MASTER DEGREE IN BIOLOGICAL ENGINEERING

DEVELOPMENT OF A YEAST BASED PLATFORM FOR THE SCREENING OF COMPOUNDS THAT MODULATE TTR TOXICITY

INSTITUTO SUPERIOR TÉCNICO, LISBON, PORTUGAL

JOANA DA COSTA BRANCO

email: joanadacostabranco@gmail.com

ABSTRACT

Familial Amyloid Polyneuropathy (FAP) disease is a hereditary amyloidosis caused by mutations in the plasma protein transthyretin (TTR). The aim of this work was to develop a tool to identify compounds that modulate phenotypes caused by TTR variants and that have the potential to become new targets for therapeutical application for FAP disease. This platform was developed using *Saccharomyces cerevisiae* as a cellular model expressing wild-type TTR and the TTR variants Leu12Pro, Val30Met, Leu55Pro and Thr119Met. Initial steps for validation and characterization of this model are presented. Toxicity of different variants of TTR was studied in wild-type yeast and in knock-out (KO) deletion strains for ER (endoplasmic reticulum) stress associated genes (*hac*1Δ, *dfm*1Δ, *ire*1Δ and *cwh*41Δ). TTR expression in yeast was confirmed and TTR toxicity assays revealed no growth delay when expressing TTR in wild-type yeast. No cell growth delay was observed in *hac*1Δ, *dfm*1Δ and *cwh*41Δ expressing TTR. *Ire*1Δ expressing TTR-L12P and V30M showed similar growth. Preliminary evidences were obtained of a slight growth delay due to WT TTR expression in *ire*1Δ but this result needs further validation. Preliminary studies were performed aiming to the development of a yeast model to investigate the cellular consequences of the interaction of TTR and the 42 amino acid peptide (Aβ42) involved in the progression of Alzheimer's disease. However, since native Aβ42 is not detected in yeast protein extracts by western blot further investigation is required using Aβ42 in fusion.

This platform has the potential to provide new insights and therapeutic strategies in FAP pathology.

Keywords: Amyloidosis, Familial Amyloidotic Polyneuropathy (FAP), Transthyretin (TTR), yeast *Saccharomyces cerevisiae*, β-amyloid (Aβ)

I. INTRODUCTION

Hereditary amyloidoses are rare and lethal disorders very difficult to treat and are mostly caused by mutations in a plasma protein, transthyretin (TTR), being familial amyloidotic polyneuropathy (FAP) the most common of these (Pepys, 2006). This disease was firstly described in Portugal by Corino de Andrade in 1952 and later associated with TTR by Costa *et al*. in 1978.

FAP is an autosomal dominant lethal neurodegenerative disorder that is characterized by a symptomatic onset of progressive sensory-motor peripheral neuropathy in the lower extremities that manifests through a selective loss of superficial sensation to pain and temperatures (Sousa & Saraiva, 2003) The onset of symptoms usually occurs between ages of 20 and 45 and is fatal 10 to 20 years after symptoms onset (Sousa & Saraiva, 2003).

Currently over 100 variants of TTR have been identified. These include Val30Met, Leu55Pro, Leu12Pro and Thr119Met, which were used in the current work (Dubrey *et al*, 2011).

The Portuguese form of FAP (type I) is associated with a TTR variant where a valine is replaced by a methionine at position 30 of the molecule TTR (Val30Met) (Sousa & Saraiva, 2003). The TTR variation Leu55Pro, substitution at the position 55 of a leucine for a proline, is the mutation associated with most aggressive type of FAP disease. This variant is described as being the one with the most amyloidogenic potential and with less tetrameric stability (Sousa & Saraiva, 2003). Leu12Pro is associated with CNS

deposition and results from the substitution of a leucine by a proline at the position 12 of the protein sequence (Brett *et al.*, 1999). Although symptoms in the CNS have not been associated with TTR-related amyloidosis, Brett *et al.* (1999) stated that patients carrying the TTR Leu12Pro variant presented clinical features of FAP and in addition amyloid deposits affecting the CNS associated with leptomeningeal amyloidosis. Finally, Thr119Met is a variant of TTR with no pathogenic role associated being denominated as non amyloidogenic (Damas & Saraiva, 2000).

Regardless all scientific breakthroughs there is still no efficient treatment for FAP. For this reason it is important to understand the mechanisms that result in pathologic gain of function of mutated TTR in order to develop therapies to fight this disease. Extracellular accumulation of TTR aggregates which lead to a neurotoxic effect seems to be related to cellular activation in the tissues surrounding the deposits by triggering calcium homeostasis, ER stress, inflammatory and apoptotic pathways resulting in cell dysfunction and death (Sousa *et al.*, 2001; Macedo *et al.*, 2007).

ER stress is caused by expression and accumulation of misfolded proteins in the ER lumen compromising ER functions (Paschen & Mengesdorf, 2005). In order to respond to this perturbation a process of ER-specific stress response known as unfolded protein response (UPR) is activated (Teixeira *et al.*, 2006). In yeast, the UPR response is only controlled by IRE1 pathway (Lai *et al*., 2007; Krysan, 2009). Then, Ire1p suffers autophosphorylation which activates an endoribonuclease which in turn removes the mRNA intron from the transcription factor Hac1p (Krysan, 2009). After Hac1p translation, the HAC1 protein goes to the nucleus and induces a response that will restore the ER functions through UPR genes (Krysan, 2009). Also associated with the ER stress and homeostasis are the proteins coded by *DFM*1 and *CWH*41 genes (Sato & Hampton, 2006; Faridmoayer & Scaman, 2007; SGD). The first codes for an ER localized protein and the second for a processing yeast α-glucosidase I, ER type II integral membrane N-glycoprotein.

The main goal of this work was to design and engineer a tool to study these effects using *Saccharomyces cerevisiae* as a cellular model. Through this yeast based platform the toxicity of different variants of TTR was studied. First the effect of expression of different TTR variants in the growth of yeast cells would be evaluated. Although TTR expression in yeast has been previously mentioned in the literature, a focused study on the cellular phenotype caused by this protein in this selected model organism has not been performed to date. Therefore, 5 isoforms of TTR were selected (TTR WT, Leu12Pro, Val30Met, Leu55Pro and Thr119Met), expressed in yeast and the resulting yeast growth phenotype evaluated. It is important to notice that all TTR variants were cloned without signal peptide as it intended to keep the protein inside the yeast cell.

Any eventual toxicity would be further studied and the most toxic isoforms would be selected for subsequent studies focused on the induction of ER stress, as described in previously.

An eventual lack of toxicity upon expression of all isoforms would shift the project into the search of genes involved in TTR-related pathways, through the expression of TTR variants in knock-out yeast strains for ER stress associated genes. These genes, selected through the Saccharomyces Genome Database (SGD) were: *HAC*1, *DFM*1, *IRE*1 and *CWH*41.

While the platform was being designed, it was decided to add to this project an initial study of TTR and Aβ42 peptide (involved in Alzheimer's disease) interaction through the evaluation of the yeast growth when expressing both proteins. This interaction between TTR and Aβ42 is being focus of several promising studies (Costa *et al*, 2008).

This work was developed in collaboration with Instituto de Biologia Molecular e Celular (IBMC), particularly with one of the leading world scientists in the field of FAP disease, Prof. Maria João Saraiva who kindly provided TTR-Leu12Pro and TTR-Thr119Met cDNA.

II. RESULTS

TTR Toxicity in Yeast

A) Construction and characterization of yeast strains expressing TTR

One of the goals of the current project was to evaluate the effects of TTR expression in yeast. All the TTR coding sequences were cloned without the signal peptide sequence to maintain this protein inside the cell avoiding the secretion to the extracellular media.In this approach a total of 5 DNA plasmids were engineered using the Gatheway® Technology to produce episomal Gal expression vectors containing the coding sequence of the protein TTR WT and its variants TTR-V30M, TTR-L55P, TTR-L12P and TTR-T119M. In order to confirm the presence and size of the inserts in the intermediate clone backbone,

restriction analysis was performed. The restriction patterns obtained were according to the expected. In agarose gel two bands were observed, one with 447 bp and other with 2500 bp (data not shown). Before moving on to the next step of this cloning method all intermediate clone constructs were additionally confirmed by sequencing to assure the integrity of the DNAs sequence. The final constructs (pBLV_TTR WT, V30M, L55P, L12P and T119M) were also confirmed by restriction analysis. Positive constructs presented two bands in agarose gel with 594 bp and 7254 bp (data not shown).All pBLV_TTRs clones and empty vector were transformed in BY4741 wild-type yeast strain.

The next step was to demonstrate that TTR is being expressed inside yeast cells. This was proven by first culturing the transformants in the presence of raffinose. Then, the culture media was shifted to galactose in order to induce TTR gene expression. As controls were used transformants containing the pBLV empty vector and TTR WT expressed also under GAL promoter in pBI vector, being the first one negative control and the second positive control. When the culture reached an exponential state the cells were harvested and further cell lysates were collected. Samples were run under SDS-PAGE conditions and analyzed by western blot (WB) using anti-human TTR antibody (Figure 1). As expected, results showed that intracellular TTR is being expressed in yeast as monomer forms of TTR were detected. TTR-T119M was expressed in a pattern similar of WT TTR, but TTR-V30M and TTR-L12P were hardly detected. The difference in their expression pattern could be attributed to inefficient protein extraction, less expression inside the cell or due to molecular mechanisms of degradation inside the cell. In order test the hypothesis of inefficient protein extraction the membrane containing the TTR extracts was blocked again and incubated with anti-GAPDH antibody since GAPDH is a housekeeping gene (Figure 1).

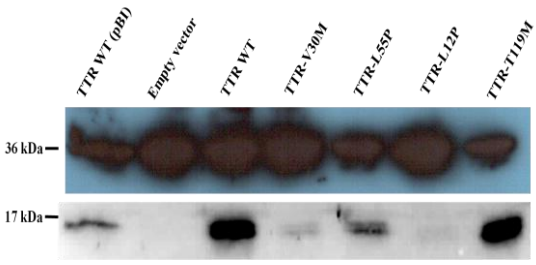

Figure 1- Expression pattern of TTR (17kDa) and GAPDH (36kDa) in BY4741 transformed with pBLV_TTRs constructs incubated at 30ºC.

The hypothesis being tested was invalidated since the result demonstrated similar secretion patterns for all lanes. Hence, this may indicate that in the case of TTR-V30M and TTR-L12P there was less expression inside the cell or that the proteins were degraded before being extracted by molecular mechanisms of degradation inside the cell.

Yeast TTR strains viability in solid media

In order to evaluate yeast viability in solid media when expressing TTR WT and its variants (V30M, L55P, L12P and T119M) the monitoring of the yeast strains growth was performed in solid media (YNB GLU and GAL). Plates were incubated at 30 and 37ºC for 3 days and yeast transformants growth was monitored during these 3 (Figure 2).

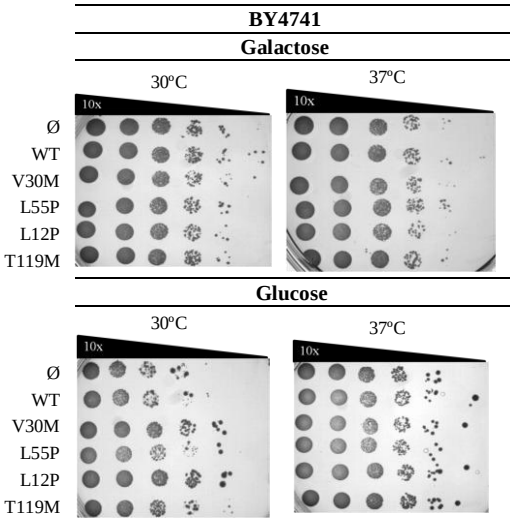

Figure 2- Assay to evaluate cell viability in solid media for BY4741 yeast strain transformed with pBLV_TTRs. (Ø: empty vector)

After 72h of incubation the transformants did not reveal a toxic phenotype upon yeast growth due to TTR expression (Figure 2).

Yeast TTR strains viability in liquid media

In addition to the previous result, the yeast cell viability in liquid media when expressing TTR WT and its variants (V30M, L55P, L12P and T119M) was also evaluated in 96 well plates incubated at 30ºC to allow the detection of minor phenotypic changes that may not be detected in solid growth. Before incubating the cultures, yeast cells grown overnight at 30°C in RAF media until OD_{600} reached the mid-log exponential phase. Cultures were incubated at three different initial ODs, 0.05, 0.1 and 0.2 for 44 hours with optical density (OD)

readings every 4 hours. This experiment was undertaken with three different initial ODs since if there is a growth defect in the studied transformant this will be exacerbated at smaller starting culture ODs, this is, at lower cell concentrations in culture. The results of this assay are presented in Figure 3, for liquid growth in glucose and galactose with starting OD_{600nm} of 0.2. The results for the other two starting ODs (0.05, 0.1) are not shown since they are very similar to the one presented.

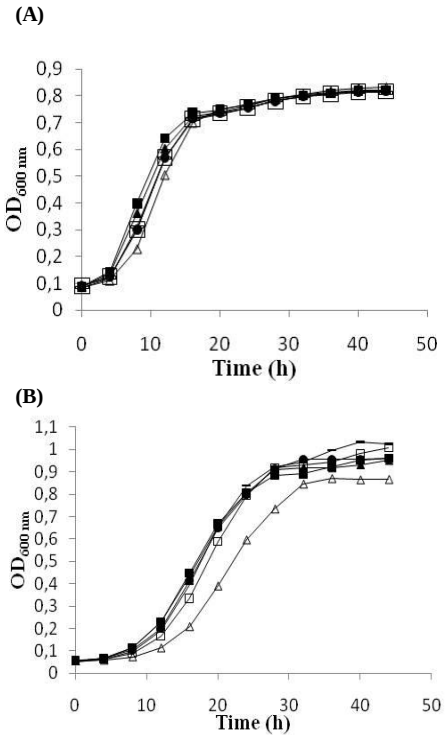

Figure 3– Cell viability in liquid media in (A) Glucose (YNB Glu His^-) **and (B) Galactose** (YNB Gal His^-) for a culture growth with a starting OD_{600nm} of 0.2 for BY4741 yeast strain transformed with: pBLV (Δ), pBLV_TTRWT (■), pBLV_TTR-V30M (▲), pBLV_TTR-L55P (▬), pBLV_TTR-L12P (□), pBLV_TTR-T119M (●).

Cell viability assay plots (Figure 3) revealed cell growth with an initial lag-phase, an exponential growth phase and stationary-phase. When grown in galactose the yeast strains expressing TTR showed delayed growth when compared with cultures grown in glucose. For cells grown in glucose media (Figure 3 A) the exponential growth phase starts around 5 hours after starting incubation whereas in galactose (Figure 3B) the exponential growth phase starts after 10 hours of incubation. In addition, the stationary-phase of the cultures in glucose is reached 18 hours after starting the assay while in galactose media this phase occurs 30 hours after. This discrepancy in growth was expected since galactose is a yeast carbon source more demanding to be metabolized than glucose (Timson, 2007). Additionally, the constructs transformed contain genes regulated by GAL promoter that are induced in the presence of galactose. The synthesis of these proteins is energy demanding to the cell which contributes to the delay in cell recover lag and mid-lag phases and reach the stationary and final phase before cell death. Comparing the growth between all transformants (pBLV_TTRs and pBLV) we can notice that they have similar growth when incubated in glucose. When grown in galactose the TTR transformants have once more similar growth. However, in the case of BY4741 transformed with pBLV a minor delay is present (Figure 3B). These results suggest that TTR expression in BY4741 yeast cells is not toxic as it results in no phenotypic alterations in cell growth in liquid media, in accordance with the results obtained in solid media. For the pBLV empty vector the obtained result can be explained by the fact that the construct pBLV, used as an empty vector, in fact expresses EGFP when in presence of galactose. This way, this construct is not the most suitable to be used as a control when evaluating cell viability.

B) Evaluating ER stress toxicity upon TTR expression

EGFP reporter gene

ER stress has been implicated in misfolded protein diseases molecular mechanisms that lead to cytotoxicity (Macedo *et al*., 2008). FAP disease is an example where this occurs (Macedo *et al*., 2008). The ER stress is a condition that occurs as a result of the accumulation of aggregated proteins and leads to cell apoptosis in chronic situations (Lai *et al*., 2007). Therefore, we proposed to evaluate if TTR expression induces ER stress using a reporter gene system and tunicamycin, a compound that induces ER stress. This evaluation started with initial tests to an ER stress vector available at BIOALVO. This vector contains two key elements: an EGFP reporter gene under the control of a KAR2 promoter and a multiple cloning site under the control of a GAL promoter. The KAR2 promoter responds to ER stress induction.

In case of detection of a toxic phenotype caused by expression of any TTR variant in BY4741 yeast strain further evaluation of specific ER stress induction will be performed. If this strategy goes

forward TTR variants cloned are meant to under the control of a Gal promoter. This way, in this experiment we used the BY4741 yeast strain transformed with the ER stress vector cloned with both KAR2 and GAL promoters. As controls were used BY4741 yeast strains transformed with ER stress vector cloned with KAR2 promoter and pYX213_ADH_yEGFP constructs. The first was used as a control of the ER stress inducer compound tunicamycin and the latter as a fluorescence control.

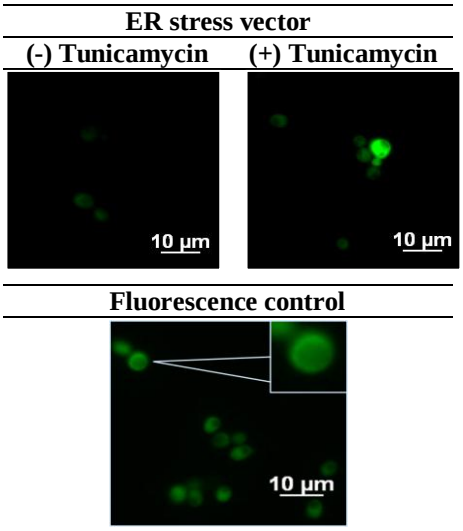

Figure 4 – Fluorescence imaging assay with an ER stress inducer (tunicamycin) of BY4741 yeast transformed with ER stress vector cloned with both KAR2 and GAL promoters and pYX213_ADH_yEGFP (control).

In order to test the ER stress vector, the EGFP fluorescent signal was evaluated after inducing the KAR2 promoter with tunicamycin. According to the results (Figure 4) GFP fluorescent signal was observed, indicating that the ER stress reporter is functional.

Since TTR expression was not toxic to yeast growth we decided not to pursuit with the cloning of TTR mutants in ER stress vector. Additionally was used an alternative strategy to approach ER stress effect in yeast cells upon TTR expression.

ER stress related knock-out yeast strains

Since TTR expression in BY4741 yeast strains did not reveal a toxic phenotype, new transformants were engineered using ER stress related yeast KO mutants (*hac*1Δ, *dfm*1Δ, *ire*1Δ and *cwh*41Δ) derivative of the *S. cerevisiae* BY4741 wild-type strain.

These constructs were engineered to express TTR in yeast in order to evaluate the relation between TTR expression and ER stress response. For this, it was decided to use the TTR-V30M and L12P variants and wild-type TTR as control. This decision was based on the fact that Val30Met mutation of TTR is the Portuguese form of FAP (type I) and that Leu12Pro mutation is associated with intracellular deposition since patients with this type of TTR mutation exceptionally have aggregates in the tissues where TTR is secreted.

After obtaining the yeast KO mutants (*hac*1Δ, *dfm*1Δ, *ire*1Δ and *cwh*41Δ) transformed with TTR constructs, cell viability in solid media was evaluated when expressing TTR. The conditions of this experiment were the same as the ones used for the assay of BY4741 transformed with TTR constructs described before. Examining the growth in solid media of the strains lacking *HAC*1 and expressing TTR no toxic phenotype was observed after 72h of incubation when comparing to the negative control which are the cells transformed with empty vector (data not shown). Similar results were observed in a strain lacking *DFM*1 (data not shown). These results suggest that these deleted genes (*HAC*1 and *DFM*1) are not essential to yeast cells viability.

The viability of strains lacking *IRE*1 and *CWH*41 in solid media when expressing TTR were also studied. In the case of these two strains more dilutions were made of the yeast strains culture when the cell viability assay was repeated for better results.

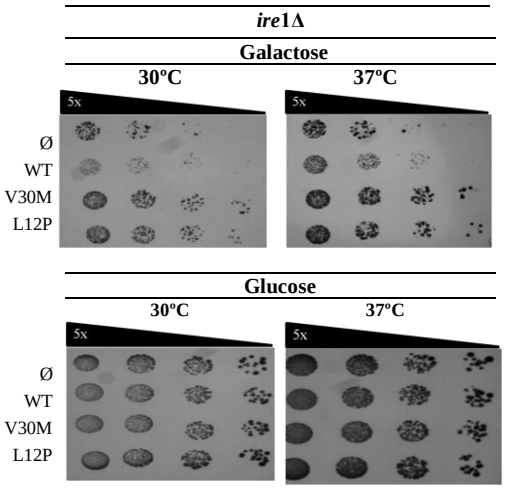

Figure 5 - Assay to evaluate cell viability in solid media for *ire*1Δ yeast deletion mutant strains transformed with pBLV_TTRs. (Ø: empty vector)

Evaluating the growth in solid media after 72h of incubation of the strain *ire*1Δ (Figure 5), we can observe that both transformants with pBLV_TTR-V30M and pBLV_TTR-L12P share the same phenotype. The same occurs between the strains transformed with pBLV and pBLV_TTR WT. As

it was mentioned above, the pBLV construct, used as an empty vector, in fact expresses EGFP when in presence of galactose. This construct is not the most suitable to be used as a control when evaluating cell viability and therefore results should be further confirmed with a more suitable control. Despite the facts mentioned previously, these results indicate that *ire*1Δ expressing TTR variants V30M and L12P have similar behaviors versus the same KO yeast strain when expressing WT TTR and EGFP.

Finally, in the *cwh*41Δ mutant expressing TTR WT, Val30Met and Leu12Pro no delay in growth was detected (data not shown).

C) TTR and Aβ42 interaction

With the goal to initiate a study on TTR and Aβ42 peptide interaction a new platform was designed so that both proteins can be co-expressed in yeast.

Both Aβ42 and TTR (WT, Leu12Pro and Thr119Met) protein coding sequences were cloned in pBI vector, having TTRs under the GAL1 promoter and Aβ42 under the GAL10 promotor. One should notice that the Aβ peptide is a cleavage product the functional protein amyloid precursor protein (APP) (Misumi *et al*., 2011). Thus, a start and stop codon in the DNA sequence that was cloned in order to induce its expression. Additionally, as in the previous chapter TTR was cloned without the signal peptide sequence. The first construct engineered with pBI vector as backbone was pBI_GAL10-Aβ42. Clone confirmation was made by restriction analysis with the enzymes E and F.

The restriction patterns obtained were according to expected confirming the constructs as after DNA electrophoresis, two bands with 132 bp and 7700 bp were observed (data not shown). This clone was also confirmed by sequencing to guarantee the integrity of the DNA sequence.

The expression pattern of Aβ42 peptide was determined. This was performed by incubating the transformant (BY4741 transformed with pBI_GAL10-Aβ42 at 30ºC in the presence of raffinose and then in galactose to induce Aβ42 expression. As control for Aβ42 expression the BY4741 yeast strain transformed with pBI_GAL1-Aβ42- mCherry construct was used. Aβ42 peptide produced *in vitro* was used as positive control. Both controls were available at BIOALVO. After the cultures grew to an exponential phase, cells were harvested and lysates were collected for analysis. The samples were then run under SDS-PAGE conditions and analyzed by western blot.

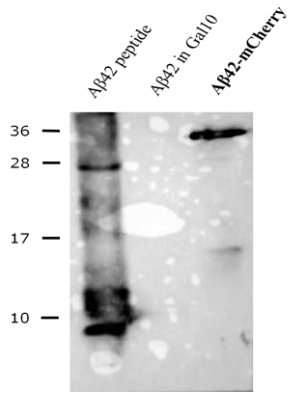

Figure 6 - Secretion pattern of Aβ42 (~4kDa) and Aβ42-mCherry (~32,8 kDa) in yeast cells transformed with pBI_GAL10-Aβ42 and pBI_GAL1-Aβ42-mCherry plasmids incubated at 30ºC.

Protein bands identified in the nitrocellulose membrane by western blotting have the expected size (Figure 6). The Aβ42 peptide has a molecular weight of 4 kDa and is detected in WB at around 6 kDa according to anti-amyloid β antibody manufacturer. In the lane were the Aβ42 peptide positive control was loaded we can observe a smear. This smear can result from too much sample loaded and due to the peptide fibril formation *in vitro*, that can be SDS-resistant (Bitan *et al.*, 2003). Results demonstrate that intracellular Aβ42 protein was only detected when expressed in fusion (Aβ42-mCherry).

Due to time management, while the previous assay to evaluate the expression of Aβ42 in yeast was being evaluated, the remaining constructs with TTR alone and with both TTR and A β42 were engineered. However, since the peptide expression was not detected unless when tagged (Aβ42-mCherry) this study was interrupted for reassessment. This way, the remaining constructs were not confirmed.

III. DISCUSSION

TTR toxicity in yeast

In this work a platform was developed using *Saccharomyces cerevisiae* as a cellular model for the screening of compounds with the potential to ameliorate or prevent the cytotoxicity caused by TTR mutations that leads to pathogenicity in FAP.

Extracellular accumulation of TTR aggregates appears to be involved in several molecular mechanisms in neurotoxicity that consequently lead to cell dysfunction and death in several neurodegenerative diseases including FAP (Hou *et al*., 2007; Gasperini *et al*., 2011; Sousa *et al*., 2001; Macedo *et al*., 2007). Between all these cellular mechanisms, the ER stress was selected to

be evaluated when expressing TTR in yeast since it has been strongly related to FAP neurotoxicity (Macedo *et al.*, 2008, Teixeira *et al*., 2006, Sato *et al*., 2007, Nikawa *et al*., 1996). Hence, an evaluation of the involvement of the ER stress pathway in the cellular toxicity caused by the expression of TTR in yeast was performed. This approach to study TTR and its role in FAP pathology is being explored in this model organism for the first time. In order to assess the toxicity of different variants of TTR the isoforms V30M, L55P, L12P and T119M were studied additionally to WT TTR.

ER stress condition in yeast due to TTR expression was induced by cloning all TTR isoforms without signal peptide as it intended to keep the protein inside the yeast cell. Since TTR in being expressed without signal peptide is not guided to the ER or the secretion pathway (Hiss & Schneider, 2009) and probably accumulates in the cytoplasm (WoLF PSORT tool). This accumulation may trigger ER stress given that TTR is a protein described as having amyloidogenic potential (Saraiva, 2002; Sousa & Saraiva, 2003). Additionally, when misfolded proteins start to accumulate other degradation pathways such autophagy and Ubiquitin Proteasome Systems (UPS) are triggered (Kraft *et al.*, 2010). Furthermore, Vidal and co-workers (2011) recently reported that alterations in the secretory pathway, particularly in the ER, are also particular features of another neuropathological disease, the Huntington's disease, which is caused by a mutation in the Huntingtin gene that code for a protein that is also called Huntingtin (Htt) protein. The Htt is a cytoplasmic protein that when mutated tends to aggregate (Vidal *et* al, 2011).

This tool was characterized by evaluating intracellular TTR expression pattern and the effect that this expression has in the viability of BY4741 yeast strain. Intracellular expression in yeast of all TTR isoforms was confirmed by Western blot. Comparing the expression patterns of the different TTR variants (Figure 1) it was evident that the TTR variant T119M was expressed with comparable efficiency as the wild type TTR. The TTR variants L55P, V30M and L12P were hardly detected, particularly the latter two. Since the expression systems are the same for all TTRs, it can be hypothesized that differences in final protein concentration might be due to differential degradation of some isoforms. This idea is supported by recent equivalent studies when expressing wild-type TTR and TTR variants V30M, D18G, T119M, A25T and E54K in mammalian cells (Sato *et al.*, 2007).This hypothesis should be further tested by treating the yeast cells with inhibitors of degradation pathways.

In order to evaluate the effect of TTR expression in yeast cells phenotype assays of cell viability, in solid and liquid media, were performed. TTR expression in yeast did not cause a growth delay. However, the pBLV Gateway® vector used as TTR expression control in fact expresses EGFP when in presence of galactose. Overexpression of EGFP can cause this protein accumulation in the cell (Katayama *et al*., 2008). Notice that the EGFP present in the TTR Gateway® constructs was not being expressed since a stop codon was inserted between the two open reading frames. Therefore, although the lack of toxicity seems to be verified, these experiments should be repeated with a more suitable control.

In yeast, when misfolded proteins start to accumulate, a response dependent on the chaperone KAR2 is activated. This way, and with the goal of evaluating if TTR expression induces ER stress, initial experiment was performed with an ER stress vector which would be used for this evaluation. This ER stress vector contains an EGFP reporter gene under the control of a KAR2 promoter. The experiment to test the ER stress vector consisted in a preliminary evaluation of the EGFP fluorescent signal after inducing the KAR2 promoter with tunicamycin. The results of this experiment indicated that the ER stress reporter is functional as a GFP fluorescent signal was observed.

As no evidence of toxic phenotype was detected when expressing TTR in yeast and because we were focusing on ER stress molecular mechanism, this project strategy shifted. This way, we performed studies in ER stress response when expressing TTR (WT, V30M and L12P) in yeast knock-out deletion strains for ER stress associated genes (*hac*1Δ, *dfm*1Δ, *ire*1Δ and *cwh*41Δ) selected through the SGD. All four strains are derivates of the *S. cerevisiae* BY4741 wild-type yeast strain. ER stress studies were performed by an assay to evaluate yeast KO cell viability in solid media. *CWH*41 and *DFM*1 genes encode proteins that are indirectly involved in ER stress by perturbing ER normal function (Hitt & Wolf, 2004; Sato & Hampton, 2006). *HAC*1 gene codes for a basic leucine zipper (bZIP) transcription factor (ATF/CREB1 homolog) that regulates the unfolded protein response (SGD). TTR expression in these three disrupted strains showed no growth

delay. The results obtained in these three KO strains assay (*hac*1Δ, *dfm*1Δ and *cwh*41Δ) suggest that the intracellular accumulation of TTR is not affecting normal cell growth. This assay results should be further confirmed with a more suitable negative control for TTR expression for all four KO yeast strains.

According to Cox and co-workers (1993), under stress conditions *IRE*1 is a gene essential for cell viability since it is an ER stress sensor which transmits the unfolded protein signal across the ER. Considering this, it makes sense that an *IRE*1-disrupted strain shows growth delay upon TTR expression.

TTR variants V30M and L12P expressed in *ire*1Δ shared similar cell growth. *Ire*1Δ expressing WT TTR grew less than V30M and L12P, suggesting that a toxic effect on expression of this specific isoform. Nevertheless, this KO yeast transformed with pBLV vector also presents a growth delay similar to WT TTR. As discussed above, this control should be improved because expression of EGFP can cause toxicity. This set of experiments will be repeated using a non-expressing plasmid as control in order to confirm the growth arrest phenotype observed in yeast transformed with WT TTR.

In yeast, the UPR pathway is linear (Lai *et al.*, 2007; Urano *et al.*, 2000). Thus, mutations in upstream components of this pathway are phenocopied by mutation in downstream ones (Urano *et al.*, 2000). This way we would expect to obtain similar results in *hac*1Δ and *ire*1Δ yeast strain when expressing wild-type TTR and TTR variants V30M and L12P. However, as it was previously described, this did not occur.

TTR and Aβ42 interaction

According to recent *in vivo* studies, transgenic mice carrying AD showed a slower progression of the disease and absence of neurodegeneration when TTR was being expressed (Costa *et al.*, 2008; Buxbaum *et al.*, 2008). Additionally, Costa and co-workers (2008) demonstrated that the co-incubation of TTR with Aβ resulted in reduction of aggregation state of the Aβ peptide. Hence, these studies suggest that Aβ/TTR interactions may lead to new clues to clarify the mechanisms that lead to pathology in AD. For these reasons it was decided that it would be interesting to include in the current project an initial study of this interaction as we proposed to investigate the cellular consequences of expressing Aβ and TTR in yeast. Therefore, a yeast platform for the study of TTR and Aβ42 interactions was developed.

All coding sequences were cloned in a yeast bi-directional epitope-tagging vector (pBI) with episomal expression in which one or two protein coding sequences can be expressed in yeast (*Saccharomyces* cerevisiae) at similar levels with two yeast promoters, GAL1 and GAL10, in opposite orientations. This vector contains as yeast-selectable auxotrophic marker leucine. Since the promoters are both GAL, the expression or co-expression of the proteins is only activated in presence of the substrate Galactose. Although some literature refers that proteins expression regulated by GAL10 is superior when compared to the ones expressed by the promoter GAL1, the difference between expression under promoters GAL1 and GAL10 has shown to be not significant (Lijima *et al.*, 2010). The construct pBI_GAL10-Aβ42 was engineered and intracellular Aβ42 expression pattern evaluated. In this experiment the Aβ42 peptide was only detected when fused to mCherry (Aβ42-mCherry). This result is consistent with what was reported by Caine *et al.* (2007) where the production of native Aβ42 in yeast was not detected. A hypothesis to explain the lack of detection of native Aβ42 is the continuous degradation of this peptide by yeast proteases while it is produced (Caine *et al.*, 2007). However, this hypothesis still needs to be tested. In order to continue with this project the Aβ42 peptide should be used in fusion.

IV. Conclusions

In conclusion, to develop a platform to study mechanisms by which TTR mutations leads to pathogenicity in FAP five TTR isoforms were used (WT, L12P, V30M, L55P and T119M)

TTR expression in yeast was confirmed and TTR toxicity assays revealed no growth delay when expressing TTR in wild-type yeast. Since in this study TTR variants expression patterns were different between the different TTR variants and wild-type TTR, this might suggest that yeast cell quality controls regulates the 5 isoforms differently.

Studies in ER stress response were performed using four yeast KO deletion strains (*hac*1Δ, *dfm*1Δ, *ire*1Δ and *cwh*41Δ) expressing WT TTR and the variants V30M and L12P. Expression of TTR WT, V30M and L12P in mutant strains *hac*1Δ, *dfm*1Δ and *cwh*41Δ did not showed cell growth delay. *Ire*1Δ transformed with pBLV vector and pBLV_TTR WT had similar growth. Similar cell growth was also observed between TTR variants V30M and L12P were expressed in this strain.

Yeast cell viability for BY4741 wild-type strain and KO mutants when expressing TTR should be further confirmed with all the transformants engineered using a more suitable negative control for TTR expression. Additionally, the growth of each KO yeast strain will be compared to wild-type yeast in both stress and non-stress conditions. This experiment will show if these four KO strains grow similar to wild-type yeast and the responsive of each to ER stress conditions. Despite needing further confirmation, initial results suggest that TTR is causing toxicity through activation of ER stress mechanisms and that this pathway can be used for the future screening of compounds that modulate this effect. Future studies should be addressed using *ire*1Δ KO yeast strains transformed with the ER stress vector expressing wild-type TTR and variants V30M and L12P. This would allow monitoring of ER stress response activation due to the reporter gene.

As described in this work, we also proposed to study TTR and Aβ42 interaction in yeast. However, native Aβ42 expression was not detected inside yeast cells in order to proceed to further studies. Hypothesis to explain this result such as continuous degradation of this peptide while it is produced by yeast proteases should be evaluated. Additionally, the absence of native Aβ42 in cell media should be confirmed to discard excretion as reason for lack of detection of native Aβ42. Since this platform may enable the study of compounds that may help to understand this interaction by co-expressing these two proteins this studies should continue using Aβ42 tagged with a fluorescent protein such as mCherry or EGFP.

The findings described in this master thesis will hopefully be useful for studies in FAP pathology or other misfolded protein diseases and provide new clues for new therapeutic strategies such as new target molecules.

V. MATERIALS AND METHODS

Plasmid construction

TTR variants Leu12Pro and Thr119Met (pCMVβ_TTR-L12P and p169ZT_TTR-T119M) were a kind gift of Prof. Maria João Saraiva (IBMC). The remaining cDNAs (pV_TTRWT, pV_TTR-V30M and pV_TTR-L55P) were available in BIOALVO's DNA clones collection. All coding sequences of the TTR variants don't have the signal peptide coding sequence.

Strains and media

E. coli XL1-Blue and DH5α strains. Strains were cultivated at 37ºC in LB media (10% NaCl, 10% Bacto peptone and 5% Yeast Extract) containing ampicilin (100µg/mL) or kanamycin (50µg/mL) as selection marker depending on the plasmid transformed. In solid media 12% agar was added to the LB media.

Saccharomyces cerevisiae: BY4741 and KO yeast strains (hac1Δ, dfm1Δ, ire1Δ and cwh41Δ). Yeast strains were cultivated as described in Guthrue & Fink (1991) either in Yeast Nitrogen Based with Glucose, Galactose or Raffinose (2% of carbon source and complete supplement mixture and all the required amino acids) or in complex medium Yeast Peptone Dextrose (YPD: 10% yeast extract, 20% bacto peptone and 20% Glucose).

Cell viability assays

Solid media: After an overnight growth and when the OD600nm reached the mid-log exponential phase, yeast cells were harvested at equal concentrations. Ten or five fold serial dilutions of each yeast strain were performed. Five dilutions were made. Each dilution (10 µl) was spotted on agar YNB Glu (2% Glucose) and Gal (2% Galactose) media plates lacking the auxotrophic marker. The plates were incubated at 30ºC and 37ºC. The colonies of yeast transformants growth was monitored for a total of 3 days and photos were taken at every 24h to compare growth.

Liquid media: After overnight growth, the yeast strains are inoculated in YNB Glu his- and YNG Gal his- at three different starting OD600nm (0.05, 0.1 and 0.2) and incubated at 30ºC. These dilutions were plated in 96 well plates (Frilabo) and growth was monitored for 44h, by OD measurement every 4 hours in BIOALVO's Robotic Unit. Each sample was plated in triple.

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