Universidade de Aveiro Departamento de Biologia
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica do Professor Doutor Arnaldo Videira, Professor Catedrático de Genética no Instituto de Ciências Biomédicas Abel Salazar

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Esta tese é especialmente dedicada ao meu avô Domingos Pinheiro

o júri

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palavras-chave Imunofilinas, PPIases, FKBPs, enovelamento de proteínas, *Neurospora crassa*

resumo

Imunofilinas são receptores intracelulares de drogas imunossupressoras, como a ciclosporina A, FK506 e rapamicina, caracterizados por apresentarem actividade de peptidil-prolil *cis-trans* isomerase. Além do seu envolvimento no enovelamento de proteínas, novos estudos demonstram o envolvimento destas proteínas em processos celulares específicos como transdução de sinal, transporte e montagem de complexos proteícos e silenciamento de rDNA. Neste estudo propusemo-nos caracterizar as funções celulares das imunofilinas do tipo FKBP (*FK506-binding proteins*) identificadas em *Neurospora crassa*. Na procura pelo papel fisiológico destas proteínas confirmamos a localização celular das proteínas FKBP12, FKBP22 e FKBP50 e determinamos a localização subcelular da proteína FKBP11. Foi também analisada a expressão destas proteínas em resposta a diversos indutores de stress, realizadas imunoprecipitações e produzidos e analisados mutantes simples e duplos para os genes *fkbp*. Através da proteína de fusão FKBP50::GFP e extractos proteicos da estirpe selvagem foi possível confirmar a localização nuclear da FKBP50, a localização endoplasmática da FKBP22 e a localização mitocondrial e citoplasmática da FKBP13. Foi também possível determinar que a FKBP11 está localizada no citoplasma. Uma análise bioquímica mais detalhada destas proteínas demonstrou que a FKBP11 não é expressa durante o desenvolvimento vegetativo do fungo mas a sua expressão é induzida por elevados níveis de cálcio e durante o desenvolvimento sexual. Através de imunoprecipitações usando um soro contra a FKBP13 foi possível co-imunoprecipitar uma proteína de aproximadamente 40 kDa. O tratamento de extractos proteícos mitocondriais e citoplasmáticos com diferentes proteases demonstrou que a FKBP13 é extremamente resistente à proteólise por proteinase K e tripsina. A análise da expressão destas proteínas em resposta a diferentes indutores de stress sugere que a FKBP50 é regulada negativamente pelo cálcio. Os níveis de FKBP22 encontram-se mais elevados em resposta ao stress osmótico e oxidativo e os níveis de FKBP13 encontram-se também aumentados na presença de elevados níveis de FKBP11, sugerindo uma possível ligação entre FKBP13 e FKBP11. Embora a disrupção dos genes *fkbp11*, *fkbp13* e *fkbp22* não conduza a num fenótipo detectável, a disrupção do gene *fkbp50* resulta num fenótipo termo-sensível. No seu conjunto, os resultados este estudo demonstram que além do envolvimento no enovelamento proteíco, estas proteínas apresentam um conjunto de características particulares, provavelmente como resultado de funções específicas. Com a caracterização das funções celulares realizadas por estas proteínas, esperamos descobrir um pouco mais sobre o papel das imunofilinas em processos celulares fundamentais comuns a todos os eucariotas.

keywords Immunophilins, PPIase, FKBP, protein folding, *Neurospora crassa*

abstract

Immunophilins are intracellular receptors of immunosuppresive drugs, as cyclosporin, FK506 and rapamycin, found to possess peptidyl-prolyl *cis-trans* isomerase activity. In addition to their involvement in protein folding, new data demonstrate the participation of these proteins in specific cellular processes as signal transduction, protein trafficking and assembly and rDNA silencing. In this study we proposed to characterize the cellular functions of the immunophilins of the FKBP-type (FK506-binding proteins) identified in *Neurospora crassa*. In a search for the physiological role of these proteins we confirmed the cellular location of proteins FKBP13, FKBP22 and FKBP50 and determined the subcellular location of the FKBP11. We also analyzed the expression of these proteins in response to several stress inducers, performed immunoprecipitations and produced and analyzed single and multiple mutants for the *fkbp* genes. Using a GFP fusion protein and wild type protein extracts we confirmed the nuclear location of FKBP50, the endoplasmic location of FKBP22 and the mitochondrial and cytoplasmic location of FKBP13. We also determine that FKBP11 is located in cytoplasm. A more detailed biochemical analysis of these proteins revealed that FKBP11 is not expressed during vegetative development of the fungus but can be induced by high levels of calcium and during sexual development. Through immunoprecipitation using an antiserum against the FKBP13 we could coimmunoprecipitate a protein of approximately 40 kDa. Treatment of wild type mitochondrial and cytoplasmic protein extracts with different proteases showed that FKBP13 is highly resistant to proteolysis by proteinase K and trypsin. Phenotypic analysis of the FKBPs expression in response to several stress inducers revealed that FKBP50 might be down regulated by calcium. The FKBP22 levels were found to be specially elevated in response to osmotic and oxidative stress and FKBP13 levels can be found notably high in the presence of high amounts of FKBP11, suggesting a possible relation between FKBP13 and FKBP11. Although disruption of the *fkbp11*, *fkbp13* and *fkbp22* genes did not conducted to a detectable phenotype, disruption of the *fkbp50* gene resulted in a temperature-sensitive phenotype. All together the results of this work showed that besides the involvement in protein folding these proteins present particular features, probably as a result of specific cellular functions. With the characterization of the cellular functions mediated by these proteins we expect to find out a little more about the role of immunophilins in fundamental cellular processes common to all eukaryotes.

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Objectives

In the last decades with the knowledge that PPIases, and in particular immunophilins, are involved in various and important cellular events, major advances have been made towards understanding the mechanism of action of these proteins in cell. Since immunophilins are extremely conserved among eukaryotes, the study of the cellular events mediated by these proteins became easier with the adoption of simple model organisms as fungi. In this study we proposed to characterize the cellular functions of the *N. crassa* FKBPs. Although the cellular location of FKBP13, FKBP22 and FKBP50 has been already established, the cellular location of FKBP11 remained to be determined. Through a biochemical analysis of these proteins and the production and characterization of *fkbp* mutant strains we expect to establish the cellular location of all the *N. crassa* FKBPs, the conditions where these proteins are (more) active, their mode of action and, if possible, their cellular partners. With this work we expect to discover a little more about the role of immunophilins in fundamental cellular processes common to all eukaryotes.

Chapter 1. Introduction

The success of organ transplantation depends mainly on the immunosuppressive therapy. The discovery and introduction in the 1980s of new and potent immunosuppressive drugs as FK506 (also known as tacrolimus), rapamycin (sirolimus) and cyclosporin A (CsA) have revolutionized allograft transplantation, leading to major advances in heart and liver transplantation. Currently, these drugs represent a powerful tool in the prevention of graft rejection after organ transplantation and treatment of autoimmune diseases (Oka and Yoshimura, 1996; Gummert *et al.*, 1999; Allison, 2000). In a search for the molecular mechanism of action underneath immunosuppression, the intracellular receptors for these immunosuppressive drugs were found (Takahashi *et al.*, 1989; Harding *et al.*, 1989; Siekierka *et al.*, 1989a; Siekierka *et al.*, 1989b). Due to their immune properties, these intracellular receptors were named immunophilins.

According to their affinity to different immunosuppressive drugs immunophilins can be classified in two families: the cyclophilins (CyPs), which bind CsA, and the FK506-binding proteins (FKBPs), which bind the macrolides FK506 and rapamycin. CsA and FK506 mode of action is identical. Both drugs when complexed with the respective ligand inhibit the activity of calcineurin, a serine-threonine fosfatase that regulates IL-2 promoter induction after T cell activation, thereby suppressing the production of IL-2 and other cytokines. In contrast, rapamycin inhibits the activity of highly conserved kinases required for cell cycling and responses to IL-2 (Gummert *et al.*, 1999; Cho *et al.*, 2003; Allison, 2000; Lee and Chapman, 2005). In addition to their immunosuppressive role, immunophilins are found to possess peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. Along with the parvulin family, these proteins make part of the larger protein superfamily of peptidyl-prolyl *cis-trans* isomerases, as they are able to catalyse the *cis-trans* isomerization of the X_{aa}-Pro peptide bonds in oligopeptides and proteins, being thus involved in the acceleration of protein folding (Galat and Metcalfe, 1995; Göthel and Marahiel, 1999; He *et al.*, 2004; Barik, 2006). In contrast to immunophilins, only a small number of parvulins or homologues are known. In opposite to the drugs CsA, FK506 and rapamycin which inhibit the enzymatic activity of immunophilins reversibly and

competitively, the enzymatic activity of several parvulins is irreversibly inhibited by jugole (Göthel and Marahiel, 1999).

Recently, a new family of isomerases presenting both FKBP and CyP domains, named FK506 and cyclosporin-binding protein (FCBP), was found in the protozoan *Toxoplasma gondi* (Adams *et al.*, 2005). In this parasite it was identified a protein of 57 kDa containing simultaneously a N-terminal FKBP domain and a C-terminal CyP domain joined by three tetratricopeptide repeats (TPR). Both domains are functional and exhibit family-specific drug sensitivity. When expressed separately both domains present about half of the FCBP PPIase activity. Three new FCBP sequences were identified; two in *Flavobacterium johnsonii* and one in *Treponema denticola*. In these new sequences the domains are present in the reverse order, being the FKBP domain located in the C-terminal portion and the CyP domain in the N-terminal portion, constituting the main difference between FCBPs of eukaryotes and prokaryotes (Adams *et al.*, 2005).

Even though cyclophilins and FKBPs are non-related in primary sequence, both families present an extraordinary sequence conservation of the PPIase domains (Galat and Metcalfe, 1995). These ubiquitous and highly conserved proteins are found in all organisms, from archaea to man, and cellular compartments (Trandinh *et al.*, 1992; Kay, 1996; Galat, 2000; Vallon, 2005). Their wide distribution and functional conservation lead to a rising curiosity and interest in their potential cellular functions. In addition to a general role in accelerating protein folding, several studies show the involvement of PPIases in several and important cellular processes as signal transduction (Bram *et al.*, 1993), protein trafficking (Stamnes *et al.*, 1991, Chambraud *et al.*, 2007), protein assembly (Wu *et al.*, 2004; Xiao *et al.*, 2006), control of transcription (Arévalo-Rodríguez and Heitman, 2005), pre-mRNA splicing (Lorkovic *et al.*, 2004), rDNA silencing (Kuzuhara and Horikoshi, 2004), apoptosis (Baines *et al.*, 2005) and cell-cycle regulation (Lu *et al.*, 1996; Vittorioso *et al.*, 1998). Characterization of the cellular functions of this group of proteins might provide us new insights of specific and complex cellular processes.

1.1 The role of immunophilins in immunosupression

In mammals, the drugs FK506 and cyclosporin bind to different target molecules, although the mechanism of action of both drugs is identical. The process begins with the binding of the drug to its intracellular receptor, a FKBP or cyclophilin. It is known that the binding site of the drugs and the PPIase activity site are the same, since this activity is completely abolished when the complex drug-immunophilin is formed. However, immunosupression is not the result of PPIase activity inhibition. In fact, there is a gain of function, being immunossupression the result of a novel activity acquired by the complex formed that is not a property of the immunophilin or the drug alone (Galat and Metcalfe, 1995; Gummert *et al.*, 1999). The formation of the complex FK506-FKBP12/cyclosporine A-cyclophilin A inhibits the activity of calcineurin, a serine-threonine fosfatase that regulates IL-2 promoter induction after T cell activation (Figure 1). The inhibition of calcineurin blocks the calcium-dependent signal transduction and inhibits cytokine gene activation by inactivating the transcription factor NF-AT (nuclear factor of activated T cells). As a consequence, the transcription of cytokines IL-2, IL-3, IL-4, IL-5, interferon-γ, tumor necrosis factor-α, granulocyte-macrophage colony-stimulating factor, and IL-2 and IL-7 receptors is suppressed by FK506 and CsA. Other *in vitro* effects of FK506 include the inhibition of T cell proliferation, the inhibition of primary or secondary cytotoxic cell proliferation and the inhibition of B cell activation. *In vivo*, the FK506 inhibits proliferative and cytotoxic responses to alloantigens and suppresses primary antibody responses to T cell-dependent antigens (Gummert *et al.*, 1999).

Although the drugs FK506 and rapamycin share the same intracellular receptor, these two drugs inhibit the immune system through two different signal transduction pathways. Contrary to the FK506, the complex rapamycin-FKBP12 does not bind to calcineurin but to a highly conserved kinase named TOR (target for rapamycin) in yeast and plants and mTOR (or FRAP, FKBP and rapamycin associated protein) in mammals, essential for cell cycle progression. The formation of the complex rapamycin-FKBP causes the arrest of the cell cycle in the G1 phase. In view of that, the TOR protein is known to be a central regulator of cell growth and proliferation. The effects of the complex rapamycin-FKBP also comprise the inhibition of the IL-2 induced T cell proliferation, the B cell Ig synthesis and antibody-dependent cellular cytotoxicity, as well as inhibition of lymphocyte-activated

killer cells and natural killer cells (Gummert *et al.*, 1999; Cho *et al.*, 2003; Lee and Chapman, 2005).

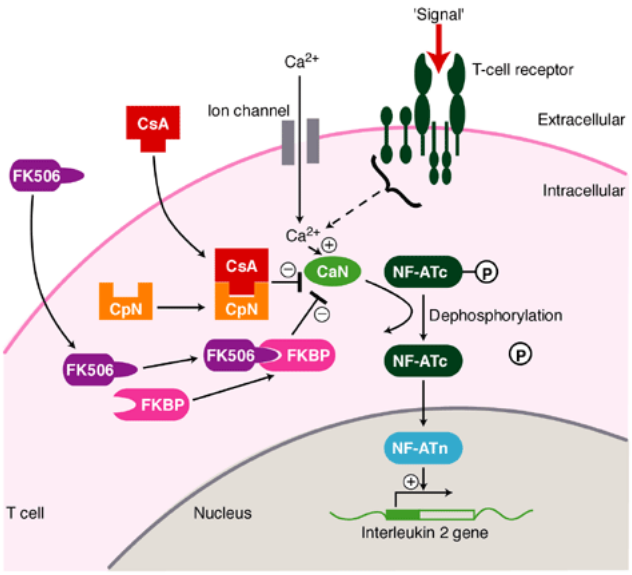

Figure 1. Mechanism of action of FK506 and cyclosporine A. The formation of FK506-FKBP/cyclosporinA (CsA)-cyclophilin (CpN) complexes inhibit the activity of calcineurin (CaN), preventing the dephosphorylation of the nuclear factor of activated T cells (NF-ATc) component and the transcription of the nuclear factor of activated T cells (NF-ATn), leading to the suppression of the transcription of several genes encoding cytokines.
(http://journals.cambridge.org/fulltext_content/ERM/ERM2_04/S1462399400001769sup004.htm)

1.2 Peptidyl prolyl *cis-trans* isomerase activity

The proper folding of the primary sequence of the nascent polypeptide chain into a well-defined three-dimensional structure is critical in determining the protein physiological function in cell. During protein synthesis and folding, the torsion angle of a peptide bond can exist either in *cis* or in *trans* conformation, being the *trans* form energetically more favorable due to steric hindrance in *cis*. The unfavorable conformational energy of the *cis* peptide bond is the main reason of its relatively unusual appearance in the native structure of proteins. The only exception applies to the amino acid proline which has a much higher

probability to form a *cis* peptide bond with the preceding amino acid residue, due to the lower activation energy barrier of 13 kDa/mol for a X_{aa}-Pro bond against 20 kDa/mol for other peptide bonds (Galat and Metcalfe, 1995). Even though the *cis-trans* isomerization of peptidyl prolyl bonds has been identified as a spontaneous event, it occurs very slowly being frequently a rate limiting step in protein folding. In order to accelerate this process some enzymes became specialized in catalyzing the reversible *cis-trans* isomerization of the prolyl peptide bond, accelerating therefore protein folding and making it compatible with the rapid pace of other intracellular events (Figure 2). Several studies suggest that these proteins, also known as PPIases, may play a critical role in protein folding and assembly of multisubunit protein complexes. In some cases it is possible to demonstrate that the isomerization of proline bonds is the rate-limiting step in protein folding *in vivo* and *in vitro* (Kiefhaber *et al.*, 1990; Steinmann *et al.*, 1991; Kern *et al.*, 1995). For instance, it has been found that SurA, a periplasmic protein in *Escherichia coli* similar to parvulin is involved in the folding of periplasmic and outer membrane proteins *in vivo* (Lazar and Kolter, 1996). It is believed that SurA maintains the outer membrane integrity since surA mutants became deformed and easily lysed during stationary phase. Other studies showed that the presence of CsA delays the collagen triple helix assembly in chicken embryo fibroblasts (Luan, 1998). Even though some PPIases present an active and crucial role in protein folding, in most of the cases it has been demonstrated that the PPIase activity is not essential for the organism survival. For example, the yeast *Saccharomyces cerevisiae* contains eight CyPs, four FKBPs and one parvulin. Disruption of all twelve immunophilins does not affect the dodecuplet mutant viability, showing that all the immunophilins are dispensable for the fungus survival (Dolinski *et al.*, 1997). Although this function has been maintained extremely conserved during evolution, new data reveal that PPIases main function may not be the protein folding but rather perform specific functions through interactions with particular partner proteins.

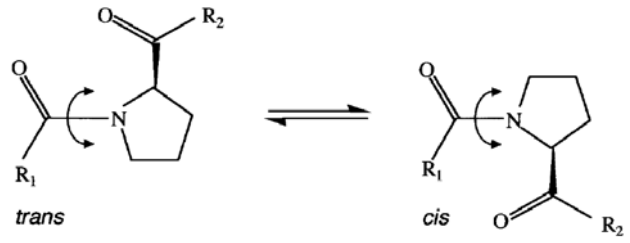

Figure 2. *Cis-trans* isomerization of a peptidyl-prolyl bond. Adapted from Göthel and Marahiel, 1999.

1.3 The FK506-binding proteins

In 1989, two independent groups presented new outstanding results regarding the immunosuppression mechanism of action (Harding *et al.*, 1989; Siekierka *et al.*, 1989a; Siekierka *et al.*, 1989b). Using a FK506 affinity matrix Harding and collaborators purified a protein of approximately 14 kDa from bovine thymus and human spleen (Harding *et al.*, 1989). This protein did not cross-reacted with antisera against cyclophilin and presented PPIase activity inhibited by FK506 (Figure 3). Although the similarity in the mechanism of immunosupression mediated by FK506 and CsA, the lack of sequence resemblance to cyclophilin lead to the establishment of a novel class of proteins with immune properties named FK506-binding proteins. Later the novel FK506-binding protein was characterized.

The archetypal 12-kDa human FKBP isolated from bovine thymus and human spleen is at the present moment the well-characterized FKBP protein. This ubiquitously expressed cytosolic protein comprises 108 amino acids representing the minimal peptide sequence harboring PPIase activity and drug binding, serving as a model for the remaining members of the FKBP family. Ten amino acids define the hydrophobic core of the protein that forms the ligand-binding pocket. The hFKBP12 structure is constituted by five-stranded antiparallel β-sheet forming two loops. One of the loops includes a short section of an α-helix. The β-sheet is twisted to give concave and convex surfaces with the concave surface made of hydrophobic residues that form the core of the protein and the hydrophilic residues projecting on the convex side making up much of the protein surface (Figure 4).

Although multiple alignment of FKBPs has shown some sequence divergence between the FKBP domains, the overall β-barrel/α-helix structure, the binding capacity to FK506 and rapamycin and the PPIase activity cleft remain conserved among different groups (Galat, 2000; Breiman and Camus, 2002).

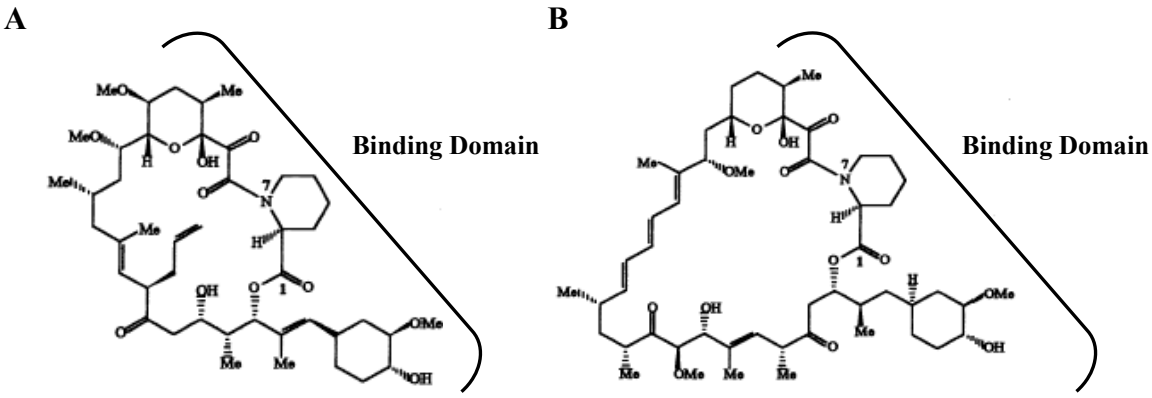

Figure 3. Molecular structure of the drugs FK506 (A) and rapamycin (B). Both macrolides FK506, a polyketide produced by *Streptomyces tsukubaesis*, a specie initially discovered in Tsukuba, Japan, and rapamycin, isolated from *S. hygroscopicus* found on Easter Island, compete for the binding to the same intracellular receptor, a FKBP.

As the cyclophilins, the FKBPs are ubiquitous proteins highly conserved during evolution, been found both in prokaryotes and eukaryotes. Using a specific nomenclature, these proteins are named using a prefix of one or two letters indicating species of origin and a suffix number indicating the molecular mass in kDa of the mature protein. Their classification into single or multiple domains is based on the number of FKBP domains. Usually small size FKBPs present only the FK506-binding domain, while high molecular weight proteins may present additional domains as TPR, WW domains, calmodulin-binding sites, DNA and RNA-binding sites and dimerization modules, important for protein interactions (Breiman and Camus, 2002; He *et al.*, 2004).

Figure 4. Human FKBP12 bounded to FK506. The FKBP12 represents the minimal sequence harboring the PPIase domain, whose activity is specifically inhibited by the macrolides FK506 and rapamycin. The human FKBP12 secondary structure is presented by different colors. (http://commons.wikimedia.org/wiki/Image:Fkbp-cartoon-1fkj.png)

1.3.1 FKBPs and disease

Since the involvement of FKBPs in several important cellular functions it is not surprising to found in recent literature new data describing the association of FKBPs and human diseases. In fact the absence or improper functioning of some of these proteins may be in the origin of quite a few severe human pathologies. The hFKBP12 is the best characterized member of the FKBP family. Several cellular functions are associated to hFKBP12. This protein was found to be tightly bound to the ryanodine receptor (RyR), a family of tetrameric intracellular calcium release channels required for excitation-contraction coupling in striated muscle, forming a stable complex with a stoichiometry of one FKBP12 molecule per each RyR molecule regulating the channel activity (Lam *et al.*, 1995; Göthel and Marahiel, 1999; Breiman and Camus, 2002; Wang and Donahoe, 2004). Studies with mutant mice showed that the absence of FKBP12 altered the single-channel properties of both cardiac RyR2 and skeletal RyR1, leading to the development of severe cardiomyophaty and ventricular septal defects identical to human congenital heart

disorders. These results revealed to be very important in line to understand the cellular mechanisms of cardiac failure as well as to establish the FKBP12-deficient mice as a promising model for testing pharmacological agents and other therapies in the treatment of human heart diseases. Along with the hFKBP12, the mammalian FKBP52 is one of the best characterized FKBPs. Contrary to the hFKBP12, the mammalian FKBP52 does not present immunosuppressive activity when complexed with FK506. A single amino acid, Lys121, correspondent to Ile90 in hFKBP12, is the responsible for the lack of binding to calcineurin (Li *et al.*, 2003; Davies and Sánchez, 2005). This lack of immunosuppressive activity is representative of the highly specialized nature of some of these proteins. Initially found as a member of an untransformed steroid receptor heterocomplexes (Wu *et al.*, 2004), it has been demonstrated that FKBP52 serves as a cochaperone for steroid hormone receptors when complexed with HSP90, promoting the proper translocation of estrogen, androgen and glucocorticoid receptors from the cytoplasm into the nucleus (Wu *et al.*, 2004; Davies and Sánchez, 2005). This function appears to be extremely important since male and female mice lacking FKBP52 display severe reproductive defects leading to infertility (Tranguch *et al.*, 2005; Tranguch *et al.*, 2006; Hong *et al.*, 2007).

New data show that immunophilins are more abundant in the nervous system than in immune tissues, suggesting neuroprotective and neurotrophic activities of these proteins. FKBP38, also known as FKBP8, is a transmembrane chaperone protein localized mainly in mitochocondria and endoplasmic reticulum that inhibits apoptosis by targeting two anti-apoptotic proteins, Bcl-2 and Bcl-x, to mitochondria. A recent study showed that FKBP38 is abundant in neurons and glial cells (Shirane et al., 2008). In this study, Shirane and collaborators created a FKBP38 nullizygous mice that soon during early stages of the embrionary development presented several neuronal defects as spina bifida, disorganization of the neuroepithelium and abnormal extension of the nerve fibers, dieing shortly after birth. Besides the neuronal defects, this mutant also display several skeletal defects as scoliosis, rib deformities, club foot and curled tail (Shirane et al., 2008). The development of neuronal defects appears to be related to an increase in frequency of apoptosis. Through a yeast two-hybrid system protrudin was identified as an interactor of FKBP38. Protrudin is a regulator of Rab11-mediate membrane trafficking being thus involved in neurite extension. In nullizygous mice, protrudin was found to be hyperphosphorilated suggesting that FKBP38 might regulate neurite outgrowth through

interaction with protrudin. All together these data indicate that FKBP38 regulates neurite extension and neuroectodermal organization during neural tube formation. As FKBP52, FKBP38 does not present immunosuppressive activity when complexed with FK506. FKBP38 lacks the conserved Trp59 in hFKBP12, important for the interaction with FK506, presenting instead a Leu residue at the correspondent position (Kang *et al.*, 2008). In addition to the involvement in calcium regulation, it has been reported that the hFKBP12 might also have an active role in nerve regeneration. Its levels were found to be significantly altered in the brain of patients with neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, dementia with Lewy bodies and in rats presenting crush injury of facial or sciatic nerves. It is proposed that hFKBP12 may degrade and prevent abnormal proteins aggregation by catalyzing protein folding, promote ubiquitination and transport of proteins through axons in a correct conformation during axon transport (Avramut and Achim, 2002).

In contrast to neurotrophins, most immunophilin ligands are highly stable, being able to readily cross the blood barrier. The development of nonimmunosuppressive immunophilin ligands may represent a potent therapeutic alternative in order to reverse the neurodegenerative process and prevent apoptotic cell death in neurons (Kang *et al.*, 2008).

1.3.2 FKBPs in Yeast

In the yeast *S. cerevisiae* genome four FKBPs were identified, Fpr1 to Fpr4. The yeast Fpr1 is the homolog of the mammalian FKBP12, presenting PPIase activity inhibited by FK506 and rapamycin. Deletion of the cytoplasmic Fpr1 leads to a slow growth phenotype, although fpr1 mutants also exhibit an increased resistance to LiCl and an enhanced recovery from α-pheromone induced cell cycle arrest when compared to the wild type strain (Dolinsky *et al.*, 1997; Arévalo-Rodrígues *et al.*, 2004). Several Fpr1 cellular partners have been identified. This protein appear to be necessary for the proper expression of the mammalian P-glycoprotein, an energy-dependent efflux pump that mediates tumor multidrug resistance by actively exporting various toxic molecules back out of the cell. In addition, Fpr1 is required for specific conformational changes in Hom3, an aspartokinase that catalysis an intermediate step in threonine and methionine biosynthesis, regulating

Hom3 feedback inhibition. Fpr1 was also identified as a cellular partner of Hmo1, a nucleolar-localized RNA polymerase I factor (Arévalo-Rodrígues *et al.*, 2004).

Fpr2 is a 13 kDa membrane associated protein localized in the ER. Fpr2 expression is induced by stress conditions, as heat shock or the presence of the antibiotic tunicamycin, and by the unfolded protein response (UPR) suggesting an active role of Fpr2 in protein transit in the ER under conditions that promote accumulation of unfolded proteins (Arévalo-Rodrígues *et al.*, 2004).

The Fpr3 and Fpr4 are nucleolar proteins. Both proteins associate with the ribosomal protein S24. Such complexes are not disrupted by FK506, suggesting that binding does not occur through the FKBP domain. Initially found in as the first phosphotyrosyl protein known to be a physiological substrate of Ptp1 (Wilson *et al.*, 1995), the 70 kDa Fpr3 was also found to be a substrate for the yeast caseine kinase II protein Cka2. These two proteins regulate the phosphorylation level of Fpr3 residue Y184 in the N-terminal nucleolin-related domain, affecting Fpr3 subcellular location (Arévalo-Rodríguez *et al.*, 2004; Wilson *et al.*, 1995). In addition, Fpr3 also prevent premature adaptation to persistent DNA damage, by inhibiting protein phosphatase 1 (PP1) activity (Hochwagen *et al.*, 2005).

In *Schizosaccharomyces pombe* it was identified a nuclear FKBP that associates with chromatin at rDNA loci, having an *in vivo* functional role in rDNA silencing, with a histone chaperone activity *in vitro*. Both activities are dependent of the N-terminal domain of the protein. Although, the C-terminal PPIase domain of the FKBP is not essential for the rDNA silencing and histone chaperone activities, it regulates rDNA silencing *in vivo* (Kuzuhara and Horikoshi, 2004). As the *S. pombe* nuclear FKBP, the *S. cerevisiae* Fpr4 also displays a novel histone chaperone activity *in vitro*. This protein facilitates nucleosome assembly in a manner identical to other acidic histone chaperones, as NAP1, and acts as a chromatin component required for transcriptional silencing at the rDNA locus *in vivo*. Structurally the Fpr4 presents 3 well-defined domains, with an acidic domain at the N-terminus, a basic domain in the middle and a PPIase domain at the C-terminus. The Fpr4 chaperone activity seems to be dependent on the acidic domain of the protein, even though the PPIase domain acts as an inhibitor of the histone chaperone activity, being this inhibition independent of the PPIase activity (Xiao *et al.*, 2006).

1.4 *Neurospora crassa*

Neurospora crassa is a bright orange filamentous fungus belonging to the Ascomycete division. The *Neurospora* genus can be found in all moist tropical and subtropical areas and in temperate areas near of agriculture areas. First documented in 1843 as a contaminant of bakeries in Paris, soon *Neurospora* was identified as a prolific contaminant of sugar cane processing plants and burned vegetation, mainly due to the requirement of heat shock activation (Davies and Perkins, 2002; Galant *et al.*, 2003). Although in past this conspicuous fungus had been known for approximately eighty four years, only in 1927 Cornelius L. Shear and Bernard O. Dodge presented for the first time the *Neurospora crassa* species description and classification (Horowitz, 1991). Even though Dodge's genetic work on *Neurospora* had contributed notably to the establishment of *Neurospora* as a model organism, only in 1941 with Edward L. Tatum and George W. Beadle landmark paper, *N. crassa* was accepted by the scientific community as a model organism. In this work, Tatum and Beadle exposed *N. crassa* to a series of random mutations caused by x-rays. The results of this study revealed the existence of flaws in some metabolic pathways caused by the mutation of specific enzymes, leading to the "one gene – one enzyme" theory, proving that genes control the fundamental processes of life. With this brilliant result, Tatum and Beadle won the Nobel Prize in physiology or medicine in 1958 (Horowitz, 1991; Davis, 2000).

Currently with the high number of model organisms documented, *N. crassa* is still accepted as an excellent model organism in what concerns the study of diverse aspects of eukaryotic cell biology. Although presenting the same basic biochemistry and cell biology of *Saccharomyces*, the more complex development with distinct, differentiated cell types and the genetic mechanisms more close to other eukaryotes make of this filamentous fungus one of the best contributors for the finding and characterization of different cellular mechanisms (Perkins and Davis, 2000).

1.4.1 *N. crassa* life cycle

In nature, *N. crassa* usually reproduces asexually with the production of bright asexual spores named conidia. In normal conditions, conidia germinate forming an initial germ tube that extends to form hyphae, which continues to grow by tip extension and branching to form a mycelium (Figure 5). The *N. crassa* mycelium presents multinucleated cells separated by perforated cross-walls allowing the flow of cytoplasm, nuclei and other organelles. In situations of nutrient exhaustion, the mycelium can produce two types of asexual spores named macro and microconidia. The macroconidia are formed by budding and constriction of aerial hyphae presenting one to several haploid nuclei, being the macroconidia with two nuclei the most frequent. In contrast, microconidia are extruded directly and serially from cells of small branched microconidiophores (Davis, 2000).

In situations of stress imposed by low luminosity, low temperature or low nitrogen sources the *N. crassa* sexual cycle is initiated. *N. crassa* is a heterotallic specie, meaning that sexual cycle requires the presence of two genetically different parents, known as mating types *A* and *a*. The mating types are determined by alternate DNA sequences at a chromosomal locus (*locus mating type*). Mating can only occur between different mating-types.

The female reproductive structure is a protoperithecium and the male fertilizing agent is normally a conidium or hyphae of the opposite mating-type. Protoperithecia are formed as a small knot of hyphae that surrounds some special cells, one of which acting as the female gamete (ascogonium). The outer hyphae of the protoperithecium form a dense protective layer through which emerges one or more specialized hyphae named trichogynes. Trichogynes respond to a pheromone emitted by conidia of the opposite mating-type by growing toward them until contact and cell fusion occurs. Upon fusion, a nucleus of the conidium travels through the tricogyne to the ascogonium in the protoperithecium. In the protoperithecium, the nuclei of both mating-type do not fuse but proliferate through a succession of divisions developing a mass of ascogenous hyphae. In the subapical cell of the crozier, a binucleate hook-shaped structure, pre-meiotic DNA replication takes place prior to nuclear fusion. Nuclear fusion completes the fertilizing process, yielding the only diploid stage of the life cycle. The diploid nucleus immediately undergoes a meiotic division. Each of the four meiotic products originated undergoes one

mitotic division resulting in the production of eight nuclei. If the parents have different alleles at one gene, asci will contain four nuclei of one parental type and four nuclei of the other. Therefore, the mating-type alleles will emerge as 4 *mat A*:4 *mat a* spore cultures. As asci form, perithecia enlarge and the walls became hard and melanized. A beaklike structure forms at the top of the perithecium containing a pore named ostiole, through which ascospores are forcibly ejected in groups of eight, each comprising the contents of an ascus. When ascospores are fully mature they germinate upon heat activation, being this one of the reasons why *Neurospora* is found in areas of burned-over vegetation (Davis, 2000).

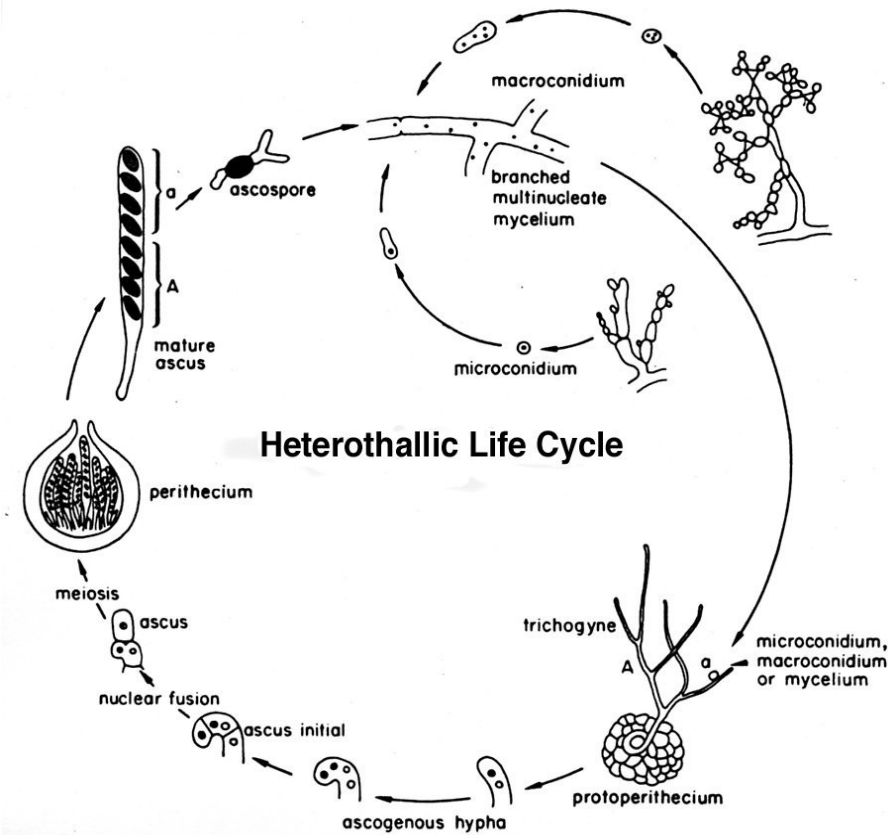

Figure 5. *N. crassa* life cycle. *N. crassa* presents a heterothallic life cycle where mating can only occur between different mating-types (*A* and *a*), leading to the development of specialized structures.

1.4.2 *N. crassa* as a model organism

The broad biological knowledge of *Neurospora*, from the molecular to the population level, makes it one of the best model organisms in the study of numerous cellular processes. At the cellular level, *N. crassa* simple life cycle, fast growth, ease of culture and simplicity in what concerns to nutritional requirements makes it a fungus easy to maintain and cultivate in laboratory. *N. crassa* is also a haploid fungus easy to manipulate genetically. This fungus presents Mendelian segregation, being the recognition of recessive loss-of-function mutations usually straightforward. This feature can be extremely useful in the study of mutant strains for particular genes. In addition *N. crassa* presents one of the most intriguing processes occurring in nature. Through a natural phenomenon named repeat-induced point mutations (RIP) it is possible to obtain mutant strains. In this process when a plasmid containing a copy of a specific gene is integrated in the *Neurospora* genome and the transformant obtained crossed with a wild type strain of the opposite mating-type, inactivation of both copies of the gene can occur during sexual development through a series of mutations involving GC to AT base pair transitions followed by DNA mutilation (Videira, 2001). This interesting process is known as a defense mechanism against selfish and mobile DNA, preventing gene innovation through gene duplication and it is extensively used to produce *N. crassa* mutant strains for specific genes (Cambareri *et al.*, 1991, Davis, 2000; Galant *et al.*, 2003).

In the Nature edition of 24th April of 2003, the *N. crassa* genome was reported as fully sequenced (Galant *et al.*, 2003). The *N. crassa* genome presents 43 megabase and encodes around 10.000 genes distributed by 7 chromosomes. Being the first filamentous fungus having its genome fully sequenced and available freely has contributed significantly to a better understanding of a great number of cellular processes as cellular signaling, growth and differentiation, metabolic activities and genome defense (Horowitz, 1991; Perkins and Davis, 2000; Davis and Perkins, 2002; Galant *et al.*, 2003).

1.4.3 *N. crassa* FKBPs

Four immunophilins belonging to the FKBP family, named FKBP11, FKBP13, FKBP22 and FKBP50 were identified in the last genome annotation of *N. crassa* (Table 1) (*Neurospora* sequencing project, Whitehead Institute/MIT Center for Genome Research). The accession numbers for the FKBP11, FKBP13, FKBP22 and FKBP50 proteins are NCU04371.3, NCU04140.3, NCU02455.3 and NCU03241.3, respectively.

The FKBP22 protein is encoded by the *fkr-5* gene, located in linkage group VII, and is constituted of 217 amino acids, with a N-terminal presequence of 20 amino acids. The mature FKBP22 presents 197 amino acids with a N-terminal PPIase domain and a C-terminal domain, with no homologies to other proteins, with an ER retention signal (HNEL) (Tropschug *et al.*, 1990). The cellular functions of FKBP22 have been recently characterized (Solscheid and Tropschug, 2000; Tremmel *et al.*, 2007; Tremmel and Tropschug, 2007). FKBP22 is a homodimeric protein located in the lumen of the ER with a novel chaperone activity. This protein interacts directly with BiP, the major HSP70 chaperone in the ER, through its FKBP domain, being BiP able to enhance FKBP22 chaperone activity. BiP and FKBP22 form a stable folding helper complex with a high chaperoning activity preventing the aggregation of unfolded rhodanese (Tremmel and Tropschug, 2007). FKBP22 appears also to form other chaperone/folding catalyst complexes with disulfide isomerases, like ERp38 and PDI, Grp170, another ER HSP70 chaperone, and Cyp23, a cyclophilin, being the organization of such complexes mediated by BiP. FKBP22 is as well involved in the development of microconidiophores (Tremmel *et al.*, 2007).

The *fkr-2* gene, located in linkage group V, encodes FKBP13, a protein of 176 amino acids with a putative N-terminal mitochondrial presequence. The FKBP13 presents only a PPIase domain (Figure 6). FKBP13 was originally isolated from a cytosolic fraction (Tropschug *et al.*, 1990). Later it was found that an identical protein can be identified in the mitochondrial matrix, indicating that FKBP13 has a dual location in *N. crassa*. Both proteins are the product of the single nuclear gene *fkr-2*. The mitochondrial protein is synthesized as a precursor with a long presequence of 56 amino acids, which is cleaved in two steps upon entry into the mitochondrial matrix (Tropschug, 1997). Mutants in *fkr-2* gene lead to the loss of FKBP13 both in the cytosol and mitochondria. These mutants are

FK506 resistant, proving that FKBP13 is the major receptor for FK506 in *N. crassa* (Barthelmess and Tropschug, 1993).

The *fkbp11* gene (*fkr-3*) is located in linkage group IV and it is composed by five exons and four large introns. This gene codes for a protein of 113 amino acids with no retention signal characterized, presenting only a PPIase domain (Figure 6). The gene *fkbp50* localized in linkage group I is composed by four exons and codes for a protein of 467 amino acids with a predicted mass of 50774.2 Da. The FKBP50 displays a conserved C-terminal PPIase domain, an N-terminal acidic domain and a basic domain in the middle (Figure 6). Previous work using a FKBP50::GFP fusion protein suggests that it is localized in the nucleus (Duarte *et al.*, 2004).

Protein	Gene	Chromosome	Exons	MW (kDa)
FKBP11	*fkr-3*	IV	5	11.18
FKBP13	*fkr-2*	V	2	19.24*
FKBP22	*fkr-5*	VII	2	23.04
FKBP50	*fkr-4*	I	4	50.77

Table 1. Main characteristics of the *fkbp* genes. The molecular weight of FKBP22 has been published previously; the predicted molecular weight of the remaining proteins was calculated using the Molecular Weight Calculator program from EnCor Biotechnology. *Molecular weight of the mature cytosolic FKBP13.

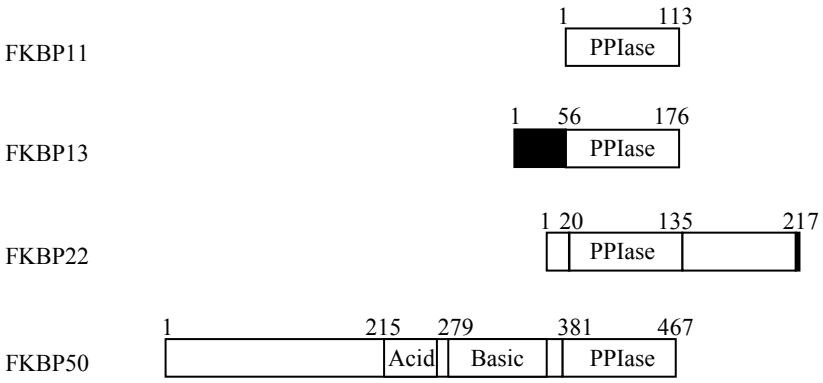

Figure 6. Schematic representation of the structures of *N. crassa* FKBPs. The numbers indicate the amino acid positions. The scan for multiple domains was performed using the ScanProsite program. FKBP13 and FKBP22 targeting sequences to mitochondria (Tropschug, 1997) and ER (Tropschug *et al.*, 1990), respectively, are shown in black.

Chapter 2. Materials and Methods

2.1 *N. crassa* strains

The fungal FGSC#6103 strain, a kind gift from Dr Michael Freitag (University of Oregon, USA), was maintained on Vogel's minimal medium (VMM) supplemented with 200µg/ml L-histidine-HCl. The strains Δfkbp11 (FGSC#15831A), Δfkbp13 (FGSC#11562A and FGSC#11563a) and Δfkbp50 (FGSC#12177a) were obtained from the Fungal Genetic Stock Center (FGSC), *Neurospora* Genome Project – Dartmouth and were maintained in VMM containing 100µg/ml hygromycin B. The mutants fkbp22^{RIP} (Tremmel *et al.*, 2007) and fkbp50^{RIP} were obtained by RIP.

2.2 Media and culture conditions

N. crassa strains were maintained in slants containing solid VMM supplemented with 1,5% sucrose and the required supplements, according to standard procedures (Davis and de Serres, 1970). For vegetative growth, strains were cultured either in liquid or solid VMM containing different substrates. Crosses between single, double and triple mutants were performed on solid Westergaard's medium (Westergaard and Mitchell, 1947). For protein isolation of cultures subjected to different stress conditions, conidia were inoculated in liquid VMM at a final concentration of 10^9cells/L and the cultures grown at 26 and 30ºC with agitation for approximately 16h. After that time the different stress inducers were added and the cultures left to grow for approximately 4h and 30min. For mating conditions, strains were inoculated in solid Westergaard's medium at a final concentration of 10^9cells/L and incubated without agitation at 26ºC for several days.

2.3 Cloning experiments

In order to determine the subcellular location of the FKBP11 protein, we induced its expression using a plasmid commonly used to obtain green fluorescent protein (GFP) fusions. For that purpose the *fkbp11* gene was amplified from the genomic DNA, using the the primers 11fwd: 5'-**GGATCC**AATGGGTGTCAACAAAATCACTC-3', 11rev: 5'-**GGATCC**CAGTGAACCGCCCAGCTACTG-3', and cloned into pCRII-TOPO vector (Invitrogen). The construct was transformed in *E. coli* DH5α strain and its sequence confrmed by DNA sequencing. The recombinant plasmid pTOPO.gene11 was digested with *Bam*HI and the gene fragment cloned into the pMF272 plasmid (FGSC). Spheroplasts of the *N. crassa* FGSC#6103 strain were transformed with the recombinant vector. Genomic DNA from several transformants was analysed by Southern blotting to identify strains with single-copy integration. One was referred as OEFKBP11 and used for further analysis.

Pre-denaturation	1st Cycle	2nd Cycle	3rd Cycle	
1' 95ºC (1x)	30" 95ºC 30" 43ºC (10x) 1' 72ºC	30" 95ºC 30" 48ºC (10x) 1' 72ºC	30" 95ºC 30" 53ºC (10x) 1' 72ºC	5' 72ºC 4ºC ∞

Table 2. PCR amplification protocol used to amplify the gene *fkbp11*.

2.4 Construction and expression of NcFKBP50::GFP fusion protein

To analyse the subcellular location of the FKBP50 protein we used strain expressing a green fluorescent protein (GFP) fused to the C-terminus of the protein previously available in the laboratory. The *fkbp50* cDNA lacking the stop codon was amplified by PCR using the primers F50fwd: 5'-**GGATCC**ATGCCTGTCGCCGTTTTTGGC-3' and F50rev: 5'-**GGATCC**ACTTGATCTCAAGGAGCTTGACG-3'. The amplified 1.4kb fragment was cloned into pGEMT-easy vector (Promega) and the resulting plasmid

digested with *Bam*HI to excise the *fkbp50* cDNA band, which was then cloned in frame with the GFP gene in pMF272 (FGSC). This expression vector was transformed into the *N. crassa* FGSC#6103 strain and a single-copy transformant stably expressing the FKBP50 protein fused to GFP was selected (Duarte *et al.*, 2004). Live cells were stained with 0,5 μM DRAQ5 (Biostatus) for visualization of nuclei and cell images were collected in a Leica Confocal SP2 using filter sets optimal for GFP and DRAQ5 red fluorescence (Leica Microsystems).

2.5 Southern blot

In order to identify the *N. crassa* fkbp11 mutants and *fkbp11* overexpressing strains, different strains were grown overnight in liquid VMM. As positive control the *N. crassa* 74A strain was included. Genomic DNA was isolated as previously described (Yarden et al., 1992). The genomic DNA of different strains was digested with *Bam*HI electrophoresed in 0,9% agarose gels (Sambrook and Russell, 2001). The gels containing the separated DNAs were washed once with water and a denaturing solution (1,5M NaCl, 0,5M NaOH) during 20 min at room temperature (RT) with agitation. After a new wash with water, the gels were washed with a neutralizing solution (1,5M NaCl, 1mM EDTA, 0,5M Tris /HCL pH 7,2) for 15 min at RT with agitation. The neutralizing step was repeated once again. After neutralization, the gels were transferred to nylon N^+ membranes (GE Healthcare) using a vacuum blotter. During transference the gels were systematically covered with 20xSSC (3M NaCl, 0,3M sodium citrate pH 7,0) to avoid dryness. The blots were pre-hybridized with a solution constituted by 5xSSC (0,75M NaCl, 75mM sodium citrate pH 7,0), 1:20 blocking agent (GE Healthcare), 0,1% sodium dodecyl sulfate (SDS) and 5% dextran sulphate, for 30 min at 65ºC with agitation. As probe it was used the *fkbp11* gene extrated from the pTOPO.gene11 plasmid digested with *Bam*HI. The *fkbp11* gene labelling was performed using the Gene Images™ Random Prime Labelling Module kit (GE Healthcare). The labelled DNA was added to the pre-hybridization solution and the blots incubated with this solution overnight at 65ºC with agitation. After hybridization, the blots were three times washed at 65ºC with 1xSSC plus 0,1% SDS; 0,5xSSC plus 0,1% SDS and 0,1xSSC plus 0,1% SDS. After the washes, the blots were washed with buffer A

(150mM NaCl, 100mM Tris/HCl pH 9,5) and buffer A plus 1:10 blocking agent for 1h at RT with gentile agitation. The blots were washed again with buffer A and incubated with a solution containing buffer A, 0,5% BSA and 1:5000 alkaline phosphatase (AP) conjugated with anti-fluorescein during 1h at RT with gentile agitation. After three 10-min washes with buffer A plus 0,3% Tween 20, the blocking agent was added and the blots exposed to a x-ray film (Kodak BioMax Light Film) during 45 min.

2.6 Organelle isolation

The wild type and OEFKBP11 nuclei isolation was performed using some protocol modifications to the procedure already described (Hautala *et al.*, 1977). Conidia from 7-day agar cultures were inoculated to a final concentration of 10^9cells/L into 1 liter of VMM and incubated with agitation at 26ºC overnight. The mycelia was collected and mechanically broken in a grinder in ice-cold buffer A (1M sorbitol, 7% Ficoll 400, 20% glycerol, 5mM $MgCl_2$, 10mM $CaCl_2$, 0,5% Triton X-100) and the homogenate centrifuged at 500 x *g* for 10 min at 4ºC. The supernatant was immediately and carefully removed avoiding the inclusion of any loosely packed pellet and a volume of buffer A equal to the volume of the supernatant removed was added to the centrifuge bottle. The suspended pellet was homogenized and centrifuged as described above. The supernatant was also collected and this procedure repeated a second time. The three supernatants were combined and centrifuged at 9,000 x *g* for 40 min to yield a crude nuclear pellet. The crude nuclear pellet was suspended in buffer B (1M sucrose, 50mM Tris pH 7,5, 5mM $MgCl_2$, 10mM $CaCl_2$, 1% Triton X-100) and homogenized in a Potter-Elvehjem tissue grinder. The nuclear suspension was layered onto a 50, 25, 12,5% sucrose gradient and the gradient centrifuged for 25 min at 8,500 rpm in a Sorvall RC5C centrifuge. After centrifugation, the nuclei were visible as a discrete white band between the 25 and 50% sucrose layers. The nuclei layer was removed using a syringe and diluted in buffer B. In order to obtain a pure nuclear pellet, the suspension was centrifuged at 5,000 x *g* for 20 min and the pellet suspended in a minimal volume of buffer B. The nuclei were stored at –80 º C.

The wild type and OEFKBP11 isolation of mitochondria, endoplasmic reticulum and cytoplasm were also performed using some protocol modifications to the methods already

described (Borgeson and Bowman, 1983). As described for nuclei isolation, conidia from 7-day agar cultures were inoculated to a final concentration of 10^9cells/L into 1 liter of VMM and incubated with agitation overnight at 26ºC. The mycelia was collected and mechanically broken in a grinder in MIM buffer (0,44M sucrose, 2mM EDTA, 30mM Tris/HCl pH 7,6). The homogenate was centrifuged for 5 min at 3,500 rpm at 4ºC in a Sorvall RC5C centrifuge. The supernatant obtained was transferred into a new centrifuge bottle and the centrifugation repeated as above. To obtain the full-extract fraction, 10 ml of supernatant was collected and added 1% Triton X-100. The remaining supernatant was centrifuged again for 50 min at 10,500 rpm at 4ºC. The supernatant obtained was saved for ER isolation and the pellet suspended in MIM buffer and centrifuged for 20 min at 16,500 rpm at 4ºC. The resulting pellet was subjected to a 30, 40, 50, 60% sucrose gradient and centrifuged for 20 min at 8,500 rpm at 4ºC. The mitochondria layer was collected, suspended in MIM buffer and centrifuged for 20 min at 16,500 rpm. The resulting mitochondria pellet was suspended in MIM buffer. The supernatant saved for ER isolation, was centrifuged for 1h at 100,000 x *g* in an Ultra Sorvall centrifuge at 4ºC. The resulting pellet corresponding to the ER was suspended in a minor volume of MIM buffer and the supernatant saved as the cytoplasm fraction.

2.7 Western blot

The cellular fractions corresponding to full-extracts, nuclei, mitochondria, ER and cytoplasm were separated on SDS-polyacrylamide gels and transferred to nitrocellulose Hybond C extra membranes (GE Healthcare) by electroblotting using a semi-dry blotter. After staining with PounceuS, the blots were washed with TBS buffer (150mM NaCl, 10mM Tris/HCl pH 7,5) and blocked with 5% milk in TBS for 1h at RT. After a wash with TBS, the blots were incubated with the respective antibodies diluted in TBS with 0,1% bovine serum albumin (BSA) at 4ºC overnight. The sera and dilutions used were: AS-FKBP11 (1:250), AS-FKBP13 (1:1000), AS-FKBP22 (1:1000), AS-FKBP50 (1:1000), AS-CIII (1:1000) and AS-COREII (1:1000). Blots were washed three times for 10 min with TBST (TBS with 0,05% Triton X-100) and incubated with a anti-rabbit goat immunoglobulin conjugated with AP (Sigma) diluted 1:7500 in TBS with 5 % milk for 1h

at RT. After three 10-min washes with TBST, the blots signal detection was performed using 1:1000 Nitro blue tetrazolium (NBT, 75mg/ml) and 1:1000 5-Bromo-4-chloro-3-indolyl phosphate (BCIP, 50mg/ml) in AP buffer (100mM NaCl, 5mM $MgCl_2$, 100mM Tris/HCl pH 9,5).

2.8 Proteases accessibility

Approximately 100µg of mitochondrial and cytoplasmic protein extracts were prepared in SEM buffer (0,25M sucrose, 2mM EDTA, 10mM MOPS/KOH pH 7,2) were solubilized with 1% Triton X-100 or 0,5% SDS in a final volume of 100µl (complete final volume with SEM buffer). The samples were incubated 3 min on ice. Each sample was diluted in fifteen times the volume in SEM K buffer (SEM buffer with 80mM KCl). To each sample 30 or 45µg/ml of proteinase K or trypsin was added and the samples incubated for 20 min on ice. Next, 2mM of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF) was added and the samples incubated 5 min on ice. The samples were centrifuged 5 min at 14,000 rpm. The pellet was washed with acetone and centrifuged again 5 min at 14,000 rpm. The pellet was suspended in 30µl of 1x GLB. The entire sample was loaded in a 17,5% SDS-PAGE gel. The gel was transferred into a nitrocellulose Hybond C extra membrane (GE Healthcare) using a semi-dry blotter. The membrane was incubated with antisera against FKBP13, FKBP22 and a mitochondrial complex III protein (CIII) overnight as described above.

2.9 Immunoprecipitation experiments

Approximately 1,5mg of *N. crassa* wild type and Δfkbp13 total extracts and mitochondrial proteins were solubilized in a buffer containing 300mM NaCl, 30mM Tris pH 7,5, 0,5mM PMSF and 0,5% Triton X-100 or 0,5% digitonin (DIG) or 0,5% n-dodecyl-β-D-maltoside (DDM). The samples were incubated on ice for 15 min, with vortexing mix every 3 min, and centrifuged at 13,500 rpm for 25 min at 4ºC. The supernatants were collected and 30µl of serum against the *N. crassa* FKBP13 were added to each sample. The

samples were incubated in an orbital mixer overnight at 4ºC. After incubation, 150µl of 62,5mg/ml Protein A immobilized on Sepharose (Sigma) in 30mM Tris pH 7,5 was added to each sample. The mixtures were incubated for 2 hours in the orbital mixer at 4ºC. After centrifugation at 13,500 rpm for 1 min at RT, the pellets obtained were six times 30-sec washed with 300mM NaCl, 30mM Tris pH 7,5 and 0,4% Triton X-100 and four times 30-sec washed with 30mM Tris pH 7,5. The final sediments were suspended in 100µl 1x gel loading buffer (2% SDS, 0,1% bromophenol blue, 10% glycerol, 50mM Tris/HCl pH 6,8). The samples were mixed for 1 hour and heated for 10 min at 65ºC. After a 10 min centrifugation the supernatants were collected. Approximately 200µg of each sample were separated on a 17,5% SDS-PAGE gel and the resulting gel stained with Coomassie Brilliant Blue.

2.10 Silver staining

The gel stained with Coomassie Brilliant Blue was washed 5 min with distillated water and fixed for 3h in a solution containing 2% ethanol and 5% acetic acid (v/v). After a new 5 min wash with distillated water, the gel was fixed again for 30 min in a 10% glutaraldehyde (v/v) solution. After fixation, the gel was five times 30-min washed with distillated water to remove glutaraldehyde excess and incubated for 10 min in a freshly made ammoniacal silver nitrate solution (per 750ml of solution: 10ml concentrated NH_4OH, 1,5ml 10M NaOH, 30 ml 1,2M $AgNO_3$). The gel was washed three times for 5 min with distillated water and the image developed with a solution containing 1% citric acid and 0,037% formaldeid. The reaction was stopped 3 min latter using a 5% acetic acid (v/v) solution.

2.11 Phenotypic analysis

i) Linear growth tests. The growth rates of several *N. crassa* strains were tested on VMM medium containing different substrates at 26 and 37ºC using race tubes. **ii) Fertility tests.** To analyze the role of the *fkbp11* gene in sexual development, both fkbp11 mutants

(mating-types *A* and *a*) were crossed to the wild type as either the female or male parent to detect dominant-specific defects. When used as male, a small drop of conidial suspension was spotted onto wild type (*A* and *a*) strains grown on plates with Westergaard's crossing medium. When used as females, the mutants were grown in plates containing the same crossing medium and fertilized three days later with wild type conidia from the opposite mating-type.

2.12 Miscellaneous

The techniques used for PCR and general cloning (Sambrook and Russell, 2001), protein determination (Bradford, 1976), SDS-polyacrylamide gel electrophoresis (Zauner *et al.*, 1985) and digestion of mitochondria with digitonin (Carneiro *et al.*, 2007) have been published before.

Chapter 3. Results and Discussion

3.1 Cloning and production of OEFKBP11

Further work showed that FKBP11 is not expressed during vegetative development of *N. crassa*. In order to determine the cellular location of this protein we constructed a strain overexpressing FKBP11. Based on the gene sequence of the *fkbp11* gene available at the Broad Institute (http://www.broad.mit.edu/annotation/genome/neurospora) we designed primers and amplified the respective gene by PCR. The sequencing results were aligned with the respective genomic sequence using the Blast program. The sequencing results related to the *fkbp11* gene showed no differences to the sequence previously annotated, although we have previously verified through *fkbp11* cDNA amplification and sequencing that three codons initially annotated as the final part of the first intron of the *fkbp11* gene are in fact the beginning of the second exon (Figure 7). Two of the codons code for the amino acids glutamate and phenylalanine (this sequence provides a recognition site for the restriction enzyme *Eco*RI) and the third codon encodes the amino acid serine (Pinto, 2006). The *fkbp11* gene amplified by PCR was cloned in *Bam*HI site of the plasmid pMF272 and the construct obtained used to transform *N. crassa*. The gene was integrated in the *his-3* locus of a *N. crassa his-3* auxotrophic mutant strain (FGSC#6103) by homologous recombination. Transformants were selected on a histidine-free Vogel's minimal medium with sorbose to induce colonial growth. Resulting prototrophic His^+ transformants were isolated and analyzed by Southern blotting to identify the correct genotype. The *fkbp11* gene extracted from the pTOPO.gene11 construct was used as probe in the Southern blotting analysis. In Figure 8A, we can see that both transformants selected (T1 and T2) present an extra copy of the *fkbp11* gene, in contrast to the wild type strain that only contains the endogenous *fkbp11*. Wild type, T1 and T2 protein full-extracts were also analysed by Western blotting using an antiserum against the FKBP11 to confirm that both strains were expressing the FKBP11 (Figure 8B). Since there were no significant differences in the FKBP11 expression between both transformants, T1 was selected to further experiments.

```
  1  atgggtgtcaacaaaatcactcacgttgcgggtaccgggccacaaccagaagctggc
  1   M  G  V  N  K  I  T  H  V  A  G  T  G  P  Q  P  E  A  G
 58  cagaccgtggttatcgagtatactggttggctgaaggactcgagccaggccgatgga
 20   Q  T  V  V  I  E  Y  T  G  W  L  K  D  S  S  Q  A  D  G
115  aagggagccgagtatgttcttttccccattgaaaaatcttgccttaccacacacgtc
 39   K  G  A  E
172  tccggttgttggtcagttttgataacactatatccag attcgacag ctcgatcggtc
 43                                        F  D  S  S  I  G
229  gtggggattttgtcacgcaaatcggggttgggagactcatcagaggtatatgcccac
 49  R  G  D  F  V  T  Q  I  G  V  G  R  L  I  R
286  catcggtccacttttttcatatatcggagactttatgctgacgtgctgcttttgtga
343  cgggcataggatgggatgaggctgtattgaaaatgaaggttggcgagaaagccactt
 64           G  W  D  E  A  V  L  K  M  K  V  G  E  K  A  T
400  tggatatttctaggtgagttattcgtgagtgaacgatgtgtcaggggaagaaattga
 80  L  D  I  S  S
457  tactgacgagacaatatgttttagtgattacggatacggagagcggtgagtaacctg
 85                           D  Y  G  Y  G  E  R
514  caggccttttttttttctttcttctgaaaatccaaaaaaatcagtctatatggtgta
571  acaccacccgcagacacgatacttctggccgtgctgatttggagttaatgcccatgt
628  gctgacccgacttattgcacaccaggggttttcacggccacattcctccgaacgcag
 92                            G  F  H  G  H  I  P  P  N  A
685  atttgatcttgtgagttgtctcctgtatgccacatatcttgctccagcttgtctcta
102  D  L  I  F
742  cagtcaaggtagcggcgggcaagatagcgtcagcaaggatacgagctaatatgtgat
799  agtgacgtatatctcaagggtctccagtag
106      D  V  Y  L  K  G  L  Q  *
```

Figure 7. Nucleotide and deduced amino acid sequence of the FKBP11. Exons and introns are in capital and lower case letters, respectively. The codons initially identified as being part of the first intron sequence but that in fact are the beginning of the second exon sequence are in bold (Pinto, 2006).

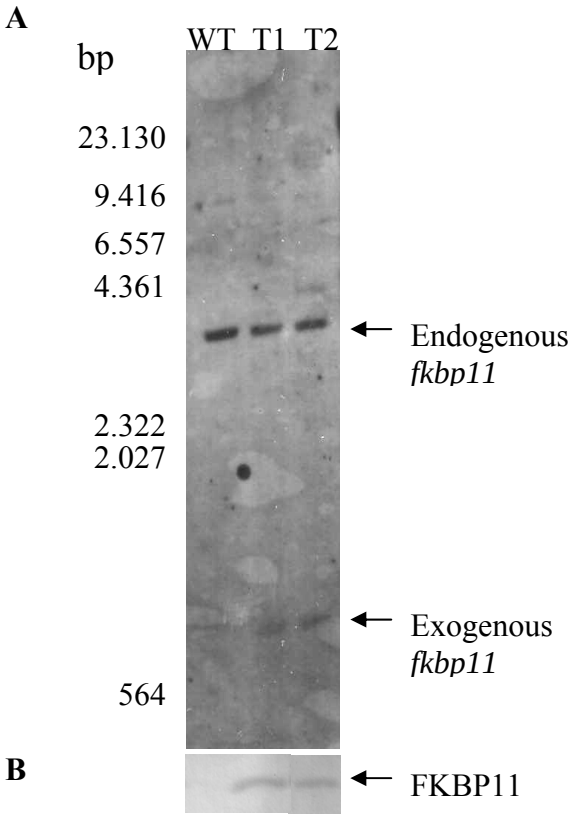

Figure 8. Identification of strains overexpressing FKBP11. A. Identification of *N. crassa* strains containing an extra copy of the *fkbp11* gene by Southern blotting using as probe the *fkbp11* gene extracted from the pTOPO.gene11 plasmid digested with *Bam*HI. B. Protein full-extracts of the same strains were analyzed by Western blotting to confirm that both transformants express the FKBP11. WT - wild type; T1 - transformant 1; T2 - transformant 2.

3.2 Cellular location of FKBPs

In this work we have confirmed the FKBP13, FKBP22 and FKBP50 cellular locations and determined the subcellular location of the protein FKBP11. To confirm the subcellular location of FKBP50, we used a strain expressing a green fluorescent protein (GFP) fusion, previously produced in the laboratory, and performed colocalization studies with different dyes specific for different organelles. To obtain this strain, the cDNA encoding FKBP50 was cloned in frame with the GFP gene, under the control of an

inducible promoter. The fusion construct was targeted to the *his-3* locus of *N. crassa* (Duarte *et al.*, 2004). By confocal microscopy analysis we observed a specific accumulation of fluorescence in small dot-like inclusions in the FKBP50::GFP strain (Figure 9), likely to be nuclei. Furthermore, these dot like structures were stained with DRAQ5 confirming the nuclear location for FKBP50::GFP.

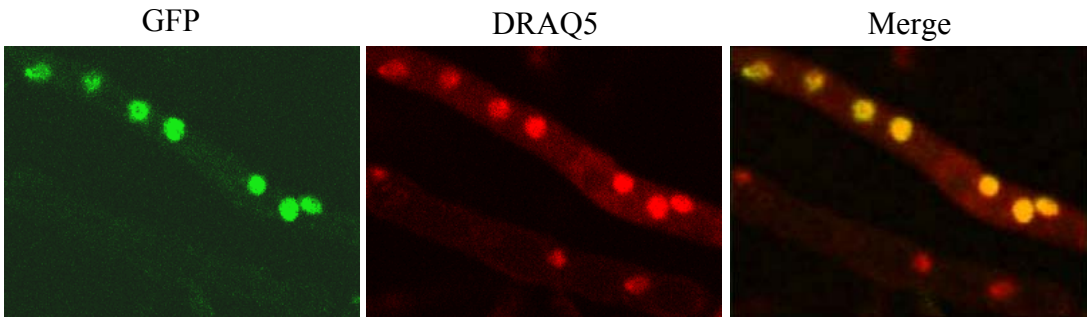

Figure 9. Localization of the fusion protein FKBP50::GFP. Conidia from *Neurospora* strain stably expressing the fusion protein FKBP50::GFP were grown overnight in minimal medium containing acetate and subsequently analysed by confocal microscopy for the presence of green fluorescence. The nuclei were stained with DRAQ5.

To confirm that the endogenous FKBP50 is also located in the nucleus we grew the wild type strain in minimal medium and isolated several organelles. Through a Western blotting analysis using specific antiserum against the FKBP50 we confirmed that the FKBP50 is indeed a nuclear protein (Figure 10). The FKBP50 appears in distinct bands possibly as a result of post-translational modifications, as phosphorylation. The upper band that appears in all lanes, including in the fkbp50 mutants (Figure 10), is a cross reaction of the antibody. As depicted in Figure 10, FKBP13 localizes in the cytoplasm and mitochondria although it is located essentially in the cytoplasm, confirming the previously described dual localization (Tropschug *et al.*, 1990; Tropschug, 1997). As control for organelle purification we used antisera against the ER protein FKBP22 (Solscheid and Tropschug, 2000) (Figure 10) and the 14 kDa mitochondrial protein of complex I (Marques

et al., 2005) (Figure 10). The cytoplasm represents what is left after cellular fractionation, so that it is possible to find remains of FKBP22 in this last fraction.

FKBP11 was not detectable in the wild type strain at different temperatures, diverse carbon sources and several stress conditions. In order to overcome this initial barrier we constructed a strain overexpressing FKBP11. The pMF272 plasmid presents an inducible promoter (*ccg-1*) that is repressed by glucose and induced by glucose absence or by stress conditions. Usually to obtain a greater protein expression we substitute the glucose carbon source by sodium acetate. In this case we observed that the *fkbp11* expression was not altered by the use of glucose or sodium acetate, so we grew the OEFKBP11 strain in minimal medium at 26ºC and isolated several organelles. Analysis of the different cellular fractions by Western blotting shows that FKBP11 is exclusively a cytosolic protein (Figure 10).

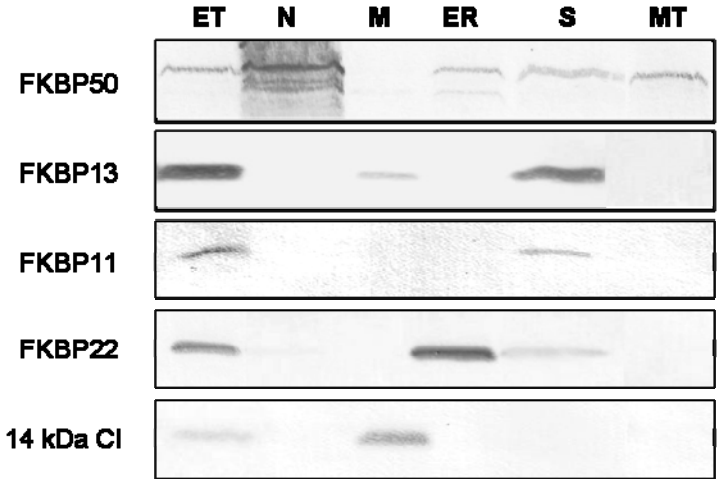

Figure 10. Cellular location of the FKBPs from *N. crassa*. Wild type cellular extracts were prepared and the cellular fractions analysed by Western blotting using specific antisera as indicated in the left side. For the location of FKBP11 the organelles were purified from the OEFKBP11 strain. The 14 kDa subunit of complex I (CI) was used as control. ET – total cellular extracts; N – nuclei; M – mitochondria; ER – endoplasmic reticulum; S – components of the cytoplasm after cellular fractionation. In each panel, MT represents total cellular extracts obtained from null-mutant strains corresponding to the

protein being analysed. Approximately 200µg of protein were loaded in the lanes corresponding to total extracts and cytoplasm and 50µg on the others.

3.3 The OEFKBP11 strain is resistant to high levels of calcium and zinc

As mentioned before, to localize the FKBP11 protein we grew the wild type strain with various carbon sources (1,5% sucrose, 44mM sodium acetate and 2% glycerol), different temperatures (26ºC and 37ºC) and different stress conditions, including starvation, osmotic stress (1M NaCl and 1M sorbitol), oxidative stress (0,5 to 2mM H_2O_2) and protein misfolding (2,5mM L-Azetidine-2-carboxylic acid). We also tested for the presence of the FKBP11 during several stages of the vegetative development as mycelium, aerial hyphae and conidia. In none of the conditions tested we detected the presence of the FKBP11.

Since the FKBP11 is not detected during vegetative development of the fungus and the Δfkbp11 mutant shows no visible phenotype we decided to test if the overexpression of the FKBP11 conducted to any alteration of the cellular functioning. To that purpose the OEFKBP11 strain was grown in plates containing solid sorbose medium (GFS) with different stress conditions induced by 1,5mM H_2O_2, 200µM paraquat, 2,5mM L-azetidine-2-carboxylic acid, 50mM LiCl, 100mM $CaCl_2$ and 4mM $ZnCl_2$. Surprisingly, the OEFKBP11 strain presented a different phenotype when grown in GFS supplemented with 100mM $CaCl_2$ and 4mM $ZnCl_2$. In both situations the OEFKBP11 strain produced larger colonies when compared to the wild type strain (Figure 11). Apparently this phenotype is not due to a chloride effect since the strain growing in 50mM LiCl did not present the same phenotype (Figure 11). Calcium is one of the most important second messengers as its levels change in response to many cellular alterations. The presence of the FKBP11 appears to give an advantage to the strain, indicating that the FKBP11 could be involved in calcium regulation. However, linear growth in race tubes is comparable in strains, wild type and OEFKBP11, in media containing $CaCl_2$ and $ZnCl_2$.

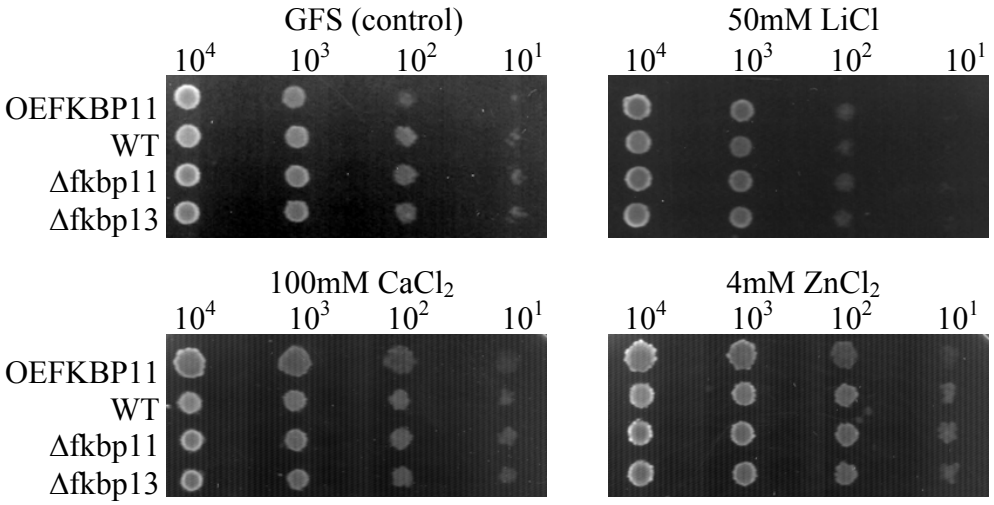

Figure 11. The OEFKBP11 strain is resistant to high amounts of $CaCl_2$ and $ZnCl_2$. Five microliters of serial dilutions of conidia from the indicated strains were inoculated (spotted) onto solid sorbose medium (GFS) supplemented with 100mM $CaCl_2$, 4mM $ZnCl_2$ or 50mM LiCl and incubated at 37ºC for approximately 48 hours.

To test if these stress agents induce the expression of the FKBP11 we grew the wild type and the OEFKBP11 strains in liquid minimal medium supplemented with 100mM $CaCl_2$ and 4mM $ZnCl_2$. The OEFKBP11 showed an increased in the expression of the FKBP11 in media containing $CaCl_2$ when compared to the control and media containing $ZnCl_2$. Interestingly, the FKBP11 was detected in the wild type growing with $CaCl_2$, but not when this strain was grown with $ZnCl_2$ (Figure 12). If the protein has an important role in the regulation of the intracellular levels of calcium or zinc we would expect to detect the protein in the wild type strain growing in the same conditions, which only happen with calcium. However, the amount of FKBP11 present in any of these situations can probably explain the phenotype observed for OEFKBP11. The overexpression of the FKBP11 appears to give an advantage to the strain in some stress conditions promoting a larger cell proliferation despite that the protein is not important to the survival of the wild type strain in the same conditions.

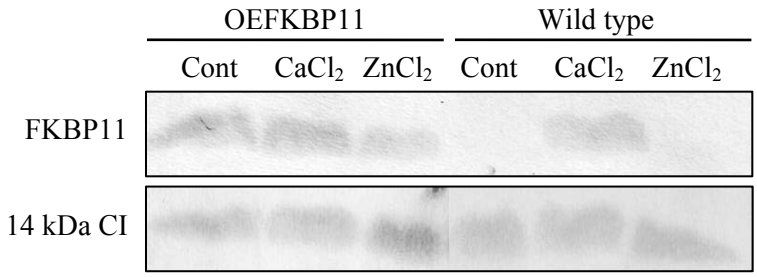

Figure 12. Calcium induces the expression of FKBP11. The wild type and the OEFKBP11 strains were grown in minimal medium supplemented with 100mM $CaCl_2$ or 4mM $ZnCl_2$. Expression of the protein FKBP11 was analysed by Western blotting using an antiserum against the protein. The 14 kDa subunit of complex I (CI) was used as loading control. Approximately 300µg of protein were loaded in each lane.

3.4 The FKBP11 is highly expressed during sexual development

N. crassa is a heterothallic filamentous fungus that presents both asexual and sexual reproduction. The decision whether to undergo asexual or sexual development is made based on environmental signals. Usually reproducing asexually, in situations of nitrogen starvation, low light and temperature *Neurospora* undergoes a complex pattern of sexual differentiation to form the female reproductive structure, the protoperithecium. After fertilization by a nucleus of the opposite mating-type, the protoperithecium develops into a fruiting body called perithecium, which contains the asci with the ascospores (Nelson and Metzenberg, 1992; Coppin *et al.*, 1997; Davies, 2000; Kim and Nelson, 2005). Since FKBP11 is not expressed during the vegetative phase of growth of *N. crassa*, we decided to investigate its expression during sexual development. For that propose we grew the wild type strain in liquid Westergaard medium at 26ºC, prepared protein full-extracts and analysed the FKBP11 expression by Western blotting. We found that FKBP11 is highly expressed in *N. crassa* growing in Westergaard medium, a medium poor in nitrogen used to promote sexual development (Figure 13). We further observed that this high expression is not dependent in the nitrogen concentration but it's prevented at 37ºC, a condition that also impairs the development of fungal perithecia (Perkins, 1987). To determine if the FKBP11 play an important role in the sexual cycle of *N. crassa* we tested the fertility of

the Δfkbp11 mutants, as female and male, with both wild type strains. When compared with the wild type, none of the displayed any defect in terms of the development of sexual structures (formation of protoperithecia and perithecia), producing viable black ascospores. Thus, although the *fkbp11* gene is expressed during the sexual development, it appears not to be a gene essential for the process. As known for other FKBPs (and cyclophilins) that are involved in multiple cellular processes, it is possible that the protein encoded by *fkbp11* has a redundant function or that its absence might be covered by the presence of other proteins. To date, no gene encoding a FKBP or cyclophilin involved in sexual development in filamentous fungi has been identified.

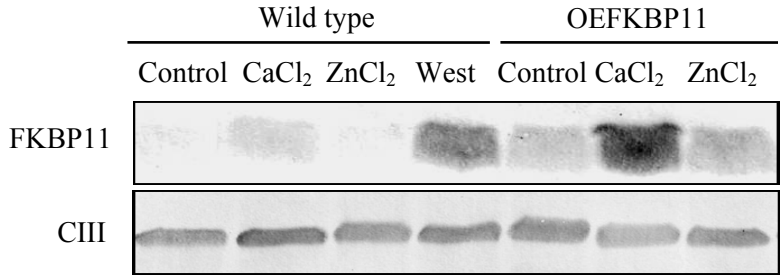

Figure 13. FKBP11 expression is also induced in Westergaard medium. The wild type and the OEFKBP11 strains were grown in minimal medium supplemented with 100mM $CaCl_2$ or 4mM $ZnCl_2$. Moreover the wild type was also grown is Westergaard medium (West). Expression of the protein FKBP11 was analysed by Western blotting using an antiserum against the protein. As loading control we used a mitochondrial complex III protein (CIII). Approximately 250µg of protein were loaded in all lanes.

3.5 FKBP13 is resistant to protease degradation

With the purpose to confirm where the FKBP13 was located in mitochondria we preformed a protease accessibility assay based on the sequential solubilization of mitochondria with digitonin (DIG) (Carneiro *et al.*, 2007). In this assay approximately 100 µg of wild type mitochondria were solubilized with increasing concentrations of DIG and the several fractions obtained subjected to a proteinase K (PK) treatment. Even though

when we analyzed the FKBP13 sequence in the PeptideCutter program we could find forty eight PK restriction sites, in this assay we verified that FKBP13 was completely resistant to PK degradation. To test if this result was due to a problem related to a deficient mitochondria solubilization, we solubilized wild type mitochondria with other detergents, as Triton X-100 and SDS, and tested the FKBP13 sensitivity to high amounts of PK and trypsin (Tryp). As control we used a mitochondrial complex III protein (CIII) and the His-FKBP13 fusion protein produced in *E. coli* (Figure 14A). In contrast to the control proteins that were completely digested by both proteases, when mitochondria were solubilized with 1% Triton X-100 the FKBP13 was found to be highly resistant to PK degradation and partially sensitive to Tryp proteolysis (Figure 14A). On the other hand, when mitochondria were solubilized with a more aggressive detergent as SDS, an anionic surfactant that disrupts all the non-covalent bonds leading to protein denaturation, we verified that FKBP13 was more sensitive to PK and Tryp degradation. In order to determine if the cytosolic FKBP13 presented the same protease sensitivity we repeated the assay using cytoplasm obtained after mitochondria isolation, for that reason we used FKBP22 as control in this assay. In opposite to the result obtained with mitochondria, the cytosolic FKBP13 was completely resistant to PK degradation in both conditions tested (Figure 14B). A higher resistance to Tryp degradation was also observed, although we could identify fifteen Tryp restriction sites in FKBP13 using the PeptideCutter program.

Proteins resistant to PK proteolysis are rare mostly due to the potency, wide pH optimum and low peptide bond specificity of this protein. Since PK is a more aggressive protease, when compared to Tryp, we would expect to find a higher or even total sensitivity of FKBP13 to this protease. The FKBP13 high stability upon protease degradation can eventually be a result of post-translational modifications. For example, it is known that the sarcoplasmic reticulum Ca^{2+}-ATPase when unphosphorylated is highly sensitive to PK, Tryp and V8 protease degradation (Danko *et al.*, 2001). In opposite, ADP-insensitive phosphoenzyme and phosphorylated state analogues display a more compact conformation being almost completely resistant to degradation by these proteins. This hypothesis is reinforced by the fact that the His-FKBP13 produced in *E. coli*, where no post-translational modifications occur, is completely sensitive to protease degradation (Figure 14 A). The differential protease resistance may represent a physiological regulatory mechanism controlling the functions of the FKBP13.

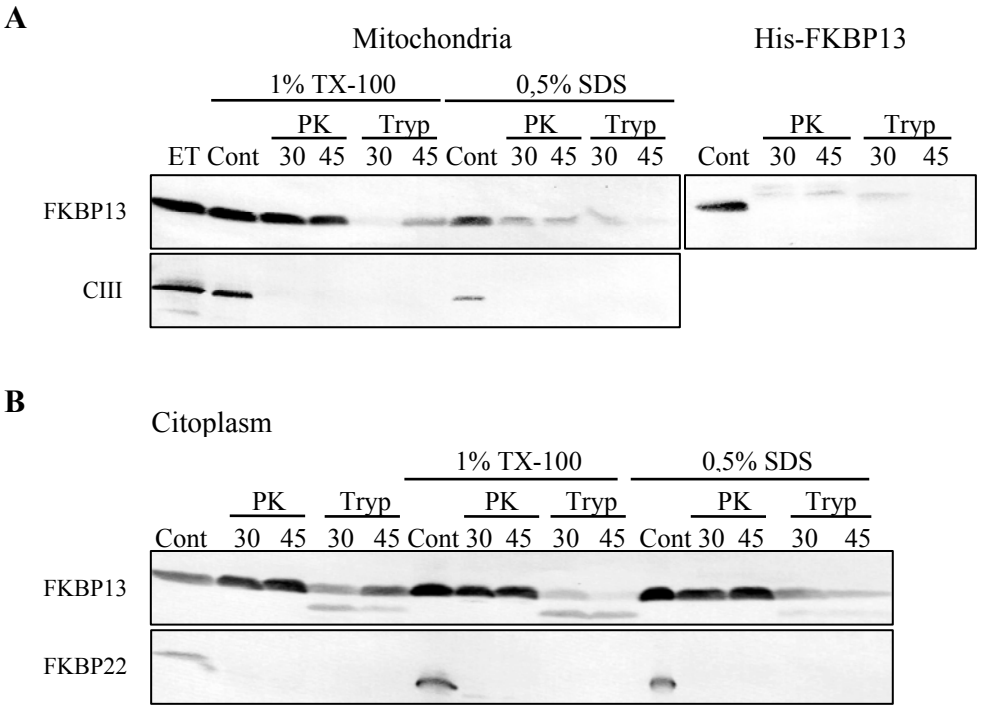

Figure 14. FKBP13 is resistant to protease degradation. A. Approximatelly 100µg of wild type mitochondria were solubilized with 1% Triton X-100 (TX-100) or 0,5% sodium dodecyl sulfate (SDS) and the FKBP13 sensitivity to protease degradation tested by different concentrations of proteinase K (PK) and trypsin (Tryp). The His-FKBP13 fusion protein and a mitochondrial complex III protein (CIII) were used as control. B The same assay was used in the determination of the sensitivity of citosolic FKBP13 to protease degradation. As control it was used the FKBP22. Cont – control; protein solubilized with the respective detergent but not subjected to protease degradation.

3.6 A protein of 40 kDa coimmunoprecipitates with As-FKBP13

In addition to their involvement in protein folding, most of the PPIases interact with restricted cellular partners, being thus involved in specific cellular processes. For that reason, the identification of the *N. crassa* FKBPs potential cellular partners is an important step in order to understand the physiological role of these proteins. In an attempt to identify the cellular partners of the *N. crassa* FKBP13 we performed immunoprecipitation experiments using an antiserum against this protein. In these experiments, the wild type and Δfkbp13 mutant full-extracts and mitochondrial proteins were solubilized with different detergents as Triton X-100, DIG and n-dodecyl-β-D-maltoside (DDM) and incubated with FKBP13 antiserum overnight. In contrast to the nonionic surfactant Triton X-100 that virtually disrupts all protein interactions, the detergents DIG and DDM are less aggressive preserving the more instable protein associations. The samples resulted from the immunoprecipitation experiments were subjected to a SDS-PAGE and the resulting gel silver stained.

In all the conditions tested it was possible to immunoprecipitate both the cytosolic and the mitochondrial FKBP13, being this result confirmed by Western blotting (Figure 15). Along with the FKBP13 it was also coimmunoprecipitated a protein of approximately 40 kDa in the fractions corresponding to full-extracts solubilized with DIG or DDM (Figure 15, indicated by the arrow). In opposition to Triton X-100, an aggressive nonionic surfactant, the mild non-ionic detergent DIG and the gentle detergent DDM are frequently used to solubilize receptors and permeabilize cellular and nuclear membranes, preserving the protein activity and the weak protein associations. This 40 kDa protein could only be immunoprecipitated using these less aggressive detergents, suggesting that FKBP13 may interact with a cytosolic protein through weak interactions. The isolation and identification of the 40 kDa protein may provide us new insights about the cellular function of FKBP13.

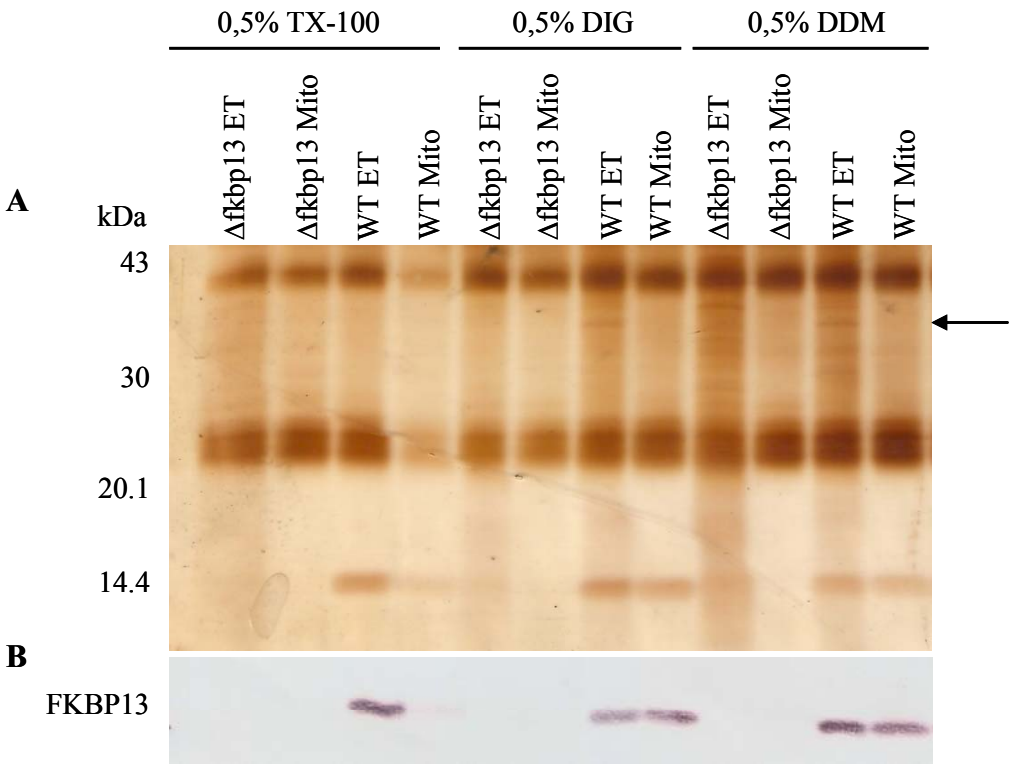

Figure 15. FKBP13 coimmunoprecipitates with a protein of approximately 40 kDa. A. Approximately 1,5 mg of wild type and Δfkbp13 full-extracts (ET) and mitochondrial (Mito) proteins were solubilized with 0,5% Triton X-100 (TX-100), 0,5% digitonin (DIG) or 0,5% n-dodecyl-β-D-maltoside (DDM) and incubated with an antiserum against the *N. crassa* FKBP13. Approximately 200µg of protein were subjected to a 17,5% SDS–PAGE and the resulting gel stained with silver. B. The same fractions were analysed by Western blotting using an antiserum against the FKBP13 protein.

3.7 *N. crassa* FKBPs amounts changes in response to stress conditions

In order to analyse the response of the *N. crassa* FKBPs to different stress conditions and the possible interaction of the FKBP11 with the remaining proteins, we grew the strains wild type and OEFKBP11 in VMM for approximately 16 hours and incubated the strains for an additional 4 hours and 30 minutes with several stress inducers. Protein full-extracts of all the conditions were prepared and analysed by Western blotting using

antisera against all the FKBP proteins. As loading control we used an antiserum against the mitochondrial protein COREII.

As shown in figures 16A and B, when compared to the control VMM, the FKBP50 can be found in slighty amounts in the wild type strain grown at 26ºC with $ZnCl_2$, the proline analogue L-azetidine-2-carboxylic (AZ) acid and sorbitol. As the heat-shock and the hyper-osmotic stresses, the incorporation of the L-proline analogue L-azetidine-2-carboxylic acid during translation induces protein misfolding. This protein levels are also increased in the OEFKBP11 strain grown in VMM and VMM supplemented with $ZnCl_2$. Interestingly, the FKBP50 is almost completely absent in the wild type and OEFKBP11 strains grown with $CaCl_2$, suggesting that *fkbp5*0 expression might be down regulated by calcium.

As the FKBP50, at 26ºC the FKBP22 can be found more expressed in stress conditions that induce protein misfolding as osmotic stress promoted by high levels of NaCl and sorbitol, oxidative stress induced by the herbicide paraquat and $ZnCl_2$ and protein misfolding caused by AZ, which correlates with its ER chaperone activity. Curiously, as the FKBP50, the FKBP22 can be detected in high amounts in the OEFKBP11 strain grown with VMM and VMM supplemented with $ZnCl_2$, but not when this strain was grown with $CaCl_2$ (Figure 16A). At 37ºC, the FKBP22 is in general more expressed possibly as an effect of the heat-shock stress, even when the OEFKBP11 was grown with $CaCl_2$.

At 26ºC, the FKBP13 levels were found to be significantly reduced when the wild type was grown with $ZnCl_2$, paraquat, LiCl or AZ. In contrast, the FKBP13 levels were considerably higher in the OEFKBP11 strain grown with $CaCl_2$, situation were the FKBP11 levels are very high, suggesting a possible relation between FKBP13 and FKBP11 (Figure 16A). At 37ºC, the FKBP13 levels are not significantly altered in any of the conditions tested (Figure 16B).

In wild type, the FKBP11 can be detected when this strain grows at 26ºC with high amounts of calcium or with Westergaard medium (Figure 16A). On the contrary, the FKBP11 cannot be detected when the wild type grows in Westergaard medium at 37ºC, correlating a possible involvement of this protein in sexual development, which does not occur or occur deficiently at high temperatures (Perkins, 1986) (Figure 16B).

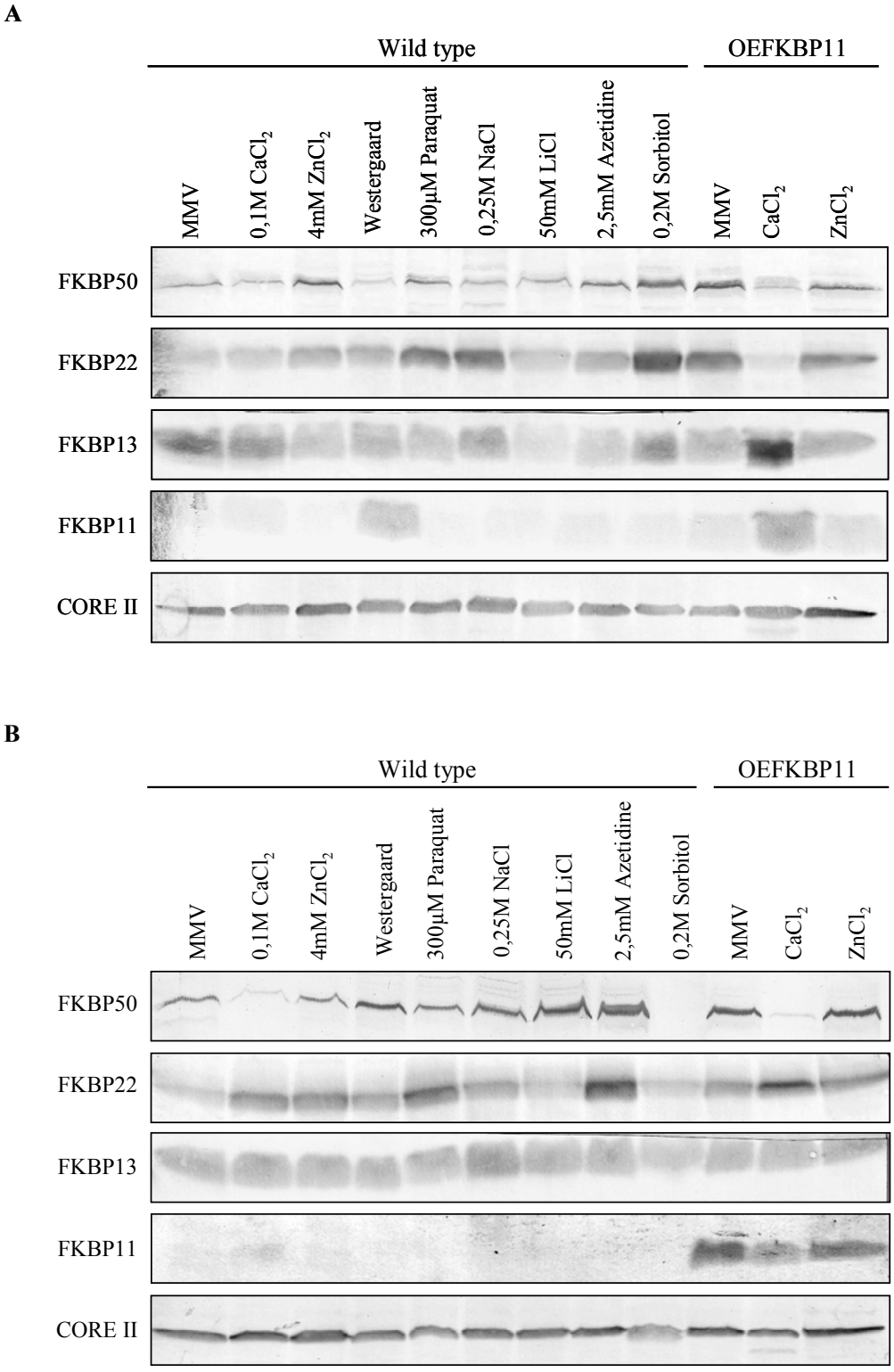

Figure 16. Effect of different stress inducers in the FKBPs levels. The wild type and OEFKBP11 strains were grow in VMM medium for approximately 16 hours and additionally incubated for 4 hours and a half with several stress inducers. The results were

analysed by Western blotting using antisera against the FKBP proteins as indicated on the left side. Approximately 250µg of protein were loaded in each lane. As loading control we used the mitochondrial protein COREII. A. Growth at 26ºC. B. Growth at 37ºC.

3.8 Production of FKBP multiple mutants

In order to investigate the specific role of FKBPs, we generated multiple mutants for the *fkbp* genes. The double, triple and quadruple mutants were obtained by crossing the single (Δfkbp11, Δfkbp13, fkbp22^{RIP} and fkbp50^{RIP}) and multiple (fkbp22/50 and fkbp13/22/50) *N. crassa* mutants in solid Westergaard medium, a synthetic medium poor in nitrogen that favours sexual reproduction (Westergaard and Mitchell, 1947). The cross between mutants was initiated with the inoculation of one of the mutants (the one serving as female) in plates containing Westergaard medium. After three days of growth at 26ºC, the second mutant of the opposite mating-type (the one serving as male) was inoculated in the same plate. After approximately two weeks of growth it was possible to observe the development of sexual structures (perithecia) and the release of the spores. Seven days after the release, the mature spores were collected and activated by heat shock at 60ºC. The activated spores were plated in GFS medium and five days later several colonies isolated. The identification of Δfkbp11 mutants among ascospore progeny of the crosses was carried out by Southern blot analysis. The multiple mutants for the genes *fkbp13*, *fkbp22* and *fkbp50* were identified by immunoblotting analysis with specific antisera against the respective proteins.

In Figure 17 are displayed some of the different mutant combinations obtained with the cross of Δfkbp13 and fkbp22/50 mutants. During the germination of spores isolated from this cross it was possible to observe the existence of both small and large colonies. Protein full-extracts from both types of colonies isolated were analyzed by Western blotting using specific antisera against the FKBP13, FKBP22 and FKBP50 proteins in order to identify the respective mutants. Interestingly, through immunoblotting it was possible to determine that all the small colonies isolated were fkbp50 mutants. Although viable, all the single, double and triple mutants lacking the *fkbp50* gene presented a spore germination delay. In contrast, none of the strains isolated from the larger colonies were

fkbp50 mutants, associating this phenotype to the mutation of the *fkbp50* gene. Since we were not able to detect the FKBP11 by Western blotting, the identification of Δfkbp11 mutants resulting from the crosses Δfkbp11 x fkbp13/50 and Δfkbp11 x fkbp13/22/50 was performed by Southern blotting as described in material and methods.

In table 3 is summarized the number and mutant genotypes obtained in the different crosses analyzed. The construction of the *N. crassa* fkbp mutant collection was relatively easy to obtain mainly due to the fact that all the genes are located in different chromosomes and none of the deletions revealed to be lethal to the different mutant strains.

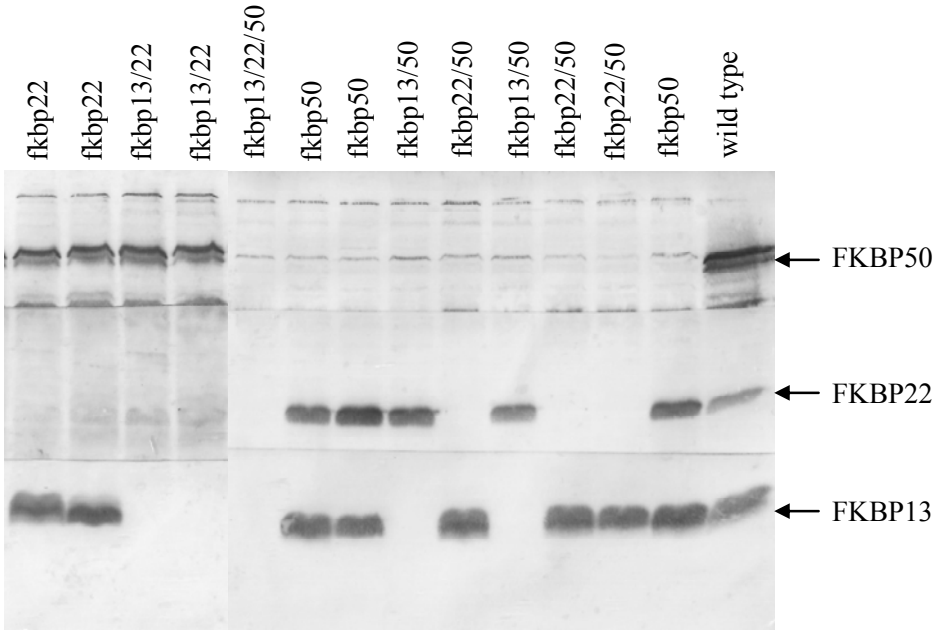

Figure 17. *N. crassa* mutants resulting from the cross Δfkbp13 x fkbp22/50. In order to obtain multiple mutants for the *fkbp13*, *fkbp22* and *fkbp50* genes, the mutants Δfkbp13 and fkbp22/50 were crossed in solid Westergaard medium. The progeny of the cross was analyzed by Western blotting using specific antisera against FKBP13, FKBP22 and FKBP50. The FKBP50 protein appears in two distinctive bands.

Crosses	Number of spores analyzed	Genotypes obtained
Δfkbp13 x fkbp50^{RIP}	8	fkbp50 (2)* fkbp13/50 (6)*
Δfkbp13 x fkbp22^{RIP}	10	WT (2) fkbp13 (4) fkbp22 (4) fkbp13/22 (1)
Δfkbp13 x fkbp22/50	21	WT (1) fkbp22 (2) fkbp50 (5) fkbp13/22 (4) fkbp13/50 (3) fkbp22/50 (4) fkbp13/22/50 (2)
Δfkbp11 x fkbp13/50	4	fkbp11 (1) fkbp13 (1) fkbp11/13 (1) fkbp11/13/50 (1)
Δfkbp11 x fkbp13/22/50	19	fkbp22 (1) fkbp50 (1) fkbp11/22 (3) fkbp11/50 (4) fkbp13/22 (1) fkbp22/50 (2) fkbp11/13/22 (2) fkbp11/22/50 (1) fkbp13/22/50 (1) fkbp11/13/22/50 (3)

Table 3. *N. crassa* fkbp mutants. In order to complete the *N. crassa* fkbp mutant collection, single and multiple mutants were crossed in Westergaard medium and the progeny analyzed by Western and Southern blotting. In the table are represented the mutants obtained in some of the crosses performed during this study. In brackets are displayed the number of mutants obtained per cross. *In this cross only small colonies were isolated.

3.9 Disruption of FKBP50 leads to a temperature-sensitive phenotype

Previous works have shown that FKBPs are not important for survival of different species. In a search for the physiological role of the *N. crassa* FKBPs we grew the single mutants Δfkbp11, Δfkbp13, fkbp22^{RIP} and fkbp50^{RIP} in race tubes with different carbon sources (1,5% sucrose, 44mM sodium acetate and 2% glycerol) and different stress conditions (1,5 to 2mM H_2O_2, 200μM paraquat, 1M NaCl, 1M sorbitol and 2,5mM L-azetidine-2-carboxylic acid) under two temperatures (26 and 37ºC). We observed that the Δfkbp11, Δfkbp13 and fkbp22^{RIP} mutants showed no detectable phenotype (Figure 18A). Since the endogenous FKBP11 protein is not detected in any of the conditions tested before, the lack of a phenotype for Δfkbp11 was expected. However, the non-existence of a visible phenotype for the Δfkbp13 mutant could be due to a redundancy of functions between similar proteins. For instance, like FKBP13 the *N. crassa* cyclophilin 20 (Cyp20) is also located both in mitochondria and cytoplasm (Tropschug *et al.*, 1988; Rassow *et al.*, 1995). Although Cyp20 is not involved in the import of preproteins into mitochondria, this protein is an active component of the protein folding machinery as it promotes protein folding in the mitochondrial matrix in an association with the molecular chaperones Hsp70 and Hsp60 (Rassow *et al.*, 1995).

Previously it was observed that the fkbp50^{RIP} mutant displayed a growth defect when grown at 26ºC. Therefore, we carried out a detailed analysis of the fkbp50^{RIP} mutant growth in race tubes containing VMM at two different temperatures. Contrary to the wild type and the other fkbp mutants, and confirming the previous result, we observed that the fkbp50^{RIP} mutant presented a notorious growth defect when grown at 26ºC (Figure 18B). Interestingly, this growth deficiency was almost completely reversed when the strain was grown at 37ºC. To verify if this phenotype was a result of the *fkbp50* disruption and not a consequence of any secondary effect of the RIPing phenomenon, we tested the Δfkbp50 mutant in the same conditions. We verified that both fkbp50 mutants displayed the temperature-sensitive phenotype at 26ºC that was almost completely overturned when the strains were grown at 37ºC (Figure 18B). It is likely that the cellular stress caused by the heat shock may partially compensate for the FKBP50 defect. FKBP50 is homologous to Fpr4 a nucleolar protein that consists of an acidic domain in the N-terminus, a basic domain in the middle and the PPIase domain at the C-terminus. Recent studies demonstrate

that Fpr4 possesses a novel histone chaperone activity, acting as a chromatin component involved in rDNA silencing (Kuzuhara and Horikoshi, 2004). The acidic domain is responsible for the histone chaperone activity while the PPIase domain inhibits this activity (Xiao *et al.*, 2006). In addition, Fpr4 was described as a histone proline isomerase, whose activity regulates methylation of lysine 38 in histone H3 and the induction of transcription (Nelson *et al.*, 2006). Based on the homology of FKBP50 and Fpr4 it is possible that the *Neurospora* enzyme is also involved in similar processes. Considering that *N. crassa* FKBP50 is involved in rDNA silencing, disruption of the *fkbp50* gene may lead to the lack of repression of the genes encoding ribosomal proteins, which conducts to an alteration of the normal cellular function and the observation of the fkbp50 phenotype. It is described that stress imposed by heat shock leads to the repression of genes encoding ribosomal proteins (Trotter *et al.*, 2002), in this way, the partial reversion of the fkbp50 phenotype may be explained by the growth of the mutant at high temperatures (37ºC). Silencing of the rDNA and/or the induction of several genes could be the reasons for the partial reversion of the growth defect by temperature increase (Nelson *et al.*, 2006).

Disruption of all four FKBP immunophilins did not affect the fungus viability, showing that all of these proteins are dispensable for *N. crassa* survival (Figure 18A). With the exception of the multiple mutants carrying the *fkbp50* mutation, which maintained the growth deficiency, none of the other mutant combinations presented a detectable phenotype in what concerns growth, temperature-sensitivity, oxidative stress or the presence of a protein misfolding inducer (L-azetidine-2-carboxylic acid).

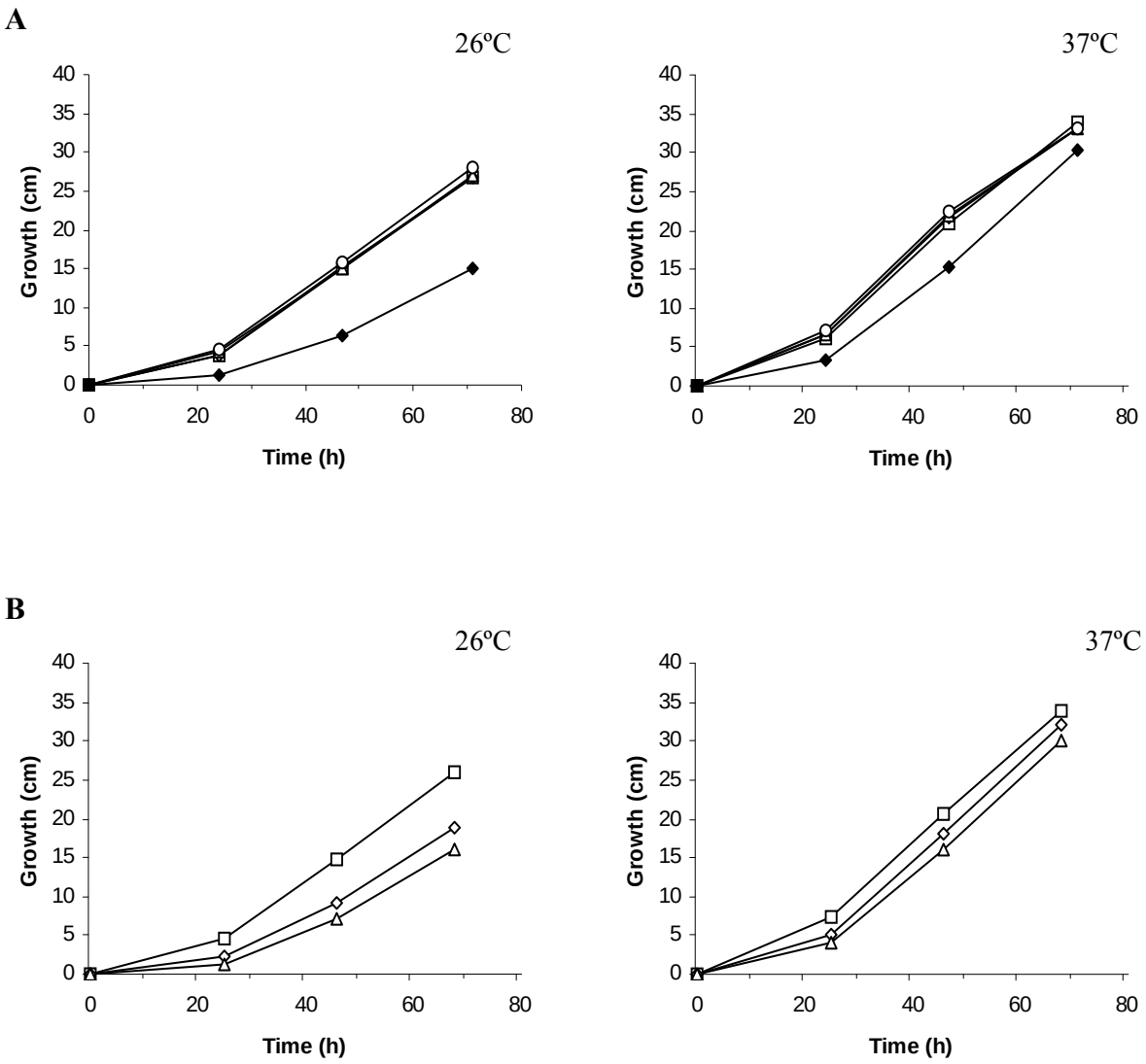

Figure 18. Effect of the temperature on the growth of different strains of *N. crassa*. A. The wild type (□) and mutants Δfkbp11 (◊), Δfkbp13 (Δ) and fkbp22^{RIP} (○) and Δfkbp11/13/22/50 (♦) were inoculated (5x10^4 conidia/5µl) in race tubes with minimal medium and grown at two different temperatures. B. Growth of fkbp50^{RIP} (◊) and Δfkbp50 (Δ) mutants compared to the wild type (□).

Chapter 4. Final remarks

In addition to their role in protein folding, several studies report the involvement of immunophilins in key regulatory mechanisms, as control of transcription, cell-cycle regulation and apoptosis (Lu *et al.*, 1996; Arévalo-Rodrígues and Heitman, 2005; Baines *et al.*, 2005). Due to their high conservation, the characterization of the cellular mechanisms mediated by these proteins using low eukaryotes as a model study might help us to understand essential cellular processes.

In this study we proposed to characterize the cellular functions of three immunophilins of the FKBP family identified in *N. crassa*. Our first goal was to establish the subcellular location of all the *N. crassa* FKBPs. Using a GFP fusion protein and wild type protein extracts we confirmed the nuclear location of FKBP50, the endoplasmic location of FKBP22 and the mitochondrial and cytoplasmic location of FKBP13. We also determine that FKBP11 is located in the cytoplasm. A more detailed analysis of these proteins showed interesting results. The FKBP11 is only expressed in particular conditions, not being detected during the vegetative development of the fungus. This protein can be detected when the wild type grows with high amounts of calcium. Interestingly, the strain overexpressing FKBP11 is more resistant to high levels of calcium, presenting a larger cell proliferation when compared with the wild type. This result may indicate the involvement of FKBP11 in calcium regulation. The FKBP11 is also found when the wild strain grows in Westergaard medium (a medium favouring sexual reproduction), but only at 26ºC. It is known that at high temperatures *Neurospora* sexual development does not occur or occurs deficiently. This result reinforces the potential involvement of FKBP11 in sexual development, although disruption of the *fkbp11* gene does not affect the development of sexual structures, as protoperithecia and perithecia, or the production of viable ascospores. As the *N. crassa* immunophilin Cyp20, the FKBP13 presents dual location, being found simultaneously in mitochondria and cytoplasm. Treatment of wild type protein extracts with different proteases showed that FKBP13 is highly resistant to proteolysis, specially the cytoplasmic FKBP13. Post-translational modifications, as phosphorylation, may be in the origin of the resistance of this protein to proteolysis. Through immunoprecipitation it

was possible to coimmunoprecipitate with FKBP13 a protein of approximately 40 kDa. Determination of the cause of resistance to protease degradation and the identification of the 40 kDa protein will help us to determine the cellular functions played by this protein. The *N. crassa* nuclear FKBP50 was the only protein which gene mutation lead to a visible phenotype. Although disruption of the *fkbp11*, *fkbp13* and *fkbp22* genes did not result in a detectable phenotype, disruption of the *fkbp50* gene resulted in a growth deficiency at 26ºC, confirmed in this work. Due to the high homology between the *N. crassa* FKBP50 and the *S. cereviseae* Fpr4 we can speculate the involvement of FKBP50 in similar cellular events as rDNA silencing. Although the quadruplet mutant presented the *fkbp50* disruption phenotype, disruption of the four *fkbp* genes did not affect the fungus viability, showing that all four proteins are non-essential for the fungus survival. A final analysis of the FKBPs expression in response to several stress inducers suggests that the FKBP50 is down regulated by calcium. The FKBP22 levels are found to be high in the majority of the stress conditions tested, correlating its function as ER molecular chaperone and the FKBP13 levels can be found notably high in the presence of high amounts of FKBP11, suggesting a possible relation between FKBP13 and FKBP11.

During the last decades a significant progress has been made towards understanding the structure-function relationship of these proteins in the cell. However, the physiological roles of the majority of these proteins remain to determine. Although we could not address the cellular functions of *N. crassa* FKBP-type immunophilins, with this work we positively contributed to a better knowledge of the factors that influence the activity of these proteins. The identification of specific natural ligands and a more profound analysis of the mutant strains may provide us new insights into the *N. crassa* FKBPs physiological role and in a more extended way the role of immunophilins in higher eukaryotes.

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Appendix

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Identification of all FK506-binding proteins from *Neurospora crassa*

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ABSTRACT

Immunophilins are intracellular receptors of immunosuppressive drugs, carrying peptidyl-prolyl cis–trans isomerase activity, with a general role in protein folding but also involved in specific regulatory mechanisms. Four immunophilins of the FKBP-type (FK506-binding proteins) were identified in the genome of *Neurospora crassa*. Previously, FKBP22 has been located in the endoplasmic reticulum as part of chaperone/folding complexes and FKBP13 has been found to have a dual location in the cytoplasm and mitochondria. FKBP11 is apparently located exclusively in the cytoplasm. It is not expressed during vegetative development of the fungus although its expression can be induced with calcium and during sexual development. Overexpression of the respective gene appears to confer a growth advantage to the fungus in media containing some divalent ions. FKBP50 is a nuclear protein and its genetic inactivation leads to a temperature-sensitive phenotype. None of these proteins is, alone or in combination, essential for *N. crassa*, as demonstrated by the isolation of a mutant strain lacking all four FKBPs.

1. Introduction

Immunophilins are intracellular receptors of immunosuppressive drugs commonly used in the prevention of graft rejection after organ transplantation and treatment of autoimmune diseases. According to their affinity to different immunosuppressive drugs they can be classified in two families: the cyclophilins (CyPs), which bind cyclosporin A (CsA), and the FK506-binding proteins (FKBPs), which bind the macrolides FK506 and rapamycin. Along with the parvulin family, these proteins make part of the larger protein superfamily of peptidyl-prolyl cis–trans isomerases (PPIases). The latter activity allows the acceleration of protein folding (Galat and Metcalfe, 1995; Göthel and Marahiel, 1999; He et al., 2004; Barik, 2006).

These ubiquitous and highly conserved proteins are found in all organisms, from archaea to man, and cellular compartments (Trandinh et al., 1992; Kay, 1996; Galat, 2000; Vallon, 2005). Their wide distribution and functional conservation lead to a rising curiosity and interest in their potential cellular functions. In some cases it is possible to demonstrate that isomerization of prolyl bonds is the rate-limiting step in protein folding *in vivo* and *in vitro* (Kiefhaber et al., 1990; Steinmann et al., 1991; Kern et al., 1995). For instance, it has been found that SurA, a periplasmic protein in *Escherichia coli* similar to parvulin is involved in the folding of periplasmatic and outer membrane proteins *in vivo* (Lazar and Kolter, 1996). It is believed that SurA maintains the outer membrane integrity since *surA* mutants became deformed and easily lysed during stationary phase. In addition to a general role in accelerating protein folding, the cellular functions of most of these proteins are only now being discovered. Recent work has shown a great number of important and exciting findings implicating these proteins in key regulatory mechanisms (Luan, 1998; Göthel and Marahiel, 1999; Breiman and Camus, 2002; Barik, 2006). PPIases are involved in several and important cellular processes as signal transduction (Bram et al., 1993), protein trafficking (Stamnes et al., 1991; Chambraud et al., 2007), protein assembly (Wu et al., 2004; Xiao et al., 2006), control of transcription (Arévalo-Rodríguez and Heitman, 2005), pre-mRNA splicing (Lorkovic et al., 2004), rDNA silencing (Kuzuhara and Horikoshi, 2004), apoptosis (Baines et al., 2005) and cell-cycle regulation (Lu et al., 1996; Vittorioso et al., 1998).

The yeast *Saccharomyces cerevisiae* contains four FKBP proteins, Fpr1 to Fpr4. All yeast prolyl isomerases, with the exception of Ess1, are individually and collectively dispensable for growth. However, deletion of the cytoplasmic protein Fpr1 leads to a slow growth phenotype (Dolinski et al., 1997). Fpr2 is a protein from the secretory pathway and Fpr3 and Fpr4 are nucleolar proteins. Four immunophilins belonging to the FKBP family, named FKBP11, FKBP13, FKBP22 and FKBP50 were also found in the last genome

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annotation of *Neurospora crassa* (Neurospora sequencing project, Whitehead Institute/MIT Center for Genome Research). The cellular functions of FKBP22 have been recently characterized (Solscheid and Tropschug, 2000; Tremmel et al., 2007; Tremmel and Tropschug, 2007). FKBP22 is a homodimeric protein located in the lumen of the endoplasmic reticulum with a PPIase and a novel chaperone activity. This protein interacts directly with BiP, the major HSP70 chaperone in the ER, through its FKBP domain, being BiP able to enhance FKBP22 chaperone activity. BiP and FKBP22 form a stable folding helper complex with a high chaperoning activity preventing the aggregation of unfolded rhodanese (Tremmel and Tropschug, 2007). FKBP22 appears also to form other chaperone/folding catalyst complexes with disulfide isomerases, like ERp38 and PDI, Grp170, another ER HSP70 chaperone, and Cyp23, a cyclophilin, being the organization of such complexes mediated by BiP. FKBP22 is as well involved in the development of microconidiophores (Tremmel et al., 2007). FKBP13 was originally isolated from a cytosolic fraction (Tropschug et al., 1990). Later it was found that an identical protein can be identified in the mitochondrial matrix, indicating that FKBP13 has a dual location in *N. crassa*. Both proteins are the product of a single nuclear gene, *fkr-2*. The mitochondrial protein is synthesized as a precursor with a long presequence of 56 amino acids, which is cleaved in two steps upon entry into the mitochondrial matrix (Tropschug, 1997). Mutants in *fkr-2* gene lead to the loss of FKBP13 both in cytosol and mitochondria. These mutants are FK506 resistant (Barthelmess and Tropschug, 1993), proving that FKBP13 is the major receptor for FK506 in *N. crassa*. Although the location of FKBP13 and FKBP22 has been already identified the location of FKBP11 and FKBP50 remained to be determined. Our goal is the characterization of the role of immunophilins in fundamental cellular processes common to all eukaryotes. In this work we present the characterization of all *N. crassa* immunophilins of the FKBP-type.

2. Materials and methods

2.1. Neurospora crassa strains and manipulations

The *N. crassa* strains were grown and handled according to standard procedures (Davis and de Serres, 1970). The fungal FGSC#6103 strain, a kind gift from Dr. Michael Freitag (University of Oregon, USA), was maintained on Vogel's minimal medium supplemented with 200 μg/ml L-histidine–HCl. The strains Δfkbp11 (FGSC#15831A), Δfkbp13 (FGSC#11562A and FGSC#11563a) and Δfkbp50 (FGSC#12177a) were obtained from the Fungal Genetic Stock Center (FGSC), Neurospora Genome Project – Dartmouth and were maintained in Vogel's minimal medium containing 100 μg/ml hygromycin B. Mutant fkbp22^{RIP} was handled as previously described (Tremmel et al., 2007).

For the generation of the fkbp50^{RIP} mutant the *fkbp50* gene was amplified from *N. crassa* genomic DNA by PCR using the primers 5′-GTGGAGTGAACCGTGAGCCT-3′ and 5′-GGATCCCCTTCCTACCCCA CGCT-3′. The amplified 2356 bp fragment was cloned in the pGEMT-easy vector (Promega). The resulting plasmid was digested with XhoI and the relevant band of 1694 bp cloned into pCSN44 treated with the same enzyme, creating plasmid pFKBP50-X. This recombinant vector was used for transformation of *N. crassa* 74-OR8-1a (FGSC) spheroplasts. Genomic DNA from transformants was analysed by Southern blotting to identify strains with single-copy integration. One transformant carrying a duplication of the *fkbp50* gene was crossed with strain 74-OR23-1A in order to inactivate this gene by RIP. Identification of fkbp50^{RIP} mutants among the progeny of the cross was carried out by Western blot analysis of total proteins with an antiserum against FKBP50.

Crosses between single, double and triple mutants were performed on Westergaard's medium (Westergaard and Mitchell, 1947). Identification of Δfkbp11 mutants among ascospore progeny of the crosses was carried out by Southern blot analysis. The multiple mutants for the genes *fkbp13*, *fkbp22* and *fkbp50* were identified by immunoblotting analysis with specific antisera against the respective proteins.

2.2. Cloning experiments

The *fkbp11*, *fkbp13* and *fkbp50* cDNAs were amplified from the M$^-$ cDNA library (FGSC) by PCR using the primers 11fwd: 5′-**GG ATCC**aatgggtgtcaacaaaatcactc-3′, 11rev: 5′-**GGATCC**cagtgaaccgcc cagctactg-3′, 13fwd: 5′-**GGATCC**gatgactattccccagcttgacg-3′, 13rev: 5′-**GGATCC**AATCCGCCTGTATCCCAGAGC-3′, 50fwd: 5′-**CATATG**GCC CCCCTTATGCCTGT-3′ and 50rev: 5′-**GGATCC**CCTTCCTACCCCACGC T-3′, respectively. All sets of primers present BamHI restriction sites, with the exception of the primer 50fwd that presents a NdeI restriction site (in bold). The PCR products corresponding to *fkbp11* and *fkbp13* cDNAs were cloned into pCRII-TOPO vectors (Invitrogen). The *fkbp50* purified cDNA was cloned into the pGEMT-easy vector (Promega). The constructs were transformed in *E. coli* DH5α strain and their sequence verified by DNA sequencing.

In order to determine the subcellular location of the FKBP11 protein, we induced its expression using a plasmid commonly used to obtain green fluorescent protein (GFP) fusions. For that purpose the *fkbp11* gene was amplified from the genomic DNA, using the same primers used to amplify the cDNA, and cloned into pCRII-TOPO vector. The recombinant plasmid pTOPO.gene11 was digested with BamHI and the gene fragment cloned into the pMF272 plasmid (FGSC). Spheroplasts of the *N. crassa* FGSC#6103 strain were transformed with the recombinant vector. Genomic DNA from several transformants was analysed by Southern blotting to identify strains with single-copy integration. One was referred as OEFKBP11 and used for further analysis.

2.3. Construction and expression of NcFKBP50::GFP fusion protein

To analyse the subcellular location of the FKBP50 protein we used a green fluorescent protein (GFP) fused to the C-terminus of the protein. The *fkbp50* cDNA lacking the stop codon was amplified by PCR using the primers F50fwd: 5′-GGATCCATGCCTGTCGCCGTT TTTGGC-3′ and F50rev: 5′-GGATCCACTTGATCTCAAGGAGCTTGA CG-3′. The amplified 1.4 kb fragment was cloned into pGEMT-easy vector and the resulting plasmid digested with BamHI to excise the *fkbp50* cDNA band, which was then cloned in frame with the GFP gene in pMF272 (FGSC). This expression vector was transformed into the *N. crassa* FGSC#6103 strain and a single-copy transformant stably expressing the FKBP50 protein fused to GFP was selected. Live cells were stained with 0.5 μM DRAQ5 (Biostatus) for visualization of nuclei and cell images were collected in a Leica Confocal SP2 using filter sets optimal for GFP and DRAQ5 red fluorescence (Leica Microsystems).

2.4. Production of polyclonal antibodies

The recombinant plasmids pTOPO.cDNA11 and pTOPO.cDNA13 were digested with BamHI and the cDNA fragments subcloned into pQE31 (Qiagen) and pET28b (Novagen) vectors, respectively. The pGEMT.cDNA50 was double digested with NdeI and BamHI and subcloned into the pET19b (Novagen) expression vector. For the expression of the His-FKBP13 recombinant protein the strain used as host was *E. coli* M15 (Qiagen). The His-FKBP11 and His-FKBP50 recombinant proteins were expressed in *E. coli* BL21(DE3) strain (Stratagene). The expression of His-tag fusion proteins was induced by 1 mM IPTG for 3 h at 37 °C and the purification of recom-

binant proteins was performed using a His-Trap™ HP column (GE Healthcare). The His-tag fusion proteins were used to produce rabbit polyclonal antisera as described previously (Videira and Werner, 1989).

2.5. Miscellaneous

The techniques used for PCR and general cloning (Sambrook and Russell, 2001), protein determination (Bradford, 1976), SDS–polyacrylamide gel electrophoresis (Zauner et al., 1985), Western (Towbin et al., 1979) and Southern blot (Southern, 1975) have been published before. The *N. crassa* preparation of total extracts and isolation of nuclei (Hautala et al., 1977), mitochondria, endoplasmic reticulum and cytoplasm was performed as described (Borgeson and Bowman, 1983). The Broad Institute Accession numbers for the FKBP11, FKBP13, FKBP22 and FKBP50 proteins are NCU04371.3, NCU04140.3, NCU02455.3 and NCU03241.3, respectively.

3. Results and discussion

3.1. Cloning and disruption of FKBP genes

For the production of antibodies against the FKBP11, FKBP13 and FKBP50 proteins, we designed primers based on the gene sequences of the three *fkbp* genes (*fkr-3*, *fkr-2* and *fkr-4*) available at the Broad Institute (http://www.broad.mit.edu/annotation/genome/neurospora) and amplified the respective cDNAs by PCR. For FKBP13 only the 120 C-terminal amino acids were considered based on the previously described sequence (Tropschug et al., 1990). The sequencing results were aligned with the respective genomic sequences using the Blast program. The sequencing results related to the *fkbp13* and *fkbp50* cDNAs showed no differences to the cDNA sequences annotated. When we analysed the amplified *fkbp11* cDNA sequence we verified that three codons initially annotated as the final part of the first intron sequence are in fact the beginning of the second exon (Fig. 1a). Two of the codons code for the amino acids glutamate and phenylalanine (this sequence provides a recognition site for the restriction enzyme EcoRI) and the third codon encodes the amino acid serine. Characteristics arising from *in silico* analysis of the different *fkbp* genes and proteins are displayed in Fig. 1b and c.

In order to investigate the specific role of FKBPs, we generated a null-mutant strain by inducing mutations in the *fkbp50* gene, as described in material and methods. Fig. 2a shows the results of Southern blots obtained with the single-copy transformant T12. Using the enzyme SacI two bands of 1.5 and 5.9 kb can be detected in the wild type DNA. The same enzyme leads to the appearance of two extra bands of 4.4 and 6.8 kb in DNA from transformant T12. With PvuII a single band of 6.4 kb in wild type and an extra band of 5.7 kb in T12 are visible. A 1.7 kb band is visible in wild type and T12 when XhoI is used (the enzyme used for cloning in pCSN44, not shown) indicating that the ectopic DNA fragment is intact. Altogether, these results indicate that a single-copy gene that has been duplicated in transformant T12 encodes the FKBP50 protein. The strain T12 was then crossed with a wild type strain to generate fkbp50 mutants that were identified between the progeny by Western blot analysis of total extracts with antisera against FKBP50 (Fig. 2b). The fkbp22 mutant was described before (Tremmel et al., 2007) and the fkbp11 and fkbp13 deletion mutants were obtained from the FGSC (Colot et al., 2006). Double, triple and quadruple mutants were obtained by crosses of the single and multiple mutants and their identification was performed by Western and/or Southern blotting analysis.

3.2. Cellular location of FKBPs

In this work we have confirmed the FKBP13 dual location and determined the subcellular location of the proteins FKBP11 and FKBP50. To determine the subcellular location of FKBP50, we first used a green fluorescent protein (GFP) fusion. The cDNA encoding FKBP50 was cloned in frame with the GFP gene, under the control of an inducible promoter. The fusion construct was targeted to the *his-3* locus of *N. crassa*. By confocal microscopy analysis we observed a specific accumulation of fluorescence in small dot-like inclusions in the FKBP50::GFP strain (Fig. 3a), likely to be nuclei. Furthermore, these dot-like structures were stained with DRAQ5 confirming the nuclear location for FKBP50::GFP. To confirm that the endogenous FKBP50 is also located in the nucleus we grew the wild type in minimal medium and isolated several organelles. Through a Western blotting analysis using specific antisera against the FKBP50 we confirmed that the FKBP50 is indeed a nuclear protein (Fig. 3b). The FKBP50 appears in distinct bands possibly as a result of post-translational modifications. The upper band that appears in all lanes, including the fkbp50 mutants (Fig. 3b), is a cross reaction of the antibody.

As depicted in Fig. 3b, FKBP13 localizes in the cytoplasm and mitochondria although it is located essentially in the cytoplasm, confirming the previously described dual localization (Tropschug et al., 1990; Tropschug, 1997). As control for organelle purification we used antisera against the ER protein FKBP22 (Solscheid and Tropschug, 2000) and the 14 kDa mitochondrial protein of complex I (Marques et al., 2005) (Fig. 3b). The cytoplasm represents what is left after cellular fractionation, so that it is possible to find remains of FKBP22 in this last fraction.

FKBP11 was not detectable in the wild type strain at different temperatures, diverse carbon sources and several stress conditions (data not shown). In order to overcome this initial barrier we amplified the *fkbp11* gene from genomic DNA by PCR. The gene fragment was cloned into the pMF272 plasmid and the construct obtained used to transform spheroplasts of a *N. crassa* histidine mutant strain. The pMF272 plasmid presents an inducible promoter (*ccg-1*) that is repressed by glucose and induced by glucose absence or by stress conditions. Usually to obtain a greater protein expression we substitute the glucose carbon source by sodium acetate. In this case we observed that the *fkbp11* expression was not altered by the use of glucose or sodium acetate (data not shown), so we grew the OEFKBP11 strain in minimal medium at 26 °C and isolated several organelles. Analysis of the different cellular fractions by Western blotting indicates that FKBP11 is exclusively a cytosolic protein (Fig. 3b).

3.3. Disruption of FKBP50 leads to a temperature-sensitive phenotype

Previous works have shown that FKBPs are not important for survival of different species. In a search for the physiological role of the *N. crassa* FKBPs we grew the single mutants Δfkbp11, Δfkbp13, fkbp22^{RIP} and fkbp50^{RIP} in race tubes with different carbon sources (1.5% sucrose, 44 mM sodium acetate and 2% glycerol) and different stress conditions (1.5–2 mM H_2O_2, 200 μM paraquat, 1 M NaCl, 1 M sorbitol and 2.5 mM L-azetidine-2-carboxylic acid) under two temperatures (26 and 37 °C). We verified that the Δfkbp11, Δfkbp13 and fkbp22^{RIP} mutants showed no detectable phenotype (Fig. 4a). Since the endogenous FKBP11 protein is not detected in any of the conditions tested before, the lack of a phenotype for Δfkbp11 was expected. However, the non-existence of a visible phenotype for the Δfkbp13 mutant could be due to a redundancy of functions between similar proteins. For instance, like FKBP13 the *N. crassa* cyclophilin 20 (Cyp20) is also located both in mitochondria and cytoplasm (Tropschug et al., 1988; Rassow et al., 1995). Although Cyp20 is not involved in the import of prep-

a

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   1  atgggtgtcaacaaaatcactcacgttgcgggtaccgggccacaaccagaagctggc
   1   M  G  V  N  K  I  T  H  V  A  G  T  G  P  Q  P  E  A  G
  58  cagaccgtggttatcgagtatactggttggctgaaggactcgagccaggccgatgga
  20   Q  T  V  V  I  E  Y  T  G  W  L  K  D  S  S  Q  A  D  G
 115  aagggagccgagtatgttcttttccccattgaaaaatcttgccttaccacacacgtc
  39   K  G  A  E
 172  tccggttgttggtcagttttgataacactatatccagattcgacagctcgatcggtc
  43                                          F  D  S  S  I  G
 229  gtggggattttgtcacgcaaatcggggttgggagactcatcagaggtatatgcccac
  49  R  G  D  F  V  T  Q  I  G  V  G  R  L  I  R
 286  catcggtccacttttttcatatatcggagactttatgctgacgtgctgcttttgtga
 343  cgggcataggatgggatgaggctgtattgaaaatgaaggttggcgagaaagccactt
  64           G  W  D  E  A  V  L  K  M  K  V  G  E  K  A  T
 400  tggatatttctaggtgagttattcgtgagtgaacgatgtgtcaggggaagaaattga
  80  L  D  I  S  S
 457  tactgacgagacaatatgttttagtgattacggatacggagagcggtgagtaacctg
  85                           D  Y  G  Y  G  E  R
 514  caggccttttttttttctttcttctgaaaatccaaaaaaatcagtctatatggtgta
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 628  gctgacccgacttattgcacaccaggggttttcacggccacattcctccgaacgcag
  92                             G  F  H  G  H  I  P  P  N  A
 685  atttgatcttgtgagttgtctcctgtatgccacatatcttgctccagcttgtctcta
 102  D  L  I  F
 742  cagtcaaggtagcggcgggcaagatagcgtcagcaaggatacgagctaatatgtgat
 799  agtgacgtatatctcaagggtctccagtag
 106     D  V  Y  L  K  G  L  Q  *
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b

Protein	Gene	Chromosome	Exons	MW (kDa)
FKBP11	*fkr-3*	IV	5	11.18
FKBP13	*fkr-2*	V	2	19.24
FKBP22	*fkr-5*	VII	2	23.04
FKBP50	*fkr-4*	I	4	50.73

c

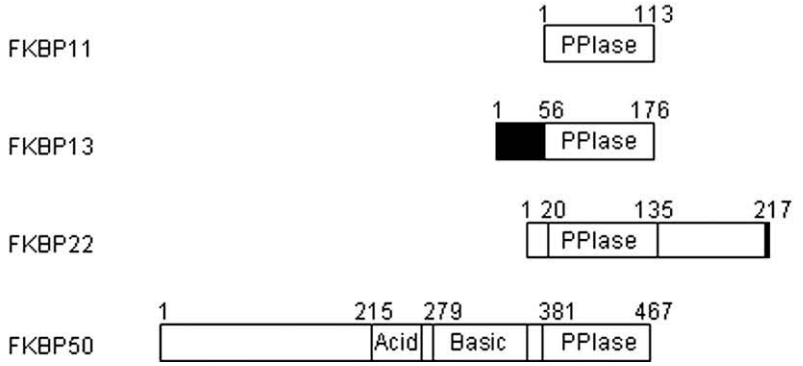

Fig. 1. Structure of *N. crassa* FKBPs. (a) Nucleotide and deduced amino acid sequence of the FKBP11. The codons initially identified as being part of the first intron sequence but that in fact are the beginning of the second exon sequence are in bold. (b) Main characteristics of the *fkbp* genes. The molecular weight of FKBP22 has been published previously; the predicted molecular weight of the remaining proteins was calculated using the Molecular Weight Calculator program from EnCor Biotechnology. (c) Schematic representation of the structures of FKBPs. The numbers indicate the amino acid positions. The scan for multiple domains was performed using the ScanProsite program. Targeting sequences are shown in black.

roteins into mitochondria, this protein is an active component of the protein folding machinery as it promotes protein folding in the mitochondrial matrix in an association with the molecular chaperones Hsp70 and Hsp60 (Rassow et al., 1995).

In contrast to wild type and the other fkbp mutants, the fkbp50^{RIP} mutant presented a notorious growth defect when grown at 26 °C (Fig. 4) and also at the lower temperature of 19 °C (data not shown). Interestingly, this growth deficiency was almost completely reversed when the strain was grown at 37 °C. To verify if this phenotype was a result of the *fkbp50* disruption and not a consequence of any secondary effect of the RIPing phenomenon, we tested the Δfkbp50 mutant in the same conditions.

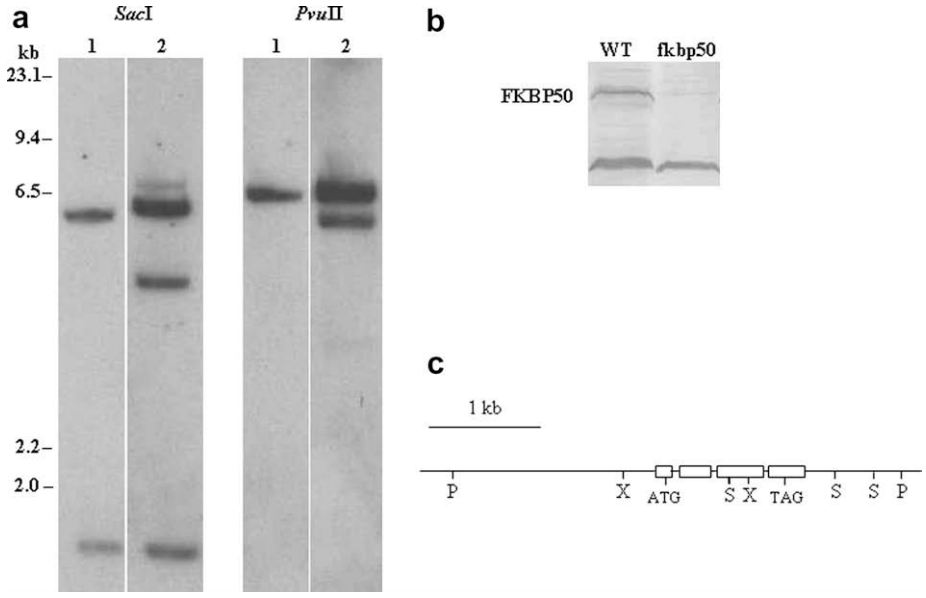

Fig. 2. FKBP50 disruption. (a) Southern blotting analysis of a *Neurospora* strain transformed with plasmid pFKBP50-X. Genomic DNA from the wild type strain (1) and transformant T12 (2) was prepared, separately digested with SacI and PvuII, electrophoresed in agarose gels and blotted onto nylon membranes. The filters were probed with the gene coding for the FKBP50 protein. (b) To identify the mutant, total proteins from *N. crassa* strains were extracted and analysed by Western blotting using antiserum against the FKBP50 protein. (c) Restriction map of the gene (P, PvuII; S, SacI; X, XhoI).

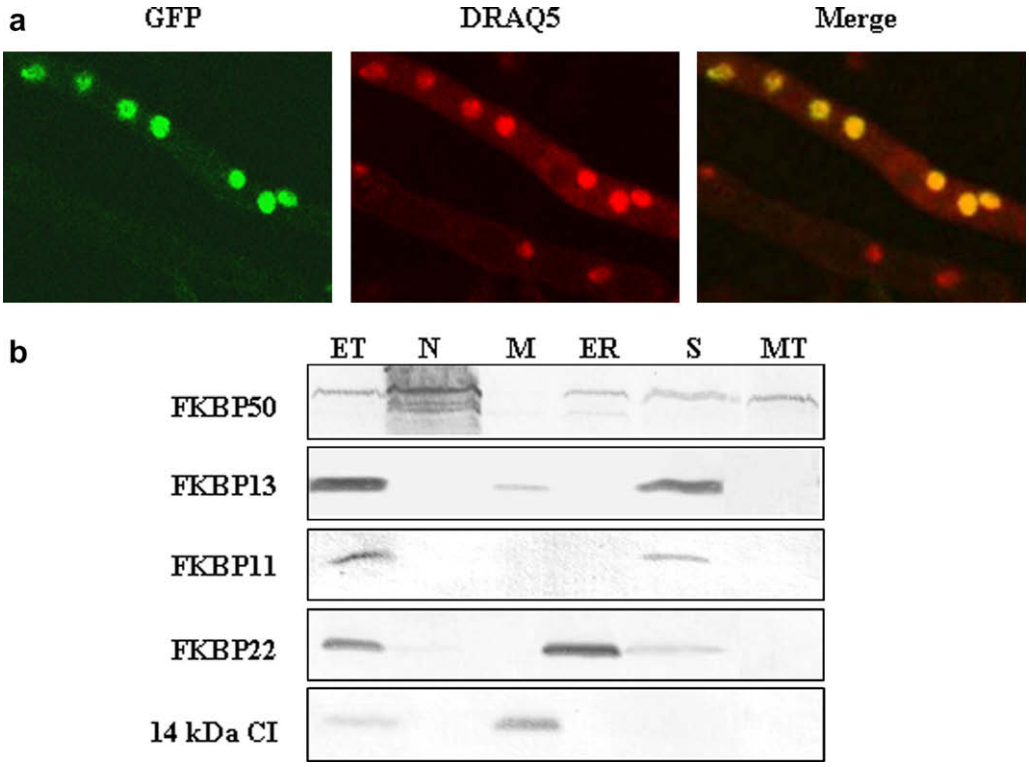

Fig. 3. FKBPs localization. (a) Localization of the fusion protein FKBP50::GFP. Conidia from *Neurospora* strain stably expressing the fusion protein FKBP50::GFP were grown overnight in minimal medium containing acetate and subsequently analysed by confocal microscopy for the presence of green fluorescence. The nuclei were stained with DRAQ5. (b) Cellular localization of the FKBPs from *N. crassa*. Wild type cellular extracts were prepared and the cellular fractions analysed by Western blotting using specific antisera as indicated in the left side. For the location of the FKBP11 the organelles were purified from the OEFKBP11 strain. The 14 kDa subunit of complex I (CI) was used as control. ET, total cellular extracts; N, nuclei; M, mitochondria; ER, endoplasmic reticulum; S, components of the cytoplasm after cellular fractionation. In each panel, MT represents total cellular extracts obtained from null-mutant strains corresponding to the protein being analysed. Approximately 200 μg of protein were loaded in the lanes corresponding to total extracts and cytoplasm and 50 μg on the others. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

We verified that both fkbp50 mutants displayed the temperature-sensitive phenotype at 26 °C that was almost completely overturned when the strains were grown at 37 °C (Fig. 4b). It is likely that the cellular stress caused by the heat shock may partially compensate the FKBP50 defect. FKBP50 is homologous to Fpr4 a nucleolar protein that consists of an acidic domain in the N-terminus, a

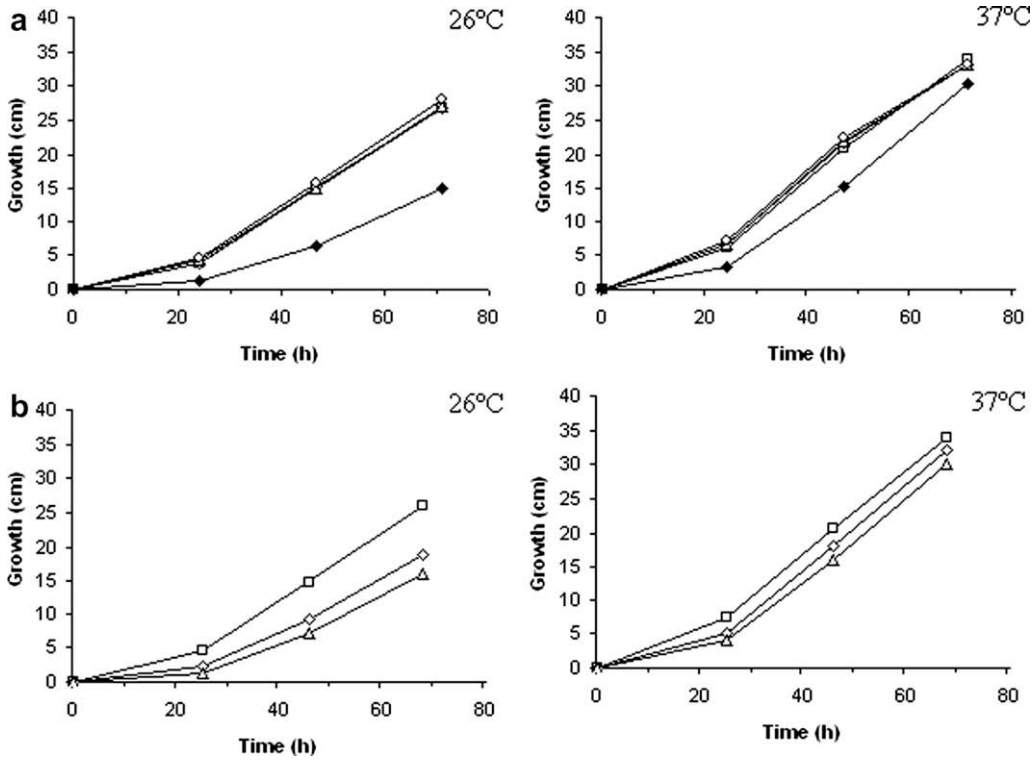

Fig. 4. Effect of the temperature on the growth of different strains of *N. crassa*. (a) The wild type (□) and mutants Δfkbp11 (◇),Δfkbp13 (Δ) and fkbp22^{RIP} (○) and Δfkbp11/13/22/50 (♦) were inoculated (5×10^4 conidia/5 μl) in race tubes with minimal medium and grown at two different temperatures. (b) Growth of fkbp50^{RIP} (◇) and Δfkbp50 (Δ) mutants compared to the wild type (□).

basic domain in the middle and the PPIase domain at the C-terminus. Recent studies demonstrate that Fpr4 possesses a novel histone chaperone activity, acting as a chromatin component involved in rDNA silencing (Kuzuhara and Horikoshi, 2004). The acidic domain is responsible for the histone chaperone activity while the PPIase domain inhibits this activity (Xiao et al., 2006). In addition, Fpr4 was described as a histone proline isomerase, whose activity regulates methylation of lysine 38 in histone H3 and the induction of transcription (Nelson et al., 2006). Based on the homology of FKBP50 and Fpr4 it is possible that the *Neurospora* enzyme is also involved in similar processes. In this way, silencing of the rDNA and/or the induction of several genes could be the reasons for the partial reversion of the growth defect by temperature increase, which also results in repression of ribosomal protein genes (Trotter et al., 2002).

Disruption of all four FKBP immunophilins did not affect the fungus viability, showing that all these proteins are dispensable for *N. crassa* survival (Fig. 4a). With the exception of the multiple mutants carrying the fkbp50 mutation, which maintained the growth deficiency, none of the other mutant combinations presented a detectable phenotype in what concerns growth, temperature-sensitivity, oxidative stress or the presence of a protein misfolding inducer (data not shown).

3.4. The OEFKBP11 strain is resistant to high levels of calcium and zinc

As mentioned before, to localize the FKBP11 protein we grew the wild type strain with various carbon sources (1.5% sucrose, 44 mM sodium acetate and 2% glycerol), different temperatures (26 and 37 °C) and different stress conditions, including starvation, osmotic stress (1 M NaCl and 1 M sorbitol), oxidative stress (0.5–2 mM H_2O_2) and protein misfolding (2.5 mM L-Azetidine-2-carboxylic acid). We also tested for the presence of the FKBP11 during several stages of vegetative development as mycelium, aerial hyphae and conidia (data not shown). In none of the conditions tested we detected the presence of the FKBP11.

Since the FKBP11 is not detected during vegetative development of the fungus and the Δfkbp11 mutant shows no visible phenotype we decided to test if the overexpression of the FKBP11 conducted to any alteration of the cellular functioning. To that purpose the OEFKBP11 strain was grown in plates containing solid sorbose medium (GFS) with different stress conditions induced by 1.5 mM H_2O_2, 200 μM paraquat, 2.5 mM L-azetidine-2-carboxylic acid, 50 mM LiCl, 100 mM $CaCl_2$ and 4 mM $ZnCl_2$. Surprisingly, the OEFKBP11 strain presented a different phenotype when grown in GFS supplemented with 100 mM $CaCl_2$ and 4 mM $ZnCl_2$. In both situations the OEFKBP11 strain produced larger colonies when compared to the wild type strain (Fig. 5). Apparently this phenotype is not due to a chloride effect since the strain growing in 50 mM LiCl did not present the same phenotype (Fig. 5). The Ca^{2+} is one of the most important second messengers as its levels change in response to many cellular responses. The presence of the FKBP11 appears to give an advantage to the strain, indicating that the FKBP11 could make part of a specific cellular mechanism. However, linear growth in race tubes is comparable in strains, wild type and OEFKBP11, in media containing $CaCl_2$ and $ZnCl_2$ (data not shown).

To test if these stress agents induce the expression of the FKBP11 we grew the wild type and the OEFKBP11 strains in liquid minimal medium supplemented with 100 mM $CaCl_2$ and 4 mM $ZnCl_2$. The OEFKBP11 showed an increased in the expression of the FKBP11 in media containing $CaCl_2$ when compared to the control and media containing $ZnCl_2$ (Fig. 6). Interestingly, the FKBP11 was detected in the wild type growing with $CaCl_2$, but not when this strain was grown with $ZnCl_2$ (Fig. 6). Since the FKBP11 protein is not detected during vegetative development of the fungus and the OFKBP11 strain displays a particular phenotype in media containing high amounts of calcium and zinc we can hypothesize the

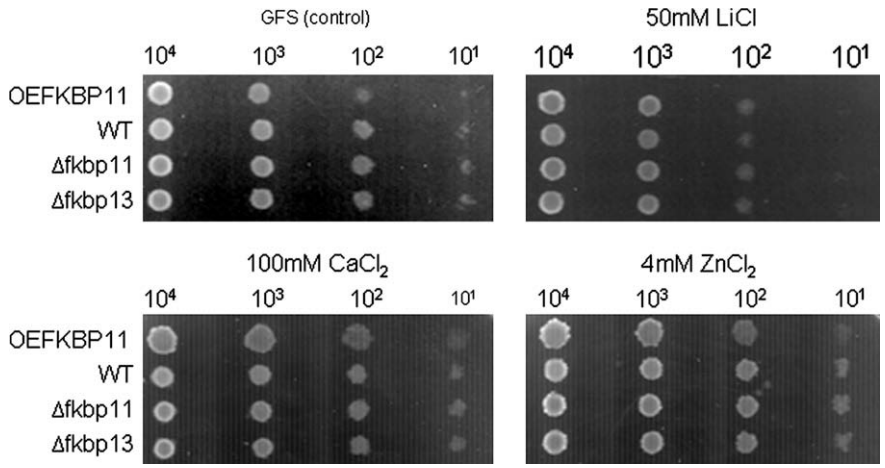

Fig. 5. The OEFKBP11 is resistant to high amounts of $CaCl_2$ and $ZnCl_2$. Five microliters of serial dilutions of conidia from the indicated strains was inoculated (spotted) onto solid sorbose medium (GFS) supplemented with 100 mM $CaCl_2$, 4 mM $ZnCl_2$ or 50 mM LiCl and incubated at 37 °C for approximately 24 h.

possible role of this protein in some particular regulatory mechanisms. If the protein has an important role in the regulation of the intracellular levels of calcium or zinc we would expect to detect the protein in the wild type strain growing in the same conditions, which only happen for calcium. However, the amount of FKBP11 present in any of these situations can probably explain the phenotype observed for OEFKBP11. The overexpression of the FKBP11 appears to give an advantage to the strain in some stress conditions promoting a larger cell proliferation despite that the protein is not important to the survival of the wild type strain in the same conditions.

3.5. The FKBP11 is highly expressed during sexual development

Later, we determined that FKBP11 is highly expressed in the wild type strain growing in Westergaard medium, a medium poor in nitrogen used to promote sexual development (Fig. 6). We further observed that this high expression is not dependent in the nitrogen concentration but is prevented at 37 °C (data not shown), a condition that also impairs the development of fungal perithecia (Perkins, 1986). Altogether, these results strongly suggest that FKBP11 is involved in sexual development. To determine if the FKBP11 play an important role in the sexual cycle of the fungus we tested the fertility of the Δfkbp11 mutants (mating-types *A* and *a*) under low-nitrogen conditions. To that propose we crossed both Δfkbp11 mutants, as female and male, with both wild type strains. When compared to the wild type, none of the mutants displayed any defect related to the development of sexual structures (formation of protoperithecia and perithecia), producing viable black ascospores (data not shown). Thus, although the *fkbp11* gene is highly expressed during the sexual development, it appears not to be a gene essential for the process. As known for other FKBPs (and cyclophilins) that are involved in multiple cellular processes, it is possible that the protein encoded by the *fkbp11* might have a redundant function or its absence might be covered by the presence of other proteins. To date, no gene encoding a FKBP or cyclophilin involved in sexual development in filamentous fungi has been identified. The FKBP11 protein presents some intriguing features. Its corresponding gene includes five large introns suggesting that this might be a highly regulated protein, apparently not necessary for the vegetative development of *N. crassa*. This much simpler fungal system, as compared with other eukaryotes, will certainly be very useful for further research on the cellular role of FKBPs.

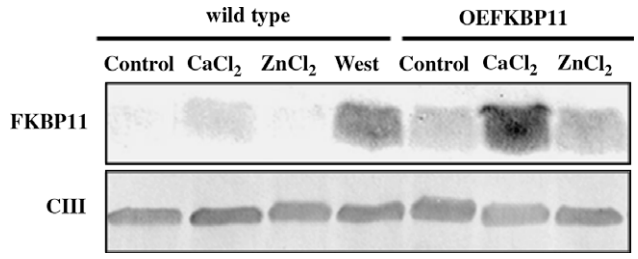

Fig. 6. FKBP11 is induced by calcium and in Westergaard medium. The wild type and the OEFKBP11 strains were grown in minimal medium supplemented with 100 mM $CaCl_2$ or 4 mM $ZnCl_2$. The wild type was also grown in Westergaard medium (West). Expression of the protein FKBP11 was analysed by Western blotting using an antiserum against the protein. Approximately 250 μg of protein were applied in each lane and a mitochondrial complex III polypeptide subunit (CIII) was used as load control.

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