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Stela Virgilio

Regulation of reserve carbohydrates metabolism in *Neurospora crassa*: responses to pH, calcium and carbon source stresses and to the biological clock

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STELA VIRGILIO

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Thesis submitted to Instituto de Química, Universidade Estadual Paulista, Programa de Pós-Graduação em Biotecnologia, for fulfillment of the requirements for the degree of Doctor in Biotechnology

Advisor: Prof. Dr. Maria Célia Bertolini

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Regulação do metabolismo de carboidratos de reserva em *Neurospora crassa*: respostas a estresses de pH, cálcio e fonte de carbono e ao relógio biológico

Tese apresentada ao Instituto de Química, Universidade Estadual Paulista, Programa de Pós-Graduação em Biotecnologia, como parte dos requisitos para obtenção do título de Doutor em Biotecnologia

Orientadora: Profª. Dr. Maria Célia Bertolini

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Araraquara, 20 de setembro de 2016.

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4. Abstracts presented in Conferences

4.1 International Conferences

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Brazilian Society for Biochemistry and Molecular Biology (SBBq), Foz do Iguaçu, PR. Abstracts Book, p. 294, 2015.

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CUPERTINO, F. B.; VIRGILIO, S.; FREITAS, F. Z.; CANDIDO, T. S.; BERTOLINI, M. C. The transcriptional repressor CRE-1 regulates glycogen metabolism in *Neurospora crassa*. In: 27th Fungal Genetics Conference, Pacific Grove, CA. Fungal Genetics Reports 60(Suppl), p. 223-224, 2013.

4.2 Nacional Conferences

VIRGILIO, S. A conexão entre relógio biológico e o rítmo do metabolismo de glicogênio no fungo *Neurospora crassa*. Abordagens biológicas e modelagens matemáticas. In: VII Simpósio de Microbiologia, São José do Rio Preto, SP. VII Simpósio de Microbiologia - Microbiologia Industrial e Aplicada, 2015.

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5. Oral Presentation

VIRGILIO, S. Correlação entre relógio biológico e metabolismo nos diferentes organismos. Instituto de Química, UNESP, Araraquara, SP, 2015.

VIRGILIO, S.; IBARRA, O.; BELL-PEDERSEN, D.; BERTOLINI, M. C. Correlação entre relógio biológico e o metabolismo de glicogênio no fungo *Neurospora crassa*: da modelagem matemática aos experimentos biológicos. In: V Congresso Farmacêutico da UNESP, Araraquara, SP. Revista de Ciências Farmacêuticas Básica e Aplicada: Supl.1 BB 10, v. 36, 2015 **(award)**.

VIRGILIO, S.; IBARRA, O.; BONI, A. C.; BELL-PEDERSEN, D.; BERTOLINI, M. C. Connection between glycogen metabolism regulation, light and the circadian clock in *Neurospora crassa*. In: Neurospora meeting, Pacific Grove, CA. Neurospora 2014 Program and Abstracts, p. 37, 2014.

6. Academic Experiences

6.1 Scientific Supervisor

2015-2016: Amanda Ventura Campos Araujo. Caracterização do fator de transcrição VOS-1 em resposta a diferentes condições de estresse e sua relação com o acúmulo de glicogênio em *Neurospora crassa*. Undergraduate student, FAPESP fellowship - Instituto de Química, UNESP, Araraquara.

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2015: Molecular Biology (75 h)

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2015-2016: Graduated Representation in the Comissão Permanente de Pesquisa

2015-2016: Graduated Representation in the Comissão de Biblioteca

2014-2016: Graduated Representation in the Conselho de Pós-Graduação em Biotecnologia

7. Awards

2015: Honor mention for 3rd position in PósMicro Scientific Text Competition, Graduation and Researcher Category, with text "A microbiologia no avanço biotecnológico", Instituto de Biociências, Letras e Ciências Exatas - IBILCE/UNESP.

2015: Award "Profª Dra Márcia da Silva" for 1st oral scientific work position in V Congresso Farmacêutico da UNESP and I Jornada de Engenharia de Bioprocessos e Biotecnologia, Faculdade de Ciências Farmacêuticas - UNESP Araraquara.

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I dedicate this thesis to God,
to my parentes Celso e Rita,
my sister Letícia and
my boyfriend Alisson,
people that I love so much!

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“If you want different results, do not do the same things” (Albert Einstein)

“The important thing is to not stop questioning. Curiosity has its own reason for existing. One cannot help but be in awe when he contemplates the mysteries of eternity; of life; of the marvelous structure of reality…”
(Albert Einstein)

ABSTRACT

The fungus *Neurospora crassa*, a model organism in studies of gene expression, metabolism, photobiology and circadian rhythm, is able to respond and adapt to different environmental stresses, such as heat shock, pH changes, nutrient limitation, osmotic stress, and others. Besides that, *N. crassa* has the genome sequenced and collections of knocked-out strains are avalaible to the scientific community. A systematic screening analysis performed with mutant strains in genes encoding transcription factors led to identify proteins involved in the glycogen metabolism regulation in this fungus. Glycogen and trehalose are storage carbohydrates that functions as a carbon and energy reserve. Trehalose can also protect membranes and proteins, increasing the tolerance to adverse conditions. In this work, some transcription factors were functionally characterized regarding their role in glycogen and trehalose metabolism regulation. The first condition investigated was the influence of the circadian clock in the glycogen metabolism. We observed that the glycogen accumulation and the expression of genes encoding glycogen synthase (*gsn*) and glycogen phosphorylase (*gpn*) are rhythmic in a wild-type strain and dependent on the FREQUENCY (FRQ) oscillator, the core component of the *N. crassa* circadian clock. The VOS-1 transcription factor, that is controlled by clock and can act in the connection between clock and glycogen metabolism, binds to *gsn* and *gpn* promoters rhythmically. However, the expression of *gsn* and *gpn* and the glycogen accumulation are still rhythmic in Δ*vos-1* strain, suggesting that not only VOS-1 but additional transcription factors could contribute to glycogen accumulation rhythmicity. Under pH and calcium stress, the PAC-3 transcription factor was investigated. First, we characterized the protein components of the pH signaling pathway, using the Δ*pal* and Δ*pac-3* mutant strains. The mutants present high melanin production and inability to grow under alkaline pH. PAC-3 undergoes only one proteolytic cleavage, binds to *pal* promoters and regulates the expression of some *pal* genes under alkaline pH. PAC-3 is predominantly nuclear under alkaline condition and is able to bind to importin-α *in vitro*. Moreover, the components of pH signaling showed high glycogen and trehalose accumulation under normal and alkaline pH when compared to the wild-type. PAC-3 binds to some glycogenic and trehalose genes, and regulates their expression. Under calcium stress, *pac-3* was induced and the carbohydrates metabolism was differently regulated. Finally, the CRE-1 transcription factor and the RCO-1 and RCM-1 cofactors, orthologs of the Mig1-Tup1-Ssn6 yeast complex, respectively, were investigated regarding their regulation of the glycogen metabolism under different carbon sources. CRE-1 is involved in catabolic repression and plays a role as repressor in glycogen regulation. CRE-1 binds *in vivo* and *in vitro* to *gsn* and *gpn* promoters, regulating their expression. This transcription factor is present in nucleus and cytoplasm in derepressed and starved conditions. RCO-1 and RCM-1 also regulated the glycogen accumulation, the glycogen synthase activity and the expression of some glycogenic genes, but do not play a major role in glycogen metabolism, while CRE-1 is the central regulator.

Keywords: glycogen, trehalose, transcription factor, circadian clock, gene expression

RESUMO

O fungo *Neurospora crassa*, um organismo modelo em estudos de expressão gênica, metabolismo, fotobiologia e ritmo circadiano, é capaz de responder e se adaptar a diferentes condições de estresse, tais como choque térmico, alterações de pH, limitação de nutrientes, estresse osmótico, entre outras. Além disso, *N. crassa* tem seu genoma sequenciado e coleções de linhagens mutantes estão disponíveis para a comunidade científica. Uma análise sistemática utilizando linhagens mutantes em genes que codificam fatores de transcrição permitiu a identificação de proteínas envolvidas na regulação do metabolismo do glicogênio neste fungo. Glicogênio, juntamente com trealose, são carboidratos de reserva que funcionam como fonte de carbono e energia. A trealose também pode proteger membranas e proteínas, aumentando a tolerância a condições adversas. Neste trabalho, alguns fatores de transcrição foram funcionalmente caracterizados em relação as suas participações na regulação do metabolismo de glicogênio e trealose. A primeira condição investigada foi a influência do relógio circadiano sob o metabolismo de glicogênio. Observamos que o acúmulo de glicogênio e a expressão dos genes codificadores das enzimas glicogênio sintase (*gsn*) e glicogênio fosforilase (*gpn*) foram rítmicos em uma linhagem selvagem do fungo, e dependentes do oscilador FREQUENCY (FRQ), principal componente do relógio de *N. crassa*. O fator de transcrição VOS-1, o qual é controlado pelo relógio e pode atuar na conexão do relógio ao metabolismo de glicogênio, se liga aos promotores *gsn* e *gpn* ritmicamente. Entretanto a expressão dos genes *gsn* e *gpn* e o acúmulo de glicogênio se mantiveram rítmicos na linhagem Δ*vos-1*, sugerindo que além de VOS-1 outros fatores de transcrição poderiam contribuir para a ritmicidade do acúmulo de glicogênio. Sob condições de estresse de pH e cálcio, o fator de transcrição PAC-3 foi investigado. Primeiro, foram caracterizadas as proteínas envolvidas na via de sinalização de pH, usando as linhagens mutantes nos genes *pal* e *pac-3*. Os mutantes apresentam alta produção de melanina e incapacidade de crescer em pH alcalino. PAC-3 sofre uma única clivagem proteolítica, se liga aos promotores dos genes *pal* e regula a expressão de alguns destes genes em meio alcalino. PAC-3 é predominantemente nuclear sob condições alcalinas e é capaz de se ligar à importina-α *in vitro*. Além disso, os componentes de sinalização de pH mostraram acumular mais glicogênio e trealose sob pH normal e alcalino quando comparado à linhagem selvagem. PAC-3 se liga a alguns genes do metabolismo de glicogênio e trealose, regulando-os. Sob estresse de cálcio, a expressão de *pac-3* foi induzida e o metabolismo de carboidratos diferentemente regulado. Finalmente, o fator de transcrição CRE-1 e os cofatores RCO-1 e RCM-1, ortólogos ao complexo Mig1-Tup1-Ssn6 de levedura, respectivamente, foram investigados na regulação do glicogênio sob diferentes fontes de carbono. CRE-1 está envolvido na repressão catabólica e atua como repressor na regulação do glicogênio. CRE-1 se liga *in vivo* e *in vitro* aos promotors *gsn* e *gpn*, regulando suas expressões. Este fator de transcrição está presente no núcleo e no citoplasma em condições de derepressão e baixa fonte de carbono. RCO-1 e RCM-1 também regulam o acúmulo de glicogênio, a atividade glicogênio sintase e alguns genes do glicogênio, mas não desempenham um papel primordial, enquanto CRE-1 mostrou ser um regulador central.

Palavras-chave: glicogênio, trealose, fator de transcrição, relógio circadiano, expressão gênica

SUMMARY

Introduction

1. *Neurospora crassa*: a model fungus

Scientists have long adopted a group of microorganism as models for detailed morphologies, genetics and evolutionaries analyses. Approximately 10% of all know living organisms are fungi, with only 70,000 different species described until now (BLACKWELL, 2011). However, it is estimated that there are around 1.5 million species of fungi (HAWKSWORTH, 2001), and the more recent prediction based on molecular approaches calculates a total of 5.1 million of fungi species (O'BRIEN et al., 2005; TAYLOR et al., 2010). Fungi are found in all niches, some in symbiotic association with plants and algae, many causing diseases, and most others playing a beneficial role in the environment. Some fungi are used for food or fermentation (reviewed in RAJU, 2009). Because fungi constitute the Kingdom most closely related to Animalia, and are exceptionally accessible experimentally, fungi are universally used as model organisms for understanding all aspects of basic cellular regulation (DUNLAP et al., 2007).

The filamentous fungus *Neurospora crassa* has been used as genetic model system since 1930 (SHEAR; DODGE, 1927; LINDEGREN; BEANFIELD; BARBER, 1939), and possesses numerous characteristics that make it a suitable model: simple nutritional requirements; fast vegetative growth; two distinct mating types; haploid vegetative phase; production of ascospores and growth on defined media (DAVIS; PERKINS, 2002). In 1941, Beadle and Tatum demonstrated the relationship between genes and proteins using *N. crassa*; they formulated what became known as the 'one gene, one enzyme' hypothesis. These findings allowed *N. crassa* became a popular experimental model for genome defense mechanisms, metabolism, circadian rhythms, gene expression, regulation of meiotic recombination, signal transduction, responses to light, post-transcriptional gene silencing and biochemistry, genetic and molecular studies (PERKINS, 1992; DAVIS, 2000; DAVIS; PERKINS, 2002; DUNLAP et al., 2007).

Neurospora can grow with only carbon source, mineral salts and one vitamin, biotin, which is absolutely required. The Vogel's salts contain Na_3 citrate, KNO_3, $(NH_4)H_2PO_4$, KH_2PO_4, $MgSO_4$, $CaCl_2$ and trace element. Many carbon sources can be used such as glucose, mannose, fructose, xylose, sucrose, maltose, cellobiose, and trehalose (METZENBERG, 1979). Its optimal growth is around pH 5.4-5.8, but is quite tolerant and can grow in a pH varying from 4.0 to 9.0 (THEDEI JUNIOR;

DOUBOWETZ; ROSSI, 1994), with growth being poor at either extreme. Vegetative growth is optimal at about 30ºC to 35ºC, although it can grow at temperatures as high as 42ºC and as low as 5ºC. Neurospora is an absolute aerobe and is considered completely nonpathogenic to humans, animals, and plants (METZENBERG, 1979).

The first record of *N. crassa* was in 1843, when an orange mould infestation in French bakeries was investigated (PAYEN, 1843; PERKINS, 1991). In nature, *N. crassa* is often found on scorched vegetation after wildfires or agricultural burns (RAJU, 2009). In Brazil, Neurospora was described as an orange fungus growing on burned vegetation (MÖLLER, 1901). The combination of Neurospora and heat is known for a long time. The ascospores are tolerant to high temperatures and remain dormant until they are exposed to heat. Activation of ascospores explains the occurrence of the fungus in bakeries or after wildfires (PERKINS, 1992).

N. crassa is haploid during the vegetative growth, when it is possible to see assexual strutures called macroconidia, or simply conidia, and microconidia, and diploid during a short time in the sexual phase, when it is possible to verify the formation of a fruiting body. As *N. crassa* is heterothallic, it exists in two mating types, designated A and a, and no mating can occur unless both are present. The two mating types are phenotypically indistinguishable. If mating type A was inoculated onto the medium first and the growth allowed for several days, it will be the female. The later arrival will function as male (METZENBERG, 1979). Both asexual development and sexual differentiation are highly influenced by environmental factors including nutrient, light, and temperature (DUNLAP et al., 2007). The *N. crassa* life cycle is shown in the Figure 1.

In the asexual cycle phase, the mycelium is composed of haploid hyphae and the reproduction occurs by conidia produced by two pathways: the macroconidiation and microconidiation (Figure 1). Both pathways require water-air interface and are suppressed in submerged cultures. The multinucleate macroconidia is promoted by apical structures called conidiophores (ADAMS; WIESER; YU, 1998; SPRINGER, 1993; BORKOVICH et al., 2004). The macroconidiation can be induced by environmental signals such as heat shock, desiccation and nutritional limitation (TURIAN; BIANCHI, 1972), and is regulated by the endogenous biological clock in *N. crassa* (LOROS; DUNLAP, 2001). In microconidiation, the microconidia are formed within the basal hypha and expelled from the wall when the maturation process is completed (PERKINS; TURNER; BARRY, 1976) or by microconidiophores

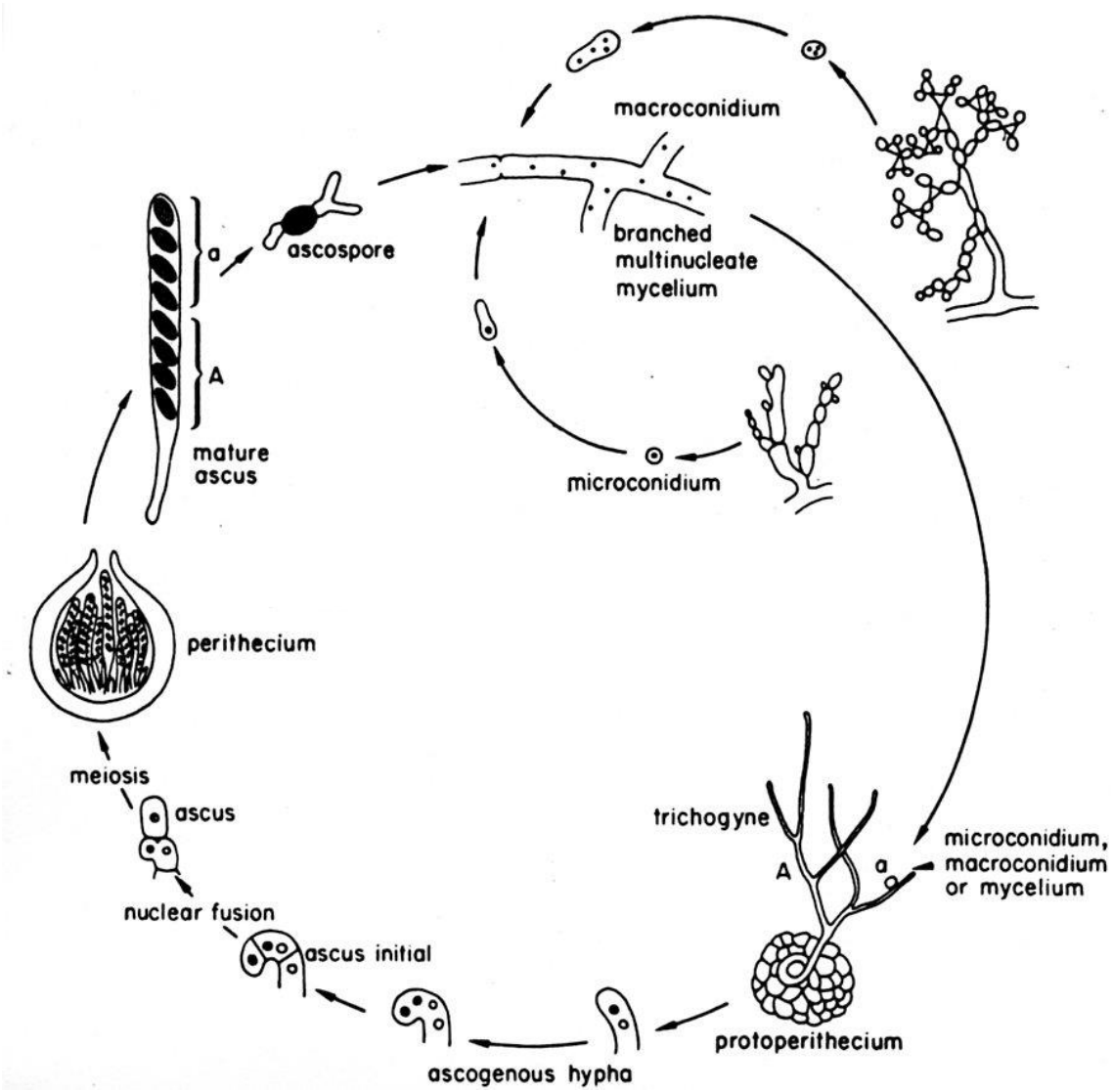

Figure 1- The life cycle of *Neurospora crassa* showing some of the many life stages and cell types in the sexual and asexual cycles (DUNLAP et al., 2007).

(SPRINGER; YANOFSKY, 1989; MAHESHWARI, 1999; BISTIS; PERKINS; READ, 2003). The microconidia are uninucleate and smaller than macroconidia. They are generally produced only in relatively small numbers (METZENBERG, 1979). Another form of asexual propagation is through arthroconidia that are multinucleate hyphae fragments produced by segmentation of pre-existing fungal hyphae (MAHESHWARI, 1999).

The complex sexual reproductive pathway occurs when any structure (A or a) is submitted by nitrogen limitation. In this case, some of the hyphae curl up into a specialized form, the ascogonium, where occurs the formation of a fruiting body called protoperithecium (female structure). The end of a specialized ascogonial hypha is differentiated into a trichogyne. When a conidium, microconidium, or even a piece of mycelium of the opposite mating type lands on the trichogyne, a donor nucleus (male) is conducted into the ascogonium, where it meets the resident nucleus (female). In this phase, the cover mycelium involving the ascogonium begins to develop a wall resulting in a structure called perithecium. When the nuclei of the two mating types fuse, meiosis proceeds promptly. The four meiotic products undergo a mitotic division to give eight sister nuclei (four for each mating type) that will result in ascospores or sexual spores. The maturing black ascospores (or spores) are contained in a sack, the ascus (Figure 1). The spores will germinate and produce hyphae resulting in colonies exactly like those produced by asexual spores (METZENBERG, 1979).

2. The sequencing of the *N. crassa* genome and its consequences

The sequencing of the *N. crassa* genome was reported by Galagan et al. (2003), which has about 40 Mb, much higher when compared to other fungi with known genomes, such as *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. A total of 10,082 protein-coding genes has been predicted with, on average, one gene per 3.7 kilobases and an average of 1.7 short introns (134 bp on average) per gene (HYNES, 2003). Its genome is organized in seven chromosomes ranging from 4 to 10 Mb (SCHULTE et al*.,* 2002) and 44% of genome are represented by genes encoding proteins. When the genome was sequenced, it was reported that only 13% of the genes encoded known proteins, 46% represented ORFs encoding hyphotetical proteins and 41% represented ORFs encoding predicted proteins that

have no significant matches to known proteins. In addition, of 1,421 *N. crassa* genes with highest matches to either plant or animal proteins, a significant number (584) have no high-scoring protein matches in either *S. cerevisiae* or *S. pombe*. Many of these proteins may be involved in determining hyphal growth and multicellular developmental structures in Neurospora, as these characteristics are not found in yeasts, suggesting good relation between filamentous fungi and upper eukaryotes, compared with yeasts and lower eukaryotes (GALAGAN et al., 2003; HYNES, 2003).

New computational analysis of the *N. crassa* genome demonstrated that 40% of genes encode proteins functionally annotated in databases and estimated 27.588 protein-protein interactions among 3,006 proteins, showing that probably each protein has, on average, 18.4 partners (WANG et al., 2011). After the genome sequencing of *N. crassa*, an overall effort was proposed by the scientific community to develop the functional genomic of Neurospora. Four projects were proposed: the generation of knockout constructs (Project 1); the annotation of genes (Project 2); the knockouts analysis by microarrays (Project 3); and cDNA libraries and SNP (Single Nucleotide Polymorphism) MAP generation (Project 4) (DUNLAP et al., 2007).

After the proposed projects, new methodologies were developed to inactivate specific genes (NINOMIYA et al., 2004) and create knockout strains (DUNLAP et al., 2007). However, there are more genes in filamentous fungi as compared to yeasts and different ways in which gene function can be eliminated in Neurospora (DUNLAP et al., 2007). One is RIP (Repeat Induced Point mutation), that utilizes a Neurospora-specific phenomenon in which duplicated sequences, when passed through meiosis, undergo frequent C to T transitions, resulting in loss of total or partial function (ROUNTREE; SELKER, 1997). Based on the natural process of *N. crassa*, inactivation of genes to construct mutant strains by RIP is based on introducing an extra copy of the target gene in the fungus. Transformants containing an additional copy of the gene introduce point mutations in the genome sequence when subjected to cross followed by meiosis (SELKER, 1990). This technique is an effective method, but requires a long time to be developed, and today is used for the partial inactivation of essential genes.

Other process for inactivate genes and generate knockout mutant strains is by replacement through standard homologous recombination. In eukaryotes, there are two mechanisms of genetic recombination: homologous recombination involves interaction between homologous sequences, whereas non-homologous

recombination involves integration of exogenous DNA into any region of the genome, independent of homology. The homologous recombination é predominant in *S. cerevisiae*, but in *N. crassa* the frequency of homologous recombination rarely approaches 100% but lies instead between a few percent and 30% depending on the gene and the length of the homologous DNA flanking the gene in the construct (DUNLAP et al., 2007).

The non-homologous recombination mechanism is evolutionarily conserved, being the predominant mechanism for upper eukaryotes such as humans, plants and insects. Among the proteins involved in this mechanism, Ku70 and Ku80 proteins form a heterodimer that binds to the DNA ends (WALKER; CORPINA; GOLDBERG, 2001). In *N. crassa, mus-51* and *mus-52* are the orthologs genes of human *KU70* and *KU80*, respectively, and a procedure based on PCR was developed to inactivate these genes and, thus, to construct strains in which non-homologous recombination process is ineffective, promoting homologous recombination (NINOMIYA et al., 2004).

The knockout approach allowed the construction of strains containing individual inactivated genes that are available to the scientific community by the Fungal Genetics Stock Center (FGSC, Kansas City, Missouri, USA). In addition, the disruption groups of genes that can form the basis of research projects are available and included, for instance, genes encoding transcription factors, genes encoding protein kinases and phosphatases, and chromatin-remodeling enzymes. In 2006, Colot et al. published the first results relating to mutant strains in genes encoding transcription factors, showing that some transcription factors highly conserved could play different roles in various fungi and 43% of the deletion mutants revealed phenotypes, with more than half of these strains possessing multiple defects.

In 2011, Park et al. published a global morphology analysis using the colletion of mutant strains in genes enconding protein kinases, showing that 71% of the serine-threonine (S/T) kinases mutated were either essential or necessary for normal growth, development, or chemical resistance underscores the central importance of S/T protein kinases to Neurospora biology. The results also revealed important differences between S/T kinases and transcription factors in the regulation of growth and development. A greater number of kinase mutants were defective in at least one phenotype compared to transcription factor mutants and, significantly, more kinase genes (40%) are involved in the regulation of two or more functions, compared to

transcription factors (18%). Thus, the data demonstrate that the impacts of kinases on fungal growth and differentiation are more dramatic than that on transcription factors, likely due to less functional redundancy in the kinases.

The annotation of the *N. crassa* genome and gene sequences has been done by association with the scientific community (Project 2), and gene expression data generated by microarray technology (Project 3) and analysis of EST sequences and SNP maps are going on, and interesting results are being published (DUNLAP et al., 2007). The use of mutant strains has become an interesting material for the early studies on the regulatory mechanism of many biological processes in *N. crassa* by the absent of a unique protein in the specific mutant strain.

3. The biological clock in *N. crassa*

The ability to sense and respond to light is critical for the survival of most organisms. *N. crassa* has been widely used as a model organism for the study of diverse biological processes ranging from metabolism to circadian rhythms and photobiology (BORKOVICH et al., 2004). This fungus is a good model system to further elucidate the connection between the clock and light to metabolism because the main components of the oscillator and input pathways of the clock have been identified and the metabolic process is less complex than in the mammals. The light activates a variety of physiological processes in Neurospora, including the biosynthesis of carotenoid pigment (HARDING; TURNER, 1981), conidiation (KLEMM; NINNEMANN, 1978; LAUTER, 1996), protoperithecium development (DEGLI-INNOCENTI; POHL; RUSSO, 1983) and the resetting of the circadian clock (CHEN; LOROS, 2009). More than 5% of *N. crassa* genes responded to the light stimulus by increasing transcript levels (CHEN et al., 2009). Genes involved in the synthesis of pigments, vitamins, cofactors, secondary metabolism, DNA processing and cellular signaling were found enriched in the early light response (high peak levels after 15-30 min light exposure). In contrast, genes involved in carbohydrate metabolism and oxidation of fatty acid were found enriched in the late light response (high peak levels after 1-2 h light exposure) (CHEN; LOROS, 2009).

The light responses in *N. crassa* are near UV/blue light, suggesting the presence of a photoreceptor dedicated to blue light sensing and signal transduction (CHEN; LOROS, 2009). It was isolated only two fully blind mutants, *wc-1* and *wc-2*,

both GATA family zinc finger transcription fator, that directly regulate the gene activation under light sensing (LINDEN; RODRIGUEZ-FRANCO; MACINO, 1997; COLLETT et al., 2002; LEE; DUNLAP; LOROS, 2003). WC-1 (White Collar-1) has a PAS domain to protein-protein interation (BALLARIO et al., 1996) and a LOV domain to FAD binding, allowing WC-1 to act as blue light photoreceptor (FROEHLICH et al., 2002). The WC-2 protein (White Collar-2) has a PAS domain (LINDEN; MACINO, 1997), and forms an obligate complex with WC-1 resulting in the White Collar Complex (WCC) that binds to specific DNA sequences (FROEHLICH; LOROS; DUNLAP, 2003). After the WCC, VIVID (VVD) is other photoreceptor intensely studied in the fungus, acting as repressor for most of all light-induced gene expression controlled by the WCC (SHRODE et al., 2001; SCHWERDTFEGER; LINDEN, 2003; CHEN et al., 2009). VVD is a small 21 kDa PAS photoreceptor consisting of a LOV domain (ZOLTOWSKI; CRANE, 2008). The activation of gene expression by light is transient and stops after a long exposure to light, when occurs the interation between VVD and WCC (Figure 2) (LAUTER; YANOFSKY, 1993; ARPAIA et al., 1999; HEINTZEN; LOROS; DUNLAP, 2001; SCHAFMEIER; DIERNFELLNER, 2011).

Light is important to synchronize the biological clock with the day-night cycle environmental (SCHAFMEIER; DIERNFELLNER, 2011). The circadian clock is an intrinsic time-tracking system, present in almost all organisms from bacteria to mammals, that provides a mechanism to predict and prepare for changes that occur in the environment, providing an adaptive advantage (DUNLAP; LOROS; DECOURSEY, 2004). The daily rhythms are generated by the biological clock and are manifested by the cyclic expression of genes or the products encoded by genes. Circadian rhythms are coordinated according to exogenous environmental cycles, allowing the species organize the metabolism and behavior appropriately (EDMUNDS, 1987). In *N. crassa*, the circadian clock consists of the WCC, the negative regulator FRQ (FREQUENCY) and FRQ-interacting RNA helicase (FRH) (HEINTZEN; LIU, 2007).

The White-Collar Complex binds to the *frq* promoter and activates gene transcription leading to FRQ accumulation. FRQ interacts with FRH forming the FRQ/FRH complex (FFC), inhibits WCC activities and, therefore, decreases the *frq* transcription (DUNLAP, 1999). Function, activity, turnover, and subcellular localization of clock proteins are tightly post-translationally regulated, and

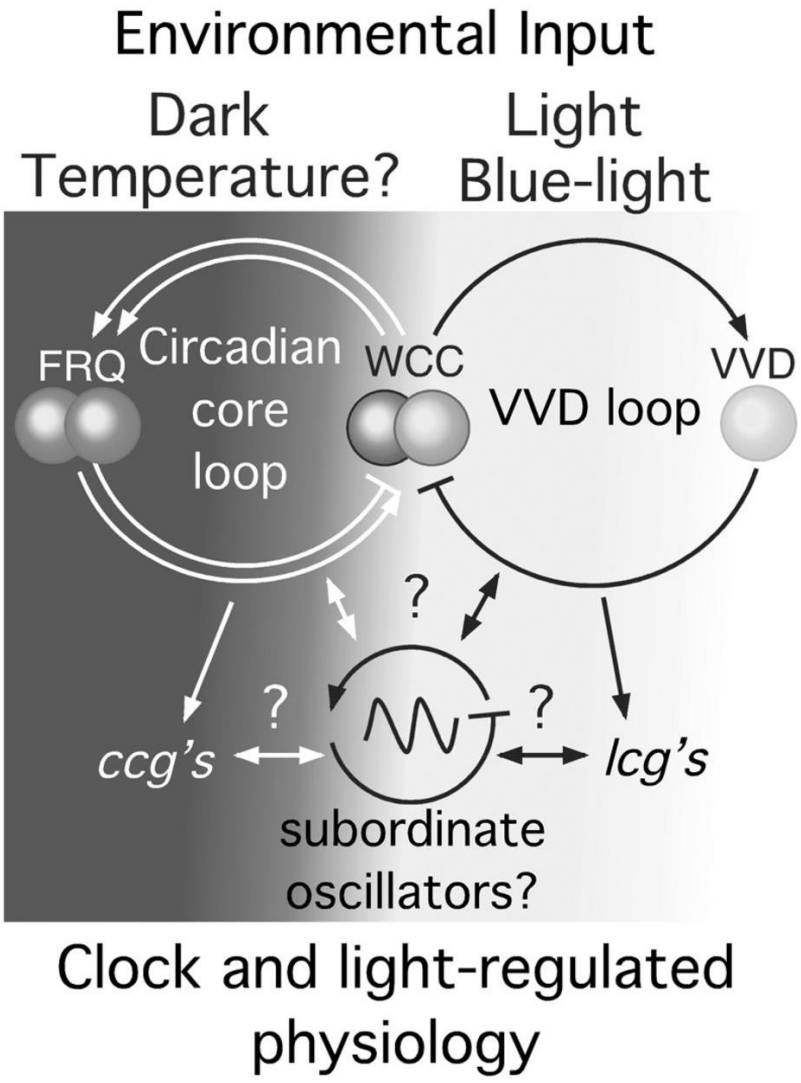

Figure 2- Interactions of the Neurospora light and circadian clock pathways. Simplified model of the Neurospora circadian system: the *frq*, *wc-1* and *wc-2* genes and their encoded proteins interact via a transcription/translation-based feedback loop that is essential for circadian rhythmicity. Light input, via WCC, induces *frq* transcription thus facilitating light-resetting and entrainment of the clock. VVD interferes with light signal-transduction pathways by repressing the WCC activity. Downstream pathways, clock- and light-controlled genes can be regulated. Dark shading indicates pathways that operate in darkness and light shading indicates pathways that are activated in the light (PRICE-LLOYD; ELVIN; HEINTZEN, 2005).

phosphorylation is crucial for clock function (REISCHL; KRAMER, 2011). Over the course of a circadian cycle, FRQ is progressively phosphorylated and then degraded via ubiquitin proteasome pathway (QUERFURTH et al., 2011). As the levels of FRQ decrease, dephosphorylation of WCC by protein phosphatase reactivates the transcription factor, thereby initiating a new circadian cycle (DUNLAP, 1999). Downstream of the clock oscillator and light-input pathways, clock- and light-controlled genes (*ccg* and *lcg*) ultimately control circadian behaviour and light-responses. Additional, subordinate feedback loops may regulate subsets of clock-controlled processes (Figure 2).

Insight into circadian clocks and metabolism started from the discovery that biological rhythms are sustained by a genetically encoded transcription network that functions as a molecular oscillator in most cell types, maintaining phase alignment in a range of behavioral, physiological, and biochemical processes with the environmental light cycle (MARCHEVA et al., 2013). In humans, the clock impacts many aspects of our lives, ranging from the regulation of our sleep/wake cycle, to cell division, and rhythms in gene expression. Therefore, defects in the clock are associated with a wide range of diseases, such as mental disorders and metabolic syndrome (ECKEL-MAHAN; SASSONE-CORSI, 2013; ASHER; SASSONE-CORSI, 2015; ZARRINPAR; CHAIX; PANDA, 2016).

Experiments performed in the last decade have highlighted the importance of connection between the circadian clock and metabolism. In mammals, many genes associated with glucose metabolism in the liver exhibit robust circadian regulation (PANDA et al., 2002; UEDA et al., 2002; MILLER et al., 2007), suggesting that the circadian clock plays a significant role in hepatic glucose metabolism. Hepatic glycogen content, which is important for glucose homeostasis, exhibits circadian rhythm that peaks during the dark-light transition in nocturnal rodents (ISHIKAWA; SHIMAZU, 1976). The activities of glycogen synthase and glycogen phosphorylase also show circadian variation, and the balance between them forms the basis for circadian variation in the hepatic glycogen content (ISHIKAWA; SHIMAZU, 1976). It was shown that CLOCK mouse transcription factor regulates the circadian variation of hepatic glycogen synthesis through the direct transcriptional activation of *Gys2*, the gene encoding the rate-limiting enzyme in glycogenesis (DOI; OISHI; ISHIDA, 2010). Another study has demonstrated the connection between biological clock and diabetes, since the mutants in clock components showed a decrease in rhythmic

oscillation of genes involved in insulin signaling and glucose detection (MARCHEVA et al., 2010).

In *N. crassa*, *ccg-9*, one of the genes controlled by the clock, encoding trehalose synthase that participates in the synthesis of the trehalose, is required for rhythmic conidiation under dark conditions (SHINOHARA et al., 2002). Correa et al. (2003) demonstrated the first correlation between clock and glycogen metabolism. They showed, according to microarray analysis, that several genes encoding enzymes involved in carbon and nitrogen metabolism showed circadian rhythms in mRNA accumulation, with peaks occurring in the late night to early morning. The glycogen phosphorylase and branching enzyme genes was circadianly regulated. Moreover, recently, many genes involved in metabolism have been shown to be clock-controlled (HURLEY et al., 2014). However, there is still much to learn about the connection between the clock and metabolism, particularly in the details of how the oscillator directs metabolic rhythms. Furthermore, the transcriptional network regulating light and clock metabolism response is increasingly being explored, putting *N. crassa* at the forefront of understanding genome wide regulation and the output from the clock.

4. Reserve carbohydrates metabolism: glycogen and trehalose

We have been investigating how *N. crassa* controls the metabolism of glycogen and trehalose, storage carbohydrates that function as a carbon and energy reserve. Glycogen is a branched polymer of glucose found in cells of different organisms (HARRIS, 1997; NELSON; COX, 2008). The glycogen is a uniform molecule characterized by glucose units linked by α-1,4 linear glycosidic bounds and α-1,6 linked glucose at the branching points, that occurs each four glycose units and are responsible to the ramification of the molecule (NELSON; COX, 2008). The synthesis and degradation of this polymer are processes conserved in eukaryotes, and three steps are involved in the synthesis, which are: initiation, elongation, and branching, and requires the activities of glycogenin, glycogen synthase, and the branching enzyme, respectively. The glycogenin is a self-glucosylate protein that uses UDP-glucose (UDPG) as the glucan donor and acts as an initiator molecule for glycogen initiation (FARKAS et al., 1991; CHENG et al., 1995). The glycogen synthase catalyzes the formation of α-1,4 glycosidic bounds, using UDPG as the

donor of the glucose residues and promotes the chain extension (LELOIR, 1971; ALONSO et al., 1995). Finally, the branching enzyme catalyzes the formation of α-1,6 glycosidic bounds, transferring approximately seven glucose residues from the nascent chain to the glucose C6 in an adjacent chain, creating a ramification point (NELSON; COX, 2008).

Degradation of glycogen requires the activities of glycogen phosphorylase, that releases glucose-1-phosphate (G1P) from a terminal α-1,4 glycosidc bond, and the activity of debranching enzyme, that carries out two distinct enzymatic functions: 1) glucosyltransferase, that transfers of three glucose residues from one branch to another, and 2) glucosidase, that breaks α-1,6 glycosidic bounds (NELSON; COX, 2008).

The trehalose, another reserve carbohydrate, is a non-reducing disaccharide composed of two linked glucose by α-1,1 glycosidic bounds and can be synthesized by bacteria, fungi, plants, and invertebrate animals. In fungi, trehalose is present mainly in spores to be further used as carbon and energy source for conidia germination (HANKS; SUSSMAN, 1969; FILLINGER et al., 2001). Trehalose biosynthesis is catalyzed by a large trehalose synthase complex consisting of trehalose-phosphate synthase and trehalose-phosphate phosphatase subunits (BELL et al., 1998). The degradation occurs by two trehalases: neutral trehalase with an optimum activity at pH 6.8-7.0 (also referred to as regulatory trehalase) and acid trehalase with an optimum activity at pH 4.5-5.0 (also referred to as nonregulatory trehalase). It is also considered that the neutral trehalase enzyme is cytosolic, while the acidic trehalose is vacuolar (cited by FRANÇOIS; PARROU, 2001). The synthesis and breakdown processes of glycogen and trehalose in *S. cerevisiae* can be seen in Figure 3.

In overall, the glucose enters the cell via passive transport, mediated by specialized proteins (OLSON; PESSIN, 1996; THORENS, 1996) and converted to glucose-6-phosphate (G6P) in a reaction catalyzed by hexokinase. G6P is first converted to glucose-1-phosphate (G1P) by the enzyme phosphoglucomutase, which serve as substrate for the production of UDPG by the action of UDP-glucose pyrophosphorylase. The UDPG molecules are direct glucose donor residues for the synthesis of glycogen and trehalose (ROACH, 2002; NELSON; COX, 2008). For glycogen synthase extend the molecule is necessary the presence of an oligosaccharide containing at least eight glucose residues, which is provided by the

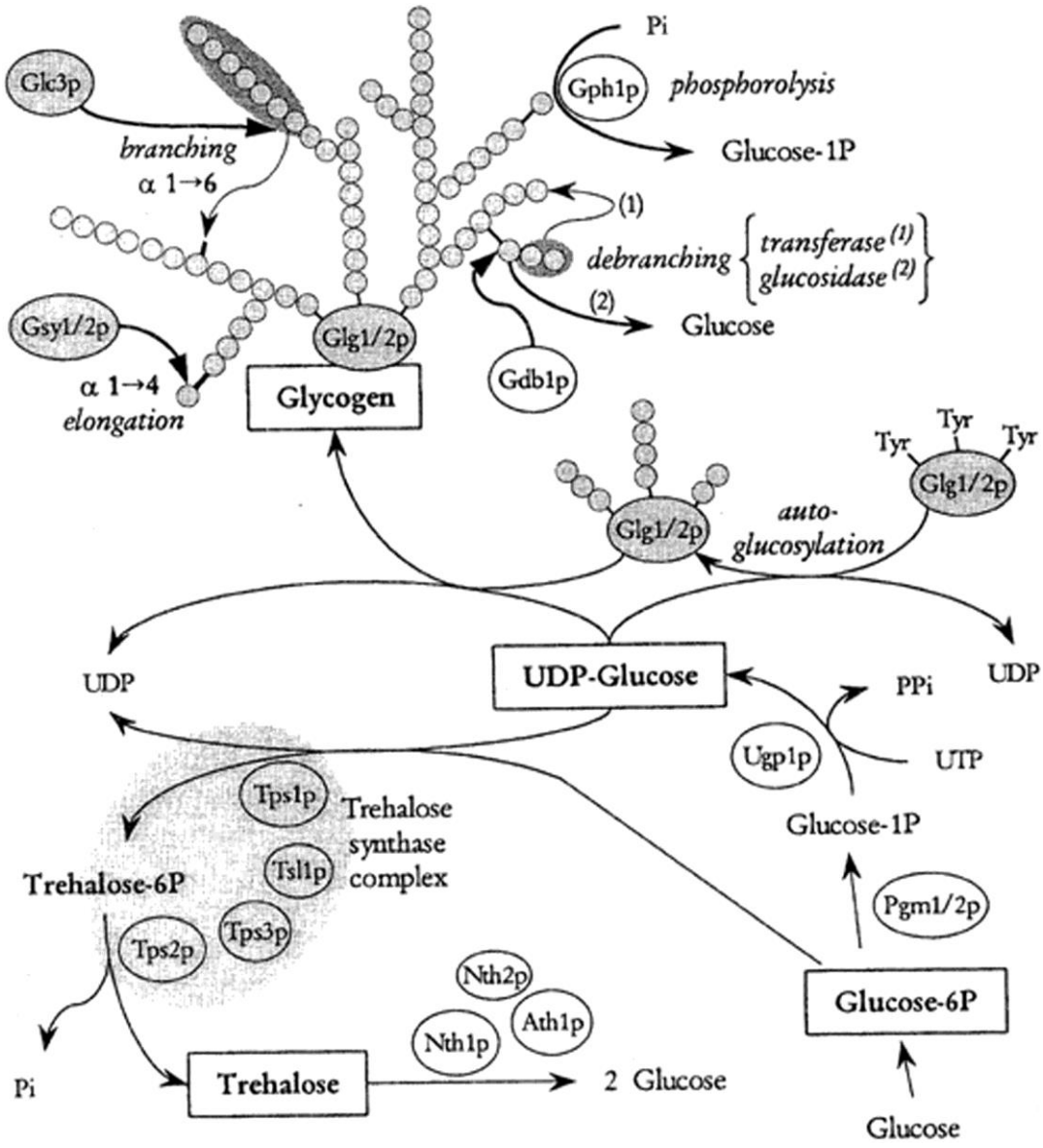

Figure 3- Schematic representation of glycogen and trehalose metabolic pathways in the *S. cerevisiae*. In glycogen process, glucose enters the cell and is converted to glucose-6P (G6P) and thus to glucose-1P (G1P) by phosphoglucomutase (Pgm1/2p). G1P is converted into UDP-glucose (UDPG), the donor molecule of glucose, by UDPG pyrophosphorylase (Ugp1p). Glycogen synthesis is initiated by glycogenin (Glg1/2p), that self-glucosylated, produces a short α (1,4)-glucosyl chain that is elongated by glycogen synthase (Gsy1/2p), through the formation of α-1,4 glycosidic bonds. The chains are ramified by the branching enzyme (Glc3p) which transfers a block of 6-8 residues from the end of a linear chain to an internal glucosyl unit and creates an α (1,6)-linkage. Glycogen degradation occurs by the combined action of glycogen phosphorylase (Gph1p) which releases glucose-1-P, and a debranching enzyme (Gdb1p) which transfers a maltosyl unit to the end of an adjacent linear α (1,4)-chain and releases glucose by cleaving the remaining α (1,6)-linkage. Trehalose biosynthesis is catalyzed by the trehalose synthase complex composed of four subunits. The trehalose-6-phosphate synthase (Tps1p) produces trehalose-6P from UDPG and G6P, which is dephosphorylated in trehalose by the trehalose-6-phosphate phosphatase (Tps2p). Tps3p and Tsl1p are two regulatory subunits that stabilize the complex. Trehalose is degraded by neutral (Nth1p) or acid (Ath1p) trehalase (FRANÇOIS; PARROU, 2001).

self-glucosylated glycogenin in their tyrosine residues. The synthesis is completed by the action of the branching enzyme, which transfers a fragment of six to seven glucose residues to the branch points (ROACH, 2002). For breakdrown of glycogen, the glycogen phosphorylase enzyme catalyzes the phosphorolysis of glycogen yielding G1P and shortened glycogen as product. The debranching enzyme transfers three glucose residues to the end of an adjacent linear chain and releases glucose by cleaving the remaining α (1,6)-linkage (Figure 3). For trehalose biosynthesis, the G6P and UDPG are used to produce trehalose-6-P by the trehalose-6-phosphate synthase. The trehalose-6-P is dephosphorylated to trehalose by the trehalose-6-phosphate phosphatase. In degradation, the disaccharide is hydrolyzed into two molecules of glucose by either the neutral or acid trehalase enzymes (Figure 3).

5. Regulation of glycogen and trehalose metabolism

Glycogen synthase and phophorylase are the rate-limiting enzymes and are subjected to different types of regulation: allosteric modulation and post-translational control by the action of protein kinases and protein phosphatases (FRANÇOIS; VILLANUEVA; HERS, 1988). The two enzymes are regulated by allosterism, where glucose-6-phophate and AMP are the allosteric effectors of glycogen synthase and glycogen phosphorylase, respectively. Glucose-6-phophate reverses the glycogen synthase inactivation by phosphorylation and AMP is the allosteric activator for the dephosphorylated glycogen phosphorylase (TÉLLEZ-IÑÓN; TORRES, 1970; MADSEN, 1986; FRANÇOIS; VILLANUEVA; HERS, 1988; JOHNSON; BARFORD, 1990; JOHNSON, 1992). In addition, they are also regulated by reversible covalent modification, in which phosphorylation activates glycogen phosphorylase and inhibits glycogen synthase. Multiple phosphorylation sites were identified in glycogen synthases, which are phosphorylated by different protein kinases, depending on the organism, whereas glycogen phosphorylase is phosphorylated in a single residue, which is modified by the phosphorylase kinase protein (HARDY; ROACH, 1993; NELSON; COX, 2008, cited in BERTOLINI et al., 2012).

In contrast to glycogen metabolism, the trehalose synthase complex is not subject to reversible phosphorylation. A peculiar property of this protein complex is its strong temperature activation, with an optimum at 42ºC to 45ºC in the presence of physiological concentrations of substrates and effectors. The trehalose synthesis can

be strongly influenced by changes in substrates concentration (G6P and UDPG), temperature and the steady-state levels of the protein. In the breakdown of trehalose, only the neutral trehalase from yeasts exhibits a N-terminal extension that contains the phosphorylation regulatory domain (NWAKA; HOLZER, 1998). Thus, this enzyme exists in two interconvertible forms by reversible phosphorylation. PKA is the only protein kinase known to directly phosphorylate and activate neutral trehalase isoform 1 (cited in FRANÇOIS; PARROU, 2001).

5.1 Environmental conditions regulating reserve carbohydrate accumulation and metabolism

Glycogen and trehalose are the two glucose stores of the cells, and the large variations in the cell content in these two compounds in response to different environmental changes indicate that their metabolism is controlled by complex regulatory systems. The amount of glycogen found in a particular situation results from the balance between glycogen synthase and glycogen phosphorylase activities. Besides reversible changes in their activities, glycogen levels are also correlated with physiological conditions through control of gene expression, and the activation of different signaling pathways affects glycogen storage (reviewed in ROACH et al., 2012).

Microorganisms and mammals synthesize and accumulate glycogen in periods of abundance of nutrients and degrade under periods of stress, such as nutritional shortage (HARRIS, 1997; LILLIE; PRINGLE, 1980). In mammalian cells, the liver and the skeletal muscle are the main depository of glycogen. In the yeast *S. cerevisiae*, the synthesis and degradation of glycogen vary with environmental conditions and stages of the life cycle (JOHNSTON; CARLSON, 1992). Cells acumulate glycogen when the culture begins the stationary phase during vegetative growth or when submitted to heat shock (NI; LAPORTE, 1995), or during the sporulation or germination induction of the spores (THEVELEIN, 1984; COLONNA; MAGEE, 1978; KANE; ROTH, 1974), or in nutrient limitation (LILLIE; PRINGLE, 1980). Such conditions induce the transcription of the *GSY2* gene (glycogen synthase isoform 2) (NI; LAPORTE, 1995). The *GSY2* encodes the predominant glycogen synthase since loss of its function resulted in a 90% reduction in both enzyme activity and glycogen levels in *S. cerevisiae* (FRANÇOIS; PARROU, 2001).

N. crassa accumulates glycogen late in the exponential phase of the vegetative growth (24 h), when the glycogen synthase activity and the expression of *gsn* (glycogen synthase) transcript are increased, and degrades it at the beginning of the stationary phase (NOVENTA-JORDÃO et al., 1996; DE PAULA et al., 2002). Besides that, glycogen levels were highly regulated on exposure of cultures to some stress conditions, such as heat shock (transfer from 30ºC to 45ºC) and carbon source limitation (sugar-free medium) (DE PAULA et al., 2002). In *N. crassa*, the glycogen is degraded under heat shock stress (NOVENTA-JORDÃO et al., 1996). The levels of *gsn* transcript, the glycogen content and the glycogen synthase activity were reduced when the mycelium was exposed to heat shock and carbon starvation, however were recovered when the cultures returned to normal growth conditions (30ºC and 2% of sugar) (DE PAULA et al., 2002; FREITAS; BERTOLINI, 2004). These results suggest that transcriptional regulation may account for the decrease in glycogen synthase activity and subsequent glycogen mobilization observed under these conditions. On the other hand, glycogen phosphorylase activity was activated under heat shock showing that reversible changes in the two regulatory enzymes were observed upon temperature shifting (NOVENTA-JORDÃO et al., 1996).

Trehalose is another reserve carbohydrate that can be mobilized under different growth conditions. Yeast cells submitted to heat shock accumulate trehalose (GRBA; OURA; SUOMALAINEN, 1975). In *N. crassa* conidia, trehalose corresponds to 10% of dry-weight, its levels decrease to a minimal value upon germination, remains at lower levels during all vegetative growth, and rises again at the end of the growth, accumulating into the conidia (HANKS; SUSSMAN, 1969). Under heat shock (from 30°C to 45°C), trehalose accumulates (DE PINHO et al., 2001; NEVES et al., 1991) and this effect may depend on trehalose-phosphate synthase enzymatic activities since it is the regulatory enzyme in trehalose metabolism (NOVENTA-JORDÃO et al., 1996). Under source carbon starvation, the levels of glycogen and trehalose content are reduced, but can be recovered after glucose additional in the medium (DE PINHO et al., 2001; NOVENTA-JORDÃO et al., 1996).

Studies of biochemistry and molecular characterization of proteins glycogenin (GNN) and glycogen synthase (GSN) were performed in *N. crassa* (DE PAULA et al., 2002, 2005a, 2005b). Only one isoform of GNN has been identified and gene inactivation by RIP completely abolished the glycogen accumulation (DE PAULA et al., 2005a). GNN has two glucosylation sites (Tyr196 and Tyr198) in the N-terminal

region, however each residue contributes differently to the self-glycosylation process, and the long C-terminal extension seems to be importante to enchance the interaction with the GSN (DE PAULA et al., 2005b). All glycogen synthases are conserved proteins among microbes and higher organisms and the differences are located mostly in the N- and C-termini of the protein, where the regulatory phosphorylation sites are located. The GSN enzyme of *N. crassa* shared much conservation with the Gsy1p and Gsy2p proteins of *S. cerevisiae* and with enzymes of mammals (rabbit muscle and human muscle) (DE PAULA et al., 2002). It was identified four putative phosphorylation sites in GSN enzyme based on a sequence alignment of different glycogen synthase and in *in vitro* phosphorylation reactions, all located at the C-terminus. However, *N. crassa* GSN seems to have an additional phosphorylation site (BARBOSA, 2007).

The decrease in glycogen content observed in *N. crassa* cells exposed to heat stress may result from down-regulation of the *gsn* gene mediated by the STRE (Stress Responsive Elements) motif within the promoter region. The *gsn* promoter has two STRE motifs and nuclear proteins activated by heat shock specifically bound DNA fragments containing both motifs (FREITAS; BERTOLINI, 2004). However, Msn2/4p homolog proteins, involved in yeast gene activation via STRE motifs, were not identified in the *N. crassa* database, suggesting the existence of a different mechanism to regulate the heat shock response (FREITAS et al., 2008) or different transcription factors acting in heat shock conditions.

5.2 Transcription factors regulating carbohydrate accumulation in *N. crassa*

The construction of a set of deletion strains, each carrying a deletion in a specific ORF, has allowed the screening for proteins linked to a particular phenotype. A transcription factor mutant strains collection available at FGSC was used to identify proteins that either directly or indirectly regulate glycogen metabolism in *N. crassa*. Transcription factors or regulatory protein modulate the activity of RNA polymerase II, by binding to gene regulatory sequences (COOPER, 2000), and are classified into general transcription factors or activators/repressors. The general transcription factors are constituents of the transcriptional machinery while the activators/repressors are specific transcriptional regulatory proteins that modulate the expression of certain genes (ALBERTS et al., 2002). The proteins, that are

transcriptional regulators but do not have the ability to bind to DNA itself, are called cofactors or transcriptional coregulators, and interact with transcription factors to activate or repress specific genes transcription (GLASS; ROSENFELD, 2000).

A systematic screening of a *N. crassa* deletion strains collection was performed to search for mutant strains having glycogen accumulation profiles different that in the wild-type strain under normal growth temperature (30ºC) and after heat shock stress (45ºC for 30 min). It was identified 17 transcription factors potentially involved in glycogen metabolism regulation. The identified proteins are classified in different families of transcription factors. Many of them are annotated as hypothetical proteins, however some of them were biochemically characterized either in *N crassa* or in other fungi, as PacC, XlnR, SUB-1, FlbC, RCO-1, CSP-1 and NIT-2 (GONÇALVES et al., 2011). Some mutant strains showed impairment in the regulation of *gsn* and *gpn* expression, suggesting a putative regulation of glycogenic genes by the transcription factors (GONÇALVES et al., 2011; BERTOLINI et al., 2012). Some transcription factors are involved in metabolism control, biological clock, and cell cycle progression, suggesting the existence of a link between glycogen metabolism and these processes. Recently, a screening of mutant strains in protein kinases revealed hyphotetical and identified kinases as controlling glycogen and trehalose storage in *N. crassa* under normal temperature and after heat shock stress *in N. crassa* (CANDIDO et al., 2014).

Many transcription factors are being investigated for their roles as regulators of many processes in *N. crassa* and other fungi. Among the transcription factors already characterized, it is noteworthy to describe NIT-2 in nitrogen metabolism regulation (FU; MARZLUF, 1990), XlnR in alternative carbon sources regulation (VAN PEIJ et al., 1998), SUB-1 in late light response gene regulation (CHEN; DUNLAP; LOROS, 2010), CSP-1 in ergosterol synthesis and fatty acid desaturases (SANCAR et al., 2011) and Seb1/SebA/SEB-1 in stress response (PETERBAUER; LITSCHER; KUBICEK, 2002; HAN; PRADE, 2002; DINAMARCO et al., 2012; FREITAS et al., 2016).

This study aims to elucidate the role of the VOS-1, PAC-3, CRE-1, RCO-1 and RCM-1 proteins in the regulation of the glycogen and/or trehalose metabolism under different environmental conditions: circadian clock, alkaline pH, calcium stress, repressing and non-repressing carbon sources.

5.2.1 VOS-1 transcription factor

The VOS-1 transcription factor is the *Aspergillus nidulans* VosA ortholog (viability of spores), which belongs to the velvet protein family and, together with VelB protein, is involved in the spore maturation. This protein controls trehalose biogenesis and cell wall completion (NI; YU, 2007; PARK et al., 2012) and is interconnected to the central regulatory genes in the conidiation cascade by a negative feedback regulation of *brlA* (NI; YU, 2007). In *N. crassa,* VOS-1 was identified as a WCC target showing that the *vos-1* expression was light induced and abolished in the Δ*wc-2* mutant strain (SMITH et al., 2010). Recent data showed that the *N. crassa* Δ*vos-1* mutant strain exhibits impairments in glycogen accumulation during vegetative growth when compared to wild-type cells (BONI, 2014), suggesting that VOS-1 may participate in the regulation of glycogen accumulation. In addition, data from Neurospora Program Project exploring genome wide ChIP-seq to define transcription binding sites have shown that several transcription factors bind to the promoters of glycogen enzymes, including VOS-1 (unpublished data). The consensus *A. nidulans* VosA DNA binding site (5'-CTGGCCAAGGC-3') (AHMED et al., 2013) was identified in the *gsn* and *gpn* promoters. This observation led us to start investigating the connection between light and circadian clock and glycogen metabolism.

5.2.2 PAC-3 transcription factor

The PAC-3 is ortholog of the *A. nidulans* PacC and *S. cerevisiae* and *Candida albicans* Rim101 transcription factors that play a central role in pH signaling pathway. In *A. nidulans*, PacC is activated by two successive proteolytic cleavage steps, leading to the active protein PacC (27 kDa) capable of binding to the promoters of pH-regulated genes (ARST; PEÑALVA, 2003), activating genes under alkaline pH and repressing under acidic pH (TILBURN et al., 1995; ESPESO et al., 1997). In *S. cerevisiae*, Rim 101 undergoes only one proteolytic cleavage and exerts its role as a repressor (LAMB; MITCHELL, 2003).

Some studies have shown the involvement of PacC in gene regulation of different cellular processes, such as the production of antibiotics, antifungal, and toxins (ESPESO; PEÑALVA, 1996; KELLER et al., 1997; MEYER; STAHL, 2002;

MORENO-MATEOS et al., 2007), extracellular enzymes (MACCABE et al., 1998), heat shock proteins (SQUINA et al., 2010), and others. PacC/Rim101 has been widely studied in model fungi and in human pathogens fungi, such as *C. albicans*, *A. fumigatus* and *Cryptococcus neoformans*, in which the pH signaling pathway is required for various functions associated to pathogenicity and virulence. Advances in the understanding of this pathway can lead potential results for therapeutic targets in antifungal strategies (CORNET; GAILLARDIN, 2014).

The *N. crassa* PAC-3 regulates the *gsn* gene under alkaline pH, leading to reduced glycogen accumulation. The *pac-3* mutant strain showed deficiencies in the development of the fungus, being unable to grow in alkaline pH. Furthermore, the mutant strain showed changes in glycogen accumulation before and after heat shock when compared to the wild-type cells, suggesting a role of the PAC-3 in glycogen regulation (CUPERTINO et al., 2012). Clear differences between *A. nidulans* and *N. crassa* regarding the role of PAC-3 were observed and some questions remain unclear in relation to the signaling cascade involving the PAC-3 transcription factor in *N. crass*a. Therefore, here we investigated the pH-signaling pathway, focusing on the characterization of the proteins components of this pathway, based on what is described in *A. nidulans*. In addition, the consensus *N. crassa* PAC-3 motif 5'-BGCCVAGV-3' (B=C/G/T; V=A/C/G) (WEIRAUCH et al., 2014) was identified in promoters of genes involved in glycogen and trehalose metabolism. There are evidences that the pH signaling pathway and calcium response pathway can act together. In yeasts, the transcriptional response to alkaline pH by Rim101 involves different signaling mechanisms, and the calcium signaling seems to have an important role in this response (SERRANO et al., 2002) and Rim101 acts in parallel to Crz1 transcription factor for adaptation to alkaline pH (KULLAS; MARTIN; DAVIS, 2007). Thus, we also investigated the glycogen and trehalose metabolism regulation under alkaline pH and calcium stress.

5.2.3 CRE-1 transcription factor and RCO-1 and RCM-1 corepressors

The *N. crassa* CRE-1 transcription factor is the ortholog of the *A. nidulans* CreA and the *S. cerevisiae* Mig1, the mediators of carbon catabolite repression (CCR), which represses the expression of a variety of genes through its interaction with protein partners. The Tup1-Ssn6 complex, orthologs to the *N. crassa* RCO-1-

RCM-1 proteins, acts together Mig1 in *S. cerevisiae* (NEHLIN; CARLBERG; RONNE, 1991). This complex represses various genes that encode proteins of alternative carbon sources utilization, such as galactose, maltose, xylose, arabinose, glycerol, etc, when glucose are present in the medium (RUIJTER; VISSER, 1997; ARO; PAKULA; PENTTILA, 2005). The Mig1 transcription factor has been reported to regulate a high number of genes by binding to the DNA motif 5'-SYGGRG-3' (S= G/C; Y=T/C; R= A/G) (STRAUSS et al., 1999). In *N. crassa*, deletion of the *cre-1* gene led to an increase in the production of hydrolytic enzymes involved in cellulose degradation (SUN; GLASS, 2011) and many Mig1 motifs were identified in glycogenic promoters.

The RCO-1 protein, the *rco-1* gene product (regulator of conidiation-1), has previously been described as involved in glycogen metabolism under normal and heat shock conditions (GONÇALVES et al., 2011), conidiation, development and cell differentiation (YAMASHIRO et al., 1996; ALDABBOUS et al., 2010) and is the downstream efector in circadian rhythms (BRODY et al., 2010). The RCM-1 protein, the *rcm-1* gene product (*regulation of conidiation and morphology*), is an essential protein and the *rcm-1*^{RIP} mutant strain showed serious defects in the vegetative and sexual development (OLMEDO et al., 2010). The RCO-1 and RCM-1 probably form a corepressor complex similar to Tup1-Ssn6 in yeast. Olmedo et al. (2010) showed that RCO-1 and RCM-1 participate in photoadaptation of genes regulated by light and Sancar et al. (2011) showed that CSP-1 transcription factor forms a transient complex with RCO-1 and RCM-1, acting as repressor for genes controlled by clock in *N. crassa*. The CRE-1, RCO-1 and RCM-1 complex has not been reported in *N. crassa*. We investigated here the regulatory role of CRE-1, RCO-1 and RCM-1 proteins in the regulation of glycogen metabolism in *N. crassa* under repressing and non-repressing carbon sources.