developmental stages the growth direction of polarized hyphae is strictly controlled. In *N. crassa* conidial germ tubes exhibit mutual attraction, directed growth towards each other and cell fusion. Similarly hyphae in the older, inner parts of the mycelial colony attract each other and fuse. During sexual development trichogynes sense mating pheromones and grow towards the mating partner.

Directed growth requires the interaction of signaling components with factors controlling cellular polarity. Signaling between fusion germlings is mediated by the MAP kinase MAK-2 (Pandey et al. 2004). During mutual attraction of two germlings MAK-2 is recruited to the cell tips in an oscillating manner. Recruitment and relase to/from the plasma membrane is highly coordinated between the fusion partners (Fleißner at al. 2009). In order to gain a better understanding on how MAK-2 signaling results in directed growth, we are analyzing additional factors involved in germling communication and fusion. Recently we identified BEM-1 as an essential factor of germling and hyphal fusion. In *Saccharomyces cerevisiae* BEM-1 functions as a scaffold linking MAP kinase signaling and polarity establishing.

In *N. crassa* BEM-1-GFP fusion constructs localize to growing tips of hyphae and germlings. In addition the protein accumulates around septal pores. During germling fusion BEM-1 concentrates at the zone of cell-cell contact and co-localizes with MAK-2 around the forming fusion pore. However no direct physical interaction between MAK-2 and BEM-1 was found so far.

Bem-1 knock out mutants exhibit a pleiotropic phenotype including shortened aerial hyphae, reduced conidiation and slowed growth. However, hyphal polarity is not affected. Interestingly cell cell interaction and fusion between germlings or between hyphae is completely abolished. No cell cell interaction between germlings or between hyphae in the inner parts of the mycelial colony are observed.

Artificial recruitment of BEM-1 to the plasma membrane of the entire cell surface has a dominant negative effect on germling fusion, but no influence on general polarity establishment and maintenance.

Studying the function of BEM-1 and its relationship to signaling and polarity factors will further our understanding of the molecular mechanisms controlling directed cellular growth.

Characterization of the *ric8* gene in *Neurospora crassa*.

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Eukaryotic organisms respond to changes in their environment via heterotrimeric G protein signaling. The heterotrimer consists of a G alpha protein, which binds and hydrolyzes GTP, and a G beta/G gamma dimer. G proteins associate with membrane-bound G protein coupled receptors (GPCRs), which regulate the G alpha subunit by serving as a guanine nucleotide exchange factor (GEF). Recently, GEF activity by a non-GPCR, RIC8, has been discovered as a novel regulator of G proteins in animals, but has not been characterized in filamentous fungi. Here we present analysis of RIC8 in *Neurospora crassa*. Deletion of *ric8* leads to defects in polar growth and asexual and sexual development, similar to phenotypes observed for a mutant lacking G alpha genes *gna-1* and *gna-3*.

Where is calcium sequestered in hyphae, and how does it get there?

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Calcium is a major signaling molecule in filamentous fungi. It has been hypothesized to play a major role in polar growth. We used a cell fractionation procedure to examine where calcium is sequestered in hyphae of N. crassa. Most of the “pelletable” calcium, presumed to be in organelles, was found in two different compartments. Roughly 30% was in the vacuole. Suspension of vacuoles in a solution of low osmolarity lyses the vacuoles and solubilizes all of the calcium. Sequestration in the vacuole is dependent on a functional cax gene, which encodes a Ca2+/H+-exchange protein in the vacuolar membrane. More than 60% of the calcium was found in “microsomes” obtained after centrifugation at 100,000 x g. Surprisingly this calcium is not solubilized by suspension in low osmotic solutions. It is solubilized in dilute acid or in 1 mM EDTA.

Large amounts of polyphosphate copurify with the calcium in both the vacuoles and the microsomes. Microsomal calcium remains high in deletion strains for cax or for the calcium-pumping ATPases nca-1, nca-2, or nca-3. In fact, deletion of NCA-2 (located in the plasma membrane) causes a significant rise in both vacuolar and microsomal calcium. The data suggest that NCA-2 pumps calcium out of the cell, maintaining a low concentration in the cytosol. In the absence of NCA-2 excess calcium in the cytosol is sequestered in vacuoles and in an insoluble form in another, unidentified, organelle.

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Maintenance of Neurospora centromeres

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Centromeres serve as the foundation for kinetochore assembly. Properly maintained centromeres are essential for attachment of spindle microtubules, which transport chromosomes into daughter nuclei during nuclear division. Neurospora is an excellent organism for the study of centromere function and evolution, as we can use its arsenal of genetic, biochemical and cytological tools to study centromere proteins and centromere DNA composition. We analyzed fungal homologs of the centromere foundation proteins CenpA (CenH3) and CenpC to identify motifs that are either under adaptive or purifying selection. CenH3 swapping experiments between different fungi are providing information about what is required for centromere function in Neurospora. To learn about centromere assembly and maintenance, we subjected *Neurospora crassa* and *Fusarium graminearum* to ChIP-sequencing with tagged CenH3 and CenpC as well as antibodies against histone modifications thought to be required for centromere function. In Neurospora, we found colocalization of CenH3, CenpC and H3 K9me3 in a 100-300 kb region on each chromosome. H3 K4me2 was not enriched at Neurospora centromeres, in contrast to results from studies with plant, fission yeast, Drosophila and human core centromeric regions. DNA methylation, almost always tightly associated with H3 K9me3 in Neurospora, was enriched only at the centromere peripheries and overlapped little with Cen protein distribution. Mutation of *dim-5*, which encodes an H3 K9 methyltransferase, resulted in partial loss of CenH3-GFP binding, mostly from the edges of the centromere regions. Similarly, in the absence of HP-1, the chromo domain protein that binds H3 K9me3, CenH3-GFP was mislocalized and restricted to the centromere core. Our findings suggest that centromere maintenance in Neurospora is qualitatively different from that in fission yeast, where small RNA and heterochromatin formation is required for the assembly but not maintenance of centromeres. We propose a model where CenH3 is maintained at the centromere core even in the absence of H3 K9me3 and HP-1, but both marks are required for normal spreading of CenH3 to the centromere peripheries.

Session II:

Microtubule plus end proteins in *Neurospora crassa*

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The microtubule (Mt) plus end proteins (Tip+) are heterogeneous factors that promote the assemblage and disassemblage of Mts dynamic end. There are two conserved families of Tip+, which function as stabilizers, such as, the End Binding 1 (EB1) and the human Lis1. We studied the dynamics of the EB1 homolog MTB-3 and the Lis1 homologs Lis1-1 and Lis1-2 in the filamentous fungus *Neurospora crassa*. Both families of Tip+ were observed as abundant fluorescent fragments in different hyphal regions. MTB-3 was present as comet-like structures homogeneously distributed along the hypha. The comets had a length of 1.6 ± 0.4 μm (mean±standard error) (n=100). The MTB-3 coments moved mainly towards the apex (speed of 1.9 μm s^{-1}), 10-folds faster than the hyphal elongation rate (0.2 μm s^{-1}). We observed instances of comets moving in retrograde direction. On the other hand, Lis1-1 and Lis1-2 were observed with variable densities through the different regions of the hypha. In distal subapical region there was little fluorescence and few individual particles can be discerned, the density of particles increases as they get closer to the tip and many of them form linear strings that grow in intensity as they get near the apex, reaching a maximum intensity around the Spk. The fast speed (0.4 μm s^{-1}) of Lis1-1 and Lis1-2 indicated that the fluorescent particles move independently of cytoplasmic bulk flow.

Both families of proteins participate in the polymerization of Mts, although each group has a different distribution and dynamics. Unlike other organisms, <I>N. crassa</I> seems unique in having duplicate homologs of <I>lis1</I> and both having the same dynamics and distribution. The organized displacement of Lis1-1 and Lis1-2 from individual particles at the base of the hypha to the filament-like pattern at the tip, suggests that Lis1-1 and Lis1-2 participate in unique fashion in the dynamics of polarized growth. The Mt-related motor protein conventional kinesin is involved in Lis1-1 and Lis1-2 transport.

Growth rate dependent branching among mutants from the Neurospora knockout library.

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The spatial distribution of branch formation in wild-type Neurospora has been shown to remain constant at different growth rates. A previous survey of classical Neurospora mutants, however, yielded a collection of strains for which slow growth acted as an environmental suppressor of hyperbranching. This study repeats that survey using the Neurospora knockout library to identify genes involved in branch initiation homeostasis. Knockouts displaying growth rate dependent branching were then crossed to confirm the phenotype was the result of the knockout. Although tip growth and branching is nearly unique to fungi, most of the genes identified have homologs in a broad range of organisms, thus play other (non-branching) roles in non-fungi. The gene functions highlighted by this screen are diverse with several emerging themes including: ubiquitin-binding proteins, presumed kinases, metal binding/metal

The apical secretory apparatus of *Neurospora crassa*

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One of the unsolved questions in fungal biology is how different types of secretory vesicles arrive at their destination to provide the components needed for cell expansion. We are currently studying vesicle dynamics and composition in *Neurospora crassa*. Earlier studies demonstrated that chitin synthase activity and glucan synthase are associated with chitosomes (microvesicles) and macrovesicles, respectively. We have labeled several cell-wall building enzymes with fluorescent proteins and followed their trajectory in Neurospora growing hyphae by confocal microscopy. We found that 4 out of the 7 reported chitin synthases, CHS-1, CHS-3, CHS-5 and CHS-6 are localized at the core of the Spitzenkörper (Spk). In contrast, GS-1, a component necessary for ß-1,3-glucan synthase activity, localizes at the external layer of the Spk. Benomyl treatment showed that CHS-1-GFP and GS-1-GFP accumulated at the hyphal apex independently of the microtubular cytoskeleton. Once leaving the Spk and prior to SNAREs recognition, secretory vesicles are presumably tethered to their target acceptor membrane in a process mediated by the exocyst. We tagged exocyst components (SEC-3, SEC-5, SEC-6, SEC-8, SEC-15, EXO-70 and EXO-84) with GFP and found fluorescence in a delimited region of the plasma membrane at the hyphal dome, the place of intensive exocytosis during polarized growth. Weak fluorescence was observed around the pore of newly formed septa, while strong fluorescence was noticeable in older septa and at sites of hyphal contact in anastomosed hyphae. These conclude that: 1) at the Spk there is a functional stratification of the machinery responsible for cell wall formation, 2) cell wall-building vesicles move along a microtubule independent cytoskeleton, and 3) exocytosis is restricted to a very delimited region of the hyphal apical plasma membrane.

Cell Biology of Colony Initiation in *Neurospora crassa.*

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Colony initiation in *Neurospora crassa* involves the formation of two types of hyphae: germ tubes that are involved in colony establishment, and conidial anastomosis tubes (CATs) that fuse to form interconnected networks of conidial germlings. These two hyphae are morphologically and physiologically distinct and under separate genetic control. Mutant screening has identified a number of signalling pathways involved in CAT induction, homing and fusion. Pharmacological evidence indicates that germ tube formation is dependent on both microtubule and F-actin function but that CAT fusion only requires F-actin. F-actin cable and patch dynamics has been imaged using Lifeact fused to either GFP or TagRFP. Asynchronous nuclear behaviour and mitosis was analyzed by live-cell imaging and indicates that *N. crassa* exhibits a form of 'closed mitosis'. CAT formation, homing, and fusion, and nuclear migration do not require microtubules, dynein/dynactin or nuclear division to occur. Nuclei undergo cell cycle arrest during CAT homing.

Towards experimental determination of the Neurospora mitochondrial proteome

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Determining the complete protein composition of any organisms, or even organelle, remains a challenge in proteomics. In the current work we have used a variety of gel electrophoretic techniques to separate the proteins in highly purified Neurospora mitochondria and sub-mitochondrial fractions (soluble proteins, membranes, protein complexes and ribonucleoproteins) and identify them by MALDI-TOF peptide mass fingerprinting. Combined with previous mass spectrometry studies from other groups, and re-evaluation of annotations, we have compiled a curated list of approximately 430 identified proteins that are likely to be bona fide mitochondrial proteins. Literature data mining and computational approaches suggest another 300 proteins not yet identified during mass spectrometric projects are also mitochondrial.

Localization of Dynein in Cytoplasmic Dynein Heavy Chain Mutants of Neurospora

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Cytoplasmic dynein is a large, microtubule-associated motor complex that facilitates minus-end-directed transport of various cargoes. The dynein heavy chain (DHC) is >4000 residues in length, with the last two- thirds of the heavy chain forming the motor head. Six domains within the dynein motor exhibit varying degrees of homology to the AAA+ superfamily of ATPases. These domains are followed by a distinct C- terminal domain and together form a ring-like structure from which a microtubule-binding domain protrudes. Using a genetic assay, we have isolated over 40 DHC mutants of Neurospora that produce full- length proteins that are defective in function. We have identified DHC point mutations in nearly all domains within the dynein motor head. To help define the mechanism(s) by which specific mutations lead to loss of dynein activity we have constructed fusions of the mCherry fluorescent protein to the dynein intermediate chain and the p150 subunit of dynactin. We have found that dynein heavy chain mutations result in four distinct mislocalization patterns. The data suggest that dynein heavy chain mutations strongly affect specific steps in the transport cycle.

Session III:

The DMM complex prevents spreading of DNA methylation from transposons to nearby genes in Neurospora crassa

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Perkins Award Recipient

Transposable elements are common in genomes and must be controlled. Many organisms use DNA methylation to silence such selfish DNA but the mechanisms that restrict the methylation to appropriate regions are largely unknown. We identified a JmjC-domain protein in Neurospora, DNA METHYLATION MODULATOR-1 (DMM-1) that prevents aberrant spreading of DNA and histone H3K9 methylation from inactivated transposons into nearby genes. Mutation of a conserved residue within the JmjC Fe(II) binding site abolished dmm-1 function, as did mutations in conserved cysteine-rich domains. Mutants defective only in dmm-1 mutants grow poorly but growth is restored by reduction or elimination of DNA methylation using the drug 5-azacytosine or by mutation of the DNA methyltransferase gene, dim-2. DMM-1 relies on an associated protein, DMM-2, which bears a DNA binding motif, for localization and proper function. HP1 is required to recruit the DMM complex to the edges of methylated regions.

Ascus dominance in Neurospora

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Our group is interested in a phenomenon known as Meiotic Silencing by Unpaired DNA (MSUD). In MSUD, genes unpaired during meiosis, as well as all homologous copies, are subject to silencing. As such, a deletion mutant is often dominant over a wild-type strain as it deprives the wild-type gene of a pairing partner. Another well-known ascus-dominant phenomenon in Neurospora is spore killing, which is a form of meiotic drive. In a cross of Spore killer x wild type (*Sk* sensitive), ascospores that do not contain the killer element are inviable, i.e. all survivors carry the killer element. We will discuss how these two seemingly unrelated ascus phenomena are linked as well as our current understanding of them.

Meiotic Silencing, Not Maple Syrup Urine Disease (MSUD)

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In Neurospora, if a segment of DNA is not present on the opposite homologous chromosome in meiosis, the resulting "unpaired" DNA segment is targeted for silencing. This situation occurs when a DNA element gets inserted at a particular chromosomal position (e.g., a situation akin to the "invasion" of a genome by transposable DNA elements). It can also occur when a normal region gets deleted. In both situations, the resulting loop of "unpaired" DNA activates a genome-wide "alert" system that results in the silencing not only of the genes present in the "unpaired" DNA segment, but also of those same genes if present elsewhere in the genome, even if they are in the paired condition. This phenomenon is called, meiotic silencing and was originally described in Neurospora crassa, but has since been observed in nematodes and mammals. In all these organisms, "unpaired or unsynapsed" regions (or chromosomes) are targeted for gene silencing. We think that meiotic silencing is a two-step process. First meiotic trans-sensing compares the chromosomes from each parent and identifies significant differences as unpaired DNA. Second, if unpaired DNA is identified, a process called meiotic silencing silences expression of genes within the unpaired region and regions sharing sequence identity. We are using a combination of genetics, molecular biology and biochemistry aimed at identifying all the molecular players of the process and at understanding how they work together. In this work we describe the genetic, molecular, cytogenetic and biochemical characterization of key components of the system. In addition, we describe and discuss how mutants in key genes required for recombination and chromosome pairing are not required for gene-specific meiotic silencing.

Diverse pathways generate microRNA-like RNAs and Dicer-independent small interfering RNAs in *Neurospora*

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A variety of small RNAs, including the Dicer-dependent miRNAs and the Dicer-independent Piwi-interacting RNAs (piRNAs), associate with Argonaute family proteins to regulate gene expression in diverse cellular processes. These two species of small RNA have not been found in fungi. Here, by analyzing small RNA associated with the *Neurospora* Argonaute protein QDE-2, we show that diverse pathways generate miRNA-like small RNAs (milRNAs) and Dicer-independent small interfering RNAs (disiRNAs) in this filamentous fungus. milRNAs are processed by at least four different mechanisms that use a distinct combination of factors, including Dicers, QDE-2, the exonuclease QIP and a novel RNAse III domain-containing protein MRPL3. In contrast, disiRNAs originate from loci producing overlapping sense and antisense transcripts, and do not require any of the known RNAi pathway components for their production. Taken together, these results uncover several novel pathways for small RNA production in filamentous fungi, shedding light on the diversity and evolutionary origins of eukaryotic small RNAs.

A fluorescence-based recombination reporter system.

Fred Bowring, Jane Yeadon and David Catcheside. School of Biological Sciences, Flinders University.

A degree of ambiguity is unavoidable when chromatid data are used exclusively to study meiotic recombination. One cannot, for instance, be certain if a spore with a non-parental ditype arose from a crossover or from gene-conversion unless the genotypes of all progeny in the ascus (the octad) are known. However, as the labor required to assemble a sufficiently large octad dataset is prohibitive, the lower power afforded by chromatid analyses is considered quite acceptable. The stunning micrographs of *Neurospora* rosettes with GFP-stained nuclei in Freitag, Hickey, Raju, Selker and Reid (2004, Fungal Genet. & Bio, 41:897) suggested to us a potential system for visual detection of octads containing recombinant spores. Here we report on the development of a recombination reporter system built using variants of the GFP-histone fusion protein and illustrate how it can be used to study meiotic recombination in *Neurospora*.

Genetic pathways and transcriptome patterns during conidiation in *Neurospora* and other fungi.

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A fundamental question concerning conidiation is how it has evolved. Was conidiation present in the last common ancestor of the Ascomycetes and then modified to give rise to the morphological diversity we see today (i.e. divergence)? Or, has conidiation arisen independently in different lineages that may share a common 'toolkit' of genes to create novel developmental pathways (i.e. convergence)? To address this question, a comparative analysis of conidiation in *Neurospora crassa* and *Aspergillus nidulans* has been performed for seven of the *A. nidulans* genes that regulate conidial development. Three of the *N. crassa* orthologs of the *A. nidulans* genes were found to be regulators of macroconidiation in *N. crassa*, one was found to be a regulator of micro- but not macroconidiation, and three were found to play no role in conidiation. In addition to examining the architecture of the regulatory pathway, we have compared the transcriptomes of both fungi during conidiation. If conidiation is conserved from an ancient process, we might expect to see conservation in gene expression patterns. Since this was not the case, we must now distinguish between convergent evolution and extensive divergence from a common ancestor. The 2.9 Gb of mRNA sequence from the *N. crassa* Illumina runs provides a wealth of data for updating gene models. This work was funded through the National Science Foundation grant IOS0716894.

PP-1 (Ste12) regulatory networks during early colony establishment

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Ste12 is a conserved transcription factor that regulates a diverse range of genes, and is required for various processes. It is the environment-dependent association of Ste12 with other proteins that leads to it's differential regulation of various subsets of targets. For example in *S. cerevisiae*, Ste12 mediates mating and filamentous growth as a result of signals from the MAP kinase Fus3 and from Cdk8. The *N. crassa* homolog of Ste12 is known as PP-1, and its mutation results in shortened aerial hyphae, female sterility, and a reduced growth rate. We have used genome profiling to analyze PP-1 regulatory networks in *N. crassa* during early colony establishment, and have studied direct targets of PP-1 using ChIPseq. A summary of the overall results will be presented.

Session IV:

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Genetic and Molecular Dissection of the Neurospora Clock: Genetics and Epigenetics

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Transcription/ translation feedback loops are central to all eukaryotic circadian clocks (Dunlap et al, Cold Spring Harbor Symp. 72: 57 – 68, 2007). In the circadian oscillator in fungi and animals the negative feedback loop drives periodic expression of proteins that feed back to reduce their own expression. While canonical clock proteins work exclusively in timing, all systems utilize additional, often essential, proteins that perform other functions in the cell. Among these in Neurospora is an essential putative RNA helicase, FRH. A novel, unbiased genetic screen for circadian negative feedback mutants uncovered a point mutation that completely complements the essential functions of FRH yet is totally arrhythmic, thus genetically separating essential functions from clock-associated roles.

Expression of the circadian negative element, *frq*, in response to light and time-of-day is driven by a PAS-heterodimer of WC-1 and WC-2. *frq* is complex, encoding alternatively spliced sense transcripts as well as a long (> 4knt) antisense transcript, *qrf*. The *frq* and *qrf* promoters show chromatin rearrangement in response to light as well as time-of-day, and deletion of all 19 genes encoding ATP-dependent chromatin-remodeling enzymes revealed only 2 genes, *clockswitch* (*csw-1* a homolog of yeast *Fun30*, mouse *Etl1* and human *SMARCAD* genes) and *chd1* (a homolog of the mammalian *mi-2, chd2* and yeast *Chd1* genes), required for remodeling at *frq* and for normal clock function. Unexpectedly and unusually, however, Δ*csw-1* and Δ*chd1* knockouts are not simply arrhythmic but rather become arrhythmic slowly, over days. The data suggest a model in which these remodeling enzymes help WC-2 to leave the *frq* locus, thereby participating in the negative arm of the feedback loop.

Defining the basis for a high-throughput bioluminescence clock-screening in Neurospora

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The *Neurospora* circadian oscillator is composed of a transcriptional translational negative feedback loop, where the phosphoprotein Frequency (FRQ) inhibits its own expression by affecting the activity of the white collar transcriptional complex (WCC). As a result, *frq* message and protein levels oscillate daily, and these rhythms can be indirectly followed by the overt circadian regulation of spore formation (conidial banding). While the circadian banding can be tracked for several days, technical limitations restrict the routine molecular analysis of *frq* message and protein levels to no more than 2-3 days. To overcome this and other limitations a fully-codon optimized luciferase reporter system for *Neurospora crassa* was developed. Thus, by putting this real-time reporter under the control of promoter regions containing circadian elements, rhythms in transcription of *frq* or clock-controlled genes (*ccg*s) can be easily tracked for over a week. Moreover, by generating FRQ-LUC translational fusion strains, rhythms in FRQ protein can be followed in a semiquantitative manner. We have combined this bioluminescence-based system with a variety of tools for gene manipulation, so that different knock out strains can be easily analyzed for circadian molecular phenotypes. Thus, the clock of strains that are phenotypically arrhythmic, or exhibiting severe growth defects can now be molecularly examined an otherwise overwhelming task when performed by classic western/northern or real-time PCR approaches. This reverse genetics approach has also been complemented with pharmacological perturbations, setting up the basis for a high-though put screening platform for *Neurospora* circadian analyses. As a result, examination of knockouts for different genes, using this new experimental setup, has started to reveal new molecular details of the Neurospora oscillator. Funding: FONDECYT 1090513

Molecular mechanism of photo-Adaptation in *Neurospora crassa*

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Light responses and photoadaptation of *Neurospora* depend on the photosensory light-oxygen-voltage (LOV) domains of the circadian transcription factor White Collar Complex (WCC) and its negative regulator Vivid (VVD). We have analyzed the interactions of WCC and VVD leading to photoadaptation. During the day, expression levels of VVD correlate with light intensity, allowing photoadaptation over several orders of magnitude. At night, previously synthesized VVD serves as a molecular memory of the brightness of the preceding day and suppresses responses to light cues of lower intensity. We show that VVD is an essential component of the circadian clock in naturally noisy and ambiguous photoperiods.

Insights into Circadian Oscillator Complexity

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Perkins Award Recipient

Circadian clocks are ubiquitous and control daily rhythms in a wide range of biochemical, cellular and behavioral activities. The formal properties of circadian clocks are: 1) persistence of rhythmicity in the absence of environmental cues, 2) the ability to be entrained by external time cues, such as the light-dark cycle, and 3) compensation of period length for changes in temperature. To date, temperature compensation remains the least understood property of the circadian system. We identified a nutrient sensor protein kinase (PSK) that is part of a mechanism for temperature compensation of the *Neurospora crassa* circadian clock. PSK is a PAS domain kinase that regulates energy flux, glycogen metabolism and protein synthesis in fungi and mammals. In *Neurospora*, *Dpsk* cells are unable to grow in medium containing galactose. Conidiation rhythms in *Dpsk* cells displayed a 4h shorter period (18 h) at 30°C, but a wild type period (22 h) at 20°C, suggesting a possible defect in temperature compensation in the mutant. We found that the core clock component WC-1 was degraded at a faster rate at 30°C, but not at 20°C, in the *Dpsk* strain as compared to wild type. Moreover, phosphorylation of the WCC was reduced in the *Dpsk* k strain at the higher temperature, suggesting that PSK regulates phosphorylation and stability of WCC. *In vitro* kinase assays demonstrated that PSK can directly phosphorylate WC-1 and, to a lesser extent, WC-2. Together, these results indicate a role for PSK in controlling aspects of temperature compensation of the *Neurospora* circadian system.

A role for the Neurospora RCO-1/RCM-1 complex in the regulation by light of gene transcription

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The activation of gene transcription by light requires the transient binding to the promoters of the photoresponsive white collar complex (WCC). We have observed a complex stimulus/response relationship for con-10 and con-6 photoactivation that led us to propose that a light-dependent repressor modifies the activity of the WCC. Deletion of several photoreceptor genes resulted in high gene photoactivation suggesting a role for secondary photoreceptors in the repression of light-dependent gene expression. We have found that strains with mutations in rco-1 or rcm-1, homologs of the yeast Tup1-Ssn6 repressor complex genes, show high and sustained accumulation of mRNAs for con 10 and other genes after long exposures to light. RCO-1 and RCM-1 accumulate in the nuclei and their localization is not altered by light exposure. Our results suggest that the Neurospora RCO 1/RCM 1 complex participates in the light-transduction pathway by repressing gene transcription after long exposures to light. Is the RCO 1/RCM 1 complex the proposed light-

The role of VIVID in light and temperature responses of the Neurospora circadian system

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The Neurospora PAS/LOV protein VIVID (VVD) is a light and temperature regulated blue-light receptor that influences light and temperature responses within the Neurospora circadian system. As such, VVD facilitates a stable alignment of the circadian clock with external time cues (entrainment). One way in which VVD controls clock time (i.e phase) involves the rapid down-regulation of transcript levels of the central clock gene *frequency* (*frq*) at the light dark boundary. We have probed the mechanism by which VVD regulates *frq* levels at dusk and found that it does not influence exosome-mediated degradation of *frq* transcript but rather inhibits *frq* transcription by interacting with the transcriptional activator and blue-light sensing White Collar complex (WCC).

Session V:

Unexpected population structure in *Neurospora crassa* from the Caribbean Basin

Christopher Ellison, Charles Hall, Angela Kaczmarczyk, David Kowbel, Juliet Welch, Rachel Brem, N. Louise Glass, John W. Taylor, University of California, Berkeley

We used solexa sequencing of mRNA to simultaneously identify Single Nucleotide Polymorphisms (SNPs) and quantify gene expression for more than sixty isolates of *Neurospora crassa* from the Caribbean Basin. Through population genomic analysis of the SNP data, we find strong support for two recently diverged populations, one endemic to Louisiana and the other distributed through Florida, Haiti and the Yucatan. We also identify a subset of genes that show the signature of positive selection and a subset that are differentially expressed between species. Based on the evidence for a recent divergence time and the presence of gene flow between these populations, we argue that this represents an ideal dataset for the study of the early stages of speciation.

The development of genetics and genomics for analysis of complex traits in the model filamentous fungus, *Neurospora crassa*.

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Our goal is to develop and make available to the community a set of strains and tools that will facilitate the rapid identification of genes

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contributing to quantifiable traits in the filamentous ascomycete Neurospora crassa, as well as identify regulatory networks on a genomic scale. In all organisms combinations of genes acting at multiple sites in the genome control many traits. The genes contributing to such complex traits are Quantitative Trait Loci (QTLs). Previous studies that map genes by linkage to phenotype have suffered from poor resolution. As a result many mapped QTLs have not resulted in the identification of the specific contributing gene(s). The Illumina short read sequencing technology and the availability of many wild isolates of *N. crassa* allows us to address this problem. RNA-seq from wild type *N. crassa* isolates gives us both sequence and expression data. This allows us to generate a dense marker map, genotype each strain at our discovered markers, and gives us a measure of gene expression from multiple strains. The dense marker map will facilitate the mapping of QTLs by association in our wild population with high resolution. Moreover, as most sequence variation in a gene will result in an altered expression level for that gene, combining QTL analyses of physiological and gene expression traits, based on co-localization of expression QTLs (eQTLs) and QTLs can directly indicate candidate genes. Furthermore, the identification of polymorphisms that control multiple eQTLs allows us to identify regulators and their regulatory networks. By this method we will be able to utilize the genetic, phenotypic, and expression variation within a population of *N. crassa* to annotate thousands of previously uncharacterized genes.

Evolutionary genomics of *Neurospora* and other Sordariales

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Comparative genomics examining shared regions of genomes can help identify functional and unannotated regions of the genomes. Here we have examined genome alignments across 10 species of Sordariales fungi including *Neurospora crassa* OR74A (Clade A), *N.crassa* D106 (Clade C), *N. intermedia*, *N. tetrasperma*, *N. discreta*, *Sordaria macrospora*, *Sporotrichum thermophile*, T*hielavia terrestris*, *Podospora anserina*, and *Chaetomium globosum*.

Among the closely related *Neurospora* species we have examined conserved genomic regions to examine rates of sequence evolution, regions with unusually high conservation, and explored regions with signatures of RIP to deduce ancestral genomic context of RIPed regions. We have scanned for evidence of positive selection and identified a select few genes that show evidence for recent positive natural selection. We see that large stretches of the genome are collinear among the *Neurospora* spp genomes while the synteny breaks down dramatically when comparing to *P. anserina* or *C. globosum*.

Comparing protein coding genes among the ten genomes, in particular contrasting the dung growing fungi with those found primarily on grasses and woody vegetation, we examined the overall gene content of the clade. There is a core set of nearly 65% of genes found in common among the species, with patterns of independent loss and gain that mark scattered distribution of the rest of gene families with a distinct set of orphan genes found in each species. Among the duplicated genes within Neurospora we were able to identify a select few cases of recent duplication within the group. We observed an increased number of gene duplication events in species outside the immediate Neurospora+Sordariales group that are anti-correlated with the amount of sequences with signatures of RIP.

Overall comparisons of gene and genome evolution in the Sordariales will provide a better evolutionary history of the *Neurospora* genomes and the influence of ecological niche and genome defense on the patterns of genome evolution.

Biofuels: Systems analysis of plant cell wall degradation by the model filamentous fungus *Neurospora crassa*

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Plant biomass, primarily composed of lignocellulose, is widely viewed as a potential feedstock for the production of liquid fuels and other value-added materials. However, the principal barriers to production of liquid fuels from lignocellulose are the high costs of pretreatment and the conversion of insoluble polysaccharides to fermentable sugars. Conversion of insoluble plant cell wall polysaccharides currently involves using hydrolytic enzymes produced by filamentous fungi. *Neurospora crassa* is commonly found growing on dead plant material in nature, particularly grasses. Using functional genomics resources available for *N. crassa*, which include a near full genome deletion strain set and whole genome microarrays, we undertook a system-wide analysis of plant cell wall, cellulose and hemicellulose degradation. As a complement to the expression data, the secretomes associated with *N. crassa* growth on *Miscanthus*, cellulose and hemicellulose were determined using a shotgun proteomics approach. To decipher the transcriptional regulatory network of *N. crassa* involved in plant cell wall deconstruction, we identified the transcriptional regulon of the carbon catabolite repressor (CRE-1) under celluloytic conditions. Strains containing deletions in genes within the CRE-1 regulon were analyzed for phenotypic changes during growth on cellulose and for cellulase activity. Finally, we are developing *N. crassa* as a platform to express genes involved in plant cell degradation. We successfully expressed and purified a functional GH5-1-GFP protein, which was shown to bind to live plant cell walls of *Arabidopsis*. The genomic tools developed in *N. crassa* allow for a comprehensive system level understanding of plant cell wall degradation mechanisms used by a ubiquitous filamentous fungus.

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NEUROSPORA 2010 POSTER ABSTRACTS

Light and Circadian Clock

1) Bioluminescent Neurospora circadian behavior under different lighting conditions.

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Using an optimized firefly luciferase gene as a reporter, several constructs of Neurospora are used to better understand the role of light on the circadian mechanism. The frequency (frq) gene, a key component of the Neurospora circadian clock, is under both circadian- and light-regulation which depends upon the blue light receptor/transcriptional complex WC-1 and WC-2. Using a frq promoter driving the luciferase gene, we can measure the detailed kinetics of the frq promoter activity. When bright lights go on, there is a rapid increase in signal that ultimately shows biphasic characteristics. In continued light, the signal ultimately drops to a high steady state level. When the lights go off, there is always a rapid drop in signal and in continued dark a stable free running rhythm develops that is phased by the "lights-off" event. When given a repeated 12 h light, 12 h dark cycle, the result is a very angular entrained rhythm with the biphasic kinetics being very different from one light cycle to the next. If a 12 h light, 12 h dark cycle is used where lights are slowly increased and slowly decreased to emulate the daily sun pattern, the angularity of the entrained rhythm is greatly reduced. If cells are exposed to a long period of dark and then placed into constant dim light, no oscillatory activity is subsequently seen. However, if cells are exposed to a long period of light and then placed into constant dim light, an obvious damped oscillatory rhythm appears. By using different segments of the frq promoter, as well as other promoter elements and constructs, we are progressively dissecting new features of the light responses dynamics.

2) *In vivo Neurospora* protein expression using the *vvd* promoter

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Filamentous fungi are often studied for their use in examining a variety of eukaryotic processes. One such example is the protein processing machinery of fungi, which is able to perform all of the extensive post- translational modification needed in the complex world of eukaryotic organisms. While there are several fungal protein expression systems in place, more could be done to exploit *Neurospora crassa*. Though some promoters such as the qa-2 promoter are used in *Neurospora* to control gene expression; the qa-2 promoter is somewhat leaky and often does not induce protein expression at levels much above those seen *in vivo*. In order to increase and control *in vivo* protein expression in *Neurospora*, we are harnessing the *vvd* gene. Expression of *vvd* is suppressed in dark conditions but is strongly induced by light reaching high levels within minutes of light induction. Interestingly, the *vvd* promoter is also auto repressed, meaning that by deleting the *vvd* gene, we could further increase expression from the *vvd* promoter. By constructing a strain in which *vvd* and its promoter have been deleted, we can replace the native promoter of a gene with the *vvd* promoter, creating a system in which any gene can be placed under the influence of the *vvd* promoter, creating a light regulated, *in vivo* protein over-expression system enabling phenotypic analysis and protein purification of *Neurospora* genes and potentially genes from other eukaryotic organisms.

3) A new mutation affecting rhythmicity in both FRQ-less and FRQ-sufficient *Neurospora crassa*. Patricia Lakin-Thomas[1], Sanshu Li[2], and Kamyar Motavaze[1]. [1]Department of Biology, York University, Toronto, Canada. [2]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT.

Although the FRQ/WCC feedback loop is said to be central to the circadian system in *N. crassa*, rhythms can still be seen under many conditions in FRQ-less (knockout) strains such as frq^{10}. For example, conidiation rhythms in *chol-1* grown with limiting choline continue in *chol-1;* frq^{10} strains, and conidiation rhythms in frq^{10} strains can be entrained to cycles of heat pulses. To identify components of the FRQ-less oscillator (FLO), we mutagenized spores of *chol-1;* frq^{10} and found a mutation (named UV90) that abolishes or strongly damps conidiation rhythms in both *chol-1;* frq^{10} and frq^{+} backgrounds. UV90 increases the phase-resetting response to pulses of both light and heat in frq^{+}, and reduces the level of FRQ protein, indicating that it reduces the amplitude of the oscillator in frq^{+}. UV90 alters entrainment to heat pulse cycles in frq^{10}, consistent with reduced amplitude of the FLO. Therefore the wild-type UV90 gene product appears to be required for sustained, high-amplitude rhythms in both frq^{+} and frq^{10}. We have previously shown that *frq* and *wc-2* mutations can affect the period of the rhythm in *chol-1*, and that *prd* mutations can also affect both FRQ-less and FRQ-sufficient rhythms. These results support a model in which the FRQ/WCC feedback loop interacts with a single FLO in an integrated circadian system. Funded by NSERC.

5) A role for the Neurospora RCO-1/RCM-1 complex in the regulation by light of gene transcription

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The activation of gene transcription by light requires the transient binding to the promoters of the photoresponsive white collar complex (WCC). We have observed a complex stimulus/response relationship for con-10 and con-6 photoactivation that led us to propose that a light-dependent repressor modifies the activity of the WCC. Deletion of several photoreceptor genes resulted in high gene photoactivation suggesting a role for secondary photoreceptors in the repression of light-dependent gene expression. We have found that strains with mutations in rco-1 or rcm-1, homologs of the yeast Tup1-Ssn6 repressor complex genes, show high and sustained accumulation of mRNAs for con 10 and other genes after long exposures to light. RCO-1 and RCM-1 accumulate in the nuclei and their localization is not altered by light exposure. Our results suggest that the Neurospora RCO 1/RCM 1 complex participates in the light- transduction pathway by repressing gene transcription after long exposures to light. Is the RCO 1/RCM 1 complex the proposed light-dependent repressor that modifies the activity of the WCC?

6) Interaction of the blue-light photoreceptor VVD with the FRQ-FRH (FFC) and White Collar Complex (WCC).

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In *Neurospora crassa*, FREQUENCY (FRQ), FRQ-interacting helicase (FRH), WHITE-COLLAR-1 (WC-1), and WC-2 are essential components of the circadian clock. In addition, the PAS/LOV protein and photoreceptor VIVID (VVD) influences both light and temperature responses of the clock. However, the molecular pathway in which VVD transmits environmental information to the oscillator is largely unknown. Here we report that VVD is both a cytoplasmic and nuclear protein that interacts with complexes central for circadian clock and blue-light signalling, namely the FRQ-FRH (FFC) and White-collar complex (WCC). Our data indicate that VVD modulates the activity of the FFC that acts within the transcriptional loop of *frq* negative feedback regulation rather than interfering with the posttranscriptional role of FFC in exosome-mediated degradation of *frq* transcript.

7) Mechanisms of circadian clock regulation of signaling pathways

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In the filamentous fungus *Neuropora*, the Osmotically Sensitive (OS) pathway is rhythmically activated and functions as an output pathway from the circadian clock. Our research aims to define the mechanism used by the FRQ/WCC oscillator to mediate rhythmicity of OS pathway activity that subsequently regulates rhythmicity of the downstream target *ccg-1*. Expression of two components in the OS pathway, the histidine phophotransferase (HPT-1) and the downstream MAPKKK (OS-4), is regulated by the FRQ/WCC oscillator. The promoter of *os-4* is rhythmically bound by the White Collar Complex (WCC), a core component of the FRQ/WCC oscillator, with a period and phase that correlate with *os-4* transcript accumulation. Interestingly, *hpt-1* expression rhythms peak at the opposite phase compared to *os-4* expression, and the *hpt-1* promoter is not bound directly by the WCC. Our working model suggests that the activity of HPT-1 inhibits pathway activation, while the activity of OS-4 promotes pathway activation; therefore, by expressing these two components in opposite phases, the clock coordinates their activity to set the activation state of the pathway. Supporting this model, a mutation of the RRG-1 response regulator, which acts as an intermediate between HPT-1 and OS- 4, disrupts rhythmicity of both OS pathway activity and *ccg-1* mRNA accumulation. Together, these data suggest that rhythmic expression of both genes is important for generating rhythms in downstream targets.

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8) Determination of functional domains and residues in Tob37 via mutant analysis in *Neurospora crassa*.

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Nuclear-encoded Mitochondrial Outer Membrane (MOM) proteins are translated on cytoplasmic ribosomes and targeted to mitochondria for integration into the MOM. The TOB complex facilitates the import and assembly of both beta-barrel and C-terminal alpha-helical anchored MOM proteins. The core TOB complex contains three proteins: Tob55, Tob37 and Tob38. For beta-barrel protein assembly, the current model suggests that Tob55 units form a ring structure spanning the MOM. Tob38 is on the cytoplasmic surface of the MOM and associates with the pore that is formed by the Tob55 ring, and exposes a beta-signal recognition sequence into the hydrophilic pore of the complex. Beta-barrel preproteins are brought within the pore where they undergo folding. Tob37 is thought to open the pentameric pore to create a "gate" to allow the release of the protein into the membrane. To gain information on the mechanism of action of Tob37, we have created sheltered disruption mutants and determined that Tob37 is essential for the viability of *N. crassa*. We have begun structure/function studies by changing and/or deleting various conserved residues or domains of the protein and testing them for rescue of the null nucleus, localization of Tob37 and effects on import of beta- barrel proteins. We have found one set of conserved residues is required for Tob37 function while another domain influences the binding of Tob37 to the MOM.

9) Septum formation is regulated by the RHO4-specific exchange factors BUD3 and RGF3 and by the landmark protein BUD4 in Neurospora crassa

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Rho GTPases have multiple, yet poorly-defined functions during cytokinesis. By screening a Neurospora crassa knockout collection for Rho guanine nucleotide exchange factor (GEF) mutants that phenocopy rho-4 defects (i.e. lack of septa, slow growth, abnormal branching and cytoplasmic leakage), we identified two strains defective in homologs of Bud3p and Rgf3 of budding and fission yeast, respectively. The function of these proteins as Rho GEFs and their specificity for RHO4 was determined by genetic and in vitro assays. Localization studies indicated that the two GEFs and their target GTPase act as two independent modules during the selection of the septation site and the actual septation process. Furthermore, we determined that the N. crassa homolog of the anillin-related protein BUD4 is required for septum initiation and that its deficiency leads to typical rho-4 defects. The localization of BUD4 as a cortical ring prior to septation initiation was independent of functional BUD3 or RGF3. These data position BUD4 upstream of both RHO4 functions in the septation process and make BUD4 a prime candidate for a cortical marker protein involved in the selection of future septation sites.

10) The Neurospora peptide:N-glycanase ortholog PNG1 is essential for cell polarity despite its lack of enzymatic activity.

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Secretory proteins are subjected to a stringent ER-based quality control system that distinguishes aberrant from correctly folded proteins. The cytoplasmic peptide:N-glycanase (PNGase) cleaves oligosaccharides from misfolded glycoproteins and prepares them for degradation by the 26S proteasome. In contrast to abundant in vitro data on its enzymatic function, the in vivo relevance of PNGase activity remains unclear. Here we show that the PNG1 ortholog from the filamentous ascomycete Neurospora crassa is an essential protein, and its deletion results in strong polarity defects. PNG1 and its predicted binding partner RAD23 have distinct functions in N. crassa and are involved in cell wall integrity and DNA repair, respectively. Moreover, wild type PNG1 has substitutions in essential catalytic amino acids, and its deglycosylation activity is lost. These substitutions are conserved in many PNG1 orthologs of the fungal kingdom, implying a so far unrecognized enzyme-independent function of PNG1 that may only become apparent in highly polar cells such as fungal hyphae.

11) Regulation of COT1 activity and function by phosporylation and associated proteins

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Ndr kinases are important for cell differentiation and morphogenesis in various organisms, yet the regulation of their activity and their integration into a cellular signaling context is still fragmentary. The Ndr kinase COT1 is essential for hyphal elongation in Neurospora crassa and important for fungal pathogenicity and differentiation in related organisms. The genetic and biochemical amenability of N. crassa has facilitated the identification of an intricate signaling network consisting of protein kinase A, MAP kinase cascades and COT1-associated proteins that coordinately regulate vegetative growth and conidiation, hyphal fusion and sexual development. COT1 requires the association with two MOB2-type proteins and phosphorylation of two conserved residues in the activation segment and the hydrophobic motif of the kinase, respecitvely, for their function. We will discuss a model of COT1 activation that involves autophosphorylation of the COT1 dimer in cis at Ser417 in the activation segment and phosphorylation of Thr589 in the hydrophobic motif by the germinal centre kinase POD6. Interestingly, in vitro activity of COT1 does not mirror the in vivo functionality of COT1. This discrepancy may be explained by a conformational change induced by the two phosphorylation events and altered localization of the COT1-MOB2 complex.

12) Growth rate dependent branching among mutants from the Neurospora knockout library

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The spatial distribution of branch formation in wild-type Neurospora has been shown to remain constant at different growth rates. A previous survey of classical Neurospora mutants, however, yielded a collection of strains for which slow growth acted as an environmental suppressor of hyperbranching. This study repeats that survey using the Neurospora knockout library to identify genes involved in branch initiation homeostasis. Knockouts displaying growth rate dependent branching were then crossed to confirm the phenotype was the result of the knockout. Although tip growth and branching is nearly unique to fungi, most of the genes identified have homologs in a broad range of organisms, thus play other (non-branching) roles in non-fungi. The gene functions highlighted by this screen are diverse with several emerging themes including: ubiquitin-binding proteins, presumed kinases, metal binding/metal metabolism proteins and catalases.

13) The apical secretory apparatus of *Neurospora crassa*

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One of the unsolved questions in fungal biology is how different types of secretory vesicles arrive at their destination to provide the components needed for cell expansion. We are currently studying vesicle dynamics and composition in *Neurospora crassa*. Earlier studies demonstrated that chitin synthase activity and glucan synthase are associated with chitosomes (microvesicles) and macrovesicles, respectively. We have labeled several cell-wall building enzymes with fluorescent proteins and followed their trajectory in Neurospora growing hyphae by confocal microscopy. We found that 4 out of the 7 reported chitin synthases, CHS-1, CHS-3, CHS-5 and CHS-6 are localized at the core of the Spitzenkorper (Spk). In contrast, GS-1, a component necessary for B-1,3-glucan synthase activity, localizes at the external layer of the Spk. Benomyl treatment showed that CHS-1-GFP and GS-1-GFP accumulated at the hyphal apex independently of the microtubular cytoskeleton. Once leaving the Spk and prior to SNAREs recognition, secretory vesicles are presumably tethered to their target acceptor membrane in a process mediated by the exocyst. We tagged exocyst components (SEC-3, SEC-5, SEC-6, SEC-8, SEC-15, EXO-70 and EXO-84) with GFP and found fluorescence in a delimited region of the plasma membrane at the hyphal dome, the place of intensive exocytosis during polarized growth. Weak fluorescence was observed around the pore of newly formed septa, while strong fluorescence was noticeable in older septa and at sites of hyphal contact in anastomosed hyphae. These conclude that: 1) at the Spk there is a functional stratification of the machinery responsible for cell wall formation, 2) cell wall-building vesicles move along a microtubule independent cytoskeleton, and 3) exocytosis is restricted to a very delimited region of the hyphal apical plasma membrane.

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14) Research of mutation of ion beam irradiation in *Neurospora*

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Ion beams are known to induce the severe injury of cellular components. Especially, its irradiation to cell mostly causes the DNA double strand breaks (DSBs). We examined the sensitivity and induced mutation spectrum caused by irradiation of ion beam to several DSB repair deficient mutants; *mus-52* (YKU80 homolog, NHEJ deficient) and *mei-3* (RAD51 homolog, HR deficient) mutant strains, and 74OR-28a (wild-type) as a control. Carbon-ion beam ($^{12}C^{5+}$; 135 MeV/u) were irradiated to conidia and forward mutations occurred in the *ad-3* loci (*ad-3A* or *ad-3B*) were detected by Jug culture. The forward mutation frequency in the *mus-52* strain was quite low and decreased approximately 2.8-fold compared to wild type, in contrast that of *mei-3* strain increased approximately 3.0-fold. Mutation types of these strains were, (1) deletions were the most common mutation observed in wild type at *ad-3A* and *ad-3B* loci, (2) transitions and deletions were together appeared in the *mei-3* strain at these loci, (3) transversions were the most common mutation observed in *mus-52* strain of the *ad-3B* locus. We also report the ion beam sensitivity of argon-ion ($^{40}Ar^{17+}$; 95 MeV/u) ion and ferrous-ion (^{56}Fe; 90 MeV/u).

15) Localization of Dynein in Cytoplasmic Dynein Heavy Chain Mutants of Neurospora

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Cytoplasmic dynein is a large, microtubule-associated motor complex that facilitates minus-end-directed transport of various cargoes. The dynein heavy chain (DHC) is >4000 residues in length, with the last two- thirds of the heavy chain forming the motor head. Six domains within the dynein motor exhibit varying degrees of homology to the AAA+ superfamily of ATPases. These domains are followed by a distinct C- terminal domain and together form a ring-like structure from which a microtubule-binding domain protrudes. Using a genetic assay, we have isolated over 40 DHC mutants of Neurospora that produce full- length proteins that are defective in function. We have identified DHC point mutations in nearly all domains within the dynein motor head. To help define the mechanism(s) by which specific mutations lead to loss of dynein activity we have constructed fusions of the mCherry fluorescent protein to the dynein intermediate chain and the p150 subunit of dynactin. We have found that dynein heavy chain mutations result in four distinct mislocalization patterns. The data suggest that dynein heavy chain mutations strongly affect specific steps in the transport cycle.

16) Activation and subcellular localization of *Neurospora crassa* Protein Kinase C

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Choline-limited growth of the *chol-1* mutant of *N. crassa* lengthens the period of the circadian conidiation rhythm and reveals conidiation rhythmicity in FRQ-less strains (*frq* null, frq^{10}). We have previously shown that *chol-1* accumulates high levels of the neutral lipid diacylglycerol (DAG), and that exogenous DAG can increase the period of the conidiation rhythm. We are therefore interested in the downstream effects of DAG. The single protein kinase C isoform in *N. crassa* (NPKC) is predicted to be activated by DAG, as are novel PKCs in animals, although this has not been directly demonstrated. Putative inhibitors and activators of NPKC affect light responses in *N. crassa* (Arpaia et al, 1999) and NPKC regulates levels of the blue-light receptor WC-1 through phosphorylation (Franchi et al, 2005). We have created a NPKC-GFP fusion protein to study the activation and subcellular localization of NPKC. We report here that NPKC localizes to tips, branch sites, and sites of septum development in actively growing hyphae. Strains overexpressing NPKC show a hyperbranching phenotype. Exogenous DAG and phorbol ester induce translocation of NPKC from the cytoplasm to the plasma membrane, as is found with mammalian novel PKC isoforms. Choline-limited *chol-1* cultures with high DAG levels show increased *in vitro* PKC activity assayed in cell extracts. We conclude that DAG does activate NPKC and induces translocation, and that NPKC plays a role in branching. Whether DAG affects FRQ-less conidiation rhythmicity by activating NPKC is still an open question. Funded by NSERC.

17) New features of the *mutagen sensitive-10* mutant indicate relationship between mitochondrial morphology and senescence in *Neurospora crassa*

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In *Neurospora crassa*, many mutants which are sensitive to DNA damaging agents have been isolated and placed into some epistasis groups. However additional mutants exist which can not be classified into these groups. One such mutant, *mus-10 (mutagen sensitive)*, was isolated as a mutant that was sensitive to the alkylating agent methyl methanesulfonate (MMS) by Käfer et al. But it is not clear why *mus-10* mutants are sensitive to DNA damaging agents. This time, we found interesting phenotypes of *mus-10* mutant other than mutagen sensitivity. The *mus-10* mutant showed senescence phenotype, that is, short lifespan and progressive accumulation of mitochondrial DNA deletions. Additionally, in *mus-10* mutant cells, mitochondria were fragmented. To determine the function of the MUS-10 protein, a gene rescue approach was used, which facilitated the cloning of the *mus-10* gene and subsequent identification of MUS-10 as a novel F-box protein. F-box proteins are generally found in SCF (Skp1, Cullin, F-box) complexes, which are involved in the degradation of substrates via the ubiquitin proteasome system. F-box motif of MUS-10 required for its function and MUS-10 interacted with components of SCF complex, suggesting MUS-10 prevents senescence by turnover of unknown substrates. Further description of MUS-10 will be discussed.

18) Characterization of temperature sensitive lethal mutants in *Neurospora crassa.*

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We have used a variety of approaches to characterize the mutation underlying temperature sensitive lethal mutants in Neurospora. Complementation approaches have allowed direct identification of five such genes and an informatics based approach is being used to identify two additional ts-lethal genes. Complementation is still the ultimate criterion used to determine if a particular ORF is responsible for the mutant phenotype. One such gene identified is being developed as a selectable marker for transformation and gene disruption. Interestingly another ORF has been identified for which different alleles encode different phenotypes. *un-7* allele T53M50(t) was complemented by NCU00651. This ORF was found by Seiler and colleagues to encode a ts-morphological mutation they called *png-1* based on it's similarity to the yeast gene. The knock-out of NCU00651 was not readily purified as a homokaryon suggesting that whatever this gene does is required for viability. That different mutations in the same gene should have different phenotypes adds value to strains generated via classical mutant hunts.

19) The roles of Mdm10 and Tom7 in the import and assembly of mitochondrial beta-barrel proteins in *Neurospora crassa*.

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The TOM (Translocase of the Outer Mitochondrial Membrane) complex and the TOB (Topogenesis of Outer Membrane beta-Barrels) complex exist in the outer mitochondrial membrane and facilitate the import and assembly of outer membrane proteins. We have investigated the relationship of Mdm10 (a potential component of the TOB complex) and Tom7 (a component of the TOM complex) in the import and assembly of outer membrane beta-barrel proteins in *N. crassa*. Previous work showed that mitochondria lacking Tom7 assemble Tom40 more efficiently, and porin less efficiently than wild type mitochondria. Analysis of *mdm10* and *tom7* single and double mutants has revealed that the effects of the mutations are additive. Loss of Tom7 partially compensates for the decrease in Tom40 assembly seen in mitochondria lacking Mdm10 while porin assembly is more severely reduced in the double mutant than in either single mutant. The additive effects observed in the double mutant suggest that different steps in beta-barrel assembly are affected in the individual mutants. Further results indicate that only a small amount of Mdm10 associates with the TOB complex. Taken together these data suggest that many aspects of Tom7 and Mdm10 function in *N. crassa* are different from those of their homologues in *Saccharomyces cerevisiae*.

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20) Coronin is a soft regulator of polar growth

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Actin remodeling, as the force behind specialized processes such as polarized growth, endocytosis and organelle trafficking is tightly regulated by several proteins. Coronin is a protein that binds to the Arp2/3 complex blocking its actin polymerization activities allowing the complex only to bind to F-actin. It localizes at sites of endocytosis and interacts with microtubules. It is involved in actin turn over affecting actin assembly and dissasembly. In this study, we analize the dynamics of coronin fuse to green fluorescent protein and characterized the coronin mutant (*cor-1*) of *Neurospora crassa*. We observed that Coronin is localized at the sub apex where endocytosis is suggested to take place and co-localized with the Arp2/3 complex and fimbrin. Hyphae of cor-1 mutant were incapable to maintain continuous polarize growth, they showed alternating periods of isotropic cell wall expansion that originated swells or new branches and actual apical growth. The Spk size and dynamics were notoriously compromised. The Spk was unable to support continuous hyphal polarized directionality and Isotropic growth was accompanied by the disassembly of the Spk and there was an accumulation of Fim:GFP and Life-act patches at the apex. Microtubules were also affected, filaments were longer and thicker at the sub-apex and during isotropic growth they were unable to reach the apex. The internalization of the lypophilic dye FM4-64 was slower in the cor-1 mutant (4:48 min) compared with the WT strain (1:18 min). In the cor-1 mutant, showed more septa than the wild type strain. These results points at coronin as an important protein for the actin-based polarized cell growth and actin-tubulin mediated roles.

21) Microtubule plus end proteins in *Neurospora crassa*

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The microtubule (Mt) plus end proteins (Tip+) are heterogeneous factors that promote the assemblage and disassemblage of Mts dynamic end. There are two conserved families of Tip+, which function as stabilizers, such as, the End Binding 1 (EB1) and the human Lis1. We studied the dynamics of the EB1 homolog MTB-3 and the Lis1 homologs Lis1-1 and Lis1-2 in the filamentous fungus *Neurospora crassa*. Both families of Tip+ were observed as abundant fluorescent fragments in different hyphal regions. MTB-3 was present as comet-like structures homogeneously distributed along the hypha. The comets had a length of 1.6 ± 0.4 μm (mean±standard error) (n=100). The MTB-3 coments moved mainly towards the apex (speed of 1.9 μm s^{-1}), 10-folds faster than the hyphal elongation rate (0.2 μm s^{-1}). We observed instances of comets moving in retrograde direction. On the other hand, Lis1-1 and Lis1-2 were observed with variable densities through the different regions of the hypha. In distal subapical region there was little fluorescence and few individual particles can be discerned, the density of particles increases as they get closer to the tip and many of them form linear strings that grow in intensity as they get near the apex, reaching a maximum intensity around the Spk. The fast speed (0.4 μm s^{-1}) of Lis1-1 and Lis1-2 indicated that the fluorescent particles move independently of cytoplasmic bulk flow. Both families of proteins participate in the polymerization of Mts, although each group has a different distribution and dynamics. Unlike other organisms, *N. crassa* seems unique in having duplicate homologs of *lis1* and both having the same dynamics and distribution. The organized displacement of Lis1-1 and Lis1-2 from individual particles at the base of the hypha to the filament-like pattern at the tip, suggests that Lis1-1 and Lis1-2 participate in unique fashion in the dynamics of polarized growth. The Mt- related motor protein conventional kinesin is involved in Lis1-1 and Lis1-2 transport.

22) Lifeact as a reporter for the actin cytoskeleton in *Neurospora crassa*

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The actin cytoskeleton plays a crucial role in hyphal growth and morphogenesis, hence the importance of visualizing its localization and dynamics in growing hyphae. The visualization of the actin cytoskeleton in *N. crassa* has proven to be difficult by GFP-tagging of the actin monomer (G-actin). Recent strategies have consisted in the utilization of actin binding proteins (ABPs) fused to fluorescent proteins. The latest reporter developed is called Lifeact, which consists of the first 17 aminoacids of the actin binding protein Abp140 of *Saccharomyces cerevisiae*. Here we present Lifeact-GFP distribution in *N. crassa* hyphae. Lifeact-GFP labels presumably all F-actin present in *N. crassa*, in the form of cables, patches and a contractile ring involved in septum formation. This idea is supported by the observations that Lifeact tags structures previously imaged using other ABPs such as, tropomyosin-GFP labeling actin cables, and fimbrin-GFP as well as components of the Arp2/3 complex for the actin patches.

23) The role of the GTPase CDC-42 in hyphal growth of Neurospora crassa

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Polarized growth in fungal cells originates at specific sites by the action of cortical markers. The subsequent apical growth is maintained by the assembly of multiple protein complexes that ensure that cellular components get incorporated into the plasma membrane, and provide precursors and enzymes required for cell growth. CDC-42 is a highly conserved member of the family of Rho GTPases that presumably functions as a cortical marker in fungal cells. Besides having a role in polarity establishment and maintenance in Saccharomyces cerevisae and Aspergillus nidulans, in the latter, Cdc42p appears to have an important role in hyphal morphogenesis. Analysis of the Neurospora crassa strain Δ*cdc-42* (FGSC# 15833) stained with FM4-64 revealed a defective morphological pattern showing hyphae with an aberrant shape and multiple septa near the apex; this was accompanied by a severe decrease in hyphal growth, which had an impact on the colony diameter. We found that CDC-42 labeled with mCherry fluorescent protein (mChFP) at the C-terminus was localized at the Spitzenкцrper and at sites of septum formation in mature hyphae. To determine the functionality of CDC-42-mChFP fusion protein, the Δ*cdc-42* strain was crossed to the *cdc-42::mchfp* strain. The resulting selected hygromycin resistant progeny (*cdc-42*$^{+}$*::mchfp*$^{+}$*::hph*$^{+}$) showed the same labeling pattern than that of the parental strain without presenting a defective growth pattern. These results suggest: 1) CDC-42 plays an important key role in hyphal morphogenesis and 2) the C-terminal region of CDC-42 is important for its anchoring within the plasma membrane. These combined results suggest that CDC-42 can exist in two cellular pools.

24) Characterization of the Neurospora VS plasmid in vivo

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The Varkud satellite (VS) plasmid is found in the mitochondria of many Neurospora strains, though no corresponding phenotype has yet been identified. The plasmid encodes a ribozyme that is abundantly transcribed, and is of significant interest as a model for RNA catalysis. We have constructed otherwise-isogenic strains that contain or lack the plasmid, and are comparing their mitochondrial proteomes. We are also comparing the growth rates of these strains under a variety of stress conditions. By these approaches we seek to determine whether the plasmid has any effect on Neurospora function.

25) Are there different populations of vesicles containing chitin synthases in *Neurospora crassa*?

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Filamentous fungi grow by apical and polarized extension. A characteristic of hyphae is a structure named Spitzenkorper (Spk), found at their growing tip. The role of the Spk is to act as a supply center of vesicles that transport the components needed for the expansion of the cell wall, including chitin synthases. These enzymes catalyze the addition of monomers of N-acetylglucosamine to growing chitin chains. Genomic analysis indicates the presence of 7 different sequences of CHS in *Neurospora crassa*. Previous studies on *N. crassa* in which CHS-1, CHS-3, and CHS-6 were tagged with GFP, showed that all of them accumulated at the core of the Spk, and also participated in the formation of septa. In this work, using a split marker technique, we endogenously labeled other chitin synthases namely *chs-2* (NCU05239.3), *chs-4* (NCU09324.3), *chs-5* (NCU04352.3) and *chs-7* (NCU04350.3) with *gfp* to study their distribution in living hyphae of *N. crassa*. We found that three of them, CHS-2, CHS-4, and CHS-7, appear solely involved in septum formation; whereas CHS-5 was localized both in the septum and the core of the Spk. Our findings raise a number of questions and possibilities. There is previous evidence that chitin synthases are carried in chitosome microvesicles. Is each CHS contained in its own chitosome or is it likely that the different CHS are grouped together in two or more types of chitosomes? Conceivably a microvesicle carrying CHS-1, CHS-3, CHS-5, and CHS-6 would account for chitin synthesis in apical growth, but for some as yet unexplained reason septum formation also requires CHS-2, CHS-4 and CHS-7. These three CHS could be supplied by a single type of chitosome dedicated to septum formation or, alternatively, all 7 CHS could be delivered to the septum in a single specialized chitosome.

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26) Use of 1D NMR to measure intracellular metabolite levels during growth and asexual sporulation in *Neurospora crassa*

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Conidiation is a response to adverse conditions and is the main mode of dispersal utilized by a fungal pathogen to reestablish itself in a more favorable environment. Heterotrimeric G proteins (consisting of the alpha, beta, and gamma subunits) have been shown to regulate conidiation in diverse fungi. Of the three G alpha-subunits in *N. crassa*, loss of *gna-3* leads to the most dramatic effects on conidiation. The goals of this research were two-fold: to catalog the metabolome of *N. crassa* under conditions affecting conidiation and to determine whether lack of *gna-3* causes a significant metabolite shift. We measured metabolite levels in vegetative hyphae and conidia from cultures grown in the presence of high or low sucrose to explore the effect of carbon deficiency and conidiation. The results demonstrate that the overall metabolome of the *delta gna-3* mutant is similar to wild-type, but there are changes in levels of specific metabolites that suggest a defect in nutrient sensing in the mutant.

27) Studying the G protein Regulator RIC8 using Suppressors and Chemical Inhibitors in *Neurospora crassa*

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RIC8 is a recently discovered Guanine Nucleotide Exchange Factor (GEF). In animals it is essential for asymmetrical cell division as well as synaptic signaling. Despite being a GEF for Galpha proteins, it is neither a receptor nor membrane bound, making it an interesting exception to canonical G protein signaling. I am currently investigating *ric8* in *N. crassa* using both classical and chemical genetics. Deletion of *ric8* induces severe pleiotropic effects and a nearly lethal growth phenotype. Through random mutagenesis, I am generating suppressor mutants which partially recover the wild-type phenotype in the *ric8* deletion background. I am using SNP-CAPS to identify the mutated gene responsible for the phenotype. In parallel, I am conducting a chemical screen for inhibitors of the interaction between RIC8 and Galpha proteins. Using compounds that specifically alter the RIC8-Galpha interaction *in vivo*, I intend to tease apart the effects of RIC8 that are dependent on its interaction with G proteins from effects that are independent of G protein signaling in *N. crassa*.

28) Identification and analysis of STE-50, a protein that interacts with the a novel GEF, RIC8

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Heterotrimeric (alpha, beta ,gamma) G proteins are essential components of signal transduction pathways that regulate environmental sensing, growth, and development in eukaryotes. RIC8 is a cystolic protein that can serve as a guanine nucleotide exchange factor (GEF) for G alpha proteins. RIC8 is required for asymmetric cell division in zygotes and priming of synaptic vesicles in *Caenorhabditis elegans*. A *ric8* homologue is present within the *Neurospora crassa* genome. We have identified proteins that interact with RIC8 via a yeast-two-hybrid cDNA library screen. One of the protein hits is STE- 50, a regulator of MAPK signaling in fungi. A construct containing the STE-50 FLAG fusion protein with the driver Nourseothricin acetyl transferase gene (Nat) as a selectable marker was obtained through yeast recombination cloning, and then transformed into a *delta mus-51 Neurospora* strain. In order to obtain homokaryons, the transformants were crossed to wildtype (74A). The presence of the FLAG sequence was confirmed with PCR and western blots. This STE-50 FLAG strain will be used will be used for identification of additional interacting proteins in complexes, with the use of mass-spectrometry (MALDI- TOF).

29) Characterization of the *ric8* gene in *Neurospora crassa*.

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Eukaryotic organisms respond to changes in their environment via heterotrimeric G protein signaling. The heterotrimer consists of a G alpha protein, which binds and hydrolyzes GTP, and a G beta/G gamma dimer. G proteins associate with membrane-bound G protein coupled receptors (GPCRs), which regulate the G alpha subunit by serving as a guanine nucleotide exchange factor (GEF). Recently, GEF activity by a non- GPCR, RIC8, has been discovered as a novel regulator of G proteins in animals, but has not been characterized in filamentous fungi. Here we present analysis of RIC8 in *Neurospora crassa*. Deletion of *ric8* leads to defects in polar growth and asexual and sexual development, similar to phenotypes observed for a mutant lacking G alpha genes *gna-1* and *gna-3*. This phenotype can be partially suppressed by introducing constitutively activated alleles of *gna-1* and *gna-3*. Similar to reports in *Drosophila*, strains lacking *ric8* have greatly reduced levels of G protein subunits. RIC8, GNA-1, GNA-2, and GNA-3 have been purified from *E. coli* and used to test the G alpha GEF activity of RIC8. Studies suggest that RIC8 may increase GTP binding of GNA-1 and GNA-3. Our results support a role for RIC8 and G proteins in the regulation of polar growth in *Neurospora*.

30) N-terminal tagging of Galpha proteins at the native locus in *Neurospora crassa*

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Heterotrimeric G proteins regulate environmental sensing and control production of second messengers in eukaryotes. G proteins consist of three subunits: alpha,beta and gamma. Regulation of the signal transduction pathway is accomplished through the alternation between binding of GDP (inactive form) and GTP (active form). Galpha-GTP can regulate downstream signaling pathways. *Neurospora crassa* has a well-characterized G protein signaling pathway. *N. crassa* has three Galpha subunits (GNA-1, GNA- 2 and GNA-3),one Gbeta (GNB-1), and one Ggamma (GNG-1). Mutating all three Galpha protein genes has been shown to severely restrict apical growth, and to cause dense premature conidiation and female sterility (Kays, Borkovich 2004). Since G proteins are so important for cellular signal transduction and the Galpha subunit is the most mobile part of the system, our laboratory has begun a project to tag N-terminus of the Galpha protein subunits (the C-terminus is a key of receptor specificity), to facilitate isolation of proteins that interact with G proteins in vivo.The tagging strategy is based on fusion into delta mus51 and delta mus52 *Neurospora* strains Galpha vectors assembled by yeast recombinational cloning of polymerase chain reaction (PCR) products. All vectors were designed as “knock-ins” that would replace the endogenous Galpha genes at the native locus. Results of tagging one of the Galpha subunits (GNA-1) will be presented.

31) Calcium signaling genes in *Neurospora crassa*

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We have identified NCU02826.2, NCU04379.2, and NCU09123.2 gene as major components of the *N. crassa* calcium signaling machinery. The NCU04379.2 gene predicted to encode a Ca2+and/or CaM binding protein of 190 amino acids that shows best overall with *Magnaporthe grisea* MgNCS1. The NCU04379.2 knockout mutant is extremely sensitive to calcium and grows slowly. We have identified NCU04379.2 homologs in other organisms including *Saccharomyces cerevisiae*. In *S. cerevisiae*, NCU04379.2 ortholog is called Frq1 that interacts with Pik1. We have also identified NCU10397.2 as the *N. crassa* ortholog of Pik1. The NCU02826.2 gene encodes a putative a Ca2+/Na+ exchanger protein, and crosses homozygous for NCU02826.2 knockout mutants are barren. The NCU09123.2 gene predicted to encode a Ca2+and/or CaM binding protein and crosses homozygous for NCU09123.2 knockout mutants show intermediate phenotype. Acknowledgements: We thank Indian Institute of Technology Guwahati , Assam, India for financial supports and Fungal Genetics Stock Center, University of Missouri, Kansas City, USA for generously providing Neurospora strains for free of charges.

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32) Two *Neurospora crassa* NDR kinases (DBF2 and COT1) perform distinct functions in regulating hyphal morphology.

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The nuclear Dbf2-related (NDR) protein kinases are comprised of two subgroups (represented in *Neurospora crassa* by DBF2 and COT1). In *N. crassa*, DBF2, (a component of the HIPPO pathway in higher eukaryotes) is involved in cell cycle regulation, glycogen biosynthesis and both sexual and asexual reproduction. Defects in other DBF2 and glycogen metabolism pathway components (*mob-1, ccr-4, mst-1* and *gsk-3*) share similar phenotypes such as mitotic arrest, massive hyphal swellings, hyphal tip bursting, glycogen leakage and impaired conidiation, suggesting that DBF2 functions as a link between the DBF2 and glycogen metabolism pathways. Dysfunction of the second *N. crassa* NDR kinase, COT1, leads to cessation of tip extension and massive induction of new branches. COT1 function is dependent on interactions with other proteins (e.g., POD6 and MOB2a/b) and its phosphorylation state. Analyses of point-mutated *cot-1* strains (mimicking non and constitutively- phosphorylated states of conserved NDR residues) indicate the involvement of COT1 phosphorylation in the regulation of hyphal morphology and asexual development by altering cell wall integrity and actin organization. Furthermore, COT1 regulates elongation and branching in an independent manner, which is determined by the phosphorylation states of COT1's activation segment (at Ser417) and the C-terminal hydrophobic motif (at Thr589). Taken together, DBF2 and COT1 share structural similarity and associate with MOB family proteins, yet they perform distinct functions in governing hyphal morphology.

33) Characterization of the central pathway regulating macroconidiation in *Neurospora crassa* and comparative analysis with *Aspergillus nidulans*

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A. nidulans and *N. crassa* are models to study the regulation of conidiation. BrlA and FL are well characterized transcription factors that are the key regulators of conidiation in these species. fl is essential to induce conidation and previous studies placed fl downstream of acon-2 and upstream of acon-3 in the genetic pathway controlling development. Here we describe the role of the N. crassa ortholog to A. nidulans flbC (acon-4) in regulation of fl and acon-3 (the ortholog of A. nidulans medA). acon-4, fl, and acon-3 had increased induction after 3 hrs of nitrogen starvation-induced conidiation in the wild type. In Δfl and Δacon-3, acon-4 expression was similar to that of the wild type. fl showed very weak induction in theΔacon-4 mutant, and had a similar expression pattern to the wild type in Δacon3. acon-3 was not induced in either Δacon-4 or Δfl. This implies that acon-4 functions upstream of fl, and acon-3 is downstream of fl. Expression of con-6, con-10, and eas in the wild type and mutants confirmed the dependencies of these genes on the fl and acon-3 genes and, as expected, showed they are dependent on acon-4. Therefore, we provide a simple model of the regulation of conidation in N. crassa. The orthology of two of the three regulators with regulators of conidiation in A. nidulans has implications for how regulatory pathways for conidiation arise during fungal evolution.

34) Comparison of *Neurospora crassa* transcription profiles before and after mycelia contact with fungi of increasing phylogenetic distance.

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We are interested in looking at whether there are transcriptional profile differences in *Neurospora crassa* when it comes into contact with fungi of different phylogenetic relatedness. We have processed over 20 lanes of RNA-seq data of *Neurospora crassa* growing with *N. crassa* of the same genotype, *N. crassa* of a different genotype, *N. discreta* and *Penicillium chrysogenum*. All experiments were done in triplicate and mycelia from *N. crassa* were collected before contact with the other fungi and after contact with the other fungi. Using the RNA-seq data we have found that there are significant differences among the transcription profiles.

35) Sun tanning in Neurospora: Characterization of wild types from the Iberian peninsula and the Canary Islands show a correlation between latitude and carotenoid accumulation.

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Neurospora is found on burned vegetations and during the summers of 2006 and 2007 the Northwestern corner of the Iberian peninsula (Galicia, Spain) and the Canary Islands (North West of Africa) suffered unusually frequent wild fires that allowed us to sample the Neurospora populations in these regions. For each sample we amplified and sequenced three noncoding loci flanking microsatellites (TMI, DMG and TML). In addition we measured the amount of carotenoids in mycelia grown in the dark or after one day of light exposure. We isolated 125 new wild type strains of Neurospora: 69 from Galicia and nearby regions, and 56 from two islands of the Canary Islands archipelago (Santa Cruz de Tenerife and Gran Canarias). Most of the strains collected in the Northwestern part of the peninsula are *N. discreta* while the majority of the strains collected in the Canary islands are *N. crassa*. In addition, most of the plants that we sampled repeatedly in the Canary Island had more than one Neurospora strain, unlike plants sampled in the Iberian Peninsula. We have observed that strains isolated from northern regions of Spain accumulated less carotenoids than strains isolated from the Canary Islands. Could environmental conditions, including light, affect the presence and distribution of Neurospora species in nature?

36) Unexpected population structure in *Neurospora crassa* from the Caribbean Basin

Christopher Ellison, Charles Hall, Angela Kaczmarczyk, David Kowbel, Juliet Welch, Rachel Brem, N. Louise Glass, John W. Taylor, University of California, Berkeley

We used solexa sequencing of mRNA to simultaneously identify Single Nucleotide Polymorphisms (SNPs) and quantify gene expression for more than sixty isolates of *Neurospora crassa* from the Caribbean Basin. Through population genomic analysis of the SNP data, we find strong support for two recently diverged populations, one endemic to Louisiana and the other distributed through Florida, Haiti and the Yucatan. We also identify a subset of genes that show the signature of positive selection and a subset that are differentially expressed between species. Based on the evidence for a recent divergence time and the presence of gene flow between these populations, we argue that this represents an ideal dataset for the study of the early stages of speciation.

37) Multiple, complementary, gel-based mass spectrometric and computational approaches to determining the mitochondrial proteome of Neurospora.

Diane DeAbreu, Andrew Keeping, Michael DiBernardo and Richard Collins. Department of Molecular Genetics, University of Toronto

Determining the complete protein composition of any organisms, or even organelle, remains a challenge in proteomics. In the current work we have used a variety of gel electrophoretic techniques to separate the proteins in highly purified Neurospora mitochondria and sub-mitochondrial fractions (soluble proteins, membranes, protein complexes and ribonucleoproteins) and identify them by MALDI-TOF peptide mass fingerprinting. Combined with previous mass spectrometry studies from other groups, and re-evaluation of annotations, we have compiled a curated list of approximately 430 identified proteins that are likely to be bona fide mitochondrial proteins. Literature data mining and computational approaches suggest another 300 proteins not yet identified during mass spectrometric projects are also mitochondrial.

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38) High-throughput gene disruption and functional analysis of serine-threonine protein kinase genes in *Neurospora crassa*

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Annotated *Neurospora* genes (approximately 10,000) have been systematically knocked out using a high throughput procedure as part of an NIH-funded Program Project (PO1). Currently, we have completed targeted disruption for 7,342 genes and over 10,000 strains have been submitted to the Fungal Genetics Stock Center. The list of submitted strains is available at the *Neurospora* genome project website. The UCR group will continue to knock-out the rest of the genes (~2,700 genes) using PCR amplification instead of Southern blot for the final verification of mutants. Currently, diagnostic PCR primers for all annotated genes have been designed. Ninety serine/threonine (S/T) protein kinase genes annotated in the *Neurospora* genome were functionally analyzed using the knockout mutant collection. Of the 90 total genes, viable gene replacement mutants were generated for 80 kinases. Phenotypic analysis showed that more than 50% of the viable kinase mutants had defects in vegetative growth, or asexual or sexual development. A majority of mutants having phenotypes (71%) exhibited defects in more than one trait, indicating that most S/T kinases regulate multiple functions. Eleven kinase mutants were more sensitive or more resistant to menadione (100μM) than wild type. Phosphorylation of the three Neurospora MAPKs was assayed in several kinase mutants under menadione and high salt treatment, and our current results will be presented.

Gene Regulation

39) *Neurospora crassa* transcription factors involved in glycogen metabolism regulation and cellular development

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The glycogen content in microorganism varies depending on the environment conditions. In *N. crassa*, glycogen reaches maximal levels at the end of the exponential growth phase. However, under heat shock condition, glycogen content and transcription of the glycogen synthase gene (*gsn*) rapidly decrease. In this work we showed that transcription factors annotated as hypothetical or predicted protein regulate glycogen metabolism and some cellular development processes. Analysis of glycogen content in *N. crassa* mutant strains individually knocked-out in genes encoding these transcription factors (ORFs NCU04390, NCU09739, and NCU03043) were performed. All mutant strains presented changes in glycogen accumulation during normal growth and under heat shock condition (from 30є to 45єC) when compared to the wild type strain. The *gsn* expression assays by Northern blot in both conditions showed that two proteins (NCU03043, and NCU04390) regulate gene expression, which led us to conclude that the proteins regulate glycogen metabolism by regulating *gsn* expression. Mutant strains conidia were analyzed by flow cytometry and two strains (NCU09739, and NCU03043) showed severe alterations in conidia morphology and in cell cycle. These analysis contribute to a better characterization and understanding the mechanisms involved in the glycogen metabolism regulatory network. Financial support: FAPESP, CNPq, and CAPES

40) The *Neurospora crassa gsn* gene expression is modulated by extracellular pH changes

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The *gsn* gene encodes glycogen synthase in *N. crassa*, the rate-limiting enzyme in the glycogen synthesis. The *gsn* expression is regulated at different levels and by different environmental conditions. In this work we have described the modulation of the gene expression by ambient pH changes. In *Aspergillus nidulans* gene expression regulation under alkaline growth conditions is mediated by the PacC transcription factor, which promotes transcription of alkaline-specific genes and repression of acid-specific genes. The *gsn* promoter contains a *cis* PacC DNA element leading us to investigate whether pH stress regulates *gsn* expression. Conidia from wild type strain were first cultivated under physiological pH condition (5,8) and then shifted to acid (4.2) and to alkaline pH media (7.8). Samples were collected at different times, the glycogen content was quantified and the *gsn* expression was analyzed by Northern hybridization. We also analyzed the expression of the gene encoding the *N. crassa* PacC homologue (NCU00090). Glycogen content increased under acid stress and decreased under alkaline stress. Glycogen results were consistent with *gsn* expression under alkaline pH, gene transcription was downregulated. In the same condition the *NcpacC* expression was upregulated. The results suggest that the NcPacC transcription factor is activated under alkaline pH and then downregulates *gsn* expression, thus characterizing *gsn* as an acid-specific gene. Financial support: FAPESP, CNPq, and CAPES

41) Analysis of the sugar transporter gene family of *Neurospora crassa.*

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Biofuel production from lignocellulosic biomass is limited in part by the high costs of breaking down cellulose into glucose. *N. crassa* strains that lack glucose uptake but secrete cellulases would obviate the need to add cell-free enzymes to achieve saccharification. The accumulated glucose could be used as a substrate for other microbes for conversion to biofuels. An understanding of both how filamentous fungi sense sugars to control transporter gene expression and the functions of the sugar transporter genes is necessary to construct Neurospora strains that do not take up glucose. There are ~36 sugar transporter homologs in Neurospora crassa. Several the several sugar transporter homologs were regulated by glucose. This includes NCU10021 (hxt-1), a major high-affinity glucose transporter and NCU01633, a major low- affinity glucose transporter. The functions of *N. crassa* glucose transporters have been tested by heterologous expression in yeast. More than half of the *N. crassa* "sugar transportome" has been assessed and we identified 14 glucose transporters so far. Gene expression profiles and yeast functional analysis will help to prioritize the genes that need to be mutagenized to create a transport-null strain. In addition, rco-3 and other deoxyglucose resistant mutants regulate sugar transporters. Combinations of regulatory mutants and glucose transporter mutants may allow elimination of glucose transport by combining a small number of genes.

Other Topics

42) Development of an inducible promoter for *Neurospora crassa*

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Inducible promoters that can conditionally express desired genes are useful tools for genetic analyses. In particular, such promoters are extremely helpful in the identification and characterization of essential genes. Unfortunately, there is currently only one conditional promoter available in *Neurospora*, the *qa-2* promoter, which drives gene expression through exposure to quinic acid. We examined the possibility of developing another heterogeneous inducible promoter for conditional gene expression in *Neurospora*. Expression from the xylanase (AN3613) promoter of *Aspergillus nidulans* is induced by the monosaccharide xylose and is under the control of the transcriptional activator XlnR. We cloned the 628 bp xylanase promoter from *A. nidulans* and placed it directly upstream of a hygromycin resistance gene (*hpt*). This construct was then introduced into wild type Neurospora. We show that in resulting transformants, addition of xylose to the growth medium induced transcription of the *hpt* gene and resulted in resistance to hygromycin. Interestingly, these phenotypes were clearly repressed when transformants were grown in the presence of glucose rather than xylose.

43) A short-patch repair variant in Neurospora?

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In general, the frequency of intragenic recombination depends on the distance between the sites used for the measurement, so when alleles are very close, prototrophic recombinants are very rare or absent. The polymorphic nature of *Neurospora crassa* laboratory strains, although frequently annoying, has led to some interesting discoveries, and what we report here is no exception. Routine strain-building generated crosses in which allelic recombination at *his-3* was so abnormal that we suspected a mistake and began again. When the repeat experiment yielded the same result, we realised we had discovered a new gene with an unexpected affect on recombination. In crosses between strains with alleles at opposite ends of *his-3* (K1201 and K480), this new gene has no obvious effect. However, when the alleles are closer, 215 bases apart (K874 and K26), if at least one strain in the cross carries the high frequency (HF+) variant, His+ progeny are 12-fold more numerous than when HF+ is absent. This increases to about 60-fold for alleles K1201 and K504, which are only 64 bases apart! At present the nature of the gene is unknown, but we speculate it may be involved in short-patch mismatch repair. In collaboration with Michael Frietag, we plan to locate the gene sequence by massively parallel sequencing. We have extracted 120 progeny from a cross between the variants and have currently identified 21 as HF+ and 24 as HF- with respect to recombination between close alleles.

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44) Biosynthesis of Metallic and Bimetallic Au/Ag Nanoparticles Using *Neurospora crassa*

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Green chemistry methods offer opportunities to design nanomaterial production processes that can reduce the environmental impact, offer waste reduction and increase the energy efficiency. In this work we have investigated the biosynthesis of metallic and bimetallic Au/Ag nanoparticles using the filamentous fungus *Neurospora crassa*. Analysis by scanning electron microscopy (SEM), energy dispersive X-ray spectroscopy (EDS) and transmission electron microscopy (TEM) confirmed the biosynthesis of nanoparticles by the fungus. The shape of nanoparticles was found to be mainly spherical with 11.1±4.4 nm and 12.9±5.9 nm diameter for silver and gold, when exposed to the aqueous solutions of 10^{-3} M of $AgNO_3$ and $HAuCl_4$, respectively. Electron diffraction and EDS results confirm the formation of alloy-type Au/Ag bimetallic nanoparticles of dimensions between 20 and 50 nm when a combination of $AgNO_3$ and $HAuCl_4$ was used. TEM images of thin sections of *N. crassa* cells confirmed the intracellular formation of silver and gold nanoparticles. The results obtained indicate that *N. crassa* can be a potential "nanofactory" for the synthesis of various metallic nanoparticles. The use of *N. crassa* for this purpose will offer several advantages since it is considered as a non-pathogenic organism, has a fast growth rate and easy and economic biomass handling.

45) Antifungal activity of chitosan in filamentous fungi depends on membrane fluidity

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The antifungal mode of action of chitosan has been studied for the last thirty years, but is still little understood. We have previously found different sensitivity to chitosan on different filamentous fungi. In this work we have found that chitosan enters cells of chitosan sensitive (*Neurospora crassa* and *Fusarium oxysporum*) but not cells of chitosan resistant (*Pochonia chlamydosporia* and *Beauveria bassiana*) fungi, indicating that the plasma membrane forms a barrier to chitosan in chitosan-resistant fungi. The plasma membranes of chitosan-sensitive fungi were shown to have more polyunsaturated fatty acids than chitosan-resistant fungi suggesting that their permeabilization by chitosan may be dependent on membrane fluidity. A fatty acid desaturase mutant of *Neurospora crassa* (Δods) with reduced plasma membrane fluidity exhibited increased resistance to chitosan. Steady- state fluorescence anisotropy measurements on artificial membranes showed that chitosan binds to negatively charged phospholipids that alter plasma membrane fluidity and induces membrane permeabilization, which was greatest in membranes containing more polyunsaturated lipids. Our findings provide a method to predict the sensitivity of a fungus to chitosan based on its plasma membrane composition.

46) High throughput identification of classically derived genetic mutations in *Neurospora crassa*

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Despite the availability of the genome sequence many classical genetic mutants of *Neurospora crassa* remain anonymous. To associate classical mutants with their respective DNA sequence, whole genome sequencing was carried out for several *N. crassa* classically derived mutant strains from the FGSC collection. This approach has allowed the tentative identification of two point mutations (*sc* and *mb-2*) and five indels (*ts*, *dot*, *fi*, *tng* and *fs-n*) associated with genetically characterized genes. Because of the high divergence among strains of *N. crassa*, especially for mutations identified decades ago, in most cases mutations had to be compared to the newly defined consensus sequences rather than to the reference genome sequence. A number of approaches are being used to reinforce the association between the identified DNA sequences and classical genetic mutants including association studies (i.e. the mutated sequence should occur in every strain carrying the mutant gene) and complementation.

47) Genetic studies to uncover rules for epigenetic regulation of *Neurospora crassa* centromeres.

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Centromeres are essential for proper segregation of chromosomes during cell division. While centromeres in budding and fission yeast are well understood, it remains unknown how centromeres of filamentous fungi are assembled or maintained. We are studying centromere proteins of closely related filamentous fungi to find conserved domains, functions and mechanisms of chromosome segregation in this large group of organisms. The centromere-specific histone H3 (CenH3) and centromere protein C (CENP-C) are foundation proteins for kinetochore assembly. Both proteins appear highly adapted, suggesting important differences in the organization of centromeres or the mechanisms of chromosome segregation. Our studies aim to improve the general understanding of CenH3 and its potential for epigenetic regulation. We showed previously that *N. crassa* (Nc) has regional, heterochromatic centromeres, and analyses of fungal CENP-C and CenH3 genes revealed a patchwork of motifs under both positive and negative evolutionary selection pressure. These results allowed us to design experiments that test the functional importance of residues with putative posttranslational modifications. Substitution of the endogenous NcCenH3 with the genes from *Podospora anserina* (Pa), *Fusarium graminearum* (Fg) and *Aspergillus nidulans* (An) revealed that ΔNcCenH3::PaCenH3-GFP strains undergo mitosis, but are unable to complete meiosis. Strains with FgCenH3-GFP or AnCenH3-GFP loci were never recovered, suggesting that these alleles are lethal. Strains harboring ΔNcCenH3::PaCenH3-GFP alleles underwent crosses with heterokaryotic strain (with mixtures of transformed AnCenH3-GFP or FgCenH3-GFP, and untransformed NcCenH3 nuclei). Homozygous NcCenH3-GFP crosses remained barren, suggesting that altering the C-terminus of CenH3 by addition of the bulky GFP adduct may change the conformation of centromeric DNA in contact with CenH3 nucleosomes. Nevertheless, homozygous NcCenH3-3xFLAG crosses were also barren, suggesting that tag size itself is not as important as potential disruption of CenH3 protein or DNA interaction motifs. We also report results from experiments with tag-less PaCenH3, FgCenH3 and AnCenH3 strains and from domain-swapping experiments that should further define important functional regions of Neurospora CenH3.

48) A SNP map of *Neurospora crassa* Mauriceville-1-c.

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With the advent of high-throughput DNA sequencing it is now simple and inexpensive to generate high-density SNP maps that can improve mapping strategies based on RFLP [1], CAPS [2,3] or RAD-mapping [4,5] analyses. Direct identification of single point mutations has been described in fission yeast [6] but in most organisms bulk segregant analyses followed by SNP mapping are used [7]. Here we present the SNP map of *Neurospora crassa* Mauriceville-1-c (FGSC2225) [8], the strain most typically used by Neurospora researchers to carry out RFLP mapping crosses. Citations: 1. Metzenberg *et al.* 1984. Neurospora Newsl. 31: 35-39. 2. Jin *et al.* 2007 Fungal Genet. Biol. 44: 455-465. 3. Lambreghts *et al.* 2009. Genetics 181: 767-781. 4. Lewis *et al.* 2007. Genetics 177: 1163-1171. 5. Baird *et al.* 2008. PLoS ONE 3: e3376. 6. Irvine *et al.* 2009. Genome Res. 19: 1077-1083. 7. Cuperus *et al.* 2010. PNAS 107: 466-471. 8. Beauchamp *et al.* 1977. PNAS 74:1172-1176.

49) The α-1,6-mannosylation of N-linked oligosaccharide present on cell wall proteins is required for their incorporation into the cell wall in the filamentous fungus *Neurospora crassa*

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The α-1,6-mannosylation of cell wall proteins is an important post-translation modification that occurs in the Golgi apparatus. The enzyme α-1,6-mannosyltransferase (OCH-1) adds an initial mannose residue to the N-linked N-acetyl glucosamine 2/Mannose 8 oligosaccharide, which is then further modified by the addition of other sugars. The *och-1* KO mutant of the filamentous fungus *Neurospora crassa* is a morphological mutant that exhibits a tight colonial phenotype. A carbohydrate analysis of the *och-1* mutant cell wall revealed an altered composition, with a ~10-fold reduction in mannose content and a total lack of 1,6-linked mannose residues. Subjecting the *och-1* mutant to cell wall stress tests verified that it had a defective cell wall. Analysis of the cell wall protein fraction from the wild type and *och-1* mutant showed that the mutant cell wall contained a reduced level of cell wall protein. MS analysis of the proteins released from the mutant into the growth medium identified a number of proteins found in the wild-type cell wall. Western blot analysis of ACW-1 and GEL-1 (two GPI-anchored proteins that are covalently integrated into the wild-type cell wall) showed that high levels of these proteins were being released into the medium by the *och-1* mutant. We also demonstrate that high levels of ACW-1 and GEL-1 are released from the mutant cell wall by a treatment with boiling in 1% SDS, indicating that the protein is not covalently integrated into the mutant cell wall. From these results we conclude that N-linked mannosylation of cell wall proteins by OCH-1 is required for their efficient covalent incorporation into the cell wall and for the biogenesis of a normal cell wall.

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50) The MAK-1 MAP Kinase Pathway: Identifying Targets and Defining Its Role in Circadian Output in *Neurospora crassa*

Lindsay Bennett, Phillip Beremand, Terry Thomas, and Deborah Bell-Pedersen

In *Neurospora crassa*, the circadian clock impinges upon the Osmolarity Sensing pathway (OS), an evolutionarily conserved mitogen activated protein kinase (MAP kinase) pathway, to regulate clock controlled genes. The clock signal is transduced through the rhythmic phosphorylation of the MAP kinase OS-2. Neurospora contains two additional MAP kinase pathways and here we show that the MAP kinases, MAK-1 and MAK-2, are also rhythmically phosphorylated, and therefore activated, in a clock dependent manner. MAK-1 is a homologue of Slt2, the MAP kinase responsible for cell wall integrity in yeast; however, little is known about the function or the genetic regulon of this pathway in *Neurospora crassa*. Using a *mak-1* deletion strain, we performed microarray analysis and identified ~600 putative targets of the MAK-1 pathway, with ~200 of these transcripts also predicted to be under control of the clock. Using these results, we hope to further characterize the mechanism by which the clock is co-opting established signaling pathways, thereby allowing circadian oscillators to efficiently regulate multiple genes that prepare the organism for daily environmental changes.

51) Chemical screening of protein kinase mutants in *Neurospora crassa*

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Serine-threonine and histidine protein kinases phosphorylate downstream target proteins that carry out cellular work in response to various environmental stimuli. The goal of this project is to determine phenotypes for 91 viable protein kinase knockout mutants under circumstances that significantly inhibit (30%) growth of the wild-type strain. These mutants have been tested under six growth inhibitory conditions: sorbitol, FK-506, fludioxonil, tert-butyl hydroperoxide, cytochalasin A and elevated temperature (42°C). Continual work to further validate previous results is underway, along with the addition of two more chemicals, benomyl and sodium chloride. Previous analysis of growth and development phenotypes demonstrated that 50 of the kinase mutants had phenotypes (UCLA Neurospora Genetics and Genomics Summer Research Institute). In this study thus far, an additional 40 mutants have been found to have defects, leaving only one kinase mutant without a phenotype. These results will help elucidate the function of each kinase. Presented herein will be current data.

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52)Mating type specific differences in *mat-A* and *mat-a* strains of *Neurospora tetrasperma* and *N. crassa*

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Sexual dimorphism among plants and animals is widespread and allows distinction between mating types like males and females during mating. In many species these traits are apparent in secondary sexual characteristics like tusks, color, body size, etc. and gamete production. Even though these differences are strikingly apparent, the genomes of males and females are nearly identical with the exception of few genes on the sex chromosomes. In fungi, sexual dimorphism is not visible to the naked eye, and in filamentous ascomycetes sexual compatibility resides on a genomic level within mating type loci (MAT, which appears as *mat-A* or *mat-a*, i.e. they have a bipolar mating-system). Our research turns to mating-type specific and mating-type enriched expression in *N. tetrasperma*, which is a pseudohomothallic species with a large region of suppressed recombination, spanning more than 6.6 Mbp around the *mat*-locus. This suppression of recombination results in numerous genes linked to mating-type in this species. Six different homokaryotic components (four *Neurospora tetrasperma* and two *N. crassa*) were grown on two different nutrient sources (SC and Vogel's medium, +/- forming of protoperithecia, respectively). The mycelia were harvested after five days and mRNA was extracted and used for competitive hybridizations on microarrays.

Our results show a larger total number of expressed genes in the protoperithecia-forming mycelium, than in the plain mycelia. Also, the total number of expressed genes per linkage group (LG) was significantly greater over all LGs in the strains grown on SC-medium when compared to the strains grown on Vogel's medium. The *mat-A* strains grown on SC showed a higher number of significantly expressed genes than the mat-*a* strains, suggesting that *mat-A* is more active than *mat-a* during protoperithecia formation. We are currently trying to characterize differentially expressed genes in *mat-A* and *mat-a* of *N. tetrasperma*.

53) Sequential phosphorylation of FRQ by CKI is crucial for establishing rhythmicity in the Neurospora clock

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The Neurospora clock protein FREQUENCY (FRQ) is rhythmically expressed under the control of the transcription factor WHITE COLLAR COMPLEX (WCC). FRQ is in complex with CASEIN KINASE I (CKI) and modulates the activity of the WCC by facilitating its phosphorylation via CKI. Additionally CKI phosphorylates FRQ, which is crucial for its maturation and subsequent degradation. Thus, abundance levels and phosphorylation state of FRQ are rhythmic and determine the circadian activity profile of the WCC. Here we show that two sites in FRQ are required to bind CK1. Mutation of either site abolishes CKI binding and results in accumulation of hypophosphorylated and hyperstable FRQ. We also show that the fate of FRQ is determined by sequential phosphorylation of distinct regions in the N-terminal and central portion of the protein.

54)The Rab GTPase-activating protein-MSB-3 in the filamentous fungus *Neurospora crassa*

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In fungal hyphae multiple protein complexes assemble at sites of apical growth to maintain cell polarity. Polarity allows the directional traffic of vesicles to the Spitzenkörper prior to fusing with the plasma membrane to provide precursors and enzymes required for cell extension and nutrition. One of these complexes is the polarisome, which in Saccharomyces cerevisiae consists of Spa2, Pea2, Bud6, Bni1 and perhaps additional proteins. One of the possible additional components of the polarisome in yeast cells is Msb3 that is a GTPase-activating protein (GAPs) that interacts directly with Spa2, a scaffold protein of the polarisome that also interacts with the formin Bni1. In yeast cells, the behavior of Msb3 during the cell cycle is similar to other polarisome components. We studied the localization and role of MSB-3 during polarity establishment and septum formation in Neurospora crassa. To our knowledge this is the first report of the MSB-3 protein in filamentous fungi. We tagged MSB-3 with the mCherry fluorescent protein (mChFP) and examined growing cells by laser scanning confocal microscopy in mature hyphae. MSB-3 was observed as a bright fluorescent accumulation in the center of the Spitzenkörper, smaller than the signal of the SPA-2-GFP. This fluorescent spot mirrored the behavior of the Spitzenkörper. During septum formation MSB-3 was transiently present. The MSB-3 knockout mutant had a strong decrease of the growth rate to one third of the wild type strain. Preliminary results show that MSB-3 could be part of the polarisome and apparently, its presence is not essential but seems to be important to maintain normal polarized growth.

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