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Cellular Signaling Mechanisms in *Neurospora crassa*

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In this journey towards obtaining a Ph.D. and becoming an independent scientist who is able to make hypotheses and build upon observations to eventually make significant and novel contributions to the scientific community, we face innumerous hurdles. One person alone cannot upturn such roadblocks and we constantly look to others to help us solve problems and trudge along this path to obtain our moments of success. In such regard I am grateful to so many people I have met on this road leading to this monumental effort towards a successful completion of my graduate career. The first and the most important person I would like to thank would be my major professor – Dr. Katherine Borkovich who through her sterling supervision, advice and outstanding scientific expertise has guided me over the last five years I have spent in her lab. Not only have I become a better researcher under her guidance and mentorship but thanks to the opportunities I have received for scientific writing, I have improved as a writer as well. I am also indebted to Dr. Jacqueline Servin for the initial years of mentorship and extraordinary training and collaboration on my first scientific publication. I would also like to thank Dr. Alexander Michkov for the innumerable nights spent in the lab training to gain expertise in polysome profiling and sucrose density centrifugation, discussing my data and various translational control related projects.

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ABSTRACT OF THE DISSERTATION

Cellular Signaling Mechanisms in *Neurospora crassa*

by

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Cellular signal transduction mechanisms are regulated at multiple different stages during an organism's life cycle as well as life span. Environmental stress and starvation responses are extremely well coordinated by cell surface receptors and internal scaffolding molecules that are routing the signal to the effector proteins such as transcription factors. It is through these transcription factors that the cell will then regulate gene expression. With the help of this study, my co-authors and me have tried to elucidate a signal transduction network, which explains various facets of cellular signaling in the model filamentous fungus – *Neurospora crassa.*

The first chapter has elucidated the role of serine/threonine and tyrosine phosphatases in growth and development in *Neurospora.* In addition, the chapter also shows that deletion of certain phosphatases lead to sensitivities to chemicals inducing osmotic stress, oxidative stress, cytoskeletal defects or ROS accumulation. Nine phosphatase mutants are also listed to have elevated levels of the active phosphorylated form of p38 mitogen activated protein kinase (OS-2 in *Neurospora*) which is a critical regulator for counteraction to osmo-stress as well as an important regulator of female sexual development. Two other interesting genes – NCU04600 (*pph-8*) and NCU08380

(*csp-6*) are also described in this chapter for their unique phenotypes. The second chapter deals with the role of the important scaffolding protein and RACK1 homolog – CPC-2 in regulation of amino acid starvation mechanisms in *Neurospora* known as cross pathway control. CPC-2 is found to regulate the bZIP transcription factor – CPC-1 via modulation of post-translational modifications on CPC-1 during amino acid starvation. This transcription factor is in turn, integral towards de-repression of amino acid biosynthetic genes under amino acid starvation conditions. This chapter provides mechanistic details on how CPC-2 is able to regulate CPC-1 protein and thereby affect cross pathway control. In chapter III, the focus is shifted towards translational regulation via heterotrimeric G proteins and the guanine-exchange factor RIC-8 to build up a novel finding revealing that G proteins and RIC-8 are an integral part of the ribosome.

Elevated phospho-eIF2α levels in the G protein and *ric-8* mutants suggest that global translation is greatly reduced in these strains. Poly-RNA-seq analyses of *gna-1, gnb-1* and *ric-8* mutants reveal certain ribosomal proteins as well as elongation factors and two serine/threonine kinases – *stk-18* and *stk-43* are greatly affected in polysomal co-migration by these gene deletions. In addition, deletion of *ric-8* leads to loss of PKC protein from the polysomes, which suggests critical translational control of PKC via RIC-8. This thesis has thus aimed to expand on current knowledge on these abovementioned topics and laid the groundwork for future advances in understanding these cellular signaling mechanisms in *Neurospora crassa.*

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Chapter I

Introduction and Background

Neurospora crassa: an ideal model organism

A model organism has forever been the cornerstone for discovery in the field of biology. Even amongst various model organisms such as the baker's yeast – *Saccharomyces cerevisiae,* the fruit fly – *Drosophila melanogaster* or the simple nematode worm – *Caenorhabditis elegans,* there are some that have been outstanding in terms of the innumerable significant discoveries critical towards advances in genetics, molecular and microbiology. *Neurospora crassa* definitely belongs to such a fine group of model of model microbes, as so eloquently put forward by Rowland Davis and David Perkins in their 2002 review (1). In a rather influential 1941 paper, George Beadle and Edward Tatum defined the intricate relationship between genes and enzymes in what was heralded the "one gene – one enzyme hypothesis" which led to their Nobel Prize in physiology or medicine in 1958. Although today the hypothesis is an oversimplification of the relationship between genes and proteins/enzymes, their findings were instrumental (among other findings) in burgeoning the concept and the field of molecular biology. Among other discoveries, the renowned geneticist – Robert Metzenberg showed that meiotic silencing of unpaired DNA can cause silencing of DNA segments homologous to the unpaired one (2), thus giving impetus to a rising number of studies describing this critical cellular phenomenon. Yet another famous *Neurospora* geneticist – Dr. David Perkins was also instrumental in identification of several genes involved in meiotic cell division and recombination in *Neurospora* and also gave significant impetus to the Fungal Genetics Stock Center, which is such a vital resource in fungal research to this very day.

Life Cycle of *N. crassa*

As a model organism, *N. crassa* leads a haploid life style with a very short diploid phase during sexual development. It is a multicellular ascomycete and a heterothallic (self-sterile) fungus, which grows vegetatively by apical extension of basal hyphae (1). The asexual phase of growth involves germination of an asexual spore (conidium), followed by polarized growth of tube-like structures called hyphae. Hyphal fusion and branching gives rise to formation of a networked multicellular structure – the mycelium. Various environmental stimuli, such as low oxygen, heat and/or nutrient deprivation, can stimulate the asexual sporulation pathway known as macroconidiation (3). Macroconidiation involves formation of aerial hyphae that bud from their tips forming conidiophores, eventually giving rise to the mature spores or macroconidia (4). Spores are spread throughout the environment via wind dispersal or mechanical agitation. These conidia can survive freezing but are still susceptible to heat (REF). Upon acquisition of appropriate nutrient conditions conidia are able to establish a new colony thereby completing the primary asexual (phase) of the life cycle (5). A secondary sporulation pathway also exists in *N. crassa* called the microconidiation pathway. In this pathway mono-nucleate spores – called microconidia form directly from the mature hyphae in response to specific stressors such as iodoacetic acid (6).

N. crassa has two different mating types (*mat a* and *mat A*) that are encoded by one locus (7). Under nitrogen starvation conditions, *Neurospora* enters into the sexual phase of development. This leads to formation of female reproductive structures known as protoperithecia (8). Chemotropic growth of a female hypha (trichogyne) from a

protoperithecium towards a male cell (conidium) of opposite mating type results in cell and nuclear fusion followed by meiosis and subsequent enlargement and development of the protoperithecium into the mature fruiting body (perithecium; (9)). As certain macroscopic changes take place, each of the asci undergoes a pair of meiotic divisions followed by single mitotic division, forming eight sexual spores termed ascospores (10). Ascospores germinate to produce hyphae under certain environmental conditions, notably heat (8). In this thesis, Chapters 2 and 3 will in part deal with such aspects of characterizing the osmosensing and amino acid starvation stress pathways and their relationship to relevant stages of the *N. crassa* life cycle.

N. crassa **as a model organism to study cellular stress**

N. crassa is an excellent model to study the response to various cellular and environmental stress conditions. It displays distinct visible morphological phenotypes, exhibits fast growth and under often shows noticeable differences under stress compared to normal growth conditions. As a haploid multicellular fungus, generation of knockouts in *N. crassa* is also relatively easy (11).

The development of new methodologies involving an increased rate of homologous recombination in the *mus* knockout backgrounds (12), has led to a great increase in generation of knockout mutants and successful completion of the *Neurospora* genome project (13). In this light, the Fungal Genetics Stock Center (FGSC, Kansas City) has become a critical resource for *N. crassa* research. This facility enables researchers to procure any specific gene knockouts they are interested in, in connection to the stress

pathways being studied. Additionally with regards to studying amino acid starvation responses, as *Neurospora* is able to synthesize all 20 amino acids, starvation for a single amino acid can be used to track gene expression changes for that particular starvation response. The *Neurospora* cell membrane and cell wall are also critical for effective regulation of stress response pathways such as regulation of Erk class – MAK-1 and MAK-2– mitogen-activated protein kinases (MAPKs) and the osmoregulatory p38 MAPK – OS-2 (14,15). A large number of studies have focused on osmoregulation, cell wall integrity in *N. crassa* (15-17).

In addition to these processes, some heterotrimeric G protein signaling components such as Gα subunits also are known to play important roles in regulation of hyperosmotic stress. The heterotrimeric G protein cycle is initiated by activation of an upstream trans-membrane G protein coupled receptor by an extracellular ligand which leads to release of the Gαβγ heterotrimer by a GDP-GTP exchange on the Gα subunit (Figure 1.4) (18). This results in a formation of Gα-GTP and free Gβγ, which are now free to regulate downstream effectors. The recycling of Gα-GTP to Gα-GDP takes place via the intrinsic GTPase activity for the G alphas or via another family of proteins called RGS (regulator of G protein signaling) which accelerate GTP hydrolysis to allow Gα-GDP formation (19). There are three Gα molecules in *Neurospora* – GNA-1, GNA-2 and GNA-3, and one Gβ – GNB-1 and one Gγ – GNG-1 (20,21). In addition to the canonical Gβ there is an alternate G-beta subunit – CPC-2 which has at least 76% homology to the G-beta subunit (21). The GNA-1 (Gα subunit in *Neurospora*) knockout strain has been shown to be sensitive to hyperosmotic stress (20), but resistant to heat stress (22). The

Δ*gna-1* mutant has low cAMP levels and this may explain its thermotolerance, as previous studies have shown that deletion of the adenylyl cyclase gene *cr-1* leads to undetectable cAMP levels and even greater thermotolerance than the *gna-1* knockout (20). The level of thermotolerance exhibited by either a *cr-1* single or a *cr-1, gna-1* double mutant is the same (20), consistent with other studies demonstrating that GNA-1 is able to positively regulate CR-1 activity (23), which could lead to increased cAMP levels negatively regulating thermo-tolerance.

Seven trans-membrane helix proteins, such as G protein coupled receptors (GPCRs) are also involved in sensing environmental changes in *N. crassa*. One such GPCR is GPR-4, which regulates carbon sensing in *Neurospora,* eventually resulting in adenylyl cyclase activity, elevated intracellular cAMP levels and activation of the catalytic subunit of PKA, which is then free to phosphorylate downstream target proteins (24). On the other hand, amino acid sensing studies in mammalian cells have mainly focused on a major signaling hub known as the mammalian target of rapamycin complex 1 (mTORC1) which receives activating signals via the PI3K-AKT signaling cascade (25). TORC1 is required for mediating cell growth in response to nutrient availability and other stresses (26,27). TOR signaling cascades regulate cell-cell adhesion in the fungus *Candida albicans* (28), while in *Neurospora* inhibition of the TOR pathway results in increased cellulase production (29). TOR is also important towards transcriptional outputs such as positive regulation of genes involved in ribosome biogenesis as well as negative regulation of genes involved in de novo synthesis of glutamine and glutamate

(30). How amino acid sensing activates the TOR cascade is yet not fully understood. The TOR pathway is known to control GCN4 activity via translational regulation of the transcription factor GCN4 in *S. cerevisiae* (31). But it is also possible that there are TOR-independent mechanisms, that bypass transcription factors for downstream amino acid biosynthetic genes, such as GCN4 (in yeast) or CPC-1 in *N. crassa* (32). It is likely that upstream GPCRs and TOR regulation might converge in an inter-dependent mode of regulation of downstream stress response pathways such as amino acid or carbon starvation.

Objectives

Understanding cellular signaling mechanisms as well as stress responses in *Neurospora crassa* requires a wide breadth of investigations into the various factors and pathways that influence the cellular changes which materialize post-stimuli or stress conditions. The objective of this thesis is to investigate several cellular signals and components, which are involved in sensing, stress or regulating their downstream factors. These include MAPKs, transcription factors and integral components of the translational machinery. This thesis attempts to provide a holistic view of the fundamental components used to counter different cellular stressors, such as hyperosmolarity, chemical inhibitors (cytoskeletal dysfunction, ROS accumulation, fungicides) and amino acid starvation. In addition, the final aim demonstrates the critical role of heterotrimeric G proteins and the guanine exchange factor – RIC-8 in regulation of the translational machinery.

Outline of Chapter 2: Characterization of Serine/Threonine and Tyrosine protein phosphatases and their role in p38 MAPK regulation

Phosphorylation of three amino acids – serine, threonine and tyrosine – regulates various biological reactions in eukaryotes (33). Such regulatory cascades involve a cycle of phosphorylation via kinases followed by removal of the phosphate group by phosphatases. An adequate balance between kinases and phosphatases is essential for appropriate cellular homeostasis (Figure 1.1A). Because of their importance to cellular processes, kinases and phosphatases are among the most extensively studied enzymes (34).

Phosphatases have been classified according to their sequence homology, structural characteristics as well as substrate specificity (35). Based on such properties, there are two major families of phosphatases: serine/threonine (S/T) protein phosphatases and protein tyrosine phosphatases. In general, protein phosphatases perform dephosphorylation of the substrate in a reaction involving nucleophilic attack on the phosphate moiety of the substrate (36). In this context, S/T phosphatases and protein tyrosine phosphatases are integral for regulating cellular processes that require switching on or off a protein's activity via removal of the phosphate groups, which have been previously incorporated by the cognate kinase. Phosphatases have been shown to be important for hyphal growth, cell-cell fusion as well as several other important cellular events such as mitosis and cell cycle checkpoints in *Neurospora crassa* as well as humans (37-39). In Chapter 2, detailed analyses and characterization of the 24 viable S/T and protein tyrosine phosphatases sheds light on the role these important proteins play in

regulation of cellular processes. Utilizing chemical sensitivity screens as well as growth under different nutritional regimens (such as yeast extract and microcrystalline cellulose), these 24 viable knockout mutants were analyzed to elucidate a putative role for these genes in the concerned stress response pathway. Additionally, because a number of the phosphatases knockouts showed variable responses to hyperosmolarity, they were also tested for the levels of phosho-OS-2, which is the terminal MAPK in the osmosensing MAPK pathway (Figure 1.1B), involved in transducing the high osmolarity response in *Neurospora crassa.*

Upstream activators of OS-2 (MAPK) include the MAPK module consisting of OS-4 (MAPKKK) and OS-5 (MAPKK) (Figure 1.1.B). The response regulator RRG-1 also functions downstream of the hybrid histidine kinase OS-1 and a histidine phosphotransfer protein – HPT-1. It is also likely that RRG-1 has other upstream regulators (40). Deletion of *os-2* leads to female sterility and hypersensitivity to osmotic stress and OS-2 phosphorylation is important for protoperithecial development as well as fungicide and osmoresistance (40). Out of the 24 phosphatase mutants that were screened, nine showed increased phospho-OS-2 levels (basal and/or induced) compared to wild type. Hence, the focus of Chapter 2 is on the role of S/T and protein tyrosine phosphatases and their role in regulating cellular responses in *Neurospora,* as well a putative role in regulation of phosphorylation of the terminal OS-2 MAPK.

Outline of Chapter 3: CPC-2/RACK1 mediated regulation of amino acid starvation via the bZIP transcription factor CPC-1

Amino acid sensing and regulation of downstream molecular mechanisms is a highly important process in the cell. The human body cannot synthesize certain essential amino acids such as histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan and valine. These amino acids must be supplied via the diet. On the other hand, the filamentous fungus *N. crassa* is able to synthesize all 20 amino acids, making it an ideal candidate for investigating the mechanisms regulating amino acid starvation in the cell. A major hub for regulating responses to amino acid starvation in *Neurospora* is the bZIP transcription factor CPC-1 [Cross Pathway Control-1; (41)]; the corresponding protein in *S. cerevisiae* is Gcn4p [general control non-derepressed-4; (42-44)]. Amino acid starvation results in upregulation of Gcn4p synthesis, which then binds to specific DNA sequences called Gcn4p-protein responsive elements (GCREs). GCREs are upstream of at least 50 target genes that include those responsible for regulation of general amino acid control and the actual amino acid biosynthetic genes.

Upon detection of amino starvation, upstream effectors transduce the starvation signal onto Gcn4/CPC-1, which is then able to de-repress the appropriate amino acid biosynthetic genes and direct cellular capacities towards countering the stress condition (Figure 1.2). Such starvation for a single amino acid is able to initiate a secondary effect in eliciting derepression for other amino acid genes in a phenomenon known as cross pathway control (*N. crassa)* or general amino acid control (in *S. cerevisiae*). Several

important trans-acting upstream effectors regulating GCN4 translation have been characterized, such as mTOR, GCN2 and eIF2α, among others (42). However one important component of cross pathway control – CPC-2 (Cross Pathway Control-2) – is still not well characterized in any fungal system. CPC-2 is a multifunctional protein and a putative alternative Gβ subunit-like protein. It is homologous to human RACK1 (Receptor for Activated C Kinase) and is known to be an important scaffolding protein with both ribosomal and extra-ribosomal functions (45). The *cpc-2* knockout strain is required for growth under amino acid starvation in *N. crassa* (46), while *cpc2/asc1* plays a negative role in general amino acid control in *S. cerevisiae* (47). The eIF2α kinase, GCN2 (in yeast) or CPC-3 (in *Neurospora*), is known to be the sensory kinase, which is necessary for efficient expression of the transcriptional activator Gcn4p under starvation conditions (48,49). To simulate amino acid starvation conditions in the cell (in vivo) one commonly used chemical is 3-amino,1,2,4,triazole (3-AT) which induces histidine starvation in the cell by acting as a competitive inhibitor of imidazoleglycerol-phosphate dehydratase (HIS3 gene and the enzyme for the sixth step in histidine production) (50). This results in starvation for histidine leading to a cross pathway effect as well. Deletion of this kinase leads to sensitivity to the chemical 3-AT treatment in yeast, however a *gcn2 cpc2* double mutant is relatively resistant to 3-AT compared to *gcn2,* and is more similar to *cpc2*. This has suggested that the *cpc2* mutation is genetically epistatic to *gcn2* in yeast (51). However no direct biochemical or molecular evidence has been provided for the same. Genetic evidence has also suggested that *cpc2/asc1* is required for repression of *gcn4* under non-starvation conditions (47). Recent studies in *Schizosaccharomyces*

pombe have shown that Cpc2p is essential for Gcn2p activation and dimerization (52), although a role in Gcn4p regulation is yet to be elucidated. As there has been no direct molecular and/or biochemical evidence demonstrating a role for CPC-2 in regulation of the bZIP transcription factor CPC-1 in *N. crassa*, this chapter deals with this investigation. In Chapter 3, a detailed phenotypic analysis of different cross pathway control mutants - *cpc-2, cpc-1* and a *cpc-2 cpc-1* double mutant- is conducted, followed by gene expression and protein level analyses. The results reveal an additional layer of CPC-1 regulation via post-translational modification (SUMOylation) and demonstrates that CPC-2 regulates CPC-1 via an eIF2α-independent mechanism beyond translational regulation of the transcription factor. The data suggest that CPC-2 affects how adequately CPC-1 is SUMOylated and that SUMOylation of CPC-1 is essential for proper functioning of the protein under amino acid starvation conditions.

Outline of Chapter 4: Role of heterotrimeric G proteins and the guanine exchange factor, RIC8 in regulation of the translational machinery

The ribosome is a critical workforce in the cell and any dysregulation of the ribosomal machinery leads to severe cellular defects either under normal or stress conditions. In this context, ribosomal proteins are crucial for proper functioning of the ribosome. The yeast 80S ribosome consists of about 79 ribosomal proteins as well as several initiation factors (eIF1-eIF6) that facilitate translation initiation and assembly of a translationally competent 80S ribosome. In eukaryotes, translation initiation begins at the 5'-7-methyl-

guanosine (5'-m7G) cap followed by AUG codon scanning (53). To continue the scanning process, repertoires of eukaryotic translation initiation factors are required, from eIF1 to eIF6 (54,55). The phenomenon of mRNA translation is highly dynamic and regulation of translation is an effective way for the cell to respond to various environmental stressors. An integral component of translational control involves phosphorylation of the α subunit of the eukaryotic initiation factor eIF2α, which leads to global repression of protein synthesis (56). This change in the cellular program helps in conserving resources and turns the focus onto a gene expression program that can overcome the existing stress conditions (Figure 1.3). There are several eIF2α kinases, each serving as the hub to transduce upstream stress signals onto the translational machinery. In mammals, there are four different eIF2α kinases - GCN2 (EIF2AK4) is induced by nutritional stresses, PKR-like endoplasmic reticulum (ER) kinase (PERK, EIF2AK3/PEK) is an ER stress sensor, heme-regulated kinase – HRI (EIF2AK1) which is activated by heme deprivation, and PKR or protein kinase R (EIF2AK2) which is activated after viral infections involving interferon cascades (29). In *N. crassa,* the sole eIF2α kinase is CPC-3 (cross pathway control-3) (48) and it is highly likely that it is able to transduce stress signals from upstream receptors. In mammals, activation of GCN2 takes place after accumulation of uncharged (deacetylated) tRNAs (in response to stress conditions) leading to eventual phosphorylation of eIF2α (57,58).

As mentioned earlier, heterotrimeric G proteins are regulated via upstream GPCRs, which are in turn activated via ligand binding (59). Activation of the GPCR leads to dissociation of the Gαβγ complex and exchange of GTP for the GDP on the Gα

subunit. In this way, GPCRs act as guanine nucleotide exchange factors [GEFs; (60)] for Gα proteins. The Gβγ dimer and the Gα-GTP are now free to regulate downstream effectors such as adenylyl cyclases, phospholipases, MAPKKKs and transcription factors. The exchange of GTP for GDP on the Gα subunit can also be carried out by non-receptor GEFs such as RIC8 (resistance to inhibitors of cholinesterase-8), which also leads to the GTP-GDP exchange and dissociation of the Gαβγ (60). This particular chapter demonstrates a putative role of heterotrimeric G proteins and RIC8 in eIF2α-phosphorylation. The idea that G proteins have an important role in translation is a novel and a striking one. Loss of a G protein or *ric8* may lead to mimicking stress conditions in the cell leading to dysregulated eIF2α-phosphorylation.

Previous findings of a former graduate student in our group – Dr. Alexander Michkov that showed that RIC8 is a ribosomal protein have raised questions regarding its possible role in translation. Chapter 4 explores a more detailed role for RIC8 in translation via analyses of polysomes from the *ric8* mutant using MuDPIT (multi-dimensional protein identification technology). Results from RNA-seq and Poly-Seq analyses of *ric8* mutant total mRNA and polysomal mRNA also suggest possible roles for RIC8 in translation. Even though there has been no direct molecular evidence linking heterotrimeric G proteins to GCN2/CPC-3 activation, such a scenario would explain how G proteins are regulating translation under normal or starvation conditions.

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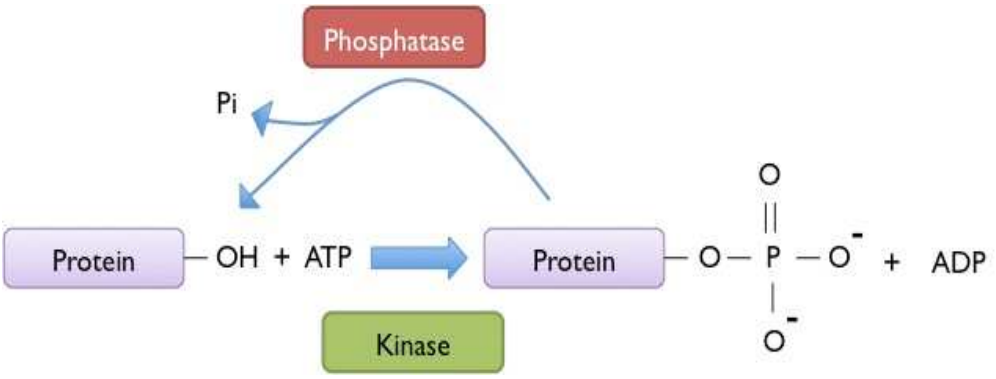
A.


Phosphatase
Pi
Protein
OH + ATP
Protein
O
P
O
+ ADP
Kinase

B.

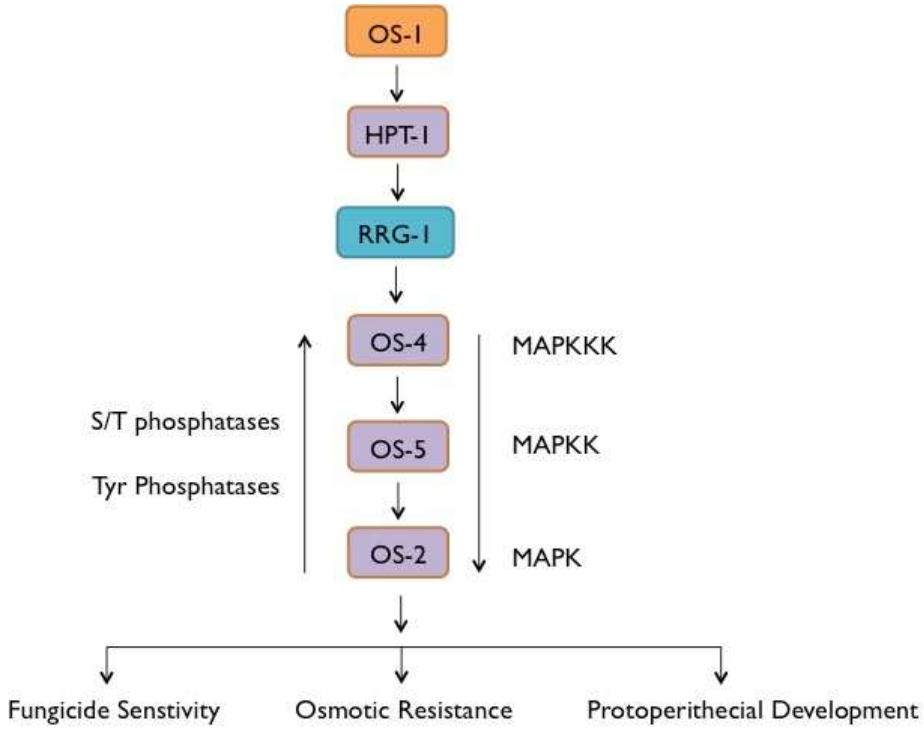

OS-1
HPT-1
RRG-1
OS-4
OS-5
OS-2
MAPKKK
MAPKK
MAPK
S/T phosphatases
Tyr Phosphatases
Fungicide Senstivity
Osmotic Resistance
Protoperithecial Development

Figure 1.1. A. Phosphatases and kinases work in cognate pathways for reversible phosphorylation of a protein. Phosphorylation leads to attachment of a phosphate moiety at the serine, threonine or tyrosine residues of the target protein via a protein kinase and utilization of energy in the form of ATP. A protein phosphatase is able to reverse the phosphorylation by removal of the phosphate moiety and release of the phosphate group from the target protein. **B.** The OS-2 MAPK (mitogen activated protein kinase) pathway in *Neurospora crassa.* The OS-1 hybrid histidine kinase is an upstream regulator of RRG-1 (response regulator-1), which in turn regulates the OS-4 MAPKKK. The cascade initiated by RRG-1 leads to sequential phosphorylation leading to activation of OS-2 MAPK (Modified from Jones *et al*, 2007, MBoC). Reversible phosphorylation of OS-2 can be mediated by the several serine/threonine and tyrosine phosphatases investigated in this study.

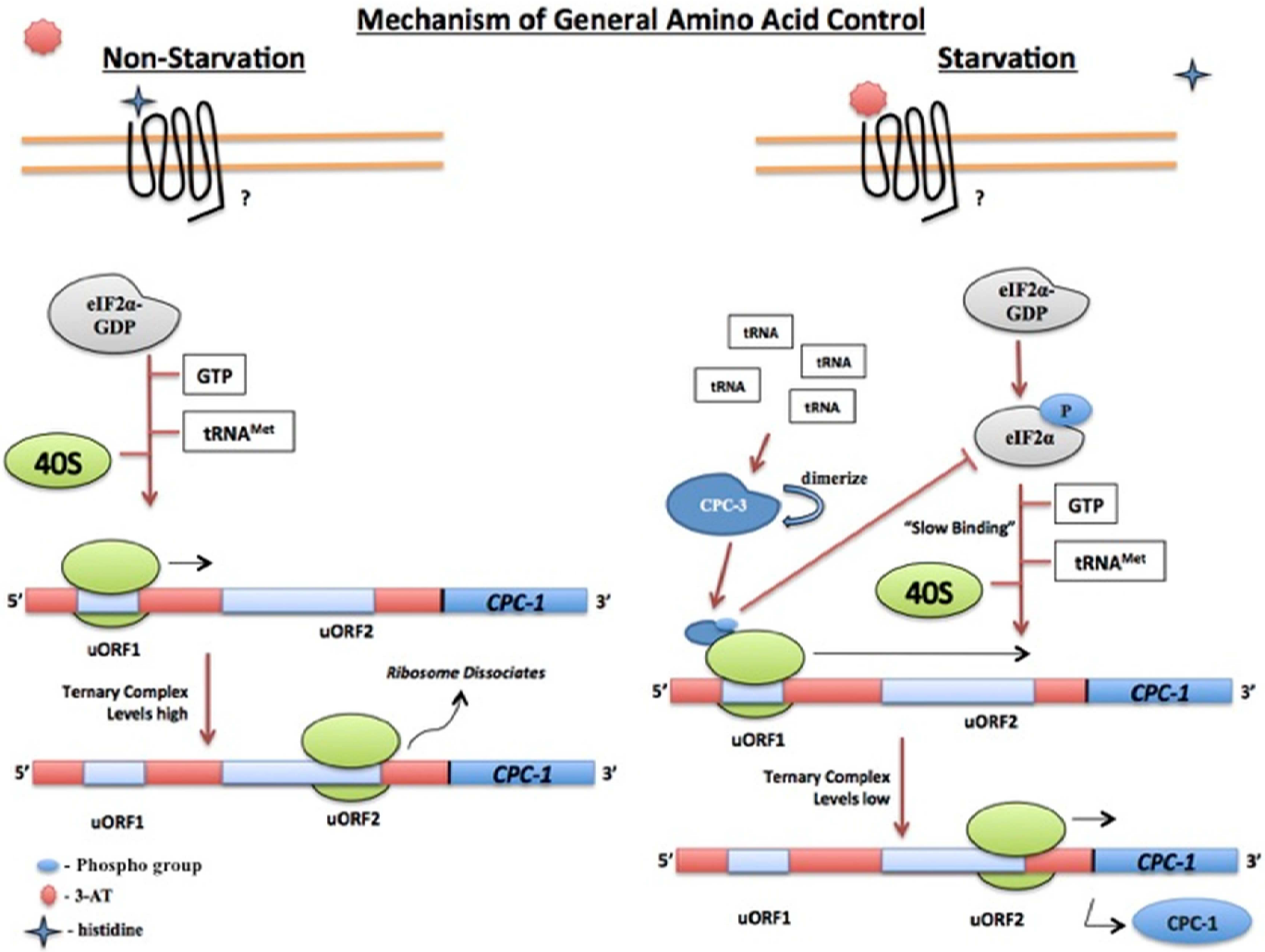

Mechanism of General Amino Acid Control
Non-Starvation
Starvation
?
?
eIF2α-GDP
GTP
tRNAMet
40S
5'
CPC-1
3'
uORF1
uORF2
Ternary Complex Levels high
Ribosome Dissociates
- Phospho group
- 3-AT
- histidine
tRNA
tRNA
tRNA
tRNA
dimerize
CPC-3
eIF2α-GDP
P
eIF2α
"Slow Binding"
GTP
tRNAMet
40S
Ternary Complex Levels low
CPC-1

Figure 1.2. Mechanism of General Amino Acid Control or Cross Pathway Control via translational regulation of Gcn4p/CPC-1. Under non-starvation conditions, cells are sensing normal levels of histidine and do not require transcription of amino acid biosynthetic genes such as *his-3* which is regulated via the bZIP transcription factor – CPC-1/GCN4. In *Neurospora crassa, cpc-1* contains two upstream open reading frames (uORFs) while yeast Gcn4 has 4 uORFs. Under normal conditions, the ribosomal ternary complex (TC) levels are high leading to greater and faster clearance of TCs from the uORFs and thus only leaky or non-existent translation of the *cpc-1* ORF. Under starvation conditions such as post-treatment with 3-aminotriazole, there is reduced binding of charged tRNAs, GTP to the initiation factor eIF2α. This is the result of activation of the eIF2α kinase GCN2/CPC-3, which phosphorylates eIF2α thus reducing the formation of the eventual ternary complex. This eventually allows TC accumulation and translation start at the GCN4/CPC-1 AUG codon.

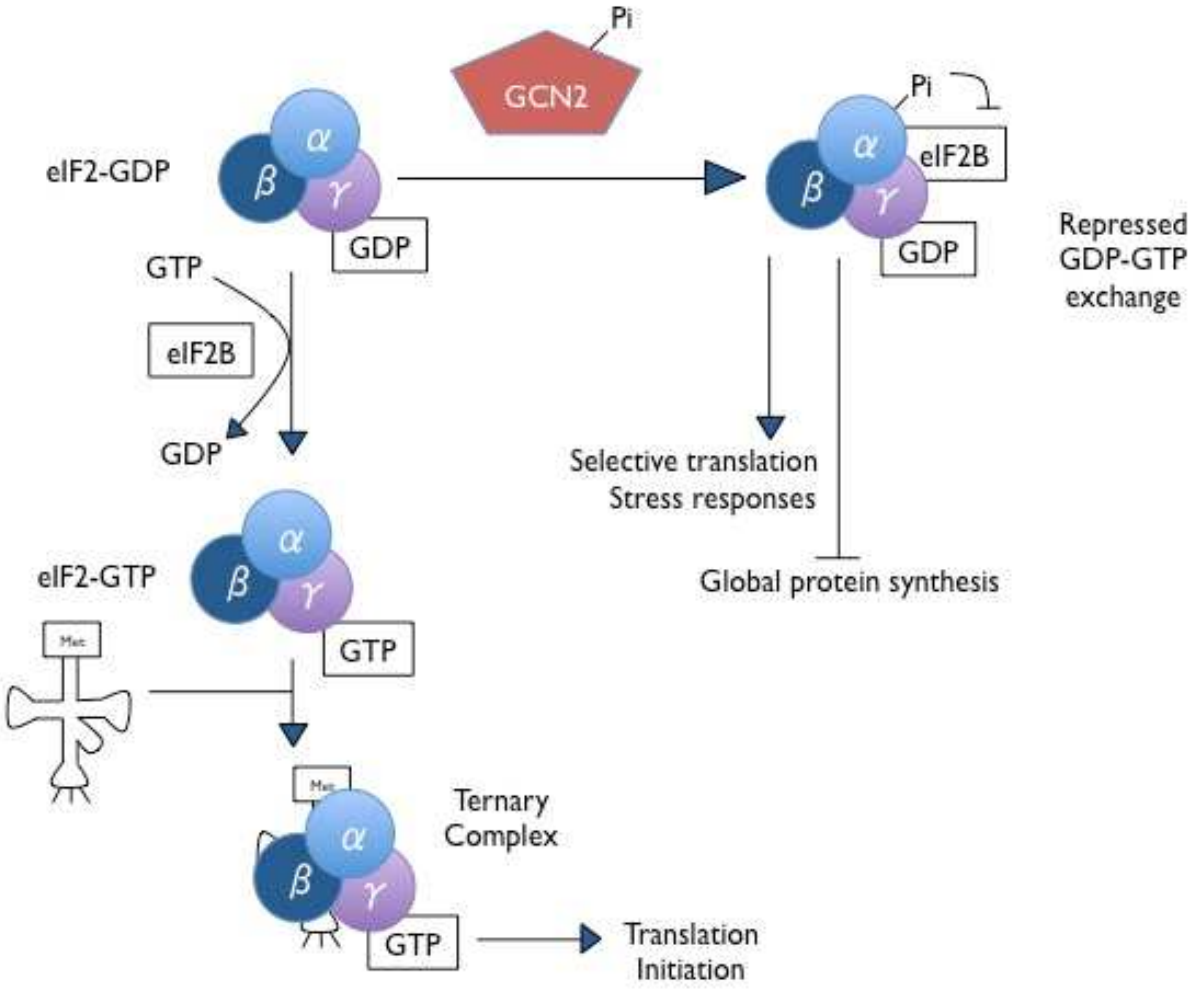
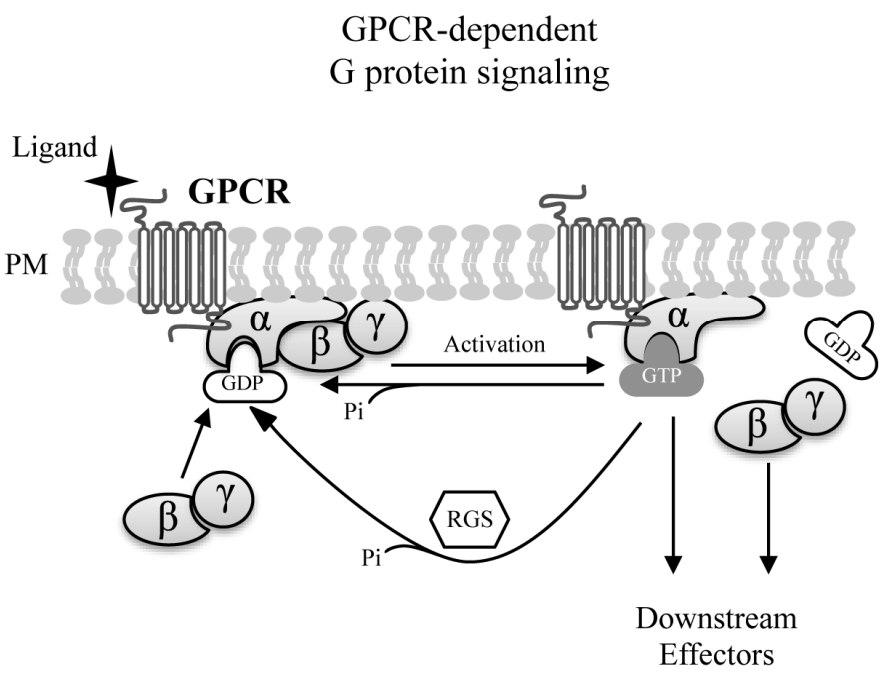

Pi
GCN2
eIF2-GDP
α
β
γ
GDP
Pi
α
eIF2B
β
γ
GDP
Repressed
GDP-GTP
exchange
GTP
eIF2B
GDP
Selective translation
Stress responses
Global protein synthesis
eIF2-GTP
α
β
γ
GTP
Met
Met
α
β
γ
GTP
Ternary
Complex
Translation
Initiation

Figure 1.3. Detailed mechanism of eIF2α phosphorylation via GCN2 in *S. cereviseae*. Upon activation of GCN2 via amino acid starvation, ER stress or UV irradiation, the eIF2-GDP complex consisting of α, β and γ subunits are phosphorylated at the α subunit. This prevents exchange of GDP for GTP on the α subunit that in turn leads to selective translation of specific mRNAs, which are able to overcome the prevailing stress conditions. The eIF2α subunit together with the eIF2β and the eIF2γ are part of the ternary complex. The ternary complex is formed after normal GDP-GTP exchange via the eIF2B guanine exchange factor (GEF) resulting in addition of GTP and a methionyl-initiator-tRNA to the eIF2 heterotrimeric complex (Modified from Holcik *et al,* 2005, *Nature Reviews Mol. Cell. Biol.*). The eIF2 complex looses GTP via hydrolyses after each round of initiation and is replenished via eIF2B GEF activity.

A.


GPCR-dependent
G protein signaling
Ligand
GPCR
PM
α
β
γ
GDP
Activation
GTP
Pi
RGS
Downstream
Effectors

B.

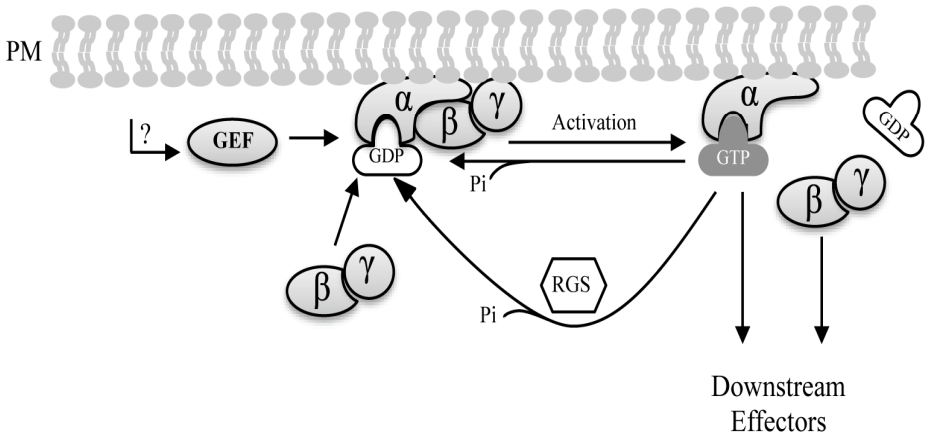

Non-receptor GEF mediated
G protein signaling
PM
?
GEF
α
β
γ
GDP
Activation
GTP
Pi
RGS
Downstream
Effectors

Figure 1.4. Overview of heterotrimeric G protein pathways. Heterotrimeric G protein signaling mechanisms in fungi are regulated via GPCR-dependent and GPCR-independent, non-receptor GEF pathways. **A.** Ligand binding to a GPCR stimulates a conformational change in the receptor, which leads to a GDP-GTP exchange on the Gα subunit protein and subsequent dissociation of the Gα from the Gβγ dimer. The Gα and Gβγ units regulate downstream effectors, such as adenylyl cyclase, MAPK cascades etc. Hydrolysis of GTP on the Gα subunit occurs via its intrinsic GTPase activity and this reaction can also be accelerated via RGS proteins. Re-association of the Gβγ dimer with the GDP bound Gα returns to the heterotrimeric G protein to the resting state in association with the GPCR. **B.** Non-receptor GEFs such as RIC8 and ARR4/GET3 can also stimulate GDP-GTP exchange on Gα proteins. In various fungal systems, RIC8 has been shown to interact with Group I and III Gα proteins to regulate cAMP signaling. *Abbreviations* – *GPCR* – G protein-coupled receptor, *PM* – plasma membrane, *GDP* – guanosine diphosphate, *GTP* – guanosine triphosphate, *RGS* – regulators of G protein signaling, *GEF* – guanine nucleotide exchange factor, *Pi* – phosphate. (Reproduced with permission from - Deka R., Ghosh A., Tamuli R., and Borkovich KA. In H. Drik (ed.), 2016. Heterotrimeric G Proteins. The Mycota III. Springer-Verlag, Berlin, Germany)

Chapter II

Global analysis of serine/threonine and tyrosine protein phosphatases catalytic subunit genes in *Neurospora crassa* reveals interplay between the phosphatases and the p38 mitogen-activated protein kinase

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Abstract

Protein phosphatases are integral components of the cellular signaling machinery in eukaryotes, regulating diverse aspects of growth and development. The genome of the filamentous fungus and model organism *Neurospora crassa* encodes 30 protein phosphatase genes. In this study, we have characterized 25 viable *N. crassa* phosphatase knockout mutants for phenotypes during growth, asexual and sexual development. We found that 92% of the mutants had defects in at least one of these traits, while 28% possessed phenotypes in all three. Chemical sensitivity screens were conducted in order to reveal additional phenotypes for the mutants. This resulted in the identification of at least one chemical phenotype for 18 phosphatase knockout mutants, including novel chemical sensitivities for two phosphatase mutants lacking a growth or developmental phenotype. Hence, a chemical or growth/developmental phenotype was observed for all 25 mutants. We investigated p38 mitogen-activated protein kinase (MAPK) phosphorylation profiles in the phosphatase mutants and identified nine potential candidates for regulators of the p38 MAPK. We demonstrated that the PP2C class phosphatase *pph-8* (NCU04600) is an important regulator of female sexual development in *N. crassa*. In addition, we showed that the Δ*csp-6* (ΔNCU08380) mutant exhibits a phenotype similar to the previously identified conidial separation mutants, Δ*csp-1* and Δ*csp-2,* that lack transcription factors important for regulation of conidiation and the circadian clock.

INTRODUCTION

S/T phosphatases are further classified into three main subfamilies: phosphoprotein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs), and aspartate-based protein phosphatases; comprised of the FCP/SCP (transcription factor IIF -interacting C-terminal domain phosphatase) and HAD (haloacid dehalogenase) classes (1,2). Protein tyrosine phosphatases are subdivided into classical protein-tyrosine phosphatases (PTPs), dual specificity phosphatases (DSPs), low molecular weight phosphatases (LMW-PTP) and CDC25- class phosphatases (3-5).

The PPP subfamily of S/T phosphatases has been implicated in a broad range of cellular processes, such as metabolism, cytoskeletal rearrangements, base excision repair, mitotic entry and regulation of membrane receptors and ion channels (6-9). Differences in relative inhibition by okadaic acid (10) have led to subdivision of the PPP subfamily into PP2A, PP2B (Calcineurin-A) and PP5 classes. In mammals, PP2A phosphatases dephosphorylate the microtubule-associated protein Tau and modulate mitogen-activated protein kinase (MAPK) signaling pathways by dephosphorylation of the component kinases (11). PPP class phosphatase calcineurin-A is a calcium-calmodulin-dependent enzyme (12,13). In animals, calcineurin dephosphorylates and activates the transcription factor NFATc, leading to T cell differentiation via interleukin-2 expression (14,15). Calcineurin is an essential gene involved in hyphal growth and maintenance of Ca^{+2} gradients in *Neurospora crassa,* (16). In *Saccharomyces cerevisiae*, *cna1* and *cna2* are functionally redundant catalytic subunits for calcineurin and while *cna1 cna2* double

mutants are viable, they are more sensitive to high levels of sodium, lithium and other ions in the growth medium (17,18). Deletion of the *cna-1* homolog is not lethal, but results in a weakly growing mutant in *Aspergillus nidulans* (19-21).

The PPM subfamily of S/T phosphatases consists of PP2C enzymes with well-documented roles in cell cycle progression (22-24) and tumorgenicity (25) in animals and as negative regulators of the abscisic acid (ABA) signaling pathway in the model plant *Arabidopsis thaliana* (26). PP2C phosphatases act on a number of MAPK pathways (27). For example, Ptc1p in *S. cerevisiae* inactivates the high osmolarity glycerol (HOG) pathway by dephosphorylating the Hog1 MAPK (28). The FCP/SCP and HAD phosphatases are an aspartate-based class of S/T phosphatases with a shared DxDxT/V sequence motif. FCP1 is an essential protein phosphatase that dephosphorylates the C-terminal domain (CTD) of the largest subunit of RNA Polymerase II (29,30). The HAD (haloacid dehalogenase) class of protein phosphatases contains important regulators of actin-cytoskeleton dynamics in mammals (31,32)

The protein tyrosine phosphatase (PTP) family is distinguished by a signature $HC(X_5)R$ catalytic motif. These proteins play important roles during meiosis and sporulation in yeast and cell adhesion, metabolism and immune cell signaling in mammals (33-35). Classical PTPs can be classified as receptor and non-receptor PTPs and these phosphatases have functions in cell-substrate and cell-cell adhesion as well as insulin signaling in animals (36). Dual specificity phosphatases (DSPs) dephosphorylate phosphotyrosine, phosphoserine and phosphothreonine residues on substrates (37,38). DSPs can be further classified on the basis of the presence (typical) or absence (atypical)

of a mitogen-activated protein kinase (MAPK)-interacting domain, (39,40). For example, in *Ustilago maydis*, the dual specificity phosphatase Rok1 is known to regulate mating and virulence by controlling the phosphorylation of Erk MAPKs Kpp2 and Kpp6 (41). Among the other classes of PTPs, CDC25-type phosphatases have essential roles in mitotic entry (42), while the low molecular weight phosphatase (LMW-PTP) is less well understood. In addition to these major classes of protein tyrosine phosphatases, SSU72 is a unique RNA Polymerase II CTD phosphatase that shares high sequence similarity with PTPs (43,44). Y-phosphatases are a lesser studied class of PTPs that seem to be unique to filamentous fungi. In *A. nidulans*, AN4426 (19) is a Y-phosphatase homologous to SIW14, a tyrosine phosphatase involved in endocytosis in *S. cerevisiae* (45).

The filamentous fungus *N. crassa* is a model system for investigations of cell growth, development, gene silencing, the circadian clock and stress responses in eukaryotic cells (46,47). *N. crassa* possesses 16 S/T phosphatases and 14 PTPs (this study). Among the previously characterized protein phosphatases in *N. crassa*, the PP2A phosphatase *pp2A* (NCU06563) is involved in hyphal growth and cell-cell fusion (48-50) while another PP2A phosphatase, *pph-1* (NCU06630), has so far been implicated in hyphal growth (48). Mutation of the *tangerine/tng* gene (NCU03436), an ortholog of the cell-shape-control protein phosphatase, *cpp-1* in *Fusarium verticillioides*, leads to swollen hyphae and hyperbranching at the colony edge (51).

Taking advantage of the publicly available *N. crassa* genome sequence (52) and the large scale gene knockout project for ~10,000 predicted genes (53), we have previously investigated the effects of mutating 86 S/T kinase genes in *N. crassa* (54,55).

In order to elucidate the functions of protein phosphatases in *N. crassa,* we initiated a systematic analysis of the 30 predicted genes. In this study, we analyzed 25 viable phosphatase mutants for defects in basal growth, asexual and sexual development. Chemical sensitivity testing has proven to be a powerful method for identification of phenotypes for gene deletion mutants and genes of unknown function, as evident from previous studies in *S. cerevisiae* (56) and our analysis of protein kinases (55). Accordingly, we tested the phosphatase mutants for altered sensitivity to several chemical stresses and growth under different nutritional regimens. We also measured phosphorylation of the p38 MAPK (OS-2) in all mutants in order to identify potential phosphatases acting on this pathway in *N. crassa*. The results reveal at least one defect for every phosphatase mutant, demonstrating the importance of these proteins to *N. crassa* biology. We present evidence linking the PP2C phosphatase *pph-8* (NCU04600) and the HAD family phosphatase *csp-6* (NCU08380) to important aspects of sexual and asexual development in *N. crassa.* We identify several protein phosphatases that influence basal or induced phosphorylation of the OS-2 MAPK.

MATERIALS AND METHODS

***Neurospora crassa* strains and growth conditions:** Wild-type strains ORS-SL6a (FGSC 4200; *mat a*) and 74-OR23-IVA (FGSC 2489; *mat A*) and phosphatase mutants produced during the knockout project (Table 2.1) were obtained from the Fungal Genetics Stock Center (FGSC; Kansas City, MO). Knockout mutants for three phosphatase genes were not available as either homo- or heterokaryons (Table 2.1). Vegetative growth and asexual development (conidiation) were analyzed using Vogel's minimal medium (VM) (57), while sexual development was assessed using synthetic crossing medium (SCM) (58). Conidia used for inoculating cultures were propagated in VM agar flask cultures grown for three days at 30° in the dark and for four days at 25° in the light. Sorbose-containing medium (FGS) was used for isolation of colonies on plates and for ascospore germination assays (59). Where indicated, VM was supplemented with hygromycin (Calbiochem, San Diego, CA) at a concentration of 200 μg/ml. In this study, the gene names for *N. crassa* phosphatases were taken from literature, the e-compendium at Leeds University (http://bmbpcu36.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm) or were assigned a name (Table 2.1).

Purification of homokaryotic phosphatase mutants from heterokaryons: Since *N. crassa* is multinucleate, primary transformants are often heterokaryons, with both mutant and wild-type nuclei (53,60,61). Therefore, transformants were crossed to wild type to purify homokaryotic meiotic progeny for the knockout project (53,54). Using the aforementioned method, one phosphatase cassette did not yield transformants (Δ*pph-9*; NCU000434) and viable ascospores could not be isolated for four phosphatase mutants: Δ*ppp-1* (FGSC 11653), Δ*cna-1* (FGSC 17929), Δ*pph-1* (FGSC 17800) and Δ*div-12* (FGSC 16654). Homokaryotic mutants for Δ*pph-1* (FGSC 17800) and Δ*div-12* (FGSC 16654) were purified after serial plating of conidia. Macroconidia from each strain were plated on FGS-hygromycin plates and incubated in the dark at 30°. The next day, one colony was picked and transferred to a VM-hygromycin agar slant and cultured for five days. Macroconidia were isolated from this slant and plated on a FGS-hygromycin plate and post-incubation, a colony was transferred onto a fresh VM-hygromycin slant. These steps were repeated twice. Diagnostic PCR with gene-specific and *hph* primers (Table S2) was used to test for the absence of the open-reading frame of the respective deleted gene (with wild type as a positive control) and the simultaneous presence of the *hph* cassette in the purified strains (54). PCR conditions were as follows : 94° C- 2 mins, 52° C 30 seconds, 72° C 2 mins repeat steps 1-2 for 35 cycles, 72° C for 5 mins, 4° C forever. These experiments confirmed that the purified strains were homokaryons.

Analysis of growth, morphological and developmental phenotypes: The 25 viable *N. crassa* phosphatase knockout mutants (Table 2.1) were analyzed for phenotypes using methods reported previously (62), with some modifications. Linear growth rates for the mutants (Table S1) were measured on VM at 25° in the dark using race tubes (25 ml plastic piptettes) in triplicates (62).Mutants were grown on VM plates for 24 h and hyphae at the colony edge were photographed using an Olympus SZX9 stereomicroscope with a C-4040 digital camera (Olympus, Lake Success, NY). VM slant tubes were inoculated with the mutant strains and grown for three days in the dark at 30°C and four days under constant light at 25° and then scored for conidial production (see Table S1). Aerial hyphal extension was measured in 2 ml VM (standing) liquid cultures (13 x 100 mm glass tubes). These cultures were inoculated at the liquid surface and incubated statically at 25° (in the dark) for 96 h. The total height of aerial hyphae above the basal hyphal cell pad was measured in mm (see Table S1). Data was subsequently tested for significance using Student's *t*-test (paired, two-tailed, independent means). How many replicates?

For analysis of female sexual fertility, strains were inoculated onto SCM slants (16 x150 mm glass tubes containing 6 ml agar medium) and incubated under constant light for 7-8 days at 25°. Cultures were scored for protoperithecia formation and then fertilized with conidia of the opposite mating type. Subsequent perithecia formation and ascospore development were scored one and two weeks post-fertilization, respectively. All scoring for female sexual fertility analysis was done using the SZX9 stereomicroscope (Olympus). For visualizing unregulated protoperithecial formation in

the Δ*pph-8* mutant (FGSC11185), the strain was inoculated on VM and SCM agar plates (100 mm x 150 mm) and incubated under constant light or dark at 25° as indicated in Fig. 2.3. A wild-type strain (FGSC 2489; *mat A*) was used as a control. Photographs were taken using the SZX9 stereomicroscope with a C-4040 digital camera Olympus at five and seven days post-inoculation and two days post-fertilization.

Conidial separation was investigated in wild type (FGSC 4200; *mat a*), Δ*csp-1* (FGSC 2555), Δ*csp-2* (FGSC 2522) and Δ*csp-6* (FGSC 20306) strains. Conidia were propagated by culturing strains in VM agar flasks (25 ml medium size flasks) for three days in the dark at 30° and four days in the light at 25°. A small amount of conidia was withdrawn from the flask, suspended in 50 µl of sterile liquid VM, and then 50 µl of Calcofluor White (Eng Scientific Inc., Clifton, NJ) was added to the suspension. A volume of 20 µl was placed on a glass slide and covered with a cover slip. Conidia were visualized using differential interference (DIC) microscopy with an IX71 inverted microscope (Olympus America, Center Valley, PA) using a 60X oil immersion objective. X-Cite® 120PC Q (Lumen Dynamics, Ontario, Canada) was used as the fluorescence microscope light source with a DAPI filter cube on the microscope. Photographs were taken using a QIClickTM digital CCD camera (QImaging, Surrey, British Columbia, Canada).

Chemical sensitivity assays and nutritional phenotypes: Chemical sensitivity assays were restricted to viable phosphatase knockout mutants with growth rates at least 50% of the wild-type strain on VM as shown in Table S1 (55). The mutants were screened for responses to a variety of chemicals at concentrations that inhibited wild-type growth by ~50-60% (Table S1). The chemicals included sodium chloride (0.35 M; EMD Chemicals, Gibbstown, NJ), sorbitol (0.8 M; Sigma), cytochalasin A (40 ng/ml; Sigma, St. Louis, MO), benomyl (92 ng/ml; Fluka, St. Louis, MO), *tert*-butyl hydroperoxide (0.13 mM; Sigma, St. Louis, MO), Menadione (100 μM; M5750, Sigma), FK-506 (50 ng/ml; LC Laboratories, Woburn, MA), and fludioxonil (2.75 ng/ml; a gift from Frank Wong and Allison Tally). Phosphatase mutants were also analyzed for nutritional phenotypes, including growth on VM supplemented with 2% yeast extract and utilization of crystalline cellulose (Avicel; PH-101, Sigma, St. Louis, MO) as a carbon source (63-65). VM plates (60 mm x 15 mm) were supplemented with the respective chemicals and one edge of the plate was inoculated and radial colony growth was measured after 20-22 h at 30°. A VM plate lacking chemical was used as a control for each of the tested strains. The percentage growth was calculated by dividing the radius with chemical by the radius in the absence of chemical for four biological replicates. Three independent experiments were performed. One-way Anova analysis (66) was used for significance testing. Knockout mutants were considered sensitive/slower growing (S) or resistant/faster growing (R) (Table 3) if there was a difference from wild type in percent growth in the presence of the chemical at $p<0.05$.

p38 MAPK assays: For analysis of MAPK profiles of the phosphatase mutants, conidia were used to inoculate 25 ml VM liquid cultures (in 125 ml volume culture flasks) at an initial concentration of 1 x 10^6 conidia/ml as previously described (67). Cultures were grown for 16 h at 30° with shaking at 200 rpm, brought to 0.8 M NaCl (for activation of p38 OS-2 MAPK) and then incubated for an additional 10 min. An untreated sample was used as an uninduced control (time zero). Following treatment, the tissue was flash frozen in liquid nitrogen and ground using 2-5 mm stainless steel beads (Qiagen), with the Qiagen Retsch TissueLyser system (Qiagen Retsch GmbH, Hannover, Germany). Depending upon the amount of tissue, 300-700 µl of extraction buffer (50 mM HEPES, pH 7.5; 2 mM EGTA; 2 mM EDTA; 1% SDS; 10% glycerol; 100 mM NaCl; 1 mM sodium orthovanadate and 1 mM sodium fluoride) was added to the powdered fungal tissue, and the mixture heated at 85° for 5 min. Afterwards, 10 µl of 100 mM PMSF and 1 µl of fungal protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO; Product #T8215) was added and the solution was centrifuged at 4000 g for 15 min. at 4°. The supernatant was collected and the protein concentration determined using the BCA protein assay (Pierce Chemical, Rockford, IL). A volume of extract containing 30 µg of protein was subjected to SDS-PAGE, followed by immunoblotting (68). Commercial antibodies directed against mammalian or *S. cerevisiae* MAPKs were used to detect phospho-OS-2 (1:600 dilution; anti-phospho-p38 #9211; Cell Signaling Technology, Beverly, MA). Primary antibody was incubated in 5% Tris base saline with 0.1% Tween 20 at 4° C overnight on a shaking incubated. Incubation with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:2000 dilution) in 5% Tris base saline with 0.1%

Tween 20 at 25° C for 2 h (Sigma Chemical, St. Louis, MO) and subsequent chemiluminescence detection was carried out as previously described (68).

RESULTS

Protein phosphatase genes in the *N. crassa* genome: We utilized the amino acid sequences of known protein phosphatases from the *Saccharomyces* Genome Database (http://www.yeastgenome.org/) as queries during a reciprocal BLAST approach for identifying corresponding homologs in the Broad Institute *N. crassa* database (http://www.broadinstitute.org/annotation/genome/neurospora; Table 1). We identified 30 genes that were classified as serine/threonine (PPP, PPM and Asp-based subfamilies) or protein tyrosine phosphatases (Classical, Dual-specificity, LMW-PTP, Cdc25-type and SSU72 subfamilies). Two genes in the tyrosine phosphatase family (NCU01010 and NCU03333) appeared to be unique to filamentous fungi.

We compared protein phosphatases across different eukaryotic species (Table 2.2), including humans; plant, *A. thaliana* (4) (69); baker's yeast, *S. cerevisiae*; and filamentous fungi, *A. nidulans* (19) and *N. crassa*. There are many more S/T phosphatases in *A. thaliana* and humans than in the three fungi (Table 2.2). Comparing the fungi, *S. cerevisiae* has the greatest number (21), followed by *A. nidulans* with 17 and *N. crassa* with 16 genes (Table 2). The observation of lower numbers of S/T phosphatase genes in *N. crassa* compared to *A. thaliana*, humans and baker's yeast is consistent with the fact that *N. crassa* has fewer serine/threonine kinase genes (55).

With regards to protein tyrosine phosphatases, the number of genes is similar in the three fungi but fewer than in humans and *A. thaliana* (Table 2). In spite of the presence of tyrosine phosphatases, fungi do not possess recognizable tyrosine kinases (46,70). The same is true for *A. thaliana* and several apicomplexan species whose genomes lack any true tyrosine kinases or receptor tyrosine kinases (69,71,72). It is now believed that protein tyrosine phosphatases evolved before tyrosine kinases due to leaky phosphorylation of tyrosine residues by serine/threonine kinases, thus providing a target for the tyrosine phosphatases (72).

As part of the *Neurospora* Genome Project, we attempted gene replacement of the 30 phosphatase genes (54). Transformants could not be recovered for ΔNCU00434 (*ptc-1*) and mutants for NCU05049 (*dsp-5*) were not available (Table 2.1). We were unable to purify three of the knockout mutants (ΔNCU03804; *cna-1*, ΔNCU00043; *ppp-1* and ΔNCU09300; *fcp-1*) to homokaryons. As mentioned above, *cna-1* has been reported as an essential gene in *N. crassa* (16). Previous work showed that homologs of *ppp-1* and *fcp-1* are essential in both *A. nidulans* (19) and *S. cerevisiae* (21,73) and our observation the two *N. crassa* mutants could not be purified to homokaryons supports *ppp-1* and *fcp-1* as essential genes in *N. crassa*. Thus for phenotypic analyses and characterization, we were able to analyze a total of 25 viable protein phosphatase mutants.

Deletion of protein phosphatase genes leads to growth and developmental phenotypes in *N. crassa:* *N. crassa* is a heterothallic (self-sterile) fungus that spends most of its life cycle in the haploid state and grows vegetatively by apical extension of basal hyphae (47). The asexual phase of growth begins with germination of an asexual spore (conidium) that undergoes polarized growth to form hyphae. Hyphal fusion and branching gives rise to the networked, multicellular body of the organism, the mycelium. Different environmental stimuli, such as desiccation, heat and/or nutrient deprivation can stimulate the asexual sporulation pathway known as macroconidiation. This leads to the differentiation of aerial hyphae, which then bud from their tips, thus forming conidiophores and eventually giving rise to the free asexual spores, macroconidia or conidia (74). Under nitrogen starvation, *N. crassa* enters into the sexual phase of development, inducing the formation of female reproductive structures known as protoperithecia (75). Chemotropic growth of a female hypha (trichogyne) towards a male cell (conidium) of opposite mating type results in cell and nuclear fusion, followed by meiosis, and enlargement of the protoperithecium into the fruiting body (perithecium). Perithecia contain the meiotic progeny known as ascospores that germinate to produce hyphae under appropriate environmental conditions (75).

In order to characterize *N. crassa* phosphatase genes, we began with phenotypic analyses of the 25 viable mutants. In terms of extension of basal hyphae, 10 mutants showed reduced growth and three displayed increased growth as compared to wild type (Fig. 2.1, Table 2.1; detailed phenotypic data in Table S1). A total of 14 mutants exhibited defects in asexual development. Among these strains, only one mutant, Δ*pph-5*,

possessed abnormalities in both growth of basal hyphae and asexual development but not in sexual development (Fig. 2.1 and Table 2.1). The *pph-5* homolog in *S. cerevisiae* (*PTC5*) is also required for normal vegetative growth (76). Mutants lacking the genes *ppt-1*, *pph-6* and *pty-2* displayed increased basal growth (compared to wild-type) as their only morphological phenotype. Interestingly, the *S. cerevisiae pty-2* homolog, *PTP1*, is a negative regulator of filamentation (77). The faster hyphal growth observed in the *N. crassa* Δ*pty-2* mutant suggests that *pty-2* and *PTP1* may have similar functions in *N. crassa* and *S. cerevisiae*.

Among the PP2A class phosphatase mutants, Δ*pph-1* was unique in that a defect in basal growth was its only growth/developmental phenotype. Similarly, mutants lacking the dual-specificity phosphatase *dsp-1*, the CDC25 phosphatase *pty-1* and the Y-phosphatase member *pty-5,* exhibited phenotypes in asexual development as their only morphological defect. Deletion of the *dsp-1* homolog *PPS1* in *S. cerevisiae* does not produce any adverse effects on growth, but overexpression of *PPS1* results in growth arrest and aberrant DNA synthesis (78).

In our previous study on *N. crassa* kinases (55), we found that 32/77 (42%) of the mutant strains exhibited defects in sexual development, with 30/77 (39%) unable to produce ascospores (female-sterile; 94% of sexual phenotypes). In contrast, among the 25 phosphatase mutants, we found that 15 had a phenotype during sexual development (60%), with four strains (Δ*tng*, Δ*pp2A,* Δ*csp-6* and Δ*dsp-2;* 16% of mutants) being female-sterile, accounting for 26% of the sexual phenotypes in the phosphatase mutants (Table 2.1, Fig. 2.1 and Appendix table S2.1). These results demonstrate that although a

greater proportion of phosphatases than kinases influence the sexual cycle, kinases are more critical for production of ascospores and absolute female fertility in *N. crassa*.

The Δ*pp2A* mutant failed to produce protoperithecia and also had reduced aerial hyphae extension (Table 2.1, Fig. 2.1; Appendix table S2.1). These phenotypes are similar to those of mutants lacking components of the two MAPK pathways in *N. crassa*: MIK-1/MEK-1/MAK-1 (cell fusion and cell wall integrity) and NRC-1/MEK-2/MAK-2 (cell fusion) (55,79,80). On the other hand, the HAD-class phosphatase knockout Δ*csp-6* was unique in that it had very few (and small) protoperithecia that were unable to mature into perithecia upon fertilization with the opposite mating type.

Among the remaining strains with defects in sexual development, four mutants – Δ*pty-3*, Δ*div-12,* Δ*dsp-2* and Δ*pph-10* – exhibited decreased numbers of protoperithecia and perithecia, as well as few or delayed shooting of ascospores (Table 2.1, Fig. 2.1 and Appendix Table S2.1). Δ*dsp-3* and Δ*dsp-4* each displayed abnormal and increased protoperithecia or perithecia formation and increased ascospore production (Table 2.1, Fig. 2.1 and Appendix Table S2.1). It is interesting to note that all of these aforementioned genes are tyrosine phosphatases, suggesting that this phosphatase class is important for regulation of sexual development in *N. crassa.* Two more mutant strains (Δ*tng* and Δ*pph-11*) produced few protoperithecia and perithecia and while one ejected no ascospores (Δ*tng*), the other produced very few (Δ*pph-11*). Another three mutants (Δ*pph-4,* Δ*cdc-14* and Δ*pph-8*) possessed defects in the timing or in the number of ascospores produced. The *pph-4* mutant developed abnormal/small protoperithecia, but normal-appearing perithecia, while Δ*pph-8* was precocious in protoperithecia formation,

leading to perithecia that were embedded in the agar surface. A null mutation in the well-characterized phosphatase *CDC14* (involved in mitotic exit and meiosis I spindle disassembly) is lethal in yeast (81), while the *N. crassa* Δ*cdc-14* mutant is viable (but with defects in all three growth/developmental pathways). Deletion of *cdc-14* in *A. nidulans* did not result in any obvious growth defects (19).

Most of the 15 mutants that had phenotypes in sexual development also exhibited defects in basal hyphae extension and asexual differentiation. However, Δ*pty-1,* Δ*dsp-1* and Δ*pty-5* demonstrated phenotypes only during asexual development and thus seem to be specific for aspects of conidiation in *N. crassa* (Fig. 2.1, Table 2.1 and Appendix Table S2.1). Overall, our results show that 23/25 mutants (~92%) display a defect in at least one of three growth/developmental pathways analyzed in this study (Fig. 2.1; Table 2.1) with seven of the 23 mutants (~30%) possessing phenotypes in all three stages. As a comparison, among the previously studied S/T protein kinase knockouts in *N. crassa* (55), 57% of the mutants possessed a defect in at least one of the growth/developmental stages, while ~45% had overlapping defects in all three. This suggests that similar to kinases, phosphatases are also important regulators of growth and development in *N. crassa*.

Chemical sensitivity assays reveal additional phenotypes for protein phosphatase mutants: Various chemical and environmental stresses have been known to influence growth and developmental outcomes in eukaryotic cells. In order to gain a better understanding of the functions of the different protein phosphatases in *N. crassa*, we subjected the phosphatase mutants to a panel of chemical treatments (see Materials and Methods) and compared their relative sensitivity to each chemical to that of wild type (Table 2.3; detailed results in Appendix Table S2.1). Strains with linear growth rates less than 50% of wild type on minimal medium were excluded from this assay in order to avoid any bias due to their slow growth.

We analyzed the relative sensitivities of the phosphatase mutants to the reactive-oxygen species (ROS) generating chemical menadione (82), while peroxide stress was introduced by exposure to tert-butyl hydroperoxide (*t*-BuOOH) (83). Similar to our previous study on kinases (55), treatment with tert-butyl hydroperoxide yielded the greatest number of phenotypes, with a total of 10 strains displaying sensitivity or resistance to peroxide treatment. Three mutants (Δ*pp2A,* Δ*pzl-1*and Δ*pph-4*) showed increased sensitivity to both tert-butyl hydroperoxide as well as menadione (Table 2.3), while Δ*pph-6*, Δ*csp-6* and Δ*dsp-2* were exclusively sensitive to peroxide. The tyrosine phosphatase mutant Δ*pty-4* was resistant to menadione treatment, while Δ*ppt-1*, Δ*pph-5*, Δ*pty-2* and Δ*dsp-1* were resistant to tert-butyl hydroperoxide. It is thus of particular interest to understand how these phosphatases might be regulating cellular responses to oxidative stress. Conidiation is influenced by ROS in *N. crassa* (84,85). In the case of the Δ*csp-6*, we have shown that this mutant is defective in conidial separation (Fig. 2.4). The

finding that it is also sensitive to peroxide stress reinforces the notion that *csp-6* is an important component of the conidiation pathway in *N. crassa.*

We used sodium chloride and sorbitol to induce salt/osmotic stress in the *N. crassa* phosphatase mutants. A total of six mutants exhibited phenotypes in these assays. The Δ*pp2A* mutant was sensitive to both sorbitol and sodium chloride, suggesting it has important roles in osmotic stress resistance. The *S. cerevisiae* homolog of *pzl-1,* PPZ1, has been characterized as an important (negative) regulator of salt stress, halotolerance and pH homeostasis (86,87). In our assays, we found that the Δ*pzl-1* mutant was resistant to both sorbitol and sodium chloride, providing evidence for similar functions for *pzl-1* in *N. crassa* as observed in baker's yeast. *S. cerevisiae* Psr1p and Psr2p are most similar to *N. crassa* PPH-11 and CSP-6, respectively (88) (Table 1). The slow growth rate of the *N. crassa* Δ*pph-11* mutant disqualified this strain for chemical sensitivity screening, but we observed that the Δ*csp-6* mutant was sensitive to sodium chloride (Table 2.3). The observation that Δ*psr1* and Δ*psr2* single mutants are normal, but that loss of both genes leads to sensitivity to salt stress in *S. cerevisiae* (88) illustrates the difference in genetic wiring between *S. cerevisiae* and *N. crassa*.

In order to decipher possible functions for protein phosphatases in cytoskeletal maintenance, we treated the mutants with cytochalasin A, which prevents polymerization and elongation of actin filaments (89) and benomyl, which binds to microtubules, thus inhibiting mitosis, meiosis and cellular transport (90). Interestingly, the only phenotype observed using these chemicals was increased resistance. Three mutants, Δ*pph-4,* Δ*pph-1* and Δ*pph-10*, were resistant to cytochalasin A, while four (Δ*pp2A*, Δ*pzl-1,* Δ*cdc-14* and

Δ*csp-6*) showed enhanced growth as compared to wild type with benomyl treatment (Table 2.3). It is possible that these missing phosphatases play important antagonistic roles in mitotic exit or in cell proliferation, perhaps through dephosphorylation of a mitotic/cell proliferation kinase. For example, *cdc-14* is known to be an important regulator of the cell cycle and mitosis in fungi and deletion of the gene in *N. crassa* imparts resistance to benomyl. Deletion of *cdc-14* leads to increased activity of *cdk-1,* which is known to promote cell proliferation and survival (in mammalian cells) via phosphorylation of the transcription factor FOXO1 (91,92). Resistance to benomyl in Δ*cdc-14* might be imparted via a similar mechanism, which in effect counteracts the inhibitory effects of the chemical.

FK506 is a macrolide lactone (93) that binds the immunophilin FKBP12 (FK506 binding protein), inhibiting the S/T phosphatase calcineurin in the calcium-signaling pathway (16). Assays with this immunosuppressant drug revealed that six mutants (Δ*pzl-1*, Δ*csp-6,* Δ*pty-2,* Δ*cdc-14*, Δ*pty-4* and Δ*pty-6*) were resistant, while one (Δ*pph-6*) was sensitive to FK506 (Table 2.3). Resistance to FK506 was the only phenotype for the Δ*pty-6* mutant.

Calcineurin A subunit mutants have been shown to have phosphatase activity with increased resistance to FKBP12-FK506 in mammalian cells (94). A similar effect is also observed in case of TOR pathway mutants in yeast (*TOR1* and *TOR2*) that display resistance to a different macrolide, sirolimus, also known as rapamycin (93,95). The TOR signaling pathway has also been implicated in regulation of microtubule structure/function and acts antagonistically to the calcineurin-signaling network (96,97).

Of the strains that were resistant to FK506, three mutants (Δ*pzl-1*, Δ*csp-6* and Δ*cdc-14*) also displayed resistance towards benomyl. It is thus tempting to speculate that these three phosphatase knockout mutants with a common resistance to benomyl and FK506 might have overlapping roles in the TOR pathway and calcineurin function in *N. crassa*.

Fludioxonil is a phenylpyrrole class fungicide (98) that stimulates the OS-2 MAPK pathway, leading to increased glycerol production and cell death in *N. crassa* (99). The OS MAPK module mutants (*os*-4/*os*-5/*os*-2) are resistant to fludioxonil, but sensitive to sodium chloride and sorbitol (55,99). We found that two mutants, Δ*csp-6* and Δ*pph-7*, are sensitive to fludioxonil, whereas the PP2A class phosphatase mutant Δ*pp2A* and CDC25 phosphatase mutant Δ*div-12* were resistant. The resistance phenotype of the latter group suggests that these gene products might have important roles in the OS-2 MAPK signaling pathway (see below). Incidentally, fludioxonil sensitivity was the only phenotype observed for the Δ*pph-7* mutant in this study.

We also analyzed the relative growth of the phosphatase mutants on medium supplemented with 2% yeast extract, which is rich in amino acids, peptides and vitamins. Only two phosphatase mutants, Δ*pzl-1* and Δ*pph-4*, exhibited a significant difference in growth relative to wild type on 2% yeast extract. Both of these strains grow less well than wild type, suggesting that nutrient sensing and/or utilization abilities are compromised in the mutants.

The 25 viable phosphatase mutants were also cultured on VM with Avicel (2%) substituted for sucrose as an alternate carbon source. We found that two phosphatase mutants (Δ*pph-4* and Δ*dsp-2*) were better able to utilize Avicel than wild type, consistent

with the corresponding genes acting as negative regulators of cellulose utilization (Table 2.4). It is interesting to note that both of these mutants display a common sensitivity to tert-butyl hydroperoxide, and that Δ*pph-4* is also sensitive to menadione. It will be of interest in future studies to determine whether sensitivity to oxidative stress could prove beneficial in up-regulating carbon metabolism genes in fungi, and even how these phosphatases could assist in the process.

Through our morphological testing, we determined that 2 of the 25 viable mutants had no obvious growth defects (Δ*pty-6* and Δ*pph-7)*. However, phenotypes were revealed for these two mutants through the chemical sensitivity assays, resulting in at least one phenotype for every phosphatase mutant analyzed. This supports the advantage of chemical testing for identifying defects for mutants that do not display growth or developmental phenotypes. Knockout strains for *pzl-1* and *pp2a* possessed the greatest number of chemical phenotypes and 18/22 tested strains exhibited at least one chemical phenotype. Also taking into account mutants that possessed either significant sensitivity or resistance to more than one chemical, we observed a total of 42 chemical phenotypes for a set of 22 phosphatase mutants.

The phospho-p38 MAPK level is elevated in a number of protein phosphatase mutants: MAPKs are a class of serine/threonine kinases present in all eukaryotic cells. As a group, they are responsible for a wide variety of cellular responses towards stress and environmental stimuli and also regulate gene expression, metabolism, mitosis, apoptosis, cellular motility and differentiation (100-104). MAPKs are highly conserved

throughout evolution and also are one of the most widely studied groups of proteins for investigation of physiological responses (105). The p38 MAPK homologs in *S. cerevisiae* (Hog1p) and *N. crassa* (OS-2) are involved in cellular responses to hyperosmolarity, as well as oxidative stress (106-108). Previous studies have shown that loss of any of the three genes in the MAPK module (*os-4, os-5* and *os-2*) in *N. crassa* does not appreciably affect basal hyphal growth, but leads to fragile conidia, increased sensitivity to hyperosmotic conditions, resistance to the fungicide fludioxonil and female sterility (99,109).

In order to identify protein phosphatases that may act on the OS MAPK cascade, we analyzed the phosphorylation status of OS-2 in the 25 viable phosphatase knockouts using 0.8 M NaCl for inducing osmotic stress in 16-hour liquid cultures (see Materials and Methods). For detecting the phosphorylated form of OS-2 in the protein samples from the cellular extracts, we used commercially available peptide antibodies raised against the mammalian MAPK phospho-p38. Similar to previous studies (109), this antibody was found to cross-react with a *N. crassa* phosphoprotein of ~41 kDa, near the predicted size of OS-2 (Fig. 2.2). We found that nine mutants had elevated basal levels of phospho-OS-2: Δ*pty-1*, Δ*pph-11*, Δ*dsp-2*, Δ*pty-3*, Δ*div-12*, Δ*pty-4*, Δ*pp2A*, Δ*pph-10* and Δ*pph-8* (Fig. 2.2; Table 2.1). However, only Δ*div*-12 exhibited higher levels of phospho-OS-2 than wild type post-induction using sodium chloride (Fig. 2.2). As mentioned above, Δ*div-12* also displays resistance to fludioxonil (Table 2.3), a phenotype similar to that of mutants lacking *os-2*, *os-4* or *os-5*. Hence, DIV-12 (a CDC25-type protein tyrosine phosphatase) may play a role in dephosphorylation of one or more of the

component kinases of the OS pathway in *N. crassa.* In budding yeast, Mih1p (a DIV-12 homolog) is involved in dephosphorylation of CDC28 (110,111) and a role in Hog1p dephosphorylation has also been suggested (112). The other CDC25-type phosphatase in *N. crassa,* PTY-1, also seems to have functions in the OS-2 pathway, as deletion of *pty-1* leads to an increase in the basal levels of phospho-OS-2. We also found that Δ*pph-10* showed elevated basal phospho-OS-2. The yeast homolog SSU72 phosphatase is primarily involved in transcription termination via removal of phospho-Ser7 marks from the RNA Pol II CTD (44). Our MAPK assays also implicate the SSU72 class of phosphatases in regulation of OS-2 phosphorylation, although such a function might also be imparted via regulation of the transcriptional machinery.

Deletion of the protein tyrosine phosphatases *pty-3* and *dsp-2* leads to increased basal levels of phospho-OS-2 as compared to wild type (Fig. 2.2). The yeast *pty-3* homolog PTP3is involved in dephosphorylation of both Hog1p and Slt2p in the cell wall integrity pathway (113,114). In contrast, Msg5p, the yeast homolog of DSP-2, is implicated in dephosphorylation of Slt2p and Fus3p in the pheromone-sensing pathway (115-117). Our results suggest that DSP-2 is also required for inactivation of the OS-2 pathway and further study of the Erk class MAPKs MAK-1 and MAK-2 in *N. crassa* may uncover similar functions for this phosphatase as seen in yeast. It is also possible that there is a high degree of crosstalk between the different MAPK cascades, leading to an overlap of function.

Our study on the low-molecular weight protein tyrosine phosphatase *pty-4* is especially unique because so far a cellular role has not been demonstrated in *S. cerevisiae* (118). The *N. crassa* Δ*pty-4* mutant exhibits decreased production of protoperithecia and resistance towards menadione and FK-506 (Table 2.1, Table 2.3; Appendix Table S2.1). This suggests that PTY-4 negatively regulates pathways involved in activation of oxidative stress responses. Deletion of *pty-4* leads to an increased basal level of phospho-OS-2 as compared to wild type (Fig. 2.2). These results suggest that PTY-4 may regulate sexual development and ROS sensitivity via OS-2 phosphorylation in *N. crassa*.

Among the PP2A class of S/T phosphatases, Δ*pp2A* was the only mutant exhibiting elevated basal phospho-OS-2 levels (Fig. 2.2). The finding that the *pp2A* mutant was sensitive to sodium chloride and sorbitol, but resistant to fludioxonil treatment, also supports a role as a major phosphatase in the OS-2 pathway (Table 3). The PP2C class phosphatase mutant Δ*pph-8* and the HAD class mutant Δ*pph-11* also exhibited elevated levels of basal phospho-OS-2 (Fig. 2.2). The *S. cerevisiae* homolog of *pp2A* is *PPG1* and that for *pph-8* is *PTC2*; the *PPG1* and *PTC2* gene products are required for glycogen accumulation and dephosphorylation of Hog1p, respectively (119,120). These results suggest that similar to their homologs in yeast, these phosphatases are bona fide regulators of OS-2 dephosphorylation and have important roles in the MAPK signaling cascade.

Taken together, our assays revealed that 9/25 viable mutants exhibit altered p38 MAPK phosphorylation. Information for a number of these phosphatases in *S. cerevisiae* indicates that there is considerable crosstalk and/or overlap in function for some of these phosphatases with other MAPK cascades, such as the cell wall integrity pathway (114,115,121,122). Our results suggest that a similar commonality in function may also exist in *N. crassa*.

Deletion of the PP2C class protein phosphatase gene *pph-8* leads to unregulated protoperithecial development in *N. crassa*: In *N. crassa*, the formation of female sexual structures (protoperithecia) is stimulated by growth on synthetic crossing medium (SCM) containing low nitrogen (123). While carrying out phenotypic analysis of the phosphatase mutants, we observed that a PP2C class phosphatase knockout mutant (Δ*pph-8*) displayed inappropriate protoperithecial formation on VM medium containing high nitrogen (Fig. 2.3). In order to further assess this unregulated protoperithecial formation, Δ*pph-8* was cultured on VM and SCM agar plates in constant light or constant darkness for 5-7 days and then fertilized with an opposite mating type wild-type strain. At five days under constant light conditions, the Δ*pph-8* mutant displayed small protoperithecia that were embedded under the agar surface of both media. At seven days, Δ*pph-8* protoperithecia differentiated on VM had enlarged to the same size seen in the wild-type strain at seven days on SCM medium, while Δ*pph-8* protoperithecia produced on SCM were smaller than wild type (Fig. 2.3A). Post-fertilization, the Δ*pph-8* mutant was able to form mature perithecia (Fig. 2.3A) and shot ascospores 10 days post-

fertilization (data not shown). Previous studies have shown that blue light is necessary for photo-induction of protoperithecial development (124). When cultured in constant darkness, wild type produced protoperithecia after seven days on SCM (but not VM), a delay of two days relative to constant light conditions (Fig. 2.3B). The Δ*pph-8* mutant exhibited no delay in protoperithecial development on either VM or SCM in constant darkness (Fig. 2.3B). Interestingly, Δ*pph-8* protoperithecia formed in constant darkness were slightly larger than those formed in light (Fig. 2.3). Perithecia were produced in wild type on SCM and the Δ*pph-8* mutant on both SCM and VM medium in constant darkness (Fig. 2.3). When formed, perithecia from wild type and the Δ*pph-8* mutant produced ascospores by 10 days after fertilization (data not shown). However, in contrast to wild type, Δ*pph-8* ascospore progeny did not germinate (data not shown).

The results presented above suggest that loss of *pph-8* significantly affects nitrogen sensing and the sexual development pathway in *N. crassa*. This mutant also exhibited multiple defects in hyphal growth and asexual sporulation (Table 2.1, Table 2.3). PPH-8 shares a high degree of homology to Ptc2p in *S. cerevisiae*. Ptc2p dephosphorylates Hog1p as well as Cdc28p and is also implicated in functioning with proteins such as RAD53 to regulate DNA damage checkpoint pathways (22,120,125). As seen from the MAPK assays, Δ*pph-8* has a high basal level of phospho-OS-2, and levels after treatment with sodium chloride are similar to those of treated wild-type. This suggests that PPH-8 is involved in dephosphorylation of the OS-2 MAPK in *N. crassa* (Fig. 2.2). Other studies have shown that the *os-4*/*os-5*/*os-2* mutants are unable to

produce protoperithecia (109), a phenotype in opposition to that observed for Δ*pph-8*. Hence, it is plausible that the PPH-8 phosphatase regulates protoperithecial development via modulation of the OS-2 MAPK pathway.

The protein phosphatase mutant Δ*csp-6* displays a conidial separation defect most similar to Δ*csp-1* mutants: Two conidial separation mutants, Δ*csp-1* and Δ*csp-2*, form major constriction chains with double crosswalls in developing conidiophores, but no free macroconidia (126). Genetic and molecular studies that have implicated a number of genes in the macroconidiation pathway place *csp-1* and *csp-2* downstream of other genes, including *acon-2* and *fl* (127,128). The *csp-1* gene encodes a light-inducible zinc finger transcription factor, and deletion of *csp-1* leads to shortening of the period length for the circadian clock by approximately one hour (129,130). Recent evidence showed that CSP-1 is a transcription repressor, with its function and abundance coupled to the circadian activity of the white-collar complex (WCC), thus constituting an important output for the clock (131,132). CSP-1 is primarily involved in ergosterol biosynthesis, modulating the lipid composition of membranes (131,132). In contrast to *csp-1*, deletion of the grainy head transcription factor gene *csp-2* lengthens the clock period by 1.5 hours in *N. crassa* (133,134). Among its functions, CSP-2 influences expression of genes involved in construction and remodeling of the cell wall (134).

Microscopic observation of conidia revealed that Δ*csp-6*, lacking a HAD class serine/threonine phosphatase, appeared to possess a conidial separation defect reminiscent of Δ*csp-1* and Δ*csp-2* mutants. Similar to the Δ*csp-1* and Δ*csp-2* strains, when slant cultures of Δ*csp-6* are agitated, no free conidia are released. In addition to a conidial separation defect, the Δ*csp-6* mutant exhibited reduced basal hyphal growth and produced few, small protoperithecia that did not develop into mature perithecia after fertilization during sexual development (Table 2.1 and Appendix Table S2.1). This contrasts with knockout mutants lacking *csp-1* or *csp-2*, which have reduced hyphal growth, but do not possess defects in female sexual development (Broad database). In order to more accurately compare and contrast the conidial separation defects of Δ*csp-1*, Δ*csp-2* and Δ*csp-6* strains, we used the fluorescent stain calcofluor white to visualize the cell wall (Fig. 2.4). Upon staining with calcofluor white, we found that the Δ*csp-2* mutant is able to form numerous double-doublets at interconidial junctions (Fig. 2.4), consistent with results from a previous studies. This suggests that the Δ*csp-2* mutant is blocked at the double-doublet stage before connective formation takes place.

Consistent with previous results, we also observed that the Δ*csp-1* mutant displays fewer double-doublets and sometimes does not form septa between macroconidial compartments (Fig. 2.4). In the case of the Δ*csp-6* mutant, we observed double-doublets (Fig. 2.4), but such structures were not as extensive as in Δ*csp-2* strains. In addition, the *csp-6* mutant sometimes lacked septa between macroconidial compartments in conidiophores (Fig. 2.4). Hence, the conidial separation defect of Δ*csp-6* is more similar to Δ*csp-1* than to Δ*csp-2.* This conclusion supports CSP-1 and CSP-6

acting in the same pathway to regulate growth and conidiation, perhaps through dephosphorylation of phosphorylated CSP-1 transcription factor (or a regulated target) by the CSP-6 protein phosphatase.

DISCUSSION

In this study, we examined the role of protein phosphatases in growth and development and regulation of p38 MAPK dephosphorylation in the filamentous fungus, *N. crassa*. We have identified 30 protein phosphatase genes in the *N. crassa* genome and found that these genes are highly conserved among humans, plants and other fungi. In particular, *N. crassa* phosphatases are similar in number to those in *A. nidulans.* Two phosphatases (*pty-5* and *pty-6*,) showed little or no homology to genes in yeast, animals or plants, while similar genes are present in *A. nidulans*, suggesting that these are specific for filamentous fungi. Our results demonstrated that Δ*pty-5* mutants possess defects in conidiation, while strains lacking *pty-6* are resistant to fludioxonil. Conidiation is observed in many filamentous fungi, but not baker's yeast. Likewise, in contrast to many filamentous fungal species, *S. cerevisiae* is naturally resistant to fludioxonil, apparently lacking the cellular target of this fungicide (99,135). Future studies will shed light on the cellular pathways impacted by these two tyrosine phosphatases in filamentous fungi.

A majority of protein phosphatase knockouts (92%) exhibited defects in basal growth, asexual or sexual development. We found that three mutants (Δ*pty-2,* Δ*pph-6* and Δ*ppt-1*) actually displayed increased basal growth rates compared to wild type. This is in contrast to our previous study with kinase mutants (55), where all mutants with a basal

hyphae growth defect exhibited reduced growth. Since in general, phosphatases impart their roles by dephosphorylation of their targets, it is likely that phosphatase mutants with increased growth may experience constitutive phosphorylation of targets, leading to unregulated cell proliferation. Interestingly, the Δ*pty-2* and Δ*ppt-1* mutants were resistant to tert-butyl hydroperoxide, perhaps suggestive of a link between increased growth and oxidative stress resistance.

Morphological analyses of two development pathways in *N. crassa* showed that certain phosphatases are specific for sexual or asexual development (Fig. 2.1). In particular, four tyrosine phosphatases (*pty-3*, *pty-4*, *div-12* and *dsp-4*) are restricted to sexual development, while another three tyrosine phosphatases (*pty-1, dsp-1* and *pty-5*) are only involved in asexual differentiation. In contrast, S/T phosphatases seem to have broader roles in fungal development (Fig. 2.1). Deletion of various S/T kinases in *N. crassa* led to a high proportion (40%) of female-sterile strains (55). In contrast, only four phosphatase mutants (Δ*tng*, Δ*pp2A,* Δ*csp-6* and Δ*dsp-2*) were female-sterile, representing 16% of the viable phosphatase mutants. This may reflect the antagonistic roles of protein phosphatases and kinases, with constitutive phosphorylation of targets in phosphatase mutants less likely to result in female sterility. Opposing functions for kinases and phosphatases are also manifested by the chemical phenotypes. Treatment of S/T kinase mutants with tert-butyl hydroperoxide only revealed strains with increased sensitivity (55), whereas 40% of the affected phosphatase mutants displayed a resistant phenotype.

Two of the analyzed protein phosphatase mutants (Δ*pph-7* and Δ*pty-6*) did not have obvious growth or developmental phenotypes. As in our previous study on kinases,

chemical sensitivity assays proved to be an effective tool in assigning a function for such mutants lacking a morphological phenotype. Deletion of *pty-6* resulted in increased resistance to the calcineurin inhibitor FK506, while the absence of *pph-7* rendered the strain sensitive to fludioxonil (which stimulates the OS-2 pathway; Table 2.3). In *S. cerevisiae*, deletion of the *pty-6* homolog SIW14 leads to cytoskeletal abnormalities and defective endocytosis (136). Hence it is possible that *pty-6* and its related phosphatases might regulate cytoskeletal organization in concert with the calcineurin-mediated signaling pathways in *N. crassa*. In the case of *pph-7*, an intron in the mRNA of the *S. cerevisiae* homolog *PTC7* is alternatively spliced, producing two protein isoforms (137). The protein derived from the spliced mRNA is localized to the mitochondrion, while that produced from the unspliced mRNA is found on the nuclear envelope. The mitochondrial protein is modified in a carbon source-dependent fashion, while mutants lacking the version on the nuclear envelope are more sensitive to latrunculin (a chemical that disrupts actin filaments) than wild type (137). In contrast to its closest yeast homolog, *N. crassa pty-6* lacks an intron in the ORF and biochemical studies have localized the protein to the mitochondrion (138). Furthermore, we did not observe altered sensitivity of the Δ*pty-6* mutant to cytochalasin A, but instead to fludioxonil, which has been shown to activate the OS-2 MAPK pathway, leading to glycerol production. No proteins involved in fludioxonil sensitivity have been localized to the mitochondrion. Our findings support a scenario in which loss of *pty-6* leads to elevated production of glycerol in *N. crassa*. This likely occurs at a point downstream of the OS-2 MAPK, as we observed that Δ*pty-6* mutants possessed normal basal and induced levels of phospho-OS-2. Concomitant loss

of *pty-6* and inappropriate activation of the OS-2 MAPK by fludioxonil would render the mutant more sensitive than wild type.

A number of studies in *N. crassa* investigating utilization of cellulose as an alternate carbon source have shown that there is an upregulation of lignocellulolytic enzymes when *N. crassa* is switched from sucrose to cellulose (63,139-141). Previous work has identified the zinc finger transcription factor CRE-1 as a carbon catabolite repressor, whereby deletion of *cre-1* leads to increased expression of cellulolytic genes when *N. crassa* is grown on the microcrystalline cellulose source, Avicel (139). We have identified two protein phosphatase mutants, Δ*pph-4* and Δ*dsp-2*, that display increased growth on Avicel, consistent with roles as negative regulators of cellulose utilization. Therefore, it is possible that these phosphatases may operate in the same pathway or play parallel roles with CRE-1 in regulating the transcriptional machinery or downstream events to influence cellulolytic activity in *N. crassa*.

The phosphatases *pp2A* (NCU06563) and *pzl-1* (NCU07489) belong to the PP2A class of S/T phosphatases, a highly conserved family of proteins with several important functions in cellular signaling, from mammals to fungi (142-144). It is therefore not surprising that deletion of these genes led to several defects in growth and development and also yielded the highest number of chemical phenotypes (six for Δ*pp2A* and seven for Δ*pzl-1*). The Δ*pp2A* mutant was sensitive to osmotic stresses, peroxide stress and reactive oxygen species, as well as displaying resistance to fludioxonil and benomyl (Table 2.3). All of these chemical phenotypes were observed with high significance and very low p values, and the p38 MAPK assays further reaffirm the authenticity of the responses for

this mutant to the respective chemicals (see Appendix Table S2.1). From the p38 MAPK assays, we found that PP2A is involved in dephosphorylation of the terminal MAPK OS-2 in the osmosensing pathway (Fig. 2.2) and its function in the OS-2 pathway is also reflected by the resistance of the mutant to fludioxonil, similar to the *os* mutants (55,99). Recent evidence from *Sordaria macrospora* suggests a role for a homolog of *pp2A* (SmPP2Ac) in regulating cell-cell fusion and sexual development as an integral component of the STRIPAK complex (145). However, the exact role of SmPP2Ac in regulation of these developmental outcomes remains to be deciphered. It will be interesting to further investigate whether the PP2A has any role in MAK-2 phosphorylation, a protein that is a major component of cell-cell fusion in *Neurospora* (49).

Another phosphatase mutant with interesting phenotypes as well as elevated phospho-OS-2 levels in this study is the PP2C class protein phosphatase mutant Δ*pph-8*. When grown on minimal medium, the mutant displayed inappropriate formation of protoperithecia (Fig. 2.3), similar to the S/T kinase mutant Δ*ime-2* (55,146). One possible scenario is that IME-2 and PPH-8 regulate two different target phospho-proteins with opposing functions on protoperithecial development. While one target protein could inhibit protoperithecial development upon dephosphorylation by PPH-8, the other one could repress it upon being phosphorylated by IME-2. Genetic epistasis studies should provide further insight into understanding the underlying mechanism of how *ime-2* and *pph-8* regulate protoperithecial formation in *N. crassa.*

With the success of the *N. crassa* gene knockout project, we have focused on analysis of phenotypes for large groups of genes with crucial roles in cellular homeostasis, including transcription factors, serine-threonine protein kinases and now, serine-threonine and tyrosine protein phosphatases. Prior to our study, most of the protein phosphatases in *N. crassa* had not been characterized. In numerous cases, we now have important clues to their functions. Further studies on these protein phosphatases should provide a greater understanding of how these proteins are able to regulate important cellular roles in *N. crassa,* related fungi and other eukaryotic organisms.

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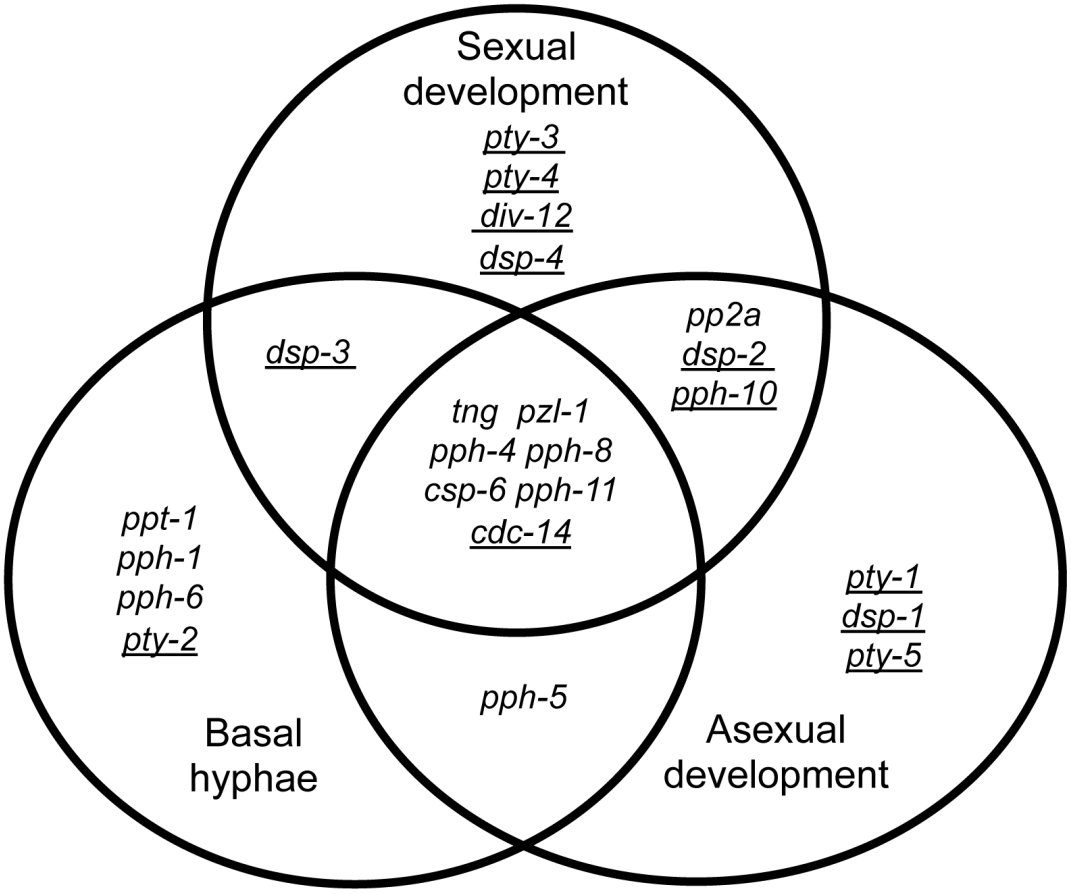
Sexual development
pty-3
pty-4
div-12
dsp-4
dsp-3
pp2a
dsp-2
pph-10
tng pzl-1
pph-4 pph-8
csp-6 pph-11
cdc-14
ppt-1
pph-1
pph-6
pty-2
pty-1
dsp-1
pty-5
pph-5
Basal hyphae
Asexual development
S/T Phosphatases
Tyrosine Phosphatases

Figure 2.1 –Venn Diagram displaying distribution of S/T protein kinase mutants with growth and developmental phenotypes. The 23 viable protein phosphatase mutants exhibiting defects in at least one major growth/developmental pathway are indicated by the names for deleted genes. The underlined gene names correspond to tyrosine phosphatases, while the remaining are serine/threonine phosphatases.

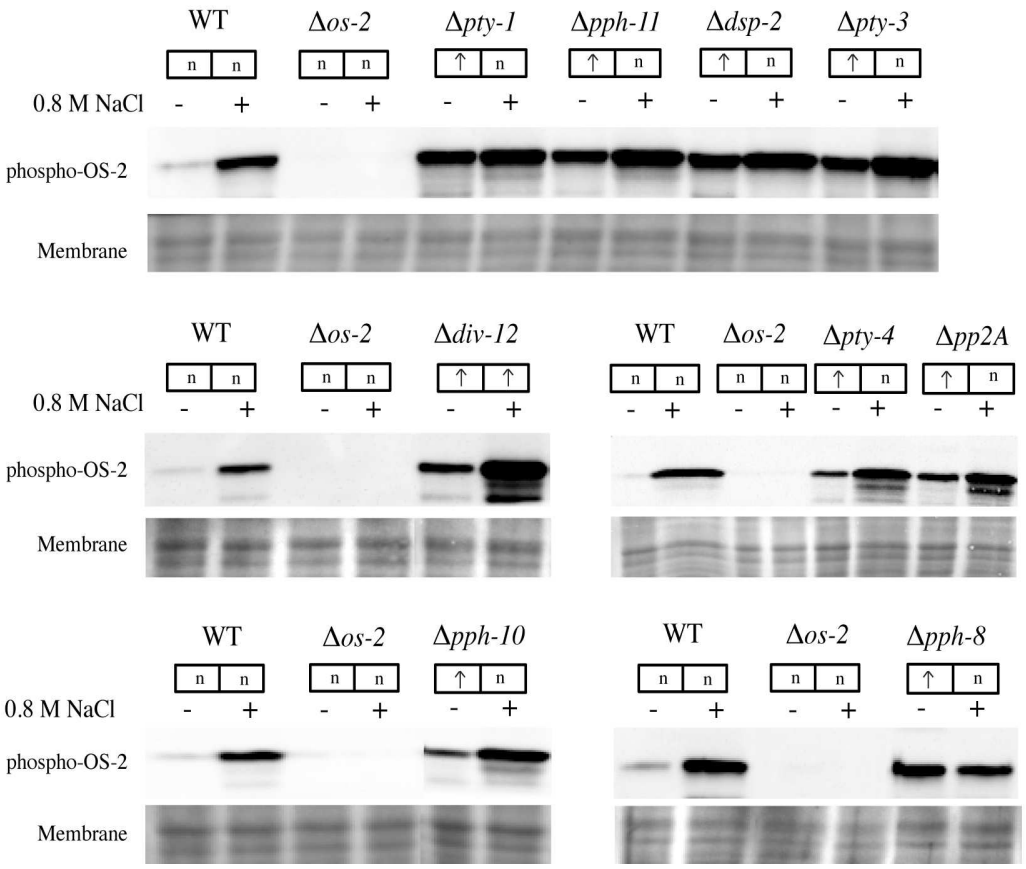
WT
Δos-2
Δpty-1
Δpph-11
Δdsp-2
Δpty-3
n n
↑ n
0.8 M NaCl
- + - + - + - + - + - +
phospho-OS-2
Membrane
WT
Δos-2
Δdiv-12
WT
Δos-2
Δpty-4
Δpp2A
n n
↑ ↑
↑ n
0.8 M NaCl
- + - + - + - + - + - +
phospho-OS-2
Membrane
WT
Δos-2
Δpph-10
WT
Δos-2
Δpph-8
n n
↑ n
0.8 M NaCl
- + - + - + - + - + - +
phospho-OS-2
Membrane

Figure 2.2 – Analysis of p38 MAPK phosphorylation. Conidia were used to inoculate shaken liquid cultures that were grown for 16 h at 30°. Cultures were left untreated or brought to 0.8 M NaCl for 10 min. to stimulate OS-2 phosphorylation. Phospho-OS-2 levels were analyzed by immunoblotting with a specific antiserum (top panels). A portion of the membrane was excised and stained using amido black to use as a loading control (bottom panels). The experiment was repeated at least three times and a representative blot is shown. The letter 'n' signifies that the levels of phospho-OS-2 were similar to wild type. The arrow signifies that the levels of phospho-OS-2 were elevated as compared to wild type.

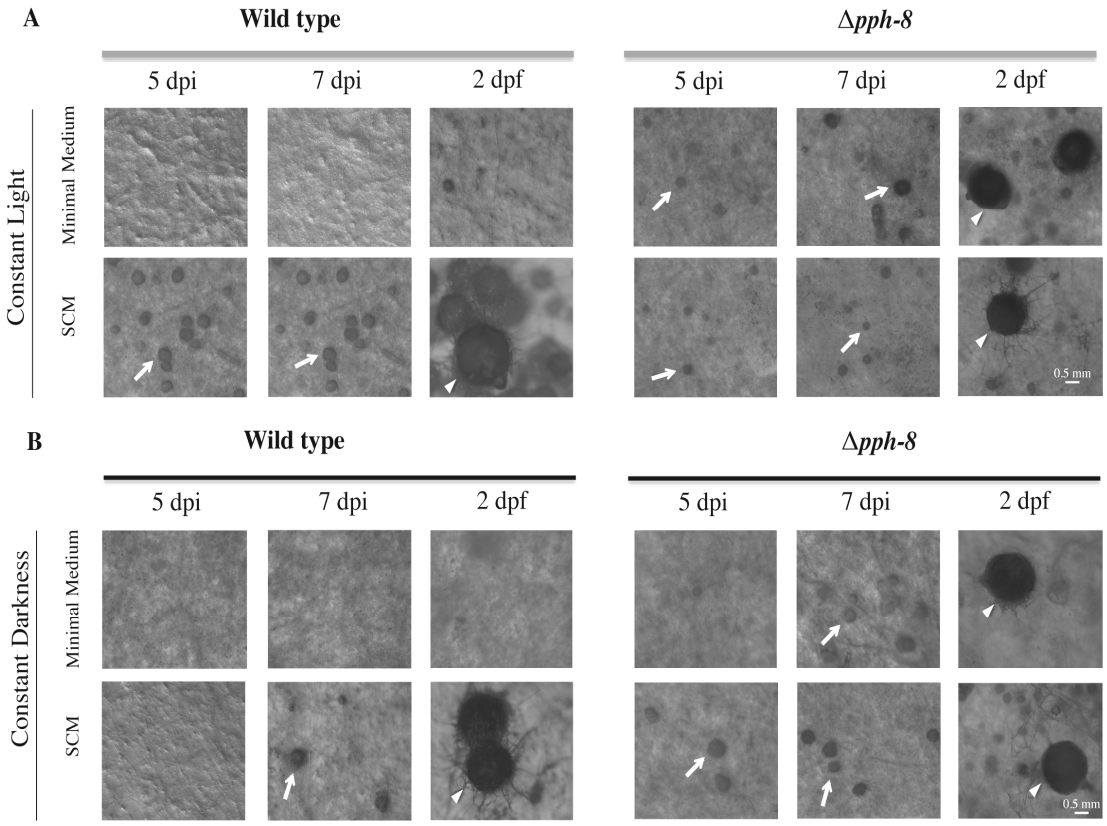
A
Wild type
Δpph-8
5 dpi
7 dpi
2 dpf
5 dpi
7 dpi
2 dpf
Constant Light
Minimal Medium
SCM
0.5 mm
B
Wild type
Δpph-8
5 dpi
7 dpi
2 dpf
5 dpi
7 dpi
2 dpf
Constant Darkness
Minimal Medium
SCM
0.5 mm

Figure 2.3. The Δ*pph-8* mutant displays unregulated protoperithecial development on minimal medium. Wild-type and Δ*pph-8* strains were cultured on VM and SCM plates under constant light (A) or constant darkness (B) and photographed at five days post inoculation (dpi), seven days dpi and two days post fertilization (dpf) with opposite mating type conidia. The white arrows point to protoperithecia, while the white arrowheads indicate mature perithecia. Scale bar = 0.5 mm.

Wild type
Δcsp-1
Δcsp-2
Δcsp-6
Calcofluor White
DIC

Figure 2.4. Δ*csp-6* displays a conidiation separation defect most similar to Δ*csp-1* strains. Wild type, Δ*csp-1*, Δ*csp-2* and Δ*csp-6* strains were cultured on VM medium for seven days under constant light and conidia were stained with calcofluor white to visualize developing crosswalls in the conidial chains. The arrow points to the conjoined conidia indicating the separation defect. Scale bar size = 10 μ.

Table 2.1: *Neurospora crassa* phosphatase gene families and summary of phenotypes and p38 MAPK levels

Family [a]	Sub-family[b]	Class/ Domain[c]	NCU#[d]	*N. crassa* gene[e]	Yeast *homolog*[f]	Phenotype Summary				Chemical Sensitivity/ Nutritional[j]	P38 MAPK
						Inviable	Linear Growth[g]	Asexual Development[h]	Sexual Development[i]		
S/T	PPP	PP2Ac	00043	*ppp-1/ pph-3*	*GLC7*	X	-[m]	-	-	-	-
S/T	PPP	PP2Ac	03436	*tng*	*SIT4*		R	AH, C	PP, P, A	N/S[n]	
S/T	PPP	PP2Ac	06563	*pp2A*	*PPG1*			C	PP, P, A	SC, S, B, M, FL, T,	B
S/T	PPP	PP2Ac	07489	*pzl-1*	*PPZ1*		R	AH, C	PP	SC, S, B, M, T, F, YE	
S/T	PPP	PP2Ac	08301	*pph-4*	*PPH3*		R	AH	PP, A	C, M, T, YE, A	
S/T	PPP	PP2Ac	06630	*pph-1*	*PPH21*		R			C	
S/T	PPP	PP2Bc	03804	*cna-1/ pph-2*	*CMP2*	X	-	-	-	-	-
S/T	PPP	PP5c	01433	*ppt-1*	*PPT1*		I			T	
S/T	PPM	PP2Cc	00958	*pph-7*	*PTC7 /AZR1*					FL	
S/T	PPM	PP2Cc	01767	*pph-5*	*PTC5*		R	AH[**]		T	
S/T	PPM	PP2Cc	03495	*pph-6*	*PTC6*		I			T, F	
S/T	PPM	PP2Cc	04600	*pph-8*	*PTC2*		R	AH, C	PP, P, A	N/S	B
S/T	PPM	PP2Cc	00434	*pph-9*	*PTC1*	na[o]	na	na	na	na	na
S/T	Asp-Based	HAD	08948	*pph-11*	*PSR1*		R	AH, C	PP, P, A	N/S	B
S/T	Asp-Based	HAD	08380	*csp-6*	*PSR2*		R	AH, C	PP, P, A	SC, B, FL, T, F	
S/T	Asp-Based	FCP/SCP	09300	*fcp-1*	*FCP1*	X	-	-	-	-	-
PTP	Classical	PTPc	02257	*pty-2*	*PTP1*		I			T, F	

PTP	Classical	PTPc	05364	*pty-3*	*PTP2 /PTP*				PP, P, A	S	B
PTP	Dual Specificity	DSPc	03246	*cdc-14*	*CDC 14*		R	AH**, C	A	S, B, F	
PTP	Dual Specificity	DSPc	03426	*dsp-1*	*PPS1*			AH**		T	
PTP	Dual Specificity	DSPc	06252	*dsp-2*	*MSG 5*			AH, C	PP, P, A	S, T, A	
PTP	Dual Specificity	DSPc	06330	*dsp-3*	*MSG 5*		R		PP, P, A		
PTP	Dual Specificity	DSPc	08158	*dsp-4*	*YVH1*				PP, A		
PTP	Dual Specificity	DSPc	05049	*dsp-5*	*SDP1*	na	na	na	na	na	na
PTP	LM-PTP	LMWPc	09841	*pty-4*	*LTP1*				PP	M, F	B
PTP	CDC-25 type	CDC25	02496	*div-12*	*MIH1*				PP, P, A	F	B, I
PTP	CDC-25 type	CDC25	06966	*pty-1*	*YCH1*			C			B
PTP	SSU72	SSU72	03114	*pph-10*	*SSU7 2*			AH**	PP, P, A	C	B
PTP	-	Y-Phosphatase3	01010	*pty-5*	-			C			
PTP	-	Y-Phosphatase2	03333	*pty-6*	*SIW1 4*					F	

[a] Family abbreviations: S/T (serine/threonine), PTP (protein tyrosine phosphatase)
[b] Subfamily abbreviations: PPP (phosphoprotein phosphatase), PPM (Mg^{2+} or Mn^{2+} dependent protein phosphatase), Asp-Based (Aspartate based phosphatase), LMW-PTP (low molecular weight protein tyrosine phosphatase), CDC25 type (cell division cycle 25 type), SSU72 (C-terminal domain RNA Pol II phosphatase).
[c] Class/Domain abbreviations: PP2Ac (protein phosphatase 2 A catalytic subunit), PP2Bc (protein phosphatase 2 B catalytic subunit), PP5 catalytic subunit (protein phosphatase 5 catalytic subunit), PP2Cc (protein phosphatase 2C catalytic subunit), HAD (haloacid dehalogenase), FCP/SCP (transcription factor IIF -interacting C-terminal domain phosphatase 1/ small C-terminal domain phosphatase), PTPc (protein tyrosine phosphatase catalytic subunit), DSPc (dual specificity phosphatase catalytic subunit), LMWPc (low-molecular weight phosphatase catalytic subunit), CDC25 (cell division cycle), SSU72 (C-terminal domain RNA Polymerase II phosphatase), Y_phosphatase3 (tyrosine phosphatase 3), Y_phosphatase2 (tyrosine phosphatase 2).
[d]Based on version 5 annotation of the Broad Institute's *Neurospora crassa* database (http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html).
[e]Phosphatase gene names are consistent with the *Neurospora* e-Compendium Project at Leeds University (http://bmbpcu36.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.html). All other gene names were named during this study in accordance with the e-Compendium system.
[f]Yeast orthologs were obtained from literature or blastp search and are consistent the *Saccharomyces* genome database (http://www.yeastgenome.org/) .
[g]R, reduced growth; I, increased growth (Significance testing done with Student's T test, paired, two-tailed; $p<0.05$, $^{**}p<0.1$)
[h]Asexual phenotypes are depicted by phenotypes in aerial hyphae (AH) or conidial development (C).
[i]Sexual phenotypes are depicted by their occurrence during protoperithecial (PP), perithecial (P) or ascospore (A) development.
[j]Chemical phenotypes are represented based on the sensitivity or resistance to sodium chloride (SC), sorbitol (S), cytochalasin A (C), benomyl (B), tert-butyl hydroperoxide (T), menadione (M), FK506 (F), fludioxonil (FL) and yeast extract (YE).
[k]Nutrional phenotypes are represented by increased growth on Avicel (A) as compared to wild-type.
[l] p38 MAPK levels are represented relative to wild-type levels as elevated basal (B) or elevated induced (I).
_[m], Phenotypic analysis could not be performed due to inviability of the knockout mutant/s.
[n]N/S, mutant strain was not analyzed for chemical screening due to poor growth compared to wild-type.[o] na, mutant not available.

Table 2.2: Serine/threonine and protein tyrosine phosphatase genes in *Homo sapiens*, *Arabidopsis thaliana*, *Saccharomyces cerevisiae*, *Neurospora crassa* and *Aspergillus nidulans*

Serine/threonine (S/T) protein phosphatase genes					
Family	***Homo sapiens*[1]**	***Arabidopsis thaliana*[1,2]**	***S. cerevisiae*[1,3]**	***Neurospora crassa***	***Aspergillus nidulans*[5]**
PPP	13	26	11	8	8
PPM	18	76	7	5	6
Asp-based	13	23	3	3	3
Total	44	125	21	16	17
Protein tyrosine phosphatase genes (PTPs)					
Classical	38	1	4	2	2
Dual-specificity	61	22	6	6	5
LMW-PTP	1	1	1	1	1
CDC25	3	-	2	2	1
SSU72	1	1	1	1	1
Y-phosphatase	-	-	-	2	1
Total	104	25	14	14	11

References- [1]Moorhead et al., 2007, [2]Kerk et al., 2008; [3]Breitkreutz et al., 2010; [4]Moorhead et al., 2009; [5]Son and Osmani, 2009.

Table 2.3: Mutants with chemical sensitivity phenotypes

NCU #	FGSC #	Deleted Gene	Sodium Chloride	Sorbitol	Cytochalasin A	Benomyl	Tert-butyl hydroperoxide	Menadione	FK506	Fludioxonil	Yeast Extract
06563	11546	*pp2a*	S	S	R		S	S		R	
07489	11548	*pzl-1*	R	R	R		S	S	R		S
08301	12454	*pph-4*			R		S	S			S
06630	17800	*pph-1*			R						
01433	15790	*ppt-1*					R				
00958	19378	*pph-7*								S	
01767	12451	*pph-5*					R				
03495	16430	*pph-6*					S		S		
08380	20306	*csp-6*	S		R		S		R	S	
02257	16060	*pty-2*					R		R		
05364	12444	*pty-3*		S							
03246	13311	*cdc-14*		R	R				R		
03426	16425	*dsp-1*					R				
06252	14464	*dsp-2*		S			S				
06330	15781	*dsp-3*									
08158	19644	*dsp-4*									
09841	18801	*pty-4*						R	R		
02496	16654	*div-12*								R	
06966	14056	*pty-1*									
03114	16337	*pph-10*			R						
01010	16679	*pty-5*									
0333	17653	*pty-6*							R		

[a]Mutants were classified as sensitive (S) or resistant (R) relative to the growth of wild-type. One-way Anova analysis was carried out to determine significance. The above results reflect strains displaying chemical phenotypes at $p<0.05$. Radial colony growth was measured and percentage growth was calculated as growth with chemical vs. growth without chemical. See Materials and Methods for details.

Table 2.4. Mutants with altered growth on 2% Avicel

Strains	Sucrose (mm/day)[a]	Avicel (mm/day)[b]	% Growth[c]	SD[d]
Wild-type (*mat a*)	33.6	18.2	54%	0.106
Δ*pph-4*	18	14	78%	0.114
Δ*dsp-2*	29.5	22.83	77%	0.100

[a]Radial growth of strains on minimal medium containing sucrose.
[b]Radial growth of strains on minimal medium containing 2% Avicel.
[c]% growth = (Radial growth on Avicel)/(Radial growth on sucrose) X 100
[d]Standard deviation for three replicates.

Chapter III

Regulation of Cross Pathway Control via the RACK1 homolog CPC-2 is coupled to the sumoylation of the bZIP transcription factor CPC-1

Abstract

Cross pathway control (also called general amino acid control) is a global response mechanism by which eukaryotic cells respond to amino acid starvation. In *Neurospora crassa*, depletion of one amino acid can lead to upregulation of numerous enzymes for other amino acid biosynthetic pathways. Increased expression of the bZIP transcription factor CPC-1 (Cross Pathway Control-1) is integral for transcriptional induction of amino acid biosynthetic genes, such as *arg-3, trp-3* and *his-3,* under starvation conditions. The WD40 protein/RACK1 homolog *cross pathway control-2* (*cpc-2*) is an important component of the cross pathway network and is also required for normal female fertility and negative regulation of submerged culture conidiation in *N. crassa*. Deletion of *cpc-2* or *cpc-1* leads to sensitivity to growth on media containing 3-amino,1,2,4 triazole (responsible for histidine starvation) as well as reduced mRNA levels for amino acid biosynthetic genes under histidine starvation. In contrast, mRNA levels for these genes are elevated in both knockout mutants under non-starvation conditions. Additionally protein levels for CPC-1 were highly decreased in Δ*cpc-2.* We provide evidence that CPC-1 is post-translationally modified via SUMOylation and that normal expression of CPC-2 is integral to this modification. CPC-2-mediated regulation of CPC-1 is also an eIF2α phosphorylation-independent process as low levels of eIF2α phosphorylation in Δ*cpc-2* do not affect CPC-1 polysomal RNA abundance with or without 3-AT stress.

Introduction

Amino acid availability is crucial for cellular homeostasis and proper sensing of amino acid levels in the environment allows eukaryotic cells to appropriately regulate the expression of downstream amino acid biosynthetic genes. The process regulating the response to starvation of amino acids is called cross pathway control in *Neurospora crassa* or general amino acid control in *S. cerevisiae* and is associated with the integrated stress response in mammals (1-3). As such this phenomenon is highly conserved across several species ranging from fungi, mammals, *Caenorhabditis elegans* as well as *Drosophila melanogaster* (4-6). A convenient method for inducing amino acid starvation in vivo is by using various inhibitors of amino acid biosynthetic enzymes such as 3-amino-1,2,4-triazole (3-AT; targets the histidine pathway), sulfometuron methyl (inhibits Ile, Val and Leu biosynthesis) and 5-methyl tryptophan (5-MT; represses tryptophan biosynthesis) (7-9). Amino acid starvation incited by 3-AT (which is a more commonly used chemical for this purpose) results from competitive inhibition of the *his-3* gene product, the enzyme imidazoleglycerol-phosphate dehydratase, which catalyzes the sixth step of the histidine biosynthetic pathway (10). Cross pathway control or general amino acid control is imparted via an intricate signaling cascade involving several molecules. An important regulator that integrates this response is the bZIP transcription factor CPC-1 (Cross Pathway Control-1) that is regulated throughout the *Neurospora* sexual cycle (11,12). CPC-1 is responsible for transcription of numerous amino acid biosynthetic

genes, including *arg-3, trp-3* and *his-3* (12-14). As a bonafide DNA-binding protein CPC-1 was also shown to be regulate its own expression at the transcriptional level by binding to upstream promoters (15). CPC-1 was found to have DNA binding sites similar to its yeast homolog – Gcn4p on amino acid gene promoters such as *arg-2* and *trp3*. However, the size of the complex was found to be larger than even the CPC-1 homodimer suggesting there are additional unknown proteins that are binding to this region (15). Transcriptional profiling of CPC-1 in *Neurospora* also revealed that CPC-1 is able to regulate 67 amino acid biosynthetic genes including 443 genes that are direct or indirect targets for CPC-1 (13). An important regulator of CPC-1 protein synthesis under amino acid starvation (via 3-AT) is a kinase named – CPC-3, deletion of which completely abolished CPC-1 protein levels under 3-AT conditions (16). However the exact mechanism for this translational control was not elucidated.

Mutation of *cpc* genes in *N. crassa* leads to similar phenotypes, including sensitivity to 3-AT (2,17). Deletion of *cpc-2* (cross pathway control-2) leads to a 3-AT sensitive phenotype and general inability to induce amino acid biosynthetic genes under 3-AT starvation in *N. crassa* (17). CPC-2 is a homolog of the receptor for activated C kinase 1 (RACK1) in mammals, which has been shown to have a multitude of roles from development, cell migration, apoptosis, circadian rhythms as well as brain functions (18,19). Recent work in *S. pombe* has demonstrated that the RACK1 homolog Cpc2 plays a crucial role in translational regulation of Gcn4 through activation of Gcn2 (via autophosphorylation). Cpc2 was shown to be a ribosomal protein, however it is not clear whether Cpc2 plays a role in Gcn4 loading on polysomes or if it affects Gcn2 kinase

activity (20). Studies in *S. cerevisiae* had shown that deletion of the RACK1 homolog *ASC1* led to increased Gcn4p-mediated transcription of amino acid biosynthetic genes under non-starvation conditions (21), presumably via destabilization of translation initiation complexes on ribosomes. This study also showed that *ASC1* is a negative regulator of general amino acid control in *S. cerevisiae.* Previous work in *S. cerevisiae* has shown that the CPC-1 homolog Gcn4p is regulated by a complex system of upstream ORFs, accumulation of uncharged tRNAs (upon amino acid starvation), the eukaryotic initiation factor eIF2α and its corresponding kinase homolog Gcn2p [Reviewed in (1,22)]. Transcriptional regulation of Gcn4p is also achieved through a feedback loop in which Gcn4p is able to bind to its own promoter elements and regulate its own transcription (22). Dysregulation of Gcn4p either by deletion of a repressor or overexpression of an activator (such as an eIF2α kinase Gcn2p) leads to a general control derepressed (GCD) phenotype, while overexpression or activation of a negative regulator such as *GCD1* (an eIF2B catalytic complex subunit) leads to a general control non-derepressible (GCN) phenotype (13). This is best exhibited by Gcn- mutations which confer sensitivity to multiple inhibitors of amino acid biosynthetic enzymes, such as 3-AT, 5-MT or sulfometuron methyl. Past studies have confirmed that Gcn4p in *S. cerevisiae* is turned over via the 26S proteasome pathway and that Gcn4p becomes ubiquitinated during this process (23-25). Pulse-chase studies have also shown that in absence of amino acid starvation, Gcn4p proteolysis begins within 5-10 minutes (steady-state half-life of the protein) (23). The same study also showed that amino acid starvation was able to stabilize Gcn4p independently of Gcn2p (the eIF2α kinase) function in *S.*

cerevisiae as the protein was stable in a *gcn2* knockout under amino acid starvation.

In this study, we have analyzed the role of CPC-2 in regulation of cross pathway control via regulation of the bZIP transcription factor. Phenotypic and chemical sensitivity analyses for the cross pathway mutants – *cpc-2* and *cpc-1* are also presented as well as the effect of these gene deletions on amino acid biosynthetic genes – *arg-3, trp-3* and *his-3*. Our results reveal a novel mode of regulation of CPC-1 via CPC-2 by modulation of post-translational modifications on CPC-1.

Materials and Methods

Strains and culture conditions

Vogel's minimal medium [VM; (26)] containing proline as the sole nitrogen source was used for selection using phosphinothricin as described (27). Synthetic crossing medium [SCM; (28)] was used to facilitate development of female reproductive structures for sexual crosses. Where indicated, media were supplemented with 10 μg/ml panthothenate 200 μg/ml hygromycin (Calbiochem; San Diego, CA) 400 μg/ml phosphinothricin (extracted from Finale, Farnam Companies INC.; Phoenix, AZ) or 50 μg/ml Nourseothricin (clonNAT; Werner Bioagents; Germany) (29,30). Sequencing of plasmid constructs was carried out at the Genomics Core Facility, Institute for Integrative Genome Biology, University of California, Riverside. For propagation of plasmids, the DH5α strain (*E. coli*) was used and bacterial transformations were carried out via electroporation (31). *N. crassa* was transformed using electroporation as described (32).

Wild type strain strain 74-OR23-IVA (FGSC 2489, *mat* A) and the *cross pathway control* mutants used in this study are listed in Supplemental Table 1. The Δ*cpc-2* mutant was constructed as part of the *Neurospora* genome project (33), while Δ*cpc-1* (FGSC 9011) was a gift from Dr. Michael Plamann (University of Missouri, Kansas City). The Δ*cpc-1* mutant contains a deletion extending from the *Sph*1 restriction site located in an intron 8 bp downstream of the ATG start site (structural gene) to a *Kpn*1 restriction site located approximately 645 bp downstream of the termination codon for the *cpc-1* structural gene (11). This region has been replaced with a *trpC-hph* cassette. The upstream open reading frames for *cpc-1* were not altered in this deletion strategy. The Δ*cpc-3* (FGSC 17934) mutant was obtained from FGSC and has been previously analyzed for phenotypes (34). For construction of a Δ*cpc-2* Δ*cpc-1* double mutant, the Δ*cpc-1* strain was used as female and crossed to the Δ*cpc-2* male (35). Sorbose-containing medium (FGS) was used for germination of ascospores resulting from the sexual crosses as described before (34).

The CPC-2-V5-GFP strain was produced by transformation of a Δ*rid-1* Δ*mus-51* strain (27) with a construct containing the pCCG-1 promoter sequence upstream of the CPC-2 open reading frame (951 bps), followed by in-frame V5 epitope tag and GFP and the *bar* selectable marker at the 3' end (27). This segment (pCCG-1-CPC-2-V5-GFP-bar) was inserted between sequence corresponding to 1 kb 5' and 3 flanking regions of the *panthothenate-2* (*pan-2*) gene (27). Transformants were selected on medium containing phosphinothricin and screened for the presence of the inserted DNA at the *pan-2* locus using PCR (listed primers in appendix table 3.3). Positive strains were crossed to wild

type *mat* A (FGSC 2489) and ascospores plated on medium containing phosphinothricin and pantothenate. Progeny were screened for panthothenate auxotrophy and the presence of the construct. A similar methodology was used for construction of a strain expressing CPC-1 tagged with V5 strain (lacking the GFP fragment) (Appendix table 3.1).

Conidia used to inoculate cultures were propagated on VM agar flasks and grown for 3 d at 30° C in the dark followed by 4 d at 25°C in the light. For analysis of basal vegetative growth, conidia were inoculated on VM agar plates and then incubated for 24 hours in the dark at 30°C. To test for sensitivity to 3-aminotriazole (3-AT; Cat#A8056, Sigma, St. Louis, MO), VM solid medium was supplemented with 0, 0.5, 1, 2 and 4 mM 3-AT and plates incubated for 24 hours in the dark at 30°C. The height of aerial hyphae was determined in cultures prepared by inoculating 3 ml standing liquid VM medium in 13x100 mm glass tubes with conidia, followed by incubation at 25°C in the dark for 96 hours. For assessment of sexual development phenotypes, the cross pathway control mutant strains and wild type were cultivated on SCM agar plates for 7 days in constant light at 25°C. For propagation of vegetative hyphae in liquid cultures, flasks containing 25 ml of liquid VM were inoculated with conidia to reach a final concentration of $1x10^6$ conidia/ml and were grown at 30°C in the dark at 200 rpm for 16 hours. Hyphae were collected from liquid cultures as previously described (36) and then flash frozen in liquid nitrogen.

RNA isolation and Northern blot analyses

Total RNA was isolated from 16 h liquid cultures (propagated as described above) using TRIzol Reagent as per manufacturer's instructions (Cat# 15596-026, Invitrogen, Carlsbad, CA). Approximately 10 μg of the isolated RNA was subjected to gel electrophoreses in a MOPS [3-(*N*-morpholino)propanesulfonic acid] gel system. The MOPS gel was subjected to overnight transfer onto a nitrocellulose membrane (as previously described (37,38)] in 20x SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) buffer. Northern analyses were carried out as previously described with modifications (38) using a 5x labeling buffer (PR-U1151, Promega, Madison, WI) and γP^{32}-dCTP as the radiolabeled isotope (NEG013H250UC, Perkin Elmer, Waltham, MA). The primers used to amplify genomic DNA regions used for probes are listed in Appendix table 3.2. The amplicons were cloned using the pGEM-T Easy Vector system (Promega, Madison, WI) and sequenced to confirm the identity of the insert. A stripping buffer (0.1 SSC, 0.5% SDS; (37,38)] was used to remove residual probe from the membrane before treatment with another probe.

Preparation of whole cell protein lysates, western analysis and SUMO protease treatment

For preparation of whole cell lysates to conduct immunoblot analysis, tissue pads collected from 16 h cultures were ground in liquid nitrogen using glass rods, re-suspended in extraction buffer [50 mM TrisCl pH 7.5, 1 mM EDTA, 6 mM $MgCl_2$, 0.1% FPIC (Product #T8215; Sigma-Aldrich, St. Louis, MO) and 2.5 mM PMSF] and mixed vigorously using a vortex mixer for 5 min. For eIF2α phosphorylation assays, the extraction buffer was brought to 1 mM sodium orthovanadate ($NaVO_4$) and 1 mM sodium fluoride (NaF). The samples were spun at 1000xg for 15 min at 4°C and the resulting supernatant collected. Protein concentration was assessed from the resulting whole cell lysate supernatants using the Bradford protein assay (Bio-Rad, Irvine, CA). A volume of the extract containing 50 μg of protein was separated by SDS-PAGE (polyacrylamide gel electrophoreses) and subsequent western analysis was carried out as previously described (37). For CPC-1 western blots, samples were separated using a 7.5% SDS PAGE gel, while 10% SDS PAGE gels were used for all other western analysis. Antibodies were used at the following concentrations and conditions: CPC-1 (rabbit polyclonal; gift from Dr. Michael Plamann and Dr. Matthew Sachs) was used at 1:2000 dilution, with overnight incubation shaking at 4°C. Phospho-eIF2α (rabbit polyclonal Cat# 1090-1, AbCam, Cambridge, UK) was used at a 1:2000 dilution, with overnight incubation at 4°C. Hog-1 (rabbit polyclonal, Cat# sc-6815, Santa Cruz Biotechnology, Santa Cruz, CA) was used at a 1:2000 dilution with overnight incubation at 4°C. A GFP antibody (rabbit polyclonal, Cat#A11122 Life Technologies, Carlsbad,

CA) was used at 1:5000 dilution with overnight incubation at 4°C. RPL6 was used at 1:1000 dilution (rabbit polyclonal; Cat# A303-586-T, Bethyl laboratories, Montgomery, TX) with overnight incubation at 4°C. The antibodies against phospho-eIF2α and GFP were incubated in Tris-buffered saline with Tween-20 (1X TBST; 50 mM Tris pH 7.6, 150 mM, 0.1% Tween 20) containing 5% BSA, while the CPC-1, Hog-1 and RPL6 antibodies were incubated in 1X TBST containing 5% milk. After incubation in the primary antibody, membranes were washed 3 times using 1X TBST and then incubated with goat anti-rabbit IgG secondary antibody (H+L)-HRP conjugate (cat. # 170-6515, Biorad, Hercules, CA) at a 1:2000 dilution for 2 h at room temperature. Membranes were then washed again 3 times using TBST, followed by chemiluminescence detection as previously described (37).

In order to determine whether the higher molecular species (50 kDa) detected during CPC-1 western analysis is a SUMOylated form of CPC-1, protein extracts were treated with the SUMO specific protease Ulp1 (39). Whole cell protein lysates isolated as described above were treated with 5 units of recombinant Ulp1 (Enzymax, LLC, Cat#84, Lexington, KY) for 2 hours at 37°C. After incubation, the samples were subjected to SDS PAGE electrophoresis and western analysis using CPC-1 antibody as described above.

Polysome profiling and RNA extraction

Isolation of polysomes was carried out following a previously established methodology (40) with certain modifications. Sucrose density gradients were prepared as 5 ml of each of the four sucrose concentrations - 15, 30, 45 and 60% (final) of 2M high-purity sucrose and made up to volume of 5 ml with 10x Sucrose salts (0.4 M Tris-HCl pH8.4, 0.2 M KCl, 0.1 M $MgCl_2$) 50 µg/ml cycloheximide and stored at – 80°C. Gradients were stored for no more than 3 months. On the day of use, the gradients were thawed at 37°C for 1 h and then cooled to 4°C for 1-2 h. Conidia from the strains used for polysome analyses were grown on VM agar flasks for a total of 7 d as described above, and then harvested for inoculation into 200 ml of liquid VM in (500 ml) Erlenmeyer flasks at $1x10^6$ conidia/ml. Hyphal tissue was cultivated and collected as indicated above, ground in liquid nitrogen using mortar and pestle and then homogenized on ice using a Dounce glass homogenizer in a polysome extraction buffer (Final concentrations – 0.2 M Tris, pH 9.0, 0.2 M KCl, 2, 0.025 M EGTA, 0.035 M $MgCl_2$, 1% detergent mix, 1% Sodium deoxycholate, 1% polyoxyethylene 10 tridecyl ether, 5mM DTT, 1mM PMSF, 50 µg/ml cycloheximide, 2 mM vanadyl ribonucleoside complex and 10 units/ml RNasin ribonuclease inhibitor). Homogenized samples were spun at 1000xg at 4°C for 15 min. The resulting supernatant was centrifuged again at 16,000xg at 4°C for 15 min to remove any remaining cellular debris. Equal amounts (A260 units ~ 800 ng/ml) of the cell lysate from the spin was loaded onto the sucrose gradient and centrifuged at 50,000 rpm for 1.5 h at 4°C. The gradients were fractionated into twelve 400 µl aliquots using a gradient

fractionator (185? ISCO, Lincoln, NE) connected to an ISCO UA-5 UV detector. RNA was extracted from fractions using the TRIzol LS reagent (company, city, state) according to the manufacturer's instructions. Samples containing 10 µl by volume of RNA were subjected to Northern analysis as described above and subsequently probed for *cpc-1* and *arg-2*.

Protein purification from polysome samples

Samples from polysome profiling experiments were pooled according to the banding pattern for the 18S rRNA (Figure 2.4A,B): fractions 1-3 (Free RNA), fractions 4-7 (Monosomes) and fractions 8-12 (Polysomes). Pooled samples were brought to a final concentration of 20% TCA (trichloroacetic acid) and incubated overnight at 4° C. Samples were centrifuged at 12,000xg for 30 min at 4°C, the resulting pellet was washed with 10% TCA and centrifuged again at 12,000xg for 15 min at 4°C. To remove residual TCA, 500 µl of acetone was added to the pellet, followed by incubation at –20° C for one hour and centrifugation at 12,000xg for 15 min at 4°C. The acetone wash was repeated once more without the additional incubation at 4°C. After removal of acetone, samples were air dried for 10 min and then re-suspended in 5x Laemelli SDS sample loading buffer (41) and stored at –80°C. Equal volumes of each of the free, monosome and polysome samples were run on SDS-PAGE gels for western analysis using GFP or RPL6 antiserum, as described above.

Nuclei Isolation

Preparation of total cell lysates for nuclei isolation via a sucrose step gradient centrifugation was carried out as described in a previous study (42) with minor modifications. Conidia propagated as described above were used to inoculate 200 ml liquid VM cultures to a final concentration of $1x10^6$ conidia/ml. Cultures were incubated for 12 h at 30°C with shaking at 200 rpm. Hyphae were collected as described above and tissue was flash frozen in liquid nitrogen. The cell pads were ground using a mortar and pestle and homogenized in 10 ml of Buffer A (1 M sorbitol, 7% w/v ficoll, 20% v/v glycerol, 5 mM $MgCl_2$, 5 mM EGTA, 3 mM $CaCl_2$, 3 mM DTT, 50 mM Tris-HCl pH 7.5) using a Bead Beater (Biospec Products, Bartlesville, OK). The chamber was kept at 4°C by using an ice water jacket. The resulting crude extract was filtered through cheesecloth, while maintaining a constant addition of ~ 10 ml of Buffer B (10% v/v glycerol, 5 mM $MgCl_2$, 5 mM EGTA, 25 mM Tris-HCl pH 7.5). The diluted homogenate was layered over a 15 ml solution containing a 1:1.7 mixture of buffer A:B in 50 ml centrifuge tubes and centrifuged at 3000xg for 7 min at 4°C. This resulted in removal of cell debris to the pellet. The supernatant was withdrawn (aliquot was saved as the whole cell extract fraction) and layered on a 5 ml step gradient (1 M sucrose, 10% v/v glycerol, 5mM $MgCl_2$, 1 mM DTT, 25 mM TrisHCl, pH 7.5) and centrifuged at 9400xg for 15 min at 4°C. The supernatant from the previous spin was collected and aliquots stored at –70° C as the cytoplasmic fraction. The final pellet is the nuclear fraction/pellet and was resuspended in 500 µl of ice-cold storage buffer (25% v/v glycerol, 5 mM $MgCl_2$, 3mM

DTT, 0.1 mM EDTA, 25 mM TrisHCl, pH 7.5) and was stored at -70°C. All of the buffers used in the isolation procedure also contained 0.1% FPIC and 50 μM PMSF.

Microscopy

For investigation of possible sexual developmental defects, strains were inoculated onto SCM plates and incubated under constant light for 7 d at 25°C (Fig. 2.1C). Protoperithecia were visualized using a SZX9 stereomicroscope (Olympus America, Center Valley, PA) and images collected with a C-4040 digital camera (Olympus America) at 7 d after inoculation. For visualization of CPC-2-GFP localization, conidia from the CPC-2-GFP strain were isolated as described (43) and a final concentration of $8x10^6$ conidia/ml was spread onto 100 mm VM agarose plates containing 10 ml of medium with or without 6 mM 3-AT supplementation. The plates were incubated at 30°C for 0 and 6 h and a slice of agar containing germinating conidia was layered onto a glass slide for imaging. The germinating conidia were visualized using differential interference microscopy on an Olympus IX71 inverted microscope (Olympus America) with a 60X oil immersion objective. For visualization of GFP fluorescence, the GFP laser was used for excitation at 400 nm. Images were captured using a QIClickTM digital CCD camera (QImaging Surrey, British Columbia, Canada).

Results

Cross pathway control genes *cpc-2* and *cpc-1* are required for normal growth under 3-AT stress and non-starvation conditions

N. crassa grows vegetatively in a haploid state via extension of basal hyphae (44). Asexual growth begins with polarized germination of an asexual spore (conidium) that continues to elongate and then branch to form the multicellular structure called the mycelium (45). A variety of environmental changes or stressors such as heat, high oxygen or nutrient deprivation stimulate, the asexual sporulation (macroconidiation) pathway, which begins with differentiation of aerial hyphae (44,45). Conidiophores bud from the tips of aerial hyphae and later give rise to the mature spores, macroconidia or conidia (44). It is well known that under nitrogen starvation conditions, *N. crassa* will enter into the sexual phase of development leading to formation of female reproductive structures known as protoperithecia (44,45). Attraction of the female hypha or trichogyne towards a male conidium (of the opposite mating type) is regulated via chemotropism and eventually leads to cellular and nuclear fusion, meiosis and formation of the perithecium. These perithecia now contain the meiotic progeny known as ascospores, which germinate to produce hyphae under the appropriate environmental conditions.

To begin our investigation of *cross pathway control*, we analyzed Δ*cpc-2* and Δ*cpc-1* single mutants and a Δ*cpc-2* Δ*cpc-1* double mutant (Appendix Table 2.1) for

basal hyphal growth, aerial hyphae height and female fertility. Deletion of *cpc-2* or *cpc-1* led to a decrease in apical extension rates on minimal medium. Of the three mutants, Δ*cpc-1* and Δ*cpc-2* Δ*cpc-1* showed the slowest growth phenotype, with ~16% of wild type, while Δ*cpc-2* was ~50% of wild type (Figure 3.1B; no 3-AT addition). Deletion of *cpc-2* led to a failure to produce protoperithecia and complete female sterility (Figure 2.1C) and inappropriate formation of conidia in liquid submerged cultures (Appendix Table 2.2). In contrast, the Δ*cpc-1* mutant was female-fertile (but produced fewer protoperithecia than wild type) and did not exhibit submerged culture conidiation. Similar to Δ*cpc-2*, the Δ*cpc-2* Δ*cpc-1* double mutant failed to produce protoperithecia and displayed submerged culture conidiation, suggesting that Δ*cpc-2* is epistatic to Δ*cpc-1* for these traits. In terms of aerial hyphae height, Δ*cpc-1* and Δ*cpc-2* Δ*cpc-1* were at ~40% of wild type, while Δ*cpc-2* showed marginally improved aerial hyphae height at ~50% of wild type.

One of the important functions for CPC-1 is regulation of amino acid biosynthetic genes under amino acid starvation conditions (13). An efficient method for stimulating amino acid starvation under laboratory conditions is to treat cells with the histidine amino acid analogue 3-aminotriazole (3-AT), a competitive inhibitor of the product of the histidine biosynthetic gene, *his-3* gene. *his-3* encodes imidazoleglycerol-phosphate dehydratase, an enzyme catalyzing the sixth step of histidine production (10). Treatment of cells with 3-AT has been shown to lead to derepression of the *his-3* gene (46,47). When the *cpc* mutant strains were grown on 3- AT supplemented media, Δ*cpc-1* was arrested at 1 mM 3-AT, while growth of the Δ*cpc-2* mutant was 80% of wild type (Figure

1B). In contrast, the Δ*cpc-2* Δ*cpc-1* double mutant was the slowest growing of the three strains, with no growth at 1 mM and 70% wild type growth at 0.5 mM 3-AT compared to ~55% for both single mutants at the same 3-AT concentration. This suggests that *cpc-2* and *cpc-1* are required for growth under 3-AT stress and that *cpc-1* may be epistatic to *cpc-2* towards assisting in overcoming the detrimental amino acid starvation conditions.

It is known that 3-AT can lead to oxidative stress responses and changes in catalase activity (48). Growth on medium containing both 3-AT and L-histidine restored growth for wild type, Δ*cpc-1,* Δ*cpc-2* and Δ*cpc-2* Δ*cpc-1* to levels comparable to growth on minimal medium (data not shown). This suggests that the growth defects observed on 3-AT media were due to histidine starvation. Additionally, apical extension of the Δ*cpc-2* mutant was also tested on medium containing sodium chloride, sorbitol and cell wall stressors, such as caspofungin and SDS. No significant difference in growth was observed for these chemicals relative to wild type (Appendix figure 3.1). This suggests specialization of CPC-2 functions in the response to amino acid starvation, female fertility and submerged culture conidiation.

Deletion of *cpc-2* and *cpc-1* leads to defects in derepression of amino acid biosynthetic genes under amino acid starvation

Northern analysis was used to observe transcriptional changes in amino acid biosynthetic genes after treatment of wild type and *cpc* knockout mutants with the amino acid analogue 3-AT. Treatment of submerged (shaking) liquid VM cultures with 3-AT was carried out as described in the Materials and Methods. Consistent with previous studies (13), we observed that when wild type cells were treated with 3-AT, there was an increase in *his-3*, *arg-3* and *trp-3* mRNA levels (Figure 2A). In contrast, in both Δ*cpc-2* and Δ*cpc-1* knockout strains, *his-3, arg-3* and *trp-3* transcript levels were elevated even under non-starvation conditions. In Δ*cpc-2,* the steady-state levels of *arg-3* transcripts showed a ~50% increase over wild type, while *his-3* and *trp-3* were elevated by ~30% fold compared to wild type. Similarly, in Δ*cpc-1,* the basal levels of *arg-3* transcripts showed a ~75% fold increase and a ~20% fold increase for *his-3* and *trp-3*. However, under 3-AT mediated starvation conditions, both mutants were unable to upregulate biosynthesis of these genes as compared to wild type (Figure 3.2A). In fact in Δ*cpc-1, arg-3* mRNA levels were actually reduced (~40%) in induced compared to basal levels. Taken together, these results suggest that two separate events are occurring under non-starvation and starvation conditions. In the single mutants, the cellular conditions may mimic starvation due to loss of a critical component of the *cpc* machinery. It is possible that this leads to upregulation of a yet unidentified transcription factor, which initiates derepression of the amino acid biosynthetic genes. This hypothesis is further supported by our observations from Northern analysis for *arg-3, his-3* and *trp-3* in the Δ*cpc-2* Δ*cpc-*

1 double mutant. Loss of both *cpc-2* and *cpc-1* leads to normal uninduced levels of the amino acid biosynthetic genes (as compared to wild type). After treatment with 3-AT, the double mutant failed to de-repress those genes to wild type levels, similar to the single mutants (Figure 2A). However there was a slight increase in the induced levels (for the three amino acid genes) compared to the basal levels in the double mutant. This increase seems to be negligible enough in the sense that it does not assist in counteraction of the prevailing 3-AT stress in the double mutant.

I next wanted to determine whether *cpc-1* and *cpc-2* themselves show transcriptional changes in response to histidine starvation. A previous microarray study by Tian *et al,* 2007 (13), showed that *cpc*-1 exhibits a 3.4 fold increase and *cpc-2* a 2.5 fold decrease in transcription after 3-AT treatment. Previous studies have also shown that levels of the *cpc-1* transcript is increased several fold upon 3-AT mediated histidine starvation (11,15,49). The results from my Northern analysis showed that *cpc-1* mRNA levels were elevated (~120% increase) when wild type cells were treated with 3-AT. In contrast, the *cpc-2* transcript did not exhibit a significant decrease in wild type cells (Figure 3.2A, B). Although the trend in the changes in *cpc-1* and *cpc-2* transcription post 3-AT treatment are similar, the apparent disparity in fold changes between our results and those of Tian *et al* (Appendix figure 2.2) can be explained by systematic differences between microarray and Northern analyses Even though *cpc-2* transcripts were down regulated by 3-AT exposure in the Tian *et al.* study, it is not immediately clear that CPC-2 can be regarded as a negative regulator in this process, especially considering that the Δ*cpc-2 N. crassa* strain is sensitive to 3-AT treatment.

Levels of *cpc-1* transcripts were elevated (~75% increase) relative to wild type in the Δ*cpc-2* mutant without 3-AT treatment, but did not increase beyond these levels after exposure to 3-AT (Figure 3.2A, B). In contrast, when we examined the status of *cpc-2* transcripts in the Δ*cpc-1* mutant, we found that *cpc-2* transcription is elevated by 100% under steady state conditions (absence of 3-AT mediated histidine starvation) (Figure 3.2) compared to wild type. After 3-AT treatment there was no significant change in levels of *cpc-2* in the Δ*cpc-1* mutant. This suggests that *cpc-1* may negatively regulate *cpc-2* transcription, but that the increased levels of *cpc-2* mRNA cannot compensate for sensitivity to 3-AT in Δ*cpc-1* strains. Hence, any secondary transcription factors that may act as co-regulators for CPC-1-mediated 3-AT resistance are not sufficient to counter the 3-AT stress on their own. From these results we were still asking the question as to why is Δ*cpc-2* sensitive to 3-AT treatment even though the mRNA levels for *cpc-1* were sufficient similar compared to wild type *cpc-1* levels (under 3-AT stress). This led us towards investigating about the CPC-1 protein levels in Δ*cpc-2* and whether the regulation of CPC-1 via CPC-2 occurs at a post-transcriptional level.

Deletion of *cpc-2* leads to inadequate CPC-1 protein expression after 3-AT treatment

We utilized a CPC-1 antibody to detect the CPC-1 protein in Δ*cpc-2* strains using western analysis (see Materials and Methods; Figure 3.3A). The sequence of CPC-1 predicts a molecular weight of ~30 kDa, and a species of this size was observed in wild type, but not the Δ*cpc-1* mutant (Figure 3.3A) After 4 h of 3-AT treatment, levels of CPC-1 protein (~30 kDa) were elevated ~ 2.5 fold in wild type. In contrast, treatment with 3-AT failed to induce CPC-1 protein expression in the Δ*cpc-2* strain compared to wild type and the basal levels of CPC-1 in Δ*cpc-2* was also similar to wild type.

Because the levels of CPC-1 were not induced in the Δ*cpc-2* strain after 3-AT treatment, we asked the question whether regulation of CPC-1 via CPC-2 could be occurring at the translational level via regulation of phosphorylation of the initiation factor eIF2α, which is known to positively regulate Gcn4p translation in *S. cerevisiae* (50,51). The *N. crassa* homolog of the eIF2α kinase is CPC-3 (16) and we used a *cpc-3* mutant as a control for eIF2α phosphorylation. For these experiments, we took advantage of a commercially available antibody for phospho-eIF2α that recognizes a protein the predicted size of eIF2α in wild type, but not the Δ*cpc-3* mutant in *N. crassa* during western analysis (~39 kDa; Figure 3.3C). Using this antibody, I observed that levels of eIF2α-Pi were down-regulated in the Δ*cpc-2* mutant, but were normal in Δ*cpc-1* compared to wild type (Figure 3.3C). This suggests that translation of CPC-1 might be affected, and the level of translation (Figure 3.3C). The strain expressing CPC-2-GFP did

not have any adverse effect on eIF2α phosphorylation, suggesting that overexpression of CPC-2 does not directly influence the process (Figure 3.3C,D). To further test whether CPC-2 exerts translational control over *cpc-1* mRNA we moved onto analysis of *cpc-1* polysomal RNA from Δ*cpc-2* extracts.

CPC-2 regulates CPC-1 independent of eIF2α phosphorylation

As mentioned above, RACK1 homologs have been demonstrated to be ribosomal proteins in several organisms, including humans, *S. cerevisiae*, *Cryptococcus neoformas* (52-54). To determine whether CPC-2 is a ribosomal protein *N. crassa*, we produced a strain expressing a GFP-tagged version of CPC-2 and conducted polysome analyses followed by TCA-acetone protein precipitation from the free (F), monosomal (M) and polysomal (P) fractions after sucrose density centrifugation and gradient fractionation (Figure 3.4A). Western blot analysis using GFP antibody demonstrated that CPC-2-GFP was present in the polysome fraction, as well as free and monosomal fractions. RACK1 and Asc1p have been known to be associated with extra-ribosomal functions as well as ribosomal ones and does exist in a ribosome free state as well (55,56). It is likely that we are seeing a similar effect via detection of a major quantity of CPC-2 in the free fractions as well. In contrast in the control strain (empty vector), the free GFP signal was weak in all fractions. Using a commercial antibody, we detected the 60S ribosomal protein RPL6 (NCU02707) only in the CPC-2-GFP monosomal and polysomal fractions, while no signal was detected in the control strain polysome fractions.

This indicated that a significant fraction of CPC-2 does co-migrate with the ribosome, similar to RACK1 in other organisms.

Next we asked whether the ribosomal association of CPC-2 is in fact responsible for decreased CPC-1 protein production via a decrease in *cpc-1* polysomal RNA abundance, which would also correlate with the low levels of eIF2α-Pi. However, when we analyzed the levels of *cpc-1* mRNA in the polysomal fractions of Δ*cpc-2* using northern analysis, we noted that there was no significant difference between *cpc-1* mRNA levels in wild type polysomes versus those from Δ*cpc-2* (Figure 3.3C, top panels). Surprisingly, *cpc-1* mRNA was already loaded on lower and higher molecular weight polysomes in Δ*cpc-2* and this distribution did not change upon 3-AT treatment. This in turn suggests that CPC-1 protein is actually translated in Δ*cpc-2* but is non-functional, thereby resulting in the 3-AT sensitivity defects observed in Δ*cpc-2.* This still left an issue of how and why are the eIF2α-phosphorylation levels severely decreased in Δ*cpc-2* but the CPC-1 protein is still translated to a certain extent. We hypothesized that this might be due to an eIF2α-phosphorylation-independent mode of regulation. Such a process has been demonstrated in other studies reporting translational control during ER stress or ribosome associated stress granule formation during heat shock independent of eIF2α-phosphorylation (57,58). To understand whether such regulation might also exist in *N. crassa* during amino acid starvation in strains deleted for *cpc-2,* we analyzed the levels of another well-studied uORF containing mRNA, *arg-2* (59,60). When we compared the levels of *arg-2* in Δ*cpc-2* against the wild type *arg-2* levels, the mRNA levels were much reduced (~20% decrease) in Δ*cpc-2* after 3-AT treatment (Figure

3.4A,B; middle panels). This suggests that the low levels of eIF2α-phosphorylation in Δ*cpc-2* strains is not specific to *cpc-1* mRNA translation under 3-AT stress but also affects other mRNAs containing uORFs. Hence, although an important regulator of eIF2α-phosphorylation, CPC-2 does not regulate CPC-1 via a CPC-1 uORF regulation/ eIF2α phosphorylation pathway.

CPC-1 is post-translationally modified via SUMOylation after 3-AT treatment

In addition to the predicted 30 kDa form of CPC-1, we also observed a higher molecular weight form of ~50 kDa during western analysis (using denaturing SDS-PAGE gels). This 50 kDa species was also reported in a previous study (16) and is observed in the wild type cells but not Δ*cpc-1*, but the origin of this apparent upshift was not explained. The 50 kDa protein accumulated to significant levels only after 3-AT treatment in wild type cells, but was not sufficiently induced in Δ*cpc-2*. A recent study has revealed that the yeast CPC-1 homolog – Gcn4p is SUMOylated after binding to its target promoters and this post-translational modification assists in clearance from its promoter binding sites (34,61). Small Ubiquitin-like Modifier or SUMO is a family of small proteins which are able to be covalently attached to target proteins leading to modification of their function (62). Post-translational modification via SUMOylation has been shown to involved in determining nuclear-cytoplasmic transport, transcriptional regulation, programmed cell death, responses to stress as well as cell cycle regulation (63). In *N. crassa,* the predicted molecular weight for CPC-1 is 28.2 kDa and that of the SUMO

protein SMT3is 10.9 kDa. Therefore the 50 kDa species that reacts with the CPC-1 antibody may correspond to the native protein covalently bound to two SMT3 proteins to form a SUMO chain. Indeed, SUMO groups have been known to form conjugated chains through a single acceptor lysine (64,65) and the *S. cerevisiae* Smt3p protein contains a predicted type II consensus sequence motif for SUMO conjugation (66,67). Therefore a SUMO-SUMO conjugation on the ~30 kDa CPC-1 explains the higher 50 kDa band detected under 3-AT stress conditions (Figure 3.3A).

As described above, previous studies on Gcn4p regulation have clearly demonstrated that the protein is SUMOylated at Lysines 50, 58 after amino acid starvation and that SUMOylation of the protein is essential for removal of Gcn4 from the promoters (61). Using different prediction software, such as SUMOsp, GPS-SUMO and SUMOplotTM (http://www.abgent.com/sumoplot/) (68,69), we identified a putative SUMOylation site in *N. crassa* CPC-1 at Lysine 227, which was predicted at high confidence levels. Interestingly all the *N. crassa* CPC-1 lysines are at the C-terminus, in contrast to *S. cerevisiae* Gcn4p. In addition, the predicted SUMOylation site for CPC-1 is (K227) within the DNA binding domain, whereas Gcn4p SUMO sites (K50 and K58) are within the activation domain (Figure 3.5A). This might reflect a differential molecular wiring between the two model organisms and thereby a different mode of regulation of the bZIP transcription factor as well as its own function. It has also been shown by Rosonina *et al* that SUMOylation of Gcn4p enhances its promoter clearance and that mutation of the K50 and K58 sites (to Arg) abolished the SUMO-modified forms of Gcn4p (61). However, when we mutated the K227 site to Arg on a CPC-1-V5 plasmid

construct, no CPC-1-V5-K227R homokaryons were recovered from the cross with wild type (data not shown). This suggested that abolishing the SUMO site on CPC-1-V5 might be lethal.

To further assess and confirm the identity of the higher molecular weight band for CPC-1 we carried out an enzyme treatment to remove the SUMO group. The most commonly used enzyme to remove SUMO conjugations from proteins is the endo-peptidase Ulp1 (70). Utilizing whole cell extracts from wild type and a CPC-1-V5 strain (Figure 3.5B) we setup up a digestion reaction with commercially available Ulp1 (as described in Materials and Methods) and then subjected the extracts to western analysis using the CPC-1 antibody (as previously described). The results demonstrated that the 50 kDa reactive species was significantly reduced in the wild type and CPC-1-V5 strains treated with 3-AT after incubation with Ulp1 (Figure 3.5B). This result is consistent with the 50kDa form as SUMOylated CPC-1. The findings support a mechanism in which CPC-1 is modified with SUMO after 3-AT-induced histidine starvation in *N. crassa*.

CPC-1-SUMO is nuclear localized and is dependent on CPC-2 function

In order to further assess the role of CPC-2 in CPC-1 regulation, we asked whether the localization of CPC-1 and/or CPC-1-SUMO levels change in a *cpc-2* mutant background. To address this question, we implemented a nuclear isolation method from wild type and Δ*cpc-2* strains with or without 3-AT treatment. As shown above, wild type cells showed a marked increase in CPC-1-SUMO as well an increase in CPC-1 (lower molecular weight form) after treatment with 3-AT. However, in the cytoplasmic fraction, the putative CPC-1-SUMO species was present at lower levels and most CPC-1 migrated with the unmodified 30 kDa form. Analysis of isolated nuclei showed that CPC-1-SUMO protein is mostly localized in the nuclear fractions of Δ*cpc-2* strains, but barely detectable in wild type cells (Figure 3.6A). This suggests that CPC-1-SUMO is predominantly a nuclear protein and only present in the nucleus after 3-AT-mediated histidine starvation. This result also suggests that CPC-1-SUMO should be the active functional form of CPC-1 that positively regulates expression of the downstream amino acid biosynthetic genes. As evidenced by the whole cell and cytoplasmic extracts (for Δ*cpc-2* after 3-AT exposure) the unmodified CPC-1 protein was ~125% and ~ ~75% down-regulated compared to wild type respectively (Figure 3.6A, 3.6B, 3.6C). Similar to the decrease in overall CPC-1 protein level in Δ*cpc-2,* we observed a reduction in CPC-1-SUMO levels under the same conditions in the nuclear fractions for the Δ*cpc-2* strain (Figure 6A,D), suggesting a critical role for CPC-2 in carrying out post-translational modifications on the nascent CPC-1 peptide. There have been several studies demonstrating that SUMO modifications are carried out in the cytoplasm and that modification of the target protein

also leads to its nuclear translocation (71-73). This led us to ask whether CPC-2 assists in CPC-1-SUMOylation in the nucleus or in the cytoplasm. To answer this question, we wanted to visualize how CPC-2 localizes post 3-AT treatment. Therefore, we constructed a strain that overexpresses GFP-tagged CPC-2 and imaged the strain during a 6 h germination assay (see Materials and Methods).

Prior to treatment with 3-AT, the CPC-2-GFP signal was localized in the cytoplasm and excluded from the nucleus (as represented by DAPI staining) (Figure 3.7). After 6 hours of germination on minimal medium, CPC-2-GFP localization did not change. Similarly, after 3-AT treatment, CPC-2-GFP was completely cytoplasmic and did did not translocate to the nucleus even after 6 hours of germination. Instead, after 3-AT treatment (6 h) we observed greatly increased vacuole-like structures. Overall these results indicate that CPC-2 does not translocate to the nucleus after 3-AT treatment. This suggests that any effect of CPC-1 on SUMOylation of CPC-1 may be carried out in the cytoplasm, possibly in vacuolar or stress granule-like compartments and the modified version of CPC-1 is then trans-located into the nucleus to transcribe amino acid biosynthetic genes. Future studies on the exact role of CPC-2 in CPC-1 SUMOylation should reveal the different components required to carry out this important post-translational modification under 3-AT stress conditions.

Discussion

In *N. crassa,* although a lot of work has been done on characterization of CPC-1 and its targets, no such study has focused on how this important bZIP transcription factor is itself regulated. One important regulator of the cross pathway control network is the scaffolding protein CPC-2 (RACK1 homolog), which thus far has not been shown to exert control over CPC-1. In this study, we have explored the possibility that CPC-2 regulates CPC-1 and have indeed demonstrated a critical role for CPC-2 in CPC-1 regulation via modulation of the post-translational machinery. RACK1 is a major scaffolding protein in mammals and is homologous to the Gβ subunit of heterotrimeric G proteins (74). It has a seven tryptophan-aspartate (WD) repeat structure and is known to bind to Gβ subunits and other WD40 repeat proteins (75). Initially identified as a protein that binds to the active conformation of Protein Kinase C βII (PKC βII), it is now known to be multifunctional (18). For example, RACK1 allows cross talk between PKC and Mitogen Activated Kinase (MAPK) pathways, by acting as a scaffold for Jun N-terminal Kinase (JNK) upon stimulation, leading to PKC-mediated phosphorylation and activation of JNK (76). Specific tyrosine and serine residues in RACK1 are required to stabilize the activity of Protein Phosphatase 2A (PP2A) and RACK1 is able to bind PP2A and β-integrins in a mutually exclusive manner [reviewed in (18). Binding of RACK1 to integrins promotes pro-survival and MAPK pathways (77). As a 40S ribosomal protein, RACK1 is able to regulate translation of various proteins such as PKC.

As a scaffolding protein, RACK1 binds both soluble signaling proteins as well as membrane-bound receptors (78) and is known to shuttle activated PKC to its appropriate

cellular location (79). It is possible that the *N. crassa* homolog of RACK (CPC-2) also demonstrates similar scaffolding capabilities. As evident from our data, deletion of *cpc-2* leads to defective up-regulation of (unmodified) CPC-1 protein upon 3-AT-mediated histidine starvation. This is confirmed by polysome analyses of Δ*cpc-2* revealing that *cpc-1* mRNA is indeed less translated in absence of *cpc-2* as compared to wild type in presence of 3-AT stress. A failure to up-regulate CPC-1 protein even in presence of existent *cpc-1* polysomes in Δ*cpc-2* suggests the lack of translational regulation of CPC-1 protein. Additionally eIF2α-phosphorylation is negatively affected in Δ*cpc-2* suggesting that CPC-2 might regulate the eIF2α kinase – CPC-3/GCN2 which in turn leads to CPC-1 regulation. Another interesting study in yeast has demonstrated that Gcn4 SUMOylation facilitates its clearance from promoters via a protein kinase and mediator complex subunit – Srb10 (61). Although blocking sumoylation caused prolonged association of Gcn4p with promoters, the study could not rule out that SUMOylation, in some way is affecting Gcn4p recruitment to promoters. Interestingly in *N. crassa,* the predicted sumoylation site for CPC-1 (lysine 227) is at the DNA binding domain instead of the dual SUMO sites in yeast (lysine 50, lysine 58) at the activation domain of Gcn4p. This might be indicative of varied/additional modes of regulation of CPC-1 in *Neurospora* as compared to that in yeast. Our results suggest that upon starvation CPC-2 is able to influence CPC-1-SUMOylation, leading to greater nuclear localization of SUMOylated CPC-1 and de-repression of amino acid biosynthetic genes.

Deletion of *cpc-2* leads to defective SUMOylation of CPC-1, as seen from our nuclear isolation experiments. In wild type nuclear fractions, CPC-1 is present solely in

the putative SUMOylated form, while the *cpc-2* mutant nuclear fraction there is severe down-regulation of SUMO-CPC-1, which may not be sufficient to counter the existing 3-AT stress conditions. This suggests that deletion of *cpc-2* does not completely abolish CPC-1 protein synthesis, but rather affects its post-translational status and the process might be occurring in a co-translational fashion as the nascent CPC-1 peptide is coming out of the ribosome exit tunnel. It will be interesting to investigate whether CPC-2 as a ribosomal protein is able to regulate possible co-translational protein (SUMO) modifications on CPC-1 as translation is ongoing or whether the modifications are occurring after the protein is translated, in which case CPC-2 might be regulating CPC-1 SUMOylation in a non-ribosomal state. Also future work should focus on whether any ubiquitination/SUMOylation related proteins are able to form a complex with CPC-2 after 3-AT treatment. Amino acid starvation could trigger CPC-2 interaction with a SUMO E3 ligase and initiate SUMO modification of CPC-1. Indeed, there have been previous reports confirming RACK1 interaction with PIAS2 proteins, which are known to have SUMO E3 ligase activities (80). Binding of PIAS proteins to transcription factors results in the SUMOylation of the factor (81,82). Additionally, SUMO modification has been documented to be associated with nuclear localization of various target proteins, such as zinc finger transcription factors (ZIC3) and the small GTPase RanGAP, intermediate filament protein IFB-1 in *C. elegans* (83-85). SUMOylation of such proteins seems to occur in the cytosol and the proteins are periodically nuclear localized to carry out their designated functions. It is highly likely that a similar process occurs during CPC-1 regulation. We hypothesize that SUMOylation of CPC-1 occurs in

the cytoplasm and is assisted by the scaffolding protein CPC-2 that is able to recruit SUMO E3 ligases in a co-translational manner upon 3-AT treatment. Modified CPC-1 is now able to translocate to the nucleus to facilitate transcription of various amino acid biosynthetic genes (Figure 3.8, Model of CPC-1 regulation via CPC-2).

A previous study had identified a Gcn2p ortholog in *N. crassa* called CPC-3 via restriction mapping and confirmed the translational start site using RT-PCR analysis (16). We have used a *cpc-3* knockout mutant (FGSC 17934) and found that it lacks the ability to phosphorylate eIF2α with or without 3-AT starvation. This confirms that CPC-3 is indeed the eIF2α kinase in *N. crassa.* On the other hand, deletion of *cpc-1* did not affect eIF2α phosphorylation with or without 3-AT, indicating that CPC-1 does not seem have a feedback effect on its own translational machinery, in contrast to the well known transcriptional feedback regulation of the GCN4 promoter via Gcn4p itself (22,46). However microarray studies have found that *cpc-3* is a target for the transcriptional activation via CPC-1 (13). Therefore as *cpc-1* deletion does not affect eIF2α phosphorylation but should affect *cpc-3* (eIF2α kinase) transcription, our hypothesis for an eIF2α phosphorylation independent mechanism of CPC-1 regulated is reinforced. This indicates that the signaling cascade is more complicated and might involved other unidentified components. Recent studies have also illustrated that RACK1 is able to associate with the elongation factor, eEF1A in *Trypanosoma brucei* (86) as well as the multimeric initiation factor eIF3 (which binds to 40S subunit along with eIF1 and eIF1A to complete ternary complex formation) in *S. cereviseae* (87). Also as eIF3 is released from the ribosome after 80S assembly, RACK1 remains associated with the polysomes.

Although there seem to be mechanistic differences between *S. cereviseae* and *N. crassa* in terms of regulation of Gcn4p translation and RACK1 function, it is possible that CPC-2 is also essential for completing the elongation phase of translation and without this important ribosomal protein, CPC-1 translation is stalled at the initiation step. This might be an alternate explanation towards the significant *cpc-1* mRNA signals observed in the *cpc-2* knockout polysomes post 3-AT stress. An inefficient elongation phase could result in mis-folded forms of CPC-1 being generated in the cell and thus leading to activation of the unfolded protein response (UPR). Recent studies in mammalian systems have shown that the UPR does have significant cross-talk with PERK and other eIF2α kinases (which regulate the CPC-1 mammalian homolog – ATF4) and that PERK is a negative influence on deSUMOylation enzymes such as SENP3 in an eIF2α phosphorylation independent manner (88,89).

Another observation that we made from our data was that the CPC-2-GFP protein accumulates in vacuolar-like structures in the cell after 3-AT treatment (Figure 7). This suggests that CPC-2 function might impinge on sequestration of *cpc-2* mRNA to stress granules during 3-AT treatment. On the other hand, it is also possible that CPC-2 protein might be localizing to stress granules to act as a scaffold to recruit various factors involved in CPC-1 regulation. This has been documented in other studies where RACK1 is sequestered into stress granules to inhibit apoptosis via suppression of MAPK pathways (90). A RNA binding protein – Nrd1 (in *S. pombe*) is known to localize to stress-induced RNA granules and bind to Cpc2 in a phosphorylation dependent manner, thereby regulating stress (arsenite/KCl/heat tolerance) resistance in fission yeast (91). It

will be interesting to investigate whether CPC-2 also has similar partners to regulate specific RNA binding proteins in order to activate CPC-1 post-translational modifications.

Overall, in this study we have addressed the role for CPC-2/RACK1 in cross pathway control via regulation of the bZIP transcription factor CPC-1. We have demonstrated a critical role for CPC-2 in post-translational modification of CPC-1 and that cells lacking this scaffolding protein are highly sensitive to 3-AT treatment. Future work detailing a ribosomal or extra-ribosomal role for CPC-2 towards SUMOylation of the nascent CPC-1 peptide should shed additional light on this newly revealed mechanism of action.

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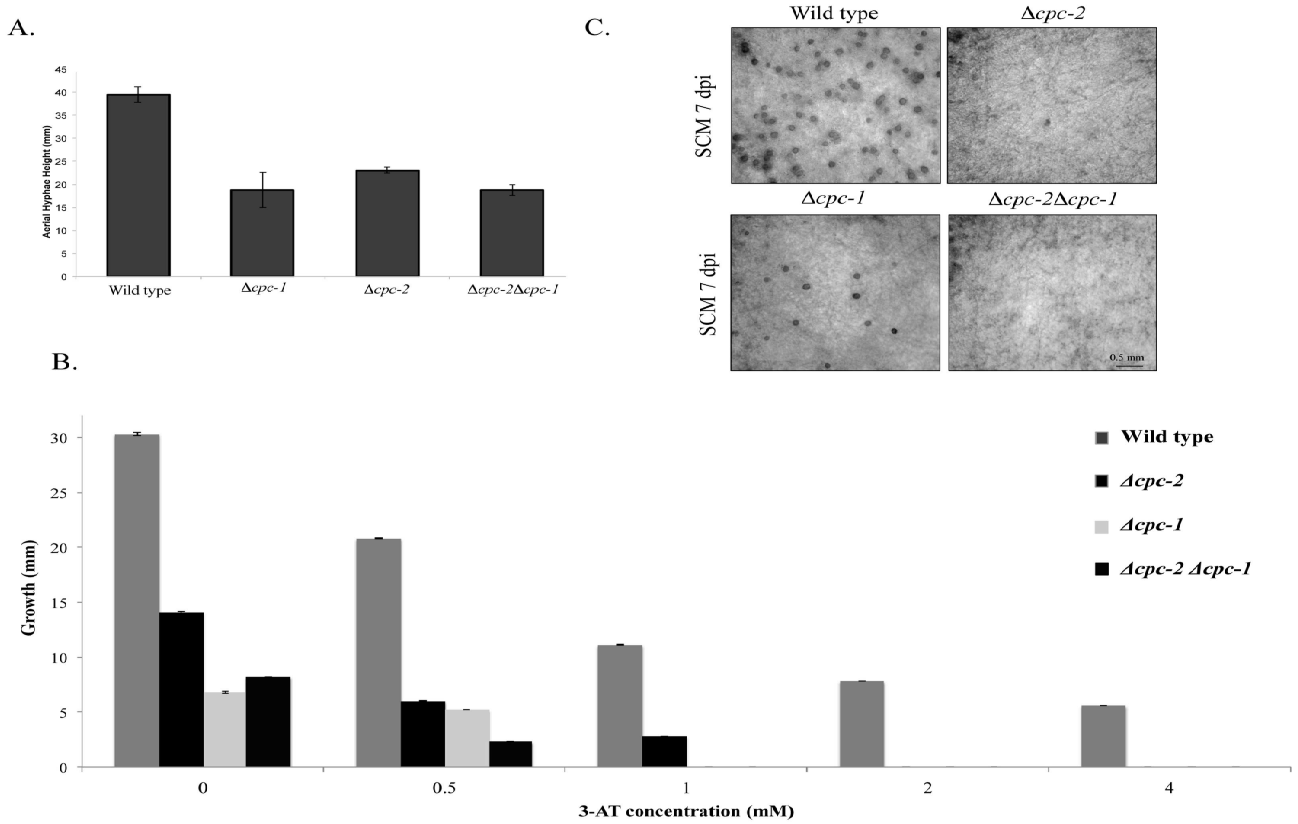

A.
Aerial Hyphae Height (mm)
45
40
35
30
25
20
15
10
5
0
Wild type
Δcpc-1
Δcpc-2
Δcpc-2Δcpc-1
C.
Wild type
Δcpc-2
SCM 7 dpi
Δcpc-1
Δcpc-2Δcpc-1
SCM 7 dpi
0.5 mm
B.
Growth (mm)
30
25
20
15
10
5
0
0
0.5
1
2
4
3-AT concentration (mM)
Wild type
Δcpc-2
Δcpc-1
Δcpc-2 Δcpc-1

Figure 3.1. CPC genes are important for normal growth and development and growth under 3-AT starvation. **A.** Aerial hyphae height for *cpc-2, cpc-1* and the *cpc-2cpc-1* double mutant. All heights are measured in mm. Cultures were grown in the dark at 25° C in 3 ml liquid VM for 3 days. **B.** Sexual development phenotypes in the cross pathway control mutants. Indicated strains were grown on SCM agar for 7 days under constant light at 25° C. Scale bar = 0.5 mm. **C.** Growth of *cpc-2, cpc-1* and *cpc-2 cpc-1* double mutant on varying concentrations of 3-amino-triazole (in milli-molar concentrations). Growth is represented in mm with wild type growing at 30 mm on VM plates 30° C in the dark overnight.

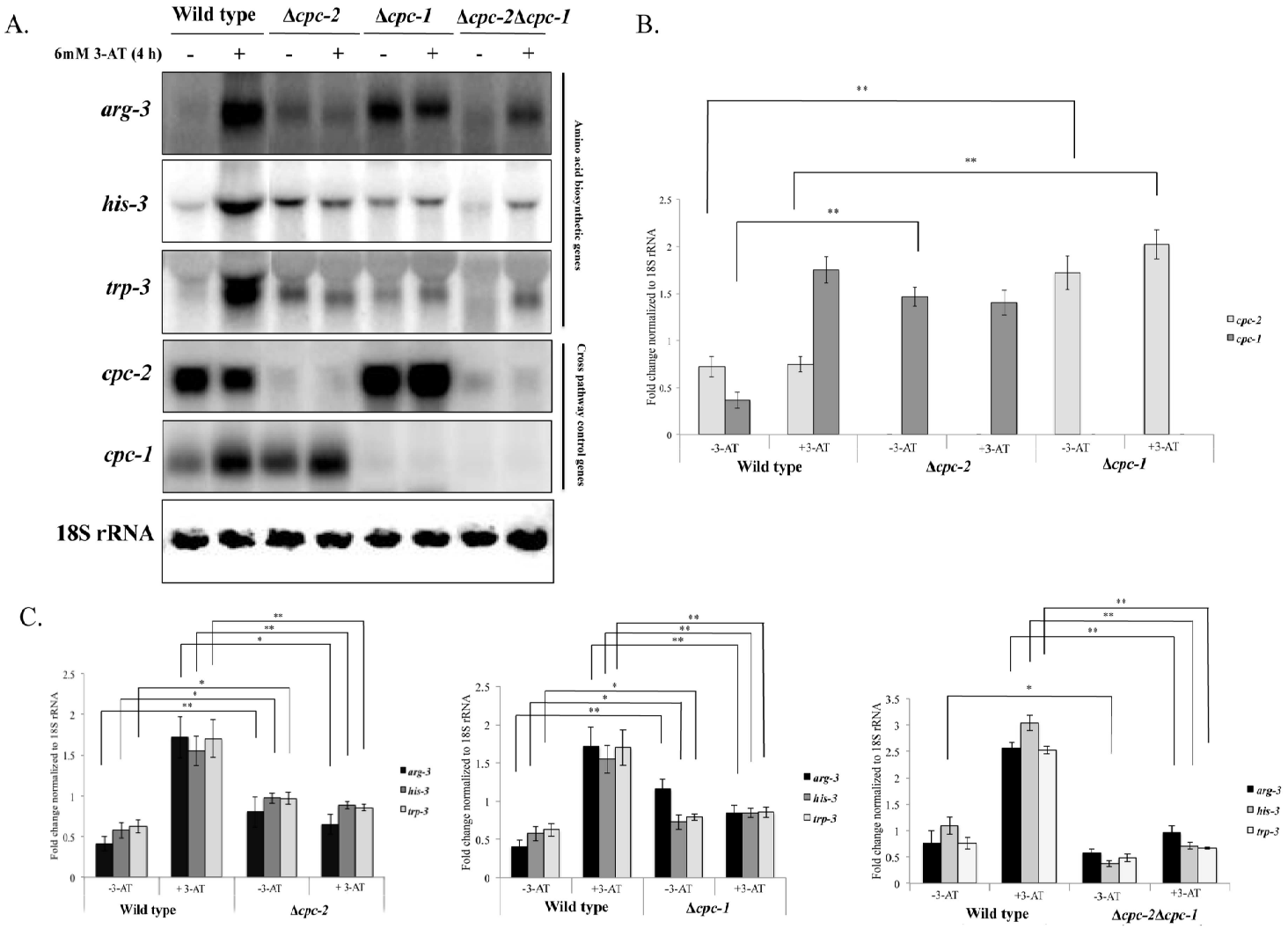

A.
Wild type
Δcpc-2
Δcpc-1
Δcpc-2Δcpc-1
6mM 3-AT (4 h)
- + - + - + - +
arg-3
his-3
trp-3
cpc-2
cpc-1
18S rRNA
Amino acid biosynthetic genes
Cross pathway control genes
B.
Fold change normalized to 18S rRNA
-3-AT
+3-AT
Wild type
Δcpc-2
Δcpc-1
cpc-2
cpc-1
C.
Fold change normalized to 18S rRNA
arg-3
his-3
trp-3
Wild type
Δcpc-2
Δcpc-1
Δcpc-2Δcpc-1

Figure 3.2. CPC-2 and CPC-1 are involved in de-repression of amino acid genes under 3-AT stress. A. Northern blot analyses of amino acid biosynthetic genes – *arg-3, his-3* and *trp-3* in the three cross pathway control mutants. Also shown are transcript levels for *cpc-2* and *cpc-1* genes in either of the mutant strains. Liquid cultures for respective strains were grown in constant darkness at 30° C for 12 h and then treated with 6 mM 3-AT concentration for an additional 4 h before collection and total RNA isolation followed by northern blotting. The 18S rRNA is shown as a loading control. **B.** Quantification of *cpc-2* and *cpc-1* transcript levels in respective mutants (as shown in the Northern analyses). All p value calculations were conducted comparing within the non-starved (-3-AT) levels or starved (+3-AT) levels. **C.** Quantification for the transcript levels of *arg-3, his-3* and *trp-3* in the respective mutants (as shown in the northern analyses) representative of three independent experiments. Students T test values denoted via - ** $p < 0.01$, *$p < 0.05$

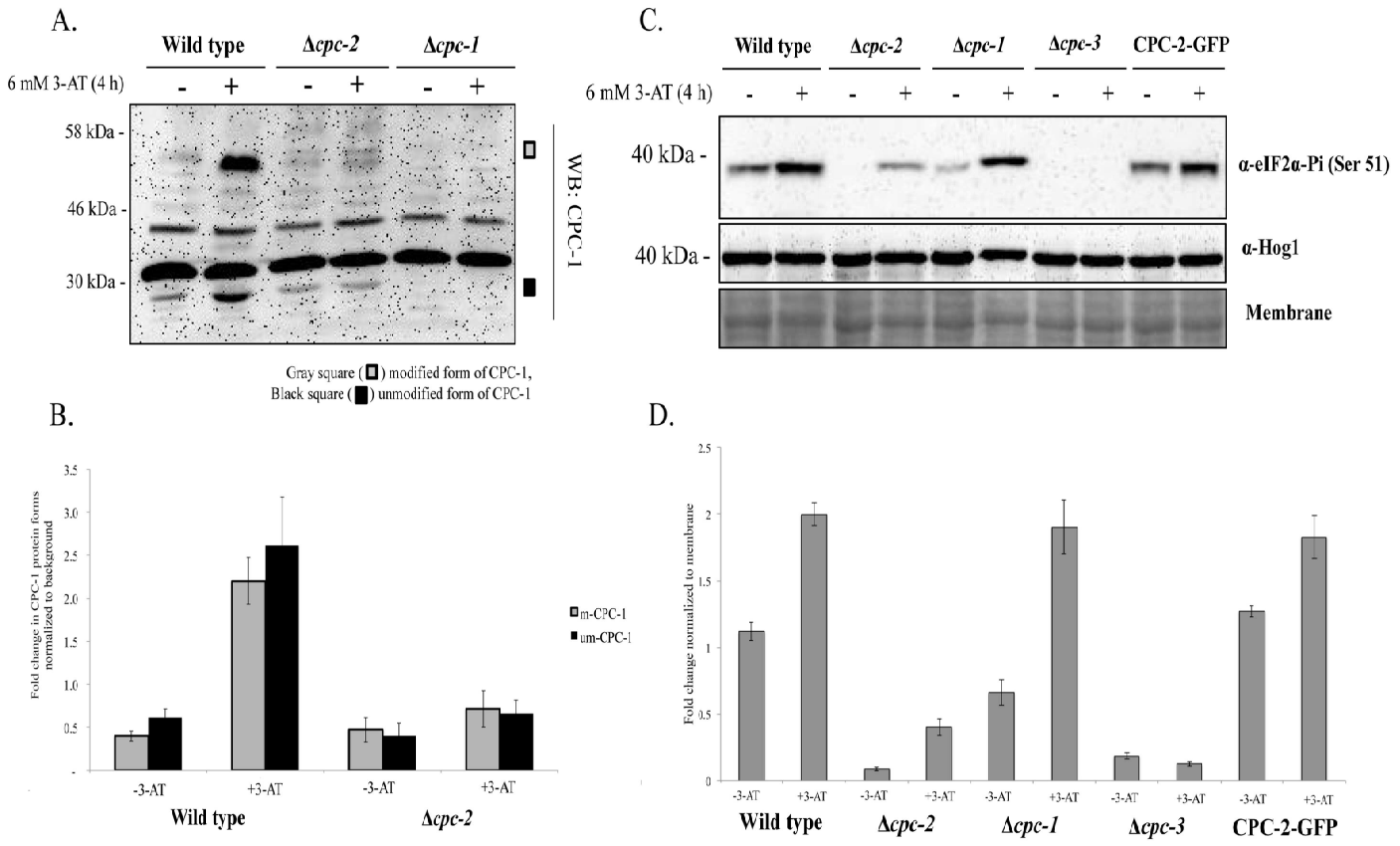

A.
Wild type
Δcpc-2
Δcpc-1
6 mM 3-AT (4 h)
- + - + - +
58 kDa -
46 kDa -
30 kDa -
WB: CPC-1
Gray square ( ) modified form of CPC-1,
Black square ( ) unmodified form of CPC-1
B.
Fold change in CPC-1 protein forms normalized to background
m-CPC-1
um-CPC-1
-3-AT
+3-AT
-3-AT
+3-AT
Wild type
Δcpc-2
C.
Wild type
Δcpc-2
Δcpc-1
Δcpc-3
CPC-2-GFP
6 mM 3-AT (4 h)
- + - + - + - + - +
40 kDa -
α-eIF2α-Pi (Ser 51)
40 kDa -
α-Hog1
Membrane
D.
Fold change normalized to membrane
-3-AT
+3-AT
Wild type
Δcpc-2
Δcpc-1
Δcpc-3
CPC-2-GFP

Figure 3.3. Δ*cpc-2* has defective CPC-1 protein levels and reduced eIF2α phosphorylation. **A.** Western blot analyses for CPC-1 protein in wild type and Δ*cpc-2* with Δ*cpc-1* as a negative control. Cultures grown for 12 h were treated with 6 mM 3-AT for 4 h to stimulate histidine starvation as previously described. Black squares indicate the unmodified ~28 kDa CPC-1 protein while the gray square indicates the post-translationally modified CPC-1 protein. **B.** Quantification of CPC-1 western blots with gray squares for modified CPC-1 (m-CPC-1) and black squares for unmodified CPC-1 (um-CPC-1). Fold change in CPC-1 levels is representative of three replicates normalized to the background band. **C.** Western blot analyses for eIF2α phosphorylation levels in the cross pathway control mutants and CPC-2-GFP strain. The kinase mutant - Δ*cpc-3* was used as a control for eIF2α phosphorylation levels. The MAPK Hog1 (OS-2 in *Neurospora*) is used as an internal control while the membrane was stained with amido black and used as a loading control. **D.** Quantification of the eIF2α phosphorylation levels in the respective strains from (C.) is representative of the fold change in three independent replicates and normalized to the membrane.

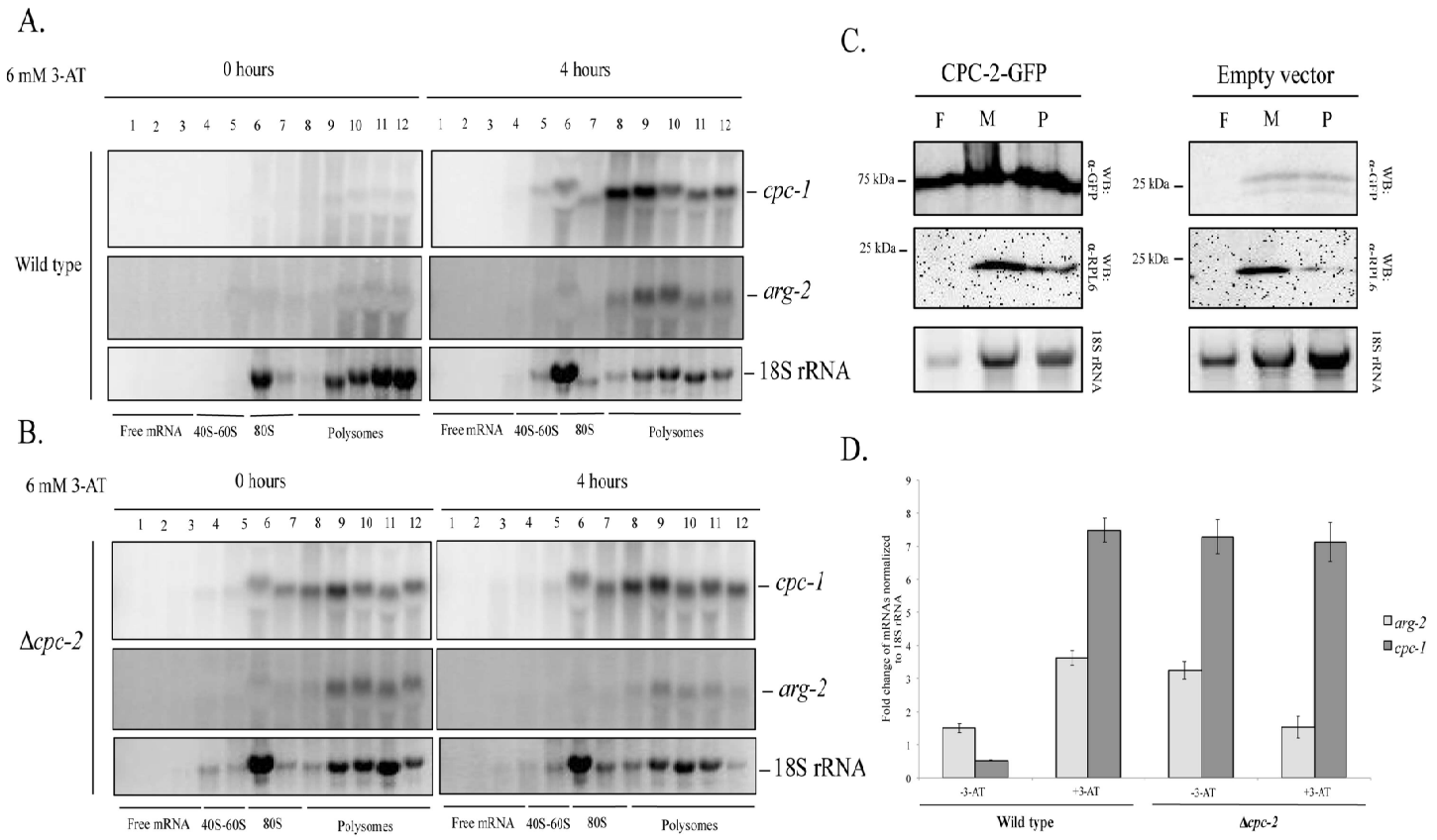
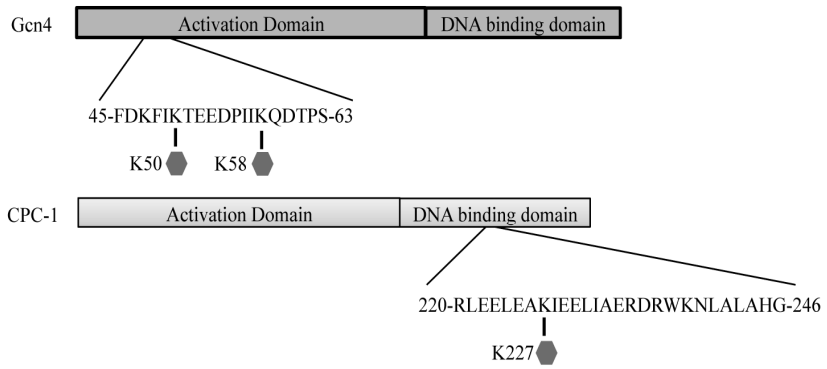

A.
6 mM 3-AT
0 hours
4 hours
1 2 3 4 5 6 7 8 9 10 11 12
Wild type
cpc-1
arg-2
18S rRNA
Free mRNA
40S-60S
80S
Polysomes
B.
Δcpc-2
C.
CPC-2-GFP
Empty vector
F M P
75 kDa
25 kDa
WB: α-GFP
WB: α-RPL6
18S rRNA
D.
Fold change of mRNAs normalized to 18S rRNA
arg-2
cpc-1
-3-AT
+3-AT
Wild type
Δcpc-2

Figure 3.4. CPC-2 regulates CPC-1 via an eIF2α phosphorylation-independent mechanism. A,B. Northern blot analysis of wild type and Δ*cpc-2* fractions 1-12 from sucrose density centrifugation for polysome analyses of *cpc-1* and *arg-2* mRNAs. Cultures were treated with 3-AT as described in the Materials and Methods and polysome profiling analysis was carried out to obtain fractions 1-3 (free mRNA), 4-7 (monosomes) and 8-12 (polysomes). RNA was isolated and subjected to northern analysis as described in the text.. The relative abundance of 18S rRNA was used to identify the various ribosomal fractions. **D.** Quantification of *cpc-1* and *arg-2* polysomal RNA levels. Values were normalized to the 18S rRNA for the respective polysomal fractions (8-12). Results shown are representative of three independent replicates. **C.** TCA-acetone precipitation was carried out on 200 µl aliquots of the samples pooled from free mRNA – F, monosomes – M and polysomes – P fractions followed by western blot analyses for GFP tagged CPC-2 and the 60S ribosomal protein – RPL-6. The other half of the samples (200 µl) was subject to RNA isolation with TRIzol LS reagent to detect ribosomal RNAs in the same pools. A strain with no insert and only GFP-V5 tags was used as the empty vector control.

A.


Gcn4
Activation Domain
DNA binding domain
45-FDKFIKTEEDPIIKQDTPS-63
K50
K58
CPC-1
Activation Domain
DNA binding domain
220-RLEELEAKIEELIAERDRWKNLALAHG-246
K227

B.

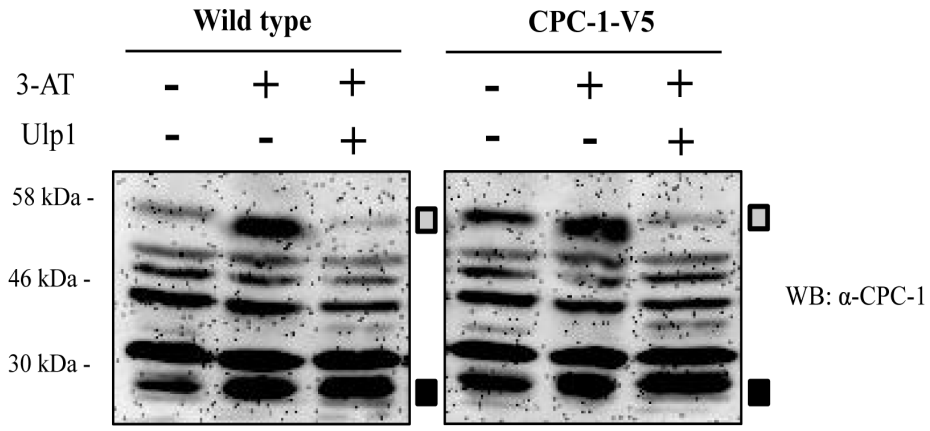

Wild type
CPC-1-V5
3-AT
Ulp1
58 kDa -
46 kDa -
30 kDa -
WB: α-CPC-1

Figure 3.5. CPC-1 is post-translationally modified via SUMOylation. A. Schematic view of the domains present on Gcn4p (*S. cerevisiae*) and CPC-2 (*Neurospora crassa*) along with the respective lysine residues required for SUMO modifications. For CPC-2 the lysine residue are present in the DNA binding domain while in yeast they are in the activation domain. **B.** Western analyses of SUMO protease – Ulp1 treated whole cell extracts with or without 3-AT treatment. Gray Square denotes SUMO-modified CPC-1 and black square denotes un-modified CPC-1. Representative of three independent replicates.

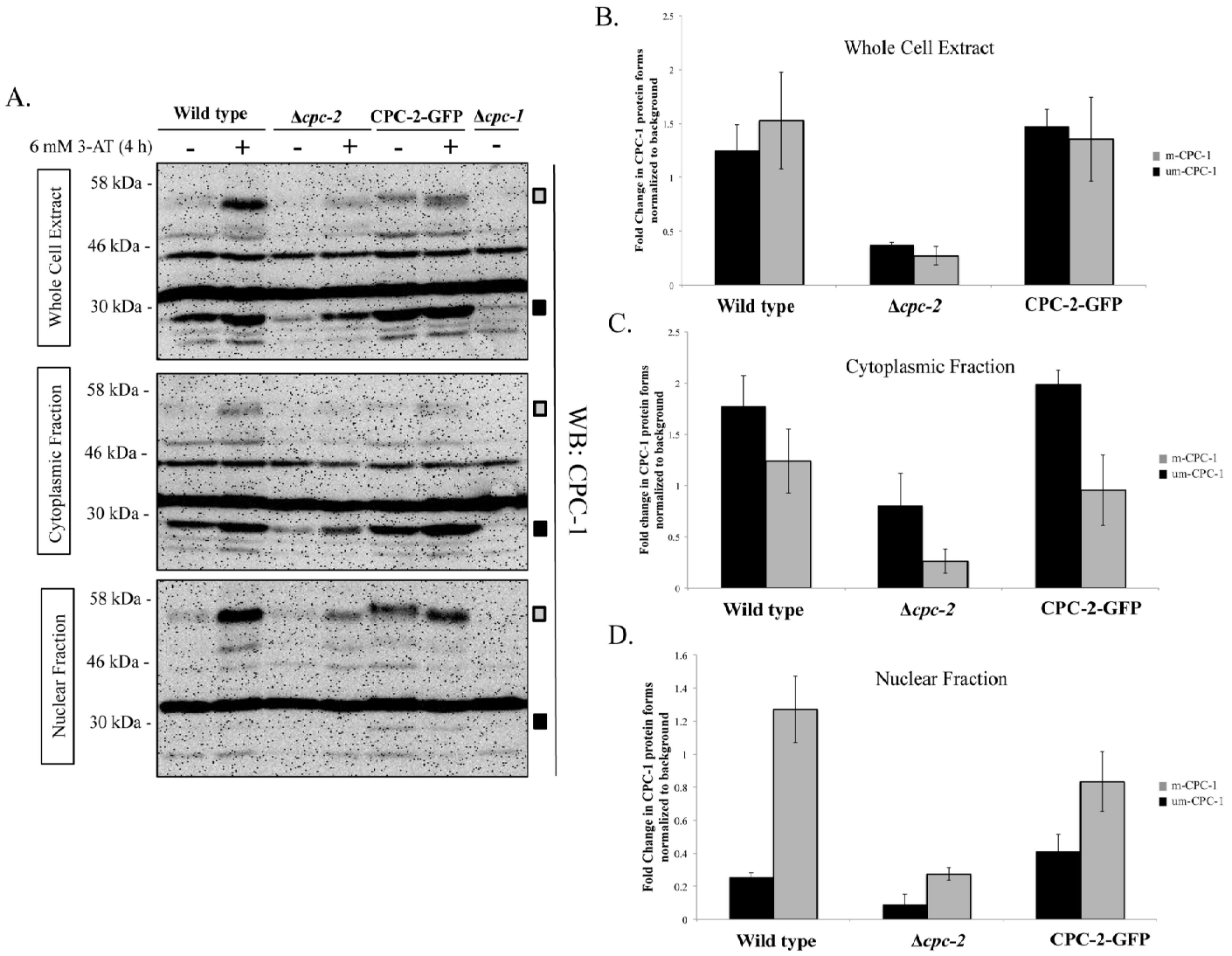

A.
Wild type
Δcpc-2
CPC-2-GFP
Δcpc-1
6 mM 3-AT (4 h)
- + - + - + -
Whole Cell Extract
Cytoplasmic Fraction
Nuclear Fraction
58 kDa -
46 kDa -
30 kDa -
WB: CPC-1
B.
Whole Cell Extract
C.
Cytoplasmic Fraction
D.
Nuclear Fraction
Fold Change in CPC-1 protein forms normalized to background
Fold change in CPC-1 protein forms normalized to background
m-CPC-1
um-CPC-1
Wild type
Δcpc-2
CPC-2-GFP

Figure 3.6. CPC-2 is required for effective SUMOylation of CPC-1. A. Western analyses for CPC-1 from whole cell, cytoplasmic and nuclear fractions after nuclei isolation (upper, middle and lower panels) in wild type, Δ*cpc-2* and CPC-2-GFP strains before and after 4 h of 3-AT treatment. Δ*cpc-1* was used as a negative control (without 3-AT treatment). **B, C, D.** Quantification of the CPC-1 protein levels unmodified (black squares) and SUMO-modified (gray squares) CPC-1 after 3-AT treatment in the indicated strains. Experiment is representative of three independent replicates.

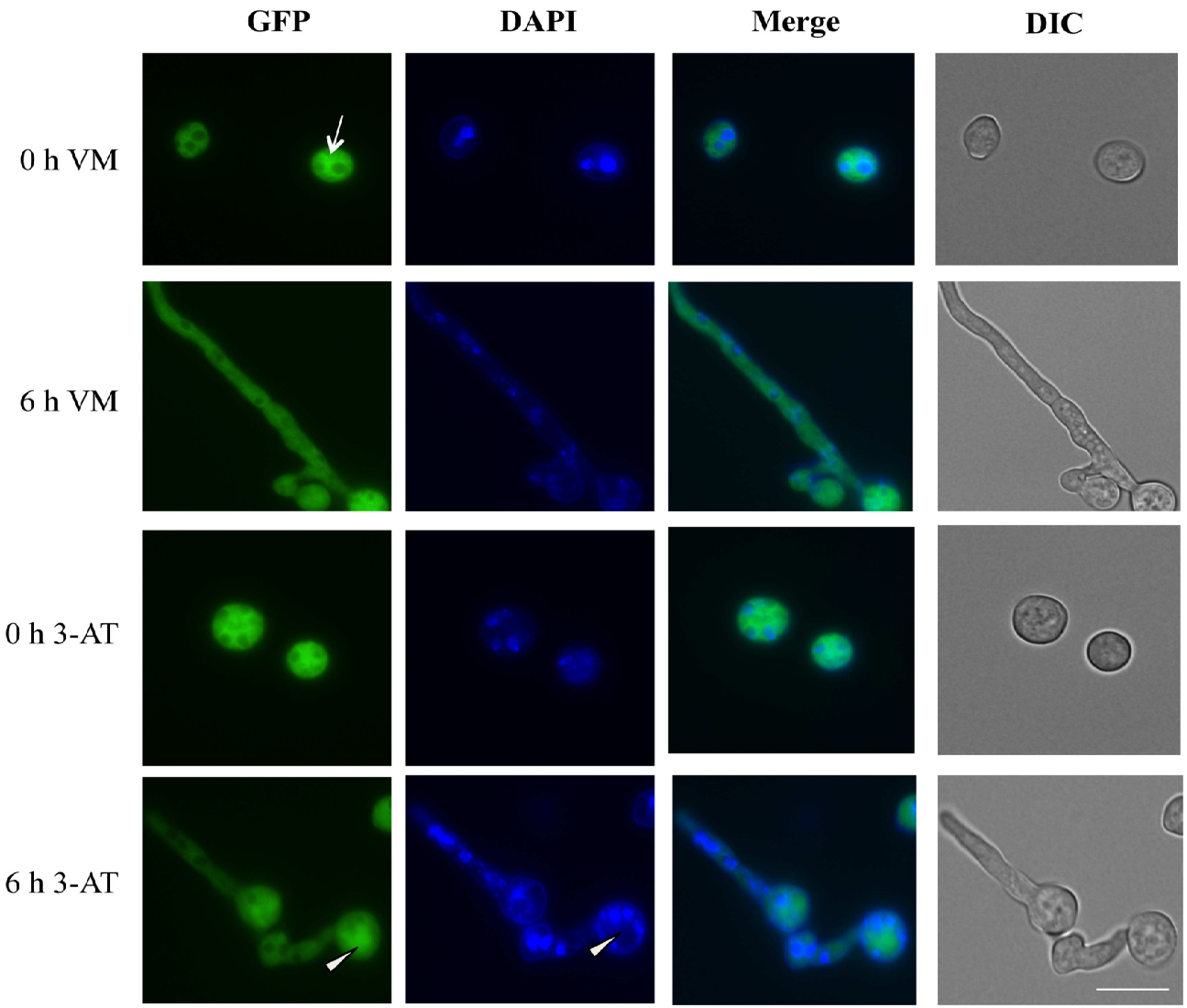

GFP
DAPI
Merge
DIC
0 h VM
6 h VM
0 h 3-AT
6 h 3-AT

Figure 3.7. CPC-2-GFP localization post 3-AT treatment. The CPC-2-GFP strain was inoculated on VM agarose or VM agarose-3-AT plates (6 mM) at a concentration of 8 x 106 conidia/ml and allowed to germinate for 0 h and 6 h. Images for GFP channel were obtained via fluorescence microscopy (Olympus IX71 inverted microscope) and also stained with DAPI to visualize the nucleus. Images for GFP and DAPI were merged using ImageJ (National Institutes of Health, Bethesda, MD). Differential interference contrast (DIC) images were taken to confirm germination capacities for the strain with or without 3-AT stress. Scale bar size = 10 μ

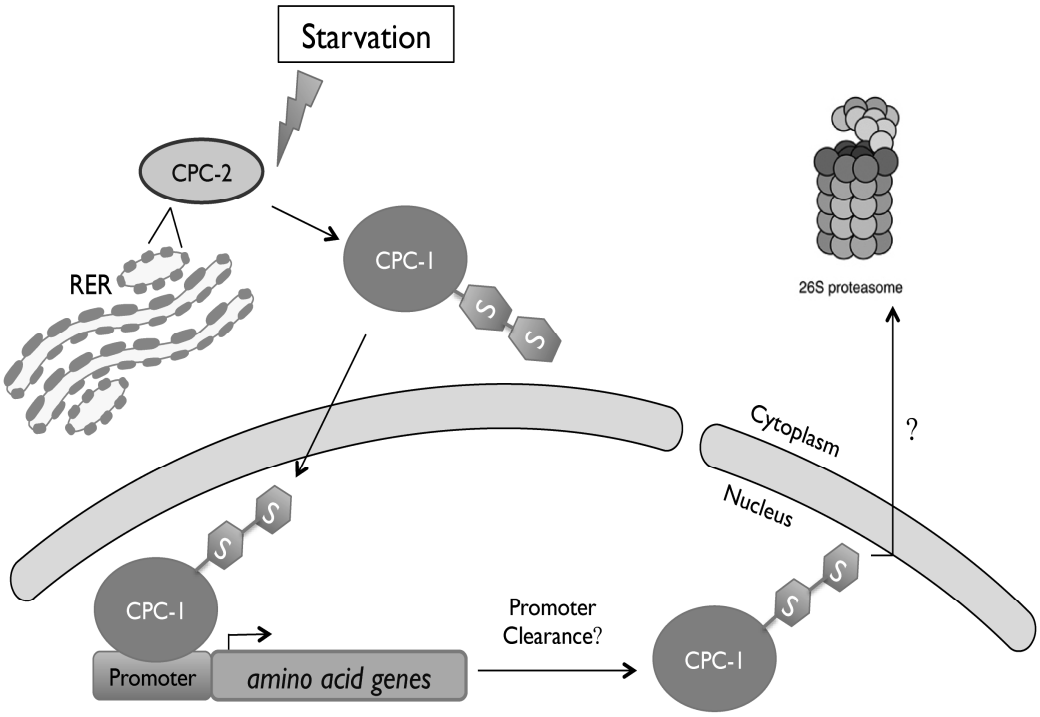

Starvation
CPC-2
RER
CPC-1
S
S
26S proteasome
Cytoplasm
?
Nucleus
S
S
CPC-1
Promoter
amino acid genes
Promoter
Clearance?
S
S
CPC-1

Figure 3.8. Hypothesis for mechanism of action of CPC-2 mediated regulation of CPC-1 and modulation of cross pathway control in *Neurospora crassa.* Under 3-AT mediated 3-AT starvation stress the ribosomal protein CPC-2 elicits a response towards effective SUMOylation of the bZIP transcription factor. SUMOylation of CPC-1 is critical towards translocation of CPC-1 into the nucleus. In the nucleus CPC-1-SUMO is able to up-regulate various amino acid biosynthetic genes, which is essential towards counteraction against the prevailing amino acid starvation stress. It is likely that CPC-1 is deSUMOylated in the nucleus and after nuclear exit is turned over by the 26S proteasome (as evidenced in several other studies on Gcn4p in yeast and ATF4 in mammals).

Chapter IV

Role of heterotrimeric G proteins and the guanine nucleotide exchange factor RIC-8 in regulation of the translational machinery

Abstract

Translation is a multi-step process that is controlled at the initiation, elongation, termination and ribosome recycling phases, with initiation being rate-limiting. Even before these three phases, formation of the pre-initiation complex and a functional ribosome that is able to scan for the translational start site is a critical step for protein synthesis. This chapter investigates the role of heterotrimeric G proteins and the guanine nucleotide exchange factor (GEF) RIC8 in regulation of translation and factors involve in translational control. Previous studies from the Borkovich group have identified the Gβ subunit as well as RIC-8 to be a part of the ribosome. The results indicate that deletion of the Gα protein subunit genes *gna-1* and *gna-3* or the *ric8* GEF leads to defects in eIF2α-phosphorylation, indicative of general translation repression. We identify numerous mRNAs whose polysome distribution is affected by deletion of these genes via RNA-seq of polysomal RNA pools. MuDPIT analysis of the polysomal protein fraction from wild type and Δ*ric8* strains revealed several proteins, including the GNB-1 Gβ subunit, the GNA-1 Gα, certain 40S and 60S ribosomal proteins and an ABC transporter, that are absent from polysomes of Δ*ric8.* From this study, we conclude that G proteins and RIC-8 have important roles to play in ribosome biogenesis and function and might also regulate translation of specific mRNAs as and when required by the cell.

Introduction

Heterotrimeric G proteins are involved in wide variety of cellular responses, ranging from transduction of information regarding environmental stresses, such as osmo-sensitivity, heat or oxidative stress, detected by the seven trans-membrane G protein coupled receptors (GPCRs) (1,2). G proteins also transmit non-chemical or peptide signals perceived by GPCRs, such as light (3,4). Upon binding of an extracellular ligand to the GPCR there is a conformational change in the protein, which leads to its eventual dissociation from the Gαβγ heterotrimer via exchange of GDP for GTP on the Gα subunit. The released activated Gα and Gβγ heterodimer are then free to regulate downstream responses, such as activation of MAPK cascades and cAMP signaling (5). Under certain circumstances, non-receptor-mediated G protein signaling is initiated with using certain guanine nucleotide exchange factors, such as ARR4, GET3 or RIC8 (6).

A previous graduate student in the Borkovich group, Dr. Alexander Michkov, had shown that RIC8 and GNB-1 are ribosomal proteins. Both RIC8 and GNB-1 were shown to be present in higher molecular weight polysomes as demonstrated by western blot analyses for the respective proteins from wild type polysome fractions. This chapter extends the previous study by investigating the mechanistic details behind the function of G proteins in translation through investigating eIF2α-phosphorylation and gene expression profiles from polysomal RNA in in G protein and Δ*ric8* mutants. As described in Chapter 3, eIF2α-Pi results in cellular stress responses as well as a reduction in global translation of genes that are not directly influencing stress responses (7,8). This leads to

effective regulation of genes containing upstream open reading frames (uORFs), such as the amino acid biosynthetic genes *arg-2* and *his-3* and the transcription factor *cpc-1*.

Although the effects of deletion of G protein subunits and *ric8* has been well documented in mammalian cell lines as well as in fungi (9-12), it is a novel finding that G proteins are somehow able to regulate a critical and highly regulated signaling mechanism in the cell such as translation (Michkov and Borkovich unpublished). It has been shown that eIF2α-Pi activates the serine/threonine kinase Akt in cells that are exposed to oxidative stress, and decreased Akt activation in eIF2α-Pi-defective cells leads to cell death (13). On the other hand, is also well established that GPCRs coupled to $G\alpha_q$ and $G\alpha_i$ and associated $G\beta\gamma$ subunits are able to activate the Akt kinase via a PI3 kinase-dependent pathway (14). Another well-known and important regulator of eIF2α-Pi is the mammalian target of rapamycin, or mTOR, which is also highly conserved in eukaryotes, including fungi (15,16). In mammals, inhibition of mTOR/TOR leads to activation of the GCN2 kinase, which is a positive regulator of eIF2α-Pi (17). Although there is no direct evidence for regulation of GCN2 via upstream factors, including GPCRs or G proteins, it has been shown that certain small G proteins, such as Rheb (Ras homologue enriched in the brain), are able to promote mTOR signaling (18). It is possible that G proteins act on such translational machineries via such intermediate proteins, such as Akt and mTOR, which are critical and central hubs in regulation of stress-mediated translational changes.

The work in this chapter shows that not only does deletion of G protein genes and/or *ric8* lead to increased eIF2α-Pi levels, but also results in dysregulation of protein-encoding genes in the respective mutant polysomes. This suggests that the defect in translation and/or global decrease in translation might be due to these differentially regulated genes (Figure 4.3). This chapter also uses MuDPIT (multi-dimensional protein identification technology) to identify proteins that could not be detected in Δ*ric8* mutant polysomes, indicating that *ric8* might be critical for translation of the indicated genes (Table4.2). Additionally we also find that deletion of *ric-8* leads to loss of GNB-1 as well as PKC (protein kinase C) on polysomes as well as reduction of CPC-2 from polysomes.

Materials and Methods

Strains and culture conditions

Strain ORS-SL6a (FGSC 4200, *mat a*) was the wild type used in this study. Other strains are listed in Appendix Table 4.1. Culture growth conditions were as described in Chapter 3, with the exception that a 500 ml liquid VM culture was used for overnight (16 h) growth of strains for polysome analyses. The inoculum concentration for 16 h cultures for polysome experiments was 1 x 10^6 conidia/ml.

Western blot analysis

For detection of eIF2α-phosphorylation levels in the indicated mutants, 16 h cultures were harvested and cell pads flash-frozen as described in Chapter 3. For protein isolation, the same methodology and extraction buffer was used as described in Chapter 3: 50 mM TrisCl pH 7.5, 1 mM EDTA, 6 mM $MgCl_2$, 0.1% FPIC and 2.5 mM mM PMSF, with 1 mM sodium orthovanadate ($NaVO_4$) and 1 mM sodium fluoride (NaF) to protect against dephosphorylation. For each sample, a volume containing 50 μg of protein was run on a 10% SDS-gel and then subjected to western analysis. A phospho-eIF2α antibody (Epitomics, rabbit polyclonal, AbCam ID ab32157, Cat# 1090-1) was used at a 1:2000 dilution [overnight incubation with shaking at 4°C, in Tris-buffered saline with 0.1% Tween-20 (TBS-T) and 5% BSA] as described earlier in Chapter 3. Membranes were incubated in blocking reagent (5% milk with TBS-T) for 1 h at room temperature. After the primary antibody incubation, membranes were washed 3 times with TBST and then

incubated for 1 hour in anti-rabbit IgG secondary antibody at a 1:2000 dilution for 2 h at room temperature (Sigma Chemical, St. Louis, MO) and followed by chemiluminescence detection as previously described (10).

Polysome profiling and RNA extraction

Isolation of polysomal fractions from mutant strains and wild type was carried out as described in Chapter 3 using a previously described methodology (19), followed by RNA extraction TRIzol LS reagent (Ambion, RNA) as per manufacturer's instructions.

RNA-seq library preparation

The RNA isolated from polysome pools for each strain was checked for quality using the 2100 Agilent Bioanalyzer (Agilent, Santa Clara, CA) at the IIGB genomics core facility at UC Riverside. The samples were then used for RNA-seq library preparation with NEBnext Illumina Ultradirectional RNA-seq kit (#NEBE7420S, New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. A poly-A mRNA enrichment was carried out using magnetic oligo-dT beads (NEBNext Poly(A) mRNA magnetic isolation module, NEB #E7490; New England Biolabs) and a magnetic rack. NEBNext Multiplex Oligos (NEB#E7335; New England Biolabs) were used for multiplexing (Index barcodes 1-8) and Agencourt AMPure xp magnetic beads (#A63881, Beckman Coulter, Inc., Fullerton, CA) were used for subsequent steps, as per manufacturer's instructions. After library preparation, the cDNA was checked for quality using a Qubit^{TM} dsDNA high sensitivity assay (#Q32854, Invitrogen) at the IIGB Genomics core facility,

UCR using manufacturer's instructions and a Qubit Fluorometer (Invitrogen). A final Bioanalyzer run was used to check for quality and quantity of each library. The prepared libraries were used for a 2 x 51 x 7 cycle HiSEQ2500 run in 2 flow cells with 2 replicates. De-multiplexing was carried out by the bioinformatics core facility group at UCR followed by EdgeR analyses by Dr. Jason Stajich of UCR.

Analysis of polysomal protein extracts

For preparation of polysomal protein extracts for MuDPIT (multi-dimensional protein identification technology) analyses of wild type and Δ*ric8* polysomes, a previously established protocol was used (20) with minor modifications. The polysome pools (fractions 8-12, 400 µl each) from the wild type and *ric8* strains were split into two 1 ml aliquots and brought up to 20% trichloroacetic acid (TCA) and incubated on ice overnight. Samples were centrifuged in 1 ml eppendorf tubes at 12,000xg for 30 min at 4°C in a Beckman Coulter tabletop centrifuge. The resulting pellets was washed with 10% TCA and centrifuged again using the same conditions. The pellets was washed with 500 µl ice cold acetone to remove residual TCA, incubated for an hour at -20°C, followed by centrifucation at 12000xg for 15 min at 4° C in a Beckman Coulter tabletop centrifuge. The pellets was washed once more with cold acetone and the pellet was allowed to dry for 10 mins and stored at 4° C until mass spectrometry analyses. The pellets was submitted to the IIGB proteomics facility at UC Riverside for combined elution of proteins for each strain (wild type and *ric8*). MuDPIT was carried out by Dr.

Songqin Pan, followed by data collection and assembly of gene IDs, counts and p values for each sample set.

Data analysis and heat map generation

For functional category analyses and pictorial representation of the RNA-seq and MuDPIT data, the MIPS catalog was used - http://mips.helmholtz-muenchen.de/funcatDB/ (*Neurospora crassa,* ORF74A – p3_p13841_Neu_crass_MIPS) to classify the functional categories (funCAT) represented in our datasets. The various NCU numbers from each funCAT from the MIPS cataloging were listed in excel and the corresponding CPM values were extracted from a master spreadsheet using VLOOK functions in Excel (Microsoft, City, WA). The CPM values for various funCATs (such as translation, translation intiation etc.) were visualized in a heatmap format using the software HeatmapGenerator (21) which utilizes R and a C++ hybrid computational pipeline.

Results

Heterotrimeric G proteins and RIC8 impact eIF2α-phosphorylation

The heterotrimeric G protein subunit mutants Δ*gna-1,* Δ*gna-2,* Δ*gna-3,* Δ*gnb-1* and the double knockout mutant Δ*gna-1* Δ*gna-3* showed a marked increase in eIF2α-phosphorylation compared to wild type (Figure 4.1). In contrast, although elevated relative to wild type, the Δ*gna-2* mutant showed the smallest increase in phosphorylation compared to the other mutants. Strains expressing GTPase-deficient, constitutively activated Gα alleles, denoted *gna-1*, gna-2** and *gna-3** (9,22,23), were also tested using this assay. In these strains, the Gα protein is constitutively bound to GTP and is not recycled to the GDP-bound inactive state (10). Of interest, the *gna-1** and *gna-3** strains exhibited increased phosphorylation of eIF2α, similar to the respective knockout mutants (Figure 4.1.C). Loss or activation of Gα proteins can lead to increased levels of free Gβγ dimer in the cell. Furthermore, the Gβγ dimer is a guanine nucleotide dissociation inhibitor [GDI; (24)] that maintains and stabilizes Gα-GDP at the membrane docked with the GPCR. In addition, in *N. crassa*, a mutant lacking *gnb-1* or *gng-1* has low levels of Gα proteins under various conditions (10,23). The observation that the Δ*gnb-1* mutant has levels of eIF2α–Pi similar to the Gα deletion and Gα* strains argues against a Gβγ-centric mechanism. Rather, the data suggest that the absence of a GDP-bound G protein leads to elevated eIF2α–Pi. The observation that the Δ*ric8* mutant displays increased levels of eIF2α-Pi (Figure 4.1.D), is consistent with the reduced amount of Gα proteins observed in this strain (9).

In terms of understanding the role of G proteins in regulating eIF2α-Pi levels, it must be considered that the exchange of GDP for GTP on the eIF2 hetero-trimer is catalyzed by the eIF2B GEF (25). It is possible that there are additional regulators and GEFs, such as RIC8, that control this process, and that deletion of *ric8* leads to reduced GDP-GTP exchange on eIF2B and increased production of eIF2α-Pi (Figure 4.1.D).

RNA-seq analysis of mRNA from wild type, Δ*gna-1,* Δ*gnb-1* and Δ*ric-8* polysomes reveals numerous differentially represented mRNAs genes

Poly-RNA-seq was carried out using RNA extracted from the polysome pools in wild type (*mat a*), Δ*gna-1,* Δ*gnb-1* and Δ*ric-8* strains, as described in the Materials and Methods. CPM values were used to generate heat maps for the FunCAT groups corresponding to genes involved in ribosome biogenesis, ribosomal protein function, translation initiation/elongation/termination as well as translational control (Figure 4.3 A-F). Overall, the MIPS analysis revealed a large subset of genes from the sequencing runs were related to metabolism and several more for combined processes in translation and translational control (Table 4.1). In this chapter, the focus is on the translation-related proteins that also show the highest degree of differential regulation in the different strains.

Several ribosome biogenesis genes were differentially regulated at the polysomal RNA/translational level in our poly-Seq experiments (Figure 4.3A). Of the mRNAs, NCU06111 [encoding the RAS-2 small GTPase; (26)] showed reduced polysomal co-

migration by 7.5 fold and 5.2 fold in Δ*gnb-1* and Δ*gna-1* respectively compared to wild type and even the Δ*ric8* mutant. Another mRNA encoding a GTPase, the Dynamin-domain containing NCU05693, was decreased in Δ*gna-1* but not Δ*gnb-1* or Δ*ric8* mutants. The translational elongation factor EF2 (NCU06278) was also reduced in the Δ*ric8* and Δ*gnb-1* mutants compared to wild type and Δ*gna-1* strains. Of the genes involved in regulation of ribosomal proteins, NCU03598 and NCU09793 showed the highest differences in the respective strains. NCU03598 is a microfibril-associate protein and is greatly down-regulated in Δ*gna-1,* while NCU09793 is a DEAD/DEAH box DNA helicase that exhibits a significant increase in Δ*ric-8* and slight upregulation in Δ*gnb-1* (Figure 4.3.B) It is not immediately evident how a microfibril-associated protein is able to have influence on ribosomal proteins. It has however been demonstrated that DEAD/DEAH box helicases are important for RNA metabolism as well as ribosome biogenesis by assisting in unwinding RNA as well as 60S and 40S subunit assembly (27). Examples of mis-regulated genes that are involved in translation initiation are NCU06149 and NCU07868 (Figure 4.3.C). An ATP-dependent RNA helicase – NCU06149 and the eukaryotic initiation factor – eIF4G1, NCU07868 exhibited a 1.5 fold decrease in co-migration with polysomes in Δ*ric8.* In terms of genes involved in regulation of translation elongation, the data suggested that the eRF (eukaryotic release factor) GTP-binding subunit and the translation activator – NCU05803 showed reduced (1.6 fold) polysomal co-migration in in Δ*ric-8* compared to the other strains (Figure 4.3.D). In case of translation termination, the eukaryotic release factor – eRF1 (NCU00410) was showed a 1.22 fold increase in Δ*ric-8* and a 1.1 fold increase in Δ*gnb-1* compared to wild type,

while there is also a 1.42 fold decrease in co-migration for NCU04790 (eRF GTP-binding subunit) in Δ*ric-8*.

Besides the 3 major phases of translation, Figure 4.3 also summarizes mRNAs encoding proteins involved in translational control. NCU04749 (DNA replication helicase) and NCU07395 (DNA binding protein, SMUBP-2) were greatly underrepresented in polysomes from Δ*ric-8* compared to wild type, and somewhat decreased in Δ*gnb-1*. Also mRNA for a Pumilo-family RNA protein, NCU06511, was decreased in Δ*gnb-1* polysomes. Helicases and DNA binding proteins are critical for unwinding RNA duplexes in an ATP-dependent reaction (28). Interestingly, the Pumilo (PUF) family of RNA binding proteins are able to bind certain sequence motifs in the 3' UTR (un-translated region) of target mRNAs to regulate translational repression (29). Therefore, it is interesting to note that GNB-1 might involved in regulating this mode of translational control via PUF family of proteins. Surprisingly we also identified two serine/threonine kinases that showed varied abundance in polysomes from the respective mutant strains (Figure 4.3.F). The *stk-18* kinase (NCU01379) showed a slight decrease in abundance on polysomes in a Δ*ric-8* strain, while *stk-43* (NCU06486) showed increased levels in Δ*ric-8*. The *stk-18* kinase is related to the SAGA complex (30), a multifunctional coactivator of eukaryotic transcription. In this context, it seems the SAGA complex and *stk* kinases might have an overarching role towards controlling factors, which are more directly involved in translational control, and hence the regulatory effect is imparted via transcriptional regulation via SAGA group of proteins. On the other hand, the *stk-43* kinase is homologous to the CAMK class of

Ca^{+2}/calmodulin kinases, which are involved in regulation of neuronal mRNA translation in mammals (31). It has been reported that CAMKs are able to phosphorylate Ser1156 of the eukaryotic translation initiation factor eIFGII to modulate translation initiation in rat hippocampal neurons (32). Considering it is highly represented in Δ*ric-8* polysomes, it is tempting to speculate that increased phosphorylation of eIF2α in the mutant might be a consequence of over-activity of *stk-43,* which may be a positive regulator of the eIF2α-kinase CPC-3 in *N. crassa*.

MuDPIT of Δ*ric8* polysomes reveals the importance of RIC8 in regulation of several translation-related factors

As described in the Materials and Methods, polysomal fractions from wild type and Δ*ric8* strains were pooled and subjected to TCA-mediated protein isolation. Samples were analyzed using MuDPIT at the IIGB genomics core facility at UC Riverside. We identified 308 proteins that were present in wild type, but absent from Δ*ric8* polysomes. Another 384 proteins were detected in wild type but not in Δ*ric-8* (Figure 4.4.B). MIPS (FunCAT) analysis of all the 993 proteins from the MuDPIT run revealed an even distribution of proteins involved in several critical cellular processes, such as cellular transport, metabolism, transcriptional control, signal transduction mechanisms, stress responses, cell cycle, cellular sensing, proteasomal degradation as well as translation (Figure 4.5). The Δ*ric8* polysomes also lacked GNB-1, GNA-1, Protein Kinase C (PKC) and several ribosomal proteins (Table 4.2). The ABC transporter NCU05226 was another protein that was absent from Δ*ric-8* polysomes in the MuDPIT analyses and was

categorized for both translational control (Table 4.2.B) and translation elongation (Table 4.2.D.). It has been observed that certain groups of proteins annotated as ABC transporters do not function as transporters, but are in fact involved in translational processes (33).

As noted above, GNB-1 and GNA-1 were not found in Δ*ric8* polysomes. Thus, these MuDPIT experiments provided a proof-of-principle control for the observation from previous studies in the Borkovich laboratory that RIC8 may be involved in regulating translation of GNB-1 and GNA-1. It will be an interesting continuation of this study to build upon these findings and decipher the exact mechanistic role RIC8 might have towards translational regulation of G proteins besides other ribosomal proteins and components of the translational machinery.

Discussion

As shown in this chapter, G proteins and RIC8 are required for normal eIF2α-phosphorylation, as well as normal co-migration of ribosomal proteins and translation factors in polysomes. It is tempting to speculate that G proteins and RIC8 regulate the CPC-3 kinase that in turn controls eIF2α-phosphorylation in *N. crassa*. It is also possible that there is an indirect mode of regulation being carried out by the upstream components to regulate other kinases such as *stk-43* (via RIC8) to eventually impinge upon CPC-3 activation. Using Poly-Seq analysis, this study also discovered that mRNAs for several integral ribosomal components are mis-regulated in the Δ*gna-1,* Δ*gnb-1* and Δ*ric8* strains. A number of these defects were present in the Δ*ric-8* mutant, indicating the significance of this GEF in regulation of translation. We also found that deletion of *ric08* leads to loss of protein kinase C – PKC from the polysomes. Previous LC-M/S analyses by Dr. Jacqueline Servin (unpublished Servin and Borkovich) had shown that RIC-8-V5 is able associate with CPC-2. This suggests that RIC-8 might be able to co-regulate PKC levels via CPC-2 regulation which is known to be a bonafide ribosomal protein. The next step in this study would be to confirm the association of RIC8 with polysomes, cementing its place as a newly found ribosomal protein as hinted at by a previous study by Dr. Alexander Michkov in the Borkovich group. It is also of importance that several *ric8* point mutant (ORFs are V5-tagged) strains be used for polysome analyses to decipher which domains within the RIC8 protein are essential for ribosomal association. This would provide us a strain expressing ribosome-free RIC-8 and enable us to

separately characterize RIC-8's ribosomal and cytoplasmic or non-ribosomal functions. This particular strain can prove critical towards future experiments to show how cytoplasmic or ribosomal forms of the protein are able to regulate development and/or translation. This will lead further specialization for RIC8 function and give a structure-function outlook to this current study.

In addition, future experiments comparing wild type and Δ*ric-8* strains for total RNA-seq versus poly-Seq will be able to give us a clearer picture regarding regulation of GNB-1 and GNA-1 via RIC8. As shown in this chapter, deletion of *ric8* abrogates GNA-1 and GNB-1 protein migration from the polysomal fractions. Demonstration of the differences in expression of these G proteins in total extract versus polysomal fractions will make for a stronger case for the role of RIC8 in translation of these specific mRNAs.

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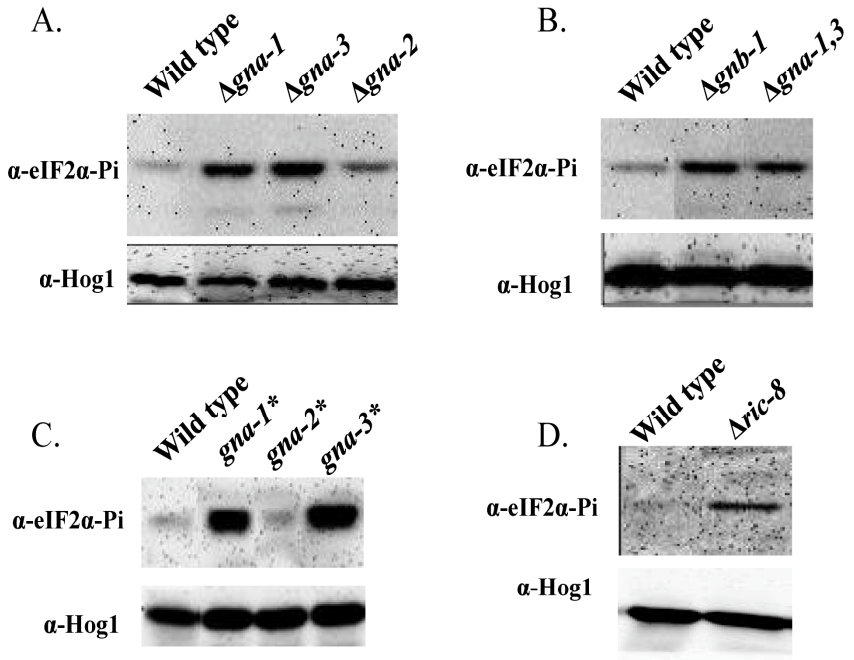
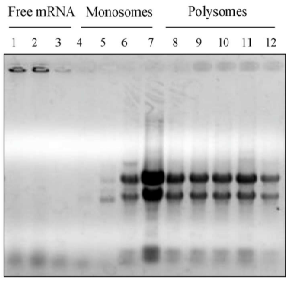
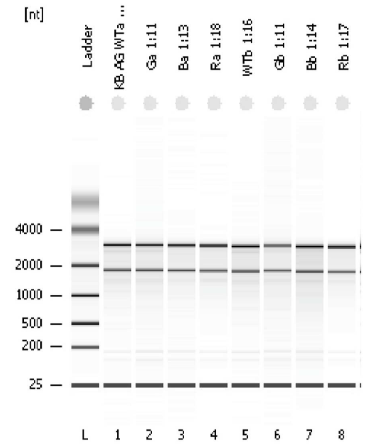

A.
Wild type
Δgna-1
Δgna-3
Δgna-2
α-eIF2α-Pi
α-Hog1
B.
Wild type
Δgnb-1
Δgna-1,3
α-eIF2α-Pi
α-Hog1
C.
Wild type
gna-1*
gna-2*
gna-3*
α-eIF2α-Pi
α-Hog1
D.
Wild type
Δric-8
α-eIF2α-Pi
α-Hog1

Figure 4.1. eIF2α-phosphorylation levels in various G protein mutants, G protein activated allele strains and Δ*ric-8*. A-D. Indicated strains were grown for 7 days on VM agar and grown for 16 h in liquid VM culture as described in materials and methods and whole cell extracts for each sample was tested for eIF2α-phosphorylation. Hog-1 MAPK was used as an internal control.

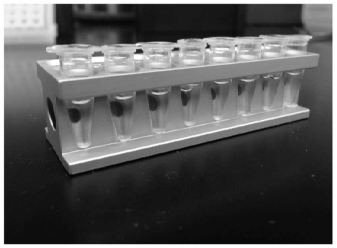
Poly-RNA seq library prep workflow



Pooled total RNA@Polysomes → Bioanalyzer Run Test RNA quality



Polysomal RNA pooled samples



poly-A mRNA isolation with oligo-dT beads (mRNA fragmentation)

1st strand cDNA synthesis

2nd strand cDNA synthesis

Purify double stranded cDNA with AMPxp pure magnetic beads

End Repair/dA-tailing of cDNA library

Adaptor ligation

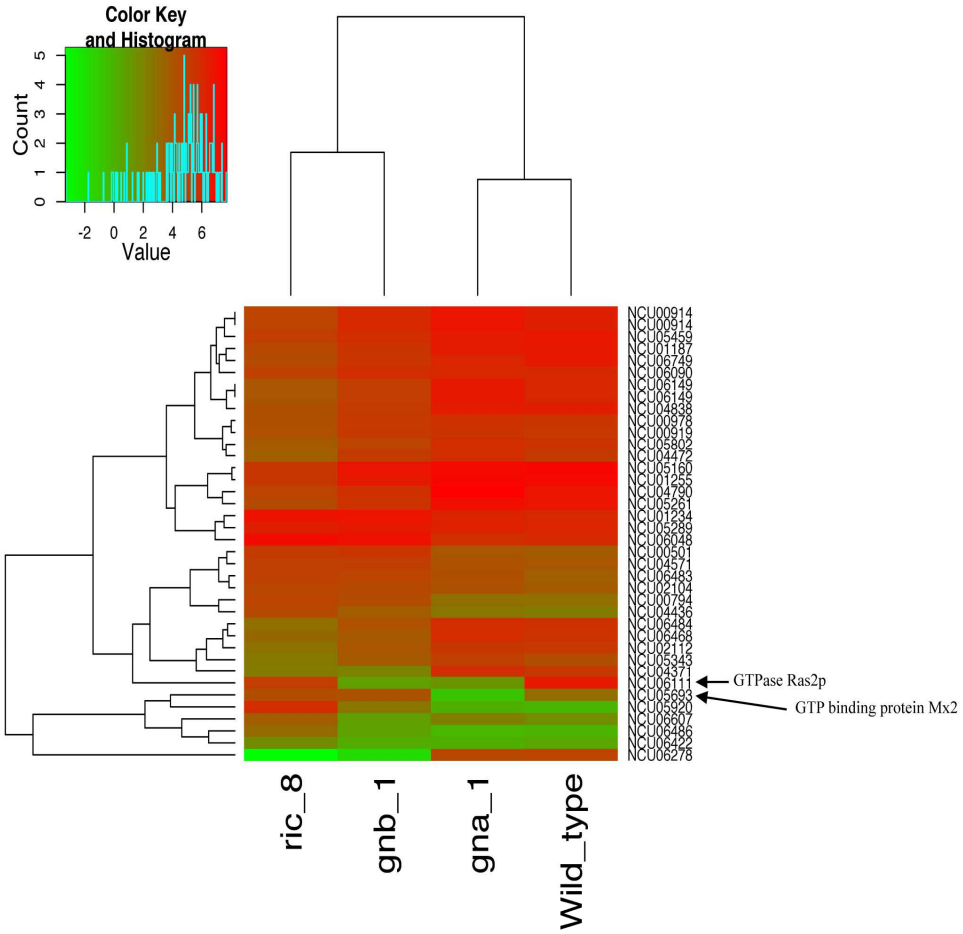
Purify ligation reaction with AMPxp beads

PCR enrichment and Index barcoding

Proceed to Qubit/Bioanalyzer run for quality control

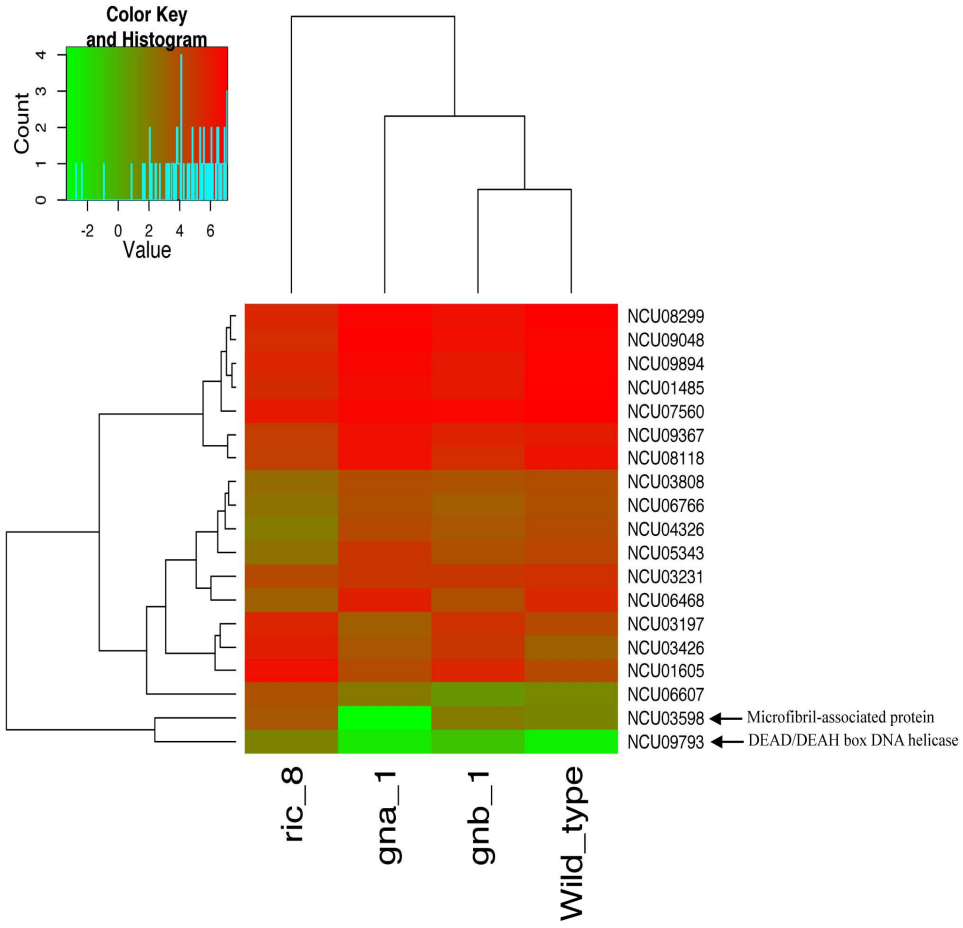
Figure 4.2. Workflow for Poly-RNA-Seq library preparation. Polysomal RNA samples were prepared using TRIzol LS RNA extraction and checked for quality using a Bioanalyzer. The pooled RNA samples were subjected to poly-A mRNA pulldown using magnetic oligo-dT beads and a magnetic rack followed by mRNA fragmentiation, 1st strand and 2nd strand synthesis. The dsDNA was purified using AMPxp magnetic beads and adaptor ligation was followed by Index barcoding and PCR enrichment. The libraries were checked for quality control using Qubit dsDNA HS assay and Bioanalyzer at the IIGB Genomics core facility at UC Riverside.

A.



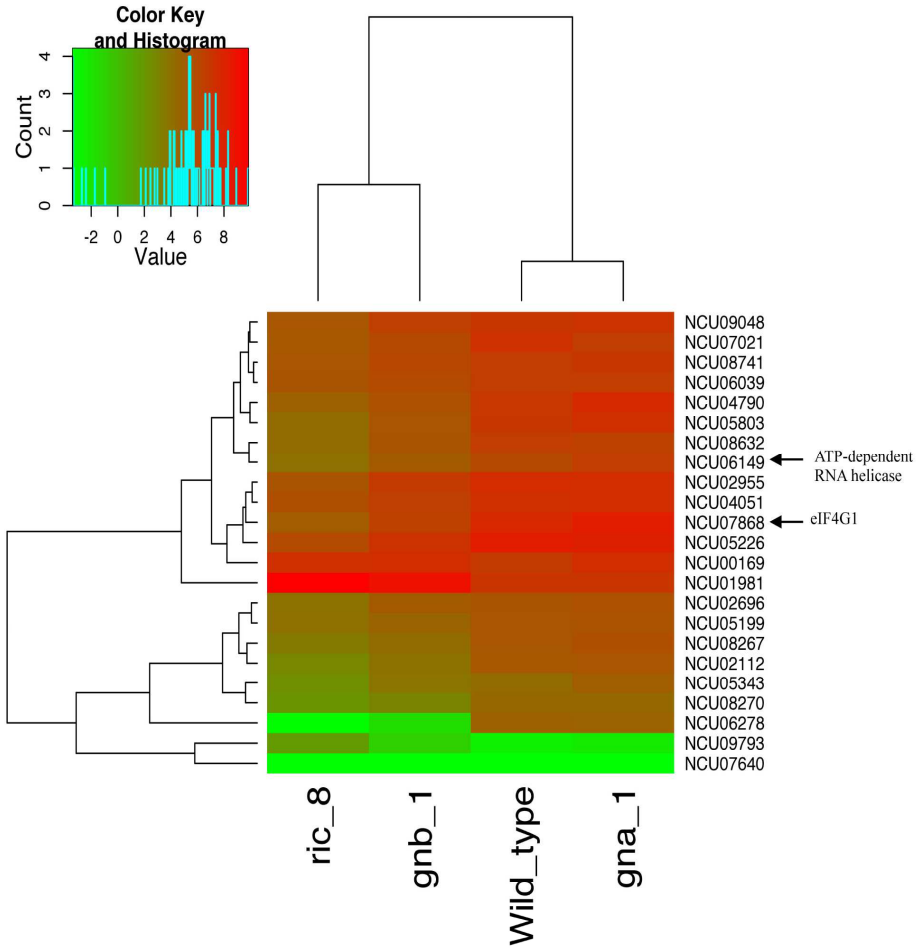
Ribosome biogenesis

B.



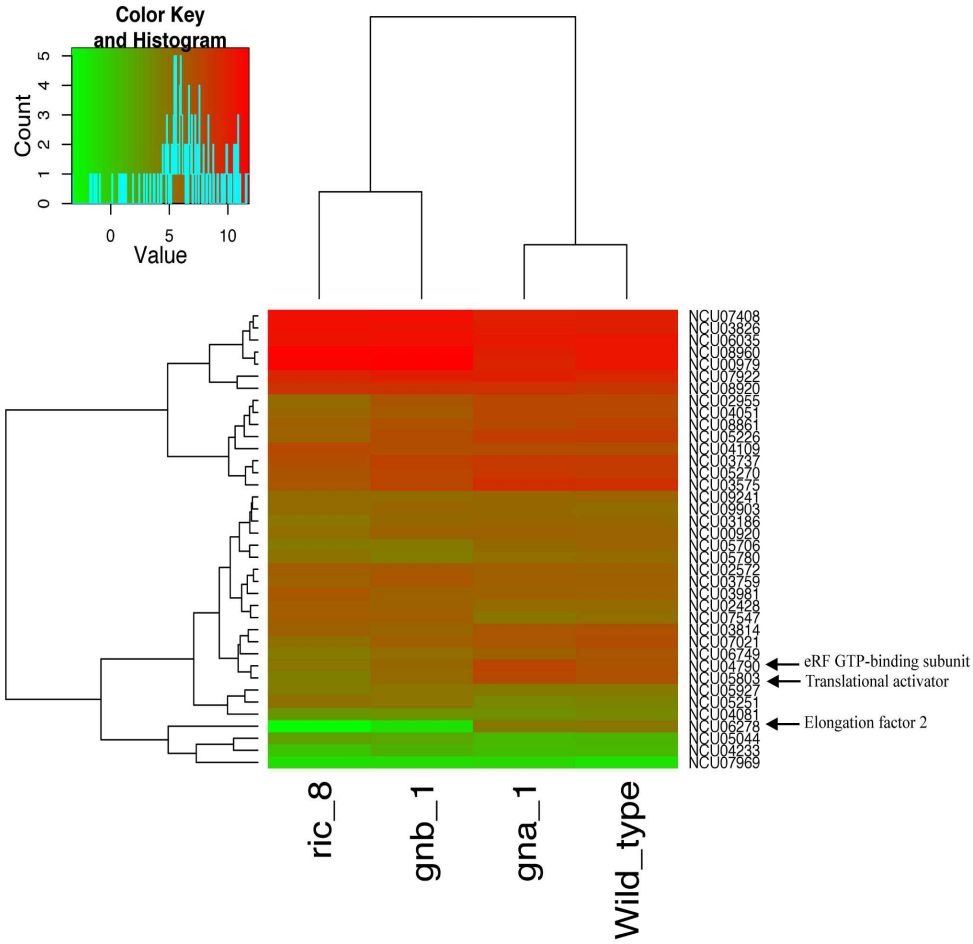
Ribosomal proteins / ribosome biogenesis

C.



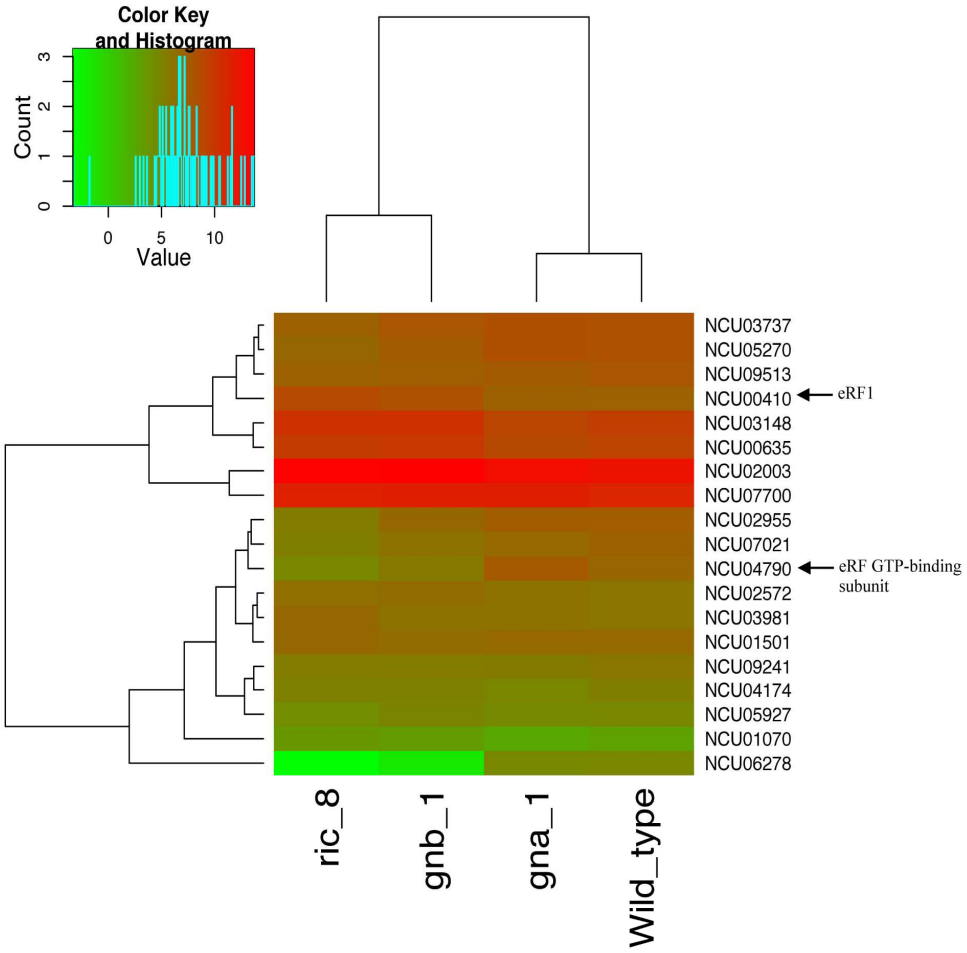
Translation initiation

D.



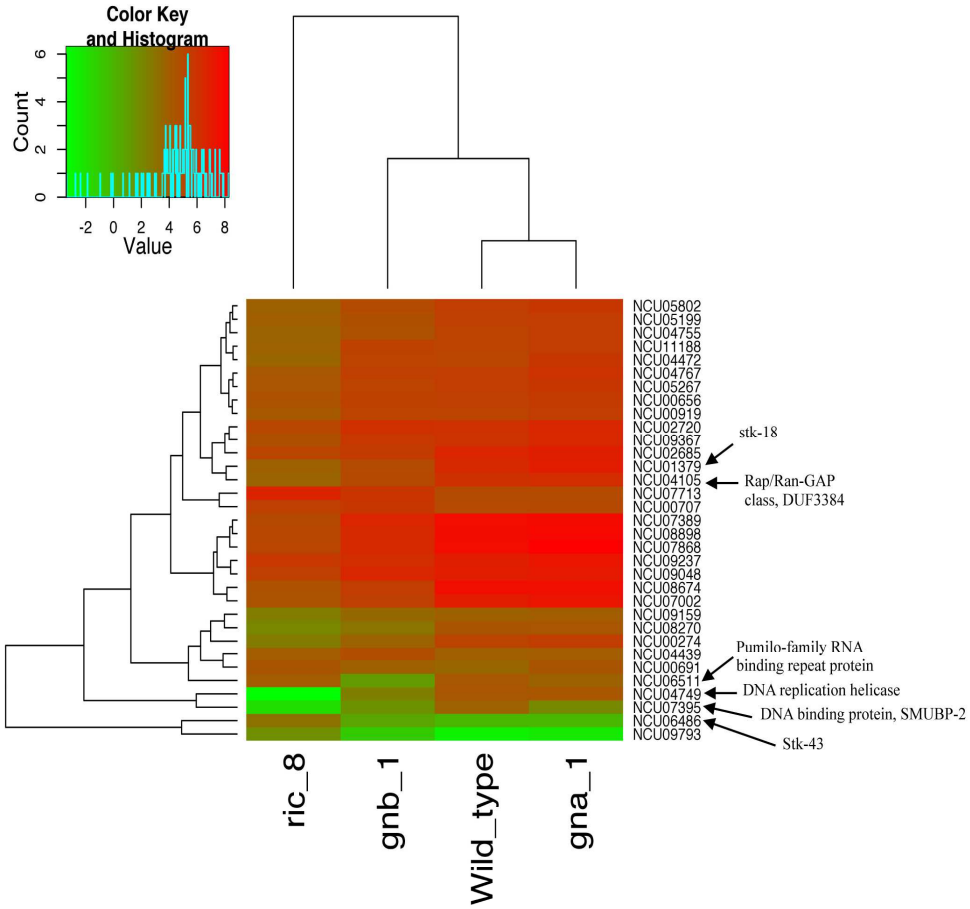
Translation elongation

E.



Translation termination

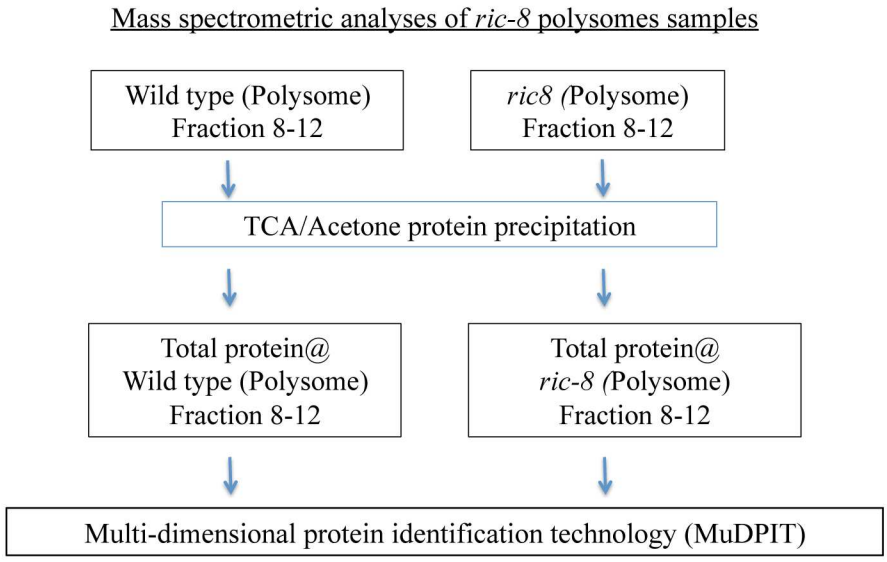
F.



Translational control

Figure 4.3. Heatmap representation of various genes that show differences in co-migration on mutant polysomes. A. Ribosome biogenesis. B. Ribosomal proteins. C. Translation initiation. D. Translation elongation, E. Translation termination, F. Translational control. (**A-F**) Different genes which are up or down regulated and known to have putative function in the indicated processes. The genes with higher relative differences are highlighted on the right side with an arrow. The legend at the top left corner represents the color key and a histogram. Red – higher value/up-regulated genes, green – lower/down-regulated genes. The annotation for FunCAT analyses was carried out with help of the MIPS database (http://mips.helmholtz-muenchen.de/funcatDB/). Heatmaps were generated using Heatmap generator software downloaded from sourceforge - https://sourceforge.net/projects/heatmapgenerator/ (requires R-based operating systems).

A.


Mass spectrometric analyses of ric-8 polysomes samples
Wild type (Polysome) Fraction 8-12
ric8 (Polysome) Fraction 8-12
TCA/Acetone protein precipitation
Total protein@ Wild type (Polysome) Fraction 8-12
Total protein@ ric-8 (Polysome) Fraction 8-12
Multi-dimensional protein identification technology (MuDPIT)

B.

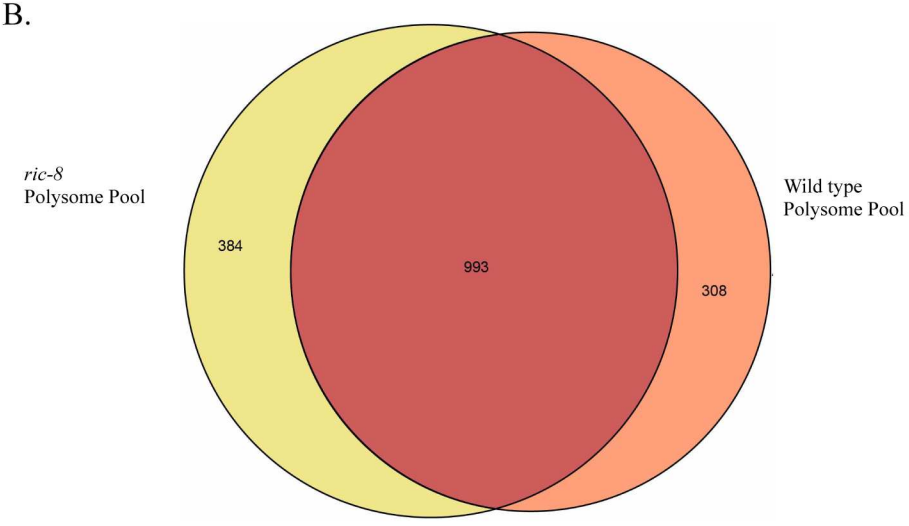

ric-8 Polysome Pool
Wild type Polysome Pool
384
993
308

Figure 4.4 Workflow for MuDPIT sample preparation and distribution of total number of proteins in the wild type and Δ*ric-8* mutant. A. Fractions 8-12 from polysome samples of wild type and *ric-8* were pooled together and TCA-acetone precipitation was carried out as described in the materials and methods. **B.** At least 993 proteins were found in both strains while 308 proteins were absent in wild type and 384 proteins were present only in Δ*ric-8.*

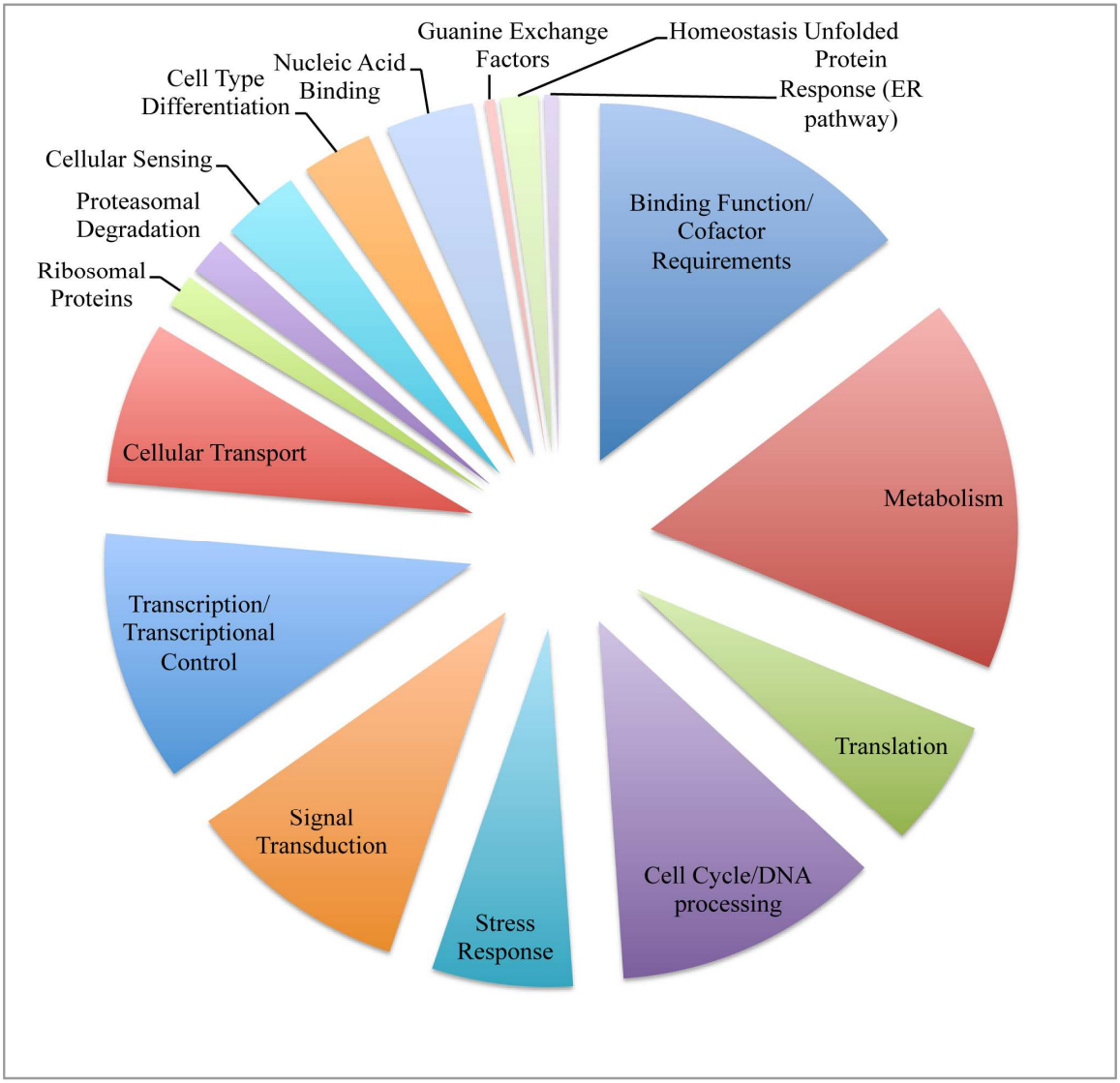

Guanine Exchange Factors
Homeostasis Unfolded Protein Response (ER pathway)
Nucleic Acid Binding
Cell Type Differentiation
Cellular Sensing
Proteasomal Degradation
Ribosomal Proteins
Binding Function/ Cofactor Requirements
Cellular Transport
Metabolism
Transcription/ Transcriptional Control
Translation
Signal Transduction
Cell Cycle/DNA processing
Stress Response

Figure 4.5. Distribution of genes that are mis-regulated in Δ*ric-8* compared to wild type. This distribution was calculated using the various gene id hits from MuDPIT analyses and followed by MIPS cataloguing for functional category analyses. The major groups of proteins were in the metabolism, cellular transport, binding factors, translation, transduction and signal transduction groups.

Table 4.1. Functional Category analyses of various roles of genes identified to be differential regulated via Poly-Seq experiments in wild type, *gna-1, gnb-1* and *ric-8* in *Neurospora crassa.* MIPS database used for analyses.

Functional Category	Counts	p value
Metabolism	3788	6.15E+69
RNA degradation	202	1.37E-03
DNA degradation	23	1.47E-01
Carbohydrate Metabolism	1983	4.98E-10
Energy	1270	5.62E-11
Respiration	488	2.50E-03
Cell cycle/DNA processing	1755	5.39E+13
Transcription	1789	3.23E+09
rRNA biogenesis	194	4.29E-03
mRNA biogenesis	1476	5.62E-13
Transcription initiation	251	2.71E-03
Transcriptional control	1359	1.02E-14
Post-transcriptional Control	56	1.01E-01
RNA processing	754	4.58E-06
mRNA processing/Splicing	553	2.71E-03
Protein folding and stability	323	1.38E-05
Proteasomal Degradation (Ub/proteasome)	384	1.25E-05
Ribosome biogenesis	549	4.60E-11
Ribosomal Proteins	258	4.85E-06
Translation initiation	175	2.12E-02
Translation elongation	43	3.46E-02
Translation Termination	19	3.55E-01
Translational Control	224	1.51E-02
Amino-acyl-tRNA synthetases	43	4.28E-01

Table 4.2. List of proteins not expressed in *ric-8* compared to wild type as deduced from MuDPIT analyses of the polysome pools from respective strains.

Table 4.2.A. Translation	
Gene_ID	Description/ class/ domain
NCU00440	*gnb-1*
NCU02428	nucleolar essential protein 1
NCU03827	40 S ribosomal protein S9
NCU03972	proteasome regulatory particle
NCU04063	protein transporter sec-13
NCU04174	hypothetical protein
NCU08678	translational machinery associated protein
NCU08552	60 S ribosomal protein L14
NCU05226	ABC transporter
NCU06279	eukaryotic translation initiation factor 3
NCU06544	Protein kinase C
NCU08357	chromatin assembly factor-1
NCU06768	60S ribosomal protein L16
NCU07011	WD repeat containing protein
NCU07280	serine/threonine protein kinase *gad8*
NCU07421	hypothetical protein
NCU01474	60 S ribosomal protein L4
NCU11357	cell cycle control protein
NCU05714	50S ribosomal subunit protein L15
NCU08952	translational regulator GCD7
NCU07991	DUF292 domain-containing protein
NCU02563	transcription elongation factor S-II
NCU03039	splicing factor u2af large subunit
NCU02781	hypothetical protein
NCU03230	mitochondrial ribosomal protein subunit S18

Table 4.2.B. Translational Control	
Gene_ID	Description/ class/ domain
NCU03768	lysophospholipase
NCU04242	ATP-dependent helicase
NCU04174	hypothetical protein
NCU05226	ABC transporter
NCU07002	THO complex component
NCU07421	hypothetical protein
NCU07866	DEAD helicase superfamily protein
NCU03039	splicing factor u2af large subunit
NCU09438	translation repressor/ antiviral protein SKI3
NCU03435	hypothetical protein

Table 4.2.C. Translation Initiation	
Gene_ID	Description/ class/ domain
NCU08678	translation machinery associated protein
NCU06279	eukaryotic translation initiation factor
NCU07421	hypothetical protein
NCU08952	translation regulator GCD7
NCU07991	DUF292 domain-containing protein
NCU03039	splicing factor u2af large subunit

Table 4.2.D. Translational Elongation	
Gene_ID	Description/ class/ domain
NCU02428	nucleolar essential protein 1
NCU05226	ABC transporter
NCU06768	60 S ribosomal protein L16
NCU02563	transcription elongation factor S-II

Table 4.2.E. G protein regulation	
Gene_ID	Description/ class/ domain
NCU06493	*gna-1*
NCU00440	*gnb-1*
NCU02788	*ric-8*

Chapter V

Conclusions and future directions

Overall summary of the thesis

The main objective of this thesis has been to expand upon the existing knowledge regarding cellular signal transduction mechanisms in the filamentous fungus *Neurospora crassa.* The various chapters delve into details of cellular signaling, ranging from 1) serine/threonine and tyrosine protein phosphatases to 2) stress responses via amino acid starvation and post-translational modifications and 3) to polysome mRNA expression profiles in various G protein mutants.

In Chapter II, global analysis of serine/threonine and tyrosine protein phosphatases is presented. A total of 24 viable mutants were tested for defects in basal growth, sexual development, aerial hyphae height and conidiation, as well as sensitivity to hyperosmolarity, cytoskeletal deformation, oxidative stress and a fungicide. The study found that 91% of the mutants had at least one growth/developmental phenotype and 29% possessed defects in all three growth/developmental pathways. Also, at least one chemical phenotype was revealed for the 17 mutants that were tested for sensitivity. With the help of such detailed tests on growth/development and chemical sensitivity, we were able to decipher a function for the vast majority of these protein phosphatases, many of which had never been previously studied. Additionally, the study also revealed that nine of the phosphatase mutants had dysregulated OS-2/p38 MAPK phosphorylation, with most of the strains showing increased OS-2 phosphorylation. This suggests that a number of these proteins are involved in dephosphorylation of OS-2-Pi. Understanding the role of

these phosphatases in MAPK regulation is an important step towards expanding this study past its current state. Future work on these phosphatases might focus on complementation and/or overexpression of the genes involved in OS-2-Pi regulation and subsequent confirmation of those with direct effects on the MAPK pathway. Additionally, understanding how overexpression changes the growth pattern in the presence of a chemical that induces hyperosmolarity will also build upon this initial study. Two other interesting phosphatase mutants that were investigated include the PP2C mutant Δ*pph-8*/NCU04600 that displayed sexual structure formation in the absence of nitrogen starvation and the conidial separation mutant – Δ*csp-6*/NCU08380 that formed chains of conidia, signifying a separation defect. Understanding the mechanism underlying increased nitrogen-independent sexual fertility in Δ*pph-8* should provide insights into how sexual development is regulated in *Neurospora crassa.* In addition, decreased conidial separation has relevance for pathogenic fungi, such as *Histoplasma capsulatum* and *Coccidioides immitis*, which are able to propagate and cause respiratory failures in humans via dispersal and inhalation of conidia (1,2).

In Chapter III, the role of the well-known scaffolding protein and putative alternate Gβ subunit CPC-2 (RACK1 homolog in mammals) in regulating cross pathway control of amino acid biosynthesis was investigated. Deletion of *cpc-2* leads to sensitivity to 3-AT, which is a competitive inhibitor of an enzyme involved in histidine biosynthesis. The *cpc-2* deletion also results in female sterility and abnormal submerged culture conidiation. This re-affirms the role of CPC-2 as a multifunctional protein that modulates growth and development in addition to functions in amino acid metabolism. The major

hub in regulation of amino acid starvation responses in *Neurospora crassa* is the bZIP transcription factor CPC-1. In this study, I found that CPC-2 has a positive role in regulation of CPC-1 and that deletion of *cpc-2* leads to defective SUMOylation of CPC-1. I also observed that CPC-2 does not itself translocate to the nucleus upon 3-AT stimulation, suggesting that SUMOylation of CPC-1 might be occurring in the cytosol, and that the modification results in translocation of CPC-1 into the nucleus. Further confirmation of this would provide a clearer understanding of this phenomenon. Mass spectrometric analyses of immunoprecipitated CPC-1 (after 3-AT treatment) from the whole cell, cytoplasmic and nuclear extracts could provide direct evidence for the cellular location of CPC-1 SUMOylation, through co-immunoprecipitation of SUMOylation associated proteins such as SUMO E3 ligases. Future experiments could also be directed at revealing interacting partners for CPC-2 with or without 3-AT treatment to determine whether any SUMO E3 ligases are interacting with CPC-2. If the latter were validated, this would suggest that CPC-2 is able to recruit SUMO E3 ligases, which in turn would add SUMO groups to CPC-1, thus inducing its nuclear translocation and activation of amino acid biosynthetic gene promoters. Another topic of interest is the clearance of CPC-1 from bound promoters after starvation has subsided. Is CPC-1 turned over via the Ubiquitin-proteasomal pathway, similar to the yeast homolog Gcn4p? Answers to these questions will build upon the data presented in this chapter, and provide a more holistic understanding of the mechanism of CPC-1 regulation under amino acid starvation.

In Chapter IV, the role of heterotrimeric G proteins and the guanine exchange factor RIC8 in translation is investigated. Previous data from the Borkovich group has

suggested that various G proteins and RIC8 are critical components of the ribosomal machinery and that RIC8 is a novel ribosomal protein. This chapter expands on these findings by analyzing the phosphorylation levels of eIF2α in the G protein and Δ*ric8* mutants. It was found that all the mutants had increased levels of eIF2α-Pi, indicating that global translation has been repressed. RNA-seq analyses of polysomes from *gna-1, gnb-1* and *ric-8* mutant polysomes revealed a subset of translation-related factors that were mis-regulated in the concerned mutants. Two proteins that were repeatedly observed in the data were the Elongation Factor-2 and SMUP2 (a DNA binding protein), which were decreased in Δ*ric8* and Δ*gnb-1* polysomes. Failure to produce sufficient amounts of EF-2 might result in faulty production of short and nascent peptides that begin to stall at the initiation step. It will be interesting to investigate what role does RIC-8 and/or GNB-1 has in regulation of EF-2 so that a stall is prevented and translation is able to continue.

Using MuDPIT analyses of Δ*ric8* mutant polysomes, we found that Protein Kinase C (PKC) was absent from Δ*ric8* and that the levels of the RACK1 scaffolding protein homolog CPC-2 were decreased. Hypothetically, without effective CPC-2 levels in Δ*ric8* ribosomes, it is possible that there is a general failure of PKC biosynthesis and this is also reaffirmed by the fact that CPC-2 was found to interact with RIC8 in LC-M/S analyses from the Borkovich group (Servin and Borkovich, unpublished). Future work on this project would be to perform LC-M/S analyses of RIC8 complexes immunoprecipitated from the polysomal fractions to confirm whether there are any overlapping hits with those from the total polysome preparations. Does RIC8 interact

with PKC on the ribosomes or does it associate with EF-2 or SMUP2? Information about these interactions will shed light on the overall mechanisms of translational control and open a new avenue for discovery of novel members at this critical juncture for regulation of gene expression.

References:

1. Inglis, D. O., Berkes, C. A., Hocking Murray, D. R., and Sil, A. (2010) Conidia but not yeast cells of the fungal pathogen *Histoplasma capsulatum* trigger a type I interferon innate immune response in murine macrophages. *Infect Immun* **78**, 3871-3882

2. Hector, R. F., and Laniado-Laborin, R. (2005) Coccidioidomycosis--a fungal disease of the Americas. *PLoS Med* **2**, e2

Appendix A. Detailed Phenotypic and Chemical sensitivity data

Overview

The following tables are relevant information in connection to chapter 2 and provide detailed quantitative description of the basal growth, aerial hyphae height, conidiation as well as sexual development phenotypes. The numbers are reflective of three independent replicates. Numbers are also displayed as percentage of wild type significant p values highlighted in gray.

Appendix Table A.1. Detailed growth and developmental data

					Basal Hyphae Linear Growth Rate		
NCU Number	FGSC Number	Gene Name	Source[1]	Mating Type	mm/day	(%WT)	p value[2]
03436	16003	*tng*	Ascospore	a	34	47%	1.00E-05
06563	11547	*pp2A*	Ascospore	A	70.9	97%	0.2436
07489	11548	*pzl-1*	Ascospore	a	43.77	60%	6.79E-06
08301	12454	*pph-4*	Ascospore	A	36.42	50%	0.0002
06630	17800	*pph-1*	Single spore	a	67.65	93%	0.0217
01433	15790	*ppt-1*	Ascospore	a	76.38	105%	0.0372
00958	19378	*pph-7*	Ascospore	a	71.84	99%	0.5738
01767	12451	*pph-5*	Ascospore	a	68.12	93%	0.0131
03495	16431	*pph-6*	Ascospore	A	76.38	105%	0.0261
04600	11185	*pph-8*	Ascospore	A	35.49	49%	2.69E-06
08948	18439	*pph-11*	Ascospore	A	34.46	47%	2.41E-06
08380	20306	*csp-6*	Ascospore	a	42.1	58%	0.0022
02257	16060	*pty-2*	Ascospore	A	78.4	108%	0.007
05364	12444	*pty-3*	Ascospore	A	71.48	98%	0.2631
03246	13311	*cdc-14*	Ascospore	a	55.82	77%	0.0001
03426	16425	*dsp-1*	Ascospore	A	73.45	101%	0.7317
06252	14464	*dsp-2*	Ascospore	A	73.23	100%	0.7874
06330	15781	*dsp-3*	Ascospore	A	59.15	81%	0.0004
08158	19644	*dsp-4*	Ascospore	a	72.34	99%	0.7157
09841	18801	*pty-4*	Ascospore	a	74.31	102%	0.3215
02496	16654	*div-12*	Single spore	A	75.13	103%	0.1761
06966	14056	*pty-1*	Ascospore	a	73.53	101%	0.7355

03114	16337	*pph-10*	Ascospore	a	72.73	100%	0.9321
01010	16679	*pty-5*	Ascospore	a	71.38	98%	0.234
03333	17653	*pty-6*	Ascospore	a	72.83	100%	0.9503
Wild type	4200			a	72.9	100%	-

Appendix Table A.1. Continued

NCU Number	FGSC Number	Gene Name	Conidia Formation	Aerial Hyphae Height		
				mm/day	(%WT)	p value
03436	16003	*tng*	Abnormal (swollen, lysed)	5.73	50%	0.0056
06563	11547	*pp2A*	Reduced	7.63	66%	0.1163
07489	11548	*pzl-1*	Slightly reduced	3.05	27%	0.0025
08301	12454	*pph-4*	Normal	5.38	47%	0.0195
06630	17800	*pph-1*	Normal	12.06	105%	0.8036
01433	15790	*ppt-1*	Normal	12.29	107%	0.6941
00958	19378	*pph-7*	Normal	12.16	106%	0.6564
01767	12451	*pph-5*	Normal	7.56	66%	0.0865
03495	16431	*pph-6*	Normal	9.97	86%	0.5308
04600	11185	*pph-8*	Slightly reduced	7.48	65%	0.0453
08948	18439	*pph-11*	Reduced (lysed)	5.56	48%	0.0045
08380	20306	*csp-6*	Reduced (puffed)	6.35	55%	0.0152
02257	16060	*pty-2*	Normal	8.66	75%	0.09
05364	12444	*pty-3*	Normal	11.97	104%	0.8184
03246	13311	*cdc-14*	Slightly reduced	8.71	75%	0.0875
03426	16425	*dsp-1*	Normal	8.95	78%	0.0985
06252	14464	*dsp-2*	Reduced	7.07	62%	0.0353
06330	15781	*dsp-3*	Normal	9.38	82%	0.4924
08158	19644	*dsp-4*	Normal	9.82	85%	0.6289
09841	18801	*pty-4*	Normal	9.12	79%	0.4043
02496	16654	*div-12*	Normal	10.66	93%	0.6672
06966	14056	*pty-1*	Slightly reduced	12.03	105%	0.7342
03114	16337	*pph-10*	Normal	9.11	79%	0.0699
01010	16679	*pty-5*	Slightly reduced	8.73	76%	0.3094

03333	17653	*pty-6*	Normal	9.88	86%	0.3605
Wild type	4200		Normal	11.5	100%	-

Appendix Table A.1 Continued

NCU Number	FGSC Number	Gene Name	Protoperithecia Formation	Perithecia Formation	Ejected ascospores
03436	16003	*tng*	Few or abnormal	Few	None
06563	11547	*pp2A*	None	None	None
07489	11548	*pzl-1*	Many	Normal	Normal
08301	12454	*pph-4*	Abnormal/Small	Normal	Few
06630	17800	*pph-1*	Normal	Normal	Normal
01433	15790	*ppt-1*	Normal	Normal	Normal
00958	19378	*pph-7*	Normal	Normal	Normal
01767	12451	*pph-5*	Normal	Normal	Normal
03495	16431	*pph-6*	Normal	Normal	Normal
04600	11185	*pph-8*	Many	Many,Embedded	Few and small
08948	18439	*pph-11*	Few	Few	Few
08380	20306	*csp-6*	Few and small	None	None
02257	16060	*pty-2*	Normal	Normal	Normal
05364	12444	*pty-3*	Few	Few	Delayed
03246	13311	*cdc-14*	Normal	Normal	Delayed
03426	16425	*dsp-1*	Normal	Normal	Normal
06252	14464	*dsp-2*	Few	Few	None
06330	15781	*dsp-3*	Many	Many	Many
08158	19644	*dsp-4*	Abnormal or large	Normal	Many
09841	18801	*pty-4*	Few	Normal	Normal
02496	16654	*div-12*	Few	Few	Delayed
06966	14056	*pty-1*	Normal	Normal	Normal
03114	16337	*pph-10*	Few	Few	Delayed
01010	16679	*pty-5*	Normal	Normal	Normal
03333	17653	*pty-6*	Normal	Normal	Normal

Wild type	4200	Normal	Normal	Normal

[1] Source reflects whether homokaryotic mutant was isolated from sexual cross (ascospore) or serial plating of conidia (single spore)
[2] p value reflecting significance levels for different chemicals.
The dark highlighting signifies $p<0.05$, while underlined values are $p<0.1$

Appendix Table A.2. Detailed chemical sensitivity phenotypes

NCU Number	FGSC Number	Gene	Sodium Chloride		Sorbitol		Cytochlasin A		Benomyl	
			%growth*	p value[2]	% Growth[1]	p value[2]	% Growth[1]	p value[2]	% Growth[1]	p value[2]
06563	11546	*pp2a*	38%	0.0014	40%	0.0001	79%	0.3775	90%	0.0026
07489	11548	*pzl-1*	69%	0.0357	86%	0.0184	75%	0.8778	85%	0.0305
08301	12454	*pph-4*	59%	0.7325	57%	0.2758	80%	0.0176	78%	0.1703
06630	17800	*pph-1*	63%	0.4215	47%	0.0759	83%	0.0185	58%	0.4039
01433	15790	*ppt-1*	57%	0.8994	71%	0.5828	72%	0.6603	65%	0.9519
00958	19378	*pph-7*	55%	0.806	67%	0.9382	72%	0.4844	61%	0.7181
01767	12451	*pph-5*	55%	0.8609	69%	0.8024	73%	0.6782	66%	0.8911
03495	16430	*pph-6*	45%	0.094	63%	0.6002	73%	0.7007	67%	0.7733
08380	20306	*csp-6*	39%	0.0024	69%	0.753	91%	0.4554	91%	0.0262
02257	16060	*pty-2*	61%	0.5653	67%	0.9296	74%	0.8683	71%	0.5493
05364	12444	*pty-3*	49%	0.3297	36%	0.0012	78%	0.1261	63%	0.9342
03246	13311	*cdc-14*	65%	0.3514	81%	0.0265	80%	0.1356	92%	0.0042
03426	16425	*dsp-1*	52%	0.5139	62%	0.4927	81%	0.1006	80%	0.1211
06252	14464	*dsp-2*	55%	0.8431	44%	0.0114	72%	0.7369	53%	0.1628
06330	15781	*dsp-3*	50%	0.3208	70%	0.6945	73%	0.9045	66%	0.8609
08158	19644	*dsp-4*	59%	0.8139	55%	0.5251	73%	0.8289	58%	0.4855
09841	18801	*pty-4*	58%	0.7726	66%	0.9672	74%	0.9634	68%	0.8511
02496	16654	*div-12*	65%	0.4045	63%	0.6257	69%	0.308	60%	0.5254
06966	14056	*pty-1*	54%	0.7393	69%	0.7348	74%	0.9487	67%	0.8264
03114	16337	*pph-10*	68%	0.5731	74%	0.2445	83%	0.0232	78%	0.2791
01010	16679	*pty-5*	53%	0.6039	65%	0.847	63%	0.5164	71%	0.2985
00333	17653	*pty-6*	59%	0.7748	65%	0.887	74%	0.8531	75%	0.1365

Wild type	4200		56%	-	66%	-	75%	-	64%	-

Appendix Table A.2. Continued

NCU Number	FGSC Number	Gene	Tert-butyl hydroperoxide		Menadione		FK506	
			% Growth[1]	p value[2]	% Growth[1]	p value[2]	% Growth[1]	p value[2]
06563	11546	*pp2a*	15%	6.00E-14	18%	0.0001	70%	0.6396
07489	11548	*pzl-1*	28%	0.0001	26%	0.0102	72%	0.0037
08301	12454	*pph-4*	11%	2.00E-13	29%	0.0303	69%	0.1988
06630	17800	*pph-1*	52%	0.5368	43%	0.6713	62%	0.3863
01433	15790	*ppt-1*	59%	0.0482	43%	0.5996	57%	0.1488
00958	19378	*pph-7*	54%	0.333	33%	0.4079	64%	0.7078
01767	12451	*pph-5*	58%	0.0186	45%	0.4216	67%	0.7114
03495	16430	*pph-6*	14%	6.00E-11	32%	0.2946	55%	6.00E-06
08380	20306	*csp-6*	30%	0.0001	38%	0.8382	91%	0.0289
02257	16060	*pty-2*	57%	0.0307	37%	0.8131	81%	0.0002
05364	12444	*pty-3*	58%	0.0635	35%	0.4444	60%	0.7934
03246	13311	*cdc-14*	50%	0.8312	45%	0.2563	85%	9.00E-09
03426	16425	*dsp-1*	64%	0.0003	43%	0.5268	66%	0.8715
06252	14464	*dsp-2*	4%	3.00E-14	32%	0.0973	61%	0.2361
06330	15781	*dsp-3*	57%	0.3237	40%	0.8633	61%	0.1396
08158	19644	*dsp-4*	48%	0.9683	40%	0.8787	65%	0.9948
09841	18801	*pty-4*	57%	0.2132	53%	0.0032	69%	0.0349
02496	16654	*div-12*	55%	0.0944	44%	0.4046	78%	0.1308
06966	14056	*pty-1*	37%	0.1718	44%	0.5161	64%	0.8111
03114	16337	*pph-10*	51%	0.809	31%	0.1265	69%	0.1981
01010	16679	*pty-5*	57%	0.3471	44%	0.4742	72%	0.2083

00333	17653	*pty-6*	50%	0.8564	46%	0.1525	72%	0.0113
Wild type	4200		48%	-	39%	-	65%	-

Appendix Table A.2. Continued

NCU Number	FGSC Number	Deleted Gene	Fludioxonil		Yeast Extract		Avicel	
			% Growth[1]	p value[2]	% Growth[1]	p value[2]	% Growth[1]	p value[2]
06563	11546	*pp2a*	63%	0.0006	117%	0.5114	41%	0.0812
07489	11548	*pzl-1*	51%	0.7171	76%	0.0159	58%	0.6671
08301	12454	*pph-4*	52%	0.8523	74%	0.0102	78%	0.0048
06630	17800	*pph-1*	60%	0.0625	101%	0.6244	71%	0.0574
01433	15790	*ppt-1*	47%	0.2192	104%	0.7661	69%	0.0578
00958	19378	*pph-7*	46%	0.0386	112%	0.8047	55%	0.949
01767	12451	*pph-5*	53%	0.9504	96%	0.4255	55%	0.9258
03495	16430	*pph-6*	49%	0.2503	115%	0.7094	55%	0.8624
08380	20306	*csp-6*	44%	0.0113	121%	0.514	59%	0.4642
02257	16060	*pty-2*	59%	0.1767	107%	0.9559	63%	0.4228
05364	12444	*pty-3*	47%	0.4789	106%	0.8633	57%	0.7882
03246	13311	*cdc-14*	49%	0.2604	99%	0.5037	70%	0.1266
03426	16425	*dsp-1*	52%	0.9464	101%	0.6113	58%	0.5086
06252	14464	*dsp-2*	61%	0.5242	104%	0.8008	77%	0.0032
06330	15781	*dsp-3*	56%	0.6457	103%	0.6895	61%	0.4787
08158	19644	*dsp-4*	57%	0.1793	95%	0.3208	86%	0.2322
09841	18801	*pty-4*	50%	0.3961	99%	0.4656	66%	0.2397
02496	16654	*div-12*	66%	0.0434	101%	0.6301	71%	0.0732
06966	14056	*pty-1*	56%	0.3462	108%	0.9887	68%	0.4165
03114	16337	*pph-10*	54%	0.8357	105%	0.8217	64%	0.4632
01010	16679	*pty-5*	58%	0.4315	101%	0.5925	64%	0.2781

00333	17653	*pty-6*	55%	0.6326	103%	0.7573	64%	0.2263
Wild type	4200		53%	-	108%	-	54%	-

Appendix B.

Overview

The following sets of figures and tables are relevant information in connection to chapter 3. Growth of *cpc-2* knockout was tested on sodium chloride, sorbitol, SDS as well as the Caspofungin to test for defects in being able to overcome cell wall stress. No significant change was found between the mutant and wild type *Neurospora.* Differences in gene expression for *cpc-2, cpc-1, arg-3, trp-3* and *his-3* (post 3-AT stress) were analyzed from the Tian *et al,* 2007 paper and graphed to provide a visual analyses and comparison to Chapter 3's northern analyses. The upregulation of *cpc-1, arg-3, his-3* and *trp-3* after 3-AT treatment was similar to the Northern analyses but *cpc-2* levels did not change in the northerns unlike the microarray data from Tian *et al,* 2007. Lastly, in order to provide further support to the hypothesis that CPC-1 is post-translationally modified, we used immunoprecipitation experiments to pull-down CPC-1-V5 in a high salt conditions to reveal whether the higher molecular weight band for CPC-1 (at 50 kDa) would remain unchanged. Indeed there was no change in the size of modified-CPC-1.

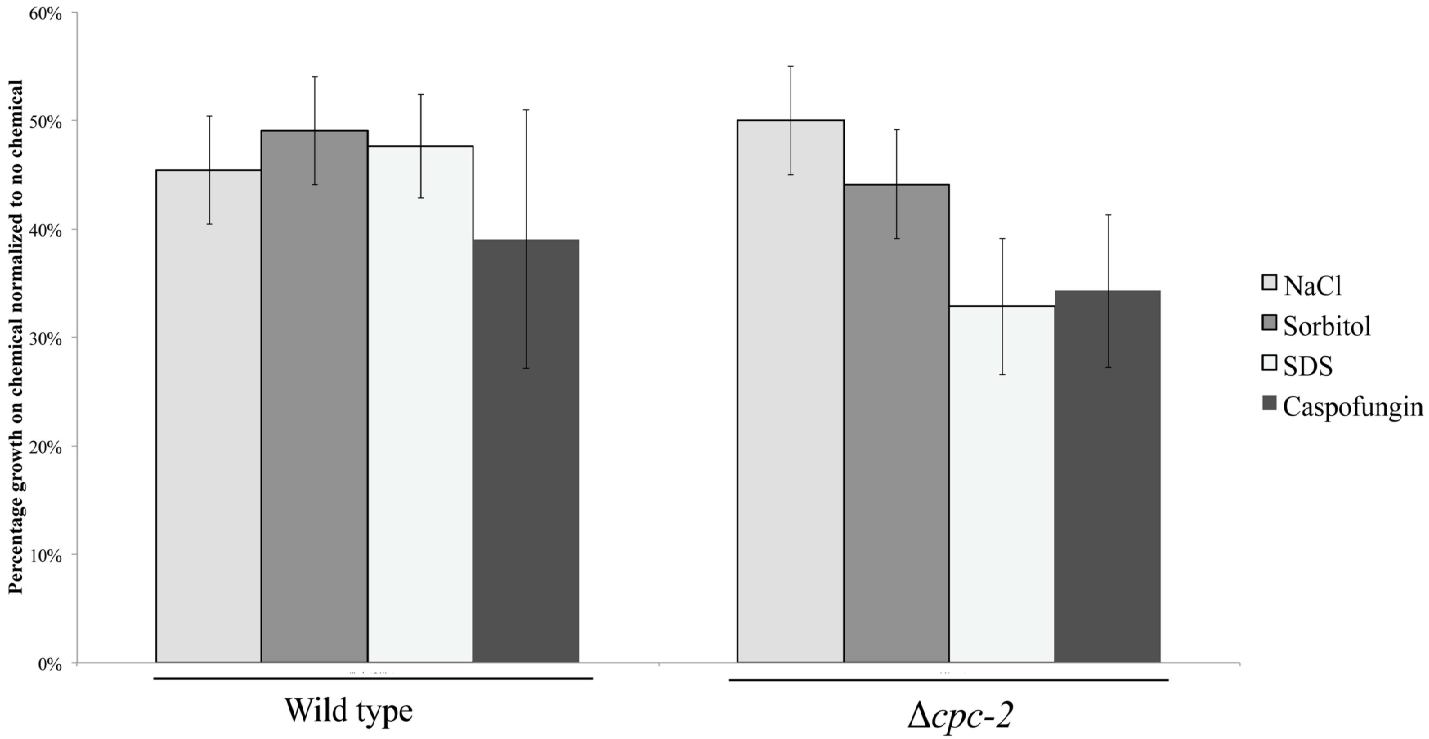
Materials and Methods –

Immunoprecipitation

For pulldown of CPC-1-V5 using V5 beads a previously well established protocol was used (1). The western analyses for CPC-1-V5 extracts was conducted as described in Chapter 3 using a V5 antibody (Bethyl laboratories, Montgomery, TX) at 1:2000 dilution in TBS-T (as in chapter 3) with 5% BSA. Secondary antibody incubation and chemilumiscence was carried out as described earlier.

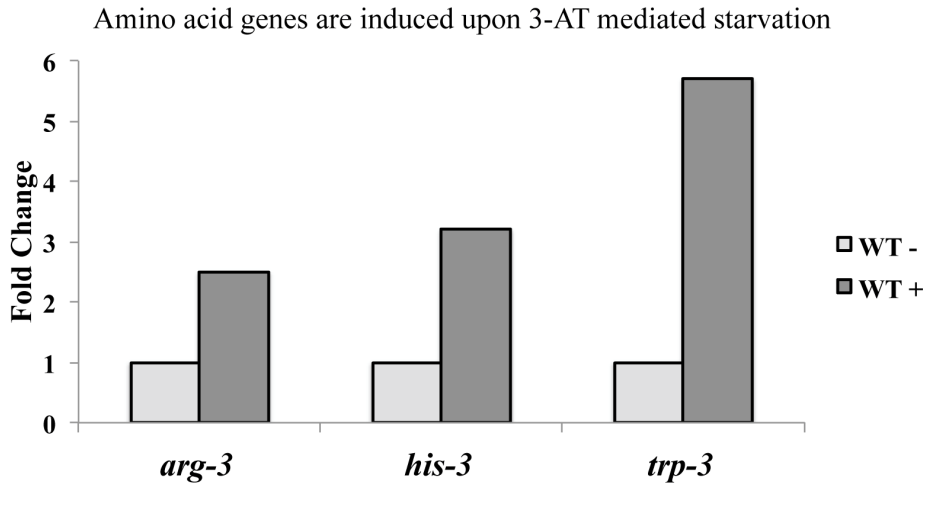
References:

1. Won, S., Michkov, A. V., Krystofova, S., Garud, A. V., and Borkovich, K. A. (2012) Genetic and physical interactions between Galpha subunits and components of the Gbetagamma dimer of heterotrimeric G proteins in *Neurospora crassa*. *Eukaryotic cell* **11**, 1239-1248

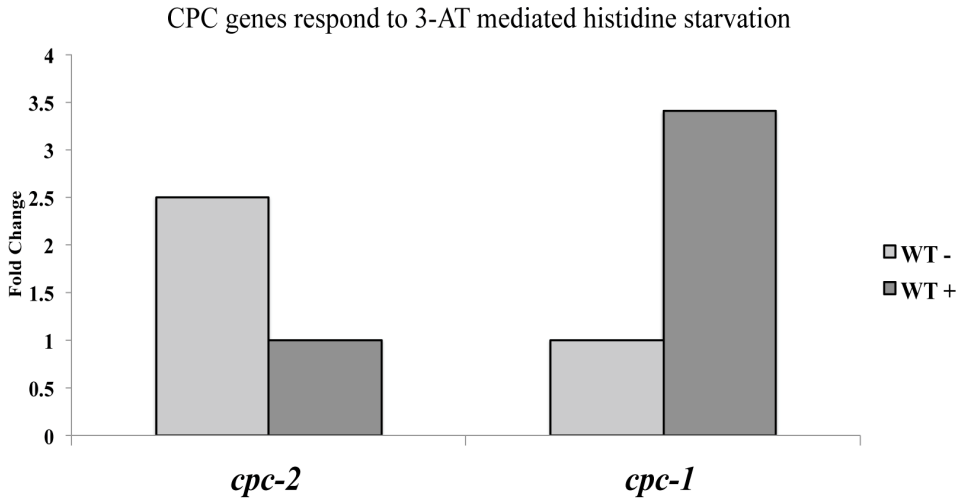

60%
50%
40%
30%
20%
10%
0%
Percentage growth on chemical normalized to no chemical
NaCl
Sorbitol
SDS
Caspofungin
Wild type
Δcpc-2

Appendix figure B.1. Growth of Δ*cpc-2* under cell wall stress conditions. The mutant Δ*cpc-2* was grown on 0.35 M sodium chloride (NaCl), 0.8 M Sorbitol, 0.005% SDS and 0.25 μg/ml Caspofungin containing VM agar media for 24 h at 30 C in the dark. Extension of basal hyphae was measure the next day in mm and percent growth was calculated for the indicated chemical compared to no chemical (VM only). Percent growth is representative of three independent experiments.

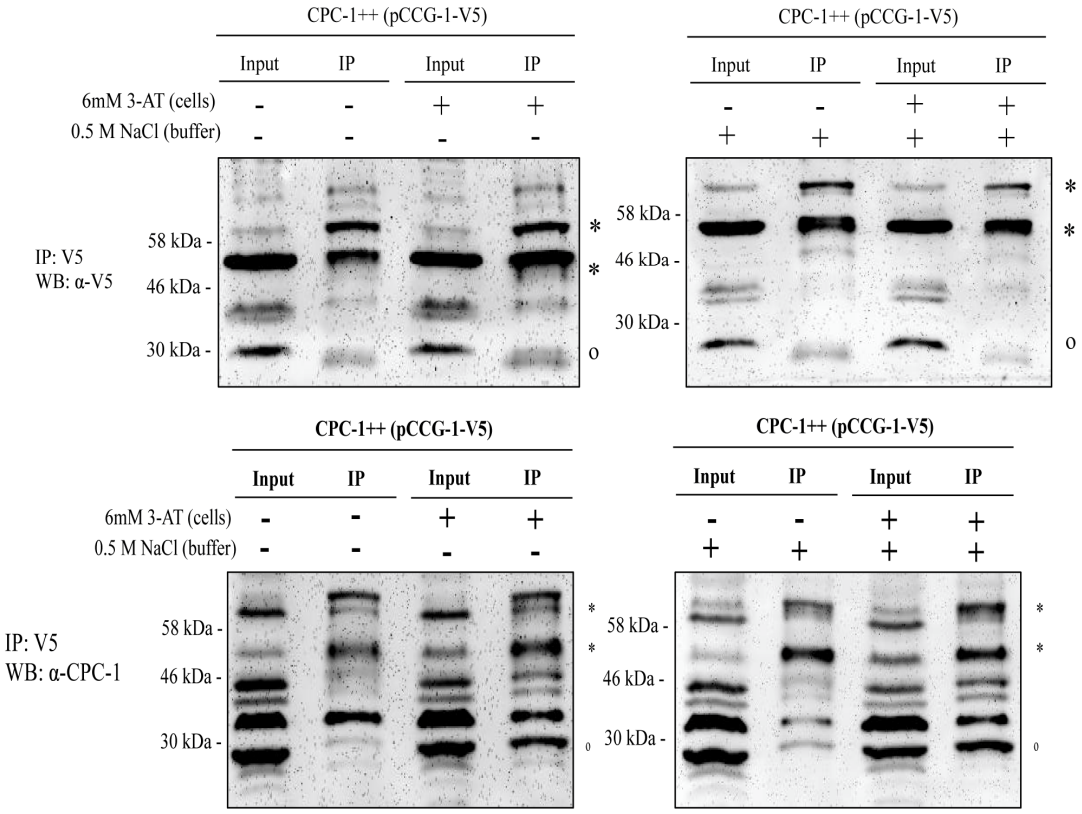
A.


Amino acid genes are induced upon 3-AT mediated starvation
Fold Change
6
5
4
3
2
1
0
arg-3
his-3
trp-3
WT -
WT +

B.


CPC genes respond to 3-AT mediated histidine starvation
Fold Change
4
3.5
3
2.5
2
1.5
1
0.5
0
cpc-2
cpc-1
WT -
WT +

Appendix figure B.2. Changes in amino acid gene expression upon 3-AT treatment from Tian *et al,* 2007. A. Microarray data from Tian *et al,* 2007 shows fold change in amino acid genes *arg-2, his-3* and *trp-3.* The genes are upregulated after 3-AT treatment in wild type (FGSC 4200, *mat a*). **B.** Fold change in *cpc-1* and *cpc-2* levels in wild type cells after 3-AT treatment.


CPC-1++ (pCCG-1-V5)
Input
IP
Input
IP
6mM 3-AT (cells)
0.5 M NaCl (buffer)
IP: V5
WB: α-V5
58 kDa -
46 kDa -
30 kDa -
*
*
o
CPC-1++ (pCCG-1-V5)
IP: V5
WB: α-CPC-1

Appendix figure B.3. Salt treatment does not affect higher molecular weight form of CPC-1. Cells from a CPC-1-V5 tagged strain were treated with 3-AT as previously described and CPC-1-V5 was immunoprecipitated with or without high salt conditions (0.5 M NaCl). Western analyses were conducted using anti-V5 antibody and an anti-CPC-1 antibody to detect whether high salt treatment would destabilize the higher molecular weight form of CPC-1 (~50 kDa).

Appendix Table B.1. List of strains used in this study

Strain	NCU	Genotype	Comment	Source/Reference
74-OR23-IVA	-	Wild type, mat A	Wild type strain	FGSC 2489
c2	NCU05810	Δ*cpc-2*:*hph*$^+$, *mat a*	Scaffolding protein/RACK1 homolog knockout	This study
790-c1	NCU04050	Δ*cpc-1*:*hph*$^+$, *mat A*	bZIP transcription factor knockout	FGSC 9011
c3	NCU01187	Δ*cpc-3*:*hph*$^+$, *mat a*	eIF2α kinase	FGSC17934
#22c2c1	-	Δ*cpc-1*Δ*cpc-2*:*hph*$^+$, *mat a*	Double knockout *(cpc-2, cpc-1)*	This study
51-4-1	-	Δ*rid-1*::*nat*; Δ*mus-51*::*nat*, *mat a*	Recipient strain	[a]Ouyang et al., manuscript in preparation
2994	-	Δ*pan-2*::P_{ccg-1}::V5::*gfp*::*bar*, *mat a*	Empty vector control	[a]Ouyang et al., manuscript in preparation
CPC-2-GFP	-	Δ*pan-2*::P_{ccg-1}::CPC-2::V5::*gfp*::*bar*, *mat a*	V5-GFP tagged CPC-2 (pantothenate auxotroph)	This study
CPC-1-V5	-	Δ*pan-2*::P_{ccg-1}::CPC-1::V5::*gfp*::*bar, mat a*	V5-tagged CPC-1 (pantothenate auxotroph)	This study

Appendix Table B.2: Submerged culture phenotypes for cross pathway control knockouts

Strain	Submerged culture conidiation
Wild type	No
Δ*cpc-2*	Yes
Δ*cpc-1*	No
Δ*cpc-2*Δ*cpc-1*	Reduced

Appendix Table B.3: List of primers used in this study

A. Primers for northern probes	
Primer name	Sequence (5'-3')
F-orf-*arg3*	CGACAAGTGGTTCCTCTACAAGC
R-orf-*arg3*	GTAACCAACCTCCTCAGCAAAC
F-orf-*his3*	CCTCGATGGGACGAGAGAAACG
R-orf-*his3*	GGACTGGAGATGCTAAGGGTAAG
F-orf-*trp3*	CCACACTGGTTCCCACAAGATC
R-orf-*trp3*	GCTCGGTATCCTTCCAGTTGG
F-orf-*cpc1*	CTGCCAACCAGACATACGATC
R-orf-*cpc1*	GCAACGACATCGGAAGGATC
F-orf-*cpc2*	CGTCCTGCAAGCTCCAGACTG
R-orf-*cpc2*	GACATGACACCCCAGGCACG
F-*arg2*	CAGTACAGCCACTGGACTGCTGTTC
R-*arg2*	GAAGAGTCCATGGGACCACCCTTAG
B. Primers for construct design	
Primer name	Sequence (5'-3')
F-C2	CCACTTTCACAACCCCTCACATCAACCAAAATGGCTGAGCAACTCATCCTCAAG
R-C2-V5G	GTTAGGGATAGGCTTTCCGCCGCCTCCGCCAGCGCGGGACATGACACCCCAGGC
FWD-pCCG1-C1	CCACTTTCACAACCCCTCACATCAACCAAAATGTTCTCAGAACTGGACCTGCTGG
REV-C1-V5yrc	GTTAGGGATAGGCTTTCCGCCGCCTCCGCCCTCCGTAGACGCACCGTGCGCCAAAG
REV-V5-BARgfpcut	GCTCCTTCAATATCATCTTCTGTCGATTACGTAGAATCGAGACCGAGGAGAGGG

Appendix Table B.4. Prediction of SUMOylation sites on CPC-1 protein

Prediction Software	Group	Position	Score / Confidence level	Method	Reference
SUMOplot™ Analysis Program	LEELE AKIE ELIAE	K247	0.79	1.Direct amino acid match to SUMO-consensus sequence. 2.Substitution of the consensus amino acid residues with amino acid residues exhibiting similar hydrophobicity.	AbGent™
seeSUMO	LEELE AKIE ELIAE	K247	85.26 (Threshold=0)	Random Forest	Teng *et al.*, Amino Acids. 2012
SUMOsp 2.0 (GPS-SUMO)	LE AKIE E	K247	0.441 (Threshold=High)	4th generation GPS algorithm, particle swarm optimization	Zhao Q *et al*, Nucleic Acids Res, 2014